Insulin-like signaling regulates glial immune responses to axon degeneration in *Drosophila melanogaster*

by

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CERTIFICATE OF APPROVAL

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Abstract

Glia are the primary immune responding cells in the brain and are thus highly attuned to changes in central nervous system (CNS) health. After any form of neural trauma, subsets of glia quickly respond by migrating to the site of damage and clearing degenerating tissue through phagocytic engulfment. This response contains the original insult and minimizes further damage to surrounding tissue. Unfortunately, the molecular mechanisms underlying these complex glial immune responses are poorly understood. Here, I provide the first direct evidence for the involvement of insulin-like signaling in the glial clearance response to acute CNS injury. Using a well established acute axotomy assay in *Drosophila*, I demonstrate that glial insulin-like signaling is critical for effective clearance of degenerating axonal debris in the adult brain. This effect is mediated through the insulin-like receptor (InR) and several downstream signaling components, all of which are highly conserved from *Drosophila* to humans. Additionally, I show that adult glial InR signaling is a positive regulator of the engulfment receptor Draper (*Drosophila* ortholog of mammalian MEGF10 and Jedi-1), a receptor that is highly upregulated in glia responding to CNS injury and plays an essential role in the glial clearance response. Furthermore, I demonstrate that forced expression of Draper in adult glia partially rescues the glial clearance defects caused by acute InR knockdown. Taken together, the work presented here demonstrates a novel yet critically important role for the insulin-like signaling pathway in innate glial immune responses. The insulin-like signaling pathway is a powerful regulator of transcription, translation, and metabolic homeostasis, and might

represent a fundamental signaling mechanism used by glia to orchestrate the complex and varied cellular steps needed to achieve an effective glial clearance response to CNS damage

Chapter 1

Introduction

Framing the study

Glia play a variety of roles critical for brain health, one of which is the phagocytic clearance of cellular debris after injury, apoptotic events, or infection. In this dissertation, I report my investigations into the regulation of the injury-induced glial clearance response by the insulin-like signaling pathway in adult *Drosophila melanogaster*. In this first introductory chapter, I discuss the importance of glial cell phagocytic activity in mammals and invertebrates and introduce molecular mechanisms underlying these functions.

Innate immunity

Organismal immunity is a biological phenomenon derived from a highly complex and varied set of cellular responses that allow a given organism to deal with insults to its tissue, whether originating from within or from without. Even though organismal immune processes are extremely diverse, much of the research conducted in the immunological fields has focused on the biological phenomena of acquired immunity and the production of antibodies targeted against harmful antigens.

While this antibody based “adaptive immunity” is one important component of the immune system in humans, it is actually a relatively recent and highly specialized evolutionary development present only in jawed vertebrates [1]. There is another much broader and far older branch of organismal immunity known collectively as innate immunity. Innate immunity does not rely upon antibody-mediated targeting and destruction of harmful material in the host

organism. Instead, innate immunity relies upon cells (and sometimes secreted factors) that can inherently recognize and destroy harmful or unwanted material through the use of a variety of highly conserved molecular pathways, many components of which are conserved from humans, to *Drosophila melanogaster*, to the nematode worm *C. elegans*. [2-4].

A very simplified outline of innate immune cell action can be seen in Figure 1.1, showing the primary steps an innate immune phagocyte must perform to accomplish its function of ridding the host of offending material, such as invading microbes, apoptotic cells, or cellular debris. The innate immune phagocyte must first find the material in question, recognize it as foreign or unwanted, destroy it, and recycle its contents. These are highly orchestrated steps that involve many sets of molecular signaling cascades that will be explored in more detail below.

Innate immunity in the central nervous system

While innate immune function is critically important throughout the body, it takes on even greater importance in the central nervous system (CNS). This is due to the fact that the CNS is largely excluded from peripheral immune cell access and therefore has a limited capacity to utilize adaptive immune responses imparted by blood-borne immune cells of the periphery [5-7]. This isolation is due in large part to the tight cellular barrier surrounding the CNS, termed the blood-brain barrier (BBB) in mammals, which is present in varying forms in all vertebrates and many invertebrates including insects, crustaceans, and

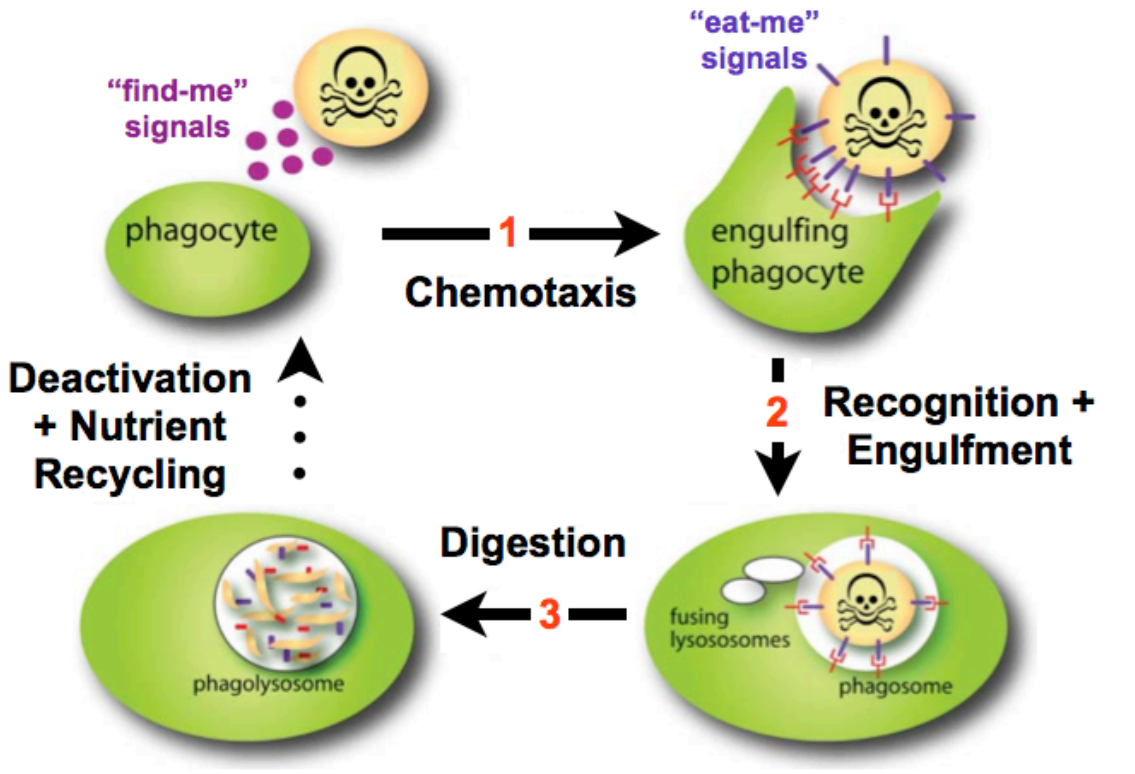


Figure 1.1. Phagocytic engulfment of targeted tissue. 1. A phagocytic immune cell must find tissue targeted for engulfment by either sensing "find-me" cues from the targeted tissue or by incidentally contacting it. **2.** The phagocyte must recognize "eat-me" signals on the surface of targeted tissue, verifying it for destruction and initiating phagocytic activity. The now activated phagocyte must then extend pseudopodial projections around the targeted tissue and internalize it into a phagosome compartment. **3.** The phagosome must then mature by fusing with acidic lysosomes, thereby digesting the engulfed material in the newly formed phagolysosome. Eventually, the phagocyte must deactivate and recycle the nutrients derived from the digested material.

cephalopods (reviewed in [8,9]). While the exclusion of peripheral immune cells from the CNS is no longer considered to be as absolute as once believed (reviewed in [10,11]), exclusion from CNS parenchymal tissue still appears to be quite robust in most physiological circumstances, at least when the immune insult does not originate in the periphery [5,6]. The upshot of this is that the CNS must frequently overcome insults without the help of the circulating peripheral immune system.

To deal with this challenge, the CNS houses its own resident innate immune cells. In mammals, these cells are mainly microglia, which appear to be CNS-resident macrophages of hematopoietic origin seeded early in development (reviewed in [12]). For the sake of simplicity, I will hereafter refer to CNS innate immune functions as glial immune functions, since glia are the primary resident immune cells of the CNS, though admittedly the relative contributions of resident glia versus invading peripheral immune cells to the overall CNS immune response is still an area of debate and probably varies to some extent depending on the exact nature and severity of the insult. It is also worth noting at this point that some aspects of the glial immune response, such as phagocytosis and clearance of injured axons, apoptotic bodies, or other regularly occurring cellular debris, are considered “housekeeping/homeostatic” functions of glia by some immunologists and therefore not strictly “immune” functions. It is my opinion that these distinctions are largely semantic, as many of the molecular underpinnings driving “housekeeping” functions also drive bona fide “immune” functions, as will be discussed in more detail later on. With this in mind, and for the sake of clarity,

I will simply refer to all these processes as “glial immune function(s),” even though not all lead to an inflammatory response. Before detailing some of the known molecular basis of glial immune functions, I will first discuss some of the roles these functions play in the healthy, acutely injured, and neurodegenerative CNS.

Glial immune functions in the healthy CNS

Ironically, less is known about glial immune functions in the healthy CNS than in circumstances of infection, injury, or disease. As is common in biological research, well functioning mechanisms have a tendency to go unnoticed until things go wrong and an abnormal phenotype can be observed. Research into glial immune function is no exception, and this has caused the field to undoubtedly miss many important but quotidian roles of glial immune function. Only recently have things begun to turn around for the glial research field, as effective tools for observing healthy cells have become more available and widespread.

One apt example is microglial function in the healthy CNS of mammals. For years, microglia have been thought to reside in one of two states: “reactive” in the case of injury, infection, or disease; or “resting” in healthy, intact tissue. Recent work in the mouse using *in vivo* two-photon imaging techniques has instead established that “resting” microglia are actually quite active, constantly probing their immediate cellular environment with highly motile processes on a minute-to-minute basis [13,14]. These microglial process movements occur

throughout the 3-dimensional territory of each microglial cell [13], and microglia are themselves 3-dimensionally tiled throughout the CNS, albeit quite heterogeneously [15]. In this way, very large regions of CNS parenchyma appear to be thoroughly and constantly probed and monitored by “resting” microglia in the healthy brain. This is clearly an energetically costly exercise, so what are these microglia doing that is worth the effort? In short, they are surveying the CNS for anything that does not belong or is no longer needed and are efficiently disposing of it when discovered.

Emerging data over the last several years in mouse studies has implicated microglia in various aspects of normal brain function at different developmental stages and into adulthood. New evidence suggests that many structures and/or cells empirically observed to “disappear” in the brain over time are actually actively phagocytosed by microglia. For example, both newly born hippocampal neurons undergoing apoptosis in the adult brain [16], and many synapses pruned during development [17] are actively eliminated by microglia. Possibly even more interesting are new studies that have found microglial roles in synaptic plasticity (reviewed in [18]). Indeed, microglial processes have been shown to associate with both neuronal and astrocytic elements *in vivo* [14], and recent high resolution electron microscopy and two-photon *in vivo* imagery has demonstrated microglial processes intimately associated with both immature dendritic spines [19] and the synaptic cleft of so-called “tri-partite” synapses in the juvenile mouse visual cortex [20]. Furthermore, the association of the microglial processes with dendritic spines and synaptic structures was shown to be activity dependent,

indicating that microglia can sense synaptic activity and might play important roles in synaptic transmission [20]. Work such as this has led some to propose the idea of a “quad-partite” synapse, composed of pre- and postsynaptic elements, astrocytic processes, and transient microglial processes [21], though it is not yet clear how common or widespread such a synaptic architecture might be in the CNS.

Collectively, in the last decade, a new view of microglial immune function in the healthy brain has begun to emerge. The contributions of this immune function to normal brain health are only beginning to be appreciated: from clearing away routine debris, to modulating synaptic activity, the list will undoubtedly only continue to grow. Importantly, microglia appear to accomplish many of these healthy basal functions using some of the same innate immune elements employed during injury and disease. For example, complement proteins classically associated with the innate immune response to microbial infection are also used to mediate microglial-associated pruning of unneeded synapses during development [17]. As another example, the chemokine receptor CX3CR1 (traditionally associated with inflammatory responses) is expressed exclusively in microglia in the CNS [22] and is involved in modulating synaptic connectivity and plasticity in postnatal mouse hippocampal neurons [23]. It is still very early days for this type of research, but future studies of glial immune pathways in the healthy CNS are likely to have direct implications for injured or diseased states and vice versa, as many of the same molecular pathways appear to be used in injury, disease, and in health.

Glial immune function in the injured CNS

In the mammalian CNS, microglia are the primary phagocytic immune responders to injured tissue. Their high level of process motility (as described above) makes them ideal surveyors for damage to the CNS. After injury to surrounding tissue, microglia undergo morphological changes, transforming from their highly branched “resting” or “surveying” state into an amoeboid “reactive” state. Reactive microglia then migrate to and/or send processes toward sites of injury and actively phagocytose cellular debris, thereby clearing the area of injured tissue (reviewed in [24]). Reactive microglia can also prune away synaptic inputs to the ablated area (inputs that are presumably newly nonfunctional due to loss of target tissue) in a process known as “synaptic stripping” [25,26]. Microglia play a clean up role after CNS injury, clearing the resulting cellular debris [27,28]. Whether or not these phagocytic actions also help (or hinder) efforts of CNS regeneration in mammals is still an area of considerable debate [28-30], as is the underlying question of why the mammalian CNS seems to have so little capacity for regeneration in the first place, especially in adults [31]. Nevertheless, glial phagocytic functions are critical for clearing damaged tissue and sparing neighboring tissue from unnecessary inflammation [27,28,32,33].

It should be noted here that astrocytes also play an important role in the injury response in mammals, mainly helping to orchestrate the actions of the primary immune responding cells and also helping to support the survival of spared neurons [34,35]. They do this through both contact dependent signaling

with primary responding cells and through the release of neuroprotective factors and soluble messaging agents like cytokines and nucleotides into their immediate extracellular environment [36,37]. In this way, they influence the fates of surrounding neurons and the behavior of the primary responding cells in the vicinity, like microglia and even peripheral immune cells (if they have gained access to the damaged area through the wound). While the astrocytic role in the glial immune responses is substantial, it is not the focus of my work presented in Chapters 2 and 3, which specifically addresses glial phagocytic function-- a role that astrocytes, though able to in perform in some circumstances [38-41], might not perform as a primary function to the same extent as some other glial subtypes like microglia (though this is still an area of some debate). As such, the astrocytic component of glial immune responses will not be covered here in any depth, though it should be noted that when astrocytes do phagocytose material they appear to use similar molecular pathways as those classically associated with phagocytosis in microglia, etc., again highlighting the importance of basic molecular pathways broadly governing phagocytic activity [40,42].

Glial immune function in the diseased CNS

In the past 20 years, there has been growing evidence that glial immune function plays important roles in the pathology of many neurodegenerative diseases such as Alzheimer's disease (AD), and Parkinson's disease (PD), among others. Microglia have been shown to phagocytose amyloid-β (Aβ) both *in vitro* and *in vivo* [43-45]. Furthermore, microglia in mouse models of AD

display significant impairment in process motility and phagocytic activity compared to age-matched controls. This impairment correlates spatially and temporally with Aβ plaque deposition [46]. Interestingly, reducing amyloid-β burden with an Aβ vaccination restores microglial function, indicating that Aβ is toxic to microglia and that part of AD pathology might be due to Aβ induced defects in microglial function [46]. Additionally, mutations in the gene for *TREM2*, coding a protein expressed in myeloid cells (including microglia) that is involved in mammalian phagocytosis [47], have very recently been identified as a risk factor for AD [48]. Furthermore, *TREM2* mutations associated with a higher risk of developing frontotemporal dementia and PD have been demonstrated to functionally impair the phagocytosis of primary microglia cultures [49]. Additionally, aggregated forms of alpha-synuclein, aberrant protein products commonly found in PD CNS parenchymal tissue, is inhibitory toward microglial phagocytic function of latex beads [50], and peripheral monocytes of PD patients also display defects in phagocytic activity *ex vivo* compared to monocytes derived from age-matched controls [51]. Again, the recurring finding is that microglial phagocytic function is disrupted in AD, PD, and other neurodegenerative diseases, though the exact mechanisms by which this happens and the ultimate consequences of this reduced function for disease pathology are not yet clear. Additionally, protein aggregates such Aβ have been demonstrated to have detrimental effects on macroglial function as well (e.g. astrocyte metabolism) [52], and the relative contribution of microglial versus

macroglial dysfunction to the pathology of neurodegenerative diseases is unclear and still very much an area of active research.

Molecular basis of the glial immune response to injury

The known molecular pathways governing glial immune functions are quite complex and can vary to some extent depending on the nature of the insult eliciting the immune response (e.g. microbial infection versus apoptotic bodies versus acute injury, etc.). I will focus on the glial immune functions underlying the response to acute injury, as this is most relevant in informing my own work presented in Chapters 2 and 3 and its implications.

The glial phagocytic immune response can be broadly broken down into discrete cellular steps of: glial recruitment to targeted tissue, positive recognition of tissue to be destroyed, phagocytic engulfment and digestion of said tissue, and ultimately deactivation and recycling of digested nutrients (a schematic of these steps can be seen in Figure 1.1). During the first step in the process, there must be “find-me” cues sensed by the responding glial immune cells that are released or expressed by the injured tissue itself and/or by neighboring cells that sense the damage directly and mediate the signal. Once the primary responding glia migrate their cell bodies and/or processes to the site(s) of injury, they must sense “eat-me” signals on the injured tissue itself, verifying it for destruction. Finally, once the targeted tissue is verified, it must be disposed of by the responding glia via phagocytic mechanisms. Below, I will provide a brief

summary of some of the molecular mechanisms currently known or suggested to underlie these steps.

"Find-me" signaling

"Find-me" signals are cues that direct phagocytes (glial cells, in this case) to tissue that is in need of phagocytic engulfment. A few examples that have been studied include nucleotides like ATP and UDP, and several chemokines such as fractalkine, CXCL10, and CCL21. These candidates "find-me" signals are discussed in more detail below.

Extracellular nucleotides as injury cues

While still relatively little is known about the signaling events involved in the migration of glia and their processes toward sites of CNS injuries, nucleotides (and in particular adenosine triphosphate (ATP)) have become leading candidates as "find-me" signals during the microglial response to acute injury. Pioneering two-photon *in vivo* work by Davalos *et al.* (2005) in the intact mouse cortex demonstrated that surveying microglial processes quickly chemotax toward microinjections of ATP in a fashion qualitatively similar to their procession toward acutely laser ablated tissue. Furthermore, the authors showed that the chemotaxis of microglial processes toward laser ablated tissue was abolished by application of either apyrase (an ATP-hydrolyzing enzyme), reactive blue 2 or PPADS (G-protein coupled purinergic receptor blockers), or flufenamic acid (a connexin channel blocker) [13]. This suggests that acute injury causes ATP to

be released, probably from the injured tissue itself, followed by additional ATP release by surrounding astrocytes [37], which together acts on microglial purinergic receptors to initiate chemotaxis. Follow up work demonstrated that cortical mouse microglia lacking G_i-coupled $P2Y_{12}$ receptors (which can bind ATP) failed to chemotax toward sites of injury during the early phases of the injury response *in vivo*, indicating that $P2Y_{12}$ receptors mediate this initial chemotaxis event. Microglial processes did eventually extend toward the injury site two hours after laser ablation, indicating that there are likely redundant pathways involved in this recruitment response which are as of yet unknown [53].

The nucleotides uridine diphosphate (UDP) and uridine triphosphate (UTP) have also been shown to initiate chemotaxis in microglia and promote their ability to phagocytose fluorescently labeled microspheres *in vitro*. These effects are blocked by an antagonist to the $P2Y_6$ receptor, a metabotropic purinergic receptor upregulated solely in microglia after kainic acid injection to the mouse hippocampus [54]. Furthermore, kainic acid treated hippocampal neurons release UTP/UDP in cell culture, and this nucleotide laden supernatant promotes microsphere phagocytosis when applied to microglial cultures [54], though ATP is also probably present in these supernatants so it is difficult to discern what effects are solely mediated by UTP/UDP and what effects are mediated by ATP. It is also difficult to know if UTP/UDP promotes phagocytosis directly (as a bona fide “eat-me” cue) or only indirectly as a chemotactic agent, allowing microglia to more efficiently find the material to be phagocytosed.

Fractalkine as a “find-me” signal

The chemokine fractalkine (also known as CX3CL1) might constitute another potential “find-me” signal, though the evidence for this is less direct than for ATP or UDP/UTP. Fractalkine is a transmembrane ligand that can be cleaved into a soluble extracellular form by ADAM metalloproteases and the cysteine protease Cathepsin S (CatS) [55-59]. Soluble fractalkine is a strong chemoattractant for monocytes (a peripheral immune cell type from which microglia are derived) *in vitro* [55], and furthermore the receptor for fractalkine, CX3CR1, is expressed exclusively on microglia in the CNS [22]. Fractalkine is expressed in neurons [60,61] and is found at elevated levels in the CSF after sciatic nerve injury. These elevated soluble fractalkine levels are dependent on CatS enzymatic activity, and CatS is upregulated in microglia following nerve injury [62]. This suggests that microglia somehow sense the injury and upregulate and secrete CatS in response. This secreted CatS then liberates fractalkine from injured neurons, which acts back on microglia as a chemoattractant. Interestingly, cultured microglia secrete CatS in response to high concentrations (mM range) of ATP sensed through the purinergic receptor P2X7 [63], providing a possible mechanism by which microglia sense the injury in the first place to then promote fractalkine signaling. Still, this effect was only observed when microglia were primed with the immune aggravant lipopolysaccharide (LPS) before ATP application, indicating that there are still other signals involved in this response that are not yet understood. The

regulation of fractalkine's subcellular localization specifically in injured neurons is also incompletely understood.

The chemokines CCL21 and CXCL10 as putative injury signals

In addition to fractalkine, the chemokines CCL21 and CXCL10 have also been proposed as potential "find-me" cues emanating from injured CNS tissue. CCL21 is released by cultured cortical neurons after glutamate induced excitotoxicity [64], and it is a known chemoattractant for cultured microglia [65] which express the receptor for CCL21, called CXCR3. Like CCL21, the chemokine CXCL10 also binds to CXCR3 and is expressed by cortical neurons in response to injury [66]. In a well established entorhinal cortical lesion model where microglia usually migrate to the site of injury and clear dendrites of affected neurons, genetic deletion of CXCR3 renders microglia unable to properly migrate and results in persistent denervated dendritic processes that would otherwise be cleared in the wild-type CNS [66]. It is unclear whether CXCL10 alone, CCL21 alone, or both of these chemokines is the bona fide injury signal in this injury paradigm.

To date, these are the most well described candidates for "find-me" signals released after CNS injury and sensed by the primary responding glia. It is still unclear how specific these cues might be to any given type of insult. There might be generalized "find-me" signals common to all injuries (such as ATP), as well as more specialized ones released only in certain types of insults. Whether

the same injury cues are used in all areas of the CNS and at all times during development and adulthood is also an unresolved issue.

“Eat-me” signaling

After a given responding glial cell locates a source of injury by following injury-induced “find-me” cues, it must then make contact with the injured tissue and actively phagocytose the material. Implicit in this task is the ability to discriminate between healthy tissue and injured tissue destined for destruction. How does a primary responding glial cell achieve this fine level of discrimination? Experimental evidence points to the existence of membrane-associated “eat-me” cues on the tissue to positively identify it for destruction, for example: phosphatidylserine and pathogen associated molecular patterns.

Phosphatidylserine as an apoptotic “eat-me” signal

Phosphatidylserine (PtdSer) is the most well-established “eat-me” signal, best known for its role in the clearance of apoptotic cells [67]. PtdSer is a core component of cellular lipid bilayers and under healthy cellular conditions it is actively moved from the outer leaflet of the plasmalemma membrane to the inner leaflet by transmembrane enzymes called phospholipid flippases [68]. This results in very little PtdSer being present on the extracellular surface of cell membranes under normal conditions [69]. When a cell undergoes apoptosis, caspases cleave phospholipid flippases, inactivating them [70]. This eventually leads to the redistribution of PtdSer molecules to the outer membrane leaflet by

the action of integral membrane proteins called scramblases [68]. The newly exposed PtdSer on the extracellular membrane surface then acts as an “eat-me” cue recognized by phagocytic cells, prompting engulfment of the PtdSer displaying cell (reviewed in [71]). While nearly all the early work investigating PtdSer’s role as an “eat-me” signal primarily assayed macrophage phagocytic activation by PtdSer, more recent work has established that microglia act similarly when exposed to PtdSer, indicating its role in immune phagocyte activation appears to be the same in the CNS as it is in the periphery [72].

Candidate phosphatidylserine “eat-me” receptors

The exact receptor(s) for PtdSer mediating its recognition by phagocytic cells is an area of intense debate [73]. The gene first described as the Phosphatidylserine Receptor (PSR) [74] turned out in later studies to be a histone arginine demethylase residing in the nucleus [75], so it is unlikely to be a receptor for PtdSer after all. Several other candidates have emerged as putative PtdSer receptors, most notably the T cell Immunoglobulin and Mucin-domain-containing molecule (TIM) family of proteins, including TIM-1, TIM-4, and TIM-3, which can bind PtdSer directly through their immunoglobulin V (IgV) domain [76,77]. While TIM-4 promotes phagocytosis of apoptotic cells, the downstream signaling in this TIM dependent phagocytosis is unclear since the transmembrane and cytoplasmic domains of TIM-4 are dispensable for phagocytosis of PtdSer presenting cells [78]. This indicates that the TIM’s might

act as tethering receptors in this phagocytic event without any inherent intracellular signaling activity themselves.

Another recently reported PtdSer receptor is Brain-specific Angiogenesis Inhibitor 1 (BAI-1) which can bind to PtdSer directly through the thrombospondin type 1 repeats in its extracellular domain [79]. Inhibition of BAI-1 leads to defects in apoptotic clearance *in vivo*, indicating that its interaction with PtdSer is functionally relevant. Importantly, BAI-1 was demonstrated to bind to and act upstream of the CrkII/Dock180/ELMO complex, a guanine nucleotide exchange factor (GEF) for the small GTPase Rac1 [79] (I will discuss these signaling molecules in more detail below).

Finally, Stabilin-1 and Stabilin-2, two scavenger receptors, have also been proposed to act as PtdSer receptors. Stabilin-1 was recently shown to directly interact with PtdSer and was demonstrated to be important for macrophage phagocytosis of aged red blood cells [80]. Similarly, exogenous expression of Stabilin-2 can turn otherwise non-phagocytosing fibroblasts into efficient engulfers of PtdSer-displaying aged red blood cells, and this effect is inhibited by either blocking PtdSer availability or by knocking down Stabilin-2 in the fibroblasts [81]. Interestingly, both Stabilin-1 and Stabilin-2 appear to require the intracellular adaptor protein GULP for their phagocytic engulfment functions [82,83]. GULP is the mammalian ortholog to the well characterized *C. elegans* engulfment protein CED-6, which will be discussed in more detail in the next section on phagocytosis.

To add further complexity to PtdSer mediated engulfment signaling, there are even some secreted proteins that can bind to PtdSer and act as bridging molecules that are themselves recognized by their cognate receptors on phagocytes. Examples of these are the Milk Fat Globule EGF factor 8 (MFG-E8) expressed by macrophages [84] and recognized by integrin complexes [85], and Growth arrest-specific 6 (Gas6) and Protein S which bind to the TAM family of proteins [86,87].

While PtdSer's role as an "eat-me" signal labeling apoptotic cells for phagocytic destruction is very well documented at this point, it is still not yet clear if PtdSer is also used as an "eat-me" signal in any other circumstances, such as in acute injury. While it is possible that acutely injured cells lose the ability to actively maintain normal PtdSer distributions (i.e. sequestering it from the exoplasmic membrane), this possibility is currently untested.

Pathogen associated molecular patterns as "eat-me" ligands

Another class of "eat-me" signals are pathogen associated molecular patterns (PAMPs). These are molecules, recognized by a host's innate immune cells, that are highly conserved in the pathogen of question [88]. These PAMPs effectively serve as molecular labels for cells that do not belong in the host. Lipopolysaccharide (LPS) is a good example of a PAMP. LPS is a core component of the outer membrane of gram-negative bacteria but is not present in the cells of most organisms that these bacteria can infect. This makes LPS an ideal molecule to be recognized by a host's innate immune cells to then elicit an

immune response to destroy the bacterial invaders. The list of characterized PAMPs is quite long but still almost certainly covers only a small fraction of all the PAMPs that are recognized by the innate immune systems of host organisms. Unfortunately, since PAMPs are generally associated with pathogenic cells foreign to a host [89], it is unlikely that they play a role in the "eat-me" signaling involved in identifying injured tissue in a host organism and therefore will not be covered further here. To date, there are simply no well established "eat-me" signals known to directly mediate the disposal of acutely damaged tissue. This is an area ripe for further scientific inquiry.

Phagocytic signaling

While knowledge of the "find-me" and "eat-me" signals involved in the glial immune response to acute injury remains woefully incomplete, details of the intracellular pathways guiding phagocytosis are somewhat more defined thanks in large part to many pioneering studies done in *Drosophila melanogaster* and *Caenorhabditis elegans*. In brief summary, two parallel phagocytic pathways have now been described that converge on the GTPase Rac1, a protein involved in actin cytoskeleton remodeling (Figure 1.2). These pathways, which are described in more detail below, are highly conserved from *C. elegans*, to *Drosophila*, to mammals.

