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Dopaminergic neurotoxicity of *S*-ethyl *N,N*-dipropylthiocarbamate (EPTC), molinate, and *S*-methyl-*N,N*-diethylthiocarbamate (MeDETC) in *Caenorhabditis elegans*

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Abstract

Epidemiological studies corroborate a correlation between pesticide use and Parkinson's disease (PD). Thiocarbamate and dithiocarbamate pesticides are widely used and produce neurotoxicity in the peripheral nervous system. Recent evidence from rodent studies suggests that these compounds also cause dopaminergic (DAergic) dysfunction and altered protein processing, two hallmarks of PD. However, DAergic neurotoxicity has yet to be documented. We assessed DAergic dysfunction in *Caenorhabditis elegans* (*C. elegans*) to investigate the ability of thiocarbamate pesticides to induce DAergic neurodegeneration. Acute treatment with either *S*-ethyl *N,N*-dipropylthiocarbamate (EPTC), molinate, or a common reactive intermediate of dithiocarbamate and thiocarbamate metabolism, *S*-methyl-*N,N*-diethylthiocarbamate (MeDETC), to gradual loss of DAergic cell morphology and structure over the course of 6 days in worms expressing green fluorescent protein (GFP) under a DAergic cell specific promoter. HPLC analysis revealed decreased DA content in the worms immediately following exposure to MeDETC, EPTC, and molinate. Additionally, worms treated with the three test compounds showed a drastic loss of DAergic-dependent behavior over a time course similar to changes in DAergic cell morphology. Alterations in the DAergic system were specific, as loss of cell structure and neurotransmitter content was not observed in cholinergic, glutamatergic, or GABAergic systems. Overall, our data suggest that thiocarbamate pesticides promote neurodegeneration and DAergic cell dysfunction in *C. elegans*, and may be an environmental risk factor for PD.

Keywords

C. elegans; molinate; EPTC; pesticide; dopamine; neurodegeneration

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease in the United States, affecting 1% of the population over the age of 55 (Lees et al., 2009). PD is characterized by the loss of selected catecholamenergic neurons in the central and peripheral nervous systems including the dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc). Loss of DAergic neurons in the SNpC gives rise to the motor symptoms observed in PD, such as bradykinesia, rigidity, resting tremors, and postural instability, with onset of symptoms occurring when ≥70% of these DAergic neurons are lost (Lees et al., 2009). Additionally, alterations in neurotransmitter systems other than dopamine (DA), such as glutamate, serotonin and acetylcholine, have been implicated in non-motor clinical symptoms in PD, including behavioral and cognitive decline (Caudle and Zhang, 2009; Politis and Loane, 2011; Yarnall et al., 2011). Mechanisms underlying the selective degeneration of the DAergic neurons leading to progressive motor disease are poorly understood, but complex interactions between an individual's age, genetics and environment are thought to contribute to the etiology of PD. Because familial forms of PD account for only 10% of PD cases (Lees et al., 2009), environmental agents have gained attention as contributing factors in the etiology of PD. Among the environmental agents examined thus far, epidemiological studies have identified a strong correlation between development of PD and the use of pesticides (Ascherio et al., 2006; Hancock et al., 2008; Li et al., 2005; Tanner et al., 1999; van der Mark et al., 2012; Wang et al., 2011a).

Pesticides from a broad range of chemical classes have been implicated in the development of PD, including paraquat (a bipyridyl derivative herbicide), dieldrin (an organochloride insecticide), and rotenone (a rotenoid ester insecticide). The extent to which these compounds model human PD in rodents is variable. Both rotenone and paraquat exposure lead to DAergic neurodegeneration, increased oxidative stress, aggregation of α-synuclein, and motor deficits in rodents, while dieldrin and cyclodiene pesticides induce oxidative stress and promote aggregation of α-synuclein but do not cause motor deficits or loss of DAergic neurons (Betarbet et al., 2000; Kanthasamy et al., 2005; Karen et al., 2001; McCormack et al., 2005; Prasad et al., 2007; Richardson et al., 2006). These differences highlight some of the difficulties inherent when trying to select endpoints for testing environmental agents in animal models of neurodegenerative diseases, in that multiple biological processes can be targeted through divergent chemical properties of test agents that cannot be predicted *a priori*. This is important to consider, as there are a large number of chemical compounds applied as pesticides and numerous mixture combinations that can arise in fields that require evaluation. While much can be gleaned from *in vivo* rodent studies, the complexity of the mammalian brain and the time required for testing compounds and mixtures for DAergic neurodegeneration limit the utility of these studies for initial evaluations. For this reason model organisms such as *Drosophila melanogastor* and *Ceanorhabditis elegans* (*C. elegans*) are gaining wider attention for their simplified nervous systems and screening potential.

The nematode *C. elegans* is a powerful genetic model organism useful for exploring DAergic vulnerability to environmental contaminants and molecular mechanisms of PD. These worms possess all of the genes required for DA neurotransmission and contain homologues of most PD-associated genes. Visualization of DAergic neurons in strains expressing green fluorescent protein (GFP) under the dopamine transporter-1 (*dat-1*) promoter is possible due to the transparent bodies of these worms. DAergic neurons in *C. elegans* are sensitive to toxicants used to model PD in rodents and cell culture, such as 1-methyl-4-phenyl-pyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA) (Braungart et al., 2004; Nass et al., 2002). In the present study we used *C. elegans* to investigate the potential of thiocarbamate and dithiocarbamate pesticides to induce DAergic neurotoxicity.

Thiocarbamate and dithiocarbamate pesticides are widely used in the United States; these compounds repeatedly appear on the U.S. Environmental Protection Agency's (US EPA) most commonly used pesticide list (Grube et al., 2011). Thiocarbamate and dithiocarbamate pesticides share many characteristics, including chemical and microbial degradation pathways, and metabolism in mammals (Mulkey, 2001; Szolar, 2007). Thiocarbamate pesticides are typically volatile and have short half-lives, reducing the chance of bioaccumulation in the food chain, while dithiocarbamate pesticides are more stable and present greater risk (Mulkey, 2001; Szolar, 2007). The major source of human exposure is usually through dermal contact, inhalation during application, and residues on food crops. Metabolism of thiocarbamates and dithiocarbamates produces electrophilic metabolites, that can covalently modify proteins and inhibit enzymes (Pentyala and Chetty, 1993; Savolainen and Hervonen, 1985; Staub et al., 1995), and has been associated with neurotoxicity in both rodents and humans (Bergouignan et al., 1988; Tonkin et al., 2004; Viquez et al., 2008). More specifically, previous studies have supported the ability of *N,N*-dialkyl dithiocarbamates to produce DAergic toxicity *in vitro* and degenerative change in the nigrostriatal pathway *in vivo* (Chou et al., 2008; Viquez et al., 2012). Covalent modification and inhibition of E1 ubiquitin activating enzyme was proposed to be a contributing mechanisms for this toxicity. Interestingly, the bioactivation pathway leading to the protein alkylating species derived from dithiocarbamates proceeds through an *S*-methyl thiocarbamate intermediate possessing the characteristic structure of thiocarbamate herbicides. This suggests that thiocarbamate herbicides may also be DAergic toxicants that are potentially more potent than the dithiocarbamates as a result of being located several steps down the activation pathway relative to dithiocarbamates.

