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Review

Perspectives on *Caenorhabditis elegans* models of human Parkinson's Disease

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Abstract

Caenorhabditis elegans is a 1 mm long nematode comprised of 959 cells in the adult hermaphrodite. Through transgenic injection, neurotoxin treatment, or isolation of mutants, this roundworm has been used as an animal model for studies of human Parkinson's disease (PD). The ability to genetically manipulate this animal, its short reproductive cycle and transparent body type have allowed it to be treated pharmacologically and toxicologically and interrogated for features of PD including loss of dopaminergic neurons, aggregation of α-synuclein protein, basal slowing responses to food, and lifespan. This short review aims to capture some of the recent studies on Caenorhabditis elegans PD models and highlight some aspects of absorption, distribution, metabolism, and excretion that make the worm a useful organism for studies in neurodegeneration.

Keywords

keyword; dopamine, nematode, transgenic model, neurodegeneration, synuclein.

Introduction

Parkinson's disease (PD) is a devastating neurological disorder for which there is currently no cure. Symptoms of the disease include severe motor impairment such as tremor, slowness of movement, rigidity, and postural instability, and involuntary movement. The disease etiology is attributed to progressive loss of dopaminergic neurons from the basal ganglia. Neuropathological hallmarks of PD consists of Lewy bodies and Lewy neurites which are proteinaceous inclusions containing α-synuclein. While there is a genetic basis for PD, the overwhelming number of cases arises sporadically. To date, at least 20 human genes (*PARK1-20*) have been identified that contribute to PD.

Several recent reviews have dealt in detail with the contributions of *Caenorhabditis elegans* (*C. elegans)* models in understanding the development and progression, and role of genetic factors in PD [1-5]. Those reviews cover in depth the genetic models available and their contributions to understanding the neuropathology of the disease. This short review aims to take a broad pharmacological perspective in order to demonstrate the utility of *C. elegans* PD models in studying not only basic mechanisms but also potential treatments.

C. elegans is a round worm nematode that naturally lives in soil. Its adult length of approximately 1 mm contains exactly 959 cells in the hermaphrodite sex and 1031 in males. Of these cells, 302 are neurons of which 8 are dopaminergic in the hermaphrodite. The male has 6 additional dopaminergic neurons in the tail. The basic body plan consists of an outer cuticle that acts as a flexible tube and an inner tube that comprises the intestine. *C. elegans* is transparent, so neurons can be visualized in living animals through differential contrast or fluorescence microscopy. The reproductive life cycle of 2.5 days and life span of 3 weeks also provides vast numbers of easily reproduced animals for experiments. Finally, in the laboratory, *C. elegans* is cultured on the surface of agar plates with *E. coli* as a food source and can also be cultured in liquid making pharmacology and toxicology studies straightforward.

The specific dopaminergic neuron toxins 6-hydroxy dopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the precursor of toxic 1-methyl-4-phenylpyridinium (MPP+) have been used widely in *C. elegans* [6,7]. The toxic effects of 6-OHDA (10 mM) have been pharmacologically shown to be via uptake through the dopamine transporter since these effects can be blocked by inhibitors D-amphetamine (10 mM) or imipramine (1 mM) [6]. More recently, dopaminergic neuron degeneration has been shown with heavy metals such as methyl mercury [8], manganese [9,10], and aluminum [11]. More general toxins directed at mitochondrial electron transport and oxidative stress includes the pesticides paraquat and rotenone, and these have been used as well in *C. elegans* to generate neurodegeneration models [12]. Transcriptomic studies have verified the role of oxidative stress and misfolded protein pathways in the toxicology of these agents [13]. In addition, the use of GFP protein expressed in dopaminergic neurons has greatly facilitated the scoring of neurodegeneration in these chemical models. This visualization in living animals allows for direct and speedy determination of the effectiveness of chemical treatments.

Of the 20 PARK genes, 11 have *C. elegans* orthologs [1-5]. The orthologs can be used to model human PD by transgenic overexpression if the mode of inheritance is dominant. Alternatively, homozygous mutants or RNAi knockdowns can be used if the mode is recessive. For PARK genes without a *C.elegans* ortholog, dominant wildtype and mutant versions of the gene can be over expressed ectopically. Use of cell type specific promoters facilitates localization of transgenic proteins. Recessive PARK genes without a *C. elegans* ortholog are not possible to study.

