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Methods to identify and analyze gene products involved in neuronal intracellular transport using *Drosophila*

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Abstract

Proper neuronal function critically depends on efficient intracellular transport and disruption of transport leads to neurodegeneration. Molecular pathways that support or regulate neuronal transport are not fully understood. A greater understanding of these pathways will help reveal the pathological mechanisms underlying disease. *Drosophila melanogaster* is the premier model system for performing large-scale genetic functional screens. Here we describe methods to carry out primary and secondary genetic screens in *Drosophila* aimed at identifying novel gene products and pathways that impact neuronal intracellular transport. These screens are performed using whole animal or live cell imaging of intact neural tissue to ensure integrity of neurons and their cellular environment. The primary screen is used to identify gross defects in neuronal function indicative of a disruption in microtubule-based transport. The secondary screens, conducted in both motoneurons and dendritic arborization neurons, will confirm the function of candidate gene products in intracellular transport. Together, the methodologies described here will support labs interested in identifying and characterizing gene products that alter intracellular transport in *Drosophila*.

INTRODUCTION

Neurons extend long axonal and often elaborate dendritic processes across which a multitude of cargoes must be efficiently transported along microtubule tracks for proper functioning of the neurons and the brain. Human neurodegenerative diseases have been shown to result from mutations in genes encoding members of the Kinesin microtubule plus-end motor protein family (Reid et al., 2002; Zhao et al., 2001), the microtubule minus-end motor protein Dynein (Weedon et al., 2011), the Dynein-associated protein complex Dynactin (Farrer et al., 2009; Puls et al., 2003), and Spastin, a protein that regulates microtubule stability (Trotta, Orso, Rossetto, Daga, & Broadie, 2004) among others (Reviewed in Millecamps and Julien (2013)). Additionally, evidence for disrupted neuronal transport has been reported for neurodegenerative diseases, but the mechanisms underlying

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disrupted transport appear less direct. For example, disrupted transport observed in models of Alzheimer's disease may stem from altered signaling pathways that change the phosphorylation status of motor proteins (Reviewed in Kanaan et al. (2013)). It is thus conceivable that disrupted trafficking and neurodegeneration could stem from changes in a range of posttranslational modifications or gene expression pathways that impact transport machinery.

Drosophila is a highly tractable genetic system and its use has led to many important discoveries that have advanced biomedical research. Greater than 60% of protein-coding genes in *Drosophila* have human homologs (Wangler, Yamamoto, & Bellen, 2015), making *Drosophila* a great model system to further our understanding of the molecular function of human genes. *Drosophila* is also highly amenable to live cell studies. Both developing and fully mature neurons can be studied in their native environment, with intact synaptic connections and normal glial cell interactions. Many powerful genetic tools are available in *Drosophila* to study gene product function including the UAS-Gal4 system for tissue specific-expression, transgenic markers for live imaging, and four RNAi libraries with greater than 85% coverage of the *Drosophila* genome for gene-specific knockdown studies (Venken, Simpson, & Bellen, 2011).

Here we present methods to perform an RNAi genetic screen in *Drosophila* devised to identify gene products that are important for intracellular transport. This screen is based on the "tail-flip" phenotype observed for mutants of the molecular motors Kinesin (Gindhart, Desai, Beushausen, Zinn, & Goldstein, 1998; Hurd & Saxton, 1996) or Dynein/Dynactin (Haghnia et al., 2007; Lorenzo et al., 2010; Martin et al., 1999), the Kinesin adaptor proteins Aplip1/Jip1 (Horiuchi, Barkus, Pilling, Gassman, & Saxton, 2005) and Syd/Jip3 (Bowman et al., 2000), and proteins required for microtubule stability in axons such as Stathmin (Duncan, Lytle, Zuniga, & Goldstein, 2013). Larvae of these mutants exhibit paralysis of the posterior abdominal segments which causes the posterior of the larvae to lift in the air. The screen makes use of *Drosophila* UAS-RNAi lines and a tissue-specific Gal4 driver to knockdown gene products in motoneurons. The restriction of target gene knockdown to a single tissue type, or at a particular developmental time, enhances the selectivity of screening and the identification of gene products that function at specific developmental times and in specific tissues. In addition, such targeted knockdown analyses can reveal tissue- and cell-specific functions for essential genes that are broadly expressed in all tissues. These specific functions of broadly expressed genes could be obscured if the disruption of gene function across all tissues results in lethality.

Further, we present methods for secondary screens to confirm the function of candidate gene products in intracellular transport. One secondary screen is to examine motoneuron intracellular transport at a cell biological level using live imaging. In motoneurons intracellular transport of a number of vesicles or organelles can be analyzed for disrupted transport using well characterized, transgenic markers for organelles and vesicles. The examination of multiple membranous cargoes allows for the determination of an organelle-/vesicle-specific transport defect versus a defect in general axonal transport. For instance, mutants of Unc-104, a Kinesin motor protein, alter motility of dense core vesicles, but do not affect motility of synaptic vesicles or mitochondria (Barkus, Klyachko, Horiuchi,

Dickson, & Saxton, 2008). In addition to assessing the individual dynamics of organelles and vesicles, there are phenotypic hallmarks of axonal transport defects in motoneurons that may be screened for visually. For example, axonal jams, which are accumulations of membranous organelles in axons, were reported for both Dynein and Kinesin mutants and associated proteins such as cargo adaptors (Bowman et al., 2000; Gindhart et al., 1998; Haghnia et al., 2007; Horiuchi et al., 2005; Hurd & Saxton, 1996; Martin et al., 1999). Further, membrane vesicle accumulation within the distal-most (terminal) boutons of neuromuscular junction synaptic terminals is another hallmark of an intracellular transport defect. These terminal boutons accumulate membrane material in motoneurons depleted of, or mutant for, Dynein/Dynactin complex subunits. One explanation is that Dynactin assists in the loading of Dynein onto microtubule plus ends via Dynactin's ability to bind the plus ends of microtubules through its CAP-Gly domain (Lloyd et al., 2012). Similarly, expression of a dominant negative form of Kinesin heavy chain, KHC^{N262S}, a human-associated hereditary spastic paraplegia 10 mutation, results in membrane accumulation in a subset of synaptic boutons (Fuger et al., 2012), the majority of which appear to be terminal boutons. It is possible that the KHC^{N262S} mutant phenotype reflects the loss of Dynein function resulting from Kinesin-mediated transport of Dynein to the microtubule plus ends in terminal boutons.

