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Effects of Chronic Manganese Exposure on Cognitive and Motor Functioning in Non-Human Primates

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Abstract

Acute exposure to manganese is associated with complex behavioral/psychiatric signs that may include Parkinsonian motor features. However, little is known about the behavioral consequences of chronic manganese exposures. In this study, cynomolgus macaque monkeys were exposed to manganese sulfate (10 –15 mg/kg/week) over an exposure period lasting 272 ± 17 days. Prior to manganese exposure, animals were trained to perform tests of cognitive and motor functioning and overall behavior was assessed by ratings and by videotaped analyses. By the end of the manganese exposure period, animals developed subtle deficits in spatial working memory and had modest decreases in spontaneous activity and manual dexterity. In addition, stereotypic or compulsive-like behaviors such as compulsive grooming increased in frequency by the end of the manganese exposure period. Blood manganese levels measured at the end of the manganese exposure period ranged from 29.4 to 73.7 μg/L (mean = 55.7 ± 10.8 (compared to levels of 5.1–14.2 μg/L at baseline (mean = 9.2 ± 2.7), placing them within the upper range of levels reported for human environmental, medical or occupational exposures. These results suggest that chronic exposure to levels of manganese achieved in this study may have detrimental effects on behavior, cognition and motor functioning.

Keywords

manganese; monkeys; cognition; motor; behavior

1. Introduction

Manganese is an essential metal found in a variety of biological tissues and is necessary for the normal functioning of a variety of physiological processes including: amino acid, lipid, protein and carbohydrate metabolism; normal immune system functioning (see Erikson et al., 2005 for review); regulation of cellular energy through complexes formed with ATP and inorganic phosphate; normal bone and connective tissue growth and normal blood clotting (see Erikson and Aschner, 2003 for review). Manganese is a constituent of metalloenzymes such as arginase and functions as a cofactor for other enzymes, such as the anti-oxidant manganese

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superoxide dismutase (Mn-SOD) (Hurley and Keen, 1987). Under normal circumstances, manganese also plays a role in the synthesis and metabolism of various neurotransmitters (ex., dopamine and serotonin) through a role in the activities of monoamine oxidase and catechol-o-methyltransferase enzymes (Golub et al., 2005).

Manganese deficiency in a variety of species, including man, can lead to multiple problems such as stunted growth, skeletal defects, abnormal glucose tolerance (Erikson et al., 2005) and seizure activity (Critchfield et al., 1993). Clinically significant manganese deficiency occurs rarely in humans (Erikson et al., 2005). In contrast, exposure to excessive amounts of manganese is more prevalent and is associated with a variety of psychiatric and motor disturbances (Calne et al., 1994;Pal, et al., 1999).

Excess manganese intake can occur from excessive dietary intake as well as occupational and environmental exposures. Excess dietary intake most typically occurs in infants fed soy-based formulas that contain higher levels of manganese than breast milk or cow's milk –based formulas (Lonnerdahl, 1994). Occupational exposures occur in workers in certain industries such as alloy production, mining, battery manufacturing and welding. Environmental exposures occur most commonly through drinking contaminated water, from exposure to organo-manganese agricultural chemicals, and more recently, from environmental deposition of methylcyclopentadienyl manganese tricarbonyl (MMT), which is used an anti-knock additive to gasoline (Vezar, 2005). The clinical effects of manganese toxicity, many of which are Parkinson-like in nature, include a movement disorder characterized by tremor, rigidity, dystonia and/or ataxia (Josephs et al., 2005) and psychiatric disturbances including irritability, impulsiveness, agitation, obsessive-compulsive behavior, hallucinations and cognitive deficits such as memory impairment, reduced learning capacity, decreased mental flexibility and cognitive slowing (Josephs et al., 2005).

Neurological deficits in humans, such as those outlined above, are usually found following high level acute exposures or following long-term or chronic exposures. However, there is relatively little known about the threshold exposure necessary for inducing such deficits. This issue has become of increasing concern in light of possible adverse effects from long-term exposure to increasing ambient levels of manganese in the environment (Aschner et al., 2005). Also, while there are many studies of manganese toxicity in rodents, there are relative few studies of the effects of manganese exposure in non-human primates, a species whose behavioral repertoire more closely resembles that generated by the human neurobehavioral system. Also, existing studies of effects of manganese exposure in non-human primates have primarily examined short-term motor or behavioral effects from relatively acute exposures. The present research was performed as part of an on-going multi-disciplinary study assessing the behavioral, neuroimaging and neuropathological consequences of chronic exposure to different levels of manganese in non-human primates. We report here our findings from cognitive, motor and behavioral assessments of monkeys exposed chronically to the lowest level of manganese used in this ongoing study.

2. Results

Animal dosing and general characteristics of animals at termination of studies

The mean ± s.e.m. cumulative Mn dose administered was 156.7 ± 9.5 mg Mn/kg body weight. The average time from the initiation of Mn administration to the end of the study was 272 ± 17 days.

Effects of Chronic Mn Exposure on Variable Delayed Response (VDR), Visual Discrimination (VD) and Object Retrieval Performance

Three animals were successfully trained to perform VDR and VD and the object retrieval task; one animal performed only VD. One control animal performed VDR, VD and object retrieval tasks. Before initiation of Mn exposure, the animals had a delay-dependent decrement in performance on the VDR task. That is, there was a significant effect of delay wherein performance at short delays (i.e., 2 or 5 s) differed significantly from performance at longer delays (10 to 60 s) [$F(2,4) = 33.67$, $p < 0.0001$]. Short duration delay trials (2 or 5 s delays) were performed almost without error (100 % ± 0 and 98% ± 1.8 correct responses, respectively). Performance declined with delays of increasing duration (20 or 30 s delay, 88.2% ± 7.0; 30 or 45 s delay, 80.1 ± 5.3; 45 to 60 s delays, 65.8% ± 3.3).

