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Mancozeb-induced behavioral deficits precede structural neural degeneration

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ABSTRACT

Manganese-containing fungicides like Mancozeb have been associated with neurodegenerative conditions like Parkinson's disease. We examined the behavioral damage and differential neuronal vulnerability resulting from Mancozeb exposure using *Caenorhabditis elegans*, an important mid-trophic level soil organism that is also a powerful model for studying mechanisms of environmental pollutant-induced neurodegenerative disease. The dopamine-mediated swim to crawl locomotory transition behavior is exquisitely vulnerable to Mancozeb, with functional impairment preceding markers of neuronal structural damage. The damage is partially rescued in mutants lacking the divalent metal transporter, SMF-1, demonstrating that some, but not all, of the damage is mediated by manganese. Increasing concentrations of Mancozeb recruit additional behavioral dysfunction, notably serotonin-mediated egg-laying behavior, but without evident serotonergic neuronal structural damage. Thus, measurements of behavioral dysfunction are a sensitive early marker of fungicide toxicity that could be exploited to examine further mechanisms of neuron damage and possible therapeutic interventions. These results also provide important insight into the consequences of fungicide use on the ecological behavior of nematodes.

1. Introduction

Increasing evidence suggests that commonly applied lawn and garden chemicals are associated with neurological conditions like manganism and Parkinsonism (McCormack et al., 2002; Domico et al., 2006; Kamel et al., 2007). A number of recent studies have established *Caenorhabditis elegans* as a viable and valuable model organism for examining the neurocellular and behavioral effects of these environmentally prevalent compounds (Easton et al., 2001; Ruan et al., 2009; Settivari et al., 2009; Negga et al., 2012). Sublethal concentrations of manganese, a potent neurotoxicant ingredient of many agricultural chemicals found to contaminate water and soil, cause degeneration of dopaminergic neurons in *C. elegans* (Benedetto et al., 2010) via the conversion of dopamine into a toxic, reactive species. The toxicity of manganese can be partially rescued in knock-out worms lacking the divalent metal transporter gene *smf-1* (Settivari et al., 2009).

Mancozeb (Bonide Products Inc., NY) is a manganese/zinc ethylene-bis-dithiocarbamate fungicide that is widely used by gardeners and in agriculture to control a broad variety of fungal infections of both vegetables and ornamental plants, including leaf spot, downy mildew and blossom end rot. In fact, it is the most commonly applied fungicide in the United States (Tsang and Trombetta, 2007). It is applied to garden plants and soil topically after being mixed with water. Mancozeb has limited solubility in water, forms a thin film on plants and penetrates into the upper layers of soil with an active half-life of 1–7 days depending on weather conditions. Mancozeb is a complex mixture of both the full manganese and zinc-complexed compound and its major metabolite, ethylene thiourea. Because multiple possible toxic compounds are present under realistic conditions, we chose to examine the effects of the applied compound, Mancozeb.

Recent work in *C. elegans* has demonstrated that Mancozeb is lethal to nematodes at concentrations experienced by agricultural workers (Negga et al., 2011) and sublethal doses lead to degeneration of neurons, including dopamine neurons (Negga et al., 2011), induction of heat shock responses (Easton et al., 2001; Leung et al., 2008) and inhibition of larval growth (Easton et al., 2001; Ruan et al., 2009). Mancozeb is considered a potential estrogen disruptor and suspected carcinogen (Shukla and Arora, 2001), and the presence of both manganese and zinc in the formulation suggest additional mechanisms by which it might exert neurotoxic effects, including production of free radicals and mitochondrial inhibition (http://environmentalcommons.org/cetos/criticalhabitat/mancozeb.pdf; Negga et al., 2012). Thus, there may be multiple mechanisms at work

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simultaneously during exposure to Mancozeb causing damage to neuron function and viability.

C. elegans is an important soil nematode and, in addition to serving as a valuable model for human and other animal diseases, plays an important role in soil ecology. Mancozeb application to soil may benefit garden plants, but in the longer term it could have negative impacts on beneficial soil organisms. We examined the influence of sublethal concentrations of Mancozeb at levels typically seen with garden application (Bonide, 2010) on a number of essential behaviors of *C. elegans*, as well as on the neuron subtypes that govern them. We explored locomotory, mechanosensory, and egg-laying behavior in response to a 24 h exposure to Mancozeb to better understand the impacts of sublethal amounts of this widely used fungicide on this important soil organism.

C. elegans move through the top layer of soil, navigating in both liquid using swimming behavior and more solid matrices using crawling, frequently transitioning from one form of locomotion, or gait, to another. The transition from swimming to crawling is controlled by dopaminergic neurons, particularly the ADE and PDE neurons, while that from crawling to swimming is mediated by VC-4, VC-5, AIM and RIH serotonergic neurons (Vidal-Gadea et al., 2011). Overall sinusoidal locomotion is mediated by cholinergic motor neurons. To navigate between and among soil particles, nematodes respond to mechanosensory cues by reversing direction around an obstacle. Gentle touch to the body is detected by five glutamatergic mechanosensory neurons, AVM, ALML/R, and PLML/R (Chalfie et al., 1985). The ability to lay eggs is governed by the mixed cholinergic/serotonergic neurons, HSNL/R (Desai et al., 1988). Egg-laying behavior can be modulated by numerous stimuli, including crowding and the presence or absence of bacterial food (Nass and Blakely, 2003; Schafer, 2005).

