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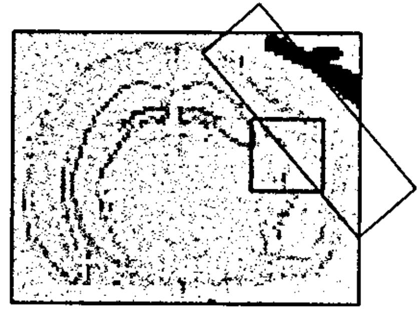

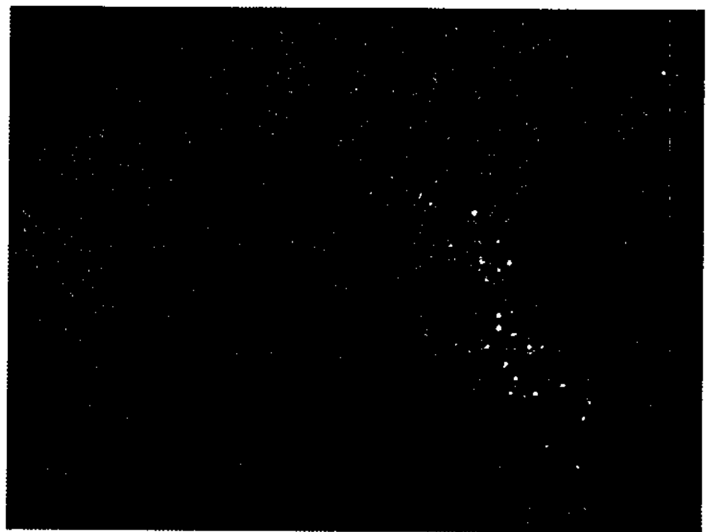

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Low-dose perinatal diethylstilbestrol exposure affected behaviors and hypothalamic estrogen receptor-α-positive cells in the mouse

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Abstract

To estimate the potential risk of perinatal exposure to estrogenic endocrine disrupters, pregnant female mice received daily oral administration of diethylstilbestrol (DES; either 0.3 or 3 μg/kg body weight) dissolved in corn oil from gestation days 11 to 17 and from postnatal days 2 to 6. Multiple behaviors that are sexually dimorphic were examined, and the numbers of estrogen receptor-α and tyrosine hydroxylase-immunoreactive (ER-IR and TH-IR) cells in some brain loci related to these behaviors were investigated. Perinatal exposure to DES caused significantly enhanced open-field activity in both males and females and significantly poorer passive avoidance performance in males. In addition, a significant increase in the number of ER-IR cells in the ventromedial hypothalamic nucleus (VMH) was demonstrated for the first time. The DES-induced increases in the sexual and aggressive behaviors, although statistically nonsignificant, and the increase in the number of ER-IR cells did not agree with those obtained in previous studies using high-dose DES, which suggests that DES may have a different effect on these endpoints depending on the dose used. The relationship between the increase in ER-IR cells and behavioral changes should be further examined.
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Keywords: Low-dose diethylstilbestrol; Sexual behavior; Aggressive behavior; Open-field activity; Passive avoidance; Estrogen receptor-α immunoreactivity

1. Introduction

Numerous man-made chemicals that are widely distributed in the environment act as estrogen receptor agonists and are suspected to cause estrogen receptor-mediated disorders in animals. Estrogen plays an important role in brain development. It modulates neural differentiation and influences cell migration, cell survival, cell death, and synaptic neuron plasticity [1,28,35], thereby affecting subsequent behavioral and cognitive function [2]. Little is known about the potential effects of estrogenic endocrine disruptors (EDs) on the developing central nervous system (CNS), which is exquisitely sensitive to estrogen.

To estimate the potential risk of perinatal exposure to estrogenic EDs, we need to know the effects of low-dose synthetic estrogen on the CNS and subsequent behavior. Most of the earlier studies on the effects of perinatal estrogen used extremely high doses, which may not be relevant to estimating the effects of EDs on humans. Diethylstilbestrol (DES) has been used as a good model compound for estrogenic EDs because it does not bind to α-fetoprotein, an estrogen-binding plasma protein, and thus can bind to estrogen receptors in fetal tissues much more efficiently than does endogenous estrogen [32]. Effect of the lower doses DES on reproductive system gave inconsistent results; while several reports suggest that prenatal [6,21,49] or postnatal exposure [4] to DES affected size of testis, epidydimis, and

Abbreviations: EDs, endocrine disruptors; CNS, central nervous system; DES, diethylstilbestrol; ERα, estrogen receptor-α; TH, tyrosine hydroxylase; GD, gestation day; PND, postnatal day; PBS, phosphate-buffered saline; BSA-PBST, bovine serum albumin with Triton X in phosphate-buffered saline; ER-IR cell, estrogen receptor-α immunoreactive cell; TH-IR cell, tyrosine hydroxylase immunoreactive cell; VMH, ventromedial hypothalamic nucleus; AM, amygdaloid; PAG, periaqueductal gray; VTA, ventral tegmental area; LC, locus coeruleus; ARH, arcuate hypothalamic nucleus; BPA, bisphenol A.

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prostate, other failed to confirm these observations with pre- and postnatal [38] or prenatal exposure [3,10].

On the other hand, a few reports are available on the behavioral effects of low-dose DES. Palanza et al. [39] and Vom Saal et al. [48] have reported an increase in aggressive behavior and urine-marking behavior in mice born to dams that were exposed during prenatal period. Takahama et al. [45] have shown a learning impairment in mice by prenatal exposure, and Kubo et al. [25] have reported an abolishment of sexual difference in the open-field behavior and in the size of locus coeruleus and reproductive organs in rats by pre- and postnatal exposure.

In the present study, we evaluated the effects of low-dose DES on multiple behaviors that are sexually dimorphic (i.e., open-field test, passive avoidance test, sexual behavior, and aggressive behavior) and analyzed the inter-relationships among these behaviors in order to characterize the pattern of neurobehavioral changes. Although it was shown that the effects of DES on the reproductive organs depend on the timing of exposure, our exposure extended to pre- and postnatal periods because both of these are critical for the development of CNS and the sexual differentiation of brain. Furthermore, neurohistological evaluations were made to explore the possible mechanisms that may induce behavioral changes. It is well known that estrogen elicits aggressive behavior and masculine sexual behavior in male rodents via estrogen receptor-α (ERα) in the brain, and that the establishment of estrogen responsiveness requires estrogen during the perinatal period [33,43]. We examined ERα-immunoreactive (ER-IR) cells in the ventromedial hypothalamic nucleus area (VMH) and the amygdaloid area (AM), which are some of the brain loci related to sexual and aggressive behaviors [22,41,42].

In addition to ERα expression, the dopaminergic system is also associated with defensive aggression [29,30], sexual behavior [33], and general activity [27]. The midbrain dopaminergic and noradrenergic neurons, which are marked by tyrosine hydroxylase (TH), are especially affected by perinatal estrogen with respect to functional and morphological maturation [7]. Therefore, tyrosine hydroxylase-immunoreactive (TH-IR) cells in the midbrain areas, including the periaqueductal gray (PAG), the ventral tegmental area (VTA), and the locus coeruleus (LC), were examined in relation to sexually dimorphic behaviors.

Several studies have revealed the effects of EDs on the CNS at the cellular and tissue levels [12,16,17], but only a few attempts have been made to examine the effects at the whole organism and tissue levels simultaneously. The behavioral effects of bisphenol A (BPA) were found to be related to the size of the brain nuclei which are known to be sexually dimorphic [24,36]. In another study, perinatal exposure to DES (15 μg/kg body weight) did not affect sexual behavior, but did affect the sexually dimorphic nucleus of the preoptic area and the estrous cycle in the female rat [26].

2. Materials and methods

2.1. Animals

Eleven timed pregnant female C57BL/6 Cr Slc mice were purchased from a commercial breeder (Japan SLC, Tokyo, Japan) on gestation day (GD) 7 (the day of confirmation of a vaginal plug was defined as GD 0). Mice were housed individually in standard polypropylene mouse cages on sawdust bedding and were fed chow with reduced amounts of estrogen and phytoestrogen; 17 β-estradiol was less than 0.05 μg/kg, genistein was less than 0.5 mg/kg, and coumestrol was less than 1 mg/kg (NIH-07PLD; based on an open formula of U.S. National Institutes of Health, Oriental Yeast, Shizuoka, Japan). Rooms were kept at 23 ± 2 °C with a cycle of 12 h of light and 12 h of darkness; the lights came on at 2000 h.

Eleven pregnant mice were divided into three groups. From GD 11 to 17 and from postnatal day (PND) 2 to 6 (the day of birth was defined as PND 1), each pregnant female received daily oral administration of DES (Sigma-Aldrich, MO, USA) dissolved in corn oil. A dose of either 0.3 or 3 μg/kg body weight of DES (referred to as DES 0.3 or DES 3, respectively) was delivered with a micropipette, which enabled delivery of an accurate volume of the solution (1μl/g body weight). The pipette tip was placed into the mouth and the mice readily consumed the corn oil without apparent stress [47]. The numbers of dams were 4, 4, and 3 in control, DES 0.3, and DES 3 group, respectively. The pregnant females were allowed to deliver the offspring and each cage was checked every morning for the presence of newborns.

On PND 2, each litter was culled to four pups (two males and two females) after recording the number of pups, the proportion of males to total pups (sex ratio), and the body weights. Pups were weaned at PND 21 and were group housed separately by sex until the behavioral tests described below were performed.

All experimental protocols were approved by the Animal Care Committee of the Graduate School of Medicine of the University of Tokyo.

