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Live Imaging Mitochondrial Transport in Neurons

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Abstract

Mitochondria are among a cell's most vital organelles. They not only produce the majority of the cell's ATP but also play a key role in Ca^{2+} buffering and apoptotic signaling. While proper allocation of mitochondria is critical to all cells, it is particularly important for the highly polarized neurons. Because mitochondria are mainly synthesized in the soma, they must be transported long distances to be distributed to the far-flung reaches of the neuron—up to 1 m in the case of some human motor neurons. Furthermore, damaged mitochondria can be detrimental to neuronal health, causing oxidative stress and even cell death, therefore the retrograde transport of damaged mitochondria back to the soma for proper disposal, as well as the anterograde transport of fresh mitochondria from the soma to repair damage, are equally critical. Intriguingly, errors in mitochondrial transport have been increasingly implicated in neurological disorders. Here, we describe how to investigate mitochondrial transport in three complementary neuronal systems: cultured induced pluripotent stem cell-derived neurons, cultured rat hippocampal and cortical neurons, and *Drosophila* larval neurons in vivo. These models allow us to uncover the molecular and cellular mechanisms underlying transport issues that may occur under physiological or pathological conditions.

Keywords

Neurodegenerative disease; Mitochondrial transport; Microtubules; Axonal transport; Embryonic neurons; iPSCs; *Drosophila*; Larva; Live imaging

1 Introduction

A neuron's complex function is mirrored by its complex form: its processes can be long and intricately branched. This complicated form makes it challenging for neurons to transport the materials required for structure and function maintenance, both in and out of cell bodies and to far-flung neurites. Mitochondria are among the most crucial organelles requiring proper transport, as they perform aerobic respiration for ATP production, provide a reservoir for Ca^{2+} ions, synthesize heme compounds and some steroids, produce reactive oxygen species (ROS), and signal apoptosis. Neurons contain many areas of sub-specialization, like the presynapse, post-synapse, soma, axon, and dendrites, so the optimal density of mitochondria differs between and within cells as demands of the neuron shift to different

subcellular domains [1]. These changing requirements for mitochondria are reflected in the fact that at any given time in a healthy neuron, 30–40% of mitochondria are in motion [2–4]. In addition to the services they provide to the cell, when mitochondria are damaged or dysfunctional, they can produce an excess of ROS, which can in turn destroy cells. Damaged mitochondria must either be replenished by newly synthesized mitochondria transported from the soma, or the entire mitochondrion must be transported back to the soma for degradation, or cleared inside neurites in situ [5–7]. Precise regulation of mitochondrial transport in neurons is therefore critical to neuronal health.

Most mitochondrial movement occurs on microtubules. Movement towards the “plus” end of the microtubule—in the case of an axon, the axon terminal—is called anterograde motion, and occurs using the kinesin motors. Movement towards the “minus” end of the microtubule—in this case, the cell body—is called retrograde motion, and occurs using the dynein motor [1, 3, 8, 9]. Some mitochondria may need to go long distances without pausing, while others may need to go short distances with frequent pausing. For example, Ca^{2+} can cause mitochondria to detach from microtubules, thus allowing mitochondria to stop in areas of high Ca^{2+} -buffering demand or low ATP supply [10–12]. In this case, the mitochondrial ability to arrest motion is just as important as its ability to move.

Given the vital importance of mitochondria to cellular health and the complexity of controlling their proper localization, it is unsurprising to learn that impaired mitochondrial motility can seriously endanger a cell. Disruptions in neuronal mitochondrial transport and distribution have been closely correlated with many psychiatric and neurodegenerative disorders, like Charcot–Marie–Tooth disease [13], amyotrophic lateral sclerosis (ALS) [14], Alzheimer’s disease [15–18], Huntington’s disease [19–21], hereditary spastic paraplegia [22, 23], Parkinson’s disease [5, 24], and schizophrenia [25–27]. Studying the fundamental principles that control mitochondrial motility in model systems may help elucidate the causes of neurological pathologies. Perhaps one of the clearest examples of this is found in the case of Parkinson’s disease, where two familial Parkinson’s disease-causing genes, *PINK1* (*PTEN-induced putative kinase 1*) and *Parkin*, have been found to function in the same pathway to arrest mitochondrial movement [5, 24]. This finding suggests that some Parkinsonian neurodegeneration may result from failure to arrest mitochondrial transport.

Using static imaging to study mitochondrial motility is insufficient for understanding how mitochondria distribution is achieved, and by what mechanisms their aberrant distribution may be pathogenic; therefore, we must live image mitochondrial transport in neurons. Huge strides have been made in live imaging axonal mitochondria in the past few years. One advantage of live imaging axons is that they have long, parallel arrays of microtubules, which makes following long distance movement of mitochondria straightforward. Also, because axons have largely uniform polarity, with “plus” ends pointing towards the axon terminals and “minus” ends towards the cell bodies, it is easy to distinguish kinesin- versus dynein-mediated movement. This homogeneity is not found in dendrites, which in mammals exhibit mixed polarity [28, 29]. Difficulties with live imaging mitochondria in axons involve finding adequate mitochondrial markers, keeping neurons alive during imaging, preventing the neuronal network from obscuring individual mitochondrial movement, and getting light to penetrate in vivo tissues.

Here, we introduce methods for live imaging mitochondrial transport in axons, and address these concerns. We describe three complementary systems that have been used successfully in our laboratory: cultured induced pluripotent stem cell (iPSC)-derived neurons, cultured rat hippocampal and cortical neurons, and *Drosophila* larval neurons which are an in vivo model. Together, these systems allow for clear observation of mitochondrial movement, with the potential for easy genetic or chemical manipulation.