An additional secondary screen can be performed in *Drosophila* class IV dendritic arborization (da) neurons to test if depletion of candidate gene products results in phenotypes that are hallmarks of disrupted microtubule transport. Class IV da neurons are a component of the peripheral nervous system and extend elaborate dendritic arbors that line the epidermis. Because the larval epidermis is semitranslucent, live cell fluorescence imaging of these neurons can be performed in fully intact larvae. The morphology of the class IV da neuron dendritic arbor has been well characterized (Grueber, Jan, & Jan, 2002), and normal arbor morphology is critically dependent on microtubule transport within dendrites. Primary dendrites contain microtubules with nearly uniform (>95%) minus end away from soma orientation (Ori-McKenney, Jan, & Jan, 2012; Rolls et al., 2007). Consistent with this polarized microtubule orientation, loss of function of Dynein results in depletion of multiple different membrane cargoes from the dendritic arbor and accumulation of dendritic membrane cargoes in the proximal axon (Satoh et al., 2008; Zheng et al., 2008). The redistribution of membrane cargoes is accompanied by a pronounced proximal shift (toward the cell body) in the position of dendrites and a decrease in total dendritic branch length (Satoh et al., 2008; Zheng et al., 2008). This dendritic arbor phenotype is also observed for Kinesin loss of function (Satoh et al., 2008), likely due to Kinesin's role in proper subcellular localization and function of Dynein.

To illustrate the phenotypes and analyses of intracellular transport defects we describe here, data for RNAi knockdown of the Dynein heavy chain subunit dhc64c will be presented for each section.

1. PRIMARY SCREEN USING RNAi TO IDENTIFY GENES IMPORTANT FOR INTRACELLULAR TRANSPORT

In the screen described here UAS-RNAi transgenes are expressed under the motoneuron driver *Ok6-Gal4* (Aberle et al., 2002; Sanyal, 2009). We recommend the use of *UAS-dicer2* coexpression with RNAi lines to increase the RNAi efficiency (Dietzl et al., 2007). However, strong knockdown of a given gene product in the nervous system may result in lethality or sick larvae. If this occurs RNAi lines can be rescreened without the use of *UAS-dicer2*, or alternatively at a reduced temperature of 18 °C or 25 °C to lower Gal4 activity and UAS-RNAi expression level (Duffy, 2002; Wilder, 2000). RNAi transgenes are known to have off-target effects; therefore it is necessary to test multiple transgenes that target different regions of the mRNA of a given gene to confirm preliminary observations. Testing of additional RNAi transgenes can either be done in the primary screen or in the secondary screen analyses.

1.1 MATERIALS AND EQUIPMENT

Incubator capable of holding constant 29 °C temperature

Stereomicroscope (e.g., Zeiss Stemi SV6)

Standard cornmeal agar food (Bloomington *Drosophila* Stock Center recipe)

60 × 15 mm Petri dishes (Fisher Scientific)

Paint brush, size 0

<i>Drosophila</i> Strain	Purpose	References
<i>Ok6-Gal4</i>	Motoneuron Gal4 driver	Aberle et al. (2002) and Sanyal (2009)
<i>UAS-dicer2</i>	Increase RNAi efficiency	Dietzl et al. (2007)
<i>UAS-RNAi</i> lines	mRNA knockdown	Multiple collections available

Example used here is UAS-dhc64c RNAi, VDRC #28054.

1.1.1 RNAi collections available

TRiP (Transgenic RNAi Project; www.flyrnai.org/Trip-HOME.html)

VDRC (Vienna *Drosophila* RNAi Center; stockcenter.vdrc.at/control/main), 2 Libraries

NIG (National Institute of Genetics, Japan; www.shigen.nig.ac.jp/fly/nigfly/)

1.1.2 Recipes—Juice agar plates (1 L), Elgin & Miller (1980)

Reagent	Quantity
Agar	20 g
Glucose	58 g
Sucrose	29 g

Yeast extract	1.8 g
Grape or apple juice	456 mL
Water	536 mL
NaOH (1.25 N)	22 mL
Acid Mix A (see recipe below)	11.2 mL

In a 1 L flask mix agar and water. In a second 1 L flask, mix glucose, sucrose, yeast extract, juice, and 11 mL NaOH. Autoclave both flasks for 20 min. After autoclaving combine the contents of the two flasks and add 11 mL NaOH and 11.2 mL of Acid Mix A and stir on a hot plate. When flask is cool to the touch, pour into 60×15 mm Petri dishes, cover, and let solidify. This recipe will yield approximately 4 sleeves of 20 dishes. When cooled, store in a plastic bag at 4 °C.

1.1.3 Acid Mix A (1 L)

500 mL water
418 mL propionic acid
41.5 mL phosphoric acid

1.2 METHODS

1.2.1 Setup crosses

1. Cross 10–15 virgin female transgenic *Drosophila* containing *Ok6-Gal4*, *UAS-dicer2* to 2–5 male transgenic *Drosophila* containing *UAS-RNAi* targeting specific genes in vials containing standard food (see Figure 1). Place vials at 29 °C, and let *Drosophila* mate for 1 day.
2. Transfer *Drosophila* to a fresh vial of food and allow females to lay eggs for 4–8 h. After the 4–8 h time period, transfer the parental *Drosophila* to a fresh food vial. This process can be repeated for several days. Controlling the time of egg laying will ensure that the larval progeny are of a similar age and not overcrowded.

1.2.2 Screen larvae for tail-flip phenotype

1. Five days post egg laying collect 10 third instar wandering larvae (larvae that have emerged from the food and are crawling on the sides of the vial) for analysis. Use a paint brush to remove larvae from the sides of the vial. Rinse briefly in water to remove food debris and blot to remove excess liquid.
2. Place each larva on a 60 × 15 mm juice agar plate and use a stereomicroscope to visually assess the larvae for a posterior paralysis phenotype while crawling (see Figure 2).

2. ANALYSES IN MOTONEURONS FOR AXONAL TRANSPORT DEFECTS

A secondary screen is routinely used to assess whether RNAi lines identified by the “tail-flip” phenotype also exhibit defects in motoneuron transport at the cellular level. The

synaptic terminals are examined for organelle or vesicle accumulation and the nerves (bundles of motor and sensory neuron axons) for axonal jams, both hallmarks of intracellular transport defects in *Drosophila*. In addition, the motility of individual organelles and vesicles can be examined directly to assess for defects in velocity, run length, pause frequency, and flux.

The UAS-Gal4 system is used to direct expression of UAS-transgenes in motoneurons. The *OK6-Gal4* (Aberle et al., 2002; Sanyal, 2009) or *OK371-Gal4* (Mahr & Aberle, 2006) driver can be used to express transgenes in multiple motoneurons, while the *SG26.1-Gal4* (Gunawardena et al., 2003), or *eve-Gal4* (Fujioka et al., 2003), driver can be used to express transgenes in a few motoneurons per nerve. Transgenically expressed markers for synaptic vesicles (Zhang, Rodesch, & Broadie, 2002), mitochondria (Pilling, Horiuchi, Lively, & Saxton, 2006), dense core vesicles (Rao, Lang, Levitan, & Deitcher, 2001), lysosomes (Pulipparacharuvil et al., 2005), autophagosomes (Takats et al., 2013), endosomes (Zhang et al., 2007), and peroxisomes (Nakayama et al., 2011) can enhance the investigator's ability to monitor the transport of organelles/vesicles using live cell imaging.

