Title:
Phosphorylation of microtubule-associated protein Tau tunes mitochondrial transport by controlling the inter-microtubule spacing.

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Date
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Summary

Mitochondrial transport within axons is crucial for axonal maintenance of neurons and its dysregulation can contribute to neurodegenerative diseases. In axons, mitochondrial movement is driven by two oppositionally-directed motor proteins, kinesin and dynein, along microtubules. The surface of microtubules is decorated with microtubule-associated proteins (MAPs). Tau serves as a predominant MAP in axons and is a filamentous protein of 441 amino acid residues (the longest human isoform). Tau is comprised of two functional regions, the N-terminal projection domain that protrudes from the surface of microtubules and the C-terminal microtubule-binding domain. Overexpression of Tau inhibits mitochondrial transport in various cells. However, the mechanism and regulation of Tau-mediated inhibition of mitochondrial transport are not understood. Tau is a phosphoprotein, the functions of which can be regulated by phosphorylation. Among Tau phosphorylation sites, Ser199, Ser202, and Thr205 (AT8 sites) are particularly interesting. These sites are located at the border of the projection domain and microtubule-binding Pro-rich region. Phosphorylation at these sites may extend the projection domain outwards from the surface of microtubules and affect mitochondrial transport. Here, I directly examined the effect of phosphorylation at the AT8 sites on mitochondrial transport using the non-phosphorylatable Ala mutant (3A) and
the constitutive phosphorylation mimic Asp mutant (3D) of Tau. Mitochondrial clumping was more pronounced in COS-7 cells when pseudo-phosphorylated Tau 3D was expressed. Overexpression of wild-type Tau, Tau 3A or 3D inhibited mitochondrial movement in the neurite processes of PC12 cells as well as the axons of mouse brain cortical neurons. However, the greatest effects on mitochondrial translocation were induced by Tau 3D. These mutations also caused expansion of the space between microtubules in Sf9 cells when membrane tension was reduced by disrupting actin filaments. These results suggest that phosphorylation at the AT8 sites affects mitochondrial transport by changing the spaces between microtubules. Tau is a major component of neurofibrillary tangles found in Alzheimer pathology. Hyperphosphorylation of the AT8 sites may contribute to axonal degeneration by disrupting mitochondrial transport in Alzheimer’s disease. The regulation of the AT8 sites phosphorylation would be a therapeutic strategy against development of Alzheimer disease.
Introduction

Neuronal axons are long processes in which organelle biogenesis is limited. Also, organelle transport is vital for the development and maintenance of axons, in which the distances between sites of organelle biogenesis, function, and recycling or degradation can be vast. Transport of cellular components such as membrane-bound organelles and cytoskeletal components into axons is also crucial for axonal extension and maintenance. Mitochondria can move within axons in a bidirectional manner (Fig. 1). In axons, mitochondria undergo bidirectional transport along microtubules (MTs) that is driven by two oppositely directed motor proteins, kinesin and dynein (Su et al., 2010). It has been well documented that kinesin moves anterogradely and dynein moves retrogradely in the neuronal cell processes. MT-based motility is controlled at several levels including control of motor-associated cargo adaptors, kinesin and dynein, and transport tracks composed of MTs and microtubule-associated proteins (MAPs) (Hollenbeck and Saxton, 2005; Bereiter-Hahn and Jendrach, 2010). Microtubule-associated proteins (MAPs), a group of filamentous proteins copurified with tubulin through repetitive cycle of depolymerization and reassembly, have various roles in the nervous system. For example, in MT promoting assembly, nucleation and flexibility (Marx et al., 2006) as well as neuronal growth. The MAPs also play a role as regulating factor in the
processive transport (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008; Stoothoff et al., 2009; Vossel et al., 2010), and one of the most established of their roles is in space-making (Shahpasand et al., 2008) which determines the inter-microtubular spaces (IMS) in the neuronal processes (Mukhopadhyay and Hoh, 2001; Jeganathan et al., 2008). Particularly MAP2, MAP1B and Tau play a critical role on the MT spacing. Six tau isoforms exist in human brain tissue, and they are distinguished by their number of binding domains. Three isoforms have three binding domains and the other three have four binding domains. The binding domains are located in the carboxy-terminus of the protein and are positively-charged (allowing it to bind to the negatively-charged microtubule). The isoforms with four binding domains are better at stabilizing microtubules than those with three binding domains. The isoforms are resulted from alternative splicing in exon 2, 3, and 10 of the Tau gene (Fig. 2).

Tau protein is a highly soluble MAP. In humans, these proteins are mostly found in neurons compared to non-neuronal cells. Tau protein is mainly concentrated in axons and determines the inter-microtubule spaces in the axonal cytoskeleton (Mukhopadhyay and Hoh, 2001; Jeganathan et al., 2008). Regarding the inter-MT spaces in the axonal cytoskeleton, MTs have to move radially in order to make room for the giant vesicle, which will not be
possible without a local alteration in the IMS (Fig. 3). It was demonstrated that the adjustable IMS are resulted from the space-making role of the MAPs (Shahpasand et al., 2008). I think that there are defined mechanisms that control dynamically the IMS for an efficient vesicular transport. MAPs bind to the surface of MTs and contribute to their polymerization and stabilization. A major MAP in axons is Tau, which inhibits mitochondrial transport when over-expressed (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008; Stoothoff et al., 2009; Vossel et al., 2010). However, the mechanism and regulation of Tau-mediated inhibition of mitochondrial transport are not understood.

Tau is a filamentous protein of 441 amino acid residues (the longest human isoform) comprising two regions, the N-terminal projection domain and the C-terminal MT-binding domain. The projection domain protrudes from the surface of MTs, providing sites for interaction with other proteins, and it likely determines the distance between MTs (Stoothoff and Johnson, 2005; Hanger et al., 2009). The MT-binding domain includes three or four tandem repeats of MT-binding sequences, and these MT-binding and assembly properties have been extensively analyzed (Watanabe et al., 1993; Morishima-Kawashima et al., 1995; Stoothoff and Johnson, 2005). Both the N-terminal and C-terminal regions are implicated in regulating mitochondrial transport, and various hypotheses have been proposed
for the mechanism underlying transport; these include competition with motor proteins in binding to MTs (Marx et al., 2006), direct interaction with motor proteins (Stamer et al., 2002), an increase in the number of MTs with concomitant reduction in the space between them (Thies and Mandelkow, 2007), and displacement of Tau-stabilized MTs to submembrane domains (Vershinin et al., 2008).

Tau contains more than 20 phosphorylation sites, and Tau function can be regulated by phosphorylation (Ishiguro et al., 1992; Planel et al., 2002). With increasing the number of phosphate groups one would expect higher rigidity of the projection domain. Therefore the steric constraints would be increased and so would the IMS (Fig. 4) provided that the MAPs still remain on the MT surface since the highly phosphorylated Tau cannot bind to MT (Planel et al., 2002). Many phosphorylated residues are found in the regions flanking the MT-binding repeats as well as a few within the repeats. Generally, phosphorylation in the MT-binding repeats inhibits interaction with MTs, whereas phosphorylation in the flanking regions just decreases the affinity for MTs. Many phosphorylation sites reside in proline-directed (Ser/Thr)-Pro sequences. These sites are moderately phosphorylated in healthy neurons but abnormally phosphorylated in degenerated brains of Alzheimer’s patients (Plattner et al., 2006). Cdk5 and GSK3β are proline-directed protein kinases that are known to phosphorylate...
these (Ser/Thr)-Pro sites in a hierarchical manner (Darios et al., 2005; Morel et al., 2010). Cdk5 is a primary kinase that phosphorylates Tau mainly at Ser202, Thr205, Ser235, and Ser404, whereas GSK3β is a secondary kinase phosphorylating Ser199, Thr231, and Ser396, although other sites may be phosphorylated according to the cellular context (Hanger et al., 2009). Hyper-activation of Cdk5 or GSK3β reduces mitochondrial movement (Darios et al., 2005; Morel et al., 2010), but it has not been clearly demonstrated whether Tau is a major downstream target of these kinases and, if so, which phosphorylation site(s) is critical.