2 Methods

2.1 Live Imaging Mitochondrial Transport in iPSC-Derived Neuronal Axons

Since their introduction in 2007, iPSCs have revolutionized our ability to explore mechanistic pathways in physiologically relevant models. To generate iPSCs, terminally differentiated somatic cells from a subject (usually skin fibroblasts) are reprogrammed to a pluripotent cell state. There are several ways to reprogram the cells; the original and most common method employs cocktails of embryonic transcription factors, as discovered simultaneously in the laboratories of Dr. James Thomson and Dr. Shinya Yamanaka [30, 31]. iPSCs have emerged as an extraordinary human disease model, as they allow us to observe and experiment in human cells, while obviating ethical qualms with embryonic stem cells, and giving more flexibility for cell type differentiation than adult stem cells. Derivation of iPSCs from patients with diseases to the cell types most vulnerable in their diseases makes findings highly translatable. Here, we briefly discuss how to derive iPSCs to dopaminergic neurons, the cell type which degenerates in Parkinson's disease, and how to image and analyze mitochondrial transport in iPSC-derived neuronal axons.

iPSC-derived neurons exhibit characteristic morphologies and after 28 days of neuronal differentiation, axons can be easily identified by their unique structure, which is long and thin with uniform diameters and a lack of branching [29, 32]. Cultured neurons can be transiently transfected with constructs encoding a mitochondrial targeting sequence fused with a fluorescent protein, such as the red fluorescent mito-DsRed, to label live mitochondria. To simultaneously visualize neuronal morphology, presynaptic or postsynaptic specializations, or other organelles, cultured neurons can be co-transfected with an additional marker fused with green or blue fluorescent protein [5, 9, 10, 33]. In this way, neurons are sparsely transfected, allowing the process of a single transfected neuron to be examined and the orientation to be determined. Dense transfection, like with viral infection, can obscure individual axons and the origin of the cell body. Mitochondrial-specific dyes can also be used to aid in visualization, but just as with dense transfection, dyes mask observation of individual mitochondrial movement since they stain all mitochondria in the dish.

Additionally, agonists, antagonists, toxins, and ionophores can be easily applied to the cultures to alter intracellular signaling or ion concentrations, trigger cell death, or mimic pathological effects. Genetic manipulation by knocking down a gene with RNAi or by over-expressing a wild type or mutant gene can also be achieved by co-transfection. Thus, the neuronal culture system is a powerful platform for revealing mechanistic principles underlying mitochondrial transport.

2.1.1 Methods for iPSC-Derived Axons

Coverslip Preparation for Neuronal Culture

Cleaning: Glass coverslips (12 mm diameter, #1.5) are used for neuronal culture. To help with cell adhesion, acid washing with 70% nitric acid is used to pre-clean and microetch the surface of the coverslips (caution: wear gloves and be extremely careful). The coverslips are placed in a 500 ml beaker and 50 ml of 70% nitric acid is added. After incubating in acid for 2 h, with occasional mild agitation by shaking, acid is carefully decanted into a waste bottle. Excess acid can be washed with running tap water, and then rinsed extensively with double distilled water five times. After washes, excess water is absorbed with paper towels, and coverslips are transferred into a beaker containing 70% ethanol to incubate for 30 min. Coverslips can be dried between sheets of filter paper (1001-070, Whatman) and kept in a clean glass container. Once the coverslips are dry, the container can be wrapped with aluminum foil and autoclaved.

Coating: Autoclaved coverslips are UV-sterilized in a culture laminar flow hood for at least 30 minutes, and then transferred to a sterile petri dish. Filter-sterilized poly-ornithine (1 mg/50 ml, Sigma) is added to cover the surface of coverslips. After 2 h of incubation at room temperature (22 °C), the solution is aspirated and coverslips are washed three times with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH = 7.4). After they are coated with poly- ornithine, coverslips are transferred into wells of a 24-well plate (Corning Life Sciences). Filter-sterilized laminin (170 µg/50 ml, Invitrogen) is then added to cover the surface of the coverslips, and they are incubated for at least 2 h at room temperature. Lastly, laminin is aspirated for removal, and coverslips are rinsed with PBS just before cell seeding.

Dopaminergic Neuron Derivation from iPSCs: iPSC lines can be converted from fibroblasts using standard procedures [31, 34–36]. Some control lines and lines from patients with neurodegenerative diseases are also available at cost in public repositories such as the Coriell Institute and the New York Stem Cell Foundation. Midbrain dopaminergic neurons are generated using a modified version of a previously described protocol [37]. For embryoid body (EB) formation, iPSC colonies are detached and cultured in suspension on non-adherent petri dishes using StemPro defined medium, without FGF2. At day 8, EBs are switched to neural induction medium containing Neurobasal medium (21103-049, Life Technologies) with GlutaMAX, Nonessential amino acids (NEAA), B27, and FGF2 (20 ng/ml) for an additional 2 days before being plated on Matrigel-coated dishes to form rosettes. The freshly formed rosettes are manually isolated and dissociated into single cells, and allowed to expand on Matrigel-coated dishes in order to generate a homogenous population of neural stem cells (NSCs) in the same neural induction medium for additional 2–5 days. For dopaminergic differentiation of NSCs, cells are cultured in Neurobasal medium supplemented with NEAA, L-glutamine (2 mM), B27, Shh (200 ng/ml), and FGF8 (100 ng/ml) for 10 days. At day 11, Shh and FGF8 are removed and replaced with brain-derived neurotrophic factor (BDNF, 20 ng/ml, 248-BD-025, R&D Systems), glial cell-derived neurotrophic factor (GDNF, 20 ng/ml, 450-10, Peprotech), transforming growth factor β-3 (TGFβ3, 1 M, AF-100-36E, Peprotech), ascorbic acid (200 µM, A5960, Sigma-Aldrich), and Dibutyl-tyl-cAMP (1 mM, D0627, Sigma-Aldrich) for an additional 3 weeks.

Alternatively, a dual-SMAD inhibition protocol is used to differentiate and maintain dopaminergic neurons [38–40]. Using this method, the neurons are maintained in Neurobasal medium supplemented with N-2 (17502-048, Life Technologies), BDNF (20 ng/ml), ascorbic acid (200 μ M), GDNF (20 ng/ml), TGF β 3 (1 ng/ml), and dibutyl-AMP (500 μ M).

NSCs can be confirmed by the expression of the neural progenitor markers Pax6, Sox1, or Nestin. Successful differentiation of dopaminergic neurons from NSCs can be confirmed by the expression of dopaminergic progenitor markers such as FoxA2 or Lmx1a/b after 14-day neuronal differentiation from NSCs, and mature dopaminergic neurons can be confirmed by the expression of β -tubulin isotype III and the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH), after 30-day neuronal differentiation from NSCs. The recommended antibody staining protocol is: Nestin (PRB-570C, Biolegend) at 1:500; Sox1 (AB5768, Chemicon) at 1:200; Lmx1a and b (139,736 and 139,726, Abcam) at 1:1500; FoxA2 (AF2400, R&D System) at 1:500; β -Tubulin isotype III (clone SDL.3D10, T8660, Sigma) 1:1000; and TH (P40101, Pel-Freez) at 1:500.