Herein, we determined the potential of a cyclic and archetypical *N,N*-dialkyl thiocarbamate herbicide, molinate and EPTC, respectively, to produce DAergic neurodegeneration in *C. elegans*. Molinate is a thiocarbamate herbicide commonly used worldwide in rice patty fields that has been banned by the US EPA due to its ability to cause peripheral neuropathies and reproductive toxicity (Cochran et al., 1997; Wickramaratne et al., 1998). EPTC was the 19th most commonly used pesticide in the United States in 1999, but its use is declining (Donaldson et al., 2002; Grube et al., 2011). An association between EPTC pesticide application and colon cancer and leukemia has been reported in male pesticide applicators in North Carolina and Iowa followed over 7–11 years (van Bemmel et al., 2008), suggesting the potential of EPTC to cause cumulative toxicities from chronic exposures. Additionally, selected metabolites of the *N,N*-dialkyldithiocarbamate DEDC were examined to evaluate the relevance of the bioactivation pathway of DEDC previously proposed to be responsible for nigrostriatal injury *in vivo*. General toxicity was assessed in the worms through determination of LD_{50} values and DAergic toxicity was determined through imaging of DAergic neurons, quantification of DA levels and measuring DA-dependent behavioral endpoints. To evaluate the selectivity of the test compounds for DAergic toxicity, morphological assessments of glutaminergic and cholinergic neurons were performed and the level of γ-aminobutyric acid (GABA) and glutamate were quantified. The data demonstrate that the *S*-alkyl thiocarbamate class of compounds exhibit substantial and selective DAergic tocxicity in this model. This finding supports further investigation into this class of compounds as a potential contributing risk factor for PD and suggests that metabolism of *N,N*-diaklydithiocarbamates to *S*-methyl thiocarbamate metabolites may contribute to the nigrostriatal injury *in vivo* and DAergic toxicity reported for these compounds *in vitro*.

Materials and Methods

Reagents

Unless otherwise stated all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Sodium *N*,*N*-diethyldithiocarbamate (DEDC) was obtained from Alfa Aesar (Ward Hill, MA), while MeDETC, EPTC, molinate, and DETC-sulfoxide were synthesized as previously described (Zimmerman et al., 2004).

C. elegans strains and handling of the worms

C. elegans strains were handled and maintained at 20°C on Nematode Growth Medium (NGM) plates seeded with OP-50 strain of *Escherichia coli*, as previously described (Brenner, 1974). The following strains were used in this study: N2, BY200 (P*dat-1*::GFP(*vtIs1*)), NC1307 (P*glr-1*::GFP(rhIs2)), LX929 (P*unc-17*::GFP(*vsIs48*)), VP596 (dvls19[pAF15(gst-4::GFP::NLS)];vsls33[dop-3::RFP]) and CB1112 (*cat-2*(*e1112*)). All strains were provided by the Caenorhabditis Genetic Center (CGC; University of Minnesota), except for the NC1307 strain, which was provided by Dr. David Miller (Vanderbilt University), and the VP596 strain, provided by Dr. Keith P. Choe (University of Florida). Synchronous L1 populations were obtained by isolating embryos from gravid worms using a bleaching solution (1% NaOCl and 0.25 M NaOH), and segregating eggs from worm and bacterial debris by flotation on a sucrose gradient, as previously described (Stiernagle, 1999).

Dose-response curves and acute pesticide treatment

The lethal dose 50% (LD_{50}) of EPTC, molinate, MeDETC, DEDC, DETC-sulfoxide, and ethyl isocyanate in *C. elegans* was determined by treating 5,000 synchronized L1 BY200 worms with doses ranging from 0.001 to 10 mM for 1 hour (h) in M9 liquid buffer at 25°C on a eppendorf tube rotator. Worms were also treated with 1% DMSO as a vehicle control. All exposures were carried out in triplicate and repeated 4 times. After treatment worms were washed 3 times with M9 buffer, transferred to OP-50-seeded NGM plates, and manually counted for lethality 24 h post treatment.

Lifespan Analysis

Aged L4 and healthy-looking worms previously treated with LD_{50} concentrations of MeDETC, EPTC, or molinate (40 per treatment, in duplicate) were transferred to new OP-50-seeded NGM plates. Worms were transferred to new plates every 2 days for feeding, and survival was assessed daily until all the worms died. Plotted curves represent averages of triplicate independent experiments.

Confocal microscopy and fluorescence quantification

To examine morphological changes in neurons as a result of test agent exposure, worms expressing GFP in DAergic, glutaminergic, or cholinergic neurons were visualized by confocal microscopy (BY200, NC1307, and LX929 strains, respectively). GFP-expressing worms were treated with LD_{50} concentrations of MeDETC, EPTC, or molinate and visualized 2, 4, and 6 days following exposure. 20–30 worms were mounted on 4% agarose pads in M9 and anaesthetized with 0.2% tricane and 0.02% tetramisole. Images were captured through Plan-Apochromat 20x objective on a LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc) scanning every 200 nm for XZ sections. Images were processed with the Zeiss LSM Image Browser. Quantification of GFP fluorescence was performed using ImageJ 1.36 software as previously described (Gavet and Pines, 2010).

Activation of SKN-1, the worm homologue of nuclear factor (erythroid-derived-2)-like 2 (Nrf2), was measured using VP596 strain, which expresses GFP under the control of the promoter for the SKN-1 target glutathione S transferase 4 (*gst-4)*. L1 VP596 worms were treated with LD_{50} concentrations of MeDETC, molinate, or EPTC for 1 h and visualized immediately, 1, 2, and 3 days following exposure. Confocal microscopy and GFP fluorescence quantification was performed as described above.

Neurotransmitter content measurement

Following a 1 h treatment with LD_{50} concentrations of molinate, EPTC, or MeDETC, 200,000 L1 worms were pelleted, the supernatant was removed, and the pellet was frozen in liquid nitrogen. The frozen pellet was re-suspended in lysis buffer and sonicated to disrupt cell membranes. DA, γ-aminobutyric acid (GABA), and glutamate levels were quantified by HPLC through services provided by the Center for Molecular Neuroscience Neurochemistry Core Laboratory at Vanderbilt University. Neurotransmitter levels were determined by a specific HPLC assays utilizing an Antec Decade II electrochemical detector (DA) or 474 scanning detector (GABA, glutamate). HPLC control and data acquisition were managed by Millennium 32 software. DA, GABA, and glutamate were quantified using internal standards after separation by HPLC and were reported as ng/mg protein. Protein levels were quantified using the BCA assay (Thermo Scientific, Sunnyvale, CA), following manufacturer's instructions.

Basal slowing response

Assessment of DA-dependent behavior was performed using the basal slowing response assay, as previously described (Sawin et al., 2000). Briefly, following exposure to LD_{50} concentrations of MeDETC, EPTC, or molinate for 1 h, N2 L1 worms were seeded on OP-50-spread plates. BY200 worms could not be used for this assay as they contain the *rol-6* gene as a balancer to the P*dat-1*::GFP, causing a rolling phenotype. N2 worms were also pre-treated with either a nonlethal dose of dopamine chloride (10 mM) for 10 min, as previously described (Benedetto et al., 2010), or 2 mM N-acetyl-L-cysteine (NAC) for 1 h, followed by 5 washes in M9 and treatment with DMSO, or LD50 concentrations of MeDETC, molinate, or EPTC for 1 h. Three and 6 days post exposure worms were washed off the plates with S basal buffer and ~10 worms were transferred to either unseeded or OP-50-seeded NGM plates. Locomotor activity was assessed as the number of body-bends per 20 seconds (s). Worms deficient in *cat-2* (homolog of mammalian tyrosine hydroxylase (TH)) were used as a positive control. Data is presented as change in body bends, which is calculated by subtracting the number of body bends of worms plated on OP-50-seeded plates from the number of body bends of worms plated on unseeded plates.

Glutathione quantification

Total intracellular glutathione (GSH) levels, i.e. reduced and oxidized GSH, were measured using the 5,5′-dithiobis-2-nitrobenzoic acid-glutathione disulfide reductase recycling method, as previously described (Rahman et al., 2006) in whole worm extracts from 50,000 BY200 worms. Worms were treated with either DMSO or LD_{50} concentrations of MeDETC, molinate, or EPTC for 1 h in the presence or absence of a 1 h pre-treatment with NAC (2 mM).

