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Caenorhabditis elegans as a model system for identifying effectors of α-synuclein misfolding and dopaminergic cell death associated with Parkinson's disease

Adam J. Harrington [a,1], Adam L. Knight [a,1], Guy A. Caldwell [a,b,*], Kim A. Caldwell [a,b]

[a] Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487, USA
[b] Departments of Neurology and Neurobiology, Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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ABSTRACT

Protein misfolding and aggregation are key pathological features observed in numerous neurodegenerative diseases, including the misfolding of α-synuclein (α-syn) in Parkinson's disease (PD) and β-amyloid in Alzheimer's disease. While this phenomenon is widely observed, the etiology and progression of these diseases is not fully understood. Furthermore, there is a lack of therapeutic treatments directed at halting the progression and neurodegeneration associated with these diseases. This demands a need for an inexpensive, easy to manipulate multicellular organism to conduct both genetic and chemical screens within to identify factors that may play a pivotal role in the pathology of these diseases. Herein, we describe methodology involved in identifying genetic modifiers of α-syn misfolding and toxicity in the nematode roundworm, *Caenorhabditis elegans*. Transgenic nematodes engineered to express human α-syn in the body wall muscles or dopaminergic (DA) neurons result in formation of cytoplasmic puncta or DA neurodegeneration, respectively. Using these models, we describe the use of RNA interference (RNAi) and transgenic gene expression to functionally elucidate potential therapeutic gene targets that alter α-syn misfolding and DA neurotoxicity.

1. Introduction

With an increase in the aging population, there is a growing concern to understand and therapeutically address diseases of protein misfolding that increase with aging, especially neurodegenerative diseases including Parkinson's disease (PD). PD is the most common human movement disorder which affects 1–2% of the population over the age of 60 and is progressively debilitating with age [1,2]. While there are several known genetic causes of PD, these monogenic mutations only account for ~10% of diagnosed cases. The common pathological hallmarks of PD include the formation of proteinaceous inclusion bodies, termed Lewy bodies, in the dopaminergic (DA) neurons in the brains of post-mortem PD patients, as well as a selective loss of over 80% of the DA neurons in the *substantial nigra pars compacta*. One of the main components of Lewy bodies is the misfolded and aggregated protein α-synuclein (α-syn) (Fig. 1), whereby mutations or multiplications of the α-syn locus are an established genetic cause of PD [3].

Although the etiology of PD is not fully understood, there is strong evidence suggesting a combination of both environmental factors and genetic susceptibility factors play a pivotal role in the onset and progression of this disease. While there are numerous models available to study PD, the nematode *Caenorhabditis elegans* (*C. elegans*) has proven to be a valuable research tool in conducting high throughput genetic screening and mutant analysis due to its small size, transparency, short reproduction time, ease of creating transgenic animals, and low cost of maintenance. The application of *C. elegans* as a model system for studying PD has proven useful in discerning pathways that are affected in this disease, and may shed light onto the identification of novel therapeutic targets for the prevention and treatment of the pathological symptoms [4,5]. Here, we will discuss the methodology involved in using *C. elegans* as a model system to identify genetic candidates that ameliorate two pathological hallmarks of PD recapitulated in worms, including both α-syn misfolding in body wall muscles and α-syn-induced DA neurodegeneration. We will discuss how these models can be used to identify modifiers of α-syn protein aggregation and proteotoxicity through targeted genetic modifications involving gene knockdown by RNA interference (RNAi) and transgenic gene overexpression in specific cell types.

* Corresponding author at: Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487, USA.
E-mail addresses: gcaldwel@bama.ua.edu, kcaldwel@bama.ua.edu (G.A. Caldwell).
[1] These authors contributed equally to the preparation of this manuscript.

