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ORIGINAL PAPER

Disruption of microtubule network by Alzheimer abnormally hyperphosphorylated tau

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Abstract Hyperphosphorylated tau has long been proposed as the key molecule disrupting normal neuronal microtubule dynamics and leading to neurofibrillary degeneration in Alzheimer disease. Here we provide a direct evidence of hyperphosphorylated tau-induced disruption of microtubule network. Using Nocodozole-treated and detergent-extracted cells, we created a neuronal environment in mouse embryonic fibroblasts, 3T3 cells, by replacing their cytoplasm with adult rat brain cytosol. By recreating neuronal microtubule network in these cells, we were able to follow the effects of hyperphosphorylated tau on microtubule dynamics in real time. Whereas recombinant human brain tau promoted assembly and bundling of microtubules, abnormally hyperphosphorylated tau isolated from Alzheimer disease brain cytosol (AD P-tau) inhibited the assembly and disrupted preformed microtubule network by sequestering normal brain tau and MAP2. This breakdown of the microtubule network was reversed by treatment of the extracted cells with protein phosphatase-2A. This study, for the first time, provides direct mechanistic insights into the molecular basis of both axonal and dendritic neurodegeneration seen in Alzheimer disease.

Keywords Alzheimer disease · Retrograde neurodegeneration · Detergent extracted cells

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Abbreviations

AD	Alzheimer disease
MT	Microtubules
MAP	Microtubule associated protein
AD P-tau	AD brain cytosolic abnormally hyperphosphorylated tau

Introduction

Alzheimer's disease (AD) is characterized by profound central neurodegeneration. While it is a recurring theme in many neurological and neuro-psychiatric disorders, the precise mechanisms leading to the loss of neuronal mass have varying etiologies. In neurofibrillary degeneration of Alzheimer type, the affected neurons exhibit a particular type of pathology that evolves in a distal-to-proximal manner. This so-called, retrograde degeneration begins distally in the axo-dendritic network disrupting normal synaptic activity. The basis for retrograde degeneration can be explained by the presence of altered microtubule (MT) dynamics.

MTs play an essential role in maintaining the structural and physiological integrity of neurons. Neurodegenerative disorders, including AD, show evidence of cytoskeletal dysfunction at both cytoarchitectural and molecular levels. Changes in neuronal shape, loss of dendrites and dendritic spines and abnormal accumulation of cytoskeletal proteins either in the form of inclusions like neurofibrillary tangles of paired helical filaments (PHF) seen in AD [24], or Lewy bodies seen in Parkinson's disease [29], constitute evidence of cytoarchitectural abnormalities. These neurons with anatomical aberrations show impaired function, which is seen as loss of synapses, decrease in synaptic conductivity and synaptic plasticity. This places the neuronal cytoskeleton as a key target for research and drug development.

In the normal brain, MT assembly is stimulated by a number of microtubule-associated proteins (MAP), the high molecular weight MAP1 and MAP2, as well as the 50–75 kDa tau proteins [37, 39]. It was previously reported by us that in AD, tau is abnormally hyperphosphorylated and aggregated into bundles of paired helical filaments (PHFs), which progressively replace the neuronal cytoskeleton [20, 21]. In situ and in vitro observations suggest that the cytosolic/non-fibrillized abnormally hyperphosphorylated tau is inhibitory to MT assembly and stability [1, 2, 3, 25, 31], whereas fibrillized tau is not [5, 9]. Moreover, only hyperphosphorylated tau, and neither MAP1 nor MAP2, has been shown to accumulate in the affected neurons in AD and related tauopathies. While it is desirable to study the effects of tau hyperphosphorylation on MT function in an in vivo system, there are several limitations surrounding direct and isolated manipulation of tau phosphorylation and its effect on MT dynamics in the living cell. Both genetic and pharmacologic means of studying mechanisms of neurofibrillary degeneration have several pitfalls. Transfected cell lines and transgenic animals overexpressing wild type, pseudophosphorylated or mutated tau do not reflect the pathology in AD in which neither any causative tau gene mutation nor increase in tau messenger RNA has been found [15, 32]. Modifying kinase and phosphatase levels/activities in the cell to achieve tau hyperphosphorylation [13] affects several other pathways in the cell [6, 10, 12, 14, 23, 40] and may not truly reflect the disease state. Consequently, the mechanisms proposed for the apparent toxicity of hyperphosphorylated tau have not yet been identified in the cell and therefore remain unestablished.

Detergent extraction of cells has been extensively employed in the literature to study components of the cytoskeleton [7]. When cells are treated with a non-ionic detergent, such as Triton X-100, approximately 70% of the cytosolic proteins are extracted [30]. Such detergent-extracted cells retain many of the morphological features of the cytoskeleton seen in intact cells, including microfilaments, microtubules, intermediate filaments and associated proteins [16, 35].

Here, we describe a model in which we disrupted the MT network of 3T3 cells (mouse embryonic fibroblasts) with Nocodazole (Noco), followed by removal of the cytoplasm by extraction with Triton X-100 (Tri), and replaced the MT network of the host cells (Noco/Tri cells) with that polymerized from adult rat brain cytosol. The MT network formed from rat brain tubulin, MAP2 and tau created a neuron-like environment in which we could directly study the effects of AD P-tau in real time. We found that AD P-tau causes breakdown of MT network by sequestering both tau and MAP2. A compromised MT network both in the axons and dendrites might be the basis of the progressive retrograde degeneration of the affected neurons seen in AD and related tauopathies.

