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Neonatal C57BL/6J and Parkin Mice Respond Differently Following Developmental Manganese Exposure: Result of a High Dose Pilot Study

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Abstract

It has been suggested that childhood exposure to neurotoxicants may increase the risk of Parkinson's disease (PD) or other neurodegenerative disease in adults. Some recessive forms of PD have been linked to loss-of-function mutations in the *Park2* gene that encodes for parkin. The purpose of this pilot study was to evaluate whether responses to neonatal manganese (Mn) exposure differ in mice with a *Park2* gene defect (parkin mice) when compared with a wildtype strain (C57BL/6J). Neonatal parkin and C57BL/6J littermates were randomly assigned to 0, 11, or 25 mg Mn/kg-day dose groups with oral exposures occurring from postnatal day (PND) 1 through PND 28. Motor activity was measured on PND 19–22 and 29–32. Tissue Mn concentrations were measured in liver, femur, olfactory bulb, frontal cortex, and striatum on PND 29. Hepatic and frontal cortex gene expression of *Slc11a2*, *Slc40a1*, *Slc30a10*, *Hamp* (liver only), and *Park2* were also measured on PND 29. Some strain differences were seen. As expected, decreased hepatic and frontal cortex *Park2* expression was seen in the parkin mice when compared with C57BL/6J mice. Untreated parkin mice also had higher liver and femur Mn concentrations when compared with the C57BL/6J mice. Exposure to > 11 mg Mn/kg-day was associated with increased brain Mn concentrations in all mice, no strain difference was observed. Manganese exposure in C57BL/6J, but not parkin mice, was associated with a negative correlation between striatal Mn concentration and motor activity. Manganese exposure was not associated with changes in frontal cortex gene expression. Decreased hepatic *Slc30a10*, *Slc40a1*, and *Hamp* expression were seen in PND 29 C57BL/6J mice given 25 mg Mn/kg-day. In contrast, Mn exposure was only associated with decreased *Hamp* expression in the parkin mice. Our results suggest that the *Parkin* gene defect did not increase the susceptibility of neonatal mice to adverse health effects associated with high-dose Mn exposure.

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Keywords

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INTRODUCTION

Physicians and public health officials increasingly recognize that Parkinson's disease (PD) involves interplay between genetic and environmental risk factors (Kalia and Lang; 2016; Schapira, 2006). Genetics likely plays a major role in about 5–20% of the patients that suffer from PD (Bonifati, 2005; Corti et al., 2011). Inheritable forms of PD are often associated with genetic mutations, among them *a-synuclein*, *DJ-1*, and *Park2* (Kitada et al., 1998, Bonifati et al., 2003). Parkin, encoded by the *Park2* gene, is part of an E3 ubiquitin ligase complex which is part of the ubiquitin-proteasome system that mediates the targeting of proteins for degradation (Vincent et al., 2012). Parkin may have an indirect role in mitochondrial respiratory chain function, cellular responses to oxidative stress, and removal of damaged mitochondria (Narendra et al., 2008; Vincent et al., 2012). Parkin has been shown to be involved in an autosomal recessive form of PD and relevant mouse models have been developed (Lee et al., 2012; Pickrell and Youle, 2015). To date, very few environmental causes of PD have been recognized and existing research has largely focused on pesticides, solvents, and other chemicals that induce mitochondrial dysfunction, alter proteasomal function, and promote aggregation of *a-synuclein* and other proteins (Calabrese et al., 2010; Chin-Chan et al., 2015; Schapira, 2006).

Hallmark clinical features of PD are the development of bradykinesia, postural instability, and other signs of motor dysfunction. These motor symptoms generally appear when greater than 60% of dopaminergic neurons are lost and dopamine content in the globus pallidus is reduced to below 20% of normal levels (Jankovic, 2008; Wirdefeldt et al., 2011). Many of these clinical features (e.g., postural instability, bradykinesia, loss of dopaminergic neurons) are also seen in people following high dose Mn exposure (Guilarte, 2013). Manganese neurotoxicity typically occurs after environmental or occupational Mn exposure and is presumed to reflect oxidative stress, mitochondrial dysfunction, and other disruptions of cellular function (Erikson et al., 2007; Farina et al., 2013). High-dose Mn exposure in people produces a form of parkinsonism that is characterized by Mn accumulation in the globus pallidus, neurobehavioral changes, impaired motor control, and loss of dopamine-containing neurons (Dorman, 2015; Guilarte, 2013).

