Chapter 3

Methodological Strategies to Evaluate Functional Effectors Related to Parkinson's Disease Through Application of *Caenorhabditis elegans* Models

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Abstract

Improvements to the diagnosis and treatment of Parkinson disease (PD) are dependent upon the identification and molecular understanding of modifiers of neuronal degeneration. Here, we describe the use of multifactorial functional analyses to exploit the experimental attributes of the nematode, *Caenorhabditis elegans*, to accelerate the translational path toward identification and characterization of modifiers of dopaminergic neurogeneration. *C. elegans* is ideal for both screening and target validation of potential modifiers. Specific assays discussed in this technical overview include in vivo analyses using whole, intact, and living nematodes with readouts for age-dependent α-synuclein-proteotoxicity and 6-hydroxydopamine-induced neurodegeneration in dopamine (DA) neurons. These methods provide an integrated approach to target characterization and functional validation in *C. elegans* that allow researchers to prioritize lead candidates for translation toward mammalian systems.

Key words: *C. elegans*, Dopamine, Parkinson's disease, Alpha-synuclein, 6-OHDA, RNAi, Neurodegeneration

1. Introduction

Caenorhabditis elegans is a rapidly cultured organism (3 days from fertilized egg to adult) with an experimentally tenable lifespan (14–17 days), and studies can be designed to take exploratory concepts to mechanistic fruition, rapidly. Moreover, this microscopic nematode, which is a millimeter in length as an adult, is grown on agar Petri dishes with a bacterial food source, and thus, experiments are inexpensively performed. Well-designed experiments can be conducted with hundreds of animals for each data point or condition desired, therefore yielding statistical power across a variety of distinct experimental paradigms.

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C. elegans has only 959 somatic cells as an adult hermaphrodite, yet it has hypodermis, intestine, muscle, glands, as well as reproductive and nervous systems. This small organism is also transparent and its cells can be readily correlated with gene and protein expression patterns using fluorescent marker constructs, including GFP (1). Thus, when examining neurodegenerative processes, such as those that occur in DA neurons, cell death can be readily observed and quantified within living organisms (Sect. 3).

The cells of *C. elegans* are also genetically invariant and anatomically defined, thereby allowing great accuracy when analyzing expression constructs. Each somatic cell in the nematode has been individually named according to lineage and is documented in the WormAtlas (Table 1). A serial-section, electron microscope-level map of the animal displaying the relationship of all cells and organs to each other is also available. Thus, cells can be followed from inception through final destination, and modifiers of cell survival are readily evaluated. Taken together, these resources have provided a unique platform for detailed cellular studies across many biological fields, including metabolism (insulin/daf signaling pathway), aging, sex determination, apoptosis, and neurodevelopment, among others. The relevance and contributions of *C. elegans*

Table 1
Key online resources for *Caenorhabditis elegans* research

Database	Web address	Description
WormBase	http://www.wormbase.org/	Gene summary pages containing functional, structural, and phylogenetic information. Links to other databases (i.e., interactome, microarray databases, expression patterns, etc.), related to query genes or ORFs. Links to published articles and *C. elegans* meeting abstracts as well
Caenorhabditis elegans WWW Server	http://elegans.swmed.edu/	Collection of hyperlinks to sites relevant to the study of *C. elegans* and other nematodes
WormBook	http://www.wormbook.org/	An online, open-access, site with peer-reviewed chapters describing nematode biology
WormAtlas	http://www.wormatlas.org/	An online database of the structural anatomy and its relationship to *C. elegans* behavior, with a complete nervous system wiring diagram at the EM level

research to that of higher eukaryotes, and humans, have been recognized at the highest levels of scientific achievement, as worm researchers were awarded the Nobel Prize in Physiology or Medicine for discovering programmed cell death (2002), RNA interference (RNAi) (2006), and the Nobel Prize in Chemistry for GFP (2008). In this context, *C. elegans* arguably represents the most well-understood and experimentally tractable animal on our planet.

C. elegans also has a fully sequenced genome and shares approximately 50% of its genes with humans, and there are many bioinformatics resources available to worm researchers, many of which can be accessed through links described in Table 1. Notably, ~70% of genes that are known to cause a genetic disease in humans have an ortholog in *C. elegans*. Application of *C. elegans* toward human disease research has already provided insights into the function of specific gene products linked to a variety of human movement disorders, such as dystonia (2), Huntington's disease (3), ALS (4), and Parkinson's disease (this volume).

C. elegans also exhibits a variety of behaviors that can be elicited using forward and reverse genetic strategies. As an example, Dr. Sydney Brenner, who pioneered the use of *C. elegans* as a model organism in the 1960s, initially worked with his group on a forward mutagenesis screen to identify mutants with abnormal locomotion (the uncoordinated, Unc, phenotype). Forward genetic screens, beginning with a phenotype of interest, often involve the use of a mutagen, such as EMS. This mutagen most often produces point mutations (G/C–A/T transitions) (5). Following successful screening for mutant phenotypes, identification for the genetic lesion(s) typically occurs. Their screen identified 77 *unc* genes that included both neuronal and muscular defects (6) in many genes that have since been identified as evolutionarily conserved components of muscle cells, synaptic transmission, or neurotransmitter release. Many forward genetic screens have been performed since this time, based on a variety of phenotypes (embryonic, morphological, reproductive, and neurological).

Reverse genetic screens require knowledge of the gene sequence of interest. Since the completion of the genome projects for *C. elegans*, humans, mouse, and other well-studied laboratory species, the field of comparative genomics has allowed *C. elegans* researchers to extensively use RNAi screening in their research to knockdown target genes and then screen for potential phenotypes. In nematodes, this method simply involves injecting, soaking, or feeding worms dsRNA that is complementary to the targeted gene of interest. The expression of the candidate gene will be greatly reduced, or silenced, in the next generation. Relevant to human movement disorders, whole genome-wide screens, as well as smaller, hypothesis-based screens, have been performed to examine a variety of phenotypes, such as aging (7), protein aggregation in ALS (4), and spinal muscular atrophy (8).

1.1. Overview of the C. elegans Nervous System

Despite its evolutionary distance from humans, the neurons of *C. elegans* display most of the hallmarks of mammalian neuronal function including ion channels, neurotransmitters (dopamine, serotonin, acetylcholine, GABA, etc.), vesicular transporters, receptors, and synaptic components (9, 10). Compared with the ~100 billion neurons of the human brain, or even the 10,000 neurons of *Drosophila*, *C. elegans* hermaphrodites have exactly 302 neurons. Thus, of the 959 somatic cells comprising the adult animal, approximately 1/3 of these cells are neurons. The nervous system has also been reconstructed in great detail to reveal the anatomy and complete connectivity of all the neurons (11). This is a remarkable accomplishment given that the cell bodies of worm neurons are approximately 2 μm in diameter (12). Worms have 118 different types of neurons that have been classified into groups based on differences in morphology, connectivity, and function, including mechanosensation, chemosensation, and thermosensation. Worms also have approximately 7,000 synapses.

