Molecular motors implicated in the axonal transport of tau and α-synuclein

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Summary

Tau and α-synuclein are both proteins implicated in the pathology of neurodegenerative disease. Here we have investigated the mechanisms of axonal transport of tau and α-synuclein, because failure of axonal transport has been implicated in the development of several neurodegenerative disorders. We found that the transport of both of these proteins depend on an intact microtubule- but not actin-cytoskeleton, and that tau and α-synuclein both move at overall slow rates of transport. We used time-lapse video microscopy to obtain images of live neurons that had been transfected with plasmids expressing proteins tagged with enhanced green fluorescent protein. We found that particulate structures containing tau or α-synuclein travel rapidly when moving along axons but spend the majority of the time paused, and these structures have similar characteristics to those previously observed for neurofilaments. The motile particles containing tau or α-synuclein colocalise with the fast-transporting molecular motor kinesin-1 in neurons. Co-immunoprecipitation experiments demonstrate that tau and α-synuclein are each associated with complexes containing kinesin-1, whereas only α-synuclein appears to interact with dynein-containing complexes. In vitro glutathione S-transferase-binding assays using rat brain homogenate or recombinant protein as bait reveals a direct interaction of kinesin-1 light chains 1 and 2 with tau, but not with α-synuclein. Our findings suggest that the axonal transport of tau occurs via a mechanism utilising fast transport motors, including the kinesin family of proteins, and that α-synuclein transport in neurons may involve both kinesin and dynein motor proteins.

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Key words: Tau, α-Synuclein, Axonal transport, Kinesin, Dynein, Glutathione S-transferase

Introduction

Tau protein is expressed predominantly in the axons of neurons where it promotes assembly and stabilisation of microtubules (Cleveland et al., 1977; Drubin et al., 1986). Tau also binds to membranes and anchors enzymes to microtubules (Brandt et al., 1995; Lee et al., 1998; Liao et al., 1998; Sontag et al., 1999). In Alzheimer's disease (AD) and related tauopathies, hyperphosphorylated tau aggregates into neurofibrillary tangles, a pathological hallmark of these disorders (Lee et al., 2001). Tau is normally transported along axons at a rate compatible with slow transport (Mercken et al., 1995; Utton et al., 2002; Zhang et al., 2004) and failure of axonal transport has been implicated as a possible mechanism for tau accumulation in the tauopathies (Praprotnik et al., 1996; Flament-Durand et al., 1979; Richard et al., 1989; Bendiske et al., 2002; Dai et al., 2002).

α-Synuclein is a pre-synaptic protein involved in maintenance of synaptic integrity and function and regulation of dopamine synthesis (Abeliovich et al., 2000; Perez et al., 2002; Cabin et al., 2002; Chandra et al., 2004). In vivo studies have shown that the majority of α-synuclein moves along axons in the slow component with the remainder travelling in the fast component of axonal transport (Jensen et al., 1998; Jensen et al., 1999). Aggregates of α-synuclein are present in brain in Parkinson's disease (Polymeropoulos et al., 1997; Mezey et al., 1998; Kruger et al., 1998), dementia with Lewy bodies (Spillantini et al., 1997), and 60-70% of AD cases (Mukaetova-Ladinska et al., 2000; Hamilton, 2000). Dysfunctional axonal transport has also been proposed as a mechanism involved in the pathogenesis of Parkinson's disease (Lach et al., 1992). The co-occurrence of tau and α-synuclein aggregates in neurodegenerative diseases (Spillantini et al., 1998; Iwai, 2000; Mukaetova-Ladinska et al., 2000; Hamilton, 2000; Masliah et al., 2001; Popescu et al., 2004), and enhancement of tau pathology by α-synuclein (Giasson et al., 2003), suggests that there may be a link between these two pathologies.

The paucity of protein synthesising machinery within axons means that neurons rely on an efficient axonal transport system and hence may be especially vulnerable to altered regulation of axonal transport. We have investigated the axonal transport of tau and α-synuclein in neurons because defective transport has been implicated as a possible mechanism involved in diseases that exhibit tau and α-synuclein pathology. We found that both tau and α-synuclein can travel at speeds in the range of fast axonal transport. We show for the first time that the fast anterograde motor kinesin-1 may be involved in transport of both tau and α-synuclein, and this is supported by our finding

of a direct interaction between tau and kinesin-1. Furthermore, we found that α-synuclein is associated with the retrograde motor dynein. These observations further our understanding of the mechanisms involved in tau and α-synuclein axonal transport, which may be important factors in development of neurodegenerative disease.

Materials and Methods

Plasmids

The human α-synuclein constructs, α-synuclein in pcDNA3.1(–) or α-synuclein-fused to the C-terminus of enhanced green fluorescent protein (EGFP), were generated as previously described (Saha et al., 2000; Saha et al., 2004). Tau-EGFP was generated as previously described (human 0N4R tau fused to the C-terminus of EGFP) (Dayanandan et al., 1999). A plasmid expressing glutathione S-transferase (GST) fused to the 0N4R isoform of tau (GST-0N4Rtau) was a kind gift from H. Mori (Osaka City University Medical School, Osaka, Japan) (Mori et al., 1989; Kawamata et al., 1998). GST-α-synuclein was generated by cloning α-synuclein into the XhoI site in pGEX-5X-2. Plasmids expressing GST-fused to kinesin-1 light chains 1 and 2 (GST-KLC1 and GST-KLC2) were kind gifts from L. Goldstein (Howard Hughes Medical Institute, La Jolla, CA, USA) (Rahman et al., 1998).

