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TAU₄₅₋₂₃₀ ASSOCIATION WITH THE CYTOSKELETON AND MEMBRANE-BOUND ORGANELLES: FUNCTIONAL IMPLICATIONS IN NEURODEGENERATION

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Abstract

The dysregulation of posttranslational modifications of the microtubule-associated protein (MAP) tau plays a key role in Alzheimer's disease (AD) and related disorders. Thus, we have previously shown that beta amyloid (A β)-induced neurotoxicity was mediated, at least in part, by tau cleavage into the tau₄₅₋₂₃₀ fragment. However, the mechanisms underlying the toxicity of tau₄₅₋₂₃₀ remain unknown. To get insights into such mechanisms, we first determined the subcellular localization of this tau fragment in hippocampal neurons. Tau₄₅₋₂₃₀ was easily detectable in cell bodies and processes extended by these neurons. In addition, cell extraction experiments performed using Triton X-100 and saponin showed that a pool of tau₄₅₋₂₃₀ was associated with the cytoskeleton and the cytoskeleton plus membrane-bound organelles, respectively, in cultured hippocampal neurons. Furthermore, they suggested that these associations were independent of the presence of full-length tau. We also assessed whether this tau fragment could alter axonal transport. Our results indicated that tau₄₅₋₂₃₀ significantly reduced the number of organelles transported along hippocampal axons. This altered axonal transport did not correlate with changes in the total number of organelles present in these cells or in motor protein levels. Together these results suggested that tau₄₅₋₂₃₀ could exert its toxic effects by partially blocking axonal transport along microtubules thus contributing to the early pathology of AD.

Keywords

cytoskeleton; mitochondria; lysosomes; axonal transport; degeneration

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INTRODUCTION

The microtubule-associated protein (MAP) tau plays an important role not only in neurite elongation but also in a group of neurodegenerative diseases known as tauopathies (reviewed by Huang et al., 2016). The most well studied tauopathy is Alzheimer's disease (AD), a progressive and irreversible neurodegenerative disorder largely affecting the elderly population (Yankner et al., 2008). AD is characterized by the aberrant extracellular deposition of aggregated amyloid beta (A β) peptides into senile plaques and the intracellular accumulation of hyperphosphorylated tau forming neurofibrillary tangles (NFT) in affected brain areas (Glennner and Wong, 1984; Hyman et al., 1984; Grundke-Iqbal et al., 1986; Kosik et al., 1986; Davies et al., 1987; Selkoe, 1994). Recent research has focused on the mechanistic link between A β deposition and tau pathology in the progression of this disease (Gotz et al., 2011). The widely accepted amyloid cascade hypothesis proposes that A β initiates the pathogenic process inducing cell death in central neurons (Hardy and Selkoe, 2002). However, degeneration induced by aggregated A β only occurred in neurons that express tau, suggesting this MAP is required for A β -induced neurotoxicity (Rapoport et al., 2002; Roberson et al., 2007).

The mechanisms by which tau mediates A β toxicity are not completely elucidated. A growing body of evidence suggests that A β can induce deleterious posttranslational tau modifications. Tau phosphorylation has been extensively studied in AD due to the composition of NFTs. More recently, a series of studies suggested that proteolytic tau cleavage also played an essential role in neurotoxicity (Canu et al., 1998; Fasulo et al., 2000; Chung et al., 2001; Gamblin et al., 2003; Park and Ferreira, 2005; Amadoro et al., 2006; Reinecke et al., 2011; Lang et al., 2014; Zhang et al., 2014). We have previously shown that exposure of mature cultured hippocampal neurons to aggregated A β oligomers increased Ca²⁺ influx via the activation of *N*-methyl-D-aspartic acid receptors (Kelly et al., 2005; Kelly and Ferreira, 2006). This enhanced Ca²⁺ influx, in turn, induced calpain-mediated tau cleavage leading to the generation of a 17 kDa tau fragment (tau₄₅₋₂₃₀) (Park and Ferreira, 2005; Kelly and Ferreira, 2006; Nicholson and Ferreira, 2009; Nicholson et al., 2011). The neurotoxic effects of this fragment have been extensively characterized. Thus, tau₄₅₋₂₃₀ induced cell death when expressed in neuronal and non-neuronal cell types or in an *in vivo* *Drosophila* model system, (Park and Ferreira, 2005; Reinecke et al., 2011). In contrast, pharmacological inhibition of calpain activity or genetic modification of the putative cleavage sites (Leu43 and Val229) that produced this toxic fragment suppressed the production of the tau₄₅₋₂₃₀ and significantly reduced A β -induced neurotoxicity (Park and Ferreira, 2005; Amadoro et al., 2006; Sinjuano et al., 2008; Reinecke et al., 2011). More recently, we have characterized the phenotype of mice expressing tau₄₅₋₂₃₀ (Lang et al., 2014). Enhanced neuronal loss, decreased number of synaptic contacts and behavioral defects were easily detected in transgenic tau₄₅₋₂₃₀ mice as compared to wild type controls (Lang et al., 2014). Collectively, these data provided strong evidence for an important role of tau₄₅₋₂₃₀ in the progression of A β -mediated neurodegeneration. However, the mechanism(s) underlying the neurotoxic effects of this tau fragment remained unknown.

In the present study, we first analyzed the subcellular distribution of tau₄₅₋₂₃₀ in cultured hippocampal neurons. We also assessed the effects of this tau fragment on the transport of

organelles along the axons extended by these neurons using time-lapse microscopy. The data obtained provided insights into a mechanism by which the tau₄₅₋₂₃₀ could induce the formation of dystrophic neurons and cell death in the context of AD and related disorders.

EXPERIMENTAL PROCEDURES

Hippocampal culture preparation

Hippocampal neuronal cultures were prepared from embryonic day 18 (E18) Sprague-Dawley rats (Taconic; n= 30 E18 pregnant rats) and from E16 C57BL/6J mice (wild type and tau knockout mice, Lang et al., 2014; n=21 E16 pregnant mice) as described previously (Banker and Goslin, 1998; Rapoport et al., 2002). In brief, hippocampi were dissected, stripped of meninges, and trypsinized (0.25%) for 15 min at 37°C. Neurons were dissociated by pipetting gently through a fire-polished Pasteur pipette and plated (~800,000 cells/60 mm dish or ~240,000/35 mm dish) in minimum essential medium (MEM) containing 10% horse serum (MEM10) on poly-L-lysine coated dishes. After 4 hr, the medium was replaced with glia-conditioned MEM containing N2 supplements, ovalbumin 0.1%, and 0.1 mM sodium pyruvate (N2 medium, Bottenstein and Sato, 1979). For immunocytochemical analysis, neurons were plated (150,000 cells/60-mm dish) onto poly-L-lysine-coated coverslips in MEM10. After 4 hr, the coverslips were transferred to dishes containing an astroglial monolayer and maintained in N2 medium.