Among GSK3β- and Cdk5-related phosphorylation sites, Ser199, Ser202, and Thr205 are particularly interesting. These sites are located at the border of the projection domain and MT-binding Pro-rich region (Fig. 5). Phosphorylation at these sites may extend the projection domain outwards from the surface of MTs (Fig. 6). These phosphorylated sites are recognized by the phosphorylation-dependent antibody, AT8 (Goedert et al., 1995; Wang et al., 2006; Hanger et al., 2009), which is commonly used as a marker of abnormal phosphorylation in brains of Alzheimer’s patients (Fig. 7). Non-phosphorylated Tau at these sites is recognized by the antibody, Tau-1, which is specific for axons. The extended projection domains may affect mitochondrial transport (Shahpasand et al., 2008), but this idea has not been experimentally confirmed.
I directly examined the effect of phosphorylation of Tau at Ser199/Ser202/Thr205 on mitochondrial trafficking using the non-phosphorylatable Tau mutant 3A (i.e., each of Ser199/Ser202/Thr205 was mutated to Ala) and the constitutive phosphorylation mimic of Tau, 3D (i.e., each of Ser199/Ser202/Thr205 was mutated to Asp. Overexpression of Tau 3D in PC12 cells or cortical neurons decreased the movement of mitochondria compared to overexpression of wild-type (WT) Tau or Tau 3A. Mitochondrial clumping was more pronounced in COS-7 cells when pseudo-phosphorylated Tau 3D was expressed. Furthermore, Tau overexpression reduced the inter-MT spaces in PC12 cell neurites while Tau phosphorylation at the AT8 sites has no further effect on the inter-MT spacing. Expression of Tau 3D in Sf9 cells expanded the inter-MT spacing in the cell protrusions, when the membrane tension resulted from actin filaments was reduced, indicating that Ser199/Ser202/Thr205 are the critical phosphorylation sites for mitochondrial transport via regulation of inter-MT spacing.
Materials and Methods

Anti-human Tau (A0024) was obtained from Dako (Glostrup, Denmark). Anti-phospho-Tau antibody AT8 was purchased from Thermo Scientific (Rockford, IL). Anti-TOM20 (FL-145) was from Santa Cruz Biotechnology (Santa Cruz, CA). MC-1 and Alz-50 anti-Tau antibodies were generous gifts from Dr. Peter Davies (Albert Einstein College of Medicine, NY). Anti-β-tubulin and latrunculin B were from Sigma-Aldrich (St. Louis, MO). Anti-His tag was from Invitrogen (Carlsbad, CA). NGF and Phos-tag acrylamide was obtained from Wako Chemicals (Osaka, Japan).

Construction of expression vectors for Tau proteins

Mutations of Tau at Ser199, Ser202, and Thr205 were introduced in the longest wild-type human Tau in a pSG5 expression vector. Double Ala mutants (2A) at Ser202A and Thr205A were constructed with PCR using primers

5’-GGCGCCCCAGGCAGCTCCCCGGAGCCGC-3’ (forward) and

5’-CCGCGGGGTCCGCGAGGGCCGTCGGCG-3’ (reverse). The condition was 30 s for denaturing time, 60 s for annealing time and 340 s for the extension time. The PCR solution composed of 2.5 μl of 10× PfuUltra buffer, 0.5μl on template cDNA (50~100 ng), 0.5 μl of
the primers (Fwd & Rev), 2.5 μl dNTPs, 0.5 μl Pfu and 18 μl DDW. For Tau 3A, an additional Ala mutation at Ser199 was constructed with PCR using Tau 2A as a template and primers 5’-AGCGGCTACAGCGCCCCCGGCGCCCCA-3’ (forward) and 5’-TCGCCGATGTCGCGGGGCAGGGGT-3’ (reverse). The condition was 30 s for denaturing time, 60 s for annealing time and 320 s for the extension time. Tau 3D was constructed by one-step PCR using Tau 3A as a template and primers 5’-AGCGGCTACAGCGACCCCGGCGACCCAGGCGATCCCGGCAGCCGC-3’ (forward) and 5’-TCGCCGATGTCGCTGGGGCCGCTGGGTCCGCTAGGGCCGTCGGCG-3’ (reverse). The condition was 30 s for denaturing time, 65 s for annealing time and 360 s for the extension time. All constructs were confirmed by DNA sequencing. Bacterial expression vectors encoding Tau 3A and Tau 3D were constructed similarly with PCR using Tau 1N4R (one N-terminal insertion and four MT-binding repeats) in the pRK172 vector.

Baculovirus expression vectors for Tau proteins were constructed as follows: Tau WT, 3A, or 3D in pSG5 was amplified with PCR using primers 5’-GGATCCATGGCTGAGCCCCGCCA-3’ (forward, with BamHI site) and 5’-GCGGCGCTCACAACCTGCCTT-3’ (reverse, with NotI site). The PCR solution composed of 32.5 μl of 2× GC buffer-1, 10.4 μl dNTPs mix (2.5 mM of each nucleotide), 1.3
μl of the primers (10 pmol/μl), 19.5 μl autoclaved water and 1 μl of template DNA. PCR products were inserted into the pCR2.1 vector. The ligation solution composed of 4.5 μl of the PCR product, 0.5 μl pCR2.1 vector and 5 μl of the ligation kit solution-1 which contains every necessary parts including ligating enzymes and nucleotides. The solution was incubated at 16°C for 3 hours. Then, 5 μl of the product was mixed with 50 μl DH5α competent cells for transformation. Tau genes were confirmed by sequencing. Tau cDNAs were digested with BamHI and NotI. In order to digest the DNA substrate with the two restriction endonucleases simultaneously (double digestion), I selected the NEBuffer 3 (to provide reaction conditions that optimize enzyme activity as well as avoid star activity associated with the enzymes that is an important consideration. Each enzyme is supplied with the optimal NEBuffer to ensure 100% activity. The solution contained 1 μl 10x Buffer, 6.5 μl H₂O, 2 μl DNA and 0.5 μl of each enzyme and was incubated for 1 hour at 37°C. Also, pFastBac Dual vector was doubly digested with the same enzymes at the same conditions. Meanwhile the agarose gel containing ethidium bromide (1 μl /100 ml) was prepared. Having done the electrophoresis, the convenient DNA bands were excited from the gel and placed in 1.5 ml tubes. Membrane binding solution was added to the gel slices (10 μl solution per 10 mg of the gel slice), voltexed and incubated at 50-60°C up to complete dissolving. Then an equal volume of
membrane binding solution was added and the total mixture transferred to the SV mini-column assembly. The mixture was incubated for 1 min at RT, then centrifuged at 16000 × g for 1 min at RT. Next, 700 μl membrane wash solution (ethanol added) was admixed with the sample and centrifuged at 16000 × g for 1 min at RT. The washing step was repeated by 500 μl washing solution. Then, centrifuged for 5 min at 16000 × g. The empty collection tube was re-centrifuged to completely evaporate the residual ethanol. 50 μl nuclease free water was used to elute the DNA by centrifugation at 16000 × g for 1 min. Tau genes and pFastBac Dual vector were ligated as mentioned before. Recombinant plasmids were used to transform competent DH10Bac cells for transposition to the bacmid shuttle vectors. The routine transformation was performed, except for that after the heat-shock and 2 min chilling, the tubes were shake for 4 hours at 225 rpm at 37°C. The cells were cultured on the LB plates containing Kanamycine (50 μg/ml), gentamycine (7 μg/ml), tetracycline (10 μg/ml), Blue-gal (100 μg/ml) and IPTG (40 μg/ml) for 48 hours. The white colonies were picked and were cultured on fresh LB containing Kanamycine (50 μg/ml), gentamycine (7 μg/ml), tetracycline (10 μg/ml), Blue-gal (100 μg/ml) and IPTG (40 μg/ml). The bacmids were isolated using Maxi-preps kit. Recombinant bacmid DNA was transfected into monolayers of Sf9 insect cells using lipofectamine 2000. Supernatants containing virus were harvested 96 h after
transfection.

Then, the viral titer was amplified and determined. In this regard, the Sf9 cells were plated in 6-well dishes and 10-fold serial dilutions of the baculoviral stock was prepared and added to the cells for 1 hour. The virus was removed and the cell monolayer overlayed with Plaquing medium. The plaquing medium contains a mixture of culture medium and agarose, and will be used to immobilize the infected cells for the plaquing assay. It’s better to prepare the plaquing medium just before the usage. The cells were incubated for 7-10 days in a humidified 27°C incubator. The cells were stained with Neutral red (high purity) for 1-2 hours just prior to the counting. Plaques will appear as clear spots on a red monolayer. The number of plaques were counted in each dilution using the following formula: Titer (pfu/ml) = number of plaques × dilution factor × (1 / ml of inoculum per well).

Cell culture and expression of Tau proteins

COS-7 cells (2 × 10^5 per ml) were grown in DMEM (Sigma) containing 10% FBS and transfected with PolyFect reagent (Qiagen, CA) (Kaminosono et al., 2008). The cells were passaged every 3 days as following: sucking the DMEM from the culture, washing the cells
with pre-warmed PBS and adding 5 ml pre-warmed PBS containing 50 μl trypsin. Then incubating for 10 min at the incubator and removing the PBS from the culture. Next, 5 ml fresh and pre-warmed medium was added to the cells and detached the cells by pipetting. The cells then were counted and seeded on the cover-slips in 35 mm culture dishes. At the time of transfection, plated cells on the cover-slips, which should be 40~80% confluent, were washed with pre-warmed PBS and the medium containing 10% FBS with no antibiotics were applied to the cells. Then, the convenient amount of cDNA admixed with 100 μl medium free of serum and antibiotics. 10 μl polyfect transfection reagent was added to the DNA solution and mixed well. The samples were incubated for 10 min at RT to allow the complex formation. 500 μl medium containing serum with no antibiotics was added to the samples and applied to the cell dishes, incubated for 4 hours in the incubator. After 4 hours, the cell’s medium was changed with the fresh one that contains serum and antibiotics. Transfected cells were kept for 24 hours in the incubator to allow the protein expression.

