Molecular analysis of axonal transport dynamics upon modulation of microtubule acetylation

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ABSTRACT

Axonal transport is used by neurons to distribute mRNAs, proteins and organelles to their peripheral compartments in order to sustain their structural and functional integrity. Cargoes are transported along the microtubule (MT) network whose post-translational modifications influence transport dynamics. Here, we describe methods to modulate MT acetylation and record its impact on axonal transport in cultured mouse cortical projection neurons as well as in motoneurons of Drosophila melanogaster 3rd instar larvae. Specifically, we provide a step-by step procedure to reduce the level of MT acetylation and to record and analyze the transport of dye-labelled organelles in projection neuron axons cultured in microfluidic chambers. In addition, we describe the method to record and analyze GFP-tagged mitochondria transport along the motoneuron axons of transgenic Drosophila melanogaster 3rd instar larvae.

Key words: Axonal transport, MT acetylation, cargo, organelles, ATAT1, HDAC6

1. INTRODUCTION

Neurons are highly polarized cells that transport cargoes along their protracted axons to supply mRNAs, proteins and organelles to the axoplasm. Intracellular trafficking of cargoes along axons is essential for neuron maturation and homeostasis as well as for their functional integration into neuronal network via synaptic activity. Cargoes are transported along MT whose structure, properties

and dynamics rely on its constitutive tubulin subtypes as well as their post-translational modifications (PTMs) that can fine-tune the trafficking of cargoes (reviewed in [1]).

While most PTMs, such as glutamylation, glycylation and detyrosination, occur on the C-terminal tails of α/β-tubulins, acetylation of α-tubulin takes place on intralumenal Lys40 (K40) of MTs. This PTM is catalyzed by the α-tubulin N-acetyltransferase 1 (ATAT1) and removed by the histone deacetylase 6 (HDAC6) [2]. The acetylation of α-tubulin modulates the mechanical properties of MTs by enhancing their flexibility, thereby protecting them from stress-induced mechanical ageing[3]. Additionally, α-tubulin acetylation has been reported to increase the recruitment and processivity of molecular motors on MTs, pinpointing a functional correlation between MT acetylation and axonal transport dynamics [4, 5]. Accordingly, some neurodegenerative diseases exhibit transport defects which correlate with decreased MT acetylation levels. Restoring the balance of K40 acetylation using overexpression of ATAT1 or inhibitors of HDAC6 subsequently rescues axonal transport, therefore strengthening the link between this MTs PTM and the regulation of axonal transport [4, 6][7]. In accordance with the role that MTs play in neuronal development and activity, mice lacking ATAT1 display dentate gyrus deformation, hypoplasia of the septum and striatum and enlarged lateral ventricles. Moreover, their somatosensory cortical neurons suffer from axon overgrowth and overbranching, further suggesting an important role for MTs acetylation during cortical maturation[8–10].

To investigate the role of ATAT1 on axonal transport dynamics and further untangle its regulatory molecular mechanisms, we assessed axonal transport dynamics in mouse cortical neurons and in third-instar larva motoneurons of *Drosophila melanogaster* upon RNA interference (RNAi)–induced knock-down (KD) of ATAT1 and Atat1/2 (the *D. melanogaster* orthologs of mouse Atat1), respectively [11]. We reported comparable axonal transport defects of membrane-bound organelles across species upon knockdown of ATAT1 that were rescued by the expression of a mutant α-tubulin (K40Q) mimicking K40 acetylation in mice or by pharmacological inhibition of HDAC6 in third-instar larvae [11].

Here, we provide an experimental paradigm to analyze the axonal transport of organelles in mouse cortical projection neurons cultured in microfluidics chambers as well as in motoneurons of live anaesthetized D. *melanogaster* third-instar larvae. These models are suitable to study the contribution of MTs acetylation to the transport dynamics of organelles and allow a comparison of these molecular parameters across species. More specifically, we provide a step-by-step method to knock down ATAT1 in mouse cortical neurons by *in utero* electroporation of short hairpin RNA (shRNA) and to record axonal transport dynamics of dye-labelled organelles in neurons cultured in microfluidic devices. As a culturing platform, microfluidics devices enable the physical isolation of axons from cell bodies and dendrites thus allowing the study of axon biology and its transport dynamics. Moreover, we describe the experimental protocol to record axonal transport of mitochondria along the motoneuron axons from anesthetized third instar larvae as well as the genetic strategies used to generate simple or compound conditional Atat1/2 and/or Hdac6 knockdown. We further emphasize the pharmacological strategy used to rescue MTs acetylation levels in larvae upon Atat1/2 KD and ultimately provide the detailed procedure to quantify the axonal transport parameters extracted from the time lapse recordings.

