

The c-Jun kinase signaling cascade promotes glial engulfment activity through activation of *draper* and phagocytic function

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After neuronal injury or death glial cells become reactive, exhibiting dramatic changes in morphology and patterns of gene expression and ultimately engulfing neuronal debris. Rapid clearance of degenerating neuronal material is thought to be crucial for suppression of inflammation and promotion of functional recovery. Here we demonstrate that *Drosophila* c-Jun N-terminal kinase (dJNK) signaling is a critical *in vivo* mediator of glial engulfment activity. In response to axotomy, we find glial dJNK signals through a cascade involving the upstream mitogen-activated protein kinase kinases Slipper and Tak1, the mitogen-activated protein kinase kinase MKK4, and ultimately the *Drosophila* activator protein 1 (AP-1) transcriptional complex composed of Jra and Kayak to initiate glial phagocytosis of degenerating axons. Interestingly, loss of dJNK also blocked injury-induced upregulation of Draper levels in glia, and glial-specific overexpression of Draper was sufficient to rescue engulfment defects associated with loss of dJNK signaling. This work identifies that the dJNK pathway is a novel mediator of glial engulfment activity and a primary role for the glial Slipper/Tak1 → MKK4 → dJNK → dAP-1 signaling cascade appears to be activation of *draper* expression after axon injury.

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Cell death occurs throughout the life of most metazoans. The presence of cell corpses and cellular debris triggers rapid responses from neighboring professional or non-professional phagocytes, ultimately promoting corpse engulfment.^{1–3} In the central nervous system (CNS) glial cells are the primary immune cell type, constantly surveying the neural environment for signs of cell death or injury. Neural injury results in rapid changes in glial cellular phenotypes – glia become ‘reactive’ whereby they exhibit dramatic changes in morphology and patterns of gene expression and they ultimately phagocytose degenerating neuronal debris.^{4–7} Reactive glial responses are an early feature of most neurodegenerative diseases^{8,9} and occur after any brain injury,^{10,11} but remarkably little is known about how glial cells sense neuronal injury, become phagocytic, and eventually engulf neuronal debris.

A key mediator of glial responses to neuronal death or injury in *Drosophila* is the engulfment receptor Draper,^{12,13} which encodes the *Drosophila* ortholog of the *Caenorhabditis elegans* cell corpse engulfment receptor CED-1.¹⁴ In the *Drosophila* embryo, glial Draper is required for efficient clearance of neuronal cell corpses from the developing CNS.¹² In the adult brain, Draper is essential for activation of glial responses after axotomy and promotion of glial phagocytosis of degenerating axonal debris. For instance, after olfactory receptor neuron (ORN) axotomy, glial cells dramatically increase levels of Draper, extend membranes to

degenerating axons, engulf axonal debris, and then terminate their responses and return to a resting state.^{13,15} In *draper* null mutants, glial cells fail to show any morphological or molecular responses to axonal injury and unengulfed axonal debris lingers in the adult brain for the lifespan of the fly.

During glial engulfment of degenerating axons, the Draper receptor appears to signal in a manner similar to the mammalian Fc, B-, and T-cell immunoreceptors with activation of Draper resulting in downstream engulfment signaling through an Src family kinase signaling cascade. Src42a phosphorylates an intracellular immunoreceptor tyrosine-based activation motif (ITAM) domain on Draper, the non-receptor tyrosine kinase Shark then binds to the Draper ITAM, and engulfment is activated.¹⁶ The phosphotyrosine-binding domain-containing protein dCed-6 also appears to signal downstream of the Draper receptor,¹⁷ but additional signaling molecules that promote engulfment activity in glia remain poorly defined. Here we show that the *Drosophila* c-Jun amino-terminal kinase (dJNK) plays a critical role in glial responses to degenerating axonal debris. Loss of glial dJNK signaling potently blocks axon injury-induced upregulation of Draper, activation of a glial phagocytic phenotype, and glial clearance of axonal debris. Our work identifies the dJNK pathway as a novel molecular cascade required for glial engulfment signaling and we propose that a primary role for dJNK is transcriptional activation of the *draper* gene after axotomy.

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Abbreviations: dJNK, *Drosophila* c-Jun N-terminal kinase; CNS, central nervous system; ITAM, immunoreceptor tyrosine-based activation motif; ORN, olfactory receptor neuron; AP-1, activator protein 1; UAS, upstream activating sequences; GFP, green fluorescent protein

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Results

Basket signaling is required in adult brain glia for engulfment of axonal debris. In an RNAi-based screen for novel genes required for glial responses to axonal injury, we identified *basket* (*bsk*), encoding dJNK, as a key regulator of glial engulfment of axonal debris. For glial-specific dJNK knockdown, we drove the expression of upstream-activating sequences (*UAS*)-*bsk*^{RNAi-104569} with the pan-glial driver *repo-Gal4* in a background where a subset of maxillary ORNs were labeled with membrane-tethered green

fluorescent protein (mCD8::GFP). We then severed ORN axons by maxillary palp ablation and assayed clearance of axonal debris 5 days after axotomy. Whereas control animals cleared the vast majority of axonal debris within 5 days of axotomy, we found that this glial engulfment activity was potently suppressed by glial *bsk*^{RNAi} (Figures 1a, b, d, and e). To confirm this phenotype was specific to *bsk*, we performed glial-specific RNAi knockdown using two additional RNAi lines targeting different regions of the *bsk* transcript (*bsk*^{RNAi-34138} and *bsk*^{RNAi-TRIIP}) and found similar results (Supplementary Figure 1). *Bsk*^{RNAi} suppression of axonal