Since PD and Mn disrupt similar cellular processes within dopaminergic neurons it is reasonable to hypothesize that Mn toxicity may exacerbate the signs of PD (Bowman et al., 2011; Dusek et al., 2015; Roth, 2014). This hypothesis is supported by the growing epidemiologic literature suggesting that chronic exposure to Mn may predispose an individual to acquire PD-like signs (Andruska and Racette, 2015; Gorell et al., 1999, Kim et al., 2002; Racette et al., 2001; Willis et al., 2010). Importantly, high dose Mn exposure can result from inhalation in occupational settings, such as mining and welding, consumption of Mn-contaminated drinking water, in patients with compromised hepatobiliary function, and in children receiving intravenous total parenteral nutrition (TPN) solutions fortified with

additional Mn or from ingestion of soy-based milk replacements (Aschner et al., 2015; Crinella, 2012; Dorman, 2015; Hardy et al., 2008; Michalke et al., 2007).

Most studies evaluating Mn exposure and PD have focused on occupational exposure of welders and other Mn workers. It is hypothesized that childhood exposure to neurotoxicants may increase the risk of PD and other neurodegenerative diseases during senescence (Berry et al., 2010). It is currently unknown whether neonatal Mn exposure is a risk factor for PD and other neurodegenerative diseases that occur in late adulthood. Our study begins to address this critical data gap by evaluating the early effect of Mn exposure in neonatal wildtype mice and others with a mutation in the *Park2* gene. Other investigators have used *Park2* mice to investigate the role of Parkin loss-of-function mutations in PD pathogenesis (Goldberg et al., 2003; Hennis et al., 2013; Kitada et al., 2012; Lee et al., 2012). Mice deficient in the *Park2* gene develop motor dysfunction and other neurologic effects during aging (Goldberg et al., 2003; Hennis et al., 2013; Hennis et al., 2014; Kitada et al., 2009) and we hypothesize that they are an appropriate model to study gene and Mn interactions in PD.

MATERIALS AND METHODS

Experimental design:

This study used wildtype (C57BL/6J) and parkin mice, an autosomal homozygous recessive knock out (*Park2^{tm1Shn}*), obtained from Jackson Laboratories (Bar Harbor, ME). On postnatal day (PND) 1, male and female parkin and wild type (C57BL/6J) littermates were sexed, uniquely identified (foot tattoo), and randomly allocated to each Mn exposure concentration (whenever possible $n = 1$ pup/sex/dose/litter). Based on our laboratory's previous developmental neurotoxicity studies conducted in neonatal rats (Brenneman et al., 1999), our initial goal was to use the following four exposure doses: 0, 11, 25, and 50 mg/kg-day. However, unlike neonatal rats, we found that neonatal mice were more susceptible to the effects of high dose Mn exposure during the postnatal period necessitating a change in our highest dose from 50 mg Mn/kg-day to 25 mg Mn/kg-day. Pups were exposed from PND 1 through PND 28. Some mice were killed on PND 29 and tissues collected for determination of tissue Mn concentrations as well as liver and frontal cortex expression of genes involved in metal homeostasis. Motor activity was also assessed on PND 19–22 and 29–32. A companion manuscript describes additional measurement of tissue iron, zinc, and copper concentrations and our analyses of associations between these metals and the expression of genes evaluated in the present study (Foster et al., 2017).

