

Effects of developmental exposure to manganese and/or low iron diet: Changes to metal transporters, sucrose preference, elevated zero-maze, open-field, and locomotion in response to fenfluramine, amphetamine, and MK-801



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ABSTRACT

Manganese overexposure (MnOE) can be neurotoxic. In humans this can occur through occupational exposure, air or water contamination, well water, soy milk, and some baby formulas. In children MnOE has been associated with cognitive and behavioral deficits. The effects of MnOE may be modified by factors such as iron status. We hypothesized that developmental MnOE would be exacerbated by iron deficiency. A diet with a 90% decrease in iron (FeD) was given to gravid female rats starting on embryonic day 15 and continued through postnatal day (P) 28. Mn (100 mg/kg) or vehicle (VEH) was administered by gavage every other day from P4–28. Metal transporters and receptors (divalent metal transporter-1 (DMT1), transferrin (Tf), transferrin receptor (TfR), and Zrt-Irt-like protein 8 (ZIP8)) were quantified in brain at P28. These markers were increased but the changes were specific: MnOE increased TfR and decreased Tf in hippocampus, whereas FeD increased TfR in neostriatum and increased TfR and DMT1 in the hippocampus, and the combination increased TfR in neostriatum (ZIP8 was unaffected). Identically treated animals were tested behaviorally at P29 or P60. The combination of FeD + MnOE increased head dips in an elevated zero-maze, reversed deficits in sucrose preference induced by MnOE alone, and increased spontaneous locomotion in an open-field. Rats were also evaluated for changes in locomotor activity after challenge with (±)-fenfluramine (FEN, a 5-HT agonist: 5 mg/kg), MK-801 (MK801, an NMDA antagonist: 0.2 mg/kg), or (+)-amphetamine (AMPH, a dopamine agonist: 1 mg/kg). Compared with VEH animals, MnOE animals were more hyperactive after amphetamine or MK-801, and were less inhibited after fenfluramine, regardless of FeD exposure. The results indicate persistent effects of developmental MnOE on brain and behavior but few interactions with dietary iron deficiency.

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1. Introduction

Manganese overexposure (MnOE) can be a developmental neurotoxin in children [79,12,39,40,50]. A recent review shows that high levels of Mn can be ingested from well water or from other environmental sources and is a common route of exposure in children. Such exposures are associated with cognitive deficits, behavioral disinhibition, decreased IQ, and decreased performance

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on school-related tasks [80]. MnOE deficits are not always dose dependent; variable concentrations in the environment above or below recommended Center for Disease Control (CDC) and US Environmental Protection Agency (EPA) levels are related to similar symptoms [74,11]. Animal models of developmental MnOE recapitulate some of the signs observed in MnOE children [64,65,38,37], but the majority of animal studies focus on striatally-dependent deficits, since these are commonly observed after adult occupational MnOE [52,51,6]. Hence, it is not clear whether children with Mn-associated cognitive deficits also have dopaminergic changes. Moreover, the effects of MnOE can be modified by iron deficiency (FeD), which is common in children of many regions.

FeD is the most common nutritional deficiency in the world [47,75]. It is prevalent in women of child bearing age, especially

pregnant women, and consequently, in infants. Increased blood Mn has been observed in children with decreased Fe stores and low ferritin, indicators of FeD [42,56,60]. Similar to MnOE [32], studies on FeD have often focused on possible effects on striatal dopamine [49]. FeD and MnOE symptoms often appear related to dopaminergic function. In both human and animal studies, these effects appear as delayed sensory-motor reflex development, social and exploratory deficits, and sometimes cognitive deficits [49].

The similarity of such deficits may be related to shared physiological pathways for Fe and Mn. Competitive inhibition has been shown between Fe and Mn, and FeD is associated with increased Mn deposition in blood and brain [3,59]. Mn and Fe share metal transport proteins, including the divalent metal transporter-1 (DMT1), transferrin (Tf), transferrin receptor (TfR), and Zrt-Irt-like protein 8 (ZIP8) [66]. These proteins activate both Fe-independent and dependent transport and have high affinities for Mn [62,34,73]. These transporters are found in both the gastrointestinal system and brain, suggesting regulatory linkage between these two tissues.

The combination of FeD and MnOE has been investigated in adult rodents [20,63,1,23,76,24,41], but in only one developmental study [27]. In this study, the combination of FeD and MnOE resulted in decreased pup weight, reduced hematocrit, decreased Fe levels, increased brain Mn, and increased DMT1 and TfR expression. No significant changes in GABA or glutamate were found in cerebellum, cortex, hippocampus, striatum, or midbrain; other neurotransmitters were not investigated. Behavioral assessments were not included in this study.

Human studies investigating possible behavioral and cognitive deficits in children who are both FeD and MnOE have not been done. Metal absorption is higher in children because of their still developing gastrointestinal system; this also occurs in animals [43,21,77]. The developing nervous system is also vulnerable to toxin deposition [61,5]. Therefore, MnOE in combination with FeD during development warrants further investigation.

We previously investigated developmental MnOE where pups were exposed to 100 mg/kg Mn every other day from postnatal day (P) 4 to P28. Exposed pups exhibit increases in monoamine neurotransmitters in multiple regions of the brain at different ages prior to P29 [68]. For the following experiments, we combined MnOE and FeD by using a diet that was 90% below a standard diet in Fe content. The FeD diet was given to gravid dams beginning on embryonic (E) day 15 and continuing until their litters were P28. We first investigated the combined treatment on metal transport protein expression in brain immediately after cessation of exposure to determine the cumulative effect of treatment. In a separate experiment, we assessed behavior: anxiety using the elevated zero-maze, anhedonia using the sucrose preference test, and probed neurotransmitter functionally using selective pharmacological agents in a locomotor test. Anxiety and activity were assessed because others showed that developmental Mn exposure alters anxiety-related measures in the elevated-plus maze and open-field [38,54]. Iron deficiency can lead to depression; therefore, the sucrose preference test was included to assess anhedonia.

2. Materials and methods

2.1. Animals

Male sires and nulliparous female Sprague–Dawley CD (IGS) rats (Charles River Laboratories, Raleigh, NC, strain #001) were habituated for at least one week in the vivarium (AAALAC-accredited) prior to breeding. Animals were maintained on a 14:10 h light:dark cycle (lights on at 600 h) with controlled temperature (19 ± 1 °C) and humidity (50 ± 10 %). Animals were housed in a barrier using the Modular Animal Caging System (Alternative Design, Siloam

Spring, AR). HEPA filtered air was supplied to cages directly (Alternative Design, Siloam Spring, AR) at 30 air changes/h. Water was provided to each cage using an automated system (SE Lab Group, Napa, CA); NIH-07 diet was provided ad libitum except during the FeD feeding period. A curved stainless steel enclosure was placed in each cage as enrichment [71]. Following cohabitation, detection of a sperm plug counted as E0. Birth was designated as P0 and offspring were weaned at P28 [10,57].

2.2. Iron deficient diet

FeD diet was based, in part, on previous studies [35,67,45,44,46]. Pregnant females were kept on a standard NIH-07 diet until E15. From E15 to birth dams, and after birth, litters were provided one of two purified diets (Land O' Lakes Purina Feed, Evansville, IN) in which only Fe content differed. Half the dams and litters were given an iron sufficient diet (350 ppm, FeS) and half an iron insufficient diet with a 90% decrease (35 ppm, FeD, [25]). Offspring were placed back on the sufficient NIH-07 diet at weaning (P28) and continued on this diet throughout the remainder of the experiment.

2.3. Manganese overexposure

Litters were culled to 12. Three male and female pairs within each litter were gavaged with 0 mg/kg Mn (VEH: 0.01 M anhydrous sodium chloride) or 100 mg/kg Mn chloride (100Mn) in a volume of 3 ml/kg as previously done [30]. Gavage was used to avoid maternal exposure and provide oral exposure. Oral intake is a common route for developmental exposure in humans [53]. Rat pups were gavaged every other day from P4 until P28.

2.4. Metal transport proteins

One group of animals was used for the metal transporter experiment. These rats were sacrificed at P29 to assess metal transporters in the hippocampus and neostriatum. Brains were dissected over ice using a 1 mm brain block (Zivic-Miller, Pittsburgh, PA) by carving out the regions of interest from each slice as described [28]; tissues were frozen at -80 °C until assayed by Western blot. Frozen brain tissues were homogenized in RIPA buffer with a protease inhibitor. Samples were spun at 4 °C for 20 min at $14,000 \times g$; protein was assayed using the BCA kit (Pierce Biotechnology, Rockford, IL) and then normalized. The following metal transporters were assayed: divalent metal transport 1 (rabbit α DMT1, 1:5000, Alpha Diagnostic International, San Antonio, TX), transferrin protein (rabbit α Tf, 1:1000, Abcam, Cambridge, MA), transferrin receptor (TfR, 1:1000, Abcam, Cambridge, MA), and zinc and iron-related protein 8 (also called Slc39A8; rabbit α ZIP8, 1:1300, Proteintech Group, Chicago, IL). Aliquots of cellular protein at either 10 μ g (DMT1, ZIP8, Tf, and TfR) or 25 μ g (Tf) and a molecular weight standard were loaded on 12% gels under reducing conditions and blotted on PVDF membranes for 90 min at room temperature using the Protean Tetra system (Bio-Rad, Hercules, CA). Blots were blocked using either 5% (DMT1, ZIP8) or 10% casein (Tf, TfR) in Tris-buffered salt with Tween20 (0.01% TBST) for 30 min at room temperature. Primary antibodies were incubated overnight at 4 °C. After being washed, blots were incubated with goat α rabbit secondary antibody conjugated with horseradish peroxidase (1:5000, Jackson Labs, West Grove, PA) for 2 h at 4 °C. Immunoreactive proteins were detected using enhanced chemiluminescence (Western Bright ECL, Advansta, Menlo Park, CA) and quantified using densitometry software (ImageJ, National Institutes of Health, Bethesda, MD). Relative density was calculated against FeS \times VEH animals (4 samples per diet \times Mn combination and run in triplicate).