Here we describe the preparation of equipment for larval dissections, the dissection process, live imaging of larval fillets, and the analysis of data to determine if there is a motoneuron transport defect.

2.1 MATERIALS AND EQUIPMENT

- Incubator capable of holding constant 29 °C temperature
- Spinning disk confocal microscope with imaging camera
- Sylgard 184 silicone elastomer kit (Dow Corning Corporation)
- Tungsten wire 0.125 mm diameter (World Precision Instruments, Inc.)
- Forceps (Durmont #5, 11 cm, extra fine tip; World Precision Instruments, Inc.)
- Forceps (Durmont assembling forceps, style NN; Electron Microscopy Sciences)
- Stereomicroscope (e.g., Zeiss Stemi SV6)
- Vannas scissors, 0.025 × 0.015 mm tips, straight, 8.5 cm long (World Precision Instruments)
- Custom-made slide dish (see Figure 3)
- Custom-made dissection and imaging platform mold (see Figure 3)
- Coverslips (24 × 40 mm, No. 1.5; VWR)
- 9 V battery
- Insulated electrical wire with the ends stripped
- Paper clip
- Packaging tape
- Razor blade

Medium binder clips, 1¼" width, (ACCO)

5 N NaOH

60 × 15 mm Petri dish (Fisher Scientific)

<i>Drosophila</i> Strain	Marker/Purpose	References
<i>UAS-Mito-GFP</i>	Mitochondria	Pilling et al. (2006)
<i>UAS-ANF-GFP</i>	Dense core vesicles	Rao et al. (2001)
<i>UAS-syt1-GFP</i>	Synaptic vesicle	Zhang et al. (2002)
<i>UAS-mCh-GFP-Atg8</i>	Autophagosomes	Takats et al. (2013)
<i>UAS-Lamp1-GFP</i>	Lysosomes	Pulipparacharuvil et al. (2005)
<i>UAS-Rab5-YFP</i>	Early endosomes	Zhang et al. (2007)
<i>UAS-Rab7-YFP</i>	Late endosomes	Zhang et al. (2007)
<i>UAS-SKL-GFP</i>	Peroxisomes	Nakayama et al. (2011)
<i>Ok6-Gal4</i>	Multiple motoneurons	Aberle et al. (2002) and Sanyal (2009)
<i>OK371-Gal4</i>	Multiple motoneurons	Mahr and Aberle (2006)
<i>SG26.1-Gal4</i>	Label single motoneurons	Gunawardena et al. (2003)
<i>eve-Gal4</i>	Label single motoneurons	Fujioka et al. (2003)
<i>UAS-RNAi</i> lines	mRNA knockdown	See Section 1.1

2.1.1 Recipes—HL3 saline (1 L), Stewart et al. (1994)

Reagent	Quantity	Final Concentration
NaCl	4.09 g	70 mM
KCl	0.37 g	5 mM
MgCl ₂	4.07 g	20 mM
NaHCO ₃	0.84 g	10 mM
Trehalose	1.89 g	5 mM
Sucrose	39.40 g	115 mM
Hepes	1.19 g	5 mM

Adjust the pH 7.2. Filter to sterilize and store at 4 °C.

2.1.2 Software

ImageJ (version 2.0.0)

MetaMorph (Molecular Devices, version 7.7.10.0)

2.2 METHODS

2.2.1 Preparing a Sylgard dish for imaging—To perform live imaging on either an upright or inverted microscope, a custom-made mold is used to create a dissection/imaging platform (Figure 3). To create this platform, prepared Sylgard is poured into a custom-made dish and the mold is then carefully placed onto the Sylgard. After the Sylgard polymerizes, the mold is then removed. This plastic/Sylgard dish will be used for live imaging of the

axons and neuromuscular junctions of *Drosophila* larvae. The dish must be prepared at least 1 day in advance and can be used several times until the Sylgard is damaged and no longer able to hold the dissection pins in place.

To prepare the dish:

1. Mix 10 parts (by weight) Sylgard silicone elastomer base with 1 part Sylgard silicone elastomer curing agent in a conical tube.
2. Put onto a platform rocker to mix for 10–15 min.
3. Pour Sylgard into custom-made slide dishes to be level with the top of the dish, avoid bubbles.
4. Place the custom-made mold on top of the Sylgard with the cut surface facing into the Sylgard.
5. Clamp the mold onto the dish using two-medium binder clips.
6. Turn the assembly over so that the mold is on the bottom and any bubbles end up on the bottom of the dish and not in the dissection platform. Put in an 85 °C oven overnight to cure.
7. The next day cool to room temperature and remove the mold to reveal the platform for dissections and imaging.
8. Place a piece of packaging tape over the custom slide containing the solidified Sylgard platform. Using a razor blade cut a square around the perimeter of the depressed area surrounding the imaging platform, then remove the tape from this region. The remaining tape will serve as a spacer to protect the dissected sample from being crushed by the coverslip.

2.2.2 Preparing dissection pins—Pins made from tungsten wire are used to pin down the larva during the dissection process and pin down the dorsal sides of the body wall on the hexagonal dissection/ imaging platform. Here we describe the pin-sharpening process (see Figure 4 for setup procedure and examples of pins before and after sharpening).

1. Using a 9 V battery, connect the negative terminal to the stripped end of one piece of insulated electrical wire, which is connected to a loop made at one end of an unfolded steel wire paper clip. Make a hook with the other end of the paper clip and clip it to the edge of a 60×15 mm Petri dish partially filled with 5 N NaOH, so that the hook is partially submerged in the NaOH (see Figure 4(B) and (C)).
2. The positive battery terminal should be connected to the end of another piece of insulated electrical wire, the end of which is stripped and wrapped around one arm of a number five forceps (see Figure 4(B)).
3. The tungsten wire (0.125 mm diameter) should be cut into approximately 4–5 mm pieces. Each piece of tungsten will become a pin.

4. The tungsten is held with the forceps and dipped into the 5 N NaOH while visually inspecting the sharpening process under the stereomicroscope. Bubbles should be observed coming from the paper clip wire in the NaOH when the circuit is closed and the pin is being sharpened. The tungsten wire may also bubble slightly.
5. Each tungsten piece should be dipped up and down in the NaOH solution approximately 25–30 times to result in a pin that is tapered and approximately 2 mm in length (see Figure 4(D)). Once each pin is sharpened it can be rinsed with water and placed in a Sylgard dish.

