Parkinson’s disease α-synuclein mutations exhibit defective axonal transport in cultured neurons

Anirban R. Saha1,*, Josephine Hill1,*, Michelle A. Utton1, Ayodeji A. Asuni1, Steven Ackerley1, Andrew J. Grierson1, Christopher C. Miller1, Alun M. Davies2, Vladimir L. Buchman2, Brian H. Anderton1 and Diane P. Hanger1,‡

1Department of Neuroscience, PO Box 38, Institute of Psychiatry, King’s College London, De Crespigny Park, London, SE5 8AF, UK
2Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Edinburgh, EH9 1QH, UK
*These authors contributed equally to this work
‡Author for correspondence (e-mail: d.hanger@iop.kcl.ac.uk)

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Summary

α-Synuclein is a major protein constituent of Lewy bodies and mutations in α-synuclein cause familial autosomal dominant Parkinson’s disease. One explanation for the formation of perikaryal and neuritic aggregates of α-synuclein, which is a presynaptic protein, is that the mutations disrupt α-synuclein transport and lead to its proximal accumulation. We found that mutant forms of α-synuclein, either associated with Parkinson’s disease (A30P or A53T) or mimicking defined serine, but not tyrosine, phosphorylation states exhibit reduced axonal transport following transfection into cultured neurons. Furthermore, transfection of A30P, but not wild-type, α-synuclein results in accumulation of the protein proximal to the cell body. We propose that the reduced axonal transport exhibited by the Parkinson’s disease-associated α-synuclein mutants examined in this study might contribute to perikaryal accumulation of α-synuclein and hence Lewy body formation and neuritic abnormalities in diseased brain.

Key words: Synuclein, Axonal transport, Lewy body, Parkinson’s disease, Aggregation, Neurodegeneration

Introduction

α-Synuclein is a 14 kDa phosphoprotein of 140 amino acids that was first identified as a presynaptic protein in rat brain (Maroteaux et al., 1988). The normal function of α-synuclein remains unknown, although it might be involved in synaptic plasticity (George et al., 1995), maintenance of the synaptic vesicle pool (Murphy et al., 2000) and regulation of dopamine biosynthesis (Perez et al., 2002).

Two pieces of evidence suggest that α-synuclein has a causal role in neurodegenerative disease: (1) two rare point mutations in α-synuclein (Ala53Thr and Ala30Pro) have been found in a few families with autosomal dominant Parkinson’s disease (Polymeropoulos et al., 1997; Kruger et al., 1998); (2) α-synuclein accumulates in Lewy bodies and other pathological inclusions in conditions including Parkinson’s disease, multiple system atrophy, neurodegeneration with brain iron accumulation and Alzheimer’s disease (Masliah et al., 1996; Spillantini et al., 1997; Lippa et al., 1998; Spillantini et al., 1998; Gai et al., 1998; Irizarry et al., 1998; Braak et al., 1999; Iwanaga et al., 1999; Hamilton, 2000), thus highlighting its importance in the pathogenesis of these neurodegenerative disorders. Furthermore, overexpression of α-synuclein in Drosophila (Feany et al., 2000) and transgenic mice (Masliah et al., 2000; Kahle et al., 2000) results in pathological accumulation of α-synuclein and neuronal dysfunction. Thus, overexpression of α-synuclein within distinct subcellular compartments might lead to the formation of perikaryal Lewy bodies and other pathological accumulations of α-synuclein.

α-Synuclein is actively transported from its site of synthesis in the cell body along axons to synaptic termini. Metabolic labelling of neuronal proteins in the rat optic nerve has shown that most α-synuclein (76%) moves in the slow component of axonal transport (Jung et al., 2000). In addition to phosphorylation of α-synuclein on serine residues, tyrosine phosphorylation of α-synuclein has also been reported, both in vitro and in cells (Nakamura et al., 2001; Ellis et al., 2001; Negro et al., 2002; Nakamura et al., 2002). However, any effects of tyrosine or serine phosphorylation of α-synuclein on its axonal transport...
have not been determined and this is one of the purposes of this study.

