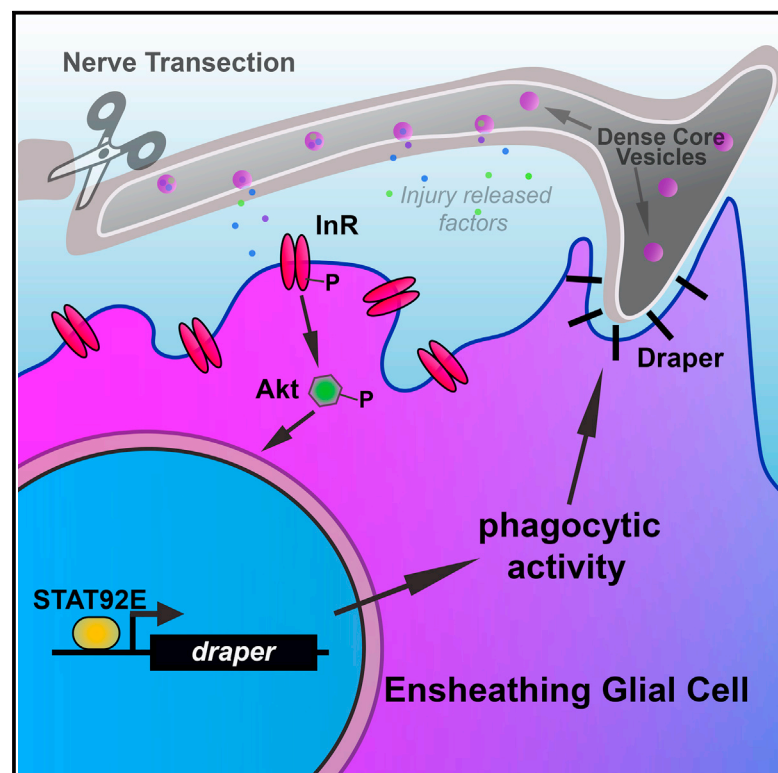


Cell Reports

Insulin-like Signaling Promotes Glial Phagocytic Clearance of Degenerating Axons through Regulation of Draper

Graphical Abstract



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In Brief

Musashe et al. find that the insulin-like signaling (ILS) pathway is stimulated in local glia after nerve axotomy thus upregulating expression of the engulfment receptor gene *draper* in a STAT92E-dependent manner. Activation of the ILS/Draper pathway is required for proper glial clearance of degenerating axonal debris.

Highlights

- InR and Akt are activated in ensheathing glia after axotomy
- InR/Akt signaling is required for upregulation *draper*
- InR/Akt induction of *draper* is required for glial clearance of degenerating axons



Insulin-like Signaling Promotes Glial Phagocytic Clearance of Degenerating Axons through Regulation of Draper

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SUMMARY

Neuronal injury triggers robust responses from glial cells, including altered gene expression and enhanced phagocytic activity to ensure prompt removal of damaged neurons. The molecular underpinnings of glial responses to trauma remain unclear. Here, we find that the evolutionarily conserved insulin-like signaling (ILS) pathway promotes glial phagocytic clearance of degenerating axons in adult *Drosophila*. We find that the insulin-like receptor (InR) and downstream effector Akt1 are acutely activated in local ensheathing glia after axotomy and are required for proper clearance of axonal debris. InR/Akt1 activity, it is also essential for injury-induced activation of STAT92E and its transcriptional target *draper*, which encodes a conserved receptor essential for glial engulfment of degenerating axons. Increasing Draper levels in adult glia partially rescues delayed clearance of severed axons in glial InR-inhibited flies. We propose that ILS functions as a key post-injury communication relay to activate glial responses, including phagocytic activity.

INTRODUCTION

Glial cells are highly sensitive to changes in neuronal health and respond swiftly to diverse forms of neural trauma. Acute injury triggers robust responses in glia, including altered gene expression, glial infiltration of damaged areas, and glial clearance of degenerating neurons through phagocytic engulfment (Logan and Freeman, 2007; MacDonald et al., 2006; Napoli and Neumann, 2009). Many of these reactive glial responses are neuroprotective. Impaired glial clearance of damaged axons and myelin can inhibit axonal regeneration after neural injury and provoke inflammation (Akiyama et al., 2000; Bamberger and Landreth, 2002; Glezer et al., 2007). Finally, there is growing evidence that defects in glial immunity are coupled to the onset and progression of numerous neuro-

degenerative diseases (Giunti et al., 2014; Hamby and Sofroniew, 2010; Scuderi et al., 2013). As a result, there is intense interest in elucidating the molecular and cellular features of glial immunity that govern how glia sense and respond to neuronal stress and damage, including the extrinsic injury cues that rapidly activate robust intrinsic immune response programs in glia.

Drosophila is a powerful model for investigating fundamental aspects of neurodegeneration and glial responses to neural damage (Ayaz et al., 2008; Cantera and Barrio, 2015; Etcheagaray et al., 2016; Fang and Bonini, 2012; Freeman et al., 2003; Kato et al., 2011; Lee and Sun, 2015; Logan and Freeman, 2007; Liu et al., 2015; Mishra et al., 2013; Rooney and Freeman, 2014; Ugur et al., 2016). Axotomy in adult flies elicits glial reactions that share many cellular hallmarks with mammalian glia responding to neurodegeneration, including altered morphology and increased phagocytic function (Kurant 2011; Logan and Freeman, 2007). Notably, conserved molecules essential for glial engulfment activity are conserved, including the Draper/MEGF10 receptor, which signals through a tyrosine-based activation motif (ITAM)/Src tyrosine kinase signaling cascade to regulate phagocytic removal of degenerating axons and apoptotic neurons (Chung et al., 2013; Logan et al., 2012; MacDonald et al., 2006; Scheib et al., 2012; Wu et al., 2009). In *Drosophila*, olfactory nerve axotomy triggers upregulation of Draper in local ensheathing glial cells, which is essential for efficient glial clearance of axonal debris (Doherty et al., 2014; Logan et al., 2012; MacDonald et al., 2006), but little is known about the upstream signals that trigger upregulation of Draper or other innate immune genes in responding glia.

The insulin-like signaling (ILS) pathway is a critical regulator of energy homeostasis, cellular growth, and cell survival during embryonic and postnatal development (Barbieri et al., 2003; Broughton and Partridge, 2009; Puig and Mattila, 2011). This highly conserved pathway is initiated by activation of the insulin receptor (IR) or insulin-like growth factor (IGF) receptors in vertebrates and the homologous insulin-like receptor (InR) in *Drosophila* to trigger downstream signaling cascades that modulate a range of molecular and cellular events, including transcription, translation, and autophagy. ILS is