Preparation of astrocyte cultures

Astrocyte cultures were prepared from the cerebral cortex of E16 mice embryos (n=5 E16 pregnant mice) as previously described (Ferreira and Loomis, 1998). Briefly, embryos were removed and their cerebral cortex dissected and freed of meninges. The cells were dissociated by trypsinization (0.25% for 35 minutes at 37° C) and then centrifuged in MEM plus 10% horse serum at 1,000 rpm for 10 minutes. The cells were resuspended in fresh MEM plus 10% horse serum, triturated with a fire-polished pipette, and plated at high density (800,000 cells/60-mm dish) on non-coated culture dishes.

Plasmid constructs and cell transfection

cDNA encoding for the longest human tau (hTau40) isoform (tau₁₋₄₄₁) and the tau₄₅₋₂₃₀ fragment were generated as described previously (Park and Ferreira, 2005). Both constructs were subcloned into the mammalian expression vector, enhanced green fluorescent protein - N1 (p-eGFP-N1) (Invitrogen) to produce C-terminal GFP-labeled full-length tau (hTau40-GFP) and tau₄₅₋₂₃₀ (tau₄₅₋₂₃₀-GFP) constructs. These constructs were nucleofected into dissociated hippocampal neurons as previously described (Park and Ferreira, 2005). Briefly, dissociated neurons were resuspended in nucleofection solution containing 3 µg of the respective constructs, transferred to an electroporation cuvette, and nucleofected using the Amaxa Nucleofection system (Lonza, Inc. Allendale, NJ) according to the manufacture protocol (program O-03). Non-transfected cells and neurons transfected with an empty p-eGFP-N1 vector were used as controls. For some experiments, astrocytes were nucleofected with the tau₄₅₋₂₃₀-GFP construct using the T-20 program (Lonza) as previously described (Paganoni et al., 2004).

A β aggregation and cell treatment

Synthetic A β ₁₋₄₀ (American Peptide, Sunnyvale, CA) was dissolved in N2 medium to a concentration of 0.5 mg/ml and incubated for 3 days at 37°C to induce its preaggregation (Park and Ferreira, 2005). Neurons kept in culture for 21 days were incubated in the presence of the preaggregated peptide at final concentrations of 20 μ M for 24 hr to induce calpain-mediated tau cleavage into the tau₄₅₋₂₃₀ fragment (Park and Ferreira, 2005; Nicholson and Ferreira, 2009). Cultures prepared from 5 E18 pregnant rats per experimental condition were used for these experiments.

Preparation of whole cell lysates and detergent-resistant subcellular fractions

To prepare whole cell lysates, hippocampal and astrocyte cultures were washed once in warmed (37°C) phosphate-buffered saline (PBS), scraped into Laemmli buffer, and homogenized by boiling in a water bath for 10 min (Laemmli, 1970). Detergent-resistant subcellular fractioning was performed to isolate membrane-bound organelle plus cytoskeleton fractions and pure cytoskeleton fractions as previously described (Paganoni et al., 2004). In brief, hippocampal neurons or astrocytes plated in 60 mm dishes were washed once with warmed PBS, and stabilized by 30s incubation with warmed microtubule stabilizing buffer (MTSB: 130 mM HEPES, 4 mM MgCl₂, 10 mM EGTA, pH 6.9). Cultures were extracted in MTSB containing 0.02% saponin (Sigma) or 0.2% Triton X-100 (Sigma) for 60 s to prepare membrane-bound organelle or cytoskeletal fractions, respectively. Extracted cells were gently rinsed with MTSB, scraped into Laemmli buffer, and homogenized in a boiling water bath for 10 min. Whole cell extracts and detergent-resistant fractions were analyzed by immunoblotting as described below. For these experiments, cellular extracts were prepared from 5 independent hippocampal cultures per experimental condition.

Electrophoresis and immunoblotting

Whole cell lysates and subcellular fraction extracts prepared from tau₄₅₋₂₃₀-GFP transfected and untransfected hippocampal cells or astrocytes as well as from A β -treated and untreated neurons were ran on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and detected by immunoblotting (Towbin et al., 1979). The following primary antibodies were used for immunodetection of specific proteins: kinesin (ubiquitous kinesin) heavy chain (1:500; Millipore); kinesin 5A (neuron-specific kinesin) heavy chain (1:500; Abcam), dynein intermediate chain 1 (1:50; Santa Cruz), α -tubulin (1:1,000; clone DM1A; Sigma), tau (1:1,000; clone Tau5; BioSource International), GFP (1:500; Millipore); and acetylated tubulin (1:1,000; clone 6-11-B1, Sigma). Incubation with secondary antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence reagent were used to detect protein expression (Yakunin and Hallenbeck, 1998). Imaging and densitometric quantification of proteins were performed using a ChemiDoc XRS system (Bio Rad Life Sciences). Lysates were loaded equally based on internal control levels of α -tubulin (for whole cell extracts) or acetylated tubulin (for subcellular fractions).

Immunocytochemistry

Hippocampal neurons cultured on coverslips were fixed in 4% paraformaldehyde in PBS containing 0.12 mM sucrose for 15 min and permeabilized in 0.3% Triton X-100 in PBS for 1 min. Coverslips were then incubated with 10% bovine serum albumin (BSA) in PBS at room temperature for 1 hr before overnight incubation with the primary antibody. The following antibodies were used: α -tubulin (clone DM1A; 1:1,000), GFP (1:500), and tau (1:100 clone tau5). Anti-mouse and anti-rabbit AlexaFluor secondary antibodies (1:200; Molecular Probes) were used for protein detection. Cultures prepared from 6 E16 pregnant mice (3 wild type and 3 tau knockout mice) were used for these experiments.

Organelle labeling

For mitochondria and lysosomal labeling, neurons were incubated in 1 nM MitoTracker Red CMXRos for 15 min or 300 nM LysoTracker Red DND-99 (Invitrogen) for 1 hr at 37°C, respectively. After labeling, cultures were washed once with N2 medium. Observations of axonal transport were performed at least 3 hr after washout of organelle probes in glia-conditioned N2 medium without phenol red in order to reduce fluorescent background.

