The role of Protein Phosphatase 4 in glial immune responses to neuronal injury.

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The role of Protein Phosphatase 4 in glial immune responses to neuronal injury.

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List Of Abbreviations

AD: Alzheimer's Disease

AMPK: AMP-Activated Protein Kinase

AP-1: Activator Protein 1

ATP: Adenosine Triphosphate

BBB: Blood Brain Barrier

Drpr: Draper

Falafel/PP4r3/SMEK: Protein Phosphatase 4 Regulatory Subunit 3

GDP/GTP: Guanosine Di/Triphosphate

GEF: Guanine Nucleotide Exchange Factor

GFAP: Glial Fibrillary Acidic Protein

GFP: Green Fluorescent Protein

GTPase: Guanosine Triphosphatase

ITAM: Immunoreceptor Tyrosine-based Activation Motif

JNK: c-Jun N-terminal Kinases

MAPK: Mitogen-Activated Protein Kinase

MMP: Matrix Metalloproteinase

MS: Multiple Sclerosis

NES: Nuclear Export Signal

NLS: Nuclear Localization Signal

NSC: Neural Stem Cell

OE: Overexpression

OPCs: Oligodendrocyte Precursor Cells

ORN: Olfactory Receptor Neuron

PP2A: Protein Phosphatase 2 A family

PP4: Protein Phosphatase 4

PP4c: Protein Phosphatase 4 catalytic subunit

PP4r2: Protein Phosphatase 4 Regulatory Subunit 2

RFP: Red Fluorescent Protein

RNAi: RNA interference

STAT: Signal Transducer and Activator of Transcription

ts: Temperature Sensitive

UAS: Upstream Activation Sequence

Common Genes		
Drosophila	*C. elegans*	Mammalian
Drpr	Ced-1	MEGF10/Jedi-1
dCrk	Ced-2	CrkII
Mbc	Ced-5	Dock 180
dCed12	Ced-12	ELMO
dCed6	Ced-6	Gulp1
DRK	Sem-5	Grab-2
DOS	Soc-1	Gab-2
SOS	SOS	SOS

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Abstract

Recent advances in glial cell research have proven that glial cells are much more than brain "glue," and that glia perform numerous important functions in vertebrate and invertebrate nervous systems. For example, glial cells are vital for maintaining brain health after injury and in disease conditions. In this dissertation, I present data collected during my time in the Logan lab where we have studied how glial cells act as immune responders in the *Drosophila* brain. Glial cell immune responses to neuronal injury include altered gene expression profiles, directed migration to injury sites, and glial clearance of damaged neurons through phagocytic engulfment. Importantly, the glial cell immune response stops further spread of disease and damage, but there are currently several gaps in our knowledge of the mechanisms underlying these protective glial reactions. In this dissertation, I will discuss our current understanding of the glial cell immune response and then present my data revealing how the evolutionarily conserved trimeric Protein Phosphatase 4 (PP4) serine/threonine phosphatase complex is a novel member of the glial response to nerve injury in the adult *Drosophila* brain. I will examine how the PP4 complex contributes to glial cell membrane dynamics after injury, and I will then study possible PP4 downstream effectors after injury. These data reveal PP4 as a novel member of the glial cell immune response signaling pathway after injury in the adult Drosophila brain.

Chapter 1: Introduction

INTRODUCTION TO GLIAL CELLS

Glial Cells in the CNS and PNS

In neuroscience, glial cells have been casually referred to as the "non-exciting" cells in the brain, but this phrase does them a great disservice. Research into glial cell functions has been overlooked, since glial cells were thought to be helper cells that only provide support to the "exciting" neurons. In the past few decades, however, researchers have realized that glial cells provide much more than trophic support to neurons, and that glial cells are vital for brain health. For example, glial cells compose the innate immune system in the brain, are necessary for brain development, and can be involved in neuronal signaling(1). I will begin my introduction with a background of the study of glial cells, since my dissertation work focuses on understanding how glial cells function as primary immune responders in the brain. For this work, I'd like to borrow the dedication from H. Ketternmann and B.R. Ransom's book *Neuroglia:* "to those who believed in glial cells during the long dark period when the neuron concept dominated brain science."(2)

Glial cells were first discovered in the mid-1800s by several scientists, however these early studies were mostly observational and lacked functional knowledge(1). Rudolf Virchow first coined the term "neuro-glia," and Henrich Muller first published drawn pictures of glial cells. Other scientists, including Theodor Schwann, Robert Remak, Camillo Golgi, Michael von Lenhossek, Louis Ranvier, and Pio del Rio-Hortega also described glial cells in the central nervous system (CNS) and the peripheral nervous system (PNS) in the 1800s. Next, several scientists categorized the various glial cells into subtypes. In 1871, Golgi defined oligodendrocytes and astrocytes, and in 1893, von Lenhossek labeled the star-shaped cells in the CNS as astrocytes. Ranvier named Schwann cells, and Rio-Hortega

identified oligodendrocytes and microglia. Of course, the powerhouse neuroscientist Santiago Ramon y Cajal, along with his numerous pupils and scholars, used silver stain to further study these “neuro-glia” (3).

There are four main glial cell types in the mammalian CNS and PNS: astrocytes, microglia, oligodendrocytes, and Schwann cells(4). Each glial cell subtype has distinct roles in the brain during development and into adulthood. There is a large body of evidence suggesting that each glial subtype can contribute to disease and neurological disorders, so studying glial cells is of the utmost importance. In the next section, I will briefly describe these different glial functions, and will then describe how these different glial cells contribute to various diseases and the CNS immune response.

Astrocytes

Astrocytes are star-shaped, multi-processed cells with numerous functions(5). For example, astrocytes form and maintain the blood brain barrier (BBB), participate in neuronal signaling, and react to injury and disease. Astrocytes impact neuronal signaling as part of the tripartite synapse, where astrocytes located close to the synapse exchange molecules with neurons. Upon the release of the excitatory neurotransmitter glutamate from neurons, astrocytes can reuptake excessive glutamate, which reduces excitotoxicity(6). Astrocytes can also extend and retract their processes towards or away from dendritic spines during neuronal firing(4). Furthermore, astrocytes facilitate calcium waves, which propagate from astrocyte to astrocyte through gap junctions(7).

Astrocytes are derived from neural stem cells (NSC) along with neurons and oligodendrocytes(8). The first wave of NSC divisions produces neurgenic cells, and the second wave produces gliogenic cells, including radial glial cells. Astrocytes can be directly produced from these radial glia, but evidence suggests that most astrocytes arise from

proliferation of recently born astrocytes(8). In humans, astrocytes are over 20-times larger and make 10-times more contacts with synapses than in rats and other mammals(9), suggesting that astrocytes play an important role in human cognition.

Astrocytes are very important during development, and disruptions in astrocytes have been associated with numerous developmental diseases. For example, Down syndrome patients have reduced myelination and increased astrocytes, which could result from increased expression of the gliogenic transcription factor olig2 during development(8). There is also evidence that astrocytic signaling during development can contribute to autism spectrum disorders. Several astrocytic markers, including glial fibrillary acidic protein (GFAP), Aquaporin 4 (AQP4) and Connexin 43 (CX43) have abnormal expression patterns in autism spectrum disorder patients, and astrocytes in these patients have been observed to be smaller with fewer branched processes(10). Disruptions in astrocytes have also been linked to psychiatric disorders. In major depressive disorder and bipolar depression, patients have been shown to have fewer glia in the anterior cingulate(11). Recently, studies have shown that astrocytes express lower levels of EAAT2, the astrocyte glutamate transporter, in deep cortical layers in patients with schizophrenia, suggesting that astrocytes play an important role in glutamate homeostasis in this disorder(12).

Oligodendrocytes and Schwann Cells

Oligodendrocytes and Schwann cells are best known for creating myelin, the fatty substance that surrounds neuronal axons to increase conduction velocity. In 1853, Virchow first used the term “myelin” to describe this sheath around axons, and Remak described these same fibers in the PNS(1). Further, Rio-Hortega argued that oligodendrocytes and Schwann cells make myelin, but this was not proven until later by electron microscopy(1).

“Oligodendrocyte” comes from Greek and refers to a cell with fewer processes than other cells. Oligodendrocytes reside in the CNS, and Schwann cells reside in the PNS.

During CNS development, oligodendrocytes are the last cell type to differentiate. Oligodendrocyte precursor cells (OPCs) either differentiate into mature, myelinating oligodendrocytes, or remain as a pool of precursors into adulthood(1). This reserve of undifferentiated OPCs is thought to act as a reservoir of cells that can differentiate to help remyelinate the CNS after injury or disease. Several studies have found evidence that oligodendrocytes are involved in psychiatric disorders, including autism, bipolar disorder, and schizophrenia(13). In post-mortem brains from patients suffering from schizophrenia, there were fewer oligodendrocytes, abnormal myelin, and reduced oligodendrocyte-expressed genes(14). There are numerous diseases related to abnormal myelin or myelination failure, including multiple sclerosis (MS) and Charcot-Marie-Tooth disease(13). In MS, activated microglia and macrophages attack oligodendrocytes that are forming myelin, leading to large areas of unmyelinated axons. In chronic MS, disease progression appears to be linked to myelin and oligodendrocyte degeneration(15). OPCs present in chronic MS are unable to differentiate into myelinating oligodendrocytes and help remyelinate the exposed axons(16). There is also a body of evidence suggesting an oligodendrocytic role in the neurodegenerative Alzheimer’s disease (AD). In approximately 50% of AD cases, there is white matter loss and decreased oligodendrocyte lineage cells, perhaps due to a toxic effects of amyloid beta on these cells(17). Further, in aged brains, there is a loss of oligodendrocyte function. Clearly, oligodendrocytes have numerous functions beyond supporting neuronal firing.

Microglia

Microglia are constantly surveying the brain for damage or disease(18). This glial subtype is the main constituent of the mammalian innate immune system of the CNS. After an injury or in disease states, microglia become active. Active microglia undergo morphological and functional changes, becoming more processed and migrating towards the site of injury or diseased area. Even when not activated, microglia are still very motile(18). They extend and retract their processes to "test" the environment, constantly searching for things that shouldn't be in the brain. Microglia origin is contested, but current evidence suggests they are derived from a mononuclear phagocyte lineage, which differentiates them from other CNS glia and neurons(19). I will describe active microglia in more depth in the next section of this introduction, where I will introduce the various glial roles in the immune response.

Glial Cell Immune Functions

Glial cells are the primary immune responders in the central nervous system. Upon insult, microglia and astrocytes undergo morphological changes, becoming more branched and migratory(20-22). These "reactive" glia migrate or extend membrane processes towards the injury site to phagocytose cellular debris(23, 24). This glial response is vital for clearing damaged cells and blocking the spread of inflammation(25, 26). In the next section of my introduction, I will describe the current understanding of how glial cells respond to insult and disease, but our knowledge is still incomplete. The primary goal of my thesis is to elucidate the signaling mechanisms underlying these glial immune responses.

Astrocytes as Immune Responders:

Astrocytes become reactive in response to various conditions, such as ischemia, trauma, and disease(21). Astrocytes can protect the brain in several ways, including by forming and

repairing the blood-brain barrier (BBB), uptaking excessive excitotoxic glutamate, and protecting cells from oxidative stress(22). One of the hallmarks of reactive astrocytes is an increase in glial fibrillary acidic protein (GFAP), an intermediate filament (IF) protein. IF proteins are a part of the cytoskeleton along with actin and microtubules. Mice lacking GFAP and vimentin, another IF protein, have slower wound healing and delayed astrocyte migration(21). In disease states, astrocytes increase production of cytokines and chemokines such as Interleukin (IL-) 1, 6, and 10, Interferon alpha and beta, and monocyte chemoattractant protein-1(27). For example, IL-6 has both positive and negative effects in the diseased CNS. IL-6 is neuroprotective and promotes astrocyte proliferation, but abnormally high levels of IL-6 can lead to BBB breakdown and neuronal death(28). Regulating the BBB is a very important immune function of astrocytes. Gimsa et al. detail the ways that astrocytes act against neuroinflammation from T-cells entering the CNS by regulating the BBB, which helps minimize damage after an insult(29). Interestingly, there is evidence that astrocytes can also activate microglia during an immune response, which I will discuss next.

Microglia as Immune Responders:

Microglia are thought to be constantly surveying the brain for damage and disease. In response to injury, microglia rapidly extend their processes towards the site of injury(20). For more than a century, scientists have observed the microglial ability to change from a resting state to an activated state upon brain insult. Microglial activation is graded in response to the severity of insult, with microglia becoming phagocytic cells in severe cases(30). These phagocytic microglia engulf damaged cells and invading micro-organisms to reduce overall inflammation and return the brain to homeostasis. Microglia respond to several signals in the brain such as complement factors, CREB, TGF-Beta1, Interleukin 1,2, 6, and ATP(30). Applying ATP to injured tissue increases the speed of the microglial

extensions towards the injured area, suggesting that microglia are attracted to signals released from the injury site, such as ATP. After insult, microglia can phagocytose damage, debris, and dead cells (31, 32). In the CNS, astrocytes and microglia work together after injury and in disease.

Microglial action is important in several diseases. In AD, microglia can internalize soluble amyloid-beta, converting some of the internalized amyloid-beta into fibrils(31). Reactive microglia surround amyloid plaques, and several studies have focused on increasing microglial phagocytosis of amyloid-beta. For example, aspirin-triggered lipoxin A4 (ATL) treatment, which increases anti-inflammatory molecules, increased microglial phagocytosis, synapse recovery, and improved cognition(33). Microglia also have a role in psychiatric disorders(34). In schizophrenia, increased activated microglia has been seen in prefrontal white matter(35). Furthermore, activation of microglia is thought to be a stressor that can lead to development of depression. Selective serotonin reuptake inhibitors (SSRIs) and norepinephrine reuptake inhibitors reduce microglial activation(36). Further clinical studies could help to determine the role of microglia in human psychiatric disorders.

Reactive Gliosis: a Double-Edged Sword?

As I mentioned, glial cell immune functions are necessary for a healthy brain, but reactive gliosis can also be a double-edged sword. During the initial stages after CNS injury, astrocytes appear to have a positive effect, but later form glial scars that can block regeneration(37). The glial scar can block the spread of degeneration from the damaged area to healthy areas of the CNS, but the scar can also block new, healthy neurons from reaching the damaged area to facilitate regeneration. However, in experiments where scar-forming reactive astrocytes were ablated, there was a spread of disease, less blood-brain barrier repair, increased tissue damage, and worse clinical recovery(22). Glial scar

formation occurs in response to very traumatic events, and the amount of reactive astrocyte response is determined by the severity of the initial damage or insult(22). The glial cell subtypes each play an important role in maintaining brain health, but there is a lack of understanding surrounding the signaling pathways underlying glial cell immune responses. To examine this glial immune signaling pathway in more detail, the Logan lab performs experiments in *Drosophila melanogaster.*

INTRODUCTION TO DROSOPHILA AS A MODEL SYSTEM:

This thesis research has been completed using a *Drosophila melanogaster* model system. The *Drosophila* model system is superb for studying the glial immune response for several reasons. Ndubaku has described *Drosophila* as "the most important animal model" for glial cell research(1). *Drosophila* have a short generation time of about 10 days and are simple to breed and house. The upkeep costs for *Drosophila* lines are very low, especially compared to other model systems. Importantly, the genetic techniques and tools available with *Drosophila* make research possibilities almost endless. For example, my thesis depends on knocking down several genes in glial cells only, specifically in adult fly brains. I am also able to genetically label a subset of neurons with green fluorescent protein (GFP). These examples are explained in more detail in Chapter 2, but serve as potent illustrations of how *Drosophila* genetics can be taken advantage of in neuroscience research.

Drosophila Glial Subtypes

Another important reason that *Drosophila* is a great model is because *Drosophila* glia and neurons are very similar to mammalian glia and neurons. The *Drosophila* glial subtypes have recently been identified(38-40). My thesis work focuses on the ensheathing glial subtype, which wraps axons and synaptic-rich regions. These ensheathing glia are reminiscent of mammalian oligodendrocytes. Another glial type in *Drosophila* are the

astrocyte-like glia. These glia have tufted membrane processes, similar to mammalian astrocytes, which are closely associated with synaptic-rich regions. The *Drosophila* astrocytes are thought to function similarly to mammalian astrocytes, and can affect synaptic transport of excitatory amino acids(41). A third glial cell subtype in *Drosophila* is the cortex glia type. These glia reside in the cortical areas of the brain and tile the area in non-overlapping regions. The function of cortex glia is less well understood, but they could be providing trophic support to the neurons they are next to in the cortex(40). One disadvantage to using *Drosophila* as a model system is that these glial subtypes do not exactly match up to mammalian glia. Notably, *Drosophila* do not have microglia, but the ensheathing glial subtype does have a similar phagocytic ability.

Drosophila Genetic Techniques

Importantly, there are glial subtype-specific genetic tools available for researchers, which have allowed me to perform most of the research presented in this dissertation. These tools, such as the Gal4-UAS system, allow for tissue- and cell-specific regulation of genes. As Brand and Perrimon explained in 1993, this system uses the yeast transcription factor Gal4 to activate transcription of target genes that contain a promoter with an upstream activation sequence (UAS) of Gal4-binding sites(42). Genes containing Gal4 binding sites within their promoters can be “turned on” with the UAS-Gal4 system in flies. Broadly, flies expressing the target gene (UAS-XXX) are crossed to flies expressing Gal4, resulting in progeny that have the target gene being activated in cells expressing Gal4(42). In my research, I use the glial-specific driver Repo-Gal4 (*Drosophila* glial cells express the *reversed polarity* (*repo*) gene) and the ensheathing glia-specific driver TIFR-Gal4. These lines drive UAS-regulated gene expression in glial cells only.

Another genetic technique I used frequently in my research is the $Gal80^{ts}$ (temperature sensitive) system, which allows for temporal gene control. Gal80 is a transcriptional repressor that binds to Gal4 to block Gal4-mediated transcription(43). At the permissive temperature of 18C, $Gal80^{ts}$ is blocking transcription, but at temperatures above 28C, $Gal80^{ts}$ is degraded, allowing for transcription of the target gene. This technique is very powerful for allowing manipulation of genes in adult flies without affecting the larvae.

An additional tool that makes *Drosophila* a great model system is the RNA interference method (RNAi), which is based on the findings of Fire and Mello that double-stranded RNAs can knock down gene activity(44). With the fly genome being fully sequenced, there are useful RNAi lines for virtually all *Drosophila* genes. Combining RNAi lines with the $Gal80^{ts}$-Gal4-UAS system has allowed me to temporally regulate knockdown and expression of my genes of interest in glial cells only in adult flies.

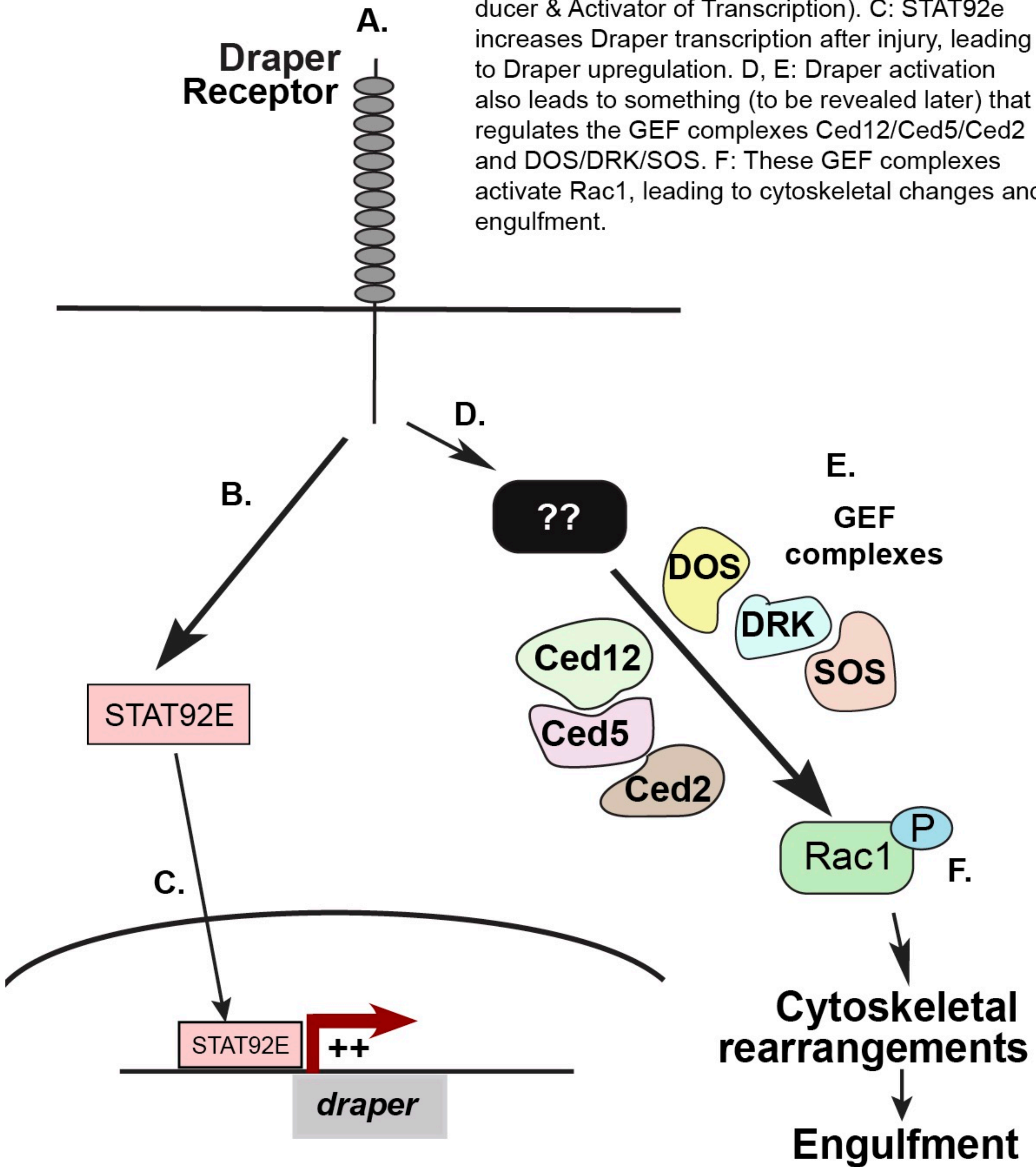
For my dissertation work, I use this superb *Drosophila* model to study the glial immune response. As described earlier, glial cells perform numerous functions, including becoming reactive in response to injury, insult, and disease. In the following sections, I will describe in more detail how *Drosophila* glial cells react during the immune response, and note how these glial responses are conserved through many species.

Glial Cell Signaling Pathways

There are four broad steps to the glial immune response to injury: 1) sensing the injury, 2) changes in immune genes and signaling molecules, 3) glial migration/membrane extension towards the site of injury, and 4) phagocytosis and degradation of debris. Currently, there are gaps in our understanding of these signaling pathways, which I will discuss below. My work in Chapter 2 will focus on filling in these holes, specifically focusing on glial migration and phagocytosis.

Figure 1

The Draper Immune Pathway. A: Draper receptor becomes activated (potentially by “eat me signals.” B: Draper leads to STAT92e activity (Signal Transducer & Activator of Transcription). C: STAT92e increases Draper transcription after injury, leading to Draper upregulation. D, E: Draper activation also leads to something (to be revealed later) that regulates the GEF complexes Ced12/Ced5/Ced2 and DOS/DRK/SOS. F: These GEF complexes activate Rac1, leading to cytoskeletal changes and engulfment.



Step 1: Glial Cells Sense the Injury:

To sense the injury, glial cells rely upon various "find me" and "eat me" signals released by the dying cells. One such signal could be ATP. Davalos et al. found that microglia robustly respond toward ATP injections in the mouse brain(20). One of the most well studied "eat me" signals is phosphatidylserine, which becomes present on the extracellular membrane surface when a cell undergoes apoptosis(45). Phagocytic cells can recognize the phosphatidylserine on the membrane and engulf the cell(46). Another interesting "eat me" signal is Pretaporter. During *Drosophila* development, Pretaporter is exposed on the surface of dying cells to induce apoptosis(47). It is worth noting here that in the context of my thesis work, we are not sure what the "find me" or "eat me" signals are.

These "find me" or "eat me" signals trigger an immune response in the glia by activating immune receptors. One well studied receptor that could be sensing a cue is Draper (MEGF10/Jedi1 in mammals, Ced1 in *C. elegans*), which resides on the membrane of glial cells. It is important to note that during development in *Drosophila*, Draper senses the "eat me" signal Pretaporter to initiate phagocytosis(47), but we currently have no evidence of a Draper ligand in the adult fly brain. Upon Draper receptor stimulation, a complex signaling pathway is activated, leading to changes in immune genes and proteins, glial cell morphological changes, and phagocytosis of the debris or foreign object (see Figure 1). In the following section, I will describe these initial signaling pathways in response to Draper activation.

Activated Draper receptor initiates a signaling cascade resulting in glial cells phagocytosing damaged cells(38, 48-50). Draper contains 15 Epidermal Growth Factor (EGF)-like repeats on its extracellular end and on its intracellular end has an immunoreceptor tyrosine-based activation motif (ITAM) and a NPxY phosphotyrosine

binding motif(48). As Figure 1 shows, the Draper pathway is complex. The intracellular adaptor protein Ced6 binds to the NPxY motif, and the non-receptor tyrosine kinase Shark and the kinase Src42A can both interact with the ITAM of Draper. During development, Ced 6 is necessary for glial pruning in *Drosophila*(51, 52). In adult flies, Shark and Src42A are both necessary for glial phagocytosis in *Drosophila* after injury(50). Downstream of Shark, the GTPase Rac1 becomes active allowing for changes in the glial cell membrane and phagocytosis of debris(53). There is evidence that Rac1 becomes activated by the GEF complex DRK/DOS/SOS, which I will discuss below(54, 55). Later in the glial migration and phagocytosis steps, I will describe a potential parallel signaling pathway to Draper, the Ced2/Ced5/Ced12 pathway, which was first identified in *C. elegans* (54, 56).

