Familial Parkinson Mutant α-Synuclein Causes Dopamine Neuron Dysfunction in Transgenic Caenorhabditis elegans*8

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Mutations in α-synuclein gene cause familial form of Parkinson disease, and deposition of wild-type α-synuclein as Lewy bodies occurs as a hallmark lesion of sporadic Parkinson disease and dementia with Lewy bodies, implicating α-synuclein in the pathogenesis of Parkinson disease and related neurodegenerative diseases. Dopamine neurons in substantia nigra are the major site of neurodegeneration associated with α-synuclein deposition in Parkinson disease. Here we establish transgenic Caenorhabditis elegans (TG worms) that overexpresses wild-type or familial Parkinson mutant human α-synuclein in dopamine neurons. The TG worms exhibit accumulation of α-synuclein in the cell bodies and neurites of dopamine neurons, and EGFP labeling of dendrites is often diminished in TG worms expressing familial Parkinson disease-linked A30P or A53T mutant α-synuclein, without overt loss of neuronal cell bodies. Notably, TG worms expressing A30P or A53T mutant α-synuclein show failure in modulation of locomotor rate in response to food, which has been attributed to the function of dopamine neurons. This behavioral abnormality was accompanied by a reduction in neuronal dopamine content and was treatable by administration of dopamine. These phenotypes were not seen upon expression of β-synuclein. The present TG worms exhibit dopamine neuron-specific dysfunction caused by accumulation of α-synuclein, which would be relevant to the genetic and compound screenings aiming at the elucidation of pathological cascade and therapeutic strategies for Parkinson disease.

Parkinson disease (PD)3 is a major neurodegenerative disease of the adulthood affecting the extrapyramidal motor system (1). The brains of patients affected with PD are characterized by a loss of neurons in the brainstem monoaminergic neurons, e.g. dopamine neurons in the substantia nigra and noradrenergic neurons in the locus caeruleus, which is accompanied by the deposition of Lewy bodies (LBs) in the cytoplasm of remaining neurons. LBs are characteristic spherical intracytoplasmic inclusions composed of filaments of ~7–10 nm in diameter and are pathognomonic for PD and related dementing disorder, dementia with Lewy bodies (DLB) (2). A small percentage of patients inherit PD as an autosomal dominant trait (familial PD; FPĐ), and missense mutations (3–5) or multiplications (6–8) of the α-synuclein gene have been identified in these families. LBs in the brains of patients with sporadic PD or DLB were shown to be composed of α-synuclein (9, 10). Glial cytoplasmic inclusions in the brains of patients with multiple system atrophy, another major sporadic neurodegenerative disease, or dystrophic neurites in Hallervorden-Spatz disease also were shown to be composed of α-synuclein, and these neurodegenerative diseases characterized by deposition of α-synuclein are collectively designated “synucleinopathies” (2). In vitro studies suggested that α-synuclein aggregates and forms filaments that are similar to those seen in PD or DLB brains, and amino acid substitutions linked to familial PD (A53T, A30P, or E46K) have been shown to enhance the aggregation of α-synuclein possibly through conformational changes (11–14), implicating deposition of α-synuclein in the pathogenesis of synucleinopathies including PD. Taken together with the gene dosage effects of α-synuclein in a subset of FPD (i.e. duplication and triplication), transgenic overexpression of α-synuclein in neurons would be a rational strategy to model neurodegeneration in PD.

A number of transgenic models overexpressing α-synuclein using heterologous organisms (mouse (15–20), Drosophila (21, 22), Caenorhabditis elegans (23), yeast (24)) have been reported, and some abnormal behavioral or pathological phenotypes have been documented in a subset of these animal models. However, an ideal model in which deposition of α-synuclein in dopamine neurons, the most vulnerable subset of neurons in PD, causes behavioral phenotypes that are inherent to the functions of dopamine neurons, has not been established yet. Here we describe a transgenic C. elegans model in which human α-synuclein overexpressed specifically in dopamine neurons causes an abnormal phenotype in food-sensing behavior that has been attributed to the function of C. elegans dopamine neurons (25), in a manner dependent on FPD-linked mutations, through reduction of neuronal dopaminergic function.

