Cognitive Deficits and Changes in Gene Expression of NMDA Receptors after Prenatal Methylmercury Exposure

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Previous studies showed learning and memory deficit in adult rats that were prenatally exposed to methylmercury chloride (MMC) in an advanced stage of pregnancy (15 days). Under these conditions, the cognitive deficits found at 60 days of age paralleled particularly changes in the N-methyl-D-aspartate (NMDA) receptor characteristics. In the present study, we report the behavioral effects of a single oral dose of MMC (8 mg/kg) administered earlier at gestational day 8. The use of different learning and memory tests (passive avoidance, object recognition, water maze) showed a general cognitive impairment in the in utero-exposed rats tested at 60 days of age compared with matched controls. Considering the importance of the glutamatergic receptor system and its endogenous ligands in learning and memory process regulation, we surmised that MMC could affect the gene expression of NMDA receptor subtypes. The use of a sensitive RNase protection assay allowed the evaluation of gene expression of two families of NMDA receptors (NR-1 and NR-2 subtypes). The result obtained in 60-day-old rats prenatally exposed to MMC, showed increased mRNA levels of the NR-2B subunit in the hippocampus but not in the frontal cortex. The data suggest that the behavioral abnormalities of MMC-exposed rats might be ascribed to a neurotoxic effect of the metal that alters the gene expression of a specific NMDA receptor subunit in the hippocampus. Key words: gene expression, learning, memory, methylmercury, NMDA receptors, prenatal exposure. Environ Health Perspect 110(suppl 5): 855–858 (2002).

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It is well established that prenatal exposure to methylmercury (MM) can have profound effects on the mental development of children. Clinical as well experimental studies indicate that the central nervous system is the main target organ of MM neurotoxicity, particularly during the fetal period.

Although high-dose exposures result in clear evidence of neurological disorders, the more subtle actions of moderate to low doses of MM on important parameters of behavioral development are becoming increasingly evident (1). Consistent with results from human studies, experimental data obtained in rodents provide information on developmental effects of MM and its mechanism of action. The consequences of in utero exposure to MM in rats range from increased rates of intrauterine death, delayed developmental growth, and altered brain cellular arrangement to more subtle effects, depending on the dose and the time of exposure during gestation.

One of the most frequent findings related to prenatal MM exposure is represented by learning and memory deficit (2–5), which was confirmed and expanded also by our groups (6,7). The learning and memory deficits were described as a consequence of a single prenatal exposure to the metal at different stages of gestation. Our previous studies performed in rat offspring after methylmercury chloride (MMC) exposure at gestational day 15 demonstrated changes in the activity of dopaminergic, cholinergic, GABAergic, glutamatergic, and opiateergic systems, at different stages of postnatal life, in parallel with altered brain functional activity. Some of these effects are transient, such as those related to the dopaminergic (6), cholinergic (7), and GABA–benzodiazepine (8) receptor systems; others, such as those related to the opiateergic system, are detectable only at the adult stage (9) and hence must be considered delayed effects. Moreover, we observed long-lasting changes in the glutamatergic system (6) that were tentatively associated with learning and memory deficits found in MMC-exposed animals. In the present study, to increase our knowledge of postnatal effects due to MM neurotoxicity and its neurochemical mechanisms, we studied the effects of a single dose of MMC administered in rats on gestational day 8.

The performance of adult offspring on different tests of learning and memory was assayed at 60 days of age in parallel with the gene expression of N-methyl-D-aspartate (NMDA) receptors subunit measured in two brain areas. It is now commonly accepted that activation of glutamatergic receptors plays a pivotal role in mediating learning and memory (10). The cloning of different glutamate receptors provides the opportunity to investigate in more detail the regulation of this system (11). The NMDA receptor is a glutamate-gated cation-specific ion channel involved in many forms of neuronal plasticity resulting in altered behavior (12,13).