2.2. General procedure

Two males and two females from each litter were used for the behavioral tests. Mice were examined at 9 weeks of age for the open-field test (male, $n=22$; female, $n=22$), at 12 weeks for the passive avoidance test (male, $n=22$; female, $n=22$), at 19 weeks for the sexual behavior test (male, $n=22$), and at 21 weeks for the aggressive behavior test (male, $n=22$). Individual offspring mice were repeatedly used for different behavioral tests. The order of the tests was chosen to minimize potential confounding effects of multiple tests.

Prior to the sexual behavior test and the aggressive behavior test, the mice were housed singly for at least 5

weeks to facilitate aggressiveness. All behavioral tests were conducted during the dark phase (3–5 h after lights off) of the light cycle.

After the completion of all the behavioral tests, brains from male mice in the DES 3 and control groups were used for immunohistochemistry.

2.3. Behavioral tests

2.3.1. Open-field test (at 9 weeks of age)

The open-field was an area of 50 × 50 cm surrounded by 50-cm-high walls. Each mouse was placed individually into the middle of the field and covered with a small box for 20 s. After removing the box, behaviors (distance of ambulation, number of rearing behaviors, and number of feces) were observed for 2 min under bright lighting (approximately 1000 lx on the floor). The apparatus was cleaned with 70% ethanol between tests. Behavioral analysis was performed with a Macintosh computer using Image OF (O'Hara, Tokyo, Japan), a modified NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.3.2. Passive avoidance test (12 weeks of age)

The passive avoidance test consisted of a single training trial that was followed 24 h later by a test trial using an avoidance shuttle box apparatus (#111, Med Associates, VT, USA). A guillotine door separated the box into two chambers; the walls of the start chamber were transparent Plexiglas, and those of the dark chamber were black opaque Plexiglas. The floor of each chamber was made of a metal grid, through which an electric shock could be delivered. For the training trial, each subject was placed in the start chamber, which was illuminated by fluorescent light (approximately 500 lx) for a 30-s adaptation period, after which the guillotine door was opened. When all four feet of the mouse had entered the dark chamber, the guillotine door was immediately closed, and the mouse received an electric shock to the feet (0.3 mA for 1 s). The mouse was then removed from the shuttle box, and the time to enter the dark chamber (step-through latency) was recorded. The apparatus was cleaned with 70% ethanol between tests. The test trial was identical to the training trial except that no electric shock was delivered. The step-through latency was recorded again. It was assumed that the prior electric shock experience would delay the entry into the dark chamber. When a mouse had not entered the dark chamber for 900 s, the trial was terminated.

2.3.3. Sexual behavior test (19 weeks of age)

The sexual behavior of the males was observed in the subject's home cage under a dim red light (approximately 10 lx). Mice were tested for 20 min after the introduction of an estrous female ($n = 11$), which was judged as proestrous by a vaginal smear on the previous day and confirmed as estrous immediately after the test. The behavior was video-recorded and subsequently analyzed. Social investigation by males (sniffing and chasing the female) and male sexual behavior (attempted mount) were observed and recorded. An attempted mount was defined as the male jumping toward the female from behind with forepaws raised [31,50]. The assignment of individual females was counterbalanced across the different treatment groups. The entire procedure was performed blindly with respect to the identification of individual mice.

2.3.4. Aggressive behavior test (21 weeks of age)

Aggressive behaviors were examined in a resident-intruder paradigm for 5 min under the same red light that was used for the sexual behavior test. A group-housed young male ($n = 8$) was placed into the subject's home cage as the intruder. The behavior was video-recorded and analyzed blindly afterward. The numbers of attacks (bites) and of species-specific threat behaviors (tail rattling and digging) were observed and recorded for 5 min after the first attack. A bite was defined as a mouse putting its head on the opponent followed by the opponent twisting its body. Tail rattling was defined as rapid undulation of the tail, and digging was defined as the animal digging the sawdust with its forepaws [34]. If the subject did not attack for 15 min, the observation was terminated. The entire procedure was performed blindly with respect to the identification of individual mice.

2.4. Immunohistochemistry

2.4.1. Tissue collection and preparation (36 and 37 weeks of age)

The mice were anesthetized with pentobarbital (60 mg/kg body weight ip) and were perfused through the left ventricle, first with 0.9% saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The brain was quickly removed and postfixed overnight in 4% paraformaldehyde at 4 °C. The fixed brain was rinsed in phosphate buffered saline (PBS) and immersed in 20% sucrose in PBS for 24 h at 4 °C before sectioning. Serial frontal 40-μm brain sections were made using a freezing microtome (Yamato Kohki Industrial Co., Saitama, Japan) at −20 °C. The sections were preserved in PBS until immunostaining.

2.4.2. Immunohistochemistry

The avidin–biotin technique was used for the immunostaining study. Free-floating sections were incubated in 3% hydrogen peroxide to eliminate endogenous peroxidase activity. After the hydrogen peroxide was rinsed off, sections were blocked by incubation in 1% bovine serum albumin with 0.1% Triton X-100 in PBS (BSA-PBST) containing 10% normal goat serum for 1 h at room temperature. The sections were subsequently incubated

with rabbit polyclonal antibody against rat ERα (dilution: 1:15,000; Upstate Biotechnology, NY, USA) or rabbit polyclonal antibody against rat TH (dilution: 1:1000; Chemicon International, CA, USA) in BSA-PBST for 60 h at 4 °C, then incubated with biotinylated anti-rabbit secondary antibody (dilution: 1:200; Vector Laboratories, CA, USA) in BSA-PBST for 1 h. Finally the sections were incubated with avidin–biotin complex (dilution; 1:100, Vector Elite Kit, CA, USA) in BSA-PBST for 1 h at room temperature. The sections were washed with PBST after each incubation. After the last wash, the sections were rinsed with sodium acetate buffer (pH 7.6), and the peroxidase activity of the avidin–biotin complex was revealed in the same buffer containing 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich, MO, USA) and 0.0025% hydrogen peroxide. The sections from the DES and control groups were put on the same plate and processed together to minimize any possible variation in staining. The sections were mounted onto Silane-coated slide glass (MSA-slides glass, Matsunami Glass, Japan), air-dried, dehydrated with graded concentrations of ethanol and xylene, and covered with a cover slip.

2.4.3. Quantification of ER-IR and TH-IR cells

The ER-IR cells were examined with a computer-assisted image analysis system (KS-300, Zeiss, Germany). For counting ER-IR cells, seven serial sections of the VMH area (start from 1.46 mm caudal to bregma) and three serial sections of the AM area (from 1.46 mm caudal to bregma) were used. The TH-IR cells were counted under microscopy three times to minimize counting errors; the mean of the three counts was used for analysis. The number of TH-IR cells in the periaqueductal gray area (PAG; six sections in every fourth section from 4.24 mm caudal to bregma), the ventral tegmental area (VTA; two sections in every second section from 3.28 mm caudal to bregma), and the locus coeruleus area (LC; two sections in every fourth section from 5.34 mm caudal to bregma) were counted. A brain atlas by Franklin and Paxinos [20] was consulted to determine the brain structures. For both ER-IR and TH-IR cells, the total number of immunoreactive cells in each area was calculated and used for analysis.

2.5. Evaluation of the genital organs (36 and 37 weeks of age)

The left testis and the preputial glands of male offspring were removed and weighed before the paraformaldehyde perfusion. Anogenital distance was measured with calipers.

2.6. Statistical analysis

The data were averaged within litter, which was used as the unit of statistical analysis. The results of the behavioral tests were analyzed with a two-way ANOVA or a one-way ANOVA followed by Scheffe's test. To evaluate the interrelationship among the different behavioral endpoints, the Pearson's correlation coefficient was calculated among all the behaviors examined. Immunohistochemistry data were analyzed using Student's t test. Unless otherwise specified, P values less than 0.05 were considered statistically significant.

3. Results

3.1. Reproductive performance of the dams and growth of offspring

The pregnancy weight gain was not different among treatment groups. The number of pups per litter, the sex ratio, and birth weights did not differ among the groups. DES exposure did not influence absolute or relative values for body weights, testis weights, or preputial gland weights in male offspring. In female offspring, although the birth weight did not differ between groups, the body weights in the DES 3 group after weaning (3–12 weeks of age) were slightly (10%) but significantly heavier than in the control group. There was no difference in anogenital distance between the DES groups and the control group in males.

3.2. The effects on activity in the open-field test

The mean distance of ambulation in the open-field is shown in Fig. 1. A two-way ANOVA showed the significant primary effect of treatment [$F(2,16)=4.38$, $P=0.030$], while neither the sex nor the interaction between treatment and sex was significant. The mean distance in the DES groups showed a dose-dependent increase, and a significant difference was found between the DES 3 group and the control group ($P=0.030$ by Scheffe's test). It should be

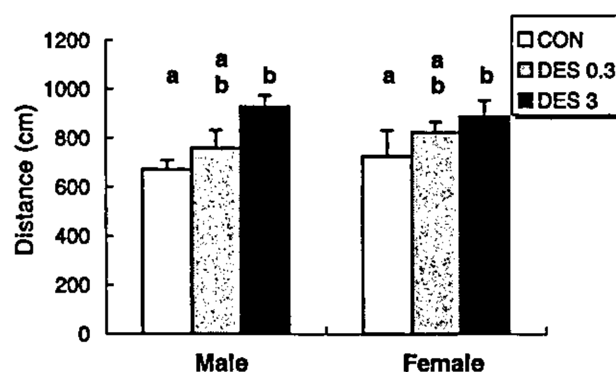

Fig. 1. Effect of perinatal exposure to DES on mean distance of ambulation (mean ± SE) for offspring of dams exposed to 0.3 μg/kg/day DES (DES 0.3), 3 μg/kg/day DES (DES 3), or corn oil (CON). In both sexes, averaged data within litter was shown. $n=4$ for CON and DES 0.3, $n=3$ for DES 3. Bars sharing the same alphabets are not significantly different with each other (by Scheffe's test).