Antibodies

The rabbit polyclonal α-synuclein antibody, α90, was raised against peptide C-AATGFVKKDQMGK, corresponding to amino acids 90-102 of rat α-synuclein with the addition of an N-terminal cysteine) conjugated to PPD with glutaraldehyde (Totterdell et al., 2004). The rabbit polyclonal α-synuclein antibody, αC, was raised against a peptide C-EGYQDYEPEA, corresponding to amino acids 131-140 of human α-synuclein. For western blotting, mouse anti-kinesin-1 monoclonal antibody (MAB1614) and mouse anti-dynein, cytoplasmic (74 kDa intermediate chain) monoclonal antibody (MAB1618) were purchased from Chemicon International Inc., Temecula CA, USA. For immunofluorescence microscopy, mouse anti-kinesin-1 antibody (ab9097) was purchased from Abcam, Cambridge, UK. The monoclonal antibody to α-tubulin (DM1A) was purchased from Sigma Chemical Co., Poole, UK. The rabbit anti-human tau antibody (code no. A0024) was purchased from DAKO, Glostrup, Denmark. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Biosciences UK Ltd, Little Chalfont, UK. Secondary antibodies linked to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Cambridge Biosciences, Cambridge, UK.

Purification of recombinant tau and α-synuclein

Recombinant tau (2N4R isoform) was purified as previously described (Scott et al., 1991; Utton et al., 1997). Samples were used from the pre-Mono-S purification stage.

Transfection of cortical cultures

DNA for transfection was prepared by an endotoxin-free maxiprep kit (Qiagen, Crawley, UK). Cortical neurons were obtained from embryonic day 18 (E18) rat embryos, cultured and transfected as previously described (Ackerley et al., 2000).

Nocodazole and cytochalasin treatment of transfected neurons

Neurons were transfected with either 0N4Rtau-EGFP or α-synuclein and were treated 3 hours after transfection with either 5 μg/ml nocodazole (Tocris, Bristol, UK) or 25 μM cytochalasin B (Sigma Chemical Co., Poole, UK). Neurons were fixed 0, 30, 60 and 90 minutes following treatment. The α-synuclein transfected neurons were immunostained using α90. The analysis of overall slow axonal transport of tau-EGFP and α-synuclein was carried out as previously described (Ackerley et al., 2000; Utton et al., 2002). The distance travelled by the exogenous protein was measured from the perimeter of the cell body along the axon to the limit of the fluorescent front. Neurons were immunostained for tubulin using DM1A and FITC-conjugated secondary antibody and for actin using phalloidin-TRITC to ensure that breakdown of the microtubule and actin cytoskeletons, respectively, had occurred under the experimental conditions.

Live microscopy of neuronally expressed tau-EGFP and α-synuclein-EGFP

Rat cortical neurons were transfected with either 0N4R tau-EGFP or α-synuclein-EGFP. Live image analysis of neurons was performed 48 hours after transfection (Ackerley et al., 2003) and images were collected at 0.5-2.5 second intervals for 60-200 seconds. The kinetic parameters of moving fluorescent structures were analysed by manual tracking using MetaMorph software. Fluorescent structures were judged to be moving if they were displaced by more than 0.3 μm between consecutive images, this value was determined empirically by manually determining the average apparent 'movement' of stationary fluorescent structures in the neurons. Displacement of less than 0.3 μm was considered most likely to be due to fluctuations in the conditions surrounding the cells rather than being due to axonal transport. Pause times were measured only for those structures that moved, paused and moved off again during the observation time.

Immunofluorescence labelling of neurons

Rat cortical neurons were fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline (PBS), permeabilised in 0.5% (v/v) Triton X-100 in PBS for 10 minutes and non-specific sites were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS. Neurons were immunostained for tau using the rabbit polyclonal antibody (DAKO) and for α-synuclein with α90. For co-localisation of tau and α-synuclein with kinesin-1, neurons were co-stained for kinesin-1 using a monoclonal antibody (Abcam, ab9097). Transfected neurons were immunostained 48 hours after transfection. Images were collected using a Zeiss Axiovert 200M microscope with Zeiss LSM 5 image software.

Immunoprecipitation

Adult rat brains or rat cortical cultures were homogenised in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis (2-aminoethyl)-N,N,N′,N′-tetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulphate, 0.5% (w/v) sodium deoxycholate, Complete protease inhibitor (Roche, Welwyn Garden City, UK)), incubated on ice for 30 minutes and centrifuged at 100,000 ***g*** for 30 minutes at 4°C. The supernatant was removed and protein content was assayed (BioRad Protein Assay, Hemel Hempstead, UK). For immunoprecipitation, 1-5 μl rabbit antibody against human tau (DAKO, Glostrop, Denmark) or 16 μl rabbit antibody against α-synuclein (α90) were added to the supernatant, and immunoprecipitates were isolated by the addition of protein A/G agarose (Amersham Biosciences UK Ltd, Little Chalfont, UK). Immunoprecipitates were washed three times with RIPA buffer and associated proteins were detected by immunoblotting the precipitates with either mouse anti-kinesin-1 monoclonal antibody or mouse anti-dynein, cytoplasmic (74 kDa intermediate chain) monoclonal antibody.

Purification of GST fusion proteins

The GST purification method was based on the manufacturer's instructions (Amersham Biosciences UK Ltd, Little Chalfont, UK). Expression of GST and GST-fusion proteins was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 2-3 hours. Bacterial pellets were resuspended in PBS containing Complete protease inhibitor and sonicated. Triton X-100 was added to a final concentration of 1% (v/v) and the suspension was incubated for 30 minutes at 4°C under constant agitation. Following centrifugation at 10,000 ***g*** for 10 minutes at 4°C the resulting supernatant, containing GST-fusion proteins, was incubated with glutathione Sepharose 4B for 1 hour at 4°C under constant agitation. GST-Sepharose beads were collected by centrifugation at 500 ***g*** for 1 minute at 4°C and washed three times by re-suspension and centrifugation with PBS containing Complete protease inhibitor. GST-protein was quantified by comparison with standard amounts of bovine serum albumin on Coomassie-Blue-R-stained polyacrylamide gels.

