Original Article

Nasal instillation of nanoparticle-rich diesel exhaust particles slightly affects emotional behavior and learning capability in rats

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ABSTRACT — In the present study, in order to reveal novel adverse effects of ultrafine particles (UFP) on the central nervous system, the effects of nanoparticle-rich diesel exhaust particles (NRDEP; count mode diameter, 21.45 nm) on emotional behavior, learning capability and brain neurotransmitter levels were studied in rats by intranasal instillation (iNI). NRDEP (10 and 50 µg/rat) was instilled into 2-week old infant, male rats once a week for 4 weeks. Spontaneous motor activity measured was observed to be inverse to the dose level. In active avoidance tests using a shuttle box, NRDEP-treated animals showed a lower avoidance performance than control animals given air-instillation. The levels of dopamine and its metabolite (DOPAC) in the medial mammillary nucleus of the brain tended to be lower in the NRDEP-treated animals. From these results, although the effects of NRDEP by iNI on the emotionality and the brain neurotransmitter levels were not fully clear, the results obtained by avoidance testing suggested involvement of UFP in learning capability.

Key words: Olfactory bulb, Avoidance test, Spontaneous activity, Nanoparticle

INTRODUCTION

The health effects of ambient particulate matter (PM) in patients have been widely studied. Exposure of patients to PM increases respiratory disorders (Neas *et al.*, 1995; Wong *et al.*, 1999), cancer (Ito and Thurston, 1996) and arrhythmia (Rich *et al.*, 2005). Also, it has been reported that a higher atmospheric black carbon concentration, a marker for traffic-generated particles, causes a decrease in cognitive function in children (Suglia *et al.*, 2008). Furthermore, causes of olfaction deficits, endothelial hyperplasia in olfactory bulbs (OB), neuronal accumulation of particles and presage of Alzheimer disease have been observed in children and young adults inhabiting urban areas (Calderón-Garcidueñas *et al.*, 2010).

Recently, the proinflammatory effects of nanoparticles, developed as industrial materials, on the brain have been

studied (Oberdörster *et al.*, 2005; Win-Shwe *et al.*, 2006, 2008a; Wang *et al.*, 2008a, 2008b). It has been suggested that particles with diameters less than 100 nm are easily translocated into the brain (Mistry *et al.*, 2009; Liu *et al.*, 2009) and impair OB and cerebral tissues, where their injurious effects are due mainly to their oxidative activity (Win-Shwe *et al.*, 2006, 2008a; Wang *et al.*, 2008b). Therefore, it has been considered that translocation of nanoparticles into the brain may have adverse effects on the central nervous system (CNS) (Wang *et al.*, 2008b; Win-Shwe *et al.*, 2008b).

The high number of atmospheric ultrafine particles (UFP), with diameters less than 100 nm and mainly exhausted from diesel engines (Biswas and Wu, 2005), their adverse effects have recently become a focus of attention on in advanced countries. In advanced countries, although the exhaust of larger particles, such as PM 2.5

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(PM with diameters less than 2.5 μm) from motor vehicles was strongly regulated, that of smaller particles, such as UFP, has not yet been regulated. Since diesel exhaust particles contain various metals and chemicals, nanoparticles also show a strong injurious activity *via* production of oxygen radicals (Vogl and Elstner, 1989). Win-Shwe *et al.* (2008b) reported that co-exposure of mice to nanoparticle-rich diesel exhaust and lipoteichoic acid caused dysfunction of spatial learning, an increase in levels of gene expression related to the memory function, and inflammation in the hippocampus. Taking these reports together, it is worthwhile investigating the effects of nanoparticle-rich diesel exhaust particles (NRDEP) on the CNS in experimental animals by intranasal instillation (iNI).

Since the "Miami Declaration" from the "G8 Environment Ministers Meeting on Children's Environmental Health," held in 1997, recommended, "the effects of environmental pollutants should be evaluated in young-aged animals" (1998), the experiment was designed to be able to evaluate the influence on young animals. Therefore, in the present study, NRDEP was administered to animals at an early stage of life, i.e., infancy, and its effects on emotional behavior, learning capability and neurotransmitter levels in the brain were evaluated during growth.