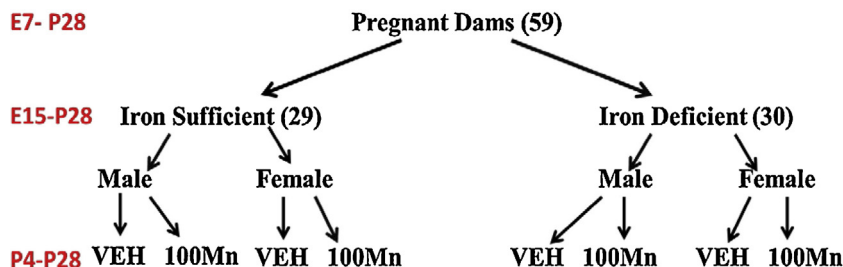


Fig. 1. Study design and number of litters per condition. FeD and MnOE paradigms are the same between Western blot and behavior experiments, but litter numbers are listed for behavioral experiments. At E15, dams were removed from NIH-07 diet and placed on either a purified FeS or FeD diet. Nutritional content differed only in Fe content (350 mg/g vs 35 mg/g). At P4, male and female offspring (3 per litter) were assigned to either 100 mg/kg MnCl₂ or isotonic VEH. Offspring were exposed via gavage every other day from P4 to P28. MnOE and FeD diet ended on P28 and offspring were placed back on NIH-07 diet for the remainder of the experiment.

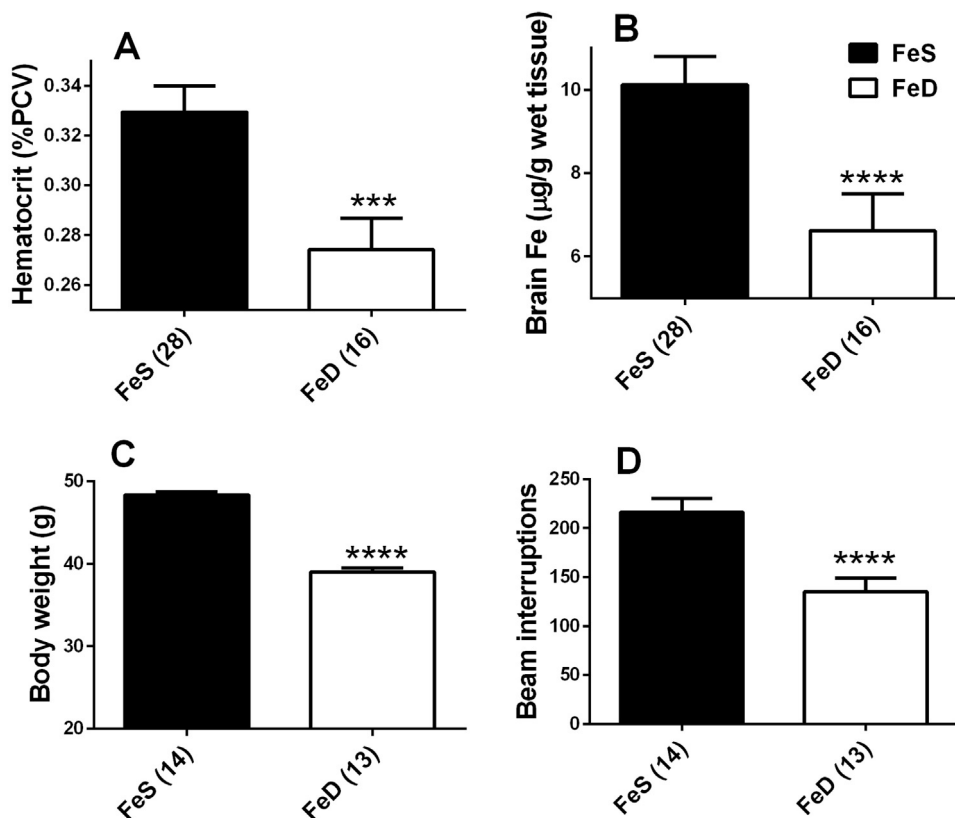


Fig. 2. Purified diet with a 90% decrease in Fe content given from E15 to P28 resulted in signs of FeD. For each panel, data were combined across sex and age during the lactation period. A, FeD diet caused a 22% decrease in hematocrit in offspring. B, Brain Fe (measured by LCP-MS), was decreased in FeD offspring compared with FeS controls. C, FeD offspring were significantly lighter than FeS controls during the preweaning period. D, During a 1 h locomotor activity test at P29, FeD offspring were less active than FeS controls. *** $p < 0.001$; **** $p < 0.0001$.

2.5. Behavior

A separate group of animals was used for the behavioral study. Litters were culled on P1 to 12, 6 males and 6 females using a random number table to assign pups to exposure groups within litters. Hence, within each litter there were 3 male/female pairs treated with Mn and 3 male/female pairs treated with vehicle. Two pairs within each litter (one Mn and one vehicle) received behavioral testing starting on P29; two pairs within each litter (one Mn and one vehicle) received behavioral testing starting on P60, and the third set of pairs were held and not tested and used if and only if one of the animals designated for behavioral testing died prior to the start of testing. The design of the behavioral study is shown

in Fig. 1. Maternal weights for these litters were taken at E21, P1, and P28. Offspring were weighed at P1, every other day throughout MnOE, and prior to behavioral testing on P29 or P60. Offspring from all groups within litters (Fig. 2) were tested in the following tests beginning at P29 (young) or at P60 (adult). Behavioral testing was conducted by personnel blind to group enrollment.

2.5.1. Elevated zero-maze

The elevated zero-maze (EZM) is a circular runway 105 cm in diameter with a 10 cm path width made of black Kydex and divided into four equal quadrants. Two opposite quadrants have black acrylic sidewalls (28 cm high) and two quadrants have 1.3 cm clear acrylic curbs to prevent falls. The runway was mounted 72 cm above

the floor [13]. Rats were placed in the center of a closed quadrant and behavior was scored live using a video camera connected to a monitor outside the test room where the experimenter scored each behavior by direct observation. The maze was illuminated by a single halogen lamp (approximately 11.7 lux at the center of the maze) and between animals the maze was cleaned with 70% ethanol. Dependent measures were: latency to open, time in open, open entries, and head dips. Time in open was defined as the animal having both front legs past the boundary of the closed area extending into an open quadrant. Data were analyzed by age.

2.5.2. Sucrose preference

This test was conducted on either P30 or P61 in separate animals. In an adapted protocol [72], animals were food and water deprived overnight for 20 h. After habituation to a new cage, animals were exposed ad libitum to two pre-weighed water bottles (one with tap water and one with 1% sucrose). After 30 min, bottles were switched with new, pre-weighed bottles and animals were allowed ad libitum access for an additional 30 min. When the new bottles were placed in the cage, the side on which the water and sucrose were placed was reversed to prevent side preference. Sucrose preference was determined by the decrease in weight of sucrose bottles over the total decrease in weight of all bottles. Data were analyzed by age.

2.5.3. Locomotor activity: baseline, saline, and drug challenge

This test was conducted the day following sucrose preference on P31 or P62–66. Rats were placed in 40 × 40 cm² automated open-field activity monitors (PAS System, San Diego Instruments (SDI), San Diego, CA) and allowed to ambulate for 1 h then briefly removed and given a subcutaneous injection of saline (SAL, 3 ml/kg), and tested another 1 h. Following the second hour, animals received a subcutaneous injection of one of three drugs at established doses: (±)-fenfluramine (FEN, a 5-HT agonist: 5 mg/kg [4,26]), MK-801 (MK801, an NMDA antagonist: 0.2 mg/kg [29]), and (+)-amphetamine (AMPH, a dopamine agonist: 1 mg/kg [33,78]). Animals were tested for 2 h after drug challenge. This paradigm allows for animals to act as their own control (both for drug and injection stress) and reduces the number of animals required for the study. Chambers were cleaned with 70% EtOH between animals.

Activity was measured by recording the total number of beam interruptions over each 5 min interval. The results of the first two hours (habituation and saline) are presented individually for each age. FEN, MK-801, and AMPH results are presented separately and within that section, effects of age and developmental exposure are discussed.

2.6. Statistical analyses

Data were analyzed using mixed linear factorial analysis of variance (ANOVA; ProcMixed, SAS v9.2, SAS Institute, Cary, NC) and analyzed by age for behavioral studies. Between subject factors were diet (FeS or FeD), Mn exposure (SAL or 100Mn), and sex (M or F). Within subject factors were day for weight and interval for locomotor activity. In order to account for litter effects, litter was treated as a randomized block factor. Randomized block designs require that subjects be assigned to subgroups (blocks) in which the variability is less within blocks than between blocks, in this case variability within litters is less than variability between litters, which makes this design well suited to control for litter effects. Then MnOE is randomly assigned within blocks, i.e., in this case within litters and these factors, MnOE and sex, are therefore treated as between subject factors since litter is already considered. Significant interactions were further analyzed using slice-effect ANOVAs as a post hoc test. Kenward-Rodgers degrees of freedom were used. Significance was set at $p \leq 0.05$. Data are presented as least square

mean ± SEM unless otherwise specified. Complete *F*-ratios are only presented for diet, Mn exposure, and their interactions; *p*-values are included for everything else.

