Age-dependent susceptibility to manganese-induced neurological dysfunction

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Short title:
Neurological effects of juvenile Mn exposure

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ABSTRACT

Chronic exposure to manganese (Mn) produces a spectrum of cognitive and behavioral deficits associated with a neurodegenerative disorder resembling Parkinson’s disease. The effects of high-dose exposure to Mn in occupational cohorts and in adult rodent models of the disease are well described but much less is known about the behavioral and neurochemical effects of Mn in the developing brain. We therefore exposed C57Bl/6 mice to Mn by intragastric gavage as juveniles, adults, or both, postulating that mice exposed as juveniles and then again as adults would exhibit greater neurological and neurochemical dysfunction than mice not pre-exposed as juveniles. Age- and sex-dependent vulnerability to changes in locomotor function was detected, with juvenile male mice displaying the greatest sensitivity, characterized by a selective increase in novelty seeking and hyperactive behaviors. Adult male mice pre-exposed as juveniles had a decrease in total movement and novelty seeking behavior and no behavioral changes were detected in female mice. Striatal dopamine levels were increased in juvenile mice but were decreased in adult pre-exposed as juveniles. Levels of Mn, Fe, and Cu were determined by ICP-MS, with the greatest accumulation of Mn detected in juvenile mice in the striatum, substantia nigra and cortex. Only modest changes in Fe and Cu were detected in Mn-treated mice, primarily in the substantia nigra. These results reveal that developing mice are more sensitive to Mn than adult animals and that Mn exposure during development enhances behavioral and neurochemical dysfunction relative to adult animals without juvenile exposure.
KEYWORDS: manganese, development, behavior, neurochemistry, metals

INTRODUCTION

Manganese (Mn) is an essential nutrient with a recommended daily intake of 2-5 mg/day for adults and 1-3 mg/day for children (NRC 1989). Mn is necessary for homeostasis in the central nervous system (CNS) and is involved in the metabolism of proteins, carbohydrates, and lipids (Keen 1984) through its role as a cofactor for multiple enzymes including mitochondrial superoxide dismutase (Hearn et al., 2003) and glutamine synthetase (Takeda 2003). Routes of exposure to Mn include inhalation in occupational settings such as steel manufacturing, welding, and mining (Bader et al., 1999; Bowler et al., 2006; Josephs et al., 2005) and dietary intake through drinking water and soy-based infant formula (Krachler et al., 2000; Wasserman et al., 2006; Woolf et al., 2002), all of which lead to Mn accumulation in the brain.

Excessive exposure can cause Mn neurotoxicity, or manganism, a neurodegenerative condition affecting cortical and basal ganglia structures, specifically the globus pallidus (Gp), striatum (St), and substantia nigra pars reticulata (SNpr) (Yamada et al., 1986). Early neuropsychological symptoms of the disorder include aggressiveness, anxiety, and decreased cognitive function (Mergler et al., 1994). These symptoms generally precede changes in motor function observed later in the progression of the disease, which are characterized by deficits that include dystonia, bradykinesia, rigidity, masked facial expression, and difficulty in walking backwards (Wolters et al., 1989). Some of these clinical features are shared with Parkinson’s disease (PD); however,
dystonia, lack of resting tremor, and gait disturbances termed “cock walk” are more characteristic of manganism (Cersosimo and Koller 2006). A variety of locomotor tests have been utilized to assess behavioral abnormalities in models of Mn neurotoxicity. Previous studies in aged mice exposed subcutaneously to Mn observed a decrease in horizontal movement (Dodd et al., 2005), whereas primates exposed to MnSO₄ intravenously displayed gait dysfunction, rigidity, bradykinesia, and facial grimacing (Schneider et al., 2006) indicating extrapyramidal motor system dysfunction.

Mn neurotoxicity causes adverse motor symptoms similar to PD that are primarily attributed to loss of dopamine in the striatum and decreased GABAergic output from the internal Gp but, unlike PD, dopaminergic neurons are largely spared (Perl and Olanow 2007). Mn-induced decreases in striatal dopamine levels have been reported in rats (Dorman et al., 2000; Hirata et al., 2001), mice (Liu et al., 2006), rabbits (Mustafa and Chandra 1971), and in non-human primates (Bird et al., 1984; Eriksson et al., 1987; Guilarte et al., 2008a; Neff et al., 1969).

