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Pre-weaning Mn exposure leads to prolonged astrocyte activation and lasting effects on the dopaminergic system in adult male rats

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Abstract

Little is known about the effects of manganese (Mn) exposure over neurodevelopment and whether these early insults result in effects lasting into adulthood. To determine if early Mn exposure produces lasting neurobehavioral and neurochemical effects, we treated neonate rats with oral Mn (0, 25, or 50 mg Mn/kg/d over PND 1–21) and evaluated 1) behavioral performance in the open arena in the absence (PND 97) and presence (PND 98) of a d-amphetamine challenge, 2) brain dopamine D1 and D2-like receptors and dopamine transporter densities in the prefrontal cortex, striatum, and nucleus accumbens (PND 107), and 3) astrocyte marker glial fibrillary acidic protein (GFAP) levels in these same brain regions (PND 24 and 107). We found that pre-weaning Mn exposure did not alter locomotor activity or behavior disinhibition in adult rats, though Mn-exposed animals did exhibit an enhanced locomotor response to d-amphetamine challenge. Pre-weaning Mn exposure led to increased D1 and D2 receptor levels in the nucleus accumbens and prefrontal cortex, respectively, compared to controls. We also found increased GFAP expression in the prefrontal cortex in Mn-exposed PND 24 weanlings, and increased GFAP levels in prefrontal cortex, medial striatum and nucleus accumbens of adult (PND 107) rats exposed to pre-weaning Mn, indicating an effect of Mn exposure on astrogliosis that persisted and/or progressed to other brain regions in adult animals. These data show that pre-weaning Mn exposure leads to lasting molecular and functional impacts in multiple brain regions of adult animals, long after brain Mn levels returned to normal.

Keywords

neonate exposure; lasting effects; dopamine; rat; astrocyte; D1; D2; DAT; GFAP

Introduction

Epidemiological studies have reported associations between childhood Mn exposure and behavioral and cognitive deficits in children, including ADHD-like deficits in executive function affecting impulse control, hyper-reactivity, behavior disinhibition, cognitive flexibility, and visual-spatial and goal-oriented behavior (Barkley, 1997; Bouchard et al., 2007a; Ericson et al., 2007; Oades et al., 2005; Takser et al., 2003; Wasserman et al., 2006; Winstanley et al., 2006; Woolf et al., 2002; Wright et al., 2006). These studies corroborate past reports dating back several decades of associations between elevated Mn exposure and learning disabilities and ADHD-like behaviors in children (Barlow, 1983; Collipp et al.,

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1983; He et al., 1994; Marlowe, 1993; Pihl and Parkes, 1977; Zhang et al., 1995). Little is known, however, about whether these reported Mn-related effects in children may be lasting and persist into adulthood.

Animal studies have reported neurobehavioral and neurochemical impacts of early-life Mn exposure, often focusing on the dopaminergic system in the basal ganglia (Brenneman et al., 1999; Carter et al., 1980; Chandra et al., 1979; Dorman et al., 2000; Kostial et al., 1978; Lai et al., 1984; Mena, 1974; Pappas et al., 1997; Seth et al., 1977). However, only a few have investigated whether early-life exposure produces neurological effects lasting into adulthood (e.g., McDougall et al., 2008; Reichel et al., 2006). The studies of Reichel et al. (2006) and McDougall et al. (2008) reported impaired acquisition of a fixed ratio 1 task and altered amphetamine- or cocaine-induced locomotion in PND 90 adults exposed to Mn over PND 1 – 21; these effects were associated with a decrease in dopamine transporter levels and a decrease in cocaine-stimulated dopamine release in the striatum of adult rats. However, it is not known whether those effects persisted/progressed from impacts present in young weanlings after Mn exposure ended, since Mn effects in young animals were not investigated.

Studies in occupationally-exposed workers have shown that chronic elevated exposure to Mn can lead to manganism, a neurodegenerative disorder affecting the basal ganglia (Huang et al., 1998; Huang et al., 1993; Kessler et al., 2003; Lucchini et al., 1999; McMillan, 1999; Mergler et al., 1994; Normandin and Hazell, 2002; Roels et al., 1987; Roels et al., 1999; Yamada et al., 1986). Several of these studies noted the importance of detecting Mn neurotoxicity at the pre-clinical stage, because once clinical neurological symptoms emerged motor and cognitive deficits tended to be irreversible or progressively worsen, even after cessation of exposure (Bouchard et al., 2007b; Huang et al., 1998; Huang et al., 1993; Roels et al., 1987; Roels et al., 1999). For example, smelting plant workers exposed to Mn exhibited symptoms of dystonic gait, poor stability, micrographia, and muscle stiffness which worsened 5 and 10 years after the cessation of exposure (Huang et al., 1993, 1998). Similarly, Roels and colleagues (Roels et al., 1987, 1999) reported that dry-alkaline battery plant workers exposed to elevated Mn showed deficits in reaction time and hand stability that remained or worsened when subjects were re-examined 8–10 years after the end of exposure. Taken together these studies suggest that elevated early-life exposure to Mn may also produce lasting and perhaps progressive neurological damage into adulthood.

