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Cellular and Molecular Mechanisms Driving Glial Engulfment of Degenerating Axons: A Dissertation

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CELLULAR AND MOLECULAR MECHANISMS DRIVING GLIAL ENGULFMENT OF DEGENERATING AXONS

A Dissertation Presented
By

Johnna E. Doherty

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This work is dedicated to my mother and father, for all of their support and encouragement.

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Abstract

The nervous system is made up of two major cell types, neurons and glia. The major distinguishing feature between neuronal cells and glial cells is that neurons are capable of transmitting action potentials while glial cells are electrically incompetent. For over a century glial cells were neglected and it was thought they existed merely to provide trophic and structural support to neurons. However, in the past few decades it has become increasingly clear that glial cell functions underlie almost all aspects of nervous system development, maintenance, and health. During development, glia act as permissive substrates for axons, provide guidance cues, regulate axon bundling, facilitate synapse formation, refine synaptic connections, and promote neuronal survival. In the mature nervous system glial cells regulate adult neurogenesis through phagocytosis, act as the primary immune cell, and contribute to complex processes such as learning and memory. In recent years, glial cells have also become a primary focus in the study of neurodegenerative diseases. Mounting evidence shows that glial cells exert both beneficial as well as detrimental effects in the pathology of several nervous system disorders, and modulation of glial activity is emerging as a viable therapeutic strategy for many diseases. Although glial cells are critical to the proper development and functioning of the nervous system, there is still relatively little known about the molecular mechanisms used by glial cells, how they exert their effects on neurons, and how glia and neurons communicate.

Despite the relative simplicity and small size of the *Drosophila* nervous system, glial cell organization and function in flies shows a remarkable complexity similar to vertebrate glial cells. In this study I use *Drosophila* as a model organism to study cellular and molecular mechanisms of glial clearance of axonal debris after acute axotomy. In chapter two of this thesis, I characterize three distinct subtypes of glial cells in the adult brain; cell body glia which ensheath neuronal cell bodies in the cortex region of the brain, astrocyte like glial cells which bear striking morphological similarity to mammalian astrocytes and share common molecular components, and ensheathing glial cells which I show act as the primary phagocytic cell type in the neuropil region of the brain. In addition, I identify dCed-6, the ortholog of mammalian GULP, as a necessary component of the glial phagocytic machinery.

In chapter three of this thesis, I perform a candidate based, *in vivo*, RNAi screen to identify novel genes involved in the glial engulfment of degenerating axon material. The Gal4/UAS system was used to drive UAS-RNAi for approximately 300 candidate genes with the glial specific *repo-Gal4* driver. Two assays were used as a readout in this screen, clearance of axon material five days after injury, and Draper upregulation one day after maxillary palp or antennal injury. Overall, I identified 20 genes which, when knocked down specifically in glial cells, result in axon clearance defects after injury.

Finally, in chapter four I identify Stat92E as a novel glial gene required for glial phagocytic function. I show that Stat92E regulates both basal and injury induced Draper expression. Injury-induced Draper expression is transcriptionally regulated through a Stat92E dependent non-canonical signaling mechanism whereby signaling through the

Draper receptor activates Stat92E which in turn transcriptionally activates *draper* through a binding site located in the first intron of Draper. Draper represents only the second receptor known to positively regulate Stat92E transcriptional activity under normal physiological conditions.

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List of Abbreviations

ALRM- Astrocyte like Leucine Rich repeat Molecule

AP-1- Activator Protein 1

ATF- Activating Transcription Factor

ATP- Adenosine Triphosphate

BBB- Blood Brain Barrier

CNS- Central Nervous System

EAAT-1- Excitatory Amino Acid Transporter-1

GAS- Gamma interferon Activation Site

GFP- Green Fluorescent Protein

GLAST- Gluatamate-Aspartate Transporter

GLT-1- Gluatamine Transporter-1

JAK- Janus Kinase

JNK- c-Jun N-terminal kinases

MAPK- Mitogen-Activated Protein Kinase

MARCM-Mosaic Analysis with a Repressible Marker

NMJ- Neuromuscular Junction

NPC- Neural Precursor Cell

ORN- Olfactory Receptor Neuron

PCD- Programmed Cell Death

PDK1- Pyruvate Dehydrogenase Kinase 1

PI3K92E- Phosphatidylinositol 3-kinase at cytological location 92E

PIP2- Phosphatidylinositol (4,5)-bisphosphate

PIP3- Phosphatidylinositol (3,4,5)-trisphosphate

PNS-Peripheral Nervous System

RNAi- Ribonucleic Acid Interference

S6K- S6 Kinase

STAT- Signal Transducer and Activator of Transcription

Stat92E- Signal Transducer and Activator of Transcription at cytological location 92E

VDRC- Vienna *Drosophila* RNAi Center

Preface

All work described in this thesis was performed at University of Massachusetts Medical School in the lab of Marc Freeman. In chapter two, Mary Logan was an equal contributor to the work and Ozge Tasdemir characterized the *alrm-Gal4* driver line. In chapter four Amy Sheehan made the *dee7-Gal4* driver line and Rachel Hackett performed several western blots and coimmunoprecipitation experiments.

CHAPTER I: Introduction

Glia-what are they?

The nervous system is composed of two major cell types-neurons and glia. Neurons are electrically excitable cells which are able to form synapses and communicate with each other via chemical and electrical signaling mechanisms. Glia collectively refers to the other major cell type in the nervous system not competent to conduct electrical impulses. For over a century the sole focus of intense study in the nervous system was directed towards neurons. All cognitive function in the brain was thought to occur exclusively by electrical impulses flowing through neuronal circuits and communicating via synapses. Additionally, neuronal dysfunction was assumed to be the primary culprit underlying all neurodegenerative disorders. These traditional neurocentric views are now being challenged by mounting evidence that glia are crucially involved in all aspects of development, maintenance and function in the nervous system (Barres, 2008).

Glia are the most abundant class of cells in the mammalian brain and they comprise up to 90% of the brain cells in some animals. Despite the generic term used to classify these cells, glia are a vastly heterogeneous population of cells with largely distinct roles in the nervous system. During development, glia are indispensible for the proper establishment of the nervous system. Foremost, glial cells are necessary for the survival of neurons. *In vivo* studies show that survival of both pre and post mitotic neurons is dependent upon factors provided by glia (Bush et al., 1999; Platel et al., 2010; Riethmacher et al., 1997; Tao et al., 2011; Woldeyesus et al., 1999). Additionally, glia communicate with neurons in a bi-directional manner to drive developmental events such as axon pathfinding, nerve fasciculation and synapse formation (Allen and Barres, 2005;

Gilmour et al., 2002; Marin and Rubenstein, 2003; McDermott et al., 2005; Tole et al., 2006). Ultimately, glial cells refine and sculpt the nervous system through specialized pruning and engulfment mechanisms (Logan and Freeman, 2007; Mallat et al., 2005; Marin-Teva et al., 2004; Paolicelli et al., 2011; Sierra et al., 2010). Glial cells are equally important for promoting homeostasis and driving complex functions in the mature nervous system. They maintain the health of neurons by secreting trophic factors, myelinating axons, and supplying energy and metabolites. Through the release of gliotransmitters, they can communicate with neurons and modulate synaptic activity which underlies complex processes such as behavior, learning and memory, and circadian rhythms (Araque et al., 2001; Araque et al., 1998b; Fellin et al., 2007; Fellin et al., 2006; Henneberger et al., 2010; Jackson, 2011; Mazzanti and Haydon, 2003; Mothet et al., 2006; Prosser et al., 1994). Glia also act as the primary immune cell in the nervous system, facilitating the isolation and removal of pathogens and harmful debris stemming from cell death and injury (Napoli and Neumann, 2009; Neumann et al., 2009).

Subtypes of glial cells in the mammalian nervous system

In order to perform these various functions within the nervous system, mammals have developed four major subtypes of glial cells which differ in their morphology, molecular profiles and functional roles. Oligodendrocytes are found in the white matter of the central nervous system. They possess slender cytoplasmic extensions which enwrap and myelinate axons to allow for saltatory conduction of action potentials. They also provide local trophic support to neurons by secreting molecules such as nerve growth factor,

brain-derived neurotrophic factor, and glial-derived neurotrophic factor (Dai et al., 2003; Ubhi et al., 2010). Schwann cells, both myelinating and non-myelinating, reside in the peripheral nervous system (PNS) where they ensheath and support peripheral nerves. In contrast to oligodendrocytes, Schwann cells are able to facilitate neuronal regeneration following injury (Torigoe et al., 1996). Astrocytes, the most abundant cell type in the brain, are star shaped, highly tufted cells which interdigitate among axons and dendrites in neuropil regions of the CNS. These cells were classically assigned to providing trophic support to neurons, maintaining ionic homeostasis and contributing to the structural and functional integrity of the blood brain barrier (BBB). However, recent discoveries have revealed exciting new roles for astrocytes in the modulation of neuronal activity (Fellin et al., 2006; Mazzanti and Haydon, 2003; Nedergaard, 1994; Panatier et al., 2011; Pascual et al., 2005). The traditional view of the ~~"s~~ynapse" involved only two cells, a pre-synaptic neuron and a post-synaptic neuron. It is now evident that astrocytes represent a third cell type which can form intimate connections with both pre- and post-synaptic neurons in what is now referred to as the ~~"tr~~ipartite synapse" (Araque et al., 1998a, 1999). Astrocytes express receptors for almost all neurotransmitters which allow them to sense neuronal activity and in turn influence neuronal excitability through the Ca^{+} dependent release of a variety of neuroactive molecules. Through this mechanism of modulating neuronal excitability, astrocytes are able to influence synaptic plasticity (Araque et al., 1999; Eroglu and Barres, 2010). Being intimately associated with neurons, astrocytes are also able to sense injury in the CNS and they respond by secreting a number of molecules which, depending on the circumstances, can further increase

neuronal cell death or alternatively, promote the survival of neurons (Halassa et al., 2007; Sofroniew and Vinters, 2010). Microglial cells, which arise from a monocytic-macrophage lineage, are phagocytic cells dedicated primarily to immune functions in the CNS. They are highly mobile cells which constantly survey the CNS and in the presence of trauma (whether it be mechanical injury, infection, chemical toxin or degenerative disease), microglia react by launching pro- or anti- inflammatory responses, recruiting astrocytes, and phagocytosing cellular debris.

Drosophila as a model organism to study glia

While it is overwhelmingly clear that glial cells contribute to most, if not all, aspects of nervous system development, maintenance, and function, we know very little about the molecular mechanisms driving glial biology. How do glia communicate and interact with neurons? How diverse are glial cell populations? What are the primary functions of glia in the mature nervous system? These questions can be addressed incisively in the *Drosophila* nervous system which is highly stereotyped, well-characterized, and can be manipulated and observed at single cell resolution. Furthermore, in comparison to vertebrates, *Drosophila* contain a smaller number of glial cells and impressively, every glial cell in the *Drosophila* embryonic CNS has been characterized (Awasaki and Lee, 2011).

Drosophila glial cells and vertebrate macroglial cells (astrocytes, oligodendrocytes and Schwann cells) originate from neural precursor cells (NPCs). In *Drosophila* the transcription factor encoded by the *glial cells missing* (*gcm*) gene is a

master switch necessary and sufficient for glial cell fate specification (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Overexpression of *gcm* is able to transform NPCs into glial cells, while loss of *gcm* results in an overabundance of neurons and a near complete loss of glial cells in the *Drosophila* embryo. Interestingly, while *gcm* genes are conserved in mammals, they appear to play no *in vivo* role in the specification of glial cells (Kim et al., 1998). However, recent evidence points to conserved epigenetic mechanisms which control glial cell fate specification through the modification of chromatin. For instance, glial differentiation from NPCs in *Drosophila* requires low levels of histone acetylation, and overexpression of histone acetyl transferases in NPCs results in downregulation of the majority of glial genes (Flici et al., 2011). Similarly, in mammals, oligodendrocyte differentiation is also dependent upon low levels of histone acetylation, and histone deacetylases directly repress genes which prevent oligodendrocyte differentiation (Liu et al., 2009a; Shen and Casaccia-Bonnefil, 2008; Ye et al., 2009). While initial events promoting glial cell fate occur by distinct molecular mechanisms in *Drosophila* and vertebrates, a number of studies reveal the similarities in later aspects of glial morphogenesis (e.g., subtype diversity, interactions with neurons, and neuronal ensheathment) (Banerjee et al., 2006; Brose et al., 1999; Dickson and Gilestro, 2006; Franzdottir et al., 2009; Freeman and Doherty, 2006; Xie and Auld, 2011) and ultimately function in the mature central nervous system (e.g., support of neurons, blood-brain barrier formation, modulation of neuronal activity, immune function) (Bhat et al., 2001; Daneman and Barres, 2005; Kazama et al., 2011; Leiserson et al., 2011; MacDonald et al., 2006; Mayer et al., 2009; Stork et al., 2009).

Glial cell types in the *Drosophila* embryo

Glial cells have been extensively studied and characterized within the *Drosophila* embryo. Here, four main subtypes of glial cells can be distinguished- cortex, neuropil, surface, and peripheral glia, and they exhibit many morphological and functional similarities to their mammalian counterparts. Cortex glia, also known as cell-body-associated glia, are in some regards similar to astrocytes, being embedded within the cortex in close contact with neurons. They extend membranes profusely around neuronal cell bodies, forming a honey-combed structure of glial processes that invade the spaces between neuronal cell bodies (Pereanu et al., 2005). Interestingly, cortex glial membranes make significant physical contact with the blood–brain barrier and oxygen-supplying tracheal elements (Ito et al., 1995; Pereanu et al., 2005). This close association of cortex glia with the major sites of gas and nutrient entry into the central nervous system (CNS) suggests they act as cellular conduits to supply gases and nutrients to target neurons, as has been proposed for mammalian astrocytes. Neuropil glia can be divided into two distinct morphological subtypes, both closely associated with the neuropil in the CNS. The first type is dedicated to ensheathing individual neuropil regions, and another extends fine processes into the neuropil to form a dense glial meshwork contacting dendrites, axons and synapses, a feature making them strikingly similar to mammalian astrocytes. The neuropil glia, both ensheathing and astrocyte-like, have been shown to promote neuronal survival and help to form properly fasciculated nerves (Booth et al., 2000; Ito et al., 1995; Klambt and Goodman, 1991). In addition to morphological similarities, *Drosophila* astrocyte-like glia have been shown to be functionally very

similar to their mammalian counterpart as they are able to tightly control neurotransmitter levels at synapses by expressing specific transporters on their membranes (Kucukdereli et al., 2011; Rival et al., 2004; Stacey et al., 2010)(Dr. Tobias Stork, personal communication). A CNS glial subtype specifically assigned to immune functions, like mammalian microglia, does not appear to be present in the *Drosophila* embryo; rather, all glia seem competent to perform immune-like functions such as engulfment of neuronal corpses during development (Freeman et al., 2003; Sonnenfeld and Jacobs, 1995). The ectoderm derived surface glia form a flat sheath around the *Drosophila* CNS blood-brain barrier, isolating neural elements from surrounding tissues and hemolymph (Edwards et al., 1993; Ito et al., 1995). Finally, in the PNS, CNS-derived peripheral glia ensheath and support peripheral nerves containing motor and sensory axons (Auld et al., 1995; Freeman et al., 2003; Leiserson et al., 2000; Sonnenfeld and Jacobs, 1995), much like mammalian Schwann cells.

Glial cells in the mammalian brain have been fairly well characterized according to morphology, gene expression and function. While *Drosophila* embryonic glial cells have been the focus of elaborate studies (Beckervordersandforth et al., 2008; Halter et al., 1995; Klambt and Goodman, 1991) very little is known about the morphological diversity of glial cells in the mature *Drosophila* brain. Similar to mammals, *Drosophila* glia play essential roles in the adult brain. They regulate complex mechanisms such as circadian rhythms, courtship behavior, synaptic plasticity and longevity (Buchanan and Benzer, 1993; Ewer et al., 1992; Jackson, 2011; Kazama et al., 2011; Ng et al., 2011; Seugnet et al., 2011), act as immune cells through the phagocytic clearance of

degenerating axons (MacDonald et al., 2006; Ziegenfuss et al., 2008), and are critical for the overall health of neurons as revealed by the finding that disruption of glia in the mature brain can lead to neurodegeneration (Bainton et al., 2005).

In chapter two of this thesis I use genetic means to identify and classify three distinct subtypes of glial cells in the adult *Drosophila* brain. These cell types share common morphological and molecular features with mammalian brain glia. Additionally, I identify the glial cell type responsible for phagocytosing degenerating axon material after injury and show that dCed-6, the *Drosophila* ortholog of *C. elegans* CED-6 is required for clearance of degenerating axon debris.

Glial phagocytosis and immune functions

A fundamental way in which glial cells exert their influence and control over the nervous system is through their phagocytic activity. Phagocytosis is a highly conserved process which arose prior to the evolution of multicellular organisms. This means of ingesting material is now used by metazoans mainly as a cellular defense mechanism to isolate and safely remove foreign objects like pathogens, dying cells, and other potentially harmful material from the body. This process protects surrounding cells from exposure to toxic contents, serves to prevent further damage by stimulating the production of anti-inflammatory molecules and can be used as a mechanism to recycle cellular materials. In addition, the process of glial phagocytosis can actively promote neuronal cell death and pruning events (Awasaki et al., 2011; Marin-Teva et al., 2004). Under normal homeostatic conditions, glial cells do not indiscriminately phagocytose healthy

endogenous tissue. Rather, glial cells express an array of receptors which are capable of recognizing ―find me", ―eat me" and ―don't eat me" cues presented by dead or dying cells (―modified-self") and molecules presented by invading pathogens (―non-self") (Armstrong and Ravichandran, 2011; Fadok et al., 1998; Fadok and Henson, 1998; Lauber et al., 2003). While the process of phagocytosis is complex and the exact mechanisms depend greatly on the particle or cell type being ingested, there are some fundamental principles common in all situations. These include receptor-mediated recognition of the object, actin polymerization at the site of ingestion, actin-dependent internalization of the object, and endocytic processing and phagolysosome maturation (Erwig and Henson, 2008).

Glial phagocytic functions during development: Apoptotic cell clearance and synaptic pruning

During development, glial engulfment activity plays a prominent role in both the clearance of apoptotic cell corpses and tissue remodeling events. During assembly of the nervous system, many more cells are born than will survive to become part of the mature nervous system. This excess cell production leads to subsequent programmed cell death (PCD) utilizing well-conserved apoptotic pathways (Truman, 1984; Williams and Herrup, 1988; Zhou et al., 1995). Programmed cell death is a common feature of development in all metazoan organisms and it serves to control cell number, remove redundant structures and eliminate unhealthy cells. Rapid clearance of apoptotic cells is necessary to maintain homeostasis and prevent toxicity to surrounding cells. Persistence

of apoptotic cells is an underlying cause of inflammatory responses and has been implicated in the progression of various neurodegenerative diseases (Yuan and Yankner, 2000).

During *Drosophila* embryonic development, approximately 500 neurons are produced per hemisegment of the ventral nerve cord and roughly 30% of these will undergo PCD (Rogulja-Ortmann et al., 2007). These cell corpses are rapidly cleared from the CNS primarily by surface glial cells which ensheath the entire CNS and send projections into the cortex regions (Sonnenfeld and Jacobs, 1995)(Fig. 1-1A). It is unclear whether the glial cells then degrade and process the dead cells or whether they transfer the corpses to macrophages in close contact with the CNS for further processing.

In mammals, almost all classes of neurons are produced in excess and up to 50% of the neurons die prior to CNS maturation (Lossi and Merighi, 2003). During development of the cerebellar cortex, Purkinje cells undergo PCD and are subsequently cleared by microglia. Interestingly, microglial phagocytosis also serves to activate the death process as selective elimination of microglia strongly reduced PCD in Purkinje cells (Marin-Teva et al., 2004). This mechanism of engulfment-mediated cell death has also been demonstrated in *C. elegans* indicating an evolutionarily conserved role for microglia in actively driving developmental cell death events (Hoeppner et al., 2001; Reddien et al., 2001).

The nervous system is arguably the most complex system in nature and proper wiring of billions of neurons requires a great degree of plasticity and refinement. During development, an excess number of neuronal projections, neurite branches, and synapses

are established. This redundancy is thought to be a mechanism by which to ensure full synaptic coverage of the CNS. However, limitations in target-derived cues and sites of innervation requires that some neuron projections get eliminated (Bagri et al., 2003; Nakamura and O'Leary, 1989; O'Leary and Koester, 1993; Truman, 1990). This process involves the breakdown of the microtubule cytoskeleton, separation of axon material from the main arbor, fragmentation of debris and ultimately engulfment of this material by glial cells. Glial cells play an evolutionarily conserved role in the synaptic pruning and clearance of neuronal debris (Awasaki et al., 2006; Paolicelli et al., 2011; Watts et al., 2004).

During *Drosophila* metamorphosis, mushroom bodies, a center of learning and memory consolidation in the brain, undergo extensive fragmentation, pruning, and remodeling events which occur independently of programmed cell death mechanisms, as the cell bodies of these neurons remain intact. Prior to the onset of these events, glial cells, stimulated by an increase in the ecdysone hormone, proliferate and infiltrate the mushroom body neuropil and ultimately phagocytose the degenerating axon material (Awasaki et al., 2006; Watts et al., 2004) **(Fig. 1-1B)**. Interestingly, in addition to sculpting and refining neuronal circuits through engulfment mechanisms, recent findings show glial cells are necessary to initiate these neuronal remodeling events. Glial cells secrete Myoglianin which in turn upregulates neuronal expression of the ecdysone nuclear receptor and this triggers neuronal remodeling in the presence of the ecdysone peak which occurs during late larval stages. Knockdown of Myoglianin specifically in

neuropil astrocyte glia and cortex glia was sufficient to inhibit mushroom body pruning (Awasaki et al., 2011).

Recently, similar mechanisms of glial engulfment of pruned axon material have been shown to occur in the hippocampus of the postnatal mouse where electron microscopy revealed the presence of both pre- and post-synaptic material within vesicles of microglial processes. Knockout of the microglial CX3CR1 fractalkine receptor led to a decrease in microglia which in turn resulted in the accumulation of synaptic puncta in the CNS, increases in the density of pyramidal neuron dendritic spines and immature synapse formation (Paolicelli et al., 2011). Synaptic pruning also occurs at the neuromuscular junction (NMJ) where growing synapses shed significant amounts of presynaptic membranes and immature synapse material. In *Drosophila,* clearance of this material is mediated by both glial cells and muscle (Fuentes-Medel et al., 2009). In the developing NMJ of mammals, synaptic material is shed from the NMJ as membrane bound remnants called ~~–a~~xosomes" which are ultimately eliminated by Schwann cells (Bishop et al., 2004).

Glial phagocytic functions in the adult *Drosophila* CNS

Phagocytic functions of glial cells during development (i.e., clearance of apoptotic cells and pruned neuronal material) are normal physiological processes which are required for the formation of a healthy nervous system. In the adult CNS, glial phagocytic activity is most commonly associated with some form of insult or dysfunction. Microglia are the primary phagocytic cell type and the first line of immune defense in the CNS. They

populate the entire CNS early during development and exist in non-overlapping territories. In a healthy brain, they remain in a resting state exhibiting a small cell body and highly ramified processes which are extremely motile and capable of surveying the entire brain approximately once every hour (Davalos et al., 2005). Microglia are highly sensitive to changes in the environment and they can sense even minor perturbations within the CNS. In response to trauma, whether it be mechanical injury, chemical exposure, invading pathogens or neurodegenerative disease, microglia transform from an immunologically silent state to an active state reflected by an amoeboid morphology, proliferation and increased expression of certain cell surface markers (Singh et al., 2011). Microglia rapidly migrate to sites of injury, release cytokines and neurotrophic factors, and phagocytose degenerating debris.

Activation of microglia is not an ~~–a~~ll-or-nothing" response, and the effects vary greatly depending on the severity and duration of the injury, the microenvironment, the age of an organism, interactions with other cells, and the downstream signaling pathways activated. In certain contexts, microglial activation is associated with the production of anti-inflammatory cytokines and neurotrophic factors which promote neuronal health. This type of reaction is typically seen during the engulfment of apoptotic cells. In other circumstances, microglial over-activation leads to the production of pro-inflammatory molecules and reactive oxygen species (ROS) which can lead to neuronal cell death, chronic inflammation and subsequent further brain damage (Neher et al., 2011; Neumann et al., 2009). This type of response generally occurs during the phagocytosis of pathogens and in the context of neurodegenerative disease. However, not much is known

about the molecular mechanisms and extrinsic factors which determine whether microglial responses will result in neuroprotection or neurotoxicity.

In the past decade there has been much debate about whether microglial activation is more harmful or beneficial to the nervous system. Microglial activation is a hallmark of almost all neurodegenerative diseases and recent studies of Parkinson‘s disease, Alzheimer‘s disease, and amytrophic lateral sclerosis point to both protective roles of microglia through the phagcotyic engulfment of misfolded proteins and toxic substances and detrimental effects mediated through increased inflammation, toxicity and indiscriminate phagocytosis of healthy neurons (Cardona et al., 2006; Fellner et al., 2011; Fiala et al., 2007; Fuhrmann et al., 2010; Heneka et al., 2010; Lasiene and Yamanaka, 2011; Lee et al., 2010).

Microglial activity also leads to ambiguous outcomes after acute injury. Inflammatory responses mediated by microglia and astrocytes after acute trauma or lesion within the CNS can lead to the formation of a glial scar which acts as a molecular and physical barrier to the regeneration of axons (Sofroniew, 2009). One study shows that impaired signaling of microglia by knockout of the CX3CR1 chemokine receptor promotes recovery after traumatic spinal cord injury in mice by inhibiting their ability to produce pro-inflammatory cytokines and recruit macrophages (Donnelly et al., 2011). Alternatively, several studies also point to the neuroprotective roles of microglial after acute trauma. *In vivo* imaging of the mouse cerebral cortex showed that microglia respond rapidly to laser induced injury and exert neuroprotective effects by shielding the injured site and rapidly phagocytosing damaged tissue (Nimmerjahn et al., 2005).

Another study showed that microglial clearance of apoptotic neurons after acute neonatal focal stroke is necessary to prevent elevated levels of chemokines and limit the severity and volume of injury (Faustino et al., 2011). Axotomy of the optic nerve in amphibians results in rapid clearance of degenerating myelin debris by microglia which is necessary for successful regeneration of the axon (Battisti et al., 1995). Additionally, in rat hippocampal brain slices, microglial cells protected neurons after ischemic tissue damage through engulfment of invading neutrophil granulocytes (Lambertsen et al., 2009). A better understanding of the molecular mechanisms driving microglial reactivity and engulfment is necessary to fully understand the ambiguity of reactive glial processes.

Drosophila as a model to study glial engulfment mechanisms

The adult *Drosophila* olfactory system provides a tractable, highly stereotyped, and well characterized model in which to study axon degeneration and glial engulfment events. The neuronal cell bodies of olfactory receptor neurons (ORNs) reside outside the CNS in peripheral sensory organs, the maxillary palps and the third antennal segments, and they extend their axon projections into the antennal lobe where they synapse onto spatially distinct glomeruli. Each ORN expresses one or two odorant receptors and all neurons expressing the same odorant receptor(s) will synapse onto the same distinct glomerulus (Vosshall et al., 2000). Removal of the maxillary palp or third antennal segment severs the cell bodies from the axons and ultimately leads to a process termed Wallerian Degeneration which is characterized by fragmentation and blebbing of the portion of the axon distal to the site of injury (Waller, 1850). Within hours of the injury, glial cells

undergo morphological changes, extend processes to the sites of injury, and initiate engulfment of the degenerating axons **(Fig. 1-1C)** (MacDonald et al., 2006). Interestingly, overexpression of the mammalian protein, Wallerian Degeneration Slow (Wld^S), can significantly delay the axon degeneration process in mice, and was sufficient to protect severed axons from degeneration in *Drosophila* as well (Lunn et al., 1989; MacDonald et al., 2006). Thus, severed axons degenerate through a conserved mechanism, and similar to mammals, glial cells are able to sense trauma within the CNS and respond rapidly to phagocytose degenerating debris.

Conserved molecular mechanisms of engulfment

A number of recent studies have reinforced the notion that molecular mechanisms driving engulfment of apoptotic cells are well conserved. In *C. elegans*, two partially redundant pathways mediate cell corpse removal, with CED-1, CED-6, and CED-7 functioning in one pathway, and the actin-regulating protein complex, CED-2, CED-5, and CED-12 acting in the other (Ellis et al., 1991; Reddien et al., 2001). These two pathways converge on CED-10/Rac1 (Kinchen et al., 2005). CED-1 is a phagocytic receptor which recognizes dying cells and initiates degradation of engulfed apoptotic cells (Yu et al., 2008; Zhou et al., 2001b). CED-6 is an adaptor protein which physically binds to CED-1 and likely acts as a signaling molecule through CED-10 to drive phagosome maturation (Liu and Hengartner, 1998; Neukomm et al., 2011; Su et al., 2002; Yu et al., 2006).

In *Drosophila,* Draper, the ortholog of *C. elegans ced-1*, is a phagocytic receptor expressed on the surface of nearly all glial cells. Similar to the role of *C. elegans* CED-1,

Draper is required for proper clearance of cell corpses during development (Freeman et al., 2003). Additionally, both Draper and dCed-6 are required by glial cells for the timely clearance of pruned axons during metamorphosis, the clearance of degenerating axons in the adult brain, and the clearance of pruned synaptic material at the neuromuscular junction (Awasaki et al., 2006; Doherty et al., 2009; Fuentes-Medel et al., 2009; MacDonald et al., 2006).

Furthermore, the mammalian homolog of CED-1, MEGF10, enhances the ability of HeLa cells to phagocytose apoptotic cells and, when overexpressed in worms under the control of the *ced-1* promoter, can partially rescue the corpse engulfment phenotype of *ced-1* mutants (Hamon *et al*., 2006). Recent studies also revealed that MEGF10 acts in satellite glial precursor cells to clear sensory neuron corpses in the dorsal root ganglion (Wu et al., 2009) and facilitates the clearance of amyloid-β in cell culture (Singh et al., 2010). Additionally, GULP, the mammalian ortholog of Ced-6, has been shown *in vitro* to enhance the engulfment of apoptotic cells by macrophages (Park et al., 2010).

Not surprising, CED-1, Draper and MEGF-10 all share common structural features. They all contain a series of EGF-like repeats in their extracellular domains, a single transmembrane domain, and two common motifs, NPXY and YXXL, in their intracellular domains. The NPXY and YXXL motifs have been shown in all three organisms, mouse, worm, and fly, to be critical for their engulfment activity. In *C. elegans* it has been shown that CED-6 binds to CED-1 through the NPXY motif and mutation of either the NPXY or YXXL in CED-1 leads to a reduction in clearance of apoptotic cells, and mutation of both leads to a complete block of engulfment activity (Su

et al., 2002; Zhou et al., 2001a). *In vitro* experiments done in HeLa cells revealed that mutation of either motif in MEGF10 led to a drastic reduction in the ability of cells to engulf amyloid-β protein (Singh et al., 2010). Additionally, mammalian Ced-6/GULP has been shown to bind to the NPXY motif of Stabilin-1, another phagocytic receptor, and mutation of this motif inhibited Ced-6/GULP binding, and led to a reduction in phagocytosis (Park et al., 2010).

Shark, a non-receptor tyrosine kinase similar to mammalian Syk, binds to Draper through the YXXL immunoreceptor tyrosine-based activation motif (ITAM), and Shark activity is essential for Draper mediated engulfment. Binding of Shark to Draper is dependent upon Src42a-mediated phosphorylation of the tyrosine residue in the ITAM motif (Fig. 1-2)(Ziegenfuss et al., 2008). The NPXY motif in Draper is likely not sufficient for engulfment, as an isoform of Draper which contains the NPXY motif but lacks the YXXL motif cannot rescue axon clearance defects in a Draper null animal (Dr. Mary Logan, personal communication). However, this motif is probably necessary for Draper engulfment activity since it likely represents the docking site for the Ced-6 adaptor protein which has been shown to be critical for Draper-mediated clearance and pruning events (Awasaki et al., 2006; Doherty et al., 2009; Fuentes-Medel et al., 2009). These data indicate that molecular features of apoptotic cell clearance are highly conserved among species and *Drosophila* is well poised to provide molecular insight into the mechanisms of glial engulfment applicable across species.

In chapter three I describe a candidate-based, *in vivo,* RNAi screen aimed at identifying novel components of the Draper engulfment pathway. Briefly, I used the

Gal4/UAS system to knock down candidate genes specifically in glial cells, and I then severed *Drosophila* olfactory axons from their cell bodies and assayed glial responses. From this screen, I identified several genes involved in Draper engulfment signaling as well as genes involved in the transcriptional and/or translational regulation of Draper. From these candidate genes, I went on to further define the role of Stat92E in glial engulfment events.

Overview of the Janus Kinase (JAK)/Signal Transduction and Activator of Transcription (STAT) pathway

The JAK/STAT signaling pathway is well conserved and regulates multiple processes of development and immunity (Agaisse and Perrimon, 2004; Arbouzova and Zeidler, 2006). In vertebrates this pathway is inclusive of four Janus kinases (JAKs), seven Signal Transducers and Activators of Transcription genes (STATs), and an array of diverse extracellular ligands and transmembrane receptors (Levy and Darnell, 2002). In canonical JAK/STAT signaling, STAT molecules act as latent transcription factors which reside in the cytoplasm. In the presence of cytokine or growth factor stimulation, JAK molecules, constitutively associated with the receptor, phosphorylate a conserved C-terminal tyrosine residue on STAT leading to its translocation to the nucleus (Darnell et al., 1994; Shuai et al., 1994). STAT molecules then bind to gamma interferon activation site (GAS) elements located in the promoter/enhancer regions of target genes to initiate transcription (Fig. 1-3) (Reich and Darnell, 1989; Schindler et al., 1992; Shuai et al., 1992). The GAS element is a palindromic consensus sequence of TTC(2-4*n*)GAA and

often they are found in multiples as STATs can oligomerize and bind to tandem sequences as tetramers (Ota et al., 2004; Soldaini et al., 2000; Xu et al., 1996). Binding of STAT molecules as tetramers increases the stability of the DNA-protein interaction at tandem low affinity sites by decreasing the off-rate of the complex (John et al., 1999; Vinkemeier et al., 1996).

Although mechanistically the JAK/STAT pathway seems basic, this simplicity is hampered by the number and diversity of ligands and receptors able to directly feed into this pathway and further exacerbated by the fact that many of the receptors can homo- and heterodimerize. Additionally, recent studies reveal that STAT signaling mechanisms are much more complex and diverse than those originally envisaged in the overly simplistic canonical paradigm (Brown and Zeidler, 2008; Li, 2008; Sehgal, 2008). In contrast to the notion that STATs exist as unphosphorylated monomers in the cytoplasm and only upon growth factor or cytokine induced stimulation are they subsequently phosphorylated and translocated to the nucleus, several studies have shown that STATs are capable of existing in the cytoplasm as unphosphorylated dimers and as part of heteromeric complexes with other proteins (Braunstein et al., 2003; Haan et al., 2000; Kretzschmar et al., 2004; Lackmann et al., 1998; Li and Shaw, 2004; Ndubuisi et al., 1999; Novak et al., 1998; Schroder et al., 2004). Furthermore, unphosphorylated STATs are capable of shuttling in and out of the nucleus and can drive transcriptional activation of genes distinct from those whose transcriptional activation is dependent upon phosphorylated STAT molecules (Chatterjee-Kishore et al., 2000; Cui et al., 2007; Yang et al., 2005). In addition to positively regulating transcriptional events, recent evidence

shows that STATs are also able to repress gene transcription. In *Drosophila,* unphosphorylated STAT molecules induce global gene repression and protect genome stability by binding to and stabilizing transcriptionally repressed heterochromatin (Shi et al., 2006; Shi et al., 2008). Evidence from mammals reveals that binding of STAT5 tetramers to DNA, while essential to drive transcriptional activation of some genes, is capable of repressing transcription of other genes through the recruitment of Polycomb repressor proteins (Farrar and Harris, 2011; John et al., 1999; Mandal et al., 2011; Meyer et al., 1997; Yamamoto et al., 2002).

In mammals, the JAK/STAT pathway is the primary signaling mechanism for a wide array of cytokines and growth factors and its dysregulation has been implicated in various malignancies and inflammatory conditions (Lacronique et al., 1997; Levine and Wernig, 2006). While this pathway has been well studied in multiple contexts (i.e., hematopoiesis, immunity, development, proliferation, migration, differentiation), the findings are often inconclusive due to redundancy in the pathway, use of *in vitro* studies which do not recapitulate *in vivo* settings, and tissue-specific adaptations of the pathway. Furthermore, due to the array of growth factors and cytokines capable of activating the JAK/STAT pathway, and the differences in transcriptional outputs resulting from the various modes of activation, many mammalian studies utilize a constitutively active form of STAT which bypasses the need for upstream receptor activation. This system has considerable drawbacks as aberrant STAT signaling is implicated in many forms of cancer and doe not mimic the physiological effects of spatially and temporally controlled ligand-induced STAT activation.

The *Drosophila* JAK/STAT pathway provides a simple alternative to the more complex mammalian pathway. In *Drosophila* there is one JAK (Hopscotch), one STAT (Stat92e), most similar to mammalian STAT3 and STAT5, and one Interleukin-6 related cytokine receptor (Domeless), activated by three known cytokine-like ligands (Upd, Upd2 and Upd3) (Binari and Perrimon, 1994; Chen et al., 2002; Gilbert et al., 2005; Harrison et al., 1998; Hou et al., 1996; Wright et al., 2011; Yan et al., 1996). The study of Stat92E signaling during *Drosophila* development has led to a great wealth of knowledge regarding its role in proliferation, migration, specification, and stem cell maintenance (Ekas et al., 2006; Ghiglione et al., 2002; Silver et al., 2005; Wang et al., 2011). Impressively, despite the relative simplicity of this pathway in *Drosophila*, its pleiotropic roles are well conserved (Arbouzova and Zeidler, 2006).

In mammals STAT signaling is activated in macrophages and glial cells in response to various forms of CNS trauma (Justicia et al., 2000; Kacimi et al., 2011; Natarajan et al., 2004; Oliva et al., 2011; Townsend et al., 2004). In many cases the inflammatory responses of these cells are regulated through STAT signaling and depending on the type and severity of the insult, STAT signaling in glial cells and macrophages can either have beneficial or detrimental effects on surrounding tissue (Amantea et al., 2011; Capiralla et al., 2012; Hao et al., 2010; Herrmann et al., 2008; Kacimi et al., 2011; Okada et al., 2006; Oliva et al., 2011; Park et al., 2003; Yang et al., 2010; Zhao et al., 2011). Several mammalian studies point to the beneficial roles of astrocytic STAT3 upregulation following focal brain lesion, traumatic brain injury, and spinal cord injury (Herrmann et al., 2008; Okada et al., 2006; Zhao et al., 2011).

