DRK/DOS/SOS Converge with Crk/Mbc/dCed-12 to Activate Rac1 during Glial Engulfment of Axonal Debris

Tsai-yi Lu  
*University of Massachusetts Medical School*

Johnna E. Doherty  
*University of Massachusetts Medical School*

Marc R. Freeman  
*University of Massachusetts Medical School*

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Nervous system injury or disease leads to activation of glia, which govern postinjury responses in the nervous system. Axonal injury in Drosophila results in transcriptional up-regulation of the glial engulfment receptor Draper; there is extension of glial membranes to the injury site (termed activation), and then axonal debris is internalized and degraded. Loss of the small GTPase Rac1 from glia completely suppresses glial responses to injury, but upstream activators remain poorly defined. Loss of the Rac guanine nucleotide exchange factor (GEF) Crk/myoblast city (Mbc)/dCed-12 has no effect on glial activation, but blocks internalization and degradation of debris. Here we show that the signaling molecules downstream of receptor kinase (DRK) and daughter of sevenless (DOS) (mammalian homologs, Grb2 and Gab2, respectively) and the GEF son of sevenless (SOS) (mammalian homolog, mSOS) are required for efficient activation of glia after axotomy and internalization/ degradation of axonal debris. At the earliest steps of glial activation, DRK/DOS/SOS function in a partially redundant manner with Crk/Mbc/dCed-12, with blockade of both complexes strongly suppressing all glial responses, similar to loss of Rac1. This work identifies DRK/DOS/SOS as the upstream Rac GEF complex required for glial responses to axonal injury, and demonstrates a critical requirement for multiple GEFs in efficient glial activation after injury and internalization/degradation of axonal debris.

Reactive glia engulfing signaling | Draper pathway | Wallerian degeneration

Activation of glia is a hallmark of nearly all neurodegenerative diseases and neural injuries. When brain insults have occurred, glia rapidly change their morphology and gene expression profiles to invade the injury site and clear pathogens and/or neuronal debris by phagocytic engulfment (1, 2). Failure to clear debris from the CNS can result in prolonged neuroinflammation and hamper the recovery of the CNS (3, 4). However, the genetic pathways promoting glial activation after neural injuries remain poorly defined.

Genetic studies of Wallerian degeneration in Drosophila have provided important insights into glial responses to axotomy (5). Olfactory neuron axotomy results in the degeneration of axons projecting into the antennal lobe of the fly brain, where local glia sense degenerating axons, and initiate a multistep process of reactivity. Reactive glia up-regulate the transcription of the engulfment receptor Draper (drpr) and extend membranes to degenerating axons (5, 6). When at the injury site, glia internalize axonal debris and degrade it through the phagolysosomal pathway (7). Finally, glia terminate their responses by withdrawing from the injury site and down-regulating Draper, and finally return to a resting state (6).

Draper is essential for all glial responses to axonal injury. In drpr-null mutants, glia fail to respond morphologically to axonal injury, and axonal debris lingers in the brain for weeks after axotomy (5). Downstream of Draper, the small GTPase Rac1 appears to be critical in executing glial activation to axon injury, as loss of Rac1 phenocopies drpr-null mutants (7). The only Rac guanine nucleotide exchange factor (GEF) known to be required for glial engulfment of axonal debris is the noncanonical GEF Crk/myoblast city (Mbc)/dCed-12. However, in contrast to loss of Rac1, animals lacking Crk/Mbc/dCed-12 signaling exhibit relatively normal activation of glia after axotomy, with glia increasing Draper expression and extending membranes to degenerating axons, but glia then fail to internalize and degrade axonal debris (7). These data argue for a specific role for the Crk/Mbc/dCed-12 complex at the internalization/degradation phase of the glial response, and suggest that an additional Rac1 GEF must act earlier during initial activation of glial responses to axonal injury.

In an RNAi-based screen for new engulfment genes, we identified downstream of receptor kinase (drk) as a gene required for efficient glial clearance of degenerating axons. drk is best known for its role in signaling downstream of the Sevenless (Sev) receptor tyrosine kinase (RTK), where it functions with daughter of sevenless (dos) and son of sevenless (sos) to activate the small GTPase Ras (8–13). More recent studies have also linked SOS to the activation of Rac1 in the regulation of axon guidance during Drosophila embryonic CNS development (14), indicating that DRK/DOS/SOS can act upstream of multiple small GTPases. Here we show that the DRK/DOS/SOS complex plays a critical role in activation of glial responses to injury and internalization of axonal debris. Moreover, we provide genetic evidence that, at the earliest stage of glial activation, DRK/DOS/SOS function redundantly with Crk/Mbc/dCed-12 to promote Rac1 activation and initiate all steps in glial responses to axonal injury.