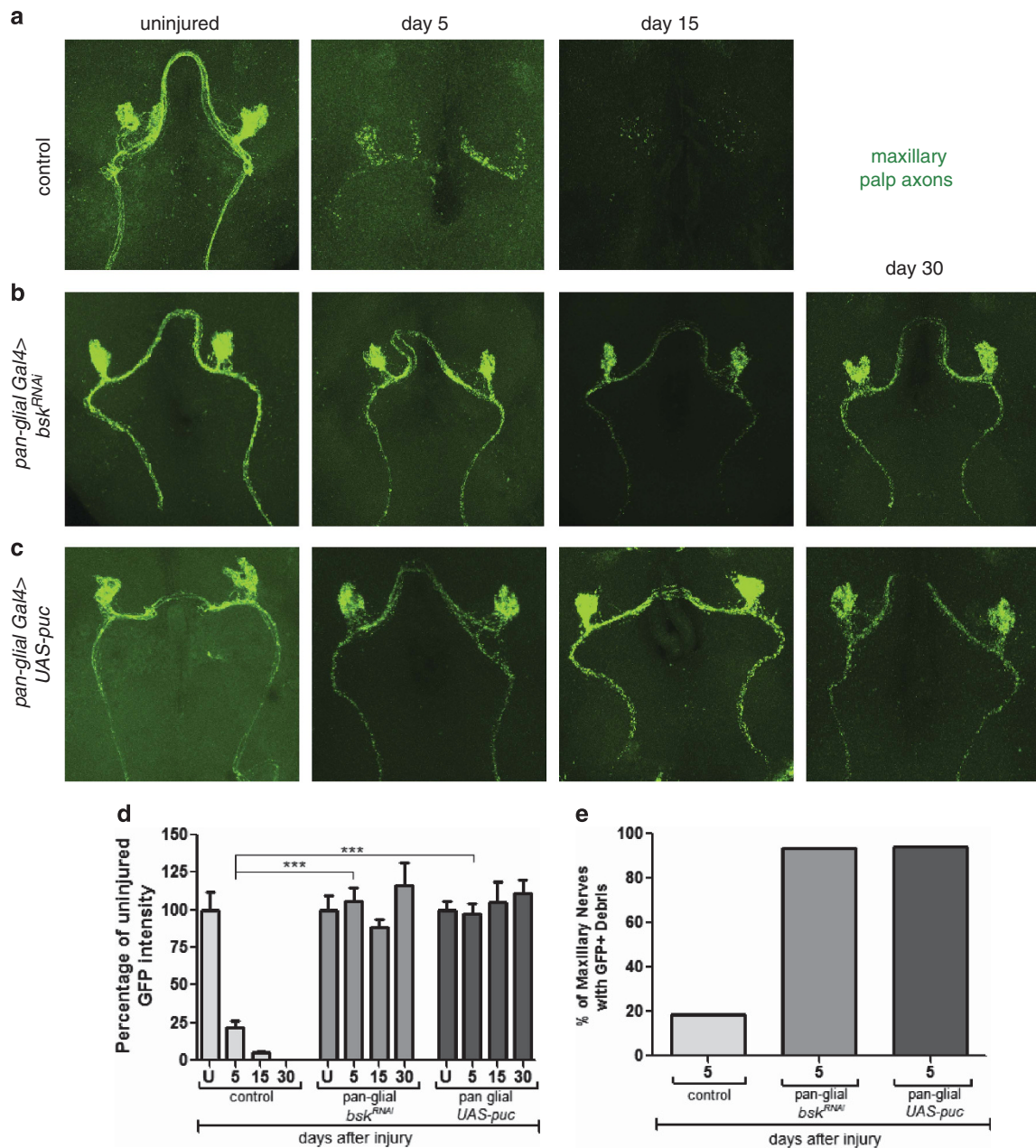


Figure 1 dJNK is required for clearance of axonal debris after nerve injury. (a) Control animals (*OR85e-mCD8::GFP/+; repo-Gal4/+*) 0, 5, and 15 days after axotomy. (b) Glial knockdown of dJNK using *repo-Gal4* to express *UAS-dJNK RNAi* (*OR85e::mCD8::GFP/UAS-bsk*^{RNAi104569}; *repo-Gal4/+*). (c) Glial overexpression of *UAS-puckered* using *repo-Gal4* (*OR85e::mCD8::GFP/+; repo-Gal4/UAS-puckered*). (d) Quantification of GFP intensity in glomeruli for (a)–(c). Error bars depict ± S.E.M. ****P* < 0.001. (e) Quantification of maxillary nerves with GFP + debris for (a)–(c)

clearance was robust (near 100%) and persistent – we observed axonal debris lingering for as many as 30 days after axotomy in glial *bsk^{RNAi}* animals, which is longer than the median lifespan of adult *Drosophila*.

We next overexpressed *puckered* (*puc*), a phosphatase that negatively regulates dJNK activity.¹⁸ Glial-specific expression of Puc phenocopied glial *bsk^{RNAi}* with nearly all axonal debris lingering in the CNS for 30 days after axotomy (Figures 1c, d, and e). We note that axonal fragmentation appears to occur normally and on schedule (i.e. within 1 day) in these backgrounds, indicating that glial Bsk function is not required for axonal degradation.

dJNK signaling is known to be important for the development of a number of cell types. We examined glial morphology, numbers, position, and expression of the engulfment genes *draper* and *dCed-6* in the CNS of glial *bsk^{RNAi}* and *UAS-puc* animals, but found no noticeable defects in glial cell development or engulfment gene regulation before injury (Supplementary Figure 2). Nevertheless, to exclude the possibility that engulfment phenotypes might arise from requirements for *bsk* in glial development, we conditionally expressed *bsk^{RNAi}* in adult brain glia using the temperature-sensitive Gal80^{TS} repressor. At 18 °C, Gal80^{TS}

represses activation of the Gal4/UAS binary system; however, at 29 °C Gal80^{TS} is inactivated, thereby derepressing Gal4/UAS. We crossed Gal80^{TS} into control and glial *bsk^{RNAi}* backgrounds, raised animals at 18 °C, shifted them to 29 °C after eclosion for 7 days, and then performed axotomies. Consistent with a role for dJNK signaling in mature glia after axotomy, we found that adult-specific knockdown of *bsk* was sufficient to block glial engulfment of degenerating axons (Figures 2a and b).

