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Differential effect of three-repeat and four-repeat tau on mitochondrial axonal transport

Will Stoothoff[*,†], **Phillip B. Jones**[*], **Tara L. Spires-Jones**[*], **Daniel Joyner**[*], **Ekta Chhabra**[*], **Kathryn Bercury**[*,‡], **Zhanyun Fan**[*], **Hong Xie**[*], **Brian Bacskai**[*], **Jon Edd**[§], **Daniel Irimia**[§], and **Bradley T. Hyman**[*]

[*]MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA

[†]University of Massachusetts Medical School, Worcester, Massachusetts, USA

[‡]University of Colorado Health Science Center, Denver, Colorado, USA

[§]BioMEMS Resource Center, Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital, Shriners Hospital for Children, and Harvard Medical School, Boston, Massachusetts, USA

Abstract

Tau protein is present in six different splice forms in the human brain and interacts with microtubules via either 3 or 4 microtubule binding repeats. An increased ratio of 3 repeat to 4 repeat isoforms is associated with neurodegeneration in inherited forms of frontotemporal dementia. Tau overexpression diminishes axonal transport in several systems, but differential effects of 3 repeat and 4 repeat isoforms have not been studied. We examined the effects of tau on mitochondrial transport and found that both 3 repeat and 4 repeat tau change normal mitochondrial distribution within the cell body and reduce mitochondrial localization to axons; 4 repeat tau has a greater effect than 3 repeat tau. Further, we observed that the 3 repeat and 4 repeat tau cause different alterations in retrograde and anterograde transport dynamics with 3 repeat tau having a slightly stronger effect on axon transport dynamics. Our results indicate that tau-induced changes in axonal transport may be an underlying theme in neurodegenerative diseases associated with isoform specific changes in tau's interaction with microtubules.

Keywords

Alzheimer's disease; axonal transport; mitochondria; tau; tauopathy

In the adult human brain, there are six isoforms of the tau gene expressed, with an approximately 50/50 ratio of isoforms containing either 3 microtubule binding repeats (not including exon 10) or 4 microtubule binding repeats (including exon 10) (Goedert *et al*. 1989; Kosik *et al*. 1989; Goedert and Jakes 1990). Although it has been postulated that the 4

Address correspondence and reprint requests to Bradley T. Hyman, M.D., Ph.D., Department of Neurology/Alzheimer Disease Research Unit, Massachusetts General Hospital, 114 16th Street, Charlestown, MA 02129, USA. bhyman@partners.org.

Supporting Information Additional Supporting Information may be found in the online version of this article:
Figure S1. Similar 3R and 4R tau over-expression levels in cortical neurons.
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repeat isoforms bind microtubules more avidly than the 3 repeat isoforms (Butner and Kirschner 1991), a direct comparison of the 3 repeat and 4 repeat isoforms on microtubule function has not been reported. The issue is of importance from a pathophysiologic perspective, because the ratio of 3 repeat to 4 repeat tau is substantially altered in many cases of autosomal dominantly inherited frontotemporal dementia, in which changes in splice acceptor domains stabilize the 4 repeat form of the molecule (Hutton 2000; Hasegawa 2006; Tsuboi 2006). A striking increase in 4 repeat to 3 repeat ratio is observed in sporadic corticobasal ganglionic degeneration and progressive supranuclear palsy as well as frontotemporal dementia (Ingelsson *et al*. 2007). In Alzheimer's disease, a subtle increase in 4 repeat tau has also been detected (Conrad *et al*. 2007).

Microtubule-dependent transport of mitochondria into the axon is a critical factor to maintain local ATP generation in distal compartments in neurons. Although true in all cells, neurons place an especially heavy burden on microtubule-dependent transport processes by motor proteins because of the extraordinary distances involved in these highly polarized cells. In previous studies, utilizing Chinese hamster ovary (CHO) cells (Ebneth *et al.* 1998), N2A cells (Mandelkow *et al.* 2004) cultured retinal ganglion cells (Mandelkow *et al.* 2004), or NB2a/d1 cells (Dubey *et al*. 2008), overexpression of full-length tau led to a consistent phenotype of diminished anterograde transport of a variety of kinesin cargos, including organelles. For example, mitochondria were noted to accumulate within the cell body, and to cluster near the microtubule organizing center (Ebneth *et al*. 1998). These studies suggested that tau competes for kinesin-mediated anterograde transport when it is bound to microtubules. In support of this model, the velocity of microtubule-dependent transport of vesicles and organelles was not observed to change, but the likelihood that organelles attach to microtubules was altered by overexpression of full-length tau (Trinczek *et al*. 1999).

Thies and Mandelkow (2007) have recently demonstrated that co-expression of the kinase microtubule associated protein/microtubule affinity-regulating kinase 2 (MARK2) can partially rescue the tau over-expression phenotype, likely because MARK2 phosphorylates tau near the microtubule binding domain repeats, removing tau from the microtubule tracks. These results also suggest that tau binding to microtubules is critical to impair transport of mitochondria, and that manipulations that alter tau binding to microtubules may therefore impact this phenotype (Thies and Mandelkow 2007).

As 3 repeat tau is believed to be less tightly associated with microtubules than 4 repeat tau (Hasegawa 2006; Tsuboi 2006), we postulated that 4 repeat tau may lead to greater alterations of organelle transport than 3 repeat tau and, via for example loss of mitochondria from axons, may pre-dispose to neurodegeneration. In this study, we therefore directly examined the effects of over-expression of 3 repeat compared with 4 repeat tau on mitochondrial transport from cell bodies into processes, and found that 4 repeat tau has a greater impact on mitochondrial transport into axons than 3 repeat tau.

We also reasoned that we may be able to detect subtle changes in anterograde or retrograde movement over several hundred microns of axons in comparison with earlier studies which focused primarily on cultured cells with relatively short processes. To facilitate these studies, we adapted for our use a novel nanochamber microfluidic device (made in house) that allows culturing of primary neurons in one chamber, and the outgrowth of their axons into straight channels that are ~1000 μm long. Using these devices, we were able to readily monitor mitochondrial trafficking in axons. We find that tau over-expression leads to changes in anterograde and retrograde transport, and that both isoforms impair transport, although the specific effects of 3 repeat and 4 repeat tau differ.

