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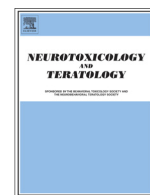

Full length article

# Developmental manganese exposure in combination with developmental stress and iron deficiency: Effects on behavior and monoamines

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ABSTRACT

Manganese (Mn) is an essential element but neurotoxic at higher exposures, however, Mn exposure seldom occurs in isolation. It often co-occurs in populations with inadequate dietary iron (Fe) and limited resources that result in stress. Subclinical FeD affects up to 15% of U.S. children and exacerbates Mn toxicity by increasing Mn bioavailability. Therefore, we investigated Mn overexposure (MnOE) in rats in combination with Fe deficiency (FeD) and developmental stress, for which we used barren cage rearing. For barren cage rearing (BAR), rats were housed in cages with a wire grid floor or standard bedding material (STD) from embryonic day (E)7 through postnatal day (P)28. For FeD, dams were fed a 90% Fe-deficient NIH-07 diet from E15 through P28. Within each litter, different offspring were treated with 100 mg/kg Mn (MnOE) or vehicle (VEH) by gavage every other day from P4-28. Behavior was assessed at two ages and consisted of: open-field, anxiety tests, acoustic startle response (ASR) with prepulse inhibition (PPI), sociability, sucrose preference, tapered beam crossing, and the Porsolt's forced swim test. MnOE had main effects of decreasing activity, ASR, social preference, and social novelty. BAR and FeD transiently modified MnOE effects. BAR groups weighed less and showed decreased anxiety in the elevated zero maze, had increased ASR and decreased PPI, and exhibited reduced sucrose preference compared with the STD groups. FeD animals also weighed less and had increased slips on the tapered beam. Most of the monoamine effects were dopaminergic and occurred in the MnOE groups. The results showed that Mn is a pervasive developmental neurotoxin, the effects of which are modulated by FeD and/or BAR cage rearing.

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## 1. Introduction

Manganese (Mn) is an essential nutrient that is neurotoxic in excess. The neurotoxic effects of Mn overexposure (MnOE) were first identified in adults following occupational exposure to high concentrations (manganism). However, Mn has neurological and cognitive effects at doses well below those causing manganism, including developmental effects in children. In children, Mn absorption is increased because gastrointestinal mechanisms restricting Mn uptake are not fully developed (Kontur and Fechter, 1988, Fechter, 1999, Yoon et al., 2011). In addition, the developing nervous system is a site of metal deposition (Suzuki, 1980, Bellinger, 2013). MnOE in children has been studied in several populations (Zhang et al., 1995, Bouchard et al., 2011, Khan et al., 2011, Khan et al., 2012, Lucchini et al., 2012, Haynes et al., 2015), and symptoms include cognitive deficits, behavioral disinhibition, decreased IQ, and decreased school performance (Zoni and Lucchini, 2013).

Oral MnOE is the most common route of exposure in children (Zoni and Lucchini, 2013). Oral exposure comes from sources such as soy-based infant formulas, polluted water and ground wells (Zoni and Lucchini, 2013). In animals, oral exposure can be modeled by adding it to drinking water, gastric infusion, or gavage (Pappas et al., 1997, Reichel et al., 2006, Beaudin et al., 2013, Vorhees et al., 2014). These models lead to increased brain Mn (Kontur and Fechter, 1988, Tran et al., 2002b, Anderson et al., 2008) and result in dopaminergic changes, including increases in dopamine (DA) and its metabolite dihydroxyphenylacetic acid (DOPAC), as well as altered DA release by in vivo microdialysis (Dorman et al., 2000, McDougall et al., 2008). Behaviorally, developmental MnOE is associated with altered locomotor activity, increased acoustic startle responses (ASR), rotorod deficits, impaired place preference, and passive avoidance deficits (Pappas et al., 1997, Dorman et al., 2000, Tran et al., 2002b, Tran et al., 2002d, Reichel et al., 2006). However, not all possible effects have been

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explored. For example, there are no data on MnOE on tests of anxiety, depression-related behavior, or sociability.

One of the perplexing findings in MnOE children is that the effects are not highly correlated with Mn exposure metrics (Bouchard et al., 2007, Khan et al., 2012). It has been hypothesized that this may be because exposure varies over time and/or because there are secondary factors that contribute to Mn sensitivity. Low iron (Fe) levels have been suggested to interact with MnOE. It is known that Mn levels in blood are increased by iron deficiency (FeD) (Rahman et al., 2013, Smith et al., 2013). However, the functional effects of such MnOE-FeD dual exposures is unknown. FeD is prevalent, affecting up to 15% of children in the United States (Lee and Okam, 2011). FeD alone has effects on brain development; it is associated with hippocampal and dopaminergic changes (Youdim, 2008). Common symptoms of developmental FeD in children are fatigue, inattention, social deficits, and learning disabilities (Lozoff, 2011).

In addition, Walker et al. reported that FeD is often found in impoverished regions and such environments are often associated with stress (Walker et al., 2007, Walker et al., 2011, Walker et al., 2012). Poverty, as defined by the United States Census Bureau in terms of socioeconomic status (SES), occurs in 11–12% of people in the United States (Center NP, 2015) and this number is even higher in other parts of the world. Low SES is associated with a number of factors, including higher rates of mental illness, anxiety, conduct disorders, and attention problems (Hackman et al., 2010, Walker et al., 2011, Reiss, 2013). There are children with MnOE living in low SES, impoverished environments (Wasserman et al., 2004, Wasserman et al., 2006, Zota et al., 2009, Haynes et al., 2011). Because FeD and low SES are often seen together with MnOE, we hypothesized that the combination of these would produce more severe adverse outcomes than each one alone.

