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Dopaminergic neuronal loss and motor deficits in *Caenorhabditis elegans* overexpressing human α-synuclein

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Abstract

Overexpression of human α-synuclein in model systems, including cultured neurons, drosophila and mice, leads to biochemical and pathological changes that mimic synucleopathies including Parkinson's disease. We have overexpressed both wild-type (WT) and mutant alanine53 → threonine (A53T) human α-synuclein by transgenic injection into *Caenorhabditis elegans*. Motor deficits were observed when either WT or A53T α-synuclein was overexpressed with a pan-neuronal or motor neuron promoter. Neuronal and dendritic loss were accelerated in all three sets of *C. elegans* dopaminergic neurons when human α-synuclein was overexpressed under the control of a dopaminergic neuron or pan-neuronal promoter, but not with a motor neuron promoter. There were no significant differences in neuronal loss between overexpressed WT and A53T forms or between worms of different ages (4 days, 10 days or 2 weeks). These results demonstrate neuronal and behavioral perturbations elicited by human α-synuclein in *C. elegans* that are dependent upon expression in specific neuron subtypes. This transgenic model in *C. elegans*, an invertebrate organism with excellent experimental resources for further genetic manipulation, may help facilitate dissection of pathophysiologic mechanisms of various synucleopathies.

Keywords: α-synuclein, model organism, motor neuron, neurodegeneration, worm transgenic.

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Synucleopathies represent a large range of neuropathologically defined conditions that include Parkinson's disease (PD), dementia with Lewy bodies, Pick's disease and multiple system atrophy (Spillantini *et al*. 1997, 1998; Baba *et al*. 1998; Takeda *et al*. 1998). PD is a neurodegenerative disorder that affects 1% of the population over the age of 50 years. PD neurodegeneration is found predominantly in dopaminergic neurons of the substantia nigra where the pathological hallmark is the appearance of intracellular inclusions termed Lewy bodies. These bodies consist of protein complexes that include neurofilaments, ubiquitin and α-synuclein (Forno 1996; Spillantini *et al*. 1997). Rare familial forms of PD have provided an opportunity to understand the pathophysiologic mechanisms of this disease and at least eight PD loci have been identified (Lansbury and Brice 2002). Autosomal dominant forms of PD have been linked to mutations in α-synuclein. Two mutations, alanine to threonine at position 53 (A53T) and alanine to proline at position 30 (A30P), have been identified that are highly penetrant (Polymeropoulos *et al*.

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Abbreviations used: A53T, mutant alanine53 → threonine; ADE, anterior deirid; CEP, cephalic neurons; GFP, green fluorescent protein; PD, Parkinson's disease; PDE, posterior deirid; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; TBSB, Tris-buffered saline with 0.5% bovine serum albumin; WT, wild type.

1997; Kruger *et al*. 1998). Another autosomal dominant form of PD is caused by a mutation in ubiquitin carboxy-terminal hydrolase (UCH-L1) (Leroy *et al*. 1998). Recently, it was discovered that UCH-L1 also contains dimerization-dependent, ubiquityl ligase activity (Liu *et al*. 2002). Mutations in *parkin* represent a gene defect that underlies PD with an autosomal recessive mode of inheritance (Lucking *et al*. 2000). Parkin, like numerous other RING finger-containing proteins, has E3 ubiquitin ligase activity and autosomal recessive juvenile-linked Parkin mutants are defective in E3 activity.

A model that describes the interactions of mutant forms of α-synuclein, Parkin and UCH-L1 has been proposed (Shimura *et al*. 2001), whereby mutation leads to over-accummulation of α-synuclein and formation of Lewy bodies. Most patients with Parkin mutations lack Lewy bodies. Nonetheless, sporadic cases constitute the substantial bulk of PD cases in which the wild-type (WT) form of α-synuclein is present. A mechanism by which dopaminergic neurons are selectively lost in PD has been proposed by Conway *et al*. (2001) and Xu *et al*. (2002). It is suggested that oxidative ligation of α-synuclein to dopamine leads to accumulation of neurotoxic adducts.

The best studied rodent and primate models of PD use the administration of neurotoxic agents such as 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which target dopaminergic neurons. *Cenorhabditis elegans* contain well defined dopaminergic neurons that are visible under fluorescence microscopy (Sulston *et al*. 1975; Nass *et al*. 2002) and have a range of locomotor activities (Thomas and Lockery 1999). 6-Hydroxydopamine has been administered to *C. elegans* whereupon dopaminergic neuron degeneration and membrane blebbing of axons and dendrites have been demonstrated (Nass *et al*. 2002).

