PERIADOLESCENT ORAL MANGANESE EXPOSURE AFFECTS CONDITIONED PLACE PREFERENCE BY COCAINE AND CONDITIONED PLACE AVERSION BY LITHIUM CHLORIDE IN RATS

A Senior Honors Thesis

By

SAMUEL MING HIN LEE

Submitted to the Office of Honors Program
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2004

Majors: Psychology and Biochemistry

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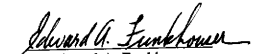
Approved as to style and content by:

Jack R. Nation
(Fellows Advisor)



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ABSTRACT

Periadolescent Oral Manganese Exposure Affects Conditioned Place Preference by Cocaine and Conditioned Place Aversion by Lithium Chloride in Rats. (April 2004)

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Manganese neurotoxicity compromises basal ganglia functions that could affect the limbic system and drug sensitivity. Male rats were orally exposed to manganese chloride (0, 100, 200 mg/kg/day Mn) for 15 days starting at postnatal day (PND) 28. In Experiment 1, conditioned place preference (CPP) was conducted in a two-compartment apparatus in which cocaine was paired with the least-preferred compartment as determined by a pretest. Animals received 0, 2.5, or 5 mg/kg cocaine HCl (i.p.) for 4 days and, alternatively, vehicle-only for 4 days. Animals exposed to 0 mg/kg/day Mn showed an increased place preference for 2.5 mg/kg cocaine and a reduced place preference for 5 mg/kg cocaine. In contrast, animals exposed to 100 mg/kg/day Mn showed an increased place preference for both 2.5 and 5 mg/kg cocaine, and animals exposed to 200 mg/kg/day Mn showed an increased place preference for 5 mg/kg cocaine only. To determine the possible effects of alterations of learning mechanisms by manganese, a conditioned place aversion (CPA) procedure was employed for Experiment 2. Animals received 40 mg/kg lithium chloride (i.p.) for 4 days, and

alternatively, vehicle-only for 4 days. Animals exposed to 100 mg/kg/day Mn and 200 mg/kg/day Mn showed an increased place aversion for 40 mg/kg LiCl when compared to animals exposed to 0 mg/kg/day Mn. However, the increase was not statistically significant. These findings are discussed within a framework of possible manganese-induced disturbance of neurochemical function relating to drug reward and learning.

ACKNOWLEDGMENTS

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INTRODUCTION

Manganese (Mn) overexposure may result in a condition referred to as manganism. Three progressive clinical stages have been described, with the most advanced stage clinically similar to the fully-developed stage in Parkinson's disease (PD), characterized by muscular rigidity (cf. Mergler et al., 1994). Although there are features that can differentiate diagnosis between manganism and PD, such as clinical symptoms, response to levodopa, and neuroimaging studies (Kim et al., 2002; Olanow, 1998), both manganism and PD produce increases in dopamine turnover during early stages (Erikson and Aschner, 2003; Sossi et al., 2002), and depletion of dopamine during late stages. These findings suggested possible parallelism in how the limbic system functions between the two diseases.

In addition to manganism's relevance to PD, a body of evidence indicates that environmental pollution can cause Mn overexposure and irreversible nervous system damage. A recent report by the Agency for Toxic Substances and Disease Registry (ATSDR) identified the main sources for Mn exposure to be the following: factories or hazardous waste sites that release significant amounts of Mn dust into the air, Mn

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This thesis follows the style and format of *Neurotoxicology*.

released into air by combustion of unleaded gasoline that contains methylcyclopentadienyl manganese tricarbonyl (MMT) as an antiknock ingredient, ingestion of contaminated drinking water, and the usage of pesticides with added Mn ingredients (ATSDR, 2000). 7,550 cases of manganism have been recorded in the literature since the first report in 1937, according to the Environmental Protection Agency (EPA) (ATSDR, 2000). Since the first diagnoses of manganism more than a century ago (as early as 1837) (Lee, 2000), a wide range of clinical symptoms have been described (ATSDR, 2000).

A current focus of Mn neurotoxicity research is the elevated levels of glutamate in the central nervous system (CNS). Mn accumulates primarily in the striatum and substantia nigra (Kobayashi et al., 2003; Liu et al., 2000) within a type of cell known as astrocytes (Chen and Liao, 2002). Mn overexposure significantly decreases antioxidant enzyme activities and interferes with glutamate uptake in astrocytes, which can cause neural cells in the striatum and substantia nigra to be more prone to excitotoxicity and oxidative stress (Chen and Liao, 2002). The dorsal striatum and the ventral striatum (nucleus accumbens) have been regarded traditionally as an interface between limbic and motor systems (Berlanga et al., 2003). These areas of the brain receive extensive dopaminergic innervation from the ventral tegmental area and the substantia nigra, which subserve primarily limbic and motor function, respectively (Berlanga et al., 2003). Since Mn neurotoxicity in the striatum and substantia nigra is linked to the depletion of dopamine and motor deficits, a substantial literature regarding Mn-induced changes in locomotive behavior and neurotransmitter levels is available. Meanwhile, treatment

with MK-801, a non-competitive NMDA (glutamate receptor subtype) antagonist, has been shown to block the general excitotoxic lesions created by Mn exposure (Brouillet et al., 1993). It was therefore suggested that Mn exerts its neurotoxic effects in the striatum and substantia nigra by an excitotoxic process which could be mediated by NMDA receptors (Brouillet et al., 1993). Since NMDA receptors have been linked to learning and memory, the potential for Mn to affect learning and memory mechanisms has also been examined in previous investigations.

In contrast, Mn-induced disturbances in dopaminergic systems strongly suggests potential changes in the functionality of the limbic system, but none of the previous investigations addressed the issue of Mn's effect on the limbic system, particularly on drug reward. This would be the first project to address the effect of Mn on the reward properties of cocaine.

