"A ROLE FOR C-JUN KINASE (JNK) SIGNALING IN GLIAL ENGULFMENT OF DEGENERATING AXONS"

A Dissertation Presented
By

Jennifer M. MacDonald

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"A ROLE FOR C-JUN KINASE (JNK) SIGNALING IN GLIAL ENGULFMENT OF DEGENERATING AXONS"

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This thesis is dedicated to my family, especially my parents, for their unwavering support and encouragement.

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ABSTRACT

The central nervous system (CNS) is composed of two types of cells: neurons that send electrical signals to transmit information throughout the animal and glial cells. Glial cells were long thought to be merely support cells for the neurons; however, recent work has identified many critical roles for these cells during development and in the mature animal. In the CNS, glial cells act as the resident immune cell and they are responsible for the clearance of dead or dying material. After neuronal injury or death, glial cells become reactive, exhibiting dramatic changes in morphology and patterns of gene expression and ultimately engulfing neuronal debris. This rapid clearance of degenerating neuronal material is thought to be crucial for suppression of inflammation and promotion of functional recovery, but molecular pathways mediating these engulfment events remain poorly defined.

Drosophila melanogaster is a genetically tractable model system in which to study glial biology. It has been shown that *Drosophila* glia rapidly respond to axonal injury both morphologically and molecularly and that they ultimately phagocytose the degenerating axonal debris. This glial response to axonal debris requires the engulfment receptor Draper and downstream signaling molecules dCed-6, Shark, and Rac1. However, much remains unknown about the molecular details of this response. In this thesis I show that *Drosophila* c-Jun kinase (dJNK) signaling is a critical *in vivo* mediator of glial engulfment activity. In response to axotomy, glial dJNK signals through a cascade involving the upstream MAPKKKs Slipper and TAK1, the MAPKK MKK4, and ultimately the *Drosophila* AP-1 transcriptional complex composed of JRA and Kayak to

initiate glial phagocytosis of degenerating axons. Interestingly, loss of dJNK also blocked injury-induced up-regulation of Draper levels in glia and glial-specific over-expression of Draper was sufficient to rescue phenotypes associated with loss of dJNK signaling. I have identified the dJNK pathway as a novel mediator of glial engulfment activity and show that a primary role for the glial Slipper/Tak1→MKK4→dJNK→dAP-1 signaling cascade is activation of *draper* expression after axon injury.

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CHAPTER I

Introduction

Glia – the "other" brain cell

The nervous system is made up of two main cell types – neurons and glia. Neurons are electrically excitable cells that are responsible for processing and transmitting information in the nervous system. These cells signal both intra- and intercellularly using specialized electrical and chemical mechanisms. The term "glial cell" comes from the Greek word for glue and is used to describe the other non-excitable cells in the nervous system. Glial cells were long thought to be merely space-fillers in the brain and the vast majority of neuroscience research was neuron-centric. However, work over the past few decades has begun to reveal the many roles that glial cells play in the brain during health and disease.

Critical roles have now been discovered for glia in nearly every aspect of brain development, maintenance, and function (reviewed in Barres, 2008). For example, glia are required for the survival of neurons during development and in the mature central nervous system (CNS) and the peripheral nervous system (PNS) (Riethmacher et al., 1997; Platel et al., 2010; Tao et al., 2011; Cui et al., 2001; Wilkins et al., 2003; Booth et al., 2000). Glial support of neuronal survival includes the maintenance of neurons through release of trophic factors to promote survival (Furuya et al., 2000; Banker, 1980), delivering metabolites to neurons (Meisingset et al., 2010; Allaman et al., 2011), and allowing for gas exchange (Iadecola and Nedergaard, 2007; Haydon and Carmignoto, 2006). Glia also increase synapse formation which promotes the formation of neuronal circuits (Ullian et al., 2001; Christopherson et al., 2005)

Notably, glial cells also act as the primary immune cell of the nervous system

during neural development and in the mature brain. Ultimately, about 50% of the neurons made during development undergo programmed cell death. Neuronal cell corpses are cleared from the nervous system by glia (Wu et al., 2009; Kurant et al., 2008; Williams and Rakic, 1988; Freeman et al., 2003). At the same time other neurons undergo axonal, dendritic, or synaptic pruning. Glial cells help sculpt connectivity in the nervous system by engulfing the pruned neurites and synapses, thereby helping to ensure that each neuron's adult targets will be properly innervated (Watts et al., 2004; Awasaki and Ito, 2004; Cantera and Technau, 1996; Logan and Freeman, 2007). In the mature brain, glia quickly respond to any pathogens or unwanted material and facilitate its elimination (Fierz et al., 1985; Dong and Benveniste, 2001; Vilhardt, 2005; Farina et al., 2007).

Beyond pruning, the assembly of neural circuits is dramatically affected by the actions of glia. Glial cells regulate axon pathfinding (Chotard and Salecker, 2004), fasciculation (Gilmour et al., 2002) and synapse formation (Ullian et al., 2001; Nägler et al., 2001; Steinmetz et al., 2006). In the mature brain, glial cells modulate neuronal activity by regulating the timely removal of neurotransmitters from the synaptic cleft, or by releasing "gliotransmitters"—glial-derived molecules are thought to directly alter synaptic function (Panatier et al., 2006; Ehinger, 1977; Suchak et al., 2003). By interacting with neurons in this way, glial cells are intricately involved in neural circuit formation and signaling which underlies complex behaviors including circadian rhythms, courtship, and learning and memory (Fellin et al., 2006; Jackson, 2011; Suh and Jackson, 2007; Mothet et al., 2006; Gibbs et al., 2008; Augustin et al., 2007;

Grosjean et al., 2008).

Subtypes of glia in mammalian nervous system

Glial cells outnumber neurons in the brains of all species, although the exact glia to neuron ratio varies significantly. There are four major subtypes of glial cells – Schwann cells, oligodendrocytes, astrocytes, and microglia. Each type is well characterized based on its location in the nervous system, morphology, and molecular markers. Myelinating and non-myelinating Schwann cells are found in the PNS. These glial cells are important for the development and maintenance of peripheral nerves, for myelination of axons and therefore the speed of signal conduction down the axons, and for the modulation of synaptic transmission at peripheral synapses such as the neuromuscular junction (NMJ) (Bunge, 1993). Interestingly, Schwann cells are essential for nerve regeneration as they secrete growth factors that stimulate axon re-growth after injury (Gulati, 1988; Hall, 1986; Ide et al., 1983).

Oligodendrocytes are similar to Schwann cells as they also wrap neurons with myelin but they are located in the CNS. Oligodendrocytes have thin processes that wrap multiple neurons in the CNS.

Astrocytes are the most abundant of the glial cells and, as their name would suggest, their cell bodies are star-shaped. These cells have extensive processes which interact with many cells including neurons, other glia and endothelial cells of the brain vasculature. Astrocytes have long been thought to be critical for providing trophic support for axons, maintaining levels of neurotransmitters and ions, and contributing to

the blood brain barrier (BBB). Additionally, recent studies have shown that astrocytes are present at synapses and are critical for the formation, maturation, and plasticity of these structures (Pfrieger, 1997; Ullian et al., 2001; Christopherson et al., 2005; Araque et al., 1999; Steinmetz et al., 2006). This is contrary to the long-held belief that synapses consist of only two cells, a pre-synaptic and a post-synaptic neuron. As a part of the "tripartite" synapse, astrocytes express receptors for a number of neurotransmitters, allowing them to sense and respond to neuronal activity (Murphy and Pearce, 1987). However, exact roles for astrocytes at the synapse and in coordinating neural signaling are still being debated (Nedergaard and Verkhratsky, 2012).

Microglia, the final type of glial cells in the CNS, are developmentally unrelated to the other glial subtypes as they stem from a hematopoietic lineage. These cells are constantly surveying the brain (Nimmerjahn et al., 2005) and are responsive to a variety of traumas - infection, neurodegenerative disease, stroke, or mechanical injury (Graeber et al., 1988), for example. When stimulated, microglia release both pro- and anti-inflammatory factors, recruit astrocytes and promote the clearance of cellular debris or pathogens. The effects of activated microglia can be both helpful and harmful to the CNS and research is ongoing to determine the molecular details of the microglial response, its effects, and how it integrates with the response of other glial cells.

The *Drosophila melanogaster* nervous system as a model for studying adult glia

While the ultimate goal of most biomedical research is to provide insight to mammalian (i.e., human) processes, the innate complexity and redundancy of mammalian systems

often makes the dissection of molecular pathways difficult. *Drosophila melanogaster* is a genetically tractable system in which to study biological processes *in vivo*. The genome has been fully sequenced and the ~14,000 genes are well annotated; about 75% of human genes are represented in the fly genome (Reiter et al., 2001). Many genetic tools exist to study *Drosophila* genes, including loss-of-function alleles and over-expression constructs. The GAL4/UAS system allows for tissue specific expression of transgenes, allowing for analysis of gene function during specific periods and in specific cell types (Brand and Perrimon, 1993). With this system, genes can be over-expressed, knocked down (with *UAS-dsRNAi*, for example), inactivated (with dominant negative constructs), or patterns can be visualized (with *UAS-mCD8::GFP* or another reporter).

Recent progress in the identification of glial subtypes and subtype-specific reagents make *Drosophila* particularly useful for the study of glial cell biology (Awasaki et al., 2008; Doherty et al., 2009). Peripheral glia in the PNS of *Drosophila* represent the functional ortholog of mammalian Schwann cells as they ensheath peripheral nerves. In the CNS, three major types of glia have been identified – surface glia, cortex glia, and neuropil glia (**Figure 1.1**).

Surface glia surround the outside surface of the brain. There are two subtypes of surface glia – perineurial and subperineurial glia. The perineurial glia are small, oblong cells around the outermost surface of the brain while the sheet-like subperineurial cells are inside of the perineurial glia. Together, the surface glia make up the blood brain barrier in the fly.

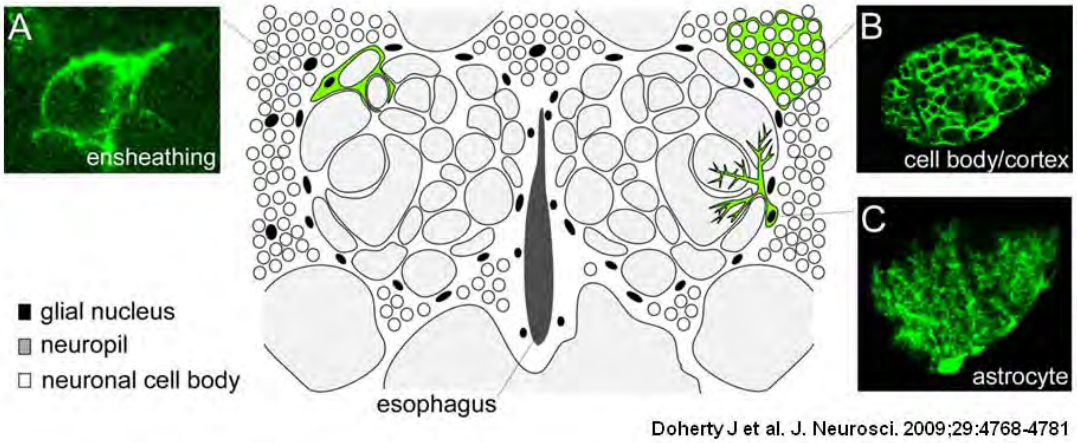


Figure 1.1
Subtypes of glia in the *Drosophila* adult CNS

Figure 1.1 Subtypes of glia in the *Drosophila* adult CNS.

Repo-GAL4 was used to label glial MARCM clones with GFP and the morphology of individual glial cells was analyzed in the adult CNS. Three major subytpes were identified:

(A) Ensheathing glia had a flattened appearance with relatively few branch points and their membranes appeared to surround and demarcate distinct compartments of the neuropil.

(B) Cortex glia resided outside the neuropil in the cortex where neuronal cell bodies are found and appeared to fully ensheath the soma of every brain neuron within its spatial domain.

(C) Astrocytes had a major stalk that projected into the neuropil and branched and ramified profusely, ultimately positioning astrocytes membrane processes in close proximity to the synapse-rich regions of the glomeruli.

(Adapted from J. Doherty et al., 2009)

Cortex glia, also known as “cell body associated” glia, are found outside of the neuropil within the cell cortex of the brain. The processes of these cells surround the neuronal cell bodies in the cortex region and one cortex glial cell can wrap multiple neuronal cell bodies (Awasaki et al., 2008). Cortex glia make contact with the blood brain barrier and trachea, which are major sites of nutrient and gas exchange in the fly brain. This positioning makes it feasible that these cells may facilitate the transfer of nutrients and gases to neurons, similar to the function of the mammalian astrocyte.

Finally, the neuropil glia are divided into two subtypes: astrocyte-like glia and ensheathing glia. Astrocyte-like glia have tufted processes that delve deep into the neuropil regions where synapses are present. In the antennal lobe, for example, astrocyte-like processes are visible within the synaptic regions of the glomeruli (Doherty et al., 2009; Awasaki et al., 2008). Interestingly, this subtype of cells expresses EAAT-1, an excitatory amino acid transporter also present in mammals that permits the cell to uptake glutamate from the synapse and therefore to modulate synaptic transmission (Doherty et al., 2009; Awasaki et al., 2008; Besson et al., 1999). In contrast to the mammalian astrocyte, *Drosophila* astrocyte-like glia do not show any obvious changes in morphology after neuronal injury and do not appear to be required for the clearance of degenerating axonal debris. The ensheathing subtype of neuropil glial cells are morphologically identifiable by their flattened shape and their location along the borders of the neuropil (Doherty et al., 2009). These cells divide the neuropil into subregions, perhaps with a functional consequence (Awasaki et al., 2008). Ensheathing glial processes can be visualized surrounding the glomeruli within the antennal lobe but not

within these structures (Doherty et al., 2009).

Reactive gliosis in mammals

Glia are constantly monitoring the nervous system for signs of trauma and quickly react to a variety of traumatic events, including excitotoxicity, anoxia, injury, and neurodegenerative disease. Though seemingly different, each of these events triggers a common response in astrocytes and microglia that is termed "reactive gliosis". In general, reactive gliosis involves dramatic alterations in glial morphology and gene expression patterns, movement of glial cells or their processes to sites of damage, and phagocytosis of dead and degenerating cells (Aldskogius and Kozlova, 1998; Singh et al., 2011). Microglia are the primary immune cell of the CNS; their processes are continuously moving to survey the brain for changes in the environment (Davalos et al., 2005). In response to a trauma, microglia rapidly respond by changing morphology (Vilhardt, 2005; Bechmann and Nitsch, 1997), increasing the expression of certain cell surface markers, and proliferating (Singh et al., 2011). These cells quickly migrate to the site of damage and begin phagocytosing debris while releasing cytokines and other pro-inflammatory factors (Halleskog et al., 2011; Huang et al., 2008). Astrocytes also respond with rapid changes to morphology (Bechmann and Nitsch, 1997) and gene expression after trauma to the CNS (Murray et al., 1990). While much work has been done to study the molecular details of reactive gliosis and many important factors have been identified, questions about this process remain unanswered.

Many of the questions currently being addressed by researchers involve reactive

gliosis in relation to a specific disease process, both as a result and a potential cause. Recent work has identified heterogeneity in the response of glial cells; that is, reactive astrogliosis after ischemic injury does not have the same molecular profile as after lipopolysaccharide (LPS)-induced neuroinflammation (Zamanian et al., 2012). Additionally the reactive process in both microglia and astrocytes is thought to be graded with the intensity of the response correlating with the severity of the injury or insult (Sofroniew, 2009). This leads to interesting possibilities: Are there subpopulations of glia within the brain that react differently to various forms of stimulation? Do the cells activate different molecular programs depending on the insult? Are different signaling pathways activated in different cells based on factors such as proximity to the injury site?

It is commonly thought that reactive gliosis has both beneficial and harmful results. The phagocytosis of degenerating neuronal debris, cell corpses, or pathogens is widely believed to prevent further tissue damage. For example, it has been shown that the clearance of apoptotic neurons after a focal stroke helps to prevent elevated levels of chemokines and to limit the severity of the injury (Faustino et al., 2011). However, reactive glia may also cause excessive damage to the surrounding tissue by releasing inflammatory cytokines and creating a glial scar which acts as a physical blockade to any potential nerve regrowth (Sofroniew, 2009). In diseases such as multiple sclerosis and Alzheimer's disease, glia appear to be trapped in this reactive state and may in fact hasten disease progression (Chang et al., 2012; Canning et al., 1993; Solito and Sastre, 2012; Huizinga et al., 2012; Prineas and Parratt, 2012). Excessively active glia have also been implicated in chronic neuropathic pain (Watkins et al., 2001; Tsuda et al., 2005; Zhuang

et al., 2005). To successfully develop targeted therapeutics for neurological disorders and brain trauma we need a thorough understanding of underlying molecular events. How do neurons and glia communicate following injury? What pathways drive glial function in the injured brain?

The signaling mechanisms that underlie both the beneficial and harmful effects of reactive gliosis are poorly understood. This is likely because much of the mammalian data regarding reactive gliosis is from *in vitro* studies which, while useful for identifying molecular players, are not ideal for studying the molecular pathways that are present in an intact animal because the intricate relationships between cells are not preserved. Additionally, the glia in culture may already be "reactive" due to the mechanical dissociation required to start a culture or stimulatory factors in the culture media. By utilizing a genetically tractable model organism, *Drosophila melanogaster*, we are able to explore reactive gliosis *in vivo*.

Studying axon degeneration and glial responses to injury in *Drosophila*.

The *Drosophila* adult olfactory system has been identified and well characterized as a tool to understand neuron-glia signaling after brain injury. Olfactory receptor neuron (ORN) cell bodies are housed in the antennae and maxillary palps and send axon projections into the antennal lobe of the brain. All ORNs expressing the same odorant receptor (OR) gene synapse on the same distinct glomerulus (Vosshall et al., 1999). Conveniently, we can use this fact to genetically label highly reproducible subsets of ORN axons. Promoters for nearly all fly OR genes have been identified and used to

generate OR-GAL4 driver lines, which we use to express membrane tethered GFP (*UAS-mCD8-GFP*) thereby allowing us to visualize subsets of ORN axons in the intact brain (Vosshall et al., 2000). When antennae or maxillary palps are surgically removed, ORN axons are severed. This induces Wallerian degeneration, a process characterized by fragmentation of the axon distal to the injury site (Waller, 1850; MacDonald et al., 2006). Within hours of the injury, nearby glial cells begin to show changes in morphology as they extend processes to the degenerating axons and begin to initiate the process of clearing this debris from the CNS (**diagrammed in Figure 1.2B**).

Glial responses to axon injury can be assayed by using *repo-GAL4* (a pan-glial driver that is not expressed in neurons) to drive expression of marker proteins (e.g. *UAS-mCD8-GFP*) or by using an antibody to the engulfment receptor Draper, an excellent marker for glial membranes as it is expressed only in glia in the adult brain (MacDonald et al., 2006; Doherty et al., 2009). Removal of the antennae causes a severe trauma to the antennal lobe by severing axons innervating 44 of the 50 glomeruli. Within one day of antennal ablation, local glial cells dramatically up-regulate Draper levels and undergo a striking expansion of their membranes as judged by membrane tethered GFP (MacDonald et al., 2006). Thus, neurons and glia in the *Drosophila* brain signal intensively to one another after axotomy and the glia respond to this injury morphologically and molecularly (**Figure 1.2A3, 1.2B**).