3. Results

3.1. Effectiveness of FeD

A validation experiment (8–10 litters/diet) was performed before the main experiment. It revealed that the FeD diet decreased hematocrit (Hct) in both FeD dams (0.37 ± 0.03 vs. FeS: 0.47 ± 0.03 , $F(1,12.5) = 4.39$, $p < 0.05$ at E18) and pups (at P7, P14, P21, and P28: $F(1,50) = 11.49$, $p < 0.001$; Fig. 2A). In pups, this resulted in a 22% decrease in Hct relative to FeS diet, consistent with clinical FeD, but not enough to qualify as anemia [8]. FeD offspring showed a significant reduction in brain Fe (repeated measures analysis at P7, P14, P21, and P28, $F(1,28) = 9.67$, $p < 0.001$, Fig. 2B, collapsed across age). The FeD diet also resulted in decreased pup weight during lactation (repeated measures analysis from P7 to P28 weekly weights: $F(1,114) = 17.39$, $p < 0.0001$, Fig. 2C, weight collapsed across age). At P28, offspring were hypoactive in a test of locomotor activity ($F(1,114) = 17.39$, $p < 0.0001$, Fig. 2D). Hematocrit and brain Fe levels returned to normal levels by P60 in the FeD animals (not shown).

3.2. Blood and brain manganese

Brain and blood Mn concentrations were reported elsewhere [68]. In that experiment, rats exposed to Mn (100 mg/kg) had significantly increased levels of Mn in the neostriatum relative to VEh-treated rats ($F(1,23) = 230.3$, $p < 0.0001$), i.e., VEh = 0.39 ± 0.12 µg/g vs. Mn100 = 2.39 ± 0.12 µg/g tissue. Serum Mn levels were somewhat elevated ($F(3,31) = 1.58$, $p < 0.10$), i.e., VEh = 11.67 ± 4.75 µg/L vs. Mn100 = 16.62 ± 4.75 µg/L.

3.3. Metal transporters

At P29, metal transporters DMT1, ZIP8, Tf, and TfR were assessed by Western blot in the hippocampus and neostriatum to investigate how the combination of FeD diet and 100Mn exposure affected these key markers in regions of interest. These two regions were chosen because Mn is closely associated in the brain with dopamine, and dopamine is in greatest abundance in neostriatum. In addition, this model is being used to assess the effects of FeD and MnOE on spatial and egocentric learning based on evidence that spatial learning is largely mediated by the hippocampus and egocentric learning is largely mediated by the striatum [14]. In the hippocampus, both DMT1 ($F(1,12) = 10.86$, $p < 0.01$) and TfR ($F(1,12) = 4.70$, $p < 0.05$) were significantly increased by the FeD diet in comparison with the FeS diet (Fig. 3A), which replicates previous studies and confirms the efficacy of our exposure method. Although short of being significant ($p < 0.07$), 100Mn exposure showed a tendency to increase DMT1, whereas TfR protein was significantly increased by 100Mn exposure ($F(1,4) = 19.09$, $p < 0.001$, Fig. 3B). Interestingly, Tf protein was differentially decreased by a Mn × diet interaction ($F(1,12) = 6.87$, $p < 0.05$, Fig. 3C). Although 100Mn or FeD decreased Tf protein, the combination of FeD × 100Mn resulted in protein levels similar to controls (Fig. 3C). ZIP8 was unaffected by either factor or the combination.

DMT1, ZIP8, and Tf protein levels were unaffected by the developmental FeD or MnOE in the neostriatum. TfR was increased by FeD ($F(1,9) = 6.55$, $p < 0.05$) in the neostriatum. In this case, TfR was affected by a Mn × diet interaction ($F(1,9) = 5.13$, $p < 0.05$), where FeD exposure increased protein levels, but the combination showed a slight but significant reduction (Fig. 3D).

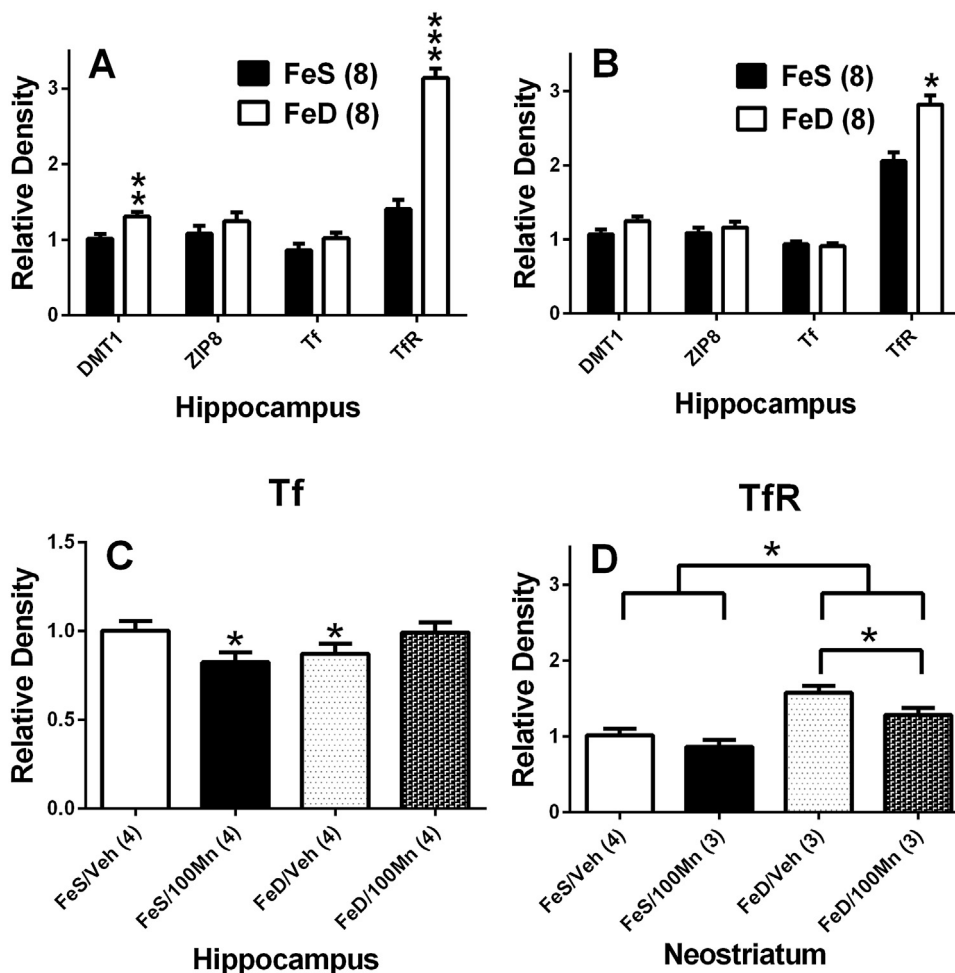


Fig. 3. Metal transporters in brain. Both FeD diet and 100Mn altered metal transport protein levels in the hippocampus and neostriatum at P29 as shown by Western blot. A, FeD diet significantly increased DMT1 and TfR protein in the hippocampus. B, 100Mn exposure significantly increased TfR in the hippocampus; DMT1 showed a nearly significant trend ($p < 0.07$), and ZIP8 and Tf protein were unaffected. C, A significant interaction of the FeD diet and 100Mn exposure on Tf in the hippocampus showed that protein levels were significantly reduced by 100Mn or FeD, but not by the combination. D, TfR protein was significantly increased by FeD irrespective of Mn exposure, however, the combination of the two partially, but significantly offset the FeD effect. Protein was measured by relative densitometry and normalized to the FeS \times VEH group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. the FeS \times VEH group.

3.4. Mortality

A total of 630 animals were used in the locomotor activity test (9–17 animals per age \times sex \times diet \times Mn \times drug). There were 69 deaths (11% overall) of which 66 (96%) were in the 100Mn group. Deaths within each diet group were not statistically different (30 FeS vs. 36 FeD pups); 61% of deaths were male.

3.5. Maternal body weight and litter measures

No significant differences in maternal weight gain were found between diets (not shown). There were also no significant differences in maternal weight during lactation (not shown). The number of pups born, sex ratio, and length of pregnancy did not differ between diet groups (not shown).

3.6. Offspring body weight

On P1, FeD pups showed a trend toward weighing less than FeS pups, 7.66 ± 0.32 g vs. 8.30 ± 0.29 g, respectively: $F(1,490) = 3.11$, $p < 0.08$. Females weighed less than males ($p < 0.0001$), but there were no interactions with diet.

Offspring body weights from P4 to P28 were analyzed using repeated measures ANOVA. Significant main effects of the FeD

diet ($F(1,619) = 97.05$, $p < 0.0001$) as well as a significant interaction with day ($F(12,6693) = 28.81$, $p < 0.0001$) were found. FeD animals were smaller than their FeS counterparts (collapsed across age: 36.33 ± 0.47 g vs. 38.69 ± 0.46 g, respectively). Across days, this decreased weight between groups became significant by P10. 100Mn animals also weighed less than their VEH counterparts (32.31 ± 0.46 g vs. VEH: 42.70 ± 0.47 g, $F(1,619) = 249.3$, $p < 0.0001$). This was also true across the exposure period ($F(12,6693) = 32.88$, $p < 0.0001$); 100Mn animals were significantly lighter by P6, the second day of exposure. A significant main effect of sex ($p < 0.001$) and sex \times day interaction ($p < 0.001$) revealed that females weighed less than males, regardless of exposure.

Pup weights were significantly affected by the combination of FeD diet and 100Mn exposure during the dosing period ($F(1,619) = 5.45$, $p < 0.05$). FeD \times 100Mn offspring were significantly lighter than FeS \times VEH animals (29.84 ± 0.69 g vs. 46.71 ± 0.62 g), but were also significantly lighter than offspring exposed to either factor (FeS \times 100Mn: 34.78 ± 0.61 g and FeD \times VEH: 38.69 ± 0.71 g). There was also a significant diet \times Mn \times day interaction ($F(12,6693) = 1.82$, $p < 0.05$). By P8, the FeD \times 100Mn offspring weighed significantly less than other groups (Fig. 4).

