研究成果の刊行一覧表

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発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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Xiaoming He, Satoshi Imanishi, Hideko Sone, Reiko Nagano, Xian-Yang Qin, Jun Yoshinaga, Hiromi Akanuma, Junko Yamane, Wataru Fujibuchi, Seiichiroh Ohsako	Effects of Methylmercury Exposure on Neuronal Differentiation on Mouse and Human Embryonic Stem Cells.	*Toxicol Lett*			in press
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研究成果の刊行物・別刷り

Prenatal Exposure to Permethrin Influences Vascular Development of Fetal Brain and Adult Behavior in Mice Offspring

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ABSTRACT: Pyrethroids are one of the most widely used classes of insecticides and show neurotoxic effects that induce oxidative stress in the neonatal rat brain. However, little is still known about effects of prenatal exposure to permethrin on vascular development in fetal brain, central nervous system development, and adult offspring behaviors. In this study, the effects of prenatal exposure to permethrin on the development of cerebral arteries in fetal brains, neurotransmitter in neonatal brains, and locomotor activities in offspring mice were investigated. Permethrin (0, 2, 10, 50, and 75 mg/kg) was orally administered to pregnant females once on gestation day 10.5. The brains of permethrin-treated fetuses showed altered vascular formation involving shortened lengths of vessels, an increased number of small branches, and, in some cases, insufficient fusion of the anterior communicating arteries in the area of circle of Willis. The prenatal exposure to permethrin altered neocortical and hippocampus thickness in the mid brain and significantly increased norepinephrine and dopamine levels at postnatal day 7 mice. For spontaneous behavior, the standing ability test using a viewing jar and open-field tests showed significant decrease of the standing ability and locomotor activity in male mice at 8 or 12 weeks of age, respectively. The results suggest that prenatal exposure to permethrin may affect insufficient development of the brain through alterations of vascular development. © 2011 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2011.

Keywords: pyrethroids; cerebral arteries; fetal exposure; mice

INTRODUCTION

Permethrin, a member of the synthetic pyrethroid family, is widely used to control insect pests in agricultural, residential, and other applications as well as for avoiding malaria. Residentially, people may be exposed through pest control operations and contaminated food and water (Gorell et al., 1998). Preschool children have been found to be potentially exposed to permethrin from several sources and through several routes in their daily environments (Tulve et al., 2006; Morgan et al., 2007; Naeher et al., 2009). Permethrin is also known as a neurotoxin (Imamura et al., 2000; Meyer et al., 2008; Shafer et al., 2008). Pyrethroids target neuronal sodium channels, increasing sodium entry into nerve cells and inducing depolarization of nerve membranes and blockage of nerve conduction at high concentrations (Narahashi, 1996). Reactive oxygen species have also been implicated in the toxicology of permethrin. In neonatal rats, behavioral changes, alterations in striatal monoamine levels, and striatal protein oxidation have been reported

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(Cantalamessa, 1993; Gabbianelli et al., 2002; Nasuti et al., 2003), and adult animals exposed to permethrin show abnormalities or degeneration of dopaminergic nerve pathways (Karen et al., 2001; Bloomquist et al., 2002; Gillette and Bloomquist, 2003; Jortner, 2006). Indeed, a mixture of the antioxidant vitamins C and E suppressed pyrethroid toxicity, suggesting that these antioxidants could protect the erythrocyte plasma membrane against the oxidative injury induced by pyrethroid exposure (Gabbianelli et al., 2004). Pups from mice administered permethrin before mating are inhibited in behavioral development (Farag et al., 2006), and neonatal exposure to permethrin alters levels of oxidative-damage molecular markers and dopaminergic locomotor behavior later in adults (Nasuti et al., 2008). Thus, prenatal exposure to permethrin induces neurotoxicity as it does in adults.

The neurotoxicity of permethrin and other pyrethroids has been thought to contribute to neurodegenerative diseases like Parkinson's disease and Alzheimer's disease as well as cognitive impairment (Nasuti et al., 2008), because oxidative stress is a common event of biological impairment in brain aging and neurodegenerative and vascular disease.

Vascular development is essential for a variety of physiological events, and abnormalities in vascular development can lead to pathological conditions (Semenza, 2007). Two processes, vasculogenesis and angiogenesis, are evident during normal vascular development. Because these processes are regulated by a complex interaction of signals, many chemicals can affect vascular development (Heldin, 2004; Tabernero, 2007; Raffetto and Khalil, 2008).

Central nervous system (CNS) abnormalities caused by chemicals often involve insufficient or abnormal vascular development (Bardosi et al., 1985a,b, 1987; Hallene et al., 2006; Bassanini et al., 2007). Prenatal exposure to methylazoxymethanol acetate (MAM) in rats causes necrosis, loss of neurons, and disturbed neural progenitor migration in offspring (Haddad et al., 1972; Johnston and Coyle, 1979; Jones et al., 1981). Although the commonly accepted mechanism of action of MAM implicates the death of neural precursors (Cattaneo et al., 1995), some reports suggest that MAM-induced abnormalities include vascular malformations in the brain (Bardosi et al., 1985a,b, 1987). Results of a more recent study show that MAM neurotoxic activity in fetal rat brain is observed transiently (Bassanini et al., 2007), but the inhibition of angiogenesis via decreased expression of vascular endothelial growth factor, aquaporin 1, and lectin B is persistent. Therefore, the occurrence of MAM-induced neural abnormalities depends on its dual action as a neurotoxin and an antiangiogenic factor. Recently, insufficient brain development caused by prenatal exposure to thalidomide, a well-known teratogen with potent antiangiogenic activity, was demonstrated to be caused by insufficient vascular development (Hallene et al., 2006). Neural development and vascular development share critical molecular signals during normal development (Yancopoulos et al., 1998). Although a close relationship between vascular development and neural development has been shown, the vascular toxicity of neurotoxins, other than MAM and thalidomide, remains unclear. Effects of prenatal exposure to permethrin on vascular development are also unknown in fetal brain and adult behavior.

Therefore, in this study, we examined the effects of prenatal exposure to permethrin on cerebral vascular development in mouse fetuses, CNS development, and on adult motor behavior later in life. Analysis of cerebral vascular development in the fetus brain was performed by anatomical and histochemical examination. CNS development was assessed by histochemical analysis and measurements of neurotransmitters in the juvenile brain of mice. Motor behaviors in adulthood were tested with a modified-SHIRPA screening.

