Caenorhabditis elegans: An Emerging Model System for Pesticide Neurotoxicity

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Caenorhabditis elegans as a Model System

An ideal model organism should generally have three essential characteristics: successful sexual crosses should be easy to perform; a fully sequenced genome should be available; and it should be easy to induce reliable and reproducible DNA mutations [1]. The nematode Caenorhabditis elegans (C. elegans), introduced to the broad scientific community by Brenner in 1974, fulfills each of these three essential guidelines [2]. Although initially used extensively by the developmental biology community, its recent adoption by the biomedical and environmental toxicology scientists has strengthened experimental design power in these fields [3]. Relative to traditional model organisms, e.g. rat, mouse, dog (mammals) and Arabidopsis (plants), the invertebrate species C. elegans has begun to be recognized as invaluable. For example, it requires a relatively small budget project compared to other possible organisms, is quite small and transparent, has a short lifespan and has relatively simple anatomy and physiology [4].

These characteristics provide for straightforward genetic manipulation, growth and observation within a laboratory environment for a wide array of experiments [5]. In particular, the small size of C. elegans allows for easy storage in small, dedicated laboratory areas, its transparency facilitates visualization under a microscope and the short life span enables assessment of multiple generations within days, making genetic studies related to inheritable disorders possible in a relatively short time frame. Furthermore, studies involving multiple generations are aided by the fact that C. elegans also exist as a self-fertilizing hermaphrodite, preventing inbreeding [5]. C. elegans have also become more widely-used in toxicology due to their autonomic and somatic neural and molecular similarities to humans [6,7], allowing for valuable insight related to multiple vertebrate systems. Furthermore, as a fully intact in vivo model organism, scientists can study multiple complex behaviors, such as searching for food and/or mates [8-11]. Interestingly, C. elegans is rarely found in the wild, although does lives in tropical and subtropical areas and is more typically found cultured in laboratories [12]. Some major areas where C. elegans is becoming increasing used include mechanistic toxicology, environmental toxicology and high-throughput screening [3]. A major methodological breakthrough came when it was recognized that C. elegans could easily be manipulated through RNA interference, or RNAi [13-15]. This technique involves the introduction of exogenous RNA to complement endogenous RNA, which then “interferes” with the translation of the target gene. The silencing of this expression potentially helps the scientific community better understand the function of the gene’s product [16-18]. Extensive experimentation with C. elegans has produced complete neural and developmental wiring diagrams that have been confirmed by numerous labs [3,19]. Furthermore, these networks are known to be similar in humans, providing additional justification for comparing two widely diverse organisms [5]. On the other hand, the developmental pattern of C. elegans is vastly different from most animals in that they display a “mosaic structure” in which the entire developmental process will not proceed correctly if any cell is removed. This is in contrast to other model organisms, such as the fruit fly, where the removal of a single cell, or even multiple cells, may have no effect on the developmental fate of the organism [5]. C. elegans has been used to further the study of neurodegenerative and transgenic diseases in humans [4], including Huntington’s, Alzheimer’s and Parkinson’s diseases [20-25]. In fact, C. elegans genes related to these three diseases are substantially comparable with human genes [1]. For example, about 40-75% of human genes identified as disease-related are analogous to those found in C. elegans [1,26]. Other similarities exist between C. elegans stem cells and those of “higher” organisms, including humans [27,28]. Thus, comparisons between worms and humans have already provided important insights into possible causes and potential treatments for many diseases. Furthermore, since C. elegans are easy to genetically manipulate, this facilitates research involving the interplay of the genetic and environmental aspects of many neurodegenerative diseases [3]. As such, C. elegans can be easily treated with or introduced to specific chemicals thought to contribute to neurodegeneration through interactions with various genetic mutations [4]. More recently, C. elegans has proven to be a beneficial model organism for studies of major pesticides classes, including organophosphates (OPs) and dithiocarbamates (DTCs). Using C. elegans, scientists can study the various effects these agrochemicals may exert on humans [3,29-31]. For example, in a study involving OPs and C. elegans, alterations in gene and protein expression were examined [32], particularly nervous system-specific endpoints.

