Molecular mechanisms of glial immune responses to neurodegeneration in the young and aged CNS

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LIST OF ABBREVIATIONS

50-terminal oligopyrimidine: TOP

A disintegrin and metalloproteinase: ADAM

Accessory mesothoracic: AMN

Activator protein 1: AP-1

Alzheimer's disease: AD

Amyloid-beta: aβ

Amyotrophic lateral sclerosis: ALS

Ascending sensory tract: AST

Autophagy-related 1: Atg1

Beta-galactoside: β-gal or beta-gal

c-Jun N-terminal kinase: JNK

Central nervous system: CNS

Complement component 4: C4

Constitutively active PI3K92E: CA-PI3K

Destabilized GFP: dGFP

Draper enhancer element 7: dee7

Drosophila sterile α/Armadillo/Toll-Interleukin receptor homology domain protein: dSarm

Empirical analysis of digital expression in R: EdgeR

Glial fibrillary acidic protein: GFAP

Green fluorescent protein: GFP

Huntingtin: Htt

Insulin-like growth factor 1: IGF-1

Insulin-like receptor: InR

Insulin-like signaling: ILS

Janus kinase/signal transducer and activator of transcription: JAK/STAT

Matrix metalloproteinase-1: MMP-1

Mesothoracic: MN

Metathoracic: MtN

Methyl-CpG-binding protein 2: MeCP2

mitochondria polymerase γ: POLG

Mitochondria-targeted GFP: mito-GFP or mito::GFP

Mitogen-activated protein kinase: MAPK

Nicotinamide mononucleotide adenylyltransferease: Nmnat

Olfactory receptor neuron: ORN

Parkinson's disease: PD

Peripheral nervous system: PNS

Phosphoinositide-3-kinase: PI3K

Phosphorylated insulin receptor: phospho-InR or phInR

Prothoracic: PN

Regulatory RNA motifs and elements finder: RegRNA

RNA-sequencing: RNA-seq

Superoxidase dismutase 1: SOD1

Temperature sensitive version of Gal80: Gal80ts or $Gal80^{ts}$

Tissue inhibitor of metalloproteinases: TIMP

Toll-like receptor 4: TLR-4

Ubiquitination factor E4B: Ube4b

Ventral nerve cord: VNC

Vesicular glutamate transporter 1: VGLUT1

Wallerian degeneration slow: Wlds or Wld^{s}

Wallerian degeneration: WD

Wild type: WT

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ABSTRACT

Glial cells refine synaptic signaling, ensheath axonal projections and, importantly, are the first immune responders in the brain after trauma or stress. During reactive gliosis, glia undergo a multi-step process of recognizing the injury, traveling to or expanding membranes at the site of injury, engulfing and destroying the debris. In *Drosophila*, the Draper/MEGF10 engulfment pathway is required for engulfment of degenerating axonal material. However, little is known about how brain aging mechanisms contribute to glial health and what other neuroprotective pathways are upregulated during reactive gliosis.

Advanced age is the most significant risk factor for neurodegenerative diseases, but it remains unclear how aging contributes to declining neuronal function, cognition and brain health. It has been proposed that altered glial immune activity in the aging brain may be coupled to the onset and progression of common neurodegenerative diseases, but it is unclear how changes in the molecular profile of aged glia are linked to an increased risk for neurodegenerative pathologies. We used *Drosophila* as a model organism to examine how key features of glial immunity are altered with age. Using a well-established acute axotomy assay in the olfactory system, we can monitor glial responses to neurodegeneration. Using this strategy, we observed that Wallerian degeneration of olfactory receptor axons, recruitment of glial membranes, and clearance of axons are all significantly delayed in aged flies. This work highlights reduced glial engulfment activity as an intriguing candidate mechanism for age-related vulnerability to neural damage and disease.

Although our work demonstrates that the Draper engulfment pathway is required for maintenance of glial health in the aging brain, we lack a complete

understanding of other genes and pathways involved in glial immune activity. In order to uncover novel genes and pathways involved in glial responses to neuronal injury, we established a novel injury paradigm that includes injury to peripheral structures (e.g. legs, wings) to compare the molecular profile of glia in uninjured ventral nerve cords (VNCs) compared to injured VNCs that contain actively degeneration axons. We performed RNA-sequencing (RNA-seq) and uncovered 500+ genes that are significantly upregulated and have human orthologues. We found that one gene in particular, *matrix metalloproteinase-1* (MMP-1) was transcriptionally upregulated by ensheathing glia and was required for glial membrane expansion and clearance of degenerating axonal material. Importantly, expression of MMP-1 in the aging brain in response to neurodegeneration was greatly attenuated. Our findings are significant because the RNA-seq screen will not only help uncover novel genes involved in glial immune responses, but also genes that are dysregulated in the aging brain. The differentially expressed genes will serve as a resource for the glial community.

Chapter 1: INTRODUCTION

GLIA

"Nature takes form, and form is a by-product of evolution," said architect Moshe Safdie while trying to explain that humans would be much better at building homes and cities if we just took the time to observe how nature has unfolded itself. After all, Earth has had over 4.5 billion years to perfect every crystal, rock, virus, plant, and animal. Why then not follow Safdie's advice when studying the brain? Although the field of neuroscience has existed for almost two centuries, scientists have generally failed to study one of the main cell types found in the brain, glia. Unlike most organs which are made of primarily one cell type (i.e. hepatocytes make up the liver, cardiomyocytes make up the heart) the brain is composed of both glia and neurons. Is it then not a betrayal to nature to just pick one cell type to study the brain and claim that we are trying to understand the entire organ as a whole?

Glial cells were first described in 1856 by the "father of modern pathology" Rudolf Virchow who named them based on the Greek word for glue, believing that their function was only to support and nourish neurons (Ndubaku and de Bellard, 2008). It is important to note that the first description of glial cells appeared almost 20 years prior when Robert Remak described what we now call Schwann Cells in his dissertation (Remak, 1838). During the roaring twenties, Pio del Rio Hortaga (a student of Santiago Ramon y Cajal) was able to distinguish and elegantly describe what today we know as the three major types of glial cells in the brain: astrocytes, microglia, and oligodendrocytes (Tremblay et al., 2015). All of this work and other glial work not described here was overshadowed starting about 1894 when Spanish anatomist Ramon y Cajal established the groundbreaking and important neuron doctrine, which specified that the neuron is the basic anatomical and physiological unit of the nervous system

(Glickstein, 2006). Although we have learned immensely about how the brain works since the neuron doctrine was described, in taking a rather "neurocentric" approach, the field of neuroscience has a fragmented understanding of the brain as an organ.

The term glia describes three very complex and distinct cell types in the central nervous system (CNS): astrocytes, microglia, and oligodendrocytes. The glial cells in the peripheral nervous system (PNS), Schwann cells, are very similar to the CNS oligodendrocytes. All of these cells are critical for brain function during development, adulthood, and in disease. In the last few decades, scientists have begun to recognize that glia are important not just for providing trophic support to neurons, but also for modulating neuronal activity, increasing the conduction velocity of axons as a result of myelination, and removing cellular debris and pathogens from the brain (Allen and Barres, 2009; Barres, 2008; Risher et al., 2014). In fact, disorders that affect the health of any of the glial subtypes leads to neurodegeneration and disrupted brain function. Although all of the different glial subtypes and their functions are described in more detail below, the research presented in this dissertation will focus specifically on glial phagocytosis of degenerating axons.

ASTROCYTES:

Astrocytes (meaning "star-like") are the most diverse and abundant of the glial subtypes. Astrocytes can have different function and even morphology depending on the area of the brain in which they are found. Astrocytes have countless roles in the brain. They are involved in maintaining brain homeostasis, building the brain architecture, forming the blood-brain barrier, controlling blood flow, etc (Khakh and Sofroniew, 2015). Most interestingly, during development astrocytes use secreted and

contact-mediated signals to control synapse formation, maturation, elimination, and engulfment (Clarke and Barres, 2013). It is not hard to imagine astrocytes having so many roles in synapse function when it has been described that one single rodent astrocyte can associate with multiple neurons and can contact up to 100,000 synapses (Chung et al., 2015).

Astrocyte dysfunction during development is associated with several neurodevelopmental disorders (Sloan and Barres, 2014). One seminal paper demonstrated just how critical astrocytes are in the progression of Rett Syndrome, a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in females. Rett Syndrome is caused by mutations in the gene *MECP2* encoding X-linked methyl-CpG-binding protein 2 (MeCP2) (Chahrour and Zoghbi, 2007). Lioy et al. 2011 demonstrated that astrocyte-specific expression of MeCP2 in global MeCP2 null mice ameliorated several phenotypes including locomotion levels, breathing patterns and extended lifespan. Importantly, the authors also showed that restoring MeCP2 in mutant astrocytes only was sufficient to restore dendritic morphology and levels of excitatory glutamate transporter VGLUT1 (vesicular glutamate transporter-1) in neurons, thus demonstrating that astrocytes can have a very strong non-cell-autonomous effect on mutant neurons (Lioy et al., 2011). Studies such as this one highlight the importance of studying glia-neuron interaction even in neuronal dysfunction disorders.

OLIGODENDROCYTES:

The main function of oligodendrocytes is to produce myelin, which insulates axons and allows for high velocity of nerve conduction. Although invertebrates also have glial cells that ensheath axons, myelin itself is only found in vertebrates (Baraban et al., 2016). Large animals that do not have myelin, such as the squid, for example, have evolved to have giant axons with diameters as large as 1mm to allow for fast nerve conduction velocities (Hodgkin and Katz, 1949). Independent of myelination, oligodendrocytes work together with astrocytes to provide trophic support to axons (Morrison et al., 2013). Oligodendrocytes shuttle lactate to neurons in order to fuel axonal ATP generation, and dysfunction in this lactate transfer leads to neurodegeneration of axons. Now that we know some of the factors that are necessary for this transfer of energy to neurons, it is imperative that more studies of neurodegenerative disorders investigate whether glial trophic support is impaired.

After Albert Einstein died in 1955, several studies were done looking at histological and morphological features of his brain in order to uncover features of what made him a genius. To much dismay, Einstein's brain was quite average. One more recent study showed that Einstein had a significantly thicker corpus callosum, meaning more white matter insulating the connections between the two brain hemispheres, and insinuated that it is "another clue to his high intelligence" (Men et al., 2014). Although it seems like an oversimplification to make correlations about unusual anatomical characteristics and intelligence when there is so much heterogeneity in brain anatomy from person to person, studies in rodents have shown that increased myelination has been associated with increased ability to learn (McKenzie et al., 2014). For example, it was recently demonstrated that in the adult mouse brain, oligodendrocytes contribute to

early and late stages of motor skill learning and that deleting a critical transcription factor in oligodendrocyte precursors impairs learning (Xiao et al., 2016). This study is important because it shows that there is a close link between oligodendrocytes and synaptic strengthening, and additionally, that oligodendrocytes generated later in life are not only functional but contribute to learning and storage of memories (Xiao et al., 2016).

MICROGLIA:

Microglia are the resident immune cells of the mammalian CNS. Microglia share several features with macrophages, but it has been recently shown that microglia have distinct developmental origins and functions that differentiate them from macrophages (Bennett et al., 2016). Even when microglia are in a resting state, they are constantly moving and investigating whether there is local debris, damage, or weak synapses that need to be removed (Nimmerjahn et al., 2005; Zuchero and Barres, 2015). Once microglia recognize an insult, they undergo changes in their molecular profile and morphology, migrate to the site of insult, phagocytose and destroy debris, and can even recruit other microglia for assistance (Aguzzi et al., 2013; David et al., 2015). Activated microglia can be heterogeneous in action depending on the insult or different area of the brain (Colton, 2009; David and Kroner, 2011). Additionally, microglia have evolved to express a plethora of recognition, phagocytosis, and immune receptors (Noda and Suzumura, 2012).

Microglia, like astrocytes, are absolutely necessary for proper circuit formation during development. If microglia do not prune weak synapses during the development

of the retinogeniculate system, then the excess neuronal connections lead to developmental defects (Schafer et al., 2012; Stephan et al., 2012; Stevens et al., 2007). Microglial dysfunction can also lead to neuropsychiatric disorders like schizophrenia (Sekar et al., 2016). More specifically, individuals that have heritable schizophrenia have varying high levels of the *complement component 4 (C4)* genes and the C4 proteins localize to entire neurons, including synapses (Sekar et al., 2016). Microglia recognize the C4 protein as an "eat me signal" and aberrantly phagocytose synapses and neurons, which implicates excessive microglia activity as drivers of reduced synapse numbers in schizophrenia (Sekar et al., 2016). Excessive microglia phagocytosis has also been shown to be a driver of synapse and neuronal loss in Alzheimer's disease (AD) (S. Hong et al., 2016), which is a very critical finding because synapse loss in AD correlates with cognitive decline (Selkoe, 2002; Terry et al., 1991). More specifically Hong S. et al., 2016 showed that in mouse models of AD or in wild-type (WT) mice that have had oligomerized amyloid-beta (Aβ) injections, microglia use the complement system to inappropriately tag synapses for engulfment. This study has important clinical implications because to date, there is no reliable biomarker for AD or drugs that stop the progression of the disease. Now that we know that microglia are active drivers of AD pathogenesis (S. Hong et al., 2016), we can start exploring potential drugs that inhibit aberrant microglia phagocytic activity.

SCHWANN CELLS:

Schwann cells are the myelinating cells of the PNS. Like oligodendrocytes, their main role is to provide trophic support as well as insulation to the axons through the

formation of myelin. Schwann cells differ from the oligodendrocytes in that one Schwann cell myelinates one single internode, while an oligodendrocyte can myelinate several internodes (Chang et al., 2016). Schwann cells and oligodendrocytes also have different molecular and genetic profiles. Unlike oligodendrocytes that originate from neuroepithelial cells (Zhou et al., 2000), Schwann cells originate from the migrating neural crest cells (Dupin et al., 1990). It is unknown where in evolution these two cells types diverged or if this is an example of convergent evolution (Zalc, 2016).

Schwann cells ensure the structural and functional integrity of peripheral axons during development, adulthood, and regeneration after injury, therefore when disease affects the health of Schwann cells, the consequences are devastating (Bhatheja and Field, 2006). During diabetes, Schwann cells are compromised in providing neurotrophic support that is essential for nerve function (Eckersley, 2002). The Center for Disease Control 2014 National Diabetes Statistics Report claims that 9.3% of the United States population have diabetes and that 1 out of the 3 remaining adults have prediabetes. Currently, there is controversy regarding whether neuropathy due to either type 1 or type 2 diabetes is primary or secondary to Schwann cell dysfunction (Mizisin, 2014; Zenker et al., 2013). Regardless of whether axonal loss or demyelination is the initial insult, there is strong evidence that Schwann cells are sensitive to varying levels of glucose and insulin (Askwith et al., 2009; Di Liu et al., 2016). Because the rates of diabetes continue to rise sharply, it is imperative that more studies address the cause of neuropathy and whether Schwann cells are good drug targets for inhibiting neuropathy during diabetes.

TRANSCRIPTOME PROFILING OF MAMMALIAN GLIA:

Researchers are just now poised to uncover the specific functions of astrocytes, microglia, oligodendrocytes, and Schwann cells. In order to better understand the distinct and overlapping roles of each glial cell type, several labs have developed methods to purify each glial cell type and visualize their transcriptome. Most recently RNA-seq technology has become the gold standard for transcriptome analysis because it allows researchers to investigate known and novel transcripts in an unbiased manner (which is great for non-model organisms with unknown genomic sequences) and has very low background (Wang et al., 2009). The Barres lab has been a leading pioneer in modifying known methods to efficiently separate glial cell types and in creating a transcriptome database of expression levels of different cell types. More specifically, they purified the following cell types from mouse cerebral cortex and created a simple point-and-click website platform for examining and comparing transcription and alternative splicing profiles: neurons, astrocytes, oligodendrocyte precursor cells, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, endothelial cells, and pericytes (Y. Zhang et al., 2014). Many labs have also looked at several of these glial cell types in comparison to neurons but this is the first that they have been globally characterized at the same time and such detail (Y. Zhang et al., 2014). The website http://web.stanford.edu/group/barres_lab/brain_rnaseq.html is an invaluable resource to neuroscientists for understanding cell-cell interactions in the brain. In the future, it is important to do the same type of detailed analysis on PNS cells. Additionally, recent data suggests that the same glial cell types might have different roles depending on the area of the brain in which the cell resides (Bayraktar et al., 2015; Molofsky et al., 2014; Schmid et al., 2002). Region-specific functional analysis is also

needed to understand such differences and how they affect cell function. Lastly, now that it is established that glial cells have critical roles during development, aging, disease and after injury, transcriptome analysis of these different states is also essential in order to better understand how these cells interact with each other in the healthy and diseased brain. Transcriptome profiling of healthy and injured CNS tissue is explored in Chapter 3 of this manuscript.

GLIAL RESPONSES TO WALLERIAN DEGENERATION

In the mid-nineteenth century, neurophysiologist August Waller was the first to describe that after the tongue nerve of a frog was transected, the portion most distal to the injury undergoes swellings followed by progressive degeneration (Waller, 1850). We now call this process Wallerian degeneration (WD) and we know that it is conserved from insects to humans (Hoopfer et al., 2006; MacDonald et al., 2006). Although WD occurs in both the PNS and the CNS, there are significant differences in the timeline and progression of degeneration between the two nervous systems. For example, mammalian PNS WD occurs very fast, occurring in a timeframe of 7-14 days, while CNS WD is considerably slower, occurring in the timeframe of months-to-years (Vargas and Barres, 2007). Although these differences are still actively being studied, it has been shown that the difference in the rates of WD between the two nervous systems is due to the CNS environment and the failure to clear myelin debris in the CNS (Vargas and Barres, 2007). Any form of trauma to the axon, whether acute or chronic, can induce WD. After axotomy, the axon undergoes an active and complex process of genetic and molecular changes. In brief, there is a large calcium influx that activates proteases, which

then degrade the cytoskeleton and cellular compartments (Conforti et al., 2014). The myelin sheath also breaks down and the degenerating neuronal and myelin debris that is most distal to the injury gets removed by immune cells (including glia) (Conforti et al., 2014).

WALLERIAN DEGENERATION IS AN ACTIVE PROCESS

For a century and a half after Waller made the critical observation that axons undergo progressive degeneration after injury, degeneration was believed to be a passive process resulting from lack of trophic factors from the severed cell body. It was only after the serendipitous discovery of a spontaneous mutation in the C57Bl/6 mouse line that we learned that WD is an active process (Lunn et al., 1989). These mutant mice appeared normal but after nerve axotomy, the researchers observed that the distal axons and nerve terminal remained intact for weeks before degenerating (Lunn et al., 1989). The delay in WD was observed in both CNS and PNS axons and extraordinarily, the axons were still capable of conducting action potentials and releasing neurotransmitters (Lunn et al., 1989). The mouse strain and the mutation were given the name Wallerian Degeneration Slow (Wld^S).

The delay in WD in the Wld^S mouse is an intrinsic property of the neuron. Despite a lot of research into these mice, the exact function and mechanisms that lead to neuroprotection after injury are still unknown. The Wld^S gene mutation is a triplication of an 85kb fragment that contains the 5´ end of *ubiquitination factor e4b (Ube4b)* and full-length *nicotinamide mononucleotide adenylyl transferase 1* (*Nmnat-1*), which encodes the enzyme responsible for synthesizing NAD+ from NADH. The mutation leads to the

expression of a novel chimeric fusion of the two Ube4b/Nmnat-1 proteins, and it is localized to the nucleus (Mack et al., 2001). The exact mechanism for protection is unknown, and even controversial, but there is evidence that overexpression of Nmnat-1 (or its effector NAD+) is neuroprotective (Araki et al., 2004; Awasaki and Ito, 2004; Mack et al., 2001).

GLIAL RESPONSE TO WALLERIAN DEGENERATION

The cellular and molecular processes through which WD occurs in both the PNS and the CNS have been examined in great detail (Gillingwater and Ribchester, 2001). In the brain and spinal cord specifically, Wallerian-like degeneration features granular disintegration of the cytoskeleton, large axonal swellings, degenerating myelin, fragmentation of distal axons and recruitment of astrocytes, microglia, and even macrophages (if there is a breakdown of the blood brain barrier) (Conforti et al., 2014). Interestingly, unlike Schwann cells in the PNS that aid after injury by clearing debris, multiplying, and recruiting macrophages (Liu et al., 1995), the oligodendrocytes in the CNS respond to WD by initiating programmed cell death (apoptosis) (Shuman et al., 1997). Astrocytes play a central role after CNS injury, and their response has been described as a "double-edged sword" because they uptake harmful contents released by the dying cells, but also release toxic inflammatory factors (Buss, 2004). After injury, astrocytes undergo a heterogeneous reaction pattern, depending on how far they are from the injury site (M. A. Anderson et al., 2014; Burda et al., 2016). The astrocytes closest to the injury site undergo proliferation, hypertrophy, and send their projections to the injury site where they form a "glial scar" (Bardehle et al., 2013; Buss, 2004).

The permanent astrocytic glial scar is detrimental to axon regeneration (Silver and J. H. Miller, 2004). However, the Sofroniew lab recently published data that disputes this view and showed that under certain conditions, the glial scar facilitates the regeneration of injured axons (M. A. Anderson et al., 2016). More specifically, they showed that blocking scar formation (by inhibiting astrocyte division or killing the reactive astrocytes) did not promote axon regrowth after spinal cord injury (M. A. Anderson et al., 2016). It has been previously described that the ascending sensory tract (AST) axons have the capacity to regrow if they can turn on intrinsic development growth programs through nerve conditioning (S. Neumann and Woolf, 1999; Omura et al., 2016; P. M. Richardson and Issa, 1984). During nerve conditioning, the peripheral nerve is lesioned at the same time or prior to the dorsal column lesion, and this conditioning provides the AST nerves with growth cues (S. Neumann and Woolf, 1999; Omura et al., 2016; P. M. Richardson and Issa, 1984). The authors showed that only in wild type (WT) situations after conditioning and when astrocytes were able to form glial scars that these growth factors were released and AST neurons regrew (M. A. Anderson et al., 2016). In the absence of the astrocytic scar, even though the mice were exposed to peripheral nerve conditioning, the AST neurons showed little or no regrowth (M. A. Anderson et al., 2016). Groundbreaking papers that publish ideas contrary to the prevailing dogma are rare, but when they do arise, they show that science can auto-correct. Because this study has not been replicated, it is too early to tell whether these results will have any direct effect on developing translational strategies to get axons to regrow over lesions in spinal cord injury patients. However, it is imperative that future research continues to address the role of glia and the molecular mechanisms required for nerve growth after CNS lesions.

PHAGOCYTOSIS OF NEURONAL DEBRIS AFTER WALLERIAN DEGENERATION IN THE CNS

Nerve injury gives rise to cellular debris such as intracellular compartments and proteins, fragmented axons and dendrites, and apoptotic cell bodies. Clearing dead cells and their processes is critical in order to limit damage to other tissues after injury (Hines et al., 2009). Glial cells have evolved to be efficient at phagocytosis not just during development, but also to maintain brain homeostasis during adulthood and aging (David et al., 2015). Although there is evidence that astrocytes can also become phagocytic after injury (Lööv et al., 2012), microglia are the professional phagocytes of the CNS (Aguzzi et al., 2013; David et al., 2015; Napoli and H. Neumann, 2009). Live imaging experiments in slices and *in vivo* in mice that have cranial windows have shown that after an insult, microglia purinergic receptors get activated and microglia quickly extend their processes to the injury site to phagocytose neuronal debris (Davalos et al., 2005; Dissing-Olesen et al., 2014). After traumatic brain injury, microglia respond to astrocytic cell death by undergoing chemotaxis to phagocytose the dead cells (Roth et al., 2014). Similar to how a 600-pound octopus can squeeze through a tube the size of a quarter (goo.gl/fFW3bj), microglia can also translocate their entire cell bodies after injury and migrate through the extracellular matrix and the densely packed brain environment to phagocytose dying cells (Roth et al., 2014). In response to injury, microglia and other cells release inflammatory factors to recruit monocyte-derived macrophages (also known as professional phagocytes) to the injury in the CNS. The presence of macrophages at the site of injury is temporary and after insult, and potentially repair, macrophages clear from the brain.

DROSOPHILA MELANOGASTER AS A MODEL TO STUDY GLIAL FUNCTION

Perhaps because the glial field is in its infancy, more questions than answers remain. Even if we just focus on one topic, such as glial responses to nerve injury, for example, several fundamental questions exist: What signals are released from axons undergoing WD? Do different parts of the injured neurons release different targets? How do glia sense an injury? What are the molecular mechanisms required for glia to become reactive? How do glia extend membranes to the injury site? How do glia distinguish between intact axons and neurodegenerative debris for phagocytosis? How do glia efficiently destroy debris after injury? The fruit fly, *Drosophila melanogaster,* is an excellent model organism that can be used to start answering some of these questions. In fact, recent work has shown that *Drosophila* neurons also undergo WD (Hoopfer et al., 2006; MacDonald et al., 2006) and fly glia use molecular mechanisms that are conserved in mammals (Doherty et al., 2009; Logan and Freeman, 2007; Logan et al., 2012; Tasdemir-Yilmaz and Freeman, 2014). Fruit flies have well defined and simple anatomy, share the majority of their genome with mammals, and there are several genetic and molecular tools that can be used to manipulate gene expression.

WALLERIAN DEGENERATION IS CONSERVED IN *DROSOPHILA*

The active program of WD is conserved in all animals, including worms, flies, fish, rodents, and humans. Work in *Drosophila* over the last decade has shown that not only is WD conserved in flies, but that we can use *Drosophila* as a genetic model to uncover Wld^S function and new genes involved in WD. In 2006, two papers showed that when *Drosophila* olfactory receptor neurons (ORNs) are transected, the neurons undergo

classic WD mechanisms and that expression of mouse Wld^S in the ORNs significantly blocks degeneration after axotomy (Hoopfer et al., 2006; MacDonald et al., 2006). Additionally, expression of the endogenous fly Nmnat in the ORNs can also delay degeneration after injury, although not as potently as Wld^S expression (Hoopfer et al., 2006; MacDonald et al., 2006). When injured axons express $Wlds^S$ or Nmnat, glia do not upregulate their phagocytic machinery to engulf the neurodegenerative debris. Interestingly, neurons must use different degeneration programs early in development, because expression of Wld^S in flies and mice does not prevent the pruning of axons and dendrites (Hoopfer et al., 2006). These results indicate that in response to injury and even disease, WD mechanisms are similar between flies and mammals, and that flies are powerful genetic model organisms that can be used to better understand the genetic and molecular mechanisms of WD.

Although there has been extensive research on WD and how Wld^S confers neuroprotection, not much is known about the active process that neurons undergo to initiate and complete neurodegeneration. Because *Drosophila* is an excellent genetic model organism, the Freeman lab took advantage of the genetic tools and performed a forward genetic screen to uncover loss-of-function mutants that display Wld^S-like protection following axonal injury (Osterloh et al., 2012). The screen lead to the discovery of *Drosophila sterile a/Armadillo/Toll-Interleukin receptor homology domain* (*dSarm*) loss of function mutants that showed a suppression of degeneration similar to the neuroprotection that the gain of function Wld^S mutation confers (Osterloh et al., 2012). The function of *dSarm* is evolutionarily conserved, because when the mammalian orthologue, *Sarm1*, was knocked out in either PNS or CNS neurons, neurodegeneration was significantly delayed (Osterloh et al., 2012). The discovery of *dSarm* is very

important because it provides evidence for endogenous genes that promote axon destruction during WD (Osterloh et al., 2012). Additionally, because the suppression of *Sarm1* in mammals leads to preservation of axons after injury, inhibition of *Sarm1* could serve as a translational approach to inhibit axonal loss in patients suffering from spinal cord injuries or other neurodegenerative diseases.

DROSOPHILA OLFACTORY AXOTOMY ASSAY TO MONITOR GLIAL IMMUNE ACTIVITY

Olfaction in *Drosophila* has been extensively studied in the past. Flies detect odors with their antennae and maxillary palps ORNs, which project their axons to odor-specific synaptic areas in the brain called glomeruli (Couto et al., 2005). Individual odors are recognized by specific odor receptor molecules that are expressed in the ORNs (Couto et al., 2005). The olfactory system of flies is very similar to that of mammals, but the number of odorant receptors is much smaller and less complex to study. For example, mice have over two million ORNs, while *Drosophila* has ~1300 ORNs, 62 odorant receptors, and ~50 glomeruli, all of which have been systematically mapped (Couto et al., 2005). Additionally, there are Gal4 lines for almost all of the individual odorant receptors and a pan-ORN reporter line (Vosshall et al., 2000).

To induce WD in the ORNs, the antennae or the maxillary palps are surgically removed with forceps. This is a non-lethal injury. Once the cell bodies are removed from the ORNs, the neurons undergo WD and glial immune responses to neurodegeneration can be monitored *in vivo* in adult flies (MacDonald et al., 2006). The antenna and

maxillary palp contain ~1200 and ~120 ORNs, respectively (Stocker, 1994). Therefore, removal or either organ will induce either a large or small injury. Subsets of olfactory receptor neurons can be visualized by crossing odorant-receptor specific Gal4 lines to transgenic lines that express a membrane-tethered green fluorescent protein (GFP) such as UAS-mCD8::GFP (Couto et al., 2005; Vosshall et al., 2000). Additionally, there are also fly lines where the olfactory receptor promoter has been directly fused to a membrane-tethered GFP, which allows researchers to use the powerful Gal4/UAS system to manipulate glial gene expression while also monitoring neurodegeneration. For example, line OR22a-Gal4, UAS-mCD8::GFP expresses membrane tethered-GFP in the OR22a ORNs, which reside in the antennae of the adult flies. Line OR85e-mCD8::GFP is a direct promoter fusion line in which the OR85e promoter fused to a membrane-tethered GFP labels a small subset of ORNs in the maxillary palps. Throughout this dissertation, I will almost exclusively use these two lines to monitor axon WD and the time course of the clearance of the debris after olfactory injury.

ADULT FLY CNS CONTAINS GLIA THAT ARE FUNCTIONALLY AND MORPHOLOGICALLY DIVERSE

Drosophila have simple and well-defined CNS anatomy that contains a variety of neuronal and glial subtypes that are similar to mammals. In the past decade, five different glial subtypes have been identified with their own characteristic location and morphology. Surrounding the entire adult CNS, there are two layers of glia: perineurial and subperineurial glia (Awasaki et al., 2008). The perineurial glia in the outermost layer are small and oblong, whereas the inner layer is composed of large "sheet like"

perineurial cells. However, most researchers refer to these two types of cells by one name: "surface glia" (Freeman, 2015). The surface glia form the *Drosophila* blood-brain barrier and protect the CNS from the surrounding potassium-rich hemolymph (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008). Deeper inside the brain, cortex glia form a honeycomb-like pattern, enwrapping individual neuronal cell bodies. Single cell analysis by mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 2001) of cortex glia has shown that an individual cortex glial cell surrounds multiple cell bodies (Awasaki et al., 2008) (similarly to how one oligodendrocyte myelinates several local axonal segments and similar to how mammalian astrocytes surround several cell bodies). Although not well studied in the adult, during development, cortex glia interact with surface glia (Pereanu et al., 2005), and it is thus inferred that cortex glia supply neurons with trophic factors. Within the synaptic rich neuropil areas are two types of neuropil glia. Ensheathing glia, although they do not form myelin, wrap individual axons and nerve bundles with multi-layered membrane sheaths. Astrocytes, like their mammalian counterparts, are largely tufted and extend their processes deep into the neuropil around synapses. Astrocytes express high-affinity excitatory amino acid transporters *EAAT1* and *EAAT2* and *glutamine synthetase2,* which are all required for the uptake of neurotransmitters, such as glutamate and aspartate, at the synaptic cleft (Doherty et al., 2009; Freeman and Doherty, 2006; Soustelle et al., 2002). Because it is now well established that the *Drosophila* adult CNS contains a variety of different glial subtypes, the fly provides a model system to examine glial development and function.

ENSHEATHING GLIA UPREGULATE *DRAPER* TO PHAGOCYTOSE DEGENERATING AXONS

Interestingly, although the fly CNS contains glial subtypes that are functionally or morphologically similar to mammalian glia, a mammalian microglia-type does not seem to exist in the fly. However, during development each of the fly glial subtypes can perform immune functions such as recognizing and engulfing apoptotic cells and debris (Awasaki and Ito, 2004; Freeman et al., 2003; Sonnenfeld and Jacobs, 1995; Tasdemir-Yilmaz and Freeman, 2014). In the adult brain, the ensheathing glia act as immune cells in response to WD. Using the acute olfactory axotomy assay described earlier, ensheathing glia undergo robust transcriptional, translational and morphological changes in response to neurodegeneration (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; Lu et al., 2014; MacDonald et al., 2006; Ziegenfuss et al., 2008; 2012). The Draper receptor is required in ensheathing glia for a proper glial response to ORN injury, and in *draper*$^{-/-}$ flies, severed axons are not cleared from the CNS (MacDonald et al., 2006). Draper is a highly conserved receptor required for phagocytic activity of immune cells, including glia, in worms, flies, and mammals. The Draper orthologue in worms is Ced-1 and in mammals, it is MEGF10 or Jedi-1. Interestingly, the mammalian homologs of Draper are abundant in glia and also function as phagocytic receptors required for glia to engulf apoptotic neurons and synapses in developing rodents (Cahoy et al., 2008; Chung and Barres, 2012; Chung et al., 2013; Wu et al., 2009). Draper can be visualized in ensheathing glial membranes undergoing hypertrophy (Doherty et al., 2009) and therefore serves as a useful marker in *Drosophila* to assess glial immune function and glial responses to axon degeneration.

After removal of either the antennae or the maxillary palps, severed ORNs release secreted neuropeptide signals through dense core vesicles (Musashe et al., 2016). Although those released factors are currently unknown, they are recognized by ensheathing glia insulin-like receptors (InR) (Musashe et al., 2016). The InR and downstream effector Akt1 are required for the transcriptional activation of the phagocytic receptor Draper (Musashe et al., 2016). Draper is transcriptionally upregulated as early as 1.5 hours after olfactory injury and peaks 3 hours post-injury (Logan et al., 2012). Not only are Draper mRNA levels upregulated in response to injury, but within several hours, Draper protein localizes to the injury site and within 24 hours Draper protein levels peak (Logan and Freeman, 2007; MacDonald et al., 2006). The ligand that activates the Draper receptor is still unknown, but several downstream effectors have recently been characterized including two Syk and Src family kinases that regulate Draper-mediated activity (Ziegenfuss et al., 2008). Once Draper is activated, it initiates the activity of Scr42A, a Src family kinase that phosphorylates a tyrosine on the intracellular domain of Draper, thus allowing Shark (a non-receptor tyrosine kinase similar to mammalian Syk) to bind Draper. Draper also works through the phosphotyrosine-binding domain adaptor protein Ced-6 to promote Draper-dependent phagocytosis of debris (Awasaki et al., 2006; Fullard et al., 2009; Reddien and Horvitz, 2004). These Draper-Src42A-Shark and Draper-Ced-6 interactions are critical for recruitment of glial membranes to severed axons and clearance of degenerating axonal debris. However, it is still unknown what cellular processes and pathways Shark and Ced-6 act on after transducing Draper activity.

JNK PATHWAY, PI3K, AND STAT92E REGULATE TRANSCRIPTIONAL ACTIVATION OF DRAPER

There are three Draper receptor isoforms (*draper-I*, *draper-II,* and *draper-III*). *draper-I* promotes engulfment of axonal debris whereas *draper-II* inhibits glial engulfment by inhibiting *draper-I* (Logan et al., 2012). As mentioned earlier, *draper-I* mRNA increases as early as 1.5 hours after axotomy. In addition, *draper-II* transcripts also increased 4.5 hours after injury which supports the idea that the differential regulation of the two isoforms allows for effective activation and temporal regulation of the phagocytic response (Logan et al., 2012). This data is very exciting because it demonstrates that fly ensheathing glial cells undergo a transcriptional program, similar to mammalian glia. Most recently it has been shown that Draper signaling activates the c-Jun N-terminal kinase (JNK) cascade and STAT92E to regulate the transcription of *draper* and other engulfment genes, thus causing an auto-regulatory feedback loop controlling Draper expression (Fig. 1). More specifically, after olfactory injury ensheathing glia upregulated several mitogen-activated protein kinases (MAPK) that lead to the *Drosophila* activator protein 1 (AP-1) transcriptional complex composed of Jra and Kayak (mammalian Jun and Fos, respectively) to initiate transcription of *draper* and phagocytosis of neurons (MacDonald et al., 2013). Blocking the expression of any MAPKs involved in the JNK pathway in glial cells specifically impeded injury induced upregulation of Draper, but glial-specific overexpression of *draper-I* was sufficient to rescue engulfment defects (MacDonald et al., 2013). Furthermore, glial phosphoinositide 3-kinase (PI3K) is critical in maintaining basal levels of Draper (Doherty et al., 2014). However, after injury, STAT92E is upregulated and controls the activity of *draper*, which contains injury-responsive regulatory elements that are directly modulated by STAT

(Doherty et al., 2014). The authors proposed that fly glia use this Draper/STAT92E/Draper auto-regulatory feedback loop as a mechanism to adjust their reactive state after injury (Doherty et al., 2014).