Materials and methods

Antibodies

The following primary antibodies were used: DM1A, (1:2000; Sigma, St Louis, MO, USA), a mouse monoclonal antibody to α tubulin; YL1/2, (Harlan Sera-Lab, Leicestershire, UK), a rat monoclonal antibody to tyrosinated/dynamic-tubulin ; anti- Glu-tubulin, (1:500, a gift from A del C. Alonso), a rabbit polyclonal antibody to detyrosinated tubulin; 134d, (1:1000 for immunocytochemistry, 1:5000 for Western blots), a rabbit polyclonal antibody to total tau [11]; SMI52, (1:200; Sternberger Monoclonals, MD, USA), a mouse monoclonal antibody to MAP2; Tau-1, (1:100, a gift from L. Binder), a mouse monoclonal antibody to un-phosphorylated tau; pSer 422, (1:100, raised in our laboratory), a rabbit polyclonal antibody to phosphorylated tau at serine 422; PHF-1, (1:5000, a gift from P. Davies), a mouse monoclonal antibody to phosphorylated tau at serine 396 and serine 404; M4, (1:2000, a gift from Y. Ihara), a mouse monoclonal antibody to phosphorylated tau at threonine 231 and serine 235; 12E8, (1:2000, a gift from D. Schenk), a mouse monoclonal antibody to phosphorylated tau at serine 262 and serine 356. The following secondary antibodies were used: Alexa 488-conjugated goat anti-mouse IgG antibody and Alexa 594-conjugated goat anti-rabbit or anti-rat IgG antibody (Molecular probes, Carlsbad, CA, USA); horseradish peroxidase-conjugated anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA).

Preparation of rat brain cytosol

Adult Wistar rats (Charles River Laboratories, Wilmington, MA, USA), 3–5 months old, were used to prepare 61,000 g brain cytosolic extract as described previously [4]. Briefly, the brain was homogenized in equal volume (*w*/*v*) of chilled PEM buffer (100 mM PIPES, 1 mM EGTA, 2 mM $MgCl_2$ at pH 6.9), containing a cocktail of protease inhibitors (4 μg/ml Aprotinine, 4 μg/ml Leupeptin, and 1 μg/ml Pepstatin). The homogenate was centrifuged at 61,000*g* at 4°C for 30 min and the supernatant was referred as the cytosol.

Preparation of Noco/Tri 3T3 cells

Mouse embryonic fibroblast (3T3) cells were plated on poly-D-lysine coated 8-well culture slides (BD Biosciences, Bedford, MA, USA) and cultured in DMEM supplemented with 10% neonatal calf serum (both from

Invitrogen, Carlsbad, CA). Upon reaching ~50% confluence, the cells were washed in PEM buffer and either extracted with 0.2% Triton X-100 for 1 min at 37°C or pretreated with 10 μM nocodazole (ICN Pharmaceuticals, Casta Mesa, CA, USA) at 37°C for 4 h to depolymerize endogenous MTs before detergent extraction. The extracted cells, thus obtained were referred to as Noco/Tri cells.

Isolation of recombinant normal human tau and AD P-tau

Recombinant human brain tau_{410} was expressed in the prokaryotic expression vector pRK172 and was purified as described previously [36], except that the perchloric acid extraction was avoided. AD P-tau was isolated from frozen autopsied AD brains, obtained within 6 h postmortem, as described [26] and further purified by Sepharose G-column chromatography (Amersham Bioscience, Arlington Heights, IL, USA).

Microtubule Assembly in Noco/Tri cells

Noco/Tri cells were rinsed with PEM buffer, and incubated with rat brain cytosol in the presence of 1 mM GTP at 37°C to study microtubule dynamics. Where indicated, recombinant tau_{410}or AD P-tau (100 μg/ml) was added and the extracted cells were analyzed for MT assembly by immunocytochemistry.

Dephosphorylation of AD P-tau by protein phosphatase-2A

Dephosphorylation of AD P-tau was carried out as described previously [19, 38]. Briefly, AD P-tau (0.2 mg/ml) was incubated in a mixture of 50 mM Tris, pH 7.0, 20 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 1.0 mM $MnCl_2$, 10 μg/ml each of leupeptin, pepstatin and aprotinin, and 1.0 U/ml protein phosphatase-2A at 37°C for 45 min. Protein phosphatase-2A holoenzyme was purified from bovine brain as described previously [38]. For Western blot analysis, the reaction was stopped by the addition of 2 × SDS-PAGE sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The blots were developed with the primary antibodies 134d, Tau-1, PHF-1, M4 and 12E8, followed by incubation with anti-mouse IgG conjugated with horseradish peroxidase and ECL detection reagents (Amersham Biosciences Corp., Piscataway, NJ).

Immunocytochemistry

After treatment, the extracted cells were fixed in a mixture of 0.3% glutaraldehyde and 0.5% NP-40 in PEM buffer at 37°C for 10 min, washed with PBS and treated with 10 mg/ml sodium borohydride (Sigma-Aldrich, St Louis, MO) for 7 min. The extracted cells were then incubated with 0.1 M glycine in PBS for 20 min, washed with PBS and blocked with 4% horse serum for 1 h at 37°C. The cells were incubated with the appropriate primary antibodies overnight at 4°C. Next morning, after extensive washing with PBS, the cells were incubated in the blocking solution for 30 min at room temperature and then incubated with Alexa 488-conjugated anti-mouse antibody and Alexa 594-conjugated anti-rabbit or anti-rat antibody simultaneously, each at a final dilution of 1:1000, for 1 h at room temperature. Images were captured with Nikon PCM 2000 Confocal Imaging System.

Analysis of microtubule assembly by Western blot

Rat brain cytosol (15%) was incubated in 100 μl of PEM buffer containing 1 mM GTP at 37°C for 1 h in the presence or absence of normal or AD P-tau. The reaction mixture was then centrifuged at 5,600*g*, at 37°C for 10 min to separate assembled MTs. The supernatant and the MT pellets were then lysed in 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol, followed by bath sonication and boiling for 5 min. Indicated volumes of supernatant and dissolved pellets were subjected to SDS-polyacrylamide gel electrophoresis and employed for Western blots on PVDF membranes as described previously [20]. The blots were developed with the primary antibody anti-α tubulin (DM1A), followed by incubation with anti-mouse IgG conjugated with horseradish peroxidase and ECL detection reagents (Amersham Biosciences Corp., Piscataway, NJ).