One of the most useful transgenic models utilizes the overexpression of α-synuclein (*PARK1*), both wildtype and mutant forms in *C. elegans* dopaminergic neurons [14-15]. In several studies, dopaminergic neuron degeneration as well as alterations on lifespan and movement has been shown. Another useful model overexpresses α-synuclein in body wall muscles [16-17]. While these are not neurons, the muscle cells are large and lend themselves for rapid and automated measurement of fluorescence desired in genetic and drug screens.

Another transgenic model is transgenic overexpression of human mutant LRRK2 (*PARK8*) using a pan neuronal promoter [12]. These animals showed increased loss of GFP fluorescence in dopaminergic neurons as well as dopamine levels. A specific mutant (G2019S) was also shown to be more vulnerable to rotenone induced neurodegeneration. Finally, knockdown of *lrk-1*, the *C. elegans* ortholog of human LRRK2, showed reduced survival compared to wild type controls when treated with the mitochondria stressor rotenone (25 μM). In addition to *lrk-1*, some mutant *C. elegans* lines are available for study: *pdr-1* (*PARK2*), *pink-1* (*PARK6*), *djr-1.1* (*PARK7*) (reviewed in [1]).

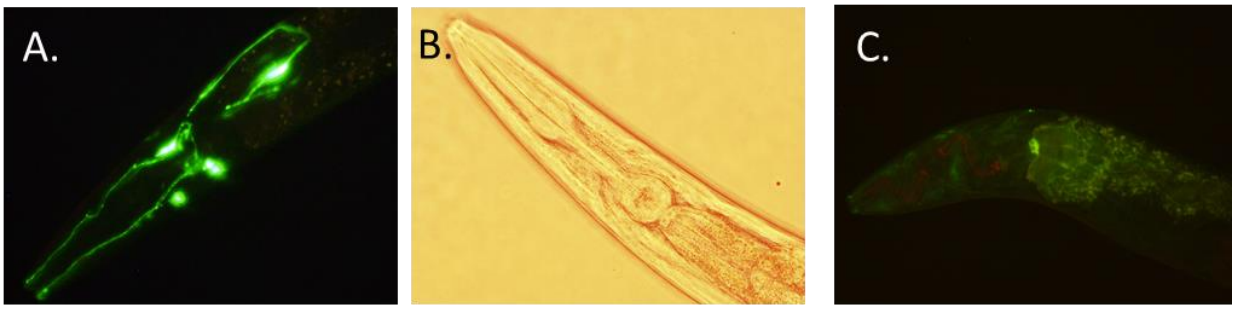

Figure 1. Microscopy images of *C. elegans* head region. A. Transgenic animal expressing GFP in dopaminergic neurons and associated processes [*dat-1p*::GFP]. B. Light micrograph of pharynx showing mouth, anterior bulb, isthmus, terminal bulb and connection to intestine. C. Transgenic animal expressing GFP in excretory pore and excretory duct cells [*hmp-1p*::*hmp-1*::GFP + *dlg-1p*::dlg-1::dsRed + pRF4(*rol*-6(su1006))].

ADMET in *C. elegans*

Absorption

C. elegans has principally 2 routes of absorption: orally via feeding and dermally through the cuticle. The worm takes food through a multi-part structure termed the pharynx which consists of: a buccal cavity, procorpus, metacorpus with an anterior bulb, istmus, terminal bulb, and valve to separate the pharynx from intestine [18]. Food in the pharynx is crushed in the terminal bulb and passed to the intestine where nutrients are absorbed. The muscle cells of the pharynx contract ~200 times per minute allowing for a large amount of food relative to its body size to be passed through per unit time. Within the intestine, proteases, peptidases, and lipases digest material passed from the pharynx [19]. The worm intestine is long, comprises about one-third of the worm mass and extends throughout the body terminating at the anal cavity. Nutrients and substances are absorbed from the intestine to the pseudocoelomic space. Similar to higher organisms, the worm intestine has microvilli structures which maximize surface area for absorption and extends into the intestinal lumen.

In contrast to the intestine, which is organized to rapidly assimilate small molecules and peptides, the cuticle is a barrier designed to keep chemicals out. The *C. elegans* cuticle is an extracellular matrix that acts like skin and covers essentially the entire *C. elegans* body as an exoskeleton. The cuticle is composed of proteins typically found in skin such as cross linked collagens [20], but also has insoluble proteins termed cuticlins. While flexible to allow for worm movement, it also acts to protect the animal from both mechanical and chemical insults. The cuticle itself is covered by an epicuticle consisting of glycoproteins and lipids. Molting during each of the 4 larvae stages of the *C. elegans* life cycle permits the cuticle to be exchanged during each stage.

