Developmental Exposure to Methylmercury Alters Learning and Induces Depression-like Behavior in Male Mice

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To investigate the long-term effects of developmental exposure to methylmercury (MeHg), pregnant mice were exposed to at 0.5 mg MeHg/kg/day via drinking water from gestational day 7 until day 7 after delivery. The behavior of offspring was monitored at 5–15 and 26–36 weeks of age using an automated system (IntelliCage) designed for continuous long-term recording of the home cage behavior in social groups and complex analysis of basic activities and learning. In addition, spontaneous locomotion, motor coordination on the accelerating rotarod, spatial learning in Morris water maze, and depression-like behavior in forced swimming test were also studied. The analysis of behavior performed in the IntelliCage without social deprivation occurred to be more sensitive in detecting alterations in activity and learning paradigms. We found normal motor function but decreased exploratory activity in MeHg-exposed male mice, especially at young age. Learning disturbances observed in MeHg-exposed male animals suggest reference memory impairment. Interestingly, the forced swimming test revealed a predisposition to depressive-like behavior in the MeHg-exposed male offspring. This study provides novel evidence that the developmental exposure to MeHg can affect not only cognitive functions but also motivation-driven behaviors.

Key Words: developmental neurotoxicity; behavior; neural stem cells.

There is an increasing concern about neurological deficits in humans. Environmental contaminants have been proposed as possible causes of learning and emotional disturbances at young age, and neurodegenerative diseases in later life (Landrigan *et al.*, 2005; NRC, 2000). Methylmercury (MeHg) is known to be an environmental neurotoxicant potentially causing neuropsychological disorders in humans (Gilbert and Grant-Webster, 1995). Furthermore, epidemiological and experimental studies have clearly shown that the developing nervous system is particularly vulnerable to MeHg toxicity. Severe neurotoxic effects of prenatal exposure to high doses of MeHg were established in humans after MeHg disasters in Japan and Iraq (Amin-Zaki *et al.*, 1979; Harada, 1995) and confirmed in animal studies (Burbacher *et al.*, 1990). Later, developmental exposure to low doses of MeHg contained in seafood was found to be a risk factor for cognitive disorders (e.g., memory, attention, and language problems) in children and adolescents in the fish-eating population of the Faroe Islands (Debes *et al.*, 2006; Grandjean *et al.*, 1997). This fact raised researchers interest in studying effects of prolonged low-dose exposure in animal models, representing a chronic pattern of exposure in humans. Toxicological studies appear to be more relevant in mice than in rats, as in mice MeHg toxicokinetics are more similar to those of humans (Young *et al.*, 2001); however, only a few studies on chronic low-dose MeHg-exposure effects in mice have been published (Goulet *et al.*, 2003; Markowski *et al.*, 1998; Weiss *et al.*, 2005).

We designed the present experiment as a model for the chronic early life exposure of mice to fairly low dose of MeHg (0.5 mg/kg/day) to analyze the long-lasting effects in young and adult animals with a focus on motor, cognitive, and motivational behavior. Several methods were used, including a recently developed automatic recording system, IntelliCage. which allows to study the behavior of mice while in a social group and within their home cage. This approach also eliminates the influence of the stress generally associated with individual testing, i.e., handling, isolation, and allows designing experimental tasks ethologically relevant for rodents.

Oxidative stress is believed to play a significant role in neural cell damage induced by MeHg (Daré *et al.*, 2000; Sanfeliu *et al.*, 2001). The ability of a cell to cope with oxidative stress is, in part, dependent on the activation of a *cis*-acting regulatory element termed the antioxidant response element (ARE). The ARE is located in the 5′-flanking region of many genes essential for both detoxification and

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maintenance of the cellular-reducing potential (Jaiswal, 2004; Nguyen *et al.*, 2003). Moreover, *in vitro* studies have shown that ARE induction can protect neural cells against oxidative insults (Kraft *et al.*, 2004). To evaluate a possible role of the ARE system in the intracellular responses to MeHg exposure, we used ARE-human placental alkaline phosphatase (hPAP) reporter transgenic mice as an experimental model.

MATERIALS AND METHODS

Animals and Treatment

All experiments were performed in accordance with the rules of the Swedish Animal Protection Legislation and approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd). ARE-hPAP transgenic mice (Johnson *et al.*, 2002) backcrossed to C57BL/6/Bkl (Scanbur BK, Sollentuna, Sweden) were used. Offspring were tail clipped for genotyping at 21 days of age. DNA was extracted from the tail clips, and PCR was performed to identify positive animals as earlier described by Johnson *et al.* (2002). Animals were kept under standard laboratory conditions (21°C, 12-h light-dark cycle with a gradual increase in illumination between 5 A.M. and 6 A.M. and decrease between 5 P.M. and 6 P.M.) with free access to food and water.

Pregnant dams ($n = 6$) received MeHg (CH_3HgOH) at the dose of 0.5 mg/kg/day (0.47 mg/kg/day of Hg) via drinking water from gestational day (GD) 7 till day 7 after delivery. This period was chosen for the following reasons: (1) the developing mouse brain is thought to be most susceptible to MeHg neurotoxicity from GD 7, when specific areas of the CNS begin to form (Rice and Baron, 2000); (2) levels of total Hg are highest in the exposed offspring at postnatal day (PND) 7, and then fall during the suckling period despite of continuing exposure via milk (Markowski *et al.*, 1998; Newland and Reile, 1999). MeHg concentration in the drinking water was adjusted to keep daily dose of exposure at the level of 0.5 mg/kg bw, resulting in a variation from 1.6 to 3.9 mg/l with median concentration of 2.3 mg/l. Control females received tap water.