Transient Transfection: Here we describe how to transfect with a single construct. Co-transfection with multiple constructs is also often used. A neuron that has absorbed one construct is likely to have taken up additional constructs [5, 10, 33]. For each well of a 24-well plate, 0.5 μ g of DNA or 5 μ l of Lipofectamine 2000 (Invitrogen) is added in serum-free Opti-MEM (Gibco) to a final volume of 50 μ l in two separate tubes, and then gently mixed and incubated for 20 min at room temperature. During the incubation time, the culture medium of neurons for transfection is replaced with 200 μ l pre-warmed Opti-MEM. After incubation, the 100 μ l of DNA- Lipofectamine complex is added onto neurons with Opti-MEM. Then the neurons are returned into a 37 $^{\circ}$ C incubator with humidified atmosphere containing 5% CO₂. After transfection for 6 h, the DNA–Lipofectamine complex in Opti-MEM is replaced with the fresh culture medium. Transfected neurons are imaged 2–4 days later.

Image Acquisition: The transfected neurons on glass coverslips are placed in a 35 mm dish with pre-warmed CO₂-independent Hibernate E Low Fluorescence medium (HE-Lf, BrainBits) on a heated stage kept at 37 $^{\circ}$ C. Images are acquired by a Leica TCS SPE confocal microscope using a 63 $\times$ /N.A.0.9 water-immersion objective and a Leica Application Suite (Version 2.6), with standard filter sets and excitation laser lines. The laser power is set minimally (1–10%) to reduce laser damage, and pinholes are opened maximally to image at a greater depth of the axon. Each individual image is 512 $\times$ 512 pixels. The objective and zoom factor results in a pixel dimension of approximately 0.23 μ m. Time-lapse movies are obtained with 2–5 s intervals. Axons longer than 50 μ m are selected for analysis.

Image Analysis: The confocal images of the time-lapse file collected by the Leica software are imported as an image stack into ImageJ (Version 1.48) using the LOCI plug-in, a Bio-Formats importer. To analyze the movement characteristics of individual mitochondria, kymographs are generated. First, the “straighten” plug-in or the “rectangular selection” function of ImageJ is used to choose and crop the axonal region to be analyzed from the

image stack. Then, the “reslice” function is used to compress each cropped image to one line. Lastly, the “z-projection” function is used to compile all time-lapse image converted lines into one single picture, with time running from top to bottom. Thus in each kymograph, the *x*-axis represents mitochondrial displacement, and the *y*-axis represents time. Vertical lines indicate stationary mitochondria and diagonal lines are motile mitochondria moving either anterograde or retrograde. With the aid of macros written in additional software such as LabVIEW and MATLAB [5, 10, 33], one can determine multiple features of movement, such as: (1) the instantaneous velocity of each mitochondrion, (2) the average velocity of those mitochondria that are in motion, (3) the percent of time each mitochondrion is in motion, (4) stop frequency, and (5) turn back frequency. The mitochondrial length and intensity can also be determined using ImageJ.

2.1.2 Expected Results for iPSC-Derived Axons—Neuronal mitochondria are labeled with the red fluorescent mito- dsRed by transient transfection. Figure 1 shows an example of an iPSC-derived neuronal axon from a healthy human subject. Movement of mito-dsRed was recorded and converted to a kymograph, which can be further used to analyze mitochondrial movement parameters such as velocity, percentage of time mitochondria in motion, stop frequency, and turn back frequency. Here, about 50% of the mitochondria are in motion within the first 100 s. This method can assist researchers in analyzing mitochondrial transport in human neurons derived from controls or patients with various neurological disorders, or in neurons with targeted genetic or chemical manipulation. It is a powerful tool for revealing regulatory mechanisms underlying mitochondrial motility, and for shedding light on pathological pathways altering mitochondrial transport in neurons.

2.2 Live Imaging Mitochondrial Transport in Rat Embryonic Neuronal Axons

Cultured rodent embryonic neurons are a well-established system and have been widely exploited for studying mitochondrial transport [2–4, 10, 41–44]. For example, both the roles of Ca^{2+} and syntaphilin in arresting mitochondrial motility have been elucidated using rat or mouse embryonic hippocampal neurons [10, 43, 44]. These neurons exhibit the key in vivo features of forming complex neuronal networks and relaying synaptic transmission [45]. After 3–4 days in vitro, axons differentiate from dendrites and exhibit unique morphological characteristics for live identification. Axons can reach a few millimeters, allowing for observation of long-range movements. The unipolarity of the microtubules in the axons makes it easy to orient and analyze mitochondrial transport [29].

Similar to the iPSC-derived neurons described in section 2.1, cultured rodent embryonic neurons can be transiently transfected with a fluorescent mitochondrial marker like mito-DsRed to label mitochondria, and with additional fluorescent markers for visualizing other structures. Both individual mitochondrial tracking and genetic or chemical manipulation can be easily accomplished. Here we describe the methods for live imaging mitochondrial transport in rat embryonic hippocampal and cortical neurons.

2.2.1 Methods for Rat Embryonic Neuronal Axons

Coverslip Preparation for Neuronal Culture: RD German Coverslips (12 mm, #1, 1943-10012A, Bellco Glass) are acid and ethanol washed, and then autoclaved as detailed in section 2.1.1 “Coverslip Preparation for Neuronal Culture”. The day before use, coverslips are coated with filter-sterilized poly-ornithine (1 mg/50 ml) and laminin (170 µg/50 ml), and left in the hood with the UV light left on overnight. On the day of the procedure, coated coverslips are washed twice with sterile water, and then once with pre-warmed Neurobasal medium (also called Plain Neurobasal or PNB). The third wash of PNB is left in wells to prevent coverslips from drying out until the cells are plated.