Materials and methods

Cloning

To create cyan fluorescent protein (CFP)-labeled tau proteins, subcloning of tau constructs of different isoforms was carried out by standard PCR and ligation procedures. Tau3 repeat minus 2 minus 3 (excluding exons 2, 3, and 10), Tau3 repeat plus 2 plus 3 (including exons 2 and 3, excluding 10), Tau4 repeat minus 2 minus 3 (excluding exons 2 and 3, including 10), and Tau4 repeat plus 2 plus 3 (including exons 2, 3, and 10) in PCDNA 3.1 were generously provided by Dr. Mike Hutton (Mayo Clinic, Jacksonville, FL, USA). The tau gene from each construct was obtained utilizing PCR (N-terminal primer 5′-CTCGAGATGGCTGAGCCCCGCCAGGAGTTCGAAG-3′), containing an Xho-1 digestion site and (C-terminal primer 5′-AATAGGATCCAAACCCTGCTTGGCCAGGGAGGCAGAC-3′) with a BamH1 digestion site. PCR products were digested with Xho-1 and BamH1 and ligated into double-digested eCFP-N1 vector (Clontech, Mountain View, CA, USA).

For TauC3 construct, the longest human isoform of tau (4 repeat tau including exons 2 and 3) was used to PCR the tau gene minus the last 60 bp (N-terminal primer 5′-TGCACCTCGAGACATGGCTGAGCCCCGCCAGG AGTTCGAAGTGCACCTCGAGACATGGCTGAGCCCCGCCAGGAGTTCGAAG-3′ and C-terminal primer 5′-TTGGATCCCTAGTCTACCATGTCGATGCTGCCG-3′). Tau PCR product was then digested, along with the pMAX-green fluorescent protein (GFP) vector (Amaxa, Walkersville, MD, USA) and ligated into the digested vector. All vectors and constructs were verified by sequence analysis.

Cell culture

H4 human neuroglioma cells were maintained in Opti-MEM (Gibco, Carlsbad CA, USA) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Cells were transiently transfected with the identified constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Primary cortical neurons were prepared from cerebral cortices of mouse embryos (day 15–17 of gestation). Cortices were dissociated by trypsinization for 5 min at 23°C and cells were resuspended in Neurobasal (NB) (Gibco) medium supplemented with 10% fetal bovine serum, 2 mM/L Gln, 100 U/mL penicillin, and 100 μg/mL streptomycin and centrifuged at 100 g for 10 min and resuspended in NB/B-27 [NB medium containing 2% (v/v) B-27 supplement], 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM/L glutamine and plated at 1×10^6 on 42 mm round coverslips (Hemogenix, Colorado Springs, CO, USA), coated with Poly-D-Lysine (20 μg/ml) (Sigma, St. Louis, MO, USA) in 60 mm cell culture dishes (Corning, Corning, NY, USA) or in microfluidic devices as described later. Neurons were grown for 6–7 days *in vitro* (DIV) and transiently transfected with the indicated constructs utilizing Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

We examined whether tau over-expression altered the actin cytoskeleton by culturing cortical neurons on poly-D-lysine-coated four-well chamber slides and differentiating neurons for 6–7 DIV. After differentiation, neurons were transfect as before, with either 3 repeat or 4 repeat CFP-tau (both containing exons 2 and 3). Cells were then fixed, 24 h post-transfection with 4% paraformaldehyde for 1 h at 23°C, washed three times with phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton-X100 in PBS for 10 min at 23°C and washed in PBS. CFP-Tau transfected neurons were fixed as before and neurons were then stained with rhodamineconjugated phalloidin and washed three times with PBS. Slides were then mounted

with Permount mounting media and allowed to dry, prior to visualization via confocal microscopy. No qualitative difference in the actin staining was observed in tau-transfected neurons.

To examine the extent to which tau transfection led to tau over-expression, neurons were transfected, fixed, and prepared as before, then incubated with the Tau5 monoclonal antibody at a dilution of 1/100 at 4°C overnight and washed thoroughly with PBS. The Tau5 primary antibody was then labeled with an Alex 594 donkey antimouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1/100 at 23°C for 1 h, washed three times with PBS and mounted. Slides were then mounted with Permount mounting media and allowed to dry, prior to visualization via confocal microscopy. Tau5 immunostaining intensity was measured along a 10-μm-line drawn across the cell body, outside the nucleus (line scan) for 10 transfected and 10 untransfected cells (see supporting information Fig. S1).

Mouse work was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and conformed to National Institutes of Health (US) guidelines.

Western blotting

Cells were collected 48 h post-transfection in 100 μL 2× sodium dodecyl sulfate (SDS) lysis buffer (0.25 M Tris–Cl, pH 7.5, 2% SDS, 5 mM EDTA, 5 mM EGTA, 10% glycerol supplemented with a protease inhibitor pellet (Gibco). Cell lysates were then sonicated for 10 s and boiled for 10 min. Samples were then centrifuged at 23°C for 10 min at 15 000 *g*. Sample concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were diluted to 1 μg/μLin 2× SDS running buffer and loaded onto precast 4–12% Tris–Glycine gradient polyacrylamide gels (Invitrogen), electrophoresed and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at 23°C in TBST (20 mM Tris–Cl pH 7.6, 137 mM NaCl, 0.05% Tween 20) and 5% milk and incubated in TBST and milk at 4°C overnight in indicated primary antibody. Membranes were then washed three times for 10 min in TBST at 23°C and incubated for 1 h with appropriate horseradish peroxidase-conjugated secondary antibody in TBST and milk. Membranes were washed three times for 10 min in TBST at 23°C and visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

Nano chamber devices

Microfluidic devices were adapted from Taylor *et al.* (2005) and manufactured in-house. The device contains two chambers which are connected by 300 or 1000 μm channels that are 3 μm high and 8 μm wide. Initial experiments used 300 μm channels, but we found that 1000 μm channels were more convenient as they allowed visualization of a longer section of axon. The device is attached to a poly-D-lysine coated coverslip, to which the cells adhere. This allows the growth of axons through the channels and ensures that only one or two axons propagate through each channel. Taylor *et al.* (2005) demonstrated that only axons propagate through the channel for longer than 150 μm and that axons will consistently grow from one major chamber to the other, with one chamber containing only cell bodies and the other containing axon terminals and growth cones.

The microfluidic devices were loaded with mouse primary cortical neurons isolated from E15 mouse embryos that were trypsinized and dissociated and placed in the chamber at an approximate density of 1×10^5 cells per device. Cells were transfected with the above tau constructs on 6 or 7 DIV. One tau construct was transfected per device.

Transfections were carried out as follows: 1.2 μg of total DNA was used per device. 0.6 μg of mitochondrial DsRed (Mito-DsRed) was used and 0.6 μg of Tau DNA was used per device.