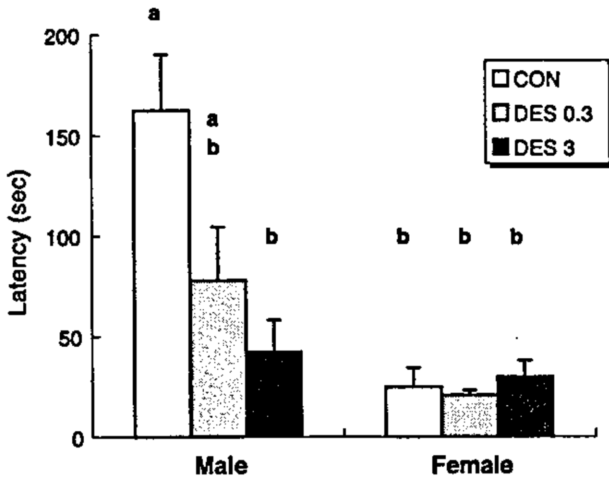

Fig. 2. Effect of perinatal exposure to DES on mean step-through latencies (mean ± SE) for offspring of dams exposed to 0.3 μg/kg/day DES (DES 0.3), 3 μg/kg/day DES (DES3), or corn oil (CON). Averaged data within litter was shown. In male, $n=4$ for DES 0.3, $n=3$ for CON and DES 3. In female, $n=4$ for CON and DES 0.3, $n=3$ for DES 3. Bars sharing the same alphabets are not significantly different with each other (by Scheffe's test).

noted that no sex difference in the distance of ambulation was observed in the control groups.

3.3. The effects on performance in the passive avoidance test

Fig. 2 shows the effects of DES on the step-through latencies in the passive avoidance test of male and female mice. One male in control group never enter into the dark chamber in the training trial, thus, we eliminate this litter from analysis. Since a two-way ANOVA showed significant interaction between treatment and sex, the results were reanalyzed with a one-way ANOVA, which showed a significant effect of the treatment [$F(5,15)=8.67$, $P=0.0001$]. Scheffe's test showed significant differences between male control group and the male DES 3 group as well as all the female groups.

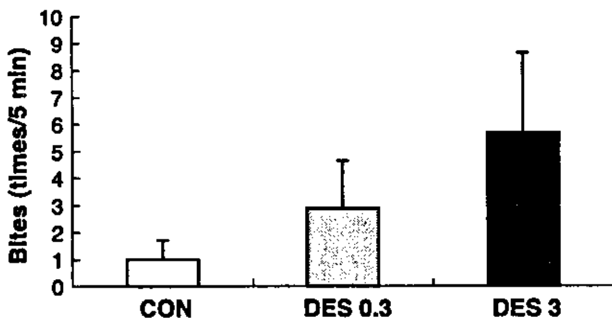

Fig. 3. Effect of perinatal exposure to DES on mean number of bites (mean ± SE) for offspring of dams exposed to 0.3 μg/kg/day DES (DES 0.3), 3 μg/kg/day DES (DES 3), or corn oil (CON). Averaged data within litter was shown. $n=4$ for CON and DES 0.3, $n=3$ for DES 3.

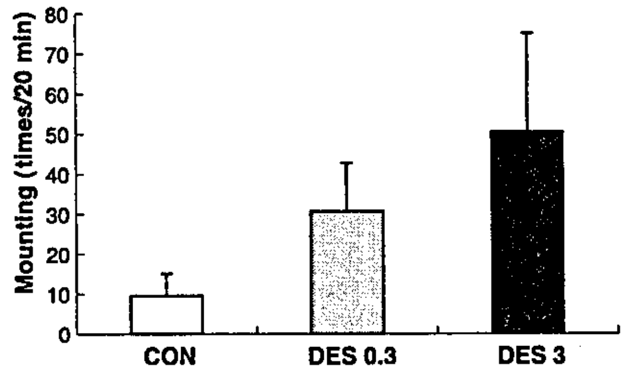

Fig. 4. Effect of perinatal exposure to DES on mean number of attempted mounts (mean ± SE) for offspring of dams exposed to 0.3 μg/kg/day DES (DES 0.3), 3 μg/kg/day DES (DES 3), or corn oil (CON). Averaged data within litter was shown. $n=4$ for CON and DES 0.3, $n=3$ for DES 3.

3.4. The effects on the aggressive and sexual behaviors of males

The DES groups tended to have a higher number of attacks, a higher proportion of individuals exhibiting attacks and, among these individuals, a shorter latency to the first attack, although the differences did not reach statistical significance ($P>0.1$; Fig. 3). The DES group also exhibited a higher frequency of attempted mounts, while not statistically significant ($P>0.1$; Fig. 4).

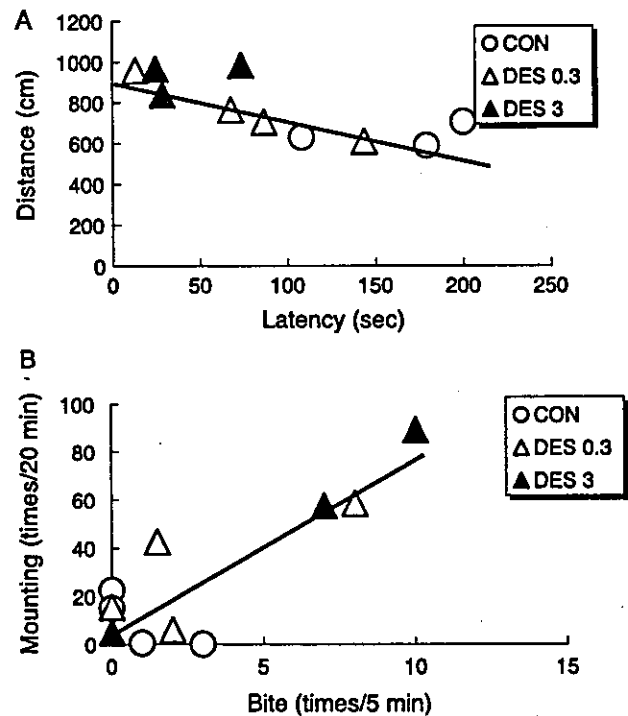

Fig. 5. Correlations among behavioral tests within individuals; between step-through latencies and distances of ambulation (A), between the number of bites and the number of attempted mounts (B). Averaged data within litter was shown. Regression lines were statistically significant ($P<0.05$).

3.5. Intraindividual correlations between different behaviors

The distance of ambulation in the open-field test and the step-through latency in the passive avoidance test were negatively correlated in males ($r = -0.77$, $P = 0.009$; Fig. 5A). In male, the step-through latency in the passive avoidance test was negatively correlated to the number of attempted mounts in the sexual behavior test ($r = -0.71$, $P = 0.022$). Also, the number of attempted mounts in the sexual behavior test was positively correlated with the number of bites in the aggressive behavior test in males ($r = .85$, $P = 0.001$; Fig. 5B).

3.6. The effects on the number of ER-IR cells and TH-IR cells

The number of ER-IR cells identified in VMH sections was significantly higher in the DES 3 group than in the control group ($P = 0.048$; Figs. 6 and 7). In AM sections, there was no significant difference in the number of ER-IR cells among the groups. No differences were observed in the number of TH-IR cells in PAG, VTA, or LC sections (data not shown).

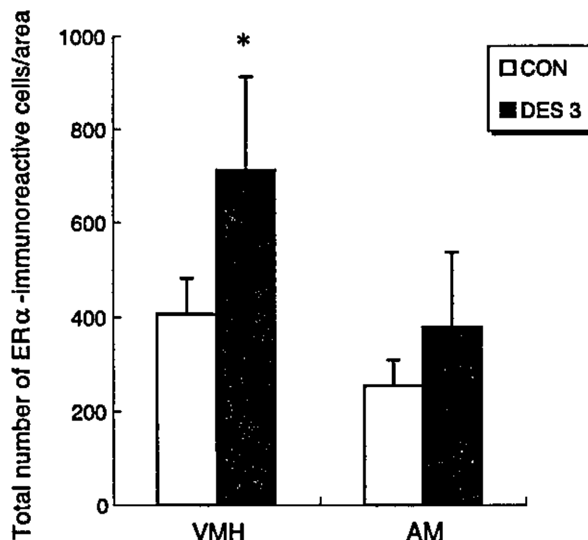

Fig. 7. Effect of perinatal exposure to DES on CNS (mean ± SE). Figure shows the number of ER-IR cells in area of VMH and AM. $n = 3$ for CON and $n = 4$ for DES 3. * $P < 0.05$, compared with the control group.