Serotonergic neurotransmission is also a suspected target of Mn in the basal ganglia, although less data are available reporting effects on this system. Serotonin (5-HT) is a monoamine neurotransmitter that is involved in maintaining emotional stability in the CNS; however, modulation of the serotonergic neurons leads to lack of emotional stability resulting in anger, depression, sleeplessness and loss of memory, (Lesch et al., 1996) all early symptoms of manganism. 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) and was reported to be decreased in the Gp of primates exposed to aerosolized MnSO₄ (Struve et al., 2007). Additionally, a decrease in 5-HT was identified in rats exposed to high dietary Mn (Kimura et al., 1978). These studies suggest that
serotonergic pathways are affected during Mn neurotoxicity but the data from these adult models are difficult to extrapolate to developmental exposures.

Studies of Mn neurotoxicity in adult animals have examined metal accumulation in the brain, behavioral changes, and alterations in neurotransmitter levels but it has not been previously investigated whether exposure early in life alters susceptibility to Mn during aging. We therefore exposed C57Bl/6 mice to Mn by intragastric gavage as juveniles, adults, or as juveniles and again as adults and examined metal accumulation in multiple brain regions and serum, as well as catecholamine and monoamine neurotransmitter levels and neurobehavioral parameters. The results indicate that developing mice are more sensitive than adult animals to Mn-induced changes in behavior and neurochemistry within the basal ganglia. Moreover, adult mice previously exposed to Mn as juveniles displayed a greater decrease in overall locomotor function and striatal catecholamines than naïve adults exposed to Mn. This study demonstrates both that developing animals are highly susceptible to changes in behavior and striatal neurochemistry and effects of exposure persist during aging that render these brain regions more vulnerable to neurotoxic insult later in life. Consistent with this hypothesis, we also identified distinct patterns of glial activation and neuroinflammation that were strongly potentiated in adult animals pre-exposed as juveniles, discussed in detail in the companion article in this issue.
Materials and Methods

Reagents. All chemical reagents were obtained from Sigma Chemical Co (St. Louis, MO) unless otherwise stated. C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Animal exposure model. Male and female C57Bl/6 mice were housed in microisolator cages (five animals per cage) and kept on 12-hr light/dark cycles with access to laboratory chow and water ad libitum. Littermates from timed pregnant dams were paired in control and Mn-exposed groups and received 0.9% normal saline, 10 or 30 mg/Kg MnCl$_2$ by intragastric gavage daily during the following time periods: juvenile exposure, day 20-34 postnatal; adult exposure, from week 12-20; and juvenile + adult exposure day 20-34 postnatal and week 12-20 (Supplementary Figure 1). Animals were weighed prior to each gavage and the amount of Mn delivered was adjusted accordingly. The amount of Mn delivered was adjusted for the molar concentration in the tetrahydrate form (MnCl$_2$•4H$_2$O) to achieve a precise dose of 10 or 30 mg/Kg. Previous studies in our laboratory using this model administered 100 mg/Kg Mn via intragastric gavage to adult female mice (Liu et al., 2006) for eight weeks but pilot studies in juvenile mice indicated a greater level of sensitivity to the neurological effects of Mn. We therefore revised the dosing paradigm downward to better test the hypothesis that low-dose juvenile exposure would potentiate later adult neurotoxicity upon subsequent exposure. All procedures were approved by the Institutional Animal care and Use Committee at Colorado State University and were performed under the supervision of veterinarians at the Laboratory Animal Resource facility.
Determination of catecholamines and monoamine neurotransmitters. Brain samples were snap frozen in liquid nitrogen the day following the last day of saline or Mn gavage for each separate group (see Supplemental Figure 1 for treatment schematic) and stored at -80°C until sample preparation. The samples were removed and kept on ice, weighed, and 300 µL of 0.2 M perchloric acid (PCA) containing 0.5 mg/mL of deoxyephinephrine (EPN) was applied per 10 mg of wet weight brain. Tissue samples were then disrupted by sonication and placed directly onto ice. 20 µL of sample was utilized for total protein concentration via BCA protein assay. The tissue sample was then centrifuged at 14,000 x g for 10 minutes at 4°C. Aliquots of supernatant were loaded into glass HPLC vials and analyzed within eight hours of sonication. Standards for all 5 compounds were processed in the same manner as the samples and 1.0 mg/mL, 0.6 mg/mL, 0.3 mg/mL and 0.1 mg/mL concentrations of standards concluded the runs for each day. The internal standard EPN was included in all runs in order to account for minute changes in detector sensitivity. The areas from the peaks in the samples were compared to those observed in the standards and values expressed as ng of neurotransmitter per mg of total protein.

