Negative regulation of glial engulfment activity by Draper terminates glial responses to axon injury

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Neuronal injury elicits potent cellular responses from glia, but molecular pathways modulating glial activation, phagocytic function and termination of reactive responses remain poorly defined. Here we show that positive or negative regulation of glial responses to axon injury is molecularly encoded by unique isoforms of the Drosophila engulfment receptor Draper. Draper-I promotes engulfment of axonal debris through an immunoreceptor tyrosine–based activation motif (ITAM). In contrast, Draper-II, an alternative splice variant, potently inhibits glial engulfment function. Draper-II signaling through a previously undescribed immunoreceptor tyrosine–based inhibitory motif (ITIM)-like domain and the tyrosine phosphatase Corkscrew (Csw). Intriguingly, loss of Draper-II–Csw signaling prolongs expression of glial engulfment genes after axotomy and reduces the ability of glia to respond to secondary axotomy. Our work highlights a novel role for Draper-II in inhibiting glial responses to neurodegeneration, and indicates that a balance of opposing Draper-I and Draper-II signaling events is essential to maintain glial sensitivity to brain injury.

Glia are highly sensitive to nervous system insults, including acute injury, infection and neurodegenerative disease. Neuronal injury triggers reactive gliosis, a robust glial response that includes dramatic changes in glial gene expression and cell morphology1,2. Reactive glia rapidly extend membrane processes toward damage sites and clear degenerating neurons through phagocytic engulfment, then return to a resting state in which pre-injury morphology and molecular profile is restored3,4.

Damaged neurons release a battery of toxic factors that threaten the health of neighboring cells. Reactive glia counteract these effects by clearing excess glutamate to prevent excitotoxicity5 and secreting protective molecules6. Glial clearance of degenerating neurons prevents large-scale release of proteases and cytotoxic antigens, which can activate inflammatory immune responses7,8. However, some reactive glial responses are highly detrimental and can actively promote the destruction of healthy neurons. For example, chronic exposure to amyloid plaques characteristic of Alzheimer’s disease causes microglia to continuously release pro-inflammatory agents9. In addition, activation of glial scavenger receptors and phagocytic activity stimulates secretion of harmful reactive oxygen species10–12. Given the powerful effects of reactive glia on neuronal survival, tight regulation of their interactions with neurons after trauma is essential to minimize damage.

In professional immune cells, such as macrophages, many immune responses are regulated by receptors that contain ITAMs, defined by a consensus sequence YXXI/LX6–12YXXI/L, that signal through non-receptor tyrosine kinase cascades13. ITAM signaling can be negatively regulated by receptors that bear a related ITIM. ITIMs recruit the Src homology 2 (SH2) domain–containing protein tyrosine phosphatases (SHP) SHP-1 and/or SHP-2, which dephosphorylate ITAM domains, key downstream signaling kinases, or adaptor molecules14,15. Notably, ITAM and ITIM signaling molecules are expressed in mammalian microglia16,17, but the role of ITAM and ITIM during glial responses to trauma remains unclear.

The Drosophila engulfment receptor Draper, which regulates glial clearance of axon debris in the injured adult brain, has recently been shown to signal through an ITAM-Src-Syk–like signaling pathway. Extension of glial membranes to injury sites and glial clearance of axonal debris requires the ITAM-bearing receptor Draper, the Src kinase Src42A and the non-receptor tyrosine kinase Shark18,19. Here we explored how glial responses to axon injury are encoded by Drosophila melanogaster Draper receptor isoforms and found that unique isoforms have strikingly different roles in glial responses to axonal injury: the Draper-I isoform activated engulfment activity, whereas the alternatively spliced Draper-II isoform potently blocked all glial responses to axonal injury. Draper-II associated with the protein tyrosine phosphatase Corkscrew (Csw) through an intracellular ITIM-like domain to negatively regulate glial responses to injury. Notably, upon loss of Draper-II–Csw signaling, glia failed to terminate responses to axonal injury and had a reduced ability to respond to secondary nerve lesions. These data argue that a balance of the opposing actions of Draper-I–ITAM and of Draper-II–ITIM-like signaling fine-tune glial responses to neurodegeneration.

RESULTS

Drosophila adult glia express three Draper isoforms

Three Draper receptor isoforms (Draper-I, Draper-II and Draper-III) are generated through alternative splicing of the draper gene transcript, and each contains a unique combination of extracellular and intracellular sequences (Fig. 1a)19. To determine which isoforms are present in the adult brain, we prepared head-protein lysates

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from control yw and draperΔ5 null mutant flies and performed western blotting using an antibody that recognizes all three Draper isoforms. We detected bands corresponding to at least two Draper isoforms (Fig. 1b and Supplementary Fig. 1): a larger, predominant band representing Draper-I (113 kDa) and a smaller band or bands at the predicted sizes of Draper-II and/or Draper-III (65 kDa and 59 kDa, respectively). Because it was difficult to resolve Draper-II and Draper-III, we performed RT-PCR and detected transcripts for all three isoforms in whole adult heads and dissected brains.