At P60 rats were weighed before the start of behavioral testing. FeD animals were now similar in weight to FeS animals, but 100Mn-exposed animals remained lighter compared with VEH animals

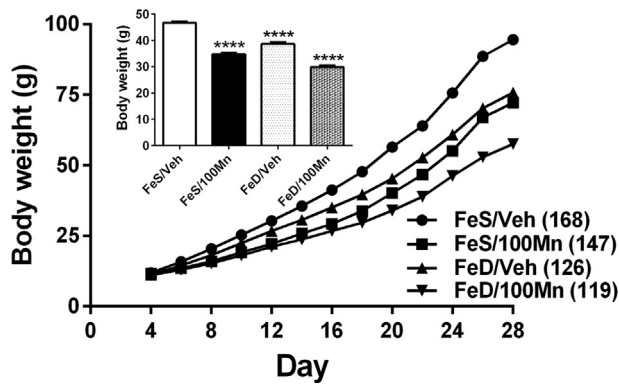


Fig. 4. Offspring body weight. Offspring weights were significantly decreased by FeD diet and 100Mn exposure and further reduced by the combination of both. Inset: average weights during the preweaning period. **** $p < 0.0001$.

(($F(1,221) = 97.75$, $p < 0.0001$): 100Mn: 258.61 ± 4.50 g vs. VEH: 290.88 ± 4.48 g). There was no significant diet \times Mn interaction on body weights at adult ages. A significant sex \times Mn interaction confirmed that both 100Mn males and females were smaller than their VEH counterparts ($F(1,221) = 5.74$, $p < 0.05$). Regardless of developmental exposure, females were smaller than males as adults ($p < 0.0001$).

3.7. Behavior

3.7.1. Elevated zero-maze

At P29, there were no significant effects of 100Mn on any of the EZM dependent variables. However, the FeD diet, regardless of 100Mn exposure, significantly affected time spent in open quadrants ($F(1,273) = 5.08$, $p < 0.05$) and number of open entries ($F(1,273) = 4.04$, $p < 0.05$). These animals spent a shorter time in the open (FeD: 83.86 ± 9.05 s vs. FeS: 110.59 ± 8.03 s) and had a decreased number of open entries (FeD: 8.11 ± 0.77 s vs. FeS: 10.14 ± 0.68 s). Interestingly, the number of head dips was significantly affected by a Mn \times diet interaction ($F(1,273) = 4.09$, $p < 0.05$). The combination of 100Mn exposure and FeD significantly decreased the number of head dips compared with other groups (100Mn \times FeD: 4.29 ± 0.80 vs. FeS \times VEH: 6.28 ± 0.69 , FeS \times 100Mn: 6.52 ± 0.71 , FeD \times VEH: 5.48 ± 0.78). Sex was not significant and no significant interactions with 100Mn or FeD were noted for any measure.

At P60, the behavioral phenotype was different for 100Mn animals, regardless of diet. At this age, these animals spent more time in the open ($F(1,214) = 27.09$, $p < 0.0001$; 100Mn: 144.82 ± 6.51 s vs VEH: 120.07 ± 6.54), had increased open entries ($F(1,214) = 14.15$, $p < 0.0001$; not shown), and although not significant, had a tendency for shorter latency to first open quadrant entry ($F(1,210) = 2.89$, $p < 0.09$). 100Mn animals also had increased head dips compared with VEH animals ($F(1,214) = 14.64$, $p < 0.0001$; 100Mn: 8.01 ± 0.81 vs VEH: 6.33 ± 0.73). FeD animals, now repleted, exhibited opposite patterns from their younger counterparts. In comparison with FeS animals, adult FeD animals spent more time in the open ($F(1,214) = 4.34$, $p < 0.05$; not shown) and had increased head dips ($F(1,214) = 4.13$, $p < 0.05$; FeD: 7.97 ± 0.77 vs FeS: 6.36 ± 0.77). There were no significant effects of the FeD \times 100Mn combination. At P60, all EZM dependent measures were significantly affected by sex ($p < 0.0001$). A significant sex \times Mn interaction ($F(1,214) = 9.13$, $p < 0.01$) showed that the significant increased time in the open in Mn animals was specific to 100Mn males (142.26 ± 6.15 s vs VEH: 102.32 ± 6.34 s).

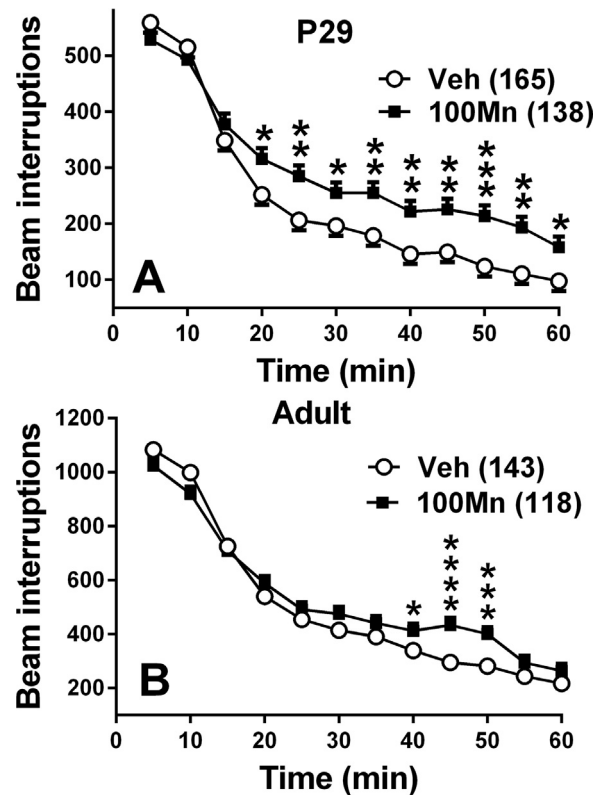


Fig. 5. Locomotor activity: Baseline. A, During the first hour of testing, the P29 100Mn group showed an increase in activity (photobeam interruptions) during the habituation phase compared with VEH group from 20 min onward ($p < 0.0001$). B, Adult 100Mn animals had significantly increased activity compared with VEH animals only during minutes 40–50 ($p < 0.0001$). Data are shown in 5 min intervals. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.7.2. Sucrose preference

Sucrose preference was not significantly affected by 100Mn exposure at P29. It was also unaffected by FeD diet. However, a diet \times Mn interaction revealed that FeS \times 100Mn animals had significantly decreased sucrose preference compared with the FeS \times VEH group ($F(1,271) = 4.06$, $p < 0.05$). There were no significant effects of sex at P29.

At P60, sucrose preference was also unaffected by 100Mn exposure or FeD diet. However, a significant sex \times diet \times Mn interaction ($F(1,200) = 7.14$, $p < 0.01$) revealed that in males, 100Mn exposure decreased sucrose preference compared with VEH animals in the FeS group (0.61 ± 0.02 vs. VEH: 0.67 ± 0.02).

3.7.3. Locomotor activity

3.7.3.1. Habituation. Animals tested for locomotor activity received three phases of testing: Baseline with no treatment; Saline with saline-only injection; and drug challenge with one of three drugs (see [29,69,70]). At young ages, there was no significant main effect of diet; there was a significant diet \times interval interaction ($F(11,3466) = 2.39$, $p < 0.01$). Further analysis revealed that FeD animals were significantly hypoactive during the initial 5 min interval (491.88 ± 19.09 vs. FeS: 595.01 ± 17.82) and maintained a lower level of activity throughout the first hour. Separately, there was a significant main effect of 100Mn exposure on activity at the younger age ($F(1,314) = 8.48$, $p < 0.01$); 100Mn animals were more active than controls. A significant Mn \times interval interaction ($F(11,3466) = 4.34$, $p < 0.0001$) revealed that 100Mn animals were more active than VEH animals during most of the test period (Fig. 5A). During this phase, females were also more active than males ($p < 0.01$). There were no other significant effects at this age.