Drug-related stimuli have been demonstrated to be important in control of the behavioral and subjective properties of drugs in animals and humans (Poulos et al., 1981). Conditioned place preference (CPP) is an appropriate procedure to study the stimulus factors that underlie drug use (Miller et al., 1999). In this preparation, drug is administered to the animal immediately before placement in an environment with unique contextual stimuli (e.g., visual, tactile). Following several pairings of the drug and the unique context, and separate pairings of a distinctively different context and the absence of the drug (vehicle), the animal is tested for preference by being allowed free access to both the drug-paired and the vehicle-paired contexts. CPP is then defined by some

measure of preference for one context over another. Cocaine has been shown to produce a robust CPP in non-exposed animals (Kuzmin et al., 1997).

The CPP procedure had been demonstrated to be sensitive not just to the stimulus effects of drug use, but also neural mechanisms of cocaine reward (Miller et al., 1999). The D_1 dopamine receptor antagonist SCH 23390 and the noncompetitive NMDA antagonist MK-801, each administered with cocaine during conditioning, has been shown to block the acquisition of cocaine CPP (cf. Miller et al., 1999). For the present investigation, Experiment 1 examined the alteration to cocaine reward by periadolescent Mn exposure using CPP.

It must also be considered that attenuation in cocaine CPP in Mn-exposed animals could derive from challenges to associative or cognitive processing, rather than neural mechanisms of drug reward. CPP is a learned phenomenon in which the contextual cues of the environment acquire secondary reinforcing properties via Pavlovian conditioning. Decades of research have indicated that the basal ganglia especially the striatum, are involved in learning and memory (Packard and Knowlton, 2002). Developmental Mn exposure is expected to cause a disturbance of NMDA receptors, glutamate and dopamine levels, which may affect the animals' associative (learning) processes. To address issues related to the actions of Mn on mechanisms of conditioning in Experiment 1, Experiment 2 employed a conditioned place aversion (CPA) procedure in which an aversive stimulus (lithium chloride, LiCl) was paired with one distinct context and vehicle only was paired with another.

Developmental exposure was chosen over adult exposure because the developing organism is more likely to accumulate higher brain Mn levels and therein more likely to experience altered brain dopamine concentrations after exposure when compared to adult rats (cf. Dorman et al., 2000). In addition, D_1 and D_2 dopamine receptor density in both striatum and nucleus accumbens are also increased significantly during adolescence (Gelbard et al., 1989). Finally, developmentally-exposed rats manifest more pronounced brain pathology than do adults, even in the face of equivalent or lesser Mn exposures (cf. Dorman et al., 2000). Accordingly, Experiment 1 and Experiment 2 examined the effects of Mn exposure had on cocaine reward and learning, respectively, during a vulnerable period (adolescence) in rats.

METHODS

Apparatus

Place conditioning and testing were conducted in seven 20×60×20 cm wooden shuttle boxes with wooden tilt floors. At the end of each box was a microswitch interfaced to an IBM compatible computer. A BASIC computer program was written to continuously record the number of times and duration the switch was activated via a tilt of the floor.

One compartment of the apparatus had smooth white walls and floor, and the other compartment had black walls with a black sandpaper floor. For conditioning sessions, the boxes were divided into two equal-sized compartments by removable partitions. On test sessions (pretest and posttest), the partitions were removed and a 20×10×5 cm wooden platform was installed 2 cm above the floor to divide the two compartments but allow free access by rats. In earlier investigations, subjects showed a strong preference for the black compartment (Miller et al., 1999; Miller et al., 2000; Miller and Nation, 1997; Smith and Nation, 2003). To counteract this preference, a 40 W light was positioned 50 cm above the black compartment of each apparatus. These seven lamps provided the only illumination in the room. Following each conditioning and test session the apparatus was cleaned with a mild soap solution. The apparatus was located in a sound-resistant room with a 40 dB white noise generator operating continuously. All conditioning and testing sessions were conducted during the light phase of the cycle.

Drugs

The Research Technology Branch of NIDA provided cocaine HCl gratis. LiCl and $MnCl_2 \times 4H_2O$ were purchased from Sigma Co. (St. Louis, MO). Cocaine HCl and LiCl were dissolved in a 0.9% w/v saline vehicle, and the doses were expressed as the salt. $MnCl_2 \times 4H_2O$ was dissolved in distilled water, and the doses were expressed as the amount of Mn^{2+} cations present in the solution.

Experiment 1: Cocaine CPP

Animals

The animal housing and testing facility was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International), and all animal maintenance and research was conducted in accordance with guidelines provided by the University Laboratory Animal Care Committee (ULACC). The health of the animals was monitored by the campus veterinarian throughout the duration of the project.

Male Sprague-Dawley rats (n = 68) were obtained from a commercial source (Harlan, Houston, TX) during PND 25. Animals were provided rat chow and purified water ad libitum, and were caged in individual plastic cages throughout the study. A 12h/12h light-dark cycle was used throughout the study.

Procedures

For the Mn pretreatment, animals were gavaged with one of the three doses of Mn (0, 100, and 200 mg/kg body weight/day Mn) in volumes of 1 ml beginning on

postnatal day (PND) 28 for 15 consecutive days. Mn was administered in the form of manganese chloride tetrahydrate ($MnCl_2 \times 4H_2O$) dissolved in distilled water. Body weights were recorded daily throughout the experiment. CPP experiment was started the day immediately after the 15th day of Mn exposure. On day 1 of the CPP experiment, animals were transferred from the colony room to the testing room for 20 minutes in an effort to habituate the animals to transportation and the sound and illumination of the room. Animals were not placed in the apparatus during the 20 minute period. On day 2, animals were placed into the CPP apparatus and allowed free access to either compartment for 15 minutes. Pretest data were measured by the amount of time animals spent in each compartment, and these data were used to determine animals' pretest preference for the white or the black compartment. On four alternate days (days 3, 5, 7, and 9), animals exposed to various levels of Mn (0, 100, 200 mg/kg/day) in pretreatment received a daily i.p. injection of one of the three doses of cocaine (0, 2.5, or 5 mg/kg body weight). The resulting interaction created 9 groups; 0 Mn-0 mg/kg coc (n = 14), 0 Mn-2.5 mg/kg coc (n = 7), 0 Mn-5 mg/kg coc (n = 6), 100 Mn-0 mg/kg coc (n = 7), 100 Mn-2.5 mg/kg coc (n = 6), 100 Mn-5 mg/kg coc (n = 7), 200 Mn-0 mg/kg coc (n = 7), 200 Mn-2.5 mg/kg coc (n = 7), and 200 Mn-5 mg/kg coc (n = 7). Animals were confined to the least-preferred compartment (defined as the compartment in which the animal spent less amount of time on the day 2 pretest) for 20 minutes immediately after injection. On the other four alternate days (days 4, 6, 8, and 10), all animals received a daily i.p. vehicle (saline) injection, and were confined to the most-preferred compartment (defined as the compartment in which the animal spent more amount of