Abbreviations

ACA	anterior cerebral arteries
AComA	anterior communicating arteries
CW	circle of Willis
CNS	central nervous system
GD	gestation day
MAM	methylazoxymethanol acetate
MCA	middle cerebral artery
PFA	Paraformaldehyde

MATERIALS AND METHODS

Animals and Chemical Administration

Animal protocols for the breeding and all other experiments were approved by NIES's Institutional Animal Care and Use Committee under the Guideline for Animal Care of NIES. ICR mice were purchased from CLEA Japan (Tokyo, Japan) and housed at a constant temperature (22°C ± 0.5°C) at 35–70% humidity with 12-h light–dark photoperiod. Mice were given standard lsb chow and water *ad libitum*. At GD10.5, the pregnant mice were divided into seven groups of three to four mice per group and administrated corn oil (Wako Pure Chemical Industries, Osaka, Japan) as the negative control and permethrin (Wako Pure Chemical Industries) at a dose of 2, 10, 50, or 75 mg/kg by oral gavage, which are 1/250, 1/50, 1/10, and 1/6.7 of the oral LD50 of mice (Miyamoto, 1976). For thalidomide (Wako Pure Chemical), a dose of 150 mg/kg was administered, respectively, at GD10.5. To determine the effects of permethrin on the development of cerebral arteries in the circle of Willis (CW), we conducted two experiments [Fig. 1(A,B)]. In the first experiment, anatomical and histological observations of cerebral arteries in the fetal mouse brain were examined at GD17.5. The second experiment investigated the later effects of prenatal exposure to

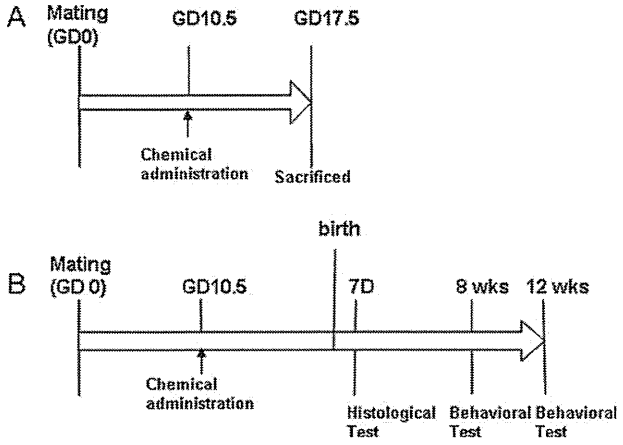

Fig. 1. Schematic experimental design. A: Regimen for prenatal exposure to permethrin and the fetal brain analysis. B: Regimen of behavioral tests.

permethrin on CNS development and neuronal transmitters at postnatal day (PND) 7 and spontaneous behavior in offspring mice at 8 and 12 weeks of age [Fig. 1(B)]. In the first experiment, at GD17.5, all the mice were sacrificed by dislocation of cervical vertebrae, and the fetuses were removed. The body weight of each fetus, without placenta and yolk sac, was measured. The fetuses were kept on iced plastic dishes, and the brains were removed. Brains were washed in phosphase buffer saline (PBS) and fixed in 4% paraformaldehyde (PFA) at 4°C overnight. The methods of the second experiment are described later.

Carbon Black Perfusion of Cerebrovasculature

Fetal mouse hearts were exposed either under anesthesia. After opening the right atrium to ambient pressure, a 26-gauge needle was inserted into the left ventricle, through which 2 mL of PBS was perfused, followed by 2 mL of carbon black ink in 4% PFA. All perfusions were performed in series at 20 rpm by using a peristaltic pump (MasterFlex, Cole-Parmer Instrument, Vernon Hills, IL). The brains were carefully removed, and the CW anatomy was visualized by using a stereomicroscope with a cooling charge-coupled device image sensor (CCD) camera (Olympus, Tokyo, Japan).

Whole-Mount Immunohistochemistry

Fixed brains were washed in PBS with 0.2% Triton-X for 30 min, permeabilized in PBS with 50% DMSO for 20 min and washed three times in PBS with 0.2% Triton-X for 30 min. The brains were incubated in PBS with 5% normal goat serum to block nonspecific immunoreactivity. After 1 h of blocking, the primary immunoreaction was performed in PBS with 5% normal goat serum and 1:200 rat anti-CD31 (PECAM-1) antibody (MEC 13.3; BD Pharmingen, San Diego, CA) overnight at 4°C. Brains were washed three times in PBS with 0.2% Triton-X for 30 min, and blocking for the secondary immunoreaction was carried out in PBS with 5% normal goat serum. The secondary immunoreaction was performed in PBS with 5% normal goat serum and 1:200 antirat IgG antibody-conjugated Alexa 546 (Invitrogen, Carlsbad, CA). After washing three times in PBS, images of the anterior half of the CW area were obtained using a cooling CCD camera under fluorescent microscopy (Olympus, Tokyo, Japan). Photographs of the whole brain were also obtained under a stereomicroscope (Olympus) to measure the length of the anterior–posterior axis. The frequencies of the abnormal location were calculated as the ratio of the number of fetus with the abnormal location versus the sample number of in each exposure group.

Histological and Anatomical Analyses

Histological and anatomical observation and image analyses of cerebrovasculature in the brain were performed using a stereo microscope (Olympus SZX16 Macroview, Sinjuku, Tokyo) and image-processing software (WinRoof, Mitani Corporation, Fukui, Japan). For the GD17.5 fetal brain, the length of the anterior–posterior axis of the whole brain, the length of the anterior cerebral arteries (ACA) or anterior communicating arteries (AComA), and the number of branches of the ACA or AComA were measured. For histological analysis of cortex layers at the Bregma, brains of mice at PND7 were fixed with 10% formalin solution, then washed in 70% ethyl alcholo-xylene, and embedded in paraffin. Those brains were cut in the coronal plane at 10 μm. Sections were collected every 50 μm, thaw-mounted onto coated slides, and stained with 0.5% Cresyl violet. The thickness of the cortical layers in the lateral part of secondary visual cortex (V2L) and the thickness of pyramidal cell layers (Py) in hippocampus and granular layers in the dentate gyrus (GrDG) were quantitatively measured at the −2 mm place from the Bregma position of brain.

Measurement of Monoamines by High-Performance Liquid Chromatography

The monoamines and their metabolites were measured using high-performance liquid chromatography (HPLC) with electrochemical detector. At PND7, the brain after removing cerebellum was rapidly dissected out, weighted, and frozen at −80°C until assay. Each frozen brain was homogenized by ultrasonic irradiation in 2 mL of 0.2 M perchloric acid/0.1 mM EDTA solution containing isoproterenol as an internal standard. The homogenates were placed on ice for 30 min and spun at 20,000 × g for 10 min

at 4°C. The supernatants were filtered through a syringe filter unit (DISMIC-3; Advantec, Japan), their pH was adjusted to 3.0 by adding 1 M sodium acetate, and then they were injected into a HPLC system (Shimadzu, Japan) equipped with an ODS column (Eicompak SC5-ODS; 3 mm i.d. × 150 mm; Eicom, Japan) and an electrochemical detector (EDC-100; Eicom) with the potential set at + 750 mV. The mobile phase was 0.1 M citric acid/0.1 M sodium acetate, pH 3.5, containing sodium-1-octansulfonate (190 μg/mL), EDTA-2Na (5 μg/mL), and 13% methanol. The flow rate was set at 0.25 mL/min.