The effect of Mn exposure on VDR performance varied from animal to animal with different animals having impaired performance at different delay durations and at different time points during the Mn exposure period. Due to this inter-animal variability, group analyses of their behavioral data showed no consistent changes in the group's performance across task delays at any time point during Mn exposure (performance at baseline vs. mean performance during wks . 4, 8, 12, 16, 20, 24, 28 of Mn exposure for 2 s delay [$F(2,7) = 0.62$, $p = 0.75$], 5 s delay [$F(2,7) = 0.68$, $p = 0.71$], 20 or 30s delay [$F(2,7) = 2.54$, $p = 0.05$], 30 or 45 s delay [$F(2,7) = 1.11$, $p = 0.41$], 45 or 60 s delay [$F(2,7) = 2.66$, $p = 0.045$]). Even though post-Mn performance was more variable than baseline performance, the effect of delay was still observed at the end of the Mn exposure period [$F(2,4) = 4.77$, $p < 0.05$]. At the end of the Mn exposure period, short duration delay trials (2 or 5 s delays) continued to be performed well (96 % ± 6.9 and 100% ± 0 correct responses, respectively). As during the baseline period, performance declined with delays of increasing duration (20 or 30 s delay, 83.6% ± 18.9; 30 or 45 s delay, 79.3 ± 18.9; 45 to 60 s delays, 58.3% ± 18.8).

The variable effects of chronic Mn exposure on VDR performance in individual animals is shown in Figure 1. To overcome limitations of averaging variable data, 95% confidence intervals were calculated based on each animal's baseline performance (Heinz et al., 1994). Changes from baseline performance were considered to be significant if means following manganese administration were outside of these intervals. Two of the three manganese-exposed animals showed decreasing performance at both short and intermediate delays on the VDR task and the third animal showed no appreciable change in task performance over time. This is in contrast to a control, vehicle-treated animal in which performance on the same task improved over time, as expected due to learning effects (Figure 1D). Considering the comparatively low (approximately chance) level of baseline performance at the longest delay interval, it was difficult to detect any meaningful post-Mn decrease in the performance of any animal at the longest delay.

Prior to receiving any Mn, visual pattern discrimination was performed at a 99.4% ± 0.8 correct level and task performance remained intact throughout the Mn exposure period (baseline performance vs. performance at wks 4, 8, 12, 16, 20, 24, 28 of Mn exposure, [[$F(3,7) = 1.86$, $p = 0.13$]]. Performance at the end of the Mn exposure period was 97.5% ± 4.6. The control animal performed this task at a 98% ± 2.7 correct level at baseline and task performance did not change (98% ± 3.5 correct responses) over at least 20 weeks of testing.

The main cognitive measure on the object retrieval task is number of barrier reaches. At baseline, animals made a mean of 0.38 ± 0.04 barrier reaches per "easy" trial and 0.99 ± 0.16 barrier reaches per "difficult" trial. Throughout the Mn exposure period, no consistent significant changes were observed in the number of barrier reaches made on trials designated as either cognitively "easy" [$F(2,7) = 2.54$, p = .065] or "difficult" [$F(2,7) = 2.20$, $p = 0.100$] (Table 1) or in task completion times for easy or difficult trials (data not shown). The lack of

consistent significant change in this behavior was apparent both when examining group data as well as individual animal performances. In the control animal, the number of barrier reaches per trial when performing the "difficult" version of the task decreased over time (1.82 ± 1.08 barrier reaches/trial at baseline vs. 0.23 ± 0.06 at last assessments) presumably as the animal became more familiar with the task. This practice effect was not seen in the Mn-exposed animals.

Effects of Chronic Manganese Exposure on Behavioral Ratings, Fine Motor Skills, General Behavior and Overall Activity

All five animals received behavioral ratings, four had fine motor skills assessed and three had general activity and behavior data recorded at baseline and throughout the manganese exposure period. Behavioral rating scores increased slightly during the manganese exposure period and became significantly different from baseline from week 20 through the end of the study (Figure 2A). The behavioral rating of the control animal did not change over the study (0.4 ± 0.5 at baseline, 0 ± 0 at last ratings). No abnormal movements (i.e, dystonia or dyskinesia) were observed in any animals at any time during the study.

On the test of fine motor skills, animals made 0.98 ± 0.80 errors/well on the "easy" board and 2.45 ± 0.91 errors per well on the "difficult" board during baseline. Over the course of the manganese exposure period, performance on the "easy" board did not significantly change whereas animals made significantly more errors on the "difficult" version of the task at the end of the Mn exposure period (6.25 ± 0.35 errors), compared to baseline performance (Figure 2C and D). Performance of the "difficult version of this task by the control animal did not deteriorate and if anything, improved somewhat over the course of the study (1.0 ± 0.8 errors at baseline vs. 0.5 ± 0.7 errors at last evaluations).

Individual animals had different levels of overall activity at baseline. Over the course of the Mn exposure period, overall activity levels varied greatly from one observation period, tended to decrease over time [$F(2,7)$, 2.33, $p = 0.08$] and became significantly different from baseline at the end of the study period (Figure 2B). In the control animal, there was no change in overall activity over the observation period (baseline: 100.0% ± 37.3; last observation: 98.0% ± 13.0).