2. MATERIALS

2.1. Axonal transport recordings in mouse cortical neurons

2.1.1. In utero electroporation

1. Micropipette puller (Sutter Instrument, #P-97)
2. Electroporator (BTX, #ECM 830)
3. Micro-injector (Eppendorf, #5252000013)
4. Isoflurane anaesthesia vaporizer (Harvard Apparatus, #34-1041)
5. Heating pad (Beurer, HK 35 Heat pad)

6. Surgical lamp with flexible arm
7. Endo-free plasmid maxi kit (Macherey-Nagel, #740420)
8. Sterile PBS
9. Fast green
10. Isoflurane
11. Analgesic: Buprecare 0.3 mg/mL injection (Animalcare)
12. Ophthalmic lubrificant: Fucithalmic Vet 10 mg/g (Aventix)
13. 3mm φ platinum electrodes (Sonidel, # CUY650P3)
14. Borosilicate Glass capillaries (Harvard Apparatus, #30-0016)
15. Syringe 1 mL
16. Needle 26GA (0.45 x 10mm)
17. Microloader tips (Eppendorf, #5242956003)
18. Scissors
19. Spring scissors
20. Ring forceps
21. Vicryl suture 4-0
22. Plasmids: pEGFP α-tubulin WT (Clontech) and pEGFP α-tubulin K40Q (Addgene, #105302)
23. Atat1 short hairpin RNA (shRNA); sequence: 5'-GCAGCAAATCATGACTATTGT-3' [12]. Scrambled-shRNA; sequence: 5'-TACGCGCATAAGATTAGGG-3'

2.1.2. Culturing and plating mouse cortical neurons in microfluidic devices

1. Vacuum desiccator
2. Glass-bottom dish (*Mattek Life Science, #*P50G-0-30-F)
3. Microfluidic chambers, 450 µm microgroove barrier (XONA microfluidics, # SND450)

4. 0,1 mg/mL Poly-D-Lysine, resuspended in sterile water, ready to use. Solution can be stored at 4°C for up to 2 years.
5. Neurobasal medium (ThermoFisher, #21103049), to be supplemented with 1% Penicillin-Streptomycin, 1% Glutamine and 2% B27 to prepare the culturing medium.
6. 200 mM Glutamine
7. 10,000 units/mL Penicillin-Streptomycin (P/S)
8. B27 (Gibco, Invitrogen, #15140122)
9. Forceps
10. Perforated spoon
11. 20μm/mL BDNF, resuspended in culturing medium and aliquoted for long term storage at -20°C (Peprotech, #450-02)
12. Mitotracker Red (ThermoFisher, # M7514)
13. Lysotracker Deep red (ThermoFisher, # L12492)
14. Ethanol 70%
15. 40 µm cell strainer
16. 30% Glucose
17. HBSS

2.2. Axonal transport recordings in *Drosophila melanogaster*

2.2.1. Fly lines and husbandry

1. Drosophila culture tubes 25x95mm (MLS QD789008)
2. Foam plug, Ø 36 x 40 mm (MLS QD330070)
3. Homemade cornmeal medium based on BDSC standard recipe (https://bdsc.indiana.edu/information/recipes/bloomfood.html)
4. Incubators

5. Fly lines of the appropriate genotype (Table 1)

2.2.2. Recording of axonal transport

1. Ether
2. 80% glycerol solution in water
3. 10% Sucrose solution in water
4. 50 mL conical centrifuge tubes
5. 12 well plate
6. Beaker
7. Parafilm
8. Forceps
9. Tissue paper
10. Standard microscope slides
11. 32x32mm microscope coverslips
12. Dissection microscope equipped with fluorescent lamp
13. Confocal microscope

2.2.3. Treating 3rd instar larvae with Tubastatin A

1. 24 well plates
2. 10% sucrose solution in water
3. Tubastatin A powder (Sigma SML0044)
4. DMSO
5. Heating bath
6. Vortex

3. METHODS

3.1 Axonal transport recording of mouse cortical neurons seeded in microfluidic devices

3.1.1. Preparation of the microfluidic chambers

1. Coat glass-bottom dishes with 0.1 mg/mL Poly-D-lysine (2mL/dish) and incubate overnight at 4°C. Rinse 3 times with sterile water and allow to air dry under a laminar flow hood for 30 minutes. Dried coated dishes can be stored at 4°C until use for up to one month.
2. Sterilize microfluidics chambers with 70% ethanol for 30 minutes, making sure microgrooves are facing up. Remove ethanol and allow to dry under laminar flow hood (see **Note 1**).
3. Assemble the microfluidic chambers with the microgrooves facing downward on the coated dishes and press gently, either with your fingers or a pipette tip; be gentle on the grooves, to prevent their clogging that would hamper the media flow from somal to axonal compartments. To remove air bubbles that might have formed within the grooves, place microfluidics in a desiccator under vacuum for 30 minutes.

3.1.2. Irrigation of the microfluidics

1. Irrigate the main channel of the somal compartment by adding into one of the two adjacent wells 200µL of Neurobasal medium complemented with 1% Glutamine, 1% P/S and 2% B27 (culturing medium). Wait 5 minutes for the media to flow through the channel and equilibrate with the other somal well. Fill up both somal wells with further 100µL of media.
2. Wait up to 1 hour to allow the media in the somal channel to flow through the microgrooves into the axonal channel thus filling the adjacent axonal wells. Keep the microfluidics in the incubator until ready to use (see **Note 2**).