Materials and Methods

Plasmid Construction—A dat-1 promoter (Pdat-1) was cloned by PCR amplification of the upstream 719-bp region containing the initiation codon ATG from genomic DNA of N2 worms, as previously reported (26). The following PCR primers were used: sense primer containing BamHI site, 5’-CCCGGATCCATGAAATGGAACTTGA-3’; antisense primer containing NotI site, 5’-GGGGCCGCGCCGATGGCGTAAATTG-3’. The PCR product was subsequently inserted into the BamHI/NotI site of the enhanced green fluorescent protein (EGFP) vector pFXneEGFP to create Pdat-1::EGFP. Full-length cDNAs

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1 The abbreviations used are: PD, Parkinson disease; LB, Lewy body; FPĐ, familial PD; DLB, dementia with Lewy bodies; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; HPLC, high-performance liquid chromatography; wt, wild-type; mt, mutant; DA, dopamine; TG, transgenic.
encoding human α- or β-synuclein were kindly provided by Dr. T. Nakajo. The A53T and A30P mutant α-synuclein cDNAs were generated by the overlapping PCR mutagenesis strategy. To create the P\textsubscript{dat},::synuclein constructs, EGFFP sequence in the P\textsubscript{dat},::EGFFP construct was removed by NotI/BglII digestion and then replaced with the NotI/BglII fragments containing human α- or β-synuclein. All constructs were sequenced using Thermo Sequenase\textsuperscript{TM} mesmersham Biosciences) on an automated sequencer (Li-Cor, Lincoln, NE).

\textit{C. elegans} Protocols—Nematodes were handled by standard methods (27). N2 Bristol is the wild-type strain. The \textit{cat-2} (e1112) strain (28) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). For generation of transgenic \textit{C. elegans} (TG worms), plasmid DNAs encoding P\textsubscript{dat},::synuclein were mixed with P\textsubscript{ges},::red fluorescent protein (each 200 µg/ml), and the mixtures were co-injected into the gonads of young adult hermaphrodite N2 worms. Transgenic progeny carrying α-synuclein and red fluorescent protein transgenes inherited as extrachromosomal arrays were selected on the basis of red fluorescent protein fluorescence expressed in the intestines. Transgenes were integrated into chromosomes by ultraviolet irradiation as described previously (29), and each isolated animal was out-crossed for two to four times. Multiple independent lines for each transgene were isolated and analyzed. For generation of transgenic animals carrying P\textsubscript{cat},::EYFP, mixture of plasmid DNAs encoding P\textsubscript{cat},::EYFP and pRF4 (rol-6) (200 µg/ml each) was co-injected, and transgenic progeny was selected based on the roller phenotype attributed to rol-6. Isolated transgenic worms were integrated and out-crossed in the same manner. Double transgenic lines carrying both P\textsubscript{dat},::synuclein and P\textsubscript{cat},::EYFP were obtained by mating each P\textsubscript{dat},::synuclein line with a P\textsubscript{cat},::EYFP line, and subsequently selection of the worms carrying both EYFP and roller markers.

\textit{Fluorescence Microscopy}—Animals were anesthetized by placing in a 10-µl drop of 50 mM sodium azide in M9 buffer (22 mM KH\textsubscript{2}PO\textsubscript{4}/22 mM Na\textsubscript{2}HPO\textsubscript{4}/85 mM NaCl/1 mM MgSO\textsubscript{4}), which is put on the solidified pads of 5% agarose laid on the slides. After mounting in a coverslip, animals were examined with Fluoview FV300 confocal microscope (Olympus, Tokyo, Japan).