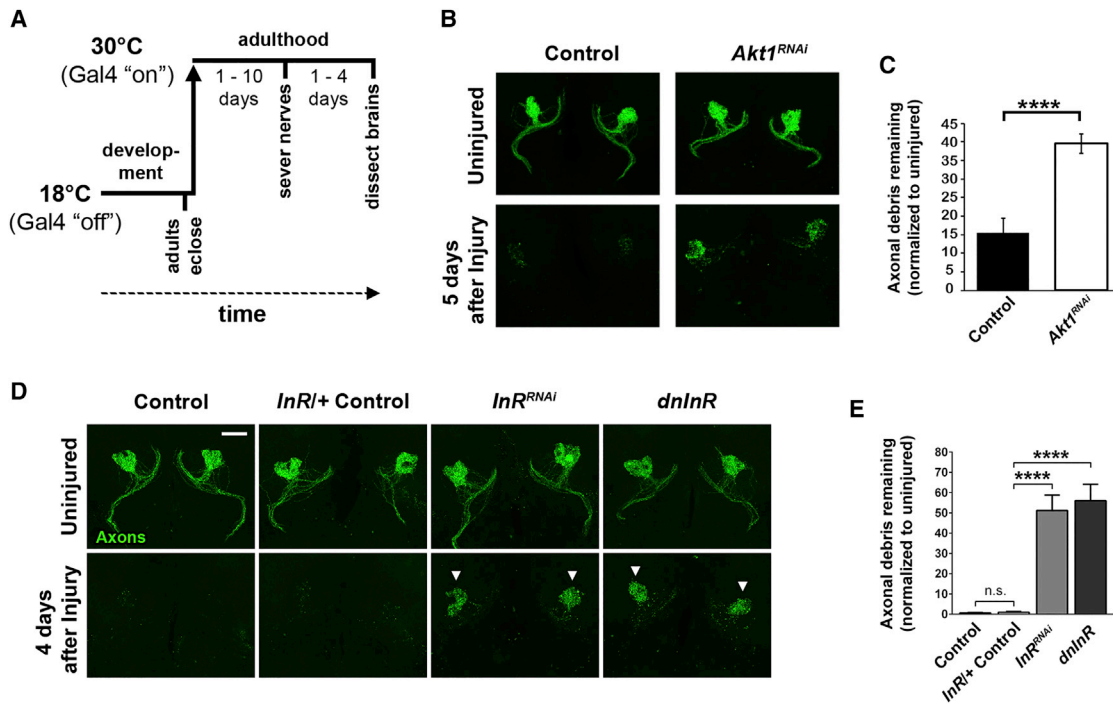


Figure 1. InR/Akt1 Is Required in Adult Glia for Proper Glial Clearance of Degenerating Axons

(A) Schematic of experimental method to temporally control UAS-driven gene expression.

(B) Maximum intensity projections of the antennal lobe region show axons of OR85e ORNs expressing membrane-tethered GFP (green).

(C) Quantification of GFP⁺ axonal material shown in (B). N ≥ 18 for each genotype and condition.

(D) Confocal projections of OR85e axons in control, *InR^{RNAi}* or dominant-negative InR (*dnInR*)-expressing flies. White arrowheads point to persistent axonal debris post-injury.

(E) Quantification of GFP shown in (B). N ≥ 22 for each genotype and condition. ****p < 0.0001. Pooled data plotted as mean ± SEM. All image scale bars, 20 μm. Genotypes: control (*OR85e-mCD8::GFP, tub-Gal80^{TS/+}; repo-Gal4/+*), *dnInR* (*OR85e-mCD8::GFP, tub-Gal80^{TS/+}; repo-Gal4, InR^{ex15}/UAS-Dominant-Negative InR*), *InR^{RNAi}* (*OR85e-mCD8::GFP, tub-Gal80^{TS/+}; repo-Gal4, InR^{ex15}/UAS-InR^{RNAi}*), *Akt^{RNAi}* (*OR85e-mCD8::GFP, tub-Gal80^{TS/+}; repo-Gal4, InR^{ex15}/UAS-Akt^{RNAi}*). See also Figure S1.

essential for development, but there is growing evidence that ILS also plays important roles in the mature brain. Boosting serum IGF-1 levels protects against various insults, including kainic acid injection (Miliadous et al., 2010) and hypoxic-ischemic injury (Guan et al., 1993). Conversely, pharmacological inhibition of IGF-1 results in greater neuronal death after trauma (Carro et al., 2003; Guan et al., 2003), as well as faster cognitive decline in neurodegenerative disease models (Carro et al., 2006; Torres-Aleman, 2007). ILS may support functional integrity and survival of neurons in diverse contexts, but the mechanisms of ILS-mediated neuroprotection are not well understood.

Here, we show that activity of the insulin-like receptor (InR) and the downstream effector Akt1 are upregulated in ensheathing glia following olfactory nerve axotomy in *Drosophila* and are required for proper glial clearance of degenerating axonal debris. Inhibition of glial InR inhibits STAT92E-dependent transcriptional upregulation of *draper* after axon injury and also prevents recruitment of the Draper receptor to degenerating axons. We also show that dense core vesicle release from severed axons may be required for proper InR activation in local ensheathing glia. Our results implicate ILS as key signaling relay that promotes

glial engulfment activity in response to axotomy through activation of STAT92E/Draper.

RESULTS

Glial InR Signaling Is Required for Glial Phagocytic Clearance of Severed Axons in the Adult *Drosophila* Brain

In an ongoing in vivo screen to identify genes required for glial engulfment of degenerating axons (Table S1), we found that RNAi against the serine/threonine kinase Akt1 (*UAS-Akt1^{RNAi}*) in adult glia inhibited glial clearance of severed olfactory receptor neuron axons. We used flies that expressed membrane-tethered GFP in a subset of maxillary palp olfactory receptor neurons (ORNs) (*OR85e-mCD8::GFP*), as well as the pan-glial driver *repo-Gal4*. We also temporally restricted activity of Gal4 to adult post-mitotic glia by expressing Gal80^{TS} under the control of tubulin (*tubulin-Gal80^{TS}*) (McGuire et al., 2003) so as not to perturb glial development (Figure 1A). Maxillary nerve axotomy triggers Wallerian degeneration of maxillary nerve ORN axons. Local ensheathing glia clear degenerating OR85e maxillary axons within ~48 hr (MacDonald et al., 2006). Expression of *Akt1^{RNAi}*