Measurement of Total Mercury Content in Brain Tissue

Total Hg content in the offspring brain tissue was measured in one to two pups from three different litters at PND 8, i.e., after the termination of MeHg exposure, and in 4-week-old mice, i.e., before the first session of behavioral experiments. Hg measurements in the brain samples were carried out using the cold vapor atomic-absorption technique following alkaline digestion according to Magos (1971).

Behavioral Studies

One or two males and females from different litters were used for behavioral testing. Spontaneous locomotion, motor coordination on the accelerating rotarod, spatial learning in Morris water maze, and depression-like behavior in forced swimming test were studied according to standard protocols. Detailed description of the tests is provided in supplementary data.

Behavioral Assessment in the IntelliCage System

Apparatus and subjects.

IntelliCage is a novel system for automated monitoring of spontaneous and learning behavior of mice in social groups (New Behavior AG, Zurich, Switzerland). Detailed description of the system is provided by Galsworthy *et al.* (2005) and Knapska *et al.* (2006). The system consists of four test chambers settled in a large cage as shown in Figure 1. To standardize the experimental conditions, control ($n = 3$) and MeHg-exposed ($n = 4$) mice were housed in the same IntelliCage. These social groups were formed soon after weaning when the mice were 4 weeks old. At the same time, animals were sc injected with sterile transponders (T-IS 8010 FDX-B; Datamars SA, Switzerland) under 4% isoflurane inhalation anesthesia. IntelliCages were controlled by a computer with installed software, executing preprogrammed experimental schedules and registering visits to corner chambers, nose pokes to the door areas and tube lickings as parameters of mouse behavior.

Experimental protocols.

Adaptation: animals were introduced to the IntelliCages at 10 A.M. and were allowed to have free access to all water sources. Place preference learning: 72 h later the water-access doors remained open only in one corner, which was least visited by all mice in the cage over the previous 3 days. Reversal: after another 72 h, the location of the open corner was changed. The previously opened corner was closed and a new, the least preferred (over the last 3 days) corner was opened for drinking. Patrolling behavior: (1) Conditioning: all water-access doors were initially closed but opened in any corner chamber when the mouse nose poked at least once on the door area. Moreover, a green light turned on in the chamber, when the animal began to drink. (2) Testing: after 2 days of
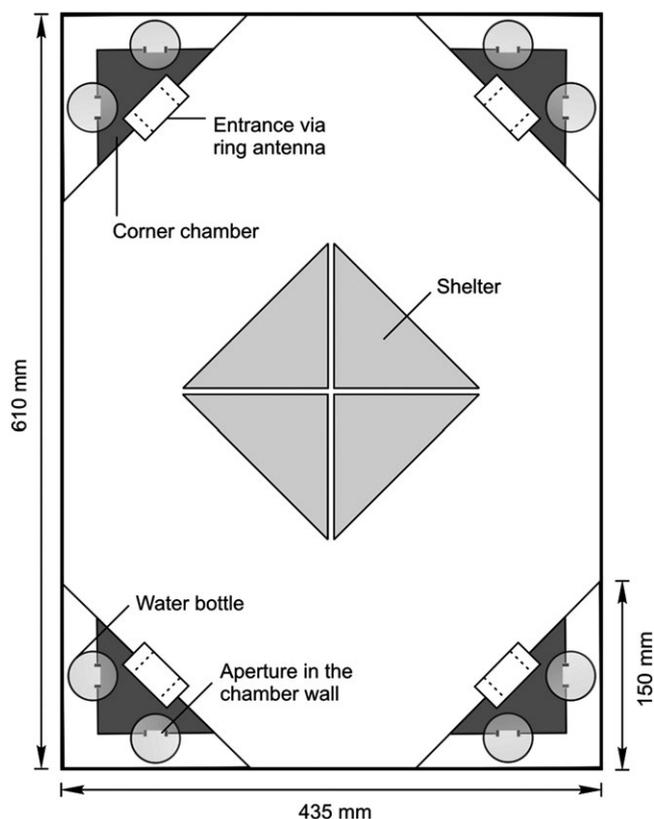

FIG. 1. Plan view of the IntelliCage. From the central cage area, mice can enter triangular corner chambers through a tubular antenna, which reads the individual animal's transponder number. Two round apertures on the walls of each chamber can remain open, providing free access to water-bottle nipples, or closed by small motorized doors to prevent access to water but allowing mice enter a corner. The inner space of the chamber can be illuminated by light-emitting diodes located on the topside. Four triangular shelters are placed in the middle of each cage and used by mice as sleeping quarters and as a stand to climb to reach food.

conditioning schedule, a patrolling protocol was applied. Mice were allowed to access water in one corner chamber of the IntelliCage at a given time. After each visit, the next corner became available for drinking in a clockwise manner. With a mouse entering the correct corner, a green light was turned on, and the doors opened after a single nose poke on the door area. No light was switched on when entering an incorrect corner and the doors could not be opened. Thus, animals had to patrol corners to find the correct chamber, where the doors will be opened.