Ensheathing glia are the primary cell type in the *Drosophila* neuropil that express molecular components of the engulfment machinery, including the Draper receptor and dCed-6 adaptor molecule, and they phagocytose axonal debris after axotomy.¹⁹ To determine whether Bsk functions in ensheathing glia, we drove the expression of *bsk^{RNAi}* or *UAS-puc* with the ensheathing glia-specific driver *mz0709-Gal4* and assayed glial clearance of degenerating axons. We found that suppression of dJNK signaling in ensheathing glia was sufficient to block the glial engulfment function (Figures 2c and d). Notably, suppressing dJNK signaling in astrocytes, the only other subtype of glial cells present in the antennal lobe neuropil, had no effect on glial clearance of axonal debris (data not shown).

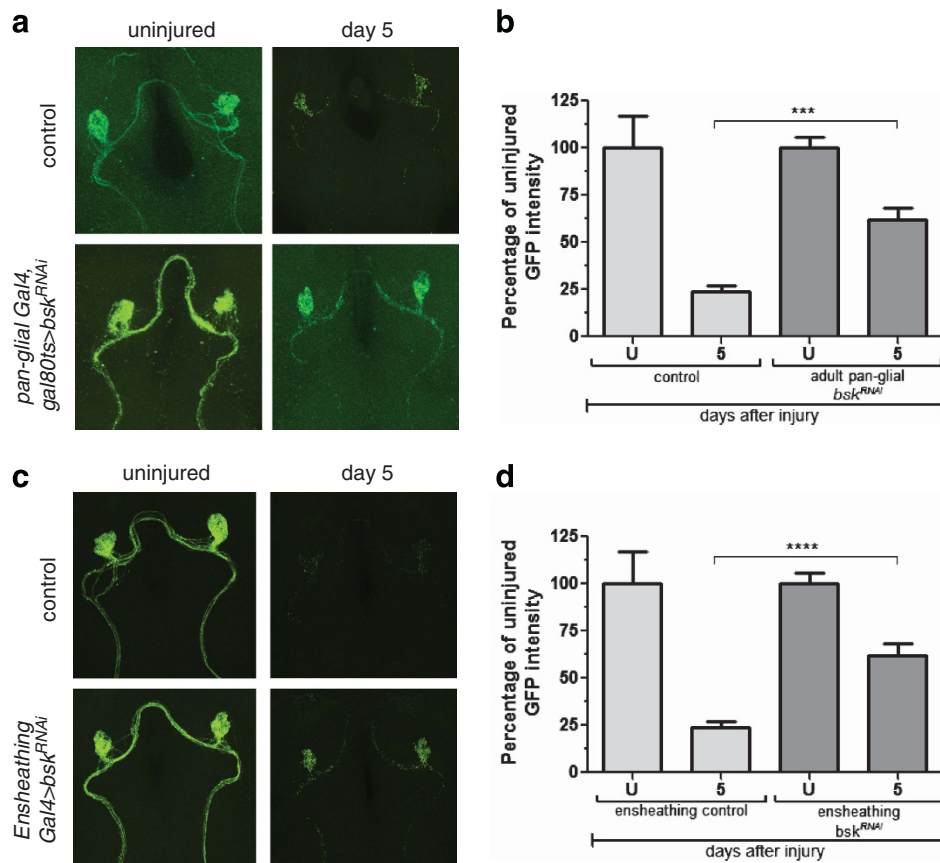


Figure 2 The requirement for dJNK in glial engulfment of axonal debris is adult- and ensheathing-glia-specific. (a) Control animals (*OR85e::mCD8::GFP, tub-Gal80^{TS}/+*; *repo-Gal4/+*) and *bsk^{RNAi}* animals (*OR85e::mCD8::GFP, tub-Gal80^{TS}/UAS-bsk^{RNAi104569}; repo-Gal4/+*) were raised at 18 °C. After eclosion, adults were shifted to 29 °C for at least 7 days, maxillary palps were ablated, and flies were kept at 29 °C for 5 days before assaying axon clearance. (b) Quantification for (a). Error bars depict mean \pm S.E.M. *** $P < 0.001$. (c) Control animals (*OR85e::mCD8::GFP/+; mz0709-Gal4/+*) or animals with ensheathing glia-specific knockdown of dJNK (*OR85e::mCD8::GFP/UAS-bsk^{RNAi104569}; mz0709-Gal4/+*) were axotomized and assayed for axon clearance 5 days after axotomy. (d) Quantification for (c). Error bars depict mean \pm S.E.M. **** $P < 0.0001$

These data identify dJNK signaling as a novel mediator of glial responses to axonal injury. Our data further showed that dJNK signaling is required in mature ensheathing glia – the same glial subtype in which Draper signals to promote phagocytosis of axonal debris.

Glial dJNK signaling involves a mitogen-activated protein kinase cascade that signals to the nucleus.

dJNK has been shown to function downstream of a wide range of receptor types and to execute signaling events with a diversity of kinases. Precisely which signaling molecules dJNK interacts with appears to be context specific.²⁰ To identify additional molecules involved in dJNK signaling during glial phagocytic clearance of degenerating axons, we assayed the roles of known components of JNK signaling pathways in *Drosophila* (Figure 3a). We found that glial-specific knockdown of the mitogen-activated protein kinase kinases Slipper and Tak1, the mitogen-activated protein kinase kinase MKK4, and the transcriptional factors Jun-related antigen (*Jra*, *Drosophila* c-Jun) and Kayak (*Drosophila* c-Fos) significantly suppressed glial clearance of degenerating axonal debris 5 days after axotomy (Figure 3a and Supplementary Figure 3). We confirmed the specificity of these RNAi transgenes by using additional RNAi lines that target non-overlapping regions of each transcript.