Alternatively, STAT3 activation in microglial glial cells following focal cerebral ischemia, exposure to lipopolysaccharides, or resulting from amytrophic lateral sclerosis has been shown to promote pro-inflammatory responses which further damage surrounding tissue (Capiralla et al., 2012; Satriotomo et al., 2006; Shibata et al., 2009). While the role of STAT signaling in mammalian glial cells and in particular, in reactive glial cells, has been well established, there is a dearth of knowledge regarding the role of Stat92e in *Drosophila* glial cells. Evidence of Stat92e activity in glial cells comes from a single study showing that cell-autonomous toxicity caused by overexpression of human tau in glial cells is mediated through Stat92E signaling (Colodner and Feany, 2010). This unexplored area has great potential to help unravel some of the key questions and controversies surrounding JAK/STAT signaling in the mammalian nervous system.

In chapter four I identify a role for Stat92E in the regulation of glial engulfment processes. I show that in the presence of injury Stat92E binds to GAS elements located in the first intron of the Draper gene to transcriptionally activate *draper.* Additionally, this occurs through an auto-regulatory loop whereby signaling through the Draper receptor leads to activation of Stat92E through downstream components Shark, Src42a, and Rac1, ultimately leading to Stat92E-dependent transcription of *draper*.

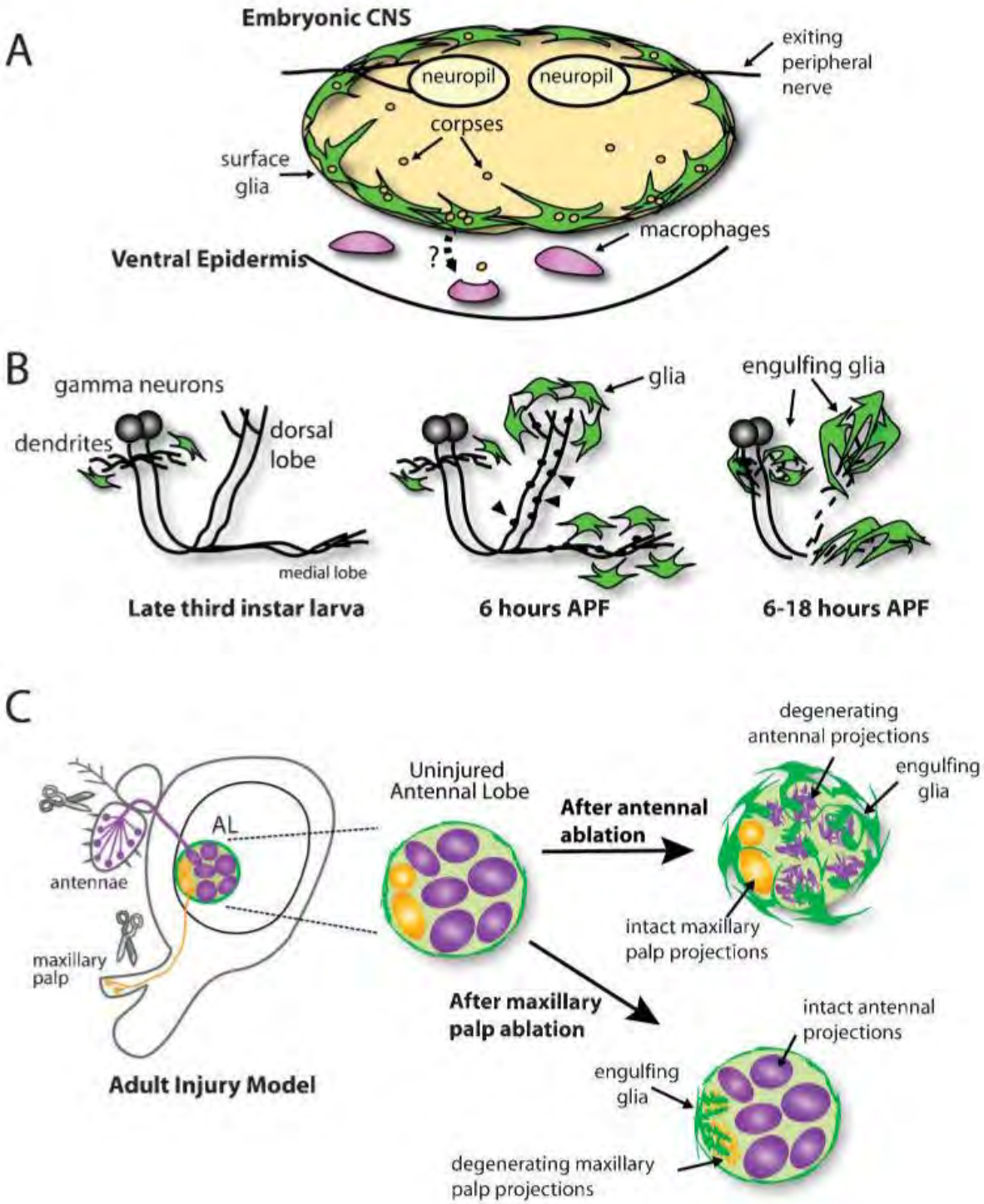
A
Embryonic CNS
neuropil
neuropil
exiting peripheral nerve
corpses
surface glia
macrophages
Ventral Epidermis
?
B
gamma neurons
dendrites
dorsal lobe
medial lobe
Late third instar larva
glia
6 hours APF
engulfing glia
6-18 hours APF
C
AL
antennae
maxillary palp
Adult Injury Model
Uninjured Antennal Lobe
After antennal ablation
degenerating antennal projections
engulfing glia
intact maxillary palp projections
After maxillary palp ablation
intact antennal projections
engulfing glia
degenerating maxillary palp projections

Figure 1-1

Fig. 1-1. Schematic models of glial engulfment in the *Drosophila* nervous system

(A) Glial cells engulf apoptotic neuron corpses in the embryo: Cartoon of a cross-section through a late stage (stage 15/16) embryonic CNS showing only surface glial subtypes (green). Most apoptotic corpses (tan) occur in surface glia (green), particularly within ventral regions of the CNS. After corpses are engulfed, they may be destroyed by glia through lysosomal destruction or possibly transferred to macrophages (pink) that reside outside of the CNS (indicated by question mark). See text for more details. (B) Glia engulf pruned neurites during metamorphosis: At late larval stages, mushroom body (MB) gamma neurons project a single axon extension that bifurcates into the dorsal and medial lobes of the MB. By 6 hours after puparium formation (APF), gamma neuron axons begin to display signs of _blebbing' (arrowheads), and glial cell bodies (green) have accumulated around the dorsal and medial lobes of the MB. Over the next 12 hours, glial membranes invade the MB lobes and engulf fragments of degenerating gamma neuron axons as they are pruned. Glia also engulf degenerating gamma neuron dendrites during this time. (C) Mature glia engulf degenerating olfactory receptor neuron (ORN) axons after injury: ORN cell bodies reside in either the third antennal segments (purple) or maxillary palps (orange) and send axonal projections into the brain where they synapse on antennal lobe (AL) glomeruli. Antennal or maxillary palp ORN axons can be selectively severed by surgical ablation of these olfactory organs. Within hours after injury, severed ORN axons fragment into smaller pieces, and glial cell membranes accumulate specifically on AL glomeruli that contain severed axons to begin engulfing fragmented axon debris. (Reproduced from Logan and Freeman, 2007)

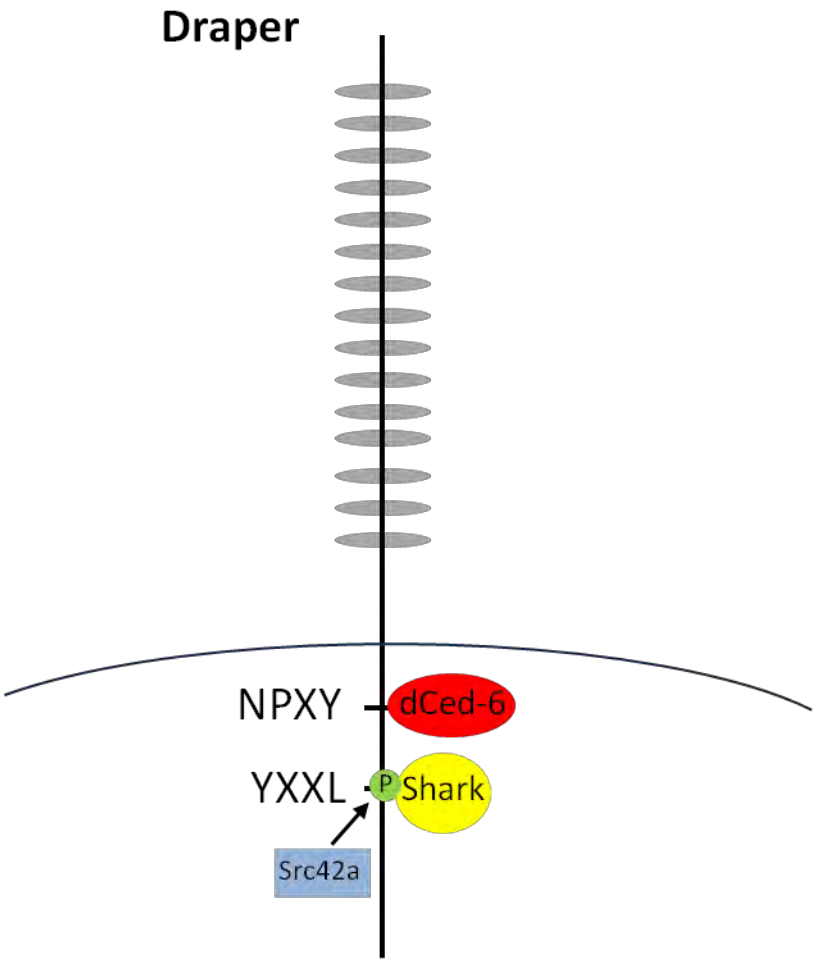

Fig. 1-2. Draper signaling pathway

Draper contains 15 EGF like repeats in its extracellular domain as well as NPXY and YXXL motifs in the intracellular region. In response to an unknown ligand, dCed-6 binds to the NPXY motif and Src42a phosphorylates the YXXL ITAM motif recruiting Shark. This signaling cascade, through unknown downstream effectors, leads to phagocytosis of cell corpses and degenerating axon material.

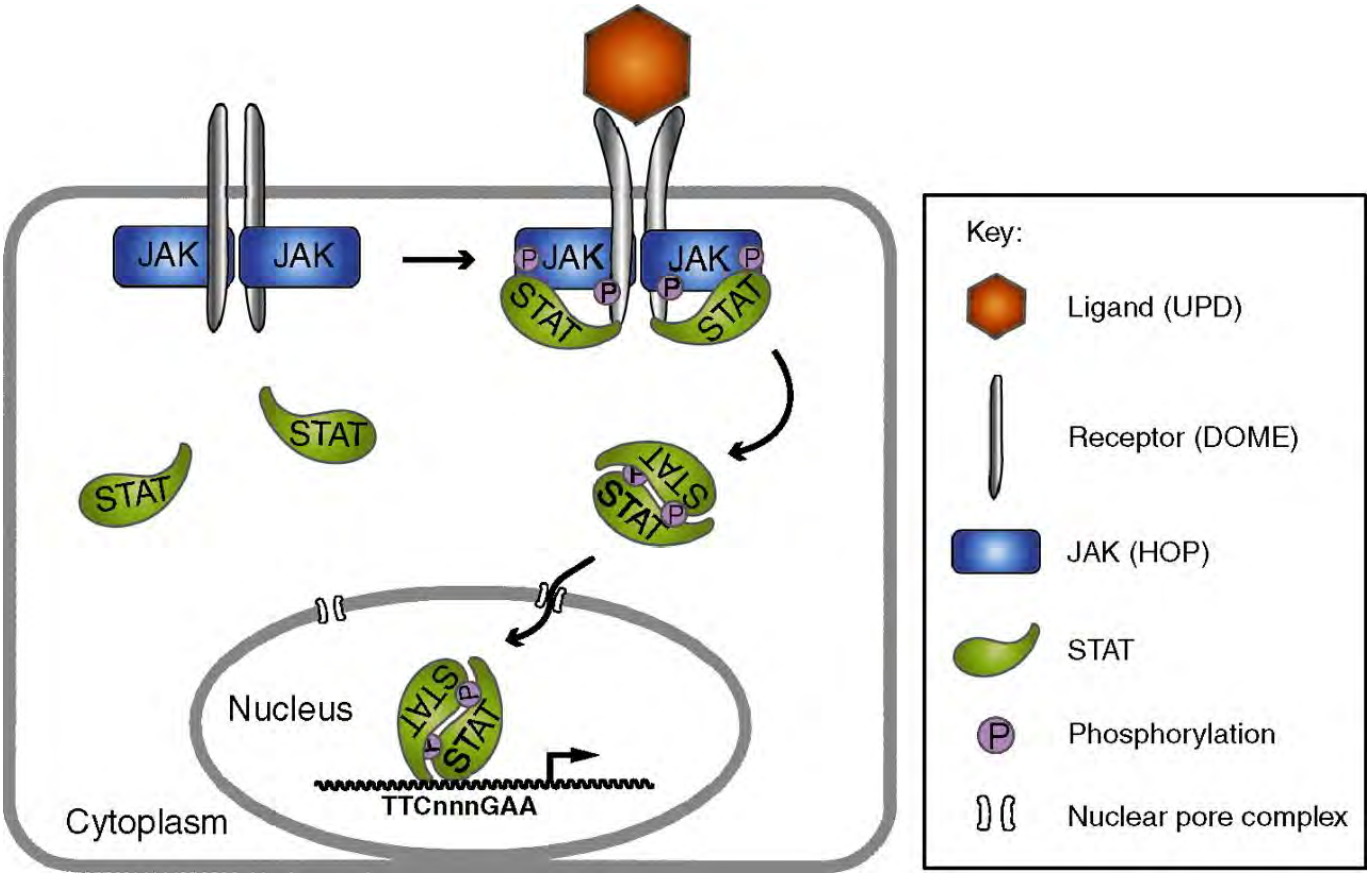

Fig. 1-3. The canonical model of JAK/STAT signaling.

Pre-dimerised complexes of a pathway receptor (grey) and JAKs (blue) are activated following ligand (red) binding. Phosphorylation (purple circles) of the JAKs and the receptors generate docking sites for the normally cytosolic STATs that are recruited to the active complex. Following phosphorylation of the STATs, STAT dimers form, which translocate to the nucleus and bind to a palidromic DNA sequence in the promoters of target genes to activate their transcription. The names of the pathway components in *Drosophila* are provided in brackets in the key. (Reproduced from Arbouzova and Zeidler, 2006)

CHAPTER II: Ensheathing glia function as phagocytes in the adult *Drosophila* brain

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* Johnna Doherty and Mary A. Logan contributed equally to this work

Abstract

The mammalian brain contains many subtypes of glia that vary in their morphologies, gene expression profiles, and functional roles; however the functional diversity of glia in the adult *Drosophila* brain remains poorly defined. Here we define the diversity of glial subtypes that exist in the adult *Drosophila* brain, show they bear striking similarity to mammalian brain glia, and identify the major phagocytic cell type responsible for engulfing degenerating axons after acute axotomy. We find that neuropil regions contain two different populations of glia: ensheathing glia and astrocytes. Ensheathing glia enwrap major structures in the adult brain, but are not closely associated with synapses. Interestingly, we find these glia uniquely express key components of the glial phagocytic machinery (e.g. the engulfment receptor Draper, and dCed-6), respond morphologically to axon injury, and autonomously require components of the Draper signaling pathway for successful clearance of degenerating axons from the injured brain. Astrocytic glia, in contrast, do not express Draper or dCed-6, fail to respond morphologically to axon injury, and appear to play no role in clearance of degenerating axons from the brain. However, astrocytic glia are closely associated with synaptic regions in neuropil, and express excitatory amino acid transporters, which are presumably required for the clearance of excess neurotransmitters at the synaptic cleft. Together these results argue that ensheathing glia and astrocytes are preprogrammed cell types in the adult *Drosophila* brain, with ensheathing glia acting as phagocytes after axotomy, and astrocytes potentially modulating synapse formation and signaling.

Introduction

Glia are the most abundant cell type in the mammalian nervous system, accounting for approximately 90% of cells in the mature brain. The different subtypes of mammalian glia—astrocytes, oligodendrocytes, microglia, and Schwann cells—have been extensively classified based on morphology, molecular markers, and position within the nervous system and are thought to play largely distinct roles in nervous system development and function. In the central nervous system (CNS), astrocytes provide trophic support for neuronal growth and regulate synapse formation and signaling. Oligodendrocytes ensheath and myelinate axons, thereby protecting axons and providing a stable ionic environment for proper conduction of action potentials. Microglia act as the resident immune cells in the brain, responding to infection and neural trauma, acting as phagocytes, and mediating post-trauma events (reviewed in Barres (2008).

The developing *Drosophila* embryonic and larval nervous systems contain unique subsets of glial cells that are morphologically and molecularly similar to their mammalian counterparts (Edenfeld et al., 2005; Freeman and Doherty, 2006; Ito et al., 1995; Logan and Freeman, 2007). *Drosophila* glia play critical roles during the formation of the nervous system, including regulation of axon pathfinding (Hidalgo and Booth, 2000; Poeck et al., 2001), engulfment of apoptotic neurons (Freeman et al., 2003; Sonnenfeld and Jacobs, 1995), and nerve ensheathment (Auld et al., 1995; Leiserson et al., 2000; Silies et al., 2007). Much less is known about adult brain glia in *Drosophila*, although exciting recent evidence suggests that glia in the adult *Drosophila* brain play a central

role in the function and health of the brain. For example, in the adult brain, Ebony, a N-β-alanyl-biogenic amine synthetase, acts in glia to modulate circadian locomotor behavior (Suh and Jackson, 2007); glial cells also signal to one another in the adult through the 7-transmembrane G-protein coupled receptor Moody to actively maintain a pleated septate junction-based blood-brain barrier that isolates the CNS from non-neuronal tissues (Bainton et al., 2005; Schwabe et al., 2005); and glial cells act as phagocytes in the injured brain and engulf degenerating axonal debris after trauma (MacDonald et al., 2006). With the exception of blood-brain barrier formation, which appears to be mediated by a population termed "surface glia", the subtypes of glia that regulate the diversity of glial functions in the adult *Drosophila* brain remain to be identified.

Here we explore the diversity of glial cell populations found in the adult *Drosophila* CNS to determine if unique subtypes of glia exist and how they might differ in morphology, location within the CNS, gene expression patterns, and function. Using the olfactory system as a model tissue, we identify three unique glial subtypes, differing in morphology, patterns of gene expression, and function. We identify "ensheathing glia" as the subtype that are molecularly and functionally competent to act as phagocytes after acute axotomy. We also identify an astrocytic subtype of glia in *Drosophila*, which does not appear to respond morphologically to axotomy or be required for clearance of degenerating axons. Thus, uniquely identifiable and functionally distinct subclasses of glia exist in the mature *Drosophila* brain, and these bear striking molecular and functional similarity to the major glial subtypes found in the mammalian brain.

Results

In an effort to identify distinct morphological subtypes of glial cells in the mature *Drosophila* brain, we used the MARCM system (Lee and Luo, 2001) to generate small clones of glial cells labeled with membrane tethered GFP. Larvae containing a *hs-flippase* allele and a wild type chromosome arm for recombination were subjected to a short heat shock (37^{o}C) and glial cells within clones were visualized by use of the pan-glial driver, *repo-Gal4*. Our analysis of glial subtype morphology is focused mainly on the adult antennal lobe region (cartoon schematic depicted in **Fig. 2-1**) owing to its well-defined histology and accessibility to genetic manipulation. In the adult brain we identified clones resembling each of the three main types of glial cells found in embryos and larvae: (1) cortex glia, which resided outside the neuropil in regions housing neuronal cell bodies, ramified dramatically to surround individual cell bodies (**Fig. 2-1B**); (2) surface glia, which appeared as large flat cells enveloping the surface of the brain, did not extend any processes into the brain (not shown); and (3) neuropil glia, which were closely associated with the neuropil, extended membranes into synaptic regions, and surrounded large bundles of axons (**Fig 2-1A,C**). As in the embryo and larva, glial cell bodies were not found within the neuropil, rather they resided at the edge of the neuropil (neuropil glia), in the cortex (cortex glia) or at the surface of the brain (surface glia).

Interestingly, the single-cell resolution provided by MARCM analysis allowed us to further subdivide neuropil glia into two distinct morphological classes, ~~–e~~nsheathing glia" and ~~–a~~strocytic glia". Ensheathing glia (**Fig. 2-1A**) appeared as flattened cells that

lined the borders of the neuropil and subdivided regions of the brain by isolating neuropil from the surrounding cortex. Within the antennal lobe, ensheathing glial membranes surrounded individual glomeruli (the functional units of the antennal lobe) but did not extend into the synaptic regions of the glomeruli. In addition, we identified an astrocyte-like cell type (**Fig. 2-1C**) that extended membrane processes deeply into the neuropil and ramified profusely in synaptic-rich regions. This latter cell type we refer to as the fly ~~–a~~strocyte," based on its striking morphological similarity to mammalian astrocytes, as well as the conserved expression of a number of molecular markers used to identify astrocytes in the mammalian brain. Mammalian astrocytes remove excess amounts of extracellular glutamate through the high-affinity excitatory amino acid transporters (EAAT), GLAST and GLT-1, which transports the glutamate into glial cells where it is then converted into glutamine by glutamine synthetase (Chaudhry et al., 1995; Lehre et al., 1995; Rival et al., 2004). We found that Drosophila astrocytes also express the transporter EAAT1 (**Supplemental Fig. 2-1**). While we used the adult antennal lobe as our primary model tissue in this study, we observed the morphological glial subtypes described above in all brain regions examined, suggesting that our results are generally applicable to glial populations throughout the adult *Drosophila* brain (**Supplemental Fig. 2-2**).

We next sought to identify Gal4 driver lines that would allow us to uniquely label and manipulate these glial populations, with our major focus being to genetically subdivide neuropil glia (i.e. ensheathing glia versus astrocytes). To accomplish this, we crossed *UAS-mCD8::GFP* to a previously described collection of embryonic and larval

glial drivers (Ito et al., 1995) , as well as a number of drivers generated in our own laboratory. We then looked in the adult antennal lobe to examine the morphology and spatial distribution of cell types marked by these drivers in a background with glial nuclei (α-Repo) and the neuropil (α-nc82) also labeled. The *repo-Gal4* driver labeled all $Repo^+$ glial subtypes in the adult brain, as evidence by α-Repo immunostaining in the nuclei of GFP^+ cells (**Fig. 2-2A**). Membrane processes from $Repo^+$ cells are found throughout the adult brain, and together they constitute the diverse collection of glial subtypes identified in our single-cell MARCM analysis. Upon examination of a single glomerulus within the antennal lobe we found that membranes from $Repo^+$ cells both surround and invade glomeruli (**Fig. 2-2A3,A4**). All GFP expression in the adult brain driven by the *repo-Gal4* driver can be suppressed by co-expression of Gal80 (a Gal4 inhibitor) under control of the repo promoter (*repo-Gal80*) (**Fig. 2-2A5**), arguing that *repo-Gal80* can efficiently block Gal4-mediated activation of UAS-reporters in all adult brain glia.

Two drivers, *mz0709-Gal4* and *alrm-Gal4*, appeared to show very specific expression in ensheathing glia and astrocytes, respectively (**Fig. 2-2B1,C1**). Glial processes labeled by *mz0709-Gal4* were found at the edge of the antennal lobe and extended deeply into the neuropil region (**Fig. 2-2B1,B2**). These flattened glial processes surrounded, but did not invade, individual glomeruli (**Fig. 2-2B3,B4**), and did not extend into the cortex region. With the exception of variable expression in a small number of neurons, all *mz0709-Gal4*-induced expression was suppressed by *repo-Gal80*, indicating that *mz0709-Gal4* is largely specific to ensheathing glia. The generation of MARCM clones labeled with the *mz0709-Gal4* driver resulted in the consistent labeling of

ensheathing glia, but not astrocytes, within the antennal lobes. Reciprocally, *alrm-Gal4* was found to be expressed exclusively in astrocytes (**Fig. 2-2C**). All cellular processes from cells labeled with *alrm-Gal4* extended into the neuropil (**Fig. 2-2C1,C2**), showed a highly branched or tufted morphology, invaded individual glomeruli (**Fig. 2-2C3,C4**), and all *alrm-Gal4*-driven expression was suppressed by *repo-Gal80* (**Fig.2- 2C5**). Additionally, we found that single cell MARCM clones labeled with the *alrm-Gal4* driver resulted in the consistent labeling of astrocytes, but not ensheathing glia. Together, these drivers are excellent tools to manipulate and functionally distinguish different subtypes of glia in the adult *Drosophila* brain.

Ensheathing glia, but not astrocytes, express the engulfment receptor Draper

What are the functional roles for each glial subtype in the adult brain? Is each subtype responsible for a unique collection of tasks, or are all glial subtypes functionally equivalent? As a first step to determining the *in vivo* functional differences between adult brain glial subtypes we explored the cell autonomy of glial phagocytic function. Severing olfactory receptor neuron (ORN) axons by surgical ablation of maxillary palps leads to axon degeneration (termed Wallerian degeneration), recruitment of glial membranes to fragmenting axons, and glial engulfment of axonal debris. These glial responses are mediated by Draper, the *Drosophila* ortholog of the *C. elegans* cell corpse engulfment receptor CED-1. In *draper* null mutants, glia fail to extend membranes to degenerating ORN axons and axonal debris is not removed from the CNS (MacDonald et

al. 2006). Thus, Draper function should be autonomously required in phagocytic glial subtypes and Draper expression is predicted to act as a molecular marker for glial cells capable of performing engulfment functions.

To define the precise cell types that express Draper, we first labeled all glial membranes with mCD8::GFP driven by *repo-Gal4*, stained with α-Draper antibodies, and assayed for colocalization of Draper and GFP (**Fig. 2-3**). As previously reported, we found extensive overlap of Draper and GFP in this background (MacDonald et al., 2006). Draper and GFP signals overlapped at the edge of the neuropil, in membranes surrounding antennal lobe glomeruli, and in all cortex glia (**Fig. 2-3A1-A3**). This labeling was specific to Draper since expression of a *UAS-draper*^{RNAi} construct with *repo-Gal4* led to the elimination of all Draper immunoreactivity in the adult brain (**Fig. 2-3A4,A5**). Thus, the entire population of cortex glia appear to express Draper and are likely to be phagocytic. However, cortex glia do not extend membranes into the antennal lobe neuropil, even after ORN axon injury (data not shown). Therefore, cortex glia are not likely responsible for clearing severed ORN axonal debris from the antennal lobe neuropil.

Interestingly, when mCD8::GFP was driven by *mz0709-Gal4* we observed extensive overlap of Draper and GFP in neuropil-associated ensheathing glia (**Fig. 2-3B1-B3**). A high magnification view of the antennal lobe revealed Draper and *mz0709-Gal4* labeled membranes colocalizing and surrounding, but not innervating individual glomeruli (**Fig. 2-3D1-D3**). Moreover, expression of *UAS-draper*^{RNAi} in ensheathing glia

with *mz0709-Gal4* led to a dramatic reduction in Draper immunoreactivity in the neuropil, but the weaker Draper immunoreactivity in the cortex remained unchanged (**Fig. 2-3B4,B5**). Conversely, we observed no overlap between Draper and GFP when we labeled astrocytic membranes using the *alrm-Gal4* driver (**Fig. 2-3C1-C3, 2-3E1-E3**). Furthermore, driving the expression of *UAS-draper*^{RNAi} in astrocytes had no obvious effect on Draper expression in the brain (**Fig. 2-3C4,C5**). These results indicate that Draper is expressed in cortex glia and ensheathing glia but not in astrocytes.

Ensheathing glia use Draper to extend membranes to degenerating axons and engulf axonal debris

The specific expression of Draper in antennal lobe ensheathing glia suggests that this glial subset is the phagocytic cell type responsible for engulfing degenerating axonal debris after ORN axotomy. To explore this possibility, we asked whether ensheathing glia or astrocytes extend membranes to severed axons after injury, and in which cell type Draper was required for clearance of axonal debris from the CNS. To assay extension of glial membranes to severed axons, we labeled glial membranes with mCD8::GFP, severed maxillary palp axons, and assayed for colocalization of Draper and GFP in glomeruli housing severed ORN axons. Within one day after injury, Repo^{+} glial membranes were found to localize to glomeruli housing severed maxillary palp axons and these membranes were decorated with Draper immunoreactivity (**Fig. 2-4A1-A4**). Similarly, we found that mz0709^{+} glial membranes also localized to severed axons and

colocalized with intense Draper immunoreactivity 1 day after injury (**Fig. 2-4B1-B4**). Knockdown of Draper with *UAS-draper*^{RNAi} using *repo-Gal4* or *mz0709-Gal4* completely suppressed the recruitment of both Draper and glial membranes to severed axons (**Fig. 2-4A5,A6,B5,B6,D**). In contrast, when astrocyte membranes were labeled with GFP we did not observe colocalization of GFP and Draper immunoreactivity 1 day after axotomy (**Fig 2-4C1-C4**). In addition, knockdown of Draper in astrocytes with *UAS-draper*^{RNAi} did not suppress the recruitment of Draper to severed axons (**Fig. 2-4C5,C6,D**). In an effort to identify any indirect role for astrocytes during the injury response, we examined the morphology of astrocytes both before and after injury to determine whether they exhibited any overt changes in morphology or retracted their membranes from the site of injury to accommodate the recruitment of ensheathing glial membranes. However, we did not detect any obvious changes in morphology or in the positions of the astrocyte glial cells in response to axon injury. Together, these data indicate that Draper is required in ensheathing glia for recruitment of glial membranes and accumulation of Draper on severed ORN axons, and suggest that *Drosophila* astrocytic glia do not undergo any dramatic changes in morphology in response to ORN axotomy.

From the above data we predicted that ensheathing glia would act as phagocytes to engulf degenerating ORN axonal debris from the CNS. To test this we labeled a subset of maxillary palp ORN axons with mCD8::GFP using the OR85e-mCD8::GFP transgene, knocked down Draper function in glial subsets using our subset-specific driver lines, severed maxillary palp ORN axons, and assayed clearance of axons 5 days after injury. We first severed GFP-labeled axons in control animals with each driver and

found that GFP^{+} axonal debris was efficiently cleared from the CNS within 5 days after injury (**Fig. 2-4E1,E2,F1,F2,G1,G2,H,I**), confirming that glial phagocytic function is not affected in the driver lines. Strikingly, RNAi knockdown of Draper using *UAS-draper^{RNAi}* in a background with *repo-Gal4* or *mz0709-Gal4* completely blocked clearance of GFP labeled axonal debris from the CNS (**Fig. 2-4E3,E4,F3,F4,H,I**), while RNAi knockdown of Draper in astrocytes with *alrm-Gal4* had no effect on axon clearance (Fig. 4G3,G4,H,I). Thus, Draper is required autonomously in ensheathing glia for the clearance of degenerating ORN axonal debris from the CNS. In addition, knockdown of Draper in ensheathing glia with *mz0709-Gal4* had no measureable effect on Draper expression in cortex glia (see **Fig. 2-3B4,B5**), arguing that cortex glia are not capable of compensating for the loss of phagocytic activity in ensheathing glia during the clearance of axonal debris from the antennal lobe neuropil after axotomy. From these data on morphogenic responses to injury and phagocytic function, we conclude that astrocytic, cortex, and ensheathing glia represent functionally distinct subsets of glial cells in the adult *Drosophila* brain.

Shark, a Src-family kinase acting downstream of Draper, is required in ensheathing glia for clearance of degenerating axons

We have recently shown that Shark, a non-receptor tyrosine kinase similar to mammalian Syk and Zap-70, is part of the Draper signaling cascade and is essential to initiate phagocytic signaling events downstream of Draper during the engulfment of degenerating

axons (Ziegenfuss et al., 2008). Our model that Draper functions exclusively in ensheathing glia for clearance of degenerating ORN axons predicts that Shark and other components of the Draper signaling cascade would also function in ensheathing glia. To determine whether Shark function is required in ensheathing glia, we knocked down Shark in glial subsets and assayed the recruitment of Draper to severed ORN axons 1 day after injury, and the clearance of degenerating axonal debris from the CNS 5 days after axotomy. Consistent with a role for Shark in ensheathing glia, we found that knockdown of Shark with *UAS-Shark*^{RNAi} (Ziegenfuss et al., 2008) driven by *repo-Gal4* or *mz0709-Gal4* strongly suppressed both the recruitment of mCD8::GFP-labeled glial membranes and Draper to severed axons (**Fig. 2-5A1,A2,B1,B2,D**), as well as the clearance of degenerating axonal debris from the CNS (**Fig. 2-5E1,E2,F1,F2,H,I**). However, knockdown of Shark with *alrm-Gal4* had no effect on the recruitment of Draper or GFP-labeled glial membranes to degenerating axons (**Fig. 2-5C1,C2,D**), nor did it inhibit the clearance of axonal debris from the CNS (**Fig. 2-5G1,G2,H,I**). Thus, Shark, like Draper, is required in ensheathing glia for efficient extension of glial membranes to degenerating axons and clearance of degenerating axonal debris from the CNS.

dCed-6 is expressed in ensheathing and cortex glia, and is required for glial clearance of degenerating axons

In *C. elegans* the PTB domain adaptor protein CED-6 acts genetically downstream of CED-1 during engulfment of apoptotic cells (Liu and Hengartner, 1998) and, during

Drosophila metamorphosis, RNAi knockdown of *ced-6* has been shown to partially suppress glial engulfment of pruned axon arbors during remodeling of larval mushroom body γ neurons (Awasaki et al., 2006). We asked whether dCed-6 was involved in glial reponses to axon injury in the adult brain. First, to determine where dCed-6 is expressed in the adult CNS we stained control animals with Draper and dCed-6 antibodies and found that Draper and dCed-6 immunoreactivity perfectly overlapped throughout the adult brain (**Fig. 2-5A1,B1,C1, D1-D4**). Since we have shown that Draper is expressed in cortex and ensheathing glia, we conclude that dCed-6 is also expressed in these glial subtypes.

Next we asked if dCed-6 was recruited to degenerating ORN axons. We performed maxillary palp or antennal ablations and compared dCed-6 and Draper recruitment 1 day after injury. We consistently found that dCed-6 colocalized with Draper at sites of severed axons after maxillary palp (**Fig. 2-6A2,B2,C2**) and antennal injury (**Fig. 2-6A3,B3,C3**), which is consistent with dCed-6 being expressed in ensheathing glia and possibly functioning downstream of Draper.

To obtain genetic evidence that dCed-6 was essential for glial clearance of degenerating axonal debris, we used the *Df(2R)w73-1* deletion chromosome, which harbors a small deletion that removes the *dced-6* gene, and assayed for genetic interactions between this *dced-6* deletion chromosome and *draper* mutants. In control animals, GFP-labeled ORN axons are largely cleared 3 days after injury (**Fig. 2-6E1,E2,I**). Interestingly, we found that while the majority of axons were cleared at this

time point in *draper*$^{\Delta 5}$/+ or *Df(2R)w73-1*/+ animals (**Fig. 2-6F1,F2,G1,G2,I**), a significant number of axons remained in *Df(2R)w73-1*/+; *draper*$^{\Delta 5}$/+ trans-heterozygous animals. (**Fig. 2-6H1,H2,I**) These data are consistent with *draper* and *ced-6* exhibiting strong genetic interactions during glial clearance of degenerating axons.

The expression pattern of dCed-6 in the adult brain suggests that it is acting in ensheathing and/or cortex glia to mediate Draper-dependent glial engulfment functions. To determine the autonomy of dCed-6 function in the adult brain, we knocked down dCed-6 in different glial subsets using a *UAS-dced-6*^{RNAi} construct and assayed Draper recruitment to severed axons and clearance of degenerating axonal debris (**Fig. 2-7**). Knockdown of dCed-6 in all glial cells using *repo-Gal4* suppressed both the recruitment of Draper to the site of injury (**Fig 2-7A1,A2,B1,B2,E**) as well as the clearance of degenerating GFP^{+} axon material five days after injury as compared to control animals (**Fig. 2-7F1,F2,G1,G2,J**). Based on our findings using Draper^{RNAi} and Shark^{RNAi}, we expected Ced-6^{RNAi} treatment using the *mz0709-Gal4* driver to also suppress glial responses to axon injury. However, we found normal levels of Draper recruited to severed axons one day after injury (**Fig. 2-7C1,C2,E**) and efficient clearance of axon material five days after injury (**Fig. 2-7H1,H2,J**). To assay the level of dCed-6 knockdown by RNAi, we stained brains with α-dCed-6 antibodies five days after maxillary palp ablation and found moderate levels of dCed-6 staining when *UAS-dced-6*^{RNAi} was driven by *mz0709-Gal4* or *alrm-Gal4*, but not when driven by *repo-Gal4* (**Fig. 2-7F3,G3,H3,I3**). Thus, *mz0709-Gal4* does not appear to provide a complete knockdown of dCed-6 in ensheathing glia. Nevertheless, based on the colocalization of

Draper, and dCed-6 in ensheathing glial subtypes in the adult brain, we propose that dCed-6 acts in ensheathing glia to promote Draper-dependent recruitment of glial membranes to severed axons and clearance of degenerating axonal debris.

Blocking endocytosis in ensheathing glia suppresses glial clearance of severed axons

Our analyses of Draper, Shark, and dCed-6 indicate that ensheathing glia express all components of the engulfment machinery and that the Draper signaling pathway is essential in ensheathing glia for efficient clearance of degenerating axons in the adult brain. These findings argue that ensheathing glia are the primary phagocytic cell type in the adult brain neuropil. Nevertheless, to further exclude any possible role for astrocytic glia in engulfing degenerating axons, we used the dominant temperature-sensitive *shibire*^{ts} molecule (*UAS-shibire*^{ts}) to conditionally block endocytosis in either astrocytes or ensheathing glia, and subsequently assayed glial responses to axon injury.

Interestingly, when we raised animals expressing *shibire*^{ts} under the control of *repo-Gal4* at 18^{o}C and then subsequently shifted the adult animals to the restrictive temperature of 30^{o}C, we observed 100% lethality within 3 days after temperature shift. Thus, suppressing glial endocytic function in all glia results in rapid adult lethality, indicating that in the healthy adult *Drosophila* brain glial cells likely perform a high level of endocytic events that are essential for viability.