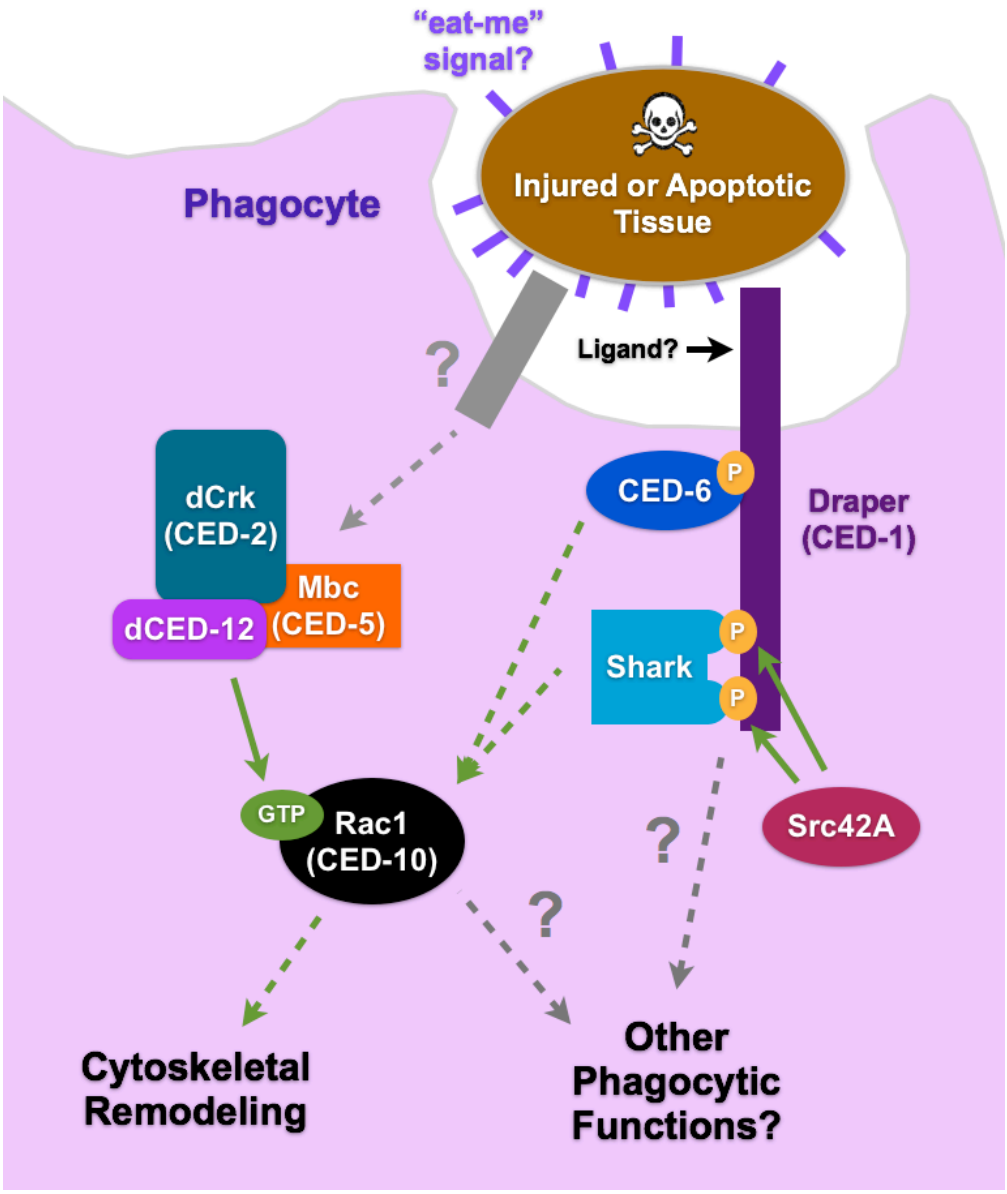


Figure 1.2. Current model of engulfment of injured and apoptotic CNS tissue in *C. elegans* and *Drosophila*. Studies in *C. elegans* and *Drosophila* have revealed two parallel pathways directing phagocytic engulfment of targeted tissue. The Draper (CED-1) pathway is involved in the early glial activation and recruitment phase of the injury response, while dCrk (CED-2), Mbc (CED-5), and dCED-12 (CED-12) are involved in the later recognition and/or engulfment stages of phagocytosis. In a parallel pathway, dCrk/Mbc/dCed-12 act in a complex as a guanine nucleotide exchange factor activating the Rho family GTPase Rac1. Rac1 is presumed to exert its effect on this phagocytic process through cytoskeletal remodeling but other roles have not been rules out. Rac1 also acts downstream of Shark in the Draper pathway. CED-6 is also presumed to act upstream of Rac1 since evidence suggests CED-6 and Shark act synergistically to influence glial engulfment.

Draper signaling

The most well characterized phagocytic receptor to date is the protein CED-1, first described in *C. elegans* for its role in engulfment of apoptotic cell corpses [90]. Further work examining the functions of this protein has been performed in *Drosophila*, whose ortholog of CED-1 is called Draper. Draper has been shown to be essential in glia for both clearance of apoptotic neurons [91] and for programmed axon pruning during development [92]. The closest mammalian orthologs of Draper, MEGF10 and Jedi-1, also play a critical role in glial clearance of apoptotic cells during mouse development, indicating that Draper's phagocytic function is very highly conserved [93].

Importantly, in addition to its pro-phagocytic role during the clearance of apoptotic cells, Draper also plays a pivotal role in glia in the clearance of severed axons after acute axotomy [94], indicating that Draper's pro-phagocytic functions are important in a variety of etiologically different situations that nonetheless require phagocytosis of whole cells and/or cellular debris.

The ligand(s) for Draper remains an area of some mystery and debate. Recent evidence suggests that the fly endoplasmic reticulum protein Pretaporter relocates from the ER to the cell surface and serves as a ligand for Draper during engulfment of apoptotic cells [95]. Additionally, one study has suggested that PtdSer might bind directly to Draper through its extracellular EMILIN-like and Nimrod-like domains and initiate phagocytosis of apoptotic cells [96], while a contradictory report proposes that Draper-mediated phagocytosis of apoptotic

cells is not dependent on PtdSer [97]. Still, while the exact ligand(s) for Draper involved in phagocytic engulfment of apoptotic cells remains an area of active debate, the ligand(s) responsible for Draper mediated phagocytosis of acutely injured tissue remains completely unknown.

Based on its amino acid sequence, Draper appears to be a pattern recognition receptor, with many cysteine rich extracellular domains including one Nimrod-like domain, one EMILIN-like domain, and 15 Epidermal Growth Factor (EGF)-like repeats [96,98]. Indeed, in at least one study Draper has been implicated in the identification and clearance of *Staphylococcus aureus* by *Drosophila* hemocytes (akin to mammalian macrophages) using lipoteichoic acid as its ligand [99].

On its intracellular tail, Draper possesses multiple signaling domains, including an NPxY phosphotyrosine binding motif and an immunoreceptor tyrosine-based activation motif (ITAM). The phosphotyrosine binding protein CED-6 (ortholog of mammalian GULP) binds the phosphorylated NPxY motif of Draper [100] and is required for clearance of apoptotic cells in *C. elegans* [101] and for programmed axon pruning by glia in *Drosophila* [92]. Additionally, the ITAM domain of Draper is also involved in mediating phagocytic signaling through its interaction with the non receptor tyrosine kinase Shark, which is orthologous to the mammalian Syk and Zap70 proteins [102]. Draper's ITAM domain is phosphorylated by the Src family kinase Src42A, and glial RNAi knockdown of either Shark or Src42A is sufficient to cause defects in glial clearance of severed axons [102]. Very recent evidence has situated the Rho

family GTPase Rac1 (also known as CED-10) downstream of Shark in this pathway, as glial expression of a constitutively active form of Rac1 can rescue the clearance defects seen in shark^{RNAi} expressing glia [103]. The current model favored by the available data is that activation of Draper by as of yet unknown injury induced ligands leads to phosphorylation of its intracellular signaling domains by Src42A (activating the ITAM domain) and other unknown tyrosine kinases (activating the NPxY domain), leading to association of CED-6 and Shark to Draper and eventual downstream activation of pro-phagocytic factors and machinery, including Rac1. Exactly what other signaling molecules lie downstream of these known players is not yet established.

dCrk/Mbc/dCed-12 complex signaling

In addition to the Draper signaling pathway, a parallel pro-phagocytic pathway has been identified in *C. elegans* that is centered around the CED-2/CED-5/CED-12 protein complex [104]. In flies the orthologous complex is called dCrk/Mbc/dCed-12, while in mammals the orthologous complex is CrkII/Dock180/ELMO. Importantly, this complex has been demonstrated in mammals to be a novel guanine nucleotide exchange factor (GEF) activating the GTPase Rac1 [105,106]. While mutations in any three of these genes in *C. elegans* leads to defects in cell corpse engulfment [107-109], their role as a complex in the glial response to acute axotomy has only very recently been described.

Both, the Draper and the dCrk/Mbc/dCed-12 pathway are essential for glial phagocytic activity, however recent studies propose that they drive distinct functions of the engulfment response. While the Draper pathway described above appears to be most important in the initial glial activation and membrane recruitment phase of the phagocytic response to injury [94,102,110], the dCrk/Mbc/dCed-12 pathway is critical for the later stages of the phagocytic process once glial membranes have already been recruited to sites of injury [103]. When glial dCrk/Mbc/dCed-12 signaling is knocked down using RNAi, glial membranes are still successfully recruited to sites of injury (albeit with a small delay) but they then fail to accomplish normal levels of phagocytosis. These recruited glial membranes linger for up to 7 days around the severed axons before eventually retracting and leaving behind significant amounts of axonal debris that would otherwise be cleared in phenotypically normal flies. Interestingly, glial expression of constitutively active Rac1 can rescue the defects caused by knockdown of components of either the Draper pathway or the dCrk/Mbc/dCed-12 pathway. This indicates that both pathways utilize Rac1, but it is used to accomplish different tasks at different times. Rac1 is important for glial activation and recruitment of membranes to sites of injury early on in the glial injury response, and it is also important for the ultimate phagocytosis of the injured debris. Exactly how Rac1 activation leads to both of these complex processes at different times in the glial injury response is not yet understood. Additionally, the upstream activators of the dCrk/Mbc/dCed-12 complex during the glial injury response remain completely unknown.

While much progress has been made in the last several years in understanding the molecular underpinnings of the glial immune response to acute injury, many questions still remain. What are the “find-me” and “eat-me” cues mediating the actions of responding glia? What are all the glial molecular pathways that allow for such a complex biological response as glial phagocytic clearance of acute injured tissue? Importantly, such a complex cellular task must involve countless cellular changes from transcription to translation. How are all these changes orchestrated? What are the master regulators in this process?

Drosophila as a model for elucidating glial immune responses

Drosophila melanogaster, also known as the common fruit fly or vinegar fly, has already been used to successfully identify and study many new proteins involved in the glial injury response. There are many practical benefits of using *Drosophila* for scientific study. The short generation times (~10 days) and the low cost of upkeep are powerful motivators to use this system. The genetic tools available in *Drosophila* to manipulate gene expression and visualize different populations of cells are simply unmatched by any other model organism, and for neuroscientific inquiry in particular the *Drosophila* CNS is quite amenable to modern microscopy techniques with very little preparation or manipulation of the tissue needed. Importantly, *Drosophila* has a complex nervous system with both neuronal and glial cellular components very similar in many respects to mammalian CNS counterparts. Recently, the glial subtypes of the adult *Drosophila* brain were characterized [110-112] and tools for glial specific

expression of gene products have become available, quickly turning *Drosophila* into a powerful system to study basic glial cell biology in ways that are otherwise much more difficult in higher vertebrate model systems. A simplified schematic showing of the glial subtypes in the central brain of *Drosophila* can be seen in Figure 1.3.

Drosophila has perineurial and subperineurial glia with long and flat morphologies that encase the entire fly brain in a tight sheath that comprises the fly blood-brain barrier system (note: these glial subtypes are not shown in Figure 1.3). In this way, the perineurial and subperineurial glia are most functionally homologous to ependymal cells in the mammalian CNS, though their cellular morphologies are quite different. Looking beyond the surface of the brain and deeper into the CNS parenchyma, *Drosophila* possesses three characterized glial subtypes. One is a seemingly novel type of glial cell located exclusively in cortical areas, having a flat and expansive morphology. These cortex glia tile *Drosophila* cortical regions, covering distinct, non-overlapping domains. Cortex glia appear to blanket the cell bodies of neurons, possibly providing direct trophic support to these cells. Another *Drosophila* glial subtype is ensheathing glia, which enwrap glomeruli and sub-glomerular structures in synapse-rich neuropil regions and also ensheath bundles of axons in nerve tracts. These ensheathing glia are most morphologically similar to mammalian oligodendrocytes, though they produce no myelin in their axonal wrappings, so it is currently unclear exactly how functionally homologous they are to their mammalian

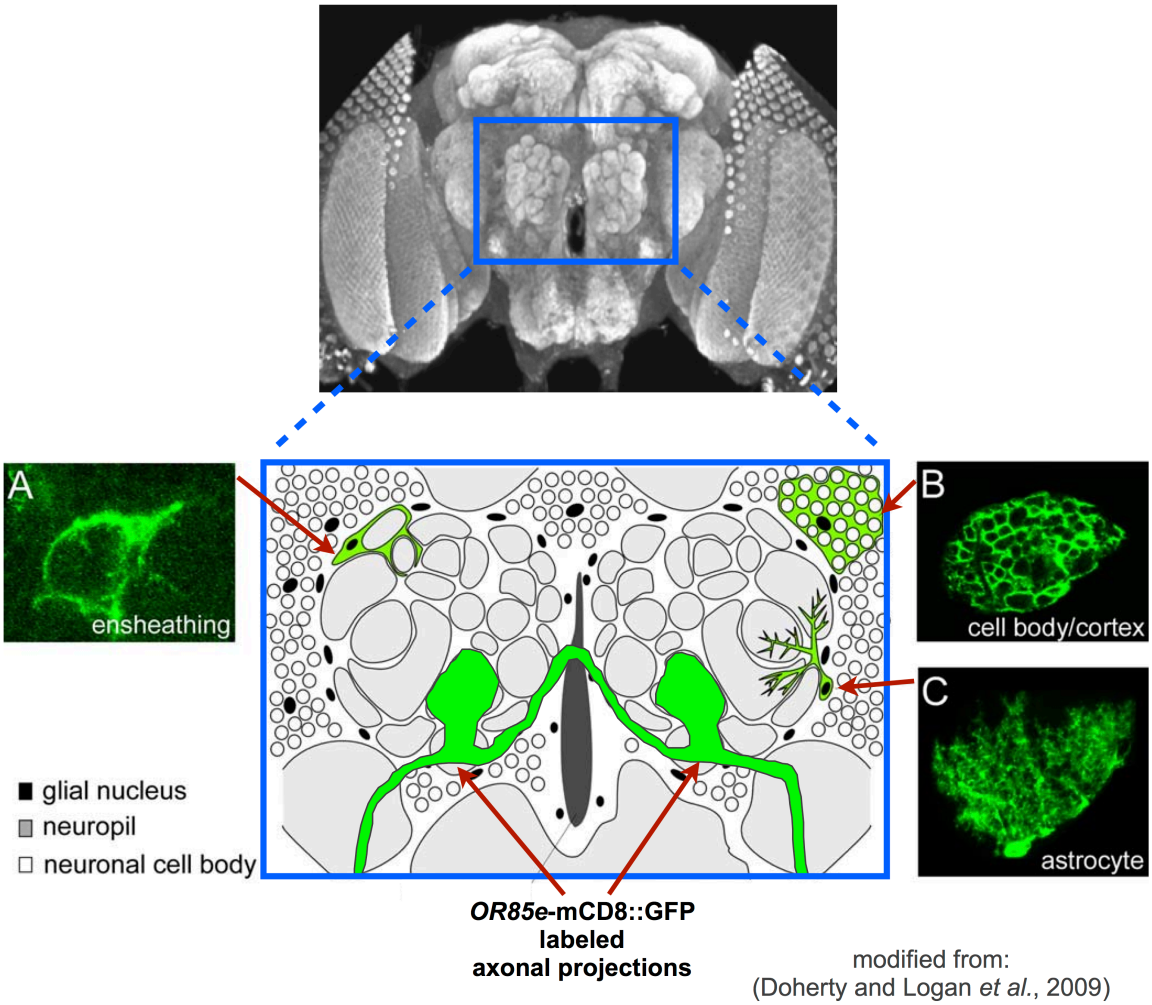


Figure 1.3. Representation of morphologically distinct glial subtypes in the *Drosophila* central brain. The pan-glial driver *repo*-Gal4 was used to generate MARCM glial clones with GFP by Doherty and Logan (2009), and glial subtypes were analyzed around the adult antennal lobe brain region. Three morphologically distinct subtypes of glial cells were identified: **(A)** ensheathing glia, **(B)** cortex glia, and **(C)** astrocytic glia, shown in their relative positions in the middle cartoon of the antennal lobe region. Ensheathing glia surround glomerular regions of neuropil with fine, apparently flattened processes. Cortex glia blanket neuronal cell bodies outside of neuropil regions, and each cortex glial cell has a distinct, non-overlapping domain. Astrocytic glia project highly branched and tufted processes into synapse-rich neuropil regions. The approximate position of *OR85e*-mCD8::GFP labeled axonal projections has also been superimposed on the schematic for orientation purposes.

oligodendrocyte counterparts. Lastly, *Drosophila* has astrocyte-like glia characterized by having highly tufted membrane processes that project into synapse-rich neuropil regions and intimately associate with synaptic connections. These cells have been demonstrated to modulate synaptic activity [113] and are presumed to play similar physiological roles as mammalian astrocytes [110-112].

It must be acknowledged that the *Drosophila* glial subtypes do not perfectly mirror those seen in mammals. More specifically, *Drosophila* do not possess a monocyte-derived microglia subtype like mammals, which appears to be an adaptation present only in vertebrates. Nevertheless, other cell types such as ensheathing glia have been demonstrated to take on the phagocytic role in the *Drosophila* CNS [110], and thus they represent an effective tool to uncover basic molecular pathways involved in glial immune function since these phagocytic pathways are likely conserved mammals. The conservation of Draper's function across evolutionary time from *C. elegans* to mammals is a prime example of this [93]. Nature does not often reinvent the wheel.

Although using *Drosophila* as a model organism has many advantages, there are also limitations that need to be taken into account. For example, *Drosophila* is not a perfect model for the entire mammalian immune system. This is due in large part to the fact that *Drosophila* does not possess an immunoglobulin family-based adaptive immune system as seen in mammals, so *Drosophila* cannot produce mammalian-like antibodies in response to infection. While the line between "adaptive" and "innate" immunity is not as clear as it was once thought to be, and *Drosophila* does show some signs of having a small to

moderate level of immune "memory" capacity [114], *Drosophila* is nevertheless a poor choice for modeling the antibody-based adaptive immunity found in humans. The flipside to this coin is that *Drosophila* still has a fully functional and effective innate immune system closely resembling the human counterpart. In fact, some fundamental elements of the human innate immune system, such as Toll-like signaling, were first discovered and elucidated in *Drosophila* [115,116]. So in many ways, *Drosophila* is actually an ideal model system for studying innate immune processes since there are no antibody-based adaptive immune components to play a role in the immune process and potentially confound results.

Drosophila olfactory injury assay

Having the ability to both spatially and temporally control gene expression in the *Drosophila* CNS opens up a wide range of ways to assay glial cell immune function at the biochemical level. To probe the underlying mechanisms of the glial response to acute injury, a simple and reliable axotomy paradigm has been developed that takes advantage of the fruit fly's unique olfactory system anatomy [94] (Figure 1.4). In this injury paradigm, subsets of olfactory receptor neurons (ORNs) are first genetically labeled with a membrane tethered green fluorescent protein (GFP). This GFP tag labels the entire cell surface of the given subset of ORNs, including their axons projecting into the *Drosophila* antennal lobes. The cell bodies of the ORNs can then be ablated in adult flies by simply removing the

Figure 1.4

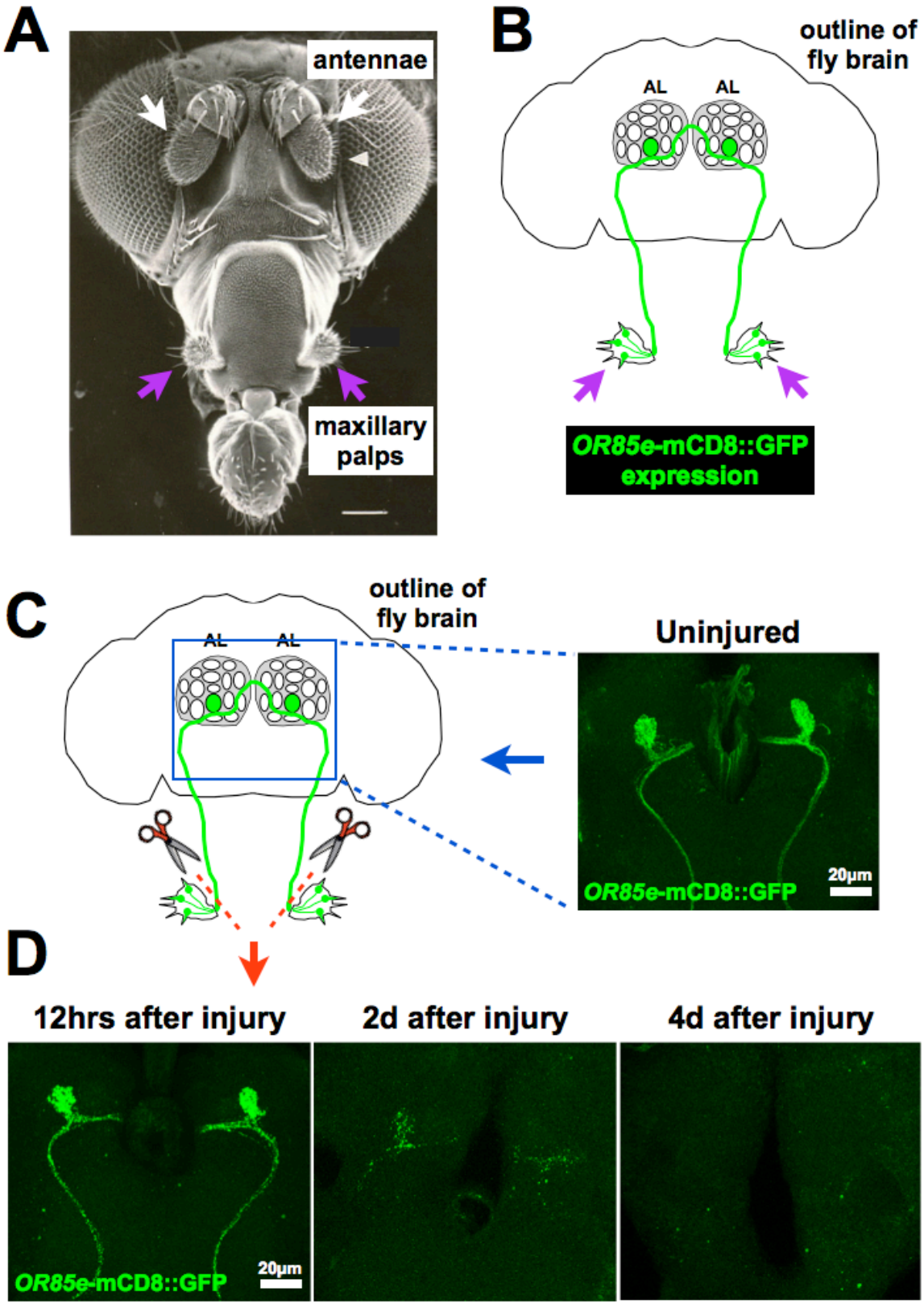

A
antennae
maxillary palps
B
outline of fly brain
AL
AL
OR85e-mCD8::GFP expression
C
outline of fly brain
AL
AL
Uninjured
OR85e-mCD8::GFP
20μm
D
12hrs after injury
2d after injury
4d after injury
OR85e-mCD8::GFP
20μm

Figure 1.4. *Drosophila* olfactory injury assay. (A) A scanning electron microscope image of a fly head shows the fly olfactory organs: the antennae (white arrows), and the maxillary palps (purple arrows). **(B)** Schematic representation of the fly brain and maxillary palps (purple arrows) shows *OR85e*-mCD8::GFP expression pattern. The cell bodies of *OR85e* expressing olfactory receptor neurons (ORNs) reside in the maxillary palp and these neurons send projections into the antennal lobes of the brain synapsing onto discrete glomeruli on both the ipsilateral and contralateral sides. **(C)** Schematic representation of maxillary palp ablation injury severing the *OR85e*-mCD8::GFP axons from their cell bodies in the maxillary palp. On right is a representative maximum intensity projected confocal z-stack image of uninjured *OR85e*-mCD8::GFP expression in the antennal lobes. **(D)** Representative maximum intensity projected confocal z-stack images of *OR85e*-mCD8::GFP signal 12 hours, 2 days, and 4 days after maxillary palp ablation. Most *OR85e*-mCD8::GFP positive axonal debris is cleared by glia with 5 days of maxillary palp ablation injury. Scale bars represent 20μm. Abbreviation: AL = antennal lobe.

entire olfactory organs housing them, these being either the antennae or the maxillary palps. Antennal ablation removes ~90% of axons innervating the antennal lobes while maxillary palp ablation removes ~10%, allowing for the selection of either a more severe or more minor injury depending on which organ is ablated [117-119]. Surgical ablation or either antennae or maxillary palps is non lethal to flies and leaves in its aftermath the now severed distal portions of the labeled ORN axons projecting into the antennal lobes onto discrete glomeruli. The severed axons undergoes classic Wallerian degeneration, with an initial latent phase of 6-8 hours, followed by axonal beading, cytoskeletal breakdown, and ultimately frank fragmentation [94].

The fragmented axonal debris is eventually cleared over the course of several days in this injury paradigm by ensheathing glia, a subtype of neuropil associated glia that intricately enwrap neuronal processes and whole glomerular structures [110]. Importantly, this injury model provides a very simple and

reliable way to assess glial clearance function by allowing the quantification of the GFP-labeled axonal debris burden at various time points during the course of the clearance event. This sets up a system where not only previously known innate immune genes can be studied in the context of acute CNS injury, but entirely new gene candidates can be identified through forward genetic screens. The work presented in Chapters 2 and 3 directly resulted from an initial genetic screen that identified the *Drosophila* insulin-like receptor (InR) as a novel component involved in the glial response to injury. With this in mind, I will now briefly introduce insulin-like signaling to provide a context for my work in the following chapters.

Insulin and IGF signaling: a brief primer

Since its discovery in 1921 by Frederick Banting, Charles H. Best, James B. Collip, and John J. R. Macleod [120,121], the understanding of insulin's importance to human physiology has steadily grown. Insulin signaling is best known for its role in Type 1 and Type 2 diabetes mellitus, diseases which are of great epidemiological concern at the present with current estimates of nearly 10% of the US population being afflicted [122] and an estimated 347 million people burdened with these diseases worldwide [123]. At its core, diabetes mellitus is characterized by abnormally high blood glucose levels resulting from defects in insulin signaling. These defects can stem from insufficient insulin production by the pancreas (Type 1 diabetes) or by reduced insulin sensitivity at the receptor level in target tissues (Type 2 diabetes), though Type 2 diabetics

often develop secondary problems with insulin production as their disease progresses [124]. The exact molecular etiology of diabetes is unknown in most cases, though Type 2 diabetes has many risk factors strongly associated with its development such as obesity, lack of exercise, family history of the disease, and race [125,126].

Insulin is a peptide hormone that in humans is produced almost exclusively by the beta cells of the islets of Langerhans in the pancreas. Insulin's primary role in the periphery is metabolic, regulating the cellular uptake of glucose from the blood and its subsequent storage as glycogen or lipids (reviewed in [127]). Insulin's action is mediated by binding to the insulin receptor, a receptor tyrosine kinase capable of signaling through many intracellular pathways, most notably the phosphatidylinositol 3-kinase complex (PI3K) and Akt (also known as Protein Kinase B) signaling pathways.

In vertebrates there also exist paralogs to insulin called insulin-like growth factors (IGF-1 and IGF-2). These are peptide hormones produced primarily in the liver [128] that bind preferentially to their own receptors in target tissue, the insulin-like growth factor receptors (IGF-1R and IGF-2R). IGF-1R and IGF-2R are paralogous to the insulin receptor, though IGF-2R has a truncated intracellular domain with no tyrosine kinase capabilities and is thought to act primarily as a modulator of extracellular IGF availability by binding to and sequestering IGF ligands [129]. IGF-2 largely has similar roles as IGF-1, as its main functional receptor target is also the IGF-1R. As its name suggests, IGF signaling is generally considered to be mitogenic, supporting the growth,

differentiation, and survival of cells. IGF signaling utilizes many of the same intracellular cascade networks as insulin signaling, and exactly how these two signals lead to different cellular outcomes in many instances is not yet completely understood [130]. The insulin and IGF pathways both share more in common than they differ. Upon activation of the insulin receptor by insulin, or the IGF-1R by IGF-1 or IGF-2, the intracellular β-subunits of these receptors auto-phosphorylate, leading to recruitment of substrate adapters, most notably the Insulin Receptor Substrate (IRS) proteins. These IRS proteins can then activate at least two fundamental signaling pathways, the PI3K/Akt pathway and the Ras/MAPK pathway. While intricate detail of all the signaling events possible in these pathways is beyond the scope of the this work, it should instead simply be noted that these pathways represent fundamental signaling nodes for cells. In short, Insulin/IGF signaling can regulate many basic aspects of cellular activity, such as transcription, translation, and metabolic homeostasis [131-134].

Insulin/IGF signaling in the CNS

Current evidence suggests that only small amounts of IGF-1 and insulin are made in the mammalian brain (reviewed in [135,136]). Nevertheless, expression of insulin and IGF receptors is widespread throughout the CNS [137-139], and both neurons and glia have been demonstrated to express these receptors [140-142]. Where are the ligands for these receptors coming from? The main source appears to be the bloodstream. Insulin, IGF-1 and IGF-2 can all enter the brain through a saturable transport mechanism across the blood

brain barrier (BBB) [143,144]. The exact nature of this transport mechanism is still a highly debated topic, but some evidence suggests that it is receptor mediated (reviewed in [145]). This is consistent with the observation that Type 2 diabetics generally have lower CSF levels of insulin, presumably due to receptor insensitivity at the BBB and therefore reduced transport into the CNS [145,146].

What role does insulin and IGF signaling play in the adult CNS? Surprisingly, while the cytoprotective and mitogenic nature of IGF signaling appears to be conserved in the CNS as in the periphery, the role of insulin is quite different. The strongly metabolic based actions of insulin in the periphery are largely absent in the CNS, which is the sole tissue in the body that regulates glucose uptake nearly independent of insulin (reviewed in [146,147]). The insulin insensitive glucose transporter GLUT-1 is highly expressed in the endothelial cells composing the mammalian BBB [148] and allows an estimated 50 times more glucose to pass into the brain than would otherwise passively enter [149]. Once in the CNS, glucose is taken up as needed by neurons and glia expressing various glucose transporters of their own, most of which are insulin insensitive [148,150]. If not heavily involved in CNS metabolism, what role is insulin playing in the brain? Over the last 20 years growing evidence suggests that insulin in the CNS is involved in regulating many higher brain functions, such as regulation of food intake, learning, and memory formation (reviewed in [151]). Additionally, IGF-1 has also been implicated in many higher brain functions similar to insulin, such as cognition, learning, and memory formation, in addition to more basic “housekeeping” roles as a growth factor critical for cell survival, differentiation

and proliferation (reviewed in [152]). However, how these signaling pathways exert their effects on higher order brain functions is still very much an area of active research.