A less well known and minor route of absorption is via sensory cilia in the *C. elegans* head. These are specialized structures of neurons which send process from the cell body to interact with the environment. Small molecules can pass from these ciliated endings toward neuronal cell bodies as evidenced by experiments using fluorescence dyes.

Distribution

Once nutrients are absorbed by the intestine they enter the pseudocoelom of the worm. This area, also known as the body cavity, is bounded by the basal lamina of hypodermal cells and the walls of the intestine and gonads [21]. Epidermal cells of the intestine and gonads form belt junctions which prevent contents once in the space from passing back out between the pseudocoelom and these structures. The

pseudocoelom is under hydrostatic pressure. Graphically, one can think of the pseudocoelom as the area between tubes in a body structure of a "tube within a tube". There is no circulatory system in *C. elegans* and it is presumed that contents in the space are moved during physical motion of the worm. There is no barrier in the pseudocoelom to neurons located in the nerve ring located between the bulbs of the pharynx or to motor neurons located in the ventral nerve cord. Therefore, once compounds pass into the pseudocoelomic space, exposure to neuronal cells can be immediate. *C. elegans* also has a family of multi-drug resistant proteins to exclude unwanted chemicals from entering cells [22].

Metabolism

Small molecule metabolism occurs through the *C. elegans* Cytochrome P-450 system (CYP). *C. elegans* contains 77 known CYP genes [23]. They are expressed in different structures including cuticle, intestine, and also in neurons. CYP genes show similar characteristics to their orthologs in higher organism: they are inducible by xenobiotics, participate in signaling molecule biosynthesis, and for a vast majority encode a protein for which the endogenous or exogenous substrate is not known [23-24]. The best characterized CYPs reside in the intestine where fat stores are located. The CYP35 family members are expressed in intestine and regulate fat storage and levels of endocannabinoids. CYP29A3 and CYP33E2 are involved in biosynthesis of fatty acid derived signaling molecules for hydroxylation and epoxylation of eicosapentaenoic acid [25]. In addition to CYPs, *C. elegans* has 5 flavin monooxygenases (FMO) [26]. The role of these genes in *C. elegans* physiology are not known, but they appear to be highly inducible (>50-fold) by exposure to methyl mercury [13].

Excretion

Excretion in *C. elegans* occurs through 2 structures. The anus is a specialized structure controlled by a specialized muscle group containing an intestinal muscle, anal sphincter, and anal depressor via a neuromuscular GABAergic junction. When feeding, *C. elegans* undergoes a regular 45 sec defecation cycle. The defecation cycle consists of: an anterior body contraction, a posterior body contraction, opening of an anal sphincter, and expulsion of anal cavity contents [27]. The excretion route is supported by another structure that terminates as an excretory pore located underneath the isthmus in the pharynx. The excretory system is made up of 4 cells: 2 excretory gland cells, an excretory duct cell, and an excretory pore cell [28]. The excretory pore cell is very large and extends 2 parallel canals along the entire length of worm body. Excretion materials are thus collected through the canals for distal sites. Excretion of contents from the excretory pore or anus includes saline fluid for osmoregulation and secondary metabolism contents. Prior to excretion, oxidized small molecules are conjugated to glutathione or glucuronic acid through a family of glutathione S transferases (GST) or glucuronosyltransferases (UGT), respectively. Like their phase I metabolism members, many of these phase II members are also highly inducible under oxidative stress conditions [13,29].

Toxicology

C. elegans is a robust organism from a biochemical perspective and can tolerate very high concentrations of toxic compounds. For example, the LD_{50} at 24h for ethanol is >50 mg/mL and atropine sulphate is >20 mg/mL [30]. Animals also have heavy metal- and multidrug resistant ABC transporters. Very good correlation (r=0.885) in acute toxicity of 21 different compounds has been shown between rat and *C. elegans*, and this correlation value was found to be even better than mouse versus rat [30]. The nematode is however very sensitive to physical environment. *C. elegans* larvae or adult worms live in a narrow temperature range near 20°C. The animals undergo heat shock at 30 °C and cannot be maintained at 4 °C,

however their embryos can be frozen in glycerol solution. Animals also desiccate easily. Under crowding, desiccation, or other extreme physical stress, *C. elegans* enters an alternative life cycle termed dauer. In this state, the animal produces a protective protein coat, moves slowly, and can extend its life span from 3 weeks to 4 months. Several excellent reviews on the topic of dauer stage and life in nature are available [31,32].