3.1.3. In utero electroporation of cortical projection neurons

1. Prepare a mix of endotoxin-free plasmids in sterile nuclease-free water in a total volume of 10 μL by diluting each plasmid at 1μg/μL with 0.1% (w/v) Fast green at 1/10 volume to visualize the injection site. To down-regulate Atat1 and decrease MTs acetylation level, you can use conditional sh-Atat1-expressing plasmid or sh-scrambled-expressing plasmid, as control, combined with a cre recombinase-expressing plasmid to drive their respective expression. To restore MTs acetylation levels, use a plasmid encoding for α-tubulin K40Q which mimics MTs acetylation or a WT tubulin-expressing plasmid, as control (see **Note 3-4** for details of the plasmids).
2. By using a Needle Pipette Puller, pull glass microcapillaries tubing with inner diameter of 0.58 mm under the following conditions: heat: 634; pull 92; velocity: 115; time: 210.
3. By using microloader tips, fill pulled microcapillaries with the plasmid mix. Use spring scissors to make progressively deeper diagonal cuts at the microcapillary tip, thus enlarging its diameter, until the plasmid solution passes through. To prevent air drying of the solution, cut the tip of microcapillaries only at the start of the surgery.
4. Anesthetize a pregnant mouse at E14.5 with a flow of 4% isoflurane; once sedated transfer it on a heating pad to quickly apply ophthalmic lubricant (Fucithalmic Vet® 5mg/g) and inject the analgesic (Buprecare 0.1mg/kg injection) subcutaneously.
5. Reduce the anesthetic flow to 2% and turn the mouse on its back. Shave the abdomen with depilatory cream and disinfect afterwards with 70% ethanol solution. Place the surgical field with rhomboidal aperture on the shaved area and wet it with warm phosphate-buffered saline (PBS) before performing a first incision of about 1.5cm along the skin and a second along the midline of the abdominal muscles.

6. Use ring forceps to pull one uterine horn at a time out of the abdomen cavity by holding it at the junction between embryos. Expose the horn on the surgical field and make sure to apply further warm saline solution during the entire procedure to prevent its drying.
7. Gently, hold an embryo steadily and by using a prepared microcapillary connected to a mouth-controlled tube or microinjector, inject 0.1-0.2 μL of DNA plasmid mix through the placenta into one of the lateral ventricles. The filling of the ventricles with the Fast-Green dye indicates the success of the injection (see **Note 5**) (Figure 1A).
8. Hold the embryo with electrode-type forceps, placing the anode at the injection site and the cathode at the opposite side of the brain and deliver 5 electric pulses of 40 V at 50-ms intervals for 950 ms (see **Note 6**) (Figure 1A).
9. After performing ventricle injection and electroporation of the remaining embryos, wet the uterine horn before placing it back into the abdominal cavity. Perform the same procedure on the second uterine horn.
10. Suture first the abdominal muscles and then the skin before placing the operated mouse into a cage kept under a warming light. Monitor closely the recovery of the mouse (see **Note 7**).

3.1.4. Culture of electroporated cortical projection neurons in microfluidic chambers

1. Prepare primary mouse cortical neurons isolated from electroporated E14.5 mouse embryos. Briefly, one day after performing *in utero* electroporation (E15.5), dissect the brains and check for fluorescence of the co-electroporated reporter plasmid under a binocular fluorescent microscope. Dissect the electroporated cerebral hemisphere in ice-cold Hank's balanced salt solution (HBSS) supplemented with 1.5% glucose. In the case of embryos electroporated with the same plasmid mix, collect the micro-dissected electroporated cortexes in the same tube. Dissociate mechanically in culturing medium by using a P1000 pipette and pipetting up and down very gently for approximately 15-20 times. Filter the cellular suspension through a

40 μm strainer, concentrate it by centrifugation at 150 x g for 5 mins and finally resuspend the cells in culturing medium at 40x10^3 cells/μL (see **Note 8**).

2. Aspirate the medium from all the four wells to leave a thin layer of medium covering the well surface (see **Note 9**).
3. Load slowly, by keeping the pipette tip at the entrance of the somal channel, 5 μL of cell suspension. To favor high seeding density within the channel, quickly after adding the cells, remove 5 μL of media from the opposite somal well and slowly load it back in the same well. This practice allows first the cell transit along the channel and then its slowing down. Quickly, observe under a microscope the cell flow through the channel until cell positioning; cells should have attached also along microgrooves entrance (see **Note 10**) (Figure 1B).
4. Without adding any additional media to the wells, incubate in a humidifier incubator for 2 hours until cells have attached.
5. Fill somal and axonal wells with fresh culture medium supplemented with 20ng/mL BDNF and 50ng/mL BDNF, respectively, to promote axon outgrowth. In order to establish a fluidically isolated system ensuring compartment-specific BDNF concentrations, keep a 50μL media volume difference between somal and axonal compartment, with the higher volume in the somal well. This practice prevents diffusion of the higher BDNF concentrated solution from the axonal to the somal well.
6. Keep the devices in a humidified incubator at 37°C, 5% CO2.
7. Check the media volume in the wells every day, and fill up if evaporation occurred. Every two days, replace half of the media with fresh BDNF-enriched media.

3.1.5. Labelling of membrane-bound organelles

1. At DIV4, label mitochondria or lysosomes by replacing half of each reservoir media with fresh culturing media containing 200 nM Mitotracker® or Lysotracker®, to reach a final concentration of 100 nM (see **Note 11**) (Figure 1C).
2. Incubate for 60 minutes at 37°C.
3. Wash twice the wells with fresh culturing media and incubate overnight. Acquisitions are performed the following day.

