Table 1. Effects of prenatal exposure to TiO_2 nanoparticles on the central dopaminergic system of offspring

Brain region	Group	Content (pg/mg protein)			
		DA	*DOPAC*	*HVA*	*3-MT*
Olfactory bulb	*Control*	4,682 ± 638	346 ± 44	1,615 ± 138	749 ± 24
	TiO_2	4,444 ± 272	346 ± 33	1,766 ± 140	719 ± 39
Prefrontal cortex	*Control*	3,121 ± 345	1,380 ± 135	3,608 ± 275	612 ± 44
	TiO_2	6,534 ± 1,123**	2,022 ± 181*	5,352 ± 342**	956 ± 78**
Neostriatum	*Control*	194,928 ± 16,063	25,293 ± 2,465	22,408 ± 2,256	22,165 ± 1,744
	TiO_2	270,793 ± 13,886*	36,239 ± 1,280*	32,510 ± 1,143*	28,015 ± 1,595
Nucleus accumbens	*Control*	196,751 ± 6,459	34,169 ± 2,495	24,305 ± 1,191	20,893 ± 1,249
	TiO_2	209,617 ± 6,956	34,420 ± 1,466	27,467 ± 710	19,717 ± 868
Hippocampus	*Control*	2,865 ± 1,408	1,002 ± 381	1,227 ± 364	1,433 ± 417
	TiO_2	1,471 ± 806	450 ± 111	1,105 ± 150	986 ± 214
Hypothalamus	*Control*	16,560 ± 955	6,462 ± 458	4,678 ± 351	2,486 ± 152
	TiO_2	15,759 ± 1,616	5,512 ± 184	4,191 ± 204	2,261 ± 117
Amygdala	*Control*	17,440 ± 1,786	3,960 ± 352	1,683 ± 181	3,490 ± 313
	TiO_2	14,475 ± 1,192	3,913 ± 252	1,676 ± 226	2,956 ± 229
Midbrain	*Control*	8,430 ± 1,056	5,185 ± 738	4,175 ± 586	1,302 ± 154
	TiO_2	9,163 ± 471	5,494 ± 274	4,964 ± 293	1,442 ± 118
Brainstem	*Control*	1,374 ± 63	1,237 ± 98	2,277 ± 127	198 ± 15
	TiO_2	1,655 ± 252	1,347 ± 232	2,744 ± 444	228 ± 20
Cerebellum	*Control*	N.D.	N.D.	576 ± 58	N.D.
	TiO_2	N.D.	N.D.	765 ± 59	N.D.

The suspended TiO_2 nanoparticles were administered subcutaneously to the pregnant ICR mice at gestation days 6, 9, 12, 15 and 18. Control animals were treated with vehicle (saline with 0.05% Tween 80). In each group, pups were weaned on postnatal day 21. Levels of DA and its metabolites (pg/mg protein) in each area of the brain. Data are presented as mean ± S.E.M. (n = 8). *$p < 0.05$, **$p < 0.01$ vs. each control group, N.D. - not detectable.

ric pathologies such as ADHD and schizophrenia (Thapar *et al.*, 2005; Wong *et al.*, 1986).

Psychiatric conditions are regarded as prenatal developmental disorders of the brain that are associated with heritable and environmental factors. Therefore, an increase in striatum levels of DA leads to enhancement of locomotor activity. Furthermore, it has been reported that the enhancement of DA metabolic turnover in the prefrontal cortex results in impairments of working spatial memory (Hirvonen *et al.*, 2005). Taken together, these findings suggest that the increase in DA observed in this study might affect motor and cognitive functions.

Many studies have shown that nanoparticles produce reactive oxygen species and cause oxidative damage to cells and tissues (Wang *et al.*, 2008a, 2008b; Hussain *et al.*, 2009). It is uncertain whether the changes in DA levels in response to prenatal exposure to TiO_2 nanoparticles resulted from an increase in the production of reactive oxygen species. Notably, we have also observed similar pathological phenomena in the brain using the rutile form of TiO_2 that we observed with the anatase form.

