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1. Introduction

Manganese (Mn), an essential nutritional element, is neurotoxic at high levels. First identified in workers following chronic inhalation exposure (Mena et al., 1967), it results in manganism, a disorder that shares some symptoms with parkinsonism. Mn overexposure (MnOE) is also seen in children and is more subtle than manganism (Bouchard et al., 2011; Khan et al., 2011; Khan et al., 2012; Lucchini et al., 2012). In children, the route of exposure can be ingestion from water (Wasserman et al., 2006; Oulhote et al., 2014), soy-based infant formulas (Tran et al., 2002a), and contaminated air and water from smelting factories (Menezes-Filho et al., 2014). Neonates that ingest Mn from infant formula retain more Mn than those breastfed (Dorner et al., 1989). MnOE in children exhibit effects such as cognitive deficits, behavioral disinhibition, and reduced school achievement (Zoni and Lucchini, 2013; Haynes et al., 2015).

In rodent models of developmental MnOE, effects include decreased passive avoidance retention, attenuated locomotor responses to cocaine, impaired rotorod coordination, and reduced striatal dopamine (Tran et al., 2002a; Tran et al., 2002b; Reichel et al., 2006; Cordova et al., 2012). Furthermore, deficits were found in rats exposed to 25 or 50 mg/kg/day Mn by gavage from postnatal day (P)1-21 with MnOE animals taking longer and making more errors than vehicle (VEH) controls in a radial arm maze (RAM) (Kern et al., 2010), an effect not seen in a study using the Morris Water Maze (MWM) or on RAM in rats exposed to 2 or 10 mg/mL in drinking water from embryonic day (E)1 through P30 (Pappas et al., 1997). Two studies show that early MnOE in rats results in reduced fine motor control on a food reaching task (Beaudin et al., 2013; Beaudin et al., 2015).

Developmental MnOE seldom occurs in isolation (Walker et al., 2011). Concomitant factors include impoverished/low socioeconomic status (SES) environments where iron deficiency (FeD), and/or stress (developmental stress) are common. FeD occurs in 15% of U.S. children (Lee and Okam, 2011) and can affect brain development (Youdim, 2008). SES is often used as a surrogate for impoverishment and stress. Children from low SES environments exhibit higher rates of anxiety, conduct disorders, and attention deficit disorders (Hackman et al.,

2010; Walker et al., 2011; Reiss, 2013). The intersection of MnOE, FeD, and low SES/stress may represent a combinatorial risk greater than each factor when acting separately (Wasserman et al., 2006). We hypothesized that cognitive development may be vulnerable to such an interaction since each affects brain regions known to be involved in learning and memory.

In this study, rats were exposed to 100 mg/kg Mn or VEH every other day from P4-28 (Amos-Kroohs et al., 2016). This regimen increases blood and neostriatal Mn and alters monoamine neurotransmitters (Vorhees et al., 2014). FeD was induced using a diet with 90% less Fe than a standard diet. The diet was given from E15-P28 and produced decreased blood hematocrits, body weight, and locomotor activity, all recognized markers of FeD. Moreover, MnOE and FeD in combination increase body weight reductions and alter anxiety in rats compared to either factor alone (Amos-Kroohs et al., 2015). Developmental stress was induced using a barren cage procedure (Avishai-Eliner et al., 2001; Vorhees et al., 2014). Rats were placed in barren (BAR) cages from E7-P28, an interval spanning most of neurogenesis (Clancy et al., 2007). After cessation of exposure, offspring were tested for cognitive ability using tests for allocentric and for egocentric learning and memory. Allocentric learning and memory was evaluated using the MWM (Morris et al., 2003; Vorhees and Williams, 2006), a test that relies on the use of extramaze cues to determine the shortest path from start to goal (a hidden platform). Egocentric learning and memory was evaluated using the Cincinnati water maze (CWM) (Vorhees, 1987; Vorhees et al., 1991; Braun et al., 2012; Braun et al., 2015). In this test, animals must use internal, self-movement cues to find the goal in a complex labyrinthine maze in complete darkness. Separate, identically treated animals were used to assess α-synuclein and long-term potentiation (LTP), the latter an established correlate of spatial learning and memory (Lisman et al., 2002; Morris et al., 2003). α-Synuclein was assessed based on its association with dopamine and Lewy body formation associated with parkinsonism.

2. Materials and methods

2.1. Animals

Nulliparous female Sprague-Dawley CD (IGS) rats (Charles River Laboratories, Raleigh, NC; strain #001), approximately 60 days old on arrival were habituated for not less than one week to the vivarium (AAALAC International accredited) before breeding by being placed with male sires of the same strain and supplier. Animals were maintained on a 14–10 h light-dark cycle (lights on 600 h) with controlled temperature (19 ± 1 °C) and humidity (50% ± 10%). Animals were housed in a barrier facility using a Modular Caging System (Alternative Design, Siloam Spring, AR). HEPA filtered air was supplied to each cage (Alternative Design, Siloam Spring, AR) with 30 air changes/h. Reverse osmosis filtered water (SE Lab Group, Napa, CA) and NIH-07 diet were provided ad libitum. A semicircular stainless steel enclosure was placed in standard cages for enrichment (Vorhees et al., 2008). Females were separated the day a sperm plug was detected and this day was designated E0. Birth was counted as P0; on P1, litters were culled to 10, five per sex, using a random number table. Pups were removed from dams on P28 into same sex cages (4/cage) and re-housed (2/cage) on P42. Maternal body weight was measured on E7, 15, 21, and P1 and 28. Pups were weighed on P1, during dosing and on P42 and P60.

2.2. Rearing

Gravid females were housed in standard cages (STD) until E7 at which time half were moved to cages without bedding or enclosures and with a wire grid floor inserted (BAR $n = 29$); the other half were continued in STD cages ($n = 30$) but moved to new STD cages on E7 to control for rehousing experience. On E21, wire floors were temporarily removed to prevent pups from slipping through the spaces between wires of the grid floor, and a 15 × 25 cm absorbent pad was placed in each cage instead (Anderson Lab Bedding, Maumee, OH). On P6, BAR cages had pads removed and grid floors reinstalled. Cages were changed daily for both types of litters. BAR cages were maintained until P28 then switched back to STD cages.

2.2.1. Diet

The FeD diet (Amos-Kroohs et al., 2015) was adapted from (Fitsanakis et al., 2009; Fitsanakis et al., 2011). Females were given standard NIH-07 diet until E15 then switched to purified NIH-07 diet (Land O' Lakes Purina Feed, Evansville, IN) with half of the dams in the BAR and STD groups given purified iron sufficient (FeS) diet and the other half purified FeD formulated diet. The FeD diet contained 35 ppm Fe and the FeS diet contained 350 ppm Fe (the standard NIH-07 diet also contains sufficient Fe). Offspring were returned to standard NIH-07 diet on P28.

2.3. Manganese

For MnOE (Vorhees et al., 2014), a split-litter design was used in which two male and two female pups per litter were gavaged with VEH (0.01 M anhydrous sodium chloride) and three male and three female pups per litter were gavaged with 100 mg/kg Mn chloride (MnOE). The extra pair of MnOE pups was included only for backup purposes. Gavage solutions were given in a volume of 3 mL/kg of VEH every other day from P4-28. Gavage was used to avoid maternal exposure and its effects on maternal-pup behavior (Graham et al., 2011). This exposure regimen produces increased serum and brain Mn (Amos-Kroohs et al., 2015) but does not increase corticosterone above that of untreated littermates (Graham et al., 2011).

2.4. Behavior

One male-female pair from each exposure group within each litter received one set of all tests shown on the left and one male-female pair from each exposure group received a second set of tests shown on the right in Fig. 1. Only the offspring learning and memory data are included here; data on the other outcomes, including body weights and litter composition, were reported separately (Amos-Kroohs et al., 2016). This design means that prior behavioral tests may have some influence on later tests. For the learning and memory tests, rats in both test sequences received straight water channel testing prior to maze testing. There were a total of 234 rats in the CWM testing arm and 216 rats in the MWM testing arm. This design resulted in approximately 12 offspring from different litters per sex per rearing condition per diet per Mn exposure group.

2.4.1. Straight Channel

This test acclimates rats to swimming, teaches escape to a hidden platform, and tests motivation and swimming coordination by measuring swim speed as latency to traverse a long straight water channel. On P60, animals were placed at one end of a 15 × 244 × 50 cm high channel filled with water to a depth of 25 cm and given four trials to reach the submerged platform at the opposite end. Latency to reach the platform was recorded.