MATERIALS AND METHODS

Reagents

3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA), homovanillic acid (HVA), noradrenalin (NA) and serotonin (5-HT) were purchased from Sigma (St. Louis, MO, USA) and used as standard substances of liquid chromatograph. Tris(hydroxymethyl)amino-methane (Tris) were purchased from Sigma. 5-perchloric acid and polyoxyethylene sorbitan monooleate (Tween80) were purchased from Wako (Tokyo, Japan). Protein Assay kit was purchased from Bio-Rad (Hercules, CA, USA). Sodium pentobarbital was purchased from Tokyo Kasei (Tokyo, Japan).

NRDEP collection and preparation for nasal instillation

NRDEP was collected at the National Institute for Environmental Studies Japan (NIES: Ibaraki, Japan). An 81-diesel engine (J08C, Hino Motors, Tokyo, Japan) was run under steady-state conditions (speed: 2,000 rpm; engine torque: 0 Nm; diesel fuel: JIS No. 2) for 7 hr. These conditions generated nanoparticle-rich exhaust (particle concentration, 3.51×10^7 counts/ml; count mode diameter $\pm$ S.D., 21.45 ± 1.46 nm) (Fujitani *et al.*, 2009). Particles were collected at approximately 10 m from the engine

with a dilution ratio of 8 (55 m^3/min) on dichloromethane-washed, gold discs (25 mm in diameter, 0.025 mm in thickness) electrostatically (-27 kvolts) at a flow rate of 20 l/min using a sampler (SSPM-100, Shimadzu, Kyoto, Japan). The composition by weight of NRDEP collected at the dilution tunnel was analyzed at NIES, and the main elements of NRDEP with diameters less than 100 nm were organic carbon (86%) and elemental carbon (12%). Furthermore, the sum of metals, including Fe, Co, Ni and Zn, was 0.14%. In addition, Mg, Ti, V, Cr, Mn, Cu and Pb were not detectable. The average single particle weight for particles less than 470 nm in diameter was calculated at NIES, and was 0.03 fg.

Seven gold discs with NRDEP (totally 4.271 mg) were provided and stored in refrigerator (-30°C). Two days before first instillation, NRDEP was dispersed from the discs into the vehicle (0.05% Tween80-saline) using an ultrasonic water bath (US-2, SND, Nagano, Japan) under ice-cold conditions for 10 min per disc. Finally, 1 mg/ml of suspended NRDEP (approximately 4.2 ml in volume) was obtained, and then, 300 μl of the suspension was diluted 5 fold with the vehicle (0.2 mg/ml). These NRDEP suspensions were divided into 4 tubes of each, and were stored in a refrigerator (-30°C) until of use. At the administration of NRDEP, the suspensions were sonicated for 10 min, before of use. Aggregation of NRDEP in suspension was observed by scanning electron microscope and it was confirmed that NRDEP formed aggregates in the range of approximately 100-200 nm in length.

In addition, 2 fresh gold discs without NRDEP were immersed in the vehicle and were sonicated for 10 min. The liquid obtained was administered to control animals to evaluate the biological effects of the vehicle.

Animals and breeding

The present study was carried out in accordance with the Instructions of the Committee for Ethical Usage of Experimental Animals in Hatano Research Institute.

Fifty infants, male Sprague-Dawley rats at 12 days of age (weighing 24.7-33.7 g) were purchased from Atsugi Breeding Center, Charles River Japan (Kanagawa, Japan), with 5 foster mothers. The animals were divided into 5 sets, each comprising 10 infants and a foster mother, and each set was housed in a single polycarbonate cage with bedding chips (35 x 40 x 18 cm in width x depth x height) until the animals were 5 weeks of age. After that, at 6 weeks of age, the animals were individually housed in grid-floored, stainless steel cages (22 x 27 x 19 cm). To reduce the stress from the environmental changes, breeding condition during administration period (2-5 weeks of age) was not changed including group breeding and cage size.