Levels of dopamine, 2-(3,4-dihydroxyphenyl)acetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) were determined by high performance liquid chromatography (HPLC) with electrochemical detection as described in previous studies from our laboratory (Liu et al., 2006), adapted from (Champney et al., 1992). Briefly, mobile phase consisted of 7% methanol, 0.946 g Na₂HPO₄, 2.8 g of citric acid, 18.6 mg of EDTA and 20mg of sodium octyl sulfate in 930 mL of Milli-Q water maintained at a pH of 4.6 and ran isocratically at a flow rate of 1.0
mL/min. An LED-6A electrochemical detector working electrode was set at +0.67 V, recorder output set at 8 nA, response output at STD or 0.5 seconds, with negative polarity for the output and polarity of electrode set at reduction. Experiments were performed through a Microsorb-MV C-18 reverse phase column with a pore size of 100Å, 5 mm size and 25 cm in length (Varian; Walnut Creek, CA). Striatal tissues were processed from 3-4 separate animals from all treatment groups and cerebral hemispheres were measured independently.

Neurobehavioral analysis. Open field activity parameters were determined using Versamax behavior chambers with an infrared beam grid detection array to assess animals movements in X, Y, and Z planes. Activity was measured in the juvenile exposure group every other day for two weeks and for the adult exposure group once a week for two months during the period of oral gavage. Activity parameters were binned every minute for a total of ten minutes and then analyzed using Versadat software (v. 4.00-127E, Accuscan Instruments, Inc., Columbus, OH). Analysis of juvenile animals compared 11-18 animals within each female and male treatment group, whereas 8-10 animals were assessed in adult treatment groups.

Levels of Mn, Cu and Fe in brain tissue. Brain regions were dissected following the last saline or Mn gavage for each separate group (see Supplemental Figure 1 for treatment schematic) based upon stereologic coordinates using 1 mm brain blocks and stored at -80°C until sample preparation. Brain samples were weighed and transferred to acid-cleaned polypropylene tubes, 100 µL of Nitric Acid was applied to each sample and cooked for 2 hours at 95°C to digest the tissue. Lipids were then digested with 50 µL of 30% Hydrogen Peroxide for 15 min at 80°C followed by 50 µL of hydrochloric acid.
incubation to complex the metals for 15 min at 80°C. Samples were then brought to a final volume of 1 mL using Milli-Q water. Analysis of the samples was performed by inductively-coupled plasma mass spectrometry (ICP-MS) on a Perkin-Elmer Elan DRC-II instrument. Standards and blank tubes underwent the same digestion procedures as the samples. The concentration of each metal analyzed in brain samples is expressed as ppm for each metal, based on wet weight of tissue. Brain tissue samples were processed from 3 separate animals from all treatment and exposure groups.

**Statistical Analysis.** Comparison of two means was performed by Student’s t-test. Comparisons three or more means was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison post-hoc test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). Alpha level for all statistical analyses was set at \( p < 0.05 \).
RESULTS

*Mn-induced locomotor changes.* Locomotor function was determined by open field activity measures to identify patterns of neurobehavioral dysfunction in developing and adult mice exposed to Mn. Time spent in the margin was decreased in juvenile males at both 10 and 30 mg/Kg of MnCl₂, whereas juvenile females displayed no change in margin time (Figure 1A-B). In contrast, mice exposed only as adults displayed no change in margin time but male mice exposed to 10 and 30 mg/Kg MnCl₂ as juveniles and again as adults had a significant increase in time spent in the margin (Figure 1E-F). Adult female mice displayed no change in margin time in any exposure group. The total number of movements was unchanged in female mice pre-exposed as juveniles (Figure 2E) but was increased in male mice exposed to 30 mg/Kg MnCl₂ (Figure 2F). The total number of movements for mice exposed only as juveniles (Figure 2A-B) and only as adults (Figure 2C-D) was unchanged at either dose of Mn. Total distance traveled and rearing movement were also assessed but no significant change was detected in any groups observed (Supplemental Figures 2 and 3).