We have investigated the effects of the Parkinson’s disease-associated A30P and A53T mutations in α-synuclein on its axonal transport in cultured rat brain cortical neurons. We have also examined the transport of α-synuclein mutants that mimic serine or tyrosine phosphorylation at three defined sites to determine whether these phosphorylation states have a role in the regulation of α-synuclein transport in neurons.

Materials and Methods
Expression constructs and site-directed mutagenesis of α-synuclein
Human α-synuclein cDNA (gift from M. Goedert, Cambridge) was cloned into the EcoRV and HindIII sites of pcDNA 3.1(−) (Invitrogen) or pEGFP-C (BD Biosciences). Site-directed mutagenesis of wild-type α-synuclein in pcDNA 3.1(−) was performed to generate mutant constructs using the polymerase chain reaction (Hemsley et al., 1989). The mutagenic oligonucleotides used were:

A30P, 5'-CAGAGGCCGCTGGAAAGA-3'
A35T, 5'-CATGGTGTCACAACAGTG-3'
S87D, 5'-TGGAGGGCGCCGGGCATTCGCA-3'
S87A, 5'-TGGAGGGCGCCGGGCATTCGCA-3'
S129D, 5'-AATCTAGTTTACGCTAGGATGCTAGGATCTGATACCTTCCCTTCATGACGGCATTTC-3'
S129A, 5'-AATCTAGTTTACGCTAGGATGCTAGGATCTGATACCTTCCCTTCATGACGGCATTTC-3'
Y125F, 5'-CTTAAGCTTTAAGGCTAGGATGCTAGGATCTGATACCTTCCCTTCATGACGGCATTTC-3'
Y125D, 5'-CTTAAGCTTTAAGGCTAGGATGCTAGGATCTGATACCTTCCCTTCATGACGGCATTTC-3'
Y125F, 5'-CTTAAGCTTTAAGGCTAGGATGCTAGGATCTGATACCTTCCCTTCATGACGGCATTTC-3'

All mutations were confirmed by DNA sequencing (Oswel Research Products). A plasmid expressing the targeting sequence from subunit VIII of cytochrome c oxidase tagged with the fluorescent protein pDsRed2-C was used to label mitochondria (BD Biosciences).

Transfection and treatment of rat cortical neuronal cultures
Cortical neuronal cultures 5 days in vitro (DIV) were prepared from embryonic day 18 rats as previously described (Davis et al., 1995). Cells were transfected with plasmids containing human wild-type or mutant α-synuclein using calcium phosphate (Xia et al., 1996), followed by 5% (v/v) glycerol, 2% (v/v) dimethyl sulfoxide (Ackerley et al., 2000) for 2 minutes (glycerol shock). In some experiments, 3.5 hours after the glycerol shock, cells were exposed to either 0.05% (w/v) sodium azide and 50 mM 2-deoxy-D-glucose for 30 minutes or 2 μg ml⁻¹ cycloheximide for the duration of the experiment (Furukawa et al., 1997).