The Nervous System of C. elegans

Overview

Starting in the mid 1990s C. elegans became a more widely-used model in the neurotoxicology community [3,29,31,32]. This was partly due to the simplicity of their nervous system, which is a relatively small neuronal network that is highly stereotypical from animal to animal [6]. A total of 302 neurons and 56 glial cells (e.g., CEP sheath glia) make up the hermaphrodite’s nervous system, whereas the males have 381 neurons and 92 glial cells [34,35]. As more in complex model organisms, their neurons are involved in multiple synaptic contacts, including chemical synapses, gap junctions [36] and neuromuscular junctions [6]. About half of the neuronal cell bodies are found in the head, surrounding the dorsal nerve ring, while the remaining soma are found along the ventral cord and in tail ganglia [34]. Furthermore,
Neurotransmitters: Tyramine

Tyramine is only synthesized in a few cells in *C. elegans* and is therefore found in small quantities [69,70]. It is required for the following functions: inhibition of head oscillations in response to being touched on the anterior portion of the body; inhibition of egg laying; and modulation of spontaneous reversals [61]. When a nematode is in starvation circumstances, tyramine is released to inhibit unnecessary functions so the worm can better focus on searching for food [71]. A newly identified tyramine receptor, SER-2, binds the neurotransmitter with high affinity and when mutations are present, behavioral defects are seen [66]. For example, SER-2 mutants fail to inhibit oscillations in response to touch [61]. Exogenous tyramine also blocks pharyngeal pumping, which is stimulated by serotonin, further supporting the idea that tyramine is released in response to food deprivation [72]. Tyramine also inhibits egg laying, but this most likely does not act through SER-2. Rather, it is more likely that tyramine serves as an intermediate for octopamine which inhibits egg laying [61].

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**Figure 1:** Nervous System of *C. elegans*. Summary of functional classification of neurons in *C. elegans* hermaphrodite. Based on [151].
Neurotransmitters: Dopamine

The neurotransmitter dopamine is found in eight neurons in the *C. elegans* hermaphrodite and in fourteen additional neurons located in the tail of the male [61,73]. The role of dopaminergic neurons in *C. elegans* is thought to be predominantly mechanosensory in nature [42]. Supporting evidence indicates a lack in the animal's ability to respond to environmental changes when dopamine is inhibited [74,75]. Dopamine signaling in *C. elegans* also has established roles in regulating locomotion and learning [61]. For example, dopamine signaling facilitates the response of *C. elegans* to environmental changes by modulating locomotion. Well-fed wild-type nematodes slow their movements when they encounter a food source, a process requiring dopamine release [76,77]. Nematodes that have their dopaminergic neurons ablated fail to exhibit this “slowing” process, further supporting the notion that these neurons are mechanosensory and that slowing is likely caused by physical stimuli [78]. Dopamine signaling also leads to more efficient searches for new food sources. After wild-type nematodes exhaust an immediate food supply, they continue to search the nearby area before expanding their exploration [79]. This localized search is referred to as “restricted searching” and is characterized by high-angled turns [61]. In *C. elegans* whose dopaminergic neurons have been ablated or inhibited, there is marked failure to exhibit these high-angled turns when searching for food sources [61]. On the other hand, nematodes exposed to exogenous dopamine exhibit increases in high-angled turns, further supporting a role for dopamine in modulating food-related locomotion [80]. Dopamine also further contributes to *C. elegans* survival by causing a decrease in forward movement, in the presence of food, increasing the likelihood that the animals will stay near the newly discovered food source [78,80]. Learning in *C. elegans* is also modulated by dopamine [81]. The first time wild-type nematodes are exposed to “plate-tapping” they react by backing up; repeated plate-tapping results in decreased movement [82]. Olfactory adaptation, another type of learning, is responsive to dopamine [83]. Over prolonged exposure to an odorant, nematodes show decreases in their responses [84].

