

Video Article

Morphological and Functional Evaluation of Axons and their Synapses during Axon Death in *Drosophila melanogaster*

Maria Paglione^{*1}, Arnau Llobet Rosell^{*1}, Jean-Yves Chatton¹, Lukas J. Neukomm¹¹Department of Fundamental Neurosciences, University of Lausanne^{*}These authors contributed equallyCorrespondence to: Lukas J. Neukomm at lukas.neukomm@unil.chURL: <https://www.jove.com/video/60865>DOI: [doi:10.3791/60865](https://doi.org/10.3791/60865)Keywords: Neuroscience, Issue 157, *Drosophila*, Neurobiology, Wallerian Degeneration, injury-induced axon degeneration, axon death, genetics, optogenetics, behavior, microscopy, fluorescence

Date Published: 3/16/2020

Citation: Paglione, M., Rosell, A.L., Chatton, J.Y., Neukomm, L.J. Morphological and Functional Evaluation of Axons and their Synapses during Axon Death in *Drosophila melanogaster*. *J. Vis. Exp.* (157), e60865, doi:10.3791/60865 (2020).

Abstract

Axon degeneration is a shared feature in neurodegenerative disease and when nervous systems are challenged by mechanical or chemical forces. However, our understanding of the molecular mechanisms underlying axon degeneration remains limited. Injury-induced axon degeneration serves as a simple model to study how severed axons execute their own disassembly (axon death). Over recent years, an evolutionarily conserved axon death signaling cascade has been identified from flies to mammals, which is required for the separated axon to degenerate after injury. Conversely, attenuated axon death signaling results in morphological and functional preservation of severed axons and their synapses. Here, we present three simple and recently developed protocols that allow for the observation of axonal morphology, or axonal and synaptic function of severed axons that have been cut-off from the neuronal cell body, in the fruit fly *Drosophila*. Morphology can be observed in the wing, where a partial injury results in axon death side-by-side of uninjured control axons within the same nerve bundle. Alternatively, axonal morphology can also be observed in the brain, where the whole nerve bundle undergoes axon death triggered by antennal ablation. Functional preservation of severed axons and their synapses can be assessed by a simple optogenetic approach coupled with a post-synaptic grooming behavior. We present examples using a *highwire* loss-of-function mutation and by over-expressing *dnmnat*, both capable of delaying axon death for weeks to months. Importantly, these protocols can be used beyond injury; they facilitate the characterization of neuronal maintenance factors, axonal transport, and axonal mitochondria.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60865/>

Introduction

The morphological integrity of neurons is essential for sustained nervous system function throughout life. The vast majority of the neuronal volume is taken by axons^{1,2}; thus life-long maintenance of particularly long axons is a major biological and bioenergetic challenge for the nervous system. Multiple axonal-intrinsic and glial-extrinsic support mechanisms have been identified, ensuring life-long axonal survival. Their impairment results in axon degeneration³, which is a common feature of nervous systems being challenged in disease, and by mechanical or chemical forces^{4,5}. However, the underlying molecular mechanisms of axon degeneration remain poorly understood in any context, making the development of efficacious treatments to block axon loss challenging. The development of effective therapies against these neurological conditions is important, as they create an enormous burden in our society⁶.

Injury-induced axon degeneration serves as a simple model to study how severed axons execute their own disassembly. Discovered by and named after Augustus Waller in 1850, Wallerian degeneration (WD) is an umbrella term that comprises two distinct, molecularly separable processes⁷. First, after axonal injury, axons separated from their cell bodies actively execute their own self-destruction (axon death) through an evolutionarily conserved axon death signaling cascade within one day after injury⁸. Second, surrounding glia and specialized phagocytes engage and clear the resulting axonal debris within three to five days. The attenuation of axon death signaling results in severed axons that remain preserved for weeks^{9,10,11,12}, while the attenuation of glial engulfment culminates in axonal debris which persists for weeks in vivo^{13,14,15}.

Research in flies, mice, rats and zebrafish revealed several evolutionarily conserved and essential mediators of axon death signaling⁸. Axon death mutants contain severed axons and synapses that fail to undergo axon death; they remain morphologically and functionally preserved for weeks, in the absence of cell body support^{9,10,12,13,16,17,18,19,20,21,22,23}. The discovery and characterization of these mediators led to the definition of a molecular pathway executing axon death. Importantly, axon death signaling is activated not only when the axon is cut, crushed or stretched^{24,25}; it also seems to be a contributor in distinct animal models of neurological conditions (e.g., where axons degenerate in an injury-independent manner⁴, yet with a range of beneficial outcomes^{4,8}). Therefore, understanding how axon death executes axon degeneration after injury might offer insights beyond a simple injury model; it could also provide targets for therapeutic intervention.

The fruit fly *Drosophila melanogaster* (*Drosophila*) has proven to be an invaluable system for axon death signaling. Research in the fly revealed four essential evolutionarily conserved axon death genes: *highwire* (*hiw*)^{11,14}, *dnmnat*^{12,26}, *dsarm*¹⁰ and *axundead* (*axed*)¹². The modification of these mediators — loss-of-function mutations of *hiw*, *dsarm* and *axed*, and over-expression of *dnmnat* — potentially blocks axon death for the life span of the fly. While severed wild type axons undergo axon death within 1 day, severed axons and their synapses lacking *hiw*, *dsarm* or *axed* remain not only morphologically, but also functionally preserved for weeks. Whether functional preservation can also be achieved through high levels of *dnmnat* remains to be determined.

Here, we will present three simple and recently developed protocols to study axon death (e.g., the morphology and function of severed axons and their synapses over time) in the absence of cell body support. We demonstrate how attenuated axon death results in severed axons which are morphologically preserved with a *hiw* loss-of-function mutation (*hiw*^{ΔN}) and how attenuated axon death results in severed axons and synapses that remain functionally preserved for at least 7 days with over-expression of *dnmnat* (*dnmnat*^{OE}). These protocols allow for the observation of individual axonal and synaptic morphology either in the central, or peripheral nervous system (CNS and PNS, respectively)^{13,14}, while the functional preservation of severed axons and their synapses in the CNS can be visualized by the use of a simple optogenetic setup combined with grooming as a behavioral readout¹².