We next sought to provide genetic support for a role for this pathway in glial engulfment signaling and to explore the effects of complete loss of function of this JNK signaling

pathway in the clearance of axonal debris. We therefore performed axotomies in *slipper* and *tak1* mutants and examined their effects on the clearance of GFP-labeled degenerating axons. Clearance of axonal debris was largely normal in *slipper*- (*slipper*^{BS506}) and *tak1*- (*tak1*²) null mutant backgrounds (not shown); however, neuronal debris persisted at significant levels after axotomy in *slipper*^{BS06}, *tak1*² double mutants (Figure 3b and Supplementary Figure 2). These data argue that the Slipper and Tak1 kinases likely function in a redundant manner in the glial cells responding to axonal injury.

Positive dJNK signaling often leads to changes in gene expression through the activator protein 1 (AP-1) complex. *Drosophila* AP-1 (dAP-1) is composed of a heterodimer of *Jra* and Kayak. The significant phenotypes in *Jra* and *kayak* RNAi backgrounds argue for a role for dAP-1 in glial transcriptional responses to axonal injury. To confirm this, we assayed activation of a *Drosophila* transcriptional reporter for dAP-1 activity, *TRE-eGFP*, which contains dAP-1 binding sites upstream of the *eGFP* gene.²¹ In uninjured brains, we detected only low levels of eGFP in the *TRE-eGFP* background, suggesting weak basal dAP-1 signaling. In contrast, 1 day after antennal ablation, we found robust upregulation of the *TRE-eGFP* reporter in ensheathing glia surrounding the antennal lobe as well as the local cortex glia (Figure 3d and Supplementary Figure 4).

Taken together, these data identify a novel role for a dJNK signaling pathway involving a Slipper/Tak1 → MKK4 → Bsk →

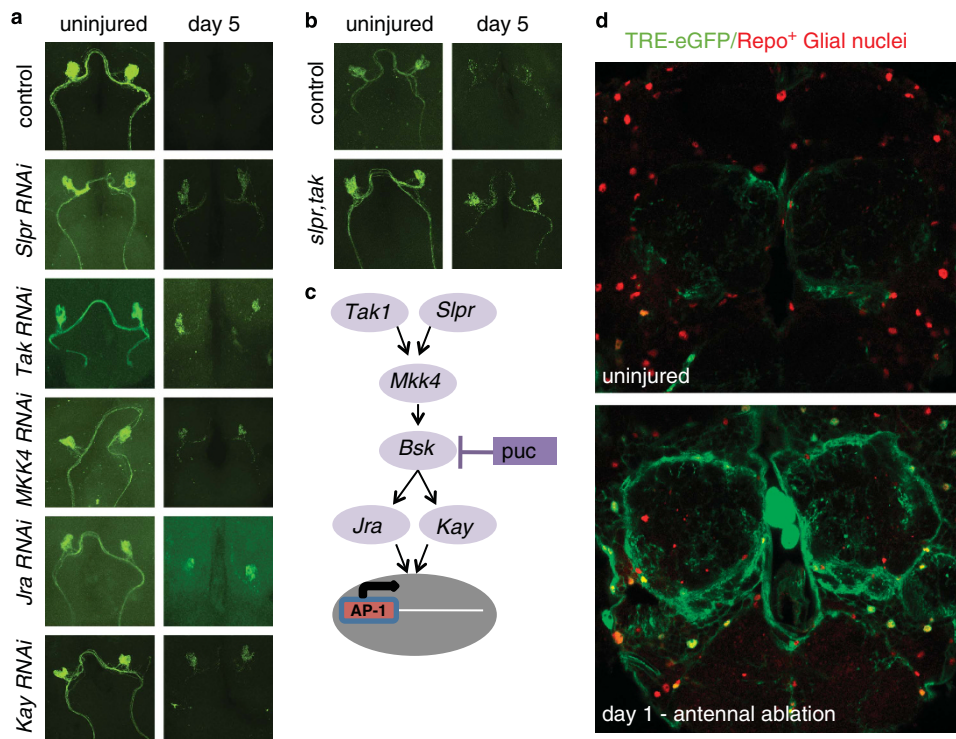


Figure 3 Glial c-Jun kinase signaling to the nucleus after axotomy involves Slpr, Tak, MKK4, Jra, and Kay. (a) *UAS-RNAi* constructs for the indicated genes were driven in glia using the *repo-Gal4* driver, axons were severed in adults, and axonal debris was scored 5 days after axotomy as above. See Supplementary Figure 3 for quantifications. (b) *Slpr, tak* double mutants (*slpr*^{BS06}, *tak1*^Y; *OR85e::mCD8::GFP/+*) were assayed for defects in axon clearance. (c) Model of dJNK signaling to the nucleus to induce engulfment gene expression. (d) Expression of the dAP-1 transcriptional reporter *TRE-eGFP* in uninjured animals (top) and animals where third antennal segments were ablated 1 day prior (bottom)

dAP-1 cascade (Figure 3c) in promoting glial engulfment of degenerating axons after axonal injury. As loss of multiple components of this pathway (i.e. Bsk, Tak1, Slpr, Jra and Kay) block the clearance of a significant amount of axonal debris, positive dJNK signaling appears to be a central regulator of glial responses to axonal injury. On the basis of the requirements for dAP-1 and its activation after axotomy (based on the TRE-eGFP reporter), we propose that dJNK signaling acts to regulate glial engulfment behavior through dAP-1-dependent transcriptional changes.