Results

DRK, DOS, and SOS Are Required for Glial Engulfment of Axonal Debris. To identify new pathways required for glial engulfment of degenerating axons, we performed an RNAi-based screen for genes required in glia for clearing axonal debris after axotomy. We expressed each of ~500 UAS-RNAi constructs from the Vienna Drosophila Resource center (15) by using the pan-glial driver repoGal4 (16). For animals that did not survive to adulthood, we

Neuronal cell death or injury leads to the production of neuronal cell corpses and cellular debris that must be cleared from the nervous system to avoid inflammation or toxicity. Glia are the primary cell type responsible for clearing neuronal debris, but precisely how these cells recognize, phagocytose, and destroy this material remains poorly defined. Here we use a simple nerve injury assay to identify genes downstream of the engulfment receptor Draper that are required for efficient glial engulfment of degenerating axons. Based on the molecular and functional conservation of this pathway in mammals, this study sheds new light on pathways potentially used by mammalian glia to react to brain injury or neurodegeneration.

Significance

Author contributions: T.-Y.L., J.D., and M.R.F. designed research; T.-Y.L. and J.D. performed research; T.-Y.L. and J.D. contributed new reagents/analytic tools; T.-Y.L. analyzed data; and T.-Y.L. and M.R.F. wrote the paper.

The authors declare no conflict of interest.

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1To whom correspondence should be addressed. Email: marc.freeman@umassmed.edu.

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further incorporated a temperature-sensitive version of Gal80 (Gal80ts) (17) in the background to temporarily control the induction of RNAi exclusively in the adult glia (SI Materials and Methods). We ablated maxillary palps (mps) in which a subset of olfactory receptor neurons (ORNs) were labeled with membrane-tethered GFP (OR85e-mCD8::GFP) (18) and scored axonal debris clearance 5 d after axotomy by quantifying GFP immunoreactivity of OR85e+ glomerulus in the antennal lobe, as previously reported (5).

In our primary screen we found that an RNAi construct (drkRNAi#105498) targeting DRK suppressed glial clearance of axonal debris. In control animals, the vast majority of axonal debris was cleared 5 d after axotomy (Fig. 1 A). However, a significant amount of the axonal debris still lingered in the brain of drkRNAi animals at day 5, and ultimately cleared in 20 d after axotomy (Fig. S1 A; quantified in Fig. S1B), which argues for a glial role for DRK in engulfment of axonal debris. DRK is known to physically interact with the adaptor protein DOS (19) and the GEF SOS (20), which can activate downstream small GTPase such as Ras and Rac1 (8, 9, 14, 21). We therefore speculated that increasing SOS activity could potentially stimulate of downstream small GTPase activity through SOS. We designed a UAS-RNAi construct (dosRNAi#3) to knock down DOS in glia and assayed for engulfment defect. Adult glia expressing dosRNAi#3 also exhibited a significant delay in engulfment of axonal debris (Fig. 1 A), arguing that DOS also plays an important role in glial engulfment of degenerating axons. To further validate this result, we repeated this experiment with an additional RNAi line (dosRNAi#12549), which targets different region of dos mRNA from dosRNAi#3, and found similar engulfment defects 5 d after injury (Fig. S2), arguing that the phenotype was not caused by the off-target effects of RNAi. We next knocked down glial SOS by using RNAi (sosRNAi#42849) and found that axonal debris remained uncleared in the CNS for as long as 20 d (Fig. 1 A and Fig. S1 A). We did not observe significant change in the expression of Draper when DRK, DOS, and SOS were knocked down respectively (Fig. S1 E and F), implying that the delayed clearance is not caused by a lower level of Draper expression. Together, these data suggest that, during glial engulfment, similar to Sev RTK signaling, DRK, DOS, and SOS interact with each other to regulate downstream small GTPase activity.