3.1.6. Recording and axonal transport analysis

After overnight incubation of the culture upon labelling with organelle-specific dies, the transport dynamics of lysosomes and/or mitochondria can be recorded. Position the glass-bottom dish within a recording chamber controlled at 37°C and 5% CO_2 of a confocal microscope (for Even et al. [11], we used a Zeiss LSM 880) and use a 63x oil objective with a working distance of 0.14 mm to acquire time-lapse movies. Given the shorter lengths of dendrites, image at the bottom of the microgrooves (approximately 450 µm from the somal channel) lining the axonal channel to unambiguously focus on axons. The MitoTracker and LysoTracker label the organelles of all cultured neurons, including the non-electroporated ones. Focus only on the electroporated neurons identified by the fluorescent reporter gene of choice. We recommend to acquire three frames per second for 60 seconds in total, as mitochondria and lysosomes trafficking follows the dynamics of the fast axonal transport (see **Note 12**) (Figure 1D).

3.2. Axonal transport recording upon modification of MTs acetylation in *Drosophila melanogaster*

3.2.1. Generation of 3rd instar larvae

1. The D42-Gal4 > UAS *mito*GFP line are maintained at 20°C and passed every 15-20 days.

2. To generate 3rd instar larvae for axonal transport, transfer stock vials in a 25°C incubator to hasten the life-cycle. Adult flies can be passed every 2 days to maximize the amount of eggs that are laid.
3. Transfer the vials with eggs in a 29°C incubator to maximize the expression of Gal4 and check regularly to monitor the growth of larvae.

3.2.2. Anesthesia and recording of axonal transport in motoneurons from D42-Gal4 > UAS *mito*GFP larvae

1. Prepare the anesthesia setup by perforating the lid of a 50mL Falcon to allow ether fumes to penetrate. Place absorbing paper at the bottom of a glass beaker and add 5mL of ether on the paper. Close the beaker hermetically with parafilm to prevent the evaporation of ether fumes (see **Note 13**).
2. When 3rd instar larvae from the D42-GAL4 > UAS-*mito*GFP line are abundant, collect and place 10 larvae in a 12 well plate filled with 10% sucrose solution (see **Note 14**). Larvae should only be kept in sucrose solution for a maximum of 1 hour. Proceed immediately with the rest of the protocol.
3. Select the larvae with the brightest GFP expression in motoneurons using a dissection microscope equipped with a fluorescent lamp and discard the other larvae (see **Note 15**).
4. Select 3 larvae and place them on absorbing paper to dry them (see **Note 16**). Transfer the larvae on the inside of the perforated 50mL falcon lid and close the falcon, making sure that the larvae rest on the inside of the falcon against the lid. Place the falcon with the lid against the paper soaked with ether to position the larvae directly on top of the ether fumes and seal with parafilm for 8 minutes (Figure 2A).
5. Prepare 3 microscope slides and add 300µL glycerol in the center. All the following steps should be performed quickly since the anesthesia lasts about 30min. After 8 minutes incubation, transfer one anesthetized larva per microscope slide. The larvae should always be

placed with the same orientation (e.g with the head facing the side of the slide reserved for annotation) to ensure that the orientation of the movies will be consistent. Use a dissection microscope and a pair of forceps to rotate the tracheas against the microscope slide so that the brain is facing up, as shown in Figure 2B. Place a 32x32mm coverslip on top of the larvae and adjust the position of the larvae by gently moving the coverslip with your finger until the brain and motoneurons are clearly visible on the dissection microscope (see **Note 17**).

6. Place the microscope slide with the coverslip facing the objective of the confocal microscope. The slide should be placed consistently so that the head of the larvae is always on the right (Figure 2C).This will allow the discrimination between anterograde and retrograde transport during the analysis of the movies.
7. Find the brain and the motoneurons emerging from the ventral nerve cord using a small magnification objective and epifluorescent illumination (see **Note 18**). Focus on the proximal segment of motor neurons emerging from the ventral nerve cord with a 60x objective as shown in the red square representing the field of view in Figure 2 C.
8. Acquire 1-minute movies with a maximum of 600ms between frames. Settings of the laser, gain and offset should be adjusted based on the setup (see **Note 19**). Multiple movies can be acquired in different motoneurons of the same larva to maximize the number of mitochondria that can be tracked. Multiple larvae should also be used for a single experiment to accurately represent inter-individual variability. Expected results and their corresponding kymograph for recordings of GFP-tagged mitochondria are shown in Figure 2 D (see **Note 20**).

3.2.3. Treatment with tubastatin A to increase MTs acetylation

1. Prepare the stock solution of Tubastatin A by diluting the powder in DMSO at a concentration of 100mM (see **Note 21**).
2. Select 3rd instar larvae as in section 3.2.2 and maintain them in 10% sucrose solution.

3. Fill a 24 well plate with 500µL 10% sucrose solution per well and divide it in two halves for the control and treated conditions. For the control group add 5µL of DMSO (see **Note 22**) and for the treated group add 5µL of the tubastatin A stock solution to reach a final concentration of 1mM.
4. Add larvae to the wells of the desired condition for 30min. The larvae should move and absorb the drug through the feeding reflex. After treatment, immediately prepare the larvae for axonal transport recording, starting from drying the larvae on absorbing paper and following the steps as previously described in section 3.2.2 (see **Note 23**).