Animals and animal husbandry:

This study was conducted under federal guidelines for the care and use of laboratory animals (National Research Council, 2011) and approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. Mice were housed in the College of Veterinary Medicine's Laboratory Animal Resources (LAR) facility using standard caging systems and appropriate materials for enrichment. Animal rooms were maintained at daily temperatures of $22 \pm 4^{\circ}\text{C}$, relative humidity of 30–70%, and an air flow rate sufficient to provide 10–15 air changes per hour. Fluorescent lighting was controlled by automatic

controls (lights on approximately 0700–1900). On arrival, mice were individually housed in polycarbonate cages and provided food and water ad libitum. A pelleted, semi-purified AIN-93G certified diet from Bio-Serv (Frenchtown, New Jersey) formulated to contain 10 ppm Mn and 35 ppm iron was fed to all animals.

Breeding:

Young adult (four-week-old) mice were purchased as breeding stock. A total of ten female and five male mice of each strain was used. All mice were quarantined for at least one week prior to breeding. Harem breeding involving one male and up to two females was used. Each breeding harem was used up to three times. All females were assessed on a daily basis for the presence of a vaginal plug or abdominal enlargement consistent with pregnancy and on a weekly basis for weight gain. Pregnant animals were removed and individually housed prior to parturition. The day that pups were born was designated as PND 0. At parturition all mice were sexed and identified using paw tattoos. Experienced laboratory animal technicians that were not blinded to treatment groups performed daily cage side observations of all animals.

Neonatal Mn exposure:

Littermates were randomly assigned to one of three dose groups (whenever possible one mouse/sex/litter/dose): control (i.e., 0 mg Mn/kg-day), low (e.g., 11 mg Mn/kg-day), and a high dose group (e.g., 25 mg Mn/kg-day). Littermates were assigned to the highest Mn dose group (one mouse/sex/litter) then the next highest (one mouse/sex/litter) and finally to the control group (one mouse/sex/litter) until all pups were allocated. Litters and their dams were weighed daily, and the individual pup weight was used to calculate the Mn exposure dose. Aqueous Mn ($\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$; Sigma Aldrich Chemical Corporation, St. Louis, Missouri) dosing solutions were prepared to provide 1 ml/kg dose volume to be given by mouth using a micropipette (Dorman et al., 2000). All surviving mice were weighed on PND 28.

Assessment of motor function:

Mice (one mouse/sex/litter/dose group) were evaluated for spontaneous motor activity between PND 19 and 32. Spontaneous horizontal and vertical movements were assessed on PND 19–22 and 29–32. For this test, individual mice were placed in a clean cage for 15 minutes during which their horizontal and vertical movements were monitored by infrared light beam sensors (Actimeter system; PanLab) and analyzed using Actitrack system software. Spontaneous locomotor activity was also assessed on PND 19. On this test, mice were placed individually in a clean cage within an infrared photobeam activity monitor (San Diego Instruments) and allowed to move freely in the dark for 60 minutes. The number of beam breaks was recorded in five-minute bins as a measure of locomotor activity.

Necropsy and tissue collection:

Necropsies were performed 20–24 hours following the gavage exposure (or equivalent) after 28 exposure days. Mice were weighed and then euthanized using carbon dioxide inhalation and subsequent exsanguination. The following tissues were collected: striatum, olfactory bulb, frontal cortex, liver, spleen, and femur for tissue Mn determination. Samples of liver

and brain were also collected for gene expression studies. Tissue samples were transferred to plastic vials, frozen in liquid nitrogen, and stored at approximately - 80°C until subsequent analyses were completed.

Chemical analyses:

Tissue samples were digested using a stepwise protocol: digestion in 0.2 mL 70% nitric acid and heating at 85°C until dry, resuspension in 0.2 mL 30% Optima grade hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) and heating at 85°C until dry, repeated resuspension in hydrogen peroxide and heating until dry, resuspension in 0.2 mL 70% nitric acid and heating until dry, then a final resuspension in 1 mL 2% nitric acid. Samples were analyzed by graphite furnace atomic absorption spectrometry (GF-AAS) using an AAnalyst 600 spectrometer (Perkin Elmer, Waltham, MA) at the Environmental Chemistry Facility at Brown University using previously described methods (Foster et al., 2015). In some analyses, the liver Mn concentrations were converted to a % change from control (for each strain individually).