Neurotransmitters: Serotonin

Another biogenic amine neurotransmitter in *C. elegans* is serotonin. It is used by the following eight classes of neurons: 20 male-specific neurons (CP 0-9, R1, R3, R9); two pharyngeal neurosecretory motor neurons (NSM), four hermaphrodite specific motor neurons (HSN and VC4-5), two amphid neurons (ADF) and three ring interneurons (RIH and AIM) [61]. Exposure to exogenous serotonin inhibits motor neurons related to locomotion and defecation, while also stimulating egg laying and pharyngeal pumping [85,86]. When food-deprived *C. elegans* move near a food source, their locomotion slows down. This response is dependent on serotonin release [61]. Bas-1 and cat-4 (serotonin biosynthetic enzymes) mutants exhibit defects in this slowing process and, when treated with exogenous serotonin, these defects are reversed. For example, a food-deprived nematode will come to a complete stop upon entering a bacterial lawn versus a well-feed nematode that will slow [61]. Serotonin signaling helps ensure that food-deprived nematodes do not leave a food source, while dopamine signaling encourages nematodes to stay within that source [61,76,80]. Synthesis of serotonin occurs in neurosecretory motor neurons (NSMs) that have sensory endings located in the lumen of the pharynx. It is hypothesized that these might play a role in sensing food. NSMs also have access to neurons outside the pharynx and pseudocelom, which facilitates communication with the rest of the animal [87-89]. Thus, serotonergic neurons are located in areas that allow for very efficient signaling that encourages nematodes to remain in food-rich areas [61].

Neurotransmitters: Γ-aminobutyric acid and glutamate

Γ-aminobutyric acid (GABA) is also an important neurotransmitter in *C. elegans* that functions primarily at neuromuscular junctions [90]. Its main functions are to relax muscles during foraging and locomotion and modulate defecation. Out of the 302 neurons in hermaphrodite *C. elegans*, 26 of them are GABAergic [91]. Interestingly, GABA is both inhibitory and excitatory in these worms [92]. The 19 ventral cord neurons comprise the inhibitory GABA neurons and their job is to inhibit muscle contractions during locomotion [91,93]. The excitatory GABAergic neurons are responsible for modulation defecation [91]. Glutamate is also responsible for both excitatory and inhibitory responses in *C. elegans* [94-96]. The majority of excitatory responses are mediated by ionotropic glutamate receptors (iGluRs) [96,97], which play a role in reversal following nose touch [98,99].

Neurotransmitters: Acetylcholine

The neurotransmitter acetylcholine (ACh) is excitatory in these worms [100,101]. It is found at neuromuscular junctions and causes contractions in the muscle wall [101]. ACh is also a modulatory neurotransmitter that plays a role in locomotion, egg laying, pharyngeal pumping, defecation cycling and male mating [90]. Locomotion is by far the most important behavior controlled by ACh, involving both crawling (surfaces) and swimming (liquid) [102,103].

Summary

Due to the relative simplicity of their nervous system, *C. elegans* provide an *in vivo* model for studying neuronal injury with respect to single neurons [3]. They have been used for studies examining mechanisms associated with metal and pesticide toxicity, as well as general neurodegeneration [1,25,29,104,105]. Since the *C. elegans* nervous system has characteristics similar vertebrates, results from these worms allow for reasonable translation to humans [96], particularly relating to pesticide neurotoxicity.
General Pesticide Neurotoxicity