In the current study, rats were exposed to 100 mg/kg Mn every other day from P4-28 as in a previous study (Vorhees et al., 2014). In that study, exposed offspring showed increases in blood and brain concentrations of Mn and exhibited changes in monoamine turnover in several brain regions. Based in part on those results, we combined MnOE with FeD and found behavioral deficits in the animals as adults (Amos-Kroohs et al., 2015). The FeD diet was a 90% reduction in Fe content that is not sufficient to induce anemia during development and causes no overt long-term symptoms. In order to include a factor related to low SES, we used barren cage rearing (BAR). For this, we used limited cage bedding as has been used previously (Gilles et al., 1996, Avishai-Eliner et al., 2001, Brunson et al., 2004) but we extended the duration of BAR cage exposure (Graham et al., 2011). Specifically, we put rats in BAR cages from embryonic day 7 (E7) to postnatal day (P)28, the period of major neurogenesis in rodents (Bayer et al., 1993, Clancy et al., 2007a, Clancy et al., 2007b). We assessed the effects in the offspring at two ages using several methods: Open-field locomotor activity (OF), elevated zero maze (EZM), sociability, sucrose preference, ASR/prepulse inhibition (PPI), the Porsolt forced swim test (FST; adults only), and tapered bridge crossing (adults only). Corticosterone was measured as an index of stress and hematocrit as an index of FeD at both ages. Monoamines were quantified in the hippocampus and neostriatum, regions known to be affected by one or more of the three variables.

## 2. Materials and methods

### 2.1. Animals

Male and nulliparous female Sprague Dawley CD (IGS) rats (Charles River Laboratories, Raleigh, NC; strain #001), approximately 60 days old when received, were habituated to the vivarium for at least 1 week prior to breeding. The vivarium is AAALAC International accredited, and the protocol was approved by the Institutional Animal Care and Use Committee and adhered to the NIH Guide on the Care and Use of Laboratory Animals in Research. All animals were maintained on a 14:10 h light-dark cycle (lights on at 600 h) with controlled temperature (19 ± 1 °C) and humidity (50% ± 10%) throughout the experiment. Animals were housed in a barrier facility using a Modular Animal Caging System (Alternative Design, Siloam Spring, AR). HEPA filtered air was supplied to each cage (Alternative Design, Siloam Spring, AR) with 30 air changes/h. Reverse osmosis filtered water (SE Lab Group, Napa, CA) and NIH-07 diet were provided ad libitum. A semicircular stainless steel enclosure was placed in cages for enrichment (Vorhees et al., 2008). Females were separated from sires the day a sperm plug was detected and this was designated E0. Birth (E22) was counted as P0. Twenty-four hours after birth, on P1, litters were culled to 10, five per sex, using a random number table. On P28, pups were removed from dams into same sex cages (4/cage) until P42 when they were re-housed (2/cage/sex). Personnel doing testing were blind with respect to group enrollment.

### 2.2. Experimental design

The overall design is shown in Fig. 1. The cage and diet factors were started prenatally, therefore, separate dams (and their litters) were assigned first. The first level of assignment was to either the standard or barren housing conditions on E7. On E15 each of these groups was subdivided into iron sufficient or iron deficient diet groups, creating 4 groups of gravid females. No further subdivisions were made until after parturition. On P4, after culling to 5 males and 5 females per litter, male/female pairs within each litter were assigned to one of two groups: Mn or vehicle. This created a 2-cage × 2-diet × 2-Mn × 2-sex design. A final subdivision within each litter was made for logistical reasons, i.e., since there were 2 male/female control and 3 male/female Mn-exposed pups in each litter, 1 male/female control and 1 male/female Mn-exposed were tested in one test sequence and the other set in a second test sequence (Fig. 1).

### 2.3. Barren cage housing

All gravid females were housed in standard cages (STD) from E0-7 at which time half were moved to cages without bedding and stainless steel enclosures but instead to cages that had a wire grid subfloor inserted (BAR). The other half of the females were moved to new STD cages with woodchip bedding and stainless steel enclosures to control for rehousing effects. On E21, wire subfloors were removed from BAR cages to prevent pups' legs from slipping through the spaces between wires of the grid floor. After removal of the wire floors, a 15 × 25 cm absorbent pad was placed in the cage (Anderson Lab Bedding, Maumee, OH); pads were changed daily. On P6, BAR cages had pads removed and wire grid subfloors were reinstalled. Clean cages were provided daily to all groups. BAR cages were maintained until P28 then switched to STD cages. Dams were removed from their litters on P28.

### 2.4. Iron deficient (FeD) diet

The FeD diet (Amos-Kroohs et al., 2015) was adapted from (Fitsanakis et al., 2009, Fitsanakis et al., 2011). Females were given standard NIH-07 diet until E15 then switched to purified NIH-07 diet (Land O′ Lakes Purina Feed, Evansville, IN) with half the dams given purified iron sufficient (FeS) and half the dams purified iron deficient (FeD) formulas. The FeD diet contained 35 ppm Fe and the FeS diet contained 350 ppm Fe. Offspring were returned to standard NIH-07 diet on P28. Note that 350 ppm Fe in the standard NIH-07 diet is more than sufficient for normal Fe dietary requirements for pregnancy and offspring growth and maintenance.

### 2.5. Manganese overexposure

For MnOE (Vorhees et al., 2014), a split-litter design was used in which two male and two female pups per litter were gavaged with

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