Precisely, eight neurons within a *C. elegans* hermaphrodite are dopaminergic. Six of these neurons are located in the anterior region of the animal (Figs. 1 and 2a, b), and are identified by their lineage names as the four cephalic (CEP) neurons and the two anterior deirid (ADE) neurons, while the other two DA neurons are in the posterior of the animal are called posterior deirid (PDE) neurons (Fig. 2a). The two dorsal CEPs are post-synaptic to the

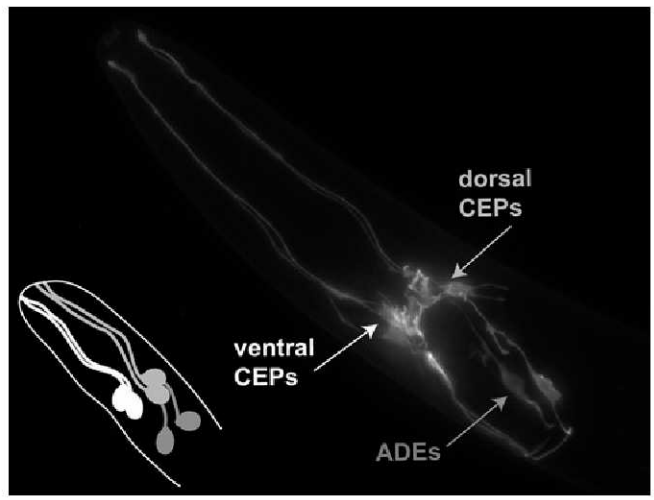

Fig. 1. A fluorescent photomicrograph depicting the anterior-most region of a *Caenorhabditis elegans* hermaphrodite. The DA neurons are illuminated using GFP driven from the DA transporter promoter ($P_{dat\text{-}1}$::GFP). The six cell bodies and neuronal processes include two pairs of cephalic (CEP) neurons and one pair of anterior deirid neurons (ADEs). The line drawing is a representation of the association of the DA neurons to each other; the dorsal CEPs *(light gray)* are post-synaptic to the ADE neurons (*darker gray*) while the ventral CEPs (*white*) are not post-synaptic to the ADE neurons.

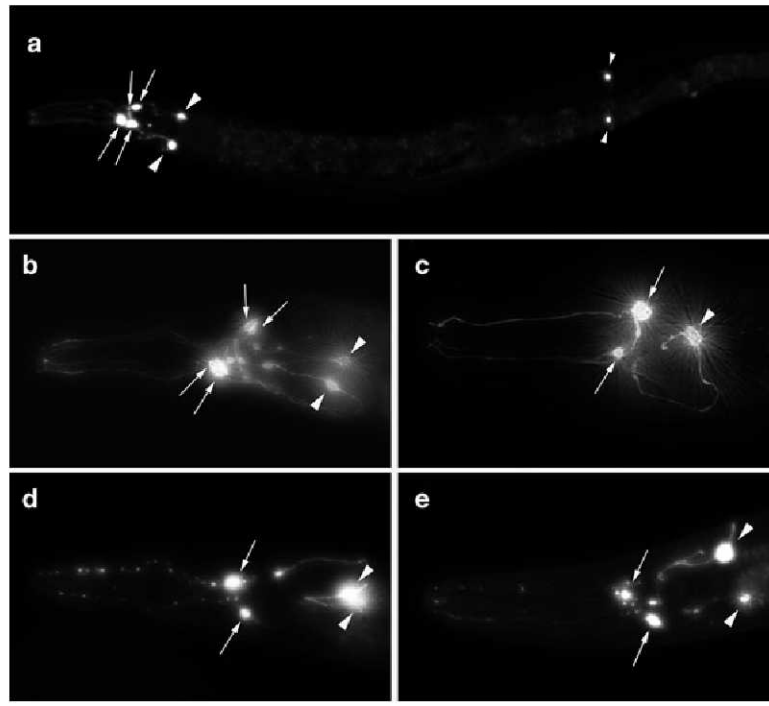

Fig. 2. Representative images of *Caenorhabditis elegans* DA neurons in normal and degenerative states. (**a**) An image depicting all eight DA neurons in a *C. elegans* hermaphrodite. The six anterior DA neurons are visible on the left (two pairs of CEP neurons (*arrows*) and one pair of ADE neurons (*large arrowheads*)), where the cell bodies and processes are highlighted. Two posterior deirid (PDE) neurons are indicated with *small arrowheads* on the right side of this image. (**b**) A magnified view of the anterior region of a worm displaying the cell bodies of the four CEP neurons (*arrows*) and the two ADE neurons (*arrowheads*). (**c**) An image displaying a 7-day-old worm that is co-expressing GFP and α-syn in DA neurons; this animal has lost three out of six anterior DA neurons. In this example, only two of the four CEP neurons (*arrows*) and one of two ADE neurons (*arrowhead*) remain. Most worms within this population are missing one or more anterior DA neurons when they are adults. (**d**, **e**) Exposure to 6-OHDA causes progressive degeneration of CEP neurons. (**d**) In this example, two of the four CEP neurons have already degenerated, while the remaining CEP neurites display blebbing and cell body rounding (*arrows*). The two ADE neurons in this animal are still intact (*arrowheads*). (**e**) This worm is undergoing further degeneration, whereby the two remaining CEP neurites are almost invisible and the associated cell bodies are also degenerating (*arrows*); the two ADE neurons are also beginning to degenerate (*arrowheads*).

ADE neurons, while the two ventral CEPs are not post-synaptic to the ADEs (Fig. 1).

There is evolutionary conservation of the pathways involved in the processing, packaging, and transport of DA, thus enabling researchers to utilize the worm for studying various cellular aspects of DA neuron biology. In this regard, several behavioral phenotypes have been identified as being specific for DA signaling in *C. elegans*. For example, upon exposure to exogenous DA, worms

exhibit a decrease in egg laying, locomotion, and defecation. Worms also normally exhibit decreased locomotion upon entering a bacterial lawn, a DA-associated behavior called the basal slowing response (13). Researchers identified that mutations in *cat-2* resulted in no basal slowing response. This gene encodes worm tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis. These studies suggest that there is a role for DA in mechanosensation and food sensing (14). The basal slowing response phenotype is a potential readout for DA neuronal function and dysfunction for *C. elegans* DA models, however, it is time consuming and does not readily lend itself to screening procedures. Notably, a recently published article described an automated worm tracking system that could potentially be modified to quantify the basal slowing response in multiple individual worms in parallel (15).