Live cell imaging

Live neurons cultured on 35 mm dishes were observed using a Nikon Eclipse Ti-U inverted microscope equipped with an INU series microscope stage incubation system (Nikon) and a Mercury-100 W lamp (Chiu Technical Corporation). Neurons were maintained at 37°C and 5% CO₂ during the course of observation. Images were collected using a 40X objective lens and a microscope-mounted Photometrics Cool Snap *fx* camera (Roper Scientific) controlled by a Lambda 10-B Smartshutter shutter system (Sutter Instruments). Neurons transfected with GFP-conjugated constructs (p-eGFP-N1 empty vector, hTau40-GFP or Tau₄₅₋₂₃₀-GFP) were identified using a 460–500 nm bandpass filter, and non-transfected neurons were used as controls. Fluorescent-labeled axonal mitochondria and lysosomes were observed using a 530–560 nm bandpass filter. Time-lapse recordings of neurons were acquired by scanning a single focal plane every 2 s for a total of 100 s. Short exposure times (250–500 ms) and neutral density filters were used to minimize photobleaching during time-lapse imaging. Easily identifiable axons as the longest process extending from the soma were selected based on the ability to observe their entire length throughout the course of the recording.

Axonal transport analysis

Time-lapse images were analyzed using MetaMorph Image Analysis software (Molecular Devices). Image stacks of fluorescent organelles within a given axonal region were processed using the MetaMorph kymograph function to generate spatiotemporal maps of organelle trafficking. Contrast was inverted and minimally adjusted on kymograph images to quantify organelle motility behaviors. Individual organelle movements were traced on the kymographs, and were assessed using MetaMorph software on movement, organelle density, direction of movement (anterograde and retrograde), distance traveled, velocity, and axonal localization. Organelles were considered “moving” if they transversed 1 μ m during the 100 s time course. If the organelle stopped and/or changed directions, it was considered a “new” movement. Motility behavior was analyzed only if the organelle movement could be traced

through the entire time course. For each control and experimental condition, a total of at least 100 lysosomes and 290 mitochondria from 15 neurons for each experimental condition and from 5 independent hippocampal cultures were observed per each experimental condition.

Statistical analysis

The data presented were obtained from at least 5 independent cultures per experimental condition, as described above. Quantitative analysis and statistical comparisons were performed using Prism 5.0 (GraphPad Software, Inc.). We tested for and found that all sampled distributions satisfied the normality criteria. Axonal transport and motility behavior of transfected neurons were compared using Kruskal-Wallis nonparametric one-way analysis of variance followed by Dunn's multiple comparison tests. Axonal locations of organelles (proximal, medial, distal from somas) within neurons were compared using one-way analysis of variance with Bartlett's test of variance, and Tukey post-test comparisons. Values of $P < 0.05$ were considered significant. Data are presented as mean $\pm$ standard error of the mean (S.E.M.).

For quantitative Western blot analysis, the compiled data were analyzed across the experimental conditions using one-way ANOVA followed by Fisher's LSD *post hoc* test. The values in the graphs represent the mean $\pm$ S.E.M. and statistical significance is indicated in the graphs.

RESULTS

Subcellular localization of tau₄₅₋₂₃₀

The neurotoxic effects of tau₄₅₋₂₃₀ have been extensively characterized in mammalian hippocampal neurons that developed either *in vivo* or in culture as well as in a *Drosophila* model of tauopathy (Park and Ferreira 2005; Reinecke et al., 2012; Lang et al., 2014). On the other hand, the molecular mechanisms underlying these toxic effects remain unknown. To get insights into such mechanisms, we first analyzed tau₄₅₋₂₃₀ subcellular localization. For these experiments, newly dissociated hippocampal neurons obtained from E16 wild type mice were nucleofected using a tau₄₅₋₂₃₀-GFP construct (Park and Ferreira, 2005). Twenty-four hr after plating, transfected hippocampal neurons were fixed and immunostained using GFP and tubulin antibodies. Untransfected cultures were used as controls. Tubulin immunoreactivity was detected in the cell bodies and all processes extended by both control and transfected hippocampal neurons (Fig. 1A & C). As expected, no GFP immunostaining was observed in non-transfected controls (Fig. 1B). On the other hand, GFP immunoreactivity was easily detectable in ~ 50% of the neurons present in transfected cultures (Fig. 1D). In these neurons, diffuse GFP immunofluorescence was uniformly distributed in the cell bodies, along the neuritic processes, and in growth cones (Fig. 1D). To analyze the subcellular distribution of tau₄₅₋₂₃₀, transfected hippocampal neurons were detergent-extracted using saponin and Triton X-100. Saponin extraction preserved membrane, membrane-bound organelles, and the cytoskeleton. Cultures treated with Triton X-100 were extracted more stringently, leaving intact only the cytoskeleton (Paganoni et al., 2004). Extracted neurons were then fixed and immunostained as described above. GFP

immunoreactivity was still easily detectable and mainly concentrated in the cell bodies of both Triton X-100 and saponin-extracted tau₄₅₋₂₃₀ transfected neurons. Faint GFP staining was also detected along the processes extended by these neurons (Fig. 1F & H). To confirm that organelles remained in saponin-extracted neurons, tau₄₅₋₂₃₀-transfected neurons were labeled with MitoTracker, a fluorescent dye that stains mitochondria, as described in the experimental procedures section. Labeled neurons were then extracted with saponin and counterstained using a GFP antibody. As expected, MitoTracker positive dots were detected in transfected neurons extracted with saponin (Fig. 1I & J). Lysates were prepared from non-extracted and detergent-extracted neurons to quantify the amount of tau₄₅₋₂₃₀ associated with the cytoskeletal or cytoskeletal plus membrane-bound organelle fractions. These lysates were analyzed by means of quantitative Western blot using GFP and tau antibodies. Immunoreactive bands detected with acetylated tubulin were used as internal controls. Since acetylated tubulin is only present in microtubules, its levels remained unchanged in whole cell extracts and in detergent-extracted fractions. In whole cell extracts, two immunoreactive bands were detected with both GFP and tau antibodies (Fig. 2A). This pattern was consistent with the presence of monomeric tau₄₅₋₂₃₀ and dimers of this fragment. An additional tau immunoreactive band of ~50 kDa molecular weight corresponding to endogenous full-length tau was detected in between the tau₄₅₋₂₃₀ monomer and dimer bands using the tau antibody but not with the GFP one (Fig. 2A). All these tau immunoreactive bands were also detected in detergent extracted cells, although the immunoreactive signal was significantly weaker (Fig. 2B). Quantitative analysis showed that similar amounts of tau₄₅₋₂₃₀ dimers were detected in cytoskeletal (3.6 % ± 1%) and cytoskeletal plus membrane-bound (5.9% ± 1.3 %) fractions (Fig. 2C). Monomeric tau₄₅₋₂₃₀ was also present in both fractions. However, the levels of tau₄₅₋₂₃₀ present in the saponin-extracted fractions were significantly higher than those detected in Triton X-100-extracted cytoskeletal fractions (8.1% ± 2.2% vs. 1.9 % ± 0.4%, respectively n=5, *t* = 2.8, *p* < 0.05) (Fig 1D).