Dissection and Culture of Rat Embryonic Neurons: All surgical tools should be autoclaved before use. A timed- pregnant Sprague Dawley Rat (day 18–19, Charles River) is anesthetized with CO₂ (first in a chamber, and then with a nose piece), and the belly is sprayed with 70% ethanol. Once unresponsive to toe pinch, an incision is made at the midline to expose the abdominal cavity. The uterine horn is removed by holding the uterus with forceps below the oviduct, cutting along the mesometrium, and pulling. The embryos are placed in a petri dish containing chilled PBS, and the anesthetized rat is then euthanized by decapitation or cervical dislocation. The embryos are separated by cutting between implantation sites and removing the muscle layer and the decidua tissue with forceps. The embryos are then decapitated with scissors, and the heads placed in chilled PBS. The brains are isolated by carefully inserting Vannas spring scissors (15000-00, Fine Science Tools) into the spinal column, making a sagittal cut from the spinal cord to the nose, peeling back the skin and the skull with forceps, and gently detaching the brain with an Iris spatula (Fine Science Tools). Each brain is placed in Hank’s Balanced Salt Solution and dissected under a dissecting microscope (at 10× or 15×) by removing the cerebellum and the olfactory bulbs, separating the hemispheres, stripping away the meninges, and removing the striatum with Dumont #5/45 Forceps (Fine Science Tools). The hippocampus and cortex are gently separated and are incubated in separate conical tubes containing chilled Hibernate E with Ca²⁺ (HE, BrainBits), penicillin (100 U/ml), and streptomycin (100 mg/ml). Once the dissection is complete, hippocampal or cortical tissue is carefully collected with a Pasteur pipette, transferred into pre-warmed Hibernate E without Ca²⁺ (HE-Ca, Brainbits), with 2 mg/ml of papain (PAP, Brainbits), and incubated at 37 °C for 10 min. The digested tissue is transferred via a Pasteur pipette into a new tube with 1 ml Hibernate E with Ca²⁺ and further dissociated by triturating 20–30 times with an autoclaved fire-polished Pasteur pipette. In order to separate the cells from the undigested tissue, the tissue is allowed to settle for 2 min, and then the supernatant without debris is transferred into a clean 15 ml conical tube and centrifuged at 1100 rpm for 1 min. The pellet is resuspended in 1 ml of filter-sterilized Neurobasal medium supplemented with penicillin–streptomycin–glutamine (100×) and B27 (50×). Cells are counted and seeded onto the prepared cover-slips described in section 2.1.1 “Coverslip Preparation for Neuronal Culture” at a density of 100,000–180,000 cells/well of a 24-well plate, and kept in an incubator at 37 °C with 5% CO₂ in a humidified environment for up to 21 days. The medium is completely replaced 1–18 h after plating, and partially replaced every 2–3 days thereafter with pre-warmed Neurobasal medium containing B27 and antibiotics. This modified culturing protocol was derived from two sources [33, 46].

Transient Transfection: Mito-dsRed DNA (0.5 µg) is added to PNB (up to 50 µl) and mixed gently. In a separate tube, Lipofectamine 2000 (2.5 µl) is added to PNB (47.5 µl) and mixed. The tubes are combined and left in the hood for a minimum of 25 min. During this time, the conditioned media from each well of cultured neurons designated for transfection are collected and left in a water bath or an incubator at 37 °C. The wells are immediately washed twice with autoclaved water and once with 500 µl of pre-warmed PNB. The last wash with 500 µl PNB is left in the well. After the mixing period, 100 µl of DNA–Lipofectamine is added to each designated well with PNB, and the neurons are returned to the incubator. After 2 h, the DNA–Lipofectamine mixture is removed and the wells are washed three times with PNB. Then, 500 µl of the conditioned medium is added back to each well. The transfected neurons are imaged 2–4 days later, following the method outlined in section 2.1.1 “Image Acquisition”, and can be analyzed following the method outlined in section 2.1.1 “Image Analysis”.

2.2.2 Expected Results for Rat Embryonic Neuronal Axons—Figure 2 shows an axon from a rat cortical neuron transfected with mito-dsRed. Mitochondrial movement labeled by mito-dsRed was recorded and transformed into a kymograph. While the majority of mitochondria are stationary, about 35% are moving during the interval shown. This model is especially advantageous for investigating the influences of genetic manipulation, neurotrophic factors, toxins, and stress conditions on mitochondrial morphology and axonal transport under normal and pathological conditions.

2.3 Live Imaging Mitochondria in Vivo: *Drosophila* Larval Neurons

Drosophila provides a great in vivo model for live imaging mitochondrial motility in axons. In addition to being cheap and easy to house—even in a non-*Drosophila* lab—access to genetic mutants is extensive. Libraries of null alleles and RNAi lines, like the Bloomington *Drosophila* Stock Center (BDSC) and Vienna *Drosophila* Resource Center (VDRC) are all searchable on Flybase (<http://www.flybase.com>) and lines can be easily obtained. If a gene of interest does not have an extant null allele and thus a knockout is required, it is much simpler to generate null alleles in *Drosophila* as compared to in mammals. Among many methods for generating targeted gene deletion, one of the most traditional is imprecise excision, whereby a *P*-element insertion in or close to the site of interest is excised by a transposon [47, 48]. New systems like the CRISPR-Cas9 system may make this process even quicker [49, 50]. Null mutations of essential genes, which cause lethality when present throughout the full body, can be turned on in just a subset of cells or tissues. Several systems, like the MARCM [51, 52], Q [53], InSITE [54], and FLP/FRT [55] enable such tissue-specific manipulation. Over-expression of a gene can be easily achieved by using the UAS/GAL4 system, for selective temporal and/or spatial expression of a gene, or selective knockdown when coupled with RNAi [56]. It is even easy to combine mutations and transgenes in one animal, allowing for identification of epistatic versus additive phenotypes in double mutants, as well as allowing for rescue analysis with transgenes.

The fly has always been a powerful model for studying genetics, development, and behavior, harkening back to their use in uncovering the chromosomal theory of inheritance by T.H. Morgan. It has now also become an established model for studies of nervous system function

and human neurodegenerative diseases. Due to the high genomic similarity between *Drosophila* and humans [57], many of the identified familial genes underlying disorders like Huntington's disease [58], Alzheimer's disease [59–63], Parkinson's disease [64, 65], and ALS [66] have been knocked out or their pathogenic forms have been expressed in *Drosophila*. For example, when the familial Parkinson's disease-causing gene *PINK1* is knocked out in *Drosophila*, the flies exhibit the locomotor defects and dopaminergic neurodegeneration also found in human patients. Significantly, *PINK1* null flies display defects in mitochondrial morphology and function and susceptibility to oxidative stress [67–72]. These studies in flies provide in vivo evidence of the cellular functions of PINK1, and point to mitochondria as critical in Parkinson's disease pathogenesis.

The nervous system of *Drosophila* larvae is highly organized [73]. The cell bodies of their central nervous system form the cortex of their brain lobes and ventral nerve cord (VNC; akin to our spinal cord). Two parallel zones of neuropil follow the midline of the VNC, and this is where the axonal and dendritic projections of cell bodies lie. Because these projections comeingle, they are difficult to image; instead, the best structures to image are the long axons issuing out of the VNC and into the body wall. These axons have their cell bodies located in the VNC, and conjoin sensory neuronal axons which extend from the body wall back into the VNC, forming axonal bundles. For this reason, it is necessary to selectively label mitochondria in a subset of neurons, whose polarity can be determined unambiguously. Researchers have expressed a mitochondrial import signal fused with GFP (termed mito-GFP) selectively in motor neurons driven by the motor neuronal D42- GAL4 [74]. Motor neurons project axons from the VNC to the neuromuscular junctions (NMJs) in the body wall muscles; however, the high density of motor neuronal axons in axonal bundles makes it difficult to trace individual mitochondrial movement without photobleaching [33, 74]. Here, we provide a method for expressing mito-GFP sparsely using crustacean cardioactive peptide (CCAP)-GAL4, which selectively labels mitochondria in almost only one neuropeptidergic axon per axonal bundle, originating from the VNC and terminating at the NMJs of muscle 12 [75, 76]. In this way, mitochondrial fluorescent intensity can be greatly reduced in axonal bundles, and tracing and analyzing individual mitochondrial motility over a long distance can be achieved. We also introduce a method to live stain mitochondria with the mitochondrial membrane potential-dependent dye TMRM (tetramethylrhodamine). These are useful tools for determining both mitochondrial motility and function.