Measurement of α-synuclein axonal transport
To determine overall rates of axonal transport, we measured the distances travelled by exogenous α-synuclein along axons following transfection into rat cortical neurons. Cultures were fixed in 4% (w/v) paraformaldehyde and permeabilized for 2 minutes in 0.1% (w/v) Triton X-100 at 30 minute intervals from 2.5-6 hours after glycerol shock, some cultures were also fixed 24 hours after transfection. Neurons transfected with untagged α-synuclein were labelled with the rabbit polyclonal antibody to α-synuclein, CP90. Expression of enhanced green fluorescent protein (EGFP)- and DsRed2-tagged constructs was detected by direct fluorescence. Axonal distribution of α-synuclein was demonstrated by double staining with a rabbit polyclonal antibody to tau, TP70 (Brion et al., 1993). Images of transfected neurons were captured digitally and axonal transport was measured using Metamorph software (Universal Imaging) as the distance travelled by the fluorescent front from the point where the axon exits the cell body to the most distal point at which fluorescence was detectable above background in the longest, most brightly stained, neurite of transfected neurons. A single measurement was obtained for each transfected neuron and this value corresponded to the furthest detectable distance travelled by α-synuclein. To eliminate potential subjectivity, transport measurements were determined by observers blinded to the experimental conditions in each case. Results are expressed as mean±s.e.m. (n), where n is the number of neurons measured under each experimental condition, and Student’s t test was used for statistical analysis. Because the neurons were transfected at 5 DIV, a time at which the neurons extend long axonal processes whose average length exceeds 700 μm (Ackerley et al., 2000), the distance travelled from the cell body by the exogenous α-synuclein in these cultures reflects α-synuclein transport rather than neurite growth rate. We have shown previously that this long neurite has axonal properties (tau positive, MAP2 negative) and that the shorter neurites surrounding the cell body are dendritic (tau negative, MAP2 positive) (Utton et al., 2002). Measurements of axonal transport were made only in neuronal cultures in which the transfection efficiency exceeded 1%. The time of glycerol shock was taken as the zero time in all experiments from which measurements were obtained (Ackerley et al., 2000). Typically, 20-30 morphologically normal, transfected neurons were measured on each coverslip and each experiment was repeated a minimum of three times. To estimate the relative amounts of α-synuclein protein expression by wild-type and mutant α-synuclein plasmids, neurons were labelled with α90 at 4 hours after transfection and the average immunofluorescence intensity of each neuronal cell body was measured using a Zeiss Axioscope (Carl Zeiss) and Metamorph software for image capture. The background intensity of an area equivalent to each cell body was subtracted from the total immunofluorescence in a minimum of 80 neurons transfected with plasmids expressing wild-type or mutant α-synuclein and the net immunofluorescence values were compared using Student’s t test. Cell death was assessed by bis-benzimide (Sigma-Aldrich) staining of nuclei and by determining the proportion of cells exhibiting nuclear condensation, fragmentation or retraction of neuronal processes. Cells expressing exogenous α-synuclein were excluded from analysis if any of these morphological signs of decreased neuronal viability were observed. For estimations of protein expression and cell death, 20-50 transfected neurons were analysed on each coverslip and at least three coverslips were counted in each experiment.

Results
Transfected wild-type α-synuclein is transported along axons in rat cortical neurons
The α-synuclein antibody used in this study (c90), labels both rodent and human α-synuclein but, under the experimental conditions described here, rat primary cortical neurons at 5 DIV express very low levels of endogenous rat α-synuclein, so we detected only exogenously expressed α-synuclein in the transfected neurons. Expression of transfected wild-type human α-synuclein in rat primary cortical neurons was first apparent in a few neuronal cell bodies and extended a short distance into the neurites at ~2.5 hours after glycerol shock (Fig. 1A). However, by 3.5 hours after transfection, we observed α-synuclein immunoreactivity at the extremities of some individual neurites, at long distances from the cell body, indicating that these accumulations represent α-synuclein that had already reached the terminals and could have done so in this time if they had travelled at a rate equivalent to that of organelles moving in the fast component of axonal transport (data not shown). However, this distally located α-synuclein represented only a minor proportion of the total exogenous α-synuclein.
**α-Synuclein mutants affect axonal transport**

The distance travelled by A30P and A53T α-synuclein is reduced

The distance travelled by A30P and A53T α-synuclein by 6 hours after transfection into cortical neurons was lower than for the wild-type protein (Fig. 2A). At 24 hours after glycerol shock, transfection of A30P α-synuclein (unlike A53T and wild-type α-synuclein) also resulted in the presence of large swellings filled with α-synuclein proximal to the neuronal cell body in some neurons (Fig. 2A). To determine whether there was a quantitative difference between the axonal transport of wild-type and the Parkinson’s-disease-associated mutant α-synucleins, we measured the distances travelled by A30P and A53T α-synuclein between 3 hours and 6 hours after transfection (Fig. 2B). The distances travelled by each of the α-synuclein expressed in the cortical neurons and so this was not included in measurements of bulk α-synuclein transport. By 6 hours after transfection, α-synuclein was present in neuronal cell bodies and also extended along the neurites (Fig. 1A); by 24 hours, α-synuclein was detected along the length of the longest neurite in transfected cells (Fig. 1A). The paucity of expression of endogenous α-synuclein in cortical neurons at 5 DIV is illustrated by the absence of α90 labelling of untransfected neurons. Discrete particles of α-synuclein were observed in approximately 25% of neurites of transfected neurons at 6 hours after transfection and the frequency of these particles gradually increased until almost all transfected neurons exhibited particulate α-synuclein along the length of the longest neurite at 48 hours after transfection. Staining of α-synuclein transfected neurons for tau, which is axonal in distribution, with antibody TP70, revealed a co-distribution of α-synuclein with tau (Fig. 1A, arrows), showing that α-synuclein is located primarily in axons and proximal regions of dendrites in transfected cortical neurons (Fig. 1A, arrowheads).

