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Behavioral Effects of Subchronic Inorganic Manganese Exposure in Rats

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Background *Manganese, an essential micronutrient, is a potential neurotoxicant in prolonged overexposure. Parkinson-like syndrome, motor deficit, disturbed psychomotor development are typical signs of neuropathological alterations due to Mn in humans.*
Methods *Young adult rats, in three groups of 16 each, received 15 and 59 mg/kg b.w. $MnCl_2$, (control: distilled water) via gavage for 10 weeks, and were kept for further 12 weeks. Correlation of $MnCl_2$ exposure to body and organ weights, neurobehavioral effects (spatial memory, exploratory activity, psychomotor performance, pre-pulse inhibition), and histopathological changes (gliosis) was sought.*
Results *By the end of treatment, Mn accumulated in blood, cortex, hippocampus, and parenchymal tissues. Body and organ weights were reduced in high dose rats. All treated rats showed hypoactivity, decreased memory performance, and diminished sensorimotor reaction. In the dentate gyrus of these, GFAP immunoreactivity increased. During the post-treatment period, body weight of the high dose group remained decreased, locomotor activity returned to control, but the lasting effect of $MnCl_2$ could be revealed by amphetamine.*
Conclusion *Using complex methodology, new data were obtained regarding the relationship between the long-term effects of $MnCl_2$ at neuronal and behavioral level.* Am. J. Ind. Med. 50:841–852, 2007. © *2007 Wiley-Liss, Inc.*

KEY WORDS: manganese; spatial learning; maze; exploratory activity; open field; psychomotor performance; sensorimotor gating; GFAP; d-amphetamine; rat

INTRODUCTION

Manganese (Mn) is, in small amounts, an essential microelement. At excessive exposure, however, it can cause possible health problems including neurodegenerative alterations.

Mn exposure in humans causes a Parkinson-like syndrome [Gorell et al., 1979], motor neuron disease [Crinella et al., 1998], and disturbed psychomotor development [Takser et al., 2003]. Massive exposure to Mn is mostly airborne and of occupational origin. The first report on the health effect of Mn containing mineral dust was published over 150 years ago [Couper, 1837]. Since then, the symptoms have been studied in hundreds of human cases (groups of highly exposed miners, and railway-, construction-, steel smelting-, and other workers) all over the word [Wennberg et al., 1991; Mergler, 1999]. The relationship found between external and internal exposure was variable. In a smelter, 0.1–3 mg/m^3 airborne Mn gave 12.5 μ/L Mn-blood level [Myers et al., 2003]. In alloy production workers, 0.89 mg/m^3 Mn in the total and 0.04 mg/m^3 Mn in the respirable dust for ca. 10 years resulted in 1.03 μg/100 ml whole blood Mn level [Mergler et al., 1994]; but in another study, 0.014–11.48 mg/m^3 total dust and 0.001–1.273 mg/m^3 respirable dust Mn resulted in 11.3 μg/L whole blood

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level [Bouchard et al., 2005]. Above 7.5 [Mergler, 1999] or 10 [Lucchini et al., 1999] μg/L blood Mn, motor and other functional damages were observed.

Health-relevant non-occupational exposure to Mn is typically dietary, as in babies fed by cow milk- or soybean-based formulas [Marlowe and Bliss, 1993], or due to the drinking water. In Greece, 50 mg/L Mn in the drinking water was associated with neurological effects [Kondakis et al., 1989]. Further environmental sources are the use of the organometal compound methylcyclopentadienyl manganese tricarbonyl (MMT) as anti-knock petrol additive [Lynam et al., 1999], and application of some organo-Mn compounds as agricultural fungicides [Ferraz et al., 1988].

In rodents exposed experimentally to Mn, the morphological changes resemble those in human Parkinson's disease [Ponzoni et al., 2000]. The Mn-induced neurological manifestations indicate a significant relationship with dopaminergic [Hirata et al., 2001] and cholinergic [Pappas et al., 1997] regulation of certain other neurotransmitters (glutamate, GABA, acetylcholine, norepinephrine; Trepper et al. [1998]). The effect of Mn on sensorimotor integration depends on the central dopaminergic-glutamatergic interactions [Calabresi et al., 1997] while the mesolimbic/mesocortical dopaminergic pathway is responsible for cognitive functions and motivation, and indirectly for locomotor activity [Alexander et al., 1990] and psychomotor performance [Wise, 1998]. The high sensitivity of different behavior types and the integration of different (motor, sensory, motivational) functions make behavioral tests an important tool in the functional toxicology of manganese.

In the present work, a complex behavioral test battery (spatial memory, spontaneous and amphetamine-induced open field (OF) activity, psychomotor performance and sensorimotor gating) was applied—together with blood and tissue Mn level determinations, and measurement of the GFAP reactivity in three hippocampal regions—on 8 weeks old male Wistar rats during 10 weeks of $MnCl_2$ treatment and a 12-week post-treatment period. This longitudinal model was supposed to provide new data, contributing to a better-based relationship between the known subchronic and long-lasting toxicological effects of Mn at CNS level, and the behavioral and histopathological outcomes of prolonged Mn exposure.

MATERIALS AND METHODS

Animals, Housing, Treatment

Adult male Wistar rats (body weight 220 ± 20 g) were used. The animals (a total of 48 at start-up) were housed under controlled conditions of temperature (22–24°C) and photoperiod (12-hr light/dark cycle with light starting at 6:00 a.m.), with free access to water and conventional rat chow. One group of the rats (16 animals) was treated with 15 mg/kg b.w. (low dose group), and another 16, with 59 mg/kg b.w. (high dose group), inorganic Mn ($MnCl_2 \cdot 4\ H_2O$, min. 99% purity; REANAL Fine Chemical Co., Budapest, Hungary) dissolved in distilled water to 1 ml/kg administration volume. These doses were approximately 1/100 and 1/25 of the oral LD_{50} for $MnCl_2$ [Physical and Theoretical Chemistry Laboratory, 2001]. Animals in the control group received distilled water. Administration was done by gavage, 5 days a week, from the 1st to 10th experimental week (time scheme of the treatment period, number of animals used and tests are summarized in Table I). From 11th to 22nd experimental week, the rats kept till that time (altogether 18) received no further treatment (1st–12th post-treatment week in Table I).