PC12 cells (2 × 10^5 per ml) were cultured on 35-mm glass-bottom dishes in DME containing 10% FBS and antibiotics (penicillin and streptomycin) at 37°C in 5% CO₂. Every 4 days, the cells were passaged as following:
Cell culture medium was aspirated and the cells were washed with pre-warmed PBS. Then, 50 μl trypsin in 5 ml PBS was added to the cells and incubated for 10 min in the incubator. 5 ml pre-warmed medium was added to the culture dish and the cells were detached by pipetting. The total 10 ml sample was centrifuged for 3 min at 300 rpm. The supernatant was then aspirated and 2 ml fresh medium was added to the cells and re-suspend the cells by pipetting. Cell were counted and seeded on the pre-coated culture dishes with poly-L lysine.

For transient transfection, the cells were washed with pre-warmed PBS and fresh medium containing 10% FBS free of antibiotics was added to the culture dishes. 10 μl lipofectamine 2000 transfection reagent was added to 250 μl medium free of serum and antibiotics and incubated for 5 min at RT. Also, the convenient amount of cDNA was admixed in 250 μl medium free of serum and antibiotics. After 5 min, the two 250 μl samples were mixed and incubated for 25 min at RT to allow the complex formation. Then, the total 500 μl sample was applied to the culture dishes. After 4 hours incubation in the incubator, the medium replaced with fresh one containing serum and antibiotics. Cells were also treated with NGF (50 ng/ml) 4 h after transfection for 3 days.

Primary neurons were prepared from 17-day-old embryonic mouse brain cerebral cortex of either sex. The heads of the pups were removed and placed in a 10cm dish. For each
head, the skin was removed and cut along the scalp in the midline with fine scissors. A similar midline cut in the calvarium was also made. The calvarium with a blunt spatula was deflected and scooped the brain into another 10cm dish containing ice-cold DM/KY. The cortices were dissected as following: the brain ventral was placed side up. The spatula was placed in the medial aspect of the ventral cortex and midbrain and the cortices were cut off. Discarded the midbrain and dissected the hippocampus and cortex. The remainder was discarded. Then, the individual cortices or hippocampi were placed in 10cm dish containing ice-cold DM/KY. Then, striped the meninges and cut the tissue into small 1mm3 pieces and transfered all of the cortical/hippocampal tissue to a 15ml conical tube. It’s better to use one 15ml tube per 5 cortices dissected. Once the tissue has settled, the extra DM/KY solution was removed. Next, mixed the cell suspension and diluted the cells with optimem/glucose solution to final count of 2.5-3 million per ml. Neurons (2 × 10^5 per ml) were seeded on 35-mm glass-bottom dishes. The medium was then changed to neurobasal medium supplemented with B27 (Invitrogen) and 1 mM L-glutamine (Endo et al., 2009). Cells were transiently transfected with Tau WT, 3A, or 3D with Lipofectamine 2000 as mentioned before. Various amounts of Tau cDNA (1–4 μg) were tested to determine the appropriate amount of cDNA. Neurons were co-transfected with Mito-GFP to label and observe mitochondrial movement.
Sf9 cells were grown at 27°C in 35-mm dishes in serum-free medium complete (Gibco) and antibiotics (penicillin and streptomycin). Monolayer cultures of Sf9 cells (4 × 10^5 per ml) were infected with baculoviruses encoding Tau cDNA at a titer of 2 × 10^7 pfu/ml. Cells were treated with 0.5 μg/ml latrunculin B from the time of infection for 72 h and were fixed with 2.5% glutaraldehyde and 2% PFA in PEM buffer (0.1 M PIPES, pH 7.2, 1 mM EGTA, 1 mM MgCl_2) for 30 min at room temperature. The samples were then subjected for staining and embedding for thin-sectioning.

**Immunostaining**

One day after transfection, COS-7 cells on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min, and permeabilized with 0.1% Triton X-100 in PBS containing 5% BSA for 20 min. Cells were incubated with anti-TOM20 antibody (1/1000), followed by Alexa 546-labeled secondary antibody. Mitochondrial clumping was examined with an LSM 5 EXCITER microscope (ZEISS) and measured with 20 cells in each sample by Image J software. In order to observe the binding of Tau to MTs in cells, COS-7 cells were treated with pre-warmed PEM buffer containing 4 M glycerol and 0.5% Triton X-100 for 10 min at 37°C. After fixation with cold methanol
(-20°C) for 3 min, cells were incubated with anti-tubulin (1:500) or anti-Tau (1:500), followed by Alexa-conjugated anti-mouse IgG or anti-rabbit IgG (1:500). Immunoflourscent stainings were examined with an LSM5 EXCITER microscope (Zeiss).

**Electron microscopy**

Sf9 cells in 35-mm culture dishes were fixed with 2.5% glutaraldehyde and 2% PFA in PEM buffer (0.1 M PIPES, pH 7.2, 1 mM EGTA, 1 mM MgCl₂) for 30 min at RT. Then washed with 10% sucrose in Cacodylate 0.1 M (pH = 7.4) for 15 min on ice. Post fixation was performed with 1% osmium tetroxide in 0.1 M Cacodylate for 1~1.5 hour on ice. Then washed with DDW to remove osmium tetroxide and block-stained with 0.5% uranyl acetate in DDW for 2 hours at RT. Dehydration of the samples was performed using an increasing amount of ethanol from 65%, 75%, 85%, 95% and 99% for 15 min of each step. Complete dehydration by 100% ethanol was done for 3 times and 20 min for each step at RT. Embedding was performed with Epon812. The mixture contains: 45.5 ml of Epon812, 31 ml DDSA, 23.5 ml MNA and 1.5 ml DMP-30. At first, the mixture without DMP-30 was prepared and well mixed for 30 min by up and down procedure. Then the DMP-30 was added and vigorously mixed for 5 min. For the embedding, I did the following procedure:
1. Epon / ethanol 100% (1:2). 60 min at RT.

2. Epon / ethanol 100% (1:1). 60 min at RT.

3. Epon / ethanol 100% (2:1). 60 min at RT.

4. Epon (-DMP-30) 3~4 times. Each step was performed for 1 hour.

5. Epon (-DMP-30) for overnight to completely evaporate the ethanol in a desicator.

6. Epon (+DMP-30), 5~6 times. Each step was performed for 1 hour.

7. Epon (+DMP-30), 24 hours at 33°C

8. Polymerization of the Epon812 at 60~65°C for 48 hours.

The samples were then processed for thin-sectioning and observation with electron microscopy (Tokuoka et al., 2000). Specimens were examined with a JEM-1010 transmission electron microscope (JEOL Tokyo, Japan). Inter-MT distances were measured with Image J software.

PC12 cells were transfected with plasmids encoding Tau and EGFP. Tau-expressing PC12 cells were identified with EGFP after fixation and their positions were marked on the base of culture dishes by a felt pen. Fixation was done as Sf9 cell fixation and embedding procedure and observed at the same manner.
Time-lapse imaging

Mitochondrial movement in NGF-treated PC12 cells and mouse brain cortical neurons was examined with an LSM 5 EXCITER microscope using an incubation chamber with 5% CO₂ at 37°C. Fluorescent images of mitochondria in the longest process of each PC12 cell and axons of cortical neurons were acquired at intervals of 5 s over a period of 300 s. In each experiment, eight mitochondria in five cells were analyzed for their movement in image stacks composed of 60 images. Individual mitochondrial movements were analyzed with ZEN 2008 software (Zeiss). Differences in the position of each mitochondrion between two frames during each 5-s interval were exported to Excel, and they were classified and scored as stationary, anterograde, or retrograde movements. Completely immotile mitochondria during 300 s observation were excluded from the counts. Kymographs were made using serial frames of the same area during the observation period.

Statistical analysis

Statistical differences in stationary phase of mitochondria among control, Tau WT, 3A and 3D were analyzed by one-way ANOVA. Holm’s multiple test was conducted on all possible pair-wise combinations. Differences in the ratios between anterograde and retrograde
movements were analyzed by ANCOVA. The dependent variable was time of anterograde movement, and the independent variables were Tau transfection samples as a fixed factor and time of retrograde movement as a covariate. After confirming no interaction between Tau transfection samples and the retrograde ratios, the anterograde/retrograde moving durations were compared using all data or data of Tau transfected samples. When p > 0.05, the difference was considered non-significant. All of the statistical analyses were carried out with version 2.13.1 of the R software package (R: A language and environment for statistical computing. R Development Core Team, Vienna, Austria, 2011, available at http://www.R-project.org).

