Maternal choline supplementation: A potential therapy for developmental Manganese exposure?

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Key terms

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Highlights

1. The MCS intervention was partially, but not completely, effective in protecting against Mn-induced deficits; the degree of benefit varied with the various functional domains. Supplementing the maternal diet with choline during pregnancy and lactation provides some benefits to the Mn-exposed animals, including lessening differences between Mn and control animals in attentional function. MCS also partially normalizes the reaction of Mn exposed animals to committing an error or not receiving an expected reward.
2. This study has uncovered the effect of developmental Mn exposure to cause lasting abnormalities in behavioral reactivity to committing an error or not receiving an expected reward. Additionally, we have replicated the effects seen in our prior animal model studies in which Mn causes deficits in attention, learning, and sensorimotor function. The Mn deficits reported here parallel many of the behavioral deficits seen in children exposed to high levels of Mn during development, establishing developmental Mn exposure as an environmental risk factor for ADHD symptoms more broadly.
3. In the absence of Mn exposure, maternal choline supplementation produced lasting offspring benefits in the area of attentional function, recapitulating benefits of MCS demonstrated in other studies.
4. These findings underscore the lasting health risks to children posed by developmental Mn exposure and provide new evidence that MCS lessens some areas of Mn-induced dysfunction.
5. In light of evidence that 90% of pregnant women consume less choline than the current Adequate Intake (AI) levels, these data provide additional support for efforts to add choline to standard prenatal vitamin regimens.

Abstract

Background: Studies in children and adolescents have associated early developmental manganese (Mn) exposure with inattention, impulsivity, hyperactivity, and fine motor deficits, and our rodent studies of early life Mn exposure have recapitulated many of these outcomes, demonstrating causality. There are currently no recognized therapies/interventions other than exposure prevention to mitigate the neurotoxic effects of developmental Mn exposure. One possible preventive treatment is to supplement the maternal diet with additional choline during pregnancy. Maternal choline supplementation (MCS) has been shown to improve offspring cognitive function in humans and animal models, and lessens dysfunction caused by various developmental insults.
Objectives: Determine whether MCS during pregnancy and lactation protects against Mn-induced impairments in attention, impulse control, learning, behavioral reactivity, and sensorimotor function.
Methods: Starting at gestational day 3 (G3), pregnant dams were given standard diet or diet with additional choline (4x the level in standard diets) throughout gestation and lactation until offspring were weaned on PND 21. Pups were exposed orally to 0 or 50 mg Mn/kg/day during early postnatal life (PND 1–21). In adulthood, animals were tested in the five-choice serial reaction time task and the Montoya staircase task, to assess impulsivity, focused and selective attention, behavioral reactivity to errors or omission of an expected reward, and sensorimotor function.
Results: MCS intervention was partially effective in protecting against the Mn-induced deficits, with the degree of benefit varying with the specific domain of function. Specifically, MCS lessens differences between Mn and control animals in attentional function and reactivity to committing an error or not receiving an expected reward. MCS does not protect against Mn-induced sensorimotor dysfunction. Finally, in the absence of Mn exposure, MCS produces lasting benefits in attentional function and reactivity to errors.
Conclusions: MCS was partially effective in alleviating Mn-induced deficits – MCS normalized Mn exposed animals in the domains of attentional function and behavioral reactivity. These findings have implications for understanding the molecular mechanisms by which both MCS and Mn produce lasting cognitive changes, and provide further evidence that MCS benefits offspring. These findings, taken with evidence from other studies demonstrating the benefit of MCS to offspring and the fact that ~90% of women consume less than the adequate intake (AI) of choline, inform the recommendation that MCS should be considered for pregnant women.

1. Introduction

Attention and impulse control dysfunction, including ADHD, is the most prevalent neurodevelopmental syndrome among children, affecting ~5 - 7% of youths up through age 18 years, and ~2 – 3% of adults[1]. There is an emerging body of epidemiological evidence showing that environmental manganese (Mn) exposure during neurodevelopment is associated with increased risk of ADHD and/or attention/impulsivity/hyperactivity symptoms in children and adolescents[2,3,12,4–11]. However, while these epidemiologic studies have played key roles in highlighting associations between elevated Mn exposure and increased risk of ADHD-related symptoms, they are unable to establish causality, in part because of challenges identifying and/or controlling for confounding factors, and in accurately assessing exposure.

Our prior rodent model studies have shown that developmental Mn exposure can cause lasting ADHD-like deficits in attention, impulse control, and sensorimotor function, and that these deficits are associated with a hypofunctioning catecholaminergic system in the prefrontal cortex and striatum, the brain regions

mediating attentional, impulse control, and sensorimotor function[13–20]. Notably, gene expression of several of the catecholaminergic system proteins altered by developmental Mn exposure are known to be regulated in part by epigenetic mechanisms[21–25], and thus Mn-induced epigenetic alterations may contribute to the lifelong alterations we see in these protein levels due to developmental Mn exposure.

While therapies such as methylphenidate (Ritalin) exist for children diagnosed with ADHD, there are no established treatments available for children exposed to high levels of Mn during development. A number of nutritional interventions, including maternal dietary supplementation with choline, folic acid, genistein, and L-methionine, have been shown to mitigate the effects of a variety of environmental insults[26,27]. In particular, maternal choline supplementation (MCS) has been shown in animal models to lessen cognitive dysfunction caused by prenatal alcohol exposure[28–30], maternal stress[31], and Down syndrome[32–34], and to normalize some gene expression and epigenetic molecular alterations caused by perinatal alcohol exposure[35,36]. In addition, MCS has been shown to improve cognitive function of typically developing human offspring[37–39] and animal models[40–48]. Consistent with this, current recommendations for nutritionally sufficient levels of maternal choline intake may be inadequate, particularly over periods of increased nutritional demand such as during fetal and neonatal development (pregnancy and lactation)[49–51]. Notably, despite the fact that the adequate intake (AI) level of choline is likely insufficient, over 90% of women do not meet this intake level of choline during pregnancy[39,49,50,52]. Thus, while numerous studies show that offspring may derive cognitive benefits from MCS, it is unknown whether MCS provides protection against the neurocognitive deficits caused by developmental Mn exposure, or whether elevated Mn exposure mitigates some of the cognitive benefits of MCS.

Here, we used a rodent model of elevated childhood Mn exposure to determine whether MCS protects against the attention, impulse control, behavioral reactivity, and sensorimotor deficits caused by developmental Mn exposure.

2. Materials and methods

Subjects

Sixty-four (64) male Long-Evans rats were used for neurobehavioral assessment. Additional littermates were used for tissue Mn analysis and choline/choline metabolite analysis. Subjects were born in-house from 16 nulliparous timed-pregnant dams (Charles River; gestational age 3; 8-9 weeks old). Twelve to 24 hours after parturition (designated PND 1, birth = PND 0), litters were weighed, sexed, and culled to eight pups per litter such that each litter was composed of as many males as possible (2-8) and the remainder females. Each treatment group contained n = 16 animals, and no more than 1-2 males per litter were assigned to a particular treatment group. Animals were housed in polycarbonate cages at a constant temperature of 21 ± 2°C. At PND 22, all pups were weaned and pair-housed with an animal of the same treatment group and maintained on a reversed 12:12 hour light/dark cycle. All aspects of behavioral testing and feeding were carried out during the active (dark) phase of the animals' diurnal cycle. All animal care and treatments were approved by the institutional IACUC (protocol Smitd1803) and adhered to National Institutes of Health guidelines set forth in the Guide for the Care and Use of Laboratory Animals.

Maternal choline supplementation

Pregnant dams were randomly selected to receive one of two concentrations of choline chloride in their diet (Dyets AIN-76A Purified Rodent Diet), both of which were provided ad libitum during gestation and

lactation, from G3 until PND 21. The unsupplemented purified diet food contained 1.1 g choline/kg, while the supplemented purified diet food contained 5.0 g choline/kg; both are reported by the manufacturer to contain 54 mg Mn/kg. Upon weaning on PND 21, all pups were provided Irradiated Teklad Global Soy Protein-Free Extruded Rodent Diet 2920, reported by the manufacturer to contain 80 mg Mn/kg and 1200 mg choline/kg. See Figure 1 for a timeline of maternal choline supplementation, Mn dosing, and behavioral testing.

Manganese exposure

Neonate animals were orally exposed to 0 or 50 mg Mn/kg/day in a vehicle solution (5% w/v stevia in Milli-Q water) from PND 1–21, as in our prior studies[13–19]. Briefly, Mn was delivered once per day directly into the mouth of each pup (~15-25 µL/dose) using a micropipette fitted with a flexible polyethylene pipette tip. This Mn exposure regimen is relevant to children exposed to elevated Mn via drinking water, diet, or both, as pre-weaning exposure to 50 mg Mn/kg/day produces relative increases in Mn intake that approximates the increases reported in infants and young children exposed to Mn-contaminated water or soy- based formulas (or both)[15,17].

Testing apparatus

Sixteen identical automated 5-Choice Serial Reaction Time Task (5-CSRTT) testing chambers fitted with odor delivery systems (#MED-NP5L-OLF, Med Associates, Inc., St. Albans, VT) were used to assess specific cognitive processes, including focused and selective attention, inhibitory control, and behavioral reactivity, as described previously[15,16,53]. Briefly, each testing chamber contained a curved wall equipped with five 2.5 × 2.5 cm response ports fitted with light-emitting diodes that served as the visual cue, an infrared beam to register nose pokes, and pneumatic inlet and vacuum outlet ports to introduce and remove air-based odor distractors. Opposite the response wall was the food magazine wall that contained a 45 mg food pellet reward dispensing port fitted with an infrared beam to register nose pokes; each unit also contained a small house light. The entire testing chamber was enclosed in a sound attenuating cubicle. Each animal was assigned to one of the 16 testing chambers, and each chamber was balanced for the four treatment conditions.