To determine the overall rate of transport of human α-synuclein in cortical neurons, we used a previously described assay that measures the bulk movement of transfected protein by analysing the distance travelled by the fluorescent front at set time points in fixed cells (Ackerley et al., 2000). This assay has been used successfully to measure axonal transport of both neurofilaments and tau protein (Ackerley et al., 2000; Utton et al., 2002; Brownlees et al., 2002). At 3 hours, most transfected neurons exhibited a single, brightly fluorescent process that extended further than the other neurites, so 3 hours after glycerol shock was selected as the earliest time at which we could obtain a reliable and reproducible measure of α-synuclein transport in neurons. Wild-type α-synuclein was transported a mean distance of 162 μm from the axon hilum between 3 hours and 6 hours after glycerol shock, equivalent to an overall transport rate of 54 μm h⁻¹ during this time, equivalent to ~1.3 mm day⁻¹. Treatment of α-synuclein-transfected cultures with the metabolic inhibitors sodium azide and 2-deoxy-D-glucose for 30 minutes at 3 hours after glycerol shock reduced the rate of transport of α-synuclein to 12±6 μm h⁻¹ (n=110) from 61±7 μm h⁻¹ (n=90) in untreated cells, measured between 3.5 hours and 4 hours after glycerol shock (Fig. 1B). Removal of azide/deoxyglucose from the cultures resulted in a delayed recovery (~1 hour) of the rate of α-synuclein transport to that observed before treatment, showing that the reduced transport was not due to cell death. Thus, as we found previously for the axonal transport of neurofilaments, α-synuclein transport is an energy-requiring process that is not due to passive diffusion (Ackerley et al., 2000).

We could detect no differences between the subcellular localization or rate of transports of wild-type and mutant EGFP-tagged α-synuclein constructs (in which EGFP was fused to the N-terminus of α-synuclein) and their untagged counterparts. However, human wild-type α-synuclein expressed as a C-terminally tagged EGFP fusion protein aggregated in transfected rat cortical neurons, suggesting that addition of EGFP to the C-terminus of α-synuclein has a deleterious effect on protein folding and resulting conformation in neurons (not shown).

**Fig. 1.** Distribution and transport of transfected α-synuclein in neurons. (A) Rat cortical neurons 5 DIV were transfected with wild-type (WT) α-synuclein and fixed at intervals after glycerol shock. Panels show the expression of exogenous α-synuclein, detected with the α90 antibody, at 2.5 hours, 6 hours and 24 hours after glycerol shock. Cells fixed at 6 hours after glycerol shock are double labelled with α90 (left) and the antibody to tau, TP70 (right). Arrows indicate co-distribution of α-synuclein and tau, arrowheads indicate dendritic extensions containing α-synuclein. Scale bars, 50 μm. (B) Rat cortical neurons (5 DIV) were transfected with wild-type α-synuclein and labelled with α90 at 30 minute intervals from 3 hours to 6 hours after glycerol shock. Neurons were either untreated (circles) or treated with the metabolic inhibitors sodium azide and 2-deoxy-D-glucose (triangles) between 3 hours and 3.5 hours after glycerol shock. Points represent mean distance travelled (in μm) from the axon hilum ± s.e.m.
mutants were significantly reduced compared with the wild-type protein at 3, 4, 5 and 6 hours after glycerol shock (Fig. 2B, $P<0.05$). Both of the mutant $\alpha$-synuclein proteins travelled $\sim$140 $\mu$m from the axon hilum by 6 hours, equivalent to a rate of $\sim$30 $\mu$m hour$^{-1}$ or 0.7 mm day$^{-1}$ measured between 3 hours and 6 hours after glycerol shock. However, we observed that the rate of transport of wild-type and mutant $\alpha$-synuclein was not linear throughout the 3-hour observation time, so we examined the rates of transport of each construct at hourly intervals after glycerol shock (Fig. 2C). We found that wild-type $\alpha$-synuclein was transported faster than both A30P and A53T $\alpha$-synuclein at 3-4 hours and 4-5 hours after glycerol shock, and this difference was statistically significant for both mutant proteins ($P<0.001$). However, the transport rate of wild-type $\alpha$-synuclein was significantly decreased from 62 $\mu$m hour$^{-1}$ at 3-4 hours after transfection to 45 $\mu$m hour$^{-1}$ at 5-6 hours ($P<0.05$); at this later time, the rates of transport of wild-type and mutant $\alpha$-synucleins were similar. This suggests that the most significant effects of the A30P and A53T mutations in $\alpha$-synuclein are exerted at the earlier time points and that, at 5-6 hours after transfection, the rates of mutant $\alpha$-synuclein transport recovers to such an extent that the effects of the mutations are reduced.