Protocol

1. Observation of Axon Morphology During Axon Death in the PNS

1. Wing injury: partial injury of axon bundles
 1. Use 5 virgin females and 5 males from the right genotype (**Figure 4A**, P₀ generation) to perform crosses at room temperature (RT). Pass P₀ into new vials every 3–4 days. Collect freshly eclosed adult progeny (F₁ generation) daily and age them for 7–14 days.
 2. Anesthetize flies on CO₂ pads. Use micro scissors to cut the anterior wing vein roughly in the middle of the wing (**Figure 1A**). Use one wing for the injury and the other wing as an age-matched uninjured control. Apply one injury per wing, and make sure to get sufficient wings injured (approximately 15 wings).
NOTE: The whole wing can be cut through, but it is sufficient to cut only the anterior wing vein. This is the strongest part of the wing.
 3. Recover the flies in food-containing vials.
2. Wing dissection and visualization of axons
 1. Spread 10 μL of halocarbon oil 27 with a pipette along a whole glass slide (**Figure 1B**).
 2. Cut off the injured, as well as, the uninjured control wing at desired time points (e.g., 1 or 7 days post injury). Use micro scissors to cut, and tweezers to grab the wing. Mount maximal 4 wings into halocarbon oil 27 (**Figure 1B**) and cover them with a cover slide.
 3. Image the wing immediately using a spinning disk microscope. Acquire a series of optical sections along the z-axis with 0.33 μm step-size and compress z-stacks into a single file for subsequent analyses.
NOTE: Do not grab the anterior wing vein where cell bodies and axons are housed. Grab the wing at the center. The tissue in wings is not fixed; keep the time from mounting wings to imaging these under 8 min.

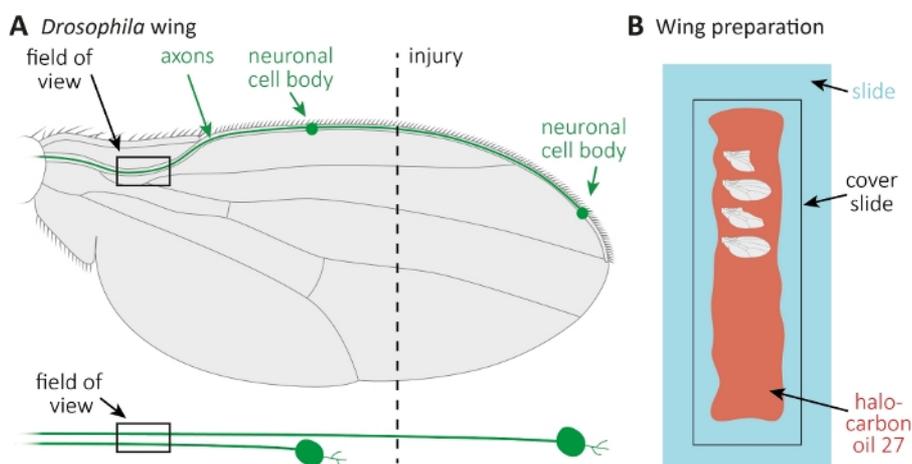


Figure 1: Observation of axon morphology during axon death in the wing. (A) Schematic fly wing with two sparsely GFP-labeled sensory neurons, which are also separately indicated below. The site of injury and the field of observation are indicated. (B) Schematic setup for wing imaging. Injured and uninjured control wings (grey) are mounted in halocarbon oil 27 (red) on a glass slide (light blue) and covered with a cover slide (black). [Please click here to view a larger version of this figure.](#)

2. Observation of Axon and Synapse Morphology During Axon Death in the CNS

1. Antennal ablation: injury of whole axon bundles
 1. Use 5 virgin females and 5 males from the right genotype (**Figure 5A**, P₀ generation) to perform crosses at RT. Pass P₀ into new vials every 3–4 days. Collect freshly eclosed adult progeny (F₁ generation) daily and let them age for 7 up to 14 days.