We next drove *shibire*^{ts} in ensheathing or astrocytic glia and assayed recruitment of Draper to severed axons 1 day after injury. At the permissive temperature of 18^{o}C, expression of Shi^{ts} in ensheathing glia had no effect on Draper recruitment to severed

axons. However, shifting animals to the restrictive temperature strongly suppressed this response (**Fig. 2-8A1-A4,C**). In contrast, expression of Shi^{ts} in astrocytes had no effect on Draper recruitment to severed axons at either permissive or restrictive temperatures (**Fig 2-8B1-B4,C**). Moreover, we found that blocking endocytic function in ensheathing glia with Shi^{ts} (at restrictive temperature) strongly suppressed glial clearance of degenerating axons from the brain, while the same treatment of astrocytes had no effect on axon clearance (**Fig. 2-8D1,D2,E1,E2,8D3,D4,F,G**). These data provide additional compelling evidence that ensheathing glia are the primary phagocytic cell type responsible for engulfing degenerating ORN axons. We also note, since blocking endocytic function in astrocytes did not affect glial clearance of severed axons (**Fig. 2-8E3,E4,F,G**), these data further argue that neither phagocytic activity nor signaling events involving endocytosis in astrocytes are essential for efficient clearance of degenerating axonal debris from the CNS.

Discussion

Molecular and morphological subtypes of glia in the *Drosophila* adult brain

We have identified three molecularly and morphologically distinct subtypes of glia in the adult *Drosophila* brain: ensheathing glia, astrocytes, and cortex glia. We note again that surface glia, which form the blood brain barrier, are another major glial subtype in the adult brain (Bainton et al., 2005), but surface glia do not make extensive contact with neurons and we have not addressed this subtype in this study. Rather we have focused only on those glial subtypes that are in direct association with CNS neurons.

The morphology of ensheathing glia, astrocytes, and cortex glia appears to be hardwired. For example, 100% of astrocytic membrane processes invade the neuropil, and we never observed astrocyte processes straying into the cortex. Likewise, ensheathing glia and cortex glia appear to respect discrete spatial boundaries within the brain. Ensheathing glia extend the membranes into the neuropil, but not into the cell cortex, and within the neuropil they appear to primarily form boundaries between neuropilar structures with brain lobes such as antennal lobe glomeruli or different regions of the mushroom body (i.e. α/β, $\alpha'\beta'$, and γ lobes), or between discrete brain centers (e.g. antennal lobe and subesophageal ganglion). Cortex glia extend profuse projections throughout the region of the brain housing neuronal cell bodies, and, impressively, appear to individually enwrap each neuronal cell body, but we never observed cortex glia extending membranes out of their unique spatial domain (the cortex) and into the neuropil. We have never found an adult brain glial cell that has the characteristics of more than one glial subtype (e.g. a cell with membranes in the cortex surrounding cell bodies that also ensheaths an antennal lobe glomerulus). Thus the morphology of ensheathing, astrocytic, and cortex glial membranes is precisely controlled, presumably by underlying molecular programs that are specific to each subtype. In the future it will be exciting to determine how these unique glial subtypes, spatial domains, and morphologies are established, and to identify the underlying molecular pathways that govern glial subtype identity and morphogenesis. In addition, we may find that some or all of these glial subtypes can be further refined into smaller populations, just as it has

long been thought that astrocytes comprise heterogeneous populations of glia (Barres, 2008; Matthias et al., 2003).

Importantly, while the gross morphology and spatial domain of any particular glial subtype is highly stereotyped, the precise morphology of each glial cell within any given class may not be preprogrammed or stereotyped. For example, in our MARCM analysis of ensheathing glia or astrocytes, each single cell clone had a unique morphology (i.e. we did not observe specific ensheathing glia or astrocytes based on position or morphology that were uniquely identifiable from animal to animal), and the position of glial nuclei and glial cell bodies at the edge of the neuropil appeared variable from one animal to the next. We suspect the precise morphology of glia within these classes is determined by cell-cell trophic interactions with neighboring glia of the same class. Our observations that each glial subtype (cortex, ensheathing, or astrocytic) distributes membranes throughout its entire spatial domain argues that regulatory pathways are in place to ensure full coverage of each spatial domain with the appropriate glial subtype. Finally, in support of our conclusion on the stereotyped morphologies of these glial populations we note that *Awasaki et al.* (2008) have recently described a very similar complement of glial subtypes using MARCM clonal analysis in the *Drosophila* adult brain.

Drosophila **adult brain glial subtypes have unique functions in the brain**

Mammalian macroglial subtypes are thought to play specific roles in CNS development, function, and health. Astrocytes are general regulators of synapse formation and physiology. They secrete synaptogenic molecules, associate closely with mature synapses, buffer ions, pH, and neurotransmitters at the synaptic cleft, and likely modulate synaptic efficacy and signaling. Oligodendrocytes physically separate axons through ensheathment, and myelinate them to allow for saltatory conduction. Microglia are the resident immune cells of the brain, rapidly responding to injury, phagocytosing dead cells and cellular debris and modulating brain inflammatory responses (Barres, 2008). Our work argues that glia found in the adult *Drosophila* brain are very similar to their mammalian counterparts in that they vary according to morphological, functional, and molecular criteria.

As a first step in defining the functional differences between glial subtypes in the adult *Drosophila* brain we focused primarily on the role of ensheathing glia and astrocytes in engulfing degenerating axons after brain injury. We have shown that ensheathing glia express all key components of the Draper signaling pathway (see below), respond morphologically to axon injury by extending membranes to severed axons, and clear degenerating ORN axons from the brain. Thus ensheathing glia appear to be the primary cell type that clears degenerating axonal debris from the neuropil and plays the role of resident phagocyte. Moreover, since ensheathing glia invade new spatial regions of the brain after injury (e.g. antennal lobe glomeruli) we cannot rule out the possibility that ensheathing glia extend membranes into the cell cortex and engulf neuronal debris after cell cortex-specific axotomy or neuronal death. However, cortex

glia may also perform phagocytic functions in the cell cortex region of the brain since they express engulfment genes (Draper and dCed-6). Unfortunately, we are unable to test these ideas directly since we are currently unable to generate reproducible brain lesions in the cell cortex.

How do ensheathing glia survey the brain for axonal injury? With respect to the olfactory system, ensheathing glia envelop both the antennal and maxillary nerves as they enter the brain and their membranes are in close proximity to axons up to the point where axons enter to terminate within individual antennal lobe glomeruli. Upon axotomy, ensheathing glia are thus uniquely positioned to sense "eat me" cues presented by degenerating ORN axons. Since ensheathing glial membranes also invade antennal lobe glomeruli, a region of the brain where they normally are absent, these axon-derived cues are clearly sufficient to drive dynamic extension of ensheathing glial membranes into new regions of the CNS.

Of the three subtypes of glia we describe the *Drosophila* astrocyte is most striking in its resemblance to its mammalian counterpart. Similar to mammalian protoplasmic astrocytes, *Drosophila* astrocytes show a highly branched and tufted morphology, extending profuse membrane specializations into regions of the fly brain that are rich in synapses. At the molecular level, these cells also appear similar to mammalian astrocytes: fly astrocytes express the high-affinity excitatory amino acid transporter, EAAT1, homologous to mammalian GLAST and GLT-1, which transport the glutamate into glial cells where it is then converted into glutamine by glutamine synthetase

(**Supplemental Fig. 2-1**). Our identification and characterization of the astrocyte-specific *alrm-Gal4* driver now opens the door to exciting genetic, cellular, and molecular analyses of astrocyte development and function in *Drosophila*.

We found no evidence that fly astrocytes in the adult brain respond to axon injury, or are required for clearance of degenerating axons from the CNS. *Drosophila* astrocytes exhibit no detectable changes in morphology after ORN axon injury, lack endogenous expression of components of the Draper signaling pathway, and they do not appear to activate the expression of these genes after axon injury. Moreover, we found that knocking down Draper signaling or blocking the majority of endocytic function in astrocytes with *shibire*^{ts} had no effect on the clearance of degenerating axons by ensheathing glia. A number of transmembrane receptors mediating cell-cell signaling require endocytosis for signaling events. The latter result therefore argues that such pathways in astrocytes must be dispensible for ensheathing glial clearance of degenerating axons from the CNS. These observations were somewhat surprising since mammalian astrocytes respond to a variety of brain injury by increasing expression of glial fibrillary acid protein (GFAP) and undergoing hypertrophy (Liu et al., 1998; Murray et al., 1990) and intercellular ATP signaling occurs between astrocytes and microglia in the mouse cortex after acute injury (Davalos et al., 2005). Since a *GFAP* gene is not present in the *Drosophila* genome we were unable to assay its expression, but fly astrocytes showed no obvious morphological changes after injury, suggesting they do not undergo axon injury-induced hypertrophy. A major proposed role for reactive astrocytes in mammals is the modulation of neuroinflammation (Wyss-Coray and Mucke, 2002),

rather than phagocytic activity. Since classical tissue inflammatory responses have not been described in *Drosophila*, modulation of neuroinflammation may be an astrocytic function specific to more complex nervous systems than those found in Diptera.

Ensheathing glia express components of the Draper engulfment signaling pathway and act as phagocytes to clear degenerating ORN axons

The Draper signaling pathway is a central mediator driving glial engulfment of neuronal cell corpses (Freeman et al., 2003), pruned axons and dendrites (Awasaki et al., 2006; Hoopfer et al., 2006; Williams et al., 2006), and axons undergoing Wallerian degeneration (MacDonald et al., 2006) in *Drosophila*. Our studies show that within the adult brain neuropil, ensheathing glial cells are the only cell type that expresses Draper. Draper expression overlaps precisely with ensheathing glial membranes (when labeled with GFP driven by the ensheathing glia-specific driver *mz0709-Gal4*), and RNAi for *draper* in ensheathing glia leads to the elimination of Draper immunoreactivity in the neuropil. Similarly, we have found that dCed-6, the fly ortholog of *C. elegans* CED-6, a PTB-domain binding protein that functions genetically downstream of worm CED-1, is expressed in the adult brain in a pattern indistinguishable from Draper. dCed-6 immunoreactivity is strongly reduced in the neuropil through *mz0709-Gal4* mediated knockdown (with *UAS-dced-6*^{RNAi}), and eliminated from the entire brain when dCed-6^{RNAi} treatment is performed with the pan-glial driver *repo-Gal4*. Thus, in the adult brain dCed-6 appears to be expressed exclusively in glia, including cortex glia and ensheathing glia of the neuropil.

Components of the Draper signaling pathway are required specifically in ensheathing glia for glial membrane recruitment to severed ORN axons, and clearance of degenerating axonal debris from the brain. We have shown that knocking down either *draper* or *shark* in ensheathing glia is sufficient to block recruitment of glial membranes and Draper to severed axons and clearance of degenerating axonal debris from the CNS. We suspect that dCed-6 is also required in ensheathing glia for a number of reasons. First, dCed-6 and Draper show perfect overlap in expression in the neuropil, and Draper is only expressed in ensheathing glia. Second, ensheathing glia are recruited to degenerating axon injury, and dCed-6, like Draper, is specifically recruited to degenerating maxillary palp ORN axons after maxillary palp ablation. Third, dCed-6 expression is dramatically increased in ensheathing glia surrounding the antennal lobe after antennal ablation, similar to what we have previously found for Draper (MacDonald et al., 2006). Surprisingly, RNAi knock down of *dced-6* in ensheathing glia (with *mz0709-Gal4*) failed to suppress the recruitment of Draper to severed ORN axons or the clearance of axonal debris from the brain. We suspect this is because *mz0709-Gal4* mediated knockdown of *dced-6* is incomplete in ensheathing glia, based on reduced but not eliminated staining in this background. Nevertheless, our observations that dCed-6 is localized with Draper in immunostains, is required in glia by RNAi knockdown with *repo-Gal4*, and that a null allele of *dced-6* genetically interacts with *draper* null mutations in axon engulfment, argues strongly for a role for dCed-6 in ensheathing glia.

Moreover, knockdown of *draper* in ensheathing glia, or suppressing glial engulfing activity by blocking endocytic function with Shibire^{ts} fully suppresses the

recruitment of Draper to severed axons and clearance of degenerating axonal debris from the brain. In contrast, astrocytes do not express Draper (or dCed-6), fail to respond morphologically to axon injury, and knockdown of *draper* in astrocytes has no effect on the recruitment of Draper to severed axons or clearance of axonal debris from the CNS. Such a separation of phagocytic function in neuropil glial cells suggests similarities to the assigned functional roles in mammalian glia, with ensheathing glia as resident phagocytes, engulfing the majority or all axonal debris and astrocytes perhaps playing a less important role in phagocytosis of degenerating axons.

We conclude that ensheathing glia are the phagocytes of the central brain, responsible for engulfing degenerating ORN axons after axotomy. Based on their expression of Draper and dCed-6 we propose that cortex glia play a similar role in the cortex, perhaps engulfing degenerating axons in this tissue, or cell corpses generated during neuronal development or after brain injury. *Drosophila* astrocytes, in contrast, are in close association with synapse rich regions of the brain and we speculate they likely play an important role in neural circuit and synapse physiology. Our work represents the first functional dissection of glial subtypes in the central brain of adult *Drosophila*, and lays the foundation for future functional studies of these diverse classes of glia.

Materials and Method

Fly Strains: The following *Drosophila* strains were used: *repo-Gal4* (Leiserson et al., 2000), *MZ0709-Gal4* (Ito et al., 1995) , *repo-Gal80* (a kind gift from T. Lee), *pUAST-*

mCD8::GFP (Lee and Luo, 2001), *OR85e-mCD8::GFP* (gift from B. Dickson), *pUAST-ced-6-RNAi* (Awasaki et al., 2006), *pUAST-shark-RNAi* (Ziegenfuss et al., 2008), *pUAST-draper-RNAi* (MacDonald et al., 2006), *draper*$^{\Delta 5}$, *Df(2R)w73-1, cn*^{1}*/CyO* (Bloomington *Drosophila* Stock Center), *UAS-shibire*^{ts}, *yw,hs-FLP; FRTG13,Tub-Gal80* (a kind gift from T. Lee)*, FRTG13;UAS-mCD8::GFP,repo-Gal4, FRTG13,UAS-mCD8::GFP;alrm-Gal4, FRTG13,UAS-mCD8::GFP;mz0709-Gal4, Eaat-Gal4*(Rival et al., 2004), *w,hs-flp;FRT82B,UAS-mCD8::GFP and w;eaat-gal4;FRT82B,Tub-Gal80*

The *alrm-Gal4* construct was generated by amplifying a 4973bp region of the CG11910 (named *astrocytic leucine-rich repeat molecule, alrm*) promoter using the following primers: 5' GATCGATCGCGGCCGCTAGTGGCGATCCTTTCGCTCG 3', and 5' GATCGGTACCGAGTTAATATGGTGGGAACTGC 3'. The resulting fragment was then cloned into the PG4PN2 vector (a gift from C. Warr), and transgenic flies were generated using standard methods by BestGene, Inc. (Chino Hills, CA).

ORN Injury Protocol. Maxillary palp and antennal ablations were performed as previously described (MacDonald et al., 2006). A detailed description of quantitative anlaysis of Draper recruitment and clearance of severed axons is provided in the Supplemental Materials (Supplemental Methods and Supplemental **Fig. 2-3**)

Shibire^{ts} Experiments. *UAS-shibire*^{ts} flies were crossed to the appropriate Gal4 driver lines. Flies were raised at 18^{o}C, shifted to 30^{o}C 2 days prior to maxillary palp ablation, and maintained at 30^{o}C until ready for immunohistochemistry.

Immunohistochemistry and Confocal Microscopy. Adult brains were dissected, fixed, and antibody stained using standard techniques (MacDonald et al., 2006). Confocal imaging and quantitation of Draper staining and GFP were performed as previously described (MacDonald et al., 2006). The following primary antibodies were used: 1:200 mouse anti-GFP (Invitrogen), 1:500 rabbit anti-GFP (Invitrogen), 1:500 rabbit anti-Draper (Freeman et al., 2003), 1:5 mouse anti-repo (Developmental Studies Hybridoma Bank), 1:1000 guinea pig anti-repo (A gift from M. Bhat), 1:20 mouse anti-nc82 (Developmental Studies Hybridoma Bank), 1:500 rat anti-dCed-6 (Awasaki et al., 2006). All anti-IgG secondary antibodies were FITC, Cy3, or Cy5 conjugated (Jackson ImmunoResearch) and used at 1:200.

MARCM Clone Production. Glial MARCM clones were made by heat shocking 36-72 hr old larvae in a 37^{o}C water bath for 30 minutes. Flies were then maintained at 25^{o}C until anti-GFP immunohistochemistry was performed.

Supplementary Methods:

Imaging and Quantification of Draper Recruitment and Clearance of Axonal Material.

Brains were mounted in BioRad Fluoroguard and imaged on a Zeiss LSM 5 Pascal confocal microscope equipped with a 63x 1.4 NA oil immersion lens. Z-stacks through the antennal lobe regions were collected at 1 micron steps. For each quantification experiment, laser intensity and gain were adjusted to prevent pixel saturation, and all

uninjured and injured brain samples were imaged with the same settings on the same day. Quantification of Draper and GFP intensity was performed in ImageJ by calculating the mean pixel intensity from a hand drawn region of interest in the central section of each OR85e-innervated glomerulus (white arrowheads in **Supplemental Fig. 2-3**). Non-specific background intensity was calculated as the mean pixel intensity of an equal size region (denoted by yellow arrowheads in **Supplemental Fig. 2-3**) and this value was subtracted from the mean intensity of the experimental region of interest to obtain a final measurement.

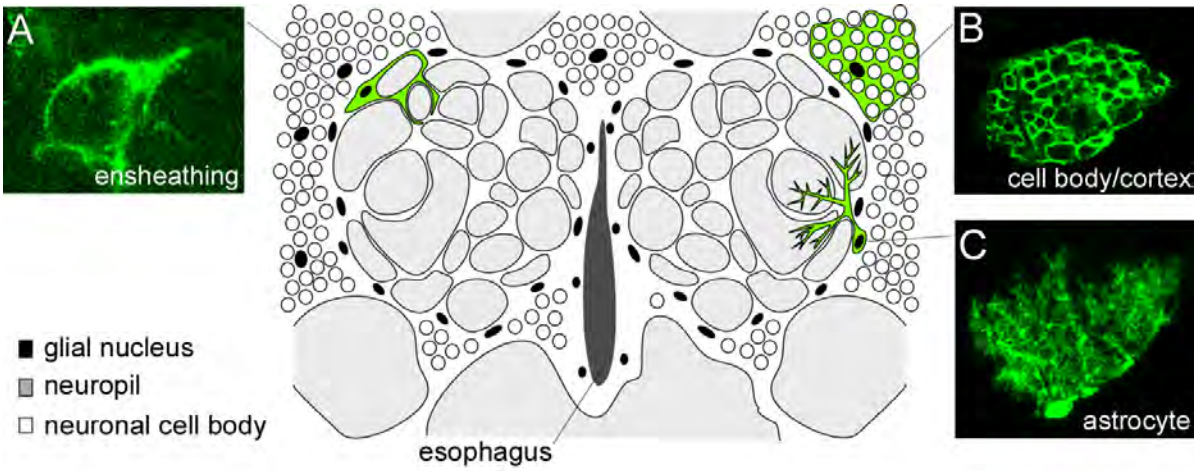


Figure 2-1. Identification of morphologically distinct subtypes of glial cells in the adult *Drosophila* brain. *repo-Gal4* was used to label MARCM glial clones with GFP, and the morphology of individual glial cells was analyzed in the adult antennal lobe brain region.We identified three major subtypes of glial cells: ensheathing glia, cortex (or cell body) glia, and astrocytes. Cartoon schematic of the adult antennal lobe brain region depicts the standard position of cell bodies and approximate sizes of each glial cell type within the brain. Confocal *Z*-stack projections of representative MARCM clones of each glial subtype are shown in ***A–C***. ***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 projected into the neuropil a major stalk that branched and ramified profusely, ultimately positioning astrocyte membrane processes in close proximity to the synapse-rich regions of the glomeruli.

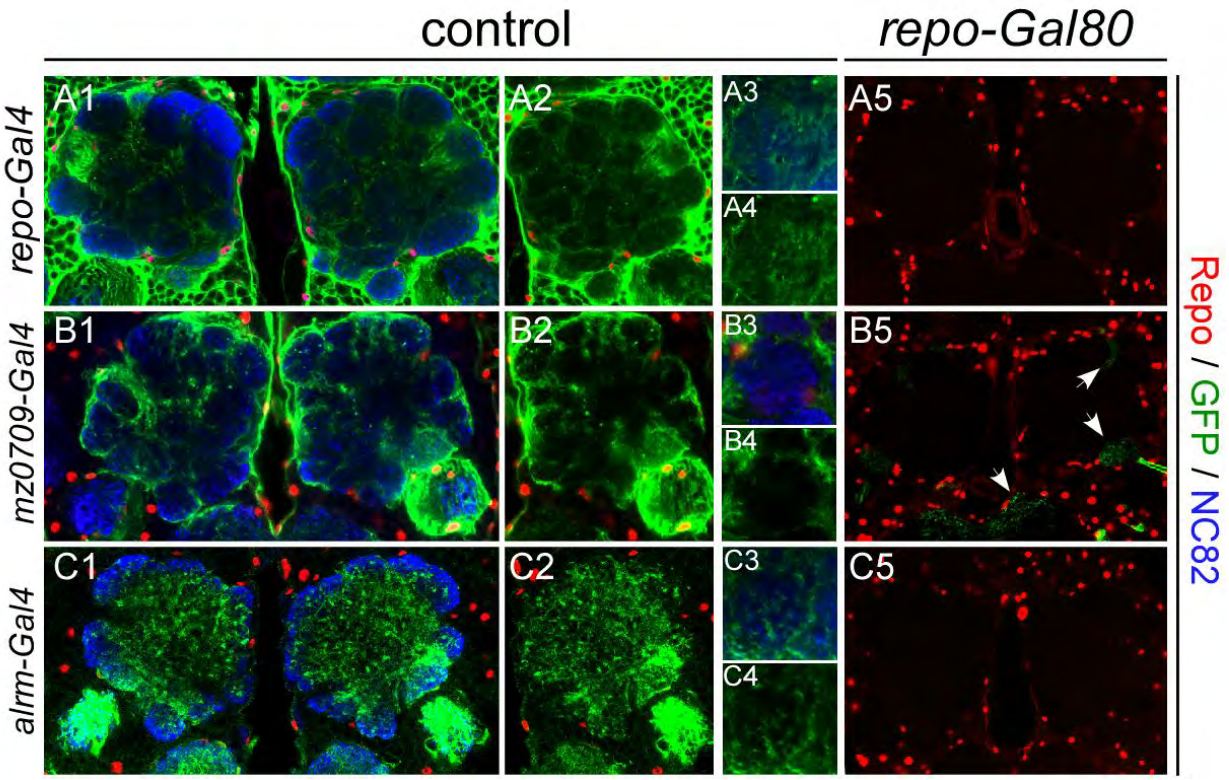


Figure 2-2. Characterization of *Gal4* drivers that uniquely label astrocytes and ensheathing glial subtypes. Individual Gal4 drivers were crossed to *UAS-mCD8::GFP*, and stained for GFP (α-GFP,green), neuropil (nc82, blue), and glial nuclei (α-Repo, red). Panels show single confocal slices of both antennal lobes (***A1***, ***A5***, ***B1***, ***B5***, ***C1***, ***C5***), a single antennal lobe without nc82 stain (***A2***, ***B2***,***C2***), or a high-magnification view of a single glomerulus (top, with nc82; bottom, without) (***A3***,***A4***,***B3***,***B4***, ***C3***, ***C4***). A minimum of 10 animals were imaged for each experiment with similar patterns of expression observed. ***A***, *repo-Gal4*-driven mCD8::GFP labeled all glial membranes (***A1***, ***A2***), which were seen surrounding and invading glomeruli (***A2–A4***). All mCD8::GFP expression was suppressed in this genetic background by *repo-Gal80* (***A5***). ***B***, *mz0709-Gal4* labeled ensheathing glia that surround glomeruli (***B1***, ***B2***), but their membranes did not invade glomeruli (***B2–B4***). Nearly all GFP expression was suppressed by *repo-Gal80* (B5), indicating that this driver is largely specific to glia. However, in some brains, a small number of neurons remained GFP labeled (arrowheads). These cells were identified as neurons by tracing axons to Repo$^-$ neuronal cell bodies. ***C***, *alrm-Gal4* labeled only astrocytes (***C1***, ***C2***), which projected ramified processes that deeply invaded glomeruli (***C2–C4***), and all GFP expression driven by this Gal4 line was suppressed by *repo-Gal80* (***C5***).

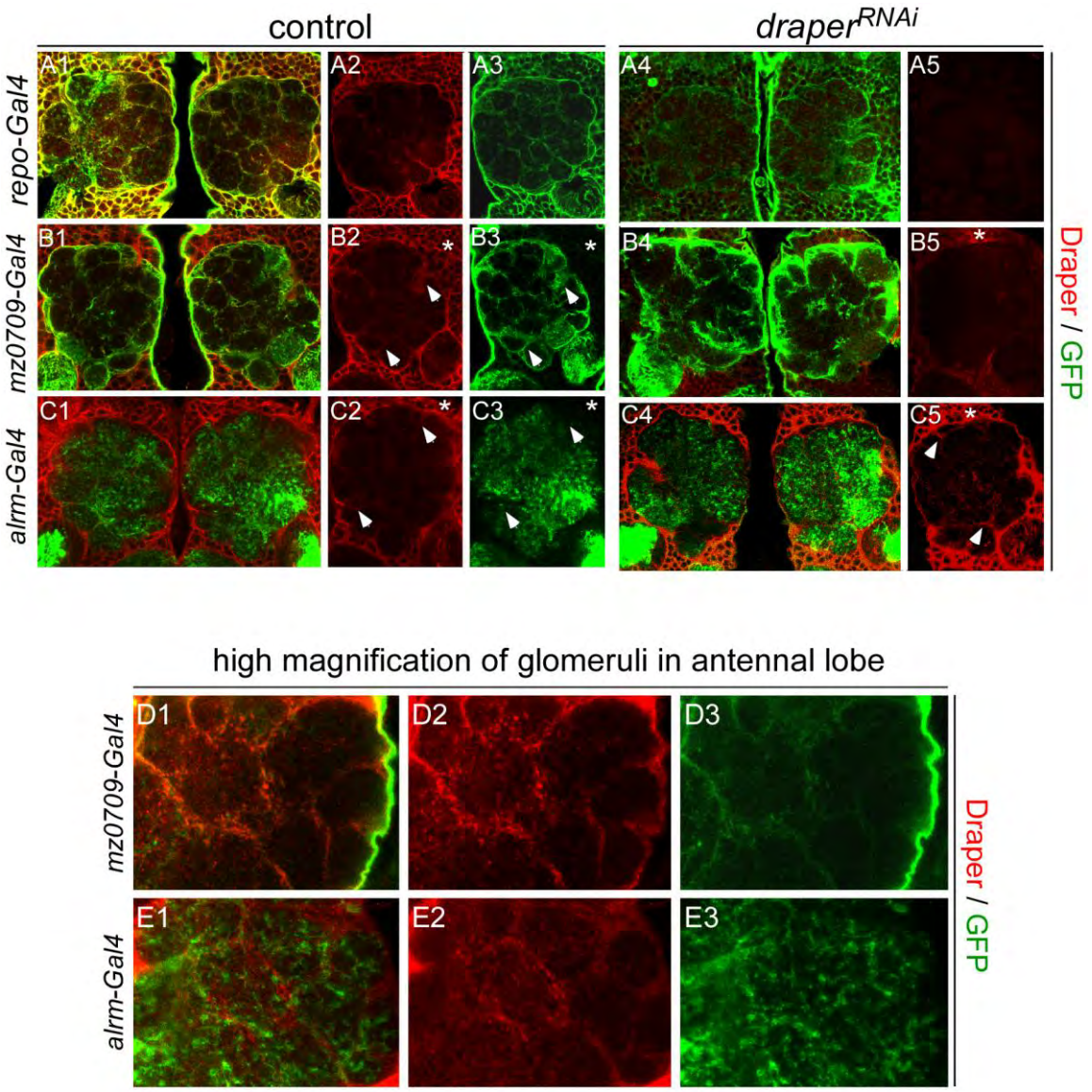

control
draper^RNAi
repo-Gal4
mz0709-Gal4
alrm-Gal4
A1
A2
A3
A4
A5
B1
B2
B3
B4
B5
C1
C2
C3
C4
C5
Draper / GFP
high magnification of glomeruli in antennal lobe
D1
D2
D3
E1
E2
E3

Figure 2-3

Figure 2-3. The engulfment receptor Draper is expressed in ensheathing and cortex glia but not in astrocytes. Flies carrying UAS-mCD8::GFP were crossed to each glial subtype driver line. Glial membranes were visualized with α-GFP (green) and assayed for colocalization of α-Draper (red). Panels show single confocal sections of merged Draper and GFP images (***A1***, ***A4***, ***B1***, ***B4***, ***C1***, ***C4***) as well as single antennal lobes showing either GFP or Draper staining alone (***A2***, ***A3***, ***A5***, ***B2***, ***B3***, ***B5***, ***C2***, ***C3***, ***C5***). A minimum of 10 animals were imaged for each genotype with similar results. ***A***, Pan-glial expression of mCD8::GFP with *repo-Gal4* resulted in extensive colocalization of Draper and GFP-labeled membranes in the neuropil and cortex of the brain (***A1–A3***). No Draper immunoreactivity was detectable when *UAS-draperRNAi* was expressed in the same genetic background (***A4***, ***A5***). ***B***, Driving mCD8::GFP with *mz0709-Gal4* resulted in extensive colocalization of Draper and GFP-labeled membranes (arrowheads) at the edges of the neuropil and surrounding individual glomeruli, but not in the cortex (asterisk; ***B1–B3***). When *mz0709-Gal4* was used to drive *UASdraperRNAi*, Draper immunoreactivity was absent immediately surrounding and within the neuropil but was still detectable in the cell cortex (***B4***, ***B5***; asterisk). ***C***, Labeling of astrocyte membranes with *alrm-Gal4*-driven mCD8::GFP resulted in no detectable colocalization of Draper and GFP (***C1–C3***; arrowheads, asterisk), and expression of *UAS-draperRNAi* in astrocytes had no effect on Draper levels in the cortex or neuropil (***C4***, ***C5***; arrowheads, asterisk). ***D***, High-magnification view of glomeruli within the antennal lobe shows Draper and *mz0709-Gal4*-driven GFP colocalizing on membranes that ensheath (***D1–D3***). ***E***, High-magnification view of glomeruli within the antennal lobe shows distinct staining patterns for Alrm+ membranes and Draper. Alrm+ membranes innervate but do not wrap around glomeruli.

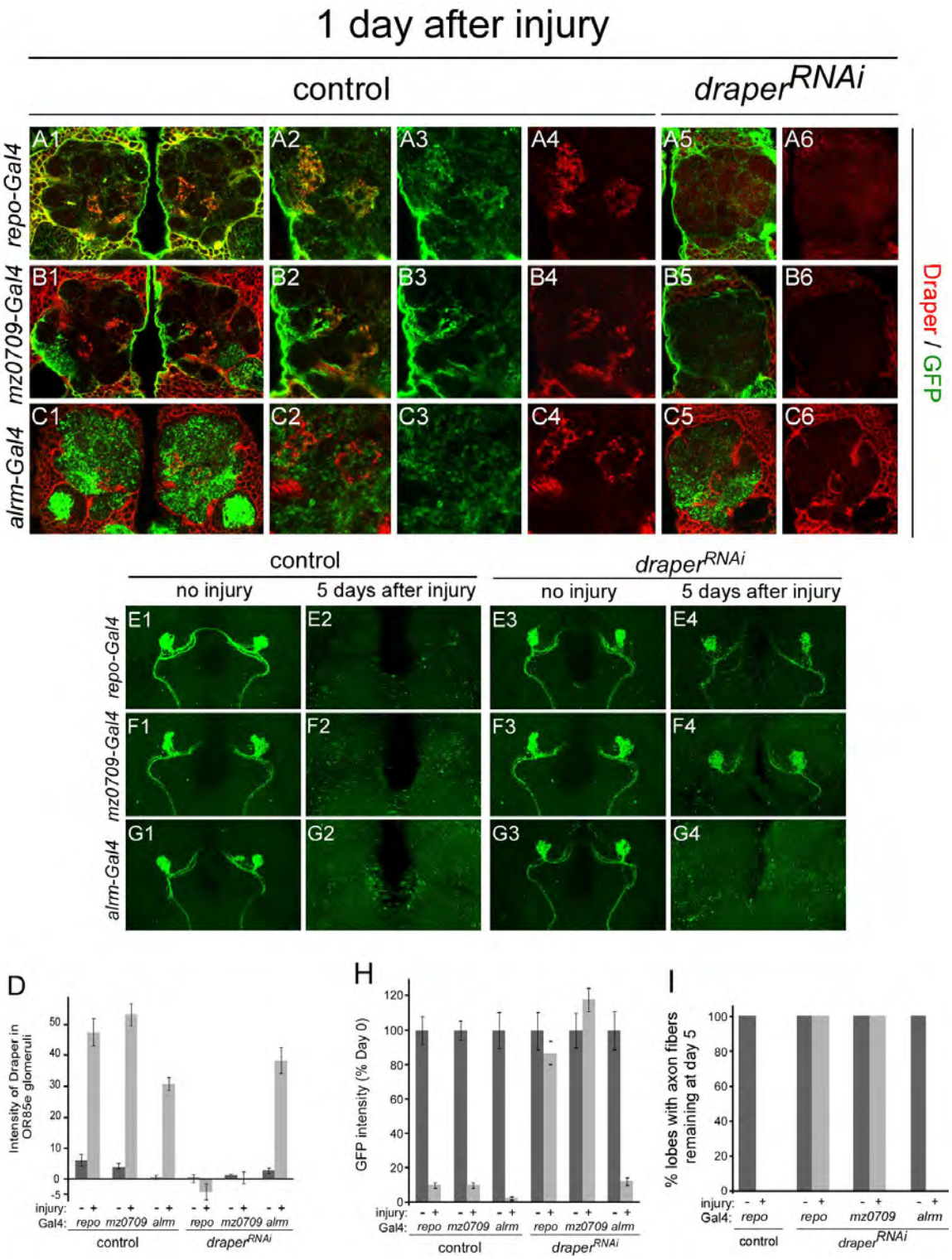

1 day after injury
control
draper^RNAi
repo-Gal4
mz0709-Gal4
alrm-Gal4
A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
B6
C1
C2
C3
C4
C5
C6
Draper / GFP
control
draper^RNAi
no injury
5 days after injury
no injury
5 days after injury
E1
E2
E3
E4
F1
F2
F3
F4
G1
G2
G3
G4
D
Intensity of Draper in OR85e glomeruli
injury:
Gal4:
repo
mz0709
alrm
control
draper^RNAi
H
GFP intensity (% Day 0)
I
% lobes with axon fibers remaining at day 5

Figure 2-4

Figure 2-4. Ensheathing glia express Draper, are recruited to severed ORN axons, and phagocytose degenerating axonal debris. ***A–D***, To assay recruitment of each glial subtype to severed axons, we ablated maxillary palps, allowed maxillary palp axons to degenerate for 1 d, and then assayed glial membrane morphology with mCD8::GFP (green) and colocalization of glial membranes with Draper (α-Draper in red). Images are single confocal slices of the antennal lobe region. ***A***, In animals with Repo^{+} glial membranes labeled with GFP we found that GFP was enriched in glomeruli housing degenerating maxillary palp ORNaxons, that these same glomeruli were decorated with Draper, and that Repo^{+} membranes colocalized perfectly with Draper (***A1–A3***). Expression of *UAS-draperRNAi* in Repo^{+} glia completely suppressed recruitment of glial membranes and Draper to severed axons (***A4***, ***A5***). ***B***, When glial membranes were labeled with mCD8-GFP using the *mz0709-Gal4* driver we found that mz0709^{+} GFP-labeled glial membranes also colocalized with Draper after maxillary palp ORNs were severed (***B1–B3***). Moreover, expression of *UAS-draperRNAi* using the *mz0709-Gal4* driver suppressed all recruitment of Draper and GFP-labeled membranes to severed axons 1 d after injury (***B4***, ***B5***). ***C***, Labeling astrocyte membranes with mCD8::GFP using the *alrm-Gal4* driver did not result in the colocalization of GFP-labeled glial membranes with severed axon-associated Draper staining (***C1–C3***), and expression of *UAS-draperRNAi* using this driver failed to suppress Draper recruitment to severed axons 1 d after injury (***C4***, ***C5***). ***D***, Quantification of data from ***A–C***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment. ***E–I***, To explore the functional requirements for Draper in glial subtypes, we labeled a subset of ORN axons with OR85e-mCD8::GFP, drove *UAS-draperRNAi* in glial subsets with the indicated drivers, severedaxons by ablating maxillary palps, and assayed axon clearance 5 d after injury. All images are confocal *Z*-stack projections of GFP^{+}OR85e axonal material. ***E–G***, In control animals (no *UAS-draperRNAi*), axons developed normally (***E1***, ***F1***, ***G1***), degenerated, and were cleared from the CNS by 5 d after injury (***E2***, ***F2***, ***G2***). Driving *UAS-draperRNAi* with *repo-Gal4* (***E3***, ***E4***) or the ensheathing glial driver *mz0709-Gal4* (***F3***, ***F4***) blocked glial clearance of severed axons. However, expression of *UAS-draperRNAi* in astrocytes with *alrm-Gal4* had no effect on glial clearance of axons from the CNS 5 d after injury (***G3***, ***G4***). ***H***, Quantification of data from ***E–G***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment. ***I***, The number of antennal lobes containing GFP-labeled axon debris 5 d after maxillary palp ablation was counted. In control animals (no *UAS-DraperRNAi*) no GFP-labeled axon debris remained 5 d after ablation. Knockdown of Draper with *repo-Gal4* or *mz0709-Gal4* resulted in GFP-labeled axon debris present in 100% of the antennal lobes counted 5 d after maxillary palp ablation. Consistent with control animals, driving *UAS-draperRNAi* with *alrm-Gal4* resulted in a complete absence of any GFPlabeled axons 5 d after injury. $n \geq 10$ antennal lobes for all.

