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Waterborne manganese exposure alters plasma, brain, and liver metabolites accompanied by changes in stereotypic behaviors

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ABSTRACT

Overexposure to waterborne manganese (Mn) is linked with cognitive impairment in children and neurochemical abnormalities in other experimental models. In order to characterize the threshold between Mn-exposure and altered neurochemistry, it is important to identify biomarkers that positively correspond with brain Mn-accumulation. The objective of this study was to identify Mn-induced alterations in plasma, liver, and brain metabolites using liquid/gas chromatography–time of flight–mass spectrometry metabolomic analyses; and to monitor corresponding Mn-induced behavior changes. Weanling Sprague–Dawley rats had access to deionized drinking water either Mn-free or containing 1 g Mn/L for 6 weeks. Behaviors were monitored during the sixth week for a continuous 24 h period while in a home cage environment using video surveillance. Mn-exposure significantly increased liver, plasma, and brain Mn concentrations compared to control, specifically targeting the globus pallidus (GP). Mn significantly altered 98 metabolites in the brain, liver, and plasma; notably shifting cholesterol and fatty acid metabolism in the brain (increased oleic and palmitic acid; 12.57 and 15.48 fold change (FC), respectively), and liver (increased oleic acid, 14.51 FC; decreased hydroxybutyric acid, −14.29 FC). Additionally, Mn-altered plasma metabolites homogentisic acid, chenodeoxycholic acid, and aspartic acid correlated significantly with GP and striatal Mn. Total distance traveled was significantly increased and positively correlated with Mn-exposure, while nocturnal stereotypic and exploratory behaviors were reduced with Mn-exposure and performed largely during the light cycle compared to unexposed rats. These data provide putative biomarkers for Mn-neurotoxicity and suggest that Mn disrupts the circadian cycle in rats.

1. Introduction

Overexposure to environmental manganese (Mn) is known to have neurological consequences with symptomology similar to Parkinson's disease (PD) (Pal et al., 1999; Cersosimo and Koller, 2006; Perl and Olanow, 2007). Both are characterized by alterations in the dopaminergic system of the basal ganglia, producing movement abnormalities and cognitive deficits (Pal et al., 1999; Cersosimo and Koller, 2006). Mn neurotoxicity is clinically distinct from PD in that onset may occur at earlier ages, movement symptoms occur bilaterally as opposed to unilaterally in PD, and the lack of response to levo-Dopa treatment (Cersosimo and Koller, 2006). Cases of Mn neurotoxicity have been reported due to occupational contact (e.g., mining, battery manufacturing, and welding) and contaminated drinking water (Crossgrove and Zheng, 2004; Wasserman et al., 2006). Challenges exist in diagnosing Mn neurotoxicity, and factors such as length or route of exposure may differentially affect symptom onset. Inhalation of Mn species leads to rapid brain Mn accumulation and is associated with increased biomarkers of oxidative stress (Erikson et al., 2007); whereas, ingested Mn accumulates in the brain at slightly lower concentrations and is associated with neurochemical alterations (Garcia et al., 2006; Anderson et al., 2008; Fordahl et al., 2010) and cognitive decline (Wasserman et al., 2006; Bouchard et al., 2011).

Mn-neurotoxicity has been linked with changes in dopamine, γ-aminobutyric acid (GABA), and glutamate (Fitsanakis et al., 2006 for review). Mn-induced changes in these neurochemicals, specifically dopamine, have been associated with hyperactivity in rodents (Kern et al., 2010), and learning/memory deficits accompanied by changes in stereotypic behaviors in primates (Schneider et al., 2006; Kern et al., 2010). Similar symptoms have been reported in Mn-exposed children (Bouchard et al., 2007; Farias et al., 2010), and it is imperative to identify symptoms of toxicity early during this critical stage of growth and neurological development.