Results

MT assembly in Noco/Tri cells

We employed 3T3 cells to study microtubule network assembly in intact cells and after extraction with Triton X-100. Compared to the intact cells (Fig. 1a–c), Triton-extracted cells displayed remarkable preservation of the microtubule network (Fig. 1d). As previously reported [8], the MT in intact cells mainly contained tyrosinated (Tyr) tubulin, a form of tubulin found predominantly in dynamic assemblies of MT. We used detergent extraction in Nocodazole treated 3T3 cells to obtain a relevant "platform" on which to study MT dynamics in the cellular context. After extraction with Triton X-100, majority of the unpolymerized tubulin was removed, while some free tubulin could be seen attached to the detergent-resistant platform of the Noco/Tri cells (Fig. 1f). Next, rat-brain cytosol was used as a source of tubulin to form MT on Noco/Tri cells (Fig. 1g–k). Only minimal MT assembly was observed at concentrations of 5–10% cytosol (Fig. 1g, h), whereas MTs readily assembled in the presence of higher concentrations of the

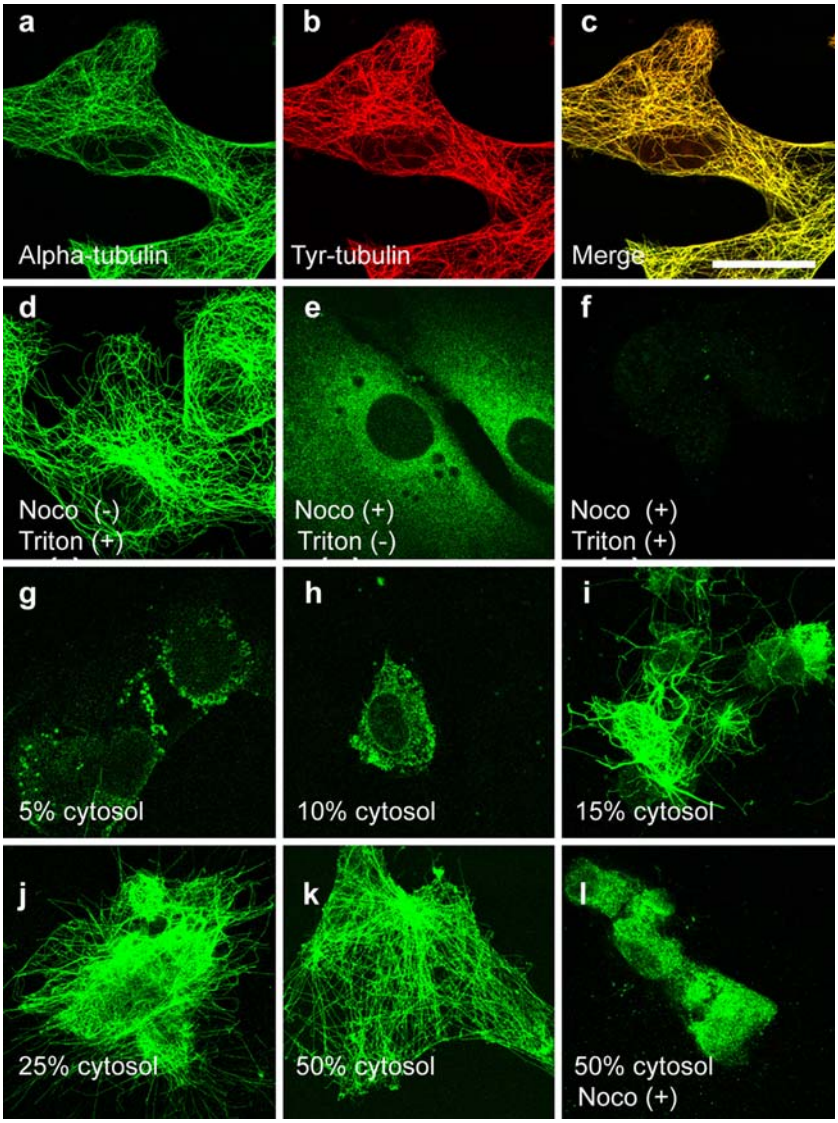

Fig. 1 Microtubule assembly in Noco/Tri cells. 3T3 cells were extracted with 0.2% Triton X-100 and used as a "cellular platform" for MT assembly. **a–c** Microtubule network in intact 3T3 cells. **d–f** detergent extracted cells with or without pretreatment with Nocodazole. MT structure is faithfully preserved in detergent extracted 3T3 cells (**d**). Pretreatment with Nocodazole destroys endogenous MT (**e**) and its components are largely lost on detergent extraction (**f**). Rat brain cytosol, at various concentrations (5, 10, 15, 25 or 50%) was added to Nocodazole pre-treated, detergent extracted 3T3 cells (Noco/Tri cells) in the presence of 1 mM GTP to study MT assembly (**g–k**). Nocodazole inhibited MT assembly when added to the rat brain cytosol on Noco/Tri cells (**l**). Alpha tubulin (DM1A, *green*), tyrosinated tubulin (YL1/2, *red*). Scale bar represents 25 μm

extract (Fig. 1i–k). Furthermore, similar to intact cells, Nocodazole treatment inhibited MT assembly in Noco/Tri cells and free tubulin could be seen attached to the Noco/Tri cells (Fig. 1l).

To investigate the dynamic course of MTs in Noco/Tri 3T3 cells, the time course of MT assembly was studied at different time intervals (from 1 min to 4 h) after incubation of the extracted cells with 25% rat brain cytosol in the presence of 1 mM GTP at 37°C. Microtubule-organizing center (MTOC)-like structures were evident as early as 1 min post incubation. Assembled MTs were observed growing from MTOC-like structures by 10 min and were stable up to 4 h (Fig. 2a).

To study the time course of MT disassembly, MTs were first assembled in Noco/Tri cells in the presence of 25% rat brain extract containing 1 mM GTP for 20 min. The extracted cells were then washed and incubated in PEM buffer, and fixed at different time intervals to study the time course of disassembly. Almost all MTs depolymerized by 1 h in this experimental paradigm (Fig. 2b).