2.2.3 Larval dissections—In order to perform live imaging of motoneurons larvae must first be dissected. To dissect larvae first identify, using a stereomicroscope, the dorsal/ventral and anterior/posterior sides of the larva. In *Drosophila* the brain is on the anterior ventral side, and thus the larva must be open on the dorsal side to reveal the brain and connected axons. The ventral surface can be distinguished by the abdominal denticle belts, while the dorsal side can be identified by the tracheal dorsal trunk tube. The anterior of the larva can be identified by the black mouth hooks and fanlike projections of the tracheal system called the anterior spiracles. The posterior dorsal surface can be identified by the tracheal openings called posterior spiracles, which are yellowish in color (see Figure 5 for illustrations of all of these anatomical features).

1. Set up crosses of transgenic *Drosophila* containing a motoneuron Gal4 driver genetically recombined with a UAS–fluorescently tagged organelle or vesicle of interest (see *Drosophila* strains in the Materials section) to transgenic *Drosophila* containing the UAS-RNAi transgene of interest. Crosses should be set up and maintained as described in Section 1.1.
2. Pick third instar wandering larvae to dissect. Rinse briefly in water.
3. Using the assembling forceps to move the pins, pin the larvae at the anterior and posterior ends to the dissection platform with the dorsal side up and dorsal tracheae centered (see Figure 6(A)). Put the pins at a 45° angle to the platform.
4. Fill the small reservoir surrounding the larva with enough HL3 saline to cover the top of the larva (0.1–0.2 mL). HL3 saline should be warmed to room temperature before use.
5. At the very posterior end of the larva make a single snip perpendicular to the anterior/posterior axis of the larva using the scissors to cut only through the dorsal cuticle layer.
6. Insert the scissors into this incision and cut through the dorsal cuticle and body wall along the anterior/posterior axis between the dorsal tracheae. While cutting, carefully pull up on the scissors to avoid damage to the internal organs. Cut until the pin at the anterior end of the larva is reached.
7. Using the assembling forceps and four additional pins, pin down the anterior and posterior edges of the exposed inner dorsal body wall. Use two pins at the

anterior and two pins at the posterior to splay open the larva to match the hexagonal shape of the dissection platform (See Figure 6(C)).

8. Use Dumont #5 forceps to detach the gut tube (hindgut, arrowhead in Figure 6(D)) and the tracheal dorsal trunk tubes at the posterior of the larva. At the anterior end detach the proventriculus (foregut immediately behind the brain, arrowhead in Figure 6(E)) from above the brain and the dorsal trachea. Use the forceps to remove all of the tissue except the brain, attached axons, body wall muscles, and imaginal discs. Removal of the imaginal discs is optional as they do not interfere with imaging. The brain and attached axons should be left undamaged.
9. Push the pins down so that the top of each of the pin heads sits below the Sylgard dissection platform level (see Figure 6(G)).
10. Wick up the HL3 media from the reservoir and replace with fresh HL3 media, covering the larva in a thin layer.
11. Lay a 24 × 40, No. 1.5 coverslip toward the posterior of the animal and gently push even with the top of the slide. Remove all excess liquid from the edges of the slide.

2.2.4 Live imaging of organelle/vesicle motility, axonal jams, and synaptic terminals

—Fast axonal transport of membranous organelles can occur at rates up to 5 mm/s (Brown, 2003). Capturing this motility requires fast imaging acquisition settings, 5–30 frames per second depending on the size of the vesicle or organelle and the spatial resolution required to resolve the event(s) of interest. At the same time the larval prep is relatively thick, and imaging thus greatly benefits from confocal microscopy. In our experience best results are achieved by using a spinning disk confocal microscope equipped with a high speed and high sensitivity camera, such as an Electron Multiplying CCD. A 60× – ×100 objective with high numerical aperture should be used for best resolution.

• **Organelle/Vesicle motility in axons:** It is important to image all samples at the same anterior/posterior position of the axons as axonal transport defects become more pronounced as the distance from the cell body increases.

1. Find the posterior end of the ventral ganglion and center it in the field of view.
2. For vesicle/organelle motility in axons move the stage to select a field of view 800–1000 μm posterior to the ventral ganglion for imaging.
3. Set up a time-lapse acquisition. The exposure time necessary will depend on the system's lasers, objective, and camera. The time interval will also need to be determined empirically as the velocity of each organelle or vesicle varies. Capture enough frames for analysis of vesicle/organelle motility. In general we capture 200 frames. When imaging note the orientation of the axon in the image with respect to proximal versus distal distance from the ventral nerve cord (VNC). This will be important for later analysis to decipher retrograde versus anterograde motility.

• **Axonal jams:** Axonal jams can be identified by eye under the fluorescence microscope as an uneven distribution of a particular vesicle or organelle. This accumulation of organelles in a restricted portion of the axon results in the swelling of the axon, sometimes to the width of the nerve (bundled axons).

1. To image axonal jams, as above, move the stage to select a field of view 800–1000 μm posterior to the ventral ganglion.
2. Set up a Z-series acquisition. Set the top slice above the nerve and the bottom slice just below. To avoid oversampling and photobleaching or under sampling and losing or distorting information the optimal slice interval should be determined based on Nyquist sampling criterion (Murphy & Davidson, 2013).

Figure 7 shows an example of axonal jams accumulating dense core vesicles when *dhc64c* is depleted in motoneurons.

• **Synaptic terminals:** Membranous material accumulates in the distal-most boutons of the synaptic terminal when Dynein/Dynactin is depleted (Lloyd et al., 2012).

1. The larval body wall musculature has a well-defined pattern that has been extensively characterized (Gorczyca & Budnik, 2006). The pattern of the muscles is repeated in abdominal segments A2–A7 (Figure 8). Abdominal segments A2–A6 are typically imaged, since the more posterior segments are easily damaged in the dissection process. The abdominal segments can be identified in two ways. First, using differential interference contrast (DIC) microscopy, the abdominal segments can be identified by the muscle pattern. Abdominal segment A1 does not have muscles 6 and 7, but a larger muscle 31 to which muscles 6 and 7 of abdominal segment A2 are attached (See Figure 8). The second way to identify abdominal segments is by using the denticle belts. The denticle belts appear as rows of teeth at the anterior of each body segment. Focus through the larva until reaching the ventral surface containing the denticle belts. Starting at the anterior and moving toward the posterior, the denticle belts of the thoracic body segments will be observed first and contain small-sized teeth. The abdominal denticle belts begin in segment A1 and include a mixture of larger black teeth as well as small teeth. The muscles directly behind the A2 denticle belt belong to body segment A2.
2. We image neuromuscular junction 4 because the synaptic terminal is relatively flat and the muscle is easily identified. Muscle 4 is in the most interior layer of muscles and therefore the first and most accessible layer of muscles as you focus into the dissected sample. Muscle 4 can be identified using DIC microscopy by first finding the herringbone or V-shaped patterned muscles (15/16/17) at the midline of the larvae. Next to muscles 15/16/17, but in the interior most layer, lie muscles 6/7. Muscles 6/7 are connected at the posterior to muscle 5, which is at a 45° angle to 6/7. The anterior of muscle 5 is connected to the anterior of muscle 4 (see Figure 8 to identify muscle 4 and abdominal segments A1/A2 using the larval musculature).