2. Anesthetize flies on CO₂ pads. Use tweezers to ablate the right 3rd antennal segment for unilateral ablation; or both left and right 3rd antennal segments for bilateral ablation (**Figure 2A-C**). This will remove GFP-labeled neuronal cell bodies, while their axonal projections remain in the CNS.
NOTE: Antennal ablation severs the whole axon bundle. If unilateral ablation is performed, the axon bundle on the contralateral side (the unablated antenna) serves as internal control. Make sure to perform sufficient antennal ablations (approximately 15 animals).
 3. Recover the flies in food-containing vials.
2. Brain dissection and visualization of axons
1. Mix silicone elastomer base (9 mL) and curing agent (1 mL) in a volume ratio of 10:1. Transfer each 5 mL mixture into a 35 mm tissue culture plate, and reduce air introduced by mixing with gentle agitation in the fume hood overnight. The mixture solidifies within 24 h.
NOTE: Dissection plates must be prepared only once and may be used multiple times.
 2. Anesthetize flies on CO₂ pads and decapitate adult heads using two tweezers at desired time points (e.g., 1 or 7 days after antennal ablation). Use one tweezer to grab the neck, and the other tweezer to fix the thorax. Gently pull the neck and head off the thorax.
NOTE: Leave decapitated heads on the CO₂ pad until the desired number is achieved, but make sure to proceed to the next step within 30 min.
 3. Transfer all heads into a 1.5 mL microcentrifuge tube containing 1 mL of fixing solution containing 4% paraformaldehyde (PFA) and 0.1% Triton X-100 in phosphate buffered saline (PBS) using tweezers that have been dipped into the fixing solution.
NOTE: Fly heads stick well on wet tweezers. It makes it feasible to transfer all heads readily into the microcentrifuge tube.
 4. Fix heads for 20 min with gentle agitation at RT. Put the microcentrifuge tube on ice, heads will gravitate to the bottom of the microcentrifuge tube. Remove the supernatant with a pipette and repeat this procedure with five 2 min washes with 1 mL of washing buffer containing 0.1% Triton X-100 in PBS with gentle agitation at RT, to remove residual fixing solution.
NOTE: Videos on how to dissect adult *Drosophila* brains are readily available²⁷.
 5. Transfer the heads with a glass pipette into a dissection plate filled with washing buffer. Use one tweezer to grab and pull the proboscis off the head, while holding the head with the other tweezer. This will leave a hole where the proboscis was attached to the exoskeleton.
 6. Use two tweezers to remove the exoskeleton between the hole and each compound eye. This will make it feasible to open the head structure with both tweezers, and to gently scratch out the brain within.
 7. Clean each brain by removing trachea or fat stuck to it (**Figure 2D**, top). Once the brain is cleaned, put it in a new microcentrifuge tube containing 1 mL of washing buffer on ice.
NOTE: Damaged or lost optic lobes will not affect the olfactory lobe in the center of the brain (**Figure 2D**, top).
 8. Replace washing buffer with 1 mL of fixing solution once all brains are collected and accumulated at the bottom of the microcentrifuge tube. Fix brains for 10 min with rocking at RT, followed by five 2 min washes in 1 mL of washing buffer with rocking at RT.
 9. Apply primary antibodies (1:500) in washing buffer overnight with rocking at 4 °C, followed by 10 washes over 2 h using 1 mL of washing buffer with rocking at RT.
 10. Apply secondary antibodies (1:500) in washing buffer 2 h with rocking at RT and wrap microcentrifuge tube in aluminum foil to block light. Keep the microcentrifuge tube covered with aluminum foil for the rest of the procedure. Apply ten washes with 1 mL of washing buffer over 2 h with rocking at RT.
 11. Remove the supernatant and use a single drop of antifade reagent to cover the brains in the microcentrifuge tube. Incubate brains for at least 30 min at 4 °C before preparing them for mounting and imaging.
 12. Prepare a cover slide, stick lab tape on it, and cut out a "T"-like shape from the tape (**Figure 2D**, bottom). The resulting space serves as area where the brain-containing antifade reagent²⁸ will be pipetted into, preferably into both chambers.
NOTE: Use a 20-200 µL pipette tip where 3 mm of the tip has been cut off to widen the opening of the pipette. This will make it feasible to pipette the brain-containing antifade reagent. Carefully cover the brains with a cover slide.
 13. Use clay to prepare two small even rolls. Ensure that the clay rolls are not higher than the height of a glass slide. Stick the clay rolls onto the glass slide (**Figure 2D**, bottom). Place the brain-containing cover slide sandwich onto the clay rolls.
NOTE: GFP-labeled axons and their synapses are located in the front of the brain. It is, therefore, easier to image them from the front. However, brains will either face up, or face down on the cover slide sandwich. Clay rolls serve as sandwich holders, and during imaging, the sandwich can be flipped upside down. This will make it feasible to acquire images from the front from every brain.
 14. Acquire a series of optical sections along the z-axis with 1.0 µm step-size using a confocal microscope, and compress z-stacks into a single file for subsequent analyses, to assess the number of axonal projections remaining intact.