Statistics

All statistical analyses were performed using Prism 5 (Graphpad software). Dose-response lethality curves and LD_{50} determination were generated using a sigmoidal dose-response model with a top constraint at 100%. Statistical analysis of significance was carried out by one-way ANOVA followed by Dunnett's post-hoc test for the neurotransmitter

quantifications, basal slowing response, and GSH quantification. One-way ANOVA followed by Tukey's post-hoc test was used for GFP fluorescence quantification. A two-tailed t-test was used to assess the statistical significance of the change in the basal slowing response between day 3 and 6. Gehan-Breslow-Wilcoxon Test was used to assess the statistical significance of the lifespan experiments. Values of $P < 0.05$ were considered statistically significant.

Results

Acute thiocarbamate toxicity to *C. elegans*

To determine whether *C. elegans* was a suitable *in vivo* model organism for investigating thiocarbamate neurotoxicity, we exposed the BY200 strain to increasing concentrations of the compounds for 1 h and generated dose-response survival curves (Figure 1). We treated worms with the dithiocarbamate DEDC, which has been implicated in DAergic dysfunction (Viquez et al., 2012), and the thiocarbamate herbicides EPTC and molinate (Figure 1A, Table 1). To evaluate the relevance of the previously proposed bioactivation pathways for *N,N*-dialkyl dithiocarbamates, we also treated worms with MeDETC, DETC-sulfoxide, and ethyl isocyanate (Figure 1B). MeDETC (LD_{50} = 0.114 mM) was found to be the most potent, followed by EPTC (LD_{50} = 0.209 mM), molinate (LD_{50} = 0.442 mM), DETC-sulfoxide (LD_{50} = 1.232 mM), DEDC (LD_{50} = 1.679 mM), and ethyl isocyanate (LD_{50} = 2.867 mM) (Table 1). The compounds that were the most toxic to *C. elegans* were the *S*-alkyl thiocarbamates. Based upon these data we used the two thiocarbamate herbicides, EPTC and molinate, and MeDETC, the most potent DEDC metabolite.

Lifespan analysis was performed to further characterize toxicity to EPTC, molinate and MeDETC. Worms treated with LD_{50} concentrations of EPTC, molinate and MeDETC had significantly decreased lifespans as compared to DMSO control treated worms (Figure 2). From day 1 to around day 6 post exposure there was little difference in treated vs. untreated worms' lifespan (Figure 2); however there was increased lethality following this lag time. Therefore subsequent neurotoxicity studies were conducted up to 6 days post exposure.

Selective DAergic neurodegeneration following pesticide exposure

To investigate the propensity of EPTC, molinate and MeDETC to cause DAergic neurodegeneration, the BY200 strain, which expresses GFP under the control of the DAergic neuron specific DAT1 promoter, *dat-1::GFP(vtIs1)*, was used to visualize DAergic neuron morphology. *C. elegans* hermaphrodites have a total of 8 DAergic neurons; 4 cephalic deirids (CEP) and 2 anterior deirids (ADE) in the head and 2 posterior deirids in the tail, all of which express *dat-1*. Worms were treated with LD_{50} concentrations of MeDETC, EPTC, molinate or DMSO vehicle control, and were visualized 2, 4, and 6 days after exposure. There were no significant changes in DA cell morphology or GFP expression prior to day 4 (Figure 3A and B). On the fourth and sixth days after exposure there were increased markers of neurodegeneration in the 4 CEP and 2 ADE head DAergic neurons (Figure 3A and B). Defects observed were primarily in neuron processes, resulting in discontinuous and punctuated GFP signals, as well as decreased size of neuronal bodies, and an overall loss of GFP intensity in worms treated with MeDETC, EPTC, or molinate as compared to DMSO control worms. Intensity of the GFP signal was quantified from the photomicrographs, revealing significant loss of GFP signals for worms treated with molinate, EPTC, and MeDETC on days 4 and 6 post treatment (Figure 3B).

The DAergic neurodegeneration following MeDETC, EPTC, and molinate was not observed in other neuronal cell types. Worms expressing GFP in cholinergic neurons (*unc-17*::GFP) or in glutaminergic neurons (*glr-1::GFP*) showed no changes in cell morphology in

response to pesticide treatment as compared to DMSO treated worms (Figure 4A and 5A). This suggests that neurodegeneration in response to MeDETC, molinate, and EPTC is DAergic neuron selective. While *unc-17* driven GFP florescence significantly increased over time, there was no difference between DMSO and pesticide treated worms (Figure 4B). There was no significant changes in GFP intensity in worms expressing *glr-1*::GFP.

Reduction in DA levels and DA-dependent behavior by thiocarbamates

As DAergic neuron morphology was significantly altered by MeDETC, EPTC, and molinate treatments, it was next investigated whether this effect was paralleled by selective loss of DA content in the worms. Levels of DA, GABA, and glutamate were measured in extracts from BY200 worms harvested immediately following treatment with LD_{50} concentrations of MeDETC, EPTC, and molinate. MeDETC, EPTC, and molinate exposure significantly decreased DA levels as compared to DMSO control (Figure 6A). There was no change in GABA or glutamate levels in worms treated with these compounds as compared to control (Figure 6B and C).

To determine whether the loss of DAergic morphology and reduction in DA levels results in a loss of DAergic-dependent function, we next analyzed the effect of MeDETC, EPTC, and molinate treatment on the basal slowing response. It has been previously shown that N2 worms significantly slow their movement on NGM plates containing a bacterial food source as compared to worms placed on unseeded NGM plates (Sawin et al., 2000). Worms deficient in CAT-2, the nematode homologue of TH, the rate-limiting enzyme for DA biosynthesis, do not show this behavior (Sawin et al., 2000). N2 worms treated with LD_{50} concentrations of MeDETC, EPTC, and molinate were assessed for the basal slowing response 3 days and 6 days after exposure. After 3 days of treatment, worms treated with DMSO showed a change in locomotor activity similar to Sawin et al (Figure 7A). Worms containing mutant *cat-2* were used as a positive control, and showed a loss of basal slowing response behavior similar to Sawin et al (Figure 7A). Both EPTC and molinate significantly decreased the basal slowing response 3 and 6 days after exposure as compared to DMSO control (Figure 7A and B). MeDETC only decreased the basal slowing response after 6 days of treatment as compared to DMSO control (Figure 7A and B). Comparing the change in body bends of worms treated with MeDETC, molinate, or EPTC, there was significantly greater deficit in DA-dependent behavior on day 6 than on day 3 (Figure 7C), suggesting that the loss of DAergic behavior was progressive.

As we observed a significant loss of DA content immediately following treatment with MeDETC, EPTC, and molinate, we next determined whether pre-treating worms with DA could restore DA-dependent behavior. N2 worms were pre-treated with 10 mM DA in M9 for 10 min, washed 5 times with M9, and then treated with LD_{50} concentrations of MeDETC, molinate, or EPTC. Basal slowing response was not significantly different between worms treated with MeDETC, molinate, or EPTC with or without the DA pre-treatment at either 3 or 6 days post treatment (Figure 7A and B). This suggests that loss of DA-dependent behavior following exposure may not be dependent on the initial loss of DA, but on protracted neurotoxicity of the thiocarbamates.

Increased oxidative stress following thiocarbamate exposure

Since oxidative stress has been implicated in carbamate pesticide-induced neurotoxicity, and increased oxidative stress and autoxidation of DA are implicated in DAergic neurodegeneration in PD (Chen et al., 2000; Jana et al., 2007; Khan et al., 2012; Valentine et al., 2009; Viquez et al., 2009), we investigated whether there was increased oxidative stress in worms treated with *S*-alkyl thiocarbamates by measuring total GSH levels. Worms treated with LD_{50} concentrations of MeDETC, EPTC, and molinate contained significantly less

total GSH than DMSO treated control worms (Figure 8A). Reduction in total GSH levels could be prevented by pre-treating the worms with 2 mM NAC (Figure 8A). As a decrease in GSH levels following thiocarbamate treatment can lead to alteration of the redox potential of the cell and increased oxidative stress, we then examined whether these compounds activated SKN-1, the worm homologue of Nrf2. Transgenic worms expressing GFP under the *gst-4* promoter were treated with LD_{50} concentrations of MeDETC, molinate, or EPTC, and GFP fluorescence was measured. Since *gst-4* is a gene up-regulated by SKN-1 under conditions of oxidative stress, GFP fluorescence serves as an indicator of oxidative stress and SKN-1 activity (Choe et al., 2009; Link and Johnson, 2002). Worms treated with MeDETC, EPTC, or molinate showed increased GFP fluorescence as compared to DMSO treated worms immediately following exposure and continuing for 2 days (Figure 8B). DMSO treatment mildly increased GFP expression in *C. elegans*, which is not surprising as DMSO has been shown to induce Nrf2 in human umbilical vein endothelial cells (Liang et al., 2011). On the third day following exposure there was no significant difference in GFP fluorescence between DMSO or thiocarbamate treated worms. This suggests that the thiocarbamate-induced oxidative stress initiates an antioxidant response in worms.