Insulin/IGF signaling in CNS injury

Insulin and IGF signaling is important for many functions of the healthy body, but it also plays a critical role during injury and disease. Interestingly, one consistent finding first observed almost 20 years ago is a local upregulation of IGF-1 signaling components at sites of CNS injury [153]. Both the IGF-1 ligand and the IGF-1R receptor are upregulated after injury in neurons, astrocytes, and microglia [153,154]. Additionally, during an ischemic insult to the mouse brain, IGF-1 is upregulated in microglia and has been shown to increase the proliferation of both microglia and invading macrophages in an apparent autocrine/paracrine mitogenic signaling event [154]. It has also been shown that an intraventricular administration of IGF-1 is neuroprotective after a hypoxia ischemia injury in the adult rat [155]. Furthermore, exercise, which leads to higher IGF-1 serum levels (and presumably in turn higher levels in the CNS), is neuroprotective against a variety of CNS insults, such as domoic acid injection, 3-acetylpyridine injection, or genetically inherited neurodegeneration. These effects are completely abrogated by application of serum antibodies to IGF-1, indicating that IGF-1 is indeed the causative agent by which exercise is exerting its neuroprotective effects [156]. Importantly, many of the studies conducted on IGF-1's neuroprotective role have not examined the precise details by which IGF-

1 exerts its effect and in what cell types its signaling is most important. It has simply been assumed that IGF-1's neuroprotective effect results from direct trophic support of neurons. This is not an unreasonable assumption since IGF-1 has been demonstrated to be neuroprotective against a variety of insults in neuronal monocultures *in vitro* with no other cell types present [157,158]. IGF-1 undoubtedly has a direct protective effect on neurons *in vivo* after an insult, but it is also possible that IGF-1 could have an additional indirect neuroprotective effect, serving to bolster glia as they respond to the insult. Unfortunately, this hypothesis has not been rigorously tested, probably due in part to the technical difficulties of performing these kinds of experiments *in vivo* in mammalian systems.

Insulin/IGF signaling in CNS disease

In addition to acute injury, insulin/IGF signaling appears to also play a role in some neurodegenerative diseases. Chronic hyperinsulinemia, like that often found in type 2 diabetes mellitus, is associated with a decline in memory-related cognitive scores in human patients and is a significant risk factor for developing Alzheimer's disease (AD) [159]. This chronic hyperinsulinemia in the periphery is actually associated with a deficiency of insulin in the CNS, presumably due to aberrant transport across the BBB in insulin resistant patients (as mentioned above). Human patients with lower levels of insulin in the CNS have an increased incidence of Alzheimer's disease, and in general CNS insulin levels decrease with age at the same time that cognitive decline rises [160].

Furthermore, in a mouse model of Alzheimer's disease, IGF-1 signaling is extremely perturbed [161]. The exact mechanistic link between AD and insulin/IGF signaling is not completely clear, but some part of AD pathology could be due to inadequate microglial clearance function [162], which itself could be influenced by aberrant IGF signaling. Consistent with this notion is the finding that serum IGF-1 is neuroprotective partly through its promotion of amyloid-β clearance in the brain [163]. The exact details of this IGF-1 induced amyloid-β clearance have not been explored, but it is almost certainly mediated through microglia as these cells are also able to clear amyloid-β deposits from the brain (discussed above and reviewed in [164]). Interestingly, macrophages derived from AD patients display a reduced ability to phagocytose amyloid-β effectively [165], further adding to the notion that there is probably a glial phagocytic defect involved in the pathology of AD. Whether or not this defect is at all due to the abnormal insulin/IGF signaling associated with AD is still an unresolved question. In general, very little work has been done to characterize glial specific roles of insulin/IGF signaling. Both the inherent complexity of the mammalian insulin/IGF signaling pathways (which contain many gene duplications, for instance) and the difficulty in performing *in vivo* examination of glial function in mammalian models has hindered progress on this front.

Drosophila insulin-like signaling as model pathway to study insulin/IGF function *in vivo*

One emerging avenue forward in the study of insulin/IGF signaling *in vivo* in various cell types is to use *Drosophila melanogaster* as a model. The insulin/insulin-like signaling pathway is very highly conserved across evolutionary time and compared to mammals, the *Drosophila* insulin-like signaling pathway is somewhat simpler, with fewer gene duplications. Nevertheless, nearly all the core components of the pathway are conserved [166,167] (Figure 1.5 and Table 1.1). *Drosophila* has a single unified receptor, called the insulin-like receptor (InR) [168], which serves the roles that both the insulin receptor and IGF-1R play in mammals. It shares nearly 35% sequence identity with the human insulin receptor, and about the same with the IGF-1R [169]. It is thought to represent a more ancestral receptor present before the insulin-like receptor gene was duplicated in the vertebrate lineage to yield multiple insulin-like receptors with more specialized functions (insulin receptor, IGF-1R, IGF-2R, etc.) [147]. *Drosophila* possesses 8 insulin-like peptides (ilp) that are expressed in various parts of the fly and serve as ligands for the InR [170-172] (Figure 1.6). Similar to mammalian insulin/IGF signaling, *Drosophila* insulin-like signaling is critical for sugar and lipid metabolism, cell growth, survival, and differentiation. Importantly, a growing body of InR research is *Drosophila* is establishing previously unknown roles for the InR pathway, such as its recently discovered role in aging and longevity [173]. Overall, studies of *Drosophila* insulin like signaling now represent a new frontier in insulin/IGF signaling research. The fly opens up

whole new avenues of inquiry, allowing for targeted, cell-type specific *in vivo* analysis of the many functions of this ancient and fundamental signaling pathway.

In the following chapters, I detail my work testing the hypothesis that insulin-like signaling is a high-level regulator of the glial clearance response to injury. The aim of this work is to better understand the molecular underpinnings of the innate glial immune response, a biological process that is critical for the integrity of the CNS in health, injury, and disease.

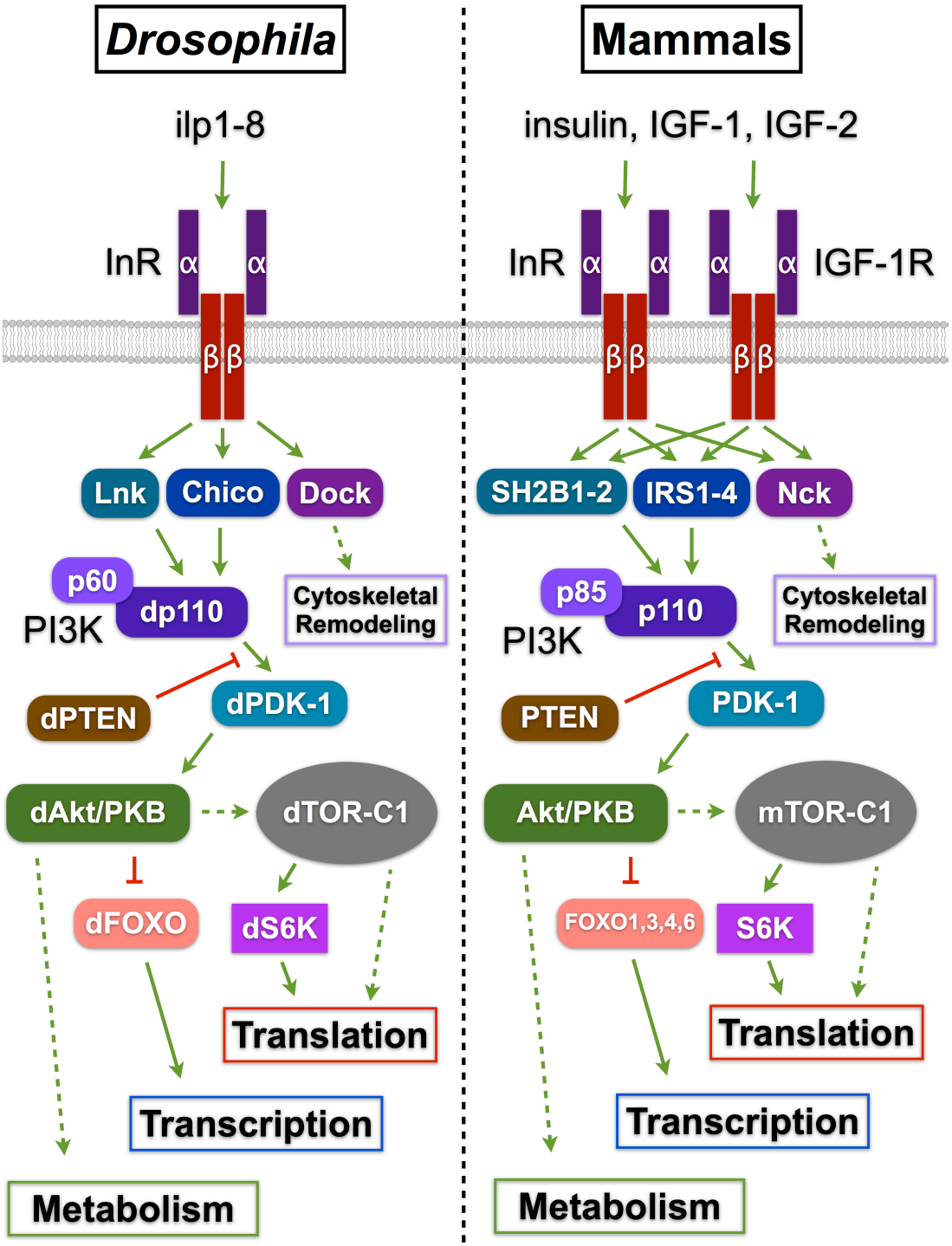


Figure 1.5. Conservation of insulin-like signaling between *Drosophila* and mammals. Condensed and simplified depiction of insulin-like signaling in *Drosophila* and in mammals, highlighting the phosphatidylinositol 3-kinase complex (PI3K) and Akt signaling components of the respective pathways. Both pathways are important regulators of transcription, translation, metabolic homeostasis, and cytoskeletal remodeling.

Table 1.1. Abbreviated list of orthologous Insulin-like signaling pathway components in mammals, *Drosophila,* and *C. elegans*.

Mammals	***Drosophila melanogaster***	***Caenorhabditis elegans***
Insulin Receptor (InR) IGF-1 Receptor (IGF1-R) IGF-2 Receptor (IGF2-R)	Insulin-like Receptor (InR)	DAF-2
Insulin Receptor Substrate (IRS) 1-4	Chico	IST-1
SH2B adaptor protein 1-2	Lnk	ABL-1
Nck	Dock	NCK-1
p85 (PI3K regulatory subunit 1a)	dp60	AAP-1
p110 (PI3K catalytic subunit 1a)	dp110	AGE-1
PTEN	dPTEN	DAF-18
PDK-1	dPDK-1	PDK-1
Akt / PKB	dAkt	Akt-1 Akt-2
FOXO1,3,4,6	dFOXO	DAF-16
CrkII	dCrk	CED-2
Dock180 / DOCK1	Mbc	CED-5
ELMO	dCed-12	CED-12
Rac1	Rac1	CED-10
MEGF10 Jedi-1	Draper	CED-1

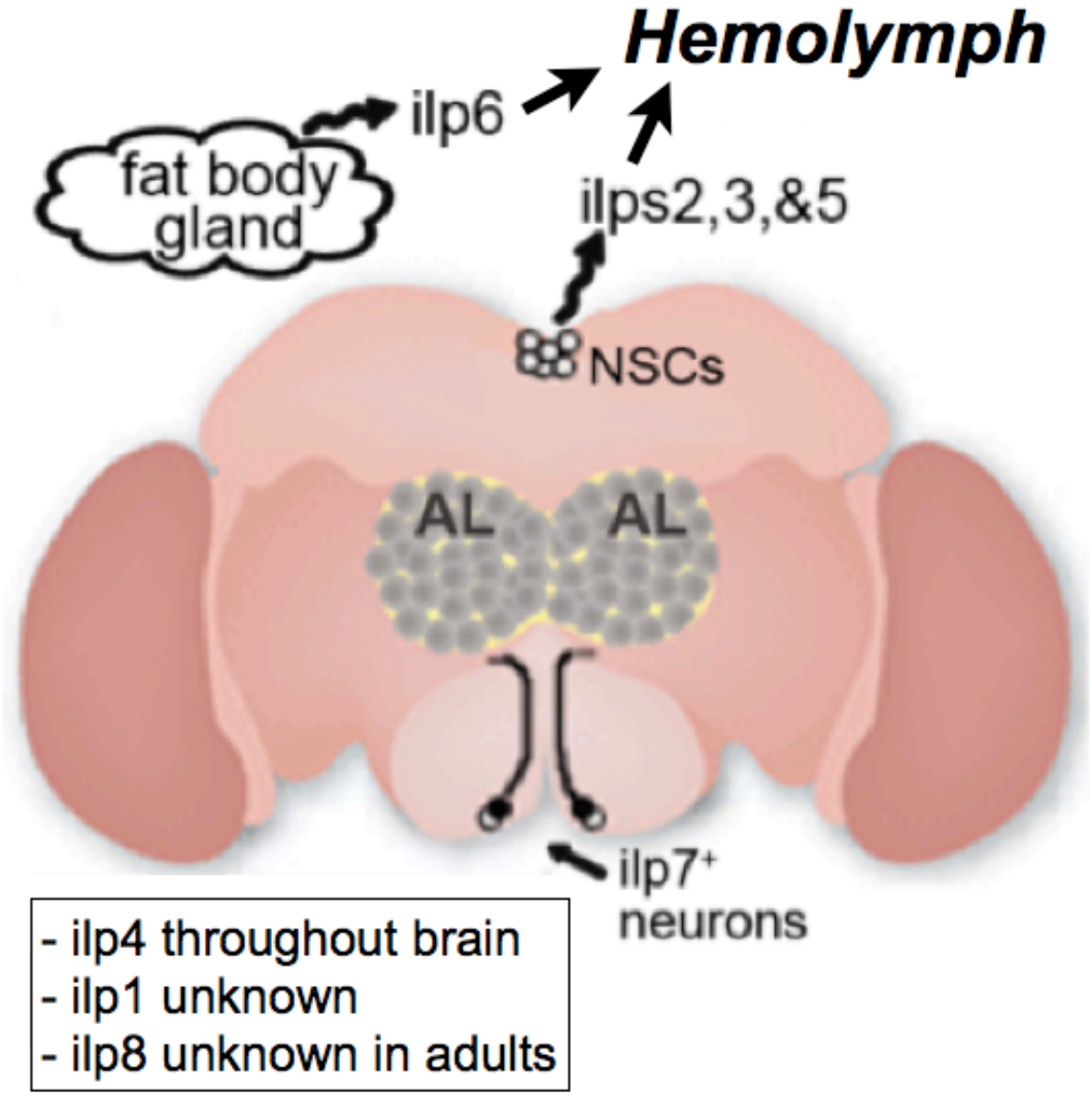


Figure 1.6. Expression pattern of insulin-like peptides in adult *Drosophila*. Eight insulin-like peptides (ilps) have been discovered in *Drosophila*. ilp6 is expressed primarily in the fat body (which serves both adipose and hepatic-like functions) and released into the circulating hemolymph. ilps 2, 3, and 5 are expressed at high levels in the median neurosecretory cells of the brain and released into the hemolymph. ilp7 is expressed in a small subset of CNS neurons in the brain and ventral nerve cord. ilp8's expression is unknown in the adult fly. ilp1 expression has never been detected, indicating that this ilp might be a pseudogene.

Chapter 2

Insulin-like signaling regulates adult glial immune response to axon degeneration in *Drosophila*

INTRODUCTION

Glia are the resident immune cells of the brain and they respond swiftly and robustly to changes in CNS health. Both chronic and acute insults can trigger a set of glial immune responses including directed glial migration to sites of damage, and clearance of degenerating neurons through phagocytic engulfment [27,28,94,162,174,175]. These complex innate glial immune responses are essential for minimizing collateral damage after acute injury and in neurodegenerative disease states by clearing damaged cells and debris before they elicit larger inflammatory responses [27,28,176,177]. Despite the important role that glial cells play in protecting brain health, we still have a poor understanding of the molecules and signaling pathways that govern glial immune responses. In order to fully harness the protective power of innate immunity, we must fully elucidate how glia detect changes in neuronal health, how the complex cellular events associated with innate immune responses are initiated and efficiently carried out and, finally, how these robust responses are shut down once an immune response is no longer required.

The insulin-like signaling (ILS) pathway, which includes insulin and insulin-like growth factor (IGF) signaling in vertebrates, is a highly evolutionarily conserved pathway that influences many important aspects of development such as regulation of cellular size and differentiation [178-181]. It is now becoming increasingly clear that ILS also contributes to mature brain function and maintenance. IGF ligands circulating in the blood serum have the capacity to cross the blood-brain barrier to activate their cognate receptors in the CNS [143].

A few intriguing studies suggest that the ILS pathway is neuroprotective. For example, reducing serum IGF-1 levels in mice through pharmacological intervention results in greater neuronal death in traumatic brain injury models and a striking decline in cognitive function in neurodegenerative disease mouse models [163,182,183]. IGF-1 injections in Alzheimer's Disease (AD) mouse models, conversely, reduce amyloid-β accumulation and restore some cognitive ability [163,184-186]; IGF also protects against acute insults, such as kainic acid injection, domoic acid injection, and 3-acetylpyridine injection, and hypoxia ischemia injury [155,156,187]. Such findings suggest that ILS pathways influence the risk for neuronal death in a variety of contexts and may be engaged after a range of neural insults, but the details of ILS-mediated neuroprotection and the physiological consequences of activating this pathway at acute injury sites are still unclear.

Insulin-like signaling pathways are highly conserved across species ranging from yeast to mammals with respect to the core signaling components and biological processes, such as cell proliferation, metabolic homeostasis, and longevity [166,167,188]. The *Drosophila* ILS pathway consists of eight insulin-like peptide (ilp) ligands and a single Insulin-like Receptor (InR) [168,170-172]. InR signaling is most commonly initiated by the kinase Chico [189,190] to activate the PI3K/Akt signaling cascade that influences gene transcription and protein translation [178,191-193], although Chico-independent signaling has also been reported to modulate cytoskeletal remodeling in migrating retinal neurons [194] and low level PI3K/Akt activation in some cell types [195,196]. The

expression of the eight *Drosophila* ilp ligands is complex during development and in adult tissues [170-172,197-199]. Select ilps are expressed at a high level in neurosecretory cells, as well as in the fat body (an adipose and hepatic-like tissue in *Drosophila*); these ilps are all released into the circulating hemolymph of the fly, although other ilps are expressed in very discrete locations including in regions of the CNS (see Figure 1.6 for an overview). Given that the *Drosophila* genome contains only a single InR, it is presumed to be ubiquitously expressed.

Little is known about ilp/InR signaling between glial cells and neurons, although recent work revealed that insulin-like signaling between neuroblasts and the glial niche governs neuroblast proliferation rates during development [200,201]. Here, I have investigated ILS in adult glia in the context of innate glial immunity and discovered that the InR is a novel regulator of glial responses to degenerating axons in the adult *Drosophila* brain. Reduced InR in adult glia inhibits the expression of a critical glial engulfment receptor, Draper, and results in delayed clearance of axonal debris from the central brain after axotomy. Notably, forced expression of Draper partially rescues the engulfment defects that result from InR inhibition. Finally, I show that InR activity is robustly upregulated in local glia after axon injury, suggesting that insulin-like signaling may represent a novel neural injury-glia communication relay that elicits a range of key innate glial immune reactions.

RESULTS

The Insulin-like receptor is required for proper glial clearance of severed axons in the adult Drosophila brain.

As part of a large-scale screening strategy, our lab discovered that the InR is required in glia for glial clearance of degenerating axons in the adult brain. Specifically, we expressed publicly available *Drosophila* RNA interference (RNAi) constructs [202] in glia using the pan-glial driver *repo*-Gal4. These flies also carried a transgene to label a subset of maxillary palp olfactory receptor neurons (ORNs) with membrane-tethered GFP (*OR85e*-mCD8::GFP). Surgical removal of the maxillary palps severs the maxillary nerves, thereby inducing Wallerian degeneration of the ORN axons and triggering an innate immune response that is ultimately leads to phagocytic engulfment of the severed axonal debris by local glial cells in the antennal lobe area [94,110]. Our lab found that expression of dsRNA against the insulin-like receptor (UAS-InR^{RNAi}) inhibited glial clearance of severed *OR85e*-mCD8::GFP labeled axons 5 days after maxillary nerve axotomy (data not shown). Since insulin-like signaling (ILS) is critical for many aspects of development, including cell proliferation and growth [188,190,199], I wanted to determine whether the glial clearance phenotype found in our initial screen was a result of an acute effect on adult glial function or a result of reduced ILS during development. Thus, I conditionally induced expression of InR^{RNAi} in adult glial cells by using a temperature sensitive variant of Gal80 ($Gal80^{ts}$) [203], which is a powerful repressor of the Gal4 transcription factor. This strategy allowed me to temporally control the expression of UAS-regulated transgenes and bypass

developmental phenotypes (Figure 2.1A). Flies were reared at the Gal80^{ts} permissive temperature (18°C) to inhibit Gal4 activity. Eclosed adults were shifted to the restrictive temperature (30°C) to induce Gal80^{ts} destruction and initiate Gal4/UAS activity (Figure 2.2A). Using this method, I expressed InR^{RNAi} in adult glia and found that this significantly inhibited glial clearance of axonal debris, as there was significantly more GFP^{+} axonal debris in the antennal lobes of glial InR^{RNAi} flies (Figure 2.2B and C). I used an alternative approach to inhibit ILS within glia by expressing a well-characterized dominant negative version of InR (UAS-dnInR) and found that this similarly inhibited glial clearance of severed axons (Figure 2.2B and C). I also performed an important control experiment in which I repeated the injury and quantification of GFP^{+} axonal debris in the same genotypes of flies that were maintained at 18°C (protocol illustrated in Figure 2.1B). I observed almost complete clearance of degenerating OR85e axons in all flies, with no significant difference between genotypes, confirming the fidelity of the Gal80^{ts} system to effectively block Gal4 activity at 18°C (i.e. during the development of the flies) (Figure 2.2D and E). Together, these results suggest that the conserved ILS pathway is required in adult glia to properly respond to and phagocytose degenerating axons.

Activation of glial InR signaling promotes faster clearance of axonal debris

Activation of certain “pro-engulfment” pathways in phagocytic cells has been shown to increase the rate of phagocytic activity and destruction of engulfment targets [204,205]. I therefore wondered if activating high levels of ILS in adult glial cells would result in faster glial clearance of degenerating axons.

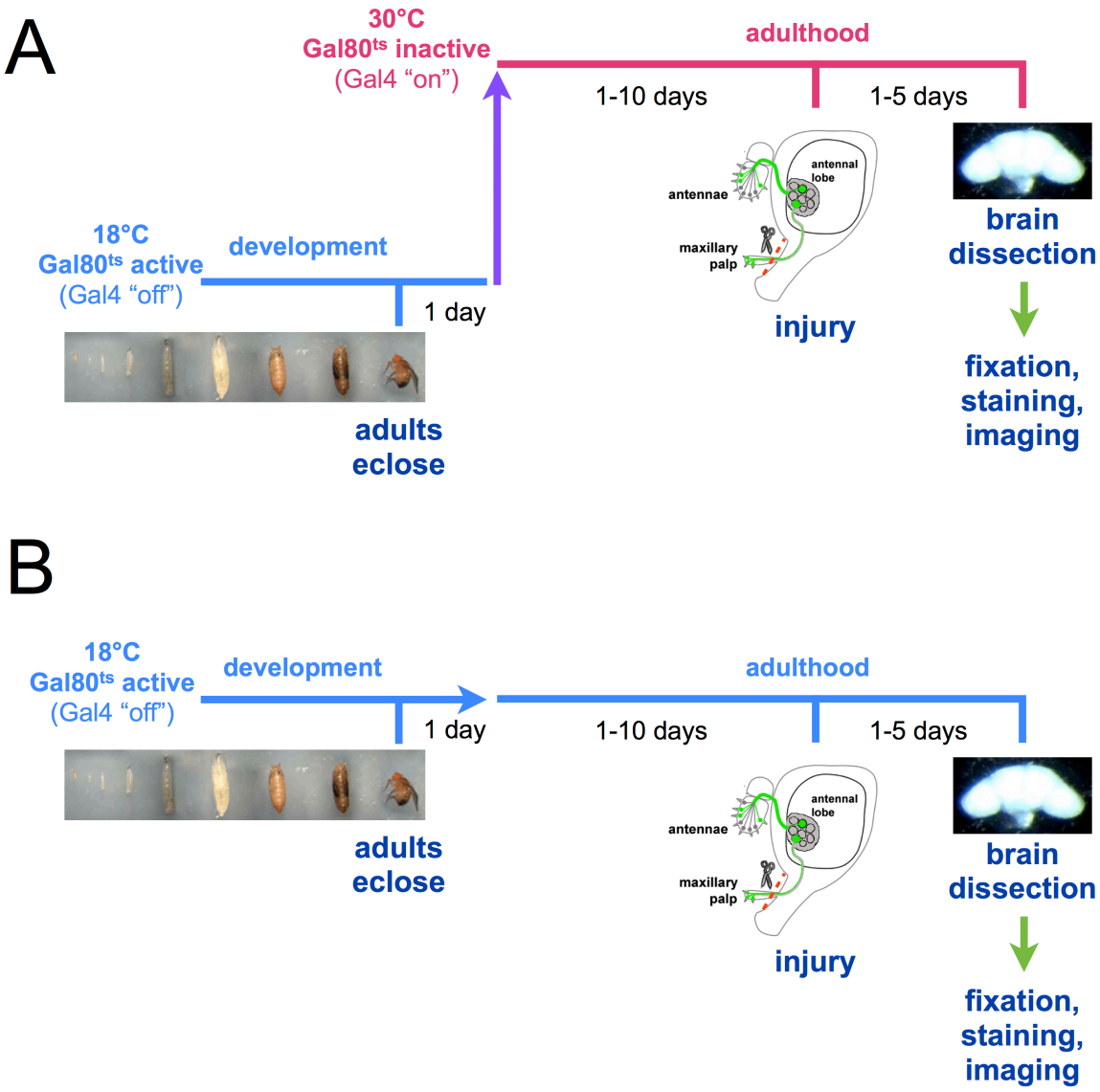


Figure 2.1. Experimental protocol using Gal80^{ts} for temporal control of Gal4/UAS expression system in olfactory injury paradigm. (A) Schematic overview of experimental protocol to activate adult-specific gene expression and time course of olfactory injury. Gal80^{ts} inhibits GAL4/UAS regulated gene expression at 18°C. Adult flies were shifted to 30°C to induce Gal80^{ts} degradation, which allows GAL4 to activate UAS-responsive transgenes. **(B)** Schematic overview of experimental protocol for 18°C olfactory injury control animals. Gal80^{ts} inhibits GAL4/UAS regulated gene expression at 18°C during development and adulthood. This paradigm tests the precision of Gal4/UAS temporal control with Gal80^{ts} expression, revealing any phenotypes induced by "leaky expression" of transgenes that might otherwise confound results.

Figure 2.2

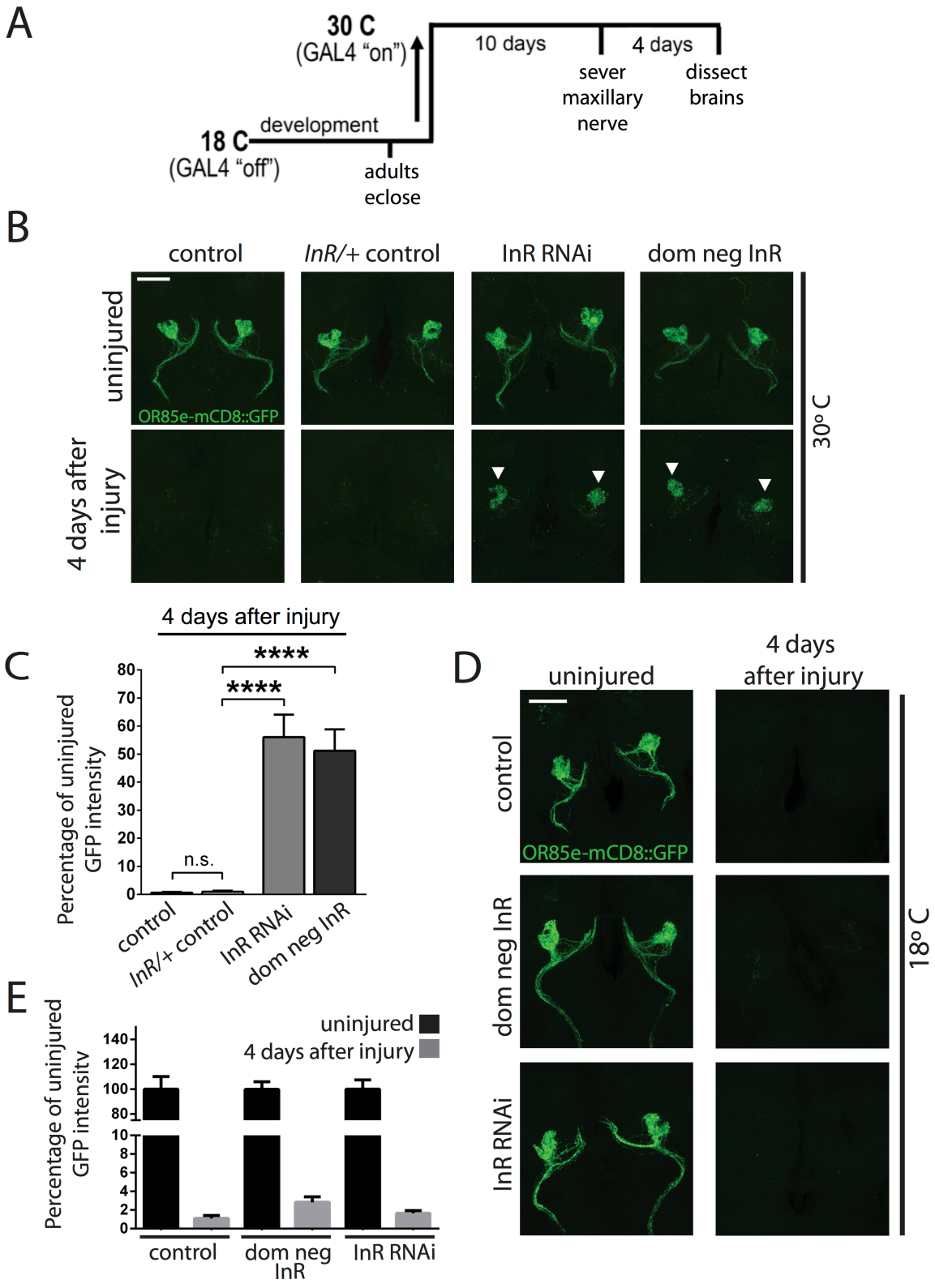

A
30 C
(GAL4 "on")
10 days
4 days
sever maxillary nerve
dissect brains
development
18 C
(GAL4 "off")
adults eclose
B
control
InR/+ control
InR RNAi
dom neg InR
uninjured
4 days after injury
OR85e-mCD8::GFP
30° C
C
4 days after injury
****
****
Percentage of uninjured GFP intensity
n.s.
control
InR/+ control
InR RNAi
dom neg InR
D
uninjured
4 days after injury
control
OR85e-mCD8::GFP
dom neg InR
InR RNAi
18° C
E
Percentage of uninjured GFP intensity
uninjured
4 days after injury
control
dom neg InR
InR RNAi

Figure 2.2. Glial InR signaling is required for proper glial clearance of degenerating ORN axons. (A) Schematic overview of experimental paradigm to activate adult-specific gene expression. Gal80^{ts} inhibits GAL4/UAS regulated gene expression at 18°C. Adult flies were shifted to 30°C to induce Gal80^{ts} degradation, which allows GAL4 to activate UAS-responsive genes. **(B)** Maximum intensity confocal projections of the antennal lobe region show that *OR85e*-mCD8::GFP maxillary palp axons (green) are normally cleared from the antennal lobe region within 4 days after severing the maxillary nerve of control animals (control). In flies that lack one copy of the InR gene (InR^{-}/+ control), degenerating OR85e axons are also cleared from the brain with 4 days after axotomy. Further inhibition of InR signaling within glia through expression of double stranded RNA against the InR (InR RNAi), or a dominant negative version of the InR (dnInR), resulted in significantly more GFP^{+} axonal debris present in the antennal lobe regions (white arrowheads). Scale bars = 20µm. **(C)** Quantification of data presented in panel B, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Ordinary 1-way ANOVA with Dunn's multiple comparisons test, **** $p < 0.0001$. N = 11 for control; N = 12 for InR^{-}/+ control; N = 15 for InR RNAi; N = 14 for dnInR. **(D)** At 18°C, clearance of severed OR85e axons in InR RNAi and dominant negative InR flies was comparable to controls, indicating the GAL80^{ts} system effectively inhibits InR^{RNAi} or dominant negative InR expression, respectively, at this lower temperature. Scale bar = 20µm. **(E)** Quantification of experiment shown in panel D, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Ordinary 1-way ANOVA with Dunnett's multiple comparisons test, n.s. $p > 0.05$. N = 10 for control uninjured; N = 7 for control injured ; N = 7 for dnInR uninjured; N = 7 for dnInR injured; N = 9 for InR RNAi uninjured; N = 8 for InR RNAi injured. Genotypes in Figure 2.2: control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4*/+. InR^{-}/+ control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/+. InR RNAi = *w*/*w;OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/*UAS-InR^{RNAi}*. dnInR = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/*UAS-dnInR*

I used the temperature sensitive Gal80^{ts} system, combined with the pan-glial *repo*-Gal4 driver, to express a constitutively active form of the InR (UAS-caInR) in adult glia for one day prior to and after axotomy and then assessed glial clearance of severed OR85e axons. Interestingly, I found that constitutive activation of ILS in adult glia resulted in clearance of significantly more GFP^{+} OR85e axonal debris one day after injury as compared to control flies one day

after injury (Figure 2.3A and B). I repeated this experiment with two additional characterized constitutively active versions of the InR and found that axonal debris was also cleared significantly faster in these flies (data not shown). Finally, I confirmed the efficacy of our Gal80^{ts} construct by repeating these experiments in flies that were maintained at the permissive temperature of 18°C and saw no effect on glial clearance of severed axons (Figure 2.3C), suggesting that the Gal80^{ts}/Gal4 system is effectively inducing caInR expression acutely in adult glia in our experimental paradigm (with 30°C shift). These results are consistent with the notion that insulin-like signaling is a positive regulator of adult glial responses to axon injury, including phagocytic engulfment activity.