Tubulin undergoes a unique post-translational modification in which a tyrosine is either attached to its C-terminal (glutamate) by a specific tubulin tyrosine ligase or is removed by tubulin carboxypeptidase. The generation of Glu-tubulin is a post-polymerization event in which detyrosination of some Tyr-MT forms Glu-MT as early as 20–25 min of polymerization. Consequently, Glu-tubulin predominates in stable assemblies of MTs present in differentiated cells such as neurons [22]. More than 75% of α tubulin in the adult mammalian brain is believed to be Glu-tubulin [33]. We found that Glu-tubulin represented the major tubulin component of assembled MT in Noco/Tri cells (Fig. 3a). MAPs are known to differ in their abilities to stimulate MT assembly; MAP2 promotes higher rates and higher levels of MT assembly and nucleation than tau [34]. We found that MAP2 predominated in assembled MT, with

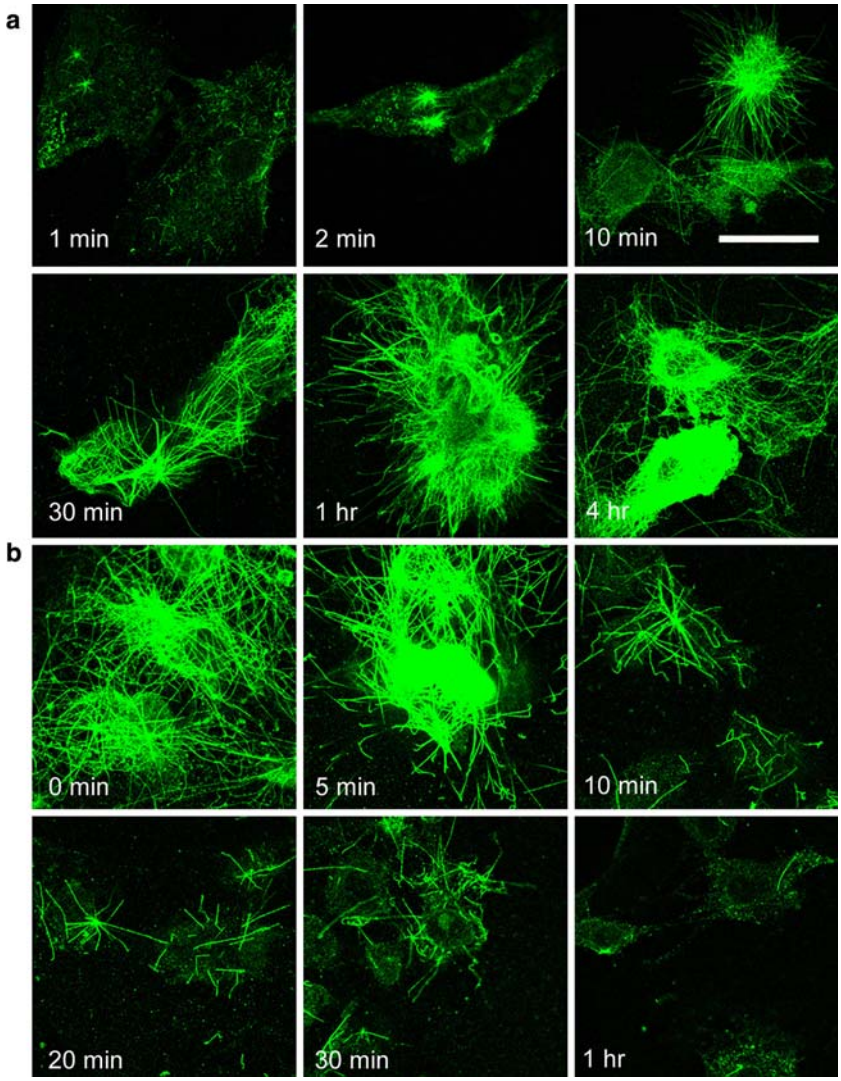

Fig. 2 Time course of MT assembly/disassembly in Noco/Tri cells. 3T3 cells were pretreated with 10 μM nocodazole prior to detergent extraction. Rat brain cytosol (25%) was then applied to the Noco/Tri cells in the presence of GTP to study MT assembly at various time points (**a**). Microtubule organizing center (MTOC)-like structures could be detected as early as 1 min after incubation with adult rat brain cytosol, whereas by 10 min, MT growth from MTOC could be observed. Assembled MTs were stable for up to 4 h studied. **b** To study disassembly, MTs were first assembled in Noco/Tri cells as described above for 20 min. The cells were then washed, incubated in PEM buffer, fixed at different time points, immunostained and analyzed by confocal microscopy. By 1 h, almost all MTs were disassembled. Anti-alpha tubulin (DM1A) was used for immunocytochemical staining. Scale bar represents 25 μm

tau co-staining with relatively fewer MT in Noco/Tri cells (Fig. 3b).

Effect of AD P-tau on MT assembly

To study the effect of AD P-tau on MT network, we added AD P-tau or as a control recombinant tau_{410} (100 μg/ml each) to rat brain cytosol and incubated Noco/Tri cells with the reaction mixture for 1 h at 37°C in the presence of phosphatase inhibitors (100 nM okadaic acid and 25 mM sodium floride). No MTs assembled in the presence of AD P-tau, and tau could be seen attached to the Noco/Tri cells (Fig. 4a). Recombinant tau on the other hand, promoted the bundling of MTs (Fig. 4a). These results suggest that AD P-tau inhibits MT assembly in Noco/Tri cells and that excess of normal tau (beyond that present in the 25% brain cytosol) causes bundling of MT.

Next, we investigated the ability of AD P- tau to breakdown preassembled MTs in Noco/Tri cells (Fig. 4b). After inducing MT assembly with rat brain cytosol for 20 min, rat brain cytosol was replaced by an equal volume of either warm PEM buffer containing 1 mM GTP alone (control), or together with recombinant tau_{410} (100 μg/ml), or AD P-tau (100 μg/ml), and incubated for 40 min. AD P-tau, caused breakdown of MTs whereas recombinant tau promoted MT bundling.