JNK signaling is required for axotomy-induced increases in glial Draper levels. To explore the potential roles of dJNK signaling in regulating the expression of glial genes, we examined the expression of the engulfment receptor Draper in *bsk^{RNAi}* and *UAS-puc* backgrounds. Before injury, Draper levels were indistinguishable from control animals when we drove *bsk^{RNAi}* or *UAS-puc* in adult brain glia as judged by immunofluorescence (Figure 4a and Supplementary Figures 2A and 5A) and western

blot (Supplementary Figure 2B). Therefore, loss of JNK signaling does not appear to affect basal levels of Draper expression.

Antennal ablation severs ~88% of all ORN axons innervating the antennal lobe, resulting in widespread axonal degeneration throughout this brain region and in turn a robust increase in Draper levels in glia surrounding the site of injury in wild-type (Figure 4a).^{13,19} Interestingly, we found a complete absence of this axotomy-induced increase in Draper levels when we drove glial-specific expression of *bsk^{RNAi}* (Figure 4a) or *UAS-puc* (Supplementary Figure 5A). Thus, loss of JNK signaling specifically blocks axotomy-induced changes in glial Draper levels after antennal ablation. These data are consistent with the notion that dAP-1 might regulate *draper* expression.

Glia with depleted dJNK signaling recruit Draper to local axonal debris. The above data raised the possibility that glia lacking dJNK signaling are incapable of responding to axonal injury – specifically, glia might be unable to extend

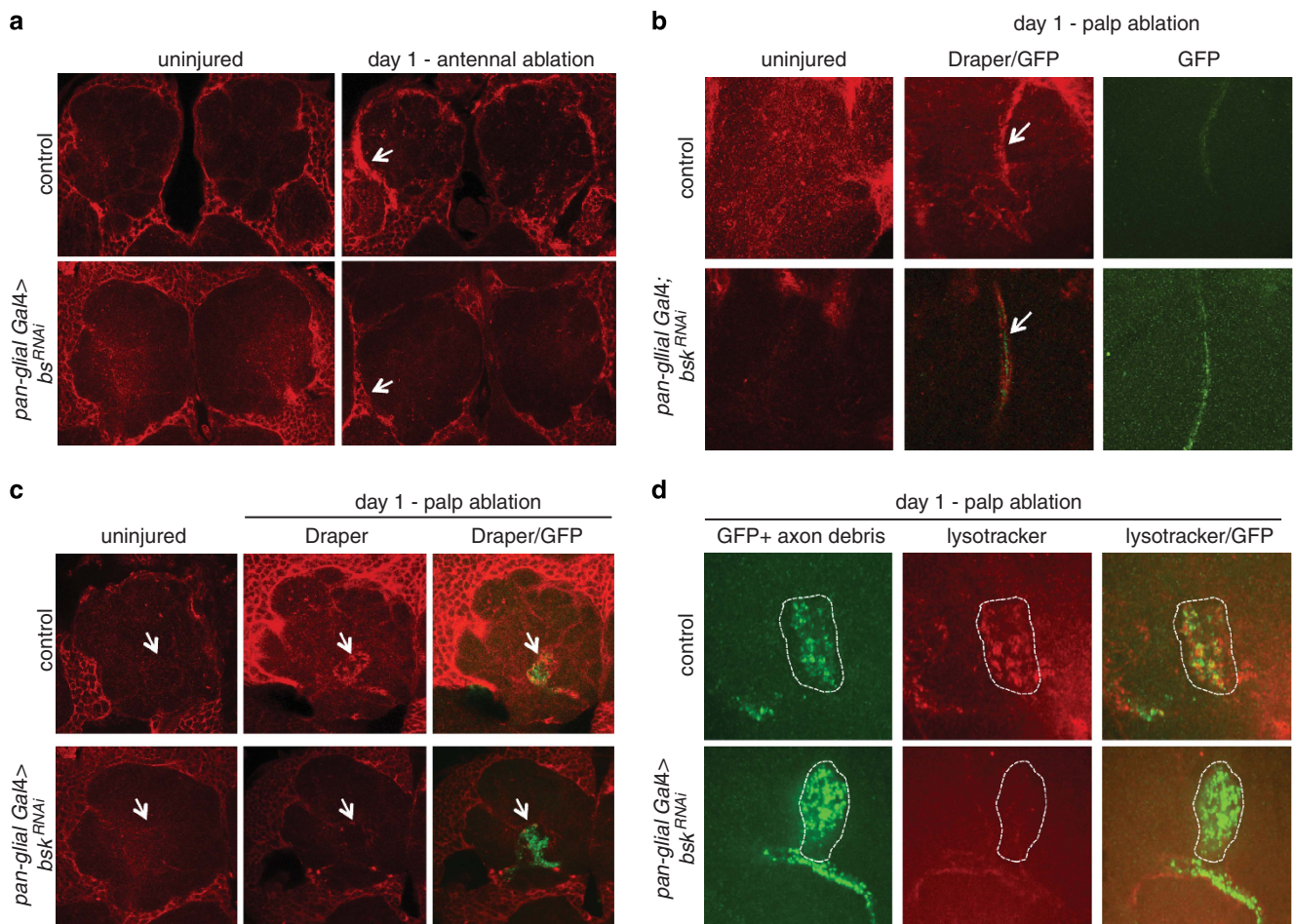


Figure 4 dJNK is required for Draper upregulation after axotomy and activation of glial phagocytic function. (a) Immunostains for Draper in control or glial dJNK^{RNAi} animals before or after antennal ablation. Arrows highlight upregulation of Draper in ensheathing glia (control) or lack thereof (*bsk^{RNAi}*). (b) Draper (red) localization along the maxillary nerve (arrows) before or 1 day after maxillary palp ablation. (c) Draper immunostains (red) in a single antennal lobe before or after axotomy. A subset of maxillary palp ORN axons are shown (green). Note the lack of Draper staining within the glomerulus of glial dJNK^{RNAi} animals compared with controls (arrows). (d) Lysotracker Red stains for lysosomal activity one day after maxillary palp ablation. Note the punctate accumulation of Lysotracker Red in the OR85e + glomerulus (green, white dotted circle) in controls but not in glial dJNK^{RNAi} animals