InR is a positive regulator of expression of the engulfment receptor Draper

Draper is a highly conserved engulfment receptor that is essential for proper glial engulfment of apoptotic cells during development as well as degenerating olfactory receptor neuron axons in the adult brain [91,94,98]. I wondered if the ILS pathway might regulate Draper expression and/or function, as this would be one viable mechanistic way in which changes in ILS could modulate glial engulfment activity. To determine how ILS activity might modulate Draper expression levels, I activated the ILS pathway by expressing a constitutively active InR in adult glia with *repo*-Gal4 and then assessed Draper levels by performing anti-Draper immunostaining on dissected brains from uninjured and injured animals. To induce injury, I severed the maxillary nerve, since within 24 hours this injury results in robust accumulation of Draper on maxillary palp glomeruli that contain actively degenerating axons. Flies also

Figure 2.3

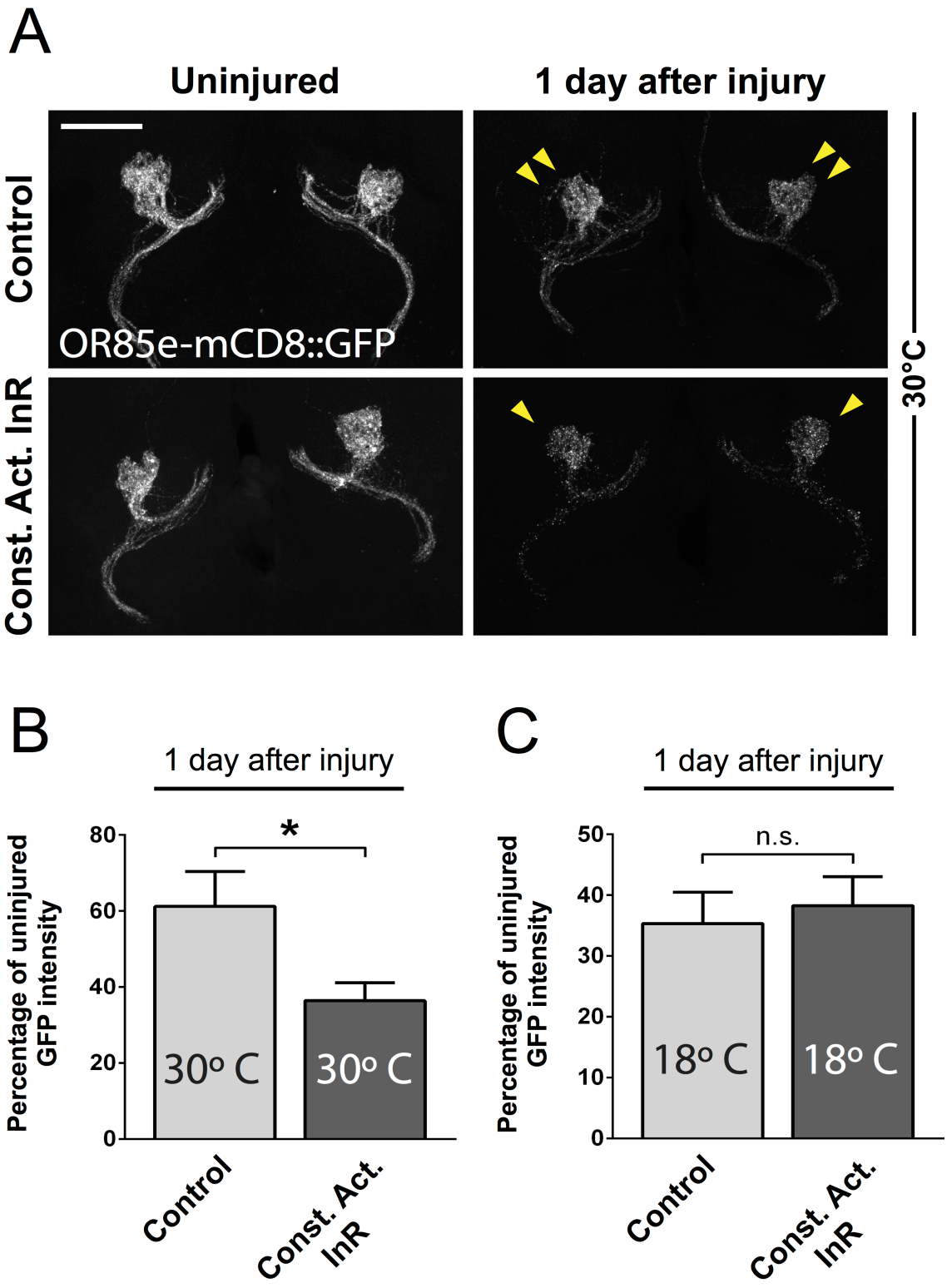
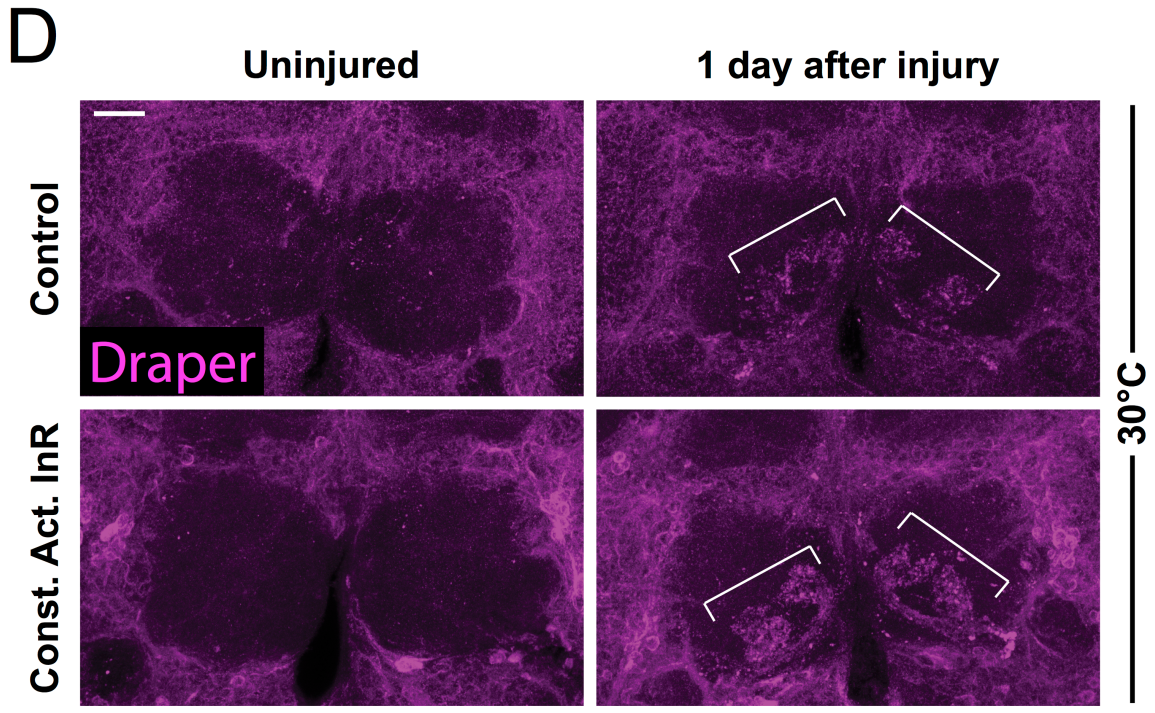

A
Uninjured
1 day after injury
Control
Const. Act. InR
OR85e-mCD8::GFP
30°C
B
1 day after injury
Percentage of uninjured GFP intensity
80
60
40
20
0
*
30° C
30° C
Control
Const. Act. InR
C
1 day after injury
Percentage of uninjured GFP intensity
50
40
30
20
10
0
n.s.
18° C
18° C
Control
Const. Act. InR

Figure 2.3 (continued)



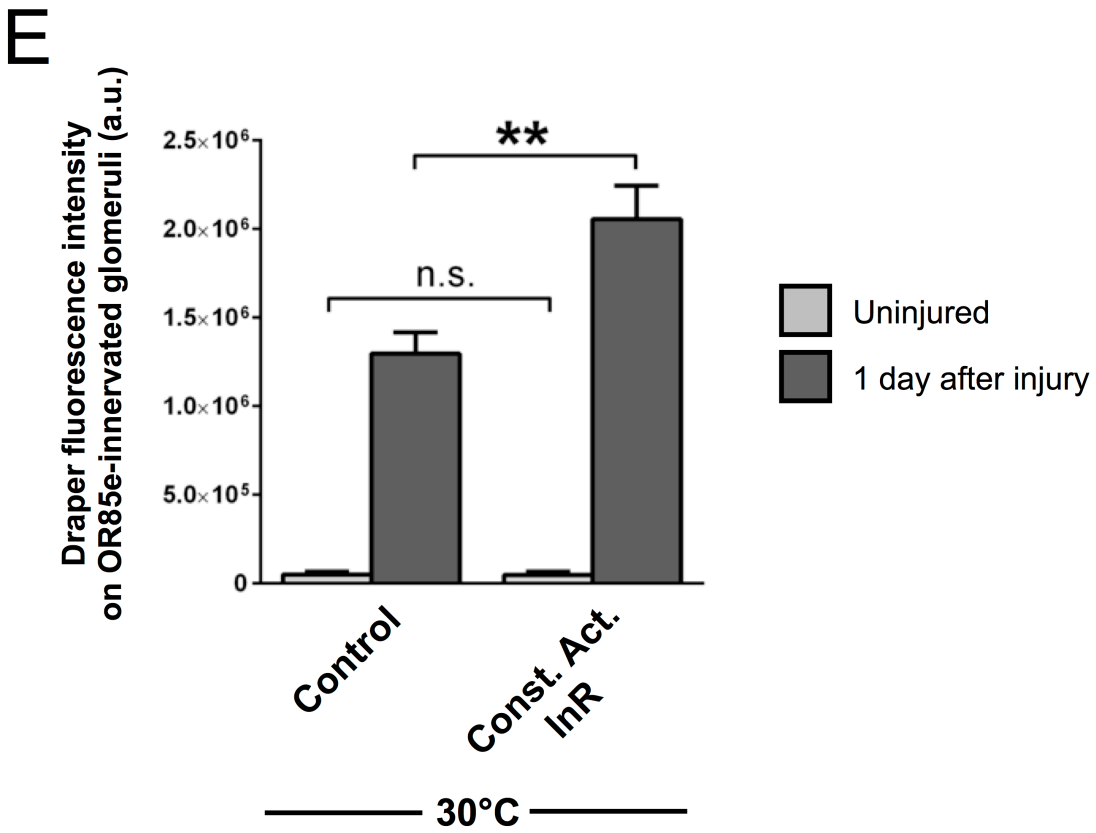


Figure 2.3. Constitutive activation of glial InR results in faster clearance of severed ORN axonal debris. (A) Maximum intensity confocal confocal Z stack projections of the central antennal lobe region show uninjured and injured OR85e axons. Acute expression of a constitutively active version of the InR (caInR) in adult glia results in significantly less GFP^{+} OR85e axonal debris in the antennal lobes 24 hours after severing the maxillary palp nerve (yellow arrowhead) as compared to control animals (double yellow arrowheads). Scale bar = 20μm. **(B)** Quantification of the experiment shown in panel A. Welch's corrected *t*-test, * $p < 0.05$. N = 8 for control; N = 9 for caInR. **(C)** Quantification of GFP^{+} OR85e debris in control and constitutively active InR when flies were maintained at 18°C, confirming fidelity of the $GAL80^{ts}$ system. Student's *t*-test, n.s. $p > 0.05$. N = 17 for control; N = 19 for caInR. **(D)** Representative single confocal slices through the antennal lobe region of uninjured and axotomized brains immunostained for Draper (magenta) are shown. There is notably stronger accumulation of Draper on OR85e-innervated antennal lobe glomeruli (brackets) in flies that express a constitutively active version of InR (caInR) in adult glia. Scale bar = 20μm **(E)** Quantification of D. Welch's corrected *t*-test, ** $p < 0.001$. N = 20 for control injured; N = 23 caInR injured. Mann-Whitney *U*-test, n.s. $p > 0.05$. N = 17 for control uninjured; N = 18 caInR uninjured. Genotypes in Figure 2.3: control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4*/+. caInR = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/ *UAS-caInR*;*repo-Gal4*/+

carried the *OR85e*-mCD8::GFP transgene that allowed me to identify the same set of OR85e glomeruli in each animal for Draper quantification. As previously shown [94], I observed an accumulation of Draper on maxillary palp glomeruli one day after maxillary nerve axotomy in control flies (Figure 2.3D). Notably, I observed what appeared to be a higher level of Draper accumulation on injured maxillary nerve axons in flies expressing glial constitutively active InR (Figure 2.3D). I quantified the fluorescence intensity of Draper specifically on OR85e glomeruli and indeed found that Draper levels were significantly increased in this region at this time point (Figure 2.3E). My observations of enhanced Draper recruitment and clearance of axonal debris in response to axon injury are consistent with ILS positively regulating various components of the innate glial immune response.

Glial InR is required for proper recruitment of Draper to degenerating axons

To determine if inhibition of ILS in adult glia would attenuate expression of Draper and/or recruitment of Draper to axon injury sites, I expressed InR^{RNAi} in adult glia, ablated maxillary palps, and quantified Draper levels on OR85e glomeruli one day after injury as described above. Control animals displayed robust accumulation of Draper on severed axons (white arrowheads, Figure 2.4A). Flies that expressed glial InR^{RNAi} showed a dramatic reduction of Draper on all maxillary glomeruli, including OR85e (green arrows, Figure 2.4A). Quantification of Draper fluorescence intensity on OR85e-innervated glomeruli confirmed there was a significant reduction when the InR was knocked down acutely in adult glia (Figure 2.4B), consistent with the idea that ILS is a positive regulator of Draper receptor expression. These results also further indicate that proper InR signaling within glia is required for orchestrating normal innate immune responses to axon injury in the adult brain.

Glial InR is required for transcriptional upregulation of Draper in response to axotomy

To determine if reducing InR signaling also inhibited the rate of Draper gene transcription, I performed quantitative PCR on dissected central brains from control flies and flies in which I acutely inhibited InR signaling by adult expression of dominant negative InR with the $Gal80^{ts}$/Gal4 system (control flies had the same genetic background minus the dominant negative InR construct).

Figure 2.4

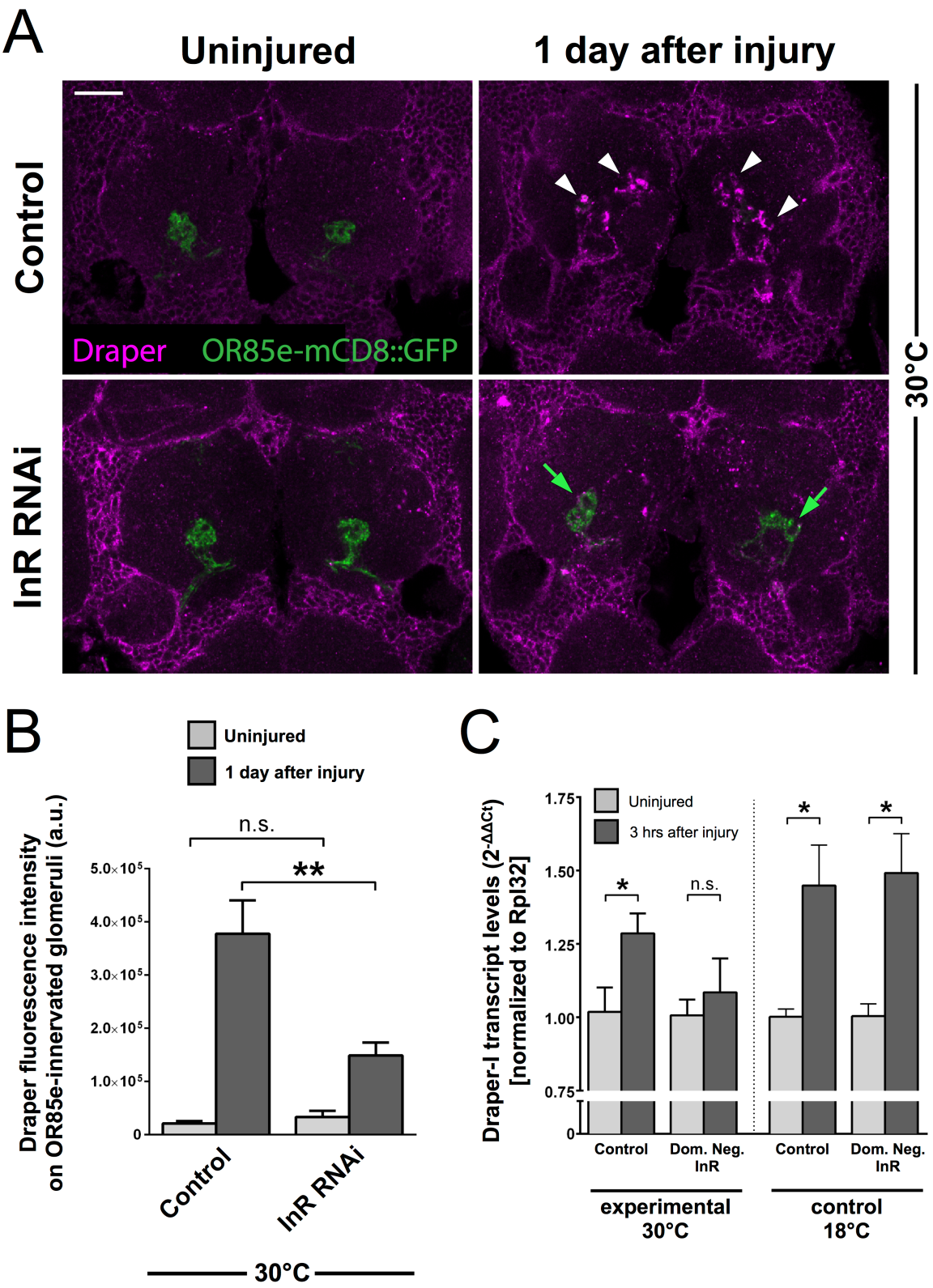

A
Uninjured
1 day after injury
Control
InR RNAi
Draper
OR85e-mCD8::GFP
30°C
B
Uninjured
1 day after injury
Draper fluorescence intensity on OR85e-innervated glomeruli (a.u.)
n.s.
**
5.0×10⁵
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
Control
InR RNAi
30°C
C
Uninjured
3 hrs after injury
Draper-I transcript levels (2-ΔΔCt) [normalized to Rpl32]
1.75
1.50
1.25
1.00
0.75
0
*
n.s.
*
*
Control
Dom. Neg. InR
Control
Dom. Neg. InR
experimental 30°C
control 18°C

Figure 2.4. Glial InR is required for proper recruitment of the Draper receptor to axon injury sites. (A) Representative single confocal z-slices of brains that were uninjured or brains 24 hours after maxillary nerve axotomy. Brains were immunostained with GFP (green) to visualize GFP-expressing OR85e maxillary palp axons and Draper (magenta). Robust accumulation of Draper is observed on maxillary palp glomeruli one day after injury in control flies (white arrowheads). Expression of InR^{RNAi} in adult glia inhibits Draper accumulation on degenerating maxillary palp ORN axons 24 hours after axotomy (green arrows). Scale bar = 20μm. **(B)** Quantification of experiment shown in panel A. Total Draper fluorescence intensity on OR85e-innervated glomeruli one day after maxillary nerve axotomy was quantified. Welch's corrected *t*-test, ** $p < 0.001$. N = 26 for injured control; N = 15 for injured InR RNAi. Mann-Whitney *U*-test, n.s. $p > 0.05$. N = 22 for uninjured control; N = 5 for uninjured InR RNAi **(C)** Quantitative PCR for the Draper-I transcript was performed on dissected adult central brains to compare Draper-I mRNA levels in uninjured brains compared to 3 hours after antennal nerve axotomy. Draper-I is significantly upregulated in control flies at this time point. Expression of dnInR in adult glia blocks injury-induced upregulation of Draper-I 3 hours after injury (30°C). Student's *t*-test and Welch's corrected *t*-test, * $p < 0.05$; n.s. $p > 0.05$. N = 6 for each sample. Genotypes in Figure 2.4A and B: control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/+. InR RNAi = *w*/*w;OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/*UAS-InR^{RNAi}*. Genotypes in C: control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/+. dnInR = *w*/*w;OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/*UAS-dnInR*

I compared uninjured animals to a cohort that had been injured with an antennal ablation 3 hours previously. In my experimental flies, which were shifted to 30°C as adults post-eclosion, I detected a significant increase in *draper-I* transcript levels in my injured control animals as previously reported [98] (Figure 2.4C). Interestingly, although inhibiting InR activity (dnInR) in adult glia did not alter basal transcript levels of Draper-I, it did prevent injury-induced upregulation of *draper-I* three hours after axotomy (Figure 2.4C). Thus, ILS may be essential for *draper-I* transcription in the context of an innate immune response, while basal *draper-I* levels may be maintained by ILS-independent signaling mechanisms.

Draper-I partially rescues clearance defects resulting from reduced glial InR activity.

My results indicate that InR signaling positively regulates both Draper expression in antennal lobe ensheathing glia and clearance of degenerating axons. Since Draper is known to be essential for proper engulfment of severed ORN axons in the adult brain [94], I asked if forced expression of Draper could rescue the InR knockdown clearance phenotype. Our lab generated flies that carried the following elements: *OR85e*-mCD8::GFP transgene to monitor axon clearance, *tubulin*-Gal80^{ts} to temporally control Gal4 activity, and the pan-glial driver *repo*-Gal4 (control flies). I used UAS-InR^{RNAi} to inhibit glial InR expression in a subset of flies, as described in the experiments for Figure 2.2 (InR RNAi flies). To determine if Draper-I could rescue the InR^{RNAi} phenotype, I also introduced a UAS-Draper-I transgene into one set of animals (InR RNAi + Draper-I rescue). Overexpression of Draper-I from the *UAS*-Draper-I transgene (Draper-I OE) was also tested to confirm that it did not slow clearance of axonal debris and obscure our analysis of the Draper-I rescue experiments. Finally, a UAS-LacZ::NLS transgene was introduced into control, InR^{RNAi} and Draper-I OE animals to control for titration of the Gal4 transcriptional regulator in our rescue samples. I surgically ablated maxillary palps to sever the maxillary nerve, waited four days, then performed anti-GFP and anti-Draper immunostains on dissected brains. As previously shown in Figure 2.2, expression of InR^{RNAi} in adult glia inhibited glial clearance as I detected significantly more GFP^{+} axonal debris in the antennal lobes compared to my injured control animals (arrowheads Figure

2.5A, Figure 2.5B). Intriguingly, co-expression of Draper-I with InR^{RNAi} in adult glia rescued this phenotype as there was almost no detectable OR85e axonal debris remaining four days after axotomy in these rescue flies (Figure 2.5A and B). Finally, I repeated this rescue experiment and assessed clearance of OR85e axons only three days after injury. At this earlier time point, I saw a modest reduction in the amount GFP^{+} axonal debris in Draper-I + InR RNAi rescue flies compared to flies expressing glial InR^{RNAi} alone, but this difference was not significant (Figure 2.5C). Together, these results suggest that reduced Draper accounts, in part, for the poor glial engulfment response following inhibition of InR signaling in adult glia. The fact that Draper-I does not completely rescue the InR knockdown phenotype suggests that InR is also essential for other as yet unknown aspects of glial immunity, in addition to regulating Draper receptor levels.