The whole study was performed under GLP conditions,[1] and in strict adherence to the requirements by the Ethical Committee for the Protection of Animals of the University.

Cognitive Behavioral Tasks

During the study, the memory and OF investigations were performed between 08:00 and 14:00, in a room of 25 lux illumination and 40 dB white background noise level, different from that used for keeping and treating the animals. The psychomotor and sensorimotor gating tests of rats were performed sequentially, under identical conditions, later in the day.

Spatial learning and memory ability of rats was tested in an 8-arm radial maze (Columbus Instruments, Columbus, OH). Throughout the tests (to obtain high motivation in the ''win-shift food search strategy''), the animals were food deprived, maintaining them at 80–85% of their growth-adjusted free-feeding body weights [Beatty and Shavalia, 1982].

In the adaptation (1st) week, all animals had 10-min training twice a day in which the rats were adapted to find food pellets in the maze arm ends. Completion of the radial maze involved the rat running down each of the 8-arms branching from the octagonal center platform, in order to obtain a food reward at the end of each arm of the maze. Perfect performance of this task required entering each arm only once.

During acquisition of the "win-shift strategy" (2nd week, see Tables I and III), with one training per day, the rats were first individually trained to learn the general cues of the task: entering each one of the 8 arms only once in a given session, with no more than one error per session in six consecutive days. The percent rate of correct responses was counted as the performance indicator.

[1] The laboratory and animal house of the Department has been GLP certified for safety neurotoxicological testing. Certification No. 3011/48/2003.

TABLE I. Time Scheme of the Whole Experiment

Time scheme of the whole experimental period	Investigations							
	Cognitive behavior tests				Measurements			
	Memory	OF	ASR PPI	Histology (GFAP reactivity)	Body weight	Organ weight	Mn levels in blood	Mn levels in tissues
10 weeks of treatment period								
1st–4th	3 × 16	—	—	—	3 × 16	—	—	—
5th	3 × 16	3 × 12	3 × 12	—	3 × 16	3 × 4	3 × 4	3 × 4
6th–7th	—	—	—	—	3 × 12	—	—	—
8th–9th	3 × 12	—	—	—	3 × 12	—	—	—
10th	3 × 12	3 × 12	3 × 12	3 × 4	3 × 12	3 × 6	3 × 6	3 × 6
12 weeks of post-treatment period								
1st	—	—	—	—	3 × 6	—	—	—
2nd	—	—	—	—	3 × 6	—	3 × 3	—
3rd–5th	3 × 6	—	—		3 × 6	—	—	—
6th	—	—	—	—	3 × 6	—	3 × 3	—
7th	—	3 × 6	3 × 6	—	3 × 6	—	—	—
8th	—	0.5 d-A 3 × 6	—	—	3 × 6	—	—	—
9th	—	—	—	—	3 × 6	—	—	—
10th	—	1.5 d-A 3 × 6	—	—	3 × 6	—	—	—
11th	—	—	—	—	3 × 6	—	—	—
12th	—	—	—	—	3 × 6	3 × 6	3 × 6	3 × 6

Tests and investigations performed are indicated in the column of the corresponding week. The numbers in the cells show the number of animals used in the different tests. OF, open field exploratory activity; ASR, acoustic startle response; PPI, pre-pulse inhibition; d-A, injection of d-amphetamine; GFAP, glial fibrillary acidic protein.

During the 2 and 4 hr working memory tests (3rd and 5th treatment week, 4th and 5th post-treatment week), the rats were one by one put for 10 min maximum in the center of the maze, but were allowed to enter only 4 of the 8 open and baited arms (at their own random selection). After visiting the 4 arms, the animal was returned to its cage and kept there for 2 or 4 hr. The rat was then put again in the maze center and allowed to complete arm choices 5–8, to obtain rewards in the 4 baited arms not visited before. In the working memory tests, aimed at retaining information useful for only one session, errors meant reentry into any of the arms visited in the first run.

In the reference memory test (4th treatment week), food reward was put only in the 4 arms preferred by the individual rats. Entering an unbaited arm constituted a reference memory error, from which performance was calculated as above.

In the rest periods (6th and 7th treatment week, 1st and 2nd post-treatment week), the animals were kept in the housing room, were treated with $MnCl_2$ (in the treatment period) but did not have any testing and were not exposed to new information.

In the long-term working memory tests (8th–10th treatment week, 3rd–5th post-treatment week), the memory return and the 2- and 4-hr working memory were observed.

Spontaneous Motility Test

An OF box was used, equipped with two arrays of infrared movement detectors (at 3 and 15 cm above floor level). The animals were placed individually into the center of the OF, and their horizontal (running), vertical (rearing), and local (stereotype grooming and scratch) activity was simultaneously recorded during a 10 min session. Movement scores were computed on the basis of the beam interruptions. For timing, see Table I. In the 8th and 10th post-treatment week, OF test was repeated, 15 min after ip. injection of 0.5 or 1.5 mg/kg b.w. of d-amphetamine (d-A). This drug is known to pass the blood–brain barrier and increase the release of catecholamines from pre-synaptic endings [Clarkson et al., 1988].