To determine whether oxidative stress may play a role in thiocarbamate-induced DAergic dysfunction, the basal slowing response was measured in worms 6 days following pre-treatment with 2 mM NAC and treatment with LD_{50} concentrations of MeDETC, molinate, and EPTC. Measuring the behavior 6 days post exposure was selected as there is the greatest loss of DA-dependent behavior from the thiocarbamates at this time point (Figure 7C). Pre-treatment with NAC significantly prevented the loss of DA-dependent behavior induced by MeDETC, molinate, and EPTC (Figure 8C). Treatment of NAC to worms deficient in CAT-2 did not significantly increase DA-dependent behavior, suggesting that the NAC treatment alone does not affect the basal slowing response.

Discussion

Dithiocarbamate and thiocarbamate pesticides are highly used in the United States; however their effects on the central nervous system (CNS) are not fully characterized. Studies in rats treated with DEDC have shown altered protein processing and inhibition of the ubiquitination cascade, pathways implicated in PD (Viquez et al., 2012). However loss of DAergic neuron function or integrity was not previously reported. Therefore, we chose to address whether dithiocarbamate and thiocarbamate pesticides could cause DAergic neurodegeneration in *C. elegans* taking advantage of their simple nervous system, short lifespan and the ease of examining neuronal cell morphology and integrity *in vivo* with GFP tagged strains. Furthermore, we evaluated the contribution of *S*-alkyl thiocarbamate metabolites to the DAergic toxicity of *N,N*-diakly dithiocarbamates.

The adverse health effects of thiocarbamate and dithiocarbamate pesticides, including enzymatic inhibition, peripheral neuropathies, and reproductive toxicity, are believed to arise from the metabolism of the parental compounds to reactive electrophilic metabolites. Parental dithiocarbamate compounds are metabolized in a series of reactions by methyl transferases and cytochrome P450 enzymes to produce *S*-alkyl thiocarbamate metabolites, such as MeDETC (Scheme 1) (Madan and Faiman, 1995; Pike et al., 2001; Staub et al., 1995). MeDETC can be further metabolized to DETC-sulfoxide and ethyl isocyanate, which can covalently modify proteins through nucleophilic addition by sulfhydryl groups. In assessing general toxicity to *C. elegans*, MeDETC was the most potent compound tested, with an LD_{50} lower than the parental compounds tested, DEDC, molinate, and EPTC. The higher potency of MeDETC in *C. elegans* compared to the parental compounds agrees with previous data showing sulfoxide metabolites of molinate being more potent testicular toxins in rats (Jewell et al., 1998). Interestingly, the other metabolites tested were less toxic than

MeDETC; both DETC-sulfoxide and ethyl isocyanate had LD_{50} concentrations higher than MeDETC (Figue 1 and Table 1). This may be due to the higher reactivity of DETC-sulfoxide and ethyl isocyanate with cysteine residues that could limit distribution to the nervous system. From these data, it can be inferred that other thiocarbamate and dithiocarbamate compounds that share the same metabolic pathway may also elicit similar toxic effects.

The *C. elegans* system has been used in studies examining the neurotoxicity of pesticides, such as rotenone, glyphosphate, mancozeb, and monocrotophos (Ali and Rajini, 2012; Anbalagan et al., 2013; Mocko et al., 2010; Negga et al., 2011; Negga et al., 2012), however, the effects of molinate and EPTC have not been evaluated in this organism. *C. elegans* expressing GFP under the *dat-1* promoter were treated with the three compounds to visualize DAergic neuron morphology. A single exposure at the LD_{50} concentrations of EPTC, molinate, or MeDETC significantly altered DAergic cell morphology in the CEP and ADE head neurons of *C. elegans* over the course of 6 days following exposure (Figure 3A). Morphological changes in DAergic neurons included punctuated processes and shrinkage of cell bodies, which are similar to the neurodegeneration previously observed in worms treated with Mn or 6-OHDA (Benedetto et al., 2010; Nass et al., 2002). EPTC, molinate, and MeDETC were shown to decrease lifespan (Figure 2), however changes in DAergic cell morphology were observed at time points where there was no significant death as compared to control worms. The concentrations of molinate and EPTC used in our studies are higher than some of the reported environmental levels. Molinate levels in rice fields vary from 3.9 μg/l to 1042 μg/l (Castro et al., 2005; Quayle et al., 2006), however concentrations have not been directly measured during peak seasonal applications. It is also important to consider that toxicity to the DAergic system from pesticide exposure can occur over the course of years, giving rise to cumulative exposures higher than environmental levels. It is important to note that in comparison to compounds previously used in *C. elegans* to induce DAergic neurodegeneration, EPTC, molinate, and MeDETC are more potent. DAergic neurotoxicity has been observed with 10–50 mM 6-OHDA, 400 mM glyphosphate, and 8.2 mM mancozeb, while MeDETC produced DAergic neurodegeneration following a 0.114 mM treatment (Nass et al., 2002; Negga et al., 2011). As 6-OHDA and mancozeb are toxic to DAergic neurons in mammals, our data suggest that the thiocarbamate pesticides and metabolites may also be potent in mammalian systems.

Quantification of P*dat-1*::GFP signal has been used as a secondary method to assess neurodegeneration in *C. elegans* (Negga et al., 2011; Negga et al., 2012). DAT is essential for proper DAergic neurotransmission, being responsible for the clearance of DA from synapses. Brain samples from PD patients have revealed that there is from 50–70% loss of the DAT (Seeman and Niznik, 1990). This can be viewed as a protective, compensatory response to the loss of DA. In our study, GFP intensity, which served as a proxy for *dat-1* gene activity, was significantly diminished following treatment with EPTC, molinate, and MeDETC, not only suggesting that there is loss of DAergic morphology, but there may also be a loss of DAergic function (Figure 3B).

DAergic signaling and function was assessed by two parameters, DA content and the basal slowing response, a DA-dependent behavior. DA levels were significantly decreased in worms treated with EPTC, molinate, and MeDETC immediately following exposure, preceding the loss of DAergic cell morphology (Figure 6). Interestingly, rats treated with DEDC showed no change in DA levels, but had decreased TH protein levels in the striatum consistent with injury, possibly through loss of DAergic terminals and an up-regulation of TH activity through phosphorylation consistent with a compensatory response to maintain DA levels (Viquez et al., 2012). Comparisons between the rat and *C. elegans* are difficult, and differences between DA levels in the two animal models may be due to species

differences, exposure protocols, or the rats were at an earlier stage of injury that could still be compensated and eventually would have decreased dopamine levels. Previous studies of Mn-containing dithiocarbamate pesticides in *C. elegans* have shown altered DA-cell morphology (Negga et al., 2011; Negga et al., 2012), however functional consequences have not been reported. Here, we observed that loss of DA-dependent behavior occurred concurrently with increased DA-ergic neurodegeneration. The DAergic neurons in *C. elegans* are mechanosensory neurons responsible for detecting and responding to changes in the environment (Goodman, 2006). The basal slowing response reflects the worm's ability to sense the presence of food and adjust its locomotor activity to allow for consumption (Sawin et al., 2000). Both molinate and EPTC treatments resulted in significant decrease in the basal slowing response 3 days after exposure (Figure 7). Six days following exposure to EPTC, molinate, or MeDETC showed both a significant loss of DAergic cell morphology, GFP signal intensity, and decrease in the basal slowing response. Similar correlations between DA content, behavior, and morphology have been observed in *C. elegans* treated with the organophosphorous insecticide monocrotophos (Ali and Rajini, 2012). Interestingly, treatment of worms with excess DA prior to MeDETC, molinate, or EPTC did not prevent loss of DA-dependent behavior 3 or 6 days after exposure, suggesting that alterations in DAergic cell morphology and behavior may not be entirely due to an initial loss of DA but to time-dependent damage.