Histochemistry

Brains of control and MeHg-treated 8-day-old pups ($n = 5$ from each group) were perfused with 4% paraformaldehyde and cut on a cryostat in 14 µm thick coronal sections. To visualize hPAP activity, tissue sections were processed as described by Johnson *et al.* (2002). Detection of DNA damage was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Detailed description of the histochemical techniques is provided in supplementary data.

In Vitro Studies

Primary culture of adult neural stem cells.

Primary cultures of neural stem cells (NSCs) were derived from adult ARE-hPAP transgenic mice brains. The anterior portion of the lateral wall of the lateral ventricles was dissected out and enzymatically dissociated in Hank's balanced salt solution (Life Technologies, Grand Island, NY) containing papain (Worthington, Biochemical Corporation) at 37°C for 30 min and subsequently mechanically dispersed. Ovomucoid inhibitor (Worthington, Biochemical Corporation, Lakewood, NJ) was added to the suspension and passed through a cell strainer to separate the cells from debris. Cells were centrifuged at 1000 rpm for 3 min, and resuspended in DMEM/F12 medium supplemented with B27, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C with 10 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN) added to the culture every 48 h. Primary neurospheres of adult NSCs formed within 6 days, after which they were passaged to single cells by dissociating with papain. Secondary neurospheres were passaged after an additional 6 days of culturing and seeded on poly-L-lysine (Sigma St. Louis, MO)–coated coverslips.

Immunocytochemistry.

After exposure, cells were fixed with cold 4% paraformaldehyde for 60 min and then washed with phosphate-buffered saline (PBS). Cells were incubated with primary antibodies (mouse anti-hPAP, 1:500, Sigma) in a humid chamber at 4°C overnight, rinsed with PBS, incubated with secondary green-fluorescent Alexa Fluor 488–conjugated antibodies (1:200, Molecular Probes, Eugene, OR) and counterstained with Hoechst33342 (30 µg/ml) (Molecular Probes). After rinsing in PBS, coverslips were mounted in Vectashield mounting medium (Vector, Burlingame, CA). Cells were examined using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany) and apoptosis (based on nuclear morphology) was assessed, scoring at least 300 cells in four microscopic fields randomly selected on each coverslip. The experiments were performed in triplicate.

Statistical Analysis

We used mixed model of ANOVA. The type of statistical method is specified for each experiment in the "Results" section. Values reported in the text and figures are represented as mean ± SEM. A *p* value < 0.05 was considered as statistically significant.

RESULTS

Mercury Levels in the Brain

The exposure of pregnant mice to MeHg resulted in the following whole-brain mercury concentrations measured on PND 8: dams: 2.60 ± 0.19 µg/g wet weight, pups: 0.93 ± 0.02 µg/g. Mercury concentration dramatically decreased to 0.045 ± 0.003 µg/g in the brains of 4-week-old offspring. Mercury concentrations in controls were below 0.006 µg/g.

Behavioral Studies

Our tests did not reveal any behavioral changes in female offspring exposed to MeHg during development. Below we report findings on male offspring behavior.

Experiments were performed in two sessions, first when the offsprings ($n = 6$–8 per group) were 5–15 weeks old, then when they were 26–36 weeks old; therefore, in the following the animals are referred to as young and adult, respectively.

Motor Function

Because of the importance of normal motor function for successful performance in the tests designed for studying learning abilities and emotional behavior in rodents, we began the analysis by evaluating motor status of animals. Locomotor activity and motor coordination of young and adult animals were studied in spontaneous locomotion and accelerating rotarod tests. Control and MeHg-exposed mice of both ages did not differ in distance covered over 1 h (Supplementary Figs. 1A and 1B). The results from the accelerating rotarod test (Supplementary Figs. 1C and 1D) showed that the motor coordination at both ages was not affected by MeHg exposure ($F_{1,12} = 0.287$; $p = 0.60$ and $F_{1,12} = 0.066$; $p = 0.80$, repeated measures ANOVA for young and adult mice, respectively).

Behavioral Parameters Studied in the IntelliCage

Novelty-induced exploration.

Young control mice showed fast and active exploration of the novel-enriched environment of the IntelliCage (Fig. 2A): the average latency to the first visit into the corner chamber was 3.3 ± 0.9 s, while it took almost three fold longer time (9.7 ± 1.9 s) for MeHg-exposed mice to enter the first corner ($F_{1,12} = 5.7$; $p < 0.05$, one-way ANOVA). Furthermore, MeHg-exposed mice made less visits over the first 30-min period after introduction into the IntelliCage (11.6 ± 0.6 and 15.0 ± 1.7 for MeHg exposed and controls, respectively; $F_{1,12} = 4.75$; $p < 0.05$, one-way ANOVA). As a result, all corners of the cage were explored more slowly by MeHg-exposed mice than by controls ($F_{1,12} = 5.5$; $p < 0.05$; one-way ANOVA). In adult

animals, no difference was found neither in initial nor in total IntelliCage exploration (Fig. 2B).

Daily activity.