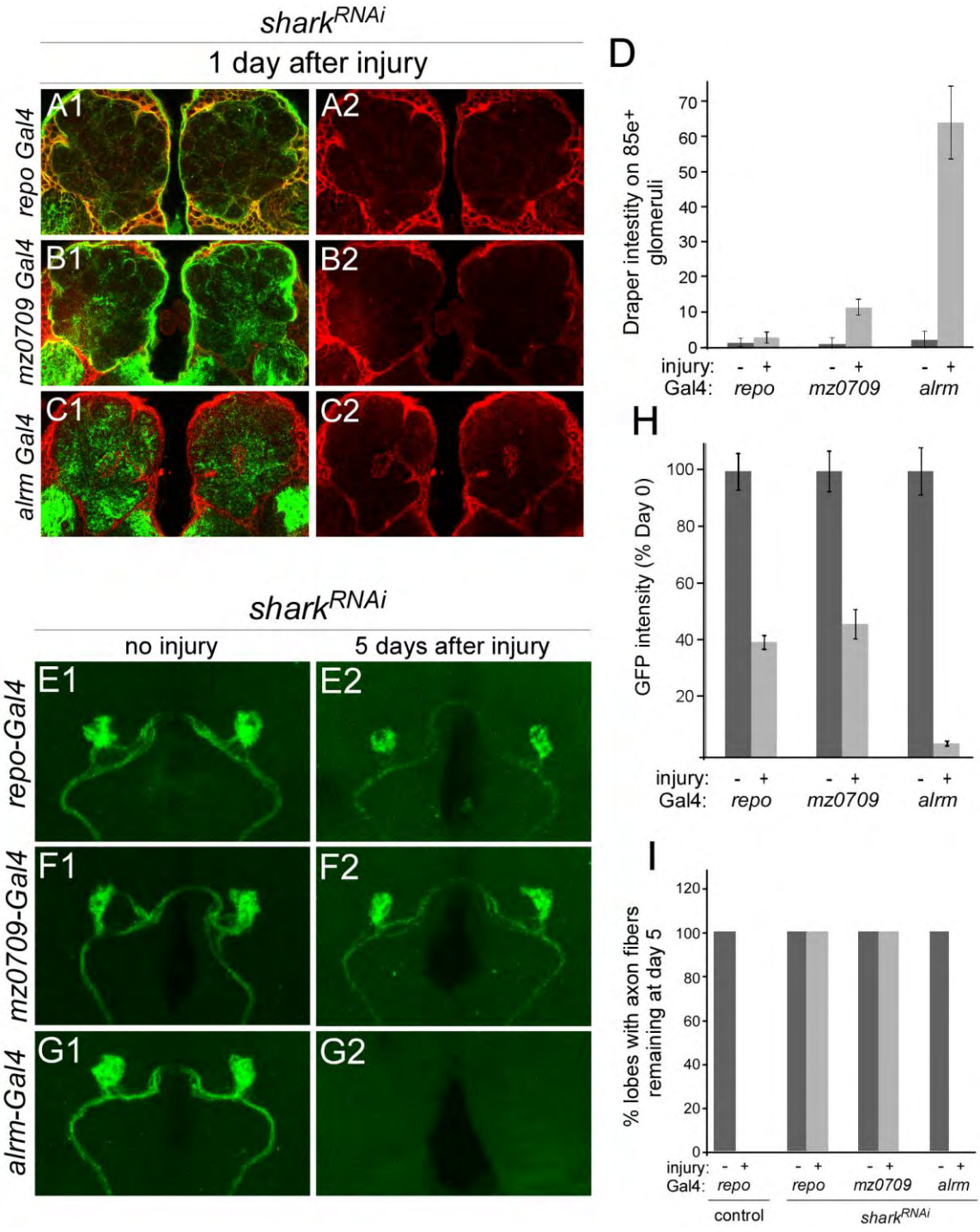

shark^RNAi
1 day after injury
A1
A2
repo Gal4
B1
B2
mz0709 Gal4
C1
C2
alrm Gal4
D
Draper intestity on 85e+ glomeruli
70
60
50
40
30
20
10
0
injury: - + - + - +
Gal4: repo mz0709 alrm
H
GFP intensity (% Day 0)
100
80
60
40
20
injury: - + - + - +
Gal4: repo mz0709 alrm
shark^RNAi
no injury
5 days after injury
E1
E2
repo-Gal4
F1
F2
mz0709-Gal4
G1
G2
alrm-Gal4
I
% lobes with axon fibers remaining at day 5
120
100
80
60
40
20
0
injury: - + - + - + - +
Gal4: repo repo mz0709 alrm
control
shark^RNAi

Figure 2-5

Figure 2-5. The non-receptor tyrosine kinase Shark functions in ensheathing glia to drive engulfment of ORN axonal debris. We knocked down Shark function in subsets of glia with *UAS-SharkRNAi* and assayed recruitment of Draper to severed maxillary palp axons 1 d after injury (***A–D***), and glial engulfment of degenerating axonal debris 5 d after injury (***E–I***). ***A1***, ***B1***, and ***C1*** show α-Draper (red) and Gal4-driven mCD8::GFP (green); ***A2***, ***B2***, ***C2*** show Draper alone. Representative single confocal slices (***A–C***) and confocal *Z*-stacks (***E–G***) are shown. ***A***, Knockdown of Shark in Repo^{+} glia suppressed recruitment of Draper to severed maxillary palp ORN axons 1 d after injury. ***B***, Knockdown of Shark in ensheathing glia with *mz0709-Gal4* also suppressed recruitment of Draper to severed axons. ***C***, Draper was strongly recruited to severed axons when *UAS-SharkRNAi* was driven in astrocytes with *alrm-Gal4*. ***D***, Quantification of data from ***A–C***. Error bars represent SEM; n ≥10 antennal lobes for each experiment. ***E***,Shark RNAi in Repo_glia suppressed glial clearance of degenerating axonal debris. ***F***, Knockdown of Shark in ensheathing glia also suppressed glial clearance of degenerating axonal debris. ***G***, Shark RNAi treatment of astrocytes failed to suppress glial clearance of degenerating axonal debris. ***H***, Quantification of data from ***E–G***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment. ***I***, The percentage of antennal lobes containing GFP^{+} axonal membranes 5 d after maxillary palp ablation. In control animals (no *UAS-sharkRNAi*), no GFP^{+} axonal material was present in any samples. Shark RNAi treatment of all glial cells, using *repo-Gal4*, or ensheathing glial cells, using *mz0709-Gal4*, resulted in perdurance of GFP^{+} axonal debris 5 d after maxillary palp ablation in 100% of the samples. Similar to control animals, driving *UAS-sharkRNAi* with *alrm-Gal4* led to a complete loss of GFP^{+} axonal material 5 d after injury in all antennal lobes. $n \geq 10$ antennal lobes for all.

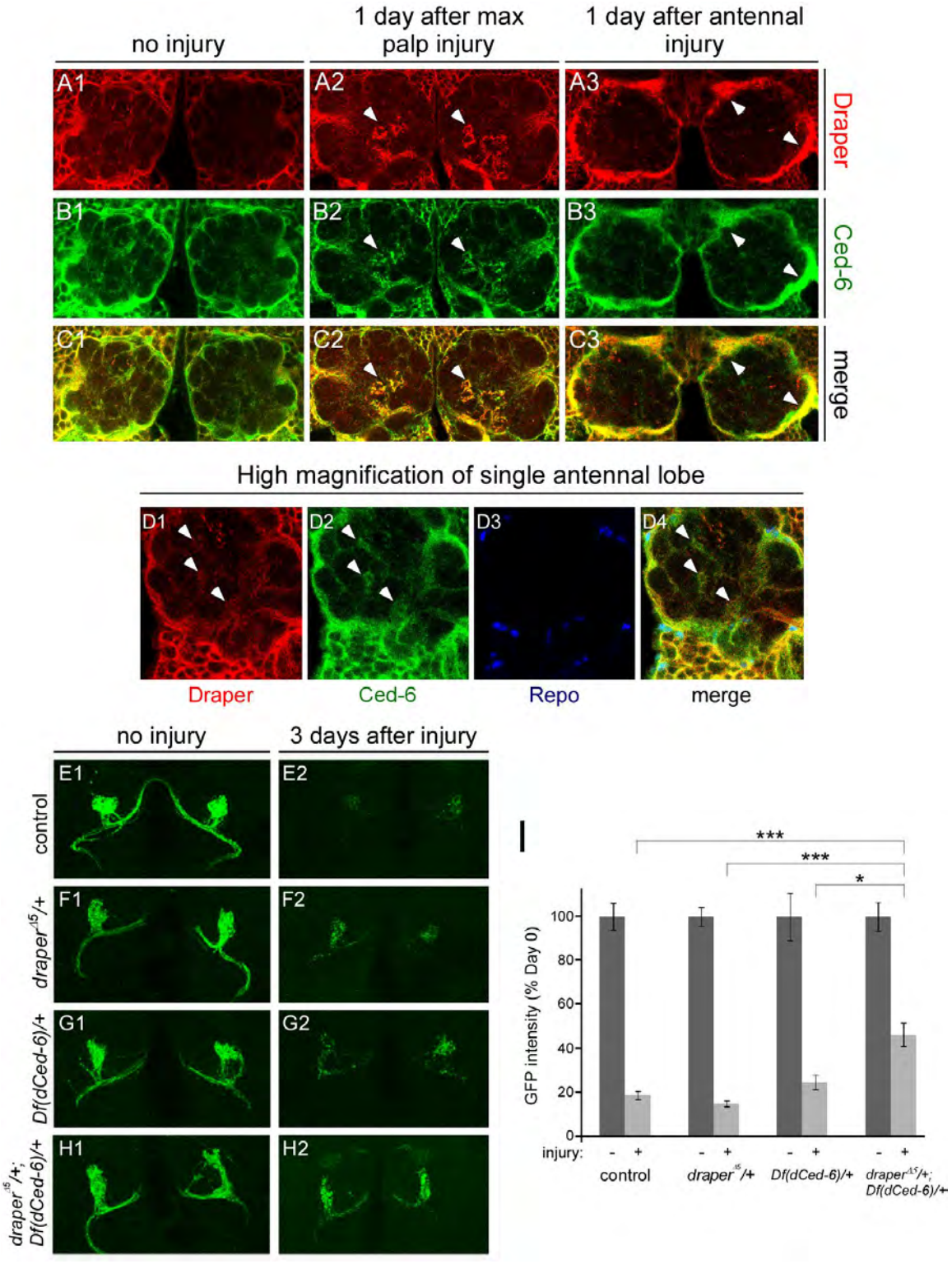

no injury
1 day after max palp injury
1 day after antennal injury
A1
A2
A3
Draper
B1
B2
B3
Ced-6
C1
C2
C3
merge
High magnification of single antennal lobe
D1
D2
D3
D4
Draper
Ced-6
Repo
merge
no injury
3 days after injury
control
draper^Δ5/+
Df(dCed-6)/+
draper^Δ5/+; Df(dCed-6)/+
E1
E2
F1
F2
G1
G2
H1
H2
GFP intensity (% Day 0)
***
***
*
100
80
60
40
20
0
injury:
control
draper^Δ5/+
Df(dCed-6)/+
draper^Δ5/+; Df(dCed-6)/+

Figure 2-6

Figure 2-6. dCed-6 is recruited to severed ORNaxons after injury and genetically interacts with Draper. ***A–C***, We compared α-dCed-6 (green) and α-Draper (red) localization in three sets of animals: control (no injury), 1d post maxillary palp ablation, and 1d post antennal ablation. Representative images of single confocal slices through the antennal lobe regions are shown. Draper (***A1***) and dCed-6 (***B1***) had overlapping patterns of expression (***C1***) in control animals,including cortex and neuropil regions of the brain. One day after maxillary palp ablation, high levels of Draper (***A2***) and dCed-6 (***B2***) immunoreactivity were colocalized (***C2***) on severed axons(arrowheads). Ablation of antennae resulted in a characteristic dramatic increase in Draper immunoreactivity in glia surrounding the antennal lobes 1 d later (***A3***). We found that dCed-6 also showed a dramatic increase in immunoreactivity in glia outlining the antennal lobes (whitearrowheads) at this time point (***B3***, ***C3***). ***D***, Single confocal slice of antennal lobe at high magnification stained with Draper (***D1***), dCed-6 (***D2***), and Repo (***D3***), and merged image (***D4***). Note colocalization of Draper and dCed-6 (arrowheads). ***E–I***, To assay axon clearance of 85e^{+}maxillarypalp ORN axons were labeled with mCD8::GFP, maxillary palps were ablated, and the amount of GFP^{+} axonal debris was quantified in control, *draper*$^{\Delta 5/}$+, *Df(dCed-6)/+*, and*Df(dCed-6)/+;draper*$^{\Delta 5}$/+ animals 3 d after axotomy. Representative confocal *Z*-stack imagesare shown. Severed axons were largely cleared 3 d after injury in control (***E***), *draper*$^{\Delta 5}$/+ (***F***),and *Df(dCed-6)/+*(***G***) animals. Less axonal debris was cleared from the CNS 3 d after injury in *Df(dCed-6)/+;draper*$^{\Delta 5}$/+ (***H***) animals compared with all other genotypes. ***I***, Quantification of data from ***E–H***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment; p values were calculated using an unpaired t-test, *$p<0.05$; ***$p<0.0001$.

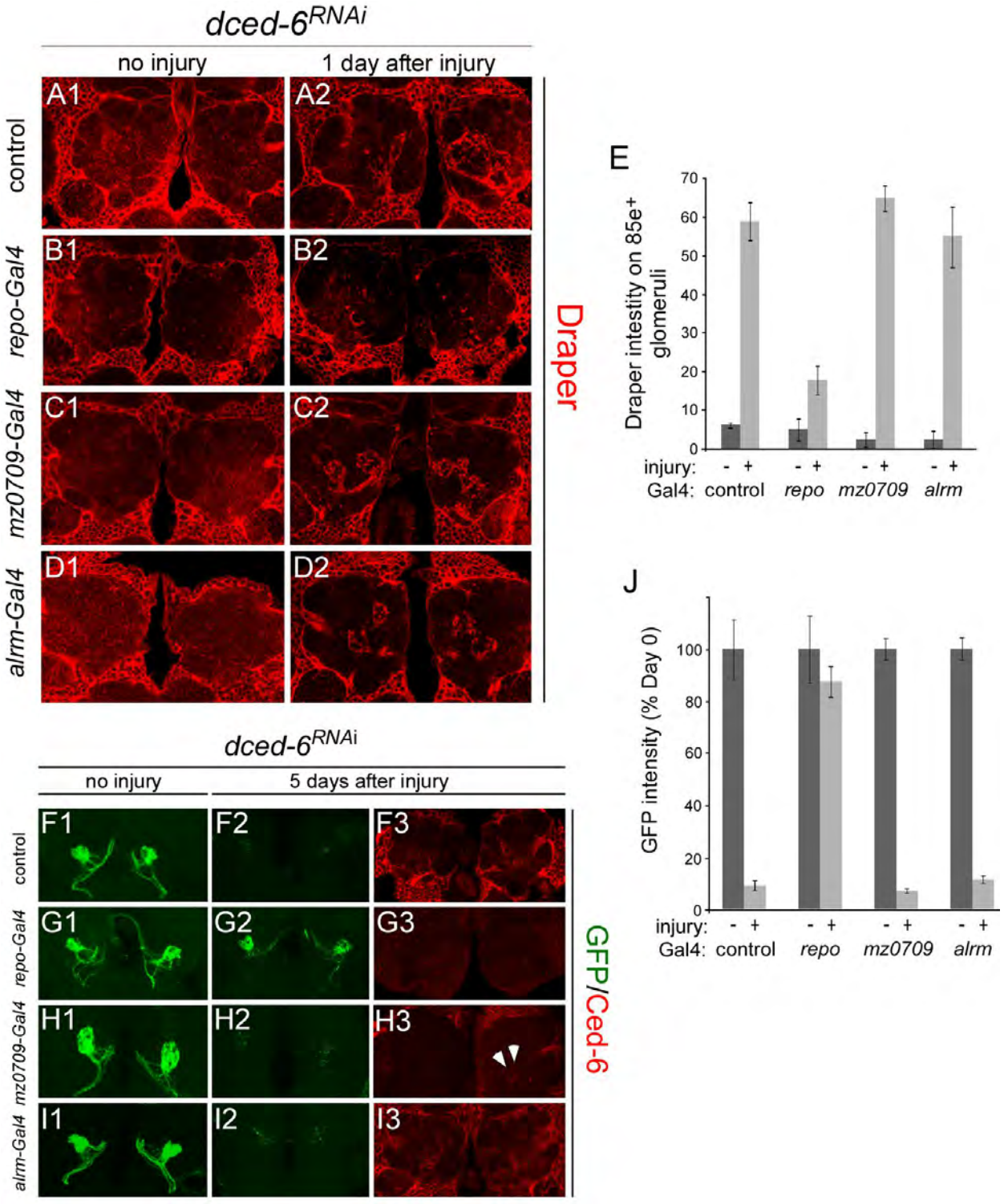

dced-6RNAi
no injury
1 day after injury
A1
A2
B1
B2
C1
C2
D1
D2
control
repo-Gal4
mz0709-Gal4
alrm-Gal4
Draper
E
Draper intestity on 85e+ glomeruli
70
60
50
40
30
20
10
0
injury: - + - + - + - +
Gal4: control repo mz0709 alrm
dced-6RNAi
no injury
5 days after injury
F1
F2
F3
G1
G2
G3
H1
H2
H3
I1
I2
I3
control
repo-Gal4
mz0709-Gal4
alrm-Gal4
GFP/Ced-6
J
GFP intensity (% Day 0)
100
80
60
40
20
0
injury: - + - + - + - +
Gal4: control repo mz0709 alrm

Figure 2-7

Figure 2-7. Glial-specific knockdown of dCed-6 suppresses clearance of degenerating ORN axonal debris. dCed-6 was knocked down in glia using a *UAS-dCed-6RNAi* construct and recruitment of Draper to severed axons (***A–E***) and clearance of degenerating GFP^+ axonal debris (***F–J***) were assayed. Representative single confocal images (***A–D***) and *Z*-stack projections (***F–I***) are shown. ***A***, In control animals (*repo-Gal4* driver alone), Draper was recruited at high levels to severed axons 1 d after injury. ***B***, Pan-glial knockdown of dCed-6 using *repo-Gal4* suppressed Draper recruitment to severed axons. ***C***, Knockdown of dCed-6 in ensheathing glia using the *mz07090-Gal4* driver did not suppress glial recruitment to severed axons. ***D***, Astrocyte-specific knockdown of Draper using *alrm-Gal4* did not affect recruitment of Draper to severed axons after axotomy. ***E***, Quantification of data from ***A–D***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment. ***F***, In control animals (*repo-Gal4* driver alone), severed axons were cleared from the CNS 5 d after injury (***F1***, ***F2***), and dCed-6 was expressed strongly throughout the adult brain (***F3***). ***G***, Knockdown of dCed-6 in all glia with *repo-Gal4* completely suppressed clearance of degenerating axons 5 d after injury (***G1***, ***G2***), and dCed-6 immunoreactivity was no longer detectable in the adult brain (***G3***). ***H***, Knockdown of dCed-6 in ensheathing glia with *mz0709-Gal4* did not suppress glial clearance of axonal debris (***H1***, ***H2***), and, notably, dCed-6 staining in the adult brain was only partially reduced in dCed-6 RNAi animals (white arrowheads in ***H3***). ***I***, dCed-6 knockdown in astrocytes with *alrm-Gal4* had no effect on clearance of axonal debris (***I1***, ***I2***) or dCed-6 staining in the adult brain (***I3***). ***J***, Quantification of axon clearance data from (***F–I***). $n \geq 10$ antennal lobes for each experiment, error bars are SEM.

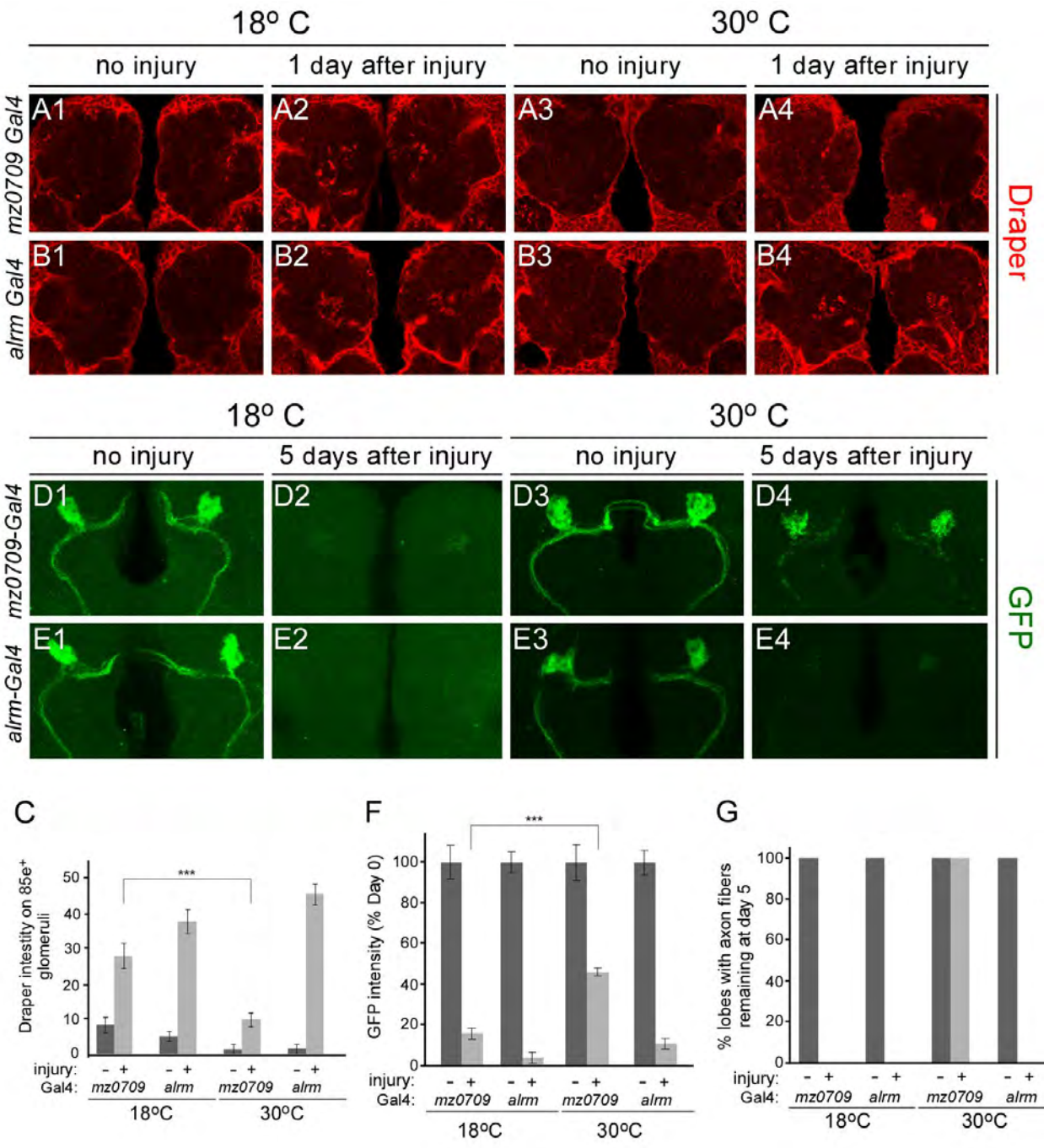
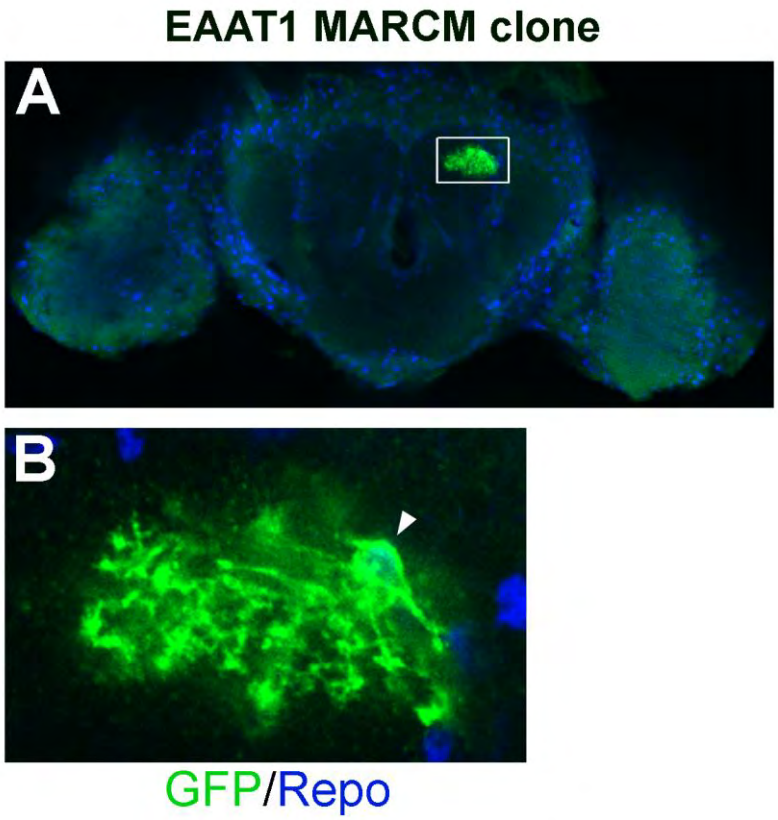

18° C
30° C
no injury
1 day after injury
no injury
1 day after injury
mz0709 Gal4
alrm Gal4
A1
A2
A3
A4
B1
B2
B3
B4
Draper
18° C
30° C
no injury
5 days after injury
no injury
5 days after injury
mz0709-Gal4
alrm-Gal4
D1
D2
D3
D4
E1
E2
E3
E4
GFP
C
Draper intestity on 85e+ glomeruli
***
injury:
Gal4:
mz0709
alrm
mz0709
alrm
18°C
30°C
F
GFP intensity (% Day 0)
***
injury:
Gal4:
mz0709
alrm
mz0709
alrm
18°C
30°C
G
% lobes with axon fibers remaining at day 5
injury:
Gal4:
mz0709
alrm
mz0709
alrm
18°C
30°C

Figure 2-8

Figure 2-8. Endocytic function is required in ensheathing glia, but not astrocytes, for glial clearance of degenerating ORN axons. The requirements for endocytic activity during phagocytosis of axons in ensheathing glia and astrocytes were determined by expressing *UAS-shibire^{ts}* with *mz0709-Gal4* and *alrm-Gal4*, respectively, and assaying recruitment of Draper to severed axons and clearance of degenerating axonal debris from the brain. ***A***, Expression of Shibire^{ts} in ensheathing glia did not block recruitment of Draper to severed maxillary palp ORN axons 1d after injury when animals were maintained at the permissive temperature of 18°C (***A1***, ***A2***), but Draper recruitment to severed axons was strongly suppressed when these animals were maintained at the restrictive temperature of 30°C (***A3***, ***A4***). ***B***, Shibire^{ts} expression in astrocytes had no effect on Draper recruitment to severed axons at either 18°C or 30°C (***B1–B4***). ***C***, Quantification of data from ***A*** and ***B***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment. p-values were calculated using an unpaired t-test, ***$p<0.0001$. ***D***, Expression of Shibire^{ts} in ensheathing glia had no effect on glial clearance of severed axons at 18°C (***D1***, ***D2***). Shifting to the restrictive temperature of 30°C strongly suppressed clearance of degenerating axons 5d after axotomy (***D3***, ***D4***). ***E***, Expression of Shibire^{ts} in astrocytes with *alrm-Gal4* had no effect on glial clearance of degenerating axons 18°C or 30°C (***E1–E4***). ***F***, Quantification of data from ***D*** and ***E***. Error bars represent SEM; $n \geq 10$ antennal lobes for each experiment; ***$p<0.0001$. ***G***, The number of antennal lobes containing GFP-labeled axon debris 5 d after maxillary palp ablation were counted and expressed as a percentage of the total number. In control animals (entire duration of the experiment performed at the permissive temperature of 18°C) no GFP-labeled axons were present in either the *mz0709-Gal4* or the *alrm-Gal4* flies. When the flies are shifted to the restrictive temperature of 30°C, 100% of the axons in the *mz0709-Gal4* flies are still GFP+, whereas none of the axons in the *alrm-Gal4* flies contain any GFP-labeled axons.



Supplemental Figure 2-1. *Eaat1* is expressed in astrocyte-like glia.
eaat1-Gal4 was used to label MARCM-generated clones with membrane-tethered GFP. (A) Confocal Z-stack of a representative *eaat1-Gal4* labeled clone (green) in a single antennal lobe shows that *eaat1*-expressing cells display a tufted, highly branched astrocyte-like morphology. Glial nuclei are labeled with anti-repo (blue). (B) High magnification view of Eaat1-positive clone shown in (A). This confocal Z-stack of selected slices shows the cell body of the clone (arrowhead) and the dendritic-like projections that extend throughout antennal lobe glomeruli.

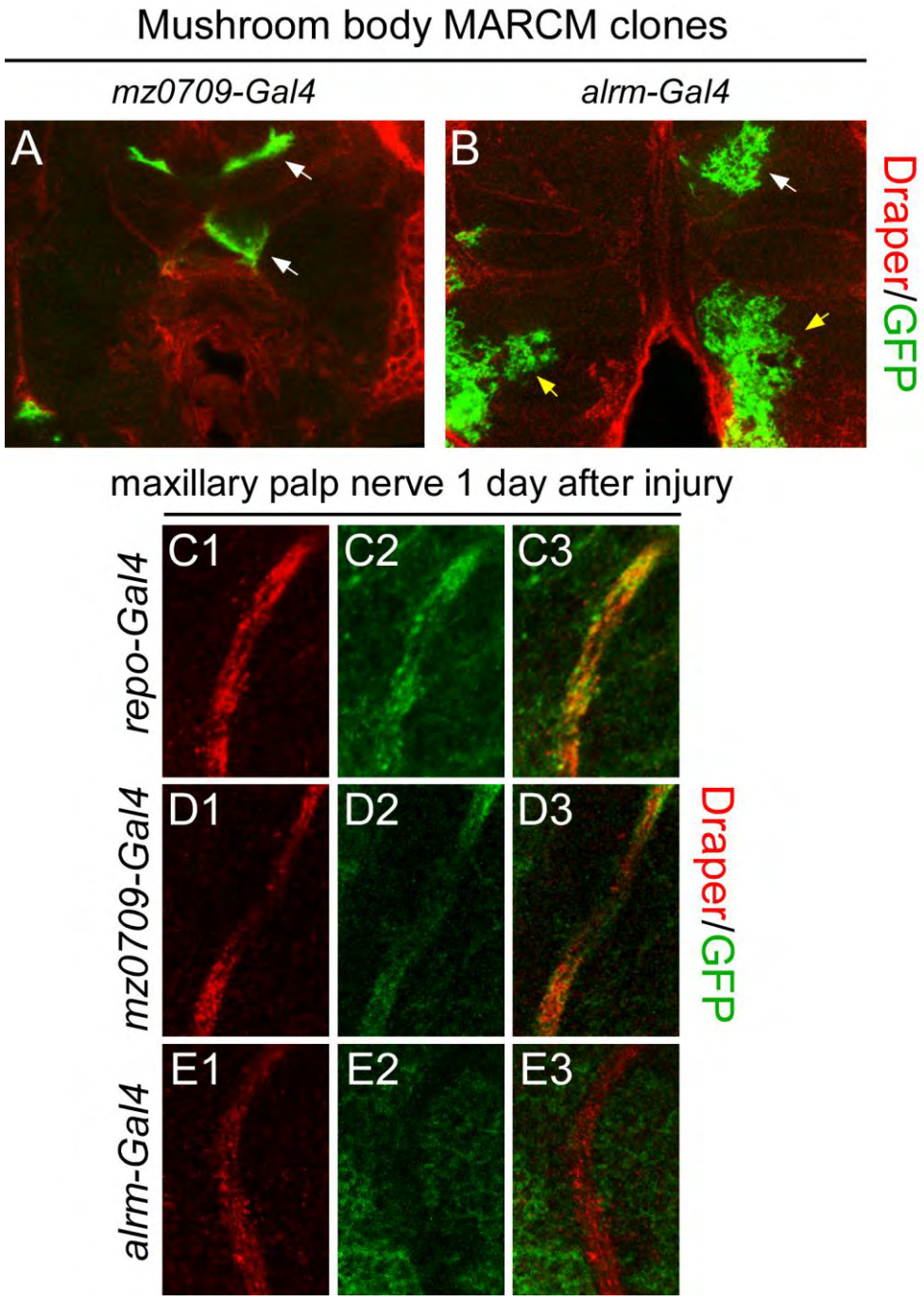
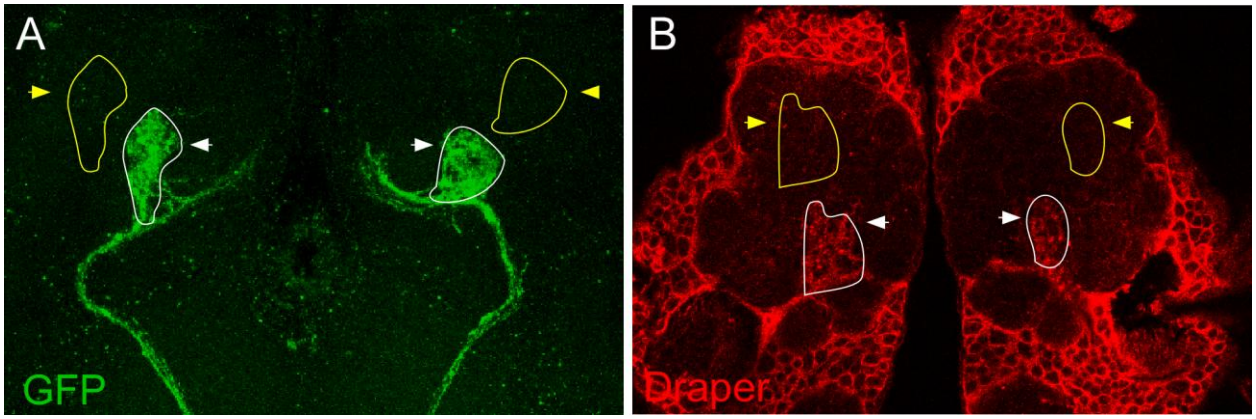

Mushroom body MARCM clones
mz0709-Gal4
alrm-Gal4
A
B
Draper/GFP
maxillary palp nerve 1 day after injury
repo-Gal4
mz0709-Gal4
alrm-Gal4
C1
C2
C3
D1
D2
D3
E1
E2
E3
Draper/GFP

Figure 2-S2

Supplemental Figure 2-2. Adult mushroom body neuropil and SOG region contains ensheathing glia and astrocyte-like glia.
MARCM clone analysis (A,B) and functional injury assay (C-E) were performed to demonstrate that astrocytic glia and ensheathing glia maintain their morphology and functional roles in areas of the brain outside of the antennal lobes, namely the neuropil region of the mushroom bodies and the subesophageal ganglion. (A) GFP labeled MARCM clones generated with *mz0709-Gal4* (white arrows) depict large flat cells within the neuropil regions of the mushroom bodies. These cells shows similar morphology and formation of spatial boundaries as those ensheathing glial cells seen in the antennal lobe. (B) *alrm-Gal4* was used to label MARCM clones generated with membrane-tethered GFP. An *alrm+* clone in the mushroom body region (white arrow) shows a strikingly similar morphology to astrocytic clones depicted below in the antennal lobe region (yellow arrows). (C-E) The Gal4/UAS system was used to label subtype specific glial membranes with GFP and colocalization of the glial membranes with Draper (using α–Draper) was examined in the subesophageal ganglion one day after maxillary palp injury. (C1-C3, D1-D3) Membranes from *repo* and *mz0709*-positive cells were recruited to severed maxillary palp axons and could be seen colocalizing with Draper. (E1-E3) Membranes from *alrm+* cells were not recruited to the injured maxillary palp nerve and no colocalization was observed between the *alrm+* cell membranes and Draper.



Supplemental Figure 2- 3. Quantification methods for clearance of GFP+ axonal materialand Draper recruitment after maxillary palp injury.
Representative confocal slice images of GFP-labeled OR85e+ axonal projections (A) or α-Draper stain (B) one day after maxillary palp injury. Regions selected for fluorescence intensity quantification are outlined in white (white arrowheads), while the yellow outlines (yellow arrowheads) depict typical regions used for background subtraction.

CHAPTER III: An *in vivo* RNAi screen to identify glial engulfment genes

The work presented in this chapter was performed in the lab of Dr. Marc Freeman and I was the sole contributor to this screen. I identified the candidate genes and carried out all experiments relating to these studies. Genes identified through this screen are currently being investigated further by other members of the lab. Jennifer Ziegenfuss is currently looking at the role of Rac1 in both axon clearance and axon pathfinding. Tsai-Yi Lu is currently investigating the role of the Sos/Dos/Drk complex in axon clearance. Jennifer MacDonald is currently looking at the role of Basket in axon clearance and regulation of Draper expression. The following publications are in progress:

Doherty J, Sheehan A, Hackett R and Freeman MR
Engulfment signaling promotes Stat92E-dependent transcriptional activation of *draper* after axotomy.

Doherty J, Lu T and Freeman MR
AKT/PI3K regulates basal levels of Draper expression in the adult Drosophila brain.

Ziegenfuss J, **Doherty J** and Freeman MR
Drosophila Crk, Mbc and Elmo mediate glial phagocytosis of severed axons.

MacDonald J, **Doherty** J, Sheehan A, Hackett R and Freeman MR
JNK mediates glial clearance of severed axons through transcriptional regulation of *draper*.

Lu T, **Doherty J** and Freeman MR
Dos, Sos and Drk mediate glial clearance of severed axons through activation of Rac1.

Introduction

One critical and conserved feature of glial cells is their ability to sense and respond to neuronal injury. During injury responses, glia exhibit robust changes in gene expression, migrate to sites of trauma, and phagocytose degenerating neuronal debris. However the molecules and signaling pathways mediating neuron-glia communication after injury remain poorly defined. The adult *Drosophila* olfactory system is a powerful *in vivo* genetic model to explore the molecular mechanisms that govern glial recognition and clearance of degenerating axons. In previous studies it was shown that glia respond robustly to axotomy of olfactory receptor neurons (ORNs) by upregulating the phagocytic receptor Draper, extending membranes to severed axons, and clearing degenerating axons from the CNS (MacDonald et al., 2006; Ziegenfuss et al., 2008). In the absence of Draper, glial membranes showed no obvious morphological changes in response to injury, they do not extend membranes to severed axons, and consequently degenerating axon material persisted throughout the life of the animal. The generation of the Vienna *Drosophila* RNAi Center (VDRC) UAS-driven RNAi collection which covers ~ 94% of the *Drosophila* genome, in combination with an array of cell-type specific Gal4 drivers, and a tractable model system, provides excellent potential for *in vivo* RNAi screening.