While there was overt DAergic dysfunction in *C. elegans*, there was no effect on glutamatergic, cholinergic, or GABAergic neurons. Both glutamate excitotoxicity and cholinergic dysfunction have been implicated in PD. Glutamate is the major fast excitatory neurotransmitter in the CNS, which in high concentrations can lead to an overload of calcium influx leading to necrotic or apoptotic cell death, termed excitotoxicity (Caudle and Zhang, 2009). DAergic neurons may be susceptible to excitotoxicity in PD due to impaired mitochondria and bioenergetics. Rats treated with rotenone, a mitochondrial complex I inhibitor, had a potentiated response to glutamate levels in the substantia nigra, increasing the cell's vulnerability to excitotoxicity (Wu and Johnson, 2009). Therefore we examined whether there was an effect of thiocarbamates on glutaminergic neurons. Morphology of glutaminergic neurons did not change following exposure to MeDETC, EPTC, or molinate, nor were there differences in intracellular glutamate levels following thiocarbamate treatment in *C. elegans* (Figures 5 and 6). Similarly, we did not observe changes in intracellular GABA levels following EPTC, MeDETC, or molinate exposure (Figure 6). Cholinergic degeneration has been observed in the basal forebrain of PD patients and has been observed in vagal motorneurons of rats exposed to 6-OHDA (Zheng et al., 2011; Ziegler et al., 2012). In addition, dithiocarbamates are well characterized inhibitors of acetylcholinesterase (Pentyala and Chetty, 1993; Savolainen and Hervonen, 1985). We therefore investigated whether there was degeneration of cholinergic neurons of *C. elegans*. Exposure to MeDETC, EPTC, and molinate did not alter cholinergic cell morphology (Figure 4). Overall, the effects of MeDETC, molinate and EPTC in *C. elegans* were DA specific, suggesting these compounds may be effective tools in examining mechanisms of DAergic cell death.

PD is a complex disease involving motor and cognitive dysfunction resulting from as diverse mechanisms as DAergic neurodegeneration, mitochondrial dysfunction, oxidative stress, inflammation, and protein aggregation (Ebrahimi-Fakhari et al., 2012; Exner et al., 2012; Sutachan et al., 2012). While characterization of thiocarbamate-induced neurodegeneration was shown to be DA specific, mechanisms that led to selective loss of DAergic neurons in *C. elegans* remain to be fully characterized. DEDC has been shown to increase lipid peroxidation and induce the antioxidant enzymes superoxide dismutase 1 (SOD1), glutathione S-transferase–α (GST-α), and heme oxygenase-1 (HO-1) in sciatic nerves of rats (Valentine et al., 2010). Sodium methyldithiocarbamate has been shown to

deplete intracellular GSH, induce antioxidant enzymes sulfiredoxin, peroxiredoxin, and isocitrate dehydrogenase, and alter the inflammatory cytokine profile of peripheral macrophages in mice (Pruett et al., 2009). Herein, we report that EPTC, molinate, and MeDETC decreased total GSH levels in *C. elegans* (Figure 8A). GSH depletion was fully reversible by pre-treating worms with NAC. These measurements were performed in whole worm extracts, and reflect the level of oxidative stress in every cell of the worm, including the glutaminergic and cholinergic neurons that were unaffected by thiocarbamate pesticide exposure. Important to note, many of the thiocabamate metabolites can react directly or be catalyzed by glutathione transferase to conjugate with GSH. Decreased glutathione levels will alter a cells' thiol status, increasing vulnerability to oxidants. Our studies demonstrate that there is increased activation of SKN-1 following thiocarbamate exposure (Figure 8B), illustrating a protective mechanism by which worms respond to thiocarbamate pesticide-induced oxidative stress. It is well established that DAergic neurons are more susceptible to oxidative stress than other types of neurons. This is thought to be due, at least in part, to the autoxidation of DA to reactive quinones and semiquinones that can redox cycle and generate ROS, resulting in a vicious cycle of increased oxidative stress and cellular damage (Berman and Hastings, 1999; Jana et al., 2007). In our studies, pre-treatment of worms with NAC increased DA-dependent behavior in pesticide treated worms (Figure 8C), suggesting that oxidative stress may play an important role in thiocarbamate-induced DAergic neurodegeneration. Cellular targets of thiocarbamate-induced oxidative stress are currently unknown. Several of the proteins involved in familial PD are involved in oxidative stress pathways, such as DJ-1, PINK-1, and parkin (Cookson, 2012; Joselin et al., 2012; Wang et al., 2011b), or increase the cell's vulnerability to oxidative stress, such as α-synuclein (Lastres-Becker et al., 2012; Wang et al., 2010). Whether thiocarbamate pesticides may interact with these PD-associated proteins remains to be determined.

In conclusion, our studies demonstrate that *C. elegans* are an effective tool for screening environmental agents for DAergic neurotoxicity. Using this model system we have characterized selective DAergic toxicity resulting from treatment of worms with thiocarbamate pesticides and dithiocarbamate metabolites. Our data demonstrate that thiocarbamates are potent DAergic toxicants, and support further investigation of this class of pesticides as a risk factor for development of PD. Additionally, we report a role for *S*-alkyl thiocarbamate metabolites in *N*,*N*-diakly dithiocarbamate-induced DAergic neurotoxicity. Further studies elucidating the underlying mechanisms of *S*-alkyl thiocarbamate DAergic toxicity will facilitate the development of structure activity relationships useful for predicting and prioritizing chemicals for assessing their potential as risk factors in PD.

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Abbreviations

6-OHDA	6-hydroxydopamine
ADE	anterior deirid

CEP	cephalic deirid
CNS	central nervous system
DA	dopamine
DAergic	dopaminergic
DAT1	dopamine transporter 1
DEDC	*N*,*N*-diethyldithiocarbamate
MeDETC	*S*-methyl-*N*,*N*-diethylthiocarbamate
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
EPTC	*S*-ethyl *N*,*N*-dipropylthiocarbamate
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GSH	glutathione
GST-α	glutathione S-transferase-α
h	hour
HO-1	heme oxygenase-1
MPP^+	1-methyl-4-phenyl-pyridinium
NAC	N-acetyl-Lcysteine
NGM	nematode growth medium
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PD	Parkinson’s disease
s	seconds
SNpC	substantia nigr pars compacta
SOD1	superoxide dismutase 1
TH	tyrosine hydroxylase
US EPA	United States Environmental Protection Agency