Grouping and iNI

Each set of 10 pups was divided into 5 groups of 2 pups each. Each of three groups were treated with the vehicle, 10 µg of NRDEP or 50 µg of NRDEP, respectively (i.e., using 25 µl of 0.05% Tween80-saline for each nostril, totally 50 µl were instilled by Eppendorf® micro-pipette); one group was given sham treatment (i.e., instillation of 25 µl of air into each nostril for the estimation of the effects by vehicle); and remained 2 pups of each were used for histopathological examination at 5 weeks of age (i.e., satellite group). For this examination, the 10 animals in the satellite group were treated with air (3 animals), vehicle (3 animals) or 50 µg of NRDEP (4 animals).

Summary of the study protocol

From 14 days (i.e., 2 weeks) of age, the animals were treated with air (sham treatment), vehicle, 10 µg of NRDEP or 50 µg of NRDEP by once per week, for 4 weeks. The day following the last treatment, the animals in the satellite group were used for histopathological examination. The behavioral tests of the 10 animals remaining in each treatment group (i.e., 50 µg of NRDEP, 10 µg of NRDEP, vehicle, and sham) were examined from 6 weeks to 11 weeks of age. At 12 weeks of age, half (i.e., 5) of the animals in each treatment group were used for pathological examination of the brain. Finally, the 5 animals remaining in each treatment group were used for the measurement of neurotransmitter levels in brain areas and organ weights. In addition, no abnormalities in general condition was observed in any animals used during the study. Also, no significant differences in body weight changes and organs weight were observed between groups.

Histopathological examination of brain in the satellite animals

The day following the last treatment (5 weeks of age), the animals in the satellite group were sacrificed by exsanguination under anesthesia (60 mg/kg of pentobarbital, PB; i.p.). Polyethylene catheter was passed into the aorta via apex of left ventricle of the heart, and axillary vein was cut bilaterally. After an initial perfusion with cacodylate-buffered saline solution for 1 min, the arteries excepting of carotid artery were ligated. Then, a mixture of 0.1 M cacodylate-buffered 2% paraformaldehyde and 1.25 glutaraldehyde (pH 7.4) was perfused at room temperature for 10 min. Perfusion was carried out at a pressure of 700-800 mmH₂O. After fixation, the brain, including the olfactory bulbs, was isolated, and then small blocks were fixed with 0.1 M cacodylate-buffered 2% osmium tetroxide and embedded in epoxy resin. Semith-

in sections were stained with toluidine blue and observed microscope. Ultrathin sections stained with uranyl acetate and lead citrate were observed with an electron microscope (H-7100, Hitachi, Tokyo, Japan). Additionally, the olfactory bulbs were examined by energy dispersive X-ray spectroscopy (H7650, Hitachi and EMAX, Horiba, Kyoto, Japan).

Measurement of spontaneous motor activity by Supermex

The horizontal (ambulation) and vertical (rearing) spontaneous motor activity of animals at 6 weeks of age was measured automatically by infrared pyroelectric sensors (Supermex, Muromachi, Tokyo, Japan). Animals were placed individually in an acrylic box (33 x 44 x 32 cm) at 13:00. Then, the measurement was started, and their ambulation and number of times rearing were cumulatively counted every 30 min for 24-hr. Feeding and drinking were taken *ad libitum* during the test.

Learning ability estimated by shuttle box avoidance test

At 9 weeks of age, animals were subjected to a shuttle box avoidance test (Unicom Inc., Chiba, Japan), comprising two compartments (50 x 20 x 25 cm). For each trial, a 3 sec conditioning stimulus (CS), comprising a buzzer and a light, was followed by 3 sec of unconditioned stimulus, comprising the CS plus a 1.0 mA electric current to the grid floor. The number of responses in which the animal moved to the other side during the CS was recorded. Sixty conditioning trials, separated by 30 sec inter-trial intervals, were given daily for 3 consecutive days.

Histopathological examination and brain dissection

After behavioral examination, half (i.e., 5) of the animals in each treatment group were sacrificed under anesthesia (60 mg/kg of PB, i.p.), at 12 weeks of age. Brain, including OB, heart, lungs, liver, kidneys, spleen and testes were isolated and immersed in 10% formaldehyde-PBS for histopathological examination by light microscopy.