GST-binding assay

Purified GST-KLC proteins (10 nM) bound to glutathione Sepharose 4B beads were incubated with adult rat brain homogenate or purified recombinant tau (10 nM) in modified RIPA buffer (mRIPA) (20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid, 0.2 mM PMSF, 1% (v/v) Nonidet 40 (NP40), Complete protease inhibitor). The GST-Sepharose beads were pelleted at 500 g for 1 minute at 4°C, the beads were washed three times with mRIPA buffer and resuspended in sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) sample buffer containing 40 mM dithiothreitol. GST-bound proteins were analysed on western blots with antibodies to kinesin-1, dynein or tau.

Results

Axonal transport of tau and α-synuclein depends on an intact microtubule- but not actin-cytoskeleton

Rat cortical neurons were transfected after culturing for five days in vitro; therefore, at this stage, the neurons already have an established neuritic network. Initially, we investigated the dependence of tau and α-synuclein on the integrity of the microtubule- or actin-cytoskeletons. We treated transfected rat cortical neuronal cultures with the microtubule-destabilising agent nocodazole or with the actin-depolymerising agent cytochalasin B. Treatment was carried out 3 hours after transfection, a time at which significant amounts of exogenously expressed proteins were present in the cell body and in the proximal regions of neurites. The rates of transport of tau-EGFP or α-synuclein in transfected neurons are determined by measuring the distances travelled by the fluorescent front of each protein at intervals of 3 and 4.5 hours after transfection. Cultures treated with nocodazole or cytochalasin B were immunostained with antibody against tubulin (DM1A) or with phalloidin-TRITC to verify disruption of the microtubule- or actin-cytoskeletons, respectively (Fig. 1A). As expected, nocodazole treatment resulted in depolymerisation of microtubules, visualised as a decreased intensity of tubulin staining and the disappearance of the microtubule network. The transport of both tau and α-synuclein was inhibited when microtubules were depolymerised with nocodazole. Although the overall effect of nocodazole on the transport of α-synuclein and tau-EGFP was similar, the onset of the disruptive effect of nocodazole on the transport of tau-EGFP was more rapid than on α-synuclein transport (Fig. 1B,C). Three-and-a-half hours after transfection (and 30 minutes after addition of nocodazole) the transport of α-synuclein appeared unaffected when compared with untreated neurons, whereas the transport of tau was significantly reduced. This differential effect on transport of tau and α-synuclein also indicates that the mechanism involved is unlikely to be mediated through neurite retraction induced by nocodazole because it would be expected that proteins that are transported at similar rates would be equally affected by such retraction. Indeed, we did not observe retraction of neurites caused by these treatments during the period of the experiments. Treatment of cortical neurons with cytochalasin B caused the dense network of fine actin threads to collapse and the intensity of actin labelling in these regions was reduced (Fig. 1A). In contrast to the effects of nocodazole, collapsing the actin cytoskeleton with cytochalasin B did not have any measurable effect on transport velocities of tau or α-synuclein in neurons (Fig. 1B,C). These results show that the slow overall rate of transport of both tau and α-synuclein is independent of an intact actin network and implicates a microtubule-based mechanism for movement of these proteins in neurons.

Particles containing tau or α-synuclein move at rates comparable with fast axonal transport

Neurons were transfected with constructs expressing tau-EGFP or α-synuclein-EGFP and the movement of tau-containing or α-synuclein-containing structures was monitored in live cells by analysing images 48 hours after transfection (Fig. 2 and supplementary material Movies 1-4). The majority of exogenous tau-EGFP was observed as continuous fluorescence throughout the axon; however, in regions of the axon where the fluorescence was sufficiently low, it was possible to distinguish discrete tau-containing structures. These particles were of various size and, in addition to rapid and bi-directional movement, they also exhibited extended periods during which they were paused. Others have also noticed that fluorescence intensity has to be sufficiently reduced to allow the detection of fluorescent structures (Okabe and Hirokawa, 1993; Okabe et al., 1993), hence we assume this is also the case for tau-EGFP, because the majority of expressed tau-EGFP decorates the axonal microtubules and is visible as continuous fluorescence along axons. Fourty-eight hours after transfection, most of the transfected α-synuclein was, similarly to tau, detected as structures of various size, some of which were moving bi-directionally at a rapid rate in addition to periods of pausing. We assume that discrete structures containing α-synuclein are more readily observed owing to the lack of decoration of microtubules by α-synuclein compared with tau-EGFP. Fig. 2A-D shows frames from time-lapse recordings of tau-containing (Fig. 2A,B) and α-synuclein-containing structures (Fig. 2C,D). Particles highlighted (*) in A and C move in an overall anterograde direction, whereas particles highlighted in B and D move in an overall retrograde direction. The α-synuclein–EGFP- and tau-EGFP-containing structures shown in Fig. 2A-D were tracked using MetaMorph image analysis software, and the direction and timing of these movements are shown in Fig. 2A′-D′. Notice that these tracked particles represent a sub-population of moving structures and