To determine whether loss of Draper-I was sufficient to suppress engulfment activity, we generated an RNA interference (RNAi) construct (UAS-draper-I RNAi) that targets the unique extracellular domain region in Draper-I (Fig. 1a)29. We first confirmed specificity by ubiquitously driving UAS-draper-IΔRNAi and performing anti-Draper immunoblotting (Fig. 2a). Next, we used repo-Gal4 to drive UAS-draper-IΔRNAi and assessed glial clearance of severed axons. Clearance of degenerating axons was substantially inhibited, to levels indistinguishable from those observed by knockdown of all Draper isoforms or in draper null mutants (Fig. 2b,c). We also noted that glial expression of UAS-draper-IΔRNAi resulted in the loss of the majority of Draper staining (Fig. 2b), suggesting that Draper-I is the predominant isoform in adult glia. These clearance phenotypes were not an artifact of activating the RNAi machinery, as repo-Gal4–driven RNAi targeting other genes did not affect clearance of severed axons (Supplementary Fig. 4). Because glial expression of Draper-II and Draper-III is retained in Draper-I RNAi flies,
Figure 2 Draper-I is required for glial clearance of severed axons. (a) Expression of UAS-draperRNAi, which targets the unique region in the extracellular region of Draper-I (see Fig. 1a), was driven ubiquitously with tubulin-Gal4. Draper western blotting (WB) was performed on adult head protein lysates to confirm specific knockdown of Draper-I (96.4 ± 0.3% reduction in Draper-I, P < 0.01; 2.1 ± 25% reduction in Draper-II and/or Draper-III, P = 0.76). Full-length blots are shown in Supplementary Figure 1. (b) repo-Gal4 was used to knock down Draper-I by driving UAS-draperRNAi or to knock down all Draper isoforms with UAS-draperRNAi. Projected confocal z stacks show that glial clearance of axonal debris (green) was inhibited by knockdown of all Draper isoforms and in draper mutants. Notably, glial clearance of severed axons was equally suppressed by selective knockdown of Draper-I. Draper (red) was barely detectable in the brain following expression of UAS-draperRNAi or UAS-draperRNAi in glia. The mean intensity of cortical Draper immunostaining in control, UAS-draperRNAi and UAS-draperRNAi flies was 82.5 ± 4.7, 39 ± 1.2, and 36 ± 1.3 units of fluorescence intensity, respectively. Scale bars, 20 μm. (c) Quantification of OR85e-mCD8:GFP shown in b. Error bars represent mean ± s.e.m. ***P < 0.001. Genotypes in a: control, tubulin-Gal4/+;draper-I RNAi, tubulin-Gal4/+;UAS-draperRNAi/+.

Genotypes and N values for b and c: control, w;OR85e-mCD8::GFP/+;repo-Gal4/+ (D0 N = 22; D3 N = 23); draper, w;OR85e-mCD8::GFP/+;draperRNAi/+ (D0 N = 12; D3 N = 10); Draper RNAi, w;OR85e-mCD8::GFP/UAS-draperRNAi;repo-Gal4/+ (D0 N = 20; D3 N = 24); Draper RNAi, w;OR85e-mCD8::GFP/+;repo-Gal4/UAS-draperRNAi (D0 N = 22; D3 N = 19).

we conclude that these isoforms are not sufficient to drive glial engulfment of degenerating axons. Together, our findings suggest that Draper-I is necessary and sufficient for glial engulfment of axonal debris after axotomy.

The Draper-I intracellular domain activates engulfment
Because Draper-I is structurally unique in both its extracellular and intracellular domain compared to Draper-II and Draper-III, engulfment-promoting activity could map to either region. To examine this, we generated chimeric receptors that contained every combination of extracellular (ex) and intracellular (int) domains from each Draper isoform (Fig. 3a) and tested the ability of each to promote glial engulfment of severed axons. We first confirmed that each construct was stably expressed in adult brains (Fig. 3b). Next, we examined...
Draper-II inhibits engulfment through an ITIM-like domain

Upon closer inspection of Draper-II, we identified a motif with a sequence reminiscent of an ITIM (VKYIYXXL, Fig. 3e). Mammalian receptors bearing ITIMs inhibit ITAM signaling cascades14,15,26, which raised the intriguing possibility that Draper-II negatively regulates Draper-I. To test this idea, we first overexpressed Draper-II in glia of wild-type flies and found that glial clearance of axonal debris was completely blocked (Fig. 4a,b). Likewise, glial overexpression of Draper-Iex-IIint blocked glial engulfment of GFP+ debris (Fig. 4a,b). In contrast, we overexpressed Draper-I or Draper-III in wild-type glia and discovered that engulfment activity was comparable to that of control flies (Fig. 4a,b). Thus, the inhibitory activity of Draper-II maps to the intracellular domain, and because the unique feature of this domain is the ITIM-like motif, we conclude that this motif confers inhibitory signaling activity.