Behavioral testing

Animals were mildly food restricted starting on PND 45 in preparation for behavioral testing, with water available ad libitum throughout behavioral assessment. Animals were provided food for 2 hours each day immediately after behavioral testing, so that their body weights were maintained at ~90–95% of free-feeding weights. All rats were weighed and tested 6 days/week throughout training and testing. Behavioral assessment occurred during the active (dark) period of the diurnal cycle at the same time each day and in the same chamber for each individual rat. A daily test session consisted of 150 trials or 50 min, whichever came first. Each trial sequence was initiated by a nose-poke in the food magazine port, and followed by a 3 sec turnaround time to allow the animal to reorient from the food magazine wall to the response wall; trial onset began after the 3 sec turnaround time. All behavioral testing was conducted by individuals blind to the treatment condition of the subjects.

Behavioral testing began on ~ PND 50, as described elsewhere[15]. Briefly, testing began with food magazine and nose-poke training for 11 days, followed by a 5-choice visual discrimination learning task with a fixed 15 sec cue duration and no pre-cue delay. Animals stayed on this task until they reached the criteria of 80% Correct trials on 2 of 3 consecutive testing days. Following this task, animals were moved to a second visual discrimination task with a fixed 1 sec cue duration and no pre-cue delay for 5 days.

This training was followed by a series of attention and behavioral reactivity tasks as described below (see Figure 1 for the timeline of testing).

5-CSRTT assessment is conducted via a series of progressively more difficult tasks, described in greater detail below, that challenge the ability of animals to sustain attention, inhibit premature responses, and ignore olfactory distractions in favor of the salient visual cue. Recorded response types for all 5-CSRTT tests included the following: 1) premature responses (responses made after trial onset but before presentation of the visual cue; in the first focused attention, second focused attention, and selective attention tasks); 2) correct response (responses made to the correct port following presentation of the visual cue); 3) incorrect response (responses made to the incorrect port following presentation of the visual cue); and 4) omissions (failure to respond within the 15 sec response interval following the visual cue). Premature and incorrect responses and omission errors were not rewarded and were immediately followed by a 5 sec time-out, in which the house light was turned off for 5 sec. In addition, the latency (sec) for correct and incorrect responses was recorded, as was the latency to retrieve the food pellet reward following a correct response. Perseverative responses, defined as additional nose pokes into the correct port after a correct response, were also recorded. The calculated response outcomes were % Correct, calculated as number of correct responses/(correct + incorrect + premature + omissions) × 100; % Incorrect, calculated as above but with incorrect responses in the numerator; % Premature, calculated as number of premature responses/(correct + incorrect + premature + omissions) × 100; and % Omissions, calculated as number of omissions/(correct + incorrect + premature + omissions) × 100. Additionally, because %Correct includes all trial response outcomes in the denominator, all error types (incorrect, premature, or omission) may contribute to differences between treatment groups. Therefore, we also assessed attentional accuracy via the %Accurate outcome, which does not include premature or omitted responses, and is calculated as number of correct responses/(correct + incorrect) × 100.

Focused attention tasks

Focused attention can be defined as the ability to maintain attentional focus on a specific task or stimulus (e.g., a visual cue). Two successive focused attention tasks (FA1 and FA2) were administered over ~PND 85-103, and PND 104-121, respectively, following completion of the visual learning discrimination task. Both of these tasks assessed the ability of animals to detect and respond to a visual cue presented unpredictably in time and location (one of the five response ports). The first focused attention task used variable pre-cue delays of 0, 1, 2, or 3 sec and a fixed visual cue duration of 0.7 sec and was administered for 15 sessions. The second focused attention task included variable pre-cue delays of 0, 3, 4, or 5 sec and variable visual cue durations of 0.5 or 0.7 sec, and was administered for 15 sessions.

Selective attention task with olfactory distractors

Selective attention can be defined as the ability to maintain a behavioral or cognitive set in the face of distracting or competing stimuli. The two selective attention tasks administered were the selective attention baseline (SAB) task and the selective attention (SA) task with olfactory distractors, from PND 126-128 and 129-140, respectively. Animals were tested in the selective attention baseline task for 3 daily test sessions. In this baseline task, the pre-cue delay varied between 3 and 4 sec, with the two delays balanced across the trials within each test daily session, and the cue duration was 0.5 sec. This task was followed by 12 sessions of the selective attention task, which was identical to the baseline task except that on one third of the trials in each session, an olfactory distractor was presented 1 or 2 sec after trial onset (i.e., 1–3 sec before the visual cue). This delay between odor presentation and cue illumination (1-3 sec) was referred to as the odor-to-cue interval (OtoC) and was included in statistical

analysis. The nine different olfactory distractors were made from liquid odorants (McCormick & Company, Inc.) diluted in propylene glycol, and delivered as scented air.

Assessment of behavioral reactivity and the reward omission task

Behavioral reactivity was assessed the second focused attention task, and again in the reward omission task. In the second focused attention task, performance (e.g., %Correct) on the current trial was assessed as a function of the outcome of the prior trail (correct or incorrect). Similarly, the reward omission task was included as an assessment of behavioral reactivity, but with the addition of an omitted reward on a subset of trials. Specifically, the food pellet reward was randomly omitted on three out of ten correct trials, and attentional performance on the current trial was assessed as a function of whether the preceding trial outcome was either (1) correct, (2) incorrect (including omission errors), or (3) correct but food reward withheld. This task used a 0.7 sec cue duration, and there was no pre-cue delay present. Animals were tested in the reward omission task for 12 sessions from PND 175-187.

Montoya Staircase Test

Montoya staircase test was used to assess sensorimotor function, as described in our prior studies[14]. In the Montoya Staircase test, the rat reaches down on the left or the right side of the central platform of the apparatus to grasp and retrieve food pellets from the descending steps of the staircase. Briefly, eight identical Plexiglas staircase devices were used, modeled after the original design of Montoya et al. (1991)[54]. Each device contained a platform and a left-and-right side staircase. The staircases contained 6 descending steps on each side, and each step was baited with three 45 mg food pellets for a total of 36 pellets available at the start of a session. To determine the step of origin and final location of each pellet, pellets were color coded with a unique color per step level[55].

The staircase study was conducted over 1 month, beginning on PND 85. Each staircase session occurred immediately following operant testing every day. Staircase training occurred for the first 11 days. During training, rats retrieved the food pellets from the descending steps of the staircases until all subjects reached asymptotic performance of total pellets retrieved. The next 19 days comprised testing phase of the task. Throughout training and testing, each subject was given one 10-min trial per day, 6 days per week. After each trial, the rat was removed, and the final distribution of colored pellets was quantified per step and side. The order and time of testing were balanced by treatment and remained the same each day for each rat.

Skilled forelimb performance was evaluated step-by-step for (1) the number of pellets taken (i.e., pellets that were grasped and removed from their step of origin), (2) the number of pellets eaten (pellets that were taken and consumed), (3) the corresponding percent of success (the ratio of the number of pellets eaten/pellets taken x 100), (4) the number of pellets misplaced (all the pellets that were grasped but ended up on a different step level than their step of origin), and (5) the number of pellets lost (pellets that ended up on the floor of the apparatus, out of reach to the animal).

Tissue Mn and choline/choline metabolite levels

For blood and brain Mn levels, tissue samples were collected from weaned pups on PND 24, and from the behaviorally-tested animals on PND 202. Briefly, animals were euthanized via CO_2 asphyxiation, and whole blood was immediately collected from the left ventricle of the surgically-exposed heart and stored in EDTA Vacutainers at − 20 °C for analyses. Whole brain was immediately removed, and the hind-brain region collected and stored at −80 °C for Mn concentration determinations. Blood and brain Mn

concentrations were determined by inductively coupled plasma–mass spectrometry (Thermo Element XR), as previously described[13,15]. The analytical detection limit for Mn was 0.01 ng/mL.

Choline/choline metabolite levels were measured in plasma, brain, and/or liver tissues from dams at weaning (plasma and liver), and/or from the same PND 24 animals (liver and hindbrain) used for Mn analyses described above. Briefly, concentrations of free choline, betaine, dimethylglycine, methionine, and trimethylamine N-oxide were measured by liquid chromatography tandem mass spectrometry according to the method of Holm et al (2003)[56], while phosphocholine, glycerophosphocholine, phosphatidylcholine, acetylcholine, and lysophosphatidylcholine were measured by liquid chromatography/mass spectroscopy according to the method of Koc et al. (2002)[57] with modifications[58].

Statistical methods

The behavioral data were modeled using structured covariance mixed models. Fixed effects included in the model were the between-subject factors MCS (two levels corresponding to the unsupplemented and MCS groups), and Mn exposure treatment (two levels corresponding to the 0 and 50 mg/kg/d groups), and the within-subject factors pre-cue delay, cue duration, session block, distraction condition, and/or previous trial outcome, depending on the outcome analyzed. In all models, rat was included as a random effect to account for correlations within observations from the same animal. Statistical tests used the Kenward-Roger correction to improve estimation of the variance-covariance of the fixed effects parameters. Plots of residuals by experimental condition were used to examine the assumption of homogeneity. Random effects with high variance in the residuals across the levels of the factor (e.g., distraction condition) were added to the model to achieve homogeneity if necessary. The distribution of each random effect was inspected for approximate normality and presence of influential outliers. The significance level was set at $p \leq 0.05$, and p-values between 0.05 and 0.10 were considered to be trending towards significance. Trending effects are presented if they aid in clarifying the nature of the Mn and/or MCS effects. Significant main effects or interaction effects were followed by single-degree of freedom contrasts to better understand the nature of the interactions, using the Student's t-test for pairwise comparisons of least squared means. Analyses were conducted using SAS (version 9.4) for Windows. For tissue Mn and choline/metabolite data, analyses were performed using one-way (for dams) or two-way (for offspring) analysis of variance and Tukey's post hoc test for pairwise comparisons, using JMP (version 16.0; SAS Institute, Inc.).