Can fly glia specifically recognize severed axons? Maxillary palps contain only ~60 ORNs and these innervate six uniquely identifiable antennal lobe glomeruli. Severing maxillary palp ORNs results in glia specifically recruiting their membranes and

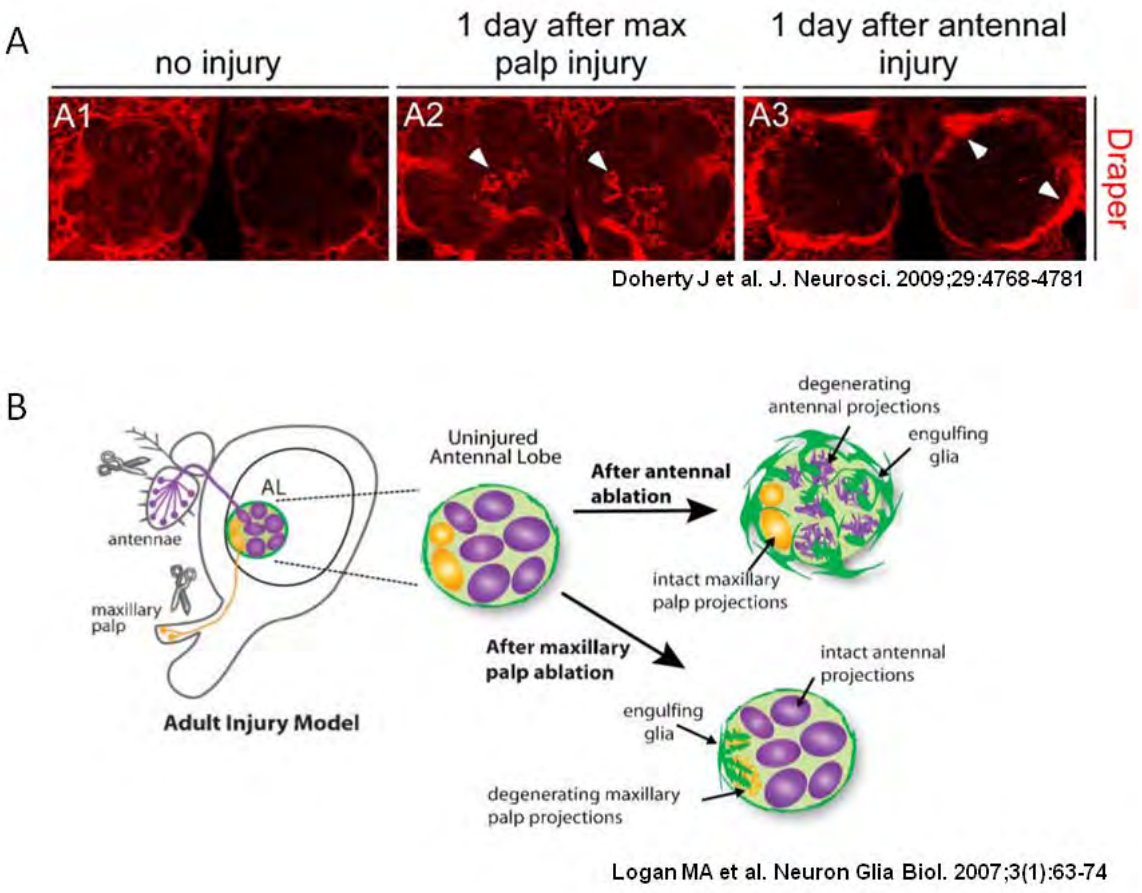


Figure 1.2
***Drosophila* adult glia respond to axonal injury**

Figure 1.2 *Drosophila* adult glia respond to axonal injury

(A) Antibody against Draper, an engulfment receptor, specifically la
bels glial membranes in the adult CNS. A1 - Before injury, Draper is visible throughout the brain. A2 – one day after maxillary palp ablation, Draper is visible specifically on the injured glomeruli (arrowheads) and along the maxillary nerve (data not shown). Neighboring glomeruli that are uninjured do not show this increase in Draper staining. A3 – Draper is dramatically increased around the antennal lobe one day after removal of the third antennal segment (arrowhead).

(Adapted from J. Doherty et al., 2009)

(B) Diagram of the adult CNS injury model in *Drosophila.* ORN cell bodies reside in the third antennal segments (purple) or maxillary palps (orange) and send projections to the glomeruli within the antennal lobe (AL). Surgical removal of the antennae or maxillary palp severs the neurons and induces a glial response to this injury. Glial membranes are recruited specifically to the injured glomeruli where they phagocytose the debris.

(Adapted from Logan and Freeman, 2007)

Draper to severed axons within hours after injury (**Figure 1.2A2, 1.2B**) (MacDonald et al., 2006). However, neighboring antennal innervated glomeruli are not covered with glial membranes or Draper staining, showing the precision of the glial response. This tells us that glial cells are able to discriminate between injured and uninjured neurons.

Together, these data tell us that *Drosophila* axons undergo Wallerian degeneration and that glia respond to injured axons with both molecular and morphological changes. The glial response to axon injury can be divided into three phases: activation, engulfment, and termination. The initial activation step involves the glial cell receiving a yet unidentified injury signal, activating engulfment genes and mobilizing glial membranes to the severed axons. The engulfment step, which involves the internalization of debris, will be discussed in depth below. Finally, the termination step involves the glial cells returning to their resting state, driven by an increase in the Draper-II protein, an alternative splice variant of *draper* that contains an inhibitory domain in its intracellular region (Logan et al., 2012). The ability of glia to respond to axonal injury, combined with the previously discussed advantages of using this genetic model system, provides a good rationale for utilizing *Drosophila* to further characterize the molecular details of glial response to neuronal injury and the subsequent engulfment of the axonal debris.

Molecular mechanisms of engulfment are conserved from mammals to *Drosophila*

Phagocytosis is a highly conserved process that is important during all life stages of a eukaryote. In the developing CNS, phagocytosis occurs as a part of the normal

developmental program as cell corpses are removed and axons are pruned. In the adult CNS, phagocytosis is typically associated with a pathological state such as infection or cell death due to trauma. The invading pathogen or cell debris (material from dying cells) must be cleared to restore the health of the organism. Work from *C. elegans* has helped us to begin to understand how cells engulf apoptotic cell corpses; orthologs for components of the *C. elegans* machinery have been revealed in *Drosophila* and in mammals, suggesting that the molecular underpinnings of phagocytosis are conserved between species (**Figure 1.3**).

There are two partially redundant signaling pathways that promote engulfment in *C. elegans* and converge on CED-10/Rac to exert their effects. The first, made up of CED-2, CED-5, and CED-12, acts to promote reorganization of the actin cytoskeleton and the engulfment of dead or dying cells (Hedgecock et al., 1983; Ellis et al., 1991). The second is composed of CED-1, CED-6 and CED-7 (Ellis et al., 1991; Hedgecock et al., 1983). CED-1 acts as an engulfment receptor that identifies dying cells and initiates their degradation after engulfment while CED-6 acts as an adaptor molecule that physically binds CED-1 and likely signals through CED-10/Rac1 to promote phagosome maturation (Neukomm et al., 2011; Su et al., 2002; Yu et al., 2006; Liu and Hengartner, 1998; Zhou et al., 2001).

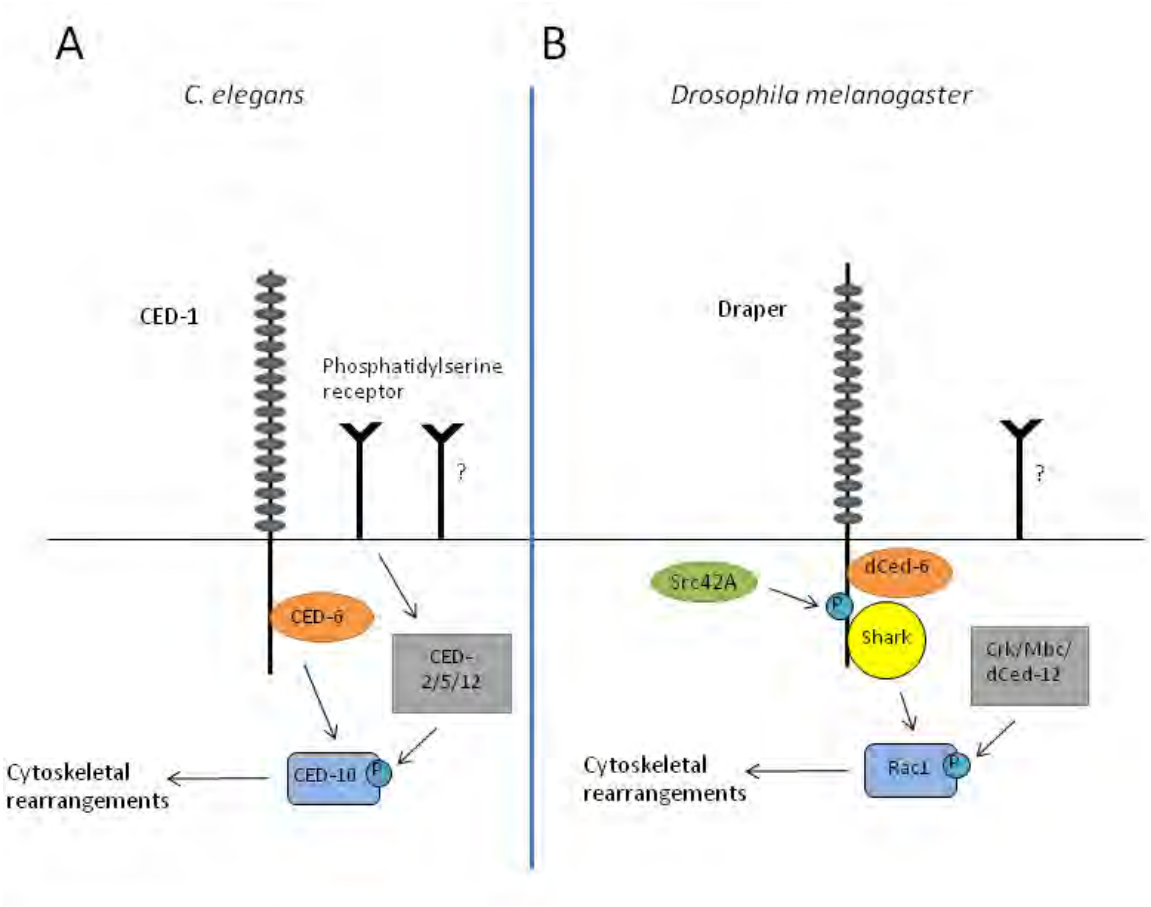


Figure 1.3

Figure 1.3 Current models of engulfment in *C. elegans* and *Drosophila*

Figure 1.3 Current models of engulfment in *C. elegans* and *Drosophila*

Studies of engulfment in *C. elegans* and *Drosophila* have unveiled conserved mechanisms underlying phagocytosis.

(A) In *C. elegans*, two partially redundant pathways converge on CED-10 to promote reorganization of the cytoskeleton and phagocytosis. One pathway is composed of the engulfment receptor CED-1, the adaptor molecule CED-6, and CED-7. The parallel pathway includes CED-2, CED-5, and CED-12.

(B) The *Drosophila* engulfment pathway is composed of Draper, a CED-1 ortholog, and adaptor molecules dCed-6 and Shark. A parallel pathway includes Crk (dCed-2), MBC (dCed-5) and dCed-12. Both of these pathways feed into Rac1, an ortholog of CED-10, to promote engulfment via cytoskeletal rearrangements.

Recent work has identified orthologous pathways in both *Drosophila* and mammals, indicating that many molecular underpinnings of engulfment have been conserved throughout evolution. The *Drosophila* CED-1 ortholog *draper* was originally identified for its role in the clearance of embryonic neuronal cell corpses (Freeman et al., 2003). Roles for Draper as an engulfment receptor have now been discovered throughout development and in the adult fly. The *Drosophila* CED-6 (dCed-6) has been shown to be required for clearance of degenerating and pruned axon debris and to act in the Draper/CED-1 pathway (Doherty et al., 2009; Awasaki et al., 2006). Draper signaling has been shown to be required for the developmental pruning of dendrites (Williams et al., 2006) and axons (Awasaki et al., 2006; Hoopfer et al., 2006), for the removal of presynaptic debris at the larval neuromuscular junction (NMJ) (Fuentes-Medel et al., 2009) and for the clearance of adult axons undergoing Wallerian degeneration (MacDonald et al., 2006). In *draper* null mutants, glia fail to respond in any way to antennal or maxillary palp ablation and severed ORN axons linger in the CNS for weeks after injury (MacDonald et al., 2006). Together, these data tells us that the dCED-1 receptor Draper and downstream signaling are critical for glia to respond to an array of dying cells– apoptotic cell corpses, developmentally degenerating axons, and traumatically injured axons.

The CED-2/5/12 pathway has been recently characterized in *Drosophila* and was shown to be required for the phagocytosis of degenerating axon debris after axotomy (Ziegenfuss et al., 2012). Notably, the *Drosophila* Crk (dCed-2), MBC (dCED-5), and dCed-12 are required in glia to activate phagocytosis and to engulf axonal debris but not

for the initial glial response to axonal injury (Ziegenfuss et al., 2012). Based on this pathway's role in *C. elegans* where it is required for cell migration, it is surprising that glia depleted of Crk/MBC/dCed-12 are able to respond and migrate to injured axons. These data indicate that while the basic engulfment pathway components are conserved from *C. elegans* to mammals, there may be some interspecies variability in the exact functions and interactions of these pathways. Alternatively, different engulfment targets may invoke slightly different engulfment pathways – degenerating axons may be biologically different from cell corpses resulting from programmed cell death.

Orthologs for components of these known engulfment pathways have also been uncovered in mammals. The closest mammalian sequence orthologs to CED-1/Draper (MEGF10 and Jedi-1) have been shown to be expressed in the satellite glial precursor cells of the dorsal root ganglion and to be important for glial clearance of cell corpses (Wu et al., 2009). Additionally, when MEGF10 is put into *C. elegans* under the control of the *ced-1* promoter, it can partially rescue the engulfment phenotype in *ced-1* null animals, indicating a conserved molecular function (Hamon et al., 2006). GULP, the mammalian ortholog to CED-6, was also recently shown to be important for phagocytosis in an *in vitro* model as it clusters around the foreign material and its knock down results in decreased clearance (Su et al., 2002; Park et al., 2010). Studies of the mammalian orthologs of the CED2/5/12 pathway (CrkII, Dock180 and Elmo) have revealed that this complex acts as a guanine nucleotide exchange factor (GEF) that activates Rac1 (Brugnera et al., 2002; Gumienny et al., 2001).

Despite recent progress, many questions remain about the glial response to nerve injury. What activates Draper? Thus far, only two potential ligands have been identified in *Drosophila*, Pretaporter in the setting of phagocytosis of apoptotic cell corpses (Kuraishi et al., 2009) and lipoteichoic acid in the immune response to *Staphylococcus aureus* infection (Hashimoto et al., 2009). However, these ligands have not been found to be involved in the clearance of degenerating axon debris. Is Draper the only engulfment receptor required for clearance of neuronal debris? What are the downstream or parallel effectors that mediate glial engulfment and debris clearance? To begin to address these questions Johnna Doherty, a former graduate student in the Freeman lab, conducted a small scale RNAi-based screen for genes specifically in glia cells that, when knocked down with RNAi, showed a persistence of degenerating axon material after injury in the CNS. One of the molecules identified in this screen as being required for glial engulfment of axon debris is the *Drosophila c-Jun NH_2-terminal kinase* (*dJNK*); in Chapter II I will describe this novel role for dJNK.

An overview of c-Jun NH_2 Kinase (JNK) signaling

Cells are constantly receiving multiple signals from the environment; responsiveness to these signals is necessary for the cell, and therefore the animal, to grow, remain healthy, and survive. One pathway that is responsible for receiving a broad range of extracellular signals is the Mitogen-activated Protein Kinase (MAPK) pathway (Kyriakis and Avruch, 2012). Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases that are responsive to many extracellular stimuli in organisms ranging

from yeast to mammals (Widmann et al., 1999). Their activation leads to a range of responses including transcriptional changes, alterations in metabolic pathways, inflammatory responses, proliferation and apoptosis (Kyriakis and Avruch, 2012). There are several subtypes of MAPKs, each of which is responsive to a distinct set of stimuli. The Jun NH_2-terminal kinase (JNK) is a MAPK that is activated by a variety of extracellular signals including growth factors and cytokines as well as by factors that stress the cell such as UV radiation (Li et al., 2010; Wang et al., 2012) and ischemia (Bogoyevitch et al., 2004; Stronach, 2005; Zeng et al., 2011). As would be expected of a protein that is responsive to such a variety of insults, the downstream effects of JNK are varied but include cell survival, growth and metabolic changes, and cell death (Kyriakis and Avruch, 2012; Stronach, 2005; Herdegen et al., 1997)

The specific proteins that act at each stage of activation of the JNK pathway depend on the stimulus and the cell type. Generally, JNK activation is downstream of the activation of a receptor and the binding of its adaptor molecules. The exact adaptor molecules involved in the activation of the MAPKKKKs are not fully known but roles for small adaptors such as TRAF, SHC, and CRK or GTP binding proteins such as Rac or Ras have been suggested (Jaffe and Hall, 2005; Hashimoto, 1999; Min and Pober, 1997a; Song et al., 1997a; Dolfi et al., 1998). Phosphorylation of a MAPKKKK activates this enzyme which then phosphorylates and activates a MAPKKK which phosphorylates and activates a MAPKK. These MAPKKs phosphorylate and activate the MAPKs which in turn phosphorylate and activate their downstream targets (such as transcription factors). The outcomes of the signaling depend on the components involved (Bogoyevitch and

Kobe, 2006). While other downstream targets have been identified, the most common result of JNK activation is phosphorylation of c-Jun which, along with c-Fos, makes up the activator protein-1 (AP-1) transcription factor (Karin, 1996; Whitmarsh and Davis, 1996). This then leads to transcriptional up-regulation of a variety of genes which help the cell respond to the triggering stressor. Alternative targets in mammals include ATF2 which heterodimerizes with c-Jun to activate expression of *c-Jun* (Gupta et al., 1995; Livingstone et al., 1995) and Elk-1 which is involved in the transcriptional activation of *c-fos*, a component of the AP-1 transcription factor (Yang et al., 1998; Whitmarsh et al., 1995). Other, non-transcription factor targets have also been identified such as the mitochondrial protein Bcl-x_L, an anti-apoptotic protein (Salameh et al., 2010), and various microtubule associated proteins (Chang et al., 2003a; Yoshida et al., 2004). Interestingly, proteins such as Elk-1 and ATF2 can also be phosphorylated by other MAPK cascades, possibly leading to integration of signals received by each of these pathways (Baan et al., 2006).