3.2.4. Transgenic fly lines to modulate MTs acetylation levels

1. Transgenic fly lines expressing interfering RNA directed against the main regulators of MTs acetylation can be used to modulate MT acetylation levels. To decrease MTs acetylation, use a double transgenic line expressing both RNAi ATAT1 and RNAi ATAT2 (see **Note 24**), conversely, to increase MTs acetylation, use RNAi against HDAC6. To record axonal transport and knock down ATAT1, ATAT2 or HDAC6, cross the D42-GAL4 > UAS-*mito*GFP with either of the RNAi expressing line. The steps to carry out the crossings are detailed below.
2. The flies used to carry out the crossings should be homozygous to ensure that the F1 larvae will all be heterozygous for the transgenic alleles. To generate homozygote flies, make sure that all flies have lost their balancer and the corresponding phenotypic marker (see **Note 25**). Males from one line should then be crossed with females from the other line.
3. Segregate males and virgin females based on their morphological characteristics (see **Note 26**).
4. Cross males from line 1 (e.g D42-GAL4 > UAS-*mito*GFP) with females from line 2 (e.g any RNAi line) to generate larvae expressing mitochondrial GFP and RNAi against the desired target in motoneurons (see **Note 27**). As in section 3.2.1, carry out crossings at 25°C and transfer adult flies every 2 days to a new vial. Transfer the vials with eggs at 29°C to maximize the expression

of mitochondrial GFP and RNAi before recording axonal transport. From this step, follow the protocol as described in point 3.2.2.

3.3. Analysis of axonal transport recordings

1. Open an image series in FIJI/ImageJ provided with the plugin Kymotoolbox [14].
2. If necessary, separate stacked channels [Image | Color | Split channels].
3. Invert the values of Slices and Frames in the image properties [Image | Properties] (e.g if the value in Slices is 1 and the value in Frames is 50, change the value of Slices to 50 and the value of Frames to 1). This step is necessary for the Kymotoolbox plugin.
4. Use the Segmented Line Tool [right-click on the Line icon | Segmented Line] to manually trace the shape of the axon. Make sure to always trace the line following the directionality of anterograde transport (starting from the soma to the tip of the axon) to ensure that the anterograde and retrograde directionality will be maintained in the kymograph.
5. Double-click on the Segmented Line Icon to select a width that encompasses all moving particles within the axon. Take note of the width and change the value back to 1. Click [t] to add the trace to the Region of Interest (ROI) Manager. Export the data set into excel to keep a trace of the axons that were tracked (see **Note 28**).
6. Use the kymoToolBox plugin to generate a kymograph [Plugin | KymoToolBox | Draw Kymo], tick [Get Kymo] and set the width to the value determined previously in step 5.
7. On the kymograph, draw a segmented line along the length of the particle's trajectories, and, as described in step 5, define their ROI and add it to the ROI Manager.
8. Analyze the displacement over time of the particles tracked by the segmented lines [Plugin | KymoToolBox | Analyse Kymo]. Set the directionality of the movement [Outward is… From left to Right] to ensure that the directionality of anterograde and retrograde transport is preserved. Set the minimum speed for movement to 0,10 μm/sec. Choose [Show colored kymo] to generate a color-coded kymograph for representative purposes where stationary

particles are shown in blue whereas motile particles moving with anterograde or retrograde directionality are shown in green and red respectively (see **Note 2**).

9. Export the Summary data set from the Result panel into Excel. [Mean_speed_Out] is the average velocity in the anterograde direction for a given particle, conversely [Mean_speed_in] is the average velocity in the retrograde direction. A particle moving in the anterograde direction, then changing its course and moving in the retrograde direction would have a value for both these parameters. [Mean_speed] represents the average speed considering both retrograde and anterograde movements of a given particle. [%_Time_Pause] is the percentage of time during which a given particle has a velocity lower than the threshold set in the previous step (0.10 μm/sec).

4. NOTES

1. For the success of the bonding it is important that the device surface is debris-free. Surface contaminants may favor the formation of suboptimal hydrophobic bonds with the glass dish surface, leading to media leaking and axons growing outside the microgrooves. Before sterilization, tape can be used to gently remove debris and cell contaminants accumulated on the chamber surface from previous usages.
2. We recommend to prepare and irrigate the microfluidic platforms a few days before the plating day. If excessive pressure was exerted on the microgrooves during the bonding procedure, a longer time would be needed for the media to flow through the microgrooves. To improve the flow through the microgrooves, pipette up and down very gently at the entrance of the somal channel, making sure of not exerting an excess of pressure that could induce the detachment of the device from the dish. In case of incomplete filling of the microgrooves, ensure that the axonal wells are irrigated with a lower volume compared to the somal ones, to create a hydrostatic pressure that would force the media to pass through the

microgrooves. Keep the microfluidic chamber in the incubator for 24-48 hours until microgrooves are filled.

3. In (Even et al.)[11] we used a pCX-cre expressing plasmid to drive the expression of the sh-Atat1/scrambled-expressing plasmids. Alternatively, a conditional Cre recombinase, e.g. a CreERT2 Tamoxifen-driven expression plasmid, can be used to modulate in a time-controlled manner the down-regulation of the protein of interest, e.g. down-regulation of a protein of interest at post-mitotic stages by injecting tamoxifen to drive cre recombination after the neurogenesis window.
4. If possible, use plasmids expressing a fluorescent reporter. E.g. In (Even et al.) we co-expressed the pair sh-Atat1/scrambled- and Cre-expressing plasmids with a plasmid expressing either pEGFP α-tubulin WT or pEGFP α-tubulin K40Q, to assess the Atat1-driven contribution of tubulin acetylation on axonal transport dynamics. If, on the other hand, the plasmids of interest do not express a fluorescent reporter, we recommend to co-inject a reporter plasmid expressing a fluorescent protein to allow the visualization of the electroporated neurons. Make sure that the fluorophore of the reporter plasmid does not overlap with the channel of the MitoTracker/LysoTracker as this would interfere with the transport dynamics recording of the fluorescently-labelled organelles.
5. A microcapillary tip optimally cut enables a precise and harmless injection into the ventricle. A sub-optimal cut could prevent a sharp tissue perforation hampering its integrity and may allow the leakage of amniotic fluid.
6. Electroporation parameters should be adapted to the experimental design, in accordance with the developmental stage of the embryos to electroporate. The parameters described here were used in (Even et al. 2019)[11]. The correct position of the positively and negatively charged electrodes with respect to the electroporated area, and their steady placement on the uterus wall is critical for the success of the electroporation. When delivering the electric pulses, pay attention not to touch the heart or the placenta with the electrodes,