2.4.2. Cincinnati water maze (CWM)

The apparatus is a 9-unit multiple-T labyrinthine water maze (Vorhees, 1987; Vorhees et al., 1991; Vorhees and Williams, 2016). Trials are run under infrared light with a submerged platform at the goal location. By testing in the absence of visible light, distal cues are eliminated which prevents rats from using spatial cues to find the escape. We have shown that learning in this maze under these conditions is severely disrupted by dopaminergic reductions in the neostriatum (Braun et al., 2012; Braun et al., 2015). The maze channels are 15 cm wide and the walls 51 cm high filled with water to a depth of 20 cm. The day after

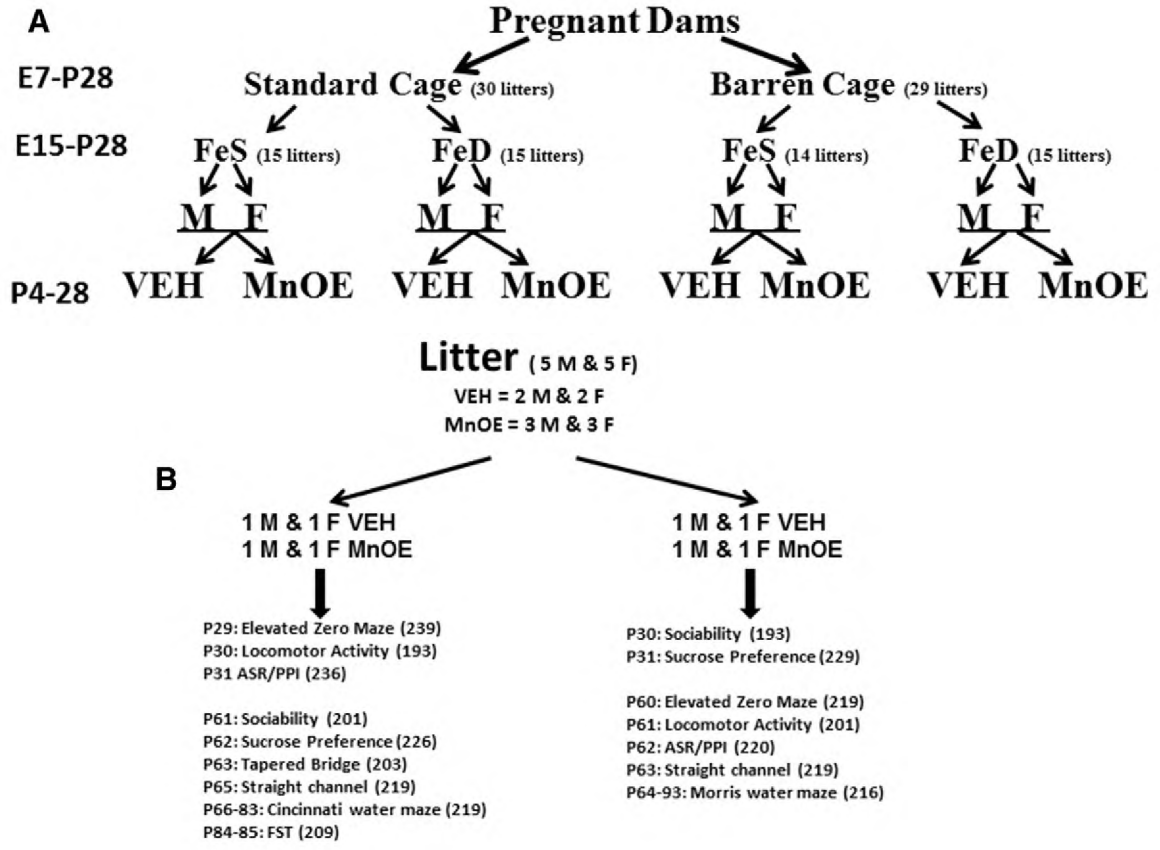

Fig. 1. Study design. **A**, Experimental paradigm showing cage, diet, and dosing exposures. All treatments ended on P28 when pups were separated from their dams. **B**, Behavioral testing layout. Litters were split into two arms; each rat was given each test. Group sizes are shown in parentheses. This report only pertains to the straight channel, CWM, and MWM data. Data on the other behaviors are reported elsewhere (Amos-Kroohs et al., 2016).

straight channel trials, animals were placed in the CWM at the farthest point from the goal and given 5 min to find the escape (Vorhees et al., 1991; Vorhees and Williams, 2014). Animals received two trials per day for 18 days. After trial-1 of each day, rats were placed in a holding cage with absorbent substrate to facilitate drying. Water temperature was approximately 20 °C. If an animal failed to locate the platform within 5 min on trial-1 of a test day it received at least a 5 min rest before trial-2. Rats that reached 5 min were removed from wherever they were (unassisted escape). Latency and errors were recorded. Errors were defined as >50% entry into the stem and/or arm of a T-shaped cul-de-sac or reentry into the start channel. To adjust for rats that stopped searching for several minutes and reached the trial time limit, these trials were given an error score equivalent to the animal making the most errors in under 5 min.

2.4.3. Morris water maze (MWM)

Allocentric (spatial) learning and memory were assessed in the MWM (Vorhees and Williams, 2006). Testing was conducted in a 244 cm diameter × 51 cm high circular pool filled halfway with water (water temperature approximately 20 °C). Beginning the day after straight channel testing, animals in this arm of the study were tested in the maze in four phases: acquisition (platform in SW position); reversal (platform in NE position); shift (platform hidden in NW position); and cued (platform moved on every trial). For the first three phases, rats received four trials per day for 6 days to find a camouflaged platform submerged 1.5 cm below the surface. If an animal did not find the platform within 2 min, it was removed and placed on the platform for 30 s (unassisted escape). On the seventh day of each phase, animals received a single 45 s probe trial with the platform removed and started from a novel position. One week after the shift probe trial, a second, delayed probe trial was given. Each hidden phase used a platform of a different size (10, 7, and 5 cm in diameter for acquisition, reversal, and shift, respectively) in order to increase spatial difficulty since transfer of training facilitates performance in phases after acquisition. Following hidden platform phases, a cued version was tested with a visible platform. For this, the platform remained submerged but had a plastic ball attached to a brass rod mounted on it that protruded 12 cm above the water. Curtains were closed around the pool to minimize visibility of extra-maze cues. Rats were given four trials per day for 2 days with the platform and start positions moved randomly on each trial to prevent use of a spatial strategy. A low-light sensitive video camera was mounted over the tank and video tracking software was used to trace performance (ANY-maze; Stoelting Instruments, Wood Dale, IL). On hidden platform trials, latency, path length, and swim speed were analyzed. We show path length because it is less prone to secondary effects of swim speed than latency, therefore, we present path length/distance to the platform (Vorhees and Williams, 2006). On probe trials multiple measures were recorded and were consistent with one another, therefore, mean distance from the former platform site data are presented. Latency was analyzed on cued trials.

2.5. Electrophysiology

Based on the behavioral findings in the MnOE group, brains from a separate group of identically treated rats were analyzed on a MED64 system (Alpha MED Scientific, Inc., Berkeley, CA) for LTP in the CA1 region. Brains were removed on P23 and placed in cold artificial cerebral spinal fluid (aCSF) and thick sections (400 μm) cut on a vibratome. This age was chosen because younger brain tissue remains responsive longer than at older ages. Slices were allowed to equilibrate at 37 °C in aCSF and placed on top of a 64 channel rat hippocampal recording chip (AutoMate, Berkeley, CA). Data were collected using Moebius

software (Alpha MED Scientific, Inc., Berkeley, CA). Field excitatory postsynaptic potentials (fEPSPs) were recorded following stimulation (60 mA) delivered from two stimulus electrodes once every 5 s during a 5-min baseline to determine connectivity. To induce LTP, a theta burst stimulation of 90 mA was delivered to one of the electrodes every 200 ms for 2 s for a total of 10 pulses; fEPSPs were recorded for 1 h post-tetanus. Data are presented as percent of baseline with 4–6 slices from different animals in the VEH and MnOE groups. These parameters are ones we have used successfully (Sun et al., 2008; Sun et al., 2010b; Sun et al., 2010a; Stottmann et al., 2016).

2.6. α-Synuclein Western blots

In another set of identically treated rats, concentrations of α-synuclein in P29 and P60 animals from the MnOE and VEH groups were assayed. Samples (20 μg of protein for P60 rats and 25 μg of protein for P29 rats/lysate) were prepared with 2× Laemmli buffer (Sigma) at a total volume of 20 μL per sample. Samples were warmed in an incubator for 5 min and then electrophoresed on 12% Mini-PROTEAN precast gels (Bio-Rad). Following electrophoresis, proteins were blotted to polyvinylidene fluoride membranes. Membranes were blocked in 5% casein in TBS-T buffer for 30 min at room temperature, and incubated with primary antibodies against α-synuclein (1:1500: purified mouse anti-alpha-synuclein from BD Transduction Laboratories) and actin (1:5000: anti-beta actin from Abcam) overnight at 4 °C. Following repeat washes in TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (Goat anti-Mouse, 1:5000) for 1 h at room temperature and washed again in TBS-T. ECL (Advansta Western Bright Peroxide) was applied to the membranes for 2 min, and exposed on Blue Basic Autorad film (BioExpress). Expression was determined using the ratio of α-synuclein to actin. Values used in the ratios constituted the normalized area of protein per lane determined by ImageJ analysis software (NIH, Bethesda, MD). Four animals per age per treatment group were analyzed, and blots were repeated three times and averaged.