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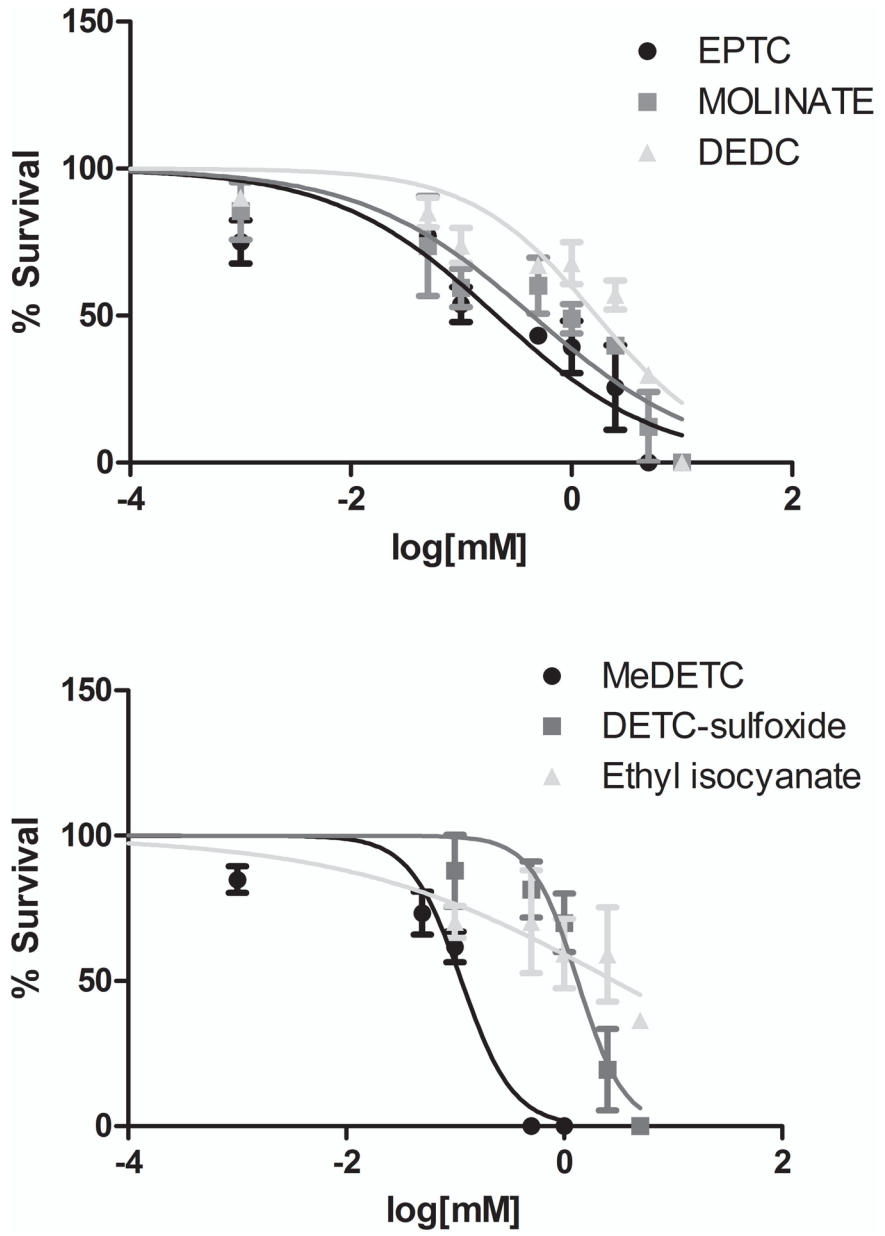


Figure 1.

Dose-response curves for selected thiocarbamate pesticides and intermediate metabolites. BY200 worms were treated for 1 h with increasing concentrations of (A) EPTC, molinate, and DEDC, or (B) the DEDC metabolites MeDETC, DETC-sulfoxide, and ethyl isocyanate. Data are expressed as means ± SEM from 4 independent experiments.

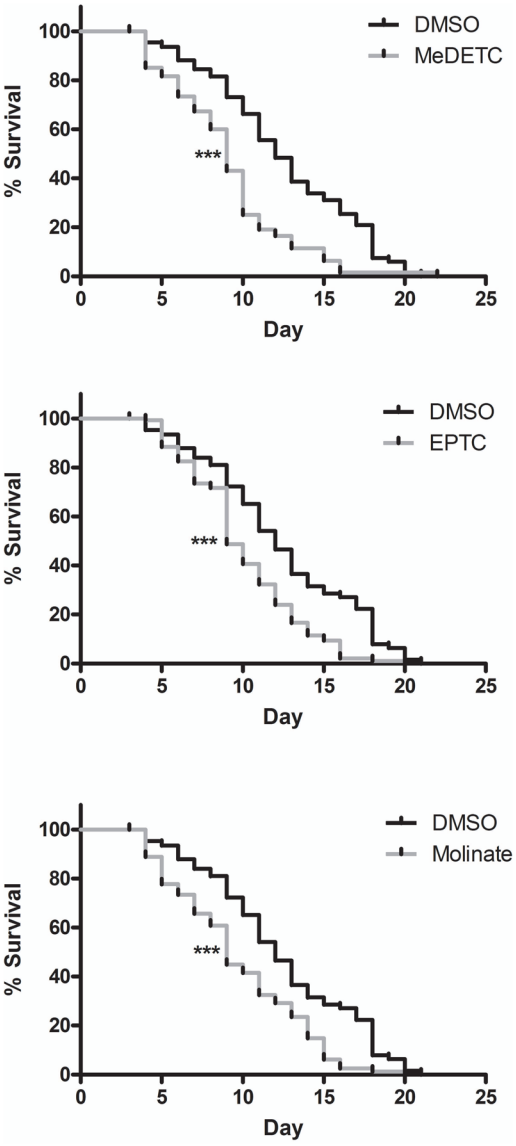


Figure 2.

Lifespan of *C. elegans* following treatment with EPTC, molinate, and MeDETC. BY200 worms were treated for 1 h with LD_{50} concentrations of (A) MeDETC, (B) EPTC, or (C) molinate or DMSO control, and worms were followed for their life time and counted for survival. Data are expressed as means ± SEM from 3 independent experiments. ***p<0.001,

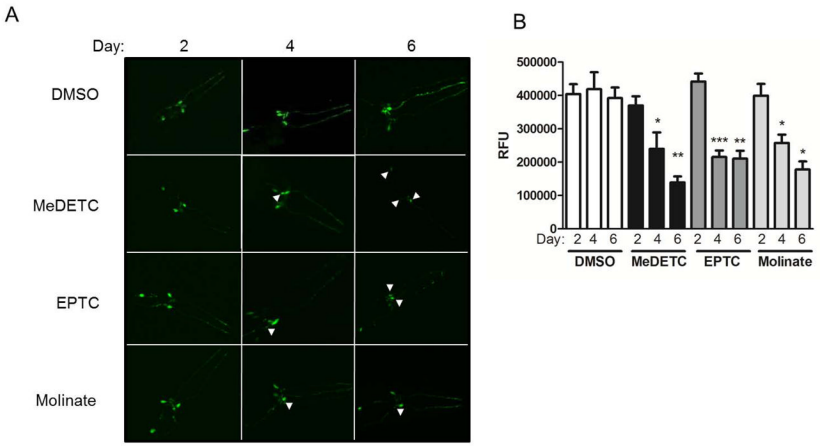


Figure 3.

Time-dependent DAergic neurodegeneration following exposure to EPTC, molinate, and MeDETC. (A) Mechanosensory CEP and ADE neurons in BY200 (*dat-1*::GFP) expressing worms were visualized 2, 4, and 6 days post exposure to LD_{50} concentrations of MeDETC, EPTC, and molinate. Worms were also treated with DMSO as a vehicle control. White arrowheads indicate shrunken cell bodies. (B) Quantification of GFP fluorescence. Data are expressed as means ± SEM from 3 independent experiments. ***p<0.001, **p<0.01, *p<0.05.

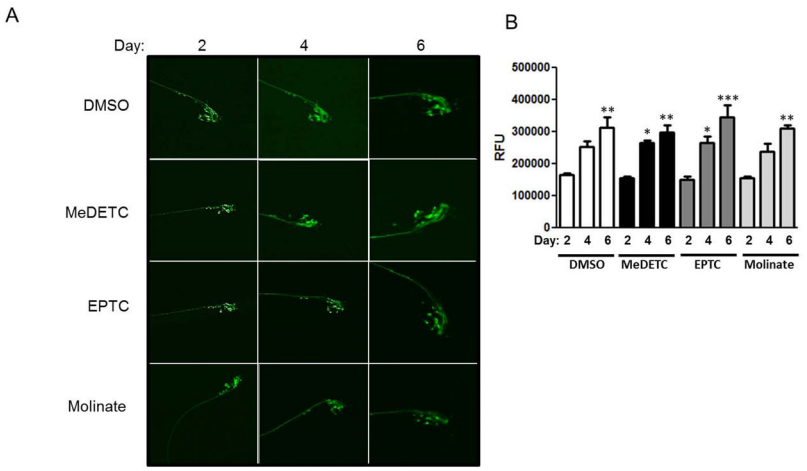


Figure 4.