Post-mortem analyses in manganism patients has revealed well-recognized neurochemical deficits of Mn exposure such as altered dopamine receptors and loss of dopaminergic cells, as well as heightened activation of immune cells of the CNS (Yamada et al., 1986). Animal model and cell culture studies also have shown that Mn exposure results in increased expression of CNS immune markers of activated microglia (OX-42) and astrocytes (GFAP), as well as increased expression of pro-inflammatory cytokines and nitric oxide (NO), suggesting that initiation and/or progression of Mn neurotoxicity involves activation of these cells in the CNS (Barhoumi et al., 2004; Chang and Liu, 1999; Filipov et al., 2005; Hazell et al., 2006; Jayakumar et al., 2004; Liu et al., 2005; Liu et al., 2006; Moreno et al., 2008; Ramesh et al., 2002; Spranger et al., 1998; Verity, 1999; Zhao et al., 2009; Zwingmann et al., 2003). Upon insult or injury to the CNS, microglial activation is rapid and leads to release of substances that subsequently activate astrocytes, which in turn can maintain a long-term pathological activation state (DeLeo et al., 2004; McMahon et al., 2005; Tanga et al., 2004). Chronic or uncontrolled release of cytokines and NO can in turn activate other immune cells, setting up a self-propagating cycle of NO release and inflammatory response that can potentially cause irreversible injury (Bronstein et al., 1995; Hunot and Hirsch, 2003; Minghetti, 2005; Minghetti et al., 2005). While it has been shown in animal models and cell culture studies that Mn exposure results specifically in astrocytic activation (Hazell, 2002;

Hazell et al., 2006; Jayakumar et al., 2004; Liu et al., 2005; Liu et al., 2006; Moreno et al., 2008; Spranger et al., 1998; Verity, 1999; Zwingmann et al., 2003), research exploring the effects of early-life Mn exposure on astrocytes and microglia is lacking.

Our previous work showed that pre-weaning oral Mn exposure over postnatal day (PND) 1 – 21 resulted in hyperactivity, disinhibition of exploratory behavior, learning deficits, and alterations in dopamine receptor and transporter density in young weanling male rats (Kern et al., 2010). In the present study, we investigated whether these early Mn effects persisted or progressed into adulthood by evaluating motor activity, behavior disinhibition, D1 and D2 dopamine receptor and dopamine transporter levels, and astrocyte GFAP expression in rats exposed to Mn over PND 1 – 21. Our results show that pre-weaning exposure to Mn levels comparable, on a relative basis, to those experienced by children consuming contaminated well-water or soy-based infant formula, produced significant lasting increases in D1 and D2 receptor levels, increased astrocyte activation, and an enhanced locomotor response to d-amphetamine challenge in adult rats. These results suggest that children exposed to elevated levels of Mn as infants may be at increased risk for neurodevelopmental deficits that may persist into adulthood.

Materials and Methods

Animals

Twenty-six primiparous pregnant Sprague-Dawley rats (gestational day 13–15) weighing 350–500g were purchased from Simonsen Laboratories (Gilroy, Ca.). At parturition, the litters were examined, sexed and weighed, and culled to adjust litter size to 10 pups composed of at least six male pups and maximum of four extra pups to complete the litter. Treatments were balanced within each litter, and only one male per litter/treatment was used in an experimental outcome. All animals were weighed daily throughout Mn exposure and at regular intervals thereafter. Animals were maintained on a reverse 12/12 hour light/dark cycle throughout the duration of the study. The animals were fed Harlan Teklad rodent chow #2018 which is reported by the manufacturer to contain 118 mg Mn/kg. Animals were given municipal tap water *ad libitum*, which contained Mn levels that were below the city's reported detection limit. All procedures related to animal care conformed to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals (NRC 1996).

Manganese Treatment

Neonate rats were orally exposed to Mn doses of 0, 25, or 50 mg Mn/kg/d over PND 1 – 21. A 150 µg Mn/mL stock solution of MnCl₂ was prepared by dissolving MnCl₂·4H₂O with Milli-Q™ water; aliquots of stock solution were diluted daily in 25% sucrose solution vehicle for oral administration to neonate pups. Oral Mn (or sucrose vehicle) was administered in a volume of ~25 µL/dose via micropipette. Control animals received only the sucrose vehicle.

These oral Mn exposure levels increased Mn intake by ~350 and 700-fold over levels consumed from lactation alone, which approximates the relative ~300 to 500-fold increases in Mn exposure experienced by infants and young children exposed to Mn-contaminated water or soy-based formulas (or both), compared to Mn ingestion from human breast milk. Human breast milk contains ~6 µg Mn/L, yielding normal infant intake rates of ~0.6 µg Mn/kg/d, based on infant daily milk consumption rates of ~0.8 L/day for a 8 kg 6–9 month old infant (Arcus-Arth et al., 2005; Dewey et al., 1991; Dorner et al., 1989; Stastny et al., 1984). By comparison rat milk Mn levels are ~200 – 300 µg Mn/L (Dorman et al., 2005; Keen et al., 1981), and pre-weaning rats consume an average of 260 mL/kg/d over PND 1–21 (Godbole et al., 1981; Yoon and Barton, 2008). Thus, pre-weanling control rats consume

~70 µg Mn/kg/d, which is ~100-times higher than normal human infant Mn intake from breast milk. Since normal daily dietary requirements for Mn are not well known for either infant humans or rats (Keen et al., 1981; Ljung and Vahter, 2007), we chose an exposure regimen that modeled the relative increase in Mn intake experienced by human infants exposed to contaminated well water or soy formulas, compared to human breast milk. For comparison, human breast milk contains an average of ~4 µg Mn/L, whereas drinking water from roughly 6% of wells monitored in the US contain over 300 µg Mn/L and can be as high as 1,500 µg Mn/L, and average Mn levels in infant formula is 400 µg Mn/L and can be as high as 1,200 µg Mn/L (Ljung and Vahter 2007; Lonnerdal 1997; Wasserman et al. 2006). These Mn exposure levels were also similar to levels used in other published studies in neonatal rats (Brenneman et al., 1999; Deskin et al., 1981a; Deskin et al., 1981b; Dorman et al., 2000; Kontur and Fechter, 1985; Tran et al., 2002a; Tran et al., 2002b).