The remaining 5 animals in each treatment group were sacrificed by decapitation at 14 weeks of age, and the brain was immediately isolated and placed on ice. OB was quickly isolated and frozen. In accordance with the methods described by Palkovits and Brownstein (1983), brain areas were isolated. The brain was placed on a brain slicer (RB-AL1-C, Muromachi), and frozen with dry ice. Then, the brain was coronally dissected in slices 1.0 mm in thickness using a razor, and the lateral hypothala-

mus (LH), medial amygdaloid nucleus (Me), ventromedial hypothalamic nucleus (VMH) and medial mammillary nucleus (MM) were punched out in accordance with a brain atlas (Paxinos and Watson, 1998). The samples were frozen and stored in a deep freezer (-90°C). In addition, liver, kidneys, adrenal glands, spleen and testes were isolated and were weighed.

Monoamines and metabolites level in brain areas

A frozen sample of a brain area was thawed and homogenized in 200 µl of perchloric acid (0.1 M, pH 3.0). Then, the sample was centrifuged (10,000 g x 15 min at 4°C, CF-15R), and the supernatant and pellet were separated. Then, the supernatant (100 µl) was injected into a high-performance liquid chromatography (HPLC) system (System-6, Shimadzu), and the amounts of NA, DA, DOPAC, HVA, 5-HT and 5-HIAA were measured with an electrochemical detector (3005, Shiseido, Tokyo, Japan). The pellet was resuspended in 100 mM Tris-HCl (pH 8.0), and the concentration of protein was measured by Bradford protein assay with Bio-Rad Protein Assay Kit.

Statistical Analysis

Values are expressed as the means $\pm$ S.E.M. of the 10 animals in each treatment group, except for organ weights and monoamine neurotransmitter levels in brain areas ($n = 5$). Two-way ANOVA was used, with Bonferroni's correction to control for multiple comparisons (Prism 4.0; Graph Pad, San Diego, CA, USA). Significance levels (p) less than 0.05 were considered to be significant. Fischer's exact probability test was used to compare incidences between two groups.

RESULTS

Spontaneous motor activity

A significantly higher spontaneous motor activity in the ambulation was observed in the first several hours of the light period (13:00-16:00) of the group treated with 10 µg of NRDEP (lower panel of Fig. 1). In these animals, activity in the rearing also tended to be higher (upper panel of Fig. 1). Furthermore, the animals in the group treated with 10 µg of NRDEP displayed greater activity in rearing during the dark period (19:00-07:00), although no statistically significant differences were observed between the groups. Conversely, the animals in the group treated with 50 µg of NRDEP displayed slightly lower activity in both directions than those in the groups given sham treatment or treated with the vehicle.

Shuttle box avoidance test

The mean daily avoidance rate of each group is shown in the upper panel in Figs. 2 (A, B and C), and scatter plots of individual avoidance rates in each group are shown in the lower panel in Figs. 2 (D, E and F). The mean daily avoidance rate in the group given sham treatment improved day by day Figs. 2 (A, B and C), accompanied by a reduction in decentralization in the individual avoidance rates in the animals Figs. 2 (D, E and F). The mean daily avoidance rate in the vehicle group also improved day by day, although complete failure in the avoidance task was observed in 1 animal during the 3 days Figs. 2 (D, E and F). The animals in the NRDEP-treated groups showed a suppression of daily improvement in the mean daily avoidance rate Figs. 2 (A, B and C). Especially, the mean daily avoidance rate in the group given 50 µg of NRDEP mostly did not improve during the 3 days. Also, a reduction of decentralization of individual avoidance rates in the animals was not observed Figs. 2 (D, E and F). Because a large decentralization of mean daily avoidance rates was observed in the animals treated with NRDEP, the number of animals in the first quartile (Q1) was counted, and it appeared that almost all the animals below Q1 were NRDEP-treated animals (Table 1).

