Molecular Pathways Mediating Glial Responses during Wallerian Degeneration: A Dissertation

Tsai-Yi Lu
University of Massachusetts Medical School
MOLECULAR PATHWAYS MEDIATING GLIAL RESPONSES DURING WALLERIAN DEGENERATION

A Dissertation Presented

By

Tsai-Yi Lu

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 14th, 2015

Interdisciplinary Graduate Program
This work is dedicated to Juan Hsiao-Chi, for being my inspiration in the past several years, and to my family, for their unconditional support.
Acknowledgements

I would like to express my greatest appreciation to my advisor, Marc Freeman, for creating the best lab in the world and showing me how to be a great scientist. I would also like to offer my special thanks to my awesome mentor, Johnna Doherty, for patiently taking my questions from fly genetics to what a furry party would look like. My sincere thanks are also extended to all the past and present Freeman lab members, including Mary Logan, Jennifer MacDonald, Jennifer Ziegenfuss, Ozge Tasdemir-Yilmaz, Kimberly Kerr, Yuly Fuentes-Medel, Michelle Avery, Jeannette Osterloh, Allie Muthukamar, Edith Plada, Timothy Rooney, Zhiqiuo Ma, Lukas Neukomm, Thomas Burdett, Jaeda Coutinho-Budd, Owen Peters, Jemeen Sreedharan, Megan Corty, Jonathan Farley, Sukhee Cho, Lizzy Lewis, Yonca Kushkuly, Jack Wong, Gaynor Smith, Rachel Bradshaw, Tobias Stork, Nicki Fox Tapper, and Amy Sheehan, for giving valuable advice and making life in Worcester so colorful. I am forever grateful to be part of the UMMS GSBS family and would like to thank all the wonderful PhD mentors I met here, including Neal Silverman, Eric Baehrecke, Carlos Lois, Vivian Budnik, Claire Benard and Tony Ip. My special thanks also go to Yongjie Yang, for being my external committee member. Finally, I wish to thank the kindness of the Taiwanese community here at UMMS, especially Chun-Ting Chen, who passed away last summer, for being a respectful and truthful friend since my day 1 at UMMS, and Yung-Chi Huang, for always being on my side and reminding me why I started this journey in the beginning and how far I have come.
Abstract

Glia are the understudied brain cells that perform many functions essential to maintain nervous system homeostasis and protect the brain from injury. If brain damage occurs, glia rapidly adopt the reactive state and elicit a series of cellular and molecular events known as reactive gliosis, the hallmark of many neurodegenerative diseases. However, the molecular pathways that trigger and regulate this process remain poorly defined. The fruit fly *Drosophila melanogaster* has glial cells that are strikingly similar to mammalian glia, and which also exhibit reactive responses after neuronal injury. By exploiting its powerful genetic toolbox, we are uniquely positioned to identify the genes that activate and execute glial responses to neuronal injury *in vivo*. In this dissertation, I use Wallerian degeneration in *Drosophila* as a model to characterize molecular pathways responsible for glia to recognize neural injury, become activated, and ultimately engulf and degrade axonal debris. I demonstrate a novel role for the GEF (guanine nucleotide exchange factors) complex DRK/DOS/SOS upstream of small GTPase Rac1 in glial engulfment activity and show that it acts redundantly with previously discovered Crk/Mbc/dCed-12 to execute glial activation after axotomy. In addition, I discovered an exciting new role for the TNF receptor associated factor 4 (TRAF4) in glial response to axon injury. I find that interfering with TRAF4 and the downstream kinase *misshapen* (*msn*) function results in impaired glial activation and engulfment of axonal debris. Unexpectedly, I find that TRAF4 physically associates with engulfment receptor Draper – making TRAF4 only second factor to bind directly to Draper – and show it is essential for Draper-dependent activation of downstream engulfment signaling, including
transcriptional activation of engulfment genes via the JNK and STAT transcriptional cascades. All of these pathways are highly conserved from *Drosophila* to mammals and most are known to be expressed in mouse brain glia, suggesting functional conservation. My work should therefore serve as an excellent starting point for future investigations regarding their roles in glial activation/reactive gliosis in various pathological conditions of the mammalian central nervous system.
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List of Abbreviations

CNS: Central nervous system

DRG: Dorsal root ganglion

ORN: Olfactory receptor neuron

GFP: Green fluorescent protein

MP: Maxillary palp

ANT: Antenna/antennal

ITAM: Immunoreceptor tyrosine-based activation motif

SH2: Src homology 2

SH3: Src homology 3

FcγR: Fc gamma receptor

GEF: Guanine nucleotide exchange factor

GTP: Guanosine-5’-triphosphate

GDP: Guanosine diphosphate

MAPK: Mitogen-activation protein kinase

MAP4K: Mitogen-activation protein kinase kinase kinase kinase

SOG: Subesophageal ganglion

GOF: Gain-of-function

RTK: Receptor tyrosine kinase

BLAST: Basic local alignment search tool

FPKM: Fragments per kilobase of transcript sequence per million mapped fragments
CHAPTER I: Introduction
What are glia? What do they do?

“Glia” (Greek: glue) is a generic name for a large group of non-neuronal cells in the nervous system. They account for an average about 60% of the cells in the human brain (Azevedo et al., 2009) but not until recently have scientists begun to appreciate their importance in maintaining the homeostasis of the nervous system: they provide nutrition support, modulate synaptic activity, defend the brain from pathogens, and remove cellular debris from the nervous system. The evolutionary origin of glia remains a mystery. Nevertheless, it is known that both arthropods and vertebrates have well-developed glia which can be classified into several subtypes according to their distinct cellular morphologies, molecular features and biological functions (for a comprehensive review regarding the evolutionary origins of glia, see Hartline, 2011). For example, in the mammalian central nervous system (CNS), glia can be categorized into three different major subtypes – astrocytes, oligodendrocytes, and microglia. Astrocytes are star-shaped glial cells that are present in both the grey matter (protoplasmic astrocytes) and white matter (fibrous astrocytes) (Andriezen, 1893; Somjen, 1988). They provide energy support to neurons (Tsacopoulos and Magistretti, 1996) and modulate synaptic activity in multiple ways (Clarke and Barres, 2013; Perea et al., 2009). Oligodendrocytes form myelin sheath around axons to facilitate the transmission of action potentials. Microglia, the resident macrophages and professional phagocytes in the brain, are constantly surveying the surrounding microenvironment to detect and eliminate invading pathogens or clear dead neurons and cellular debris (Davalos et al., 2005; Nimmerjahn et al., 2005; Rio-Hortega, 1993). Recent reports also demonstrate the microglial involvement of
synaptic pruning during normal brain development (Paolicelli et al., 2011; Stevens et al., 2007).

**Reactive gliosis, neural injury and clearance of cellular debris by glia**

Microglia are highly sensitive to the brain microenvironment and react rapidly to brain injury by changing dramatically at both the cellular and molecular levels. However, they are not the only type of glial cells that can respond to brain injury. In fact, both astrocytes and a particular type of oligodendrocyte precursor cells, NG2 glia, participate in the multi-cellular response to CNS insults, collectively known as reactive gliosis (Burda and Sofroniew, 2014). Reactive gliosis can be induced by various kinds of brain insults, including ischemic stroke, local traumatic brain injury, pathogen infection, tumor, autoimmune response, neurodegeneration, etc. Whether reactive gliosis is beneficial or detrimental to the recovery of the nervous system has been a long debate. Recent opinions incline to portrait the nature of reactive gliosis as a double-edged sword – the glial responses evoked after brain insults help to reduce the damage but also hinder the regenerative power of the nervous system (Barres, 2008; Neumann et al., 2009; Sofroniew, 2005).

Neural injury usually gives rise to cellular debris that needs to be cleared from the nervous system. Delayed or hampered clearance of the debris can be observed in the aging brain and in Alzheimer’s disease, and efficient removal of neuronal debris has been viewed as a key factor to create a pro-regenerative environment for the nervous system (Dirnagl et al., 2009; Neumann et al., 2009; Vargas and Barres, 2007). In the peripheral
nervous system, both Schwann cells and macrophages can rapidly remove neural debris after injury. On the contrary, since the CNS is immune-privileged, the clearance of axonal debris relies heavily on the resident professional phagocytes – microglia. Live imaging studies of vertebrate microglia have demonstrated their abilities to respond to brain injury within hours and migrate toward the lesion site to perform phagocytosis (Davalos et al., 2005; Nimmerjahn et al., 2005; Peri and Nüsslein-Volhard, 2008). Questions regarding how these glia sense different kinds of injury, what molecular cue(s) are used, what molecular pathways are activated in these glia, and how these glia know when to terminate the responses have fascinated scientists trying to characterize glial responses to brain injury. This dissertation study aims to answer these questions by studying a simple axon injury model called Wallerian degeneration.

Glial responses during Wallerian degeneration in the CNS

Wallerian degeneration is named after a British neurophysiologist, Augustus V. Waller (1816 – 1870), who severed nerve fibers in frog tongues and found axons distal to the severe site disintegrated into small particles 5 to 6 days after section (Waller, 1850). Wallerian degeneration had long been thought as a passive process resulted from the loss of cell bodies, thus the nutrition supports, but the characterization of Wld שעון (Wallerian degeneration-slow) mutant mouse (Lunn et al., 1989; Perry et al., 1990a, 1990b) sparked the idea that axon destruction after axotomy was driven by an axon-autonomous program. Axons expressing Wld שעון protein preserve axon integrity for more than 7 days in DRG (dorsal root ganglion) cell culture (Buckmaster et al., 1995; Glass et al., 1993), more than
2 weeks in mice sciatic nerve transection (Lunn et al., 1989), and 20-30 days in fruit fly olfactory nerve injury model (MacDonald et al., 2006). Wallerian-like degeneration has been described in many murine neurodegenerative disease models, in some cases the expression of Wld<sup>S</sup> delayed disease progression, suggesting that similar axon destruction program is also deployed in selected pathological axon degeneration (Conforti et al., 2014).

During Wallerian degeneration, axon destruction elicits multiple glial reactions in the CNS: Astrocytes near the injury site become reactive and dramatically upregulate GFAP (glial fibrillary acidic protein) (Ludwin, 1990a). At the later stage, astrocytes form glial scar and occasionally undergo mitosis at the injury site (Bignami and Ralston, 1969). Microglia become highly active and are recruited to the injury site to phagocytose axonal debris (Bignami and Ralston, 1969; Ludwin, 1990b). The role of oligodendrocyte during Wallerian degeneration has been elusive but they seem to play little role in clearing axonal debris (Bignami and Ralston, 1969; Chew, 2007; Ludwin, 1990b). However, though these cellular phenomena are well described by the field, the exact molecular pathways/mechanisms regulating glial responses during Wallerian degeneration in the CNS remain unclear and elusive. This is in part due to a lack of glial markers, but also because screening for genes required to drive Wallerian degeneration in mouse is very labor-intensive. A simple model organism that would allow for large-scale genetic screens would greatly facilitate the rapid genetic dissection of the pathways regulating glial response to axotomy. In particular it would be exciting to identify genes required autonomously in glia for their activation after axon injury and clearance of axonal debris.
To this end, I have used the fruit fly *Drosophila melanogaster* to study the molecular basis of glial responses during Wallerian degeneration.

**Drosophila as a model organism to study Wallerian degeneration**

*Drosophila* has been used as a model organism to study genetic interactions in various biological processes for more than a hundred years. A cross-genomic analysis revealed that ~75% of human disease genes have fly homologs (Reiter et al., 2001; Rubin et al., 2000), making it an excellent model organism to study the conserved genetic networks activated in a number of pathological conditions. Recent work has demonstrated that Wallerian degeneration is also well conserved in *Drosophila* (MacDonald et al., 2006; Neukomm et al., 2014; Osterloh et al., 2012; Xiong et al., 2012). First, severed axons distal to the injury site fragmented into small pieces within 24 hours after injury, which is morphologically similar to Wallerian degeneration in mammals (MacDonald et al., 2006; Neukomm et al., 2014). Second, expressing Wld^5 in axons *in vivo* suppressed the fragmentation of severed axons (MacDonald et al., 2006; Neukomm et al., 2014), which is the genetic definition of Wallerian-like degeneration. Third, genes that are required to initiate Wallerian degeneration, such as Nmnat/Nmnat1 (nicotinamide mononucleotide adenyllytransferase 1) (Avery et al., 2009; MacDonald et al., 2006; Mack et al., 2001; Xiong et al., 2012), dSarm1/Sarm1 (sterile alpha and TIR motif containing 1) (Osterloh et al., 2012) and Highwire/Phr1 (PAM-Highwire-Rpm-1) (Neukomm et al., 2014; Xiong et al., 2012), are all functionally conserved between *Drosophila* and mammals (Neukomm and Freeman, 2014). Lastly, axonal debris produced after axotomy is also eventually
cleared from the nervous system by the surrounding glial cells (MacDonald et al., 2006; Neukomm et al., 2014), similar to the microglial response to Wallerian degeneration in mammals (Bignami and Ralston, 1969; Ludwin, 1990b). It therefore appears that Wallerian degeneration in *Drosophila* is not only morphologically reminiscent of Wallerian degeneration in mammals but that genes and molecular machinery are also very likely to be conserved. As a result, together with the powerful genetic toolbox available for *Drosophila* biologists, flies present a unique opportunity to study the genetic interactions during Wallerian degeneration *in vivo*, especially the interactions between degenerating axons and the surrounding glial cells.

**Glial responses during Wallerian degeneration in the olfactory nerve injury model**

The robust glial responses during Wallerian degeneration in *Drosophila* have been best demonstrated in the olfactory nerve injury model (MacDonald et al., 2006). The *Drosophila* olfactory receptor neurons (ORNs) reside in the third antennal segment and maxillary palps (**Figure 1.1A**). Each olfactory neuron expresses specific odorant receptor gene (Vosshall et al., 2000) and projects axon into the spatially invariant synaptic field, called glomerulus, to form synapses with other neurons. There are in total 50 glomeruli clustered in a region called antennal lobe. 44 of 50 glomeruli are innervated by ORN axons projected from the antenna, and 6 of them are innervated by maxillary ORN axons projected from the maxillary palps (MacDonald et al., 2006). Different subsets of the olfactory axons can be easily observed under the microscope by using membrane-tethered green fluorescent protein (mCD8::GFP) fused with corresponding
odorant receptor gene promoter and hence the axon degeneration events can be monitored and easily quantified. For example, OR85e-mCD8::GFP labels a subset of the maxillary palp nerves, as shown in Figure 1.1B, and when maxillary palps are ablated by a non-invasive surgical removal of the entire maxillary palps, OR85e+ axons lose their connections to the cell bodies, initiate Wallerian degeneration program and begin to lose membrane integrity within 24 hours after injury (Figure 1.1B, 1dpmp). Glial responses to axon degeneration are immediate and include a transcriptional increase of glial engulfment gene, draper, within the first 1.5 hours after axon injury (Logan et al., 2012). Glial membranes also undergo hypertrophy and can be found at the injured axons as early as 4 hours after axotomy, coinciding with the fragmentation of the axons (MacDonald et al., 2006). At the injured axons, glial membranes are found to form small vesicles with an average radius about 1-2 μm (Ziegenfuss et al., 2012). They only appear after axon injury, and often times (49.85% ± 0.08, Tsai-Yi Lu, unpublished data) contain axonal debris within the enclosed region. The number of vesicles are correlated with the engulfment efficiency of glia, and some of the vesicles can also be stained with Lysotracker, the fluorescent probe that labels acidic organelles, such as lysosomes (Lu et al., 2014; Ziegenfuss et al., 2008). As a consequence, these vesicles are thought to be the internalization structure for glia to engulf axonal debris and later fused with lysosomes in the cytoplasm to degrade the debris (Ziegenfuss et al., 2012). Indeed, 5 days after maxillary palp ablation, very little OR85e+ axonal debris remains in the brain, a result from the active glial engulfment of axonal debris during this period of time (Figure 1.1B, 5dpmp).
The diversity of glial cell types in *Drosophila*

Like mammals, *Drosophila* also has many different subtypes of glial cells that resemble mammalian glial cells in morphology and biological functions. For example, the antennal lobe region is highly compact with dendrites, axons and glial membranes that ensheath and infiltrate the glomeruli. The major subtypes of glial cells surrounding the antennal lobe – astrocytes, ensheathing glia, and cortex glia – are functionally and morphologically diverse and have been well characterized in detail in Awasaki et al., (2008) and Doherty et al., (2009) (Figure 1.1A). They all express the glia-specific homeodomain protein, Repo (reverse polarity). Astrocytes, as shown in Figure 1.1A, protrude fine fibrous processes extensively into the antennal lobe and infiltrate nearby glomeruli. It has been shown that astrocytes can promote synapse formation during development and regulate circadian rhythms (Muthukumar et al., 2014; Ng et al., 2011). They exclusively express GABA (gamma-aminobutyric acid) transporter (GAT) (Stork et al., 2014) to facilitate glutamate recycling at the synaptic regions (Muthukumar et al., 2014). Although astrocytes can engulf neural debris after pruning during pupal stage (Tasdemir-Yilmaz and Freeman, 2014), they do not exhibit engulfment activity to clear axonal debris within the antennal lobe after axotomy in the adult (Doherty et al., 2009). Ensheathing glia extend their bulbous processes to wrap axon bundles and glomeruli in the antennal lobe and are the major phagocytic glia that engulf axonal debris during Wallerian degeneration (Doherty et al., 2009). Ensheathing glia are highly responsive to axon injury: their membranes undergo dramatic changes, such as hypertrophy and
forming small phagocytic vesicle, and they internalize and degrade axonal debris. Draper is strongly expressed in the ensheathing glia and required for all known glial responses to axon injury including ensheathing glial membranes recruitment to the injured glomeruli after maxillary palp ablation (Doherty et al., 2009). The third type of glial cells around the antennal lobe region is cortex glia. They have honeycomb-shaped morphology and form a large mesh-like network with the neuronal cell bodies (mostly interneurons) they enwrap outside the antennal lobe. Adult cortex glia also strongly express Draper, but they do not engulf axonal debris after injury (Doherty et al., 2009). The physiological functions of cortex glia remain largely unclear, although they have been implicated in balancing extracellular ion concentrations and determining seizure susceptibility (Melom and Littleton, 2013).

**Draper-mediated glial engulfment of axonal debris**

*Drosophila* glia also use highly conserved molecular machinery to clear axonal debris during Wallerian degeneration. The first gene identified responsible for glial responses to axon injury is the evolutionarily conserved engulfment receptor Draper (*Drpr*). In *drpr* null animals, glia fail to induce membrane hypertrophy, glial membranes are absent from the injured axons 1 day after injury, and >80% of the axonal debris still linger in the brain 5 days after maxillary palp ablation (MacDonald et al., 2006), indicating Draper is required for glia to activate injury response and clear axonal debris.

Draper is highly conserved from worms to mammals. Draper homolog in worms, CED-1, is among the first several genes identified in the genetic screen for cell death...
pathways and later found to be required for the neighboring cells to engulf the apoptotic
cells (Zhou et al., 2001). The mouse homologs of Draper, MEGF10 and Jedi-1, are
required for satellite glial cells to phagocytose neuronal corpses (Wu et al., 2009) and for
CNS astrocyte to prune synapses during development (Chung et al., 2013), suggesting
that Draper is an ancient molecule that glial cells continue to exploit for engulfment of
neuronal debris since early in evolution. Draper is a single-pass transmembrane protein
with multiple atypical EGF-like repeats outside the plasma membrane, although their
roles in Draper-mediated glial engulfment of axonal debris are not clear yet. In the
intracellular domain, Draper has two important signaling motifs: one is the NPXY motif
and the other is ITAM (immunoreceptor tyrosine-based activation motif). The NPXY
motif is also present in the worm homolog (Caenorhabditis elegans) and the mammalian
homolog of Draper. It is thought to be the binding site for dCed-6, an evolutionarily
conserved engulfment gene that is also required for Drosophila glia to clear axonal debris
(Doherty et al., 2009; Fujita et al., 2012). dCed-6 is also involved in the engulfment of
apoptotic cells in worms, axon pruning in Drosophila, and glial phagocytosis of apoptotic
neurons in mouse (Awasaki et al., 2006; Doherty et al., 2009; Liu and Hengartner, 1998;
Su et al., 2002; Sullivan et al., 2014).