Since this subcellular localization resembled the one described for full-length tau, we assessed next whether the association of tau₄₅₋₂₃₀ with the cytoskeleton and/or membrane-bound organelles could be mediated by its binding to full-length tau. For these experiments, we prepared tau-depleted hippocampal cultures from knockout mice and neurons were transfected, extracted, and processed for immunocytochemistry and Western blot analysis as described for the experiments performed using wild type mice. Tau₄₅₋₂₃₀ was detected both in Triton X-100- and saponin-extracted tau-depleted hippocampal neurons (Fig. 3D, F, H & I). Furthermore, quantitative Western blot analysis showed similar levels of both dimers and monomers of tau₄₅₋₂₃₀ in cytoskeletal and cytoskeletal plus membrane-bound organelle fractions to those detected in full-length tau expressing hippocampal neurons transfected with this tau fragment. As in the case of tau expressing neurons, the levels of tau₄₅₋₂₃₀ monomers were significantly higher in saponin-extracted tau-depleted neurons as compared to Triton X-100-extracted ones (10.3% ± 2.9% vs. 2.0% ± 0.6%, respectively. n=5, *t* = 2.8, *p* < 0.05) (Fig. 4A–D). Together these results indicated that a pool of tau₄₅₋₂₃₀ was associated with the cytoskeleton and membrane-bound organelles independently of the presence of full-length tau.

To assess whether this tau₄₅₋₂₃₀ subcellular localization was observed in non-neuronal cell types, we transfected tau₄₅₋₂₃₀-GFP into cultured astrocytes. Quantitative Western blot

analysis showed that tau₄₅₋₂₃₀ monomers and dimers were present in whole cell extracts. However, no tau₄₅₋₂₃₀ was detected in Triton X-100 and saponin-extracted fractions (data not shown).

We determined next whether endogenously generated tau₄₅₋₂₃₀ was present also in these subcellular fractions. For these experiments, the generation of the tau₄₅₋₂₃₀ fragment was induced by treating 21 days in culture hippocampal neurons with aggregated A β as previously described (Park and Ferreira, 2005; Nicholson and Ferreira, 2009). Twenty-four hr after the addition of the aggregated peptide, detergent-resistant cellular fractions were prepared as described above. Western blot analysis did not detect tau₄₅₋₂₃₀ in whole cell lysates obtained from non-treated control hippocampal neurons in agreement with previously described results (Fig. 5A) (Park and Ferreira, 2005). In contrast, a strong 17 kDa tau immunoreactive band was detected in whole cell extracts obtained from A β -treated neurons (Fig. 5A). Quantitative analysis of immunoreactive bands showed that $25.5\% \pm 3.5\%$ of the endogenously cleaved tau₄₅₋₂₃₀ fragment remained in the saponin-resistant extract, and $13.3\% \pm 1.12\%$ in the Triton X-100-resistant extract in subcellular fractions obtained from A β -treated neurons. Statistical analysis showed that the levels of tau₄₅₋₂₃₀ were significantly higher in saponin-extracted A β -treated neurons when compared to Triton X-100-extracted ones ($n=5$, $t=3.32$, $p<0.01$) (Fig. 5B).

Visualization of axonal transport in transfected hippocampal neurons

The association of tau₄₅₋₂₃₀ with both the cytoskeleton and membrane-bound organelles raised the possibility of its role in the transport of such organelles. To determine the potential effects of tau₄₅₋₂₃₀ on axonal transport, dissociated hippocampal neurons were transfected with the empty p-eGFP-N1 vector, full-length tau (htau40-GFP), or tau₄₅₋₂₃₀ (tau₄₅₋₂₃₀-GFP). Neurons expressing GFP or the GFP-conjugated constructs were identified, and clearly distinguishable axon structures were chosen to examine organelle axonal transport (Fig. 6A). Fluorescence from the GFP-tagged proteins appeared diffuse along the processes. In agreement with previous studies, the expression of tau₄₅₋₂₃₀ induced morphological changes indicative of neurodegeneration (Park and Ferreira, 2005). These changes included the presence of varicosities along the axon (Fig. 6A, arrowheads), formation of tortuous processes, and retraction of neurites. Dystrophic morphology was not observed in non-transfected neurons (control), p-eGFP-N1-, or htau40-GFP-expressing neurons (Fig. 6A).

In order to assess axonal transport, organelles were labeled with fluorescent lysosomal or mitochondrial-specific dyes in non-transfected controls and neurons transfected as described above (Fig. 6B, arrows). Analysis of the distribution of organelles in non-transfected control neurons showed that lysosomes were evenly dispersed throughout the axon with $38.2\% \pm 4.84\%$, $23.2\% \pm 3.38\%$, and $38.7\% \pm 4.58\%$ located proximal, medial and distal from cell somas, respectively. Mitochondria were also uniformly distributed with $34.5\% \pm 2.24\%$, $32.6\% \pm 1.15\%$, and $30.8\% \pm 1.67\%$ located proximal, medial and distal from cell somas, respectively. These patterns of organelle localization within neuronal axons were consistent with previous reports (Miller and Sheetz, 2004; Lee et al., 2011). The overall density and distribution of organelles was unchanged in the p-eGFP-N1, htau40-GFP, and the tau₄₅₋₂₃₀-GFP expressing neurons when compared to non-transfected controls (Tables 1 and 2).