EPTC, molinate, and MeDETC do not cause degeneration of cholinergic neurons. (A) Worms expressing GFP in cholinergic neurons (*unc-17*::GFP) were visualized 2, 4, and 6 days post exposure to LD_{50} concentrations of MeDETC, EPTC, and molinate. Worms were also treated with DMSO as a vehicle control. (B) Quantification of GFP fluorescence. Data are expressed as means ± SEM from 3 independent experiments. ***p<0.001, **p<0.01, *p<0.05.

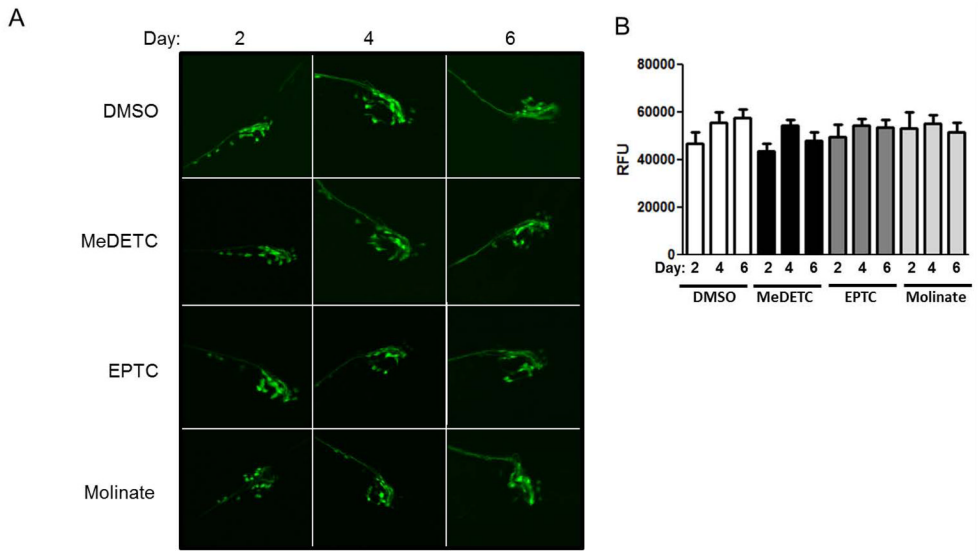


Figure 5.

EPTC, molinate, and MeDETC do not cause degeneration of glutaminergic neurons. (A) Worms expressing GFP in glutaminergic neurons (*glr-1*::GFP) were visualized 2, 4, and 6 days post exposure to LD_{50} concentrations of MeDETC, EPTC, and molinate. Worms were also treated with DMSO as a vehicle control. (B) Quantification of GFP fluorescence. Data are expressed as means ± SEM from 3 independent experiments.

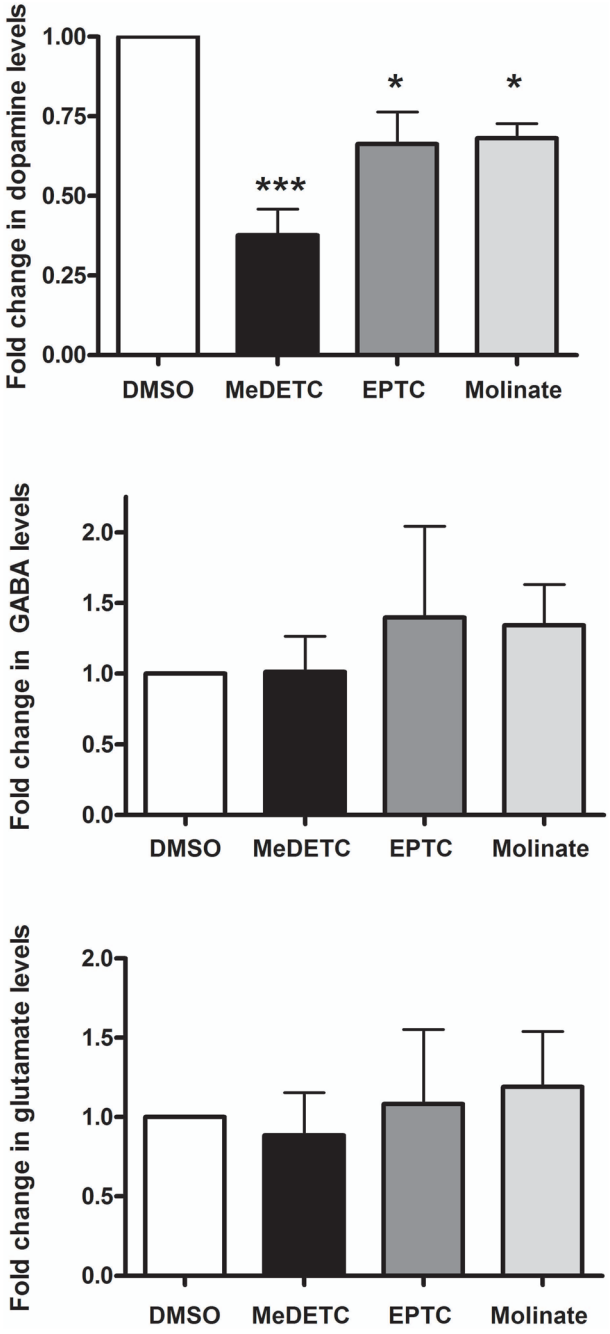


Figure 6.

Exposure to EPTC, molinate, and MeDETC decrease DA levels, but not glutamate or GABA levels in *C. elegans*. (A) DA, (B) GABA, and (C) glutamate levels were measured immediately following exposure to LD_{50} concentrations of MeDETC, EPTC, and molinate. Worms were also treated with DMSO as a vehicle control. Data are expressed as means ± SEM relative to DMSO controls from 3 independent experiments. **p<0.01, *p<0.05.

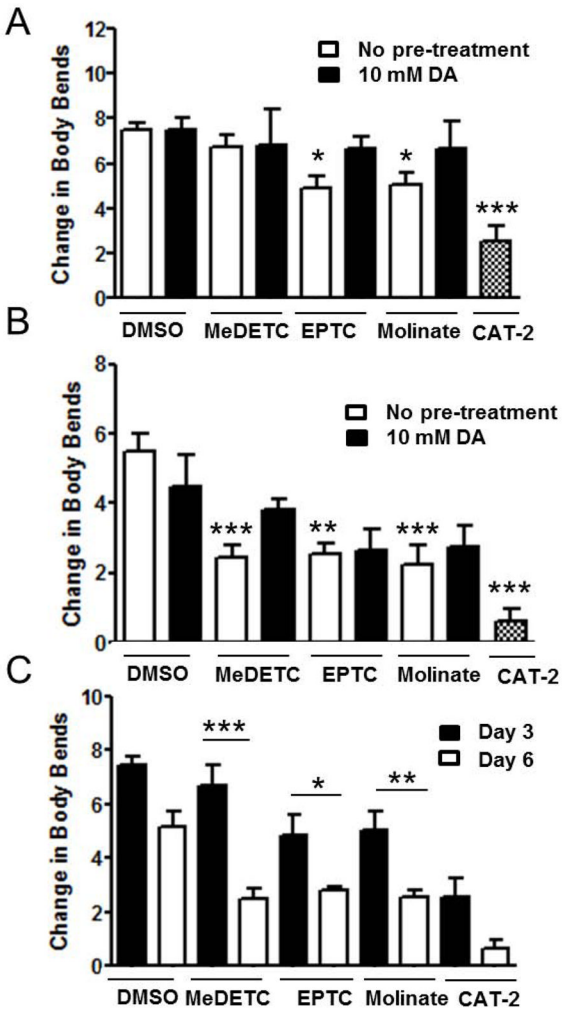


Figure 7.