Neurons were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. The only change made was that the cells were transfected in Dulbecco's modified Eagle's medium, which was left on neurons for 2 h and then medium was changed back to Neurobasal with B-27 supplement. Cells were left for 24 h and imaged the next day. Neurons transfected with CFP-3R tau or CFP-4R tau were examined 24 h after transfection and the amount of CFP signal, quantitated on a Zeiss 510 inverted microscope (Carl Zeiss, Jena, Germany), was equal, suggesting that 3 repeat tau and 4 repeat tau were equally well translated and equally stable under these conditions.

Coverslips containing the microfluidic device were examined using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss) encased in a 37°C, 5% CO_2 environmental chamber equipped with a Zeiss LSM 510 META (Zeiss, Jena, Germany) confocal scanhead using 488 and 543 nm lasers and a Coherent (Santa Clara, CA, USA) Chameleon Ti/Saph at 800 nm ~140 fs pulse width at 90 MHz, ~20–30 mW average power with linear polarization at the sample, for CFP and GFP imaging. All images were acquired using a Zeiss 63× 1.2 NA C-APo-Plan NEOFLU water immersion lens (Carl Zeiss), mounted on the microscope described before.

Calculation of mitochondrial distribution and clustering

H4 cells and primary neurons were co-transfected with GFP or one of the GFP or CFP-tau constructs (Fig. 1) and Mito-DsRed to label mitochondria. Images of cell bodies and, in the case of cultured neurons, axons, were acquired using a Zeiss 63× 1.2 NA C-APo-Plan NEOFLU water immersion lens, mounted on the microscope described before (n = 45 H4 cells and 35 neurons per transfection condition). In Image J (free software from the National Institutes of Health, Bethesda, MD, USA), the green (for GFP) or blue (for CFP constructs) and red channels were thresholded separately (to the edge of the intensity histogram). The cell body was outlined in the GFP or CFP channel and the analyzed particles' function was used on the red channel to find the percentage area occupied by mitochondria. A decrease in the percentage of cytoplasm occupied by mitochondria is interpreted to mean the mitochondria are clustering near the nucleus instead of being transported to the cell periphery.

Generation of kymographs and calculation of mitochondrial dynamics

Kymographs were obtained from time series images obtained on the Zeiss LSM 510 META. Images were acquired every 2 s and 100 images were acquired for a total of 200 s. Movies were then input into Metamorph and kymographs were generated using the kymograph function of the software, based on tracing a line along the length of travel of a mitochondrion along an axon by hand. Kymographs were then analyzed using a specialized macro created in Matlab (Mathworks, El Segundo, CA, USA) that calculated the velocity in the anterograde and retrograde directions. The macro also calculated the percentage of time traveled in the antero- and retrograde directions per mitochondrion. In each condition, at least 35 mitochondria were imaged, from at least 10 individual neurons from at least three separate neuron preparations. Each condition was pooled and analyzed as described next. As a result of the low transfection efficiency (typically 1–2% in differentiated neurons), the incidence of transfected axons spanning the microfluidic nanochannel is relatively low. Therefore, only a few axons per device are able to be utilized to analyze mitochondrial behavior.

Statistics

Normality of data was assessed using a Shapiro–Wilks test. As datasets were not normally distributed, non-parametric analyses were used. For two group comparisons, Mann–Whitney tests were used (control vs. tau and 3 repeat vs. 4 repeat) and for multiple group comparisons (tau isoforms), Kruskal–Wallis tests were used. Data are presented as box plots, which display the median value (line inside the box), upper quartile (top of the box), lower quartile (bottom

of the box), 90th percentile (top whisker), 10th percentile (bottom whisker), and all values below the 10th and above the 90th percentile (potential outliers) shown as dots.

Results

Previous studies have clearly demonstrated that over-expression of full-length tau leads to the clustering of organelles in the cell (Ebneth *et al*. 1998; Trinczek *et al*. 1999; Seitz *et al*. 2002; Stamer *et al*. 2002; Thies and Mandelkow 2007; Dixit *et al*. 2008; Dubey *et al*. 2008; Ittner *et al*. 2008). The percentage of cytoplasmic area in which mitochondria are located is substantially changed by over-expression of tau. For example, in CHO cells, the percent of cellular area populated by mitochondria is more than 50%, and this diminishes to below 20% in the presence of over-expressed full-length tau (Mandelkow *et al*. 2004). We utilized this same mitochondrial clustering assay to initially examine the effects of different isoforms of tau (Fig. 1), including the two major human isoforms: the shortest isoform containing 3 microtubule binding domain repeats and missing the two small amino terminal exons 2 and 3 (3 repeat tau minus 2 minus 3) and full-length 4 repeat tau which includes exons 2, 3 and 10. We also examined the effects of other isoforms (e.g., 4 repeat minus exon 2 and exon 3; 3 repeat minus exon 2 and exon 3) as well as a carboxy terminal truncation at position 421, mimicking a postulated caspase cleavage product of tau (Krishnamurthy *et al*. 2000). These constructs therefore provide physiologically relevant alterations of the amino terminal domain (exons 2 and 3), the microtubule binding domain (exon 10, 3 repeat vs. 4 repeat), and the carboxy terminal domain (truncation at position 421). We first utilized human H4 neuroglioma cells, which, like CHO cells, are relatively easy to transfect and have a flat, easy to image cytoplasm. The CFP-Tau constructs along with the Mito-DsRed construct were co-transfected into H4 cells. As expected, tau over-expression markedly decreased the overall area occupied by mitochondria (Fig. 2a). Separate transiently transfected H4 cells were collected and western blotted to verify construct expression and protein properties. Expression was similar in all conditions and all constructs migrated as expected on SDS–Polyacrylamide gel electrophoresis, indicating that the tau constructs express and produce the expected tau isoform.

Tau over-expression led to a clear phenotype of clustering of the mitochondria within the cell body 24 h after transfection causing a 50% decrease in the area of the cell body occupied by mitochondria (Mann–Whitney test $p < 0.0001$) (Fig. 2b). Although all tau isoforms had a statistically significant effect on mitochondrial transport to the cell periphery, interestingly all constructs containing 4 microtubule binding repeats had a more profound effect than constructs containing only 3 repeats (Mann–Whitney test 3 repeat vs. 4 repeat split by presence of tau; tau group $p < 0.0001$). Manipulation of the amino terminal exons 2 and 3, or deletion of the carboxyl terminal 20 amino acids to mimic caspase cleavage, did not further impact the tau-induced change in mitochondrial localization in H4 cells (Fig. 2b). Experiments using untagged Tau constructs cotransfected with Mito-DsRed showed similar results (data not shown), indicating that the presence of the CFP or GFP tag on tau constructs did not contribute to the mitochondrial clustering phenotype. Tau over-expression in these experiments was verified by immunocytochemistry following the fixation of the cells.