3. Results

For The 5-CSRTT measures, we focus primarily on outcomes of %Premature, %Correct, and %Accurate responses because these measures have historically best differentiated control vs Mn animals, and did so as well in this study. Results for each outcome are plotted in the way that best illustrates the Mn and MCS effects. When there is a significant two-way but not three-way statistical interaction, we plotted both the two-way (shown in an insert panel) and the three-way interaction to show the performance of each of the four treatment groups under specific conditions due to our *a priori* hypotheses relating to the Mn effect under both diet conditions.

3.1 Visual learning

Visual learning in the 5-choice task is not altered by Mn or MCS

Visual learning (i.e., learning to make a nosepoke in the illuminated port) is foundational to preparing the animals for subsequent, more challenging 5-CSRTT tasks. Animals were trained on a visual discrimination learning task over PND 58-79, and there was no effect of early postnatal Mn exposure or MCS on visual learning. Specifically, there was no main effect of Mn or MCS on trials to criterion or days to criterion, and no Mn x MCS interaction for these two outcomes (P's > 0.295). Moreover, there also was no effect of Mn or MCS on the number of animals per treatment group to reach the learning criterion. The lack of treatment group differences in learning the task criterion indicates that visual acuity and motivation did not differ between groups and therefore cannot account for group differences seen in subsequent 5-CSRTT tasks.

3.2 Impulsivity

Developmental Mn exposure did not affect impulse control, but did impair the animals in learning to wait for the visual cue, and this effect is not rescued by MCS

We evaluated premature responses, a measure of impulse control in the 5-CSRTT, as a percent of total responses in the two successive focused attention tasks and the selective attention task (SA).

In the first focused attention task, Mn animals are impaired in learning to withhold premature responses, and MCS does not alter this effect.

In the focused attention 1 task, there is a significant Mn x session block interaction [$F(2, 435.8) = 11.07$, $p<0.0001$] for %Premature responses. While both control and Mn groups commit a relatively high level of premature responses at the start of the task (~ 50%), the Mn animals improve less rapidly than controls. As a result, the Mn animals commit significantly more premature responses than controls in session block 3 ($p=0.0084$; Figure 2a1). There is also a significant MCS x session block interaction [$F(2, 435.8) = 17.06$, $p<0.0001$], and while the MCS and unsupplemented groups don't statistically differ from each other in any block, the interaction likely appears because MCS animals have slightly fewer premature responses in block 1 but more premature responses in block 3. Refer to Figure 2A, which depicts differences between Mn and controls under each of the two diet conditions (three-way interaction $p = 0.69$).

In the second focused attention task, Mn and MCS don't interact to alter premature responses.

In the subsequent focused attention task, there is a Mn x session block interaction [$F(2, 933.7) = 5.53$, $p = 0.0041$], although there are no significant contrasts between control and Mn animals in any session block (P's > 0.40; Figure 2b1). Refer to Figure 2B, which depicts differences between Mn and controls under each of the two diet conditions (three-way interaction $p = 0.40$).

There is also a MCS x pre-cue delay interaction [$F(2, 932.8) = 3.18$, $p = 0.0422$], though again, none of the specific contrasts between unsupplemented and MCS animals reached significance at any pre-cue delay (P's > 0.21, Figure 2c1). However, MCS animals had non-significantly lower premature responses across pre-cue delays compared to unsupplemented animals, with the apparent difference being greatest at the shorter (and less challenging) 3 sec pre-cue delay. Refer to Figure 2C, which depicts differences between Mn and controls under each of the two diet conditions (three-way interaction $p = 0.24$).

In the selective attention task, MCS lessens the difference in premature responses between treatment groups in early session blocks.

In the selective attention task with olfactory distractors, there is a Mn x MCS x session block interaction for %Premature responses [$F(3, 1665) = 4.44$, $p = 0.0041$], although none of the specific contrasts between treatment groups or diet conditions reached significance for any session block (P's > 0.12, Figure 2D). Here, the significant three-way interaction arises because the difference in premature responses between Mn and control groups in the first two session blocks is greater for unsupplemented animals than for the MCS animals.

3.3 Attention

Mn impairs focused attention, and while MCS lessens the difference between control and Mn animals on focused attention, the nature of the MCS effect differs in control vs. Mn animals

As described earlier, the two focused attention tasks were used to assess whether Mn exposure or MCS alters the ability of animals to maintain attentional focus to detect the visual cue. We evaluated %Correct and %Accurate responses, which assess attentional function in the 5-CSRTT.

In the first focused attention task, developmental Mn impairs attention, and MCS alters the rate of performance improvement differently in control compared to Mn animals.

In the focused attention 1 task, there is a significant Mn x session block interaction for %Correct responses [$F(2, 595.6) = 4.12$, $p = 0.0167$]. This reflects that performance in the Mn animals improves more slowly than control animals across session blocks, such that the Mn animals have significantly fewer %Correct responses than the controls during blocks 2 and 3 ($p=0.007$ and 0.002, respectively, figure 3a1). Refer to Figure 3A, which depicts differences between Mn and controls under each of the two diet conditions (three-way interaction $p = 0.22$).

Consistent with the %Correct outcome noted above, there is a significant Mn x session block interaction for %Accurate responses [$F(2, 601.3) = 3.33$, $p = 0.0365$; not shown], and a trending three-way interaction of Mn x MCS x session block for %Accurate responses [$F(2, 601.3) = 2.58$, $p = 0.0769$] (Figure 3B). For %Accurate responses, the Mn deficit emerges under session blocks 2 and 3 (p's = 0.018 and 0.049 respectively under MCS; Figure 3B). This three-way interaction reflects the fact that the difference in performance of the Mn and controls was greater in the MCS condition than the unsupplemented condition; this appears to be because the controls benefitted more from the MCS than the Mn rats, in terms of learning rate.

In the second focused attention task, MCS improves performance of both the Mn and control animals, but in different functional domains.

In the focused attention 2 task, there is a significant Mn x MCS x pre-cue delay interaction for %Correct responses [$F(3, 1277) = 7.32$, $p < 0.0001$] (Figure 3C). For animals on the unsupplemented diet, the Mn-exposed rats have significantly fewer %Correct responses than controls at the 3 sec delay ($p = 0.0477$), and this Mn deficit is also trending at the 4 and 5 sec delays (p's = 0.0518 and 0.0699, respectively). However, in the MCS animals, the Mn deficit at the 3 sec delay is only trending ($p = 0.0762$), and the deficit is no longer present under the 4 and 5 sec pre-cue delays (p's = 0.27 and 0.69, respectively). Thus, the Mn x MCS x pre-cue delay interaction reflects the fact that the differences between control and Mn animals at the longer delays is smaller under MCS than under the unsupplemented diet. Additionally, control animals that received MCS have significantly more correct responses at the 0 sec delay compared to unsupplemented controls ($p = 0.0113$), reflecting a benefit of MCS to control animals for trials when the cue was presented immediately at trial onset, with no pre-cue delay.

We also assessed attentional accuracy for the second focused attention task, and again observed a significant Mn x MCS x pre-cue delay interaction for %Accuracy [$F(3, 1271) = 3.95$, $p = 0.0081$] (Figure 3D), reflecting a pattern in attentional performance similar to what was seen in the %Correct outcome described above. Under the unsupplemented diet, there is a trending Mn deficit at the 3 and 4 sec pre-cue delays (p's = 0.058 and 0.067, respectively). In contrast, under MCS, this Mn deficit is still present at the 3 sec delay (0.043), but not at the 4 sec pre-cue delay ($p = 0.23$). Although there was no significant Mn deficit at the 5 sec pre-cue delay under the unsupplemented diet, MCS also appears to reduce the difference between control and Mn animals at this longer delay ($p = 0.11$ under the unsupplemented diet and 0.53 under MCS; Figure 3D).

While there was a significant benefit of MCS to controls at the 0 sec pre-cue delay for %Correct responses ($p = 0.0113$), for %Accurate this benefit did not reach significance ($p = 0.129$). There was, however, a trending Mn deficit that emerged at the 0 sec delay under MCS ($p = 0.09$, Figure 3D).

Collectively, this evidence suggests that the lasting attentional deficits caused by developmental Mn exposure persist into the second focused attention task. Additionally, MCS improves performance of the controls but not the Mn animals for trials with a 0 sec pre-cue delay, whereas it reduces the difference between Mn and control animals at the longer delays. The MCS benefit derived by the controls at the 0 sec delay appears to be due to a lessened reaction to committing an error on the prior trial (discussed below; Figure 5), whereas the benefit derived by the Mn animals at the longer delays appears to reflect improved attention.

MCS reduces the Mn effect on distraction trials in the selective attention task

To determine the effects of Mn exposure and MCS on selective attention, we assessed animal performance on a selective attention task with olfactory distractors randomly presented either 1, 2, or 3 sec prior to the onset of the visual cue (referred to as the odor-to-cue interval, 'OtoC').

Mn causes a deficit in attention, but not selective attention specifically, and MCS reduces the difference between Mn and control animals on trials with olfactory distractors.

For %Correct responses in the selective attention task, there was a significant Mn x OtoC interaction for %Correct [$F(3, 239.2) = 2.94$, $p = 0.0339$], where the Mn animals were more impaired relative to controls for trials without a distractor ($p = 0.0096$; Figure 4a1), than for distraction trials. The Mn x MCS x OtoC interaction for %Correct responses was not significant ($p = 0.269$) (Figure 4A). However, qualitatively based on a plot of this interaction, it appears that under the MCS diet, Mn and control animals have comparable performance under the three distraction conditions (Figure 4A). In light of this, we conducted follow-up analyses where the three trial conditions with olfactory distractors (i.e., -1, -2, -3 OtoC) were collapsed into a single "distraction" trial condition, thus creating only two distraction conditions (distractor and no-distractor trials; Figure 4B). In this follow-up analysis, there was a trending interaction of Mn x MCS x distractor [$F(1, 52.16) = 3.29$, $p = 0.0754$]. This trend reflects that for nondistraction trials there is a Mn deficit under both diets, but for distraction trials there is only a Mn deficit for unsupplemented animals (Figure 4B).