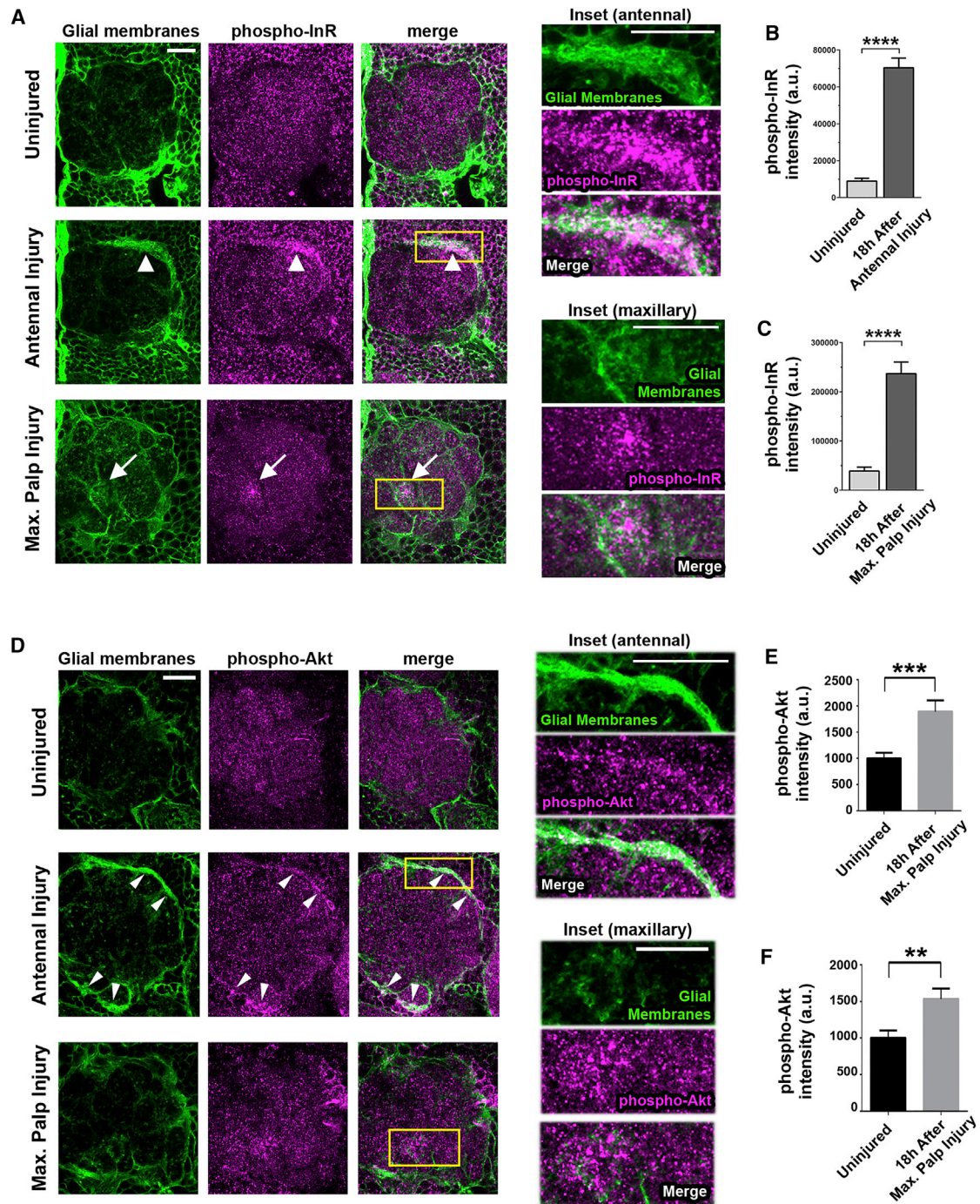


Figure 2. InR and Akt1 Are Acutely Activated in Local Glia Responding to Axon Injury

(A) Single confocal slice images of the antennal lobe region in uninjured, antennal nerve axotomized, and maxillary nerve axotomized animals. Phospho-InR signal (magenta) overlaps with GFP-labeled glial membranes (green) expanding after antennal nerve axotomy (white arrowheads) or accumulating on severed maxillary glomeruli post-maxillary nerve axotomy (white arrowheads). Insets: magnified view of outlined area (yellow rectangle).

(B) Phospho-InR signal in the dorsal half of antennal lobes was quantified after computationally segmenting to GFP (antennal injury in A). $N \geq 20$ for each condition.

(C) Quantification of phospho-InR signal in OR85e-innervated glomeruli in (A) (maxillary injury). $N \geq 16$ for each condition.

(D) Single confocal slice images of antennal lobes. Phospho-Akt (magenta) intensity is increased in ensheathing glia (green) responding to antennal (white arrowheads, middle panels) or maxillary nerve injury (lower panels). Insets: magnification of yellow boxed areas.

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in adult glia significantly inhibited glial engulfment of OR85e axonal debris (Figures 1B and 1C). We screened candidate RNAi lines against receptors known to signal via Akt-dependent cascades and discovered that knockdown of the insulin-like receptor (InR) with *UAS-InR^{RNAi}* phenocopied the *Akt1^{RNAi}* clearance phenotype. Significantly more GFP⁺ axonal debris was present in glial *InR^{RNAi}*-expressing brains 4 days after axotomy (white arrowheads in Figures 1D and 1E). To complement our RNAi analysis, we independently inhibited InR activity by expressing a dominant-negative version (*UAS-dnInR*) (Demontis and Perrimon, 2009) and similarly observed impaired glial clearance of GFP⁺ debris post-injury (Figures 1D and 1E). These manipulations were performed in a *InR* heterozygous mutant background (*InR^{ext15}*) (Song et al., 2003), because it is well established that numerous compensatory mechanisms exist to strengthen ILS activity when inhibited (Ferguson et al., 2012; Kannan and Fridell, 2013; Marr et al., 2007). Efficacy of Gal80^{TS}/Gal4 temporal regulation was confirmed by testing each genotype maintained at 18°C (Figures S1A–S1D). Importantly, we also confirmed that delayed glial clearance of OR85e axonal debris was rescued in glial *Akt1^{RNAi}* and glial *InR^{RNAi}* animals with expression of *UAS-Akt1* (Wang et al., 2011) or *UAS-InR* (Martín-Peña et al., 2006) (Figures S1E–S1H). Together, these results reveal that InR/Akt1 activity is required in adult glia for proper phagocytic clearance of degenerating axons after injury.

InR and Akt1 Activity Is Increased in Local Glia after ORN Axotomy

Basal changes in ILS can alter the metabolic state of cells but activation of ILS cascades also serve as local signaling relays to intrinsically alter events such as transcription, translation and/or cell motility (Fernandez and Torres-Alemán, 2012; Gu et al., 2014; Hua et al., 2009; Luckhart and Riehle, 2007). To determine if InR activity is altered in local glia following olfactory nerve axotomy, we took advantage of a well-characterized phospho-specific InR antibody that recognizes the activated form of *Drosophila* InR (Root et al., 2008). We used flies that transgenically expressed membrane-tethered GFP in glia, performed bilateral antennal nerve axotomy and then stained brains with anti-phospho-InR and anti-GFP at various time points after axotomy. Within 24 hr, glial cells surrounding the antennal lobes expand their membranes as they invade the antennal neuropil (MacDonald et al., 2006) (Figure 2A). We also observed a striking increase in phospho-InR signal on these glial membranes (Figures 2A and 2B). Maxillary nerve axotomy severs a smaller cohort (~20%) of ORNs that project into the antennal lobes, but this still elicited a significant increase in phospho-InR signal at sites where glial membranes were accumulating on degenerating maxillary axons (Figures 2A and 2C). Similarly, we detected a significant increase in phosphorylated Akt1 (phospho-Akt1) levels in ensheathing glia responding to antennal or maxillary nerve axotomy (Figures 2D–2F). Our findings sug-

gest that InR/Akt1 activity increases in activated antennal lobe glia within 1 day after axon injury, supporting the notion that the ILS pathway functions as a local injury communication cascade required for proper innate glial immune responses to axon degeneration.

InR Signaling Is Required for Axotomy-Induced Upregulation and Recruitment of Glial Draper to Injury Sites