To further evaluate the effects of the 3 repeat and 4 repeat tau constructs in neurons, we utilized a new microfluidic nanochamber device. This device has a reservoir for neuronal cell bodies, a series of rather long, thin channels that are only 3 μm high, 8 μm wide, and either 300 or 1000 μm long that contain axons, and a second reservoir chamber which contains only axon terminals. The design for these chambers was adapted from Taylor *et al*. (2005) (Fig. 3a). Neurons were grown in the reservoir and, as expected, axons grew down the channels over the course of the first week in culture. Each channel is wide enough to accommodate only a few axons at a time, and staining of neurons on a device with Mitotracker Green, reveals some channels with multiple axons (typically two or three), and a majority with only one axon

present. This is not an issue in the experiments described below, as the likelihood of two cells being transfected and sending axons through the same channel is minimal, and CFP signal showed only a single transfected axon in each channel examined (Fig. 3b). Neurons were co-transfected with CFP-Tau and Mito-DsRed, allowing observation of the percentage of cell soma occupied by mitochondria, the density of mitochondria in axons, and (via generation of time-lapse confocal optical section stacks) data on mitochondrial transport, direction and velocities.

To determine relative levels of expression of 3 repeat and 4 repeat tau in neurons, we transfected neurons with 3 repeat or 4 repeat tau tagged with CFP and quantified the intensity of CFP fluorescence in processes from 10 cells per condition by immunostaining with an anti-tau antibody. We found that intensity of 3 repeat and 4 repeat tau stains do not differ, indicating similar expression levels of 3 repeat and 4 repeat tau with transfection. Measurement of both endogenous and expressed CFP-tagged tau, showed that both 3 repeat and 4 repeat exogenous tau are expressed at ~1.6-fold, the level of the endogenous tau (see supporting information Fig. S1).

To examine whether tau over-expression alters the normal actin filaments in transfected cells, differentiated neurons were transfected with either the 3 repeat or 4 repeat CFP-tau and stained with rhodamine phalloidin. No difference was observed between the CFP-tau transfected and untransfected neurons.

We next examined the percentage of the neuronal cell body occupied by mitochondria as a function of tau transfection (Fig. 4a). Again, a marked and highly statistically significant effect of tau on mitochondrial clustering in the cell body was observed ($p < 0.0001$, Mann–Whitney test control vs. tau). Although both 3 repeat-containing isoforms and 4 repeat-containing isoforms had a strong and statistically significant effect on mitochondrial clustering, there was a clear isoform difference in which 4 repeat forms had a stronger effect on mitochondrial localization than 3 repeat forms ($p < 0.0001$, Mann–Whitney test 3 repeat vs. 4 repeat, tau group). Within the 3 repeat isoforms, the inclusion or exclusion of the N-terminal exons 2 and 3 had no further effect on mitochondrial distribution ($p > 0.05$, *post hoc* Mann–Whitney test 3 repeat minus 2 minus 3 vs. 3 repeat plus 2 plus 3). However, 4 repeat tau without exons 2 and 3 (4 repeat minus 2 minus 3) had less effect on mitochondrial distribution than the longer 4 repeat plus 2 plus 3 or 4 repeat C3 isoforms ($p < 0.0001$, *post hoc* Kruskal–Wallis test on 4 repeat isoforms, $p < 0.0001$ *post hoc* Mann–Whitney tests comparing 4 repeat minus 2 minus 3 to 4 repeat plus 2 plus 3 and $p < 0.0001$ *post hoc* Mann–Whitney test comparing 4 repeat minus 2 minus 3 to 4 repeatC3). Overall, these data show substantial similarities in the effect of tau isoforms on mitochondrial translocation to the periphery of the cell between H4 cells and neurons, and suggest that the number of microtubule binding repeats influences this process.

To examine whether the increased clustering of mitochondria in the neuronal cell body was associated with impaired trafficking of mitochondria to axons, we measured the density of mitochondria in axons. As expected, tau transfection led to a significant reduction in the area of mitochondria in axons ($p < 0.0001$, Mann–Whitney test control vs. tau), and this effect was more pronounced for 4 repeat compared with 3 repeat tau ($p < 0.0001$ *post hoc* Mann–Whitney test 3 repeat vs. 4 repeat, tau group only). The area of mitochondria per axon examined was reduced by about 33% by 4 repeat tau over-expression, and by about 20% by 3 repeat tau overexpression (Fig. 4b).

This result suggests that mitochondria are less likely to reach axons and engage the axonal microtubular tracks in the presence of tau. To examine whether the mitochondria that do engage axonal microtubular tracks then move normally, or if tau also alters trafficking of mitochondria

in axons, we measured the kinetics of mitochondrial trafficking in axons using microfluidic channels. Several technical difficulties confound measurement of organelle transport in axons in primary neurons in culture. In routinely used 35 mm dishes, it is often somewhat difficult to distinguish axons from dendrites without immunostaining which, of course, precludes live-cell imaging. Processes tend to cross each other innumerable times, making it difficult to observe a long expanse of a relatively straight process. We utilized microfluidic devices to overcome these issues. The configuration of the channels precludes short dendritic processes from growing down the channel (Taylor *et al*. 2005).

We constructed our chambers to be 300 or 1000 μm in length, allowing relatively long, straight expanses of each axon to be imaged (Fig. 3). Mouse primary neurons were cultured for 6–7 days in the device, followed by the transfection of the neurons with either CFP or CFP-Tau along with Mito-DsRed. The axons were imaged in an environmental chamber which enclosed the entire microscope, holding temperature at 37°C with 5% CO_2 and optimal humidity for long-term experiments. We found that, under these conditions, we could monitor both anterograde and retrograde movement and calculate velocity. From these transfected cells, time-lapse movies were made and kymographs generated (Fig. 5a). Transfecting CFP alone, average mitochondrial anterograde velocity was 0.24 μm/s, and retrograde velocity was 0.22 μm/s, which is in agreement with recent data by Gerencser and Nicholls (2008). Transfection with either 3 repeat or 4 repeat tau slowed average velocity (both anterograde and retrograde averaged together: transfected axons, $p < 0.0001$ Mann–Whitney test control vs. tau). The effect on anterograde velocity was larger than the effect on retrograde velocity (significant reduction of anterograde velocity with tau over-expression, $p = 0.03$ Mann–Whitney test; no significant effect on retrograde velocity $p = 0.08$ Mann–Whitney test), leading to a net decrease in the anterograde to retrograde ratio. Surprisingly, the effect on anterograde velocity was more pronounced with 3 repeat tau than 4 repeat tau ($p < 0.0001$ *post hoc* Mann–Whitney test 3 repeat vs. 4 repeat, tau group). Thus, 3 repeat tau slow down motors to a greater extent than 4 repeat tau, although both decrease the anterograde velocity ratios leading to a bias against plus-end directed transport.