*Striatal neurotransmitter levels.* Levels of striatal catecholamines and monoamines were determined in each exposure group by HPLC using electrochemical detection. Juvenile mice exposed to 30 mg/Kg MnCl₂ had a significant increase in striatal dopamine compared to both the control and 10 mg/Kg MnCl₂ groups (Figure 3A). In mice exposed to Mn only as adults, dopamine was decreased in the 30 mg/Kg MnCl₂ group (Figure 3B) but was decreased in both the 10 and 30 mg/Kg MnCl₂ treatment groups in adult mice pre-exposed as juveniles (Figure 3C). The dopamine metabolite, DOPAC, was decreased
at 30 mg/Kg MnCl₂ in all three exposure groups but in adults pre-exposed to Mn as juveniles DOPAC levels were also decreased at 10 mg/Kg MnCl₂ (Figure 4A-C). The ratio of DOPAC to dopamine (DA), a general indicator of dopamine turnover, was increased in juvenile mice at 30 mg/Kg MnCl₂ (Figure 5A) but was unchanged in any dose group in mice receiving Mn only as adults. The DOPAC/DA ratio was not different from controls in adult mice pre-exposed as juveniles, although an increasing trend was noted (Figure 5C; p = 0.08). No significant change in 5-HT was identified in any of the animals exposed to Mn (Figure 6A-C) and only the juvenile exposure group had a significant increase in the serotonin metabolite, 5-HIAA, in the 30 mg/Kg dose group (Figure 7A). No change was detected in 5-HIAA in other exposure groups (Figure 7B-C).

**Determination of tissue levels of Mn, Fe, and Cu in various brain tissues.** Levels of Mn, Fe, and Cu were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) in multiple brain regions and serum to determine whether differences in uptake of Mn or perturbations in other transition metals associated with the alterations in behavioral and neurochemical parameters in Mn-exposed mice. In juvenile mice, there was an increase in Mn accumulation in the striatum (St) and substantia nigra (SN) at both 10 and 30 mg/Kg MnCl₂ (Table 1). There was also an increase in cortical Mn levels in the 30 mg/Kg dose group. Mice exposed only as adults had an increase in Mn in the St at 10 mg/Kg MnCl₂ and a trend towards increase at 30 mg/Kg in the St and SN (Table 1). Levels of Fe and Cu were also determined in the same brain regions and serum. A significant increase of Fe was observed in the SN of juvenile mice exposed to 30 mg/Kg MnCl₂ and in cortex at both 10 and 30 mg/Kg MnCl₂ (Table 2). Mice exposed to 30
mg/Kg MnCl₂ as adults had an increase of Fe only in the hypothalamus (Table 2). Although Cu levels were largely unaffected, some accumulation was detected in the SN of juvenile mice exposed to 10 and 30 mg/Kg MnCl₂ (Table 3). Interestingly, no change in serum Mn, Fe, or Cu was detected in any exposure group at the relatively low doses of Mn used in this study.

DISCUSSION

Manganism is primarily described as a disorder of the basal ganglia, with Mn accumulating preferentially in the St, Gp and SNpr (Liu et al., 2006; Morello et al., 2008), although cortical structures are affected as well (Guilarte et al., 2008b). To better understand the phenotype of the disorder and the comparative sensitivity of the developing and adult brain to neurobehavioral and neurochemical deficits, mice were exposed to Mn both during development and adulthood and assessed for multiple indices of neurological dysfunction. The purpose of this treatment paradigm was to address a poorly studied aspect of Mn neurotoxicity, namely, how developmental exposure impacts neurological function during aging and whether neurotoxic exposures early in life can potentiate later susceptibility to disease.

Locomotor activity was notably decreased in male adult mice pre-exposed as juveniles (Figure 2F), whereas no significant changes were observed in the absence of juvenile exposure (Figure 2D), suggesting that this period of postnatal development may represent a window of sensitivity to Mn exposure. The age at which juvenile mice were exposed to Mn corresponds approximately to a pre-pubertal age in humans (Flurkey K...
2007). Female mice appeared refractory to the relatively low doses of Mn used in these studies, an interesting finding consistent with the known neuroprotective effects of estrogen (D’Astous et al., 2004; Tripanichkul et al., 2007). Decreased locomotor activity has also been described in Mn-exposed workers, non-human primate and rodent models of Mn neurotoxicity, (Wolters et al., 1989; Dodd et al., 2005), suggesting that low-dose exposure to Mn during critical developmental periods may potentiate susceptibility to Mn and possibly other environmental toxicants later in life.