In this study I performed a candidate based, *in vivo,* RNAi screen, using the Gal4/UAS system (Brand and Perrimon, 1993), to identify molecules involved in the glial engulfment of degenerating axon material. Approximately 300 UAS -RNAi strains were crossed to the glial specific driver line, *repo-Gal4,* and two readouts were assayed:

Draper recruitment to the sites of injury one day after either maxillary or antennal injury, and clearance of GFP^+ degenerating axon material five days after maxillary palp injury, a time point at which the vast majority of GFP^+ material in control animals has been cleared. For RNAi lines which resulted in lethality when driven with *repo-Gal4*, a temperature sensitive repressor of Gal4, *tubulin-Gal80^{ts}*, was used to temporally restrict the expression of the RNAi to adult flies, bypassing any developmental requirement for the genes of interest.

Results

The *Drosophila* antennal lobe is highly stereotyped and well characterized. It consists of 50 spatially invariant glomeruli which are innervated by ORN axons whose cell bodies lie out in peripheral sensory organs, the maxillary palps and third antennal segments. The majority of glomeruli in the antennal lobe are innervated by axons projecting from the third antennal segments while only a small portion are innervated by axons projecting from the maxillary palps **(Fig. 3-1A)**. Thus, ablating the third antennal segment induces a large scale injury and removing the maxillary palp induces a much smaller more localized injury. These different grades of injuries are reflected by the responsiveness of the engulfment receptor Draper. One day after antennal injury, Draper is upregulated around the entire antennal lobe but one day after maxillary palp injury, Draper is localized only to those glomeruli directly affected **(Fig. 3-1B)**. Regardless of the severity of the injury, the axon material is generally cleared from the CNS by glia within five days **(Fig. 3-1B)**.

The main objective of this screen was to identify genes which, when knocked down specifically in glial cells, would prevent the upregulation of Draper at sites of injury, and/or would inhibit the clearance of axon material from the CNS. Interestingly, four distinct phenotypes emerged: 1) an axon pathfinding phenotype, 2) an axon clearance phenotype, 3) a phenotype consistent with a reduction in basal Draper levels (i.e. Draper levels in an uninjured animal), and finally, 4) a phenotype characterized by a reduction in both basal Draper levels as well as injury-induced Draper upregulation. Additionally, RNAi knockdown of many of the genes identified in this screen resulted in more than one of the above phenotypes.

Genes affecting axonal pathfinding

There is a well established role for glial cells during axon pathfinding events in *Drosophila* (Hidalgo and Booth, 2000; Jacobs, 2000; Sepp et al., 2001). In some instances glia act as permissive substrates for the migration of growing axons and in other situations they secrete molecules which act as permissive or repulsive cues to actively guide migrating neurites to the appropriate target. However, it has been noted that glial cells play no role in axon pathfinding in the adult antennal lobe presumably because the axons reach their targets prior to the infiltration of glial cells (Ng et al., 2002). Interestingly, several of the RNAi lines, *stat92e*, *rac1*, *basket*, *pi3k92e* and *raptor*, which were expressed by the glial specific *repo-Gal4* driver, exhibited axon pathfinding defects in the antennal lobe (**Table 3-1, Fig. 3-2A-E** (*basket* not shown)). In order to determine if these pathfinding defects occur during development of the antennal lobes, I utilized

tubulin-Gal80^{ts} to temporally restrict the expression of the RNAi to adult flies. The flies were raised at the permissive temperature of 18^{o}C throughout development and upon eclosion, the flies were shifted to the restrictive temperature of 30^{o}C for at least 7 days prior to dissection to allow for the RNAi to take effect. Not surprisingly, I found that for all of the genes, *stat92e*, *rac1*, *basket*, *pi3k92e* and *raptor*, these axon pathfinding defects manifest during development and not in the adult as adult-specific knockdown of these genes did not lead to aberrant axon pathfinding defects (**Fig. 3-2F**, *stat92e^{RNAi}*). The developmental nature of this axon pathfinding defect limited my identification of this phenotype to only those RNAi lines which were viable when driven with *repo-Gal4*. Therefore, it is possible that some of the genes I examined in this study may play a role in axon pathfinding but due to the developmental requirement of these genes in glial cells for viability, they went undetected. While this axon pathfinding defect is not related to engulfment, at least not in the adult brain, it is nevertheless interesting as it further highlights the importance of glial cells in the developing nervous system.

It will be interesting to determine the mechanisms by which glial cells aid in axon pathfinding in the antennal lobe. Glial cells perform axon pruning during development and it may be possible that secondary effects stemming from axon pruning deficiencies lead to the aberrant pathfinding defects in axons migrating into the antennal lobes. It is also a possibility that glial cells act as a physical scaffold for the migration of neurons into the antennal lobe. Or, it is likely that glial cells secrete chemo-attractants or chemo-repellents in order to guide the axons. Furthermore, it is a possibility that glial cells exert their effects on axon pathfinding in the antennal lobes through various different

mechanisms and knockdown of the different genes result in different underlying causes of aberrant axon pathfinding. All of the RNAi lines which display an axon pathfinding phenotype correspond to genes which are involved in a number of pleiotropic roles so it is likely that various factors contribute to the axon pathfinding defects. However, it is worth noting that several of the axon pathfinding defects resulted in a similar pattern where the axons projected up and around the periphery of the antennal lobe, never crossing the midline, and approximately halfway down they extended in towards their target glomerulus and terminated in a bulbous structure, similar to innervations of an ectopic glomerulus. This consistency in the phenotype among the various RNAi lines may indicate that the underlying mechanisms resulting in axon pathfinding are the same. Jennifer Ziegenfuss in the lab is further investigating the cellular and molecular mechanisms underlying this phenotype.

Genes affecting clearance of axonal debris

In **Table 3-1**, I list twenty genes which I identified in this screen as being required in glial cells for the timely and efficient clearance of degenerating axons. The axon clearance phenotype is typically not characteristic of an all-or-nothing outcome and there was a wide range of variability among the axon clearance phenotypes exhibited by the different candidate genes. Typically, in control animals, all GFP^{+} axon material is cleared from the antennal lobe five days after axotomy. Knockdown of *stat92e*, *rac1* and *basket* resulted in robust axon clearance phenotypes in which the majority of the axon material was still present five days after injury (**Fig.3-3**, $rac1^{RNAi}$).

In order to rule out the possibility that the axon clearance phenotypes exhibited by these RNAi lines was a result of developmental defects, I used *tubulin-Gal80^{ts}* to temporally restrict the expression of the RNAi to adult flies. The flies were raised at the permissive temperature of 18^{o}C throughout development and upon eclosion, the flies were shifted to the restrictive temperature of 30^{o}C for at least 7 days prior to axon injury and the flies were kept at 30^{o}C for 5 days following the injury. For all three genes, *stat92e*, *rac1* and *basket*, the axons were still present five days after injury indicating that the axon clearance phenoytpe is not the result of a developmental defect but is specific to glial cells acting in the adult brain. In addition, I confirmed these phenotypes through additional non-RNAi means. The *rac1* phenotype was confirmed by overexpression of a dominant negative Rac1 allele, Rac1^{N17}, the *basket* phenotype was confirmed by overexpression of an inhibitor of Basket activity, Puckered, and *stat92e* was confirmed using Stat92E transcriptional reporters. I am further investigating the role of Stat92E in glial responses to injury and this will be discussed in detail in chapter four. Jennifer MacDonald is further examining the role of Basket in glial responses to injury and Jennifer Ziegenfuss is currently looking at the role of Rac1 in both axon pathfinding and glial responses to injury.

I identified three genes from this screen which, when knocked down in glial cells, result in very strong axon clearance phenotypes. I also identified several others which gave weaker, more subtle, phenotypes (see **Table 3-1**). Of these genes, several are involved in the PI3K signaling pathway, *akt*, *pi3k92e*, *pi3k21b*, *s6k*, *raptor*, *14-3-3zeta* and *pdk1,* and will be discussed further in the next section. In addition, protein products

of three of the genes, *sos, drk* and *dos,* are known to act together in a complex and function as a guanine nucleotide exchange factor for Rac1. Tsai-Yi Lu is currently examining the role of *sos*, *dos* and *drk* in glial responses to injury.

The reason for the weaker phenotypes exhibited by many of the RNAi lines could be due to a number of factors: perhaps the RNAi is not fully effective, the candidate gene may aid in making the process of engulfment more efficient but might not be a crucial player, and there is always the possibility that knockdown of the gene is compromising the health of the glial cells and they are overall not as efficient in their roles thus engulfment occurs more slowly. The remaining genes identified in this screen will need to be examined further to confirm that the phenotypes are in fact real and not an artifact of the RNAi, and to determine that the phenotypes are adult-specific and not the result of developmental defects. Adult-specificity of the phenotype has already been confirmed for those RNAi lines that resulted in lethality when driven with *repo-Gal4*.

Genes controlling the expression of Draper

In another distinct phenotypic class identified in this screen, the readout used for glial reactivity, Draper itself, was reduced, which ultimately led to axon clearance defects. This axon clearance defect was mild for most genes. Within this phenotypic class, two subclasses were identified, one class which resulted in a loss of only basal Draper expression (i.e., the Draper levels normally present in the uninjured brain), and another class which displayed a loss of both basal Draper expression as well as injury-induced Draper upregulation.

Many of the RNAi lines for genes which resulted in a loss of basal Draper expression were genes involved in the PI3K/Akt signaling pathway (*akt*, *pi3k92e*, *pi3k21b*, *s6k*, *raptor*, *14-3-3ζ* and *pdk1*) **(Fig. 3-4).** The PI3K/Akt signaling cascade is an evolutionarily conserved pathway involved in a number of critical cellular processes including cell growth and survival, metabolism, protein synthesis, transcription, and receptor-mediated phagocytosis. In the canonical PI3K/Akt signaling cascade, PI3K is activated by an upstream receptor and converts PI-4,5-biphosphate (PIP2) into PI-3,4,5-triphosphate (PIP3). PIP3 then recruits the serine/threonine kinase Akt to the membrane where it is activated through phosphorylation by pyruvate dehydrogenase kinase 1 (PDK1) and ultimately leads to downstream events which may include protein synthesis mediated through S6 kinase (S6K). Raptor forms part of a complex which drives a feedback loop that normally keeps PI3K activity in balance and 14-3-3ζ binds to and inhibits an inhibitor of the PI3K/Akt signaling pathway thereby keeping the pathway activated. Glial-specific knockdown of components of this pathway resulted in significant reductions in basal levels of Draper in the brain. However, upon antennal ablation, Draper levels increased around the antennal lobes. As previously discussed, these animals displayed axon clearance phenotypes as well but they were mild and most likely represent a delay in clearance resulting from low initial Draper levels **(Fig. 3-4)**. These results suggested that the PI3K/Akt pathway is involved in transcriptionally and/or translationally regulating Draper expression. To further examine this possibility, a constitutively active *pi3k92e* construct, *UAS-pi3k92e*^{CAAX}, was expressed under the control of *repo-Gal4* and the effect on Draper expression levels was assayed. Strikingly,

Draper levels were significantly increased in the activated PI3K92E animals. Western blot analysis on *Drosophila* brains confirmed these findings **(Fig 3-5)**.

As previously noted, an additional phenotypic class resulting in changes in Draper expression was identified. However, this phenotype was characteristic of a reduction of both basal Draper expression and injury-induced Draper expression. Not surprisingly, this condition led to a robust axon clearance defect as Draper is required for clearance of degenerating axons. Only a single gene yielding this phenotype was identified in this screen, *stat92E*, and its role in engulfment signaling will be discussed in detail in chapter four.

Conclusions

Of the ~300 RNAi lines tested in this screen, I identified 20 potential candidates affecting glial engulfment processes **(Table 3-1)**. Four phenotypes were distinguished, an axon pathfinding phenotype, an axon clearance phenotype, a reduction in basal Draper expression and a reduction in both basal and injury-induced Draper expression. Many RNAi lines displayed more than one phenotype. For instance, loss of Draper expression likely results in a mild axon clearance defect as well. While aberrant axon pathfinding events are not related to engulfment, nonetheless they provide evidence of a new role for glia in developmental pathfinding in the antennal lobe. A robust axon clearance phenotype was observed in three lines, *rac1*, *basket* and *stat92E*. These genes are currently being examined further and phenotypes have been confirmed by other non-RNAi means. In addition, several components of the PI3K/Akt pathway were identified

in this screen and all gave a similar phenotype; loss of basal Draper expression, upregulation of Draper expression after injury, and mild clearance defects.

Finally, it is also worth noting that there were a few RNAi lines which displayed adult specific lethality shortly after induction of RNAi. The adult specific glial requirement of these genes for viability is intriguing and could provide some insight into new roles for glial cells in the mature brain.

Materials and Methods

Fly strains

The following fly strains were used: OR85e-mCD8::GFP (Couto et al., 2005) (B. Dickson), *repo-Gal4, tubulin-gal80^{ts}*. Unless otherwise noted all RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC) and flies were raised and maintained at 25^{o}C.

Temperature shift experiments

To perform the temperature shift assay all flies were raised at the restrictive temperature of 18^{o}C until eclosion. The flies were then shifted to the permissive temperature of 30^{o}C for 7 days prior to maxillary palp ablation to allow for induction of the RNAi and they were kept at 30^{o}C for an additional 5 days following maxillary palp ablation prior to dissection.

Olfactory neuron injury protocol, immunohistochemistry, and confocal microscopy. Maxillary palp and third antennal segment ablations, adult brain dissections, and antibody stainings were performed using previously described methods (MacDonald et al., 2006; Vosshall et al., 2000). Samples were mounted in Vectashield (Vector Laboratories) antifade reagent and viewed on a Zeiss LSM5 Pascal confocal microscope.

Generation of the *dos* RNAi construct

The dos^{RNAi} construct was made by PCR amplification of a 486bp fragment of the *dos* gene from the gold collection SD02517 clone. The following primers were used: Forward 5‘-gatcactagtcaccagcagccttactacaa-3‘ , Reverse 5‘-gatctctagatctcttataggcattcggtttgc-3‘. The PCR product was purified using a Qiagen Gel Purification kit, directionally cloned into the pWIZ vector (Lee and Carthew, 2003) and transformed into DH5α cells. Colonies were prepped using the Qiagen miniprep kit and the construct was sequence-verified. Transgenic flies were generated by Best Gene (Chino Hills, CA) using standard methods.

Western Blot

Drosophila brains of the indicated genotype were dissected in PBS and homogenized in SDS loading buffer (60mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue). For Western analysis, samples containing approximately 2 brains were loaded onto 10% SDS-PAGE gels (BioRad), transferred to a nitrocellulose membrane (BioRad), and probed with rabbit α-Draper antibody, 1:1000 (Freeman et al.,

2003), diluted in PBS/0.01% Tween-20/5% BSA . The blots was 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. The blot was then reprobed with mouse α-Repo (1: 20).

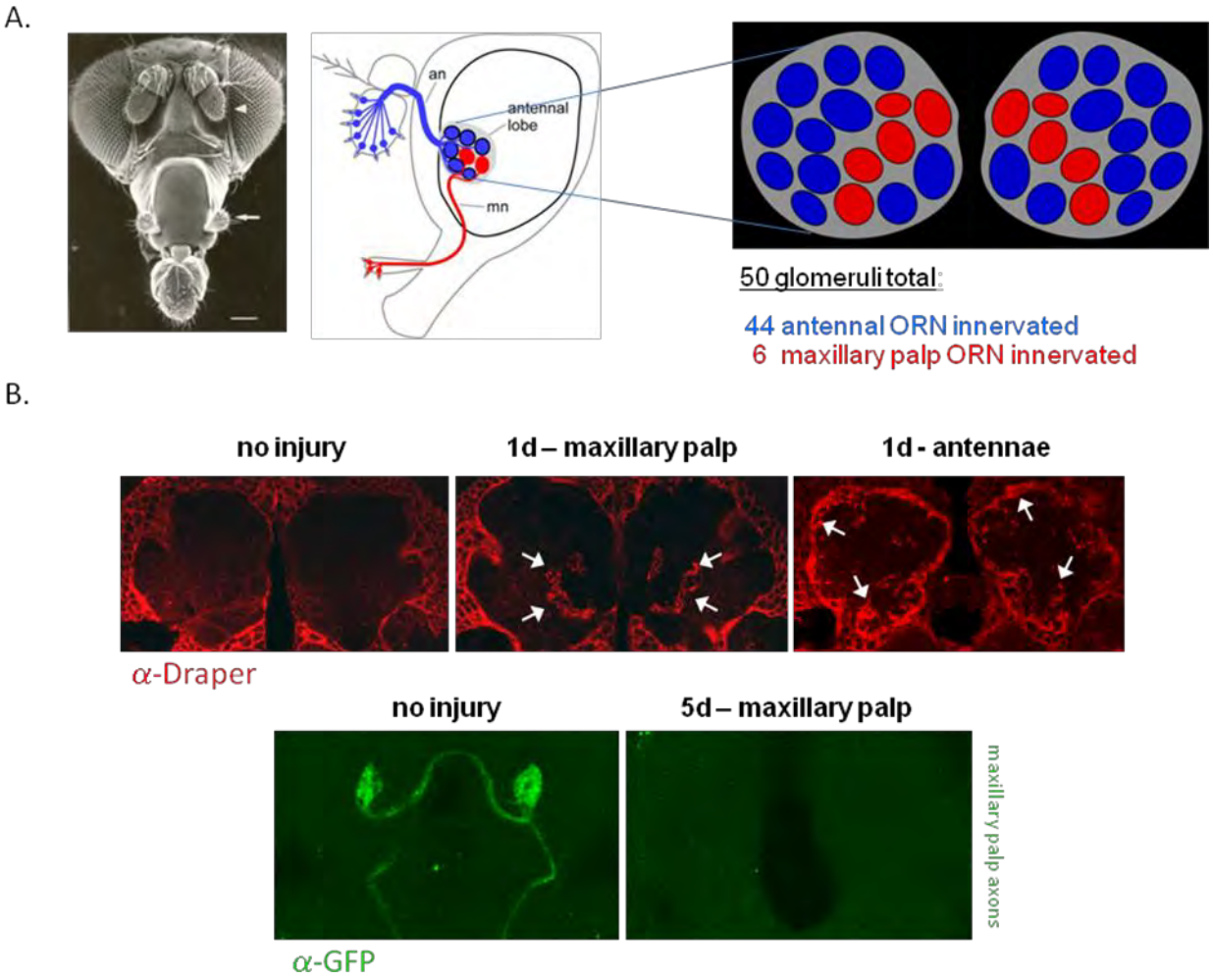


Figure 3-1. Diagram of ORNs projecting into the antennal lobes and overview of injury assay and markers

(A)Frontal view of *Drosophila* head, cartoon showing sagittal section of Drosophila head, antennal axons (blue), maxillary palp axons (red) project into the antennal lobe region of the brain and synapse onto bundled structures termed glomeruli, the majority or glomeruli are innervated by axons projecting in from the antennae while only a few are innervated by axons projecting in from the maxillary palp. (B) Draper staining of the antennal lobe region in an uninjured animal, white arrows, Draper upregulation one day after maxillary palp and one day after antennal injury. OR85e+ maxillary palp axons labeled with GFP in an uninjured animal and 5 days after removal of the maxillary palps, note all of the GFP+ material has been cleared.

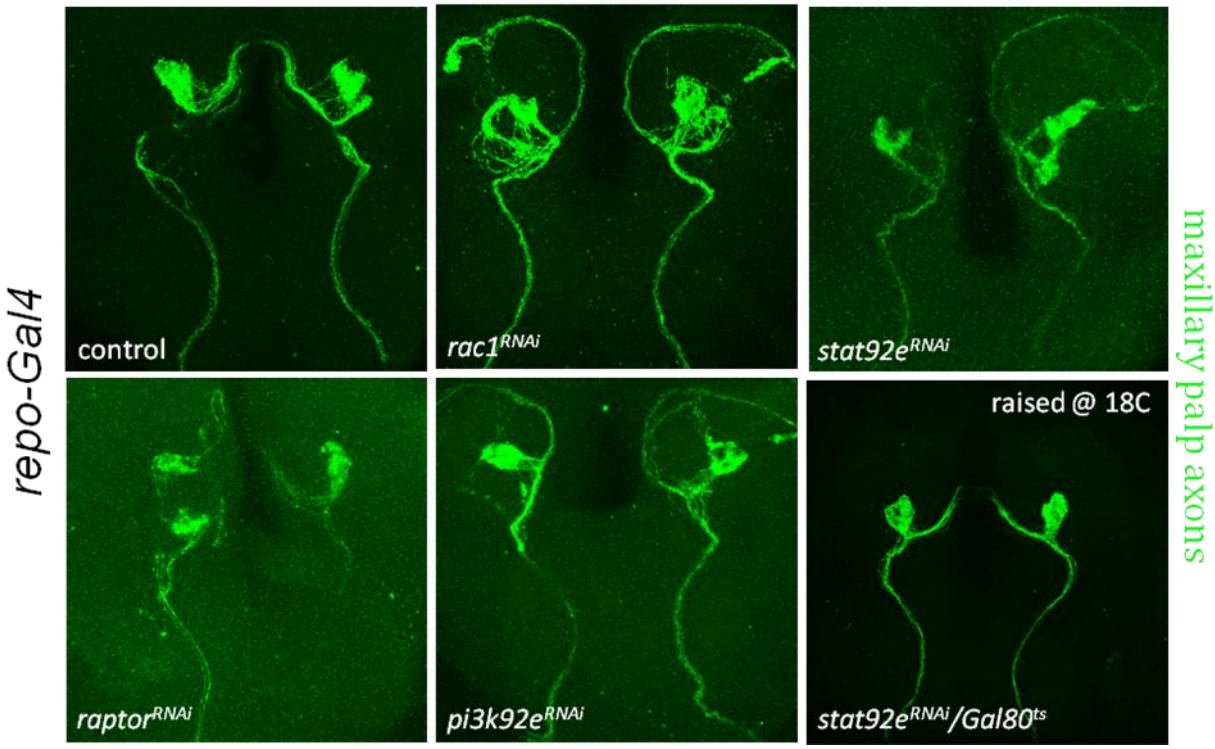


Figure 3-2. Glial cells play a critical role in axon pathfinding in the antennal lobes OR85e+ axons in (A) control, (B) a *repo-gal4>rac1*^{RNAi}, (C) a *repo-gal4>stat92E*^{RNAi}, (D) a *repo-gal4>raptor*^{RNAi}, (E) a *repo-gal4>pi3k92E*^{RNAi}, and (F) a *repo-gal4>stat92E*^{RNAi} in a background with *Gal80*^{ts}, these flies were raised at 18^{o}C to keep the RNAi off during development and shifted to 30^{o}C upon eclosion. The axon pathfinding defects occur during development as adult specific knockdown of stat92E does not result in axon pathfinding defects.

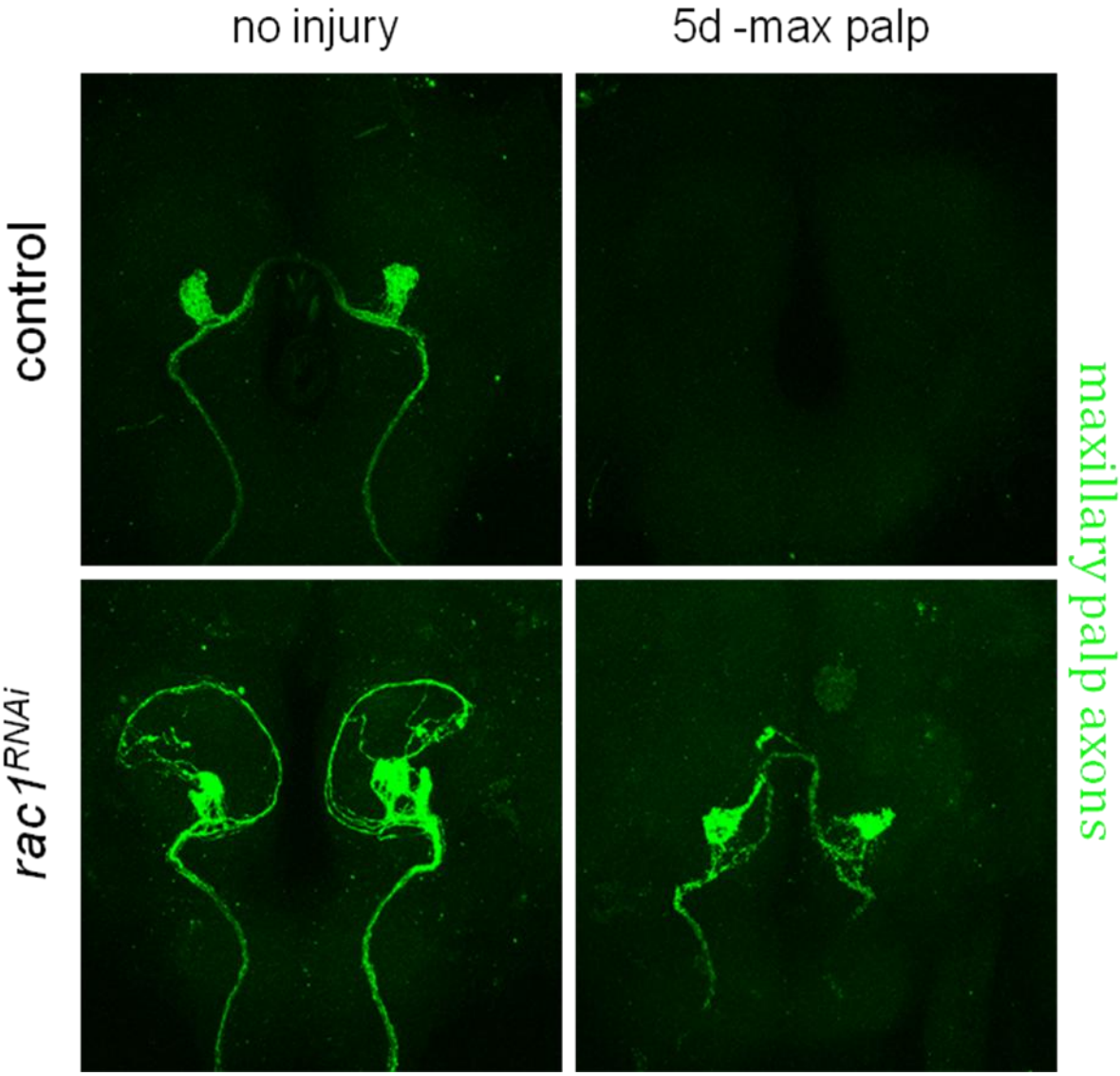


Figure 3-3. Glial specific knockdown of *rac1* inhibits the clearance of degenerating axons OR85e+ axons (green) five days after maxillary palp injury in a control animal and a *repo-gal4>rac*^{RNAi} animal. Loss of Rac1 in glial cells leads to an axon clearance defect as indicated by the persistence of GFP+ axon material left at day 5.

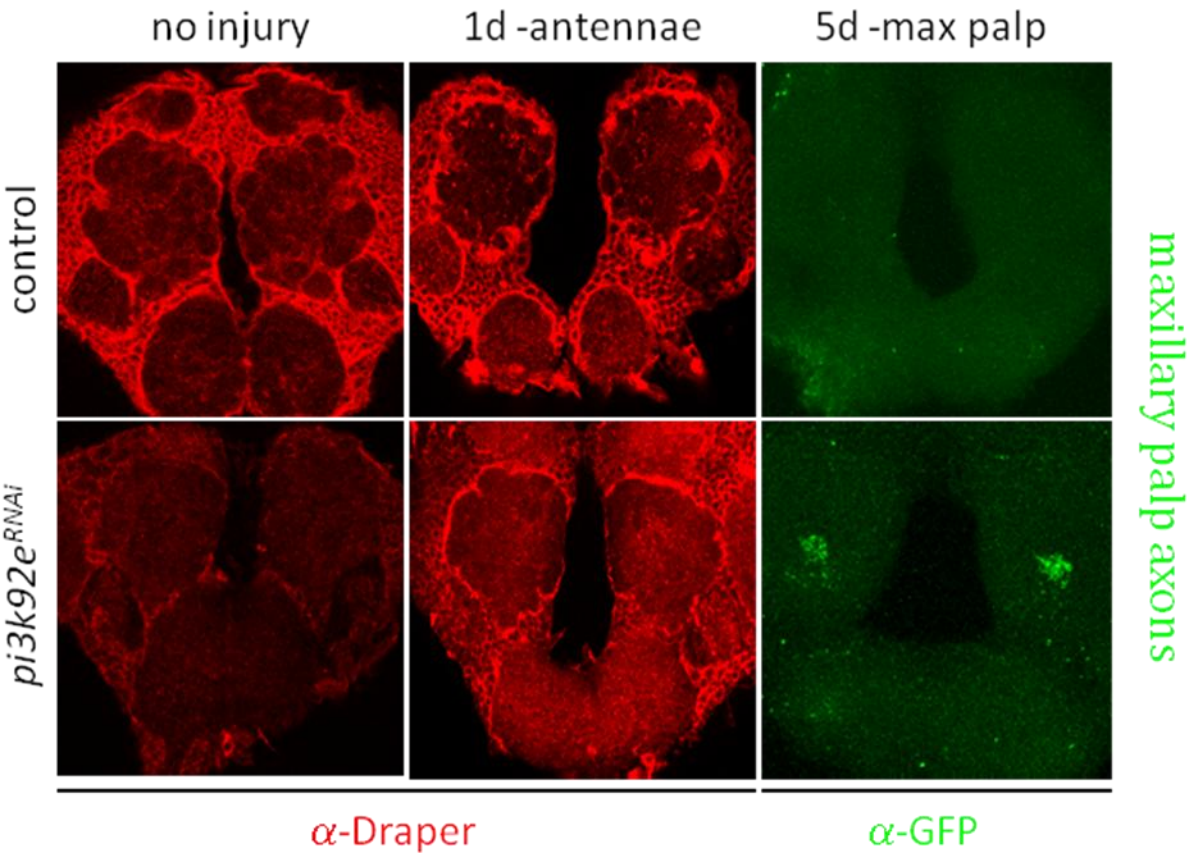


Figure 3-4. Glial specific knockdown of *pi3k92e* leads to reduction in basal Draper levels and axon clearance phenotype Draper staining (red) in an uninjured animal and one day after antennal injury. In uninjured animals Draper staining is seen throughout the brain and one day after antennal injury it is increased around the antennal lobe. In *repo-gal4>pi3k92e*^{RNAi} Draper staining is significantly reduced in the uninjured animal but it increases upon antennal injury. OR85e+ axons (green) 5 days after injury in control and *repo-gal4>pi3k92e*^{RNAi} animals. GFP+ axon material persists in the *repo-gal4>pi3k92e*^{RNAi} animal

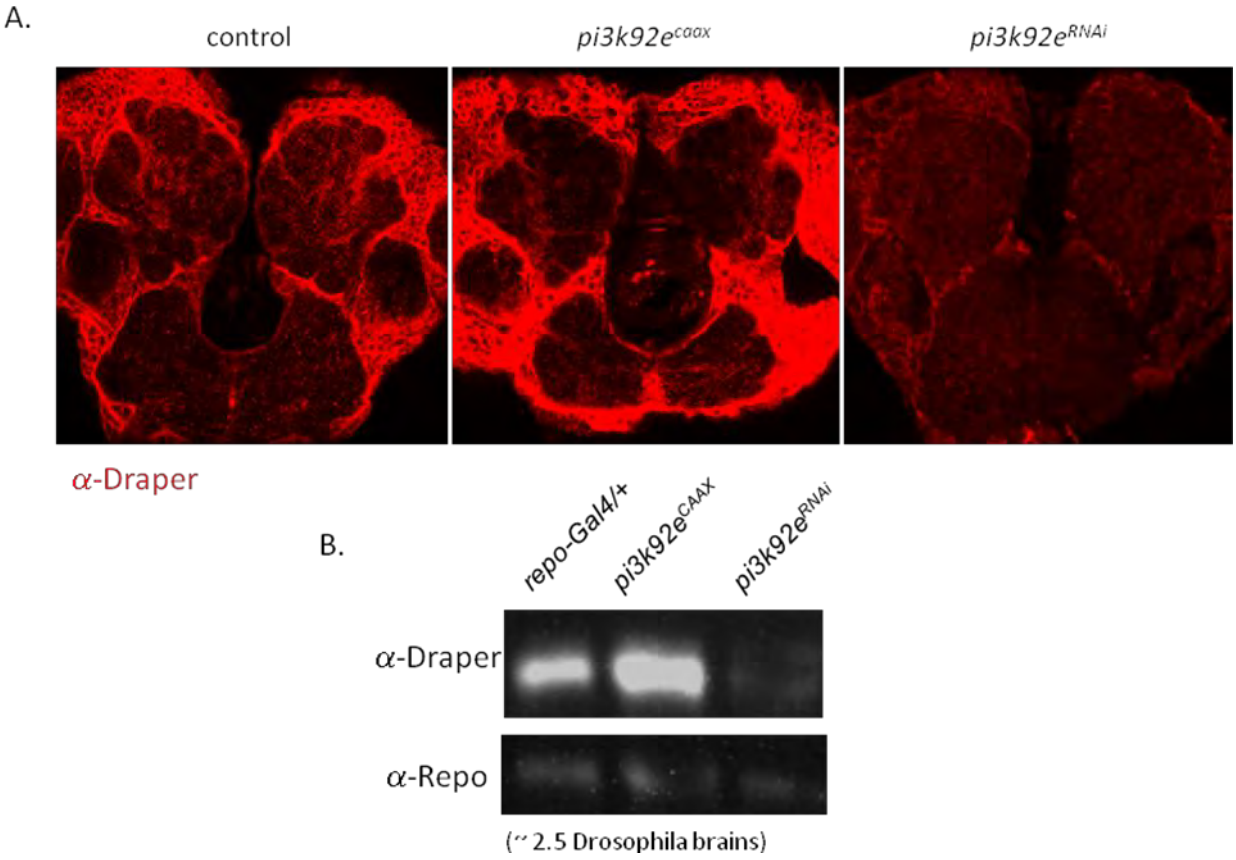


Figure 3-5. PI3K92E regulates Draper protein levels
Draper (red) staining in a control animal, *repo-Gal4>pi3k92e*^{CAAX} and *repo-Gal4>pi3k92e*^{RNAi}. Overactivation of PI3K92E leads to increased Draper expression and knock down of PI3K92E leads to a reduction of Draper expression. α-Draper western blot showing Draper levels in control, *repo-Gal4>pi3k92e*^{CAAX} and *repo-Gal4>pi3k92e*^{RNAi}.

CG#	Name	VDRC Trans#	Lethal with *repo-gal4*	Axon pathfinding defect	Axon clearance defect	Decrease in basal Draper
CG1044	dos	Self made #3	n	n	mild	n
CG1210	pdk1	18736	n	n	mild	y
CG2248	rac1	49247	n	y	severe	n
CG2699	pi3k21b	33556	n	n	mild	y
CG3605		26252	y	n/a	mild	n
CG4006	akt	2902	y	n/a	mild	y
CG4141	pi3k92e	38986	n	y	mild	y
CG4257	stat92e	43866	n	y	severe	y
CG4320	raptor	13112	n	y	mild	y
CG4931	sra1	34908	n	n	mild	n
CG5680	basket	34138	n	y	severe	n
CG5771	rab11	22198	y	n/a	mild	y
CG6033	drk	105498	y	n/a	mild	n
CG7793	sos	42849	y	n/a	mild	n
CG10539	s6k	18126	n	n	mild	y
CG10960		8359	n	n	mild	y
CG13345	tumbleweed	17145	n	n	mild	y
CG17870	14-3-3zeta	48724	n	n	mild	y
CG33106	mask	29541	n	n	mild	y
CG43443	hts	103631	y	n/a	mild	y
UAS-pi3k92e^{caax}	pi3k92e		n	n	n/a	increase

mild- <50% of axon material left at day 5
severe- >50% of axon material left at day 5

Table 3-1

CHAPTER IV: Engulfment signaling promotes Stat92E-dependent transcriptional activation of *draper* after axotomy

The work conducted in this chapter was performed in the lab of Dr. Marc Freeman. My contribution to this work included identification of Stat92E as a transcriptional regulator of Draper, generation of the mutant *dee7*-Gal4 line, all work associated with generation of stocks and maintenance of crosses, all injury assays, all *in situ* antibody stainings, and all confocal microscopy. Amy Sheehan contributed to work presented in this chapter by generating the original *dee7*-Gal4 line. Rachel Hackett contributed to the work presented in this chapter by performing western blot analyses. The following publication is in progress:

Doherty J, Amy Sheehan, Rachel Hackett and Freeman MR
Engulfment signaling promotes Stat92E-dependent transcriptional activation of *draper* after axotomy.

Abstract

Glial cells exhibit dramatic morphological and molecular responses to brain injury, but signaling mechanisms that activate glial cells after injury remain poorly defined. Here we show that the *Drosophila draper* gene is transcriptionally activated in glial cells in response to axonal injury through a Stat92E-regulated injury-responsive enhancer element. Glial-specific inhibition of Stat92E function eliminates nearly all Draper expression, and suppresses glial engulfment of axonal debris. Surprisingly, additional components of the canonical JAK/STAT signaling pathway do not regulate *draper* expression or glial activation after injury. Rather, Stat92E transcriptional activity after axotomy is promoted in a novel way by Draper/Src42a/Shark/Rac1 engulfment signaling. This identifies Stat92E as a novel glial gene required for glia phagocytic function, and Draper as only the second receptor capable of activation of Stat92E-dependent transcriptional changes. We propose this Draper→Stat92E→*draper* gene auto-regulatory loop is activated in glia by axonal injury, leads to increased expression of engulfment factors, and ultimately enhances the ability of glial cells to clear cellular debris after injury.

Glial cells are extraordinarily sensitive to even minor disruptions in central nervous system (CNS) homeostasis and exhibit an impressive ability to respond to a diversity of neural injuries including hypoxia, chemical insults, and mechanical injury (e.g. axotomy, or traumatic brain injury) (Bignami and Dahl, 1976; Deng et al., 2011; Sofroniew, 2009; Weigart, 1895). Glial "reactive" responses after injury can be beneficial and promote recovery. For example, reactive glia clear degenerating neuronal debris to avoid nervous system inflammation, and can facilitate remyelination (Fancy et al., 2011; Logan and Freeman, 2007; Napoli and Neumann, 2009; Neumann et al., 2009). However, reactive glia can also exacerbate damage in the CNS by driving inflammation and actively destroying healthy cells (Block et al., 2007; Brenner et al., 2001; Nagai et al., 2007; Neniskyte et al., 2011; Rogers et al., 2007; Swanson et al., 2004). Whether reactive gliosis is ultimately more beneficial or harmful to the nervous system remains an open question, and the neuron-glia signaling pathways that modulate reactive glial responses remain largely undefined.