time on the day 2 pretest) for 20 minutes immediately after injection. All injections were given at a volume of 1 ml/kg. Animals were run in squads of seven, counterbalanced by group assignments. On day 11, posttest data were obtained using the same procedure as the day 2 pretest. Animals were rendered unconscious with 60 mg/kg sodium pentobarbital (i.p.) 21 days after posttest, decapitated, and their brains dissected and frozen for subsequent analysis.

Experiment 2: LiCl CPA

Animals

Male Sprague-Dawley rats (n = 23) were obtained from a commercial source (Harlan, Houston, TX) during PND 25. Animals were provided rat chow and purified water ad libitum, and they were caged in individual plastic cages throughout the study. A 12h/12h light-dark cycle was used throughout the study.

Procedures

The procedure was precisely as described for Experiment 1, with the following exceptions. On four alternate days (days 3, 5, 7, and 9), animals exposed to various levels of Mn (0 mg/kg body weight/day, n = 8; 100 mg/kg body weight/day, n = 7; 200 mg/kg body weight/day, n = 8) in pretreatment received a daily i.p. injection of LiCl (40 mg/kg body weight) instead of cocaine, and were confined to the most-preferred compartment instead of the least-preferred compartment. On the other four alternate days (days 4, 6, 8, and 10), animals received a daily i.p. vehicle (saline) injection, and were confined to the least-preferred compartment instead of the most-preferred

compartment. Animals were rendered unconscious with 60 mg/kg body weight sodium pentobarbital (i.p.) immediately after posttest, decapitated, and their brains dissected and frozen for subsequent analysis. All other aspects of the study were as described on Experiment 1.

Concentrations of Mn in Brain

A random sample of rat brains from Experiment 1 (n = 35, 21 days after completing cocaine CPP testing) and Experiment 2 (n = 16, immediately after completing LiCl testing) were dissected into 3 parts: the striatum, the posterior basal ganglia (which contained the substantia nigra), and the rest of the brain. Each dissected sample was digested with nitric acid and with hydrogen peroxide and then analyzed using mass spectrometry (Hewlett-Packard model 4500 ICP/MS). To assure accuracy in measurements of Mn levels, spiked samples were spaced intermittently (about 1 per 20 experimental samples) and reference material (DORM-2, 1577b, and 8414) were also digested to ensure adequate recovery. Additional details of the analytical procedure have been described previously (cf. Dearth et al., 2003).

Statistical Analysis

The pretest data from day 2 and the posttest data from day 11 were examined. The conditioning scores were defined by the number of minutes spent on the drug-conditioned compartment on posttest trial minus the number of minutes spent on the same compartment in the pretest trial in both experiments.

RESULTS

Growth and Development

Animals assigned to the three Mn exposure groups (0 mg/kg/day Mn, n = 42; 100 mg/kg/day Mn, n = 26; and 200 mg/kg/day Mn, n = 29) were counterbalanced by weight before Mn treatment in both Experiments 1 and 2. Weight differences on the first day of exposure (PND 28) were not significant as tested by one-way ANOVA ($F(2, 94) = 1.34$, $p = 0.27$). Weight differences on the last day of Mn exposure (PND 42) were significant across all three groups ($F(2, 94) = 49.36$, $p < 0.01$; 0 mg/kg/day Mn mean = 191.7, SEM = ±2.3; 100 mg/kg/day Mn mean = 169.1, SEM = ±2.2; 200 mg/kg/day Mn mean = 160.3, SEM = ±2.6), with both 100 mg/kg/day Mn ($t(66) = 5.21$, $p < 0.01$) and 200 mg/kg/day Mn ($t(69) = 6.2046$, $p < 0.01$) associated with lower body weight when compared to 0 mg/kg/day Mn. Weight differences 10 days after Mn exposure has ended remained significant across all three groups ($F(2, 94) = 24.34$, $p < 0.01$; 0 mg/kg/day Mn mean = 249.3, SEM = ±2.6; 100 mg/kg/day Mn mean = 228.3, SEM = ±2.9; 200 mg/kg/day Mn mean = 223.9, SEM = ±3.2), with both 100 mg/kg/day Mn ($t(66) = 5.21$, $p < 0.01$) and 200 mg/kg/day Mn ($t(69) = 6.20$, $p < 0.01$) associated with lower body weight when compared to 0 mg/kg/day Mn (See Fig. 1).

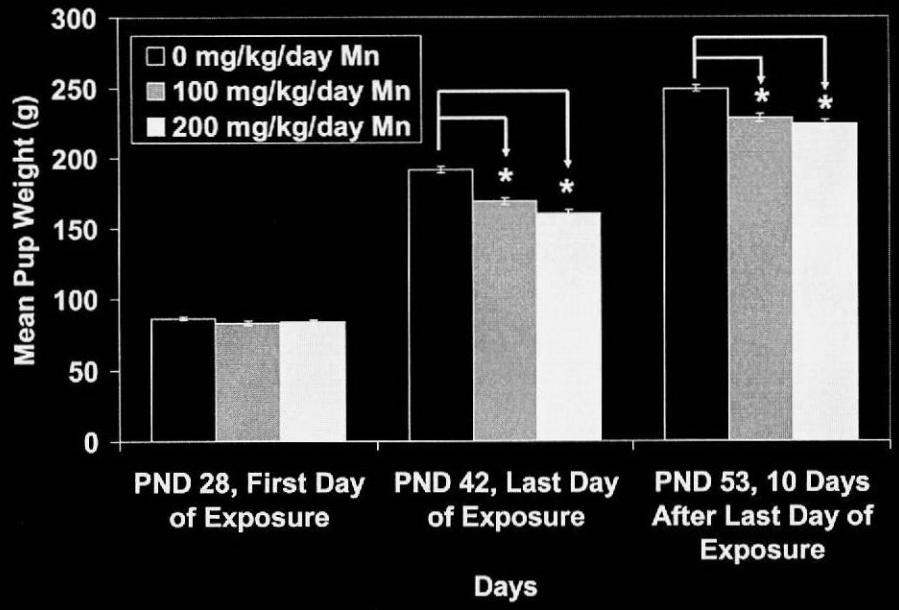

Fig. 1 Mean (±SEM) body weights (measured in grams) for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals during the first day of exposure (PND 28), the last day of exposure (PND 42), and 10 days after exposure (PND 53). Asterisks (*) indicate that the mean weights for exposed animals (100 mg/kg/day Mn and 200 mg/kg/day Mn) were significantly different from non-exposed animals ($P < 0.05$).