Progressive loss of DAergic behavior following exposure to EPTC, molinate and MeDETC. Wild type N2 worms were treated with LD_{50} concentrations of MeDETC, EPTC, and molinate in the presence or absence of a 10 min pre-treatment with DA (10 mM), and the basal slowing response was measured (A) 3 and (B) 6 days following exposure. Worms expressing mutant *cat-2* were used as a negative control. (C) Comparison of the change in the basal slowing response between worms treated with EPTC, molinate, and MeDETC for 3 and 6 days. Data are expressed as means ± SEM from 5 independent experiments. ***p<0.001, **p<0.01, *p<0.05.

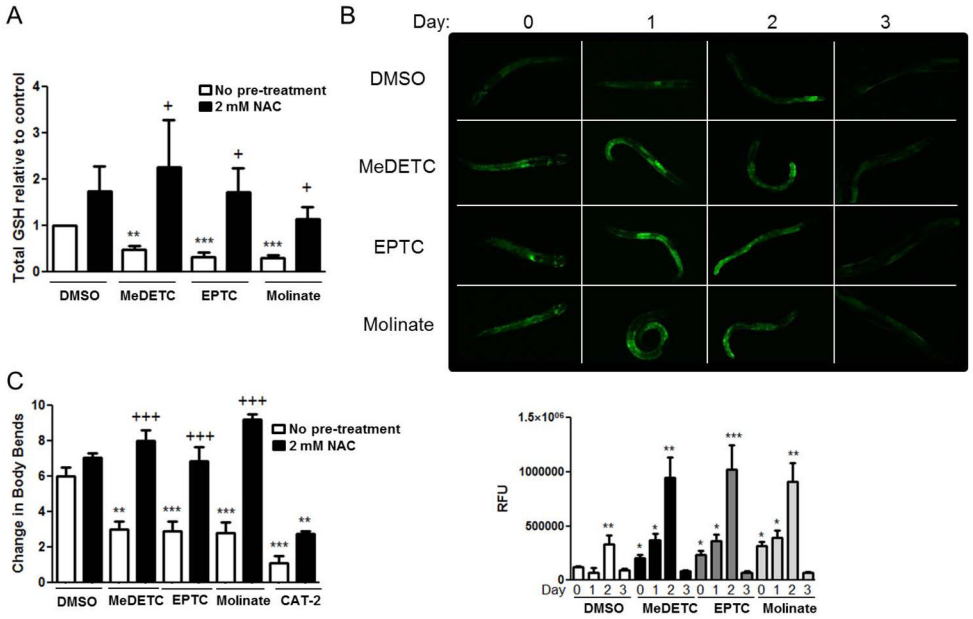
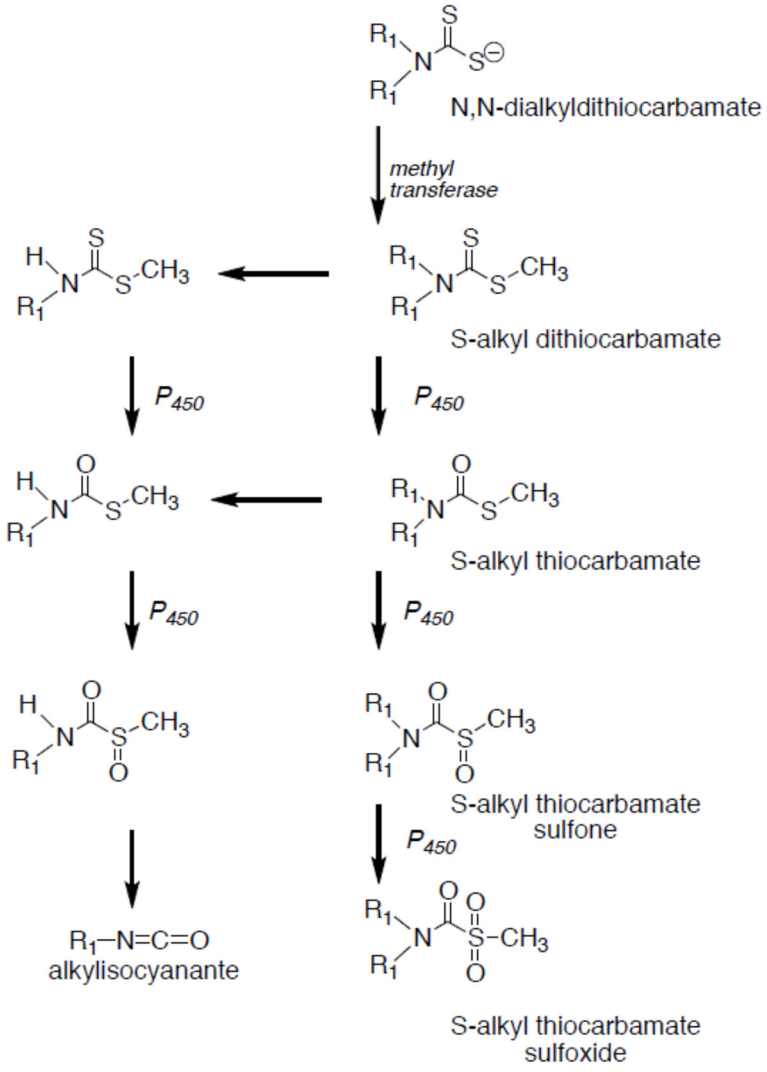


Figure 8.

Oxidative stress is involved in thiocarbamate pesticide-induced loss of DA-dependent behavior. (A) Total GSH levels were measured at the end of a 1 h exposure to LD_{50} concentrations of MeDETC, EPTC, and molinate in the presence or absence of a 1 h pre-treatment with NAC (2 mM). Worms were also treated with DMSO as a vehicle control. (B) Photomicrographs and quantification of GFP fluorescence of transgenic worms expressing GFP under the *gst-4* promoter treated with DMSO, MeDETC, EPTC, or molinate immediately (time 0), 1, 2, and 3 days following exposure. (C) Basal slowing response in N2 worms treated with LD50 concentrations of MeDETC, molinate, EPTC in the presence or absence of a 1 h pre-treatment with NAC (2mM). Worms expressing mutant cat-2 were used as a negative control. Data are expressed as means ± SEM relative to DMSO controls from 4 independent experiments. ***p<0.001, **p<0.01, *p<0.05. vs. DMSO treated worms. $^{+++}$p<0.001, $^{+}$p<0.05 vs. respective MeDETC, molinate, or EPTC treatment.



Scheme 1.

Recognized metabolic pathways of *N*,*N*-dialkyldithiocarbamates leading to the generation of reactive electrophilic species. *N*,*N*-dialkyldithiocarbamates are *S*-methylated and undergo oxidation to generate *S*-alkyl thiocarbamate metabolites that are homologues of commercial thiocarbamate herbicides. *S*-alkyl thiocarbamate metabolites can then be further oxidized to electrophilic *S*-alkyl thiocarbamate sulfoxide and sulfone metabolites capable of reacting with nucleophiles to form covalent adducts. Alternatively *N*,*N*-dialkyldithiocarbamate metabolites can be dealkylated and oxidatively metabolized to the N-monalkyl sulfoxide that can either react directly or undergo facile decomposition to an alkylisocyante prior to reacting with a nucleophile.

Table 1

LD_{50} concentrations and structures of thiocarbamate pesticides and metabolites.

Compound	Structure	LD_{50} (mM)
MeDETC		0.114
EPTC		0.209
Molinate		0.442
DETC-sulfoxide		1.232
DEDC		1.679
Ethyl isocyanate	CH_3CH_2-N=C=O	2.867