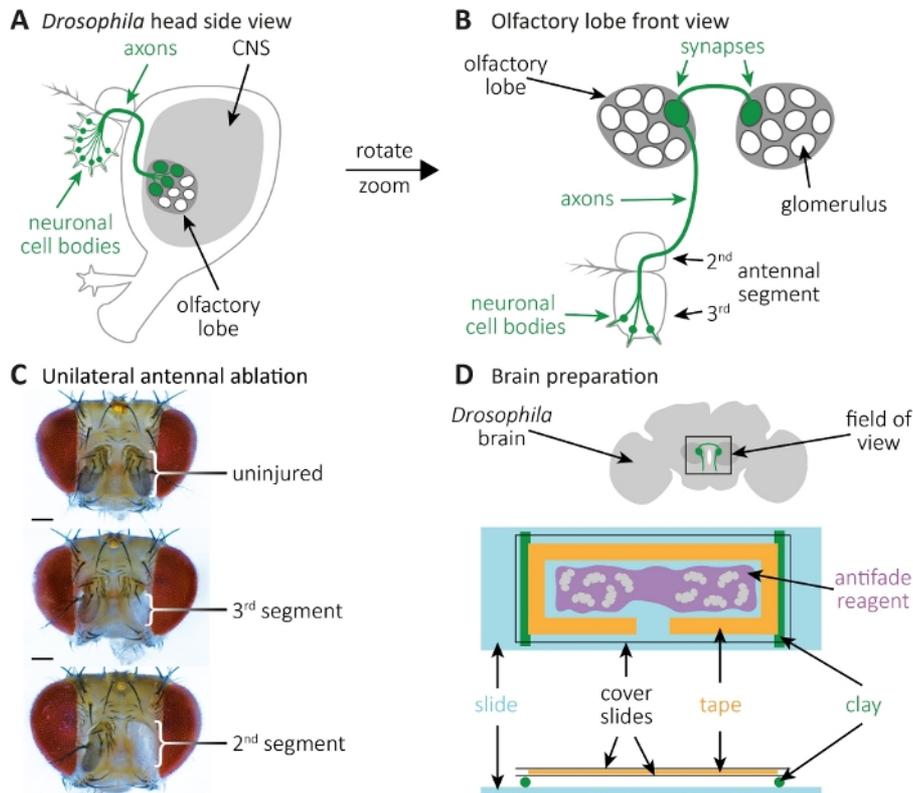


Figure 2: Observation of axon and synapse morphology during axon death in the brain. (A) Side view of a schematic fly head with GFP-labeled cell bodies, axons and synapses. (B) High-magnification front view of GFP-labeled olfactory receptor neurons and their axons and synapses. Cell bodies are housed in the 3rd antennal segment, and their axons project into the CNS. Axons form synapses in a glomerulus in the left olfactory lobe, cross the midline and form synapses in the glomerulus on the contralateral olfactory lobe. (C) Examples of fly heads with unilateral antennal ablation. Top: Uninjured control. Middle: Ablation of the 3rd antennal segment. Bottom: Ablation of the 2nd (and thus also 3rd) antennal segment. (D) Brain preparation. Top: Schematic dissected fly brain with indicated olfactory lobes and axonal projections in the field of view. Bottom: Schematic setup for brain imaging. Two clay rolls (green) are mounted onto a glass slide (light blue), they carry a cover slide sandwich, which contains fly brains (grey). Brains are mounted in antifade reagent (purple), surrounded by a lab tape (orange), and covered by two cover slides (black). [Please click here to view a larger version of this figure.](#)

3. Grooming Induced by Optogenetics as a Readout for Axon and Synapse Function

1. Optogenetic setup

1. Perform the optogenetic experiment in a dark room. Ensure that the setup consists of an 850 nm infrared (IR) LED spotlight to illuminate flies in the dark (Figure 3A), a flashing 660 nm red LED spotlight to activate neurons expressing CsChrimson, and a monochrome camera with a 700 nm longpass filter, which prevents the recording of red light flashes.
2. Use a 3D printer to generate a tiny circular behavior chamber with a diameter of 1 cm, cover it with a cover slide, and place an 860 nm emitter coupled to the red LED spotlight next to the chamber (Figure 3B).
NOTE: The emitter indicates when the red LED spotlight is on, thus activating the neurons.
3. Mount the LED spotlights and the camera on top of the chamber (Figure 3A, C).
4. Activate neurons by 10 Hz flashes during 10 s. The duration of activation can be adjusted according to the experimental design.

2. Preparing flies for optogenetics

1. Melt fly food in a microwave. After the food cooled down, before solidification, add 1:100 of 20 mM all trans-retinal in ethanol (EtOH) to a final concentration of 200 μ M. Mix well, and pour the food immediately into empty vials.
NOTE: Avoid adding all trans-retinal to hot food, this could result in less efficient optogenetics.
2. Cover vials containing solidified food with plugs or cotton balls. Wrap vials with aluminum foil. Then, store the food-containing vials in a dark, cold room.
3. Use 5 virgin females and 5 males (Figure 6A, generation P₀) from the right genotype to perform crosses at RT. Pass P₀ into new vials every 3–4 days. Collect freshly eclosed adult progeny (generation F₁) on a daily base and let them age for 7 up to 14 days in aluminum-covered vials containing 200 μ M all trans-retinal in fly food.
4. Collect flies by tapping them from food-containing vials into an empty vial with no food. Cool the vial down in ice-containing water for approximately 30 s. Flies will fall asleep. Put individual flies rapidly into small chambers covered with a cover slide (Figure 3B).
NOTE: As soon as flies warm up, they wake up. It is crucial to quickly spread individual flies into single chambers each. Avoid CO₂ pads to anaesthetize flies, this will impact their behavior.

5. Perform optogenetics to elicit antennal grooming. Here, the protocol consists of the following intervals: 30 s where the red light is absent, followed by 10 s of red-light exposure at 10 Hz. Repeat this procedure three times in total, followed by an additional 30 s interval where the red light is absent^{12,29,30}.
NOTE: This protocol can be adjusted according to the experimental preference.
6. Collect individual flies from each chamber on CO₂ pads. Subject them to antennal injury. Ablate both the left and the right 2nd antennal segments (**Figure 2C**). This will remove the cell bodies of Johnston's organ (JO) neurons, while the axonal projections remain in the CNS. Recover the flies in aluminum-covered vials containing 200 μ M all trans-retinal.
NOTE: For antennal grooming induced by optogenetics, the sensory neuron cell bodies are housed in the 2nd antennal segment (**Figure 2C**).
7. At corresponding time points (e.g., 7 days post antennal ablation), subject flies to another grooming assay (go back to step 3.2.4).

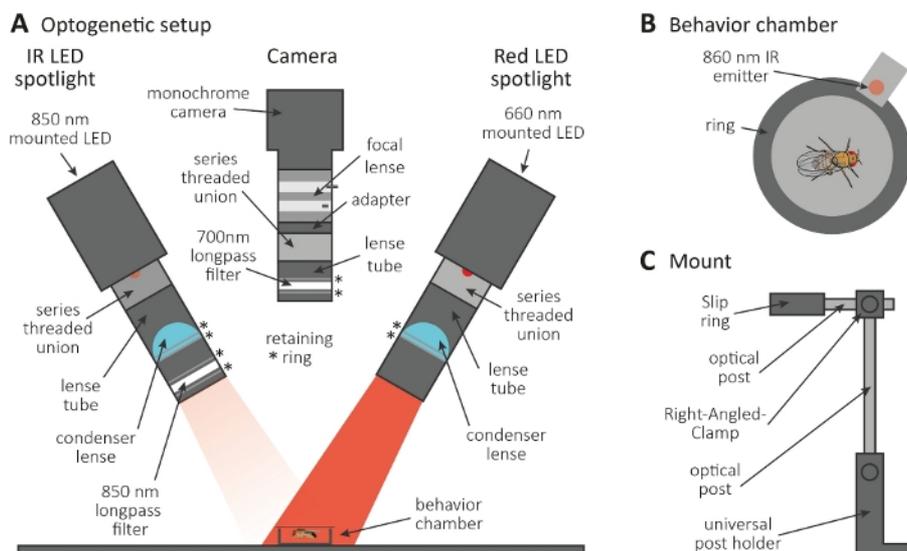


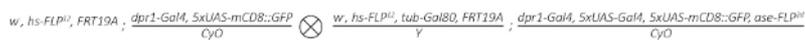
Figure 3: Optogenetic setup to induce grooming as a readout for axon and synapse function. (A) Illustration of assembled components required for optogenetics. Infrared (IR) LED spotlight, camera and red LED spotlight (from left to right, respectively). The components including a detailed description are listed in the **Table of Materials**. (B) Top view illustration of a behavior chamber including an IR emitter to indicate red LED spotlight activation. (C) Illustration of a single mount setup. A total of three mount setups are required for the two LED spotlights and the camera, respectively. [Please click here to view a larger version of this figure.](#)