In CFP-transfected neurons, 33% (median) of mitochondria move in the anterograde direction and 16% move in the retrograde direction, while in tau-transfected neurons 17% of mitochondria move in the anterograde direction and 49% in the retrograde direction, a significant decrease and increase, respectively ($p < 0.0001$ Mann–Whitney test control vs. tau for both percent anterograde moving and percent retrograde moving). These values are medians from at least 35 neurons analyzed over three preparations per conditions. As illustrated in the kymograph in Fig. 5a, there are numerous mitochondria that do not move appreciably in the time course observed, thus the percent mitochondria moving in either direction do not add up to 100%. These changes indicate an imbalance in transport which likely contributes to the accumulation of mitochondria in the cell body. There is no difference between 3 repeat and 4 repeat isoforms on percent anterogradely moving mitochondria, but 3 repeat tau has a more pronounced effect on increasing the percent retrogradely moving mitochondria ($p < 0.0001$, post hoc Mann–Whitney test 3 repeat vs. 4 repeat, tau group). The shortest isoform, 3 repeat minus 2 minus 3 has a more pronounced effect than 3 repeat plus 2 plus 3 ($p = 0.003$, post hoc Mann–Whitney test). Within the 4 repeat group, the 4 repeat minus 2 minus 3 isoform has more of an effect than either of the longer 4 repeat isoforms used ($p = 0.001$ for 4 repeat minus 2 minus 3 vs. 4 repeatC3; and $p < 0.0001$ for 4 repeat minus 2 minus 3 vs. 4 repeat plus 2 plus 3, *post hoc* Mann–Whitney tests), indicating a more pronounced effect on the percent retrogradely moving mitochondria without the two N-terminal inserts. The total percentage of mitochondria moving in either direction is decreased by tau over-expression from 21.4% to 13.3% (median, $p = 0.001$, Mann–Whitney test tau vs. control), with no difference between 3 repeat and 4 repeat tau ($p > 0.05$, *post hoc* Mann–Whitney test 3 repeat vs. 4 repeat tau group). Thus, the amino terminal domain of tau may impact this aspect of organelle trafficking.

Together, these data indicate that tau leads to a decrease in mitochondria translocating to the axon, a decrease in the percentage of mitochondria moving in the axon, a pronounced shift in the balance of anterograde to retrograde transport with fewer mitochondria moving away from the cell body and more moving toward it (Mandelkow *et al*. 2003), and a decrease in the velocity of mitochondria, with a more pronounced effect on slowing mitochondria moving away from the cell body while allowing normal speed of transport toward the cell body. Overall, 4 repeat tau has a more profound effect than 3 repeat tau on mitochondrial trafficking in primary neuronal axons.

Discussion

Four-repeat isoforms are over-represented in autosomal dominantly inherited cases of frontotemporal dementia, but the physiologically important consequence of 4 repeat tau (as compared with 3 repeat tau) that leads to neurodegeneration remains uncertain (Mandelkow *et al*. 2007; van Swieten and Spillantini 2007; Spires-Jones *et al*. 2009; Wolfe 2009). A major alteration in 4 repeat to 3 repeat ratio has also been demonstrated in sporadic forms of other neurodegenerative diseases, including corticobasal ganglionic degeneration, progressive supranuclear palsy (Ingelsson *et al*. 2007) and, to a lesser extent, Alzheimer's disease (Conrad *et al*. 2007). Moreover, previous studies using a variety of non-neuronal or reduced preparations have suggested that tau might have an effect on organelle transport by reducing the attachment frequency of motors to microtubules but not altering velocities once attached (Trinczek *et al*. 1999; Seitz *et al*. 2002) or by differentially regulating kinesin and dynein based transport (Vershinin *et al*. 2008). We therefore re-examined the effects of 3 repeat and 4 repeat tau on mitochondrial transport in central nervous system cells, to evaluate (i) whether differential effects of 3 repeat and 4 repeat tau could be uncovered and, (ii) whether the primary effect of tau was via competition for kinesin/dynein-mediated transport or by impacting the properties of microtubular transport after it had been initiated.

It is well established that tau strongly impacts mitochondrial translocation to and transport in axons (Sato-Harada *et al*. 1996; Mandelkow *et al*. 2004). Tau over-expression leads to a clear phenotype of clustering of mitochondria in the perinuclear area in multiple cell types, including CHO cells and retinal ganglion cells (Ebneth *et al*. 1998; Stamer *et al*. 2002; Mandelkow *et al*. 2004; Gotz *et al*. 2006; Dubey *et al*. 2008), suggesting an impact on transport of organelles toward the cell periphery which has been attributed to competition for kinesin-based anterograde transport between tau and other kinesin cargoes. Our studies show that, in both H4 neuroglioma cells and in primary neurons, tau overexpression leads to a decrease in mitochondrial transport to the periphery of the cell body. Although both 3 repeat and 4 repeat tau have a strong effect on mitochondrial trafficking to axons, the 4 repeat-containing isoforms have a markedly greater influence than the 3 repeat-containing isoforms.

Previous studies in cells suggested that full-length 4 repeat tau primarily changed the likelihood that kinesin cargoes were transported, with little effect on velocities and transport characteristics (Ebneth *et al*. 1998; Stamer *et al*. 2002; Mandelkow *et al*. 2003). By contrast, elegant *in vitro* single molecule studies of motor proteins moving along microtubules (Dixit *et al*. 2008) showed that kinesin tended to detach at patches of bound tau, whereas dynein tended to reverse direction. In another set of studies, Vershinin *et al*. (2007, 2008) examined the influence of tau on the kinesin and dynein motor proteins in their movement of cargo along microtubules utilizing taxol-stabilized microtubules and an optical trap with motor proteins bound to polystyrene beads as cargo (Vershinin *et al*. 2007, 2008). High levels of tau binding to microtubules significantly decreased, or abolished kinesin binding to microtubules, while the same tau concentrations had far less of an effect on dynein binding to microtubules. Tau-binding microtubules also decreased the distance traveled by individual motor proteins along microtubules, again with kinesin being affected to a far greater extent than dynein. In accord

with our observations, 3 repeat tau had a more significant effect than 4 repeat tau on both kinesin and dynein binding to and movement along stabilized microtubules (Vershinin *et al*. 2007, 2008). Our data do not distinguish between the possibility that velocity of organelle transport is affected by tau, or that there is a difference in the rate at which organelles attach and detach from microtubules so that periods of short movement could be averaged with stationary periods. Regardless, it seems clear from our results as well as those of other studies (Thies and Mandelkow 2007; Vershinin *et al*. 2007, 2008), that tau has multiple effects on mitochondrial trafficking and localization within neurons.