Because the amounts of $\alpha$-synuclein expressed by the wild-type, A30P and A53T $\alpha$-synuclein constructs might influence their relative rates of transport in neurons, we measured the intensity of immunofluorescence as a measure of the amount of each exogenously expressed protein in the neuronal cell body at 4 hours after transfection. We found that neurons transfected with the plasmid expressing A30P $\alpha$-synuclein exhibited more immunofluorescence than those expressing wild-type or A53T $\alpha$-synuclein at this time (Fig. 2D, $^*P<0.05$). A53T $\alpha$-synuclein exhibits reduced axonal transport and both wild-type and A53T $\alpha$-synuclein are expressed in equal amounts, which suggests that the decreased transport of A53T $\alpha$-synuclein is not due to altered expression or turnover of the mutant protein. Furthermore, because the amount of cellular A30P $\alpha$-synuclein was increased and transport of A30P $\alpha$-synuclein was decreased compared with the wild type, the neuronal inclusions observed after A30P $\alpha$-synuclein expression might be caused by a combination of decreased metabolism and/or reduced transport of this mutant $\alpha$-synuclein expression.

Fig. 2. $\alpha$-Synuclein mutants associated with Parkinson’s disease exhibit reduced transport in neurons. (A) Rat primary neuronal cortical cultures were transfected at 5 DIV with wild-type (WT), A53T or A30P $\alpha$-synuclein and fixed 6 hours or 24 hours after transfection. $\alpha$-Synuclein expression was detected with anti-$\alpha$90. Arrows indicate the extent of $\alpha$-synuclein migration in the longest neurite extending from each neuron. Swellings containing A30P mutant $\alpha$-synuclein are indicated by arrowheads. Scale bars, 50 $\mu$m. (B) Human wild-type (circles), A30P (squares) and A53T (triangles) $\alpha$-synuclein were transfected into 5 DIV rat cortical cultures and labelled with $\alpha$90 at 3 hours, 4 hours, 5 hours and 6 hours after glycerol shock. Points represent mean distance travelled (in $\mu$m) from the axon hilum ± s.e.m. The differences between the distances moved by wild-type and mutant $\alpha$-synucleins were statistically significant at each time point examined ($P<0.05$ at 3 hours, $P<0.01$ at 4 hours, 5 hours and 6 hours after glycerol shock (n=40-124 neurons)). (C) $\alpha$-Synuclein was transfected into 5 DIV rat cortical cultures as above and rates of transport were calculated at hourly intervals during the time of observation. Bars show mean rates ± s.e.m. for wild-type (black), A30P (white) and A53T (grey) $\alpha$-synuclein. $^*P<0.001$ compared with wild-type $\alpha$-synuclein. (D) Rat cortical neurons were transfected with human $\alpha$-synuclein plasmids, fixed 4 hours after glycerol shock and labelled with $\alpha$90. The average intensity of immunofluorescence within the neuronal cell body was measured for wild-type (n=96 neurons), A30P (n=138 neurons), and A53T (n=82 neurons) $\alpha$-synuclein. The asterisk indicates $P<0.05$ for A30P $\alpha$-synuclein average immunofluorescence intensity compared with the wild type. Bars represent mean intensity of immunofluorescence ± s.e.m.
form of the protein leading to an increased cellular concentration of α-synuclein.