Behavioral Tests

For behavioral tests, pregnant ICR mice at GD10.5 were administrated permethrin dissolved in corn oil at a dose of 0, 2, or 50 mg/kg. Three litters were used in each group. The mean body weight of all groups was approximately equal. At 7 days after parturition, three males and three females were reserved from each litter. Motility was examined at 8 and 12 weeks using selected items from a modified-SHIRPA test (Rogers et al., 2001; Masuya et al., 2005; Jin et al., 2008a). We tested body weight, body position, grooming, locomotor activity, transfer arousal, trunk curl, contact righting reflex, and negative geotaxis using a clear perspex cylindrical viewing jar (14-cm diameter × 18-cm height), a clear perspex arena (60-cm length × 37-cm width × 18-cm height with a the floor marked with 15 squares), and a grid (40 cm × 20 cm with 12-mm mesh) at 8 and 12 weeks of age. For quantitative analysis of motor activity, we counted the number of leaning-against wall events for 5 min in the viewing jar to test body-position activity, and we counted the number of squares in the floor of the arena that the mice ran through for 1 min to test locomotor activity. After the test at 12 weeks of age, mice were sacrificed, and their brains were removed. Brains were fixed in 4% PFA after being weighed. After overnight fixation, the brains were photographed under a dissection microscope and used in image analysis.

Statistical Analyses

Statistical analysis was performed using SAS software (version 9.1 with Enterprise 4.0, SAS Institute, Cary, NC). Differences among doses were determined for each endpoint. Data were tested for normality using the Shapiro–Wilk test and for homogeneity using Bartlett's test for experiments using multiple dose levels of a test chemical. When the data were found to be homogeneous ($P > 0.05$), Dunnett's multiple analysis was performed. If the data were from experiments using a single dose level of a test chemical and the initial variance of the data were homogeneous, the data were analyzed by the Student's t test. Each evaluation was by two-tailed tests with 0.05, 0.01, or 0.001 as the levels of significance.

TABLE I. Effects of prenatal exposure to thalidomide or permethrin on dam body weights, litter sizes and fetal body weights at GD17.5

Group	Number of Dams	Average Body Weight of Dams	Litter Size	Fetal Body Weight
Control				
Total	5	68.1 ± 1.7	19.4 ± 0.7	0.775 ± 0.005
Male			10.2 ± 1.2	0.788 ± 0.007
Female			9.2 ± 0.5	0.762 ± 0.007
Thalidomide (150 mg/kg)				
Total	4	62.7 ± 1.8	16.0 ± 0.7	0.787 ± 0.006
Male			9.5 ± 1.0	0.797 ± 0.008
Female			6.5 ± 0.6	0.773 ± 0.007
Permethrin (10 mg/kg)				
Total	5	62.4 ± 1.1*	15.6 ± 0.9*	0.827 ± 0.022*
Male			6.8 ± 0.7*	0.846 ± 0.029
Female			8.8 ± 1.2	0.811 ± 0.031

*$P < 0.05$ from correspondent control.

RESULTS

Detection of Vascular Malformation in Fetus Brains Prenatally Exposed to Thalidomide or Permethrin

The effects of prenatal exposure to chemicals on dam body weights, litter sizes, and fetal body weights at GD17.5 were shown in Table I. Mice were prenatally exposed to corn oil as vehicle, thalidomide, or permethrin. Thalidomide was used as positive controls that induce abnormal patterning of the cerebral arteries in the CW and its surrounding arteries. Permethrin exposures significantly decreased the body weight of dams and litter sizes, but increased the fetal body weight. As a positive agent, thalidomide did not show significant alterations for the body weight of dams, litter sizes, and the fetal body weight.

Fetal brains were analyzed to determine alterations in the development of cerebral arteries in the CW [Fig. 2(A)]. Figure 2(A) shows a schematic picture of arteries of the CW in the fetal mouse brain; the left side of the brain represents normal patterning of cerebral arteries, and the right side depicts abnormal patterning. For the GD17.5 fetal brain, the half length of the anterior–posterior axis of the whole brain, the length of the ACA [ab in Fig. 2(A)] or the length AComA [bc in Fig. 2(A)], and the number of branches of the ACA or AComA were measured as shown in Figure 2(A). These observations were summarized in Table II. When more than three branches in the right and left ACA or AcomA were observed, the vascular patterns were assessed as abnormal locations [Fig. 2(C–F)]. Prenatal exposure to thalidomide significantly increased frequencies of abnormal locations in the right ACA [Fig. 2(C,D)]. In