InR is acutely activated in glia responding to injury

Since my quantitative PCR results suggested that ILS is essential for activation of an injury response program in adult glia after axotomy, I wanted to explore the possibility that the InR may be acutely activated in local antennal lobe glia responding to axonal injury. To do this I utilized a well-characterized phospho-InR specific antibody that specifically recognizes the activated form of the *Drosophila* InR [206]. I used flies that expressed membrane-tethered GFP in all glia (*repo*-Gal4*,* UAS-mCD8::GFP), performed antennal nerve injury, and then stained dissected brains with anti-phospho-InR at 16 hours after axotomy. Consistent with previous work [94], I saw that local ensheathing glia, which

Figure 2.5

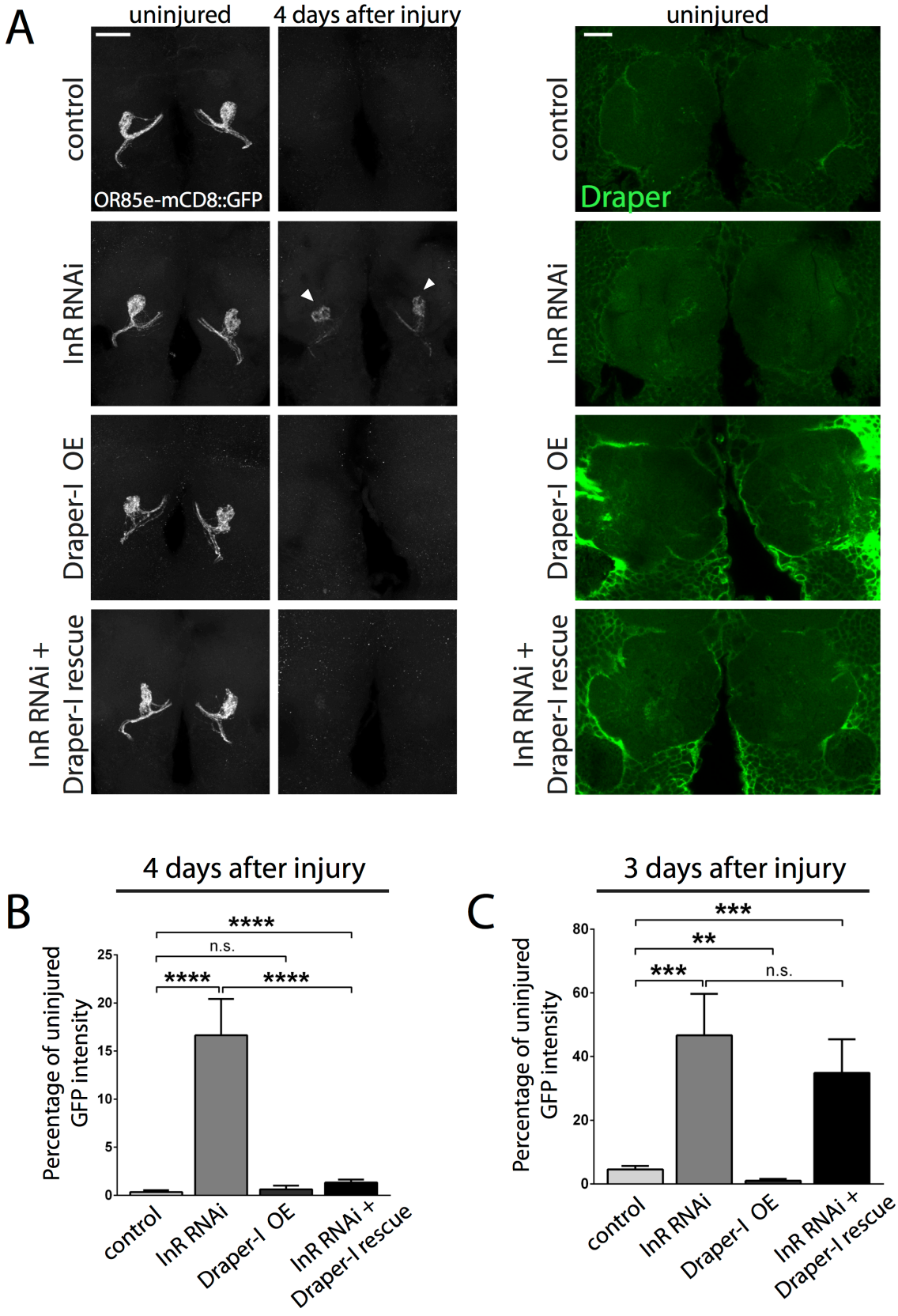

A
uninjured
4 days after injury
uninjured
control
OR85e-mCD8::GFP
Draper
InR RNAi
Draper-I OE
InR RNAi +
Draper-I rescue
B
4 days after injury
Percentage of uninjured
GFP intensity
****
n.s.
****
****
25
20
15
10
5
0
control
InR RNAi
Draper-I OE
InR RNAi +
Draper-I rescue
C
3 days after injury
Percentage of uninjured
GFP intensity
***
**
***
n.s.
80
60
40
20
0
control
InR RNAi
Draper-I OE
InR RNAi +
Draper-I rescue

Figure 2.5. Forced glial expression of Draper-I partially rescues the InR^{RNAi} clearance phenotype. (A) Maximum intensity projected confocal Z-stacks of the antennal lobe region show OR85e maxillary palp axons. Glial expression of InR^{RNAi} inhibits glial clearance of degenerating OR85e axons 4 days after maxillary nerve axotomy (white arrowheads). Forced co-expression of Draper-I and InR^{RNAi} in adult glia rescues clearance of axonal debris at this time point. (InR RNAi + Draper-I rescue). Overexpression of Draper-I did not inhibit clearance of axonal debris (Draper-I OE). Representative single confocal slices of Draper (green) immunostained brains confirm glial expression of the *UAS-Draper-I* transgene in Draper-I OE and InR RNAi + Draper-I rescue samples. Scale bar = 20µm. **(B)** Quantification of OR85e axon clearance four days after maxillary nerve injury (shown in panel (A)). Ordinary 1-way ANOVA with Holm-Sidak's multiple comparisons test, **** $p < 0.0001$; n.s. $p > 0.05$. N = 18 for control; N = 20 for InR RNAi; N = 16 for Draper-I OE; N = 20 for InR RNAi + Draper-I rescue. **(C)** Quantification of OR85e axon clearance three days after maxillary nerve injury. Ordinary 1-way ANOVA with Sidak's multiple comparisons test, *** $p < 0.001$; ** $p < 0.01$; n.s. $p > 0.05$. N = 9 for control; N = 8 for InR RNAi; N = 9 for Draper-I OE; N = 6 for InR RNAi + Draper-I rescue. Genotypes in Figure 2.5: control = *w*/*w*;*OR85e-mCD8*::*GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/+. InR RNAi = *w*/*w;OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/UAS-LacZ::NLS;*repo-Gal4, InR^{ex15}*/*UAS-InR^{RNAi}*. Draper-I OE = *w*/*w*;*OR85e-mCD8*::*GFP*, *UAS-Draper-I/ tubulin-Gal80^{ts}*;*repo-Gal4, InR^{ex15}*/ *UAS-LacZ::NLS*. InR RNAi + Draper-I rescue = *w*/*w;OR85e-mCD8::GFP*, *UAS-Draper-I*/*tubulin-Gal80^{ts}*; *repo-Gal4, InR^{ex15}*/*UAS-InR^{RNAi}*

enwrap ORN axonal projections, expanded their membranes after antennal nerve injury (white arrowheads in green channel, Figure 2.6A). I detected puncta of phospho-InR signal throughout uninjured and injured brains, which was not unexpected since some InR activity is likely important for basal metabolic activity in most cells. Interestingly, after antennal nerve injury, I observed a significant increase in phospho-InR signal that overlapped with the regions of ensheathing glial membrane expanding around the antennal lobes (merge panel, Figure 2.6A and B). I quantified phospho-InR in responding glial membranes by computationally segmenting to the glial membrane signal (GFP) and found that the phospho-InR signal was significantly higher in ensheathing glia post-axotomy

Figure 2.6

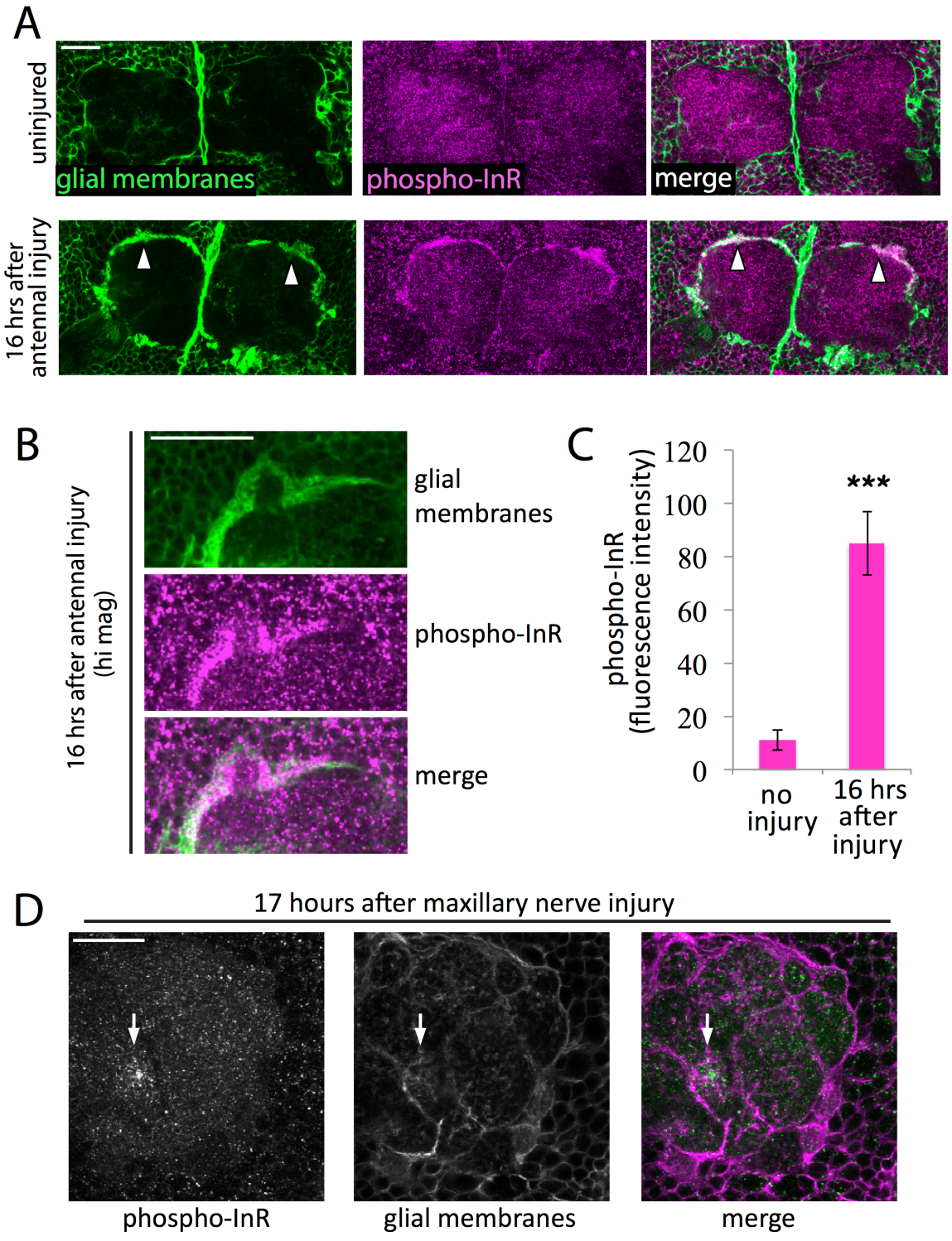

A
uninjured
glial membranes
phospho-InR
merge
16 hrs after antennal injury
B
16 hrs after antennal injury (hi mag)
glial membranes
phospho-InR
merge
C
phospho-InR (fluorescence intensity)
120
100
80
60
40
20
0
***
no injury
16 hrs after injury
D
17 hours after maxillary nerve injury
phospho-InR
glial membranes
merge

Figure 2.6. InR is stimulated on ensheathing glia within 16 hours after ORN axon injury. (A) We transgenically labeled glial membranes *in vivo* with *repo-Gal4, UAS-mCD8::GFP*, ablated antennae and stained dissected brains with anti-phospho-InR. Representative single confocal slice images show robust expansion of ensheathing glial membranes (green) in injured flies (white arrowheads). We observed a dramatic increase in phospho-InR staining in injured flies that co-localized with expanding glial membranes around the antennal lobes (white arrowheads, merged panel). Scale bar = 20μm **(B)** High magnification single confocal slice view of expanding ensheathing glial membranes after antennal nerve injury and robust phospho-InR signal. Scale bar = 20μm **(C)** Quantification of phospho-InR intensity on segmented glial membranes revealed a 5.3-fold increase in glial phosphor-InR signal 16 hours after antennal injury. Student's *t*-test, *** $p < 0.001$. **(D)** Glial membranes were labeled with RFP (*repo-LexA, LexAOp-mCD2::RFP*), maxillary palps were ablated and dissected brains were stained with phospho-InR 17 hours after injury. We observed greater phospho-InR signal that appeared to overlap with glial membranes localized to maxillary palp glomeruli that housed degenerating axons (white arrow). Scale bar = 20μm

(Figure 2.6C). I performed a similar experiment injuring the maxillary nerve in flies expressing glial membrane-tethered RFP (*repo*-LexA*, LexAop*-mCD2::RFP*)* and immunostained for phospho-InR 17 hours after axotomy. I observed a similar increase in phospho-InR signal (green in merge panel, Figure 2.6D) that overlapped with areas of glial membrane accumulation on maxillary glomeruli (magenta in merge panel, Figure 2.6D). These observations suggest that activity of the InR is acutely upregulated in activated antennal lobe glia within one day after axon injury, suggesting that the ILS pathway may be a local injury communication relay that governs innate glial immune responses to axon degeneration.

DISCUSSION

Here I show that Insulin-like Receptor (InR) signaling in glia is required *in vivo* for proper activation of the engulfment receptor gene, Draper, and for timely removal of axonal debris after axotomy of olfactory system nerves in the adult *Drosophila* brain. I also provide evidence that ORN axon injury triggers robust activation of the InR in local ensheathing glial cells that carry out innate immune responses and clear degenerating axons in the antennal lobe region.

InR positively regulates glial engulfment of severed axons in the adult brain.

I found that inhibiting expression or activity of the InR in adult glia prevents proper glial clearance of damaged ORN axons in the adult *Drosophila* brain. Interestingly, I also discovered that stimulating InR through the use of constitutively active forms of the receptor significantly boosts the engulfment activity of antennal lobe glia resulting in more rapid clearance of injured axonal debris. These findings highlight insulin-like signaling as a novel and essential pathway to explore to target innate immune responses of glial cells in acute as well as chronic neurodegeneration and trauma conditions. There are intriguing hints in the literature that suggest insulin-like signaling pathways are neuroprotective in many contexts (reviewed in [163,182,183]); our work now suggests that ILS-mediated control of glial immunity may be one mechanism that accounts for the beneficial effects of ILS in the mature brain.

I found that InR activity also regulates Draper levels in the antennal lobes after axon injury. Constitutive activation of InR in adult glia resulted in greater Draper accumulation on actively degenerating axons one day after injury. This finding supports a model in which InR positively regulates Draper expression in glia responding to axon injury. However, these higher levels of Draper are unlikely to account for faster clearance of OR85e axonal debris in constitutively active InR expressing flies, since overexpression of Draper-I does not speed up glial clearance of severed axons in this assay. ILS likely targets other molecules and pathways to promote clearance of injured tissue in addition to modulating Draper expression. Glial cells undergo striking morphological and functional changes in response to axon injury, including dramatic changes in membrane morphology, rapid turnover of molecules during phagocytosis, and activation of internal destruction pathways to dispose of engulfed material [28,103,162,174]. These responses undoubtedly place a substantial energy demand on the glial cells and ILS activation of metabolic pathways and/or enhanced protein translation are reasonable candidate pathways that ILS may target to help drive an efficient immune response from glia. Reactive glial membranes likely also must grow to make contact with and ultimately encompass all the injured tissue. Since ILS has a well established role in cellular growth, this might represent another way that ILS promotes glial clearance function. Future work to identify Draper-independent ways in which ILS drives glial engulfment activity will enhance our understanding of this novel post-injury communication network.

InR directs Draper protein levels in ensheathing glia after axon injury

Interestingly, inhibiting InR in adult glia in uninjured brains did not significantly inhibit Draper gene transcription or protein levels in uninjured brains; however, I found that glial InR is required for axotomy-induced upregulation of *draper-I* transcript and for normal accumulation of the Draper engulfment receptor on degenerating ORN axons. It remains to be determined if the lack of transcriptional upregulation of *draper* exclusively accounts for the attenuated levels of Draper protein at injury sites. Alternatively, ILS may independently control Draper at the gene and protein level, as ILS is well known to directly control translational machinery in addition to its transcriptional regulation capabilities [207-210]. It is therefore possible that activation of glial InR after injury stimulates Draper translation and/or promotes Draper stability. Could InR directly regulate *draper* transcription in the context of injury? Future work will reveal if there are insulin signaling responsive promoter elements in the draper gene or, alternatively, if a certain level of InR signaling is required for proper expression of another as yet unidentified receptor/signal transduction cascade that converges on positive *draper* transcription in glia responding to axon injury.

Draper is required for phagocytic activity of glial cells (as well as other cells types) in several contexts, including glial clearance of apoptotic neurons undergoing programmed cell death and removal of degenerating axons and dendrites during the normal neuronal circuitry remodeling that occurs during metamorphosis [91,92,94,98,211,212]. It will be interesting to explore whether InR activity in glia is also required for Draper-dependent engulfment activity

during these developmental events; if so, then insulin-like signaling may represent an exciting new master regulatory function of innate immunity events in a variety of scenarios – and perhaps even in various tissues outside the CNS.

Glial InR is acutely activated in ensheathing glia after ORN axotomy

Using an antibody that specifically recognizes the activated (phosphorylated) version of the InR, I discovered that ensheathing glial InR is robustly activated within 16 hours after severing either the large antennal nerve or the smaller maxillary palp nerves that project into the antennal lobes. These results suggest that activation of InR signaling cascades in glia may represent an exciting new innate glial immunity pathway that is required for several features of the glial response to trauma. It is important to point out that the increase in glial phospho-InR signal that I see after injury could potentially be due in part to a simple increase in the amount of glial membranes around injury sites—membrane that could be bringing phosphorylated-InR with it to begin with. Future experiments assessing activation of downstream players in the InR signaling cascade (that are not themselves transmembrane proteins) should be able to resolve issue. Preliminary work with assessing phospho-Akt levels in glia before and after injury has indeed shown an acute increase in the activation of this signaling pathway in responding glia, but more experiments will be needed to substantiate these results.

One pressing question that remains is: What is the source of the ligand that triggers glial InR acutely after axon injury? Both neurons and glia are known

to release insulin-like peptide ligands, and thus, there are multiple cell types within the antennal lobe region that are reasonable candidates, including the injured axons themselves, local astrocyte-like glia, and other ensheathing glia. Additionally, one or more ilps circulating in the hemolymph may be preferentially transported across the blood brain barrier following injury to then bolster glial immune responses. Regardless, this work reveals a previously undescribed role for insulin-like signaling as an acute activator of glial innate immunity that may function within a neuron-glial, inter-glial, or hemolymph-glial communication relay after axonal injury and degeneration.

MATERIALS AND METHODS

Fly Stocks.

The following *Drosophila melanogaster* strains were used (separated by bullet points to preserve clear nomenclature punctuation): *repo*-Gal4 • w^{1118}; *OR85e*-mCD8::GFP, *tubulin*-Gal80^{ts}; [103] • InR^{ex15} [194] • w^{1118}; *OR85e*-mCD8::GFP, *tubulin*-Gal80^{ts}/+; *repo*-Gal4, InR^{ex15}/+ • y^1 w^{1118}; UAS-InR.K1409A (dominant negative InR, Bloomington stock 8253) • y^1 w^{1118}; UAS-InR.del (constitutively active InR, Bloomington stock 8248) • y^1 w^{1118};; UAS-InR.R418P (constitutively active InR, Bloomington stock 8250) • y^1 w^{1118};; UAS-InR.A1325D (constitutively active InR, Bloomington stock 8263) • w^{1118};; UAS-mCD8::GFP, *repo*-gal4 • y^1 v^1; UAS-InR^{RNAi} (Bloomington stock 31037).

Olfactory Neuron Injuries, Dissection, and Analysis. I performed maxillary palp ablations, adult fly brain dissections, and whole brain antibody staining using

previously described methods [94]. To quantify clearance of *OR85e*-mCD8::GFP labeled maxillary palp olfactory receptor neurons, I computationally reconstructed entire *OR85e*-innervated glomeruli and performed blinded volumetric quantification of above threshold GFP^+ fluorescent signals, with background fluorescence subtraction, using Volocity software (Perkin Elmer).

To quantify Draper immunofluorescence in responding glia after maxillary palp injury, I first volumetrically segmented my area of quantification to the OR85e-innervated glomeruli using the *OR85e*-mCD8::GFP signal as a guide. I then blindly quantified above threshold $Draper^+$ fluorescent signals in this volume using Volocity software, as above.

Olfactory Neuron Injuries, Dissection, and Analysis. I performed maxillary palp and third antennal segment ablations, adult fly brain dissections, and whole brain antibody staining using previously described methods [94]. To quantify clearance of *OR85e*-mCD8::GFP labeled maxillary palp olfactory receptor neurons, I computationally reconstructed *OR85e*-innervated glomeruli and performed a volumetric quantification of above threshold GFP+ fluorescent signal, with background fluorescence subtraction, using Volocity software (Perkin Elmer). For Draper injury response experiments, I quantified Draper recruitment after axotomy as previously described [94,98]. Basal levels of Draper and Repo were quantified volumetrically using Volocity software (Perkin Elmer). To quantify phospho-InR intensity in antennal lobe glia, I computationally segmented to the *repo*-mCD8::GFP signal (glial membranes) and then quantified total phospho-InR intensity within these GFP+ regions at selected sites.

Antibody Use and Dilutions. The following antibodies were used at the indicated dilutions: 1:200 mouse anti-GFP (Invitrogen), 1:1000 chicken anti-GFP (Life Technologies), 1:10 mouse anti-Repo (Developmental Studies Hybridoma Bank), 1:500 mouse anti-Draper (Abmart Inc.), 1:500 rabbit anti-Draper [91], 1:1000 rabbit anti-phospho-InR β (Tyr1146) (Cell Signaling), 1:400 Alexa 488-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch), 1:400 Alexa Fluor 488-conjugated donkey anti-chicken IgY (H+L) (Jackson Immunoresearch), 1:400 Rhodamine Red-X-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch), 1:400 Alexa Fluor 647-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch)

Draper Antibody Production. Our lab generated a monoclonal mouse anti-Draper antibody against the epitope: NPIVYNESLK. This antibody was designed and produced by Abmart Inc. using their Protein Surface Epitopes Targeted by Monoclonal Antibody Library (SEAL) technique. The mouse anti-Draper antibody used for experiments in this chapter was derived from an affinity-purified ascites fluid provided by Abmart.

Confocal Microscopy. All immunostained brains were imaged on a Zeiss LSM 700 with a Zeiss 40X 1.4NA oil immersion plan-apochromatic lens. Brains within a single experiment (i.e. those being directly compared for quantification) were whole mounted under a single #1.5 cover glass in either Vectashield mounting media (Vector Laboratories) or CFM3 mounting media (CitiFluor). All brains being directly compared were imaged on the same day with the same confocal

microscope settings (laser power, photomultiplier tube gain, offset, filter configuration, etc.). The sampling interval between Z-stacks in all experiments was 0.76 microns, while the pixel size ranged from 100nm - 230nm.

Real-Time Quantitative PCR. I manually dissected central brains in Jans' saline (0.5 mM Ca^{2+}) and immediately froze them on dry ice. I extracted total RNA in Trizol reagent (Invitrogen), isolated the aqueous phase with a Phase Lock Gel kit (5 Prime) and passed RNA over a RNA Clean & Concentrator-5 column (Zymo Research). I treated RNA with DNase (Ambion *DNA-free* kit), and determined the final RNA concentration with a Qubit Fluorometer (Invitrogen). Total RNA (~25 ng) was reverse-transcribed with the qScript cDNA SuperMix kit (Quanta Biosciences) for 30 minutes at 42°C.

I performed relative quantitation of gene expression on an Applied Biosystems StepOne Real-Time PCR System. The TaqMan Fast Advanced Master Mix kit (Applied Biosystems) and the following TaqMan assays were used: (i) Ribosomal Protein L32 (Applied Biosystems premade assay Dm02151827_g1), (ii) Draper-I custom assay: F-primer, TGTGATCATGGTTACGGAGGAC; R-primer, CAGCCGGGTGGGCAA; probe, CGCCTGCGATATAA. The raw threshold cycle (C_t) of the normalization control (RpL32) did not vary by more than 0.5 cycles across all time points and genotypes analyzed. Statistical analysis was performed on $2^{-\Delta\Delta Ct}$ values.

Statistical Analysis. GraphPad Prism software was used to perform: Ordinary 1-way Analysis of Variance tests, Kruskall-Wallis 1-way Analysis of Variance

tests, two-tailed Student's *t*-tests, two-tailed Welch's *t*-tests, two-tailed Mann-Whitney *U*-tests, Dunnett's multiple comparisons *post hoc* tests, Holm-Sidak multiple comparisons *post hoc* tests. Assumptions of normality were tested with the D'Agostino-Pearson normality test. Where applicable, outliers were identified using the ROUT method. In some analyses, log-transformations were uniformly performed on otherwise non-Gaussian data sets to allow for the appropriate use of parametric tests. When assumptions of normality could not be met for a given data set, non-parametric tests were used. Each N = 1 sample number represents pooled measurements taken from completely independent animals.

Acknowledgements

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Chapter 3

Insulin-like receptor substrates Chico, Lnk, and Dock regulate adult glial immune responses to axon degeneration in *Drosophila*

INTRODUCTION

All vertebrates and many invertebrates (including insects, crustaceans, and cephalopods) possess a tight, cellular barrier between the central nervous system and all other tissues [8]. In vertebrates this is termed the blood-brain barrier (BBB), and while the exact cellular composition of this barrier system varies to some degree between different organisms, its main function to chemically insulate the CNS from the rest of the body is highly conserved [9]. One major consequence of the BBB is that it largely prevents peripheral immune cells from entering the CNS, thereby endowing the CNS with so-called "immune privileged" status [5-7]. While recent evidence suggests that exclusion of peripheral immune cells from the CNS as a whole is not nearly as absolute as once believed [10,11], CNS parenchymal tissue, in particular, still appears to be robustly excluded from peripheral immune cell access.

This presents a unique challenge to CNS parenchymal tissue (i.e. neurons and glia) dealing with injury or infection because these cells cannot rely upon the peripheral immune system for assistance in dealing with insults. To cope with this problem, certain glia act as resident innate immune cells in the brain. In mammals, this task falls to microglia [213,214] and also to a certain extent astrocytes [35,38]. In *Drosophila*, ensheathing glia are the primary responders to neural injury [110], although astrocyte-like glia also utilize similar signaling pathways to phagocytose degenerating neurons and neuronal debris that must be cleared during metamorphosis [215,216]. These glia can identify and phagocytose both "altered-self" material, such as cellular debris [28,110],

apoptotic bodies [72,217], and even aberrant extracellular protein products (e.g. amyloid-β aggregates) [43,218], as well as “non-self” material such as invading bacteria [24,219]. Recent work has demonstrated that glia accomplish many of these tasks using similar molecular components as the peripheral innate immune system, including the complement system [220], pattern recognition receptor (PRR) pathways (e.g. toll/toll-like receptor signaling) [221], and phagocytic engulfment machinery [222]. Nevertheless, the field of glial immunity is in its infancy, and much work is still needed to provide a more complete mechanistic model of the molecular basis of innate glial immune function. For example, it is still unclear what molecular pathways govern glial phagocytosis from start to finish. What are the master regulators initiating and coordinating the various aspects of the glial immune response, from initial activation and migration to targeted tissue, to the phagocytosis the target tissue itself, to the deactivation of responding glia once the immune response is not longer needed? These are still very much open questions in the field.

To investigate these questions in more detail, I used a well-established *Drosophila* olfactory axotomy assay [94] and demonstrated that glial clearance function can either be enhanced by activating the Insulin-like Receptor (InR) pathway or attenuated by inhibiting InR signaling (see Chapter 2). Furthermore, I was able to demonstrate that the InR acts as a positive regulator of Draper (*Drosophila* ortholog of mammalian MEGF10 and Jedi-1), a well-defined engulfment receptor required for glial phagocytic clearance of apoptotic and degenerating neurons [92,94,97,211]. Importantly, forced expression of Draper

partially rescues the glial clearance defects caused by genetically induced inhibition of insulin-like signaling (ILS). The work presented here in Chapter 3 begins to address what signaling cascades downstream of the InR are involved in the glial immune response to acute injury.

Chico, Lnk, and Dock: three InR substrates characterized in *Drosophila*

The *Drosophila* insulin-like signaling pathway contains only one unified receptor, the insulin-like receptor (InR) [168], with 8 known insulin-like peptide (ilp) ligands [170-172] (see Figure 1.5 and Figure 1.6 for overview). While over a dozen insulin/IGF receptor substrates have been reported in mammals (reviewed in [169]), to date, only three direct intracellular substrates have been confirmed in *Drosophila*: Chico, the single fly ortholog of the mammalian Insulin Receptor Substrate proteins (IRS) 1-4 [223]; Lnk, the sole Src Homology 2B (SH2B) adaptor protein family member in *Drosophila* [224]; and Dock (Dreadlocks), the fly ortholog of mammalian Nck (non-catalytic region of tyrosine kinase adaptor protein) [225-227].

The biological functions of these three substrates are not well defined. The functions of Chico have been best studied as it appears to be the main InR substrate responsible for canonical activation of the class-1A phosphatidylinositol 3-kinase complex (PI3K) and, in following, activation of Akt (also known as Protein Kinase B). This PI3K/Akt signaling cascade represents a fundamental intracellular signaling node and has been shown to regulate cell survival, growth, proliferation, metabolism, transcription and translation (reviewed in [133,134]).

Consistent with its putative role in regulating PI3K/Akt activity, existing studies of Chico function have established its importance in the determination of cellular size, proliferation, and lipid metabolism [223]. Similar to the InR itself [173], Chico has also been shown to be an important determinant of lifespan, with *chico* mutant flies living nearly 50% longer than their wild-type counterparts [189]. This observed extension of lifespan is partially-dependent on the *Drosophila* Forkhead Box O (Foxo) transcription factor [228], which is described in more detail below.

The second characterized *Drosophila* InR substrate, Lnk, appears to play an interconnected role with Chico in ILS. *lnk* mutant flies display similar phenotypes to *chico* mutants, showing a dramatic reduction in body size and cell number, and displaying defects in lipid metabolism [224]. While both *lnk* and *chico* mutant flies are viable, *lnk*;*chico* double mutants are lethal, similar to *InR* null mutants, suggesting that these two proteins play partially redundant roles and transduce most of the InR signaling essential for organismal survival [224]. Viability can be restored to *lnk*;*chico* double mutants by reducing levels of the lipid phosphatase, PTEN, which antagonizes PI3K action, suggesting that both Lnk and Chico primarily act upstream of PI3K [224]. More recent work suggests that Lnk serves as a scaffolding protein to mediate the subcellular localization and interaction between the InR and Chico in *Drosophila* salivary gland cells [196], though more work will need to be done to confirm this function in other cell types.

The third known *Drosophila* InR substrate, Dock, has been shown to act as a downstream signaling protein to guide axon outgrowth of photoreceptor

neurons during development [194]. Furthermore, Dock's ability to properly guide photoreceptor cells is dependent on its SH2 and SH3 domains [227], which have been shown to interact with the InR *in vitro* and *in vivo* [194]. The fact that the InR, through its interactions with Dock, can mediate cytoskeletal dynamics in neurons raises the intriguing possibility that it might also regulate outgrowth of glial processes toward sites of injury during reactive gliosis. Taken together, the three substrates Chico, Lnk and Dock are all able to activate the insulin receptor and lead to downstream signaling.

Forkhead Box O transcription factor

InR activation leads to active sequestration of the sole *Drosophila* Forkhead Box O (Foxo) transcription factor in the cytosol (as a result of phosphorylation by Akt), thereby inhibiting Foxo-mediated transcription. Foxo's influence on gene transcription is quite complex. In addition to directly promoting the transcription of hundreds of genes, Foxo can also act in complexes with other nuclear proteins to both upregulate and downregulate various genes [229-231]. Importantly, Foxo represents a major node of transcriptional regulatory output for ILS, accounting for the vast majority of detectable ILS-mediated transcriptional regulation in a variety of cell types [230]. However, while the Foxo proteins have been studied extensively *in vitro* and *in vivo*, very little work has been done to establish Foxo protein functions in adult glia. Because of this, and bearing in mind the role of insulin-like signaling in the glial response to injury described in the previous chapter, I was curious to know if dFoxo played any role in the InR-mediated glial clearance response.

Here I show for the first time that the InR substrates Chico, Lnk, and Dock play a role in the glial response to axon injury. Additionally, defects in Chico signaling lead to excessive accumulations of Draper around sites of injury, indicating a possible defect in Draper turnover and phagocytic activity. Lastly, Foxo inhibition in adult glia does not alter the ability of glial cells to clear axonal debris, suggesting that dFoxo may not mediate the InR's influence on glial innate immune responses after axotomy.

RESULTS

Chico is essential in adult glia for effective clearance of axonal debris

To date, the role of Chico has never been specifically examined in glia, much less in adult glia responding to an acute injury. Since Chico is the single fly ortholog of the mammalian IRS1-4 proteins, which are the primary InR substrates responsible for the receptor's canonical activation of the PI3K pathway, I wanted to test whether Chico is involved in the adult glial immune response to injury. To do this, our lab first generated transgenic fly strains that allowed for conditional manipulation of *chico* expression in adult glial cells. Thus, we generated a strain that carried the following genetic elements: the pan-glial driver *repo*-Gal4, *tubulin*-Gal80ts to temporally manipulate Gal4 activity in glia, and *OR85e*-mCD8::GFP to monitor clearance of maxillary palp axons after maxillary palp ablation. By crossing the above strain to a publicly available [202] *UAS-chico*^{RNAi} line, I drove chico^{RNAi} specifically in adult glial cells. These flies were reared at a nonpermissive temperature of 18°C (i.e. no transgene

expression) before adult flies were shifted to 30°C for ten days prior to axotomy. I ablated the maxillary palps and allowed the flies to recover at 30°C for 5 days then fixed, stained, and imaged brains via whole mount confocal microscopy techniques.