**Protein purification, motor–MT binding assay, and in vitro phosphorylation of Tau by Cdk5-p25**

Tau WT, 3A, or 3D was expressed in *E. coli* and purified as below:

The pRK172 bacterial expression vector containing recombinant human wild-type and mutant Tau cDNAs (encoding a tau protein with one N-terminal insert and four repeat domains (1N4R), WT, 3A, or 3D) was used to transform competent *E. coli* BL21-DE3 cells. Tau protein expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactoside for 2 h at
37 °C. Bacterial cells collected by centrifugation at 6,000 × g for 15 min at 4 °C were lysed in MOPS buffer (10 mM MOPS (pH 6.8), 0.1 mM EGTA, 0.1 mM EDTA, 10 µg/ml leupeptin, and 0.2 mM Pefabloc SC) by sonication. The supernatant obtained by centrifugation at 15,000 × g for 15 min at 4 °C was adjusted to 0.5 M NaCl and boiled for 10 min. After centrifugation at 100,000 × g for 30 min at 4 °C, the supernatant was dialyzed against MOPS buffer overnight at 4 °C. The Tau proteins were purified with a Mono S FPLC column (Amersham Biosciences) used after dialysis against MOPS buffer (Fig. 8). The amount of Tau protein was estimated by Coomassie Brilliant Blue (CBB) staining of gels using bovine serum albumin as the standard (Sakaue et al., 2005).

Tubulin was prepared from porcine brains by three cycles of temperature-dependent polymerization/depolymerization. Tubulin was separated from MT-associated proteins using a phosphocellulose column (Fig. 9) (Whatman P-11, Brentford, UK) (Sakaue et al., 2005). Kinesin RK430-AviTag-KRC-His₆ was expressed and purified from E. coli as following:

Plasmids of the kinesin was transformed into BL21 Star (DE3) (Invitrogen) or BL21-CodonPlus (DE3) RIL (Stratagene) host cells for expression in bacteria. Cells were grown in LB medium supplemented with 50 µg/ml ampicillin. Expression was induced at A₆₀₀ = 0.7 with 0.1 mM isopropyl- β-D-thiogalactopyranoside (expressed as final concentration).
Cells were grown for a further 4~6 h at 20 °C and then harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at -80°C. Proteins were purified as described previously (Stoothoff and Johnson, 2005), except that the gel filtration process was performed on an NAP-5 column (GE Healthcare) and that the final MT affinity purification step was modified for SNAP-Pkl1F and AviTag-Pkl1F due to the low recovery rate; proteins were co-sedimented with MTs, and the precipitate was re-suspended and placed on ice for use in further assays. GST-Pkl1N was purified by GST·bind resin (catalog number 70541; Novagen).

SNAP-vitro 647 labeling of SNAP-Pkl1F was performed at a mixing ratio of 1:3 (Pkl1F head/SNAP-vitro 647) for 30 min at room temperature described (Furuta et al., 2008), except that ATP was not added to the preparation buffers. Tubulin (0.5 μM) was assembled in PEM containing 1 mM GTP and 10 μM Taxol in the presence of various concentrations of Tau WT, 3A, or 3D (0.05 μM, 0.1 μM, or 0.15 μM) and 0.1 μM RK430 at 37°C for 45 min. Polymerized MTs were collected by centrifugation at 100,000 × g for 60 min at 37°C. The supernatants and pellets were subjected to SDS-PAGE followed by immunoblotting.

Cdk5-p25 was purified from Sf9 cells that had been infected with baculovirus encoding Cdk5 and p25 (Saito et al., 2003). Phosphorylation of Tau by Cdk5-p25 was performed in vitro by incubating 50 μg/ml Tau WT, 3D, or 3A with Cdk5-p25 at 37°C for 2 h.
**SDS-PAGE and immunoblotting**

SDS-PAGE and immunoblotting were performed as described (Sakaue et al., 2005). Phos-tag SDS-PAGE was performed with 7.5% polyacrylamide and 50 μM Phos-tag as below:

7.5% polyacrylamide gel containing 50 μM Phos-tag and 50 μM MnCl₂ was prepared and the routine electrophoresis was performed. The gel then washed with 30 ml submarine buffer containing EDTA (2 mM) for 30 min at RT. Next, the gel was washed with the submarine buffer for 15 min to remove the EDTA from the gel. Submarine transfer was performed for overnight. On the next day, the routine western-blotting was done on the membrane as described (Hosokawa et al., 2010).

Dot blot was performed with MC-1 and Alz-50 antibodies by the method reported (Jicha et al., 1997). All experiments were performed at least three times, and representative results are shown. All experiments were performed at least three times, and representative results are shown.
Results

Phosphorylation of Tau mutants and their binding to MTs

Tau can be phosphorylated at multiple sites in vitro and in vivo. To assess the effects of site-specific phosphorylation on mitochondrial transport, I used the Tau mutants 3A (nonphosphorylatable) and 3D (phosphorylation mimic), each of which was mutated at the AT8 Alzheimer phosphorylation sites (Ser199, Ser202, and Thr205) (Fig. 5). Recombinant Tau WT, 3A, and 3D were phosphorylated in vitro by Cdk5-p25 and immunoblotted with AT8, which recognizes Tau that is phosphorylated at Ser202 and Thr205 (Goedert et al., 1995) or Ser199, Ser202, and Thr205 (Hanger et al., 2009). Phosphorylated Tau WT was immunoreactive to AT8, whereas phosphorylated Tau 3A and 3D were not (Fig. 10), confirming that Cdk5 indeed phosphorylated the sites recognized by AT8 (Takahashi et al., 2003) and that these phosphorylation sites were disrupted in 3A and 3D constructs. These Tau constructs were expressed in COS-7 cells, and phosphorylation was examined by immunoblotting with AT8. Immunodetection of Tau WT but not 3A nor 3D clearly demonstrated that Tau WT was phosphorylated at the AT8 recognition sites in COS-7 cells (Fig. 11).

To determine if the mutations affected phosphorylation of Tau at other sites,
Tau expressed in COS-7 cells was detected by immunoblotting with a phosphorylation-independent Tau antibody after Phos-tag SDS-PAGE. Phos-tag SDS-PAGE is a recently developed method that detects phosphorylated proteins via an expanded mobility shift on SDS-PAGE (Kinoshita et al., 2006; Hosokawa et al., 2010). Multiple Tau bands indicated the presence of various phosphorylated forms of Tau in COS-7 cells (Fig. 12). As a whole, the decreased mobility of Tau WT compared to Tau 3A indicated the higher level of phosphorylation of Tau WT, including the AT8 site. The relative upward shift of all the Tau 3D bands was apparently due to the increased negative charge of the three Asp residues at positions at 199, 202, and 205. Phosphorylated Tau 3A and 3D had similar, but not identical, banding patterns, suggesting that these mutants underwent similar phosphorylation at other sites (Fig. 13). These results indicated that by using Tau 3A and 3D, I could examine the role of Tau phosphorylation at the AT8 sites (Ser199/Ser202/Thr205) on mitochondrial transport.

To see whether the 3D mutant mimics the phosphorylation state at the AT8 sites, I performed dot blot of Tau constructs with MC-1 and Alz-50 antibodies after phosphorylation with Cdk5-p25. MC-1 and Alz-50 are monoclonal antibodies that recognize conformation of Tau when phosphorylated at both AT8 and PHF-1 (Ser396 and Ser 404) sites (Jicha et al., 1999). Cdk5-phosphorylated Tau WT and Tau 3D, but not Tau 3A, were immunoreactive to
MC-1 and Alz-50 (Fig. 14). The results indicated that Tau 3D in fact mimics phosphorylation at the AT8 sites.

To assess Tau 3A and 3D binding to MTs, COS-7 cells were transfected with vectors that mediated expression of WT, 3A, or 3D forms of Tau. The Tau-expressing cells were then incubated in a MT-stabilizing buffer containing 0.5% Triton X-100 and 4 M glycerol to remove soluble tubulin and Tau, and then stained with anti-tubulin and anti-Tau (Bershadsky et al., 1978; Kaminosono et al., 2008). In all three cases, there was wide co-distribution of Tau and MT (Fig. 15), suggesting that Tau phosphorylation at the AT8 sites does not inhibit the binding of Tau to MTs in cells, as reported (Rankin et al., 2005; Hanger et al., 2009). Further, in vitro studies indicated that both recombinant pure Tau 3A and 3D bound to MTs similarly to Tau WT (data not shown).