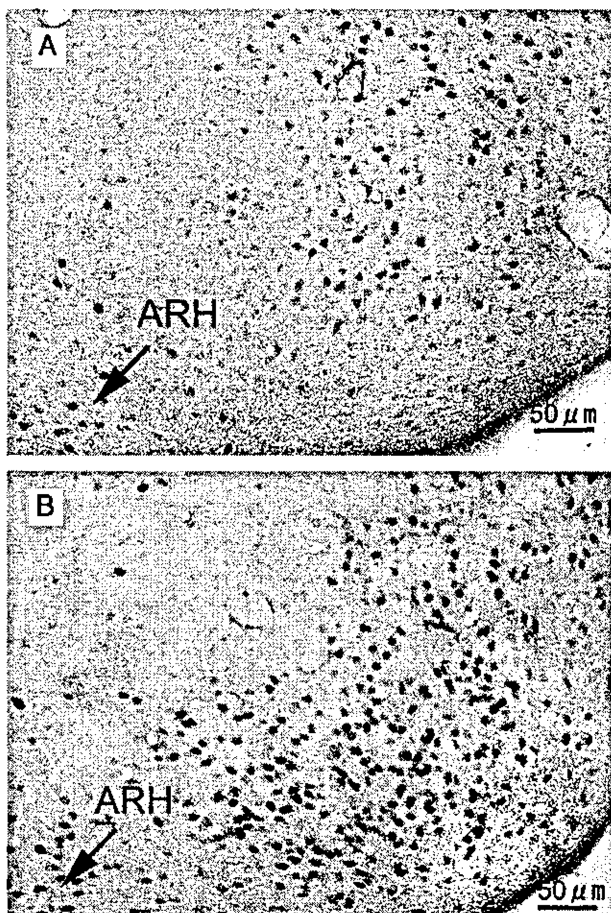

Fig. 6. Photomicrographs showing ER-IR cells in the ventrolateral part of the VMH for male offspring of dams exposed to corn oil (A), or 3 μg/kg/day DES (B). ARH = arcuate hypothalamic nucleus. The scale bars represent 50 μm.

4. Discussion

The present study demonstrated that perinatal exposure to DES in mice caused enhanced open-field activity in both males and females and poorer passive avoidance performance in males. In addition, a significant increase in the number of ER-IR cells in the VMH was demonstrated for the first time.

The behavioral changes demonstrated in the present study were consistent with previous studies using low-dose DES. The effects of low-dose DES on open-field activity (increased in males) and on sexual behavior (not statistically significant but slightly elevated frequency in mount behavior in males) were reported by Kubo et al [25]. Takahama et al. [45] have shown a poorer performance in passive avoidance test. These two experiments used pre- and/or postnatal exposure to approximately 6.5 and 3 μg DES/kg body weight respectively. Palanza et al. [39] and Vom Saal et al. [48] have reported an increase in aggressive behavior and urine-marking behavior, a type of reproductive behavior, in mice born to dams that were exposed to 2 μg DES/kg body weight during prenatal period, although the change of aggressiveness and sexual behavior did not reach statistical significance in the present study.

On the other hand, the behavioral changes induced by estrogenic EDs such as BPA and nonylphenol are not consistent with DES studies. Various studies using the dose below the NOAEL of BPA and nonylphenol were conducted to evaluate many endpoints. Aggressiveness in male and

female rats was not influenced by pre- and postnatal BPA administration [18]. Male sexual activity showed a slight impairment in terms of latency and frequency of intromissions [18]. These changes do not agree with that induced by DES exposure [39,48]. BPA abolished the sexual differences of the open field behavior and passive avoidance test [24,25], which appeared to be due to the increased activity in the open-field test and the poorer performance in males. These findings were similar to the results in the present study and other DES studies [24,45], while no effects on the activity in open-field test were reported by one study [15]. We could not find any studies that showed effects of nonylphenol on behaviors such as open-field behavior and other sexually dimorphic behaviors [19,37].

Causal relationships between the behavioral and neurohistological changes could not be elucidated in this study. It has been known that estrogen is related with open-field activity [40,51], and VMH is known as a brain site influencing the locomotor, sexual, or aggressive activity in rodents [5,11,41]. Although it is possible that the neurohistological change in the VMH underlies the observed behavioral changes, neither the role of ERα of VMH in such behaviors, nor the mechanism by which an increase of ERα links to behavior is currently understood, and in the present study, the correlations between the immunohistological results and behavioral results within individual were not statistically significant (data not shown) presumably due to the small sample size used. The relation between the observed changes in ER-IR and behavioral changes should be further examined. It should be noted that no effect of DES on the number of TH-IR cells was found in the regions examined.

Previous studies using higher doses of DES, estrogen, or testosterone (will be converted to estrogen in the brain), (e.g., greater than 500 times the doses used in the present study), resulted in the suppression of sexual and aggressive behaviors and no change in open-field activity in male rodents [8,9,13]. These results do not agree with our results. The discrepancies in behavioral changes induced by DES may be due to the differences in doses among these studies. It should be noted that Khurana et al. [23] demonstrated that ERα expression decreased in the medial basal hypothalamus in male rats with neonatal exposure to DES. The dose used in the study by Khurana et al. [23] (5μg DES/animal/day sc for 5 days) was approximately 60 times higher than that used in our study. Therefore, results to date suggest that DES and estrogen at high doses can suppress both ERα expression and sexual and aggressive behaviors, while DES at low doses may have the opposite effect.

The increased activity in the DES-treated males may be responsible for their decreased step-through latency, the latter of which basically confirmed the observation by Takahama et al. [45]. In intraindividual correlations, locomotion in the open-field test negatively correlated with performance in the passive avoidance test. Thus, the poorer performance in the passive avoidance may be due to the increased exploratory behavior in novel environment. This possibility should be further examined.

Sexually dimorphic behaviors have been the major targets in evaluating the toxicity of estrogenic compounds, since perinatal estrogen has an important role in the sexual differentiation of the brain. In the present study, the DES-exposed males performed poorer in the passive avoidance test, becoming closer to female's performance, which could be regarded as feminization. On the other hand, male sexual behavior and aggressive behavior, although statistically not significant, tended to be enhanced by DES, which may be indicative of masculinization rather than feminization. As discussed above, these behavioral changes are consistent with several preceding studies [25,39,48]. Other than these endpoints, low-dose DES induced several changes in male rodents that could be interpreted as feminization, including the open-field behavior in rats [25,44] and reduced testis size [4,25], while it did not change the size of sexually dimorphic nuclei in rodents [14,16,46]. Therefore, the DES-induced changes could be either feminization or masculinization, depending on the examined endpoint. Although explanation of such an apparent discrepancy awaits further research, it is possible that DES exerted its effects via several pathways, resulting in mixed responses in terms of sexual dimorphism.

In conclusion, this study showed that perinatal exposure to low-dose DES induced a significant increase in open-field activity in both sexes, a significant decrease in performance of the passive avoidance test in male mice. Furthermore, a significant increase in the number of ER-IR cells in the VMH area of the DES-exposed male mice was demonstrated for the first time. The relationship between the increase in ER-IR cells and behavioral changes should be further examined.

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Susceptibility of Metallothionein-Null Mice to the Behavioral Alterations Caused by Exposure to Mercury Vapor at Human-Relevant Concentration

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While recent human studies suggested adverse neurobehavioral outcomes of low-level exposure to mercury vapor (Hg^0) as found among those having dental amalgam fillings and dental personnel, past animal experiments only dealt with exposure at much higher mercury concentrations. The present study aimed to examine neurobehavioral effects of prolonged, low-level Hg^0 exposure in mice and to evaluate the protective role of metallothionein -I,II (MT-I,II) against Hg^0-induced neurotoxicity, using a knock-out strain of mice. Adult female metallothionein-I,II-null (MT-null) and wild-type OLA129/C57BL6 mice were exposed to 0.06 mg/m^3 of Hg^0 for 8 h per day for 23 weeks. Neurobehavioral effects were evaluated at 12 and 23 weeks of exposure using open-field test and passive avoidance test. Subcellular distribution of mercury and the induction of MT were also assessed. The Hg^0 exposure resulted in significantly enhanced locomotion in the open-field test and poorer performance in the passive avoidance test at a brain Hg concentration less than 1 ppm. These effects were slightly exaggerated in MT-null mice, which showed less induction of MT, lower brain Hg concentration, and lower calculated concentration of MT-unbound cytosolic Hg. The results showed, for the first time, that a concentration of Hg^0 relevant to human exposure level could cause neurobehavioral effects in adult mice. The higher susceptibility of MT-null mice suggested that MT-I,II have protective roles in the metal-induced neurobehavioral toxicity, which cannot be entirely explained by kinetic mechanisms, thus suggesting an involvement of nonkinetic mechanisms.

Key Words: mercury (elemental); low-level exposure; metallothionein; knock-out mouse; behavior; learning.

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Two types of mercurials could potentially exert neurotoxic effects that are relevant to the general public: methylmercury and metallic mercury (Hg^0). Of these, exposure to the latter occurs in occupational and nonoccupational settings, and the exposure at higher levels, as found among mercury miners, results in neurotoxicity such as tremor, ataxia, and psychic disturbances (WHO, 1991).

Prolonged, lower-level exposures occur among dental personnel handling Hg-containing amalgams or among those who have dental amalgam fillings, for which health consequences have not been well documented. While recent human studies suggested that such a low-level exposure might be related with adverse neurobehavioral outcomes (Bittner *et al.*, 1998; Echeverria *et al.*, 1998), animal experiments only dealt with exposure at higher mercury concentrations, except for those evaluating perinatal exposure (Danielsson *et al.*, 1993; Fredriksson *et al.*, 1992). Thus, experiments using adult rats or rabbits employed Hg^0 concentrations far exceeding 1 mg/m^3, resulting in brain mercury levels more than 1 µg/g (wet weight), while the LOAEL (Lowest-Observed-Adverse-Effect Level) and RfC (Reference Concentration) adopted by EPA (U.S. EPA, 2001) were 0.025 and 0.0003 mg/m^3, respectively, and the brain mercury concentrations found among the dental personnel or among those with dental fillings only reach up to 0.3 µg/g (Nylander and Weiner, 1991). Therefore, experimental data corresponding for the low-level exposures found among adult human population is apparently lacking.