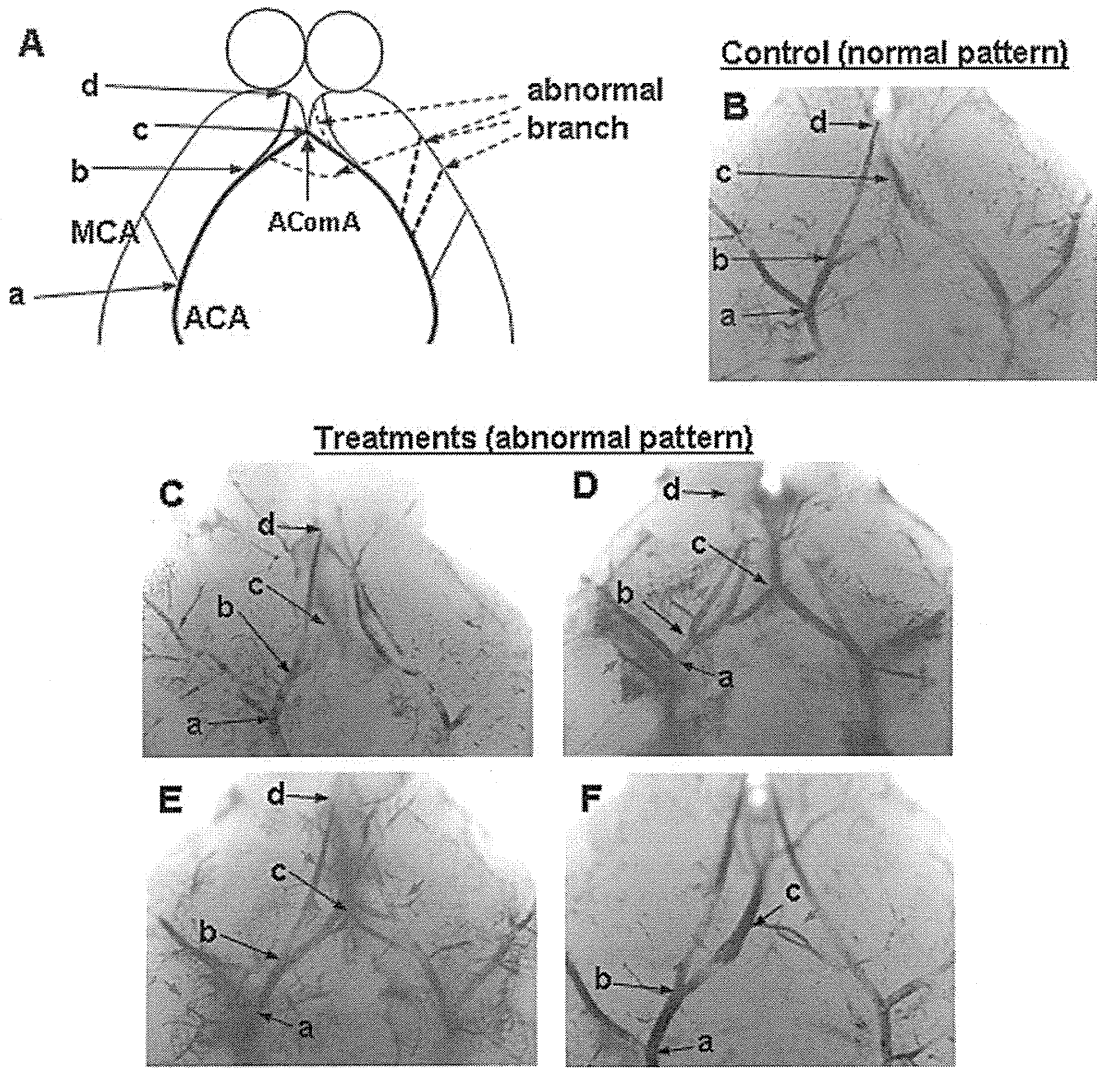

Fig. 2. Drawing and representative photographs of the anterior half of CW in the fetal mouse brain. Drawing (A), control (B), 150 mg/kg thalidomide (C and D) and 10 mg/kg permethrin (E and F) groups. a, b, c, and d in the drawing and each photograph indicate the branching point of MCA and ACA, the branching point of AcomA and ACA, the fusion point of AcomA, and the end of ACA, respectively. Prenatal exposure to thalidomide or permethrin causes various malformations of the CW of fetal brains. Vascular networks were filled with a fourfold dilution of Chinese liquid ink. Arrows indicate malformation sites detected. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the case of permethrin, frequencies of abnormal locations in the AcomA [Fig. 2(E,F)] were increased. Different patterning of the CW was observed between thalidomide and permethrin-exposed fetal brain.

Dose-Range Effects of Permethrin on Cerebral Arteries in CW of the Mouse Brain

To confirm the effects of prenatal exposure to permethrin on cerebral arteries in the CW, its dose-response relationship was assessed. Branching patterns in the CW of brains were detected by immunohistochemical examinations, which were performed with anti-PECAM-1 antibody. Typical immunohistochemical observations in each group were shown in Figure 3. As we mentioned previously, in the control group, ACAs were connected by AComAs that were fused on the anterior side of the optic chiasma. Major cerebral arteries including the MCA and ACA showed few small branches in the control group [Fig. 3(A)]. In contrast, branching of cerebrovasculars in the CW of brains isolated from permethrin-treated fetuses [Fig. 3(B–E)] showed no

TABLE II. Prenatal exposure to thalidomide or permethrin causes various malformations of the CW of fetal brains

		Frequencies of Abnormal Locations			*P* Value of the Chi Square Test		
Group	Number of Tested Fetus	Right ACA	Left ACA	AcomA	Right ACA	Left ACA	AcomA
Control							
Total	25	0.20	0.32	0.24	–	–	–
Male	14	0.21	0.36	0.21	–	–	–
Female	11	0.18	0.27	0.30	–	–	–
Thalidomide (150 mg/kg)							
Total	21	0.48*	0.38	0.24	0.047	0.666	0.988
Male	11	0.45	0.36	0.21	0.201	0.973	0.943
Female	10	0.50	0.40	0.30	0.122	0.537	0.916
Permethrin (10 mg/kg)							
Total	25	0.28	0.44	0.96**	0.508	0.382	0.009
Male	13	0.31	0.46	0.92*	0.580	0.581	0.037
Female	12	0.25	0.42	0.92	0.692	0.469	0.122

*$P < 0.05$ and **$P < 0.001$ from correspondent control. More than three branches in the right and left ACA or AcomA were assessed as abnormal locations of the CW area. The frequencies of the abnormal location were calculated as the ratio of the number of fetus with the abnormal location versus the sample number of fetus in each exposure group.

AComAs and many small and slender branches from the ACA. In addition, insufficient fusion of the AComAs was also observed in some cases as shown in Figure 3(C,E).

To quantify these observations in terms of the permethrin influence, we determined the number of branches in the area [bc as shown in Fig. 2(A)] of the AComA significantly increased in fetal brains exposed to permethrin at a dose of 2 ($P < 0.05$), 10 ($P < 0.001$), 50, or 75 mg/kg ($P < 0.01$) [Fig. 4(A)]. Furthermore, the length of the AComA was reduced significantly in the fetuses exposed to permethrin at a dose of 10 mg/kg ($P < 0.05$) [Fig. 4(B)] while the lengths of the ACA [ab and bd as shown in Fig. 2(A)] or branch numbers on it was neither affected by the prenatal exposure to permethrin. In summary, the abnormalities of CW were caused by 2 mg/kg of permethrin, which the lowest dose we experimented, and reached the plateau at 10 mg/kg. Therefore, we use the samples from the one exposure group, 50 mg/kg, in histological analysis and measurement of neurotransmitter.