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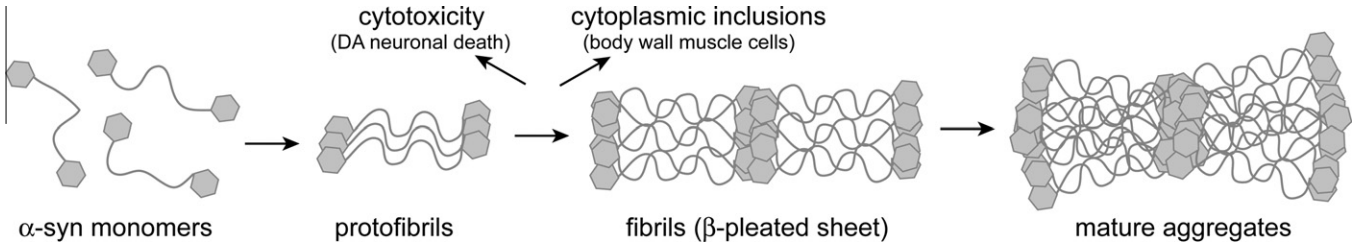

Fig. 1. The proposed pathway for the progression of α-syn toxicity and aggregation. Unfolded, yet soluble, α-syn monomers can associate into small, soluble, protofibrils that can then be folded into β-sheet-containing fibrils. Transformation into mature, higher molecular weight, insoluble aggregates is also observed.

2. Methods

2.1. α-Syn misfolding in body wall muscles

2.1.1. Background information

C. elegans body wall muscles provide an attractive *in vivo* model for studying protein aggregation and proteotoxicity due to their large size and accessibility to RNAi screening. This model has been utilized to examine the cellular mechanisms for a variety of disease-associated aggregation-prone proteins, including beta amyloid toxicity and protein aggregates associated with polyglutamine expansion [6,7]. These models have resulted in the identification of numerous proteotoxicity modifiers [8,9]. We have exploited *C. elegans* to investigate the pathological mechanisms underlying α-syn misfolding in PD [10–12]. To this end, we expressed human α-syn fused to GFP in the body wall muscles and observed that there is an age-dependent increase in both the number and size of aggregates (Fig. 2A). Since we wanted to screen for effectors of α-syn misfolding using RNAi, we reasoned that this genetic background might not be sensitive enough to detect moderate changes affecting the formation of α-syn aggregates because of their abundance. We therefore co-expressed the *C. elegans* homolog of torsinA (TOR-2), a chaperone-like protein, along with α-syn::GFP in the body wall muscles. Coexpression of TOR-2 attenuated the amount of α-syn misfolding (Fig. 2B), and thus allowed for RNAi examination of factors that influence the level of misfolding (Fig. 2C and D). The presence of TOR-2 also resulted in a background whereby immature aggregates are produced; these non-ubiquitinated species are believed to be the more toxic form of misfolded protein. Taken together, our model of α-syn::GFP + TOR-2 co-expression in body wall muscles provides a sensitive method for rapidly screening through potential genetic and pharmacological modifiers of α-syn aggregation and proteotoxicity.

2.1.2. Experimental procedures

2.1.2.1. Construction of transgenic lines. The making of general lab reagents (i.e., M9) and worm growth media (i.e., NGM agar plates) is assumed or can be referenced in Caldwell et al., 2006 [13]. The α-syn::GFP fusion was created by cloning human α-syn cDNA (gift from Philipp Kahle, University of Tubingen, Germany) into the gfp-containing plasmid pPD95.75 (gift from Andrew Fire, Stanford University). TOR-2 cDNA was amplified from *C. elegans* using standard PCR techniques using high-fidelity polymerase. We used Gateway technology (Invitrogen) to clone the α-syn::GFP fusion and TOR-2 cDNA into a vector that contains the *unc-54* promoter, which drives expression in body wall muscle cells [10]. The use of Gateway technology (Invitrogen) enables rapid cloning of genes from a single entry vector subsequently into various expression vectors through site-specific recombination with the lambda recombination system. In this regard, the *C. elegans* community is currently producing Gateway vectors for all worm open reading frames (ORFeome v3.1) [14]. Alternatively, traditional subcloning techniques may be used to insert your gene of interest into tissue-specific expression vectors. PCR fusion between a cell-specific promoter and gene of interest can also drive target gene expression in certain cell types [15].