2.7. Data analyses

Data were analyzed using mixed linear factorial analysis of variance in a randomized block design (ANOVA; Proc Mixed, SAS v9.3, SAS Institute, Cary, NC). A randomized block design was used to account for litter and is based on the fact that variance is generally lower within than

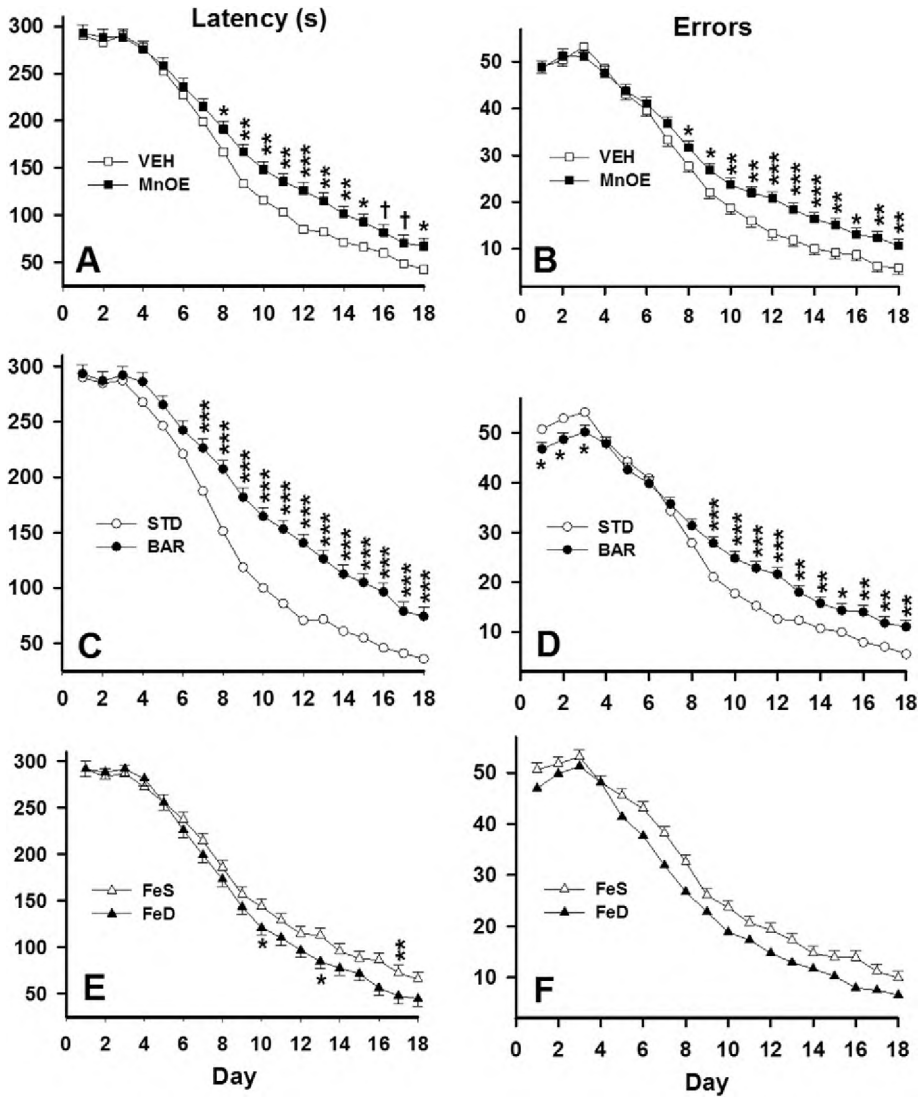

Fig. 2. Cincinnati water maze: latency to escape and errors as a function of MnOE (**A,B**), cage rearing (**C,D**), and dietary iron (**E,F**). Rats were tested starting on P66. VEH = vehicle-treated controls; MnOE (100 mg/kg) was by gavage every other day from P4-28; STD = standard cage; BAR = barren cage; FeS = Fe sufficient diet; FeD = Fe deficient diet (90% reduction relative to FeS diet). Note that although the general pattern of effect on errors is similar to that found on latency, there are differences, including that BAR cage effects were similar in magnitude to those seen on latency for MnOE but the BAR effect was less pronounced for errors than on latency. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; †$p < 0.10$ compared with VEH, STD, or FeS.

between litters. Between-subject factors were rearing condition (STD vs. BAR), diet (FeS vs. FeD), exposure (VEH vs. MnOE), and sex. For data, such as maze learning, day was a repeated measure factor in the model. Significant between X within interactions were further analyzed using slice-effect ANOVAs. Kenward-Rodger adjusted degrees of freedom were used in repeated measure ANOVAs. The significance was $p \leq 0.05$. Data analyzed by mixed models are presented as least square means ± SEM for purposes of inference.

3. Results

3.1. Straight channel

A MnOE × trial interaction occurred ($F(3,1264) = 2.57$, $p < 0.05$) as well as a MnOE main effect ($F(1422) = 14.16$, $p < 0.001$). For the main effect, means and SEMs were MnOE: 16.7 ± 0.3 s vs. VEH: 14.8 ± 0.3 s. Comparisons by trial showed that on trial-1, and to a lesser extent on trial 3, MnOE animals took slightly longer than VEH animals to reach the goal. However, on trial-4, the last trial before maze testing, there were no group differences. For rearing condition, BAR animals had longer latencies compared with STD animals (BAR: 16.7 ± 0.3 s vs. STD: 14.9 ± 0.3 s, main effect: $F(1422) = 17.9$, $p < 0.001$). This difference also disappeared by trial-4. There was no effect of diet.

3.2. Cincinnati water maze

For latency, there were main effects of rearing ($F(1202) = 25.52$, $p < 0.0001$) and MnOE ($F(1202) = 6.97$, $p < 0.01$) and interactions with day. Exposure interacted with day such that MnOE groups had longer latencies on days 8–18 compared with VEH groups ($F(17,3469) = 2.54$, $p < 0.001$, Fig. 2A). Rearing condition also interacted with day in which BAR groups had longer latencies on Days 7–18 compared with STD ($F(17,3469) = 6.61$, $p < 0.0001$, Fig. 2C). FeD also interacted with day ($F(17,3469) = 1.66$, $p < 0.05$, Fig. 2E) and showed that the FeD groups had slightly shorter latencies on three days than FeS groups near the end of the test. There were interactions of day × rearing × diet × sex ($F(17,3469) = 1.74$, $p < 0.05$) and day × diet × sex × MnOE ($F(17,3469) = 1.81$, $p < 0.05$), but post hoc tests failed to show meaningful differences.

Errors were also significantly affected by rearing condition (main effect: $F(1202) = 6.61$, $p < 0.01$), diet ($F(1202) = 11.82$, $p < 0.001$) and MnOE (main effect: $F(1202) = 10.25$, $p < 0.01$). There were significant

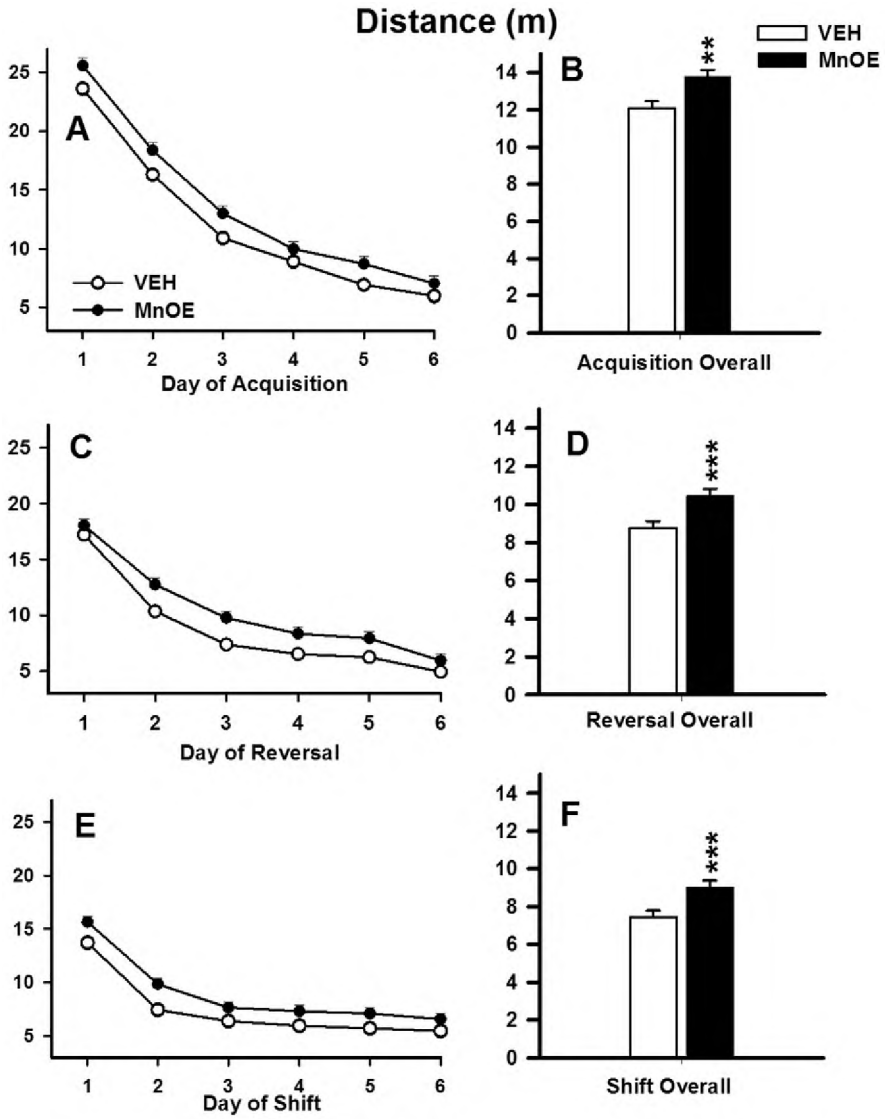

Fig. 3. MWM path length (m) for three phases of testing following developmental MnOE: MWM-acquisition (initial learning); MWM-reversal (platform moved to the opposite quadrant); MWM-Shift (platform moved to an adjacent quadrant relative to the one used during reversal). Platform sizes were 10, 7, and 5 cm in diameter during acquisition, reversal, and shift, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$; compared with VEH.