Effects of Permethrin on Histological Observations and Neurotransmitter at Early PN Development

We next investigated how prenatal exposure to 50 mg/kg of permethrin influences the processes of neuronal

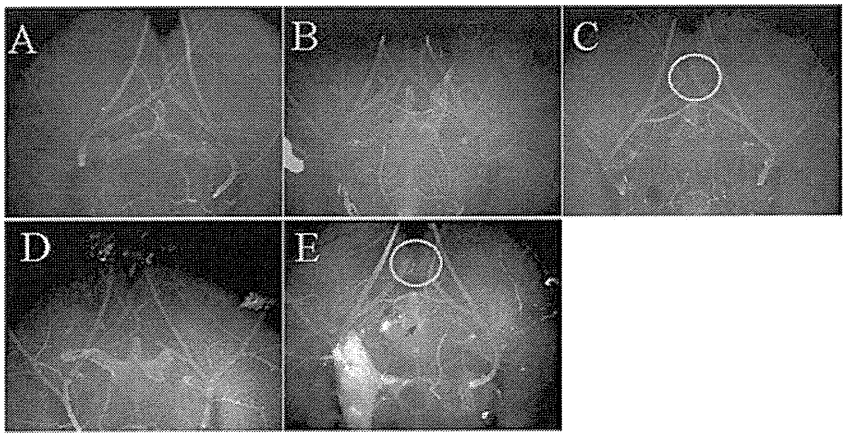

Fig. 3. Arteries of CW of fetal brains are affected by prenatal exposure to permethrin. Representative photographs of the anterior half of the CW of fetal brains; prenatal exposure to permethrin causes various malformations of the CW. Control (A), 2 mg/kg permethrin (B), 10 mg/kg permethrin (C), 50 mg/kg permethrin (D), and 75 mg/kg permethrin (E) groups. Arrows and circles indicate malformation sites detected. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

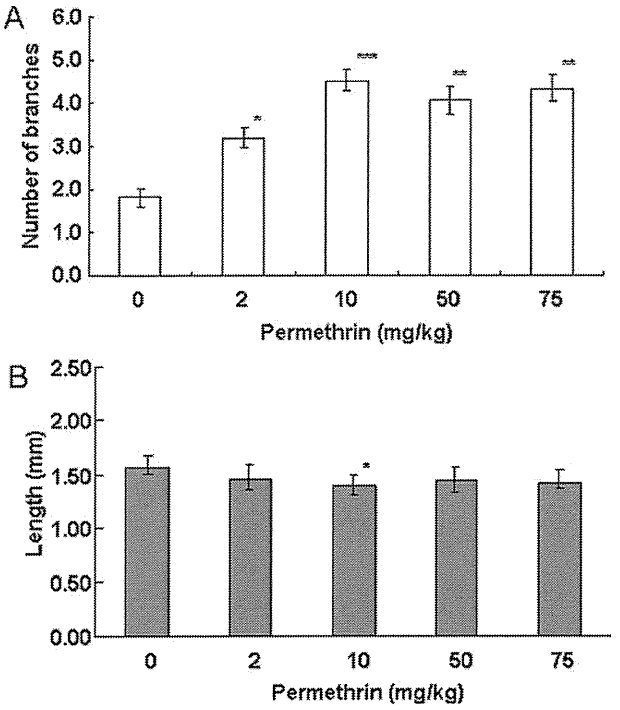

Fig. 4. Prenatal permethrin exposures alter vascular pattern in the CW area. Number of branches from the anterior half of ACA [bd as shown in Fig. 2(A) picture] and the length of AcomA [bc as shown in Fig. 2(A) picture] were measured. Dose-response relationship between the permethrin exposure and abnormal branches (A) in the observed area and the length of AcomA (B). Values are mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

commitment and differentiation during early PN development. Because it was known that prenatal stress alters spine density and dendritic length and hippocampus neurons in rat offspring (Tseng et al., 2008; Lu et al., 2009), we assessed the thickness of cortical layers in the V2L, Py, and GrDG in the hippocampus. Notably, the significant differences were found reduced layer I, Py, and GrDG in mice prenatally exposed to permethrin in compared to control [Fig. 5(E,F)]. However, there were no significant differences for the other layers (II–VI) and the thickness between the hippocampus and the dentate gyrus near the Bregma [Fig. 5(E,F)]. These data indicate that prenatal exposure to permethrin influenced CNS development at the later PN period.

Second, we sought to determine whether influences of permethrin to CNS development were due to premature contents of neurotransmitters. Levels of norepinephrine (NA), dopamine (DA), DOPAC, HVA, 5HT, and 5HIAA at PND7 were determined in controls and the mice after prenatal exposure to permethrin (Fig. 6). NA and DA in the permethrin-exposed mice were significantly increased in compared to control. However, there were no significant differences in the other neurotransmitters between controls and the exposed mice. Together, these results indicate that prenatal exposure to permethrin affects cortical layers and development of dopaminergic and adrenergic neurons in the hippocampus region.

Effects on Motor Behavior in Adulthood

To evaluate behavioral activity during the adulthood of mice prenatally exposed to permethrin, the body-position test and the open-field arena test were conducted at 8 and 12 weeks of age. In the body-position test, no difference was detected in either females or males at 8 weeks of ages. However, at 12 weeks of age, the number of leaning-against wall events for the body-position test that occurred in the 5-min observation period in males was significantly decreased at a dose of 2 mg/kg (Fig. 7). For the confirmation of the effect of permethrin on locomotor activity, motor behavior was observed in an open-field arena for 1 min. In the arena test, the number of squares that the mice passed through significantly decreased at 50 mg/kg of permethrin in males, but not in females. Similarly, at 12 weeks of age, 2 mg/kg permethrin significantly decreased the locomotor activity of male mice, but not female mice (Fig. 8); however, at 50 mg/kg of permethrin, the decrease was not significant because of marked data variation.

Prenatal exposure to permethrin did not affect body weight at 8 and 12 weeks of age. No difference was detected in the ratio of brain/body weight in both males and females. The length of the CW also was not affected (data not shown).

DISCUSSION

In mouse brain development, the internal carotid arteries can be recognized at GD 10.5, which is when the pregnant mice were administered permethrin in the present study. Internal carotid arteries give rise to the ACA, from which the AComA branch. At the same time, the posterior cerebral arteries and posterior communicating arteries branch from the basilar artery, which is derived from the vertebral arteries. All the components of the CW are formed by GD13.5 (Kaufman, 1995). In neural tissues, the cephalic region of the neural tube begins developing rapidly at GD10.5, and the fetal brain is formed by GD13.5. Therefore, GD10.5 is thought to be the critical window for brain vascular development. Taking into consideration brain vascular development, this study was conducted using exposure to permethrin on GD10.5. Our results demonstrated that the prenatal exposure to permethrin altered patterning of the vascular formation in portions of the CW in fetal mice, including a shortening of the AComA and an increased number of small branches of the AComA. In some cases examined in this study, insufficient fusion of

