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The neurotoxic effects of heavy metal exposure on GABAergic nervous system in nematode *Caenorhabditis elegans*

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ABSTRACT

The number of cell body or synapse made by *Caenorhabditis elegans* GABAergic neurons is constant during development. The neurotoxic effects of metal (Pb, Hg, Cu, Cd, Cr, and Mn) exposure on GABAergic motor neurons were investigated in *C. elegans*. Exposure to examined metals could not alter the position of GABA neurons, whereas exposure to high concentrations (75 μM and 200 μM) of metals caused noticeable axonal degeneration and neuronal loss in nerve cords, suggesting neurodegeneration will be induced by metal exposure to different degrees. In addition, exposure to Pb, Hg, Cu, and Cd at the low concentration (2.5 μM) could also induce obviously neuronal loss. Moreover, exposure to high concentrations (75 μM and/or 200 μM) of most of examined metals significantly reduced the relative size and fluorescent intensities of AVL, RMEs, and RIS neurons. Therefore, the neurodegeneration and abnormal structures may be formed in GABAergic motor neurons after metal exposure, and the endpoint of neuronal loss will be useful for the neurotoxicity assessment from trace metal exposure.

1. Introduction

Caenorhabditis elegans, a free-living soil nematode, has already been explored as a valuable bioindicator, since it is one of the best-characterized model animals at the genetic, physiological, molecular, and developmental levels (Riddle et al., 1997). It has also been proved that *C. elegans* serves as an excellent candidate for studying the development and functions of nervous system and the neurotoxicology, since it has only 302 neurons and the complete writing diagram for chemical and electrical connections is available (Shen and Wang, 2005; Ye et al., 2008a; Leung et al., 2008). *C. elegans* has already been used to test the environmental contaminants known to have neural toxic effects. Williams and Dusenbery (1990a) indicated that lead, mercury, and two organophosphate pesticides displayed concentration–response dynamics consistent with neurotoxicity. The movement or locomotion behavior could be monitored using a computer tracking system after metal exposure in nematodes, and movement was decreased in a concentration-dependent fashion by metals such as Pb and Cd (Dhawan et al., 2000; Boyd et al., 2003; Anderson et al., 2004). The endpoints of head thrash, body bend, and basic movements (forward sinusoidal movement (forward turn), reversal movement (backward turn), and turn) have further been systematically examined in nematodes exposed to heavy metals, and endpoints of head thrash, body bend, and forward turn can establish a fast and economic way to assess the presence of acute toxicity from heavy metal exposure (Wang et al., 2007a,b; Wang and Wang, 2008a,b; Wang and Xing, 2008). *C. elegans* was also used for the test of organophosphate-induced mammalian neurotoxicity by computer tracking for acute behavioral toxicity and anticholinesterase activity assay (Cole et al., 2004).

Nevertheless, so far the possible alterations of nervous system in nematodes exposed to heavy metals have never been reported, although occupational exposure to toxic airborne metal dust usually will cause adverse effects on the central nervous system (Mergler et al., 1994, 1999; Myers et al., 2003). Exposure to Ni, Zn, Co, and Pb can induce severe learning or memory defects in nematodes (Wang et al., 2007a,b; Wang and Wang, 2008b; Ye et al., 2008b), suggesting the possible alterations of nervous system of exposed nematodes. In *C. elegans*, 26 neurons express γ-aminobutyric acid (GABA) (White et al., 1986), and killing the DD and VD GABAergic motor neurons causes a locomotory behavior known as "shrinking", in which the animal simultaneously hypercontracts both ventral and dorsal body muscles (Hodgkin, 1983; McIntire et al., 1993). Visualization of the GABAergic nervous system has been successfully used to evaluate the progressive axonal degeneration and neuronal loss in *C. elegans* (Kraemer et al., 2003). In the present study, we investigated the possibly neurotoxic effects of heavy metal exposure on the GABAergic nervous system in nematodes. We show in this paper that metal exposure will alter the

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GABAergic nervous system and induce the axonal degeneration and neuronal loss in *C. elegans*.

2. Materials and methods

2.1. Reagents and strains

The metal concentrations used in this study were selected as previously described (Wang and Yang, 2007a,b). Three concentrations of $CdCl_2$, $CrCl_2$, $CuSO_4$, $HgCl_2$, $MnCl_2$, and $Pb(NO_3)_2$ solutions were used in the current work, and they were 2.5 μM, 75 μM, and 200 μM, respectively. Metal concentrations of exposed solutions were analyzed by atomic absorption spectrophotometry (AAS; Pye-Unicam model SP9, Cambridge, UK). All the chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Nematodes used in the present study were wild-type N2 and *oxIs12* [P*unc-47*::GFP; *lin-15*(+)] labeling the GABAergic nervous system (Bamber et al., 1999), originally obtained from the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resource, USA). They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C as described (Brenner, 1974). Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45N NaOH, 2% HOCl). Age synchronous populations of larva (L4-stage) were obtained by the collection as described (Donkin and Williams, 1995). The L4-larva stage nematodes were washed with double-distilled water twice, followed by washing with modified K medium once (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) (Williams and Dusenbery, 1990a). Exposures were performed in 12-well sterile tissue culture plates as described (Mutwakil et al., 1997). All exposures were 24-h long and were carried out in 20 °C incubator in the absence of food.