2.3.1 Methods for *Drosophila* Larval Neurons

Fly Stocks and Culture: Wild type, *CCAP-GAL4* [75], and *UAS-mito-GFP* [74] flies are used. Flies are cultured at 25 °C on molasses/yeast medium (6.7% molasses, 1.1% yeast, 5.5% cornmeal, 0.5% agar, and 0.13% *p*-hydroxybenzoic acid methyl ester).

Dissection of Live Larvae: Late third instar wandering larvae are dissected in freshly prepared Schneider's medium (pH 7.4, S9895, Sigma) with 5 mM EGTA. Larval dissection is as previously described [77] in a chamber glass slide coated with a thin layer of Sylgard (Dow Corning Corporation, MI). The larva is opened along the dorsal midline with Vannas spring scissors (15000-00, Fine Science Tools), then the body wall muscles are pinned on

the slide and the main guts and salivary glands are carefully removed. The larva is then washed gently three times using fresh Schneider's medium with 5 mM EGTA, and kept in the same medium for imaging. For TMRM staining, 20 nM TMRM (Molecular Probes) is added to Schneider's medium with 5 mM EGTA and mixed evenly, and then applied to the dissected larva for 20 min in the dark. After TMRM staining, the larva is washed three times again with fresh Schneider's medium with 5 mM EGTA and kept in the same medium with 5 nM TMRM for imaging.

Imaging Acquisition and Analysis: The freshly dissected larva is maintained at 22 °C and imaged within an hour of dissection. Time-lapse images of 100 s are taken every 2 s from each axon on a Leica DFC365 FX CCD camera for SPE II system with a Leica N2.1 filter LP 590 nm or an I3 filter LP 515 nm (JH Technologies). Kymograph conversion and motility analysis are similar to those described in section 2.1.1 "Image Analysis."

2.3.2 Expected Results for *Drosophila* Larval Neurons—Two different methods can be applied for monitoring mitochondrial transport in fly larval axons. Mitochondria can either be fluorescently labeled by mito-GFP in CCAP neurons, or stained with TMRM, a mitochondrial membrane potential-dependent dye. Individual mitochondrial movement can be traced in axons of *CCAP-GAL4, UAS-mito-GFP* larvae (Fig. 3), because CCAP neurons project almost only one axon per axonal bundle [75, 76]. TMRM labels all mitochondria in axons with a polarized membrane potential, and thus long-range mitochondrial movement is hard to follow. This caveat can be circumvented by imaging the distal segment of the axon close to the NMJs, where fewer mitochondria are present. Combined with the power of fly genetics, these methods allow analysis of mitochondrial motility in a semi- intact in vivo system, to reveal the regulatory roles of genes and cellular pathways. In addition, TMRM intensity can be quantified and compared among different genetic backgrounds to determine the mitochondrial membrane potential as an indicator of mitochondrial function.

3 Conclusion

Increasing evidence suggests that mitochondrial transport defects may underlie many neurological disorders [5, 13–27], and therefore it is vital to understand the fundamental mechanisms controlling mitochondrial movement. Here, we have described how to live-image and analyze mitochondrial motility in axons in both cultured and in vivo systems.

It is difficult to explore physiological or pathological regulatory mechanisms in intact human or rodent brains due to the complexity and heterogeneity of the central nervous system and multiple inputs to individual regions. Cultured neuronal systems provide alternative models that can be used to study regulatory mechanisms without effects being masked by complicated circuitry. The advancements of modern microscopy and neuronal culturing systems have greatly facilitated our ability to image axonal transport of mitochondria in live neurons. The two cultured neuronal systems we have introduced here are iPSC-derived neurons and rat embryonic neurons. The rat neurons are easier to access, while the iPSC-derived neurons lend translational relevance to findings.

These cultured neuronal systems may allow us to bypass the complexity of the central nervous system, but we still need to dissect molecular mechanisms in an in vivo system to relate our findings to living organisms. *Drosophila* larvae are the optimal system for live imaging mitochondrial transport in vivo. They are easy to obtain and care for, easy to dissect, easy to image, and allow for easy genetic manipulation of molecular pathways.

These systems are especially powerful when used together, and will significantly forward our understanding of the mechanisms by which mitochondrial motility is regulated, and how its disruption can contribute to psychiatric and neurodegenerative diseases.

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References

1. Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. *J Cell Sci.* 2005; 118(Pt 23): 5411–5419. DOI: 10.1242/jcs.02745 [PubMed: 16306220]
2. Chen S, Owens GC, Crossin KL, Edelman DB. Serotonin stimulates mitochondrial transport in hippocampal neurons. *Mol Cell Neurosci.* 2007; 36(4):472–483. DOI: 10.1016/j.mcn.2007.08.004 [PubMed: 17904380]
3. Overly CC, Rieff HI, Hollenbeck PJ. Organelle motility and metabolism in axons vs dendrites of cultured hippocampal neurons. *J Cell Sci.* 1996; 109:971–980. [PubMed: 8743944]
4. Waters J, Smith SJ. Mitochondria and release at hippocampal synapses. *Pflugers Arch.* 2003; 447(3):363–370. DOI: 10.1007/s00424-003-1182-0 [PubMed: 14556074]
5. Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, Rice S, Steen J, LaVoie MJ, Schwarz TL. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell.* 2011; 147(4):893–906. DOI: 10.1016/j.cell.2011.10.018 [PubMed: 22078885]
6. Ashrafi G, Schlehe JS, LaVoie MJ, Schwarz TL. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. *J Cell Biol.* 2014; 206(5):655–670. DOI: 10.1083/jcb.201401070 [PubMed: 25154397]
7. Hoye AT, Davoren JE, Wipf P, Fink MP. Targeting mitochondria. *Acc Chem Res.* 2008; 41(1):87–97. [PubMed: 18193822]
8. Schnapp BJ, Reese TS. Dynein is the motor for retrograde axonal transport of organelles. *Proc Natl Acad Sci U S A.* 1989; 86(5):1548–1552. [PubMed: 2466291]
9. Chang DTW, Honick AS, Reynolds JJ. Mitochondrial trafficking to synapses in cultured primary cortical neurons. *J Neurosci.* 2006; 26(26):7035–7045. DOI: 10.1523/JNEUROSCI.1012-06.2006 [PubMed: 16807333]
10. Wang X, Schwarz TL. The mechanism of Ca²⁺-dependent regulation of kinesin-mediated mitochondrial motility. *Cell.* 2009; 136(1):163–174. DOI: 10.1016/j.cell.2008.11.046 [PubMed: 19135897]
11. Saotome M, Safiulina D, Szabadkai G, Das S, Fransson A, Aspenstrom P, Rizzuto R, Hajnoczky G. Bidirectional Ca²⁺-dependent control of mitochondrial dynamics by the Miro GTPase. *Proc Natl Acad Sci U S A.* 2008; 105(52):20728–20733. DOI: 10.1073/pnas.0808953105 [PubMed: 19098100]
12. Macaskill AF, Rinholm JE, Twelvetrees AE, Arancibia-Carcamo IL, Muir J, Fransson A, Aspenstrom P, Attwell D, Kittler JT. Miro1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses. *Neuron.* 2009; 61(4):541–555. DOI: 10.1016/j.neuron.2009.01.030 [PubMed: 19249275]