to prevent administering a shock to the dam. For more practical information check (Turchetto et al.) [15].

7. To increase the survival rate of the operated mouse and electroporated embryos, it is crucial to perform the surgery within 45-50 minutes.
8. To increase cell survival rates, reduce the time required to prepare the cell suspension by dissociating the tissue mechanically without previous enzymatic digestion. This reduces the risk of cell damage that would induce cell aggregation and heterogeneous seeding along the axonal channel.
9. It is very important that both somal wells contain the same volume of media. As follows, the addition of cells into a somal well would generate a hydrostatic pressure between the two somal wells driving the cell to transit along the axonal channel.
10. If 5 μL cell suspension does not ensure 60-70% cell confluency along the somal channel, repeat the loading a second time. Be careful to not load more than three times, as high confluency in the somal compartments would compromise survival of the cells in the channels.
11. Multiple fluorescent organelle dyes can be simultaneously added to a given culture.
12. To prevent neuron photodamages and loss of the fluorescent signal over time, set the lasers of the microscope at a low power to enable the visualization of the labelled cargos for the entire acquisition time.
13. This step should be performed inside a fume hood to prevent the inhalation of ether fumes.
14. 3rd instar is the last stage of larval growth. At this stage, larvae wander on the edge of the vials before starting pupation. To collect 3rd instar larvae, use a pair of forceps to grab motile wandering larvae on the edge of a vial.
15. The D42 driver allows the expression of Gal4 in motoneurons but also in salivary glands. The GFP signal can be strong in the salivary glands bordering the brain but should not be taken

into consideration when selecting the larvae. Focus instead on the brightness of the motoneurons emerging form the ventral nerve cord of the brain.

16. Incomplete drying of larvae will result in inconsistent anesthesia by the ether fumes.
17. If the coverslip is moving during transport or imaging, use nail polish on the sides of the coverslip to seal it.
18. The brightest mitochondria tend to remain stationary whereas the motile mitochondria tend to be smaller and dimmer. The settings of the microscope should be adjusted accordingly in order to capture most motile mitochondria.
19. Axonal transport should always be recorded in the same region to ensure consistent results. We typically record in the proximal portion of the motoneurons, close to the ventral nerve cord.
20. We typically use a Nikon A1R confocal microscope in resonant mode.
21. Tubastatin A is a specific HDAC6 inhibitor which can be used to increase MTs acetylation in models with reduced MTs acetylation [13]. The Tubastatin solution should be clear in DMSO. If some precipitate can be observed, incubate the solution at 37°C and vortex intermittently until it turns clear.
22. We have observed a slight effect of 1% DMSO solution on axonal transport velocity which explains why when treating with tubastatin A, the control group must be treated with DMSO as well.
23. We usually treat 3 larvae at a time with either the DMSO vehicle or the tubastatin A and anesthetize all 3 after the end of the treatment to record axonal transport. The timing of the experiment has to be followed rigorously and the anesthesia and recording of axonal transport should be carried out as quickly as possible following treatment to minimize experimental variability.
24. ATAT1 is the major enzyme responsible for promoting the acetylation of Lysine 40 of MTs in mammals [16]. In consequence, its loss results in strong reduction of MTs acetylation.

Drosophila Melanogaster has two orthologs of the ATAT1 gene, ATAT1 and ATAT2 which have a redundant function, meaning that downregulation of one or the other will result in partial loss of MTs acetylation whereas combined downregulation will result in an almost complete loss of MTs acetylation.

25. Balancer chromosomes are modified chromosomes which prevent homologous recombination, carry a dominant phenotype marker mutation and are recessive lethal. Based on these properties, flies can be identified base on the phenotypic marker associated to their balancer chromosome and cannot carry two copies of the same balancer chromosome. For instance, flies carrying the CyO balancer will exhibit curled wings (Cy marker) while flies carrying the TM6B balancer will be shorter and thicker than wild-type flies at both the larval and pupal stages (Tubby marker).
26. Males have a thin abdomen, slightly curved inwards with visible genitals appearing as a large brown mass in the postero-ventral part of the abdomen whereas females are bigger than males and their abdomen is round and white. Virgin females are slightly translucent and can be recognized by the meconium, a dark spot in the upper left abdomen persisting up to 3 hours after eclosion. The presence of the meconium guarantees that the female is a virgin since female flies are only fertile 8 hours after eclosion.
27. Both sexes can be taken from each line for the crossing if the genes of interest are carried on the somatic chromosomes (1, 2 or 3). If a transgenic allele is carried by the X chromosome, only virgin females should be used to ensure that all the progeny will receive one transgenic allele.
28. If an axon harbors a high number of static cargoes which are cluttering the kymograph, it is possible to choose a smaller width which would allow to exclude some static cargoes to focus only on motile ones. We also recommend to trace segmented lines of the same length along all the axons/motoneurons considered for the analysis, for standardized calculations of vesicles run length.