For %Accurate responses, there was a significant Mn x OtoC interaction [$F(3, 235.7) = 3.14$, $p = 0.026$], where the Mn deficit was significant for trials without a distractor and trending for trials in the -3 sec distractor condition (p's = 0.0084 and 0.098; Figure 4c1); the groups were not different for trials with a -1

or -2 distractor. The three way interaction (Mn x MCS x OtoC) was not significant [$F(3, 235.8) = 0.8$, $p = 0.495$]; however, we see a similar pattern of group differences as noted above for %Correct, where MCS reduces the difference in %Accuracy between control and Mn animals under the distractor conditions (Fig 4C). However, neither the original (Figure 4C) nor collapsed (not shown) %Accurate models revealed a significant Mn x MCS x odor interaction (p's > 0.31).

Collectively, this evidence demonstrates that MCS lessens the difference between control and Mn animals for trials with olfactory distractors.

3.4 Behavioral reactivity

Mn alters behavioral reactivity compared to controls, and MCS differentially affects control vs Mn animals in this domain

To determine if developmental Mn exposure and/or MCS alter reactivity to committing an error or not receiving an expected reward, we analyzed performance outcomes on the current trial based on the outcome of the preceding trial for the second focused attention and reward omission tasks.

For the second focused attention task, control animals that received MCS had significantly more %Correct responses than their unsupplemented counterparts at the 0 sec pre-cue delay (reported above in Figure 3C). We hypothesized that this might be due to MCS reducing the behavioral reaction to making an error on the prior trial, as trials without a pre-cue delay are closest temporally to the prior trial. If the outcome of the prior trial was disruptive to the animal, it would be most apparent for trials for which the cue was presented immediately at trial onset. Therefore, we assessed %Correct responses on current trials with a 0 sec pre-cue delay, as a function of the outcome of the prior trial.

The animal's reaction to committing an error on the prior trial was also assessed in a subsequent Reward Omission task designed to assess behavioral reactivity to two different types of potentially disruptive events: (1) making an error on the previous trial (accompanied by a timeout and no reward), or (2) making a correct response but not receiving an expected reward (see Methods). The results of both tasks are described below.

In the second focused attention task trials with a 0 sec delay, MCS improves performance in control animals following an error, and Mn animals do not exhibit behavioral reactivity to an error under unsupplemented or MCS diet.

In the second focused attention task, analysis revealed a significant Mn x MCS x previous trial outcome interaction for trials with a 0 sec pre-cue delay [$F(1, 273.2) = 6.13$, $p = 0.0139$]. This interaction reflects that the reaction to committing an error varied as a function of both Manganese exposure and prenatal maternal diet. Specifically, under the unsupplemented diet, only the controls exhibited a lower percent correct for trials following an error (relative to after a correct response); the Mn animals showed no decline in performance due to making an error on the prior trial ($p < 0.0001$ for control and 0.79 for Mn animals comparing error vs. correct; Figure 5). In contrast, for animals born to dams given the choline supplemented diet, both groups showed a reduction in percent correct for trials following an error (relative to after a correct response; $p = 0.074$ for control and 0.17 for Mn), and the magnitude of this disruptive effect was similar in the two groups.

In the control animals, MCS significantly increased %Correct responses after an error, but not following a correct response (p's=0.05 and 0.50, respectively) (Figure 5). In Mn animals, MCS did not improve performance following either a correct response ($p = 0.34$) or error ($p = 0.73$) compared to unsupplemented animals.

In contrast to trials with a 0 sec pre-cue delay, trials with a 3 sec pre-cue delay did not have a significant interaction of Mn x MCS x previous trial outcome [$F(1, 273.8) = 1.72$, $p = 0.19$] (not shown). Therefore, the effect of MCS to improve performance of the control group animals and mitigate behavioral reactivity on trials following an error is most pronounced on trials that are closest in time to the prior trials (i.e., trails with a 0 sec pre-cue delay).

MCS and developmental Mn exposure differently alter behavioral reactivity in response to an error or omitted reward on the prior trail in a reward omission task

In the reward omission task, we introduced a novel trial condition in which the food pellet reward is unexpectedly omitted on a subset of correct response trials, and assessed how this affects performance on the subsequent trial. This was done because we wanted to explore the effect of another type of behaviorally disruptive stimuli (i.e., reward omission) in contrast to the disruptive effect of an incorrect response. Specifically, we assessed performance on the current trial depending on whether the preceding trial response was either (1) correct with a food pellet delivery, (2) an error (incorrect, no food pellet delivery + 5 sec time out), or (3) correct but with food pellet reward omitted (RO; occurred pseudo-randomly on three out of each ten consecutive correct trials).

Under the unsupplemented diet, Mn eliminates the reactivity to an error that is seen in control animals, but causes reactivity to the omission of an expected reward. MCS eliminates the deficit following an omitted reward in Mn animals and improves performance in control animals following an error.

In the reward omission task, there was a Mn x MCS x previous trial outcome interaction for %Correct [$F(2, 410.6) = 4.51$, $p = 0.0116$] (Figure 6). This interaction reflects that both prenatal diet and Mn exposure affects reactivity to these two conditions. In the unsupplemented controls, committing an error on the prior trial (versus a correct response) caused a reduction in %Correct responses on the subsequent (current) trial ($p < 0.0001$), whereas the unexpected omission of the food reward on the prior correct trial had no effect on the performance on the subsequent trial ($p = 0.20$). In contrast, a different pattern of post–error performance was seen in the unsupplemented Mn animals. Notably, for these animals, committing an error on the prior trial did not affect their performance on the subsequent (current) trial ($p = 0.152$), compared to trials following a correct response (Figure 6). In contrast, performance of the unsupplemented Mn animals was significantly decreased on trials following the omission of the expected food reward, compared to a correct response ($p = 0.0031$). These findings demonstrate that, for offspring of dams fed an unsupplemented diet, animals exposed to Mn early in life respond differently than controls (no Mn) to both committing an error and not receiving an expected reward.

A different pattern was seen for animals born to dams on a choline supplemented diet. In MCS control animals, there was a trend for MCS to lessen the disruptive effect of committing an error on the prior trial; i.e., improve performance on trials that followed an error (relative to the unsupplemented controls) ($p = 0.071$, Figure 6). However, within the MCS controls, %Correct responses on trials following an error was still lower than on trials that followed a correct response ($p < 0.0001$). Furthermore, in the MCS controls, the unexpected omission of a food reward on the prior trial also impaired performance compared to

following a correct response ($p = 0.0013$), an effect that was not seen in the unsupplemented animals. In addition, for the Mn animals, MCS normalized performance on trials following a reward omission (Figure 6).

Finally, under the MCS diet, performance on trials following a correct response was significantly worse in the Mn animals than controls ($p = 0.0176$), a deficit that was not present in the unsupplemented groups due to the fact that the controls benefited from MCS for trials following a correct response but the Mn animals did not.

3.5 Sensorimotor function

Early life Mn exposure causes a lasting deficit in sensorimotor function that is not altered by MCS.

To determine whether MCS can mitigate the lasting sensorimotor deficits caused by developmental Mn exposure, animals were assessed using the Montoya staircase test of skilled forelimb performance. As expected, Mn exposure caused lasting deficits in sensorimotor function, as demonstrated by a significant Mn x step level interaction for number of pellets taken [$F(5, 229.3) = 10.66$, $p < 0.0001$], number of pellets eaten [$F(5, 232.6) = 4.57$, $p = 0.0005$], %Success [$F(5, 231.4) = 3.26$, $p = 0.0073$], and number of pellets misplaced [$F(5, 356.7) = 5.76$, $p < 0.0001$]. Specifically, there was a significant Mn deficit on steps 4 and 5 of the staircase, where the unsupplemented Mn animals ate significantly fewer pellets and misplaced more pellets compared to unsupplemented control counterparts (pellets eaten, p's = 0.0017 and 0.0002 for steps 4 and 5 respectively; pellets misplaced, p's = 0.0005 and 0.002) (Figure 7a1 and 7b1, respectively). However, MCS did not alter sensorimotor function in either the controls or the Mn groups, based on findings that the main effect of MCS (P's >0.16), or any interactions involving MCS and Mn were not significant for any of the outcomes (e.g., Mn x MCS, P's >0.32; Mn x MCS x step level, P's >0.72); see figure panels 7A and 7B for the Mn x MCS x step plots for pellets eaten and misplaced).

3.6 Choline and choline metabolite levels

MCS measurably altered choline and choline metabolite levels in dams and offspring.

To confirm that oral MCS over gestation and lactation altered levels of choline and/or its metabolites in dams and their offspring as expected, and whether oral Mn exposure altered choline/metabolite status in the offspring of MCS-treated dams, we measured levels of choline and selected metabolites in dam and offspring tissue (dam plasma and liver upon completion of MCS at PND 21, offspring liver and brain at PND 24). As expected, MCS produces measurable changes in choline and some of its metabolites in dam plasma and liver, and PND 24 pup liver and brain. See supplemental table 1 for choline and metabolite measurements in dams, and supplemental table 2 for measurements in pups.

3.7 Blood and brain Mn levels

Oral Mn dosing increased blood and brain Mn levels in weanlings, and returned to control levels in adulthood.

Mn exposure from postnatal days (PND) 1-21 increased levels of Mn in both blood and brain tissue in PND 24 weanlings. Specifically, oral Mn significantly increases blood Mn levels approximately 8-fold, from ~30 to 250 ng/mL in control vs. Mn exposed groups [$F(1, 45) = 398.4$, $p < 0.0001$] (Figure 8A). There was also a significant interaction of Mn x MCS on blood Mn levels [$F(1, 42) = 8.37$, $p = 0.0059$];

however, Tukey's contrasts revealed no significant difference between blood Mn levels in unsupplemented vs. MCS animals that received Mn.

