Methylphenidate alleviates attentional and sensorimotor dysfunction induced by developmental manganese exposure: Assessment of catecholaminergic receptor involvement

Stephane A. Beaudin[1], Shanna Howard[1], Nicholas Santiago[1], Barbara J Strupp[2]* and Donald R Smith[1]*

1. Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz, Santa Cruz, California, USA;
2. Division of Nutritional Sciences, and Department of Psychology, Cornell University, Ithaca, New York, USA

* Co-corresponding authors

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Abstract

BACKGROUND: Environmental manganese (Mn) exposure is associated with impairments in attention and psychomotor function as well as impulsivity/hyperactivity in children and adolescents. We have shown previously that developmental Mn exposure can cause these same areas of dysfunction in a rat model. Although treatment with methylphenidate (MPH) is well documented to lessen impairments in attention, impulse control, and sensorimotor function in children, it is unknown whether this treatment is effective in improving these areas of dysfunction induced by developmental Mn exposure.

OBJECTIVES: Determine if oral MPH therapy ameliorates the lasting attention and sensorimotor impairments caused by developmental Mn exposure and elucidate the underlying mechanisms of Mn neurotoxicity and MPH effectiveness, if observed. Rats were given 50 mg Mn/kg/d orally over PND 1 - 21 and assessed as adults in a series of attention, impulse control and sensorimotor tasks during chronic oral MPH treatment (0, 0.5, 1.5, or 3.0 mg/kg/d), followed by continued behavioral testing in the presence of selective catecholaminergic receptor antagonists (SCH 23390, raclopride, and BRL 44408 s.c.) and MPH treatment, using a quasi-Latin-Square design.

RESULTS: Developmental Mn exposure caused persistent attention and sensorimotor impairments in adult rats. MPH therapy at 0.5 mg/kg/d completely ameliorated the Mn-induced attentional dysfunction; notably this benefit was not seen following acute administration, but was apparent after prolonged (i.e., >8 days) treatment. The Mn-induced sensorimotor dysfunction was also ameliorated by MPH treatment but only at the 3 mg/kg/d MPH dose. Furthermore, selectively antagonizing D1, D2, or $\alpha 2_A$ receptors had no effect on the Mn-induced attentional deficits, or MPH efficacy to ameliorate those deficits. However, antagonism of D2R attenuated the sensorimotor deficits produced by Mn, whereas the efficacy of MPH to ameliorate those sensorimotor deficits was diminished by D1R antagonism.

CONCLUSIONS: These findings demonstrate that MPH treatment is effective in treating the lasting attention and sensorimotor deficits caused by developmental Mn exposure, and they clarify the mechanisms underlying developmental Mn neurotoxicity and MPH efficacy. Given that the cause of attention and co-morbid psychomotor deficits in children is often unknown, these findings may have implications for the treatment of environmentally-induced attentional and psychomotor dysfunction in children more broadly.

Highlights

- Exposure to manganese (Mn) in early life causes lasting impairments in attentional and sensorimotor (reaching and grasping) abilities.
- Prolonged oral methylphenidate (MPH, Ritalin) treatment ameliorates the Mn-induced deficits in attention and sensorimotor function, with the most effective MPH dose varying by domain of behavioral dysfunction.
- The efficacy of MPH for improving attentional dysfunction was not seen following acute administration, but was apparent after prolonged (i.e., >8 days) of treatment.
- Selective dopamine D1, D2, or noradrenergic $\alpha 2_A$ receptor antagonists did not alter the Mn-induced attentional dysfunction or the MPH efficacy to ameliorate the attention deficits. However, the D1R and D2R antagonists altered impulse control and sensorimotor (grasping) ability depending on the Mn exposure and MPH dose.
- These findings have important implications for understanding and treating attention and co-morbid psychomotor deficits caused by environmental Mn exposure in childhood.
- Given that the cause of attention and co-morbid psychomotor deficits in children is often unknown, these findings have implications for the treatment of environmentally-induced attentional and psychomotor dysfunction in children more broadly.

Introduction

Numerous epidemiological studies have demonstrated associations between environmental manganese (Mn) exposure and the prevalence of attention-deficit/hyperactivity disorder (ADHD) and/or attention-impulsivity/hyperactivity symptoms in children and adolescents. For example, in a recent population-based registry study of over 600,000 Danish children, Schullehner et al.(2020) reported that early exposure to increasing levels of Mn in drinking water was associated with an increased risk of ADHD-inattentive subtype. Environmental Mn exposure has also been linked to ADHD-related behavioral problems in children and adolescents in Korea (Bhang et al., 2013; Hong et al., 2014), Spain (Capelo et al., 2022), Italy (Broberg et al., 2019), and Canada (Bouchard et al., 2007; Oulhote et al., 2015). Although suggestive, these studies do not allow causal inferences due to the possibility that these associations reflect uncontrolled, or poorly controlled, confounding.

Animal model studies are a crucial addition to human investigations of environmental Mn exposure in this regard. First, they are essential for testing the causal relationship between well-defined Mn exposures and these neurobehavioral symptoms. Second, they can facilitate determining the underlying neurobiological bases for the symptoms, which can inform the findings from pediatric epidemiologic studies and guide treatment options (Smith & Strupp, 2023). Our prior animal model studies have shown that developmental oral Mn exposure at levels corresponding to the exposure risk faced by newborns and young children can produce abnormalities in attentional processes and sensorimotor function, as well as hypofunctioning of the catecholaminergic fronto-cortico-striatal system that lasts into adulthood (Beaudin, Nisam, & Smith, 2013; Beaudin, Strupp, Strawderman, et al., 2017a; Conley et al., 2020a; Lasley et al., 2020a). These studies have enhanced our understanding of the role of Mn exposure in the development of ADHD symptoms and attentional/sensorimotor deficits in general, and their biological foundations. They also provide a model system for testing the efficacy of potential therapies.

Methylphenidate (MPH) is an effective ADHD medication that can lessen core behavioral symptoms in both children and adults (Coghill et al., 2014; Faraone et al., 2021; Volkow et al., 2005) and also alleviate manual skill impairments in ADHD children with co-morbid developmental coordination disorder (DCD)(Bart et al., 2013). Pre-clinical and clinical studies have implicated a number of mechanisms underlying MPH efficacy, ranging from the acute pharmacologic action of MPH to enhance basal synaptic dopamine and norepinephrine levels leading to activation of specific dopamine and norepinephrine receptors within the fronto-cortico-striatal system (Arnsten, 2006, 2009; Spencer & Berridge, 2019), to longer-term adaptations in catecholaminergic and Wnt- and mTOR-signaling pathways with chronic MPH treatment (Ohki et al., 2020; Zetterström et al., 2022). Overall, however, the specific mechanism(s) responsible for MPH's efficacy in treating ADHD and related symptoms remains poorly understood.

Our prior studies on the effectiveness of MPH for treating Mn-induced neurobehavioral deficits have demonstrated that 16 days of daily oral treatment at a dose of 2.5 mg/kg/d

can completely ameliorate Mn-induced impairment in sensorimotor function (Beaudin et al., 2015). However, this MPH regimen did not lessen the attentional deficits caused by Mn in adult rats (Beaudin et al., 2017). These studies, while encouraging, did not identify an MPH dose that can lessen the attentional dysfunction caused by Mn, or provide mechanistic insights into Mn-induced behavioral symptoms and MPH efficacy.

Here, we used our rodent model of developmental Mn exposure producing lasting sensorimotor and ADHD-like symptoms to investigate 1) the efficacy of a range of clinically-relevant 30-day oral MPH treatment regimens for ameliorating the lasting attention and sensorimotor impairments caused by developmental Mn exposure, and 2) the underlying mechanistic role of dopamine D1, D2, and noradrenergic $\alpha 2_A$ receptors in developmental Mn neurotoxicity and MPH efficacy. Notably, we used behavioral tests that have been widely employed to assess ADHD-like symptoms and MPH efficacy in rat models of fronto-cortico-striatal dysfunction (Bari et al., 2008; Kloth et al., 2006; Montoya et al., 1991; Robbins, 2002; Whishaw et al., 1987). Our findings demonstrate the efficacy of MPH for treating the lasting attention and sensorimotor deficits caused by developmental Mn exposure, and they clarify the mechanisms underlying developmental Mn neurotoxicity and MPH efficacy.

Methods

Subjects

A total of 128 Long-Evans male rats were used in the study. Animals were born at the University of California, Santa Cruz from 28 nulliparous timed-pregnant rats (Charles River; gestational age 16). Twelve to 24 hours after parturition (designated PND 1, birth = PND 0), each litter was culled to eight pups, with five to six males and the balance females. Only one male/litter was assigned to a particular treatment condition. The study used a 2 x 4 factorial design, with two Mn exposure levels (0 and 50 mg/kg/d) and four oral MPH treatment levels (0, 0.5, 1.5, and 3.0 mg/kg/d), yielding 8 independent treatment groups: Control + 0 MPH, Control + 0.5 MPH, Control + 1.5 MPH, and Control + 3.0 MPH, and Mn + 0 MPH, Mn + 0.5 MPH, Mn + 1.5 MPH and Mn + 3.0 MPH. The final sample size was 115 animals, 14-15 per treatment group. The final catecholaminergic receptor antagonist phase, entailing four receptor antagonist conditions (vehicle, D1R, D2R, and $\alpha 2_A$R), was a within-subject, quasi Latin-square design.