Our studies, using microfluidic devices that allow monitoring of mitochondrial movement in axons, support the idea that tau over-expression alters both the likelihood that mitochondria are trafficked to axons and the properties of anterograde and retrograde movement. For example, in strong agreement with Thies and Mandelkow (2007) and other reports, tau profoundly impacts mitochondrial localization and leads to clustering in the cell body. However, our studies also suggest that the velocity of anterograde transport is significantly impacted by tau expression, contrasting in this regard with Thies and Mandelkow (2007). This difference may be because of technical issues such as the use of hippocampal and cortical neurons, use of the microfluidic devices, or use of a genetically encoded mitochondrial marker compared with MitoTracker dyes (Thies and Mandelkow 2007). Interestingly, there appears to be a net effect of diminishing the anterograde to retrograde ratio, leading to a lower level of anterograde net flux. Thus, mitochondria may cluster in the cell soma for two reasons: competition for kinesin-based transport that leads to diminished translocation to the axonal microtubules (or, in H4 cells, the cell periphery), and a stronger effect on kinesin compared to dynein-mediated transport mechanisms leading to a bias favoring retrograde transport for mitochondria that reach the axon. Four-repeat tau may have a more profound influence on the competition for kinesin-based mitochondrial transport, whereas our data suggest that 3 repeat tau may impact movement within the axon to a greater extent. Our detailed kymograph analyses of tau effects on mitochondrial trafficking in axons of living neurons match very well with predictions from single molecule studies of dynein and kinesin motor proteins *ex vivo* (Dixit *et al*. 2008), extending these observations to an *in vitro* trafficking of mitochondria.

Our current results are also in accord with recent studies of axonal transport in tau over-expressing mice, where in some models there is a reduced synaptic supply of transported cargoes, such as tyrosine hydroxylase delivery to the striatum in a model that over-expresses K369Z mutant tau in the substantia nigra (Ittner *et al*. 2008).

The effects of tau on axonal transport in our current studies are independent of the formation of fibrillar tau inclusions. The type of changes we observe in both axonal translocation and in axonal transport due to tau over-expression may help explain the phenotype of rTg4510 mice, in which tau P301L over-expression leads to tangle formation, neuronal loss, and cognitive impairments (SantaCruz *et al*. 2005; Spires *et al*. 2006). In this model, suppressing tau over-expression led to restoration of behavioral function despite the continued presence of neurofibrillary tangles, arguing that the neural dysfunction was a consequence of soluble tau expression rather than the presence of neurofibrillary lesions. If so, the current results suggest that one consequence of overexpression of soluble tau (as opposed to fibrillar tau accumulation) is likely to be defects in microtubule trafficking to axons and in anterograde and retrograde axonal transport. Thus, tau-induced changes in axonal transport may be a major underlying theme in neurodegenerative diseases associated with genetic or pathophysiologic alterations that lead to changes in tau's interaction with microtubules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

CFP	cyan fluorescent protein
CHO	Chinese hamster ovary
DIV	days *in vitro*
GFP	green fluorescent protein
Mito-DsRed	mitochondrial DsRed
NB	neurobasal
PBS	phosphate-buffered saline
RT	room temperature
SDS	sodium dodecyl sulfate
TBST	20 mM Tris–Cl pH 7.6, 137 mM NaCl, 0.05% Tween 20

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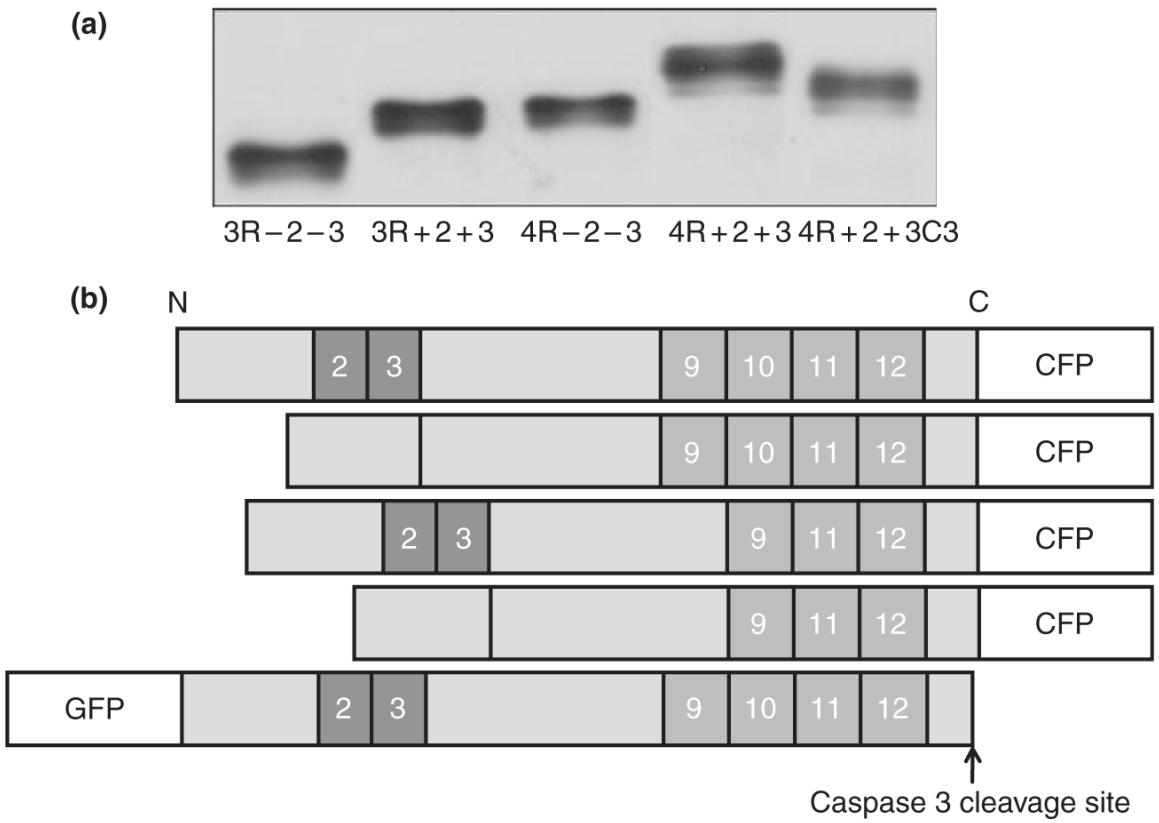

Fig. 1.