Cycloheximide treatment of neurons transfected with wild-type α-synuclein at 3.5 hours after glycerol shock failed to affect α-synuclein transport (not shown), showing that reducing α-synuclein synthesis in neurons does not inhibit its transport. Taken together, these results suggest that the reduced transport of A30P and A53T α-synuclein that we observed in neurons is not due to altered levels of protein expression.

The calcium phosphate transfection protocol used for primary neuronal cultures compromises the viability of a small proportion of neurons, but we found no significant differences between the viability of neurons expressing exogenous wild-type or mutant α-synucleins (not shown). Furthermore, transport measurements were made only on neurons with a normal cellular morphology and bisbenzimide staining was used to exclude any neurons with nuclear abnormalities. Thus, the reduced transport of A30P and A53T mutant α-synucleins was not accounted for by impaired cell viability. To address any potential toxic effect of mutant α-synuclein relative to the wild-type protein in rat cortical neurons that might affect axonal transport more generally, we also investigated the distribution of mitochondria in transfected neurons. A plasmid expressing the targeting sequence from subunit VIII of cytochrome c oxidase tagged with DsRed2 (MITO) or co-transfected with the mitochondrial marker plus EGFP-tagged wild-type (WT) or A30P mutant α-synuclein (A30P), and fixed 24 hours after transfection. Protein expression was detected by direct fluorescence of DsRed2-cytochrome c oxidase (MITO), EGFP-tagged wild-type (WT) or A30P mutant (A30P) α-synuclein. Scale bar, 50 μm.

**Fig. 3.** α-Synuclein expression does not perturb axonal transport of mitochondria in rat cortical neurons. Rat primary neuronal cortical cultures (5 DIV) were transfected with a plasmid expressing the targeting sequence from subunit VIII of cytochrome c oxidase tagged with DsRed2 (MITO) or co-transfected with the mitochondrial marker plus EGFP-tagged wild-type (WT) or A30P mutant α-synuclein (A30P), and fixed 24 hours after transfection. Protein expression was detected by direct fluorescence of DsRed2-cytochrome c oxidase (MITO), EGFP-tagged wild-type (WT) or A30P mutant (A30P) α-synuclein. Scale bar, 50 μm.

**Fig. 4.** Mutants that mimic permanent serine phosphorylation of α-synuclein exhibit reduced transport in rat cortical neurons. α-Synuclein mutants that mimic permanent states of phosphorylation (S87D, n=124 neurons, and S129D, n=56 neurons) or dephosphorylation (S87A, n=50 neurons, and S129A, n=53 neurons) of human α-synuclein, or wild-type human α-synuclein (WT, n=124 neurons) were transfected into DIV rat cortical neurons. Bars represent mean rate of transport (μm hour⁻¹) ± s.e.m. for each protein between 3 hours and 6 hours after glycerol shock. Transfection of the phosphorylation mimics of α-synuclein, S87D or S129D, resulted in statistically significant reductions in the rate of transport compared to wild-type α-synuclein (*P<0.001).

**Phosphorylation mimics of Ser87 or Ser129 reduce α-synuclein transport in neurons**

To investigate serine phosphorylation in α-synuclein as a possible mechanism involved in regulation of α-synuclein transport, plasmids expressing the following mutants were transfected into rat cortical neurons: serine residues 87 and 129 of human α-synuclein were individually mutated to mimic permanent states of phosphorylation (S87D and S129D) or dephosphorylation (S87A and S129A). There are now many examples in which introducing a charged residue accurately mimics the functional effect of phosphorylation of the protein (Leger et al., 1997; Antonsson et al., 1998). Measured between 3 hours and 6 hours after glycerol shock, S87D α-synuclein migrated at a rate of 31±2 μm hour⁻¹ (n=124) and S129D α-synuclein at 40±4 μm hour⁻¹ (n=56), compared with 54±2 μm hour⁻¹ (n=124) travelled by the wild-type protein. Hence, the transport of S87D was reduced by 43% and S129D transport by 26%, and both of these reductions were statistically significant compared with the movement of wild-type α-synuclein (Fig. 4, *P<0.001). By contrast, both S87A and S129A α-synuclein migrated at rates similar to the wild-type protein between 3 hours and 6 hours after glycerol shock (52±3 μm hour⁻¹, n=53, and 49±4 μm hour⁻¹, n=50, for S87A and S129A, respectively, P>0.05). Hence, mimicking α-synuclein phosphorylation by replacing either S87 or S129 with aspartate reduces its rate of axonal transport but, by contrast, inhibiting phosphorylation at these sites by mutating serine to the non-phosphorylatable residue alanine does not appear to alter α-synuclein transport. Physiological phosphorylation of S87 and/or S129 in neurons might therefore have a role in the regulation of α-synuclein axonal transport.
Mutations of Y125 does not affect α-synuclein transport in neurons