Representative Results

Above, we described three methods to study the morphology and function of severed axons and their synapses. The first method allows for high-resolution observation of individual axons in the PNS. It requires clones generated by the MARCM technique^{14,31}. Here, we performed crosses to generate wild type and *highwire* mutant MARCM clones (**Figure 4A**). A simple cut in the middle of the wing induces axon injury of neurons housed distal (e.g., at the outer side of the wing), while proximal neurons (e.g., between the cut site and the thorax) remain uninjured. This approach makes it feasible to observe axon death side-by-side of uninjured control axons in the same nerve bundle (**Figure 1A**, **Figure 4B**). Here, we used a genetic background resulting in low numbers of GFP-labeled clones (e.g., two in each experiment¹⁴). We present examples of 1 and 7 days after injury of wild type axons, to provide examples of control axons, axons undergoing axon death, and axonal fragments being cleared by surrounding glia, respectively. In addition, we repeated axonal injury in *highwire* mutants where we analyzed the outcome 7 days after injury.

Uninjured control wings harbor two wild-type clones, thus two GFP-labeled wild-type axons (**Figure 4B**, wild type, uninjured control). One day after cutting the middle of the wing by the use of micro scissors, axon death is induced in GFP-labeled axons where cell bodies are distal to the cut site, while axons from proximally housed cell bodies serve as an internal control within the same nerve bundle (**Figure 4B**, wild type, 1 day post injury). Note the axonal debris trace in the upper part indicated by the arrow. 7 days after axonal injury, GFP-labeled axonal debris is cleared by surrounding glia, while GFP labeled uninjured control axons remain in the nerve bundle (**Figure 4B**, wild type, 7 days post injury, arrow). In contrast, *highwire* mutant axons that have been severed for 7 days remain morphologically preserved, consistent with previous findings^{11,14} (**Figure 4B**, *highwire*, 7 days post injury, arrow). These results demonstrate the powerful visual resolution of the *Drosophila* wing. Axon death can be observed side-by-side of uninjured controls in the same nerve bundle. While wild-type axons undergo axon death within 1 day after injury and the resulting debris is cleared within 7 days, axon death deficient *highwire* mutants remain morphologically preserved for 7 days.

A Wild type:



highwire:

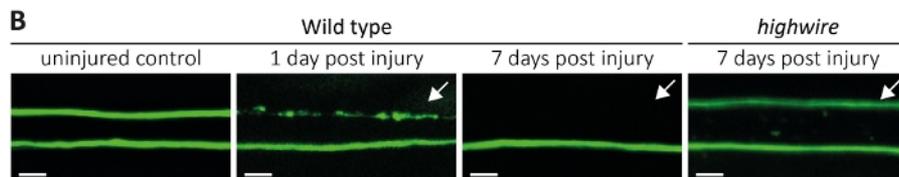
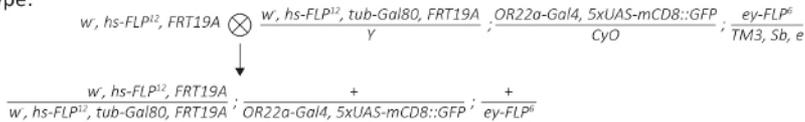


Figure 4: Approach to study axon death of GFP-labeled sensory neuron axons in the wing. (A) Schematic crosses to generate wild type and *highwire* clones in the wing (P₀ and F₁ generation, respectively). Virgin females are on the left, males on the right. See **Table of Materials** for genotype details. (B) Examples of control and injured GFP-labeled axons. The field of view is indicated in (Figure 1A). From left to right: uninjured wild type control axons, wild type axons 1-day post injury, wild type axons 7 days post injury, *highwire* mutant axons 7 days post injury, respectively. Arrows indicate severed axons. Scale bar = 5 μm. [Please click here to view a larger version of this figure.](#)

The second method describes how to visualize whole axon bundles projecting into the CNS where they form synapses, which belong to neurons housed in both left and right antennae (Figure 2A-C). Here, we performed crosses to generate wild type and *highwire* mutant MARCM clones (Figure 5A). Uninjured, GFP-labeled axons and their synapses can be visualized over the course of days to weeks, in the absence of injury (Figure 5B, Wild type, uninjured control). Alternatively, animals can be subjected to 3rd antennal segment ablation, and severed GFP-labeled axons and their synapses can be observed during a time course over hours to days. We focused on 7 days after antennal ablation, because at this time point, axons and their synapses have undergone axon death, and resulting debris is cleared by surrounding glia. If unilateral ablation of the right antenna is performed, then the right axon bundle is severed and will disassemble and the resulting debris is fully cleared 7 days after injury (Figure 5B, wild type, unilateral ablation, 7 days post injury, arrows), consistent with previous findings¹³. Alternatively, both the right and the left antennae can be ablated, which will sever both axon bundles, and 7 days after injury, axons and their synapses disappeared (Figure 5B, wild type, bilateral ablation, 7 days post injury, arrow). In contrast, unilateral ablation of the right antennae in *highwire* mutants results in severed axons that remain preserved 7 days post injury, consistent with previous findings^{11,14} (Figure 5B, *highwire*, unilateral ablation, 7 days post injury, arrow). These results demonstrate that severed wild-type axons undergo axon death and the resulting debris is cleared within 7 days, while axon death deficient *highwire* mutants fail to undergo axon death and remain morphologically preserved for 7 days.