Several different α-synuclein-overproducing transgenic mouse models and a rat virally transduced model have been produced (Kahle *et al*. 2000; Masliah *et al*. 2000; van der Putten *et al*. 2000; Kahle *et al*. 2002; Kirik *et al*. 2002; Lee *et al*. 2002; Richfield *et al*. 2002). Although many of the results obtained in these models indicate neuronal abnormalities, the studies report differences in time course, progression of pathologic effects, effects elicited by WT or mutant forms, and localization of pathologic changes. In order to explore the effects of α-synuclein in a less complex genetic environment, with a better defined neuronal system, and to obtain better control of the transgene expression, we have overexpressed human WT and a mutant form of α-synuclein in *C. elegans*. We have taken advantage of different promoters to direct expression of the transgenes into specific subsets of neurons. This transgenic *C. elegans* model that overexpresses α-synuclein might help to elucidate the pathophysiology of synucleopathies and facilitate the development of novel therapeutic strategies.

Materials and methods

Plasmid constructs

The transgenic and neuronal markers $P_{dat\text{-}1}$::green fluorescent protein (GFP) (Nass *et al*. 2001; Nass *et al*. 2002) and P_{aex3}::GFP (Iwasaki *et al*. 1997) were constructed as described previously. Human α-synuclein was amplified by RT–PCR (Finnzymes, Helsinki, Finland) from human brain RNA using primers (5′-AAAGGAATTCATTAGCCATG; 3′-GGGAGCAAAGATATTTCTTA). The cDNA was cloned into pGEM-TEasy (Promega, Madison, WI, USA) and the sequence matched precisely the coding region for GenBank entry XM_003494. The insert was cloned into the *Sma*I site of TJ644 (Iwasaki *et al*. 1997) to create $P_{aex\text{-}3}$::WT. The A53T mutant form of α-synuclein was prepared by the overlapping PCR-mutagenesis strategy (Ausubel *et al*. 1997) and cloned into the *Msc*I site of TJ1078 (Iwasaki *et al*. 1997) to create $P_{aex\text{-}3}$::A53T. The *acr-2* promoter (Hallam *et al*. 2000) was cloned into the *Hind*III–*Bam*HI site of pPD49.26 (Professor Andy Fire, Carnegie Institute of Washington, Baltimore, MD, USA) and the cDNA for wild-type α-synuclein was cloned to the *Nco*I site, whereas mutant α-synuclein was cloned to the *Nco*I–*Sac*I site, to create $P_{acr\text{-}2}$::WT and $P_{acr\text{-}2}$::A53T respectively. The *unc-30* promoter (Allyson McCormick, University of Washington, Seattle, WA, USA) was cloned into the *Hind*III–*Xba*I site of pPD49.26, and the cDNA for wild-type α-synuclein was cloned to the *Nco*I site and that for A53T mutant α-synuclein to the *Nco*I–*Sac*I site to create $P_{unc\text{-}30}$::WT and $P_{unc\text{-}30}$::A53T respectively. The DAT-1 promoter was amplified and cloned into the *Hind*III–*Bam*HI site of pPD95.73 (Andy Fire). This promoter was exchanged with the *unc-30* promoter of $P_{unc\text{-}30}$::WT and $P_{unc\text{-}30}$::A53T by *Hind*III–*Sma*I digestion and ligation to create $P_{dat\text{-}1}$::WT and $P_{dat\text{-}1}$::A53T. All plasmids were sequenced to verify cDNA identity, cloning sites and correct orientation.

Transgenics

Transgenic animals were generated using microinjection techniques as described previously for *C. elegans* (Mello and Fire 1995). The standard DNA concentration injected was 70 ng/μL per construct. pBSK plasmid (Stratagene, La Jolla, CA, USA) was used as carrier DNA for controls. Co-injection of either $P_{aex\text{-}3}$::GFP or $P_{dat\text{-}1}$::GFP was used as a marker for transgenic animals, and simultaneously to help visualize and locate neurons. A Nikon (Tokyo, Japan) SMZ800 microscope equipped with a P-FLAP fluorescence attachment was used for routine selection of transgenic animals. Transgenic animals collected for the assays were generally from F2–F5 generations.