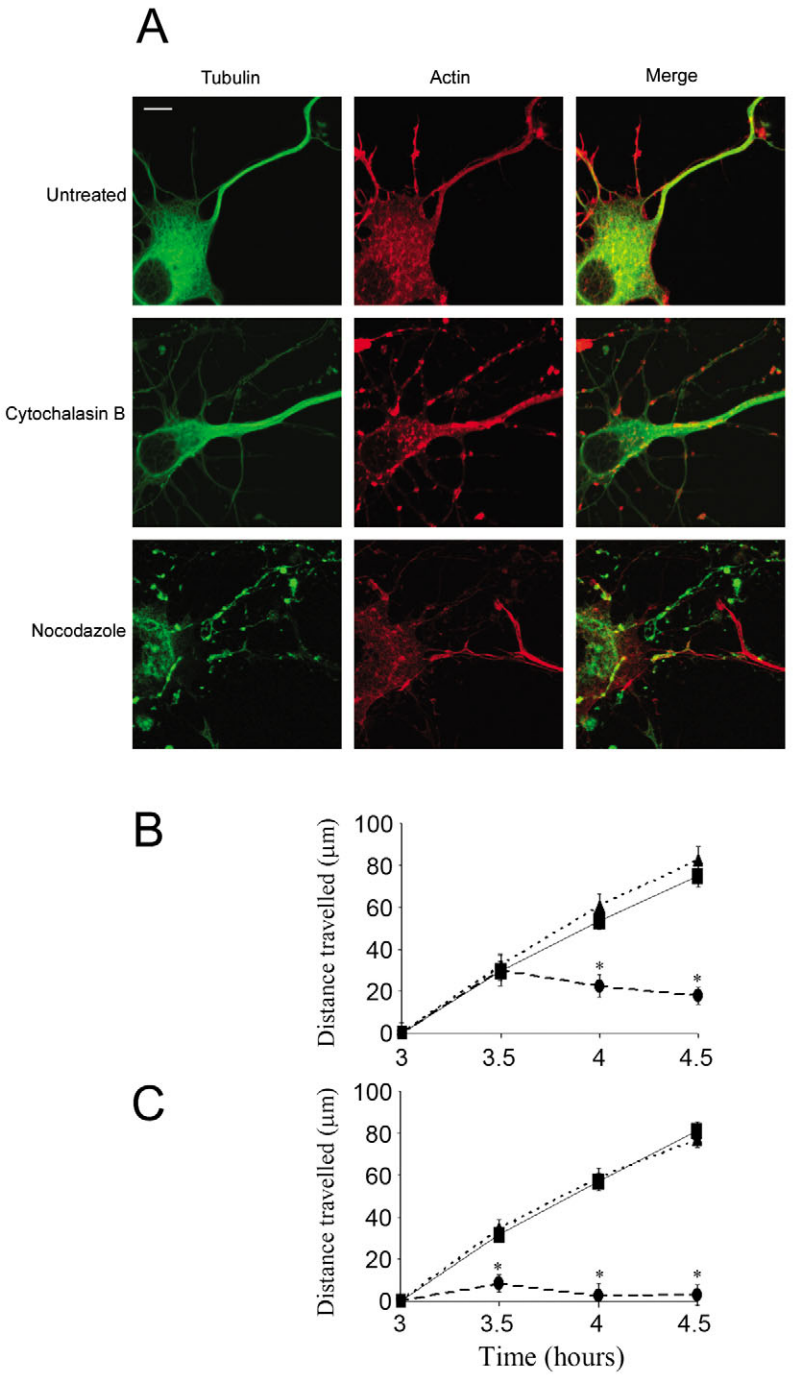


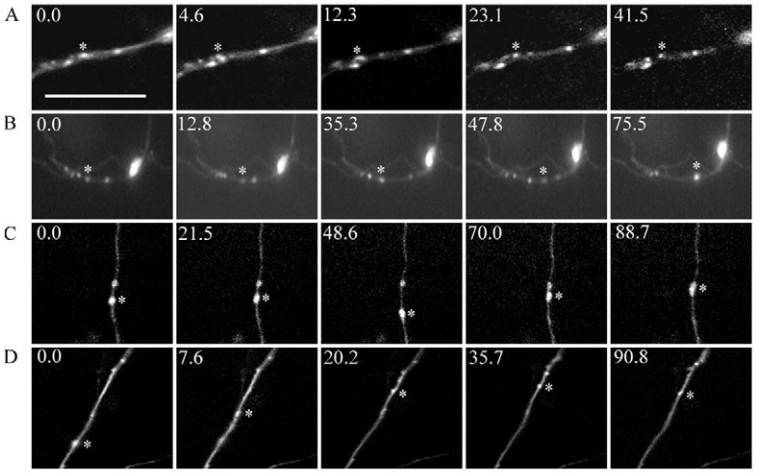
Fig. 1. Tau and α-synuclein transport depends on an intact microtubule cytoskeleton. (A) Rat cortical neurons were either not treated or treated with cytochalasin B to disrupt the actin cytoskeleton or with nocodazole to depolymerise microtubules. Tubulin was visualised with antibody to α-tubulin and FITC-conjugated secondary antibody (green) and actin was detected with phalloidin-TRITC (red). Merged images of tubulin and actin are shown on the right. Bar, 10 μm. (B,C) Neurons were transfected with α-synuclein (B) or tau-EGFP (C) and cells were fixed at 30-minute intervals after transfection. Neurons were either not treated (■) or treated with nocodazole 3 hours after transfection (●) or cytochalasin B (▲). Neurons transfected with α-synuclein were immunostained for α-synuclein using α90 antibody and tau was detected by direct fluorescence of EGFP. The distance travelled by the exogenous protein was measured from the perimeter of the cell body along the axon to the limit of the fluorescent front. Each point represents the mean±s.e.m. (n=60-130 measurements). Treatment with nocodazole inhibited the transport of both proteins (*P<0.01 using one-way ANOVA), whereas transport of neither of the proteins was affected by treatment with cytochalasin B (P>0.05), indicating that an intact microtubule network is essential for the transport of tau and α-synuclein.

are not representative of the total visible particles, the majority of which are paused during the periods of observation (see below).