After creating the expression vectors, the plasmids are microinjected, along with a phenotypic marker (the *rol-6* gene, a dominant collagen mutation that causes worms to roll) into the distal tip of the gonad within the wild-type N2 strain [22]. This produced stable, transgenic lines that express α-syn::GFP alone or α-syn::GFP + TOR-2 in body wall muscle cells. Stable, transgenic lines express the transgene at different levels among siblings and

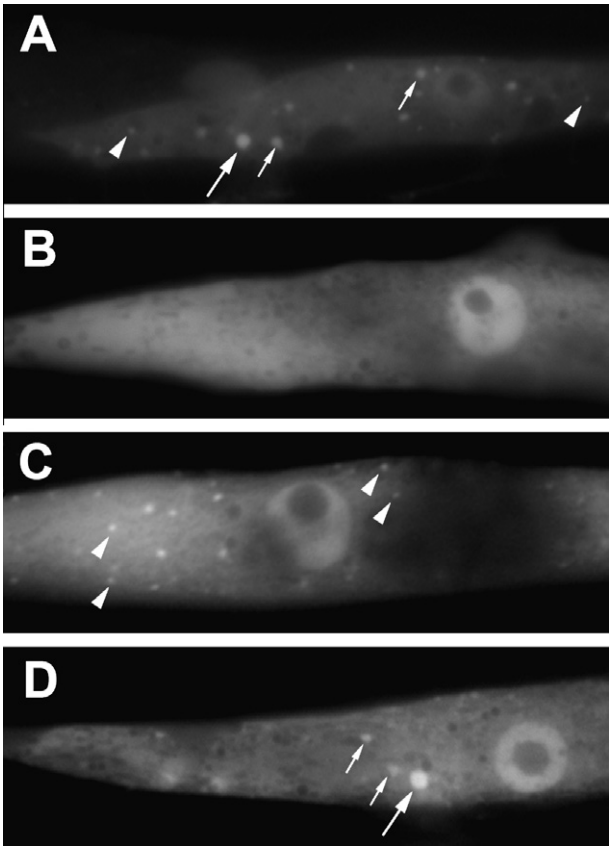

Fig. 2. Photomicrographs of *C. elegans* body wall muscle cells exhibiting varying amounts of α-syn misfolding and aggregation. (A) A cell expressing α-syn::GFP under control of the body wall muscle cell promoter *unc-54* ($P_{unc\text{-}54}$::α-syn::GFP). A moderate number of aggregates are visible and display a range of sizes from small (arrowhead), medium (small arrow), and large aggregates (arrow). (B) A body wall muscle cell from a worm expressing TOR-2 and α-syn::GFP ($P_{unc\text{-}54}$::α-syn::GFP + -$P_{unc\text{-}54}$::*tor-2*). The presence of this chaperone attenuates α-syn misfolding, thereby generating an ideal background for screening enhancers of misfolding. (C and D) Following RNAi of specific gene targets in worms expressing TOR-2 + α-syn::GFP, misfolded α-syn::GFP is revealed. The α-syn::GFP aggregates can display a variety of phenotypes such as the examples shown here where there the aggregates are small (arrowhead) and abundant (C) or sparsely distributed with medium (small arrow) and large (arrow) aggregates (D).

only in a select portion of the offspring; therefore, transgenic animals must be selected by identifying progeny that express the transgenic marker which was co-injected with the gene of interest. Once a stable line has been created, the transgenes (which are expressed as an extrachromosomal array) should be integrated into the genome of the worms by UV or gamma irradiation [16,17]; this induces breaks in the genome allowing for the extrachromosomal plasmid to be inserted into the genome via cellular DNA repair mechanisms. Integration of transgenes allows for uniform expression of the target gene of interest and 100% transmission of the transgene among progeny. Once integrated, transgenic lines must be outcrossed at least three times to remove any extraneous mutations that may have been induced during the irradiation process. After integration, these isogenic strains are available for RNAi screening for effectors of α-syn misfolding.