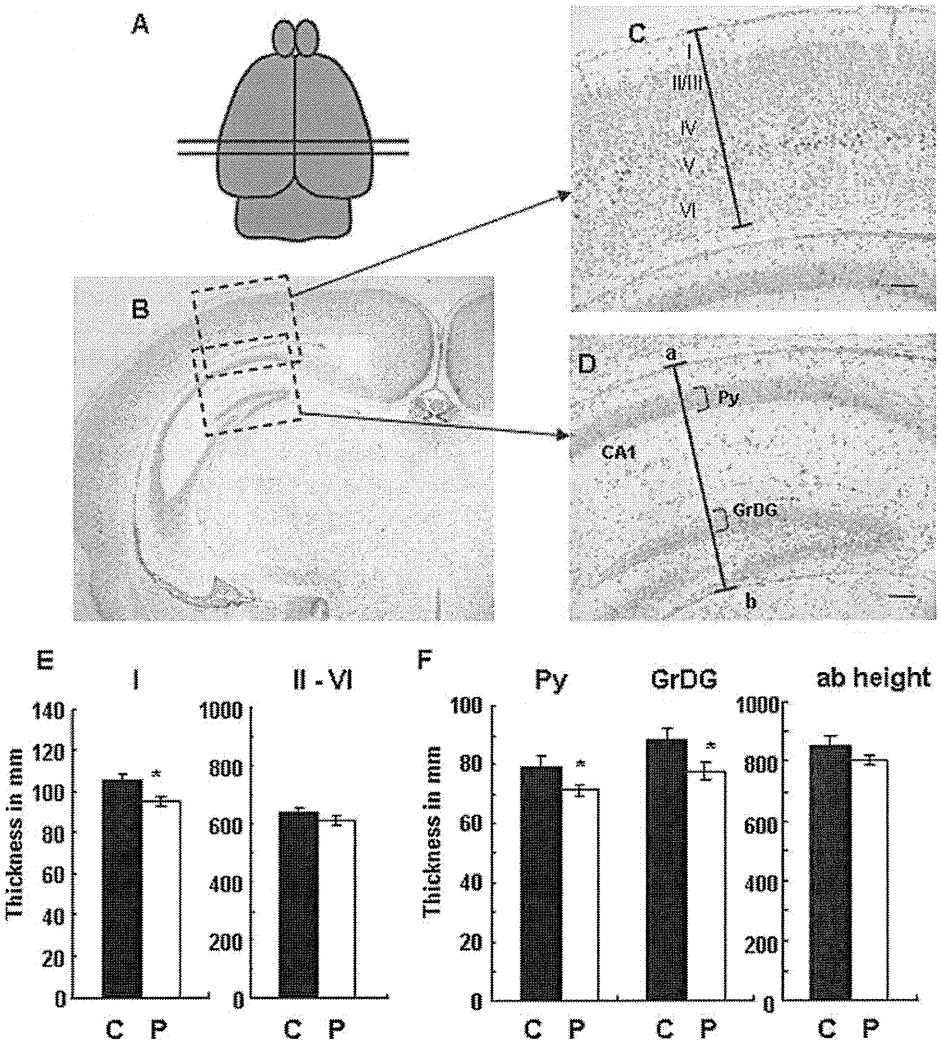

Fig. 5. Morphological alteration of the developing midbrain in the mice prenatally exposed to 50 mg/kg of permethrine. Quantitative analysis reveals a significant reduction of cortical layers and hypocampus in the permethrine-exposed brain in compared with the vehicle control at PND7. A: The schematic drawing of the mouse brain. B: Cresyl violet staining of cross-sectional brain at PND7. Squares with dot lines indicate area that quantitatively analyzed. C: Cortical layers in the lateral part of secondary visual cortex at the −2 mm place from the Bregma position of brain. D: Pyramidal cell layers (Py) in hyppocampus and granular layers in the dentate gyrus (GrDG). D: Quantitative analysis of cortical layers. E: Quantitative analysis of Py layers, GrDG layers, and the height (ab) between hyppocampus and the dentate gyrus. Data values represent mean ± SE for 12–16 individual samples per each group. *$P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the AComA was observed. Furthermore, the adult behavioral examination revealed that prenatal exposure to permethrin caused changes in motor behavior.

In this study, the lowest dose 2 mg/kg showed significant influences on vascular development in fetus and adult offspring behaviors, indicating that prenatal exposure to permethrin is more susceptible rather than the postnatal exposure. Considering doses of permethrin used in this study, we used 2, 10, 50, and 75, which are 1/250, 1/50, 1/10, and 1/6.7 of the oral LD50 of mice (Miyamoto, 1976), because less than 2mg/kg permethrin increased levels of dopamine transporter protein in the striatum of adult mice (Bloomquist et al., 2002) and higher doses of permethrin induced a significant risk to the offspring following treatment of F0-mice before mating (Farag et al., 2006). Although there is no direct evidence for absorption to fetus

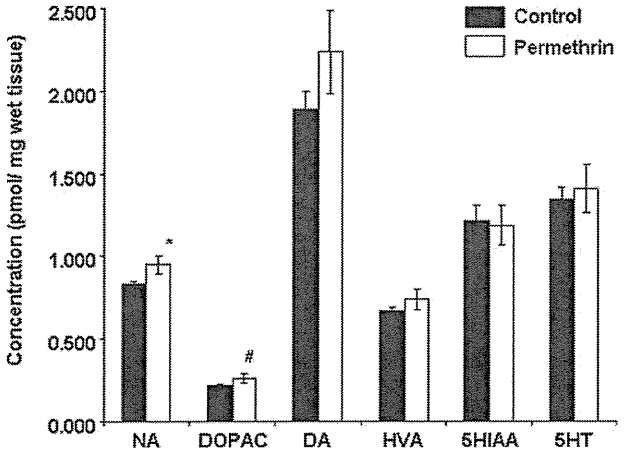

Fig. 6. Levels of norepinephrine (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxy-4-hydroxyphenylacetic acid (HVA) homovanillic acid (HVA), serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), in mid brain of mice from control group, and permethrin-treated groups (50 mg/kg). Data are presented as mean ± SE for six mice per each group. $^*P < 0.05$ versus control (C).

from the dam in rodent experiments, human cord blood concentrations of permethrin could be detected about 30 ng/mL nonlipid adjusted geometric mean (Neta et al., 2010). These levels of permethrin in cord blood were associated with the decrease of the anti-inflammatory cytokines IL-10 (Neta et al., in press). Therefore, it becomes crucial to verify the probable absorbed quantity of permethrin or other pyrethroids if vulnerable populations such as pregnant women would be exposed to higher permethrin or pyrethroids.