interactions as well. There was a MnOE × day interaction ($F(17,3469) = 3.26$, $p < 0.0001$) in which MnOE animals made more errors on days 8–18 compared with VEH animals (Fig. 2B). There was also a rearing condition × day interaction ($F(17,3469) = 7.27$, $p < 0.0001$) in which BAR animals made more errors on days 9–18 than STD animals (Fig. 2D). There was no diet × day interaction on errors (Fig. 2F). The pattern for errors was similar to that for latency for MnOE and BAR, but not for FeD. The small FeD effect found on latency was not significant for errors. There was a day × diet × sex × MnOE interaction ($F(17,3469) = 2.05$, $p < 0.01$) in which FeS-MnOE animals made more errors than other diet-MnOE groups on several days but these differences were small (not shown).

3.3. MWM-acquisition

MnOE resulted in longer path lengths on acquisition compared with VEH animals (main effect: $F(1292) = 10.03$, $p < 0.01$ (Fig. 3A, B)). There were no significant effects on swim speed (not shown). Also on the platform trials, there was a significant rearing × diet interaction ($F(1292) = 14.16$, $p < 0.001$, Fig. 5A) in which STD-FeD and BAR-FeS animals had longer path lengths compared with the STD-FeS animals. On the acquisition probe trial, average distance to the platform site did not differ between MnOE and VEH groups (Fig. 4A), between BAR and STD groups (Fig. 4D), or between FeD and FeS groups (not shown).

3.4. MWM-reversal

MnOE resulted in longer path lengths on reversal trials (main effect: $F(1257) = 15.26$, $p < 0.0001$, Fig. 3C,D). There was a sex × rearing × diet interaction ($F(1266) = 6.14$, $p < 0.05$) in which BAR-FeS females had longer path lengths than BAR-FeD females (BAR-FeS: 12.7 ± 0.8 m vs. BAR-FeD: 10.3 ± 0.7 m) and BAR-FeS females had longer path lengths than STD-FeS females (BAR-FeS: 12.7 ± 0.8 m vs. STD-FeS: 10.5 ± 0.7 m). Secondly, there was a sex × rearing × MnOE interaction ($F(1262) = 3.94$, $p < 0.05$), in which BAR-MnOE females had longer path lengths than BAR-VEH females (BAR-Mn: 13.2 ± 0.7 m vs. BAR-VEH: 9.8 ± 0.7 m); and STD-MnOE males had longer path lengths than STD-VEH males (STD-Mn: 8.3 ± 0.7 m vs. STD-VEH: 6.6 ±

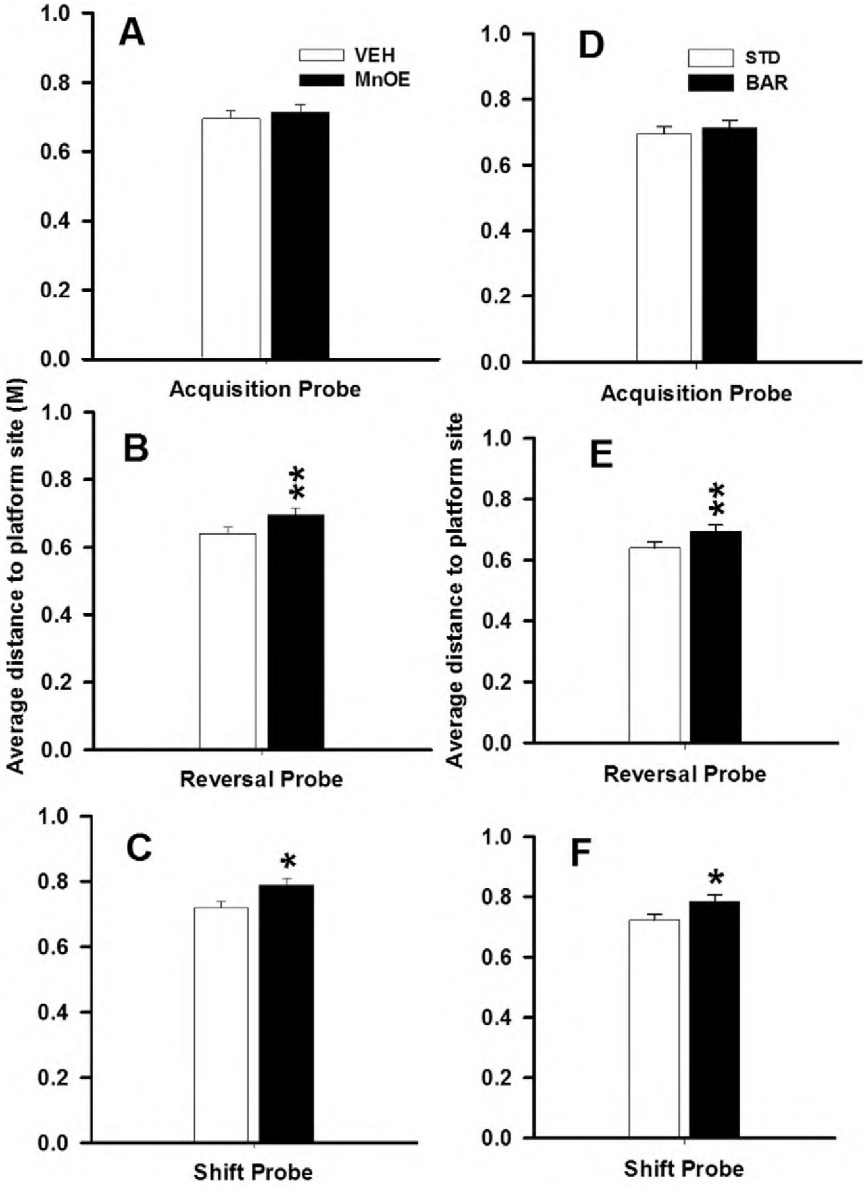

Fig. 4. Morris water maze probe: **A,B,C**: effect of MnOE on average distance to the former platform site during acquisition (**A**), reversal (**B**), and shift (**C**) probe trials of each phase. For each phase the 45 s probe trial was given on day-7 with the platform removed. **D,E,F**: Effect of rearing condition on probe performance during acquisition (**D**), reversal (**E**), and shift (**F**), respectively. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with VEH or STD cage controls.

0.6 m). Thirdly, rearing condition interacted with day ($F(5929) = 3.96$, $p < 0.01$) in which on day-1, BAR animals had shorter path lengths than STD animals, but were similar on subsequent days. There were no swim speed differences on reversal trials (not shown).

On reversal probe, MnOE animals had greater average distance from the platform site compared with VEH animals ($F(1145) = 7.26$, $p < 0.01$, Fig. 4B), and BAR animals had increased average distance from the platform site compared with STD animals (main effect: $F(1145) = 9.67$, $p < 0.01$, Fig. 4E). The BAR × FeD interaction seen on the acquisition probe trial was not seen on the reversal probe trial (Fig. 5B). However, there was a diet × MnOE interaction on average distance from the platform site ($F(1145) = 9.67$, $p < 0.01$) in which FeD-MnOE animals had increased distance from the site compared with controls (Fig. 5D). There was also a sex × MnOE interaction ($F(1145) = 3.87$, $p < 0.05$) in which MnOE males were further from the platform site compared with VEH males (not shown).