Videotaped analyses of behavior showed an increased frequency of certain stereotypical behaviors such as grooming and licking/biting fingers, over the course of the manganese exposure period (Figure 3). The frequency of other behaviors such as exploration of the cage and climbing/swinging decreased by the end of the study, consistent with the observed decrease in overall activity noted above. Behaviors were analyzed in regard to the percent of the overall activity contributed by a specific behavior during the videotaping session. For example, during baseline, exploratory behavior and climbing accounted for 23.6% ± 5.6 and 12.4% ± 3.4, respectively, of the recorded activity whereas at the end of the manganese exposure period, these behaviors only accounted for 13.3% ± 3.1 ($p < 0.05$ vs. baseline) and 1.5% ± 0.5 of activity ($p < 0.01$ vs. baseline), respectively. Behaviors such as grooming and licking/biting fingers accounted for only very small amounts of the recorded activity during baseline (1.7% ± 0.8 and 1.5% ± 0.4, respectively, whereas at the end of the study, these behaviors accounted for 48.3% ± 9.1 and 9.3% ± 2.4 of recorded activity, respectively ($p < 0.01$ vs. baseline for each). The control animal showed a marked decrease in exploratory behavior over the study period (81% of total activity at baseline; 6% of total activity at last observation), presumably due to familiarity with the observation cage. Stereotypic behaviors did not increase in frequency to the extent observed in manganese exposed animals (Figure 3).

Metal Analysis in Whole Blood and Brain Tissue

Analysis of whole blood showed a significant effect of manganese exposure on whole blood manganese concentrations ($F_{3,11}$= 4.73; p= 0.024). No significant effect of Mn exposure was observed on whole blood Cu, Fe or Zn concentrations (Table 2). The mean ± sem of whole blood Mn concentrations in μg/L were as follows: Baseline: 9.2 ± 2.7 (n=3; range: 5.1– 14.2 μg/L, midpoint of Mn exposure period: 67.1 ± 13. 7 (n=4; range: 42.9–106.1 μg/L); end of Mn exposure period: 55.7 ± 10.8 (n=4; range: 29.4–73.7 μg/L).

Post-mortem brain tissue from Mn-exposed and control animals was also analyzed for metal content. There was a significant increase in Mn concentrations (Treatment effect: $F_{1,20}$ = 7.06; p= 0.015) in all brain regions examined (Table 3). We also found a significant effect of Mn exposure ($F_{1,20}$= 8.38; p= 0.009) on brain Cu concentrations (Table 3) with no significant effect on Fe or Zn concentrations.

3. Discussion

In the present study, we describe cognitive, behavioral and motor abnormalities resulting from chronic manganese exposure in non-human primates. At the level of manganese exposure used in the present study, subtle effects on tests of cognitive and motor functioning were observed along with behavioral changes suggestive of increased stereotypical or compulsive-like behaviors. Whole blood manganese levels achieved during the exposure period were within the upper range of those reported in children or adults receiving environmental, medical or occupational exposures (see Gulson et al., 2006 and table within), making our findings relevant to these human exposure conditions. Additionally, a study by Takser et al., (2003) found that approximately 50% of 112 cord blood samples obtained at birth in a French population had Mn concentrations greater than 40 μg/L. Therefore, there are human exposure conditions that produce whole blood Mn concentrations within the range of those measured in the present study. In addition, our finding of apparent changes in motor dexterity is consistent with reports in the clinical literature of changes in motor coordination, reaction time and finger tapping with low level manganese exposures (Iregren 1990;Roels et al., 1987), and may suggest the utility of such measures as early indicators of manganese toxicity.

Individual animals had different sensitivities to manganese administration and developed different degrees of cognitive impairment at different times post manganese exposure. Such differences in individual sensitivity to manganese made group analyses of some of the cognitive data difficult with the current small cohort of animals. Nonetheless, data from individual animals suggested disruptive effects of chronic manganese exposure on aspects of cognition. Inter-animal variability to the effects of manganese exposure have been described previously in other non-human primate studies of acute and chronic manganese exposures (ex., Newland and Weiss, 1992;Olanow et al., 1996). Wide variation in individual susceptibility to the behavioral effects of manganese exposure has also been described in humans (Iregren, 1990).

Anticipating possible effects of chronic manganese exposure on dopamine neurotransmission (Racette et al., 2005), we initially chose to study behaviors that, based on our previous experience with monkeys exposed to the dopaminergic neurotoxin MPTP, would be sensitive to disruption of dopaminergic function (Schneider and Kovelowski, 1990;Schneider and Pope-Coleman, 1995;Schneider et al., 1999). However, aspects of behavior believed to be most sensitive to dopaminergic dysfunction and dysfunction of the frontostriatal axis, such as attention, working memory, problem solving, and impulsivity were either unaffected or minimally affected by the manganese exposure in the current study. In post mortem studies performed on the brains of the animals used in this study (Guilarte et al., 2006), no significant loss of dopamine was found in either the caudate or putamen. In view of this finding, the lack of severe cognitive or motor dysfunction on the parameters assessed in this study may not be

surprising. It is possible that more consistent and severe cognitive deficits might be observed with either more prolonged Mn exposure or by examining performance of tasks that tap into different cognitive domains. In particular, preliminary results from NMR spectroscopy studies performed on these animals suggest the presence of damage in the parietal cortex (Guilarte et al., 2006a). Thus, it is possible that greater or more consistent deficits might be observed in performance of tasks that are dependent upon the integrity of more posterior cortical regions.

An apparent increase in certain stereotypic or compulsive-like behaviors was observed in manganese-exposed monkeys. Ventral prefrontal cortical regions are involved in response inhibition processes (Rosvold and Mishkin, 1961;Diamond, 1990) whereas dorsal prefrontal cortical regions are more involved in spatial working memory processes (Fuster, 1989). Stereotypic and compulsive-like behaviors in manganese intoxicated monkeys may be related to dysfunction of ventral frontostriatal circuits. That our monkeys had relatively intact performance on tasks that involve dorsal fronto-striatal connections suggest that with the type of manganese exposure used in this study, orbito-fronto-striatal circuits may be more vulnerable.