Interestingly, the ITAM found in Draper is not present in CED-1, but both
MEGF-10 and Jedi-1 have functional ITAM domains. An ITAM domain is composed of
a series of conserved amino acid sequence (typically YxxI/L-X_{6-12}-YxxI/L) and
commonly found in the immunoreceptors. The phosphorylation of the tyrosine residue(s)
within ITAM recruits SH2 (Src homology 2) domain-containing proteins to activate
downstream pathways and induce phagocytosis. During Fc receptor (FcR)-mediated phagocytosis, the ITAMs of FcγR are phosphorylated by Src family kinase, which then allows the Src family kinase Syk (or Zap-70) to bind. Interestingly, Drosophila Src family kinase, Src42A, can phosphorylate Y934 and Y949 in the ITAM of Draper, and the phosphorylation of Y949 is important for Shark, the Drosophila version of Syk family tyrosine kinase to bind (Ziegenfuss et al., 2008). Knocking down Src42A and Shark in glia respectively affects glial ability to clear axonal debris after injury, implying that Drosophila glia use a mechanism and/or molecular machinery during the engulfment of axonal debris similar to mammalian macrophages when phagocytosing non-self particles. MEGF-10 and Jedi-1 also employ Src24A and Shark mammalian homologs to engulf apoptotic neurons via their ITAMs (Scheib et al., 2012), underlining the highly conserved nature and the importance of the Draper pathway.

In addition to Draper, dCED-6, Src42A and Shark, many other genes have been reported to be important for glia to clear axonal debris (Doherty et al., 2014; MacDonald et al., 2013; Ziegenfuss et al., 2012). Their roles in glial responses to axon injury are summarized in Figure 1.2, and will be discussed in details in Chapter II and Chapter III. In brief, the axonal debris resulting from axon degeneration signals glial cells via an unknown mechanism, and Draper becomes activated through the ITAM phosphorylation by Src42A and the binding of Shark. The function of dCed-6 in Drosophila glial engulfment is not clear yet, but mammalian studies suggest it might be involved in clathrin-dependent phagocytosis (Sullivan et al., 2014). Draper, Src42A, Shark and dCed-6 are all required for glial membranes to be recruited to the injured axons (Doherty
et al., 2009; MacDonald et al., 2006; Ziegenfuss et al., 2008), which is thought to be mediated by the small GTPase Rac1 (Lu et al., 2014; Ziegenfuss et al., 2012). The activation of Rac1 requires GEF (guanine nucleotide exchange factor) to facilitate the binding of Rac1 to GTP, and one of the GEFs known to be upstream of glial Rac1 activation is the non-canonical GEF complex Crk/Mbc/dCed-12 (C. elegans: CED-2/CED-5/CED-12; mammals: CrkII/Dock180/Elmo) (Ziegenfuss et al., 2012). Knocking down Crk/Mbc/dCed-12 suppresses glial clearance of axonal debris but, unlike rac1 RNAi phenotype, does not completely abolish the recruitment of glial membranes to degenerating axons, leading to the hypothesis that there may be more than one GEF upstream of Rac1 during glial responses to axon injury. I identify this alternative GEF in Chapter II.

**Activation of glial transcriptional responses by JNK and STAT after axon injury**

Glia also react to axon injury by changing their gene expression profiles through intracellular signaling pathways. As mentioned earlier, the mRNA transcripts of Draper I, the major isoform of Draper that positively regulates glial engulfment, is significantly increased within hours after injury (Logan et al., 2012). Doherty et al. shows that it is due to the activation of the non-canonical STAT (signal transducer and activator of transcription) signaling pathway (Doherty et al., 2014). Normally, after receiving extracellular signals, generally cytokines or growth factors, Janus kinase (JAK) associated with receptor will be autophosphorylated and then recruits STAT. STAT is then phosphorylated by JAK upon tyrosine residues and dimerized to enter into the
nucleus. Dimerized STATs bind to specific DNA sequences in the genome to initiate transcription of certain genes in response to the extracellular signals. However, during *Drosophila* glial responses to axon injury, the activation of STAT-dependent transcriptions does not require Hopscotch (*Drosophila* JAK) but requires Draper, as well as other engulfment genes such as Src42A, Shark, and Rac1 (Doherty et al., 2014). The exact mechanism of how Draper regulates STAT signaling activation is unknown, but it appears that glia form a positive-feedback loop to upregulate Draper expression through Draper itself to increase glial engulfment activity during Wallerian degeneration, since increasing Draper expression in glia is sufficient to rescue the engulfment defect caused by the lack of STAT in glia (Doherty et al., 2014).

JNK (c-Jun N-terminal kinases) signaling cascade is also important in glial clearance of axonal debris. JNK is one of the MAPK (mitogen-activated protein kinase) family members that respond famously to cellular stress. *In vivo* glial RNAi experiments suggest that glia activate JNK signaling cascade after axon injury through Slipper (Slpr)/TAK1 (TGF-beta activated kinase 1) → MKK4 (mitogen-activated protein kinase kinase 4) → Basket (*Drosophila* JNK) → dAP-1 (*Drosophila* activator protein 1) and induce downstream dAP-1-dependent gene transcriptions (MacDonald et al., 2013). Depletion of JNK in glia results in axonal debris clearance defect and the lack of Draper upregulation after axon injury (MacDonald et al., 2013). Nevertheless, how glia activate JNK signaling pathway and how JNK pathway regulates Draper expression remains unknown, which will be the main topic in Chapter III.
To summarize, glia are indispensible brain cells for the development, maintenance, and repair of the nervous system. Glial reactivity has been observed in nearly every pathological condition, and the molecular mechanisms of reactive gliosis are receiving more attention from the field. Yet their intrinsically complicated nature has impeded our unraveling of the molecular mechanisms induced in glia in response to CNS insults. *Drosophila* has proven a powerful model in which to study fundamental mechanisms of glial roles in Wallerian degeneration. In the work presented in Chapter II, I further expand our understandings of glial engulfment of axonal debris by characterizing a new Rac1 GEF complex, DRK/DOS/SOS, and show that, together with the previously identified Crk/Mbc/dCed-12, these two GEF complexes act redundantly upstream of Rac1 to efficiently activate glia during Wallerian degeneration. In Chapter III, I will describe how the conserved adaptor protein, TRAF4, is an exciting new binding partner for Draper and is required for glia to clear axonal debris and to activate the JNK and STAT signaling pathways in response to axon injury through MSN. TRAF4 therefore provides the missing link between Draper and the activation of glial transcriptional responses after axotomy.
Figure 1.1. Using *Drosophila* olfactory nervous system to study Wallerian degeneration. (A) The wiring diagram of the adult *Drosophila* ORNs and the architecture of the antennal lobe. See text for details. (B) OR85e+ maxillary nerves labeled with membrane tethered GFP (mCD8::GFP) undergo Wallerian degeneration after maxillary palp ablation. Dashed lines roughly circled the antennal lobes. Arrow indicated a glomerulus innervated by OR85e+ maxillary nerves. 1dpmp: 1 day after maxillary palp ablation. 5dpmp: 5 days after maxillary palp ablation. Representative z-stack images were shown. Scale bar = 10 µm.
Figure 1.2. Genes and molecular pathways involved in *Drosophila* glial responses during Wallerian degeneration. See text for details.
CHAPTER II: DRK/DOS/SOS Converge with Crk/Mbc/dCed-12 to activate Rac1 during Glial Engulfment of Axonal Debris

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Introduction

Activation of glia is a hallmark of nearly all neurodegenerative diseases and neural injuries. When brain insults have occurred, glia rapidly change their morphology and gene expression profiles to invade the injury site and clear pathogens and/or neuronal debris by phagocytic engulfment (Davalos et al., 2005; Nimmerjahn et al., 2005). Failure to clear debris from the CNS can result in prolonged neuroinflammation and hamper the recovery of the CNS (Neumann et al., 2009; Vargas and Barres, 2007). However, the genetic pathways promoting glial activation after neural injuries remain poorly defined.

Genetic studies of Wallerian degeneration in Drosophila have provided important insights into glial responses to axotomy (MacDonald et al., 2006). Olfactory neuron axotomy results in the degeneration of axons projecting into the antennal lobe of the fly brain, where local glia sense degenerating axons, and initiate a multistep process of reactivity. Reactive glia up-regulate the transcription of the engulfment receptor Draper (drpr) and extend membranes to degenerating axons (Logan et al., 2012; MacDonald et al., 2013). When at the injury site, glia internalize axonal debris and degrade it through the phagolysosomal pathway (Ziegenfuss et al., 2012). Finally, glia terminate their responses by withdrawing from the injury site and down-regulating Draper, and finally return to a resting state (Logan et al., 2012).

Draper is essential for all glial responses to axonal injury. In drpr null mutants, glia fail to respond morphologically to axonal injury, and axonal debris lingers in the brain for weeks after axotomy (MacDonald et al., 2006). Downstream of Draper, the small GTPase Rac1 appears to be critical in executing glial activation to axon injury, as
loss of Rac1 phenocopies *drpr* null mutants (Ziegenfuss et al., 2012). The only Rac guanine nucleotide exchange factor (GEF) known to be required for glial engulfment of axonal debris is the noncanonical GEF Crk/Myoblast city (Mbc)/dCed-12. However, in contrast to loss of Rac1, animals lacking Crk/Mbc/dCed-12 signaling exhibit relatively normal activation of glia after axotomy, with glia increasing Draper expression and extending membranes to degenerating axons, but glia then fail to internalize and degrade axonal debris (Ziegenfuss et al., 2012). These data argue for a specific role for the Crk/Mbc/dCed-12 complex at the internalization/degradation phase of the glial response, and suggest that an additional Rac1 GEF must act earlier during initial activation of glial responses to axonal injury. In an RNAi-based screen for new engulfment genes, we identified *downstream of receptor kinase (drk)* as a gene required for efficient glial clearance of degenerating axons. DRK is best known for its role in signaling downstream of the Sevenless (Sev) receptor tyrosine kinase (RTK), where it functions with daughter of sevenless (dos) and son of sevenless (sos) to activate the small GTPase Ras (Herbst et al., 1999; Olivier et al., 1993; Raabe et al., 1996; Rogge et al., 1991; Simon et al., 1991, 1993). More recent studies have also linked SOS to the activation of Rac1 in the regulation of axon guidance during *Drosophila* embryonic CNS development (Yang and Bashaw, 2006), indicating that DRK/DOS/SOS can act upstream of multiple small GTPases. Here we show that the DRK/DOS/SOS complex plays a critical role in activation of glial responses to injury and internalization of axonal debris. Moreover, we provide genetic evidence that, at the earliest stage of glial activation, DRK/DOS/SOS
function redundantly with Crk/Mbc/dCed-12 to promote Rac1 activation and initiate all steps in glial responses to axonal injury.

**Results**

**DRK, DOS, and SOS are required for glial engulfment of axonal debris**

To identify new pathways required for glial engulfment of degenerating axons, Johnna Doherty performed an RNAi-based screen for genes required in glia for clearing axonal debris after axotomy. She expressed each of ∼500 UAS-RNAi constructs from the Vienna Drosophila Resource center (Dietzl et al., 2007) by using the pan-glial driver *repo-Gal4* (Sepp et al., 2001). For animals that did not survive to adulthood, she further incorporated a temperature-sensitive version of Gal80 (*Gal80ts*) (McGuire et al., 2004) in the background to temporally control the induction of RNAi exclusively in the adult glia. She ablated maxillary palps in which a subset of olfactory receptor neurons (ORNs) were labeled with membrane-tethered GFP (*OR85e-mCD8::GFP*) (Couto et al., 2005) and scored axonal debris clearance 5 days after axotomy by quantifying GFP immunoreactivity of OR85e+ glomerulus in the antennal lobe, as previously reported (MacDonald et al., 2013).

In her primary screen, we found that an RNAi construct (*drkRNAi#105498*) targeting DRK suppressed glial clearance of axonal debris. In control animals, the vast majority of axonal debris was cleared 5 d after axotomy (**Figure 2.1A**). However, a significant amount of the axonal debris still lingered in the brain of *drkRNAi* animals at day 5, and
ultimately cleared in 20 d after axotomy (Figure 2.2A, quantified in Figure 2.2B), which argues for a glial role for DRK in engulfment of axonal debris. DRK is known to physically interact with the adaptor protein DOS (Feller et al., 2002) and the GEF SOS (Olivier et al., 1993), which can activate downstream small GTPase such as Ras and Rac1 (Nimmual et al., 1998; Rogge et al., 1991; Simon et al., 1993; Yang and Bashaw, 2006). She therefore designed a UAS-RNAi construct ($dos^{RNAi#3}$) to knock down DOS in glia and assayed for engulfment defect. Adult glia expressing $dos^{RNAi#3}$ also exhibited a significant delay in engulfment of axonal debris (Figure 2.1A), arguing that DOS also plays an important role in glial engulfment of degenerating axons. To further validate this result, I repeated this experiment with an additional RNAi line ($dos^{1044R-3}$), which targets different region of $dos$ mRNA from $dos^{RNAi#3}$, and found similar engulfment defects 5 days after injury (Figure 2.3), arguing that the phenotype was not caused by the off-target effects of RNAi. I next knocked down glial SOS by using RNAi ($sos^{RNAi42849}$) and found that axonal debris remained uncleared in the CNS for as long as 20 days (Figure 2.1 and Figure 2.2A, 2.2D). I did not observe significant change in the expression of Draper when DRK, DOS, and SOS were knocked down respectively (Figure 2.2E-F), implying that the delayed clearance is not caused by a lower level of Draper expression. Together, these data suggest that, during glial engulfment, similar to Sev RTK signaling, DRK, DOS, and SOS interact with each other to regulate downstream small GTPase activity. During Sev signaling, DRK and DOS couple RTK activation to stimulation of downstream small GTPase activity through SOS. I therefore speculated that increasing SOS activity could potentially compensate for the depletion of
DRK and DOS. To test this hypothesis, I explored the effect of a gain-of-function (GOF) SOS allele (Sos$^{JC2}$) (Karlovich et al., 1995; Rogge et al., 1991) on the ability of glia to clear axonal debris when DRK or DOS was knocked down. Sos$^{JC2}$/+ animals did not exhibit any discernible clearance defect (Figure 2.1), nor did they clear debris faster than controls (Figure 2.4). However, I found the delay in clearance of axonal debris caused by $drk^{RNAi}$ and $dos^{RNAi}$ was completely rescued by Sos$^{JC2}$/+ (Figure 2.1). These data indicates that the DRK/DOS/ SOS complex is required for efficient engulfment of axonal debris by glial cells, and that activation of SOS is sufficient to drive glia to engulf axonal debris when DRK or DOS is depleted, consistent with the notion that SOS acts downstream of DRK and DOS.

**Glial DRK is recruited to degenerating axons after injury**

After axon injury, Draper is up-regulated in glial cell and recruited to sites where glia actively engulf axonal debris (Logan et al., 2012; MacDonald et al., 2006). I sought to determine whether DRK was also expressed in glia, as our RNAi data would suggest, and whether it was recruited to injury sites during glial engulfment. I used α-DRK polyclonal antibodies (Olivier et al., 1993) to detect DRK expression in the adult brain. I first examined DRK localization along the maxillary nerve in the subesophageal ganglion (SOG), through which GFP-labeled mp ORN axons are projected to the antennal lobe. In control brains, I observed widespread DRK expression and, interestingly, 1 day after mp ablation, DRK was enriched along the maxillary nerve which was absent when I drove $drk^{RNAi}$ in glia (Figure 2.5A), indicating that glial DRK is recruited to severed axons.
Moreover, I found that Draper is required for the recruitment of DRK after injury because I could not detect up-regulation of DRK 1 day after axotomy in drpr null (drpr$^{A5}$) animals (Figure 2.6), suggesting that the activation of DRK/DOS/SOS requires Draper. I next sought to determine if DRK expression was increased after antennal ablation, which leads to a dramatic increase in Draper expression and hypertrophy of glial membranes (MacDonald et al., 2006). I labeled glial membranes with mCD8::GFP by using the repo- Gal4 driver, and assayed glial morphology and DRK expression in control animals and animals where the third antennal segments had been ablated 1 day earlier. Consistent with our findings in the SOG, DRK immunoreactivity was dramatically increased around the antennal lobe 1 day after antennal ablation in control flies (Figure 2.5B). The increase in DRK is likely a result of recruitment of DRK to glial membranes at sites of axon injury rather than up-regulation of drk gene transcription and/or translation, as DRK protein levels did not increase significantly after ablation of olfactory organs (Figure 2.7). When we drove drk$^{RNAi}$ in glia, there was a significant decrease of DRK immunoreactivity in the hypertrophic ensheathing glia (Figure 2.5B, arrows) but not in neurons, confirming that the increase of DRK immunoreactivity comes from glia. Together, these results are consistent with the model that DRK acts downstream of Draper to promote engulfment of axonal debris.

**DRK-SOS activation helps glia to internalize axonal debris and activate the phagolysosomal program**
After axotomy, glial membranes are recruited to severed axons, where they internalize and degrade axonal debris (Ziegenfuss et al., 2012). As the first step to determine where DRK impinged on glial engulfment of axonal debris, I examined glial membrane dynamics after mp injury. In healthy, uninjured animals, glia usually wrap each glomerulus with their fibrous membranes. After axon injury, glia form large numbers of membranous vesicles throughout the field of degenerating axons (Figure 2.8A); these vesicles often contain internalized axonal debris, presumably to become acidic thereafter for degradation (Ziegenfuss et al., 2012). However, I found that, unlike in control animals, knocking down DRK in glia by using repo-GAL4 resulted in a failure of glia to efficiently form enclosed vesicles 1 day after injury, with total vesicle number in \( \text{drk}^{\text{RNAi}} \) animals being reduced to \(~30\%\) of that found in the controls (Figure 2.8B). DRK activity in glial membrane vesicle formation appears to act through SOS, as \( \text{Sos}^{JC2/+/} \) was sufficient to suppress the loss of glial membrane vesicles in the \( \text{drk}^{\text{RNAi}} \) background.

I next sought to determine whether reduced DRK function affected the ability of glia to internalize axonal debris. I labeled ensheathing glia with \( \text{UAS-mCD4::tdTomato} \) by using the \( \text{TIFR-Gal4} \) driver (Ziegenfuss et al., 2012) and compared internalization of \( \text{OR85e-mCD8::GFP} \)-labeled ORN axons in control and \( \text{drk}^{\text{RNAi}} \) animals. Internalized axonal debris was scored when \( \text{GFP}^+ \) axonal debris was found within \( \text{tdTomato}^+ \) membrane-enclosed vesicles. Before injury, \( \text{tdTomato} \)-labeled glial membrane vesicles were not detectable in the \( \text{OR85e}^+ \) glomerulus. However, in controls, 1 day after mp ablation, glial membranes invaded the glomerulus and \( \text{tdTomato} \)-labeled vesicles containing \( \text{mCD8::GFP}^+ \) axonal materials were found throughout this glomerulus.
Expression of \( drk^{RNAi} \) in TIFR\(^+ \) glia significantly reduced the number of glial vesicles containing axonal debris compared with the control (Figure 2.8D). Activating SOS signaling in the \( drk^{RNAi} \) background through GOF \( Sos^{JC2} \) resulted in glial internalization of axonal debris at wild-type levels. \( Sos^{JC2} \) itself did not increase the total number of glial vesicles, nor did it alter the number of vesicles internalizing degenerating axons, arguing that the rescuing effect did not result from a \( Sos^{JC2} \)-dependent general increase in vesicle number. I therefore concluded that DRK-SOS signaling plays an important role in forming phagocytic vesicles during glial engulfment of axonal debris.