Binding of AD P-tau to normal MAPs in Noco/Tri cells

In order to investigate whether the breakdown of MT network by AD P-tau induced its interaction with normal MAPs, we immunostained Noco/Tri cells, which were treated similar to Fig. 4a, with specific antibodies against un-phosphorylated, normal tau (Tau-1) and AD P-tau (pSer 422). Control and recombinant tau_{410}-treated Noco/Tri cells showed robust staining of MT with Tau-1 indicating that the majority of normal tau associated with MTs (compare with Fig. 4a). AD P-tau-treated Noco/Tri cells displayed

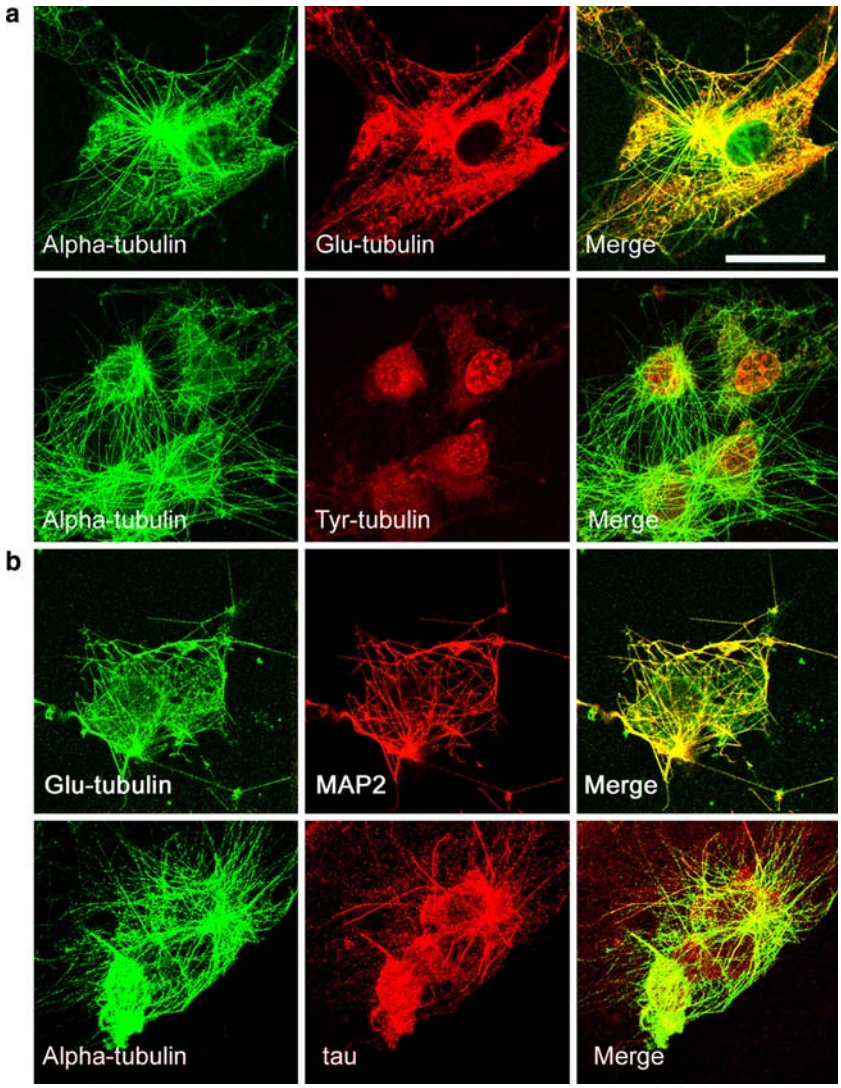

Fig. 3 Characterization of MTs assembled in Noco/Tri cells. MTs were assembled in Noco/Tri cells using 25% rat brain extract. The cells were then stained for modified alpha tubulin and various MAPS. **a** Majority of assembled MTs had detyrosinated (Glu-tubulin) tubulin (α-tubulin, *green*; Glu-tubulin, *red*). Tyrosinated tubulin was largely absent from assembled MTs (Tyr-tubulin, *red*). **b** MAP2 was the major MAP in assembled MTs whereas tau co-localized considerably less with single MTs. MAP2 *green*, tau *red*. Scale bar represents 25 μm

remarkable co-localization of Tau-1 with pSer422 indicating that AD P-tau had sequestered normal tau from the participating pool of MAPs (Fig. 5a). Similarly, AD P-tau also co-stained with MAP2 (Fig. 5b), indicating that it sequestered MAP2 from the system. Together, these results indicate that AD P-tau causes breakdown of MT network by sequestering normal tau and MAP2.

In order to find out whether the effect of AD P-tau on MT dynamics was dependent on its concentration, we next confirmed the effects of AD P-tau on MT assembly using Western blots. MT assembly was studied in vitro by incubating rat brain cytosol with varying concentrations (6.25–100 μg/ml) of recombinant tau_{410} or AD P-tau. After incubation for 1 h, the sample was centrifuged and supernatant and pellet were analyzed by Western blots to compare the amount of tubulin in both fractions (Fig. 6). The proportion of tubulin in the pellet reflected its assembly into MTs. About 60% of tubulin assembled into MTs in 15% rat brain cytosol. AD P-tau decreased the assembly of MTs in a dose-dependent manner with concentrations of 50 μg/ml and above totally abolishing MT assembly. Tau_{410} by itself had no significant effect on MT assembly.

Dephosphorylation of AD P-tau by PP-2A restores normal MT dynamics in Noco/Tri cells

Tau is a phospho protein and its biological activity is regulated by the degree of phosphorylation [27]. Hyperphosphorylation alone is enough to confer tau with inhibitory activities towards MT assembly. The activities of protein kinases and phosphatases are therefore, critical to the regulation of tau phosphorylation. We have previously shown that AD P-tau can be dephosphorylated by protein phosphatases [17–19] and that this treatment restores its biological activity in vitro [38]. Protein phosphatase 2A, which is present in the brain and is relevant to AD pathology [28], has the highest efficiency of restoring MT assembly-promoting activity of AD P-tau in vitro [38]. We next studied