It is also possible that chronic manganese exposure at the levels achieved in the current study may have had a detrimental effect on learning ability, although that was not specifically assessed in the current study. Reduced learning capacity in humans exposed to manganese has been reported (Josephs et al., 2005) and it is possible that manganese exposure in monkeys may have similar effects. Data from the control animal used in this study as well as preliminary data from another ongoing study in our laboratory suggest that monkeys trained to perform the same spatial working memory task as used in this study and tested as frequently as the animals reported in the current study show improved performance over time, presumably due to learning effects or over-training. Such learning effects were not observed in the manganese exposed animals in this study. The possibility of impaired learning capacity in manganese-exposed monkeys will need to be examined more directly in future studies.

Manganese sulfate was used in this study since it is one of the main combustion products of MMT (Ressler et al., 1999;Zayed et al 1999a), an antiknock additive to unleaded fuel that contributes to environmental deposition of manganese and in consideration of the enhanced awareness that manganese may play a role in neurologic diseases (Aschner et al., 2005). To the best of our knowledge, this is the first study to assess the behavioral effects of chronic manganese sulfate administration in non-human primates. Other studies of manganese toxicity in non-human primates primarily used either manganese chloride or manganese oxide and focused mostly on imaging parameters, post-mortem pathological changes and effects on gross motor behavior (Olanow et al., 1996;Shinotoh et al., 1995;Eriksson et al., 1987;Eriksson, et al., 1992, Eriksson et al., 1992a). For example, manganese chloride, administered intravenously at doses of 10 – 14 mg/kg once per week for up to seven consecutive weeks in three monkeys caused significant clinical features of basal ganglia dysfunction (i.e., marked and generalized bradykinesia, dystonia and rigidity and extensor posturing) in two animals and had no clinical effect on a third animal (Olanow et al., 1996). The nigrostriatal pathway was judged not to be impaired in these animals (Shinotoh et al., 1995). Manganese oxide, administered to monkeys subcutaneously (0.4 g manganese/injection) periodically over 4 months resulted in unsteady gait, clumsiness and hypoactivity (Eriksson et al., 1992a), with longer exposure resulting in changes in striatal dopaminergic markers (Eriksson et al., 1992). In an effort to study behavioral effects of manganese at cumulative doses below those evoking clinical neurological signs, Newland and Weiss (1992) administered $MnCl_2$ intravenously to cebus monkeys at doses of 5 or 10 mg/kg of manganese (calculated as the base), once per week (or less frequently), for up to 450 days. Action tremor reportedly appeared after a cumulative dose of 40 mg/kg manganese, although disrupted performance on a previously learned behavior (i.e., fixed ratio responding) appeared earlier (Newland and Weiss, 1992). Interestingly, these authors

described manganese's initial effects on behavior as increased variability in responding, an affect also seen in our animals.

The observations in other studies of overt neurological signs such as tremor, rigidity or dystonia in manganese intoxicated monkeys at lower cumulative doses of manganese than achieved in our study is of interest and may relate to the form of manganese administered. Most of these other studies used $MnCl_2$ whereas we used $MnSO_4$ and different physiological effects of different chemical forms of manganese must be considered (Aschner et al., 2005). In rodent studies, different manganese compounds had different effects on brain monoamine levels and behavior (Komura and Sakamoto, 1992) and manganese metabolism or initial valence states have been suggested to be important determinants of manganese retention in the body (ATSDR, 2000).

In summary, chronic manganese exposure in macaque monkeys led to behavioral alterations consisting of compulsive-like behaviors, decreased activity levels, problems in fine motor functioning and variable sensitivity to cognitive deficits such as impaired spatial working memory. Additional work is necessary to understand the long-term effects of different doses and dosing regimens of manganese on cognitive and motor functioning in non-human primates.

4. Experimental Procedure

Six research naïve young adult male M. fascicularis monkeys (approximately 5 to 6 years of age at the start of the study) were used: five received manganese exposure and one served as a control animal that was treated exactly the same as the manganese-exposed animals except received only vehicle injections. All animal studies were reviewed and approved by the Thomas Jefferson University Animal Care and Use Committee.

Following quarantine, animals were trained to perform cognitive tasks and their baseline motor functioning was assessed. Once animals achieved a stable performance baseline, they were temporarily transferred to Johns Hopkins University for in vivo imaging studies (animals were transported again to Johns Hopkins University for additional imaging studies performed approximately midway through the manganese exposure period and again at the end of the exposure period), results to be reported elsewhere). Upon their return, a stable level of behavioral performance was confirmed and Mn exposure was initiated. Animals received intravenous injections of manganese sulfate ($MnSO_4$) (10 mg/kg/week for 5 weeks and then 15 mg/kg/week for the remainder of the study period) into the saphenous vein under light isofluorane anesthesia. Manganese sulfate was prepared fresh for each injection (50 mg/ml in sterile saline), filtered and warmed to 37º C prior to use. A needle and catheter were inserted into the vein and flushed with sterile saline. $MnSO_4$ was then administered at a rate of 0.5 ml/minute over an approximately 4 to 6 minute period, depending upon the total volume to be injected. Vital signs were monitored during the manganese administration. At the end of the $MnSO_4$ infusion, at least 1.0 ml of sterile saline was pushed through the catheter. Animals were then returned to their home cage and observed for any possible adverse events.

Cognitive Training and Testing

Apparatus—Animals were trained and tested on a number of tasks while seated in a restraining chair placed inside a Wisconsin General Test Apparatus (WGTA). For some assessments (object retrieval task, general behavior, activity, fine motor skills) testing was performed while animals were freely moving in an observation/testing cage. When inside the WGTA, the monkey was positioned behind an opaque screen which when raised, allowed the animal access to a sliding tray containing two food wells with sliding Plexiglas covers that served as stimulus plaques. The animals were trained to displace the Plexiglas covers in order

to obtain rewards from the food wells. All animals were food restricted overnight prior to testing.