Step 2: Immune Gene and Protein Changes:

Within hours after injury, the immune gene Draper is upregulated. In *Drosophila* brains, Draper transcript levels increase as early as 1.5 hours after injury, peak at 3 hours, and remain elevated at 4.5 hours when measured via real-time PCR(48, 57). Increases in Draper protein levels can be seen with an anti-Draper immunostain within hours after injury, peak at one day, and remain raised for two days(38). This increase in Draper gene and protein is necessary for the glial immune response to occur: $\textit{Draper}^{RNAi}$ flies have no glial cell membrane extension and no phagocytosis of damaged axons(38). Anti-Draper reveals the localization of the Draper receptor to be along glial cell membranes that are reacting to the injury. Currently, the glia field recognizes that Draper must be activated to initiate the immune response, but there are missing pieces in the signaling pathway downstream of the Draper receptor. My thesis work in Chapter 2 reveals a new player in this pathway.

In response to injury, Draper transcription is upregulated via the signal transducer and activator of transcription (STAT) transcription factor (Figure 1)(53). In *Drosophila*, the

Draper promoter has STAT92e (*Drosophila* STAT) binding elements, and *STAT92e*^{RNAi} flies have reduced phagocytosis(53). Interestingly, STAT92e-mediated Draper transcription is dependent upon Rac1. In *Rac1*^{RNAi} flies, a reporter of STAT92e activity showed decreased STAT92e(53). Further, there is evidence that Draper activates c-Jun-N-terminal kinase JNK signaling, which regulates the STAT92e pathway(58). The authors of this study suggest that the primary role of *Drosophila* JNK is to regulate Draper transcription in glia after injury. Glial knockdown of dJNK blocks Draper upregulation after injury. After injury, JNK signals through mitogen-activated protein kinases (MAPKs) to increase Draper transcription by the heterodimer *Drosophila* activator protein 1 (AP-1). Interestingly, STAT and AP-1 have been shown to be involved in reactive mammalian astrocyte and microglia initiation after various insults including ischemia and Alzheimer's disease(59-62). I will describe my work examining STAT activity in more detail in Chapter 2.

Step 3: Glial Migration/Membrane Changes:

As mentioned above, glial cells are highly motile after injury or in disease conditions. Glial cells dramatically change shape and size, becoming more branched, and migrate towards the injury site. These glial changes are conserved across species. Davalos et al. has shown that microglia rapidly migrate towards a laser ablation in mice brains, while Pekny describes the dramatic cell shape changes astrocytes undergo in CNS pathologies(20, 21). In *Drosophila,* these glial cell changes are conserved, which I will describe in more detail below.

After Draper receptor activation and upregulation, the glial cells undergo dramatic morphological changes. Glial cells extend their membranes towards the site of injury to phagocytose debris. This glial membrane extension can be viewed by genetically labeling the glial cell membranes with a green or red fluorescent protein (GFP/RFP) and imaging the

brains of injured flies (more on this technique later in Chapter 2)(38). Chapter 2 has a heavy focus on glial membrane changes, so I will spend extra space introducing the molecular players involved in this process.

In *Drosophila* glia, there is evidence that Rac1 is necessary for the glial cell membrane dynamics that allow the cell to reach towards the damage and phagocytose the debris. In chapter 2, I will discuss the role of Rac1 in the glial immune pathway. Rac1 is a small GTPase (guanosine triphosphatase) in the Rho family of GTPases and is involved in several cellular processes including cytoskeletal organization, transcriptional regulation, protein kinase activation, and cell migration(63). Rac1 was discovered in 1989 as Ras-related C3 botulinum toxin substrate 1(64). Rac1 is expressed in all cell types in two forms: inactive (GDP-bound) and active (GTP-bound) form. Upon various signals, guanine nucleotide exchange factors (GEFs) activate Rac1 and GTPase activating proteins (GAPs) inactivate Rac1 (see Figure 2).

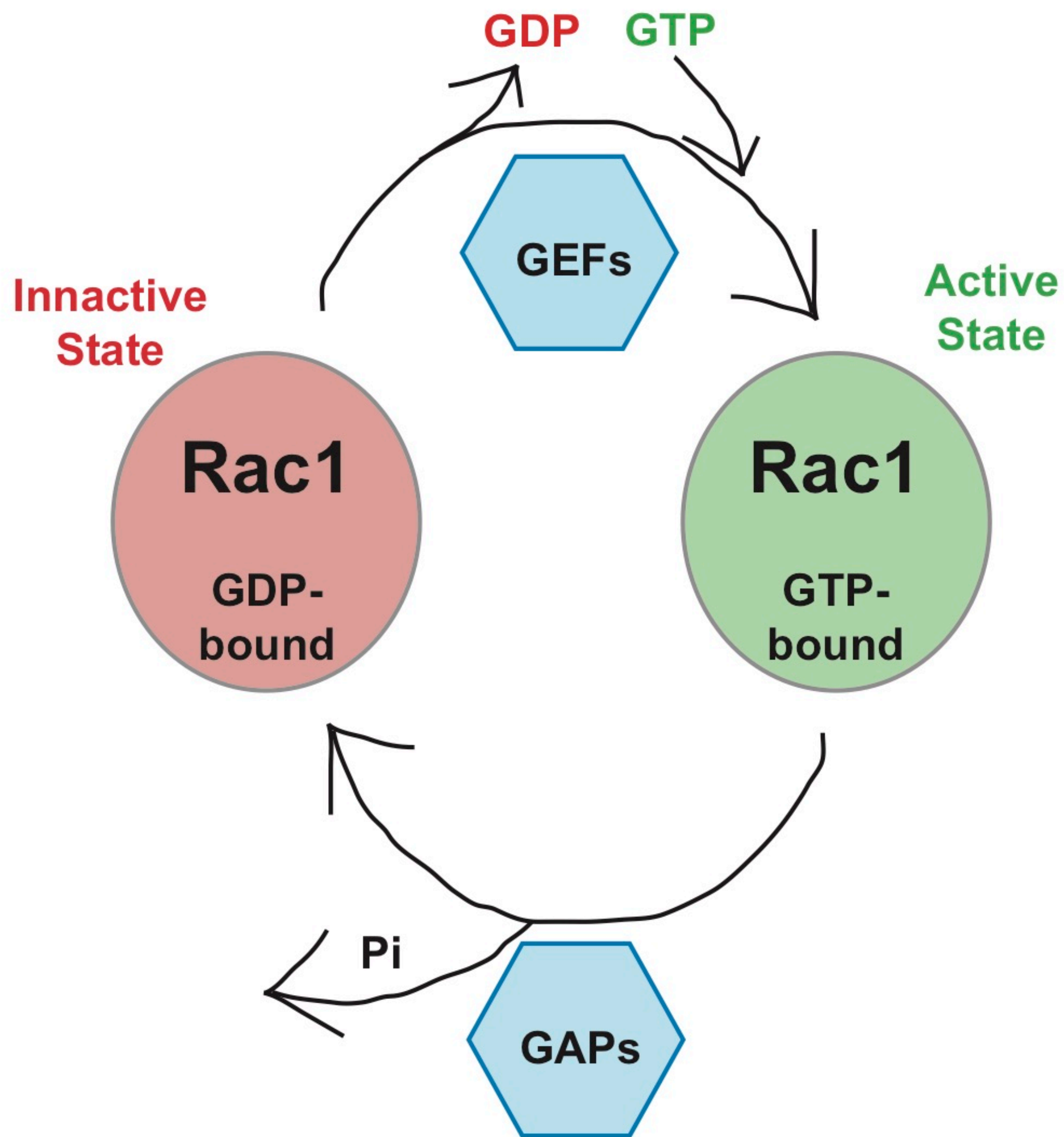
There are two GEF complexes that I will focus on in my dissertation: DRK/DOS/SOS and Crk/Mbc/Ced12. In other species, there is evidence that both of these GEFs can activate Rac signaling. SOS is typically considered an exchanger for the small GTPase Ras, however Nimnual et al. found that SOS can also activate Rac in fibroblast-like cos cells (65). Mbc is the *Drosophila* orthologue of Dock180, and is thought to activate Rac1 in a complex with Ced12 (mammalian Elmo)(66). Ced12/Elmo is a scaffolding protein with no apparent catalytic activity on its own(66). There are studies showing that Dock180 is only active towards Rac1 when coupled to Elmo, which could explain why Ced12/Elmo has been shown to activate Rho and Rac signaling pathways in *C. elegans(67, 68)*.

Rac1 is thought to be downstream of Draper in the glial immune response to injury, but there is currently a gap in knowledge about how Rac1 becomes activated in this pathway.

Rac1^{RNAi} knockdown flies phenocopy *Draper*^{RNAi} flies; there is no glial cell membrane extension and no axonal clearance(54). In 2014, Lu et al. wrote that the GEFs DRK/DOS/SOS and Crk/Mbc/Ced12 activate Rac1 in this injury paradigm and are necessary for proper glial immune functions. The authors found that expressing a constitutively active SOS (*SOS*^{JC2}) was sufficient to partially rescue axonal clearance (by 50%) in a Rac1 fly line. They also found that expressing *SOS*^{JC2} in the dominant-negative Rac1 line led to a 30% rescue of Draper protein recruitment after injury. Also, the Crk/Mbc/Ced12 mammalian orthologues, CrkII/Dock180/ELMO, have been shown to act as a GEF complex for Rac1 in mammals(68, 69). Ziegenfuss et al. found that constitutively active Rac1 can rescue axonal clearance in *Crk*^{RNAi} flies, although expressing the constitutively active Rac1 killed flies in our hands. Currently, there is a gap in evidence for how Rac1 is being activated after injury in glial cells, and my thesis work sheds new light on how else Rac1 could be activated. In chapters 2 and 3, I argue for a novel regulator of Rac1 function in glial cells.

Rac1 has several cellular functions. For example, Rac1 can affect gene transcription by activating NFkB, JNK, and MAPK, which then induce activator protein 1 (AP1) transcription factors(70). For cell migration, Rac1 is involved with lamellipodia formation and is found at the leading edge of migrating cells(63). Rac1 activates the WAVE complex, which activates the Arp2/3 complex, which leads to actin polymerization and cell membrane extensions(71). During microtubule growth, Rac1 can be activated to lead to lamellipodia extension. During neural development, Rac1 is necessary for growth cone formation and neurite extension(71). Astrocytes migrate in a Rac1-dependent manner by cell elongation, which is a microtubule based movement(70). Rac1 is also necessary for the final step in the immune pathway, phagocytosis.

Figure 2: Rac1 Activation Cycle



Rac1 activation cycle. Rac1 is inactive when bound to GDP. GEF complexes facilitate the exchange of GDP for GTP, to activate Rac1. GAP complexes "turn off" (active) Rac1 by helping to hydrolyze the GTP and return Rac1 to its inactive state.

Step 4: Phagocytosis:

Our final readout of the glial immune response is phagocytosis. In response to injury or insult, glial cells phagocytose the cellular debris or foreign objects. Phagocytosis occurs when cells internalize large particles (>0.5um), such as apoptotic cells(72). The object to be phagocytosed is recognized by receptors on the phagocytic cell, which then binds and engulfs the foreign object. As there are numerous objects that phagocytic cells will engulf, there are also numerous receptors on the phagocytic cells to recognize the various foreign objects and initialize phagocytosis. These receptors include pattern-recognition receptors, such as CD14, opsonic receptors, such as CD45, and apoptotic corpse receptors, such as TIM-1 and TIM-4(72). Once the foreign object is recognized, the next step of phagocytosis is internalization via an actin-driven process. Important regulators of this internalization process are the rho GTPases, such as Rac1(73). During phagocytosis, Rac1 acting with Cdc42 causes actin polymerization which allows for membrane extensions to engulf particles (72). In Swiss 3T3 fibroblasts, dominant-negative Rac1 transfection inhibits phagocytosis(73).

In *Drosophila*, dCrk/Mbc/dCed12 (Ced2/Ced5/Ced12), have been shown to be necessary for glial phagocytosis after injury(54). In dCrk/Mbc/dCed12 RNAi flies, glial membranes extend correctly, but phagocytosis is still reduced(54). Ced1 is necessary for apoptotic corpse phagocytosis in *C. elegans*(74). Draper and the mammalian orthologues, MEGF10 and Jedi1, are involved during development for phagocytosis of apoptotic neurons and developmental pruning(52, 75). There have been several large-scale studies performed in the *Drosophila* cell line, S2 cells, to identify molecules involved in phagocytosis. I will discuss these S2 cell studies in more detail in the introduction to Chapter 3.

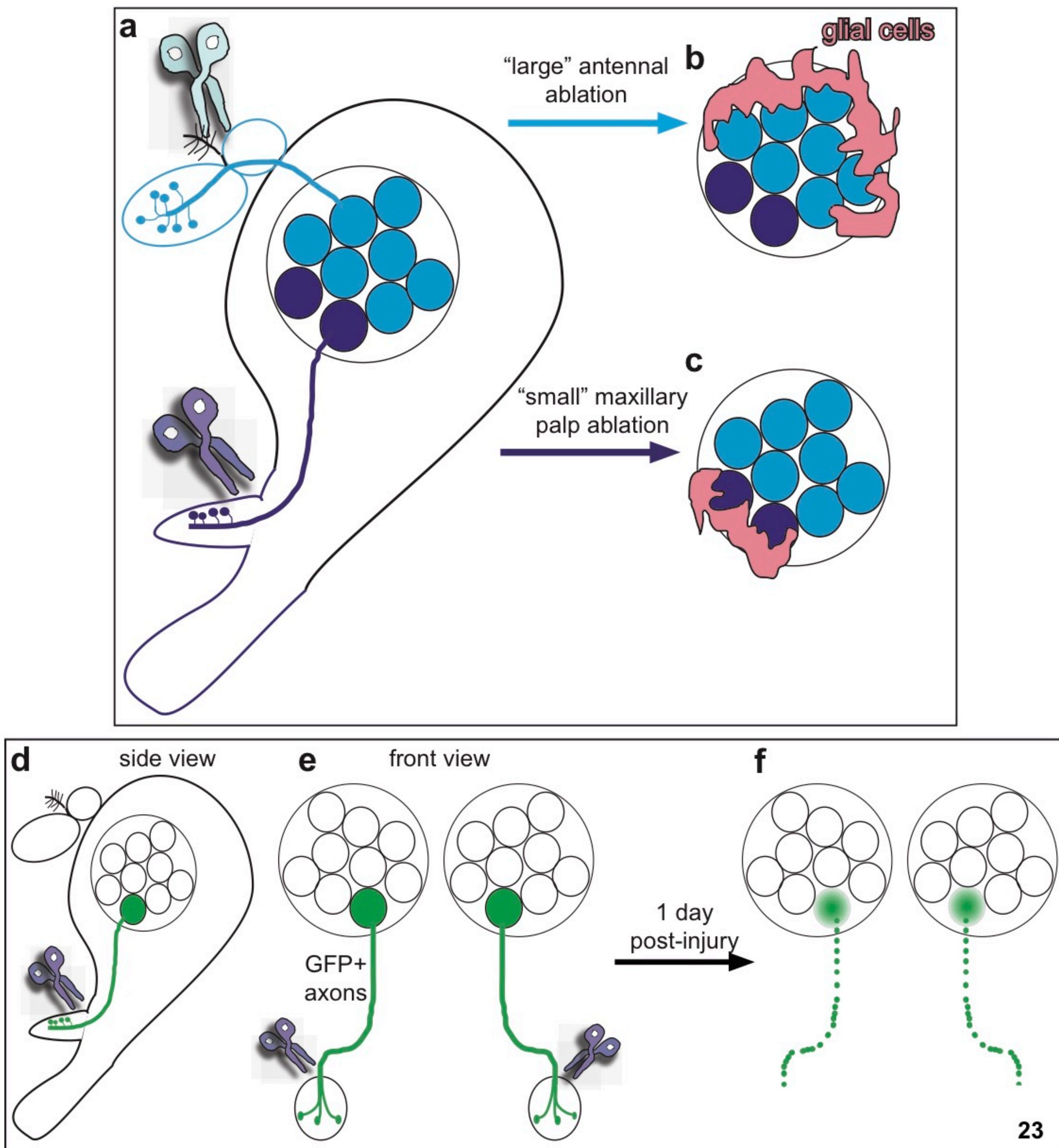
Parallel Immune Signaling Pathway:

The Draper/Ced6/Shark pathway and the parallel Crk/Mbc/Ced12 pathway appear to converge upon Rac1 and glial cell cytoskeletal changes to promote glial cell membrane extension and phagocytosis(55). These two partially parallel pathways are essential for the glial immune response, but their exact physiological roles are still under investigation. The Draper pathway appears to be most important for initiation of the immune response, while the Ced2/Ced5/Ced12 pathway has emerged as more important for later stages of phagocytosis(38, 50, 52, 54). In Draper null flies, glial cell membranes do not extend towards the injury site and phagocytosis is blocked(38). There is evidence that both of these pathways converge on Rac1. Overexpression of Rac1 can rescue phagocytosis defects in experiments with knockdowns in both the Draper and Ced2/Ced5/Ced12 pathways(54, 55). I will discuss this in more detail in Chapter 2 as I examine the role of Rac1 in the immune response.

Drosophila Olfactory System

To elicit a glial immune response in *Drosophila*, we injure the olfactory neurons using an axotomy assay. The *Drosophila* olfactory system consists of olfactory receptor neurons (ORNs) located on the peripheral olfactory organs, including the antennae and the maxillary palp(76). The ORNs extend their axons into the central brain where they synapse in glomeruli. In adult *Drosophila*, there are approximately 1300 ORNs, 62 odorant receptors, and about 50 glomeruli, allowing for creation of an olfactory map. Couto et al. have created such a map, and constructed reporter lines for all 62 odorant receptor promoters(76). These lines consist of a membrane-tethered GFP (mCD8-GFP), and each projects to a distinct glomerulus.

Figure 3: *Drosophila* Axotomy Assay. a: Side view cartoon of a *Drosophila* head, showing the antenna (blue) and maxillary palp (purple) structures with corresponding ORN axons and glomeruli in the antennal lobes of the CNS (blue, purple circles). b,c: 1 day after antennal ablation (b) or after maxillary palp ablation (c), the local ensheathing glia (pink) extend their processes to phagocytose debris around the damaged glomeruli. d: Side view cartoon of a *Drosophila* head, showing GFP+ ORN axons in the maxillary palp and the corresponding 85e GFP+ glomeruli in the antennal lobes of the CNS. e: front view of the antennal lobes, showing GFP+ ORN axons from the maxillary palp and the 85e GFP+ glomeruli. f: 1 day after maxillary palp injury, the GFP+ ORN axons and 85e glomerulus start to degenerate, and are eventually phagocytosed by ensheathing glia.



Axotomy Assay

To elicit an immune response, we injure the maxillary palp or antennal structures, severing the axons of the ORNs (see Figure 3). The antennal ablation injures about 80% of ORNs, while the maxillary palp ablation is a milder injury, affecting about 20% of ORNs. This smaller injury results in more acute, local changes in the responding glial cells. We can genetically label a subset of the ORNs using the membrane-tethered GFP lines described above. After injuring the antenna or maxillary palp, we dissect out the brains, perform antibody staining, and image the brains using a confocal microscope. Using this technique, we can image the degeneration of the damaged GFP+ ORN axons. These axons undergo Wallerian degeneration, where the axon distal to the injury fragments. The damaged axons are then phagocytosed by the surrounding ensheathing glia within 1-5 days(49). We analyze the ensheathing glial phagocytosis of the damaged ORNs by quantifying the GFP fluorescence of the axons. We can also use an antibody against the Draper immune receptor to visualize changes in Draper receptor localization after injury. We observe an increase in Draper receptor along the ensheathing glial membranes at the site of injury. After an antennal ablation, we observe an increase in Draper around the antennal lobes. Ablating the maxillary palp structure injures the axons that innervate the 85e glomeruli (labeled by Couto et al.), and we see Draper accumulation along the glial membranes surrounding this injured glomerulus (38). Further, we can genetically label the membranes of the glial cells using a membrane-bound red fluorescent protein (mcd2::RFP), and use this technique to image glial membrane extension towards the site of injury. Therefore, the axotomy assay provides us with a straightforward way to analyze the various steps of the glial immune response: changes in immune proteins, glial membrane dynamics, and phagocytosis of debris. In Chapters 2 and 3, I use this axotomy assay to examine the role of a novel signaling member, the Protein Phosphatase 4 complex.

Introduction to protein phosphatase 4

Protein Phosphatase 4 Structure:

The protagonist of this thesis is the Protein Phosphatase 4 (PP4) complex, which, in Chapter 2, I identify as a novel signaling member of the glial immune cascade. PP4 is a ubiquitous phosphatase in the protein phosphatase 2A (PP2A) family. PP4 is a serine/threonine phosphatase, involved in numerous cellular mechanisms, and regulates cellular processes independently of other PP2A family members(77). As Cohen et al. describe in their 2005 minireview, PP4 consists of a catalytic subunit (PP4c) and regulatory subunits (R1, R2, and a variable R3 in mammals), which control the specificity and action of PP4c (77). In mammals, R1 and R2 can form a complex with PP4c, or R2 and R3 can form a complex with PP4c. In *Drosophila*, there are two regulatory subunits, R2 and Falafel (PP4r3), which form a complex with PP4c. Gingras et al. showed that R2 and R3/Falafel must form a complex before they can interact with PP4c(78). Complex specificity is thought to be due to the interaction of the regulatory subunits with the catalytic subunit. Without the regulatory subunits, PP4c alone is more promiscuous. It has been speculated that the regulatory subunits are inhibiting the catalytic subunit, as opposed to narrowing substrate specificity(78).

PP4 has been studied in numerous systems and cell types and has been shown to be involved in several cellular functions. In the next section of this introduction, I will describe the current literature about the roles of PP4. It is worth noting that there is little evidence of a PP4 functional role in glial cells, so my work in this thesis sheds light on a novel PP4 function. Furthermore, much PP4 research has focused on development or cancer cells, while my work has used *in vivo,* adult *Drosophila*. Importantly for my thesis work, several recent studies have shown a role of PP4 in immune responses. I will also discuss the current

evidence that PP4 could be regulating Rac1. This background is important for understanding the significance of my findings in Chapter 2.

Protein Phosphatase 4 Functional Roles in…

Cell Cycle Regulation and Development:

Two of the most well-studied PP4 topics are cell cycle regulation and development. PP4c is necessary for centrosome maturation in *Drosophila* and *C. elegans(79).* PP4 is localized to the centrosome during cell division in *Drosophila, C. elegans*, and humans(80-82). Studies in *C. elegans* have shown that PP4 is present at the centrosome and has two main roles: it recruits pericentriolar material constituents to the centrosome and is necessary for microtubule activation(82). Further, Toyo-oka et al. found that at the centrosome, PP4c negatively regulates Cdk1 activity, which phosphorylates NDEL1. PP4 disruption in MEF cells in mice leads to microtubule disorganization. This data suggests that PP4c coordinates with Cdk1 for proper microtubule organization during cell division(83). PP4r3 was named as Falafel in *Drosophila* due to its null phenotype – loss of Falafel lead to dorsal tube closure defects, which one could guess looked like falafel in a pita(84). This pivotal experiment shows the importance of PP4 during development, which presented a challenge to me during the genetic manipulations I will discuss in Chapter 2.

Cancer, DNA Damage Repair, and Cell Survival:

There are also numerous studies showing the roles of PP4 in cancer, DNA damage repair, and cell cycle. PP4 has been shown to interact with Cisplatin, a cancer treatment drug that damages DNA. Deletion of PP4 subunits in yeast and *Drosophila* lead to increased cisplatin sensitivity(78, 85). In a morphological study, Wang et al. found that PP4 is overexpressed in a human breast cancer cell line, where it could be responsible for aberrant protein phosphorylation states associated with tumors(86). Mohammed et al. found that

PP4c overexpression or knockdown affect survival of breast cancer cells in two separate cell lines, suggesting that PP4 is involved in breast cancer development(87).

There is a body of evidence that PP4 may be affecting DNA repair. A yeast two-hybrid screen identified Psy2 (the yeast Falafel homolog) as interacting with Rad53, which is critical for DNA damage repair(78, 88). Further, PP4 in yeast can associate with transcription elongation factors Spt4 and 5, which are linked to DNA damage-induced dephosphorylation of Pol II, and which leads to a transcriptional shutdown(89). In human cells, PP4 dephosphorylates γH2AX in response to DNA damage, which is a necessary step for DNA repair(90).

There is conflicting evidence to the role of PP4 in apoptosis and cell survival. One study suggesting that PP4 is pro-apoptotic found that depleting endogenous PP4c mRNA and protein levels by 50% resulted in less apoptosis by dexamethasone and UV radiation(91). In HEK-293 cells, reduced PP4 levels led to increased phosphorylation of apoptotic regulators Bad and PEA-15, leading to less cell death (92). However, in *Drosophila* and *C. elegans* embryos, it was found that depleting PP4 lead to reduced survival(82). The differences in these reports could be due to use of different cell types, or could reflect the complicated balance of regulators of cell survival.

JNK and Insulin Signaling:

PP4 can also interact with the c-Jun N-terminal kinase (JNK) pathway: expression of PP4 activated the JNK pathway, but there is no evidence that PP4 directly interacts with JNK(93). In human prostate cancer cells transfected with PP4, there was increased JNK activity(94). These studies are relevant to my work with glial cells since we know that *Drosophila* JNK is necessary in glial cells for the immune response, although I have no evidence for a PP4-JNK link in my studies.