2.1.2.2. RNAi screening for effectors of α-syn misfolding.

1. Streak RNAi bacterial feeding clones (Geneservice, Cambridge, UK) onto LB plates containing tetracycline and ampicillin, and grow 14 h at 37 °C.
2. Strip 2 plates of dauer stage worms {UA50 [*baInl13*; P_{unc-54}::α-syn::GFP, P_{unc-54}::*tor-2*, *rol-6* (*su1006*)]} onto normal worm plates and store them at 25 °C the day before transferring them to RNAi plates.
3. Inoculate RNAi bacterial cultures in LB with 100 mg/mL ampicillin, and grow 14 h at 37 °C, shaking.
4. Spread isopropyl β-D-thiogalactoside (IPTG) onto one medium size (60 mm) and one small size (35 mm) plate per gene for final concentration of 1 mM (0.25 mM for lethal genes) and allow IPTG to soak into the plate for at least 1 h.
5. Add RNAi bacteria to both plates (100 μl to 35 mm and 200 μl to 60 mm) and allow cultures to dry.
6. Transfer five L4 hermaphrodites UA50 worms onto each medium plate and incubate at 25 °C for 44 h.
7. Synchronize offspring for analysis by transferring the original gravid adults onto the corresponding small RNAi plates, and allow worms to lay eggs for 9 h. The adults should then be removed from the plate to maintain a synchronized population.
8. Keep the worms in the 25 °C incubator until they are ready for analysis at the young adult stage (∼44–48 h).
9. On days of α-syn misfolding analysis in body wall muscle cells, prepare 2% agarose pads on microscope slides that will be used to mount the worms. This is achieved by placing two spacer slides (with a piece of tape on the slides) next to a third empty slide. Transfer a drop a molten 2% agarose on the middle slide, place a fourth slide perpendicular to the other slides on top of the molten agarose drop, and slightly press down (Fig. 3). After a few seconds, the agarose pad will solidify, and the top slide can be gently removed, leaving an agarose pad that can be used to mount worms.
10. Place an 8 μl drop of 3 mM levamisole (an anesthetic) on a 22 × 30 mm cover glass and transfer ∼25–30 transgenic worms to the drop. The worms will be paralyzed after several seconds once exposed to the levamisole. Invert the cover slip and place the worms on the 2% agarose pad prepared in step 9. Repeat for each worm strain to be analyzed.
11. Analyze α-syn misfolding by qualitatively scoring the number (none/few/many) and size (small/medium/large) of aggregates for 20 worms per RNAi treatment (Fig. 4A). A worm is scored as having significant aggregation if it has multiple muscle cells with increased quantity and size of aggregates (Fig. 2A vs. C and D). With experience, this qualitative measure of α-syn misfolding yields consistent and reproducible data following the analysis of multiple RNAi targets and animals when compared to controls. For example, in Fig. 2, compare the cells within animals exhibiting aggregates of varying sizes (small, medium, and large) in 2A with the animal in 2C wherein the cells exhibit small aggregates only. In contrast, the animal displayed in 2D has cells with a mixture of medium and large aggregate sizes. Bacterial RNAi clones are scored as positive genetic candidates if 80% ($\geqslant$16/20) of the worms exhibit significant aggregation. It is important to note that aggregation varies within an animal, and it is common for some muscle cells

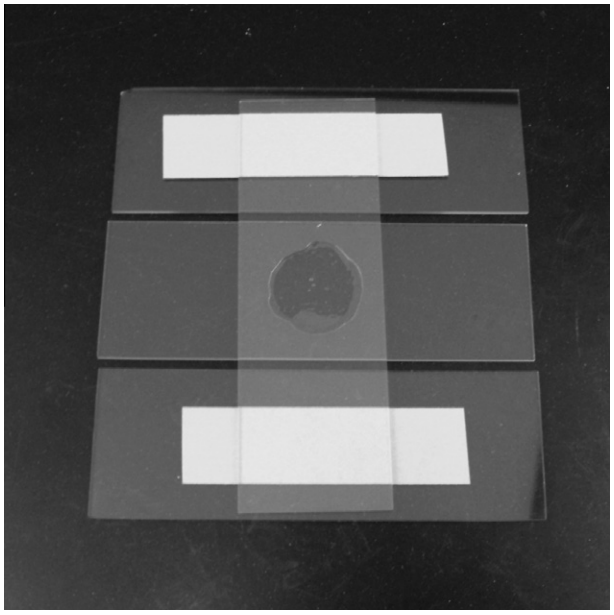

Fig. 3. An image depicting the microscope slide layout used to create agarose pads to mount *C. elegans* for fluorescent microscopy analysis. Following careful removal of the top slide, a pad for mounting worms will be revealed.