Because α-synuclein has also been demonstrated to be phosphorylated on tyrosine residues, we investigated the potential role of phosphorylation at Y125 to regulate axonal transport of α-synuclein. We either replaced Y125 with aspartate (Y125D) or with phenylalanine (Y125F) to mimic permanent dephosphorylation, and each of these constructs was transfected into rat cortical neurons. The distributions of the tyrosine mutants Y125D and Y125F in rat cortical neurons were identical to the wild-type protein at all time points examined between 3 hours and 6 hours after transfection (Fig. 5A, 4.5 hours). The rates of transport of Y125D, Y129F and wild-type α-synuclein measured between 3 hours and 4.5 hours after glycerol shock were 49 μm hour⁻¹, 56 μm hour⁻¹, and 51 μm hour⁻¹, respectively, and the rates were also not significantly different at other intervals between 3 hours and 6 hours after transfection, demonstrating that the tyrosine mutants moved at the same rate as wild-type α-synuclein (Fig. 5B, P>0.05). These results suggest that the phosphorylation state of tyrosine 125 in α-synuclein is not involved in the regulation of its axonal transport.

Discussion

In this study, we have used transfected rat cortical neurons as a model to investigate α-synuclein transport following a method developed previously in this laboratory to study axonal transport of neurofilaments and tau protein (Ackerley et al., 2000; Uutton et al., 2002; Brownlee et al., 2002; Ackerley et al., 2003). One condition of using such a method, however, is that the results obtained from cultured cells should correspond to those obtained from in vivo studies.

Axonal transport of α-synuclein has been studied previously in the rat optic nerve and most α-synuclein has been shown to travel in the slow component of axonal transport at 1.9-2.4 mm day⁻¹ (Jensen et al., 1999). Here, we have used a simple cellular system and found that the bulk of α-synuclein is transported along axons of transfected neurons at an average rate equivalent to ~1.3 mm day⁻¹. Our results in transfected cells are thus broadly similar to those observed in vivo in the optic nerve. For cytoskeletal protein transport, a slow overall rate of movement is now known to be due to intermittent conventional fast transport (Wang et al., 2000; Wang et al., 2002) and this might well be the case for α-synuclein. These results therefore indicate that our system is a good cellular model, comparable to in vivo models, for determining the parameters of α-synuclein axonal transport.

Axonal transport of both A30P and A53T, α-synuclein mutations associated with rare forms of familial Parkinson’s disease, was significantly attenuated compared with the wild-type protein. Hence, reduced axonal transport of mutant forms of α-synuclein might explain, at least in part, the finding of A30P and A53T α-synuclein accumulation in transgenic mice (Sommer et al., 2000; Kahle et al., 2000; Van der Putten et al., 2000; Lee et al., 2002; Lo et al., 2002). The rates of transport of wild-type compared with A30P and A53T mutant α-synuclein differed most at the earlier time points in this study, possibly representing an increased lag phase in transport of the mutant proteins. This suggests to us that the rate of association of A30P and A53T α-synuclein with other proteins involved in access to the neuronal transport machinery might be affected by the presence of the A30P and A53T mutations in α-synuclein. One possibility is that the rate of association/disassociation of α-synuclein with the motor and/or accessory proteins involved in its initial transport from the cell body might be a crucial rate-determining step in α-synuclein axonal transport, and this might be disrupted by the two causal Parkinson’s disease mutations. At later times following transfection, the transport rate of mutant α-synuclein was less affected than at earlier times, when the newly synthesized mutant protein was starting to leave the cell body and travel along the proximal axon. This result is therefore in line with our hypothesis that the rate of α-synuclein egress from the cell body might be impeded by the presence of mutations.