Measurement of dopaminergic neurons

L4 transgenic animal lines produced by co-injection with $P_{dat\text{-}1}$::GFP as a marker were selected by fluorescence microscopy a day before the assay and transferred to a fresh plate. On the day of the assay, 10–15 worms at a time were transferred to an agar pad and examined using an Olympus AX70 fluorescence microscope with an Olympus (Tokyo, Japan) UPlan Apo 60X/0.90 objective. The different classes of dopamine neurons [cephalic neurons (CEP), anterior deirid (ADE) and posterior deirid (PDE)] were clearly visible, and the locations and dendritic patterns agreed with those described previously (White *et al*. 1986; Nass *et al*. 2002). Presence of a cell body, dendritic morphology and positions were scored individually for each worm. Photographs were taken with a Sensys

camera (Photometrics, Roper Scientific, Tucson, AZ, USA) that was connected to the microscope. Cell bodies and dendrites were scored as present if fluorescence could be seen. Dendrites were scored as abnormal if they had breaks or were barely visible.

Thrashing assay

L4 transgenic animals were selected by fluorescence microscopy a day before the assay and transferred to a fresh plate. On the day of the assay, animals were placed on to a 10-μL drop of M9 buffer on a standard microscope slide and allowed to equilibrate for ∼30 s. Animals were scored for the number of times the head crossed an axis drawn across the length of the body in 30 s (Miller *et al*. 1996; Thomas and Lockery 1999).

Immunohistochemistry and immunoblotting

The whole-mount freeze-cracking method was carried out according to Crittenden and Kimble (1999) with a few modifications. Worms were collected into 0.5 mL of M9 medium from starved agar plate and transferred to a centrifuge tube. Worms were spun for 2 min at 225 *g* and extra medium was removed. Worm suspension (8 μL volume) was transferred on to an object glass that was covered with subbing solution. No paraformaldehyde was added. A coverslip was placed on top of the animals and extra liquid was removed with Whatman #1 paper. After freeze-cracking, worms were blocked in Tris-buffered saline with 0.5% bovine serum albumin (TBSB) for 30 min at 22°C, incubated in 50 μL primary antibody (1 : 300 dilution, mouse IgG_1 anti-α-synuclein; BD Transduction Laboratories, San Jose, CA, USA) in phosphate-buffered saline overnight at 4°C or 2 h at room temperature. Worms were washed three times each for 15 min with 200 μL TBSB and incubated with 100 μL secondary antibody (1 : 200 dilution, donkey anti-mouse IgG-RRX; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) in TBSB for 1 h at room temperature under a light cover. After washing again as described above, mounting medium was added (FluorSave™ Reagent; CalBiochem, LaJolla, CA, USA) and a coverslip applied. Worms were viewed under epifluorescence with either an Olympus AX70 microscope or Nikon Eclipse/Ultra VIEW confocal microscope.

For immunoblot analysis, 20-μg samples of cell extracts were fractionated on polyacrylamide gels under denaturing conditions and transferred to nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH, USA). α-Synuclein was detected with α-synuclein antibody (1 : 250; BD Transduction Laboratories), and immunocomplexes were visualized by using rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1 : 1000; Amersham Biotech, Amersham, UK) and chemiluminescent detection (Luminol; Sigma, St Louis, MO, USA).

Results

Transgenic worms were generated to provide a model with which to investigate the impact of α-synuclein expression on dopaminergic neuron integrity and motor performance. Promoters that direct pan-neuronal (*aex-3*), dopaminergic (*dat-1*), or motor neuron-specific expression (*acr-2, unc-30*) were fused to WT and A53T mutant α-synuclein cDNAs and injected. Transgenic worms carrying constructs of promoters fused to GFP or a stop codon at the second amino acid position of α-synuclein had thrashing values similar to N2 wildtype worms (Table 1). Transgenic worms overexpressing WT and A53T forms of α-synuclein under control of a dopaminergic promoter (*dat-1*) also displayed thrashing values similar to N2 worms. Transgenic worms overexpressing WT and A53T α-synuclein under control of a pan-neuronal (*aex-3*) or motor neuron promoter (*acr-2*, *unc-30*) displayed significantly lower thrashing values in comparison to controls. An example of transgenic worms carrying a GFP marker only or GFP marker with $P_{aex\text{-}3}$::WT is shown in Figs 1(a) and (b).