After internalization of axonal debris, glia activate the phagolysosomal pathway to degrade engulfed axonal materials (Ziegenfuss et al., 2012). To explore if activation of the phagolysosomal pathway was also affected by altered DRK-SOS signaling, I examined the formation of acidified phagolysosomes surrounding severed axons by using LysoTracker red DND-99. In control uninjured brains, the area surrounding OR85e\(^+ \) axons did not exhibit detectable LysoTracker\(^+ \) puncta (Figure 2.9A). One day after injury, LysoTracker\(^+ \) puncta appeared robustly along the degenerating OR85e\(^+ \) axons. Knocking down DRK in ensheathing glia resulted in \(~75\%\) reduction in the number of LysoTracker\(^+ \) puncta (Figure 2.9B), indicating that the loss of DRK function severely impedes glial activation of phagolysosomal pathway. I detected a slight increase in the efficiency of phagolysosome maturation with \( Sos^{JC2} \), as revealed by a \(~1.5\)-fold increase in the number of LysoTracker\(^+ \) puncta compared with the controls, suggesting that activation of SOS promotes phagolysosome formation. However, I found that \( Sos^{JC2} \) was
not sufficient to rescue the phagolysosome maturation defect caused by \( drk^{RNAi} \). This lack of rescue by \( Sos^{JC2} \) could indicate that the \( Sos^{JC2} \) allele is not sufficiently strong to overcome the absence of DRK activity during phagolysosome formation, or that another molecule might act in a redundant fashion with DRK during this specific signaling step. In summary, these data argue that DRK-SOS signaling plays a critical role during glial internalization of axonal debris and activation of the phagolysosomal program for degradation of axonal materials.

**Rac1, but not Ras, is the main small GTPase effector downstream of SOS in activating the engulfing glia**

SOS is an evolutionarily conserved GEF for the small GTPase Ras, and DRK/DOS/SOS were initially identified as key molecules coupling the Sev RTK to Ras activation (Herbst et al., 1996; Olivier et al., 1993; Raabe et al., 1996; Rogge et al., 1991; Simon et al., 1991, 1993). I therefore explored the possibility that Ras might act as the downstream effector after SOS activation to promote glial clearance of axonal debris. I expressed a dominant-negative (DN) form of Ras (\( Ras85D^{N17} \)) (Lee et al., 1996) specifically in adult glia and assayed glial engulfment after axotomy. In these animals, 5 days after mp ablation, I found that the vast majority of axonal debris was cleared, as <10% of axonal debris was left 5 days after injury (Figure 2.10). This phenotype is extremely mild compared with what we observed with \( dos, drk \) and \( sos \) RNAi. Although I could not exclude the possibility that Ras is activated by SOS after injury and plays a minor role in
clearance of axonal debris, I suspected additional GTPases might be the key downstream effectors of SOS during glial engulfment.

The small GTPase Rac1 is a potent regulator of membrane ruffling and lamellipodia formation, and is required for glial phagocytosis of degenerating axons (Ziegenfuss et al., 2012). Interestingly, SOS possesses Rac1-specific GEF activity in mammals and Drosophila through its conserved N-terminal Dbl homology domain (Nimnual et al., 1998; Yang and Bashaw, 2006). I therefore assayed for genetic interactions between Rac1 and SOS. In Drosophila eye development, overexpression of a DN Rac1 (Rac1\(^{N17}\)) results in a rough eye phenotype that can be suppressed by supplying excess upstream GEF complex proteins, Mbc and dCed-12 (Geisbrecht et al., 2008). I therefore reasoned that over-activation of SOS by Sos\(^{JC2}\) might suppress Rac1\(^{N17}\) phenotypes during glial engulfment of axonal debris. Adult-specific overexpression of Rac1\(^{N17}\) in glia resulted in a potent suppression of axonal debris clearance as previously described (Ziegenfuss et al., 2012) (Figure 2.11A). However, when I overexpressed Rac1\(^{N17}\) in Sos\(^{JC2}/+\) background, I observed a \(~50\%\) suppression of clearance defect compared with animals expressing Rac1\(^{N17}\) alone (Figure 2.11B). As Rac1\(^{N17}\) also blocks glial membrane extension to the site of injury (Ziegenfuss et al., 2012), I also examined whether Sos\(^{JC2}\) could rescue this defect by scoring recruitment of Draper to severed axons 1 day after axotomy (Figure 2.11C). I found that, consistent with our previous study, glial expression of Rac1\(^{N17}\) completely suppressed the recruitment of Draper to severed axons, but over-activation of SOS using Sos\(^{JC2}\) partially restored (to \(~30\%\) control levels) glial recruitment of Draper to axonal debris. The simplest
interpretation of these data, based on interactions between SOS and RAC1 in other signaling contexts such as the Sevenless pathway, is that the GEF component SOS acts upstream of the small GTPase RAC1. However, I cannot exclude the formal possibility that SOS acts downstream of RAC1 to activate another small GTPase during glial engulfment of axonal debris.

**Both DRK/DOS/SOS and Crk/Mbc/dCed-12 activation contribute to Rac1 activity in glial response to axon injury**

Ziegenfuss et al. argued that the GEF complex Crk/Mbc/dCed-12 acts upstream of Rac1 in glial clearance of axonal debris (Ziegenfuss et al., 2012), but there were significant differences in the requirements for Rac1 vs. Crk/Mbc/dCed-12. Specifically, depletion of Rac1 activity by RNAi or use of DN constructs completely blocked glial activation: there was no recruitment of glial membranes or Draper to sites of axon injury, along with severe defects in axonal debris clearance. In contrast, depletion of Crk, Mbc, or dCed-12 resulted in a slight delay in glial recruitment to severed axons, suggesting a complementary GEF acting upstream of Rac1 might also play a role in glial response to axon injury. Based on the similarity of the phenotypes associated with inhibition of Crk/Mbc/dCed-12 and DRK/DOS/SOS (i.e., reduced vesicle formation, failure to activate the phagolysosomal pathway, and an axonal debris clearance defect), I speculated that Crk/Mbc/dCed-12 and DRK/DOS/SOS activation might act redundantly downstream of Draper to activate Rac1 not only at the early phases of the glial response (when glial membranes are recruited to severed axons), but also at later phases (during internalization
and degradation of axonal debris). This predicts that simultaneous depletion of
Crk/Mbc/dCed-12 and DRK/DOS/SOS should result in a complete suppression of glial
activation. To test this model, I simultaneously knocked down SOS and MBC, both of
which possess the enzymatic GEF activity for Rac1, specifically in adult glia, and
examined the recruitment of glial membranes to the severed OR85e+ axons 1 day after
injury (Figure 2.12A). Interestingly, I found that sosRNAi or mbcRNAi resulted in a slightly
reduced recruitment of Draper-decorated glial membranes to severed axons. However,
when sosRNAi and mbcRNAi were simultaneously expressed, the recruitment of glial
membrane to injured OR85e+ glomerulus was completely blocked 1 day after injury
(Figure 2.12B). The additive nature of the sosRNAi and mbcRNAi phenotypes also extended
to clearance of axonal debris as well as glial hypertrophy response (Figure 2.13). Thus,
simultaneous blockade of DRK/DOS/SOS and Crk/Mbc/dCed-12 signaling phenocopied
drpr null mutants (MacDonald et al., 2006), and inhibition of Rac1 signaling (Ziegenfuss
et al., 2012). I further explored if SOS activation could partially substitute for
Crk/Mbc/dCed-12 during glial clearance of axonal debris by crossing SosJC2 into dCed-
I2RNAi background. As previously shown (Ziegenfuss et al., 2012), glial knockdown of
dCed-12 function strongly suppressed glial clearance of axonal debris 5 days after injury
(Figure 2.12C). However, in a SosJC2/+ background, the effect of dCed-12RNAi was
attenuated (Figure 2.12D), indicating that activation of SOS can partially compensate for
the reduced activity from Crk/Mbc/dCed-12 to promote glial clearance of axonal debris.
I conclude that Crk/Mbc/dCed-12 and DRK/DOS/ SOS act in a partially redundant
fashion downstream of Draper to activate Rac1 and thereby glia, and promote glial
clearance of axonal debris.

**Summary and discussion**

In this chapter, I identify *Drosophila* DRK, DOS, and SOS as new molecules required for
glial responses to axonal injury. I show that glial depletion of DRK, DOS, or SOS results
in a delay in glial responses to ORN axotomy and reduced efficiency of glial
internalization and digestion of axonal debris. I observe no obvious alterations in glial
morphology or expression of engulfment machinery (e.g., Draper), and demonstrate that
adult-specific knockdown of DRK/DOS/SOS leads to defects in glial clearance of
degenerating axons. These data indicate that DRK/ DOS/SOS promote engulfment
signaling in mature glia, and argues against a developmental defect causing the
phenotypes I observe. Based on my observation that a dominant GOF allele of SOS
(*Sos^{JC2}* ) can partially suppress depletion of DRK and DOS, I propose that SOS acts
genetically downstream of DRK and DOS. Previously, Ziegenfuss et al. demonstrated a
key role for the *Drosophila* GEF Crk/Mbc/dCed-12 in glial engulfment activity, with
elimination of this signaling complex from glia resulting in normal glial activation (e.g.,
recruitment of glial membranes to axonal debris), but a failure to engulf axonal debris
(Ziegenfuss et al., 2012). Here I provide strong evidence that DRK/DOS/SOS and
Crk/Mbc/dCed-12 act redundantly downstream of Draper at two key steps in the
engulfment process (**Figure 2.14**). First, based on the fact that simultaneous depletion of
both signaling complexes phenocopies Rac1 loss of function, I propose that these complexes act redundantly to activate Rac1 and glial responses, including Draper up-regulation and extension of glial membranes to degenerating axons. Second, after glia have arrived at axonal debris, both complexes are required for the elimination of axonal debris. At this step, DRK/DOS/SOS and Crk/Mbc/dCed-12 appear to act in a non-redundant fashion to promote glial internalization of axonal debris and activation of the phagolysosomal program for degradation of internalized axonal material.

DRK/DOS/SOS signaling has been studied most intensively for its role downstream of the RTK Sev, where it acts to activate small GTPase Ras (Herbst et al., 1999; Olivier et al., 1993; Raabe et al., 1996; Rogge et al., 1991; Simon et al., 1991, 1993). However, consistent with my findings, in vitro and in vivo studies have also demonstrated a role for SOS in activating Rac1. In cell culture studies, SOS stimulates guanine nucleotide dissociation from Rac1 but not Cdc42 (Nimnual et al., 1998). In some cases, such as axon guidance in the *Drosophila* embryo, SOS action as a Rac1 GEF is independent of Ras activation (Yang and Bashaw, 2006), but, in other situations SOS activation of Rac1 is coupled to stimulation of Ras (Nimnual et al., 1998). Based on my observation that glial expression of a dominant-negative Ras (*Ras85D^{N17}*)) only very weakly suppresses clearance of degenerating axons, SOS activation of Rac1 during glial responses to axonal injury is likely largely independent of Ras activation. The increase of DRK localization to glial membrane processes engulfing axonal debris, and the consequences of DRK depletion by RNAi all argue for an early role for DRK (and likely DOS and SOS) in engulfment events. Although DRK is expressed in neurons and glia
(see Figure 2.5), our glial-specific RNAi clearly demonstrates that DRK function is required in glia during engulfment. Western blot analysis of the brain lysates before and after axotomy suggests the increase of DRK in the glial membrane processes is likely a result of recruitment of DRK other than up-regulation of gene transcription/translation (Figure 2.7). I also found that Draper is responsible for DRK localization to the site of injury, as DRK does not localize to severed nerves in drpr null animals, suggesting DRK/DOS/SOS activation requires Draper. It seems Draper activation or Draper-mediated recruitment of DRK is necessary for DRK/DOS/SOS to execute their functions, as we found SosJC2 failed to rescue the debris clearance defect in drpr null animals (Figure 2.15). Does DRK interact directly with Draper? Despite considerable efforts, I was unable to detect physical interactions between DRK and Draper. DRK may therefore interact very transiently or indirectly with Draper or DRK might associate with another yet unknown glial receptor that localizes to degenerating axonal materials. The requirements I describe for DRK/DOS/SOS appear to be conserved in mammals in the context of phagocytic activity. Mammalian DRK (growth factor receptor-bound protein 2, Grb2) is accumulated at the phagocytic cup during leukocyte phagocytosis (Kantonen et al., 2011). Mammalian DOS (Grb2-associated binding protein 2, Gab2) also plays a critical role during Fcγ receptor-mediated phagocytosis (Gu et al., 2003). Recently, MEGF10 (mouse Draper) is expressed in mammalian astrocytes and essential for synaptic pruning during refinement of dorsal lateral geniculate nucleus connectivity (Chung et al., 2013). The work I present here also argues that DRK/DOS/SOS and Crk/
Mbc/dCed-12 are exciting candidates downstream of MEGF10 to promote synaptic pruning.
Materials and methods

Fly strains and antibodies

The following *Drosophila* strains were used: *OR85e-mCD8::GFP/CyO* (gift from B. Dickson, Research Institute of Molecular Biology, Vienna, Austria) (Couto et al., 2005), *UAS-mCD8::GFP* (Lee and Luo, 2001), *UAS-mCD4::tdTomato* (Han et al., 2011), *repo-Gal4/TM3* (Sepp et al., 2001), *tub- Gal80ts* (gift from S. Waddell, University of Oxford, Oxford, United Kingdom) (McGuire et al., 2004), *TIFR-Gal4/TM3* (gift from H. Hing, University of Illinois, Urbana, IL), *SosJC2/CyO* (gift from G.M. Rubin, Janelia Farm, Ashburn, VA), *UAS-Rac1N17* (gift from L. Luo, Stanford University, Stanford, CA) and *UAS-Ras85D* (purchased from Bloomington Drosophila Stock Center, Bloomington, IN). The following UAS-RNAi lines were from Vienna Drosophila Resource Center (Vienna, Austria): *UAS-drkRNAi#105498*, *UAS-sosRNAi#42849*, *UAS-mbcRNAi#16044* and *UAS-dCed-12RNAi#104555*. *UAS-dosRNAi#3* was generated by Johnna Doherty, who cloned daughter of sevenless (dos) cDNA fragment (nucleotide 1,629–2,112) from *Drosophila* Genomics Resource Center cDNA Stock Center clone SD02517 into pWIZ vector. Injection of pWIZ-dosRNAi into fly embryos was performed by BestGene (ChinoHills, CA) to make transgenic flies. The following fly stocks were obtained from NIG-FLY Stock Center: *UAS-drk6033R-2* and *UAS-dos1044R-3*. To study genetic interactions, the following strains were generated following standard procedure: *tub-Gal80ts*, *OR85e-mCD8::GFP/CyO*; *repo-Gal4/TM3*, *tub-Gal80ts*, *repo-Gal4*, *UAS- mCD8::GFP/TM3*, *OR85e-mCD8::GFP*, *UAS-mCD4::tdTomato/CyO* and *UAS-drkRNAi#105498*, *SosJC2/CyO*. 
Rabbit anti-DRK polyclonal antibodies (1:500) was a gift from M. A. Simon (Stanford University, Stanford, CA). Rabbit anti-Draper antisera (1:500) was raised as previously described (Freeman et al., 2003). Mouse anti-GFP monoclonal antibody (1:200) was purchased from Molecular Probes (A11120). Cy3 anti-rabbit IgG and FITC anti-mouse IgG were purchased from Jackson ImmunoResearch and used at 1:100.

**Injury protocols and adult fly brain dissection**

Standard maxillary palp and antennal ablations were performed as previously described (MacDonald et al., 2006; Wu and Luo, 2006). For experiments that required tub-Gal80$, flies were raised at 18 °C before eclosion and then transferred to 29 °C at least 5 days before injury. After injury, flies were grown at 29 °C until the day of dissection. Standard methods were used for dissection, fixation, and antibody staining of *Drosophila* adult brain (MacDonald et al., 2006). Fly brains were eventually mounted in Vectashield Mounting Medium (H-1000; Vector Labs) and stored at 4 °C in the dark before confocal microscopy analysis within 2 weeks.

**Lysotracker staining in Drosophila adult brains**

Flies were aged and injured as described in the previous section. Heads, after having been removed from the bodies, were immediately immersed and dissected in chilled PBS solution. Dissected brains were stained by LysoTracker Red DND-99 (L-7528; Molecular Probes)/PBS solution at a dilution of 1:500 at room temperature for 15 min with constant rocking, followed by five quick washes in PBS solution within 15 min, and
then fixed for another 30 min at room temperature with 4% formaldehyde/PBS solution/0.1% Triton X-100. To visualize OR85e+ axons, fixed brains were further stained with mouse anti-GFP antibody at 1:200. Mounted brains were kept in the dark for 1 h before confocal analysis and imaged on the same day to minimize the decay of LysoTracker signals.