BRAIN AGING

According to the US Census, by 2060 more than 20% of the total US population will be 65 and older (Colby and Ortman, 2014). The older population will burgeon between the years 2011 and 2029 when the "baby boom" generation reaches age 65. At the same time, it is pivotal that progress continues in the development of strategies for neuroprotection in the aging brain and in age-related neurological conditions such as AD and Parkinson's disease (PD). Brain aging occurs in every person and is associated with a decrease in cognition, which is marked by a decline in the ability to learn new tasks and remember information (Ardila et al., 2000). The cause behind this age-related decrease in mental function has been attributed to shrinking of certain brain areas (e.g. hippocampus and prefrontal cortex) (Golomb et al., 1993; Jernigan et al., 1991), decrease in blood flow to the brain (Pappas et al., 1996), decrease in neurotransmission due to reduced myelination (Peters, 2002), an increase in free radicals (Hsieh and C.-M. Yang, 2013), increased inflammation (Ransohoff, 2016), and the formation of protein aggregates (i.e. plaques and tangles). Although there is evidence that these age-related changes can lead to disease pathogenesis, it is not well understood how or why these changes occur at a molecular level.

AGING MECHANISMS ARE CONSERVED BETWEEN FLIES AND MAMMALS

Although flies have a relatively brief lifespan, they are a great model system to study the complex aging process. Flies undergo conserved physiological and genetic changes with aging. For example, flies undergo an age-related decline in cardiac performance, which varies based on environmental and genetic factors (Wessells and Bodmer, 2007). Fly cardiac activity can be monitored using electrical pacing, and when aged flies are put under stress, heart failure rates increase with age from 20-35% in 1-2 week old flies to 65-85% in 5-7 week old flies (Ocorr et al., 2007). Muscle loss or deterioration is another common sign of aging. Researchers are still trying to understand the genetic and environmental mechanisms that lead to muscle deterioration, but it is well established that locomotor activity declines with age in flies (Augustin and Partridge, 2009). For example, after young flies are tapped to the bottom of a vial, they quickly climb up the vial, against gravity, because of their natural negative geotaxis behavior. In aged flies, negative geotaxis progressively decreases with age and flies cannot climb up the vial (Arking and Wells, 1990; Gargano et al., 2005; Goddeeris et al., 2003). Lastly, like mammals, flies undergo neurological decline with age. For example, flies show age-related changes in circadian rhythm (Metaxakis et al., 2014), learning, and cognition (Tamura et al., 2003), and they undergo age-dependent changes in olfaction (Chihara et al., 2014). As further discussed below, *Drosophila* are an excellent model organism to study aging as they undergo conserved genetic brain aging mechanisms. Additionally, because of a plethora of genetic and molecular tools available, flies offer the opportunity to study age-related changes in a tissue-specific manner.

Genome-wide studies in *C. elegans*, *D. melanogaster*, mouse, non-human primates, and humans have unraveled several genetic pathways that have conserved age-

dependent expression changes. These studies demonstrate a (1) decrease in mitochondrial function with age due to reduced expression of genes involved in mitochondria metabolism (Ishii et al., 2013); (2) decrease in autophagy-related genes and protein turnover (Partridge et al., 2011); and (3) decrease in insulin and insulin growth factor signaling (IGF). These pathways are not only conserved amongst organisms, but when modulated by either increasing or decreasing their expression, can lead to the expansion or shortening of lifespan (Blagosklonny, 2012; Blagosklonny et al., 2010). Nonetheless, how these evolutionarily conserved pathways affect the onset or the progression of age-related neurodegenerative disorders remains unclear.

MITOCHONDRIA METABOLISM

The mitochondria theory of aging proposes that during normal metabolism, mitochondria produce free radicals or reactive oxygen species that can accumulate with age and cause oxidative damage (Mattson et al., 2008; Payne and Chinnery, 2015). The brain is especially susceptible to oxidative damage because it has high energy and oxygen demands. Mitochondria are involved in the pathology of common age-related disorders such as AD, PD, and amyotrophic lateral sclerosis (ALS) (Schon and Manfredi, 2003). AD patients show a reduction in brain energy metabolism, have defects in mitochondrial metabolic enzymes, and defects in electron transport chain complexes (Cunnane et al., 2011). Previous *Drosophila* studies of AD have shown that aβ42 induces mitochondrial mislocalization, which contributes to aβ42-induced neuronal dysfunction (Iijima et al., 2008). The role of mitochondrial deficiency in PD pathology has been supported by genetic studies that show that mutations in POLG (mitochondria

polymerase γ) lead to early onset PD (Davidzon et al., 2006). Additionally, other genes involved in familial forms of PD (*LRRK2*, *HTRA2*, *PARK2*, *PARK7*, *PINK1*, *SNCA*) encode proteins that either directly or indirectly interact with mitochondria (Nuytemans et al., 2010). Importantly, extensive studies in fly models of PD have been pivotal in determining the mechanism between mitochondrial dysfunctions and genes that cause PD (Auluck, 2001; Feany and Bender, 2000; Whitworth, 2011). Lastly, although less than 10% of ALS disease is inherited, the majority of familial cases are linked to mutations in the gene encoding *superoxide dismutase* (*SOD1*) (Chen et al., 2013). Fly and mouse models that overexpress mutant SOD1 have damaged the mitochondria, although the mechanisms that cause toxicity remain controversial (Picher-Martel et al., 2016; Watson et al., 2008). Clearly, there is amassing evidence from invertebrate and mammalian studies that suggest a causative link between mitochondrial dysfunction and age-related disorders. However, it remains unclear whether age-related mitochondrial dysfunction affects WD dynamics. Because mitochondria are active drivers of WD, in Chapter 2 of this dissertation I will monitor mitochondrial fission and fusion dynamics after nerve injury to determine whether axonal fragmentation dynamics change with age.

AUTOPHAGY

Macroautophagy (referred to as autophagy) is a cellular process during which cytoplasmic cargo is delivered to the lysosome via the intermediary of a double membraned-autophagosome that fuses to the lysosome for degradation to form the phagolysosome. Most of the 30 autophagy genes are found in all eukaryotes (Mizushima, 2007), and importantly they decrease with age across model organisms (F. Yang et al., 2014), causing an increase in cellular components in aged cells and tissues. Extensive research has shown that autophagy is a protective cellular mechanism (Radad

et al., 2015). Impairing lysosomal function in *Drosophila* and mice leads to an accumulation of autophagosomes and lysosomes and increased neurodegeneration (Komatsu et al., 2006; Mulakkal et al., 2014). Additionally, autophagy is protective in neurodegenerative and age-related disorders. (Ghavami et al., 2014) For example, in the *Drosophila* model of the polyglutamine disease spinobulbar muscular atrophy, inhibiting autophagy greatly enhanced the expected degeneration (Pandey et al., 2007). Similarly, in amyloid precursor protein mouse models of Alzheimer's that have a heterozygous mutation in a critical autophagy gene, there is an increase in accumulation of toxic proteins and neurodegeneration (Pickford et al., 2008). Additionally, autophagy is critical for neuronal homeostasis. Flies and mice that have either developmental or conditional knockouts of autophagy genes in neurons die prematurely and have extensive neurodegeneration (Cecconi et al., 2007; M. Kim et al., 2013). And conversely, overexpression of autophagy genes in several species prolongs lifespan and improves several features of aging, including motor function (Pyo et al., 2013).

INSULIN SIGNALING

The aging process is often metabolically characterized by insulin resistance and a decrease in the evolutionarily conserved insulin pathway. In yeast, worms, flies, and mammals, insulin plays a key role in aging because the insulin pathway regulates longevity (Piper et al., 2008). Attenuating the insulin pathway genetically or via caloric restriction can extend lifespan significantly, raising the possibility that aging mechanisms are evolutionarily conserved (Barbieri et al., 2003). Interestingly, although lowering circulating levels of insulin and insulin growth factors are beneficial to the

organism; in the brain, increasing levels of insulin are neuroprotective (Duarte et al., 2012). Not only are high levels of insulin critical for regeneration after injury, but increasing insulin levels in the brain has recently been demonstrated to enhance cognition (Hölscher, 2014). However, the mechanism behind this observation is not known. Importantly, along with a large aging population, disorders involving insulin dysregulation (such as obesity and diabetes) are also increasing. Metabolic disorders such as diabetes are major non-genetic risk factors for AD, but the mechanisms by which brain insulin resistance can increase the risk of AD are still unknown.

Survival of neurons depends on the ability of maintenance and repair mechanisms to keep up with damage and wear and tear associated with aging. Interestingly, although the majority of research on brain aging has focused on neuronal aging, several hallmarks of reactive gliosis are prevalent in the aged brain. For example, glial expression of the reactive marker glial fibrillary acidic protein (GFAP) (Goss et al., 1991; Kohama et al., 1995; O'Callaghan and D. B. Miller, 1991), as well as several chemokines and cytokines (induced by oxidative stress) (Li et al., 2015), are substantially increased in the brain of older populations. Dysfunctional glial immune responses in the aged brain are associated with enhanced neuronal death and reduced neuroprotection in models of neurodegenerative disease. Importantly, because glia support neuronal function and health, it is pivotal to understand how aging affects glial function (Conde and Streit, 2006; Streit, 2005). As stated previously, the molecular mechanisms involved in mitochondria metabolism, autophagy and insulin signaling are critical in understanding brain aging, but little research has been done on how these pathways affect glial function. In Chapter 2 of this dissertation, I will explore how aging affects

glial phagocytic function and whether insulin signaling and autophagy in glial cells contribute to age-related phagocytic dysfunction.

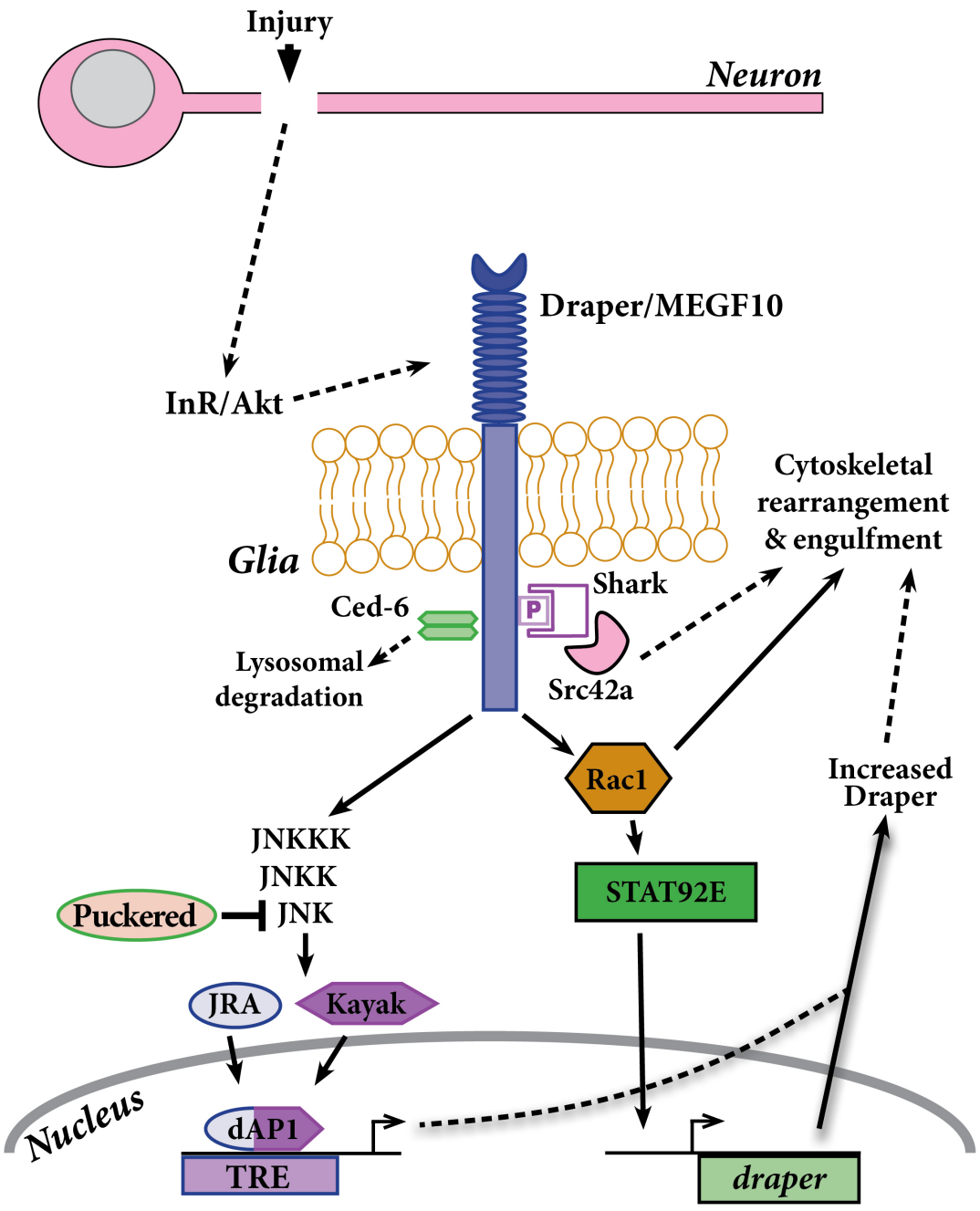

Figure 1. The InR, JNK, and STAT92E pathways regulate transcriptional activation of *draper* in response to injury. After olfactory injury, the insulin-like signaling pathway is stimulated in ensheathing glia to upregulate the expression of *draper* in a STAT92E-dependent manner. In addition, injury also activates the JNK/AP-1 pathway to increase levels of Draper and phagocytic engulfment activity (most likely through STAT92E). Lastly, Draper signals through the Src family kinase signaling cascade and Rac1 to transcriptionally activate its own expression (and possibly other engulfment genes) in a STAT92E-dependent fashion. The dotted lines represent unclear mechanisms of action. Adapted from: MacDonald et al., 2013, Doherty et al., 2014, and Musashe et al., 2016.

CHAPTER 2: DELAYED GLIAL CLEARANCE OF DEGENERATING AXONS IN AGED *DROSOPHILA* IS DUE TO REDUCED PI3K/DRAPER ACTIVITY

Modified from: Purice, M. D., Speese S.D., Logan M.A. (2016) Delayed glial clearance of degenerating axons in aged Drosophila is due to reduced PI3K/Draper activity. *Nat. Commun*. 7:12871 doi: 10.1038/ncomms12871

ABSTRACT

Advanced age is the greatest risk factor for neurodegenerative disorders, but the mechanisms that render the senescent brain vulnerable to disease are unclear. Glial immune responses provide neuroprotection in a variety of contexts. Thus, we explored how glial responses to neurodegeneration are altered with age. Here we show that glia–axon phagocytic interactions change dramatically in the aged *Drosophila* brain. Aged glia clear degenerating axons slowly due to low phosphoinositide-3-kinase (PI3K) signaling and, subsequently, reduced expression of the conserved phagocytic receptor Draper/MEGF10. Importantly, boosting PI3K/Draper activity in aged glia significantly reverses slow phagocytic responses. Moreover, several hours post-axotomy, early hallmarks of Wallerian degeneration (WD) are delayed in aged flies. We propose that slow clearance of degenerating axons is mechanistically twofold, resulting from deferred initiation of axonal WD and reduced PI3K/Draper-dependent glial phagocytic function. Interventions that boost glial engulfment activity, however, can substantially reverse delayed clearance of damaged neuronal debris.

INTRODUCTION

Glial cells are highly sensitive to neuronal stress and damage, and they respond swiftly to trauma by extending membrane projections to injury sites, upregulating essential immune genes, and clearing damaged neurons efficiently through phagocytic engulfment. Rapid clearance of dying cells and degenerating neuronal projections thwarts secondary inflammatory responses that can expedite tissue damage and cell death. Given the critical link between glial engulfment activity and neuronal health, it has been proposed that glial phagocytic function may decline with age and, as a result, make the aged brain more sensitive to acute trauma and chronic neurodegenerative conditions (Mosher and Wyss-Coray, 2014; Niccoli and Partridge, 2012; Sokolowski and Mandell, 2011); however, the intrinsic and extrinsic factors that influence age-dependent changes in glial phagocytic function *in vivo* are still largely unclear.

In aged mice, microglia and astrocytes often display enlarged soma and shorter projections as compared with their young counterparts (Damani et al., 2010; Hefendehl et al., 2014; Streit et al., 2004; Tremblay et al., 2012). These age-dependent changes in morphology suggest that aged glia may be compromised in their ability to sense and/or respond to neuronal stress and degeneration and, in fact, *in vitro* studies have suggested that microglia harvested from older animals display defective phagocytic activity (Floden and Combs, 2011). Although there is growing evidence that the transcriptional profile of glia changes with age (Hickman et al., 2013; Orre et al., 2014) few *in vivo* functional studies have pinpointed specific glial factors that contribute to altered engulfment activity and typical age-related decline in neuroprotection.

Studies in Drosophila have provided critical insight into the molecular, biochemical, and systemic changes that govern aging and longevity. For example, reduced insulin-like signaling, mitochondrial fatigue, defects in autophagy, and altered transcription/translation have all been confirmed to influence senescence across species (Broughton and Partridge, 2009; Fontana et al., 2010; Guarente and Kenyon, 2000). Thus, *Drosophila* offers a well-established genetic model system to rapidly interrogate the molecular intricacies of aging physiology and identify specific pathways that can be targeted to delay age-related decline and extend lifespan. Here we use an acute axotomy assay in the olfactory system of adult *Drosophila* to investigate how aging alters glial responses to axon degeneration *in vivo*. We demonstrate that glial clearance of damaged axons is significantly delayed in aged animals due to an age-dependent decline in the translation of the critical glial recognition engulfment receptor Draper due to reduced phosphoinositide-3-kinase (PI3K) signaling. Importantly, in aged glia, Draper levels fall below a critical threshold required to activate a Draper-dependent STAT92E transcriptional program in local glia in response to axon injury.

RESULTS

CLEARANCE OF SEVERED AXONS IS DELAYED IN AGED *DROSOPHILA*

We used an acute axotomy assay in the olfactory system of adult flies to explore how aging influences glial responses to axon degeneration *in vivo*. Bilateral removal of the third antennal segments or maxillary palps triggers Wallerian degeneration (WD) of olfactory receptor neuron (ORN) axons that project into the antennal lobes of the central brain (Hoopfer et al., 2006; MacDonald et al., 2006). Local ensheathing glia extend

membranes to accumulate on degenerating axons, upregulate key immune factors, and efficiently clear axonal debris through phagocytic engulfment (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006; Ziegenfuss et al., 2012). We compared glial clearance of degenerating ORN axons in young and aged flies by severing the maxillary nerves of flies expressing membrane-tethered green fluorescent protein (GFP) in a subset of maxillary palp ORNs (OR85e-mCD8::GFP) and quantifying GFP. Axonal debris in OR85e-innervated glomeruli at various time points post-axotomy. Clearance of GFP-labelled axon material was significantly delayed in aged animals after injury (Fig. 1a–c).

We observed higher basal levels of GFP in the OR85e glomeruli of aged OR85e-mCD8::GFP animals (Fig. 1d). Thus, we took an alternative approach to label ORN axons and ensure that age-dependent changes in axonal GFP levels did not influence clearance post-axotomy. Specifically, we used the robust GAL4/UAS system to express membrane-tethered GFP in a subset of ORNs (OR22a-Gal4, UAS-mCD8::GFP), which resulted in comparable basal GFP levels in young and aged neurons (Fig. 1h and Fig. 2), and then quantified clearance of GFP+ axonal debris after antennal nerve axotomy. Again, we observed significantly more GFP+ axonal debris lingering in the brains of aged flies at each time point (Fig. 1e–g and Fig.2). For example, 10 days post-injury, identifiable tracts of fragmented axons were visible in almost half of the aged flies, whereas no tracts were detectable in young brains (Fig. 1g). Delayed removal of OR22a GFP+ material in aged animals, despite the fact that GFP levels are comparable in uninjured young and aged flies, suggests that differences in OR22a axonal debris clearance are not simply due to the fact that glia must manage higher axonal GFP load in aged brains.

GLIA ARE NOT RECRUITED TO AGED DEGENERATING AXONS

Efficient clearance of degenerating olfactory axons requires that local ensheathing glia expand their membranes to accumulate on actively degenerating nerves (Doherty et al., 2009; Logan et al., 2012; MacDonald et al., 2006; Ziegenfuss et al., 2012). We used flies that expressed glial membrane-tethered GFP (Repo-Gal4, UAS-mCD8::GFP) to quantify recruitment of glial membranes to injured maxillary ORN axons 24 h after maxillary nerve injury and found that accumulation of glial membranes was significantly reduced in aged flies (maxillary injury; Fig. 3a,b). Similarly, expansion of ensheathing glial membranes around the antennal lobes was attenuated after severing the antennal nerves in aged animals (antennal injury; Fig. 3a,c). We also immunostained aged and young brains for the pan-glial marker Repo and, consistent with a previous study (Trunova and Giniger, 2012), found no reduction in the number of glial cells in the central brain regions adjacent to the antennal lobes (Fig. 4), suggesting that poor glial membrane recruitment and phagocytic clearance of damaged axons in the aged brain is not a result of glial cell death.

DRAPER IS REDUCED IN AGED *DROSOPHILA* GLIA

Draper/MEGF10 is a highly conserved phagocytic receptor required for glial engulfment of apoptotic cells and pruned neural projections during development (Logan et al., 2012) and is essential for glial clearance of adult degenerating axons (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006; Neukomm et al., 2014). Thus, we asked how glial Draper expression and/or function might change with age. Indeed, western blotting (Fig. 5a,b) and

immunostaining (Fig. 5c and top panels in Fig. 5e) revealed that Draper protein levels were significantly reduced in the aged central nervous system, although *draper-I* transcript levels were unchanged (Fig. 5d). Importantly, we found that injury-induced upregulation of Draper protein in local ensheathing glia was also attenuated. In young flies, Draper accumulates on maxillary palp-innervated glomeruli within 24 h after maxillary nerve axotomy (Doherty et al., 2009; Logan et al., 2012; MacDonald et al., 2006; Ziegenfuss et al., 2012), but we found this response to be virtually undetectable in aged brains (middle panels in Fig. 5e,f). Even after eliciting a substantially larger injury by severing the majority of ORNs by antennal nerve axotomy, Draper upregulation was still significantly reduced in aged ensheathing glia (lower panels in Fig. 5e,g).

ACTIVATION OF GLIAL STAT92E IS ATTENUATED IN AGED GLIA

Recent work has shown that following olfactory nerve axotomy in young animals, the STAT92E transcription factor is activated in local ensheathing glia in a Draper-dependent manner to boost *draper-I* transcript levels (Doherty et al., 2014). This positive auto-regulatory feedback loop ensures that Draper levels are sufficiently increased when the glial phagocytic load is high following axon degeneration (Doherty et al., 2014). Thus, we hypothesized that STAT92E activity may be attenuated in aged animals after axon injury. The in vivo transcriptional reporter 10XSTAT92E-dGFP, which contains ten tandem STAT92E consensus binding sites controlling the expression of destabilized GFP (dGFP), serves as a reliable readout of ensheathing glial STAT92E activity before and after axon injury (Doherty et al., 2014). We found that induction of dGFP after maxillary or antennal nerve injury on aged 10XSTAT92E-dGFP flies was

almost undetectable 24 h after injury, in contrast to young animals (Fig. 5h), suggesting that weak upregulation of Draper in aged glia following axotomy may result from reduced activation of STAT92E-dependent transcriptional cascades.

CLEARANCE OF INJURED AXONS IS DELAYED IN DRAPER HETEROZYGOTES

Notably, basal Draper levels are significantly reduced in young Draper heterozygous (*draper*$^{+/-}$) flies and, in fact, are comparable to the levels in aged *draper*$^{+/+}$**.** brains (Fig. 6a,b). We compared clearance of degenerating OR85e axons in young and aged *draper*$^{+/+}$ and draper$^{+/-}$ adults. Strikingly, the clearance defect of young *draper*$^{+/-}$ and aged *draper*$^{+/+}$ animals were similar, and loss of one copy of Draper further exacerbated this phenotype in aged animals (Fig. 6c,d). Finally, we compared activation of 10XSTAT92E-dGFP reporter activity in young and aged animals following maxillary nerve axotomy and, surprisingly, detected no increase in 10XSTAT92E-dGFP reporter activity in young animals that lacked one copy of draper (Fig. 6e,f). These results indicate that loss of Draper in adult glia below a critical threshold can significantly compromise downstream immune responses, including injury-responsive STAT92E transcriptional cascades and phagocytic function.

DRAPER REVERSES AXOTOMY RESPONSE PHENOTYPES IN AGED ANIMALS

As maintaining proper Draper levels is essential for efficient glial clearance of degenerating axons, we reasoned that restoring Draper in aged glia might rescue engulfment phenotypes in older animals. We used a temperature-sensitive version of

Gal80 (Gal80ts) (McGuire et al., 2004) to temporally control activation of a UAS-Draper-I transgene and provide a short pulse of Draper expression in aged glia immediately following maxillary nerve axotomy (Fig. 7a). This paradigm increased Draper levels in aged glia at both the protein (Fig. 7b,c) and transcript level (Fig. 8a). Strikingly, re-expression of Draper in aged glia (Aged rescue) restored phagocytic clearance of degenerating OR85e axons 3 days after axon injury (Fig. 7d,e) and also rescued injury-induced activation of 10XSTAT92E-dGFP in aged animals (Fig. 7f,g). We should note that modestly raising Draper levels in young glia above endogenous levels does not result in faster glial clearance and, in fact, forcing higher levels of Draper expression can ultimately inhibit glial clearance of axonal debris (Logan and Freeman, 2007) (Fig. 8). Our results suggest that loss of the Draper receptor is a key causal factor in delayed glial responses to degenerating axons in the aged brain.

PI3K REVERSES AXOTOMY RESPONSE PHENOTYPES IN AGED ANIMALS

In young animals, PI3K92E is required for proper basal Draper expression in glial cells and for timely clearance of degenerating ORN axonal debris (Doherty et al., 2014), and glial overexpression of a well-characterized constitutively active version of PI3K92E (CA-PI3K) (UAS- CAPi3K92Ecaax) in young flies increases Draper levels (Doherty et al., 2014). We expressed this CA-PI3K in the glial cells of young flies that lacked one copy of draper (*draper*$^{+/-}$) and found that this was sufficient to rescue delayed phagocytic clearance of OR85e axonal debris 3 days after axotomy (Fig. 9a,b) and also significantly raised basal Draper levels in the heterozygous background (Fig. 9c,d). Thus, we hypothesized that age-dependent decline in PI3K92E signaling may account

for the loss of Draper and delayed glial responses to degenerating axons in older animals. We expressed CA-PI3K acutely in aged glial cells (Fig. 10a). Raising PI3K activity restored basal Draper levels (Fig. 10b,c) and also rescued delayed clearance of degenerating OR85e axons (Fig. 10d,e). Notably, basal levels of OR85e-mCD8::GFP are higher in aged animals (Fig. 1d). Thus, we cannot rule out the possibility that this may partially contribute to the differential clearance of GFP-labelled OR85e material in young versus aged brains. Together, our findings indicate that age-dependent changes in Draper expression and downstream signaling pathways contribute to delayed destruction of severed axons in older animals.

Next, we assessed STAT92E activity in aged flies following expression of CA-PI3K by monitoring 10XSTAT92E-dGFP induction 1 day after antennal nerve axotomy. Although we do not detect an increase in dGFP in aged glia post-injury (Fig. 10f,g, Aged), dGFP levels are significantly increased when PI3K signaling is constitutively activated (Fig. 10f,g, Aged CA-PI3K rescue). Together, these findings suggest that with age reduced PI3K activity causes Draper to fall below a critical threshold required for ensheathing glia to activate a STAT92E-dependent transcriptional program post-injury, which ensures adequate upregulation of engulfment genes and efficient clearance of neuronal debris.

TOR IS REQUIRED FOR GLIAL DRAPER AND SEVERED AXON CLEARANCE

To determine whether there is an age-dependent basal reduction in *draper* at the transcriptional level, we compared transcript levels of Draper-I in young versus aged brains by quantitative real-time PCR and detected no significant difference (Fig. 5d).

Thus, we reasoned that reduced translation might account for reduced Draper protein levels in aged brains, in particular as PI3K is a well-known positive regulator of target of rapamycin (TOR)-mediated translation (Krab et al., 2008; Patursky-Polischuk et al., 2009; Stolovich et al., 2002) and glial knockdown of PI3K92E does not alter *draper-I* transcript levels in young flies (Doherty et al., 2014). We performed glial-specific knockdown of TOR (UAS-TOR RNAi) or expressed a dominant-negative version of TOR (UAS-D/N TOR) in young flies and found that in both instances Draper levels were reduced (Fig. 11a,b) and glial clearance of severed OR85e axons was significantly delayed (Fig. 11d,e). Notably, although Draper protein levels were lowered by inhibiting the expression/function of TOR, *draper-I* transcript levels were unchanged (Fig. 11c). These findings support the notion that loss of Draper following inhibition of PI3K results from a TOR-dependent decline in translation.

DRAPER TRANSLATION IS REDUCED IN AGED BRAINS

To compare Draper translation in young versus aged brains, we analyzed the polysome load of the *draper-I* transcript by sucrose gradient centrifugation. Polysome profiles from young and aged brain lysates were recorded and fractionated (Fig. 11f), and we performed quantitative PCR to determine *draper-I* transcript levels in each fraction. In aged brains, we observed a reduction in heavy polysomal-associated *draper-I* messenger RNA and a corresponding increase in the light polysome fraction, as compared with young animals (Fig. 11g), which indicates reduced translation initiation of *draper-I* in aged animals. Transcript and protein levels of our housekeeping gene Rpl32 did not significantly change with age (Fig. 11h–j). Although TOR controls global

protein synthesis, transcripts that contain 50-terminal oligopyrimidine (TOP) motifs are particularly sensitive to TOR regulation (Patursky-Polischuk et al., 2009; Stolovich et al., 2002; Tang et al., 2001). We performed a computational analysis of the *draper-I* mRNA sequence using the RegRNA (Regulatory RNA Motifs and Elements Finder) program (Huang et al., 2006) and found that the *draper-I* transcript contains three TOP domains in the 5′-untranslated region. It remains to be determined how each of these TOP motifs contribute to age-dependent changes in *Draper-I* translation but, interestingly, translational activation of TOP-containing mRNAs is highly dependent on PI3K activity (Stolovich et al., 2002; Tang et al., 2001). Together, our findings suggest that with age, *draper* gene transcription is unaltered but Draper receptor levels are attenuated due to reduced translation rates, likely to be through inhibition of PI3K/TOR function.

AXONAL FRAGMENTATION IS DELAYED POST-AXOTOMY IN AGED ANIMALS

In aged mice, crushed or severed peripheral nerves fragment more slowly as compared with young animals (Tanaka et al., 1992; Vaughan, 1992). Therefore, we wondered whether *Drosophila* olfactory nerves are similarly delayed in initiating a WD program in aged flies and, potentially, in releasing signals that elicit innate responses from local ensheathing glia within the first ~24 h post-injury. First, we compared fragmentation rates of young and aged GFP-labelled OR85e axons 6 h after maxillary nerve axotomy. Fragmentation did appear to be more pronounced in young axons at this time point (Fig. 12a); however, we also detected more glial infiltration into the glomeruli (denoted by Draper) at this early time point in young flies (white arrows, Fig. 12a). We were concerned that faster glial recruitment of young glia would obscure our

ability to detect differences in intrinsic axon fragmentation rates in young versus aged animals over an extended time course. Therefore, we examined axon fragmentation rates in flies expressing UAS-Draper RNAi under the control of Repo-Gal4, to ensure that recruitment/infiltration of glial cells after axon injury was blocked (MacDonald et al., 2006). Using this strategy, we compared fragmentation rates of young and aged OR85e axons. We applied a blind scoring analysis to images of axons 0, 24, 48 and 72 h post-maxillary nerve injury, to assess the extent of axonal beading and fragmentation over a fixed distance, and found that aged axons appeared to fragment more slowly (Fig. 12b,c). For example, 72 h post-injury (the last time point analyzed), continuous axonal projections could be traced in ~50% of aged brains, whereas no continuous projections were visible in young brains (Fig. 12c). Next, we compared mitochondrial morphology in young and aged axons post-injury, as mitochondria undergo rapid fragmentation after axotomy and, in fact, are active factors in driving WD (Avery et al., 2012; Conforti et al., 2014; Knott and Bossy-Wetzel, 2008; Rooney and Freeman, 2014). We genetically expressed mitochondrial targeted GFP (UAS-mito::GFP) in a subset of ORNs (OR22a-Gal4). Mito-GFP patterns appeared similar in young and aged uninjured animals and included both discrete puncta and continuous tubular structures, which represent ongoing mitochondrial fission and fusion (no injury, Fig. 12d). Ten hours after severing antennal nerves, the mito-GFP signal became highly punctate in young axons (arrows, Fig. 12d). Conversely, in aged axons, mito-GFP distribution appeared more tubular, with fewer discrete puncta, even compared with aged uninjured axons (arrowheads, Fig. 12d). Together, these findings suggest that initiation of WD after axotomy is qualitatively different and/or slowed in aged flies. Delayed axon-to-glia signaling after

injury in aged brains may explain why Draper is not sufficient to reverse glial clearance defects in the first 24 h post-axotomy.

SUPPLEMENTAL RESULTS

DRAPER NEUROPIL AGGREGATES CO-LOCALIZE WITH RAB11

Although we detected significantly lower levels of Draper in the cortex (by immunostaining, Fig. 5c) and by Western blot on whole brain lysates (Fig. 5a,b) in aged animals, we also consistently noticed an increase in Draper-positive puncta in neuropil regions, which are devoid of cell bodies and contain only axonal, dendritic and glial projections (yellow arrows, Fig. 10f). These puncta were rarely present in aged CA-PI3K rescue flies (Fig. 10f). Since PI3K signaling can influence many aspects of intracellular transport, receptor recycling and protein quality, (Wymann and Pirola, 1998) we wondered if these puncta might represent Draper aggregates that form due to age-dependent changes in receptor transport. Thus, we co-immunostained aged brains with Draper and markers for autophagy/degradation (Ubiquitin, Ref (2)p) and cell compartments/endosomal trafficking (Lava lamp, Rab4, Rab5, and Rab11). The GTPase Rab11 was the only factor that co-localized with Draper aggregates in the neuropil of aged brains (Figs. 14 and 15). Rab11 is associated with endosomal recycling of transmembrane receptors (Maxfield and McGraw, 2004), suggesting that Draper endocytic transport and/or recycling within ensheathing glia projections becomes dysregulated with aging. Future studies are necessary to uncover which cell compartments Draper is trafficked through and whether rescuing Draper trafficking is

sufficient to ameliorate the Draper recruitment and glial clearance of axons in the aging brain.

ACTIVATION OF PI3K OR INHIBITION OF ATG1 LEADS TO FASTER CLEARANCE IN YOUNG ANIMALS

Doherty et al., 2014 showed that glial knockdown of *PI3K92E* and other key components of the PI3K signaling pathway (i.e. *raptor* or *pdk-1)* lead to impaired clearance of axonal debris 5 days after axotomy in young animals. Additionally, overexpression of *PI3K92E* in young uninjured animals leads to increased basal level of Draper (Doherty et al., 2014). Our data suggests that loss of PI3K/Draper is a primary reason for declining innate glial immune function in senescent animals and that increasing PI3K levels rescues Draper levels and glial function in the aged brain. However, it is well established that Class-I PI3K/Dp110 such as PI3K92E acts through TOR to inhibit autophagy (X. Yu et al., 2015). We wondered whether PI3K activity in glia regulates autophagy and whether autophagy flux in glia affects glial function after injury.

We first increased *PI3K92E* activity in young animals and monitored glial clearance of degenerating axons. We expressed CA-PI3K in the glial cells of young animals for two days prior and one day after injury, and found that it was sufficient to increase clearance of axons at this time point (Fig. 16a,c). Interestingly, this short pulse of PI3K did not increase basal levels of Draper, but injury induced Draper was significantly reduced (Fig. 16b). Next, we performed adult glial-specific knockdown of Atg1 (autophagy-related 1) (UAS-Atg1 RNAi) and found that inhibiting autophagy in young

animals leads to faster clearance of axons (Fig. 16d,e). We also observed that there is an increase in Draper aggregates in the neuropil regions of these animals suggesting that Draper receptor recycling involves autophagy (Fig16d, yellow arrows). These results indicate that preventing autophagy in glial cells by either increasing activity of PI3K or by inhibiting Atg1 both lead to faster clearance of degenerating ORN materials in young animals. These results suggest that inhibiting autophagy initiation leads to faster degradation of engulfed debris after injury and therefore faster clearance. Further research investigating the relationship between glial autophagic flux and phagocytic debris recycling after injury in the healthy and aged brain need to be explored.