Thigmotaxis, or margin time, was altered in mice exposed to Mn. This tendency to stay near the margin of the chamber is typically associated with either anxiety or a lack of novelty seeking in an animal model of PD (George et al., 2008). Male mice exposed only as juveniles to either 10 or 30 mg/Kg MnCl₂ displayed a decrease in margin time compared to controls (Figure 1B), a sign of increase in novelty seeking or hyperactivity behavior in juvenile male mice (Helms et al., 2008), a symptom reported in children exposed to excessive levels of Mn through drinking water (Wasserman et al., 2006; Woolf et al., 2002). Although the present data support a correlation between Mn and hyperactivity, additional behavioral studies in animal models, as well as larger clinical epidemiology studies, will be required to better understand the mechanisms underlying observed increases in hyperactivity in the developing and adult brain. To our knowledge this is the first report of locomotor effects from exposure to Mn between weaning and early adulthood.

The impact of early Mn exposure on striatal neurotransmitters was examined to better understand the basis for the observed neurobehavioral alterations in juvenile and adult mice. Striatal dopamine levels were decreased in mice exposed only as adults and in
adults pre-exposed as juveniles but dopamine levels were decreased to a greater degree in
the latter group and at a lower dose of Mn (10 mg/Kg MnCl₂), whereas dopamine was
only decreased in naïve adult mice at 30 mg/Kg MnCl₂ (Figure 3B,C). These data
demonstrate that developmental exposure to Mn increases vulnerability of the striatal
system to dopamine loss during aging. Mice exposed to 30 mg/Kg Mn as adults had a
significant decrease in DOPAC, the dopamine metabolite, and similar to dopamine, the
mice exposed as juveniles and adults had a decrease in DOPAC at both doses of Mn,
further supporting that developmental exposure to Mn enhances susceptibility to
alterations in striatal neurochemistry during aging. Loss of striatal dopamine due to Mn
exposure in the adult brain is well established in other Mn toxicity models and is a
pathological indicator of basal ganglia dysfunction (Liu et al., 2006).

Juvenile mice exposed to Mn showed an opposite effect from adult mice with
respect to the striatal dopamine levels. A significant increase in striatal dopamine was
detected at 30 mg/Kg MnCl₂ (Figure 3A) and levels of DOPAC were decreased (Figure
4A). The DOPAC/dopamine ratio was also decreased in juvenile mice exposed to 30
mg/Kg MnCl₂ but no significant changes were detected in either adult exposure groups
(Figure 5), suggesting a greater sensitivity of dopamine catabolism in the developing
basal ganglia. Previous studies in neonatal mice exposed to Mn by oral gavage reported a
similar increase in striatal dopamine but noted an increase in DOPAC (Cotzias et al.,
1976; Dorman et al., 2000). The decreased ratio of DOPAC to dopamine in juvenile
mice in the studies reported here (Figure 5) may represent a decrease in overall dopamine
turnover, most notably in adults sensitized by prior juvenile exposure.
Previous studies of Mn toxicity reported a decrease in the serotonin metabolite 5-HIAA in the pallidum of monkeys exposed to Mn (Struve et al., 2007; Golub et al., 2005), indicating that Mn affects serotonergic, as well as dopaminergic, pathways. In the studies reported here, juvenile mice exposed to 30 mg/Kg of Mn were the only group that had a significant decrease in 5-HIAA (Figure 7A). The finding of low levels of 5-HIAA in cerebral spinal fluid (CSF) is a phenomenon found in children who suffer with attention deficit disorder (Miczek et al., 2002), which is consistent with the observed hyperactivity and loss of 5-HIAA in juvenile mice exposed to Mn in the present studies.

Accumulation of Mn was most prominent in juvenile mice, occurring in the cortex, St and SN (Table 1), and in adult mice to a lesser degree in the St. Levels of Fe and Cu were essentially unchanged in adult animals but increased somewhat in the SN of juvenile mice exposed to 30 mg/Kg MnCl\(_2\) (Tables 2 and 3), suggesting both that this brain region is particularly vulnerable in developing animals and that Mn accumulation seems to alter homeostatic regulation of other transition metals known to associate with oxidative damage (Uversky et al., 2001). These findings are consistent with previous studies in non-human primates exposed to Mn intravenously and by inhalation, and with rodent models using exposure via ingestion, that reported increased Mn levels in various brain regions (Dorman et al., 2000; Dorman et al., 2006a; Guilarte et al., 2006; Struve et al., 2007). Interestingly, it appeared that Mn levels in several brain regions in control mice from the juvenile + adult exposure group were somewhat higher than Mn levels in the corresponding brain regions in naïve adults (Table 1), due possibly to either stress resulting from juvenile gavage or to experimental variation between these two study groups. Because this is the first report of such findings, we cannot rule out either
possibility and must rely on subsequent studies to confirm these findings. The variability in dose-related accumulation of Mn could also be explained in part by the robust elimination pharmacokinetics of Mn in rodent brain. It was previously reported that dietary Mn does not increase brain accumulation of inhaled Mn$_3$O$_4$ (Dorman et al., 2002), suggesting that once saturation is reached in brain tissue, further accumulation is difficult, particularly with chronic low-dose exposure. However, low dose exposure to Mn at critical times during development might elicit other persistent changes, such as glial inflammatory activation, that amplifies the effects of even low doses of Mn upon subsequent exposures.