Variable Delayed Response—In this task which has both attentional and spatial working memory components (Schneider et al., 1999), animals were trained to retrieve food after observing the experimenter bait one of two wells. Right and left wells were baited in a balanced order. Five different delay lengths (ranging from 2 to 60 secs.) were randomly distributed in blocks of 8 trials over the 40 trials that made up a daily testing session. The delay conditions were selected to yield performance ranging from approximately 90% correct performance at the shortest delay and approximately chance performance at the longest delay.

Visual Pattern Discrimination—This task, which assesses reference memory, requires animals to discriminate between two distinct black patterns on a white background. One stimulus was designated as the positive stimulus (i.e., animal was rewarded for choosing that stimulus) and it appeared over the right or left food wells in a pseudo-randomized way. Animals were trained to perform 20 trials per day to a 90% criterion level.

Object Retrieval Task—This task, which has fronto-executive and motor components (Schneider et al., 1998) required monkeys to reach into a clear Plexiglas box (15 x 15 x 5 cm) with one open side, presented just outside of the observation cage, to retrieve a food reward. The open side of the box could face front, left, or right relative to the monkey; and the reward could be placed at the front, center or rear edge of the box. The box could also be centered in front of the monkey or placed to the far left or right of the testing cage. Each of 30 trials per test session differed in the combination of reward placement, location of the open side of the box, and position of the box, thus affecting the cognitive and motor difficulty of the trial. Response times (i.e., time between presentation of the box and a correct retrieval response) were recorded as were the number of “barrier” reaches. Barrier reaches (i.e., cognitive errors) occurred when the open side of the box faced away from the animal and it reached for and hit a closed side of the box rather than making an appropriate detour movement.

Behavioral Ratings—Throughout this study, animals were rated for a variety of behaviors using a rating scale based on a Parkinson symptom rating scale for non-human primates (Schneider and Kovelowski, 1990). The scale evaluated several items including: posture, balance, climbing, tremor (initiation/resting), freezing during movement, upper and lower limb movement, fine motor skills and eye blink rate. Each item was rated as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe). A score of 33 represented the highest possible disability score. The presence of dystonia or dyskinesia were also noted and if present, the severity of these abnormal movements were rated on the same 0 – 3 scale described above for left/right, upper/lower extremity, face, neck , trunk/tail. A score of 21 represented maximum severity.

Fine Motor Skills—Fine motor skills were assessed using 2 boards made of clear Plexiglas that contained 16 wells of small (14 mm) or large diameter (22 mm), all of which were 17 mm deep (Schneider and Pope-Coleman, 1995). An “easy” version of the board consisted of 12 large and 4 small wells while a “difficult” version of the board consisted of 12 small and 4 large wells. Animals were required to retrieve a standard reward pellet from each well as quickly as possible. The primary measure obtained was the number of errors made per well (i.e., the number of attempts to remove a pellet from a well and/or the number of times a pellet was dropped).

General Activity Monitoring—The level of overall activity during 2 – 3 consecutive 24 hr. periods was recorded prior to manganese exposure (1 –3 separate sessions) and following manganese exposure (1 session approximately every 2 – 4 weeks) using a personal activity

monitor (Actitract; IM Systems, Inc.) placed into a pocket on the back of a jacket worn by the animals during the evaluation period. All animals were adapted to the jacket prior to data collection.

Videotaped Analysis of Natural Behavior—Since human manganism is often accompanied by psychiatric and behavioral abnormalities (Greger, 1999), an attempt was made to characterize any possible changes in natural behaviors or emergence of abnormal or stereotyped behaviors that might occur following long-term manganese exposure in our monkeys. Animals were placed in an observation cage in a quiet room separate from the colony, allowed to acclimate to the cage, and videotaped for 45 successive minutes on each of 4 consecutive days during the week in which data were collected. Recordings were obtained at baseline, after approximately 15 weeks of manganese exposure and at the end of the manganese exposure period. Videotapes were analyzed by scoring the duration (in seconds) of selected behaviors per 3 min. epoch for the duration of each recording period. The behaviors that were scored were: rubbing or pulling floor/bars of the cage, exploratory behavior, licking the bars of the cage, climbing, licking/biting fingers, visual tracking, and grooming.

Metal Analysis of Blood and Brain Tissue Using High-Resolution Inductively-Coupled Plasma Mass Spectrometry (HR-ICP-MS)—Whole blood samples were obtained under fasting conditions at baseline, midpoint of the manganese exposure period and at the end of the manganese exposure period. Manganese levels measured at mid-point and end of exposure period were taken approximately 3 weeks after the last manganese dosing. Concentrated nitric acid (HNO_3) (Suprapur, Merck) was added to dried whole blood and brain samples. Blood samples were placed at room temperature for 24 hours and digested on a heat block for 1 hr at 70 ºC, 1 hr at 100 ºC and 1 hr at 110 ºC. Brain samples were placed at room temperature for 24 hr and digested either on a heat block (QBT4, Grant) for 3 hrs at 70 ºC or using a microwave oven (Multiwave 3000, Anton Paar) using ramp 200W for 10 min and then held for 10 min. Samples were then diluted with 0.6 M HNO_3 with 18.2 MΩ water. Blood and brain samples were analyzed for metal content by HR-ICP-MS using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany), according to a published protocol (Erikson et al., 2004) except that radio frequency power was set at 1250W: ^{55}Mn ^{57}Fe, ^{63}Cu, and ^{67}Zn were measured at medium resolution.