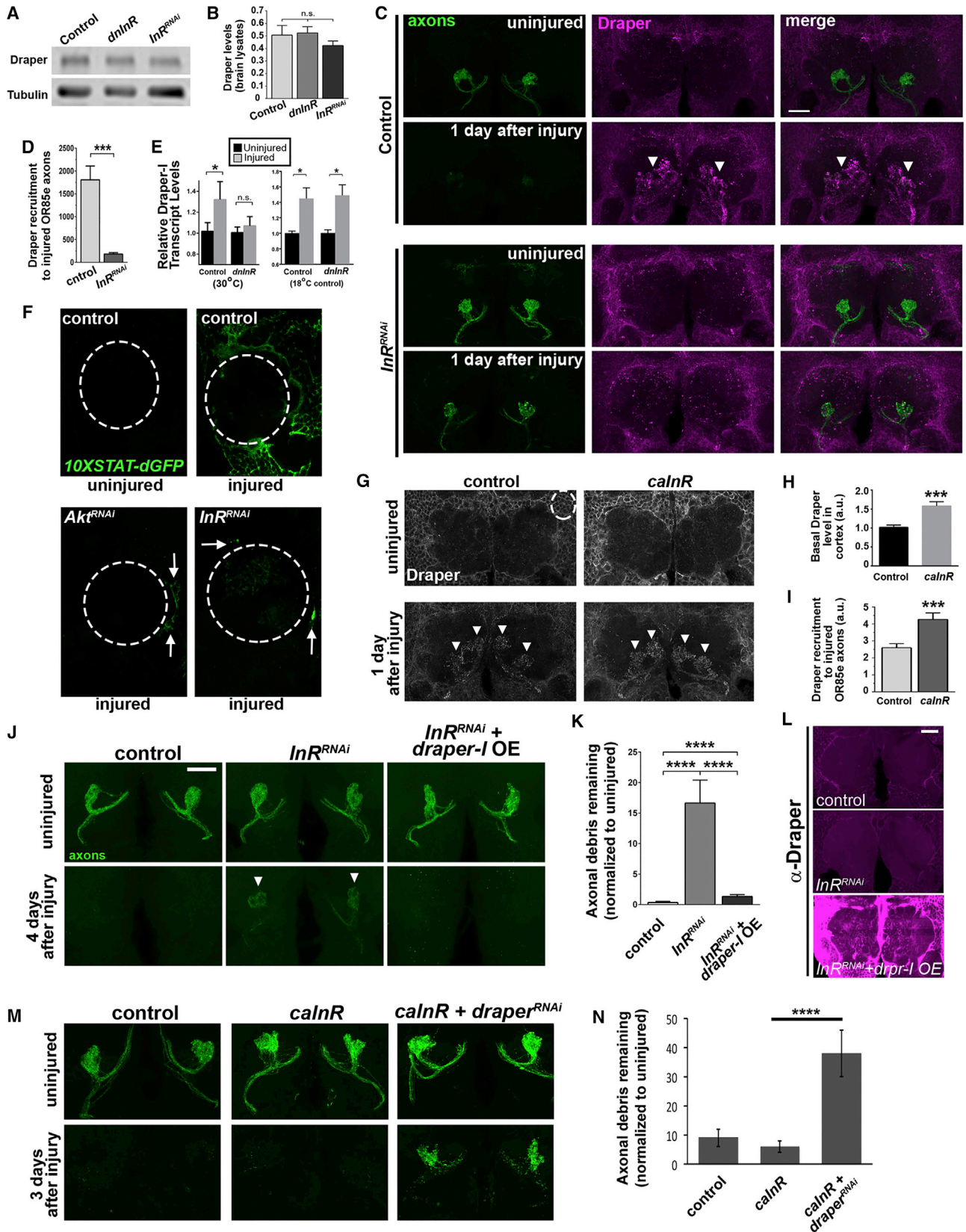
The Draper receptor is essential for glial clearance of severed ORN axons (Logan et al., 2012; MacDonald et al., 2006). *draper* is transcriptionally upregulated in local glia within hours after injury, and ~24 hr later, robust accumulation of Draper protein is visible on olfactory glomeruli that contain degenerating axons (Doherty et al., 2014; Logan et al., 2012; MacDonald et al., 2006). We wondered if InR signaling might regulate phagocytic activity of glia by influencing Draper levels. First, we compared basal levels of Draper in central brain lysates of control flies compared to *dnInR* or *InR^{RNAi}*-expressing flies and found no difference in basal Draper levels (Figures 3A and 3B). Instead, we found that Draper accumulation on degenerating OR85e axons was dramatically reduced 1 day after maxillary nerve injury when adult glia were depleted of InR (arrowheads in Figures 3C and 3D). This phenotype did not result from glial cell death as we detected no change in the number of Repo⁺ nuclei in the central brain region following 10 days of *InR^{RNAi}* expression (Figures S3A and S3B), and gross glial morphology appeared normal when InR activity was inhibited in adult glia for 10 days (Figure S3C). Finally, to confirm specificity of our RNAi constructs, we confirmed that reduced Draper recruitment was reversed in glial *InR^{RNAi}* and *Akt1^{RNAi}* following expression of *UAS-InR* or *UAS-Akt1*, respectively (Figures S4B–S4E).

Next, we performed qPCR for *draper-I* transcript on dissected central brains from uninjured flies and 3 hr post-axotomy. There are three predicted splice variants of *draper*, Draper-I is the activating form essential for glial phagocytic activity in adults (Logan et al., 2012). As previously reported (Logan et al., 2012), *draper-I* was significantly increased after antennal nerve axotomy in control animals; however, *draper-I* was not upregulated in *dnInR*-expressing flies (Figure 3E). Surprisingly, basal levels of *draper-I* transcript were not altered by inhibition of glial InR (2^{-ΔΔCt}) values in uninjured control and uninjured *dnInR* were 0.01908 ± 0.00155 and 0.01614 ± 0.000859, respectively; p = 0.22). The Draper promoter contains several binding sites for the STAT92E transcription factor, which are required for injury-induced upregulation of *draper* (Doherty et al., 2014). Activation of a *10XSTAT-dGFP* in vivo reporter, which contains ten tandem STAT92E binding sites driving expression of destabilized GFP (dGFP), mirrors Draper upregulation in local ensheathing glia following olfactory nerve axotomy (Doherty et al., 2014). Consistent with our finding that ILS is required to upregulate *draper* in glia after axon injury, we found that activation of the *10XSTAT-dGFP* reporter was largely inhibited 24 hr post-axotomy in adult

(E) Quantification of phospho-Akt signal in dorsal antennal lobes after computationally segmenting to GFP. N ≥ 18 for each condition.

(F) Quantification of phospho-Akt signal in OR85e-innervated glomeruli in (D) (bottom panels). N ≥ 20 for each condition. **p < 0.01, ***p < 0.001, ****p < 0.0001. Pooled data plotted as mean ± SEM. All image scale bars, 20 μm. Genotypes: *UAS-mCD8::GFP*, *repo-Gal4/+*.

See also Figure S2.



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glia depleted of InR or Akt1 (Figure 3F). Together, these results suggest that basal InR signaling does not appreciably influence glial Draper levels at the transcriptional or protein level but instead is essential for activation of a STAT92E-dependent transcriptional program to upregulate the phagocytic receptor gene *draper* at times when glial phagocytic loads are high.

Activation of Glial InR Is Sufficient to Raise Draper Levels

To determine if activation of the ILS could alter Draper expression levels in adult glia, we used *tubulin-Gal80^{ts}* combined with the *repo-Gal4* driver to express a constitutively active form of the InR (*UAS-InR.del*, referred to below as *calnR*) (Werz et al., 2009) specifically in adult glia for 24 hr prior to and after maxillary nerve axotomy (by shifting flies to 30°C). We found that, even in the absence of injury, expression of *calnR* resulted in significantly higher levels of basal Draper in the central brain region ($p < 0.001$) (Figures 3G and 3H) and significantly higher levels of Draper on maxillary palp glomeruli that contained degenerating axons 1 day after axotomy ($p < 0.001$) (Figures 3G and 3I), further supporting InR signaling as a positive regulator of Draper in adult glial cells.