Confocal microscopy, image analysis and statistic

Confocal microscopy settings were always kept constant throughout the same set of experiments. For axon debris clearance, brains were imaged in 0.85-µm steps with a Zeiss LSM5 Pascal confocal microscope under 63× oil objective lens. Pixel intensity of GFP or Draper immunoreactivity at each OR85e+ glomerulus was measured by using ImageJ (National Institutes of Health) as previously described (MacDonald et al., 2006). Glial membrane vesicles and LysoTracker+ puncta were detected by spinning-disk confocal microscope (Carl Zeiss) under 63× oil objective lens and analyzed in Volocity (PerkinElmer). Statistics were all carried out in GraphPad Prism 6 (GraphPad Software).
Figure 2.1. Glial clearance of axonal debris requires DRK, DOS, and SOS. (A) Glia failed to clear OR85e+ axonal debris in 5 days after axotomy in \(drk^{RNAi}\), \(dos^{RNAi}\) and \(sos^{RNAi}\) animals, and the clearance defects in \(drk^{RNAi}\) and \(dos^{RNAi}\) animals were completely rescued by a GOF allele of \(sos\), \(Sos^{JC2}\). Axons from a subset of maxillary palp (mp) ORNs were labeled by \(OR85e\)-\(mCD8::GFP\) and the integrity of axons 5 days after mp ablation (5 days after injury) was examined under confocal microscope. Representative images (z-stack) are shown. Scale bar: 30 µm. (B) Quantification of OR85e+ axonal debris remaining in the brain 5 days after injury. GFP immunoreactivity of each OR85e+ glomerulus was measured and normalized to uninjured, age-matched cohorts (as 100%). Control: \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\); \(repo-Gal4/+\). \(drk^{RNAi}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(UAS-drk^{RNAi\#105498}\); \(repo-Gal4/+\). \(dos^{RNAi}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(UAS-dos^{RNAi\#3}\); \(repo-Gal4/+\). \(sos^{RNAi}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(UAS-sos^{RNAi\#42849}\); \(repo-Gal4/+\). \(Sos^{JC2/}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(Sos^{JC2/}\); \(repo-Gal4/+\). \(drk^{RNAi}\), \(Sos^{JC2/}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(UAS-drk^{RNAi\#105498}\), \(Sos^{JC2/}\); \(repo-Gal4/+\). \(Sos^{JC2/}\): \(OR85e\)-\(mCD8::GFP\), \(tub-Gal80^{ts/}\)/\(Sos^{JC2/}\); \(repo-Gal4/UAS-dos^{RNAi\#3}\). Error bars represent SEM throughout. n.s., not significant. (*) \(P < 0.05\), (**) \(P < 0.005\), and (***) \(P < 0.0001\); one-way ANOVA and Bonferroni post hoc throughout unless otherwise mentioned.)
Figure 2.2. The depletion of DRK, DOS, and SOS in glia results in delay of axonal debris clearance after axotomy. (A) Representative images (z-stack) of OR85e+ axons from control, \(drk^{RNAi}\), \(dos^{RNAi}\) and \(sos^{RNAi}\) animals before and after mp ablation (at days 5, 10, and 20). Scale bar: 30 µm. (B–D) Quantification of axonal debris remaining in the antennal lobe after axotomy in \(drk\) RNAi (B), \(dos\) RNAi (C), and \(sos\) RNAi (D) animals (\(n = 10\) for all except \(sos^{RNAi}\) at day 20, \(n = 4\) because of poor survival of animals). Student t test. Control: \(OR85e-mCD8::GFP, tub-Gal80^{ts}/+; repo-Gal4/+\). \(drk^{RNAi}\): \(OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-drk^{RNAi\#105498}; repo-Gal4/+\). \(dos^{RNAi}\): \(OR85e-mCD8::GFP, tub-Gal80^{ts}/+; repo-Gal4/UAS-dos^{RNAi\#3}\). \(sos^{RNAi}\): \(OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-sos^{RNAi\#42849}; repo-Gal4/+\). (E) Draper protein expression does not differ between control, \(drk^{RNAi}\), \(dos^{RNAi}\) and \(sos^{RNAi}\) animals. Anti-Draper antibody was used to detect the protein level of Draper in western blot with approximately 5 dissected brains per lane. RNAi was induced for at least 5 days at 29 °C before dissection. \(\alpha\)-Tubulin was used as the internal control. Representative images are shown. The intensity of anti-Draper signals were quantified and normalized and were shown in (F) (\(n = 3\) for all). Control: \(tub-Gal80^{ts}/+; repo-Gal4/+\). \(drk^{RNAi}\): \(tub-Gal80^{ts}/UAS-drk^{RNAi\#105498}; repo-Gal4/+\). \(dos^{RNAi}\): \(tub-Gal80^{ts}/+; repo-Gal4/UAS-dos^{RNAi\#3}\). \(sos^{RNAi}\): \(tub-Gal80^{ts}/UAS-sos^{RNAi\#42849}; repo-Gal4/+\).
Figure 2.3. Alternative RNAi of *drk* and *dos* caused similar engulfment defects in adult glia. (A) The clearance assay was performed as described in Figure 2.1. Scale bar: 30 µm. (B) Quantification of data in (A). GFP immunoreactivity of injured OR85e+ glomerulus in RNAi animals was normalized to uninjured, age-matched controls (as 100%) as a result of insufficient number of RNAi animals collected during experiment (n = 10 for all). Control: *OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+.* *drk*<sup>6033R-2</sup>: *OR85e-mCD8::GFP, tub-Gal80ts/UAS-drk<sup>6033R-2</sup>; repo-Gal4/+.* *dos*<sup>1044R-3</sup>: *OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/UAS-dos<sup>1044R-3</sup>.*
Figure 2.4. Gain-of-function allele of sos ($Sos^{JC2}$) alone does not change the efficiency of glial clearance of axonal debris. (A) Representative images of OR85e$^+$ axons in control and $Sos^{JC2}$/+ animals before and 1 day after mp ablation. Scale bar: 30 µm. (B) Quantification of (A) (n = 10 for all). Student t test. Control: OR85e- mCD8::GFP, tub-Gal80$^/$/++; repo-Gal4/+; $Sos^{JC2}$/+: OR85e-mCD8::GFP, tub-Gal80$^$/Sos$^{JC2}$/; repo-Gal4/+. 

![A](image)

![B](image)
**Figure 2.5.** DRK is recruited to glial membranes surrounding the injured axons. (A)

Endogenous DRK (red, α-DRK) in glia was recruited to the severed mp nerves labeled with *OR85e-mCD8::GFP* (green, α-GFP) 1 day after axotomy. Before injury, DRK expression was evenly distributed in the SOG but not co-localized with the OR85e+ maxillary nerve. One day after mp injury, DRK expression was increased around the degenerating maxillary nerves (arrowheads), which was not seen in *drkRNAi* animals. Representative images (single slice) are shown. Scale bar: 10 µm. Control: *OR85e-mCD8::GFP, tub-Gal80^ts/+; repo-Gal4/+.* *drkRNAi: OR85e-mCD8::GFP, tub-Gal80^ts/UAS-drkRNAi#105498; repo-Gal4/+.*

(B) Endogenous DRK expression in glia was increased 1 day after axotomy. Glial membranes were labeled with mCD8::GFP by glia-specific *repo-Gal4* driver. Antennal ablation (removal of the third segment of antennae) was performed to induce a greater extent of axon degeneration in the antennal lobe (AL). Normally, thin glial membranes ensheath (arrows) the antennal lobe and each glomerulus (no injury, control). However, 1 day after antennal ablation, ensheathing glia became hypertrophy (1d after injury, dashed lines), and strong DRK immunoreactivity (red) was found in hypertrophic region of ensheathing glia (yellow), but not when *drkRNAi* was expressed by *repo-Gal4*, indicating that the increase of DRK is glia-specific.

Representative images (single slice) are shown. C, cortex. Scale bar: 10 µm. Control: *tub-Gal80^ts/+; repo-Gal4, UAS-mCD8::GFP/+.* *drkRNAi: tub-Gal80^ts/UAS-drkRNAi#105498; repo-Gal4, UAS-mCD8::GFP/+.*
Figure 2.6. Draper is required for DRK to be recruited to the severed axons.

Maxillary nerves were labeled with mCD8::GFP (green) and DRK immunoreactivity before and after injury was determined by α-DRK polyclonal antibodies (red). Compared with the increase of DRK around the severed maxillary nerves (arrows) 1 day after injury in Draper heterozygous null animal (drprΔ5/+), no detectable increase of DRK was found around the severed maxillary nerves in drpr homozygous null animals (drprΔ5).

Representative images are shown (single slice). Scale bar: 10 µm. drprΔ5/+: OR85e-mCD8::GFP/CyO, drprΔ5/TM6. drprΔ5: OR85e-mCD8::GFP/CyO; drprΔ5.
Figure 2.7. DRK protein level in the brain is unaltered 1 day after axonal injury.

(A) Western blots of adult brain lysates (yw) without injury (no injury) or 1 day after removal of the third segments of the antenna and maxillary palps (1d after injury). No significant change of DRK level was observed. α-Tubulin was used as the internal control. Approximately 5 brains were used per lane. (B) Quantification of (A). (n = 3; Student t test)
Figure 2.8. DRK-SOS activation is required for glia to internalize axonal debris. (A) Glia failed to form membrane vesicles 1 day after axotomy in drkRNAi animals, which was rescued by SosJC2. Glial membranes were labeled by repo-Gal4 driving mCD8::GFP. Without injury, glial membranes normally loosely wrap each glomerulus (delineated by dotted lines) in the antennal lobe. However, 1 day after axon injury, glial membranes invaded the field of injured glomerulus and formed enclosed membrane vesicles (arrowhead). These membrane vesicles were largely absent in animals in which DRK expression was knocked down by repo-Gal4 (drkRNAi). SosJC2 alone did not increase the number of vesicles formed 1 day after injury, but rescued the defect of glial membrane vesicle formation caused by drkRNAi. Representative images (single-slice) are shown. Scale bar: 10 µm. Control: tub-Gal80/; repo-Gal4, UAS-mCD8::GFP/+. drkRNAi: tub-Gal80/; UAS-drkRNAi105498, repo-Gal4, UAS-mCD8::GFP/+. SosJC2/+: tub-Gal80/; SosJC2, repo-Gal4, UAS-mCD8::GFP/+. SosJC2/+: tub-Gal80/; SosJC2, repo-Gal4, UAS-mCD8::GFP/+. (B) Quantification of the number of glial membrane vesicles in (A). Because there were hardly any detectable glial membrane vesicles without axon injury, we quantified only the number of vesicles in animals 1 day after axon injury (n ≥ 3 for all). (C) Internalization of OR85e-mCD8::GFP-labeled axonal debris was reduced when drk was knocked down in ensheathing glia but restored by SosJC2. Ensheathing glial membranes were labeled with mCD4::tdTomato (red) driven by TIFR-Gal4. OR85e+ axons were labeled with mCD8::GFP (green). Degenerating OR85e+ axons were often found inside glial membrane vesicles (arrows). Representative images (single-slice) are shown. Scale bar:
10 µm. Control: OR85e-mCD8::GFP, UAS-mCD4::tdTomato/+; TIFR-Gal4/+. drkRNAi: OR85e-mCD8::GFP, UAS-mCD4::tdTomato/UAS-drkRNAi#105498; TIFR-Gal4/+.

SosJC2/+: OR85e-mCD8::GFP, UAS-mCD4::tdTomato/SosJC2; TIFR-Gal4/+. drkRNAi, SosJC2/+: OR85e-mCD8::GFP, UAS-mCD4::tdTomato/UAS-drkRNAi#105498, SosJC2; TIFR-Gal4/+.

(D) Quantification of the number of glial vesicles containing GFP-labeled axonal debris 1 day after axotomy in (C). n ≥ 3 for all.
Figure 2.9. DRK-SOS is required for glia to activate phagolysosomal program. (A) Phagolysosome formation 1 day after axotomy was suppressed when drk was knocked down in ensheathing glia (drkRNAi), as assayed by LysoTracker staining. SosJC2 enhanced phagolysosomal activities (SosJC2/+), although it was unable to rescue the effect of drkRNAi on phagolysosome formation (drkRNAi, SosJC2/+). Representative images (z-stack) are shown. Scale bar: 10 µm. (B) Quantification of the amount of LysoTracker+ puncta formed in (A). n ≥ 5 for all. Control: OR85e-mCD8::GFP/+; TIFR-Gal4/+. drkRNAi: OR85e-mCD8::GFP/UAS-drkRNAi#105498, TIFR-Gal4/+. SosJC2/+: OR85e-mCD8::GFP/SosJC2, TIFR-Gal4/+. drkRNAi, SosJC2/+: OR85e-mCD8::GFP/UAS-drkRNAi#105498, SosJC2, TIFR-Gal4/+. 
Figure 2.10. Ras has little effect on glial clearance of axonal debris. (A)

Overexpression of dominant-negative Ras85D (Ras85D<sup>N17</sup>) in adult glia only mildly affected axonal debris clearance. Representative images (z-stack) are shown. Control: +/+; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/+; repo-Gal4/+. Ras85D<sup>N17</sup>; UAS-Ras85D<sup>N17</sup>/+; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/+; repo-Gal4/+ . Scale bar: 30 µm. (B) Quantification of data in (A) (n = 10 for all; Student t test).
Figure 2.11. SOS modulates Rac1 activity in glial response to axonal injury. (A) 
SosJC2 suppressed the inhibitory effects of dominant-negative Rac1 (Rac1N17) on glial 
clearance of axonal debris. Scale bar: 30 µm. (B) Quantification of data in (A). n = 10 
for all. (C) SosJC2 partially rescued Rac1N17 and increase glial membrane (red, α-Draper) 
recruitment to severed OR85e+ glomerulus (green, α-GFP) 1 day after axotomy. Scale 
bar: 10 µm. (D) Quantification of Draper immunoreactivity at OR85e+ glomerulus in 
(C). a.u., arbitrary unit. n = 10 for all. Control: OR85e-mCD8::GFP, tub-Gal80ts/+; 
SosJC2/+ : OR85e-mCD8::GFP, tub-Gal80ts/SosJC2; repo-Gal4/+ . SosJC2/+ ; Rac1N17: 
OR85e-mCD8::GFP, tub-Gal80ts/SosJC2; repo-Gal4/UAS-Rac1N17.
Figure 2.12. DRK/DOS/SOS and Crk/Mbc/dCed-12 are redundant pathways in activating glial clearance of axonal debris. (A) Simultaneous knockdown of SOS and Mbc completely blocked glial membrane (red, α-Draper) recruitment to injured OR85e+ glomerulus (green, α-GFP) 1 day after injury, whereas individual RNAi expression showed mild inhibitory effects. Scale bar: 30 µm. Control: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+. sosRNAi: OR85e-mCD8::GFP, tub-Gal80ts/UAS- sosRNAi#42849; mbcRNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/UAS- mbcRNAi#16044. sosRNAi; mbcRNAi: OR85e-mCD8::GFP, tub-Gal80ts/UAS-sosRNAi#42849; repo-Gal4/ UAS-mbcRNAi#16044. (B) Quantification of data in (A). n = 10 for all. (C) SosJC2 partially rescued the clearance defect caused by dCed-12RNAi. The clearance assay was performed as described in Figure 2.1, and the results were quantified in (D). n = 10 for all. Control: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+. dCed-12RNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/UAS-dCed-12RNAi#10455. SosJC2/+: OR85e-mCD8::GFP, tub-Gal80ts/SosJC2; repo-Gal4/+ . dCed-12RNAi: OR85e-mCD8::GFP, tub-Gal80ts/SosJC2; repo-Gal4/UAS-dCed-12RNAi#10455.
Figure 2.13. Glial activation and the clearance of axonal debris require SOS and Mbc. (A) Knocking down sos and mbc simultaneously in adult glia resulted in more severe axonal debris clearance defects compared with single RNAi. Control: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+. sosRNAi: OR85e-mCD8::GFP, tub-Gal80ts/UAS-sosRNAi#42849; repo-Gal4/+; mbcRNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/UAS-mbcRNAi#16044. sosRNAi; mbcRNAi: OR85e-mCD8::GFP, tub-Gal80ts/UAS-sosRNAi#42849; repo-Gal4/UAS-mbcRNAi#16044. Scale bar: 30 µm. (B) Quantification of data in (A). n ≥ 10 for all. (C) Glial hypertrophy was blocked in sosRNAi and mbcRNAi double RNAi animals. Glial membranes (labeled with mCD8::GFP) surrounding the antennal lobe (AL) undergo hypertrophy 1 day after antennal ablation, as indicated by arrows in control. However, glial knockdown of SOS and Mbc slightly reduced the hypertrophy, respectively, and the reduction was more severe when SOS and Mbc were knocked known simultaneously in adult glia. Representative images (single slice) are shown. C: cortex. Scale bar: 10 µm. Control: tub-Gal80ts/+; repo-Gal4, UAS-mCD8::GFP/+; sosRNAi: tub-Gal80ts/UAS-sosRNAi#42849; repo-Gal4, UAS-mCD8::GFP/+; mbcRNAi: tub-Gal80ts/+; repo-Gal4, UAS-mCD8::GFP/UAS-mbcRNAi#16044; sosRNAi; mbcRNAi: tub-Gal80ts/UAS-sosRNAi#42849; repo-Gal4, UAS-mCD8::GFP/UAS-mbcRNAi#16044.
Figure 2.14. The proposed model of glial response to and clearance of axonal debris after axon injury in *Drosophila* adult CNS. Upon axon injury, glia receive a yet unknown signal(s) to activate a series of events to clear axonal debris from the CNS, which requires Src42A to phosphorylate tyrosine residue(s) in the intracellular domain of Draper, allowing Shark to bind (Ziegenfuss et al., 2008) (step 1). dCed-6, which is required for clearance of axonal debris through an undetermined mechanism, can also be recruited (Doherty et al., 2009). After activation, glial membranes become hypertrophic and Draper is up-regulated. Meanwhile, glia start to extend their processes to the injured axons (MacDonald et al., 2006) (step 2). At this step, glia activate DRK/DOS/SOS and Crk/Mbc/dCed-12 pathways to efficiently drive RAC1 function, as knocking down each pathway results in delay of glial membrane recruitment toward the injured axons (Ziegenfuss et al., 2012 and Figure 2.12). The clearance of axonal debris is mediated by glial membrane vesicles, which enclose and internalize axonal debris (step 3). DRK/DOS/SOS and Crk/Mbc/dCed-12 contribute to the formation of glial membrane vesicles (Ziegenfuss et al., 2012 and Figure 2.8), possibly through regulating Rac1 activity. Later, the internalized axonal debris is degraded through phagolysosomal pathway (step 4), which also requires DRK, SOS, and Crk/Mbc/dCed12.
Step 1. activation

injury

Draper

axonal debris

Step 2. glial membranes recruitment

Step 3. debris internalization

Step 4. degradation of debris

RAC1

GTP

GDP

Src42A

dCed-6

DOS

DRK

SOS

ELMO

MBC

CRK

acidification
degradation
Figure 2.15. DRK/DOS/SOS requires Draper to function. (A) $Sos^{JC2}$ failed to rescue the clearance defect in $drpr$ null animals. Representative images (z-stack) are shown. Scale bar: 10 µm. (B) Quantification of (A). $n = 10$ for all. Control: $OR85e$-$mCD8::GFP/+$: $Sos^{JC2}$/+: $OR85e$-$mCD8::GFP/Sos^{JC2}$. $drpr^{A5}$/+: $OR85e$-$mCD8::GFP/+; drpr^{A5}$/+: $Sos^{JC2}$/+: $OR85e$-$mCD8::GFP/Sos^{JC2}$; $drpr^{A5}$/+. $drpr^{A5}$: $OR85e$-$mCD8::GFP/+$; $drpr^{A5}$. $Sos^{JC2}$/+: $drpr^{A5}$: $OR85e$-$mCD8::GFP/Sos^{JC2}$; $drpr^{A5}$. $drpr^{A5}$.
CHAPTER III: TRAF4 is an Adaptor Protein for Draper to Activate Downstream JNK and STAT Signaling Pathways during Wallerian Degeneration

The work presented in this chapter was conducted with the help from Jennifer M. MacDonald, who made the initial discovery, Rachel Bradshaw, who helped with S2 cell culture and co-immunoprecipitation experiments, and Amy Sheehan, who cloned TRAF4 cDNA. This chapter is in preparation to be submitted for publication in a peer-reviewed scientific journal.
Introduction

Neural injury in the central nervous system (CNS) elicits reactive gliosis, where the surrounding glia rapidly adopt a proinflammatory state, act as phagocytes, and manage post-injury events. Reactive gliosis is a hallmark of nearly all neurodegenerative diseases, but whether it is beneficial or detrimental to the CNS recovery remains elusive. Also, the identities of the molecular pathways that promote glial activation and reactive functions remain poorly defined. In this chapter I examine molecular pathways downstream of the engulfment receptor Draper in glial activation and clearance of axonal debris during Wallerian degeneration.

After axotomy in Drosophila, glia near severed axons become hypertrophic, invade the injury site, and clear axonal debris within 5 days after injury (MacDonald et al., 2006). The conserved glial receptor Draper is a central regulator of glial hypertrophy, infiltration of the injury site, and internalization and degradation of axonal debris (MacDonald et al., 2006). The transcription of Draper is increased within hours after injury (Logan et al., 2012), likely through the JNK (c-Jun N-terminal kinase) and STAT (signal transducer and activator of transcription) signaling pathways. For instance, transcriptional reporters of both STAT and dAP-1 are strongly activated in glia throughout the brain (Doherty et al., 2014; MacDonald et al., 2013). Moreover, transcriptional activation of the draper gene itself appears to require STAT, and occurs through an enhancer element that is rich in STAT and AP-1 binding sites (Doherty et al., 2014; MacDonald, 2012). Nevertheless, how glia activate JNK and STAT signaling after
injury downstream of Draper remains unclear. Either pathway could be directly downstream of Draper signaling, or in a parallel genetic pathway.

