Original Article

Neurobehavioral changes and alteration of gene expression in the brains of metallothionein-I/II null mice exposed to low levels of mercury vapor during postnatal development

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(Received July 1, 2011; Accepted July 15, 2011)

ABSTRACT — This study examined the neurobehavioral changes and alteration in gene expression in the brains of metallothionein (MT)-I/II null mice exposed to low-levels of mercury vapor (Hg^0) during postnatal development. MT-I/II null and wild-type mice were repeatedly exposed to Hg^0 at 0.030 mg/m³ (range: 0.023-0.043 mg/m³), which was similar to the current threshold value (TLV), for 6 hr per day until the 20th day postpartum. The behavioral effects were evaluated with locomotor activity in the open field (OPF), learning ability in the passive avoidance response (PA) and spatial learning ability in the Morris water maze (MM) at 12 weeks of age. Hg^0 -exposed MT-I/II null mice showed a significant decrease in total locomotor activity in females, though learning ability and spatial learning ability were not affected. Immediately after Hg^0 exposure, mercury concentrations in the brain did not exceed 0.5 $\mu\text{g/g}$ in any animals. Hg^0 exposure resulted in significant alterations in gene expression in the brains of both strains using DNA microarray analysis. The number of altered genes in MT-I/II null mice was higher than that in wild-type mice and calcium-calmodulin kinase II (Camk2a) involved in learning and memory in down-regulated genes was detected. These results provide useful information to elucidate the development of behavioral toxicity following low-level exposure to Hg^0 .

Key words: Mercury vapor, Low-level exposure, Behavior, Microarray, Gene expression

INTRODUCTION

The mammalian fetus and neonate are well known to be particularly susceptible to the toxic effects of heavy metals and other chemical substances and subsequently the potentially adverse effects appear in developing individuals (Kacew *et al.*, 1984; Counter and Buchanan, 2004). Exposure of the neonate to toxic metals occurs in the living environment through nursing and intake of metal-containing foods and water, and respiration in contaminated atmospheres.

Because mercury vapor (Hg^0) has high lipid solubility, inhaled Hg^0 is easily absorbed from the alveolus and

is then soon oxidized to inorganic mercury, accumulating in various organs. However, some Hg^0 can penetrate the blood-brain barrier and accumulates in the brain, the target organ. Significant exposure of the neonate to Hg^0 results in greater accumulation and retention of mercury in neonates than in adults (Yoshida *et al.*, 1989). Exposure to Hg^0 occurs not only in occupational populations, but also in the general population, which is primarily exposed to mercury from dental amalgam and diet. The exposure of the general population is generally low, but may occasionally be raised to the level of occupational exposure such as exposure of Hg^0 generated from a broken liquid mercury thermometer (Waffarn *et al.*, 1979). By expo-

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sure to prolonged low-levels of Hg^0 , neurobehavioral and psychological effects have been proven in human being (Echeverria *et al.*, 1998; Osborne and Albino, 1999). In animal experiments, low-level exposure to Hg^0 , at levels (0.05–0.10 mg/m^3) relevant to human exposure caused neurobehavioral effects (Yoshida *et al.*, 2004). Further, it was reported that neurobehavioral changes were caused in mice prenatally exposed to Hg^0 vapor at about a 20 times higher level than the current TLV (Yoshida *et al.*, 2005). Children are also known to be particularly vulnerable to mercury intoxication, which may lead to impairment of the developing central nervous system. Recently, children who assist their parent in gold-mining operations or live in gold-mining settlements and are exposed to Hg^0 vapor and its health effects, especially neurobehavioral toxicity due to the pediatric Hg^0 exposure, have been of concern. Thus, although Hg^0 exposure in childhood is a growing international public health concern, the neurobehavioral toxicity caused by pediatric Hg^0 exposure has not been studied enough.