During Sev signaling, DRK and DOS couple RTK activation to stimulation of downstream small GTPase activity through SOS. We therefore speculated that increasing SOS activity could potentially compensate for the depletion of DRK and DOS. To test this hypothesis, we explored the effect of a gain-of-function (GOF) SOS allele (SosIC2−) (8, 22) on the ability of glia to clear axonal debris when DRK or DOS was knocked down. SosIC2−/− animals did not exhibit any discernable clearance defect (Fig. 1 A), nor did they clear debris faster than controls (Fig. S3A; quantified in Fig. S3B). However, we found the delay in clearance of axonal debris caused by drkRNAi and dosRNAi was completely rescued by SosIC2− (Fig. 1 A; quantified in Fig. 1B). These data indicates that the DRK/DOS/SOS complex is required for efficient engulfment of axonal debris by glial cells, and that activation of SOS is sufficient to drive glia to engulf axonal debris when DRK or DOS are depleted, consistent with the notion that SOS acts downstream of DRK and DOS.

**Glial DRK Is Recruited to Degenerating Axons After Injury.** After axon injury, Draper is up-regulated in glial cell and recruited to sites where glia actively engulf axonal debris (5, 6). We sought to determine whether DRK was also expressed in glia, as our RNAi data would suggest, and whether it was recruited to injury sites during glial engulfment. We used α-DRK polyclonal antibodies (11) to detect DRK expression in the adult brain. We first examined DRK localization along the maxillary nerve in the sub-esophageal ganglion (SOG), through which GFP-labeled mp ORN axons are projected to the antennal lobe. In control brains, we observed widespread DRK expression and, interestingly, 1 d after mp ablation, DRK was enriched along the maxillary nerve,
Draper expression and hypertrophy of glial membranes (5). We labeled glial membranes with mCD8::GFP by using the repo-Gal4 driver, and assayed glial morphology and DRK expression in control animals and animals where the third antennal segments had been ablated 1 d earlier. Consistent with our findings in the SOG, DRK immunoreactivity was dramatically increased around the antennal lobe 1 d after antennal ablation in control flies (Fig. 2B). The increase in DRK is likely a result of recruitment of DRK to glial membranes at sites of axon injury rather than up-regulation of drk gene transcription and/or translation, as DRK protein levels did not increase significantly after ablation of olfactory organs (Fig. S5). When we drove drkRNAi in glia, there was a significant decrease of DRK immunoreactivity in the hypertrophic ensheathing glia (Fig. 2B, arrows) but not in neurons, confirming that the increase of DRK immunoreactivity comes from glia. Together, these results are consistent with the model that DRK acts downstream of Draper to promote engulfment of axonal debris.

**Fig. 2.** DRK is recruited to glial membranes surrounding the injured axons. (A) Endogenous DRK (red, α-DRK) in glia was recruited to the severed mp nerves labeled with OR85e-mCD8::GFP (green, α-GFP) 1 d after axotomy. Before injury, DRK expression was evenly distributed in the SOG but not colocalized with the OR85e+ maxillary nerve. One day after mp injury, DRK expression was increased around the degenerating maxillary nerves (arrowheads), which was not seen in drkRNAi animals. Representative images (single slice) are shown. (Scale bar: 10 μm.) (b) Endogenous DRK expression in glia was increased 1 d after axotomy. Gial membranes were labeled with mCD8::GFP by glia-specific repo-Gal4 driver. Antennal ablation (removal of the third segment of antennae) was performed to induce a greater extent of axon degeneration in the antennal lobe (AL). Normally, thin glial membranes ensheath (arrows) the antennal lobe and each glomerulus (no injury, control). However, 1 d after antennal ablation, ensheathing glia became hypertrophy (1 d after injury, dashed lines), and strong DRK immunoreactivity (red) was found in hypertrophic region of ensheathing glia (yellow), but not when drkRNAi was expressed by repo-Gal4, indicating that the increase of DRK is glia-specific. Representative images (single slice) are shown. C, cortex. (Scale bar: 10 μm.)