Histopathological examination

In the animals subjected to pathological examination on the day following the last day of treatment (5 weeks of age), an increased number of corpuscles densely stained with toluidine blue was observed by light microscopy in the glomerulus of OB in the animals treated with 50 µg of NRDEP. By transmission electron microscopy, electron dense lamellar bodies were observed in the microglial cells of the glomerulus (Fig. 3A). However, changes were very slight. Also in the area close to the glomerulus, a marked accumulation of electron dense granules with diameters of approximately 20 nm was observed in the axonal cytosole (Fig. 3B). Examination using energy dispersive X-ray spectroscopy revealed large granules containing phosphorus (Fig. 3C). In addition, no marked abnormalities were observed in the brain and nasal cavity of animals treated with 50 µg of NRDEP. Also, no abnormalities were observed in the heart, liver, kidneys and spleen in NRDEP-treated animals at 9 weeks of age by light microscopy. In addition, no changes related to the deposition of NRDEP in the lung were observed.

Fig. 1. Spontaneous motor activity in animals measured by Supermex at 6 weeks of age. The spontaneous motor activity of the animals was automatically measured for 30-min intervals over a 24-hr period using Supermex. The cumulative counts of movements in rearing and ambulation at each interval are indicated in the upper and lower panels, respectively. Also, the scales for the counts during the light and dark periods are indicated on the left and right sides, respectively. Data are expressed as means $\pm$ S.E.M. of the 10 animals in each treatment group. * $p < 0.01$, ** $p < 0.001$, significantly different from the mean values in the vehicle treatment group (by Bonferroni's post-hoc test).

Fig. 2. Changes in daily avoidance rate of the animals subjected to shuttle box tests for 3 consecutive days at 9 weeks of age. Mean daily avoidance rate of each group is shown in the upper panels. Data are expressed as means $\pm$ S.E.M. of the 10 animals in each treatment group. Scatter plots of each group are shown in the lower panels. Bars indicate the average of each group. Numbers in symbols indicate animal number.

Table 1. The number of animals in each treatment group below the first quartile (the 25th percentile; Q1) of all animals examined (10 out of 40 animals).

	Treatments	Number of animals	P value (versus vehicle)
Day-1	Sham	1	0.5000
	Vehicle 50 μ l	2	-
	NRDEP 10 μ g	4	0.3142
	NRDEP 50 μ g	3	0.5000
Day-2	Sham	1	0.5000
	Vehicle 50 μ l	2	-
	NRDEP 10 μ g	2	0.7090
	NRDEP 50 μ g	5	0.1749
Day-3	Sham	0	0.5000
	Vehicle 50 μ l	1	-
	NRDEP 10 μ g	4	0.1517
	NRDEP 50 μ g	5	0.0704

Q1 (n = 10) was calculated from the avoidance performances in all animals examined (n = 40). After that, the animals in each group below Q1 were counted. At Day- 3, the animals displaying a lower avoidance ratio than Q1 were mostly observed in the NRDEP-treated animals.

Neurotransmitter levels in brain areas

Results are summarized in Fig. 4. Neurotransmitter levels of brain areas varied widely by area. In some combinations of area and monoamines or its metabolite, such as NE in VMH, DA and DOPAC in MM, HVA in MM and 5-HT in MM, neurotransmitter levels in the vehicle group were greatly different from those in the sham-treatment group, although a statistical significance between these two groups was not observed. The average of only two samples was used for the HVA levels in VMH because the amount of HVA in 3 of the samples was below the lower limit (0.1 ng/injection), i.e., the samples from the groups treated with 10 μ g of NRDEP or the vehicle and the group given sham treatment. In MM, DA levels in the animals treated with 10 μ g of NRDEP tended to be decreased (Fig. 4B). Also, DOPAC and HVA levels in MM in the NRDEP-treated animals tended to be decreased (Figs. 4C and D). However, significant decreases of DA and DOPAC levels were only observed in MM in the NRDEP-treated animals when compared with the animals given sham treatment. Also, 5-HT level in MM in the animals treated with 10 μ g of NRDEP tended to be decreased (Fig. 4E).