1.2. C. elegans as a Model for Parkinson's Disease

Orthologs of six familial Parkinson disease (PD) genes have been identified in *C. elegans* and mutant alleles are available for these *C. elegans* PD gene orthologs (Table 2), thus enabling genetic analyses. For example, in a recent paper, *C. elegans* researchers examined double mutants in LRRK2 and PINK1 (*lrk1* and *pink1*, respectively), and determined that the gene products have an antagonist role in cellular stress response and regulation of neurite outgrowth (16). In this manner, single and double mutants of *C. elegans* PD gene orthologs provide an opportunity to evaluate pathways and interrelationships, as well as screen for potential modifiers of PD. Notably, the *C. elegans* genome does not have a homolog for human α-synuclein (α-syn) (Table 2). This exclusion has allowed researchers to overexpress wildtype (WT) or mutant α-syn in the nematode α-syn null genetic background without concerns for endogenous α-syn or the risk of eliciting a dominant negative effect (17–22).

1.2.1. Use of C. elegans as a Screening Tool to Identify Putative PD Susceptibility Genes

An expeditious route toward discerning genetic contributors to PD involves the use of animal systems amenable to screening methods to identify potential genetic modifiers. In this regard, the use of *C. elegans* has the potential to greatly accelerate the discovery of neuroprotective factors for PD (23) and other diseases of protein misfolding. These assays consist of forward and reverse genetic screens, as well as chemical screens. An example of a relevant forward genetic screen in *C. elegans* was performed for enhancers of polyglutamine aggregation in muscle cells, whereby it was determined that a general imbalance in protein homeostasis in post-synaptic muscle cells can occur if there is an increase in acetylcholine (ACh) signaling, or defective GABA signaling (24). Another example of a forward genetic analysis involved screening *C. elegans* DA neurons for insensitivity to 6-hydroxydopamine (6-OHDA), whereby new alleles of the DA transporter (DAT-1) were identified (25).

Table 2
Summary of human familial PD genes, corresponding *Caenorhabditis elegans* orthologs and mutants

PD Gene	PD Protein	*C. elegans* ortholog	E-Value	*C. elegans* chromosome location	C. elegans allele name	Type of mutation
PARK1	SNCA/α-syn	n/a				
PARK2	PRKN/parkin	*pdr-1*	3.4e-38	III	gk448	Knockout allele; superficially wildtype
PARK5	UCHL-1	*ubh-1*	1.2e-33	V	n/a	
PARK6	PINK1	*pink-1*	7.8e-53	II	ok3538	Knockout allele
PARK7	DJ-1	*djr-1.1* *djr-1.2*	1.6e-45 8.9e-36	II V	tm918 tm1346	Both are knockout alleles; double mutant created
PARK8	LRRK2	*lrk-1*	5.5e-66	I	tm1898	Deletion; homozygous viable
PARK9	ATP13A2	*catp-6*	2.5e-180	IV	ok3473	Knockout allele; homozygous viable
PARK11	GIGYF2	n/a				
PARK13	HTRA2	n/a				

There are many examples of reverse genetic, RNAi, screens using the full genome in *C. elegans*. Several of these screens scored phenotypes related to protein misfolding. For example, worms expressing α-syn::YFP in body wall muscle cells were screened for a modulation in protein misfolding and 80 candidates were identified (18). The candidates clustered into a few biological categories. Notably, quality control and ER/Golgi vesicle trafficking gene products were identified as those that, when knocked down, increased protein aggregation. Another category of gene products identified were aging related, but these were suppressors of protein aggregation.

Recently, candidate RNAi screens, based on the mature bioinformatics available within the *C. elegans* field, have become more common. Our laboratory performed a hypothesis-based screen for genetic modifiers of α-syn::GFP misfolding in body wall muscle cells and identified 20 candidate genes. Five of these candidates were subsequently validated as having a protective role in DA neurons as well (17). The candidates identified from this screen included orthologs of known recessive PD genes, DJ-1 and PINK1, VPS41, a vesicular protein necessary for lysosomal trafficking and biogenesis, ATG7, an autophagy-associated regulatory gene, and ULK2, a conserved serine-threonine kinase also related to the yeast autophagy protein, Atg1p.

The outcome of this screen represented an exciting group of gene products with implications for PD, both as potential susceptibility markers and novel targets for therapeutic development. For example, knockout of the lysosomal protein ATG7 has been reported to produce a neurodegenerative phenotype in mice (26). Another neuroprotective gene product identified from our screen, VPS-41, is the nematode homolog of mammalian and yeast VPS41, a protein implicated in trafficking from the Golgi to the vacuole/lysosome in yeast (27). Little was known about the function of VPS41 in mammalian systems except that it is strongly expressed within DA neurons of the *substantia nigra pars compacta*. However, subsequent mammalian studies have since revealed that VPS41 over-expression is protective against rotenone- and 6-OHDA-induced toxicity in SHSY5Y cells (28). Finally, *ULK2* was one of six genes significantly associated with single nucleotide polymorphisms in a genome-wide association study (GWAS) of PD patients (29). Therefore, it is clear that establishing a functional screening paradigm for modifiers of PD-related phenotypes in *C. elegans* has successfully yielded an intriguing collection of effectors that demonstrate the predictability of the nematode model to identify targets with high translational potential.

1.2.2. Use of C. elegans to Validate Therapeutic Targets and Chemicals

Our ability to successfully predict the probability of PD among individuals is dependent upon knowledge about genetic susceptibility factors that render certain populations at risk. In attempting

to discern genetic factors associated with PD, scientists working with a variety of different organisms have generated many lists of candidate genes and proteins, but most of these leads remain mechanistically undefined. In this regard, application of *C. elegans* in a directed manner can facilitate the functional evaluation of leads originally identified in other species (yeast, cell culture, mouse, and human genomic studies). As described previously, a distinct advantage of using *C. elegans* for functional investigation of gene activity and validation is that large populations of isogenic animals can be propagated and analyzed, leading to an unequivocal level of accuracy when evaluating neurodegeneration. As shown in Fig. 2, we have established isogenic lines of transgenic *C. elegans* that overexpress human α-syn in DA neurons ($P_{dat\text{-}1}$::α-syn + $P_{dat\text{-}1}$::GFP) that enable rapid evaluation of factors (drugs and genes) that protect or enhance DA neurodegeneration. This worm strain has been successful in rapidly predicting genes that have significant consequences for neuronal survival in mammalian systems. For example, we investigated three Rab gene products, involved in regulating vesicular trafficking, that were originally identified from yeast screens as effectors of α-syn-dependent toxicity (30, 31). Suppression of cytotoxicity was recapitulated in yeast cells, *Drosophila*, rat neuronal cell cultures, and in *C. elegans*, where we showed that elevated expression of specific Rab GTPases rescue DA degeneration induced by α-syn overexpression. For example, overexpression of mammalian Rab1 successfully by-passed an α-syn-associated block of ER to Golgi trafficking (30). New molecules that impact this cellular mechanism have since been discovered in yeast and subsequently validated across multiple organisms, including *C. elegans* (32).