Quantitative polymerase chain reaction (qPCR):

To measure gene expression, all kits, reagents, and instruments were obtained from ThermoFisher and used according to the manufacturer's recommendations. RNA was isolated from thawed mouse tissues samples using Trizol then incubated with DNase I to remove genomic DNA. cDNA was synthesized from 1 µg RNA per sample using a High Capacity cDNA Reverse Transcription Kit. Gene expression levels were measured on cDNA aliquots using Taqman Gene Expression Master Mix and Gene Expression Assays on a Viia7 Real-Time PCR System using a relative standard curve approach. The following gene expression assays were used: *Actb* (Mm02619580), *Gapdh* (Mm99999915), *Hamp* (Mm04231240), *Hprt* (Mm03024075), *Park2* (Mm01323528), *Slc11a2* (Mm00435363), *Slc30a10* (Mm01315481), and *Slc40a1* (Mm01254822). In some analyses, the gene expression data were converted to a % change from control (for each strain individually) and a grand mean calculated across the three data sets normalized to different housekeeping genes (*Gapdh*, p-actin, and *Hprt*).

Statistical analysis:

Individual data that appeared to be outliers were critically evaluated using a Dixon-type test for discordancy of an upper or lower outlier (Barnett and Lewis, 1984). Statistical analyses were conducted using SAS Statistical Software (JMP, SAS Institutes, Inc., Cary, NC). Tissue metal concentrations, change in liver gene expression, and the total distance traveled and total number of rears measured in the Actimeter system were initially measured using a mixed model with strain, sex, and dose as fixed effects and subject as the random effect. With few exceptions (e.g., body weight on PND 28) a significant sex effect was not seen and data were pooled across sex in subsequent analyses. All data were subsequently analyzed individually by strain. Tissue Mn concentrations, % change in liver and frontal cortex gene expression, body weights, and total distance traveled and total number of rears were inter-compared for the three Mn exposure groups by ANOVA or with a Welch ANOVA for unequal variances followed by a Tukey's honestly significant difference test to perform pairwise multiple comparisons. Distance traveled in the photobeam activity monitor was

assessed using a repeated-measures analysis with exposure as a grouping factor and test period and test session time as within-subject factors (MANOVA). Correlation analyses between % change in liver Mn and % change in liver gene expression were calculated by linear regression of the plotted data points. A probability value of < 0.05 was used as the critical level of significance for all statistical tests. Unless otherwise noted, data presented are mean values $\pm$ standard error of the mean (SEM).

RESULTS

Clinical effects:

Mice given 50 mg Mn/kg-day demonstrated high mortality rates and severe weight loss necessitating a change in our highest dose from 50 mg Mn/kg-day to 25 mg Mn/kg-day (data not shown). Mice given the highest Mn exposure dose (25 mg Mn/kg-day) had decreased body weight on PND 28 (Figure 1). In addition, male C57BL/6J and female parkin mice given 11 mg Mn/kg-day also developed an approximate 15% decrease in body weights on PND 28. Fecundity was relatively low in both strains: average numbers of live births ($\pm$ SD) was 6.2 ± 2.3 and 5.7 ± 2.2 pups in the C57BL/6J and parkin mice, respectively. This limited our ability to allocate littermates to each Mn dose group.

Motor activity:

Spontaneous motor activity was assessed using two automated systems in Mn-exposed and untreated mice of both strains (controls). Mice given the highest Mn exposure dose (25 mg Mn/kg-day) had decreased total distance traveled on PND 19–22 (Table 1). C57BL/6J given 11 or 25 mg Mn/kg-day also had decreased motor activity on PND 29–32. C57BL/6J given 25 mg Mn/kg-day also had fewer total rears on PND 19–22 (Table 1). Total activity was also assessed on PND 19 using a second motor activity system. Reduced motor activity was seen in PND 19 C57BL/6J mice given either 11 mg Mn/kg-day ($p = 0.0027$) or 25 mg Mn/kg-day ($p < 0.001$) when compared with controls (Figure 2). Although parkin mice given Mn also had lower total motor activity on PND 19, this did not reach statistical significance ($p = 0.0677$; Figure 2).