Metallothionein (MT) protects against the toxicities of various metals. We examined the neurotoxicity and developmental toxicity of Hg^0 in MT-I/II knockout (MT-I/II null) mice and demonstrated the susceptibility of this genetically manipulated strain to Hg^0 toxicity (Yoshida *et al.*, 2004). Since a small subgroup with an impaired ability to induce MT synthesis is reported to exist among Japanese (Yoshida *et al.*, 1998; Kita *et al.*, 2006), such an MT-dependent susceptibility to Hg^0 toxicity is of practical important once.

The purpose of the present study was to examine whether the neurobehavioral changes were caused in MT-I/II null and wild-type mice by low-level exposure to Hg^0 during the suckling period. An exposure level closed to the TLV recommended by the WHO was chosen. The effects were evaluated by employing neurobehavioral paradigms, i.e., the open field test (OPF) and passive avoidance test (PA), which could detect the toxic effects of such low-level exposure in our previous study (Yoshida *et al.*, 2004). The Morris water maze (MM), a common behavioral test for the evaluation of spatial learning (Morris, 1984), was also used in the present study. Recently, genomic analysis using an array of gene alteration has been utilized to elucidate the mechanism of toxicity after Hg^0 exposure (Liu *et al.*, 2003). We also investigated the alteration of gene expression in the brains of wild-type mice and MT-I/II null mice exposed to Hg^0 during postnatal development.

MATERIALS AND METHODS

Animals and exposure to mercury vapor (Hg^0)

MT-I/II 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-hr, temperature of $24 \pm 1^\circ\text{C}$, 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) aged 10 weeks were mated. Pregnancy was confirmed by the presence of a vaginal plug the following morning and females were housed until giving birth. On day one postpartum, the litter size was adjusted to six (three males and three females if possible). Afterward, the dams and neonates of both strains were placed in the exposure chamber (60 x 60 x 60 cm) and the exposure to mercury vapor was started. Animals were repeatedly exposed to mercury vapor for 6 hr per day until the 20th day postpartum in the manner described previously (Yoshida *et al.*, 1986). The concentration of mercury in the exposure chamber was measured every day using a mercury survey meter (Type; EMP-1A, Nippon Instruments Co., Tokyo, Japan). The mean concentration found was 0.030 mg/m^3 (range: 0.023–0.043 mg/m^3), which was equivalent to a LOAEL of 0.025 mg/m^3 . The control animals were housed identically in a chamber, but without exposure to mercury vapor. Immediately after exposure to Hg^0 , a male and female offspring born to each mother were sacrificed to measure mercury concentration in the organs and the remaining offspring were housed with dams. After weaning, the offspring were housed into group cages containing three to four animals each. After 10 weeks, six to seven mice from each group were tested for locomotor activity in the open field test, and for learning in the passive avoidance task and Morris water maze. After the behavioral tests were completed end, these animals were killed under diethylether anesthesia. The brain, lungs, liver, and kidneys were removed immediately and stored at -80°C until analysis.

Animals received humane care throughout the experiment according to the National Institute for Environmental Studies' guidelines for animal welfare (Notification of the Prime Minister Office, 1980).

Behavioral analysis

Behavioral functions were evaluated with three commonly used methods: the open field test, passive avoidance test, and Morris water maze as described above.

OPF 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 x 10 cm) of the open field (50 x 50 cm), and covered with a black Plexiglas box (10 x 10 x 10 cm). After 20 sec, the box was gently removed, and the behavior of the mouse was video-recorded for the following 5 min. The video images were analyzed with Image OF, software for image analysis (O'hara Co. & Ltd., Tokyo, Japan). Two parameters of activity were calculated: the distance (in cm/5 min) moved by the mouse and the positioning of the mouse. For the latter, 25 squares (each 10 x 10 cm) were classified as either peripheral (the 16 squares adjacent to the wall) or central (the nine remaining squares in the center).