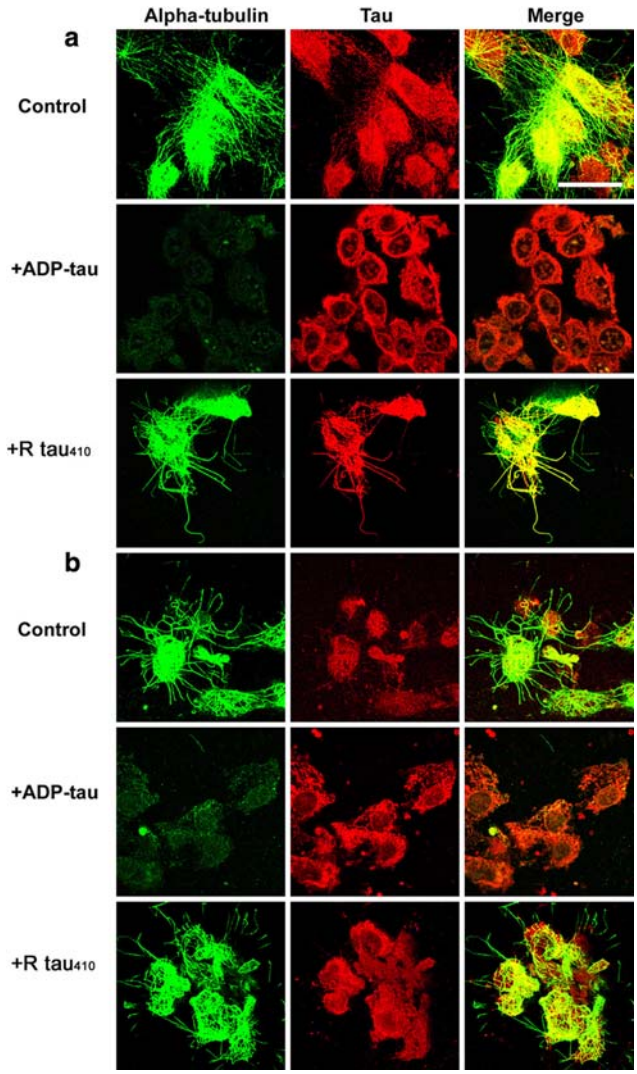

Fig. 4 Effect of AD P-tau on MT dynamics in Noco/Tri cells. MT assembly was induced with 25% adult rat brain extract in Noco/Tri cells in the presence or absence of 100 μg/ml of either recombinant human brain tau_{410} or AD P-tau. **a** AD P-tau, but not tau_{410}, completely inhibited MT assembly. Tau_{410} promoted assembly and bundling of MT. **b** MT were assembled on Noco/Tri cells for 20 min after which rat brain cytosol was replaced with either warm PEM buffer containing 1 mM GTP alone (*control*), or 100 μml of tau_{410} or AD P-tau and incubated for 40 min. AD P-tau, but not tau_{410}, destroyed assembled MTs. Scale bar represents 25 μm. (α-tubulin *green*, total tau *red*)

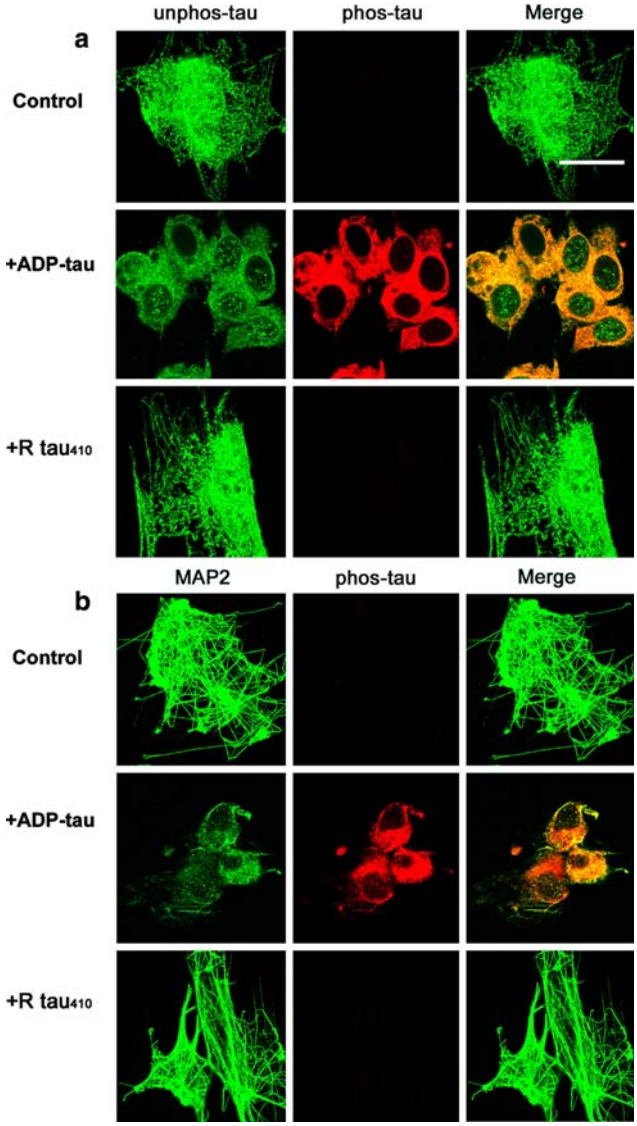

Fig. 5 Co-localization of AD P-tau with normal tau and MAP2 in Noco/Tri cells. MT assembly was induced with 25% adult rat brain extract in Noco/Tri cells in the presence or absence of 100 μg/ml of either recombinant human brain tau_{410} or AD P-tau. AD P-tau sequestered normal tau and MAP2 as seen by co-localization of AD P-tau (stained with antibody to pSer 422, a site only phosphorylated in AD P-tau) with normal, unphosphorylated tau (**a**) and MAP2 (**b**). Scale bar represents 25 μm

whether treatment of AD P-tau with PP-2A can counteract its inhibitory activity towards MT assembly in Noco/Tri cells. For this purpose, we did two sets of experiments: in the first set of experiments, AD P-tau was treated with PP-2A for 45 min at 37°C prior to its incubation with rat brain cytosol for MT assembly in Noco/Tri cells. PP-2A releases ~50% of inorganic phosphate from AD P-tau within 45 min [38]. So the incubation mixture for MT assembly contained partially dephosphorylated AD P-tau. Treatment of AD P-tau with PP-2A partially restored its activity towards MT assembly (Fig. 7a–c).