3. After identifying the body segment and muscle 4, images of NMJ4 can be obtained for all hemisegments (both to the left and right of the larval midline) of A2–A6. We often find that phenotypes are more pronounced in the posterior body segments and it is thus important to note which hemisegment is imaged for consistent analysis. Z-stacks of the NMJ should be obtained by setting the top slice above the synaptic terminal and the bottom slice just below. As discussed above, the optimal slice thickness for Nyquist sampling will need to be determined for the objective being used.

An example of dense core vesicle accumulation in motoneuron terminal boutons resulting from *dhc64c* depletion is shown in Figure 9.

2.2.5 Analyses of organelle/vesicle motility and accumulation in distal boutons

• **Kymograph analyses of organelle/vesicle motility In axons:** A kymograph is a 2D plot of time versus distance and can be created from time-lapse images of organelle or vesicle transport. Kymographs are used to quantify transport dynamics and directionality. We use the MetaMorph software to create kymographs of organelle or vesicle motility in a single axon. MetaMorph provides standard routines that have been developed for the expressed purpose of analyzing motility data. While several MATLAB programs have been developed to automate such measurements, in our hands we have not found the accuracy of such automated measurements to be satisfactory. Therefore, we employ a significant user interface in the motility measurements. Based on the parameters captured in the kymograph, the run length of a given organelle or vesicle, the segmental velocity, the flux, and the pause frequency may be calculated. These parameters when compared to wild-type controls assist in characterizing the intracellular transport defect. Results from kymographs made from multiple larvae imaged under the same setting can be compiled for analysis.

1. First open the time-lapse image to be analyzed in MetaMorph. Then under the Measure tab select calibrate distances. Under this dialog box select the objective used or enter in the pixel size for x,y in micrometer. Hit the Apply button to apply these settings to the open image. Under the Stack tab select Set Plane Time Increment. In this dialog box enter in the time increment used for capturing images. Hit the Apply button to apply these settings to the open image.
2. Under the Stack tab select kymograph. A kymograph dialog box will open. Draw a line along the axon of interest from the region of the axon proximal to the VNC to the more distal region. In the kymograph dialog box hit the create button. A kymograph will then be created from the selected line region. Diagonal lines from the upper left to lower right are organelles/vesicles moving in an anterograde direction (see Figure 10 for examples of kymographs).
3. For analysis purposes the Open Log button within the kymograph dialog box will open an excel file to save the data collected. The Configure Log button allows for the selection of which data will go into the excel file Log. We collect x,y coordinates, distance, time, and velocity. The line tool can be used to select a run or a segment as defined below. Once all of the runs or segments for anterograde

or retrograde have been drawn, the lines can be selected and pushing “F9” on the keyboard will record the log information for that particular line. Analyses for anterograde and retrograde runs/segments are collected into separate excel files.

Run length: The run length is the net distance traveled for any organelle or vesicle and is calculated separately for movements in the anterograde versus retrograde directions. This parameter is calculated for only those vesicles that appear in both the first and last frame of the kymograph, and includes organelles/vesicles that momentarily pause during the run. In the schematic of a kymograph in Figure 10(C) the run length for a retrograde moving vesicle would be calculated by drawing a diagonal line from point A to point B. The average velocity for each run will also be provided by MetaMorph and can be analyzed separately.

Segmental velocity: The segmental velocity is the average velocity of all of the segments and is also determined separately for movements in the anterograde versus retrograde directions, respectively. In the kymograph schematic in Figure 10(C) retrograde segmental velocities would include the velocity from point C to D plus D to E and all of the other segments moving in a retrograde direction. Segment E–F is moving in an anterograde direction and although part of the same total run as C–D and D–E, segment E–F would be added separately to the anterior segmental velocities. It is important to note here that this calculation does not take into account segments where the vesicle or organelle is not in motion. The run length of each segment will also be provided by MetaMorph and can be separately analyzed.

Flux: Flux is the number of moving particles crossing a certain position (X) during a given time period and is usually expressed as a ratio of particles per unit time (e.g., number of particles/minute). Organelle or vesicle flux can be determined by bleaching a region of a nerve or axon and counting the number of organelles/vesicles that move into the bleached area in a retrograde or anterograde direction over time (Moughamian & Holzbaur, 2012; Pilling et al., 2006; Shidara & Hollenbeck, 2010). However, not all spinning disk confocals allow for regional bleaching of the nerve or axon so flux must be determined by other means. For example, the very edge of a kymograph made from single axons can be designated as the reference position for flux analysis. The number of vesicles that cross the reference point as they enter or exit the image, can be scored as a function of time and reported as the anterograde or retrograde flux, respectively. This results in a flux rate calculation for each kymograph. In the example in Figure 10(C), designating the position for flux analysis at the left-hand side of the kymograph, the anterograde flux would be 3 vesicles/min and the retrograde flux would be 2 vesicles/min.

Stationary/paused vesicles: Stationary vesicles are not in motion during the entire acquisition time, while paused vesicles stop momentarily during a run. In a kymograph, a stationary or paused vesicle appears as a straight vertical line (Figure 10(C), point G is a stationary vesicle). One can determine the number of stationary vesicles/organelles for the time duration of the kymograph and then separately report the percentage of stationary vesicles as a function of the total number of vesicles in each kymograph. For vesicles or

organelles that pause, the average pause time and frequency can be calculated for comparison between genotypes.

• **Distal bouton accumulation analysis:** To quantify the percentage of terminal boutons that exhibit an accumulation of particular organelles or vesicles, we compare the mean fluorescence intensity of the terminal bouton to that of the next proximal bouton in the synaptic terminal. ImageJ software is used to measure the fluorescence intensities. As an example, the results of quantifications of distal bouton accumulation of dense core vesicles for wild type and *dhc64c* depleted motoneurons are given in Figure 9(C).