All animals (dams and weaned pups) were fed Harlan Teklad rodent chow #2018 (reported by the manufacturer to have 118 mg Mn/kg), housed in polycarbonate cages at a constant temperature (21 ± 2 °C). After weaning on PND 22, weanlings were pair-housed by treatment group assignment and kept on a reversed 10:14 hrs light/dark cycle with lights off at 6:00 AM and on at 8:00 PM. All aspects of testing, feeding, and treatment were conducted during the active (dark) phase of the animals' diurnal cycle, approved by the institutional IACUC and adhered to NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

Developmental Postnatal Manganese Exposure regimen

The method used for early postnatal Mn exposure was identical to that described in our prior studies (Beaudin et al., 2017b; Kern et al., 2010). Briefly, neonates were orally exposed to 0 or 50 mg Mn/kg/d from PND 1-21. Manganese (Mn) was delivered once daily directly into the mouth of each pup (~ 25 µL/dose) via a micropipette fitted with a flexible polyethylene pipet tip (Fisher Scientific, Santa Clara, CA, USA). This Mn exposure regimen produces relative increases in Mn intake that approximate the increases reported in infants and young children exposed to Mn via drinking water, diet, or both (Kern & Smith, 2011). Our prior studies and those of others have shown that this, and similar, oral Mn exposure regimens impaired attention, learning, impulse control, locomotor activity, and forelimb sensorimotor function, and reduced evoked-release of dopamine and norepinephrine and expression of key receptors/transporters in the frontostriatal catecholamine system in adult rats (Beaudin et al., 2013; Beaudin et al., 2017b; Conley et al., 2020; Kern & Smith, 2011; Lasley et al., 2020; McDougall et al., 2008; Reichel et al., 2006).

Oral Methylphenidate Treatment regimen

Methylphenidate hydrochloride (MPH) (Sigma-Aldrich Inc., St. Louis, Missouri) was administered orally once daily for 30 days from PND 109-143 at dosages of 0, 0.5, 1.5, and 3.0 mg/kg/d, as in our previous study (Beaudin et al., 2015). Control- and Mn-exposed animals were randomized into the four MPH dosage conditions prior to the start of the MPH treatment phase of the study. MPH treatment was delivered using a food wafer delivery method (Beaudin et al., 2015; Ferguson et al., 2009), in which quartered pieces of wafer (Mini-Vanilla, Nabisco Inc) were adulterated daily with vehicle or MPH solutions (volume >20-30 µL) to achieve the targeted daily MPH dose per subject. The MPH solutions were prepared each day, and the time and order of treatment were the same each day for every rat. Personnel delivering wafers confirmed that the rat consumed the whole piece within 10 seconds of delivery. MPH treatment always occurred 30 minutes before testing on the attention tasks, and >1h before the start of staircase testing. This, or similar, MPH dosing regimens have been shown to be safe to adolescent rats and juvenile monkeys, to produce a blood MPH half-life of ~2 h in adult rats, and to improve attention, working memory, inhibitory control, and forelimb skill deficits in animal models (Beaudin et al., 2015, 2017; Cao et al., 2012; Kuczenski & Segal, 2002; Mohamed et al., 2011; Thanos et al., 2015; Zhu et al., 2007), thereby mimicking the human pharmacokinetic profile and clinical use of the drug.

Antagonist treatment regimen

In the last phase of behavioral testing, animals received a subcutaneous injection of one of the antagonists (D1R, D2R, $\alpha 2_A R$), or vehicle (saline) 10 minutes before MPH administration and 40 minutes before behavioral testing. D1R, D2R, and $\alpha 2_A R$ antagonists (SCH 23390, raclopride, BRL 44408) were purchased from Sigma Aldrich (St Louis, Missouri) and dissolved in 0.9% sterile saline solution before being administered in a volume of 1 mL/kg. Vehicle-treated animals received only the saline solution.

Receptor antagonist treatments were delivered according to a within-subject crossover Latin square design, in which all subjects received the four different antagonist treatments

three times over consecutive treatment cycles. To minimize any potential antagonist carry-over effect, the antagonist treatments were separated by 2-day washout breaks, with the first washout day consisting of no MPH and antagonist drug treatment and no testing, and the second day consisting of MPH treatment only (no antagonist) and testing on the third focused attention task. The antagonist treatment period lasted a total of 36 days (PND 144 to 179) given that there were three cycles, each lasting 12 days. By the start of the antagonist treatment phase, the animals had been treated with MPH for 30 days.

The antagonists, doses, and timing of treatment were chosen based on previous research demonstrating that these drugs antagonize the effect of acute MPH treatment on behavioral and physiological measures of frontostriatal function relevant to ADHD in animal models (Arnsten et al., 2005; Bandyopadhyay et al., 2005; de Oliveira et al., 2006; Granon et al., 2000; Hemsley et al., 1999; Hoffman & Donovan, 1994; Kiss et al., 1995; Newman et al., 2008). The selective receptor antagonists to D1R *(*SCH23390, K_i = 0.2 nM vs >1000 nM for D2) (Bourne, 2001), D2R (raclopride, K_i = 1.8 nM vs >1000 nM for D1) ((Hall et al., 1989; Seeman et al., 1987), or α2AR (BRL 44408, K_i = 1.7 nM vs >150 nM for other α1B,C and α2 receptors) (Kiss et al., 1995; Young et al., 1989) were delivered to transiently inhibit receptor function while undergoing MPH or vehicle treatment and behavioral testing. The K_i's of these receptor antagonists exceed by >100-fold the binding affinities of the receptors' natural ligands (DA and NE), and the pharmacokinetics of all three are well defined (Alexoff et al., 2003; Alstrup et al., 2011; Hall et al., 1989; Han & Gu, 2006; Janhunen et al., 2011).

Because SCH23390 and raclopride have been shown to decrease the number of response trials in the 5-CSRTTT (Koskinen and Sirvio, 2001 and Shoaib and Bizzaro, 2005), the final dose selection for these drugs was based on the behavioral results of a pilot experiment. In the pilot study, D1R, D2R, and $\alpha 2_A$R receptor antagonist treatments were given to control and Mn-exposed rats 40 minutes before behavioral assessment on the selective attention baseline task. The pilot study found that doses of 0.005 mg SCH23390/kg and 0.015 mg raclopride/kg had little to no effect on response rate when compared to vehicle, and notably higher response rates compared to the initially selected higher doses of 0.25 mg/kg and 0.50 mg/kg of the same drugs; the initial dose of 1.0 mg BRL 44408/kg, like lower doses, had no effect on response rate in animals. Based on these findings, dosages of 0.005 mg SCH23390/kg, 0.015 mg raclopride/kg, and 1.0 mg BRL 44408/kg were utilized in the receptor antagonist phase of the study.

Behavioral testing

As adults, all animals were tested in the 5-CSRTT task and the Montoya staircase test to evaluate learning, attention, impulse control, and sensorimotor functions. These tasks were chosen because they assess specific cognitive and sensorimotor control processes, such as focused and selective attention, inhibitory control, and forelimb reaching and grasping skills that are relevant to epidemiologic research, and which have previously been shown to be impaired as a result developmental Mn exposure in our prior animal studies (Beaudin et al., 2017a; 2017b; 2015; 2013).

5-CSRTT testing Apparatus

Sixteen identical automated 5-CSRTT testing chambers fitted with odor delivery systems (#MED-NP5L-OLF, Med Associates, Inc., St. Albans, VT) were used. Each testing chamber had a curved aluminum wall equipped with five 2.5 × 2.5 cm response ports positioned 2 cm above the grid floor; each port was fitted with a light-emitting diode 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 had a 45 mg food pellet dispensing port fitted with an infrared beam to register nose pokes. The two side walls and ceiling were polycarbonate, and the floor was a grid of stainless steel rods; each unit also had a small house light. The entire testing chamber was enclosed in a sound attenuating cubicle.

5-CSRTT Procedure

On PND 47, behavioral testing began with a week of food magazine and nose-poke training, followed by two five-choice visual discrimination learning tasks with a fixed cue duration and no pre-cue delay, followed by a series of visual attention tasks, as previously described (Beaudin, et al., 2017a). All animals were kept on a modest food restriction schedule with water available *ad lib*, as described previously (Beaudin, et al., 2017a). They were weighed and evaluated 6 days/week (Sunday-Friday) during the active dark period of the diurnal cycle throughout the study. The order and time of testing were balanced by treatment and stayed the same each day for every 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, 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 personnel blind to the treatment condition of the subjects.

Focused Attention Tasks

The focused attention tasks were administered after the last day of training on the visual discrimination two task to evaluate rats' capacity to identify a brief light presented randomly in space and time, as well as to inhibit pre-potent responses over variable pre-cue delays (i.e., nose poking at 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 0.700 sec and was administered for 12 testing sessions from PND 80-93. The second focused attention task included longer pre-cue delays of 0, 3, 4, or 5 sec and variable visual cue durations of 0.4 or 0.7 sec and was administered for 12 testing sessions from PND 95-108. During these tests, the animals were not given MPH.

Selective Attention Task with Olfactory Distracters

The final two tasks administered were the (1) third focused attention task and the (2) selective attention task with olfactory distractors. This latter task was designed to evaluate the rat’s ability to maintain a behavioral or cognitive set in the face of distracting or competing olfactory stimuli (Beaudin et al., 2017a). In the selective attention task, the pre-cue

delay varied between 3 and 4 sec and cue duration was 700 milliseconds. In addition, on one third of the trials in each daily testing session, an olfactory distractor was presented 1–3 sec before presentation of the visual cue. The third focused task included the same pre-cue delays and cue duration as used in the selective attention task, and thus provided a baseline with which to compare performance in the selective attention task, and thereby determine whether the unpredictable presentation of the odor distractors in that selective attention task produced a generalized disruption of performance, which extended into the non-distraction trials. Animals were tested in the third focused attention task for 18 days (testing sessions) while receiving for the first time daily oral MPH treatment from PND 109 – 129. All animals were assessed on the selective attention task for 12 test sessions while they continued to receive daily oral MPH treatment from PND 130-143 (for a total of 30 days in phase two).