JNK signaling and the resulting biological outcomes are complex: a wide variety of "stress" signals are able to activate the pathway, multiple proteins are able to act at each step of the signal transduction and the combination determines the end signaling result(s). Transcriptional activation downstream of JNK may lead to the upregulation of other transcription factors, thereby propagating and amplifying the signaling ongoing in the cell. Alternatively, JNK may be activated for a short, specific period of time (Rallis et al., 2010). All of this signaling may be occurring in a specific subcellular location and in the context of other signaling mechanisms (Lowes et al., 2002a; Meng and Xia, 2011).

Together, these factors combine such that JNK signaling results in a variety of biological outcomes ranging from increased survival to cell death and a number of processes in between (Kyriakis and Avruch, 2012).

JNK in *Drosophila melanogaster*

In order to study complex signaling pathways such as JNK *in vivo*, some researchers have turned to model organisms. *Drosophila melanogaster* is particularly useful as there is only one JNK (compared to 3 in mammals). The amino acid sequence of dJNK, encoded by the gene *basket*, is 65-70% identical to the human JNK1, including critical conserved regions: the TPY motif required for activation and key residues in the kinase domain (Sluss et al., 1996; Riesgo-Escovar et al., 1996). In an *in vitro* model, DJNK was shown to phosphorylate both c-Jun and d-Jun indicating that the specificity of the enzyme has been conserved throughout evolution (Sluss et al., 1996). When Hemipterous (HEP, a MAPKK homolog) is added to the assay, the phosphorylation of d-Jun is significantly increased, indicating HEP is an upstream activator of JNK and that they are both involved in a MAPK signal transduction cascade (Sluss et al., 1996). Together these data show that JNK has been evolutionarily conserved from mammals to *Drosophila*, therefore allowing researchers to use this model system to elucidate the details of JNK signaling in various biological situations

Developmental and immunological roles for *dJNK*

One of the key roles identified for dJNK is in dorsal closure during embryonic development (Jacinto et al., 2002). The initiating signal(s) for dorsal closure remains unknown but it is thought that small GTPases such as RHO, Rac1 and Cdc42 are activated and they activate downstream kinases such as Misshapen and Slipper (Neisch et al., 2010; Garlena et al., 2010). This leads to phosphorylation of HEP, the MAPKK upstream of JNK, which then phosphorylates JNK (Glise et al., 1995). JNK activation specifically in the leading edge cells leads to transcriptional activation of *dpp* (which encodes decapentaplegic, a TGF-B related secreted factor) and *puc* (which encodes puckered, a dual-specificity phosphatase), signaling molecules whose presence defines the leading edge cells during dorsal closure (Glise and Noselli, 1997; Martin-Blanco et al., 1998; Hou et al., 1997).

Additional proteins have been identified as being involved in dorsal closure but their exact placement in the signaling cascade has not been fully elucidated. SRC42A, a tyrosine protein kinase (Tateno et al., 2000) and Shark, a nonreceptor tyrosine kinase (Fernandez et al., 2000), have been shown to function upstream of JNK during dorsal closure but exactly how they are interacting with other pathway members is unclear. Interestingly, Shark and SRC42A have been shown to be critical for Draper dependent glial phagocytosis of neuronal debris after axotomy in the adult CNS (Ziegenfuss et al., 2008), making it possible that a signaling cascade similar to that in dorsal closure is activated during the engulfment process.

As in mammals, a role for dJNK has been identified in the *Drosophila* immune

response. In the early work identifying the *Drosophila* homolog of JNK, it was noted that this protein was increased in response to LPS stimulation in S2 cell culture (Sluss et al., 1996). Further studies revealed that dJNK acts downstream of the MAPKKK TAK1 in a signaling pathway that leads to the upregulation of genes such as cytoskeletal proteins (that might help in wound repair, for example) and proapoptotic genes (Boutros et al., 2002). TAK1 may also feed into the NF-kB pathway that upregulates Relish, a protein that when phosphorylated and cleaved can enter the nucleus and induce expression of genes including antimicrobial peptides (Silverman et al., 2003; Kim et al., 2007). Interestingly, Relish can inhibit JNK signaling by degrading TAK1, providing crosstalk that may help to determine the cell's fate (Park et al., 2004). Ongoing studies of the role for dJNK in *Drosophila* immunology may be useful for identifying receptors and dJNK interacting molecules involved in glial phagocytosis.

In vitro evidence that JNK may be involved in glial phagocytosis

Is JNK required for mammalian glia to sense and respond to axon injury? Data from *in vitro* studies provide compelling evidence that JNK is required for each of the three components of reactive gliosis: proliferation, inflammation, and changes in gene expression. In several culture preparations, JNK has been shown to mediate astrocyte proliferation downstream of molecules such as Angiotensin II and Endothelin-1 (Clark et al., 2008; Gadea et al., 2008). A previous study showed that the proliferative effects of Angiotensin II in cultured astrocytes were mediated by Src, making it likely that Src is upstream of JNK in this pathway (Clark and Gonzalez, 2007). In primary microglial

cultures after application of lipopolysaccharide (LPS, a bacterial component that causes an immune reaction), JNK mediates inflammatory changes that include metabolic changes and induction of *TNF-α*, *Cox-2*, *IL-6*, and other AP-1 target genes (Waetzig et al., 2005). In an *in vivo* rat model of neuropathic pain, there is an increase in phosphorylated-JNK (P-JNK) after partial sciatic nerve ligation (Ma and Quirion, 2002). Together, these studies argue that JNK may play a role in the glial response to nerve injury but the details of this involvement and the other signaling molecules involved remain unknown. In Chapter II I will discuss an *in vivo* role for dJNK in glial phagocytosis and will identify other members of the dJNK signaling cascade that are required for the clearance of degenerating axonal debris.

CHAPTER II

The d-Jun kinase signaling cascade promotes glial engulfment activity through activation of *draper* and phagocytic function.

All work presented in this chapter was conducted in the lab of Marc Freeman, Ph.D.. Johnna Doherty contributed by identifying dJNK in a candidate-based RNAi screen for genes required for glial clearance of degenerating axon debris. Rachel Hackett contributed by performing western blot analyses. I confirmed the dJNK phenotype and carried out all other experiments.

A modified version of this work is currently submitted to *Cell Death and Differentiation* as:

MacDonald JM, Doherty J, Hackett R, Freeman MR
The d-Jun kinase signaling cascade promotes glial engulfment activity through activation of *draper* and phagocytic function

Abstract

After neuronal injury or death, glial cells become reactive, exhibiting dramatic changes in morphology and patterns of gene expression and ultimately engulfing neuronal debris. Rapid clearance of degenerating neuronal material is thought to be crucial for suppression of inflammation and promotion of functional recovery, but molecular pathways mediating these engulfment events remain poorly defined. Here we demonstrate that *Drosophila* d-Jun kinase (dJNK) signaling is a critical *in vivo* mediator of glial engulfment activity. In response to axotomy, we find glial dJNK signals through a cascade involving the upstream MAPKKKs Slipper and TAK1, the MAPKK MKK4, and ultimately the *Drosophila* AP-1 transcriptional complex composed of JRA and Kayak to initiate glial phagocytosis of degenerating axons. Interestingly, loss of dJNK also blocked injury-induced up-regulation of Draper levels in glia and glial-specific over-expression of Draper was sufficient to rescue phenotypes associated with loss of dJNK signaling. Thus, the dJNK pathway is a novel mediator of glial engulfment activity and a primary role for the glial Slipper/TAK1→MKK4→dJNK→dAP-1 signaling cascade is activation of *draper* expression after axon injury.

Introduction

Cell death occurs throughout the life of most metazoans. The presence of cell corpses and cellular debris triggers rapid responses from neighboring professional or non-professional phagocytes, ultimately promoting corpse engulfment (Gumienny and Hengartner, 2001; Giorgi and Deri, 1976; Sears, 2003). In the central nervous system (CNS) glial cells are the primary immune cell type, constantly surveying the neural environment for signs of cell death or injury. Neural injury results in rapid changes in glial cellular phenotypes—glia become "reactive" whereby they exhibit dramatic changes in morphology and patterns of gene expression and they ultimately phagocytose degenerating neuronal debris (Aldskogius and Kozlova, 1998; Sofroniew, 2009; Zamanian et al., 2012; Murray et al., 1990). Reactive glial responses are an early feature of most neurodegenerative diseases (Gerber et al., 2012; Perry, V.H., Nicoll, J.A. & Holmes, 2010) and occur after any brain injury (Loane and Byrnes, 2010; Nedergaard and Dirnagl, 2005), but remarkably little is known about how glial cells sense injury, become phagocytic, and eventually engulf neuronal debris.

A key mediator of glial responses to neuronal death or injury in *Drosophila* is the engulfment receptor Draper (MacDonald et al., 2006; Freeman et al., 2003) which encodes the *Drosophila* ortholog of the *C. elegans* cell corpse engulfment receptor CED-1 (Zhou et al., 2001). In the *Drosophila* embryo glial Draper is required for efficient clearance of neuronal cell corpses from the developing CNS (Freeman et al., 2003). In the adult brain Draper is essential for activation of glial responses after axotomy and

promotion of glial phagocytosis of degenerating axonal debris. For example, after olfactory receptor neuron axotomy glial cells dramatically increase levels of Draper, extend membranes to degenerating axons, engulf axonal debris, and then terminate their responses and return to a resting state (MacDonald et al., 2006; Logan et al., 2012). In *draper* mutants, glial cells fail to show any morphological or molecular responses to axonal injury and unengulfed axonal debris lingers in the adult brain for the lifespan of the fly.

During glial engulfment of degenerating axons the Draper receptor appears to signal in a manner similar to the mammalian Fc, B-, and T-cell immunoreceptors, with activation of Draper resulting in downstream engulfment signaling through an Src family kinase signaling cascade. Briefly, SRC42A phosphorylates an intracellular ITAM domain on Draper, the non-receptor tyrosine kinase Shark then binds to the Draper ITAM, and engulfment is activated (Ziegenfuss et al., 2008). The phosphotyrosine-binding (PTB) domain-containing protein dCed-6 also appears to signal downstream of the Draper receptor (Liu and Hengartner, 1998), but additional signaling molecules that promote engulfment activity in glia remain poorly defined. Here we show that the *Drosophila* c-Jun amino terminal kinase (dJNK) plays a critical role in glial responses to degenerating axonal debris. Loss of glial dJNK signaling potently blocks axon injury-induced up-regulation of Draper, activation of a glial phagocytic phenotype, and glial clearance of axonal debris. Our work identifies the dJNK pathway as a novel signaling pathway required for glial engulfment signaling and we propose that a primary role for dJNK is transcriptional activation of the *draper* gene after axotomy.

Results

Basket signaling is required in adult brain glia for engulfment of axonal debris

In an RNAi-based screen for novel genes required for glial responses to axonal injury we identified *basket* (*bsk*), encoding *Drosophila* c-Jun Kinase (dJNK), as a key regulator of glial engulfment of axonal debris. For glial-specific dJNK knockdown we drove expression of *UAS-bsk*$^{RNAi\text{-}104569}$ with the pan-glial driver *repo-GAL4* in a background where a subset of maxillary olfactory receptor neurons (ORNs) were labeled with membrane-tethered GFP (mCD8::GFP). We then severed ORN axons by maxillary palp ablation and assayed clearance of axonal debris 5 days after axotomy. Whereas control animals cleared the vast majority of axonal debris within 5 days after axotomy, we found that this glial engulfment activity was potently suppressed by glial *bsk*^{RNAi} (**Figure 2.1A-B**). To confirm this phenotype was specific to *bsk*, we performed glial specific RNAi knockdown using two additional RNAi lines targeting different regions of the *bsk* transcript (*bsk*$^{RNAi\text{-}34138}$ and *bsk*$^{RNAi\text{-}TRiP}$) and found similar results (**Supplementary Figure 2.1**). *bsk*^{RNAi} suppression of axonal clearance was robust (near 100%) and persistent—we observed axonal debris lingering for as many as 30 days after axotomy in glial *bsk*^{RNAi} animals, which is longer than the median life-span of adult *Drosophila*.

We next overexpressed *puckered* (*puc*), a phosphatase that negatively regulates dJNK activity (Martin-Blanco et al., 1998). Glial-specific expression of PUC phenocopied glial *bsk*^{RNAi}, with nearly all axonal debris lingering in the central nervous system (CNS) for 30 days after axotomy (**Figure 2.1C**). We note that axonal fragmentation appears to occur normally and on schedule (i.e. within 1 day)

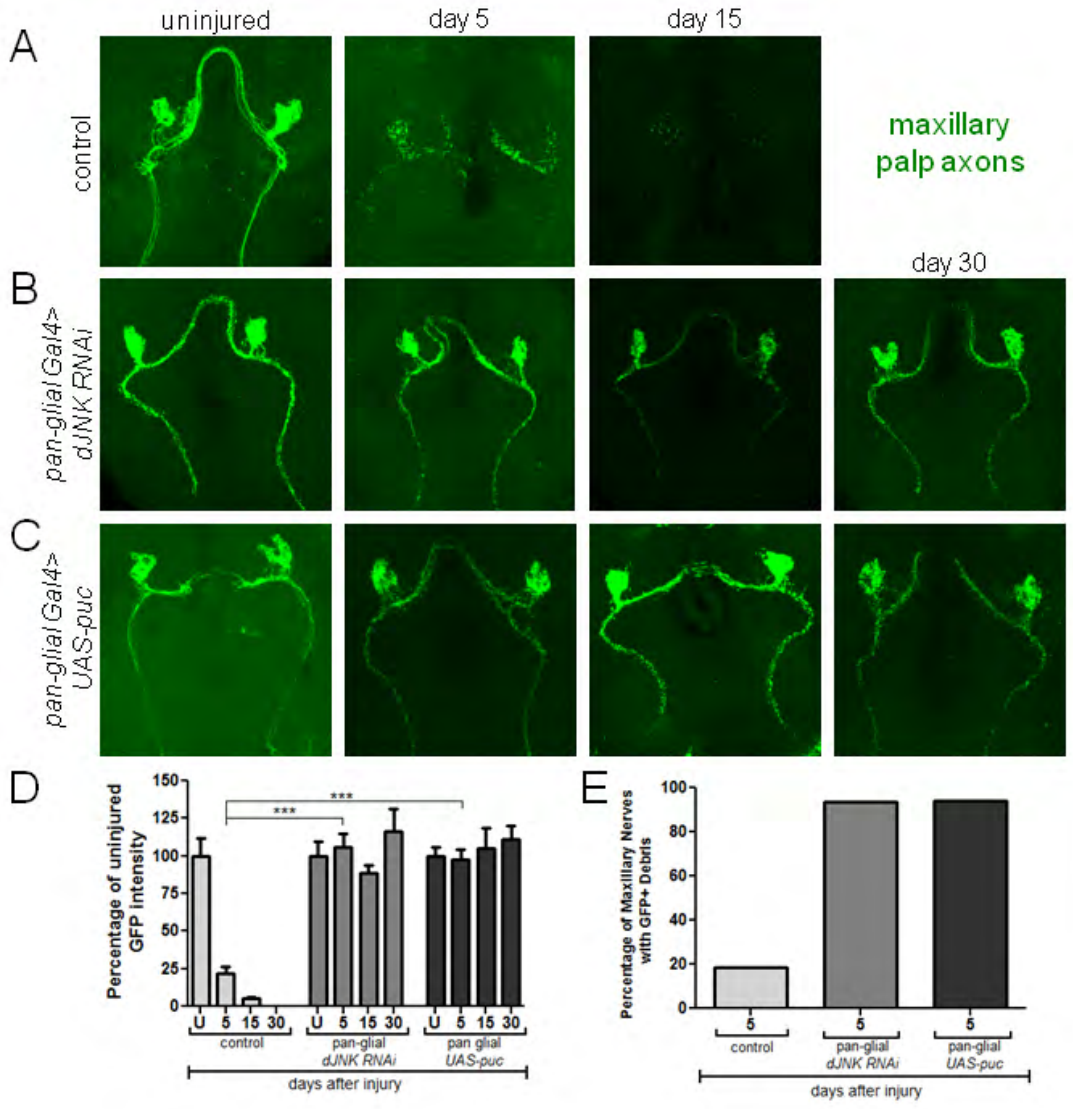


Figure 2.1
dJNK is required for clearance of axonal debris after nerve injury.

Figure 2.1 dJNK is required for clearance of axonal debris after nerve injury.

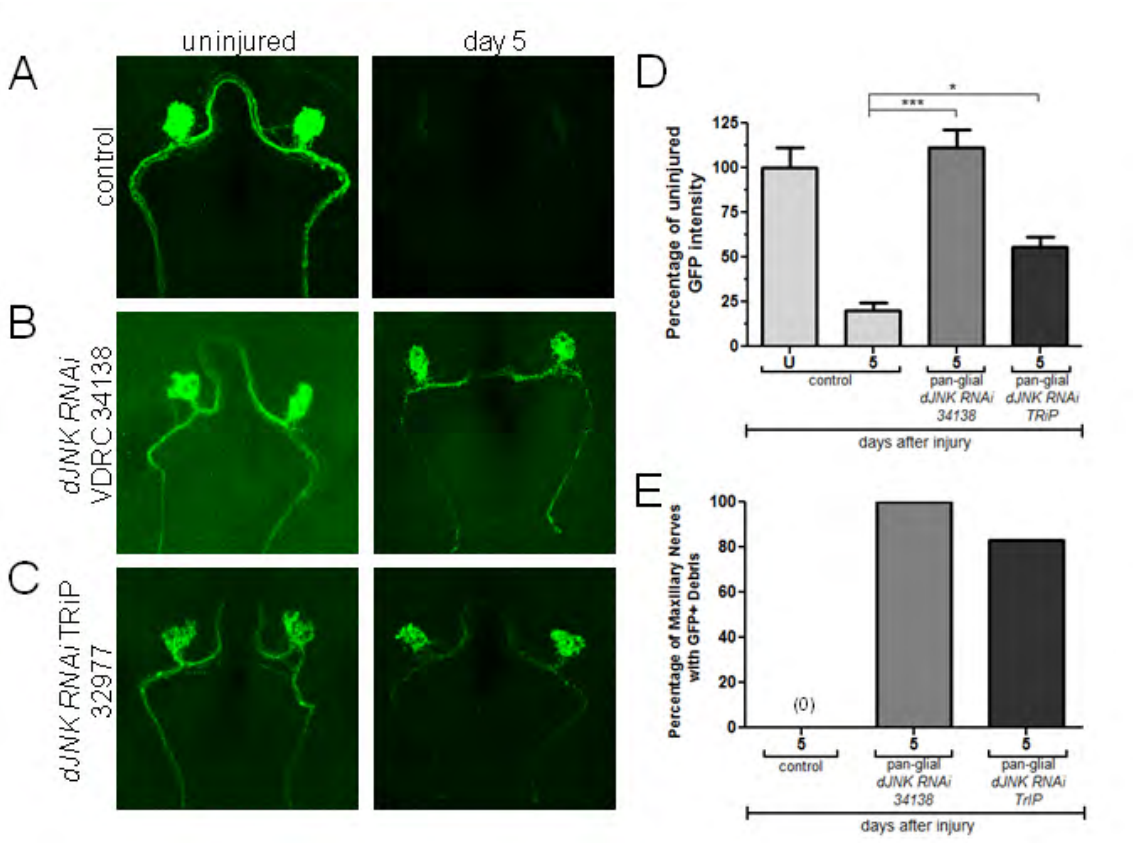
(A) Control animals (*OR85e-mCD8::GFP/+; repo-GAL4/+*) 0, 5, and 15 days after axotomy.