Experiment 1: Cocaine CPP

The two-way ANOVA conducted on cocaine CPP conditioning scores (see Fig. 2) revealed significant main effect of Mn dose ($F(2, 60) = 9.98$, $p < 0.01$; 0 mg/kg/day Mn mean = 5.25, SEM = ±0.57; 100 mg/kg/day Mn mean = 2.29, SEM = ±0.44; 200 mg/kg/day Mn mean = 2.83, SEM = ±0.77), and also a significant main effect of cocaine dose ($F(2, 60) = 5.31$, $p < 0.01$; 0 mg cocaine/kg mean = 2.95, SEM = ±0.56; 2.5 mg cocaine/kg mean = 4.26, SEM = ±0.84; 5 mg cocaine/kg mean = 3.95, SEM = ±0.63). There was a significant Mn dose × cocaine dose interaction ($F(4, 60) = 10.91$, $p < 0.01$).

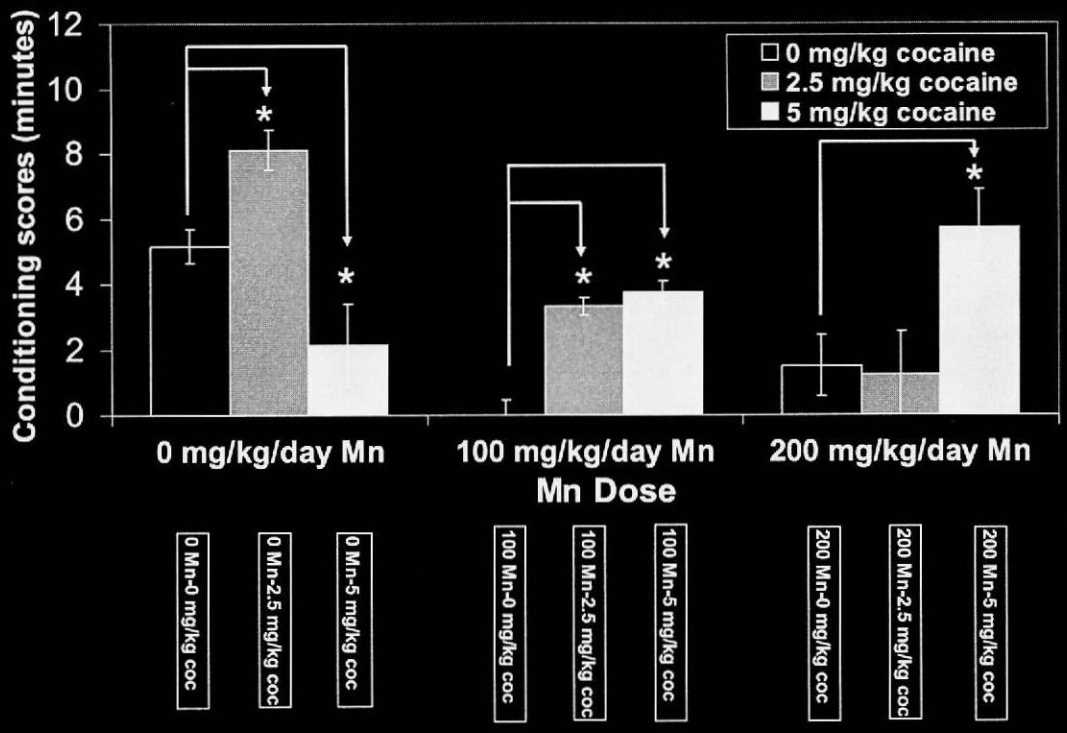

Fig. 2 Mean (±SEM) conditioning scores (measured in minutes) for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals CPP produced by 0 mg/kg, 2.5 mg/kg, and 5 mg/kg cocaine. Asterisks (*) indicate that the CPP conditioning scores produced by 2.5 mg/kg and 5 mg/kg cocaine were significantly different from CPP conditioning scores produced by 0 mg/kg cocaine (vehicle) within their respective Mn dose. ($p < 0.05$)

Post hoc tests were performed on group means. 0 mg/kg/day non-exposed Mn animals showed a significant simple effect in cocaine CPP conditioning scores across three doses of cocaine ($F(2, 24) = 12.00$, $p < 0.01$). 100 mg/kg/day Mn-exposed animals also showed a significant simple effect in cocaine CPP conditioning scores across three doses of cocaine ($F(2, 17) = 30.04$, $p < 0.01$). And, 200 mg/kg/day Mn-exposed animals showed a significant simple effect in cocaine CPP conditioning scores across three doses of cocaine ($F(2,18) = 4.95$, $p = 0.02$).

0 mg/kg cocaine animals showed a significant simple effect in cocaine CPP conditioning scores across three doses of Mn ($F(2, 25) = 19.41$, $p < 0.01$). 2.5 mg/kg cocaine animals showed a significant simple effect in cocaine CPP conditioning scores across three doses of Mn ($F(2, 17) = 15.86$, $p < 0.01$). 5 mg/kg cocaine animals, demonstrated a marginally significant simple effect in cocaine CPP conditioning scores across three doses of Mn ($F(2, 17) = 3.32$, $p = 0.06$).