The kinetic parameters of particles containing tau-EGFP or α-synuclein-EGFP were determined using MetaMorph image analysis software (Table 1). These results show some similarities in the movement characteristics of tau-EGFP and α-synuclein-EGFP-containing structures, for example, the time spent pausing was approximately 70-80% for both proteins. The mean rate of transport between pauses for tau-EGFP structures was 31.4 mm/day, thus resembling that of neurofilaments at ~45 mm/day (Wang et al., 2000; Roy et al., 2000; Wang and Brown, 2001) and peripherin at 27 mm/day (Helfand et al., 2003). The range of transport rates determined for tau-EGFP-containing structures, 14-124 mm/day, is also similar to that reported for peripherin (7-129 mm/day). When the tau-EGFP-containing structures did move they did so in an anterograde direction for 57% of their motile time and in a retrograde direction for 43% of their movement time. α-Synuclein-containing structures moved in an anterograde direction for 48% of the movement time and in a retrograde direction for 52% of the movement time. The mean rate of transport of α-synuclein-containing structures was much faster than tau at 161 mm/day and the range of transport rates also differed at 111-645 mm/day. These results suggest that different molecular mechanisms might be involved in the transport of α-synuclein compared to that of cytoskeletal proteins, such as tau and neurofilaments.

Tau-EGFP and α-synuclein-EGFP containing structures colocalise with kinesin-1

Since both tau-EGFP and α-synuclein-EGFP-containing structures were observed moving at rates comparable with fast transport, we determined whether known fast motors, such as kinesin-1, can be involved with this movement, because it has been previously shown for proteins such as peripherin (Helfand et al., 2003). Co-localisation with kinesin-1 was investigated in rat cortical neurons transfected with tau-EGFP or α-synuclein-EGFP, fixed after 48 hours and immunostained for kinesin-1 (Fig. 3). A proportion of the structures containing tau-EGFP or α-synuclein-EGFP were identified by laser confocal immunofluorescence microscopy as being associated with kinesin-1 (Fig. 3, arrows). Not all of the tau- or α-synuclein-containing structures colocalised with



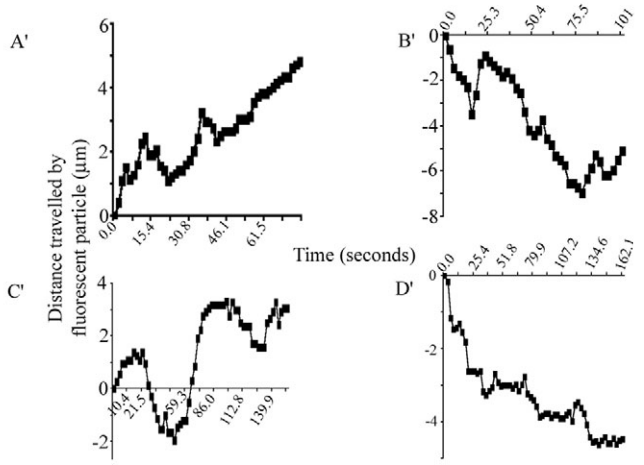


Fig. 2. Tau- and α-synuclein-containing structures move at rates comparable with fast transport. (A,B) Rat cortical neuron transfected with 0N4Rtau-EGFP, 48 hours after transfection. The movement of tau-containing structures were monitored using live image analysis. The frames, taken at the indicated time intervals in seconds, show live images of tau-containing structures moving at a rate comparable with that of fast transport. The frames show overall anterograde (A) and retrograde (B) movement of a tau-containing structure (*). See also supplementary material Movies 1 and 2. (A′,B′) Tracking of the tau-containing structures shown in A and B. (C,D) As A and B, showing overall anterograde (C) and retrograde (D) movement of α-synuclein-containing particles. See also supplementary material Movies 3 and 4. (C′,D′) Tracking of the α-synuclein-containing particles shown in C and D. Bar, 10 μm.

kinesin-1 but, since the majority of the structures are paused at any given time, this observation was to be expected.

Tau and α-synuclein both co-immunoprecipitate with kinesin-1, whereas only α-synuclein co-immunoprecipitates with dynein

Since both tau-EGFP and α-synuclein-EGFP exhibited co-localisation with kinesin-1, we next determined if tau or α-synuclein are present in complexes with known molecular motors of fast axonal transport, such as kinesin and dynein. Immunoprecipitation experiments from adult rat brain were conducted using antibodies recognising tau (Fig. 4, left panels) or α-synuclein (Fig. 4, right panels). Immunoprecipitates were analysed on western blots using antibodies to kinesin-1 (Fig. 4, upper panels) or dynein intermediate chain (Fig. 4, lower panels). Tau and α-synuclein co-immunoprecipitated with kinesin-1, the tau antibody bringing down ~15% of total tau and ~1% of total kinesin-1, and the α-synuclein antibody bringing down ~60% of total α-synuclein and ~1% of total kinesin-1. The co-immunoprecipitation of tau and kinesin-1 was not significantly affected by the addition of 500 mM NaCl, indicating that the interaction is robust (data not shown). Only α-synuclein co-immunoprecipitated with dynein intermediate chain, with ~0.5% of total dynein intermediate chain co-immunoprecipitating under these experimental conditions. Thus, both tau and α-synuclein can exist in a complex with kinesin-1 in the adult rat brain. These results are in agreement with previous studies suggesting direct or indirect interactions between tau and kinesin (Jancsik et al., 1996) and between α-synuclein and dynein (Zhou et al., 2004).

Association of GST-tau and GST-α-synuclein with kinesin-1

The presence of kinesin-1 in complexes with tau and α-synuclein was also investigated using purified GST, GST-tau or GST-α-synuclein bound to glutathione-Sepharose beads incubated with adult rat brain homogenate. Bound proteins were separated by SDS-PAGE and analysed on western blots with antibodies to kinesin-1 or dynein (Fig. 5). Kinesin-1 immunoreactivity was observed in the precipitates when either GST-tau or GST-α-synuclein was used in pull down

Table 1. Kinetic parameters of motile structures containing tau-EGFP or α-synuclein–EGFP in transfected rat cortical neurons

	Rate between pauses* [Range] (mm/day)	Time moving† (% of total)	Time pausing (% of total)	Time moving anterograde (% of total)	Time moving retrograde (% of total)
Tau	31.4±0.8 (*n*=193) [14-124]	27	73	57	43
α-Synuclein	161±5 (*n*=185) [111-645]	20	80	48	52

The movement characteristics of individual fluorescent structures containing tau-EGFP or α-synuclein–EGFP were measured 48 hours after transfection of rat cortical neurons.