Therefore, we tested the hypothesis that the cognitive dysfunctions observed after MMC exposure in utero correlate with changes in the levels of expression of various NMDA receptor subtypes. The gene expression of two families of NMDA receptors (R) NR-1 and NR-2 and their subtypes were simultaneously evaluated in frontal cortex and hippocampus of rats prenatally exposed to MMC. Here we report that the learning and memory deficits found in rats at postnatal day 60 parallel changes in the gene expression of NMDA receptors.

Materials and Methods

Animals

We used Sprague-Dawley female rats (Harlan Italy, Udine, Italy) weighing 220–250 g. They were maintained in a climatized room (20–22°C) with free access to food and water and exposed to a light cycle of 12 hr/day. Pairs of females were placed with single male rats in late afternoon. Vaginal smears were taken the next morning at 9:00 AM. The day on which sperm was present was designated day 1 of gestation. Females (n = 20) were randomly assigned to two equal experimental groups and individually housed in standard cages. On day 8 of gestation, pregnant females were administered saline or a single dose of MMC (8 mg/kg) in the volume of 2 mL/kg of saline by oral gavage. After birth, all 20 litters were culled to six pups per litter. They were weighed once weekly and weaned at 26 days after birth.

All procedures involving animals were performed in accordance with the guidelines of the Italian (14), European legislation (15), and the U.S. National Institutes of Health (16).

Open Field Test

The tests were performed by placing each animal in the center of a square arena (100 × 100 × 50 cm high) with a black floor. Rats were continuously filmed for 10 min with a video camera connected to a computerized system (Motion Analyzer BM 800, Biomedica Mangoni, Pisa, Italy), by which the following parameters were recorded:

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number of crossings of 10 × 10 cm squares, total length of the rat ambulatory activity, and time spent in immobility. An observer unaware of treatment recorded, by means of an external video, the number of rearings (when the rat reared upon its hind feet).

Passive Avoidance Test

Passive avoidance behavior was evaluated using a step-through method (passive avoidance apparatus; Ugo Basile, Comerio, Varese, Italy). The test apparatus consisted of an illuminated compartment connected to a dark chamber via a guillotine door. In brief, the rat was placed in the illuminated closed compartment; after 2 min the door was opened and the latency (approach latency in the first trial) to enter the dark compartment was recorded. Animals that do not step through the door within the cutoff time (300 sec) were not used. Two hours later, the rat was again placed in the illuminated chamber and allowed to enter the dark compartment: its latency (approach latency in the second trial) to step through the door was recorded. This last trial was followed by single 2-sec unavoidable foot shock (90 mV) immediately after entering the dark compartment. The retention of the passive avoidance response was tested 24 hr after the learning trial. The animal was placed in the illuminated compartment and the latency to reenter (avoidance latency) the dark compartment was recorded. The retention session had a maximal observation time of 300 sec.

Object Recognition Test

The test was carried out according to the procedure of Ennaceur and Delacour (17) with minor modification. Briefly, the rat was individually placed in Plexiglas cage (60 × 36 cm) illuminated by a 60-W lamp suspended 50 cm above the box. The objects to be discriminated were cubes and pyramids made of gray-painted wood. The day before testing, the rats were allowed twice (in the morning and in the afternoon) to explore the box for 5 min. On the day of the test, a session of two trials separated by an intertrial interval of 5 min was carried out. In the first trial (T1), lasting 3 min, two identical objects were presented in two opposite corners of the cage. Exploration was considered to be directing the nose at a distance of <2 cm from the object and/or touching it with the nose. During the second trial (T2), a new object replaced one of the objects presented in T1 and the rat was left in the box for 5 min. The time spent in exploration of the familiar (F) and the new object (N) was recorded separately, and the difference between the two exploration times was taken as discrimination index (D = N − F). To avoid olfactory stimuli, the objects to be discriminated were cleaned carefully.