A possible mechanism for the reduced rates of transport of A30P α-synuclein at early time-points might be related to inhibition of its interaction with lipid membranes and vesicles, which might slow the rate at which α-synuclein can associate with components involved in neuronal transport (Jensen et al., 1998; Perrin et al., 2000; Jo et al., 2002). It is possible that the rate of association and/or disassociation of α-synuclein with the proteins involved in its initial export from the cell body is a crucial rate-determining step in its axonal transport. Thus, it is feasible that a transport control mechanism might exist in which proteins are gated through a region of the axon close to the cell body, although further studies are required to confirm this hypothesis.
Phosphorylation of α-synuclein is known to occur on serine residues 87 and 129 as well as tyrosine residues (Okochi et al., 2000; Pronin et al., 2000; Nakamura et al., 2001; Ellis et al., 2001; Negro et al., 2002; Nakamura et al., 2002). Here, we have demonstrated that mimicking protein phosphorylation by mutating specific serine residues to aspartate might have a direct influence on α-synuclein axonal transport as each of the S87D and S129D mutations reduced the rate of α-synuclein transport. Serine 87 is unique to human α-synuclein and lies within the non-Aβ component of Alzheimer’s disease amyloid portion of α-synuclein, close to a region of α-synuclein that is pivotal in its ability to aggregate in vitro (Giasson et al., 2001). Phosphorylation of serine 87 might therefore have a significant influence on the interaction of α-synuclein with proteins involved in its transport. It has been reported recently that phosphorylation of serine 129 in α-synuclein promotes the formation of α-synuclein filaments in vitro (Fujiwara et al., 2002). α-Synuclein-containing lesions in human brain and in transgenic mice overexpressing human α-synuclein are highly phosphorylated at serine 129, suggesting that increased phosphorylation at this site might cause it to accumulate in diseased neurons (Fujiwara et al., 2002; Kahle et al., 2002). We therefore propose that at least some α-synuclein accumulations might result from mechanisms causing reduced transport of the phosphorylated form of the protein.

We found no effect of α-synuclein on axonal transport when tyrosine 125 was replaced by either aspartate or phenylalanine, suggesting that a mutation in α-synuclein at its C-terminus does not cause a generalized non-specific inhibitory effect on its transport in neurons. The Y125D tyrosine mutant used in this study is likely to have caused significant alterations in the structure adopted by α-synuclein in this region of the molecule and this might therefore have been expected to affect α-synuclein transport owing to conformational changes. In keeping with this assumption, the presence of the negatively charged aspartate residue in place of tyrosine did result in a slightly retarded electrophoretic mobility of Y125D α-synuclein on polyacrylamide gels, suggesting that the Y125D mutant α-synuclein does exhibit a different conformation to the wild-type protein (J.H. and D.P.H., unpublished). Successful mimics of permanent tyrosine phosphorylation have been reported using substitution of tyrosine for aspartate residues in proteins (Wu et al., 1998; Bonvini et al., 2001), although we cannot exclude the possibility that, in the case of α-synuclein, replacing tyrosine (a large hydrophobic amino acid) with aspartate is not an accurate mimic of phosphorylation in our model system. However, because neither Y125D nor Y125F α-synuclein affected transport rates in neurons, we conclude that there is no evidence for the involvement of tyrosine 125 in regulation of its axonal transport.

In conclusion, our results show that both of the Parkinson’s disease-associated mutations in α-synuclein significantly reduce its rate of transport in neurons and that phosphorylation also appears to have an important role to play in the regulation of α-synuclein axonal transport. Defects in α-synuclein or its processing enzymes might be responsible for inhibition of normal transport processes in neurons in neurodegenerative disease and this could lead to the development of Lewy pathology and/or neuronal death. Using the model cellular system described here, we can now begin to examine the mechanisms involved in α-synuclein transport in neurons, including identifying the motor proteins. This is a key step in expanding our understanding of potential mechanisms involved in the pathogenesis of neurodegenerative disease.

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