Finally, because Gal4–UAS-driven Draper-II levels were much higher than endogenous Draper levels, we reasoned that high concentrations of Draper-I might overcome the inhibitory activity of Draper-II. We expressed Draper-I and Draper-II together in glia using repo-Gal4 but found that glial engulfment of axonal debris was still potently suppressed (Fig. 4a,b). Thus, providing roughly equivalent concentrations of Draper-I and Draper-II is insufficient to overcome the inhibitory activity of Draper-II, which may explain the predominance of Draper-I in wild-type brains.

Corkscrew preferentially binds Draper-II

In vertebrates, the tyrosine phosphatases SHP-1 and SHP-2 typically bind ITIMs14,27. We explored the possibility that Draper-II might associate with Csw, the Drosophila homolog of SHP-1 and SHP-2. We transfected Drosophila S2 cells with plasmids for wild-type Csw (Csw WT) and hemagglutinin (HA)-tagged versions of Draper-I or Draper-II, then immunoprecipitated with anti-Csw antibody and performed western blotting of these samples. We consistently detected robust association of Csw with Draper-II, and, notably, significantly more HA-tagged Draper-II co-immunoprecipitated with Csw compared to Draper-I (Fig. 5a, lane 4 versus lane 5, 24±1.7-fold increase, P < 0.0001), indicating that Csw preferentially associates with Draper-II.

Catalytically inactive phosphatases can bind target substrates, but, because they cannot dephosphorylate the phosphotyrosine residues required for dissociation, they fail to release, thereby ‘trapping’ the substrates28–30. As an alternative strategy to show Draper-II–Csw
Corkscrew associates preferentially with and dephosphorylates Draper-II. (a) HA-tagged Draper constructs and wild-type Corkscrew (CswWT) were transfected into Drosophila S2 cells. Anti-Csw immunoprecipitation (IP) samples were analyzed by anti-HA western blot (WB) to compare Csw association with Draper-I and Draper-II. Anti-HA WB was performed on cell lysates to confirm Draper-I HA transfection. (b) Draper-II: HA was coexpressed with CswWT or CswCS. Anti-HA WB was performed on anti-HA IP samples to compare Draper-I: HA association with CswWT versus CswCS. Blots were re-probed for Csw. Anti-HA WBs on lysates confirmed Draper-II:: HA expression. (c) Cells were transfected with the indicated constructs, and anti-HA IP was performed on cell lysates. Anti-phosphotyrosine (PY) and anti-HA WBs were performed on IP samples. Coexpression of CswWT with Draper-II substantially reduced the tyrosine phosphorylation level of bands corresponding to the sizes of Shark and Draper-II (lane 7 versus 8). CswWT did not affect phosphorylation of Draper-I: HA or Shark in the absence of Draper-II:: HA (lane 5 versus 6). (d) Anti-PY, anti-HA and anti-Myc WBs were performed on anti-HA IP samples from cells transfected with indicated constructs. When coexpressed with Shark or Draper-II, CswCS increased the phosphorylation status of each (lane 6 versus 7) but did not substantially alter Draper-I or Shark when coexpressed in S2 cells (lanes 4 versus 5). (e) Anti-Myc IP was performed on cells transfected with Draper-II:: HA, Myc:: Shark and noted versions of Csw. Anti-phosphotyrosine, anti-HA, and anti-Myc WBs on IP samples are shown. Tyrosine phosphorylation of Shark was reduced by CswWT (lane 2 versus 3) but increased by CswCS (lane 2 versus 4). See full-length blots in Supplementary Figure 1.