Water Maze Test

The maze, as previously described by Giurgea and Moutarieux-Lesuisse (18), consists of a rectangular pool (120 × 50 × 40 cm deep) made of stainless steel with four L-shaped partition walls that create four blind compartments. The pool is filled to 24 cm with 20°C water. The rat is placed into the maze at the starting point, which is illuminated by 60-W lamp, located at the distance of 30 cm over the water surface. The animal may escape from the water by finding (swimming to and climbing onto) a grid, 45° inclined, placed in the exit point of the pool. The test was carried out daily for 5 consecutive days. For each trial, the experimenters evaluated three parameters: a) number of animals who found the exit grid within 10 min, b) exit latencies, and c) number of errors for each rat (errors were entrance into blind compartments or swimming in the direction opposite to the exit grid). The trials lasted for a maximum observation time of 10 min. In the first trial only, the rats that did not find the exit grid within 10 min were manually guided toward it and placed upon it for 1 min.

RNA Preparation

Rats were sacrificed by decapitation. The brain regions were rapidly dissected, frozen in liquid nitrogen, and stored at −70°C for further analysis. Tissues from rat hippocampus and frontal cortex were homogenized in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate, pH 7.5; 0.5% sarcosyl; and 0.1% 2-mercaptoethanol), and total RNA was isolated by phenol–chloroform extraction (19). Quantification was carried out by absorption at 260 nm, and RNA was re-precipitated in ethanol for RNase protection assay.

Probe Preparation and RNase Protection Assay

The cDNAs for the different NMDA receptor subunits were arranged to obtain proper templates for the in vitro transcription of cRNA probes to be used in the RNase protection assay (20). The cRNA probes for NMDA receptor subunits and the relative protected fragment (p.f.) were the following: NR-1 = 438, p.f. = 414; NR-2A = 187, p.f. = 171; NR-2B = 310, p.f. = 264; NR-2C = 244, p.f. = 213, pTRI-actine-Rat (Ambion-Austin, TX, USA) containing a portion of rat β-actin cDNA of 195, with a p.f. = 126.

The RNase protection assay was performed on a 10–15 µg sample of total RNA as previously described (21). Briefly, after ethanol precipitation, total RNA, obtained from different tissues, was dissolved in 20 µL of hybridization solution (80% formamide; 40 mM PIPES, pH 6.4; 400 mM sodium acetate, pH 6.4; 1 mM EDTA) containing 150,000 cpm of each 32P-labeled cRNA probe (specific activity > 108 cpm/sg). After being heated at 85°C for 10 min, the cRNA probes were allowed to hybridize the endogenous RNA overnight at 45°C. At the end of the hybridization, the solution was diluted with 200 mL of RNase digestion buffer (300 mM NaCl; 10 mM Tris HCl, pH 7.4; 5 mM EDTA, pH 7.4) containing a 1:200 dilution of an RNase cocktail (1 mg/mL RNase A, 20 U/mL RNase T1) and incubated for 30 min at 30°C. Protease K (10 µg) and sodium dodecyl sulfate (10 µL of 20% stock solution) were then added to the sample, and the mixture was incubated at 37°C for an additional 15 min. At the end of the incubation, the sample was extracted with phenol–chloroform and precipitated by ethanol.

The pellet (containing the RNA:RNA hybrids) was dried and resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), boiled at 95°C for 5 min, and separated on a 5% polyacrylamide gel under denaturing conditions (7 M urea). The protected fragments were visualized by autoradiography.

RNA Calculation and Statistical Analysis

The levels of mRNA for NMDA receptor subunits were obtained by densitometry measurements of autoradiographs analyzed with a Biorad GS-690 scanner (Biorad, Milan, Italy). Optical densities from all experimental groups were analyzed by analysis of variance (ANOVA). Individual group differences were further analyzed by the Dunnett t-test. Optical densities from experimental groups were than expressed and presented as mean percentage of control values for graphic clarity; that is, NMDA receptor subunit/β-actin levels were expressed as a percentage of control animals. β-Actin was employed as internal standard for RNase protection assay and did not differ across experimental groups. Statistical differences were considered significant when p > 0.05 (ANOVA and Dunnett t-test).

Statistical Analysis

The results obtained in the behavioral experiments are expressed as the mean ± SEM. The comparison between saline and MMC-exposed rats was carried out by means of the Mann–Whitney U-test. The data obtained by both groups of rats in the different sessions of water maze testing were analyzed using one-way ANOVA followed by the Newman–Keuls post hoc test.