Psychomotor Performance Test

Sensorimotor control was assessed by means of the acoustic startle response (ASR), the contraction of the whole body musculature on a sudden auditory stimulus [Koch, 1999]. The rats were placed individually on a metal plate with built-in piezoelectric accelerometer and surrounded by a cage inside a sound- and light-attenuated chamber. After a 10-min accommodation, the rats received a series of 10

consecutive tones (5 kHz, 110 dB, 200 ms, 15 s interval) as test stimuli. A whole body twitch resulting in more than 50 g force to the cage floor was accepted as ''noise positive response,'' of which latency, time to peak, and peak force amplitude were measured. Startle plasticity (tested by pre-pulse inhibition, PPI) of the animals was examined in a second series, after 15 min rest. This time, the startling test pulses were preceded by inhibiting (non-startling) pre-pulses (1 kHz, 73 dB, 500 ms, 200 ms delay between pre-pulse and startling pulse), similarly to the method published by Graham [1975].

Tissues Sampling, Measurements of Mn Level, Body Weight, and Organ Weights

On the 0th, 5th, and 10th treatment and 2nd, 6th, and 12th post-treatment weeks (having finished all tests of the days involved), blood samples were taken from the tail vein (see Table I for the number of animals sampled) into heparinized vacutainers of 7.0 ml capacity, and stored at 4°C until analyzed. On the 5th and 10th treatment week, and 12th post-treatment week, an overdose of Nembutal was given ip. after the blood collection, the thorax of the rat was opened, and transcardial perfusion was applied with 500 ml saline to remove blood from the organs. The brain was removed whole, without the meninges, and was, under a stereomicroscope, halved and dissected to isolate the hippocampus. The whole hippocampus, and 0.3 g pieces of the cortex, liver, kidney, and femur, were taken as samples and were kept at −18°C until analyzed. Manganese content was determined at the accredited laboratory of the National Center for Public Health, Budapest, by flameless graphite furnace atomic absorption photometry.

In the first 11 weeks of the experiment, body weight of the rats was measured each workday, always at 6 to 7 a.m., and weekly averages were calculated. Later, weighing was done on Mondays only and 2-weekly averages were calculated.

In the rats sacrificed at the end of the 5th and 10th treatment and 12th post-treatment week, the weight of the brain, liver, kidneys, femur, and heart was measured (before taking tissue samples). Form these data, relative organ weights, related to body weight, were calculated.

Immunohistochemistry

At the end of the 10th week, the four animals with the lowest memory performance were identified in each group (altogether 12 rats), and were selected for histology. Fixation was carried out by transcardial perfusion performed as above, with 50 ml saline and then with 4% paraformaldehyde fixative dissolved in 0.1 M phosphate buffer (pH 7.4). After removal, post-fixation and cryoprotection of the brain, coronal sections were cut on a cryostat microtome at 50 μm thickness and used for glial fibrillary acidic protein (GFAP) immunostaining.

Immunohistochemical reactions were performed on free floating sections using the routine avidine–biotine technique with nickel-enhanced 3,3′-diaminobenzidine as a chromogen. The density of GFAP immunoreactivity (ir) was determined in the hippocampal CA1 stratum radiatum and stratum oriens, and for the hilus of the dentate gyrus. The area of GFAP-ir structures was determined by video imaging using the Image Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD). Sections were examined under bright field with an Olympus microscope and a 10× objective. Images were recorded with a SONY 950-P CCD camera (Sony Corp., Tokyo, Japan) and digitized. The program expressed the area occupied by GFAP-ir elements as number of pixels having densities above the threshold. Measurements were taken in a blinded fashion from at least 16 sections for each animal group, and averaged.

Data Processing

The number of animals giving ''noise positive response'' in the ASR and PPI was evaluated with the χ^2 test. Other data were checked for normality by the Kolmogorov–Smirnov test. Depending on the distribution, the statistical analyses were carried out by two-way ANOVA or Kruskal–Wallis one-way ANOVA for the OF results in the post-treatment period (3×3; with $MnCl_2$ doses and d-A administration as independent variables). One-way ANOVA was used to evaluate the results of the ASR and PPI numerical data, Mn level, relative organ weights, body weight and of OF in the treatment period. Post-hoc analysis was performed by the Scheffe's or by Duncan's test. Group comparisons following the Kruskal–Wallis test were performed by the Mann–Whitney U-test. The limit was $P < 0.05$.

RESULTS

Manganese Levels in the Blood and Tissue Samples

Significant increase of tissue Mn levels was seen only in the group receiving the high dose. In the 5th treatment week, Mn content in the tail vein blood, and kidney and femur samples, was significantly elevated compared the controls and low dose rats. By the 10th treatment week, Mn levels in all tissue samples (brain and parenchymal organs) were significantly higher in high dose group than in the control and low dose animals. In the 12th post-treatment week, the liver, kidney, and femur samples still showed significant and dose-dependent Mn accumulation, whereas in the blood and brain tissues the Mn levels were no more significantly different from the control. Mn levels of all tissue samples collected and analyzed are given in Table II.

TABLE II. Total Manganese Content (μg/g; mean ± SD) in the Tissue Samples Taken at Different Times During the Experiment