Recent insights on PD using *C. elegans*

While there is currently no cure for PD, current human therapy aims at treating symptoms and providing supportive therapy. The first line medications used to improve the main symptoms of PD are levodopa, dopamine agonists, monoamine oxidase-B inhibitors, and catechol-O-methyltransferase inhibitors. Recently, surgical procedures have proven to be enormously successful and include deep brain stimulation. As an experimental model system, novel compounds have been shown to attenuate many of neuropathological hallmarks of PD. Thus the aims of recent pharmacological studies in *C. elegans* have been to prevent or attenuate dopamine neuron cell death. Some studies have also measured effectiveness towards symptoms such as uncoordinated movement and changes in life-span. Below, we describe these studies.

Pharmacological stuedies

Spermidine is a polyamine that is present endogenously and acts in cell proliferation and differentiation presumably via an autophagic pathway. Previous studies have shown that it can extend life span and therefore it was tested as an anti-neurodegenerative compound. Spermidine (5 mM) supplemented in food was shown to attenuate the loss of dopaminergic neurons caused by overexpression of α-synuclein [33]. The authors also showed concurrent increased production of autophagosomes in treated animals suggesting a mechanism for spermidine actions.

Valproic acid, an approved anti-epileptic drug that acts via GABAergic signaling and also has effects on voltage gated sodium channels and T-type calcium channels. It was shown to attenuate dopaminergic neurodegeneration caused by transgenic overexpression of α-synuclein [34]. Valproic acid, dissolved in the agar plates used to culture animals, was effective at both 2 and 3 mM concentrations, but not 1 mM. Using RNAi, the authors could negate the effect of valproic acid with knockdown of the *mek-2* gene suggesting a pharmacological action involving the ERK-MAPK signaling pathway.

Acetylcorynoline is a major alkaloid component of the traditional Chinese medical herb *Corydalis bungeana*. It has indications as an anti-inflammatory agent for upper respiratory tract infections, bronchitis, tonsillitis, and acute nephritis. Acetylcorynoline dissolved in the agar (5mM) was able to decrease aggregation of α-synuclein expressed in muscle cells [35]. At the same concentration, it was also able to protect dopaminergic neurons from degeneration elicited by 6-OHDA. Furthermore, in 6-OHDA treated animals, it restored the basal slowing response on food, a *C. elegans* behavior mediated through dopaminergic neurons.

n-Butylidenephthalide is an organic extraction product of *Angelica sinensis*, and has been shown to have anti-inflammatory properties. With results similar to acetylcorynoline, it was also shown at 2 mM and 5 mM concentrations in agar to reduce α-synuclein aggregation in muscle cells, rescue loss of dopaminergic neurons and reverse loss of basal slowing response in 6-OHDA treated animals [36].

Synthetic medicinal chemistry approaches have also been used with *C. elegans* models providing an assay for novel compounds. 3-Arycoumarin-tetracyclic tacrine hybrids have been developed as potential

anti-PD agents [37]. Many members of this series of compounds are able to significantly decrease α-synuclein aggregation in muscle cells. Molecular modeling of interaction between active compounds and α-synuclein suggests that more potent and selective derivatives might be possible as well as provide insight into the mechanism of anti-aggregation. Xyloketal derivatives have also been developed as potential neuroprotective agents in PD [38]. In *C. elegans* animals with dopaminergic neuron degeneration produced by 1-methyl-4-phenylpyridinium (MPP+), the survival rate could be significantly increased with several derivatives.

Genetic studies

Genetic screens take advantage of the available genetic resources of the *C. elegans* community and the rapid reproductive cycle [39]. These screens aim to identify genes that when expressed enhance or suppress a PD phenotype in a *C. elegans* model. One RNAi screen using α-synuclein-GFP fusion expressed in body wall muscle cells, identified 80 genes which influence α-synuclein inclusion formation in muscle cells during aging [17]. Among the genes identified that suppress inclusion formation was *Sir2.1*. Not all 80 gene products could be druggable, but the RNAi screening approach demonstrates perhaps the large number of possible proteins that affect a biochemical process such as protein aggregation.