Because the neurons that govern each of these behaviors are well-understood, *C. elegans* is a powerful model to explore directly the behavioral dysfunctions resulting from neuronal damage. Our goal was to examine the possible differential sensitivity of behaviors governed by different subsets of neurons, as well as elucidate the nature of the cellular changes in damaged neurons. Our results indicate that behavioral damage, which might influence the overall fitness of nematodes in their soil environment, is a sensitive early indicator of neuronal damage that precedes overt structural impairment.

2. Materials and methods

2.1. Worm strains

The following strains were obtained from the *C. elegans* Genetics Center: wild-type Bristol N2, RB1491 (*smf-1*(ok1748)), OH7547 ((otIs199)[*cat2*::GFP + rgef-1(R25B3.3)::dsRed + *rol-6*(su1006)]); LX929((vsIs48)[*unc-17*::GFP]), and BC12648 (*dpy-5*(e9077/*dpy5*/e907); [sls11686 IrCesF42G9.9a::GFP + pCeh361]). UL1164 (*bas-1*::GFP + *rol-6*(sw1006)) strain was a gift from Dr. Ian Hope (University of Leeds, London). Nematodes were maintained at 20 °C on NGM petri plates with small lawns of bacterial strain OP50 (Stiernagle, 2006). Small-scale synchronized cultures were prepared for Mancozeb exposure by transferring 10–15 gravid adults to fresh NGM plates with OP50 bacteria. Worms were allowed to lay eggs for approximately 2 h and then adults were removed. Eggs therefore developed into small (30–50 worms) synchronized cultures.

2.2. Mancozeb (MZ) exposure

We examined the effects on young adult worms of a 24 h exposure to Mancozeb. This time period was chosen to encompass the beginning of production of eggs without substantial aging within the nematodes. In addition, we wanted to operate within the t½ of Mancozeb in water of 1–2 days (Extoxnet, 1997). Within our time frame, the majority of the Mancozeb mixture should be Mancozeb, although there is also the presence of the major metabolite, ethylene thiourea. We first verified that under our conditions we obtained a similar lethality profile as reported by Negga and colleagues (2011; data not shown). We then selected three concentrations for our study (0, 0.5, 0.75 and 1.5%, v/v). Mancozeb solutions were prepared in distilled water to approximate concentrations applied to garden soils. The two higher concentrations are slightly above the recommended application amount, but within the LC50 of nematode sensitivity (Negga et al., 2011). Fifty microliters of each solution was spread on the surface of 2 cm NGM agar plates using a sterile angled glass rod and the resulting mixture was allowed to soak into the agar, after which the bacterial lawn was applied. Control plates were prepared the same way, except only water and then bacteria were applied. Late stage L4 worms were transferred to MZ-treated or control plates for 24 h. For behavioral measurements, worms were transferred to plates without MZ for testing. For microscopic analysis, 4–7 worms were transferred to 2% agarose pads on individual microscope slides in a drop of M9 buffer containing 2.5 mM NaN_3.

2.3. Behavioral measurements

For each behavioral assay, MZ-exposed and unexposed nematodes were tested on the same day to minimize day-to-day variability in behavior. In addition, more than one investigator conducted each of the behavioral tests to minimize investigator bias. All behavioral tests were conducted at ambient temperature (22 °C) with the lid off the petri plate. Nematodes were visualized with an Olympus SZ-1 dissecting microscope.

2.3.1. Locomotion

Nematodes were transferred individually with a flame-sterilized flattened platinum wire to a fresh measurement plate lacking *Escherichia coli* food. After a few seconds to allow acclimation to the plate, full body bends consisting of sinusoidal cycles initiated by head oscillations with full waves propagated along the length of the worm, were counted for a thirty-second interval and reported as body bends per 30 s. The transition from crawling on the agar surface to body bends suspended in solution (swimming) was measured by placing a 10 μl drop of M9 buffer on the worm, waiting 2 s for the worm to initiate swimming oscillations and then counting oscillations for 30 s. To measure the transition back to crawling from swimming, the drop was carefully wicked with a kimwipe. Again, body bends were counted for 30 s beginning 2 s after the droplet was removed.

2.3.2. Mechanosensation

Individual worms ($n = 40$) were transferred from treatment plates to fresh plates without food. Following a few seconds to acclimate to the new conditions, the touch reversal response was initiated by stroking alternately the anterior and posterior thirds of the worm body and recording whether or not a reversal occurred. Worms were stroked gently perpendicular to the longitudinal axis of the worms with an eyebrow hair glued to a toothpick, as described by Gordon et al. (2008).

2.3.3. Egg-laying behavior

Late L4 nematodes were transferred to MZ-treated or untreated plates with OP50 bacterial lawns for a 24 h exposure. Individual exposed worms were then transferred to fresh, untreated plates