Tau₄₅₋₂₃₀ disrupted axonal transport in cultured hippocampal neurons

To analyze the effects of tau₄₅₋₂₃₀ on the transport of labeled organelles, we recorded their movements in both anterograde and retrograde directions using live cell microscopy (Fig. 7). The motility of organelles transported through axons of control and transfected hippocampal neurons was quantified using kymographs generated from acquired time-lapse image stacks (see Experimental Procedures Section) (Fig. 7). In these kymographs, stationary organelles appear as horizontal lines, and moving organelles as slopes. The baseline axonal transport levels for lysosomes ($64.0\% \pm 5.33\%$, $n=163$) and mitochondria ($37.2\% \pm 4.56\%$, $n=494$) in non-transfected control neurons were in agreement with previous studies (Fig. 7) (Vossel et al., 2010; Lee et al., 2011). Expression of the empty p-eGFP-N1 vector or htau40-GFP construct had no effect on the percentage of organelles transported along the axons (Tables 1 and 2). However, the percentage of organelles transported was significantly decreased in neurons expressing tau₄₅₋₂₃₀-GFP when compared to other experimental conditions including non-transfected controls (Fig. 7, Table 1). Quantitative analysis of the subpopulation of moving organelles showed that transport was inhibited by ~24% for both lysosomal (24.4% decrease) and mitochondrial (23.6% decrease) trafficking when compared to either empty p-eGFP-N1 vector or htau40-GFP-transfected neurons and non-transfected controls (Table 1). Furthermore, these changes were not due to alterations in number of organelle cargo, as there was no significant effect on organelle density within the axons (Tables 1 and 2).

To further investigate how tau₄₅₋₂₃₀ disrupted specific aspects of axonal transport, motility behavior of the moving organelles including directionality (anterograde vs. retrograde), distance traveled, and velocity (calculated from the slopes of organelles in kymographs) was analyzed for all experimental conditions. To ensure results were not skewed by the significant decrease in the number of organelles moving observed in the tau₄₅₋₂₃₀-GFP transfected neurons (% of organelles moving), the distance and velocity measurements were collected per excursion. Anterograde and retrograde measurements of motility were also analyzed separately to investigate potential directional machinery-specific changes. The trafficking of lysosomes occurred primarily in the retrograde direction ($73.9\% \pm 4.13\%$ of total movements), while mitochondrial transport occurred equally in the anterograde ($49.9\% \pm 5.76\%$ of total movements) and retrograde ($55.9\% \pm 5.81\%$ of total movements) direction (Fig. 7, Table 1). The percentage of anterograde vs. retrograde movement of organelles in control neurons was consistent with previous investigations (Hollemberck and Saxton, 2005; Lee et al., 2011), and expression of empty p-eGFP-N1, htau40-GFP or tau₄₅₋₂₃₀-GFP had no effect on directionality. Interestingly, hTau40-GFP and tau₄₅₋₂₃₀-GFP had no significant effects on any specific motility behavior of lysosomal and mitochondrial transport in the anterograde direction (Tables 1 and 2). On the other hand, a slight decrease in retrograde excursion length of lysosomes and mitochondria was detected in tau₄₅₋₂₃₀-transfected neurons when compared to non-transfected controls or full-length tau-transfected neurons (Table 1 and 2).

Disruption of axonal transport was not associated with changes in motor protein expression

Previous investigations of the molecular pathology of AD and related neurodegenerative diseases have shown that toxic proteins could induce degradation of synaptic and motor proteins (Kelly et al., 2005; Roselli et al., 2005; Stokin et al., 2005). To determine if tau₄₅₋₂₃₀-induced defects in axonal transport were associated with degradation of essential transport proteins, we examined the protein levels of ubiquitous and neuron-specific kinesin (primary anterograde motor proteins), and dynein (primary retrograde motor protein) in transfected neurons (Fig. 8A & B). Quantitative Western blot analysis of immunoreactive bands showed that the expression of tau₄₅₋₂₃₀-GFP had no significant effects on motor protein levels compared to non-transfected control neurons (Fig. 8C). These data suggested that tau₄₅₋₂₃₀-induced disruptions in axonal transport were not due to the degradation of the transport machinery.

DISCUSSION

Tau₄₅₋₂₃₀ accumulates in degenerating central neurons in the context of multiple tauopathies (Park and Ferreira, 2005; Amadoro et al., 2006; Nicholson and Ferreira, 2009; Ferreira and Bigio, 2011; Reinecke et al., 2011). Furthermore, a growing body of evidence strongly suggests that this fragment does induce cell death and is not merely a marker of a degenerative process (Park and Ferreira, 2005; Amadoro et al., 2006; Reinecke et al., 2011; Lang et al., 2014). On the other hand, the mechanisms linking this tau fragment and neurodegeneration have yet to be elucidated. The results presented above indicated that a pool of tau₄₅₋₂₃₀ associates with membrane-bound organelles and the cytoskeletal network. This subcellular distribution could have some bearings on the partially blocked axonal transport detected in the presence of tau₄₅₋₂₃₀ in hippocampal neurons. Together, these data provide the first insights into a mechanism underlying the neurotoxic effects of tau₄₅₋₂₃₀.

In this study, we first assessed the subcellular localization of this tau fragment using tau₄₅₋₂₃₀-GFP-transfected hippocampal neurons as well as neurons in which this fragment was endogenously generated under experimental conditions that induced neurodegeneration. The data obtained showed that tau₄₅₋₂₃₀ was present mostly in the cytosol of hippocampal neurons. However, a pool of this fragment was recovered in detergent-extracted fractions. In both experimental conditions, monomeric tau₄₅₋₂₃₀ levels were significantly higher in saponin-extracted neurons than in Triton X-100-extracted ones. These results indicated that this fragment associated not only with the cytoskeleton preserved in extractions performed using either detergent but also with membrane-bound organelles present only in saponin-extracted neurons. We also detected the presence of tau₄₅₋₂₃₀ dimers in transfected neurons. These results are consistent with our previous observations indicating that this tau fragment could form small aggregates (Ferreira and Bigio, 2011). In contrast to monomeric tau₄₅₋₂₃₀, similar levels of tau₄₅₋₂₃₀ dimers were detected in fractions extracted with saponin and Triton X-100. These results strongly suggested that these dimers were associated with the cytoskeleton but not with membrane-bound organelles. It is worth noting that no tau₄₅₋₂₃₀ dimers were identified in hippocampal neurons treated with A β . The presence of these dimers could be masked due to their similar molecular weight to those of the adult full-

length tau isoforms expressed in mature hippocampal neurons (Ferreira et al., 1997). Alternatively, the lack of tau₄₅₋₂₃₀ dimerization in A β -treated neurons could reflect a differentially regulated process depending on specific stages of development (young vs. mature neurons) or levels of tau₄₅₋₂₃₀ accumulated under these experimental conditions.