Organophosphates

Agricultural pesticide poisoning is a major public health problem in the developing world, resulting in the deaths of at least 250,000–370,000 people annually [106]. Although numerous pesticide classes are used throughout the world, some of the more common are organophosphates (OPs) and dithiocarbamates (DTCs). Taken together, OPs and DTCs are the most used pesticides for pest control [107]. OPs, which contain phosphorous derived from phosphoric acid, are generally the most toxic of all pesticides to vertebrates [108] and exposure is a serious global health problem. It is estimated that there are, on average, more than 3 million poisonings reported per year [109]. OPs are absorbed rapidly via all routes (respiratory, gastrointestinal, ocular and dermal) and exposure effects can be immediate and/or long term [109,110]. The most prominent effect of OP exposure is excessive accumulation of ACh in the synaptic cleft resulting from the irreversible inhibition of acetylcholinesterase (AChE), the enzyme responsible for ACh catabolism [107]. The standard treatment for OP poisoning is the administration of atropine, a competitive ACh antagonist that reverses the effects of excess ACh at the synapses, or the use of nucleophilic oxime, pralidoxime or obidoxime, to regenerate AChE [111,112]. Other proposed mechanisms of OP toxicity include production of or increasing oxidative stress [113]. Either increasing reactive oxygen species (ROS) or decreasing antioxidants result in oxidative stress [109,114]. ROS have been implicated in inflammation, aging, mutations, carcinogenesis, degenerative and many other diseases. OPs induce oxidative stress and deplete ATP in vivo, as well as alter antioxidants enzymes such as superoxide dismutase (SOD) [115]. In vitro, markers such as an increased ROS (hydroxyl, superoxide, or lipid peroxide) and reduced glutathione levels have been observed in cultured lymphocyte cells exposed to OPs, further supporting the hypothesis that oxidative stress plays a prominent role in OP mechanisms of action [108].

Carbamate pesticides

DTCs are widely-used pesticides with typically low acute and chronic toxicity in humans [116-119]. Similar to OPs, carbamates are esters of phosphoric acid, phosphotheic acid, or carbamic acid. There is considerable structural diversity, however, in the side chains and it is typically these moieties that determine the toxicokinetics and toxicodynamics of pesticide poisoning [109]. Carbamate poisoning is less severe than that of OP exposure [120-122] even though carbamates may also inhibit AChE [115,123]. The latter, however, cause reversible inhibition [114]. Thus, AChE inhibition by carbamates lasts only minutes or hours, whereas the effects of OPs may last for 3 to 4 months. Thus, acute intoxication by carbamates generally resolves within a few hours, unless their exposure is combined with that of OPs [123]. It is likely that carbamates may also be involved in oxidative stress [124]. For example, the carbamate carbofuran increases oxidative stress in rat brains by inducing lipid peroxidation and diminishing antioxidant defenses [125]. Because AChE inhibition is reversible in carbamate poisoning, therapeutic treatment is less effective than treatment for OP poisoning [123]. Oxime antidote therapy is usually not needed for these patients and atropine treatment is usually adequate [122].

Organophosphate Pesticides in C. elegans

Movement, behavior and LC50s

Although early research with C. elegans and pesticides focused on the actual toxicity of agrochemicals in the soil nematode, more recent work has shifted to include C. elegans as the model organism in toxicity testing. Since then, C. elegans has been valuable in generating data in neurotoxicity studies involving OPs [126,127]. Importantly, studies have shown that the toxicity rank and mechanism of action in C. elegans are comparable to those observed in more traditional mammalian models [128]. For example, Cole et al. [105] found that 15 OPs elicited behavioral EC50 rankings in C. elegans consistent with LD50 rankings observed in rats and mice. In addition, five of six OPs known to exhibit AChE activity in mammals (dichlorvos, fensulfothion, methidathion, methyl parathion, parathion) similarly reduced cholinesterase activity in C. elegans. Interestingly, glyphosate and ethylene dibutyl ether did not exhibit significant AChE activity and these two compounds are not strong cholinesterase inhibitors in mammals. Lethality, movement and AChE activity have all been used as endpoints to examine OP neurotoxicity [128]. Exposure to each of ten specific OPs resulted in significant movement reduction, supplying additional evidence that the mechanism of toxicity (AChE inhibition) in C. elegans is comparable to that in mammals. Furthermore, the LC50s for the ten OPs showed a significant degree of correlation with the previous LD50s for rats [128]. This study also demonstrated that utilizing LC50s and movement as toxic endpoints do not present some of the challenges involved in using AChE activity as an endpoint. Additionally, the former were more consistent than solely relying on measurements of AChE activity [128].