The present study has two objectives. The first was to examine the effects of prolonged low-level Hg^0 exposure on neurobehavioral functions of mice. Two behavioral paradigms, open-field test and passive avoidance test, were used for the evaluation. These two behavioral tests were chosen primarily because they are easy to conduct and often very sensitive to many

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environmental manipulations, and also because the functions examined by these tests (i.e., response to a novel environmental stimuli and retention of a learned response, respectively) have some resemblances to those affected by low-level exposure to mercury vapor in human. That is, long-term, low-level exposure to mercury vapor is associated with symptoms of erethism, including irritability, excitability, and loss of memory (WHO, 1991).

The second objective was to test the hypothesis that mice lacking metallothionein-I,II (MT-I,II), the metal-binding proteins (MT-null), show enhanced susceptibility to the neurobehavioral effects of the Hg^0 exposure, if any. Alleviation of heavy-metal toxicity by MT-I,II is well established, and we and others have shown that MT-null mice exhibited enhanced renal (Satoh *et al.*, 1997) or pulmonary (Yoshida *et al.*, 1999) toxicity of inorganic mercury or cytotoxicity of methylmercury (Yao *et al.*, 2000). Since brain is a unique organ, in that it expresses a brain-specific MT, MT-III (Hidalgo *et al.*, 2001; Satoa and Kondoh, 2002), such a protective role of MT-I,II might be obscured in case of neurotoxicity.

MATERIALS AND METHODS

Animals and exposure to mercury (Hg^0) vapor (Yoshida et al., 1986). Eight-week-old female MT-null mice and wild-type OLA129/C57BL6 control mice were kindly provided by Dr. A. Choo. The animal facility was maintained under a light/dark cycle of 12-h, temperature of 24 ± 1°C, relative humidity of 55 ± 10%. The mice received laboratory chow (CE-2, Japan Crea, Tokyo, Japan) and filtered tap water *ad libitum* with humane care according to the National Institute for Environmental Studies' guidelines for animal welfare. The total mercury content of the laboratory chow was routinely analyzed by the manufacturer and reported to be less than their detection limit (i.e., < 10 ppb). Sixteen MT-null and fourteen wild-type female mice were exposed to mercury vapor for 8 h/day for 23 weeks starting at 8-weeks old. Briefly, mercury vapor was generated by passing air through a tandem flask containing Hg^0, mixed with fresh air, and introduced into a plastic exposure chamber (50 × 50 × 70 cm). The median concentration of mercury vapor in the exposure chamber was 0.06 mg/m^3 (range: 0.031–0.119 mg/m^3), which was comparable to the LOAEL of 0.025 mg/m^3 (USEPA; as described above). The control animals were treated identically but with no mercury in the flask. Animals were subject to two behavioral tests, at both 12 and 23 weeks of exposure period. After completion of all the behavioral evaluations, all animals were killed under diethyl ether anesthesia. Brain, lung, liver, and kidney were removed immediately and stored at −80°C until analysis.

Gel filtration of tissue supernatant. Details of the procedure are given elsewhere (Yoshida *et al.*, 1999). Briefly, tissues were homogenized in ice-cold KCl under a N_2 atmosphere and centrifuged (105,000 × *g* for 60 min). The obtained supernatant was filtered, and an aliquot of the supernatant was applied to a Superdex 75 HR 10/30 column (Pharmacia Biotech, Tokyo, Japan) equilibrated with phosphate-buffered saline (PBS). The sample was eluted with the same buffer at 4°C, and 1-ml fractions were collected at a flow rate of 0.5 ml/min.

Analysis of mercury concentrations in tissue. Mercury concentrations in the tissues were measured with a cold atomic absorption spectrophotometer (RA-2A Mercury Analyzer; Nippon Instruments, Tokyo, Japan) after digestion with a concentrated acid mixture ($HNO_3/HClO_4$ 1:3 [v/v]). The detection limit was 0.5 ng Hg with an intra-assay coefficient of variation (n = 10) of 4%.

Analysis of MT in tissue. Metallothionein levels in the maternal and fetal liver were determined using the mercury-binding method (Naganuma *et al.*, 1987). Briefly, tissue homogenate in Tris-HCl (pH 7.6) was mixed with diethylmaleate and incubated at room temperature for 20 min. After the addition of $CdCl_2$, the samples were heated to 100°C for 3 min and centrifuged (3000 × *g* for 5 min). $HgCl_2$ was added to the supernatant, and metallothionein-unbound mercury was removed by adding ovalbumin. Afterward the ovalbumin was removed with trichloroacetic acid and centrifugation. After filtration, the amount of metallothionein -bound mercury was measured as above.

Open-field test. Both of the behavioral experiments were conducted by a person who was blind to the treatment allocation. The locomotor activity was assessed using the open-field apparatus after Tanaka *et al.* (2004) with slight modifications. The apparatus consisted of the floor (50 × 50 cm) surrounded by a 50-cm-high opaque wall. A CCD camera fixed above the apparatus was connected to a Macintosh computer, and the movement of the [image of] mouse was analyzed using Image OF (O'Hara & Co., Ltd., Tokyo, Japan), a modified NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In this software, the position of the animal was defined as the position of the gravity center of the image of the animal, which was calculated in every 0.5 s. The total distance moved by the gravity center was calculated and converted into centimeters. Also, the area of floor was divided into 25 squares (10 × 10 cm), and the location of the animal was classified either as central (nine areas that did not have direct contact with the wall) or peripheral (other sixteen areas). Behavior was monitored for 10 min after placing the mice on the center of the floor. Between each trial, the floor and wall were cleaned with 70% alcohol followed by wet cotton. The test was conducted in 2 days; the wild type was tested on the first day, and the MT-nulls on the second.

Passive avoidance. The apparatus (model PA-2010A, O'Hara & Co., Ltd, Tokyo, Japan) consisted of a dark and an illuminated compartment, which were separated by a sliding door. On the first day (training trial), the mouse was placed in the illuminated compartment for 30 s, and then the door was opened. When the mouse entered the dark compartment, it received an unavoidable scrambled electric shock to its foot (4 mA × 2 s). Latency was defined as the interval between the opening of the door and the entry of the mouse to the dark chamber. Immediately after the mouse escaped to the illuminated compartment, the door was closed and the mouse was removed from the apparatus. The animal was placed once again in the illuminated compartment, 24 h after the training trial (retention trial), and the latency (avoidance latency) was recorded. The retention session lasted a maximum ("cut-off") of 300 s; when the latency of an animal exceeded 300 s, the latency was recorded as 300 s for later analyses. The test was conducted in 2 days immediately following the open field test; the wild type was tested on the first day, and the MT-nulls on the second.

Statistical analysis. Student's *t*-test was used to compare the nonexposed control with the exposed group, except for the passive avoidance test, in which Wilcoxon's nonparametric test was used because the distribution was skewed due to the existence of cut-off time. In either of these tests, the significance level was set to $p < 0.05$.

RESULTS

At 12 weeks of exposure, the mice were placed in the open-field apparatus for a 10-min observation to evaluate general activity levels in a novel environment (Fig. 1A). Regardless of the mice strain, the Hg-exposed mice showed enhanced locomotor activity when compared with the unexposed (control) mice. At the completion of the exposure (23 weeks), the enhanced activity was observed again, although the effect of the mercury was significant only for MT-null groups, presumably due to the small variation in the nonexposed MT-null group (Fig. 1B).

Performance in the passive avoidance test, a learning task motivated by strong aversive stimuli, was not affected at 12 weeks of exposure (Fig. 2A). When these mice were tested

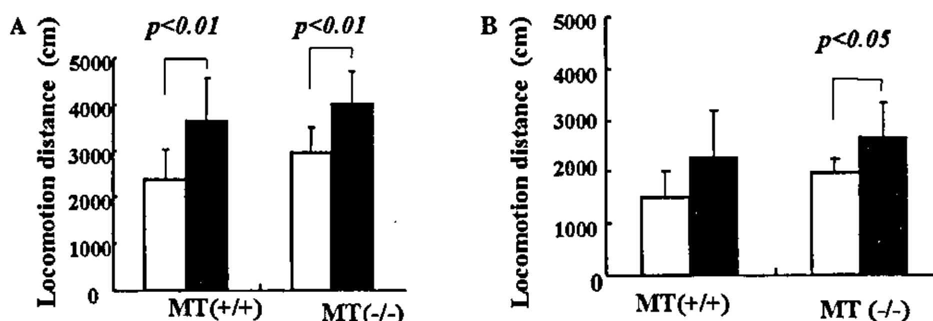

FIG. 1. Locomotion activity of metallothionein-null (MT-null) and wild-type mice at 12 (A) or 23 (B) weeks of exposure to mercury vapor in the open-field task. MT(+/+) and MT(−/−) indicate the wild-type and MT-null mice, respectively. White bars and black bars stand for unexposed and mercury-exposed groups, respectively. Values are means ± standard deviations. Results of *t*-test are shown, where the difference is significant.