Recorded response types for all attention tests included the following: premature responses (responses made after trial onset but before presentation of the visual cue); correct response (responses made to the correct port following presentation of the visual cue); incorrect response (responses made to the incorrect port following presentation of the visual cue); and omissions (failure to respond within the 10 sec response interval following the visual cue). In addition, the latency for correct responses was recorded, as was the latency to collect the food pellet reward following a correct response. The calculated response outcomes were percent correct, calculated as number of correct responses/(correct + incorrect + premature + omissions) × 100; percent incorrect, calculated as above but with incorrect responses in the numerator; percent accuracy, calculated as number of correct responses/(correct + incorrect) × 100; percent premature, calculated as number of premature responses/(correct + incorrect + premature + omissions) × 100; and percent omissions, calculated as number of omissions/(correct + incorrect + premature + omissions) × 100.

Staircase Sensorimotor Testing Apparatus

Eight Plexiglas staircase devices were used to assess forelimb reaching and grasping skills in adult rats, as described previously (Beaudin et al., 2013). Each device was made of a left and right sliding staircase that had 6 descending steps on each side, a central platform where the rat stood, and a restriction box. The first step was situated 12mm below the platform, and the distance between each step was 6mm, so the lowest step 6 was situated 42mm below the platform.

Staircase Procedure

The staircase test was conducted daily 10 minutes after completion of 5-CSRTT testing and used a colored-coded counting procedure, as used in our earlier studies (Beaudin et al., 2015; 2013). Each rat was given one 10-min trial per day, 6 days per week while receiving daily oral MPH treatment. The order and time of testing were balanced by treatment and stayed the same each day for each rat. Each step was baited with different colored 45mg food pellets prior to testing to determine the final distribution of pellets and quantified forelimb skill performance per step and side.

Skilled forelimb performance was evaluated step-by-step for (1) the number of pellets taken, (2) the number of pellets eaten, (3) the corresponding percent of success, and (4) the number of pellets misplaced. The number of "pellets taken" per step describes pellets that were grasped and removed from their step of origin and was calculated as [number of initial pellets - number of pellets remaining per step]. The number of "pellets eaten" describes those pellets that were taken and consumed, and was calculated as [number of initial pellets - (number of pellets remaining + number of pellets misplaced + the number of pellets lost)]; for this, the "pellets misplaced" describes all the pellets
that were grasped but ended up on a different step level. Finally, the "percent grasping success" was calculated as the ratio of the number of pellets eaten/pellets taken x 100.

Blood and Brain Mn Levels

Blood and brain Mn concentrations were determined in littermates as well as the study animals at the completion of neurobehavioral testing [~ PND 192, as previously described (Beaudin et al., 2013; Kern et al., 2011; 2010). Briefly, whole blood was digested using trace clean metal methods and analyzed for Mn by inductively coupled plasma–mass spectrometry (Thermo Element XR). The analytical detection limit for Mn was 0.04 ng/mL.

Statistical Methods

The behavioral data were modeled by way of structured covariance mixed models and according to the 2 x 4 x 4 factorial design. Fixed effects included in the model were Mn exposure treatment (2 levels corresponding to the two exposure treatment groups), MPH treatment (4 levels corresponding to the 4 MPH-dose groups), and antagonist treatment (4 levels corresponding to the 4 receptor antagonist treatments depending on the stage of the study. In addition, the model also included pre-cue delay, cue duration, day, testing session block, and/or distraction condition, and step level depending on the outcome analyzed. Testing session blocks were 3 day long test sessions each. In all models, the random effect was rat to account for correlations within observations from the same animal. Statistical tests used a Kenward-Roger of freedom estimate correction. Plots of residuals by experimental condition were used to examine the assumption of homogeneity. Other random effects with high variance in the residuals across the levels of the factor (e.g., distraction condition) were added to achieve homogeneity if needed. The distribution of each random effect was inspected for approximate normality and presence of influential outliers. Blood and brain Mn data were analyzed using a one-way analysis of variance and Tukey's *post hoc* test for pairwise comparisons.

The significance level was set at $p \leq 0.05$, and p-values between 0.05 and 0.10 were trends and are reported if the pattern of findings aided in clarifying the nature of the Mn, MPH, or antagonist treatment effects. Significant main effects or interaction effects were followed by single-degree of freedom contrasts to clarify the nature of the interactions, using the Student's t-test for pairwise comparisons of least squared means. All behavioral analysis were conducted using SAS (version 9.4) for Windows and Mn concentration data were analyzed using JMP (version 11.0; SAS Institute, Inc.).

Results

Here, we focus on the outcome measures which most clearly revealed the effects of Mn exposure and MPH treatment; namely, percent accurate and premature responses for the attention task series, and the number of pellets taken and misplaced in the Montoya staircase test of sensorimotor function.

Mn exposure during development causes long-term deficiencies in attention and learning in adults.

To confirm that early postnatal Mn exposure caused lasting focused attention and impulse control deficits in adulthood, as shown in several of our prior studies (Beaudin et al., 2016; 2017b), animals were tested on two successive focused attention tasks that differed in the duration of randomly presented delays prior to cue presentation (i.e., 0 - 3s in FA 1, 0 - 5s in FA 2), increasing the demands on both inhibitory control and focused attention.

Manganese exposure impairs attention in tasks requiring focused attention. The ability to maintain attentional focus to brief visual cues presented randomly in space (i.e., response port) and time is impaired by Mn exposure. The analysis of percent accurate responses in the first focused attention task reveals a significant interaction between Mn exposure and testing session block (3 sessions per block) ($F(3, 434)=6.75$, $p=0.0002$), reflecting that the Mn group improved less across session blocks compared to controls (Figure 1A); they were also impaired relative to controls in all four blocks (all $p <0.05$). In the second focused attention task, an interaction emerges between Mn exposure and pre-cue delay ($F(3, 520)=3.67$, $p=0.012$), reflecting that the Mn rats, in clear contrast to the controls, did not significantly improve attentional accuracy at the longer delays relative to the 0 sec delay. The Mn animals were also impaired at all pre-cue delays relative to controls (all p's<0.05) (Figure 1B).

In the second focused attention task, manganese exposure impairs learning to withhold responses until the cue was presented. While premature responses (i.e., a nose-poke into a port prior to presentation of the visual cue) did not differ between the Mn and control groups in the first focused attention task (Figure 1C), impairment in inhibitory control emerges in the second focused attention task with the imposition of longer pre-cue delays (Figure 1D). Specifically, the analysis of percent premature responses reveals a border-line interaction between Mn exposure and testing session block ($F(3, 376)=2.22$, $p=0.085$), reflecting that the Mn group learned to withhold premature responses more slowly than controls across successive blocks of testing (Figure 1D). As a result, the Mn group committed significantly more premature responses than controls during session blocks 2 and 4 (p's=0.048 and 0.006, respectively) (Figure 1D).

Prolonged MPH therapy at the lowest MPH dose ameliorates Mn-induced attention impairments and improves inhibitory control.

Our prior studies using a selective attention task revealed that rats exposed to Mn during early development are more disrupted than controls by unpredictable olfactory distractors,

manifested as reduced response accuracy and increased premature responses on distraction trials (Beaudin et al., 2017a; 2017b). To determine whether MPH treatment alleviates these areas of dysfunction, animals were dosed with oral MPH and tested on this selective attention task, and a preceding selective attention 'baseline' task that was identical in terms of cue onset/duration but did not include olfactory distractors (see Methods); this baseline task is referred to as the third focused attention task.

Mn exposure impairs attention in the third focused attention task, and prolonged low dose MPH therapy ameliorated this Mn attentional deficit. The major findings in the third focused attention task are: (1) impairment in attentional accuracy produced by Mn exposure, (2) efficacy of the oral 0.5 mg MPH/kg/d dose to ameliorate the Mn-induced attentional dysfunction, but only after prolonged MPH treatment, and (3) the two higher MPH doses had little to no benefit (relative to no MPH) in ameliorating attentional dysfunction induced by Mn. These findings are supported by the significant Mn x MPH x session block interaction for percent accuracy ($F(15, 747)=4.82$, $p<0.0001$). Specifically, the Mn+0 MPH group was significantly less accurate than controls over all testing session blocks (p's <0.001) (Figure 2A). Notably, for the Mn group treated with 0.5 mg MPH/kg/d, there is little or no MPH efficacy over the initial series of testing session blocks (i.e., initial ~9d of testing and MPH treatment), but complete efficacy by session block six, in which the Mn-exposed animals with this MPH dose perform like unexposed controls (Figure 2A). Contrasts show that the Mn+0.5 MPH group performed significantly better than the Mn+0 MPH group during session blocks 4 – 6 (p's=0.03, 0.01, and 0.04), and was not different than the control+0 MPH group during session blocks 3 – 5 (p's=0.21, 0.13, 0.38) (Figure 2A). In contrast, the higher MPH doses of 1.5 and 3.0 mg/kg/d showed little or no efficacy, i.e., attentional accuracy of the Mn+1.5 MPH group and the Mn+3.0 MPH group was not different from the Mn+0 MPH group and was significantly worse than the control+0 MPH group across many session blocks (all p's<0.05) (Figure 2A).