In the second set of experiments, AD P-tau was first added to the rat brain cytosol in order to inhibit MT assembly in Noco/Tri cells (Fig. 7d). After 10 min, PP-2A was added to the Noco/Tri cells and the samples were incubated for another 20 min. PP-2A activity was then inhibited by adding phosphatase inhibitors (100 nM Okadaic acid and 25 mM NaF) and the cells were further incubated for 20 min in the presence of fresh GTP and thereafter fixed. A twenty-minute treatment of AD P-tau by PP-2A releases ~30% phosphate per mole of AD P-tau [38]. There was clear evidence of at least some MT "re-assembly" as seen

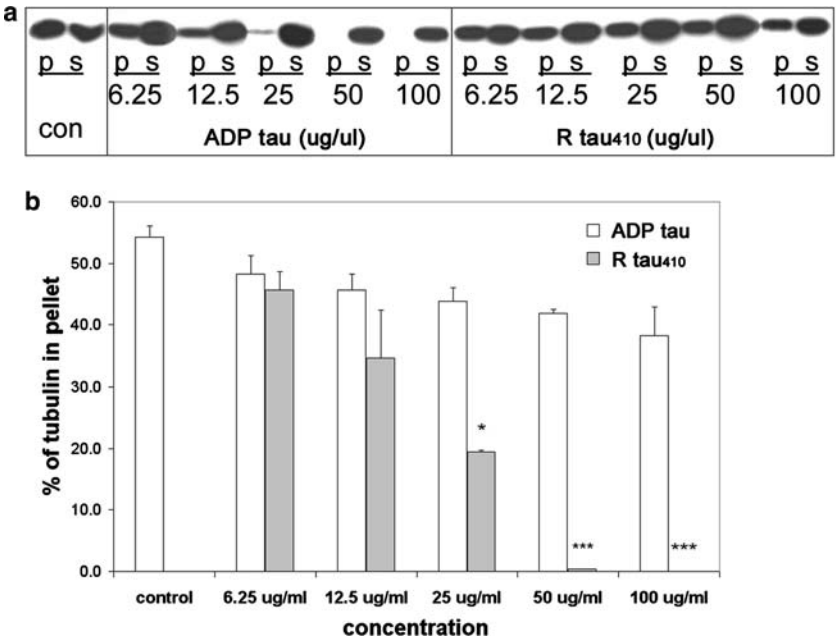

Fig. 6 Concentration-dependent effect of AD P-tau on MT assembly from rat brain cytosol. Fifteen percent rat brain cytosol containing 1 mM GTP was used as a source of tubulin and MAPs for MT assembly. Recombinant tau_{410} or AD P-tau was added at different concentrations (6.25–100 μg/ml) and incubated for 1 h at 37°C. Pellets and supernatants were separated by centrifugation at 5,600*g*. **a** After dissolving pellets in 0.4% SDS, both supernatants and pellets were analyzed with anti-tubulin (DM1A) by Western blots. **b** Quantitations were done by scanning chemiluminiscent (ECL) Western blots. AD P-tau decreased MT assembly in a dose dependent manner, whereas tau_{410} had no significant effect. Data in **b** represent mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

by tubulin staining of the Noco/Tri cells (Fig. 7d–f). The extent of MT assembly in this set of experiments was less than that in the first set of experiments probably because of a relatively less dephosphorylation of AD P-tau.

Western blot analysis of AD P-tau pretreated with PP-2A for 45 min at 37°C, showed its dephosphorylation at multiple phosphorylation sites, as evident by immunostaining with Tau-1, PHF-1, M4 and 12E8 phospho-dependent tau antibodies (Fig. 7g).

These results indicate that AD P-tau competes with normal tau to inhibit MT assembly and that PP-2A-induced dephosphorylation of AD P-tau restores it to a normal-like protein.

Discussion

Dysregulation of MT dynamics leads to retrograde degeneration of neurons in Alzheimer disease and related tauopathies. With a disrupted MT network, many subcellular processes, which are vital for neuronal physiology are shut down, synapses are lost and the affected neurons die. One of the key elements in retrograde degeneration of Alzheimer type is the hyperphosphorylation of tau. Observations from in vitro studies suggest that hyperphosphorylated tau is inhibitory to MT assembly and stability. However, the precise mechanism by which hyperphosphorylated tau affects MT dynamics in the cell has been difficult to study. This is due to limitations in carrying out such studies without disturbing other cellular processes. In this study, we have employed a model in which we have replaced the cytoplasm of mouse embryonic fibroblasts with adult rat brain cytosol, creating a mature neuron-like environment of MT network made up of mostly Glu-tubulin, MAP2 and tau. Using this model, we show that AD P-tau causes a breakdown of the MT network by sequestering normal tau and MAP2, and that this inhibitory activity of AD P-tau can be reversed by its dephosphorylation by PP-2A.

Detergent extraction of cells with a non-ionic detergent like Triton X-100 removes all "soluble components" of the cytoplasmic ground substance. What remain are the elements of the cytoskeleton and the nuclear remnant. When intact cells are treated with Nocodazole to disassemble existing MT network, free tubulin is extracted along with the cytoplasmic ground substance. The remaining detergent extracted cells can now be employed as a "cellular platform" to create different cellular environments. By replacing cytosolic ground substance of the host cells with adult rat brain cytosol, a neuron-like environment is created in which the assembled MT not only structurally resemble neuronal MT (being enriched in Glu-tubulin) but also faithfully recapitulate the dynamics of MT in intact neurons. Apart from acting as a source neuronal tubulin, 25% rat brain cytosol also provides the necessary MAPs to promote