Tissue Mn concentrations:

Tissue Mn concentrations are presented in Figure 3. Increased striatum, olfactory bulb, frontal cortex, femur, and liver Mn concentrations were seen in PND 29 C57BL/6J mice given either 11 or 25 mg Mn/kg-day when compared with controls. Increased striatum, olfactory bulb, frontal cortex, and femur Mn concentrations were seen in PND 29 parkin mice given either 11 or 25 mg Mn/kg-day when compared with controls. Manganese exposure in C57BL/6J, but not parkin mice, was associated with a negative correlation between striatal Mn concentration and motor activity (Figure 4). Increased liver Mn concentration was also seen in parkin mice given 25 mg Mn/kg-day when compared with controls. Control parkin mice had increased liver Mn and femur Mn concentrations when compared with control C57BL/6J mice.

Liver gene expression:

The effects of Mn on hepatic and frontal cortex gene expression (normalized to p-actin) are presented in Figure 5. As expected, liver and frontal cortex Park 2 gene expression was decreased in the parkin mice when compared with the C57BL/6J mice. No other strain difference in gene expression was observed. Decreased *Slc30a10*, *Slc40a1*, and *Hamp* gene expression (normalized to p-actin) were seen in PND 29 C57BL/6J mice given 25 mg Mn/kg-day when compared with controls. Decreased *Slc30a10* gene expression (normalized to p-actin) was also seen in C57BL/6J mice given 11 mg Mn/kg-day when compared with controls. Decreased *Hamp* gene expression (normalized to p-actin) was seen in parkin mice given > 11 mg Mn/kg-day when compared with controls. Liver and frontal cortex gene expression data normalized to other housekeeping genes are provided in Supplemental Tables 1 and 2.

Gene expression was also normalized to % control and then averaged across all housekeeping genes to produce a composite estimate of the change in gene expression. No Mn-treatment effect was seen on frontal cortex gene expression (data not shown). Analysis of the liver gene expression data (as % control) is presented in Figure 6. High dose Mn exposure was associated with decreased hepatic *Slc30a10*, *Slc40a1*, and *Hamp* expression in C57BL/6J mice. Manganese exposure to > 11 mg Mn/kg-day was associated with decreased hepatic *Hamp* expression in parkin mice.

We also evaluated whether a linear relationship existed between liver Mn concentration (as % control) and normalized liver gene expression (as % control). Significant negative linear correlations were seen between liver Mn concentration and *Slc30a10* and *Slc40a1* expression in C57BL/6J mice (Figure 7). Near significant negative linear correlations ($p < 0.06$) were also seen between liver Mn concentration and *Slc30a10* and *Slc40a1* expression in parkin mice (Figure 7).

DISCUSSION

To date, most rodent pharmacokinetic studies with Mn have used rats and far less data are available for any mouse strain. One important challenge with this study centered on dose selection. We initially based our dose selection on a developmental neurotoxicity study that we performed in rats (Brenneman et al., 1999). Brenneman et al (1999) exposed rat pups to 0, 25, or 50 mg Mn/kg/day from PND 1 to 49 and found limited effects on body weight gain or motor activity in neonatal rats given 25 mg Mn/kg/day. Other investigators have examined the effect of even higher oral Mn doses in mice. For example, Liu et al (2006) exposed twelve-week-old female C57BL/6 mice to 100 mg Mn/kg/day for 8 weeks and reported no effect on body weight in Mn-exposed mice. Unexpectedly, developmental exposure of mice was associated with a toxicologically significant decrease in body weight gain suggestive of systemic toxic effects. Thus, interpretation of other findings in the present study must be done cautiously since systemic toxicity can affect motor activity and other evaluated endpoints.