Fig. 3. Histopathological observations in the olfactory bulbs of animals treated intranasally with 50 μ g of NRDEP. (A) Electron dense lamellar bodies were observed in the microglial cells of the glomerulus (by transmission electron microscopy, scale indicates 5 μ m). (B) The accumulation of electron dense granules (arrows) with a diameter of approximately 20 nm was observed in the axonal cytosol (by transmission electron microscopy, scale indicates 1 μ m). (C) Representative result of analysis using energy dispersive X-ray spectroscopy. Large granules containing phosphorus were observed.

The effects of nanoparticle-rich diesel exhaust particles on CNS

Fig. 4. Monoamine neurotransmitter levels in the brain areas at 14 weeks of age. Areas measured were LH, Me, VMH and MM. Also, neurotransmitters and its metabolites measured were DOPAC, 5-HIAA, DA, HVA, NA, 5-HT. Data are expressed as means $\pm$ S.E.M. of 5 animals in each treatment group, except HVA in VMH (# in panel D: $n = 2$). Statistical differences were not observed in mean values between the vehicle group and the NRDEP-treated groups (by Bonferroni's post-hoc test).

DISCUSSION

In biochemical and pathological examinations, the changes observed in the NRDEP-treated animals were not clear. Therefore, it is difficult to explain the mechanisms underlying the changes observed in behavioral examinations, based on the effects on the central nervous system. However, NRDEP subtly affected the emotionality and learning capability of rats in the present study.

Kaur *et al.* (2005) reported that personal exposure to ultrafine particles at the center of London, an urban area, was in the range of 60,000-100,000 particles/cm³. Also, in general, human daily respiratory volume is considered to be 20 m³ (Andelman, 1985). From this information, it is considered that the amount of daily personal inhala-

tion of ultrafine particles is approximately 36-60 µg/day ($1.2-2.0 \times 10^{12}$ particles/day or 0.5-0.9 µg/kg; if human body weight is assumed to be 70 kg). From these calculations, the doses used in the present study were very high. However these were judged to be reasonable, since the instillation of NRDEP was only performed 4 times. In addition, using of large amounts of test substances in experiments is frequently done to elucidate hidden adverse effects or novel mechanisms of toxicity.

In the active avoidance test using a shuttle box, daily improvements of avoidance performance were observed in the animals given sham treatment and vehicle. However, failure of daily improvement was observed in 4 and 6 animals in the groups treated with 10 and 50 µg of NRDEP, respectively. Furthermore, the avoidance rate

of animals treated with NRDEP was divided into two extremes, “excellent” and “poor.” Consequentially, almost all of the animals under the first quartile were NRDEP-treated animals.

A very important question that remained was why the suppression in avoidance performance was only observed in approximately half of the animals treated with NRDEP. It is possible that the distribution of particles in the brain is individually different. Wang *et al.* (2008a) observed translocation of nano-sized TiO_2 by iNI into the hippocampus, a component related to learning and memory functions. The TiO_2 content in the hippocampus at 30 days of treatment indicated large differences. This may be the cause for the different effects on the learning and memory functions observed in the animals.

The spontaneous motor activity in the animals treated with 10 μg of NRDEP was significantly increased during the initial 3 hr of the measurement, while that in the animals treated with 50 μg tended to decrease. The measurement of spontaneous motor activity of the animals was carried out early in the post-administration period. It is possible that iNI of small amounts of NRDEP caused injury mainly in OB at an early stage without marked injuries in the brain. OB is not only a primary center of olfaction but also a ruler of emotions and learning in animals (Ueki *et al.*, 1972; Leonard, 1984; Durand *et al.*, 1998). It was reported that olfactory bulbectomy caused imbalances in brain neurotransmitters, including noradrenalin, dopamine and serotonin (Edwards *et al.*, 1977; Song and Leonard, 2005), and also caused marked and sustained hyperactivity in rats (van Riezen and Leonard, 1990). Taken together, this information and the present results, it is possible that a small amount of NRDEP enhanced spontaneous activity of the animals through its proinflammatory activity on OB.

Conversely, a high dose of NRDEP (50 μg) tended to suppress spontaneous activity. In the measurement of neurotransmitter levels in brain areas, the levels of DA and its metabolites in NRDEP-treated animals tended to decrease in MM. This might have caused the suppression of emotionality in the animals. However, the effects of NRDEP were examined by only two dose levels in the present study. To reveal the effects of high and low dose levels, examination using 4 or more dose levels are necessary.