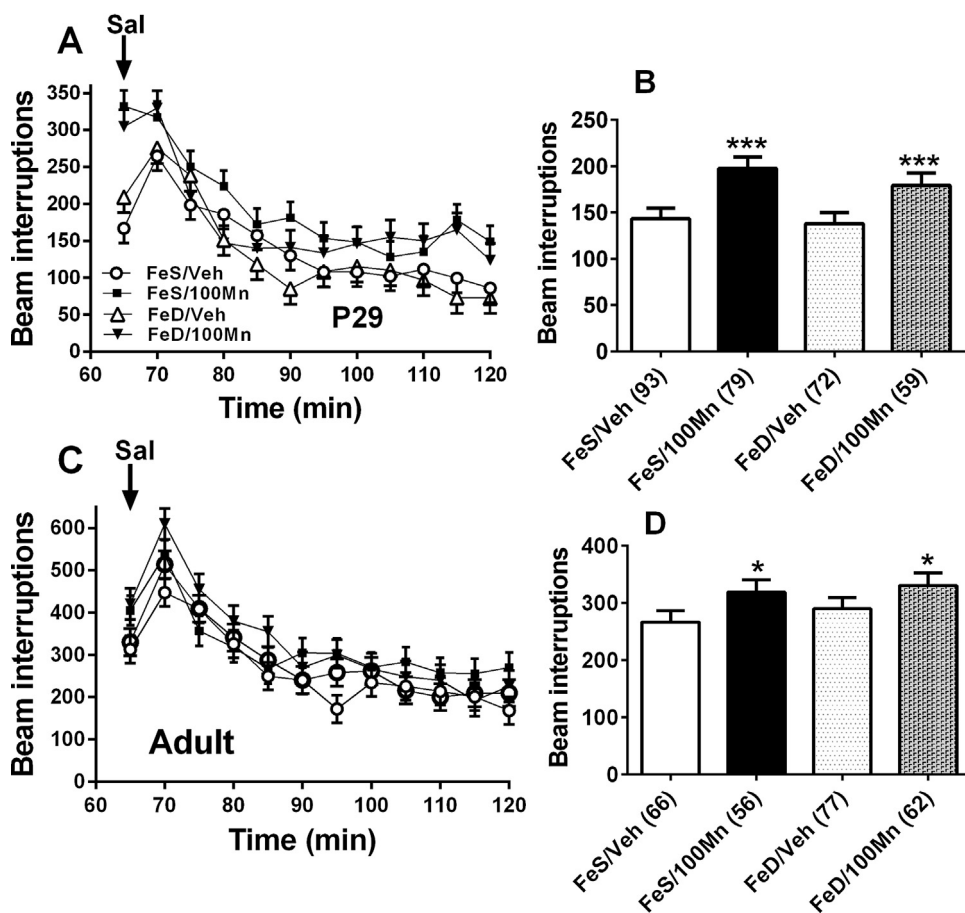


Fig. 6. Locomotor activity: Saline. During the second hour of locomotor activity after a subcutaneous injection of saline, A, P29 rats in the 100Mn group continued to show increased activity ($p < 0.0001$) compared with the VEH group. B, The P29 data collapsed across intervals ($p < 0.001$). C, The adult 100Mn group remained more active than the VEH group. D, the adult data collapsed across intervals ($p < 0.05$). * $p < 0.05$; *** $p < 0.0001$ vs. controls.

As adults, there were no significant main effects of FeD diet or 100Mn exposure. However, there was both a significant diet \times interval interaction ($F(11,2563) = 1.95$, $p < 0.05$) and a Mn \times interval interaction ($F(11,2563) = 5.43$, $p < 0.0001$). FeD animals were now more active than FeS animals, especially during initial intervals (not shown). Adult 100Mn animals exhibited the same pattern of activity as young 100Mn animals, i.e., a higher level of activity during the latter half of the test session (Fig. 5B). During this phase, females were also more active than males ($p < 0.001$). There were no other significant effects.

3.7.3.2. Saline. Prior to the second hour of testing, animals were removed briefly and injected with saline. This was done to control for the stress of removal and injection. At the young age, 100Mn animals maintained increased activity similar to the habituation phase, as both a main effect ($F(1,314) = 14.54$, $p < 0.001$) and as an interaction with interval ($F(11,3466) = 3.34$, $p < 0.0001$). Although activity levels decreased over the second hour, analysis at the last 5 min interval revealed that 100Mn animals remained significantly more active compared with VEH animals (Fig. 6A). There was no significant main effect of FeD. Females were more active than males but sex did not interact with Mn (sex \times interval interaction: $p < 0.01$). A significant diet \times sex \times interval interaction ($F(11,3466) = 1.81$, $p < 0.05$) was found, but post hoc analysis using slice effects ANOVAs revealed no significant differences between groups at any interval. The combination of FeD and 100Mn had no effect at this age, and no other significant effects were found.

As adults, there was a significant main effect of 100Mn ($F(11,3466) = 1.81$, $p < 0.05$); 100Mn animals were more active compared with VEH animals (324.53 ± 15.48 vs. 278.14 ± 13.85). However, there was no interaction with interval; 100Mn and VEH groups were similar by the end of hour 2 (Fig. 6B). FeD did not significantly affect activity levels at adult ages. Females were more active than males as before (sex \times interval interaction: $p < 0.01$) but sex did not interact with diet or Mn. No combination effect of FeD and 100Mn was seen.

3.7.3.3. Drug challenge. Activity was significantly affected by drug challenge across diet, Mn, age, and sex ($p < 0.0001$). This overall effect was attributable to the general decrease in activity after FEN dosing and increase after MK-801 or AMPH. Accordingly, separate analyses were performed for each drug.

3.7.3.4. Fenfluramine. At the younger age, the FeD diet had no differential effect on activity after FEN treatment. However, 100Mn animals had greater activity compared with the VEH group during the challenge period (150.08 ± 23.76 vs. VEH: 76.78 ± 20.65 , $F(1,95) = 5.42$, $p < 0.05$). There was no significant interaction between FeD and 100Mn treatment (Fig. 7A). Sex did not affect activity, but there was a significant sex \times diet \times interval interaction ($F(23,2185) = 1.7$, $p < 0.05$). Post hoc analysis using slice effect ANOVAs revealed that during initial intervals FeD females were more active compared with other groups, but this effect dissipated during the later intervals.

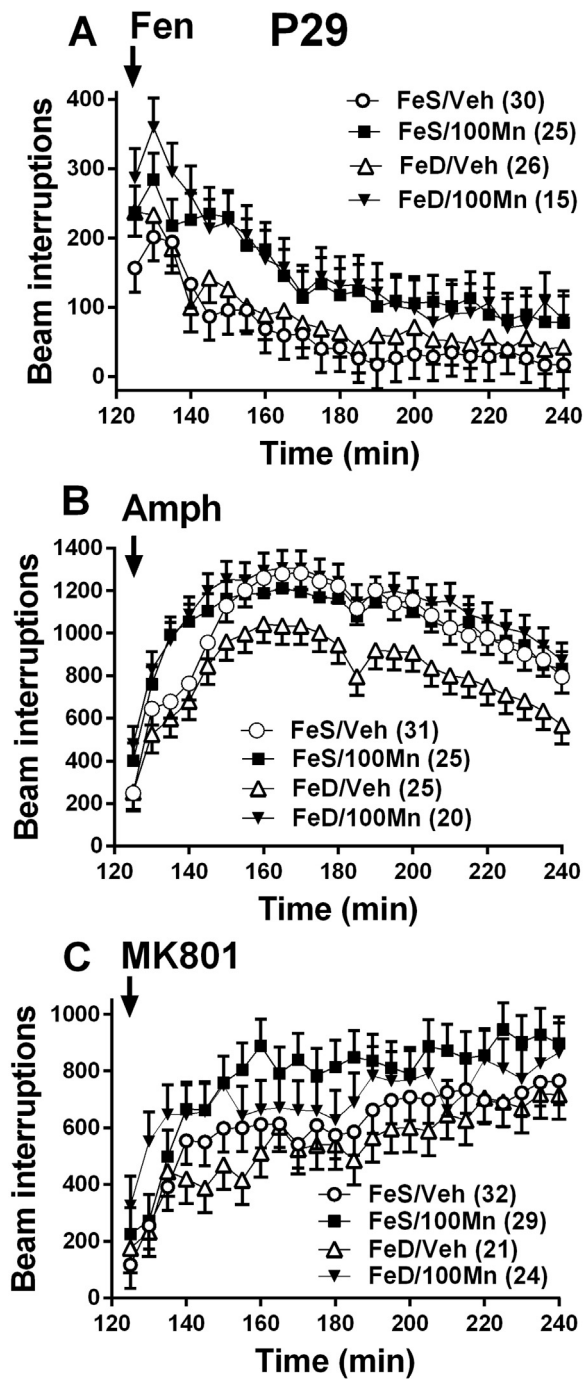


Figure 7

Fig. 7. Locomotor activity at P29: Drug challenge: During hours 3 and 4, activity was monitored following subcutaneous injection with one of three drugs. A, Challenge with (\pm)-fenfluramine (FEN), a 5-HT agonist. The 100Mn groups had significantly increased activity compared with VEH animals, regardless of diet ($p < 0.05$); no other effects were significant. B, Challenge with the indirect dopaminergic agonist (+)-amphetamine (AMPH). The 100Mn groups, regardless of diet, had significantly increased activity compared with VEH animals ($p < 0.05$). C, Challenge with the NMDA receptor irreversible antagonist MK-801. The 100Mn groups showed significantly increased activity compared with VEH groups regardless of diet ($p < 0.05$).

As adults, there were no significant effects of FeD diet or 100Mn exposure after FEN treatment (not shown). There was also no significant diet \times Mn interaction. Although there was no significant main effect of sex, there was a significant sex \times interval interaction ($p < 0.001$); females were more active during most intervals than males.

3.7.3.5. Amphetamine. In younger animals, FeD did not significantly affect activity after AMPH treatment. However, 100Mn exposure significantly increased activity after AMPH more than in controls (main effect: $F(1,106) = 4.97$, $p < 0.05$, 100Mn: 1066.68 ± 51.98 vs. VEH: 902.74 ± 51.98). This was also true across intervals (Mn \times interval interaction: $F(23,2438) = 3.16$, $p < 0.0001$). A diet \times Mn interaction approached significance ($F(1,106) = 3.48$, $p < 0.07$; (Fig. 7B)). Although sex was not significant, there was a significant sex \times interval interaction ($p < 0.0001$) showing that females maintained higher levels of activity after AMPH than males. There were no other significant main effects or interactions.

As adults, there were no significant differential effects of FeD, 100Mn, or the interaction of the two as a function of AMPH-induced hyperactivity (not shown). Sex was not significant, but there was a significant sex \times interval interaction ($p < 0.0001$) that showed females were more active after AMPH than males. There were no other significant main effects or interactions.

3.7.3.6. MK-801. In the younger animals, there was no significant effect of FeD diet on activity after MK-801 treatment. A significant main effect of 100Mn exposure ($F(1,96.9) = 5.88$, $p < 0.05$) revealed that 100Mn animals were more hyperactive after MK-801 than controls (733.79 ± 51.53 vs. VEH: 569.69 ± 43.88), but there was no interaction with interval. There was also no interaction between FeD and 100Mn following MK-801 (Fig. 7C). A significant main effect of sex revealed that males had increased beam breaks compared with females after MK-801 treatment ($p < 0.05$) and that this was true over time ($p < 0.0001$). No other interactions were significant.