Draper-II is dephosphorylated by Corkscrew

To determine whether Csw alters the tyrosine phosphorylation status of Draper, we transfected S2 cells with plasmids encoding CswWT, CswCS and HA-tagged Draper-I or Draper-II and performed anti-HA immunoprecipitation followed by anti-phosphotyrosine immunoblotting. Coexpression of CswWT caused a striking reduction in the intensity of the anti-phosphotyrosine band for Draper-II:: HA (Fig 5c, lane 7 versus lane 8, 28 ± 4% decrease, P < 0.01). In contrast, Draper-II phosphorylation was significantly higher when CswCS was coexpressed (Fig 5d, lane 6 versus lane 7, 330 ± 90% increase, P < 0.01), arguing that Draper-II is a phosphatase substrate for Csw. Tyrosine phosphorylation of Draper-I was unaffected by Csw, as we found no significant changes in the phosphorylation status of Draper-I:: HA when coexpressed with CswWT (Fig 5c, lane 5 versus lane 6, 6 ± 3% decrease, P = 0.13) or CswCS (Fig 5d, lane 4 versus lane 5, 40 ± 60% increase with CswCS, P = 0.85). We note that it remains possible that Draper-I is a Csw substrate in vivo, given that expression of constructs in cultured cells does not fully mimic an engulfment event. Nevertheless, these results suggest that Draper-II is a physiological substrate for Csw, whereby Csw dephosphorylates the intracellular domain of Draper-II, perhaps to facilitate the dissociation of Csw or other signaling molecules.

We wondered whether Draper-II:: HA and Csw signaling might affect the tyrosine phosphorylation status of Shark, which is required for Draper-mediated engulfment in vivo18. We therefore expressed Draper-I:: HA or Draper-II:: HA and N-terminal Myc-tagged Shark (Myc:: Shark) with or without CswWT in S2 cells and performed anti-HA immunoprecipitation. We found that CswWT significantly reduced the intensity of the Shark anti-phosphotyrosine band in the presence of Draper-II:: HA (Fig 5e, lane 7 versus lane 8, 32 ± 2.6% decrease, P < 0.01), but not in the presence of Draper-I:: HA (Fig 5d, lane 5 versus lane 6, 7 ± 12% decrease with CswWT, P = 0.59). Anti-phosphotyrosine Shark levels were higher in the presence of CswCS compared to CswWT when these were coexpressed with Draper-II:: HA (Fig 5d, lane 6 versus lane 7, 520 ± 140%-fold greater for Shark, P < 0.05). Notably, tyrosine phosphorylation of Shark was not significantly altered by CswCS in the presence of Draper-I:: HA (Fig 5d, lane 5 versus lane 4, 9 ± 40% increase, P = 0.82). We also performed reciprocal experiments in which we coexpressed Draper-II:: HA and Myc:: Shark with CswWT or CswCS (or no Csw), immunoprecipitated Shark using anti-Myc and analyzed the tyrosine-phosphorylation status of Shark. Again, we found that phosphorylation of Myc:: Shark was consistently reduced when it was coexpressed with CswWT (Fig 5e, lane 2 versus lane 3, 10% ± 3% decrease, P < 0.05), but increased in the presence of CswCS (Fig 5e, lane 2 versus 4, 21% ± 8% increase, P < 0.05). Finally, we...
Figure 6 Draper-II inhibition of glial engulfment of severed axons is mediated through Corkscrew. (a) The pan-glial driver repo-Gal4 was used to overexpress UAS-draper-II in a wild-type background or in flies that carried one copy of csw\textsuperscript{va199}, a dominant-negative allele of corkscrew (DN csw). Reduced Csw activity in csw\textsuperscript{va199} heterozygotes partially rescued the engulfment phenotype resulting from glial overexpression of Draper-II. Images of GFP-labeled OR85e axons before and 3 d after maxillary palp ablation are shown as confocal z-stack projections. (b) Quantification of experiment in a. Error bars represent mean ± s.e.m. ***P < 0.0001. Genotypes and N values in a, b: DN csw, csw\textsuperscript{va199}/+ (D0 N = 10; D3 N = 10); Draper-II overexpression, w;OR85e-mCD8::GFP/UAS-draper-II;repo-Gal4\textsuperscript{+} (D0 N = 14; D3 N = 10); Draper-II overexpression + DN csw, csw\textsuperscript{va199}/w;OR85e-mCD8::GFP/UAS-draper-II;repo-Gal4\textsuperscript{+} (D0 N = 10; D3 N = 16). (c) repo-Gal4 was used to drive UAS-draper-II in flies that also carried UAS-csw\textsuperscript{RNAi} to knock down Csw expression. Clearance of GFP-labeled OR85e axons was examined 3 d after maxillary palp ablation. Confocal z-stack projections show GFP\textsuperscript{+} axonal material in the antennal lobes in each genotype before and after injury. Overexpression of Draper-II in wild-type flies blocks glial engulfment of degenerating axons, but this engulfment phenotype is significantly reduced when Csw expression is knocked down by RNAi (P < 0.001). (d) Quantification of the experiment shown in a. Error bars represent s.e.m. ***P < 0.001. Genotypes and N values in c, d: Draper-II overexpression, w;OR85e-mCD8::GFP/UAS-draper-II;repo-Gal4\textsuperscript{+} (D0 N = 10; D3 N = 14); Draper-II overexpression + csw RNAi, UAS-csw\textsuperscript{RNAi}/w;OR85e-mCD8::GFP/UAS-draper-II;repo-Gal4\textsuperscript{+} (D0 N = 12; D3 N = 16). Scale bars, 20 μm.