Mn dosing also causes an approximately 3-fold increase in brain Mn levels in PND 24 offspring, from approximately 3.8 to 11.8 µg/g in control vs. Mn-dosed animals, as demonstrated by a significant main effect of Mn [$F(1, 36) = 129.7$, $p < 0.0001$] (Figure 8B). There is also a trending main effect of MCS on brain Mn levels in offspring [$F(1, 36) = 3.96$, $p = 0.0543$], as well as a trending Mn x MCS interaction [$F(1, 36) = 3.13$, $p = 0.085$]. Tukey's pairwise comparisons revealed that, of animals that received Mn, MCS animals had significantly higher levels of brain Mn than unsupplemented animals.

Finally, blood and brain Mn return to baseline levels in PND 200 behaviorally tested animals. However, there is a statistically significant but biologically unremarkable effect of MCS [$F(1, 27) = 7.87$, $p = 0.0092$] to reduce blood Mn levels (Figure 8C). In the brain tissue of PND 200 animals, there was a trending effect of MCS to reduce Mn levels [$F(1, 20) = 3.19$, $p = 0.089$]; however, Tukey's pairwise comparisons revealed no significant difference between any groups (Figure 8D).

4. Discussion summary

Studies in children and adolescents have associated early developmental Mn exposure with inattention, impulsivity, hyperactivity, and sensorimotor dysfunction. There are currently no recognized therapies/interventions, other than exposure prevention, to mitigate the neurotoxic effects of elevated developmental Mn exposure. In this study, we used a rodent model of developmental Mn exposure to determine whether MCS protects against the lasting Mn-induced deficits in attention, behavioral reactivity, learning, impulsivity, and sensorimotor function. Our results demonstrate that developmental Mn exposure causes lasting deficits in attention, behavioral reactivity, learning, and sensorimotor function, and that the MCS intervention was partially effective in protecting against Mn-induced deficits, particularly in the domains of attentional function and behavioral reactivity. Collectively, these findings demonstrate that MCS may hold promise as nutritional intervention to protect against the neurotoxic impacts of elevated Mn exposure, as has been seen previously for other early insults. Given that recent evidence suggests that 90% of pregnant women consume less choline than the current recommended Adequate Intake (AI) levels, these data provide additional support for efforts to add choline to standard prenatal vitamin regimens. These findings are discussed in more detail below.

4.1 Developmental Mn exposure causes lasting abnormalities in behavioral reactivity, and replicates the effects seen in our studies in which Mn causes deficits in attention, learning, and sensorimotor function. In this study, Mn exposure caused deficits in attentional function (Figs 3 and 4), learning to withhold impulsive responses (Fig 2), and sensorimotor function (Fig 7) in ways that are largely consistent with our prior studies[14–16,59]. In addition, we established that elevated exposure to Mn during development leads to lasting abnormalities deficits in behavioral reactivity (Figs 5 and 6), including how animals respond to an incorrect response, as well as the omission of an expected reward.

To understand the MCS effects on behavioral reactivity in control and Mn exposed rats, it is important to first describe the effects of Mn in the absence of MCS. While control animals exhibit fewer correct responses on trials following an error than on trials following a correct response, as previously observed [34,53,60–63], Mn blunts this 'normal' reactivity to an error; Mn animals perform the same on trials that follow an error vs. a correct response in both the second focused attention and reward omission tasks (Fig 5,

6). However, in contrast to the lack of effect on performance for trials that follow an error, Mn exposed animals exhibit more errors on trials that follow the unexpected omission of a reward than on trials following a correct response, whereas control animals exhibit no performance deficit under this condition (relative to trials following a correct response) (Fig 6).

4.2 MCS provides some benefits to the Mn-exposed animals by lessening the difference between control and Mn animals on behavioral reactivity and attention.

MCS in some instances normalized the behavioral reactivity of the Mn-exposed animals. Specifically, the Mn animals in the MCS condition respond like controls to the unexpected omission of reward; i.e. they do not show a decrement in performance relative to trials after a correct response. Note that control animals also derive a benefit from MCS, which improves performance in trials following an error compared to the unsupplemented diet (Fig 5, 6); there is still a drop in correct responses after an error (compared to after a correct response), but it is less pronounced. These findings support that developmental Mn exposure produces lasting disruptions in arousal and behavioral reactivity – not in ways that simply blunt or augment behavioral reactivity, but rather modify interpretation/neural processing of committing an error or not receiving an expected reward.

In addition to normalizing some aspects of behavioral reactivity in Mn animals, MCS provides benefits in the domain of attentional function by lessening differences between Mn and control animals on the key measures of attention (%Correct and %Accurate). This is seen in the focused attention 2 task, particularly at the longer and more challenging pre-cue delays (Figure 3C and D), and in the selective attention task, where MCS reduces the difference between Mn and control animals on distraction trials (Figure 4).

4.3 Mn exposure mitigates some of the benefits of MCS.

It is noteworthy that developmental Mn exposure diminishes some of the cognitive benefits of MCS seen in control animals, particularly in learning rate in the first focused attention task. MCS facilitates learning the rules of this new task to a greater extent for the controls than the Mn rats. The improvement in %Accurate responses between block 1 and 2, under MCS, was greater in controls than Mn animals, and thus a Mn deficit emerges under blocks 2 and 3 (significant difference between control and Mn). This is interpreted as a learning benefit provided by MCS to control animals, but this benefit is not seen in the Mn animals.

4.4 MCS benefits control animal cognitive function.

Maternal choline supplementation in the absence of Mn exposure provided a number of cognitive benefits. For example, in control animals, supplementing the maternal diet with additional choline during gestation and lactation lessens the disruptive effect of committing an error (Fig 5, 6; discussed above in section 4.2) and improves attentional function (Fig 3C). The benefit to attention is statistically significant at the 0 sec delay in the FA2 task (Fig 3C), which may also be due in part to the benefit to behavioral reactivity described above. In addition, there is a pattern effects that did not quite reach statistical significance, but further provide evidence of a benefits of MCS on attention. This included the MCS benefit to control attentional function in FA1(3A/B) task, as well as increasing correct responses in the RO task on trials following a correct response (Fig 6). These benefits show that in the absence of environmental exposure, MCS provides cognitive benefits to offspring, [i]which recapitulate benefits seen in prior studies in humans and animal models[37–40,42,45,46,48].

4.5 Conclusion

In conclusion, these results demonstrate that MCS is beneficial to both the control and Mn exposed animals. MCS normalizes, to some degree, the Mn deficits in the areas of attention and behavioral reactivity. These findings also underscore the lasting neurobehavioral health risks to children posed by developmental Mn exposure. Finally, in light of the low choline intakes of most pregnant women, these results provide additional support for adding choline to standard prenatal vitamin regimens.

Figures

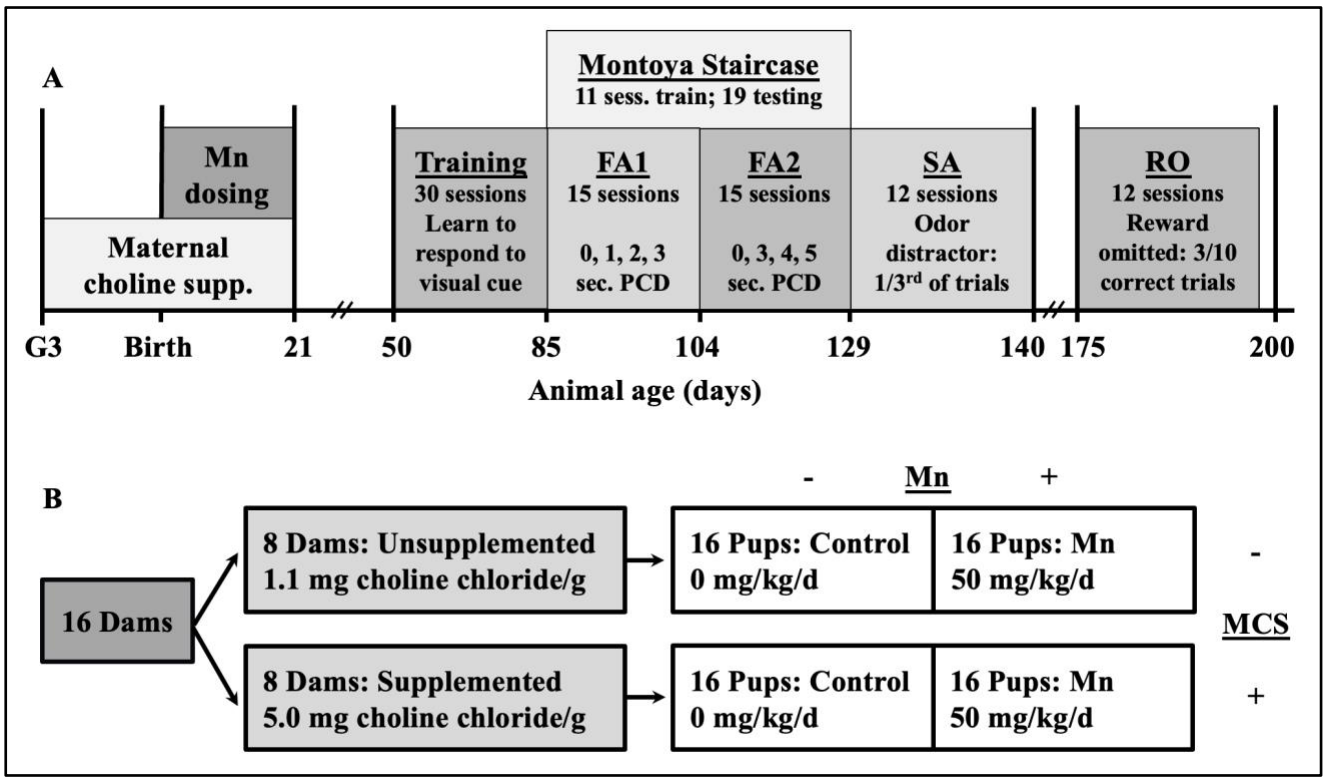

Figure 1. Study timeline with task parameters, and study design. (A) Study timeline, including MCS and Mn dosing, as well as tasks with parameters. X-axis is days of age (PND). (B) 2x2 study design, ending with 16 pups per treatment group that were used for behavioral testing.