\textit{Immunohistochemistry and Immunoblotting}—Animals were sedimented, washed three times by M9 buffer, and then fixed overnight at 4 °C with 4% paraformaldehyde, dehydrated in ethanol, sectioned at 3 µm thick. Sections were first blocked with 10% calf serum in phosphate-buffered saline, pH 7.4, and sequentially reacted with primary and secondary antibodies (biotin-conjugated goat anti-rabbit or horse anti-mouse IgG, Vector Laboratories; Alexa 488- or Alexa 594-linked goat anti-rabbit or horse anti-mouse IgG). Biotin-labeled antibodies were enhanced with Vectastain ABC elite kit (Vector Laboratories) to form avidin-biotin complex conjugated to horseradish peroxidase and visualized with diaminobenzidine, followed by counterstaining with hematoxylin. Human α-synuclein was detected with mouse monoclonal antibodies LS509, SYN211, or a rabbit polyclonal antibody number 259 (10, 18), and α-synuclein phosphorylated at Ser-129 was detected by a rabbit affinity-purified antibody anti-PSer129 (30). Human β-synuclein was detected with antibody SYN102 that cross-reacts with both α- and β-synucleins at the C terminus. EGFFP or EYFP was detected with an anti-GFP antibody (Molecular Probes). For immunoblot analysis of the transfected human α-synuclein, worms in ~40-µl suspension were sedimented and ultrasonicated in the presence of equal amount of Tris–HCl, 150 mM NaCl (pH7.6). Thus prepared lysates were ultracentrifuged (100,000 × g, 15 min), and the supernatants were collected. Approximately 40 µg of proteins were loaded on each lane, separated by SDS-PAGE, and analyzed by immunoblotting with LB509 as previously described (10).

\textit{Preembedding Immunoelectron Microscopy}—Worms were pelletled and fixed overnight at 4 °C with 4% paraformaldehyde containing 0.3% glutaraldehyde in phosphate-buffered saline (pH7.4) and then cryoprotected in 20 and 25% sucrose. After freezing by liquid nitrogen, the pellets were cut at 20 µm on a cryostat and then reacted with LB509 and then anti-mouse IgG secondary antibody conjugated with 1 nm of colloidal gold. After postfixation in 1% glutaraldehyde, the specimen was silver-intensified as described (31), postfixed in 2% OsO\textsubscript{4}, and dehydrated, embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed on JEOL1200EXII as described.

\textit{Food-sensing Behavior Assay}—The food-sensing behavior assay was performed to assess the function of \textit{C. elegans} dopaminergic neurons using a previously described method (25) with slight modifications. Briefly, well fed, synchronized young adult animals (3-day old) were washed with M9 buffer and then transferred to the center of an assay plate with or without bacterial lawn in a drop of M9 buffer. Five minutes after transfer, the locomotory rate of each worm was counted in 20-s intervals. Assay plates were prepared by spreading the \textit{Escherichia coli} strain HB101 overnight at 37 °C in a ring with an inner diameter of 1 cm and an outer diameter of 8 cm on N2 agar (27) in 9-cm, large diameter Petri plates, to prevent the worms from reaching the edge of the plate during the trial. The slowing rate was calculated as the percentage of the locomotory rate in bacteria (+) lawn as compared with that in bacteria (−) lawn. The average slowing rate among five animals was defined as the result of each trial. In all assays, plates were coded so that the experimenter was blind to the genotype of the animal.

Dopamine pretreatment was conducted as described (25). Dopamine hydrochloride (Alrich) was prepared fresh in M9 buffer, and 300 µl of solution was added to each 6-cm agar plate with bacteria to obtain final concentration of 2 mM. The plates were allowed to dry for 1 h at room temperature, and then the animals were transferred to the plate. After incubation for 6 h at 20 °C, the locomotory rate of each animal was measured as described above.

\textit{Measurement of Dopamine Level}—The dopamine levels in animals of each genotype were measured by using high-performance liquid chromatography (HPLC) analysis coupled to chemiluminescence reaction detection as described previously (32). Animals were washed three times with M9 buffer, and then worm pellets were temporarily frozen at ~80 °C. Extracts were prepared by resuspending frozen worm pellets in buffer A (0.2 M perchloric acid, 0.002 M EDTA-2Na, 0.002 M sodium metabisulfate) and sonicating. The sonicates were centrifuged at 10,000 × g for 5 min, and the supernatants were subjected to HPLC analysis.