In contrast to RNAi screens, forward genetic screens using random mutagenesis identify fewer genetic interactors, but assure that the gene product has been changed on the structural level. The mutagen ethyl methanesulfonate was used to randomly mutagenize the worm genome and identified tetraspanin (TSP-17) as a protector of dopaminergic neuron degeneration elicited by 6-OHDA [40]. Tetraspanin was shown to inhibit the function of the dopamine transporter and this provided insight into dopamine neurotransmission. The non- targeted approach of screens highlights the insight and potential for new target discovery in PD.

A more targeted approach has utilized existing knowledge that oxidative stress and its associated pathways play a key role in neurodegenerative processes. Under oxidative conditions, the thioredoxin and glutaredoxin systems act to reduce disulfide bonds. These systems contain multiple proteins in both humans and *C. elegans*. Mutants that lack thioredoxin reductase 1 (*trxr-1)* were shown to be more vulnerable to 6-OHDA mediated dopaminergic cell death [41]. Similarly, loss of glutaredoxin (*grx-1*) gene exacerbated dopaminergic neurodegeneration in a human LRRK2 mutant *C. elegans* model [42]. This effect was also seen in α-synuclein and tyrosine hydroxylase overexpressing animals. Another perspective has been taken where oxidative stress via exposure to hydrogen peroxide (1mM) for 30 minutes has been shown to induce the transcription factor *hlh-13*, the *C. elegans* ortholog of p48 [43]. *Hlh-13* mutant animals were more vulnerable to dopaminergic cell loss 7 days after hydrogen peroxide treatment suggesting a protective role for this transcription factor.

Finally, RNAi knockdown of *C. elegans* phosphotidylserine decarboxylase, an enzyme used in the synthesis of phosphatidylethnolamine, accelerates degeneration of α-synuclein over expressing dopaminergic neurons [44]. This effect can be rescued by ethanolamine which can be converted to phosphatidylethanolamine. These results highlight the importance of mitochondrial membrane integrity in PD but also may suggest ways to alleviate any deficiencies.

Future perspectives

It is now becoming clear that multiple model organism systems will be needed to tackle the challenging problems in understanding and ultimately treating PD. Although at least 20 PARK genes have been identified [1], their expression or mutation in *C. elegans* has provided insight into disease etiology only with the assistance of and often in conjunction with other systems such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*. Several examples we highlight in this review follow this pattern. Moreover, validation of *C. elegans* findings in human cell culture is proving to be more necessary, not only because PD is a human disease, but also because the genetic environment of a human cell more closely mimics that of the transgene.

At the same time, while PD is an age-related neurodegenerative disease, it shares many of the same pathological features of Alzheimer's and Huntington's diseases. Essentially, one principle feature of all these diseases is the presence of toxic protein aggregates. Therefore, more attention has been paid to the ability of neuronal cells to maintain structural integrity of proteins in terms of folding, as well as aggregation and elimination. This field has been termed proteostasis and findings in this area will likely shed light across many age related disorders [45].

While genetic manipulation of *C. elegans* has made it a powerful model to study, chemical manipulation to mimic environmental risk factors in neurodegenerative disorders have now advanced to the forefront. In addition to heavy metals and pesticides reviewed earlier, other toxic compounds humans are likely to encounter during their lifetime have been administered to *C. elegans* with the aim to access any damage to neuronal systems. These include nanoparticles, plasticizers, and cyanobacteria toxins [46-48]. Considering the ease at which toxicity studies can be conducted, and the high correlation between acute toxicity for compounds between rat and *C. elegans*, it is somewhat surprising that this list is not currently more extensive.

Finally, the wealth of genomic, transcriptomic, and proteomic data available in public databases will help to better design and validate studies using *C. elegans* in PD [49,50]. A paradigm is emerging in neurodegenerative disease research where human GWAS and transcriptomic data informs on gene candidates that can be investigated in *C. elegans*. Genetic studies in *C. elegans* is then validated or complemented by similar studies in other organisms. Hypothesis generation from these studies to look retrospectively at human disease databases then completes the investigative loop. Ultimately, for a simple model organism, *C. elegans* has in the past and will continue into the future to inform on a human neurodegenerative disease with currently no cure.

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