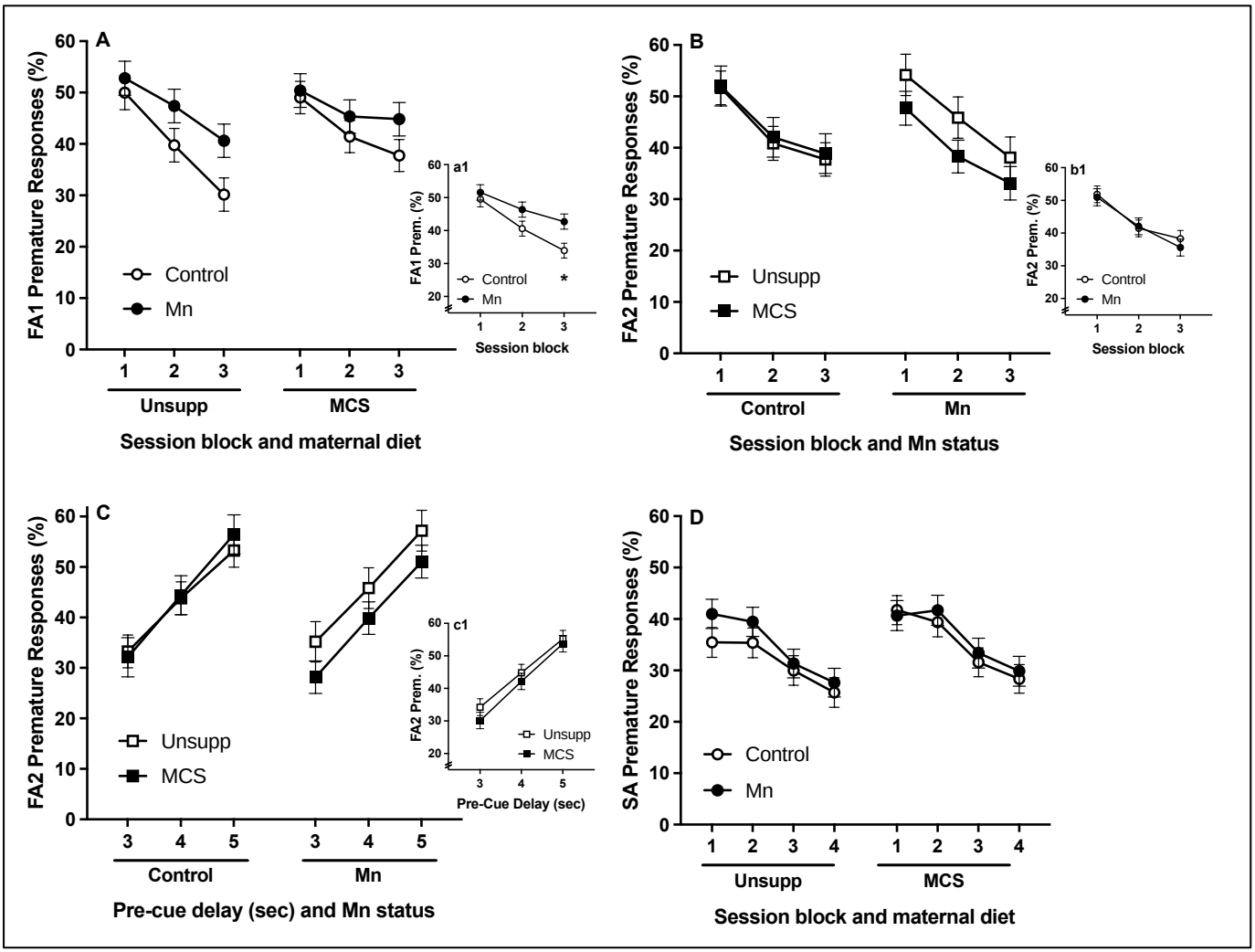

Figure 2. Mn animals are impaired in learning to withhold premature responses, and MCS lessens some differences between control and Mn treatment groups. Premature responses (%) in the first focused attention task as a function of Mn treatment, maternal diet, and session block (A), and a function of Mn treatment and session block (a1). Premature responses (%) in the focused attention 2 task as a function of Mn treatment, maternal diet, and session block (B), and a function of Mn treatment and session block (b1). Premature responses (%) in the focused attention 2 task as a function of Mn treatment, maternal diet, and pre-cue delay (sec) (C), and a function of MCS treatment and pre-cue delay (d1). Premature responses (%) in the SA task as a function of Mn treatment, maternal diet, and session block (D). The order of testing was the focused attention 1 task followed by the focused attention 2 task and then the selective attention task. * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-16/group).

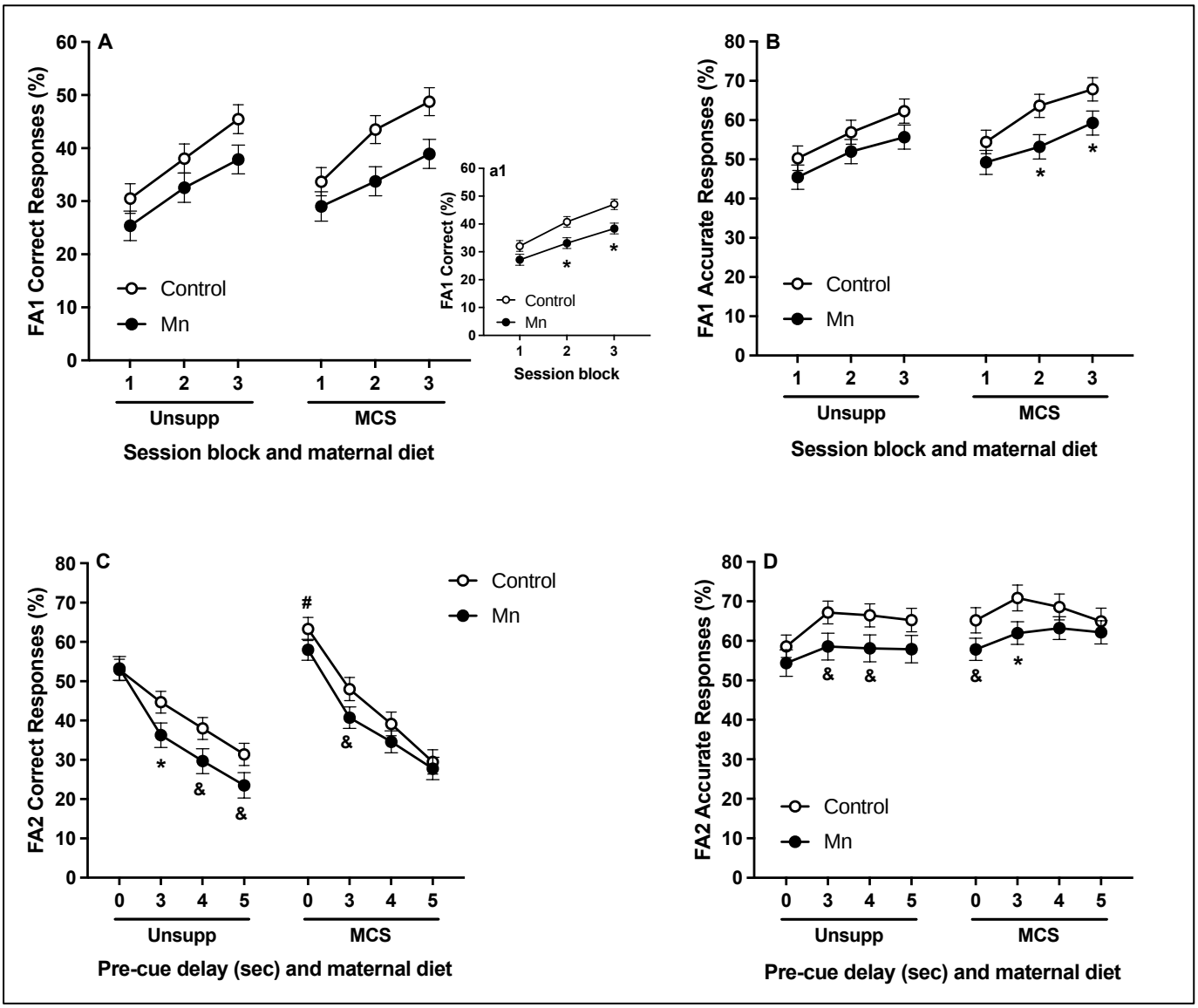

Figure 3. Mn animals exhibit an impairment in focused attention, and MCS differentially affects control vs. Mn animal performance. Focused attention 1 task %Correct (A, a1) and %Accurate (B) responses as a function of Mn treatment, maternal diet, and session block. Focused attention 2 task %Correct (C) and %Accurate (D) responses as a function of Mn treatment, maternal diet, and pre-cue delay in sec. * and & indicate $p \leq 0.05$ and $p \leq 0.10$ versus controls, respectively; # indicates $p \leq 0.05$ versus unsupplemented animals. Data are lsmeans ± SEM (n=14-16/group).