Design and Tissue Collection

Animals were sacrificed on PND 24 for measurement of blood and brain Mn levels (males and females, n = 8 – 12 animals/gender/treatment), and brain dopamine D1 and D2-like receptors, dopamine transporter, and astrocyte activation marker glial fibrillary acidic protein (GFAP) levels by immunohistochemical staining (n= 4 – 7 males/treatment). On PND 97 and 98 animals were evaluated in the open arena (n = 15 – 20 males/treatment). Animals were sacrificed on PND 107 for measurement of blood and brain Mn levels (n=10 males/treatment), brain dopamine D1 and D2-like receptors, dopamine transporter, and GFAP levels by immunohistochemical staining (n= 4–7 males/treatment).

Animals were sacrificed via decapitation and blood and brain tissues were collected. Whole blood was collected into heparinized containers and stored at –20°C for Mn concentration determinations. The brain was bisected into hemispheres; the left hemisphere was immediately frozen on dry ice and then transferred to storage at –80°C until Mn concentration analyses. The right hemisphere was used for immunohistochemical analysis; at collection it was immediately immersed in 4% paraformaldehyde (PFA, M.W.=90.1, #150146, MP Biomedicals) and fixed overnight at 4°C, then changed to a 10% sucrose 0.1M PBS solution and fixed overnight at 4°C, and then changed to a 30% sucrose 0.1M PBS solution for two days at 4°C for cryoprotection. Right hemisphere samples were then embedded in freezing medium and stored at –70°C until further processing.

Open Arena

Spontaneous locomotor activity in the full arena and defined center and perimeter zones was assessed on PND 97 and after d-amphetamine challenge on PND 98 using activity chambers in connection with an automated video tracking system from San Diego Instruments (SMART System). For the d-amphetamine challenge, rats were isolated in holding cages and administered 1.5 mg d-amphetamine/kg body weight via i.p. injection 20 minutes before placing in the arena for SMART analysis. All animals were placed individually in 60 × 60 × 30 cm open enclosure arenas in a darkened testing room and their movement was video-tracked for 30 minutes using a digital video camera under infrared light. Individual tracks were collected and analyzed in the SMART software for distance traveled. For center and perimeter zone locomotor activity measures, individual tracks were collected and center and perimeter zones were defined in the SMART software and analyzed for time and distance spent in each zone.

Blood and Brain Mn Levels

Aliquots of 180 µL whole blood were digested overnight at room temperature with 360 µL 16N HNO₃ (Optima grade, Fisher Scientific). Digestion was complete after addition of 180 µL H₂O₂ and 1,080 µL Milli-Q™ water. After 10 minutes, digestates were centrifuged

(15,000 × g for 15 min.) and the supernatant was collected for Mn analysis. Aliquots (100 mg wet weight) of homogenized brain tissue were digested with ultrapure 16N HNO₃ (Optima grade, Fisher Scientific) and redissolved in 1N HNO₃ for analyses, as previously described (Smith et al., 1992). Manganese levels were determined using a Perkin-Elmer 4100ZL Zeeman graphite furnace atomic absorption spectroscopy, with external standardization using certified SPEX standards. National Institutes of Standards and Technology SRM1577b (bovine liver) was used to evaluate procedural accuracy. The analytical detection limit for Mn was 0.1 ng/mL.

Immunohistochemistry for Dopamine Receptors D1 and D2, Dopamine Transporter, GFAP, β -tubulin, and DAPI

For immunohistochemical analysis in PND 107 animals, the PFA-fixed right brain hemisphere was microtome sliced (Leica Microsystems, Inc. model CM30505) in preparation for antibody fluorescent staining for dopamine receptors D1 and D2, dopamine transporter, and GFAP. Frozen brains were sectioned coronally at −20°C in 20 μ m slices, mounted on superfrost/Plus slides, and stored at −20°C. Brain slices were arranged so that all three Mn treatments were represented on each slide. Overall, 72 (for prefrontal cortex) and 144 (for striatum and nucleus accumbens) 20 μ m brain slices per region per animal per treatment were mounted on slides.

For immunostaining, mounted brain slices were blocked with 4% normal goat serum (Jackson ImmunoResearch) and permeabilized with 0.1% Triton X100, washed with phosphate buffered saline (PBS), and incubated with primary antibody (DAT, SC Biotech Rabbit polyclonal IgG anti-DAT (H-80) sc-14002 1:50; D1, SC Biotech Rabbit polyclonal IgG anti-D1 (H-109) sc-14001 1:50; D2, Chemicon Rabbit polyclonal IgG anti-D2 AB5084P, 1:50; GFAP, BD Pharmingen mouse monoclonal anti-GFAP 1:50; Tau 46 SC Biotech mouse monoclonal IgG anti-tubulin (46) sc-32274 1:50) overnight at 4°C, after which tissues were washed with PBS, PBST (PBS with 0.1% Triton), and incubated with secondary antibody (Molecular Probes Alexa Fluor 488 goat anti-rabbit IgG highly cross-adsorbed A11034 1:1000; Molecular Probes Alexa Fluor 555 goat anti-mouse IgG highly cross-adsorbed A21424 1:500) for 1 hour. Next, slides were washed again with PBST and DAPI-stained for 10 minutes (Invitrogen D21490/DAPI-Fluoro-Pure Grade, 300nM working solution). Slides were then loaded with Fluoromount GTM (Southern Biotech) and coverslipped prior to analyses by confocal microscopy.