\textbf{RESULTS}

\textit{Generation and Characterization by Immunohistochemistry and Immunoblotting of α-Synuclein Transgenic \textit{C. elegans}}—We constructed transgenes comprised of a promoter sequence of \textit{dat-1}, a dopamine transporter of \textit{C. elegans}, fused to wild-type (wt) or A30P or A53T familial Parkinson mutant (mt) human α-synuclein, or to wt human β-synuclein or EGFFP as controls, and injected them into the gonads of \textit{C. elegans}. We screened worms that express transgenes extrachromosomally (extrachromosomal lines) by detection of fluorescence of P\textsubscript{ges},::RFP that was co-injected as a marker plasmid and expressed in intestine. We then obtained integrant lines by inserting the transgenes into chromosomes by UV irradiation. The P\textsubscript{dat},::EGFFP fluorescence was correctly detected in the eight dopamine neurons (six in the head, i.e. CEP and ADE neurons, and two in the posterior regions, i.e. PDE neurons, respectively) as well as their anterior
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and neurites of dopamine neurons was detected by two anti-human α-synuclein-specific antibodies, LB509 and Syn211, in an identical pattern. Positive immunoreactions within the neuronal cell bodies were often observed diffusely within the cytoplasm of dopamine neurons, but they sometimes exhibited multiple, irregularly shaped or round clumps that occupy restricted areas of the cytoplasm (Fig. 1, B–D), compared with the widespread positive reaction of EYFP throughout the cell bodies (Fig. 1, F and G). Dopamine neurons in N2 (non-transgenic) lines did not show any positive immunoreactions upon staining with anti-α-synuclein antibodies, confirming the specificity of the α-synuclein reactivities (supplemental Fig. S1). Human β-synuclein also showed a similar staining pattern in dopamine neurons (Fig. 1F). In addition, α-synuclein positive structures in neurites, that extend in anterior direction from the CEP neurons (CEP dendrite), often exhibited a spherical or dot-like appearance, in contrast to the linear EYFP fluorescence within the neurites (Fig. 1H; also see B–D). These characteristics were similar between TG worms overexpressing wt and FPD-mt (A30P or A53T) α-synuclein (Fig. 1, B–D, F, and G). We next examined whether phosphorylation of α-synuclein at Ser-129, which is characteristic of α-synuclein deposited in Lewy bodies and other synucleinopathy lesions in human (30) as well as in transgenic mice (33, 34) brains, occurs in the dopamine neurons of transgenic C. elegans. No dopamine neurons were positive for anti-PSer129 in 3- or 9-day-old worms, whereas a subfraction of dopamine neurons (1.90, 2.26, and 7.09% of dopamine neurons in TG worms expressing wt, A30P, and A53T mt α-synuclein, respectively; n = 105–141) showed positive reaction in 15-day-old worms, which was most prominent in TG worms expressing A53T FPD mt α-synuclein (Fig. 1I). These positive reactions for phosphorylated α-synuclein were similarly observed with another Ser(P)-129-specific monoclonal antibody (PSyn#64), and were not observed in 15-day-old N2 worms (supplemental Fig. S1). We further examined the ultrastructure of the α-synuclein accumulated within the cell bodies and dendrites of dopamine neurons by immunoelectron microscopy. α-Synuclein immunoreactivities detected by LB509 were diffusely distributed within the cytoplasm of dopamine neurons without formation of abnormal fibrils or aggregates (Fig. 1J), as well as in dendritic cytoplasm where they were detected between microtubular arrays (Fig. 1K, arrows). We finally compared the levels of α-synuclein protein expression by immunoblotting of the Tris-soluble fractions of TG worms and confirmed that all six lines expressing wt or FPD-mt α-synuclein express comparable levels of human α-synuclein detected by LB509 at ≥15 kDa (Fig. 1L).