GLIAL INR SIGNALING IS ATTENUATED IN THE AGED BRAIN

The previous supplemental results investigated potential downstream components that are affected by constitutive activation of PI3K. However, we also asked why do we see decreased levels of PI3K/Draper in the aging brain? To better understand what could cause immune genes expression to decrease with age, we turned to the highly conserved and neuroprotective insulin-like signaling (ILS) pathway that has also been shown to decrease with age in all animals. Additionally, it is well established that PI3K92E is directly downstream of the insulin receptor (InR). Our lab has recently discovered that InR activity positively regulates Draper expression in young animals, and in fact inhibiting InR activity in young animals leads to similar phenotypes observed in aged animals (i.e. decreased basal levels of Draper expression and delayed clearance of injured axons) (Musashe et al., 2016).

The *Drosophila* InR gene is homologous to mammalian insulin and IGF-I (insulin-like growth factor 1) receptors (Barbieri et al., 2003). The highest sequence homology occurs in the β region, which includes the transmembrane and ligand-activated tyrosine kinase domains. We used an antibody that recognizes a conserved InR β tyrosine that is phosphorylated upon activation of the receptor (Fernandez et al., 1995). To determine how decreased levels of ILS in aged animals might affect activation of the InR after inducing neurodegeneration, we performed immunohistochemistry using the phosphorylated InR (phospho-InR) antibody on young and aged flies after antennal axotomy. These flies were also expressing GFP in glial membranes using the pan-glial driver Repo-Gal4. In young animals, there is a very dramatic increase in phospho-InR around the antennal lobe region that houses the degenerating ORNs, and the phospho-InR activation co-localizes with the glial membrane expansion (Fig. 17a,b). In aged animals, we observed that the phospho-InR response is greatly attenuated (788% ± 63 in young vs. 220% ± 31 in aged, $p<.0001$) and more variable (Fig. 17a,b). This discovery now points to ILS as a key player in glial responses to acute neural injury and an important contributor to age-related decline in glial immune function.

Past research on *Drosophila* ILS has focused on metabolic control, dietary restriction and lifespan expansion (Kannan and Fridell, 2015) but there is little characterization of ILS in the aging brain. We first compared basal levels of phospho-InR in young and aged brains by immunostainings and Western blot in order to determine whether basal InR levels are altered in aged animals. We observed a significant decrease of the phospho-InR by both methods (Fig. 18 a,b shows Western; Fig. 17a, no injury and Fig. 18c shows immunostaining). Interestingly we observed that the overall phospho-InR staining pattern in aged animals is more aggregated/clumped than in young

animals (Fig. 17a), possibly indicative of receptor dysfunction. We should note that we understand that using an antibody that only recognizes the activated form of the InR is a limitation, but to date, there is no known InR antibody available in *Drosophila.* Previously we showed that *draper-I* transcript levels were unchanged even though Draper protein levels were significantly attenuated. We therefore wondered whether InR transcript levels change with age, and Q-PCR revealed a significant decrease in basal levels of the InR in aged animals (Fig. 18d). To establish whether other direct InR/PI3K effectors (InR>PI3K>Akt>S6K) are attenuated with age, we compared levels of phospho-Akt and phospho-S6k by Western blot. We observed a decrease in both proteins (Fig. 18 e-h), which demonstrates that the activity of effector proteins downstream of InR/PI3K, which are known to regulate the transcription and translation of genes, is greatly attenuated. These results have identified specific features of glial immunity that are affected by age-related decline of ILS and serve as basis for future work to define how activation of the ILS pathway in glia might promote innate immune functions that are essential to maintaining brain health and fitness.

MATERIALS AND METHODS

ORN AXOTOMY ASSAY

WD was induced in Drosophila ORN axons of the antennal nerve or the maxillary nerve by bilateral removal of the third antennal segment or the maxillary palp structures with forceps, respectively (Logan and Freeman, 2007; MacDonald et al., 2006). Unless shifted to 30°C to inhibit Gal80ts activity, flies were maintained at 22°C–23°C. For aged Draper rescue, we generated flies that carried Repo-Gal4, a UAS-Draper-I

transgene, and OR85e-mCD8::GFP to monitor clearance of maxillary palp ORNs. These flies also expressed a temperature-sensitive version of Gal80 (tubulin-Gal80ts) to temporally regulate the activity of Gal4. After raising flies at the permissive temperature (23°C), we severed the maxillary nerves of young and aged flies, and then shifted flies to the restrictive temperature (30°C) for 45 min to induce glial expression of Draper-I. Flies were returned to 23°C and analyzed 3 days later for clearance of axonal debris. Control flies were carried through the same temperature shift protocol. A similar protocol with varying heat shock times was used to induce expression of genes of interest in adult glia (i.e. UAS-CA-PI3K, UAS-D/N TOR, UAS-TOR RNAi, UAS-Atg1 RNAi).

IMMUNOLABELLING

Adult Drosophila heads were fixed (1xPBS, 0.01% Triton X-100, 4% paraformaldehyde) at room temperature for 16 min. Samples were then washed 1x1min and 2x5min while rocking in PBSTx01 (1xPBS, 0.01%Triton X-100) at room temperatures. Fixed heads were kept on ice, while brains were dissected at room temperature in PBSTx01. Tissues were post-fixed for 16 min in PBSTx1 (1xPBS, 0.1% Triton X-100), washed 2x2min and incubated overnight with primary antibodies in PBSTx1. The next day, brains were washed 4x30 min with PBSTx1 and incubated with secondary antibodies (in PBSTx1) for 2 h at room temperature. Brains were then washed 4x30 min with PBSTx1 and mounted on slides in VECTASHIELD mounting media (Vector Labs).

CONFOCAL MICROSCOPY AND ANALYSIS

All brains were imaged on a Zeiss LSM 700 with a Zeiss x40 1.4 numerical aperture oil-immersion plan-apochromatic lens. Brains within a single experiment (that is, those being directly compared for quantification) were whole mounted under a single #1.5 cover glass in VECTASHIELD. All brains in a given experiment were imaged on the same day with the same confocal microscope settings. Volocity 3D Image Analysis Software (Perkin Elmer) was used for fluorescence quantification and GraphPad Prism was used for statistical analysis. Quantification of GFP+ axonal debris from OR22a GFP labeled axons and OR85e GFP-labelled glomeruli was performed on three-dimensional volumes segmented to the GFP signal in Volocity. Total intensity measurements were calculated and background fluorescence was subtracted. T quantify basal Draper levels in the cortex of adult brains, total intensity measurements were calculated in regions of interest (representative regions in young uninjured panel, white dotted line circle, Fig. 5e). See Fig. 5e panels for representative regions of interest (white dotted lines) selected to quantify Draper levels in glia responding to maxillary nerve and antennal nerve axotomy. Glial membrane recruitment to severed maxillary nerves was quantified by measuring total GFP fluorescence intensity in regions of interest similar to those outlined in Fig. 5e, maxillary palp injury (Fig. 3). Glial membrane expansion after antennal nerve axotomy was quantified by measuring the thickness of GFP+ ensheathing glial membranes at several locations around each antennal lobe on single confocal slices at a consistent anterior depth of 4 mm into the brain.

ANTIBODIES

Primary antibodies were used at the following dilutions: chicken anti-GFP (#A10262 from ThermoFischer) at 1:1,000; mouse anti-Draper (hybridoma supernatants 8A1 and 5D14, now publicly available at Developmental Studies Hybridoma Bank) at 1:400 and mouse anti-Repo (8D12 from Developmental Studies Hybridoma Bank) at 1:20 mouse anti mono- and poly-ubiquitin (clone FK2 from Enzo Life Sciences) at 1:100, rabbit anti-Ref (2)P (a kind gift from Dr. Tor Erik Rusten) at 1:1000, rabbit anti-Rab4 (ab87802 from Abcam) at 1:200, rabbit anti-Rab5 (ab31261 from Abcam) at 1:250, rabbit anti-Lava Lamp (a kind gift from Dr. Philip Copenhaver) at 1:250, mouse anti-Rab11 (BD Biosciences) at 1:100 and rabbit anti-phospo InR at 1:20 (#3021 from Cell Signaling Technology). Secondary antibodies (715-295-150 and 703-545-155 from Jackson Immunoresearch) were used at a dilution of 1:400.

WESTERN BLOT ANALYSIS

Whole adult heads were homogenized in 4 ml 1xLB (Loading Buffer) per head. Protein lysate of four to five heads was loaded onto 4–20% Tris-Glycine gels (Lonza) and transferred to Immobilon-FL (Millipore). For the Draper and Rpl32 blots, after transfer, total protein density per lane was measured using MemCode Reversible Protein Stain (Pierce ThermoFisher). For all other blots, actin was used as a loading control. Blots were probed with the following antibodies: rabbit anti-Draper (Logan et al., 2012) (1:1,000 kind gift of Marc Freeman), rabbit anti-Rpl32 (1:1,000, kind gift of Matthias Hentze), rabbit anti-phospho InR at 1:200 (#3021 from Cell Signaling Technology), rabbit anti-phospho Akt at 1:200 (#4054 from Cell Signaling Technology), rabbit anti-phospho S6K at 1:200 (#9209 from Cell Signaling Technology), mouse anti-actin at 1:1000 (JLA20 from

Developmental Studies Hybridoma Bank) and incubated overnight at 4°C, washed several times with 1xPBS/0.01% Tween 20 and probed with appropriate fluorophore-conjugated antibodies secondary antibodies (713-625-147 and 711-655-152 from Jackson Immunoresearch) for 2 h at room temperature. Additional washes were performed with 1xPBS/0.01% Tween 20 and a final wash in 1xPBS. Total protein stain blots were imaged on G:BOX F3 Imaging System and analyzed with ImageJ; fluorescent blots were imaged on Li-cor Odyssey CLx quantitative western blot imaging system and data were quantified using LiCor Image Studio software. Blot images in Figs. 5 and 11 have been cropped for presentation. Full blot images are presented in Fig. 13.

DROSOPHILA STOCKS

For all experiments, young flies were between 7 and 14 days old, whereas aged flies were between 56 and 63 days old. The following Drosophila genetic insertions were used in the paper: OR85e-mCD8::GFP/CyO (Couto et al., 2005), OR22a-Gal4/Cyo (Dobritsa et al., 2003), UAS-mCD8::GFP (Bloomington Stock 5137), UAS-mCD8::GFP (Bloomington Stock 5130), Repo-Gal4 (MacDonald et al., 2006), tubulin-Gal80ts (Bloomington Stock 7108), UAS-Draper-I/CyO (Logan and Freeman, 2007), UAS-caPi3K92Ecaax (Bloomington Stock 8294), UAS TOR-RNAi (Bloomington Stock 33951), UAS-mito-HA-GFP (Bloomington Stock 8443), 10XSTAT92E-dGFP (Bach et al., 2007) and UAS-Atg1 RNAi (Bloomington Stock 44034).

QUANTITATIVE REVERSE TRANSCRIPTASE–PCR ANALYSIS

Total RNA from heads was extracted using Trizol LS (ThermoFisher), collected via RNA Clean & Concentrator-5 Kit (Zymo Research) and subject to DNAse digestion using Ambion DNA-free kit. Complementary DNA was prepared using qScript cDNA SuperMix kit (Quanta Biosciences). Total RNA was quantified using the Qubit RNA HS assay kit and Qubit Fluorometer, and equal amounts of RNA were added to cDNA synthesis reaction. Quantitative gene expression was carried out on an ABI 7,500 Fast Real-Time PCR machine using Taqman master mix (Applied Biosystems) and the following TaqMan assays: (i) RibosomalProtein L32 (Applied Biosystems premade assay Dm02151827_g1), (ii) Draper-I custom assay: F-primer, 5′-TGTGATCATGGTTACGGAGGAC-3′; R-primer, 5′-CAGCCGGGTGGGCAA-3′; probe, 5′-CGCCTGCGATATAA-3′.

POLYSOME FRACTIONATION

Wild-type young and aged fly heads were manually homogenized with mortar and pestle in polysome lysis buffer (10mM Tris-HCl, 150mM NaCl, 5mM MgCl2,0.5mM dithiothreitol, 100 mG cycloheximide, EDTAfree protease inhibitor (Roche) and 40Uml1 Superase-in (Ambion)). The homogenate was centrifuged at 2,000 g for 10 min at 4 C, to clear the cuticle debris, and 1% NP-40 was added to the supernatant before being incubated on ice for 10 min. Lysate was then cleared a second time by centrifugation at 16,000 g for 10 min at 4 C. Lysates were layered onto a 10–60% sucrose gradient, centrifuged at 40,000 r.p.m. (SW-41Ti rotor) for 2 h at 4 C and sampled on a Biocomp gradient station/Gilson fraction collector with constant monitoring of optical density at 254nm (Martin et al., 2014; Thoreen et al., 2012). Eleven 1ml fractions were collected,

each sample was spiked with 20 ng of luciferase RNA and total RNA was extracted from each fraction using the methods described above in quantitative reverse transcriptase–PCR analysis. Draper transcript levels in each fraction were normalized to Rpl32 ($2^{\Delta Ct}$) and the percentage of Draper transcript in each fraction was adjusted using the Ct of luciferase, which reflected the RNA recovery rate in each fraction.

DISCUSSION

Our studies provide new mechanistic insight into how aging alters glial–axon interactions and glial responses to neural injury. We provide direct *in vivo* evidence that dysfunctional glial engulfment in aged *Drosophila* is largely due to downregulation of the Draper receptor at the protein level as a result of decreased PI3K92E activity and translation efficiency. Our findings are consistent with previous reports from other species that reduced translation and/or protein degradation, as opposed to reduced transcriptional activity, is an important feature of aging, coupled to declining cellular and organismal function (Rogina et al., 1998; Taylor and Dillin, 2011; Vilchez et al., 2014). Here we show that forced activation of PI3K signaling rescues both reduced Draper expression and delayed glial clearance of severed axons several days after axotomy, which implicates PI3K-dependent signaling as a critical age-sensitive cascade that strongly influences glial responses to axon degeneration. Importantly, upregulation of glial Draper after axon injury also significantly rescues glial clearance defects in aged animals. Thus, although many glial proteins are undoubtedly affected by the age-dependent decline in translation/stability, we propose that loss of the Draper receptor

specifically inhibits the engulfment activity of aged glia, which is a critical neuroprotective feature of glia.

Draper is essential for glial membrane expansion/hypertrophy following axotomy and subsequent phagocytic removal of damaged axons in adult flies (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006; Ziegenfuss et al., 2012). Our results are consistent with reports of diminished injury-induced motility of vertebrate microglia and *in vitro* experiments that suggest aged microglia have reduced phagocytic capacity (Damani et al., 2010; Floden and Combs, 2011; Hefendehl et al., 2014; Streit, 2012; Tasdemir-Yilmaz and Freeman, 2014; Tremblay et al., 2012). Therefore, our findings may be extrapolated to mammals, as Draper and the mammalian homolog MEGF10/Jedi signal through highly conserved tyrosine kinase cascades and are required for glial engulfment of degenerating axons, synapses and/or apoptotic neurons in a variety of contexts (Awasaki et al., 2006; Freeman et al., 2003; Logan et al., 2012; MacDonald et al., 2006; Scheib et al., 2012; Tasdemir-Yilmaz and Freeman, 2014; Ziegenfuss et al., 2012). Moreover, recent work has shown that *Drosophila* glia can internalize pathogenic human Huntingtin protein (Pearce et al., 2015) and neurotoxic Aβ42 peptides (A. Ray and M.A. Logan, unpublished) in a Draper-dependent manner, which further bolsters the notion that age-related decreases in engulfment activity are coupled to an increased risk for age-related neurodegenerative disorders, including Alzheimer's disease (Guerreiro et al., 2013; Schmid et al., 2002). Finally, loss of Draper results in shortened lifespan and increased risk of neuronal apoptotic death in adult *Drosophila* (Draper et al., 2014; Etchegaray et al., 2016). Together, this body of work highlights the Draper/MEGF10 pathway as an exciting new therapeutic candidate to

boost innate glial immune activity, including phagocytosis, to enhance neuroprotection with advanced age.

Recent work from Doherty et al., 2014 identified PI3K92E as a positive regulator of Draper in young glia and also showed that STAT92E was required to upregulate *draper-I* in young glia responding to injury. Importantly, our work reveals that PI3K-dependent reduction of Draper translation is a key limiting factor responsible for delayed phagocytic responses to neural injury in the aged brain. Stimulating PI3K activity and/or raising Draper levels largely rescues poor clearance of degenerating axons and defects in Draper/STAT92E-dependent transcriptional activity that are typically observed in aged animals. These results indicate that despite the many fundamental biological shifts that occur in the aging brain, loss of PI3K/Draper is a primary reason for declining innate glial immune function in senescent animals. Moreover, we show that in young animals, Draper is haploinsufficient with regard to activation of STAT92E transcription and glial phagocytic clearance of degenerating axons post-injury. Collectively, our work highlights Draper-dependent signaling pathways as a molecular linchpin for proper glial immune responses across ages.

We also show that in the aged *Drosophila* brain, transcriptional and translation levels of the InR decrease, as well as downstream effectors proteins Akt and S6k, suggesting that the decrease in PI3K/Draper could be due to a decrease in insulin receptor expression/activation. Additionally, we show that in aged animals, glial phospho-InR activation is greatly attenuated in response to neurodegeneration. We propose that the InR/PI3K signaling pathway promotes glial health and immune activity in the young and aged brain. However, previous studies also show that neurons produce and can respond to insulin or IGF-I (Rodriguez-Perez et al., 2016). We cannot

exclude the possibility that in the aged brain, neuron insulin production decreases, which it turn might decrease InR/PI3K activity in glia. The olfactory system in *Drosophila* offers a great *in vivo* system to better understand the neuroprotective mechanisms of the InR/PI3K pathway on both neurons and glia in the aging brain.

Aside from gene translation, the InR/PI3K92E pathway regulates numerous cell functions including, cellular trafficking, gene transcription, autophagy and cell cycle regulation (Vanhaesebroeck et al., 2010). We show that in the aged animals Draper aggregates in neuropil regions where it colocalizes with Rab11, but after a boost of constitutively active PI3K activity, the Rab11/Draper+ puncta are depleted. Rab11 has several functions including transporting cargo proteins via the recycling endosome to cell junctions, phagosomes, and cellular protrusions (Guichard et al., 2014). Interestingly, Rab11a regulates the trafficking and the downstream activation of the innate immune receptor Toll-like receptor 4 (TLR-4) in response to phagocytosis of *E. coli*. (Husebye et al., 2010) Although how Draper is trafficked in the cells remains unknown, we propose that in the aging brain Draper transport is dysfunctional and its transport becomes associated with Rab11 trafficking. Boosting PI3K activity in the aging brain restores not only Draper translation but also trafficking or recycling of the receptor.

Our work demonstrates that increasing PI3K activity in young animals, which is known to inhibit autophagy, leads to faster clearance of degenerated axons. Similarly, glial specific knockdown of autophagy initiation through Atg1 RNAi also leads to faster clearance of injured ORNs. We speculate that in young animals, inhibition of Atg1 leads to a pause in the degradation and recycling of cellular components, therefore allowing the cell to monopolize the lysosomal compartments to more efficiently destroy phagocytic debris. Recent work from Etchegaray et al., 2016 demonstrated that *Draper*

null flies undergo age-dependent neurodegeneration due to developmental phagosome maturation defects that cause accumulation of apoptotic neurons. Additionally, they showed that inhibiting a subset of autophagy genes in glia is sufficient to rescue the apoptotic neuron buildup and neurodegeneration phenotype, independent of Draper function (Etchegaray et al., 2016). Future research is needed to address how glial cells use canonical and non-canonical autophagy pathways during normal function, aging, or when they perform their phagocytic function.

Finally, our results are also the first to suggest that WD is initiated more slowly in aged *Drosophila* olfactory nerves immediately after axotomy, as has been described for peripheral nerves in mammals (Tanaka et al., 1992; Vaughan, 1992). The mechanisms of WD appear to be well conserved between flies and vertebrates (Rooney and Freeman, 2014). As such, age-related changes in WD programs and axon–glia signaling events may also be conserved across species. The specific molecules released by severed axons in adult Drosophila remain to be identified, but this *in vivo Drosophila* model offers a tractable platform to identify new axon-glia injury cues that are required to elicit glial responses and may be altered by normal aging events.

In summary, our work highlights the importance of maintaining PI3K-dependent signaling and Draper in aged glia to maintain glial immunity and also suggests that cooperative input from both degenerating axons and local glia are required for efficient glial clearance of axonal debris in the senescent brain.

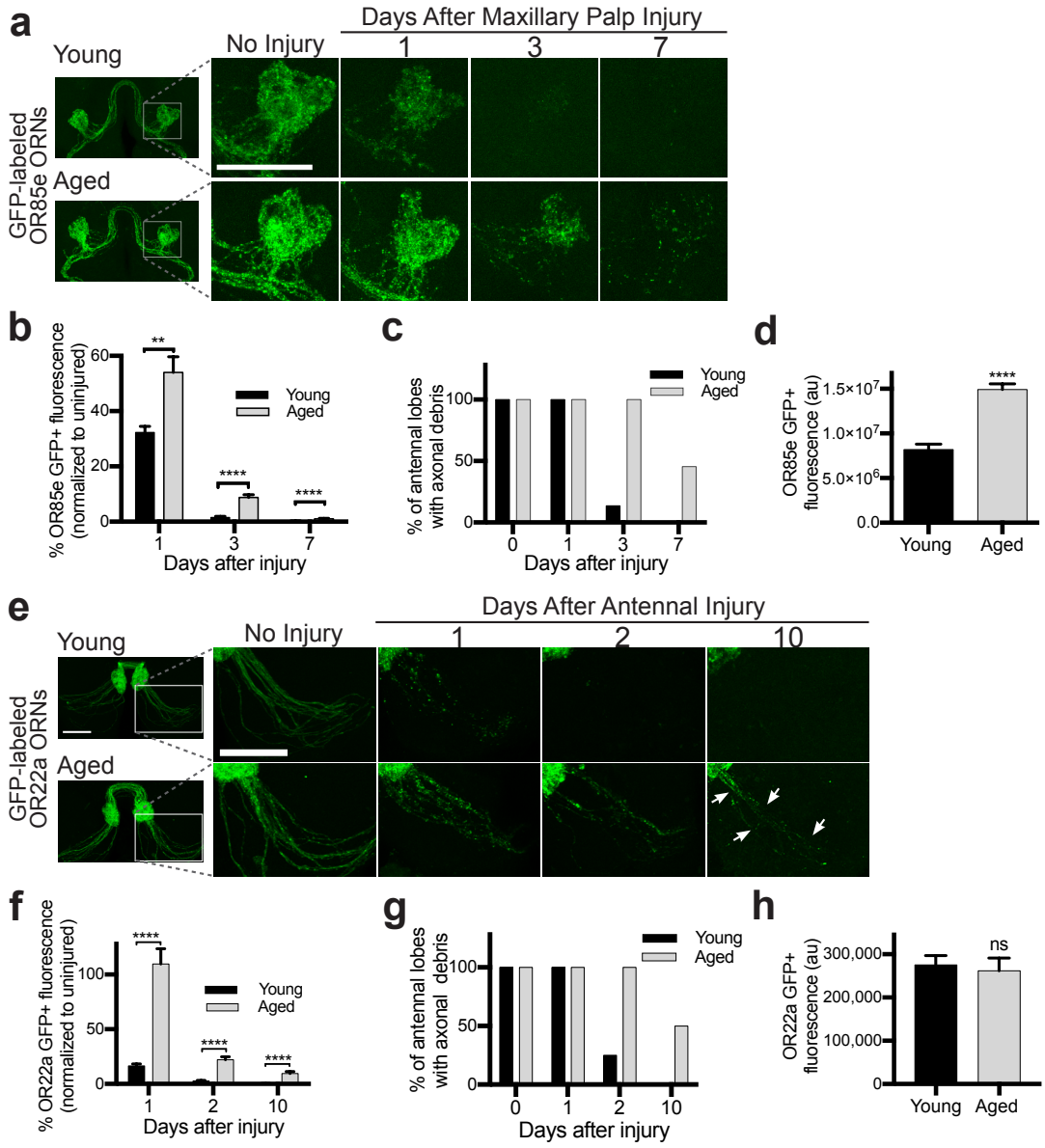

Figure 1 (continued below).

Figure 1. Aged glia fail to efficiently clear degenerating axons in the adult *Drosophila* brain. (**a**) GFP-labeled maxillary ORN axons before and after maxillary nerve axotomy in young and aged flies. Zoomed images show OR85e-innervated glomeruli. (**b**) Quantification of GFP+ debris in OR85e glomeruli; mean ± s.e.m. plotted; **P<0.01, ****P < 0.0001, Mann-Whitney post-hoc test. (**c**) Percentage of antennal lobes containing visible GFP+ OR85e debris after maxillary nerve axotomy. (**d**) Total GFP fluorescence in OR85e glomeruli; mean ± s.e.m. plotted; ****P<0.0001, unpaired t-test. (**e**) GFP-labeled antennal ORN axon projections before and after antennal nerve axotomy in young and aged flies. GFP+ axonal material was still present in aged brains 10 days after antennal nerve injury (white arrows). (**f**) Quantification of OR22a axonal debris in (e); mean ± s.e.m. plotted; ****P < 0.0001, Mann-Whitney post-hoc test. (**g**) Percentage of antennal lobes containing visible tracts of antennal GFP^+ axons. (**h**) Total GFP fluorescence of OR22a axons; mean ± s.e.m. plotted; ns=not significant, unpaired t-test. N ≥20. Scale bar = 30 um. Representative confocal Z-stacks shown for all panels in (a) and (e). Genotypes: Fig. 1a-d: w^{1118};OR85e-mCD8::GFP/+. Fig. 1e-h: w^{1118};OR22a-Gal4, UAS mCD8::GFP/+.

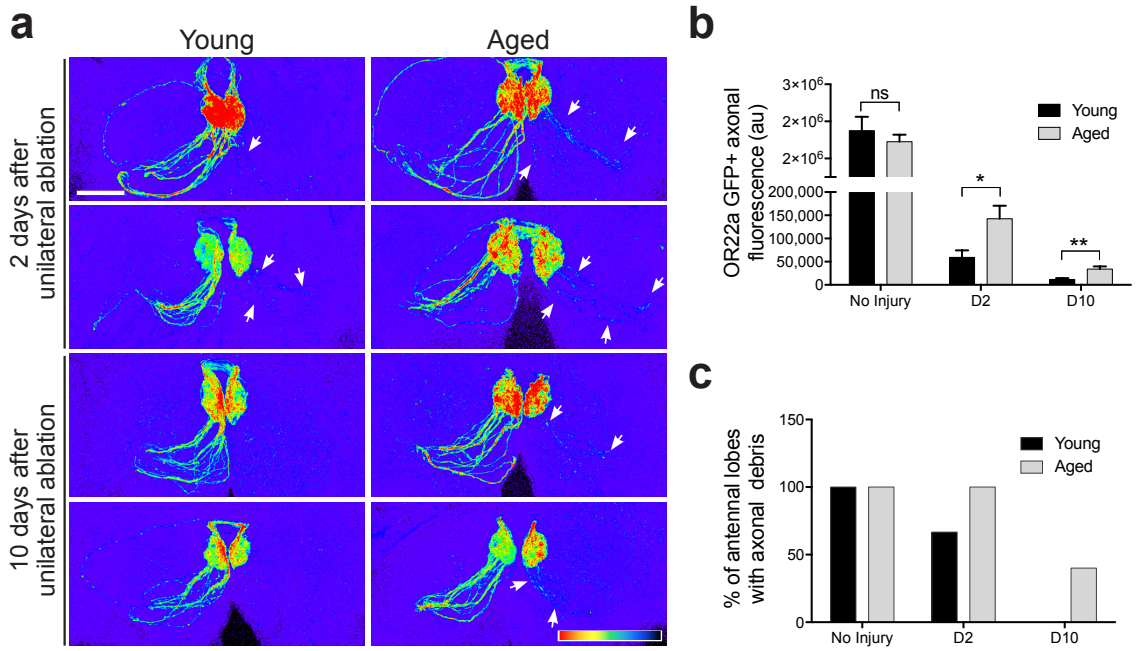

Figure 2. Aged glia fail to efficiently clear degenerating axons in the adult Drosophila brain after unilateral antennal injury. (**a**) Two examples of GFP-labeled antennal ORN axon projections 2 and 10 days after unilateral antennal nerve axotomy in young and aged flies. GFP+ axonal material was still present in young animals 2 days after injury and persisted in aged brains 10 days after antennal nerve injury (white arrows). Images are converted to heat scale (red to black, high to low fluorescence respectively) to show that there is high variability within the GFP+ OR22a glomeruli in both young and aged animals, but not within the axons, which are quantified in (b) and (c). (**b**) Quantification of raw OR22a axonal debris fluorescence in (a); mean ± s.e.m. plotted; *P <0.05, **P<0.01, unpaired t test. N≥7 antennal lobes. (**c**) Percentage of antennal lobes containing visible tracts of antennal GFP+ axons after unilateral injury. Scale bar = 30 um. Genotype: w1118;OR22a-Gal4, UAS mCD8::GFP/+.

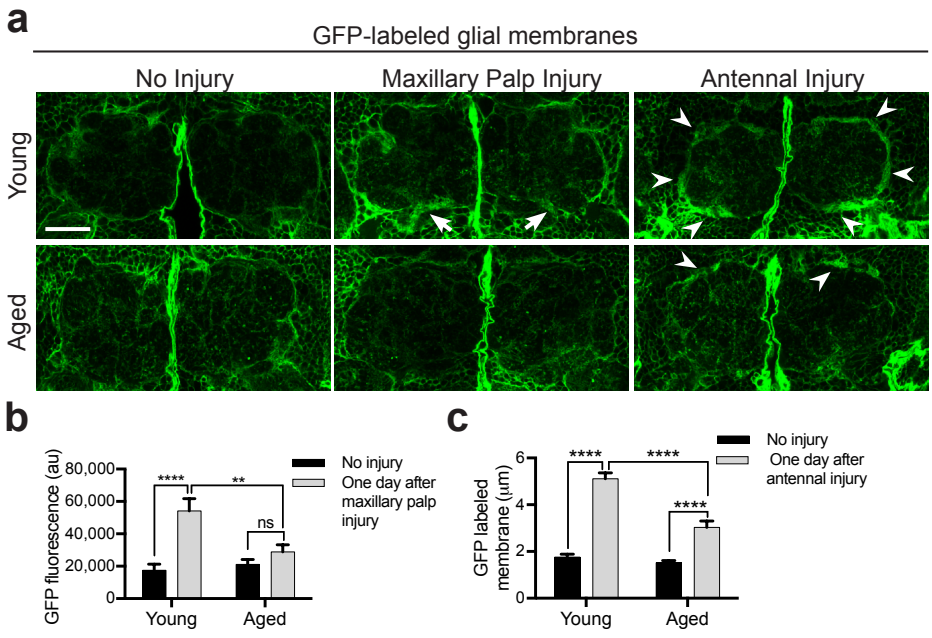

Figure 3. Glial membrane expansion to sites of axon injury is attenuated in aged flies. (**a**) Glial membranes were labeled in vivo with membrane-tethered GFP (repo-Gal4, UAS-mCD8::GFP). Robust accumulation of glial membranes was observed on degenerating axons in young flies after maxillary (arrows) or antennal nerve (arrowheads) axotomy. Representative confocal slices shown. (**b**) Quantification of GFP+ fluorescence on degenerating maxillary palp axons one day after axotomy; mean ± s.e.m. plotted; **P<0.01, ****P < 0.0001. (**c**) Quantification of GFP+ glial membrane expansion after antennal nerve injury; mean ± s.e.m. plotted; ****P < 0.0001. N ≥20 antennal lobes for each time point. Scale bar = 30 um. Two-way ANOVA with Sidak post hoc test was performed. Genotype: w1118;repo-Gal4, UAS-mCD8::GFP/TM3.

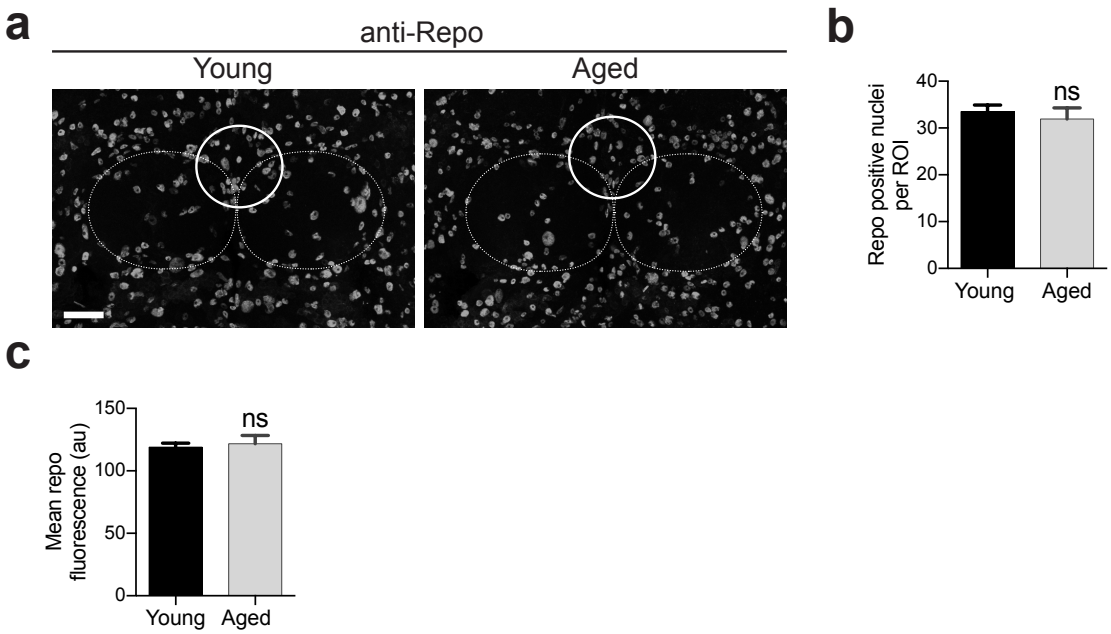

Figure 4. Glial cell numbers in the antennal lobe region do not change with age. (**a**) Representative compressed 20 um confocal Z-stack images of young and aged brains immunostained for the glial-specific nuclear transcription factor Repo. Antennal lobes outlined with a dotted line. (**b**) Counts of Repo+ nuclei in the central brain region adjacent to the antennal lobes. (**c**) Quantification of Repo fluorescence in the cortex of young and aged brains. (b) and (c) ROI used for counting and fluorescence measurements shown in the solid white circle. N=15; mean ± s.e.m. plotted; n.s.= not significant from unpaired t-test. Scale bar = 20 um. Genotype: w1118;repo-Gal4, UAS-mCD8::GFP/TM3.

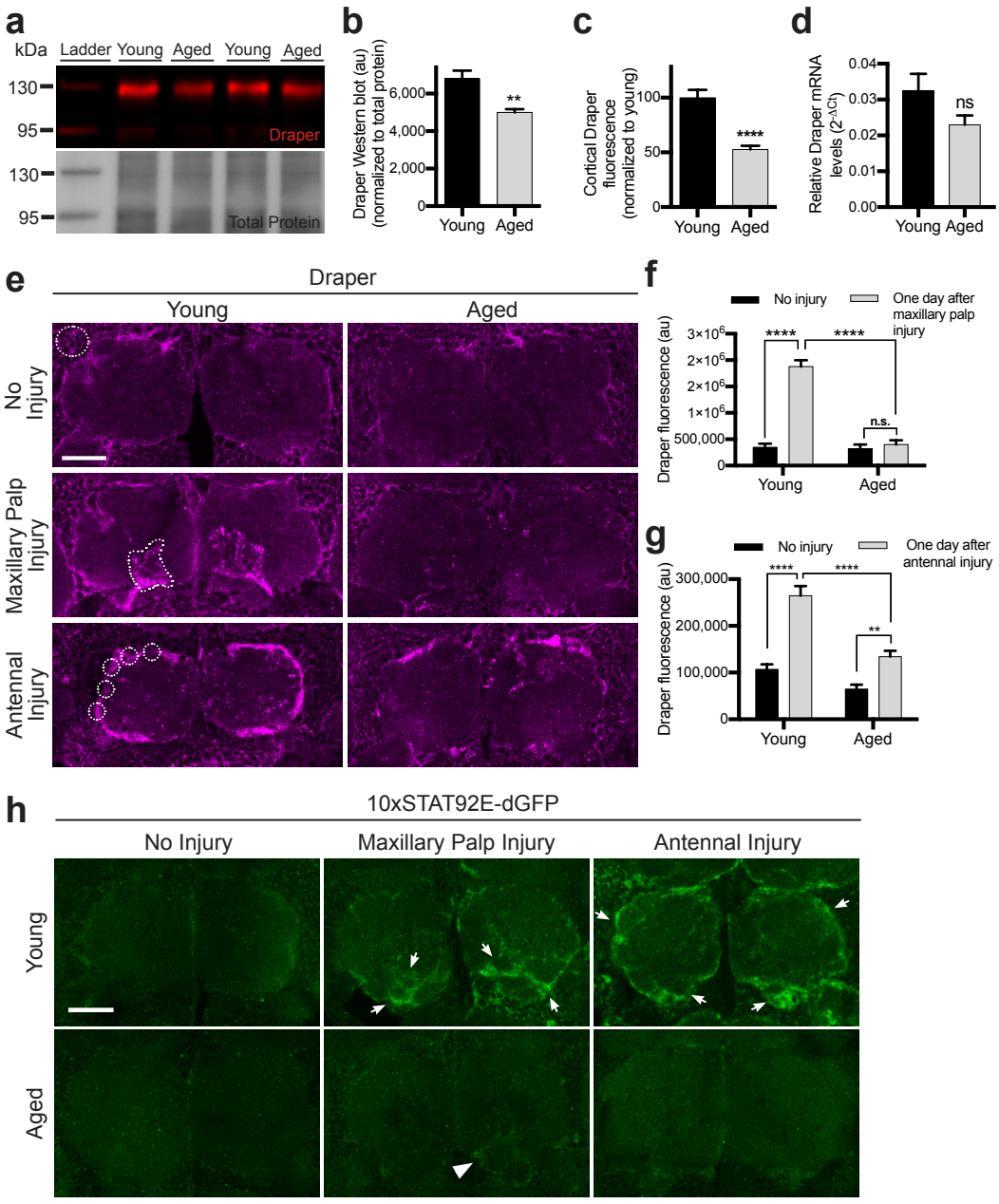

Figure 5 (continued below).