The *C. elegans* dopamine transporter promoter (P*dat-1*) was fused to green fluorescent protein (GFP) and injected to mark dopaminergic neurons in worms under epifluoresence microscopy. The same promoter was fused to α-synuclein

Table 1 Transgenic overexpression of α-synuclein causes impairment in a thrashing assay which is a test of motoric movement

Construct	Location of expression	No. of lines	Thrashes per 30 s	*n*
$P_{aex\text{-}3}$::GFP	Pan-neuronal	3	96 ± 6	86
$P_{dat\text{-}1}$::GFP	Dopaminergic	4	91 ± 10	105
$P_{aex\text{-}3}$::STOP	Pan-neuronal	6	91 ± 7	96
$P_{dat\text{-}1}$::WT	Dopaminergic	15	98 ± 14	144
$P_{dat\text{-}1}$::A53T	Dopaminergic	8	96 ± 5	90
$P_{aex\text{-}3}$::WT	Pan-neuronal	5	56 ± 19*	127
$P_{aex\text{-}3}$::A53T	Pan-neuronal	3	48 ± 5†	116
$P_{unc\text{-}30}$::WT	Motor neurons	7	77 ± 18	135
$P_{unc\text{-}30}$::A53T	Motor neurons	6	65 ± 19*	86
$P_{acr\text{-}2}$::WT	Motor neurons	7	36 ± 17†	128
$P_{acr\text{-}2}$::A53T	Motor neurons	5	48 ± 12†	131

Adult worms (∼ 4 days old) were transferred from Petri dishes to microscope slides containing isotonic buffer. Each time the worm moved across its body axis a thrash was counted (Thomas and Lockery 1999). Thrashes per 30 s are shown as the mean ± SD. The total number of worms scored is shown (*n*). STOP, a stop codon was placed at amino acid position 2 of human α-synuclein WT cDNA. *$p < 0.05$, †$p < 0.001$ versus $P_{aex\text{-}3}$::GFP (Student *t*-test).

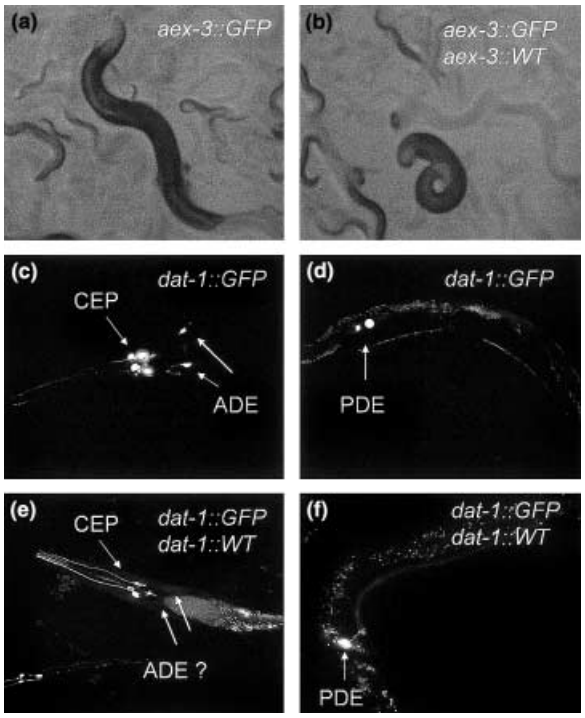

Fig. 1 Transgenic *C. elegans* overexpressing GFP markers and human α-synuclein. Worms were injected with $P_{aex\text{-}3}$::GFP, $P_{aex\text{-}3}$::WT and $P_{dat\text{-}1}$::GFP. (a) Transmitted light microscope image of an adult carrying the transgenic marker $P_{aex\text{-}3}$::GFP only. (b) Image of an adult carrying the transgenic marker and $P_{aex\text{-}3}$::WT. (c) Epifluorescence image of a transgenic worm injected with $P_{dat\text{-}1}$::GFP. The two pairs (four neurons) of CEP and one pair (two neurons) of ADE are indicated by arrows. Magnification 400 ×. (d) Image of a PDE neuron pair (two neurons). Magnification was 200 ×. (e) Image of $P_{dat\text{-}1}$::GFP; $P_{aex\text{-}3}$::WT-injected worm showing CEP but missing ADE neurons. (f) Image of $P_{dat\text{-}1}$::GFP; $P_{aex\text{-}3}$::WT worm showing only one of two PDE neurons.