My results showed that upon genetic knockdown of Chico in adult glia, glial clearance of degenerating axons was severely impaired, with a 2.25-fold increase in the amount of axonal debris left 5 days after injury compared to injured controls (Figure 3.1). This defect was not due to any developmental effects brought on by so-called "leaky" expression of the transgenes because the 18°C control groups showed normal clearance in all the genotypes, indicating that my control of conditional expression was quite precise.

InR substrates Lnk and Dock appear to play a minor role in the adult glial clearance response

After Chico, I next wanted investigate the possible role of other known InR substrates in the glial response to injury. The proteins Lnk and Dock are the only other direct InR substrates that have been described in Drosophila, and I made use of publicly available RNAi lines targeting both of these genes. To test whether these proteins are important for the adult glial immune response, I employed the same experimental setup as described above except this time I crossed in RNAi genetic constructs targeting either Lnk or Dock. The results of these initial experiments can be seen in Figure 3.2 and Figure 3.3. I found that knocking down Lnk in adult glia resulted in a significant, yet fairly minor clearance

Figure 3.1

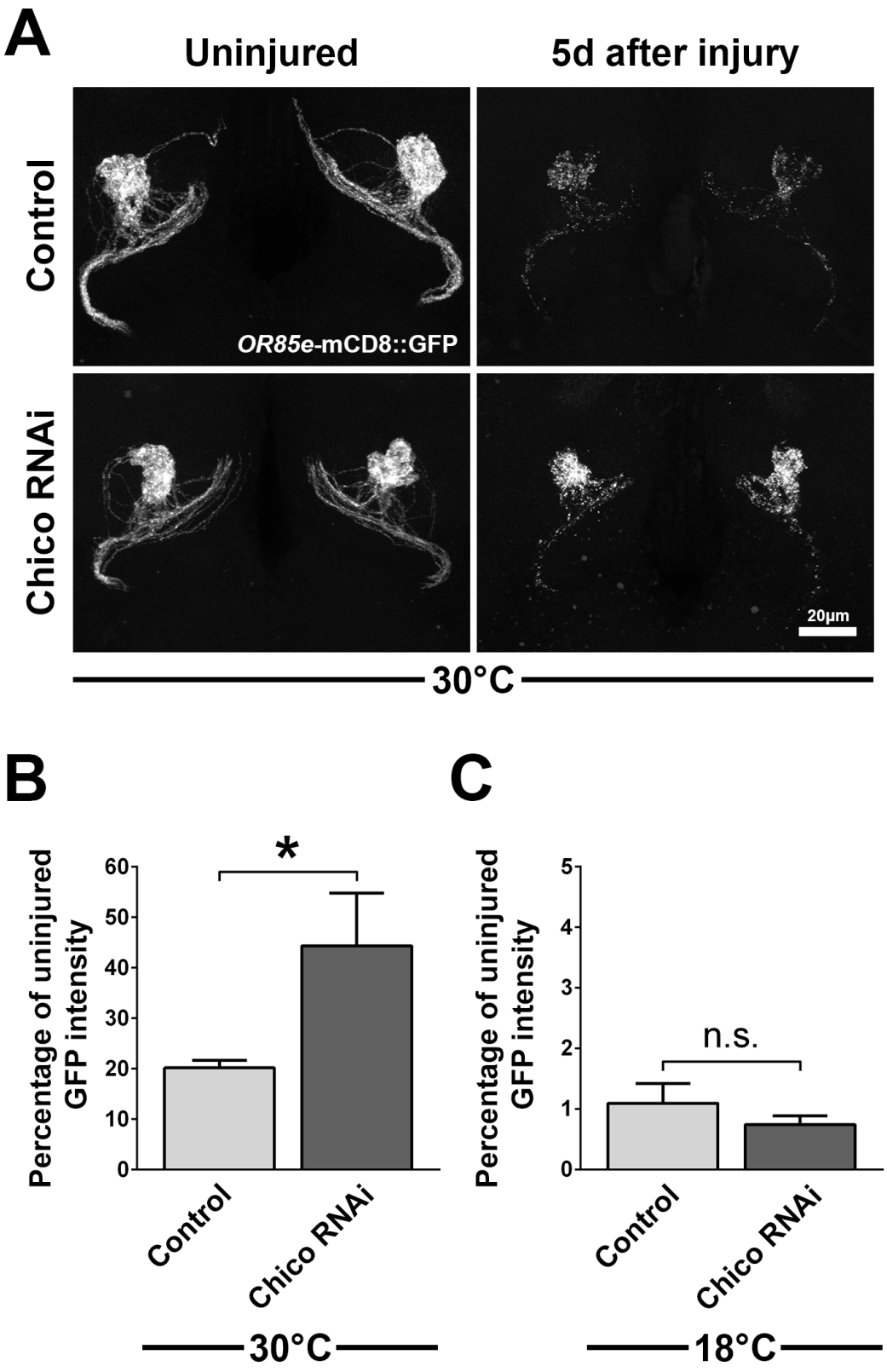

A
Uninjured
5d after injury
Control
Chico RNAi
OR85e-mCD8::GFP
20µm
30°C
B
C
Percentage of uninjured GFP intensity
*
n.s.
Control
Chico RNAi
30°C
18°C

Figure 3.1. Glial Chico is required for proper glial clearance of degenerating ORN axons. (A) Maximum intensity confocal projections of the antennal lobe region of the fly brain show that most *OR85e*-mCD8::GFP maxillary palp axons (green) are normally cleared from the antennal lobe region within 5 days after severing the maxillary nerve of control animals (Control). Compared to controls, adult glial expression of double stranded RNA against the Chico gene (Chico RNAi) resulted in significantly more GFP^{+} OR85e axonal debris present in antennal lobe regions 5 days after severing the maxillary nerve. Expression of the RNAi transgene was induced in adult flies with a temperature shift 30°C, thereby degrading $Gal80^{ts}$ and disinhibiting Gal4 action on transgene expression. Scale bar = 20μm. **(B)** Quantification of data presented in panel A, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Welch's corrected *t*-test, * $p < 0.05$. N = 17 for Control; N = 10 for Chico RNAi. **(C)** Quantification of a parallel experiment to that shown in A and B where flies were kept at 18°C and therefore transgene expression was never activated, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Student's *t*-test, n.s. $p > 0.05$. N = 7 for Control; N = 5 for Chico RNAi. Genotypes in Figure 3.1: Control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/+. Chico RNAi = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/*UAS-chico*^{RNAi}

defect when assessed 5 days after injury (Figure 3.2A and B). Flies that were reared and kept at 18°C during the whole experimental time course displayed normal clearance (Figure 3.2C), indicating that there was no premature expression of the UAS-Lnk^{RNAi} constructs during development, ruling out a potential developmentally derived cause for the observed Lnk clearance phenotype.

Unlike Lnk, my initial attempts at knocking down Dock did not appear to hinder clearance when assessed at this 5 day post-injury time point (data not shown). Several months after completing this initial knockdown experiment, a new RNAi fly line against Dock was made available through the Harvard Medical School's Transgenic RNAi Project (TRiP). Compared to the RNAi lines from the

Figure 3.2

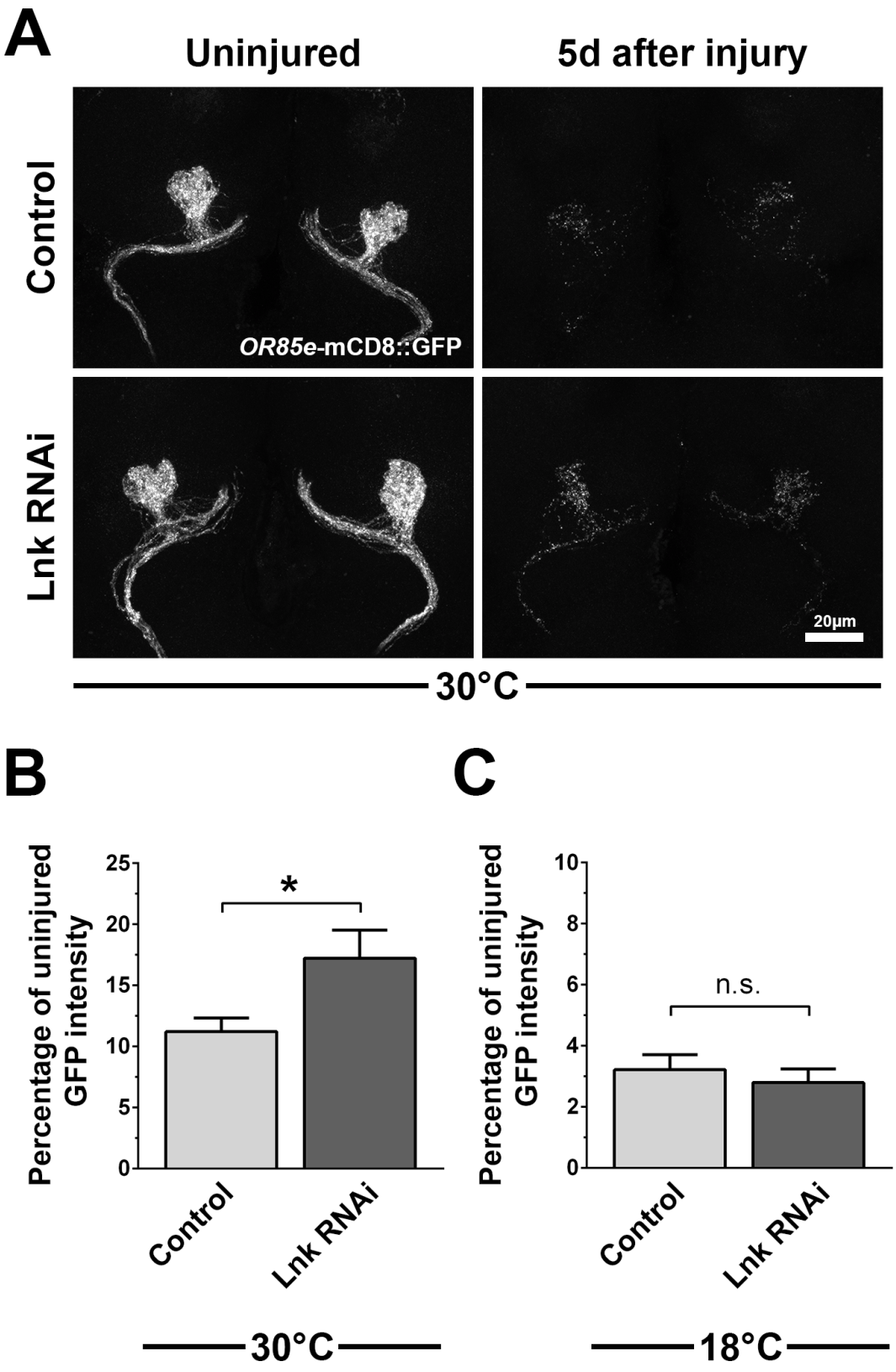
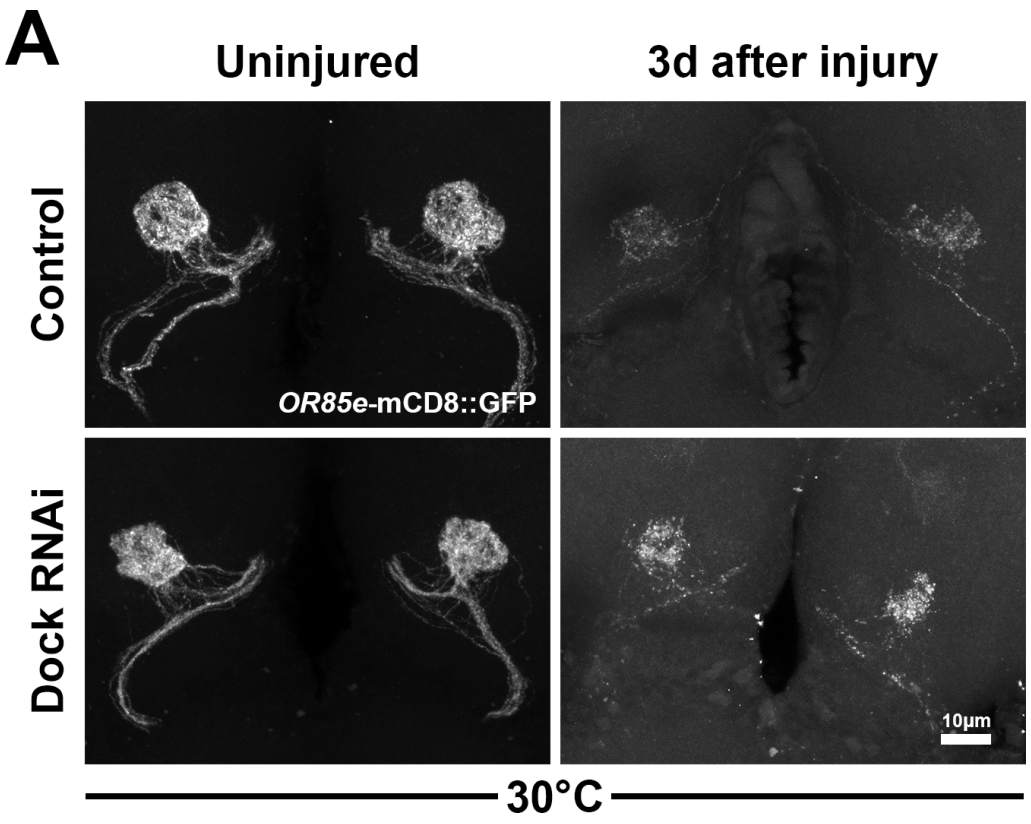

A
Uninjured
5d after injury
Control
Lnk RNAi
OR85e-mCD8::GFP
20μm
30°C
B
Percentage of uninjured GFP intensity
25
20
15
10
5
0
*
Control
Lnk RNAi
30°C
C
Percentage of uninjured GFP intensity
10
8
6
4
2
0
n.s.
Control
Lnk RNAi
18°C

Figure 3.2. Glial Lnk knockdown inhibits clearance of degenerating ORN axons. (A) Maximum intensity confocal projections of the antennal lobe region of the fly brain show that most *OR85e*-mCD8::GFP maxillary palp axons (green) are normally cleared from the antennal lobe region within 5 days after severing the maxillary nerve of control animals (Control). Compared to controls, adult glial expression of double stranded RNA against the Lnk gene (Lnk RNAi) resulted in significantly more GFP^{+} OR85e axonal debris present in antennal lobe regions 5 days after severing the maxillary nerve. Expression of the RNAi transgene was induced in adult flies with a temperature shift 30°C, thereby degrading $Gal80^{ts}$ and disinhibiting Gal4 action on transgene expression. Scale bar = 20µm. **(B)** Quantification of data presented in panel A, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Ordinary 1-way ANOVA with Dunnett's multiple comparisons test, * $p < 0.05$. N = 28 for Control; N = 16 for Lnk RNAi. **(C)** Quantification of a parallel experiment to that shown in A and B where flies were kept at 18°C and therefore transgene expression was never activated, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Ordinary 1-way ANOVA with Dunnett's multiple comparisons test, n.s. $p > 0.05$. N = 12 for Control; N = 19 for Lnk RNAi. Genotypes in Figure 3.2: Control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-*$Gal80^{ts}$/+;*repo-Gal4*/+. Lnk RNAi = *w*/*yw*;*OR85e-mCD8::GFP*, *tubulin-*$Gal80^{ts}$/+;*repo-Gal4*/*UAS-lnk*^{RNA}

Vienna Drosophila Resource Center (VDRC) used in the first experiment, these Harvard TRiP lines use a slightly more advanced approach to inserting the RNAi element into the Drosophila genome using standardized (and therefore non-random) integration sites that guarantee more uniform expression of inserted transgenes. These newer lines are also less dependent on Dicer activity for their efficacy. In practice, they are thought to generally be more effective than the VDRC lines. Because of this, I wanted to try another axonal debris clearance experiment using this newly available $dock^{RNAi}$ line. Upon performing the experiment again with this new line, I found that knocking down Dock did cause a very small but significant defect in glial clearance compared to injured control

Figure 3.3


A
Uninjured
3d after injury
Control
Dock RNAi
OR85e-mCD8::GFP
10μm
30°C

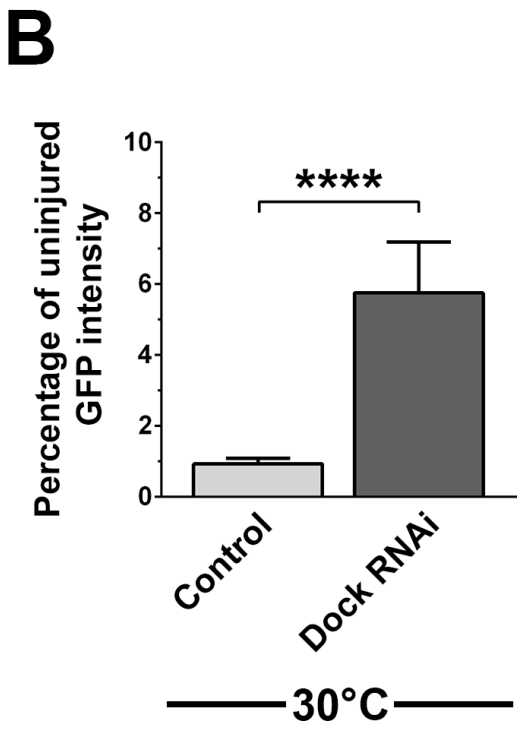

B
Percentage of uninjured GFP intensity
10
8
6
4
2
0
****
Control
Dock RNAi
30°C

Figure 3.3. Glial Dock knockdown inhibits clearance of degenerating ORN axons. (A) Maximum intensity confocal projections of the antennal lobe region of the fly brain show that the majority of *OR85e*-mCD8::GFP maxillary palp axons (green) are normally cleared from the antennal lobe region within 3 days after severing the maxillary nerve of control animals (Control). Compared to controls, adult glial expression of double stranded RNA against the Dock gene (Dock RNAi) resulted in significantly more GFP^{+} OR85e axonal debris present in antennal lobe regions 5 days after severing the maxillary nerve. Expression of the RNAi transgene was induced in adult flies with a temperature shift 30°C, thereby degrading $Gal80^{ts}$ and disinhibiting Gal4 action on transgene expression. Scale bar = 20μm. **(B)** Quantification of data presented in panel A, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Mann-Whitney *U*-test, **** $p < 0.0001$. N = 19 for Control; N = 21 for Dock RNAi. Genotypes in Figure 3.3: Control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/+. Lnk RNAi = *w*/*yw*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/*UAS-dock*^{RNAi} (from BL27728).

flies (Figure 3.3), suggesting a role for Dock in engulfment signaling through the InR.

Loss of Chico results in abnormally high accumulations of Draper at sites of injury

Since the clearance phenotype of the Chico knockdown flies was so profound, I wanted to investigate Chico's involvement in this pathway further. Specifically, I wanted to see if signaling through Chico might be regulating any other known players in the glial phagocytic clearance pathway. For both practical and biological reasons, I chose to start by investigating any potential links between Chico signaling and regulation of the well characterized innate immune receptor, Draper. In practical terms, *draper* was the most accessible gene in the glial phagocytic pathway because our lab already had many reagents targeting it, both at the protein and mRNA level. More importantly, in biological terms,

Draper has been demonstrated to be one of the most important molecular nodes in the glial clearance response. Indeed, when Draper is eliminated, glial clearance of axonal debris is almost completely stalled [94,98].

Due to my previous findings illustrated in Chapter 2 which indicated that insulin-like signaling at the receptor level is a positive regulator of Draper, I expected to find that, if anything, knocking down chico would result in reduced Draper levels in adult glia responding to injury. Surprisingly, I found quite the opposite: Draper immunofluorescence levels are significantly higher around sites of injury in chico^{RNAi} flies compared to injured controls (Figure 3.4A and B). I also quantified levels of axonal debris clearance in these same brains and found that, consistent with previous experiments shown in Figure 3.1, adult glial chico^{RNAi} expression resulted in clearance defects despite the elevated Draper levels (Figure 3.4C and D). Lastly, I quantified the volumetric overlap of Draper and GFP axonal debris at the sites of injury and found significantly more spatial overlap in injured chico^{RNAi} expressing flies than in injured controls (Figure 3.4E and F).

Insulin-like receptor mediated clearance effects do not appear to signal through the transcription factor dFoxo

The *Drosophila* Forkhead Box-O (dFoxo) protein is the most well characterized transcription factor regulated by the insulin-like signaling pathway. dFoxo's phosphorylation by Akt (also known as Protein Kinase B) at several conserved residues sequesters it from the nucleus, thereby inhibiting dFoxo's

Figure 3.4

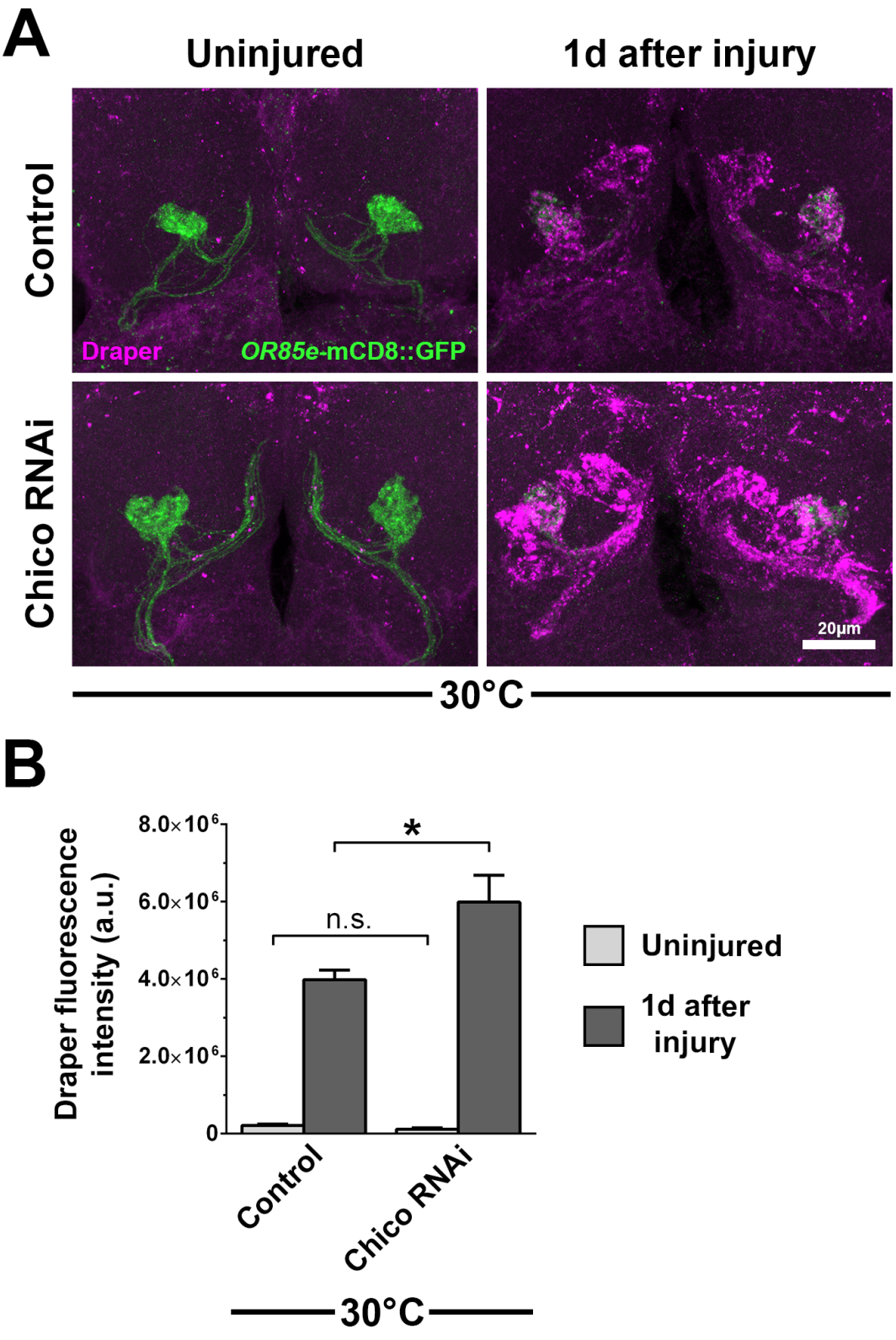
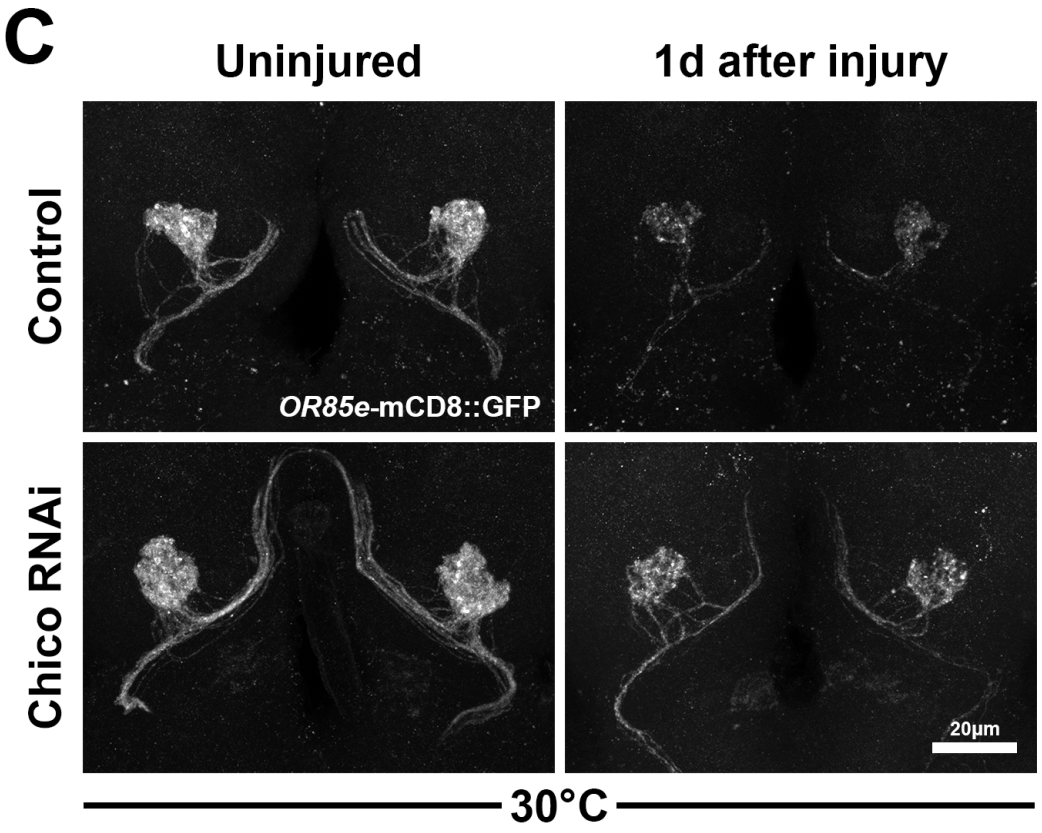

A
Uninjured
1d after injury
Control
Chico RNAi
Draper
OR85e-mCD8::GFP
20μm
30°C
B
Draper fluorescence intensity (a.u.)
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
*
n.s.
Control
Chico RNAi
30°C
Uninjured
1d after injury

Figure 3.4 (continued)



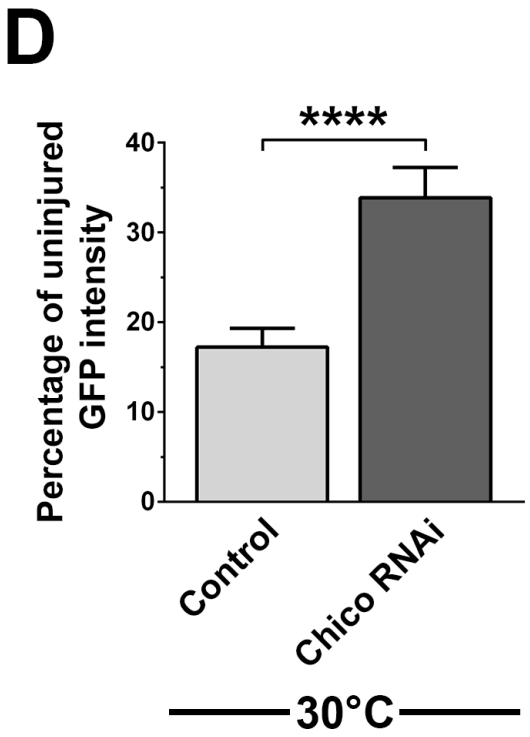
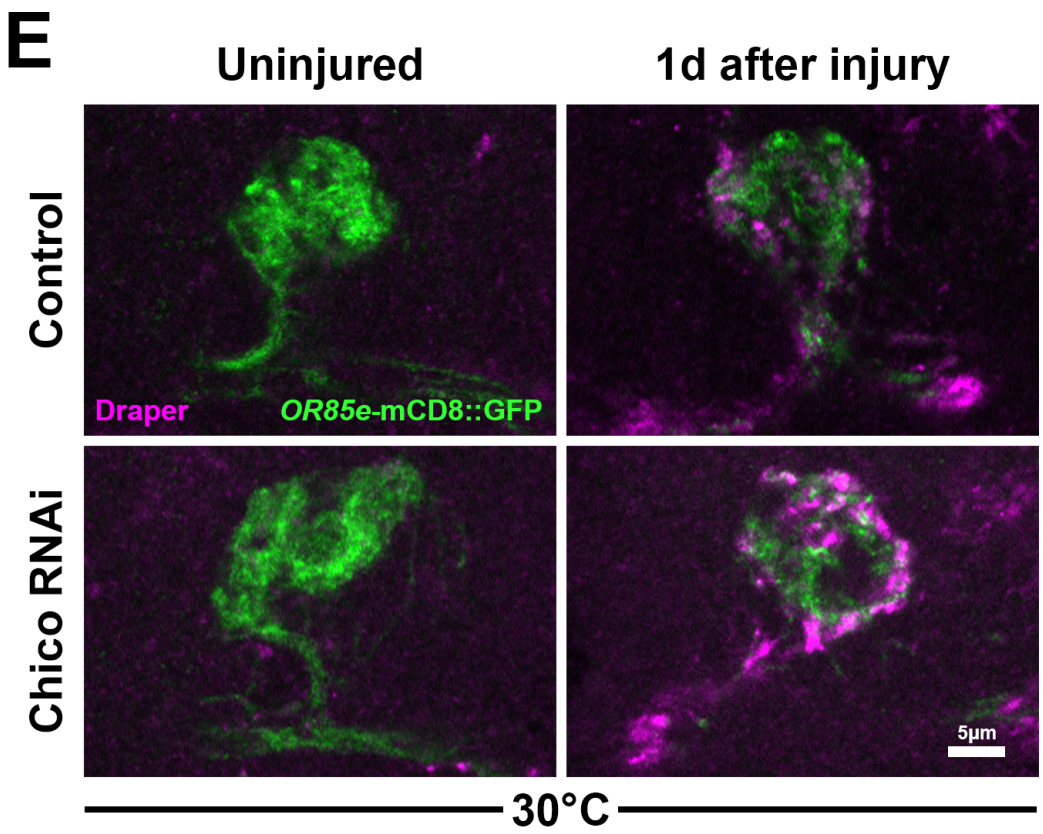