Figure 5. Axotomy-induced activation of STAT92E and upregulation of Draper decline with age. (**a**) Western blot with α-Draper (red, top panel) and MemCode general protein stain (bottom panel) of head lysates from young and aged flies. (**b**) Quantification of Draper western blot. **P<0.01, unpaired t-test. (**c**) Quantification of Draper fluorescence in the cortex of young and aged brains. Representative ROI in the young uninjured panel in (e). mean ± s.e.m. plotted; ****P<0.0001, unpaired t-test. (**d**) Q-PCR of Draper-I transcript levels in young and aged brains. mean ± s.e.m. plotted, young N=9, aged N=10, unpaired t-test. (**e**) Representative single confocal slices of Draper immunostained brains. White dotted outlines on injured images show ROIs used for quantification of Draper in (c), (f) and (g). (**f**) Quantification of Draper fluorescence in OR85e maxillary palp glomeruli before and after maxillary nerve injury; N ≥22. mean ± s.e.m. plotted; **P<0.01,****P < 0.0001. Two-way ANOVA with Sidak post-hoc test. (**g**) Quantification of Draper fluorescence in antennal lobe ensheathing glia before and after antennal nerve injury; N ≥22. mean ± s.e.m. plotted; **P<0.01,****P < 0.0001. Two-way ANOVA with Sidak post-hoc test. (**h**) STAT92E-dependent activation of dGFP (destabilized GFP) in the antennal lobe region. Robust reporter activity is observed in young flies 24 hours after maxillary or antennal nerve injury (arrows) but virtually undetectable in aged animals (arrowhead). Scale bars = 30 um. Genotypes: Fig. 2a-g: w1118. Fig. 2h: 10xSTAT92E-dGFP/+. ns=not significant, au=arbitrary units.

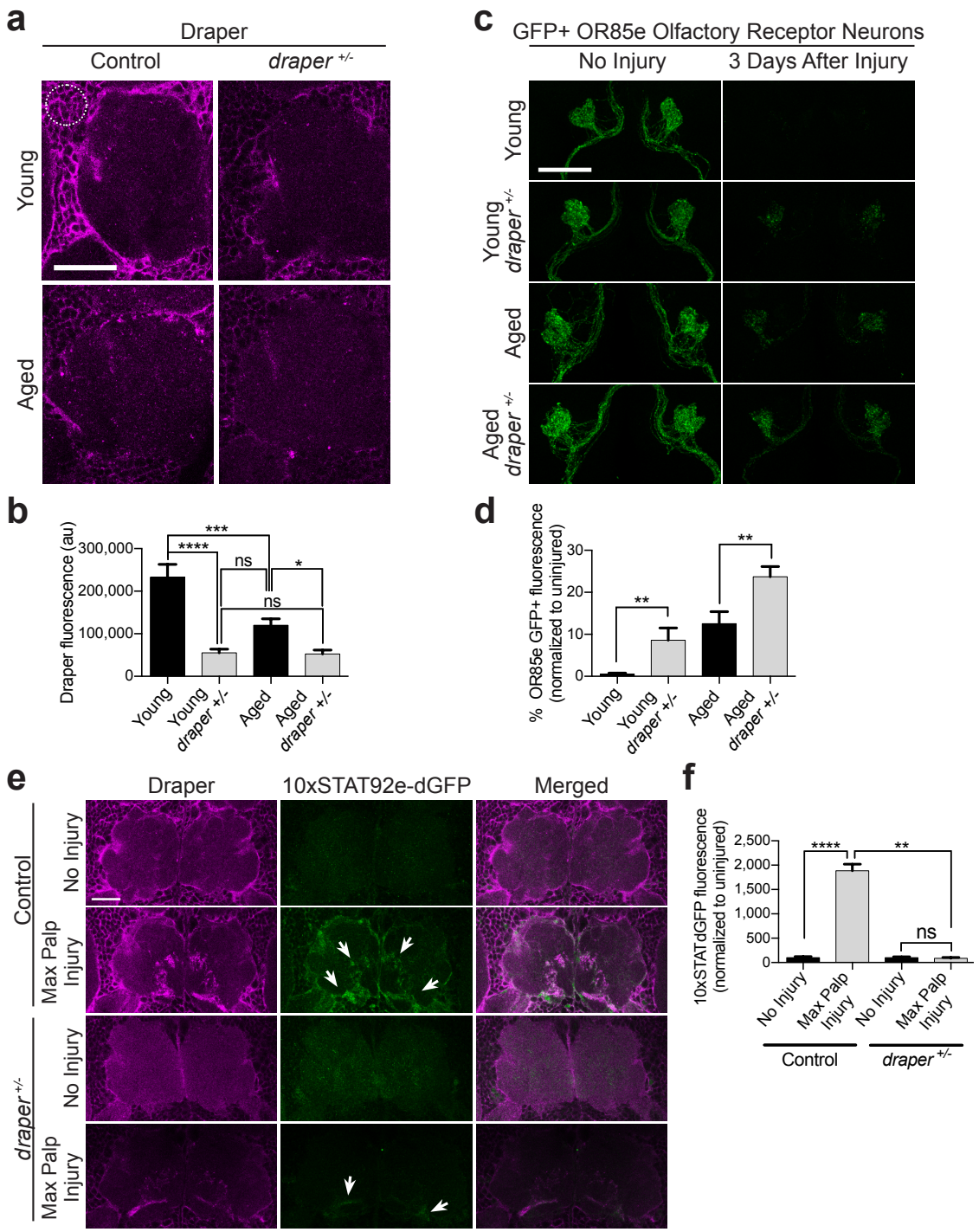

Figure 6 (continued below).

Figure 6. Glial responses to degenerating axons are attenuated in draper heterozygotes. (**a**) Representative single confocal slices of Draper immunostained brains: young (OR85e-mCD8::GFP), young *draper*$^{+/-}$ (OR85e-mCD8::GFP; draper$^{\Delta 5}$/+), aged (OR85e-mCD8::GFP), aged *draper*$^{+/-}$ (OR85e-mCD8::GFP; draper$^{\Delta 5}$/+). White dotted outline indicates ROI used for quantification of basal Draper in (b). (**b**) Quantification of cortical Draper immunostainings shown in (a); mean ± s.e.m. plotted; ns=not significant, *P<0.05, ***P < 0.001, ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥16. (**c**) Representative confocal projections of GFP-labeled OR85e axons. (**d**) Quantification of axon clearance in (c); mean ± s.e.m. plotted; **P < 0.01; unpaired t test; N ≥14. (**e**) Single confocal images of the antennal lobes region showing STAT92E-dependent activation of dGFP (green) and Draper (magenta) in Control (10xSTAT92E-dGFP/+) and draper$^{+/-}$ (10xSTAT92E-dGFP/+; draper$^{\Delta 5}$/+) animals. Arrows show expected upregulated of 10xSTAT92E-dGFP reporter activity after injury. (**f**) Quantification of dGFP on maxillary glomeruli shown in (e); mean ± s.e.m. plotted; ns=not significant, **P<0.01, ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥18. Scale bars = 30 um.

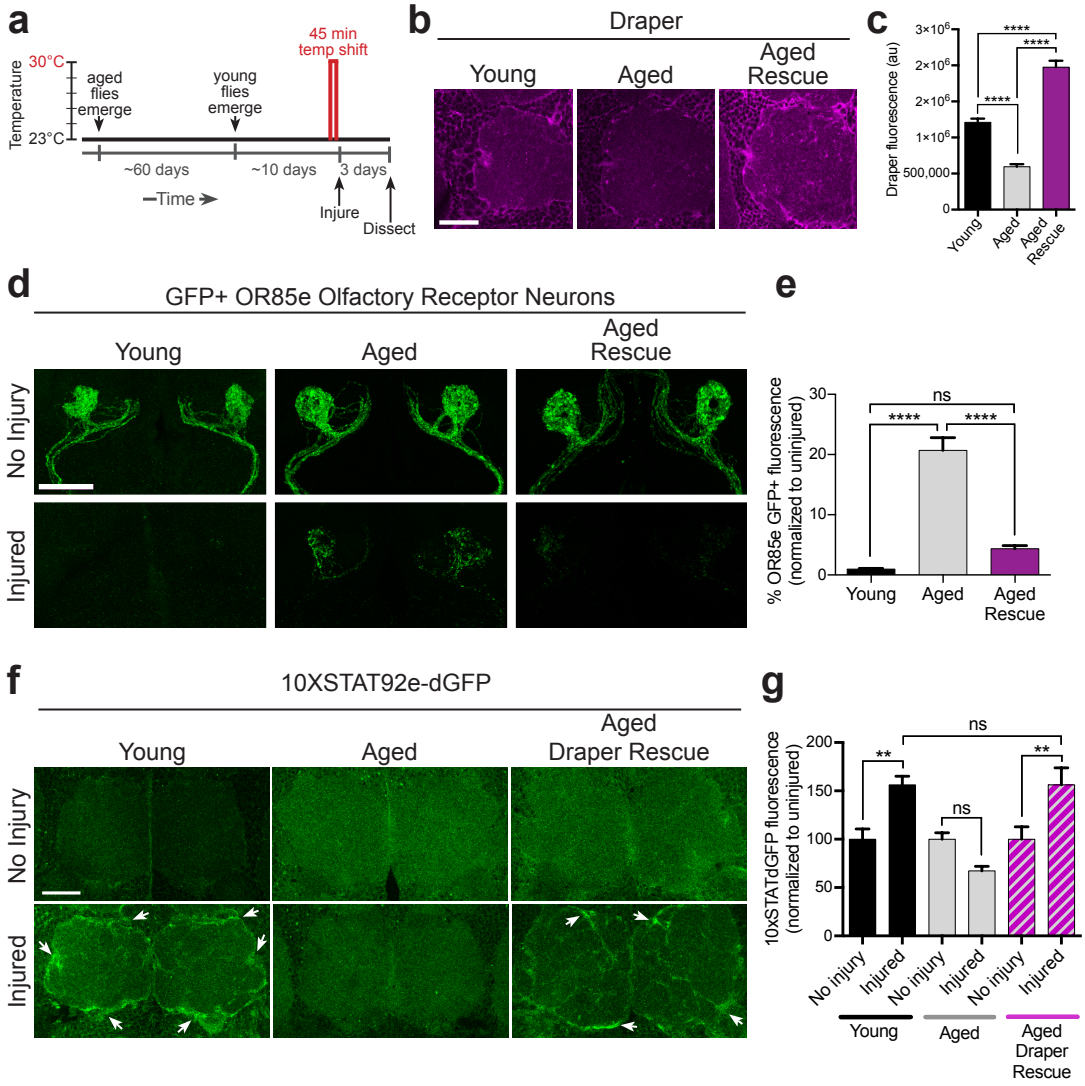

Figure 7 (continued below).

Figure 7. Expression of Draper in aged glia rescues glial clearance of axonal debris. (**a**) Schematic of the Gal80ts temperature-regulated paradigm to acutely induce expression of Draper-I in aged glia after injury. (**b**) Representative images of Draper (magenta) immunostained brains. (**c**) Quantification of Draper fluorescence in the cortex of Young, Aged and Aged Draper-I Rescue brains. mean ± s.e.m. plotted; ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥20. (**d**) Confocal projections of OR85e GFP+ axonal projections in uninjured flies versus 3 days after maxillary nerve axotomy. Expression of Draper-I in aged glia (Aged Rescue) restored normal clearance of axonal debris. (**e**) Quantification of axonal clearance shown in (d); mean ± s.e.m. plotted,ns = not significant, ****P < 0.0001. One-way ANOVA with Sidak post hoc test. N ≥14. (**f**) Representative images of antennal lobes showing expression of destabilized GFP under the control STAT92E activity (10XSTAT92E-dGFP) immunostaining (green) in Young, Aged, and Aged Draper-I Rescue animals. (**g**) Quantification of dGFP shown in (f); mean ± s.e.m. plotted; ns = not significant, **P<0.01; One-way ANOVA with Sidak post hoc test; N ≥18. Scale bars = 30 um. Genotypes: Fig. 4b-e, Young = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged = w1118;OR85emCD8:: GFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged Rescue = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/UAS-Draper-I; repo-Gal4/+. Fig 4f,g, Young = w1118; 10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged = w1118; 10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged Draper Rescue = w1118; 10xSTAT92E-dGFP, tubulin-Gal80ts/ UAS-Draper-I; repo-Gal4/+.

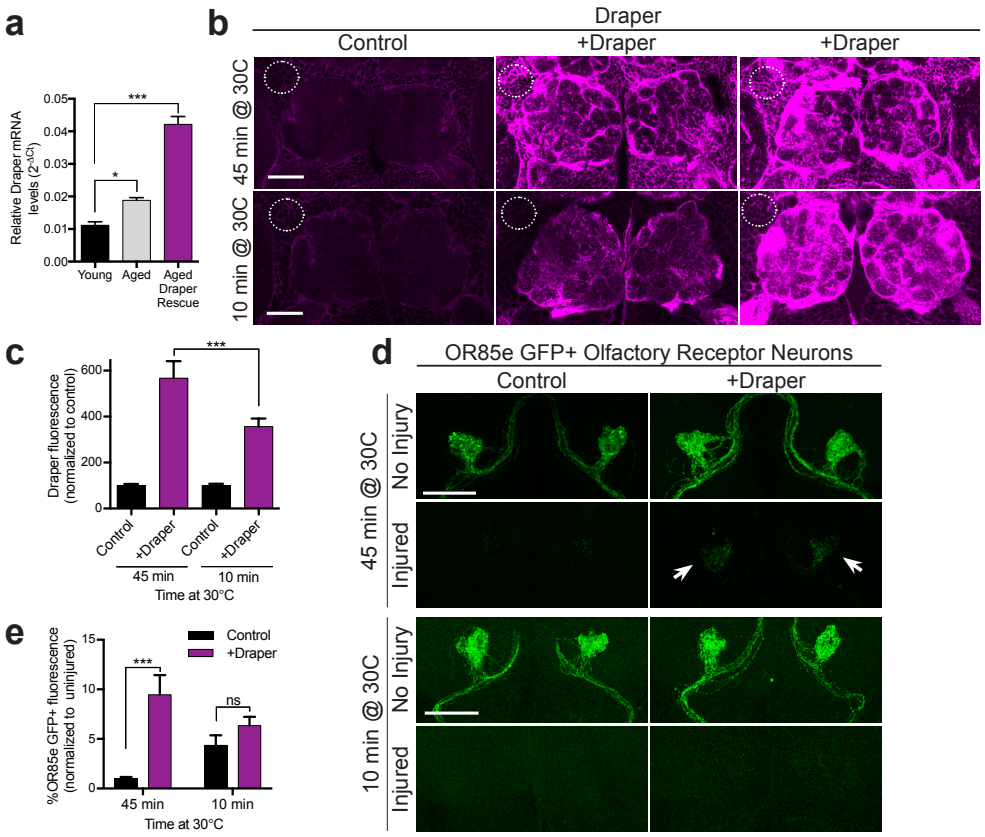

Figure 8. Increasing Draper-I levels in young glia does not result in faster clearance of degenerating ORN axons. (a-e) The Gal4/Gal80ts system was used to acutely upregulate Draper-I expression in glia of young flies at the time of maxillary nerve injury, as described in Fig 4a. (**a**) Q-PCR of Draper-I transcript levels in young, aged and aged Draper rescue brains. mean ± s.e.m. plotted, *P<0.05, ***P < 0.001; One-way ANOVA with Bonferroni post hoc test. N=3 biological replicates/group. (**b-e**) Draper immunostainings on uninjured young brains confirmed glial upregulation of Draper-I but showed a delay in clearance of GFP+ OR85e debris in the antennal lobes 3 days after injury in flies that overexpress glial Draper-I after the 45 min shift to 30°C (d, top half). The experiment was repeated and the young flies were shifted for only 10 min, showing that it is sufficient to increase Draper levels, but not cause a delay in clearance of GFP+ debris. (**b**) Representative Draper immunostainings of control and UAS-Draper-I overexpression (two examples shown) flies that were shifted to 30°C for either 45 min (using the same protocol as in Fig 4a) or 10 min. White dotted outline on single confocal slice images shows representative ROI used for quantification of basal Draper in (c). (**c**) Quantification of cortical Draper immunostainings shown in (b) normalized to the control; mean ± s.e.m. plotted; ***P < 0.001, One-way ANOVA with Sidak post hoc test; N=20 hemibrains. (**d**) Representative confocal Z-stack projections show small amounts of GFP+ OR85e axonal debris persist in the antennal lobes 3 days after injury in flies that overexpress glial Draper-I for 45 min but not 10 min. (**e**) Quantification of the experiment shown in (d). Mean ± s.e.m. plotted; ns=not significant, ***P<0.001; unpaired t test. N ≥24 antennal lobes. Scale bars: 30 um. Genotypes: Control Young and Aged = w1118; OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged Draper Rescue = w1118; OR85e-mCD8::GFP, tubulin-Gal80ts/UAS-Draper-I; repo-Gal4/+.

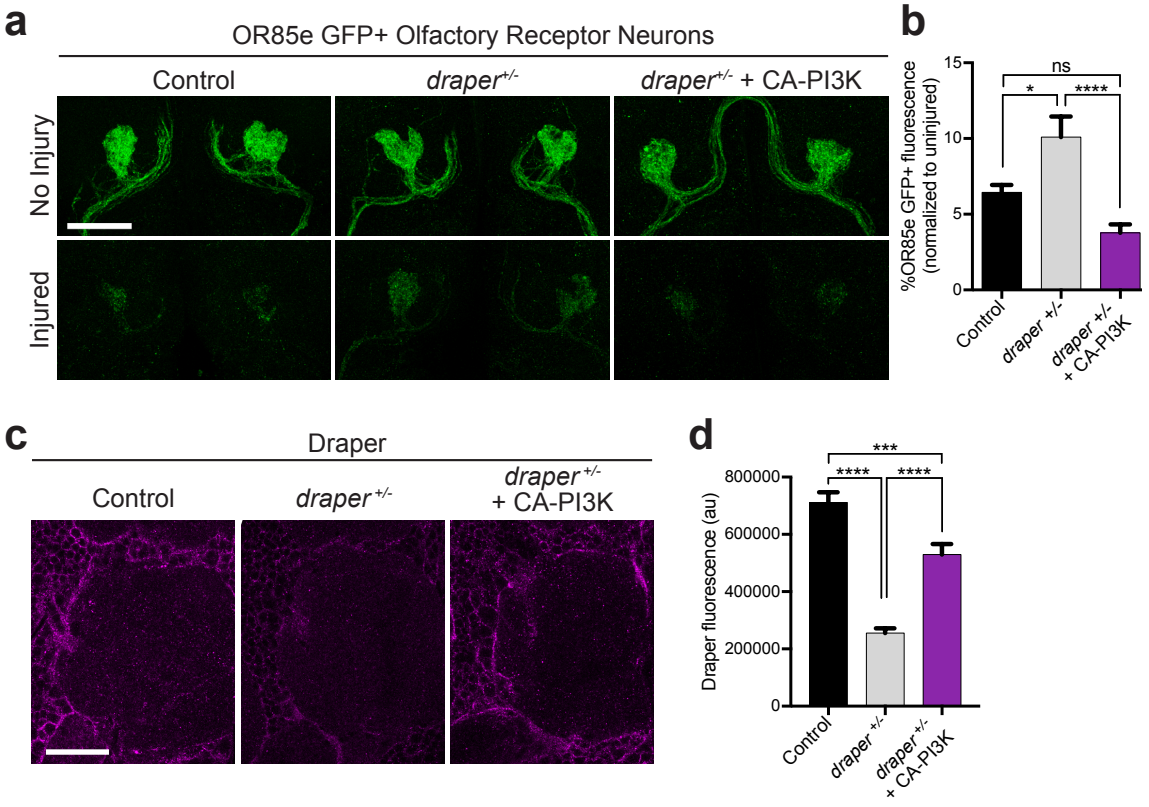

Figure 9. Expression of CA-PI3K in draper heterozygotes rescues glial clearance of axonal debris. (**a**) OR85e GFP+ axonal projections in uninjured flies versus 2 days after maxillary nerve axotomy. Expression of CA-PI3K in glia lacking a copy of Draper restored normal clearance of OR85e axonal debris. Representative Z-stack projections shown. (**b**) Quantification of axonal clearance in (a); mean ± s.e.m. plotted; ns = not significant, *P<0.05, ****P < 0.0001. One-way ANOVA with Sidak post hoc test. N ≥18 antennal lobes. (**c**) Representative images of Draper immunostainings. (**d**) Quantification of Draper fluorescence in the cortex of animals shown in (c); mean ± s.e.m. plotted; ***P<0.001, ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥19 hemibrains. All scale bars = 30 um. Genotypes: Control = w1118; OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Draper+/- = w1118; OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/ draperΔ5. Draper+/- + CA-PI3k = UAS-PI3K92eCAAX/ w1118; OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/draperΔ5.

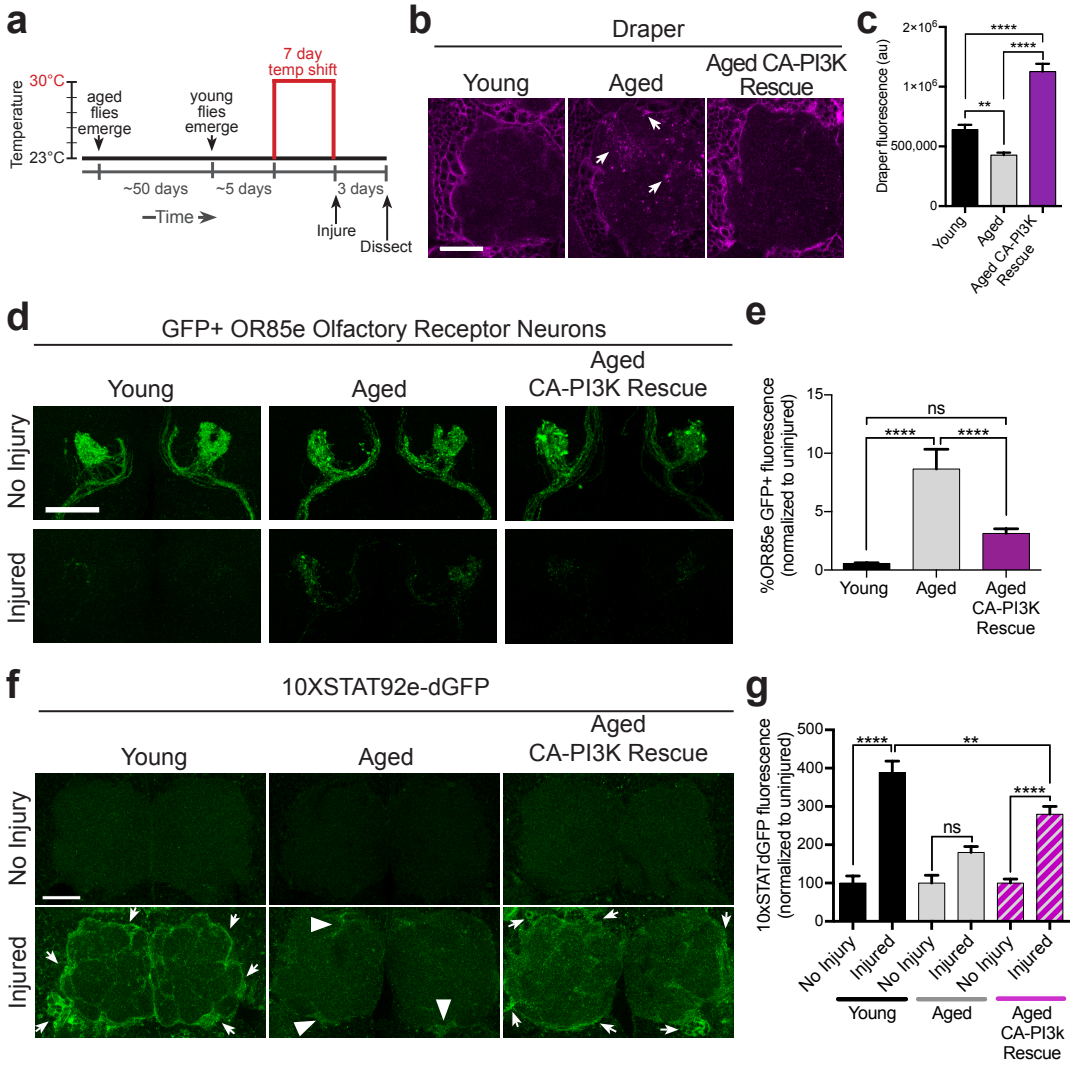

Figure 10 (continued below).

Figure 10. Expression of CA-PI3K in aged glia rescues glial clearance of axonal debris. (**a**) Schematic of experimental paradigm to induce expression of constitutively active-PI3K (CA-PI3K) in aged glia. (**b**) Representative Draper immunostainings of Young, Aged and Aged CA-PI3K Rescue brains. (**c**) Quantification of Draper fluorescence in the cortex of Young, Aged and Aged CA-PI3K Rescue brains shown in (b). mean ± s.e.m. plotted; **P<0.01, ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥26. (**d**) GFP+ OR85e axons before and 3 days post-axotomy. Representative Z-stack projections shown. (**e**) Quantification of GFP+ axonal debris in (d). mean ± s.e.m. plotted; ns = not significant, ****P < 0.0001. One-way ANOVA with Sidak post-hoc test. N ≥20. (**f**) Representative images of antennal lobes showing expression of destabilized GFP under the control STAT92E activity (10XSTAT92E-dGFP) immunostaining (green) in Young, Aged and Aged CA-PI3K Rescue. (**g**) Quantification of dGFP shown in (f). ns=not significant, **P<0.01, ****P < 0.0001; One-way ANOVA with Sidak post hoc test; N ≥18. Scale bars = 30 um. Genotypes: Fig. 5b-e, Young = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged CA-PI3K Rescue = UAS-PI3K92eCAAX; OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. Fig 5f,g, Young = w1118;10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged = w1118; 10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/+. Aged CA-PI3K Rescue = UAS-PI3K92eCAAX; 10xSTAT92E-dGFP, tubulin-Gal80ts/+; repo-Gal4/+.

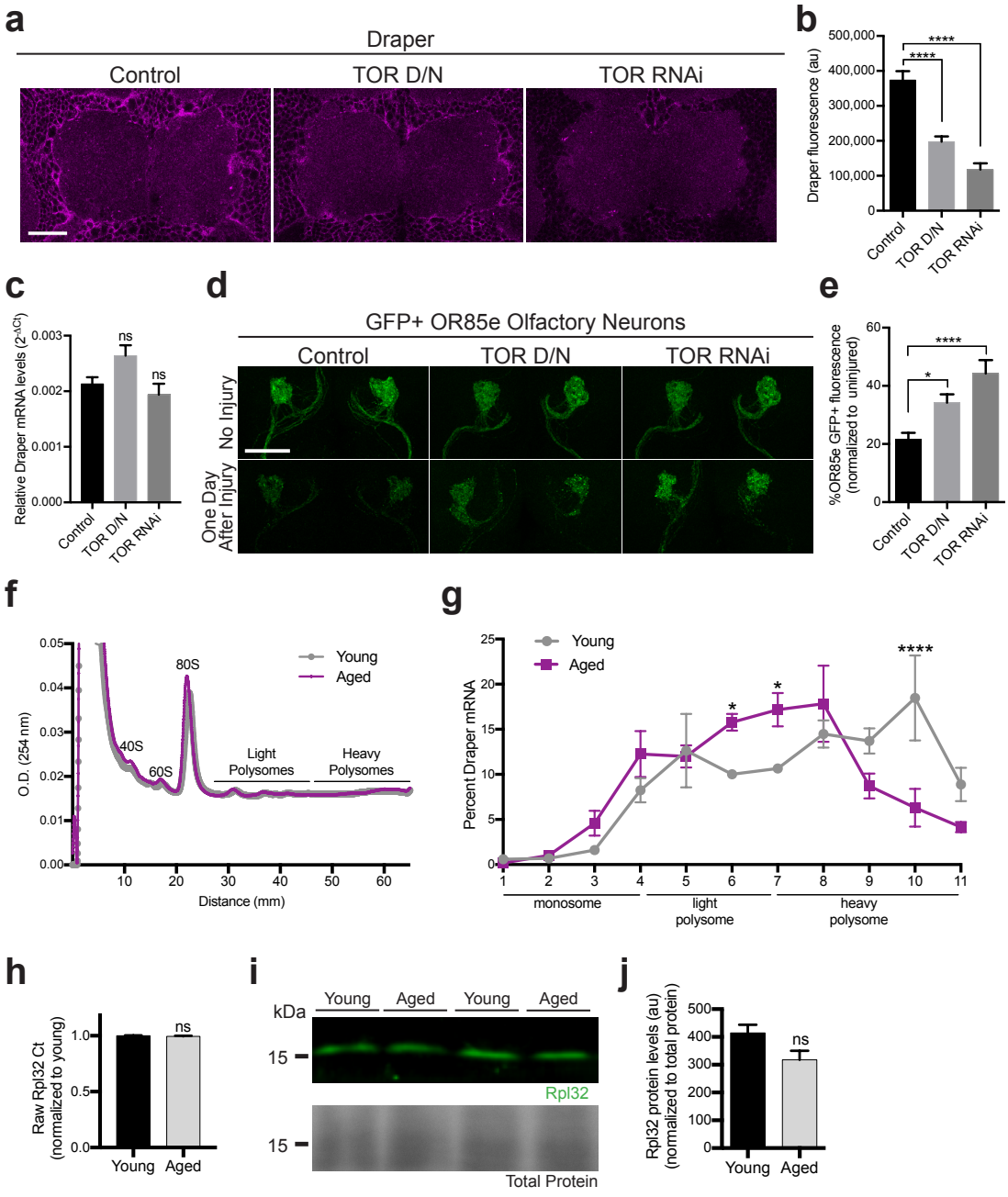

Figure 11 (continued below).

Figure 11. Inhibition of TOR in adult glia inhibits Draper and glial clearance of axon debris in young animals. (**a**) Single confocal slices of antennal lobes region. Brains immunostained with Draper. (**b**) Quantification of cortical Draper immunostainings shown in (a); mean ± s.e.m. plotted; ****P < 0.0001, One-way ANOVA with Sidak post hoc test; N ≥16. (**c**) Q-PCR analysis of draper-I transcript levels in adult heads; mean ± s.e.m. plotted; ns = not significant, One-way ANOVA with Sidak post hoc test; N=3 biological replicates. (**d**) Representative Z-stack projections of OR85e GFP+ axons. (**e**) Quantification of GFP+ axonal debris shown in (d); mean ± s.e.m. plotted; *P<0.05, ****P < 0.0001, One-way ANOVA with Sidak post hoc test. N = 22. (**f**) Polysome profiles for young and aged whole head lysates resolved on a 10–60% sucrose gradient. Absorbance continuously monitored at 254 nm during fractionation shown. (**g**) draper-I mRNA in each fraction was quantified by qPCR and normalized to the housekeeping gene Rpl32 and luciferase (control for RNA recovery). mean ± s.e.m. plotted; *P<0.05, ****P < 0.0001, Two-way ANOVA with uncorrected Fisher's LSD post-hoc test. N=3 groups of 30 w118 fly heads/age. (**h**) Normalized Rpl32 Ct values pooled from four experiments on young and aged w118 brain lysates. mean ± s.e.m. plotted; ns=not significant, unpaired t-test. N=17 biological replicates/age. (**i**) Representative Western blot for Rpl32 (green) and MemCode total protein stain (bottom panel) performed on head lysates from young and aged flies. (**j**) Quantification of Rpl32 Western blots; unpaired t-test. N=4 biological replicates/age. Genotypes: Fig 6a-e, Control = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/+. D/N TOR= w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/UAS-D/N TOR. TOR RNAi = w1118;OR85e-mCD8::GFP, tubulin-Gal80ts/+; repo-Gal4/UAS-TOR RNAi. Fig 6f-j, w1118. Scale bars = 30 um.

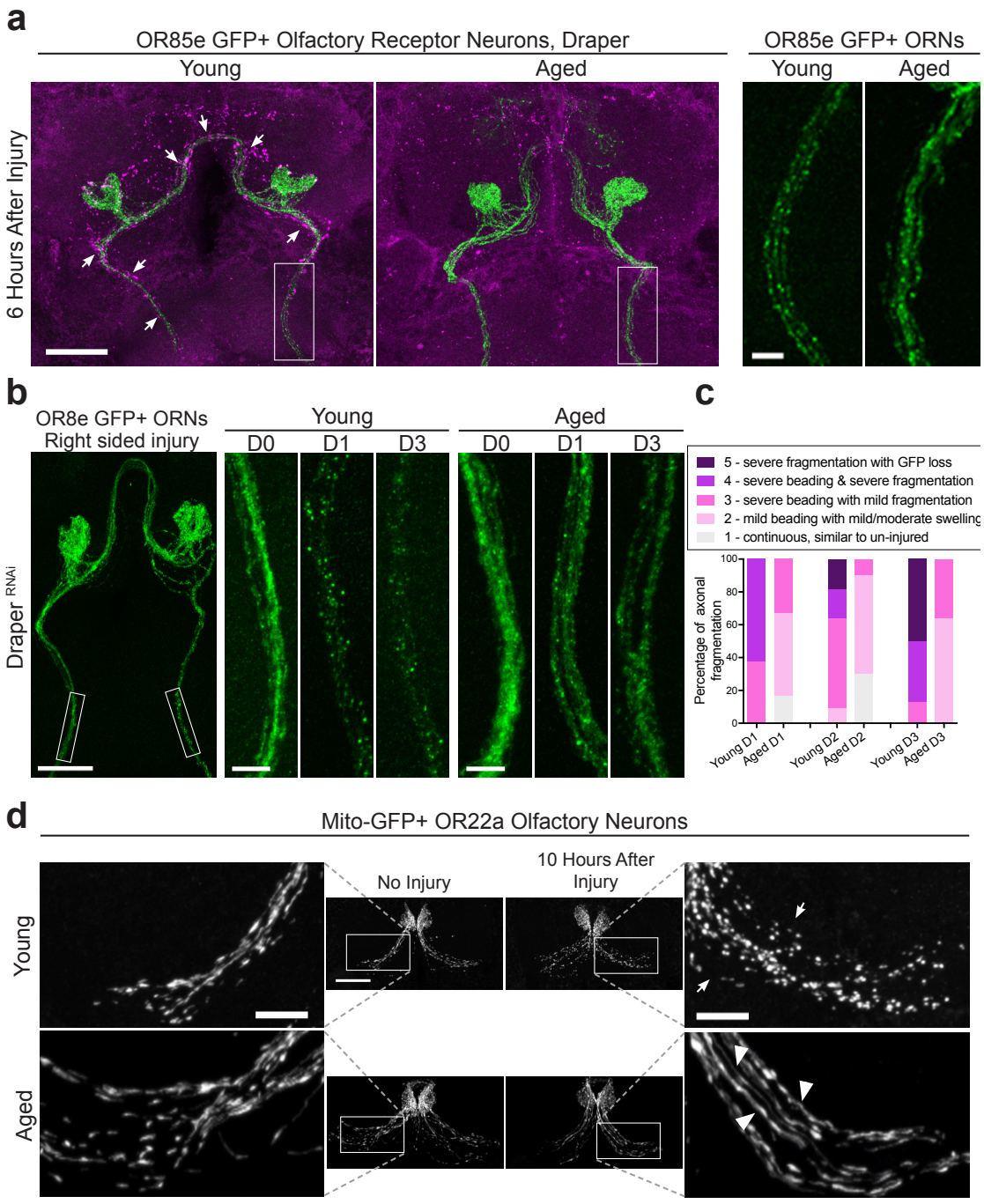

Figure 12 (continued below).