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SosJC2 mediated a slight increase in the efficiency of phagolysosome maturation. However, we found that SosJC2 suggested that activation of SOS promotes phagolysosome formation. OR85e-mCD8::GFP-labeled axonal debris was reduced when Drk expression was knocked down by repo-Gal4 (drkRNAi). SosJC2 alone did not increase the number of vesicles formed 1 d after injury, but rescued the defect of glial membrane vesicle formation in rac1RNAi. Representative images (single-slice) are shown. (Scale bar: 10 μm.) (B) Quantification of the number of glial membrane vesicles in A. Because there were hardly any detectable glial membrane vesicles without axon injury, we quantified only the number of vesicles in animals at day 1 after axon injury (n ≥ 3 for all). Internalization of OR85e-mCD8::GFP-labeled axonal debris was reduced when Drk was knocked down in ensheathing glia but restored by SosJC2. Ensheathing glial membranes were labeled with mCD8::tdTomato (red) driven by TifR-Gal4. OR85e+ axons were labeled with mCD8::GFP (green). Degenerating OR85e+ axons were often found inside glial membrane vesicles (arrows). Representative images (single-slice) are shown. (Scale bar: 10 μm.) (D) Quantification of the number of glial vesicles containing GFP-labeled axonal debris 1 d after axotomy in C (n ≥ 3 for all).

LysoTracker+ puncta (Fig. S6A). One day after injury, LysoTracker+ puncta appeared robustly along the degenerating OR85e+ axons. Knocking down Drk in ensheathing glia resulted in ~75% of reduction in the number of LysoTracker+ puncta (Fig. S6B), indicating that the loss of Drk function severely impedes glial activation of phagolysosomal pathway. We detected a slight increase in the efficiency of phagolysosome maturation with SosJC2, as revealed by a ~1.5-fold increase in the number of LysoTracker+ puncta compared with the controls, suggesting that activation of SOS promotes phagolysosome formation. However, we found that SosJC2 was not sufficient to rescue the phagolysosome maturation defect caused by drkRNAi. This lack of rescue by SosJC2 could indicate that the SosJC2 allele is not sufficiently strong to overcome the absence of DRK activity during phagolysosome formation, or that another molecule might act in a redundant fashion with DRK during this specific signaling step.

In summary, these data argue that DRK-SOS signaling plays a critical role during glial internalization of axonal debris and activation of the phagolysosomal program for degradation of axonal materials.
Discussion

In this work, we identify Drosophila DRK, DOS, and SOS as new molecules required for glial responses to axonal injury. We show that glial depletion of DRK, DOS, or SOS results in a delay in glial responses to ORN axotomy and reduced efficiency of glial internalization and digestion of axonal debris. We observe no obvious alterations in glial morphology or expression of engulfment machinery (e.g., Draper), and demonstrate that adult-specific knockdown of DRK/DOS/SOS leads to defects in glial clearance of degenerating axons. These data indicate that DRK/DOS/SOS promote engulfment signaling in mature glia, and argues against a developmental defect causing the phenotypes we observe. Based on our observation that a dominant GOF allele of SOS (Sos^{C2}) can partially suppress depletion of DRK and DOS, we propose that SOS acts genetically downstream of DRK and DOS.

Our previous work demonstrated a key role for the Drosophila GEF Crk/Mbc/dCed-12 in glial engulfment activity, with elimination of this signaling complex from glia resulting in normal a complementary GEF acting upstream of Rac1 might also play a role in glial response to axon injury. Based on the similarity of the phenotypes associated with inhibition of Crk/Mbc/dCed-12 and DRK/DOS/SOS (i.e., reduced vesicle formation, failure to activate the phagolysosomal pathway, and an axonal debris clearance defect), we speculated that Crk/Mbc/dCed-12 and DRK/DOS/SOS activation might act redundantly downstream of Draper to activate Rac1 not only at the early phases of the glial response (when glial membranes are recruited to severed axons), but also at later phases (during internalization and degradation of axonal debris). This predicts that simultaneous depletion of Crk/Mbc/dCed-12 and DRK/DOS/SOS should result in a complete suppression of glial activation. To test this model, we simultaneously knocked down SOS and MBC, both of which possess the enzymatic GEF activity for Rac1, specifically in adult glia, and examined the recruitment of glial membranes to the severed OR85e+ axons 1 d after injury (Fig. 5A). Interestingly, we found that sos^{RNAi} or mbc^{RNAi} resulted in a slightly reduced recruitment of Draper-decorated glial membranes to severed axons. However, when sos^{RNAi} and mbc^{RNAi} were simultaneously expressed, the recruitment of glial membrane to injured OR85e+ glomerulus was completely blocked 1 d after injury. The additive nature of the sos^{RNAi} and mbc^{RNAi} phenotypes also extended to clearance of axonal debris as well as glial hypertrophy response (Fig. S8). Thus, simultaneous blockade of DRK/DOS/SOS and Crk/Mbc/dCed-12 signaling phenocopied drpr-null mutants (5), and inhibition of Rac1 signaling (7).