This study is the first to evaluate Mn pharmacokinetics in neonatal parkin and C57BL/6J mice. As expected, we observed increased Mn concentrations in the brain (striatum), liver,

and femur of PND 29 mice exposed to a high (25 mg/kg-day) oral dose of Mn from PND 1 through 28. Manganese concentrations seen in the striatum in the high dose exposure group were approximately four- to five-fold higher than control values. We also observed decreased liver iron concentrations whereas Mn treatment did not affect copper or zinc concentrations in any evaluated tissues (Foster et al., 2017). The increase in brain Mn concentration observed in mice in the present study is larger than that seen with our previous studies with neonatal rats exposed similarly from PND 1–21 with 25 mg Mn/kg-day (Dorman et al., 2000) suggesting that neonatal mice may be more sensitive than neonatal rats to Mn. Additional information in support of this conclusion includes our observation that neonatal mice given 50 mg Mn/kg-day had a high mortality rate that was not seen in similarly exposed neonatal rats (data not shown). As mentioned earlier, neonatal mice unlike similarly aged rats also had significant decreases in end of exposure body weights following exposure to 25 mg Mn/kg-day (Dorman et al., 2000).

Our study also evaluated motor activity in Mn-exposed mice. We observed a consistent statistically significant decrease in motor activity in neonatal C57Bl/6J mice given either 11 or 25 mg Mn/kg-day. We also observed a transient decrease in motor activity in PND 19–22 parkin mice given 25 mg Mn/kg-day. In contrast, Mn-exposed parkin mice did not demonstrate decreased motor activity at PND 29–32 suggesting a strain difference in response to Mn. We also found that manganese exposure in C57Bl6 mice, but not parkin mice, was associated with a negative correlation between striatal Mn concentration and motor activity. Our previous studies with Mn exposed neonatal rats (given up to 50 mg Mn/kg-day) failed to identify statistically significant treatment-related effects on overall motor activity between PND 13–21 (Dorman et al. 2000) which may suggest that species differences may also be of importance in rodents.

Like Mn, iron metabolism is also under tight homeostatic regulation. To explore homeostatic mechanisms involved in Mn regulation we evaluated changes in hepatic gene expression of *Hamp*, *Slc11a2*, *Slc30a10*, and *Slc40a1*. We found that Mn exposure was not associated with changes in frontal cortex expression of *Hamp*, *Slc11a2*, *Slc30a10*, or *Slc40a1* in either strain of mice. We did find several changes in hepatic gene expression following Mn exposure. We found that neonatal parkin mice given either 11 or 25 mg Mn/kg-day had decreased hepatic *Hamp* expression. In contrast, decreased hepatic *Hamp* expression was only seen in the C57Bl6 mice following exposure to 25 mg Mn/kg-day. Changes in *Hamp* expression showed a positive correlation with liver iron concentration (Foster et al., 2017) and in the case of the C57Bl6 mice a nearly significant ($p = 0.052$) negative correlation with liver Mn concentration. The protein product of *Hamp* (also known as hepcidin antimicrobial peptide), hepcidin, is involved in iron homeostasis, regulates iron storage in macrophages, and is critical for intestinal iron absorption (Ganz 2005; Lesbordes-Brion et al., 2006). Hepcidin is released in response to increased iron status and binds to the cellular iron efflux protein, ferroportin (i.e., MTP1), causing ferroportin to be degraded, thereby reducing dietary iron absorption. Other investigators have shown marked increases in *HAMP* mRNA expression in the liver of rats fed a diet high in iron (Zhang et al., 2016). Decreases in hepcidin gene expression have also been observed in Mn-exposed welders (Fan et al., 2016).

We found that neonatal mice exposed to Mn did not have altered hepatic expression of *Slc11a2* (also known as solute carrier family 11 member 2; divalent cation transporter 1, DMT1). DMT1 transports a number of divalent metals and is mainly present in the plasma membrane of epithelial cells found in the duodenum, kidney, and choroid plexus (Forbes and Gros, 2003) where it is essential for the absorption of divalent metals (Przybyszewska and Zekanowska, 2015; Skjørringe et al., 2016). Recent studies in DMT1 knockout mice have shown that intestinal DMT1 is not required for the intestinal transport of Mn (Shawki et al., 2015). In contrast with our results, dietary supplementation of Mn is associated with decreased DMT1 expression in the liver of chickens (Bai et al., 2014) and the blood of welders (Fan et al., 2016). These inconsistent results might be due to the differences in Mn concentration and experimental animal model.