In conclusion, these studies indicate that the period of development in mice spanning weaning to early adulthood represents a critical window of sensitivity to the neurobehavioral effects of ingested Mn and that male mice are more severely affected than females. In adult mice, not only were pre-exposed animals more sensitive to Mn toxicity than naïve mice not exposed early in life, but this pre-exposure also resulted in greater effects on both dopaminergic and serotonergic neurochemical parameters in the striatum. Sex differences in susceptibility were also noted in adult animals, with only males displaying neurobehavior abnormalities. Additional studies will be required to determine the basis for these differences and to examine the possible neuroprotective role of estrogen in mitigating the neurotoxicity of Mn. Differences in accumulation of Mn and dysregulation of other transition metals, such as Fe and Cu, do not alone explain the observed potentiation of neurobehavioral deficits in adult mice pre-exposed to Mn as juveniles, suggesting that other cellular or molecular systems are affected that lead to persistent changes in the neurochemistry and function of the basal ganglia.
Neuroinflammatory signaling in glial cells is a potential pathway affected by Mn exposure that could account for the persistence of the neurobehavioral deficits observed in these mice. Enhanced inflammatory activation of glia was noted in adult mice pre-exposed as juveniles that co-localized with increased expression of inducible nitric oxide synthase and nitrosative stress in neurons of the Gp and SNpr (see companion article, this issue). Thus, Mn exposure in juvenile mice appears to result in complex changes in both neurochemistry and behavior that enhance sensitivity to Mn and possibly other neurotoxicants during aging. The mechanism underlying these changes in neurobehavioral parameters is not entirely clear but may involve inflammatory responses of glial cells that persist into adulthood.
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FIGURE LEGENDS

Figure 1. Developing and adult mice are differentially sensitive to Mn-induced effects on anxiety and novelty-seeking behavior. Time spent in the margin was recorded in open-field activity chambers and determined for each animal following exposure to Mn. Male juvenile mice (day 20-34) exposed to 10 and 30 mg/Kg MnCl₂ had a significant decrease in time spent in the margin (B) while female juvenile mice had no significant change. (C) Female and (D) male mice exposed only as adults (week 12-20) were not vulnerable to Mn-induced neurobehavioral changes. (E) Female mice pre-exposed to Mn as juveniles and then again as adults (day 20-34 and week 12-20) had no change whereas pre-exposed males (F) did have a significant increase in time spent in the margin at both 10 and 30 mg/Kg MnCl₂, compared to controls. (*p < 0.05).

Figure 2. Developmental exposure to Mn increases sensitivity to locomotor dysfunction in adult male mice upon subsequent exposure. Total movement number was recorded in open-field activity chambers for each animal following exposure to Mn. No significant changes were detected in (A) female or male (B) juvenile (day 20-34) mice exposed to 10 and 30 mg/Kg MnCl₂ or in (C) female or (D) male mice exposed to MnCl₂ only as adults (week 12-20). Likewise, there we no detectable change in total movement number in adult female mice pre-exposed as juveniles (E) but total movement number was decreased in adult male mice pre-exposed to 30 mg/Kg MnCl₂ as juveniles, relative to controls (day 20-34 and week 12-20) (F). (*p < 0.05).
Figure 3. Mn differentially modulates striatal dopamine levels in developing and adult mice. Juvenile mice (day 20-34) exposed to 10 and 30 mg/Kg MnCl₂ had a significant increase in striatal dopamine (A) but no significant change was detected in mice exposed only as adults (week 12-20) (B). Dopamine levels were decreased in the striatum of adult mice pre-exposed to MnCl₂ as juveniles (day 20-34 and week 12-20) (C). (* p < 0.05).