Forced Expression of *draper* Reverses Glial Clearance Defects in InR Knockdown Animals

Upregulation of innate immunity factors, including engulfment receptors, occurs in many activated phagocytes; this provides a positive feedback loop that ensures an adequate number of receptors are available to recognize engulfment targets and efficiently shuttle them through phagolysosomal destruction pathways (Kingsolver et al., 2013; A-Gonzalez and Hidalgo, 2014; Doherty et al., 2014). Because our findings indicated that InR is required for glial cells to upregulate Draper as phagocytic demands increase in response to local axon degeneration, we

wondered if boosting glial Draper levels might reverse axon clearance defects in InR/Akt1-inhibited flies. We expressed *UAS-InR^{RNAi}* and *UAS-draper-1* in adult glia under the control of *repo-Gal4* and *tubulin-Gal80^{ts}* in flies that also carried *OR85e-mCD8::GFP*. Notably, overexpression of Draper-1 in a glial *InR^{RNAi}* background partially restored clearance of degenerating axons 4 days post-axotomy ($p < 0.0001$) (Figures 3J–3L). Conversely, we also used a well-characterized RNAi transgenic line to knock-down Draper (*UAS-Draper^{RNAi}*) in glial cells (MacDonald et al., 2006; Doherty et al., 2009; Logan et al., 2012) while also overexpressing *calnR* and then assessed clearance of severed OR85e axons. Notably, we found that inhibiting Draper expression was sufficient to block clearance of axonal debris, despite activating glial ILS ($p < 0.0001$) (Figures 3M and 3N). Taken together, our data suggest that glial InR/Akt1 activity promotes proper phagocytic clearance of damaged axons through STAT92E-dependent transcription of the engulfment receptor Draper.

ILS Is Required in Adult Ensheathing Glia and Not Astrocytes for Proper Clearance of ORN Axonal Debris

In the adult CNS, neuropil regions, including the antennal lobes, contain two major glial subtypes: ensheathing glia, which envelop projections, and astrocytes (Awasaki et al., 2008; Doherty et al., 2009; Edwards and Meinertzhagen, 2010; Omoto et al., 2015). Draper is expressed in ensheathing glia, as well as the cell body glia that surround neuronal cell bodies in the cortex of the brain, but is not detectable in adult astrocytes (Doherty et al., 2009). To determine if ILS activity is increased in ensheathing glia and/or astrocytes following ORN axotomy, we expressed membrane-tethered GFP (*UAS-mCD8::GFP*) in astrocytes with *alrm-Gal4* (Doherty et al., 2009) or in ensheathing glia with *TIFR-Gal4* (Yao et al., 2007; Ziegenfuss et al., 2012) and immunostained for phospho-InR 18 hr after antennal nerve axotomy. We found that the increased phospho-InR signal largely overlapped

Figure 3. Glial InR Signaling Positively Regulates Draper Expression and Draper Recruitment to Severed Axons

- (A) Western blot of fly brain lysates.
 (B) Quantification of normalized Draper in (A).
 (C) Representative images of adult antennal lobes. Brains stained with Draper (magenta) and GFP to visualize OR85e axons (green). Robust Draper accumulation typically observed post-injury (white arrowheads) is attenuated following glial *InR^{RNAi}* expression.
 (D) Volumetric quantification of Draper fluorescence intensity on injured OR85e axons (arrowheads) in (C).
 (E) Q-PCR analysis of *draper-1* transcript in central brains. $2^{-\Delta\Delta Ct} \pm$ SEM values are plotted. $n = 6$ independent samples of pooled animals for each time point and genotype.
 (F) Images of single antennal lobes (dotted line) show activation of 10XSTAT92E-dGFP (destabilized GFP) reporter 18 hr after antennal nerve axotomy in control animals and glial *Akt^{RNAi}* or *InR^{RNAi}*-expressing flies.
 (G) Antennal lobe region of uninjured brains (single confocal slice) and 1 day post axotomy (maximum intensity projection) immunostained for Draper.
 (H) Volumetric quantification of basal Draper levels in the cortex region of uninjured flies (region of interest [ROI] shown with white dotted circle in G).
 (I) Volumetric quantification of Draper accumulation on injured maxillary palp glomeruli (see arrowheads in G).
 (J) Glial overexpression (OE) of Draper-1 reverses glial clearance defects in InR-depleted animals. Representative Z stack projections of OR85e-innervated glomeruli show GFP⁺ OR85e axons.
 (K) Quantification of GFP in (J).
 (L) Images of each experimental genotype shown in (J) immunostained for Draper.
 (M) OR85e axons (green) before and after axotomy.
 (N) Quantification of GFP⁺ OR85e axonal debris in (M).
 Genotypes: (A–E) control (*OR85e-mCD8::GFP, tub-Gal80^{ts}/+; repo-Gal4/+*), *dnInR* (*OR85e-mCD8::GFP, tub-Gal80^{ts}/+; repo-Gal4, InR^{ex15}/UAS-Dominant-Negative InR*), *InR^{RNAi}* (*OR85e-mCD8::GFP, tub-Gal80^{ts}/+; repo-Gal4, InR^{ex15}/UAS-InR^{RNAi}*); (F) control (*10XSTAT92E-dGFP/+; repo-Gal4/+*), *Akt^{RNAi}* (*10XSTAT92E-dGFP/+; repo-Gal4/UAS-Akt^{RNAi}*), *InR^{RNAi}* (*10XSTAT92E-dGFP/+; repo-Gal4/UAS-InR^{RNAi}*); (G–I) control (*+tub-Gal80^{ts}/+; repo-Gal4*), *calnR* (*UAS-calnR/tub-Gal80^{ts}/+; repo-Gal4*); (J–L) control, *InR^{RNAi}* (*OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-LacZ; repo-Gal4, InR^{ex15}/UAS-InR^{RNAi}*), *InR^{RNAi}* + *draper-1* OE (*OR85e-mCD8::GFP, UAS-Draper-1/tub-Gal80^{ts}; repo-Gal4, InR^{ex15}/UAS-InR^{RNAi}*); (M and N) control (*OR85e-mCD8::GFP/+; repo-Gal4/+*). *calnR* (*OR85e-mCD8::GFP/UAS-LacZ::NLS, UAS-calnR; repo-Gal4/+*). *calnR* + *draperRNAi* (*OR85e-mCD8::GFP/UAS-draperRNAi, UAS-calnR; repo-Gal4/+*). * $p < 0.05$; **** $p < 0.001$. **** $p < 0.0001$. All pooled data are represented as mean \pm SEM. All scale bars, 20 μ m. $N \geq 18$ for each condition. See also Figures S3 and S4.