		Measured Mn concentration (μg/g; mean ± SD) in the tissue samples on the:		
Tissue samples	**Groups**	**5th treatment week (n = 4)**	**10th treatment week (n = 6)**	**12th post-treatment week (n = 6)**
Total blood	Control	0.0314 ± 0.009	0.0118 ± 0.002	0.0138 ± 0.001
	15 mg/kg	0.0537 ± 0.012	0.0164 ± 0.005	0.0167 ± 0.003
	59 mg/kg	0.3167 ± 0.136**,##	0.0364 ± 0.016**,##	0.0165 ± 0.004
Cortex	Control	0.2167 ± 0.029	0.3134 ± 0.015	0.3130 ± 0.032
	15 mg/kg	0.2140 ± 0.032	0.3220 ± 0.056	0.3095 ± 0.056
	59 mg/kg	0.2823 ± 0.031	0.4420 ± 0.107*,#	0.2872 ± 0.029
Hippocampus	Control	0.1710 ± 0.012	0.4604 ± 0.035	0.5897 ± 0.102
	15 mg/kg	0.2607 ± 0.085	0.4680 ± 0.050	0.6022 ± 0.166
	59 mg/kg	0.2307 ± 0.063	0.6813 ± 0.138**,##	0.5662 ± 0.126
Liver	Control	2.2480 ± 0.313	2.1748 ± 0-235	1.6545 ± 0.144
	15 mg/kg	2.4790 ± 0.248	2.5650 ± 0.211	2.1248 ± 0.367**
	59 mg/kg	3.2220 ± 0.708	3.9834 ± 1.678*,#	2.7058 ± 0.237***,##
Kidney	Control	0.7913 ± 0.073	0.7532 ± 0.139	0.5427 ± 0.068
	15 mg/kg	0.8803 ± 0.276	0.7650 ± 0.151	0.5443 ± 0.039
	59 mg/kg	1.3747 ± 0.292*,#	2.2752 ± 1.465*,#	0.7758 ± 0.084***,###
Femur	Control	0.3453 ± 0.009	0.3453 ± 0.043	0.2617 ± 0.025
	15 mg/kg	0.3663 ± 0.031	0.3829 ± 0.054	0.2935 ± 0.045
	59 mg/kg	0.6160 ± 0.058***,###	0.6908 ± 0.299**,#	0.5152 ± 0.135***,###

n = number of animals sampled in each group.
*,**,*** $P < 0.05$ or $P < 0.01$ or $P < 0.001$ treated groups versus control group.
#,##,### $P < 0.05$ or $P < 0.01$ or $P < 0.001$ high versus low dose group.

Spatial Learning and Memory

During acquisition, and in the short term 4 hr working and reference memory and in all of the long-term working memory tests, both $MnCl_2$-treated groups showed, compared to control animals, a significant, dose-dependent decrease in the average mean memory performance, as seen in Table III.

Open Field Activity

OF behavior results of the treated and control animals in the treatment and post-treatment period are shown in Table IV. In 5th and 10th treatment week, both treated groups showed significant hypoactivity. First of all, the animals' horizontal exploration was reduced, but and in the 5th week also the vertical, and in the 10th week also the local activity. In the 7th post-treatment week, the difference between the control and both treated groups was minimal in all three forms of exploratory activity. On the 8th post-treatment week, 0.5 mg/kg d-A was injected ip., and OF activity was tested 15 min later. Locomotor activity increased to some extent but the effect was moderate and not significantly different in the treated and control animals (compared to results of 7th post-treatment week). On the 10th post-treatment week, amphetamine test was repeated with a 1.5 mg/kg d-A ip. injection. This time, local activity decreased in both treated groups ($P < 0.01$). Horizontal activity showed an increasing trend in both groups versus control but this effect was significant only in the low dose group ($P < 0.05$). The high dose group also showed clearly (albeit not significantly) increased vertical activity after 1.5 mg/kg d-A.

Acoustic Startle Response and Pre-Pulse Inhibition

In the 10th treatment week, $MnCl_2$ dose-dependently decreased the number of acoustic responses ($P < 0.01$; Fig. 1A). In the 7th post-treatment week, the number of responses was in each group similar to the 10th week's results, and the reaction of the treated rats was still significantly different from the control ($P < 0.05$). The alterations in the measurable parameters of the noise positive ASR responses in the 10th treatment and 7th post-treatment week were moderate (Fig. 1B,C). Onset latency increased significantly in both treated groups versus control ($P < 0.05$) in the 10th treatment week, but in the 7th post-treatment week, only in the high dose group. Peak time was significantly longer only in the 7th post-treatment week and only in the high dose group ($P < 0.001$).

In the 10th treatment week, the effect of PPI was significantly reduced in both treated groups (especially in the

TABLE III. Spatial Learning and Memory Performance of the Animals, During the Whole Course of the Experiment (1st–10th Treatment Week and 1st–5th Post-Treatment Week)

			Weekly memory performance (%)			
Weeks of the experiment	**Spatial learning and memory task periods in the 8-arm radial maze**		**Control Mean**	**15 mg/kg Mean**	**59 mg/kg Mean**	**Statistics**
10 weeks of the treatment period						
1st		Adaptation	—	—	—	—
2nd	Short-term retention tests (n = 16)	Acquisition	94.70 ± 4.42	88.80 ± 4.64**	89.83 ± 6.19*	$F(2;15) = 7.51$ $P < 0.01$
3rd		Working memory 2 hr	77.11 ±1.44	73.45 ± 1.83	67.55 ± 1.05	$F(2;12) = 1.10$; $P > 0.05$
4th		Reference memory	85.36 ± 4.46	76.18 ± 3.97**	69.41 ±9.70***,#	$F(2;12) = 24.68$; $P < 0.001$
5th		Working memory 4 hr	81.68 ± 3.18	71.11 ±5.26*	62.52 ± 3.12***	$F(2;12) = 16.85$; $P < 0.001$
6th–7th	Rest period	—	—	—	—	—
8th	Long-term memory (n = 12)	Return	95.49 ± 1.48	91.77 ± 2.67	69.41 ±9.70**	$F(2;12) = 7.86$; $P < 0.01$
9th		Working memory return, 2 hr	91.28 ± 4.16	77.49 ± 5.91**	67.14 ± 6.20***,#	$F(2;12) = 26.14$; $P < 0.001$
10th		Working memory return, 4 hr	93.42 ± 2.34	73.83 ± 4.72***	66.86 ± 2.20***,##	$F(2;12) = 79.40$; $P < 0.001$
5 weeks from the post-treatment period						
1st–2nd	Rest period[(e)]	—	—	—	—	—
3rd	Long-term memory[(e)] (n = 6)	Return[(e)]	91.77 ± 5.37	90.04 ± 4.77	86.79 ± 7.12	$F(2;12) = 0.94$; $P > 0.05$
4th		Working memory return[(e)], 2 hr	96.67 ± 2.36	74.8 ± 3.88***	68.54 ± 2.73***,##	$F(2;12) = 116.3$; $P < 0.001$
5th		Working memory return[(e)], 4 hr	97.39 ± 3.26	78.01 ±5.63***	65.11 ±4.55***,###	$F(2;12) = 62.77$; $P < 0.001$

Performance (see Materials and Methods Section for calculation) is given as weekly average of group averages. Mean ± SD, n = number of animals per group.