A Wild type:



highwire:

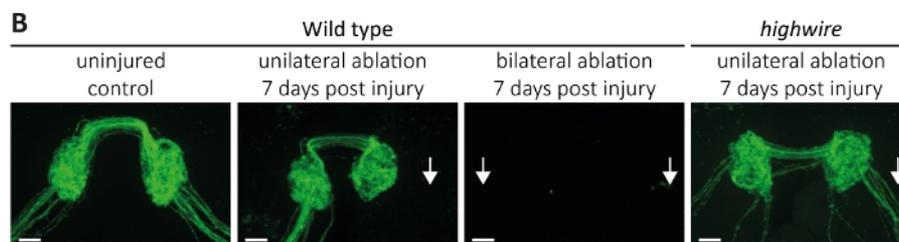
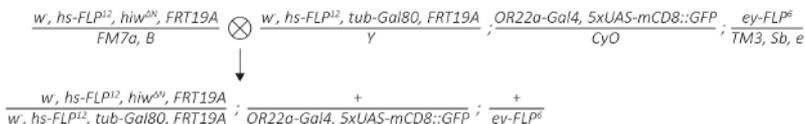


Figure 5: Approach to study axon death of GFP-labeled sensory neuron axons in the brain. (A) Schematic crosses to generate wild type and *highwire* clones in the brain (P₀ and F₁ generation, respectively). Virgin females are on the left, males on the right. See **Table of Materials** for genotype details. (B) Examples of control and injured GFP-labeled axons. From left to right: uninjured wild type controls, wild type 7 days post unilateral antennal ablation, wild type 7 days post bilateral antennal ablation, and *highwire* mutants 7 days post unilateral antennal ablation, respectively. Arrows indicate severed axon bundles. Scale bar = 10 μm. [Please click here to view a larger version of this figure.](#)

The third method allows for the observation of functional preservation of severed axons and their synapses in the CNS. It relies on the manipulation of a subset of JO neurons housed in the 2nd antennal segment which are sufficient to induce antennal grooming. Expression of a red-shifted channelrhodopsin (CsChrimson) in JO neurons, combined with dietary supplementation of all trans-retinal, is sufficient to elicit a simple post-synaptic grooming behavior upon red light exposure^{12,30}. Here, we performed crosses to generate wild type JO neurons, and JO neurons over-expressing *dnmnat* (*dnmnat*^{OE}) (Figure 6A). Wild type flies or flies containing JO neurons with attenuated axon death (*dnmnat*^{OE}), both harbor a potent grooming behavior before injury. However, 7 days post injury (e.g., bilateral ablation of the 2nd antennal segment), grooming fails to be elicited by optogenetics in wild type flies due to injury-induced axon and synapse degeneration, while animals with attenuated axon death continue to groom (Figure 6B, Movie 1,2). Attenuated axon death is therefore capable of functionally preserving severed axons and their synapses for 7 days.

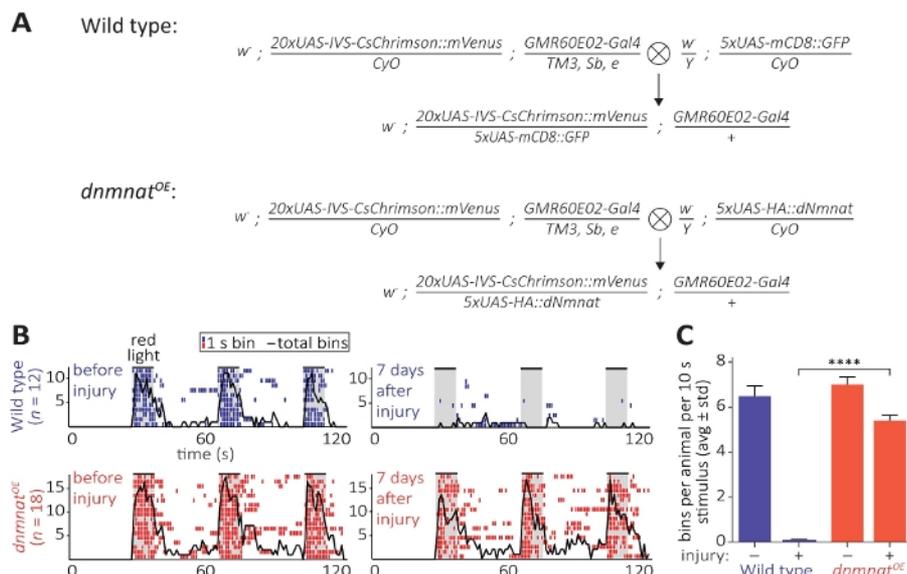


Figure 6: Approach to visualize axonal and synaptic function after axotomy. (A) Schematic crosses to generate wild type and *dnmnat* over-expressing JO sensory neurons (P₀ and F₁ generation, respectively). Virgin females are on the left, males on the right. See **Table of Materials** for genotype details. **(B)** Individual ethograms of grooming behavior induced by optogenetics. Top: individual ethograms of wild type flies before and 7 days after injury (blue). Bottom: individual ethograms of flies over-expressing *dnmnat* (*dnmnat*^{OE}) in JO neurons before and 7 days after injury (red). Each bin indicates at least 1 grooming behavior within 1 s. The black line indicates the sum of all bins. **(C)** Quantification of grooming behavior. Data is shown as average ± standard deviation, $p > 0.001$ (one-way ANOVA, multiple comparison with Tukey's post hoc test). [Please click here to view a larger version of this figure.](#)

Movie 1: Representative wild type grooming behavior elicited by optogenetics before and 7 days after antennal ablation. [Please click here to download this video.](#)

Movie 2: Representative grooming behavior elicited by optogenetics in flies over-expressing *dnmnat* in JO neurons before and 7 days after antennal ablation. [Please click here to download this video.](#)

Discussion

The protocols described here allow for the robust and reproducible observation of morphology as well as function of axons and their synapses separated from their cell bodies in *Drosophila*. The wing assay facilitates the observation of axon death side-by-side of uninjured control axons in the PNS¹⁴, while the antennal assay facilitates the observation of whole nerve bundles of GFP-labeled axons and their synapses, to assess both morphology and function in the brain (CNS)¹². There are critical steps and certain advantages for each approach to study morphology that have to be taken into consideration when designing experiments.