SLC30A10 (solute carrier family 30 member 10) also plays an important role in Mn transport (Chen et al., 2015). *SLC30A10* was originally categorized as a zinc transporter; however, it has a broader role in transporting Mn, iron, copper and other divalent cations (Roth, 2014). Some of the diseases associated with *SLC30A10* include hypermanganesemia with dystonia, polycythemia, and cirrhosis and dystonia/parkinsonism (DeWitt et al., 2013; Quadri et al., 2012; Tuschl et al., 2008). We found that neonatal C57Bl/6J mice exposed to Mn at > 11 mg Mn/kg-day had decreased expression of *Slc30a10* in the liver. We also observed decreased *Slc30a10* in the liver of Mn-treated parkin mice; however, this change did not meet statistical significance ($p = 0.249$). We also observed a statistically significant negative correlation between liver expression of *Slc30a10* in C57Bl/6J mice and hepatic Mn concentration. A similar negative correlation approached significance ($p = 0.053$) in the parkin mice. We also observed a positive correlations between liver iron concentration and hepatic *Slc30a10* gene expression in the C57Bl6 mice (Foster et al., 2017). Our finding of decreased *Slc30a10* expression in the liver is surprising and seems to run contrary to the predicted importance for this organ and transporter in Mn homeostasis (Quadri et al., 2012; Sreedharan et al., 2011).

SLC40A1 (solute carrier family 40 member 1) controls production of the iron exporter ferroportin (Ward and Kaplan, 2012). The lack of ferroportin results in iron accumulation in iron-exporting tissues. For example, loss of ferroportin in transgenic mice results in stunted growth and early onset anemia associated with excess accumulation of iron in enterocytes, macrophages, and hepatocytes (Donovan et al., 2005). We found that neonatal C57Bl/6J mice exposed to Mn at 25 mg Mn/kg-day had decreased expression of *Slc40a1* in the liver. We also observed a statistically significant negative correlation between liver expression of *Slc40a1* in C57Bl/6J mice and liver manganese concentration. A negative correlation between liver expression of *Slc40a1* and liver iron concentration was also seen in C57Bl/6J mice (Foster et al., 2017). Similar results have been seen in Mn-exposed chickens, which developed decreased ferroportin expression in the liver (but not duodenum) (Bai et al., 2014). In vitro studies in an inducible human embryonic kidney (HEK293T) cell model have shown that ferroportin expression is increased following exposure to Mn and this change in protein level has a cytoprotective effect (Yin et al., 2010). Studies in mice have also implicated ferroportin in Mn transport (Seo and Wessling-Resnick, 2015; Seo et al., 2016).

Our pilot study has several important limitations including Mn exposures that resulted in significant decreases in body weight suggestive of systemic effects. Future studies evaluating the developmental neurotoxicity of Mn in these mice strains should use considerably lower Mn exposures. Our study also evaluated gene expression changes in frontal cortex rather than striatum - future investigators may want to focus on changes in the globus pallidus of mice. We also found that high dose Mn exposure influenced iron tissue concentrations and led to changes in hepatic expression of several genes involved in iron and Mn regulation. Changes in the tissue metal concentrations may be linked to the alterations of hepcidin, ferroportin, and Mn transporter expression in the liver. It is currently unknown whether these changes in gene expression are the result of an initial change in Mn concentration or a response to the concomitant decrease in iron concentrations that occur secondarily - or a combination of the two.

In conclusion, oral administration of high dose Mn to neonatal mice resulted in Mn accumulation in several tissues, reduced body weight, and effects on motor activity. The effects of high dose Mn on motor activity appeared to be more consistent in C57Bl/6J mice when compared with parkin mice. A comparison of our results to previous findings reported by our laboratory also suggest that neonatal mice are more sensitive than similarly aged rats; however, additional studies using concurrent exposures are needed to more directly address this question. These changes were most robust in C57Bl/6J mice when compared with parkin mice. Our studies lay an important groundwork for future studies in mice and highlight the need to consider multiple metals when assessing Mn pharmacokinetics, neurotoxicity, and homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Body weight in PND 28 C57BL6/J and parkin mice following neonatal exposure to either 0, 11, or 25 mg Mn/kg-day from PND 1–28.