To examine whether accumulation of wt or FPD-mutant α-synuclein caused neuronal loss, we then counted densities of CEP dopamine neurons in 3- and 15-day-old worms and compared them with those expressing wt or FPD-mutant α-synuclein and EYFP as a control, on immunostained sections with anti-α-synuclein or EYFP antibodies (Fig. 2). We found that the average number of dopamine neurons observed per worm was at similar levels (~1.4 at 3-day-old and ~1.1 at 15-day-old) in lines expressing wt or FPD-mutant α-synuclein or EYFP (Fig. 2). We invariably detected four EYFP-positive CEP neurons (of which up to two are visible in tissue sections) by fluorescence observation of whole-mounted worms at 3- and 15-days old (data not shown), suggesting that the relative decrease in neuronal densities on sections of EYFP TG worms reflects the growth of bodies of worm relative to neurons but not decrease in neuronal numbers. Thus, we concluded that TG worms expressing wt or FPD-mutant α-synuclein do not develop neuronal loss beyond that normally observed, at least when aged to 15 days.

However, we observed a constant decrease in EYFP labeling of the CEP dendrites, which was co-expressed by a monoamine neuron-spe-
cific promoter, cat-1, in 3-day-old TG worms expressing A30P (~90% decrease) or A53T (~80% decrease) FPD mutant α-synuclein, compared with the complete preservation of this labeling in worms expressing β-synuclein, and moderate decrease in worms expressing wt α-synuclein (~35% decrease), despite similar intensities of labeling for EYFP or α-synuclein in the cell bodies (Fig. 3). These results suggest the occurrence of some abnormality in dendrites of dopamine neurons expressing FPD mutant α-synuclein.

Failure in Food-sensing Behavior of Transgenic C. elegans Expressing Familial Parkinson Mutant α-Synuclein Is Associated with Dopamine Loss and Recovered by Dopamine Administration—It has been shown that mutant worms in cat-2 gene, an isologue of tyrosine hydroxylase in C. elegans, lack dopamine synthesis in dopamine neurons (28) and show a specific failure in food-sensing behavior (25). C. elegans transport themselves by bending movement of the body, and the frequency determines the speed of movement. When worms come across food (i.e., bacteria), they slow down to feed themselves more effectively, by decreasing the bending frequency. However, cat-2 (e1112) worms show failure in this deceleration response upon perception of bacteria. Thus, one of the functions of dopamine neurotransmission in C. elegans is related to this adaptive behavior in food-sensing. We examined whether overexpression of wt- or FPD mt α-synuclein in DA neurons of C. elegans causes abnormality in this function in 3-day-old TG worms that were synchronized for age (Fig. 4). Non-TG, wt worms (N2) showed an ~35% decrease in bending frequency upon exposure to bacteria. Transgenic worms that overexpress EGF, human β-synuclein or wt human α-synuclein showed slightly attenuated but almost similar extents of decrease in bending movement (~30%) compared with N2 worms. In contrast, cat-2 mutant worms exhibited a dramatic failure in decrement (~10%). Independent two integral lines each of TG worms overexpressing A53T or A30P mutant α-synuclein in dopamine neurons showed significant reduction in this decremental response of bending frequency upon exposure to bacteria compared with wild-type (N2) worms (p < 0.01) at an intermediate levels between those in N2 and cat-2 mutant. The degree of the failure in decremental response was at similar levels between TG worms expressing wild-type or FPD mt α-synuclein out-crossed two or four times (supplemental Fig. S2).

We then quantitated the level of dopamine in 3-day-old N2 or TG worms that were synchronized for age (Fig. 5). N2 worms contained ~5 ng/g worms of dopamine, whereas the dopamine levels in TG worms overexpressing wt human α-synuclein was ~3 ng/g, which was ~60%
that of N2. In contrast, dopamine levels in TG worms overexpressing FPD mt (A53T or A30P) α-synuclein were markedly reduced to ~1 ng/g worms.

We finally tested whether the abnormality in feeding behavior (i.e. failure in deceleration upon exposure to bacteria) can be rescued by the administration of dopamine (Fig. 6). The administration of 2 mM of dopamine in the agar plate ameliorated the failure in bending response of TG worms expressing FPD mt α-synuclein, suggesting that accumulation of FPD mt form of α-synuclein caused reduction in dopamine content, which was treatable by supplementation of exogenous dopamine.