Constructs expressing five different isoforms of tau tagged with fluorescent proteins were generated with relative mobilities shown in a denaturing western blot (a). Constructs differed in the inclusion or exclusion of the splice changeable exons 2, 3 and 10 as well as one construct cleaved at amino acid 421 (4R + 2 + 3C3 construct) mimicking caspase 3 cleavage of tau (Gamblin *et al*. 2003). A schematic in (b) shows which exons are included in each construct.

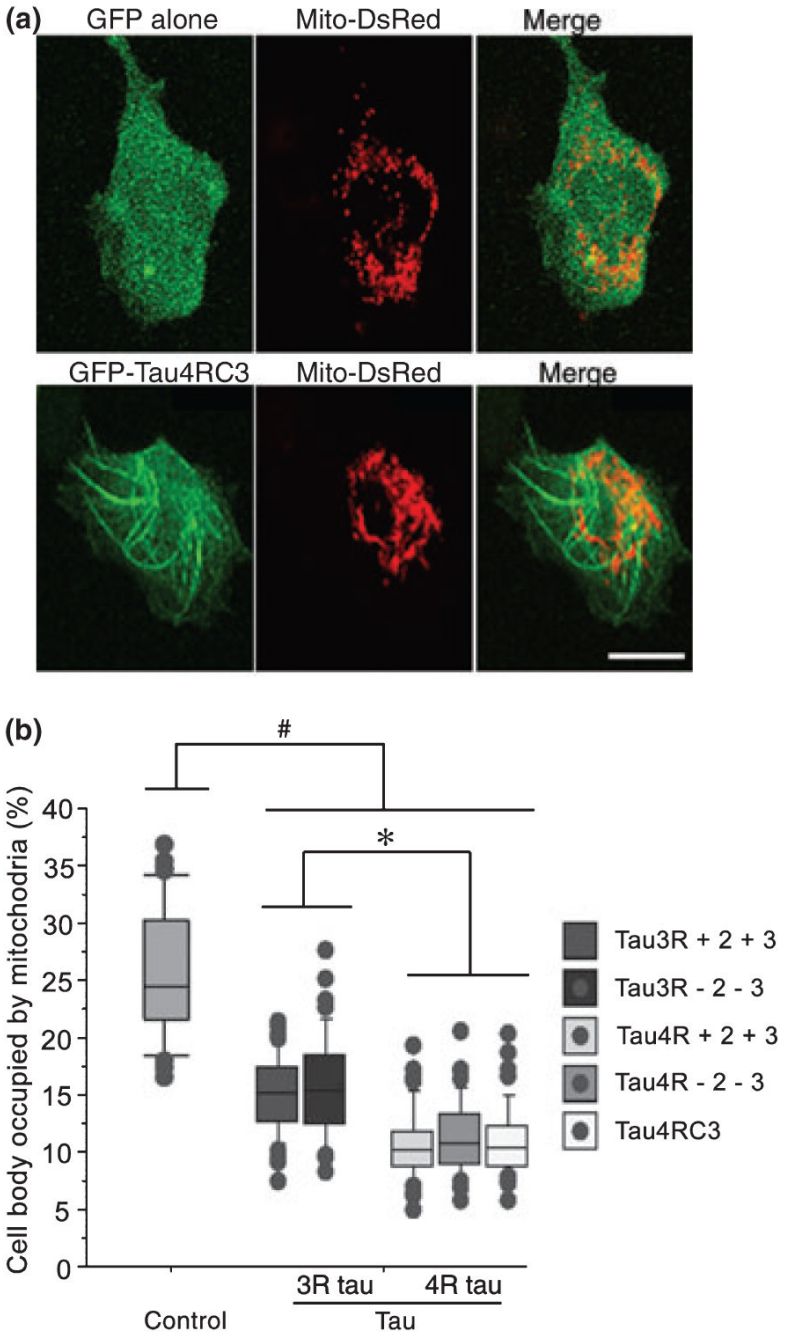

Fig. 2.

Mitochondrial distribution in H4 cells is altered by tau expression. Representative photomicrographs (a) show H4 cells transfected with either GFP or GFP-Tau 4 repeat plus 2 plus 3 lacking the last 20 aa (Tau4RC3). Cells were co-transfected with GFP or GFP-Tau4RC3 (green) and Mito-DsRed (red) to label mitochondria demonstrating the clumping of mitochondria around the nucleus in Tau-transfected cells. Quantification of mitochondrial distribution (b) shows a significant reduction in the area of cell soma occupied by mitochondria with overexpression of tau (# $p < 0.0001$ Mann–Whitney test tau vs. control). Four-repeat tau isoforms had a significantly more pronounced effect on mitochondrial clumping (* Mann–Whitney test 3 repeat vs. 4 repeat $p < 0.0001$). Within the 3 repeat and 4 repeat groups, addition

of the N-terminal inserts or truncation at the caspase cleavage site (C3) did not have a further effect on mitochondrial distribution. The data are non-parametric thus are presented as box plots, which display the median value (line inside the box), upper quartile (top of the box), lower quartile (bottom of the box), 90th percentile (top whisker), 10th percentile (bottom whisker), and all values below the 10th and above the 90th percentile (potential outliers) shown as dots. n = 45 cells per condition. Scale bar 20 μm.

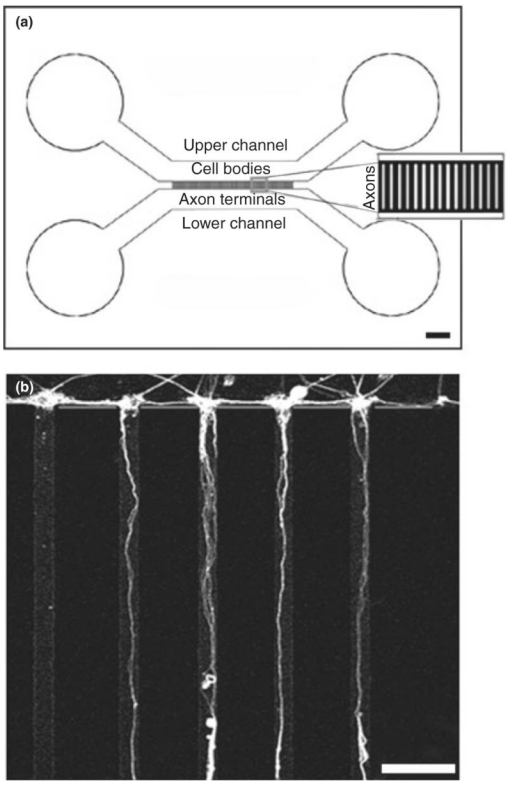

Fig. 3.