Here I provide evidence that TRAF4 (tumor necrosis factor receptor-associated factor 4) and its downstream MAP kinase kinase kinase kinase (MAP4K), MSN (*misshapen*), are important for glia to activate JNK and STAT signaling pathways in response to axon degeneration, upregulation of Draper expression, and engulfment axonal debris after axotomy. Depletion of TRAF4 or inhibition of MSN suppressed the activation of JNK signaling cascade, and in turn prevented STAT from activating downstream gene transcriptions. Intriguingly, I found that TRAF4 physically interacts with Draper and directly couples Draper to the activation of JNK signaling pathways through MSN. Thus Draper is capable of regulating both cytoskeletal changes required for engulfment of axonal debris and directly mediates glial activation of both JNK and STAT transcriptional pathways.

**Results**

During our candidate screen for genes important for glial engulfment of axonal debris during Wallerian degeneration, Jennifer M. Macdonald found that when *UAS-traf4IR*, an RNAi line targeting TRAF4 (Igaki et al., 2006), was driven by the pan-glial driver repo-*Gal4*, glia failed to clear axonal debris 5 days after maxillary palp ablation (5dpmp) compared to age-matched control animals (*Figure 3.1A and B*), suggesting TRAF4 is involved in glial engulfment pathway. Interestingly, in *traf4IR* animals, Draper
expression was reduced for ~50% even before axon injury (Figure 3.1C and D), a phenotype similar to stat92eRNAi animals in which the engulfment activity can be restored by co-expressing Draper I cDNA in glia (Doherty et al., 2014). However, when I co-expressed Draper I-HA (Draper I cDNA C-terminally tagged with HA sequence) (Figure 3.2) in glia with UAS-traf4IR, no significant rescue of axonal debris clearance was observed in 5 days after maxillary palps ablation (Figure 3.1E and F), arguing that the clearance defect caused by TRAF4 RNAi is not simply due to the insufficient amount of Draper in glia and TRAF4 is necessary for glia to clear axonal debris during Wallerian degeneration.

In Drosophila, TRAF4 has been shown to be upstream of JNK signaling pathway (Cha et al., 2003; Liu et al., 1999), which is activated in glia after axon injury and is essential for glial engulfment of axonal debris (MacDonald et al., 2013). I therefore assayed whether TRAF4 is involved in glial activation of JNK signaling pathway by measuring TRE reporter (a transcriptional reporter for dAP-1) activity in vivo using control and traf4IR animals. The TRE reporter is available with either eGFP or RFP cDNA fused with the binding sequences of AP-1 (tetradecanoylphorbol acetate response element, TRE) and is a standard assay for transcriptional activation downstream of the JNK signaling pathway (Chatterjee and Bohmann, 2012).

In the uninjured brains, very little eGFP expression was detectable from TRE-eGFP reporter. However, 1 day after antennal ablation (1dpant), which severed ~90% of the axons in the antennal lobe (MacDonald et al., 2006), robust expression of eGFP was seen surrounding the antennal lobe (Figure 3.1G), as previously reported by MacDonald
et al. (2013). The extrinsic activating signal for JNK signaling to the nucleus remains unknown. To determine whether axon degeneration was required for activation of transcriptional targets of dJNK, I assayed the activation of TRE reporter in highwire mutants, where axon degeneration is completely suppressed (Xiong et al., 2012). Remarkably, almost no TRE reporter activity was detected 1 day after antennal ablation in highwire null (hiw\textsuperscript{ΔN}) animals (Figure 3.3).

I next expressed UAS-traf4\textsuperscript{IR} in glia and found that TRE reporter activation was reduced for ~50% compared to control animals 1 day after antennal ablation (Figure 3.1G and H), arguing that TRAF4 is upstream of dAP-1 activation of transcriptional targets in glia during Wallerian degeneration. Liu et al. suggested that TRAF4 activates JNK signaling pathways by binding and then activating MSN (Liu et al., 1999), a Drosophila MAP4K which then phosphorylates the downstream MAP3K Slipper (Garlena et al., 2010). As Slipper is also one of the genes require for glia to engulf axonal debris (MacDonald et al., 2013), I therefore explored the possibility that MSN might also participate in glial responses to axon degeneration. First, I expressed dominant-negative version of MSN (msn\textsuperscript{DN}) that lacks the central proline-rich region of the gene (Houalla et al., 2005; Mathew et al., 2011) in glia, and found only ~50% of the axonal debris was cleared in 5 days after maxillary palp ablation (Figure 3.4A and B). Second, I found TRE reporter activity was strongly suppressed 1 day after antennal ablation by glial expression of MSN\textsuperscript{DN} (Figure 3.4C and D), arguing that MSN is also upstream of JNK activation in glia during Wallerian degeneration. Taken together, these
results support the notion that MSN and TRAF4 play important roles in both activating JNK signaling pathway as well as glial engulfment of axonal after injury.

The increase of Draper expression that is always found surrounding the edge of antennal lobe was absent when MSN\textsuperscript{DN} was expressed in glia (Figure 3.4E). Since glial membranes still accumulated normally around the antennal lobe (i.e. they were undergoing hypertrophy) (Figure 3.5), I conclude that blocking MSN function affects glial ability to upregulate Draper in response to axon injury. This is consistent with the previous observation that JNK activity is required for glia to upregulate Draper expression after injury (MacDonald et al., 2013). However, unlike in \textit{traf4}\textsuperscript{IR} animals, baseline levels of Draper expression were normal when MSN\textsuperscript{DN} was expressed in glia before injury (Figure 3.4C), indicating that MSN does not play a role in establishing baseline levels of Draper and therefore glial responsiveness to axotomy.

The fact that TRAF4 affects basal level Draper expression (Figure 3.1C and D) and MSN affects only injury-induced Draper upregulation (Figure 3.4E) was surprising in light of the fact that Doherty et al. recently found the transcription of \textit{draper} is under the control of the non-canonical STAT signaling pathway independent of the canonical STAT signaling receptor Domeless (fly cytokine receptor) and kinase Hopscotch (fly homolog of JAK) (Doherty et al., 2014). Moreover, three STAT92E (fly homology of STAT) binding sites were identified in the enhancer element of \textit{draper}, and both basal level of Draper expression and injury-induced Draper upregulation require STAT92E (Doherty et al., 2014). I therefore explored the possibility that TRAF4 and MSN might regulate Draper expression upstream of the STAT signaling pathway.
To quantify STAT92E transcriptional activity, I measured the amount of GFP expression from the STAT transcription reporter 10xSTAT92E-GFP (Bach et al., 2007) in uninjured adult brain lysates. Western blot analysis showed that, consistent with previous study, STAT92E is active even in uninjured adult brains, as GFP was readily detectable from the brain lysates (Figure 3.6A). However, the amount of GFP was significantly decreased in traf4IR animals (Figure 3.6B), supporting the notion that TRAF4 is required for glia to maintain STAT activity and thus the basal level of Draper expression. Does TRAF4 also affect injury-induced STAT transcriptional activity? To answer this question, I used the degradable GFP reporter 10xSTAT92E-dGFP (Bach et al., 2007) to monitor transient activation of STAT92E. In control brains, a noticeable increase of GFP expression was detected around the antennal lobe 1 day after injury (Figure 3.6C). However, glial expression of traf4IR strongly suppressed this increase of GFP expression after axon injury (Figure 3.6D), suggesting that TRAF4 is upstream of the injury-induced activation of STAT signaling pathway and the upregulation of Draper after axon injury.

Since TRAF4 activates JNK signaling cascade after injury (Figure 3.1G), I went on to test if JNK activation is also required for glia to enhance STAT activity during Wallerian degeneration. Surprisingly, when I knocked down Basket (the only fly homolog of JNK) in glia, there was very little dGFP detectable in the brain 1 day after antennal ablation (Figure 3.6C and D). Furthermore, glial expression of JNK inhibitor, Puckered, also phenocopied basketRNAi with respect to activation of STAT transcriptional reporters (Figure 3.6C and D), suggesting that during glial responses to axon degeneration, phosphorylated JNK is required for glia to activate STAT signaling
pathway. Does JNK directly activate STAT transcriptional activity or indirectly through AP-1-dependent gene transcription? To answer to this question, I further knocked down Kayak (fly homolog of Fos), one of the components of Drosophila AP-1, in glia and found the injury-induced STAT activity was decreased 1 day after axon injury (Figure 3.6C and D), suggesting that Drosophila AP-1 is important in activating STAT-dependent gene transcriptions during glial responses to axon degeneration. Taken together, the above results suggest that after axon injury, glia activate JNK signaling cascade through TRAF4 to upregulate Draper expression by increasing STAT activity, which is required for glia to clear axonal debris after injury.

Intriguingly, Doherty et al. also showed Draper is upstream of the injury-induced STAT activity, and glia failed to activate the transient 10xSTAT92E-dGFP reporter 1 day after antennal ablation in draper null (drprΔ5) animals (Doherty et al., 2014). To determine whether Draper is also upstream or downstream of JNK activation after axotomy, I compared TRE-eGFP activation after antennal ablation between wild-type (+/+) and draper null animals (Figure 3.7A). While eGFP expression was robustly increased in wild-type animals 1 day after injury, animals lacking Draper failed to activate JNK signaling cascade after antennal ablation (Figure 3.7B), suggesting that Draper is required for JNK activation after injury.

I next sought to determine whether Draper is required cell-autonomously for glial cells to activate JNK, and therefore used MARCM (mosaic analysis with a repressible cell marker) technique (Lee and Luo, 1999) to generate draper null glial cell clones in the heterozygous animal background. In these animals, cells that lose draper due to
MARCM recombination were labeled with mCD8::GFP and other GFP cells retained at least one copy of *draper*, and therefore should exhibit phenotypes similar to wild-type animals. With this technique, I could also examine JNK signaling activation and the requirement of Draper in different subtypes of glial cells around the antennal lobe, including astrocytes, ensheathing glia, and cortex glia (Doherty et al., 2009).

Astrocytes, which express little, if any, Draper after entering the adult stage, did not activate JNK signaling pathway after axotomy (Figure 3.8). On the other hand, both ensheathing (Figure 3.7C, +/+) and cortex glia (Figure 3.7D, +/+) reacted to axon degeneration by robust induction of TRE-dependent RFP expression. When I examined *draper* null glial cells clones produced by MARCM, ensheathing glia, the major phagocytic glia that undergo membrane hypertrophy and engulf axonal debris after injury (Doherty et al., 2009), failed to undergo hypertrophy and activate JNK signaling cascade in the absence of draper (Figure 3.7C, *drpr*Δ5), suggesting that Draper acts cell-autonomously in ensheathing glia during Wallerian degeneration. Moreover, to our surprise, cortex glia that were located some distance from the injured axons (i.e. not in physical contact) also failed to activate TRE reporter 1 day after antennal ablation without Draper (Figure 3.7D, *drpr*Δ5). These data argue that cortex glia are also inherently sensitive to axon injury and use Draper cell-autonomously as the major receptor to sense axon degeneration event – even when signaling at some distance. This raise the intriguing possibility that Draper might be activated at some distance by a soluble ligand released from severed axons, or that glia are coupled and capable of
responding at a population level (through Draper) and activate transcriptional targets of STAT and dAP-1.

The above findings provided an attractive model whereby Draper, the receptor, might act through TRAF4, an adaptor protein, to activate JNK signaling cascade and in turn STAT-dependent gene transcription in glia. To explore this possibility, I performed co-immunoprecipitation (co-IP) using *Drosophila* S2 cell lysates to determine if Draper and TRAF4 physically interact. Myc-tagged TRAF4 (TRAF4-myc) and HA-tagged Draper I (Draper I-HA) were co-expressed in S2 cells for 48 hours and then the cell lysates were harvested for co-IP. Lysates were precipitated by anti-HA antibody and then subjected to western blot analysis. Interestingly, I found significant levels of TRAF4-myc in the precipitates, supporting the idea that TRAF4 is physically associated with Draper (*Figure 3.9A*).

To identify the interacting region, I generated two constructs: the N-terminal TRAF4 (ZF, a.a. 1-927), which contains 7 zinc-finger domains, and the C-terminal TRAF4 (TD, a.a. 928-1458) (Liu et al., 1999; Zapata et al., 2000), which encompasses the entire TRAF domain (*Figure 3.9B*). Both constructs were C-terminally tagged with 6x myc and co-expressed with Draper I-HA in S2 cells, respectively. Co-IP experiment suggested that only the N-terminal, zinc-finger domain-enriched region was capable of binding Draper I, but not the C-terminal TRAF domain (*Figure 3.9C*), which was thought as the binding site for MSN (Liu et al., 1999). In an attempt to identify the TRAF binding sequences on Draper, I made serial deletions in the intracellular domain of Draper I (*Figure 3.9D*), and performed co-IP with full-length TRAF4-myc in S2 cells. I
found that amino acid (a.a.) 826-894 (Δ826-894) was critical for Draper I to bind TRAF4 (Figure 3.9E), but the deletion of a.a. 826-854 and a.a. 855-894, respectively did not interrupt the binding between Draper I and TRAF4 (Figure 3.10A and B). Therefore the evolutionarily conserved NPXY motif, which engulfment protein CED-6 is associated with (Su et al., 2002; Sullivan et al., 2014), is dispensable in Draper-TRAF4 interaction, consistent with the observation that dCED-6 did not participate in JNK signaling activation (data not shown). The phosphorylation of Y949 in ITAM (immunoreceptor tyrosine-based activation motif) mediated by Src42A and bound by kinase Shark (Ziegenfuss et al., 2008), was also not required for TRAF4 to bind Draper (Figure 3.10C). However both Src42A RNAi and Shark RNAi in glia interfered with TRE reporter activation 1 day after injury (Figure 3.11), suggesting Src42A and Shark are required for glia to activate JNK signaling pathway. Finally, to determine whether the binding of TRAF4 affects Draper-Shark interaction, I performed co-IP with Draper I (Δ826-894)-HA and Shark-myc, and Shark-myc was still detectable when precipitated with anti-HA antibody (Figure 3.10D), suggesting TRAF4 and Shark bind to Draper independently. Taken together, these data indicate that TRAF4 physically associates with Draper to activate the downstream JNK signaling cascade, likely through its known interaction with MSN, and in turn STAT-dependent transcription during glial responses to axon injury.
Summary and discussion

Here I use both genetic and biochemical analysis to show TRAF4 is an adaptor protein for Draper required to activate downstream pathways and engulf axonal debris during Wallerian degeneration. Knocking down TRAF4 and inhibiting the downstream MAP4K MSN function suppresses glial engulfment of axonal debris as well as the activation of JNK signaling pathway. Before injury, TRAF4 moderately activates STAT transcriptional activity to maintain basal level Draper expression. Upon axon injury, I propose that Draper-TRAF4-MSN activation turns on JNK signaling cascade, which in turn upregulates Draper expression by boosting STAT transcriptional activity. The interaction between Draper and TRAF4 is mediated through the N-terminal zinc-finger domain-enriched region of TRAF4 (a.a. 1-927) and the intracellular domain of Draper proximal to the transmembrane region (a.a. 826-894). Whether the interaction is mediated through the zinc-finger domains of TRAF4 remains to be determined. Using modified Draper I construct, I demonstrated the TRAF4-Draper interactions is independent of NPXY motif and ITAM, sequences previously identified as the binding sites for engulfment genes dCED-6 and Shark, respectively (Su et al., 2002; Ziegenfuss et al., 2008). Nevertheless, Shark is still required for glia to activate JNK signaling cascade, since knocking down Shark in glia results in a failure to induce the TRE reporter.

Knocking down Src42A, the kinase that phosphorylates ITAM and thus recruits Shark binding to Draper, also decreases TRE reporter activity after injury, implicating that the recruitment of Shark to Draper is important in JNK signaling activation in glia during Wallerian degeneration. These data are consistent with the previous observation
that injury-induced activation of STAT requires Shark (Doherty et al., 2014). Moreover, Shark has also been reported to be upstream of JNK activation in Drosophila embryonic dorsal closure and follicle cells engulfment of apoptotic cells (Etchegaray et al., 2012; Fernandez et al., 2000). Although the precise mechanism is still not clear, a simple model would be that Shark activates JNK through Rac1 (Etchegaray et al., 2012) since full activation of Slpr requires dual inputs from MSN and Rac1 (Garlena et al., 2010). Indeed, the preliminary data shows that when Rac1 activity is affected in glial cells by the expression of Rac1\(^{N17}\), a dominant-negative form of Rac1, TRE reporter activity is significantly suppressed 1 day after injury (Figure 3.11), supporting the notion that Shark is required for JNK signaling activation through Rac1 (model summarized in Figure 3.12). Recent work has demonstrated a conserved role for MEGF10, the mouse ortholog of Draper, in satellite glial cell engulfment of cell corpse (Wu et al., 2009) and astrocyte engulfment of pruned synapses (Chung et al. 2013). Moreover Syk and Src family kinases appear to activate MEGF10 through an ITAM-based signaling mechanism (Scheib et al., 2012). We therefore suspect our model for Draper-Traf4-Shark signaling to the cytoskeleton and nucleus will be functionally conserved in glial responses to neural injury in mammals.

**Materials and methods**

**Fly strains**
Drosophila strains used in this chapter of study include: OR85e-mCD8::GFP/CyO (Couto et al., 2005), repo-Gal4/TM3 (Sepp et al., 2001), UAS-traf4IR (gift from T. Xu, Yale U) (Igaki et al., 2006), UAS-drprI(24127)-HA (M. Logan, Oregon Health and Science U, unpublished) (Logan et al., 2012), TRE-eGFP-16 (Chatterjee and Bohmann, 2012), TRE-RFP(dsRed.T4)-16 (Chatterjee and Bohmann, 2012), tub-Gal80\(^\delta\), OR85e-mCD8::GFP/CyO (Lu et al., 2014), UAS-\(msn\)\(^{DN}\) (gift from M. Leptin, Cologne U, Cologne) (Mathew et al., 2011), 10xSTAT-GFP; repo-Gal4/TM3 (Doherty et al., 2014), 10xSTAT-dGFP; repo-Gal4/TM3 (Doherty et al., 2014), UAS-puckered (Bloomington Stock Center), \(drpr^{A5rec9}\) (Neukomm et al., 2014), repo-\(FLP^{6-2}\) (II) (Stork et al., 2014), FRT2A, FRT82B (III) (Neukomm et al., 2014), repo-Gal4, UAS-mCD8::GFP/TM3 (Doherty et al., 2014), \(Highwire^{\deltaN}\) (gift from C.A. Collins, U Michigan) (Wu et al., 2005), tub-Gal80\(^\delta\); repo-Gal4, UAS-mCD8::GFP/TM3 (Lu et al., 2014), and UAS-shark\(^{RNAi\#6b}\) (Ziegenfuss et al., 2008). The following Drosophila strains were generated during the study according to standard procedure: TRE-eGFP-16, tub-Gal80\(^\delta\) (II), \(drpr^{A5rec9}\), FRT2A/TM6, TRE-RFP-16, UAS-mCD8::GFP (II), tub-Gal80, FRT2A, repo-Gal4/TM3, UAS-drprI-HA, \(drpr^{A5rec9}/TM6\) and repo-Gal4, \(drpr^{A5rec9}/TM6\). UAS-src42A\(^{RNAi\#26019}\), UAS-basket\(^{RNAi\#34138}\) and UAS-kayak\(^{RNAi\#6212}\) were obtained from Vienna Drosophila Resource Center.