There is a growing body of literature demonstrating the utility of *C. elegans* for pharmacological research. A variety of compounds have been uncovered, ranging from those associated with modulating neurotransmitter activity (33, 34), anesthetics (35), lifespan extension (36), and a new calcium channel antagonist (37). Our laboratory has used *C. elegans* to conduct a small molecule screen to identify compounds that positively modulate the activity of the neuroprotective chaperone-like protein, torsinA, in vivo (38). Deficit in torsinA function is responsible for a human movement disorder termed early-onset torsion dystonia, and is linked to an in-frame 3-bp deletion in the human *DYT1* gene. These same molecules were later shown to restore functional capacity of torsinA activity in human DYT1 dystonia patient fibroblasts and reverse behavioral abnormalities in a DYT1 knock-in mouse model of early-onset torsion dystonia (38). Of relevance to PD, worms are being exploited as a system for therapeutic drug screening (39) and functional validation of drugs with a therapeutic potential for PD that have been identified in other systems (32, 40, 41).

2. Large-Scale RNAi Screening Using an α-syn Misfolding Phenotype in Non-neuronal Cells

Central to the formation of Lewy bodies, a primary pathological hallmark of PD, is α-syn (42, 43). Importantly, it was demonstrated that genomic multiplication of the WT α-syn gene results in PD, indicating that overexpression of this protein alone could lead to the disease (44). A recent GWAS on approximately 800 familial PD cases further supports that the *SCNA* (α-syn) locus is a major susceptibility factor (45). The effects of overexpression, mutation, misfolding of α-syn has led to the hypothesis that the cellular clearance of this small, aggregation-prone protein is critical to avoiding the neurodegenerative state.

As displayed in Table 3, there are two models of α-syn misfolding in *C. elegans* body wall muscle cells that can be evaluated for the consequences of α-syn overexpression and misfolding in vivo. In both models, α-syn is fused at the C-terminus to either GFP or YFP, and inclusions are visualized in body wall muscle cells. These two transgenic models were used in independent RNAi screens for gene products that modulate protein misfolding (17, 18). In both transgenic models, the aggregates become more abundant as the animals get older and can be scored over time as worms develop and age (Fig. 3).

At first, it might appear to be a daunting task to assess differences in α-syn misfolding and aggregation in body wall muscle cells visually and objectively using a compound fluorescent microscope since these aggregates are quite small. However, researchers have successfully obtained data from RNAi screens using these transgenic worm strains. In this regard, the strategies utilized were different, but equally effective, as described below. One strategy that can be used is a qualitative assessment of aggregates to compare treatment with controls. This can become second nature over time using the following criteria. The general number (none/few/many) and size of the aggregates (small/medium/large) can be scored relative to control animals. This analysis should be performed for ~20 worms per RNAi treatment (46). A worm is scored as having significant aggregation if it has multiple muscle cells with increased quantity and size of aggregates (Fig. 3a vs. Fig. 3c, d). With experience, reproducible data will consistently result from this qualitative measure of α-syn misfolding analysis. An alternative strategy is to perform a quantitative analysis of the number of aggregates, whereby only a few anterior muscle cells in each of ten animals are scored for inclusions; positive animals will have significantly more inclusions than controls (18). In both of these strategies, the level of protein misfolding in the control, α-syn, transgenic animals is directly compared to animals treated with RNAi, whereby the observer is attempting to uncover factors that, when depleted, cause an enhancement in aggregation.

Table 3
***Caenorhabditis elegans* models of α-syn expression and their corresponding phenotypes**

α-syn expression pattern	Transgenic construct(s)	Phenotypes	Reference
WT α-syn expression patterns			
Body wall muscle cells	P_{unc-54}::α-*syn*::*GFP*	α-syn accumulation visualized by misfolded GFP; used in RNAi screen	(17)
	P_{unc-54}::α-*syn*::*YFP*	α-syn accumulation visualized by misfolded YFP; used in RNAi screen	(18)
Pan neuronal	P_{aex-3}::α-*syn*	Reduced motor movement; DA neurodegeneration visualized by GFP	(19)
	P_{snb-1}::α-*syn*	Mitochondrial stress	(20)
	P_{unc-51}::α-*syn*	Endocytosis defects; motor/developmental defects	(21)
DA neuron specific	P_{dat-1}::α-*syn*	DA neurodegeneration visualized by GFP	(19)
	P_{dat-1}::α-*syn*	DA neurodegeneration visualized by GFP	(22)
	P_{dat-1}::α-*syn*	Slightly reduced DA levels; α-syn accumulation in DA neurons	(21)
Mutant α-syn expression patterns			
Pan neuronal	P_{aex-3}::α-*syn A53T*	Reduced motor movement; DA neurodegeneration visualized by GFP	(19)
	$P_{unc-119}$::α-*syn A53T*	Mitochondrial stress	(20)
	P_{unc-51}::α-*syn A30P* and *A53T*	Endocytosis defects; motor/developmental defects	(21)
DA neuron specific	P_{dat-1}::α-*syn A53T*	DA neurodegeneration visualized by GFP	(19)

Our laboratory utilized a third strategy for RNAi screening (Fig. 4a). We reasoned that the misfolding in the control α-syn genetic background might not be sensitive enough to detect moderate aggregate changes because of their normal abundance (Fig. 3a). Therefore, we co-expressed a chaperone (TOR-2, a worm ortholog of torsinA) that ameliorated the formation of the fluorescent misfolded α-syn::GFP proteins (Fig. 3b), which extended prior observations on torsinA chaperone activity (47, 48). These α-syn + TOR-2 animals were then used as the starting genetic background in an RNAi screen, which provided for a much easier qualitative analysis of aggregate levels versus controls (Fig. 3b vs. Fig. 3c, d).