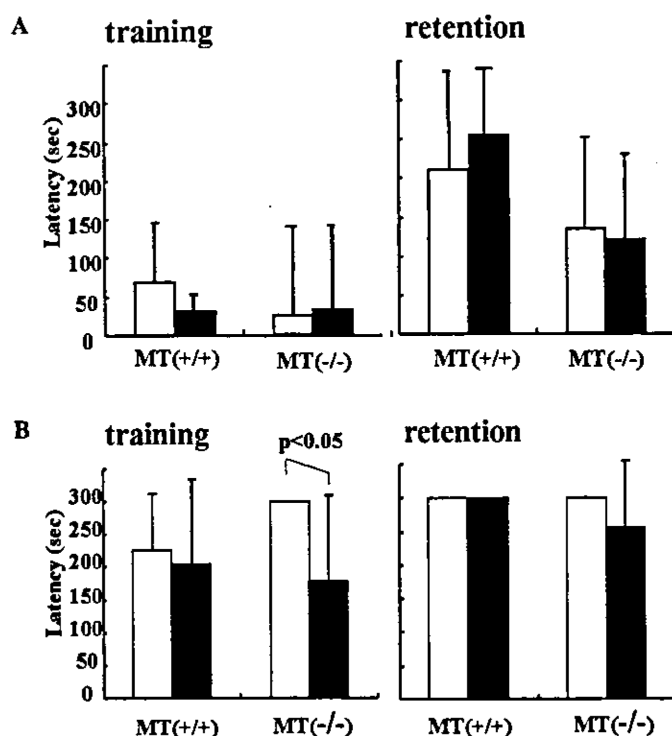

FIG. 2. Avoidance latency of metallothionein-null (MT-null) and wild-type mice at the "trial" (1st day) and the "retention" (2nd day) session of 12 (A) or 23 (B) weeks of exposure to mercury vapor in the passive avoidance task. MT(+/+) and MT(−/−) indicate wild-type and MT-null mice, respectively. White bars and black bars stand for unexposed and mercury-exposed groups, respectively. Values are means ± standard deviations. Results of Wilcoxon test are shown, where the difference is significant.

again at 23 weeks, the step-through latency at the "training" trial (i.e., the first session) was significantly shorter in exposed MT-null than in the unexposed MT-null mice. Such an effect of Hg exposure was not observed in the wild-type mice (Fig. 2B). In both the strains, these step-through latencies at 23 weeks were much longer than those in the training trial at 12 weeks, indicating the memory of learning experience at 12 weeks was retained at 23 weeks. In the retention trial (the second session),

TABLE 1
Mercury Concentration in the Brain of MT-Null and Wild-type Mice

	MT-null	Wild type
Whole brain (μg/g tissue)	0.66 ± 0.08*	0.97 ± 0.07
Supernatant (% of whole tissue)	65 ± 4*	75 ± 2
Supernatant (μg/g tissue)[a]	0.43	0.73
Pellet (μg/g tissue)[a]	0.23	0.24
MT-bound (% of supernatant)	80 ± 1	79 ± 4
MT-bound (μg/g tissue)[a]	0.35	0.58
MT-unbound (μg/g tissue)[a]	0.08	0.15

Note: Values are means ± standard deviations.
*Significant difference from the exposed wild-type mice at $p < 0.05$.
[a]Calculated from the mean values.

virtually all the mice stayed in the starting (light) chamber up to 300 s, the cut-off time, and no between-group difference was detected.

Tissue Hg levels in the exposed groups evaluated at 23 weeks of exposure showed MT-null accumulated significantly less mercury in all the examined organs (lung, heart, and kidney) except for liver (data not shown) than the wild type, which were consistent with previous observations in lung (Yoshida *et al.*, 1999) or in kidney (Satoh *et al.*, 1997). In the brain, the mean Hg concentrations were 0.97 ± 0.07 and 0.66 ± 0.08 μg/g (wet weight) for the wild type and MT-null, respectively. Only the trace amounts (<0.01 μg/g) could be detected in the brains of unexposed groups.

Possible induction of brain MTs by mercury exposure was evaluated. As expected, brain total MT level of unexposed mice was significantly higher in wild-type mice than in MT-null mice (119 ± 17 and 91 ± 6 nmol/g tissue, respectively). The Hg^0 exposure significantly increased the total MT both in wild type and, to a lesser extent, in MT-null (167 ± 11, 106 ± 5 nmol/g tissue, respectively).

As shown in Table 1, a significantly larger portion of total Hg was found in supernatant of the wild type than that of MT-null mice. The proportion of MT-bound Hg against total supernatant

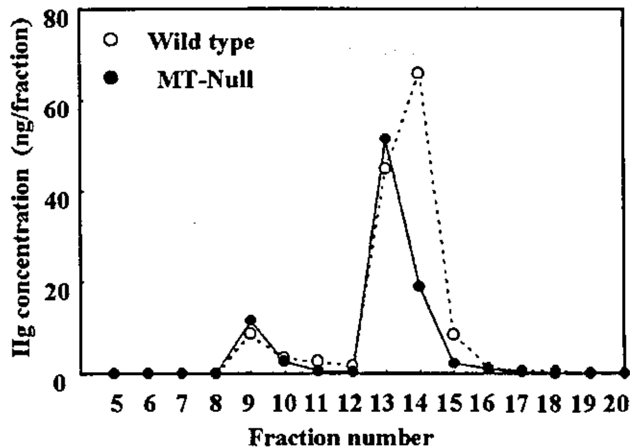

FIG. 3. Gel filtration profile of mercury in brain cytosol from MT-null and wild-type mice at 23 weeks of exposure to mercury vapor. The mercury eluted in fractions 13 to 16 is considered to be bound to MT.

Hg, however, was similar between the strains, leaving more "free" (MT-unbound) Hg in wild-type than in MT-null mice.

Typical gel filtration elution patterns of mercury in the brain cytosol of the MT-null and wild-type mice are shown in Figure 3. A major portion of the mercury eluted was detected in the MT fraction (fraction numbers 13 to 15), both in MT-null and the wild-type mice. The retention time for the MT-null peak was slightly shorter than that for the wild-type, presumably reflecting the binding to different MT isoforms (MT-I,II vs. MT-III). A small amount was also eluted in the high-molecular-weight (HMW) protein fractions (fraction numbers 9–10).

DISCUSSION

To our knowledge, this is the first study to show that prolonged, low-level exposure to mercury vapor, which resulted in brain Hg level less than 1 μg/g (ppm), caused behavioral alterations in adult rodents. It is also the first in providing *in vivo* evidence suggesting that MT-I,II null mice might be more susceptible than the wild-type mice to metal-induced neurotoxicity, although this differential susceptibility was not so distinct as we have reported before for renal or pulmonary toxicity of mercury (Satoh *et al.*, 1997; Yoshida *et al.*, 1999).

In the earlier study on rats (Kishi *et al.*, 1978), the behavioral abnormality induced by mercury vapor exposure (17 mg/m^3 × 2 h/day for 30 days) was associated with the brain Hg exceeding 10 μg/g. When the behavioral effects disappeared after the cessation of the exposure, the brain still contained approximately 5 μg/g of Hg, which is almost ten times the brain Hg in the Hg-exposed MT-null mice exhibiting behavioral abnormality. In rabbits, two of six animals exposed to 4 mg/m^3 of Hg^0 (6 hr/day, 4 days/week) for 13 weeks developed tremor; unfortunately, this study lacked the control group, and no correlation was found between the effects and the brain Hg levels (Fukuda, 1971). The brain mercury level in humans could reach as high as 33 μg/g in retired mercury miners (Takahata *et al.*, 1970), 0.3 μg/g in a group of dentists exposed to low levels of Hg^0 (while median is only 0.03 μg/g) (Nylander and Weiner, 1991), or more than 0.1 μg/g resulting from exposure through dental amalgam fillings, but less than 0.01 μg/g is expected in the absence of any known exposure source (WHO, 1991). Therefore, the present study denotes the fact that behavioral effects in mice were associated with the brain Hg levels found in humans occupationally exposed to low-levels of Hg^0. Since it is unlikely that brain Hg level at 12 weeks of exposure was higher than the level at 23 weeks, the enhanced open-field locomotion observed at 12 weeks must be associated with further low level, especially for MT-null mice.

Perinatal exposure of rats to mercury vapor leads to behavioral alterations with brain Hg concentrations much lower than the present study. Thus, *neonatal* exposure of newborn rats to Hg^0 at 0.05 mg/m^3, 4 h/day between postnatal day 11 to 17 *increased* locomotion activity and depressed spatial learning (Fredriksson *et al.*, 1992), whereas *prenatal* exposure (gestation day 11–14 and 17–20) at 1.8 mg/ m^3 for 1 or 3 h *decreased* locomotion activity and depressed spatial learning (Danielsson *et al.*, 1993). In these studies, brain mercury concentrations well below 0.1 μg/g were reported, suggesting that fetus and neonates are more susceptible to Hg^0 than adults are, as they are in case of methylmercury (WHO, 1990). These concentrations, however, were measured several days after the end of exposure, and the peak concentrations were not determined.

The higher susceptibility of the MT-null mice compared to the wild-type mice indicated that MT-I,II had a protective role in mercury-induced neurobehavioral toxicity, which apparently could not be explained by brain Hg level. Alleviation of metal-induced toxicity by MTs is widely recognized and often ascribed to their binding to (sequestering of) toxic metals; thereby the metals could not interact with other important biological molecules (Morgan *et al.*, 2002; Waalkes, 2002). In the present study, since the MT determination method did not distinguish different MT species, the brain MT levels in MT-null mice presumably reflected the presence of MT-III, the brain-specific MT (Hidalgo *et al.*, 2001; Palmiter *et al.*, 1993). Therefore, the increase of total MT in wild type and the less pronounced increase in the MT-null are consistent with previous studies showing induction of MT-I and II and less pronounced induction of MT-III in the brain of Hg-exposed rats (Palmiter *et al.*, 1993; Yasutake *et al.*, 1998). The induction of MT-I,II, the cytosol-rich proteins, influenced the subcellular distribution of Hg by providing its binding site, resulting in similar Hg levels in the pellet fraction (despite the different brain Hg levels) of both the strains. The concentrations of "free" Hg in the cytosol, which is unbound to MT and thus can interact with HMW proteins, however, was calculated to be higher in the wild-type than MT-null mice. Similar observations were obtained in our previous studies in kidney (after $HgCl_2$ injection) (Satoh *et al.*, 1997) or in lung (after Hg^0 exposure) (Yoshida *et al.*, 1999)

of MT-null mice. Therefore, the observed protective effect of MT-I,II are not entirely ascribed to kinetic mechanism (sequestering), suggesting an involvement of nonkinetic mechanisms, such as elimination of radicals (Hidalgo *et al.*, 2001; Satoh, 2002).