**Antibodies**

For primary antibodies used in this study: Mouse anti-GFP (Molecular Probes. 1:200 for IFA; 1:2000 for WB), rabbit anti-Draper (1:500 for IFA and 1:1000 for WB after pre-
absorbed with yw embryos overnight) (Freeman et al., 2003), mouse anti-α-tubulin (DM1A) (Sigma-Aldrich, 1:1000), rat anti-mCherry (Invitrogen, 1:1000), rat anti-HA (3F10) (Roche, 1:2000 for WB), mouse anti-myc (9E10) (Millipore, 1:5000), mouse anti-Highwire (6H4) (Developmental Studies Hybridoma Bank, 1:100) and mouse anti-Gat (C-term) 1:5000 (Stork et al., 2014). FITC anti-mouse IgG, Cy3 anti-rabbit IgG, Cy3-anti-rat IgG, Cy3 anti-mouse IgG and Cy5 anti-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories (all 1:100), as well as HRP (horseradish peroxidase)-conjugated anti-rat antibodies (1:6000). Both HRP-conjugated anti-mouse, and anti-rabbit IgG were from Abcam (1:6000).

**Injury protocol and adult brain dissection**

*Drosophila* cultures were kept at 25 °C except for the experiments involving *UAS-msn*\(^{DN}\) and *UAS-src42A*\(^{RNAi\#26019}\), due to the lethality they caused when overexpressed using *repo-Gal4*. Temperature-sensitive GAL80 (*Gal80\(^{ts}\)*) (McGuire et al., 2004) was used to inhibit the activity of GAL4 at 18 °C before fly eclosion and then deactivated at 29 °C for at least 5 days before injury. Flies continued to grow at 29 °C until the day of dissection. Standard maxillary palp and antennal ablations were performed as previously described (MacDonald et al., 2006). For immunocytochemistry, heads were fixed and dissected following standard procedure (Ziegenfuss et al., 2008). For western blots analysis, heads from adult flies aged at 25 °C for at least 5 days after eclosion were isolated and immediately dissected in chilled PBS. Only the central brains (without optic lobes) were collected and prepared for western blot analysis described in Muthukumar et al. (2014).
Confocal microscopy and image analysis

Confocal microscopy settings were always kept constant throughout the same set of experiments. Imaging of axonal debris clearance was performed as previously described (Lu et al., 2014). Zeiss LSM5 Pascal confocal microscope was used for all axonal debris clearance, TRE-eGFP reporter (except for UAS-msn\textsuperscript{DN}), and STAT reporter assays using Zeiss Plan-Apochromat 63x/1.4NA oil objective lens. Glial MARCM clones and TRE-eGFP reporter activity in msn\textsuperscript{DN} animals were analyzed using Zeiss Axio Imager.M2 spinning-disk confocal microscope using Zeiss C-Apochromat 40x/1.2NA water objective lens. All images collected were quantified and processed in ImageJ (National Institutes of Health), as previously described (Doherty et al., 2009).

DNA cloning and making deletions in the Draper I intracellular domains

To clone TRAF4 cDNA, forward primer containing KpnI and reverse primer containing SacII restriction site (all the primer sequences are listed in Table 3.1) were used to amplify TRAF4 cDNA from BDGP (Berkeley Drosophila Genome Project) cDNA clone LD20987. An approximate 1.6 kb cDNA was amplified by Phusion High-Fidelity DNA polymerase (New England BioLabs), purified with QIAquick PCR Purification (QIAGEN) and then digested by KpnI and SacII. Digested DNA fragment was ligated to pUAST-CT-myc vector that has 6x myc sequence (MEQKLISEEDLNE) at the C-terminus, using T4 DNA ligase (New England BioLabs). The N-terminal (ZF, a.a. 1-309) and the C-terminal TRAF domain (TD, a.a. 310-494) of TRAF4 were isolated from
pUAST-TRAF4-myc using the primers listed in Table 3.1. Amplified PCR products were digested with both BglII and SacII respectively and then ligated to pUAST-CT-myc vector. Internal deletions in the Draper I intracellular domain were generated using GeneArt® Seamless Cloning and Assembly Enzyme Mix (Life Technologies). See Table 3.1 for the exact sequences of the primers used to amplify Draper I fragments from pAc5-DraperI-HA. pAc5 vector was linearized by KpnI and XbaI, purified with QIAquick PCR Purification (QIAGEN), and then incubated with purified Draper I-HA fragments in 1x Seamless enzyme mix at room temperature (R.T.) for 15 min followed by standard bacterial transformation procedure. To make C-terminally truncated Draper I (Δ953-1101), the forward primer (containing a KpnI restriction site and the sequences matching Draper I nucleotide (nt.) 1-16), and the reverse primer (containing a XbaI restriction site, a stop codon (TAG), 1x HA DNA sequence and Draper I nt. 2838-2856) were used to amplify Draper I cDNA from pAc5-Draper I. PCR product was purified and digested with KpnI and XbaI, and then ligated to pAc5 vector. All restrict enzymes used were purchased from New England BioLabs.

**Schneider 2 (S2) cell culture and co-immunoprecipitation**

*Drosophila* S2 cells were cultured and maintained in 10% HyClone™ Fetal Bovine Serine/SFX-Insect™ Media (Fisher Scientific) with 1% Penicillin-Streptomycin solution (Sigma-Aldrich) at 25°C. For immunoprecipitation of Draper I-HA, $2 \times 10^6$ S2 cells were seeded per well in a 6-well plate the day before DNA transfection, where 100 ng of each appropriate DNA vector were mixed and incubated with Effectene Transfection
Reagents (QIAGEN), as described in manufacturer’s instruction. Two days after transfection, cells were lysed in our modified NP-40 lysis buffer (1% NP-40, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 30 mM Na$_4$P$_2$O$_7$, 50 mM NaF, 100 µM Na$_3$VO$_4$, 5 µM ZnCl$_2$ and protease inhibitor cocktail purchased from Roche) (Logan et al., 2012). Rat anti-HA monoclonal antibody (0.3 µg per sample) was incubated with Protein G beads (Sigma-Aldrich) at 4°C for 3 hrs before incubated with pre-cleared cell lysates at 4°C overnight. After washed at least 8 times to reduce non-specific binding, proteins on beads were eluted in SDS Loading Buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol and 0.01% bromophenol blue) and boiled at 100°C for 5 min, following a 2-min centrifugation at 16,000g. Supernatants were loaded into 4-15% gradient Tris-HCl ReadyGel (Bio-Rad). SeeBlue® Pre-stained Protein Standard (Life Technologies) was used (10 µl per well) to determine protein molecular weight. Samples were transferred to nitrocellulose membrane (Bio-Rad) after electrophoresis. Before incubated with corresponding primary antibodies at 4°C overnight, membrane was blocked in 5% milk/0.01% Tween-20/PBS at R.T. for 1 hour. Membrane was then washed with 0.01% Tween-20/PBS 2 times in 30 min and then probed with corresponding secondary antibodies at R.T. for 1 hr, followed by another 2 washes in 30 min. Immunoreactivity was detected using Clarity™ Western ECL Substrate (Bio-Rad) or ECL Prime Western Blotting Detection Reagent (Amersham) for TRAF4-myc after co-IP. Image acquisition and protein quantification were conducted using ChemiDoc™ MP system (Bio-Rad) and signal saturation was avoided during acquisition. All results showed were representative blots after at least 3 repeats.
Statistical analysis

All error bars represent S.E.M. Student’s t test, one-way ANOVA, and Tukey’s post-hoc analysis were carried out using GraphPad Prism 6 (GraphPad Software).
Figure 3.1. TRAF4 is necessary for glia to engulf axonal debris and activate JNK signaling cascade in response to axon injury. (A) Knocking down TRAF4 in glia (traf4IR: OR85e-mCD8::GFP/UAS-traf4IR; repo-Gal4/+; OR85e-mCD8::GFP/+; repo-Gal4/+). Maxillary nerves were labeled by OR85e-mCD8::GFP. Scale bar = 10 µm throughout. (B) Quantification of OR85e+ axon materials remained in (A). 5 days after injury. N = 10 each. ****: P < 0.0001. Student’s t. (C) Draper expression was reduced when TRAF4 was knocked down in glia, shown in western blot. Protein samples were extracted from adult central brains. α-tubulin was used as internal control. Control: repo-Gal4/+. traf4IR: UAS-traf4IR; repo-Gal4/+. (D) Quantification of (C). **: P < 0.005. Student’s t. N = 3. (E) Overexpression of Draper I did not rescue the clearance defect caused by traf4IR. Draper immunoreactivity in the cortex region was shown by anti-Draper antibody. Control: OR85e-mCD8::GFP/+; repo-Gal4/+. traf4IR: OR85e-mCD8::GFP/UAS-traf4IR; repo-Gal4/+; drprI-HA: OR85e-mCD8::GFP/UAS-traf4IR; repo-Gal4/UAS-drprI-HA. (F) Quantification of OR85e+ axon materials remained in (E). 5 days after injury. n.s.: not significant. One-way ANOVA. Tukey’s post-hoc. (G) TRE reporter activity was suppressed in TRAF4 RNAi animals (traf4IR) 1 day after antennal ablation (1dpant). Anti-GFP antibody was used to detect TRE-eGFP reporter activity. Anti-Draper antibody labeled glial membranes surrounding the antennal lobe. Control: TRE-eGFP-16/+; repo-Gal4/+. 
*traf4IR*, *TRE-eGFP-16/UAS-traf4IR; repo-Gal4/+*. (H) Quantification of the increase of eGFP immunoreactivity in (G). N = 50 each. Student’s t.
Figure 3.2. The C-terminal HA tag does not affect Draper I function in the clearance of axonal debris. (A) Draper I-HA rescued the clearance defect in the draper null (drpr<sup>Δ5</sup>) animals. In drpr<sup>Δ5</sup> animals, more than 80% of the OR85e<sup>+</sup> axonal debris was uncleared 5 days after maxillary palp ablation (5dpmp) (Lu et al., 2014; MacDonald et al., 2006). However, when UAS-Draper I-HA was driven by repo-Gal4, the majority amount of axonal debris was cleared within 5 days after injury, comparable to draper heterozygous null animals (drpr<sup>Δ5</sup>/+) where no clearance defect was found. drpr<sup>Δ5</sup>/+: OR85e-mCD8::GFP/CyO; drpr<sup>Δ5</sup>/TM6B, Tb, Hu, e. drprI-HA, drpr<sup>Δ5</sup>/+: OR85e-mCD8::GFP/+; repo-Gal4, drpr<sup>Δ5</sup>/UAS-drprI-HA, drpr<sup>Δ5</sup>. (B) Quantification of data in (A). Student’s t. N = 10 each.
Figure 3.3. Glial activation of JNK signaling cascade is specific to the degeneration event. (A) Highwire, the protein that is required for axon degeneration after axotomy, was highly enriched in the synaptic region in the antennal lobe, but not in the surrounding glial cells (repo-Gal4, UAS-mCD8::GFP/+). (B) Axotomy by antennal ablation (1dpant) dramatically induced TRE reporter activity (anti-RFP, red) in highwire heterozugous null animals (hiwΔN/+), but not in highwire null animals (hiwΔN). Glial membrane also did not undergo hypertrophy, nor was Draper increased after axotomy (anti-Draper). Dotted line delineated the edge of the antennal lobe.
Figure 3.4. MSN is required for glia to engulf axonal debris, activate JNK signaling cascade, and increase Draper expression around the antennal lobe after axotomy.

(A) The expression of dominant-negative MSN (msn$^{DN}$) suppressed glial clearance of axonal debris. Control: OR85e-mCD8::GFP, tub-Gal80$^{ts}$/++; repo-Gal4/+. msn$^{DN}$: OR85e-mCD8::GFP, tub-Gal80$^{ts}$/UAS-msn$^{DN}$; repo-Gal4/+. (B) Quantification of data in (A). Control: N = 10; msn$^{DN}$: N = 9. Student’s t. (C) TRE reporter activity was inhibited 1 day after antennal ablation by glial expression of MSN$^{DN}$. Control: TRE-eGFP-16, tub-Gal80$^{ts}$/++; repo-Gal4/+. msn$^{DN}$: TRE-eGFP-16, tub-Gal80$^{ts}$/UAS-msn$^{DN}$; repo-Gal4/+. (D) Quantification of the increase of eGFP immunoreactivity in (C). N = 30 each. Student’s t. (E) Blowups of the corresponding dashed squares in (C) showed the lack of Draper increase 1 day after antennal ablation in msn$^{DN}$ animals.
Figure 3.5. The expression of MSN$^{DN}$ did not affect glial membrane hypertrophy after axotomy. (A) Glial membranes (mCD8::GFP) were accumulated around the antennal lobe 1 day after antennal ablation in both control (tub-Gal80$^{ts}$/++; repo-Gal4, UAS-mCD8::GFP/+) and msn$^{DN}$ animals (tub-Gal80$^{ts}$/UAS-msn$^{DN}$; repo-Gal4, UAS-mCD8::GFP/+). Dotted lines delineated the edge of the antennal lobe (AL). The edge of the cortex region (C) was delineated by dash-dot lines (--) to highlight the expansion of glial membranes around the antennal lobe 1 day after antennal ablation. (B) Quantification of the amount of glial membranes surrounding the antennal lobe. Control (no injury and 1dpant): N = 6. msn$^{DN}$ (no injury): N = 4. msn$^{DN}$ (1dpant): N = 6. One-way ANOVA. Tukey’s post-hoc.
Figure 3.6. TRAF4 and JNK signaling cascade is required for STAT transcriptional activity in glia during Wallerian degeneration. (A) Knocking down TRAF4 in glia interfered with normal STAT transcriptional activity. Western blots of central brain lysates showed less GFP expression in TRAF4 RNAi animals (10xSTAT-GFP/UAS-traf4IR; repo-Gal4/+), compared to control animals (10xSTAT-GFP/+; repo-Gal4/+). (B) Quantification of data in (A). *: P < 0.05. Student’s t. N = 3. (C) Injury-induced glial STAT activation was hindered when JNK signaling cascade was blocked. STAT-mediated dGFP (degradable GFP) expression was induced 1 day after antennal ablation in control animals (10xSTAT-dGFP/+; repo-Gal4/+), but blocked in animals where JNK signaling cascade was interrupted by glial expression of traf4IR (10xSTAT-dGFP/UAS-traf4IR; repo-Gal4/+), msnDN (10xSTAT-dGFP/UAS-msnDN; repo-Gal4/+), basketRNAi (10xSTAT-dGFP/UAS-basketRNAi#34138; repo-Gal4/+), and puckeredWT (10xSTAT-dGFP/+; repo-Gal4/UAS-puckered). Knocking down kayak in glia (kayakRNAi:10xSTAT-dGFP/+; repo-Gal4/UAS-kayakRNAi#6212) also reduced the STAT-mediated dGFP expression. (D) Quantification of the average increase of dGFP immunoreactivity in (C). traf4IR: N = 30 each. basketRNAi, puckeredWT and kayakRNAi: N = 50 each. Student’s t for each group.
Figure 3.7. Draper is cell-autonomously required for glia to activate JNK signaling in response to axon degeneration. (A) Glia failed to activate TRE-eGFP reporter 1 day after antennal ablation in *draper* null (*drpr*Δ5) animals. +/+: *TRE-eGFP-16; +/+*. *drpr*Δ5: *TRE-eGFP-16; drpr*Δ5. (B) Quantification of the increase of eGFP immunoreactivity in (A). N = 30 each. Student’s t. (C) Ensheathing glial MARCM clones (arrows, green) responding to axotomy at day 1 post antennal ablation. Control clone (+/) became hypertrophic and exhibited strong TRE-RFP expression 1 day after injury, while *draper* null clone (*drpr*Δ5) remained inactive compared to the neighboring ensheathing glia in red. +/+: *TRE-RFP-16, UAS-mCD8::GFP/repo-FLP6-2; FRT2A, FRT82B/tub-Gal80, FRT2A, repo-Gal4. *drpr*Δ5: *TRE-RFP-16, UAS-mCD8::GFP/repo-FLP6-2; *drpr*Δ5, FRT2A/tub-Gal80, FRT2A, repo-Gal4. (D) TRE-RFP reporter activity in cortex glial MARCM clones (dotted line, green) 1 day after antennal ablation. Similar to ensheathing glia, cortex glial clone that lacks Draper (*drpr*Δ5) failed to induce TRE-RFP expression compared to the neighboring cortex glial cells. Animal genotypes are the same as in (C).
Figure 3.8. Astrocytes do not activate JNK signaling cascade after axon injury.

Wild-type astrocyte MARCM clone was labeled with mCD8::GFP (green). Anti-GAT antibody (blue) labeled all astrocyte processes. At day 1 post antennal ablation, no TRE-RFP reporter activation (red) was observed in astrocytes. Animal genotype: TRE-RFP-16, UAS-mCD8::GFP/repo-FLP^{6-2}; FRT2A, FRT82B/tub-Gal80, FRT2A, repo-Gal4.
**Figure 3.9. TRAF4 zinc-finger domain-enriched region physically interacts with Draper I a.a. 826-894.**  
(A) Western blots showed TRAF4-myc was associated with Draper I-HA. S2 cells, 2 days after co-transfected with TRAF4-myc and Draper I-HA, were harvested and immunoprecipitated (IP) with anti-HA antibody, followed by western blotting.  
(B) Schematic drawing of *Drosophila* TRAF4 and predicted functional domains. ZF: zinc-finger domain-enriched. TD: TRAF domain.  
(C) Draper I interacted with the ZF region of TRAF4 but not the TRAF domain, as indicated by the western blots where only ZF-myc, but not TD-myc, was identified in the samples after immunoprecipitation by anti-HA antibody.  
(D) Schematic drawing of Draper I intracellular domain deletions in the corresponding constructs. The extracellular domain was omitted (//) due to space constraint.  
(E) The deletion of a.a. 826-894 significantly interrupted the association between Draper I and TRAF4. Immnoprecipitation was performed as described in (A). All the Draper I constructs were C-terminally tagged with HA. V: vector alone.
Figure 3.10. Deletion of a.a. 826-854, deletion of a.a. 855-894, and the interruption of Y949 phosphorylation in Draper I do not affect the interaction between Draper I and TRAF4, which is independent of Draper I-Shark association. (A) Draper I deletion constructs, Δ826-854 and Δ855-894, bound TRAF4-myc equally well in the western blots from S2 cell lysates after immunoprecipitation by anti-HA antibody, suggesting these 2 regions are equally important in mediating the binding between TRAF4 and Draper I. (B) Schematic drawing of the deletions made in Draper I Δ826-854 and Δ855-894. (C) The phosphorylation of Y949 did not alter the binding ability of Draper I-HA to TRAF4-myc, indicating that the interaction between TRAF4 and Draper I is independent of Shark. (D) The deletion of a.a.826-894 in Draper I, which disrupts TRAF4-Draper I interaction, was still associated with Shark.
Figure 3.11. Src42A, Rac1 and Shark are required for glia to activate JNK signaling cascade in response to axon degeneration.  (A) Knocking down Src42A (src42ARNAi) and blocking Rac1 activity (Rac1N17) in glia suppressed TRE-eGFP reporter activation 1 day after antennal ablation.  Control: TRE-eGFP-16, tub-Gal80ts/+; repo-Gal4/+. src42ARNAi: TRE-eGFP-16, tub-Gal80ts/UAS-src42ARNAi#26019; repo-Gal4/+. Rac1N17: UAS-Rac1N17, TRE-eGFP-16, tub-Gal80ts/+; repo-Gal4/+.  (B) Quantification of the increase of eGFP immunoreactivity in (A).  Control: N = 8.  src42ARNAi: N = 7.  Rac1N17: N = 6.  One-way ANOVA and Bonferroni post hoc.  (C) Knocking down Shark in glia (sharkRNAi) almost completely suppressed the activation of TRE-eGFP reporter 1 day after antennal ablation.  Control: TRE-eGFP-16/+; repo-Gal4/+. sharkRNAi: TRE-eGFP-16/+; repo-Gal4/UAS-sharkRNAi#6b.  (D) Quantification of the increase of eGFP immunoreactivity in (C).  N = 30 each.  Student’s t.
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Figure 3.12. The proposed model of Draper-mediated JNK and STAT signaling pathway activations during glial responses to axon injury. Before injury (black arrows), TRAF4 modulates basal Draper expression through STAT (STAT92E in *Drosophila*) signaling pathway. Upon axon injury (red arrows), a yet-unknown signal(s) triggers glial responses and activates Draper. TRAF4 recruits MAP4K MSN and then in turn MSN phosphorylates MAP3K Slpr, the activation of which also requires inputs from small GTPase Rac1. Rac1 is believed to be downstream of Shark recruitment to the phosphorylated Draper intracellular ITAM, the target of tyrosine kinase Src42A. Fully activated Slpr induces JNK (Basket in *Drosophila*) phosphorylation through MAP2K (not shown here for simplicity) and eventually Kayak and Jra (Jun-related antigen) heterodimerizes to form dAP-1 to activate downstream gene transcription. The formation of dAP-1 somehow is required for the injury-induced STAT activation as the depletion of Kayak blocks injury-induced STAT activation. The upregulation of STAT transcriptional activity increases Draper expression after axon injury to facilitate the engulfment of axonal debris. TRAF4 may have an additional role during Draper-mediated engulfment although the mechanism requires further investigation.
Table 3.1. Primer sets used for this study. Restriction sites are underlined. Fw: forward primer. Rv means reverse primer.