In HEK293 cells, PP4 was found to interact with insulin receptor substrate protein 4 (IRS-4) after stimulation with TNF-a, and that PP4 downregulated IRS-4(95). Zhao et al. found that PP4 can form a complex with IRS-1 in HepG2 cells, which results in less IRS-1 expression by a JNK-mediated phosphorylation event(96). Our lab has previously published work showing that insulin-like signaling contributes to the immune response, so this PP4-insulin link is intriguing(97).

Immune Defense

Interestingly, the PP4 complex has been implicated in immune defense in numerous cell types other than glia. In chapter 2, I discuss my evidence for a novel PP4 immune function in glial cells, and I will now introduce the other studies suggesting that PP4 has an immune function. In human embryonic kidney (HEK-293) cells, PP4 has been shown to interact with the transcription factor nuclear factor kB (NF-kB), which is involved in the immune response and also tumorigenesis(98). In mouse macrophage RAW264 cells, Chen et al. found PP4 negatively regulates TRAF-6 and LPS-mediated NF-kB activation by suppressing the ubiquitination of TRAF6 (99). TRAF-6 is necessary for proper LPS signaling during immune responses. The authors found that PP4 interacts with TRAF-6 in a yeast two-hybrid screen(99). In *C. elegans*, Smek1 (PP4r3/Falafel) has been shown to be involved in various forms of stress resistance, including innate immune responses(100). The authors found that decreased *smk-1* expression in worms exposed to the pathogenic bacterium *P. aeruginosa* reduced worm lifespan. In RAW264 cells and HEK-293 cells, Zhan et al. identified PP4 to be involved in suppressing type I interferon production induced by virus by PP4 dephosphorylating TBK1(101). TBK1 is essential for modulating type I interferon during the innate immune response. The authors also found that knocking down PP4c in mice *in vivo* suppressed the innate immune response to injected virus. These studies together paint

a picture of the functional role PP4 plays in the innate immune response in various cell types.

There have also been several studies examining the role of PP4 in T cells. Liao et al. found that PP4 is necessary in T cells for adaptive immunity(102). Depleting PP4 in T cells led to reduced T cell expansion and proliferation. The authors also found elevated AMPK levels in these PP4c-depleted T cells, that PP4 co-immunoprecipitated with AMPK, and PP4 overexpression inhibited AMPK phosphorylation(102). AMPK is an energy sensor kinase with known roles in regulating cell polarity and motility, and its location varies between the nucleus and cytoplasm dependent upon stress and signals from the MEK/ERK pathway(103). There are also several studies suggesting that AMPK can regulate Rac1 activity(104-107). There is, however, conflicting evidence for AMPK activating Rac1 and cell migration, or for the opposite. Yan et al. found that increased AMPK activity inhibits cell migration by phosphorylating Pdlim5, which suppresses Rac1 activity (107). Bae et al. found that increased AMPK leads to more phagocytosis and more Rac1 activation in macrophages infected with *E. coli*(104). Kou et al. and Lee et al. also found that increases in AMPK activity lead to increases in Rac1 activity(105, 106). In chapter 3, I will discuss my preliminary work researching AMPK in the glial immune response.

Liao et al. found that PP4 is necessary for proper gut immunity in mice (108). These mice had T-cell specific ablation of PP4c, which resulted in less T-cell proliferation, lower levels of IL-10 and other immune signals, and increased bacteria associated with colitis(108). Shui et al. also found that mice with T-cells depleted of PP4 had reduced T-cells proliferation and decreased immune responses (109). These results suggest that PP4 is necessary for proper T-cell function during the innate immune response in the

periphery(109). With these studies in mind, I decided to examine the role of PP4 in glial cells in the innate immune response, which I will discuss in depth in Chapters 2 and 3.

Rac1 Signaling:

As I mentioned earlier, Rac1 is a necessary signaling component of the glial immune response. Interestingly, a study led by Martin-Granados found a novel link between PP4 and Rac1 function in human cells (110). The authors found that cells with 70% PP4c depletion have decreased migration towards a chemoattractant. They also found that these cells with 70% PP4c depletion have significantly less activated Rac1 levels before and after stimulation with EGF compared to control cells. This study suggests that PP4 can regulate Rac1 activation levels. These 70% PP4c depleted cells also showed decreased F-actin localization in cell extensions (lamellipodia-like structures), suggesting that PP4 is necessary for Rac1 mediated control of actin during cell migration. I will discuss this work in relation to my own findings in Chapter 2.

Protein Phosphatase 4 Nuclear Location and Functions:

Purportedly, PP4 function is tied to its location – the regulatory subunit PP4r3 (Falafel, SMEK) is thought to be able to translocate the nucleus and confer substrate specificity. PP4 is primarily located in the nucleus of many species(111, 112), including *Drosophila* (113). PP4r3 has three Nuclear Localization Signal (NLS) motifs, one Nuclear Export Signal (NES), and an EVH1 domain, which are all believed to contribute to the nuclear localization of the entire PP4 complex. In Chapter 3, I discuss how PP4 localization could be contributing to its function in the glial immune response.

Nuclear PP4c and PP4r1 have been shown to dephosphorylate histone deacetylase 3 (HDAC3), and depleting PP4c was shown to increase HDAC3 deacetylase activity(114). This suggests that the nuclear PP4 complex plays a role in histone acetylation and chromatin

remodeling. As mentioned earlier, Nakada et al. and Chowdhury et al. showed another nuclear function: PP4 is necessary for DNA damage repair, and that the complex phosphorylates γ-H2AX, which stabilizes proteins that repair the break site(111).

In *Dictyostelium*, the regulatory subunit SMEK is cytoplasmic in vegetative cells, but translocates into the nucleus upon receiving starvation signals(112). The EVH1 domain of SMEK is necessary and sufficient for SMEK's cortical location in vegetative cells, and the NLS domain targets SMEK to the nucleus in response to starvation signals. These signals lead to increased transcription of the MEK/ERK1 pathway. Mendoza et al. found that SMEK had to be nuclear for these changes in MEK/ERK1 transcription to occur, suggesting that SMEK is able to influence transcription (112).

In yeast, Oler et al. showed that nuclear PP4 dephosphorylates nuclear Maf1, leading to acute RNA polymerase III repression (115). This study suggests that PP4 must be nuclear to have these effects, and that the interaction of PP4 and Maf1 functions to ensure proper cell nutrition and stress responses(115). In Chapter 3, I examine PP4 nuclear location and function to determine if PP4 nuclear translocation affects the glial immune response.

UNRESOLVED QUESTIONS:

There are several unresolved questions about how glial cells respond to injury, such as what are the "find me" and "eat me" signals, what affects transcription of immune genes like Draper, and what is upstream of Rac1 to activate glial cell membrane extension and phagocytosis? My thesis work has focused on furthering our understanding of the signaling pathways underlying this immune response, specifically concentrating on glial cell membrane dynamics. In Chapter 2, I will examine the question of how the PP4 complex,

which has known immune functions, is involved in the glial cell response to injury, and I will also work to answer how Rac1 becomes activated after injury. In Chapter 3, I examine how the localization of the PP4 regulatory subunit Falafel could be regulating PP4 complex targets and contributing to the immune response, and I delve into what these PP4 targets could be. Together, these chapters reveal the novel role of the Protein Phosphatase 4 complex in the glial immune response to neuronal injury.

Chapter 2. Protein phosphatase 4 coordinates glial membrane recruitment and phagocytic clearance of degenerating axons in *Drosophila*

Modified from: Lilly M. Winfree[1], Sean D. Speese[1] and Mary A. Logan[1,*]. Protein phosphatase 4 coordinates glial membrane recruitment and phagocytic clearance of degenerating axons in *Drosophila.* Cell Death and Disease, Submitted Nov 19, 2016.

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Abstract

Neuronal damage induced by injury, stroke, or neurodegenerative disease elicits swift immune responses from glial cells, including altered gene expression, directed migration to injury sites, and glial clearance of damaged neurons through phagocytic engulfment. Collectively, these responses hinder further cellular damage, but the mechanisms that underlie these important protective glial reactions are still unclear. Here, we show that the evolutionarily conserved trimeric Protein Phosphatase 4 (PP4) serine/threonine phosphatase complex is a novel set of factors required for proper glial responses to nerve injury in the adult *Drosophila* brain. Glial-specific knockdown of PP4 results in reduced recruitment of glia to severed axons and delayed glial clearance of degenerating axonal debris. We show that PP4 functions downstream of the glial engulfment receptor Draper to drive glial morphogenesis through the guanine nucleotide exchange factor (GEF) SOS and the Rho GTPase Rac1, revealing that PP4 molecularly couples Draper to Rac-1-mediated cytoskeletal remodeling to ensure glial infiltration of injury sites and timely removal of damaged neurons from the CNS.

INTRODUCTION

Glial cells continuously survey the brain and respond swiftly to any form of stress or damage (24). Acute insults, as well as chronic neurodegenerative conditions, trigger robust immune responses from glia (21, 22). Reactive glia undergo striking morphological changes and infiltrate injury sites to rapidly phagocytose cellular debris (49, 116). Glial changes in cell shape, size and migration in response to neurodegeneration are highly conserved hallmark responses to trauma in species ranging from *Drosophila* to humans. In all instances, glial cells either migrate to injury sites or, in instances where the cell soma remains in a fixed location, reactive glia send dynamic membrane projections into regions that house damaged neurons (20, 49, 117). Importantly, inhibiting these glial morphogenic responses delays phagocytic clearance of neurotoxic cellular debris, which can attenuate post-injury neuronal plasticity and trigger secondary inflammatory reactions to exacerbate damage (118-121). Despite the fact that glial cells undergo significant changes in size and shape in order to access trauma sites and clear damaged cells, the molecular mechanisms responsible for glial migration and directed extension of processes are not entirely understood.

Acute axotomy of the olfactory nerve in adult *Drosophila melanogaster* is a well-established injury paradigm to investigate the molecular mechanisms that govern glial morphogenesis and phagocytic function in response to axon degeneration (38, 48-50, 54, 55, 58, 116, 122). *Drosophila* glia are morphologically and functionally similar to mammalian glia, and fly glial responses to axon injury mirror those that occur in vertebrate models (40, 116, 123, 124). After severing adult maxillary palp or antennal olfactory nerves, local ensheathing glia extend membrane projections to infiltrate antennal lobe neuropil and phagocytose degenerating axonal debris; glial invasion of the antennal lobes

requires the highly conserved glial immune receptor Draper/MEGF10 (49). Activated Draper signals via Src family kinases, which leads to activation of Rac1 and cytoskeletal remodeling (50, 53-55, 58). Recent work has identified two guanine nucleotide exchange factors (GEFs), Crk/Mbc/Ced-12 and DRK/DOS/SOS, that can activate Rac1 in this context, directly associating with Rac1 to catalyze the exchange of GDP for GTP(54, 55), but we still have a poor understanding of the molecular effectors that couple Draper to Rac1-mediated cytoskeletal changes.

The evolutionarily conserved serine/threonine protein phosphatase 4 (PP4) complex contributes to diverse cellular functions, including cell proliferation and apoptosis, and is required for proper embryonic development across species (77, 112, 125-130). The PP4 complex consists of three subunits: one catalytic subunit (PP4c), which is required for dephosphorylation of target proteins, and two regulatory subunits (PP4r2 and Falafel/PP4r3), which control subcellular localization of the complex and specificity of phosphatase target association (77, 78). Interestingly, PP4 is also linked to cell migration and tumor invasiveness in several organisms and cell types. For example, in the slime mold *Dictyostelium discoideum,* PP4 phosphatase activity is necessary for chemotaxis, and in human colorectal carcinoma cells, PP4 activity promotes cell migration (112, 131). PP4 is also a positive regulator of Rac1-dependent cell movement in cultured Hek293 cells (110, 112, 131). The role of the PP4 complex in glial responses to neural injury, however, has never been explored. Here, we demonstrate a novel role for PP4 in glia as they respond to severed axons. We propose that PP4 is a downstream effector of the Draper receptor that signals through the SOS GEF complex and the GTPase Rac1 to promote proper glial membrane infiltration of injury sites and clearance of degenerating neuronal material.

RESULTS

PP4 is required for proper glial clearance of severed axons

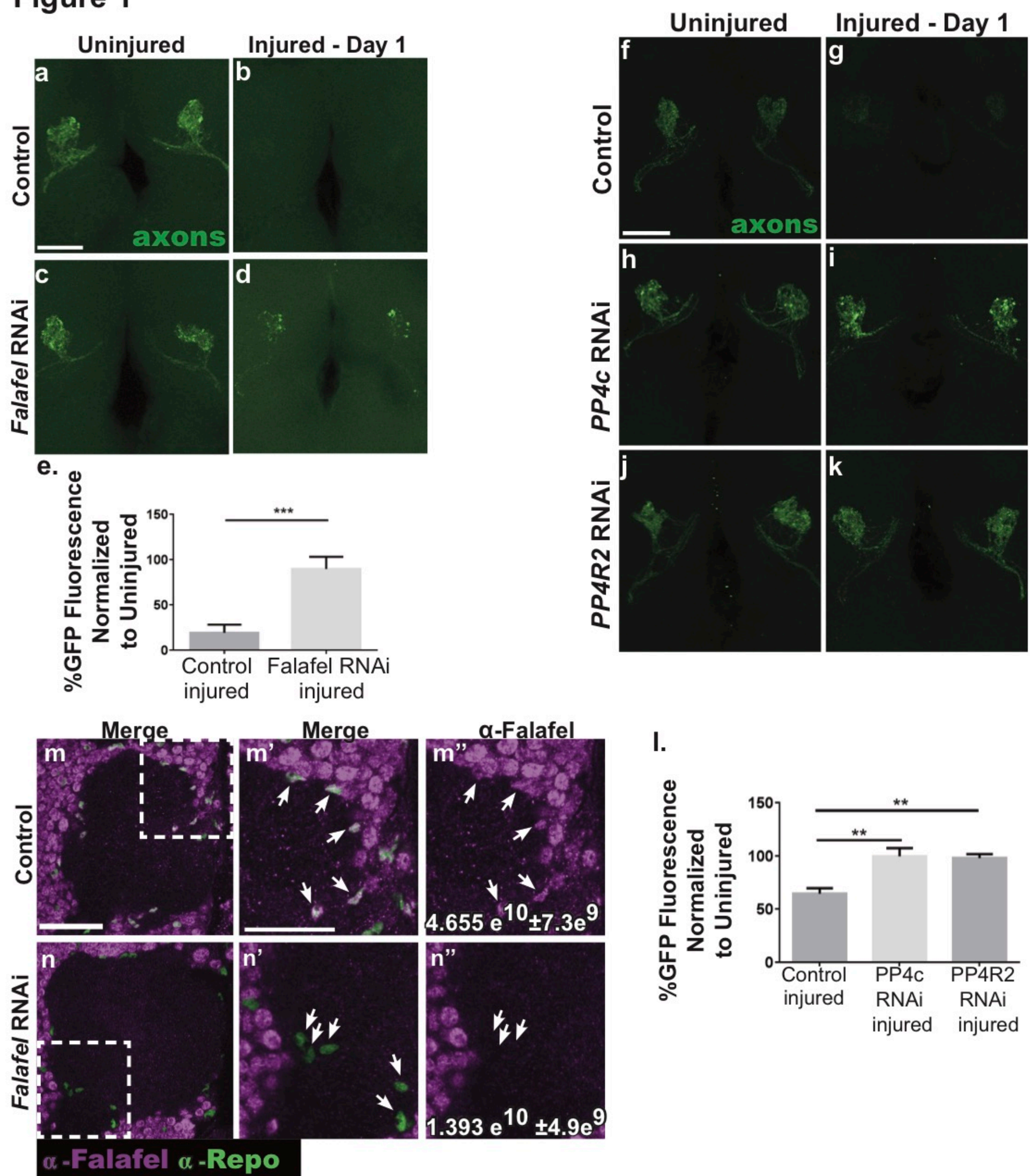
To inhibit PP4 function in adult *Drosophila* glia, we expressed *UAS-falafel*^{RNAi} with the pan-glial driver *repo-Gal4* and assayed clearance of degenerating olfactory receptor neuron (ORN) axons. Importantly, because PP4 phosphatase activity is critical for proper development, these flies also carried the tubulin-Gal80^{ts} transgene, which allows us to temporally regulate GAL4 activity and express *falafel*^{RNAi}, specifically in adult glia (132). To monitor clearance of axonal debris, a subset of maxillary palp olfactory receptor neurons (ORNs) were labeled with a membrane-tethered version of GFP (*OR85e-mCD8::GFP*). We severed the maxillary nerves that project into the antennal lobes and then quantified axonal GFP+ fluorescence in each OR85e glomerulus one day after axotomy using previously published methods (38, 48, 116). In controls, most GFP+ axonal debris was cleared within 1 day (Fig 1a,b). In *falafel*^{RNAi} flies, significantly more GFP+ axonal material was present 1 day after injury (Fig 1c-e, $p<0.001$), suggesting that Falafel is necessary for proper glial engulfment of severed axons. To confirm efficacy of *falafel*^{RNAi}, we performed immunostaining against Falafel and the glial-specific transcription factor Repo on adult brains. In control animals, Falafel appeared to be localized, or enriched, in glial nuclei (Fig 1m), and we detected a 70% reduction of glial nuclear Falafel fluorescence in *Falafel*^{RNAi} flies (Fig 1m,n).

In addition to Falafel, the PP4 complex contains a second regulatory subunit, PP4r2, and a catalytic phosphatase subunit, PP4c. To determine if a complete PP4 complex is necessary for proper glial engulfment of debris, we again used the *Gal4/Gal80*^{ts} system to knockdown PP4c (*UAS-PP4c*^{RNAi}) and PP4r2 (*UAS-PP4r2*^{RNAi}) independently in adult glia. One day after severing maxillary nerves, we observed significantly more OR85e GFP+ axonal debris lingering in the antennal lobes in *PP4c* and *PP4r2*-depleted flies (Fig 1f-l, $p<0.01$). To confirm efficacy of our *Gal4/Gal80*^{ts} experiments, we repeated these clearance

assays while maintaining flies at the permissive temperature of 22°C, and observed normal clearance (Supplemental Fig 1a-i). Finally, we performed a short time course to assess clearance delays in PP4c-depeleted flies and found that significantly more axonal material persisted in the brain for at least a week after axotomy (Supplemental Fig 1j). Together, these results indicate that the PP4 serine/threonine phosphatase complex is essential for efficient glial engulfment of axonal debris in the adult brain.

Figure 1: The PP4 complex subunits Falafel, PP4c, and PP4r2 are required for glial clearance of degenerating axons. a-d: Representative maximum intensity projection confocal images (z-stack, 15 um) show GFP-labeled OR85e axonal projections (green) in antennal lobes of uninjured (a,c) and injured (b,d) adult flies. e: Quantification of OR85e GFP fluorescence from experiment in panels a-d normalized to uninjured; mean ± s.e.m. plotted; 1-way ANOVA ***$P < 0.001$. f-k: Representative maximum intensity projection images (z-stack, 15um) shown in antennal lobes of uninjured (f,h,j) and injured (g,i,k) adult flies. l: Quantification of OR85e axonal debris (GFP fluorescence) from experiment in panels f-k normalized to uninjured conditions; mean ± s.e.m. plotted; 1-way ANOVA **$P < 0.01$. m-n: Representative antennal lobe regions (z=3um) show cells stained with anti-Falafel (magenta) and anti-Repo (glia nuclear marker, green) in control (m) and Falafel RNAi (n). Falafel fluorescence intensity in Repo+ glia nuclei was quantified from the entire central brain. See values (white font) in panels m'' and n''. Unpaired t-test ****$P < 0.0001$. Scale bars = 20 um.

Figure 1



Genotypes: Control = OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/+. Falafel RNAi = OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/UAS-FalafelRNAi. PP4c RNAi = OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4cRNAi; repo-Gal4/+. PP4r2 RNAi = OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4r2RNAi; repo-Gal4/+.

PP4 is essential for proper recruitment of Draper and glial membranes to severed axons

Following ORN axotomy, local ensheathing glial cells robustly upregulate the Draper receptor and extend their membranes into the antennal neuropil regions to phagocytose axonal debris (49), and, in fact, Draper is essential for these cells to invade the antennal lobe neuropil and access degenerating nerves (5,18). To determine if recruitment/accumulation of Draper and glial membranes was altered in PP4-depleted flies, we first expressed RNAi against Falafel (*UAS-falafel*^{RNAi}), PP4c (*UAS-PP4c*^{RNAi}), or PP4r2 (*UAS-PP4r2*^{RNAi}) in adult glia using the *Gal4/Gal80*^{ts} system, severed maxillary nerves, and immunostained brains for Draper. In controls, one day after maxillary nerve axotomy, we observed a significant increase in Draper on maxillary ORN-innervated glomeruli (Fig 2b,i), but this response was significantly attenuated in *falafel*^{RNAi}, *PP4c*^{RNAi}, and *PP4r2*^{RNAi} animals (Fig 2d,f,h,i $p<0.0001$), suggesting that the PP4 complex is essential for proper recruitment of Draper to severed nerves.

Next, because Draper accumulation on injured nerves is tightly coupled to the process of glial infiltration of neuropil, we assessed glial membrane responses in control and PP4c knockdown flies. To visualize glial membranes, we used a fly line expressing membrane-tethered red fluorescent protein (RFP) (*UAS-mCD4::RFP*) under the control of *repo-Gal4*, and again employed the *tubulin-Gal80*^{ts} system to express *PP4c*^{RNAi} specifically in adult glia. One day after antennal nerve axotomy, we observed a striking increase in ensheathing glial membrane RFP+ fluorescence around the antennal lobes (RFP in gray scale, Fig 3b,b',e $p<0.01$) which represents expansion of responding glial membranes (49). Importantly, in flies expressing glial *PP4c*^{RNAi}, there was no detectable increase of glial membrane RFP after injury (Fig 3d,d',e). Similarly, in control animals, maxillary palp ablation results in accumulation of glial membranes on the maxillary nerve (Fig 3f-g',j, $P<0.0001$). We did not detect an increase in glial membrane expansion along injured

maxillary nerve in *PP4c^{RNAi}* flies (Fig 3h-j). Finally, in uninjured flies, we did not observe any obvious changes in gross glial morphology following PP4 knockdown, nor did we detect a decrease in glial cell numbers (Supplemental Fig 2). These results indicate that the PP4 complex is required in adult glia to activate a program that drives dynamic glial membrane responses to axotomy.

Figure 2: PP4 is required for proper recruitment of Draper to degenerating axons. a-h: Representative single z-slice (1um) show anti-Draper fluorescence in one antennal lobe of uninjured (a,c,e,g) and injured (b,d,f,h) adult flies. White dotted outlines show OR85e glomeruli (visualized with OR85e-mCD::GFP, not shown) and representative areas of Draper fluorescence quantification. i: Draper fluorescence quantified in z-stack of 15um, normalized to uninjured conditions. Uninjured Draper set at a value of 1; mean ± s.e.m. plotted; 1-way ANOVA ****P < 0.0001. Scale bars = 20 um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80^{ts}/+; repo-Gal4/+.* *Falafel RNAi = OR85e-mCD8::GFP,tub-Gal80^{ts}/+; repo-Gal4/UAS-Falafel^{RNAi}.* *PP4c RNAi = OR85e-mCD8::GFP,tub-Gal80^{ts}/UAS-PP4c^{RNAi}; repo-Gal4/+. PP4r2 RNAi= OR85e-mCD8::GFP,tub-Gal80^{ts}/UAS-PP4r2^{RNAi}; repo-Gal4/+*.