membranes to degenerating axons and activate a phagocytic phenotype. To determine whether glial cells lacking dJNK signaling were capable of recognizing an injury and recruiting Draper specifically to severed axons, we assayed axon clearance and Draper localization after maxillary palp ablation. Surgical removal of maxillary palps severs only ~12% of all ORN axons which innervate six uniquely identifiable antennal lobe glomeruli. This modest injury does not lead to detectable increases in Draper levels in glia surrounding the antennal lobe, but one can examine Draper recruitment to (1) degenerating axons within identifiable antennal lobe glomeruli or (2) ORNs housed within the maxillary nerve as it projects to the antennal lobe (Figures 4b and c).¹³ Assaying antennal lobe glomerular localization of Draper is especially useful for determining whether Draper or other components of the engulfment machinery can be recruited to axonal debris, as the engulfing cells must extend membranes into glomeruli to reach engulfment targets (see below). In contrast, ensheathing glia found along the maxillary nerve are already immediately adjacent to their engulfment targets (i.e. they do not have to migrate), so the maxillary nerve is useful for determining whether glia exhibit any changes in Draper localization after axotomy even in backgrounds where glia are incapable of migratory behavior.¹³

One day after maxillary palp ablation in control animals, we found a strong increase in Draper staining along the maxillary nerve (Figure 4b) and Draper levels returned to baseline levels within 7 days after axotomy. Surprisingly, we found that glial expression of either *bsk^{RNAi}* or *UAS-puc* did not suppress increases in Draper around the maxillary nerve: in both *bsk^{RNAi}* and *UAS-puc* backgrounds Draper accumulated on severed maxillary palp ORN axons, although at levels slightly lower than controls (Figure 4b and Supplementary Figures 5B, E, and 6B). Thus, glial cells with depleted dJNK signaling are capable of detecting axonal injury and responding by recruiting the Draper receptor to severed axons. This is notably different from what is observed when the Draper signaling pathway is inactivated – elimination of the non-receptor tyrosine kinase Shark or Src family kinase Src42a from glia completely suppresses Draper recruitment to severed axons along the maxillary nerve. This indicates that dJNK signaling acts downstream of initial recruitment of Draper to axonal debris.

Interestingly, while we observe initial recruitment of Draper to degenerating maxillary ORN axons is normal in animals deficient in glial dJNK signaling, glial cells appear incapable of terminating their responses to axonal injury. Even 30 days after axotomy, Draper levels along the maxillary nerve in glial *bsk^{RNAi}* or *UAS-puc* backgrounds remained elevated (data not shown). We interpret these data to mean that in addition to its role in engulfment of axonal debris, dJNK signaling is also critical termination of glial responses to axonal injury.

Loss of dJNK signaling suppresses extension of glial membranes to degenerating axons and activation of phagocytic function. We next asked whether loss of dJNK signaling blocked the extension of glial membranes to degenerating axons. We therefore assayed glial recruitment of Draper to degenerating maxillary ORNs in the antennal

lobe, where glia must extend membranes into glomeruli to reach their engulfment targets. In control animals, Draper immunoreactivity was detectable one day after maxillary palp ablation throughout the glomeruli housing degenerating axonal debris (Figure 4c and Supplementary Figures 5 and 6). In contrast, while we observed an increase in Draper immunoreactivity around glomeruli in glial *bsk^{RNAi}* or *UAS-puc* animals, Draper immunoreactivity failed to accumulate in central regions of these structures (Figure 4c and Supplementary Figures 5 and 6). Thus, dJNK signaling is essential for glial membrane extension to engulfment targets.

Once glia have inserted membranes into glomeruli, they activate a phagocytic program – glia become Lysotracker positive (a marker for lysosomal activity) and internalize axonal fragments.²² When we examined Lysotracker activity 1 day after maxillary palp ablations, we observed strong punctate Lysotracker staining within glomeruli containing degenerating axons in control animals. However, Lysotracker staining was absent from these glomeruli in glial *bsk^{RNAi}* animals (Figure 4d). Taken together, these data indicate that dJNK signaling is required for glial membrane extension into glomeruli for engulfment of degenerating axonal material and for activation of lysosomal activity during glia phagocytic function.

Re-expression of *draper* in glia is sufficient to bypass requirements for dJNK signaling. dJNK is known to function upstream of the transcription factor (dAP-1). We have shown activation of dAP-1 reporters in glia respond to axonal injury and that loss of dAP-1 components suppresses glial engulfment of degenerating axons. In addition, we observed that glia responding to axonal injury in *bsk^{RNAi}* or *UAS-puc* animals failed to upregulate Draper in response to antennal ORN axotomy. Taken together, these observations raised the intriguing possibility that glial dJNK signaling might promote glial responses to axon injury through activation of *draper* expression. To explore this possibility, we drove the expression of Draper-I in control, glial *bsk^{RNAi}*, and *UAS-puc* animals, and assayed clearance of degenerating axonal debris. Draper-I, the longest isoform of Draper (there are three in total) and the only isoform capable of promoting glial engulfment of axonal debris, was made by cloning the full-length cDNA GH24127 into the pUAST vector.¹⁵ Expression of Draper-I in control animals did not affect axon clearance as axonal debris was cleared within 5 days (Figure 5). As described above, axonal debris remained 5 days after axotomy in glial *bsk^{RNAi}* or *UAS-puc* animals. However, when we expressed Draper-I in glial *bsk^{RNAi}* or *UAS-puc* animals, we found that engulfment defects were completely rescued (Figure 5b). Thus, increased expression of Draper-I is sufficient to overcome engulfment defects in animals with depleted glial dJNK signaling. These data argue that a primary role for dJNK signaling is increasing levels of Draper expression to promote axon clearance after axotomy.