3.5. MWM-shift

MnOE resulted in longer path lengths on hidden platform trials (main effect: $F(1228) = 13.21$, $p < 0.001$, Fig. 3E,F). A significant sex × MnOE interaction showed that the hidden platform effect was most pronounced in MnOE females compared with VEH females ($F(1146) = 6.77$, $p < 0.01$; not shown). There was also a diet × rearing condition interaction ($F(1233) = 7.94$, $p < 0.01$) that followed the same pattern as that seen on acquisition (Fig. 5C). A sex × rearing condition × diet interaction ($F(1236) = 4.1$, $p < 0.05$) occurred in which STD-FeD females had increased path lengths compared with STD-FeS females (STD-FeD: 11.3 ± 0.7 m vs. STD-FeS: 9.8 ± 0.7 m), and BAR-FeS females had increased path lengths compared with BAR-FeD females (BAR-FES: 11.3 ± 0.7 m vs. BAR-FeD: 8.5 ± 0.6 m). A sex × rearing condition × diet × day interaction was also significant ($F(5920) = 2.71$, $p < 0.05$). It showed that on day-1, BAR-FeD males had shorter path lengths compared with males in all other groups (not shown). In females (Fig. 5E), both STD-FeD and BAR-FeS animals had longer path lengths than BAR-FeD and STD-FeS animals that were similar to one another, especially on Days 3 and 4 with only STD-FeD females showing an effect on Day 6. Finally, there was a rearing × day interaction ($F(5920) = 2.91$, $p < 0.05$) in which BAR animals had longer path lengths than VEH animals on the final day of learning (not shown). Swim speed was unaffected by any of the factors (not shown).

On the shift probe trial, there was an effect on average distance from the platform site ($F(1146) = 6.77$, $p < 0.01$, Fig. 4C) in which MnOE animals were farther from the platform site compared with VEH animals. For rearing, BAR animals had increased distance from the platform site on the shift probe trial compared with STD animals (main effect: $F(1146) = 5.31$, $p < 0.05$, Fig. 4F). No significant differences were found for diet or for any of the factors on the 1-week delayed probe trial.

3.6. MWM-cued

MnOE animals took longer to find the visible platform than VEH animals (main effect $F(1152) = 4.75$, $p < 0.05$), and there was a significant sex × MnOE × day interaction ($F(1199) = 5.81$, $p < 0.05$). For the latter, on day-1, MnOE males took longer to find the platform than VEH males (Fig. 5F), but on day-2 all groups were comparable.

3.7. α-Synuclein

α-Synuclein in the hippocampus and neostriatum was measured 24 h after the last MnOE treatment on P29 and on another group 32 days later on P60 (Figs. 6–7). Two-way ANOVA on P29 data showed a significant MnOE × region interaction ($F(1,20) = 9.43$, $p < 0.01$). Slice-effect ANOVAs for each region showed an increase in MnOE animals in

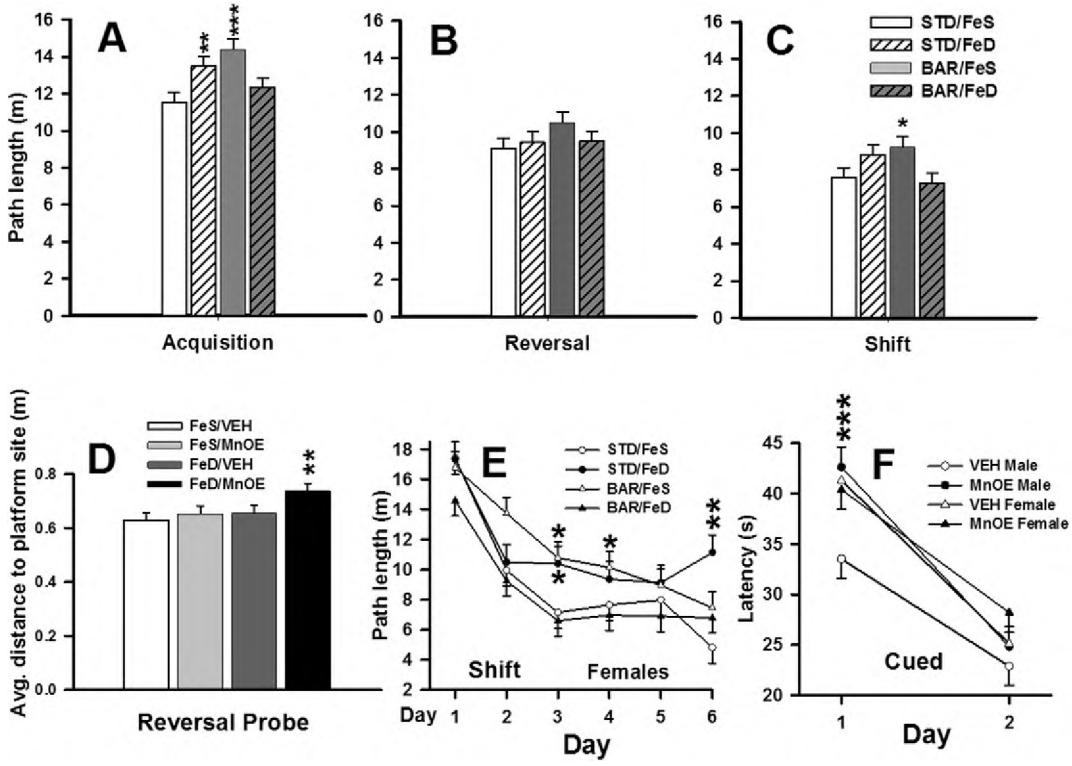

Fig. 5. Morris water maze interactions. **A,B,C**: path length (m) showing the interaction between cage condition and diet in the MWM during acquisition (**A**), reversal (**B**), and shift (**C**) phases of testing. **D**, interaction of MnOE and diet on reversal probe performance for average distance to the platform site (m). **E**, shows the result for the MWM Shift phase in females for the MnOE × sex interaction (female results shown) as a function of BAR vs. STD cage rearing. **F**, shows the result for MWM cued (proximal cue) learning as a function of MnOE and sex conducted after hidden platform testing to determine if rats could find a visible platform moved randomly from trial-to-trial and with curtains closed around the pool to obscure distal cues. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the relevant control condition in each panel.

the hippocampus ($p < 0.05$) and neostriatum ($p < 0.05$) compared with VEH animals on P29. On P60 there was also a significant MnOE × region interaction ($F(1,20) = 4.35$, $p < 0.05$). Slice-effect ANOVAs for each region at this age showed that MnOE animals had a decrease in the neostriatum compared with VEH animals ($p < 0.01$) and increase in the hippocampus that was not significant ($p < 0.09$).

3.8. Electrophysiology

LTP in the CA1 region of the hippocampus showed a significant MnOE × time interaction ($F(1,59 = 4.58$, $p < 0.05$, Fig. 8). MnOE animals had depressed LTP induction inasmuch as controls rose to nearly 400% of baseline following the tetanus and remained at approximately 200% of baseline for 35 min, whereas MnOE animals rose no higher than 150% of baseline. LTP maintenance beyond 35 min in MnOE animals was similar to VEH at 150% of baseline from 35 to 60 min suggesting that this phase of LTP was not affected by Mn exposure.

4. Discussion

We tested the neurocognitive effects of developmental MnOE alone and in combination with developmental stress and FeD, factors that often co-occur with MnOE in children using a model of MnOE we have used previously and for which we reported serum and brain Mn concentrations (Vorhees et al., 2014). There we showed that the same dose as used here resulted in Mn concentrations in neostriatum of VEH = 0.39 ± 0.12 μg/g tissue vs. MnOE = 2.39 ± 0.12 μg/g tissue and serum levels of VEH = 11.67 ± 4.75 μg/L vs. MnOE = 16.62 ± 4.75 μg/L. It was hypothesized that the combination would interact to exacerbate the effects of MnOE and if correct, account for some of the variability seen in epidemiological studies of MnOE in children. Interactions were found, but they were not simple exaggerations of MnOE-related effects.

By itself, developmental MnOE caused egocentric (Fig. 2) and allocentric learning and memory deficits (Figs. 3–4). The two tests used, CWM and MWM, reflect neostriatal and hippocampal-entorhinal cortex learning and memory, respectively, and both of these findings are new, never having been reported after developmental MnOE, although other effects have been reported (Pappas et al., 1997; Kern et al., 2010). MnOE increased latency and errors in the CWM (Fig. 2), indicating that neostriatal egocentric-mediated pathways subserving route-based navigation were affected (Buzsaki and Moser, 2013). This finding is consistent with our data that neostriatal dopaminergic dysfunction can cause CWM deficits (Braun et al., 2012; Braun et al., 2015) and that this MnOE model alters neostriatal dopamine (Amos-Kroohs et al., 2016). The data are similar to those seen after developmental methamphetamine exposure (Vorhees et al., 2009), suggesting that the neonatal period is a critical period in brain development in rats for the proper development of brain systems that underlie egocentric learning. MnOE also impaired allocentric learning in the MWM, including acquisition, reversal, and shift phases of assessment (Fig. 3). These data suggest that hippocampal-entorhinal-cortex networks (Buzsaki and Moser, 2013) that mediate spatial navigation are also in a critical phase of development during the neonatal period. This is important since the neonatal period in rodents for hippocampal development is roughly equivalent to third trimester hippocampal development in humans (Clancy et al., 2007). Reference memory as reflected by performance on probe trials was also impaired by MnOE at the end of reversal and shift trials (Fig. 4).