*Rates are shown as mean±s.e.m. (*n*), where *n*=number of periods of movement detected.

†*n*=number of structures observed (tau, *n*=17; α-synuclein, *n*=19).

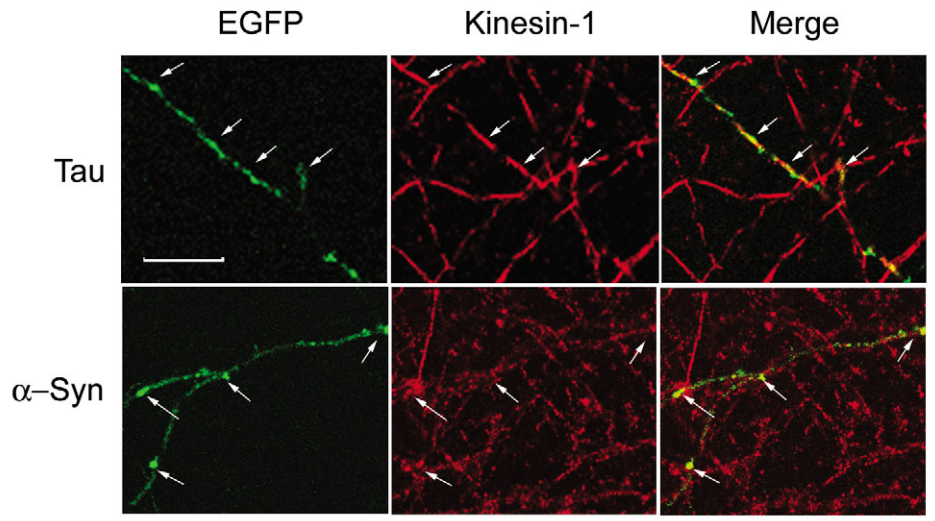


Fig. 3. Tau-EGFP and α-synuclein-EGFP-containing structures colocalise with kinesin-1. E18 rat cortical neurons were transfected with tau-EGFP or α-synuclein-EGFP, fixed after 48 hours, immunostained for kinesin-1 and visualised by laser confocal microscopy. The merged images show yellow areas (arrows) that indicate colocalisation of tau or α-synuclein with kinesin-1 immunoreactivity. Bar, 10 μm.

experiments whereas no detectable immunoreactivity was seen in controls with purified GST alone (Fig. 5A,B). Dynein immunoreactivity was observed when GST-α-synuclein beads were incubated with the brain homogenate, whereas no dynein immunoreactivity was observed using GST-tau as bait (Fig. 5C,D). This is therefore a further indication of a direct or indirect association of both tau and α-synuclein with kinesin-1, and it suggests that α-synuclein might also be present in a complex with dynein.

Further assessment of the components of the kinesin-1 complex that interact with tau

Kinesin-1 light chains have been shown to bind many of the cargoes transported by kinesin, such as amyloid precursor protein (Kamal et al., 2000). To assess the ability of GST-kinesin-1 light chain proteins to interact with tau and α-synuclein, purified GST, GST-kinesin-1 light-chain 1 (GST-KLC1) or GST-kinesin-1 light-chain 2 (GST-KLC2) bound to glutathione-Sepharose beads was incubated with adult rat brain homogenate. Bound proteins were separated by SDS-PAGE and analysed on western blots with antibodies against tau (Fig.

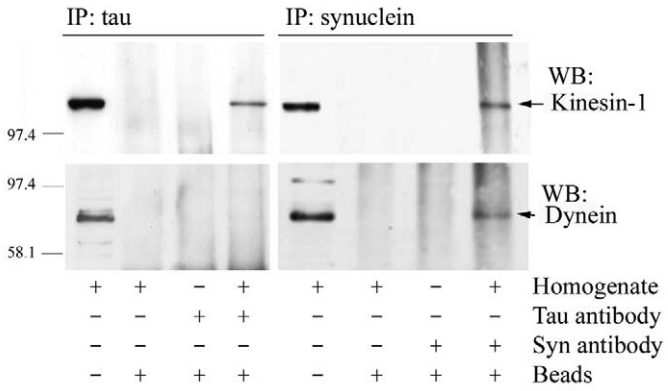


Fig. 4. Tau and α-synuclein each co-immunoprecipitate with kinesin-1, whereas only α-synuclein co-immunoprecipitates with dynein. Rat brain homogenate was immunoprecipitated using antibodies recognising tau (left panels) or α-synuclein (right panels). Immunoprecipitates were analysed on western blots with antibodies to kinesin-1 (upper panels) or dynein (lower panels). The tau antibody co-immunoprecipitated kinesin-1 but not dynein. The antibody to α-synuclein co-immunoprecipitated both kinesin-1 and dynein. Molecular mass markers are shown on the left (kDa).