Feeding, growth and Reproduction

While numerous studies have focused on the effects of OPs on C. elegans movement, effects on feeding have also been examined [129,130]. In a study using the OP chlorpyrifos, feeding decreased steadily as the worms were exposed to successively higher concentrations. EC50 values, concentrations necessary to decrease C. elegans feeding by 50%, ranged from 1.0-2.2 μM [129]. Worms exposed to comparable concentrations of chlorpyrifos also showed greater sensitivity to the pesticide when feeding and/or reproduction were used as endpoints [130,131]. Exposure also results in adverse effects on growth in a concentration dependent manner. Even though nematodes exposed to concentrations below 30 μM developed to the adult stage, they exhibited a decrease in body size [132]. Nematodes exposed to concentrations greater than 30 μM did not develop into adults and, at the highest concentration of chlorpyrifos tested (75 μM), growth was arrested at the L2 larval stage [132]. A link may also exist regarding reductions in nematode growth via the disruption of neural development, although exposure levels were not established [104,132]. Another OP, parathion, is capable of significantly reducing reproductive output in C. elegans [131]. In reproduction assays using numerous agrochemicals, including diquat and paraquat, parathion more potently inhibited reproduction [129-131]. Here, the EC50s related to the reproductive output of C. elegans, determined by counting the number of offspring produced by worms exposed to the compounds from the L4 larval stage to adulthood. With EC50s ranging from 1.14 - 2.17 μM, parathion was more toxic than any other compound tested by at least two orders of magnitude [129,131].

Changes in gene and protein expression

C. elegans has been used to examine changes in gene and protein expression induced by these OPs [32,104]. These agrochemicals are known to target genes and their protein products, specifically associated with neuronal and muscle tissue, as well as genes involved in apoptosis. Exposure of worms to fenamiphos and dichlorvos caused detectable changes in expression of 87 of genes. Decreased expression levels were observed for hsp-6 (heat shock 70 protein), map-2 (methionine...
aminopeptidase) and dhp-2 (dihydropyrimidinase), while expression of trx-1 (thioredoxin), gst-15 (glutathione s-transferase) and tmd-2 (trompomodulin) increased [32]. Along with fluctuating levels of gene expression, 34 proteins also exhibited changes in expression [32]. For example, increases in the expression of annexin and ubiquitin c-terminal hydrolase were observed.

OPs are also known to alter the expression of certain genes regulated by the mediator subunit MDT-1 and the GATA transcription factor ELT-2, as well as genes located downstream of daf-16 [104]. Determination of this dysregulation has been facilitated by the fact that the C.elegans genome has been sequenced. Loss of function of these transcription factors can lead to abnormal induction of genes related to generalized toxic stress responses, innate immunity, detoxication and response to ingested material [104]. OP exposure can also lead to apoptotic events in both mammals and C. elegans [133-135] and numerous genes and proteins involved in apoptosis have been shown to have altered expression levels. For instance, an anti-apoptotic gene found in humans (map-2) was down-regulated and the abundance of a protein involved in the engulfment stage of apoptosis (NEX-1) increased [32]. Mutation in deg-3, which codes for a nicotinic AChR, leads to neurodegeneration similar to that observed in C. elegans after OP exposure. In human astrocytes, expression levels of numerous genes are dysregulated [136,137] and these results have been duplicated in C. elegans [32,104]. Finally, medium and high-throughput screenings utilizing the complex objective parametric analyzer and sorter (COPAS) flow sorting system have been used to assess the toxicity of OPs on C. elegans feeding, growth, reproduction and locomotion [130,131]. While this high-throughput technique is not unique to work with OPs, it further demonstrates the ability of using in vitro sorting techniques with a whole model organism to obtain vast quantities of data in relatively short periods of time.

**Carbamate Pesticides in C. elegans**

**Movement and reproduction**

C. elegans has also been used to examine the order and mechanism of toxicity of carbamates. In a study by Melstrom and Williams [138], toxicity rankings determined from movement-concentration curves displayed a high degree of correlation to oral acute LD₅₀ rankings in rats and mice. Similar to observations in mammalian models, carbamate exposure also resulted in AChE inhibition, as indicated by a reduction in movement. For example, studies involving the carbamate insecticide aldicarb produced results consistent with compounds exhibiting AChE inhibition [139]. Aldicarb, an AChE inhibitor, considerably reduced tail thrashing and overall movement in the nematode. Additionally, reproduction was negatively affected, as worms treated with aldicarb displayed a decrease in brood size compared to control [139].