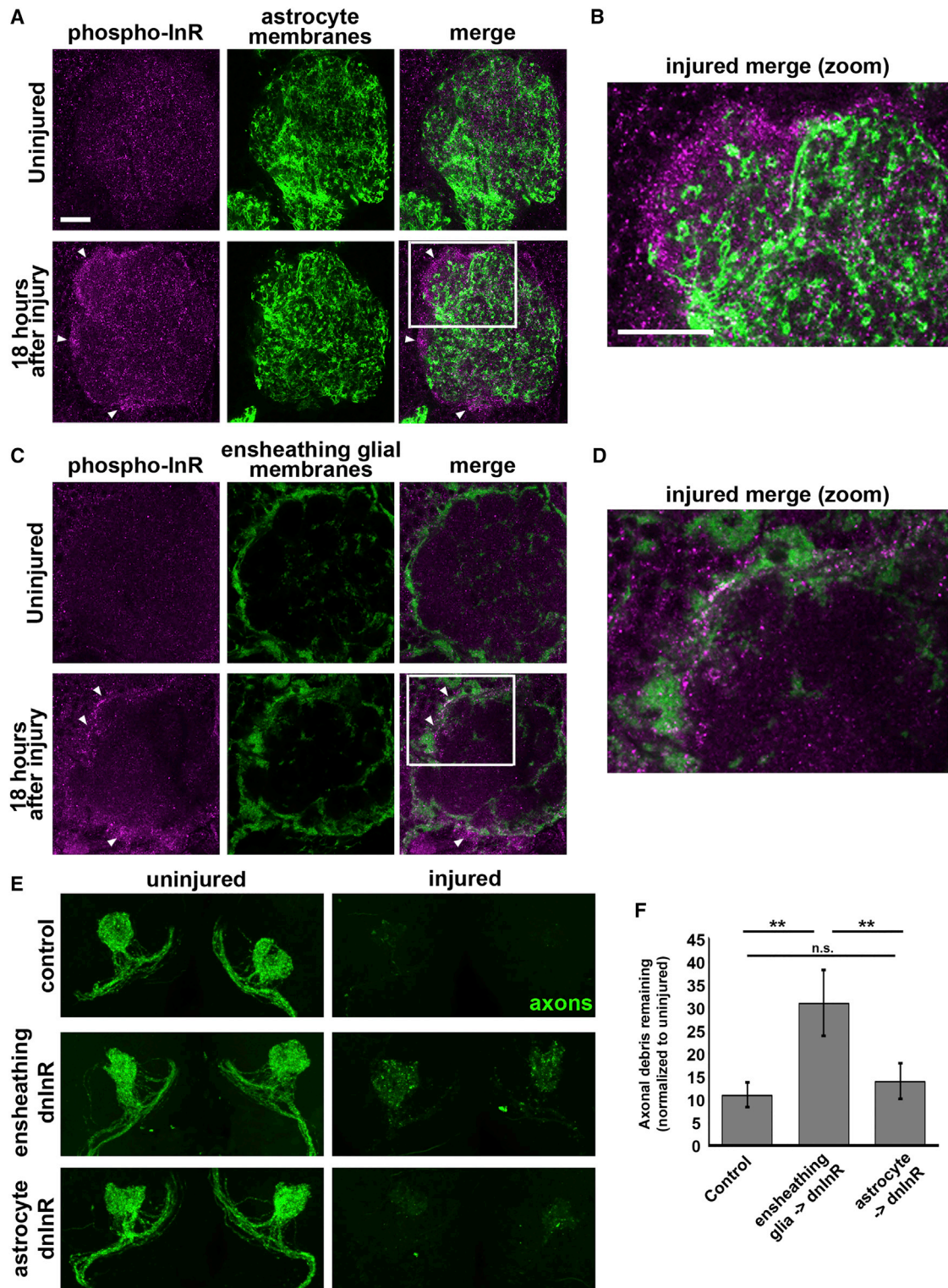


Figure 4. InR Is Required in Ensheathing Glia, Not Astrocytes, for Proper Clearance of Degenerating Axonal Debris

(A) Representative confocal images of single antennal lobes. Brains immunostained with anti-phospho-InR (magenta) reveal little overlap between increased InR activity (white arrowheads) and astrocyte membranes labeled with mCD8::GFP (green). (B) Zoomed image of region outlined in (A) (white box).

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with ensheathing glial membranes and not astrocytic membranes (Figures 4A and 4B, white arrowheads). Next, we expressed *dnInR* in each subtype and assessed clearance of GFP-labeled OR85 axons after maxillary nerve axotomy and, importantly, found that significantly more axonal debris persisted following ensheathing glial, but not astrocyte, expression of *dnInR* (Figures 4E and 4F). Together, these results indicate that InR activity is specifically required in ensheathing glia and may be selectively increased in this particular glial subtype after axon injury to drive Draper-dependent engulfment of degenerating axons in the adult brain.

Dense Core Vesicles Are Distributed along Adult Olfactory Receptor Neuron Axons

The *Drosophila* InR can be activated by eight unique insulin-like peptide (ilp) ligands (ilp1–ilp8) (Garelli et al., 2012; Grönke et al., 2010). Several neuropeptides are expressed in adult antennal lobes (Carlsson et al., 2010), and transcripts for *ilp6* and *ilp8* have been detected in adult ORNs (Menuez et al., 2014). Moreover, *ilp4* is detectable by immunostaining in the adult brain, including the antennal lobes (Grönke et al., 2010). We wondered if severed ORN axons might release ilps to activate InR on ensheathing glia. First, we employed a common strategy to visualize neuropeptide distribution in ORN axons before and after axotomy. Neuropeptides, including ilps, are packaged into dense core vesicles (DCVs) for release. Atrial natriuretic factor (ANF)-tagged GFP (ANF::GFP) is loaded into and released from DCVs along with endogenous neuropeptides, and reductions in ANF::GFP-positive vesicles track with high levels of neuropeptide release (Husain and Ewer, 2004; Rao et al., 2001). We used *OR22a-Gal4* to express ANF::GFP in antennal ORNs and imaged uninjured and injury brains. ANF::GFP was highly enriched at synaptic terminals in OR22a glomeruli (Figure 5A, brackets). We also observed discrete GFP⁺ puncta along the length of the axons (arrows). We observed a 65% reduction in ANF::GFP⁺ vesicles along OR22a axonal tracts 30 min after antennal nerve axotomy (Figure 5A), suggesting that DCVs are released from injured axons shortly after injury.

amontillado and *Cadps* Are Required in ORNs For Glial InR Activation and Draper Upregulation after Injury

To identify additional insulin-like peptides (ilps) expressed in adult ORNs, we performed RT-PCR for ilps1–7 on RNA isolated from the third antennal segments of adult *Drosophila*, which houses all of the antennal ORN cell bodies (~1,200). We detected transcripts for all seven ilps, with the exception of *ilp5* (Figure 5B). Next, we knocked down the expression of each ilp ligand in ORNs by expressing dsRNA with the pan-ORN driver

orco-Gal4 (Larsson et al., 2004). Although we observed a trend toward attenuated Draper upregulation after olfactory nerve axotomy in some genotypes, these results were not statistically significant (not shown).

Notably, there are robust compensatory mechanisms in place to boost the activity of ILS in flies when suppressed. One notable strategy is to raise the levels of one or more ilp ligands when the expression of other ilps is genetically inhibited (Broughton et al., 2008; Sousa-Nunes et al., 2011; Wigby et al., 2011). Thus, we took an alternative approach to broadly inhibit ilps/neuropeptides in ORNs. Ilps, like many neuropeptides, are initially formed as inactive precursors, which require proteolytic processing to form biologically active ligands. We tested two different RNAi lines that target unique regions of subtilisin-like proprotein convertases 2 (PC2), also known as *amontillado* in flies, which is involved in ilp proprotein cleavage (Rayburn et al., 2003; Rhea et al., 2010). We expressed each RNAi (BI 28583 or VDRC 110788) under the control of *orco-Gal4* and *tubulin-Gal80^{ts}* to temporally restrict Gal4 activity in adult ORNs. We found that expression of PC2^{RNAi} significantly inhibited phospho-InR increases and Draper upregulation in ensheathing glial regions after antennal nerve axotomy (Figures 5C–5F). Next, we targeted calcium-activated protein for secretion (*Cadps*), which is essential for DCV release (Renden et al., 2001; Wong et al., 2012, 2015). We expressed *Cadps*^{RNAi} (VDRC 110055) in all ORNs with *orco-Gal4* and found that phospho-InR and Draper increases after axon injury were also attenuated (Figures 5C and 5D). Collectively, these results suggest that severed ORNs may be an important source of secreted neuropeptide signals that elicit InR-dependent glial responses to axon degeneration.