Figure 12. Wallerian degeneration of severed ORNs is delayed in aged animals. **(a**)Representative Z-stack projections shown from young and aged brains 6 hours post-axotomy. Draper (magenta) accumulation is visible in young axons at this time point in young animals (white arrows). Scale bar = 30 um. Zoomed images of axons (right) are from white, boxed regions. Scale bar = 10 um. (**b**) Representative images of young and aged axons expressing membrane-tethered GFP from uninjured (D0) animals and one day (D1) or three days (D3) after injury in glial Draper-depleted flies. Scale bar = 30 um in the left panel and 5 um in higher magnification panels. (**c**) Blind scoring analysis of fragmentation status of young and aged axons one day (D1), two days (D2) or three days (D3) post-axotomy shown in (b). (**d**) Representative projections of young and aged flies expressing mitochondrial GFP (mito-GFP) in a subset of olfactory axons before and 10 hours after axotomy. mito-GFP pattern appeared punctate after injury in young animals (white arrows) but continuous in aged axons (white arrowheads). Scale bar = 30 um in central panels and 10 um for magnified images. Genotypes: Fig. 7a: w1118; OR85emCD8:: GFP/+. Fig. 7b,c: w1118; OR85emCD8:: GFP/+; repo-Gal4/UAS-DraperRNAi. Fig. 7d: OR22a-Gal4/UAS-mito-HA-GFP.

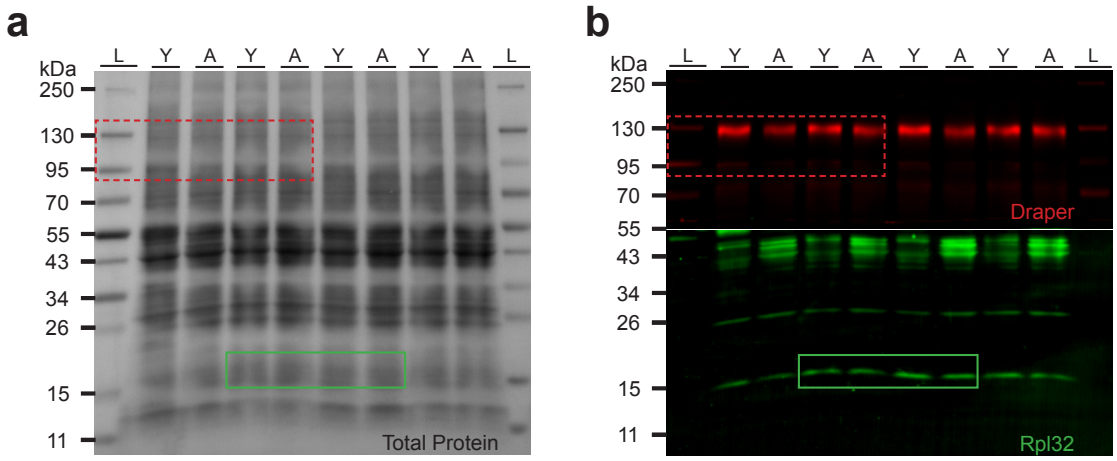

Figure 13. Uncropped blot of the Westerns shown in Figures 5a and 11i. (**a**) Total protein blot displaying four biological replicates for young (Y) and aged (A) samples. (**b**) Fluorescence image of the blot displayed in (a) probed for Draper (red, top half) and Rpl32 (green, bottom half). The red, dotted boxes highlight the cropped areas in Fig 2a and the green boxes highlight the cropped areas shown in Fig 6i.

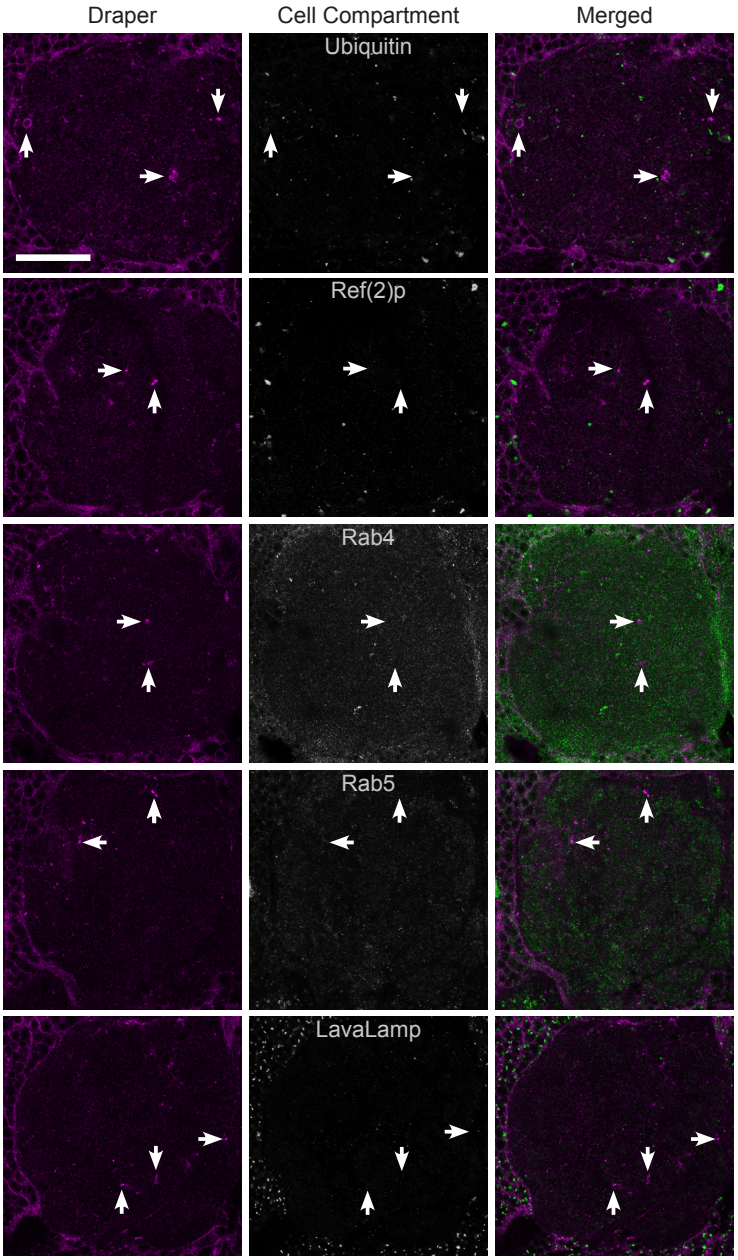

Figure 14: Draper aggregates in aged brains do not associate with known cell compartment markers. Aged w^{1118} brains were immunostained with Draper and Ubiquitin, Ref (2)P (autophagy marker), Rab4, Rab5 and Lava Lamp (Golgi marker). Arrows point to Draper aggregates in the antennal lobe region. Scale bar = 30 um.

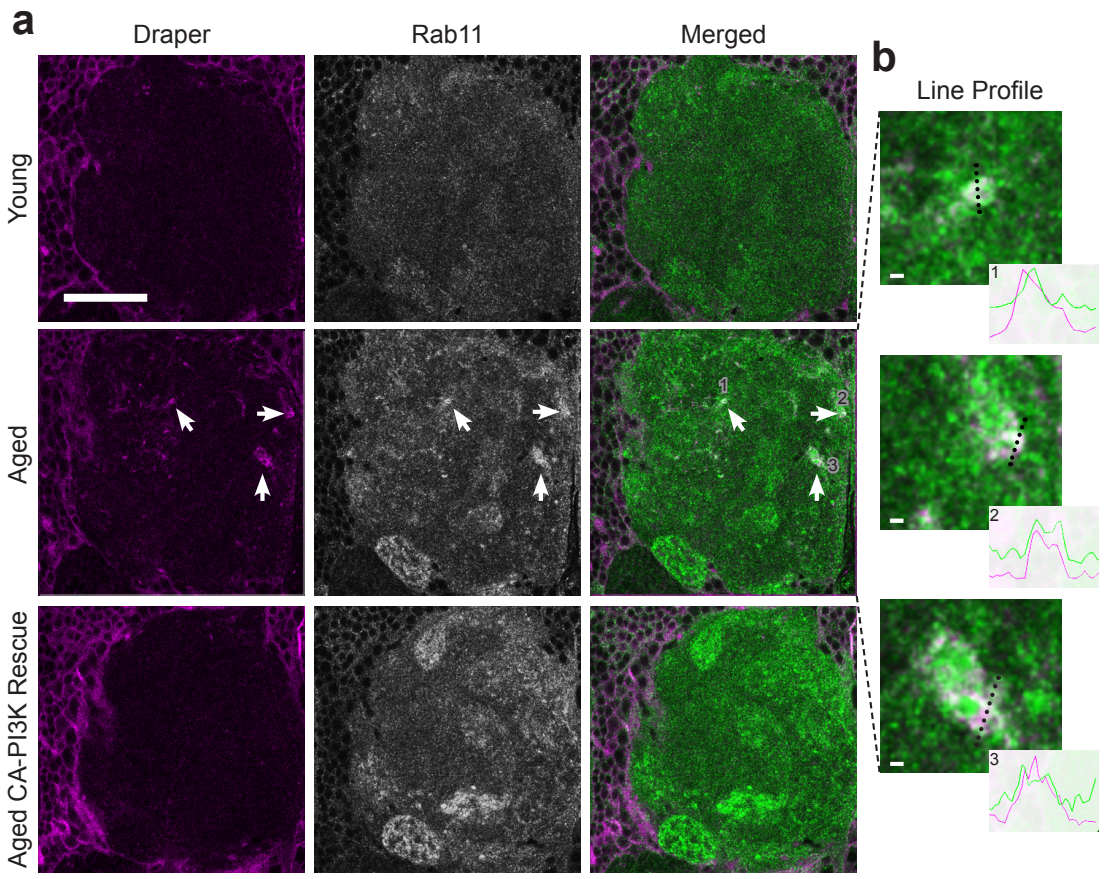

Figure 15: Increasing PI3K levels in aged glia leads to a decrease in Draper aggregates associated with Rab11. (**a**) Draper and Rab11 immunostainings of antennal lobes of young, aged and aged brains that are acutely expressing PI3K in aged glia under the control of repo-Gal4. Arrows in aged brains depict Draper aggregates that are also Rab11 positive. Scale bar = 30 um. (**b**) Zoomed in regions numbered in panel (a). Magnification line profile graphs depict the Draper and Rab11 immunofluorescence intensity along the positioned dotted lines in aged brains. Scale bars = 1 um.

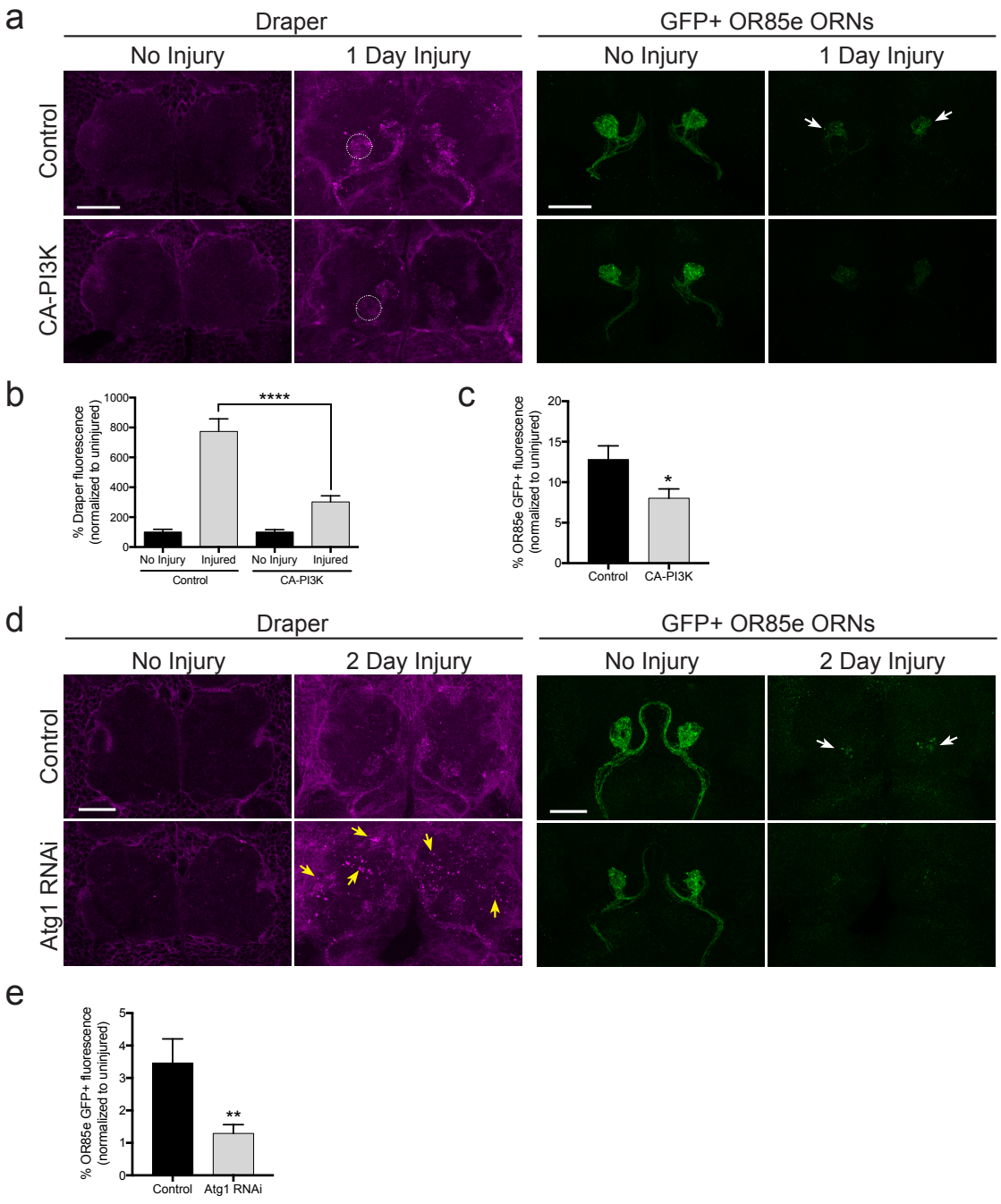

Figure 16 (continued below).

Figure 16. Activation of PI3K or inhibition of Atg1 leads to faster clearance in young animals. (**a**) Representative images of Draper (magenta) immunostained brains. Dotted circles in Draper injury response represents ROI used for quantification in (b). On the right, confocal projections of OR85e GFP^+ axonal projections in uninjured flies versus 1 day after maxillary nerve axotomy. Expression of CA-PI3K in young flies leads to faster clearance of axonal debris. (**b**) Quantification of Draper fluorescence after injury. mean ± s.e.m. plotted; ****P < 0.0001. Studen's t-test. N ≥24. (**c**) Quantification of axonal clearance shown in (a); mean ± s.e.m. plotted, *P < 0.05. Student's t-test. N ≥24. (**d**) Representative images of Draper (magenta) immunostained brains. In young flies overexpressing Atg1, there are Draper aggregates in the neuropil areas (yellow arrow). On the right, confocal projections of OR85e GFP^+ axonal projections in uninjured flies versus 2 days after maxillary nerve axotomy. Inhibition of Atg1 in young flies leads to faster clearance of axonal debris. (**e**) Quantification of axonal clearance shown in (d); mean ± s.e.m. plotted, **P < 0.01. Student's t-test. N = 18. Scale bars = 30 um. Genotypes: Fig. 16 a-d, Control = w^{1118};OR85e-mCD8::GFP, tubulin-$Gal80^{ts}$/+; repo-Gal4/+. CA-PI3K = CA-PI3K; OR85emCD8:: GFP, tubulin-Gal80ts/+; repo-Gal4/+. Fig. 16d,e: Control = w^{1118}; 10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/+. Atg1RNAi = w^{1118}; 10xSTAT92e-dGFP, tubulin-Gal80ts/+; repo-Gal4/UAS-Atg1RNAi.

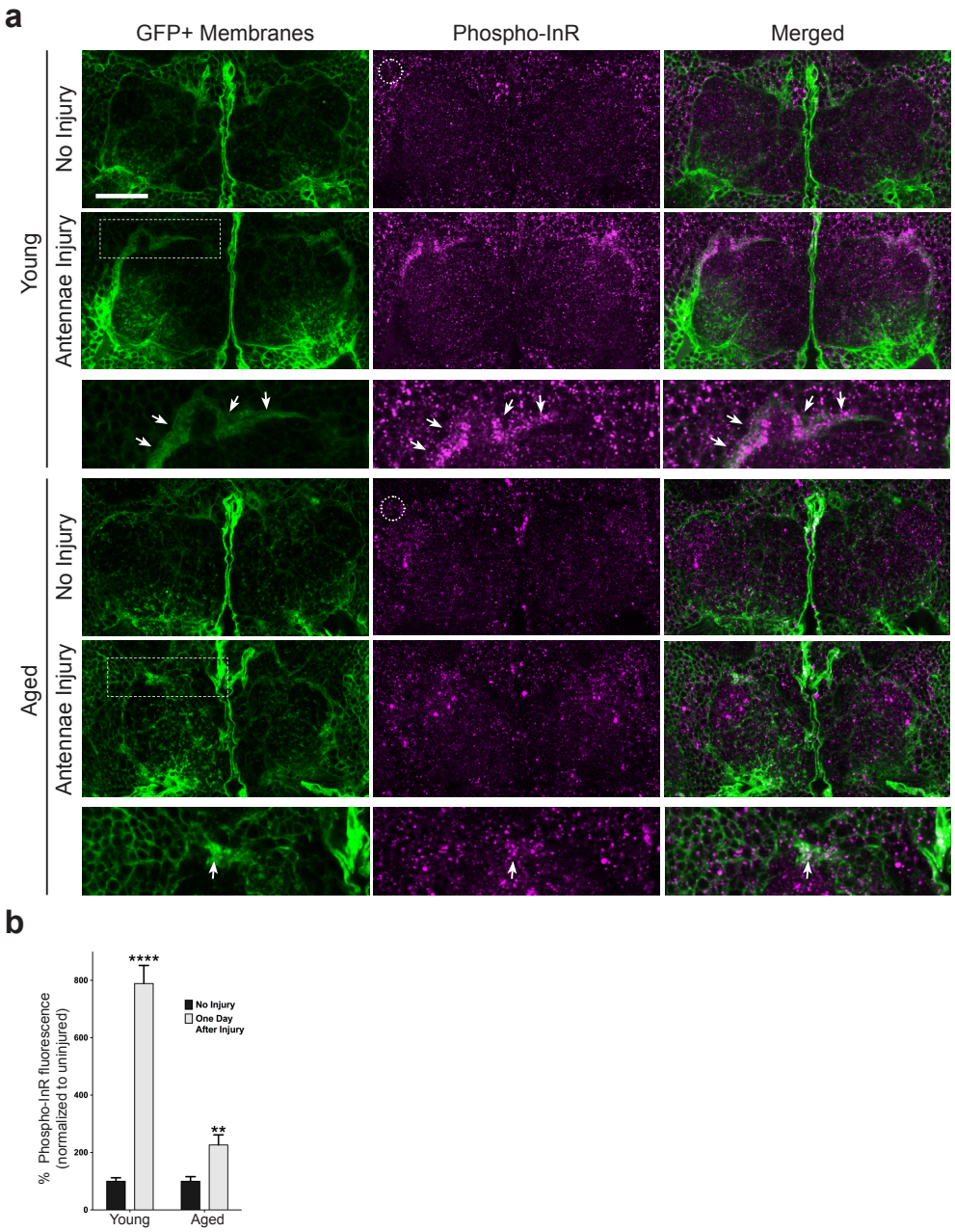

Figure 17. Glial insulin receptor activity is reduced in aged animals after injury. (**a**) Representative images of phospho-InR (magenta) and GFP+ glial membranes in young and aged flies, before and 18 hours after bilateral antennal nerve axotomy. Dotted boxes in young and aged, injured, represent areas that are used for high magnifications. Arrows depict overlap in glial membrane expansion and the phospho-InR. There is a large attenuation in glial membrane hypertrophy and InR activation in aged animals. (**b**) Quantification of phospho-InR in response to injury. mean ± s.e.m. plotted; ****P < 0.0001. Studen's t-test. N≥18. Scale bar = 30 um. Genotypes: Repo-Gal4, UAS-mcD8::GFP/TM3.

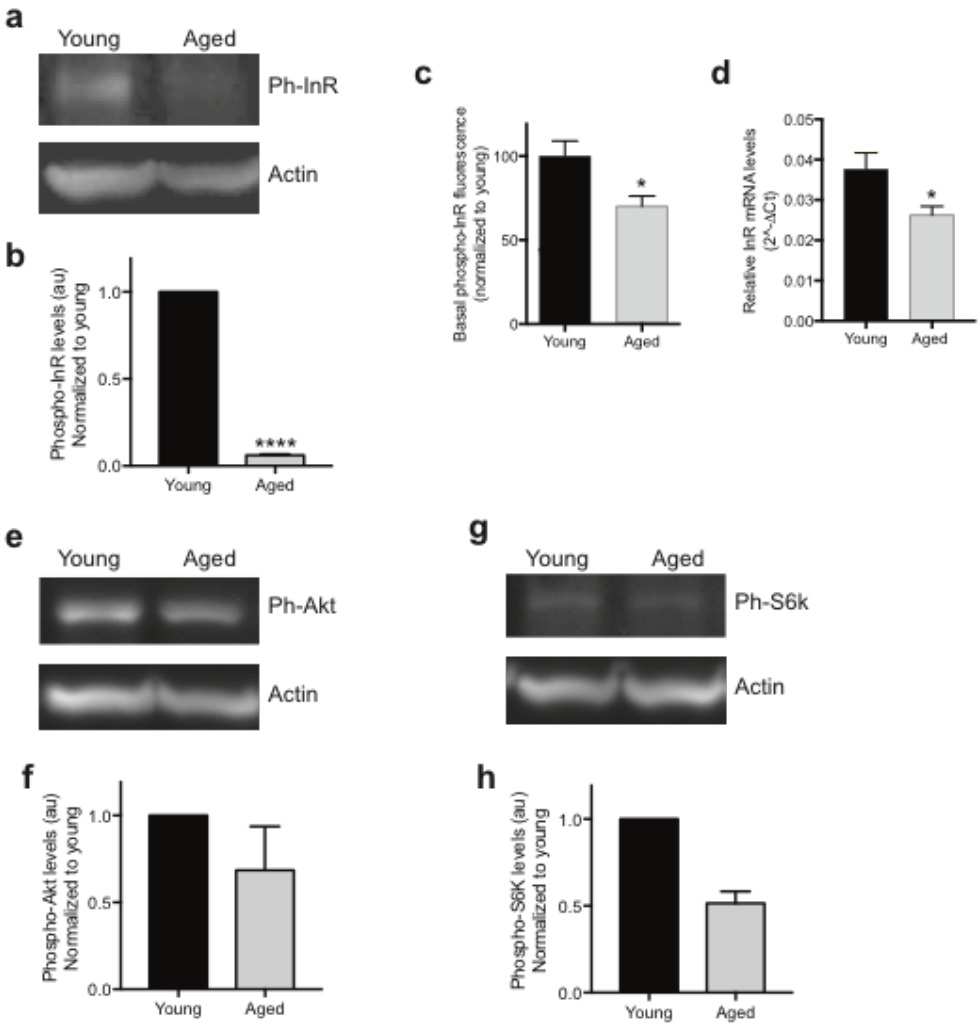

Figure 18. Insulin-like signaling is decreased in the aged brain. (**a**) Western blot with α-phospho-InR (top panel) and actin (bottom panel) of head lysates from young and aged flies. (**b**) Quantification of phospho-InR western blot. ****P<0.0001, unpaired t-test. N=3 biological replicates/age group (**c**) Quantification of phospho-InR fluorescence in the cortex of young and aged brains. Representative ROI in the young uninjured panel in Fig. 17 a. mean ± s.e.m. plotted; *P<0.05, unpaired t-test. N≥18. (**d**) Relative *InR* mRNA levels in young and aged animals. mean ± s.e.m. plotted; *P<0.05, unpaired t-test. N=10 biological replicates/group. (**e**) Western blot with α-phospho-Akt (top panel) and actin (bottom panel) of head lysates from young and aged flies. (**f**) Quantification of the phospho-Akt western blot. N=2. (**g**) Western blot with α-phospho-S6k (top panel) and actin (bottom panel) of head lysates from young and aged flies. (**h**) Quantification of the phospho-S6k western blot. N=2. Genotypes: w^{1118}

CHAPTER 3: TRANSCRIPTIONAL PROFILING IN A NOVEL *DROSOPHILA* INJURY MODEL REVEALS MMP-1 IS A GLIAL TARGET OF THE DRAPER PATHWAY AND REQUIRED FOR CLEARANCE OF DEGENERATING AXONS

Modified from: Purice, M. D., Ray A., Muenzel E.J., Klimpert N., Pope B., Park D., Speese S.D., Logan M.A. (2016) *In preparation*.

ABSTRACT

Glial cells are the first immune responders to neuronal stress and damage. Neural injury triggers swift responses from glia, including glial migration to injury sites and glial clearance of damaged neurons through phagocytic engulfment. This complex set of responses is orchestrated in part by injury-induced changes in glial gene expression, but our knowledge of the transcriptional programs that govern glial responses to neuronal injury is still incomplete. Many molecular and cellular hallmarks of glial responses to degenerating neurons are conserved across species. Here, we describe a novel adult *Drosophila* injury assay that includes severing peripheral nerves to elicit widespread glial responses throughout the fly ventral nerve cord (VNC). We profiled injury-induced changes in VNC gene expression by RNA-seq and found that acutely upregulated genes fall into discrete signaling classes, including Toll-like signaling, phagocytic activity, and extracellular matrix remodeling. Here we report matrix metalloproteinase-1 (MMP-1) is upregulated in *Drosophila* ensheathing glia responding to axon degeneration in both the central brain and the VNC. We also show that glial induction of MMP-1 requires the highly conserved Draper engulfment receptor and transcription factors AP-1 and STAT92E. In MMP-1 depleted flies, glia do not properly infiltrate neuropil regions after axotomy and, as a consequence, fail to clear degenerating axonal debris. This work identifies MMP-1 as a novel Draper-dependent transcriptional target required for efficient glial activation post-axotomy and proper clearance of axonal material.

INTRODUCTION

Glial cells exhibit swift and dramatic responses to any form of neural trauma. These reactions provide neuroprotection and minimize further damage to the CNS. Altered glial gene expression is one highly conserved feature of the glial response to various forms of insults (Allen and Barres, 2009; Barres, 2008; Chung et al., 2013; Doherty et al., 2009; Logan and Freeman, 2007; Logan et al., 2012; Ziegenfuss et al., 2008). Specifically, the janus kinase/signal transducer and activator of transcription (JAK/STAT) is a central player in the initiation of reactive astrocytic and microglial responses to a range of insults (Ben Haim et al., 2015; Herrmann et al., 2008; O. S. Kim et al., 2002; K. W. Park et al., 2016). Glial activation of the JNK pathway and the downstream transcriptional heterodimer AP-1, which consists of c-Fos and c-Jun, has also been reported in various injury and disease models (A. J. Anderson et al., 1994; Pennypacker et al., 1994; A. C. Yu et al., 1995). However, it remains unclear how transcriptional programs drive complex glial responses to neural injury.

Reactive glia often also display prominent changes in cell shape, size, or motility. Glial cells can migrate substantial distances to reach trauma sites (Roth et al., 2014). In other instances, the glial soma remains in a fixed location while the cell extends elongated processes to rapidly enter regions that houses damaged neurons (Davalos et al., 2005; Dissing-Olesen et al., 2014). These striking morphogenic responses ensure that glial cells gain access to sites of damage to release protective factors and clear apoptotic cells and degenerating projections through phagocytic engulfment. However, we but still have an incomplete understanding of how dynamic responses are elicited and carried out in reactive glia.

Glial responses to injury and disease share common features across species. In *Drosophila*, acute axotomy triggers reactions from local glia that are strikingly similar to those observed in mammals. Specifically, severing adult olfactory nerves that project into the antennal lobes of the central brain initiates a Wallerian degeneration (WD) program in olfactory receptor neuron (ORN) axons (Hoopfer et al., 2006; MacDonald et al., 2006). Ensheathing glia respond by first activating STAT92E and JNK/AP-1 cascades (Doherty et al., 2014; MacDonald et al., 2013). Next, over the course of several days, these cells extend membrane projections into the antennal lobe neuropil to phagocytose degenerating axonal and synaptic debris (Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006; Ziegenfuss et al., 2012). Recent work has shown that transcriptional pathways triggered in vertebrates (e.g. AP-1 and STAT92E) are similarly activated in *Drosophila* ensheathing glia in response to axon injury (Doherty et al., 2014; MacDonald et al., 2013). Other molecular similarities have been recently identified between fly and mammalian glia (Awasaki et al., 2006; Lu et al., 2014; Ziegenfuss et al., 2008). For example, the highly conserved glial engulfment receptor Draper/MEGF10 is required in flies and mammals for proper glial engulfment of degenerating neuronal projections and apoptotic neurons (Awasaki et al., 2006; Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006). Notably, Draper is also required to activate AP-1 and STAT92E in *Drosophila* ensheathing glia following nerve injury (Doherty et al., 2014; MacDonald et al., 2013). Together, this work validates the fly as a useful model to probe the basic mechanisms driving innate glial immune reactions.

In response to axotomy, activation of glial Draper triggers a positive auto-regulatory loop, which includes transcriptional upregulation of *draper* in a STAT92E-dependent manner (Doherty et al., 2014). This response ensures that high levels of Draper are

present at the cell surface of phagocytic reactive glia. Aside from STAT92E targeting of the *draper* locus, virtually nothing is known about the transcriptional programs invoked in *Drosophila* glia responding to neural injury. Here, we describe a new non-lethal nerve injury model which elicits widespread glial responses to neurodegeneration in the ventral nerve cord (VNC). Using this assay, we carried out an RNA-Seq analysis of VNC tissue to identify axotomy-induced changes in gene expression. We also performed a detailed *in vivo* functional analysis of the secreted protease matrix metalloproteinase-1 (MMP-1), a novel gene we found to be acutely upregulated in ensheathing glia responding to axon degeneration. Interestingly, we show that MMP-1 induction in ensheathing glia requires Draper/STAT92E/AP-1 signaling. Moreover, MMP-1 is required for proper glial recruitment to severed axons and, subsequently, timely clearance of degenerating axonal debris. Together, this work demonstrates an essential role for MMP-1 in glial recruitment and phagocytic responses post-axotomy and reveals a novel role for Draper/STAT92E/AP-1 as a transcriptional pathway that targets genes required for altered glial membrane dynamics in response to nerve injury.

RESULTS

DRAPER IS ROBUSTLY UPREGULATED IN VNC ENSHEATHING GLIA AFTER AXOTOMY

We sought to develop an *in vivo* injury model that would allow us to transcriptionally profile genes upregulated in *Drosophila* glia responding to axon injury. Recent studies revealed that peripheral sensory neurons in the wing undergo a classic WD program and are cleared by glia in a Draper-dependent manner after axotomy,

(Fang et al., 2012; Fullard and Baker, 2015) suggesting that glial cells of the VNC and the central brain use common pathways to sense and respond to degenerating axons. In adult flies, axons extending to and from the front, middle, and rear sets of legs project through the prothoracic (PN), mesothoracic (MN), and metathoracic (MtN) neuropil regions of the VNC, respectively. Sensory axons from the wing project through the VNC accessory mesothoracic (AMN) neuropil (Fig. 1a,b). First, we asked if Draper was upregulated in various axotomy paradigms in the VNC by surgically removing select peripheral tissues and then performing Draper immunostaining on dissected VNCs one day after injury. In uninjured animals, Draper is detectable around neuropil and throughout the cortex of the VNC (Fig. 2a, no injury panels). In various injury paradigms, Draper was consistently upregulated selectively on severed axons 24 hours after injury. For example, removal of a single wing or bilateral ablation of the front legs resulted in strong Draper immunofluorescence in the neuropil that contained degenerating axonal projections corresponding to each injured structure(s) (Fig. 2a). Similarly, decapitation elicited robust Draper upregulation throughout the VNC (Fig. 2a).

The adult *Drosophila* brain contains several glial subtypes that vary in location, gene expression and function (Freeman, 2015). Cortex glia enwrap neuronal cell bodies throughout the cortex, while neuropil regions contain two unique types of glia: ensheathing glia and astrocytes (Logan and Freeman, 2007). In the adult olfactory system, ensheathing glia, but not astrocytes, respond to degenerating olfactory receptor neurons by upregulating *draper*, invading the neuropil and clearing axonal debris (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006). We used several well-characterized glial subtype drivers to express membrane-

tethered GFP (UAS-mCD8::GFP) and assess expression patterns in the adult VNC and found that labeling recapitulated patterns of the central brain. The pan-glial driver Repo-Gal4 appeared to label most, if not all, VNC glia (Fig. 2b). The astrocyte driver alrm-Gal4 also specifically labeled glia immediately surrounding the neuropil and they displayed a classic astrocyte-like, highly branched pattern (Fig. 2b). Finally, TIFR-Gal4 labeled glia in an ensheathing-like pattern; we detected high levels of GFP in neuropil-associated ensheathing glia and, as in the central brain, low expression in some cortex glia (Fig. 2b).

To determine if Draper is upregulated in VNC ensheathing glia and/or astrocytes post-injury, we repeated a VNC nerve injury in flies expressing membrane-tethered GFP in each subtype. Removal of a single wing resulted in an expansion of ensheathing glial membrane and striking upregulation of Draper around the AMN neuropil on the injured side (Fig. 2c). Notably, these signals overlapped substantially (merged, Fig. 2c). We did not detect any astrocyte membrane expansion, nor any overlap of Draper with astrocytes in the same injury paradigm (Fig. 2c). Our results suggest that VNC ensheathing glia, and not astrocytes, respond to degenerating axons in the VNC.

DRAPER IS REQUIRED FOR CLEARANCE OF SENSORY NEURON AXONS IN THE ADULT VNC

Draper is essential for clearance of degenerating ORN axons in the adult central brain (Doherty et al., 2009; Logan et al., 2012; Logan and Freeman, 2007; MacDonald et al., 2006). To determine if Draper is similarly required in the VNC, we labeled Gr22c gustatory neurons with membrane-tethered version of GFP (GR22c-Gal4 x UAS-mCD8::GFP). A single Gr22c neuronal cell body resides on each front leg and projects

through the PN neuropil. We unilaterally transected the right front leg adjacent to the thorax and imaged Gr22c axons in dissected VNCs after 48 hours. At this time point, no Gr22c GFP+ axonal material was visible in the right (injured) neuropil region, while the contralateral Gr22c projection was intact (Fig. 3a). Fragmentation of the severed Gr22c axons was blocked by co-expression of Wlds (UAS-Wlds) (Fig. 3a), indicating that these axons undergo a classic WD program post-severing. Notably, degenerating Gr22c axons were still detectable in *draper* mutant flies (Fig. 3a), indicating that Draper is required in multiple regions of the CNS for efficient clearance of cellular debris.

AP-1 AND STAT92E TRANSCRIPTIONAL ACTIVITY IS STIMULATED IN THE VNC AFTER INJURY

In the olfactory system, ORN axotomy stimulates the activity of two highly conserved transcription factors: 1) the Jra/kayak heterodimer, which is the *Drosophila* version of the AP-1 complex (MacDonald et al., 2013) and 2) STAT92E (Doherty et al., 2014). To first determine if AP-1-dependent transcription is triggered in the VNC after peripheral nerve injury, we used a well-characterized Jra/kayak *in vivo* transgenic reporter (TRE-eGFP) to monitor the activity of AP-1 in the VNC. TRE-eGFP contains 10 tandem AP-1 binding sites upstream of eGFP (Chatterjee and Bohmann, 2012). In uninjured VNC, we observed almost no eGFP signal, but after bilateral ablation of the front legs and wings, we saw robust upregulation of eGFP specifically in the PN and AMN neuropil and surrounding cortical regions (Fig. 3b). Next, we assessed STAT92E activity in the VNC by using the *in vivo* reporter 10XSTAT92E-dGFP, which contains 10 STAT92E binding sites upstream of destabilized GFP (dGFP) (Bach et al., 2007). dGFP

was undetectable in uninjured VNCs, but following ablation of front legs and wings, we observed a striking increase in dGFP expression in the corresponding neuropil regions and surrounding cortex (Fig. 3b). Together, these results indicate that AP-1 and STAT92E are activated in VNC glia after axotomy and also suggest that common transcriptional programs are stimulated throughout the adult nervous system in response to nerve injury.