The MPH dose-response for attentional accuracy follows an inverted U-shape in Mn animals but not controls. To evaluate whether the Mn and control groups exhibit different MPH dose-response functions, we calculated the change (i.e., improvement) in percent accurate responses from test session block one to session block six within each control/Mn and MPH treatment groups in the third focused attention task (shown in Figure 2A, discussed above). Qualitatively, the patterns reveal that the Mn animals exhibited an inverted U-shaped MPH dose-response, with the two lower MPH doses, but not the highest dose, improving performance relative to vehicle. In contrast, the control animals exhibited a U-shaped MPH dose-response, with the two lower MPH doses producing no improvement or slight impairment in performance relative to the vehicle and the highest MPH dose groups (Figure 2B). These findings further illustrate that the MPH dose-response in the Mn animals is fundamentally different than controls.

Mn exposure impairs attentional accuracy in the selective attention task with olfactory distractors, and prolonged 0.5 mg/kg/d MPH administration fully ameliorates the dysfunction. The selective attention task with olfactory distractors reveals continued dysfunction

in attention in the Mn animals versus controls, and that the 0.5 mg/kg/d MPH dose completely ameliorating the Mn-induced attentional dysfunction (Figure 2C). Supporting this, analysis of percent accuracy uncovers a significant Mn x MPH x distraction condition interaction ($F(9, 558)=2.18$, $p=0.022$), which reflects several treatment group differences. First, the Mn+0 MPH group exhibited significantly lower percent accurate responses than the control+0 MPH group in all distraction conditions (p's<0.05), reflecting attentional dysfunction. Second, the Mn impairment is most apparent when the olfactory distractor is presented 1 second before the visual cue (i.e., the -1s condition), indicating additional dysfunction in the domain of selective attention (Figure 2C). Note too that the -1s distraction condition is the condition at which the olfactory distractor caused the greatest reduction in attentional accuracy for all groups. (Figure 2C).

The significant 3-way interaction of Mn exposure, MPH dose, and distraction condition also reflects that MPH treatment at the 0.5 mg/kg/d dose (but not higher doses) fully ameliorates the Mn attentional dysfunction in this task. Specifically, the Mn+0.5 MPH group performed identically to the control+0.5 MPH and control+0 MPH groups under all distraction conditions, and significantly better than the Mn+0 MPH group under most distraction conditions (p's=0.01, 0.003, 0.055, and 0.041 for the no odor, -1, -2, and -3 s distraction conditions, respectively) (Figure 3A). Also, the Mn+0.5 MPH group performed indistinguishably from the control+0 MPH group across all distraction conditions (p's=0.69, 0.47, 0.15, and 0.66) (Figure 3A). In contrast, the higher MPH doses produced intermediate or no improvement in attentional accuracy in the Mn animals. For example, attentional accuracy of the Mn+1.5 MPH and Mn+ 3.0 MPH groups were not significantly different than the Mn+0 MPH group for any distraction condition, though for some distraction conditions within the 1.5 and 3.0 MPH dose groups the Mn animals were also not different from controls (Figure 3A). This latter finding suggests an intermediate efficacy of these higher MPH doses in the Mn animals.

In the third focused attention task, prolonged MPH administration at 0.5 mg/kg/d improves impulse control in the Mn-exposed rats. Analysis of premature responses in the third focused attention task reveals a significant Mn x MPH x testing session block interaction ($(15, 576)=2.41$, $p=0.002$). This reflects that MPH treatment at the 0.5 mg/kg/d dose offers some benefit to decrease premature responses in the Mn animals compared to the Mn+0 MPH group in session blocks 5 and 6 (p's=0.041 and 0.050, respectively), and the control+0.5 MPH group in session block 6 ($p=0.033$) (Figure 2D).

Finally, we compared the MPH dose-response functions for the control and Mn rats, using improvement in percent premature responses across sessions, as described above for percent accuracy. Again, the Mn animals exhibited an inverted U-shaped MPH dose-response, whereas the control animals exhibited a U-shaped MPH dose-response function (Figure 2E). This again further supports that the Mn animals respond differently to MPH than controls, implicating lasting effects of developmental Mn exposure on neural systems which respond to MPH.

In the selective attention task, prolonged 3.0 mg/kg/d MPH treatment increases impulsivity in the Mn rats. As shown in our prior studies (Beaudin et al., 2017a; 2017b), the presentation of olfactory distractors prior to the visual cue significantly increases premature responding in both control and Mn groups ($F(3, 399)=541.87$, $p<0.0001$), particularly at the longer -2 and -3s intervals between the distractor and presentation of the visual cue (p's<0.0001) (Figure 2F). Further, several notable interactions are observed for premature responses; specifically, interactions of Mn, MPH, and session block ($F(9, 791)=2.36$, $p=0.012$), as well as between Mn, MPH, and distraction condition ($F(9, 506)=2.55$, $p=0.007$). The latter 3-way interaction reflects that the Mn+3.0 MPH group committed significantly more premature responses than the Mn+0 MPH group at the -3 s distraction condition ($p=0.050$) (Figure 2F), whereas the interaction of Mn, MPH, and session block indicates that this effect of the highest MPH dose on Mn animals emerged particularly in the last block of testing ($p=0.030$, data not shown). Collectively, these findings indicate that early postnatal Mn exposure did not cause lasting deficits in impulse control in these animals, and that the Mn animals responded differently to MPH under some trial conditions, particularly following prolonged MPH treatment to the 3.0 mg/kg/d dose (Figure 2F).

Specific D1R, D2R, or $\alpha 2_A$R involvement in Mn-induced attention deficit, impulse control, and MPH efficacy.

Mn-induced attentional dysfunction and MPH therapeutic efficacy are maintained throughout the catecholaminergic receptor antagonist study phase. To confirm that the Mn-induced attention deficit and MPH efficacy persisted into the 36 day receptor antagonist phase of the study, we first assessed attentional accuracy in control and Mn animals treated with the receptor antagonist vehicle. Results show that both the Mn attentional dysfunction and the efficacy of the 0.5 mg/kg/d MPH dose persisted into the receptor antagonist treatment phase of the study. Specifically, attentional accuracy in the Mn+0.5 MPH group was significantly better than the Mn+0 MPH group ($p=0.0008$) and was indistinguishable from the control+0 MPH group ($p=0.98$) at the -1 s distraction condition (Figure 3A).

Mn-induced attentional dysfunction and the efficacy of MPH to ameliorate the attention deficits are not singly mediated by catecholaminergic D1, D2, or $\alpha 2_A$ receptors. Given the efficacy of the 0.5 mg MPH/kg/d dose to fully ameliorate the Mn attention deficits in the selective attention task, we focused subsequent analyses on this MPH dose to determine whether D1R, D2R, or $\alpha 2_A$R antagonism altered the Mn attentional dysfunction or MPH efficacy. The results show that none of the specific D1R, D2R, or $\alpha 2_A$R antagonists altered the Mn-induced attention deficit or the efficacy of the lowest MPH dose to improve attentional function. The analysis uncovered a significant 3-way interaction of Mn, MPH, and distraction condition ($F(3, 212)=3.32$, $p=0.021$), but no significant interactions involving Mn, MPH, and antagonist treatment ($F(3, 670)=0.97$, $p=0.41$). This reflects that, in the absence of MPH treatment (i.e., 0 MPH), the Mn+0 MPH group performed significantly worse than the control+0 MPH group under the vehicle, D1R, D2R, and $\alpha 2_A$R antagonist

treatment conditions (p=0.021, 0.070, 0.015, and 0.036) (Figure 3B), and that attentional accuracy of the Mn+0.5 MPH group was indistinguishable from that of the control+0 MPH group across all receptor antagonist treatment conditions (Figure 3B). We can conclude from this that specific D1R, D2R, or $\alpha 2_A$R are not singly driving the Mn-induced attentional dysfunction, or the therapeutic effect of MPH treatment for this area of dysfunction.

D1R and D2R antagonists influence impulsivity and correct response latency under certain Mn/MPH treatment conditions. The effect of the 3.0 mg/kg/d MPH dose to increase premature responses under the -3s odor distractor condition in the selective attention task (reported above) was not sustained into the antagonist treatment phase of the study. Premature responses by the Mn+3.0 MPH group did not significantly differ from the Mn+0 MPH group for any distraction condition (p's>0.05), and the Mn+3.0 MPH group exhibited a similar level of premature responses compared to the control+0 MPH group across distraction conditions (p's>0.05) (Figure 4A).

Nonetheless, due to this prior effect of the 3.0 mg/kg/d MPH dose on impulse control in the Mn group in the selective attention task, we conducted analyses to determine whether any of the antagonists altered impulse control in the control and Mn groups treated with this 3.0 mg/kg/d MPH dose. We also examined correct response latency (i.e., the speed of correct responses), because a reduction in the propensity for premature responses produced by dopamine receptor antagonists has been reported to be accompanied by a delay in the initiation of responding (van Gaalen et al., 2006).

The results reveal a borderline interaction of Mn, MPH, and antagonist for percent premature responses (F(3, 590)=2.34, p=0.072), reflecting different effects of the catecholaminergic receptor antagonists depending on the Mn exposure and MPH dose. First, the effect of the D2R antagonist on premature responses in control rats varied as a function of MPH dose. For example, for control rats not treated with MPH, the D2R antagonist significantly reduced premature responses compared to the vehicle (p=0.0006), while having no effect on controls chronically treated with 3.0 MPH versus their respective vehicle (p=0.99) (Figure 4B). Moreover, the control+3.0 MPH rats treated with the D2R antagonist had significantly higher premature responses compared to their control+0 MPH counterparts treated with the D2R antagonist (p = 0.02) (Figure 4B). In contrast, the effects of the D2R antagonist in the Mn-exposed rats exhibited a different pattern. For the Mn animals not treated with MPH, the D2R antagonist had no impact on premature responding (p=0.19), but significantly reduced impulsivity in the Mn animals chronically treated with 3.0 MPH (p = 0.05) (Figure 4B).