As adults, there were no significant differential effects of FeD diet or 100Mn exposure on activity following MK-801 (not shown). There was also no interaction between the FeD diet and 100Mn on activity after MK-801. A significant main effect of sex revealed that males again had increased hyperactivity after MK-801 compared with females ($p < 0.01$) and that this was true across intervals ($p < 0.0001$). A significant diet \times sex interaction ($F(1,73) = 4.59$, $p < 0.05$) showed that FeD males had the highest activity compared with other sex \times diet groups (1228.39 ± 93.65 vs. FeS males: 942.68 ± 90.49 , FeS females: 846.44 ± 90.48 , and FeD females: 736.82 ± 94.27). This higher activity was maintained across intervals as shown by a significant sex \times diet \times interval interaction ($F(23,1675) = 2.01$, $p < 0.01$). No other interactions were significant.

4. Discussion

This study examined the interaction between FeD and MnOE in a rat model of relevant childhood exposures. Both FeD and MnOE cause neurocognitive and behavioral deficits in children, hence investigating the combination is important. While others have examined FeD and MnOE in adult animals [19,23], there is a dearth of developmental data on this combination. This set of experiments sought to examine how FeD and MnOE interact during development to affect metal transport protein status and behavior.

We found evidence for FeD and MnOE interaction on metal transport proteins in the hippocampus. A significant diet \times Mn interaction for Tf protein in the hippocampus showed that FeD \times 100Mn increased protein levels back to FeS \times VEH levels. This

was an unanticipated result and suggests that increased Mn exposure during FeD binds Tf, elevating circulating protein levels even when the Fe is low. However, the TfR protein was not significantly changed by a FeD \times 100Mn interaction showing that receptor number was unaffected.

Animals in the behavioral experiment were weighed prior to weaning as a measure of general health. Although FeD and Mn exposed animals were lighter than controls during nursing, and the combination exacerbated the decrease in body weight. These results are similar to previous studies [27,35,67,46]. Smaller body weights in young children are correlated with suboptimal neurodevelopmental outcomes [2]. This interaction was transient, however, and disappeared by P60.

At P29, FeD \times 100Mn animals had decreased head dips in the EZM, but no other effects were noted on this test. FeD \times 100Mn animals had decreased sucrose preference at P30, and this was also seen in FeD \times 100Mn males at P61. Taken together, the data suggest that the combination of exposures results in increased anxiety, decreased exploration, and anhedonia.

Overall, treatment with fenfluramine decreased locomotor activity. MnOE attenuated this fenfluramine-induced activity reduction resulting in the MnOE animals remaining more active than the non-MnOE groups. The FeD diet and 100Mn combination did not significantly affect locomotor activity habituation or the response to saline in the open-field test. Likewise, the combination did not cause differences in response to FEN or MK-801 on locomotor activity at either age. Although only a trend, FeD \times VEH animals had decreased locomotor activity compared with other diet \times Mn groups after AMPH at P29. The FeD \times Mn combination had the greatest effect on activity during the early intervals after FEN injection. Looked at another way, the addition of MnOE to animals on the FeD diet ameliorated their reduced activity after fenfluramine. The reasons behind this are unclear, but align with the pattern seen with Tf protein levels. This was not seen at adult ages. Also, at P29, amphetamine increased activity in all groups, however, further along in testing, the FeS-MnOE group remained less hyperactive in response to amphetamine than all other groups.

The FeD diet alone had modest effects. FeD increased DMT1 and TfR in the hippocampus and TfR in the neostriatum. This is similar to previous studies [27] and suggests that Fe stores were low in the brain and expression was increased in order to compensate and transport more Fe into these regions. As expected, FeD animals were smaller during lactation. After weaning, FeD animals were placed back on a FeS diet, and they showed recovery of body weight by P60, indicating Fe repletion. This was confirmed by hematocrit and brain Fe levels that were similar to FeS animals at P60. The effects of Fe repletion were also seen in behavioral measures, particularly in the EZM. At younger ages, FeD animals were hypoactive in the EZM based on the decrease in the number of open entries and time spent in the open quadrants that suggested increased anxiety. We also found hypoactivity in locomotor testing and this was shown before [67]. Some of the changes seen at P29 were no longer present at P60, i.e., FeD animals at P60 exhibited increased time in the open and increased head dips in the EZM compared with FeS animals; also, adult FeD animals were hyperactive compared with FeS animals during the first hour of the open-field test. FeD repletion can reverse many effects of neonatal FeD in humans and animals [35,67,9,16].

Pharmacological challenge revealed no effects of FeD on neurotransmitter functionality regardless of age. There were some sex-specific effects, but these were transient. Our paradigm was intended to mimic moderate FeD [46,45,44], and dietary iron levels reflect this, being sufficient for growth (except during lactation). This moderate deficiency may explain the lack of effects on behavior despite other FeD indicators.

Mn exposure, on the other hand, resulted in a number of adverse effects. First, Mn exposure increased offspring mortality, increased DMT1 in the hippocampus with no effect in the neostriatum. Second, Mn-exposed animals were lighter than VEH animals. There were no significant effects of developmental MnOE on EZM at P29. This makes the P60 phenotype more striking; in this test 100Mn animals spent significantly more time in the open, and had increased open entries; this suggests hyperactivity or decreased anxiety. Increased activity during the initial phase of open-field testing lends support to the idea of hyperactivity. 100Mn animals had increased head dips in comparison with VEH animals which also suggest a reduction in anxiety. However, being in open areas longer exposes an animal to potential threats so this is not necessarily a beneficial response. This is important to remember in light of previous work suggesting that MnOE is associated with behavioral disinhibition [38]. 100Mn animals also had a decreased sucrose preference, suggesting anhedonia, which is a characteristic not typically associated with decreased anxiety. This may suggest behavioral disinhibition, but neophobia could also explain these results. Decreases in anxiety and other evidence of behavioral disinhibition in the literature on developmental MnOE vary. Several studies found no differences between Mn and controls in the elevated plus maze (EPM) [55,38], but others report decreased anxiety in EPM or increased center activity in the open-field [38,54]. Mn is known to have increased deposition in the basal ganglia [17], which may contribute to reduced anxiety and behavioral disinhibition.

100Mn animals had increased ambulation compared with VEH animals in both the habituation and saline phases of the open-field. This fits with previously published data [38]. Young 100Mn animals retained this hyperactivity after being challenged with FEN; the opposite was seen in VEH animals. Sustained ambulation suggests a decrease in neurotransmitter release or a decrease in receptor function. Either way, this study is the first to link MnOE and serotonergic alterations. IQ decreases and cognitive deficits in children have been linked to dysfunctional serotonergic systems [48].

100Mn animals also exhibited exacerbated hyperactivity after challenge with MK-801 at younger ages. MK-801 induced hyperactivity has been linked to increases in dopaminergic function as well as NMDA receptor inactivation [15,58]. Mn^{2+} is a competitive antagonist of NMDA receptors [22,36], excess Mn^{2+} likely permeate ion channels and inhibit receptor function [31]. In this case, hyperactivity following MK-801 challenge could be the result of increased DA as shown previously [68].

Increased DA function is supported by the increased ambulation after AMPH challenge at young ages in 100Mn animals. Developmental MnOE is known to alter DA receptors and transporters [64,65,38,37]. Greater ambulation in young MnOE animals may suggest presynaptic changes consistent with excess DA in the nerve terminals. Others have reported increased extracellular DA in MnOE animals [18,7].

The results suggest that developmental MnOE produces alterations on neurotransmitter systems at younger ages. However, we saw no differences between 100Mn and VEH animals as adults in the pharmacological challenge experiment. Mn deposition in the brain could be responsible for the age-dependent effects; younger animals have higher Mn concentrations due to the proximity of testing to the end of dosing. This implies that neurotransmitter function returned to normal at adult ages after a recovery period although the EZM and sucrose preference results show that some effects persist.

In conclusion, the intention was to investigate the combination of FeD and MnOE on brain development and function. Although we saw some deficits related to an interaction, most of our results were related to the effects of each factor acting separately. The FeD diet produced transient effects which dissipated following Fe repletion. More importantly, we report that developmental MnOE

alters neurotransmitters beyond dopamine, i.e., MnOE also affected serotonergic and glutamatergic function using pharmacological methods. Children with MnOE exhibit deficits in a wide range of behaviors and cognitive functions, suggesting that modeling such effects could be used to investigate the mechanisms behind such effects.