DISCUSSION

Here, we show that the insulin-like signaling (ILS) pathway is required for two key events associated with glial responses to acute axon injury in the adult *Drosophila* brain: upregulation of the conserved engulfment receptor Draper and efficient phagocytic engulfment of degenerating axons. Previous studies ranging from invertebrates to humans have linked ILS and innate immunity. In *Drosophila*, the ILS pathway is an important homeostatic regulator of hemocyte responses to injury. Similar to our findings, repression of ILS inhibits responses to epidermal damage, including hemocyte recruitment to injury sites (Karpac et al., 2011). IGF-1 treatment of human phagocytes in vitro also boosts phagocytic activity. For example, in cultured neutrophils, IGF-1 induces upregulation of CD11b, a component of complement receptor 3, which functions as a widespread phagocytic pattern recognition receptor to facilitate recognition and engulfment of

(C) Representative confocal images of single antennal lobes. Brains immunostained with anti-phospho-InR (magenta) reveal overlap between increased InR activity (white arrowheads) and ensheathing glial membranes labeled with mCD8::GFP (green).

(D) Zoomed image of region outlined in (C) (white box).

(E) Maximum intensity projections of OR85e axons.

(F) Quantification of GFP⁺ OR85e axonal debris in (E).

**p < 0.01. N ≥ 16 for each condition. Scale bar, 20 μm. Pooled data plotted as mean ± SEM. Genotypes: (A and B) (*UAS-mCD8::GFP/+; alrm-Gal4/+*); (C and D) (*UAS-mCD8::GFP/+; TIFR-Gal4/+*), *InR*^{RNAi}; (E and F) control (*OR85e-mCD8::GFP; tub-Gal80^{ts}/+; InR^{ex15}/+*), ensheathing *dnInR* (*OR85e-mCD8::GFP; tub-Gal80^{ts}/+; TIFR-Gal4*), *InR*^{ex15}/*UAS-Dominant-Negative InR*), astrocyte *dnInR* (*OR85e-mCD8::GFP; tub-Gal80^{ts}/+; alrm-Gal4*), *InR*^{ex15}/*UAS-Dominant-Negative InR*).

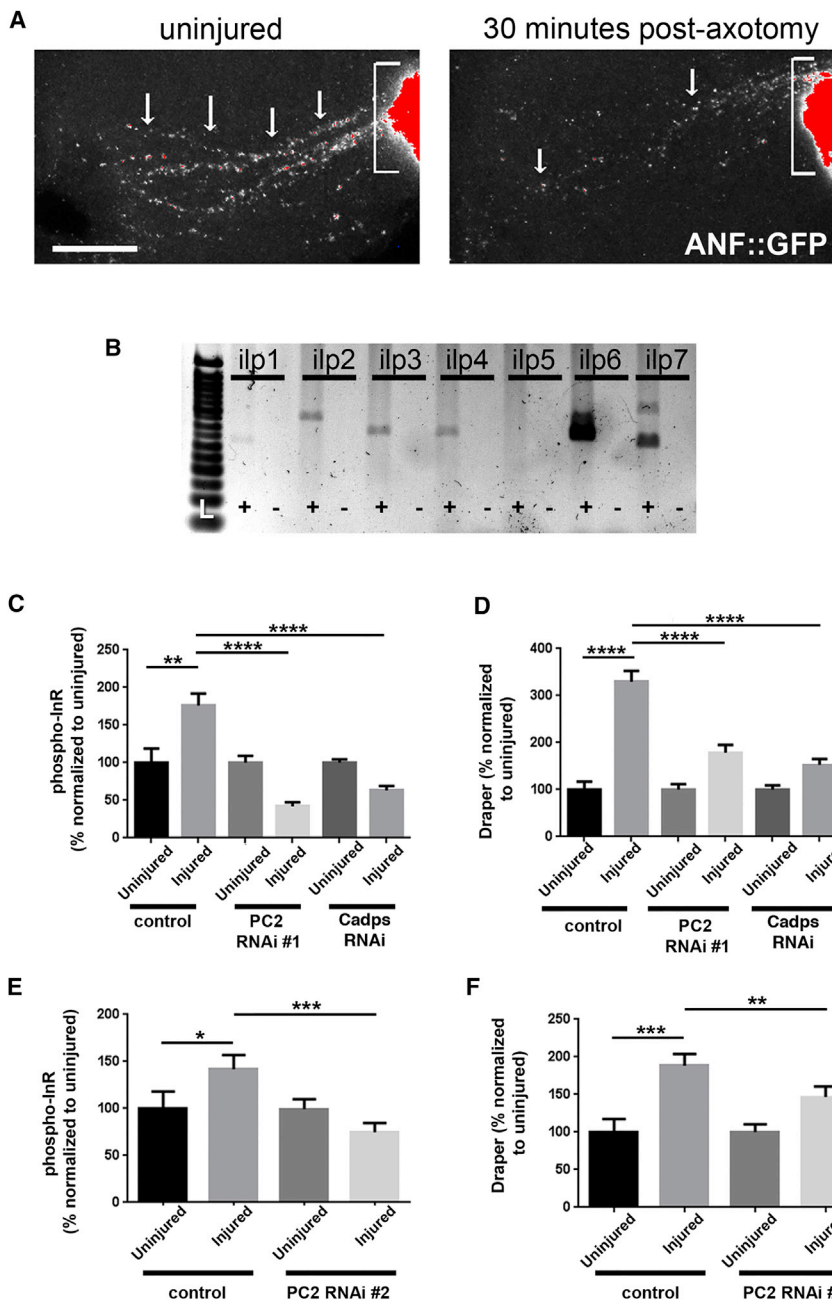


Figure 5. Knockdown of PC2 or Cadps in OR-factory Neurons Attenuates Phospho-InR Activation and Draper Upregulation in Ensheathing Glia

(A) GFP-tagged atrial natriuretic factor (ANF::GFP) neuropeptide distribution along uninjured OR22a axons (white arrows). Grayscale converted confocal images (red represents maximum intensity) are shown. Fewer ANF::GFP+ puncta (65%) are observed 30 min after antennal nerve axotomy. Synaptic rich OR22a glomeruli are highlighted with brackets. Genotype: *y,w, UAS-ANF::GFP/+; OR22a-Gal4/+*; N > 15 brains for each condition. Scale bar, 20 μ m.

(B) PCR analysis of *ilp1–7* ligands in *w¹¹¹⁸* adult third antennal segments. +/– correspond to + and – RT samples. L, molecular ladder.

(C and E) Quantification of phospho-InR in dorsal antennal lobe ensheathing glia 18 hr after antennal nerve axotomy. N \geq 18 for each condition.

(D and F) Quantification of Draper levels in dorsal antennal lobe ensheathing glia 18 hr after antennal nerve axotomy. N \geq 21 for each condition.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Pooled data plotted as mean \pm SEM. Genotypes: (B–E) control (*UAS-dicer2/+; orco-Gal4/+*), PC2 RNAi #1 (*UAS-dicer2/UAS-PC2^{RNAi} VDRC 110788; orco-Gal4/+*); PC2 RNAi #2 (*UAS-dicer2/+; orco-Gal4/UAS-PC2^{RNAi} B128583*); *Cadps^{RNAi}* (*UAS-dicer2/UAS-Cadps^{RNAi} VDRC 110055; orco-Gal4/+*).

bacteria and protein aggregates (Bjerknes and Aarskog, 1995; Inoue et al., 1998). Our work now provides in vivo evidence that ILS positively drives phagocytosis by regulating expression of a key pro-engulfment factor and suggests that this is an evolutionarily conserved signaling mechanism to regulate both professional and unprofessional phagocytes.