The analysis of correct response latency for animals treated chronically with the 3.0 MPH dose revealed a significant Mn x antagonist x distraction condition interaction for latency of correct responses (F(9, 619)=2.16, p=0.023) (Figure 4C). This interaction reflects that for these animals, the correct response latency was significantly increased by the D1R antagonist (vs. vehicle), under the no odor condition for the Mn group (p=0.009), but not for controls (p=0.18) (Figure 4C). This contrasts sharply with the D1R antagonist's effect on increasing latencies to initiate a correct response (versus vehicle) in the control group

when a distractor was presented -1 s or -2 s before presentation of the visual cue (p's=0.001 and 0.067, respectively), effects that were not observed in the Mn group (p's=0.52 and 0.96) (Figure 4C). As a result, when animals were treated with the D1R antagonist in the presence of the olfactory distractors, the control+3.0 MPH group took longer to initiate a correct response than the Mn+3.0 MPH group (p's=0.076 and 0.006). Based on these findings, we can conclude that the 30 days of oral 3.0 mg MPH/kg/d treatment that preceded the receptor antagonist phase of the study altered the number and/or binding sensitivity of the D1 and D2 receptors in the Mn group.

Mn exposure causes lasting impairments in sensorimotor reaching and grasping skills, and these deficits are ameliorated by prolonged treatment with MPH.

Developmental Mn exposure caused lasting deficits in sensorimotor skills to reach and grasp food pellets on descending steps of the Montoya staircase, and both deficits are ameliorated by the higher doses of MPH. This is shown by the significant Mn x MPH x step interaction for the number of pellets taken ($F(15, 1552)=5.17$, $p<0.0001$) and the number of pellets misplaced ($F(15, 1614)=2.95$, $p=0.0001$). The 3-way interaction for pellets taken reflects that the Mn+0 MPH group took significantly fewer pellets than their control+0 MPH counterparts at steps 5 and 6 (p's=0.024 and 0.0041, respectively), and that this Mn deficit was ameliorated by the 1.5 and 3.0 MPH doses (Figure 5A). Moreover, the Mn+3.0 MPH group retrieved significantly more pellets than the Mn+0 MPH group at step 6 of the staircase ($p=0.05$), and a similar number of pellets compared with the control+0 MPH group at the same step ($p=0.33$) (Figure 5A). In contrast, the lower 0.5 mg MPH/kg/d dose had no efficacy in alleviating this sensorimotor deficit; i.e., the number of pellets taken by the Mn+0.5 MPH group was not different from the Mn+0 MPH group at step 6 ($p=0.51$) (Figure 5A).

Similarly, the significant Mn x MPH x step interaction for the number of pellets misplaced reflects a Mn deficit in sensorimotor grasping skills, and the efficacy of the higher MPH doses to alleviate the Mn deficit. Specifically, the 3-way interaction reflects that the Mn+0 MPH group misplaced significantly more pellets than the control+0 MPH group from staircase steps 2, 3 and 4 (p's=0.030, 0.013, and 0.0001, respectively), and that this deficit was also ameliorated by the 1.5 and 3.0 MPH doses (Figure 5B).This is supported by contrasts showing that the Mn+1.5 MPH group misplaced significantly fewer pellets than the Mn+0 MPH group at steps 2 and 4 ($p=0.008$ and 0.050), with a similar trend seen in the 3.0 mg MPH/kg/d group at step 4 ($p=0.069$) (Figure 5B). In contrast, the 0.5 mg MPH/kg/d showed little or no efficacy to alleviate the sensorimotor deficits, in that the number of pellets misplaced by the Mn+0.5 MPH group was not significantly different from the Mn+0 MPH group at steps 2-4 (p's=0.49, 0.14, and 0.20) (Figure 5B).

Specific D1R and D2R involvement in Mn-induced sensorimotor impairments and MPH effectiveness.

The Mn-induced sensorimotor impairments and MPH efficacy persist throughout the antagonist treatment phase of the study. We first sought to confirm that the Mn sensorimotor

reaching and grasping deficits, and the efficacy of prolonged 3.0 mg MPH/kg/d treatment persisted into the 36 day receptor antagonist phase of the study. For this, we analyzed the number of pellets taken and the number of pellets misplaced in the control/Mn groups treated with MPH under the antagonist vehicle treatment condition, which revealed a significant Mn x MPH x step interaction for the number of pellets taken and the number of pellets misplaced ($F(15, 1419)=1.78$, $p=0.032$, and $F(15, 1453)=3.25$, $p<0.0001$, respectively). The 3-way interaction for pellets taken reflects that the Mn+0 MPH group took significantly fewer pellets than their control +0 MPH counterparts at staircase steps 4-6 (p's=0.002, 0.006, and 0.005). Further, the interaction reflects the continued efficacy of MPH treatment; both the Mn+1.5 MPH and the Mn+3.0 MPH groups took significantly more pellets than the Mn+0 MPH group at step 5 ($p=0.046$) or step 6 ($p=0.019$), respectively (Figure 6A), consistent with our findings in the preceding phase of the study without receptor antagonists (described above). This confirms that the Mn *grasping deficit* and MPH efficacy persisted into the receptor antagonist phase of the study. This is further supported by the fact that Mn+1.5 MPH group misplaced significantly fewer pellets than the Mn+0 MPH group from steps 1 and 4 (p's=0.030 and 0.002), with a similar pattern of MPH efficacy seen at the 3.0 mg MPH/kg/d dose from staircase steps 1, 2, and 4 ($p=0.003$, 0.019, and 0.046, respectively) (Figure 6B).

None of the specific receptor antagonists we studied directly mediate either the Mn reaching deficit or the efficacy of MPH to alleviate this deficit. Given the lasting reaching and grasping Mn-induced deficits and efficacy of the 3.0 mg MPH/kg/d dose to alleviate these deficits at steps 4-6, we focused subsequent analyses regarding the effect of selective antagonist treatments on this MPH dose and step levels. The analysis of the number of pellets taken revealed a trending 3 way interaction of Mn, MPH, and step ($F(3, 116)=2.35$, $p=0.10$), but no significant interactions between Mn and antagonist ($F(3, 1478)=0.96$, $p=0.41$) or between Mn, MPH, and antagonist ($F(3, 1478)=0.25$, $p=0.86$). These findings reflect that the Mn+0 MPH group took significantly fewer pellets than the control+0 MPH under the vehicle, D1R, D2R, and $\alpha 2_A$R antagonist treatment conditions ($p=0.005$, 0.009, 0.007, and 0.005, respectively) (Figure 6C). Additionally, the number of pellets taken by the Mn+3.0 MPH group was not different than that of the control 0 MPH group across antagonist treatment conditions (p's=0.23, 0.55, 0.30, and 0.15 for the vehicle, D1R, RD2R, and $\alpha 2_A$R antagonist, respectively) (Figure 6C). We conclude from these results that the 3.0 mg/kg/d MPH dose ameliorated the sensorimotor Mn reaching deficit in pellets taken, and that none of the specific receptor antagonists directly mediate either the Mn reaching deficit or the efficacy of MPH to alleviate this deficit.

The D2R antagonist ameliorates the sensorimotor Mn deficit in grasping skill, while the D1R antagonist partially blocks the efficacy of MPH therapy to ameliorate the Mn grasping deficit. The analysis of the number of pellets misplaced, a measure of sensorimotor grasping skill, revealed a significant Mn x MPH x antagonist interaction ($F(3, 1478) = 3.81$, $p=0.009$). This interaction reflects differential effects of Mn exposure, depending on the MPH and antagonist treatment condition. Specifically, in the absence of MPH treatment,

the selective D2R antagonist had opposing effects on the Mn and control groups, improving grasping abilities in the Mn group by significantly decreasing the number of pellets misplaced (p=0.022), but impairing these abilities in the controls by significantly increasing the number of pellets misplaced (p=0.043), relative to antagonist vehicle treatment (Figure 6D). In contrast, treatment with the D1R and $\alpha 2_A$R antagonists showed no measurable effect on the number of pellets misplaced) in the Mn or control groups, compared to vehicle (Figure 6D).

Furthermore, the significant Mn x MPH x antagonist interaction for the number of pellets misplaced reported above indicates that the D1R antagonist attenuates the efficacy of MPH treatment to ameliorate the Mn-induced grasping deficit. Specifically, in the presence of MPH, treatment with the D1R antagonist substantially increased the number of pellets misplaced in the Mn group (p=0.007), but not in the controls (p=0.65). As a consequence of the D1R antagonist's effect on the Mn+3.0 MPH group, there was no difference in the number of pellets misplaced between this group and the Mn+0 MPH treated with vehicle (p=0.13) or the D1R antagonist (p=0.37), demonstrating the re-emergence of the grasping Mn-induced impairment following D1R antagonism.