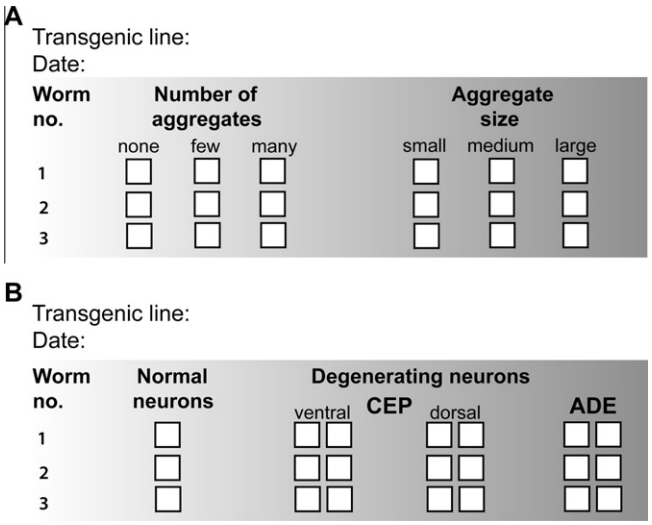

Fig. 4. Sample analysis sheets for scoring α-syn-induced aggregation in body wall muscles (A) and α-syn-induced degeneration of anterior DA neurons (B). (A) Qualitative analysis of the number of aggregates in each worm (none, few, or many), as well as the average size of the aggregates (small, medium, or large), can be scored. (B) Every neuron within a single animal can be individually annotated for degenerative changes. Any change, such as retraction of a dendrite, or cell body rounding, is considered abnormal. This method provides unequivocal accuracy in obtaining quantitative data on the total number of neurons degenerating in a population (number of worms examined × 6 anterior DA neurons per worm). It also allows for quantitation of the number of worms within a population that exhibit degenerating neurons.

to exhibit significant misfolding while others will not. Therefore, the entire worm should be surveyed (rather than focusing on a specific region) during analysis to ensure accuracy.
12. Each experiment should be repeated at least in duplicate (20 worms/screen × 2 screens = 40 total worms analyzed/RNAi target). Increasing the replicate number should make the data more consistent.

2.2. α-Syn-induced DA neurodegeneration

2.2.1. Background information

While identification of genes that alter α-syn misfolding may represent potential therapeutic targets to prevent aggregate formation, there is some controversy in the field as to whether or not intracellular α-syn aggregates are serving as protective inclusions or acting as the toxic species to cause neuronal cell death. Therefore, to further validate if potential candidate genes that reduce α-syn misfolding are good therapeutic targets for PD, these genes should be tested for protection of DA neurons against α-syn-induced neurotoxicity. While there is not an identifiable α-syn homolog encoded in the *C. elegans* genome, several labs have established worm PD models that recapitulate pathological features seen in human patients by overexpressing the human α-syn gene in worm neurons, including DA neurons [18–20]. Here, we will focus on validating target genes by overexpressing them in an isogenic line of *C. elegans* that express the green fluorescent protein (GFP) and human α-syn in the DA neurons [under the DA transporter promoter (*dat-1*)] and exhibit both age- and dose-dependent neurodegeneration. This method enables researchers to identify different target proteins that modulate α-syn neurotoxicity [10].

2.2.2. Experimental procedure

In order to generate transgenic animals expressing the candidate gene of interest in DA neurons, an expression plasmid must be constructed that will drive expression of the gene under a DA neuron-specific promoter (*dat-1, cat-2*, etc.) [21]. These plasmids are generated by amplifying candidate genes (worm or human cDNA) using standard PCR techniques with a high-fidelity polymerase and further cloning into an expression vector using Gateway technology. After making the expression vectors, transgenic animals are generated via microinjection along with a transgenic marker (P_{unc-54}::mCherry, P_{myo-2}::mCherry, etc.) and the transgene is subsequently integrated by UV or gamma irradiation. Construction of plasmids and production of transgenic worms are described in detail in Section 2.1.2.