(B) Glial knockdown of dJNK using *repo-GAL4* to express *UAS-dJNK RNAi* (*OR85e::mCD8::GFP/UAS-dJNK RNAi*^{104569}*; repo-GAL4/+*).

(C) Glial overexpression of *UAS-puckered* using *repo-GAL4* (*OR85e::mCD8::GFP/+; repo-GAL4/UAS-puckered*).

(D) Quantification of GFP intensity in glomeruli for **A-C**. Error bars depict ± SEM. ***p<0.001.

(E) Quantification of maxillary nerves with GFP+ debris for **A-C**.



Supplementary Figure 2.1
Additional RNAi lines targeting dJNK suppress glial clearance of degenerating axons.

Supplementary Figure 2.1 Additional RNAi lines targeting dJNK suppress glial clearance of degenerating axons.

(A) Control animals (*OR85e-mCD8::GFP/+; repo-GAL4/+*) 0, 5, and 15 days after axotomy. Two additional non-overlapping *UAS-dJNK RNAi* lines: (B) *UAS-dJNK RNAi*^{34138} and (C) *UAS-dJNK RNAi*^{HMS00777}.

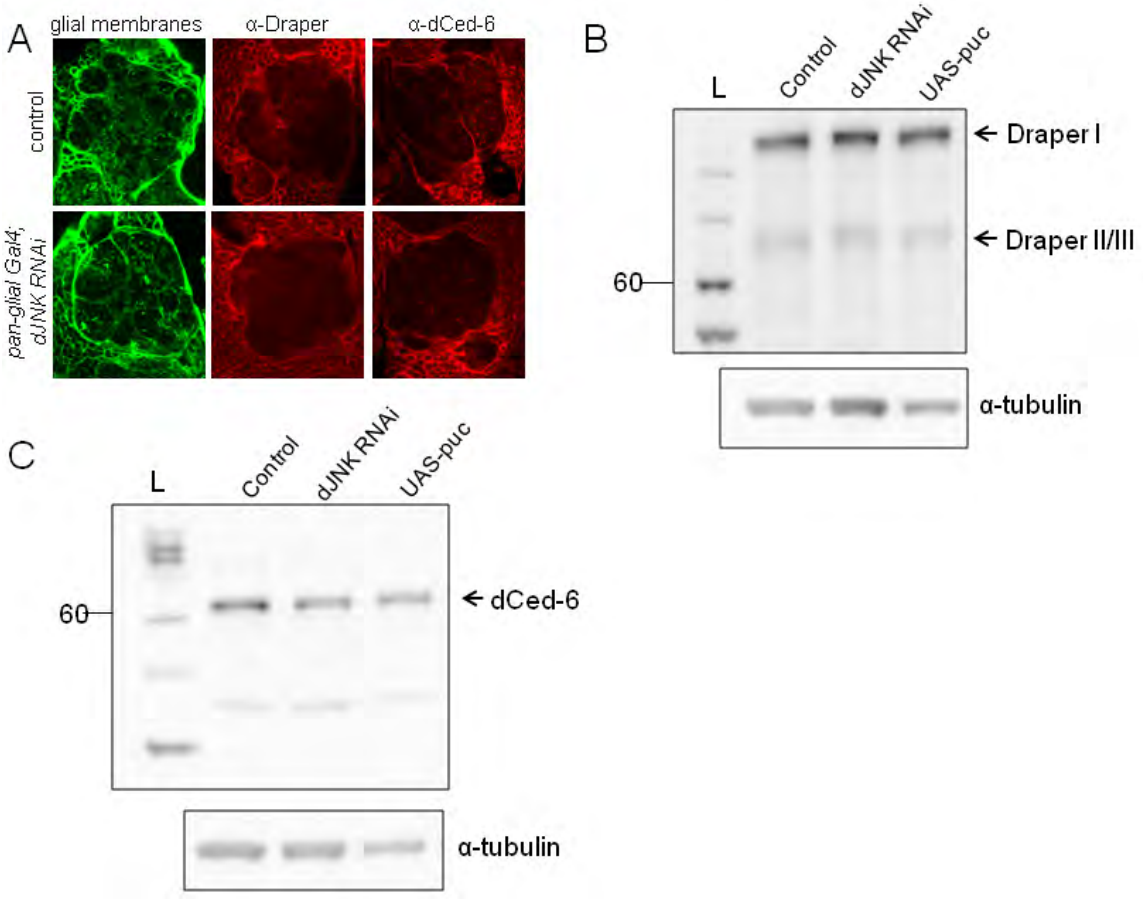
(D) Quantification of GFP+ axon material in experiments shown in A-C. Error bars depict mean ± SEM. *p<0.05, ***p<0.001.

(E) Quantification of maxillary nerves with GFP+ debris visible in experiments shown in A-C.

in these backgrounds, indicating glial BSK function is not required for axonal degradation.

dJNK signaling is known to be important for the development of a number of cell types. We examined glial morphology, numbers, position, and expression of the engulfment genes *draper* and *dCed-6* in the CNS of glial bsk^{RNAi} and *UAS-puc* animals but found no noticeable defects in glial cell development or engulfment gene regulation prior to injury (**Supplementary Figure 2.2**). Nevertheless, to exclude the possibility that engulfment phenotypes might arise from requirements for *bsk* in glial development we conditionally expressed bsk^{RNAi} in adult brain glia using the temperature sensitive $GAL80^{ts}$ repressor. At 18ºC $GAL80^{ts}$ represses activation of the GAL4/UAS binary system; however, at 29ºC $GAL80^{ts}$ is inactivated, thereby allowing for activation of GAL4/UAS. We crossed $GAL80^{ts}$ into control and glial bsk^{RNAi} backgrounds, raised animals at 18ºC, shifted them to 29ºC after eclosion for 7 days, and then performed axotomies. Consistent with a role for dJNK signaling in mature glia after axotomy, we found that adult-specific knockdown of *bsk* was sufficient to block glial engulfment of degenerating axons (**Figure 2.2A-B**).

Ensheathing glia are the primary cell type in the *Drosophila* neuropil that express molecular components of the engulfment machinery, including the Draper receptor and dCed-6 adaptor molecule, and they phagocytose axonal debris after axotomy (Doherty et al., 2009). To determine whether BSK functions in ensheathing glia we drove expression of bsk^{RNAi} or *UAS-puc* with the ensheathing glia-specific driver *mz0709-GAL4* and assayed glial clearance of degenerating axons. We found that suppression of dJNK



Supplementary Figure 2.2
Glial development and expression of engulfment genes is normal in glial dJNK^{RNAi} animals.

Supplementary Figure 2.2 Glial development and expression of engulfment genes is normal in glial dJNK^{RNAi} animals.

(A) Glial morphology (membrane tethered GFP, green), Draper (red) and dCed6 (red) immunostains in control and glial dJNK^{RNAi} animals.

(B) Draper protein levels are normal in brains dissected from controls and animals expressing glial dJNK^{RNAi} or glial overexpression of PUC. α-Tubulin was used as a loading control.

(C) dCed-6 protein levels are normal in brains dissected from controls and animals expressing glial dJNK^{RNAi} or glial overexpression of PUC. α-Tubulin was used as a loading control.

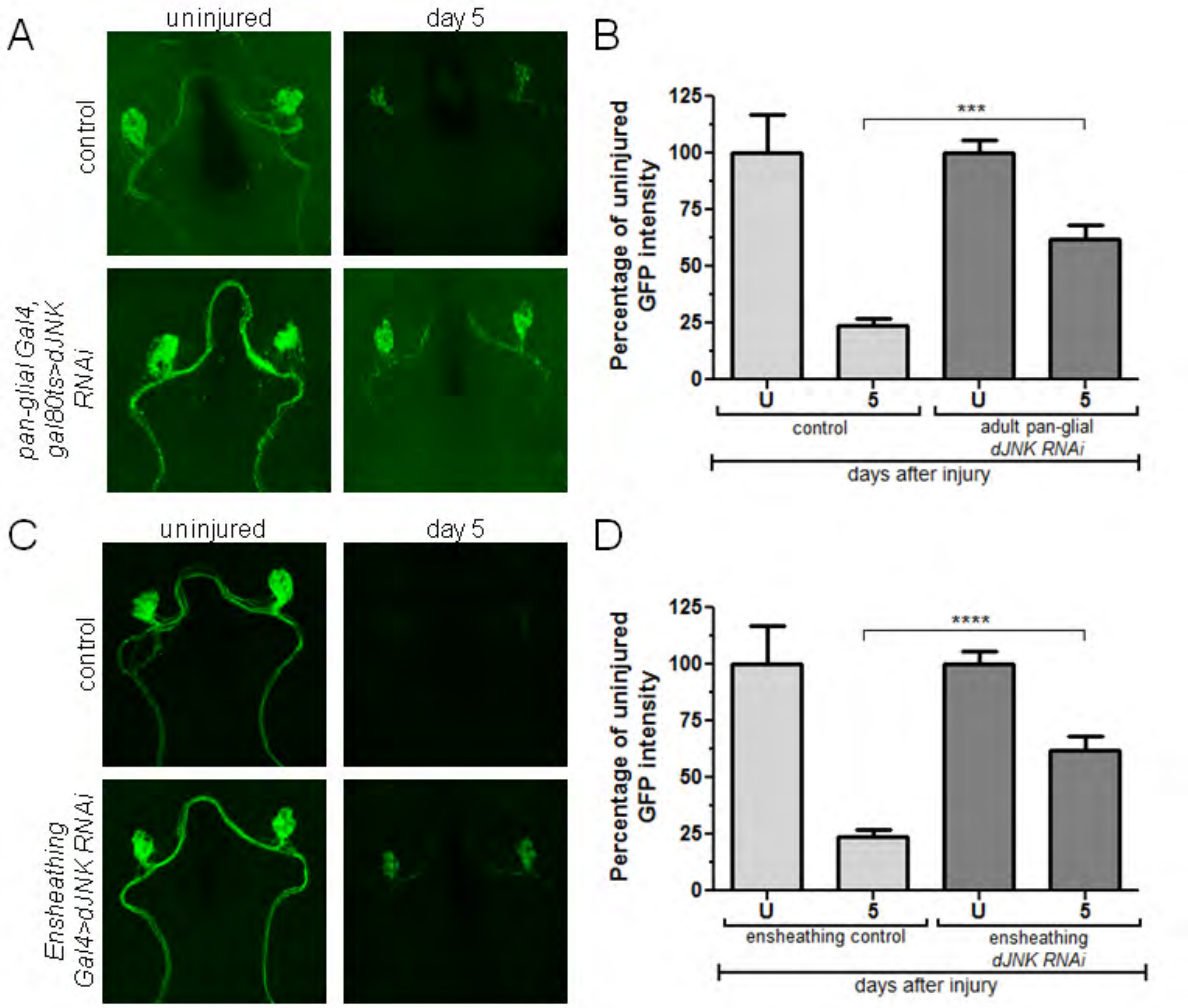


Figure 2.2
The requirement for dJNK in glial engulfment of axonal debris is adult- and ensheathing-glia specific.

Figure 2.2 The requirement for dJNK in glial engulfment of axonal debris is adult- and ensheathing-glia specific.

(A) Control animals (*OR85e::mCD8::GFP, tub-GAL80^{ts} /+; repo-GAL4/+*) and *dJNK^{RNAi}* animals (*OR85e::mCD8::GFP, tub-GAL80^{ts} /UAS-dJNK RNAi^{104569}; repo-GAL4/+*) were raised at 18^{o}C. After eclosion, adults were shifted to 29^{o}C for at least 7 days, maxillary palps were ablated and flies were kept at 29^{o}C for 5 days prior to assaying axon clearance.

(B) Quantification for A. Error bars depict mean ± SEM. ***p<0.001, ****p<0.0001.

(C) Control animals (*OR85e::mCD8::GFP/+; MZ0709-GAL4/+*) or animals with ensheathing glia-specific knockdown of dJNK (O*R85e::mCD8::GFP/UAS-dJNK RNAi^{104569}; mz0709-GAL4/+*) were axotomized and assayed for axon clearance 5 days after axotomy.

(D) Quantification for C. Error bars depict mean ± SEM. ***p<0.001, ****p<0.0001.

signaling in ensheathing glia was sufficient to block glial engulfment function (**Figure 2.2C-D**). Notably, suppressing dJNK signaling in astrocytes, the only other subtype of glial cells present in the antennal lobe neuropil, had no effect on glial clearance of axonal debris (data not shown).

These data identify dJNK signaling as a novel mediator of glial responses to axonal injury. Our data further show dJNK signaling is required in mature ensheathing glia—the same glial subtype in which Draper signals to promote phagocytosis of axonal debris.

Glial dJNK signaling involves a MAP kinase cascade which signals to the nucleus

dJNK has been shown to function downstream of a wide range of receptor types and to execute signaling events with a diversity of kinases. Precisely which signaling molecules dJNK interacts with appears to be context specific (Stronach, 2005). To identify additional molecules involved in dJNK signaling during glial phagocytic clearance of degenerating axons we assayed the roles of known components of JNK signaling pathways in *Drosophila* (**Figure 2.3A**). We found that glial-specific knockdown of the MAPKKKs Slipper and TAK1, the MAPKK MKK4, and the transcriptional factors Jun-Related Antigen (JRA, *Drosophila* c-Jun) and Kayak (KAY, *Drosophila* c-Fos) significantly suppressed glial clearance of degenerating axonal debris 5 days after axotomy (**Figure 2.3A, Supplementary Figure 2.3**). We confirmed the specificity of these RNAi transgenes by using additional RNAi lines that target non-overlapping regions of each transcript.

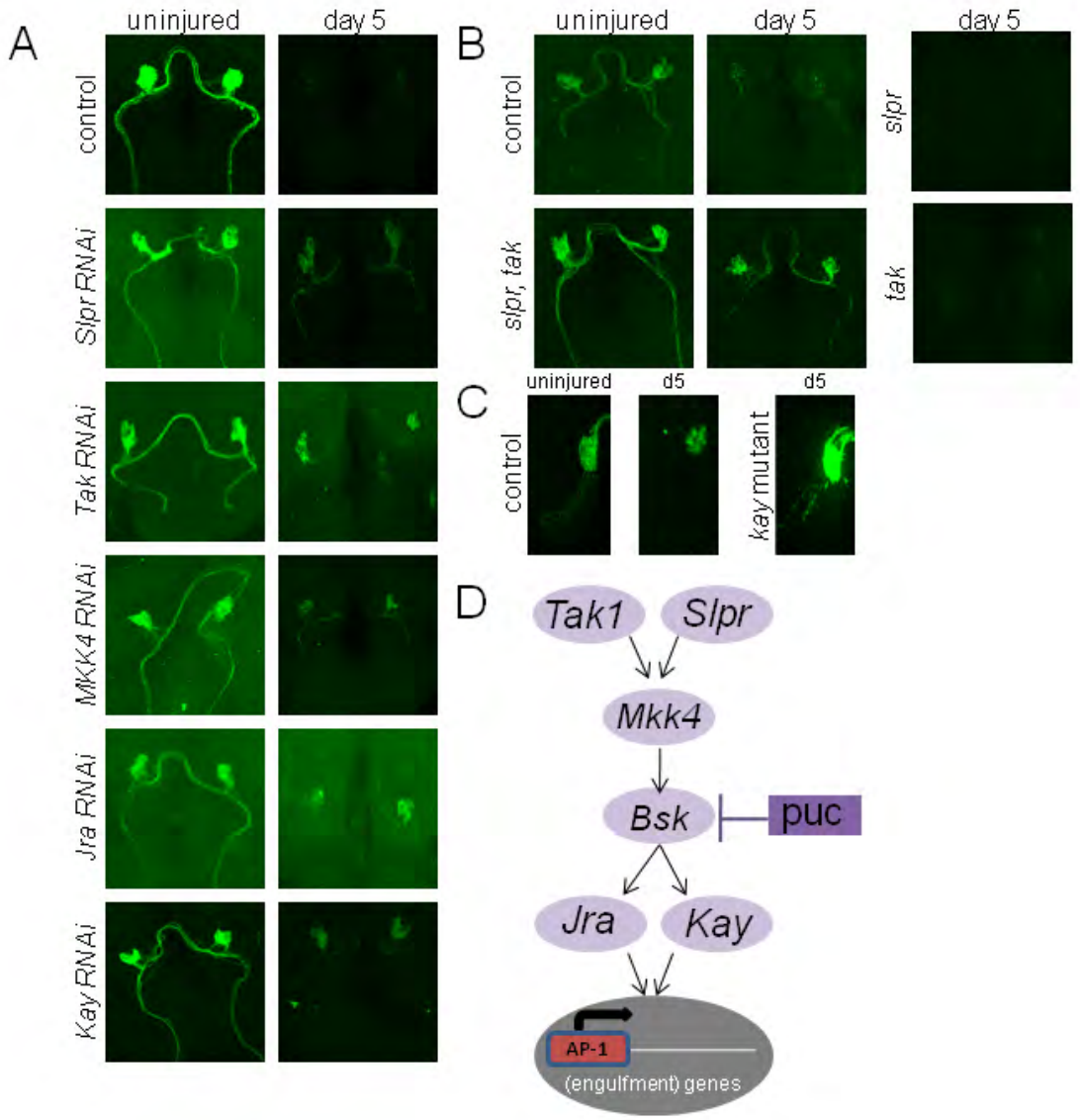


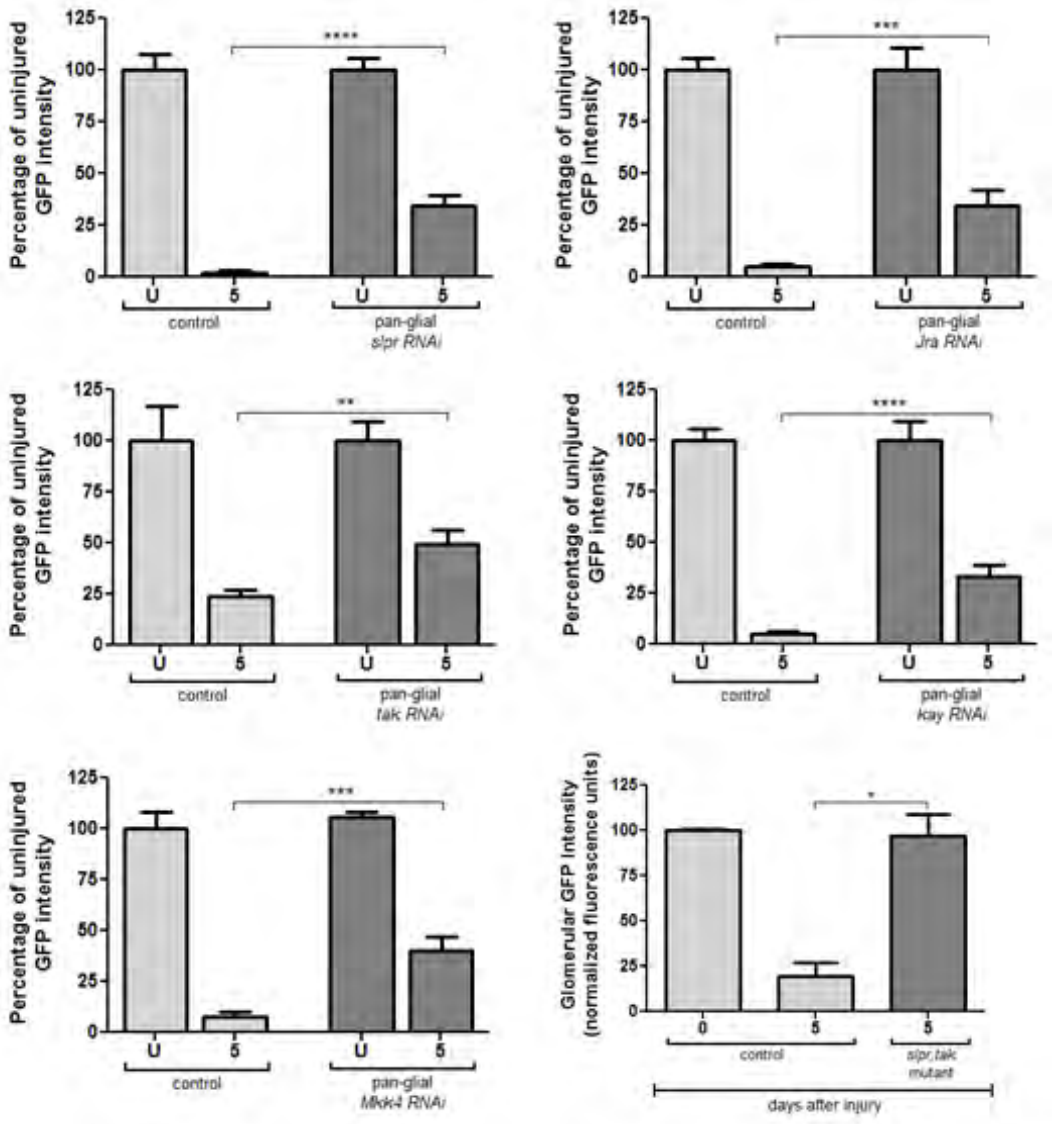
Figure 2.3
Glial c-Jun kinase signaling to the nucleus after axotomy involves SLPR, Tak, MKK4, JRA and KAY.