1. In ImageJ, a Z-stack taken as either an 8 bit or 16 bit image is opened and made into a 32 bit Z-projection of the type sum of slices.
2. On this image the oval tool is used to draw a circle that encompasses the entire terminal bouton and the mean intensity of this area is measured. The background fluorescence intensity is then measured for the same circular area in an adjacent location outside of the synaptic terminal and subtracted from the terminal bouton intensity.
3. Next, the fluorescence intensity of the neighboring, proximal bouton is measured in a similar fashion. Again, a circular template is drawn that encompasses the entire proximal bouton and the mean fluorescence intensity is determined. As above, the mean background intensity is also quantified in the adjacent area and subtracted from the mean proximal bouton intensity.
4. After collecting the data for all of the terminal and proximal bouton pairs, we calculate the ratio of the terminal bouton mean intensity to the neighboring proximal bouton mean intensity. Here we consider a ratio of intensity greater than 1.5 in the terminal bouton compared to the neighboring bouton to be accumulation of an organelle. The terminal bouton accumulation results for RNAi depleted larvae should always be compared to wild-type, outcrossed controls for the same organelle that has been imaged using comparable imaging conditions.

3. ANALYSIS OF DISRUPTED INTRACELLULAR TRANSPORT IN CLASS IV DENDRITIC ARBORIZATION NEURONS

In this secondary screen we test whether RNAi targeting of candidate genes induces phenotypes in the class IV da neuron consistent with loss of function of the microtubule motor Dynein or Kinesin. Specifically we monitor the dendritic arbor morphology and subcellular distribution of membrane cargoes.

UAS–transgene expression is directed to class IV da neurons using the driver *ppk-Gal4*, which expresses Gal4 under control of the *pickpocket* (*ppk*) gene promoter (Grueber et al., 2007). The transgene *ppk-CD4tdGFP* fluorescently labels the class IV da neuron plasma membrane (Han, Jan, & Jan, 2011), and is also observed on endomembranes. CD4tdGFP consists of the transmembrane domain of the human CD4 protein fused to a tandem dimer of GFP.

Analysis is performed in a subtype of class IV da neurons termed ddaC (Grueber et al., 2002). The ddaC neurons are located on the dorsal side of larvae, and each body segment contains two ddaC neurons positioned on opposite sides of the dorsal midline. Typically these neurons are imaged in abdominal body segments A2 –A6. Analysis is performed using wandering, late third instar larvae, a developmental stage when the dendritic arbor of class IV da neurons is fully formed.

3.1 MATERIALS AND EQUIPMENT

Incubator capable of holding constant 29 °C temperature

Stereomicroscope (e.g., Zeiss Stemi SV6)

Confocal microscope equipped with motorized stage and CCD camera (ORCAER, Hamamatsu)

Glass slides (Gold Seal Micro Slides)

Glass coverslips (24 × 40, No. 1.5; Fisherbrand)

Double-sided tape (Permanent Double Sided tape, 12.7 mm wide, 3M Scotch®)

Forceps (Durmont assembling forceps, style NN; Electron Microscopy Sciences)

<i>Drosophila</i> Strain	Purpose	References
<i>ppk-Gal4</i>	Class IV da neuron driver	Grueber et al. (2007)
<i>ppk-CD4tdGFP</i>	Fluorescent plasma membrane marker	Han et al. (2011)
<i>UAS-dicer2</i>	Increase RNAi efficiency	Dietz et al. (2007)
<i>UAS-RNAi lines</i>	mRNA knockdown	See Section 1.1

3.1.1 Software

MetaMorph v.7.1.7 (Molecular Devices)

ImageJ v.1.49m (Wayne Rasband, National Institutes of Health, USA)

Simple Neurite Tracer (Longair, Baker, & Armstrong, 2011)

Sholl analysis v.3 (Ferreira et al., 2014)

Photoshop CS2 (Adobe Systems Incorporated)

3.2 METHODS

3.2.1 Preparation and mounting of larvae for imaging

1. Set up crosses of transgenic *Drosophila* containing *ppk-Gal4* and *ppk-CD4tdGFP* to transgenic *Drosophila* containing the *UAS-RNAi* transgene of interest. Crosses should be set up and maintained as described in Section 1.1.
2. Pick a single, wandering third instar larva and wash the larva briefly in water.
3. Attach two pieces of double-sided tape to a glass slide. The pieces of tape should be separated by approximately 18 mm (see Figure 11).

4. Use blunt tip forceps to gently transfer the washed larva to the glass slide. The larva should be centered between the two pieces of tape, with the dorsal side of the larva up and the anterior–posterior axis of the larva perpendicular to the long edge of the glass slide.
5. Place a 24 × 40 mm coverslip over the larva and push down on the edges of the coverslip to firmly attach the coverslip to the double-sided tape. The pressure applied to the larva will flatten and fully spread open body segments and dendritic arbors of class IV da neurons. Be careful not to apply excessive pressure to the coverslip, which will cause the larva to rupture. Immediately commence imaging of the larva; imaging should be completed within 20 min of mounting.

3.2.2 Live imaging of ddaC class IV da neurons

1. Mount the slide on the stage of a confocal microscope and use a 20× objective to locate the green fluorescent CD4tdGFP signal of a ddaC class IV da neuron in body segment A2. To do this, begin by adjusting the focus until the CD4tdGFP signal of a ddaC neuron in any body segment is in view. Then, adjust the stage X–Y position until the most anterior body segment is in view. Finally, using the anterior segment as reference, move the field of view posteriorly until the fifth body segment from the anterior end is reached; this is A2. Note the anterior border of body segments is delineated by rows of large dorsal hairs. These dorsal hairs, although slightly out of focus, are visible in the focal plane of the ddaC neuron dendritic branches (see Figure 12).
2. Capture Z-stacks to document the morphology of the dendritic arbor. Choose one of the two ddaC class IV da neurons in body segment A2 to image. Image the CD4tdGFP signal using a 20× objective with high numerical aperture. Z-stacks should cover a 20 mm range, 10 mm above and 10 mm below the focal plane containing most dendritic branches. Use an optical slice interval appropriate for the numerical aperture of the 20× objective to achieve Nyquist sampling. To document the full area of the dendritic arbor, 12–14 Z-stacks capturing different fields of view are required. The fields of view of adjacent Z-stacks should slightly overlap to facilitate later arbor reconstruction. MetaMorph computer software is used to control microscope image acquisition performed in this step and the next.
3. Image the ddaC class IV da neuron axon at high magnification. Switch to a high magnification objective (60–100×) and perform Z-stack image capture of the axon over a depth of 20 mm. Note that the axon extends inwardly into the larva; thus only the portion of the axon proximal to the soma is imaged.
4. Generate maximum intensity projections for all Z-stack files captured in step 2 and 3 above. Save the max intensity projection files in 16 bit tiff format. MetaMorph software is used for these image processing operations.