Data Analyses—Means and standard deviations were calculated for the various cognitive measures at baseline (weekly means for the 3 weeks prior to the first Mn exposure) and at weeks 4, 8, 12, 16, 20, 24 after the first Mn exposure and at the conclusion of the study. Task performance prior to and following Mn exposure was compared by repeated measures analysis of variance for each task and for sub-components of various tasks where appropriate. Animals served as their own controls. Pairwise post-hoc comparisons (Newman-Kuhls t test; Dunnetts post hoc t test) were performed to assess changes between conditions (baseline, weeks 4, 8, 12, 16, 20, 24, 28 after Mn) and specifically between baseline and each post Mn observation period, respectively. Differences between baseline and post-Mn behavioral rating data were compared using a non-parametric Wilcoxon signed rank test. Videotaped behavioral data were analyzed with repeated measure ANOVA followed by post-hoc comparisons using Newman-Kuhls t test.

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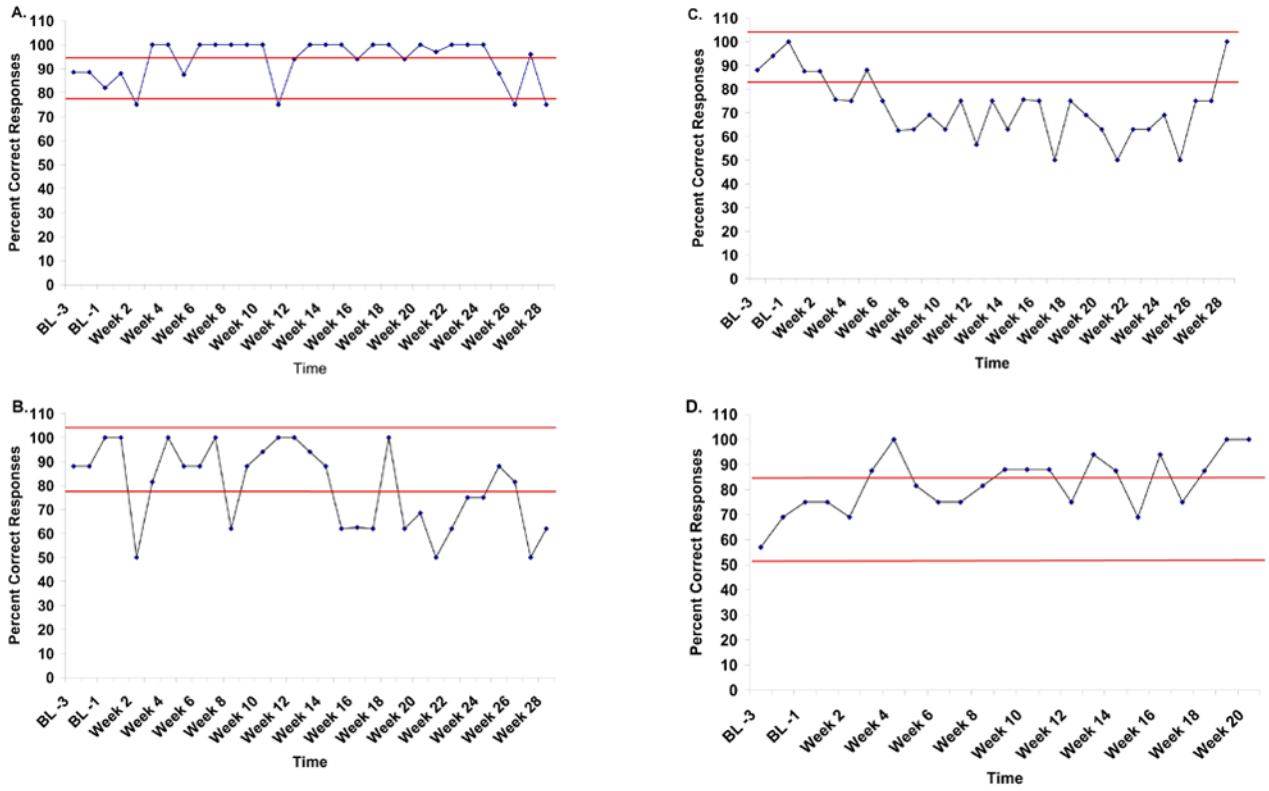

Figure 1.

Variable delayed response performance at intermediate delays (30 to 45 secs.) of 3 individual manganese-exposed animals (A – C) and a control, vehicle-treated animal (D). Graphs show each animal's baseline performance over the 3 weeks prior to start of manganese exposure and mean performance by week to the end of the study period. Horizontal lines show 95% confidence intervals based on the baseline means. While 2 animals developed deficits with task performance at these delays (B, C), 1 animal's performance (A) was resistant to disruption by the manganese administration. The performance of the control animal tended to improve over time (D).