Figure 2

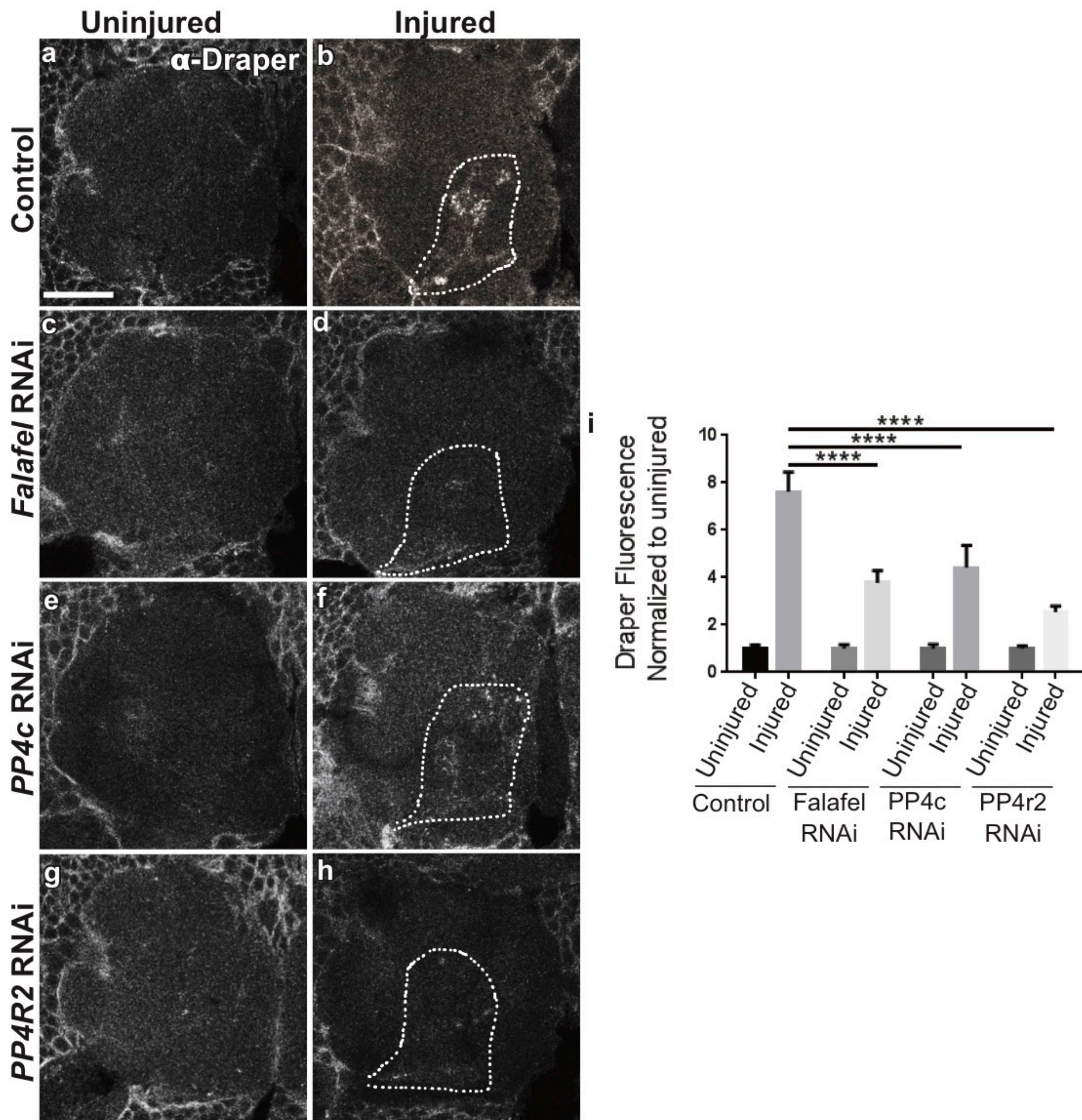
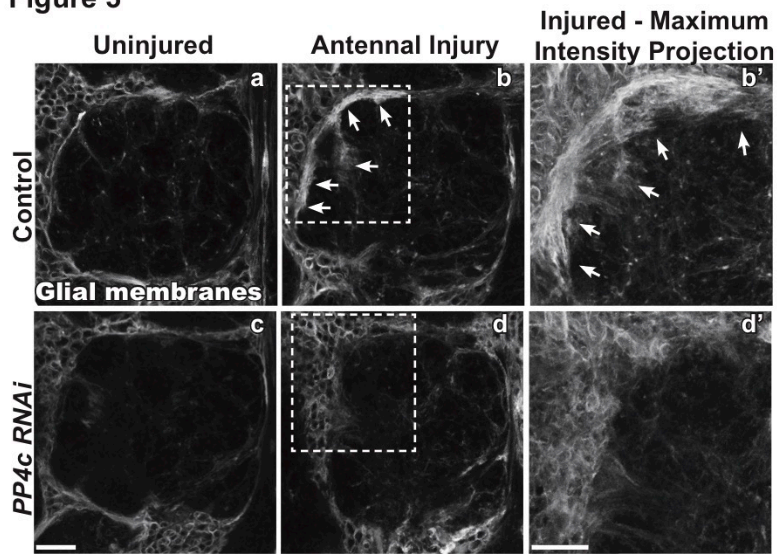


Figure 3: PP4 is necessary for glial membrane expansion and recruitment to severed axons after nerve injury. a-d: Representative antennal lobe z-stacks (3um) show glial membrane-RFP in greyscale in uninjured (a,c) and injured (b,d) adult flies. b', d': Zoomed in view of white boxed regions in b and d. e: Quantification of RFP+ glial membrane fluorescence, normalized to uninjured condition. Uninjured RFP fluorescence set to 1; N: control: 4, control injured: 6, PP4c: 18, PP4c injured: 24; individual data points with mean ± s.e.m. plotted; 1-way ANOVA $**P < 0.01$. f-i: Representative antennal lobe z-stacks (15 um) show RFP-labeled glial membranes (greyscale) and OR85e (green) in uninjured (f,h) and injured (g,i) adult flies. f', g', h', i': Zoomed in view of blue boxed regions in f-i show glial membrane accumulation on severed maxillary nerves in control animals (g'). j: RFP membrane fluorescence quantified, normalized to uninjured condition. Uninjured RFP fluorescence set to 1; individual data points with mean ± s.e.m. plotted; 1-way ANOVA $****P < 0.001$. Scale bars = 20 um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80*^{ts}*/+, repo-Gal4/repo-LexA, LexAop-mCD2::RFP. PP4c RNAi = OR85e-mCD8::GFP,tub-Gal80*^{ts}*/PP4c*^{RNAi}*; repo-Gal4/repo-LexA, LexAop-mCD2::RFP*.

Figure 3



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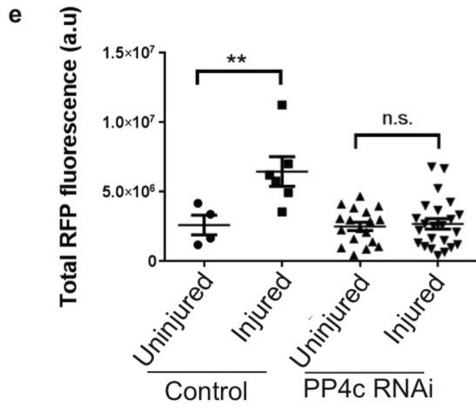
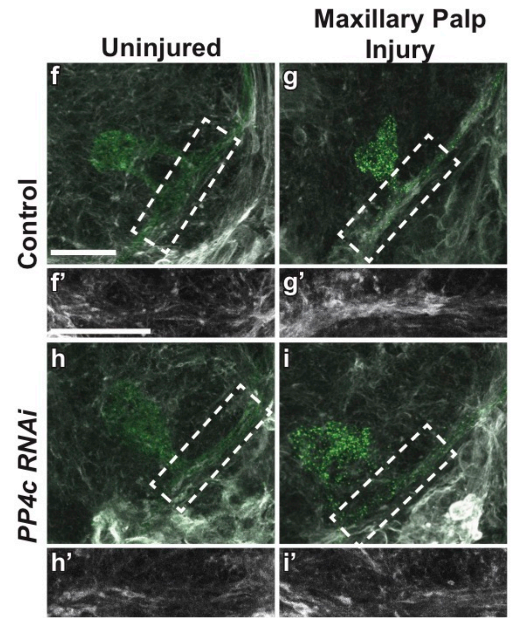
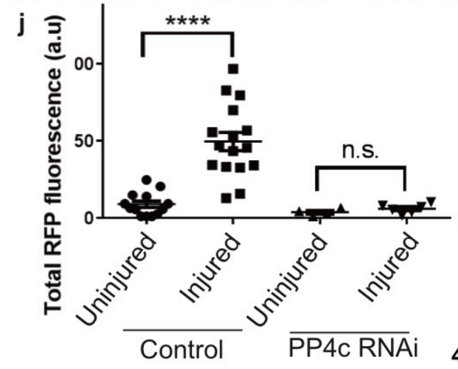






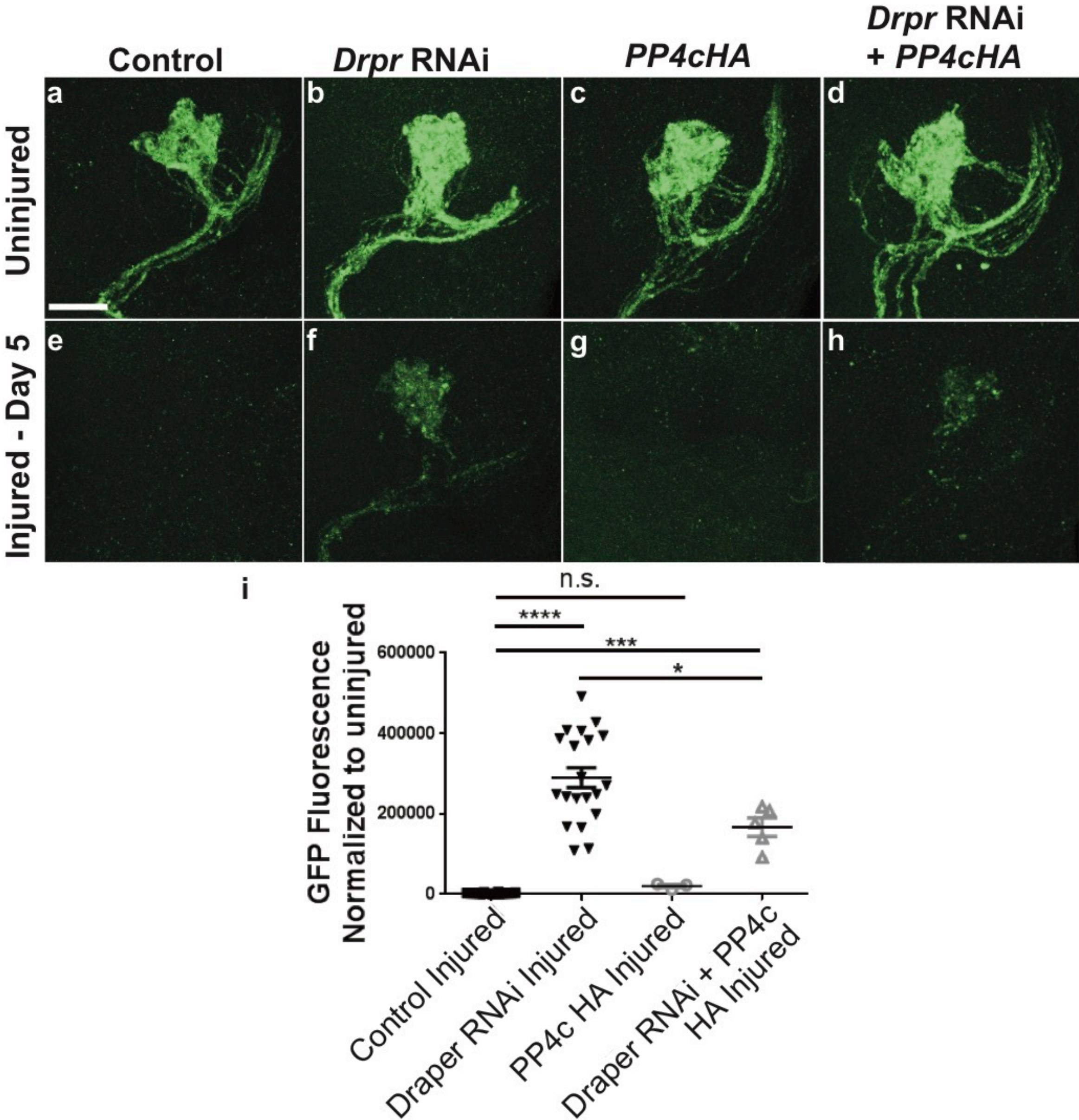
PP4 functions downstream of Draper

Simply reducing basal levels of glial Draper is sufficient to delay Draper recruitment to severed nerves and clearance of axon debris in the adult brain (5, 59). To examine if basal Draper levels were reduced in PP4 knockdown flies, we quantified Draper in the areas immediately adjacent to the antennal lobes in control flies and animals expressing *Falafel*^{RNAi}, *PP4c*^{RNAi}, or *PP4r2*^{RNAi} in adult glia. Draper levels were unchanged in *Falafel*^{RNAi} and *PP4r2*^{RNAi} flies (Supplemental Fig 3a). Basal levels of Draper were unexpectedly increased in PP4c^{RNAi} flies (Supplemental Fig 3a), as confirmed by Western blot analysis (Supplemental Figure 3b,c).

Because PP4 inhibition did not lower Draper levels in the adult brain, we reasoned that the PP4 complex may function downstream of Draper to promote glial infiltration of antennal lobes and clearance of severed olfactory axons. To further explore this, we overexpressed PP4c (*UAS-PP4cHA*) and *Draper*^{RNAi} in adult glia and assayed OR85e axonal clearance. Glial depletion of Draper significantly inhibits clearance of severed axons as compared to controls (5) (Figure 4a,b,e,f,I, $p<0.0001$). Glial expression of PP4c partially, but significantly, reversed this clearance defect in *Draper*^{RNAi} flies (Fig 4d,h,I, $p<0.05$), which indicates that boosting PP4c can partially bypass the requirement for Draper and supports a model in which PP4 functions downstream of Draper in this injury paradigm.

Figure 4: PP4c overexpression partially reverses axon clearance defects in Draper RNAi animals. a-h: Representative maximum intensity projection confocal images (z-stack, 15 um) show GFP-labeled OR85e axonal projections (green) in antennal lobes of uninjured (a,b,c,d) and injured (e,f,g,h) control (a,e), Draper RNAi alone (b,f), PP4cHA alone (c,g), Draper RNAi + PP4cHA(d,h). i: Quantification of OR85e axonal debris (GFP) normalized to uninjured conditions. Uninjured GFP fluorescence values set at 1. Individual data points with mean ± s.e.m. plotted; 1-way ANOVA. *P < 0.05, ***P < 0.001, ****P < 0.0001. Scale bar = 20 um. Genotypes: control = *OR85e-mCD8::GFP,tub-Gal80*^{ts}*/+; repo-Gal4/+.* *Drpr RNAi = OR85e-mCD8::GFP,tub-Gal80*^{ts}*/UAS-Draper RNAi ; repo-Gal4/UAS-LacZ. PP4cHA = OR85e-mCD8::GFP,tub-Gal80*^{ts}*/UAS-LacZ ; repo-Gal4/UAS-PP4cHA.* *Drpr RNAi + PP4cHA = OR85e-mCD8::GFP,tub-Gal80*^{ts}*/UAS-Draper RNAi; repo-Gal4/UAS-PP4cHA.*

Figure 4

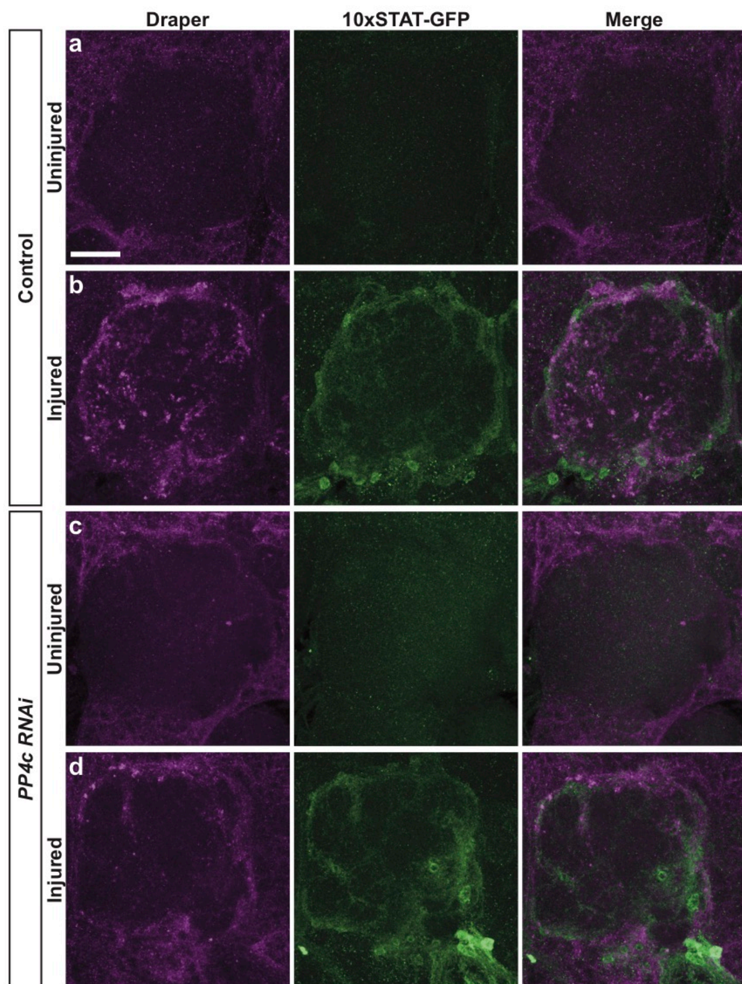


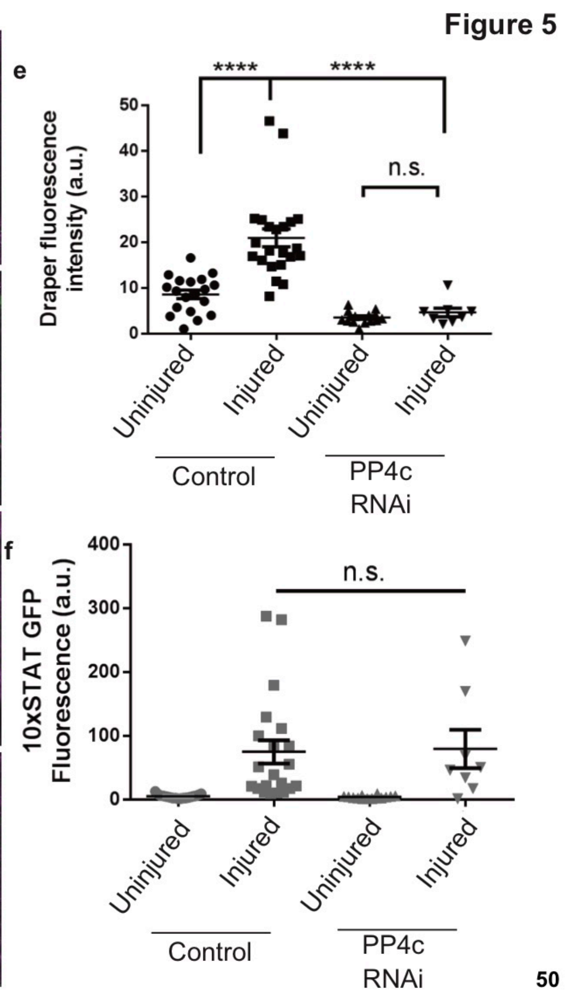
PP4 is dispensable for injury-induced activation of STAT92E in ensheathing glia

Although Draper is basally expressed in glia in the healthy adult brain, axon injury triggers transcriptional upregulation of *draper*, which ensures that adequate levels of the receptor are available to drive the dramatic morphogenic changes in glial cell morphology and phagocytic function in the days after axotomy. Upregulation of *draper* after nerve injury requires the transcription factor STAT92E, and requisite STAT92E binding elements have been defined in the *draper* promoter (53). Activation of STAT92E in glia can be easily tracked in adult brains by monitoring the activation of a *10XSTAT92E-dGFP* reporter (53), which contains 10 tandem canonical STAT92E binding sites that control expression of a destabilized form of cytosolic GFP (133). To determine if STAT92E signaling requires the PP4 complex, we severed the antennal nerves of flies expressing glial $PP4c^{RNAi}$ as well as the *10XSTAT92E-dGFP* transgene and quantified GFP levels one-day post-injury. Notably, activation of *10XSTAT92E-dGFP* was indistinguishable from control animals (Fig 5a-d,f), although the robust increase in Draper protein typically observed in control animals (Fig 5a,b,e, $P<0.0001$) was inhibited in glial PP4c-depleted animals (Fig 5c,d,e). This finding that STAT92E-dependent transcription appears unchanged in $PP4c^{RNAi}$ animals, combined with our PP4cHA rescue experiment (Fig 4), further supports a model in which the PP4 complex is acting downstream of Draper to drive glial membrane infiltration of neuropil regions to access severed axons.

Figure 5: Injury-induced activation of STAT92E in ensheathing glia does not require PP4c. a-d: Expression of the STAT92E transcriptional reporter *10XSTAT92E-dGFP*. Representative z-stacks (10um) show anti-GFP (green) and anti-Draper fluorescence (magenta) in one antennal lobe of uninjured (a,c) and injured (b,d) adult flies. e: Quantification of Draper, normalized to uninjured conditions. Uninjured Draper set at a value of 1. f: Quantification of dGFP levels, normalized to uninjured conditions. Uninjured dGFP set at a value of 1. Individual data points with mean ± s.e.m. plotted; 1-way ANOVA, ****$P < 0.0001$. Scale bar = 20 um. Genotypes: Control = *10XSTAT92E-dGFP,tub-Gal80*^{ts}*/+; repo-Gal4/+. PP4c RNAi= 10XSTAT92E-dGFP,tub-Gal80*^{ts}*/UAS-PP4c*^{RNAi}*; repo-Gal4/+*.

Figure 5





PP4 promotes glial membrane recruitment and clearance of degenerating axons via the SOS GEF complex and Rac1

The Rho GTPase Rac1 is necessary for glial cell cytoskeletal remodeling and membrane recruitment to injured axons in adult *Drosophila* (54, 55), and *in vitro* work has shown that PP4 can modulate Rac1 activity to influence cell migration of HEK293 cells (110). Therefore, we wondered if PP4 might promote glial membrane recruitment to severed axons in adult *Drosophila* glia through regulation of Rac1 activity. We overexpressed wild type Rac1 (*UAS-Rac1*) in adult glia while also knocking down PP4c by RNAi and, interestingly, found that the Draper recruitment phenotype typically observed in $PP4c^{RNAi}$ animals was rescued (Fig 6a-g, $P<0.05$, yellow outlines). Next, we quantified clearance of GFP+ OR85e axons after maxillary nerve injury, and found that overexpression of Rac1 in $PP4c^{RNAi}$ flies also significantly rescued delayed removal of axonal debris (Fig 6h-n, $P<0.05$). Notably, overexpression of *Rac1* alone did not result in faster clearance of severed axons or increased Draper accumulation after axon injury (Supplemental Fig 4). Together, these results suggest that the PP4 complex functions upstream of Rac1, or potentially in a partially redundant parallel pathway, to drive glial infiltration of injury sites and proper glial clearance of degenerating nerves.

Two independent GEF complexes (DRK/DOS/SOS and Crk/Mbc/Ced-12) reportedly activate Rac1 in ensheathing glia post-axotomy (54, 55). To determine if PP4 is coupled to activation of the SOS GEF complex, we used *repo-Gal4* to overexpress SOS (*UAS-SOS-Myc*) and $PP4c^{RNAi}$ flies in adult glia, severed maxillary palp nerves, and then assayed Draper accumulation on maxillary palp glomeruli that house severed axons. Interestingly, SOS overexpression significantly increased Draper recruitment in $PP4c^{RNAi}$ animals (Fig 7a-I,

P<0.01). We performed comparable experiments in an attempt to manipulate the expression of PP4 and the Mbc complex in glia, but this resulted in pupal lethality. Therefore, although the mechanistic connection between PP4, the Mbc GEF complex, and Rac1 in reactive glia is still unclear, our findings highlight the DRK/SOS/DOS complex as one key mediator that promotes Rac1-mediated dynamics in reactive glia required for proper Draper accumulation at injury sites.

Rac1 localization is often coupled to its activity within a cell. We performed Rac1 immunostaining on PP4c glial knockdown and control brains before and one day after maxillary nerve axotomy. Significant Rac1 accumulation was visible on injured axons in control animals (Fig 7j,l,n,p,s, P<0.01), but not in $PP4c^{RNAi}$ brains (Fig 7k,m,o,q,s).   We also confirmed that PP4c-depletion did not alter basal Rac1 levels in the central brain by quantifying Rac1 fluorescence in regions immediately adjacent to the antennal lobes (Fig 7r). To further explore the connection between PP4c and glial cytoskeletal remodeling after antennal nerve injury, we performed phalloidin stains to visualize filamentous actin (F-actin). One-day after antennal nerve axotomy, phalloidin levels were dramatically increased in the antennal lobe neuropil regions of controls (Fig 7t,u,x, P<0.0001), but almost undetectable in $PP4c^{RNAi}$ flies (Fig 7u,w,x). Collectively, these experiments indicate that PP4 does not influence basal expression of Rac1 in adult glia but instead bolster the notion that PP4 activates Rac-1-mediated cytoskeletal remodeling via DOS/SOS/DRK to promote glial responses to nerve injury.

Figure 6: Forced glial expression of Rac1 rescues axonal clearance and Draper recruitment defects in PP4 knockdown flies. a-f: Representative single z-slice (1um) show anti-Draper fluorescence in one antennal lobe of uninjured (a,c,e) and injured (b,d,f) adult flies. Yellow dotted outlines show representative areas of Draper fluorescence quantified in OR85e glomeruli. g: Draper fluorescence quantification in z-stack of 15um, normalized to uninjured conditions. Uninjured Draper set at a value of 1; mean ± s.e.m. plotted; 1-way ANOVA, ***P < 0.001. h-m: Representative maximum intensity projection confocal images (z-stack, 15 um) show GFP-labeled OR85e axonal projections (green) in antennal lobes of uninjured (h,j,l) and injured (I,k,m) adult flies. n: GFP fluorescence quantification, normalized to uninjured condition. Uninjured GFP fluorescence set to 1; mean ± s.e.m. plotted; *P < 0.05, ****P < 0.0001. Scale bars = 20 um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80^{ts}/+; repo-Gal4/+. PP4c RNAi = OR85e-mCD8::GFP,tub-Gal80^{ts}/UAS-PP4c^{RNAi}; repo-Gal4/UAS-LacZ. PP4c RNAi + UAS Rac1 = OR85e-mCD8::GFP,tub-Gal80^{ts}/UAS-PP4c^{RNAi}; repo-Gal4/UAS-Rac1*.