The presence of tau₄₅₋₂₃₀ in membrane-bound organelle fractions resembled the subcellular localization of full-length tau in neurons. Thus, it has been previously shown that the N-terminal domain of full-length tau associated with membranous organelles like mitochondria and the plasma membrane (Brandt et al., 1995; reviewed by Kolarova et al., 2012). Since most of this domain is present in tau₄₅₋₂₃₀, it is tempting to speculate that the same region could mediate the binding of this tau fragment to membrane-bound organelles. Tau₄₅₋₂₃₀ also contains the proline-rich domain of this MAP. This domain could mediate the formation of tau₄₅₋₂₃₀ homodimers or tau₄₅₋₂₃₀/full-length tau heterodimers. Through this last mechanism, tau₄₅₋₂₃₀ could indirectly bind to these organelles. However, experiments performed using tau-depleted hippocampal neurons transfected with tau₄₅₋₂₃₀-GFP showed similar subcellular distribution than the one observed in full-length tau-expressing neurons. These results ruled out the formation of tau₄₅₋₂₃₀/full-length tau heterodimers as a mechanism of binding to membrane-bound organelles.

As full-length tau, tau₄₅₋₂₃₀ was also present in the cytoskeletal fraction, albeit at much lower concentration (Kanai et al., 1992). Thus, while ~40% of full-length tau was associated with the cytoskeleton in central neuron kept in culture for 24 hr, less than 15% of tau₄₅₋₂₃₀ was recovered in this fraction (Ferreira et al., 1989). This differential association with the cytoskeleton could be the result of the absence of the microtubule-binding domains in this fragment. Furthermore, these results suggested an indirect mechanism of binding of tau₄₅₋₂₃₀ to microtubules. As in case of the binding of tau₄₅₋₂₃₀ to membrane-bound organelles, tau₄₅₋₂₃₀ binding to cytoskeleton took place even in full-length tau-depleted neurons strongly suggesting that protein(s) other than full-length tau could mediate this indirect binding. The absence of this fragment in detergent-extracted tau₄₅₋₂₃₀-transfected astrocytes suggested that the indirect binding of this fragment to the cytoskeleton could be mediated by a neuron-specific protein(s). Further studies will be necessary to identify such protein(s).

The expression of tau₄₅₋₂₃₀ in young cultured hippocampal neurons induced dystrophic changes and neurodegeneration. These results are consistent with our previous observations in mature cultured hippocampal neurons (Park and Ferreira, 2005). On the other hand, neurons transfected with either GFP-empty vector or full-length tau-GFP did not display any morphological changes when compared to non-transfected controls. In addition to the morphological changes described above, our data showed that the expression of tau₄₅₋₂₃₀ in otherwise healthy hippocampal neurons induced a significant decrease in the subpopulation of moving organelles. In contrast, no defects in axonal transport were detected in hippocampal neurons transfected with GFP or full-length tau. These findings are in agreement with recent *in vitro* and *in vivo* studies that found that full-length tau (htau40) expression had no effect on baseline axonal transport (Yuan et al., 2008; LaPointe et al., 2009; Vossel et al., 2010).

Deficient transport along neuronal process is a common event in most known neurodegenerative diseases including AD, amyotrophic lateral sclerosis (ALS), Huntington's disease, and Parkinson's disease (Stokin et al., 2005; Morfini et al., 2009; Perslson et al., 2010). Alterations in axonal transport have also been detected in hippocampal neurons exposed to A β oligomers (Pigino et al., 2009; Decker et al., 2010). Building on these data, our results suggested that the altered axonal transport observed in the presence of the tau₄₅₋₂₃₀ fragment could initiate and/or exacerbate degenerative processes leading to neuronal cell death.

The data presented here extended recent studies on the effects of recombinant tau proteins and tau fragments that demonstrated that the N-terminal region of the protein was required to produce tau-mediated inhibition of transport in squid axoplasm. In addition, our results provided the first insights into an inhibitory effect on axonal transport induced by an endogenously formed fragment located in the N-terminal half of the tau molecule (tau₄₅₋₂₃₀) in central neurons.

Tau₄₅₋₂₃₀ could induce defects in axonal transport by regulating motor protein levels. Several reports have suggested that the overexpression of full-length tau or recombinant tau isoforms disrupted axonal transport by modulating motor protein function; particularly the plus-end directed motor kinesin (Ebner et al., 1998; Dixit et al., 2008). This does not seem to be the case in tau₄₅₋₂₃₀-transfected neurons since we observed no selective disruption of transport in a given type of organelle or the anterograde vs. retrograde direction that would suggest alterations in motor protein function. In addition, the expression of tau₄₅₋₂₃₀ had no effect on motor protein levels. These results suggested that tau₄₅₋₂₃₀ effects on axonal transport are not mediated through changes in the expression and/or half-life of motor proteins.

Alternatively, tau₄₅₋₂₃₀ could disrupt motor protein binding to membrane-bound organelles or microtubules causing a direct mechanical blockade on microtubule tracks and disrupting transport. The inhibitory effects of tau₄₅₋₂₃₀ on axonal transport observed in hippocampal neurons resembled those described for other pathogenic proteins in neurodegenerative diseases. For example, mutant forms of neurofilaments causing the inherited peripheral neuropathy, Charcot-Marie-Tooth disease, can alter both anterograde and retrograde axonal transport inducing neurite degeneration (Perez-Orte et al., 2005). In addition, studies examining early pathology of Huntington's disease have revealed similar patterns of axonal transport inhibition in the presence of accumulations of a pathogenic form of polyQ polypeptides (Szebenyi et al., 2003). The axonal blockage induced by pathogenic polyQ proteins in squid axoplasm and neuronal cell lines was due to the inhibition of both kinesin- and dynein-based motility. Furthermore, the distribution of kinesin and dynein motors was not altered by the polypeptides, and an accumulation of the pathogenic polyQ polypeptides was enriched in the membrane fractions (Szebenyi et al., 2003). These results suggested that the toxic protein accumulations might block transport and/or motor protein association with microtubules. It is tempting to speculate tau₄₅₋₂₃₀ might be promoting neurodegeneration in a similar pattern, however further research is needed to investigate its effects on motor protein binding, microtubule polarity, and/or interactions with intermediate filaments.