After the integrated, transgenic line has been generated, these worms can be crossed into a *C. elegans* model of PD to look at the effect of overexpression of the candidate gene on α-syn-induced DA neurodegeneration. For these experiments, we will be using the pre-existing worm strain generated in our lab, UA44 (*baIn11*; P_{dat-1}:: α-syn, P_{dat-1}::gfp), which overexpresses both the wild-type human α-syn cDNA and GFP driven under the *dat-1* promoter [10,19]. The following methodology describes the process of crossing transgenic animals expressing the candidate gene into worms expressing α-syn in DA neurons.

2.2.2.1. Transgenic line construction.

1. Transfer three L4 staged transgenic hermaphrodites expressing the candidate gene of interest to a 60 mm NGM (nematode growth medium) plate with a small (not large) bacterial lawn (~30 μl *Escherichia coli* OP50), which will allow for a higher incidence of mating.
2. Transfer 8–10 young-adult stage transgenic UA44 males to the same mating plate with the three hermaphrodites. Males arise naturally at a very low frequency due to non-disjunction of the X-chromosome. However, males can be generated by either heat-shocking L4 hermaphrodites at 34 °C for 4–5 h or by using RNAi to knockdown *him* genes (i.e., *him-8*) that yield a high incidence of males. Both of these methods will result in progeny containing a good percentage of males. These males can be crossed into the original strain to propagate more male progeny or maintain a male stock for further crossing.
3. Allow worms to mate at 20 °C for 48 h, and then remove males to prevent the parental males from interacting with the F1 progeny.
4. Allow worms to grow at 20 °C until the F1 progeny have reached the L4 stage. If the cross was successful, a high percentage (~30–50%) of males should be present within the population. Furthermore, many of the progeny should express both transgenic markers (candidate gene transgenic marker and P_{dat-1}::GFP from UA44 strain).
5. If cross was successful, transfer eight F1 heterozygous hermaphrodites (L4 stage) expressing both transgenic markers to 60 mm NGM plates seeded with *E. coli* OP50.
6. Incubate worms at 20 °C, allowing the F1 heterozygotic hermaphrodites to self-fertilize and generate F2 progeny. For faster growth, worms may be grown at 25 °C with the caveat that some worm strains are lethal when exposed to this temperature.
7. Monitor the F2 generation until L4 staged progeny appear (~3–4 days). Transfer single L3-L4 staged F2 hermaphrodite progeny that express both transgenic markers to individual 35 mm, seeded, NGM plates. Transfer individual worms to 8–10 separate plates, allowing for selection of homozygous clones.
8. Allow worms to self-fertilize and produce the F3 generation. Screen the F3 generation for 100% transmission of both transgenes of interest (GFP and transgenic marker). Only plates where the transgenes exhibited 100% transmission should be used for analyzes.

2.2.2.2. Analyzing target gene candidates. As with PD in humans, α-syn-induced DA neurodegeneration in *C. elegans* is age dependent. When analyzing the transgenic animals expressing a target gene candidate, it is important to schedule DA neuron analysis when worms reach specific ages as the amount of degeneration increases over time. For example, if the candidate gene is expected to enhance neurodegeneration, worms should be analyzed at earlier stages, allowing for enhanced neurotoxicity to be observed. This correlates to 6 and 8 days post-hatching, if the worms are grown at 20 °C (i.e., 2 and 4 day adults). When examining candidate genes that might be protective against α-syn-induced toxicity, older worms should be analyzed [7 and 10 days post-hatching (i.e., 3 and 6 day adults, respectively)]. By using these time points, most potential effects that a candidate gene could have on α-syn-induced neurotoxicity should be readily observed [23]. The following procedure describes the method involved in synchronizing and analyzing transgenic *C. elegans* modifiers of α-syn-induced DA neurodegeneration.