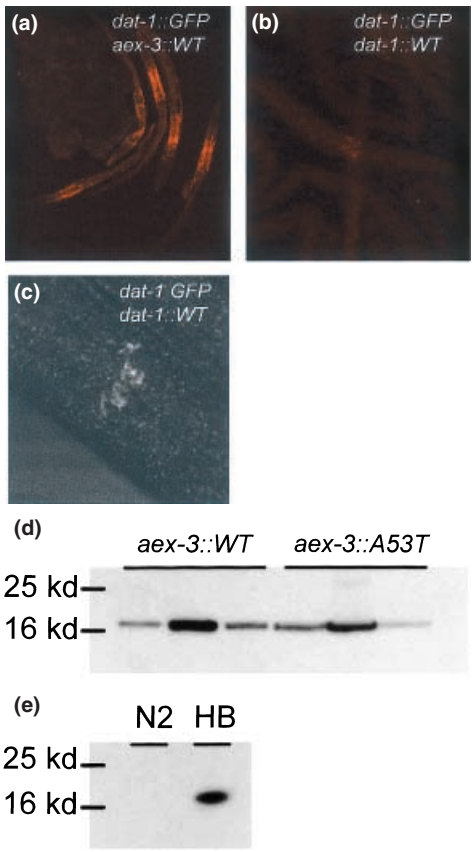

Fig. 2 Immunofluorescence images of *C. elegans* carrying $P_{aex\text{-}3}$::WT or $P_{dat\text{-}1}$::WT constructs. Worms (L2–L4 stage) were reacted with mouse anti-human-α-synuclein antibody and donkey anti-mouse IgG secondary antibody. (a) Reactivity can be seen with neurons in nerve ring and other head neurons. (b) Reactivity can be seen in CEP and ADE neurons. (c) Confocal image of transgenic *C. elegans* worm carrying $P_{dat\text{-}1}$::WT. Expression of α-synuclein as discrete intracellular inclusions can be seen. (d) Western blot of human α-synuclein proteins. Samples containing 20 μg protein per lane from three independently injected $P_{aex\text{-}3}$::WT or $P_{aex\text{-}3}$::A53T non-integrated transgenic worm lines were blotted. Sizes in kilodaltons (kd) are as indicated. (e) western blot of α-synuclein proteins. Protein (20 μg) from N2 worms carrying only a transgenic marker (*dat-1*::GFP) was compared with human brain cerebrum proteins (HB; 5 μg protein) as a control. No visible band can be observed from N2 worms. Size markers are shown.

WT and α-synuclein A53T, and co-injected with the marker plasmid. Hermaphrodite worms have four pairs of dopaminergic neurons: two pairs of CEP, one pair of ADE and one pair of PDE. These neurons were visible in worms injected with the marker plasmid (Figs 1b–e).

Immunohistochemical analysis demonstrated that α-synuclein was produced in dopaminergic cells when expression was driven by the *dat-1* promoter (Fig. 2b) and in many unspecified neurons within the nerve ring when the expression was driven by the *aex-3* promoter (Fig. 2a). Confocal microscopy demonstrated that, in some dopaminergic neurons, the *dat-1* promoter-driven expression appeared as intracellular inclusions (Fig. 2c). This event was rare and was observed in four of ∼200 neurons screened. Immunoblot analysis revealed anti-α-synuclein immunoreactivity in α-synuclein-expressing transgenic worms (Fig. 2d). Immunoreactivity varied between the lines carrying non-integrated WT and A53T α-synuclein constructs. Transmission of transgene expression to each generation was ∼20–50% and lines were maintained by regular selection. Immunoreactivity was observed in a human cerebrum lysate (BD Transduction Laboratories) but not in worms carrying the marker construct $P_{dat\text{-}1}$::GFP only (Fig. 2e).