In sum, the novel findings presented here provided insights into a neurotoxic mechanism by which tau₄₅₋₂₃₀ induces, at least in part, neuronal death in the context of neurodegenerative diseases. In concert with other aberrant pathways including activation of cell signaling cascades, degradation of synaptic complexes, and dysregulation of cellular stress signaling, tau₄₅₋₂₃₀-induced inhibition of axonal transport could underline the early events involved in the progression of these diseases.

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ABBREVIATIONS

MAP	Microtubule Associated Protein
Aβ	Beta Amyloid
AD	Alzheimer's disease
NFT	Neurofibrillary Tangles
MEM	Minimum Essential Medium
GFP	Green Fluorescent Protein
PBS	Phosphate Buffered Saline
MTSB	Microtubule Stabilizing Buffer
SDS	Sodium Dodecyl Sulfate
BSA	Bovine Serum Albumin
kDa	kilo Dalton
E18	Embryonic Day 18
E16	Embryonic Day 16

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- The neurotoxic tau₄₅₋₂₃₀ associates both microtubules and membrane-bound organelles in hippocampal neurons
- These associations are independent of the presence of full-length tau
- Tau₄₅₋₂₃₀ impairs anterograde and retrograde axonal transport
- Defects in organelle transport are not associated with changes in motor protein levels

Figure 1. Subcellular localization of tau₄₅₋₂₃₀ in cultured hippocampal neurons

(A & B) Control and (C & D) tau₄₅₋₂₃₀-transfected hippocampal neurons were double-stained with tubulin (A & C) and GFP (B & D) antibodies. No immunostaining for GFP was detected in control neurons. On the other hand, abundant (~50%) GFP-immunoreactive neurons were detected in transfected cultures. (E–J) Tau₄₅₋₂₃₀-transfected neurons were extracted with Triton X-100 (E & F) or saponin (G–J) prior to fixation to preserve only the cytoskeleton and membrane-bound organelles plus the cytoskeleton, respectively. Cells were double-stained using tubulin (E & G) and GFP (F & H) antibodies. Note the presence of GFP staining in the cell bodies and faint immunoreactivity along the processes of extracted neurons. (I & J) Tau₄₅₋₂₃₀-transfected neurons labeled with MitoTracker were extracted with saponin and immunostained using a GFP antibody (I). (J) High-power magnification of the boxed area in I showing MitoTracker positive dots indicative of the presence of mitochondria in saponin-extracted neurons (arrows). Scale bars: A–I = 20 μm; J = 10 μm.

Figure 2. Tau₄₅₋₂₃₀ was present in cytoskeleton and cytoskeleton plus organelle fractions (A & B) Immunoblots of whole cell, Triton X-100-, and saponin-extracted lysates from tau₄₅₋₂₃₀-transfected hippocampal neurons were reacted with tau and GFP antibodies. Blots were normalized to acetylated tubulin as a protein control. (C & D) Quantitative analysis of the levels of dimerized (C) and monomeric (D) tau₄₅₋₂₃₀ present in the saponin- vs. Triton X-100-extracted lysates. Values represent the mean $\pm$ S.E.M. from 5 independent experiments per condition. The levels of tau₄₅₋₂₃₀ in lysates of non-extracted cells were considered 100%. * $p < 0.05$

Figure 3. The subcellular localization of tau₄₅₋₂₃₀ in hippocampal neurons is independent of the expression of full-length tau

(A & B) Tau-depleted control and (C & D) tau₄₅₋₂₃₀-transfected hippocampal neurons were double-stained with tubulin (A & C) and GFP (B & D) antibodies. No immunostaining for GFP was detected in tau-depleted control neurons. On the other hand, abundant (~50%) GFP-immunoreactive neurons were detected in transfected cultures. (E–J) Tau₄₅₋₂₃₀-transfected neurons were extracted with Triton X-100 (E & F) or saponin (G–J) prior to fixation to preserve only the cytoskeleton and membrane-bound organelles plus the cytoskeleton, respectively. Cells were double-stained using tubulin (E & G) and GFP (F & H) antibodies. Note the presence of GFP staining in the cell bodies and faint immunoreactivity along the processes of extracted neurons. (I & J) Tau₄₅₋₂₃₀-transfected neurons labeled with MitoTracker were extracted with saponin and immunostained using a GFP antibody (I). (J) High-power magnification of the boxed area in I showing MitoTracker positive dots indicative of the presence of mitochondria in saponin-extracted neurons (arrows). Scale bars: A–I = 20 μm; J = 10 μm.

Figure 4. The presence of tau₄₅₋₂₃₀ in cytoskeleton and cytoskeleton plus organelle fractions is independent of the expression of full-length tau

(A & B) Immunoblots of whole cell, Triton X-100-, and saponin-extracted lysates from tau₄₅₋₂₃₀-transfected tau-depleted hippocampal neurons were reacted with tau and GFP antibodies. Blots were normalized to acetylated tubulin as a protein control. (C & D) Quantitative analysis of the levels of dimerized (C) and monomeric (D) tau₄₅₋₂₃₀ found in the saponin- vs. Triton X-100-extracted lysates. Values represent the mean $\pm$ S.E.M. from 5 independent experiments per condition. The levels of tau₄₅₋₂₃₀ in lysates of non-extracted tau-depleted cells were considered 100%. * $p < 0.05$

Figure 5. Subcellular localization of tau₄₅₋₂₃₀ in A β -treated cultured hippocampal neurons
 (A) Representative immunoblots of whole cell and detergent-extracted lysates from 21 DIV hippocampal neurons pre-incubated in the absence or presence of A β (20 μ M) for 24 hr reacted with a tau antibody. (B) Quantitative analysis of the levels of the tau₄₅₋₂₃₀ fragment found in the saponin- vs. Triton X-100-extracted lysates. Blots were normalized to acetylated tubulin as a protein control. Values represent the mean $\pm$ S.E.M. from 5 independent experiments per condition. The total levels of tau₄₅₋₂₃₀ in lysates of non-extracted A β -treated neurons were considered 100%. * $p < 0.01$

Figure 6. Time-lapse imaging of axonal transport of lysosomes in cultured hippocampal neurons (A) Representative live-cell fluorescent images of hippocampal neuronal cultures expressing empty p-eGFP-N1 vector, htau40-GFP, or tau₄₅₋₂₃₀-GFP constructs (green) merged with images of endogenous lysosomes stained with LysoTracker Red fluorescent dye (red). (A, B) Signs of degeneration including varicosities along the axon (arrowheads) were observed only in neurons expressing tau₄₅₋₂₃₀-GFP. (B) Zoomed view of live cell time-lapse images of axons shown in A. Lysosomes moved through the axon (numbered arrows) over a 100 sec period. Scale bar: 5 μm.