Discussion

Epidemiological studies have identified environmental Mn exposure as a risk factor for ADHD and/or attention/impulsivity/hyperactivity symptoms in children and adolescents (Bhang et al., 2013; Karatela et al., 2022; Liu et al., 2020; Lucchini et al., 2012; Schullehner et al., 2020), although these studies were not able to determine causality, elucidate underlying mechanisms, or identify effective treatments. Here, using an animal model, we demonstrate that early developmental Mn exposure can cause lasting impairments in attention, learning, and sensorimotor functions, and that prolonged but not acute, oral MPH treatment is effective in lessening all of these impairments, although the most efficacious MPH dose varies by area of dysfunction. Finally, we found that antagonism of specific D1, D2, or $\alpha 2_A$ receptors does not alter either the degree of attentional impairment caused by Mn exposure, or the MPH efficacy to improve Mn-induced attentional dysfunction, indicating that none of these receptor types singly mediates the Mn attentional dysfunction or MPH efficacy. In contrast, antagonism of D2R lessened Mn-induced deficits in sensorimotor grasping skills in the absence of MPH, while antagonism of D1R attenuated MPH efficacy to ameliorate those deficits. These findings significantly advance understanding of the causal relationship between developmental Mn exposure and lasting attentional and sensorimotor deficits and their underlying mechanisms, and they demonstrate that a clinically-relevant oral MPH regimen is fully efficacious in ameliorating these deficits. The basis for each of these conclusions is provided below.

Mn-induced neurobehavioral deficits and efficacy of oral MPH treatment

A short period of developmental postnatal Mn exposure produced impairments in learning, attention, and sensorimotor function, paralleling the domains of dysfunction seen in

children exposed to elevated Mn levels (Bhang et al., 2013; Carvalho et al., 2018; Karatela et al., 2022; Lucchini et al., 2012; Oulhote et al., 2015; Schullehner et al., 2020). Notably, these impairments lasted well beyond the period of developmental Mn exposure, indicative of persistent or irreversible neurologic dysfunction as a result of the early exposure. For the four behavioral tasks administered here, the Mn group, in the absence of MPH, consistently responded less accurately to brief visual cues than controls, indicating impairment in attention as a result of the early Mn exposure, as previously reported (Beaudin et al., 2017a). Importantly, treatment with the lowest dose of oral MPH (0.5 mg/kg/d) completely ameliorated the attentional dysfunction of the Mn animals, though this benefit only arose after prolonged MPH treatment (9 days).

The fact that the efficacy of this low MPH dose was seen only after prolonged, but not acute, treatment suggests that the efficacy is not strictly due to the acute pharmacologic action of the drug to increase synaptic dopamine and norepinephrine levels, but may also be due to adaptive neuromolecular changes that emerge with prolonged treatment. Consistent with this inference, Ohki et al. (2020) present evidence that chronic MPH treatment alters a number of intracellular signaling pathways, including Wnt- and mTOR signaling, with downstream changes in catecholaminergic function. These actions of MPH, perhaps in addition to its pharmacologic effects as a DAT/NET antagonist to increase synaptic dopamine and norepinephrine levels, may account for MPH efficacy in the treatment of ADHD-related symptoms (Coghill et al., 2014; Faraone et al., 2021). Irrespective of the precise mechanisms, our findings demonstrate the effectiveness of prolonged low-oral dose MPH treatment to ameliorate the lasting attention deficits caused by developmental postnatal Mn exposure, consistent with clinical studies showing effectiveness of MPH in the treatment of ADHD symptoms (Coghill et al., 2014; Faraone et al., 2021).

Methylphenidate was also efficacious in ameliorating the Mn-induced sensorimotor impairments seen in the Montoya staircase test. In this test, the Mn group (0 MPH) took fewer food pellets from the most challenging staircase steps than did controls, indicating impaired execution of forelimb and paw sensorimotor function as a result of the developmental Mn exposure, as previously reported (Beaudin et al., 2013). Methylphenidate treatment improved these sensorimotor abilities in the Mn-exposed rats, but only at the highest (3.0 mg/kg/d) dose. Based on these findings, we can infer that the 30 days of oral 3.0 mg MPH/kg/d treatment altered the number and/or binding sensitivity of the D1 and D2 receptors in the Mn group, which have been shown to be downregulated and upregulated, respectively, within the fronto-cortico-striatal system of adult rats by the same developmental Mn exposure regimen used here (Conley et al., 2020; Kern et al., 2010; 2011b). Finally, this therapeutic effect of MPH recapitulates our previous findings (Beaudin et al., 2015), and is consistent with clinical studies showing that MPH treatment can attenuate the manual dexterity deficits in children with ADHD-DCD (Bart et al., 2013; Brossard-Racine et al., 2015; Flapper et al., 2006; Soleimani et al., 2017).

There are some prior rodent findings which also indicate that the dose of MPH needed to improve PFC functions is lower than that needed to affect striatal regions. Specifically,

rodent studies have shown that acute low and clinically relevant MPH doses (e.g., 0.25 - 1.0 mg/kg) improved prefrontal cortex-dependent functions, such as attentional processes and working memory, by selectively increasing synaptic levels of dopamine and norepinephrine within the prefrontal cortex (Berridge et al., 2006; Schmeichel & Berridge, 2013; Spencer & Berridge, 2019). At relatively higher MPH dosages, dopamine and norepinephrine levels are increased across numerous brain areas, including the striatum (Berridge et al., 2006; Schmeichel & Berridge, 2013), which is consistent with the relatively higher MPH doses needed to improve sensorimotor versus attentional function observed in the present study. Overall, the pattern of findings in clinical and animal model studies, which are corroborated by the present study, suggests that the most efficacious dose of oral MPH differs depending on the behavioral domain of function and/or the brain region(s) subserving those different functions.

Insights into the mechanisms underlying Mn neurobehavioral toxicity and MPH efficacy

There is compelling clinical evidence that ADHD-related symptoms are mediated, at least in part, by catecholaminergic system dysfunction in children (Arnsten, 2009, 2011; Del Campo et al., 2011; Faraone et al., 2021). Consistent with this, we have shown that developmental postnatal Mn exposure in rats, which causes lasting attentional and sensorimotor symptoms akin to ADD/DCD, also causes lasting hypofunctioning of the catecholaminergic system, including reduced synaptic release of norepinephrine and dopamine, and changes in protein levels of tyrosine hydroxylase, D1R, D2R, and dopamine and norepinephrine transporters in fronto-cortico-striatal brain regions, but no changes in $\alpha 2_A$ noradrenergic receptor ($\alpha 2_A R$) levels (Conley et al., 2020; Kern & Smith, 2011; Lasley et al., 2020). However, the receptor-specific antagonism findings from the present study suggest that neither D1R, D2R, or $\alpha 2_A R$ are singly responsible for the lasting Mn deficits in attention and sensorimotor function, or the efficacy of MPH to ameliorate those deficits. The fact that these receptor-specific antagonists did not measurably alter the Mn deficits or MPH efficacy cannot be attributed to using antagonist dose levels insufficient to antagonize the receptor and elicit a behavioral response, because there is clear evidence in the present study that antagonism of D1R and D2R did alter specific behaviors, including inhibitory control of premature responses (Figure 5B), latency to initiate a correct response (Figure 5C), and grasping abilities (Figure 6). Collectively, these findings imply that numerous catecholaminergic and/or non-catecholaminergic receptor systems are involved in the Mn attention deficit and MPH effectiveness. This conclusion is consistent with research demonstrating that both ADHD symptoms and their effective treatment include several receptor systems (e.g., catecholaminergic, cholinergic, GABAergic, and glutamatergic) (Chan et al., 2016; Del Campo et al., 2011; Gamo & Arnsten, 2011; Huss et al., 2016; Purkayastha et al., 2015; Rizzo & Martino, 2015; Wigal et al., 2010).

In contrast to the null findings on the effects of the receptor antagonists on attentional function, the D1R-specific antagonist, but not the D2R or $\alpha 2_A R$ antagonists, reduced the effectiveness of the 3.0 mg/kg/d MPH dose in alleviating the sensorimotor grasping impairment caused by development Mn exposure. Specifically, Mn animals treated with this

MPH dose performed significantly better than their Mn+Veh counterparts, and this improved sensorimotor performance in the Mn+3.0 MPH group was attenuated by the D1R-specific antagonist (SCH 23390) (Figure 6B). These findings indicate that the D1R plays an important role in MPH efficacy to improve the lasting sensorimotor deficits caused by developmental Mn exposure.

Interestingly, in the absence of MPH, antagonism of D2R with raclopride ameliorates the sensorimotor grasping dysfunction caused by Mn (Figure 6D), demonstrating that this domain of sensorimotor Mn deficits is mediated directly or indirectly by D2 receptor activity. This inference is supported by previous findings that the developmental Mn exposure regimen used here leads to lasting increases in D2R levels in the prefrontal cortex (Conley et al., 2020b; C. H. Kern & Smith, 2011b), as well as findings in the current study that antagonism of D2R impairs rather than improves grasping abilities in control rats (Figure 6D). Combined, these findings suggest that D2R may play a role in sensorimotor impairments caused by Mn possibly via the dopaminergic mesocortical system.

Strengths and limitations

There are a number of notable strengths of the present study. First, the nature of the behavioral tests allowed us to uncover specific cognitive and sensorimotor impairments produced by Mn – something that is often not possible to establish in human epidemiological studies. Second, because our rodent model of Mn exposure recapitulated the areas of behavioral dysfunction reported in children and adolescents exposed to elevated levels of Mn, we had a sensitive animal model to test potential therapies and elucidate underlying neural mechanisms. Third, we utilized multiple prolonged oral doses of MPH, at clinically relevant doses. Finally, we employed specific catecholaminergic receptor antagonists to assess MPH efficacy and underlying neuromechanisms.