observed that Myc::Shark co-immunoprecipitated equally with Draper-I::HA and Draper-II::HA, regardless of the phosphorylation status of Myc::Shark (Fig. 5d, lanes 4–7). Together, these data support a model whereby Draper-II and Csw inhibit glial engulfment activity by reducing the activity of Draper-I signaling effectors, including Shark, through targeted dephosphorylation.

Corkscrew is required for Draper-II inhibitory activity

To determine whether Csw was required for Draper-II signaling \textit{in vivo}, we assayed the effects of \textit{csw} mutants and RNAi on Draper-II–dependent inhibition of glial engulfment. Glial overexpression of Draper-II completely blocked engulfment of severed axons, but this phenotype was significantly suppressed in heterozygous \textit{cswva199} flies (Fig. 6a,b, P < 0.0001). Glial engulfment activity was normal in \textit{cswva199}/+ flies, indicating that Csw is not a positive regulator of glial engulfment. To determine the cell autonomy of Csw function, we used \textit{repo-Gal4} to drive UAS-csw\textsuperscript{RNAi} expression in glia (Supplementary Fig. 5) and found that this significantly suppressed the inhibitory activity of Draper-II (Fig. 6c,d, P < 0.0001). Thus, Csw, like Draper-II, functions in glia to inhibit engulfment activity.

Figure 7 Corkscrew signaling is required for normal termination of glial responses to axon degeneration. (a) Real-time PCR analysis of \textit{draper-I} and \textit{draper-II} mRNA in adult central brains before and after antennal ablation. Values represent mean normalized cycle threshold (CT) ± s.e.m. from at least 3 independent RNA isolations. (b) Csw was knocked down in glia by driving UAS-csw\textsuperscript{RNAi} with repo-Gal4. ORN axons were severed by ablatting the third antennal segments. Brains were immunostained for Ced-6 at 0, 1, 5, 7 or 10 d after injury to compare glial activation after axotomy in control and Csw\textsuperscript{RNAi} flies. Representative single confocal slices shown for 0, 1 and 10 d. (c) Quantification of experiment in b. Error bars represent mean ± s.e.m. **P < 0.01. Genotypes and N values: Control, w;repo-Gal4\textsuperscript{+} (D0 N = 20; D1AA N = 20; D5AA N = 24; D7AA N = 20; D10AA N = 20); Csw\textsuperscript{RNAi}, UAS-csw\textsuperscript{RNAi}/w; repo-Gal4\textsuperscript{+} (D0 N = 20; D1AA N = 20; D5AA N = 24; D7AA N = 20; D10AA N = 20). Scale bars, 20 μm.
Draper-II–Csw terminates glial responses to axon injury

Our findings suggest that Draper-I and Draper-II each have important, but opposing, roles in glial responses to axotomy. Previous work has shown that Draper immunoreactivity is robustly upregulated in antennal lobe glia within 24 h after ORN axon injury. We performed real-time quantitative PCR (qRT-PCR) on central brains at 0, 1.5, 3, 4.5, 6, 12 and 24 h and 7 d after axon injury. We found that draper-I mRNA abundance was increased within 1.5 h after axotomy (Fig. 7a, P < 0.05). Notably, draper-II transcripts also increased (P < 0.05), although these levels did not peak until 4.5 h after ablation (Fig. 7a). csw levels also appear to be modulated by injury and were significantly decreased (P < 0.05) at 4.5 h, in coordination with Draper-II increases (Supplementary Fig. 6). Thus, draper-I and draper-II transcript levels are regulated in response to injury, with upregulation of the pro-engulfment isoform preceding that of the inhibitory Draper-II isoform.

As Draper-II–Csw negatively regulates glial engulfment activity, we suspected that Draper-II–Csw signaling might function to down-regulate glial responses to injury after axon clearance. We therefore assayed the efficiency of initiation and termination of glial responses to antennal nerve axotomy in wild-type and Csw RNAi flies. We first assayed the efficiency of initiation and termination of glial responses we suspected that Draper-II–Csw signaling might function to down-regulate responsiveness to axonal injury. Our work reveals a previously undescribed role for Draper and ITIM-like signaling in maintaining glial responsiveness to neural trauma, because efficient termination of an initial glial response seems to be crucial for glia to respond subsequent neural injury.