Dopaminergic neurons were scored after injection of marker ($P_{dat\text{-}1}$::GFP plasmid) plus carrier, marker plasmid plus α-synuclein WT or marker plasmid plus α-synuclein A53T. A significant reduction in the number of dopaminergic neurons was observed in all sets after co-injection of the α-synuclein constructs (Fig. 3a). Neurons were scored from 30 worms each from four independently injected lines (120 worms) for each construct set. Data shown are mean ± SEM

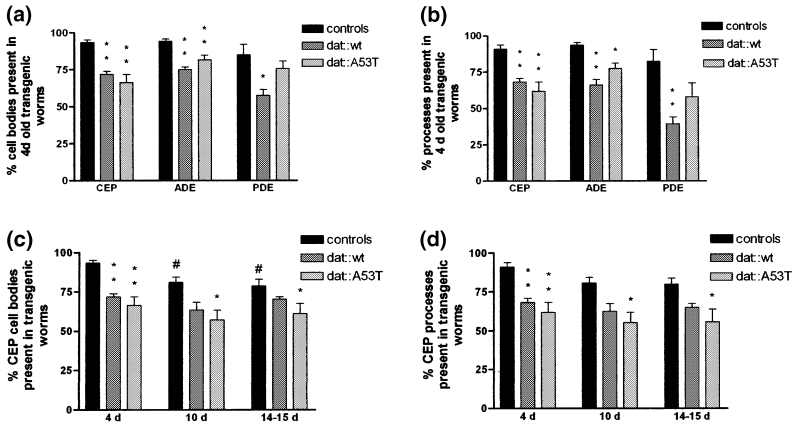

Fig. 3 Quantitation of dopaminergic neurons and dendritic processes in transgenic *C. elegans* carrying marker ($P_{dat\text{-}1}$::GFP) and carrier DNA (control), marker and $P_{dat\text{-}1}$::WT, or marker and $P_{dat\text{-}1}$::A53T. Neurons were quantitated under epifluorescence microscopy. (a, b) Analysis of dopaminergic neurons and processes from CEP, ADE and PDE sets. (c, d) Presence of dopaminergic neurons and processes at different ages. Values are mean ± SEM from four independently injected lines, 30 worms per line. The maximum score for each line is 120, 60 and 60 for CEP, ADE and PDE neurons respectively. $^*p < 0.05$, $^{**}p < 0.001$ versus controls from same neuron set and age; $\#p < 0.05$ versus control from same neuron set at 4 days (two-way ANOVA followed by Dunnett's post-hoc test).

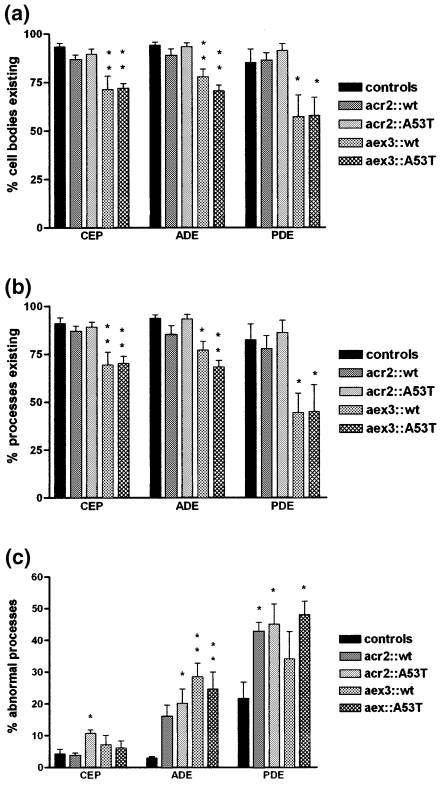

Fig. 4 Quantitation of dopaminergic neurons and dendritic processes in transgenic *C. elegans* carrying $P_{dat\text{-}1}$::GFP (marker) only, or marker with $P_{acr\text{-}2}$::WT, $P_{acr\text{-}2}$::A53T, $P_{aex\text{-}3}$::WT or P-$_{aex\text{-}3}$::A53T. (a) Neuron sets CEP, ADE and PDE were quantitated under epifluorescence microscopy. (b,c) Percentage processes existing and percentage abnormal processes were quantitated as described in the Methods section. Values are mean ± SEM from 3–6 independently injected lines, 30 worms per line. $^*p < 0.05$, $^{**}p < 0.01$ versus controls from same neuron set and age (one-way ANOVA followed by Dunnet's post-hoc test).