There are also a number of limitations to our study. First, our conclusion that the optimal MPH dose for ameliorating the attentional dysfunction is lower than that needed for improving sensorimotor function must be tempered by the fact that the time interval between the daily oral MPH dosing and behavioral testing was different for the attention task and the sensorimotor testing (~20 and 90 min, respectively), possibly leading to different MPH pharmacokinetic profiles and brain MPH levels during these two tasks. Similarly, the receptor antagonism findings should be viewed within the context of their receptor binding properties. For example, while the binding affinities of the D1R (SCH23390, K_i = 0.2 nM) (Bourne, 2001; Chou et al., 1999) , D2R (raclopride, K_i = 1.8 nM) (Hall et al., 1989), and α2AR (BRL 44408, K_i = 1.7 nM) (Kiss et al., 1995) all exceed by >100-fold the binding affinities of the receptors' natural ligands (dopamine and norepinephrine), raclopride-D2R binding is readily displaced by stimulated dopamine release, whereas SCH23390-D1R binding is not (there are no comparable data on BRL 44408-α2AR displacement with stimulated norepinephrine release) (Chou et al., 1999; Finnema et al., 2013; Gifford et al., 2000; Stokes et al., 2010). Thus, the apparent absence of D2R involvement in MPH

efficacy may be a consequence of the D2R antagonist being displaced by dopamine under MPH treatment, which may have negated the antagonist effect on the MPH response of the Mn group.

Summary

In summary, our findings demonstrate that early developmental Mn exposure can cause persistent attentional and sensorimotor dysfunction in adulthood, and that prolonged oral MPH treatment fully ameliorates those deficits, although the most efficacious MPH dose depends on the domain of neurobehavioral impairment. Further, selectively antagonizing D1, D2, or $\alpha 2_A$ receptors had no effect on the Mn-induced attentional deficits, or MPH efficacy to ameliorate those deficits. In contrast, antagonism of D2R attenuated the sensorimotor deficits produced by Mn, whereas the efficacy of MPH to ameliorate those sensorimotor deficits was diminished by D1R antagonism. Collectively, these findings have significant implications for the mechanistic understanding and therapeutic treatment of environmentally-induced attentional and psychomotor dysfunction in children.

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FIGURES

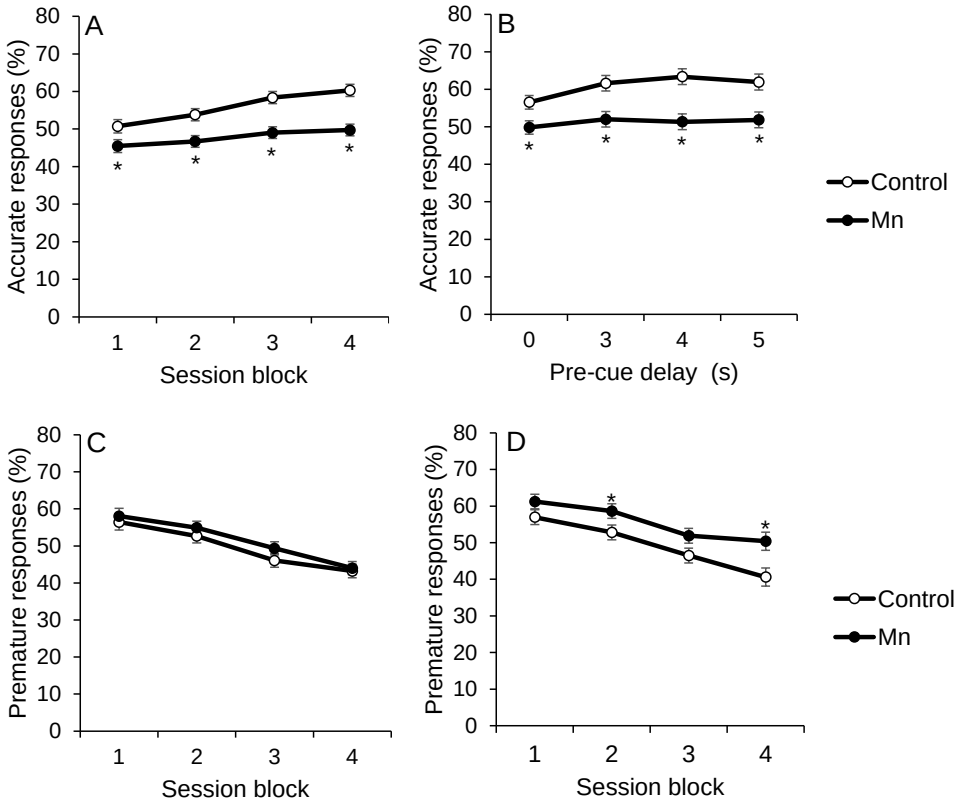

Figure 1A-D. Developmental postnatal manganese exposure produces deficits in attention and learning in the focused attention tasks with variable pre-cue delays. Accurate responses (%) for the control and the Mn groups, as a function of (A) test session block in the first focused attention task and (B) increasing pre-cue delay in seconds (s) in the second focused attention task. Premature responses (%) for the control and the Mn groups, as a function of test session block in (C) the first and (D) the second focused attention tasks. Data are lsmeans ± SEM of the Mn and control group (*n* = 61–63/group). *p ≤ 0.05 versus controls. Each test session block comprised three daily test sessions.

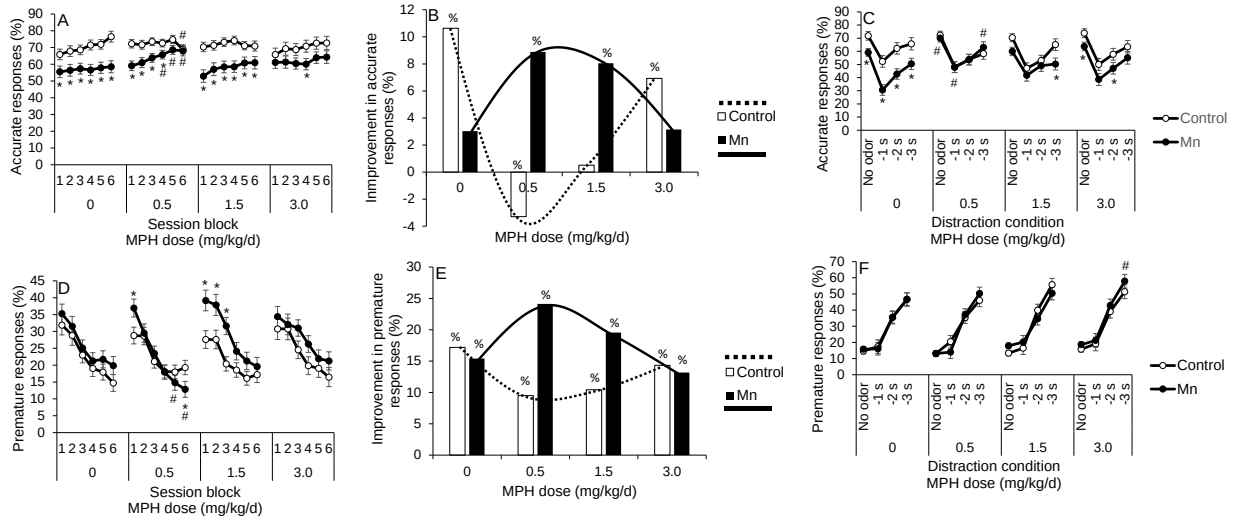

Figure 2A-F. Panels A-C. Prolonged methylphenidate treatment to 0.5 mg/kg/d alleviates Mn attention deficits in the third focused attention task and the selective attention tasks with distractors. (A) Accurate responses (%) for the control and the Mn groups, as a function of test session block and MPH dose in the third focused attention task. Each test session block comprised three daily test sessions. (B) Dose-response curves for improvement in attentional accuracy[1] is an inverted U-shape for the Mn group, unlike the U-shape for the controls. (C) Accurate responses (%) for the control and the Mn groups, as a function of distractor and increasing MPH doses in the selective attention task. **Panels D-F**. Chronic low dose methylphenidate treatment decreases impulsivity in the Mn-exposed rats in the baseline task but heightens impulsivity at the highest dose in the selective attention task with distractors. (D) Premature responses (%) for the control and the Mn groups, as a function of test session block and increasing MPH doses in the baseline task. Each test session block was 3 daily test session long. (E) Dose-response curves for improvement in inhibitory control[1] is an inverted U-shape for the Mn group, unlike the U-shape for the controls. (F) Premature responses (%) for the control and the Mn groups, as a function of distractor and increasing MPH doses in the selective attention task. Data are lsmeans ± SEM of the Mn and control group (n = 14–15/group). *$p \leq 0.05$ versus controls and $^{\#}p \leq 0.05$ versus 0 mg MPH/kg/d dose.

[1]Improvement in percent accurate and premature responses in B and E was calculated for each Mn-exposed and MPH-treated groups by subtracting the group mean responses in session block 6 from the group mean responses in session block 1 (i.e., delta). Improvement scores for premature responses are expressed positively. Higher scores reflect greater improvement in performance. $^{\%}$ in panels B and E denotes percent accurate responses in session block 6 was different than block 1 at $p \leq 0.05$.