Although we did not investigate the effect of TiO_2 nanoparticles in the adult animals, it has been reported that TiO_2 nanoparticles do not usually enter the brain of adult

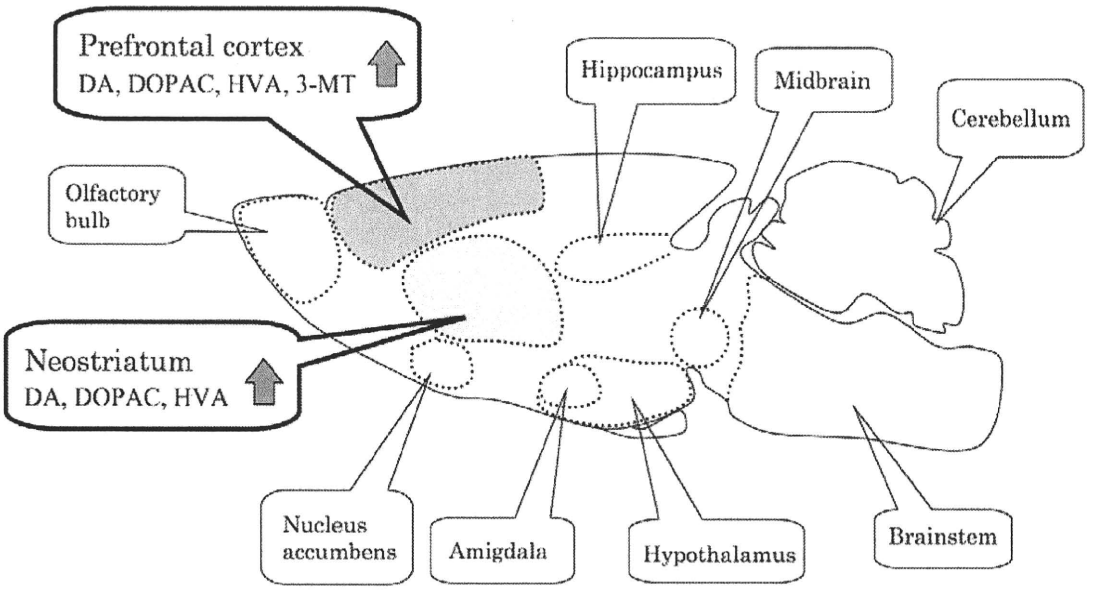

Fig. 3. Summary of changes in the levels of DA and its metabolites in 10 regions of the brain following prenatal exposure to TiO_2 nanoparticles.

animals (Fabian *et al.*, 2008). Since the blood brain barrier is not fully developed in embryos, the developing brain is sensitive to foreign chemicals during the embryonic stage. We have previously reported the penetration of TiO_2 nanoparticles into the brain, and stenosis of peripheral blood vessels of the cerebral cortex and hippocampus in the offspring of female mice exposed to TiO_2 nanoparticles during pregnancy (Takeda *et al.*, 2009). These findings strongly support the hypothesis that prenatal exposure to TiO_2 nanoparticles can affect the development of the central nervous system through the dissemination of nanoparticles into the brain.

TiO_2 nanoparticles were easily aggregated and agglomerated. We also obtained the aggregated TiO_2 nanoparticles around 2 μm in the present study (Fig. 1). However, several investigators have shown that the aggregated TiO_2 nanoparticles around 1.4 μm exerted toxicity (Bermudez *et al.*, 2004; Ferin *et al.*, 1992). Moreover, Sager *et al.* (2008) reported that intratrachial administration of TiO_2 nanoparticles, which agglomerated a diameter of 200-300 nm, cause the pulmonary inflammatory responses. Furthermore, the aggregated/agglomerated TiO_2 nanoparticles have revealed more toxicity than their larger counterparts (Ferin *et al.*, 1992; Sager *et al.*, 2008). These findings propose a hypothesis that TiO_2 nanoparticles may be able to affect the central dopaminergic neuron regardless of aggregation/agglomeration. On the other hand, we observed TiO_2 nanoparticles with a diameter of less than 300 nm in the brain of offspring (Takeda *et al.*, 2009). Recently Wick *et al.* (2010) showed that fluorescent polystyrene particles up to a diameter of 240 nm were taken up and were able to cross the placental barrier without affecting the viability of the explant using the *ex-vivo* human placental perfusion model. Therefore, intact or smaller parts of agglomerate TiO_2 nanoparticles might be able to selectively transfer and affect the DA levels in the brain of offspring.