Confocal Microscopy and Image Analysis

A total of 36 (prefrontal cortex) or 72 (striatum and nucleus accumbens) immunostained brain slices per brain region per animal per treatment were analyzed for all proteins (i.e., every other brain slice was selected for analysis). Immunostained brain slices were analyzed using a Zeiss LSM 5 Pascal Laser Scanning Microscope. All images on each slide were taken at 20X magnification using the same detector gain and amplifier offset settings for fluorescent image comparison. Subsets of 18 brain slices per region per animal were qualitatively scored for protein fluorescence, and a representative brain slice per region per animal was selected for quantification. Images from 4 – 7 animals per treatment, per protein, per brain region were selected for quantification and analyzed for treatment-based comparisons of fluorescent density within each slide using Metamorph software (Molecular Devices Corporation, MetaXpress™, MetaMorph 7, multiwavelength cell scoring and count nuclei module). For these analyses, brain regions were selected using standard landmarks (e.g. lateral ventricles, corpus callosum, anterior commissure) and average gray values were calculated to quantify grayscale intensity (pixel brightness). For dopamine receptors D1 and D2 and dopamine transporter, average grayscale values were obtained by dividing the sum of the gray values by the number of pixels detected within the defined threshold for each

slide. Fluorescence density in the Mn-treated animals for each slide was compared to the average of all control animals in that brain region to determine Mn effects. For GFAP, total grayscale values were obtained by summing all of the grayscale values for all objects detected within the defined threshold for each slide.

Data Analyses

Summary data are expressed as mean $\pm$ standard error (SE). Data were analyzed to test specific hypotheses using one-way analysis of variance (ANOVA) using JMP software (Version 7.0, 2007, SAS Institute Inc.). Multivariate analysis of variance (MANOVA) was employed to evaluate treatment effects on the behavioral endpoints, incorporating possible covariates of performance (e.g., body weight, cohort, open arena activity bin #), all of which had no influence on the Mn effect. Individual treatment comparisons were done with Tukey's or Dunnett's post-hoc tests. If data were non-normal after transformation, they were analyzed in their original form using the non-parametric Kruskal-Wallis test (rank sums) with one-way Chi Square approximation. If data showed unequal variances, they were analyzed using the WELCH ANOVA test. A p-value of less than or equal to 0.05 for the various outcomes was considered statistically significant.

Results

Pre-weaning Mn exposure did not result in elevated blood or brain Mn levels in PND 107 adults

Blood and brain Mn levels in PND 107 adults exposed to Mn pre-weaning (PND 1 – 21) did not statistically differ from controls (blood: Kruskal Wallis Chi square 0.2400, DF 2, $p=0.88$; brain: $F(2,28) = 2.87$, $p=0.07$; $n= 10$ per treatment) (Table 1). Comparison with the elevated blood and brain Mn levels in PND 24 animals and only slightly elevated brain Mn levels in PND 36 animals reported in our previous study (Kern et al., 2010) suggests that brain Mn levels decreased relatively rapidly within weeks of the cessation of Mn exposure on PND 21.

Pre-weaning Mn exposure enhanced open arena activity in d-amphetamine-challenged adults, but did not alter activity or behavioral disinhibition in the absence of drug challenge

Several studies have shown that early life Mn exposure alters spontaneous motor activity in young animals (Brenneman et al., 1999; Chandra et al., 1979; Golub et al., 2005; Kern et al., 2010; Pappas et al., 1997), though few have evaluated whether early exposure causes lasting effects on motor activity in adult animals (McDougall et al., 2008; Reichel et al., 2006). Our results show that while pre-weaning Mn exposure significantly increased open arena motor activity in PND 24 animals (Figure 1a, data from Kern et al., 2010), pre-weaning Mn exposure did not affect motor activity of PND 97 adult animals ($F(2,54)=0.08$, $p=0.92$) (Figure 1b, solid bars). Similarly, there was no evidence of a Mn effect on motor activity in PND 97 animals when total time spent in the center versus perimeter zones of the open arena was considered (data not shown).

The catecholamine agonist d-amphetamine, which produces increased levels of dopamine in the synaptic cleft (Glaser et al., 2005), was used to help assess effects of early Mn exposure on dopaminergic functionality and on susceptibility to exposures of other neurotoxic drugs/agents experienced later in life. Here, adult (PND 98) animals exposed to Mn pre-weaning exhibited enhanced motor activity in response to a single d-amphetamine challenge (1.5mg/kg i.p.) compared to no-Mn-exposed controls (total distance over 5–30 minutes: $F(2,53)=3.58$, $p=0.03$) (Figure 1b, open bars). Moreover, assessment of motor activity over the 30 minute duration of testing showed that Mn-exposed animals challenged with d-

amphetamine were more active than no-Mn controls throughout the entire test period (total distance traveled in d-amphetamine-treated animals MANOVA 5–30 min. $F(2,53)=3.58$, $p=0.03$) (Figure 1c).

Pre-weaning Mn exposure did not alter center-zone activity in the open arena

—The open arena is an established paradigm for assessing an animal's reaction to stressful stimuli related to emotion or affective state (Prut and Belzung, 2003). Increased activity the center-zone of the arena indicates increased exploratory behavior. Normally, introduction of the animal to a novel environment (the arena) elicits inhibition of exploratory behavior so that proportionally more activity is concentrated in the perimeter of the arena. To determine whether pre-weaning Mn exposure altered the behavioral response to the open arena, activity tracks of each animal were analyzed to determine the total distance traveled and time spent in defined perimeter versus center zones of the open arena enclosure. Pre-weaning Mn exposure had no effect on center zone activity of drug-naïve (PND 97) or d-amphetamine challenged (PND 98) adults, based on the ratio of center distance/total distance traveled over 5 – 30 minutes ($F(2,51)=0.10$, $p=0.90$; and $F(2,54)=0.86$, $p=0.43$, respectively) (Figure 2).