We have previously shown that Draper, a glial-expressed immuno-receptor, is a central regulator of glial responsiveness to axotomy in the adult *Drosophila* brain (Doherty et al., 2009; MacDonald et al., 2006; Ziegenfuss et al., 2008). Within hours after axotomy, Draper protein levels dramatically increase in brain regions housing degenerating axons. Glial membranes are then recruited to severed axons where Draper mediates the engulfment of axonal debris. Based on the robust increase in Draper levels observed following axotomy, we suspected the *draper* gene might be transcriptionally activated in response to axonal injury. We therefore sought to identify enhancer elements

in the *draper* locus that were responsive to axotomy, with the goal of using these to dissect signaling pathways governing injury-induced changes in glial gene expression.

Results

We focused our search on a ~40 kb region centered around the *draper* locus (**Fig. 4-1a**). avoiding the region directly upstream of the Draper locus as this contained the open reading frame for the *oseg4* gene. We cloned 9 different potential *draper* enhancer elements (termed *dee2-dee10*) from primarily intronic sequences into the *Gal4*-based *pBGW* vector (Karimi et al., 2002) and inserted these elements into identical genomic locations (**Fig. 4-1a**). Each *dee-Gal4* element was then used to drive two copies of *UAS-mCD8::GFP in vivo* and expression patterns were examined in the adult brain both before and after injury. A localized axonal injury was induced in the antennal lobe of the brain by ablating either 3rd antennal segments or maxillary palps, which results in Wallerian degeneration of olfactory receptor neuron (ORN) axons (**Fig. 4-1b**). No glial expression was observed with *dee2-6-* or *dee8-10-Gal4* lines either before or after axonal injury (data not shown). Similarly, *dee7-Gal4* did not drive GFP expression prior to axon injury in ensheathing or cortex glia, those adult brain glia which normally express Draper (Doherty et al., 2009). We note that we did observe low level expression of this element in astrocyte-like glia which were randomly distributed in the neuropil (**Fig. 4-1b**). However, following antennal ORN axotomy we noticed a striking increase in glial expression of mCD8::GFP in the *dee7-Gal4* reporter background (**Fig. 4-1b**). Interestingly, injury-induced reporter expression was strongest in ensheathing glia

surrounding the antennal lobe, those glia that normally engulf degenerating ORN axons, but the reporter expression also increased dramatically in cortex glia throughout the brain. This widespread activation of the reporter indicates that glia even at locations distant from the injury site, can respond molecularly to axonal damage. Notably, we found the severity of the axonal injury appeared to dictate the responsiveness of *dee7-Gal4*. For example, ablation of maxillary palps, which results in far fewer ORN axons being severed in the antennal lobe, resulted in a smaller and highly localized increase in mCD8::GFP in cortex glia located in the ventral region of the antennal lobe (**Fig. 4-1b**).

Sequence analysis of the 2619bp *dee7* element led to the discovery of 3 consensus Stat92E binding sites (TTC3n/4nGAA) (Yan et al., 1996), the sole member of the Signal Transducer and Activator of Transcription (STAT) family of molecules in *Drosophila* (Hou et al., 1996; Yan et al., 1996). Of these 3 Stat92E sites, two were also present in *dee6-Gal4*, which was not responsive to axonal injury (**Fig. 4-1a**). We therefore mutated the Stat92E binding site specific to the *dee7-Gal4* element, integrated this *dee7^{mut}-Gal4* construct into the same genomic location used for the previously-generated reporter lines, and examined its responsiveness to axonal injury. Strikingly, while baseline levels of mCD8::GFP expression were similar to *dee7-Gal4*, *dee7^{mut}-Gal4* exhibited a dramatic decrease in the injury-induced expression of mCD8::GFP (**Fig. 4-1b-d**). We conclude that *dee7* contains a glial regulatory element which is injury-responsive, and our data suggest that Stat92E is required for maximal activation after axotomy.

We next sought to determine whether Stat92E was required for modulating glial responses to axonal injury and clearance of degenerating axonal debris. Stat92E function

was knocked down specifically in glia by expressing a *UAS-Stat92E*^{RNAi} construct with the pan-glial *repo-Gal4* driver. We labeled a subset of olfactory receptor neurons (ORNs) with GFP, severed axons, and assayed clearance of GFP-labeled ORN axonal debris 5, or 15 days after axotomy. In controls the majority of GFP^{+} axon material was cleared from the CNS 5 days after injury (**Fig. 4-2a-c**). However, in *Stat92E*^{RNAi} animals, GFP^{+} axonal debris persisted even 15 days after injury (**Fig. 4-2a-c; Fig. 4-3**). We were able to confirm the *UAS-Stat92E*^{RNAi} line efficiently targets *Stat92E*, since glial co-expression of a GFP-tagged Stat92E molecule with the *Stat92E*^{RNAi} construct eliminated all Stat92E-GFP expression compared to controls (**Fig. 4-4**). These observations identify Stat92E as a novel regulator of glial engulfment activity in the adult brain.

Based on our identification of a Stat92E-dependent injury-responsive element in the *draper* gene, we predicted Stat92E would modulate glial phagocytic activity by regulating *draper* expression after axotomy. Draper is normally expressed in ensheathing and cortex glia throughout the brain and is dramatically up-regulated around the antennal lobe after antennal ORN axotomy (**Fig. 4-2d**). Strikingly, we found glial-specific knockdown of Stat92E lead to nearly undetectable levels of Draper expression even prior to injury (termed basal Draper levels) (**Fig. 4-2d-f**). We confirmed this widespread loss of Draper by performing Western blots on dissected adult brains from control, *Stat92E*^{RNAi} , and *draper*^{RNAi} animals and probing with α-Draper antibodies (**Fig. 4-2f**). Knockdown of Stat92E in glia was also sufficient to completely suppress glial activation of *draper* after axotomy. *Stat92E*^{RNAi} animals exhibited no detectable increase in Draper

levels after antennal ablation compared to controls. In addition, while Draper was localized specifically to severed ORN axons after maxillary palp ablation, we found no detectable Draper expression in *Stat92E*^{RNAi} animals (**Fig. 4-2d,e**). These data suggest that Stat92E renders glial cells competent to respond to axon injury by controlling the expression of Draper.

STAT signaling is involved in multiple cellular processes including cell survival, differentiation, motility and immunity (Baksa et al., 2002; Brown et al., 2006; Flaherty et al., 2010; Ghiglione et al., 2002; Kim et al., 2007; Liu et al., 2009b; Silver and Montell, 2001; Yan et al., 2011). To exclude the possibility that the defects we observed in *Stat92E*^{RNAi} animals resulted from abnormalities in glial cell development we used the conditional Gal80^{ts} system to specifically activate *Stat92E*^{RNAi} at adult stages. When *stat92E*^{RNAi} animals with Gal80^{ts} in the background were raised and tested at 18^{o}C, we found that glia efficiently cleared axonal debris and expressed normal levels of Draper (**Fig. 4-5**). However, when they were shifted to and tested at the restrictive temperature during adult stages (thereby activating the RNAi construct only after development was complete), we found that *stat92E*^{RNAi} animals exhibited reduced expression of Draper and failed to clear degenerating axons (**Fig. 4-5a,b**). Although we did not perform a detailed comparison of glial cell numbers between control animals and stat92E knock down animals, overall glial cell morphology appear grossly normal in these animals, arguing that these phenotypes are not the result of glial cell loss in *stat92E*^{RNAi} backgrounds (**Fig. 4-6**). Moreover, we found that adult specific activation of the RNAi was reversible, as shifting these animals back to 18^{o}C (thereby turning the RNAi off) re-established normal

levels of Draper and initiated clearance of axonal debris (**Fig. 4-5**). Together these data indicate that Stat92E functions in adult brain glia, where it modulates Draper expression and glial phagocytosis of degenerating axons.

To explore the dynamics of Stat92E signaling in adult brain glia we examined the expression patterns of transcriptional reporters for Stat92E activity (Bach et al., 2007). These reporters have been previously shown to accurately reflect Stat92E transcriptional activity during development as well as in the adult (Bach et al., 2007; Gilbert et al., 2009; Vidal et al., 2010). We first used the *10XStat92E-GFP* reporter, which harbors ten Stat92E binding sites driving expression of enhanced GFP. In co-stains with α-Draper and α-Repo (a glial nuclear marker) we found robust activation of the Stat92E reporter in uninjured controls (**Fig. 4-7a**). Moreover, after ablation of antennae we found strong GFP labeling of antennal lobe glia, and the GFP signal completely overlapped with Draper (**Fig. 4-7a**). After ablation of maxillary palps we found GFP signals colocalizing with Draper around glomeruli housing severed axons (**Fig. 4-7a**). Thus, Stat92E activates transcriptional reporters in adult brain glia.

The GFP driven by the *10XStat92E-GFP* reporter is quite stable and can perdure in cells for ~20 hours after activation which precludes our use of this construct to examine dynamic changes in Stat92E transcriptional activity. We therefore used a second reporter, *10XStat92E-dGFP*, which drives the expression of destabilized GFP, which is rapidly degraded, thereby allowing for increased temporal resolution of Stat92E activity. We were unable to detect any expression of this Stat92E transcriptional reporter in uninjured adult brains (**Fig. 4-7b**). However, beginning ~16 hours after antennal

ablation we detected *10XStat92E-dGFP* expression in cells surrounding the antennal lobe (**Fig. 4-7b**). GFP intensity peaked at ~24 hours after antennal ablation and disappeared by 48 hrs after axotomy (**Fig. 4-7b**). Consistent with our observations of the *dee7-Gal4* driver, we found that injury led to widespread activation of the *10XStat92E-dGFP* in glia throughout the brain (**Fig. 4-7c,d**), further supporting the notion that glial cells in the adult brain respond as a network, with glia some distance from the injury changing patterns of gene expression in response to axonal injury.

To confirm that activation of the *10XStat92E-dGFP* reporter after axotomy was Stat92E-dependent and glial specific we knocked down Stat92E specifically in glia, severed axons and assayed *10XStat92E-dGFP* activity. We found that glial-specific knockdown of Stat92E completely suppressed the axotomy-induced activation of the *10XStat92E-dGFP* transcriptional reporter (**Fig. 4-7d**). Together these data indicate that Stat92E can transiently increase the transcriptional activation of target genes in glia after axonal injury.

STAT is regulated by the JAK signaling platform, and this pathway is conserved in all higher metazoans. The *Drosophila* JAK/STAT signaling pathway consists of a single JAK molecule, Hopscotch (hop) (Binari and Perrimon, 1994), and the cytokine like receptor Domeless (Dome) (Brown et al., 2001; Chen et al., 2002). To determine if Stat92E-dependent activation of *draper* was mediated through canonical JAK/STAT signaling we drove RNAi constructs targeted against *hop*, and a dominant negative Domeless molecule (Silver and Montell, 2001), Domeless$^{\Delta CYT}$, in glial cells, and assayed Draper expression and clearance of severed axons (**Fig. 4-8a,b**). Surprisingly, in each of

these backgrounds we found Draper levels were similar to control animals and axons were efficiently cleared 5 days after injury (**Fig. 4-8a,b**). Reciprocally, we found a gain-of-function allele of *hop*, hop^{TUM}, which has been shown in numerous assays to activate Stat92E transcriptional activity failed to activate the*10XStat92E-dGFP* reporter in the adult brain. Notably, expression of an activated version of Stat92E, $Stat92E^{\Delta N \Delta C}$ (Ekas et al., 2010), led to strong activation of the *10XStat92E-dGFP* reporter (**Fig. 4-8c**). $Stat92E^{\Delta N \Delta C}$ has previously been shown to require phosphorylation at Y711 for activation (Ekas et al., 2010). We therefore expressed a version of $Stat92E^{\Delta N \Delta C}$ with a Y711F mutation in glia, and found it was insufficient for activation of the *10XStat92E-dGFP* reporter (**Fig. 4-8c**). Together these data argue that Dome- and Hop- dependent JAK/STAT signaling is not required for glial responses to axon injury and activated Hop is not sufficient to activate Stat92E in glial cells.

During canonical Stat92E signaling events, extracellular cytokines bind to the Domeless receptor and activate Stat92E transcriptional activity. Interestingly, *domeless* has been shown to be a direct transcriptional target of Stat92E and activation of the Stat92E pathway has been shown to increase Domeless expression forming an auto-regulatory loop (Arbouzova and Zeidler, 2006; Brown et al., 2001; Hombria et al., 2005; Xi et al., 2003). While a role for Draper signaling in modulating glial gene expression has never been described, we wondered whether a similar Draper-Stat92E auto-regulatory loop might function to dynamically modulate glial responsiveness to axonal injury. We therefore assayed *10XStat92E-dGFP* activation after injury in $draper^{\Delta 5}$ mutants. Intriguingly, we found a complete lack of *10XStat92E-dGFP* transcriptional activity after

axotomy in *draper*$^{\Delta 5}$ animals (**Fig. 4-9a**). Consistent with this requirement for Draper signaling in activation of Stat92E target genes in glia after axotomy, we also found a complete lack of activation of the *dee7-Gal4* reporter in *draper*$^{\Delta 5}$ backgrounds (**Fig. 4-9b**). These findings indicate that an auto-regulatory loop exists in which Draper signaling is necessary for injury induced activation of Stat92E and in turn for upregulation of Draper itself.

Next we explored whether other identified components of the Draper signaling pathway modulate activation of Stat92E after injury. Upon axon injury, Draper is thought to be phosphorylated by Src42a, initiating binding of the non-receptor tyrosine kinase Shark, which together with the PTB domain-containing protein dCed-6 promote engulfment (**Fig. 4-10d**) (Doherty et al., 2009; MacDonald et al., 2006; Ziegenfuss et al., 2008). Rac1 was identified in the *in vivo* RNAi screen described in chapter three and evidence from our lab indicates that Draper signaling feeds into the Rac1 signaling pathway to promote engulfment (Jennifer Ziegenfuss, personal communication). Interestingly, we found that glial-specific knockdown of Shark, Src42a, and Rac1 completely blocked injury-induced activation of the*10XStat92E-dGFP* transcriptional reporter (**Fig. 4- 9d**). However, while knockdown of dCed-6 eliminated dCed-6 immunoreactivity and has been previously shown to suppress clearance of degenerating axons (**Fig. 4-11**) (Doherty et al., 2009), axotomy-induced activation of *10XStat92E-dGFP* was still detectable (**Fig. 4- 9c**). Thus Draper, Src42a, Shark, and Rac1, but not dCed-6, are essential for Stat92E-dependent activation of transcriptional targets in glia responding to axonal injury. Interestingly, while there is no increase in Draper upon

injury in *src42a*, *shark* or *rac1* RNAi animals, basal Draper levels are normal. This suggests that Stat92E regulates basal (i.e. expression levels before injury) and injury-induced levels of Draper through distinct mechanisms.

While JAK is the primary kinase which activates STAT, other mammalian kinase molecules (e.g. src, tyk) or receptor tyrosine kinases (i.e. insulin receptor, EGFR) can phosphorylate STAT molecules. Since Src42a is known to signal downstream of Draper we sought to determine whether Src42a activity was sufficient to activate Stat92E transcriptional reporters. Strikingly, expression of a constitutively active Src42a molecule ($Src42a^{CA}$) was sufficient to induce robust *10XStat92E-dGFP* reporter activity throughout brain (**Fig. 4-9e**). The simplest interpretation of these data are that Src42a is the kinase that acts downstream of Draper to activate Stat92E signaling after axonal injury.

Draper and dCed-6 are both required for glial engulfment of degenerating axons and are expressed exclusively in glial cells in the adult brain (Doherty et al., 2009). To determine if Stat92E is a general regulator of engulfment gene expression we assayed dCed-6 levels in the adult brain in animals expressing $Stat92E^{RNAi}$ in glia. While knockdown of Stat92E led to a dramatic reduction of Draper, we did not observe significant changes in dCed-6 levels (**Fig. 4-10a**). However, western blot analysis on brains will need to be performed in order to confirm this finding. We also note that while dCed-6 levels appeared normal, dCed-6 immunoreactivity was not recruited to severed maxillary palp axons 1 day after axotomy (**Fig. 4-10a**) indicating that Draper and/or Stat92E is necessary for dCed-6 recruitment to severed axons. However, *dced-6* does not

appear to be an *in vivo* target for Stat92E regulation. These data are the first to demonstrate a functional divergence between dCed-6 and Draper/Src42a/Shark function during engulfment signaling.

Together our data indicate that *draper* is a direct transcriptional target of Stat92E after axotomy. To further explore this novel relationship we over-expressed Draper in a *Stat92E*^{RNAi} background and asked whether resupplying Draper was sufficient to overcome the engulfment deficit observed in Stat92E knock-down animals. Indeed, expression of Draper in *Stat92E*^{RNAi} animals led to a striking rescue of the engulfment defect (**Fig. 4-10b,c**). Thus Draper appears to be a primary transcriptional target of Stat92E during glial responses to axonal injury.

Discussion

This study identifies a novel injury-induced auto-regulatory loop whereby activation of Draper in glial cells responding to axonal injury leads to downstream signaling through Src42a/Shark/Rac1, and in turn Stat92E-dependent transcriptional activation of the *draper* locus (**Fig. 4-10d**). This is the first work that demonstrates a role for engulfment signaling pathways (i.e. Draper) in the regulation of transcription, and shows Stat92E is downstream of a novel non-canonical signaling pathway mediated by Draper which promotes Stat92E-dependent activation of transcriptional targets. Despite the fact that Stat92E signaling is essential for a wide range of vital processes, Draper is only the second wild-type receptor (Domeless being the first) known to positively regulate Stat92E transcriptional activity (Brown et al., 2001; Chen et al., 2002; Makki et al.,

2010). Similarly, the *draper* gene represents a novel target for Stat92E—only three other genes, *eve*, *crb* and *dome*, have been shown by *in vivo* analyses to be directly regulated by Stat92E (Lovegrove et al., 2006; Rivas et al., 2008; Sotillos et al., 2010). Although we show that Draper is a critical target of Stat92E in glia responding to injury and that Draper overexpression can rescue Stat92E phenotypes, it is possible that Stat92E also regulates other engulfment genes in a similar way.

It is widely accepted that reactive glial responses are graded according to the severity of the brain injury—more severe injuries induce more robust responses from glia. Here we have shown that *Drosophila* glia also respond in a graded way to ORN injury: axotomy of a small number of ORN axons by maxillary palp ablation led to a small increase in *dee7-Gal4* activity, while severing the majority of ORNs (~85%) by antennal ablation led to a more dramatic increase in reporter activation. The Draper receptor→Stat92E→*draper* gene auto-regulatory loop we describe provides a simple molecular mechanism by which glial cells can dynamically modulate their ability to clear cellular debris according to the severity of the injury. We propose that relatively mild injuries promote only modest signaling through the Draper pathway, and thus weak activation of the *draper* gene. However more severe injuries promote enhanced signaling through the Draper pathway, and more dramatic activation of *draper* expression. Presumably upregulation of engulfment factors enhances the ability of glia to clear neuronal debris (**Fig. 4-10d**). Such a mechanism whereby glial transcriptional responses are activated downstream of the very pathways that drive glial phagocytic activity would allow glia to directly modulate their engulfment capacity according to the strength of

signaling through the Draper pathway. Since it is likely that Draper ligands are present on engulfment targets, transcriptional activation of glial engulfment genes would ultimately be regulated by extracellular levels of ~~–e~~at me" cues on degenerating axons.

Materials and methods

Fly Strains and molecular biology

(1) *UAS-mCD8::GFP*(Lee and Luo, 2001) (II), (2) *UAS-mCD8::GFP* (Lee and Luo, 2001) (III), (3) OR85e-mCD8::GFP (Couto et al., 2005) (B. Dickson), (4) *UAS-Stat92E*^{RNAi} , VDRC 43866, (5) *UAS-drpr*^{RNAi} (MacDonald et al., 2006), (6) *repo-Gal4,* (7) *UAS-Stat92E-GFP* (Karsten et al., 2006) (M. Zeidler) (7) *tubulin-Gal80*^{ts}, (8) *repo-Gal4,UAS-mCD8::GFP*, (9) OR67b-GFP (10) 10XStat92E-GFP (Bach et al., 2007) (E. Bach), (11) 10XStat92E-GFP (Bach et al., 2007) (E. Bach) , (12) *UAS-hop*$^{RNAi(a)}$, VDRC 40037, (13) *UAS-hop*$^{RNAi(b)}$, VDRC 102830, (14) *UAS-domeless*$^{\Delta CYT}$ [33] (E. Bach), (15) Hop^{Tum-1} (Bloomington stock center), (16) *UASp-Stat92E*$^{\Delta N\Delta C}$ (Ekas et al., 2010) (E. Bach), (17) *UASp-Stat92E*$^{\Delta N\Delta CY711F}$ [35](DNA provided by E. Bach, transgenic flies generated by Best Gene using standard methods), (18*) UAS-src42a*^{RNAi} VDRC 26019, (19) *UAS-rac1*^{RNAi} VDRC 49247, (20) *UAS-shark*^{RNAi} [16] (21) *UAS-dced6*^{RNAi} (Awasaki et al., 2006), (22) *UAS-DrprI* (M. Logan, unpublished), (23) *drpr*$^{\Delta 5,}$ (Freeman et al., 2003; MacDonald et al., 2006), (24) *yw,* (25) *UAS-src42a*^{CA} .

The *dee7-Gal4* construct was made by PCR amplification of the 2619bp fragment from the Draper BACR17K18 clone using the following primers: Forward 5'caccagacctactcttagctctgatggagg-3', Reverse 5'-gtttgtgtttccatggattcaggcttggg-3'. The

PCR product was purified using a Qiagen Gel Purification kit, directionally cloned into the Invitrogen pENTR/D-TOPO vector and transformed into One Shot Competent Cells using the pENTR/D-TOPO Cloning Kit (Invitrogen catalog # K2400-20). Colonies were prepped using the Qiagen miniprep kit. The *dee7* fragment was then shuttled into the pBGUw destination vector (Karimi et al., 2002) using the Invitrogen Gateway LR Clonase Enzyme and transformed into heat shock competent DH5α cells. The construct was sequence verified and transgenic flies were generated by Best Gene Inc. (Chino Hills, CA) using the PhiC31 targeted integration system.

To generate the *dee7*^{MUT}-*GAL4* construct, the *dee7*/TOPO construct was used as a template and PCR was carried out using Invitrogen Quick Change II Site-Directed Mutagenesis Kit (Cat #200523) with the following primers (mutation sites indicated in red): Forward-5‘ CTG TGC CGA ACA CGT TAA CCA TTG AAA AAT CTC GC 3‘, Reverse-5‘ GCG AGA TTT TTC AAT GGT TAA CGT GTT CGG CAC AG 3‘. A DpnI digestion was performed and DNA was transformed into XL-1 Blue super competent cells and plated. Colonies were prepped using the Qiagen miniprep kit and the mutations were verified by sequencing (Genewiz). The *dee7*^{MUT} enhancer fragment was then shuttled into the pBGUw vector using methods described above and transgenic flies were generated by Best Gene using PhiC31 targeted integration.

Olfactory neuron injury protocol, immunohistochemistry, and confocal microscopy. Maxillary palp and third antennal segment ablations, adult brain dissections, and antibody stainings were performed using previously described methods (MacDonald et al., 2006;

Vosshall et al., 2000). Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) antifade reagent and viewed on a Zeiss LSM5 Pascal confocal microscope. In all experiments, laser settings were kept identical for all brains imaged as part of that experiment. For Figure **4-1d**, single slice confocal images of approximately the same depth in the brain were identified and total intensity of GFP in the brain was measured. The minimum threshold was set at 3 to eliminate most background and the maximum threshold was set at the maximum value of 255. Measure of GFP intensity within maxillary palp glomeruli were performed as previously described (MacDonald et al., 2006; Vosshall et al., 2000). For Figure **4-2e**, Draper expression after maxillary palp injury was measured from single confocal slices at the depth of the OR85e-innervated glomerulus. A circle was drawn around the area of the OR85e innervated glomerulus and total intensity of Draper was measured. Draper expression after antennal injury was measured from single confocal slices about half way through the antennal lobe. A fixed area rectangle at the edge of the antennal lobe was used to measure total intensity of Draper. All quantification of measurements was performed using Image J software. The following antibodies were used: 1:200 mouse anti-GFP (Invitrogen), 1:500 rabbit anti-Draper (Freeman et al., 2003), 1:500 rat anti-dCed6 (Awasaki et al., 2006), 1:200 FITC anti-mouse IgG, 1:200 Cy3 anti-rabbit IgG, 1:200 Cy3 anti-rat IgG (Jackson ImmunoResearch).

Temperature shift experiments

To perform the temperature shift assay all flies were raised at the restrictive temperature of 18^{o}C until eclosion. One group of flies was kept at 18^{o}C throughout the experiment to ensure the Gal80^{ts} was efficiently repressing the Gal4 driven RNAi. Another group of flies was shifted at the same time to the permissive temperature of 30^{o}C for 7 days prior to maxillary palp ablation to allow the RNAi to turn on and they were kept at 30^{o}C for an additional 5 days following maxillary palp ablation prior to dissection. The last group was shifted all at the same time to the permissive temperature of 30^{o}C for 7 days to turn on the RNAi and then shifted back to the restrictive temperature of 18^{o}C for 7 days to turn off the RNAi prior to maxillary palp ablation. They were kept at 18^{o}C for 5 days following maxillary palp ablation and the brains were dissected.

Western Blots

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% β-mercaptoethanol, 0.01% bromophenol blue). For Western analysis, sample containing approximately 2 brains were loaded onto 10% SDS-PAGE gels (BioRad), transferred to nitrocellulose membranes (BioRad), and probed with rabbit α-Draper (Freeman et al., 2003) antibody at 1:1000 diluted in PBS/0.01% Tween-20/5% BSA . 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 reprobed with mouse α-tubulin (Sigma), 1:1000.

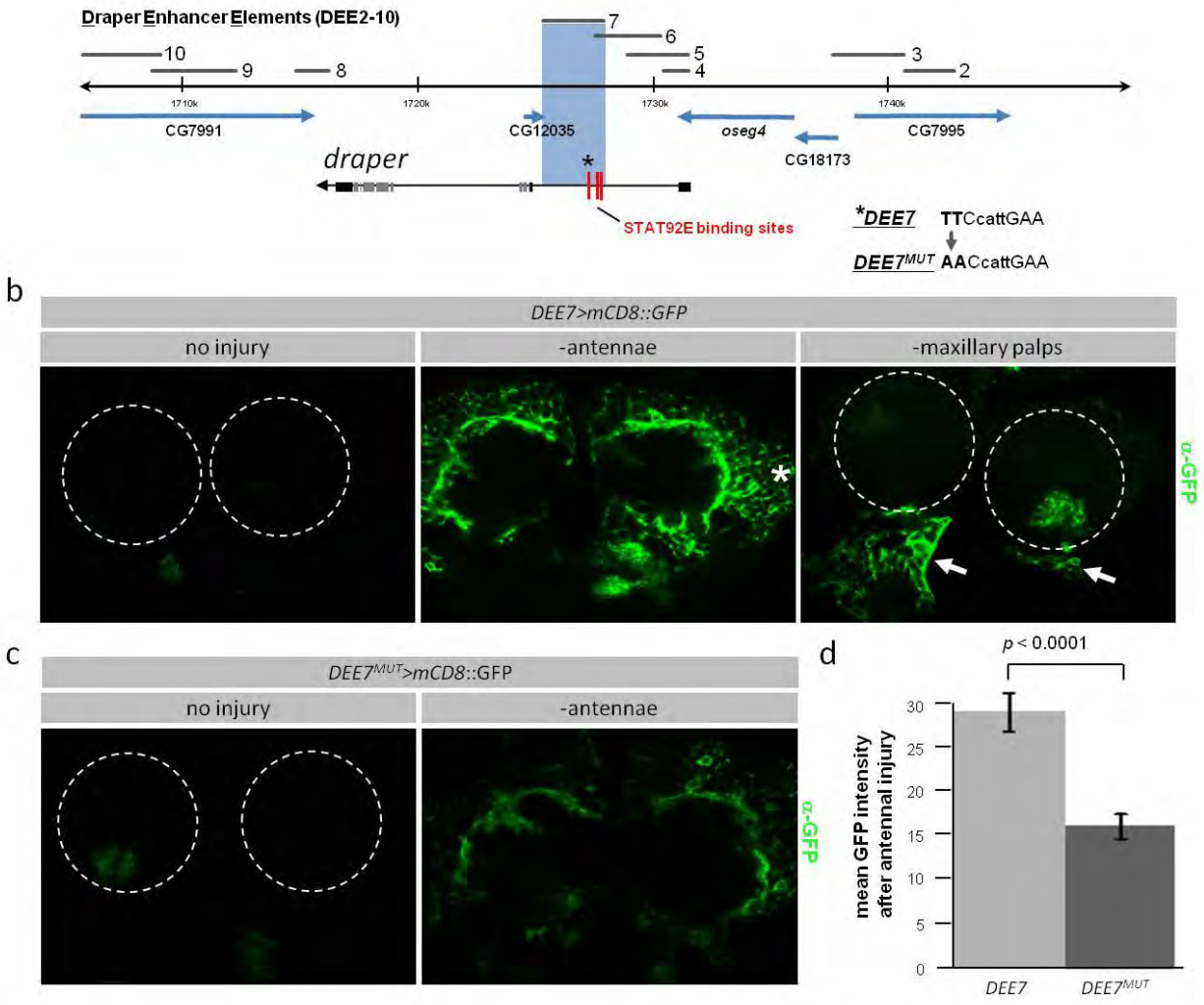


Figure 4-1. The *draper* locus contains a Stat92E-regulated injury responsive element

(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 in DEE7. $DEE7^{MUT}$ contains two point mutations in Stat92E binding site.

(b,c) Single slice confocal images of antennal lobe regions (b) *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. Asterisk, cortex glia responding to axotomy. Arrows, areas in ventral region of antennal lobe responding to maxillary palp injury. (c) $DEE7^{MUT}$ *–Gal4* driving two copies of *UAS-mCD8::GFP*. Injuries as indicated in (b). (d) Quantification for b and c. *p* values were calculated using Student's t-test. Error bars represent s.e.m, n≥17 for both.

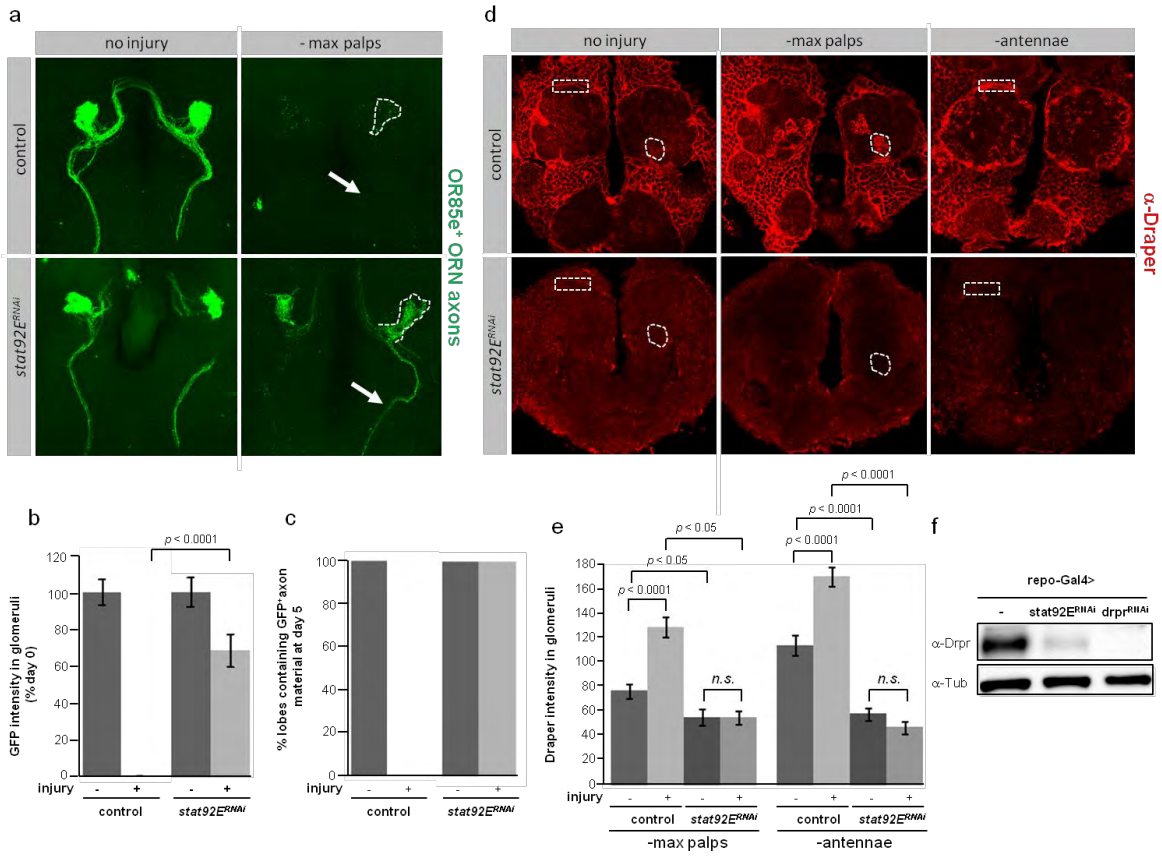


Figure 4-2. Stat92E functions in glia to promote axon clearance and Draper expression

(a) Z-stack confocal images; *repo-Gal4* was used to drive *UAS-stat92E^{RNAi}* and OR85e^{+} axons were labeled with GFP (*OR85e-GFP*) in control (*OR85e-GFP/+; repo-gal4/+)* or *stat92E^{RNAi}* (*stat92E^{RNAi}/OR85e-GFP;repo-Gal4*/+) backgrounds. Uninjured, and 5 days after maxillary palp ablation are shown. Dotted lines indicate 85e^{+} glomerulus used for quantification of GFP remaining. Arrows indicate position of maxillary nerves. (b) Quantification of GFP intensities in 85e^{+} glomeruli from (a). *p*-values were calculated using Student's t-test. Error bars represent s.e.m, n≥10 (c) Quantification of maxillary nerves with GFP+ axon fibers from (a), n≥10. (d) Single slice confocal images; Draper antibody stains in adult brain of control (*repo-gal4*/+) or *stat92E*^{RNAi} (*stat92E*^{RNAi}/+;*repo-gal4*/+) backgrounds; no injury, one day after maxillary palp injury (-maxillary palp) and, one day after antennal ablation (-antenna). Dotted circles and boxes represent regions used for quantification of Draper immunoreactivity. (e) Quantification of data from (d). *p*-values were calculated using one -way ANOVA followed by Bonferroni multiple comparison test, *n.s.,* not significant. Error bars represent s.e.m., n≥10. (f) α-Draper western blot of indicated genotypes. α-Tubulin used as a loading control.

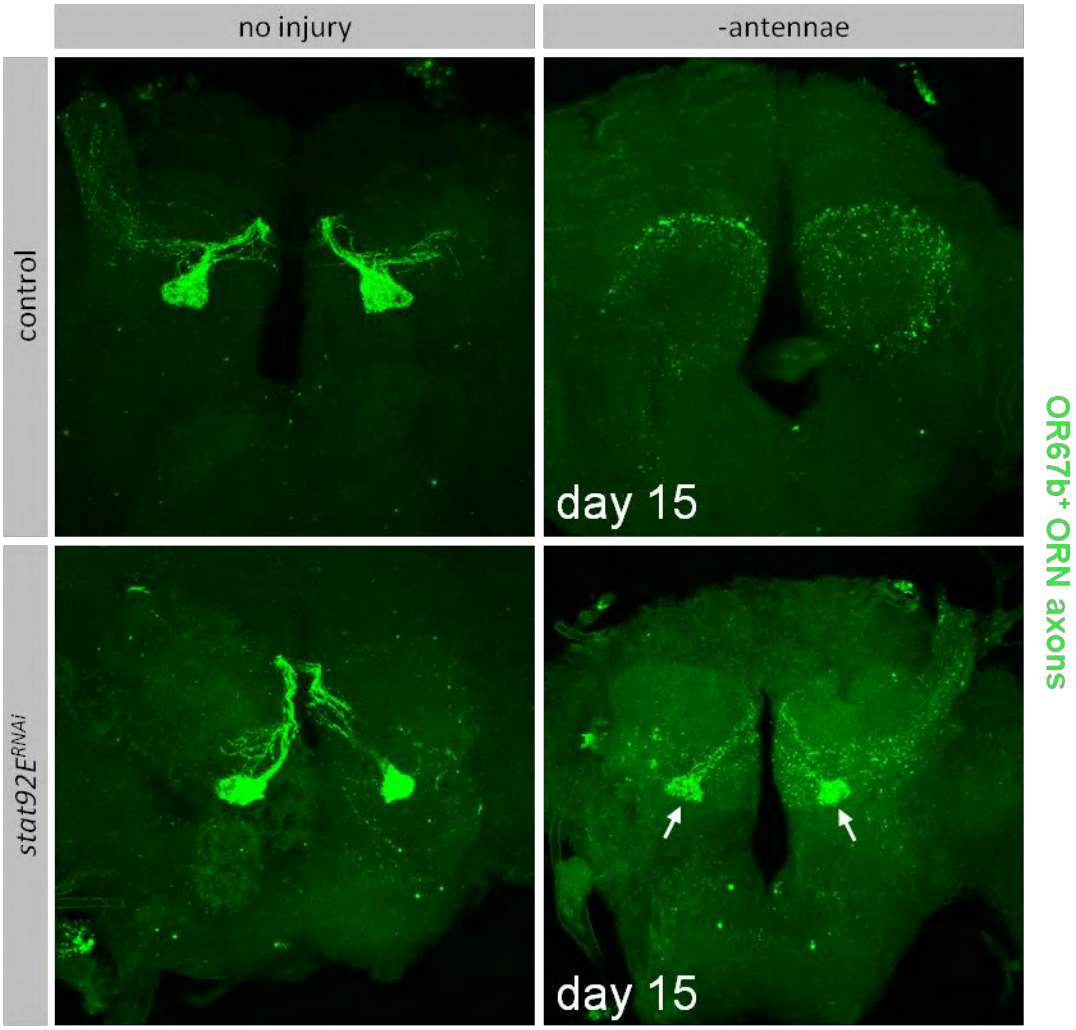


Figure 4-3. Axons persist for 15 days after antennal ablation in a Stat92E knockdown animal

(a) Confocal z-stack images of indicated genotypes, control (*OR67b-GFP/+;repo-gal4/+*) and *stat92E*^{RNAi} (*OR67b-GFP/UAS-stat92E*^{RNAi}*;repo-gal4/+*) with no injury and 15 days after antennal ablation.