13. Baloh RH, Schmidt RE, Pestronk A, Milbrandt J. Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. *J Neurosci.* 2007; 27(2):422–430. DOI: 10.1523/JNEUROSCI.4798-06.2007 [PubMed: 17215403]
14. De Vos KJ, Chapman AL, Tennant ME, Manser C, Tudor EL, Lau KF, Brownlee S, Ackerley S, Shaw PJ, McLoughlin DM, Shaw CE, Leigh PN, Miller CC, Grierson AJ. Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. *Hum Mol Genet.* 2007; 16(22):2720–2728. DOI: 10.1093/hmg/ddm226 [PubMed: 17725983]
15. Pigino G, Morfini G, Pelsman A, Mattson MP, Brady ST, Busciglio J. Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. *J Neurosci.* 2003; 23(11):4499–4508. [PubMed: 12805290]
16. Rui Y, Tiwari P, Xie Z, Zheng JQ. Acute impairment of mitochondrial trafficking by beta-amyloid peptides in hippocampal neurons. *J Neurosci.* 2006; 26(41):10480–10487. DOI: 10.1523/JNEUROSCI.3231-06.2006 [PubMed: 17035532]
17. Thies E, Mandelkow E-M. Misrouting of tau in neurons causes degeneration of synapses that can be rescued by the kinase MARK2/Par-1. *J Neurosci.* 2007; 27(11):2896–2907. DOI: 10.1523/JNEUROSCI.4674-06.2007 [PubMed: 17360912]
18. Calkins MJ, Reddy PH. Amyloid beta impairs mitochondrial anterograde transport and degenerates synapses in Alzheimer's disease neurons. *Biochim Biophys Acta.* 2011; 1812(4):507–513. DOI: 10.1016/j.bbdis.2011.01.007 [PubMed: 21241801]
19. Trushina E, Dyer RB, Ji JDB, Ure D, Eide L, Tran DD, Vrieze BT, Legendre-guillemain V, McPherson PS, Mandavilli BS, Houten BV, Zeitlin S, McNiven M, Aebbersold R, Hayden M, Parisi JE, Seeborg E, Dragatsis I, Doyle K, Bender A, Chacko C, McMurray CT. Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. *Mol Cell Biol.* 2004; 24(18):8195–8209. DOI: 10.1128/MCB.24.18.8195 [PubMed: 15340079]
20. Chang DTW, Rintoul GL, Pandipati S, Reynolds IJ. Mutant huntingtin aggregates impair mitochondrial movement and trafficking in cortical neurons. *Neurobiol Dis.* 2006; 22:388–400. DOI: 10.1016/j.envpol.2005.09.025 [PubMed: 16473015]
21. Shirendeb U, Reddy AP, Manczak M, Calkins MJ, Mao P, Tagle DA, Reddy PH. Abnormal mitochondrial dynamics, mitochondrial loss and mutant huntingtin oligomers in Huntington's disease: implications for selective neuronal damage. *Hum Mol Genet.* 2011; 20(7):1438–1455. DOI: 10.1093/hmg/ddr024 [PubMed: 21257639]
22. Ferreira F, Quattrini A, Pirozzi M, Valsecchi V, Dina G, Broccoli V, Auricchio A, Piemonte F, Tozzi G, Gaeta L, Casari G, Ballabio A, Rugarli EL. Axonal degeneration in paraplegin-deficient mice is associated with abnormal mitochondria and impairment of axonal transport. *J Clin Invest.* 2004; 113(2):231–242. DOI: 10.1172/JCI200420138 [PubMed: 14722615]
23. Kasher PR, De Vos KJ, Wharton SB, Manser C, Bennett EJ, Bingley M, Wood JD, Milner R, McDermott CJ, Miller CCJ, Shaw PJ, Grierson AJ. Direct evidence for axonal transport defects in a novel mouse model of mutant spastin-induced hereditary spastic paraplegia (HSP) and human HSP patients. *J Neurochem.* 2009; 110(1):34–44. DOI: 10.1111/j.1471-4159.2009.06104.x [PubMed: 19453301]
24. Liu S, Sawada T, Lee S, Yu W, Silverio G, Alapatt P, Millan I, Shen A, Saxton W, Kanao T, Takahashi R, Hattori N, Imai Y, Lu B. Parkinson's disease-associated kinase PINK1 regulates Miro protein level and axonal transport of mitochondria. *PLoS Genet.* 2012; 8(3):e1002537.doi: 10.1371/journal.pgen.1002537 [PubMed: 22396657]
25. Atkin TA, MacAskill AF, Brandon NJ, Kittler JT. Disrupted in Schizophrenia-1 regulates intracellular trafficking of mitochondria in neurons. *Mol Psychiatry.* 2011; 16(2):122–124. DOI: 10.1038/mp.2010.110121 [PubMed: 21079610]
26. Atkin TA, Brandon NJ, Kittler JT. Disrupted in Schizophrenia 1 forms pathological aggregates that disrupt its function in intracellular transport. *Hum Mol Genet.* 2012; 21(9):2017–2028. DOI: 10.1093/hmg/dds018 [PubMed: 22291444]
27. Ogawa F, Malavasi ELV, Crummie DK, Eykelenboom JE, Soares DC, Mackie S, Porteous DJ, Millar JK. DISC1 complexes with TRAK1 and Miro1 to modulate anterograde axonal mitochondrial trafficking. *Hum Mol Genet.* 2014; 23(4):906–919. [PubMed: 24092329]