PA test

Passive avoidance learning, a learning task motivated by strong aversive stimuli, was assessed by a step-through procedure; the details of which are given elsewhere (Yoshida *et al.*, 2004). The apparatus (PA-2010A; O'Hara & Co., Ltd., Tokyo, Japan) consisted of a dark and 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 sec, 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, "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 for the retention session was 300 sec.

MM 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 (with negligible toxicity) to prevent the animal from seeing the submerged platform. In the "hidden platform" trials, a round 10-cm-diameter platform made of white Plexiglas 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 four randomly selected positions near the

wall and facing the wall. The latency, defined as the time from the release of the mouse to climbing on the platform, was recorded. When the mouse could not find the platform within 60 sec from the time of release, it was led to the platform and placed on it for 20 sec before being removed. In such a case, a latency of 60 sec 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 marker was placed on the submerged platform, was performed after the hidden platform trial was ended.

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) after digestion with a concentrated acid mixture [HNO₃/HClO₄ 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%.

Isolation of RNA

Total RNA was extracted from the liver in the MT-III null mice and wild-type mice after Cd injection using the QuickGene RNA tissue kit S II (Fujifilm, Tokyo, Japan), according to the manufacturer's protocol and stored at -80°C.

DNA microarray analysis

Total RNA (5 µg) was applied to an OpArray™ Mouse V4.0 slide, that had 35,852 genes registered (Operon Technologies, Alameda, CA, USA). We used a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies Inc., Santa Clara, CA, USA) to synthesize complementary RNA (cRNA) from double-stranded cDNA as a template. A primer containing poly dT and T7 polymerase promoters was annealed to the poly A⁺ RNA. Reverse transcriptase was then added to the reaction to synthesize the first and second strands of cDNA. Next, the double-stranded cDNA from wild-type mice or MT-III null mice was transcribed in the presence of the cyanine (Cy) 3 or Cy5-labeled nucleotide, respectively. These two sets of fluorescence-labeled cRNA were mixed and hybridized to an OpArray™ slide for 16 hr at 42°C using a Lucidea SlidePro Hybridizer (GE Healthcare UK Ltd., Buckinghamshire, England). A fluorescent image

of the OpArray slide was recorded with CRBIO (Hitachi Software Engineering, Tokyo, Japan). The digitized image data were processed with DNASIS Array software (Hitachi Software Engineering). After global normalization, the data were filtered to exclude genes with low expression levels. The ratios of the intensity of Cy5 (MT-III null mice exposed to Hg⁰) to that of Cy3 (wild-type mice exposed to Hg⁰) were then calculated and a 2-fold change difference was applied to select up-regulated and down-regulated genes. Information on each gene on the slide was obtained from the National Center for Biotechnology Information (NCBI) database.

Statistical analysis

Data for the behavioral tests were analyzed statistically with two-way ANOVA. When the ANOVA revealed significant main effect(s), it was followed by a post-hoc multiple comparison (Tukey). When the ANOVA revealed a significant interaction (strain x exposure) regardless of the significance of the main effect(s), the results of the two strains were tested separately with Student's t-test (between exposed and non-exposed). For the between-strains comparison of biochemical data in the exposed groups, the Student's t-test was separately applied for each period. A p-value less than 0.05 were considered statistically significant.

RESULTS

The locomotor activity of MT-I/II null and wild-type mice in the open field task is shown in Fig. 1. In wild-type mice, there were no significant differences in the locomotion distance (cm) for a 5-min observation between the control and exposed male and female mice. On the other hand, the locomotive activity levels of exposed MT-I/II null mice females were significantly lower than those of the control ($p < 0.05$), but there were no differences among females. The percentage of central entries of MT-I/II null and wild-type mice in the open field task is shown in Fig. 2. The exposed female mice showed significantly more central locomotion than the controls in both strains. The results of the training and 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 both strains was observed. The latency in the Morris maze test for five consecutive days is shown in Fig. 4. There were no significant differences in the escape time between the exposed group and the control males and females of both strains.