Figure 4. Mn differentially modulates striatal DOPAC levels in developing and adult mice. Juvenile (day 20-34) (A) and adult (week 12-20) (B) mice exposed to 30 mg/kg MnCl₂ had significant decreases in striatal DOPAC levels. DOPAC levels were also decreased at both 10 and 30 mg/Kg Mn in adult mice pre-exposed to MnCl₂ as juveniles (day 20-34 and week 12-20) (C). Differing letters denote statistical significance (p < 0.05).

Figure 5. The DOPAC/DA ratio is selectively altered in juvenile mice following exposure to manganese. The ratio of DOPAC/dopamine was decreased in juvenile mice (day 20-34) exposed to 30 mg/Kg MnCl₂ (A) but no changes were detected in either naïve adult mice exposed to 10 or 30 mg/Kg MnCl₂ (B) or in adult mice pre-exposed to 10 or 30 mg/Kg MnCl₂ as juveniles (C). Differing letters denote statistical significance (p < 0.05).
Figure 6. Striatal serotonin levels are unaffected by manganese in developing and adult mice. Mice were exposed to 10 and 30 mg/Kg MnCl$_2$ as (A) juveniles (day 20-34), (B) adults (week 12-20), and (C) juveniles + adults (day 20-34 and week 12-20). No changes in levels of striatal serotonin (d-hydroxytryptamine; 5-HT) were detected in any exposure group. Significance is denoted by differing letters ($p > 0.05$).

Figure 7. Striatal levels of the serotonin metabolite, 5-hydroxyindoleacetic acid, are decreased in juvenile mice exposed to manganese. Levels of 5-hydroxyindoleacetic acid (5-HIAA) were decreased in (A) juvenile mice (day 20-34) exposed to 30 mg/Kg MnCl$_2$ but no changes in 5-HIAA levels were detected in mice exposed to MnCl$_2$ as (B) adults (week 12-20) or (C) juveniles + adults (day 20-34 and week 12-20). Differing letters denote statistical significance ($p > 0.05$).
Table 1. Manganese in brain and serum of exposed juvenile and adult mice (ppm)

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>Treatment (MnCl₂•4H₂O)</th>
<th>Striatum</th>
<th>Substantia nigra</th>
<th>Cortex</th>
<th>Hypothalamus</th>
<th>Serum</th>
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<tbody>
<tr>
<td><strong>Juvenile</strong></td>
<td>0 mg/kg</td>
<td>0.26±0.01</td>
<td>0.5±0.07</td>
<td>0.48±0.08</td>
<td>0.63±0.12</td>
<td>0.004±0.0003</td>
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<tr>
<td></td>
<td>10 mg/kg</td>
<td>0.49±0.06†</td>
<td>1.0±0.65†</td>
<td>0.47±0.32</td>
<td>1.4±0.17</td>
<td>0.009±0.0009</td>
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<td></td>
<td>30 mg/kg</td>
<td>0.70±0.01†</td>
<td>2.5±0.45†</td>
<td>1.4±0.13†</td>
<td>1.1±0.51</td>
<td>0.004±0.0002</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td>0 mg/kg</td>
<td>0.69±0.07</td>
<td>0.48±0.18</td>
<td>0.45±0.15</td>
<td>0.50±0.21</td>
<td>0.006±0.0007</td>
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<tr>
<td></td>
<td>10 mg/kg</td>
<td>2.1±0.10†</td>
<td>2.5±0.11</td>
<td>3.1±3.5</td>
<td>1.7±0.36</td>
<td>0.008±0.0008</td>
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<tr>
<td></td>
<td>30 mg/kg</td>
<td>1.1 ±0.12</td>
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<td>2.05±0.43</td>
<td>1.5±0.16</td>
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<td><strong>Juvenile + Adult</strong></td>
<td>0 mg/kg</td>
<td>1.7±0.19</td>
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<td>1.8±0.13</td>
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<td>1.4±0.10</td>
<td>0.006±0.0004</td>
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<tr>
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<td>30 mg/kg</td>
<td>1.7±0.09</td>
<td>1.3±0.15</td>
<td>1.42±0.19</td>
<td>1.3±0.20</td>
<td>0.012±0.0008</td>
</tr>
</tbody>
</table>

Data shown are means ± SEM (n ≥ 3). Significance compared to controls within a specific brain region per exposure group is denoted by † (p<0.05).
Table 2. Iron in brain and serum of juvenile and adult mice exposed to manganese (ppm)