In conclusion, the present data provide evidence that prenatal exposure to TiO_2 nanoparticles can influence the DA levels of brain in mice (Fig. 3). Further investigation is necessary to fully understand the molecular mechanisms of TiO_2 nanoparticle-mediated alterations of the central nervous system.

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Effects of fetal exposure to carbon nanoparticles on reproductive function in male offspring

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Objective: To investigate the effects of fetal nanoparticle exposure on reproductive function in male mice offspring.
Design: Animal study.
Setting: Academic research laboratory.
Animal(s): Forty pregnant ICR mice and 120 male offspring.
Intervention(s): Two hundred μg of 14-nm carbon nanoparticles was administered intratracheally on days 7 and 14 of gestation, and reproductive function of male offspring was determined at ages 5, 10, and 15 weeks after birth.
Main Outcome Measure(s): Maternal and fetal growth, histologic changes in the testes, and daily sperm production (DSP).
Result(s): Histologic examination showed partial vacuolation of seminiferous tubules. and cellular adhesion of seminiferous epithelia was reduced at all three ages. In addition, DSP was significantly decreased in fetal carbon nanoparticle–exposed mice. The DSP in the fetal carbon nanoparticle–exposed mice decreased by 47% at the age of 5 weeks, by 34% at the age of 10 weeks, and by 32% at the age of 15 weeks. On the other hand, nanoparticle administration had no marked effect on body weight, testicle weight, epididymis weight, or serum testosterone concentration.
Conclusion(s): These findings suggest that fetal nanoparticle exposure affects the reproductive function of male offspring. In the future, it would be necessary to clarify the onset mechanisms of nanoparticle-induced male reproductive disorders. (Fertil Steril® 2010;93:1695–9. ©2010 by American Society for Reproductive Medicine.)

Key Words: Male reproduction, daily sperm production, in utero exposure, nanoparticle

Diesel exhaust (DE) and DE particles (DEP) are one of the compounds responsible for air pollution. Several studies reported that DE and/or DEP aggravated respiratory and allergic diseases (1, 2). We previously reported that the DE aggravated the male reproductive function of male mice, with marked seminiferous tubule damage, regressive degeneration of Leydig cells, increased incidence of spermatogenetic cell apoptosis, and reduced daily sperm production (DSP) (3). Watanabe et al. (4, 5) reported that the Sertoli cell counts, sperm counts, serum FSH concentration, and DSP were significantly decreased with DE exposed in utero. We also found that exposure of DE to pregnant mice induced the weight of accessory reproductive gland in male offspring (6). A human study that investigated the effects of exhaust on the reproductive system of male highway tollbooth workers has shown that automobile emission particles reduced sperm motility (7). These findings indicate that auto emissions have a negative impact on human spermatozoa; however, causative agents have not been identified.

Recently, the effects of nanoparticle (particles <100 nm in mass median aerodynamic diameter, such as DEP) have been examined. Studies investigating the effects of nanoparticles on the human body have shown that nanoparticles exacerbate lung injury (8), that nasally administered nanoparticles accumulate in the brain via the olfactory nerve and exacerbated inflammatory reactions (9), and that nanoparticles affect the circulatory system by altering heart rate (10). We previously conducted a study to determine the effects of nanoparticles on the reproductive function of adult mice and documented increased detachment of seminiferous epithelia and elevated serum T (11). However, at present, the effects of fetal nanoparticle exposure on the male reproductive system have not been elucidated.