A tendency to reduced levels of DA and its metabolite, DOPAC, in the MM was observed in the NRDEP-treated animals examined at 14 weeks of age. Block *et al.* (2004) reported selective toxicity of nano-sized DEP to dopaminergic neurons *in vitro* in co-culture with microglia. Initially, glial cells phagocytized nano-sized DEP, then the dopaminergic neurons were injured by reactive oxygen

species, which were produced by the glial cells.

Because of the lower content of glutathione, an antioxidant tripeptide, dopaminergic neurons are less resistant to oxidative stress than other types of neurons, such as GABAergic neurons. Therefore, the decrease in DA level in the brain might be involved in the behavioral changes, such as suppression of the spontaneous motor activity in 50 μg -NRDEP-group and failure in the avoidance task in both, NRDEP-groups. Thus, a lowered content of DA and DOPAC in MM in the present study looks like a reasonable result.

However, similar tendencies in DA and DOPAC content were also observed in vehicle-treated animals. It is difficult to explain why iNI of the vehicle (0.05% Tween80-saline solution) changed neurotransmitter levels in the brain areas. Since Tween80 was reported to have a low cytotoxicity (Arechabala *et al.*, 1999), it is inconceivable that iNI of the vehicle injured OB or the brain. The measured areas of the brain in the present study were only 4 areas. Thus, it is possible that NRDEP suppressed avoidance performance by injuring other areas of the brain, which were not measured in the present study, such as the striatum. In addition, it is also possible that iNI of the vehicle moistened the nasal cavity and caused, thereby, deposition of small dust particles from the bedding chips or feces. However, to clarify these hypotheses, further examination is necessary.

It was reported that hyperactivity of bulbectomized rats was abolished or attenuated when the animals were placed in an area surrounded by transparent walls (Kelly *et al.*, 1997). The spontaneous motor activity of the animals was measured in a clear acrylic cage in the present study. This might, therefore, have attenuated the enhancement of spontaneous motor activity in the animals. For this reason, it is considered that further study by other methods, such as an open-field test, is necessary to evaluate the effects on spontaneous motor activity of NRDEP by iNI. Also, the animals were placed in a novel environment during the measurement, different from their home cages (stainless steel grid-floor cages). In general, spontaneous motor activity during the initial period in a novel environment is high, since the animal exhibits exploratory behavior (Takeda *et al.*, 2005). Increased motor activity in the animals treated with 10 μg of NRDEP might represent enhanced emotionality related to exploration.

An examination for translocation of NRDEP was made by energy dispersive X-ray spectroscopy in the 5-week old animals, and no marked evidence was found in the present study. Since the amount of metals, such as Fe, Ni, Cu or Zn, contained in NRDEP was very low, specifica-

tion of the NRDEP in the small field of specimens was very difficult. However the accumulations of lamella in microglia and of phospholipid granules in neuronal axons in the glomerular layer in the main OB were observed by electronmicroscopy at 5 weeks of age. From these facts and the results, it was considered that our present result does not deny the translocation of NRDEP into the OB.

In the present study, the weights of organs in the animals treated with NRDEP were not different from those in the animals given sham treatment or treated with the vehicle. It was reported that DEP contains various chemicals, including endocrine-disrupting chemicals (Izawa *et al.*, 2007). Since no differences were observed in the weights of testes and adrenal glands, involvement of an endocrine-disrupting effect of NRDEP in the present study was negligible.

Several environmental factors that might affect emotionality, temperament, learning capability or cognitive function in children were considered (Rush and Callahan, 1989; Brouillette and Quirion, 2008; Ljung *et al.*, 2009). Although the effects of NRDEP observed in the present study were very slight, the substance might be one of those environmental factors. Even if the adverse effects of individual environmental pollutants are slight, a combination of pollutants could obviously affect a child's life.

In conclusion, the present results suggest that NRDEP potentially affects emotionality and learning capability in animals in later life via modulation of monoamine neurotransmitter levels in CNS.

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