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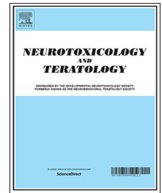
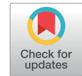


Brief communication

Transport of a manganese/zinc ethylene-bis-dithiocarbamate fungicide may involve pre-synaptic dopaminergic transporters



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ABSTRACT

Mancozeb (MZ), an organic-metal fungicide used predominantly on vegetables and fruits, has been linked to neurodegeneration and behavioral disruptions in a variety of organisms, including humans. Both γ-aminobutyric acid and dopamine neurons appear to be more vulnerable to MZ exposure than other neuronal populations. Based on these observations, we hypothesized that MZ may be differentially transported into these cells through their presynaptic neurotransmitter transporters. To test this, we pretreated *Caenorhabditis elegans* with transporter antagonists followed by exposure to various concentrations of MZ. Potential neuroprotection was monitored via green fluorescence associated with various neuron populations in transgenic worm strains. Neurodegeneration associated with subacute MZ treatment (30 min) was not altered by transporter antagonist pretreatment. On the other hand, pretreatment with a dopamine transporter antagonist (GBR12909) appeared to protect dopaminergic neurons from chronic (24 h) MZ treatment. These results are consistent with other reports that dopamine transporter levels or activity may modulate toxicity for neurotoxicants.

1. Introduction

Recent epidemiological studies have focused on Mn toxicity from exposure to the fungicide mancozeb (MZ) (Barraza et al., 2011; Mora et al., 2014; van Wendel de Joode et al., 2014; van Wendel de Joode et al., 2016). MZ is an ethylene-bis-dithiocarbamate (EBDC) fungicide containing both Mn and zinc (Zn). Exposure to this agrochemical by pesticide applicators, agricultural workers, and people living near fields, where spraying is particularly heavy, often results in intoxication, which manifests as movement disorders and memory or attention deficits (Colosio et al., 2007; Corsini et al., 2005; Nordby et al., 2005; van Wendel de Joode et al., 2016).

MZ exposure can lead to neurodegeneration, endocrine disruption, dermatitis, reproductive toxicity, and developmental delays (Runkle et al., 2017). In the case of neurodegeneration, our laboratory and others have shown that dopaminergic (DAergic) and γ-aminobutyric acidergic (GABAergic) neurons in *Caenorhabditis elegans* (*C. elegans*) are particularly vulnerable to MZ (Negga et al., 2012; Todt et al., 2016). Our initial studies determined that concentrations corresponding to the LC_{25}, LC_{50}, and LC_{75} in *C. elegans* were also consistent with concentrations recommended by the manufacturer for field application (Bonide, 2010). Since we did not observe overt toxicity or developmental delays, but rather neuronal and behavioral changes associated with DAergic neurodegeneration (Brody et al., 2013; Negga et al., 2011; Negga et al., 2012), we hypothesized that MZ exposure led to this neurotoxicity via entry of MZ through presynaptic neurotransmitter transporters. Since *C. elegans* can be easily experimentally manipulated, share numerous genetic similarities with humans (Shaye and Greenwald, 2011), have only 302 neurons (Corsi et al., 2015), and are often used to model human disease (Calahorro and Ruiz-Rubio, 2011), we continued our work in this important model organism. Here, we sought to test this hypothesis by determining the effects of GABA or DA transporter antagonists on the neurotoxicity of MZ in *C. elegans*. Because we had not documented any changes in serotonergic neurons, we also tested a serotonin transporter (SERT) antagonist as a negative control.

2. Materials and methods

2.1. Chemicals, worm strains, and bacterial strains

C. elegans strains were acquired from the *Caenorhabditis* Genetics Center (CGC; University of Minnesota). BZ555 worms ([*dat-1p::gfp*]) are genetically engineered such that all dopaminergic (DAergic) neurons

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are tagged with green fluorescent protein (GFP). EG1285 worms ([*unc-47p*::*gfp* + *lin-15*(+)]) express GFP in only γ-aminobutyric acid (GABA) neuronal populations. NW1229 worms ([*F25B3.3*::*gfp* + *dpy-20*(+)]) are engineered so that all neurons are tagged with GFP. *C. elegans* were either fed NA22 *E. coli* (for 8P plates) or OP50-1 *E. coli* (for nematode growth media (NGM) plates). Both bacterial strains were acquired from the CGC. Mancozeb Flowable with Zinc (37.0% manganese/zinc ethylene-bis-dithiocarbamate [Mn/Zn-EBDC]) was purchased commercially from Bonide®. Based on previous studies in *C. elegans* (Chase and Koelle, 2007; Jorgensen, 2005), we used clomipramine HCl, a serotonin transporter (SERT) antagonist, nipecotic acid, a GABA transporter (GAT) antagonist, GBR12909, a dopamine transporter (DAT) antagonist. Antagonists and all other chemicals were purchased from VWR International at a purity ≥95% unless otherwise noted.

2.2. Synchronization

Worms were grown at 20 °C on 8P plates (51.3 mM NaCl, 25.0 g bactoager/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 500 μM KH_2PO_4, 68 mM NaCl) seeded with NA22 *E. coli* (grown in 16 g tryptone/L, 10 g yeast extract/L, 85.5 mM NaCl) until they were gravid. Synchronization followed standard procedures (Brenner, 1974) previously published by our lab (Negga et al., 2011). Briefly, worms were washed from 8P plates and centrifuged until the supernatant was clear. Following the final wash, worms were incubated in a bleaching solution (0.55% NaOCl, 500 μM NaOH) to promote the release of eggs from gravid hermaphrodites. Following bleaching, M9 buffer (20 mM KH_2PO_4, 40 mM Na_2HPO_4, 68 mM NaCl) was added. Eggs and worm debris were further washed with M9, and eggs were differentially separated using a 30% sucrose solution. After the addition of M9, eggs were placed on a nutator and incubated for 18 h at 20 °C.