from the four lines. The percentage of CEP neurons present was 93 ± 1.9, 72 ± 2.0 and 66 ± 5.2% for control, WT and A53T respectively. Similar reductions were observed in ADE neurons (94 ± 1.4, 75 ± 1.8 and 82 ± 3.0% respectively) and PDE neurons (85 ± 7.0, 57.5 ± 4.0 and 76 ± 4.8% respectively). There were no observable differences between worms injected with WT or A53T forms of α-synuclein (two-way ANOVA). There were no apparent effects of age as similar results were obtained when worms were scored at 4, 10 or 14/15 days (Fig. 3c and d). Different worms were used for each time point. Loss of dendritic processes from dopaminergic neurons mirrored cell loss (Fig. 3b). Processes present in CEP neurons were (mean ± SEM): 91 ± 2.9, 68 ± 2.6 and 62 ± 6.4% for control, WT and A53T respectively. Similar values were observed in ADE processes (94 ± 1.7, 66 ± 3.8 and 78 ± 3.7% respectively) and PDE processes (83 ± 8.2, 40 ± 4.6 and 58 ± 9.7% respectively). Older worms showed similar results to those scored at 4 days (Fig. 3d).

Significant reductions in dopaminergic neurons were also observed when worms were transfected with α-synuclein constructs driven by the *aex-3* promoter but not by the motor neuron promoter *acr-2*. The *aex3*::WT and *aex-3*::A53T transgenic worms had mean ± SEM values of 71 ± 6.7 and 73 ± 4.3% for CEP neurons, 78 ± 4.0 and 74 ± 5.0% for ADE neurons, and 57 ± 11 and 56 ± 8.7% for PDE neurons respectively (Fig. 4a). Similar reductions were observed for the existence of dendrites in CEP and ADE dopaminergic neurons (Figs 4 b and c).

Discussion

In the present study, we produced transgenic *C. elegans* overexpressing human α-synuclein directed to different sets

of neurons. Motor deficits, as well as loss of dopaminergic neurons, loss of dendrites and increases in neuronal process breaks, were observed in transgenic worms expressing both WT and A53T forms of human α-synuclein.

Synucleopathies are age-related neurodegenerative conditions, whereas in this *C. elegans* transgenic model overexpressing α-synuclein, synucleopathy appeared to be a developmental process; no increase in dopaminergic neuronal loss was observed between young and old adults. Dopamine and dopamine transporters, which have been implicated in the neurotoxicity of α-synuclein (Conway *et al*. 2001; Lee *et al*. 2001; Xu *et al*. 2002), are present early on and are apparently sufficient for the observed dopaminergic neuron losses. As *C. elegans* has a short lifespan, approximately 21 days under laboratory conditions, longer-lived animals may have an increase in neurotoxicity. The results in *C. elegans* support the hypothesis that complex, accumulative, age-related processes that occur during etiology and pathogenesis are important for full development and progression of human synucleopathies. Mechanisms such as oxidative stress, mitochondrial dysfunction and environmental influences (Olanow and Tatton 1999) occurs in both humans and nematodes, but varies in *C. elegans* due to growth under controlled laboratory conditions.

α-Synuclein expression as inclusion bodies was rare and aggregation of α-synuclein was not observed in western blots (data not shown). This contrasts to Lewy bodies which are known to contain fibrils of α-synuclein (Spillantini *et al*. 1997, 1998). It is possible that *C. elegans* does not contain sufficient cellular components, such as polyamines, that promote fibrillization (Antony *et al*. 2003) or modifying enzymes (Junn *et al*. 2003). Alternatively, *C. elegans* may be more efficient at suppressing aggregation by expressing kinases (Negro *et al*. 2002; Seo *et al*. 2002), torsinA or heat-shock proteins (McLean *et al*. 2002). Finally, it has been reported that concentration greatly affects fibril formation (Uversky *et al*. 2001) and surviving dopaminergic neurons may not express sufficient α-synuclein for aggregation to take place.