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>Treatment (MnCl₂•4H₂O)</th>
<th>Striatum</th>
<th>Substantia nigra</th>
<th>Cortex</th>
<th>Hypothalamus</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile</td>
<td>0 mg/kg</td>
<td>6.7±1.4</td>
<td>10.1±2.02</td>
<td>14.8±9.6</td>
<td>17.2±10.4</td>
<td>2.53±0.32</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>8.05±1.9</td>
<td>18.3±12.8†</td>
<td>10.7±8.5</td>
<td>23.04±8.87</td>
<td>3.32 ±1.29</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>9.3±1.1</td>
<td>37.7±1.23†</td>
<td>32.1±1.23†</td>
<td>71.64±90.6</td>
<td>4.79±0.23</td>
</tr>
<tr>
<td>Adults</td>
<td>0 mg/kg</td>
<td>26.7±2.23</td>
<td>14.9±6.3</td>
<td>13.1±4.7</td>
<td>19.8±10.1</td>
<td>3.15±0.27</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>34.6±1.32</td>
<td>41.2±10.5</td>
<td>50.4±14.1</td>
<td>28.7±5.9</td>
<td>6.23±0.62</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>33.2±1.57</td>
<td>43±24</td>
<td>44.2±3.35</td>
<td>41±3.4†</td>
<td>5.13±0.35</td>
</tr>
<tr>
<td>Juvenile + Adult</td>
<td>0 mg/kg</td>
<td>26.6±1.9</td>
<td>32.5±0.35</td>
<td>23.3±2.6</td>
<td>25.4±2.28</td>
<td>2.91±0.27</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>35.4±1.2</td>
<td>34.5±1.83</td>
<td>34.3±1.18</td>
<td>31.0±1.24</td>
<td>3.4±0.20</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>34.7±0.43</td>
<td>24.9±2.5</td>
<td>24.3±2.6</td>
<td>20.9±1.86</td>
<td>6.2±0.56</td>
</tr>
</tbody>
</table>

Data shown are means ± SEM (n ≥ 3). Significance compared to controls within a specific brain region per exposure group is denoted by †(p<0.05).
Table 3. Copper in brain and serum of juvenile and adult mice exposed to manganese (ppm)

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>Treatment (MnCl₂•4H₂O)</th>
<th>Striatum</th>
<th>Substantia nigra</th>
<th>Cortex</th>
<th>Hypothalamus</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Juvenile</strong></td>
<td>0 mg/kg</td>
<td>1.8±0.07</td>
<td>2.0±0.13</td>
<td>4.07±0.68</td>
<td>4.1±0.75</td>
<td>0.24±0.004</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>1.9±0.16</td>
<td>3.8±0.93†</td>
<td>2.8±0.50</td>
<td>5.5±0.54</td>
<td>0.22±0.002</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>2.5±0.06</td>
<td>9.9±†</td>
<td>7.7±1.23</td>
<td>3.04±0.81</td>
<td>0.23±0.003</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td>0 mg/kg</td>
<td>8.1±0.91</td>
<td>4.1±1.85</td>
<td>2.9±0.82</td>
<td>5.5±2.73</td>
<td>0.32±0.009</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>10.1±0.52</td>
<td>8.4±0.46</td>
<td>13.5±4.03</td>
<td>8.9±2.4</td>
<td>0.34±0.028</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>9.5±0.58</td>
<td>10.2±1.58</td>
<td>13.2±1.27†</td>
<td>11.9±1.47</td>
<td>0.34±0.008</td>
</tr>
<tr>
<td><strong>Juvenile + Adult</strong></td>
<td>0 mg/kg</td>
<td>8.7±0.72</td>
<td>8.0±0.14</td>
<td>5.6±0.52</td>
<td>7.6±0.73</td>
<td>0.21±0.015</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>9.6±0.42</td>
<td>9.7±0.52</td>
<td>7.08±1.18</td>
<td>8.3±0.52</td>
<td>0.26±0.007</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>10.1±0.28</td>
<td>7.6±1.03</td>
<td>5.86±0.76</td>
<td>5.18±0.35</td>
<td>0.25±0.01</td>
</tr>
</tbody>
</table>

Data shown are means ± SEM (n ≥ 3). Significance compared to controls within a specific brain region per exposure group is denoted by †(p<0.05).
Figure 1
203x254mm (300 x 300 DPI)
Figure 2
145x217mm (300 x 300 DPI)
Figure 3
85x197mm (300 x 300 DPI)
Figure 4
87x197mm (300 x 300 DPI)
Figure 5
85x197mm (300 x 300 DPI)
Figure 6
85x197mm (300 x 300 DPI)
Figure 7

87x197mm (300 x 300 DPI)