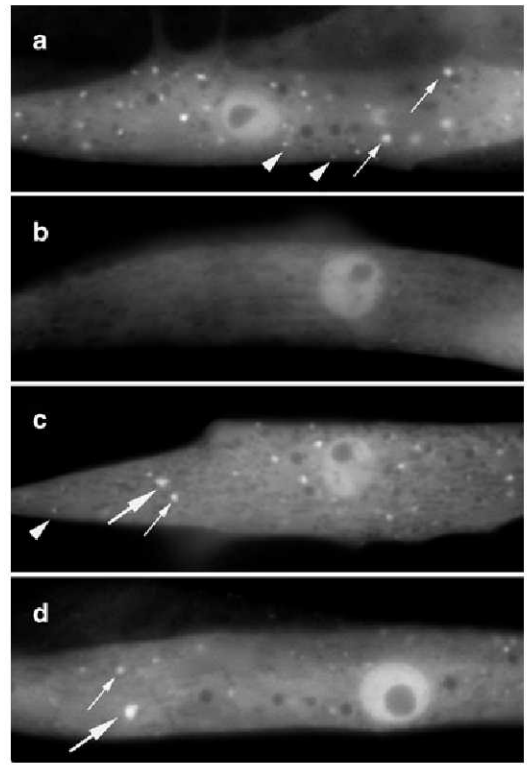

Fig. 3. α-syn misfolding and aggregation within *Caenorhabditis elegans* body wall muscle cells. (**a**) A single body wall muscle cell expressing α-syn::GFP where a moderate number of aggregates are visible following expression from the P_{unc-54}::α-syn::GFP construct. The aggregate sizes vary, and are typically small (*arrowhead*) or medium (*small arrow*). (**b**) A body wall muscle cell from a worm co-expressing the chaperone protein, TOR-2, and α-syn::GFP (P_{unc-54}::*tor*-2 + P_{unc-54}::α-syn::GFP). α-syn misfolding is attenuated in the presence of TOR-2. (**c, d**) Single muscle cells wherein misfolded α-syn::GFP is revealed in the TOR-2 + α-syn::GFP transgenic background following RNAi knockdown of specific gene targets. The cells exhibit α-syn::GFP aggregates of varying sizes. (**c**) In this example, the aggregates are mostly small (*arrowhead*) and medium (*small arrow*) and abundant (**c**), yet there is a large aggregate in this cell (*arrow*). (**d**) In this example, the cell has only a few sparsely distributed aggregates of medium (*small arrow*) and large (*arrow*) aggregates.

Furthermore, the presence of the chaperone, which likely maintained α-syn nearer a threshold of protein misfolding, potentially assisted with the identification of genetic factors that affect the formation of misfolded oligomers versus more mature aggregates.

Notably, these *C. elegans* models of α-syn protein misfolding display an age-associated increase of inclusion formation. Since PD is a disease of aging, an age-related aggregation phenotype provided an opportunity to further select positive candidates based on candidate aggregation-inducing potency. Indeed, we were able to differentially identify modifiers of α-syn misfolding by scoring aggregation at distinct time points in the worm lifespan.

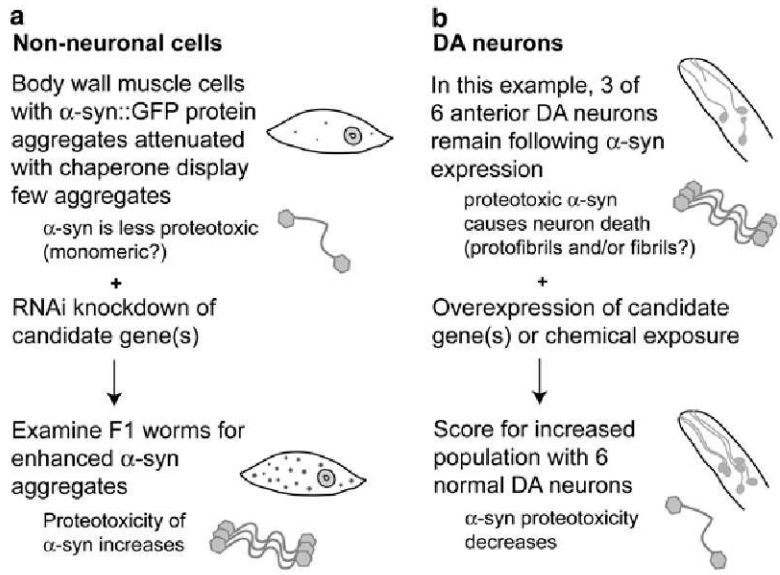

Fig. 4. Identification of factors influencing α-syn proteotoxicity in *Caenorhabditis elegans*. (**a**) RNAi screening in body wall muscle cells. A chaperone protein, TOR-2, is co-expressed with α-syn to attenuate the proteotoxicity of α-syn. The resulting expression pattern of α-syn::GFP in the muscle cells is diffuse. Following RNAi knockdown of candidate genes that affect protein misfolding, a return of the oligomeric α-syn::GFP will be visible in the muscle cells. (**b**) Target gene or chemical validation in DA neurons. Expression of α-syn in DA neurons is toxic and results in an age- and dose-dependent degeneration of the DA neurons. Over-expressed candidate genes or compounds can be evaluated for therapeutic value in this model to determine, if exposure decreases the proteotoxicity associated with α-syn.

In this regard, following RNAi knockdown, 20 genes strongly induced α-syn misfolding at an early (larval) stage of development, while there was a larger set of genes (125) that were effectors at a later chronological age (17).

3. Induction of DA Neurodegeneration in *C. elegans* and Assessment of PD Susceptibility Genes

In the era of modern "omics"-based science, technologies such as RNAi screens typically uncover many more candidates than can be readily studied. Therefore, it is useful to combine such datasets with secondary assays that exploit distinct criteria to further delineate and define functional modifiers. With respect to modeling PD, acute and chronic methods for inducing DA neurodegeneration *C. elegans* using the neurotoxin 6-OHDA or through overexpression of α-syn, respectively, are well studied. While the mechanism of action of these two neurotoxic insults is different, there are common therapeutic interventions that can be examined (Fig. 5).