Motor deficits, as measured by a thrashing assay, were dependent on the location of transgene expression. Deficits were observed when α-synuclein was expressed in motor neurons driven by *acr-2*, *unc-30* or *aex-3* promoters, but not when expression was limited to dopaminergic neurons. The *acr-2* gene encodes a non-α-subunit nicotinic acetylcholine receptor and the promoter directs expression to ventral cholinergic motor neurons (Hallam *et al*. 2000). The *unc-30* gene encodes a homeodomain transcription factor that is expressed in GABAergic D type motor neurons (Jin *et al*. 1994). Although several studies in mice and one in rat have demonstrated motor deficits following α-synuclein overexpression, it is not clear whether this was due to significant loss of dopaminergic terminals or motor neuron pathology. Motor neurons have been shown to be especially vulnerable to α-synuclein in transgenic mice (Sommer *et al*. 2000; van der Putten *et al*. 2000). A general neuronal promoter (Thy-1) was used to direct expression in these studies, so it remains to be determined whether axonal damage or denervation of neuromuscular junctions occurs by direct expression of α-syncuclein in motor neurons. The results presented here suggest that perturbations elicited by α-synuclein depend directly upon the subset of neurons in which the proteins are expressed. Thus, motor deficits were observed in this *C. elegans* model when α-synuclein was expressed in motor neurons.

Motor deficits were not observed in transgenic α-synuclein worms when the expression was directed to dopaminergic neurons. In *C. elegans*, dopaminergic neurons are thought to be mechanosensory and to modulate, but do not control motor responses (reviewed in Nass and Blakely 2003). Previous studies have indicated that ablation of all dopaminergic neurons is required for a differential locomotor response to bacteria (Sawin 1996). Moreover, *cat-1* mutants that are largely devoid of dopamine have similar thrashing rates to N2 WT worms (Duerr *et al*. 1999). Taken together with our data that show ∼30% loss of GFP-positive dopaminergic neurons in worms that overexpress α-synuclein, and a paucity of α-synuclein expression as cellular inclusions, the lack of correlation between dopaminergic cell loss and motor deficits may be expected.

Significant dopaminergic neuron loss and dendritic breaks were seen in worms injected with α-synuclein WT and A53T when dopaminergic (*dat-1*) or pan-neuronal (*aex-3*) promoters drove the expression, but not with a motor neuron promoter (*acr-2*). The promoters used, *dat-1* and *aex-3*, drive expression that can be detected in early larval stages (Iwasaki *et al*. 1997; Nass *et al*. 2002; data not shown) and both neuronal loss and motor deficits were observed at the young adult stage. These findings suggest that the cellular machinery needed to mediate the effects of α-synuclein is already present at an early stage. Moreover, because loss of dopaminergic neurons has been observed in transgenic overexpressing models in nematodes in this study, and flies, mice and human cell cultures in other studies (Feany and Bender 2000; Zhou *et al*. 2000, 2002; Xu *et al*. 2002), a common cellular mechanism may ultimately be involved in α-synuclein toxicity.

Similar effects were seen with WT and mutant A53T forms of α-synuclein in both motoric and neuronal measures. To date, it has been unclear in model systems whether the effects of WT and A53T forms can be differentiated. The expression of the A53T form leads to a highly penetrant PD phenotype in humans, whereas in rats the A53T form is the WT form, apparently with no unusual pathology. Neuronal pathology has been observed in transgenic mouse models expressing both WT and mutant forms of α-synuclein in

some (Kahle *et al*. 2000; Kirik *et al*. 2002) but not all (Richfield *et al*. 2002) studies.

To our knowledge, a worm synuclein ortholog in *C. elegans* has not been identified; however, some other proteins known to be involved in synucleopathies, such as ubiquitin and 14-3-3, have been previously described in *C. elegans* (Ostrerova *et al.* 1999; Wang and Shakes, 1997; Graham *et al*. 1989). It has already been suggested that oxidative stress and mitochondrial dysfunction may ultimately underly neurodegeneration in PD (Olanow and Tatton 1999; Hsu *et al*. 2000) and this might be further investigated in this model (Nass *et al*. 2001, 2002; Nass and Blakely 2003).

C. elegans has been proposed as a model for various neurodegenerative diseases (Culetto and Sattelle 2000; Link 2001; Nass *et al*. 2001; Baumeister and Ge 2002). Although the cardinal features of human PD (bradykinesia, rigidy, resting tremor) cannot be recapitulated realistically in nematodes, loss of dopaminergic neurons suggests that *C. elegans* may provide some insights into the cellular pathology of various synucleopathies. To our knowledge, this is the first published example of an overexpressing α-synuclein *C. elegans* model. Genetic crosses with null-mutant alleles and forward genetic screens (Jorgensen and Mango 2002) of this model should further help us to understand the role of dopamine and other molecules involved in α-synuclein interactions and ultimately in evolution of synucleopathies.

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