DRAPER IS TRANSCRIPTIONALLY UPREGULATED IN THE VNC AFTER NERVE INJURY

Doherty et al. recently identified a 2619 bp region of the *draper* promoter that is specifically activated in ensheathing glia following ORN injury, referred to as draper enhancer element 7 (*dee7*) (Doherty et al., 2014). The dee7-Gal4 flies express Gal4 under the control of this fragment. The expression is quiescent in the uninjured adult brain but activated in olfactory ensheathing glia after ORN axotomy (Doherty et al., 2014). Notably, this promoter fragment contains several requisite STAT92E binding sites for injury-induced activation (Doherty et al., 2014). We tested the dee7-Gal4 driver line in our VNC peripheral nerve injury model by crossing this strain to UAS-mCD8::GFP flies. As reported in the central brain, we detected random expression of this reporter in astrocyte-like glia in uninjured VNCs (Doherty et al., 2014). However, one day after bilateral ablation of front legs and wings, we consistently observed robust activation throughout the anterior VNC (Fig. 3b), which suggests that similar promoter elements in the *draper* locus in targeted in VNC glia in response to nerve injury. Next, we performed a quantitative PCR (Q-PCR) time course against *draper-I* in VNC tissue 0, 1.5, and 5

hours after ablation of all legs, wings, and/or head. *draper-I* was significantly upregulated in the VNC at all time points in both injury paradigms (Fig. 3c). Collectively, our results indicate that peripheral nerve injury induces transcriptional upregulation of *draper* in the adult VNC and further support the notion that common transcriptional programs, which include *draper*, are induced in various regions of the adult nervous system.

TRANSCRIPTOME PROFILING OF ADULT VNCS AFTER INJURY

Once we determined that STAT92E/AP-1 activity, as well as *draper-I* transcription, are strongly enhanced in the VNC after nerve injury, we assessed genome-wide changes in the adult VNC post-injury by performing RNA-seq on dissected VNC tissue after severing legs, wings and heads of adult animals. The analysis was performed on 5 biological replicates for each time point (uninjured and five hours post-injury). We obtained on average 14.5 million ± 973,394, 100 bp reads per sample. On average, 85.2% ± 0.39% of the fragments mapped to the *Drosophila* genome, with an average of 41.2% ± 2.6% mapping to exons. To identify differentially expressed genes, we utilized the Empirical Analysis of Digital Expression in R (EdgeR) package in GeneSifter®. After correcting for multiple comparisons (Benjamini-Hochberg procedure), we identified 1,539 genes that were differentially expressed by 1.2-fold or greater (647 upregulated, 892 downregulated) (p-value ≤ 0.01).

VALIDATION OF DIFFERENTIALLY EXPRESSED GENES IN THE VNC

Next, we performed Q-PCR against a subset of genes predicted to be transcriptionally altered in the VNC post-nerve injury. We selected eight upregulated genes and found that all eight genes were significantly increased in the VNC 5 hours after severing legs, wings and heads when compared to uninjured samples. We also tested two putative downregulated genes, Notch, and CG6277. Downregulation of Notch, but not CG6277, was confirmed by Q-PCR. Correlation analysis for all 10 genes (RNA-seq versus Q-PCR) revealed a Pearson's coefficient of r=0.5227. Exclusion of lowly expressed genes (Ets21C, CG6277, PGRP-SA, Ninjurin A) in either the control or injured conditions produced a Pearson's coefficient of r=0.9935 ($p<0.0001$), which may reflect experimental challenges in accurately quantifying low abundance genes. Overall, our secondary Q-PCR analysis confirms the validity of our RNA-seq transcriptional comparison of control and injured VNC tissue.

RNA-SEQ ANALYSIS REVEALS FUNCTIONALLY DISCRETE GROUPS OF INJURY-INDUCED GENES

To identify signaling pathways activated (or inhibited) in the adult VNC in response to nerve injury, we performed KEGG pathway analysis, as well as a GO biological process term analysis. Figure 4c provides a graphical representation of one KEGG analysis of upregulated genes. Interestingly, this revealed enriched JNK/AP-1 signaling components, which supports published work that the JNK/AP-1 pathway is required for proper glial recruitment/phagocytic responses to axonal injury in the adult *Drosophila* olfactory system (MacDonald et al., 2013) (Fig. 4c). Several members of the

Spatzle/Toll pathway were also upregulated in our RNA-seq (Fig. 4c). The Toll pathway, including the downstream transcription factors Cactus and Dorsal, are implicated in innate immune responses (Valanne et al., 2011).

RNA-SEQ REVEALS NOVEL CANDIDATE TRANSCRIPTIONAL TARGETS IMPLICATED IN GLIAL MIGRATION

Widespread axon degeneration triggers striking changes in glial cell size, shape, and membrane dynamics (M. A. Anderson et al., 2014; Bardehle et al., 2013; S. Hong and Stevens, 2016; MacDonald et al., 2006; Napoli and H. Neumann, 2009). These responses ensure that glia quickly access degenerating nerves to clear cellular debris and locally release protective factors (Hines et al., 2009). We took a two-step approach to investigate how nerve injury altered expression of VNC genes implicated in cell migration, membrane remodeling, and related events. First, we used the DRSC Integrative Orthologue Prediction Tool (DIOPT) program (Hu et al., 2011) to identify the closest orthologues for all upregulated genes in other organisms. Next, for all upregulated genes that had clear mammalian orthologues, we utilized Annokey (D. J. Park et al., 2014) to cross reference these genes from *Drosophila*, mouse, and human against NCBI Entrez Gene databases and Pubmed articles linked to select search terms (see Table 1A). Table 1B displays the top 10 most prevalent hits for each upregulated gene from the RNA-seq screen and the highest ranked associated search term.

One gene identified by Annokey as a putative gene associated with "migration" or "invadopodia" across all three species (*Drosophila*, mouse, and human) was *matrix metalloproteinase-1* (MMP-1)/MMP-14. MMPs are proteases implicated in extracellular

matrix remodeling, cell migration, and metastasis of cancer cells (Nagase and Woessner, 1999; Rosenberg, 2002; Verma and Hansch, 2007; Vu, 2000). A detailed analysis of MMP function has been hindered by the fact that there are over 23 partially redundant MMP genes in mammals (Page-McCaw et al., 2007). In contrast, the Drosophila genome contains only 2 MMP genes: MMP-1, a secreted protease, and MMP-2, which is predicted to be GPI anchored (Page-McCaw et al., 2003). Therefore, the fly offers a powerful system to explore MMP-1 regulation and function in an *in vivo* context.

MMP-1 IS UPREGULATED IN THE ADULT NERVOUS SYSTEM FOLLOWING AXOTOMY

To assess MMP-1 protein levels and localization before and after injury, we used an MMP-1-specific antibody to visualize MMP in the adult VNC. First, we used flies that expressed membrane-tethered GFP (UAS-mCD8::GFP) specifically in ensheathing glia (TIFR-Gal4), performed a unilateral wing ablation, and then stained tissue with anti-MMP-1 one day after injury. We observed a robust increase in MMP-1 in the AMN (wing-innervated) neuropil region on the injured side, and a subset of MMP-1 signal clearly overlapped with ensheathing glial membranes (Fig. 5a, white arrows). We also detected a significant increase in MMP-1 by Western blot on dissected VNC tissue following bilateral ablation of legs and wings (Fig. 5b, c).

Next, we refocused our analysis on the adult olfactory system. The *Drosophila* olfactory system contains two sets of external structures, the antennae and maxillary palps, which house ORNs that project to the antennal lobes in the central brain (Fig. 5d). Removal of the antennae or maxillary palps severs the antennal or maxillary nerves,

respectively, and local ensheathing glia respond by infiltrating the antennal lobes and clearing degenerating ORN material (Doherty et al., 2009). One notable advantage of the olfactory system nerve injury assay is that it permits analysis of degenerating sensory axons projecting into the antennal lobes with no interference from injured efferent projections to the antennae or maxillary palps.

First, we performed a comparative Q-PCR analysis of a subset of upregulated genes identified in our RNA-seq screen. All three queried genes, which were significantly upregulated in the VNC after injury (*Hairy*, *MMP-1*, and *Ets21C*), were also upregulated in the central brain 3 hours after severing the antennal and maxillary nerves (Fig. 5e), further suggesting that common transcriptional cascades are stimulated in central brain and VNC glia after neural injury. Next, we assessed MMP-1 levels by immunostaining 24 hours after severing olfactory nerves. In uninjured brains, MMP-1 was detectable at low levels on the tracheal network (arrows in Fig. 5f). MMP-1 levels were dramatically increased in the antennal lobe regions after bilateral antennae removal, which transects ~80% of ORN axons projecting into the antennal lobes (Fig. 5f, antennae injury). We also observed a striking increase in MMP-1 specifically on maxillary palp glomeruli 24 hours after bilateral maxillary nerve axotomy (arrowheads in Fig. 5f). MMP-1 contains a catalytic domain and hemopexin domain for which there are separate antibodies. To better understand which domains might be involved in glial response to injury, we performed bilateral antennae injury and probed for the different domains. We observed that both the catalytic and hemopexin domains are highly expressed in response to injury, although the hemopexin domain appears more lowly expressed in the trachea of uninjured animals (Fig. 5g).

MMP-1 UPREGULATION AFTER AXON INJURY REQUIRES DRAPER/STAT92E/AP-1 ACTIVITY

Since ORN axotomy initiates a positive autoregulatory feedback loop by which activated Draper triggers transcriptional upregulation of the *draper-I* locus (Doherty et al., 2014), we wondered if Draper might also be required for MMP-1 upregulation after nerve injury. Indeed, we found that the local increase of MMP-1 in the VNC after unilateral ablation of a single leg typically observed in control flies (white arrowheads, Fig. 6a) is undetectable in *draper* null mutants (Fig. 6a). Similarly, in the olfactory system, MMP-1 upregulation was completely blocked in *draper*$^{-/-}$ flies (Fig. 6b), indicating that Draper is essential for the glial production of MMP-1 in a variety of contexts post-injury.

Following axon injury, Draper stimulates STAT92E, as well as the JNK/AP-1 signaling pathway, to ensure that *draper-I* is sufficiently upregulated in responding glia (Doherty et al., 2014; MacDonald et al., 2013). To determine if the transcription factors STAT92E or AP-1 are required to upregulate MMP-1 after axotomy, we performed *in vivo* knockdown experiments and assessed MMP-1 levels post-injury. Specifically, we used the pan glial driver Repo-Gal4 to express RNAi against STAT92E (UAS-STAT92ERNAi) or each subunit of the AP-1 heterodimer, kayak and Jra (UAS-kayRNAi or UAS-JraRNAi). In addition, these flies carried a *tubulin-Gal80*^{ts} transgene, which allowed us to temporally control the activity of GAL4 and, thus, specifically express each RNAi construct in post-mitotic adult glia. Interestingly, we found that glial depletion of STAT92E, kayak, or Jra resulted in significant inhibition of MMP-1 induction 24 hours after maxillary nerve axotomy (Fig. 6c-f). Collectively, these results suggest that axon injury stimulates the Draper receptor to activate MMP-1 gene expression in glia, in a STAT92E/AP-1 dependent manner. In addition, aside from the

positive transcriptional feedback loop that has been described for *draper* (Doherty et al., 2014), *MMP-1* now represents the first injury-responsive gene downstream of the Draper/MEGF10 receptor, which suggests that a broader Draper/STAT92E/AP-1 transcriptional program is activated in adult glia in response to axon injury.

MMP-1 EXPRESSION AFTER INJURY IS ATTENUATED IN AGED FLIES

We recently showed that in the aging brain there are decreased basal levels of Draper and that after injury, there is reduced activation of STAT92E (Purice et al., 2016). Since MMP-1 expression after injury requires Draper and STAT92E activity, we wondered whether aging affects injury induced MMP-1 expression after injury. We injured young and aged flies and found that MMP-1 induction after antennal nerve injury is significantly reduced one day after injury, in contrast to young animals. These results suggest that upregulation of MMP-1 in the aged brain is dependent on Draper and STAT92E activity.

DRAPER ACTIVATES MMP-1 PRODUCTION IN ENSHEATHING GLIA AFTER NERVE INJURY

In the adult *Drosophila* CNS, neuropil regions contain two major subtypes of glia: ensheathing glia and astrocytes (Freeman, 2015; Freeman and Doherty, 2006; Logan and Freeman, 2007). Ensheathing glia respond to axotomy by upregulating *draper*, invading injured neuropil areas and clearing degenerating neuronal debris (Doherty et al., 2009). After antennal or maxillary nerve injury, we observe a dramatic increase in MMP-1

staining in a pattern that is strikingly similar to the ensheathing glial membrane expansion (Fig 2c). However, since MMP-1 is a secreted molecule, localization of the protein may not reflect the cell in which it's produced in response to axon injury. We took advantage of a previously characterized *in vivo* transcriptional reporter that expresses cytosolic beta-galactosidase (β-gal) under the control of a 4.7 kb fragment of the MMP-1 promoter (MMP-1-LacZ) (Uhlirova and Bohmann, 2006). We analyzed activation of this reporter in flies that also carried the STAT92E or AP-1 transcriptional reporters, 10XSTAT92E-dGFP or TRE-GFP, which are both selectively activated in olfactory ensheathing glia after antennal nerve injury. We found that in MMP-1-LacZ flies, β-gal was almost undetectable in uninjured brains, but levels were dramatically upregulated around the antennal lobes 24 hours after ORN axotomy (Fig. 8a, b). Notably, we detected a striking overlap between increased ensheathing glial GFP expression in the 10XSTAT92E-dGFP and TRE-GFP flies and β-gal after injury (Fig. 8a, b). Thus, we hypothesized that degenerating axons trigger MMP-1 upregulation specifically in ensheathing glia in a Draper-dependent manner. To test this idea, we used the ensheathing glial driver TIFR-Gal4 to knock down Draper by RNAi (UAS-Draper^{RNAi}). Strikingly, MMP-1 production was completely blocked one day after antennal nerve injury after depleting ensheathing glia of Draper (Fig. 8c, d).

MMP-1 IS ESSENTIAL FOR PROPER CLEARANCE OF SEVERED DEBRIS:

Ensheathing glia wrap individual axons and nerve bundles. After injury, glia must infiltrate the antennal neuropil and extend membrane projections to injured axons to efficiently clear degenerating axonal material (Doherty et al., 2009; Logan and Freeman,

2007; MacDonald et al., 2006). Thus, we wondered if glial clearance of severed axons would be inhibited by loss of MMP-1. We performed glial specific knockdown of MMP-1 in adult flies, using $Gal80^{ts}$ to temporally control activation of UAS-MMP-1^{RNAi}. These flies also carried an OR85e-mCD8::GFP transgene, which labels a subset of maxillary olfactory receptor neurons (OR85e) with membrane-tethered GFP. We performed bilateral maxillary nerve axotomy and quantified GFP^+ axonal debris 1 and 3 days after injury. Significantly more OR85e axonal material lingered in the brain of MMP-1^{RNAi} flies (Fig. 9a, b) but only 3 days after injury (Fig. 9a, b). We also confirmed efficient knockdown of MMP-1 by immunostaining (Fig. 9a arrows, c). To complement our RNAi analysis, we used an independent method to inhibit MMP-1 activity. Tissue inhibitor of metalloproteinase (TIMP) is an endogenous inhibitor of MMP-1 by binding to the catalytic domain of MMP-1 and inhibiting its function (Brew and Nagase, 2010; Page-McCaw et al., 2003). We expressed UAS-TIMP in adult glial cells, quantified OR85e GFP^+ axons 1 and 3 days after nerve injury, and, importantly, found that TIMP overexpression also delayed clearance of degenerating axons, but only 3 days after injury (Fig. 9d, e). Finally, we wondered whether the delay in clearance of degenerating debris persists past 3 days after injury, so we monitored clearance of synapses in control and MMP-1^{RNAi} flies 6 days after bilateral antennal injury. We observed that significantly more NC82-labeled presynaptic debris persists when MMP-1 function is disrupted in adult glial cells (Fig. 9f, g). These results suggest that in the absence of MMP-1, glia are able to initially perform their phagocytic function, most likely because they are already in contact with the degenerating ORNs. However, several days after injury, when MMP-1 function is blocked, glia are unable to invade the neuropil region, and are therefore inefficient in clearing the remaining degenerating axonal and synaptic debris.

MMP-1 IS REQUIRED FOR DYNAMIC GLIAL RESPONSES TO AXOTOMY

Our findings reveal MMP-1 a novel Draper transcriptional target required to promote glial membrane remodeling and phagocytic responses to nerve injury. Next, we wanted to monitor glial membrane expansion in the absence of MMP-1. We used flies that expressed glial membrane-tethered GFP (Repo-Gal4, UAS-mCD8::GFP) to quantify recruitment of glial membranes to injured ORN axons 1 and 3 days after antennal nerve injury. We also monitored MMP-1 expression and interestingly, we observed that glial membrane expansion after injury follows MMP-1 expression. More specifically, 1 day after injury, we observe the largest MMP-1 response and glial membrane expansion around the antennal lobe border (Fig. 10a, b). Three days after injury, MMP-1 expression is localized inside the antennal lobe, in the neuropil region, where we also observe glial membrane loops (Fig. 10a, b and Fig. 11a). We found that accumulation of glial membranes was significantly reduced in MMP-1 RNAi flies at both time points (Fig. 10c and Fig. 11a). Previous studies of tumor cell invasion demonstrate that oncogene Src activates JNK/AP-1 and MMP-1 expression to drive actin remodeling during cell migration (Rudrapatna et al., 2013). It has already been established that Src family kinases regulate Draper activity during phagocytosis (Ziegenfuss et al., 2008). We thus wondered whether loss of glial MMP-1 after injury leads to inhibition of dynamic cytoskeletal changes that are usually observed in tumor invasive process. To monitor actin polymerization, we stained the brains with phalloidin:TRITC, a fluorescent derivative of the toxin of *Amanita phalloides,* that selectively binds to filamentous actin (Wulf et al., 1979). In control animals, we observe that 1 and 3 days after antennal injury, the phalloidin pattern follows the MMP-1/glial membrane expansion pattern – strongly localized to the antennal lobe border 1 day after injury and moving to the inside of the

antennal lobes 3 days after injury (Fig. 10d). Interestingly, flies in which MMP-1 RNAi was expressed specifically in ensheathing glia (TIFR-Gal4), there is a strong reduction in the phalloidin 3 days after injury (Fig. 10d). The phalloidin pattern around the antennal lobe border at day 1 appears comparable to control animals. Together these results demonstrate that ensheathing glial membrane expansion and actin dynamics are greatly attenuated in the absence of MMP-1.

MATERIALS AND METHODS

PERIPHERAL NERVOUS SYSTEM (PNS) AXOTOMY ASSAY

Peripheral nerve injury was induced in adult *Drosophila* by removing legs, wings, and/or head with Vannas scissors (World Precision Instruments # 500260-G) while flies were anesthetized with CO_2. Legs were severed at the midpoint of the femur, and wings were severed at longitudinal vein 6. Injured flies were placed dorsal side down on 1% agarose pads in a covered petri dish. In experiments where the head was also removed, the head was removed prior to the legs and wings. We found it was critical to include a wet Kim wipe in the dish to prevent desiccation of the flies. Control flies were also placed in 1% agarose vials and both control and experimental (injured) animals were kept at room temperature for 5 hours. VNCs were dissected in Jan's Saline (0.5 mM Ca^{2+}) and immediately frozen on dry ice. Injured animals that did not move the remaining proximal portion of the leg in response to gentle forcep manipulation were discarded. For immunohistochemical experiments where only single peripheral organs were removed, the flies were placed back in food vials after injury.

OLFACTORY RECEPTOR NEURON (ORN) AXOTOMY ASSAY

ORN axotomy was induced in adult *Drosophila* by surgical ablation of the third antennal segments or maxillary palp structures. Flies were maintained at 22-23°C. For adult specific knock down or overexpression of genes, flies expressing a temperature-sensitive version of Gal80 (*tubulin-Gal80^{ts}*) were shifted to 30°C for one week to induce glial expression of each gene of interest and returned to 30°C after ablating maxillary palps until dissection. Control flies for these experiments were treated with the same temperature shift protocol.

IMMUNOLABELING

Adult *Drosophila* whole flies or heads were fixed (1xPBS, 0.1% Triton X-100, 4% PFA) at room temperature for 16 minutes. Samples were then washed 1 X 1 minute and 2 x 5 minutes while rocking in PBSTx.1 (1xPBS, 0.1% Triton X-100) at room temperature. Fixed samples were maintained on ice while VNCs or brains were dissected at room temperature in PBSTx.1. Tissue was post-fixed for 16 minutes in PBSTx.1, washed 2 x 2 minutes, and incubated overnight with primary antibodies in PBSTx.1. The next day, samples were washed 4 x 30 minutes with PBSTx.1 and incubated with secondary antibodies (in PBSTx.1) for 2 hours at room temperature. Samples were then washed 4 x 30 minutes with PBSTx.1 and mounted on slides in VECTASHIELD mounting media (Vector Labs).

CONFOCAL MICROSCOPY AND ANALYSIS

All samples were imaged on a Zeiss LSM 700 with a Zeiss 40X 1.4NA oil immersion plan-apochromatic lens. VNCs and brains within a single experiment (i.e. those being directly compared for quantification) were whole mounted under a single #1.5 cover glass in VECTASHIELD. All samples in a given experiment were imaged on the same day with the same confocal microscope settings. Volocity 3D Image Analysis Software (Perkin Elmer) was used for fluorescence quantification and GraphPad Prism was used for statistical analysis. Quantification of OR85e GFP-labeled glomeruli was performed on 3D volumes segmented to GFP signal in Volocity. Total intensity measurements were calculated and background fluorescence was subtracted. To quantify MMP-1 and NC82 levels in adult brains after injury, total intensity measurements were calculated in regions of interest made around the entire antennal lobe. Glial membrane expansion one day after antennal nerve axotomy was quantified by measuring the thickness of GFP^+ ensheathing glial membranes at several locations around each antennal lobe on single confocal slices at a consistent anterior depth of 6 µm into the brain.

ANTIBODIES

Primary antibodies were used at the following dilutions: chicken anti-GFP (#A10262 from ThermoFisher) at 1:1000; mouse anti-Draper (hybridoma supernatants 8A1 and 5D14) at 1:400, guinea pig anti-Draper (gift from E. Kurant) at 1:10000, mouse anti-MMP-1 (Developmental Studies Hybridoma Bank 14A3D2, 3A6B4, 3B8D12, 5H7B11) at 1:50 used at 1:1:1:1 ratio, Phalloidin-TRITC (Sigma #P1951) at 1:250. All

secondary antibodies (#s: 715-295-150, 703-545-155 and 706-605-148 from Jackson Immunoresearch) were used at a dilution of 1:400.

WESTERN BLOT ANALYSIS

VNCs were dissected in Schneider's *Drosophila* Medium (ThermoFisher, catalog #21720001) and homogenized in 3µL 1x Loading Buffer per VNC. Protein lysate of 5-6 VNCs was loaded onto 4-20% Tris-Glycine gels (Lonza, catalog #59517) and transferred to Immobilon-FL (Millipore, catalog # IPFL00010). After transfer, total protein density per lane was measured using MemCode™ Reversible Protein Stain (ThermoFisher, catalog # 24585). Blots were probed with mouse anti-MMP-1 (1:100 at a 1:1:1:1 ratio) and incubated overnight at 4°C, washed several times with 1xPBS/0.01% Tween 20, and probed with appropriate fluorophore-conjugated antibodies secondary antibody at 1:2000 (#715-625-150 from Jackson Immunoresearch) for 2 hours at room temperature. Additional washes were performed with 1xPBS/0.01% Tween 20 and a final wash in 1xPBS. Total protein stain blots were imaged on G:BOX F3 Imaging System and analyzed with ImageJ; fluorescent blots were imaged on Li-cor Odyssey CLx quantitative western blot imaging system and data was quantified using LiCor Image Studio software. Images in Fig. 5 has been cropped for presentation. Full-size image is presented in Fig. 12.

SAMPLE PREPARATION FOR RNA-SEQ

For each biological replicate 60-80 *w*^{1118} flies (3-5 days old) with equal numbers of males and females were injured, and 50 VNCs were used per biological replicate for RNA extraction. For injured samples, all six legs, both wings, and the head were severed with Vannas scissors while flies were anesthetized with CO_2 as described above. Frozen tissue was crushed in 500ul of Trizol with glass beads and a pestle and then stored at -80 degrees until all samples were ready for RNA extraction. A total of 5 biological replicates were collected for each condition (injured and uninjured). RNA extraction was performed by first spinning crushed material at 11,000xg for 10 minutes at 4°C to pellet cuticle and lipids. Trizol supernatant was transferred to a fresh tube, 1/5 volume of chloroform was added to each sample and mixed in a 5' Prime- Heavy Phase Lock Gel, and samples were then centrifuged (12,000xg for 15 minutes). The aqueous phase was removed and RNA was isolated on RNA Clean & Concentrator™-5 columns (catalog # R1016). DNAse digestion using Ambion DNA-free kit (catalog # AM1906) was performed and RNA was quantified using the Qubit fluorometer.

RNA-SEQ SCREEN

Total RNA samples were sent to the Massively Parallel Sequencing Shared Resource (MPSSR) at Oregon Health and Science University (OHSU) for library preparation. Briefly, total RNA concentration and sample integrity were assessed using an Agilent RNA 6000 Pico chip on an Agilent Technologies 2100 Bioanalyzer instrument. Following quantification and quality control, 325ng of total RNA was subjected to ribosomal RNA reduction via Epicenter's Ribo-Zero™ Gold kit (Human/Mouse/Rat).

The output was then used with Illumina's TruSeq® RNA Sample Preparation Kit v2, beginning at the RNA fragmentation step. Poly (A) selection was not performed due to limited starting material. Barcode indexing adapters were ligated and all 10 samples (5 control and 5 injured) were sequenced (100 bp single reads) on a single flow cell lane on the Illumina HiSeq 2500 Sequencer. Across samples, an average of 85.2% of the fragments mapped to the *Drosophila* genome; 41.2% of these mapped to exons and 9% to introns. Notably, 22% of reads corresponded to ribosomal RNA (rRNA) and small nuclear RNA (snRNA). Enhanced ribosomal RNA mapping and reduced exon mapping may have been influenced by the fact that our ribosomal RNA depletion kit was not specific to *Drosophila*.

RNA-SEQ INFORMATICS ANALYSIS

RNA-seq analysis was performed with PerkinElmer® Geospiza GeneSifter® Analysis Edition (GSAE). Briefly, HiSeq 2500 reads were uploaded to Geospiza's server for mapping of the reads to the *Drosophila melanogaster* genome (Release 5.57). Normalization of gene expression across biological replicates and conditions was accomplished by normalizing to mapped reads. Statistical analysis was performed in GeneSifter® via the Empirical Analysis of Digital Gene Expression Data in R (EdgeR) package, which is available at http://bioconductor.org (Robinson et al., 2009). False discovery rate was corrected via the Benjamini-Hochberg procedure (Benjamin and Hochberg, 1995). A p-value of 0.01 or lower was set as the cut-off for considering differential gene expression significant.

For significantly up or downregulated genes, GO term analysis, KEGG, and PANTHER pathway analyses were accomplished with DAVID (Database for Annotation, Visualization, and Integrated Discovery). An additional KEGG analysis was conducted with GeneSifter®. Genes that were significantly changed in the injured condition were also converted to their closest orthologues using DIOPT (DRSC Integrative Orthologue Prediction Tool) (Hu et al., 2011) and provided in. Finally, Annokey (D. J. Park et al., 2014) analysis was performed on upregulated genes to identify factors previously implicated in cell movement, migration, and invasion (Table 1A,B).

QUANTITATIVE REVERSE TRANSCRIPTASE–PCR ANALYSIS

Total RNA was extracted and quantified as described for the RNA-seq screen. For cDNA synthesis, 60 ng of DNAse-treated total RNA was reverse transcribed using qScript™ cDNA SuperMix (Quantabio, catalog # 95048-100). The resulting cDNA was diluted 1:5 and 5ul were used for a single RT-PCR reaction. All real-time assays were performed using TaqMan® gene expression assays (Thermofisher) and PerfeCTa® FastMix® II Rox (Quantabio, catalog # 95119-250) on a StepOne™ Real-Time PCR system (Thermofisher). Ribosomal Protein L28 (Rpl28), TaqMan® assay Dm01804541_g1 was used as a control housekeeping gene. Raw Ct values of Rpl28 were unchanged between the uninjured (28.31, SEM.3037, n=3) and 5hr post-injury (28.34, SEM.2030, n=3) samples. Our RNA-seq results confirmed that Rpl28 is an appropriate housekeeping gene (Digital gene expression levels of Rpl28 (n=5): No Injury – 68.784 (SEM=2.787) and Injured – 66.957 (SEM=3.494)).

Additional TaqMan® gene expression assays were utilized: (Ets21c-Dm01814139_m1; PGRP-SA-Dm01837990_g1; MMP-1-Dm01820359_m1; Relish-Dm02134843_g1; Ninjurin A-Dm01798347_g1; Hairy- Dm01822363_m1; Rac2-Dm01840631_s1; Cactus-Dm01807760_m1; Notch-Dm01841974_g1; CG6277 – Dm02369365_s1).

DROSOPHILA STOCKS

For all experiments, flies were between 3-10 days old. The following Drosophila genetic insertions were used: OR85e-mCD8::GFP/CyO (gift from B. Dickson), UAS-mCD8::GFP (Bloomington Stock 5137), UAS-mCD8::GFP (Bloomington Stock 5130), Repo-Gal4 (MacDonald et al., 2006), tubulin-Gal80^{ts} (Bloomington Stock 7108), 10XSTAT92E-dGFP (Bach et al., 2007), TRE-GFP (Chatterjee and Bohmann, 2012), Gr22c-Gal4 (Bloomington 57605), TIFR-Gal4 (Yao et al., 2007), alarm-Galf (Doherty et al., 2009), Dee7-Gal4 (Doherty et al., 2014), Draper$^{\Delta 5}$rec9, UAS-MMP-1RNAi (Uhlirova and Bohmann, 2006), UAS-MMP2RNAi (Chatterjee and Bohmann, 2012), UAS-TIMP (Bloomington Stock 58708), w1118 (Bloomington Stock 5905), UAS-DraperRNAi (MacDonald et al., 2006), MMP-1-LacZ (Chatterjee and Bohmann, 2012), UAS-STAT92ERNAi (Vienna Drosophila Resource Center 43866), UAS-kayakRNAi (Bloomington 31391), UAS-JraRNAi (Bloomington 31595).

Chapter 3: DISCUSSION

In this study, we have shown that the VNC can be used as an *in vivo* model system to study robust glial immune activity in response to axon injury. Our studies demonstrate that in response to neurodegeneration, *Drosophila* ensheathing glia upregulate and release MMP-1 in a Draper-dependent manner to promote glial membrane expansion. We provide *in vivo* evidence that fly glia require the secreted extracellular protease MMP-1 to extend membranes to sites of injury for proper clearance of neurodegenerative material. These findings are important because MMPs have been recognized to be significant contributors to reactive gliosis. For example, reactive astrocytes produce MMP9 at injury sites (Candelario-Jalil et al., 2009), which suggests that it is essential for astrocyte migration and glial scar formation. Because mammalian MMPs exhibit genetic redundancy and functional compensation, the role of MMP9 and other MMPs needs to be better investigated in order to better understand how glial cells use MMPs to remodel the ECM during reactive gliosis, especially now that it has been shown that preventing astrocytic scar formation significantly diminishes stimulated axon regrowth after spinal cord injury (M. A. Anderson et al., 2016).

Here we show that MMP-1 activity requires transcription factors AP-1 and STAT92E. We found that glial knock down of STAT92E or the AP-1 heterodimer (kay or Jra) resulted in significant inhibition of MMP-1 induction after neuronal injury. Using an *in vivo* transcriptional reporter line that expresses cytosolic β-gal under the MMP-1 promoter, we demonstrate that MMP-1 activity, along with reporters for AP-1 and STAT92E, is upregulated specifically in ensheathing glia. Furthermore, when ensheathing glial cells are depleted of Draper, MMP-1 upregulation in response to injury

is blocked. These results demonstrate that MMP-1 is activated cell autonomously in ensheathing glia in a Draper/STAT92E/AP-1 dependent manner.

The ensheathing glial cell response to injury parallels that of tumorigenic cells. For example, *Drosophila* epithelial tumor cells become invasive only upon JNK/AP-1 pathway activation, which promotes MMP expression, basement membrane degradation, and cell invasion (Brumby and H. E. Richardson, 2003; Pagliarini and Xu, 2003). As there is no basement membrane in the adult fly antennal lobes, future studies are needed to define the extracellular matrix proteins that are being degraded by MMP-1 in order to promote membrane expansion.

Although past MMP research has focused on their roles during injury (Candelario-Jalil et al., 2009), disease, or cancer progression (Gialeli et al., 2010; Rosenberg, 2002), MMPs have also been shown to play critical roles in structural and functional remodeling at the synaptic cleft in the healthy brain (Fujioka et al., 2012). For example, levels of MMP9 are significantly increased in the CA1 region of the hippocampus after induction of late-phase LTP (L-LTP) at CA1 (Huntley, 2012). Blocking MMP9 function via inhibitors or genetic deletion blocks not only L-LTP but also the typical spine enlargement that is observed during persistent LTP (Huntley, 2012). The role of MMPs during normal synaptic function and remodeling has interesting implications for changes in synaptic architecture after glia respond to an injury event. Too little or too high of levels of MMP activity/expression could be coupled to maladaptive wiring after injury and in disease states. Because of excellent electrophysiological and optogenetic tools to study circuit function *in vivo* (Clowney et al., 2015; E. J. Hong and Wilson, 2015), the *Drosophila* olfactory system offers a platform to further research the role of MMPs on

synaptic function in the healthy or diseased/aged brain, during learning and memory and after injury.

To date, the transcriptome of glial cells responding to injury in *Drosophila* has never been explored, which in part may have been due to limitations in isolation procedures (i.e. fluorescence-activated cell sorting). Here, we performed transcriptional profiling of the entire VNC tissue after peripheral nerve injury, which likely included genes differentially expressed in both neurons and glia post-axotomy. Future comparative analysis of upregulated genes in our study and previously published mammalian transcriptome profiling of specific glial subtypes post-injury will help reveal glial-specific changes in transcription. Notably, after spinal cord or ischemic injury in mammals, CNS axons do not regenerate unless intrinsic neuronal growth programs are turned on through neuron preconditioning (S. Neumann et al., 2002; S. Neumann and Woolf, 1999; Qiu, 2005). Thus, because injured motor neurons are included in our VNC injury assay, our RNA-seq transcriptome data may also provide novel insight into regeneration preconditioning conditions.

In summary, our work reveals a novel non-lethal injury paradigm that can be used to study glial immune changes in response to WD. Our work also highlights that our RNA-seq analysis can be used to uncover genes like MMP-1 that are transcriptionally required for proper and efficient glial function.

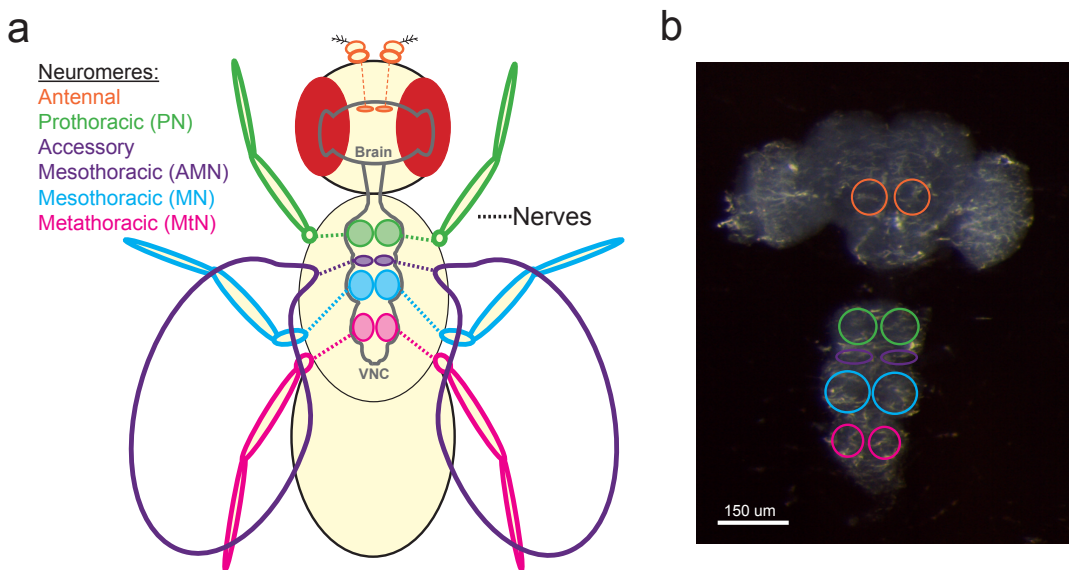

Figure 1. Drosophila VNC as a new acute axotomy assay to monitor glial immune activity. (**a**) Schematic representation of Drosophila brain and VNC depicting synaptic regions (neuromeres) in which glia respond to injury after acute axotomy of the corresponding peripheral organs. (**b**) DIC image of an adult brain and VNC with the corresponding neuropil regions from (a) highlighted.