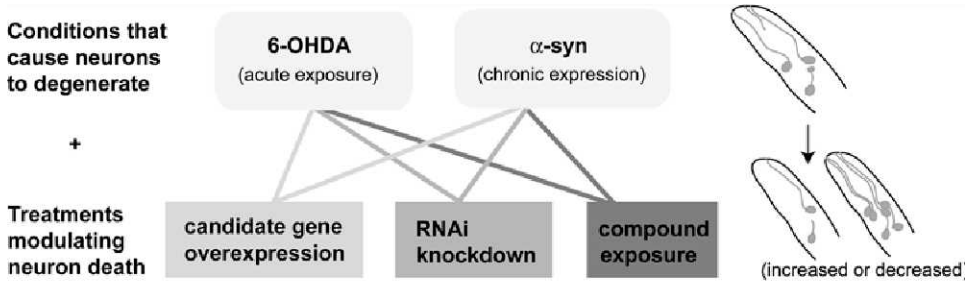

Fig. 5. Common exposure paradigms in *Caenorhabditis elegans* for examining DA neurodegeneration. 6-OHDA is an acute exposure that will cause neurodegeneration within 24 h, while transgenic overexpression of α-syn is chronic and will not result in significant degeneration until adulthood. Regardless of the condition used to induce degeneration, analysis of the neurons, and potential downstream applications and treatments, are similar. These include candidate gene overexpression, RNAi knockdown, and compound exposure. In all cases, *C. elegans* DA neurons can be examined for a decrease in neurodegeneration as a means of discovering therapeutic modalities or an increase in degeneration, which might provide information on cellular mechanisms of action.

The analysis of DA neurons in *C. elegans* is more time-consuming than protein aggregation studies, however; and therefore, researchers usually perform small-scale screening for neuroprotective agents, or validate genes or chemicals identified by other, often more high-throughput, means.

3.1. 6-OHDA-Induced Dopaminergic Toxicity

Although the precise mechanisms underlying selective neuronal vulnerability remain to be fully elucidated, the tendency of DA to induce oxidative damage in neurons renders this neurotransmitter a potential contributory factor to the degenerative process in PD. The toxicity of 6-OHDA is mediated through the formation of ROS by the generation of hydrogen peroxide and hydroxide radicals via a non-enzymatic auto-oxidation process (49, 50). After exposure to 6-OHDA, *C. elegans* DA neurons exhibit a characteristic dose-dependent pattern of apoptotic cell death that was confirmed by ultrastructural analysis (51). 6-OHDA is selectively taken up by the *C. elegans* DA transporter, DAT-1, and *dat-1* mutant worms are resistant to degeneration. Degeneration can be monitored in living animals by co-expression of GFP and occurs within a few hours of exposure (22, 51). Detailed methods for successfully performing this procedure can be found in several publications (22, 51, 52).

6-OHDA solutions (6-OHDA plus ascorbic acid, which will stabilize the 6-OHDA) must be made immediately prior to worm exposure. If the solution immediately turns pink, it has oxidized, and it will not cause DA neurodegeneration. For experimental consistency, it is important to use the same chemical supplier and lot number for 6-OHDA, as there can be vast changes in the amount of degeneration observed. Thus, for each new lot number, the 6-OHDA should be tested for DA neurodegenerative efficiency before commencing with experimental procedures. In general,

since 6-OHDA oxidizes quickly, normal oxidation will usually occur after the 1-h incubation. Exposed worms should also be developmentally synchronized to the late L3–L4 staged; treatment of younger and older worms with 6-OHDA increases lethality or resistance to the toxin, respectively.

As described previously, the hermaphrodite has six anterior DA neurons (Fig. 2a, b). Most *C. elegans* laboratories analyze the six anterior DA neurons for neurodegeneration following 6-OHDA treatment (Fig. 2d, e). Worms are evaluated by scoring these anterior dopaminergic neurons as either "normal" [i.e., WT] or "degenerative" for ~50 worms per round of toxin exposure (repeat 2×). Following 6-OHDA treatment, degenerating neurons may exhibit several morphological defects, including neurite blebbing (Fig. 2d), cell body rounding (Fig. 2e), and complete cell loss.

A primary application of this strategy has been to investigate whether the toxicity associated with 6-OHDA exposure can be rescued via transgene expression, gene depletion (RNAi or mutant analysis), or chemical exposure (Fig. 5). Enhanced survival of DA neurons would indicate a mechanism, whereby DA-associated toxicity can be attenuated. For example, the 6-OHDA assay was used to screen through 11 mammalian DAergic, GABAergic, and glutamatergic receptor agonists or antagonists. Two of the 11 compounds, bromocriptine and quinpirole, protected against 6-OHDA toxicity in a dose-dependent manner (39). Likewise, we previously reported that overexpression of a chaperone-like protein (torsinA) in *C. elegans* DA neurons results in dramatic suppression of neurodegeneration following 6-OHDA treatment (22). Further investigations into this neuroprotective mechanism of action indicated that torsinA, an ER luminal protein, was influencing the turnover of DAT-1, which is a polytopic membrane protein, thus torsinA indirectly attenuated cell death by limiting the access of 6-OHDA to the DA neurons.

6-OHDA-induced DA neurodegeneration is both rapid and acute and may represent an effect that is too strong to accurately evaluate the neuroprotective influence of certain gene products or chemical modifiers. As an alternative to 6-OHDA exposure, a more subtle means of inducing neurodegeneration involves the intracellular overproduction of DA. We have also generated transgenic lines of animals that exhibit neurodegeneration by selective overexpression of TH, the *cat-2* gene product in DA neurons (22). Given the complexities of DA signaling, it may be more mechanistically challenging to interpret modifiers in this experimental context; however, this may be a suitable alternative for some lines of experimentation.

3.2. α-syn Proteotoxicity in DA Neurons

As discussed in Sect. 2, *C. elegans* transgenic lines expressing α-syn in the large body wall muscle cells display readily observable protein aggregation (using a compound microscope). Notably, however, the neuronal α-syn models do not (Table 3). The difference between the body wall muscle and neuronal models described

herein is that in the former, α-syn is directly fused, at the C-terminus, with a fluorescent protein, while in the latter α-syn is not fused to a fluorescent marker protein, and is thus undetectable in live animals. Rather, the DA neurons are separately marked with GFP (independently expressed) as a means of examining neurodegeneration. Because the cell bodies of *C. elegans* neurons are very small (2 μm), the likelihood of accurately scoring differences in α-syn aggregation in these cells is minimal; therefore, neurodegeneration is a more robust phenotype. Notably, there are several *C. elegans* models of neuronal α-syn proteotoxicity, and they all exhibit a quantifiable phenotype (Table 3). Some of the models overexpress α-syn panneuronally, while others are DA specific. Most of the models overexpress WT α-syn and not the mutant forms. This situation correlates with human PD, where multiplication of the WT α-syn locus leads to familial PD (44), and only exceedingly rare PD cases are associated with intrinsic α-syn mutation. It should be noted that a common phenotype noted among all the transgenic lines expressing α-syn specifically in DA neurons is neurodegeneration. Thus, these models can be used to examine factors that enhance or reduce the basal level of degeneration in vivo (Fig. 4b).