Independent sample t-tests were performed on cocaine CPP conditioning scores between groups (Tables 1 and 2). Comparisons between 100 Mn-2.5 mg/kg coc and 200 Mn-2.5 mg/kg did not show significant difference ($t(11) = 1.44$, $p = 0.18$). However, both Kruskal-Wallis ($H = 4.59$, $p < 0.05$) and Mann Whitney ($U = 6$, $p < 0.05$) tests showed significant differences between these two groups, with 100 Mn-2.5 mg/kg coc's cocaine CPP conditioning score significantly lower than that of the 200 Mn-2.5 mg/kg.

Table 1 Independent sample t-test on mean (±SEM) conditioning scores (measured in minutes) produced by 0 mg/kg, 2.5 mg/kg, and 5 mg/kg cocaine within their respective Mn dose. Asterisks (*) indicate that mean conditioning scores between two groups were significantly different ($p < 0.05$).

Mn Dose	Compared Cocaine Doses (and mean CPP scores ± SEM, minutes)	t Value	Probability
0 mg/kg/day	0 Mn-0 mg/kg coc (5.15 ± 0.51) < 0 Mn-2.5 mg/kg coc (8.11 ± 0.612)	$t(19) = -3.40$	$p < 0.01$*
	0 Mn-0 mg/kg coc (5.15 ± 0.51) > 0 Mn-5 mg/kg coc (2.14 ± 1.27)	$t(18) = 2.67$	$p < 0.05$*
	0 Mn-2.5 mg/kg coc (8.11 ± 0.61) > 0 Mn-5 mg/kg coc (2.14 ± 1.27)	$t(11) = 4.33$	$p < 0.01$*
100 mg/kg/day	100 Mn-0 mg/kg coc (-0.02 ± 0.47) < 100 Mn-2.5 mg/kg coc (3.31 ± 0.26)	$t(11) = -5.90$	$p < 0.01$*
	100 Mn-0 mg/kg coc (-0.02 ± 0.47) > 100 Mn-5 mg/kg coc (3.72 ± 0.35)	$t(12) = -6.41$	$p < 0.01$*
	100 Mn-2.5 mg/kg coc (3.31 ± 0.26) < 100 Mn-5 mg/kg coc (3.72 ± 0.35)	$t(11) = -0.93$	$p = 0.37$
200 mg/kg/day	200 Mn-0 mg/kg coc (1.50 ± 0.92) > 200 Mn-2.5 mg/kg coc (1.24 ± 1.31)	$t(12) = 0.17$	$p = 0.27$
	200 Mn-0 mg/kg coc (1.50 ± 0.92) > 200 Mn-5 mg/kg coc (5.73 ± 1.15)	$t(12) = -2.88$	$p < 0.05$*
	200 Mn-2.5 mg/kg coc (1.24 ± 1.31) < 200 Mn-5 mg/kg coc (5.73 ± 1.15)	$t(12) = -4.50$	$p < 0.05$*

Table 2 Independent sample t-test on mean (±SEM) conditioning scores (measured in minutes) between 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals within their respective cocaine dose. Asterisks (*) indicate that the CPP conditioning scores between the two groups were significantly different ($p < 0.05$). Double asterisks (**) indicate that although mean conditioning scores between two groups were not significantly different using the independent sample t-test ($p \geq 0.05$), they were tested to be significantly different using the Kruskal-Wallis and the Mann-Whitney tests ($p < 0.05$).

Cocaine Dose	Compared Mn Doses (and mean CPP scores ± SEM, minutes)	t Value	Probability
0 mg/kg	0 Mn-0 mg/kg coc (5.15 ± 0.51) > 100 Mn-0 mg/kg coc (-0.02 ± 0.47)	t(19) = 6.42	p < 0.01*
	0 Mn-0 mg/kg coc (5.15 ± 0.51) > 200 Mn-0 mg/kg coc (1.50 ± 0.92)	t(19) = 3.76	p < 0.01*
	100 Mn-0 mg/kg coc (-0.02 ± 0.47) < 200 Mn-0 mg/kg coc (1.50 ± 0.92)	t(12) = -1.48	p = 0.17
2.5 mg/kg	0 Mn-2.5 mg/kg coc (8.11 ± 0.61) > 100 Mn-2.5 mg/kg coc (3.31 ± 0.26)	t(11) = 6.20	p < 0.01*
	0 Mn-2.5 mg/kg coc (8.11 ± 0.61) > 200 Mn-2.5 mg/kg coc (1.24 ± 1.31)	t(12) = 4.68	p < 0.01*
	100 Mn-2.5 mg/kg coc (3.31 ± 0.26) > 200 Mn-2.5 mg/kg coc (1.24 ± 1.31)	t(11) = 1.44	p = 0.18 **
5 mg/kg	0 Mn-5 mg/kg coc (2.14 ± 1.27) < 100 Mn-5 mg/kg coc (3.72 ± 0.35)	t(11) = -1.29	p = 0.22
	0 Mn-5 mg/kg coc (2.14 ± 1.27) < 200 Mn-5 mg/kg coc (5.73 ± 1.15)	t(19) = -2.11	p = 0.06
	100 Mn-5 mg/kg coc (3.72 ± 0.35) < 200 Mn-5 mg/kg coc (5.73 ± 1.15)	t(12) = -1.68	p = 0.12

Experiment 2: LiCl CPA

One-way ANOVA conducted on the CPA conditioning scores produced by 40 mg/kg LiCl (see Fig. 3) did not reveal significant difference in the means of the groups ($F(2, 20) = 1.13$, $p = 0.34$).

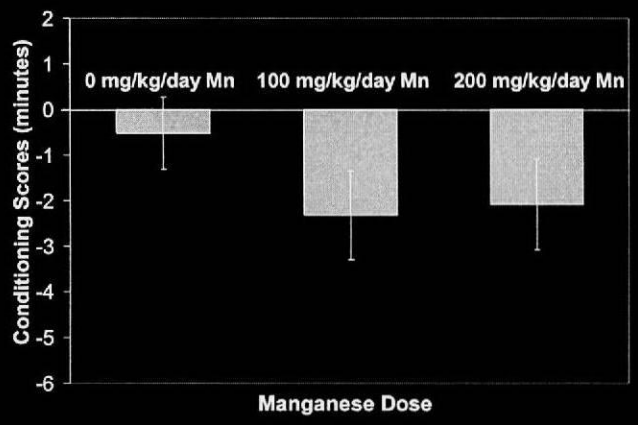

Fig. 3 Mean (±SEM) conditioning scores (measured in minutes) for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals CPA produced by 40 mg/kg LiCl.