All animals found the water sources soon after the introduction into the IntelliCage and consumed the same daily amounts of water, as measured by the number of licks made by each mouse (data not shown). However, as it has been seen throughout the study, mice visited corner chambers not only for quenching thirst but also out of curiosity, exploration, etc. The ratio between number of visits with and without drinking over the first day in the IntelliCage was different in control (21%:79%) and MeHg-treated (32%:68%) young animals. Later, after they adapted to the cage, animals of both groups entered corners for drinking in 40–50% of visits.

Activity of animals during light and dark periods was different; therefore, the statistical analysis of behavior for these periods was performed separately. Young control animals had clear peaks of exploratory activity during "sunset" and "sunrise" time as well as between 2200 h and 2300 h in the night. Young MeHg-exposed animals had activity peaks at a similar time but were less active during "sunset" and "night" (Fig. 2C). As shown in Table 1, the number of visits during the dark period was lower in MeHg-treated mice than in controls, both in the new environment (first day) ($F_{1,12} = 6.8$; $p < 0.05$, repeated measures ANOVA) and in the familiar home environment (third day in the IntelliCage) ($F_{1,12} = 5.0$; $p < 0.05$, repeated measures ANOVA). Groups of adult animals differed in exploratory behavior when the environment was new (Fig. 2D) but the difference disappeared when the environment became familiar (Table 1).

Place learning and reversal.

Both groups of young animals showed a similar gradual increase in preference of visiting correct (reinforced with water) corner when they were assigned to place-learning and reversal tasks (Fig. 3A). Although daily dynamics of learning in adult animals were quite similar in both groups (Fig. 3B) and MeHg-treated mice succeeded in finding the new open water source during reversal phase, a closer analysis of behavior over the first hours of reversal learning revealed that treated animals acquired the new place preference slower than control animals (Fig. 3C).
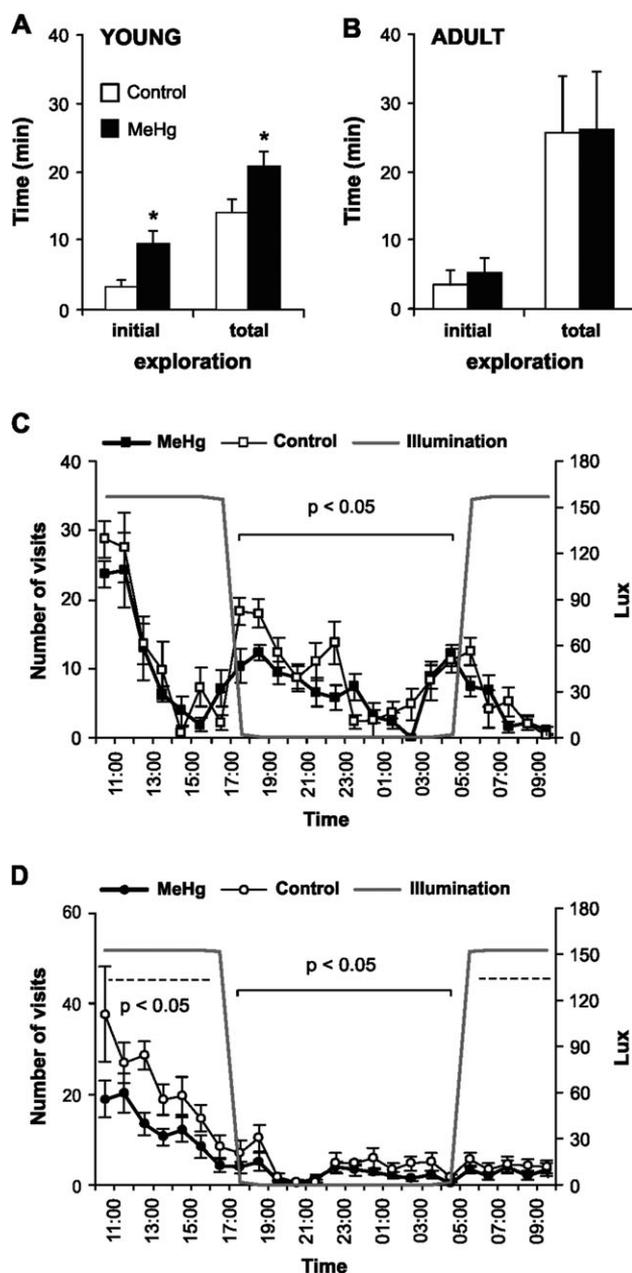

FIG. 2. (A, B) Novelty-induced exploration after introduction into the IntelliCage. Initial exploration shows latency to the first visit to the corner chamber. Total exploration shows time spent for visiting all four corner chambers. *$p < 0.05$, one-way ANOVA. Circadian activity over first 24 h after introduction of young (C) and adult (D) mice into the IntelliCage. *$p < 0.05$, repeated measures ANOVA. Control $n = 6$; MeHg treated $n = 8$.