As noted above, the effects seen in the CWM suggest a possible role of neostriatal dopamine changes caused by MnOE (Braun et al., 2012; Braun et al., 2015). Dopaminergic involvement is consistent with previous developmental MnOE studies that found dopaminergic changes

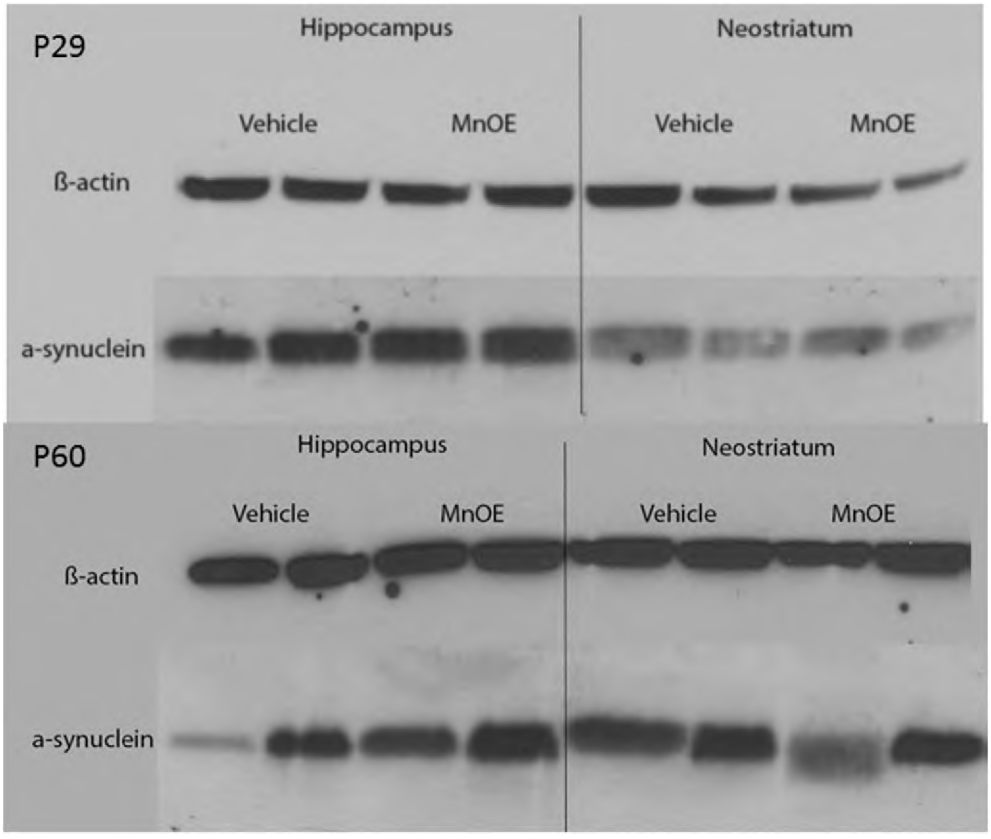

Fig. 6. Western blots of α-synuclein in hippocampus and neostriatum of VEH and MnOE rats at two ages: P29 and P60. Semiquantitative analysis of band density using ImageJ software and expressed against actin are presented in Fig. 7. Top panel: P29; bottom panel: P60. Left panels: hippocampus; right panels: neostriatum. $N = 4$/age (males).

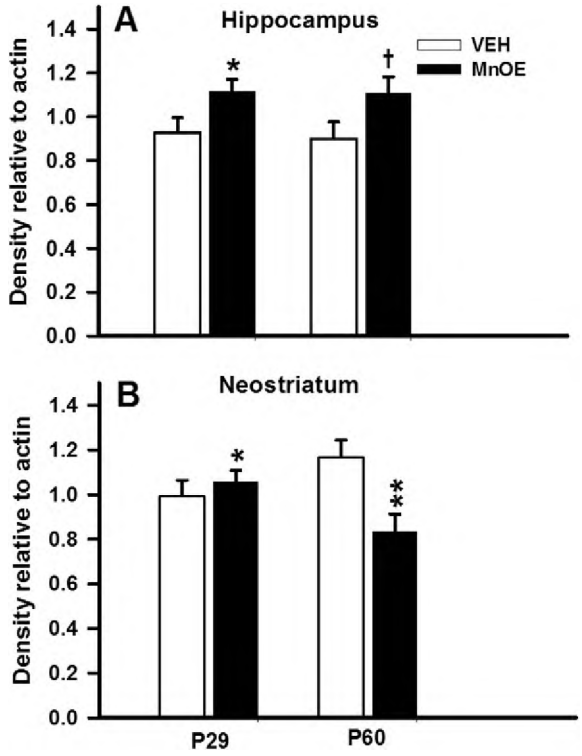

Fig. 7. Semiquantitative concentration estimates of α-synuclein in two brain regions and at two ages in a group of naïve animals from MnOE or VEH groups. **A**, α-synuclein relative band density by Western analysis in hippocampus in MnOE and VEH treated rats gavaged every other day from P4-28 and assessed on P29 or P60. **B**, α-synuclein relative density in neostriatum of the same animals as in **A**. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\dagger}p < 0.10$ trend in MnOE rats compared with VEH controls.

(Moreno et al., 2009; Kern and Smith, 2011; Amos-Kroohs et al., 2016). As for the allocentric learning and memory deficits found with the MWM, these are consistent with the data that LTP is affected by changes in dopamine (Edelmann and Lessmann, 2013) and our finding that MnOE impaired LTP induction while not affecting LTP maintenance (Fig. 8).

It is worth noting that, while different, manganism and parkinsonism share several features, among them changes in dopaminergic signaling and α-synuclein. Herein, we found α-synuclein changes in the hippocampus that are consistent with the pattern of learning and memory deficits found in the MnOE animals in the MWM (Figs. 6–7). Since

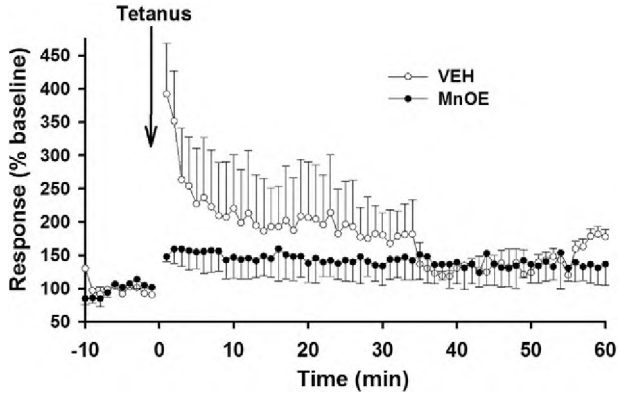

Fig. 8. Long-term potentiation (LTP). LTP in the CA1 region of the hippocampus in rats exposed to Mn by gavage every other day from P4-22 and brain slices analyzed on P23 following a tetanizing stimulus. There was a significant interaction of MnOE × Time (min). As can be seen, most of the effect of Mn was to blunt the induction phase of LTP during the first 35 min. After 35 min, LTP continued but the two groups' responses converged at approximately 150% above pre-tetanus baseline.

α-synuclein is associated with dopaminergic release, and we found α-synuclein increased in MnOE animals' hippocampi, these changes may cause dysregulation of dopamine signaling and disrupt allocentric learning. While these observations are only associations at this stage, their consistency suggests that further investigation of such connections may be worthwhile. It is less clear why α-synuclein expression changes in the neostriatum differed by age with increases at P29 and reductions 31 days later at P60. Perhaps early, shortly after the end of MnOE, α-synuclein accumulated whereas 31 days later the reduction reflects a pathophysiological downstream effect from cell loss. A direct test of this idea may resolve the point.

The observation that CA1 LTP induction was impaired along with α-synuclein accumulation in the hippocampus following developmental MnOE is noteworthy especially since the α-synuclein accumulation in this region persisted long after exposure. However, unlike Parkinson's disease where the neurodegenerative process results in continuous α-synuclein accumulation, in the present model the effect appeared relatively static indicating that developmental MnOE does not trigger progressive neurodegeneration.

The effect of developmental stress in this study was induced using a barren cage rearing model (BAR). It was found that BAR cage rearing increased latency and errors in the CWM (Fig. 2), indicating that egocentric learning is sensitive to the effect of early stress. Preweaning stress by this method also interacted with MnOE to affect neostriatal dopamine as reported elsewhere (Vorhees et al., 2014). The data are consistent with effects of developmental stress reported by others inasmuch as prenatal restraint stress has been shown to alter midbrain dopamine (Baier et al., 2012). There is also evidence that prenatal stress alters dopamine receptors, dendritic morphology, and basal and stimulated dopamine release by in vivo microdialysis in the striatum (Silvagni et al., 2008; Martinez-Tellez et al., 2009; Lovic et al., 2013). While not the same as the stressor we used, such data suggest a link between developmental stress and dopaminergic function that could affect egocentric learning.