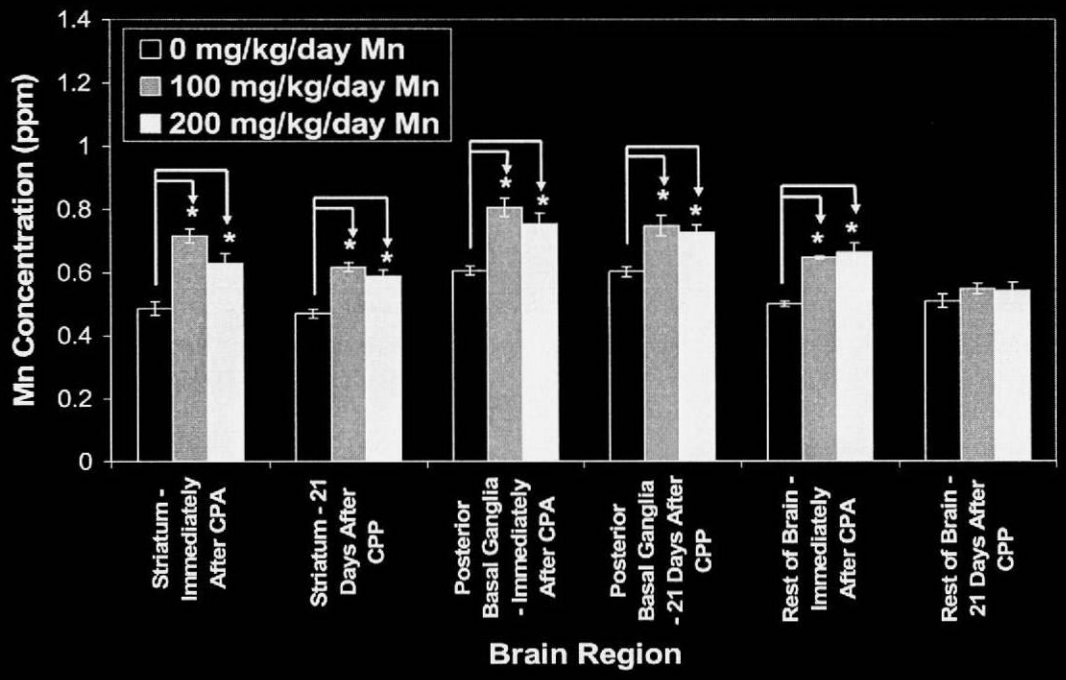

Fig. 4 Mean (±SEM) concentration of Mn detected by mass spectrometry in various brain regions (measured in ppm) for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals from Experiment 1 (21 days after completion of CPP testing) and Experiment 2 (immediately after completion of CPA testing). Asterisks (*) indicate that mean concentration of Mn for exposed animals were significantly different from non-exposed animals. ($p < 0.05$)

Concentrations of Mn in Brain

The one-way ANOVA conducted on the concentrations of Mn in brain regions isolated from Experiment 1 21 days after CPP testing (see Fig. 4) revealed significant differences between animals exposed to different doses of Mn (0, 100, 200 mg/kg/day Mn) in the striatum ($F(2, 26) = 32.94$, $p < 0.01$) and in the posterior basal ganglia ($F(2, 26) = 10.52$, $p < 0.01$). However, significant differences in Mn concentration in the rest of the brain was not found 21 days after CPP experiments ($F(2, 26) = 1.05$, $p = 0.36$). Additional independent sample t-tests were performed between groups (see Table 3). 100 mg/kg/day and 200 mg/kg/day Mn-exposed animals, when compared to 0 mg/kg/day Mn non-exposed animals, showed significantly greater Mn concentration in the striatum and the posterior basal ganglia 21 days after CPP testing.

Table 3 Independent sample t-test on mean (±SEM) concentration of Mn (measured in ppm) as measured by mass spectrometry in various brain regions for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals from Experiment 1 21 days after CPP testing. Asterisks (*) indicate that the mean concentration of Mn between the groups were significantly different ($p < 0.05$).

Brain Region	Compared Mn Doses (Mean Mn concentrations ± SEM, ppm)	t Value	Probability
Striatum	0 mg/kg (0.47 ± 0.01) < 100 mg/kg (0.62 ± 0.02)	t(21) = -7.23	p < 0.01*
	0 mg/kg (0.47 ± 0.01) < 200 mg/kg (0.59 ± 0.02)	t(21) = -4.72	p < 0.01*
	100 mg/kg (0.62 ± 0.02) > 200 mg/kg (0.59 ± 0.02)	t(22) = 1.15	p = 0.26
Posterior Basal Ganglia	0 mg/kg (0.60 ± 0.02) < 100 mg/kg (0.75 ± 0.03)	t(21) = -3.95	p < 0.01*
	0 mg/kg (0.60 ± 0.02) < 200 mg/kg (0.73 ± 0.02)	t(21) = -4.37	p < 0.01*
	100 mg/kg (0.75 ± 0.03) > 200 mg/kg (0.73 ± 0.02)	t(22) = 0.53	p = 0.60
The Rest of the Brain	0 mg/kg (0.51 ± 0.02) < 100 mg/kg (0.55 ± 0.02)	t(21) = -1.45	p = 0.16
	0 mg/kg (0.51 ± 0.02) < 200 mg/kg (0.54 ± 0.03)	t(21) = -0.86	p = 0.40
	100 mg/kg (0.55 ± 0.02) > 200 mg/kg (0.54 ± 0.03)	t(22) = 0.24	p = 0.81

The one-way ANOVA conducted on the concentrations of Mn in brain regions isolated from Experiment 2 immediately after CPA testing (see Fig. 4) revealed significant differences between animals exposed to different doses of Mn (0, 100, and 200 mg/kg/day Mn) in the striatum ($F(2, 13) = 22.64$, $p < 0.01$), in the posterior basal ganglia ($F(2, 13) = 24.51$, $p < 0.01$), and in the rest of the brain ($F(2, 13) = 41.48$, $p < 0.01$). Additional independent sample t-tests were performed between groups (see Table 4). 100 mg/kg/day and 200 mg/kg/day Mn-exposed animals, when compared to 0 mg/kg/day Mn non-exposed animals, showed significantly greater Mn concentration in the striatum, the posterior basal ganglia, and also the rest of the brain, immediately after CPA testing.