Maintenance of basal Draper levels requires phosphatidylinositol-3 kinase (PI3K) signaling (Doherty et al., 2014). Although the InR can signal through PI3K, we did not detect a significant reduction in basal Draper levels (at the transcript or protein level) following InR knockdown/inhibition in adult glia, which argues against InR as the primary upstream receptor maintaining basal

Draper expression via PI3K. Instead, we find that glial InR is required for transcriptional induction of *draper* after axotomy and for recruitment of Draper on severed axons, revealing the InR to be an upstream regulator of Draper expression in the context of neural injury. The transcription factor STAT92E is similarly required to boost Draper levels in local ensheathing glia post-injury through a positive Draper/STAT92E autoregulatory loop (Doherty et al., 2014). Interestingly, in addition to the highly conserved canonical PI3K/Akt cascade, STAT has also been identified as a signaling effector of the insulin receptor and the IGF-1 receptor

(Himpe and Kooijman, 2009; Taniguchi et al., 2006); coupling of canonical and non-canonical signal transduction pathways provides spatial and temporal refinement of insulin-like signaling cascades. Because we find that basal Draper levels are normal in glial InR-depleted flies, it is unlikely that the failure of glia to up-regulate *draper-1* or activate STAT92E-dependent transcription (Figure 3) is due to deficient pre-injury Draper levels. It will be important to define the signaling cascades, direct and indirect, that connect activation of glial InR/Akt1 with acute *draper* up-regulation, Draper recruitment to degenerating axons and timely phagocytic removal of cellular debris. Based on the strong conservation of ILS and the Draper/MEGF10/Jedi glial engulfment

pathway across species, we speculate that ILS could similarly be a positive regulator of mammalian glial phagocytic activity, in part through regulated expression of MEGF10 and/or Jedi.

Our analysis of ANF::GFP, combined with our *Cadps*^{RNAi} results, supports a model in which DCVs are released from severed axons. DCVs can be released extra-synaptically, including along axons following robust stimulation protocols (van de Bospoort et al., 2012; Matsuda et al., 2009), and CAPS1 is required for DCV fusion from extrasynaptic sites in mammalian neurons (Farina et al., 2015). Future work will provide important insight into the intrinsic axonal changes, including calcium-dependent mechanisms, that may promote DCV and neuropeptide release after injury. Our PC2 and *Cadps* loss of function results indicate that perturbing neuropeptide processing or DCV release attenuates InR signaling and Draper upregulation in local ensheathing glia. These phenotypes were not replicated in *ilp*-depleted flies, but it will be important to determine if systematic disruption of a single *ilp* (or even several *ilp* ligands) in ORNs triggers compensatory responses, like those described in other areas of the CNS. For example, medial neurosecretory cells in the dorsal region of the fly brain express *ilp2*, *ilp3*, and *ilp5*, but knockdown of *ilp2* in these cells results in increased production of *ilp3* and *ilp5*, which functionally compensates for the loss of *ilp2* (Broughton et al., 2008). Moreover, it remains to be determined if *ilps* are expressed uniformly throughout the antennal lobes or if different *ilps* are variably expressed in subsets of ORNs. We also cannot exclude that an as yet to be identified neuropeptide activates ensheathing glial InR after ORN axotomy. Finally, it will be important to also explore the possibility that glia-glia ILS communication, for example between local astrocytes and ensheathing glia, occurs after injury.

In invertebrates and mice, ILS is required in axons to execute a normal Wallerian degeneration program through the production of reactive oxygen species following neurotoxic insults (Calixto et al., 2012). It is interesting to speculate that the ILS pathway serves multiple roles at CNS trauma sites to simultaneously ensure that (1) axons degenerate promptly, and (2) local glia respond robustly to efficiently clear degenerating material. Whether this occurs in a variety of contexts (axotomy, neurodegenerative disease, etc.) remains to be explored. Nevertheless, there is growing interest in understanding the relationship between adult ILS pathways and neurological disorders because defects in insulin pathways and metabolic diseases are associated with increased risk for neurodegenerative diseases (Bassil et al., 2014; Fernandez and Torres-Alemán, 2012; Russo et al., 2005). Long-term changes in insulin/IGF signaling (e.g., ILS resistance) likely alter basal health and metabolic integrity of the nervous system (Bassil et al., 2014; Broughton and Partridge, 2009; Piriz et al., 2011), but our findings now provide *in vivo* evidence that highlights the importance of the glial ILS pathway as a critical neuroprotective signaling system in the damaged brain.

EXPERIMENTAL PROCEDURES

Fly Stocks

The following fly strains were used: *repo-Gal4* (Leiserson et al., 2000); *w*¹¹¹⁸, *OR85e-mCD8::GFP*, *tubulin-Gal80^{ts}* (Ziegenfuss et al., 2012); *InR^{ex15}* (Song et al., 2003); *y*¹ *w*¹¹¹⁸, *UAS-InR.K1409A* (dominant-negative InR, BI 8253) (De-

montis and Perrimon, 2009); *w*¹¹¹⁸, *UAS-mCD8::GFP*, *repo-gal4*; *y*¹ *v*¹, *UAS-InR^{RNAi}* (BI 31037); *w*¹¹¹⁸, *UAS-LacZ::NLS* (BI 3955); *y*¹ *v*¹, *UAS-Akt^{RNAi}* (BI 31701), *10XSTAT92E-dGFP* (Bach et al., 2007); *y*¹ *w*¹¹¹⁸, *UAS-Akt1* (BI 8191); *y*¹ *w*¹¹¹⁸, *UAS-InR* (Bloomington stock 8262); *y*¹ *w*¹¹¹⁸, *UAS-InR.del* (constitutively active InR, Bloomington stock 8248); *w*¹¹¹⁸, *TIFR-Gal4* (Yao et al., 2007); *y*¹ *w*¹¹¹⁸, *Draper^{RNAi}* (MacDonald et al., 2006); *UAS-ANF::GFP* (BI 7001); *OR22a-Gal4* (Dobritsa et al., 2003); *UAS-PC2^{RNAi}* (BI 28583); *UAS-PC2^{RNAi}* (VDR 110788) (Dietzl et al., 2007); *orco-Gal4* (BI 23292) (Larsson et al., 2004); *UAS-dicer2* (BI 24650) (Dietzl et al., 2007); and *UAS-Cadps^{RNAi}* (VDR 110055).

Olfactory Neuron Injuries, Dissection, and Analysis

Maxillary and antennal nerve transections, adult fly brain dissections, and whole brain antibody staining were performed using previously described methods (MacDonald et al., 2006). See the Supplemental Experimental Procedures.

Confocal Microscopy

All immunostained brains were imaged on a Zeiss LSM 700 with a Zeiss 40× 1.4NA oil immersion plan-apochromatic lens. Brains within each experiment were mounted under a single 1.5 cover glass and imaged on the same day with the same confocal microscope settings (laser power, photomultiplier tube gain, offset, filter configuration). The Z step in all experiments was 0.76 μm (pinhole 1.5-μm optical slice). Pixel dimensions ranged from 100–230 nm.

Statistical Analysis

GraphPad Prism software was used to perform ordinary one-way ANOVA tests, Kruskal-Wallis one-way ANOVA 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, and 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 datasets to allow for the appropriate use of parametric tests. When assumptions of normality could not be met for a given dataset, non-parametric tests were used. Each n = 1 sample number represents pooled measurements taken from independent animals.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.07.022>.

AUTHOR CONTRIBUTIONS

D.T.M. conceived and performed experiments, analyzed data, and wrote the manuscript. M.D.P. designed and performed experiments and analyzed data. S.D.S. designed and performed experiments, analyzed data, and wrote the manuscript. J.D. performed experiments. M.A.L. conceived the project, designed and performed experiments, analyzed data, and wrote the manuscript.

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