Figure 2.

Motor activity in PND 19 C57BL6/J (A) and parkin (B) mice following neonatal exposure to either 0, 11, or 25 mg Mn/kg-day. * Total number of movements was reduced in both the 11 mg Mn/kg-day ($p = 0.0027$) and 25 mg Mn/kg-day ($p < 0.001$) exposure groups when compared with controls. No statistically significant difference was observed between Mn treatment groups in the parkin mice ($p = 0.0677$).

Figure 3.

Mean ($\pm$ SEM) tissue manganese (Mn) concentrations in PND 29 C57Bl6J (wt) and parkin mice following neonatal exposure to either 0, 11, or 25 mg Mn/kg-day from PND 1–28. Asterisk indicates different from control (0 mg Mn/kg-day). † High dose significantly different when compared with low-dose Mn group. Bracket and ‡ indicates significant difference between C57Bl6J and parkin mice. $p < 0.05$.

Figure 4.

Linear correlations between striatal Mn concentration and spontaneous motor activity in PND 29 C57Bl6 (top) and parkin mice (bottom) following neonatal Mn exposure from PND 1–28. Asterisk indicates significant correlation. $p < 0.05$.

Figure 5.

Mean ($\pm$ SEM) liver (A) and frontal cortex (B) gene expression (normalized to β -actin) in PND 29 C57Bl/6J (wt) and parkin mice following neonatal exposure to either 0, 11, or 25 mg Mn/kg-day from PND 1–28. Asterisk indicates different from control (0 mg Mn/kg-day). Bracket and ‡ indicates significant difference between C57Bl/6J and parkin mice. $p < 0.05$.

Figure 6.

Mean ($\pm$ SEM) liver gene expression (as % of control averaged across three measurements using different housekeeping genes) in PND 29 C57Bl/6J (wt) and parkin mice following neonatal Mn exposure from PND 1–28. Asterisk indicates different from control. $p < 0.05$.

Figure 7.

Linear correlations between hepatic gene expression (as % of control averaged across three measurements using different housekeeping genes) in PND 29 C57Bl6 (A) and parkin mice (B) following neonatal Mn exposure from PND 1–28. Asterisk indicates significant correlation. $p < 0.05$.

Table 1.

Mean ($\pm$ SEM) total distance traveled and number of rears during a 15-minute observation period in C57Bl6J and parkin mice following neonatal exposure to either 0, 11, or 25 mg Mn/kg-day. Bold text indicates different from control (0 mg Mn/kg-day). $p < 0.05$.

Age	Endpoint	C57Bl6J			parkin		
		Manganese exposure (mg Mn/kg-day)			Manganese exposure (mg Mn/kg-day)		
		0	11	25	0	11	25
19–22	Distance traveled (m)	3.51 $\pm$ 0.19	2.94 $\pm$ 0.20	2.65 $\pm$ 0.37	3.32 $\pm$ 0.18	3.21 $\pm$ 0.17	2.58 $\pm$ 0.24
	Number of rears	76 $\pm$ 8	73 $\pm$ 10	39 $\pm$ 11	75 $\pm$ 9	82 $\pm$ 8	56 $\pm$ 9
	Sample size	23	22	13	19	26	24
29–32	Distance traveled (m)	4.06 $\pm$ 0.14	3.26 $\pm$ 0.13	3.36 $\pm$ 0.19	4.21 $\pm$ 0.15	3.96 $\pm$ 0.17	3.81 $\pm$ 0.28
	Number of rears	157 $\pm$ 12	139 $\pm$ 9	120 $\pm$ 12	166 $\pm$ 10	155 $\pm$ 12	142 $\pm$ 12
	Sample size	26	28	15	17	23	22