TABLE 1
Circadian Activity of Mice in the Novel and Familiar Environment of the IntelliCage

| | Number of corner visits | | | |
| | Novel environment (day 1) | | Familiar environment (day 3) | |
Group	Light phase	Dark phase	Light phase	Dark phase
Control, young	101.5 ± 8.1	127.2 ± 7.6	33.0 ± 7.8	104.8 ± 8.7
MeHg treated, young	87.6 ± 12.6	92.1 ± 10.1*	23.6 ± 5.0	80.6 ± 6.7*
Control, adult	168.0 ± 27.0	52.7 ± 10.0	24.8 ± 4.9	31.7 ± 7.6
MeHg treated, adult	98.6 ± 18.7*	28.4 ± 5.1*	25.3 ± 5.3	25.3 ± 5.8

*$p < 0.05$, repeated measures ANOVA.

Patrolling behavior.

Contrary to the place-learning paradigm, with the reinforced corner remaining the same over the testing period, in this test the location of the open corner changed after each visit. Hence, mice had to patrol corners in order to find the correct one, marked with green light and providing access to the water. As mentioned above, drinking was not the only motivation for visiting corners. Therefore, nose poking at the door area but not visiting was chosen as a sign of animal's attempt to reach the water. Consequently, the percentage of nose pokes in the correct corners was considered as a parameter of successful performance in this test.

Over the first day of the patrolling schedule, young control and MeHg-treated mice made 24.0 ± 3.5% and 23.0 ± 4.6% correct nose pokes, respectively (chance level 25%). A significant increase in percentage of correct nose pokes (compared with own initial performance) occurred on the fourth day of experiment in the control group, and 1 day later in MeHg-treated one (Fig. 4A). Moreover, control mice made further progress in learning during the second week, while MeHg-exposed mice remained at the same performance level till the end of the experiment.

When adult animals were assigned to the same schedule during the second session of experiments, controls learned the task very fast and showed significantly better performance already on the first day, while MeHg-exposed mice started from the same basal level as the first time (Fig. 4B).
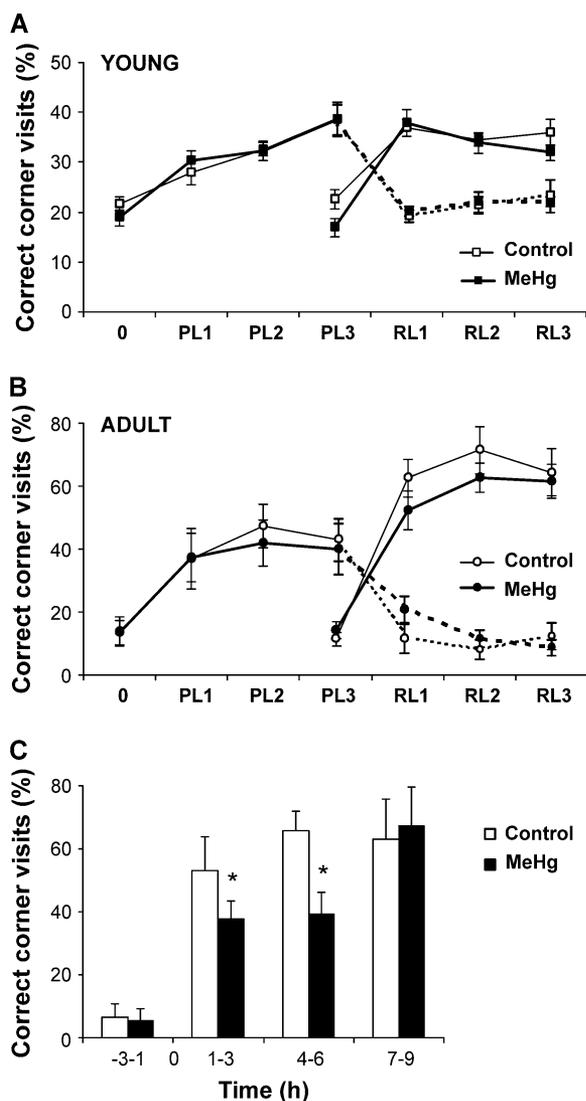
Spatial Learning in the Morris Swim Maze

The results of the pretraining session confirmed that all animals could navigate to the visible platform and treated animals did not differ from controls in this ability. Repeated measures ANOVA showed that MeHg exposure did not cause significant changes in escape latency or swim length estimated over five consecutive days of training. Furthermore, no significant difference between groups was found in these parameters during retention test performed 24 h after the last training session (Supplementary Figs. 2A and 2B).

Forced Swimming Test

This paradigm evaluates animal's response to inescapable aversive situation (placement in a beaker of water), inducing an

FIG. 3. Place learning and reversal for young (A) and adult (B) animals evaluated by the preference for visiting reinforced corner. Dotted lines show percentage of visits to the previous reinforced corner when a new corner (previously least preferred) was open. PL1-3: days 1–3 of place learning; RL1-3: days 1–3 of reversal. (C) Early phase of reversal in adult mice. Zero *x* value refers to a time point when location of the reinforced corner was changed. *$p < 0.05$, one-way ANOVA. Control $n = 6$; MeHg treated $n = 8$.
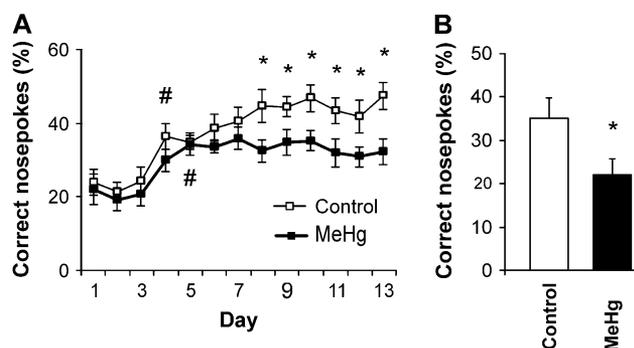