Results

Locomotor Behavior

The results obtained in the open field test (Table 1) failed to show any general motor deficits in MMC-exposed rats because they

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performed the same as control rats in total length of ambulatory activity. It is noteworthy, however, that the number of rearings was significantly reduced during the 10-min test in MMC-exposed rats, suggesting an impaired exploratory behavior.

**Learning and Memory Tests**

The data obtained in the passive avoidance test (Table 2) indicated that approach latencies in the first and second trial were not influenced by MMC exposure, whereas the avoidance latency was significantly shorter than that in control animals.

Regarding the object recognition test, control rats were able to discriminate, after a 5-min intertrial interval, between a familiar and a new object, as demonstrated by the longer time spent in exploring the new rather than the familiar one (Table 3). On the contrary, no difference in the exploration time of the two objects was observed in MMC-exposed rats. Consequently, discrimination index, which is the difference between the exploration time of the new and the familiar objects, was significantly reduced in MMC-exposed rats compared with controls (Mann-Whitney U-test).

The learning curve (i.e., the number of errors in the five daily trials) obtained in the water maze test is presented in Figure 1. All rats showed a progressive reduction in the number of errors during the 5 days. However, MMC-exposed rats made a significantly increased number of errors during the performance on the second day compared with controls. Moreover, the number of rats that reached the exit was lower in treated rats (66%) than in the control group (93%). On the third trial, although the number of rats that exited from the water maze was practically equal in the two experimental groups, the number of errors (Figure 1) and the time of performance were greater in the MMC-exposed group than in the control group (113 ± 11.2 vs. 76 ± 6.8 sec).

**Gene Expression of NMDA**

The results obtained in the study of the gene expression of NMDA receptors, measured by an RNase protection assay of NMDA receptor subunits in frontal cortex and hippocampus of control and MMC-exposed rats at 60 days of age, are shown in Figure 2. In the autoradiogram, the increase in NR-2B expression in lane 2, related to the hippocampus of MMC-exposed rats, is evident in comparison with that of controls (lane 1). No difference, on the other hand, is noted in the frontal cortex of MMC-exposed rats and controls (lanes 3 and 4). The levels of mRNA expression of the NR-2B subunit, expressed as a percentage of control, are shown in Figure 3. The levels of mRNA expression of the other NMDA receptor subunits NR-1 and NR-2A measured in both hippocampus and frontal cortex did not show statistically significant differences (Figure 2).

**Discussion**

MM is known to affect the central nervous system of children exposed to the metal during fetal development. Several neurological symptoms and mental retardation have been described in children as a consequence of

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**Table 1.** Locomotor activity in 60-day-old rats prenatally exposed to MMC compared with controls during open field test.

| Exposure | Total route (cm) | No. of crossings | No. of rearings | Time spent in immobility (sec) |
|----------|-----------------|-----------------|----------------|-----------------------------|
| Saline   | 7,252.5 ± 449.6 | 179.7 ± 13.9    | 48.3 ± 5.0     | 128.1 ± 13.2               |
| MMC      | 7,242.1 ± 498.6 | 147.0 ± 15.2    | 31.4 ± 3.8*    | 143.9 ± 12.0               |

The parameters were measured as described in "Materials and Methods." The values are expressed as mean ± SEM (n = 10). Statistical analysis was performed using the Mann-Whitney U-test. *p = 0.05 vs. saline.

**Table 2.** Effect of prenatal MMC exposure on the performance of 60-day-old rats in a passive avoidance task.

| Exposure | Approach latency (sec) | Avoidance latency (sec) |
|----------|------------------------|-------------------------|
| Saline   | 74.6 ± 20.2            | 10.1 ± 8.6              |
| MMC      | 51.3 ± 7.8             | 14.2 ± 6.1              |

The values are expressed as mean ± SEM (n = 15). Statistical analysis was performed using the Mann-Whitney U-test. *p = 0.05 vs. saline.