*,**,*** $P < 0.05$ or $P < 0.01$ or $P < 0.001$ treated groups versus control group.

#,##,### $P < 0.05$ or $P < 0.01$ or $P < 0.001$ high dose group versus low dose group.

[(e)]Indicates elimination, that is, post treatment, period.

TABLE IV. Effects of $MnCl_2$ on the Spontaneous and d-Amphetamine (d-A)-Induced Open Field (OF) Activity of the Animals During the Treatment and Post-Treatment Period

			OF activity of rats (mean±SD)		
Weeks	**Groups**	**d-A (mg/kg)**	**Horizontal (length run, cm)**	**Local (grooming, counts)**	**Vertical (rearing, counts)**
5th treatment week (n = 12)	Control	—	523.94 ± 103.93	263.24 ± 34.70	34.92 ± 6.81
	15 mg/kg		362.83 ± 73.25**	244.57 ± 46.02	18.78 ± 5.95**
	59 mg/kg		338.24 ± 55.74**	222.95 ± 43.64	21.11 ±9.29**
10th treatment week (n = 12)	Control	—	443.65 ± 78.51	259.59 ± 67.24	35.78 ± 9.52
	15 mg/kg		362.96 ± 57.86*	185.14 ± 27.12*	30.56 ± 5.38
	59 mg/kg		372.89 ± 53.01*	184.94 ± 34.70*	34.78 ± 8.09
7th post-treatment week (n = 6)	Control	—	342.84 ± 65.25	254.68 ± 37.85	26.20 ± 3.03
	15 mg/kg		371.76 ± 83.76	225.31 ±45.35	24.40 ± 11.52
	59 mg/kg		402.21 ±72.74	237.67 ± 20.84	26.80 ± 4.02
8th post-treatment week (n = 6)	Control	0.5	666.47 ± 231.20	339.55 ± 67.79	38.60 ± 21.43
	15 mg/kg		749.93 ± 173.45	327.64 ± 34.57	43.20 ± 21.74
	59 mg/kg		556.97 ± 119.52	292.64 ± 61.43	37.80 ± 13.29
10th post-treatment week (n = 6)	Control	1.5	765.45 ± 115.70	460.96 ± 94.54	73.80 ± 14.65
	15 mg/kg		1024.58 ± 44.99*	318.35 ± 69.85*	72.60 ± 16.73
	59 mg/kg		870.63 ± 164.21	340.38 ± 49.91*	92.00 ± 18.34

*,** $P < 0.05$ or $P < 0.001$ treated groups versus control.

low dose group) versus control (Fig. 2A). It was, in fact, changed to facilitation, that is, there were more responses than without pre-pulse ($P < 0.01$). At the same time, latency of the responses decreased ($P < 0.01$) without clear changes in the peak time (Fig. 2B,C). In the 7th post-treatment week, there was no more significant difference between the effect of the pre-pulse in the $MnCl_2$-treated and control groups.

Body Weight and Relative Organ Weights

In the 6th–10th treatment week, the weekly average body weight in the high dose group was significantly ($P < 0.01$) reduced compared to the controls. In the low dose group, the body weight deficit was significant versus control in the 8th, 9th, and 10th weeks of the treatment period. One week after cessation of $MnCl_2$ administration, the average body weight was in both treated groups still significantly reduced ($P < 0.01$). In the high dose group, the body weight deficit remained significant during the whole post-treatment period (not shown). The relative organ weights, compared to body weight, in the 10th treatment and 12th post-treatment weeks are given in Table V.

Immunohistochemistry

In the density of GFAP immunoreactive structures, no differences were seen between the control and treated animals in the hippocampal CA1 region of the animals, In the hilar part of the dentate gyrus, however, treated animals showed an increase of GFAP-ir density (Fig. 3). The effect was significantly dose dependent (control vs. low dose, $P < 0.05$; control vs. high dose, $P < 0.01$).

DISCUSSION

Oral $MnCl_2$ administration, 15 and 59 mg/kg b.w. for 10 weeks, resulted in considerable internal exposure, first of all in the high dose group; indicated by the Mn levels in the samples of blood, some organs (liver, kidney, femur), and two brain-regions (cortex and hippocampus). Mn levels in the control rats' brains, exposed only to Mn present in standard rodent food and drinking water, were similar to literature data [Rehnberg et al., 1982].

Typically, the human internal exposure, indicated by blood Mn levels of exposed workers [Mergler et al., 1994; Lucchini et al., 1999; Mergler, 1999; Myers et al., 2003], is lower than what was seen in our treated rats. Also, the threshold for symptoms seems to be lower, between 7.5 and 10 μg/L [Lucchini et al., 1999; Mergler, 1999]. Some recent studies indicate, however, that many factors, including age, sex, co-exposure, nutritive influence, familial sensitivity or length of exposure, have more influence on the development and severity of neurological sequelae than doses, external or internal [Desole et al., 1994; Lucchini et al., 1999; Bowler et al., 2006].