Mitochondrial clumping in COS-7 cells expressing Tau mutants

Previous studies have demonstrated that overexpression of full-length Tau leads to the clustering of mitochondria in cells (Ebneth et al., 1998; Trinczek et al., 1999; Mandelkow et al., 2004). Moreover, the clustering induced by Tau was also shown to be regulated by phosphorylation (Mandelkow et al. 2004; Tatebayashi et al., 2004). However, the exact
phosphorylation site(s) that control the clustering are not known yet. I examined here the
effect of phosphorylation of Tau at Ser199/Ser202/Thr205 on mitochondrial clumping by
expressing Tau WT, 3A or 3D in COS-7 cells. Mito-GFP was cotransfected into COS-7 cells
for mitochondrial observation. Mito-GFP transfection did not affect the mitochondrial
distribution, which was similar to those in cells stained with anti-TOM20 antibody (Fig. 16
and 17). Overexpression of any Tau induced the clumping of mitochondria (Fig. 16). The
percentage of cell area where mitochondria occupy was previously used as an index of the
clumping (Trinczek et al., 1999; Mandelkow et al. 2004). I also measured the cytoplasmic
area occupied by mitochondria and the relative ratio to the whole cell area was compared
among cells expressing mutant Tau. Overexpression of any Tau markedly decreased the
overall area occupied by mitochondria (Fig. 17). The average area occupied by mitochondria
was reduced from 50.0 % in control cells to 29.3 % by Tau WT, 29.0 % by Tau 3A and
16.3 % by Tau 3D. Tau 3D showed a more profound effect on mitochondrial clumping than
Tau WT and 3A (Fig. 17). These results indicate that phosphorylation of Tau at the AT8 site
is involved in regulation of the mitochondrial movement and distribution along MTs.

Expression of Tau constructs in PC12 cells and cultured cortical neurons
Expression levels of Tau WT, 3A and 3D in PC12 cells and cultured neurons were measured after immunofluorescent staining. The immunostainings of neurons are shown in Figure 18. The staining intensity increased with concentrations of plasmids from 2 μg to 3 μg used for transfection (Fig. 19 and 20). When 2.5 μg of plasmids were used, the fluorescent intensities were ~1.7 and ~1.5 fold of untransfected PC12 cells (Fig. 19) and cultured neurons (Fig. 20), respectively. Expression levels were almost similar among Tau WT, 3A and 3D constructs (Fig. 19 and 20). The effect of Tau on mitochondrial movement was dependent on the amount of Tau plasmid used for transfection. While transfection with less than 2 μg did not significantly affect mitochondrial movement, more than 3 μg had a substantial (and similar) effect for all Tau constructs (data not shown). Differences were observed with 2.5 μg DNA, which was used in subsequent experiments.

**Tau phosphorylation reduces mitochondrial motility in neuritic processes of PC12 cells**

To determine the effect of Tau mutants on mitochondrial movement in parallel bundles of MTs, I first used NGF-treated PC12 cells, which have been previously employed for MT-mitochondria studies (Tatebayashi et al., 2004; Morel et al., 2010). I cotransfected Tau constructs and Mito-GFP into PC12 cells and observed the movement of GFP-labeled
mitochondria in neurite-like processes 3 days after NGF treatment. Mitochondria were distributed throughout the processes of PC12 cells (left panels of Figure 21). Mitochondrial motility was recorded over 300 s in randomly selected processes and examples are shown as kymographs in Figure 21. The number of motile mitochondria was higher in control PC12 cells compared with Tau-overexpressing cells. The percentages of stationary phase of mitochondria, direction of movement, and velocity were quantified (Fig. 22 and 23). The ratio of stationary phase was 23.0% in control PC12 cells but 47.6% in cells expressing Tau WT and 44.1% for 3A; for 3D, however, the percentage was higher still, 61.3% (Fig. 22). Moving mitochondria in neurites overexpressing Tau 3D were significantly fewer than those in Tau WT or 3A-expressing neurites, indicating the stronger inhibitory activity of Tau 3D. Effect of Tau overexpression on anterograde and retrograde movements is shown in Figure 22. Overexpression of each Tau construct specifically decreased the anterograde movement of mitochondria (compare Cont with WT/3A/3D in Figure 22). However, neither Tau 3D nor 3A had further preferential effect on the direction of mitochondrial movement (Fig. 22).

Tau overexpression also affected the velocity of mitochondrial movement in both directions. In control PC12 cells, the velocity was normally distributed with a peak at 0.2–0.3 μm/s (Fig. 23). The mean velocities were around 0.29 ± 0.03 μm/s and 0.31 ± 0.04 μm/s for
anterograde and retrograde movement, respectively. Tau overexpression shifted the peak to less than 0.1 μm/s. The percentages of the total anterograde- and retrograde-moving mitochondria moving less than 0.1 μm/s were 53.3% and 57.7% in Tau WT–expressing PC12 cells, 54.2% and 62.6% in Tau 3A–expressing PC12 cells, and 54.3% and 56.8% in Tau 3D–expressing PC12 cells, respectively. The mean velocities for anterograde and retrograde movement were reduced to 0.19 ± 0.056 μm/s and 0.17 ± 0.035 μm/s by Tau WT overexpression, 0.20 ± 0.02 μm/s and 0.17 ± 0.07 μm/s by Tau 3A overexpression, and 0.22 ± 0.06 μm/s and 0.18 ± 0.04 μm/s by Tau 3D overexpression, respectively. Notably, for Tau 3D, there was a second peak of mitochondrial movement with a velocity of approximately 0.4–0.6 μm/s, particularly in the anterograde direction. Thus, there may be a population of mitochondria that is less affected by Tau 3D. The results that Tau overexpression increased the stationary phase of mitochondria are consistent with previous reports (Trinczek et al., 1999; Thies and Mandelkow, 2007; Stoothoff et al., 2009). My novel finding was that phosphorylation mimic mutation (3D) at the AT8 Alzheimer sites had an additional effect on mitochondrial motility compared with Tau WT and 3A in neuron-like processes of PC12 cells.
Increased pausing and decreased velocity of mitochondria in axons of cultured neurons expressing Tau 3D

Tau is predominantly expressed in neuronal axons. I, therefore, compared the effects of Tau 3D with those of Tau WT or 3A on mitochondrial movement in axons of cultured cortical neurons. Mitochondrial distribution in axons transfected with Tau and Mito-GFP are shown in left panels of Figure 24. During the 300 s observation period, mitochondria exhibited complex motile behavior such as anterograde and retrograde movements with frequent pausing. The duration of stationary phase was 31.6% in control neurons, which was higher than that observed in PC12 cells, consistent with a previous report (Morel et al., 2010). A significant increase in stationary phase was observed after transfection with Tau WT (48.5%) or 3A (46.6%); however, a higher stationary state (64.4%) was caused by Tau 3D (Fig. 25). The stational phase induced by Tau 3D was significantly higher than those induced by Tau WT and 3A, indicating that Tau 3D has stronger inhibitory activity against mitochondrial movement. Direction-dependent inhibition of mitochondrial movement by Tau is summarized in Figure 25. Tau protein dramatically reduced the population of anterograde-moving mitochondria, consistent with previous reports (Stamer et al., 2002; Dixit et al., 2008; Vershinin et al., 2008; Stoothoff et al., 2009). However, the ratio of mitochondria moving
anterogradely and retrogradely was not different among three Tau constructs, indicating that phosphorylation at the AT8 sites does not affect the direction of mitochondrial movements.

Tau overexpression also reduced the velocity of mitochondria in axons. In control neurons, mitochondria showed a peak velocity of 0.2–0.3 μm/s in both directions, and a substantial proportion also moved faster than 0.6 μm/s (Fig. 26). Tau overexpression shifted the peak to a slower rate, with peak velocities of 0.05 to 0.3 μm/s, depending on the Tau construct. The relative ratio of mitochondria with velocities less than 0.3 μm/s in the anterograde and retrograde directions were 31.0% and 38.2% in control neurons, 88.3% and 84.4% in Tau WT–expressing neurons, 86.9% and 82.6% in Tau 3A–expressing neurons, and 84.7% and 84.4% in Tau 3D–expressing neurons. The mean anterograde and retrograde velocities were 0.55 ± 0.08 μm/s and 0.45 ± 0.03 μm/s, respectively, in control cells, but they were reduced to 0.17 ± 0.03 μm/s and 0.18 ± 0.06 μm/s in Tau WT–overexpressing neurons, 0.18 ± 0.03 μm/s and 0.19 ± 0.07 μm/s in Tau 3A–overexpressing neurons, and 0.18 ± 0.03 μm/s and 0.18 ± 0.04 μm/s in Tau 3D–overexpressing neurons. Velocities faster than 1 μm/s, which accounted for 15% and 11% of anterograde and retrograde movements, respectively, in control neurons, were not observed in any Tau-overexpressing neurons. As in PC12 cells, neurons expressing Tau 3D had a small proportion of mitochondria that moved at
approximately 0.5–0.6 μm/s, particularly in the anterograde direction, whereas neurons expressing Tau WT or 3A did not. Thus, Tau with phosphorylation mimic mutation (3D) at the AT8 sites suppressed mitochondrial movement in axons of cultured cortical neurons more than Tau WT or 3A.