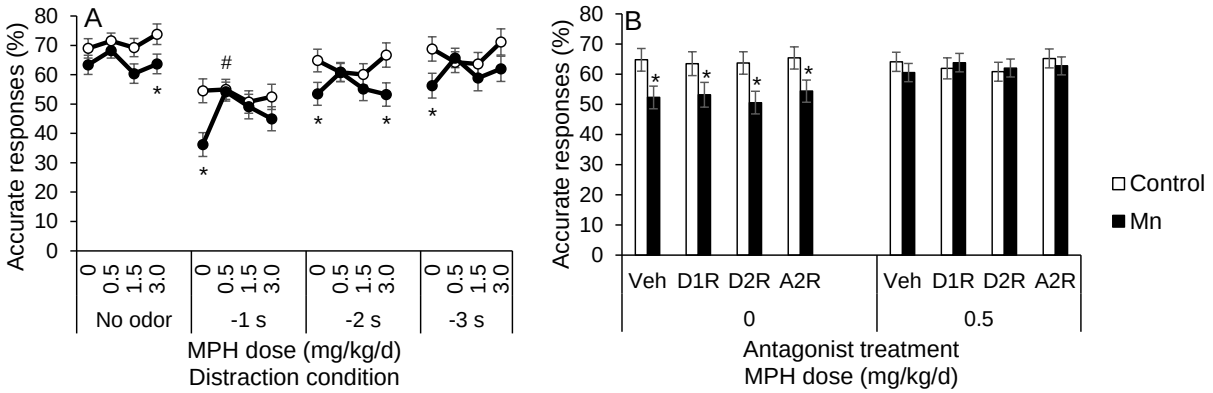

Figure 3A, B. The attention deficit induced by Mn and MPH efficacy to alleviate dysfunction at the lowest 0.5 mg/kg/d dose are sustained into the antagonist treatment phase in the selective attention task but are not altered by specific D1R, D2R, or α2$_A$R antagonist treatments. (A) Accurate responses (%) for the control and the Mn groups, as a function of MPH dose and distraction condition. (B) Accurate responses (%) for the control and the Mn groups, as a function of antagonist treatment and MPH dose. Data are lsmeans ± SEM of the Mn and control group (*n* = 14–15/group). *p ≤ 0.05 versus controls and $^{\#}$p ≤ 0.05 versus the 0 mg/kg/d MPH dose. Note: Data in A are lsmeans ± SEM of the Mn and control group under the vehicle antagonist treatment.

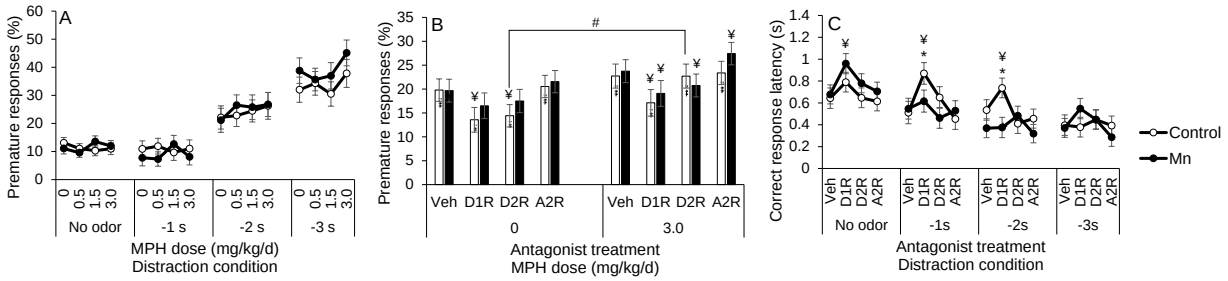

Figure 4A-C. D1R or D2R antagonist treatment decreases premature responses and increases latency of correct responses depending on MPH dose and Mn exposure level. (A) Premature responses (%) for the control and the Mn groups, as a function of MPH dose and distraction condition. (B) Premature responses (%) for the control and the Mn groups, as a function of antagonist receptor treatment and MPH dose. (C) Latencies of correct responses in second for the control and the Mn groups, as a function of antagonist treatment and distraction condition. Data are lsmeans ± SEM of the Mn and control group (*n* = 14–15/group). *p ≤ 0.05 versus controls, $^{\#}$p ≤ 0.05 versus 0 mg/kg/d MPH dose, and $^{¥}$p ≤ 0.05 versus vehicle antagonist treatment within MPH condition. Notes: Data in A are lsmeans ± SEM of the Mn and control group under the vehicle antagonist treatment and data in C are lsmeans ± SEM of the Mn and control group under the 3.0 mg/kg/d MPH dose.

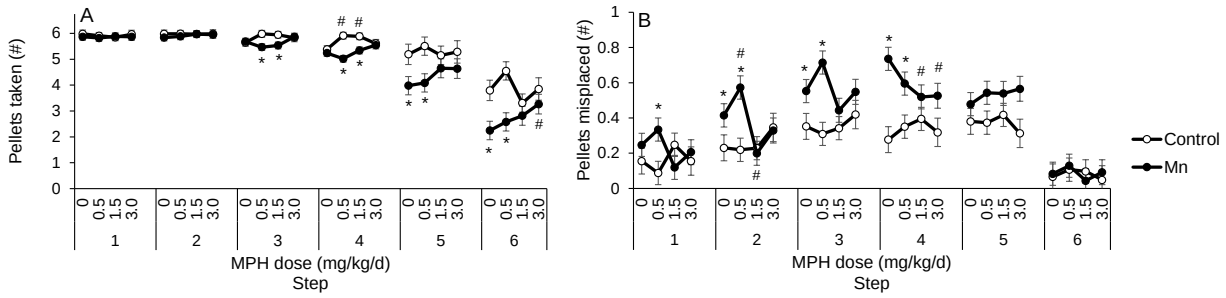

Figure 5A-B. Developmental postnatal manganese exposure causes lasting sensorimotor deficits in the staircase test and prolonged methylphenidate treatment at the highest 3.0 mg/kg/d dose alleviates those deficits. (A) Pellets taken (#) and pellets misplaced (#) (B) for the control and the Mn groups, as a function of MPH dose and staircase step. Data are lsmeans ± SEM of the Mn and control group (*n* = 14–15/group). *p ≤ 0.05 versus controls and #p ≤ 0.05 versus 0 mg/kg/d MPH dose.

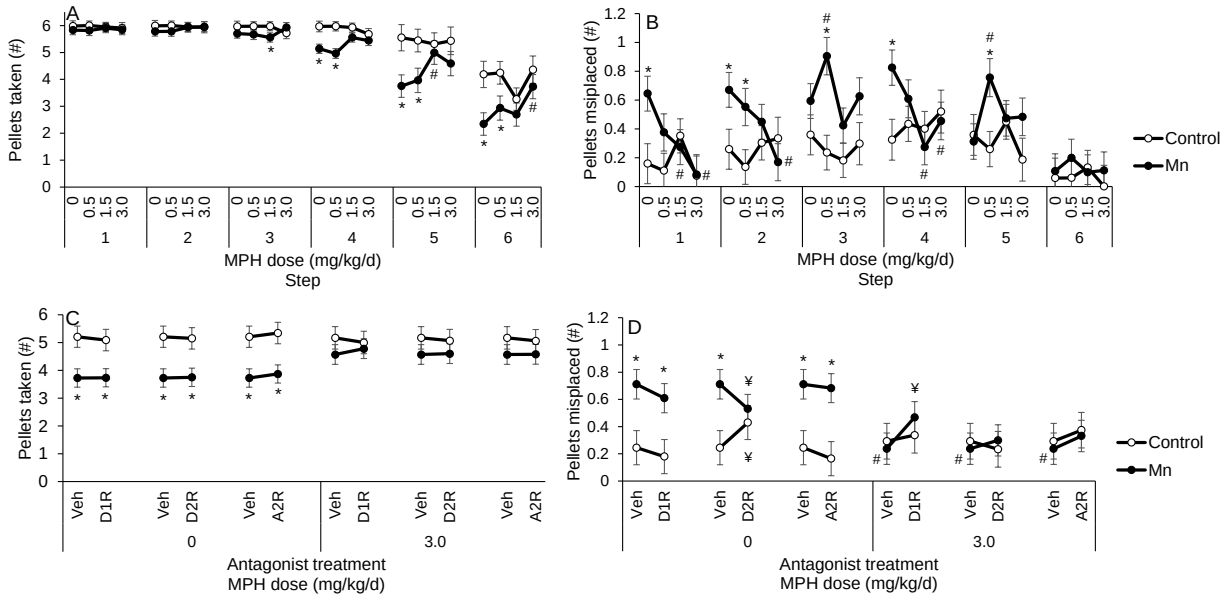

Figure 6A-D. The sensorimotor deficits induced by Mn and MPH efficacy to alleviate those deficits at the highest 3.0 mg/kg/d dose are sustained into the antagonist treatment phase in the staircase test (A and B), with D2R antagonism improving Mn-induced grasping deficit in the absence of MPH (D) and D1R antagonism attenuating MPH efficacy to alleviate Mn grasping deficit at the 3.0 mg/kg/d dose (D). (A) Pellets taken (#) and (B) pellets misplaced (#) for the control and the Mn groups, as a function of MPH dose and staircase step. (C) Pellets taken (#) and pellets misplaced (D) for the control and the Mn groups, as a function of antagonist treatment and MPH dose. Data are lsmeans ± SEM of the Mn and control group (n = 14–15/group). *p ≤ 0.05 versus controls, #p ≤ 0.05 versus 0 mg/kg/d MPH dose, and ¥p ≤ 0.05 versus vehicle antagonist treatment. Notes: Data in A and B are lsmeans ± SEM of the control and Mn-exposed groups under the vehicle antagonist treatment condition. The statistical model for C and D included, respectively, staircase step data 4-6 or 1-4 where there was an apparent effect of Mn exposure or MPH treatment.