In our laboratory, we have established that overexpression of WT human α-syn under control of a DA-specific promoter [$P_{dat\text{-}1}$::α-syn] results in age- and dose-dependent neurodegeneration (17, 22). These transgenic worms reproducibly demonstrate a high level of DA degeneration as the animals age; by day 5 of adulthood only 15% of the population has a normal complement of anterior DA neurons (Fig. 2b, c). We have used this well-characterized invertebrate model of neurodegeneration to assess gene products and chemicals for neuroprotective properties because it reproduces an important characteristic of PD – progressive dopaminergic degeneration. In this manner, large populations of isogenic animals can be propagated and analyzed, leading to an unequivocal level of accuracy in evaluating factors influencing neurodegeneration.

When analyzing transgenic animals expressing a target gene candidate, DA neuron analysis should be scheduled when synchronized worm populations reach specific ages, because degeneration increases over time. An example timeline for use with our α-syn transgenic animals follows. If the candidate gene is hypothesized to decrease neurodegeneration, worms should be analyzed at later stages, allowing for increased neurotoxicity in the control, α-syn only lines. This correlates to 7 and 10 days post-hatching, if the worms are grown at 20°C (i.e., 3- and 6-day adults). When examining candidate genes that might enhance α-syn-induced toxicity, younger worms should be analyzed [6 and 8 days post-hatching (i.e., 2- and 4-day adults, respectively)]. By judicially considering time points for analysis, and modifying them empirically through several rounds of analysis, potential effects for candidate gene products can be uncovered (53).

The analysis of DA neurons for α-syn neurodegeneration is similar to the procedure described for 6-OHDA-induced degeneration, whereby the six anterior DA neurons are analyzed. However, the phenotypes of degenerating neurons are somewhat different following this proteotoxic insult (Fig. 2c vs. Fig. 2d, e). Degenerating α-syn neurons can vary slightly and range from neurite retraction, cell rounding, and cell loss (Fig. 2c); in our experience, we have never observed the neurite blebbing that is commonly seen following 6-OHDA exposure (Fig. 2d). Each experiment should be repeated in triplicate (30 worms/screen × 3 rounds of analysis = 90 total worms analyzed/transgenic strain; with three separate transgenic lines/experiment for a total of 270 worms analyzed per gene product).

Detailed protocols for creating expression vectors and transgenic animals are available from other sources (46, 52, 54). Two sources of online videos describing the production of transgenic *C. elegans* and scoring of DA neurons are available from the *Journal of Visualized Experiments* (JoVE) (53, 54). In considering other useful tips in the application of worm PD models, it is important to consider that α-syn-induced DA neurodegeneration experiments require that adult worms stay alive for many days. Since these hermaphrodites self-propagate frequently and worm plates can become very crowded with offspring, transfer ~100 young adult stage transgenic hermaphrodites to fresh plates containing 0.04 mg/ml 5-fluoro-2′-deoxyuridine (FUDR) (Sigma-Aldrich; F-0503). FUDR will prevent the need to continuously transfer adult worms, because it is a nucleotide analog that inhibits DNA synthesis and self-reproduction. Importantly, we have determined that the presence of progeny and over-crowding can lead to stressed worms and inconsistent DA neurodegeneration data. Additionally, depending on the nature of the study, it might be useful to score ventral and dorsal CEPs separately, because the dorsal CEP neurons synapse onto the ADE DA neurons while the ventral CEP neurons do not (Fig. 1). In our $P_{dat\text{-}1}$::α-syn worms, dorsal CEPs consistently degenerate significantly more often than the ventral CEPs. ADEs also degenerate significantly more than either dorsal or ventral CEP neurons (52).

4. RNAi in Neuronal Cells

Until recently, targeted knockdown in *C. elegans* neurons, and especially DA neurons, has been notoriously difficult due to selective resistance to RNAi (55) (Fig. 6a), even in supersensitive backgrounds. We have had some success with knockdown of genes in pharmacologically-sensitized backgrounds with neurons (i.e., GABAergic, cholinergic) anatomically positioned near the worm

intestine and body wall, where exposure to dsRNA from feeding or soaking would be expected to be in greater concentration (56, 57). Overall, the limitation in neuronal efficacy of *C. elegans* RNAi has led most researchers to rely on the availability of worm strains carrying desired mutant alleles of specific genes to examine cellular processes within neuronal backgrounds. While many mutations are available, targeted knockdown of specific genes would greatly expand the repertoire of targets and/or provide stronger effects when the strength of existing alleles is either unknown or weak.

A significant exception to this limitation includes a study by Kuwahara et al. (21), wherein these researchers generated worm strains overexpressing WT and mutant α-syn pan-neuronally in a mutant background reported to be supersensitive to RNAi in worm neurons (*eri-1*). These animals did not exhibit neurodegeneration or other neuronal dysfunction, thereby indicating sub-pathological threshold levels of α-syn were expressed; moreover, DA neurons were not being directly evaluated in this study. However, differential phenotypic effects from a WT strain expressing the same α-syn constructs could be revealed following RNAi against established neuronal targets. Therefore, through an RNAi screen that targeted knockdown of 1,673 candidate genes implicated in nervous system function, ten positives were identified, primarily consisting of components from the endocytic pathway that exhibited growth and motor abnormalities. These data provided an interesting link between α-syn overexpression with defects in synaptic vesicles trafficking.

Notably, a recently published paper described a new method that results in substantially enhanced neuronal RNAi in worms (58). We have investigated application of this procedure for a single dopaminergic target gene and observed robust knockdown for this initial "proof of concept" target. To generate worms that are hypersensitive to RNAi pan-neuronally, the Chalfie lab expressed a gene encoding the ATP-independent double-stranded RNA channel, SID-1, necessary for systemic RNAi, in all neurons under control of *unc-119*, a gene required for neuronal development ($P_{unc\text{-}119}$::SID-1), in *sid-1* mutant animals, which are resistant to RNAi (Fig. 6b, c). This allowed for cell-specific targeted knockdown of genetic modifiers in neurons, while leaving other cells in the nematode resistant to RNAi (Fig. 6c). In addition to enhancing neuronal selective RNAi, this new method enables evaluation and screening of otherwise lethal genes.