Discussion

This study reveals that axonal injury in the *Drosophila* brain activates a glial signaling pathway composed of Slipper and Tak, MKK4, dJNK and the dAP-1 complex, which ultimately

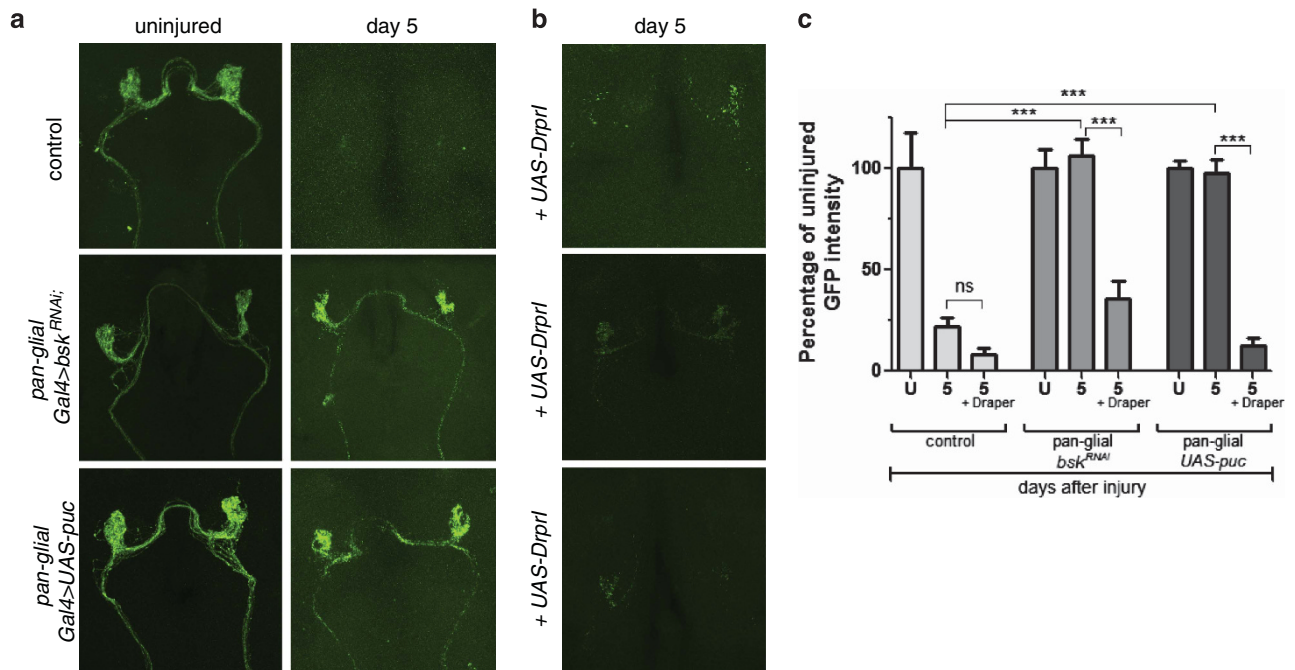


Figure 5 Overexpression of Draper rescues engulfment defects in dJNK knockdown animals. (a) Control, glial dJNK^{RNAi}, or glial expressed Puc animals before and 5 days after axotomy. (b) Control, glial dJNK^{RNAi}, or glial expressed Puc animals 5 days after axotomy with *UAS-Draper-I*. (c) Quantification from (a) and (b). Error bars depict mean \pm S.E.M. *** $P < 0.001$

leads to increased levels of Draper in glia and activation of phagocytic function. Interestingly, we have recently found that *draper* gene expression is transcriptionally upregulated after axonal injury;¹⁵ however, the signaling mechanisms that transduce injury signals from Draper to the nucleus have remained unclear. dAP-1 is, to our knowledge, the first transcriptional regulator shown to modulate glial phagocytic activation and clearance of degenerating axons, and, based on our rescue experiments, it likely does so through the regulation of Draper levels.

Loss of glial dJNK, or suppression of dJNK signaling by overexpression of the phosphatase Puc, resulted in axonal debris lingering in the brain for at least 30 days after axotomy (i.e. longer than the mean lifespan of *Drosophila*). This is among the strongest glial engulfment phenotypes observed in *Drosophila*; hence, dJNK is a critical *in vivo* regulator of glial engulfment activity. We suspect that activation of dJNK is downstream of Draper activation. Loss of Draper or its immediate downstream signaling molecules Src42A, Shark and Rac1^{16,22} also block injury-induced upregulation of Draper, and Rac1^{23,24} and Src molecules²⁵ have been shown to be capable of activating the Slipper/Tak complex upstream of dJNK. In addition, we note that loss of JNK signaling closely phenocopies *draper*-null mutants. In *draper* null animals, glia fail to respond morphologically to axonal injury, do not activate phagolysosome maturation, and axons are not cleared from the brain.^{13,22} However, it remains an open possibility that dJNK signaling is activated by a yet-to-be-identified additional engulfment receptor that is stimulated by axonal injury.

In animals with depleted glial dJNK, glia successfully recruit the small amount of Draper that is present in these cells before injury (referred to as basal Draper) to severed axons in the

maxillary nerve, indicating that dJNK is not required for glial cells to receive the injury signal or for Draper accumulation on degenerating axons. However dJNK-deficient glia fail to increase Draper levels in response to injury, to activate phagocytic pathways, and to engulf axonal debris. We interpret these data to mean that dJNK-dependent increases in levels of Draper after axotomy are critical for axon clearance. Multiple lines of evidence support this notion. First, we show activation of a dAP-1 reporter in glia after axotomy and a requirement for the dAP-1 transcriptional complex in glial engulfment of axonal debris. Second, we have recently demonstrated that *draper* is transcriptionally upregulated after axonal injury.¹⁵ Finally, we show that overexpression of Draper is sufficient to rescue dJNK^{RNAi} or Puc misexpression phenotypes. We propose that basal Draper (i.e. that before injury) is present in glia in the healthy brain to sense injury; upon activation, dJNK signaling promotes dramatic increases in Draper through the cascade described above and increased Draper after injury allows efficient clearance of axonal debris.