Pre-weaning Mn exposure altered levels of dopamine receptors D1 and D2, but not dopamine transporter in adult rats

We previously reported that pre-weaning Mn exposure over PND 1 – 21 resulted in decreased levels of dopamine D1-like receptor and dopamine transporter in the striatum and nucleus accumbens, and increased levels of dopamine D2-like receptor in the prefrontal cortex in PND 24 weanlings (Table II, Kern et al., 2010). To determine whether pre-weaning Mn exposure led to lasting changes of these dopamine system proteins in adult animals, D1-like and D2-like dopamine receptors and dopamine transporter were measured using immunohistochemical staining in the prefrontal cortex, dorsal striatum, and nucleus accumbens in PND 107 adults. Results show that pre-weaning Mn exposure produced lasting changes in D1 and D2 protein levels in the nucleus accumbens and prefrontal cortex, respectively, compared to controls (Table II, Figure 3). Specifically, nucleus accumbens D1 receptor levels were significantly increased in the 25 mg Mn/kg/d group to ~160% of control levels ($F(2,12)=4.71$, $p=0.03$), while prefrontal cortex D2 receptor levels were significantly increased in the 50 mg Mn/kg/d group to ~800% of controls ($F(2,6)=5.35$, $p=0.01$) (Table II, Figure 3). There were no differences in dopamine transporter levels across treatment groups in the striatum or nucleus accumbens of PND 107 adults. Brain regions histochemically stained for DAPI (indicator for overt CNS cell loss) and β -tubulin (negative control for general CNS protein effects) did not show measurable differences between treatments (data not shown), indicating that Mn treatments did not cause overt cell loss or non-specific effects on protein levels in the striatum, nucleus accumbens, or prefrontal cortex.

Pre-weaning Mn exposure resulted in increased expression of the astrocyte activation marker GFAP in PND 24 and PND 107 rats

Animal model and cell culture studies have shown that Mn exposure may contribute to the initiation/progression of neuroinflammation, including activation of astrocytes (Chen et al., 2005; Hazell et al., 2006; Liu et al., 2006; Yin et al., 2008; Zhao et al., 2009), though data on the effects of developmental Mn exposure on astrocyte activation is lacking. Our results show that pre-weaning Mn exposure over PND 1 – 21 significantly increased astrocyte activation in PND 24 weanlings, and that astrocyte activation persisted or progressed into adulthood (PND 107). Specifically, prefrontal cortex GFAP levels were significantly increased in PND 24 weanlings in both the 25 and 50 mg Mn/kg/d groups to ~200% of controls ($F(2,18)=7.99$, $p=0.003$), and they remained elevated in PND 107 adults (50 mg/kg/d group) to ~260% of controls ($F(2,11)=10.54$, $p=0.003$) (Table III, Figure 4). In contrast,

GFAP levels in the lateral and medial striatum and nucleus accumbens of PND 24 weanlings did not measurably differ between Mn-exposed and control groups (lateral striatum $F(2,18) = 0.19$, $p=0.83$; medial striatum $F(2,18) = 0.0519$, $p=0.95$; nucleus accumbens $F(2,17) = 1.62$, $p=0.23$), though GFAP levels were significantly increased in the latter two brain regions in PND 107 adults (Table III, Figure 4). In the accumbens of PND 107 adults GFAP levels were increased to 210% of controls (50 mg Mn/kg/d group; $F(2,11)=3.88$, $p=0.05$). Assessment of GFAP levels in the striatum of PND 107 adults showed a clear Mn effect that was specific to sub-regions within the striatum; GFAP levels were elevated only in the medial striatum bordering the lateral ventricle of both the 25 and 50 mg Mn/kg/d groups (to ~250% and 320% of controls, respectively ($F(2,11)=24.79$, $p<0.0001$), but not different in the lateral striatum ($F(2,11)=0.49$, $p=0.63$) (Table III, Figure 4).

Discussion

We investigated whether early-life (PND 1–21) exposure to Mn produced lasting impacts on motor activity, behavior disinhibition, and expression of dopamine-related proteins and astrocyte activation (GFAP) into adulthood. Our findings show that pre-weaning Mn exposure resulted in 1) enhanced motor activity response to a d-amphetamine challenge in adult rats, 2) lasting increases in dopamine D1 and D2 receptors in the nucleus accumbens and prefrontal cortex, respectively, in adults, and 3) increased astrocyte activation in prefrontal cortex of PND 24 weanlings that progressed to include increased astrocyte activation in prefrontal cortex, medial striatum, and nucleus accumbens in adults (PND 107). These lasting impacts occurred in the absence of elevated Mn levels in adults, and they extend results from our prior study showing that pre-weaning Mn exposure caused behavioral hyperactivity and disinhibition, learning deficits, and alterations in dopamine-related proteins in young weanling rats (Kern et al., 2010). Collectively, these data demonstrate that pre-weaning exposure to environmentally relevant levels of Mn produces lasting alteration of the dopaminergic system, as well as progressive astrocyte activation into adulthood in brain regions that mediate executive function. These outcomes suggest that children exposed to elevated levels of Mn as infants may be at increased risk for neurodevelopmental deficits that may persist into adulthood.

While pre-weaning Mn exposure did not cause lasting hyperactivity and behavior disinhibition in PND 98 adults, as it did in their younger (PND 24) counterparts, adult animals did exhibit an increased motor activity response to a d-amphetamine challenge (Figure 1), consistent with the lasting changes in dopamine-related proteins in adults (Table II, Figure 3). D2 receptor levels were increased a significant ~500 – 800% of controls in the prefrontal cortex, while D1 was increased ~160% of control in the nucleus accumbens of adult rats exposed to Mn pre-weaning. We previously reported that pre-weaning Mn exposure produced significant ~30 – 50% decreases in D1 receptor and DAT protein levels in the striatum and nucleus accumbens, and significant ~400% increases in D2 receptor protein levels in the prefrontal cortex of PND 24 weanlings (Kern et al., 2010, Table II). Our present results show that those early effects of Mn on D2 receptor protein levels persisted into adulthood, while the early effect on D1 receptor levels was directionally reversed, or in the case of DAT, fully resolved (Table II). It is noteworthy that these lasting changes occurred a full 86 days after the cessation of Mn exposure when brain and blood Mn levels had long since returned to background levels (Table I).