**DISCUSSION**

In this study, we have established transgenic C. elegans (TG worms) that expresses human wt or FPD-linked mt α-synuclein specifically in dopamine neurons and exhibit dopamine neuron dysfunction caused by accumulation of A53T or A30P FPD mt α-synuclein. These abnormal behavioral phenotypes were rescued by the administration of exogenous dopamine and accompanied by a reduction in dopamine levels. TG worms expressing wt α-synuclein did not exhibit overt behavioral abnormalities but did show moderate levels of dopamine loss, suggesting that these TG worms also develop abnormalities similar to but milder than those in worms expressing mt α-synuclein that are subthreshold for manifesting behavioral phenotypes. Our TG worm represents the initial animal model that exhibits abnormal behavioral phenotypes specifically attributable to impairment of dopamine neurons caused by accumulation of FPD mutant α-synuclein.

A variety of heterologous transgenic animal models that express human α-synuclein in neurons, e.g. transgenic mice (15–20), Drosophila (21, 22), C. elegans (23), and yeast (24) have been reported, although none of these exhibited abnormal phenotypes directly attributable to the dysfunctions of dopamine neurons. Transgenic mice that overexpress A53T mutant human α-synuclein in catecholaminergic neurons using a tyrosine-hydroxylase promoter did not show any motor dysfunction despite robust expression of α-synuclein in nigral neurons (17). Pan-neuronal overexpression of FPD mt α-synuclein, including dopamine neurons, led to motor dysfunction in a subset of transgenic mice (18, 20) or Drosophila (21), although the abnormal phenotypes cannot be specifically related to dopamine neuron dysfunction. Targeted overexpression of FPD mt α-synuclein in dopamine neurons of transgenic Drosophila elicited robust decrease in the number of dopamine neurons (21, 22), which can be rescued by up-regulation of molecular chaperones (22, 35, 36), although motor disturbances caused by loss of dopamine neurons have not been clearly defined in Drosophila. Our present C. elegans model that exhibits abnormalities in food-sensing behavior linked to dopamine neuron function, which is caused by accumulation of FPD-linked mutant α-synuclein in these neurons, should serve as a useful model relevant to the investigations into the molecular pathways leading to degeneration of dopamine neurons, taking advantage of the merit of C. elegans in genetic analysis, as well as for the screening of small molecule compounds that ameliorate these dysfunctions.

The mechanisms whereby dopamine neurons develop dysfunction associated with dopamine loss through overexpression of α-synuclein, especially the FPD-linked mutant forms, await further determination. It has been shown that overexpression of wt or A53T mt α-synuclein in mammalian dopaminergic cell lines reduced tyrosine hydroxylase activity and dopamine synthesis through direct binding of α-synuclein and tyrosine hydroxylase (37). However, wt and A53T mt α-synuclein showed similar extent of binding to tyrosine hydroxylase as well as inhibition of dopamine synthesis in mammalian cultured cells, whereas the phenotypes including reduction in dopamine content were more severe in TG worms overexpressing α-synuclein harboring the two FPD-linked mutations in our study. Because we directly confirmed that the protein expression of α-synuclein in each line was at similar levels by immunoblotting, despite the relative paucity of dopamine neurons (8 among 302 neurons), the differences in the severity of dopamine neuron dysfunction should be attributable to effects of FPD mutations of α-synuclein, but not to the difference in expression level of α-synuclein proteins. The following data also lend support to this view. 1) The immunolabeling intensities for α-synuclein of dopamine neurons as assessed by quantitation of confocal images labeled by LB509 were at similar levels in different TG lines (supplemental Fig. S3), and 2) the behavioral, morphological, and biochemical changes were constantly observed in multiple, integrant TG lines overexpressing wt or each of the mutant α-synuclein. In our TG model, we have not detected formation of Lewy body-like structured inclusions, and the immunoreactivities for α-synuclein were diffusely distributed throughout the neuronal cytoplasm as determined by immunogold electron microscopy, except for the clump- or dot-like distribution of α-synuclein in a fraction of dopamine neurons. Thus, it is reasonable to speculate that FPD-mutant α-synucleins, which are more prone to display altered conformation or to aggregate (11, 12), formed misfolded species or oligomeric intermediates that are more toxic to neurons but hard to detect by morphological analysis, thereby impairing the neuronal functions including the dopamine synthetic pathways and leading to the FPD mt-specific abnormalities in TG worms. Indeed, recent studies have implicated...
these non-fibillar forms of α-synuclein in the cause of neuronal death in PD (38, 39). Taken together, our observations in TG C. elegans partially recapitulate pathological changes of α-synuclein in human PD. Interestingly, administration of dopamine did not aggravate, but rather ameliorated behavioral deficits, which apparently is not consistent with the observation that dopamine enhances protofibril formation of α-synuclein in vitro (38). The reason for this discrepancy is unknown at present, but it is possible that exogenous dopamine does not effectively promote α-synuclein oligomerization or protofibril formation in C. elegans. We did not observe a correlation between phosphorylation of α-synuclein and behavioral/biochemical changes, because the latter abnormalities were already observed in young (3-day-old) TG worms in which phosphorylation of α-synuclein is not apparent. Although recent data in Drosophila (39) and human neuroblastoma cell lines (40) implicate phosphorylation of α-synuclein at Ser-129 in neuronal death or aggregate formation, phosphorylation of α-synuclein in a subfraction of dopamine neurons in our TG worms may represent a later event in the progression of α-synuclein accumulation.