Figure 6

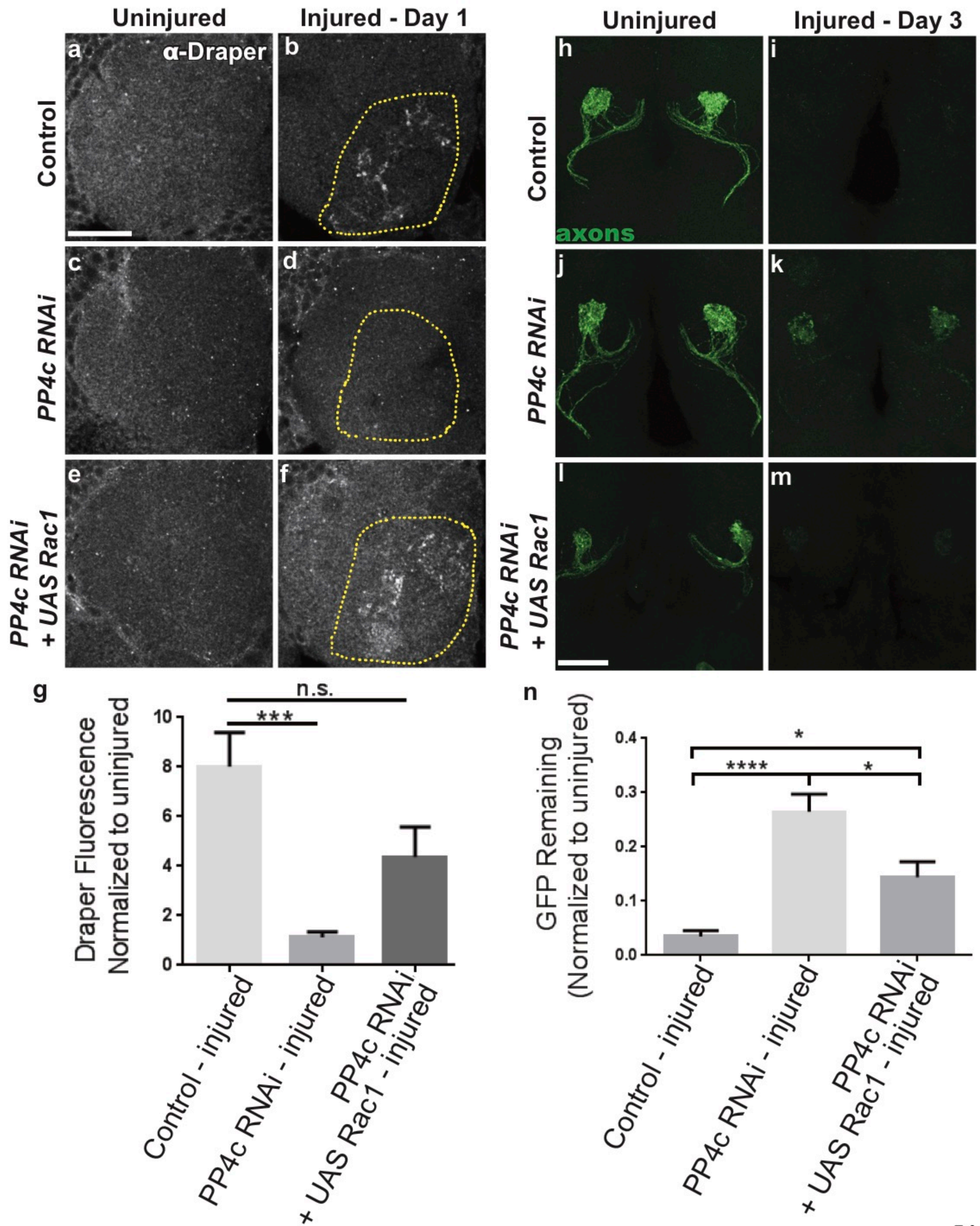


Figure 7: PP4 is an upstream effector of SOS and Rac-1-mediated cytoskeletal remodeling. a-h: Representative confocal images of Draper staining in single antennal lobes of control (a,b), *PP4c RNAi* (c,d), *UAS-SOS* (e,f), and *PP4c RNAi + UAS-SOS* (g,h) uninjured and injured brains. Single 1um slice shown. Dotted lines show region of interest (OR85e glomerulus) for Draper quantification. i: Quantification of Draper fluorescence from experiment shown in a-h, on 15 um Z-stack, normalized to uninjured conditions. Mean ± s.e.m. plotted; 1-way ANOVA, Sidak's comparison test. **P < 0.01, ****P < 0.0001. Scale bar = 20 um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/+. PP4c RNAi= OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4c RNAi; repo-Gal4/LacZ. SOS = OR85e-mCD8::GFP,tub-Gal80ts/UAS-SOS-Myc; repo-Gal4/+. PP4c RNAi + SOS = UAS-SOS-Myc/UAS-PP4c RNAi; repo-Gal4/tub-Gal80ts.* j-q: Representative confocal images of anti-Rac1 in single antennal lobes of control (j,l,n,p) and PP4c RNAi (k,m,o,q) uninjured (j-m) and injured (n-q) adult flies. Merge panels (j,k,n,o) show anti-Rac1 (greyscale) and OR85e glomeruli (green). l,m,p,q panels show the Rac1 channel alone. r: Quantification of basal anti-Rac1 levels from cortical areas. s: Quantification of anti-Rac1 fluorescence in region of interest (dotted line around 85e glomeruli) Z-stack of 15um. Mean ± s.e.m. plotted; 1-way ANOVA. *P < 0.05, Scale bars = 20 um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80*^{ts}*/+; repoGal4/+. PP4c RNAi = OR85e-mCD8::GFP,tub-Gal80*^{ts}*/UAS-PP4c RNAi; repoGal4/+.* t-w: Phalloidin F-actin (greyscale) staining on control and PP4c RNAi brains, uninjured (t,v) and 1 day after antennal nerve injury (u,w). Representative antennal lobes shown. 20um z-stacks. x: Phalloidin fluorescent intensity quantification. Dotted outline in (t) shows representative area of quantification (single antennal lobe). Mean ± s.e.m. plotted; 1-way ANOVA *P < 0.05, ****P < 0.0001. Scale bar: 20um. Genotypes: Control = *OR85e-mCD8::GFP,tub-Gal80*^{ts}*/+; repoGal4/+. OR85e-mCD8::GFP,tub-Gal80*^{ts}*/UAS-PP4c RNAi; repoGal4/+*.

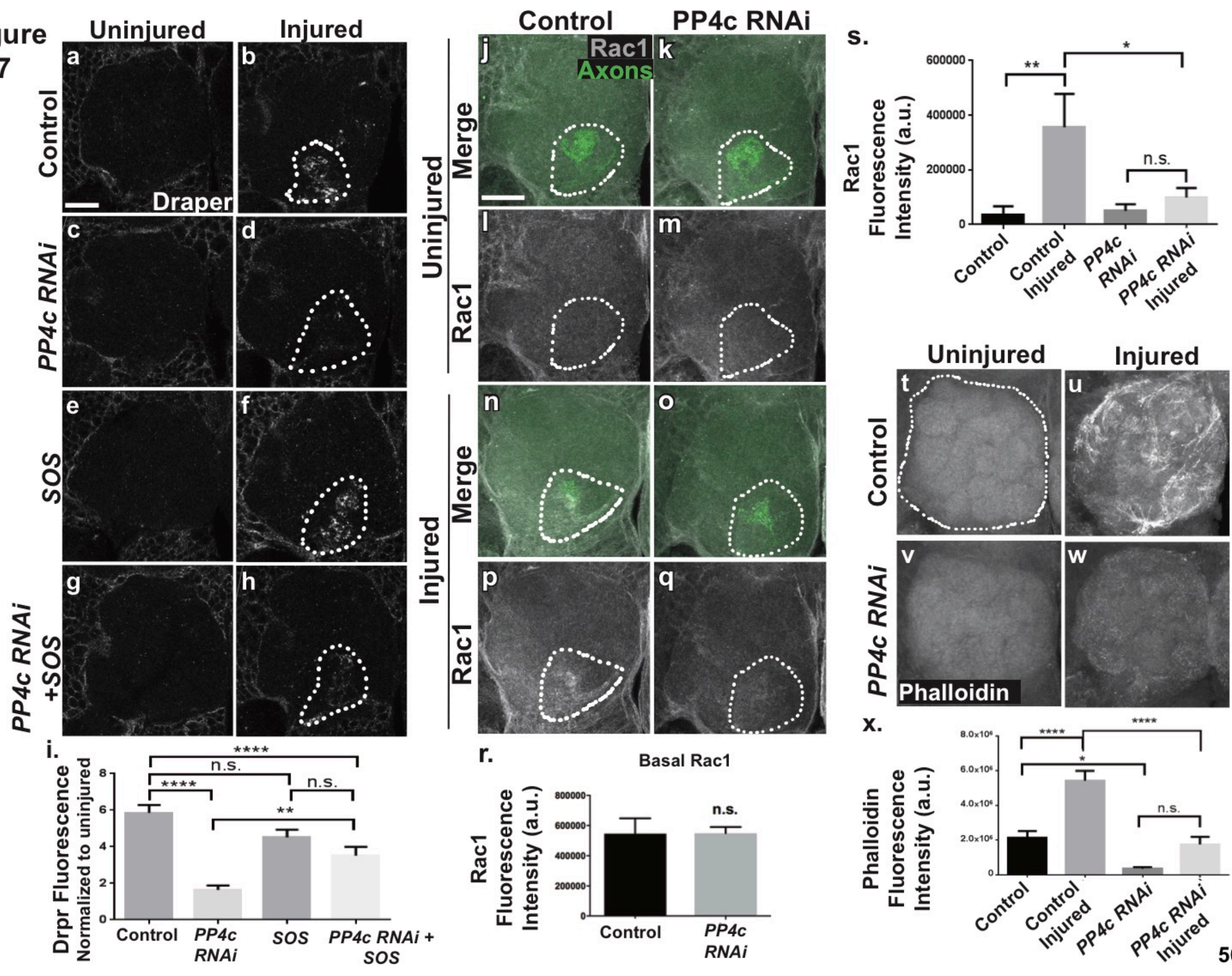


Figure 7
Uninjured
Injured
Control
PP4c RNAi
SOS
PP4c RNAi +SOS
Draper
a
b
c
d
e
f
g
h
i.
Drpr Fluorescence
Normalized to uninjured
****
n.s.
****
n.s.
**
Control
PP4c RNAi
SOS
PP4c RNAi + SOS
Control
PP4c RNAi
Rac1
Axons
Uninjured
Injured
Merge
Rac1
j
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m
n
o
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q
r.
Basal Rac1
Rac1 Fluorescence Intensity (a.u.)
n.s.
Control
PP4c RNAi
s.
Rac1 Fluorescence Intensity (a.u.)
600000
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Control Injured
PP4c RNAi
PP4c RNAi Injured

Axotomy results in reduced nuclear Falafel expression in responding glia.

The regulatory subunits Falafel and PP4r2 regulate PP4 phosphatase complex activity by influencing subcellular localization and substrate recognition (78, 113, 126). Translocation of Falafel between the nucleus and cytoplasm to access targets for de-phosphorylation has been reported in various species and cell types (112, 113, 126, 127). Thus, we wondered if Falafel might exit the nucleus in glia responding to axotomy to facilitate PP4 complex activity. We expressed nuclear beta-galactosidase (*UAS-LacZ::NLS*) under the control of the ensheathing glial driver *TIFR-Gal4* to label ensheathing glial nuclei, performed antennal nerve axotomy, and then immunostained brains with anti-β-gal and anti-Falafel. Comparing uninjured and injured animals, we quantified nuclear Falafel levels by computationally segmenting to beta-gal, and found that Falafel fluorescence was significantly decreased at 3 hours and 6 hours post-injury (Fig 8a-d, $P<0.05$). Notably, we repeated this experiment labeling the nuclei of cortex glia (*NP2222-Gal4, UAS-LacZ::NLS*) adjacent to the antennal lobes, but did not detect a significant change in nuclear Falafel levels after axon injury (Fig 8e-i), indicating that Falafel location and/or levels are specifically influenced in the ensheathing glia responding to axotomy Antennal lobe astrocytes did not express any detectable Falafel (Fig 8j,k).

Figure 8: Nuclear levels of Falafel decrease in ensheathing glia after axon injury. a-c: Representative confocal images of antennal lobe regions. Brains were stained with anti-Falafel and with anti-beta-gal to visualize ensheathing glial nuclei. Dotted squares in a, b, c outline higher magnification images in a’, a’’, b’, b’’, c’, c’’. White boxed regions in a’’, b’’, and c’’ show Falafel fluorescence in isolated glial cells. Arrows identify representative glial cells that were quantified. d: Quantification of Falafel fluorescence in ensheathing glial nuclei. e-h: Representative images of Falafel fluorescence in cortex

glia, identified by anti-beta-gal expression. i: Quantification of Falafel fluorescence in cortex glial nuclei. j-k: Representative images of Falafel fluorescence in astrocyte nuclei. 11um z-stacks. Mean ± s.e.m. plotted; 1-way ANOVA. *P < 0.05. Scale bar: 20um Genotypes: a-d, *UAS-LacZ::NLS; TIFR-Gal4. e-i, UAS-LacZ::NLS; NP2222-Gal4. j,k, UAS-LacZ::NLS; alrm-Gal4.*

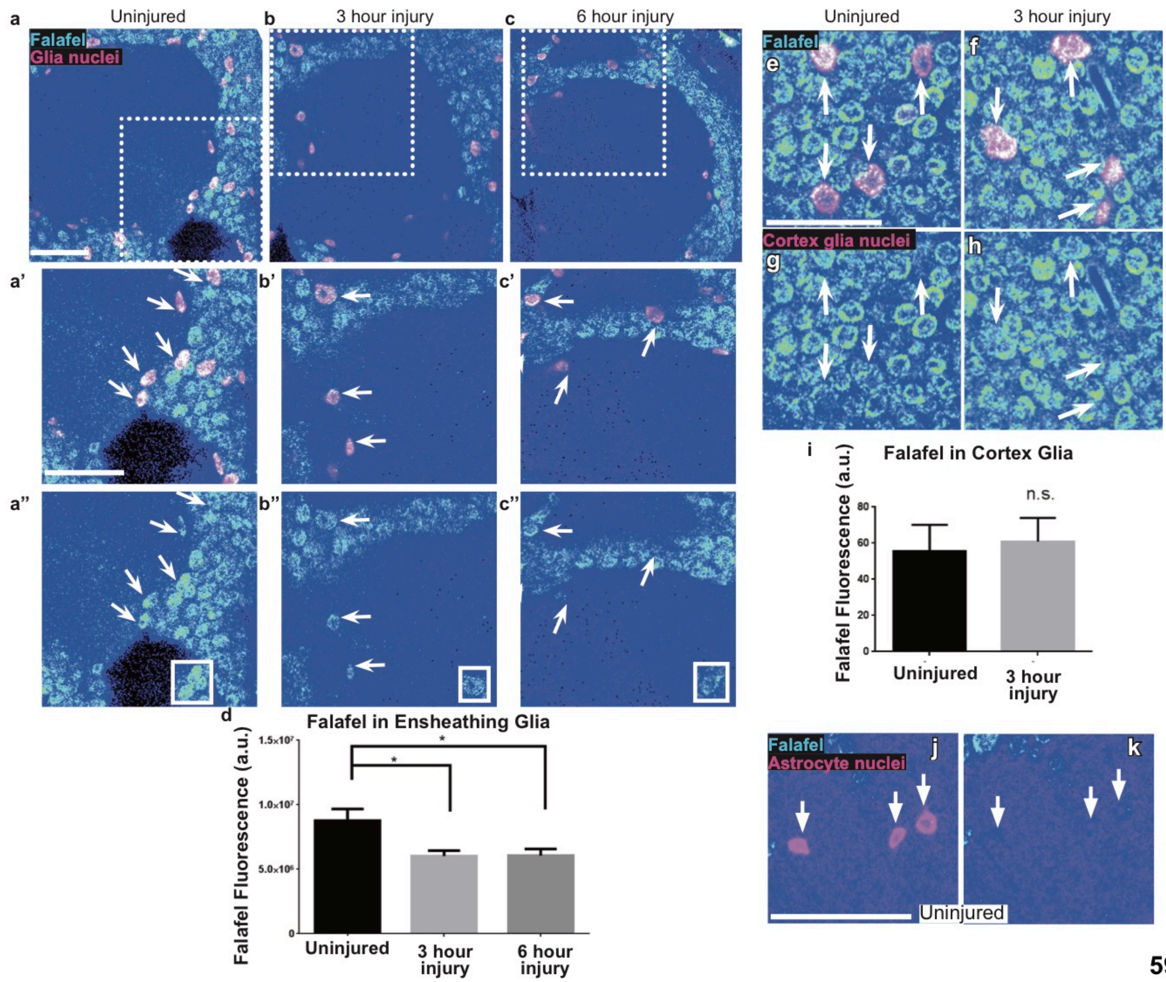


Figure 8
a
Uninjured
b
3 hour injury
c
6 hour injury
Falafel
Glia nuclei
a'
b'
c'
a''
b''
c''
Uninjured
3 hour injury
Falafel
e
f
Cortex glia nuclei
g
h
i
Falafel in Cortex Glia
n.s.
Falafel Fluorescence (a.u.)
80
60
40
20
0
Uninjured
3 hour injury
d
Falafel in Ensheathing Glia
Falafel Fluorescence (a.u.)
1.5x10⁷
1.0x10⁷
5.0x10⁶
0
Uninjured
3 hour injury
6 hour injury
Falafel
Astrocyte nuclei
j
k
Uninjured

DISCUSSION

The Draper receptor is essential for proper initiation of dynamic glial responses to axotomy in the adult *Drosophila* olfactory system (49). Ensheathing glia fail to infiltrate neuropil regions after olfactory nerve axotomy in *draper* mutant animals due to inadequate Rac1 activity (54). The mechanisms that couple activation of Draper to Rac-1-mediated cytoskeletal remodeling, glial recruitment to injury sites, and phagocytic clearance of severed axons are poorly understood. Our results now implicate the PP4 phosphatase complex as a critical molecular effector that functions downstream of Draper to activate the DOS/SOS/DRK GEF complex and Rac1 to promote dynamic cytoskeletal rearrangements in glia responding to axotomy.

The PP4 phosphatase complex influences diverse cellular functions, including mitosis, DNA strand break repair, and differentiation (77, 83, 90, 92, 110, 111). Our work now highlights a previously unexplored role for PP4 in governing innate glial immune responses to neurodegeneration and poses interesting questions for future efforts aimed at understanding precisely how PP4 activity promotes cell migration. We show that forced SOS GEF expression rescues loss of PP4c (Fig 7a-i), suggesting that the SOS GEF complex is a key effector downstream of PP4 required for proper Rac1 activation in responding glia. Targeted serine/threonine de-phosphorylation of the SOS GEF complex has never been reported, nor have direct biochemical interactions between PP4 and GEF complexes in any species. Thus, it is unlikely that the SOS/DOS/DRK complex is directly targeted by PP4 in glia; future screening efforts will be required to delineate the complete signaling pathway that couples PP4 to the SOS complex. We also cannot rule out the possibility that additional GEF complexes (e.g. Crk/Mbc/Ced-12) converge on glial Rac1 to coordinate the assorted dynamic reactions required for glia to access and dispose of degenerating axonal debris.

Finally, because glial activation (e.g. recruitment of glial membranes) and phagocytic clearance are tightly coupled, it is unclear if delayed removal of axonal material in PP4-depleted flies is exclusively a result of inadequate glial membrane invasion of the neuropil or if the PP4 complex independently regulates phagocytic internalization of axonal debris via SOS/Rac1 or a Rac1-independent mechanism.

In *Drosophila*, Falafel and PP4r2 are the exclusive regulatory subunits that associate with the catalytic subunit PP4c to form a functional trimeric complex. Mammalian genomes contain 6 or more genes that encode regulatory PP4 subunits, which enhances the capacity for combinatorial control over PP4 activity across cell types and biological states(77). PP4 complex activity can be regulated, in part, by subcellular localization of the regulatory subunits. For example, in starving *Dictyostelium*, the Falafel homolog SMEK translocates from the cytoplasm into the nucleus where it activates PP4c to facilitate cell stress responses (112). *Drosophila* PP4 complex components also cycle between the nucleus and cytoplasm of proliferating neural precursors, which is essential for PP4 to selectively associate with key targets in the nucleus and cytoplasm and facilitate the proper distribution of cell fate determinants during asymmetric cell division (113, 126). Our observation that nuclear levels of Falafel decrease significantly in ensheathing glial cells surrounding the antennal lobes within hours after olfactory nerve injury, suggests that expression and/or function of PP4 is modified in glia as innate glial immune responses are elicited. We favor the model that Falafel is translocated out of the nucleus, but we cannot exclude the possibility that it becomes incorporated into a complex that hinders antibody accessibility or becomes degraded. We did not detect a significant increase in Falafel levels in glial cytoplasm post-injury, but this may reflect *in vivo* imaging limitations while attempting to visualize low concentrations of Falafel distributed throughout the cell.

The serine/threonine protein phosphatase 4 (PP4) complex was recently identified as a requisite factor for proper immune responses in T cells, B cells, and macrophages (101, 102, 134). Although the role of PP4 in glial cell immunity has not been investigated, increased PP4 expression has been reported in glial tumors, suggesting a connection between PP4 function and glial cell invasiveness (135). Notably, Draper is a highly conserved receptor essential for glial clearance of damaged and dying neurons across species. The mammalian homolog, MEGF10, is required for glial clearance of apoptotic neurons, as well as developmental axonal/synaptic pruning in mammals (38, 48-50, 52-55, 58, 116, 122, 136-141). The high conservation of GEF/Rac1-mediated control of cell migration is also well documented(56, 68, 70, 71). Our work now reveals that the PP4 phosphatase complex unifies these two conserved molecular signaling pathways in the context of glial immunity and may also provide new molecular insight into glial tumor cell migration.

Acknowledgements

We would like to thank the Bloomington *Drosophila* Stock Center at Indiana University, the Vienna *Drosophila* Resource Center, Marc Freeman, and Greg Bashaw for flies and the Developmental Studies Hybridoma Bank at the University of Iowa for antibodies. We would also like to thank Bill Chia for the kind gift of Falafel antibody and flies. This work was supported by the National Institutes of Health (NIH) Grant R01 NS079387-01 (M.A.L), NIH New Faculty Recruitment Grant P30NS069346 P30 (M.A.L.), the Medical Research Foundation of Oregon (S.D.S and M.A.L), the Fred W. Fields Foundation (M.A.L), and the Ken and Ginger Harrison Scholar Award (M.A.L.).

Materials and Methods

***Drosophila* Stocks:** The following *Drosophila* strains were used: *repo-Gal4/TM3*(48). *OR85e-mCD8::GFP/CyO* (76). *w; Sp/CyO; Repo-LexA, LexAop-mCD2::RFP/TM3*. *UAS-LacZ::NLS,tub-Gal80^{ts}/CyO* (BL 7108). w; UAS-mCD8::GFP; UAS-LacZ::NLS. w1118; P(UAS-lacZ.NZ) (BL3955). *w^{1118}* (BL5905). *TIFR-Gal4(38). alrm-Gal4(38). y1 v1; P(TRiP. JF02802)qattP2/TM3, Sb* (*Falafel^{RNAi}*, BL31961). *w; P(UAS-Rac1.W)3* (BL6293). *P(GD9561)v25317* (*PP4c^{RNAi}*, VDRC). *P(KK100895)VIE-260B* (*PP4r2^{RNAi}*, VDRC). UAS-SOSmyc/Cyo; TM2/Sb (a kind gift from Greg Bashaw(142)). NP2222 (60).

Adult Fly Brain Injury, Dissection, and Immunostaining: Maxillary palp and antennal ablations were performed on adult flies as previously described (49). Maxillary or antennal nerves were severed by removing maxillary palps or third antennal segments, respectively, with forceps as described(143). One to five days post-injury, heads were removed and fixed in 4% PFA + 0.01% Triton-X for 15 minutes, followed by washing with 1XPBS + 0.01% Triton-X for 3x2 minutes. Brains were dissected in 1XPBS + 0.01% Triton-X in glass well plates, and then fixed in 4% PFA + 0.1% Triton-X for 15 minutes. Brains were washed in 1XPBS + 0.1% Triton-X for 3x2 minutes, then placed in primary antibody diluted in 1XPBS + 0.1% TritonX overnight at 4°C. Brains were then washed in 1XPBS + 0.1% Triton-X for 3x2 minutes and placed in secondary antibody diluted in 1XPBS + 0.1% Triton-X for 2 hours at room temperature. Brains were washed in 1XPBS + 0.1% Triton-X for 3x2 minutes then placed in CitiFluor CFM-I mounting media (Electron Microscopy Sciences) for 30 minutes before being mounted on glass slides. Flies with *tub-Gal80^{ts}* were raised at 22°C, shifted to the restrictive temperature 30°C post-eclosion for 3-7 days, injured, and then returned to 30°C until dissection. Each genotype had equal amounts of male and female flies. The following antibodies were used: mouse anti-Draper (1:400, Developmental Studies Hybridoma Bank); rat anti-Falafel (1:1000, a kind gift from Bill Chia); chicken anti-GFP (1:1000, LifeTechnologies); mouse anti-Repo (1:10, Developmental Studies Hybridoma

Bank); Phalloidin-TRITC (1:250, Sigma #P1951); mouse anti-Rac1 (1:250, BD Biosciences #61650); chicken anti-beta Galactosidase (1:2000, Abcam #ab9361). All secondary antibodies (Jackson Immunoresearch) were used at a concentration of 1:400.