To observe axon morphology in the PNS in the wing, experiments can be readily performed, because of the transparency of the wing: it allows to bypass dissection and immunohistochemistry. However, due to the lack of fixation, the wings have to be imaged immediately after mounting¹⁴. Currently, two distinct Gal4 drivers are frequently used, either *ok371Gal4* or *dpr1Gal4*, and both references offer distinct approaches to quantify degeneration^{14,26}. Sparse labeling of a few neurons is recommended, by using "Mosaic Analysis with a Repressible Cell Marker (MARCM)"^{14,31}, as the resolution of axonal morphology is unprecedented. In contrast, the observation of synapses is not possible in wings, they are located in the ventral nerve cord inside the thorax of the flies. Furthermore, additional axonal markers cannot be visualized by immunohistochemistry: the waxy cuticle makes it impossible for the diffusion of fixatives and antibodies into the underlying tissue.

To observe axon and synapse morphology in the CNS, brain dissections have to be performed. They offer the advantage of visualizing additional axonal and synaptic markers by the use of immunohistochemistry, and synapses can be observed alongside axons in the same field of view^{10,13}. A large collection of characterized olfactory receptor neuron (ORN) Gal4 drivers is readily available³², and frequently, *OR22aGal4* is the driver of choice. For antennal ablation, cell bodies of OR22a neurons are housed in the 3rd segment (Figure 2B). A fluorescence intensity-based quantification is used to quantify the degeneration of either axons or synapses¹³. Conversely, experiments are time consuming due to brain dissection and antibody staining.

To visualize axonal and synaptic function after axotomy, optogenetics is used to trigger antennal grooming: it serves as a readout for functional preservation of severed axons and their synapses¹². The grooming circuit and corresponding sensory, inter- and motoneuron Gal4 drivers have been thoroughly described^{29,30}. *GMR60E02Gal4* labels a subset of Johnston's organ (JO) sensory neurons, which are required and sufficient for grooming^{29,30}. For antennal ablation, cell bodies of JO neurons are housed in the 2nd antennal segment (**Figure 2B**). An optogenetic setup can readily be built from scratch, or an existing setup adjusted. Importantly, experiments have to be performed in a dark room, and flies thus visualized with an infrared (IR) LED spotlight. When using CsChrimson as a channel, it is crucial to supply the food with all trans-retinal and a red LED spotlight to activate JO neurons²⁹. Alternatively, blue light-sensitive channels and a blue LED spotlight, or the TrpA1 channel and temperature can be used for neuronal activation^{29,33}. The quantification of grooming behavior has already been described^{12,29}.

When these assays are used to specifically study axon death, it is important to note that the phenotype of morphological or functional preservation should be robust over time. There are cases where axon death leads to a consistent yet less pronounced phenotype in morphological preservation^{34,35}, and whether such a phenotype translates into functional preservation remains to be determined.

Axon death phenotypes have also been observed in neurons during development of *Drosophila* larvae, where nerves were crushed rather than injured^{11,23}. Here, we specifically focused on adult *Drosophila* neurons which completed development. In this context, the use of RNA interference³⁶, or tissue-specific CRISPR/Cas9³⁷ can readily be implemented. Importantly, the above techniques can be used in an axon death independent context: they facilitate the characterization of neuronal maintenance factors³⁸, axonal transport³⁹, age-dependent axonal mitochondria changes⁴⁰, and morphology of axonal mitochondria⁴¹.

Disclosures

The authors declare that they have nothing to disclose.

Acknowledgments

We would like to thank the entire Neukomm lab for contributions. This work was supported by a Swiss National Science Foundation (SNSF) Assistant Professor award (grant 176855), the International Foundation for Research in Paraplegia (IRP, grant P180), SNSF Spark (grant 190919) and by support from the University of Lausanne and the Department of Fundamental Neurosciences (État de Vaud) to L.J.N.

References

1. Matsuda, W. et al. Single Nigrostriatal Dopaminergic Neurons Form Widely Spread and Highly Dense Axonal Arborizations in the Neostriatum. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. **29** (2), 444-453 (2009).
2. Wedel, M. J. A Monument of Inefficiency: The Presumed Course of the Recurrent Laryngeal Nerve in Sauropod Dinosaurs. *Acta Palaeontologica Polonica*. **57** (2), 251-256 (2012).
3. Mariano, V., Dominguez-Iturza, N., Neukomm, L. J., Bagni, C. Maintenance mechanisms of circuit-integrated axons. *Current Opinion in Neurobiology*. **53**, 162-173 (2018).
4. Conforti, L., Gilley, J., Coleman, M. P. Wallerian degeneration: an emerging axon death pathway linking injury and disease. *Nature reviews Neuroscience*. **15** (6), 394-409 (2014).
5. Neukomm, L. J., Freeman, M. R. Diverse cellular and molecular modes of axon degeneration. *Trends in Cell Biology*. **24** (9), 515-523 (2014).
6. Gustavsson, A. et al. Cost of disorders of the brain in Europe 2010. *European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology*. **21** (10), 718-779 (2011).
7. Waller, A. Experiments on the Section of the Glossopharyngeal and Hypoglossal Nerves of the Frog, and Observations of the Alterations Produced Thereby in the Structure of Their Primitive Fibres. *Philosophical Transactions of the Royal Society of London*. **140**, 423-429 (1850).
8. Rosell, A. L., Neukomm, L. J. Axon death signalling in Wallerian degeneration among species and in disease. *Open Biology*. **9** (8), 190118 (2019).
9. Mack, T. G. et al. Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nature Neuroscience*. **4** (12), 1199-1206 (2001).
10. Osterloh, J. M. et al. dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. *Science (New York, NY)*. **337** (6093), 481-484 (2012).
11. Xiong, X. et al. The highwire ubiquitin ligase promotes axonal degeneration by tuning levels of nmnat protein. *PLoS Biology*. **10** (12), e1001440 (2012).
12. Neukomm, L. J. et al. Axon Death Pathways Converge on Axundead to Promote Functional and Structural Axon Disassembly. *Neuron*. **95** (1), 78-91 (2017).
13. MacDonald, J. M. et al. The *Drosophila* cell corpse engulfment receptor Draper mediates glial clearance of severed axons. *Neuron*. **50** (6), 869-881 (2006).
14. Neukomm, L. J., Burdett, T. C., Gonzalez, M. A., Zuchner, S., Freeman, M. R. Rapid in vivo forward genetic approach for identifying axon death genes in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*. **111** (27), 9965-9970 (2014).
15. Lu, T.Y. et al. Axon degeneration induces glial responses through Draper-TRAF4-JNK signalling. *Nature Communications*. **8**, 14355 (2017).
16. Lunn, E. R., Perry, V. H., Brown, M. C., Rosen, H., Gordon, S. Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. *The European Journal of Neuroscience*. **1** (1), 27-33 (1989).
17. Adalbert, R. et al. A rat model of slow Wallerian degeneration (Wld(S)) with improved preservation of neuromuscular synapses. *The European Journal of Neuroscience*. **21** (1), 271-277 (2005).
18. Martin, S. M., O'Brien, G. S., Portera-Cailliau, C., Sagasti, A. Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. *Development (Cambridge, England)*. **137** (23), 3985-3994 (2010).
19. Feng, Y. et al. Overexpression of Wld(S) or Nmnat2 in Mauthner Cells by Single-Cell Electroporation Delays Axon Degeneration in Live Zebrafish. *Journal of Neuroscience Research*. **88** (15), 3319-3327 (2010).