**Table 3.** Effect of prenatal MMC exposure on object recognition test in 60-day-old rats compared with controls.

| Exposure | Exploration time (sec) | N | D |
|----------|------------------------|---|---|
| Saline   | 5.5 ± 1.2              | 135.5 ± 2.5*             | 7.9 ± 2.4 |
| MMC      | 10.3 ± 4.9             | 126.2 ± 7.2              | 2.3 ± 2.0* |

Abbreviations: F, exploration time of familiar object; N, exploration time of new object; D, discrimination index (N–F). The values are expressed as mean ± SEM (n = 10). Statistical analysis was performed using Mann-Whitney U-test.

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**Figure 1.** Learning curves of MMC-exposed rats in water maze test in comparison with controls. The data reported show the numbers of errors made by rats during the five consecutive daily trials. The values are expressed as mean ± SEM obtained by groups of 15 rats each. Statistical analysis was performed using one-way ANOVA followed by the Newman-Keul post hoc test. *p < 0.001 vs. matched controls.

**Figure 2.** Representative autoradiogram of an RNase protection assay of NMDA receptor subunits in frontal cortex and hippocampus of control and MMC-treated rats at 60 days of age. The autoradiogram shows the levels of mRNA expression of the NR-2B subunit in MMC-exposed rats (lane 2) compared with controls (lane 1). The X-ray film was exposed for 6 hr at −70°C with an intensifying screen.
maternal ingestion of MM-contaminated food during gestation. The developing nervous system seems to be extremely susceptible to MM, and this metal has been shown to induce subtle behavioral abnormalities at dose levels below those associated with overt symptoms of neurotoxicity. In this context, it is worth noting that children with evident neurological symptoms are born from asymptomatic mothers exposed to MM. Evidence have been provided by many researchers that a single prenatal administration of MM to rats during late gestation produces cognitive deficits, represented mainly by learning and memory disturbances (2,3,6,7,9).

The cognitive deficits found in adult rats prenatally exposed to MMC are associated with changes in several neurotransmitter systems, comprising mainly the opiateergic and the glutamatergic systems, which seem to undergo long-lasting alterations. In particular, changes in the characteristics of NMDA receptors (6) and one of its potential endogenous ligands, quinolinic acid (22), have been described. These results prompted us to test whether prenatal exposure to MMC would affect the above-mentioned neurotransmitter systems even when the metal is administered during an early stage of gestation.

The results of the present study, using different behavioral tests, confirm that a single prenatal exposure of rats to MMC on day 8 of gestation induces learning and memory deficits still present at 60 days of age. In particular, the water maze test showed a worsening ability to learn in the MMC-exposed rats because the deficit was recorded in the first part of the test. On the other hand, the passive avoidance and object recognition tests demonstrated the ability of MMC to impair memory processes. This effect apparently was not attributable to motor impairment because the statistical analysis of locomotor behavior did not show changes in three of four parameters considered in the open field test. On the other hand, the reduction in the number of rearing appears to reflect mainly a decreased exploratory activity. These data seem to suggest that learning and memory processes are impaired by prenatal MMC exposure independent of the period of gestation.

It is well known that the activation of the glutamatergic system is implicated in the modulation of learning and memory processes. Our previous demonstrations that MMC exposure changes the characteristics of NMDA receptors (6) and increases the levels of quinolinic acid, an endogenous agonist of these receptors that potentially exerts neurotoxic effects, seem to implicate this excitatory neurotransmission system in the neurological brain dysfunction associated with MMC exposure. The altered level of NR-2B mRNA in the hippocampus may reflect a modification of the ontogenetic regulation of NMDA receptor type 2 subunit NR-2B. Indeed, NR-2B increases rapidly during the first week of postnatal life compared with the NR-2A subunit, which at this time is expressed to low levels, indicating that NR-2B will probably be dominant in determining the NMDA properties during the first period of postnatal life (17). Our data can provide a molecular correlate of properties of NMDA receptors and the impaired cognitive deficit of rats prenatally exposed to MMC.

The present data showing that prenatal exposure to MMC in an early stage of gestation induces an altered gene expression of NMDA receptors in the hippocampus, a brain area highly involved in cognitive function regulation, seem to confirm the above suggested hypothesis.

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