Table 4 Independent sample t-test on mean (±SEM) concentration of Mn (measured in ppm) as measured by mass spectrometry in various brain regions for 0 mg/kg/day Mn non-exposed animals, 100 mg/kg/day Mn-exposed animals, and 200 mg/kg/day Mn-exposed animals from Experiment 2 immediately after CPA testing. Asterisks (*) indicate that mean concentration of Mn between the two groups were significantly different ($p < 0.05$).

Brain Region	Compared Mn Doses (Mean Mn concentrations ± SEM, ppm)	t Value	Probability
Striatum	0 mg/kg (0.49 ± 0.02) < 100 mg/kg (0.72 ± 0.02)	t(10) = -6.62	p < 0.01*
	0 mg/kg (0.49 ± 0.02) < 200 mg/kg (0.63 ± 0.03)	t(10) = -3.77	p < 0.01*
	100 mg/kg (0.72 ± 0.02) > 200 mg/kg (0.63 ± 0.03)	t(6) = 2.25	p = 0.07
Posterior Basal Ganglia	0 mg/kg (0.61 ± 0.01) < 100 mg/kg (0.81 ± 0.03)	t(10) = -7.40	P < 0.01*
	0 mg/kg (0.61 ± 0.01) < 200 mg/kg (0.76 ± 0.03)	t(10) = -4.93	P < 0.01*
	100 mg/kg (0.81 ± 0.03) > 200 mg/kg (0.76 ± 0.03)	t(6) = 1.17	p = 0.29
The Rest of the Brain	0 mg/kg (0.50 ± 0.01) < 100 mg/kg (0.65 ± 0.00)	t(10) = -11.21	p < 0.01*
	0 mg/kg (0.50 ± 0.01) < 200 mg/kg (0.66 ± 0.03)	t(10) = -6.90	p < 0.01*
	100 mg/kg (0.65 ± 0.00) < 200 mg/kg (0.66 ± 0.03)	t(6) = -0.54	p = 0.61

DISCUSSION

Growth and Development

Periadolescent Mn exposure produced pronounce decrease in weight gain, which was consistent with previously observed Mn-induced decrease in weight gain in other developmental Mn exposure studies (Dorman et al., 2000; Pappas et al., 1997).

Behavioral Test Results

Periadolescent oral $MnCl_2 \times 4H_2O$ exposure is associated with significant behavioral effects. In Experiment 1, 100 Mn-2.5 mg/kg coc and 200 Mn-2.5 mg/kg coc produced a significant decrease in CPP when compared to 0 Mn-2.5 mg/kg coc. Although independent sample t-test did not reveal significant differences between CPP produced by 100 Mn-2.5 mg/kg coc and 200 Mn-2.5 mg/kg coc, both the Kruskal-Wallis and Mann-Whitney tests revealed that 200 Mn-2.5 mg/kg coc produced significantly lower CPP when compared to 100 Mn-2.5 mg/kg coc. Also, although 0 Mn-2.5 mg/kg coc and 100 Mn-2.5 mg/kg coc showed significant increases in CPP when compared to their respective 0 mg/kg cocaine counterparts (0 Mn-0 mg/kg coc and 100 Mn-0 mg/kg coc), 200 Mn-2.5 mg/kg coc did not show significant differences in CPP when compared to 200 Mn-0 mg/kg coc. These data suggest that increased amounts of Mn exposure attenuated CPP produced by 2.5 mg/kg cocaine.

In contrast, 100 Mn-5 mg/kg coc did not show significant differences in CPP when compared to 0 Mn-5 mg/kg coc. However, 200 Mn-5 mg/kg coc showed a marginal significant increase in CPP when compared to 0 Mn-5 mg/kg ($p = 0.06$). Also, 0 Mn-5 mg/kg coc showed a significant decrease in CPP when compared to 0 Mn-0

mg/kg coc, suggesting a possible aversive effect at 5 mg/kg cocaine in 0 mg/kg/day Mn non-exposed animals. In contrast, 100 Mn-5 mg/kg coc and 200 Mn-5 mg/kg coc exhibited significant increases in CPP when compared to their respective 0 mg/kg cocaine counterparts (100 Mn-0 mg/kg coc and 200 Mn-0 mg/kg coc), suggesting that increased amounts of Mn exposure enhanced CPP produced by 5 mg/kg cocaine.

An issue that must be addressed was that in Experiment 1, 5 mg/kg cocaine administration appeared to produce a possible aversive effect in 0 mg/kg/day Mn non-exposed animals, even though other studies have demonstrated cocaine CPP at much higher cocaine doses of cocaine in non-exposed rats (Campbell et al., 2000). The decrease in CPP produced by 5 mg/kg cocaine can be attributed to the biphasic changes in cocaine-induced regional cerebral blood flow reported in brain regions such as basolateral and corticomedial amygdale, olfactory tubercule, medial habenula, rostral nucleus accumbens septi, bed nucleus of stria terminalis and ventral pallidum (cf. Belzung et al., 2000), along with biphasic changes reported in local cerebral glucose utilization in the medial prefrontal cortex and the lateral habenula (cf. Belzung et al., 2000), all of which are mechanisms that can affect cocaine reward (Belzung et al., 2000). If biphasic effects of cocaine are observed on cocaine-induced cerebral activation, biphasic effects may be observed when testing for cocaine reward as well (Belzung et al., 2000). Mn exposure could potentially attenuate these biphasic effects, thus antagonizing the aversive effect evident in 0 Mn-5 mg/kg coc, allowing 5 mg/kg cocaine to produce significant increases in CPP in 100 Mn-5 mg/kg coc and 200 Mn-5 mg/kg coc.