2.2. Analysis of the axonal degeneration and neuronal loss of GABAergic neurons

The method was performed as described (Kraemer et al., 2003). GABA motor neurons were visualized using a GFP construct (*oxIs12*) in control and metal exposed wild-type animals. The axonal degeneration could be directly observed from both nerve cords. The number of ventral and dorsal cord gaps was also quantified to reflect the axonal degeneration. In addition, the neuronal loss was examined by comparing the number of cell bodies in nervous system of control and metal exposed animals. The images were photographed and examined on the same day to avoid effects of light source variance on fluorescence intensity. At least 30 animals were examined for each trial.

2.3. Fluorescence quantification

To quantify fluorescence intensities, fluorescence images of neurons in control and exposed young adult animals were captured with a Zeiss Axiocam MRm camera on a Zeiss Axioplan 2 Imaging System with 40× objective using SlideBook software (Intelligent Imaging Innovations). Images were acquired with a Quantix cooled CCD camera, and illumination was provided by a 175 W xenon arc lamp and GFP filter sets. Exposure times were chosen to fill the 12-bit dynamic range without saturation, and out-of-focus light was removed with a constrained iterative deconvolution algorithm. Background fluorescence from the coverslip and from nonspecific tissue autofluorescence was removed by subtracting an image filtered with a low pass Gaussian filter. The relative fluorescence intensity of particular fusion proteins at the cell bodies was obtained by integrating pixel intensity in at least 30 animals. The images were photographed and examined on the same day to avoid effects of light source variance on fluorescence intensity.

2.4. Statistical analysis

All data in this article were expressed as means ± S.D. Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). One-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test was used to determine the significance of the differences between the groups. The probability levels of 0.05 and 0.01 were considered statistically significant.

3. Results

3.1. Effects of metal exposure on morphology of GABA motor neurons in nerve cords of nematodes

As shown in Fig. 1, the morphologies of GABA motor neurons in nematodes exposed to metals of Pb, Hg, Cu, Cd, Cr, and Mn at concentrations of 2.5 μM, 75 μM, and 200 μM were examined. Exposure to all examined six metals at different concentrations could not obviously alter the position of GABA neurons compared to control. However, exposure to the examined metals could induce the axonal discontinuities to different degrees. In *oxIs12*, both ventral and dorsal nerve cords are continuous and neuronal morphology is normal, whereas exposure to examined metals at concentrations of 75 μM and 200 μM caused noticeable axonal discontinuities and abnormal neuronal morphology. In addition, exposure to 2.5 μM of Pb, Hg, Cu, Cd, and Cr could also result in the moderate axonal discontinuities. No axonal discontinuities or abnormal neuronal morphology could be observed in nematodes exposed to 2.5 μM of Mn.

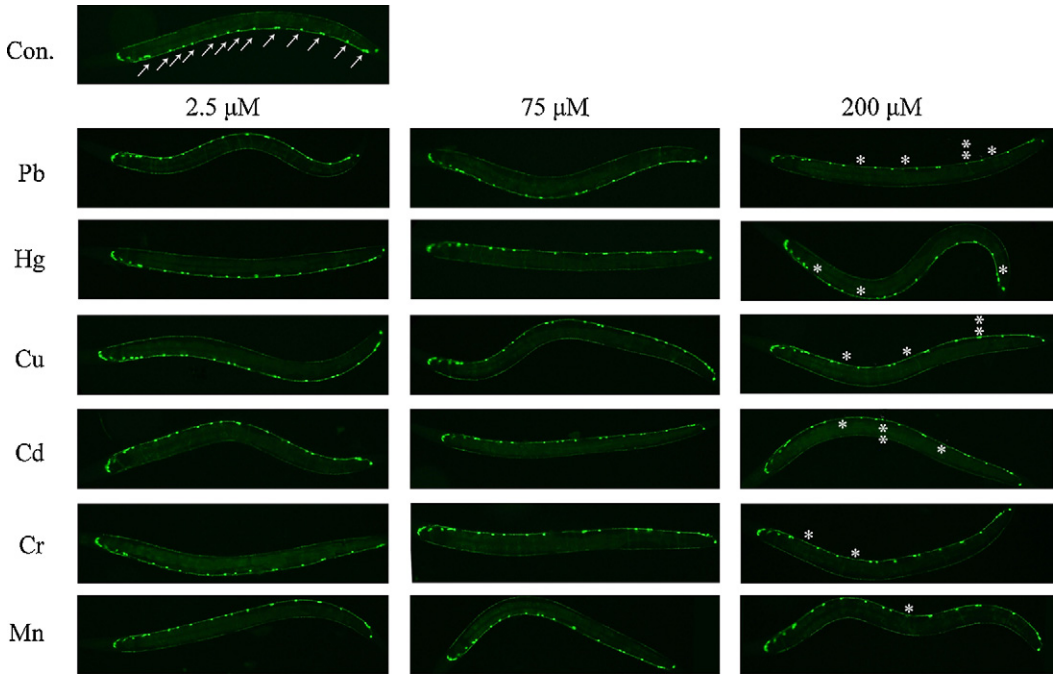

Fig. 1. Morphology of the GABA nervous system in control and metal (Pb, Hg, Cu, Cd, Cr, and Mn) exposed nematodes. GABA motor neurons were visualized using a GFP construct (*oxIs12*) in wild-type nematodes. In all images, anterior is left. Arrowheads indicate the position of cell bodies in control nematode. The positions of neuronal loss (*) and abnormal neuron (**) were indicated in 200 μM of metal exposed GABAergic motor neurons. Con., control.