Figure 2.3 Glial c-Jun kinase signaling to the nucleus after axotomy involves SLPR, Tak, MKK4, JRA and KAY.

(A) *UAS-RNAi* constructs for the indicated genes were driven in glia using the *repo-GAL4* driver, axons were severed in adults and axonal debris was scored 5 days after axotomy as above. See Supplementary Figure 2.3 for quantifications.

(B) *slpr, tak* double mutants (*slpr*^{BS06}*, tak1/Y; OR85e::mCD8::GFP/+*) and (C) *y*^{1} *w*^{67c23}*; P{EPgy2}kay*^{EY00283} homozygous mutants were assayed for defects in axon clearance. *slpr* and *tak1* mutants were also assayed but no clearance defect was noted. Note—for *kayak* mutants clearance of antennal ORN axons was assayed using 22a-GAL4/UAS-mCD8-GFP.

(D) Model of dJNK signaling to the nucleus to induce engulfment gene expression.



Supplementary Figure 2.3
Glial c-Jun kinase signaling to the nucleus after axotomy involves SLPR, Tak, MKK4, JRA and KAY.

Supplementary Figure 2.3 Glial c-Jun kinase signaling to the nucleus after axotomy involves SLPR, Tak, MKK4, JRA and KAY.

Quantification of experiments in Figure 2.3. Percentage of uninjured glomerular GFP intensity is plotted and error bars depict mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

We next sought to provide genetic support for a role for this pathway in glial engulfment signaling and to explore the effects of complete loss of function of this JNK signaling pathway in the clearance of axonal debris. We therefore performed axotomies in *slipper*, *tak1*, and *kayak* mutants and examined their effects on the clearance of GFP-labeled degenerating axons. Clearance of axonal debris was largely normal in *slipper* (*slipper*^{BS506}) and *tak1* (*tak1*^{2}) null mutant backgrounds (**Figure 2.3B**); however, neuronal debris persisted at significant levels after axotomy in *slipper*^{BS06} *, tak1*^{2} double mutants (**Figure 2.3B, Supplementary Figure 2.3**). These data argue that the Slipper and TAK1 kinases likely function in a redundant fashion in the glial cells responding to axonal injury.

Positive dJNK signaling often leads to changes in gene expression through the AP-1 complex. *Drosophila* AP-1 (dAP-1) is composed of a heterodimer of JRA and Kayak. The significant phenotypes in *Jra* and *kayak* RNAi backgrounds argue for a role for dAP-1 in glial responses to axonal injury. To confirm this we assayed clearance of axonal debris in *y*^{1} *w*^{67c23}*; P{EPgy2}kay*^{EY00283} homozygous mutant mutants. We found that 7 days after axotomy the vast majority of axonal debris from severed antennal ORN axons remained in the CNS (**Figure 2.3C, Supplementary Figure 2.3**), indicating a strong blockade of glial engulfment activity.

Together, these data identify a novel role for a dJNK signaling pathway involving a Slipper/TAK1→MKK4→BSK→dAP-1 cascade (**Figure 2.3D**) in promoting glial engulfment of degenerating axons after axonal injury. Since loss of multiple components of this pathway (i.e. BSK, TAK1, SLPR, and KAY) block the clearance of a significant

amount of axonal debris, positive dJNK signaling appears to be a central regulator of glial responses to axonal injury. Based on the requirements for dAP-1, we propose that dJNK signaling acts to regulate glial engulfment behavior through dAP-1-dependent transcriptional changes.

JNK signaling is required for axotomy-induced increases in glial Draper levels

To explore potential roles for dJNK signaling in regulating the expression of glial genes we examined expression of the engulfment receptor Draper in bsk^{RNAi} and *UAS-puc* backgrounds. Prior to injury, Draper levels were indistinguishable from control animals when we drove bsk^{RNAi} or *UAS-puc* in adult brain glia as judged by immunofluorescence (**Supplementary Figure 2.2A, Figure 2.4A**) and Western blot (**Supplementary Figure 2.2B**). Therefore loss of JNK signaling does not appear to affect baseline levels of Draper expression.

Antennal ablation severs axons ~88% of all ORN axons innervating the antennal lobe, resulting in widespread axonal degeneration throughout this brain region and in turn a robust increase in Draper levels in glia surrounding the site of injury in wild type (**Figure 2.4A**) (MacDonald et al., 2006; Doherty et al., 2009). Interestingly, we found a complete absence of this axotomy-induced increase in Draper levels when we drove glial-specific expression of bsk^{RNAi} (**Figure 2.4A**) or *UAS-puc* (**Supplementary Figure 2.4A**). Thus, loss of JNK signaling suppresses axotomy-induced changes in glial Draper levels after antennal ablation. These data are consistent with the notion that dAP-1 might regulate *draper* expression.

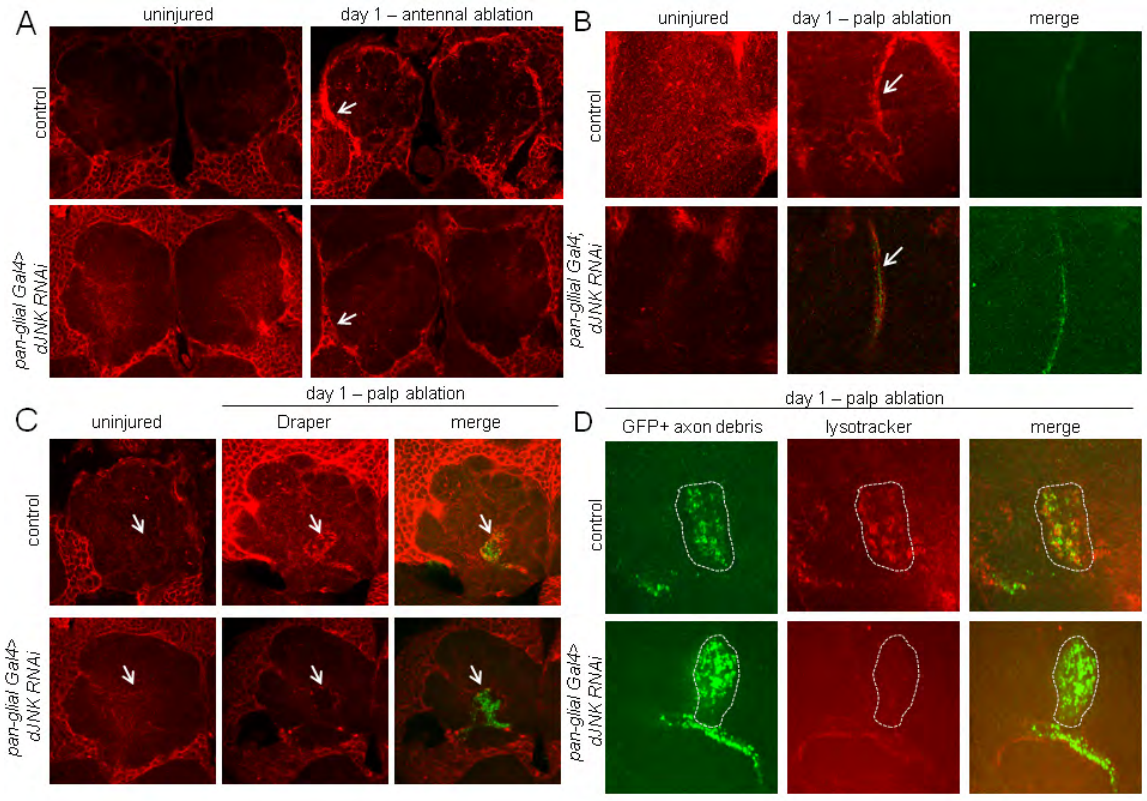


Figure 2.4
dJNK is required for Draper up-regulation after axotomy and activation of glial phagocytic function.

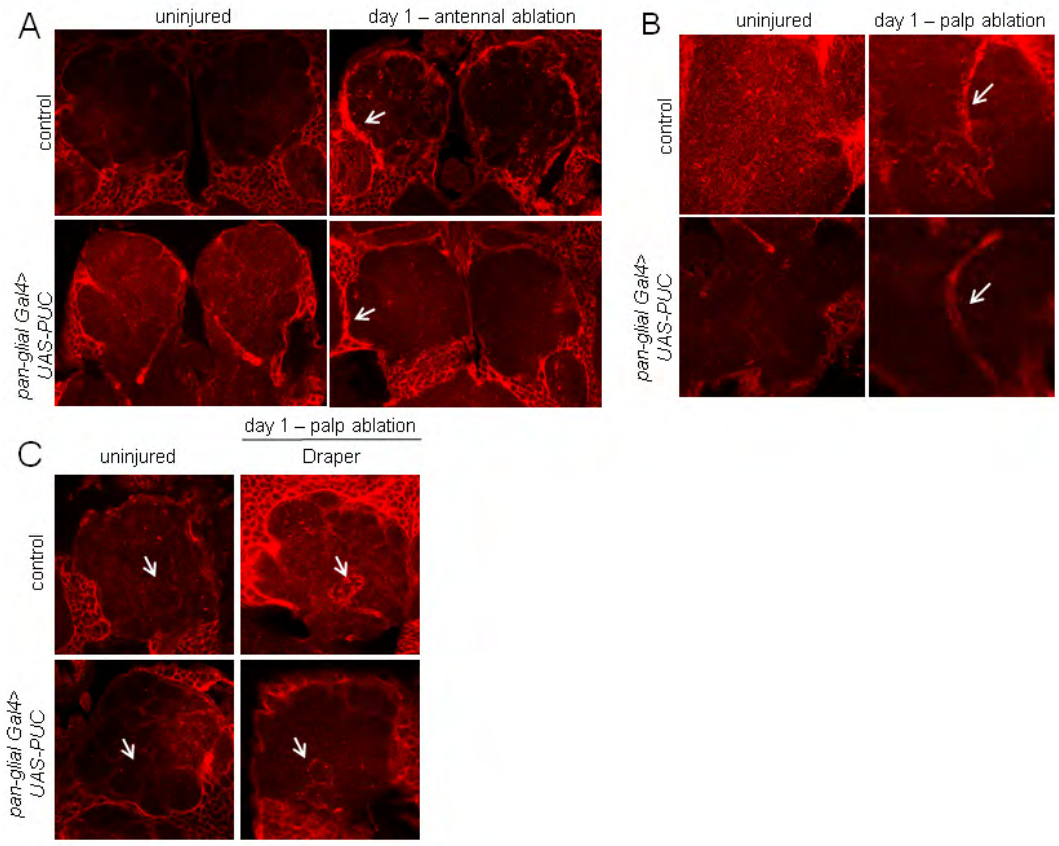
Figure 2.4 dJNK is required for Draper up-regulation after axotomy and activation of glial phagocytic function.

(A) Immunostains for Draper in control or glial $dJNK^{RNAi}$ animals before or after antennal ablation. Arrows highlight up-regulation of Draper in ensheathing glia (control) or lack thereof ($dJNK^{RNAi}$).

(B) Draper (red) localization along the maxillary nerve (arrows) before or 1 day after maxillary palp ablation.

(C) Draper immunostains (red) in a single antennal lobe before or after axotomy. A subset of maxillary palp ORN axons are shown (green). Note the lack of Draper staining within the glomerulus of glial $dJNK^{RNAi}$ animals compared to controls (arrows).

(D) Lysotracker Red stains for lysosomal activity one day after maxillary palp ablation. Note the punctate accumulation of Lysotracker Red in the OR85e+ glomerulus (green, white dotted circle) in controls, but not in glial $dJNK^{RNAi}$ animals.



Supplementary Figure 2.4
dJNK is required for Draper up-regulation after axotomy and activation of glial phagocytic function.

Supplementary Figure 2.4 dJNK is required for Draper up-regulation after axotomy and activation of glial phagocytic function.

(A) Immunostains for Draper in control or glial *UAS-PUC* animals before or after antennal ablation. Arrows highlight up-regulation of Draper in ensheathing glia (control) or lack thereof (*UAS-PUC*).

(B) Draper (red) localization along the maxillary nerve (arrows) before or 1 day after maxillary palp ablation.

(C) Draper immunostains (red) in a single antennal lobe before or after axotomy. Note the lack of Draper staining within the glomerulus of glial *UAS-PUC* animals compared to controls (arrows).

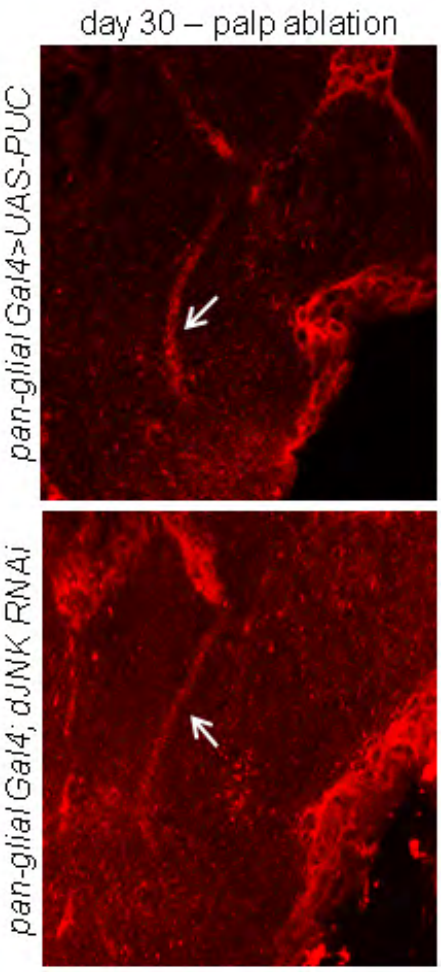
Glia with depleted dJNK signaling recruit Draper to local axonal debris

The above data raised the possibility that glia lacking dJNK signaling are incapable of responding to axonal injury—specifically, glia might be unable to extend membranes to degenerating axons and activate a phagocytic phenotype. To determine whether glial cells lacking dJNK signaling were capable of recognizing an injury and recruiting Draper specifically to severed axons we assayed axon clearance and Draper localization after maxillary palp ablation. Surgical removal of maxillary palps severs only ~12% of all ORN axons, which innervate 6 uniquely identifiable antennal lobe glomeruli. This modest injury does not lead to detectable increases in Draper levels in glia surrounding the antennal lobe but one can examine Draper recruitment to (1) degenerating axons within identifiable antennal lobe glomeruli or (2) ORNs housed within the maxillary nerve as it projects to the antennal lobe (**Figure 2.4B-C**) (MacDonald et al., 2006). Assaying antennal lobe glomerular localization of Draper is especially useful for determining whether Draper or other components of the engulfment machinery can be recruited to axonal debris, since the engulfing cells must extend membranes into glomeruli to reach engulfment targets (see below). In contrast, ensheathing glia in the maxillary nerve are already immediately adjacent to their engulfment targets (i.e. they don't have to migrate), so the maxillary nerve is useful for determining whether glia exhibit any changes in Draper localization after axotomy even in backgrounds where glia are incapable of migratory behavior (MacDonald et al., 2006).

One day after maxillary palp ablation in control animals we found a strong increase in Draper staining along the maxillary nerve (**Figure 2.4B**) and Draper levels

returned to baseline levels within 7 days after axotomy. Surprisingly, we found that glial expression of either *bsk*^{RNAi} or *UAS-puc* did not suppress increases in Draper around the maxillary nerve: in both *bsk*^{RNAi} and *UAS-puc* backgrounds Draper accumulated on severed maxillary palp ORN axons, though at levels slightly lower than controls (**Figure 2.4B, Supplementary Figure 2.4B**). Thus, glial cells with depleted dJNK signaling are capable of detecting axonal injury and responding by recruiting the Draper receptor to severed axons. This is notably different from what is observed when the Draper signaling pathway is inactivated—elimination of the non-receptor tyrosine kinase Shark or Src family kinase SRC42A from glia completely suppresses Draper recruitment to severed axons along the maxillary nerve. This indicates that dJNK signaling acts downstream of initial recruitment of Draper to axonal debris.

Interestingly, while we observe initial recruitment of Draper to degenerating maxillary ORN axons is normal in animals deficient in glial dJNK signaling, glial cells appear incapable of terminating their responses to axonal injury. Even 30 days after axotomy Draper levels along the maxillary nerve in glial *bsk*^{RNAi} or *UAS-puc* backgrounds remained elevated (**Supplementary Figure 2.5**). We interpret these data to mean that in addition to its role in engulfment of axonal debris, dJNK signaling is also critical for termination of glial responses to axonal injury.