DISCUSSION

Here we show how a single receptor, Draper, can positively or negatively influence glial responses to axonal injury. Our work reveals a crucial role for ITAM and ITIM-like signaling events in regulating the activation, termination and maintenance of engulfment signal transduction cascades during glial responses to axonal injury. In addition, we provide direct evidence that negative regulation of glial responses

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**Figure 8** Corkscrew signaling is required for proper glial clearance of severed axons. (a) Csw was knocked down in glia by driving UAS-cswRNAi with repo-Gal4. A ‘single injury’ entailed ablating the maxillary palps to sever ORN axons and then assessing clearance of GFP+ maxillary palp ORNs 5 d later. ‘Double-injury’ flies were pre-injured by ablating the third antennal segments 5 d before ablating the maxillary palps and then analyzing clearance of degenerating GFP+ maxillary palp ORNs after 5 d. Representative confocal z-stack projections are shown. (b) Quantification of GFP intensity in OR85e-innervated maxillary palp glomeruli from experiment in a. Error bars represent mean ± s.e.m. ***P < 0.001. (c) The number of antennal lobes that contained visible tracts of GFP+ maxillary palp axonal debris (arrowheads in a) were scored in control and Csw RNAi flies at the end of the double-injury experiment. GFP+ axon tracts were faintly visible in 42% of control brains, whereas the majority of CswRNAi brains (83%) contained visible axonal tracts with persistent GFP+ debris. Genotypes and N values: Control, w; repo-Gal4/+ (single injury N = 10; double injury N = 12); CswRNAi, UAS-cswRNAi; w; repo-Gal4/+ (single injury N = 20; double injury N = 18). (d) Projected confocal z stacks of GFP-labeled OR85e ORNs in control and CswRNAi flies. Images were collected from uninjured flies and 10 d after antennal ablation to compare the morphology of intact OR85e axons. (e) Quantification of GFP levels in OR85e-innervated glomeruli in experiment depicted in d. Error bars represent mean ± s.e.m. Scale bars, 20 μm.
to neurodegeneration is essential for glia to reset their responses after an initial injury, and thereby remain competent to respond efficiently to subsequent brain trauma.

The Draper intracellular domain directs engulfment

The unique intracellular domains of Draper-I and Draper-II determine their effects on glial responses to axonal injury. Whereas Draper-I promotes engulfment of axonal debris, Draper-II completely inhibited glial clearance of degenerating axons, and the inhibitory activity mapped to a Draper-II–specific intracellular motif that contains an ITIM-like domain. We note that this insertion also produces two ITAMs in Draper-II, raising the possibility that one or both of these may function as an inhibitory ITAM (ITAMI). Recent work has shown that some ITAMs function in a dual manner, recruiting activating or inhibitory effectors in response to changes in receptor configuration 33. However, we favor a model in which Draper-II acts exclusively as an inhibitory ITIM-like receptor, as the ITIM-like Draper-II domain is not a functional activator in any context we have examined in vivo.

There are two unique Draper extracellular domains that are likely to be involved in recognition of engulfment targets (Fig. 1a). These are fully interchangeable in our engulfment assay, indicating that neither extracellular domain contains inherent inhibitory activity. It is possible that both domains recognize the same molecule, perhaps a ligand presented by degenerating axons. Alternatively, each extracellular domain may recognize a unique ligand. Identifying specific factors that associate with the extracellular region of each Draper isoform after axotomy will provide key insight into these post-injury neuron-glia communication events.

Draper-II–Corkscrew signaling reprimed glia after injury

Following axotomy, engulfment molecules (Draper and Ced-6) are robustly upregulated in responding glia 8,22 and return to baseline levels once axonal debris has been cleared (Fig. 7). Notably, we found that Csw signaling is essential to restore Draper and Ced-6 to basal levels as glia terminate responses to axotomy and that glia lacking Draper-II–Csw signaling fail to respond to secondary injuries in the brain. These data highlight previously unknown in vivo requirements for Draper-I and Draper-II–Csw signaling in coordinating the activation, termination and maintenance of glial cellular responses to axonal injury.