Our results are consistent with previous studies of early postnatal insult with agents such as lead, amphetamine, or Mn that reported lasting impacts on the dopaminergic system as well as altered executive function behaviors in adults (McDougall et al., 2008; Reichel et al., 2006; Cory-Slechta et al., 1992; Decker et al., 2005; Nowak et al., 2001; Widzowski et al., 1994). Cory-Slechta et al. (1992) found that developmental lead exposure in rats led to

altered dopamine receptor binding associated with changes in D2 receptor sensitivity in an operant drug discrimination test in adult (PND 60) animals. More recently, Reichel et al. (2006) and McDougall et al. (2008) exposed rats to Mn levels up to 750 µg Mn/d over PND 1 – 21 and observed impaired acquisition of a fixed ratio 1 task, altered amphetamine- or cocaine-induced locomotion, reductions in dopamine transporter levels in the striatum and nucleus accumbens, and a decrease in cocaine-stimulated dopamine release in the striatum of PND 90 adults, ~70 days after the end of Mn exposure. It is not clear in these latter studies whether similar deficits were present in young weanlings at the end of Mn exposure, when one would expect peak blood and brain Mn levels, since young animals were not investigated. Collectively, these findings suggest that early life insults that result in lasting alterations of dopamine-related proteins, as observed here with Mn, may also produce functional and behavioral deficits in adults.

The lasting increase in dopamine D2 receptor in the prefrontal cortex in adult rats observed in this study may indicate that pre-weaning Mn exposure caused either alteration of available dopamine and/or altered expression of receptors in dopaminergic pathways during neurodevelopment. The majority of development of the dopaminergic synaptic environment in rats occurs in the first few postnatal weeks. During this time the developmental expression of dopamine D1 and D2 receptors and dopamine transporter are interdependent, though the specific timing of development is different for each (Antonopoulos et al., 2002; Broaddus and Bennett, 1990a; Broaddus and Bennett, 1990b; Tepper et al., 1998; Thomas et al., 1998). Broaddus and Bennett (1990a, b) found that D2 receptor and the dopamine transporter display delayed developmental profiles during the first postnatal month compared to D1 receptor, and that complete development of D2 receptor sites was sensitive to disruptions of dopamine terminal ingrowth. Extending these observations, the study of Archer and Fredriksson (1992) showed that blocking dopamine D3 receptor during PND 1–12 resulted in increased dopamine D2 receptor levels in the nucleus accumbens and increased response to apomorphine at PND 60, providing an example of important long-term effects stemming from altered interdependent development of the dopamine receptors. Thus, disruption of proper levels of any one of these dopamine-related proteins during development, as a consequence for example of direct or indirect effects of early Mn insult, could produce long-term impacts on the development and functionality of the dopaminergic system due to lasting alteration of receptor ratios (Archer and Fredriksson, 1992; Broaddus and Bennett, 1990a; Broaddus and Bennett, 1990b; Thomas et al., 1998).

More generally, it has been suggested that dopamine receptors function to mediate the extent of neuron terminal sprouting response after CNS insult and that dopamine D2 autoreceptors act to regulate delivery of dopamine in regeneration via dopamine storage, synthesis, and density of dopamine terminals (Parish et al., 2001). Developmental compensatory effects have also been demonstrated in dopamine D1 receptor knockout mice where a compensatory increase in dopamine D2 receptor levels accompanies impaired dopamine release (Parish et al., 2001). Studies have also shown that neuronal sprouting after nigrostriatal injury is proportional to lesion size except in dopamine D2 receptor knockouts, suggesting that dopamine D2 receptors play a regulatory role in compensatory sprouting (Cooper et al., 1996; Parish et al., 2001). In light of this, the significant increase of prefrontal cortex dopamine D2 receptor levels in our animals that persists into adulthood may be a regulatory response to altered dopamine levels and/or disruption of the development of the dopaminergic synaptic environment as a result of pre-weaning Mn exposure.

Synaptic dopamine release affects the selective strengthening or weakening of synaptic connections in the cortico-striato-thalamo-cortical loop through dopamine receptor influence on membrane ion channel permeability and intracellular signaling pathways (Arnsten, 2006; Arnsten and Goldman-Rakic, 1998; Carr et al., 1999; Hallett et al., 2006; Hernandez-Lopez

et al., 2000; Pattij et al., 2007; Russell, 2003; Snyder et al., 2000; Surmeier et al., 2007). The nature of this affect is highly variable and depends on the dopamine receptor ratios present in the synaptic environment (Pattij et al. 2007; Surmeier et al., 2007; Thomas et al., 1998). Proper dopamine receptor ratios are essential for normal stimulus-response associations of learning and memory and behavior inhibition (Girault and Greengard, 2004; Hurley and Jenner, 2006). Consistent with this, McDougall et al. (2008) showed that pre-weaning Mn exposure (750 µg Mn/d over PND1–21) resulted in a reduction of striatal dopamine transporter protein levels and a reduction in cocaine-stimulated striatal dopamine release in PND 90 adult rats, and that these changes were associated with deficits in procedural learning in a fixed ratio task. Collectively, these observations support results presented here suggesting that early Mn exposure may alter synaptic dopamine release and/or dopamine receptor ratios, which may then contribute to lasting functional deficits.