FIG. 4. Patrolling behavior test. (A) Young MeHg-exposed mice showed slower learning of the given complex task than control animals. #, significant difference with own initial level of performance occurred for the first time; *, significant difference between the groups ($p < 0.05$, Fisher's protected least significant difference *post hoc* after one-way ANOVA). (B) The performance of adult MeHg-exposed animals was significantly worse than controls' level on the first day of the test during the second session of the experiments. *$p < 0.05$, one-way ANOVA.
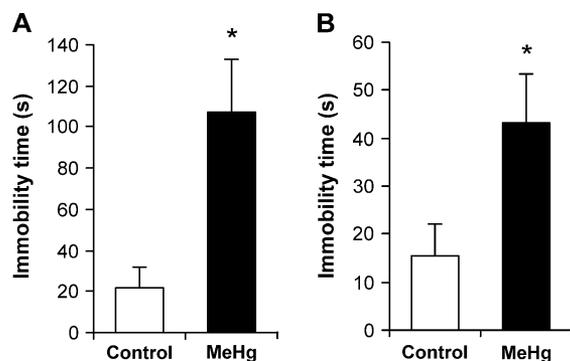
FIG. 5. Forced swimming test in young (A) and adult (B) mice. *$p < 0.05$, one-way ANOVA. Control $n = 6$; MeHg treated $n = 8$.

active (swimming, climbing on the walls) or inactive (floating) behavior. The latter is interpreted as a measure of depressive-like behavior (Porsolt *et al.*, 1977). Both young and adult MeHg-exposed mice displayed significantly longer immobility time (passive floating without limb movements) than control animals ($F_{1,11} = 8.336$; $p < 0.05$ and $F_{1,11} = 4.991$; $p < 0.05$, one-way ANOVA for young and adult animals, respectively) (Fig. 5).

Histochemical Analysis of ARE Mice

To evaluate the possible occurrence of MeHg-related ARE activation, sections from the brains of control and MeHg-exposed 8-day-old pups were processed to visualize hPAP. None of the brain regions examined, including hippocampus and cerebellum known to be MeHg targets, exhibited hPAP positive staining. Moreover, the TUNEL staining to detect apoptotic cells performed on adjacent sections did not reveal a difference in number of positive cells between control and MeHg-treated animals (data not shown).

Studies on Primary Cultures of NSCs from ARE Mice

NSCs represent an important target for developmental neurotoxicants and appear to be more sensitive to MeHg than many other *in vitro* models used for evaluating neurotoxicity (Tamm *et al.*, 2006). Therefore, we used NSCs obtained from adult ARE-hPAP transgenic mice with verified genotype to evaluate ARE activation as well as the occurrence of cell death after exposure to different concentrations of MeHg. Immuno-cytochemical staining of hPAP revealed presence of hPAP protein in cells after 24 h exposure to 0.1 or 0.5μM of MeHg (Fig. 6). Staining with Hoechst33342 demonstrated a significant increase in pyknotic nuclei in cells exposed to MeHg (Fig. 7). To further assess the time period required for MeHg to induce activation of ARE, we exposed NSCs to 0.5μM of MeHg for 1, 3, 6, and 12 h. As observed by immunofluorescent staining, ARE activation only occurred after 12 h of exposure (data not shown).

DISCUSSION

Results from the developmental exposure experiments presented here provide evidence for long-lasting effects of MeHg on learning ability and motivational behavior, with a persistent predisposition to depressive behavior. Interestingly, these effects were observed only in male mice. Earlier epidemiological studies of children (Grandjean *et al.*, 1998; McKeown-Eyssen *et al.*, 1983) as well as experimental animal studies (Gimenez-Llort *et al.*, 2001; Rossi *et al.*, 1997) have also reported greater developmental effects in males than in females. The mechanisms underlying such gender-related differences may be due to different factors (Vahter *et al.*, 2006) and need to be further clarified.