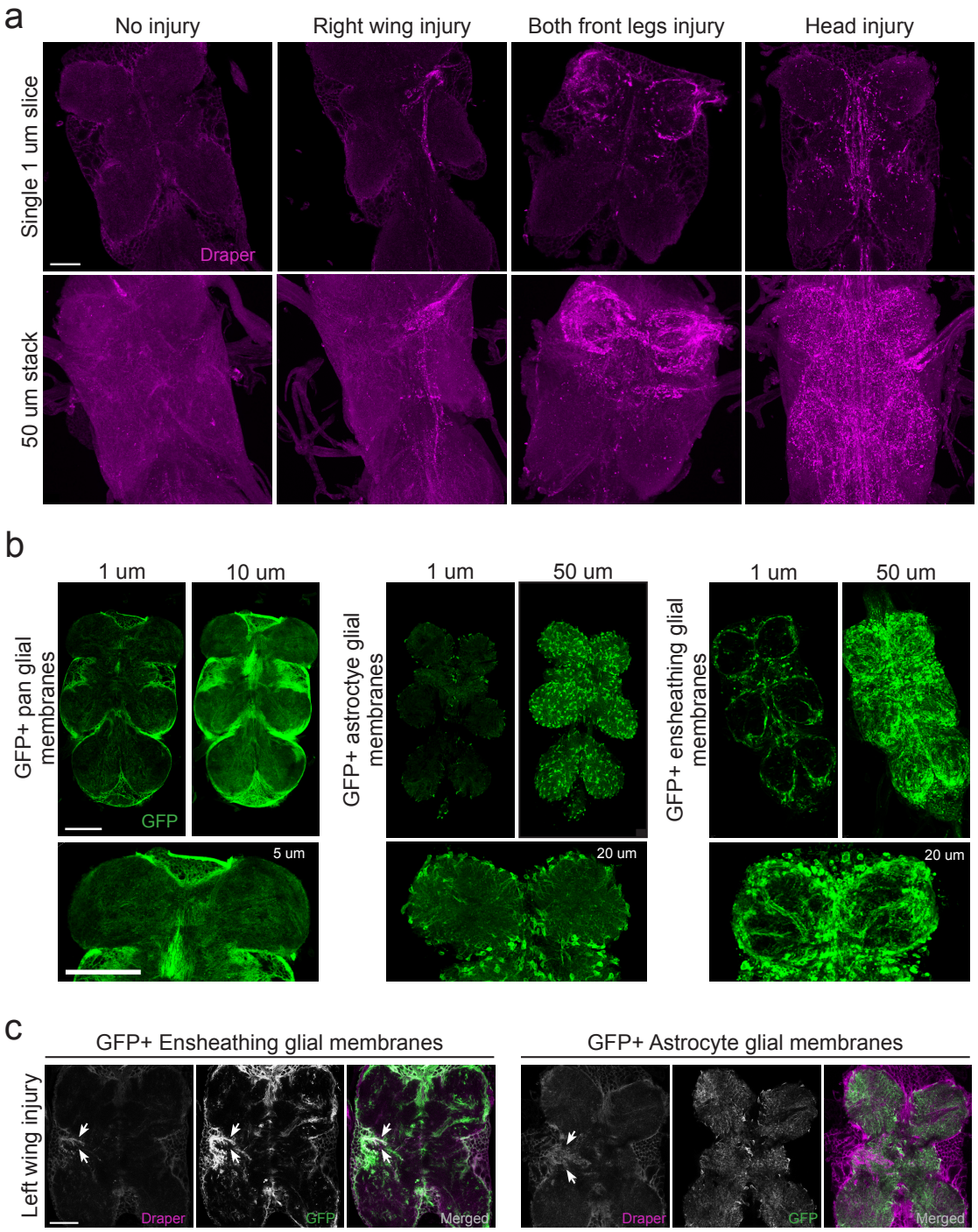

Figure 2 (continued below).

Figure 2. Draper and ensheathing glial membranes are acutely recruited in the VNC in response to peripheral nerve injury. (**a**) Representative Draper immunostainings of uninjured VNC and one day after right wing injury, both front legs injury, and head removal. Images depict the same VNC but single one-micron slice is shown on top and 50 um maximum intensity projections is displayed on the bottom. (**b**) Glial membranes were labeled *in vivo* with membrane-tethered GFP. Depicted left to right are glial membrane morphology in the VNC using a pan glial driver, astrocyte driver and ensheathing glia driver to drive expression of UAS-mCD8::GFP. Single micron slice depicts expression ~15 um into the VNC. Z-stack projection of either 10 (for pan-glial membranes) or 50 um for astrocytes and ensheathing glia. The bottom image depicts 5 or 20 um stack of the zoomed in prothoracic neuromeres. Scale bars = 30 um. (**c**) Ensheathing glial membrane expansion in the AMN co-localizes with Draper upregulation after single left wing injury. In flies where astrocyte membranes are labeled with GFP, there is Draper upregulation in response to the single left wing injury but no astrocyte membrane expansion is observed. One-micron slices shown. Genotypes: Fig. 2a: w^{1118}. Fig. 2b,c pan glial: Repo-Gal4, UAS-mCD8::GFP/TM3; astrocytes: UAS-mCD8::GFP/Cyo; alarm-Gal4/TM3; ensheathing glia: UAS-mCD4::GFP/Cyo; TIFR-Gal4/TM3.

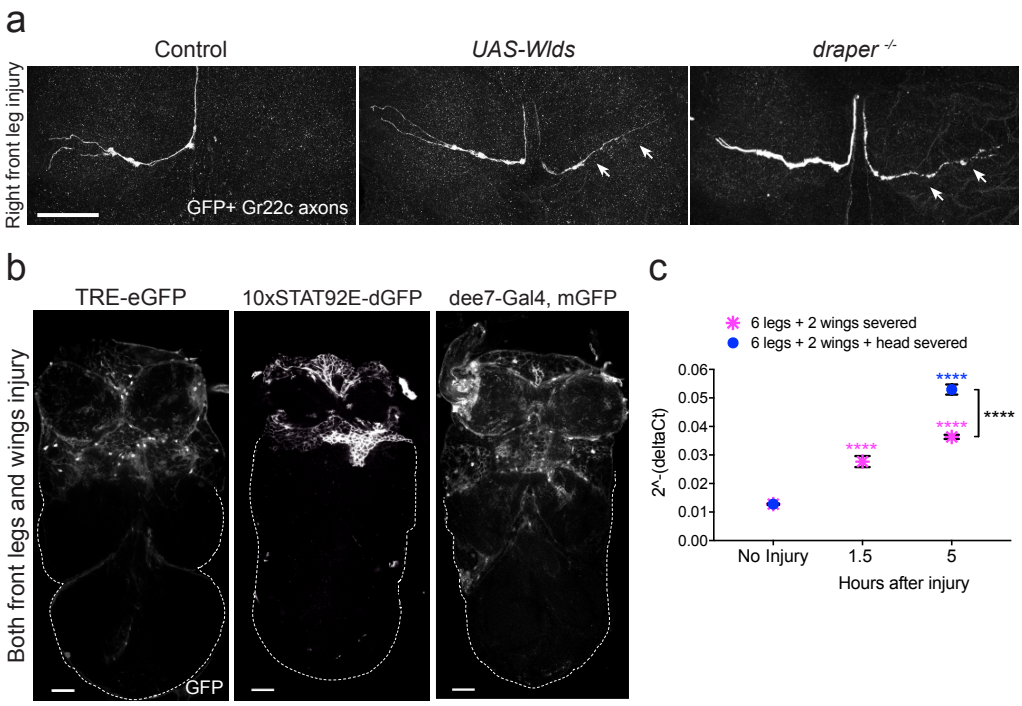

Figure 3. Draper is required for clearance of sensory neuron axons in the adult VNC. (**a**) GFP-labeled Gr22c gustatory axons in the prothoracic neuromeres of control flies, flies expressing Wlds, and *draper* mutant flies. Clearance was monitored two days after the front left right was axotomized. Gr22c axons are quickly phagocytosed in control animals, but not when Wlds was expressed or when flies are depleted of Draper. Arrows depict axons that are not phagocytosed. (**b**) Representative 1 micron confocal images of adult VNCs from flies that carry the AP-1 reporter TRE-eGFP, 10XSTAT92E-dGFP, and the Draper reporter dee7-GFP. Robust activation of TRE-eGFP only in the neuromeres affected by peripheral injury; Stat92E-dependent activation of dGFP (destabilized GFP) in the PN and AMN region after both front legs and both wings were injured; upregulation of GFP in the PN and AMN region after both front legs and both wings were injured in flies expressing membrane-tethered GFP under the Draper Gal4 promoter dee7; For each genetic line, the remaining intact neuromeres (dotted line) serve as the uninjured controls. (**c)** Quantitative real-time PCR analysis of normalized expression levels of draper-I transcript in VNCs that have had all legs and wings severed (magenta stars) and VNCs from flies that have had all legs, wings and head severed (blue circles). Draper threshold cycle (Ct) values were normalized to ribosomal protein L32 and results are presented as 2^-(deltaCt). Biological replicates: 6 legs + 2 wings: No Injury N=8; 1.5 hours N=3; 5 hours N=3. 6 legs + 2 wings + head: No Injury N=8; 5 hours N=7. Mean ± SEM plotted; ****P<0.0001; Two-way ANOVA with Sidak post hoc test. Each injury group was compared to uninjured in the same group. Black asterisks depict comparison between the two injury groups at the 5-hour time point. Scale bars = 30 um. Genotypes: Fig 3a: Control: Gr22c-Gal4/+; UAS-mCD8::GFP/+; UAS-Wlds: UAS-Wlds/+; Gr22c-Gal4/+; UAS-mCD8::GFP/+; draper$^{-/-}$: Gr22c-Gal4/UAS-mCD8::GFP/; draper$^{\Delta 5}$rec9/ draper$^{\Delta 5}$rec9. Fig. 3b: TRE-eGFP: TRE-eGFP/TRE-eGFP (on II); 10xSTAT92E-dGFP: 10xSTAT92E-dGFP/10xSTAT92E-dGFP (on II); dee7-Gal4, mGFP: dee7-Gal4, UAS-mCD8::GFP/Cyo. Fig 3c: w^{1118}.

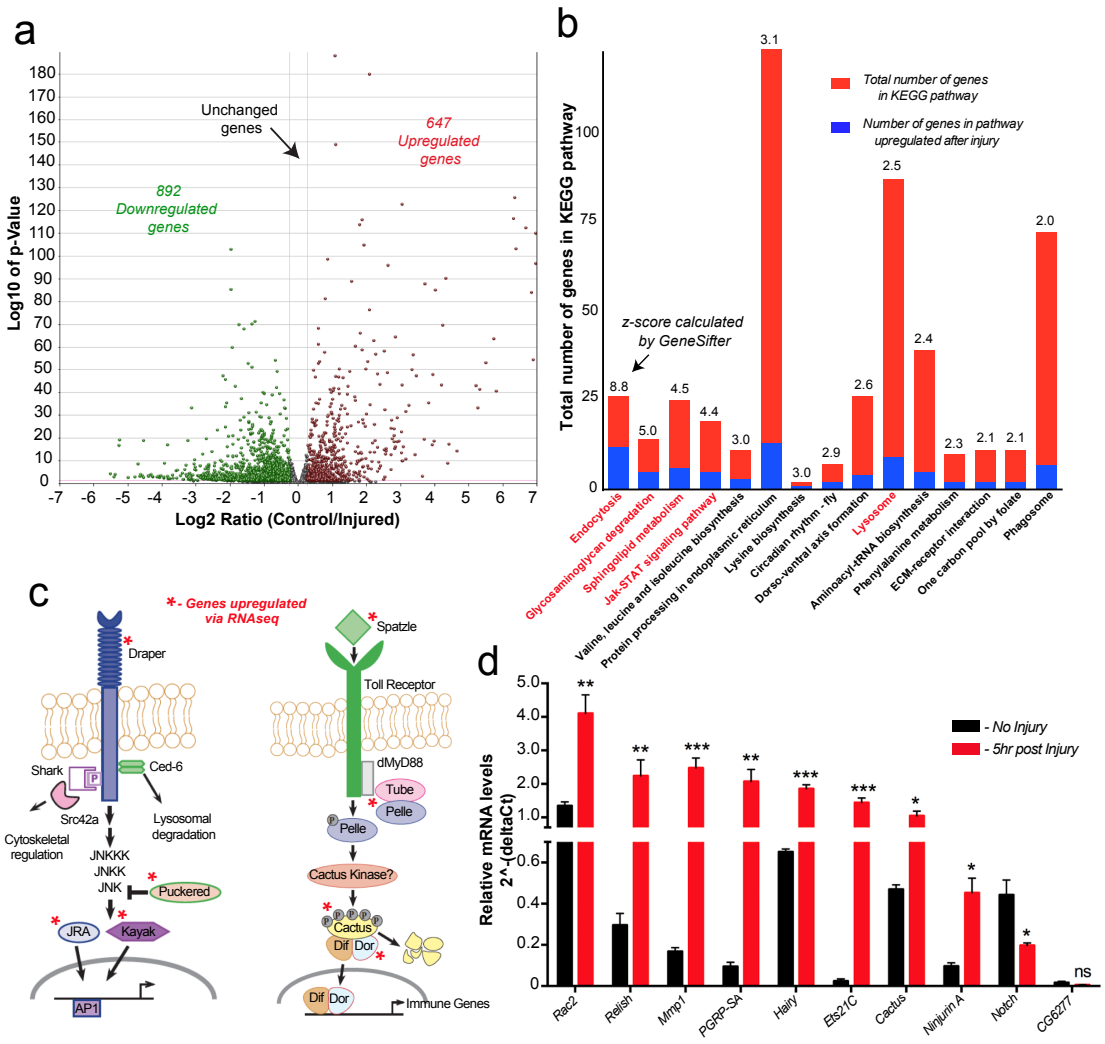

Figure 4. RNAseq analysis reveals functionally discrete groups of injury-induced genes. (**a**) Log scale volcano plot analysis shows that after injury, most genes are differentially changed - 892 significantly downregulated genes and 647 significantly upregulated genes. (**b**) KEGG pathway analysis of upregulated genes shows several pathways in the screen. Red shows the total number of genes in the KEGG pathway and blue depicts the number of genes in the pathway that were upregulated after injury. (**c**) Transcriptome analysis reveals several members of the highly conserved Draper/AP-1 and Toll pathways are upregulated in response to injury. Red asterisks represent the genes significantly upregulated in the screen. (**d**) Q-PCR validation of several genes that were upregulated or downregulated in the RNA-seq screen.

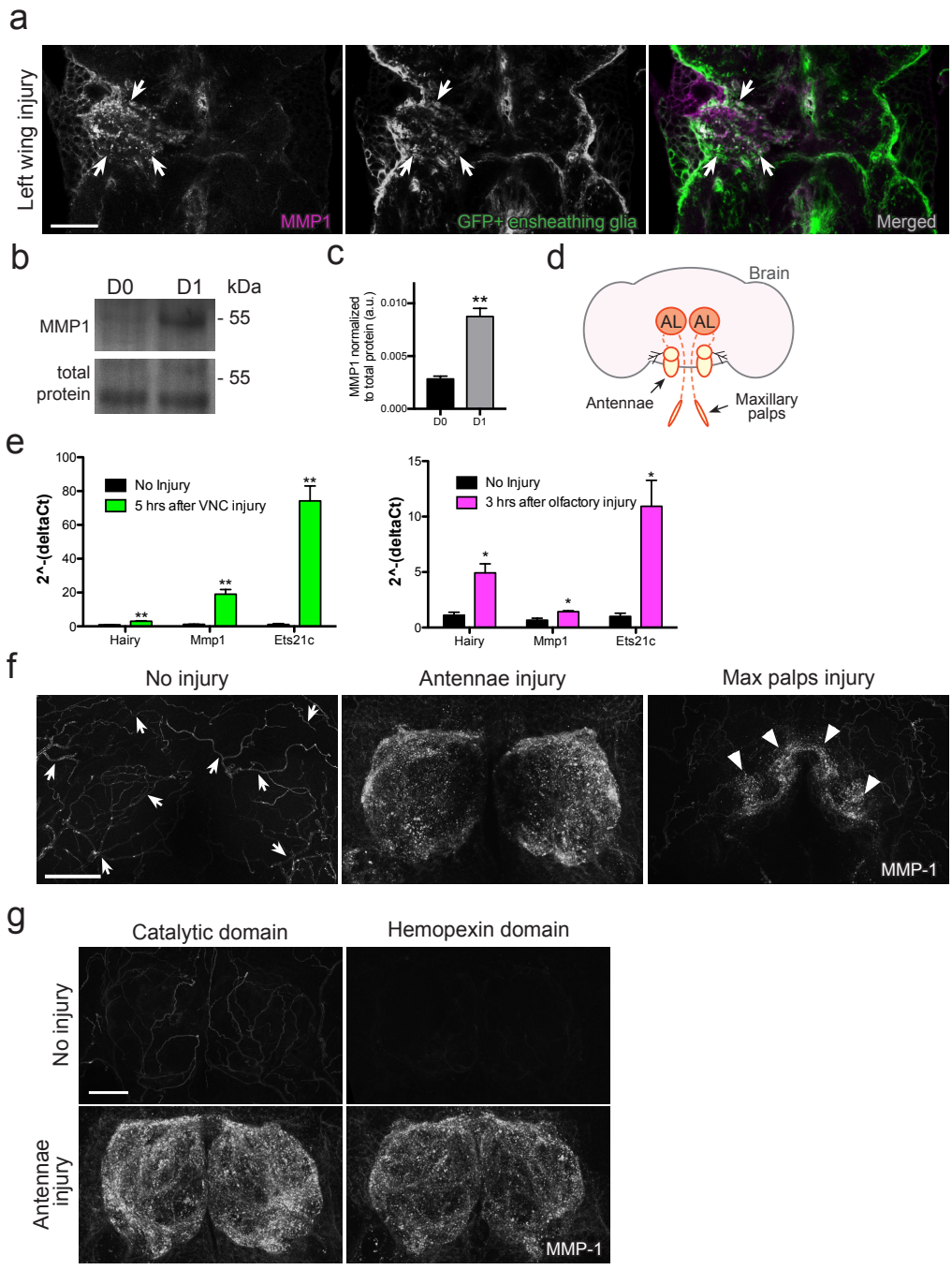

Figure 5 (continued below).

Figure 5. MMP-1 is upregulated by ensheathing glia in the VNC and the brain in response to neurodegeneration. (**a**) Ensheathing glial membrane expansion in the AMN colocalizes with MMP-1 upregulation after single left wing injury. One-micron slices shown. (**b**) Western blot with anti-MMP-1 (top panel) and total protein stain (bottom) of VNC lysates from uninjured and injured (all legs and both wings) control flies (**c**) Quantification of the MMP-1 western blot in (b). **P<0.01; unpaired t-test; 4 biological replicates. (**d**) Schematic representation of the well-established olfactory axotomy. Olfactory neurons within both the antennas and the maxillary palps project into the antennal lobes. Ablation of these peripheral structures induces Wallerian degeneration of the neurons and glial immune responses within the antennal lobe region (dark orange circles within the brain) can be monitored. (**e**) Quantitative real-time PCR analysis of normalized expression levels of *hairy, MMP-1* and *ets21c* in VNCs flies that have had all legs, wings and head severed (left) and the expression levels of the same genes in brains after double olfactory injury (right). Ct values were normalized to ribosomal protein L32 and results are presented as fold induction relative to uninjured. N≥3 biological replicates; mean ± SEM plotted; **P<0.01; *P<0.05; students t-test. (**f**) MMP-1 expression in the antennal lobe region. Robust MMP-1 activity is observed in flies after a bilateral antennal injury and bilateral maxillary palp injury. In uninjured animals, MMP-1 expression is localized to the trachea. Maximum intensity projections shown (25 um). (**g**) Robust expression of both the catalytic and hemopexin domains of MMP-1 is observed in response to bilateral third antennal segment injury. Maximum intensity projections shown (25 um). Scale bars = 30 um. Genotypes: Fig. 5a: UAS-mCD8::GFP/Cyo; TIFR-Gal4/TM3; Fig. 5b-g: w^{1118}.

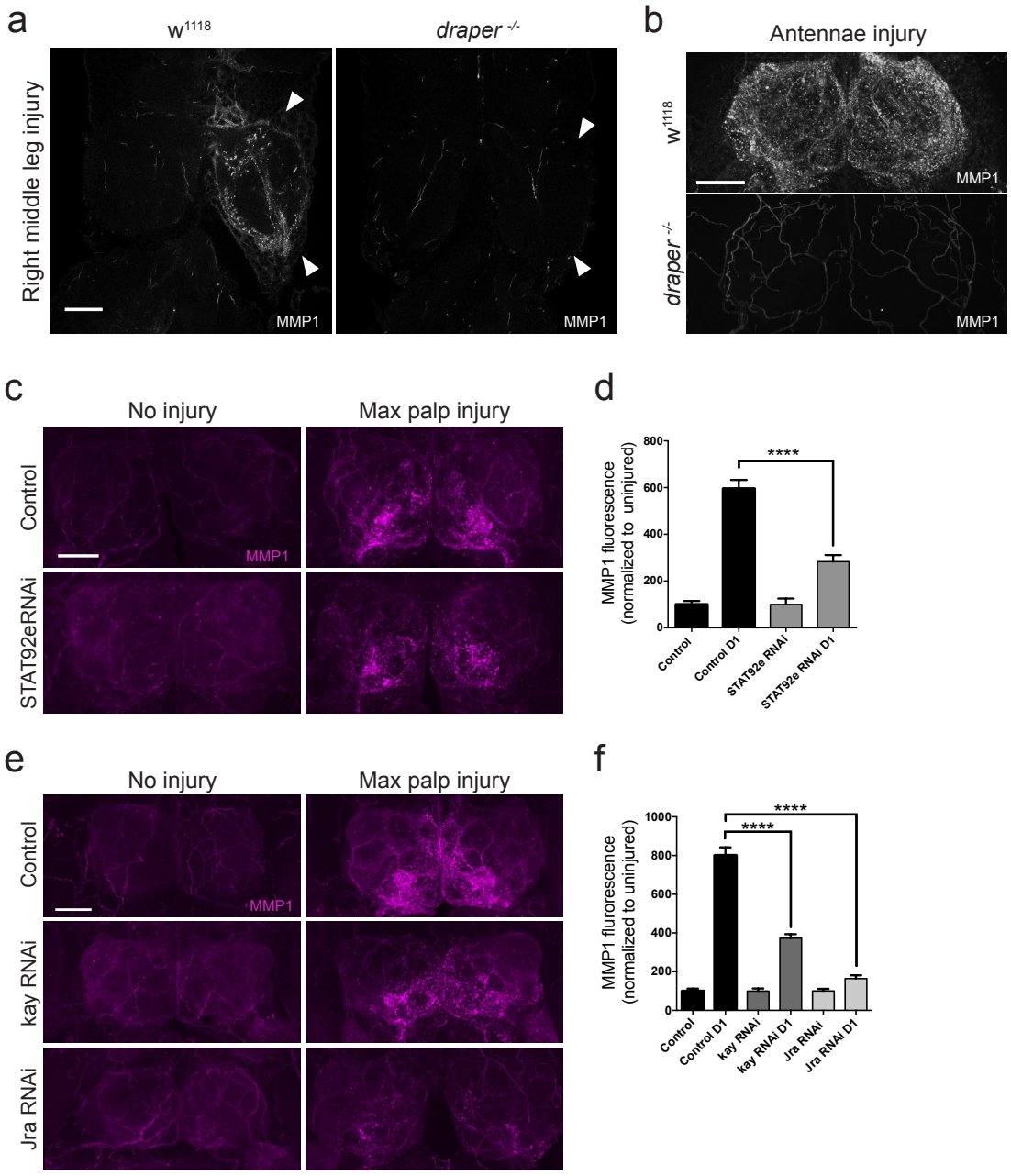

Figure 6 (continued below).

Figure 6. Draper/STAT92E/AP-1 pathway is required for MMP-1 upregulation after injury. (**a**) Representative images of control and *draper* null animals one day after a right middle leg injury. One-micron slices shown. Arrowheads show injured area. (**b**) Representative images of control and *draper* null animals one day after bilateral antennae injury. Maximum intensity projections shown (25 um). (**c**) Adult specific knockdown of STAT92E in glial cells leads to attenuated MMP-1 response to maxillary palp injury. Maximum intensity projections (25 um) of MMP-1 immunostainings in control and STAT92E RNAi flies. (**d**) Quantification of MMP-1 fluorescence in C. N ≥10 antennal lobes; mean ± SEM plotted; ****P < 0.0001; One-way ANOVA with Sidak post hoc test. (**e**) Adult specific knockdown of kay and Jra in glial cells leads to attenuated MMP-1 response to maxillary palp injury. Maximum intensity projections (25 um) of MMP-1 immunostainings in control and RNAi flies. (**f**) Quantification of MMP-1 fluorescence in F. N ≥22 antennal lobes; mean ± SEM plotted; ****P < 0.0001; One-way ANOVA with Sidak post hoc test. Scale bars = 30 um. Genotypes: Fig. 6a,b: w1118 and w1118; *draper* $^{-/-}$. Fig. 6c-f: Control: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/+; STAT92E RNAi: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/ UAS-STAT92E RNAi; Repo-Gal4/+. kay RNAi: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/UAS-Kayak RNAi; Jra RNAi: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/UAS-Jra RNAi.

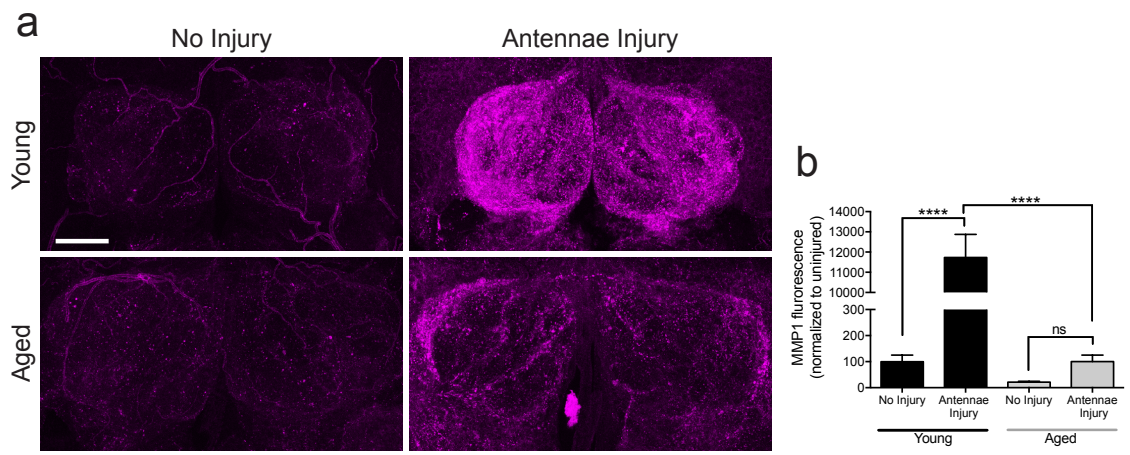

Figure 7. MMP-1 response to injury is significantly attenuated in aged flies. (**a**) Representative immunostaining of MMP-1 expression in young and aged animals before and one day after bilateral antennae axotomy. Young animals were 7-14 days old and aged animals were 56-63 days old. 25um z-stack shown. (**b**) Quantification of (a). N ≥8 antennal lobes; mean ± SEM plotted; ns=not significant, ****P < 0.0001; One-way ANOVA with Sidak post hoc test. Scale bar = 30 um. Genotypes: Repo-Gal4, UAS-mCD8::GFP/TM3

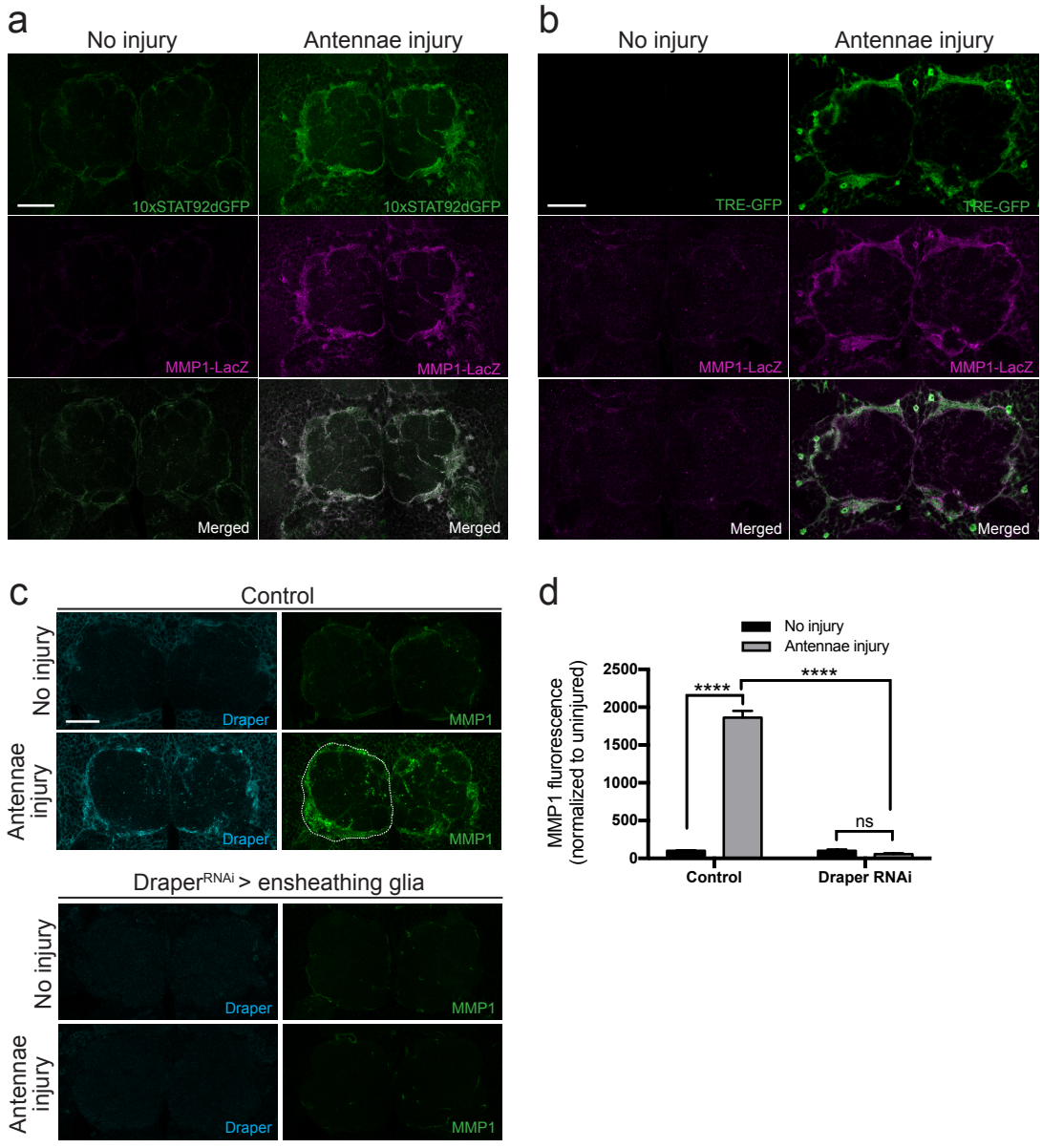

Figure 8 (continued below).

Figure 8. Ensheathing glia produce MMP-1 in response to injury. (**a**) Single confocal slices of the antennal lobes region showing STAT92E-dependent activation of dGFP (green) and MMP-1-LacZ transcriptional activity (β-gal in magenta) within the same cells one day after antennae injury. (**b**) Single confocal slices of the antennal lobes region showing dAP-1 activation of GFP (green) and MMP-1-LacZ transcriptional activity (β-gal in magenta) within the same cells one day after antennae injury. (**c-f**) Draper expression in ensheathing glia is required for MMP-1 upregulation after injury. (**c**) Representative Draper and MMP-1 immunostainings in control animals before and one day after antennae injury. Bottom panels: Representative Draper and MMP-1 immunostainings in flies that express Draper^{RNAi} in ensheathing glia. (**d**) Quantification of MMP-1 before and one day after injury in control and Draper^{RNAi} expressing flies (outline is representative ROI quantified). N≥21 antennal lobes; mean ± SEM plotted; ****P<0.0001; ns=not significant; students t-test. Genotypes: Fig. 8 a: 10xSTAT92e-dGFP/MMP-1-LacZ; Fig. 8b: TRE-GFP/MMP-1-LacZ**;** Fig. 8c: Control: TIFR-Gal4/+; Draper RNAi: TIFR-Gal4/UAS-Draper RNAi.

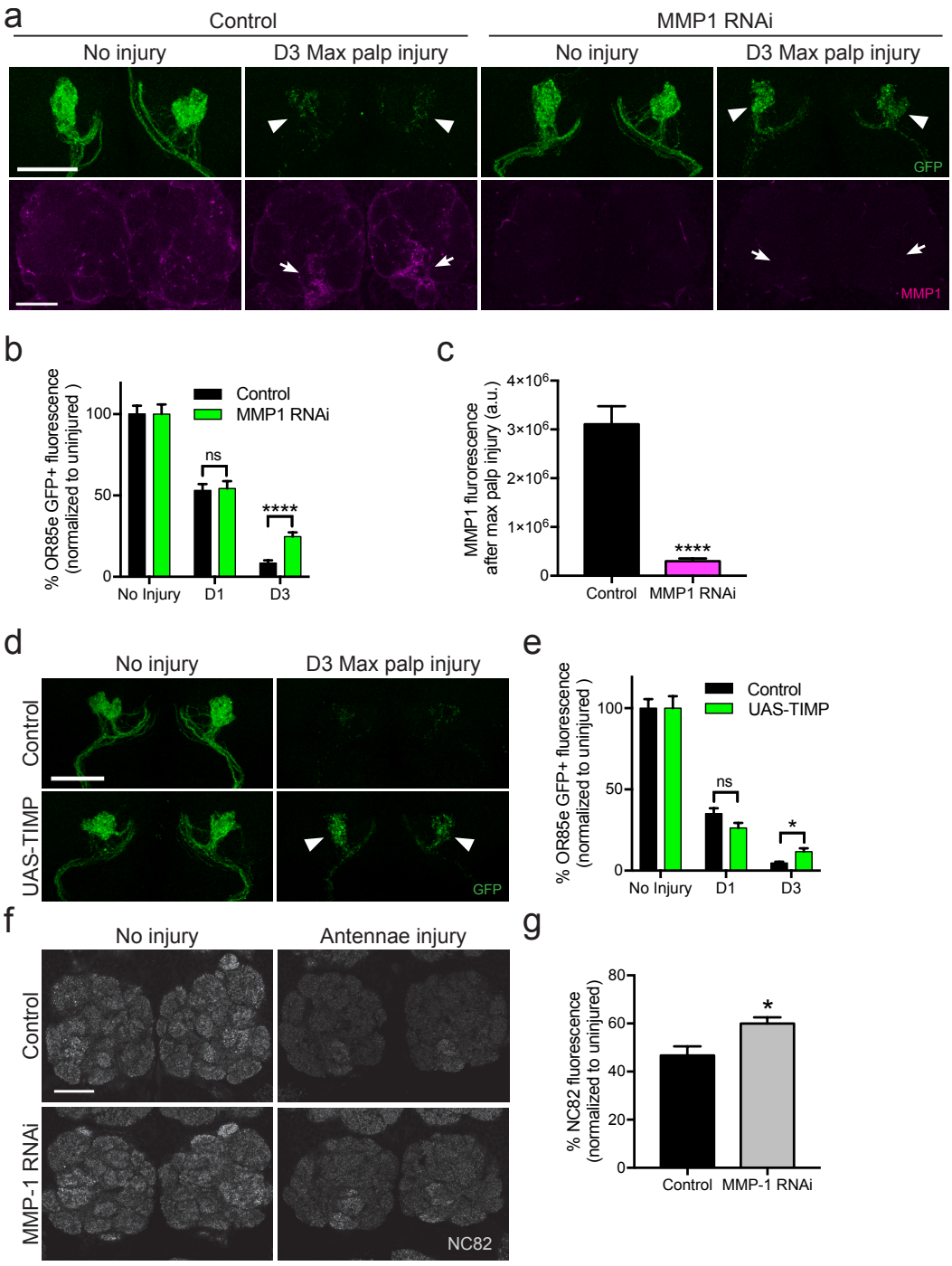

Figure 9 (continued below).