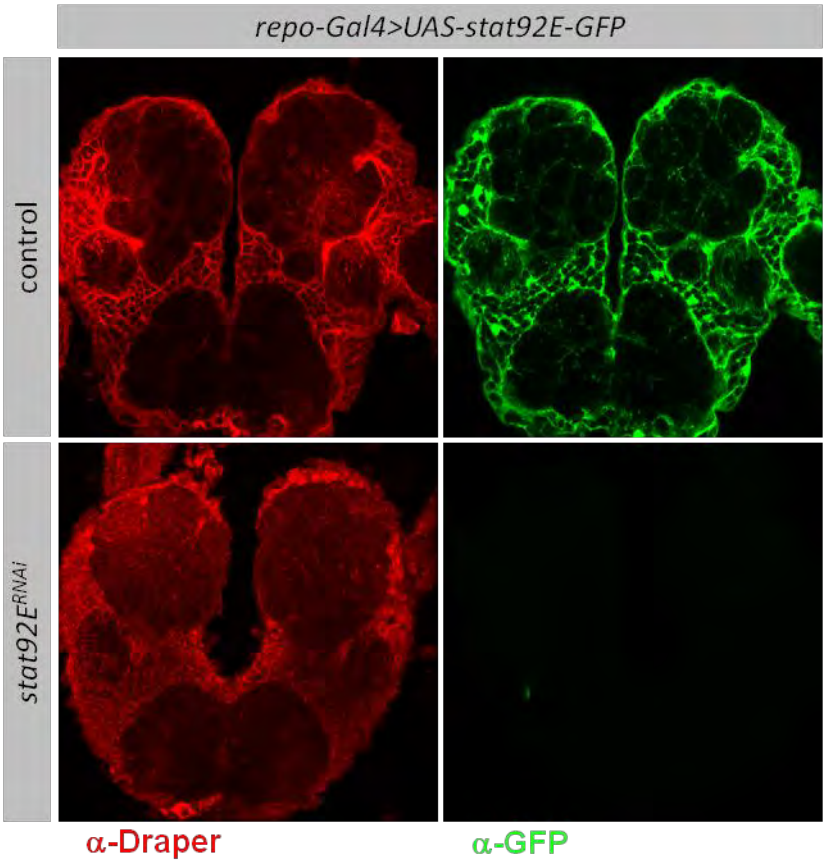


Figure 4-4. *UAS-Stat92E*^{RNAi} efficiently targets *stat92E*
(a) Single slice confocal images of indicated genotypes, control (*UAS-Stat92E-GFP/repo-gal4*) *stat92E*^{RNAi} (*UAS-stat92E*^{RNAi}; *UAS-stat92E-GFP/repo-gal4*). Glial specific *stat92E*^{RNAi} completely knocks down overexpressed Stat92E::GFP and leads to a reduction in Draper levels.

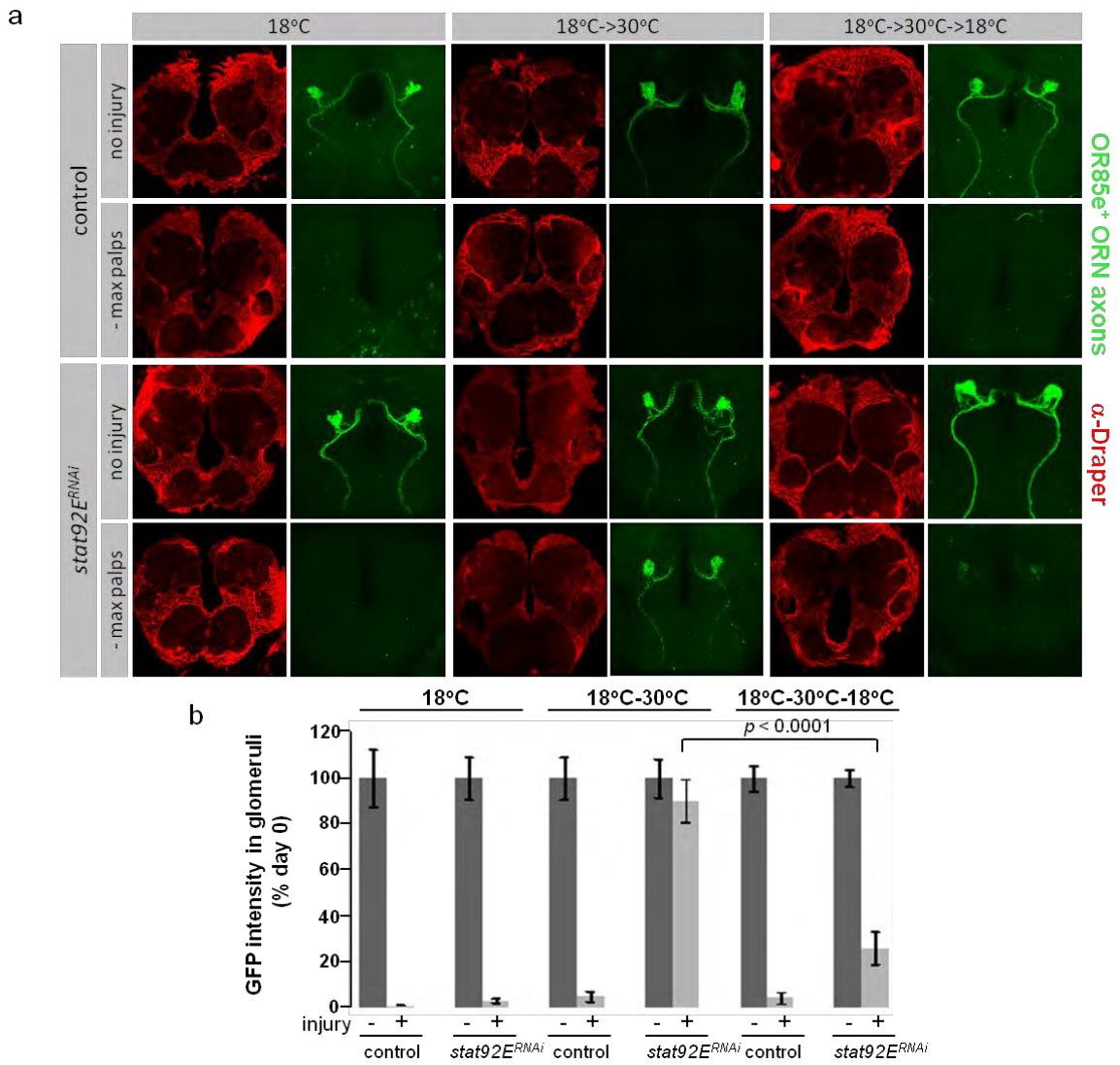


Figure 4-5. Stat92E is required in the adult brain for Draper expression and glial engulfment functions

(a) Z-stack confocal images of OR85e-GFP axons, single slice confocal images of Draper antibody stain; *UAS-Stat92E*^{RNAi} was driven in all glia using *repo-Gal4* in a background containing the temperature sensitive Gal4 repressor, Gal80^{ts}. Axons (OR*85e-GFP*, green) and Draper levels (red) are shown in control (*OR85e-GFP,Gal80*^{ts}*/+;repo-gal4/+)* or *Stat92E*^{RNAi} knockdown (*OR85e-GFP,Gal80*^{ts}*/Stat92E*^{RNAi}*;repo-Gal4*/+) animals. Temperature shifts were performed as follows: 18°C-30°C indicates that flies were raised at 18°C and then shifted to 30°C as adults for 7 days prior to and 5 days following maxillary palp ablation. 18°C-30°C-18 °C indicates that flies were raised at 18°C, shifted to 30°C as adults for 7 days, and then shifted back to 18°C for 7 days prior to and 5 days following maxillary palp ablation. (b) Quantification of mean intensity of GFP remaining in glomeruli five days after injury. *p*-value was calculated using one-way ANOVA followed by Student's t-test. Error bars represent s.e.m., n≥10.

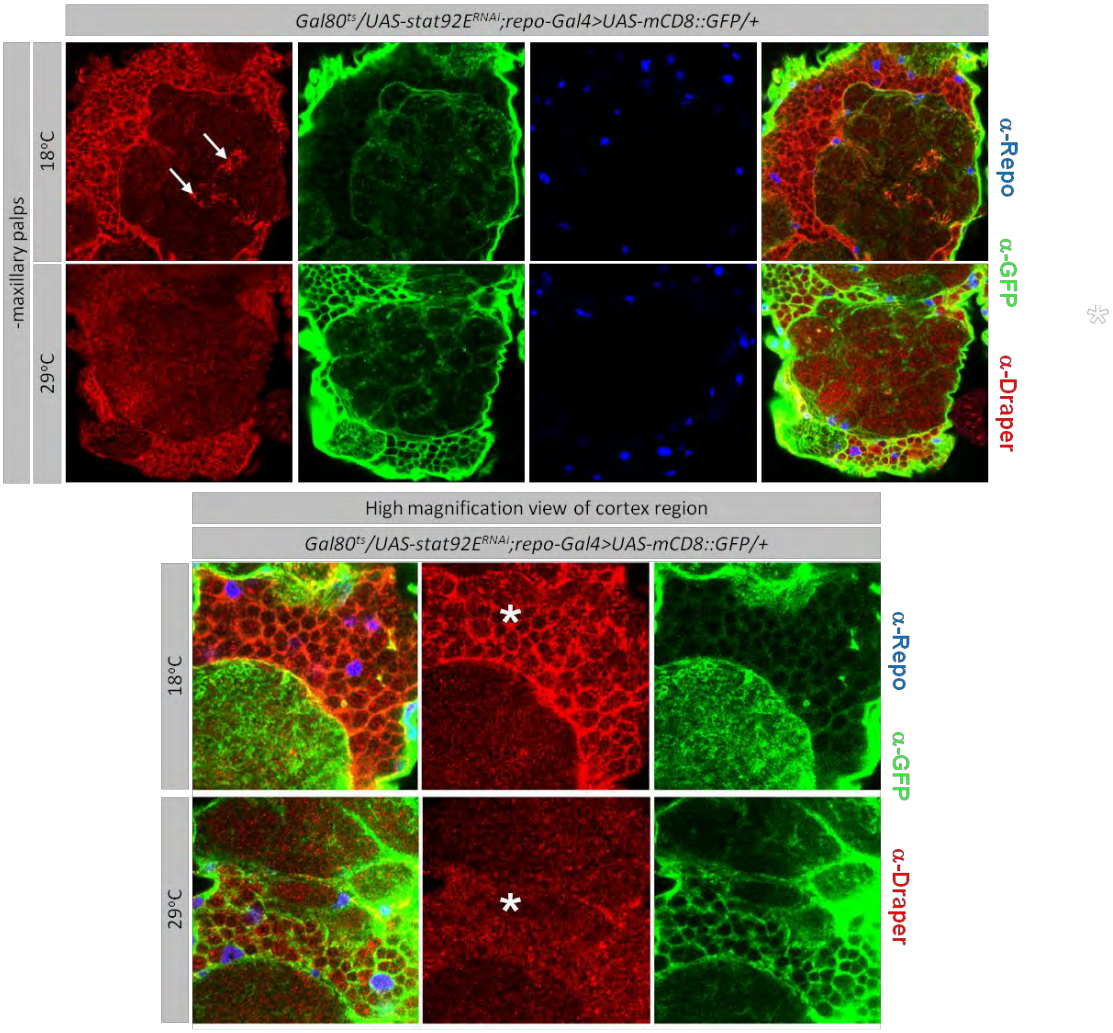

Gal80ts/UAS-stat92ERNAi;repo-Gal4>UAS-mCD8::GFP/+
-maxillary palps
18°C
29°C
α-Repo
α-GFP
α-Draper
High magnification view of cortex region
Gal80ts/UAS-stat92ERNAi;repo-Gal4>UAS-mCD8::GFP/+
18°C
29°C
α-Repo
α-GFP
α-Draper

Figure 4-6

Figure 4-6. Glial morphology and cell number appear normal in a *stat92E*^{RNAi} animal

(a) Single slice confocal images of adult brains of indicated genotype, (UAS-*stat92E*^{RNAi}*/+;repo-Gal4,UAS-mCD8::GFP/+)* one day after maxillary palp injury. Glial nuclei are labeled with anti-Repo (blue), glial membranes are labeled with GFP (green) and anti-Draper staining is shown (red). Flies were raised at 18^{o}C and either kept at 18^{o}C throughout the experiment or shifted to 29^{o}C for 7 days prior to dissection. At the restrictive temperature of 18^{o}C some *repo-Gal4* driven GFP is detectable but Draper staining looks grossly normal and glia are able to respond to injury one day after maxillary palp ablation. In flies shifted to the restrictive temperature, Draper staining is significantly reduced and no glial membranes are recruited to severed axons one day after maxillary palp injury. However, glial cell nuclei and membranes appear grossly normal in the *stat92E*^{RNAi} flies. (b) Single slice confocal image of adult brain, high magnification view of cortex region of the brain. Asterisk indicates that while Draper is absent in the *stat92E*^{RNAi} animals, glial morphology is normal.

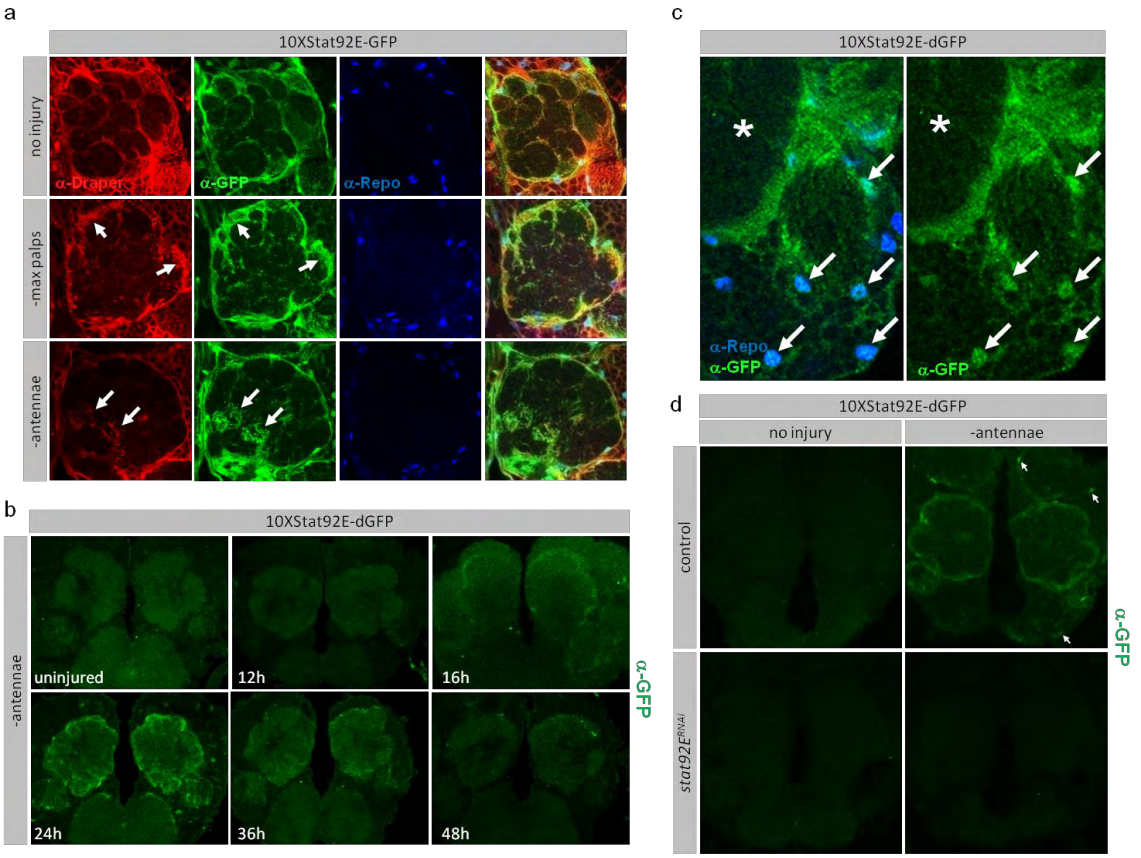


Figure 4-7. Stat92E transcriptional reporters are expressed in glia and are transiently activated after axotomy

(a-d) Single slice confocal images; (a)*10XStat92E-GFP* reporter expression in adult brain in uninjured control animals, or after severing antennal or maxillary palp ORN axons. Glial nuclei are labeled with α-Repo (blue), glial membranes with α-Draper (red), and STAT transcriptional reporter activity with α-GFP (green). (b) Timecourse of activation of the *10XStat92E-dGFP* (destabilized GFP) reporter following antennal ablation. (c) High magnification image showing colocalization of Repo^{+} positive glial nuclei (arrows) and *10XStat92E-dGFP* reporter activity in cortex glial cells following antennal ablation. Asterisks mark antennal lobe region. (d) The pan-glial *repo-Gal4* driver was used to drive *UAS-Stat92E*^{RNAi}. Single slice confocal images of control (*10XStat92E-dGFP*/+;*repo-gal4/+)* or *stat92E*^{RNAi} (*UAS-stat92E*^{RNAi}*/10XStat92E-dGFP; repo-Gal4*/+) brains assaying *10XStat92E-dGFP* activation (green) before and after injury.

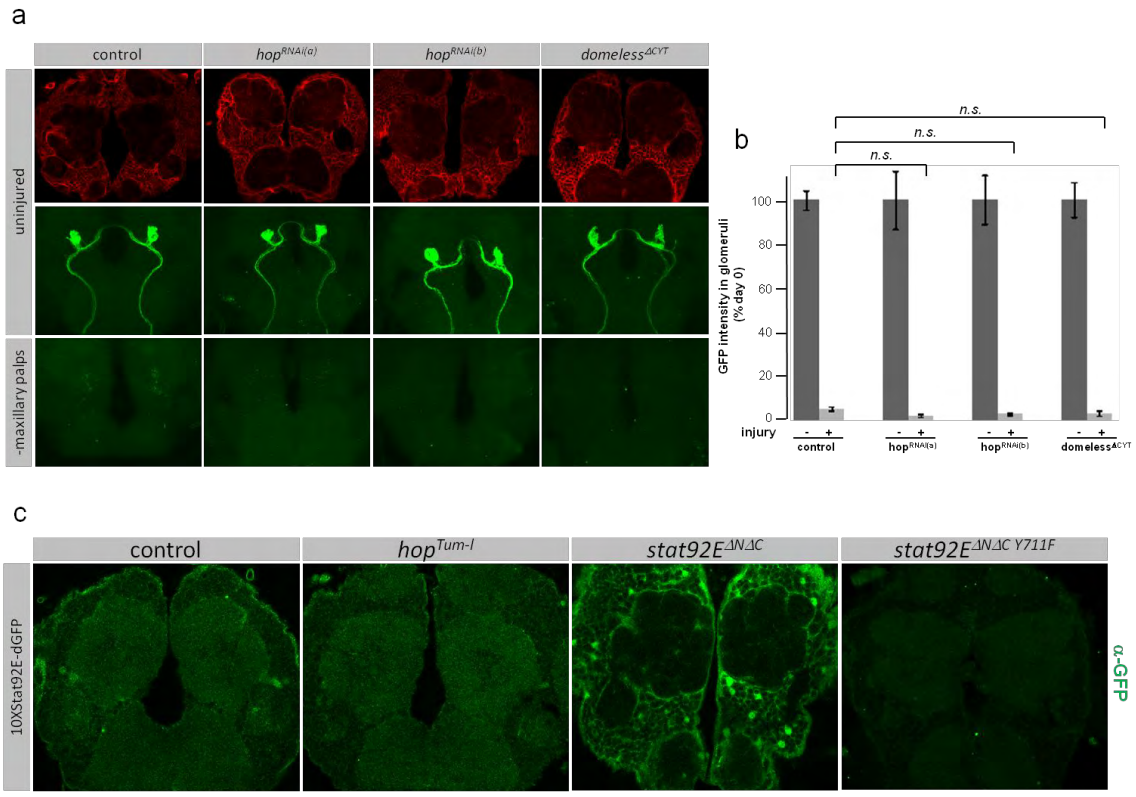


Figure 4-8. Glial activation of Stat92E activity after axotomy is not mediated by canonical JAK/STAT signaling

(a) *repo-Gal4* was used to drive two independent UAS-*hop*^{RNAi} constructs and a UAS dominant negative domeless allele; single slice confocal images of Draper antibody stain and Z-stack confocal images of OR85e-GFP^{+} axons in control (OR85e-GFP/ *hop*$^{RNAi(a)}$;*repo-Gal4*/+), *hop*$^{RNAi(a)}$ (OR85e-GFP/+;*repo-Gal4*/+) *hop*$^{RNAi(b)}$ (OR85e-GFP/*UAS- hop*$^{RNAi(b)}$;*repo-Gal4*/+), *domeless* $^{\Delta CYT}$ (OR85e-GFP/*UAS-domeless*$^{\Delta CYT}$;*repo-Gal4*/+) (b) Quantification of data from (a). *p*-value was calculated using one-way ANOVA followed by Bonferroni multiple comparison test, *n.s.,* not significant. Error bars represent s.e.m., n≥10. (c) Single slice confocal images of indicated genotype in *10XSTAT-dGFP* background; control (*10XStat92E-dGFP*/+;*repo-Gal4/+), hop*$^{Tum-1}$(*hop*$^{Tum-1}$ */X;10XStat92E-dGFP*/+;*repo-Gal4*/+),*UAS-stat92E*$^{\Delta N\Delta C}$ (*10XStat92E-dGFP*/+;*repo-Gal4/UAS-stat92E*$^{\Delta N\Delta C}$), *UAS-stat92E*$^{\Delta N\Delta CY711F}$ (*10XStat92E-dGFP/ UAS-stat92E*$^{\Delta N\Delta CY711F}$;*repo-Gal4*/+)

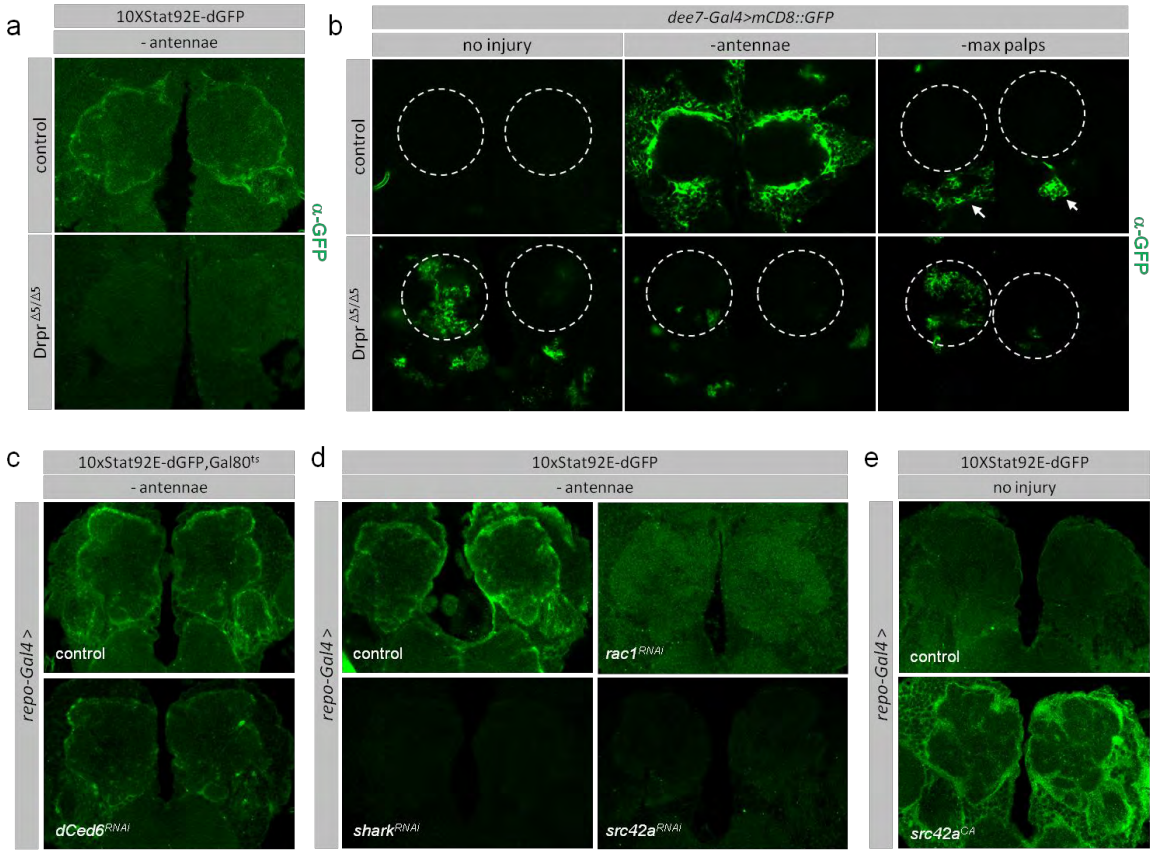

a
10XStat92E-dGFP
- antennae
control
Drpr Δ5/Δ5
α-GFP
b
dee7-Gal4>mCD8::GFP
no injury
-antennae
-max palps
control
Drpr Δ5/Δ5
α-GFP
c
10xStat92E-dGFP,Gal80ts
- antennae
repo-Gal4 >
control
dCed6RNAi
d
10xStat92E-dGFP
- antennae
repo-Gal4 >
control
rac1RNAi
sharkRNAi
src42aRNAi
e
10XStat92E-dGFP
no injury
repo-Gal4 >
control
src42aCA

Figure 4-9

Figure 4-9. Injury-induced activation of Stat92E is downstream of the Draper signaling cascade

(a,b,d) Single slice confocal images; (a) *10XStat92E-dGFP* reporter activity in the indicated genotypes before or after antennal ORN axotomy. Control (*10XStat92E-dGFP/+*) and Draper$^{\Delta 5/\Delta 5}$ (*10XStat92E-dGFP/+, draper$^{\Delta 5}$/ draper$^{\Delta 5}$*). (b) *dee7-Gal4* activity in the indicated genotypes before or after ORN axotomy. Control (*dee7-Gal4,UAS-mCD8::GFP/+*) and Draper$^{\Delta 5/\Delta 5}$ (*DEE7-Gal4,UAS-mCD8::GFP/+*, *draper$^{\Delta 5}$/ draper$^{\Delta 5}$*). Dotted circles indicate antennal lobes. (c) Glial-specific knockdown of dCed6 in the *10XStat92E-dGFP* reporter background. A *UAS*-regulated RNAi construct targeting *dCed6* was driven by *repo-Gal4*, flies were raised at the restrictive temperature of 18^{o}C to keep the RNAi off during development and shifted to the permissive temperature of 30^{o}C upon eclosion, antennal ORN axons were axotomized, and reporter activity was measured 1 day later. Control (*10XStat92E-dGFP,Gal80^{ts}/+;repo-Gal4/+*) and *dCed6^{RNAi} (10XStat92E-dGFP,Gal80^{ts}/ dCed6^{RNAi};repo-Gal4),* (d) Glial-specific knockdown of components of the *draper* pathway in the *10XStat92E-dGFP* reporter background. *UAS*-regulated RNAi constructs targeting *src42a*, *shark* and *rac1* were driven by *repo-Gal4*, antennal ORN axons were axotomized, and reporter activity was measured 1 day later. Control (*10XStat92E-dGFP/+*; *repo-Gal4/+*), *src42a^{RNAi}* (*src42a^{RNAi}/10XStat92E-dGFP; repo-Gal4/+*), *rac1^{RNAi}* (*rac1^{RNAi}/10XStat92E-dGFP; repo-Gal4/+*), *shark^{RNAi}* (*shark^{RNAi}/ 10XStat92E-dGFP; repo-Gal4/+*). (e) Glial-specific overexpression of constitutively active *src42a* in the *10XStat92E-dGFP* reporter background. Control (*10XStat92E-dGFP/+*; *repo-Gal4/+*), *src42a^{CA}* (*src42a$^{const\ active}$/10XStat92E-dGFP; repo-Gal4/+*).

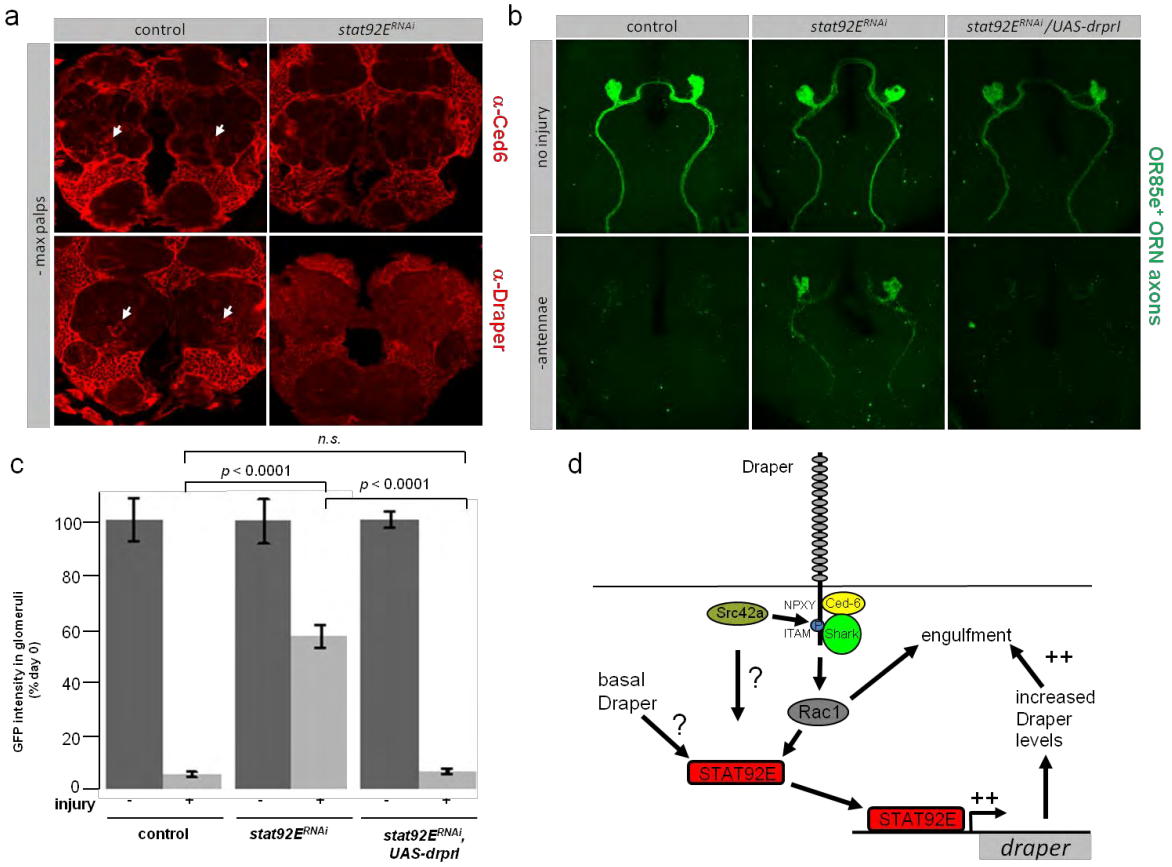

a
control
stat92E^RNAi
- max palps
α-Ced6
α-Draper
b
control
stat92E^RNAi
stat92E^RNAi/UAS-drprl
no injury
-antennae
OR85e+ ORN axons
c
n.s.
p < 0.0001
p < 0.0001
GFP intensity in glomeruli (% day 0)
100
80
60
40
20
0
injury
-
+
control
stat92E^RNAi
stat92E^RNAi, UAS-drprl
d
Draper
Src42a
NPXY
ITAM
Ced-6
Shark
engulfment
++
basal Draper
?
?
Rac1
increased Draper levels
STAT92E
STAT92E
++
draper

Figure 4-10. Stat92E regulates a subset of engulfment genes, and *draper* is a critical target

(a) Single slice confocal images; *repo-Gal4* was used to drive *UAS-Stat92E*^{RNAi} in a *Gal80*^{ts} background. *Stat92E*^{RNAi} was activated in adults by growing animals at 18^{o}C and shifting them as adults to 30^{o}C for 7 days. Draper and dCed6 levels were determined by antibody staining in uninjured animals, and one day after maxillary palp injury.
(b) Z-stack of confocal images; *repo-Gal4* was used to drive *UAS-Stat92E*^{RNAi} in the presence or absence of *UAS-Draper-I* in a background containing the temperature sensitive Gal4 repressor, Gal80^{ts}. All flies were raised at 18^{o}C and shifted to 30^{o}C as adults for 7 days prior to injury or dissection. OR85e^{+} axons were labeled with GFP in the indicated genetic backgrounds, and GFP levels were determined in uninjured animals and 5 days after axotomy. Control (*OR85e-GFP,Gal80*^{ts}/+;*repo-gal4/+), stat92E*^{RNAi} knockdown (*OR85e-GFP,Gal80*^{ts}*/Stat92E*^{RNAi};*repo-Gal4*/+), and *stat92E*^{RNAi} */UAS-draper-I*(*OR85e-GFP*,*Gal80*^{ts}*/stat92E*^{RNAi};*repo-Gal4*/*UAS-draper-I*). (c) Quantification of data from (b). *p*-values were calculated using one-way ANOVA followed by Student's t-test, *n.s.,*not significant. Error bars represent s.e.m., n≥10. (d) Proposed model for the Draper→Stat92E→*draper* signaling pathway. Upon activation of the Draper receptor by an unknown "eat me" cue, Stat92E is activated through the Src family kinase signaling cascade Src/Shark and Rac1 and transcriptionally activates the *draper* gene. Increased Draper levels likely enhance the ability of glia to engulf axonal debris. Stat92E also appears to regulate basal levels of Draper, based on the loss of Draper prior to injury in Stat92E^{RNAi} animals, but through an unidentified mechanism independent of Draper signaling.

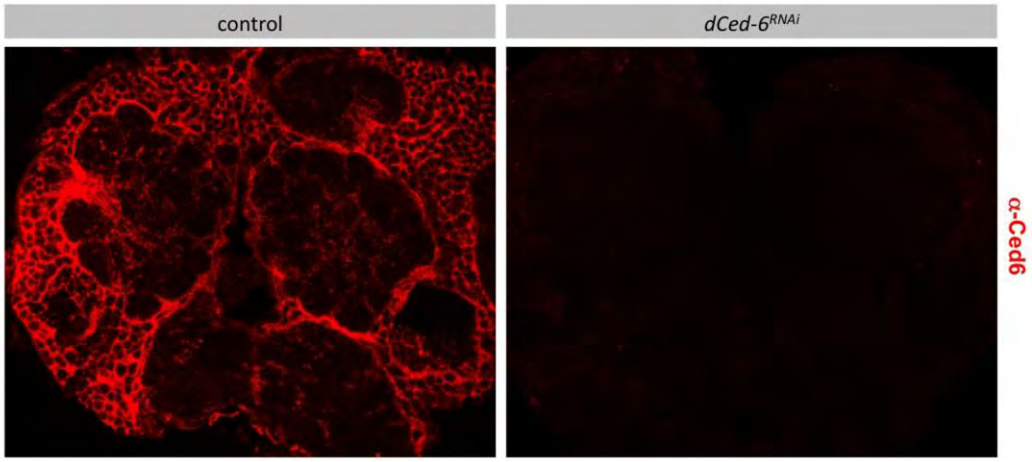


Figure 4-11. *dced-6*^{RNAi} efficiently knocks down dCed-6 protein levels
(a) Single slice confocal images of the adult brain showing anti-dCed6 protein levels. Control, (*Gal80*^{ts}*/+ ; repo-gal4/+*), *dced-6*^{RNAi} (*Gal80*^{ts}*/dCed-6*^{RNAi}*;repo-gal4/+*).

CHAPTER V: Discussion

In this dissertation, I explore the cellular and molecular biology of glial cells in the adult *Drosophila* brain with a primary focus on the mechanisms by which they engulf and eventually clear axonal debris after injury. Intrinsic to the use of invertebrate models in research is the assumption that the findings will provide relevant insight into the workings of vertebrate systems. Despite some of the more obvious differences between *Drosophila* and vertebrate glia (i.e., lack of myelination, different specification mechanisms, and far fewer glial cells relative to neurons in *Drosophila*), evidence reveals that the fundamental aspects of glial cellular and molecular biology are strikingly similar between vertebrates and flies.

Cellular Dissection of Glial Responses to Injury

Ensheathing glia in the adult brain express Draper and engulf degenerating axon material

In chapter two of my thesis I identify and characterize three morphologically and functionally distinct subtypes of glial cells in the adult central brain: cell body, ensheathing, and astrocyte-like glia. I show that the ensheathing glia are the primary cell type responsible for the clearance of degenerating axon debris in the neuropil regions of the antennal lobe and that clearance is mediated through the Draper pathway. Interestingly, in previous studies, surface glia have been shown to engulf cell corpses during embryonic development (Sonnenfeld and Jacobs, 1995), and astrocyte-like glia and cell body glial cells were shown to engulf pruned axon material during larval

development (Awasaki et al., 2011). Thus, the phagocytic competence of glial cells appears to be developmentally and/or context specific. However, a common feature in all of these engulfment events is that they utilize the Draper signaling pathway. While Draper is expressed in astrocytes during development, we could not detect any Draper expression in adult astrocyte-like glial cells, nor could we find any role for astrocytes in the engulfment of degenerating axon debris in the adult brain. Astrocyte-like glial cells displayed no obvious morphological changes after injury and inhibition of Dynamin-dependent endocytic events as well as knockdown of the Draper receptor in these cells had no effect on axon clearance. This strongly argues that *Drosophila* astrocytes in the adult brain are not directly involved in phagocytosing neuronal debris and have little or no influence on the engulfment functions of the ensheathing cells.

In mammals, astrocytes undergo a broad spectrum of molecular, cellular, and functional changes in response to all types of CNS damage, even subtle insults (Sofroniew and Vinters, 2010). In response to some forms of trauma, astrocytes exhibit phagocytic competence (Nguyen et al., 2011; Persson and Englund, 2011; Sokolowski et al., 2011). Furthermore, it has been proposed that astrocytic phagocytosis may represent a compensatory mechanism implemented once microglial cells have reached the limits of their phagocytic capacity (Magnus et al., 2002). The severity of reactive astrogliosis depends largely on the extent and type of trauma and can range from minor changes in morphology with little or no increase in gene expression, termed isomorphic astrogliosis, to hypertrophy, increased gene expression, proliferation, and glial scar formation, termed anisomorphic astrogliosis (Rodriguez et al., 2009; Sofroniew and Vinters, 2010). Lesions

which are associated with breakdown of the blood brain barrier generally lead to ansiomorphic astrogliosis, while those caused by toxins, chemical insults, or Wallerian degeneration, resulting in selective damage of neurons, result in isomorphic astrogliosis (Bignami and Dahl, 1976; Bignami and Ralston, 1969; Mansour et al., 1990). Considering the developmental role of astrocytes during axon pruning in *Drosophila,* and the crucial roles astrocytes play in immune functions in the mammalian nervous system, including phagocytosis, it was surprising to find that in our assay, inhibition of astrocytes had no effect on the engulfment of degenerating axon material.