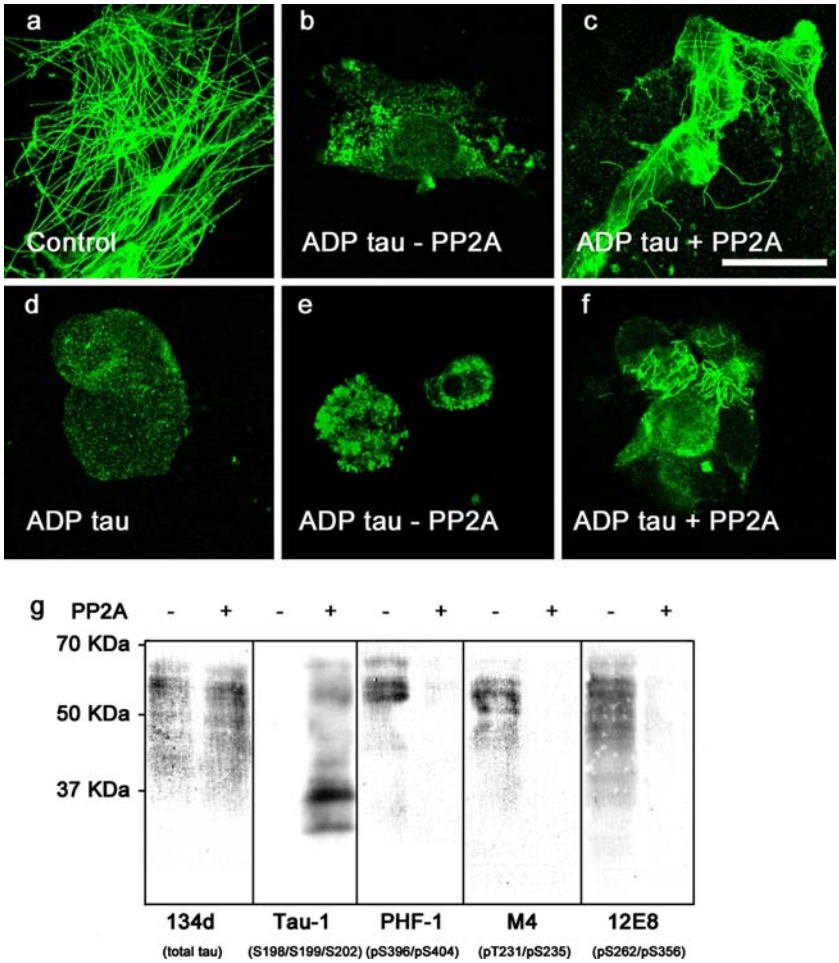

Fig. 7 Effect of dephosphorylation of AD P-tau on MT dynamics in Noco/Tri cells. AD P-tau was either treated with PP-2A prior to incubation for MT assembly (**b**, **c**) or during the incubation (**d**–**f**). In **b**, **c** AD P-tau (50 μg/ml) was treated with PP-2A (1 U/ml) for 45 min at 37°C prior to its incubation with 25% rat brain cytosol for MT assembly in Noco/Tri cells; cells treated with the brain cytosol in the absence of AD P-tau were used as a control. Dephosphorylation of AD P-tau with PP-2A partially restored its activity to promote MT assembly (compare **c** with **b**). In **d**–**f**, 25% rat brain extract was incubated with AD P tau (50 μg/ml) on Noco/Tri cells for 10 min to inhibit MT assembly (**d**). PP-2A was then added to the incubation mixture for 20 min before phosphatase inhibitors to inhibit PP-2A activity (**f**). Fresh GTP was supplied at this time and the cells were fixed after another 20 min incubation and stained with α-tubulin antibody. MT partially "re-assembled" in PP-2A treated Noco/Tri cells (**f**). PP-2A-catalyzed dephosphorylation of AD P-tau was confirmed by Western blot (**g**). AD P-tau (50 μg/ml) was treated with PP-2A (1 U/ml) for 45 min at 37°C and subjected to Western blot analysis. A decrease in tau phosphorylation at Tau-1, PHF-1, M4 and 12E8 epitopes is evident. Standard bar represents 25 μm

MT assembly. Under these conditions, MT assembly is seen as early as 1 min after incubation in the rat brain cytosol and the MT network so formed is stable for upto 4 h. When rat brain cytosol is replaced by buffer, all MTs disassemble within 1 h. Immunostaining of Noco/Tri cells shows that the assembled MTs closely resemble neuronal MT network. They are enriched in Glu-tubulin, which is found in stable assemblies of MT in differentiated cells. Although both MAP2 and tau co-stain with the MT network, MAP2 is the predominant MAP owing to its greater ability in promoting MT assembly [34]. When the reaction is carried out in the presence of AD P-tau, no MTs are seen, suggesting that AD P-tau inhibits MT assembly. Moreover, when AD P-tau is added to preassembled MT, all MT are destroyed. The effect of AD P-tau on MT assembly is dose dependant, with concentrations of 50 μg/ml and above totally abolishing MT assembly. Normal tau in the brain cytosol, on the other hand, promotes MT assembly and additional unphosphorylated tau causes bundling of MTs. AD P-tau causes inhibition of MT assembly by sequestering normal tau and MAP2, as shown by co-staining of pSer422 immunoreactive tau (an epitope of AD P-tau) with Tau-1 immunoreactive tau (an epitope of normal tau), and with MAP2. When a protein phosphatase like PP-2A is introduced in the system, AD P-tau gets dephosphorylated and is now available for MT assembly. This is evidenced by observing MT "re-assembly" in the AD P-tau-treated Noco/Tri cells in which PP-2A has been added.

In summary, AD P-tau inhibits MT assembly by a process, which involves sequestration of normal MAPs, including MAP2 and tau. This inhibitory activity is reversible, as MT re-assemble when normal tau becomes available following dephosphorylation of AD P-tau with PP-2A. Since tau is the major MAP associated with axonal MT whereas MAP2 is localized mainly in the dendritic pool of MT, removal of participating MAPs (tau and MAP2) from dynamic assemblies of MT forms, the basis of retrograde degeneration of both dendrites and axons seen in AD and related tauopathies. The Noco/Tri cells employed in this study provide a simple and relevant platform to study the neuronal cytoskeleton in a neuron-like environment. As a further extension, this model can be used to develop rapid screening assays for compounds with the potential to prevent or reverse neurofibrillary degeneration.

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