3.2.3 Reconstruction of the dendritic arbor

1. Open the 12–14 dendritic arbor Z-stack projection tiff files in Photoshop. Apply consistent adjustments to the upper and lower pixel intensity bounds of the pixel intensity histogram until the dendrites are visually distinguishable from background.
2. Open a new Photoshop file having the same resolution (pixels per inch) as each Z-stack projection but with 4× greater X–Y dimensions. This file will serve as a canvas to reassemble the full dendritic arbor as a montage of Z-stack max intensity projections.
3. Copy and paste the max intensity projections onto the new canvas. Each max intensity projection is now a unique layer on the canvas. Align layers by locating and matching the regions of dendritic branch overlap contained in max intensity projections of adjacent fields of view. The alignment process is facilitated by adjusting layer opacity and forward–backward arrangement. When the layers are properly aligned, flatten the image. Save this montage containing light dendrites on a dark background as a 16 bit tiff file for dendritic arbor analysis in the next step. For display of the arbor, we typically invert the image Lookup Table (LUT) so dendrites are dark on a light background.

Figure 12 shows the reconstructed dendritic arbors of control and dhc64c-depleted ddaC class IV da neurons.

3.2.4 Analysis of da neuron morphology

1. Open the dendritic arbor montage in ImageJ and then open the plugin “Simple Neurite Tracer.” Use this plugin to trace all dendritic branches for the neuron of interest. To facilitate accurate tracing of distal dendrites that border the dendrites of neighboring class IV da neurons, reference the original Z-stack files to view dendritic paths in three dimensions. When tracing is complete, export the data in csv file format. Then select “Make Line Stack” under the “Analysis” tab. This will convert all traced branches into a black and white binary image of the arbor. Save this binary image in tiff file format.

Figure 13(A) shows the black and white binary images of control and dhc64c-depleted neurons.

2. Perform Sholl analysis on the dendritic arbor binary image. In Sholl analysis (Sholl, 1953) a series of circles with increasing radii are centered on the soma, and the number of dendritic branch intersections for each circle is measured (see Figure 13(B)). Open the dendritic arbor binary image in ImageJ and set the image scale using the pixel–distance conversion factor specific to the microscope camera, objective and binning setting used for imaging of the arbor. Draw a line segment starting at the center of the soma and extending to the edge of the image. Open the plugin “Sholl analysis.” Set the starting radius and radius step size to 10 μm , and select “Intersections” for Sholl method. Press “OK” to run analysis. A Sholl profile

which plots number of intersections versus distance from soma will be generated. This data can be saved and plotted in alternative graphing software.

Figure 13(C) shows Sholl analysis plots that quantify the pronounced proximal shift in dendritic branches caused by *dhc64c* RNAi expression.

3. Calculate total dendritic branch length. Open the csv file from step 1 above in spreadsheet software such as Microsoft Excel. The file contains distance data in pixel units for all traced dendrites. Sum the pixel data and then multiply this value by the pixel–distance conversion factor. The resulting value equals total dendritic branch length for the arbor in micrometer units.

In Figure 13(D), the effect of *dhc64c* RNAi to decrease total branch length is shown in bar graph format.

4. To visualize accumulation of membrane material in the axon, open the axon Z-stack max intensity projection tiff files in Photoshop. Apply consistent adjustments to the upper and lower pixel intensity bounds of the pixel intensity histogram until the axon of the wild-type neuron is visually distinguishable from background.

Figure 14 shows the effect of *dhc64c* RNAi to induce proximal axon accumulation of endomembranes labeled with the general membrane marker, CD4tdGFP. The membrane localization defect can be further characterized using transgenes that label specific dendritic organelles and proteins (Zheng et al., 2008).

CONCLUSION

We described here methods to perform primary and secondary genetic screens in *Drosophila* to identify gene products that impact neuronal transport. Together these genetic and cell biological screening methodologies should result in a high confidence list of candidate gene products important for efficient neuronal transport. Further detailed molecular characterization of these gene products should lead to a greater understanding of the molecular pathways impacting intracellular transport in healthy and disease states.

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FIGURE 1. The cross scheme for an RNAi screen to identify gene products involved in intracellular transport

Transgenic virgin female *Drosophila* carrying a motoneuron driver, *OK6-Gal4*, and *UAS-dicer2* are crossed to male transgenic *Drosophila* carrying a *UAS-RNAi* insertion. The resulting larvae from this cross are depleted of the gene product targeted by expression of the *UAS-RNAi* transgene in motoneurons. The larval progeny are screened for the posterior paralysis, “tail-flip” phenotype.

FIGURE 2. The “tail-flip” phenotype is observed when Dynein is depleted in motoneurons
Depletion of dynein in motoneurons, using the RNAi transgene, *UAS-dhc64c RNAi*, and the motoneuron driver, *OK6-Gal4*, results in the “tail-flip” posterior paralysis phenotype. The paralysis resulting from deletion of *dhc64c* causes the larva to lift its posterior in the air as it crawls (B). This phenotype is not observed in control larvae lacking the *UAS-dhc64c RNAi* transgene (A).

FIGURE 3. Creating an imaging platform using custom dissection dishes and imaging molds
 Dissection dishes and imaging platform molds were custom milled and heat polished by a commercial machine shop using the following diagrams. Both the dissection dish and mold were milled out of acrylic plastic and heat polished to improve smoothness and transparency. The size and shape of the dissection dish were chosen to fit the microscope stage being used and to allow for DIC imaging. Orthogonal views of the dissection dishes are given in (A) and a 3D image is given in (A'). Orthogonal views of the imaging platform mold and dimensions are given in (B) and a 3D image of the mold is in (B'). (C) A dissection dish with the resulting Sylgard platform. (C') The Sylgard dissection platform with surrounding well.

FIGURE 4. Preparation of dissection pins

To prepare pins for dissection a 60×15 mm petri dish is filled with 5N NaOH and set under a stereomicroscope to visually inspect the sharpening process (A). A steel metal paperclip is unfolded and one end is made into a small loop, while the other is made into a hook. The loop end of the paper clip is connected to the stripped end of insulated electrical wire connected to the negative electrode of a 9V battery (B). A Durmont #5 forceps is wrapped with stripped electrical wire on one end and connected to the positive electrode of the battery on the other end (B). The hooked end of the paper clip is clipped to the side of the petri dish to submerge the tip in 5N NaOH (C). Tungsten wire is cut into 4–5 mm pieces and held with the Durmont forceps while being dipped in the NaOH bath to sharpen them into 2 mm pins. (D) A piece of tungsten before sharpening and a pin after sharpening are shown next to a millimeter ruler. Each hash mark indicates 1 mm.

FIGURE 5. Anatomical features of larvae used to distinguish the anterior/posterior and dorsal/ventral surfaces

At the anterior the black mouth hooks used for feeding, and the anterior spiracles, finger-like projections of the tracheal system. The posterior can be identified by the posterior spiracles, which are yellow in color and located on the dorsal posterior side of the larvae. The denticle belts or black teeth-like protrusions at the anterior of each abdominal body segment can be used to distinguish the ventral surface. As *Drosophila* larvae are translucent, the dorsal surface can be identified by the internal dorsal trunk tracheal tubes that run dorsally along the anterior/posterior axis from the posterior spiracles to anterior spiracles.