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References

- [1] J.G. Anderson, S.C. Fordahl, P.T. Cooney, T.L. Weaver, C.L. Colyer, K.M. Erikson, Extracellular norepinephrine, norepinephrine receptor and transporter protein and mRNA levels are differentially altered in the developing rat brain due to dietary iron deficiency and manganese exposure, *Brain Res.* 1281 (2009) 1–14.
- [2] T. Arcangeli, B. Thilaganathan, R. Hooper, K.S. Khan, A. Bhide, Neurodevelopmental delay in small babies at term: a systematic review, *Ultrasound Obstet. Gynecol.* 40 (2012) 267–275.
- [3] M. Aschner, J.L. Aschner, Manganese transport across the blood-brain barrier: relationship to iron homeostasis, *Brain Res. Bull.* 24 (1990) 857–860.
- [4] C.S. Aulakh, K.M. Wozniak, J.L. Hill, D.L. Murphy, Long-term imipramine treatment differentially affects fenfluramine-induced suppression of food intake and locomotor activity, *Pharmacol. Biochem. Behav.* 31 (1988) 97–101.
- [5] D.C. Bellinger, Prenatal exposures to environmental chemicals and children's neurodevelopment: an update, *Saf. Health Work* 4 (2013) 1–11.
- [6] A. Benedetto, C. Au, M. Aschner, Manganese-induced dopaminergic neurodegeneration: insights into mechanisms and genetics shared with Parkinson's disease, *Chem. Rev.* 109 (2009) 4862–4884.
- [7] A. Benedetto, C. Au, D.S. Avila, D. Milatovic, M. Aschner, Extracellular dopamine potentiates mn-induced oxidative stress, lifespan reduction, and dopaminergic neurodegeneration in a BHL-3-dependent manner in *Caenorhabditis elegans*, *PLoS Genet.* 6 (2010).
- [8] F. Bermejo, S. Garcia-Lopez, A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases, *World J. Gastroenterol.* 15 (2009) 4638–4643.
- [9] M.M. Black, A.M. Quigg, K.M. Hurley, M.R. Pepper, Iron deficiency and iron-deficiency anemia in the first two years of life: strategies to prevent loss of developmental potential, *Nutr. Rev.* 69 (2011) S64–S70, Suppl. 1.
- [10] E.M. Blass, M.H. Teicher, *Suckling Sci.* 210 (1980) 15–22.
- [11] M. Bouchard, F. Laforest, L. Vandeland, D. Bellinger, D. Mergler, Hair manganese and hyperactive behaviors: pilot study of school-age children exposed through tap water, *Environ. Health Perspect.* 115 (2007) 122–127.
- [12] M.F. Bouchard, S. Sauve, B. Barbeau, M. Legrand, M.E. Brodeur, T. Bouffard, E. Limoges, D.C. Bellinger, D. Mergler, Intellectual impairment in school-age children exposed to manganese from drinking water, *Environ. Health Perspect.* 119 (2011) 138–143.
- [13] A.A. Braun, M.R. Skelton, C.V. Vorhees, M.T. Williams, Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague–Dawley rats: effects of anxiolytic and anxiogenic agents, *Pharmacol. Biochem. Behav.* 97 (2011) 406–415.
- [14] A.A. Braun, D.L. Graham, T.L. Schaefer, C.V. Vorhees, M.T. Williams, Dorsal striatal dopamine depletion impairs both allocentric and egocentric navigation in rats, *Neurobiol. Learn. Mem.* 97 (2012) 402–408.
- [15] L.J. Bristow, P.H. Hutson, L. Thorn, M.D. Tricklebank, The glycine/NMDA receptor antagonist, R-(+)-HA-966, blocks activation of the mesolimbic dopaminergic system induced by phencyclidine and dizocilpine (MK-801) in rodents, *Br. J. Pharmacol.* 108 (1993) 1156–1163.
- [16] L.M. De-Regil, M.E. Jefferds, A.C. Sylvestry, T. Dowswell, Intermittent iron supplementation for improving nutrition and development in children under years of age, *Cochrane Database Syst. Rev.* 12 (2011) CD009085.
- [17] A.W. Dobson, K.M. Erikson, M. Aschner, Manganese neurotoxicity, *Ann. N.Y. Acad. Sci.* 1012 (2004) 115–128.
- [18] K.M. Erikson, C.E. John, S.R. Jones, M. Aschner, Manganese accumulation in striatum of mice exposed to toxic doses is dependent upon a functional dopamine transporter, *Environ. Toxicol. Pharmacol.* 20 (2005) 390–394.
- [19] K.M. Erikson, Z.K. Shihabi, J.L. Aschner, M. Aschner, Manganese accumulates in iron-deficient rat brain regions in a heterogeneous fashion and is associated with neurochemical alterations, *Biol. Trace Elem. Res.* 87 (2002) 143–156.
- [20] K.M. Erikson, K. Thompson, J. Aschner, M. Aschner, Manganese neurotoxicity: a focus on the neonate, *Pharmacol. Ther.* 113 (2007) 369–377.
- [21] L.D. Fechter, Distribution of manganese in development, *Neurotoxicology* 20 (1999) 197–201.
- [22] V.A. Fitsanakis, C. Au, K.M. Erikson, M. Aschner, The effects of manganese on glutamate, dopamine and gamma-aminobutyric acid regulation, *Neurochem. Int.* 48 (2006) 426–433.
- [23] V.A. Fitsanakis, K.N. Thompson, S.E. Deery, D. Milatovic, Z.K. Shihabi, K.M. Erikson, R.W. Brown, M. Aschner, A chronic iron-deficient/high-manganese diet in rodents results in increased brain oxidative stress and behavioral deficits in the morris water maze, *Neurotox. Res.* 15 (2009) 167–178.
- [24] V.A. Fitsanakis, N. Zhang, J.G. Anderson, K.M. Erikson, M.J. Avison, J.C. Gore, M. Aschner, Measuring brain manganese and iron accumulation in rats following 14 weeks of low-dose manganese treatment using atomic absorption spectroscopy and magnetic resonance imaging, *Toxicol. Sci.* 103 (2008) 116–124.
- [25] V.A. Fitsanakis, N. Zhang, M.J. Avison, K.M. Erikson, J.C. Gore, M. Aschner, Changes in dietary iron exacerbate regional brain manganese accumulation as determined by magnetic resonance imaging, *Toxicol. Sci.* 120 (2011) 146–153.
- [26] P.J. Fletcher, J. Sinyard, G.A. Higgins, The effects of the 5-HT(2C) receptor antagonist SB242084 on locomotor activity induced by selective, or mixed, indirect serotonergic and dopaminergic agonists, *Psychopharmacology (Berl)* 187 (2006) 515–525.
- [27] S.J. Garcia, K. Gellein, T. Syversen, M. Aschner, A manganese-enhanced diet alters brain metals and transporters in the developing rat, *Toxicol. Sci.* 92 (2006) 516–525.
- [28] C.E. Grace, T.L. Schaefer, D.L. Graham, M.R. Skelton, M.T. Williams, C.V. Vorhees, Effects of inhibiting neonatal methamphetamine-induced corticosterone release in rats by adrenal autotransplantation on later learning, memory, and plasma corticosterone levels, *Int. J. Dev. Neurosci.* (2010).
- [29] D.L. Graham, R.M. Amos-Kroohs, A.A. Braun, C.E. Grace, T.L. Schaefer, M.R. Skelton, M.T. Williams, C.V. Vorhees, Neonatal (+)-methamphetamine exposure in rats alters adult locomotor responses to dopamine D1 and D2 agonists and to a glutamate NMDA receptor antagonist, but not to serotonin agonists, *Int. J. Neuropsychopharmacol.* 16 (2012) 377–391.
- [30] D.L. Graham, C.E. Grace, A.A. Braun, T.L. Schaefer, M.R. Skelton, P.H. Tang, C.V. Vorhees, M.T. Williams, Effects of developmental stress and lead (Pb) on corticosterone after chronic and acute stress, brain monoamines, and blood Pb levels in rats, *Int. J. Dev. Neurosci.* 29 (2011) 45–55.
- [31] T.R. Guilarte, M.K. Chen, Manganese inhibits NMDA receptor channel function: implications to psychiatric and cognitive effects, *Neurotoxicology* 28 (2007) 1147–1152.
- [32] T.R. Guilarte, M.K. Chen, J.L. McGlothlin, T. Verina, D.F. Wong, Y. Zhou, M. Alexander, C.A. Rohde, T. Syversen, E. Decamp, A.J. Koser, S. Fritz, H. Goncz, D.W. Anderson, J.S. Schneider, Nigrostriatal dopamine system dysfunction and subtle motor deficits in manganese-exposed non-human primates, *Exp. Neurol.* 202 (2006) 381–390.
- [33] J.M. Gulley, J.J. Stanis, Adaptations in medial prefrontal cortex function associated with amphetamine-induced behavioral sensitization, *Neuroscience* 166 (2010) 615–624.
- [34] L. He, K. Girijashanker, T.P. Dalton, J. Reed, H. Li, M. Soleimani, D.W. Nebert, ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties, *Mol. Pharmacol.* 70 (2006) 171–180.
- [35] J.R. Hunt, C.A. Zito, J. Erjavec, L.K. Johnson, Severe or marginal iron deficiency affects spontaneous physical activity in rats, *Am. J. Clin. Nutr.* 59 (1994) 413–418.
- [36] P. Karki, E. Lee, M. Aschner, Manganese neurotoxicity: a focus on glutamate transporters, *Ann. Occup. Environ. Med.* 25 (2013) 4.
- [37] C.H. Kern, D.R. Smith, Prewaning Mn exposure leads to prolonged astrocyte activation and lasting effects on the dopaminergic system in adult male rats, *Synapse* 65 (2011) 532–544.
- [38] C.H. Kern, G.D. Stanwood, D.R. Smith, Prewaning manganese exposure causes hyperactivity, disinhibition, and spatial learning and memory deficits associated with altered dopamine receptor and transporter levels, *Synapse* 64 (2010) 363–378.
- [39] K. Khan, P. Factor-Litvak, G.A. Wasserman, X. Liu, E. Ahmed, F. Parvez, V. Slavkovich, D. Levy, J. Mey, G.A. van, J.H. Graziano, Manganese exposure from drinking water and children's classroom behavior in Bangladesh, *Environ. Health Perspect.* 119 (2011) 1501–1506.
- [40] K. Khan, G.A. Wasserman, X. Liu, E. Ahmed, F. Parvez, V. Slavkovich, D. Levy, J. Mey, G.A. van, J.H. Graziano, P. Factor-Litvak, Manganese exposure from drinking water and children's academic achievement, *Neurotoxicology* 33 (2012) 91–97.
- [41] J. Kim, P.D. Buckett, M. Wessling-Resnick, Absorption of manganese and iron in a mouse model of hemochromatosis, *PLoS ONE* 8 (2013) e64944.
- [42] Y. Kim, B.K. Lee, Iron deficiency increases blood manganese level in the Korean general population according to KNHANES 2008, *Neurotoxicology* 32 (2011) 247–254.
- [43] P.J. Kontur, L.D. Fechter, Brain regional manganese levels and monoamine metabolism in manganese-treated neonatal rats, *Neurotoxicol. Teratol.* 10 (1988) 295–303.
- [44] C.L. Kwik-Urbe, D. Gietzen, J.B. German, M.S. Golub, C.L. Keen, Chronic marginal iron intakes during early development in mice result in persistent changes in dopamine metabolism and myelin composition, *J. Nutr.* 130 (2000) 2821–2830.
- [45] C.L. Kwik-Urbe, M.S. Golub, C.L. Keen, Chronic marginal iron intakes during early development in mice alter brain iron concentrations and behavior despite postnatal iron supplementation, *J. Nutr.* 130 (2000) 2040–2048.
- [46] C.L. Kwik-Urbe, M.S. Golub, C.L. Keen, Behavioral consequences of marginal iron deficiency during development in a murine model, *Neurotoxicol. Teratol.* 21 (1999) 661–672.