29. By convention, anterograde transport is represented from left to right on representative kymographs and has a positive value for velocity whereas retrograde transport is represented from right to left and has a negative value for velocity.

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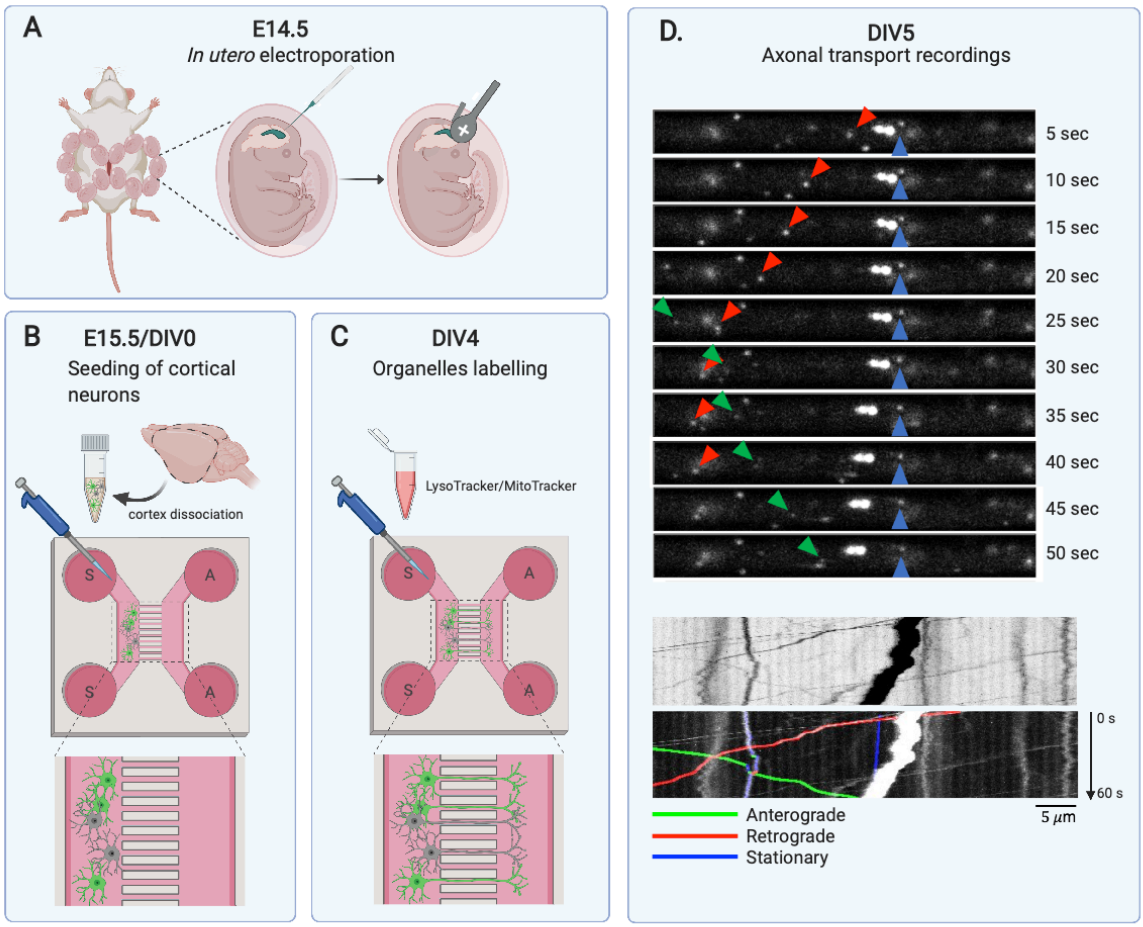

Figure 1: Axonal transport recordings of mouse cortical neurons seeded in microfluidics chambers.

(A) *In utero electroporation of E14.5 mouse embryos with a solution of DNA plasmids to modulate MTs acetylation level. Inject one lateral ventricle with the plasmid mix until the injection site is decorated by the fast green. Electroporate the injected plasmids into the neocortex by positioning the cathode (positively charged electrode) towards the target area and the anode (negatively charged electrode) at the opposite side of the brain.* ***(B)*** *Seeding of E15.5 cortical neurons dissociated from electroporated brains. Dissect and dissociate the electroporated hemisphere to prepare a cell suspension to be plated in the somal compartment (S) of the microfluidic chambers. Note that the seeded culture encompass also non-electroporated neuron.* ***(C)*** *Labelling of membrane-bound organelles. At DIV4, label seeded neurons with organelle-labelling dyes (e.g. LysoTracker for lysosomes and MitoTracker for mitochondria) for overnight incubation.* ***(D)*** *Recording of the axonal transport at DIV5 to follow the movements of labelled organelles. Wash somal (S) and axonal (A) wells with fresh medium and use a*

confocal microscope to record axonal transport dynamics of labelled organelles along the lower microgrooves segment lining the axonal channel.