28. Baas PW, Deitch JS, Black MM, Banker GA. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc Natl Acad Sci U S A*. 1988; 85(21):8335–8339. [PubMed: 3054884]
29. Baas PW. Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J Cell Biol*. 1989; 109(6):3085–3094. DOI: 10.1083/jcb.109.6.3085 [PubMed: 2592416]
30. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318(5858):1917–1920. DOI: 10.1126/science.1151526 [PubMed: 18029452]
31. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131(5):861–872. DOI: 10.1016/j.cell.2007.11.019 [PubMed: 18035408]
32. Cord BJ, Li J, Works M, McConnell SK, Palmer T, Hynes MA. Characterization of axon guidance cue sensitivity of human embryonic stem cell-derived dopaminergic neurons. *Mol Cell Neurosci*. 2010; 45(4):324–334. DOI: 10.1016/j.mcn.2010.07.004 [PubMed: 20637284]
33. Wang X, Schwarz TL. Imaging axonal transport of mitochondria. *Methods Enzymol*. 2009; 457(09):319–333. DOI: 10.1016/S0076-6879(09)05018-6 [PubMed: 19426876]
34. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008; 451(7175):141–146. DOI: 10.1038/nature06534 [PubMed: 18157115]
35. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. 2008; 322(5903):949–953. DOI: 10.1126/science.1164270 [PubMed: 18845712]
36. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science*. 2008; 322(5903):945–949. DOI: 10.1126/science.1162494 [PubMed: 18818365]
37. Swistowski A, Peng J, Liu Q, Mali P, Rao MS, Cheng L, Zeng X. Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions. *Stem Cells*. 2010; 28(10):1893–1904. DOI: 10.1002/stem.499 [PubMed: 20715183]
38. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol*. 2009; 27(3):275–280. DOI: 10.1038/nbt.1529 [PubMed: 19252484]
39. Fasano CA, Chambers SM, Lee G, Tomishima MJ, Studer L. Efficient derivation of functional floor plate tissue from human embryonic stem cells. *Cell Stem Cell*. 2010; 6(4):336–347. DOI: 10.1016/j.stem.2010.03.001 [PubMed: 20362538]
40. Nguyen HN, Byers B, Cord B, Shcheglovitov A, Byrne J, Gujar P, Kee K, Schule B, Dolmetsch RE, Langston W, Palmer TD, Pera RR. LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. *Cell Stem Cell*. 2011; 8(3):267–280. DOI: 10.1016/j.stem.2011.01.013 [PubMed: 21362567]
41. Chada SR. Mitochondrial movement and positioning in axons: the role of growth factor signaling. *J Exp Biol*. 2003; 206(12):1985–1992. DOI: 10.1242/jeb.00263 [PubMed: 12756280]
42. Chada SR, Hollenbeck PJ, Lafayette W. Nerve growth factor signaling regulates motility and docking of axonal mitochondria. *Curr Biol*. 2004; 14:1272–1276. DOI: 10.1016/j [PubMed: 15268858]
43. Kang JS, Tian JH, Pan PY, Zald P, Li C, Deng C, Sheng ZH. Docking of axonal mitochondria by syntaphilin controls their mobility and affects short-term facilitation. *Cell*. 2008; 132(1):137–148. DOI: 10.1016/j.cell.2007.11.024 [PubMed: 18191227]
44. Chen Y, Sheng ZH. Kinesin-I- syntaphilin coupling mediates activity- dependent regulation of axonal mitochondrial transport. *J Cell Biol*. 2013; 202(2):351–364. DOI: 10.1083/jcb.201302040 [PubMed: 23857772]
45. Kaech S, Banker G. Culturing hippocampal neurons. *Nat Protoc*. 2006; 1(5):2406–2415. DOI: 10.1038/nprot.2006.356 [PubMed: 17406484]

46. Kim KM, Vicenty J, Palmore GT. The potential of apolipoprotein E4 to act as a substrate for primary cultures of hippocampal neurons. *Biomaterials*. 2013; 34(11):2694–2700. DOI: 10.1016/j.biomaterials.2013.01.012 [PubMed: 23352042]
47. Voelker RA, Greenleaf AL, Gyurkovics H, Wisely GB, Huang SM, Searles LL. Frequent imprecise excision among reversions of a P element-caused lethal mutation in *Drosophila*. *Genetics*. 1984; 107(2):279–294. [PubMed: 17246216]
48. Daniels SB, McCarron M, Love C, Chovnick A. Dysgenesis-induced instability of rosy locus transformation in *Drosophila melanogaster*: analysis of excision events and the selective recovery of control element deletions. *Genetics*. 1985; 109(1):95–117. [PubMed: 2981758]
49. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013; 339(6121): 819–823. DOI: 10.1126/science.1231143 [PubMed: 23287718]
50. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. *Nat Methods*. 2013; 10(10):957–963. DOI: 10.1038/nmeth.2649 [PubMed: 24076990]
51. Lee T, Luo L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*. 1999; 22(3):451–461. [PubMed: 10197526]
52. Zong H, Espinosa JS, Su HH, Muzumdar MD, Luo L. Mosaic analysis with double markers in mice. *Cell*. 2005; 121(3):479–492. DOI: 10.1016/j.cell.2005.02.012 [PubMed: 15882628]
53. Potter CJ, Tasic B, Russler EV, Liang L, Luo L. The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell*. 2010; 141(3):536–548. DOI: 10.1016/j.cell.2010.02.025 [PubMed: 20434990]
54. Gohl DM, Silies MA, Gao XJ, Bhalerao S, Luongo FJ, Lin CC, Potter CJ, Clandinin TR. A versatile in vivo system for directed dissection of gene expression patterns. *Nat Methods*. 2011; 8(3):231–237. [PubMed: 21473015]
55. Golic KG, Lindquist S. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell*. 1989; 59(3):499–509. [PubMed: 2509077]
56. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 1993; 415:401–415.
57. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferreira S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacle JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Siden-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskaas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissbach J, Williams SM, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC. The genome sequence of *Drosophila melanogaster*. *Science*. 2000; 287(5461):2185–2195. [PubMed: 10731132]