Astrocyte response

To determine if our observed effects of pre-weaning Mn exposure on dopamine-related proteins were associated with broader evidence of CNS insult, we investigated GFAP protein expression levels as evidence of astrocyte activation in young weanling (PND 24) and adult (PND 107) rats. Notably, we observed that pre-weaning Mn exposure produced a significant ~200% of control increase in prefrontal cortex GFAP expression in PND 24 weanlings, which progressed to ~200 – 300% of controls increased GFAP expression in prefrontal cortex, striatum (medial region), and nucleus accumbens in PND 107 adults (Table III). These results are consistent with building evidence from cell culture and adult animal model studies showing that Mn exposure promotes glial activation and increased release of pro-inflammatory cytokines, which can damage nearby neurons (Barhoumi et al., 2004; Chang and Liu, 1999; Filipov et al., 2005; Hazell, 2002; Ramesh et al., 2002; Spranger et al., 1998; Jayakumar et al., 2004; Liu et al., 2005; Liu et al., 2006; Moreno et al., 2008; Verity, 1999; Zhao et al., 2009; Zwingmann et al., 2003).

Astrocytes play a significant supportive and protective role in the CNS by releasing neurotrophic compounds that mediate proper neuronal development, and by nurturing and maintaining normal synaptic environments (Aldskogius and Kozlova, 1998; Bronstein et al., 1995; Heales, 2004). Astrocytes have also been implicated in chronic neurodegenerative diseases such as Alzheimer's disease, prion disease, and Parkinson's disease (PD) (Campbell, 2004; Heales et al., 1999; Heales, 2004; McGeer and McGeer, 2002; Perry, 2004). Microglia and astrocytes become immunoreactive following CNS insult; when activated, astrocytes proliferate, increase in size, increase cytoplasmic organelles, and release inflammatory mediators such as nitric oxide, which is potentially toxic to neurons (Giulian et al., 1993; Aldskogius and Kozlova, 1998; Heales et al., 1999; Heales, 2004; Perry, 2004). In the present study, the early astrocytic response in Mn-exposed PND 24 weanlings may reflect heightened neurotrophic activity in response to Mn-induced alteration of synaptic development, but the significant persistent GFAP expression in the prefrontal cortex and progressive GFAP expression that emerged in the striatum and nucleus accumbens in adults indicates chronic astrocyte activation, which may contribute to a neurotoxic synaptic environment.

Although the astrocytic response in the prefrontal cortex is associated with a significant increase in dopamine D2 receptor levels in both young and adult animals exposed to Mn as pre-weanlings, it is unclear if these effects are related. For the striatum and the nucleus accumbens we observed a significant decrease in both dopamine D1 receptor and dopamine transporter levels in PND 24 weanlings that resolved by adulthood, whereas we observed no increase in GFAP expression in these brain areas at that early age, yet found increased GFAP expression in adults. Upregulation of GFAP in young Mn-exposed weanlings, who

possessed elevated brain and blood Mn levels, possibly indicates astrocytic activation in response to Mn insult. However, it is unclear why this early increase in GFAP expression persisted in the prefrontal cortex but latently emerged in the striatum and nucleus accumbens in adult animals well after the end of Mn exposure. Nonetheless, our results indicating that early-life Mn exposure increases glial activation in young animals that progresses in adult animals to involve multiple other brain regions important in executive function behaviors is further evidence suggesting that early life exposure to Mn may have lasting impacts on CNS health long after brain Mn levels have returned to normal.

In summary, adult rats exposed to Mn pre-weaning showed an enhanced locomotor response to d-amphetamine challenge, and significantly increased D1 and D2 receptor levels in the nucleus accumbens and prefrontal cortex, respectively. Pre-weaning Mn exposure also led to increased astrocyte activation in the prefrontal cortex of PND 24 weanlings, which progressed to include increased activation in prefrontal cortex, striatum, and nucleus accumbens in PND 107 adults, indicating lasting astrogliosis. Notably, these lasting impacts occurred in the absence of elevated Mn levels in adults. These data, along with results from previous animal studies, show that neonatal Mn exposure causes lasting alteration of the dopaminergic system, as well as progressive astrocyte activation into adulthood in brain regions that mediate executive function behaviors (Brenneman et al., 1999; Chandra et al., 1979; Dorman et al., 2000; Kern et al., 2010; McDougall et al., 2008; Pappas et al., 1997; Reichel et al., 2006; Tran et al., 2002b). Together, these results suggest that children exposed to elevated levels of Mn as infants may be at increased risk for neurodevelopmental deficits that may persist into adulthood.

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Figure 1.

Pre-weaning Mn exposure increased open arena locomotor activity of PND 24 weanlings (panel 'a', ANOVA $p=0.01$, data from Kern et al., 2010), but not drug-naïve PND 97 adult male rats (panel 'b', solid bars, $p=0.92$). However, pre-weaning Mn exposure significantly increased the locomotor response to a d-amphetamine challenge (1.5 mg/kg) in PND 98 adults (panel 'b', open bars, $p=0.03$) (data are averages ($\pm$ SE) of total distance traveled over 5–30 min). Superscripts denote significant differences between treatments based on Tukey's post-hoc analysis ($p\leq 0.05$). Panel 'c': Locomotor activity measured per minute over the entire 30 min test period for drug-naïve (no d-amphetamine, PND 97) and d-amphetamine-challenged (PND 98) adults; data show consistent increased activity over the entire test period after the d-amphetamine challenge (MANOVA $p=0.03$). Data are averages (error bars in 'c' omitted for clarity). $N=15$ – 20 rats per treatment. Activity was measured in $60\text{ cm} \times 60\text{ cm} \times 30\text{ cm}$ open enclosures using the SMART video tracking system (San Diego Instruments).

Figure 2.

Pre-weaning Mn exposure did not alter center zone activity of drug-naïve (PND 97, solid bars, $p=0.90$) or 1.5 mg/kg d-amphetamine-challenged (PND 98, open bars, $p=0.43$) adult male rats, based on the ratio of center distance/total distance traveled over 5 – 30 min. Values are averages ($\pm$ SE), $n=15$ – 20 rats per treatment. Activity was measured in 60 cm $\times$ 60 cm $\times$ 30 cm open enclosures using the SMART video tracking system (San Diego Instruments).