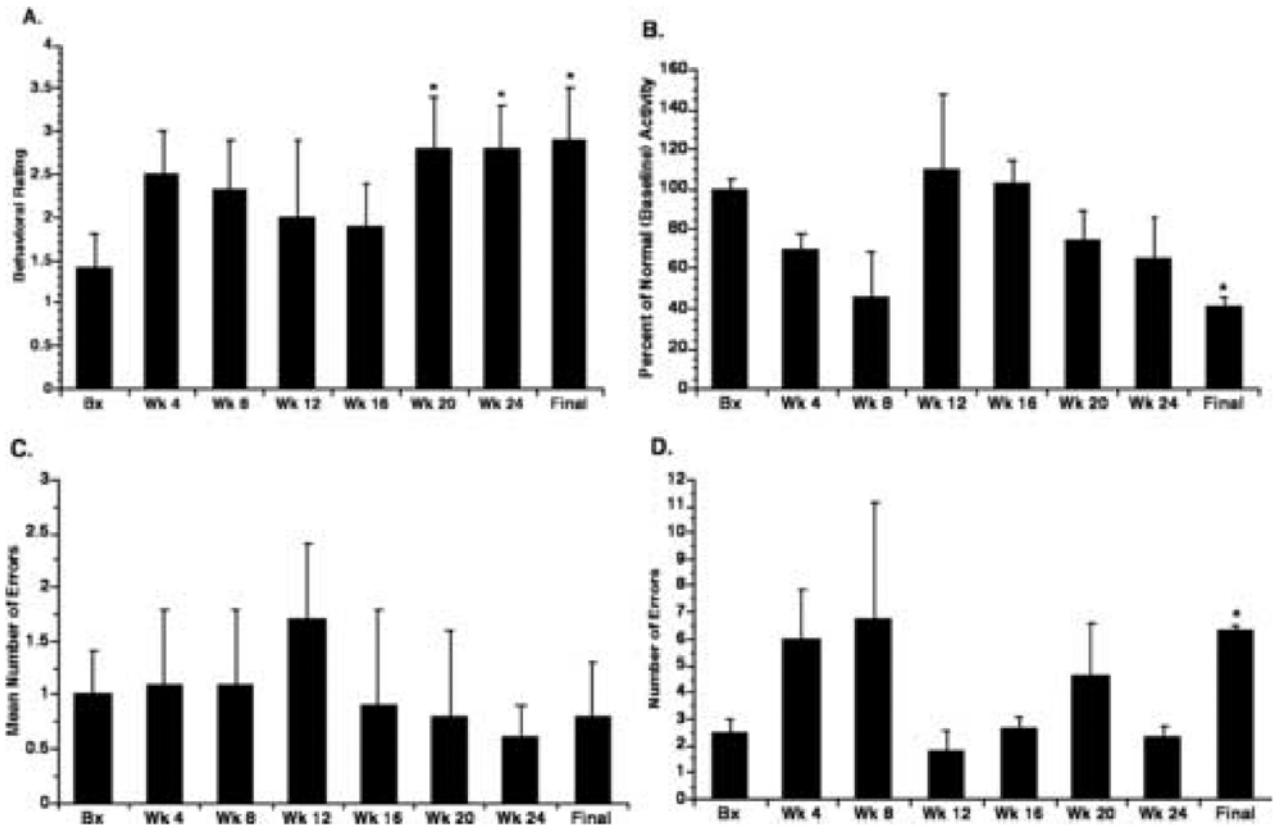

Figure 2.

Manganese effects on motor functioning. A) Behavioral ratings, based on a rating scale previously used to rate Parkinson-like behaviors in non-human primates, showed statistically significant changes from baseline from week 20 of the manganese exposure period through the end of the study. Even though a statically significant increase in rating scores was observed, these clinically subtle changes were not considered to be clinically significant since animals appeared grossly normal at the end of the study. B) Overall activity levels varied over the course of the study with an initial decrease in activity followed by a rebound in activity levels and finally a decrease in overall activity at the end of the study. C) Performance of the "easy" version of the test of fine motor skills did not substantially change throughout the study period. D) Performance of the "difficult" version of the fine motor skills test became more variable after manganese exposure and at the end of the study period, animals made significantly more errors on this task than at baseline, suggesting the presence of difficulties with fine motor dexterity. *$p < 0.05$ versus baseline (Bx).