Curiously, 0 Mn-0 mg/kg coc showed a significant increase in CPP compared to 0 Mn-100 mg/kg coc and 0 Mn-200 mg/kg coc. In other words, saline produced a significant place preference for 0 mg/kg/day Mn non-exposed animals but not for 100 mg/kg/day and 200 mg/kg/day Mn exposed animals. Pilot studies have shown that this effect could be eliminated if non-exposed rats were placed inside the CPP apparatus during day 1 of the CPP experiment, thus permitting additional time for animals to acclimate to and explore the apparatus (data not shown here). These data suggest that non-exposed animals might not have been fully habituated to the environment during the day 2 pretest. Moreover, the lack of acclimation and exploration with the apparatus possibly led to place preference in 0 mg/kg/day Mn non-exposed animals unrelated to drug conditioning. This phenomenon did not compromise our results, however, since 0 Mn-2.5 mg/kg coc clearly produced a significant increase in CPP when compared to 0 Mn-0 mg/kg coc ($p < 0.01$).

In summary, Mn exposure reduced the ability of 2.5 mg/kg cocaine to produce CPP but enhanced the ability of 5 mg/kg cocaine to produce CPP. These data suggest that the significant preference produced by the low dose of cocaine (2.5 mg/kg cocaine) in 0 mg/kg/day Mn non-exposed animals was matched by the high dose of cocaine (5 mg/kg cocaine) to produce CPP after high-level Mn exposure (200 mg/kg). Mn exposure, therefore, apparently produced a rightward-shift in the cocaine CPP dose-effect curve. With respect to interpretive issues, then, developmental Mn exposure appeared to decrease cocaine sensitivity.

In Experiment 2, 40 mg/kg LiCl did not produce statistically significant differences in CPA for 100 mg/kg/day and 200 mg/kg/day Mn-exposed animals when compared to 0 mg/kg/day Mn non-exposed animals. However, as can be seen in Fig. 2, there was an apparent trend for 40 mg/kg LiCl to produce higher CPA for 100 mg/kg/day and 200 mg/kg/day Mn-exposed animals when compared to 0 mg/kg/day Mn non-exposed animals. Although the neural mechanism responsible for the CPA effects observed here was unclear, it must be considered that the elevated levels of glutamate induced by the Mn exposure paradigm in these experiments might actually enhance associative learning, thus increasing LiCl CPA observed in Mn-exposed animals. In any event, it seems unlikely that the attenuated cocaine CPP patterns in Mn-exposed animals in Experiment 1 were due to challenges to associative learning.

Mn-induced Disturbances in Neurochemical Function

Disturbances in dopamine levels may possibly explain the apparent decrease in cocaine sensitivity. Studies have shown that Mn exposure causes depletion of dopamine in rodents (Pappas et al., 1997; Tran et al., 2002). That is, chronic Mn exposure attenuates dopamine synthesis and secretion in adult nonhuman primates (cf. Tasker et al., 2003). Decreased synthesis of dopamine could be attributed to Mn-induced reductions in homovanillic acid and 3,4-dihydroxyphenylacetic acid, two major metabolites of dopamine (cf. Normandin and Hazell, 2002). Meanwhile, Mn exposure has been demonstrated to induce autoxidation of dopamine (cf. Takeda, 2002) and to stimulate dopamine-sulfating sulfotransferases (Ranasinghe et al., 2000), thus decreasing dopamine bioavailability.

In addition, the apparent decrease in cocaine sensitivity may be attributed to Mn-induced disturbances in dopamine receptors and transporter sites. Chronic Mn exposure has been demonstrated to decrease binding of D_1 dopamine receptors and presynaptic dopamine transporter sites in the caudate nucleus and putamen (cf. Normandin and Hazell, 2002), accompanied by decreased binding of dopamine D_2 receptors in the striatum and substantia nigra (Kobayashi et al., 2003).

Although the results from our behavioral experiments were consistent with the bulk of the prior literature in the area, the precise neurochemical mechanisms underlying the attenuation of cocaine reward as the result of Mn exposure necessarily remain speculative until further investigations are completed. Further microdialysis or histological studies performed under a similar Mn exposure paradigm will be helpful with respect to interpreting the mechanisms of the observed phenomena. It also may be beneficial to conduct further experiments on whether chronic low-dose Mn exposure can produce similar disturbances in cocaine CPP that were observed with acute high-dose Mn exposure in this project. Such experiments may demonstrate similar Mn-induced behavioral effects, thus providing an even more clinically relevant model of manganism.

Concentrations of Mn in Brain

Oral Mn exposure was associated with significant increases in Mn concentration in the central nervous system. In addition, these data suggest that the striatum and the posterior basal ganglia, in contrast to the rest of the brain, are more vulnerable to Mn accumulation, and the effects persist even after an extended period of non-exposure.

Drug Use and Clinical Significance

This project has demonstrated that periadolescent Mn exposure produces attenuation of cocaine-induced place preference, suggesting that Mn neurotoxicity decreases cocaine sensitivity. Although the mechanism is not clear, desensitization to cocaine reward can be an additional clinical feature of manganism. Meanwhile, although there are features that can differentiate diagnosis between manganism and PD, both manganism and PD produce increases in dopamine turnover during early stages (Erikson and Aschner, 2003; Sossi et al., 2002), and depletion of dopamine during late stages, which suggest possible parallelism in how the limbic system functions between the two diseases. However, manganism and PD seem to affect the dopamine system differently. PD is primarily linked to the impairment of nigrostriatal pathway. In contrast, studies have shown that the integrity of the nigrostriatal dopaminergic pathway is preserved after Mn exposure, and that Mn intoxication may cause parkinsonism by damaging output pathways downstream to the nigrostriatal dopaminergic pathway (Shinotoh et al., 1995). Due to these differences, further examination is required before behavioral changes may be generalized to PD. Because a substantial literature exists regarding significant species differences in the neurotoxicity of Mn, any generalization between Mn-induced behavioral effects on rodents and humans must be made with caution (Dorman et al., 2000).

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