FIGURE 6. The dissection technique to fillet larvae for imaging of motoneurons

(A) To dissect, third instar wandering larvae are pinned along the long axis of the Sylgard platform. Using a single pin in the anterior end of the larvae, near the mouth hooks, and a single pin on the posterior of the larvae at the posterior spiracles, push the pin through the larvae and into the Sylgard adjacent to the platform. (B) A larva after the dorsal side has been cut open. (C) A dissected larva with the inner dorsal surface pinned to the hexagonal platform. (D) A zoomed in view of the posterior of the dissected larva showing the hindgut (arrowhead). (E) A zoomed in view of the anterior of the dissected larva showing the brain lobes (arrows) and proventriculus (arrowhead). (F) A dissected larva after the removal of the gut, dorsal tracheae, and fat body. (G) A diagram of pins inserted in the Sylgard around the dissection platform before and after pushing the pins down so that the pin sits below the platform. Arrowheads in (A, B, and C) indicate dorsal tracheae, arrows in (C, F) indicate the brain.

FIGURE 7. Axonal jam analysis in nerves

Axonal jams, containing dense core vesicles labeled with ANF-GFP, result when *dhc64c* is depleted by RNAi (B), axonal jams are indicated by arrowheads). These axonal jams are rarely seen in wild-type nerves at the same distance from the ventral nerve cord (A). Bar: 10 μm.

FIGURE 8. The musculature of *Drosophila* larvae is used to identify abdominal segments and muscles for imaging

(A) A cartoon drawing of the musculature of hemisegments A1, A2, and A3 of a dissected larva. The musculature can be used to identify which abdominal segments and muscles are being examined. The midline of the larvae can be determined by the herringbone pattern or V-shaped patterned muscles 15/16/17. The most interior layer of muscles is illustrated in white, while the next layer is shown in gray. The most interior layer of muscles that lie next to the herringbone pattern of muscles are muscles 6/7. Abdominal segment A1 does not have muscles 6/7 but a wider muscle 31, which is attached to muscles 6 and 7. To find muscle 4, to image NMJ4, one first must find muscles 6/7. Muscles 6/7 are connected at the posterior to muscle 5 at a 45° angle. Muscle 5 is attached to the anterior of muscle 4. (B) The musculature of hemisegments A1 and A2 in a dissected larval prep with muscles 31, 6, 7, 5, and 4 denoted. The ventral nerve cord (VNC) of the brain is also denoted.

FIGURE 9. Analysis of terminal bouton accumulation in motoneurons

Depletion of Dynein in motoneurons results in terminal bouton accumulation of ANF-GFP vesicles (B compared to A, wild type), as has been reported by Lloyd et al. (2012). (C) Terminal bouton accumulation was quantified for 17 synaptic terminals from 4 larvae in abdominal segments A4-A6 of wild type (outcrossed) *OK6-Gal4, UAS-ANF-GFP* ($n = 40$ boutons) or *dhc64c* depleted *OK6-Gal4, UAS-ANF-GFP, UAS-dhc64c RNAi* larvae ($n = 27$ boutons). This quantification was done in ImageJ to determine the ratio of the mean intensity in the terminal bouton to the mean intensity of the neighboring proximal bouton in the synaptic terminal (see insets in A and B for examples of terminal boutons and neighboring proximal boutons). Bar: 10 μ m.

FIGURE 10. Representative kymographs for wild-type and Dynein-depleted motoneuron axons and a kymograph schematic to illustrate parameters analyzed

Examples of kymographs for ANF-GFP labeled dense core vesicles in wild-type axons and axons depleted of dynein by expression of *UAS-dhc64c RNAi*. The *SG26.1-Gal4* driver was used to express *UAS-ANF-GFP* in a subset of motoneurons. Motility of ANF-GFP vesicles was imaged 800–1000 μm from the VNC. MetaMorph software was used to make a kymograph of a selected single axon. (A) An axon from a wild-type motoneuron shows many motile ANF-GFP vesicles while (B) an axon from a motoneuron depleted of dynein shows many fewer vesicles and many stationary or paused vesicles. (C) A schematic representation of a kymograph. A run length calculation can be determined for a vesicle found in the first and last frame such as segment A–B. Retrograde segmental velocities can be measured for segments C–D and D–E, while the anterograde segmental velocity can be calculated for segment E–F. A stationary vesicle is illustrated by point G. The flux calculated at the left edge of the kymograph is 3 vesicles/min anterograde and 2 vesicles/min retrograde.

FIGURE 11. A 3rd instar larva mounted between a coverslip and a glass slide using double-sided tape

The larva is positioned on the glass slide dorsal side up between two pieces of double-sided tape approximately 18 mm apart. The anterior–posterior axis of the larva is perpendicular to the long edge of the slide. Attachment of the coverslip to the double-sided tape compresses the larva and spreads open body segments allowing full imaging of class IV da neuron dendritic arbors.

FIGURE 12. Reconstructed class IV da neuron dendritic arbors from wild type and dhc64c RNAi larvae

The dendritic arbors are reassembled as a montage of Z-stack max intensity projections. In the left panel, the dendrites extending from a wild-type neuron cover most of the area contained in the panel. In contrast, in the panel at right, the majority of dendrites extending from a dhc64c-depleted neuron are located close to the soma. The boxed area in the upper right corner of the right panel highlights dorsal hairs that serve as useful markers of the anterior-posterior borders of body segments. Bar: 100 μ m.

FIGURE 13. Analysis of class IV da neuron dendritic arbor morphologies

(A) Black and white binary images of traced dendritic arbors are generated using the ImageJ plugin "Simple Neurite Tracer." These binary images are then analyzed using the ImageJ plugin "Sholl analysis." (B) Cartoon illustrating Sholl analysis methodology. A series of circles with increasing radii are centered on the soma. The number of times dendritic branches intersect each circle is measured. (C) Sholl analysis profiles show that in wild-type neurons the highest dendritic branch complexity is found approximately 230 μm from the soma. In contrast, dhc64c RNAi expressing neurons show the most dendritic branch complexity approximately 50 μm from the soma. (D) Total branch length is also reduced by dhc64c RNAi.

FIGURE 14. Analysis of endomembrane accumulation in the proximal axon

CD4tdGFP labels the plasma membrane of the somatodendritic and axonal compartments, and is also present on endomembrane material in wild-type and *dhc64c*-depleted neurons. Robust accumulation of the CD4tdGFP-labeled endomembrane material is observed in the proximal portion of the axon when Dynein is depleted but not in wild-type neurons (arrowheads). This is consistent with the reported effect of loss of Dynein function to cause dendritic membrane material to accumulate in the proximal axon. Asterisks mark the cell body. Bar: 10 μ m.