Figure 9. Adult specific knockdown or inhibition of MMP-1 in glia leads to incomplete clearance of degenerating axonal material several days after injury. (**a**) GFP-labeled maxillary ORN axons before and three days after maxillary palp nerve axotomy in control and adult specific knockdown of MMP-1 (green). Arrowheads show area quantified in b. Corresponding MMP-1 expression is shown on the bottom (magenta). Arrows show the MMP-1 upregulation three days after injury that is quantified in c. (**b**) Quantification of axon clearance at one and three days after injury. N ≥22; mean ± s.e.m. plotted; ****P < 0.0001; unpaired t test. (**c**) Quantification of MMP-1 fluorescence in a. N ≥27; mean ± s.e.m. plotted; ****P < 0.0001; unpaired t test. (**d**) GFP-labeled maxillary ORN axons before and three days after maxillary palp nerve axotomy in control and adult specific overexpression of TIMP (an endogenous MMP-1 inhibitor). Arrowheads show significantly more debris remaining three days after injury. (**e**) Quantification of axon clearance at one and three days after injury, normalized to uninjured. N ≥20; mean ± s.e.m. plotted; *P < 0.05; unpaired t test. (**f**) Presynaptic active zones labeled with NC82 in control and MMP-1 RNAi flies, before and six days after bilateral antennae injury. Significantly more debris remains in MMP-1 RNAi flies. (**g**) Quantification of presynaptic NC82+ clearance at one and six days after injury, normalized to uninjured. N ≥16; mean ± s.e.m. plotted; *P < 0.05; unpaired t test. Scale bars = 30 um. Genotypes: Fig. 9a and f: Control: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/+; MMP-1 RNAi: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/UAS-MMP-1 RNAi; Repo-Gal4/+; Fig. 9d: Control: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/+; UAS-TIMP: w1118; OR85e-mCD8::GFP, tubulin-Gal80^{ts}/+; Repo-Gal4/UAS-TIMP.

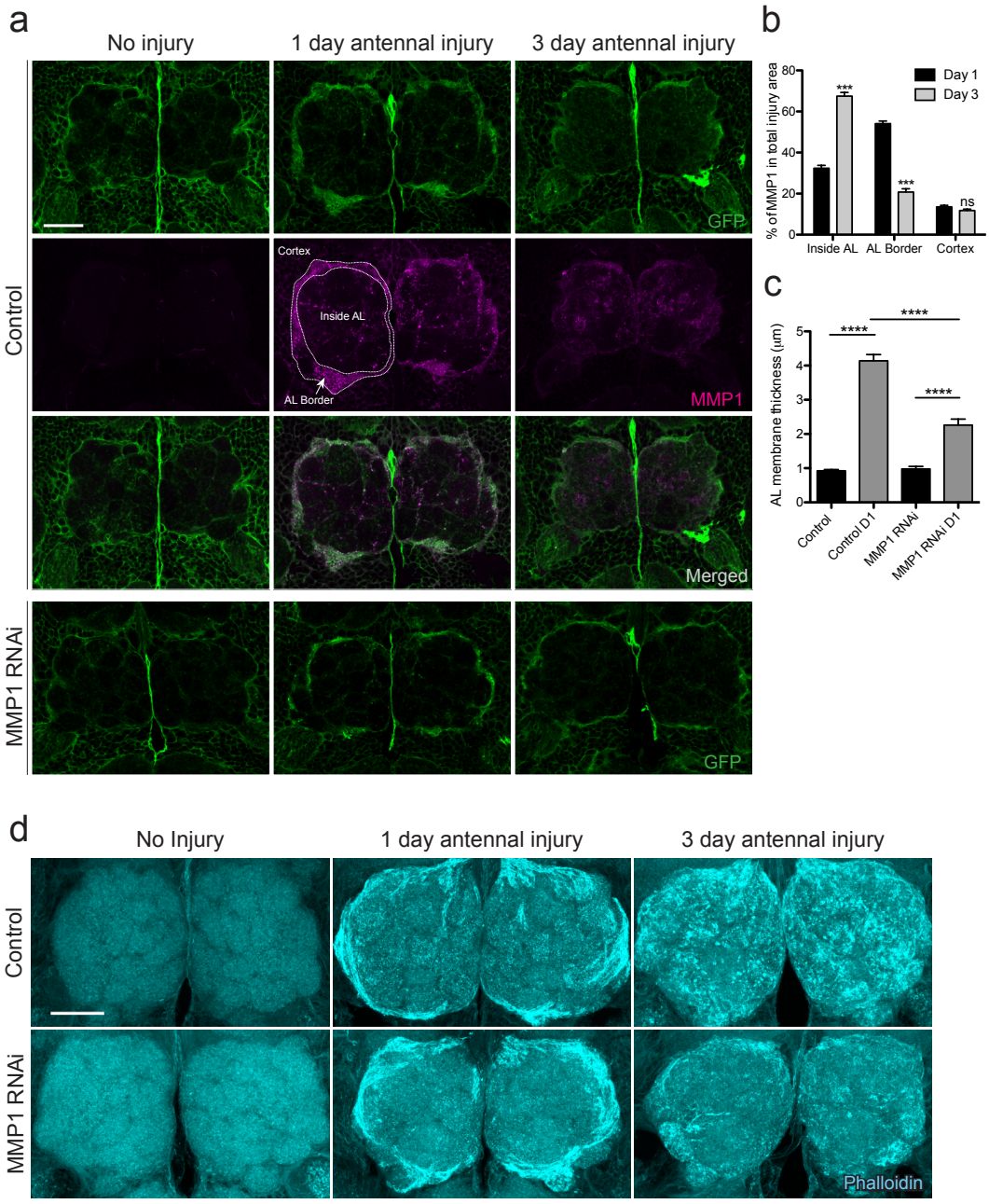

Figure 10 (continued below).

Figure 10. Glial membrane expansion and actin dynamics are attenuated after antennal injury in glial knockdown of MMP-1. (**a**) Glial membranes were labeled *in vivo* with membrane-tethered GFP. In control animals, there is a large glial membrane expansion one day after injury and a corresponding increase of MMP-1 in the antennal lobe (AL) border. Three days after injury MMP-1 expression is highest in the AL and it corresponds to an increase in glial membrane loops within the AL. In flies that express MMP-1 RNAi, glial membrane expansion at the AL border one day after injury is attenuated and three days after injury, less glial membrane loops are observed. One-micron slices shown. (**b**) Quantification of MMP-1 expression one and three days after injury inside the antennal lobe, within the AL border and in the cortex. Representative regions of interest are shown in the MMP-1 immunostained control brain one day after injury. N≥21 antennal lobes; mean ± SEM plotted; ***P<0.001; ns=not significant; students t-test. (**c**) Quantification of GFP+ glial membrane expansion one day after antennal nerve injury in control and MMP-1 RNAi flies; N ≥20 antennal lobes; mean ± SEM plotted; ****P < 0.0001; One-way ANOVA with Sidak post hoc test. (**d**) Representative immunostainings of Phalloidin-TRITC in control and ensheathing glial knockdown of MMP-1 one and three days after antennal injury. Maximum intensity projections shown (15 um). Unless otherwise noted, scale bars = 30 um. Genotypes: Fig. a-c: Control: Repo-Gal4, UAS-mCD8::GFP/+; MMP-1 RNAi: UAS-MMP-1 RNAi/+; Repo-Gal4, UAS-mCD8::GFP/+; Fig. e: Control: TIFR-Gal4/+; MMP-1 RNAi: TIFR-Gal4/UAS-MMP-1 RNAi.

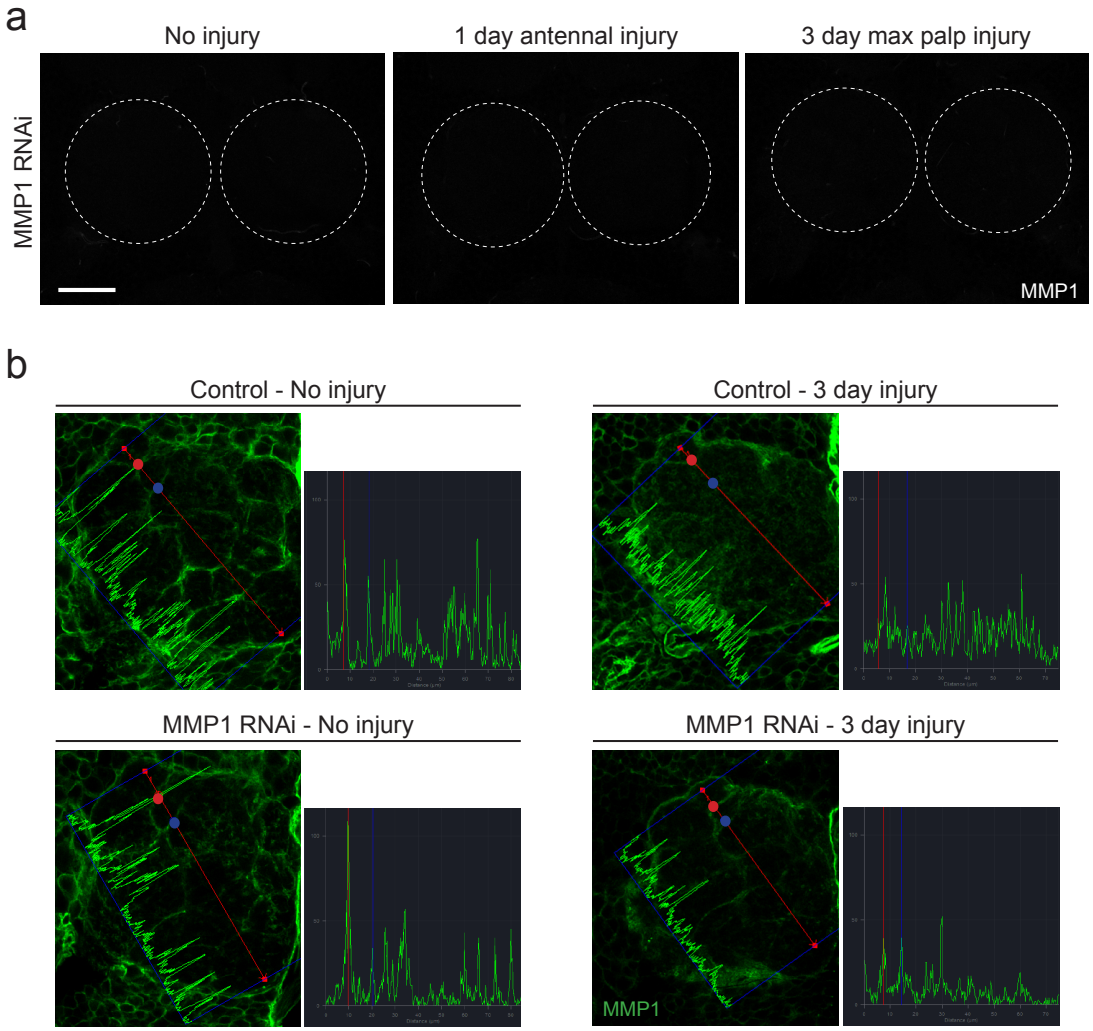

Figure 11 (continued below).

Figure 11 (related to Fig. 10). Glial membrane expansion in the neuropil region is attenuated three days after antennal injury in glial knockdown of MMP-1. (**a**) Single confocal slices of the antennal lobes region showing MMP-1 fluorescence in brains that are uninjured and one and three days after injury of flies expressing MMP-1 RNAi. Dotted circles outline the antennal lobe region. Scale bar = 30 um. (**b**) Representative antennal lobes of control and MMP-1 RNAi animals that have had their glial membranes genetically labeled with GFP. Line scan through the antennal lobe shows fluorescence intensity. Red and blue dots in the image correspond to the red and blue lines in the corresponding traces. The red and blue dots show fluorescence intensity at the same two glomeruli. In uninjured control and MMP-1 RNAi animals, glial membranes inside the antennal lobes are restricted to wrapping around entire glomeruli. In control animals, three days after injury the glial membranes form small loops and the distinct glomerular pattern is not observed. In MMP-1 RNAi animals, three days after injury the glial membrane loops are visible, but so are the glial membranes around the glomeruli. Genotypes: Fig. 6 a,b: Control: Repo-Gal4, UAS-mCD8::GFP/+; MMP-1 RNAi: UAS-MMP-1 RNAi/+; Repo-Gal4, UAS-mCD8::GFP/+.

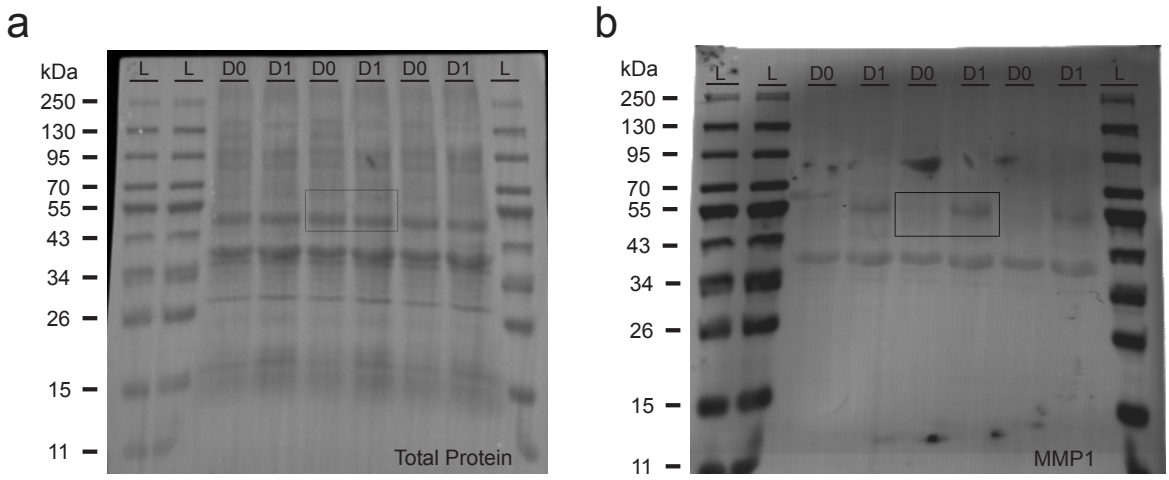

Figure 12. Uncropped blot of Westerns shown in Figure 5b. (**a**) Total protein blot displaying three biological replicates for uninjured (D0) and injured (D1) samples. The injured animals had all legs and wings removed 24 hours prior to dissections. (**b**) Fluorescence image of the blot displayed in (a) probed MMP-1 catalytic and hemopexin domains. The boxes highlight the cropped areas in Fig. 5b.

Table 1A

Annokey Search Terms
[Ff]ilopodia
[Ff]ilopodium
[Ii]nvadopodia
[Ii]nvadopodium
[Pp]odosome(s)
[Mm]igration
ECM
[Ee]xtracellular matrix
[Ii]nvadosome
[Ii]nvasive
[Mm]etastasize
[Mm]etastasis
[Cc]ell invasion

Table 1B

Top Ten Annokey Results								
Drosophila			Mouse			Human		
Gene	*Highest Rank Term*	*Total Matched Entries*	*Gene*	*Highest Rank Term*	*Total Matched Entries*	*Gene*	*Highest Rank Term*	*Total Matched Entries*
mys (Itgb1)	[Ff]ilopodia	187	Itgb1	[Ff]ilopodia	553	ITGB1	[Ff]ilopodia	1038
Fas3 (Cadm2)	[Ff]ilopodia	123	Rac1	[Ff]ilopodia	292	RAC1	[Ff]ilopodia	708
Moe (Msn)	[Ff]ilopodia	87	Tubb3	[Ff]ilopodia	252	**MMP14**	**[Ii]nvadopodia**	**609**
Pvr (Flt1)	[Ff]ilopodia	86	Apc	[Mm]igration	200	PTGS2	[Mm]igration	594
Cdc42 (Cdc42)	[Ff]ilopodia	82	**Mmp14**	**[Ii]nvadopodia**	**179**	NFKB1	[Ff]ilopodia	482
rhea (Tln1)	[Mm]igration	72	Cdc42	[Ff]ilopodia	164	SERPINE1	[Ff]ilopodia	270
Act5C (Actg1)	[Ff]ilopodia	69	Serpine1	[Mm]igration	115	CDC42	[Ff]ilopodia	261
puc (Dusp10)	[Ff]ilopodia	65	Selp	[Mm]igration	104	BRCA1	[Mm]igration	244
Stat92E (Stat5b)	[Ff]ilopodia	61	Nfkb1	[Mm]igration	101	MAPK14	[Mm]igration	239
Mmp1 (Mmp14)	**[Mm]igration**	**59**	Cdk5	[Ff]ilopodium	100	IGF1R	[Mm]igration	225

Table 1. Annokey analysis reveals several evolutionarily conserved genes associated with glial membrane expansion and movement. (**a**) List of terms used in Annokey analysis. (**b**) Top ten hits associated with our Annokey key terms in *Drosophila*, mouse, and human. MMP-1 (highlighted) is found on the top ten lists of each species.

Chapter 4: CONCLUSION AND FUTURE DIRECTIONS

Glial phagocytic responses to nerve injury in the CNS are neuroprotective (He et al., 2013; Neher et al., 2011; Sokolowski and Mandell, 2011). In this dissertation, I used *Drosophila* as a model organism to investigate molecular mechanisms involved in glial responses to neurodegeneration in the healthy and aged CNS. In Chapter 2, I used a well-established *Drosophila* olfactory system axotomy assay to explore how glial immune activity is altered with age. My studies showed that in the aging brain, there is reduced glial engulfment activity due to decreased PI3K92E activity and reduced translation efficiency of the engulfment receptor Draper. In addition, my results demonstrate that aged *Drosophila* olfactory nerves are delayed in initiating WD after injury, similar to what has been previously shown in mammals. Importantly, I showed that increasing Draper/PI3K92E activity in the aging brain was sufficient to rescue both reduced Draper expression and delayed glial clearance of severed axons several days after injury. In Chapter 3, I explored novel genes involved in glial immune responses to neurodegeneration by establishing a new injury paradigm. I severed the fly peripheral nerves (i.e. wings, legs) to elicit large glial responses throughout VNC, performed transcriptome analysis, and discovered that MMP-1 is transcriptionally upregulated by ensheathing glia for efficient membrane expansion and clearance of degenerating axonal material. These results demonstrated that an RNA-seq screen can be used to uncover novel genes and pathways involved in glial responses to neurodegeneration, and furthermore that it can be used as a resource for investigating pathways that may become dysfunctional with age.

REVERSING AGE-RELATED DECLINE IN GLIAL IMMUNE ACTIVITY

Aging is a very multifaceted process and the greatest risk factor for neurodegenerative disorders. Reactive gliosis is often associated with neuroprotection and in Chapter 2, I explored whether normal brain aging affects glial immune responses. I found that in response to either a small or a large injury, glial clearance of severed axons is delayed in aged flies due to poor glial membrane recruitment and phagocytic activity. Surprisingly, I found that this defect is not due to age-related glial cell death. Instead, I found that protein levels of the highly conserved phagocytic receptor, Draper, are significantly decreased in the aging brain. It is well established that Draper is absolutely necessary for glial clearance of adult degenerating ORN axons. In response to neurodegeneration, injury-induced Draper upregulation was significantly reduced in aged ensheathing glia due to reduced activation of STAT92E-dependent transcriptional cascades. I speculated that in the aging brain, basal levels of Draper fall below a certain threshold, causing glial phagocytic responses and injury-responsive STAT92E transcriptional cascades to become dysfunctional. To further test this hypothesis, I monitored glial immune responses in young Draper heterozygous animals that have basal levels of Draper comparable to aged control animals, and found that young Draper heterozygous animals have similar phenotypes to aged animals. Upregulation of Draper in aged glia was sufficient to rescue the dysfunctional glial clearance normally observed in the aged brain. Remarkably, upregulation of PI3K92E, which positively regulates Draper expression, was sufficient to rescue reduced Draper expression, STAT92E activity and delayed glial clearance of severed axons in aged animals and young Draper heterozygous animals. PI3K92E activates TOR to regulate gene transcription and translation, and I found that TOR is required for glial Draper and severed axon

clearance. Additionally, because basal transcription of Draper is not changed in the aging brain, I monitored Draper translation via polysome fractionation and uncovered that Draper translation is impaired in the aged brain. These results highlight the importance of monitoring protein translation in conjunction with transcriptome screens, especially during aging. I propose that even though the PI3K92E/TOR pathway regulates many aspects of cell function, loss of Draper specifically hinders the critical neuroprotective engulfment activity of aged glia. Lastly, in the PI3K/Draper overexpression experiments, I observed a rescue in clearance of severed axons several days after injury, however, there was no significant change in clearance just 24 hours after axotomy. I thus explored whether WD dynamics are changed with age. I found that initiation of WD is markedly delayed, just like it has been previously described for peripheral nerves in mammals. This is the first evidence to show that this delay is conserved in invertebrates. This chapter provides new mechanistic insight into how aging affects glial–axon interactions and glial responses to neurodegeneration.

HOW DOES PI3K92E REGULATE LEVELS OF DRAPER AND PHAGOCYTOSIS IN THE AGING BRAIN?

Downstream of the InR, the PI3K92E/Akt/TOR pathway regulates a diverse group of cellular functions including, gene transcription and translation, cell proliferation, intracellular trafficking, survival, and motility. Doherty et al., 2014 identified PI3K92E as a positive regulator of basal Draper expression in young glia and I showed in Chapter 2 that increasing PI3K92E activity in aged glia or young glia haploinsufficient for Draper was sufficient to rescue basal levels of Draper, STAT92E

activity, and delayed glial clearance of severed axons. Additionally, in the Supplemental Results of Chapter 2, I showed that InR activity decreases with age in flies, which further supports my conclusions that PI3K92E activity decreases with age. In addition, I showed that increasing PI3K92E activity in aged glia eliminates the Rab11-postive Draper aggregates observed in the synaptically rich areas of the aged fly brains. It is important to note that I did not observe Draper aggregates in the young Draper heterozygous flies, suggesting that the aggregates form with age, and not due to decreased basal levels of Draper. Since it appears that PI3K92E might regulate the trafficking of Draper, it would be interesting to do epistasis experiments using RNAi lines against the different regulators of cell compartments to uncover how Draper get trafficked from the nucleus to the membrane and how Draper gets recycled/degraded.

The InR/PI3K92E/TOR pathway is also known to inhibit autophagy, the cellular process responsible for degradation and recycling of cellular components. Although it is well established that impairing autophagy leads to neurodegeneration, whether defective glial autophagy leads to neurodegeneration is severely understudied. I explored the role of autophagy in glia of young animals and found that glial knockdown of autophagy initiation promotes faster clearance of injured axons. Activating PI3K92E in young glia also lead to the same results. These studies suggest that when the phagocytic load of glial cells is high, that attenuating autophagy allows for more efficient destruction of debris. In the future, it is imperative to understand how glial cells balance debris degradation during phagosome and autophagosome formation in response to injury in the young and aged brain. It would be interesting to re-do the PI3K92E rescue experiments in aged animals while also monitoring autophagic flux in order to uncover whether inhibiting autophagy also contributes to the rescue observed.

In addition, since inhibiting autophagy initiation in young glia lead to faster clearance of severed axons, it is important to explore whether inhibiting autophagy in aged glia would lead to the same results.

GLIAL-MEDIATED SYNAPSE ELIMINATION IN THE AGING BRAIN

The Draper mammalian homologs MEGF10 and Jedi signal through highly conserved tyrosine kinase cascades and have also been shown to be required for glial engulfment of neurodegenerative material and synapses (Scheib et al., 2012). For example, during the mammalian postnatal development of the visual system, MEGF10 and another engulfment protein, MERTK, are required for astrocyte-mediated phagocytosis of synapses during for activity-dependent synapse elimination (Chung et al., 2013). Inhibition of astrocyte engulfment leads to wiring defects (Chung et al., 2013). In addition, MEGF10 and MERTK-dependent engulfment of excitatory and inhibitory synapses by astrocytes continues in the adult CNS (Chung et al., 2013). In *Drosophila,* Draper is also required for synapse engulfment during development (Tasdemir-Yilmaz and Freeman, 2014) and our lab has uncovered that in the adult CNS, Draper is required for activity-dependent engulfment of pre-synaptic terminals (Muenzel EJ and Logan MA, in preparation). These findings suggest that Draper/MEGF10 have critical roles in continually remodeling synapses during adulthood. In the future, it would be interesting to investigate how aging affects MEGF10 function in mammals and whether age-related Draper/MEGF10 dysfunction leads to an increase of weak synapses or circuits in the aged brain. Additionally, what are the consequences of having lingering connections in the aged brain, and does it make the brain more susceptible to disease?

WHAT IS THE ROLE OF DRAPER/MEGF10 IN NEURODEGENERATIVE DISORDERS?

In Chapter 2, I explored Draper-dependent glial immune function in the healthy aging brain and found that phagocytosis of neurodegenerative material is neuroprotective. However, it would be interesting to explore the role of Draper/MEGF10 and glial phagocytosis during neurodegenerative disorders such as AD, PD, ALS, and Huntington's disease (HD). These disorders have several common cellular and molecular mechanisms related to misfolded and aggregated proteins. Recent work on HD has demonstrated that *Drosophila* ensheathing glia require Draper to internalize pathogenic human Huntingtin (Htt) protein expressed in neurons (Pearce et al., 2015). However, the phagocytosed mutant neuronal Htt aggregates can cause wild-type Htt proteins in the glial cell cytoplasm to aggregate, which suggests that glial clearance of neuronal Htt aggregates can actually contribute to the spread of pathogenic protein aggregates (Pearce et al., 2015). These results show how glial phagocytosis during neurodegenerative disorders can act like a double-edged sword. Glial phagocytic removal of neuronal protein aggregates has been shown to be neuroprotective (Streit, 2012), yet aggregates in glial cells can cause glia to become compromised, rendering them dysfunctional in their ability to clear other debris (Garden and La Spada, 2012). In mammals, another important component of CNS glial innate immune response includes the complement cascade, whose role of phagocytosis during neurodegenerative disease is also controversial. Although it has been shown that microglia use the complement pathway to phagocytose toxic Aβ-peptides, recent studies in mammals have shown that too much complement-dependent phagocytosis by microglia during AD can be detrimental and can actually drive disease progression. These studies highlight the

importance of better understanding neuron to glia communication, the molecular mechanisms that drive glial immune responses, and the intracellular pathways involved in debris internalization and phagocytosis in the young, aged, and diseased brain. Additionally, it would be interesting to explore whether glial phagosome efficiency can be altered during neurodegenerative disorders such that aggregated proteins will quickly get eliminated instead of causing glial cells to become dysfunctional. For example, can inhibiting autophagy in glial cells of disease model systems lead to more efficient destruction of phagocytosed protein aggregates?

AGE-ASSOCIATED DELAYED AXONAL FRAGMENTATION OF INJURED AXONS

By monitoring the time course of degeneration, as well as mitochondrial dynamics in young and aged flies, I uncovered that WD is initiated more slowly in aged olfactory nerves in response to injury. Although a delay in axonal fragmentation has also been described in aged mammals before, there are no recent studies that have further explored this observation. During WD, membrane disruption occurs due to large calcium influx, calpain-mediated cleavage, and axonal transport impairment (Lingor et al., 2012). It would be interesting to explore whether any of these events are disrupted in aged axons. *Drosophila* is a great model organism to investigate the mechanisms behind this age-related delay in axonal fragmentation because of the many genetic and molecular tools the fly offers. In addition, in this situation, it is beneficial that fly glia do not produce myelin because in mammals, it is difficult to distinguish between axon and myelin degeneration. Interestingly, there is little is known about axon–glia signaling events in both flies or mammals. The signal that olfactory neurons release after injury to

communicate with glia that they have been injured is unknown. Recent data published from our lab showed strong evidence that neurons release dense core vesicles filled with some unknown neuropeptide along the length of the axons to activate glial InR activity and Draper-dependent phagocytosis (Musashe et al., 2016). Since insulin signaling decreases with age in all animals, it would not be surprising that there would be a decrease in the neuropeptide levels in aged axons. This can be explored by first uncovering which insulin-like peptide are released by the injured axons and whether the levels change with age. These findings and future research are very important because the molecular mechanisms involved in delaying the initiation of WD could be neuroprotective in young axons.

Chapter 4: TRANSCRIPTIONAL PROFILING SHOWS MMP-1 IS A NEW DRAPER-DEPENDENT TARGET IN GLIAL RESPONSE TO AXONAL INJURY

When adult *Drosophila* glial cells respond to axonal injury, they undergo a transcriptional upregulation of the pro-engulfment isoform of *draper-I* followed by the inhibitory *draper-II* isoform, suggesting that fly glia, like mammalian glia, undergo a transcriptional program in response to neurodegeneration (Logan et al., 2012). In addition, injury-induced activation of Draper triggers the transcriptional upregulation of *draper* in a STAT92E-dependent manner, generating a positive auto-regulatory feedback loop of Draper expression that ensures appropriate levels of Draper are present regardless of injury size (Doherty et al., 2014). Besides STAT92E targeting of the Draper locus, no other transcriptional programs are known to be activated in response to neurodegeneration in *Drosophila* glia. To uncover other essential immune genes involved

in glial clearance of damaged neurons, I was not able to use the well-established olfactory axotomy assay because it did not give me a large enough increase in *draper* transcript by Q-PCR. Instead, I established a new non-lethal injury model in which I induced degeneration of motor and sensory neurons by removing peripheral structures that send their projections into VNC. Before doing the RNA-seq screen, I first validated that the same Draper-dependent transcriptional pathways are utilized by VNC ensheathing glia as observed in the brain. I also validated that WD mechanisms are conserved by VNC neurons. Removing the legs, wings, and head of adult flies provided a significant four-fold increase in *draper* mRNA after injury, and I thus used this injury paradigm for RNA-seq analysis. The transcriptome screen uncovered 500+ genes that are significantly upregulated and have human orthologues, and many more that were significantly downregulated.

I next shifted my focus on one gene, MMP-1, that was associated with cell migration, had clear mammalian orthologues, but its role in glial response to injury in *Drosophila* was unknown. MMP-1 was highly upregulated by ensheathing glia in response to both olfactory and VNC injury. Axotomy assays in *draper* null flies and flies expressing RNAi against STAT92E and each subunit of the AP-1 heterodimer demonstrated that MMP-1 upregulation after axon injury requires Draper/AP-1/STAT92E. This finding was very important because it suggests that there could be an entire network of signaling proteins upregulated after injury that is dependent on Draper receptor activity. Additionally, beyond phagocytic activity, Draper might have other roles in regulating transcription of genes required for glial function and glial immune responses. To uncover the role of MMP-1 in response to injury, I knocked down MMP-1 expression via RNAi and by expressing TIMP, an endogenous inhibitor of

MMP-1, and observed that MMP-1 is critical for proper clearance of severed debris several days after injury, but not during the initial 24 hours after injury, most likely because the glia are already in contact with the degenerating ORNs. After injury, ensheathing glia expand their membranes and invade the neuropil region to clear the degenerating axonal and synaptic material. I monitored glial membranes in response to injury and observed that ensheathing glial membrane expansion and actin dynamics are greatly attenuated in the absence of MMP-1. These results suggest that MMP-1 deficient glia are unable to invade the neuropil region to efficiently engulf debris. These findings are important because previous mammalian research has been controversial regarding the role of MMPs during reactive gliosis – MMPs can either be beneficial or detrimental in response to CNS injury (Rosenberg, 2002). A lot of the controversy stems from the fact that there are over 23 partially redundant MMP genes and four TIMP genes in mammals that can act redundantly via compensatory mechanisms. Because there are only two MMPs in *Drosophila*, and because there are several genetic tools to control their expression, the fly is an excellent model organism to further explore what aspects of glial MMP expression are favorable or damaging in response to neurodegeneration.

WHAT COMPONENTS OF THE ECM IS MMP-1 INTERACTING WITH DURING GLIAL IMMUNE RESPONSES?

MMPs are a large family of calcium-dependent zinc-containing endopeptidases that are evolutionarily conserved, from plants to mammals (Maidment et al., 1999), whose function it is to break down components in the ECM (Verma and Hansch, 2007). In Chapter 3, I demonstrated that several days after injury, ensheathing glial cells use MMP-1 to ensure invasion of glial membranes deep into the antennal lobe region to

drive efficient clearance of degenerative axonal and synaptic debris. The MMP-1 expression pattern followed the glial membrane expansion and actin polymerization pattern – highly expressed around the antennal lobes initially, almost forming a ring, and several days later, highly localized deep in the antennal lobe region. These results demonstrate that MMP-1 drives glial membrane dynamics in response to injury. There was still some glial membrane expansion and actin polymerization when glial cells are depleted of MMP-1, so I cannot rule out that other proteins such as MMP-2, ADAM (a disintegrin and metalloproteinase) proteins, which are a large family of transmembrane and secreted metalloendopeptidases (Wolfsberg et al., 1995), or other proteases are also expressed. Even though MMP-2 was not upregulated in the RNA-seq screen, it could compensate for depletion of MMP-1. Additionally, Kuzbanian, an ADAM protein, was significantly upregulated in our screen, along with other uncharacterized genes that were predicted to have metalloproteinase activity (CG11951, CG7649, CG31019, CG3097). In the future, it will be important to uncover what other ECM-degrading proteins are also expressed by glia in response to injury.

The ECM is formed by a large group of extracellular macromolecules secreted by cells that provide structural support and create the cellular environments required during development, morphogenesis, and overall function (Maidment et al., 1999; Nagase and Woessner, 1999). One major question remaining in Chapter 3 is: what ECM proteins is MMP-1 interacting with or degrading to allow invasion of glial membranes in the neuropil regions after injury? Because MMPs can degrade almost all component of the ECM, their main function has been believed to be remodeling of the ECM, however, research shows that MMPs also break down ECM molecules to allow for cell migration (Vu, 2000). My data in Chapter 3 suggested that MMP-1 is required for glial membrane

expansion and invasion after nerve injury, however, I cannot exclude that after injury MMP-1 is also required for remodeling the ECM after the neurodegenerative debris has been engulfed. In *Drosophila*, during *in vivo* tumor invasion, MMP-1 degrades collagen IV rich basement membrane, but the precise substrates for this degradation are undetermined (Srivastava et al., 2007). A two-hybrid screen that used the hemopexin domain of MMP-1 identified seven candidate interactors, but none of them were ECM proteins or signaling molecules (S. Zhang, 2006). One of the proteins identified that interacted with MMP-1 was Ninjurin A, an evolutionarily conserved transmembrane protein that has been shown to mediate cell adhesion and is also upregulated in rats after peripheral nerve injury. Ninjurin A was 16-fold upregulated in response to injury in the RNA-seq screen, however depleting adult glial cells of Ninjurin A did not lead to clearance deficits (data not shown). It is important to note that the efficiency of the Ninjurin RNAi line was not tested and I did not test whether the neurons are contributing the Ninjurin A. In the near future, it would be important to do Western blots on healthy and injured VNCs from control and MMP-1 RNAi animals and probe the blots for Ninjurin A to uncover whether there are cleavage products in the injured animals, that are absent when MMP-1 is knocked down. Additionally, to uncover whether MMP-1 has other substrates, there are several *Drosophila* specific ECM component antibodies available from the Developmental Studies Hybridoma Bank that should be assessed, including spectrin, coracle, WASP, profilin, htsRC, neuroglian, sidestep, integrin-α and integrin-β.

THE FLY VNC AS A NEW MODEL OF ASSESSING GLIAL RESPONSES TO NEURODEGENERATION

In this study, I presented a novel *in vivo* model to study glial responses to axonal degeneration. Using this model, I have uncovered that (1) VNC axons undergo classic WD mechanisms; (2) VNC ensheathing glial cells undergo conserved transcriptional, translational, molecular, and morphological changes in response to injury; and (3) ensheathing glial cells use the Draper/STAT92E/AP-1/MMP-1 program to drive clearance of degenerative debris. This work further establishes *Drosophila* as an excellent model system to study neurodegeneration and glial immune responses. This VNC injury model can be used to study transcriptional changes in glia in response to aging or disease. Additionally, because flies are very resilient, this non-lethal injury model offers the possibility of studying small, large and even reoccurring insults. For example, it would be interesting to define whether glial profiles change in response to injury in the same anatomical area by removal of the leg via individual segments (i.e. remove the tarsal segments first, then tibia, femur etc.). This injury paradigm could elucidate potential genetic and molecular mechanisms involved in reoccurring brain injuries. In addition, because there are Gal4 drivers for each of the glial subtypes in the adult CNS and there are new methods of doing cell-type specific mRNA purification (such as Ribo-trap (Heiman et al., 2014; 2008), for example), this VNC injury model can be used to uncover specific contributions of each glial cell type to injury. This novel injury model in *Drosophila* will be extremely valuable in defining the genetic and molecular mechanisms involved in axonal degeneration and glial immune responses to neurodegeneration.

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