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| pUAST-TRAF4-myc    | Fw: 5’- ttaaa
ggtcCTGTCGATGAGTTGCTTGGCAGCTGGA -3’  
Rv: 5’- aatctataagacggGATGGCAGCTGGA -3’ |
| pUAST-TRAF4(ZF)-myc| Fw: 5’- atcgagatctATGTGGAGCTGGA -3’  
Rv: 5’- aacgggattacgccGATGGCAGCTGGA -3’ |
| pUAST-TRAF4(TD)-myc| Fw: 5’- atcgagatctATGTGGAGCTGGA -3’  
Rv: 5’- aacgggattacgccGATGGCAGCTGGA -3’ |
| pAc5-DraperI(Δ826-894)-HA | a.a. 1-825:  
Fw: 5’- cccggatcgggatcCTGTCGATGAGTTGCTTGGCAGCTGGA -3’  
Rv: 5’- CTGCCACT ACGTCGGGTAGTA -3’ |
|                     | a.a. 895-1011:  
Fw: 5’- GTGAGCTGGA -3’  
Rv: 5’- gaagggcctctacgTTCGTAATCTCGG -3’ |
| pAc5-DraperI(Δ855-933)-HA | a.a. 1-854:  
Fw: 5’- cccggatcgggatcCTGTCGATGAGTTGCTTGGCAGCTGGA -3’  
Rv: 5’- TCGTGGTA GTGAAGTTGCTTGGCAGCTGGA -3’ |
|                     | a.a. 934-1011:  
Fw: 5’- ACTTCGAC TACGAGCATCGAAGCTTGGCAGCTGGA -3’  
Rv: 5’- gaagggcctctacgTTCGTAATCTCGG -3’ |
| pAc5-DraperI(Δ895-993)-HA | a.a. 1-894:  
Fw: 5’- cccggatcgggatcCTGTCGATGAGTTGCTTGGCAGCTGGA -3’  
Rv: 5’- AGCGTGGT GGATTTGCTGACGTCTCGG -3’ |
|                     | a.a. 994-1011:  
Fw: 5’- ATCGCAGATCGGCTTGGCAGCTGGA -3’  
Rv: 5’- gaagggcctctacgTTCGTAATCTCGG -3’ |
| pAc5-DraperI(Δ953-1011)-HA | Fw: 5’- atcgagatctATGTGGAGCTGGA -3’  
Rv: 5’- aatctataagacggGATGGCAGCTGGA -3’  
Rv: 5’- CTAAGCGTAATCTCGGACGTGATCTCGTATCAGC -3’ |
CHAPTER IV: Summary and Discussion

In this dissertation, I provide experimental evidence that DRK/DOS/SOS and TRAF4-MSN are two important molecular pathways mediating glial responses to axon injury during Wallerian degeneration. DRK/DOS/SOS act together with Crk/Mbc/dCed-12 to activate small GTPase Rac1 and drive glial membrane remodeling during glial engulfment of axonal debris. TRAF4 is a Draper-binding protein that bridges Draper to the activation of downstream signaling pathways via MAP4K MSN. A primary role for Draper-TRAF4 interactions is transcriptional activation downstream of Draper through STAT and dAP-1. In addition, since overexpression of Draper I in a TRAF4 background did not rescue loss of TRAF4 (though it does after depletion of STAT or dJNK), TRAF4 also likely plays additional roles in the engulfment process directly. Since mammalian glia also use Draper pathway homologs to clear neural debris (Chung et al., 2013; Scheib et al., 2012; Sullivan et al., 2014; Wu et al., 2009), this dissertation thus provides many exciting new molecules for researchers using mammals to study glial activation and clearance during axon degeneration. Below I will revisit the data presented in previous chapters, discuss in details about the potential of these findings as well as the experimental limitations, and provide advice for future investigators who wish to pursue this study.

The redundancy of DRK/DOS/SOS and Crk/Mbc/dCed-12 in Rac1 activation

In Chapter II, I identify DRK/DOS/SOS as the upstream GEF for Rac1 during glial engulfment of axonal debris. Before this study, DRK/DOS/SOS have been known to be
downstream of RTK Sevenless to activate small GTPase Ras during *Drosophila* eye development (Herbst et al., 1999; Olivier et al., 1993; Raabe et al., 1996; Rogge et al., 1991; Simon et al., 1991, 1993). DRK acts as an adaptor protein to bind the phosphorylated tyrosine residue in Sevenless, and recruits DOS and SOS, where SOS stimulates the release of GDP from Ras, binding to GTP, and thereby Ras activation (Feller et al., 2002; Moressis et al., 2009; Raabe et al., 1995; Simon et al., 1993). However, biochemical analysis has also shown that the N-terminal region of SOS can catalyze the GTP-GDP exchange of Rac1 (Nimnual et al., 1998), providing the possibility that the assembly of DRK/DOS/SOS complex also leads to Rac1 activation. Interestingly, the study of FcγR-mediated phagocytosis reveals that mammalian homologs of DRK and DOS, Grb2 and Gab2 respectively, are critical for macrophage phagocytosis of opsonized particles (Gu et al., 2003; Kantonen et al., 2011), the cellular process that requires Rac1 (Massol et al., 1998).

In our olfactory nerve injury model, glia require activated Rac1 to induce membrane hypertrophy and recruit membranes to the degenerating axons after axotomy in order to clear axonal debris, and Crk/Mbc/dCed-12 has been implicated as one of the GEF complexes upstream of Rac1 activation (Ziegenfuss et al., 2012). However, unlike the overexpression of the dominant-negative Rac1 or *UAS-rac1RNAi*, loss of Crk/Mbc/dCed-12 only results in a delay rather than a complete blockade of glial membrane recruitment to the degenerating axons, implying that there is an alternative GEF that is upstream of Rac1 and redundant to Crk/Mbc/dCed-12 for recruitment of glial membranes to the injury site (Ziegenfuss et al., 2012). The results I presented in Chapter
II have provided convincing evidence that DRK/DOS/SOS is the GEF that acts redundantly with Crk/Mbc/dCed-12 during glial engulfment of axonal debris. First, knocking down DRK, DOS, and SOS in glia delayed the clearance of axonal debris (Figure 2.1 and 2.2). Second, the gain-of-function allele of SOS partially rescues the clearance defects caused by dominant-negative Rac1 as well as dCed-12 RNAi (Figure 2.11 and 2.12). Third, knocking down both SOS and Mbc at the same time blocks glial membrane recruitment to the injured glomeruli, which phenocopies glial expression of the dominant-negative Rac1 (Figure 2.12). It is interesting to note that redundancy of engulfment/phagocytic machinery is strongly conserved from worms to mammals (Reddien and Horvitz, 2004; Stuart and Ezekowitz, 2008), which underlines the biological importance of the efficient removal of the dying cells from a living organism.

Are there more GEFs upstream of Rac1 other than DRK/DOS/SOS and Crk/Mbc/dCed-12? There are ~22 Rho family-specific GEFs predicted in the Drosophila genome (Bashaw et al., 2001). Thus it is reasonable to suspect that there are more than two GEFs upstream of Rac1 in glia. The data presented in this study are insufficient to answer this question because even though the complete blockage of glial membrane movement by sosRNAi and mbcRNAi phenocopies Rac1DN at day 1 post injury, data from later time points has to be collected to know if sosRNAi and mbcRNAi completely suppress glial membrane activity or only cause a delay in activation. The former result would argue in favor of the idea that DRK/DOS/SOS and Crk/Mbc/dCed-12 are the only two GEFs upstream of Rac1. However the later result must be interpreted with caution because the efficiency of neither RNAi stain has been tested directly as antibodies are not
available for these molecules, and a delayed phenotype can be simply due to lower expressions of SOS and Mbc in glia during Wallerian degeneration. To get a clear answer to this question, genetic analysis in the null background is necessary. However both sos and mbc null alleles result in premature death of the animals, rendering genetic analysis in adult animals difficult.

The role of DRK in glial engulfment of axonal debris

DRK is considered to be an adaptor protein that functions through binding to the activated receptor and recruiting the downstream effectors. The protein structure of DRK contains two SH3 (Src homology 3) domains at the N- and the C- terminus respectively, flanking a central SH2 domain. The N-terminal SH3 domain binds to the proline-rich domain in SOS (Olivier et al., 1993; Raabe et al., 1995; Simon et al., 1993), and the C-terminal SH3 domain serves as the binding region for DOS (Feller et al., 2002). The central SH2 domain of DRK is known to interact with the phosphotyrosine of the activated RTK Sevenless. The interaction brings DOS and SOS close to the membrane, which is an important step for SOS to function (Aronheim et al., 1994). Consistent with this working model, in Chapter II, I show that DRK is enriched with glial membranes surrounding the injured axons (Figure 2.5), suggesting DRK functions similarly in glial engulfment of axonal debris, which is to bring DOS and SOS to the site of action. This enrichment of DRK is absent in drpr null animals (Figure 2.6), suggesting the activation of DRK/DOS/SOS requires Draper. Since Draper contains many phosphorylated tyrosine residues in the intracellular domain (Ziegenfuss et al., 2008), it will be
interesting to explore whether DRK physically associates with Draper. I was unable to
detect any interaction between DRK and Draper in co-IP experiments using *Drosophila*
S2 cells co-expressing DRK-myc and Draper I-HA. However, the biochemical
interaction between Draper and DRK may be too weak for the co-IP condition to detect,
or require activation through some pathway that is lacking in S2 cells. Yet another
plausible explanation is that DRK interacts with another membrane receptor during glial
responses to axon injury. In fact, recently Grb2 is reported to bind MERTK (c-mer
proto-oncogene tyrosine kinase) (Shelby et al., 2013), which is one of the two most
critical engulfment receptors in mouse astrocytes to eliminate synapses during CNS
development (Chung et al., 2013). MERTK would therefore be a potential candidate as
the upstream receptor of DRK. There is no clear MERTK homolog/ortholog in
*Drosophila* reported yet, but the BLAST (basic local alignment search tool) result
comparing MERTK protein sequence (NP_032613.1) with annotated *Drosophila*
peptides suggests insulin-like receptor (InR) has the best sequence similarity to MERTK.
Encouragingly, knocking down InR in glia using RNAi indeed results in axonal debris
clearance defect (Dr. Mary A. Logan, personal communication).

**The role of DOS in glial engulfment of axonal debris**

DOS is an evolutionarily conserved protein that functions as a scaffold for other
downstream effectors to be recruited to the activated RTK (Abbeyquaye et al., 2003). It
closely associates with DRK C-terminal SH3 domain through two proline-rich sequences
(PxxRxxKP). Mutations that disrupt these proline-rich sequences eliminate DOS
function during Sevenless signaling, suggesting DRK-DOS interaction is critical to stimu-
late downstream Ras activity (Feller et al., 2002). Consistent with this working model, knock-
ding down DOS in glia using RNAi resulted in similar engulfment defect as \( drk^{RNAi} \) (Figure 2.1-2.3). Moreover, the engulfment defect caused by \( dos^{RNAi} \) is completely rescued by having one copy of GOF SOS in the background (Figure 2.1), suggesting that during glial engulfment, DOS functions together with DRK through SOS-dependent mechanism. To explore this possibility further and the cellular basis of DOS function, it would be important to test if both \( dos^{RNAi} \) strains (\( UAS-dos^{RNAi\#3} \) and \( UAS-dos^{1044R-3} \)), when expressed in glia, phenocopy \( drk^{RNAi} \) in vesicle formation as well as phagolysosome formation. Previous studies show that when activated by Sevenless, DOS is phosphorylated on tyrosine residues and changes its subcellular localization (Herbst et al., 1996; Raabe et al., 1996). It is thus worth exploring whether DOS is also recruited to glial membranes at the injury site in the same manner as DRK. Anti-DOS antibodies were made before, but were not available during my study (Raabe et al., 1996). One may consider generating anti-DOS antibody following the original design to define the precise localization dynamics for DOS activity during Wallerian degeneration.

Currently there is no evidence suggesting DOS directly acts on SOS, not clarity on the mechanism by which DOS stimulates small GTPase Ras activity during Sevenless signaling. It is possible that DOS indirectly modulates Rac1 activity through other signaling pathways, and when DOS is depleted from glia, GOF SOS is able to compensate other signaling pathways by increasing Rac1 activity. There is strong evidence that DOS interacts with phosphotyrosine phosphatase Corkscrew (CSW) during
Sevenless signaling to activate Ras and promote photoreceptor development (Herbst et al., 1996). However, during glial engulfment of axonal debris, CSW is found to participate in terminating glial responses to axon degeneration, rather than acting as a positive regulator of engulfment. Glial knockdown of CSW does not affect glial clearance of axonal debris (Logan et al., 2012), and hence CSW is unlikely to be the alternative pathway to modulate SOS or Rac1 activity in this scenario. Nevertheless, DOS is a multi-adaptor protein and highly phosphorylated on tyrosine residues when expressed in S2 cells (Herbst et al., 1996), making it a good binding partner for many SH2-containing proteins. A small RNAi screen targeting potential binding partners of DOS (Friedman et al., 2011; Guruharsha et al., 2011) may be able to shed light on how DOS modulates SOS or Rac1 activity during engulfment in the future.

The role of SOS in glial engulfment of axonal debris

Although SOS was initially discovered as the GEF activating small GTPase Ras, later it was shown that the N-terminal region of SOS contains a DH domain capable of activating Rac1 (Nimnual et al., 1998). Indeed, knocking down SOS in glia with RNAi partially phenocopies Rac1 RNAi and the overexpression of dominant-negative Rac1: there was decreased glial membrane recruitment to the injured glomerulus (Figure 2.12) and more axonal debris lingered in the brain 5 days after maxillary palp ablation (Figure 2.1-2.2). Interestingly, one copy of GOF sos allele enhanced Rac1-mediated glial clearance of axonal debris when Rac1 activity was reduced (Figure 2.11). Given the fact that Ras had little effect on glial clearance of axonal debris (Figure 2.10) and the
inhibition of Rac1 activity suppressed glial activity after injury almost 100% (Ziegenfuss et al., 2012), it is plausible that SOS acts directly as the Rac1 GEF during glial clearance of axonal debris based on the principle of Occam's razor. However to obtain more experimental evidence, one should examine if SOS and Rac1 are co-localized during glial engulfment of axonal debris by immunohistochemistry or perform epistasis analysis to look if $Sos^{JC2}$ fails to rescue $drk^{RNAi}$ and $dos^{RNAi}$ in $rac1$ null animals or if gain-of-function $rac1$ allele rescues loss-of-function $sos$ regarding glial clearance of axonal debris. The alternative model is that SOS activates other small GTPases that can functionally compensate for Rac1 to promote glial engulfment activity. One of the potential small GTPase that can compensate Rac1 function is $cdc42$, which is also thought to be important for phagocytosis (Massol et al., 1998). However, biochemical analysis suggests SOS does not stimulate guanine nucleotide dissociation from Cdc42 (Nimnual et al., 1998). Therefore it is unlikely that SOS acts on Cdc42 during glial engulfment. The other possible candidates are the other two Rac-like proteins in $Drosophila$, Rac2 and Mtl (Mig-two-like). Whether SOS can stimulate the disassociation of GDP from Rac2 or Mtl remains to be determined. A pilot experiment to test if Rac2 or Mtl functions in glia to promote engulfment by expressing dominant-negative versions of these two proteins in glia may help to determine if Rac2 or Mtl is also involved in glial engulfment during Wallerian degeneration.

Understanding the nature of $Sos^{JC2}$, the GOF allele of $sos$ that rescues $Rac1^{N17}$ phenotypes, may help to illuminate the mechanism of SOS-mediated activation of Rac1. Surprisingly, the molecular lesion in $Sos^{JC2}$ remains unidentified. $Sos^{JC2}$ was produced
by ethyl methanesulfonate (EMS)-induced mutagenesis during an attempt of finding
genes downstream of Sevenless (Rogge et al., 1991). Western blot analysis suggested
$Sos^{JC2}$ is an overactive form of SOS instead of an allele overexpressing the wild-type
SOS peptide (Karlovich et al., 1995). The activity of $Sos^{JC2}$ is dose-dependent, as two
copies of $Sos^{JC2}$ suppress hypomorphic sevenless phenotype twice as efficiently as
compared to one copy (Rogge et al., 1991). It would thus be interesting to test whether
two copies of $Sos^{JC2}$ rescue $Rac1^{N17}$ and $dCed-12^{RNAi}$ better than merely one copy.
Though admittedly, the interpretation of this result is hindered by too many variables,
such as the tissues of interest, the strength of the alleles ($UAS-Rac1^{N17}$ vs. $UAS-dCed-
12^{RNAi}$ vs. hypomorphic sevenless), the nature of the signaling pathway (Crk/Mbc/dCed-
12 also contributes to Rac1 activation in glia compared to SOS-specific Ras activation in
photoreceptor cells), etc. Hence the best way to understand how $Sos^{JC2}$ rescues the Rac1
phenotype would be to purify and sequence $Sos^{JC2}$ transcript to identify the mutation(s) in
$Sos^{JC2}$.

The role of TRAF4 in glial engulfment of axonal debris

In Chapter III, I present the data supporting the notion that TRAF4 is a novel adaptor
protein for Draper required to activate the downstream JNK signaling cascade in glia
after axon injury. Co-IP results indicate the interaction between TRAF4 and Draper is
mediated through the zinc finger (ZnF) domains-enriched region of TRAF4 (Figure 3-
9C). Whether these ZnF domains are required for Draper-TRAF4 interactions in vivo
remains to be determined. ZnF domains typically are found in transcription factors that
bind DNA to regulate gene transcriptions. However increasing evidence suggest ZnFs can mediate protein-protein interactions as well (Brayer and Segal, 2008; Gamsjaeger et al., 2007). In contrast to the well-conserved TRAF domain, the functions of the ZnF domains found in almost every TRAF protein family member have been understudied. It would therefore be interesting to explore precisely how the ZnFs of TRAF4 interact with residues of Draper I by more detailed site-directed mutagenesis.