Supplementary Figure 2.5
Glia fail to terminate their response to axonal injury in animals lacking dJNK

Supplementary Figure 2.5 Glia fail to terminate their response to injured axons in animals lacking dJNK

Immunostains for Draper in glial UAS-dJNK^{RNAi} and glial *UAS-PUC* animals 30 days after maxillary palp ablation. Arrows highlight up-regulation of Draper along the maxillary nerve. In control animals, Draper levels return to baseline within 10 days of injury.

Loss of dJNK signaling suppresses extension of glial membranes to degenerating axons and activation of phagocytic function

We next asked whether loss of dJNK signaling blocked the extension of glial membranes to degenerating axons. We therefore assayed glial recruitment of Draper to degenerating maxillary ORNs in the antennal lobe, where glia must extend membranes into glomeruli to reach their engulfment targets. In control animals, Draper immunoreactivity was detectable one day after maxillary palp ablation throughout the glomeruli housing degenerating axonal debris (**Figure 2.4C**). In contrast, while we observed an increase in Draper immunoreactivity around glomeruli in glial bsk^{RNAi} or *UAS-puc* animals, Draper immunoreactivity failed to accumulate in central regions of these structures (**Figure 2.4C, Supplementary Figure 2.4C**). Thus, dJNK signaling is essential for glial membrane extension to engulfment targets.

Once glia have inserted membranes into glomeruli they activate a phagocytic program—glia become Lysotracker positive (a marker for lysosomal activity) and internalize axonal fragments (Ziegenfuss et al., 2012). When we examined Lysotracker activity 1 day after maxillary palp ablations we observed strong punctuate Lysotracker staining within glomeruli containing degenerating axons in control animals. However, Lysotracker staining was absent from these glomeruli in glial bsk^{RNAi} animals (**Figure 2.4D**). Together, these data indicate that dJNK signaling is required for glial membrane extension into glomeruli for engulfment of degenerating axonal material and for activation of lysosomal activity during glia phagocytic function.

Re-expression of *draper* in glia is sufficient to bypass requirements for dJNK signaling

dJNK is known to function upstream of the transcription factor (dAP-1), and we have shown that loss of dAP-1 components suppress glial engulfment of degeneration axons. In addition, we observed that glia responding to axonal injury in bsk^{RNAi} or *UAS-puc* animals failed to up-regulate Draper in response to antennal ORN axotomy. Together these observations raised the intriguing possibility that glial dJNK signaling might promote glial responses to axon injury through activation of *draper* expression. To explore this possibility we drove the expression of Draper-I in control, glial bsk^{RNAi} and *UAS-puc* animals and assayed clearance of degenerating axonal debris. Expression of Draper-I in control animals did not affect axon clearance as axonal debris was cleared within 5 days (**Figure 2.5**). As described above, axonal debris remained 5 days after axotomy in glial bsk^{RNAi} or *UAS-puc* animals. However, when we expressed Draper-I in glial bsk^{RNAi} or *UAS-puc* animals we found engulfment defects were completely rescued (**Figure 2.5**). Thus, increased expression of Draper-I is sufficient to overcome engulfment defects in animals with depleted glial dJNK signaling. These data argue that a primary role for dJNK signaling is increasing levels of Draper expression to promote axon clearance after axotomy.

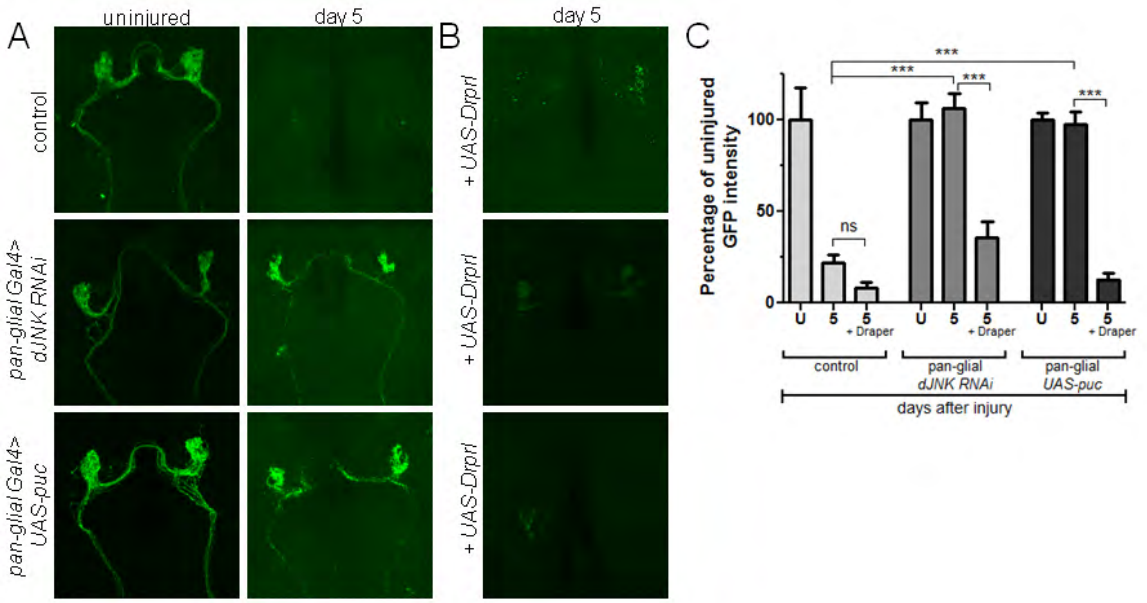


Figure 2.5
Over-expression of Draper rescues engulfment defects in dJNK knockdown animals.

Figure 2.5 Over-expression of Draper rescues engulfment defects in dJNK knockdown animals.

(A) Control, glial dJNK^{RNAi}, or glial expressed PUC animals before and 5 days after axotomy.

(B) Control, glial dJNK^{RNAi}, or glial expressed PUC animals 5 days after axotomy with *UAS-Draper-I*.

(C) Quantification from A,B. Data was quantified using a one-way ANOVA and Tukey's Multiple Comparison Test. Error bars depict mean ± SEM. ***$p<0.001$.

Discussion

This study reveals that axonal injury in the *Drosophila* brain activates a glial signaling pathway composed of Slipper and TAK1, MKK4, dJNK, and the dAP-1 complex which ultimately leads to increased levels of Draper in glia and activation of phagocytic function. Interestingly we have recently found that *draper* gene expression is transcriptionally up-regulated after axonal injury (Logan et al., 2012); however, the signaling mechanisms that transduce injury signals from Draper to the nucleus have remained unclear. dAP-1 is, to our knowledge, the first transcriptional regulator shown to modulate glial phagocytic activation and clearance of degenerating axons, and, based on our rescue experiments, it likely does so through regulation of Draper levels.

Loss of glial dJNK, or suppression of dJNK signaling by over-expression of the phosphatase PUC, resulted in axonal debris lingering in the brain for at least 30 days after axotomy (i.e. longer than the mean lifespan of *Drosophila*). This is among the strongest glial engulfment phenotypes observed in *Drosophila*, hence dJNK is a critical *in vivo* regulator of glial engulfment activity. We suspect that activation of dJNK is downstream of Draper activation. Loss of Draper, or its immediate downstream signaling molecules SRC42A, Shark, and Rac1 (Ziegenfuss et al., 2008, 2012) also block injury-induced up-regulation of Draper, and Rac1 (Nagata et al., 1998; Teramoto et al., 1996) and Src molecules (Tateno et al., 2000) have been shown to be capable of activating the Slipper/TAK complex upstream of JNK. In addition, we note that loss of JNK signaling closely phenocopies *draper* null mutants. In *draper* null animals glia fail to respond

morphologically to axonal injury, do not activate phagolysosome maturation, and axons are not cleared from the brain (MacDonald et al., 2006; Ziegenfuss et al., 2012). However, it remains an open possibility that dJNK signaling is activated by a yet-to-be-identified additional engulfment receptor that is stimulated by axonal injury.

In animals with depleted glial dJNK, glia successfully recruit the small amount of Draper that is present in these cells prior to injury (referred to as basal Draper) to severed axons in the maxillary nerve, indicating that dJNK is not required for glial cells to receive the injury signal or for Draper accumulation on degenerating axons. However dJNK-deficient glia fail to increase Draper levels in response to injury, to activate phagocytic pathways, and to engulf axonal debris. We interpret these data to mean that dJNK-dependent increases in levels of Draper after axotomy are critical for axon clearance. This notion is supported by multiple lines of evidence. First, we show a requirement for the dAP-1 transcriptional complex in glial engulfment of axonal debris. Second, we have recently demonstrated that *draper* is transcriptionally up-regulated after axonal injury (Logan et al., 2012). Finally, we show that overexpression of Draper is sufficient to rescue JNK^{RNAi} or PUC misexpression phenotypes. We propose that basal Draper (i.e. that before injury) is present in glia in the healthy brain to sense injury; upon activation, dJNK signaling promotes dramatic increases in Draper through the cascade described above and increased Draper after injury allows efficient clearance of axonal debris.

Requirements for c-Jun kinases (JNK) signaling appear to be conserved in mammalian reactive gliosis. JNK signaling (as measured with P-JNK antibodies) appears to be activated in response to a broad range of neural injuries including sciatic nerve

lesion (Ma and Quirion, 2002), accumulation of glial fibrillary acidic protein (GFAP) in models for Alexanders' disease (Tang et al., 2006), or focal demyelination (Gadea et al., 2008). To date, the primary roles for JNK in mammalian glia appear to include modulating astrocyte proliferation or pro-inflammatory responses after neural injury. For example, JNK is activated in astrocytes in response to focal demyelination and in primary astrocyte cultures JNK appears to act upstream of injury-induced glial proliferation (Gadea et al., 2008). Additionally, LPS treatment of astrocytes *in vitro* leads to JNK-dependent increases in CPEB1 phosphorylation and activation of reactive oxygen species which promote inflammation (Kim et al., 2011). Our data argue that a primary role for dJNK activation in *Drosophila* glia after axonal injury is increasing levels of Draper and promoting phagocytic activity of engulfing glia. Such roles have not been assayed for in mammalian astrocytes, but post-injury changes in astrocyte expression of a number of engulfment factors are well established (Zamanian et al., 2012) and we predict that the AP-1 transcription factor is involved in some of these injury-regulated transcriptional events.

Experimental procedures

Drosophila stocks

Fly crosses were performed on standard fly media at 25^{o}C with the exception of the *slpr^{BS06}*, *tak1^{2527}/FM7* crosses which were grown on semi-defined medium, a rich medium described by Backhaus et al. (Backhaus et al., 1984).

The following *Drosophila* strains were used: *repo-GAL4* (Leiserson et al., 2000), *OR85e-mCD8::GFP* (gift from B. Dickson, Research Institute of Molecular Pathology, Vienna, Austria), *mZ0709-GAL4* (Ito et al., 1995), *pUAST-mCD8::GFP* (Lee and Luo, 2001), *OR22a-GAL4* (Dobritsa et al., 2003), *OR85e-GAL4* (kindly provided by J. Carlson), *slpr^{BS06}*, *tak1^{2527}/FM7* (gift from B. Stronach), and *pUAST-Draper-I* (Logan, 2012).

The following RNAi stocks were obtained from VDRC: 104569 *(bsk),* 34138 *(bsk),* 34898 *(tak),* 33516 *(slpr),* 33518 *(slpr),* 106449 *(slpr),* 26928 *(MKK4),* 26929 *(MKK4),* 108561 *(MKK4),* 10835 *(Jra),* 107997 *(Jra),* 6212 *(kay).*

The following stocks were obtained from the Bloomington Stock Center: y^{1} w^{67c23}; P{EPgy2}kay^{EY00283}, *y-w-;UAS-puckered* (pucScer\UAS.cMa), and *y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00777}attP2*.

Immunolabeling and confocal microscopy

Standard methods were used for dissection, fixation, and antibody labeling of the adult *Drosophila* brain (MacDonald and Beach, 2006; Vosshall et al., 2000). Primary antibodies were used at the following concentrations: mouse anti-GFP, 1:250 (Life Technologies); rabbit anti-Draper, 1:500 (Freeman et al., 2003); FITC anti-mouse IgG, 1:200; Cy3 anti-rabbit IgG, 1:200; (Jackson ImmunoResearch)

Lysotracker staining was performed as described in Ziegenfuss et. al.(Ziegenfuss et al., 2012). Briefly, brains were dissected in PBS, Lysotracker Red was added (1:5000) and samples were rocked in the dark at room temperature for 15 minutes. Brains were washed with PBS 5 times quickly then rocked in PBS for 15 minutes at room temperature in the dark. Brains were then fixed with 4% formaldehyde in PBS/Triton for 30 minutes, rocking at room temperature in the dark. The standard antibody staining protocol was then performed as described above. Vectashield was applied to the brains and they were stored at 4^{o}C for one hour before imaging on an Intelligent Imaging Innovations Everest spinning disk confocal microscope.

Images were quantified as previously described in MacDonald et. al. (2006). Statistics were performed using GraphPad Prism. In all figures except for Figure 2.1, Supplementary Figure 2.1, and Figure 2.5, data was analyzed using a two-tailed Student's t-test. In Figure 2.1, Supplementary Figure 2.1, and Figure 2.5, a one-way ANOVA and Tukey's Multiple Comparison Test was used.

Western blot

Drosophila brains of the indicated genotype were dissected in PBS and homogenized in SDS loading buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% -mercaptoethanol, 0.01% bromophenol blue). For Western analysis, sample containing approximately 4 brains were loaded onto 10% SDS-PAGE gels (BioRad), transferred to nitrocellulose membranes (BioRad), and probed with rabbit anti-Draper (Freeman et al., 2003) or rabbit anti-dCed-6 (Awasaki et al., 2006) antibody at 1:1000 diluted in PBS/0.01% Tween-20/5% dry milk. Blots were incubated overnight at 4 degrees, washed several times in PBS/0.01% Tween-20 and probed with the appropriate HRP conjugated secondary antibody for 2 hours at room temperature. Additional washes were performed and the blot was developed using chemiluminescence (Amersham ECL Plus), and detected with a Fujifilm Luminescent Imager. The protein blot was stripped with mild stripping buffer (0.2M glycine, 0.1% sodium dodecyl sulfate, 1% Tween, pH 2.2) at room temperature followed by washes in 1XPBS and 1XPBS + 0.01% Tween-20 and then re-probed with mouse anti-tubulin (Sigma), 1:1000.

CHAPTER III

Discussion and Conclusions

Until recently, relatively little was known about glial biology as the majority of neuroscience research focused on the neuron. Our knowledge of the molecular details of glial biology has been aided by work done in model organisms such as *Drosophila melanogaster*. Despite obvious differences between mammals and *Drosophila* (such as the ratio of glial cells to neurons which is much lower in *Drosophila*, differences in specific glial subtypes, and the lack of myelination in *Drosophila*), it is clear that many basic molecular underpinnings of glial cell biology are conserved between vertebrates and flies.

In this dissertation I contribute to the understanding of how glial cells in the CNS respond to neuronal injury and phagocytose the resultant debris. Prior to this work, it was known that Draper (dCed-1), an engulfment receptor, is required for glia to receive an injury signal from degenerating axons and to engulf degenerating axon debris (MacDonald et al., 2006). Activation of Draper signaling involves SRC42A (a Src family kinase, SFK), Shark (a Syk-like non-receptor tyrosine kinase), and dCed-6 in a process similar to mammalian immunoreceptor- SRC42A - Shark signaling events (Ziegenfuss et al., 2008; Doherty et al., 2009). Concurrent with my work, it was found that Rac1, a small GTPase, is also required for glial cells to become activated downstream of Draper activation (Ziegenfuss et al., 2012). The activation of Rac1 is mediated by its phosphorylation by a Guanine Exchange Factor (GEF) composed of dCed-2 (Crk), dCed-5 (MBC) and dCed-12 (Ziegenfuss, Doherty, and Freeman 2012). Concurrent with my work, it was also found that STAT92E acts downstream of Draper

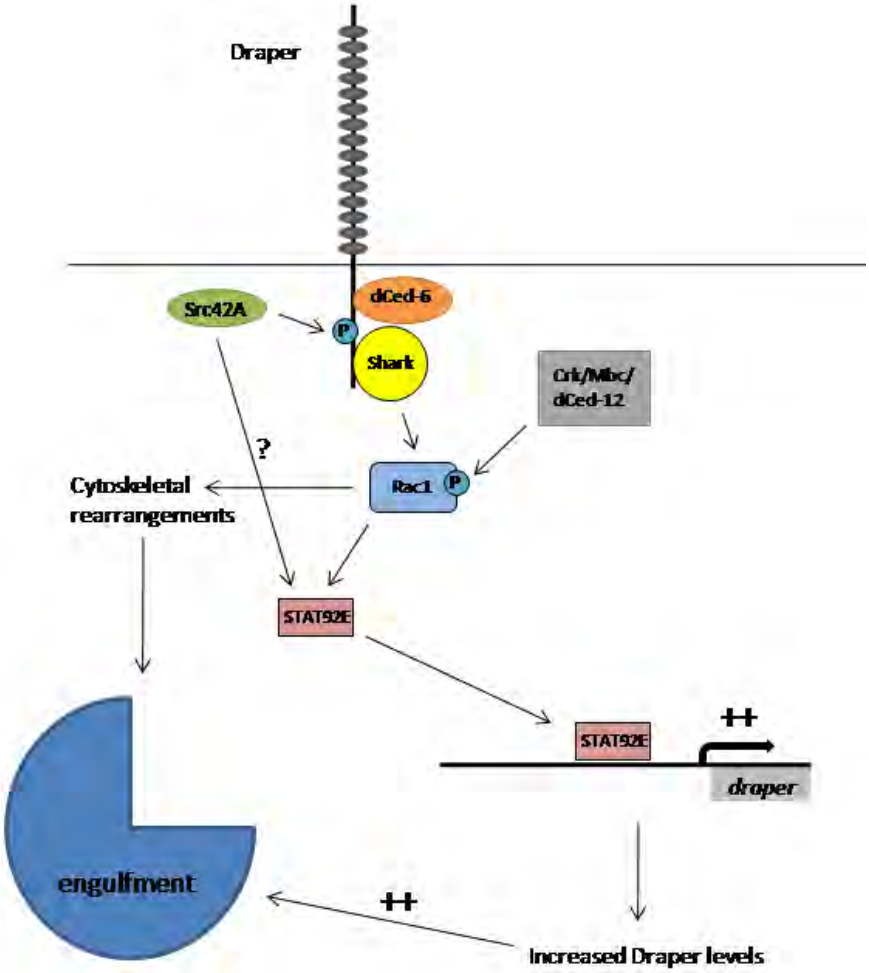


Figure 3.1
Current proposed model of glial engulfment in *Drosophila*

Figure 3.1 Current proposed model of glial engulfment in *Drosophila*

Activation of the engulfment receptor Draper initiates the glial reaction to axon injury. Draper signaling requires dCed-6, SRC42A and Shark and leads to the activation of Rac1. Rac1 may also be activated by phosphorylation by the Crk/MBC/dCed-12 complex. Rac1 activates downstream targets that promote engulfment. Concurrent with my work, it was discovered that STAT92E is required for glial engulfment to occur. STAT92 may be activated downstream of Rac1 or possibly SRC42A. STAT92E activation contributes to the transcriptional increase of Draper that occurs in glial cells in response to axotomy. Ultimately, activation of signaling downstream of Draper leads to cytoskeletal rearrangements and engulfment of degenerating axonal debris.

and Rac1 activation to contribute to the transcriptional increase in Draper that occurs in glial cells after axotomy. Ultimately, activation of Draper leads to the migration of glial membranes to degenerating debris, an increase in Draper protein levels, and the eventual phagocytosis and clearance of the axonal material.