Table 1 shows the mercury concentrations in the brains, kidneys and livers of MT-I/II null and wild-type

mice exposed during postnatal periods. Immediately after Hg⁰ exposure, mercury concentrations in the brains, kidneys and livers of males and females of both strains were significantly higher in the exposed groups than in the controls. However, the mercury concentrations in the brains and kidneys in the exposed MT-I/II null mice were significantly lower than that in the exposed wild-type mice. Ten weeks after Hg⁰ exposure, there was no difference in the mercury concentration in the organs of wild-type mice between the exposed and control animals. Although the mercury concentration in the kidneys and livers of MT-I/II null mice showed no significant difference between the exposed and control groups, the brain mercury concentration of males and females was slightly higher in the exposed than in the control animals.

Table 2 and 3 shows specific gene expression changes in the brains of both strains of mice exposed to mercury vapor during the lactation period. Increased genes are those which expression was altered more than 2-fold and decreased genes are those for which expression was altered less than 0.5-fold. Among 30,000 genes examined, Hg⁰ exposure increased the expression of genes in wild-type male mice and the expression of 12 genes in MT-I/II null mice (2 genes in males and 10 genes in females), respectively. While the expression of 14 genes in wild-type mice (9 genes in males and 5 genes in females) and 81 genes in MT-I/II null mice (47 genes in males and 34 genes in females) was depressed to below 50% of the controls. The number of genes changed by exposure to Hg⁰ was higher in MT-I/II null mice of males and females than in wild-type mice together. Down-regulated genes were also observed more than up-regulated genes in both strains.

DISCUSSION

It is known that the effects of mercury are particularly detrimental to the developing central nervous system of a fetus, as well as those of neonates and young children. When mothers and neonates were exposed to Hg⁰ under the same conditions, the uptake of mercury in the brain was at higher levels in neonates than in the mothers (Jugo *et al.*, 1976; Yoshida *et al.*, 1989). Consequently, the central nervous system is considered to be more severely damaged in neonates than in adults. Fredriksson *et al.* (1992) exposed to newborn male rats to much lower-levels of Hg⁰ (0.05 mg/m³ x 1 or 4 hr/day) for 7 days (day 11-17 postnatal) and reported enhanced locomotion in the OPF test and poorer performance in the radial maze at 2 months or 4 months. They reported that Hg present in the central nervous system during the critical period

Neurobehavioral changes and alteration of gene expression by postnatal Hg⁰ exposure

Fig. 1. Total locomotor activity of MT-I/II null and wild-type mice exposed to Hg⁰ during postnatal development in the open field task. Data shown are the mean $\pm$ S.D. for exposed (■) and control (□) mice. *Significant difference from control animals at $p < 0.05$.

Fig. 2. The percent of central entry of MT-I/II null and wild-type mice exposed to Hg⁰ during postnatal development in the open field task. Data shown are the mean $\pm$ S.D. for exposed (■) and control (□) mice. *Significant difference from control animals at $p < 0.05$.

of brain development generates changes in adult brain function. In this experiment, Hg⁰-exposed female MT-I/II null mice showed a significant decrease in total locomotor activity in the OPF test as compared with control mice. On the other hand, learning ability in the PA test and spatial learning ability in the MM test in Hg⁰-exposed mice did not show significant differences in either strain. Immediately after the exposure ceased, brain concentrations of mercury in MT-I/II null mice were significantly lower than that in wild-type mice, and were considerably less than 1.0 $\mu\text{g/g}$ in either strain. However, the effects on behavior from the results in MT-I/II null female mice, indicate that postnatal exposure of MT-I/II null mice to

Hg⁰ makes them more susceptible than wild-type mice to neurobehavioral toxicity, as well as prenatal Hg⁰ exposure.