We examined this strain for sensitivity to RNAi in DA neurons by knocking down the endogenous DA transporter (*dat-1*). As described in Sect. 3.1, DAT-1 is responsible for the selective re-uptake of DA, as well as neurotoxic DA analogs, such as 6-OHDA. Following RNAi knockdown of *dat-1* in *sid-1* mutant animals, as well as in *sid-1* mutant animals over-expressing $P_{unc\text{-}119}$::SID-1, we treated worms with 6-OHDA. We utilized two different worm

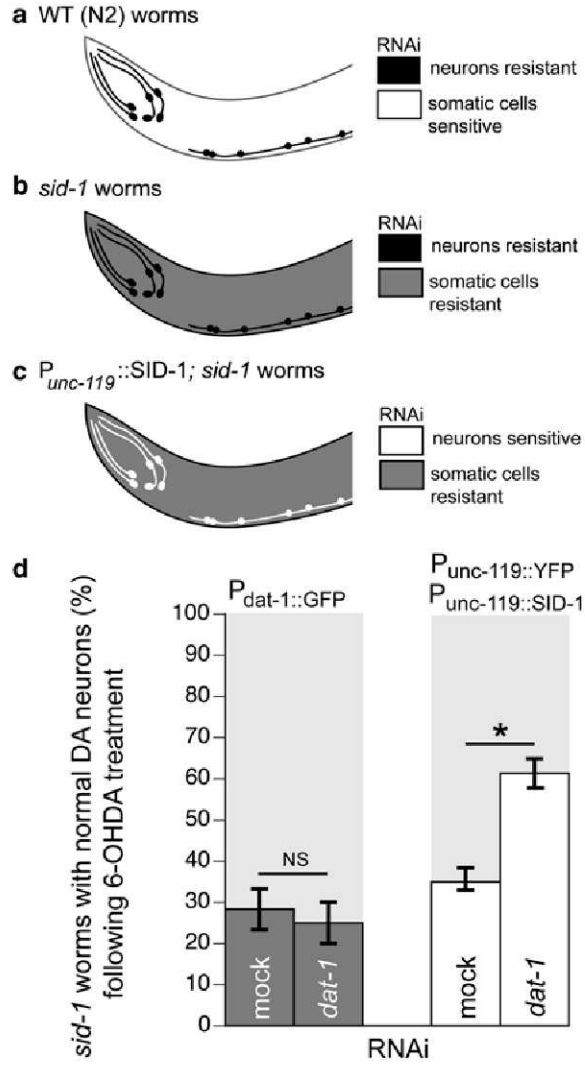

Fig. 6. Neuronal-specific RNAi in *Caenorhabditis elegans*. (**a**–**c**) Illustration depicting the mechanism of action for this method. (**a**) In normal, WT (N2) worms, non-neuronal somatic cells are sensitive to RNAi while neurons are typically resistant to RNAi. (**b**) *sid-1* mutant worms are resistant to RNAi in all cells, because the normal function of the *sid-1* gene product is to facilitate the uptake of double-stranded RNA. (**c**) In transgenic *sid-1* mutant worms over-expressing the SID-1 protein pan-neuronally ($P_{unc-119}$::*sid-1; sid-1*), the neurons are selectively sensitive to RNAi. Notably, in this transgenic strain, the non-neuronal somatic cells are still resistant to RNAi, because SID-1 is required cell autonomously for RNAi. (**d**) Proof of principle experiment demonstrating DA neuron-specific RNAi in P_{unc119}::YFP + $P_{unc-119}$::SID-1; *sid-1* worms using 6-OHDA exposure. The left side of the graph shows data collected from *sid-1* mutant worms expressing GFP specifically in DA neurons (P_{dat-1}::GFP; *sid-1*), while the right side of the graph shows data from *sid-1* mutant worms expressing YFP and SID-1 pan-neuronally. DA neurons were examined 24 h after 6-OHDA treatment. All worms were exposed to mock RNAi (empty vector) or *dat-1* RNAi to knockdown the endogenous DA transporter. Following knockdown, worms were exposed to 6-OHDA and examined for resistance to this toxin 24 h after treatment. Notably, only P_{unc119}::YFP + $P_{unc-119}$::SID-1; *sid-1* worms exposed to *dat-1* dsRNA (*far right column*) displayed resistance to 6-OHDA, demonstrating that DAT had been knocked down in these animals. $^*P < 0.05$.

strains for this experiment, one that was not sensitive to RNAi and simply expressed GFP in DA neurons, as well as the neuronally supersensitive strain (that over-expressed SID-1 pan neuronally). Since both worm strains expressed GFP in DA neurons, we were able to assess DA neurodegeneration. Following *dat-1* RNAi, we treated L4-staged worms with 30 mM 6-ODHA and scored DA neurons 24 h later. As shown in Fig. 6d, over-expression of SID-1 pan-neuronally (right columns) resulted in robust resistance of DA neurons to 6-OHDA, while the non-sensitive strain exhibited extensive degeneration from 6-OHDA exposure. Thus, this experiment validates that the $P_{unc\text{-}119}$::SID-1 strain is sensitive to RNAi in the DA neurons. In this regard, use of this supersensitive RNAi strain alone, or in combination with worms expressing α-syn, exposure to neurotoxins, or with existing PD mutant alleles, will be a valuable tool for continued PD research in the *C. elegans* field.

5. Summary

Here, we have highlighted genetic approaches to investigate cellular mechanisms underlying neurodegeneration associated with PD in the nematode model organism, *C. elegans*. The techniques discussed included the use of transgenic *C. elegans* for assessing α-syn proteotoxicity and DA neurodegeneration resulting from α-syn overexpression, as well as 6-OHDA toxicity. Advantages and disadvantages of these models center upon differential readouts and means by which cellular stress and neurodegeneration are induced. In particular, the α-syn protein aggregation assay (in body wall muscle) is well suited to large-scale RNAi screening methodologies. Conversely, assays performed in DA neurons (RNAi, 6-OHDA, or α-syn-induced neurodegeneration) are more tedious, but are an appropriate platform for target validation or hypothesis-based screening for smaller numbers of genes or compounds. As the pathological basis for many neurodegenerative diseases is complex, the alternate screening strategies described herein may or may not target the same cellular mechanisms involved in PD. Nevertheless, the identification of multiple gene and/or drug candidates through such assays may provide previously unreported avenues for therapeutic intervention. Validation across assays and systems is equally essential for optimal translational value. Continued application of *C. elegans* for PD research represents an outstanding opportunity to conduct mechanistic studies using mutants (or RNAi knockdown) in gene and cellular pathways, as well as accelerate positive clinical outcomes for PD.

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