The binding of kinesin to MTs is not affected by Tau in any phosphorylation state

Tau impairs mitochondrial movement by inhibiting the interaction of kinesin with MTs (Hagiwara et al., 1994). To delineate whether the reduced mitochondrial movement caused by Tau 3D expression was due to increased inhibition of the motor-MT interaction by Tau 3D, I examined the binding of the kinesin motor domain fragment RK430 to MTs in the presence of Tau WT, 3A, or 3D. I used the head domain of kinesin because the tail domain also has a MT-binding domain, which competes with Tau in *in vitro* MT-binding experiments (Seeger and Rice, 2010). Histidine-tagged kinesin (kinesin-His) that was bound or not bound to MTs was detected by Coomassie staining (Fig. 27-A) and Western blotting (Fig. 27-B) of the MT pellets and supernatants after centrifugation. Increasing the amount of Tau WT or Tau mutants did not alter the binding of RK430 kinesin-His to MTs even though excess Tau was added such that unbound Tau appeared in the supernatant. Thus, the reduced mitochondrial
movement was not caused by inhibiting the interaction between the kinesin head domain and MTs.

The phosphorylation mimic Tau 3D increases MT spacing in processes of Sf9 cells

Tau is a space-making protein between MTs (Chen et al., 1992; Frappier et al., 1994; Marx et al., 2000). I examined the effect of phosphorylation at the AT8 sites on MT spacing in vitro. MTs were polymerized in the presence of Tau and pelleted by centrifugation. Thin-section electron micrographs of MTs cut perpendicularly to the MT axis are shown in Figure 28. The inter-MT distances were measured from wall to wall of nearest-neighbor MTs. The distances were typically less than 10 nm in the MT pellets polymerized with Tau WT, 3A, and 3D (Fig. 28), indicating that the phosphorylation-mimicking mutation at Ser199/Ser202/Thr205 did not affect the inter-MT spacing in pelleted MTs.

To determine if this was the case in cells, I employed an Sf9 cell overexpression system, which was used for Tau-induced MT bundle formation (Kanai et al., 1989; Frappier et al., 1994). I measured the distances between the nearest-neighbor MTs in the MT bundles formed in the process of Sf9 cells after infection of cells with baculovirus encoding Tau. Electron micrographs of processes, in which most MTs were cut perpendicularly, are shown
in Figure 29 (upper panels), and the inter-MT distances are shown in Figure 29 (lower panel).

Of the 85 inter-MT distances measured, there was no significant difference between cells infected with the various Tau proteins (Fig. 29). Because only a small number of processes were formed by Tau overexpression alone, however, the number of inter-MT distances I counted was not sufficient.

I therefore treated Tau-overexpressing Sf9 cells with latrunculin B, which disrupts actin filaments, to increase the number of processes (Knowles et al., 1994). When the processes were stained with anti-Tau antibody, we noticed that Sf9 cells expressing Tau 3D had larger and longer processes than cells expressing Tau WT or 3A (Fig. 30). The mean length was $106.5 \pm 3.7 \mu m$ for Tau WT, $97.2 \pm 5.3 \mu m$ for Tau 3A and $119.1 \pm 4.3 \mu m$ for Tau 3D (Fig. 7N). The mean diameter was $1.09 \pm 0.04 \mu m$ for Tau WT, $1.04 \pm 0.09 \mu m$ for Tau 3A and $1.39 \pm 0.07 \mu m$ for Tau 3D (Fig. 30). I then observed MTs in processes by electron microscopy (Fig. 31). The inter-MT distances were greater in Tau 3D–overexpressing cells compared to those overexpressing WT or 3A (Fig. 31). Although most distances fell in the range of 15–25 nm in Tau WT– and 3A–expressing cells (mean distance = $26.8 \pm 2.7$ nm and $27.2 \pm 2.0$ nm, respectively), almost same as the previous results (Frappier et al., 1994), that range was 35–45 nm in Tau 3D–expressing cells (mean distance =
37.1 ± 2.3 nm) (Fig. 31). Because latrunculin B reduces membrane tension by depolymerizing submembranous actin filaments, these results suggested that, under reduced tension, phosphorylation of Tau at the AT8 sites increases the space between MTs.

**Tau overexpression reduces the inter-MT distances in neurites of PC12 cells**

I wanted to know how expression of Tau 3A or 3D affects the inter-MT distance in axons or neuritic processes. I performed the experiments with PC12 cells, in which mitochondrial movements were affected by Tau expression as was observed in neuronal axons. Typical electron micrographs are shown in Figure 32. More MTs were found in neurites of PC12 cells overexpressing Tau. The inter-MT distances were reduced to ~35 nm in Tau-expressing processes from ~45 nm in the control untransfected processes (Fig. 32). In contrast, there was no significant difference in the inter-MT distances among Tau WT, 3A and 3D overexpression (Fig. 32). The mean distance was 33.1 ± 2.4 nm for Tau WT, 34.1 ± 2.6 nm for Tau 3A and 34.19 ± 2.8 for 3D.
**Discussion**

Tau is a major MAP in axons and plays a role in regulating organelle transport and the dynamics of axonal MTs. Many reports have described the inhibition of mitochondrial transport by overexpressing Tau (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008; Dubey et al., 2008; Stoothoff et al., 2009; Vossel et al., 2010). However, the molecular mechanism has not been determined. I studied the effect of AT8 Alzheimer phosphorylation (Ser199/Ser202/Thr205) of Tau on mitochondrial movement and found that the phosphorylation mimetic form, Tau 3D, inhibited mitochondrial transport to a greater degree than Tau WT and Tau 3A. Based on these findings together with the observation that the inter-MT distance was greater in MT bundles containing Tau 3D, I would like to propose that phosphorylation of Tau at the AT8 sites affects the transport of mitochondria along MTs by changing the inter-MT spaces.

Tau is a phospho-protein with multiple phosphorylation sites; mass spectroscopic analysis indicates ten and five major sites in fetal and adult rat brains, respectively (Watanabe et al., 1993; Morishima-Kawashima et al., 1995; Planel et al., 2002). Major phosphorylation sites are in the Ser-Pro and Thr-Pro sequences, and most of them are in the region flanking the MT-binding domain. Phosphorylation at these sites reduces, but does not abolish, Tau
binding to MTs, leading to more dynamic MTs (Wada et al., 1998; Liu et al., 2008). However, site-specific functions have not been completely investigated. Among the many Ser/Thr phosphorylation sites, Ser199, Ser202, and Thr205, which contain the recognition epitope for the AT8 monoclonal antibody, are particularly interesting. The AT8 sites are not only physiological phosphorylation sites (Kimura et al., 2007; Verwer et al., 2007) but also a marker of hyperphosphorylation in Alzheimer’s disease (Plattner et al., 2006). The Ser199/Ser202/Thr205 sites are present in the border between the N-terminal projection region and the MT-binding region. According to the paperclip structural model of Tau (Jeganathan et al., 2006), the site near Ser199/Ser202/Thr205 folds and the N-terminal region positions close to the MT-binding repeats. When the Ser199/Ser202/Thr205 sites are phosphorylated, the N-terminal domain swings away from the C-terminal domain, resulting in a conformation that extends from the MT wall (Jeganathan et al., 2008). The extended projection may increase the distance between MTs, although this idea has not been validated. By observing MT bundles in Sf9 cell processes treated with latrunculin B, we found that the inter-MT distance in MT bundles containing Tau 3D was longer than that in MT bundles containing Tau WT or Tau 3A.

Tau overexpression inhibits mitochondrial movement in various cell types
(Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008; Stoothoff et al., 2009; Vossel et al., 2010). I also observed an inhibition of mitochondrial movement in PC12 cells and cortical neurons. Tau WT overexpression increased the pausing frequency from 31.6% to 48.5% in neurons, which is almost identical to previous results (Stamer et al., 2002). Overexpression of either Tau WT, 3A or 3D reduced anterograde movement of mitochondria in PC12 cells and cortical neurons, as reported (Mandelkow et al., 2004; Hollenbeck and Saxton, 2005; Dixit et al., 2008). Furthermore, the velocity was reduced similarly in both directions by Tau overexpression regardless of its phosphorylation state, although the inhibition profile differed slightly among the three Tau constructs. Thus, Tau clearly inhibits mitochondrial transport independent of its phosphorylation state. Several models have been proposed for Tau-mediated inhibition of mitochondrial transport: overstabilization of MTs (Shemesh et al., 2008), competition between motor proteins for interaction with the MT surface (Hagiwara et al., 1994), and inhibition of motor protein access to MTs (Seeger and Rice, 2010). Another mechanism proposed recently involves the distance between MTs (Thies and Mandelkow 2007). When Tau is overexpressed in cortical neurons, tubulin synthesis is upregulated, and MTs become more numerous and densely packed, resulting in inhibition of mitochondrial movement. This observation was in dendrites,
but similar Tau overexpression–induced increases in MTs were reported in axons (Sudo and Baas, 2010). We observed here that Tau overexpression increased the number of MTs and reduced the inter-MT spaces in neurites of PC12 cells. My observation is consistent with the last model described above. Limited spacing between MTs may block mitochondrial movement in neuritic processes such as axon and dendrites.