Comparing relevance of different animal models to human MeHg exposure, it should be noted that models of prolonged low-level exposure are more appropriate to simulate neurotoxicant effects occurring in high fish-consuming populations. Due to the species difference in MeHg toxicokinetics, it is difficult to correlate doses used in animal studies to human exposure; therefore, Hg content in the target organ and related signs of the behavioral disorders should be taken into consideration. Hg brain concentrations found in Seychelles
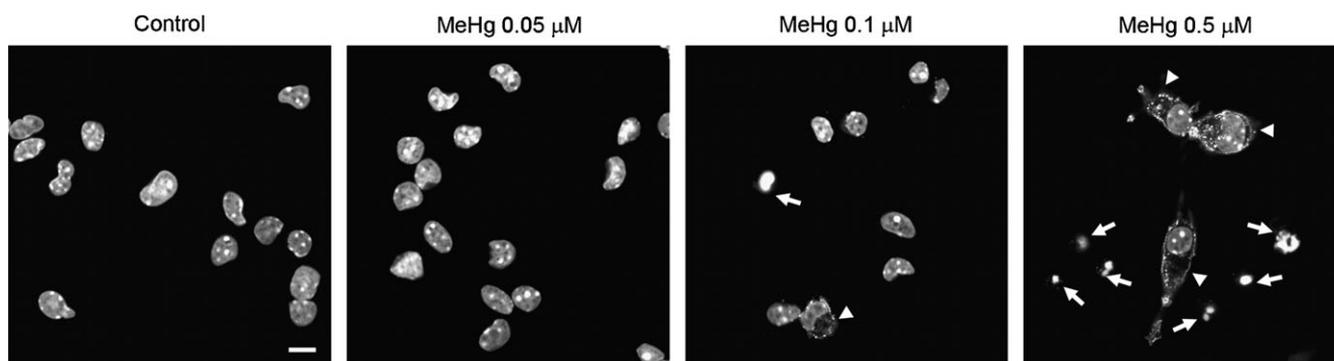
FIG. 6. MeHg-induced hPAP expression in primary cultures of adult NSCs derived from ARE-hPAP reporter mice. Cells were exposed to MeHg, then stained with Hoechst33342 to visualize apoptotic nuclei (arrows) and anti-hPAP antibody to detect hPAP protein (arrowheads). Scale bar = 10 μm.
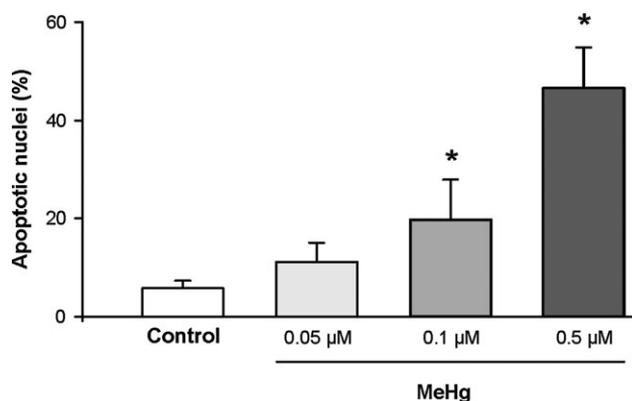
FIG. 7. Chromatin condensation in adult NSCs exposed to different concentrations of MeHg for 24 h. *$p < 0.05$, one-way ANOVA, Scheffe *post hoc* test.

infants varied from 0.026 to 0.295 μg/g but no cognitive dysfunction was found in children from the same population (Davidson *et al.*, 2004; Lapham *et al.*, 1995). In New Zealand children, mental disturbances detected using a battery of psychological tests were revealed at a maternal hair Hg level of 6 μg/g (Kjellström *et al.*, 1986) that can correspond to 0.3 μg/g in fetal brain. However, in animal studies levels of Hg up to 3 μg/g after developmental exposure to MeHg were referred to as low brain levels and caused only subtle behavioral effects (Burbacher *et al.*, 1990). Therefore, the treatment protocol used in the present experiments, which resulted in Hg brain level of approximately 0.9 μg/g, can be considered as an appropriate model for studying effects of low-dose developmental exposure to MeHg.

Our results show that prenatal and early postnatal exposure of mice to a 0.5 mg/kg (2–3 mg/l) of MeHg caused no significant changes either in locomotion or in motor coordination in young and adult offspring. Similar results were reported by Goulet *et al.* (2003) who found no difference in locomotor activity in male mice after chronic developmental exposure to 4–8 mg/l of MeHg. The lack of effect on motor coordination of MeHg-exposed mice in the rotarod test is consistent with literature data showing that low-dose exposure to MeHg does not lead to gross motor deficit in mice (Goulet *et al.*, 2003; Stringari *et al.*, 2006).

In addition to traditional tests, we employed the IntelliCage system, a new approach to study home cage behavior of mice in social groups as well as reaction to novelty. According to Lister (1990), "behavior of mice exposed to a novel situation results from a competition between an exploratory tendency (motivated by curiosity or boredom) and a withdrawal tendency (motivated by fear)." In the given test, hereditary instincts to explore holes and dark areas, as probable shelter places, lead mice to enter the corner chambers, but the unknown area might also be considered as potentially dangerous and can arouse fear to different extent. The results of the study show that MeHg-exposed young mice hesitate more than controls to encounter

a novel environment, which can possibly be a "freezing" type of reaction to novelty. Also, during the habituation period, control and MeHg-exposed mice appeared to be differently motivated to visit activity corners. Besides physiologically motivated (drinking) visits, young control mice made almost four times more visits out of curiosity (without drinking). This is considered to be a normal type of behavior for a junior age. Conversely, the low exploratory activity (one drinking visit vs. two nondrinking ones) observed in the MeHg-exposed mice points to a lower level of curiosity. Since motor function appeared normal in both groups, the data suggest that young MeHg-exposed mice are lacking interest in exploration of a novel environment. Previous studies have shown normal level of locomotion and decreased exploratory activity in young and adult male rats prenatally exposed to MeHg (Carratù *et al.*, 2006; Rossi *et al.*, 1997).