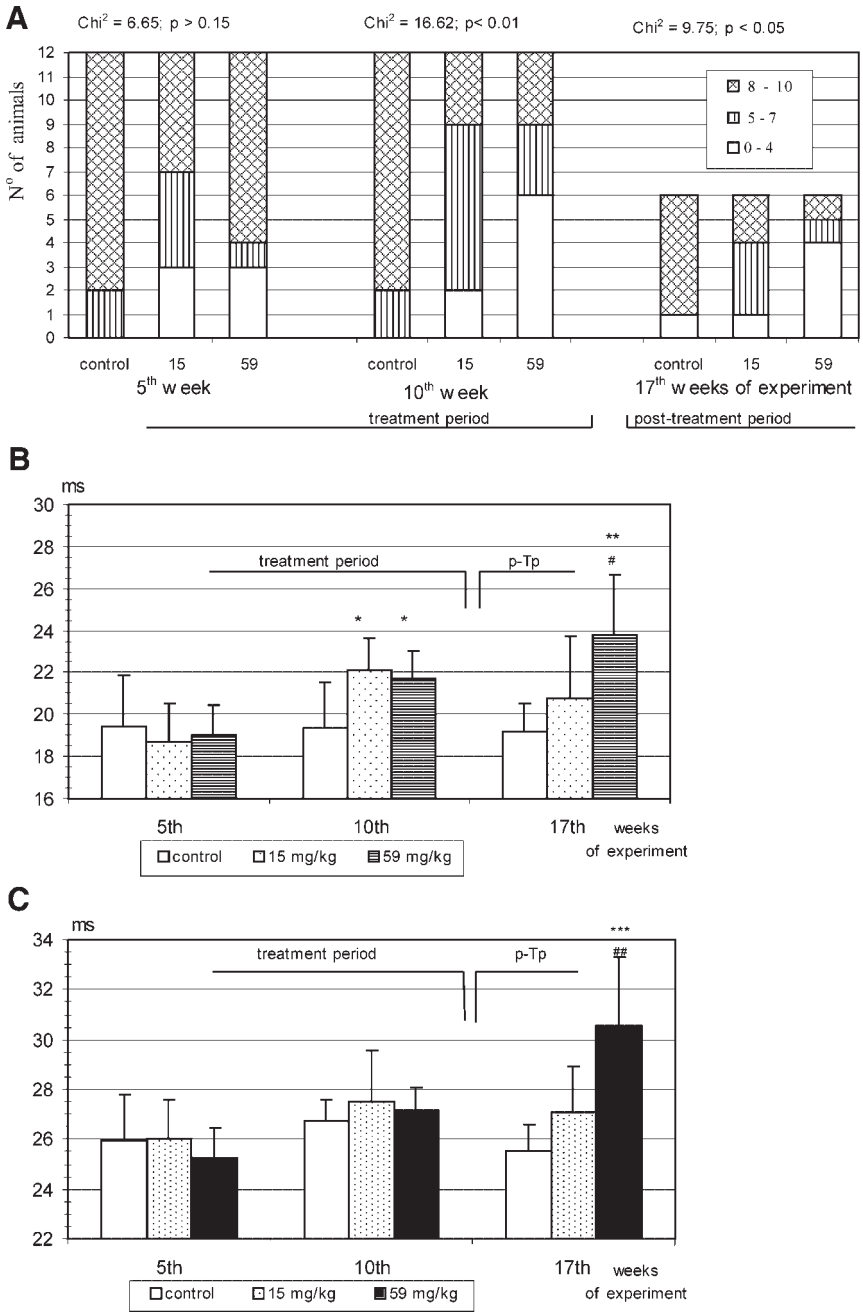

FIGURE 1. Number (**A**) and measured parameters (**B**,**C**) of the noise positive responses in the ASR test in the 5th and 10th treatment week and 7th post-treatment week. A: The bars show the distribution of rats according to the number of noise-positive responses given (see inset). Significance by χ^2 test is given above the bar groups. B: Onset latency, and (C) peak time of the responses (ordinate; mean + SD). See inset in B for groups. p-TP: post-treatment period. $^{*,**,***}P < 0.05$ or $P < 0.01$ or $P < 0.001$ treated groups versus control group. $^{\#,\#\#}P < 0.05$ or $P < 0.01$ high dose versus low dose group.

Once absorbed, Mn alters its own turnover in the tissues, possibly by a mechanism linked to calcium flux [Mertz et al., 1994]. The preferential sites of deposition are the mitochondria-rich tissues (e.g., liver and pancreas), and—after passing the blood–brain barrier—the CNS (there is also a slow shift of Mn from the parenchymal organs to the CNS). In a study in adult male rats, 21-day oral exposure to 50 mg/kg $MnCl_2$ increased the striatal, cerebellar, and cortical Mn concentrations [Dorman et al., 2000], similarly to what was found in our experiment. In the femur and kidney, increased Mn level