TRAF4 provides the first direct link between the Draper engulfment receptor and transcriptional activation of target genes in glia after axotomy. The data that supports this model comes mainly from the RNAi assays, where expressing UAS-traf4IR in glia causes glial clearance defect, inhibition of TRE reporter activation, and suppression of injury-induced STAT transcriptional activity. However, the off-target effect of RNAi has always been a concern when investigating gene function. To address this concern, I have tested different UAS-RNAi constructs currently available, including UAS-traf43048R (targeting nt. 12-511), UAS-traf4RNAi#110766 (nt. 373-1071), UAS-traf4GLC01843 (nt. 471-982) and UAS-traf4RNAi#21214 (nt. 1036-1374), and only UAS-traf43048R exhibited similar effects on glial clearance of axonal debris compared with UAS-traf4IR (nt. 1-521) when expressed in glia (data not shown). It does not clear the concern of off-target effect since 98% of nucleotide sequences that UAS-traf4IR and UAS-traf43048R target are identical. The other way to address this concern is to measure glial clearance efficiency or TRE reporter activation in glia after axon injury in traf4 null animals. Since TRAF4 null animals do not survive into adulthood, I generated traf4 null (traf412) glial MARCM clones (Wang et al., 2006) and assayed for TRE reporter activation 1 day after antennal
ablation. The preliminary data suggests that TRE reporter induction after injury may be attenuated in *traf4* null ensheathing glial clones, which remains to be determined by thorough quantifications. However, most of the *traf4* null cortex glial clones showed normal induction of TRE reporter 1 day after antennal ablation (data now shown), suggesting TRAF4 is not cell-autonomously required for cortex glia to activate JNK signaling pathway or *traf4* null cortex glial clones develop a compensatory pathway to compensate for the loss of TRAF4 during development. Therefore, TRAF4 proteins and RNA levels should be determined to verify the RNAi efficiencies of all the UAS-RNAi constructs tested above.

The TRAF domain of TRAF4 at the C-terminus is known to bind to the central proline-rich domain of MSN and thereby activates JNK (Liu et al., 1999). Consistent with this finding, I also showed that MSN was required for JNK activation in glia after axon injury (Figure 3.4). This observation fits into the model proposed in Figure 3.12, where TRAF4 binds Draper using the N-terminal ZnF region after axon injury and brings MSN to the site of action with the C-terminal TRAF domain. In my hands, TRAF4 is associated with Draper I in S2 cells without axon injury stimulation. This was also found to be true of Shark-Draper interactions, as overexpression of receptors in S2 cells often leads to some basal level of activation. Whether TRAF4 binds Draper in the absence of an injury signal remains to be determined. Unfortunately, molecules directly capable of activating Draper signaling by application of S2 cells remain to be identified.

TRAF4 appears to modulate Draper levels even before injury (Figure 3.6A and B). Interestingly, TRAF4 does not seem to regulate basal level Draper through MSN and
other JNK signaling pathway component, as when I and MacDonald et al. interrupted MSN and JNK kinase activities, basal levels of Draper expression did not differ from control animals (Figure 3.4 and MacDonald et al., 2013). This could indicate that non-transcription-based signaling downstream of Draper (through GEFs, and cytoskeletal components) is required to modulate baseline Draper levels and this involves TRAF4. Alternatively, other unidentified effector proteins might be involved in regulation of basal Draper levels but this will require further investigations.

Unlike previous studies (Doherty et al., 2014; MacDonald et al., 2013), simply supplying functional Draper I in glia does not rescue the engulfment defect caused by \textit{traf4IR} (Figure 3.1E and F). This result suggests in addition to activating JNK signaling cascade, TRAF4 also functions in a non-transcriptional role, likely during internalization and degradation of axonal debris. To date, there has been no study yet that links TRAF4 to phagocytosis. However, mammalian homolog of TRAF4 binds to phosphatidylinositol 3,4,5-trisphosphate (\textit{PI[3,4,5]P(3)}) and phosphatidylinositol 3-phosphate (\textit{PI[3]P}) with the highly conserved TRAF domain \textit{in vitro} (Rousseau et al., 2013). Both \textit{PI[3,4,5]P(3)} and \textit{PI[3]P} are important phosphoinositides required for phagocytosis (Gillooly, 2001). Further exploration of how TRAF4 regulates engulfment/phagocytosis through interacting with membrane phospholipids could prove extremely interesting.

The role of MSN in glial engulfment of axonal debris

MSN is a MAP4K that can phosphorylate downstream MAP3Ks to activate JNK. In Chapter III, I show that when MSN function is interfered in glia with dominant-negative
version of MSN (UAS-msn\(^{DN}\)), glia failed to clear axonal debris in 5 days, to induce JNK signaling cascade in 1 day, and to up-regulate Draper expression after axotomy. The dominant-negative version of MSN used in this study lacks the central proline-rich region (a.a. 330-768) that interacts with TRAF and Dock (Liu et al., 1999; Ruan et al., 1999). When overexpressed in Drosophila eye, UAS-msn\(^{DN}\) blocks photoreceptor cell nuclear migration, which requires normal function of MSN (Houalla et al., 2005). The exact mechanism of how this msn\(^{DN}\) allele interferes with wild-type MSN function is not clear yet. However, another version of msn where the central proline-rich region is also deleted (a.a. 332-667) and which therefore is unable to bind TRAF4 and Dock, can activate JNK constitutively in cell culture and rescue the dorsal closure defect in msn mutants (Su et al., 2000). One possible explanation of these two seemingly contradictory results is that msn constructs lacking the central regulatory region render MSN kinase constitutively active when overexpressed, and then prematurely activate the inhibitory feedback loop of JNK signaling cascade, for example, increasing the amount of Puckered. Interestingly, when overexpressing wild-type MSN in glia, I also observed axonal debris clearance defect, suppression of TRE reporter and inhibition of injury-induced STAT transcriptional activity after axotomy (data not shown). To test this hypothesis, one can perform biochemical assay to see if the Msn\(^{DN}\) protein phosphorylates JNK in cell culture or measure Puckered expression in vivo when UAS-msn\(^{DN}\) is expressed. Since UAS-msn\(^{DN}\) when overexpressed may exert neo-functional outcome that affects the interpretation of the model proposed in this dissertation, the result from the experiments
discussed above would help to address if MSN is indeed upstream of glial clearance of axonal debris, as well as JNK and STAT signaling activation after axon injury.

MSN has been implicated in playing roles in embryonic dorsal closure, cell migration, and axon targeting that involve cell shape change and membrane reorganization (Ruan et al., 2002; Su et al., 1998, 2000; Treisman et al., 1997). However, I did not observe any morphological abnormality from glial cells expressing MSN\textsuperscript{DN} and glia expressing MSN\textsuperscript{DN} underwent hypertrophy normally when compared to control animals (Figure 3.5), indicating that MSN in glia functions mainly on activating JNK in the cytoplasm, possibly through MAP3K Slipper, but not on membrane movement.

The cross talk between JNK and STAT signaling pathway

It is very interesting that in glial responses to axon injury, JNK signaling activation is required for subsequent STAT-dependent transcriptions. Both Basket RNAi and overexpression of Puckered inhibited the activation of STAT reporter 1 day after axotomy (Figure 3.6), suggesting JNK kinase activity is required for glia to activate STAT-dependent transcriptions. The formation of dAP-1 complex downstream of JNK activation is important for STAT to activate downstream transcriptions since when Kayak, one of the components of dAP-1, is knocked down in glia, STAT reporter activity was also suppressed (Figure 3.6). How does dAP-1 affect STAT-dependent transcriptions? There are several possible mechanisms. For example, dAP-1 can indirectly increase the expression of other genes that affect STAT transcriptional activity, which remains to be identified in future studies (see below). Another possibility is that
dAP-1 may bind to DNA sequences near the chromosomal position where the STAT reporter is inserted and therefore affects the transcriptional activation of the STAT reporter. In this case, an alternative transgenic fly strain that bears STAT reporter in different chromosome arm would be useful to test if knocking down Kayak in glia still inhibits STAT reporter activation 1 day after axotomy. In my literature search, there has not been any study clearly showing how AP-1 formation affects STAT transcriptional activity. However, similar cross-talk between JNK and STAT signaling pathway has been reported in cancer cell lines (Kim et al., 2010; Liu et al., 2006; Turkson et al., 1999). One of the proposed molecular mechanisms is that JNK phosphorylation of the serine residues on STAT can further enhance STAT transcriptional activity and promote oncogenesis (Turkson et al., 1999). It is therefore possible that in glial responses to axon degeneration, STAT92E is phosphorylated by JNK to increase transcriptional activity. To test this hypothesis, anti-phospho-STAT on serine residues would help to elucidate the mechanism of JNK-dependent activation of STAT transcriptional activity during glial responses to axon injury.

The axon injury signal and Draper-mediated glial activation

Data from Chapter III suggests Draper is upstream of injury-induced JNK and STAT signaling activation in glia (Figure 3.7 and Doherty et al., 2014). Before axon injury, Draper is highly expressed in ensheathing and cortex glia. JNK cascade activity is extremely low in the uninjured brains based on reporter levels. When axons begin to degenerate, Draper-expressing glial cells are activated – membranes undergo
hypertrophy, JNK and STAT transcriptional signaling cascades are engaged, and Draper expression is increased to clear degenerating axons (Figure 3.3). What do glia sense from degenerating axons to activate Draper-mediated engulfment of axonal debris? We have known from previous studies and this work that the signal(s) from degenerating axons is able to spread a certain distance to alert glia that do not physically in contact with the degenerating axons, i.e. cortex glia. This notion is supported by activation of the TRE reporter, STAT reporter, and Draper enhancer reporter throughout the brain after axonal injury of only ORNs in the antennal lobe (Doherty et al., 2014; MacDonald et al., 2013 and Chapter III of this dissertation). The intensity of glial transcriptional responses also appeared to be scaled accordingly with the severity of the injury. Doherty et al. compare the number of glia activated in animals where one maxillary palp (60 ORNs), two maxillary palps (120 ORNs), one antennae (600 ORNs), or two antenna (1200 ORNs) are ablated, and found the number of glia activated was tightly correlated with the number of axons injured (Doherty et al., 2014), suggesting the strength of the injury signal(s) is in proportion to the amount of axons degenerating. It is thus reasonable to surmise that the degenerating axons produce signal(s) that alerts the surrounding glial cells to respond to axon injury. In vertebrates, ATP has been suggested as the activating signal to induce microglial responses towards laser-induced acute brain injury (Davalos et al., 2005; Sieger et al., 2012). However, the homolog of purinergic receptor in Drosophila remains to be identified and characterized, especially P2Y receptors that vertebrates microglia use to mediate ATP-induced injury responses (Haynes et al., 2006; Sieger et al., 2012).
My MARCM analysis where I showed that *draper* null clones at a distance from the injury site fail to activate the TRE reporter indicates that Draper is cell-autonomously required for glia to be activated, at least in terms of activating JNK signaling pathway (Figure 3.7C and D). Does Draper directly sense the signal coming from degenerating axons? One reported ligand for Draper is Pretaporter (Prtp) during Draper-mediated apoptotic cell engulfment (Kuraishi et al., 2009), but no evidence supports a role for Prtp in Wallerian degeneration. In addition, Wallerian degeneration and apoptosis appear to be entirely different cellular processes and use different molecular machineries (Osterloh et al., 2012), suggesting that different ligands might be produced in each context. Hence the ligand for Draper during Wallerian degeneration is still not clear, but could be a protein, lipid, sugar, change in ions, reactive oxygen species, or none of the above. There also remains the possibility that activation of Draper is accomplished by association with a co-receptor that dictates engulfment target specificity, and that Draper is a widely used receptor to drive a common engulfment process. This would be consistent with the observation that Draper is required for engulfment of bacteria, which would likely present a very different “eat me” cue. A comprehensive genetic screen looking for genes that do not affect axon degeneration and Draper function but prevent glia from being activated or recognizing severed axons could help to solve this mystery.

**Gliial subtypes and their responses during Wallerian degeneration**

Among the three different subtypes of glial cells found around the antennal lobe region, only ensheathing glia are responsible for clearing axonal debris after injury (Doherty et
al., 2009). However, later studies suggest that cortex glia also respond to axon injury by activating intracellular signaling transduction pathways (Doherty et al., 2014; MacDonald et al., 2013). What do cortex glia do during Wallerian degeneration? I have investigated several transcription reporters that are known to be downstream of JNK and STAT signaling activation, including *drosomycin*, *decapentaplegic*, and *unpaired*, but have not observed any significant increase in their transcription after axon injury (data not shown). Cortex glia-specific TRAP (translational ribosome affinity purification) that can isolate cell type-specific transcripts associated with ribosomes (Heiman et al., 2014) would allow for the comparison of the enriched transcripts in cortex glia before and after injury, which may help to elucidate the roles of cortex glia during axon injury. Adult astrocytes also do not engulf axonal debris or become activated (Doherty et al., 2009) and I did not detect any signs of JNK signaling activation in astrocytes during this study. In line with the above observations, Draper appears to not be expressed in adult astrocytes (Doherty et al., 2009). However it remains an open possibility that astrocytes respond to axon degeneration potentially signaling to ensheathing or cortex glia to help mediate engulfment events and brain recovery after injury.

It is interesting to note that similar to mammalian glia, different subtypes of *Drosophila* glial cells also display subtype-specific responses and hence perform unique functions during Wallerian degeneration. As mentioned in Chapter I, microglia phagocytose axonal debris during Wallerian degeneration (Bignami and Ralston, 1969; Ludwin, 1990b). In *Drosophila* CNS, this function is performed by ensheathing glia in our olfactory nerve injury model. However, mammalian microglia are differentiated...
from hematopoietic stem cells and hence have mesodermal origin, which is distinct from neurons, astrocytes and oligodendrocytes (Chan et al., 2007). On the other hand, *Drosophila* ensheathing glia and astrocytes are originated from the same progenitor cells (Omoto et al., 2015). Therefore ensheathing glia are unlikely to be the ancient prototype of microglia, but can be considered to be the counterpart performing similar functions, which again emphasizes the basic need for animals to clear degenerating axons and cell corpses from the CNS after injury. Interestingly, the molecular machinery *Drosophila* ensheathing glia employ to engulf axonal debris is conserved in mammalian macrophages (as discussed in Chapter II), which is of the same origin as microglia, addressing the value of studying glial responses during Wallerian degeneration in *Drosophila*.

*Drosophila* astrocytes are highly similar to mammalian astrocytes in morphology and functions (Stork et al., 2014). In contrast to *Drosophila* astrocytes (see Chapter III and Doherty et al., 2009), mammalian astrocytes undergo significant changes during Wallerian degeneration (Bignami and Ralston, 1969; Ludwin, 1990a). It was therefore surprising that we did not observe any obvious injury response from astrocytes in our olfactory nerve injury model. Nevertheless, recent research suggests adult *Drosophila* ensheathing glia and astrocytes are derived from the same progenitors propagated during metamorphosis (Omoto et al., 2015). It thus gives rise to an interesting theory that in *Drosophila*, ensheathing glia are, to some extent, astrocyte-like cells that are specialized to axon injury response. In fact, accumulating evidence suggests that *Drosophila* cortex glia possess some characteristics similar to mammalian astrocytes (Dr. Jaeda Coutinho-Budd, personal communications), which may help explain the reactivity of cortex glia
observed during Wallerian degeneration. Thus when comparing *Drosophila* and mammalian glia, one must keep in mind that the functions that are performed by a single subtype of mammalian glial cell may be shared or presented by different subtypes of *Drosophila* glial cells.

The expression of DRK, DOS, SOS, TRAF4, and MSN homologs in the mouse CNS

The Barres group recently established a mouse brain RNA-sequencing database which allows the examination of gene expression and the splicing variants in different neuronal and non-neuronal cells, including astrocytes, oligodendrocytes at different stages, microglia, and endothelial cells (Zhang et al., 2014). Among the new engulfment genes identified in this work, DRK homolog, Grb2, and DOS homolog, Gab2, are enriched in microglia (>80 FPKM, fragments per kilobase of transcript sequence per million mapped fragments, and >10 FPKM, respectively), compared to other brain cells. Mouse microglia are derived from bone marrows and as mentioned in Chapter II, Grb2 and Gab2 are found to play important roles in macrophage phagocytosis. These data are in support of the idea proposed in this dissertation that mammalian microglia may also use Grb2 and Gab2 to phagocytose neuronal debris when necessary. SOS has two mammalian homologs, Sos1 and Sos2. Although both of them are low in microglia (<2 FPKM), the amount of protein produced may still be enough to activate Rac1 in microglia to engulf debris. Traf4 is highly expressed in oligodendrocytes precursor cells (OPC, >100 FPKM) but also moderately expressed astrocytes. MSN is thought be similar to 3 different mammalian genes, Map4k4 (mitogen-activated protein kinase kinase kinase
kinase 4), Mink1 (misshapen-like kinase 1), and Tnik (Traf2 and Nck-interacting kinase). Map4k4 is enriched in mature oligodendrocytes while also being highly expressed in other brain cells (>100 FPKM in oligodendrocytes and ~50 FPKM in others). Mink1 is enriched in both oligodendrocytes and astrocytes (>30 FPKM). Tnik is enriched in astrocytes (~30 FPKM). Traf4 has previously been implicated in myelin formation (Blaise et al., 2012), which explains the enrichment of Traf4 and MSN homologs in oligodendrocytes. Nevertheless, Traf4 and MSN homologs in mammals can also play important roles during reactive astrogliosis, especially in activating JNK signaling pathways to respond to brain insults. There has been in vitro evidence suggesting that demyelination increases astrocyte GFAP expression via JNK activation (Gadea et al., 2008). Based on the conserved expression of many Drosophila genes I have characterized in mammalian glial lineages, future investigations of the functional roles of these molecules could prove valuable and uncover important roles for the activation of the JNK signaling cascade as well as the roles of Traf4 and MSN homologs in vivo during reactive astrogliosis.

**Significance and concluding remarks**

Neural injury leads to the production of neuronal corpses and cellular debris that must be cleared from the nervous system to avoid inflammation or toxicity (Neumann et al., 2009). Glia play essential roles in post-injury events and undergo dramatic cellular and molecular changes to ameliorate nervous system damage and restore normal functions of the nervous system. Understanding glial responses to neural injury is thus important for
us to help patients suffering from brain injuries or neurodegeneration. It is thought that much of the cellular destruction occurring in these neurological conditions is a direct result of the pro-destructive effects of reactive gliosis. If one could decrease the destructive effects of reactive glia and enhance the reparative ones, this could go a long way toward promoting neural recovery.

*Drosophila* and mammalian glia are strikingly similar in functions and behave similarly when axotomy occurs. Using *Drosophila* Wallerian degeneration as a model to study glial responses to axon injury has led to the discovery of several molecular pathways downstream of the conserved engulfment receptor Draper, the central receptor mediating glial responses to axon injury. Mammalian glia also use Draper homologs to eliminate apoptotic neurons and unwanted synaptic material, emphasizing the molecular conservation of the pathways I have studied, and the need to understand in detail how Draper functions at the genetic and the molecular level. In Chapter II, I expand the current knowledge of Draper-mediated glial engulfment of axonal debris by revealing the new function of DRK/DOS/SOS complex to be the upstream Rac1 GEF alternative to Crk/Mbc/dCed-12. In Chapter III, I identify TRAF4 as only the second identified binding partner of Draper and the key molecule for Draper to activate the two downstream signaling pathways after axon injury – JNK and STAT signaling pathways. TRAF4 acts through kinase MSN to induce JNK phosphorylation with the help from Rac1, and JNK phosphorylation leads to dAP-1 activation, which is required for glia to increase Draper expression through STAT-mediated changes in gene transcription. In Figure 4.1 I incorporate previous studies of Draper-mediated glial engulfment of axonal
debris with the new findings from this dissertation work to generate a comprehensive model for Draper-mediated activation of glia and clearance of axonal debris. This lays the groundwork for future investigators to explore these pathways in mammalian glial cells during reactive gliosis.
Figure 4.1. The newly proposed model of Draper-mediated glial engulfment of axonal debris after injury. Compare to Figure 1.2. for differences.
Reference List