The enhanced susceptibility of MT-null mice regarding the behavioral effects of Hg^0 has dual implications for mercury neurotoxicology. First, as mentioned above, although there have been several reports showing the higher susceptibility of MT-null to lethal, renal, pulmonary, or cytotoxicity of metals including cadmium, mercury, or arsenic (Satoh and Kondoh, 2002; Yao, 2000; Yoshida, 1999), the present results appear to be the first demonstration of enhanced sensitivity of MT-null animal to metal-induced neurotoxicity. The findings indicated that the MT-I,II also play some roles in alleviating the brain mercury toxicity, where MT-III exists and potentially plays a similar role.

Second, it has a practical implication in human risk assessment. In the autopsy kidney samples of Japanese adults, MT concentrations increased with age, which is associated with accumulation of Cd with age; however, a subgroup did not show such induction of MT, although their Cd levels were high enough to trigger the induction (Yoshida *et al.*, 1998). The MT-null mice in the present study, showing genetically determined susceptibility, may be regarded as a model of these "slow-responder" to low-level (nonoccupational) metal exposure, although no genetic analyses have been done for these autopsy samples.

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Neurobehavioral changes in metallothionein-null mice prenatally exposed to mercury vapor

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Abstract

We studied the neurobehavioral effects of prenatal exposure of MT-null and wild-type mice to elemental mercury vapor (Hg^0). Pregnant mice of both strains were repeatedly exposed to Hg^0 vapor at 0.50 and 0.56 mg/m^3 for 6 h/day until the 18th day of gestation. The behavioral effects were evaluated with locomotor activity in the open field, learning ability in the passive avoidance response and spatial learning ability in the Morris water maze at 12 weeks of age. Hg^0-exposed MT-null mice showed a significant decrease in total locomotor activity in males, and a learning disability in the passive avoidance response and a retarded acquisition in the Morris water maze in females as compared with the control. In contrast, Hg^0-exposed wild-type mice did not differ from controls in the three behavioral measurements. The results indicate that MT-null mice would be more susceptible than wild-type mice to the behavioral neurotoxicity of prenatal Hg^0 exposure. Mercury concentrations in the brain of both strains were slightly higher in the exposed group than in the control group, indicating the retention of residual mercury even 12 weeks after the cessation of the exposure. Brain concentrations of mercury were also significantly higher in the exposed-females than exposed-males in either strain. From these results, we suggest that the increased susceptibility of MT-null females to behavioral changes caused by prenatal Hg^0 exposure is due to a greater retention of mercury and lack of MT-I,-II in the brain.
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Keywords: Mercury vapor; Metallothionein; Behavior; Prenatal exposure

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1. Introduction

Methylmercury (MeHg) and mercury vapor (Hg^0) are two types of mercury species that could have major health consequences in the general public. Since both of them can cross the placental barrier with relative ease and accumulate in fetal organs (Clarkson et al., 1972; Yoshida et al., 1986), prenatal exposure to these substances may exert congenital and/or developmental effects; in the case of MeHg, the effects typically manifest as congenital Minamata disease (Harada, 1968; Bakir et al., 1973).

Exposure to Hg^0 occurs not only in occupational populations such as mercury miners or dentists but also in the general population through diet and dental amalgam fillings. Particularly, Hg^0 released from amalgam fillings in the teeth of pregnant women is a potential source of exposure to Hg^0 for the fetus (Vimy and Lorsheider, 1985; Aronsson et al., 1989). Recently, gold mine workers with exposure to high levels of mercury vapor in mercury amalgamation and burning amalgam has raised great concern in developing countries (Ikingura and Akagi, 1996; Drasch et al., 2001). An experimental study showed that the release of Hg^0 from dental amalgam fillings results in a relatively high uptake of mercury in the maternal and fetal brain (Takahashi et al., 2001). While the placental transfer of Hg^0 and its distribution in fetal organs have been relatively well examined, little is known about the later consequences of in utero exposure to Hg^0.

Metallothionein (MT), a cysteine-rich and low-molecular-weight intracellular protein, is known to play an important role as a modifying factor of mercury kinetics and toxicity. Regarding the toxicity, MT-I and -II gene knockout mice (MT-null mice) show higher sensitivity to pulmonary toxicity of Hg^0 than do wild-type mice (Yoshida et al., 1999a). Also, we have recently shown that MT-null mice are more sensitive to long-term, low-level exposure to Hg^0 in terms of neurobehavioral toxicity than the wild-type mice (Yoshida et al., 2004). Regarding kinetics, MT is known to be present in the placenta, modulating the maternal-to-fetal transfer of essential and non-essential metals (Lau et al., 1998; Goyer and Cherian, 1992). It was shown that fetal mercury levels were significantly higher in MT-null mice than in wild-type mice after in utero exposure to Hg^0. Thus, it is possible that MT-null mice were more sensitive to the developmental effects of in utero Hg^0 exposure.

To test this hypothesis, the present study examined the neurobehavioral changes in mouse offspring that do not express MT-I and MT-II exposed in utero to a moderate level of mercury vapor. Although two preceding studies have examined the developmental toxicity of in utero Hg^0 exposure (Danielsson et al., 1993; Fredriksson et al., 1992), neither of them addressed the issue of genetic susceptibility. This issue has importance for human populations since we have identified a subpopulation with a limited ability to induce MT synthesis in Japanese (Yoshida et al., 1998).

2. Materials and methods

2.1. *Animals and exposure to mercury (Hg^0) vapor*

MT-null mice and wild-type OLA129/C57BL6 control mice were provided by Dr. A. Choo. The animal facility was maintained under a light/dark cycle of 12 h, temperature of 24 ± 1°C, relative humidity of $55 \pm 10\%$, and negative atmospheric pressure. The mice received mouse chow (CE-2, Japan Crea, Tokyo, Japan) and filtered tap water ad libitum. Males and females (one pair per cage) of age 8 weeks were mated. Pregnancy was confirmed by the presence of a vaginal plug the following morning (defined as gestational day 0 = GD0). Afterward, pregnant MT-null and wild-type mice were placed in the exposure chamber (60 cm × 60 cm × 60 cm) and the exposure to mercury vapor was started. Five pregnant MT-null and wild-type mice were repeatedly exposed to mercury vapor for 6 h per day until GD18 in a manner described previously (Yoshida et al., 1986). The mercury concentration in the exposure chamber was measured once a week by the air sampling method (Lindstedt and Skerfving, 1972) and was found to be between 0.50 and 0.56 mg/m^3. After the exposure for 6 h, the mercury vapor was forced out of the chamber by exchanging it with mercury-free air. The pregnant mice were housed in the exposure chamber until GD18. This relatively moderate concentration (about eight times higher than that in our previous report using adults (Yoshida et al., 2004) and 20 times higher than the current TLV) was chosen as a dose that would result in observable developmental neurotoxicity, because one primary object of this study

was to evaluate the possible modifying effect of MT in terms of developmental neurotoxicity. Animals were then moved back to their home cage to avoid further exposure and were allowed to give birth. The control animals were housed identically in the exposure chamber without exposure to mercury vapor. On day one postpartum, litter size was adjusted to six (three males and three females if possible). After weaning, the offspring were housed in group cages containing one to three animals each. After 12 weeks, all mice of each group were tested for locomotor activity in the open field test, and for learning in the passive avoidance task and in the Morris water maze. After the behavioral tests had ended, all animals were killed under diethylether anesthesia. The brain, lung, liver, and kidney were removed immediately and stored at −80 °C until analysis.

Throughout the experiment, animals received humane care according to the National Institute for Environmental Studies' guidelines for animal welfare.

2.2. *Behavioral analysis*

Behavioral functions of the mice were evaluated with three commonly used methods; the open field test, passive avoidance test, and Morris water maze. The rationale for choosing the former two tests are described elsewhere (Yoshida et al., 2004). The third method, the Morris water maze, was developed for the evaluation of spatial memory, with which the animals required to learn the spatial location of a hidden submerged platform in a water pool (Morris, 1984).

2.2.1. *Open field test*

The locomotor activity of mice was assessed using an open field, for which the methodological details are given elsewhere (Yoshida et al., 2004). Briefly, each mouse was moved from its home cage to the center square (10 cm × 10 cm) of the open field (50 cm × 50 cm), and covered with a black Plexiglas box (10 cm × 10 cm × 10 cm). After 20 s, the box was gently removed, and the behavior of the mouse was video-recorded for the following 10 min. The video image was analyzed by Image OF, software for image analysis (O'hara Co. & LTO, Tokyo, Japan). Two parameters of activity were calculated; the distance (in cm) moved by a mouse and the positioning of the mouse. For the latter, the 25 squares (each 10 cm × 10 cm) were classified as either peripheral (the 16 squares adjacent to the wall) or central (the nine remaining squares in the center).