The third independent variable investigated in this study was FeD. We did not use a diet that induced iron-deficiency anemia, but rather a moderate level of FeD. We chose this because it more closely resembles the condition found among children described as subclinical FeD that often occurs in lower SES conditions. In rats, we found that developmental FeD exposed groups had slightly reduced latencies in the CWM compared with FeS animals (Fig. 2). This may reflect a rebound effect, i.e., rather than FeD inducing a lasting effect, it may have produced changes that during Fe repletion resulted in a rapid compensatory increase in neurogenesis in regions that mediate learning. This is supported by evidence that in humans FeD followed by repletion temporarily improves learning and memory. However, this only occurs after moderate FeD, not after Fe-deficiency anemia, but this is exactly what we did, i.e., we induced moderate FeD followed by repletion. The FeD diet used here was not associated with any changes in MWM performance, although there were rearing condition × diet interactions on MWM acquisition and shift phases of the test (Fig. 5). FeD diet and BAR cage rearing had the effect of increasing path lengths compared with VEH animals, whereas both together did not show this effect. This suggests an interaction in which one effect offsets the other. How such a compensatory interaction might be mediated is unknown.

Only one dose of MnOE, one level of FeD, and one kind of stress were tested here therefore we cannot generalize to other models. Also, only one exposure period was assessed; earlier or later exposure periods may have different effects. We used two tests of learning and memory, but other tests may be worth investigating along with other neurochemical markers. Strengths of the study include testing 'real-world' factors found in exposed children. Experiments such as this one are challenging because of the need to control for litter effects, have adequate sample sizes, and maintain consistency over the months it takes to conduct an experiment of this kind. However, interaction studies may assist in modeling environments that more closely resemble

human conditions and in so doing provide perspective compared with studying one factor at a time.

In conclusion, developmental MnOE had long-term adverse effects on egocentric learning and memory in the CWM on indices of errors and latency, adverse effects on allocentric learning and memory across three phases in the MWM on multiple indices during acquisition, reversal, and shift and on probe trials and impaired hippocampal LTP and affected α-synuclein levels in neostriatum and hippocampus. BAR had its own effect as did FeD but in terms of interactions with MnOE, effects were very limited. For CWM, it was unexpectedly the MnOE-FeS group that made the most errors rather than the MnOE-FeD group. There were no MnOE × BAR interactions in CWM performance and no triple interactions. In the MWM there was one MnOE × BAR interaction and one MnOE × FeD interaction and no triple interactions. These interactions only occurred during reversal trials, not during acquisition or shift. On reversal, the MnOE-BAR females had longer path lengths than VEH-BAR females on platform trials. On the reversal probe trial, the MnOE-FeD group had increased average distance to the platform site than VEH-FeS controls. Overall, the data did not support strong interactions between BAR, FeD, and MnOE on egocentric or allocentric learning and memory, but the data did show striking effects of developmental MnOE alone on both types of learning and memory.

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References

Amos-Kroohs, R.M., Bloor, C.P., Qureshi, M.A., Vorhees, C.V., Williams, M.T., 2015. Effects of developmental exposure to manganese and/or low iron diet: changes to metal transporters, surcrose preference, elevated zero-maze, open-field, and locomotion in response to fenfluamine, amphetamine, and MK-801. Toxicol. Rep. 2, 1046–1056.

Amos-Kroohs, R.M., Davenport, L.L., Gutierrez, A., Hufgard, J.R., Vorhees, C.V., Williams, M.T., 2016. Developmental manganese exposure in combination with developmental stress and iron deficiency: effects on behavior and monoamines. Neurotoxicol. Teratol. 56, 55–67.

Avishai-Eliner, S., Gilles, E.E., Eghbal-Ahmadi, M., Bar-El, Y., Baram, T.Z., 2001. Altered regulation of gene and protein expression of hypothalamic-pituitary-adrenal axis components in an immature rat model of chronic stress. J. Neuroendocrinol. 13, 799–807.

Baier, C.J., Katunar, M.R., Adrover, E., Pallares, M.E., Antonelli, M.C., 2012. Gestational restraint stress and the developing dopaminergic system: an overview. Neurotox. Res. 22, 16–32.

Beaudin, S.A., Nisam, S., Smith, D.R., 2013. Early life versus lifelong oral manganese exposure differently impairs skilled forelimb performance in adult rats. Neurotoxicol. Teratol. 38, 36–45.

Beaudin, S.A., Strupp, B.J., Lasley, S.M., Fornal, C.A., Mandal, S., Smith, D.R., 2015. Oral methylphenidate alleviates the fine motor dysfunction caused by chronic postnatal manganese exposure in adult rats. Toxicol. Sci. 144, 318–327.

Bouchard, M.F., Sauve, S., Barbeau, B., Legrand, M., Brodeur, M.E., Bouffard, T., Limoges, E., Bellinger, D.C., Mergler, D., 2011. Intellectual impairment in school-age children exposed to manganese from drinking water. Environ. Health Perspect. 119, 138–143.

Braun, A.A., Graham, D.L., Schaefer, T.L., Vorhees, C.V., Williams, M.T., 2012. Dorsal striatal dopamine depletion impairs both allocentric and egocentric navigation in rats. Neurobiol. Learn. Mem. 97, 402–408.

Braun, A.A., Amos-Kroohs, R.M., Gutierrez, A., Lundgren, K.H., Seroogy, K.B., Skelton, M.R., Vorhees, C.V., Williams, M.T., 2015. Dopamine depletion in either the dorsomedial or dorsolateral striatum impairs egocentric Cincinnati water maze performance while sparing allocentric Morris water maze learning. Neurobiol. Learn. Mem. 118, 55–63.

Buzsaki, G., Moser, E.I., 2013. Memory, navigation and theta rhythm in the hippocampal-entorhinal system. Nat. Neurosci. 16, 130–138.

Clancy, B., Finlay, B.L., Darlington, R.B., Anand, K.J., 2007. Extrapolating brain development from experimental species to humans. Neurotoxicology 28, 931–937.

Cordova, F.M., Aguiar Jr., A.S., Peres, T.V., Lopes, M.W., Goncalves, F.M., Remor, A.P., Lopes, S.C., Pilati, C., Latini, A.S., Prediger, R.D., Erikson, K.M., Aschner, M., Leal, R.B., 2012. In vivo manganese exposure modulates Erk, Akt and Darpp-32 in the striatum of developing rats, and impairs their motor function. PLoS One 7, e33057.

Dorner, K., Dziadzka, S., Hohn, A., Sievers, E., Oldigs, H.D., Schulz-Lell, G., Schaub, J., 1989. Longitudinal manganese and copper balances in young infants and preterm infants fed on breast-milk and adapted cow's milk formulas. J Nutr]–>Br. J. Nutr. 61, 559–572.

Edelmann, E., Lessmann, V., 2013. Dopamine regulates intrinsic excitability thereby gating successful induction of spike timing-dependent plasticity in CA1 of the hippocampus. Front. Neurosci. 7, 25.

Fitsanakis, V.A., Thompson, K.N., Deery, S.E., Milatovic, D., Shihabi, Z.K., Erikson, K.M., Brown, R.W., Aschner, M., 2009. 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, 167–178.

Fitsanakis, V.A., Zhang, N., Avison, M.J., Erikson, K.M., Gore, J.C., Aschner, M., 2011. Changes in dietary iron exacerbate regional brain manganese accumulation as determined by magnetic resonance imaging. Toxicol. Sci. 120, 146–153.

Graham, D.L., Grace, C.E., Braun, A.A., Schaefer, T.L., Skelton, M.R., Tang, P.H., Vorhees, C.V., Williams, M.T., 2011. Effects of developmental stress and lead (Pb) on corticosterone after chronic and acute stress, brain monoamines, and blood Pb levels in rats. J Dev Neurosci]–>Int. J. Dev. Neurosci. 29, 45–55.

Hackman, D.A., Farah, M.J., Meaney, M.J., 2010. Socioeconomic status and the brain: mechanistic insights from human and animal research. Nat. Rev. Neurosci. 11, 651–659.

Haynes, E.N., Sucharew, H., Kuhnell, P., Alden, J., Barnas, M., Wright, R.O., Parsons, P.J., Aldous, K.M., Praamsma, M.L., Beidler, C., Dietrich, K.N., 2015. Manganese exposure and neurocognitive outcomes in rural school-age children: the communities actively researching exposure study (Ohio, USA). Environ. Health Perspect. 123, 1066–1071.

Kern, C.H., Smith, D.R., 2011. Preweaning Mn exposure leads to prolonged astrocyte activation and lasting effects on the dopaminergic system in adult male rats. Synapse 65, 532–544.

Kern, C.H., Stanwood, G.D., Smith, D.R., 2010. Preweaning manganese exposure causes hyperactivity, disinhibition, and spatial learning and memory deficits associated with altered dopamine receptor and transporter levels. Synapse 64, 363–378.

Khan, K., Factor-Litvak, P., Wasserman, G.A., Liu, X., Ahmed, E., Parvez, F., Slavkovich, V., Levy, D., Mey, J., Van, G.A., Graziano, J.H., 2011. Manganese exposure from drinking water and children's classroom behavior in Bangladesh. Environ. Health Perspect. 119, 1501–1506.

Khan, K., Wasserman, G.A., Liu, X., Ahmed, E., Parvez, F., Slavkovich, V., Levy, D., Mey, J., Van, G.A., Graziano, J.H., Factor-Litvak, P., 2012. Manganese exposure from drinking water and children's academic achievement. Neurotoxicology 33, 91–97.