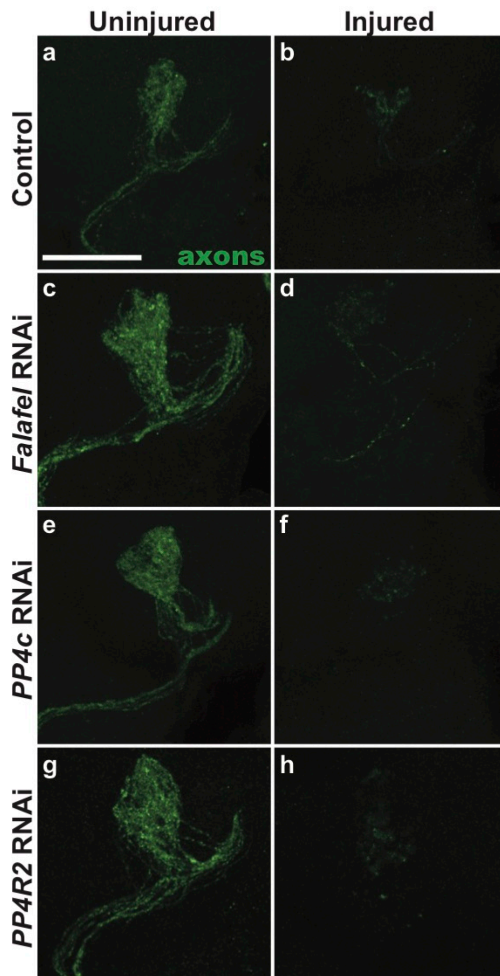
Western Blot Analysis: Central brains (optic lobes manually removed) were homogenized in 4 mL 1xLB (Loading Buffer) per brain. Lysates were loaded into 4-20% Tris-Glycine gels (Lonza) and transferred to Immobilon-FL (Millipore). Blots were probed with rabbit anti-Draper (1:1000 kind gift of Marc Freeman) and sheep anti-tubulin (Cytoskeleton, #ATN02). Blots were incubated with primary antibodies overnight at 4C, washed 3X with 1xPBS+0.01%Tween 20, and then incubated with secondary antibodies (713- 625-147 and 711-655-152 from Jackson Immunoresearch) for 2 hours at room temperature. Blots were then washed 3X with 1xPBS+0.01%Tween 20 and 1X with 1xPBS. Blots were imaged on LI-COR Odyssey CLx quantitative western blot imaging system, and data was quantified with Li-COR Image Studio software.

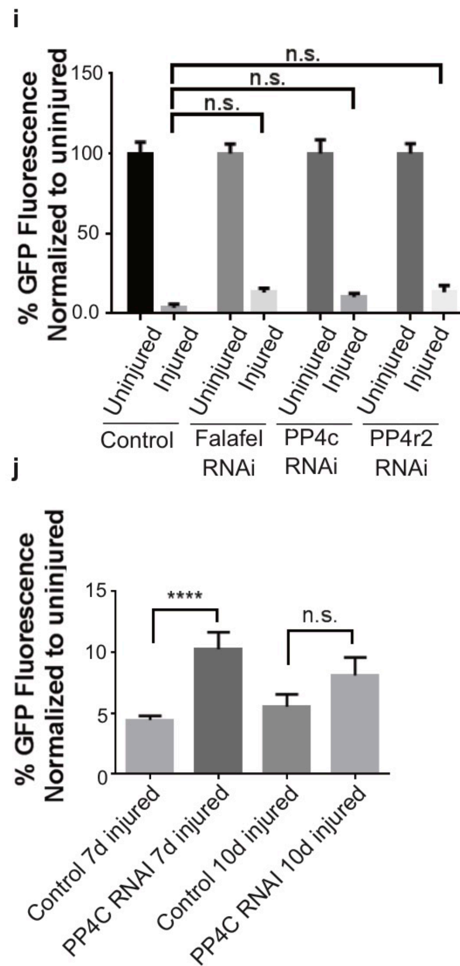
Confocal Microscopy and Image Analysis: Brains were mounted in CFM-I mounting medium and imaged using a Zeiss LSM 710 confocal microscope. Brains were imaged in 1 micron steps with a 40x 1.4 NA oil immersion plan apochromatic lens. Brains in a single experiment were imaged in the same day on the same slide with the same confocal settings. **Axonal clearance:** Quantification of GFP was performed in 3D volumes of OR85e glomeruli of 15um z-stacks. **Draper recruitment:** Draper pixel intensity was quantified in 3D regions of interest in the antennal lobe of 15um z-stacks (see dotted outline in Figure 2). These dotted regions were selected because they correspond to OR85e- glomeruli, which was visualized by the introduction of a OR85e-mCD8::GFP transgene. **Membrane expansion:** Glial membrane expansion after antennal ablation was measured as RFP+ intensity in 3D regions of interest in the antennal lobe of 15um z-stacks. **Falafel translocation:** Falafel

translocation experiments were quantified by segmenting to the beta-Galactosidase positive nuclei (either ensheathing glia or astrocytes) and then measuring mean Falafel fluorescence in these glial nuclei only using Volocity. All image analysis was performed using Volocity image analysis software (PerkinElmer).

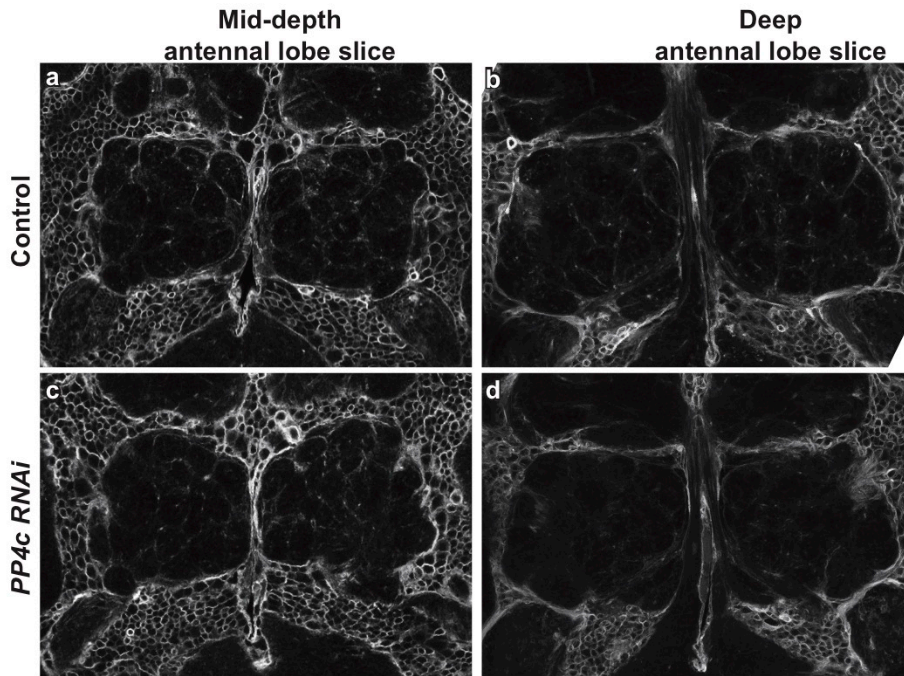
Statistics: All statistics were performed in GraphPad Prism 6. T-tests and one-way ANOVAs were performed as appropriate (see figure legends). All experiments were repeated in full at least three times and post-hoc power tests were run to ensure sample size adequacy. Experiments were not blinded. N for each genotype for each experiment: *Fig1a-d)* control: 17, control injured: 12, Falafel: 23, Falafel injured: 19, *Fig1f-k*) control: 10, control injured: 12, PP4c: 20, PP4c injured: 20, PP4r2: 14, PP4r2 injured: 16. *Fig1m-n)* control: 6, Falafel: 6. *Fig2:* control: 18, control injured: 18, Falafel: 12, Falafel injured: 16, PP4c: 12, PP4c injured: 13, PP4r2: 20, PP4r2 injured: 12. *Fig3:* Antennal Injury: control: 4, control injured: 6, PP4c: 18, PP4c injured: 24. Maxillary Palp Injury: N: control: 13, control injured: 16, PP4c: 4, PP4c injured: 6. *Fig4:* control: 20, control injured: 18, Draper RNAi: 16, Draper RNAi injured: 20, Draper RNAi + PP4cHA: 4, Draper RNAi + PP4cHA injured: 3, PP4cHA: 6, PP4cHA injured: 5. *Fig5:* control: 19, control injured: 22, PP4c: 14, PP4c injured: 8. *Fig6*: control: 19, control injured: 19, PP4c: 14, PP4c injured: 12, PP4c + Rac1: 10, PP4c + Rac1 injured: 10**.** *Fig7a-h)* control: 26, control injured: 27, PP4c: 16, PP4c injured: 20, SOS: 8, SOS injured: 10, PP4c+SOS: 15, PP4c+SOS injured: 12. *Fig7j-q)* control: 22, injured: 25, PP4c: 11, PP4c injured: 28. *Fig7t-w)* control: 18, injured: 24, PP4c: 21, PP4c injured: 26. *Fig8a-c)* uninjured: 13, 3 hour injured: 12, 6 hour injured: 12. Fig8e-h) uninjured: 20, 3 hour injury: 17.

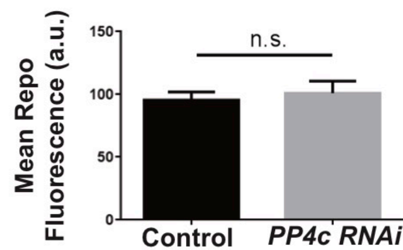
SUPPLEMENTAL FIGURES



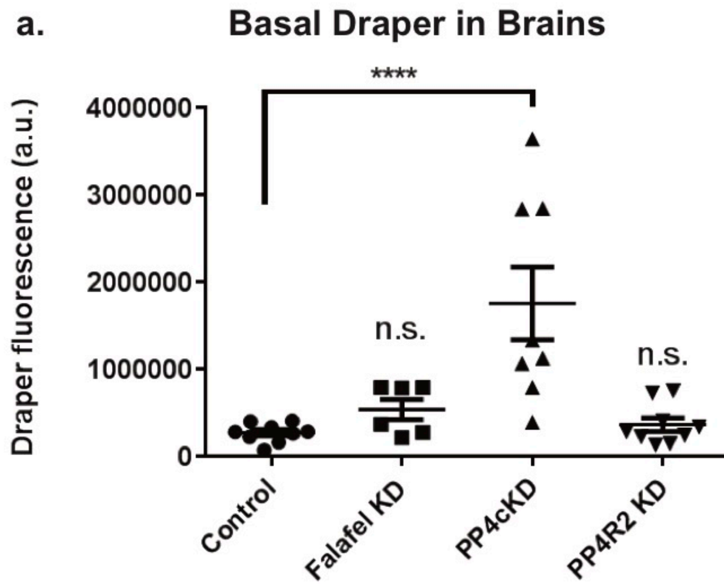


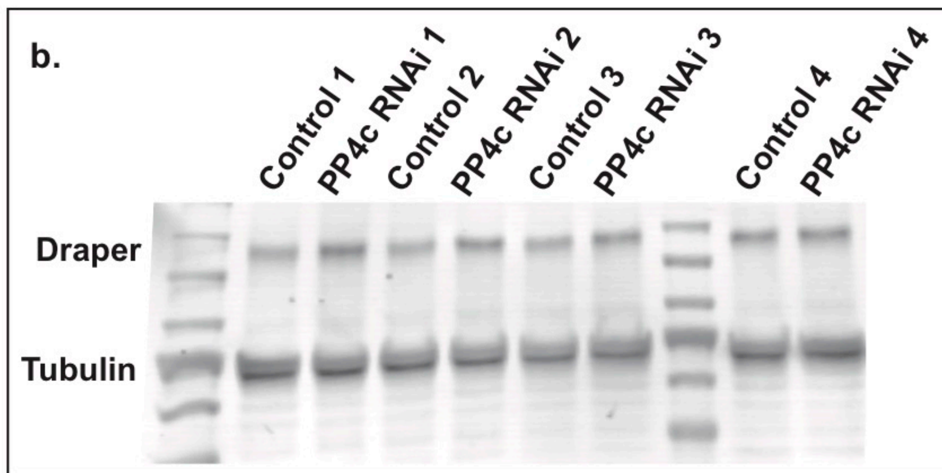
Supplemental Figure 1: Axonal clearance is unaltered in room temperature controls for adult-specific RNAi lines. a-h: Representative maximum intensity projection confocal images (z-stack, 15 um) show GFP-labeled OR85e axonal projections (green) in antennal lobes of uninjured (a,c,e,g) and injured (b,d,f,h) adult flies. i: Quantification of OR85e axonal debris GFP fluorescence normalized to uninjured conditions. Uninjured GFP fluorescence values set at 1. j: Clearance timeline. Quantification from room temperature day 7 and day 10 injuries of OR85e axonal debris GFP fluorescence normalized to uninjured. Z-stack: 15um. n>9 for each experiment; mean ± s.e.m. plotted; 1-way ANOVA. Scale bars = 20 um. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts/+; RepoGal4/+. OR85e-mCD8::GFP,tub-Gal80ts/+; RepoGal4/UAS-Falafel RNAi. OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4c RNAi; RepoGal4/+. OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4r2 RNAi; RepoGal4/+

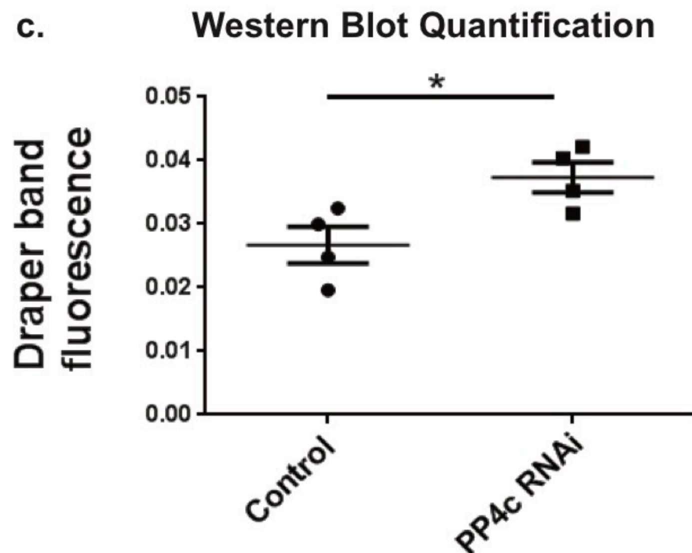




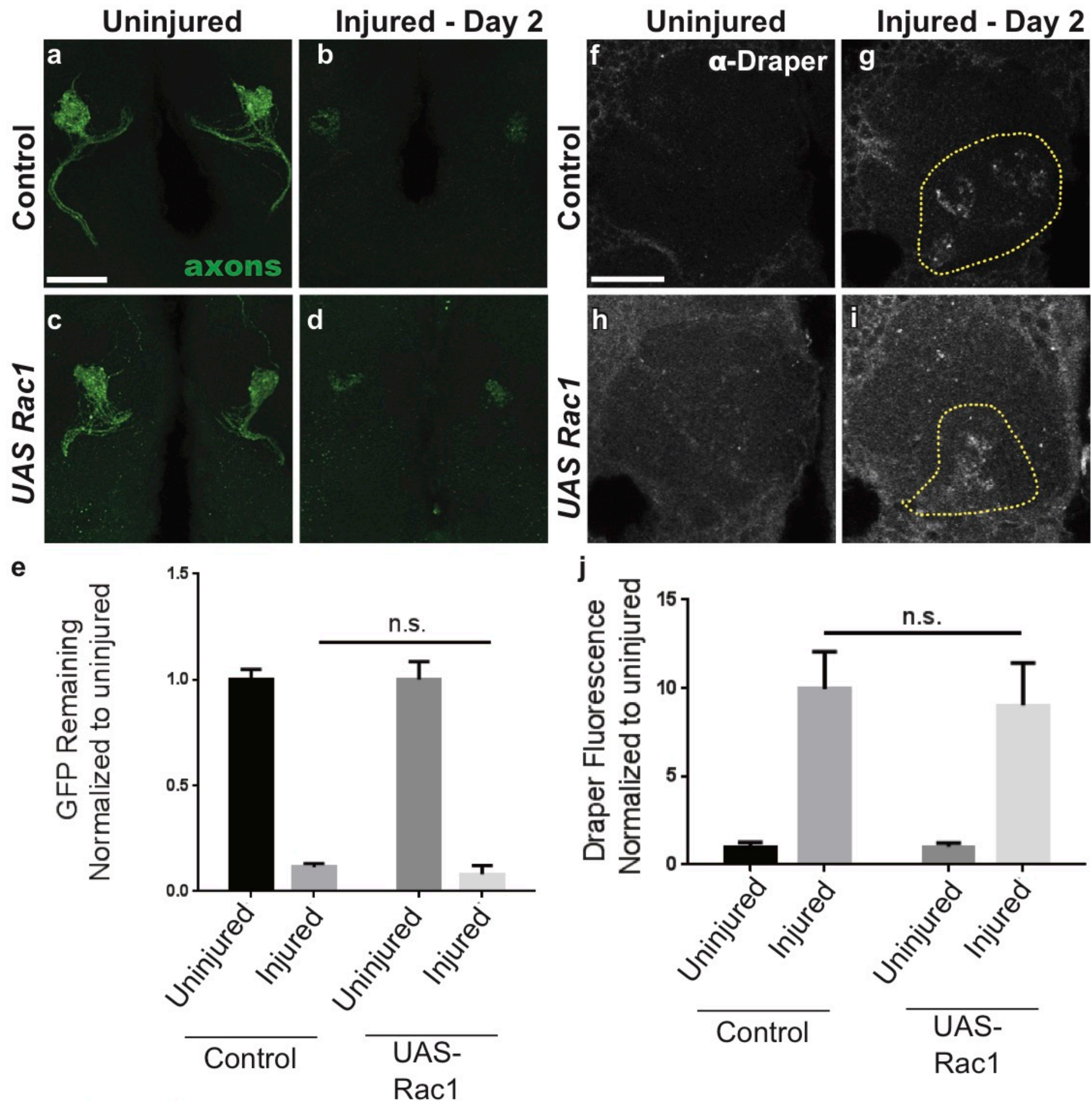
Supplemental Figure 2: Basal glial membranes are morphologically similar in PP4c knockdowns. Representative antennal lobe single slices (1um) show basal glial membrane-RFP in greyscale in control (a,b) and PP4c RNAi (c,d) adult flies. Images from mid-depth (a,c) antennal lobes shown and from deep (posterior) (b,d) antennal lobes. Scale bars = 20 um. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts /+; RepoGal4/ Repo-LexA, LexAop-mCD2::RFP. OR85e-mCD8::GFP,tub-Gal80ts/PP4c RNAi; RepoGal4/ Repo-LexA, LexAop-mCD2::RFP.
e: Average number of glial cells does not change in PP4c knockdown compared to controls. Mean Repo (glial nuclei) fluorescence quantified. N: control: 12, PP4c RNAi: 11. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts/+; Repo-Gal4/+. OR85e-mCD8::GFP,tub-Gal80ts/PP4c RNAi; Repo-Gal4/+







Supplemental Figure 3: Analysis of basal Draper levels in PP4 subunit knockdown flies. a: Quantification of basal Draper fluorescence from cortical areas in control brains compared to glial expression of Falafel RNAi, PP4c RNAi, or PP4r2 RNAi. b: Western blot of uninjured control and PP4c RNAi central brain tissue probed for Draper and tubulin. c: Quantification of Draper bands on Western blot, normalized to tubulin bands, N: 14 heads/ sample. Genotypes: Control = OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/+. Falafel KD = OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/UAS-Falafel RNAi. PP4c KD= OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4c RNAi; repo-Gal4/+. PP4r2 KD = OR85e-mCD8::GFP,tub-Gal80ts/ UAS-PP4r2 RNAi; repo-Gal4/+.



Supplemental Figure 4: Rac1 overexpression does not alter axonal clearance or Draper recruitment. a-d: Representative maximum intensity projection confocal images (z-stack, 15 um) show GFP-labeled OR85e axonal projections (green) in antennal lobes of uninjured (a,c) and injured (b,d) adult flies. e: Quantification of OR85e axonal debris GFP fluorescence normalized to uninjured conditions in 15um z-stacks. Uninjured GFP fluorescence values set at 1; n>11 for each experiment; mean ± s.e.m. plotted. Scale bars = 20 um. f-i: Representative single z-slice (1um) show anti-Draper fluorescence (grey) in one antennal lobe of uninjured (f,h) and injured (g,i) adult flies. Yellow dotted outlines show representative areas of Draper fluorescence quantified in OR85e glomeruli. j: Draper fluorescence quantified, normalized to uninjured conditions in 15um z-stacks. Uninjured Draper set at a value of 1; n>9 for each experiment; mean ± s.e.m. plotted; 1-way ANOVA. Scale bars = 20 um. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts/+; RepoGal4/+. OR85e-mCD8::GFP,tub-Gal80ts+; RepoGal4/UAS-Rac1

Chapter 3. Investigating PP4 complex location to reveal functional downstream effectors.

L Winfree performed all experiments. M Logan and S Speese contributed ideas, guidance, writing and editing.

ABSTRACT

As mentioned earlier in this dissertation, the PP4 complex has numerous cellular functions and various possible downstream effectors, however, we are currently unsure what PP4 is dephosphorylating in response to injury in glial cells. In the following sections, I will describe the current knowledge about possible PP4 substrates and the experiments we performed in the attempt to deduce potential PP4 signaling effectors in the glial response to injury.

Since it is thought that PP4 regulatory subunit localization determines PP4 complex target specificity, I investigate Falafel localization in glia before and after injury. I use Venus-tagged Falafel fly lines to determine if Falafel needs to be nuclear, or able to translocate the nucleus, for the PP4 complex to have a role in the glial immune response. To further study Falafel location and phagocytosis, I turn to the S2 cell model. Finally, I examine candidate PP4 downstream effectors that reside in the cytoplasm to investigate if there is a link between the PP4 complex and Rac1 activity that could explain the glial cell cytoskeletal defects I see in PP4c knockdowns after injury.

Introduction

Protein Phosphatase 4 (PP4) is necessary for the glial immune response after neuronal injury. PP4 functions by regulating glial cytoskeletal dynamics after injury, but we are uncertain of the PP4 downstream effectors for dephosphorylation during this immune response.

To begin our search for a PP4 downstream effector, we examine PP4 location. It is known that the PP4 regulatory subunits influence substrate specificity. For example, as mentioned in Chapter 2, cytoplasmic Falafel directs the PP4 complex to dephosphorylate a transcription factor in starving *Dictyostelium*(112). Therefore, we assess the location of the PP4 regulatory subunit Falafel and examine how Falafel location could impact the glial immune response. To study Falafel location and Falafel nuclear translocation effects, we transition from *in vivo* experiments to *in vitro* experiments in the *Drosophila* S2 cell line. Our results suggest that Falafel is initially nuclear, but becomes cytoplasmic after injury, potentially bringing the PP4 complex to molecular targets outside of the nucleus.

There are two potential PP4 signaling effectors we examine in the following chapter: AMP-activated protein kinase (AMPK) and matrix metalloproteinase 1 (MMP1). There is evidence that PP4 can regulate AMPK-mediated Rac1 activity and cell migration. Additionally, in a study of cancer cells, MMPs were increased upon PP4 overexpression. These potential downstream molecules will be discussed in further detail along with the experimental evidence we collected in our *Drosophila* model system.

RESULTS

Falafel location is important for the glial immune response.

Since PP4 complex specificity is dependent on PP4 regulatory subunits, we decided to investigate how the glial immune response would be affected in flies in which we altered the location of the regulatory subunit Falafel. As I mentioned in my thesis introduction, Falafel has 3 Nuclear Localization Signal (NLS) motifs, 1 Nuclear Export Signal (NES) and is localized to the nucleus in numerous cell types in the majority of species (including glia in *Drosophila*). Dr. Bill Chia kindly gifted our lab fly lines expressing a Venus-tagged Falafel construct in several forms: one line contains wild type Venus-Falafel and one line contains Venus-Falafel with mutated nuclear localization signals and an additional nuclear export signal (Δ3NLS + 2NES). We examined axonal clearance in these fly lines. Since the injured axons contain GFP, we were unable to distinguish the injured 85e glomerulus from the Venus-Falafel, so we analyzed axonal clearance along the nerve where there is less Venus-Falafel (Figure 1). This injured nerve degenerates and is phagocytosed by local ensheathing glia in a similar manner to the 85e glomerulus. Compared to control flies, there was more GFP fluorescence from the axon remaining in cytoplasmic-bound Venus-Falafel$^{\Delta 3NLS + 2NES}$ flies, but there was no change in clearance in Venus-Falafel flies (Figure 1b,d,f,g). This data suggests that location of the PP4 complex regulatory subunit Falafel is important for the glial immune response.