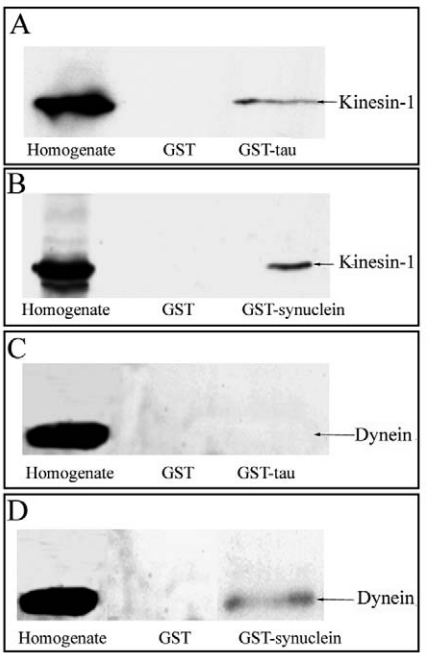


Fig. 5. GST-tau and GST-α-synuclein each interact with protein complexes containing kinesin-1, whereas only GST-α-synuclein interacts with protein complexes containing dynein. Western blots of rat brain homogenate incubated with GST, GST-tau or GST-α-synuclein bound to glutathione-Sepharose beads. Bound proteins were analysed on western blots with antibodies against kinesin-1 (A, B) or dynein (C,D). Panels A and B show an interaction of GST-tau and GST-α-synuclein with kinesin-1 in brain homogenate, respectively. There is no detectable interaction between GST-tau and dynein (C), whereas GST-α-synuclein interacts with dynein (D). No interactions were detected in brain homogenate incubated with purified GST alone.

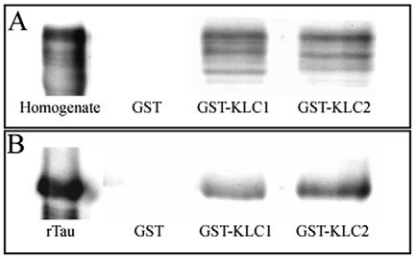


Fig. 6. Tau interacts with GST-KLC1 and GST-KLC2. (A,B) Western blots of rat brain homogenate (A) or recombinant human tau (B) probed with a polyclonal antibody to tau after incubation with purified GST, GST-KLC1 or GST-KLC2 bound to glutathione-Sepharose beads. Both recombinant human tau and endogenous rat brain tau, or complexes containing rat brain tau, bind to GST-KLC1 and GST-KLC2, but not to purified GST.

6). In rat brain homogenate, tau appears as multiple bands representing different splice isoforms in various phosphorylation states (Fig. 6A). Multiple tau species were also detected in pull-downs from rat brain homogenate using either GST-KLC1 or GST-KLC2 (Fig. 6A). No tau immunoreactivity was observed in controls with purified GST. These experiments indicate that tau exists in a complex with kinesin-1 but they do not address whether tau binds directly to kinesin-1 or whether this is an indirect interaction through other proteins. To investigate this, recombinant tau protein was incubated with purified GST, GST-KLC1 or GST-KLC2, and bound tau was detected on western blots. Significant binding of recombinant tau to both GST-KLC1 and GST-KLC2 was observed (Fig. 6B), with no detectable binding of tau to purified GST. These results indicate that tau can bind directly to both KLC1 and KLC2. By contrast, we could detect no binding of α-synuclein from rat brain homogenate or recombinant α-synuclein to GST-KLC1, or GST-KLC2 under these conditions (data not shown). These results serve as an additional control, indicating that the binding of tau to KLC1 and KLC2 is specific, and suggest that the association of α-synuclein with kinesin-1 in rat brain is mediated through additional proteins. Further analysis is necessary to determine the interaction of α-synuclein with potential accessory protein-components within a motor complex responsible for α-synuclein transport.

Discussion

We have shown showed previously that tau and α-synuclein transfected into cortical neurons are transported along axons at a rate consistent with slow axonal transport (Utton et al., 2002; Saha et al., 2004). Here, we present the first demonstration that transport of both tau and α-synuclein requires an intact microtubule network as demonstrated by the cessation of transport of both of these proteins in the presence of nocodazole. Transport of tau is significantly reduced within 30 minutes of commencing nocodazole treatment, indicating that during this time nocodazole ablates the microtubule network and causes the movement of tau to cease. In contrast to the effects on tau transport, movement of α-synuclein initially appears to be less susceptible to nocodazole treatment but 60 minutes after the commencement of treatment, α-synuclein transport was halted and we found this difference in the timing of the effect of nocodazole on the transport of tau and α-synuclein to be a robust observation. Similar delayed effects on blocking of axonal transport of neurofilaments have previously been observed 20-25 minutes after application of nocodazole (Yabe et al., 2001). A slightly delayed inhibitory effect of nocodazole on axonal transport of mitochondria has also been reported previously, such that mitochondrial movement was significantly reduced after 12-18 minutes of nocodazole treatment and mitochondrial motility was halted after 30 minutes (Ligon et al., 2000). Thus, although an intact microtubule network is undoubtedly required for axonal transport of various proteins and organelles, the differential temporal response to nocodazole on their movements suggests different mechanisms that are susceptible to the state of microtubule integrity to different degrees, which deserves further investigation.

Other groups have demonstrated that the neurofilaments peripherin and tubulin, which like tau and α-synuclein are transported principally in the slow component, exhibit rapid bi-directional movement of a sub-fraction with the bulk of the proteins remaining stationary during the period of observation (Prahlad et al., 1998; Yabe et al., 1999; Prahlad et al., 2000; Wang et al., 2002; Helfand et al., 2003; Brown, 2003). The net transport rate is thus a summation of a small fraction moving rapidly in both directions for short periods while most of the protein is paused, i.e. the duty ratio determines the net transport rate (Brown, 2003). We therefore undertook live-cell video-microscopy to investigate transport of tau and α-synuclein. Using video imaging, we were able to observe directly the movement of individual particles of tau and α-synuclein during the process of axonal transport in live neurons transfected with plasmids expressing fluorescently tagged proteins. In the case of both tau and α-synuclein, we detected structures that moved infrequently, but bi-directionally, along axons at rates consistent with fast transport; however, particle movement was interrupted by frequent pauses. By immunoprecipitation, we also found that tau and α-synuclein are each associated, either directly or indirectly with complexes containing the fast anterograde motor kinesin-1, and α-synuclein is also present in a complex with the fast retrograde motor dynein. We were unable to detect co-immunoprecipitation of dynein with tau under the experimental conditions used here. However, because we observed retrograde movement of tau in live neurons, it seems likely that tau does associate with factors controlling retrograde transport. We therefore cannot exclude the possibility that, compared to α-synuclein, there may be weak and/or transient interactions of tau with dynein or with dynein-linker proteins that are disrupted during immunoprecipitation. In the case of α-synuclein, it is of interest that recent reports have indicated an association of dynein with α-synuclein-containing inclusions (Zhou et al., 2004; Hasegawa et al., 2004). Notably, cellular aggregates of α-synuclein share some properties with aggresomes, these structures originate in the cytoplasm and subsequently form larger perinuclear inclusions after microtubule-based, dynein-mediated transport to the centrosome (Seo et al., 2002; Tanaka et al., 2004).