Figure 3.4 (continued)



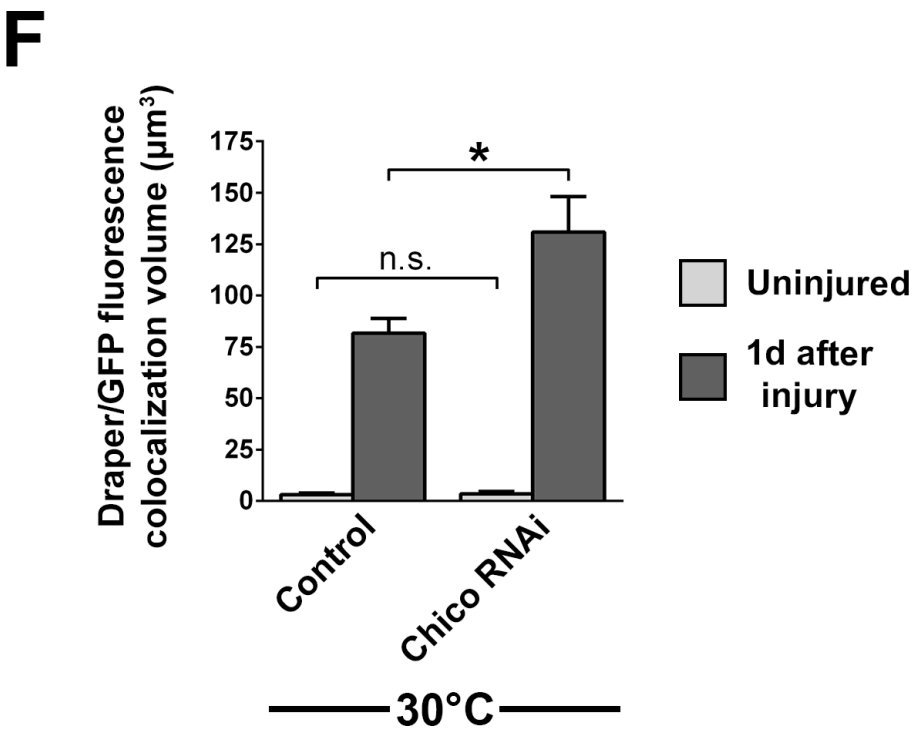


Figure 3.4. Glial Chico knockdown results in excessive accumulations of Draper protein at sites of degenerating ORN axons. (A) Maximum intensity confocal z-projections of the antennal lobe region of the fly brain show upregulation of the Draper receptor (magenta) at sites of degenerating OR85e axons (green) 1 day after severing the maxillary nerve (right column), compared to uninjured fly brains that display only very low baseline levels of Draper around OR85e glomeruli and axons (left column). Control animals display a stereotypically moderate level of Draper injury signal while Chico RNAi animals display strikingly more Draper signal around the injured axons. Scale bar = 20μm. **(B)** Quantification of data presented in panel A, plotting the total Draper fluorescence intensity localized to OR85e glomeruli. Welch's corrected *t*-test, * $p < 0.05$. N = 27 for Control injured; N = 16 for Chico RNAi injured. N = 22 for Control uninjured; N = 16 for Chico RNAi uninjured. Student's *t*-test, n.s. $p > 0.05$. **(C)** Using the same brains shown in parts A and B, clearance of OR85e axon material (white) 1 day after injury is significantly reduced in Chico RNAi expressing brain compared to controls, consistent with the previous findings shown in Figure 3.1. Scale bar = 20μm. **(D)** Quantification of data presented in panel E, plotting the percentage of GFP^+ axonal material in injured OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Student's *t*-test, **** $p < 0.0001$. N = 27 for Control; N = 16 for Chico RNAi. **(E)** Magnified view of single representative z-slices of the same brains used in A-G showing colocalization of the Draper receptor (magenta channel) and the degenerating OR85e axons (green channel). Whiter color represents more colocalization. Scale bar = 5μm. **(F)** Quantification of data presented in panel F, plotting the total volume of colocalized GFP^+ axonal signal with $Draper^+$ glial membrane signal. Chico RNAi expressing flies display significantly higher accumulations of $Draper^+$ glial membrane over presumably unphagocytosed GFP^+ axonal debris. Welch's corrected *t*-test, * $p < 0.05$. N = 27 for Control injured; N = 16 for Chico RNAi injured. Mann-Whitney *U*-test, n.s. $p > 0.05$. N = 22 for Control uninjured; N = 16 for Chico RNAi uninjured. Genotypes in Figure 3.4: Control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/+. Chico RNAi = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}*/+;*repo-Gal4, InR^{ex15}*/*UAS-chico^{RNAi}*.

transcriptional activity. I was curious to know if dFoxo signaling was involved in the InR mediated glial response to acute injury. To test this, I employed a similar experimental approach to that seen multiple times above where I knocked down our particular gene of interest (in this case, dFoxo) specifically in adult glia, then monitored clearance of GFP-labeled axonal debris after an acute axotomy. Since activation of the InR normally results in an inhibition of dFoxo's

transcriptional effects, I reasoned that knocking down dFoxo levels through the use of dFoxo^{RNAi} would be akin to activating the InR pathway. Because of this, I assessed whether or not dFoxo^{RNAi} expression in adult glia leads to faster clearance of axonal debris 1 day after maxillary palp ablation. I found that dFoxo^{RNAi} expression does not drive faster glial clearance of axonal debris, with clearance in these flies being indistinguishable from controls (Figure 3.5).

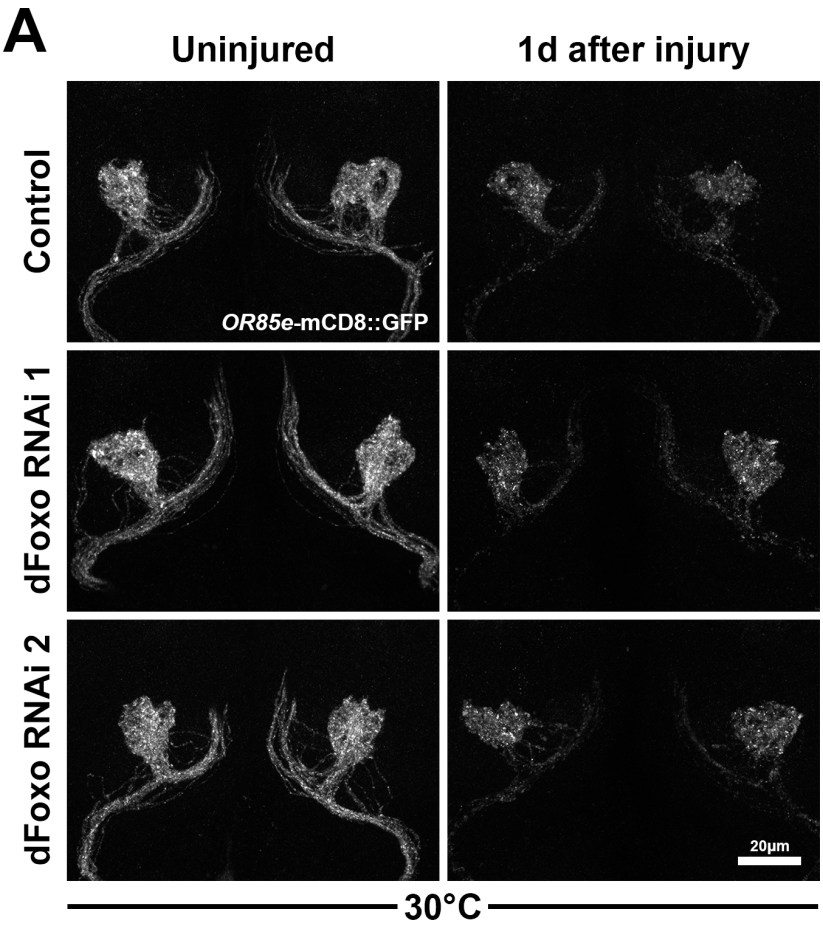
DISCUSSION

In this chapter, I show that the InR substrate Chico (homolog of mammalian Insulin Receptor Substrates 1-4) is critically important for proper glial clearance of axonal debris after injury. I demonstrate that inhibition of Chico in adult glia by means of chico^{RNAi} expression leads to abnormally high accumulations of Draper at sites of injury. Additionally, I show that adult glial expression of RNAi targeting either Lnk or Dock, two other characterized InR substrates in *Drosophila*, leads to a small but significant defect in glial clearance after injury. Finally, I show that adult glial expression of RNAi targeting dFoxo, a well-characterized transcription factor regulated by the InR pathway, has no apparent effect on glial clearance.

Chico signaling is critical for proper phagocytosis during the adult glial immune response

Although my results suggest that Chico is critical for a proper glial innate immune response to injury (Figure 3.1), it is still unclear exactly how Chico exerts

Figure 3.5


A
Uninjured
1d after injury
Control
dFoxo RNAi 1
dFoxo RNAi 2
OR85e-mCD8::GFP
20μm
30°C

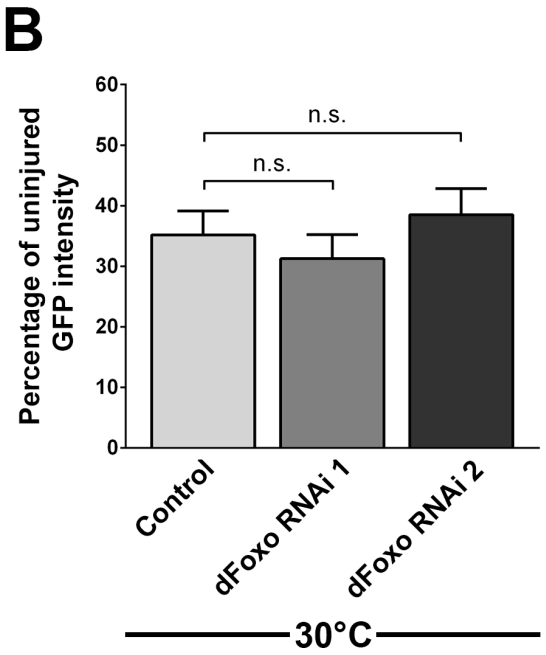

B
Percentage of uninjured GFP intensity
60
50
40
30
20
10
0
n.s.
n.s.
Control
dFoxo RNAi 1
dFoxo RNAi 2
30°C

Figure 3.5. Glial dFoxo knockdown does not augment clearance of degenerating ORN axons. (A) Maximum intensity confocal projections of the antennal lobe region of the fly brain show that that a moderate amount of *OR85e*-mCD8::GFP maxillary palp axons (green) are normally cleared from the antennal lobe region within 1 day after severing the maxillary nerve of control animals (Control). Compared to controls, adult glial expression of two separate double stranded RNA transgenes targeted against dFoxo (dFoxo RNAi 1 and 2) did not result in significantly less GFP^{+} OR85e axonal debris present in antennal lobe regions 1 day after severing the maxillary nerve. Expression of each RNAi transgenes was induced in adult flies using a temperature shift from 18°C to 30°C, thereby degrading $Gal80^{ts}$ and disinhibiting Gal4 action on transgene expression. Scale bar = 20μm. **(B)** Quantification of data presented in panel A, plotting the percentage of GFP^{+} axonal material in OR85e-innervated glomeruli normalized to GFP levels in uninjured brains of each respective genotype. Ordinary 1-way ANOVA with Dunnett's multiple comparisons test, n.s. $p > 0.05$. N = 21 for Control; N = 20 for dFoxo RNAi; N = 21 for dFoxo RNAi 2. Genotypes in Figure 3.5: Control = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/+. dFoxo RNAi 1 = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/*UAS-dFoxo*^{RNAi} (from BL25997). dFoxo RNAi 2 = *w*/*w*;*OR85e-mCD8::GFP*, *tubulin-Gal80*^{ts}/+;*repo-Gal4*/*UAS-dFoxo*^{RNAi} (from BL27656)

this effect. Due to the results presented in Chapter 2 showing the insulin-like receptor to be a positive regulator of Draper, I expected Chico knockdown to result in lower Draper immunofluorescence levels at sites of injury. Instead, I found that chico^{RNAi} expressing glia displayed higher levels of Draper immunofluorescence at sites of injury 24 hours after axotomy, despite persistent defects in ultimate glial clearance.

This unexpected finding, coupled with the results of Chapter 2 showing that adult glial knockdown of the InR leads to both defective glial clearance and lower levels of Draper, hints at a possible fork in the ILS signaling pathway at the level of Chico during the mediation of this glial clearance response. In other words, signaling at the level of the InR and signaling at the level of Chico are somewhat separable here since they are leading to distinct outcomes. Both glial

InR and glial Chico signaling appear to be critical for ultimate clearance of degenerating axons, but InR signaling is crucial for the initial upregulation of Draper around sites of injury while Draper is still robustly upregulated even when Chico signaling is inhibited. Indeed, accumulations of Draper around sites of injury are even higher in glia experiencing Chico inhibition compared to controls, suggesting that Chico's effect on the glial immune response to injury is somehow distinct from the InR's effect alone. So if not playing the same regulatory role for Draper as the InR, what exactly is Chico doing during this injury response, and how can the seemingly contradictory finding reduced clearance in Chico-deficient flies despite higher levels of injury induced Draper be explained?

It is first important to note that my analysis does not distinguish between a role for Chico in regulating Draper expression, versus Draper turnover during the innate immune response. My observation of significantly increased levels of Draper on actively degenerating axons within 24 hours after axon injury in chico^{RNAi} expressing flies could therefore be explained in several ways. One explanation for this result is that Chico-deficient glia overproduce Draper in response to injury at the transcriptional and/or translational level. Alternatively, the Draper receptor may accumulate at sites of injury in chico^{RNAi} animals due to a defect in Draper turnover. I favor the latter model that Draper turnover is attenuated in responding glia following glial knockdown of Chico for several reasons. First, expression of chico^{RNAi} in glia does not appear to noticeably alter basal levels of Draper, at least in my casual observations, suggesting that Chico inhibition probably does not simply drive higher levels of Draper expression to

begin with, though rigorous quantification of basal Draper will be needed to substantiate these observations. Second and more importantly, I found a significant increase in volumetric overlap between Draper-labeled glial membranes and GFP-labeled axonal debris 24 hours after maxillary palp injury (Figure 3.4E and F). In contrast, I observe only modest overlap between Draper immunofluorescence signals and GFP-labeled axonal debris in control flies (Figure 3.4E and F), suggesting that phenotypically normal responding glia (labeled by Draper) quickly and efficiently phagocytose GFP-labeled axonal debris once they have made contact with it. Knowing that GFP fluorescence is very rapidly quenched upon acidification induced by phagocytic uptake [232], I conclude that in our assay, the timeframe of visible GFP/Draper overlap is usually quite short in healthy brains. The fact that I see more Draper/GFP colocalization in the adult glial chico^{RNAi} expressing brains argues that phagocytosis has stalled after the glial membranes have been recruited to injured axons.

Consistent with this idea, a recent paper by Ziegenfuss and colleagues (2012) has shown that the different phases of the adult glial clearance response to axotomy are genetically separable events [103]. As described in Chapter 1, there are two partially redundant parallel pathways that control phagocytic engulfment of apoptotic cells in developing worms and flies (Figure 1.2). Interestingly, when Ziegenfuss and colleagues analyzed these two pathways in the context of glial clearance of degenerating axon debris they found that these respective pathways control unique steps of the glial response to axotomy.

Specifically, the Draper pathway is essential for the initial activation and recruitment of glial membranes to sites of injury, while the guanine nucleotide exchange factor complex dCrk/Mbc/dCed-12 pathway, along with the small GTPase Rac1, is critical for the actual phagocytic event itself [103]. Importantly, knockdown of dCrk/Mbc/dCed-12 phenocopies my chico^{RNAi} result, with extra Draper lingering around injured axons followed by reduced clearance of axonal debris. This finding opens up the intriguing possibility that Chico might be signaling, at least in part, through this dCrk/Mbc/dCed-12 complex and/or Rac1, and therefore knocking down Chico levels could lead to aberrant phagocytosis of axonal debris while still allowing for phenotypically normal glial recruitment to sites of injury.

Another possible way to explain the observed increase in Draper immunofluorescence at sites of injury after Chico knockdown is that the increased Draper signal could be a result of increased levels of the Draper-II isoform, which has previously been shown to inhibit glial clearance activity [98]. The alternative splicing event that yields Draper-II turns the intracellular ITAM domain present in Draper-I into an ITIM domain, thus uncovering an inhibitory function of this Draper isoform [98]. To test the idea that chico^{RNAi} might be increasing levels of Draper-II, I will perform real time quantitative PCR experiments assaying Draper-II transcript levels specifically after adult glial knockdown of Chico by RNAi. Increased Draper-II transcript levels in chico^{RNAi} expressing animals would be a very interesting result as it would point to a novel role for Chico in regulating acute splicing events in cells undergoing innate

immune responses. At this point, assaying protein levels of Draper-II at sites of injury is not feasible due to the lack of a Draper-II specific antibody. Furthermore, I cannot probe Draper-I versus Draper-II levels via western blotting techniques in our olfactory system axotomy assay because few (perhaps only several dozen) out of the thousands of Draper expressing glia in the *Drosophila* brain respond to this small injury event. More robust assays, such as ventral nerve cord axotomies that induce Wallerian degeneration in many nerves and thus a more widespread response from a higher percentage of glia, may allow for the assessment of Draper-I versus Draper-II expression differences by western blot analysis. If I find that Draper-II is indeed upregulated after injury in chico^{RNAi} expressing glia, then increases in Draper-II levels would be a plausible explanation for the enhanced accumulation of Draper immunofluorescence after maxillary palp injury, despite the reduced clearance of degenerating axons.

The magnitude of the role of Lnk and Dock in glial clearance of degenerating axons remains unclear

Expression of double-stranded RNA (dsRNA) against Lnk or Dock in adult glia resulted in a very weak phenotype with regard to glial clearance of degenerating ORN axons. Two possibilities could account for these observations: 1) Lnk and Dock play minor roles in glial phagocytosis of degenerating axonal debris, or 2) our RNAi constructs are not efficacious enough to knock down Lnk and Dock to levels at which a more robust clearance phenotype could be observed. Unfortunately, there are no effective and reliable

antibodies available to experimentally test the efficacy of these Lnk and Dock RNAi knockdown methods in adult glial cells.

To follow on from my work above and investigating the role of Lnk and Dock in glial immune function, our lab will repeat these RNAi experiments with more advanced Lnk and Dock RNAi lines that recently became publicly available through the Transgenic RNAi Project (TRiP) at Harvard University. Additionally, our lab recently acquired an *Lnk* null mutant fly strain that produces homozygous mutant adults, albeit at very low frequency. We will test these mutants in our glial response assays to assess glial recruitment, Draper upregulation, and clearance of degenerating axonal debris. Together, these experiments will reveal the impact of Lnk and Dock in this complex cellular innate immune response.

dFoxo signaling may not be essential for the InR mediated glial injury response

My results suggest that the InR signaling cascade exerts its effects on glial immunity independent of the transcription factor dFoxo. Since ILS normally leads to the inhibition of dFoxo's transcriptional activity, I expected that, if dFoxo is involved in this glial clearance response, knockdown of dFoxo through RNAi expression would result in faster glial clearance of axonal debris after injury, mimicking the effect of expressing a constitutively active form of the InR seen in Chapter 2 (Figure 2.3). I did not observe this effect for dFoxo knockdown, suggesting that dFoxo might not involved in the glial injury response. However, I should note that assessing glial function by probing for faster clearance of axonal

debris 1 day after injury is a new technique that, to my knowledge, has never been performed before in published studies. While I had success with this particular technique in assessing clearance of constitutively active InR expressing glia (seen in Chapter 2), I cannot yet verify the robustness of this assay to gauge enhanced clearance in all circumstances. For instance, it is possible that reduced dFoxo signaling is most important in later stages of the glial clearance response and that assessing clearance differences so early (1 day) into the response would not capture this effect. For this reason it would be interesting to over-express an existing UAS-dFoxo construct [233] in glia in my injury model and assess for reduced clearance several days after injury (the gold standard of glial clearance assays). Additionally, an even more pointed way to probe this question might be to develop a constitutively active UAS-dFoxo construct, possibly having mutations that restrict its ability to be exported from the nucleus. This constitutively active dFoxo could be expressed in adult glia as in my previous experiments and their ability to clear severed axons could be scored many days after injury. Using either of these two techniques might be a more robust way to probe dFoxo's possible role in the glial clearance response, as the timeframe for observing a phenotype might potentially be longer. If dFoxo is normally playing a role in the glial immune response, it is most likely an inhibitory one, helping to keep glia in a non-reactive state when no insult has occurred.

Insulin-like signaling as a regulator of different aspects of the glial immune response to injury

I believe the model best supported by my data is one where the insulin-like signaling pathway in adult glia regulates multiple aspects of the glial immune response to injury. In particular, I hypothesize that activation of the insulin-like receptor itself, along with one or more of its substrates, acts to upregulate innate immune factors necessary for both reactive glial process recruitment to sites of injury and phagocytosis of targeted material. The fact that the InR, acting through its substrate Dock (but not Chico), has previously been reported to play a role in photoreceptor cell axon guidance [194], lends credence to the idea that InR signaling could be orchestrating a functionally similar recruitment of reactive glial membranes to sites of injury during an acute axotomy event. Additionally, I hypothesize that, through Chico signaling, the InR serves to stimulate the phagocytic maturation needed to ultimately engulf and eliminate cellular debris. It is possible that this Chico dependent phagocytic maturation might be mediated by the dCrk/Mbc/dCed-12 complex pathway and/or Rac1, but further experiments testing this idea are needed. In particular, it will be very interesting to see if glial over-expression of either the dCrk/Mbc/dCed-12 complex or Rac1 can fully rescue my observed chico^{RNAi} mediated clearance defects. If so, this would firmly place these factors downstream of Chico signaling and further support the notion that Chico is most critical for the later stages of the glial clearance response as opposed to glial process recruitment to sites of injury.

Another line of experimentation I would like to pursue in the future is to monitor the glial morphological changes taking place after injury to see how different proteins in the InR signaling pathway might influence this aspect of the glial response. So far, our lab has used Draper immunofluorescence as a proxy label for reactive glial membranes, and while this is a useful technique and has been shown in the past to track fairly well with independently labeled responding glial membranes [94,110], it is possible that this does not hold true in all circumstances. I would predict that inhibiting InR signaling at the receptor level would lead to a defective glial morphological response to injury, while inhibiting ILS at the level of Chico would lead to phenotypically normal recruitment of glial membranes to sites of injury.

While glial recruitment to sites of injury did not appear to be diminished in chico^{RNAi} expressing flies, I cannot yet completely rule out a role for Chico in this early stage of the innate immune response. This is due to a unique feature of the *Drosophila* InR not present in its mammalian orthologs: namely, that the *Drosophila* InR's intracellular C-terminus contains a 368 amino acid extension that shares a high level homology with the domain of Chico that binds to and activates PI3K [195]. Indeed, due to the presence of this additional "Chico-like" domain, the *Drosophila* InR appears to be able to partially circumvent the need for Chico in activating PI3K by instead simply directly activating PI3K itself [195,196]. The fact that in *Drosophila* the InR itself can partially fulfill the signaling role of Chico (at least in activating the PI3K signaling cascade), opens up the possibility that in our injury paradigm Chico could be playing PI3K-

dependent role that might not be readily revealed by simply looking for a phenotype after chico^{RNAi} expression. In other words, it is possible that Chico could normally be playing a small to moderate role in the early stages of the glial innate immune response that can also be duplicated by the "Chico-like" domain on the InR's C-terminal tail in the absence of Chico (as in my own chico^{RNAi} experiments). While this caveat is somewhat academic when only considering this response in the context of fruit flies, it becomes a much more important consideration when thinking about the implications for mammals, whose insulin and IGF receptors do not have a domain that can directly activate PI3K. That is to say, it is possible that Chico might be playing a role in early glial activation that can also be performed by the *Drosophila* InR directly, while this same role might be played solely by an IRS protein in humans.

It should also be noted that the existence of this partial redundancy between the InR itself and Chico in activating the PI3K signaling cascade lends support to the idea that the clearance phenotype seen in adult glial chico^{RNAi} expressing flies is most likely due to a non-PI3K dependent effect of Chico. This fits nicely with the notion that Chico may be playing a rather unique role in the later stages of the glial injury response, such as promoting phagocytic maturation through the regulation of the dCrk/Mbc/dCed-12 complex pathway and/or Rac1. Again, this is consistent with the idea of a fork in the ILS pathway at the level of Chico, at least in the context of the glial clearance response. On one side of the fork, signaling at the receptor level (probably mediated through the InR's "Chico-like" domain) appears to be critical for the initial activation of pro-phagocytic

components like Draper after injury. On the other side of the fork, Chico might play a distinctly different role during glial immune responses, not in the injury induced upregulation of Draper (and possibly other pro-phagocytic factors), but rather in the later stages of the glial clearance response such as phagocytic engulfment.

Collectively, the work presented in this chapter showcases an important new role for the insulin-like receptor substrate protein Chico, and while the exact mechanistic connections of its role(s) in the glial innate immune response remain unclear, the data presented here open exciting new pathways of inquiry into unraveling the details of Chico's contribution to this complex cellular response. Additionally, this work implicates the two other confirmed *Drosophila* InR substrates, Lnk and Dock, in the glial immune response. While the absolute importance of these two effectors on the glial injury response is still unclear, they are both certainly well poised to play significant roles based on molecular knowledge of their mammalian orthologs. Overall, the InR appears to be very well situated as a high level orchestrator of the complex molecular changes that must undoubtedly take place to accomplish a glial immune response, and it would not be surprising if all three substrates outlined here end up being important in their own way toward this end.

MATERIALS AND METHODS

Fly Stocks. The following *Drosophila melanogaster* strains were used (separated by bullet points to preserve clear nomenclature punctuation): *repo*-Gal4 • *OR85e*-mCD8::GFP, *tubulin*-Gal80^{ts}; *repo*-Gal4 [103] • *InR*^{ex15} [194] • *w*^{1118}; *OR85e*-mCD8::GFP, *tubulin*-Gal80^{ts}; *repo*-Gal4, *InR*^{ex15} • UAS-chico^{RNAi} (Vienna *Drosophila* Resource Center line 7777) • UAS-lnk^{RNAi} (VDRC line 103646) • UAS-dock^{RNAi} (VDRC line 37524) • UAS-dock^{RNAi} (Bloomington stock 27728) • UAS-dFoxo^{RNAi} (Bloomington stock 25997) • UAS-dFoxo^{RNAi} (Bloomington stock 27656).

Olfactory Neuron Injuries, Dissection, and Analysis. I performed maxillary palp ablations, adult fly brain dissections, and whole brain antibody staining using previously described methods [94]. To quantify clearance of *OR85e*-mCD8::GFP labeled maxillary palp olfactory receptor neurons, I computationally reconstructed OR85e-innervated glomeruli and performed blinded volumetric quantification of above threshold GFP^{+} fluorescent signals, with background fluorescence subtraction, using Volocity software (Perkin Elmer). To quantify Draper immunofluorescence in responding glia after maxillary palp injury, I first volumetrically segmented my area of quantification to the OR85e-innervated glomeruli using the *OR85e*-mCD8::GFP signal as a guide. I then blindly quantified above threshold Draper^{+} fluorescent signals in this volume using Volocity software, as above.

Antibody Use and Concentrations. The following antibodies were used: 1:200 mouse anti-GFP (Invitrogen), 1:1000 chicken anti-GFP (Life Technologies), 1:200 mouse anti-Draper (Abmart Inc.), 1:400 Alexa 488-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch), 1:400 Alexa Fluor 488-conjugated donkey anti-chicken IgY (H+L) (Jackson Immunoresearch), 1:400 Rhodamine Red-X-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch), 1:400 Alexa Fluor 647-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch)

Draper Antibody Production. Our lab generated a monoclonal mouse anti-Draper antibody against the epitope: NPIVYNESLK. This antibody was designed and produced by Abmart Inc. using their Protein Surface Epitopes Targeted by Monoclonal Antibody Library (SEAL) technique. The particular mouse anti-Draper antibody used here was derived from a hybridoma cell tissue culture supernatant provided by the Developmental Studies Hybridoma Bank (University of Iowa) to whom we entrusted the hybridoma cell line purchased from Abmart.

Confocal Microscopy. All immunostained brains were imaged on a Zeiss LSM 700 with a Zeiss 40X 1.4NA oil immersion plan-apochromatic lens. Brains within a single experiment (i.e. those being directly compared for quantification) were whole mounted under a single #1.5 cover glass in either Vectashield mounting media (Vector Laboratories) or CFM3 mounting media (CitiFluor). All brains being directly compared were imaged on the same day with the same confocal microscope settings (laser power, photomultiplier tube gain, offset, filter configuration, etc.). The sampling interval between Z-stacks in all experiments

was 0.76 microns, while the pixel size ranged from 100nm - 230nm.

Statistical Analysis. GraphPad Prism software was used to perform: Ordinary 1-way Analysis of Variance tests, Kruskall-Wallis 1-way Analysis of Variance tests, two-tailed Student's *t*-tests, two-tailed Welch's *t*-tests, two-tailed Mann-Whitney *U*-tests, Dunnett's multiple comparisons *post hoc* tests, Holm-Sidak's multiple comparisons *post hoc* tests. Assumptions of normality were tested with the D'Agostino-Pearson normality test. Where applicable, outliers were identified using the ROUT method. In some analyses, log-transformations were uniformly performed on otherwise non-Gaussian data sets to allow for the appropriate use of parametric tests. When assumptions of normality could not be met for a given data set, non-parametric tests were used. Each N = 1 sample number represents pooled measurements taken from independent animals.