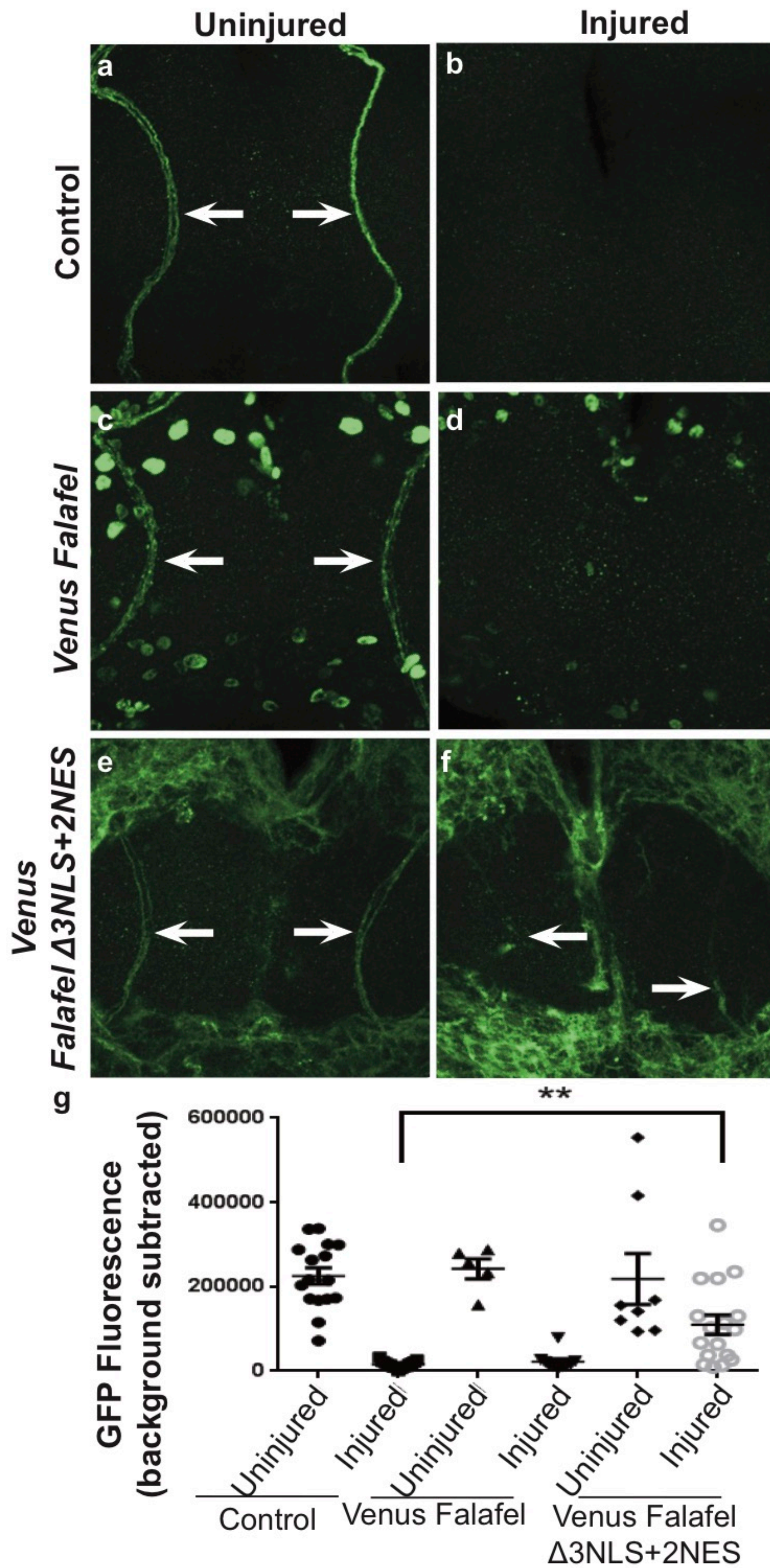


Figure 1: Axonal clearance is altered in flies expressing cytoplasmic-bound Falafel.
a-b: Maxillary palp axonal tracts in uninjured (a) and injured (b) control flies. c-d: Maxillary palp axonal tracts in uninjured (c) and injured (d) flies over-expressing Venus-Falafel. e-f: Maxillary palp axonal tracts in uninjured (e) and injured (f) flies over-expressing cytoplasmic Venus-Falafel (Δ3NLS+2NES). g: Axonal tract GFP quantified, normalized to uninjured and background subtracted. N: control: 16, injured: 15, Venus-Falafel: 5, Venus-Falafel injured: 10, Δ3NLS + 2NES: 8, Δ3NLS + 2NES injured: 17. $P < 0.01$, 1-way ANOVA. Mean ± SEM with individual points plotted. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4/+. OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4;UAS-V-flfl. OR85e-mCD8::GFP,tub-Gal80ts/+; repo-Gal4;UAS-Venus-FalafelΔ3NLS + 2NES

Examining phagocytosis and translocation in S2 cells

To further study this nuclear translocation, I turned to the *Drosophila* S2 cell line. S2 cells are derived from the hemolymph of *Drosophila* embryos and are highly phagocytic. The Franc lab has extensively studied how S2 cells phagocytose apoptotic cellular debris, and they have found that S2 cells phagocytose in a Draper-dependent manner(144). Further, Stroschein-Stevenson et al. performed a large-scale RNAi screen in S2 cells to find regulators of phagocytosis, and found that Falafel dsRNA treatment reduced phagocytosis of *c. albicans*(145). The S2 cell model allowed us to examine Falafel location by staining the cells with anti-Draper and anti-Falafel. We found nuclear Falafel localization (as we did in brains) and a punctate Draper stain (Figure 2a).

To stimulate phagocytosis in S2 cells, we co-cultured cells with pH-sensitive, fluorescent *S. aureus* particles called pHrodo Bioparticles (Life Technologies). pHrodo particles are non-fluorescent at neutral pH, when they are outside the lysosome for example, but fluoresce green upon phagocytosis into vesicles with low pH. This allowed us to track phagocytic engulfment of pHrodo-tagged particles in unfixed S2 cells. We quantified percent phagocytosis in S2 cells by counting green fluorescence inside the cells (Figure 2b-d). We found about one third of cells had phagocytosed at least one pHrodo particle after 1 hour compared to cells kept at 4C to block phagocytosis machinery, which had less than 5% phagocytosis (Figure 2e).

Next, we wondered if phagocytosis would be altered in cells lacking PP4. We used the potent PP4 inhibitor okadaic acid and assayed percent phagocytosis of pHrodo particles. Okadaic acid is derived from marine dinoflagellates and causes diarrheic shellfish poisoning in humans(146). Okadaic acid inhibits many phosphatases, such as the PP2A family, including PP4, and readily enters live cells. Okadaic acid has an IC_{50} of 0.1 nM for inhibiting

PP4, and is more selective than other PP2A inhibitors, such as Calyculin A. With this model system, we examined Falafel location and found evidence that Falafel translocates from the nucleus to the cytoplasm. We found that okadaic acid effectively blocked phagocytosis of pHrodo particles, suggesting that the PP4 complex is necessary for phagocytosis in S2 cells (Figure 2e).

We next examined Falafel localization in S2 cells upon exposure to pHrodo particles. As mentioned above, Falafel is nuclear under basal conditions. After stimulating phagocytosis by the addition of pHrodo particles, Falafel fluorescence became more cytoplasmic and less nuclear (Figure 2f-h). This outcome mirrors the result we found *in vivo*.

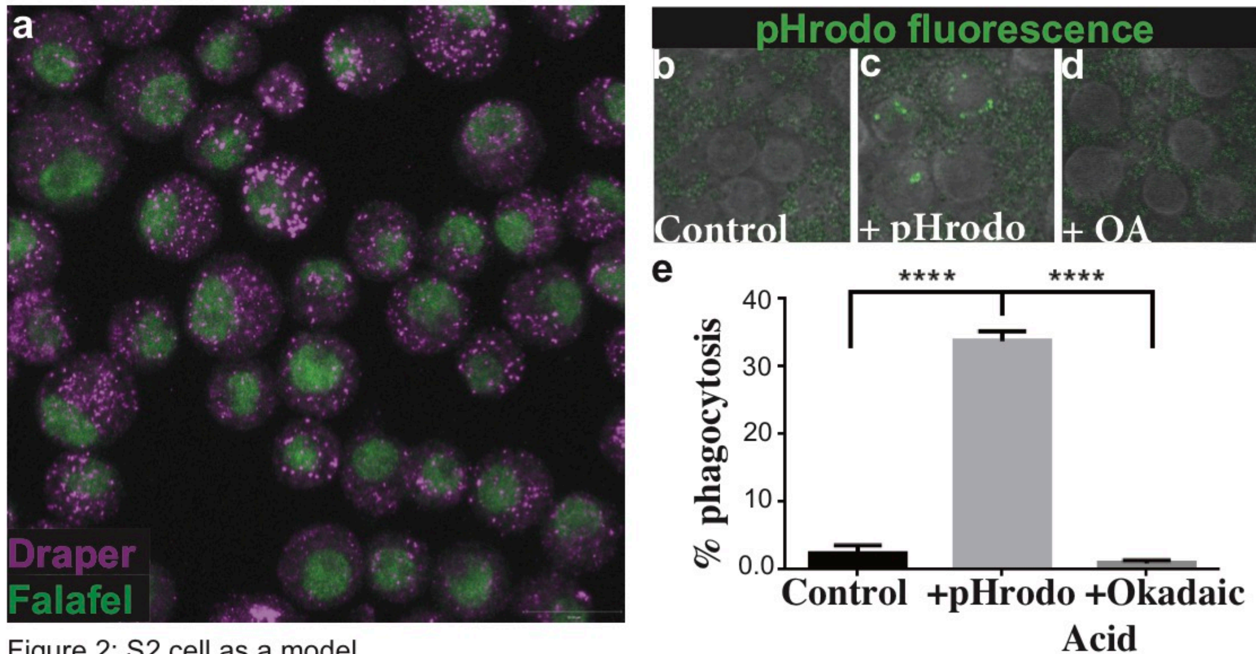
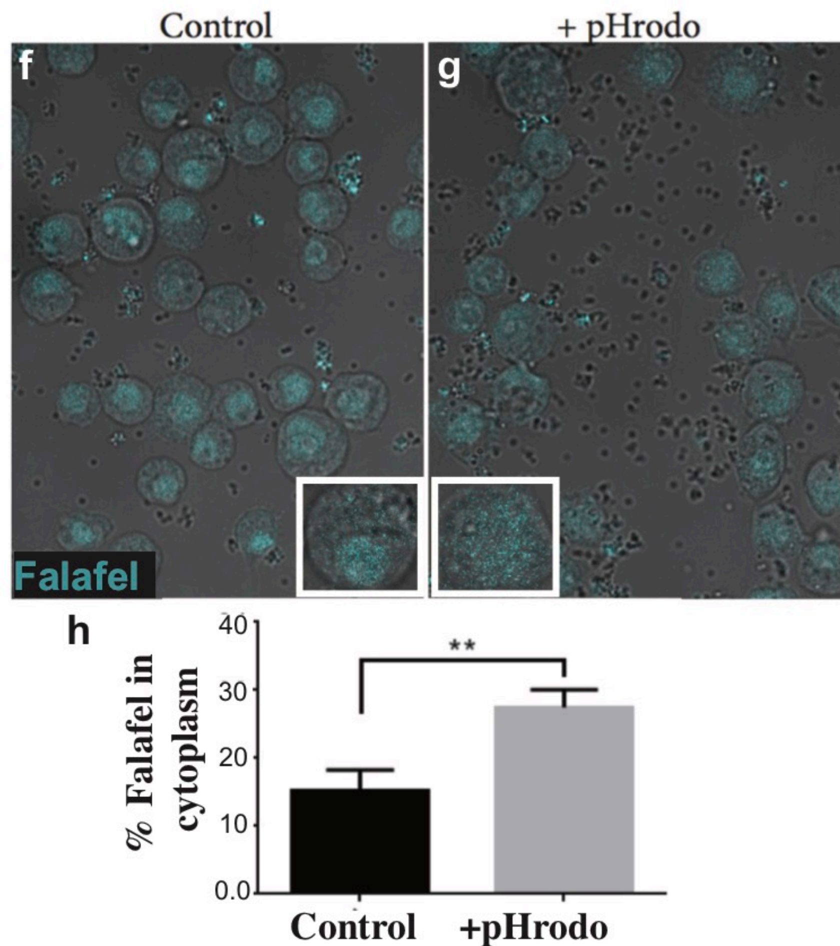
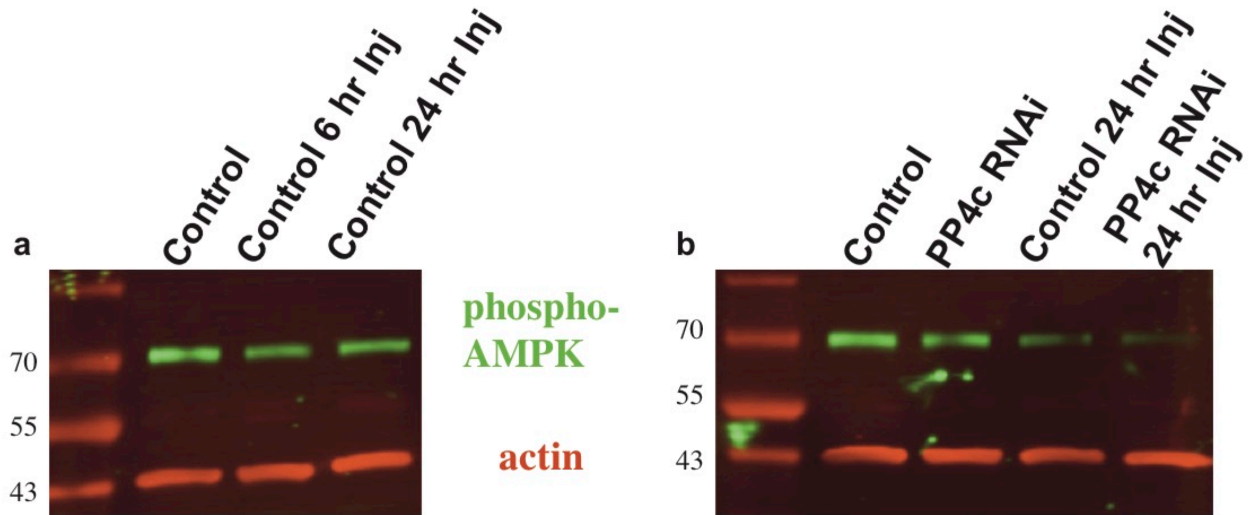


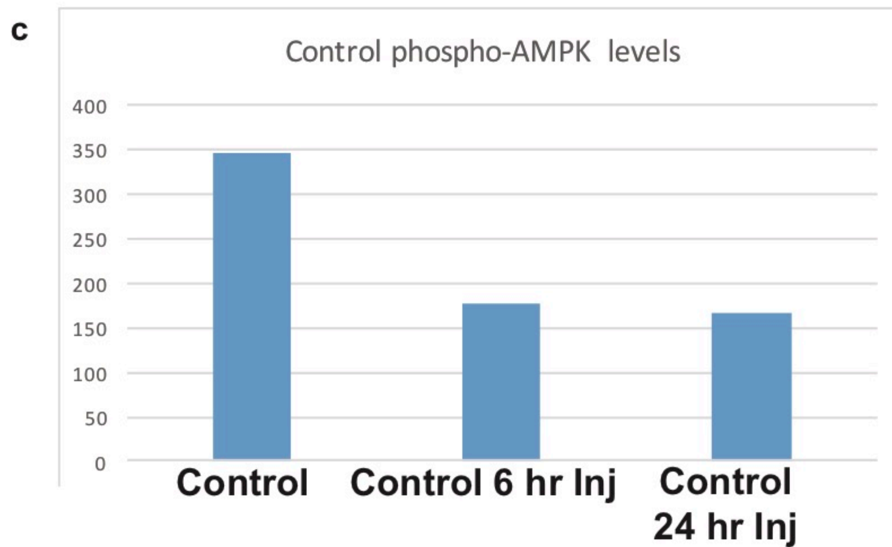
Figure 2: S2 cell as a model system. a: Falafel and Draper localization in S2 cells. Falafel (green) is nuclear localized in S2 cells, and Draper (purple) is punctate. b-e: S2 cell phagocytosis is reduced with the PP4 inhibitor Okadaic Acid. b-d: S2 cells phagocytosing pHrodo s. aureus particles. b: S2 cells kept at 4 degrees C as controls. c: S2 cells at room temperature. d: S2 cells treated with Okadaic Acid to inhibit PP4. Representative images of cells plated in 96 well plates. e: Quantification of percent phagocytosis (# of cells with pHrodo/total # of cells). N cells/slide on average: control: 49, pHrodo: 52, okadaic acid: 70. P<0.0001. f-h: Falafel becomes more cytoplasmic during phagocytosis. f-g: Falafel antibody stain in S2 cells with (f) or without (g) pHrodo treatment. h: Percent of cells with Falafel present in cytoplasm (non-nuclear localized). N cells/slide on average: control: 50, pHrodo: 65. P=.0069.



Potential PP4 targets for dephosphorylation in glial cells.

With our updated knowledge about Falafel location and translocation during phagocytosis, we examined if active AMP-activated protein kinase (phospho-AMPK) could be a PP4 substrate for dephosphorylation. AMPK is a noteworthy candidate for PP4 target activity. Recently, a study found that PP4 could be regulating AMPK activity in immune cells(102, 107). Liao et al. showed that T cells depleted of PP4c had higher levels of AMPK, that PP4 co-immunoprecipitated with AMPK, and that PP4 overexpression inhibited AMPK phosphorylation. Further, there are several studies revealing a relationship between Rac1 and AMPK(104, 107), which led me to investigate if AMPK could be is a link between the PP4 complex and Rac1 activity. We performed a western blot in *PP4c* ^{RNAi} flies and control flies, before and after maxillary palp and antennal injury. We found reduced basal phospho-AMPK levels in *PP4c* ^{RNAi} brains (Figure 3b,d). However, in both genotypes, we found a decrease in phospho-AMPK signal after injury suggesting that there is no injury-induced difference in phospho-AMPK in PP4 knockdowns (Figure 3).





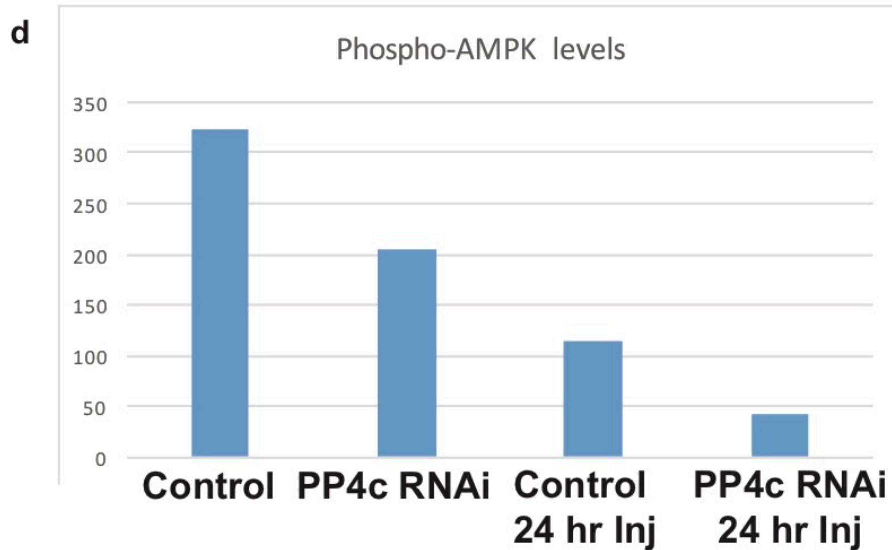


Figure 3: AMPK activity is reduced in PP4c RNAi flies. a: Western blot of uninjured and injured (6 or 24 hours) Control brains b: Western blot of Control and PP4c RNAi brains, uninjured and 24 hours post-injury. Actin band is approximately 45kD, Phospho-AMPK band is approximately 70kD. c: Quantification of blot in (a), P-AMPK band normalized with actin band for each sample. d: Quantification of blot in (b), P-AMPK band normalized with actin band for each sample. N: 10 brains/sample. Genotype: OR85e-mCD8::GFP,tub-Gal80ts/+; RepoGal4/+. OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4c RNAi; RepoGal4/+.

Another potential target is matrix metalloproteinase 1 (MMP1). MMPs are involved in matrix remodeling and cell migration. *Drosophila* contain 2 MMPs, while in mammals there are 23 partially redundant MMP genes(57). Li et al. found that overexpressing PP4c increased MMP-2 and MMP-9 expression in human colorectal carcinoma cells(131). Recently, our lab has shown that MMP1 is downstream of Draper and STAT92E in the glial cell immune response(57). Purice et al. observed that MMP1 is found along ensheathing glial membranes and in the tracheal network. Further, MMP1 is upregulated in ensheathing glia after injury, in a similar staining pattern to Draper. This upregulation is blocked in Draper null flies and in $STAT92E^{RNAi}$ flies. They also found that MMP1 is necessary for glial membrane expansion.

We assessed if PP4 is required for injury-induced upregulation of MMP1 by performing an MMP1 antibody stain one day after antennal ablation in $PP4c^{RNAi}$ flies. In control flies, there was an increase in MMP1 fluorescence around the injury site (Figure 4a,b; as seen before by Purice et al.). In $PP4c^{RNAi}$ flies, there was also an increase of MMP1 after injury, which was statistically the same as in the control flies (Figure 4d,e), suggesting no difference in MMP1 activity in PP4 knockdowns.

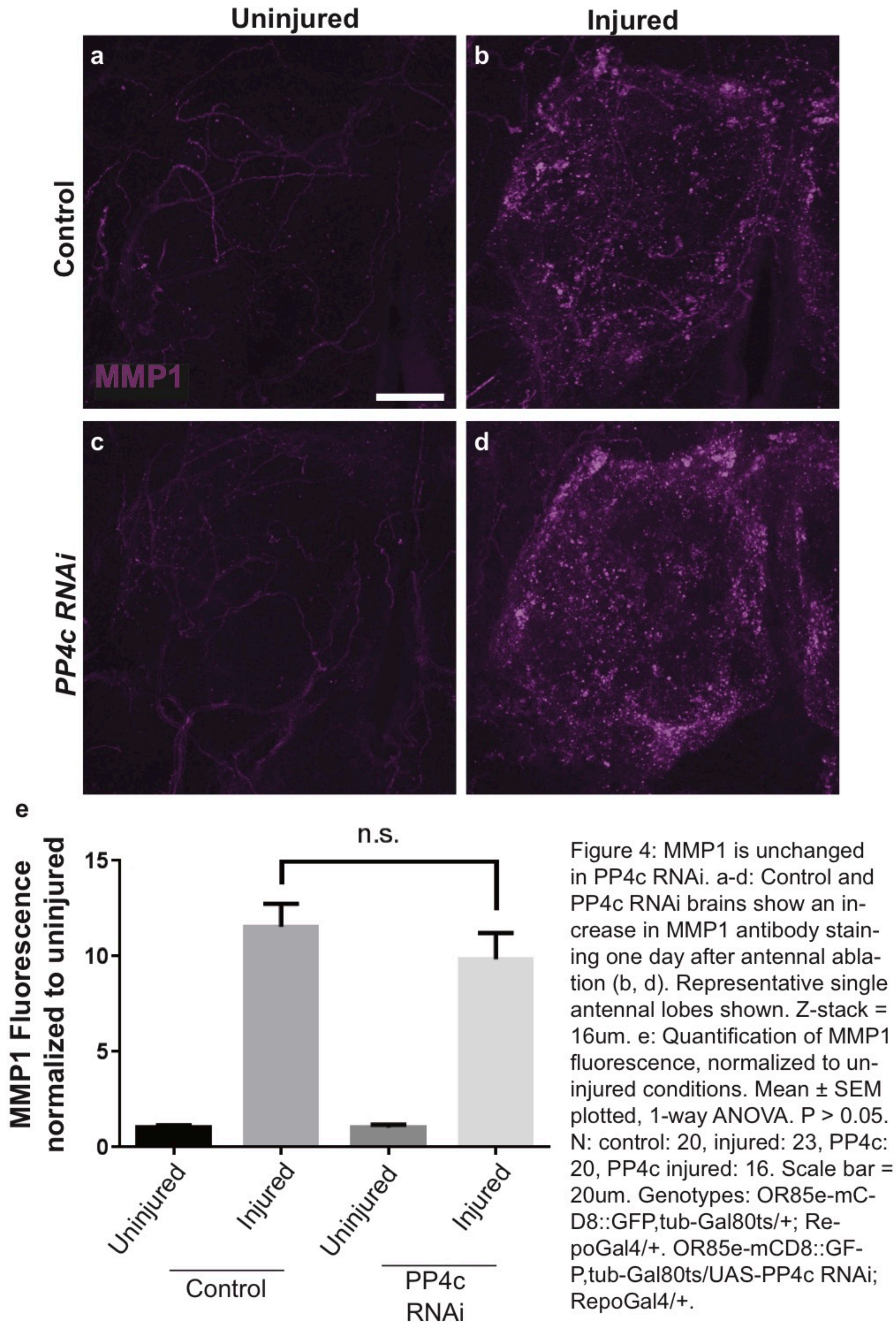


Figure 4: MMP1 is unchanged in PP4c RNAi. a-d: Control and PP4c RNAi brains show an increase in MMP1 antibody staining one day after antennal ablation (b, d). Representative single antennal lobes shown. Z-stack = 16um. e: Quantification of MMP1 fluorescence, normalized to uninjured conditions. Mean ± SEM plotted, 1-way ANOVA. P > 0.05. N: control: 20, injured: 23, PP4c: 20, PP4c injured: 16. Scale bar = 20um. Genotypes: OR85e-mCD8::GFP,tub-Gal80ts/+; RepoGal4/+. OR85e-mCD8::GFP,tub-Gal80ts/UAS-PP4c RNAi; RepoGal4/+.

DISCUSSION

In the previous chapter, we showed that Falafel is nuclear, but appears to leave the nucleus or is degraded after injury. We proposed that Falafel must be either nuclear or must translocate the nucleus in response to injury for the PP4 complex to function in the glial cell immune response. There is strong evidence that the PP4 regulatory subunit Falafel confers substrate specificity, so we examined what would happen in glial cells with altered Falafel location. We found that flies with cytoplasmic-bound Falafel (Venus- Falafel$^{\Delta 3NLS + 2NES}$) had reduced clearance of axonal debris, suggesting that Falafel needs to be nuclear or translocate from the nucleus to the cytoplasm for a proper glial immune response. I also examined Draper recruitment in the Venus-Falafel fly lines and found a trend towards reduced Draper recruitment in Venus- Falafel$^{\Delta 3NLS + 2NES}$ flies, but the difference was not significant. These experiments raise some interesting questions; such as why would Falafel need to be nuclear? Or does Falafel simply need to translocate the nucleus/take something out of the nucleus? Answering these questions could help identify the glial target of the PP4 complex after injury.