Despite the recent advances that have been made in our understanding of the molecular processes underlying glial engulfment, many important questions remain. How does Draper-SFK-Syk activation translate to changes in the cell that promote activation and engulfment? How does Draper receptor activation lead to an increase in Draper protein? Does Draper signaling reach the nucleus to activate transcription of injury responsive genes and therefore promote phagocytosis? Are there other pathways downstream of or in parallel to Draper signaling that lead to activation and engulfment? My work, stemming from an RNAi screen for genes required for glial response to axonal injury, has provided answers to some of these questions. Specifically, I have identified a novel *in vivo* role for the c-Jun NH_2-terminal kinase (JNK) signaling cascade in the glial phagocytic program after axonal injury and have shown that Draper activation leads to signaling to the nucleus via the AP-1 transcription factor downstream of dJNK.

dJNK is required for glia to phagocytose neuronal debris in the CNS

In Chapter II of this thesis I show that the dJNK signaling cascade is required in glial cells for phagocytosis of degenerating axonal debris (**Figure 3.2, yellow**). Specifically, the MAPKKKs Slipper and TAK1, the MAPKK MKK4, the MAPK dJNK and the transcription factor dAP-1 are each required for phagocytosis to occur. When each of

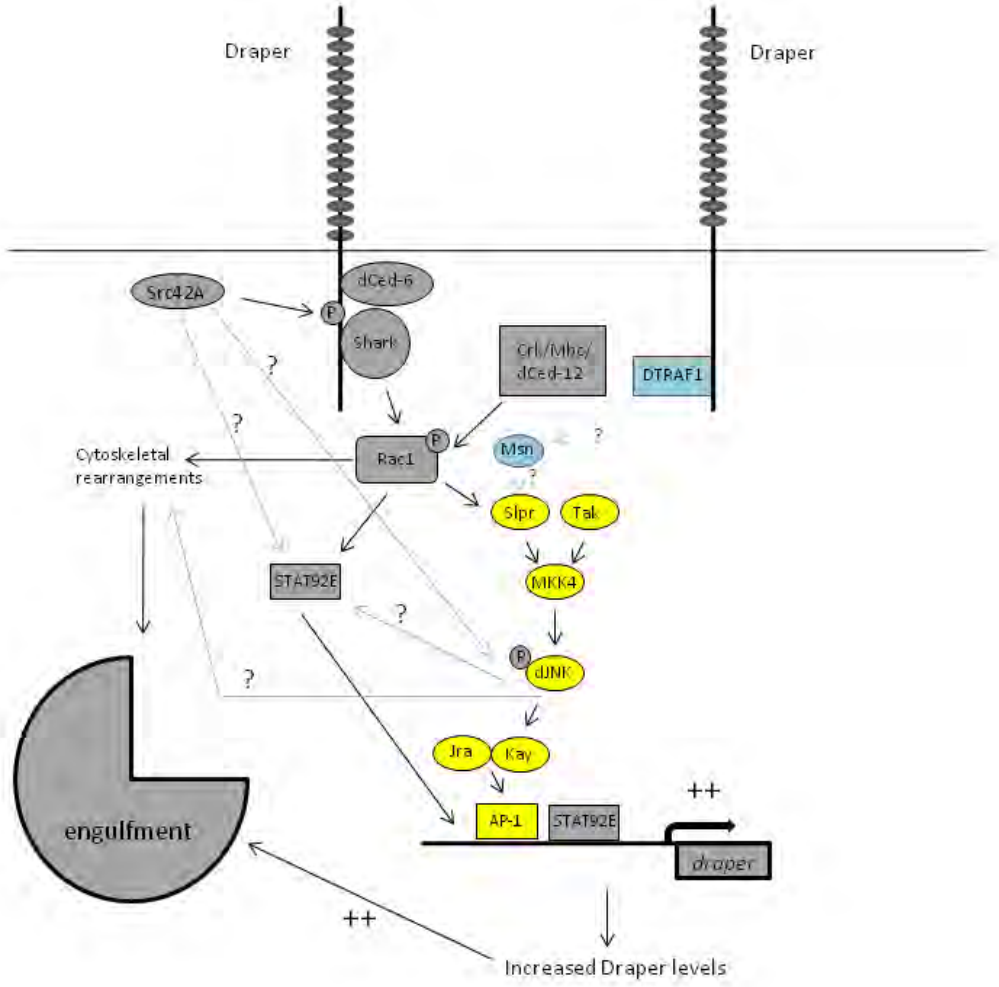


Figure 3.2
dJNK signaling is involved in the glial response to axotomy

Figure 3.2 dJNK signaling is involved in the glial response to axotomy

dJNK signaling is required downstream of Draper signaling for glia to engulf degenerating axon debris. When the components in yellow are knocked down specifically in glial cells, glia fail to become phagocytic and to clear degenerating axonal debris from the CNS (see text for details). Arrows with question marks indicate potential interactions between molecules that are supported by evidence in the literature. Components in blue have been shown preliminarily to be involved in the glial response to axotomy.

these components is knocked down, axonal debris persists significantly longer than in control animals. Glial cells in close proximity to injured axons in the maxillary nerve recruit Draper to the injured nerve, indicating that glia are able to receive and respond to an axonal injury signal. However, when dJNK is knocked down we do not see a dramatic increase in Draper protein on the injured glomeruli or around the antennal lobe after antennal ablation. Glial cells do not phagocytose degenerating axonal debris and the debris is ultimately not cleared from the CNS.

To initiate dJNK signaling the MAPKKKs SLPR and TAK1 are activated. Interestingly, when I performed experiments with the *slpr* and *Tak1* null alleles independently we did not see a clearance defect. However, when I performed the assay with a double null animal, a significant amount of axonal debris remained (**Figure 2.3B)**. This indicates redundancy at this level of the signaling pathway, which has also been briefly mentioned in a previous study on dorsal closure (Polaski et al., 2006). To my knowledge the redundancy and compensation of the MAPKKKs SLPR and TAK1 have not been fully explored, making this an interesting area for future study.

Next, I found that the MAPKK MKK4 is required for glial phagocytosis of axonal debris. There are two known *Drosophila* MAPKKs: MKK4 and MKK7 (also known as HEP). These have been shown to have parallel, non-redundant roles in *Drosophila* development and immune response (Geuking et al., 2009). However, *in vitro* studies have indicated that MKK4 and MKK7 may act together to activate dJNK; for example, both are required for robust JNK phosphorylation in immune cell culture assays (Chen et al., 2002; Lawler et al., 1998; Fleming et al., 2000). Because of this, and because

MKK7/HEP was tested only with RNAi knockdown, we cannot definitively rule out HEP as a component of the glial dJNK signaling cascade involved in phagocytosis. However, when I expressed a constitutively active HEP construct in the glia of animals which were also expressing UAS-Drpr^{RNAi}, we did not see clearance. That is, constitutively active HEP is not sufficient to overcome the lack of Draper in glial cells by activating the pathway downstream of this receptor. This, along with the lack of *in vivo* data supporting redundant or complementary roles for MKK4 and HEP, makes this a less likely possibility.

dJNK is activated by phosphorylation by MKK4. JNK activation has many potential downstream effects including transcriptional activation of genes via the transcription factor AP-1 which is a heterodimer of JRA (d-Jun) and KAY (d-Fos). When either JRA or KAY is knocked down specifically in glia using RNAi, we see an engulfment defect; this is replicated in a *kay* homozygous mutant animal. Together, these data indicate that dJNK is activated and acts through the AP-1 transcription factor to promote engulfment.

Interestingly, recent work showed that there was an increase in *draper* gene expression after acute nerve injury that correlates with the visible increase in Draper protein levels (Logan et al., 2012). This indicates there must be some signaling to the nucleus to induce these changes, but the mechanisms by which this transcriptional activation occurred remained unknown. Since knocking down dJNK signaling led to a loss of the increase in Draper protein levels after injury and since this increase in Draper

staining is due to a transcriptional activation of *draper*, it is likely that the dJNK cascade is activating Draper transcription via AP-1 binding and activation.

Together, the data presented in Chapter II show that the dJNK signaling cascade is required for glial engulfment of neuronal debris and that this signaling acts via AP-1 to activate *draper* transcription after injury which promotes phagocytosis of axonal debris.

SRC42A, Rac1, and Shark are potential upstream activators of JNK signaling downstream of Draper

SRC42A (a Src family kinase, SFK) is known to be required for glial activation and phagocytic activity in response to axonal injury; it has been shown to phosphorylate the intracellular domain of Draper upon activation of the receptor (Ziegenfuss et al., 2008). This phosphorylation allows the association of the Syk-like non-receptor tyrosine kinase Shark with the Draper receptor and promotes downstream signaling. Developmentally, SRC42A has been shown to act upstream of JNK activation and the levels of JNK activation were correlated with the amount of SRC42A (Tateno et al., 2000). *In vitro*, Src has also been shown to be required for JNK activation (Nagao et al., 1999; Nagao, 1998). These studies provide evidence that JNK may be activated downstream of the Draper receptor via SRC42A but they do not suggest a direct mechanism by which this may occur.

Shark has been shown to be required for glial phagocytosis downstream of Draper activation (Ziegenfuss et al., 2008). Interestingly, it has also been shown to increase JNK activation and AP-1 activity in two immunological culture systems (Jacinto et al., 1998; Takada and Aggarwal, 2004). Rac was found to act synergistically in this system and

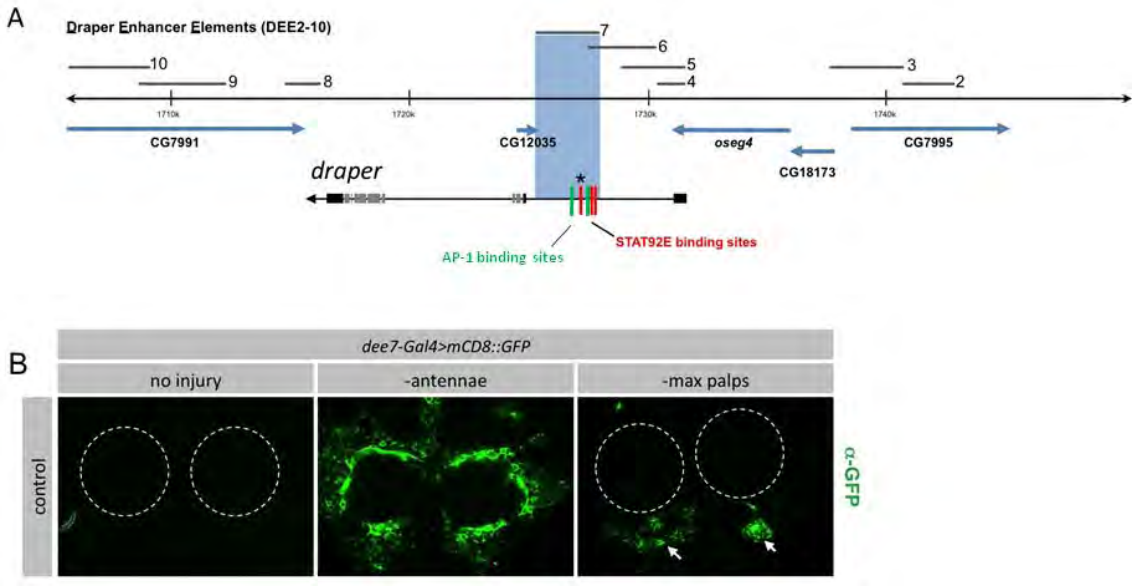
stimulation of both Syk and Rac led to potent JNK activation (Jacinto et al., 1998). However, like with SRC42A, no direct mechanism of activation has been proposed.

Rac1 activation has also been shown to be critical for both glial activation and phagocytosis (Ziegenfuss et al., 2012). Rac1 has previously been shown to mediate JNK activation as well as to directly impact cell migration via actin rearrangement (among other functions) (Raftopoulou and Hall, 2004; Burridge, 2004). It is likely that Rac is acting in both of these functions in glial cells responding to injured axons via Draper activation. Biochemical and genetic experiments indicate Rac1 can activate the dJNK cascade through SLPR (Stronach and Perrimon, 2002; Su et al., 1998). To activate SLPR, Rac binds to the CRIB domain which releases the protein's auto-inhibition. MSN also interacts with SLPR by binding to the LZ-CRIB domain which also releases the auto-inhibition (Garlena et al., 2010). While both Rac and MSN can activate SLPR alone, there is a significant increase in the downstream JNK activity when both Rac and MSN are activated (Garlena et al., 2010). It has been proposed that Rac is the primary activator and that its binding and the subsequent release of SLPR's auto-inhibition exposes the binding site for MSN (Garlena et al., 2010). It is feasible that this mechanism of SLPR activation is taking place in glial cells downstream of Draper activation. Although we did not discover a clearance phenotype when knocking down MSN in glial cells, there are many caveats to the experiment. Namely, the RNAi constructs used may not be effectively knocking down the protein. Since Rac1 may be the primary activator of SLPR activation, the knockdown achieved with the MSN RNAi construct may not have been sufficient to decrease activation of SLPR and transduction

of the signal along the dJNK pathway. Alternatively, MSN may enhance signaling initiated via Rac1 through SLPR but it may not be necessary for it to occur, so a phenotype may not be seen if Rac1 signaling is intact. An alternative method for determining a role for MSN in activating SLPR in glia would be to perform the clearance assay with a trans-heterozygous *rac1* and *msn* mutant animal to see if there is any additive phenotype observed. However, because small amounts of either protein may be sufficient to induce SLPR activation and therefore signal transduction through the dJNK cascade, we may not be able to see a failure to clear axons even when both Rac and MSN are knocked down.

Regulation of *draper* transcription in glial cells after axonal injury

Recent work by Johnna Doherty, another graduate student in the Freeman Lab, has explored the regulation of both basal and injury-induced *draper* expression (Doherty et al., submitted). In order to study the transcriptional regulation of *draper*, a series of 9 potential draper enhancer elements (termed dee2-dee10) were created (Doherty et al., submitted). The fragments, primarily intronic sequences from the region beginning ~40kB upstream of the *draper* locus, were cloned into the GAL4-based pBGW vector (Karimiet al., 2002) and inserted into identical genomic locations (**Figure 3.3A**). Each construct was used to drive the expression of *UAS-mCD8::GFP* and its expression before and after injury was assayed. No glial expression was visible either before or after injury with *dee2-, 3-,4-,5-, or 6-GAL4 or dee8-,9- or 10-GAL4. dee7-GAL4*, composed of a



(Adapted from J. E. Doherty et al., submitted)

Figure 3.3
The *draper* locus contains an injury responsive element

Figure 3.3 The *draper* locus contains an injury responsive element

(A) Schematic representation of the draper locus and regions used to generate Draper Enhancer Elements 2-10 (DEEs). Blue box highlights DEE7 region located in the first intron of the draper gene. Red lines indicate Stat92E binding sites and green lines indicate AP-1 binding sites in DEE7

(B) Single slice confocal images of antennal lobe regions. *dee7-GAL4* driving two copies of *UAS-mCD8::GFP*; no injury, one day after antennal ablation (-antenna), one day after maxillary palp injury (-maxillary palp). Dashed circles outline antennal lobes, axonal terminations of injured neurons. Arrows show areas in ventral region of antennal lobe responding to maxillary palp injury.

(adapted from J. E. Doherty et al., submitted)

sequence located within the first intron of the *draper* locus, showed low-level expression before injury in randomly distributed astrocyte-like glia throughout the neuropil but not in the cortex or ensheathing glia. This element showed a dramatic increase in GFP expression one day after antennal ablation and a smaller, more specific, increase one day after maxillary palp ablation (Doherty et al., submitted) (**Figure 3.3B**). This reporter tells us that the sequence in the *dee7* fragment is responsive to injury and that this responsiveness is graded – more severe injury results in a more robust response. This tool provides us with a way to study the effects of upstream signaling on *draper* transcriptional activation.

The AP-1 transcription factor is known to bind preferentially to the sequence TGACTCA in order to activate gene transcription. To determine if *draper* is activated by the binding of the AP-1 transcription factor to an enhancer segment known to respond after injury, we searched the *dee7* sequence for an AP-1 consensus binding site. Within the 2619 base pair region, I identified two AP-1 consensus binding sites (TGACTCA) (**Figure 3.3A**). In order to determine whether AP-1 binding is critical for the activation of *dee7-GAL4* and therefore for the activation of *draper* after injury, I mutated the AP-1 binding sites to prevent the transcription factor from binding. These experiments are ongoing but I expect that when both AP-1 sites are mutated we will see a significant decrease in *dee7-GAL4* driven GFP expression after injury when compared with control animals. This result would provide direct evidence that AP-1 is required for the transcriptional increase in *draper* that occurs in glial cells in response to neuronal injury

and it would identify the exact region within the *draper* enhancer that is activated by AP-1.

Transcriptional activation of *draper* as a point of integration for signaling cascades

In order to respond appropriately to the many extracellular signals being recognized by a cell, crosstalk between signaling pathways has been shown to occur. This integration of signals can occur at multiple levels – the receptor (Lowes et al., 2002b), various points along the signaling pathway (Jacinto et al., 1998), or at the promoter of a responsive gene (Chen et al., 1999). This integration of signaling cascades can either enhance or inhibit the cell's response (Kim et al., 2005; Su et al., 1994).

In the case of glial cells responding after neuronal injury, such integration may be required to determine the severity of the injury and the intensity of the response required. Experiments by Johnna Doherty using the *dee7-GAL4* element show that there is a graded response to neuronal injury in *Drosophila*. When antennae are ablated, therefore injuring the majority of ORNs projecting into the CNS, there is a dramatic increase in the amount of *dee7-GAL4* activity as shown by GFP expression; maxillary palp injury, affecting a smaller number of ORNs, leads to a corresponding smaller amount *dee7-GAL4* expression (Doherty et al., submitted). How do the glial cells sense the difference between the two injuries? The integration of signaling pathways at the site of transcription is one possibility.