Prenatal exposure to teratogens such as MAM or thalidomide at nonteratogenic doses has been reported to produce cortical development malformation (Hallene et al., 2006; Bassanini et al., 2007). Prenatal exposure to thalidomide at GD15 induced significant morphological alterations in the cortical and hippocampal regions of rats along with vascular malformations and a leaky blood–brain barrier. These malformations were suggested due to inhibition of vascular development and neurogenesis by MAM or thalidomide. In the present study, the shorter length and the increased number of small branches were also detected in the GD10.5 thalidomide-treated group as a positive control although the positions of vascular lesions were different from those in the permethrin group. Thalidomide also inhibits vasculogenesis in various cancer and reumatitis (Sleijfer et al., 2004). Therefore, angiogenetic blockers commonly may play an inhibitory role in cerebular vascular development during the prenatal period.

The failure of fusion was similar to the abnormal formation of ACA observed in rats treated with MAM (Bardosi et al. 1985a,b, 1987; Bassanini et al., 2007). Thus, our observations *in vivo* suggest that permethrin exhibits vascular toxicity, leading indirectly to an inhibition of brain development. To examine the inhibitory action of permethrin in cerebral artery development, the *in vitro* tubular formation assay, using human brain microvascular endothelial cells, was performed in our laboratory. A concentration of 10^{-6} M or 10^{-5} M of permethrin decreased the distance between branches and increased unattached branches (data not shown). These *in vitro* results are consistent with our *in vivo* mouse study, suggesting that permethrin acts directly on endothelial cells and disrupts angiogenesis in the fetal brain. However, other hypotheses must be considered: (1) the neurotoxicity of permethrin directly inhibited brain development and then follows inhibitory of vascular development and (2) the dual action of permethrin, including neurotoxicity and vascular toxicity, inhibited directly brain development. Another possibility of effects of prenatal exposure to permethrin should be considered to be nutritional factors or alterations of general metabolisms from dams, because body weights of dams and litter sizes were significantly decreased, and fetal body weights at GD17.5 were significantly increased (Table I). This point for nutritional factors needs further studies to clear relationships with vascular developments in fetal brain.

On the other hand, regions of the observed vascular abnormality were different between thalidomide and permethrin. Although thalidomide influenced the ACA region, permethrin caused abnormalities of AcomA. It indicated that permethrin affected on vascular later than thalidomide, because the formation of AcomA follows to the ACA formation. A possible hypothesis is that the different mechanisms of the chemicals might result in such a time lag. But it is unlikely because *in vitro* tubular formation assay revealed the similar effect of thalidomide and permethrin at the same timing (data not shown). Another possible hypothesis is that the different kinetics of them in the pregnant mice and the fetus produced such a time lag. The longer time might need to reach to the toxic concentration of permethrin in the fetal brain than thalidomide. However, at least in our knowledge, no information is available about the kinetics of permethrin in the pregnant mice and the fetus. Further research is needed on the similarities or differences of permethrin and thalidomide.

Midbrain DA neurons send one of their largest cortical projections to the superficial layers of the lateral entorhinal cortex where they target principal cell islands (Björklund, 1984; Fallon, 1987). The large dopaminergic projection to the prefrontal cortex is known to regulate cellular processes related to working memory (Goldman-Rakic, 1999), and dopaminergic inputs to the lateral entorhinal cortex are also likely to affect mechanisms of sensory and mnemonic function (Seamans and Yang, 2004).

Previous studies reported that permethrin inhibits the activities of various neurons *in vitro*, including neurons of the frontal cortex, spinal cord (Shafer et al., 2008), and

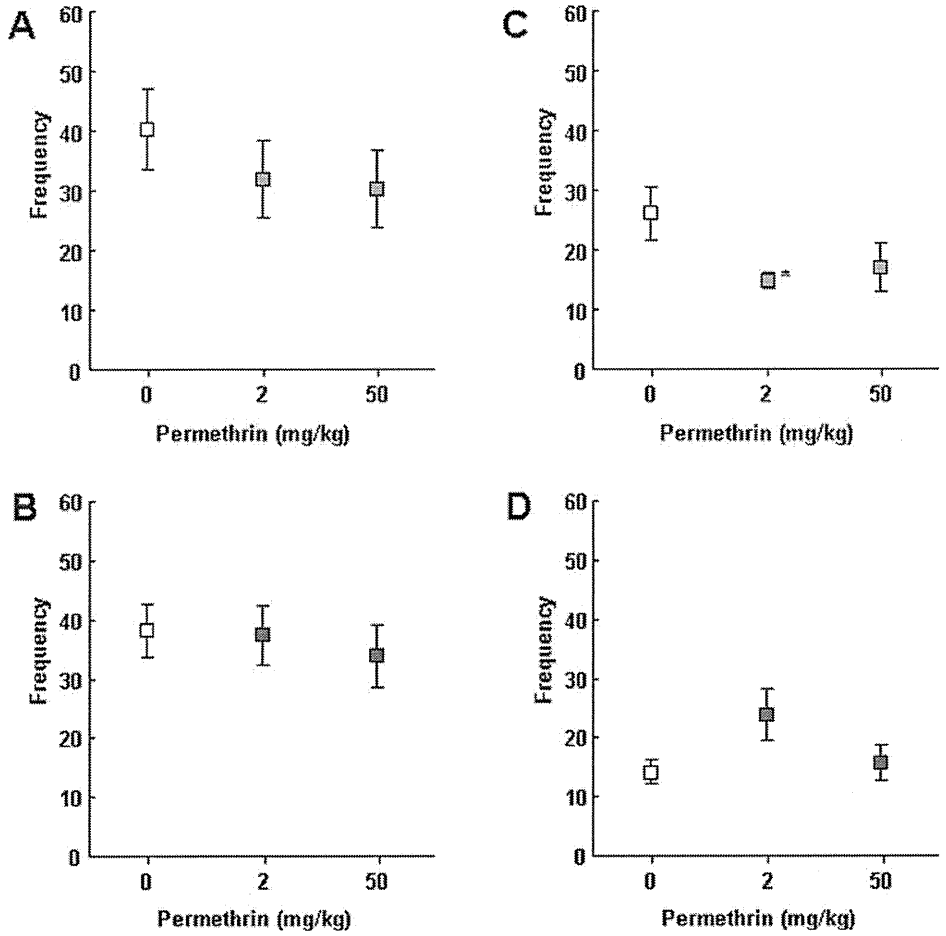

Fig. 7. Prenatal exposure to permethrin affects locomotor activity, as assessed using the body-position test. A: Male at 8 weeks of age. B: Female at 8 weeks of age. C: Male at 12 weeks of age. D: Female at 12 weeks of age. Data values represent mean ± SE. $^*P < 0.05$.