Abbreviations: Mn, manganese; PD, Parkinson's disease; GABA, γ-aminobutyric acid; MRI, magnetic resonance imaging; PET, positron emission tomography; MnSOD, manganese superoxide dismutase; GP, globus pallidus; LC–TOFMS, liquid chromatography–time of flight mass spectrometry; GC–TOFMS, gas chromatography–time of flight mass spectrometry; Fe, iron; ELISA, enzyme linked immunosorbent assay; HCS, Home Cage Scan; Cu, copper; FC, fold change; TDT, total distance traveled; AD, Alzheimer's disease; Cn, control; NAA, *N*-acetylaspartate; HMDB, Human Metabolome Database.

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Early symptom identification and removal from Mn exposure can improve the prognosis of Mn neurotoxicity. The use of magnetic resonance imaging (MRI) has been demonstrated to accurately reflect brain Mn deposits (Dorman et al., 2006; Fitsanakis et al., 2008), and when used in conjunction with positron emission tomography (PET) can identify biological alterations in neurotransmission (Kim et al., 1999). While MRI and PET technologies have advanced the identification of Mn neurotoxicity, the practical application and cost of these tools may preclude widespread use. Moving forward, it is important to establish cost effective diagnostic measures that correspond with brain Mn accumulation similar to MRI. Identifying biomarkers of Mn neurotoxicity in biological fluids may provide an alternative solution to confirm the extent of brain Mn accumulation.

To date, few reliable markers exist to measure the extent of brain Mn accumulation. Prospective compounds such as lymphocytic manganese superoxide dismutase (MnSOD) and arginase were suggested as biomarkers over a decade ago; however, each possessed diagnostic limitations (Davis and Greger, 1992; Brock et al., 1994). More recently, Dorman et al. (2008) screened for potential Mn exposure biomarkers using a liquid chromatography–mass spectrometry method to identify metabolomic changes in the blood and urine of monkeys exposed to airborne $MnSO_4$. Of the 27 metabolites significantly altered by Mn, three blood metabolites corresponded with Mn accumulation in the globus pallidus (GP): phenylpyruvate, disaccharides, and guanosine (Dorman et al., 2008). While these markers show promise, additional studies are needed to confirm their potential as consistent biomarkers.

The study of metabolomics is emerging as a reliable approach to identify potential biomarkers in diseased states including cancer (Kim et al., 2008) and amyotrophic lateral sclerosis (Pradat and Dib, 2009), among other potential applications (Oresic et al., 2006). Methods using liquid and gas chromatography, coupled with mass spectrometry (LC–MS, GC–MS), enable the detection of thousands of metabolites in a biological sample (Halket et al., 2005). These methods are ideal for monitoring changes in metabolite byproducts due to altered cellular metabolism in either a diseased state or after application of selected therapies. The goal of this study was to identify potential biomarkers of Mn neurotoxicity, and to link any changes in the metabolome with biological alterations associated with Mn-exposure. Additionally, we wanted to monitor any changes in behavior or locomotor activity indicative of neurotoxicity. While previous studies have examined the effects of Mn-exposure on behavior over short observational periods, to date no study has examined the effects of Mn on locomotor and circadian behaviors longitudinally over a 24 h period in a home-cage environment. A 24 h time frame allows for analysis of diurnal and nocturnal behaviors not normally captured with other behavioral tests.