We propose that Draper-I, acting via Src42A and Shark, promotes the expression of engulfment genes after axotomy and phagocytosis of degenerating axons; such upregulation of engulfment genes is probably essential for rapid clearance of degenerating axons. We also propose that Draper-II and Csw then negatively regulate Draper-I signaling to terminate reactive glial responses and allow glia to return to a resting state. Our data showing differential regulation of Draper-I and Draper-II transcripts, with Draper-I preceding Draper-II by several hours, supports the idea that Draper-II–Csw signaling may sequester axonal debris; such upregulation of engulfment genes is a hallmark of nearly all neurodegenerative diseases 9,16 and there is growing evidence that glia help to promote disease pathology in mouse models 43–46. The Draper-II–Csw signaling pathway is remarkably specific in its negative regulation of glial responses to axonal injury, and it provides an exciting molecular entry point to understanding how glial cells terminate cellular and molecular responses to neuronal trauma.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.A.L. and M.R.F. developed the overall concept and design of the project. M.A.L. performed, analyzed and interpreted the majority of the experiments and wrote the initial version of the manuscript. R.H. performed the immunoprecipitation and western blot experiments with S2 cells and adult flies. J.D. performed the experiments with cswnull and provided intellectual input for the study. S.D.S. performed and analyzed the qRT-PCR time course of Draper-I, Draper-I and Csw expression after injury and assisted with double injury experiments. A.S. generated the extracellular-intracellular Draper domain swap constructs.
ARTICLES

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Fly lines and molecular biology. The following Drosophila strains were used: yw, draper [25] (ref. 8), repo-Gal4 (ref. 47), repo-Gal4, draper [25] (ref. 21), OR52e- mCD8::GFP (gift from B. Dickson, Research Institute of Molecular Pathology, Vienna, Austria), UAS-draper [48] (ref. 8), UAS-draper [50] (ref. 20), UAS-cswRNAi (VDRC transformant 21756) (ref. 48), csw [50] (ref. 32), UAS-neurog2 [51] (VDRC transformant 44236) (ref. 48), UAS- LDLR [51] (VDRC transformant 27242) (ref. 48). We confirmed efficacy of UAS-draper [RNAi] by western blot analysis (Supplementary Fig. 8).

To generate pUAST-Draper-I, pUAST-Draper-II and pUAST-Draper-III fly lines, we obtained BDGP full-length cDNAs (GH42147, GH03529 and RH13935, respectively) and cloned them into the XhoI site of pUAST. To generate the Draper extracellular- and intracellular-domain swap constructs pUAST-Draper-I-II, pUAST-Draper-I-III, and pUAST-Draper-II-III-I, each pUAST-Draper construct was cut with KpnI and SphI, which excised a portion containing the 5′-UTR, the extracellular domain and a portion of the intracellular domain common to all three isoforms just following the NXPY motif. Each Draper fragment was cloned into pUAST-Draper vectors digested with KpnI and SphI to generate domain swap constructs. We sequence-verified all constructs, and transgenic flies were generated by BestGene Inc.

Full-length cDNA sequences encoding Draper-I, Draper-II and Draper-III were excised from pUAST with NotI and XbaI and cloned into pAc/5′-HisA (Invitrogen) to generate pAc-Draper-I, pAc-Draper-II, and pAc-Draper-III.

RT-PCR. We isolated total RNA from Drosophila embryos (stage 13–16), L3 larval brains, L3 larval body wall, whole adult heads and adult dissected brains using Trizol reagent and generated first-strand cDNA (Superscript). We performed PCR with Draper isoform specific primers as previously described [21].

Real-time PCR. We manually dissected central brains in Jans saline (1.8 mM Ca[2+] and immediately froze them on dry ice. We extracted total RNA in Trizol and the aqueous phase was passed over an Omega Bio-Tek E.Z.N.A MicroElute RNA Clean-Up Column. RNA concentration was determined on a Nanodrop 2000c spectrometer (Thermo Scientific). RNA was diluted to equal concentration and treated with DNase (Ambion DNA-free kit), and 125 ng of total RNA was reverse-transcribed with the SuperScript VILO cDNA synthesis Kit for 2 h at 42 °C.

Relative quantification of gene expression was carried out on an ABI 7500 Fast Real-Time PCR machine. The following Taqman assays (Applied Biosystems) were used: (i) Ribosomal protein L32 (ABI pre-made assay Dmo21518271_1_g) (ii) Draper-I custom assay, F-primer, TGTGATCATGGTTACGGAGGAC; R-primer, CAGGCCGGTTGGGCAAA; probe, CGCCCTGGGATAA (iii) Draper-II custom assay, F-primer, CAAGCCAAGGGGGCTACAA; R-primer, CATCCTCAGGAAAACAAAATCTT; probe, TTCCTCGTAAATATATAGC and (iv) Corkscrew (ABI pre-made assay Dmo18211096_g_1). Assay efficiencies were experimentally determined (Rpl32, 102%; Draper-I, 103%; Draper-II, 102% and Corkscrew, 98%) using a 5-point dilution series of cDNA spanning a 20-fold range in concentration. The raw threshold cycle (Ct) of the normalizing control (Rpl32) did not vary by more than 0.5 cycles across all time points analyzed. Statistical analysis was performed on 2-ΔΔCt values. In Figure 7a, Draper Ct values were normalized to ribosomal protein L32 and results are presented as fold induction relative to uninjured levels.