We further explored if SOS activation could partially substitute for Crk/Mbc/dCed-12 during glial clearance of axonal debris by crossing Sos^{C2} into dCed-12^{RNAi} background. As previously shown (7), glial knockdown of dCed-12 function strongly suppressed glial clearance of axonal debris 5 d after injury (Fig. 5C). However, in a Sos^{C2/+} background, the effect of dCed-12^{RNAi} was attenuated (Fig. 5D), indicating that activation of SOS can partially compensate for the reduced activity from Crk/Mbc/dCed-12 to promote glial clearance of axonal debris. We conclude that Crk/Mbc/dCed-12 and DRK/DOS/SOS act in a partially redundant fashion downstream of Draper to activate Rac1 and thereby glia, and promote glial clearance of axonal debris.
glial activation (e.g., recruitment of glial membranes to axonal debris), but a failure to engulf axonal debris (7). Here we provide strong evidence that DRK/DOS/SOS and Crk/Mbc/dCed-12 act redundantly downstream of Draper at two key steps in the engulfment process (Fig. S9). First, based on the fact that simultaneous depletion of both signaling complexes phenocopies Rac loss of function, we propose that these complexes act redundantly to activate Rac1 and glial responses, including Draper up-regulation and extension of glial membranes to degrading axons. Second, after glia have arrived at axonal debris, both complexes are required for the elimination of axonal debris. At this step, DRK/ DOS/SOS and Crk/Mbc/dCed-12 appear to act in a nonredundant fashion to promote glial internalization of axonal debris and activation of the phagolysosomal program for degradation of internalized axonal material.

DRK/DOS/SOS signaling has been studied most intensively for its role downstream of the RTK Sev, where it acts to activate small GTPase Ras (8–13). However, consistent with our findings, in vitro and in vivo studies have also demonstrated a role for SOS in activating Rac1. In cell culture studies, SOS stimulates guanine nucleotide dissociation from Rac1 but not Cdc42 (21). In some cases, such as axon guidance in the Drosophila embryophony, SOS action as a Rac1 GEF is independent of Ras activation (14), but, in other situations, SOS activation of Ras is coupled to stimulation of Ras (21). Based on our observation that glial expression of a DN Ras only very weakly suppresses clearance of degenerating axons, SOS activation of Rac1 during glial responses to axonal injury is likely largely independent of Ras activation.

The increase of DRK localization to glial membrane processes engulfs axonal debris, and the consequences of DRK depletion requires Draper. It seems Draper activation or Draper-dependent engulfment activity by Draper is essential for the elimination of axonal debris. At this step, DRK/DOS/SOS and Crk/Mbc/dCed-12 appear to act in a nonredundant fashion to promote glial internalization of axonal debris and activation of the phagolysosomal program for degradation of internalized axonal material.

Materials and Methods

Olfactory axon injury, adult brain dissection, sample preparation, and image analysis were previously described in ref. 5. Lysotracker staining in Drosophila adult brain was previously described in ref. 7. Fly strains and antibodies used in this study are provided in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Fly Strains and Antibodies. The following Drosophila strains were used: OR85e-mCD8::GFP/CyO (1) (gift from B. Dickson, Research Institute of Molecular Biology, Vienna, Austria), UAS-mCD8::GFP (2), UAS-mCD8::tdTomato (3), repo-Gal4/TM3 (4), tub-Gal80ts (5) (gift from S. Waddell, University of Oxford, Oxford, United Kingdom), TIFR-Gal4/TM3 (gift from H. Hing, University of Illinois, Urbana, IL), SosRNAi/CyO (gift from G. M. Rubin, Janelia Farm, Ashburn, VA), UAS-Rac1RNAi (5) (gift from L. Luo, Stanford University, Stanford, CA), and UAS-Ras85D RNAi (purchased from Bloomington Drosophila Stock Center, Bloomington, IN). The following UAS-RNAi lines were from Vienna Drosophila Resource Center (Vienna, Austria): UAS-dosRNAi#10498, UAS-sosRNAi#23849, UAS-mbRNAi#10643, and UAS-cDcd-12RNAi#10855. UAS-dos RNAi was generated by cloning daughter of sevenless (dos) cDNA fragment (nucleotide 1,629–2,112; SD2517; Drosophila Genomics Resource Center cDNA Stock Center) into pWIZ vector. Injection of pWIZ-dos RNAi into fly embryos was performed by BestGene (Chino Hills, CA) to make transgenic flies. The following fly stocks were obtained from NIG-FLY Stock Center: UAS-drkts#535D2 and UAS-dosRNAi#580. To study genetic interactions, the following strains were generated following standard procedure: tub-Gal80ts, OR85e-mCD8::GFP/CyO; repo-Gal4/TM3, tub-Gal80ts, repo-Gal4, UAS-mCD8::GFP/TM3, OR85e-mCD8::GFP, UAS-mCD4;tdTomato/CyO, and UAS-drkts#535D2, SosRNAi#105498, SojRNAi#105498.