1. Worms should be synchronized by allowing ~50 transgenic, gravid adult hermaphrodites to lay eggs on a 100 mm seeded NGM plate for ~5 h at 20 °C. This should result in sufficient embryos to conduct the DA neuron analysis. Alternatively, if large numbers of worms are required, a plate of gravid adult hermaphrodites can be synchronized by bleaching worms with a 20% bleach solution (with 0.5 M KOH) for 5 min. The bleach solution degrades the worms, releasing the embryos which are resistant to the bleaching process. Embryos must be washed with M9 buffer before transferring to NGM plates.
2. After eggs have been laid, the adults should be removed to maintain a synchronized population of embryos. Development of these eggs into adult worms will take 4 days at 20 °C.

3. Once the worms have reached the young adult stage (4 days), transfer ~100 transgenic hermaphrodites to fresh NGM plates containing 0.04 mg/ml 5-fluoro-2'-deoxyuridine (FUDR) (Sigma–Aldrich; F-0503). FUDR is a nucleotide analogue that inhibits DNA synthesis and self-reproduction, preventing the need to continuously transfer adult worms. In this regard, the presence of progeny can cause over-crowding, which leads to stressed worms. In our experience, stressed worms result in inconsistent data from DA neurodegeneration analyzes.
4. Continue growing worms at 20 °C until analysis (6–10 days post hatching).
5. Mount ~35–40 worms on agarose pads as described in Section 2.1.2.2 (steps 9 and 10; Fig. 3).
6. Analyze the six anterior DA neurons (four CEP and two ADE) for degeneration by annotating the individual neurons that are either normal [wild type (WT)] or degenerative for 30 worms/strain. Neurons are considered normal if they exhibit unchanged morphology vs. controls (i.e., GFP without an experimental modifying transgene), wherein cell bodies appear robust and the neurites are intact and fully extended (Fig. 5A). The morphology of the degenerating neurons can vary slightly, and range from neurite retraction, cell body rounding, and complete cell loss (Fig. 5B). With the neurons in separate focal planes, the analyzer must scan the entire depth of the nematode to ensure that all neurons have been observed and scored. No GFP signal will be observed for dead neurons that have fully retracted (Fig. 5B).
7. Each experiment should be repeated in triplicate (30 worms/screen × 3 rounds of analysis = 90 total worms analyzed/strain). Increasing the replicate number should make the data more consistent. A sample scoring sheet for neurodegeneration assays is depicted in Fig. 4B.

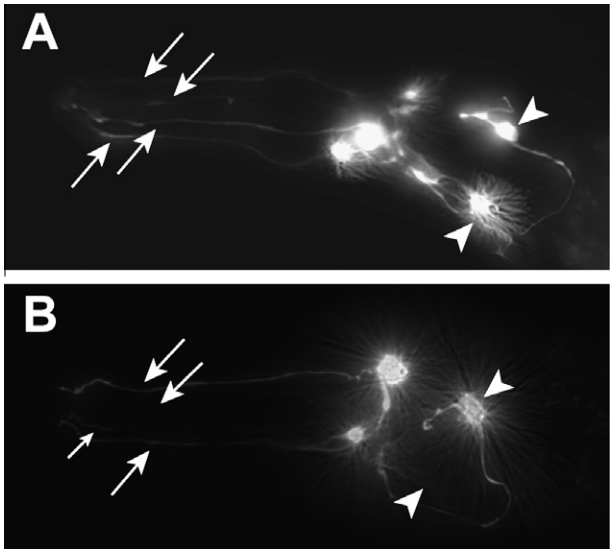

Fig. 5. Differential sensitivity to α-syn-induced degeneration is observed in the anterior DA neurons of *C. elegans*. (A) The six DA neurons in the anterior region of a *C. elegans* hermaphrodite are readily observed using a transgenic construct whereby GFP is driven from the DA transporter promoter, P_{dat-1}::GFP. The DA neurons can be sub-classified as four CEP neurons and two ADE neurons. The dendrites of the CEPs are indicated with large arrows and the cell bodies of the ADEs are shown with arrowheads. (B) In worms expressing both P_{dat-1}::GFP + P_{dat-1}::α-syn, the CEP and ADE neurons degenerate in an age-dependent manner. In this example of a 7-day old worm, the nematode displays two intact CEP neurons (large arrows), one retracting (degenerating) CEP dendrite (small arrow), and one intact ADE neuron (arrowhead). The other CEP (1) and ADE (1) neurons have completely degenerated and are no longer visible in any focal plane (grey arrow and arrowhead represent normal locations of the CEP and ADE, respectively).