Figure 3.

Representative immunohistochemistry photomicrographs showing that pre-weaning Mn exposure increased levels of D1 and D2 receptor proteins compared to controls in the nucleus accumbens (25 mg Mn/kg/d group) and prefrontal cortex (50 mg Mn/kg/d group) of PND 107 adult male rats (images reflect data summarized in Table II). Slides were prepared and stained with three animals/slide balanced by treatment and photographed at 20X magnification under defined illumination conditions (see text for details). Scale bar = 100 μ m.

Figure 4.

Representative immunohistochemistry (IHC) photomicrographs showing that pre-weaning Mn exposure increased levels of the astrocyte marker GFAP in prefrontal cortex of PND 24 rats, and the prefrontal cortex, medial striatum, and nucleus accumbens of PND 107 male rats. Panel 'a': GFAP expression in the prefrontal cortex of a representative 50 mg/kg/d Mn-exposed animal compared to control (40X magnification). Panel 'b': Diagram to indicate different striatal areas imaged for data shown in panel 'c' (Paxinos and Watson, 1998). Panel 'c': IHC fluorographs of data summarized in Table III (20X magnification). IHC slides were prepared and stained with three animals/slide balanced by treatment and photographed under defined illumination conditions (see text for details). Scale bar = 100 μ m.

Table 1

Pre-weaning Mn exposure increased Mn levels in blood and brain in PND 24 rats, but by PND 107 Mn levels were not different from controls.

Mn		Dose→		
		Control	25 mg/kg	50 mg/kg
PND 24	Blood Mn	45 ± 9	135 ± 6**	209 ± 11**
	Brain Mn	0.43 ± 0.01	0.84 ± 0.05**	1.16 ± 0.10**
PND 107	Blood Mn	11 ± 1.6	11 ± 1.7	11 ± 2.0
	Brain Mn	0.35 ± 0.02	0.33 ± 0.01	0.31 ± 0.01

Mn concentrations in blood (ng/mL) and brain (µg/g dry wt.) of PND 24 and PND 107 male rats. Data in PND 24 rats from Kern et al. (2010) and are included for comparison. Values are mean (± SE, n=10 rats/treatment). Significant differences between Mn-treated and control group indicated by ** (p≤0.01), based on Dunnett's test.

Pre-weaning Mn exposure altered dopamine receptor and transporter protein levels in PND 24 and PND 107 male rats.

Table II

Brain Region	Mn dose→	PND 24		PND 107				
		Control	25 mg/kg	50 mg/kg	Control	25 mg/kg	50 mg/kg	
D1	Prefrontal cortex		Weak staining		Weak staining			
	Striatum	100 ± 5	71 ± 7 *	54 ± 9 **	100 ± 11	136 ± 15	114 ± 13	
	Accumbens	100 ± 6	83 ± 14	62 ± 9 *	100 ± 10	158 ± 19 *	128 ± 8	
	Prefrontal cortex		100 ± 28	200 ± 37	429 ± 37 **	100 ± 33	490 ± 220	820 ± 150 **
	Striatum	100 ± 11	123 ± 14	103 ± 12	100 ± 14	120 ± 13	106 ± 13	
	Accumbens	100 ± 20	134 ± 18	103 ± 20	100 ± 17	113 ± 8	103 ± 9	
DAT	Prefrontal cortex		Weak staining		Weak staining			
	Striatum	100 ± 7	95 ± 7	67 ± 11 *	100 ± 18	96 ± 13	103 ± 9	
	Accumbens	100 ± 6	85 ± 9	61 ± 5 **	100 ± 16	106 ± 17	103 ± 11	

Levels of D1-like and D2-like dopamine receptors and dopamine transporter (DAT) in the prefrontal cortex, dorsal striatum, and nucleus accumbens of Mn-exposed male rats, expressed as percent of control group animals (±SE, n= 4-7/treatment). Data in PND 24 rats are from Kern et al. (2010) and are included for comparison. Protein levels were determined using fluorescence immunohistochemical staining and a Zeiss LSM 5 Pascal Laser Scanning Microscope, and quantified using Metamorph™ software (see text for details). Asterisks indicate significantly different from respective control group(* p≤0.05, ** p ≤ 0.01, based on Dunnett's comparison with control versus each Mn group). Striatal β-tubulin was immunostained as a negative control and showed no measurable change with Mn treatment (data not shown).

Pre-weaning Mn exposure altered levels of the astrocyte activation marker GFAP in PND 24 and PND 107 male rats.

Table III

Brain Region	Mn dose→	PND 24		PND 107	
		Control	25 mg/kg	Control	25 mg/kg
Prefrontal cortex		100 ± 15	198 ± 24**	100 ± 15	159 ± 24
			204 ± 22**		264 ± 36**
Striatum - lateral		100 ± 13	113 ± 19	100 ± 17	108 ± 11
Striatum - medial		100 ± 18	94 ± 16	100 ± 21	246 ± 15***
Accumbens		100 ± 20	126 ± 19	100 ± 21	132 ± 15
					210 ± 47*

Levels of GFAP in the prefrontal cortex, lateral and medial striatum, and nucleus accumbens of Mn-exposed male rats, expressed as percent of control group animals (±SE, n= 4–7/treatment). GFAP protein levels were determined using fluorescence immunohistochemical staining and a Zeiss LSM 5 Pascal Laser Scanning Microscope, and quantified using Metamorph™ software (see text for details). Asterisks indicate significantly different from respective control group (* p≤0.05, ** p ≤ 0.01, based on Dunnett's comparison with control versus each Mn group). Striatal β-tubulin was immunostained as a negative control and showed no measurable change with Mn treatment (data not shown).