- [47] A.I. Lee, M.M. Okam, Anemia in pregnancy, *Hematol. Oncol. Clin. North Am.* 25 (2011) 241–259, vii.
- [48] K.P. Lucchini, J. Waider, Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders, *Neuron* 76 (2012) 175–191.
- [49] B. Lozoff, Early iron deficiency has brain and behavior effects consistent with dopaminergic dysfunction, *J. Nutr.* 141 (2011) 740S–746S.
- [50] R.G. Lucchini, S. Guazzetti, S. Zoni, F. Donna, S. Peter, A. Zacco, M. Salmistraro, E. Bontempi, N.J. Zimmerman, D.R. Smith, Tremor, olfactory and motor changes in Italian adolescents exposed to historical ferro-manganese emission, *Neurotoxicology* 33 (2012) 687–696.
- [51] I. Mena, J. Court, S. Fuenzalida, P.S. Papavasiliou, G.C. Cotzias, Modification of chronic manganese poisoning. Treatment with L-dopa or 5-OH tryptophane, *N. Engl. J. Med.* 282 (1970) 5–10.
- [52] I. Mena, O. Marin, S. Fuenzalida, G.C. Cotzias, Chronic manganese poisoning. Clinical picture and manganese turnover, *Neurology* 17 (1967) 128–136.
- [53] J.A. Menezes-Filho, M. Bouchard, P.N. Sarcinelli, J.C. Moreira, Manganese exposure and the neuropsychological effect on children and adolescents: a review, *Rev. Panam. Salud Publica* 26 (2009) 541–548.
- [54] R.M. Molina, S. Phattananarudee, J. Kim, K. Thompson, M. Wessling-Resnick, T.J. Maher, J.D. Brain, Ingestion of Mn and Pb by rats during and after pregnancy alters iron metabolism and behavior in offspring, *Neurotoxicology* 32 (2011) 413–422.
- [55] B.A. Pappas, D. Zhang, C.M. Davidson, T. Crowder, G.A. Park, T. Fortin, Perinatal manganese exposure: behavioral, neurochemical, and histopathological effects in the rat, *Neurotoxicol. Teratol.* 19 (1997) 17–25.
- [56] M.A. Rahman, B. Rahman, N. Ahmed, High blood manganese in iron-deficient children in Karachi, *Public Health Nutr.* 16 (2013) 1677–1683.
- [57] R.S. Redman, L.R. Sweney, Changes in diet and patterns of feeding activity of developing rats, *J. Nutr.* 106 (1976) 615–626.
- [58] A.M. Redmond, A. Harkin, J.P. Kelly, B.E. Leonard, Effects of acute and chronic antidepressant administration on phencyclidine (PCP) induced locomotor hyperactivity, *Eur. Neuropsychopharmacol.* 9 (1999) 165–170.
- [59] J.A. Roth, M.D. Garrick, Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese, *Biochem. Pharmacol.* 66 (2003) 1–13.
- [60] E.A. Smith, P. Newland, K.G. Bestwick, N. Ahmed, Increased whole blood manganese concentrations observed in children with iron deficiency anaemia, *J. Trace Elem. Med. Biol.* 27 (2013) 65–69.
- [61] K. Suzuki, Special vulnerabilities of the developing nervous system, in: P.S. Spencer, H.H. Schaumburg (Eds.), *Experimental and Clinical Neurotoxicology*, Williams and Wilkins, Baltimore, 1980, pp. 48–61.
- [62] A. Takeda, S. Ishiwatari, S. Okada, Manganese uptake into rat brain during development and aging, *J. Neurosci. Res.* 56 (1999) 93–98.
- [63] K. Thompson, R.M. Molina, T. Donaghey, J.E. Schwob, J.D. Brain, M. Wessling-Resnick, Olfactory uptake of manganese requires DMT1 and is enhanced by anemia, *FASEB J.* 21 (2007) 223–230.
- [64] T.T. Tran, W. Chowanadisai, F.M. Crinella, A. Chicz-Demet, B. Lonnerdal, Effect of high dietary manganese intake of neonatal rats on tissue mineral accumulation, striatal dopamine levels, and neurodevelopmental status, *Neurotoxicology* 23 (2002) 635–643.
- [65] T.T. Tran, W. Chowanadisai, B. Lonnerdal, L. Le, M. Parker, A. Chicz-Demet, F.M. Crinella, Effects of neonatal dietary manganese exposure on brain dopamine levels and neurocognitive functions, *Neurotoxicology* 23 (2002) 645–651.
- [66] K. Tuschl, P.B. Mills, P.T. Clayton, Manganese and the brain, *Int. Rev. Neurobiol.* 110 (2013) 277–312.
- [67] E.L. Unger, T. Paul, L.E. Murray-Kolb, B. Felt, B.C. Jones, J.L. Beard, Early iron deficiency alters sensorimotor development and brain monoamines in rats, *J. Nutr.* 137 (2007) 118–124.
- [68] C.V. Vorhees, D.L. Graham, R.M. Amos-Kroohs, A.A. Braun, C.E. Grace, T.L. Schaefer, M.R. Skelton, K.M. Erikson, M. Aschner, M.T. Williams, Effects of developmental manganese, stress, and the combination of both on monoamines, growth, and corticosterone, *Toxicol. Rep.* 1 (2014) 1046–1061.
- [69] C.V. Vorhees, D.L. Graham, A.A. Braun, T.L. Schaefer, M.R. Skelton, N.M. Richtand, M.T. Williams, Prenatal immune challenge in rats: altered responses to dopaminergic and glutamatergic agents, prepulse inhibition of acoustic startle, and reduced route-based learning as a function of maternal body weight gain after prenatal exposure to poly IC, *Synapse* 66 (2012) 725–737.
- [70] C.V. Vorhees, D.L. Graham, A.A. Braun, T.L. Schaefer, M.R. Skelton, N.M. Richtand, M.T. Williams, Prenatal immune challenge in rats: effects of polyinosinic-polycytidylic acid on spatial learning, prepulse inhibition, conditioned fear, and responses to MK-801 and amphetamine, *Neurotoxicol. Teratol.* 47 (2015) 54–65.
- [71] C.V. Vorhees, N.R. Herring, T.L. Schaefer, C.E. Grace, M.R. Skelton, H.L. Johnson, M.T. Williams, (2008) Effects of neonatal (+)-methamphetamine on path integration and spatial learning in rats: effects of dose and rearing conditions, *Int. J. Dev. Neurosci.* 26 (2008) 599–610.
- [72] S.H. Wang, Z.J. Zhang, Y.J. Guo, H. Zhou, G.J. Teng, B.A. Chen, Anhedonia and activity deficits in rats: impact of post-stroke depression, *J. Psychopharmacol.* 23 (2009) 295–304.
- [73] X. Wang, D.S. Miller, W. Zheng, Intracellular localization and subsequent redistribution of metal transporters in a rat choroid plexus model following exposure to manganese or iron, *Toxicol. Appl. Pharmacol.* 230 (2008) 167–174.
- [74] G.A. Wasserman, X. Liu, F. Parvez, H. Ahsan, D. Levy, P. Factor-Litvak, J. Kline, G.A. van, V. Slavkovich, N.J. Loiacono, Z. Cheng, Y. Zheng, J.H. Graziano, Water manganese exposure and children's intellectual function in Arahazar, Bangladesh, *Environ. Health Perspect.* 114 (2006) 124–129.
- [75] WHO, World Health Organization (WHO): Micronutrient Deficiencies, Iron deficiency anemia, 2015.
- [76] B.B. Williams, G.F. Kwakye, M. Wegrzynowicz, D. Li, M. Aschner, K.M. Erikson, A.B. Bowman, Altered manganese homeostasis and manganese toxicity in a Huntington's disease striatal cell model are not explained by defects in the iron transport system, *Toxicol. Sci.* 117 (2010) 169–179.
- [77] M. Yoon, J.D. Schroeter, A. Nong, M.D. Taylor, D.C. Dorman, M.E. Andersen, H.J. Clewell, III, Physiologically based pharmacokinetic modeling of fetal and neonatal manganese exposure in humans: describing manganese homeostasis during development, *Toxicol. Sci.* 122 (2011) 297–316.
- [78] M. Zack, R.E. Featherstone, S. Mathewson, P.J. Fletcher, Chronic exposure to a gambling-like schedule of reward predictive stimuli can promote sensitization to amphetamine in rats, *Front. Behav. Neurosci.* 8 (2014), 36.
- [79] G. Zhang, D. Liu, P. He, Effects of manganese on learning abilities in school children, *Zhonghua Yu Fang Yi Xue Za Zhi* 29 (1995) 156–158.
- [80] S. Zoni, R.G. Lucchini, Manganese exposure: cognitive, motor and behavioral effects on children: a review of recent findings, *Curr. Opin. Pediatr.* 25 (2013) 255–260.