2. Materials and methods

2.1. Animals

Male weanling (post-natal day 21) Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) ($n = 12$) were individually housed and randomly divided into two treatment groups: control (AIN-93G diet (35, 10, and 6 mg/kg Fe, Mn, and Cu, respectively) with deionized water) and Mn-exposed (AIN-93G diet with deionized water containing 1 g Mn (as $MnCl_2$)/L). Formulated diet was obtained from Dyets Inc. (Bethlehem, PA). This Mn-exposure protocol has been used previously in our lab to achieve consistent brain Mn accumulation producing neurochemical changes indicative of toxicity after 6 weeks of exposure (Anderson et al., 2007; 2008; Fordahl et al., 2010). Based on average water consumption for rats (10–12 mL per 100 g body weight (Harkness and Wagner, 1989)), Mn ingestion was approximately 100 mg/kg per day. Water levels were monitored to examine consumption, and no avoidance of Mn-containing water was observed. Because intestinal Mn absorption in rodents is estimated at 1–5% (Hurley and Keen, 1987), the systemic Mn burden was approximately 1–5 mg. Human exposure to waterborne Mn has been reported at >700 μg/L in children (Wasserman et al., 2006) leading to cognitive impairment, and up to 14 mg/L in 25 Japanese adults (Kawamura et al., 1941) resulting in neurotoxicity ($n = 23$) and death ($n = 2$). Although 100 mg Mn/kg is considerably higher than documented human exposure, it should be noted that Sprague Dawley rats have a higher threshold for toxicity than humans withstanding Mn doses of 200 mg/kg/day for 2 yrs and 2251 mg/kg/day for 6 months before fatality (NTP, 1993; Gianutsos and Murray, 1982). Rats had free access to food and water 24 h/day, with the lights off between 1800 and 600 h and room temperature maintained at 25 ± 1 °C. During the seventh week of the study, after an overnight fast with access to water, the rats were rendered unconscious in a CO_2 chamber, euthanized via decapitation, brains and liver tissue removed, and trunk blood was collected for analysis. Dissected tissues were immediately placed on dry ice then stored at −80 °C until analysis. For metal analysis, sections of the globus pallidus (GP) and striatum, two regions known to accumulate Mn, were removed, and the remaining brain tissue was used for metabolomic analysis. The University of North Carolina at Greensboro Animal Care and Use Committee approved all of the animal procedures.

2.2. Hematology

Trunk blood from each rat was collected in heparinized tubes and stored on ice until processed. Hematocrit was determined by centrifugation of heparinized micro-hematocrit capillary tubes (Fisher Scientific, Waltham, MA). Remaining whole blood samples were centrifuged for 15 min at 1000×*g* to separate plasma for iron (Fe) status assays, metabolomic analysis and metal quantification. Plasma was stored at −80 °C. Plasma ferritin and transferrin were determined using enzyme linked immunosorbent assay (ELISA) kits from (ICL, Inc., Newberg, OR) and (GenWay Biotech, Inc., San Diego, CA), respectively.

2.3. Behavior analysis

Behavior analysis was conducted using Clever Systems Home Cage Scan (HCS) system (Reston, VA) rather than a rating scale system, which are generally time-consuming and provide ordinal data (Flagel and Robinson, 2007). The HCS system utilizes video images from the home cage acquired at 30 frames per second. Software algorithms then categorize the images into a set of behaviors by extracting the image of the animal movements. Based on the sequential postures of the animal and position of body parts in space, behaviors are assigned using pre-trained data sets as a reference (Flagel and Robinson, 2007). Agreement between behaviors identified by the HCS and manual assessments has been found to be ≥90% (Steele et al., 2007). During weeks four, five, and six of the dietary protocol, animals were placed in individual shoebox cages with food, water, and minimal bedding. The animals were allowed to acclimate to the novel environment for a 24 h period to ensure that any behavior alterations captured were treatment effects. After the acclimation period the animals were monitored by video surveillance and their behaviors were analyzed for an additional 24 h period to capture the entire light and dark cycle. Cameras were mounted onto tripods and placed parallel to the shoebox cages. Red lighting was utilized during the dark phase to provide an appropriate background for the HCS system to analyze movement. Behaviors were scored by the HCS system and data exported to MS Excel 2007 for analysis. The following behaviors were examined: total distance traveled, repetitive turning (turning), sniffing, rearing, and grooming.

2.4. Metal analyses

Mn, Fe, and copper (Cu) concentrations were measured with graphite furnace atomic absorption spectrometry (Varian AA240,