Recent work by Johnna Doherty has shown that the *Drosophila* Signal Transducer and Activator of Transcription protein, STAT92E, is responsible for regulating both basal

and injury induced *draper* expression. Upon sequence analysis, the *dee7-GAL4* element described above contains three STAT92E consensus binding sites (Doherty et al, submitted). It was determined that activation of this element by STAT92E was critical for maximal activation after axotomy; when one site was mutated and STAT92E could no longer bind and activate the fragment, GFP expression was decreased by about 50% when compared with controls. Notably, the GFP expression was not completely absent. There are multiple possible explanations for this. First, STAT92E may bind to multiple sites in order to activate *draper* expression and removing just one site may lessen but not abolish the activation. Ongoing experiments are addressing this possibility with a *dee7-GAL4* fragment that has all three STAT sites mutated. A second possibility is that STAT may require the binding of a co-factor or to be a part of complex in order to fully activate *draper* expression. AP-1 has been shown to act in a complex with STAT to modulate immune signaling (Kim et al., 2007). It has also been shown *in vitro* that AP-1 binding facilitates STAT binding to promote cytokine-induced immune responses in microglia and macrophages (Qin et al., 2007). The STAT and AP-1 sites are in close proximity – the third STAT site is flanked by AP-1 binding sites which are 85 base pairs away on either side. Because both sites are present within the injury-responsive element, it remains an interesting possibility that STAT and AP-1 are acting synergistically at the site of transcription factor binding to increase *draper* transcription.

Interestingly, JNK has also been shown to be required for STAT activation. *In vitro*, it has been shown that JNK can phosphorylate STAT to activate its transcriptional activity (Turkson et al., 1999; Liu et al., 2006; Miyazaki et al., 2008). In addition to its

role in phosophorylating and activating AP-1 components, JNK may be phosphorylating and activating STAT in reactive glial cells, thereby enhancing the transcription of *draper*. To test this, we could utilize the *10XStat92E-dGFP* reporter which has been shown to reflect STAT92E transcriptional activity both during development and in the adult (Doherty et al., submitted). One day after injury, flies expressing this reporter show an increase in GFP expression surrounding the antennal lobes and throughout the brain (Doherty et al, submitted). If JNK is required for full activation of STAT transcriptional activity, we would expect to see less GFP expression one day after injury when compared with control animals.

Integration between STAT and JNK signaling may provide glial cells with a mechanism to determine the strength of response required. In the case of the smaller injury invoked by maxillary palp ablation, Draper signaling may be moderately activated, leading to an activation of JNK and STAT that promotes weak activation of *draper* transcription. It is possible in this situation that JNK selectively activates its downstream targets so that the phagocytosis of axonal debris occurs but signaling is not amplified; STAT would not be phosphorylated in this situation. Alternatively, all downstream targets may be activated but to a lesser extent than after antennal injury. In the case of a severe injury caused by antennal ablation, JNK and STAT may be strongly activated. JNK may then activate STAT in addition to activating its other targets, thereby amplifying the glial response to axonal injury.

Why is basal Draper not enough?

It has been shown that even very low levels of basal (pre-injury) Draper are sufficient for glial cells to receive an injury signal. Glial dJNK knockdown animals have normal levels of Draper before injury; these cells are able to respond to maxillary palp ablation by recruiting Draper to the maxillary nerve (**Figure 2.4B**). Knocking down the PI3K/Akt signaling pathway, in contrast, results in significantly decreased levels of basal Draper but these animals show a dramatic increase of Draper levels after antennal ablation, indicating that the basal Draper that is present is sufficient for the glial cells to sense neuronal injury (Doherty, 2011). These data tell us that even small amounts of Draper are sufficient for glia to activate potent responses to an axonal injury signal.

Work from the *C. elegans* field has recently shown that CED-1, the ortholog of Draper, plays a role in phagosome maturation and degradation of debris as well as in its initial engulfment (Yu et al., 2008). This novel function appears to regulate the recruitment and function of RAB-7 through an unidentified mechanism involving DYN-1, an ortholog of *dynamin*, and CED-6. Rab GTPases are involved in many vesicle trafficking events; Rab7 has specifically been implicated in endocytosis and phagosomal maturation (Vieira et al., 2002).

This study provides evidence that CED-1 may be involved in both recognition and engulfment of debris as well as its subsequent phagocytosis. This may provide us with a model to explain the requirements for *draper* transcription in glial phagocytosis of degenerating axon debris. It is possible that basal Draper/dCed-1 is required for the recognition of axonal debris; this activates downstream signaling pathways that activate

the glial cell and promote engulfment through mechanisms that include *draper* transcription. However, this basal amount of Draper may not be sufficient to promote membrane extension, engulfment, and phagocytosis; a transcriptional increase in *draper*, downstream of Draper signaling, is required for these steps to occur and for axonal debris to be cleared from the CNS. My data shows that the over-expression of Draper can rescue the clearance defects in dJNK knockdown animals. Perhaps this transgene-supplied excess of Draper provides the cell with enough of the protein to efficiently engulf and phagocytose of axonal debris and the requirement for a dJNK-governed transcriptional increase is overridden.

It is also possible that the newly transcribed Draper is somehow different from basal Draper. This variation could occur through post-transcriptional modifications, for example. While it is known that the intracellular domain of Draper I is critical for engulfment and phagocytosis (Logan et al., 2012), a thorough structural analysis of basal Draper and injury-induced Draper has not been conducted, to my knowledge; this might lead to interesting insights into the various roles played by this molecule before and after Draper receptor activation.

Non-transcriptional responses downstream of Rac and dJNK in glial cells after axonal injury

In addition to the transcriptional activation of *draper* and other injury responsive genes, it is likely that there are other effects of Rac1 and dJNK activation that directly allow the glial cell to respond to and engulf the axonal debris. The role of Rac1 in processes such as cell migration, chemotaxis, cytoskeletal rearrangement and phagocytosis is well

established (Reddien and Horvitz, 2000; Sun et al., 2004; Sepp, 2003). In developing *Drosophila* embryos, Rac has been shown to mediate peripheral glial migration via effects on actin fibers (Sepp, 2003). The movement of glia in response to axon injury may be similarly regulated by Rac; it is likely that Rac1 is exerting its effects on actin while also activating downstream signaling pathways such as JNK.

While best known for its effects on transcription, other non-transcriptional targets of JNK activation, such as neurofilament and microtubules, have been reported (Waetzig and Herdegen, 2003). Evidence for JNK's effects on microtubules has come from several studies. In a culture of Cerebellar Granule Neurons, a large pool of activated JNK appears to be cytoplasmic; this localization would not allow transcriptional activation to be the primary downstream target of this pool of enzyme (Coffey et al., 2000). Activated JNK has also been found to co-localize with tubulin in the cytoplasm of cultured embryonic mouse neurons (Kawauchi et al., 2003). The knockdown of JNK has been shown to lead to a loss of microtubule integrity and dynamics, both of which lead to alterations in morphology or neurodegeneration (Chang et al., 2003b; Kawauchi et al., 2003). These studies suggest potential candidates for non-transcriptional targets of activated JNK that may be activated in glial cells in response to axonal injury; the identification of others could provide insight into the diversity of JNK's effects within the cell.

The effect of glial dJNK knockdown on the termination of the glial response to axotomy

The glial response to axotomy can be divided into three phases: activation, engulfment, and termination. In the termination phase, glial cells return to their "resting" state as Draper levels return to normal and glial membranes appear to return to their original locations. Recently it was shown that this termination step requires the function of Draper II, an isoform of the Draper engulfment receptor formed through alternative splicing (Logan et al., 2012). The intracellular portion of Draper II contains an inhibitory domain that drives the termination of the glial response to axotomy (Logan et al., 2012). Interestingly, Draper II (like Draper I) is transcriptionally activated after axotomy. While the relative mRNA expression of Draper I is evident by 1.5 hours after injury and peaks around 3 hours after injury, the transcription of Draper II peaks around 4.5 hours after injury (Logan et al., 2012). Draper II activity is critical for the "repriming" of glial cells. When Draper II is absent, clearance of injured axons occurs normally. However, when a subsequent axotomy is performed in these animals, glia are not able to clear the debris from the CNS (Logan et al., 2012). Draper II function appears critical for the "resetting" of glia after an initial response to axonal injury.

Interestingly, when I knock down dJNK in glial cells, I see a persistence of glial activation. When I look at the maxillary nerve, I can see Draper staining for at least 15 days after injury; in control animals, this staining is not visible after 5 days post-injury. Recent work has demonstrated that the presence of axonal debris does not cause glial cells to remain in an activated state (Ziegenfuss et al., 2012). For example when the Crk/MBC/dCed-12 complex is knocked down, glial cells respond to the site of injury, fail

to clear the debris, and return to their resting positions. I hypothesize that dJNK is acting to regulate Draper II transcription. Draper II is likely regulated by the same enhancer region as Draper I, which contains AP-1 binding sites as described previously. When dJNK is knocked down, Draper II is not transcriptionally activated and therefore the engulfment response is not terminated. To test this, we can perform qRT-PCR for Draper II in dJNK knock down animals to see if an increase in relative mRNA levels is detected. The effect of dJNK on the termination of the glial response has many potential implications. As discussed briefly in the introduction, hyperactive glial cells have been implicated in human pathological conditions such as multiple sclerosis and chronic neuropathic pain. Understanding how glial cells are "turned off" by dJNK and other transcriptional regulators would be an exciting path for future study.

Future directions for studying the role of dJNK in glial engulfment and phagocytosis

Visualizing AP-1 activity *in vivo*

Until very recently, we have been unable to visually monitor AP-1 activity *in vivo* after axotomy. However, the recent development of a transgenic fluorescent transcriptional reporter has provided a means of doing this (Chatterjee and Bohmann, 2012). These constructs were designed by utilizing the known AP-1 preferred binding site sequences. Four copies of this so called TRE element were placed upstream of a basal promoter and a fluorescent protein; this construct was integrated into a specific genomic locus so that positional effects would be avoided (Chatterjee and Bohmann, 2012). The constructs have been shown to accurately represent known patterns of JNK

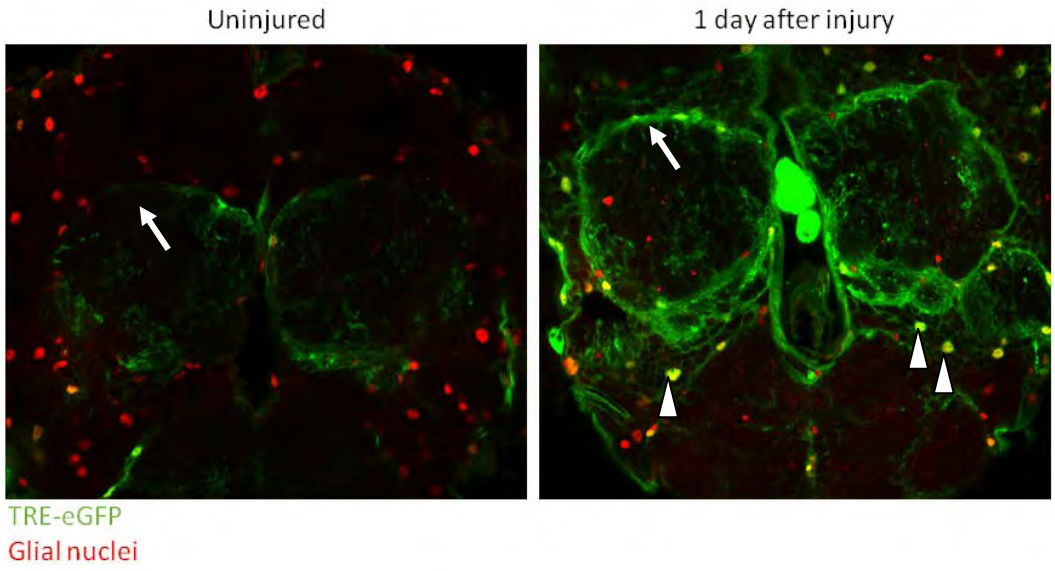


Figure 3.4
An AP-1 fluorescent reporter is increased in glia after axon injury

Figure 3.4 An AP-1 fluorescent reporter is increased in glia after axon injury

An AP-1 transcriptional reporter has recently been created by cloning four AP-1 binding sites ("TRE elements") in front of a fluorescent protein. Using flies homozygous for this reporter, I surgically removed both antennae and maxillary palps to induce a large injury in the CNS. Flies were kept at 25^{o}C for one day before brains were fixed and dissected using a standard protocol (MacDonald et al., 2006). Brains were stained with α-Repo antibody (1:5) to label glial nuclei. Brains were mounted in Vectashield and imaged on a Zeiss LSM5 Pascal confocal microscope. One day after injury there is a dramatic increase in GFP in the injured brains when compared with the uninjured brains. GFP is seen surrounding the antennal lobe (arrows). There also appears to be an increase in Repo/GFP co-labeled nuclei (which appear yellow, arrowheads).

signaling (Chatterjee and Bohmann, 2012). To test whether AP-1 is activated after nerve injury in the *Drosophila* CNS, I performed antennal and maxillary palp ablations on flies expressing this transgene. Preliminary experiments indicate that there is an increase in GFP expression one day after axotomy and that this increase is co-localized to glial cells (**Figure 3.4**). This reinforces our model that dJNK signaling is activated and inducing transcriptional changes in reactive glial cells in response to axotomy.

This newly described AP-1 reporter will allow in-depth analysis of dJNK signaling in glial cells in response to nerve injury. For example, we have yet to directly link Draper receptor activation with dJNK and AP-1 activity. By using this reporter in a *draper* null background, we can determine if the Draper receptor acts upstream of AP-1 activation. The reporter can also be used to determine the role of other known engulfment genes (such as Rac, and Crk/MBC/dCed-12) in activating AP-1 activity. The exact role for dCed-6 has not been elucidated; by utilizing this reporter and performing axotomy, we can assess AP-1 activity to determine if dCed-6 acts upstream. Utilization of this new tool will provide insight to the signaling that occurs in glia after axotomy.

DTRAF1 as a possible link between the Draper receptor and dJNK signaling

One major question that remains unanswered is how the Draper receptor activates downstream effectors. We know that SRC42A and Shark are involved, but the molecular details of downstream target activation are unknown. To begin answering this question I chose several candidates molecules that were known to activate JNK downstream of

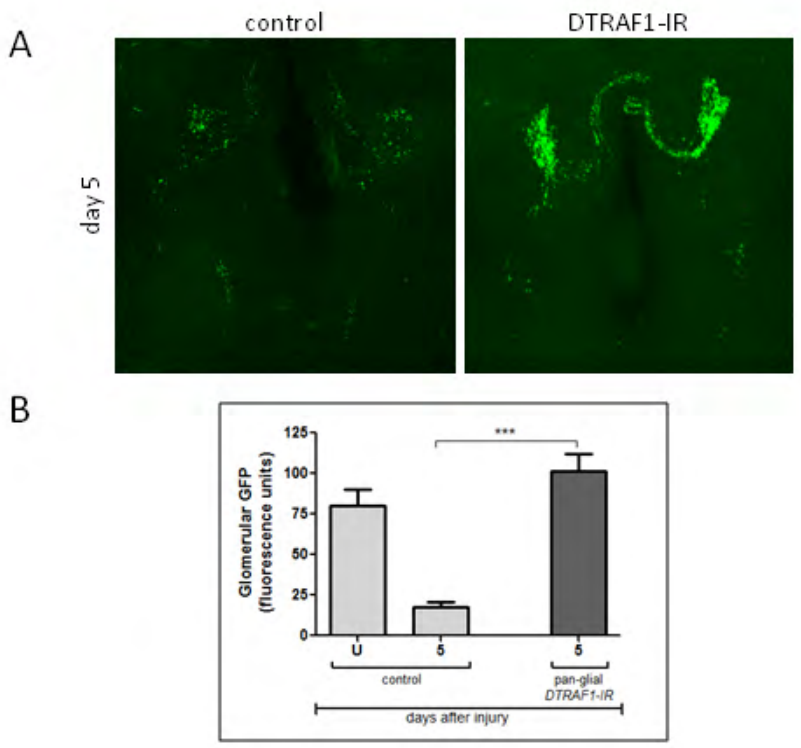


Figure 3.5
DTRAF1 is involved in Draper signaling in glial cells after axotomy

Figure 3.5 DTRAF1 is involved in Draper signaling in glial cells after axotomy

To test if DTRAF1 is required in glial cells for the clearance of degenerating axons I expressed *UAS-DTRAF1-IR* (IR = inverted repeats) in all glial cells of flies expressing *OR85e-mCD8::GFP* using *repo-GAL4*. Flies were raised at 25^{o}C and aged for at least 7 days after eclosion. Maxillary palps were ablated and flies were kept at 25^{o}C for 5 days. Brains were fixed and dissected using a standard protocol (MacDonald et al., 2006). Brains were stained with α-GFP antibody overnight at 4^{o}C and with FITC anti-mouse IgG at room temperature for 3 hours. Brains were mounted in Vectashield and imaged on a Zeiss LSM5 Pascal confocal microscope. (A) shows a control brain 5 days after maxillary palp ablation with very little GFP+ axonal debris remaining. The right panel shows a brain with DTRAF1 knocked down in glial cells. There is a significant amount of GFP+ axonal material remaining; this is quantified in (B).

various receptors and knocked them down in glia using *UAS-RNAi* constructs. One molecule, DTRAF1, gave an engulfment phenotype; a significant amount of GFP+ axonal debris remained 5 days after axotomy when DTRAF1 was knocked down in glia by RNAi (**Figure 3.5A-B**)

Tumor necrosis factor receptor-associated factors (TRAFs) are adaptor proteins that are typically signal transducing molecules that interact with receptors of the TNF or interleukin-1 receptor/Toll families (Muzio et al., 1998; Cao et al., 1996). In mammals the activation of the six known members of the TRAF family and the resultant downstream signaling appears to be context dependent, but JNK activation is one known result (Min and Pober, 1997b; Song et al., 1997b; Kim et al., 1999). In one culture system, the activation of JNK by TRAF involves Rac, although the exact mechanism is not reported (Min and Pober, 1997b). In *Drosophila*, two TRAFs that are alternate splice forms of the same genetic locus have been identified (Liu et al., 1999; Zapata et al., 2000; Preiss et al., 2001). DTRAF2 has only 19% identity to mammalian TRAFS (Preiss et al., 2001). It, like TRAF6, interacts with Pelle and ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) to activate NF-KB signaling (Kopp et al., 1999; Cha et al., 2003). DTRAF1 is most similar to TRAF4 (45% identical). It was shown bind to MSN, a JNKKKK, in a yeast two-hybrid screen; this binding to MSN was shown to be critical for TRAF1 to activate JNK *in vitro* (Liu et al., 1999). Later experiments *in vivo* also indicated that DTRAF1 acts upstream of JNK activation. Could it be interacting with the Draper receptor to activate dJNK in glial cells? This preliminary finding that knocking down DTRAF1 in glial cells with RNAi leads to a defect in the

clearance of degenerating axonal debris should be followed up to potentially uncover an exciting new role for DTRAF1 and a new component of the Draper signaling pathway.

Conclusions

The work performed in this thesis provides insight to the molecular processes underlying the engulfment and phagocytosis of degenerating axon debris by glial cells in *Drosophila melanogaster*. My work uncovers a new *in vivo* role for the well known dJNK signaling cascade; the MAPKKKs Slipper and TAK1, the MAPKK MKK4, the MAPK dJNK and the transcription factor dAP-1 are each required for phagocytosis to occur.

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