In this study, we observed marked differences in the relative associations of tau and α-synuclein directly with molecular

motors because tau interacts with a kinesin-1 complex binding to KLC1 and KLC2, whereas α-synuclein did not bind to either KLC1 or KLC2 under our experimental conditions. These results demonstrate that although both tau and α-synuclein are most probably transported through fast motors, there are clear differences between the mechanisms of their interactions with such motors, with tau being capable of directly binding to kinesin-1 whereas α-synuclein probably requires other accessory proteins to form a motile complex.

The rapid, yet infrequent, motility of tau-containing structures is consistent with reports of other non-membrane bound particles containing cytoskeletal proteins, such as neurofilaments (Prahlad et al., 2000), vimentin (Prahlad et al., 1998), peripherin (Helfand et al., 2003) and tubulin (Wang and Brown, 2002), that also display this behaviour and move rapidly along microtubules. These recent findings show that cytoskeletal proteins, which were previously thought to be transported only in the slow component of axonal transport, are probably moved by fast motors including kinesin and dynein (Yabe et al., 1999; Yabe et al., 2000; Shah et al., 2000; Wang et al., 2002; He et al., 2005; Theiss et al., 2005). Other studies of neurofilament transport show that, despite these rapid movements, neurofilaments spend ~80% of the time in a paused state and hence their net overall rate of transport is slow (Wang et al., 2000; Roy et al., 2000; Wang et al., 2001; Brown, 2003). Similarly, we found here that tau was paused for 73% of the time. The kinetic parameters of EGFP-tau movement in neurons bear some similarity to those of neurofilaments and peripherin. In particular, the reported velocity between pauses for EGFP-tau ranges from 14-124 mm/day, which is comparable to transport of neurofilaments at 2-160 mm/day (Wang and Brown, 2001), indicating the existence of similar mechanisms for the transport of cytoskeletal components.

The rapid movement of α-synuclein is also consistent with earlier reports showing that a proportion of synuclein can move in the fast component of axonal transport (Jensen et al., 1999). Since α-synuclein can be associated with rat brain vesicles (Jensen et al., 1998), it is not surprising that its transport parameters differ from those of cytoskeletal proteins. In particular, the average velocity of α-synuclein-containing particles (160 mm/day) is considerably faster than that of tau-containing structures (30 mm/day), further suggesting that different mechanisms, for example variable association with specific molecular motors, might underlie the regulation of α-synuclein and tau axonal transport. The variation in transport rates for proteins that have been shown to associate with kinesin is very wide, for example, the average transport rate for neurofilaments is approximately 30 mm/day (Wang et al., 2000), whereas for amyloid precursor protein the average transport rate approaches 400 mm/day (Kaether et al., 2000), hence the variation between the rates of tau and α-synuclein transport seen here is not unexpected.

Although this study has focused on the axonal transport of tau and α-synuclein themselves, recent studies have brought to the forefront the importance of tau in the regulation of axonal transport of other organelles. For example, overexpression of tau in culture models results in inhibition of transported components such as mitochondria (Sato-Harada et al., 1996; Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Seitz et al., 2002). It has been proposed that the mechanism involved in this inhibition of transport by tau may be caused by competition of tau with kinesin for binding sites on microtubules (Stamer et al., 2002; Seitz et al., 2002). Since our data suggest that tau and kinesin can interact directly, an alternative explanation is that, when overexpressed, tau could sequester kinesin, preventing the motor from binding to other cargoes, and allowing the accumulation of neuronal components such as mitochondria in the cell body. Indeed when tau is overexpressed, although mitochondrial transport is ablated, tau does not inhibit its own transport along axons (Stamer et al., 2002; Utton et al., 2002), suggesting that movement of tau is not inhibited by excessive coating of the microtubules by the overexpressed tau.

The physiological and functional significance of the directly observed fast transport of tau and α-synuclein has yet to be fully determined. In disease conditions, however, where the amount of tau may be increased (Khatoon et al., 1992; Savaskan et al., 2001), or the ratio of tau bound to microtubules may be altered, for example due to altered tau phosphorylation or tau mutations, the regulatory effects of tau as well as the transport of tau itself may be affected. In the case of α-synuclein, it has already been shown that mutant (A30P) α-synuclein, which causes a form of familial Parkinson's disease, abolishes α-synuclein-binding to vesicles (Jensen et al., 1998). We have reported that A30P α-synuclein exhibits significantly reduced transport in cultured neurons compared to wild-type α-synuclein (Saha et al., 2004), although another recent study did not detect any effects on slow axonal transport in peripheral nerves of transgenic mice expressing human A30P α-synuclein (Li et al., 2004). Perturbations in the axonal transport rates of tau and α-synuclein, such as may occur in neurodegenerative disease, could therefore result in the formation of intracellular aggregates and eventual cell death in affected neurons.

In conclusion, this study has begun to elucidate the mechanisms involved in the axonal transport of two proteins, tau and α-synuclein, both of which are known to be involved in neurodegenerative disease. It has also highlighted the fact that, similar to several other transported proteins, the overall slow rate of axonal transport of tau and α-synuclein may be mediated through fast transport motors, including kinesin and dynein.

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