Rabbit anti-downstream of receptor kinase (DRK) polyclonal antibodies (1:500) was a gift from M. A. Simon (Stanford University, Stanford, CA). Rabbit anti-Draper antisera (1:500) was raised as previously described (6). Mouse anti-GFP monoclonal antibody (1:200) was purchased from Molecular Probes (Karlsruhe, Germany). Anti-mouse IgG and FITC-conjugated anti-rabbit IgG were purchased from Jackson ImmunoResearch and used at 1:100.

Injury Protocols and Adult Fly Brain Dissection. Standard maxillary palp (mp) and antennal ablations were performed as previously described (7). For experiments that required tub-Gal80ts, flies were raised at 18°C before eclosion and then transferred to 29°C at least 5 d before injury. After injury, flies were grown at 29°C until the day of dissection. Standard methods were used for dissection, fixation, and antibody staining of Drosophila adult brain (7). Fly brains were eventually mounted in Vectashield Mounting Medium (H-1000; Vector Labs) and stored at 4°C in the dark before confocal microscopy analysis within 2 wk.

Lysotracker Staining in Drosophila Adult Brains. Flies were aged and injured as described in Injury Protocols and Adult Fly Brain Dissection. Heads, after having been removed from the bodies, were immediately immersed and dissected in chilled PBS solution. Dissected brains were stained by Lysotracker Red DND-99 (L-7528; Molecular Probes) PBS solution at a dilution of 1:500 at room temperature for 15 min with constant rocking, followed by five quick washes in PBS solution within 15 min, and then fixed for another 30 min at room temperature with 4% formaldehyde/PBS solution/0.1% Triton X-100. To visualize OR85eGFP axons, fixed brains were further stained with mouse anti-GFP antibody at 1:200. Mounted brains were kept in the dark for 1 h before confocal analysis and imaged on the same day to minimize the decay of Lysotracker signals.

Confocal Microscopy and Image Analysis. Confocal microscopy settings were always kept constant throughout the same set of experiments. For axon debris clearance, brains were imaged in 0.85-μm steps with a Zeiss LSM 5 Pascal confocal microscope under 63× oil objective lens. Pixel intensity of GFP or Draper immunoreactivity at each OR85eGFP glomerulus was measured by using ImageJ (National Institutes of Health) as previously described (7). Glia membrane vesicles and LysoTracker puncta were detected by spinning-disc confocal microscopy (Carl Zeiss) under 63× oil objective lens and analyzed in Velocity (PerkinElmer). Statistics were all carried out in GraphPad Prism 6 (GraphPad Software).