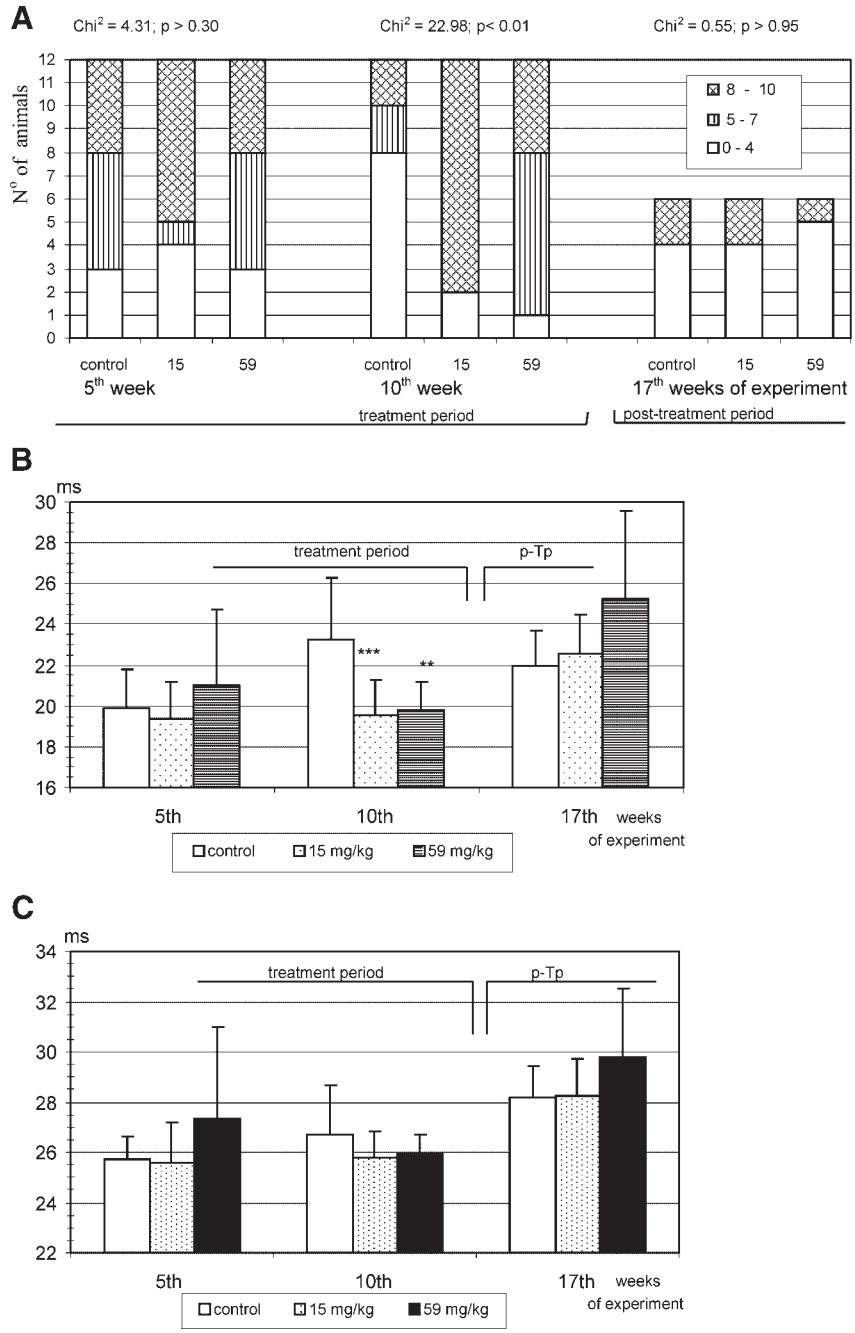

FIGURE 2. Number (**A**) and measured parameters (**B**,**C**) of the noise positive responses of the rats, tested with pre-pulse inhibition, in the 5th and 10th treatment week and 7th post-treatment week. Displayed as in Fig. 1.

and relative weight alteration was in parallel (Tables II and V).

In the 10th treatment week, Mn concentration in the high dose rats (compared control and low dose) was significantly elevated in the cortex and hippocampus, similarly to what was described in the literature [Takeda et al., 1998; Dorman et al., 2000]. Hippocampus, together with the medial prefrontal cortex, amygdala, striatum, pallidum, and pedunculopontine tegmental (PPTg) area, constitute the ''limbic CSPP'' circuitry [Swerdlow et al., 2001]. This circuitry converges with the primary acoustic startle (ASR) circuit [Lee et al., 1996] at the level of the nucleus reticularis pontis caudalis (PnC). Reducing the ascending auditory glutamatergic input by glutamate antagonists, or attenuating the psychomotor stimulant rewarding effects of mesolimbic and mesocortical dopaminergic

TABLE V. Relative Organ Weights (Mean ± SD, n = Animals Sampled in Each Group)—Related to 100 g Body Weight—in the Treated and Control Rats on the 5th and 10th Treatment and on the 12th Post-Treatment Weeks

Organs	Groups	5th treatment week (n = 4)	10th treatment week (n = 6)	12th post-treatment week (n = 6)
Liver	Control	3.5735 ± 0.1661	3.2073 ± 0.2500	3.0009 ± 0.4257
	15 mg/kg	3.7677 ± 1.5359	2.9741 ±0.2679	2.7695 ± 0.3431
	59 mg/kg	3.2331 ±0.4187	3.1495 ± 0.1828	2.8559 ± 0.2091
Kidney	Control	0.7317 ± 0.0726	0.8369 ± 0.0388	0.7108 ± 0.0621
	15 mg/kg	0.9257 ± 0.0569*	0.0695 ± 0.0.0695*	0.7022 ± 0.0512
	59 mg/kg	0.9075 ± 0.0873*	0.7390 ± 0.0749	0.7101 ±0.0505
Femur	Control	0.1857 ± 0.0104	0.2531 ±0.0403	0.2949 ± 0.0256
	15 mg/kg	0.2315 ± 0.0179*	0.2354 ± 0.0079	0.2653 ± 0.0364
	59 mg/kg	0.2328 ± 0.0262*	0.2938 ± 0.0191$^{*,\#}$	0.2728 ± 0.0311
Heart	Control	0.2962 ± 0.0179	0.2981 ±0.0104	0.2700 ± 0.0331
	15 mg/kg	0.3452 ± 0.0473	0.2749 ± 0.0062*	0.2786 ± 0.0123
	59 mg/kg	0.3321 ±0.0523	0.3220 ± 0.0178$^{*,\#\#\#}$	0.2749 ± 0.0306

n = number of animals sampled in each group.
*$P < 0.05$ treated groups versus control group.
$^{\#,\#\#\#}P < 0.05$ or $P < 0.001$ high dose versus low dose group.

projections by selective dopamine reuptake inhibitors given into the nucleus accumbens [Wise, 1998], resulted in reduced ASR. In our study, significant dose-dependent decrease in the magnitude of the ASR was seen in the $MnCl_2$-treated rats in the 10th treatment and 7th post-treatment weeks, in agreement with Dorman et al. [2000].

Locomotor activity depends on mesolimbic and mesocortical dopaminergic neuronal transmission [Fink and Smith, 1980] with the involvement of motor control centers such as the globus pallidus, substantia nigra, and deep cerebellar nuclei [Oberlander et al., 1987]. In the ventral tegmental area, dopamine-containing neurons are thought to play (by connection of D2 and $GABA_A$ receptors) an important role in motivation, emotion, and ASR amplitude [Gifkins et al., 2002]. In chronic manganese exposure, liver failure (together with hyperammonemia) can affect the substrate supply for the synthesis of the monoamine neurotransmitters [Verity, 1999]. Another probable outcome, NMDA-mediated neurotoxicity, results from ammonia-dependent elevation of extracellular glutamate [Butterworth, 1998] and Mn-dependent inhibition of astrocytic glutamine synthetase [Aschner et al., 1999].