JNK signaling (as measured with P-JNK antibodies) is activated in response to a broad range of neural injuries in mammals including sciatic nerve lesion,²⁶ accumulation of glial fibrillary acidic protein in models for Alexander's disease,²⁷ or focal demyelination.²⁸ To date, the primary roles for JNK in mammalian glia appear to include modulating astrocyte proliferation or proinflammatory responses after neural injury. For example, JNK is activated in astrocytes in response to focal demyelination, and in primary astrocyte cultures, JNK appears to act upstream of injury-induced glial proliferation.²⁸ In addition, lipopolysaccharide treatment of astrocytes *in vitro* leads to JNK-dependent increases in CPEB1 phosphorylation and activation of reactive oxygen

species, which promote inflammation.²⁹ Intriguingly, peripheral nerve injury also leads to activation of JNK signaling in mammalian Schwann cells through the p38 mitogen-activated protein kinase cascade, apparently promoting Schwann cell de-differentiation as a part of nerve repair.³⁰ Moreover, expression of mammalian Draper, MEGF10, is dramatically upregulated in Schwann cells after sciatic nerve lesion.³¹ Thus, activation of glial JNK signaling and ultimately expression of Draper/MEGF10 appears to be a conserved glial response to axonal injury. Our data would argue that a primary role for dJNK activation in *Drosophila* glia after axonal injury is increasing levels of Draper and promoting phagocytic activity of engulfing glia. Whether the same is true in mammalian astrocytes is unclear, but it is an exciting possibility awaiting exploration.

Materials and Methods

Drosophila stocks. The following *Drosophila* strains were used: *repo-GAL4*,³² *OR85e-mCD8::GFP* (gift from B Dickson, Research Institute of Molecular Pathology, Vienna, Austria), *mz0709-GAL*,³³ *pUAST-mCD8::GFP*,³⁴ *OR22a-GAL4*,³⁵ *OR85e-GAL4* (kindly provided by J Carlson, Yale University, New Haven, CT, USA), *slpr*^{B506}, *tak1*²⁵²⁷/*FM7* (gift from B Stronach, University of Pittsburgh, Pittsburgh, PA, USA), and *pUAST-Draper-I*.¹⁵ The following RNAi stocks were obtained from VDRC³⁶: 104569 (*bsk*), 34138 (*bsk*), 34898 (*tak*), 33516 (*slpr*), 33518 (*slpr*), 106449 (*slpr*), 26928 (*MKK4*), 26929 (*MKK4*), 108561 (*MKK4*), 10835 (*Jra*), 107997 (*Jra*), and 6212 (*kay*). The following stocks were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA): *y-w::UAS-puckered* (*pucScer*\UAS.cMa), and *y[1] scf¹ v[1]; P[y[+ t7.7] v[+ t1.8]= TRiP.HMS00777]attP2*.

Immunolabeling and confocal microscopy. Standard methods were used for dissection, fixation, and antibody labeling of the adult *Drosophila* brain.^{13,37} Primary antibodies were used at the following concentrations: mouse anti-GFP, 1:250 (Life Technologies, Grand Island, NY, USA); rabbit anti-Draper, 1:500;¹² FITC anti-mouse IgG, 1:200 (Jackson ImmunoResearch, West Grove, PA, USA); and Cy3 anti-rabbit IgG, 1:200 (Jackson ImmunoResearch).

Lysotracker staining was performed as described in Ziegenfuss *et al.*²² Briefly, brains were dissected in PBS, Lysotracker Red was added (1:5000), and samples were rocked in the dark at room temperature for 15 min. Brains were washed with PBS five times quickly, and then rocked in PBS for 15 min at room temperature in the dark. Brains were then fixed with 4% formaldehyde in PBS/Triton for 30 min, rocking at room temperature in the dark. The standard antibody staining protocol was then performed as described above. Vectashield was applied to the brains and they were stored at 4 °C for 1 h before imaging on an Intelligent Imaging Innovations Everest spinning disk confocal microscope.

Images were quantified as previously described in MacDonald *et al.*¹³ Statistics were performed using GraphPad Prism (San Diego, CA, USA). In all figures except for Figure 1, Figure 5, and Supplementary Figure 1, data were analyzed using a two-tailed Student's *t*-test. In Figure 1, Figure 5, and Supplementary Figure 1, a one-way ANOVA and Tukey's multiple comparison test was used.

Western blot. *Drosophila* brains of the indicated genotype were dissected in PBS and homogenized in SDS loading buffer (60 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 1% mercaptoethanol, 0.01% bromophenol blue). For western analysis, sample containing approximately four brains were loaded onto 10% SDS-PAGE gels (Bio-Rad, Hercules, CA, USA), transferred to nitrocellulose membranes (Bio-Rad), and probed with rabbit anti-Draper¹² or rabbit anti-dCed-6³⁸ antibody at 1:1000 diluted in PBS/0.01% Tween-20/5% dry milk. Blots were incubated overnight at 4 °C, washed several times in PBS/0.01% Tween-20, and probed with the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Additional washes were performed and the blot was developed using chemiluminescence (Amersham ECL Plus, GE Healthcare, Pittsburgh, PA, USA), and detected with a Fujifilm Luminescent Imager (Minato-ku, Tokyo, Japan). The protein blot was stripped with mild stripping buffer (0.2M glycine, 0.1% sodium dodecyl sulfate, 1% Tween, pH 2.2) at room temperature, followed by washes in 1 × PBS and 1 × PBS + 0.01% Tween-20, and then re-probed with mouse anti-tubulin (Sigma-Aldrich, St. Louis, MO, USA), 1:1000.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)