Lee, A.I., Okam, M.M., 2011. Anemia in pregnancy. Hematol. Oncol. Clin. North Am. 25 (241–259), vii.

Lisman, J., Schulman, H., Cline, H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 3, 175–190.

Lovic, V., Belay, H., Walker, C.D., Burton, C.L., Meaney, M.J., Sokolowski, M., Fleming, A.S., 2013. Early postnatal experience and DRD2 genotype affect dopamine receptor expression in the rat ventral striatum. Behav. Brain Res. 237, 278–282.

Lucchini, R.G., Guazzetti, S., Zoni, S., Donna, F., Peter, S., Zacco, A., Salmistraro, M., Bontempi, E., Zimmerman, N.J., Smith, D.R., 2012. Tremor, olfactory and motor changes in Italian adolescents exposed to historical ferro-manganese emission. Neurotoxicology 33, 687–696.

Martinez-Tellez, R.I., Hernandez-Torres, E., Gamboa, C., Flores, G., 2009. Prenatal stress alters spine density and dendritic length of nucleus accumbens and hippocampus neurons in rat offspring. Synapse 63, 794–804.

Mena, I., Marin, O., Fuenzalida, S., Cotzias, G.C., 1967. Chronic manganese poisoning. Clinical picture and manganese turnover. Neurology 17, 128–136.

Menezes-Filho, J.A., de Carvalho-Vivas, C.F., Viana, G.F., Ferreira, J.R., Nunes, L.S., Mergler, D., Abreu, N., 2014. Elevated manganese exposure and school-aged children's behavior: a gender-stratified analysis. Neurotoxicology 45, 293–300.

Moreno, J.A., Yeomans, E.C., Streifel, K.M., Brattin, B.L., Taylor, R.J., Tjalkens, R.B., 2009. Age-dependent susceptibility to manganese-induced neurological dysfunction. Toxicol. Sci. 112, 394–404.

Morris, R.G., Moser, E.I., Riedel, G., Martin, S.J., Sandin, J., Day, M., O'Carroll, C., 2003. Elements of a neurobiological theory of the hippocampus: the role of activity-dependent synaptic plasticity in memory. R Soc Lond B Biol Sci]–>Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 358, 773–786.

Oulhote, Y., Mergler, D., Barbeau, B., Bellinger, D.C., Bouffard, T., Brodeur, M.E., Saint-Amour, D., Legrand, M., Sauve, S., Bouchard, M.F., 2014. Neurobehavioral function in school-age children exposed to manganese in drinking water. Environ. Health Perspect. 122, 1343–1350.

Pappas, B.A., Zhang, D., Davidson, C.M., Crowder, T., Park, G.A., Fortin, T., 1997. Perinatal manganese exposure: behavioral, neurochemical, and histopathological effects in the rat. Neurotoxicol. Teratol. 19, 17–25.

Reichel, C.M., Wacan, J.J., Farley, C.M., Stanley, B.J., Crawford, C.A., McDougall, S.A., 2006. Postnatal manganese exposure attenuates cocaine-induced locomotor activity and reduces dopamine transporters in adult male rats. Neurotoxicol. Teratol. 28, 323–332.

Reiss, F., 2013. Socioeconomic inequalities and mental health problems in children and adolescents: a systematic review. Soc. Sci. Med. 90, 24–31.

Silvagni, A., Barros, V.G., Mura, C., Antonelli, M.C., Carboni, E., 2008. Prenatal restraint stress differentially modifies basal and stimulated dopamine and noradrenaline release in the nucleus accumbens shell: an 'in vivo' microdialysis study in adolescent and young adult rats. J Neurosci]–>Eur. J. Neurosci. 28, 744–758.

Stottmann, R.W., Driver, A., Gutierrez, A., Skelton, M.R., Muntifering, M., Stepien, C., Knudson, L., Kofron, M., Vorhees, C.V., Williams, M.T., 2016. A heterozygous mutation in tubulin, Beta 2b (Tubb2b) causes cognitive deficits and hippocampal disorganization. Genes Brain Behav.

Sun, Y., Witte, D.P., Ran, H., Zamzow, M., Barnes, S., Cheng, H., Han, X., Williams, M.T., Skelton, M.R., Vorhees, C.V., Grabowski, G.A., 2008. Neurological deficits and glycosphingolipid accumulation in Saposin B deficient mice. Hum. Mol. Genet. 17, 2345–2356.

Sun, Y., Ran, H., Zamzow, M., Kitatani, K., Skelton, M.R., Williams, M.T., Vorhees, C.V., Witte, D.P., Hannun, Y.A., Grabowski, G.A., 2010a. Specific saposin C deficiency: CNS impairment and acid beta-glucosidase effects in the mouse. Hum. Mol. Genet. 19, 634–647.

Sun, Y., Liou, B., Ran, H., Skelton, M.R., Williams, M.T., Vorhees, C.V., Kitatani, K., Hannun, Y.A., Witte, D.P., Xu, Y.H., Grabowski, G.A., 2010b. Neuronopathic Gaucher disease in the mouse: viable combined selective saposin C deficiency and mutant glucocerebrosidase (V394L) mice with glucosylsphingosine and glucosylceramide accumulation and progressive neurological deficits. Hum. Mol. Genet. 19, 1088–1097.

Tran, T.T., Chowanadisai, W., Crinella, F.M., Chicz-Demet, A., Lonnerdal, B., 2002a. Effect of high dietary manganese intake of neonatal rats on tissue mineral accumulation, striatal dopamine levels, and neurodevelopmental status. Neurotoxicology 23, 635–643.

Tran, T.T., Chowanadisai, W., Lonnerdal, B., Le, L., Parker, M., Chicz-Demet, A., Crinella, F.M., 2002b. Effects of neonatal dietary manganese exposure on brain dopamine levels and neurocognitive functions. Neurotoxicology 23, 645–651.

Vorhees, C.V., 1987. Maze learning in rats: a comparison of performance in two water mazes in progeny prenatally exposed to different doses of phenytoin. Neurotoxicol. Teratol. 9, 235–241.

Vorhees, C.V., Williams, M.T., 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat. Protoc. 1, 848–858.

Vorhees, C.V., Williams, M.T., 2014. Value of water mazes for assessing spatial and egocentric learning and memory in rodent basic research and regulatory studies. Neurotoxicol. Teratol. 45, 75–90.

Vorhees, C.V., Williams, M.T., 2016. Cincinnati water maze: a review of the development, methods, and evidence as a test of egocentric learning and memory. Neurotoxicol. Teratol.

Vorhees, C.V., Weisenburger, W.P., Acuff-Smith, K.D., Minck, D.R., 1991. An analysis of factors influencing complex water maze learning in rats: effects of task complexity, path order and escape assistance on performance following prenatal exposure to phenytoin. Neurotoxicol. Teratol. 13, 213–222.

Vorhees, C.V., Herring, N.R., Schaefer, T.L., Grace, C.E., Skelton, M.R., Johnson, H.L., Williams, M.T., 2008. Effects of neonatal (+)-methamphetamine on path integration and spatial learning in rats: effects of dose and rearing conditions. J Dev Neurosci]–>Int. J. Dev. Neurosci. 26, 599–610.

Vorhees, C.V., Skelton, M.R., Grace, C.E., Schaefer, T.L., Graham, D.L., Braun, A.A., Williams, M.T., 2009. Effects of (+)-methamphetamine on path integration and spatial learning, but not locomotor activity or acoustic startle, align with the stress hyporesponsive period in rats. J Dev Neurosci]–>Int. J. Dev. Neurosci. 27, 289–298.

Vorhees, C.V., Graham, D.L., Amos-Kroohs, R.M., Braun, A.A., Grace, C.E., Schaefer, T.L., Skelton, M.R., Erikson, K.M., Aschner, M., Williams, M.T., 2014. Effects of developmental manganese, stress, and the combination of both on monoamines, growth, and corticosterone. Toxicol. Rep. 1, 1046–1061.

Walker, S.P., Wachs, T.D., Grantham-McGregor, S., Black, M.M., Nelson, C.A., Huffman, S.L., Baker-Henningham, H., Chang, S.M., Hamadani, J.D., Lozoff, B., Gardner, J.M., Powell, C.A., Rahman, A., Richter, L., 2011. Inequality in early childhood: risk and protective factors for early child development. Lancet 378, 1325–1338.

Wasserman, G.A., Liu, X., Parvez, F., Ahsan, H., Levy, D., Factor-Litvak, P., Kline, J., Van, G.A., Slavkovich, V., Loiacono, N.J., Cheng, Z., Zheng, Y., Graziano, J.H., 2006. Water manganese exposure and children's intellectual function in Araihazar, Bangladesh. Environ. Health Perspect. 114, 124–129.

Youdim, M.B., 2008. Brain iron deficiency and excess; cognitive impairment and neurodegeneration with involvement of striatum and hippocampus. Neurotox. Res. 14, 45–56.

Zoni, S., Lucchini, R.G., 2013. Manganese exposure: cognitive, motor and behavioral effects on children: a review of recent findings. Curr. Opin. Pediatr. 25, 255–260.