Gene expression profiling by microarray is considered useful for understanding the mechanism of toxicity and identifying marker genes in the risk assessment of toxic metals (Honda *et al.*, 2010; Tokumoto *et al.*, 2011). Liu *et al.* (2003) demonstrated that Hg⁰ exposure caused a significant alteration in gene expression in the lungs using DNA microarray analysis; these altered gene expressions could play integrated roles in developing an adaptive response to cope with Hg toxicity. In the present study, low-level exposure to Hg⁰ during lactation induced sig-

Fig. 3. Avoidance latency of MT-I/II null and wild-type mice exposed to Hg⁰ during postnatal development in the open field task. Data shown are the mean $\pm$ S.D. for exposed (■) and control (□) mice.

Fig. 4. Latency to reach the platform in the Morris water maze for MT-I/II null and wild-type mice exposed to Hg⁰ during postnatal development. Data shown are the mean $\pm$ S.D. for exposed (●) and control (○) mice.

Table 1. Mercury concentrations in the brains, kidneys and livers of MT-I/II null and wild-type mice exposed to mercury vapor during the postnatal period.

		Wild-type		MT-I/II null	
		Control	Exposed	Control	Exposed
Immediately after exposure					
Male	Brain	1.7 ± 0.4	271 ± 49*	2.2 ± 0.5	85 ± 16*#
	Kidney	4.8 ± 0.6	2064 ± 432*	3.8 ± 0.2	416 ± 68*#
	Liver	4.4 ± 1.9	213 ± 36*	3.5 ± 0.6	127 ± 28*#
Female	Brain	1.6 ± 0.3	286 ± 20*	3.0 ± 0.6	104 ± 15*#
	Kidney	4.6 ± 0.9	907 ± 767*	3.8 ± 0.2	459 ± 165*#
	Liver	2.8 ± 0.3	119 ± 68*	3.2 ± 0.7	140 ± 56**
10 weeks after exposure					
Male	Brain	5.4 ± 2.4	5.8 ± 1.9	3.8 ± 0.5	4.8 ± 0.5*
	Kidney	68.8 ± 24.9	80.9 ± 20.5	63.6 ± 7.3	53.0 ± 12.5
	Liver	14.5 ± 0.6	9.9 ± 2.0	10.4 ± 1.8	9.7 ± 0.9
Female	Brain	5.2 ± 1.2	6.9 ± 1.3	5.0 ± 0.4	7.0 ± 1.2*
	Kidney	33.1 ± 0.6	46.3 ± 8.5	36.9 ± 4.6	39.0 ± 5.8
	Liver	14.5 ± 0.6	19.3 ± 3.9	20.0 ± 2.8	20.5 ± 1.9

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

Table 2. Gene expression in the brains of wild-type mice exposed to Hg⁰

Male mice				Female mice			
Up-regulated genes		Down-regulated genes		Up-regulated genes		Down-regulated genes	
Gene Name	Ratio	Gene Name	Ratio	Gene Name	Ratio	Gene Name	Ratio
Xist	3.12	Def8	0.36	-	-	Def8	0.23
		Akt1s1	0.42			Hsf2	0.25
		Tes	0.42			Tes	0.32
		D11Ertd498e	0.46			Ppp3cb	0.37
		Pde1a	0.46				
		Pcdhb4	0.48				
		Tmem7	0.48				
		Gt(ROSA)26asSor	0.50				
		4933424N09Rik	0.50				

nificant alterations in gene expression in the brains of both strains. The number of altered genes in MT-I/II null mice, however, was higher than those in wild-type mice, which may be associated with a sensitivity difference in the neurobehavioral toxicity between the strains following Hg⁰ exposure. When MT-I/II null and wild-type mice were exposed to Hg⁰ prenatally, a significant learning disability on the PA test and retarded acquisition in the MM test were observed in MT-I/II null females as compared with the controls (Yoshida *et al.*, 2005). In an experiment using calcium-calmodulin kinase II (Camk2a) mutant mice, Alcino *et al.* (1992) reported that Camk2a, a synap-