Fig. S1. The depletion of DRK, DOS, and son of sevenless (SOS) in glia results in delay of axonal debris clearance after axotomy. (A) Representative images (z-stack) of OR85e+ axons from control, drk RNAi, dos RNAi, and sos RNAi animals before and after mp ablation (at days 5, 10, and 20). (Scale bar: 30 μm.) (B–D) Quantification of axonal debris remaining in the antennal lobe after axotomy in drk RNAi (B), dos RNAi (C), and sos RNAi (D) animals (n = 10 for all except sos RNAi at day 20, n = 4 because of poor survival of animals). Student t test. Control: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+; drk RNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+; dos RNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+; sos RNAi: OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4+. (E) Draper protein expression does not differ between control, drk RNAi, dos RNAi, and sos RNAi animals. Anti-Draper antibody was used to detect the protein level of Draper in Western blot with approximately five dissected brains per lane. RNAi was induced for at least 5 d at 29 °C before dissection. α-Tubulin was used as the internal control. Representative images are shown. The intensity of anti-Draper signals were quantified and normalized and are shown in F (n = 3 for all). Control: tub-Gal80ts/+; repo-Gal4/+; drk RNAi: tub-Gal80ts/+; repo-Gal4/+; dos RNAi: tub-Gal80ts/+; repo-Gal4/+; sos RNAi: tub-Gal80ts/+; repo-Gal4/+.
Fig. S2. Alternative RNAi of drk and dos caused similar engulfment defects in adult glia. (A) The clearance assay was performed as described in Fig. 1. (Scale bar: 30 \( \mu \)m.) (B) Quantification of data in A. GFP immunoreactivity of injured OR85e\(^{+}\) glomerulus in RNAi animals was normalized to uninjured, age-matched controls (as 100%) as a result of insufficient number of RNAi animals collected during experiment (\( n = 10 \) for all). Control: OR85e::GFP, tub-Gal80\( ^{ts} \)/+; repo-Gal4+/+. drk\(^{6033R-2}\): OR85e::GFP, tub-Gal80\( ^{ts} \)/UAS-drk\(^{6033R-2}\); repo-Gal4+/+. dos\(^{1044R-3}\): OR85e::GFP, tub-Gal80\( ^{ts} \)/+; repo-Gal4//UAS-dos\(^{1044R-3}\).

Fig. S3. Gain-of-function allele of sos (Sos\(^{JC2}\)) alone does not change the efficiency of glial clearance of axonal debris. (A) Representative images of OR85e\(^{+}\) axons in control and Sos\(^{JC2}\)/+ animals before and 1 d after mp ablation. (Scale bar: 30 \( \mu \)m.) (B) Quantification of A (\( n = 10 \) for all). Student t test. Control: OR85e::GFP, tub-Gal80\( ^{ts} \)/+; repo-Gal4+/+. Sos\(^{JC2}\)/+: OR85e::GFP, tub-Gal80\( ^{ts} \)/Sos\(^{JC2}\); repo-Gal4+/+.

Fig. S4. Draper is required for DRK to be recruited to the severed axons. Maxillary nerves were labeled with mCD8::GFP (green) and DRK immunoreactivity before and after injury was determined by anti-DRK polyclonal antibodies (red). Compared with the increase of DRK around the severed maxillary nerves (arrows) 1 d after injury in Draper (drpr) heterozygous null animal (drpr\(^{\Delta 5}/+\)), no detectable increase of DRK was found around the severed maxillary nerves in drpr-null animals. Representative images are shown (single slice). drpr\(^{\Delta 5}/+\): OR85e-mCD8::GFP/Cyo, drpr\(^{\Delta 5} \)/TM6; drpr\(^{\Delta 5}\): OR85e-mCD8::GFP/Cyo; drpr\(^{\Delta 5}\). (Scale bar: 10 \( \mu \)m.)

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Fig. S5. DRK protein level in the brain is unaltered 1 d after axonal injury. (A) Western blots of adult brain lysates (yw) without injury (no injury) or 1 d after removal of the third segments of the antenna and mps (1 d after injury). No significant change of DRK level was observed. α-Tubulin was used as the internal control. Approximately five brains were used per lane. (B) Quantification of A (n = 3; Student t test).
Fig. S6. DRK-SOS is required for glia to activate phagolysosomal program. (A) Phagolysosome formation 1 d after axotomy was suppressed when drk was knocked down in ensheathing glia (drkRNAi), as assayed by LysoTracker staining. SosJC2 enhanced phagolysosomal activities (SosJC2/+), although it was unable to rescue the effect of drkRNAi on phagolysosome formation (drkRNAi, SosJC2/+). Representative images (z-stack) are shown. (Scale bar: 10 μm.) (B) Quantification of the amount of LysoTracker+ puncta formed in A (n ≥ 5 for all). Control: OR85e-mCD8::GFP/+; TIFR-Gal4+. drkRNAi: OR85e-mCD8::GFP/UAS-drkRNAi#105498; TIFR-Gal4+. SosJC2+: OR85e-mCD8::GFP/SosJC2; TIFR-Gal4+. drkRNAi, SosJC2+: OR85e-mCD8::GFP/UAS-drkRNAi#105498, SosJC2; TIFR-Gal4+.
Fig. S7. Ras has little effect on glial clearance of axonal debris. (A) Overexpression of dominant-negative Ras85D (Ras85D<sup>N17</sup>) in adult glia only mildly affected axonal debris clearance. Representative images (z-stack) are shown. Control: +/+; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/<sup>+</sup>; repo-Gal4+. Ras85D<sup>N17</sup>: UAS-Ras85D<sup>N17</sup>/+; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/+; repo-Gal4+. (Scale bar: 30 μm.) (B) Quantification of data in A (n = 10 for all; Student t test).