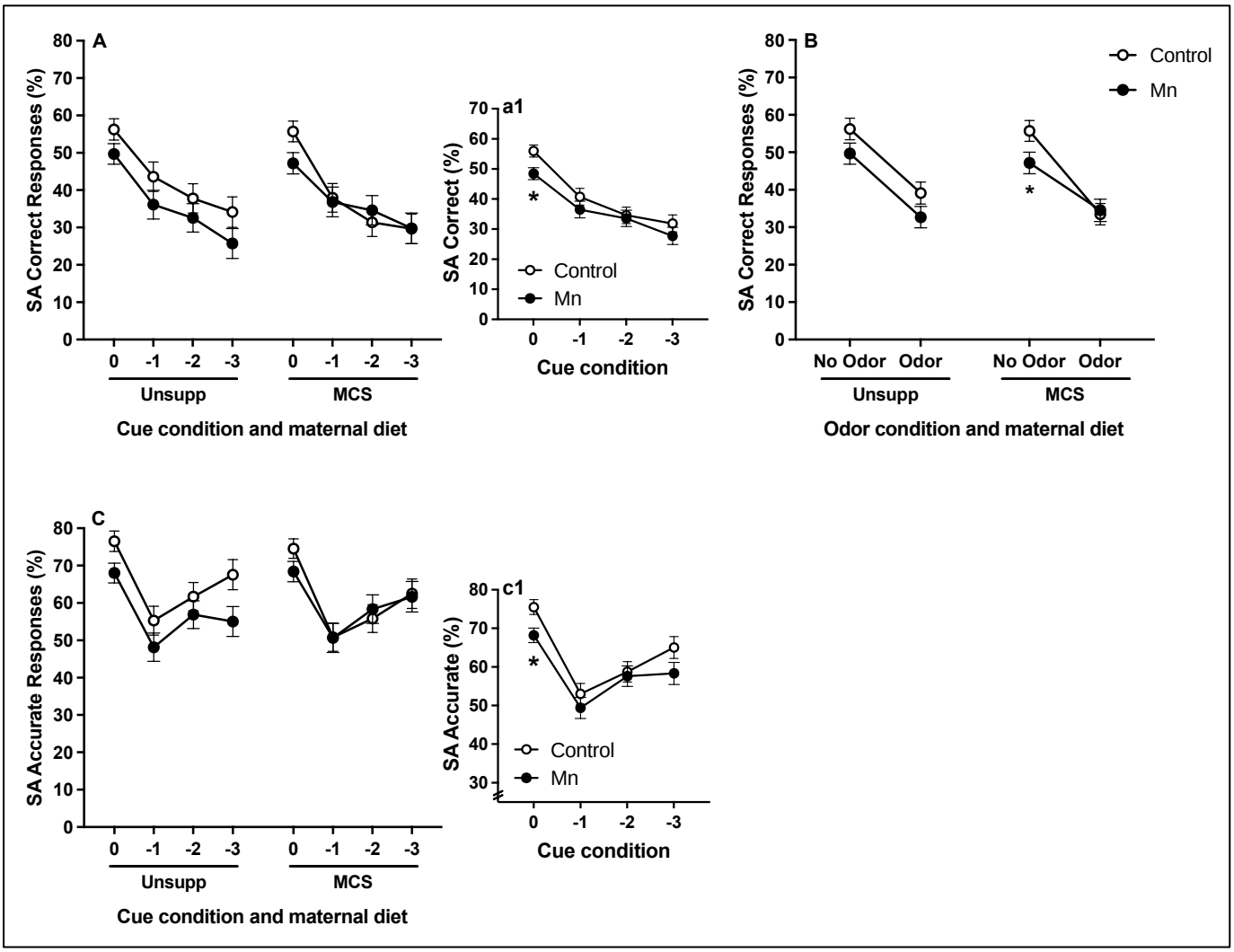

Figure 4: Mn causes a deficit in attention, but not selective attention specifically, and MCS reduces the difference between Mn and control animals under odor conditions. SA task %Correct responses as a function of Mn treatment, maternal diet, and distraction condition (no distractor, odor distractor 1, 2, or 3 sec prior to the cue illuminating) (A), Mn treatment and distraction condition (a1), or Mn treatment, maternal diet, and odor presence (no distractor, odor distractor present) (B). %Accurate responses as a function of Mn treatment, maternal diet, and distraction condition (C), and Mn treatment and distraction condition (c1). * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-16/group).

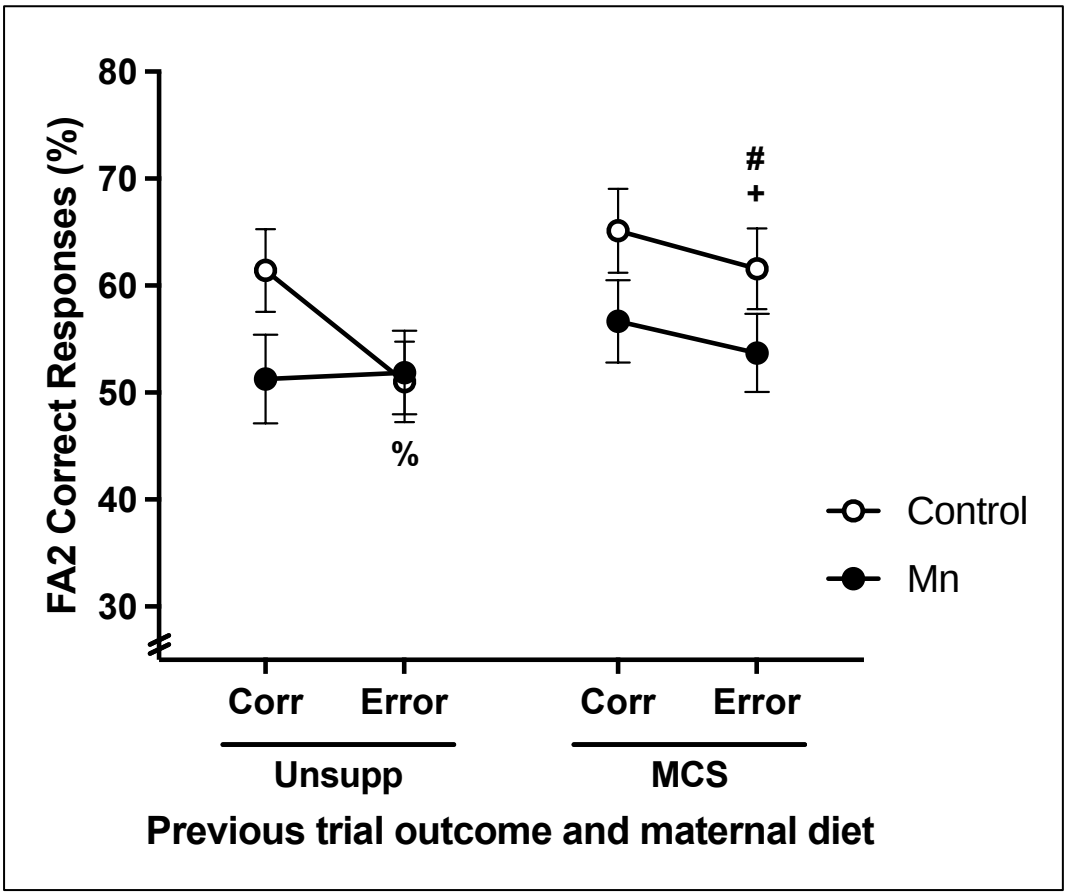

Figure 5: Under the unsupplemented diet, Mn blunts error reactivity. MCS improves performance in control animals following an error. FA 2 task %Correct responses at trial conditions with a 0 sec PCD, as a function of Mn treatment, maternal diet, and previous trial outcome. Corr = correct, Error = incorrect, premature, or omission error. % and + indicate $p \leq 0.05$ and $p \leq 0.10$ versus following a correct response, respectively; # indicates $p \leq 0.05$ versus unsupplemented animals. Data are lsmeans ± SEM (n=14-16/group).

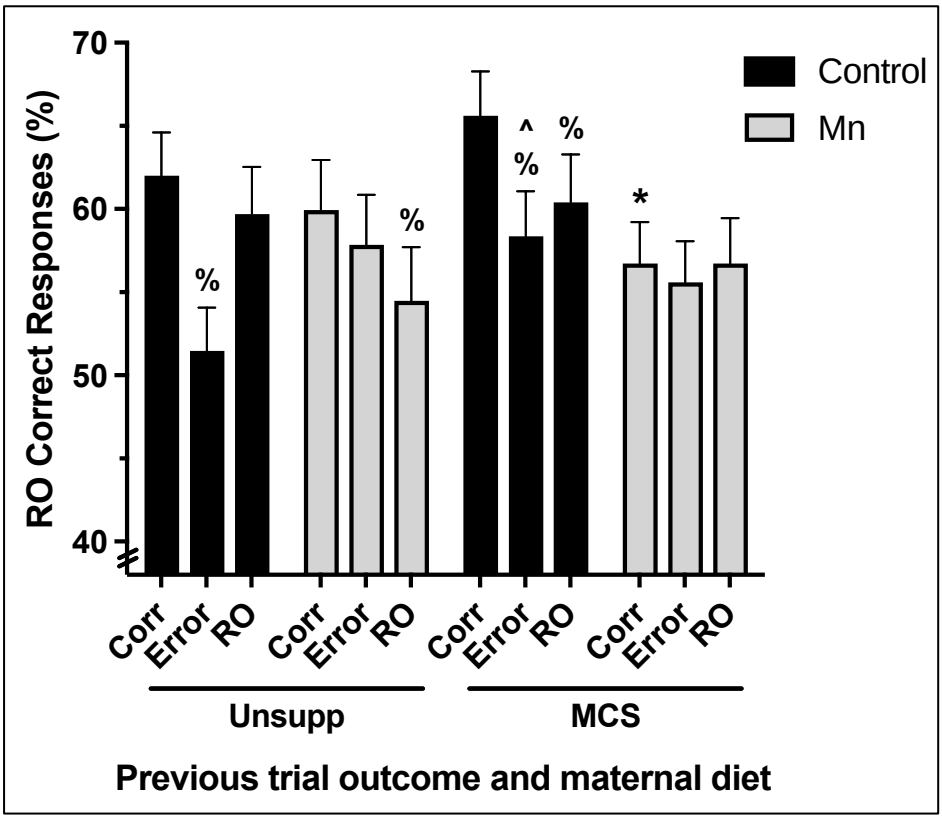

Figure 6: Mn blunts error reactivity, but causes reactivity to an omitted reward, which is normalized by MCS. RO task (no reward on one-third of the correct response trials) %Correct responses as a function of Mn treatment, maternal diet, and previous trial outcome. Corr = correct, Error = incorrect or omission error, RO = reward omitted. % indicates $p \leq 0.05$ versus following a correct response; * indicates $p \leq 0.05$ versus controls; ^ indicates $p \leq 0.10$ versus unsupplemented animals. Data are lsmeans ± SEM (n=14-16/group).