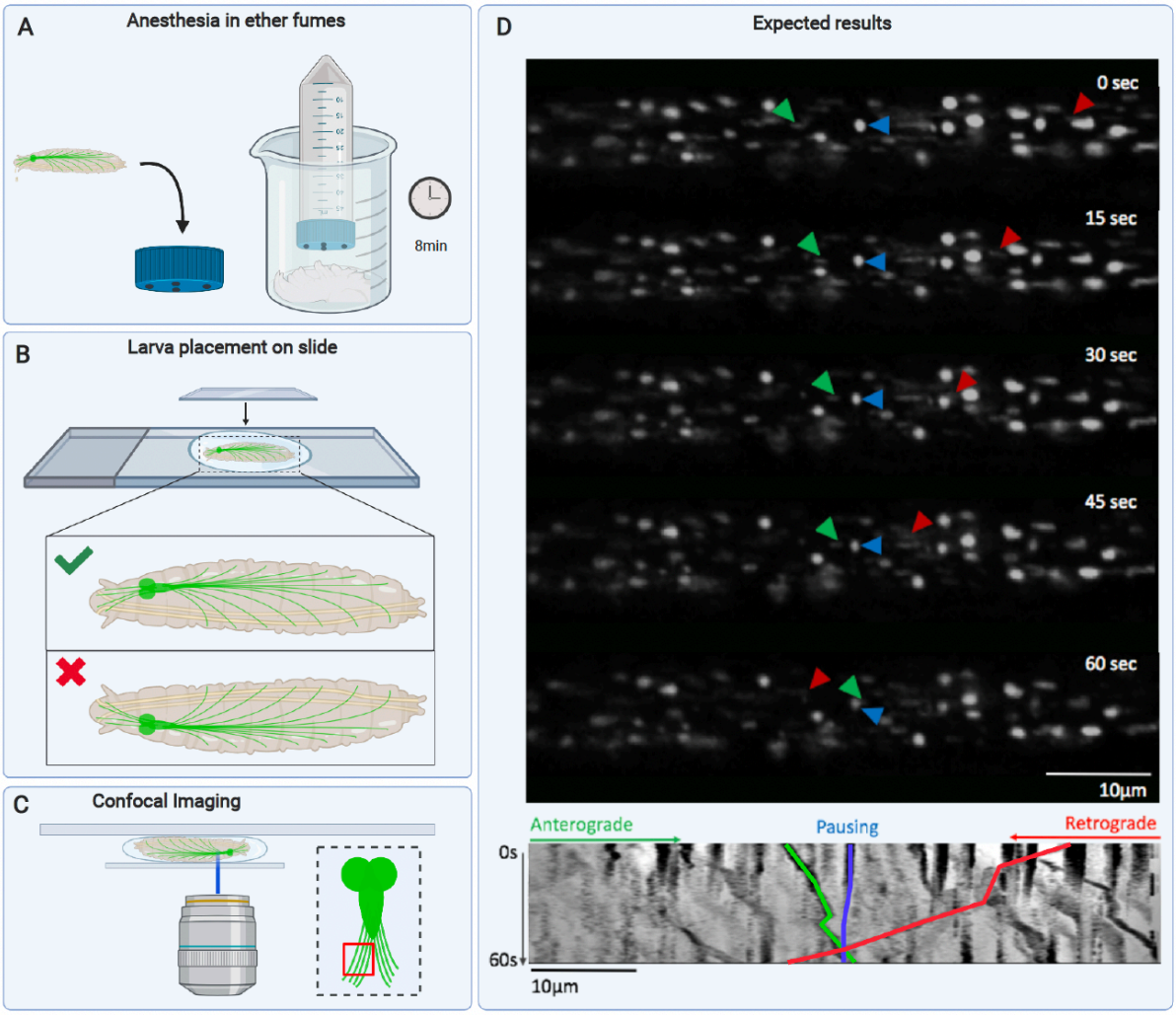

Figure 2: Axonal transport recording in 3rd instar larvae

(A) *The fly larvae are placed on the inside of a perforated 50mL falcon lid. The falcon is then closed with the larvae on the inside and placed in a beaker with ether-soaker tissue paper for 8 minutes.* ***(B)*** *The anesthetized larva is placed in a drop of 80% glycerol on a microscope slide with the ventral side facing up and is then covered by a 32x32mm glass coverslip.* ***(C)*** *A confocal microscope is used to record motile mitochondria in the proximal segment of the axon in motoneurons emerging from the ventral nerve cord, as shown in the red square depicting the field of view.* ***(D)*** *Motile mitochondria can be visualized as shown in the expected results and the corresponding kymograph. Arrows track moving mitochondria*

which are highlighted on the kymograph below. Panel D is adapted from Le Bail et al. with permission [17].

Table 1: Fly lines used in this protocol with their origin and purpose

Fly line	***Origin***	***Purpose***
D42-GAL4 > UAS-*mito*GFP	Bloomington Drosophila Stock Center (BDSC) #42737	Expression of Gal4 in motor neurons [11]
UAS-RNAi HDAC6	BDSC #34072	UAS-dependent expression of interfering RNA (RNAi) against the HDAC6 enzyme to increase acetylation levels [11]
UAS-RNAi ATAT1	VDRC #106247	UAS-dependent expression of RNAi against the ATAT1 enzyme to decrease acetylation levels [11]
UAS-RNAi ATAT2	VDRC #101273	UAS-dependent expression of RNAi against the ATAT2 enzyme to decrease acetylation levels [11]
UAS-RNAi ZPG	VDRC #33277	Control line using UAS-dependent expression of RNAi against ZPG, a protein expressed in gonads [11]

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