Fig. S8. Glial activation and the clearance of axonal debris require SOS and myoblast city (Mbc). (A) Knocking down sos and mbc simultaneously in adult glia resulted in more severe axonal debris clearance defects compared with single RNAi. Control: OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/+; repo-Gal4+. sos<sup>RNAi</sup>; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/+; repo-Gal4+; tub-Gal80<sup>ts</sup>/+; repo-Gal4/UAS-mbc<sup>RNAi#16044</sup>; sos<sup>RNAi</sup>; mbc<sup>RNAi</sup>; OR85e-mCD8::GFP, tub-Gal80<sup>ts</sup>/UAS-sos<sup>RNAi#42849</sup>; repo-Gal4/UAS-mbc<sup>RNAi#16044</sup>. (Scale bar: 30 μm.) (B) Quantification of data in A (n ≥ 10 for all). (C) Glial hypertrophy was blocked in sos<sup>RNAi</sup> and mbc<sup>RNAi</sup> double RNAi animals. Glial membranes (labeled with mCD8::GFP) surrounding the antennal lobe (AL) undergo hypertrophy 1 d after antennal ablation, as indicated by arrows in control. However, glial knockdown of SOS and Mbc slightly reduced the hypertrophy, respectively, and the reduction was more severe when SOS and Mbc were knocked known simultaneously in adult glia. Representative images (single slice) are shown. C., cortex. (Scale bar: 10 μm.) Control: tub-Gal80<sup>ts</sup>/+; repo-Gal4, UAS-mCD8::GFP<sup>+/-</sup>; sos<sup>RNAi</sup>; tub-Gal80<sup>ts</sup>/UAS-sos<sup>RNAi#42859</sup>; repo-Gal4, UAS-mCD8::GFP<sup>+/-</sup>; mbc<sup>RNAi</sup>; tub-Gal80<sup>ts</sup>/+; repo-Gal4, UAS-mCD8::GFP<sup>+/-</sup>; UAS-mbc<sup>RNAi#16044</sup>.
The proposed model of glial response to and clearance of axonal debris after axon injury in Drosophila adult CNS. Upon axon injury, glia receive a yet unknown signal(s) to activate a series of events to clear axonal debris from the CNS, which requires Src42A to phosphorylate tyrosine residue(s) in the intracellular domain of Draper, allowing Shark to bind (1) (step 1). dCed-6, which is required for clearance of axonal debris through an undetermined mechanism, can also be recruited (2). After activation, glial membranes become hypertrophic and Draper is up-regulated. Meanwhile, glia start to extend their processes to the injured axons (3) (step 2). At this step, glia activate DRK/DOS/SOS and Crk/Mbc/dCed-12 pathways to efficiently drive RAC1 function, as knocking down each pathway results in delay of glial membrane recruitment toward the injured axons (Fig. 5) (4). The clearance of axonal debris is mediated by glial membrane vesicles, which enclose and internalize axonal debris (step 3). DRK/DOS/SOS and Crk/Mbc/dCed-12 contribute to the formation of glial membrane vesicles (Fig. 3) (4), possibly through regulating Rac1 activity. Later, the internalized axonal debris is degraded through phagolysosomal pathway (step 4), which also requires DRK, SOS, and Crk/Mbc/dCed12.


**Fig. S10.** DRK/DOS/SOS requires Draper to function. (A) Sos^{C2} failed to rescue the clearance defect in drpr-null animals. Representative images (z-stack) are shown. (Scale bar: 10 μm.) (B) Quantification of A (n = 10 for all). Control: OR85e-mCD8::GFP+. Sos^{C2}/+: OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+: OR85e-mCD8::GFP+; drpr^{AT}+/+, Sos^{C2}/+; OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+, Sos^{C2}/+; OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+, Sos^{C2}/+; OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+, Sos^{C2}/+; OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+, Sos^{C2}/+; OR85e-mCD8::GFP/Sos^{C2}, drpr^{AT}+/+.