A potential role for astrocytes in injury responses

While our data argue against a direct role for astrocytes in clearance of axon debris, there is still the possibility that they play a more subtle and indirect role in glial responses to injury within the nervous system. Astrocytes are highly branched cells which densely infiltrate the neuropil, and while we could not detect any obvious topographical changes in astrocytes upon axotomy, our assay of nerve injury does not lead to a breach of the blood brain barrier, therefore it is likely astrocytes may exhibit subtle changes which were beyond our resolution of imaging. In the mammalian brain, astrocytes —tile" with themselves and form unique domains exhibiting very little interdigitation with each other (Bushong et al., 2004; Haber and Murai, 2006). Interestingly, this tiling is also characteristic of astrocytes in the *Drosophila* embryo (Dr. Tobias Stork, personal communication).

Several mammalian studies suggest that reactive astrocytes undergo hypertrophy and invade each other‘s domains (Kimelberg and Norenberg, 1989; McGraw et al., 2001; Silver and Miller, 2004; Sofroniew, 2005). However, a high resolution, three-dimensional assessment of non-reactive and reactive astrocytes in the lesioned cortex and denervated dentate gyrus of the mouse shows that both the overlap of domains, as well as the overall size, of non-reactive and reactive astrocytes, is similar. The only morphological change detected among reactive astrocytes was hypertrophy of the main cellular processes (Wilhelmsson et al., 2006). Inconspicuous morphological changes such as these could have gone undetected in our assay, as identification of individual astrocytes or changes in the sizes of individual branches could not be distinguished. In mammals, the physiological implications of such subtle astrocytic changes remain elusive.

A definitive way to determine if astrocytes play a critical role in injury responses would be to ablate the astrocytes and assay for clearance of degenerating axons. However, overexpression of the pro-apoptotic Hid or Reaper molecules in astrocytes during development results in lethality (Dr. Tobias Stork, personal communication) and adult-specific expression of Hid or Reaper is not as effective at inducing apoptosis. To further investigate the role of astrocytes in injury responses, it would be interesting to examine the effects of impaired astrocyte signaling, using a temperature sensitive Dynamin construct, on the injury-induced activation of the *10xStat92E-dGFP* reporter and the *dee7*-Gal4 reporter. These tools may allow for better detection of subtle changes

in the extent of glial reactivity, based on transcriptional activation of genes, which are not sufficient to affect the final outcome of axon clearance.

Additionally, it is possible that astrocytes exert their influence over injury responses through both Dynamin and Draper independent mechanisms. In our assay we utilized a temperature sensitive Dynamin construct to block endocytic activity of astrocytes and then assayed clearance of degenerating axons as a read-out for astrocytic contributions to injury responses. We were unable to detect any effect of impaired astrocyte signaling on the clearance of degenerating axons. However, at the time of the experiment it was assumed that inhibiting Dynamin activity would effectively block all endocytic events and essentially prevent astrocytic communication with other cells. Recent evidence from mammals revealed a Dynamin-independent endocytic pathway in astrocytes which is regulated by Rab5. This pathway is tightly controlled through adenosine triphosphate (ATP) and glutamate dependent regulation of intracellular calcium levels (Jiang and Chen, 2009).

Contrary to the previous notion that only those cells in the immediate vicinity of the area respond to injury in the *Drosophila* brain, I showed, using the *10XStat92E-dGFP* and the *dee7*-Gal4 reporters, that glial cells throughout the entire brain respond to large scale injuries such as antennal ablation. In mammals, astrocytes are coupled with neighboring astrocytes through gap junctions forming large networks (Brightman and Reese, 1969). In the presence of injury, ATP released from surrounding tissue activates purinergic receptors on astrocytes and this in turn leads to ATP-induced ATP release mediated by gap junctions composed of connexin hemichannels (Anselmi et al., 2008;

Cotrina et al., 1998; Cotrina et al., 2000; Stout et al., 2002). ATP release through gap junctions leads to the propagation of calcium waves which allows for astrocytes to communicate over long distances and modulate the activity of adjacent cells. It has been shown both *in vitro* and *in vivo* that extracellular ATP is necessary for microglial activation and migration to sites of injury (Davalos et al., 2005; Honda et al., 2001; Inoue, 2002; Samuels et al., 2010). Furthermore, local injection of ATP into the cerebral cortex of a mouse was sufficient to induce robust microglial activation. Interestingly, inhibition of connexin channels, which are highly expressed on astrocytes, inhibits microglial activation and migration in the presence of injury (Davalos et al., 2005). These data indicate that it is possible that astrocytes, through the release of ATP and propagation of calcium waves, communicate with and activate microglial cells in the presence of injury. This model also offers an explanation for graded responses to injury as the severity of the insult determines the initial amount of ATP released into the extracellular space and ultimately, via astrocytic ATP release and calcium wave propagation, would regulate the extent of microglial activation both in terms of area and duration. Furthermore, it is possible that astrocytes communicate with microglia in an ATP- and calcium- independent manner through the release of cytokines, growth factors, or other secreted molecules.

Alternatively, the signal given off by injured axons could act as a direct, long-range, diffusible cue capable of activating glial cells far from the site of injury without any involvement of astrocytes. To date only one ligand for the Draper receptor has been identified, Pretaporter. This molecule resides in the endoplasmic reticulum and is

relocated to the cell surface of apoptotic cells through a caspase-dependent mechanism. Pretaporter does not act as the ligand for Draper in the context of developmental axon pruning and, based on the caspase-dependent activation of this molecule in apoptotic cells, it is unlikely to be the ligand produced by degenerating axons (Kuraishi et al., 2009). To gain a better understanding of neuron-to-glia and glia-to-glia signaling mechanisms, future studies should focus on identifying the injury-induced ligand for Draper and developing more sensitive assays to determine the specific contributions of the different glial subtypes in the presence of trauma.

The potential of subtype-specific glial Gal4 driver lines

The identification of glial subtype-specific drivers, *mz0709*-Gal4 and *alrm*-Gal4, will undoubtedly pave the way for more detailed analysis of the functions and molecular profiles of ensheathing glial cells and astrocyte-like glial cells respectively. Future studies of ensheathing glial cells in the context of phagocytosis will likely provide valuable insight into the molecular mechanisms used by vertebrate microglial cells during injury responses and in the context of neurodegenerative disease. With the powerful array of genetic tools available in *Drosophila*, it is possible to differentially label and manipulate two distinct populations of cells at the same time (Awasaki and Lee, 2011; Potter and Luo, 2011). In recent years, a number of *Drosophila* models of neurodegenerative disorders and diseases have been established including Parkinson‘s disease, Alzheimer‘s disease, spinocerebellar ataxia, Huntington‘s disease, glioma, and epilepsy (Feany and Bender, 2000; Jackson et al., 1998; Marley and Baines, 2011; Read

et al., 2009; Warrick et al., 1998; Ye et al., 1999). Most of these studies have focused on the cell-autonomous neuronal dysfunction caused by overexpression of mutant genes. However, it is clear from vertebrate studies that both microglia and astrocytes play crucial roles which influence the prognosis of trauma to the nervous system and the progression of several neurodegenerative diseases (Berry et al., 2001; Ramaglia et al., 2012; Sheng et al., 1995; Sofroniew and Vinters, 2010; Stefanova et al., 2011). Through differential labeling and manipulation of neurons and glial cells, it will be possible to observe, at single-cell resolution even, the responses of glial cells to various forms of neuronal insult and dysfunction. Another powerful technique, which can be used in concert with the glial-specific subtype drivers to provide valuable molecular insight into astrocyte-like glia and ensheathing glial cells, is the method of TU (4-Thiouracil/ Uracil phosphoribosyltransferase) tagging, which allows for cell type-specific RNA isolation from intact tissues (Miller et al., 2009). This method would allow for the identification of molecules enriched in astrocytes or ensheathing cells and furthermore, manipulations could be performed which would allow for analysis of RNA from tissue both before and after injury as well as at different stages of development.

Molecular Dissection of Glial Responses to Injury

An *in vivo* RNAi screen to identify glial genes involved in engulfment of degenerating axon material

In the third chapter of my thesis I describe a candidate based *in vivo* RNAi screen to identify molecules required for the glial engulfment of degenerating axons. The assays used in this screen have several advantages. First, axon degeneration events induced by the injury are conserved as shown by the inhibition of axon degeneration in *Drosophila* expressing the Wld^{s} gene. Second, using simple genetic techniques we can visualize both the degenerating axons as well as the glial membranes allowing us to assay two outputs at once. And finally, the *in vivo* approach allows for an accurate readout of physiologically relevant findings.

Many of the genes that I chose as candidates for this screen were identified based on evidence from mammalian literature of their involvement in phagocytosis and reactive gliosis. One caveat associated with candidate-based screening approaches, is that they generally exclude the possibility of finding novel genes. The majority of mammalian studies on reactive gliosis have been performed in cell culture where the complexity of the intact nervous system cannot be reproduced. While these studies have been relatively successful at identifying genes differentially regulated in reactive astrocytes and microglial cells, they are limited in their ability to further analyze the functional implications of these genes. The studies I performed in *Drosophila* have the potential to both confirm results stemming from studies performed in mammalian cell culture lines as well as uncover the functional relevance of these genes in the context of reactive gliosis.

As a result of this screen, I identified twenty genes involved in glial clearance of degenerating axons. The phenotypes resulting from the RNAi knockdown of genes in glial cells could be classified into four main phenotypic classes: 1) axon pathfinding

defects, 2) impaired axon clearance, 3) loss of basal Draper and, 4) loss of both basal and injury-induced Draper expression. While *in vivo* RNAi screens can present the caveat of off-target effects, several of the identified genes have been confirmed by other non-RNAi methods and ten of the identified genes are currently being investigated by myself and other members of the lab.

Transcriptional mechanisms controlling axotomy-induced Draper upregulation

As a result of the *in vivo* RNAi screen described in chapter three, I identified Stat92E as being necessary for the efficient clearance of degenerating axons. However, complicating this result was the fact that Stat92E knockdown also resulted in decreased levels of both basal as well as injury-induced Draper protein. In previous studies, we showed that Draper is necessary for the clearance of degenerating axons (Doherty et al., 2009; MacDonald et al., 2006; Ziegenfuss et al., 2008). Therefore, it was possible that knockdown of *stat92E* indirectly resulted in an axon clearance phenotype through the loss of Draper expression. In order to determine if Stat92E was directly involved in regulating glial responses to injury, I utilized the transcriptional reporters *dee7*-Gal4 and *10XStat92E-dGFP*.

In chapter four, I identified the *dee7* injury-responsive enhancer element located within the first intron of the *draper* gene. In addition to providing valuable insight into the cellular responses of glial cells to injury, examination of the DNA sequence of the *dee7* enhancer region also contributes to our understanding of the molecular mechanisms driving glial responses to injury. Within the 2619 base pair region, I identified three

Stat92E consensus binding sites located within 500 base pairs of each other. Dismissing the possibility of base content bias, the chance of either a 3*n* (TTC(3*n)*GAA) or 4*n* (TTC(4*n)*GAA) Stat92E binding site occurring randomly within the genome is approximately 1 in every 2000 base pairs (Sotillos et al., 2010). In addition, mammalian STAT5 binding sites are generally found within the first intron of genes and similarly, in *Drosophila*, Stat92E exerts its transcriptional effects on *domeless*, *socs36e* and *crb* through binding sites located in the first intron (Karsten et al., 2002; Nelson et al., 2004; Rivas et al., 2008). Collectively, these data support a model in which Stat92E binds to sites located within the first intron of the *draper* locus to drive transcriptional activation of Draper after injury.

Within the *dee7* enhancer region, there is a 4*n* Stat92E binding site which resides by itself, and two Stat92E binding sites which occur in tandem, a 3*n* and a 4*n* site. Tandem STAT binding sites are characterized as those residing within ~20 base pairs of each other and generally these represent binding sites for STAT tetramers (Soldaini et al., 2000). To analyze the role of these potential Stat92E binding sites in regulating injury responses, I first mutated the single 4*n* site. Of the three Stat92E binding sites, this was the only one specific to the *dee7*-Gal4 reporter, as the tandem sites are also located in the *dee6*-Gal4 reporter which did not show responsiveness to injury. Mutation of this site resulted in an approximate 50% loss of *dee7*-Gal4 reporter activity after antennal ablation suggesting that Draper is indeed directly transcriptionally regulated by Stat92E and mutation of this single Stat92E binding site has a significant effect on injury-induced Draper upregulation. One possible explanation for the reduction rather than complete

inhibition of the injury-induced reporter activity is that this binding site alone is not sufficient for transcriptional activation of Draper but instead acts synergistically with the other tandem Stat92E binding sites to induce maximal transcriptional activation of Draper after injury. To test this hypothesis I have generated two additional *dee7*-Gal4 mutant lines, one line contains a construct with mutations in both of the tandem Stat92E binding sites and the other line contains a construct with mutations in all three of the Stat92E binding sites. These lines will provide additional insight into the means of Stat92E-dependent activation of Draper transcription after injury.

There are a number of different mechanisms which govern STAT transcriptional events. One mechanism used by STAT molecules to modulate transcriptional activity is the ability to bind to some sites more strongly than others. It has been shown that Stat92E preferentially binds to 3*n* sites over 4*n* sites both *in vitro* and *in vivo*. Mutation of 4*n* sites to 3*n* sites, leads to increased expression of Stat92e target genes. However, many of the known Stat92E target genes are transcriptionally regulated through multiple 4*n* binding sites or combinations of 3*n* and 4*n* binding sites (Rivas et al., 2008). Therefore, it is likely that cooperation of multiple Stat92E binding sites is necessary to reach maximal Draper upregulation after injury. The presence of weak 4*n* binding sites may represent a mechanism to selectively activate genes only when a certain threshold of pathway activation is reached. This regulation could determine the distance from the source of a ligand at which a given STAT target gene is activated. In the presence of high concentrations of ligand, close to the source, pathway activation would be maximal and STAT would be capable of binding to these weak affninity sites. At areas more

distant from the source of the ligand, activation of the pathway would not be sufficient to induce STAT transcriptional activation of the gene. This model is consistent with the graded responses exhibited by the *dee7*-Gal4 reporter in response to antennal and maxillary palp injury.

STAT molecules are also capable of oligomerizing and binding to tandem sites as tetramers. STAT tetramers lead to more stable DNA-protein complexes than dimers and result in functional cooperation between two low affinity binding sites (Lerner et al., 2003; Soldaini et al., 2000 Bergad et al., 1995; Ota et al., 2004; Xu et al., 1996). In mammals, the binding of STAT molecules as tetramers is required for the transcriptional activation of several genes (Hou et al., 2003; John et al., 1999; Meyer et al., 1997). In addition, STAT tetramer binding can also lead to the recruitment of additional transcription factors and this represents another mechanism by which the transcriptional activity of STAT molecules can be modulated in response to external stimuli (John et al., 1999; Kornfeld et al., 2008). Interestingly, the *dee7* enhancer element contains two tandem Stat92E binding sites in addition to two binding motifs for a known STAT transcriptional co-factor complex, Activator Protein-1 (AP-1). In the ~37kb region of the *draper* locus that was analyzed for injury responsive elements, there are only four AP-1 binding sites, two of which are located in the *dee7* enhancer element.

AP-1 is a heterodimeric transcription factor composed of c-Jun, c-Fos and members of the Activating Transcription Factor (ATF) families. Both c-Jun and c-Fos are phosphorylated and transcriptionally activated by c-Jun N-terminal kinases (JNKs). Furthermore, studies from mammals show that JNKs are required in some instances to

promote the full activation of STAT molecules via phosphorylation of the conserved serine residue on STAT molecules (Aznar et al., 2001; Turkson et al., 1999). Interestingly, in chapter three I showed that the *Drosophila* homolog of JNK, *basket*, is required in glial cells for the clearance of degenerating axon debris and since then Jennifer MacDonald, a member of the lab, has identified the *Drosophila* homologs of c-Fos and c-Jun, *kayak* and *Jra*, as being cell-autonomously required for glial clearance of axon debris (unpublished data). Studies from mammals demonstrate that STAT and AP-1 act together in reactive microglial cells to mediate the upregulation of inflammatory proteins (Chang et al., 2008; Kim et al., 2002; Qin et al., 2007). Together, these data suggest a model in which STAT tetramerization leads to the recruitment of members of the AP-1 complex to the injury-induced *draper* enhancer element where they act in concert to promote transcription of the *draper* gene after axon injury. Additionally, it is likely that Basket is activating Kayak and Jra through tyrosine phosphorylation and may be contributing to the activation of STAT through serine phosphorylation. Jennifer MacDonald is currently mutating the AP-1 binding sites in the *dee7* enhancer element and will do further studies to determine the role of the JNK signaling cascade in glial responses to injury. It remains an interesting and likely possibility that combinations of Stat92E binding sites, AP-1 binding sites, and binding sites for other unidentified co-factors are involved in modulating levels of Draper expression depending on the severity of the injury.

Draper expression is not regulated by components of the canonical JAK/STAT signaling pathway

Further evidence that Stat92E is activated in glial cells upon injury to promote transcription of injury response genes is revealed by the increased activity of the *10XStat92E-dGFP* transcriptional reporter in response to maxillary palp or antennal injury. This increase in reporter activity is completely lost when *stat92E* RNAi is expressed specifically in glial cells indicating that most, if not all, of the Stat92E transcriptional activity induced after axon injury stems from glial cells.

Interestingly, in our assay, Stat92E does not appear to act through the canonical signaling pathway. Overexpression of a dominant negative Domeless construct, Domeless$^{\Delta CYT}$, as well as RNAi knockdown of *domeless* and *hop* did not lead to a decrease in Draper expression nor did they result in an axon clearance defect. Additionally, while overexpression of a constitutively active Stat92E molecule is capable of activating the *10xStat92E-dGFP* reporter in the adult *Drosophila* brain, a temperature-sensitive gain-of-function Hop allele, Hop^{Tum-l}, which has been shown in other assays to be sufficient to activate the *10XStat92E-dGFP* reporter, shows no increase in 10x*Stat92E-dGFP* transcriptional reporter levels in the adult brain. Given that this allele is a point mutation in the endogenous locus of *hop,* and not an overexpression allele, it might suggest that Hop is not expressed in glial cells in the brain. Alternatively, it may be that the endogenous levels of Hop are not sufficient to activate the reporter. Taken together, these data suggest that the role of Stat92E in glial clearance of axonal debris

and/ or *draper* expression is independent of the canonical upstream factors of STAT signaling. How then is Stat92E activated in this context?

Stat92E regulates injury-induced Draper upregulation through a non-canonical auto-regulatory loop

In the canonical JAK/STAT signaling pathway, Domeless and Stat92E form an autoregulatory loop whereby signaling through Domeless activates Stat92E which in turn transcriptionally activates *domeless.* To determine if a similar auto-regulatory loop exists between Draper and Stat92E, I looked at the expression of the *dee7*-Gal4 reporter in a *draper* null mutant background. If signaling through Draper were necessary to activate its own transcription after injury, then no transcriptional activation of the *dee7*-Gal4 reporter should be witnessed upon injury in a Draper null animal. Interestingly, loss of Draper resulted in a complete loss of transcriptional activation of the *dee7*-Gal4 reporter after injury. This strongly suggests that an auto-regulatory loop exists whereby Draper leads to activation of Stat92E which in turn leads to the transcriptional activation of *draper*. This represents a novel non-canonical signaling mechanism for Stat92E.

To further analyze the mechanisms by which Stat92E is activated downstream of Draper, glial-specific RNAi was used to knock down known components of the Draper signaling pathway, *src42a, shark, dCed-6 and rac1*, and *10xStat92E-dGFP* reporter activity was examined after injury. Interestingly, RNAi knockdown of *src42a, shark* and *rac1* all resulted in a complete loss of detectable induction of *10xStat92E-dGFP* reporter activity after injury **(Fig 4-9d)**. It is unlikely that these molecules represent a linear

pathway leading to the activation of Stat92E but instead, it is probable that these molecules have different roles in the regulation of Stat92E activity.

Possible roles of Src42A and Shark in the activation of Stat92E downstream of Draper

Evidence from mammals shows that among various other kinases, Syk kinases are capable of phosphorylating tyrosine residues on STAT proteins and Src family kinases can phosphorylate both serine and tyrosine residues on STAT proteins (Klejman et al., 2002; Ng et al., 2009; Okutani et al., 2001; Olayioye et al., 1999; Read et al., 2004). Serine phosphorylation is required by some STAT proteins for maximal activation and this phosphorylation occurs at a conserved motif, P(M)SP (Friedbichler et al., 2012; Haq et al., 2002; Wen et al., 1995; Yokogami et al., 2000). Although *Drosophila* Stat92E does not contain this conserved serine phosphorylation motif, it does not rule out the possibility that other serine residues may be phosphorylated by Src molecules, or by JNK as previously discussed. Additionally, in mammals, Src family kinases and JAK molecules can act together to promote maximal activation of STAT proteins through phosphorylation of different tyrosine residues on STAT molecules (Okutani et al., 2001). In *Drosophila*, there is only a single study, showing that a Src-dependent overgrowth phenotype in the eye is dependent upon downstream Stat92E and JNK signaling, which suggests that Src molecules may be capable of phosphorylating and/or activating Stat92E (Read et al., 2004). Together these data suggest that it is possible that Src42a and/or Shark are activating Stat92E through phosphorylation of tyrosine and/or serine residues

in a Draper-dependent manner. I showed that overexpression of a constitutively active Src42a in glial cells results in increased *10xStat92E-dGFP* reporter activity, indicating that Src42a is capable of activating Stat92E likely through phosphorylation. However, further experiments will need to be done to determine if Src42a and/or Shark act downstream of Draper to phosphorylate and activate Stat92E in the presence of injury.

Possible role of Rac1 in the activation of Stat92E downstream of Draper

In addition to *src42a* and *shark*, RNAi knockdown of *rac1*also results in the loss of *10xStat92E-dGFP* reporter activity after injury. While it was not a surprise to find that Rac1 was necessary for glial clearance of degenerating axons based on its well-established roles in cytoskeletal rearrangement, cell migration, chemotaxis, and phagocytosis, it was interesting to discover a role for Rac1 in the regulation of Stat92E transcriptional events. Recent evidence from mammalian studies shows that Rac1 and the GTPase activating protein, MgcRacGAP, are capable of promoting phosporylation of STAT3 and shuttling it to the nucleus (Kawashima et al., 2009; Kawashima et al., 2006). In this model, upon cytokine stimulation, GTP-bound Rac1 and MgcRacGAP form a ternary complex with STAT3 which is necessary for the phosphorylation of STAT3. Upon phosphorylation of STAT3, this ternary complex is translocated to the nucleus via a nuclear localization signal present on MgcRacGAP and shuttled across the nuclear envelope by an importin α/β–dependent mechanism **(Fig. 5-1)**. Interestingly, in chapter three, I identified the *Drosophila* homolog of MgcRacGAP, *tumbleweed*, as being necessary for the efficient clearance of axons. RNAi knockdown of *tumbleweed* in glial

cells resulted in a slight axon clearance defect. However, this finding still needs to be confirmed by other (non-RNAi) methods.

While the proposed mechanism of Rac1/Tumbleweed regulation of STAT transcriptional activity seems plausible in the context of glial injury responses, further studies will need to be performed in order to confirm or negate this model. The use of a constitutively active Rac1 molecule could be used to determine if Rac1 leads to phosphorylation of Stat92E and furthermore, antibody stainings could be performed *in vivo* to look at localization of Stat92E in a Rac1 RNAi background and a constitutively active Rac1 background. Additionally, coimmunoprecipitation studies could be performed from brains in these different backgrounds to show direct binding of Rac1 and/or Tumbleweed to Stat92E.

Stat92E transcriptional activation of Draper is not dependent upon dCed-6 activity

In chapter two, I identified *ced-6*, the *Drosophila* ortholog of *C. elegans ced-6* and mammalian GULP, as being required in glial cells for the clearance of degenerating axon material. Additionally, in chapter four I show that while knockdown of *src42a*, *shark*, and *rac1* all result in the loss of *10xStat92E-dGFP* reporter activity, knockdown of *dCed-6* only leads to a reduction in *10xStat92E-dGFP* reporter activity and not a complete loss **(Fig. 4-9c)**. While it has already been established in *C. elegans* and mammals that CED-6/GULP acts downstream of CED-1/MEGF10 during engulfment events, the precise role of CED-6 during phagocytosis has not yet been revealed.

Evidence from *C. elegans* suggests that it may act to mediate phagolysosomal maturation (Yu et al., 2008). However, the evidence for this, intermediate delay of PI(3)P accumulation on phaglysosomal surfaces in *ced-6* hypomorphic animals, is not definitive. It is likely that *Drosophila* dCed-6 regulates steps prior to phagolysosomal maturation during glial responses to injury as we do not observe any migration of glial membranes to the sites of injury when *dCed-6* is knocked down. We have evidence from our lab (Jennifer Ziegenfuss, personal communication) that indicates migration of glial membranes and phagocytosis of axonal debris occur through two distinct pathways which converge on Rac1, with the Draper/Shark/Src42a pathway being necessary for migration, and these two events, migration and phagocytosis, are separable. Therefore, while it may be possible that dCed-6 contributes to phagolysosomal maturation, it is also critical for earlier steps of glial migration.

Studies from mammals show that the mammalian homolog of dCed-6, GULP, is involved in the regulation of cholesterol homeostasis in macrophages during phagocytosis of apoptotic cells (Kiss et al., 2006b). However, cholesterol efflux from macrophages in the context of phagocytosis appears to be dependent upon the activation of lipid receptors, as the uptake of apoptotic cells through an FcR-mediated mechanism did not enhance cholesterol efflux. Furthermore, only apoptotic cells and not necrotic cells were able to induce cholesterol efflux from phagocytosing macrophages (Kiss et al., 2006a). Thus, it is unlikely that dCed-6 exerts its effects on glial cells, during engulfment of degenerating axons, through the regulation of cholesterol homeostasis. However, GULP has also been shown to regulate the migration of cells through its physical

interaction with and positive regulation of GTP bound Arf6, a small GTPase (Ma et al., 2007). We have preliminary data from our lab (Jeannette Osterloh, personal communication) which reveals that glial-specific knockdown of Arf79F, a closely related homolog of mammalian Arf6, results in an axon clearance defect. Taken together, these data indicate that dCed-6 may be acting downstream of Draper to positively regulate Arf79F and promote glial migration after injury through a pathway independent of Src42, Shark and Rac1.

The PI3K/Akt pathway and Stat92E converge to regulate basal levels of Draper expression

I have shown several lines of evidence supporting the notion that Stat92E transcriptionally activates *draper* after injury, 1) RNAi knockdown of Stat92E leads to a loss of injury-induced Draper expression, 2) the *dee7* injury-induced enhancer element located in the first intron of the *draper* gene contains three Stat92E binding sites, 3) mutation of a single Stat92E binding site in the *dee7* enhancer element leads to >50% reduction in *dee7-Gal4* activity upon injury, 4) the *10XStat92E-dGFP* transcriptional reporter is activated in glial cells upon injury, and finally , 5) activation of both the *10XStat92E-dGFP* reporter and the *dee7-Gal4* reporter is lost in a *draper* null animal. However, I also showed that knockdown of Stat92E not only inhibits Draper upregulation after injury but it also results in a significant reduction in basal Draper protein levels as well. Surprisingly, neither the *dee7-Gal4* reporter nor the *10XStat92E-dGFP* reporter, which are both activated upon injury, show any significant expression in uninjured

brains. Additionally, overexpression of a constitutively active *stat92e* construct leads to a significant increase in *10XStat92E-dGFP* reporter activity but does not result in any detectable increase in Draper protein levels (data not shown). In the context of glial responses to injury, I show that Stat92E likely acts downstream of Src42a, Shark and Rac1 to promote injury-induced Draper transcriptional activation. However, RNAi knockdown of *src42a*, *shark* or *rac1* does not affect basal Draper protein levels. Thus, it appears that Stat92E is acting through an additional non-canonical pathway to regulate basal Draper protein levels.

I showed in chapter three that the PI3K92E/Akt pathway is involved in the regulation of basal Draper levels as RNAi knockdown of several components of this pathway results in a reduction in basal but not injury-induced Draper protein expression. Furthermore, overexpression of a constitutively active PI3K92E in glial cells results in an increase in basal Draper protein levels. Together, these findings suggest that the PI3K/AKT pathway and Stat92e likely converge at some point to regulate basal Draper protein levels.

In mammalian cell culture it has been shown that STAT5 activity in glioma cells promotes glioma cell invasion through two independent pathways, one requiring Stat5 phosphorylation and transcriptional activity and another independent of Stat5 phosphorylation and transcriptional activity. U87 glioma cells display basal levels of migration and these levels are enhanced upon EGF stimulation. Both the basal levels of migration and the EGF-induced enhanced migratory properties of the cells are dependent upon Stat5 activity and siRNA directed against Stat5 results in the loss of all U87 glioma

cell migration. However, while EGF stimulation of U87 glioma cells resulted in phosphorylation of STAT5, in unstimulated U87 glioma cells no phosphorylated STAT5 could be detected (Cao et al., 2011). Furthermore, expression of a decoy oligonucleotide containing STAT5 consensus binding sequences, which only interferes with STAT5 transcriptional activity, resulted in the loss of EGF-induced phosphorylation of STAT5 as well as EGF-induced enhanced migratory properties of the cells but it did not affect basal migration of the cells. This study suggests that in the absence of stimulation, unphosphorylated Stat5 acts within U87 glioma cells to promote migration through a mechanism independent of transcription. However, in the presence of EGF, STAT5 is phosporylated and promotes enhanced migratory properties of U87 glioma cells through mechanisms dependent on STAT5 transcriptional activity (Cao et al., 2011). Considering we are unable to find any evidence which suggests that Stat92E is transcriptionally active in the uninjured brain, it is possible that Stat92E is acting in a manner independent of its transcriptional activity to promote the expression of basal Draper protein levels.

Several lines of evidence from mammals show that STAT molecules are capable of activating the PI3K/Akt pathway through both transcriptional mechanisms and cytoplasmic signaling roles. Oncogenic activated STAT5 has been shown to accumulate in the cytoplasm and activate Akt via direct protein-protein interaction with the scaffold protein Gab2 and with p85, the regulatory subunit of PI3K (Harir et al., 2007; Kornfeld et al., 2008; Nyga et al., 2005). Another study has shown that STAT5-mediated activation of Akt is necessary for glucose uptake which promotes the survival of T-cells and in this context STAT5 and Akt act in a linear pathway, with STAT5-mediated transcription

leading to Akt activation. The authors suggest that STAT5 may alter the expression of genes involved in activation or feedback inhibition of the PI3K/AKT pathway (Wofford et al., 2008). Furthermore, it has been shown that STAT5 is a physiological substrate of the insulin receptor and insulin promotes rapid tyrosine phosphorylation and DNA binding of STAT5 in a JAK-independent manner, possibly via phosporylation and activation of STAT5 by the insulin receptor directly (Chen et al., 1997; Sawka-Verhelle et al., 1997).

Interestingly, there is evidence from our lab that the *Drosophila* insulin receptor, InR, acts upstream of the PI3K/Akt pathway to regulate basal levels of Draper protein expression (Dr. Mary Logan, personal communication). RNAi knockdown of the insulin receptor results in a significant reduction in Draper levels and conversely, overexpression of a constitutively active insulin receptor leads to increases in Draper consistent with those seen in a constitutively active PI3K background (Dr. Mary Logan, personal communication). I have preliminary data showing that RNAi knockdown of Stat92E in a consitutively active PI3K background still results in increases in Draper protein levels. Furthermore, although constitutive activation of PI3K leads to increases in basal Draper levels, it does not lead to activation of the *10xStat92E-dGFP* reporter. This data suggests Stat92E is regulating basal Draper expression through a mechanism independent of direct transcriptional activation of Draper.

In conclusion, I propose a model in which Stat92E regulates both basal as well as injury-induced Draper expression through two independent mechanisms. It is likely that Stat92E acts together with the InR/PI3K/Akt pathway to regulate Draper protein

expression in a manner independent of direct transcriptional activation. In this model, Stat92E could act as a cytoplasmic signaling molecule downstream of the insulin receptor leading to the activation of the PI3K/Akt pathway or, Stat92e could act as a transcription factor for components of the PI3K/Akt pathway or for activators of the PI3K/Akt pathway (such as insulin). In this model it is possible that Draper expression could be regulated by transcriptional, translational, or post-translational mechanisms. Furthermore, I propose that Stat92E acts downstream of Draper, Src42A, Shark and Rac1 to transcriptionally activate injury-induced upregulation of Draper. These two pathways both represent novel, non-canonical Stat92E signaling mechanisms and they provide the potential to shed light on STAT signaling mechanisms in mammals. Understanding the mechanisms by which STAT molecules regulate gene expression will allow for the generation of more effective and targeted therapeutic approaches.

Conclusions

The studies performed here highlight the role of glial cells in the adult *Drosophila* brain. In chapter two, I characterized three different subtypes of glial cells in the adult *Drosophila* brain, identified the cell type responsible for phagocytic engulfment of degenerating axon material, show that *Drosophila* astrocytes bear striking morphological and molecular similarity to their mammalian counterparts, and identified *dced-6* as a necessary glial engulfment gene. In chapter three, I performed an *in vivo* RNAi screen which uncovered twenty genes required for the efficient glial phagocytosis of degenerating axon debris. And lastly, in chapter four I characterize the role of Stat92E in

glial engulfment events and identify a novel non-canonical signaling pathway for Stat92E.

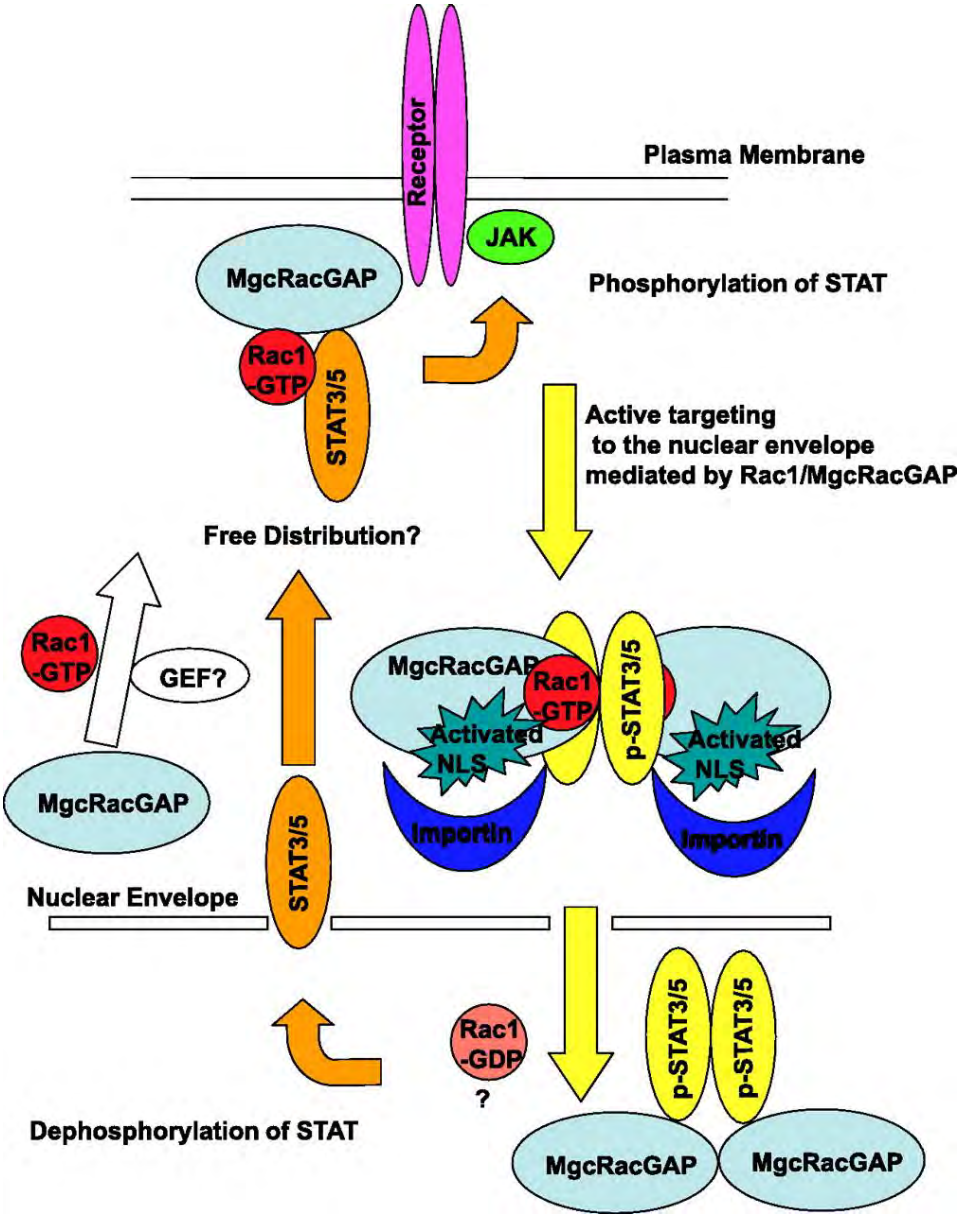

Receptor
Plasma Membrane
JAK
MgcRacGAP
Phosphorylation of STAT
Rac1
-GTP
STAT3/5
Active targeting
to the nuclear envelope
mediated by Rac1/MgcRacGAP
Free Distribution?
Rac1
-GTP
GEF?
MgcRacGAP
MgcRacGAP
Rac1
-GTP
p-STAT3/5
Activated
NLS
Activated
NLS
Importin
Importin
STAT3/5
Nuclear Envelope
Rac1
-GDP
?
p-STAT3/5
p-STAT3/5
Dephosphorylation of STAT
MgcRacGAP
MgcRacGAP

Figure 5-1

Figure 5-1. A current model of nuclear import of p-STATs and a working hypothesis for membrane targeting and phosphorylation of STATs.

In the present work, we demonstrated that the NLS of MgcRacGAP accompanied by GTP-bound Rac1 is essential for nuclear translocation of p-STATs via importin α/β. We also propose that binding of MgcRacGAP to STATs is required for their tyrosine phosphorylation after cytokine stimulation. Interestingly, the mutants that preferentially bind MgcRacGAP become constitutively active. Altogether, we conclude that MgcRacGAP critically functions both as a mediator of STAT's tyrosine phosphorylation and as an NLS-containing nuclear chaperone of p-STATs. (Reproduced from (Kawashima et al., 2009))

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