To further analyze Falafel location and translocation, we switched to the *in vitro* model system, S2 cells. A previous study from the Franc lab revealed that S2 cells phagocytose in a Draper dependent manner, and we confirmed that our S2 cell line expressed Draper and Falafel with immunofluorescence. One other paper showed a similar Draper staining pattern to the punctate pattern we found(147). To imitate an injury response *in vivo*, we added pHrodo particles to the S2 cells to stimulate phagocytosis. We found that phagocytosis of pHrodo particles was about 33%, meaning about 1 in 3 cells had phagocytosed pHrodo particles. However, even with this low percentage, we could still observe a marked decrease in phagocytosis with the addition of okadaic acid. Interestingly,

we did find that Falafel levels become less nuclear and more dispersed throughout the cell after phagocytosis has been induced. This result mirrors the result we see *in vivo*, however, *in vivo* we could not determine if Falafel was entering the glial cell cytoplasm or being degraded. These S2 cell experiments argue that Falafel is indeed becoming more cytoplasmic after injury, but we cannot rule out that Falafel is being degraded in the nucleus in response to injury.

We were encouraged by previously published research to examine if PP4 could be interacting with AMPK in glial cells after injury. We found decreased phospho-AMPK levels in uninjured *PP4c*^{RNAi} brains compared to uninjured control brains by western blot. Interestingly, we found a decrease in phospho-AMPK levels after injury in both control and *PP4c*^{RNAi} brains, suggesting that regulation of AMPK is not altered after injury in *PP4c*^{RNAi} brains. We immunostained brains with anti-phospho-AMPK but did not find a specific staining pattern or any noticeable differences between controls and *PP4c*^{RNAi} brains. We are uncertain why *PP4c*^{RNAi} brains have decreased basal phospho-AMPK activity, or what functional significance, if any, this decrease could have in glial cells. It would be interesting to examine if overexpressing Rac1 could rescue basal phospho-AMPK levels in *PP4c*^{RNAi} brains, as there is evidence to a relationship between Rac1 and AMPK. However, these Rac1-AMPK studies are contradictory, so more research would need to be done to determine if there is a relationship between PP4c, AMPK, and Rac1 in glial cells after injury.

We next examined if PP4 could be interacting with MMP1 after injury. While there is evidence suggesting that increased PP4c leads to increased MMPs in human cancer cells, we did not find any change in MMP1 levels in *PP4c*^{RNAi} brains after injury compared to controls. Our finding suggests that glial MMP1 function is not altered by PP4c knockdown.

The studies presented in Chapter 3 suggest that Falafel is initially nuclear, but translocates into the cytoplasm after injury, potentially to direct the PP4 complex to various substrates. We are still uncertain about what these targets could be, and future work will need to be performed to answer this question.

MATERIALS AND METHODS

Drosophila Stocks:

The following *Drosophila* strains were used: *repo-Gal4/TM3*(14). *OR85e-mCD8::GFP/CyO. UAS-LacZ::NLS,tub-Gal80ts/CyO* (BL 7108); *w1118* (BL5905), *y1 v1 P(TRiP.JF02802)qattP2/TM3, sb (Falafel^{RNAi}, BL31961). P(GD9561)v25317* (*PP4c^{RNAi}*, VDRC). *;;UAS-V-flfl / TM6b* and *;;UAS-Venus-Falafel$^{\Delta 3NLS + 2NES}$* lines were a kind gift from Dr. Bill Chia. Dr. Chia informed us that the 3NLS + 2NES line was created by adding sequences corresponding to the NES of the Protein Kinase A inhibitor, LALKLAGLDI(148) to both termini of *UAS-Falafel*.

Adult Fly Brain Injury, Dissection, and Immunostaining:

Brain injury, dissection, and immunostaining were performed as described in Chapter 2. For the Falafel translocation experiments, we injured flies and then fixed and dissected brains at 3 or 6 hours after injury.

Western Blot Analysis:

Western blots were performed as described in Chapter 2. Primary antibody used for the AMPK blot was rabbit anti-Phospho-AMPK (1:1000, Cell Signaling #2535, Thr172).

S2 cell Maintenance and Antibody Staining:

S2 cells were a kind gift from Dr. Cathy Galbraith. S2 cells were passaged every 3-4 days at a rate of 1:4 in T25 flasks with a plug seal. Cells were kept at 25 C in Schneider's insect medium supplemented with 10% FBS and 1% Glutamax 100X (Gibco).

S2 cell staining: Cells were plated in 96 well plates as described above. Cells were fixed with 4% PFA + 0.1%TritX for 15 minutes, then washed with 1XPBS + 0.2%TritX + 0.2% fish gelatin three times for two minutes. Cells were further blocked in 1XPBS + 0.2%TritX + 0.2% fish gelatin for 15 minutes before the addition of primary antibody diluted in block. Primary antibody was added for one hour at room temperature, followed by three two-minute washes with block. Secondary antibody was diluted in block and added for one hour at room temperature, then washed three times for two-minute with block. Cells were post-fixed with 4% PFA + 0.1%TritX for 15 minutes, then washed with block three times for two minutes. Cells were then imaged using an inverted Zeiss LSM 780 confocal microscope.

S2 Cell Phagocytosis Assay and Okadaic Acid Treatment:

Drosophila S2 cells were grown and maintained in Schneider's media supplemented with 10% FBS and 1% Glutamax 100X (Gibco). Cells were plated at a density of 6x10^5 in 96 well plates with glass bottoms and allowed to adhere for 1 hour. PHrodo Green S. aureus particles (Life Technologies P35366) were added to cells for 1.5 hours, then imaged immediately using a Zeiss LSM 780 inverted confocal with a 63x oil immersion lens. Control cells were kept at 4C to block phagocytosis before imaging. Percent phagocytosis was calculated as # of pHrodo positive cells / # of total cells.

Okadaic Acid Treatment: Okadaic Acid (OA) was added to cells in T25 flasks for 3 hours before plating the cells in 96 well plates for the phagocytosis assay. OA was added from at a

concentration of 50nM from a 100uM stock. During imaging, cells morphology was examined to confirm cell health.

Antibodies:

The following antibodies were used in brains: rat anti-Falafel (1:1000, a kind gift from Bill Chia); chicken anti-GFP (1:1000, Life Technologies); mouse anti-Repo (1:10, Developmental Studies Hybridoma Bank); chick anti-betaGal (1:2000); mouse anti-MMP-1 (Developmental Studies Hybridoma Bank 14A3D2, 3A6B4, 3B8D12, 5H7B11) at 1:50 used at 1:1:1:1 ratio. All secondary antibodies (Jackson Immunoresearch) were used at a concentration of 1:400.

The following antibodies were used in S2 cells: rat anti-Falafel (1:250), mouse anti-Draper (1:250). All secondary antibodies (Jackson Immunoresearch) were used at a concentration of 1:250.

Confocal Microscopy, Image Analysis, and Statistics:

Imaging was performed using a LSM 710 confocal microscope as described in Chapter 2. Image analysis was performed on Volocity imaging software (Perkin-Elmer) and statistics were performed in Prism (GraphPad) as described in Chapter 2.

Chapter 4. Discussion and Future Directions

CONCLUSIONS FROM CHAPTER 2:

In Chapter 2, I identified a novel member of the glial immune signaling pathway: the PP4 complex. I showed that all three *Drosophila* subunits of PP4 (Falafel, PP4c, PP4r2) are necessary in adult flies for glial mediated clearance of axonal debris after an injury. I also presented evidence that PP4 is necessary for proper glial cell membrane dynamics after injury, and hypothesized that glial cells lacking PP4 are unable to properly extend their membranes to the site of injury to phagocytose the neuronal debris. I proposed that after the Draper receptor becomes activated in response to injury, PP4 acts upon the GEF complex DRK/DOS/SOS to activate Rac1 to allow for proper glial membrane extension and phagocytosis of debris.

I found reduced levels of Draper receptor along the responding glial membranes in PP4c knockdown flies, but normal activity of the Draper transcription factor STAT92e, suggesting that this decrease in Draper receptor is due to a reduction in glial cell membranes and not due to a direct interaction between PP4 and Draper. Further, I determined that PP4c overexpression cannot rescue *Draper^{RNAi}* -induced clearance defects, and that *Draper^{RNAi}* brains did not have decreased Falafel levels. This evidence suggests that Draper and PP4 are not directly interacting in the glial immune response. I attempted to assay clearance in an experiment in which Draper I was overexpressed and PP4c was knocked down, but the Draper I OE flies had too much Draper which caused a clearance defect, as was also seen by Purice et al(149). In the future, it would be interesting to make a new fly line using the LexA-LexAop system along with Gal80ts-Gal4-UAS to separately control the expression of *PP4c^{RNAi}* and *UAS-Draper I* in the hopes of inducing a smaller amount of Draper I overexpression.

In Chapter 2 I also found further evidence that PP4 is necessary for glial membrane dynamics. In *PP4c*^{RNAi} brains, I found reduced glial cell membrane expansion at the site of injury compared to controls. I also observed reduced F-actin and reduced Rac1 levels after injury via immunofluorescence. Additionally, I revealed that Rac1 overexpression could rescue the *PP4*^{RNAi} clearance and Draper defects after injury. Finally, I discovered that overexpression of the putative Rac1 GEF member, SOS, could partially rescue clearance in *PP4c*^{RNAi} flies. Together, this data supports the idea that PP4 is upstream of Rac1 in the glial immune response.

In the future, it would be interesting to further examine if PP4 is activating Rac1 after injury in glial cells. The evidence I provide in Chapter 2, suggests there is an interaction between PP4 and Rac1, but it would be nice to have stronger evidence of a direct interaction. One experiment that could examine this would be a Rac1 activation assay. I performed a trial Rac1 activation assay with an old, borrowed kit (thank you Michael Cohen!) however I was unable to collect meaningful data. These kits (from Cytoskeleton Inc.) measure the active, or GTP-bound forms of Rac1 in tissue after various stimulations. I used S2 cells with the kit from the Cohen lab, although I think using homogenized *Drosophila* brain tissue would be a good option for the future. To simulate an injury response, I added pHrodo particles to the S2 cells. I collected cell lysates and added them to a 96-well plate from the kit that contains a powder that binds active Rac1. After addition of Rac1 primary and secondary antibodies, I measured the luminescence in the wells. Wells with more active Rac1 have a higher luminescence. I did not observe any meaningful difference between controls and S2 cells treated with okadaic acid to block PP4, but I think this was due to the age of the kit and the small number of samples I was able to use. We decided to not re-do this experiment with a new kit because the cost of the kit is very high.

In the future, it could be interesting to perform a similar experiment to determine if PP4 is directly activating Rac1, although our current evidence strongly suggests that this is so.

CONCLUSIONS FROM CHAPTER 3:

In Chapter 3, I explored potential PP4 targets for dephosphorylation during the immune response and the role that PP4 location plays when determining these targets. I found evidence that the location of Falafel is important for a proper glial immune response, and that Falafel is likely translocating from the nucleus into the cytoplasm after injury. I next examined two potential PP4-targets, however I did not confirm that either one was involved in the immune response.

I found that $PP4c^{RNAi}$ brains had reduced basal levels of phospho-AMPK, but a similar decrease in phospho-AMPK after injury compared to control brains. AMPK is an energy sensor kinase that has recently been shown to have immune functions and can regulate cell polarity and motility(102, 103). Interestingly, there are numerous studies revealing a relationship between AMPK and Rac1, although there is conflicting evidence to the nature of this relationship(105, 106). In the future, it would be interesting to further study why $PP4c^{RNAi}$ brains have reduced AMPK activity in uninjured brains, and assess if knockdowns of the other two PP4 subunits result in a similar reduction. My results contradict the study by Liao et al. in T-cells, where the authors found decreased phospho-AMPK after PP4c overexpression, therefore more work needs to be done to determine the cause of this contradiction. It is also interesting to consider why active AMPK levels might decrease after injury. Studies have shown that AMPK activity is tied to cell migration, so it is possible that glial cells must reduce AMPK activation for proper glial cell movements in response to injury.

Since PP4 function is thought to be linked to the location of PP4 regulatory subunits, I examined Falafel location and nuclear translocation after injury. In Chapter 2 I found that Falafel, which is normally nuclear, seems to leave the nucleus after injury. I was unable to confirm that Falafel is actually entering the cytoplasm *in vivo*, however, in Chapter 3 I discussed my *in vitro* evidence that Falafel translocates the nucleus into the cytoplasm.

In the future, it would be interesting to determine if we could observe Falafel entering the cytoplasm *in vivo*. In our previous experiments, we have been limited by imaging resolution, as the glial cell cytoplasm is very thin. I expressed cyto-GFP in glial cells to try to segment my quantification to the cytoplasm, but was unable to detect Falafel antibody signal in the cytoplasm at a level above the fluorescent background noise. While more work needs to be completed to further investigate this translocation, it seems likely that Falafel is leaving the nucleus after injury to influence PP4 target localization in the cytoplasm. Further, it would be nice to have a PP4c antibody that worked in brains so I could examine PP4c location and also quantify percent knockdown in my RNAi flies.

In Chapter 3, I was able to study the effects to axonal clearance after expressing a cytoplasmic-bound Falafel in glia. Thanks to the generous gift from Dr. Bill Chia, I had the use of fly lines expressing Venus-Falafel or Venus-Falafel$^{\Delta 3NLS+2NES}$. After injury, I found reduced clearance of axonal debris and a trend towards reduced Draper recruitment in the fly line expressing cytoplasmic-bound Falafel. This result suggests that Falafel must be nuclear or able to translocate the nucleus in glial cells for a proper immune response. The biggest caveat to this experiment was the difficulty in quantifying the GFP+ axonal debris due to the fluorescence from the Venus-Falafel. To overcome this difficulty in the future, it would be useful to have a line with olfactory receptor neuron drivers tagged with RFP, resulting in RFP+ axons (i.e. OR85e-RFP). Therefore, one could distinguish between the RFP

axons and the Venus-Falafel with more acuity. It is interesting to guess at the functional implications of the Venus-Falafel$^{\Delta 3NLS+2NES}$ line. Is it possible that Falafel re-enters the nucleus after injury? We examined Falafel location at 3 and 6 hours after injury and found reduced nuclear levels, but it could be useful to check Falafel location at a later time point as well.

It is interesting to speculate about what Falafel could be doing in the nucleus and cytoplasm. In my introduction, I mentioned that JNK and insulin signaling are important players in the glial immune response. There is some evidence that PP4 can interact with both JNK and insulin in other cell types (94). In human cancer cells, increasing PP4 is correlated with increased JNK activity. In HEK293 cells, PP4 has been shown to downregulate insulin receptor protein 4 (IRS-4)(95). Recently, Musashe et al. and MacDonald et al. have shown that insulin-like signaling and JNK signaling play vital roles in the glial immune response in *Drosophila(58, 97).* These studies provided us with the idea that PP4 could be interacting with JNK and insulin signaling in glial cells after injury.

To examine this idea, I performed two preliminary experiments. First, I used the Tre-GFP reporter line, which is a transcriptional reporter of *Drosophila* AP-1 activity, which is downstream of JNK signaling in *Drosophila* glia(58). I expressed Tre-GFP while knocking-down PP4c in glia, however these fly lines did not survive well, so I had too low of an N to make proper conclusions. In the future, it would be interesting to repeat this experiment and try to increase my N. I also examined insulin signaling in *PP4c^{RNAi}* flies, however I also had low survival and could not come to a conclusion. Instead, I tried to increase insulin signaling in S2 cells. I inhibited the PP4 complex with okadaic acid, then incubated the cells with additional insulin, and analyzed pHrodo phagocytosis. I did not see any improvement in pHrodo phagocytosis in the okadaic acid condition with the addition of insulin, but I also

had low phagocytosis in my insulin-only controls. In the future, this protocol could be enhanced to ensure that insulin is not having deleterious effects on phagocytosis in S2 cells. Due to the literature, I think there could be a connection between PP4 and JNK or insulin signaling after injury in glial cells, so this would be a noteworthy future question to explore.

S2 CELL FUTURE DIRECTIONS:

There are several reasons why I did not pursue more experiments in S2 cells, although I think the lab could benefit from using this system in the future. The S2 cells were very simple to grow and maintain, and importantly, they readily phagocytose various objects.

One drawback that I encountered for the S2 cells was inconsistent staining results. I was rarely able to successfully stain the cells for Draper, although I consistently had a nice Falafel stain. I trouble-shot the staining protocol several times, varying the block, fix, wash solution, antibody concentration, and cell confluency, but never settled on a perfect method. My "best" method is listed in the methods section of Chapter 3. More work in this area would be necessary for the lab to consistently use S2 cells for antibody staining.

Another issue with my S2 cell experiments was using okadaic acid to inhibit the PP4 complex. Initially, I planned on using dsRNAs to knockdown each PP4 subunit, but this proved difficult and expensive. We ordered dsRNAs from the *Drosophila* RNAi Screening Center (DRSC) at Harvard, and I proceeded to test the functionality of Falafel dsRNA in the S2 cells since I could examine knockdown effectiveness with an anti-Falafel stain. Literature suggests anywhere from a one- to seven-day dsRNA incubation, with a single dose or several doses of dsRNA(150, 151). I performed a 3-day and 7-day incubation, with the 7-day incubation having two additions of dsRNA at 10ug/ml concentration (day 1 and day 4). Upon Falafel immunostaining, I did not observe any change in Falafel antibody, suggesting

that I was not sufficiently knocking down Falafel with the dsRNA. Further work would need to be completed for each dsRNA to optimize this technique in the lab. Therefore, we proceeded with the okadaic acid inhibition. Okadaic acid has been characterized as being specific for inhibiting PP4 versus other protein phosphatases, such as PP1. Swingle et al. have produced an IC50 for okadaic acid, and I used this information to select an okadaic acid concentration that should only inhibit PP4, however I cannot confirm the specificity of this inhibition. To be certain, I would need to perform an IC50 of my own, perhaps using phagocytosis as a readout of PP4 activity. For further okadaic acid studies in the lab, I would suggest performing this IC50 curve.

A potential benefit of using S2 cells in the lab in the future would be live imaging of cell movement and membrane dynamics. S2 cells are not very motile under basal conditions, so it would be interesting to observe changes in the actin cytoskeleton during phagocytosis after the addition of pHrodo particles. For example, it would be interesting to measure membrane movements in control S2 cells compared to S2 cells with okadaic acid or PP4 dsRNAs. Live imaging of S2 cells is also straightforward: S2 cells survive well at room temperature and at normal atmospheric oxygen levels(152). In the future, I think S2 cells could prove a valuable model system for the Logan lab.

Finally, while this is beyond the scope of my dissertation work, it is interesting to consider what Falafel is doing in neurons. I observe nuclear Falafel in neurons, but have never performed experiments to determine the role of Falafel in neurons. Since I performed experiments that only affected glial cells (i.e. glial-specific knockdowns) it is unlikely that neuronal Falafel was having any affect in my experiments. There are a small number of studies examining Falafel during neuronal development. Sousa-Nunes et al. identified Falafel during a larval neuroblast clonal screen as being important for interphase and

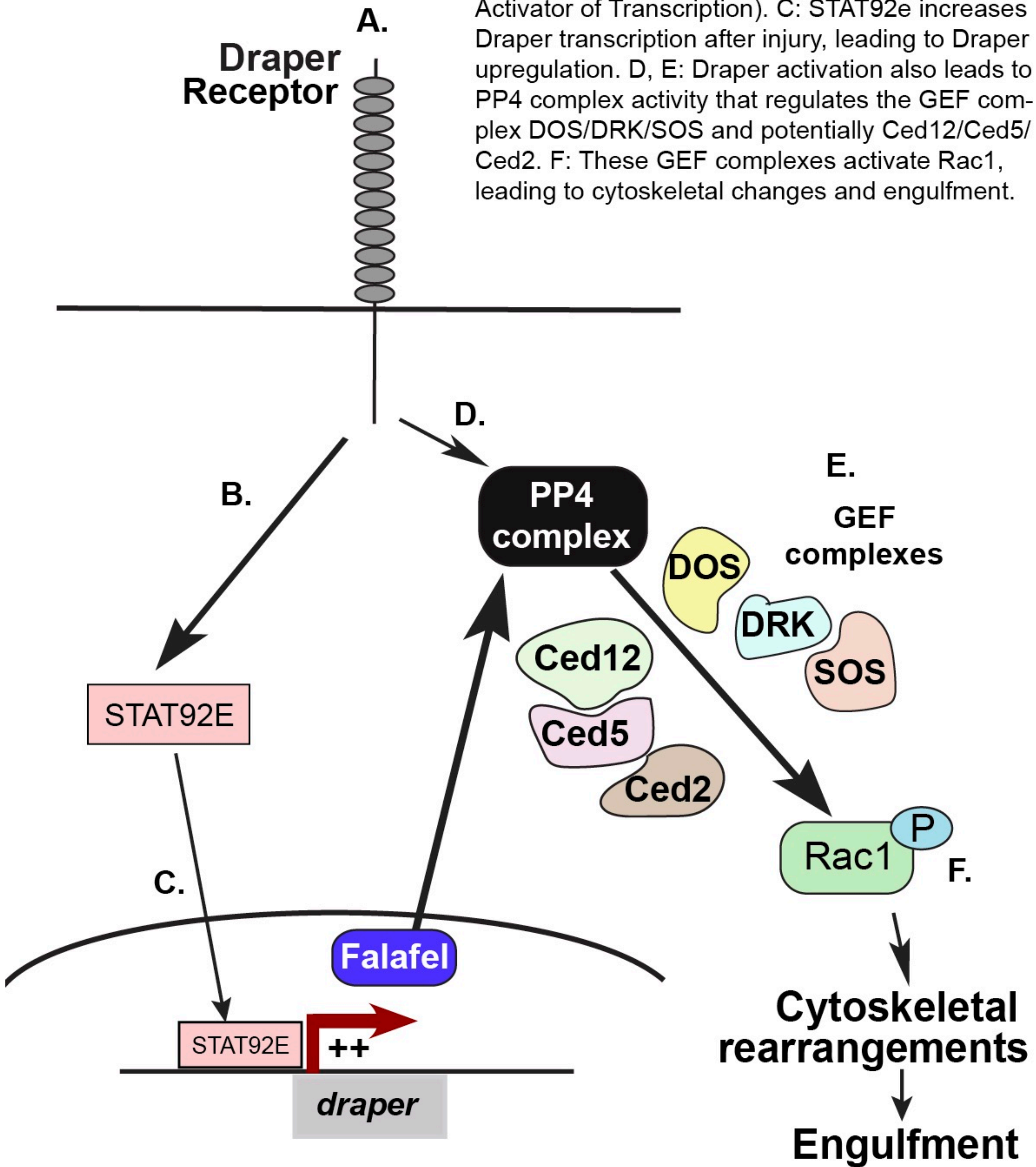
mitosis(113). Knockdown of Falafel, PP4c, or PP4r2 lead to incorrect localization of various cell fate determinants. The authors suggest that Falafel was targeting the PP4 complex to interact with the anchoring protein Miranda to allow for proper neuronal cell division. The authors propose a model in which Falafel becomes cytoplasmic after nuclear envelope breakdown to dephosphorylate Miranda, although they were unable to show evidence for this dephosphorylation step(153).

An additional study by Lyu et al. finds that Smek1 (mouse Falafel) promotes differentiation of neural precursor cells (NPC) and quells NPC proliferation(126). The authors find that Smek1 suppresses activity of Par3, a cell polarity protein, to increase neuronal differentiation. Further, the authors show that Smek1 is initially nuclear but becomes cytoplasmic during mitosis, where it interacts with cytoplasmic Par3. The authors suggest that Falafel targets the PP4 complex towards cytoplasmic Par3, where PP4c dephosphorylates Par3 to regulate neurogenesis. These studies show a role for Falafel and the PP4 complex in neurons during development, however they do not answer the question of what, if anything, Falafel is doing in adult *Drosophila* neurons.

My Favorite Model

Due to my many years of presenting at Insect Brunch, I have been taught to always end with "my favorite model" of what I think is going on inside the cell. My favorite model is that Falafel is initially nuclear, but that Falafel monitors transcriptional activity of Draper and other engulfment genes after injury. Upon a signal (maybe transcription of a certain engulfment gene) Falafel translocates from the nucleus and directs PP4c and PP4r2 to DRK/DOS/SOS to activate Rac1. DRK (mammalian Grb2) is an adaptor protein, thought to bind to phosphorylated receptors (such as Draper). DRK contains SH2 and SH3 domain sites. SOS can bind the N-terminal SH3 domain, while DOS can bind the C-terminal SH3

domain(154). Therefore, it is likely that DRK/DOS/SOS are closely associated with the activated Draper receptor and are close to the cell membrane. So, in my model, Falafel would bring PP4c and PP4r2 towards the Draper receptor to act upon SOS. SOS then activates Rac1, which leads to cytoskeletal changes (see Summary Figure). It is also possible that Rac1 could itself then affect transcription of genes. There is some evidence that Rac1 can act as a transcription factor, for instance acting at Wnt responsive promoters, and evidence that Rac1 promotes the translocation of STATs to affect transcription(155, 156). It is possible that Rac1 could be affecting STAT92e-mediated Draper transcription in a feedback loop after injury, although we currently have no evidence that PP4 is responsible for this Rac1 action.



Summary Figure. A: Draper receptor becomes activated (potentially by “eat me signals.” B: Draper leads to STAT92e activity (Signal Transducer & Activator of Transcription). C: STAT92e increases Draper transcription after injury, leading to Draper upregulation. D, E: Draper activation also leads to PP4 complex activity that regulates the GEF complex DOS/DRK/SOS and potentially Ced12/Ced5/Ced2. F: These GEF complexes activate Rac1, leading to cytoskeletal changes and engulfment.

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