I found that Tau 3D more potently inhibited mitochondrial transport than Tau WT or Tau 3A. Phosphorylation of Tau at the AT8 sites has an additional inhibitory action on mitochondrial movement over the Tau molecule itself. I hypothesize that the inhibition caused by Ser199/Ser202/Thr205 phosphorylation relates to the distance between MTs. The inter-MT distance was the same (15-25 nm) in MT bundles formed by Tau WT, 3A, and 3D in Sf9 cell processes, but it was expanded to 35-45 nm in processes expressing Tau 3D when actin filaments were disrupted by latrunculin B. Actin filaments are abundant in submembranous regions, providing tension to plasma membranes. As reported (Knowles et al., 1994), disassembly of actin filaments increases the number of processes induced by Tau overexpression. Phosphorylation-dependent expansion of the space between MTs was observed only with reduced membrane tension. The force produced by outward extension of the projection domain may not be strong enough to push surrounding MTs against the
membrane, which may explain why phosphorylation-induced expansion of the inter-MT distance has not been reported.

Axons are long processes that extend ~1 m or more. To maintain axonal structures to over 80 years in humans, the axoplasm is filled with cytoskeletal components, and the outer surface is surrounded by thick myelin. These features may indicate that the axoplasmic MT milieu is under the strong tension (Yu and Baas, 1994; Rochlin et al., 1996). Phosphorylation of Tau at the AT8 sites tends to increase the inter-MT distance, but under strong tension the inter-MT distance cannot expand (Fig. 32), and instead, the repulsive forces between MTs increase (Fig. 33). Increased repulsive forces between MTs would generate a stronger reactive resistance against mitochondria moving inside MT bundles (Yu and Baas, 1994). The AT8 sites are physiological sites for phosphorylation, but they are not always phosphorylated (Kimura et al., 2007; Verwer et al., 2007). The AT8 sites may be interconverted between phosphorylated and dephosphorylated states depending on the cell’s need for mitochondrial movement. Dephosphorylation ahead of moving mitochondria would reduce the repulsive force between adjacent MTs for mitochondrial passage, and rephosphorylation of Tau behind mitochondria may facilitate the directional movement of mitochondria (Shahpasand et al., 2008). Of course, phosphorylation-dependent tunnel opening and closing for mitochondrial
movement is expected to be coordinated with activities of motor proteins, protein kinases, and protein phosphatases. This is my working hypothesis, which I will explore further in the future.

I used PC12 cells and cultured cortical neurons in this study, in which MTs are major cytoskeletal components. In matured or aged neurons, however, Tau may not be the only space making protein that affects mitochondrial movement in a phosphorylation-dependent manner. The C-terminal tail domains of neurofilament M and H subunits extrude outward from core filaments, as does the projection domain of Tau, to make spaces between neurofilaments (NFs) (Hisanaga and Hirokawa, 1989). NFs are highly phosphorylated in aged axons and AD neurodegenerative disease (Uchida et al., 2004; Rudrabhatla et al., 2010). Hyperphosphorylation of the tail domains would increase the inter-NF distances to expand the NF domain in axons (Kumar et al., 2002; Kanungo et al., 2011), giving higher pressure to the MT domain. This would suppress mitochondrial transport along MTs by restricting radial displacement of MTs. Thus, mitochondrial movements in aged and neurodegenerative axons would be affected by a more complicated manner.

AT8 reactivity has been frequently used as an indicator of hyperphosphorylation of Tau in AD brains or other Tauopathies (Stoothoff and Johnson, 2005; Hanger et al., 2009).
Impairment of mitochondrial traffic is also a feature of Tauopathies (Stokin et al., 2005; Lippens et al., 2007). An unanswered issue is whether abnormal Tau phosphorylation is caused by impaired organelle trafficking or if blocked transport is a consequence of abnormal phosphorylation. Using the phosphorylation mimic Tau 3D, I showed that Tau phosphorylation within the AT8 sites inhibited mitochondrial transport more effectively than in experiments carried out with Tau WT and Tau 3A. Because Tau 3A and 3D have similar phosphorylation profiles at other sites, the observed effect is likely caused by phosphorylation at Ser199/Ser202/Thr205. Thus, my results suggest that the increased phosphorylation of the AT8 sites in brains of Alzheimer’s patients decreases mitochondrial transport in axons, leading to axonal degeneration. My current study not only leads us to focus on the AT8 sites with regard to Alzheimer’s therapeutics but also indicates the effectiveness of a similar strategy addressing other abnormal phosphorylation sites on mitochondrial movement.
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Figure legends

Figure 1. Schematic representation of axonal mitochondrial transport. It is being driven by two oppositely motor proteins and microtubules play a role as a track for the transport.

Figure 2. Tau is a filamentous protein provided from a single gene through different splicing and comprises of six isoforms in adult human brain.

Figure 3. Schematic representation of inter-microtubule spaces rearrangement for movement of mitochondria.

Figure 4. Proposed regulation of MAP polymer brush by phosphorylation and dephosphorylation. Dephosphorylation reduces the intramolecular repulsion, causing the projection domains to become more compact and allows the microtubules to move closer. Phosphorylation of the projection domains causes the projection domains to expand due to an increase in intramolecular repulsion. This in turn causes the distance between adjacent microtubules to increase.
Figure 5. Schematic representation of the longest human isoform of Tau composed of 441 amino acid residues. Tau consists of the N-terminal projection domain and C-terminal MT-assembly domain including four MT-binding repeats (R1–R4). Two proline-rich regions (P1 and P2) are present in the middle region. The Ser199/Ser202/Thr205 sites are located in the N-terminal side of P2 at the border of the projection domain and MT-binding domain. This molecular structure was drawn based on the description by Jeganathan et al. (2006). Amino acids that can be phosphorylated were replaced with Asp or Ala to generate the phosphorylation mimic (3D) or nonphosphorylated (3A) Tau constructs, respectively.

Figure 6. Change in global conformation of Tau upon phosphorylation. A, phosphorylation at AT8 sites (S199E _ S202E _ T205E) loosens the paperclip structure by swinging the N-terminal domain away from the C-terminal domain. B, phosphorylation at PHF1 sites (S396E _ S404E) also loosens the paperclip structure by swinging the C-terminal tail away from the repeats. C, double phosphorylation of AT8 _ PHF1 sites tightens the paperclip structure by swinging the N-terminal domain toward repeat domain. D, combined phosphorylation at AT8* _ AT100 _ PHF1 sites tightens the paperclip structure by swinging both the N- and C-terminal domains toward the repeat domain.
Figure 7. Immunostaining of human nervous system tissues with monoclonal antibody AT8. A section of 8-week (gestational age) spinal cord is shown in A and a section through the subiculum of the hippocampus from an Alzheimer disease patient is shown in B. Note the white matter staining in A and the staining of neurofibrillary tangles, neuropil threads, and the senile plaque neurites in B.

Figure 8. SDS-PAGE gel electrophoresis indicating of Tau purification using its heat-stable nature. See Material & Methods section for further details.

Figure 9. SDS-PAGE gel electrophoresis indicating of tubulin purification through repetitive cycles of depolymerization and reassembly followed by anion-exchange chromatography. Right side bands indicating of protein concentration as standard reference.

Figure 10. Cdk5-dependent *in vitro* phosphorylation at the AT8 sites of Tau WT, but not Tau 3A or 3D. Tau WT, 3A, and 3D were phosphorylated *in vitro* with Cdk5-p25. Immunoblots were performed with anti-human Tau (h-Tau) or AT8.
Figure 11. Phosphorylation at the AT8 sites of Tau WT, but not 3A or 3D, in COS-7 cells. Tau WT, 3A, or 3D was expressed in COS-7 cells. Cell lysates were subjected to immunoblotting for h-Tau or AT8.

Figure 12. The lower panel shows evaluation of phosphorylation state-dependent mobility shifts of Tau WT, 3A, or 3D expressed in COS-7 cells by Phos-tag SDS-PAGE. Tau WT, 3A, and 3D separated by Phos-tag SDS-PAGE were immunoblotted with anti-human Tau.

Figure 13. Alignment of Tau 3A and 3D to compare their banding patterns in Phos-tag SDS-PAGE. Phos-tag immunoblots of Tau 3A and 3D in (C) were aligned by adjusting the fastest moving band.

Figure 14. Tau 3D takes conformation similar to phosphorylated Tau WT. After in vitro phosphorylation of Tau constructs with Cdk5-p25, dot blots were performed with phosphorylation-independent antibody h-Tau (upper panel) and with phosphorylation-dependent antibodies, MC-1 (middle panel) and Alz-50 (lower panel).
Figure 15. Colocalization of Tau WT, 3A, or 3D to MTs in COS-7 cells. Tau WT, 3A, or 3D vectors were used to transfect COS-7 cells. Soluble Tau and tubulin were removed by treatment with PEM containing 4 M glycerol and 0.5% Triton X-100 for 10 min at 37°C, and cells were double-stained with anti-tubulin (green in middle panels) and anti-Tau (red in left panels), followed by fluorescently labeled secondary antibodies. Merged images are shown in the right panels. Bar, 20 μm.