Hypoactivity of the serotoninergic systems was also implied in the pathogenesis of hepatic encephalopathy in animals with liver disease [Yourdaydin et al., 1990]. In our experiments, a decrease in the spontaneous horizontal and local activity developed after 10 weeks of $MnCl_2$ exposure, indicating that both serotoninergic and dopaminergic control

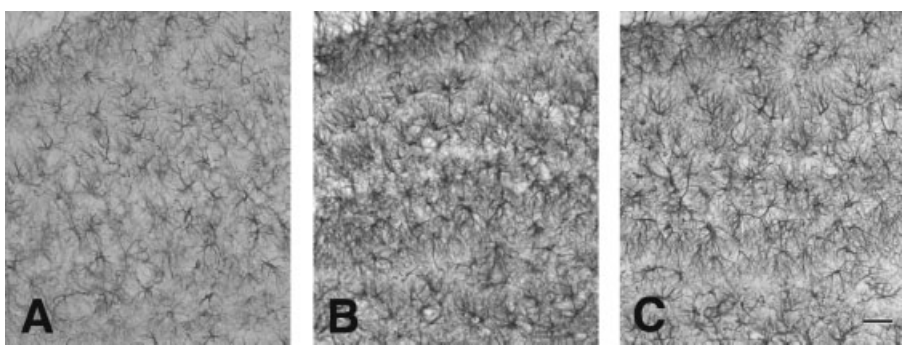

FIGURE 3. GFAP immunoreactive astrocytes in the hilus of the dentate gyrus of the control (**A**), low dose (**B**), and high dose (**C**) Mn-treated rats, sacrificed on the 10th experimental week. Electron micrograph, nickel-enhanced 3,3′-diaminobenzidine staining. Bar represents 50 μm.

was probably affected by Mn [Subhash and Padmashree, 1991]. Mn-induced lesion of the nuclei in the hypothalamic lateral area could have contributed to body weight loss and altered motility [Dorman et al., 2000], observed simultaneously from the 6th treatment week to the 1st post-treatment week.

In the 7th post-treatment week, repeated spontaneous OF activity test showed no significant difference between controls and treated groups. By giving 1.5 mg/kg d-A in the 10th post-treatment week, massive increase of the horizontal activity, but no alteration in rearing, was induced in the low dose group, while in the high dose group the increase of rearing was higher than that of the horizontal activity. Passing the blood–brain barrier, d-A (in the doses applied by us) increases the release of catecholamines from the synaptic endings in the nigrostriatal and in the mesolimbic/mesocortical pathways. During the 10 weeks of $MnCl_2$ exposure, dopamine may have been depleted and the efficacy of dopaminergic transmission lost. In such a situation, the sensitivity of the post-synaptic dopamine receptors could increase which would explain why, in the animals treated with d-A, locomotor activity returned to or beyond control level.

Cholinergic control is also known to exist in the spontaneous locomotor activity. Rodents, deficient for M1 muscarinic receptor, had increased spontaneous locomotion, combined with an increased response to the stimulatory effect of amphetamine [Mattsson et al., 2004]. In our experiments, the performance in acquisition, and in reference and short term (4 hr) and long-term working memory, was significantly decreased in the treated animals (Table III). In the initial acquisition and retention in a spatial learning task, the cholinergic septohippocampal and magnocellular forebrain-cortical projections, and the dentate gyrus (important in the acquisition of new information and possibly an integral neural substrate for spatial reference and spatial working memory; Eyre et al. [2003]), play a major role, which can be weakened due to the Mn-induced decrease in choline acetyltransferase activity [Lai et al., 1981].

Neurotoxic damage to the granule cells in the dentate gyrus of the human or rodent brain, detectable by immunoreactive staining, can significantly increase the error rate during radial maze learning. In our experiments, gliotic degeneration in the dentate gyrus and impaired working memory performance were seen together in the 10th treatment week. Selective loss of dentate granule cell populations, leading to persistent impaired acquisition and radial maze performance, and alterations of the ASR, could also be obtained by colchicine injected into dentate gyrus [Walsh et al., 1986].

In the 5th week, the 4-hr working memory performance, that is, the rats' ability to keep information for a shorter period [Beatty and Shavalia, 1982] was significantly reduced in both treated groups versus control, and the difference of high versus low dose group was also significant. At this time, elevated Mn levels (vs. control) were measured in the cortex samples of high dose rats, and in the hippocampus in both treated groups (significance failed because of the low number of animals dissected). In the 10th week, long-term working memory deteriorated further in both treated groups versus control, although significantly elevated Mn levels were seen only in the cortex and hippocampus samples of the high dose rats.

Oral $MnCl_2$ treatment resulted in significant increase of the blood, liver, kidney, femur, cortex, and hippocampus Mn levels. During the treatment period, alterations in maze learning performance, OF activity and ASR/PPI magnitude with increased gliosis in the hilar part of the hippocampal dentate gyrus indicated the decrease of neurotransmitter levels in the structures responsible for these behavioral functions, such as the glutamatergic auditory pathway and the nigrostriatal dopaminergic system. In the post-treatment period, Mn levels in all tissue samples returned to control. The alterations of the OF activity and the ASR reaction failed to cease, however, indicating a long-lasting effect of $MnCl_2$ on these functions. Taking into account that some of the effects proved to outlast the period of exposure, and the importance of the probably involved systems in the higher-order nervous functions of humans, the neurotoxic risk of occupational (and environmental) $MnCl_2$ exposure seems to deserve ongoing attention.

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