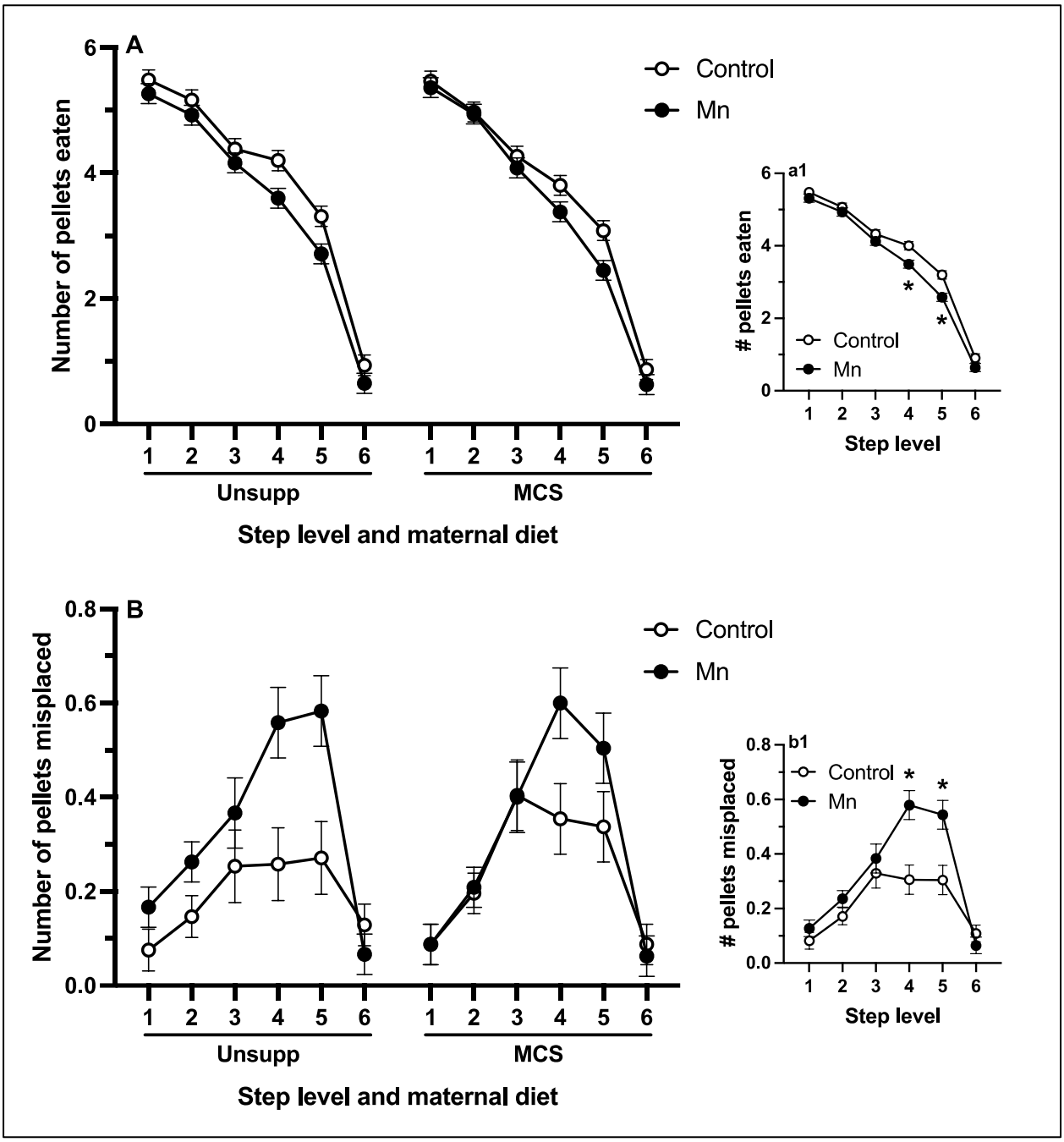

Figure 7: Mn exposure causes a lasting deficit in fine motor function that is not rescued by MCS. Number of pellets eaten by control and Mn-exposed groups that received unsupplemented or supplemented maternal choline, as a function of step level (A), number of pellets eaten as a function of Mn and step level (a1). Number of pellets misplaced by control and Mn-exposed groups that received unsupplemented or supplemented maternal choline, as a function of step level (B), number of pellets misplaced as a function of Mn and step level (b1). * indicates $p \leq 0.05$ versus controls. Data are lsmeans ± SEM (n=14-16/group).

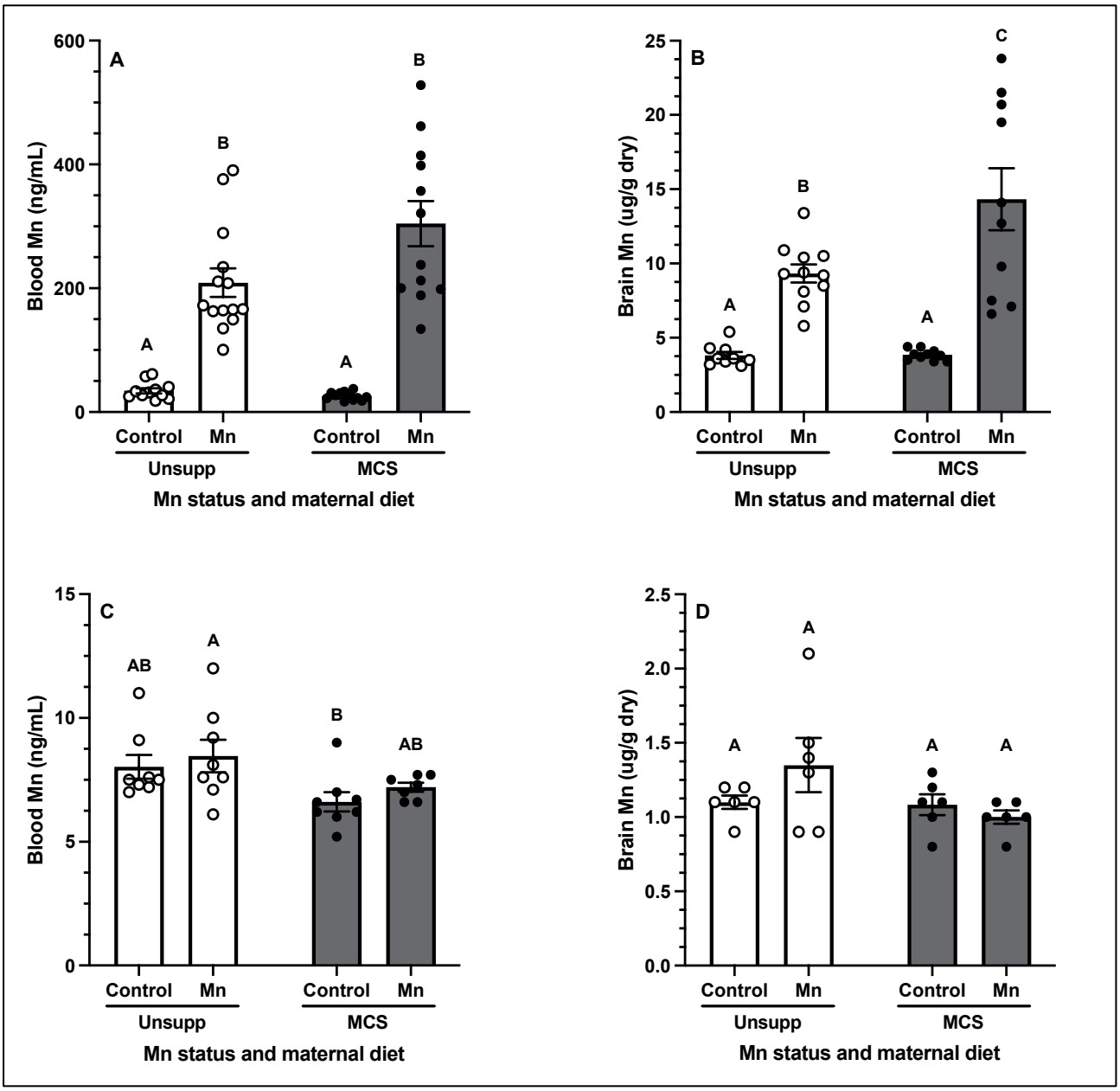

Figure 8: **Oral Mn dosing increased blood and brain Mn concentrations in weanling littermates of the behaviorally tested animals (PND 24), and returned to baseline in the behaviorally tested animals at sacrifice (PND 200)**. PND 24 animal blood (n = 11-14) (A) and brain (n = 9-11) (B) measurements show that Mn was increased in Mn-exposed animals. PND 200 animal blood (n = 7-8) (C) and brain (n = 6) (D) measurements show that Mn levels return to baseline. Graphs show arithmetic means with standard error. PND 24 blood and brain statistics were performed on log-transformed data; PND 200 statistics were performed on non-transformed data. Within an age group and tissue, treatment groups with different capital letter superscripts are statistically different from one another ($p < 0.05$), based on Tukey's *post hoc* test.

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Conflicts of interest (COI)

The authors declare no actual competing or potential financial interests.

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