Microfluidic devices with a design adapted from Taylor *et al*. (2005) were used to plate primary cortical neurons to isolate axons from cell bodies and dendrites [schematic shown in (a)]. (b) A photomicrograph of a microfluidic device with cultured neurons expressing CFP shows axons penetrating the microchannels. Scale bars 3 mm (a) 50 μm (b).

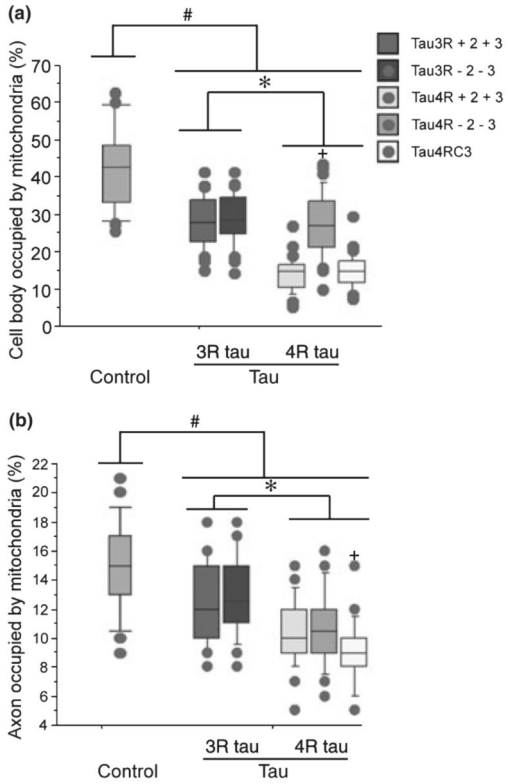

Fig. 4.

Mitochondrial distribution in neurons plated on microfluidic devices is altered by expression of human tau. In neuronal cell bodies (a), expression of tau significantly decreased the area of the cell body occupied by mitochondria (# $p < 0.0001$, Mann–Whitney test control vs. tau). Four-repeat isoforms had a more pronounced effect than 3 repeat isoforms (* $p < 0.0001$, Mann–Whitney test 3 repeat vs. 4 repeat, tau group). Within the 4 repeat isoforms, the shortest 4 repeat construct, 4 repeat minus 2 minus 3, had less of an effect than the two longer isoforms 4 repeat plus 2 plus 3 and tau truncated at caspase cleavage site 421, 4 repeatC3 (+ $p < 0.0001$, *post hoc* Mann–Whitney tests 4 repeat minus 2 minus 3 vs. 4 repeat plus 2 plus 3 and 4 repeatC3), $n = 35$ cells. In the axons (b), expression of tau similarly decreases the area occupied by mitochondria, in the axon (#), and 4 repeat tau still has a more pronounced effect than 3 repeat tau (*). Within the 4 repeat isoforms, the 4 repeat tau construct truncated at caspase cleavage site 421 has a more pronounced effect than the other 4 repeat isoforms (+ $p < 0.02$, post hoc Mann–Whitney test 4 repeat C3 vs. 4 repeat plus 2 plus 3 or 4 repeat minus 2 minus 3). As in Fig. 2, the number of individual mitochondria was not quantified, only the amount of cytoplasm occupied by mitochondria in either the cell body (a) or the axon (b). The same cells were used in (a) and (b), first the axon was imaged, and followed back to cell body, which was then imaged. $n = 35$ cells per condition.

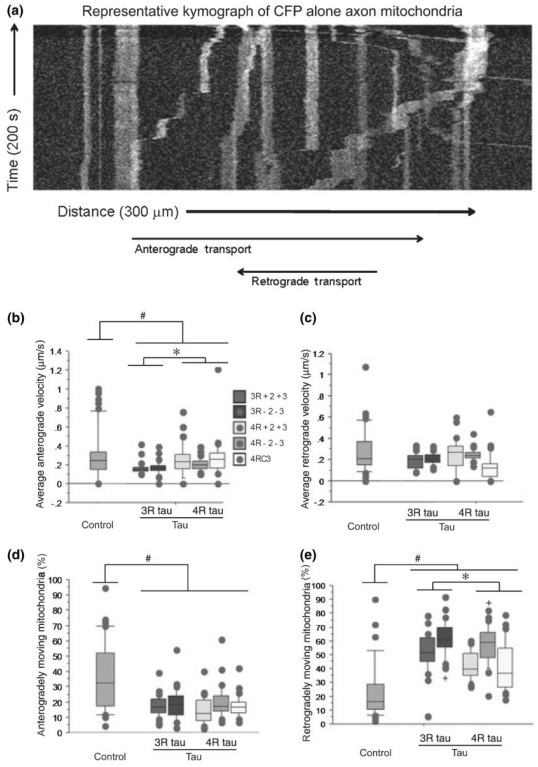

Fig. 5.

Tau over-expression increases the net movement of axonal mitochondria toward the neuronal cell body. (a) Shows a kymograph generated from a time series of mitochondria moving along an axon. Images were taken every 2 s and a total of 100 images were taken, for a total imaging session of 200 s. (b) Quantification of mitochondrial movements from the kymographs shows that velocity of mitochondria moving anterogradely is decreased (# $p = 0.03$, Mann-Whitney test control vs. tau), and that this reduction is more pronounced with 3 repeat tau than 4 repeat tau (* $p < 0.0001$ *post hoc* Mann–Whitney test). Average retrograde velocity (c) is not affected by tau over-expression. The percentage of mitochondria moving away from the cell body (d) is decreased with tau overexpression (# $p < 0.0001$ Mann-Whitney test). Conversely, the percentage of mitochondria moving retrogradely is increased with tau over-expression (# $p < 0.0001$, Mann-Whitney test), with 3 repeat tau having a more pronounced effect (* $p < 0.0001$ *post hoc* Mann–Whitney test) and with isoforms lacking N-terminal exons 2 and 3 having more of an effect than the other isoforms with the same number of repeats (+ $p < 0.01$ *post hoc* Mann–Whitney tests 3 repeat minus 2 minus 3 vs. 3 repeat plus 2 plus 3; 4 repeat minus 2 minus 3 vs. 4 repeat plus 2 plus 3; and 4 repeat minus 2 minus 3 vs. 4 repeat C3), $n = 35$ mitochondria from at least 10 individual neurons for each condition. The same kymographs were used to generate the data for (b), (c), and (d).