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Chapter 4

Summary and discussion

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Here I have shown for the first time a new role for the insulin-like signaling (ILS) pathway in the glial immune response to acute CNS injury in adult *Drosophila*. I have demonstrated that ILS in glia is critical for effective clearance of axotomized tissue. Acutely activating ILS in adult glia augments the clearance of axonal debris, while inhibiting glial ILS causes significant clearance defects. Furthermore, ILS is a positive regulator of Draper, an engulfment receptor critical for an effective glial clearance response. Much of the InR's clearance promoting effects appear to be mediated by Draper, as Draper re-expression almost fully rescues the clearance defects seen after knocking down the InR with RNAi, at least at the 4 day time point after injury. Nevertheless, the rescue is not complete, indicating that the InR still plays other roles independent of Draper to promote glial clearance of axonal debris. Finally, I identified a potential split in the ILS-mediated pro-glial clearance signaling event at the level of the insulin receptor substrate Chico. In particular, while signaling at the receptor level appears to be critical for upregulation of Draper after injury in responding glia, Chico signaling appears to play a different role in the glial clearance event, possibly in the later stages of clearance such as phagocytic maturation.

Chico as a non-canonical signaler in the ILS mediated glial clearance event?

Arguably the most interesting (certainly the most unexpected) result presented here is the finding that adult glial Chico knockdown does not interfere

with injury induced Draper upregulation at sites of injury (unlike InR knockdown), yet still causes dramatic defects in glial clearance of axonal debris (Figure 3.4). Indeed, not only did Chico knockdown fail to impede injury induced upregulation of Draper, it actually resulted in higher accumulations of Draper at sites of injury. So on the one hand, glial ILS at the receptor levels is critical for Draper upregulation after injury (as seen Chapter 2), but on the other hand, inhibition at the level of Chico does not appear to reduce levels of Draper, yet still inhibits clearance. This hints at a potential fork in the glial clearance promoting insulin-like signaling event at the level of Chico, where the InR signals through as of yet unknown Chico-independent means to bring about Draper upregulation, while Chico itself is essential for non-Draper dependent aspects of the glial clearance response. This is quite intriguing because it potentially represents a non-canonical signaling paradigm for Chico and simultaneously a somewhat unusual Chico-independent signaling paradigm for the InR in this response. This begs the questions of what exactly Chico is doing to promote glial clearance if not acting to mediate the InR's upregulation of Draper (and possibly other phagocytic components as well)? These findings also beg the related question of how the InR might be signaling in non Chico-dependent ways to promote glial clearance? In the next few sections below, I will address these questions and propose some possible explanations.

Chico as a regulator of dCrk/Mbc/dCed-12?

Perhaps the biggest conundrum in my data is the finding that adult glial Chico knockdown leads to more, not less, Draper at sites of injury, even while simultaneously resulting in defective glial clearance despite the high Draper levels. While on its face this finding seems somewhat counterintuitive, one potential explanation is that Chico is actually regulating the later stages of glial phagocytosis after the membrane processes of reactive glia have already been recruited to sites of injury. In this way, the excess Draper signal around injury sites might be explained by a reduction in phagocytic turnover of the responding glial membranes (and hence a gradual build-up of membrane associated pro-phagocytic proteins like Draper). Importantly, inhibition of these later phagocytic steps of the glial clearance response would still lead to ultimate persistence of axonal debris, even if the expression of early responding phagocytic factors (like Draper) were not inhibited. In this way, a defect in Chico-dependent phagocytic maturation might explain the seemingly disparate observations I found in glial chico^{RNAi} expressing flies.

How might Chico regulate the later stages of the glial clearance response? Luckily, there are some hints in the literature pointing to at least one possible explanation. Of particular salience is the finding that one of the mammalian orthologs of Chico, called Insulin Receptor Substrate 4 (IRS-4), has been demonstrated to physically interact with CrkII (mammalian ortholog of *Drosophila* dCrk) in 293 HEK cells upon stimulation of the IGF-1 receptor by IGF-1 [234]. The functional significance of this interaction is not known, but it does potentially

provide an intriguing direct biochemical link between Chico and the dCrk/Mbc/dCed-12 complex, which has been demonstrated to act as a guanine nucleotide exchange factor (GEF) for Rac1 [105,106] and play an essential role in the late stages of the glial phagocytic clearance process after glial membranes have already been recruited to sites of CNS injury [103]. Whether Chico can interact directly with dCrk to influence dCrk/Mbc/dCed-12 activity in *Drosophila* (and thereby influence Rac1 activation) is not known. Nevertheless, the possibility is quite tantalizing as it might explain some of the chico^{RNAi} induced clearance defects. In particular, a regulatory role of Chico for the dCrk/Mbc/dCed-12 complex could explain the failure of chico^{RNAi} expressing glia to clear axonal debris, even while glial recruitment to the debris appears intact, as this mirrors the defects seen in flies with attenuated dCrk/Mbc/dCed-12 signaling [103]. If Chico is directly regulating the dCrk/Mbc/dCed-12 complex and hence regulating the later stages of phagocytosis, then this might also explain why Draper is highly accumulated around sites of injury in chico^{RNAi} expressing glia even while the final clearance of the injured debris is defective. These observations could be indicative of reduced Draper turnover, which might be a result of defective dCrk/Mbc/dCed-12 complex signaling through Rac1 and hence faulty maturation of the phagocytic response. In future experiments, it will be interesting to test whether expression of constitutively active Rac1 in adult glia can rescue chico^{RNAi} clearance defect. If so, this will suggest that Chico is indeed acting through Rac1, and will lend credence to the idea of Chico serving as an upstream regulator of the dCrk/Mbc/dCed-12 GEF complex.

Chico as a regulator of Draper-II?

Another potential, albeit less likely, way to explain the seemingly disparate findings of my chico^{RNAi} experiments is that Chico signaling might regulate levels of Draper-II, an isoform of Draper that has previously been shown to powerfully inhibit the glial clearance functions of the Draper-I isoform [98]. Importantly, the Draper antibody used in my experiments cannot distinguish between the Draper-I and Draper-II isoforms, so the increased Draper signal seen around sites of injury in chico^{RNAi} expressing glia could potentially be due in part to an increase in Draper-II levels. If this were the case, it stands to reason that increased Draper-II expression in chico^{RNAi} knockdown could result in reduced clearance even with an apparent increase in overall Draper immunofluorescence signal from the antibody I used. To resolve this, I plan to perform quantitative PCR experiment on fly central brains with adult glial chico^{RNAi} expression versus genetic background controls, and assess mRNA transcript levels of *draper-II* 4.5 hours after an antennal injury, a time point which has previously been demonstrated to be the peak of *draper-II* expression after this kind of insult [98]. This will tell me if indeed *draper-II* expression (and therefore presumably Draper-II protein expression) is increased in chico^{RNAi} expressing glia responding to injury. If Chico does influence Draper-II levels, this would represent an exciting new role for the InR signaling pathway in regulating mRNA splicing events after acute CNS injury, though admittedly there is no known precedent for IRS proteins regulating splicing, so this possibility is perhaps unlikely based solely on

past work. The quantitative PCR experiment detailed above should nonetheless provide ultimate clarity on this.

Is Chico also involved in the early, late, or multiple phases of the glial injury response?

The fact that chico^{RNAi} expression in adult glia does not appear to interfere with glial recruitment to sites of injury, at least as measured by Draper positive glial membrane recruitment 1 day after injury, seems to indicate that Chico is not involved in the early stages of the glial injury response (e.g. chemotaxis toward "find-me" signals, upregulation of "eat-me" recognition components, initial upregulation of phagocytic machinery components, etc.). One must use caution when making this logical leap though, as *Drosophila* insulin-like signaling is unique in one important way compared to mammalian ILS. The difference is in the InR receptor itself, which in *Drosophila* carries a unique intracellular C-terminal extension that is homologous to the region of Chico that binds to and regulates the phosphatidylinositol 3-kinase (PI3K) complex [195]. Importantly, functional analysis of the receptor has revealed that it can activate PI3K signaling (albeit at a lower level) through this "Chico-like" domain upon stimulation with insulin, even in the absence of intracellular Chico [195]. What are the consequences of this for my findings? Well, it is possible that this additional ability of the *Drosophila* receptor to directly activate PI3K might be compensating for the loss Chico signaling (in activating PI3K) during genetic knockdown of Chico with RNAi during the glial clearance event. This could effectively mask a

real role for Chico, where Chico might normally activate PI3K signaling during the glial immune response when Chico is present even though the “Chico-like” domain of the InR can partially fill in for it when absent. As mentioned in the discussion of Chapter 3, this distinction becomes more important for the ILS human situation as none of the mammalian insulin-like receptors (insulin receptor, IGF-1R, etc.) contain this extra Chico/IRS like domain on their C-termini like the *Drosophila* receptor does.

Alternatively, Chico might really not be important for the early phases of the glial clearance response (such as the transcriptional and/or translational activation of pro-engulfment factors like Draper). In this scenario, the InR alone might signal through its own “Chico-like” domain to activate PI3K signaling and subsequent downstream cascades. This InR “Chico-like” domain is most similar to the PI3K binding domains of the human IRS-1 and IRS-2 proteins, though IRS-4 is also not too far behind these in terms of similarity. It could very well be that this “Chico-like” domain of the *Drosophila* InR is playing the role that one of IRS proteins normally plays in a human glial immune response, while Chico itself could be playing a role that another IRS protein plays in the later stages of the ILS mediated glial response. Chico shares nearly equal sequence identity (and similarity) with human IRS-1, -2, and -4, so it is difficult to speculate about which of these human IRS proteins might be most functionally homologous to Chico during this glial clearance event. Nevertheless, the finding that IRS-4 can physically interact with CrkII [234], and therefore also presumably with the CrkII/Dock180/ELMO complex that is so critical during the late stages of glial

clearance, makes it tempting to hypothesize that Chico might be serving a role similar to IRS-4 in regulating this GEF complex to activate Rac1 in the glial clearance response. IRS-1 and IRS-2 are ubiquitously expressed in mammals and appear to mediate the majority of the insulin-dependent signaling action of the mammalian InR (reviewed in [235]), while IRS-4 expression is much more restricted, but nevertheless is also expressed in the brain like IRS-1 and IRS-2 (note: IRS-3 is only present in rodents) [236]. Still, there is no apparent evidence in the literature linking different IRS protein functions with different aspects of innate immune responses, so it is difficult to speculate at this point as to which IRS proteins might be involved in which processes during ILS mediated glial immune responses. Nevertheless, this apparent spit in ILS mediated signaling at the level of Chico during glial clearance does strongly hint that the "Chico-like" domain of the InR, and Chico itself, might be functionally playing the roles of different IRS proteins during this complex response. Future experiments assessing the glial clearance response in flies expressing C-terminally truncated forms of the InR lacking the "Chico-like" domain might shed light on the relative contributions of this "Chico-like" domain versus Chico itself in bringing the various aspects of a proper glial clearance response.

Is the role of Lnk similar to that of Chico in the glial injury response?

While my results suggest that the InR substrate Lnk plays a role in the glial clearance of severed axons, the exact details of its molecular function in this

process are still unknown. I suspect that Lnk probably plays a similar role as Chico to promote glial clearance of axonal debris since Lnk has been shown to serve largely similar functional roles as Chico *in vivo* [224], most likely through Lnk's regulation of Chico's subcellular localization and Chico's interactions with the InR [196]. Going forward, it will be interesting to know if robust inhibition of Lnk yields a similar phenotype as chico^{RNAi} expression in regards to the excess Draper accumulations observed at sites of injury. If so, this would argue strongly for Lnk signaling through Chico to exert its effects on glial clearance.

Potential Draper-independent roles of insulin-like signaling in the glial clearance response

Forced adult glial expression of Draper-I did not rescue all the glial clearance defects brought on by InR knockdown, indicating the ILS plays roles to mediated the glial clearance response that are independent of Draper. What might some of these be? The most straightforward answer is that ILS might simply be regulating other innate immune components (some of them probably unknown) involved the glial clearance response, possibly in a similar fashion to its regulation of Draper. Since the insulin-like signaling pathway can affect cytoskeletal remodeling, cellular metabolism, the transcription of scores of genes, and the translational machinery of the cell [207-210,230,237], the possible ways for InR signaling to regulate the glial clearance response are myriad. Just a few of these possibilities will be discussed in more detail below.

Does ILS regulate the cytoskeleton during glial immune responses?

One way that the InR might be acting to promote innate glial immunity is by directly mediating some of the cytoskeletal changes necessary to initiate and/or complete a directed phagocytic response. The InR's role in photoreceptor axon guidance in the developing *Drosophila* visual system lends credence to this idea, as it appear to act as a guidance receptor in this particular instance, signaling through its substrate protein Dock [194]. The fact that Dock appears to be involved in the glial clearance response in my own investigations (albeit only to a modest degree) further adds to the speculation that InR signaling might indeed play a cytoskeletal regulatory role in the glial clearance response. Further experimentation will be needed to resolve this and it will be interesting to see if knocking down Dock with newly available, more efficacious RNAi constructs will yield a more robust clearance defect; a result which would further cement Dock's role in this clearance response. Directly assessing glial morphological response after the knockdown of Dock will also help to establish if Dock is important in the early recruitment stages of the glial injury response or later phagocytic phases.

Additionally, it would be interesting to know if adult glial expression of constitutively active Rac1 can rescue InR^{RNAi} mediated clearance defects in the same way that forced expression of Draper-I is able to. This would add further support for a role of the InR in cytoskeletal remodeling during glial clearance, as Rho family GTPases like Rac1 have a well established role in cytoskeletal regulation [238].

Does ILS regulate glial metabolic changes during the innate immune response?

Another potential role for glial insulin-like signaling in the glial response to injury is a metabolic one. Reactive gliosis must be quite energetically taxing for the activated/responding glia, and it stands to reason that a pathway such as ILS with its many inputs into metabolic homeostatic control might be used to orchestrate the intracellular signaling needed to keep up with the metabolic demands associated with mounting an effective clearance response to injury. While most recent evidence suggests that in the mammalian brain cellular uptake of glucose is largely independent of insulin (reviewed in [146,147]), there is still some CNS expression of insulin-sensitive glucose transporters in mammals (GLUT-4 and GLUT-8), though expression of these has mainly been identified in neurons, not glia [148,150]. Nevertheless, the expression of glucose transporters specifically in reactive glia responding to injury has not been studied, so it is unclear if the exact complement of glucose transporters expressed in the context of reactive gliosis might shift to those that are sensitive to insulin. Even if insulin-like signaling is not involved in the uptake of extracellular sugar by glia during the clearance response, it could still play important metabolic roles during the glial clearance event. This is due to insulin-like signaling's other main metabolic contribution, which is the synthesis of lipids (i.e. lipogenesis).

Lipids are of course essential to countless cell biological processes. Indeed, the very existence of cellular organisms in the first place owes a great deal to lipids since they serve as fundamental building blocks of cellular

structure. Within a cell, lipids are important for intracellular signaling, trafficking, and energy production, all events that are undoubtedly critical to the glial clearance response. Additionally, a hugely important role of lipogenesis that is, frankly, easy to overlook because it seems so fundamental as to be taken for granted, is the role of serving to provide the raw material necessary to synthesize new phospholipid membranes. This seems particularly relevant in the context of the glial clearance response, as phagocytosis is known to require new synthesis of plasma membranes [239]. Whether or not ILS plays a role in the production of new membranes during the glial clearance response is not yet clear, but future experiments investigating the effect of insulin-like signaling specifically on the morphological responses of glial membranes to degenerating tissue might provide an opportunity to clarify this issue, as glial membrane dynamics can be directly monitored in these types of experiments.

Is Ras/MAPK signaling involved in the glial clearance response?

One aspect of the insulin-like signaling pathway that I have not tested for its potential glial clearance function is the Ras/MAPK signaling cascade. Ras/MAPK signaling is an important regulator of cellular growth and proliferation (reviewed here [240]) and might represent another critical function in ILS's influence over the glial injury response. Indeed, it is not difficult to image some aspect of cellular growth being necessary in glia responding to injury, since these activated glia must not only extend long processes toward sites of damage, but must also fully encompass and internalize often substantial amount of cellular

debris. *Drosophila* possesses orthologs to nearly all the core signaling proteins in the Ras/MAPK cascade, and there are even publicly available RNAi lines targeting each of the major components of this pathway. In the future, it will be interesting to knock down some of the genes in the Ras/MAPK pathway in the context of the *Drosophila* olfactory axotomy assay to determine whether or not Ras/MAPK signaling is involved in the glial clearance response. If it is, it would represent yet another aspect of ILS that is fundamentally important for innate glial immunity.

What ligands activate the InR during the glial immune response?

One follow-up question that my work here poses is what are the ligands that activate the InR in the glial immune response and where are they coming from? The short answer is that I simply do not know. I have very little experimental evidence to answer this question. Presumably the activating ligand is one or more of the insulin-like peptides (ilps) as they are the only endogenous *Drosophila* ligands known to activate the InR. Which ilp could be responsible for the observed InR-dependent glial immune responses seen in my work? As is illustrated in the schematic drawing shown in Figure 1.6, the *Drosophila* ilps are differentially expressed in different cell populations throughout the fly's body [170-172,197-199]. Exactly which ilp (or ilps) are responsible for InR-mediated glial immune activation probably depends on what the source of the ilp or ilps is in the first place. For example, if the injury activated ilp is coming from other CNS parenchymal tissue, it seems more likely to be ilp4 or ilp7, whose

expression have both been demonstrated in the CNS [170,197]. If the ilp is derived from the circulating hemolymph, it seems more likely to be ilp2, ilp3, ilp5, or ilp6.

Recently, single ilp deletion fly lines were generated by Linda Partridge's lab [199] and have been kindly shared with the scientific community. Our lab tested these lines for defects in Draper upregulation at sites of injury in the *Drosophila* olfactory axotomy assay and, interestingly, found preliminary evidence that either *ilp4* or *ilp7* deletion led to a decrease in the Draper injury response (data not shown). These data are very preliminary, but, if substantiated, would indicate that ilp4 and/or ilp7 might be acting as local injury cues in the context of this axotomy event, since the known expression pattern of these two ilps appear to be CNS specific. Unfortunately, the expression and functions of ilps can be quite interdependent, as some ilps have been demonstrated to compensate for and/or regulate the expression of other ilps when they are genetically deleted [199]. Because of this, caution is advised when making broad interpretations of ilp knockout data.

Potential sources of the ilp(s) responsible for glial immune InR activation

The three most likely sources of the ilp or ilps that activate the InR in glia responding to acute axotomy are: 1) the injured axons themselves, 2) other glia in close proximity to the injured axons such as astrocytes and/or other

ensheathing glia, or 3) ilps crossing the blood brain barrier from the circulating hemolymph.

There is the least amount of biological precedent for the first possibility, that of ilps acting directly as an injury cue emanating from degenerating axons. Nevertheless, expression of a well-characterized transgenic neuropeptide marker [241] in *Drosophila* OR85e ORNs reveals that these neurons do traffic neuropeptide laden dense core vesicles (DCVs) down the length of their axons projecting into the antennal lobes (data not shown). Whether or not these DCVs contain ilps is not yet clear, but severed axons undergoing Wallerian degeneration do exhibit a significant rise in intra-axonal calcium ion concentrations [242,243], and dense core vesicle release requires only a relatively low intracellular calcium ion concentration (compared synaptic vesicle release at least) [244]. This combination could provide a possible mechanism by which DCV exocytosis could take place on degenerating axons, thereby releasing the neuropeptide cargo of the in-transit and terminal DCVs into the immediate extracellular milieu. While this type of release is currently unproven, it would represent an exciting and novel delivery mechanism for an injury cue activating innate immune responding glia. RNAi lines for each of the individual ilps are now available, and I plan test the idea of an ilp-mediated degenerating neuron-to-glia communication event by knocking down ilp expression in ORNs and assessing the glial injury response. Presumably, if a given ilp is acting as a direct injury cue emanating from severed ORN axons, then effective knockdown

of that ilp in those ORNs would abrogate the signaling event and lead to defective clearance.

Another potential source of ilps responsible for glial InR activation during the injury response could be other nearby glia that sense the injury directly and release ilps to activate/recruit responding phagocytes. As discussed in Chapter 1, there is already evidence that ischemic insult in the mouse brain can lead to upregulation of IGF-1 in microglia that then serves to increase the proliferation of other surrounding microglia and invading macrophages in an apparent autocrine/paracrine signaling event [154]. Additionally, both IGF-1 and IGF-2 mRNA and protein levels have been shown to increase in rat microglial cell cultures activated into an amoeboid state by application of lipopolysaccharide [245]. The purpose of this IGF-1 and IGF-2 increase in activated microglia has not been examined, but it is possible that these IGF ligands are acting back on other microglia to stimulate innate immune responses in an autocrine/paracrine fashion as stated above. This scenario is at least consistent with the idea that in the *Drosophila* olfactory axotomy assay, local glia could be releasing ilps into their immediate extracellular surroundings to stimulate the activity and possible recruitment of nearby phagocytic glia.

The third potential source of ilps activating phagocytic glia during the clearance response to axotomy is the circulating hemolymph. In flies, like in mammals, most of the insulin-like peptides produced are released into the circulatory system [199]. This large circulating pool of ilps would certainly make for a convenient source of ligands for activation of InR signaling on responding

glia. Interestingly, intraperitoneal injection of lipopolysaccharide in mice increases permeability of the blood-brain barrier to insulin by around 3.5 fold. This effect is independent of any physical BBB disruption, indicating that there is actually increased active transport of insulin across the BBB after a peripheral innate immune challenge [246]. While it is unclear if a purely CNS localized insult can also increase BBB permeability to insulin, these findings in the mouse do at least indicate that insulin (and perhaps IGF) transport into the CNS is actually responsive to innate immune insults. This opens up the possibility that in my fly model, acute CNS insults might be able to increase the permeability of the fly BBB to circulating ilps, thereby allowing a larger influx of ilps into the CNS to bolster innate immune function in glia responding to CNS damage.

A working model of insulin-like signaling in the glial injury response

My current model of insulin-like signaling in glial clearance can be found in Figure 4.1. Acute activation of the glial insulin-like receptor is induced by one or more insulin-like peptide ligands originating from either injured CNS tissue, other glia in close proximity to the injury, circulating hemolymph, or some combination of these sources. The activated InR then promotes the upregulation of phagocytic factors such as Draper at the transcriptional and/or translational level, most likely through the activation of the PI3K/Akt signaling pathway. This signaling event may be mediated through the InR substrate proteins Chico, Lnk, and Dock, or might be mediated in part by the *Drosophila* InR's "Chico-like"

Figure 4.1

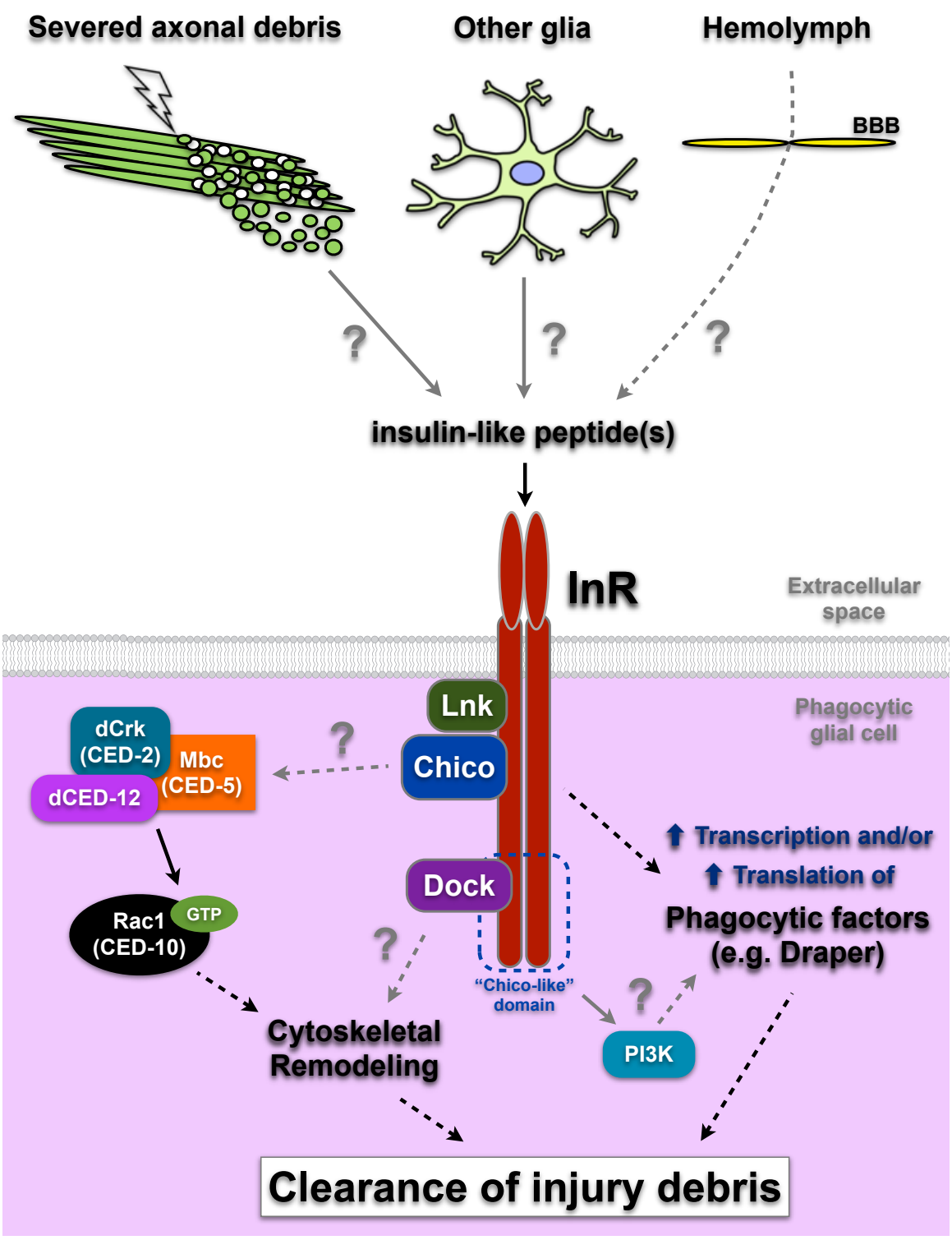
Severed axonal debris
Other glia
Hemolymph
BBB
?
?
?
insulin-like peptide(s)
InR
Extracellular space
Phagocytic glial cell
Lnk
Chico
dCrk (CED-2)
Mbc (CED-5)
dCED-12
?
Rac1 (CED-10)
GTP
Dock
"Chico-like" domain
Transcription and/or
Translation of
Phagocytic factors (e.g. Draper)
?
?
Cytoskeletal Remodeling
PI3K
Clearance of injury debris

Figure 4.1. Model of insulin-like signaling in the glial clearance response in *Drosophila*. After acute axotomy, one or more insulin-like peptides (ilps) activate the insulin-like receptor (InR) on responding glia. The exact source and identity of the activating ilp(s) is unknown. Three potential sources for these ilp(s) are: 1) the injured axons themselves, 2) other glial cells around the sites of injury, and 3) the hemolymph (after crossing the blood-brain barrier). Once activated, the InR promotes glial clearance of cellular debris through its substrates Chico, Lnk, and Dock. Chico plays a role in later stages of the glial clearance response once glial activation and recruitment have taken place, possibly acting upstream of the dCrk/Mbc/dCed-12 complex with activate the Rho family GTPase Rac1. Involvement of Chico in the early phases of the clearance response have not been ruled out as a "Chico-like" domain exists on the InR that can partially activate PI3K signaling even in the absence of Chico and might mask bona fide roles of Chico in the clearance response. Dock has a previously known InR-mediated role in cytoskeletal dynamics and might influence cytoskeletal changes occurring during the glial injury response. Lnk is known to augment Chico's localization to the InR after receptor activation and might act in a similar way during the glial clearance response. The InR signaling positively regulates Draper expression after injury (and possibly other phagocytic components) through as of yet unknown mechanisms. Abbreviation: BBB = blood-brain barrier.

C-terminal extension, which can activate PI3K directly. Activated Chico, Lnk, and Dock may also regulate some of the cytoskeletal remodeling events necessary for an effective glial clearance response. This influence over cytoskeletal dynamics may be accomplished in part through the direct regulation by Chico of the guanine nucleotide exchange factor dCrk/Mbc/dCed-12, which activates the Rho family GTPase Rac1. In this way, InR signaling exerts control over multiple aspects of the glial clearance response, helping to orchestrate the various cellular signaling events necessary to accomplish efficient clearance of cellular debris.

Many question still remain. Which ilp or ilps activate the InR in glia after injury and what is their source? What are the signaling events mediating the

InR's regulation of Draper? Does ILS regulate other phagocytic components aside from Draper? Does the insulin-like signaling pathway mediate cytoskeletal, hypertrophic, or metabolic aspects of the glial clearance response to CNS injury? Does ILS contribute to both early glial activation and recruitment to injury sites as well as the later stages of the injury response that mediate phagocytosis, and if so how? Hopefully some of the future experiments outlined in this chapter will shed light on these mysteries.

While my work presented here firmly places insulin-like signaling as a critical component in the glial clearance response, this may just be the tip of the iceberg for this pathway's involvement innate glial immunity. Indeed, one would be hard-pressed to find a signaling pathway with more potential to regulate the myriad cellular events required to achieve an effective glial response to CNS damage. Insulin-like signaling is perfectly situated as a master regulator of this complex cellular response, and future work will serve to uncover the true extent of this pathway's influence over innate glial immune biology.

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Appendix

Before joining Mary Logan's lab and undertaking the work presented in the dissertation above, I was a member of Doris Kretzschmar's lab. In the Kretzschmar lab I studied the basic biology of the amyloid precursor protein (APP) in the CNS using *Drosophila melanogaster* as a model system for my investigations. The *Drosophila* APP protein is called "APP-like" (APPL), and is a neuron specific transmembrane protein sharing significant sequence similarity with human APP. I was specifically interested in uncovering basic biological roles of APPL as there has been frustratingly little progress in understanding the normal roles of this protein in the brain.

My work in the lab contributed to two separate publications in which I share middle authorship. The first paper [247] established a new paradigm of drug testing for Alzheimer's disease (AD) whereby candidate drugs are first screened for neuroprotective efficacy in a neuroblastoma cell culture line, before moving on to testing in invertebrate models of AD, before finally moving into mouse models of AD. This system greatly streamlines the process of testing new drugs for the treatment of AD by first vetting drug candidates in less costly (both in time and money) models of AD before moving onto mammalian models and ultimately human trials. As a proof of principle, we identified the anti-hypertensive calcium channel blocker, isradipine, as promising new drug candidate for the treatment of AD.

The second paper to which I contributed [248] identified a novel neuroprotective role for the secreted α-cleaved ectodomain of APPL in variety of etiologically

different neurodegenerative models. This broad neuroprotection endowed by the secreted α-cleaved ectodomain of APPL indicated that this protein fragment has a very general neuroprotective effect in the CNS. This finding stands in stark contrast to the common conception of APP being a deleterious protein in the brain and begs for a more nuanced view of APP— a protein that has the capacity to be both harmful and beneficial, depending largely on how it is processed. This work opens the door to understanding APP function in a fresh light and potentially harnessing APP's latent neuroprotective capacity to ameliorate some of its deleterious effects uncovered in AD.