Both wild-living and laboratory rodents are known to be nocturnal animals; therefore, behavioral studies during the dark phase can provide important information about actual level of activity. We found that MeHg-exposed mice were less active than controls during the dark period, suggesting disturbance of the sleep-waking pattern. Arito *et al.* (1983) showed that short-time exposure of adult rats to MeHg led to a significant increase in both slow-wave sleep and paradoxical sleep during dark phase, as well as long-lasting sleep-waking changes.

Several studies have reported learning disabilities in the passive avoidance test performed in young and adult rats after developmental exposure to MeHg (Cagiano *et al.*, 1990; Kakita *et al.*, 2000; Sakamoto *et al.*, 2002) but data about spatial learning in water maze test are controversial (Daré *et al.*, 2003; Fredriksson *et al.*, 1996; Kim *et al.*, 2000; Rossi *et al.*, 1997). We found no significant difference either in acquisition or in retention of spatial-learning task in the water maze between MeHg-exposed and control mice.

Similarly, in the IntelliCage system, MeHg-exposed mice were able to find and learn the location of the reinforced corner, i.e., they did not have impairment in spatial learning under these conditions either. However, in older animals, the process of relearning was slower than in the control mice. This might be due to difficulties in spatial learning but more likely to subtle disturbances in the process of identification and memorizing a new condition of reinforcement, e.g., changed location of the water source in the given test, thus suggesting reference memory disturbances in MeHg-treated mice.

Learning a task in the patrolling behavior test in the IntelliCage requires visual discrimination, reference and working memory, presenting a more complicated challenge than the previous test. An animal with an unimpaired reference memory can distinguish between a rewarded illuminated chamber, where drinking is allowed, and dark chambers with barred access to the water. An animal with an unimpaired working memory can predict manner of rewarding, when water becomes available in the corner next to the already visited and rewarded one. Again, MeHg-exposed animals were able to

fulfill the requirements eventually, but they solved this integrated task slower than controls.

When results from experiments in the IntelliCage are discussed, it should be considered that animals housed in groups can benefit from this ''natural environment'' in different ways, based on their social ranking. Therefore, hierarchic interactions may influence the results in animals that remain in social groups over the testing period. However, it has been shown in other studies that even results from individual testing can be affected by animal's social status (Barnard and Luo, 2002; Fitchett et al., 2005).

We also analyzed the effects of MeHg on emotional behavior, a topic never addressed in previous studies. We used the forced swimming test, generally considered as an animal model of depression (Porsolt, 2000). In this test, both young and adult MeHg-exposed mice showed significantly longer immobility time that represented a behavioral despair response to an aversive situation and therefore a depression-like type of behavior. Depressive syndromes have been reported in humans later in life after occupational exposure to inorganic mercury (Grum et al., 2006). Further in-depth studies on potential effects of developmental exposure to MeHg on emotional behavior are needed.

The neurochemical basis of MeHg-induced behavioral alterations may be due to disturbances in a number of neurotransmitter systems, initially occurring during exposure and followed by long-lasting changes in brain functioning (Castoldi et al., 2001). Abnormalities in neuronal functioning, in turn, can be due to intracellular MeHg toxicity, which includes alteration in Ca^{2+} homeostasis, cytoskeletal damage, and induction of oxidative stress (Sarafian and Verity, 1991; Yee and Choi, 1994). ARE that transcriptionally regulates genes encoding detoxification enzymes and antioxidant proteins is known to play an important role in the cellular defense system (Lee et al., 2005; Nguyen et al., 2003). In particular, specific induction of ARE-driven genes in culture of human NSCs was shown to protect against oxidative stress-induced cell death (Li et al., 2005). However, the stage and degree of intracellular oxidative stress triggering antioxidant defense induction remain unclear. In the present study, MeHg exposure of primary cultured NSCs led to dose-dependent expression of hPAP, an ARE-driven transgene product, with a maximal expression after exposure to 0.5μM MeHg that caused apoptotic death in almost 50% of cells. Since the reporter protein was only detected after 12 h of MeHg exposure, the defense system driven by ARE in NSCs is activated most likely at later stages of oxidative stress rather than in the initial phase. Our in vivo exposure model that resulted in brain Hg concentrations of 0.9 μg/g did not cause ARE activation, as shown by the lack of detection of hPAP protein in brain tissue samples obtained from the offspring in the end of MeHg exposure. On the other hand, the level of DNA fragmentation considered as a sign of apoptotic cell death was not higher in MeHg-exposed mice than in controls. These data are in line with previous observations suggesting that low-dose exposure to MeHg under the schedules similar to the one used here causes abnormalities in neurodevelopmental processes (e.g., cell migration) but not cell death (Markowsky et al., 1998; Sakamoto et al., 2002).

A number of relevant developmental processes may be affected by exposure to toxic agents during development, i.e., neurogenesis, migration, neurotransmitter and receptor activity, but the consequences may be difficult to detect. The outcome of a prenatal damage may not necessarily be apparent until a critical age when a neurodevelopmental defect may be unmasked or precipitated by a subsequent insult. Thus, it is important to apply sensitive type of analyses when screening the effects of potential neurotoxic substances. In this respect, behavioral analysis is certainly a very powerful tool, as shown by the present study.

In conclusion, we have shown that developmental exposure to low level of MeHg induces alteration in learning and depression-like behavior. These findings point to early exposure to environmental contaminants as a possible risk factor for neurodevelopmental disorders.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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