tic protein enriched in the hippocampus, has a prominent role in spatial learning and memory. In microarray analysis of gene expression, the expression of 45 out of 30,000 genes was altered in the brains of MT-I/II null female mice after prenatal exposure to Hg⁰ (10 genes were up-regulated and 35 genes down-regulated) (Table 3). Among the down-regulated genes examined, alteration of gene expression of Camk2a involved in learning and memory was detected. Recently, Yoshida *et al.* (2008) demonstrated the delayed emergence of neurobehavioral toxicity due to perinatal methylmercury (MeHg) exposure, which developed after brain MeHg concentrations had leveled

Table 3. Gene expression in the brains of MT-I/II null mice exposed to Hg⁰

Male mice				Female mice			
Up-regulated genes		Down-regulated genes		Up-regulated genes		Down-regulated genes	
Gene Name	Ratio	Gene Name	Ratio	Gene Name	Ratio	Gene Name	Ratio
Zfp51	2.41	D730039F16Rik	0.20	Zfp51	2.98	D730039F16Rik	0.27
L1Md-A3	2.14	1300012D20Rik	0.21	L1Md-A3	2.62	Hmg20b	0.33
		2810405F18Rik	0.21	L1Md-A1	2.59	Rusc2	0.33
		Dhx30	0.22	1700082M22Rik	2.46	Tcf20	0.33
		Pftk1	0.23	4930435F18Rik	2.42	Slc8a1	0.38
		2310061A22Rik	0.25	L1Md-Tf18	2.39	Fuca	0.39
		Brd3	0.26	Pcdhga5	2.15	Nr1i2	0.39
		Mrps22	0.28	BC027231	2.10	Ppp3cb	0.40
		2210008A03Rik	0.28	D8Ert531e	2.10	Htr5b	0.41
		D230019K20Rik	0.30	C330026N13Rik	2.07	Prm1	0.42
		Ptpn4	0.30			Nfat5	0.43
		Abcb10	0.32			Bsn	0.44
		8030445B08Rik	0.32			Ik	0.44
		Ltf	0.33			Mrps22	0.45
		5830457J20Rik	0.33			Baiap1	0.46
		Def8	0.34			Clu	0.46
		Erp29	0.34			Pgd	0.46
		Hmg20b	0.34			Pyy	0.46
		Kcnip4	0.35			Sncb	0.46
		Tnfrsf10b	0.35			Golga2	0.47
		4930465P12Rik	0.36			Epn3	0.48
		MGC38922	0.37			Sdf4	0.48
		Zfyve26	0.37			Cox6a1	0.49
		Igfbp2	0.39			Fbxw1b	0.49
		Trim27	0.39			H2afx	0.49
		Cited1	0.40			Keo4	0.49
		1700030E15Rik	0.41			MOr245-13	0.49
		2210409D07Rik	0.42			Tes	0.49
		Nphp1	0.43			Tnfrsf10b	0.49
		Pgd	0.43			3100002L24Rik	0.49
		Ppp3cb	0.43			Camk2a	0.50
		2610034N03Rik	0.43			Cybb	0.50
		Aspser1	0.44			Emr4	0.50
		A330049M08Rik	0.44			Slc26a3	0.50
		Stx6	0.44				
		Btg2	0.45				
		Hoxb4	0.45				
		Adprtl3	0.47				
		Uqcrfs1	0.47				
		2900075A18Rik	0.47				
		Bmx	0.48				
		Mrps35	0.48				
		Rdh14	0.48				
		BC011468	0.49				
		Emr4	0.49				
		Nfat5	0.49				
		Golga2	0.50				

off. Although learning ability and spatial learning ability in the exposed MT-I/II null female mice was normal, the altered expression of Camk2a may indicate the possibility of the emergence of neurobehavioral effects on the aging process.

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