We have also observed a decrease in the number of GFP-labeled dendrites specifically in TG worms overexpressing FPD-linked mt α-synuclein. Decrease in the labeling of dendritic processes in TG worms overexpressing wt or A53T mutant α-synuclein driven by dat-1 promoter has also been described previously (23). Also ~30% loss in the number of GFP-positive cell bodies of dopamine neurons has been documented in the same study. This disagrees with our present observation that loss of dopamine neurons does not occur in the dat-1-driven α-synuclein TG worms. We have avoided double overexpression of GFP and α-synuclein using an identical promoter, because we have experienced that the level of co-expression of GFP driven by the same dat-1 promoter is frequently down-regulated, leading to underestimation of the number of positive structures, possibly by saturation of the promoter function because of competition for transcription factors required for a given promoter, in transgenic C. elegans (41). One of the reasons for this propensity of C. elegans neurons to be easily saturated for promoter function would be that multiplicity (often >100) copies of transgenes are incorporated into C. elegans (42). This is why we have chosen to visualize the dopamine neurons and neurites by GFP expression using a different promoter, cat-1, the latter being specifically expressed in dopamine and serotonin neurons. We have confirmed that double expression using these two different promoters does not lead to saturation of promoter function upon expression of β-synuclein (as a control protein) and GFP, whereas control data are lacking in the aforementioned study (23) where co-expression experiments using dat-1 and aex-3 (pan-neuronal) promoters also were performed. Nonetheless, Lakso and colleagues (23) have clearly shown that overexpression of α-synuclein in motor neurons or in a pan-neuronal fashion (but not by expression in dopamine neurons) causes motor deficits in threshold assays, suggesting that α-synuclein neurotoxicity is not specific to dopamine neurons.

By careful morphometric analysis based on α-synuclein immunoreactivity of dopamine neurons, we concluded that neuron loss is not present in our TG worms, whereas dendrites of CEP dopamine neurons overexpressing FPD mutant α-synuclein often failed to be GFP-positive. The reason for this failure in GFP labeling remains to be clarified. One possibility is that the dendritic processes have been retracted and lost, but it is also possible that the transport of cytosolic proteins (e.g. GFP) into dendrites has been disrupted by as yet unknown mechanisms, because of overexpression of the more toxic FPD mutant α-synuclein, contributing to the dysfunction of dopamine neurons. Rigorous electron microscopy examination would be needed to clarify this point.

The present α-synuclein TG worms, which exhibit functional disturbance caused by accumulation of familial Parkinson mt α-synuclein in dopamine neurons, will facilitate the elucidation of the mechanism of α-synuclein neurotoxicity, taking advantage of the versatility of C. elegans in genetic analysis, as well as the screening of molecules that block neuronal dysfunction and degeneration and thus open up a way to disease-modifying therapy in PD.

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