20. Gilley, J., Coleman, M. P. Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. *PLoS Biology*. **8** (1), e1000300 (2010).
21. Babetto, E., Beirowski, B., Russler, E. V., Milbrandt, J., DiAntonio, A. The Phr1 ubiquitin ligase promotes injury-induced axon self-destruction. *Cell Reports*. **3** (5), 1422-1429 (2013).
22. Gerdtz, J., Summers, D. W., Sasaki, Y., DiAntonio, A., Milbrandt, J. Sarm1-mediated axon degeneration requires both SAM and TIR interactions. *The Journal of Neuroscience*. **33** (33), 13569-13580 (2013).
23. Gerdtz, J., Brace, E. J., Sasaki, Y., DiAntonio, A., Milbrandt, J. SARM1 activation triggers axon degeneration locally via NAD⁺ destruction. *Science (New York, NY)*. **348** (6233), 453-457 (2015).
24. Bridge, P. M. et al. Nerve crush injuries--a model for axonotmesis. *Experimental Neurology*. **127** (2), 284-290 (1994).
25. Maxwell, W. L., Bartlett, E., Morgan, H. Wallerian Degeneration in the Optic Nerve Stretch-Injury Model of Traumatic Brain Injury: A Stereological Analysis. *Journal of Neurotrauma*. **32** (11), 780-790 (2015).
26. Fang, Y., Soares, L., Teng, X., Geary, M., Bonini, N. M. A novel Drosophila model of nerve injury reveals an essential role of Nmnat in maintaining axonal integrity. *Current Biology*. **22** (7), 590-595 (2012).
27. *Janelia Farm Adult Drosophila Brain Dissection*. <https://www.janelia.org/project-team/flylight/protocols> (2015).
28. Mowiol mounting medium. *Cold Spring Harbor Protocols*. **2006** (1), (2006).
29. Seeds, A. M. et al. A suppression hierarchy among competing motor programs drives sequential grooming in Drosophila. *eLife*. **3**, e02951 (2014).
30. Hampel, S., Franconville, R., Simpson, J. H., Seeds, A. M. A neural command circuit for grooming movement control. *eLife*. **4**, e08758 (2015).
31. Lee, T., Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*. **22** (3), 451-461 (1999).
32. Vosshall, L. B., Wong, A. M., Axel, R. An olfactory sensory map in the fly brain. *Cell*. **102** (2), 147-159 (2000).
33. Hampel, S., McKellar, C. E., Simpson, J. H., Seeds, A. M. Simultaneous activation of parallel sensory pathways promotes a grooming sequence in Drosophila. *eLife*. **6**, (2017).
34. Farley, J. E. et al. Transcription factor Pebbled/RREB1 regulates injury-induced axon degeneration. *Proceedings of the National Academy of Sciences of the United States of America*. **23** (6), (2018).
35. Wang, H. et al. Rapid depletion of ESCRT protein Vps4 underlies injury-induced autophagic impediment and Wallerian degeneration. *Science Advances*. **5** (2), (2019).
36. Dietzl, G. et al. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*. **448** (7150), 151-156 (2007).
37. Port, F. et al. A large-scale resource for tissue-specific CRISPR mutagenesis in Drosophila. *bioRxiv*. **102**, 636076 (2019).
38. Vagnoni, A., Hoffmann, P. C., Bullock, S. L. Reducing Lissencephaly-1 levels augments mitochondrial transport and has a protective effect in adult Drosophila neurons. *Journal of Cell Science*. **129** (1), 178-190 (2016).
39. Vagnoni, A., Bullock, S. L. A cAMP/PKA/Kinesin-1 Axis Promotes the Axonal Transport of Mitochondria in Aging Drosophila Neurons. *Current Biology*. **28** (8), 1265-1272, (2018).
40. Cao, X. et al. In vivo imaging reveals mitophagy independence in the maintenance of axonal mitochondria during normal aging. *Aging Cell*. **16** (5), 1180-1190 (2017).
41. Smith, G. A. et al. Glutathione S-Transferase Regulates Mitochondrial Populations in Axons through Increased Glutathione Oxidation. *Neuron*. **103** (1), 52-65 (2019).