hippocampus (Meyer et al., 2008) as well as cerebellar granule cells in culture (Imamura et al., 2000). In concordance with those reports, our current studies showed the NA and DA concentrations in mice prenatally exposed to 50 mg/kg of permethrin were significantly decreased concurrently with a decreasing cortical layers and Py and GrDG in hippocampus (Figs. 5 and 6). These alterations at the early postnatal period of PND7 indicate a weak suppression of CNS development. In permethrin-treated adult animals, abnormalities of the dopaminergic system and degeneration of dopaminergic neurons have been observed, indicating a possible involvement of permethrin in Parkinson's disease (Karen et al., 2001; Bloomquist et al., 2002; Jortner, 2006). The prenatal exposure to permethrin before the mating decreased motor activities such as self-righting reflex, geotaxis reflex, cliff-avoidance reflex, swimming performance, open-field activity, and social interaction in F_1 mouse pups (Farag et al., 2006). Thus, the neurotoxic activity of permethrin may influence histological neurogenesis and CNS-related behaviors. The adult behavioral examination in our present study revealed that prenatal exposure to permethrin caused changes in motor behavior (Figs. 7 and 8). Taken together, our observations suggest that prenatal exposure to permethrin results in an insufficient development of the brain because of either the vascular toxic activity or neurotoxicity or both.

For the motor behavioral tests in the present study, we used a modified-SHIPRA test for screening. Quantitative analysis was performed by counting the number of leaning-against wall events and the number of squares passed through in the body position and arena test, respectively, to clarify the influences of the permethrin exposure. At 8 weeks of age, prenatal exposure to 50 mg/kg permethrin in the males resulted in significantly decreased locomotor activity in the arena test. At 12 weeks of age, prenatal exposure to 2 mg/kg permethrin significantly decreased locomotor activity both in the body-position test and the arena test in males, but not in females. In female mice, a significant increase was observed in the body-position test at 2 mg/kg permethrin (Figs. 7 and 8).

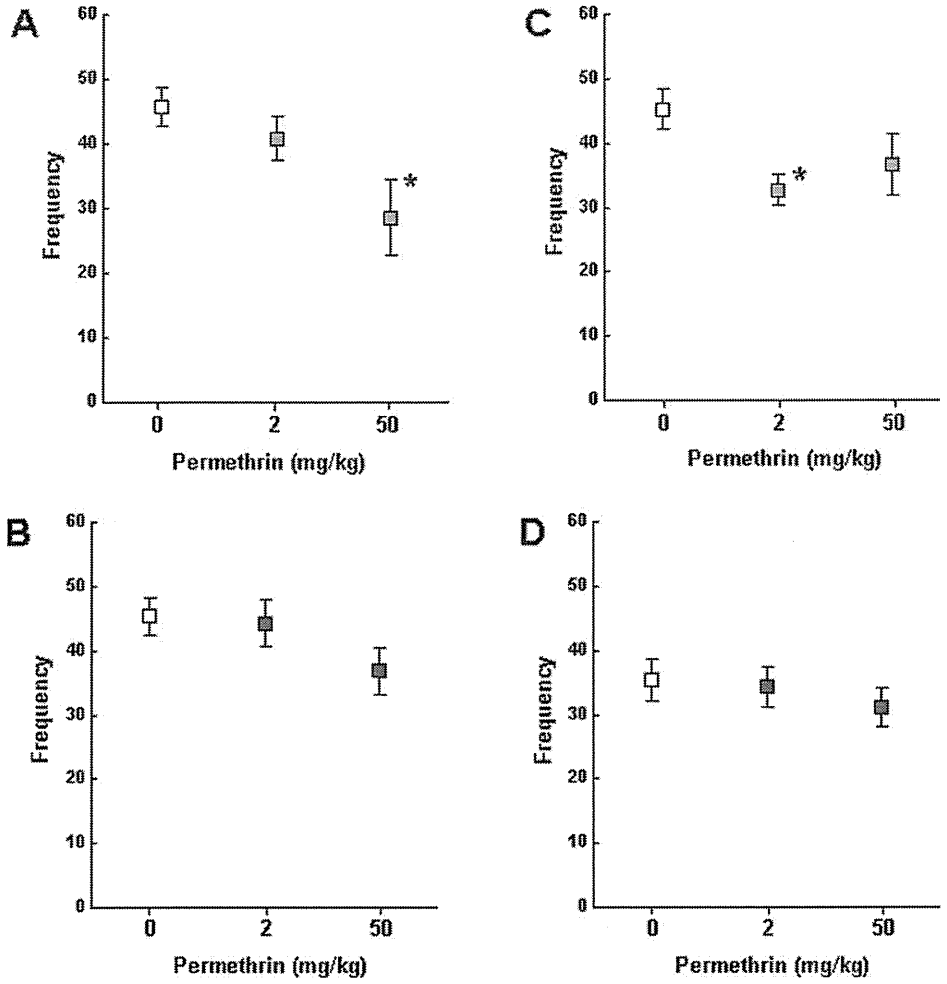

Fig. 8. Prenatal exposure to permethrin affects locomotor activity, as assessed using the arena test. A: Male at 8 weeks of age. B: Female at 8 weeks of age. C: Male at 12 weeks of age. D: Female at 12 weeks of age. Data values represent mean ± SE. *$P < 0.05$.

These sex and age differences may relate to sexual maturation. Sexual differentiation in the mouse brain is critical in fetal and perinatal periods and depends on the specific activity of androgen in the brain. Because many studies have indicated an estrogenic or antiandrogenic activity of permethrin (Garey and Wolff, 1998; Kim et al., 2004; Kojima et al., 2005; Dhooge et al., 2006; McCarthy et al., 2006; Jin et al., 2008b), it is possible that permethrin exposure *in utero* disrupts sexual differentiation of the brain and affects behavior during the period of sexual maturation.

Although prenatal exposure to permethrin on GD10.5 caused abnormal motor activity in mice at 12 weeks, brains grew to a normal size, and vascular malformations in the CW were not detected at our gross observation (data not shown). These results suggest that the recovery of morphological malformations of the vasculature during the postnatal period enabled normal growth of the brain, even though the vascular malformation had occurred during early brain development. However, the mice were unable to recover from the functional abnormality resulting from insufficient early development of the brain.

In this study, we showed that permethrin exposure *in utero* caused fetal brain vascular malformations and changes in motor behavior in adult mice. Although the relation between these abnormalities is unclear, our data show that prenatal exposure to permethrin can be a risk for health in adulthood. However, toxicological information concerning prenatal exposure to permethrin is insufficient. Further research is needed on the effect of prenatal exposure to permethrin.

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