Figure 16. Mitochondrial distribution in control COS-7 cells. COS-7 cells were incubated in the presence of anti-TOM20, followed by Alexa 546–conjugated secondary antibody. The cell membrane is indicated by a dotted white line. Mitochondrial distribution as shown by Mito-GFP fluorescence. Mitochondrial distribution in COS-7 cells expressing Tau WT (C–E), Tau 3A (F–H), or Tau 3D (I–K). COS-7 cells were cotransfected with Mito-GFP and Tau WT, 3A, or 3D, and 24 h after transfection mitochondrial distribution was examined with Mito-GFP (D, G, and J). Tau was stained with anti-human Tau (C, F, and I). Merged images are shown in E, H, and K. Blue in merged images is DAPI staining. Bar, 20 μm.

Figure 17. Quantification of mitochondrial distribution in COS-7 cells. The area of the cell
occupied by mitochondria was measured with Image J software and expressed as the percent of the total cell area (mean ± SEM, n = 20 for each Tau construct, Student’s t-test, * P < 0.01; **P < 0.001).

Figure 18. Immunostaining of cortical neurons overexpressing Tau with h-Tau. Plasmids encoding Tau at concentrations of 2, 2.5 and 3 μM were co-transfected with EGFP vector into cultured cortical neurons at DIV6, and these neurons were stained at DIV11. Transfected axons are indicated with arrowhead and un-transfected axons with arrow. Bar, 5 μm.

Figure 19. Quantification of Tau expressed in PC12 cell neurites (n = 20 for each of PC12 cells and neurons, *, p < 0.01, one-way ANOVA).

Figure 20. Quantification of Tau expressed in axon of cortical neurons. (n = 20 for each of PC12 cells and neurons, *, p < 0.01, one-way ANOVA).

Figure 21. Mitochondrial distribution and movements in neurite process of PC12 cells. PC12 cells were transfected with a Mito-GFP vector alone as a control (Cont) or cotransfected with
a Mito-GFP and either Tau WT, 3A or 3D vectors. PC12 cells were treated with NGF for 72 h after transfection. Mitochondrial distribution is shown by fluorescence of Mito-GFP (left panels). Right panels are kymographs of mitochondria moving in a process of a PC12 cell. Bar, 20 μm.

Figure 22. Upper graph indicates of the percent ratio of pausing mitochondria in PC12 cell processes. Mitochondrial movement was recorded in the neurite-like processes at 5 s intervals over a 300 s period. Any mitochondrion that translocated at least 0.1 μm between two image frames was considered to be moving. The pausing time was expressed as the percentage of the total observation time (n = 35, *, p < 0.01, one-way ANOVA). Lower graph shows effect of Tau WT, 3A, or 3D on anterograde or retrograde movement of mitochondria. The vertical axis indicates the ratio of anterogradely moving time to total moving duration. Statistical analysis was performed by ANCOVA as described in Materials and Methods. The ratio was significantly different between control and Tau-overexpressing PC12 cells (*, p < 0.01), but was not significant between three Tau constructs (p = 0.75).
Figure 23. Effect of Tau WT, 3D, or 3A on the velocity of mitochondria. Relative frequency of mitochondria moving at the indicated velocities in the anterograde (black) or retrograde (gray) direction for control, WT, 3A, or 3D. n = 35 mitochondria per sample.

Figure 24. Mitochondrial distribution and movements in axons of neurons. Cultured neurons at DIV6 were transfected with Mito-GFP vector alone (Cont), or cotransfected with Mito-GFP and either Tau WT, Tau 3A or Tau 3D vectors. Mitochondrial distribution is shown by fluorescence of Mito-GFP (left panels). Bar, 20 μm. Right panels are kymographs of mitochondria moving in axon of neurons.

Figure 25. Upper graph shows the percent ratio of pausing mitochondria in axon of cultured neurons. The pausing duration was expressed as the percentage of total observation period (n = 35, *, p < 0.01, one-way ANOVA). Lower graph indicates the effect of Tau mutants on anterograde or retrograde movement of mitochondria. The vertical axis is the ratio of anterogradely moving duration to total moving period. The results were analyzed statistically by ANCOVA as described in Materials and Methods. The ratio was significantly different between control and Tau-overexpressing neurons (p < 0.01), but was not different between
three Tau constructs ($p = 0.55$).

Figure 26. Effect of Tau mutants on the velocity of mitochondria. Relative frequencies of mitochondria moving at the indicated velocities are shown in control neurons or in neurons expressing Tau WT, Tau 3A, or Tau 3D. Black represents anterograde movement, and gray represents retrograde movement (n = 35 mitochondria per sample).

Figure 27. Tau does not inhibit the binding of kinesin to MTs. (A) Coomassie staining of an SDS-PAGE gel to show the binding of kinesin to MTs independent of Tau-binding. MTs polymerized in the presence of 0.05, 0.1 or 0.15 μM of Tau WT, 3A or 3D were incubated with 0.1 μM kinesin head domain-His, and after separation of MTs by centrifugation, the MT pellet (p) and supernatant (s) were subjected to SDS-PAGE. The right side three lanes are tubulin, Tau WT and kinesin head domain-His, respectively, for references. (B) An immunoblot confirming the binding of kinesin to MTs. MT pellet (p) and supernatant (s) shown in (A) are immunoblotted with anti-His antibody for detection of kinesin head domain-His.
Figure 28. Electron micrographs of cross-sections of MT pellets polymerized *in vitro* with Tau WT (A), 3A (B), or 3D (C). Bar, 100 nm. The wall-to-wall distances between nearest-neighbor MTs were measured and expressed as the percent of the total number of counted MTs. The mean distance was $11.7 \pm 1.7$ nm for Tau WT ($n = 48$), $11.8 \pm 2.1$ nm for Tau 3A ($n = 51$), and $11.9 \pm 1.6$ nm for Tau 3D ($n = 69$).

Figure 29. Electron micrographs of processes of Sf9 cells (untreated (-)) expressing Tau WT (E), 3A (F), or 3D (G). Inter-MT distances were measured between nearest-neighbor MTs and expressed as the percent of the total number of counts. The mean distance was $15.7 \pm 2.2$ nm for Tau WT ($n = 30$), $17.5 \pm 2.7$ nm for Tau 3A ($n = 27$), and $16.3 \pm 2.2$ nm for Tau 3D ($n = 28$).

Figure 30. Immunostaining of Sf9 cells overexpressing Tau 3D (a), Tau 3A (b), or WT (c) with anti-Tau. Bar 20 μm. Graphs showing that Sf9 cells overexpressing Tau 3D formed longer and wider processes compared to those expressing Tau WT or 3A. Left graph indicates the length distribution of Sf9 cell processes overexpressing Tau constructs. The mean length was $106.5 \pm 3.7$ μm for Tau WT, $97.2 \pm 5.3$ μm for Tau 3A and $119.1 \pm 4.3$ μm for Tau 3D ($n$}
= 20 for each Tau construct). Right graph shows the width distribution of Sf9 cell processes overexpressing Tau constructs. The mean width was 1.09 ± 0.04 μm for Tau WT, 1.04 ± 0.09 μm for Tau 3A and 1.39 ± 0.07 μm for Tau 3D (n = 20 for each Tau construct).

Figure 31. Electron micrographs of processes of latrunculin B–treated (+) Sf9 cells expressing Tau WT (I), 3A (J), or 3D (K). Sf9 cells were treated with 0.5 μg/ml latrunculin B for 72 h after infection with baculovirus expressing each Tau. Inter-MT distances were measured and expressed as the relative ratio of the total number of counts. The mean distance was 26.8 ± 2.7 nm for Tau WT (n = 60), 27.2 ± 2.0 nm for Tau 3A (n = 80), and 37.1 ± 2.3 nm for Tau 3D (n = 75).

Figure 32. The distance between MTs in PC12 cell neurites overexpressing Tau constructs. Cross-sectional electron micrographs of PC12 cell neurites, control or overexpressing either Tau WT, 3A or 3D are shown in the upper pannels. Bar, 100 nm. Inter-MT distances were measured between nearest-neighbor MTs and expressed as the percent of the total number of counts. The mean distance was 45.1± 2.2 (n = 54) for control PC12 cells and 33.1 ± 2.4 nm (n
= 49), 34.1 ± 2.6 nm (n = 44) and 34.19 ± 2.8 nm (n = 62) for PC12 cells overexpressing Tau WT, 3A and 3D, respectively.

Figure 33. Schematic representation of the greater impact of Tau phosphorylation on mitochondrial transport compared to nonphosphorylated Tau. (A) Tau is a space-making protein attached to the MT surface. When Tau is not phosphorylated at Ser199/Ser202/Thr205, the N-terminal domain is folded. This conformation does not produce much resistance force against mitochondrial movement because MTs are in relatively close proximity. (B) Tau phosphorylation at Ser199/Ser202/Thr205 may extend the projection domain away from the MT surface, increasing the repulsive forces between MTs. In this conformation, mitochondria encounter greater MT resistance, to the slowing or arresting their movement.
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Figure 29

Sf9 (-)

E  F  G

WT  3D  3A

Percentage (%)
Figure 31
Figure 32
Figure 33
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