Olfactory receptor injury protocol, immunohistochemistry and confocal microscopy. We performed maxillary palp and third antennal segment ablation experiments, adult brain dissections, and antibody staining using previously described methods [4]. Confocal imaging and quantification of GFP intensity within maxillary palp glomeruli using Image J were performed as previously described [2,2]. We quantified cortical Draper staining for Figure 2b with ImageJ; single confocal slices at the depth of OR85e-innervated glomeruli were identified, a fixed-size rectangular region adjacent to each antennal lobe was selected and total intensity measurements were calculated. The following antibodies were used: 1:200 mouse anti-GFP (Invitrogen), 1:500 rabbit anti-Draper [29], 1:500 rat anti-Ced [6] (ref. 21), 1:200 FITC anti-mouse IgG, 1:200 Cy3 anti-rabbit IgG, 1:200 Cy5 anti-rabbit IgG, 1:200 Cy5 anti-rat IgG (Jackson ImmunoResearch).

Equipment and Settings. All immunostained brains were imaged on a Zeiss LSM 5 Pascal with a 63X, 1.4NA apochromatic lens. Brains within a single experiment (being compared for quantification) were mounted in Vectashield and imaged on the same day with the same confocal settings (laser power, PMT gain, offset, filter configuration). Analysis and quantification of confocal and western blot images was performed in ImageJ. See relevant Methods sections for more details on quantification procedures. No post acquisition filtering was performed on displayed images.

Immunoprecipitation and immunoblotting. Insect Schneider S2 cells were seeded into 6-well plates (2 × 10^6 cells per well) and transfected (Effectene; QIAGEN) 24 h later with the appropriate expression vectors: pAc-Draper-I, pAc-Draper-II, pAc-Draper-III, pUAS-Myc :: shark [49], pAc-Gal4, pAT-Hygro-csw [52], and pAT-Hygro-csw [53] (ref. 30). Three wells were used for each experimental condition. Forty-eight hours after transfection, we lysed cells in buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM Na_2P_2O_5, 50 mM NaF, 100 mM NaVO_4, 5 μM ZnCl_2, and protease inhibitor cocktail (Complete Tablets; Roche). For immunoprecipitation experiments, we pre-cleared cell lysates with Protein G beads (Sigma) and then incubated with antibody-conjugated beads at 4 °C overnight. We washed beads the following day 4 times with lysis buffer and then resuspended in 45 μl of lysis buffer plus 10 μl SDS loading buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 1% b-mercaptoethanol, 0.01% bromophenol blue) and then eluted protein complexes from beads by boiling for 5 min. For western analysis, 15 μl of each sample was loaded onto 10% SDS-PAGE gels (BioRad), transferred to nitrocellulose membranes (BioRad), and probed with the appropriate antibodies: 1:1,000 rabbit anti-Draper, 1:500 rat anti-PA-HA (Roche), 1:1,000 mouse anti-phosphotyrosine (Millipore), 1:500 rabbit anti-Draper [30], 1:1,000 mouse anti-Myc (Gowance), 1:10,000 mouse anti-tubulin (Sigma). HRP-conjugated secondary antibodies (Abcam) were used at 1:6,000. Antibodies were diluted in PBS + 0.01% Tween-20 + 5% BSA, with the exception of anti-phosphotyrosine, which we diluted in PBS + 0.01% Tween-20 + 5% dry milk. We incubated blots overnight rocking at 4 °C, washed 6 times for 20 min, probed with appropriate HRP-conjugated secondary antibody for 2 h at room temperature, washed 6 times for 20 min, developed using chemiluminescence (Amersham ECL Plus) and detected with a Fujifilm Luminescent Imager. Protein blots were stripped by rocking in mild stripping buffer (0.2M glycine, 0.1% SDS, 1% Tween, pH 2.2) at room temperature for 10 min and washing in 1× PBS followed by 1× PBS + 0.01% Tween-20. All biochemical experiments were performed independently 3–6 times. ImageJ was used to quantify protein and anti-phosphotyrosine band intensities. For Draper–Csw association experiments, the mean intensity of each Draper band was normalized to the mean intensity of the Csw band in each immunoprecipitation sample. The mean intensity of anti-phosphotyrosine bands for Draper and Shark were normalized to the mean intensity of anti-Draper or anti-Myc bands, respectively. Draper Westerns were performed on whole head lysates as previously described [29]. For Figure 2a, ImageJ was used to normalize the intensity of Draper-I and Draper-II/III bands to anti-tubulin band intensities for three independent experiments.

Statistical analysis. GraphPad Prism was used to perform ANOVA and Dunnett’s post hoc test and two-tailed Student’s t-tests.

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