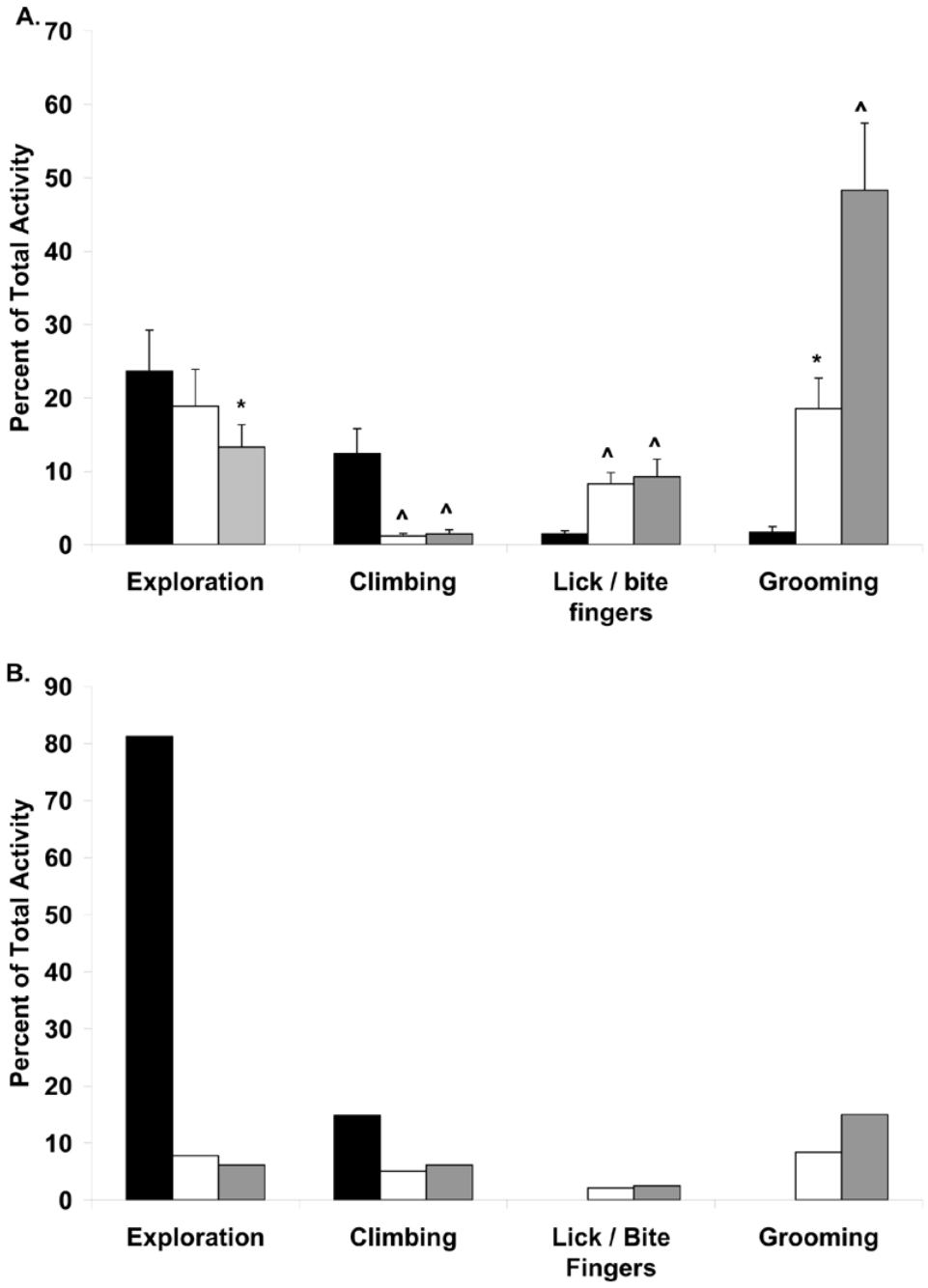

Figure 3.

Effects of manganese exposure on analyses of videotaped behavior. Animals were placed in an observation cage and videotaped on four separate occasions for 45 min. each at baseline (black bars), at the mid-point of the study (white bars) and at the conclusion of the study (gray bars). Each behavior (exploration of the cage, climbing, licking/biting fingers, grooming) was scored for its frequency of occurrence during each 3 min. epoch during the 45 min. recording period. Results were then expressed as the percent of total activity occupied by that behavior. A. Exploration of the cage and climbing behavior decreased over time in manganese-treated monkeys. Stereotypic or compulsive-like behaviors such as licking/biting fingers and grooming significantly increased after manganese exposure. In particular, grooming behavior,

which consumed less than 2% of these animal's overall activity when normal accounted for almost 50% of their overall activity in the observation cage at the end of the study. B. In a vehicle control animal, exploration of the cage and climbing behavior also decreased over time, presumably due to increased familiarity with the observation cage. However, in contrast to the manganese-exposed animals, compulsive behaviors did not dominate the control animal's total activity. *$p < 0.05$ compared to baseline; ^$p < 0.01$ compared to baseline.

Table 1

Mean (± SD) Number of Barrier Reaches Per Trial Made on Object Retrieval Task During Baseline and Manganese Exposure

	"Easy" Trials	**"Difficult' Trials**
Baseline	0.38 ± 0.04	0.99 ±0.16
Week 4	0.84 ± 0.65	1.56 ± 0.6
Week 8	0.42 ± 0.22	0.79 ± 0.37
Week 12	0.32 ± 0.19	1.17 ± 0.84
Week 16	0.34 ± 0.22	1.15 ± 0.37
Week 20	0.65 ± 0.24	1.05 ± 0.45
Week 24	0.28 ± 0.13	0.67 ± 0.37
End of Study	0.42 ± 0.11	1.18 ± 0.55

Table 2

Mean (± SEM) Whole Blood Metal Concentrations (μg/L)

Baseline	Mn-1	Mn-2
Manganese Concentrations (μg/L)		
9.2 ± 2.7 (n = 3)	67.1 ± 13.7* (n = 4)	55.7 ± 10.8* (n = 4)
Copper Concentrations (μg/L)		
572.3 ± 41.1 (n = 4)	553.0 ± 20.8 (n = 4)	501.3 ± 26.9 (n = 4)
Iron Concentrations (μg/L)		
398,500.0 ± 48,394.6 (n = 4)	365,675.0 ± 40,584.5 (n = 4)	409,200.0 ± 26,146.0 (n = 4)
Zinc Concentrations (μg/L)		
15,975.0 ± 5,691.5 (n = 4)	17,307.5 ± 4,635.9 (n = 4)	16,817.5 ± 3,333.7 (n = 4)

*Significantly different from control at $p<0.05$

Table 3

Mean (± SEM) Brain Metal Concentrations (μg/g tissue)

	Globus Pallidus	Caudate	Putamen	Frontal White Matter
Manganese Concentrations (μg/g tissue)				
Naïve control (n = 3)	0.72 ± 0.14	0.38 ± 0.05	0.48 ± 0.07	0.17 ± 0.02
Mn-exposed (n = 4[#])	3.30 ± 0.52[*]	1.18 ± 0.18[*]	1.50 ± 0.11[*]	0.57 ± 0.02[*]
Copper Concentrations (μg/g tissue)				
Naïve control (n = 3)	7.70 ± 0.58	5.90 ± 0.87	6.08 ± 1.20	2.65 ± 0.12
Mn-exposed (n = 4)	8.83 ± 0.60[*]	7.51 ± 0.46[*]	8.39 ± 0.46[*]	2.77 ± 0.48
Iron Concentrations (μg/g tissue)				
Naïve control (n = 3)	214.2 ± 7.7	77.6 ± 20.1	92.1 ± 20.9	27.31 ± 2.57
Mn-exposed (n = 4)	169.0 ± 30.5	66.8 ± 2.6	77.6 ± 6.8	23.58 ± 3.52
Zinc Concentrations (μg/g tissue)				
Naïve control (n = 3)	15.9 ± 2.1	19.5 ± 2.2	18.8 ± 2.1	9.23 ± 0.62
Mn-exposed (n = 4)	14.6 ± 1.3	18.1 ± 1.2	15.5 ± 0.2	8.51 ± 1.43

[#] n = 3 in the caudate;

[*] Significantly different from control at $p<0.05$