2.2.2. *Passive avoidance response test*

Passive avoidance learning was assessed by a stepthough procedure; the details are also given elsewhere (Yoshida et al., 2004). The apparatus (PA-2010A, O'Hara & Co., Ltd, Tokyo, Japan) consisted of a dark and an illuminated compartment, which were separated by a sliding door. On the first day (training trial), the mouse was placed in the illuminated compartment for 30 s, and then the door was opened. When the mouse entered the dark compartment, it received an unavoidable brief electric shock to the foot, and escaped immediately to the illuminated compartment. The door was closed after the mouse re-entered the illuminated compartment, and the mouse was removed. Twenty-four hours later (the retention trial), the test was repeated again but without giving the electric shock. In both trials, the "latency" was defined as the interval between the opening of the door and the entry of the mouse into the dark compartment. The cut-off time of the retention session was 300 s.

2.2.3. *Morris water maze test*

Spatial learning was assessed using a Morris-type water maze test. The water maze was a circular plastic pool 100 cm in diameter and filled with water to a depth of 20 cm. The water was kept at room temperature (23 ± 1 °C) and was made opaque by adding white paint to prevent the animal from seeing the submerged platform. In the "hidden platform" trials, a round 10 cm-diameter platform made of white Plexigas was placed 1 cm below the water surface in the center of one of the four quadrants. A mouse was released in the water at one of the four randomly selected positions near the wall and facing the wall. The latency, defined as the time from the release of the mouse to the climbing on the platform, was recorded. When the mouse could not find the platform within 60 s from the time of release, it was led to the platform and placed on it for 20 s before being removed. In such case, a latency of 60 s was recorded. Each mouse received four trials on each of five consecutive days. The pool was fixed at the same position in the room, and the investigator always stood at the same position beside the pool during the experiment. Around the pool were also situated a video

device, animal steel racks and waterworks for animals. All were visible from the inside of the pool, and served as distant visible cues for the mouse. A visible platform trial, in which a mouse found the position by placing a marker on the submerged platform, was performed after a hidden platform trial was ended.

2.3. Analysis of mercury concentrations in tissue

Mercury concentrations in the tissues were measured with the cold vapor atomic absorption spectrophotometer (RA-2A Mercury Analyzer; Nippon Instruments, Tokyo, Japan) after digestion with a concentrated acid mixture [$HNO_3/HClO_4$ 1:3 (v/v)] (Satoh et al., 1997). The detection limit of this method was 0.5 ng Hg with an intra-assay coefficient of variation ($n = 10$) of 4%.

2.4. Statistical analysis

Data were analyzed statistically with Student's t-test or Wilcoxon's t-test for comparison between the non-exposed control and the exposed group with a preset probability level of $p < 0.05$ or 0.01.

3. Results

There was no abortion or stillbirth among the control and Hg^0-exposed groups of both strains during the pregnancy period. Macroscopic examination of neonates after birth also revealed no malformations among the control and exposed groups.

Effects of prenatal Hg^0 exposure on the locomotor activity in the open field at 12 weeks of age are shown in Fig. 1. In wild-type mice, there are no significant difference in the locomotion distance (cm) between the control and exposed mice in males and females. On the other hand, the locomotor activity levels of males of the exposed MT-null mice were significantly lower than those of the control ($p < 0.05$), but there was no difference among females. The exposed male MT-null mice also exhibited significantly more peripheral locomotion and less central locomotion than did the controls as shown in Fig. 2. The results of the retention trials in the passive avoidance test are shown in Fig. 3. No difference in avoidance latency between the control and exposed mice in males and females of the wild-

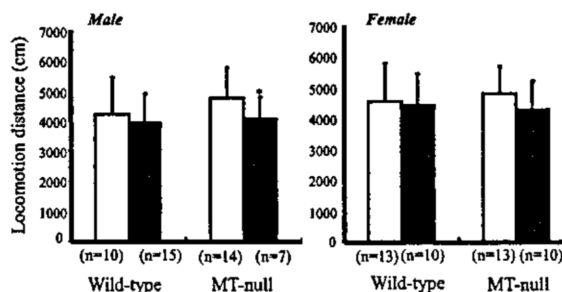

Fig. 1. Total locomotor activity of MT-null and wild-type mice exposed in utero to mercury vapor in the open field task. Data shown are the mean ± standard deviation for exposed (■) and control (□) mice. The number of animals is shown in parentheses. *Significant difference from control animals at $p < 0.05$.

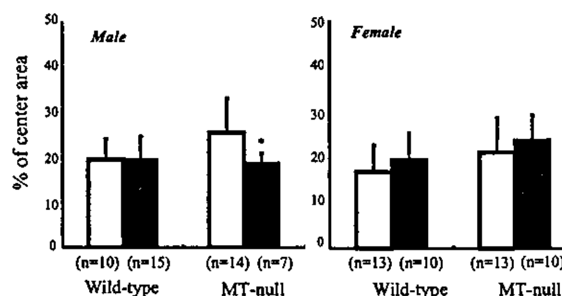

Fig. 2. The percentage of central entry of MT-null and wild-type mice exposed in utero to mercury vapor in the open field task. Data shown are the mean ± standard deviation for exposed (■) and control (□) mice. The number of animals is shown in parentheses. *Significant difference from control animals at $p < 0.05$.

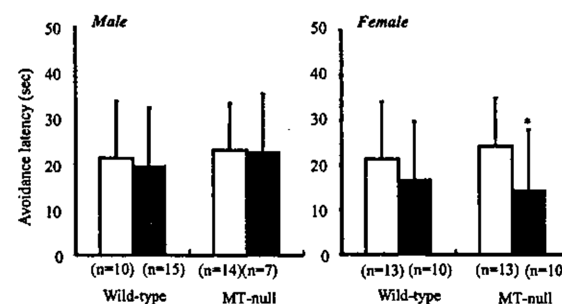

Fig. 3. Avoidance latency of MT-null and wild-type mice exposed in utero to mercury vapor in the passive avoidance task. Data shown are the mean ± standard deviation for exposed (■) and control (□) mice. The number of animals is shown in parentheses. *Significant difference from control animals at $p < 0.05$.

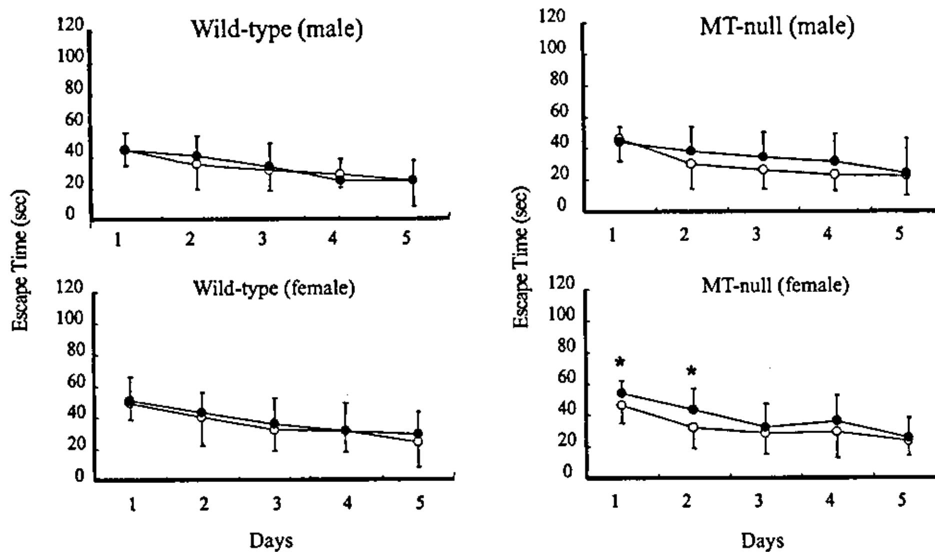

Fig. 4. Latency to reach the platform in the Morris water maze for MT-null and wild-type mice exposed in utero to mercury vapor. Data shown are the mean ± standard deviation for exposed (●) and control (○) mice. *Significant difference from control animals at $p < 0.05$.

type was observed. On the other hand, while there was no difference in the latency between the controls and exposed groups in MT-null males, the value was significantly shorter in the exposed group than in the controls of the MT-null females.

The latency in the Morris water maze test for five consecutive days is shown in Fig. 4. In males of both strains and in wild-type females, there were no significant differences in the escape time between the control and Hg^0-exposed group. In MT-null females, the latency was significantly shorter for the exposed group than the controls only in day 1 and day 2. In a visible platform trial, there were also no significant differences in the escape time between the control and Hg^0-exposed group in males and females of both strains (data not shown).

Table 1 shows the mercury concentrations in the brain and kidney of MT-null and wild-type mice exposed in utero to mercury vapor. Mercury concentrations in the brain and kidney of males and females of both strains were significantly higher in the exposed groups than in the controls. In the brain, mercury concentrations in the exposed males were not significantly different between the two strains, but the exposed MT-null females had significantly higher levels of mercury than the wild-type females. Brain concentrations

Table 1
Mercury concentration in the brain and kidneys of MT-null and wild-type mice exposed in utero to mercury vapor

		Wild-type		MT-null	
		Control	Exposed	Control	Exposed
Male	Brain	6.8 ± 1.2	8.2 ± 0.7*	4.9 ± 1.4	8.5 ± 0.9**
	Kidney	32.3 ± 5.2	43.0 ± 5.0**	29.2 ± 5.3	42.4 ± 5.4**
Female	Brain	7.3 ± 1.4	9.3 ± 1.1*	7.5 ± 0.9	11.3 ± 1.1**,#
	Kidney	20.2 ± 6.9	30.9 ± 4.0**	21.2 ± 6.3	42.6 ± 17.4*

Mercury concentration is expressed as ng Hg/g tissue. Values are means ± standard deviations.
* Significant difference from control animals at $p < 0.05$.
** Significant difference from control animals at $p < 0.01$.
# Significant difference from the exposed wild-type animals at $p < 0.05$.