Figure 7. Tau₄₅₋₂₃₀ disrupted axonal transport in cultured hippocampal neurons

(A) Representative kymographs of lysosomal (top panels) and mitochondrial (bottom panels) transport in axons of non-transfected (control) or transfected (empty p-eGFP-N1 vector, htau40-GFP, or tau₄₅₋₂₃₀-GFP) hippocampal neurons. Note the marked decrease in organelle movement in neurons expressing tau₄₅₋₂₃₀-GFP. Kymographs were generated from stacks of axonal images taken every 2 sec for total of 100 sec. (B) Quantification of total lysosomal and mitochondrial movements in control and transfected neurons. Expression of htau40-GFP had no effect on transport. In contrast, organelle movement was significantly reduced in neurons expressing the tau₄₅₋₂₃₀-GFP construct compared to all conditions (lysosomes, $n=163$; mitochondria, $n=494$). Values represent the mean $\pm$ S.E.M. Moving organelles was expressed as a percentage of number of organelles moving $1 \mu\text{m}$ compared the total number of respective organelle * $p < 0.05$; ** $p < 0.01$. Neurons ($n=15$) for each experimental condition and from 5 independent hippocampal cultures were observed.

Figure 8. Motor protein levels were not affected by tau₄₅₋₂₃₀ expression in cultured hippocampal neurons

(A & B) Representative immunoblots of ubiquitous kinesin (kinesin), neuronal kinesin (kinesin 5A) and dynein motor proteins in whole cell lysates prepared from control (C) and tau₄₅₋₂₃₀-transfected (T) neurons kept in culture for 1 day (DIV). Blots were normalized to α -tubulin as a protein control. (C) Quantitative immunoblot analysis revealed no significant differences in motor protein levels when tau₄₅₋₂₃₀-transfected neurons were compared to non-transfected controls. Samples obtained from 5 independent culture preparations per experimental condition were used in this analysis.

Table 1

Motile behavior of axonal lysosomes in transfected hippocampal neurons.

Measurement	Control	Transfection condition		
		p-eGFP-N1	htau40-GFP	Tau ₄₅₋₂₃₀ -GFP
Total lysosomes (<i>n</i>)	163	106	149	129
Moving lysosomes (%)	62.2 ± 5.24	71.8 ± 4.46	65.6 ± 6.03	37.8 ± 5.41 ^{**1}
Lysosome density (<i>n</i> /μm)	0.16 ± 0.02	0.12 ± 0.02	0.19 ± 0.03	0.14 ± 0.02
Anterograde movements (%)	26.1 ± 4.13	18.0 ± 4.22	16.6 ± 6.28	20.7 ± 6.32
Retrograde movements (%)	73.9 ± 4.13	82.0 ± 4.22	83.1 ± 6.4	72.2 ± 8.26
Average anterograde excursion length (μm)	3.43 ± 0.60	2.43 ± 0.52	2.22 ± 0.41	2.56 ± 0.33
Average retrograde excursion length (μm)	4.00 ± 0.39	4.92 ± 0.92	3.62 ± 0.50	2.84 ± 0.38 ^{*2}
Average anterograde excursion velocity (μm/sec)	0.45 ± 0.09	0.27 ± 0.13	0.38 ± 0.13	0.41 ± 0.07
Average retrograde excursion velocity (μm/sec)	0.38 ± 0.03	0.42 ± 0.05	0.34 ± 0.05	0.41 ± 0.19
Axonal location in relation to soma:				
Proximal (%)	38.2 ± 4.84	38.7 ± 5.58	26.7 ± 5.34	38.9 ± 5.96
Medial (%)	23.2 ± 3.38	26.8 ± 4.38	30.6 ± 5.75	30.0 ± 6.34
Distal (%)	38.7 ± 4.58	32.7 ± 6.02	45.2 ± 4.8	31.1 ± 7.18

The numbers represent the means ± S.E.M. 1: n=5, *t*: 3.2. 2: n=5, *t*: 2.8.

* *p* < 0.05, different from non-transfected controls;

** *p* < 0.01, different from non-transfected controls.

Neurons (n=15) for each experimental condition and from 5 independent hippocampal cultures were observed.

Table 2

Motile behavior of axonal mitochondria in transfected hippocampal neurons.

Measurement	Control	Transfection condition		
		p-eGFP-N1	htau40-GFP	Tau ₄₅₋₂₃₀ -GFP
Total mitochondria (<i>n</i>)	335	340	298	293
Moving mitochondria (%)	37.2 ± 4.56	44.3 ± 8.62	36.8 ± 8.62	13.6 ± 3.60 ^{**1}
Mitochondria density (<i>n</i> /μm)	0.44 ± 0.03	0.48 ± 0.06	0.44 ± 0.03	0.57 ± 0.05
Anterograde movements (%)	49.9 ± 5.77	43.2 ± 6.15	48.9 ± 4.03	39.0 ± 11.1
Retrograde movements (%)	55.9 ± 5.81	55.2 ± 3.60	50.0 ± 3.70	61.1 ± 11.1
Average anterograde excursion length (μm)	2.47 ± 0.39	3.10 ± 0.55	2.89 ± 0.39	2.28 ± 0.47
Average retrograde excursion length (μm)	2.38 ± 0.19	3.58 ± 0.32	3.10 ± 0.55	3.06 ± 0.56
Average anterograde excursion velocity (μm/sec)	0.23 ± 0.04	0.29 ± 0.03	0.19 ± 0.05	0.27 ± 0.02
Average retrograde excursion velocity (μm/sec)	0.39 ± 0.04	0.48 ± 0.05	0.33 ± 0.05	0.19 ± 0.06 ^{**2}
Axonal location in relation to soma:				
Proximal (%)	34.5 ± 2.24	38.5 ± 1.89	32.4 ± 2.52	30.8 ± 1.39
Medial (%)	32.6 ± 1.15	31.6 ± 1.58	35.5 ± 3.24	34.9 ± 1.21
Distal (%)	30.8 ± 1.67	31.5 ± 2.63	32.1 ± 4.38	35.6 ± 2.01

The numbers represent the means ± S.E.M. 1: n=5, *t*: 4.1. 2: n=5, *t*: 2.8.

* *p* < 0.05, different from non-transfected controls;

** *p* < 0.01, different from non-transfected control.

Neurons (n=15) for each experimental condition and from 5 independent hippocampal cultures were observed.