**Oxidative stress and reactive oxygen species**

The herbicide paraquat and the pesticide rotenone have been studied in C. elegans [3]. Paraquat, which is known to cause oxidative stress [140] in vertebrates through the production of ROS, also does so in C. elegans. Furthermore, mutant C. elegans strains lacking superoxide dismutase (SOD) enzymes SOD-1 and SOD-2 showed increased vulnerability to paraquat toxicity. In contrast, those containing greater levels of SOD, as well as increased expression of the α-class glutathione transferase (gst-1) showed decreased susceptibility to the toxic effects of paraquat [3], implying a role of ROS or oxidative stress. The role of rotenone as a contributing factor to the etiology of Parkinson’s disease has also been studied in C. elegans. Rotenone disrupts NADH-dehydrogenase in mitochondrial complex I. The role of complex I in preventing rotenone toxicity has been demonstrated in C. elegans, as strains with mutations in mitochondrial complex I showed increased vulnerability to the toxicity of rotenone [3]. Worms containing modified genes associated with Parkinson’s disease were also more susceptible to oxidative damage due to rotenone exposure [141,142]. This illustrates the importance of these genes in the prevention of rotenone toxicity and oxidative stress in dopaminergic neurons.

**Manganese-containing dithiocarbamate fungicides**

Manganese-containing dithiocarbamate fungicides, such as maneb and mancozeb, have been researched heavily due to their neurotoxicity [143-145]. Manganese exposure by itself has been shown to result in a variety of detrimental effects in C. elegans [146-148]. One study demonstrates that even short exposures (30 min) to manganese result in production of ROS, as evidenced by a drastic increase in glutathione levels [143]. In this study, worms exposed to 50 mM of MnCl₂ experienced a two-fold increase in the amount of ROS production. Additionally, acute Mn exposure can lead to mitochondrial inhibition by disrupting mitochondrial membrane potential (∆Ψm) and oxygen consumption. Oxidative stress also resulted in the neurodegeneration of dopaminergic (DAergic) neurons, indicated by the fact that treated worms showed a significant decrease in DA concentration compared to untreated worms [143]. Similar neurodegeneration has been shown following exposure to mancozeb [144,145].

C. elegans studies have also confirmed that manganese exposure may have an additive effect on DAergic neurodegeneration in neurons containing α-synuclein protein aggregates. Various labs have concluded that the C. elegans SMF-1 transporter, an analogue of the divalent metal transporter (DMT-1) found in humans, is important in the degeneration of these neurons [146,147,149,150]. Results in C. elegans indicate that this transporter plays a significant role in neurotoxicity by transporting manganese into the cell where it can induce numerous intracellular changes.

**Discussion and Conclusions**

C. elegans has continued to gain in popularity in toxicological research as a model organism over the last three decades. This is partially because these worms are only a millimeter in length and are relatively inexpensive to maintain. Furthermore, data generated in C. elegans has successfully complemented and supplemented data obtained from traditional mammalian model organisms. The ability to easily manipulate its genome via interfering RNA (irNA), allows for exploration of both gene and protein function without the difficulties associated with more traditional knock-out and knock-down techniques. Other techniques previously available for in vitro models, i.e. flow sorting, green-fluorescent tags, histochemical analysis, can now be used with ease in the transparent C. elegans. In this review, we examined how this nematode has been used to examine the toxic effects of organophosphate and dithiocarbamate pesticides. End points include gene expression, LC₅₀s, various EC₅₀s, movement, feeding and/or brood size have been studied, emphasizing the variety of rich data that can be obtained using this simple organism. While more labs are beginning to incorporate C. elegans into their research, it is likely that the toxicology community is only just beginning to utilize the power of this important organism.

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