58. Green EW, Giorgini F. Choosing and using *Drosophila* models to characterize modifiers of Huntington's disease. *Biochem Soc Trans.* 2012; 40(4):739–745. DOI: 10.1042/BST20120072 [PubMed: 22817726]
59. Prussing K, Voigt A, Schulz JB. *Drosophila melanogaster* as a model organism for Alzheimer's disease. *Mol Neurodegen.* 2013; 8:35.doi: 10.1186/1750-1326-8-35
60. Mhatre SD, Paddock BE, Saunders AJ, Marenda DR. Invertebrate models of Alzheimer's disease. *J Alzheimers Dis.* 2013; 33(1):3–16. DOI: 10.3233/jad-2012-121204 [PubMed: 22886023]
61. Bonner JM, Boulianne GL. *Drosophila* as a model to study age-related neurodegenerative disorders: Alzheimer's disease. *Exp Gerontol.* 2011; 46(5):335–339. DOI: 10.1016/j.exger.2010.08.004 [PubMed: 20719244]
62. Moloney A, Sattelle DB, Lomas DA, Crowther DC. Alzheimer's disease: insights from *Drosophila melanogaster* models. *Trends Biochem Sci.* 2010; 35(4):228–235. DOI: 10.1016/j.tibs.2009.11.004 [PubMed: 20036556]
63. Iijima-Ando K, Iijima K. Transgenic *Drosophila* models of Alzheimer's disease and tauopathies. *Brain Struct Funct.* 2010; 214(2–3):245–262. DOI: 10.1007/s00429-009-0234-4 [PubMed: 19967412]
64. Guo M. *Drosophila* as a model to study mitochondrial dysfunction in Parkinson's disease. *Cold Spring Harb Perspect Med.* 2012; 2(11)doi: 10.1101/cshperspect.a009944
65. Whitworth AJ. *Drosophila* models of Parkinson's disease. *Adv Genet.* 2011; 73:1–50. DOI: 10.1016/B978-0-12-380860-8.00001-X [PubMed: 21310293]
66. Bonini NM, Gitler AD. Model organisms reveal insight into human neurodegenerative disease: ataxin-2 intermediate-length polyglutamine expansions are a risk factor for ALS. *J Mol Neurosci.* 2011; 45(3):676–683. DOI: 10.1007/s12031-011-9548-9 [PubMed: 21660502]
67. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Ba H, Guo M. *Drosophila* pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature.* 2006; 441(7097):1162–1166. DOI: 10.1038/nature04779 [PubMed: 16672981]
68. Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, Bae E, Kim J, Shong M, Kim JM, Chung J. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature.* 2006; 441(7097):1157–1161. DOI: 10.1038/nature04788 [PubMed: 16672980]
69. Yang Y, Gehrke S, Imai Y, Huang Z, Ouyang Y, Wang JW, Yang L, Beal MF, Vogel H, Lu B. Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of *Drosophila* Pink1 is rescued by Parkin. *Proc Natl Acad Sci U S A.* 2006; 103(28):10793–10798. DOI: 10.1073/pnas.0602493103 [PubMed: 16818890]
70. Liu W, Acin-Perez R, Geghman KD, Manfredi G, Lu B, Li C. Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission. *Proc Natl Acad Sci U S A.* 2011; 108(31):12920–12924. DOI: 10.1073/pnas.1107332108 [PubMed: 21768365]
71. Vos M, Esposito G, Edirisinghe JN, Vilain S, Haddad DM, Slabbaert JR, Van Meensel S, Schaap O, De Strooper B, Meganathan R, Morais VA, Verstreken P. Vitamin K-2 is a mitochondrial electron carrier that rescues pink1 deficiency. *Science.* 2012; 336(6086):1306–1310. DOI: 10.1126/science.1218632 [PubMed: 22582012]
72. Vilain S, Esposito G, Haddad D, Schaap O, Dobrev MP, Vos M, Van Meensel S, Morais VA, De Strooper B, Verstreken P. The yeast complex I equivalent NADH dehydrogenase rescues pink1 mutants. *PLoS Genet.* 2012; 8(1):e1002456.doi: 10.1371/journal.pgen.1002456 [PubMed: 22242018]
73. Gorczyca M, Budnik V. Appendix: anatomy of the larval body wall muscles and NMJs in the third instar larval stage. In: Vivian B, Catalina RC, editors *International review of neurobiology* Vol. 75. Academic; New York, NY: 2006 367373 doi:[http://dx.doi.org/10.1016/S0074-7742\(06\)75016-4](http://dx.doi.org/10.1016/S0074-7742(06)75016-4)
74. Pilling AD, Horiuchi D, Lively CM, Saxton WM. Kinesin-1 and dynein are the primary motors for fast transport of mitochondria in *Drosophila* motor axons. *Mol Biol Cell.* 2006; 17:2057–2068. DOI: 10.1091/mbc.E05 [PubMed: 16467387]
75. Park JH, Schroeder AJ, Helfrich-Forster C, Jackson FR, Ewer J. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development.* 2003; 130(12):2645–2656. [PubMed: 12736209]

76. Vomel M, Wegener C. Neurotransmitter-induced changes in the intracellular calcium concentration suggest a differential central modulation of CCAP neuron subsets in *Drosophila*. *Dev Neurobiol*. 2007; 67(6):792–808. DOI: 10.1002/dneu.20392 [PubMed: 17443825]
77. Budnik V, , Gorczyca M, , Prokop A. Selected methods for the anatomical study of *drosophila* embryonic and larval neuromuscular junctions. In: Vivian B, , Catalina RC, editors *International review of neurobiology* Vol. 75. Academic; New York, NY: 2006 323365 doi: [http://dx.doi.org/10.1016/S0074-7742\(06\)75015-2](http://dx.doi.org/10.1016/S0074-7742(06)75015-2)

Fig. 1.

Mitochondrial movement in an iPSC-derived neuronal axon. This representative iPSC-derived neuronal axon from a healthy subject was transfected with mito-dsRed. **(A)** The first frame of the time-lapse image series is shown above of a kymograph generated from the movie. The x -axis corresponds to mitochondrial position and the y -axis corresponds to time (progressing from *top* to *bottom*). Vertical white lines represent stationary mitochondria and diagonal lines are moving mitochondria. Scale bar = 10 μm . **(B)** A hand-drawn depiction of the kymograph shown in **(A)**. Black lines label mitochondria when they are static, and blue or red lines label mitochondria when moving anterograde or retrograde, respectively

Fig. 2. Mitochondrial movement in a rat cortical neuronal axon. This representative cortical neuronal axon is transfected with mito-dsRed at 8 days in vitro. The kymograph is generated as in Fig. 1A. Scale bar = 10 μ m

Fig. 3.

Mitochondrial movement in a fly larval axon. Mito-GFP is expressed in a CCAP-expressing neuropeptidergic axon of a third instar larva. The representative segmental nerve passes hemisegment A3, with the VNC to the left. The kymograph is generated as in Fig. 1A. Scale bar = 10 μ m