3. Discussion

We have described methodologies for the application of *C. elegans* models toward investigating two of the primary pathological hallmarks of PD: α-syn-mediated neurotoxicity and aggregation. As the molecular mechanism for α-syn toxicity in PD is largely unknown, we sought to generate a model that could increase our understanding of this complex neurodegenerative disease. We successfully recapitulated α-syn proteotoxicity in *C. elegans* and took advantage of the salient features of this model organism to visualize real-time protein aggregation and DA neurodegeneration *in vivo*. More specifically, we showed that α-syn overexpression in muscle cells and DA neurons results in the progressive accumulation of α-syn and causes age-dependent aggregation and neurodegeneration, respectively. Using our model of α-syn + TOR-2 coexpression in the body wall muscles and the methods discussed above, we previously conducted a high-throughput RNAi screen for genetic targets that enhance α-syn aggregation [10]. Given that worm DA neurons are recalcitrant to RNAi, and that it is both a labor- and time-intensive process to generate transgenics, we propose the method of using body wall muscles to rapidly pre-screen potential modifiers of α-syn-induced DA neurodegeneration in this prior study. After pre-screening our list of candidate genetic targets in the body wall muscles, we then overexpressed several of these genes in the DA neurons to determine whether they could protect from α-syn-induced neurodegeneration. We hypothesize that identifying candidates that affect the phenotype in both our models represent a more promising therapeutic angle, and have found the combinatorial application of our methodologies to be a fruitful platform for deciphering genetic modifiers of aggregation and DA neurodegeneration caused by α-syn.

Although there are many advantages to using *C. elegans* to model α-syn aggregation and DA neurodegeneration, there are also limitations to this system. For example, there is no α-syn homolog in *C. elegans*. However, it has been demonstrated that a variety of toxic gain-of-function disease-associated proteins can be accurately modeled in *C. elegans* and in other systems, due to the conservation of genes, pathways and their associated proteotoxic mechanisms. Others and we have demonstrated that it is possible to recapitulate some of the pathological hallmarks of PD in worms through overexpression of human α-syn [5]. It is also important to note that we do not observe α-syn aggregation in our DA neuron model – however, this may be attributed to the small size of the worm DA neurons and challenges of reproducible immunostaining in *C. elegans* (due to the thick cuticle). Nonetheless, we still see DA neurodegeneration and have demonstrated that this model is a predictive indicator for modifiers of α-syn neurotoxicity that have also been successfully validated as neuroprotective in mammalian systems [11,12,24–27]. It is noteworthy that other labs have also generated *C. elegans* models to examine α-syn proteotoxicity. van Ham and colleagues [28] applied the biophysical technique of fluorescence recovery after photobleaching (FRAP) to a similar model of α-syn overexpression in body wall muscles (without TOR-2 coexpression), and performed a genome-wide RNAi screen for genes that modified the formation of α-syn inclusions. Kuwahara et al. [29] generated *C. elegans* models that overexpress wildtype or mutant α-syn pan-neuronally, and used RNAi to screen for neuronal genes that affected the resultant behavioral phenotype. While each of these models have proven to be informative, the combined application of these *C. elegans* α-syn models to examine the consequences of putative genetic or pharmacological modifiers on various phenotypes (i.e., aggregation, neurodegeneration, behavior) is likely to be the most effective route for discerning α-syn toxicity in PD.

Collectively, these *C. elegans* models have provided valuable insight into the mechanisms of α-syn pathogenicity, and their application towards screening for proteotoxicity modifiers has led to the

identification of several novel candidate genetic and pharmacological targets. Continued analysis to validate these targets in mammals will likely further reveal the importance of these models towards elucidating α-syn toxicity in PD. In an era of complex bioinformatics and genome-wide association studies (GWAS), these methodologies are promising for discerning the functional significance of single-nucleotide polymorphisms (SNPs) on α-syn toxicity and disease susceptibility. Using these methodologies, in concert with a hierarchy of model organisms (from yeast to mammals), holds exciting potential for the investigating the root causes of PD and more effective therapeutics for disease modification.

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