2.3. Pretreatment with antagonists

Antagonist pretreatments took place 18 h post-synchronization. For each pesticide concentration, 5000 synchronous worms were pretreated for 7 min with the respective neurotransmitter transporter antagonists: 4.3 μM clomipramine HCl; 2.5 μM nipecotic acid; or 125 nM GBR 12909. Worms were followed under the microscope for behaviors indicative of excess synaptic neurotransmitter (Chase and Koelle, 2007; Jorgensen, 2005) to verify that neurotransmitter transporter antagonism was successful. Following behavioral assessment, worms were washed of the respective antagonists before MZ treatment to limit any direct interactions of the neurotransmitter transporter antagonists with the fungicide.

2.4. MZ Treatment

Immediately following removal of the respective transporter antagonist, worms were treated subacutely (30 min) or chronically (24 h) with concentrations of MZ used in our previous studies (Bailey et al., 2016; Todt et al., 2016), and were equivalent to the LC_{25}, LC_{50}, or LC_{75} for the respective subacute or chronic studies. All concentrations were well within manufacturer recommendations for different field and/or environmental conditions (Bonide, 2010). Subacute treatments included low (0.1% Mn/Zn-EBDC; LC_{25}), medium (7.5% Mn/Zn-EBDC; LC_{50}), or high concentrations (17.0% Mn/Zn-EBDC; LC_{75}) of the fungicide. In chronic treatments, worms were also exposed to a low (0.1% Mn/Zn-EBDC; LC_{25}), medium (1.0% Mn/Zn-EBDC; LC_{50}), or high concentration (1.5% Mn/Zn-EBDC; LC_{50}). Consistent with manufacturer-recommended directions, final concentrations of the active ingredient (Mn/Zn-EBDC) were achieved by diluting commercial Manzate™ (37.0% Mn/Zn-EBDC) with water. Control and antagonist-treated control worms were an equivalent amount of water (vehicle for the fungicide) in lieu of fungicide treatment. In both chronic and subacute studies, worms were incubated in microcentrifuge tubes for 30 min with the respective MZ concentrations. Worms in the subacute studies were washed with water to remove MZ, and then placed on NGM plates (51.3 mM NaCl, 17.0 g bactoager/L, 2.5 g bactopeptone/L, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 500 μM KH_2PO_4 [pH 6.0], 12.9 mM cholesterol [in 95% ethanol], 1.25 mL nystatin/L, 200 mg streptomycin/L) seeded with OP50-1. Chronically-treated worms were placed on NGM plates seeded with OP50-1 without additional washes to maintain MZ exposure. Both groups were incubated for an additional 24 h at 20 °C (subacute in the absence of MZ; chronic in the presence of MZ).

2.5. Fluorescence determination

Following the 24-h incubation, worms were washed from NGM plates into centrifuge tubes, and washed free of bacteria (or MZ, in the case of chronic studies) until supernatant was clear. Worms were then counted and standardized to yield solutions of 4 worms/μL (for NW1229 and EG1285 strains) or 6 worms/μL (for BZ555). Standardized solutions of 250 μL were then added to 96-well plates in triplicate for each pretreated and non-pretreated group. Plates were read by a Promega™ GloMax-Multi+ Detection System with an emission spectrum of 510–570 nm and an excitation of 490 nm. Readings from water were subtracted from the raw data to eliminate background fluorescence.

2.6. Data analysis

Data were analyzed by two-way ANOVA (MZ treatment with antagonist treatment) using GraphPad Prism (version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). When appropriate, Sidek's posttest was used to determine whether differences existed between no antagonist and antagonist pretreatment groups. Each study represents $N \geq 3$ separate synchronizations ($n = 3–4$ intraexperimental replicates of 1000–1500 worms/well). Data are presented as mean ± standard error of the mean (SEM), and the data were considered statistically significantly different when $p \leq 0.05$.

3. Results

3.1. Clomipramine pretreatment does not protect neurons from MZ exposure

Following exposure of worms to the SERT antagonist, clomipramine, worms expressing GFP in all neurons (NW1229 strain) were treated subacutely or chronically with varying MZ concentrations (Fig. 1). In both cases, clomipramine pretreatment did not result in statistically significant differences in fluorescence between the control for non-pretreated versus pretreated worms. When the fluorescence from worms treated only with MZ (black bars) was compared to our previously published results (Negga et al., 2011), the dose-dependent decreases in fluorescence were similar to what we had observed. Two-way ANOVA of results from the subacute study indicated that both MZ treatment ($DF = 3$, $F = 13.36$, $^{****}p < 0.0001$) and antagonist pretreatment ($DF = 1$, $F = 7.219$, $^{**}p = 0.008$) contributed to the observed variation in the data. There was not statistically significant interaction between the two conditions, however. Furthermore, clomipramine did not provide any protection from the MZ-induced decreased fluorescence (Fig. 1A). In contrast, when the data from worms in the chronic study was analyzed via two-way ANOVA, the only statistically significant source of variation was due to MZ treatment ($DF = 3$, $F = 10.39$, $^{****}p < 0.0001$). As such, pretreatment with clomipramine had no effect on neuron-associated fluorescence (Fig. 1B).

3.2. Nipecotic acid pretreatment does not protect GABAergic neurons from MZ exposure

In order to assess the effect of the GAT antagonist nipecotic acid on

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