In the present study, we investigated the adverse effects of nanoparticle exposure to pregnant mice on the reproductive system of male offspring. For this purpose, 14-nm carbon nanoparticles (CB) were administered to pregnant mice, and DSP, sperm characteristics, serum T, and histologic analysis were analyzed at the ages of 5, 10, and 15 weeks after birth in male offspring.

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TABLE 1

Effects of carbon nanoparticle (CB) exposure on dams and fertility.

	Control	CB
Fertility	100%	90%
Gestation length (d)	18.1 ± 0.5	18.1 ± 0.5
Litter size	13.8 ± 3.0	13.7 ± 3.3
Male:female ratio	1.04 ± 0.30	0.92 ± 0.34

Note: Results are presebted as mean ± SD.

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MATERIALS AND METHODS

Animals

Forty pregnant ICR mice purchased from Clea Japan (Tokyo) were used (induction at day 5 postcoitum (pc; the day the plug was found was considered to be day 0 pc)). Mice were divided into two groups of 20 mice each: the nanoparticle and control groups. Up to day 16 pc, each cage housed five mice, and starting on day 17 pc, each mouse was caged separately. The mice were kept under the following conditions: temperature 23 °C–25 °C, humidity 50%–70%, and 12-hour light/dark cycle. The mice had free access to food (CE-2 solid food; Clea Japan) and water. The study was conducted in accordance with the Laboratory Animal Facility Usage Guidelines compiled by the Ohita University of Nursing and Health Science.

Nanoparticles

The present study used 14-nm CB, and microbial toxins, lipopolysaccharide, and β-glucan were eliminated by heating the CB at 350 °C for 30 minutes.

Particle Administration

The CB were suspended in a normal saline solution (Otsuka Co., Kyoto, Japan) containing 0.05% Tween 80 (Wako, Osaka, Japan) for instillation. The instillation dose of the CB was 0.2 mg/ mouse. Before administration, CB was sonicated for 5 minutes. The pregnant mice were intratracheally (IT) instilled with CB under anesthesia with 4% halothane (Takeda Chemical, Osaka, Japan) using a polyethylene tube on days 7 and 14 of gestation. The control mice were instilled IT with 0.1 mL of normal saline solution containing 0.05% Tween 80. The amount of 0.2 mg/mouse in the lungs of a single mouse was eight times the amount of the Japanese national air quality standard of suspended particulate matter (0.1 mg/m^3) accumulated in the lungs of a mouse/day.

Offspring

Mice that were delivered with an average gestation of 18 ± 0.5 days were used. At the age of 12 days, six male mice (from each mother mouse) were selected. Mice were weaned at 25–26 days after birth, and each cage housed eight mice.

Body and Organ Weights

At age 5, 10, or 15 weeks, male mice were anesthetized using pentobarbital sodium, and body weight was measured and a blood sample collected from the heart. Male mice are sexually mature at 7 or 8 weeks (therefore, 5-week-old mice were sexually immature, and 10- and 15-week-old mice were sexually mature). The mice were then killed to remove testicular and epididymal tissues. Both left and right tissues were separately weighed. The right testis was stored frozen at −80 °C and was used to measure DSP. The right epididymis was used to evaluate sperm characteristics. The left testis was used for histologic analysis.

Sperm Characteristics

In 1 mL physiologic saline, the tail of the epididymis was chopped 100 times using ophthalmologic scissors and filtered using 100-mesh membrane to obtain a sperm suspension. Using a cytometer, the number of spermatozoa in the tail of the epididymis was counted. In addition, smear samples were prepared to observe spermatozoa. Abnormal spermatozoa were defined as cells without heads or tails, and the ratio of abnormal spermatozoa was calculated. A sperm analyzer (SCA; NeuroScience Inc., Tokyo, Japan) was used to determine the concentration of sperm in the epididymis and to

TABLE 2

Effects of fetal carbon nanoparticle (CB) exposure on offspring body, testis, and epididymis weights.

	Body weight (g)		Testis (mg)		Epididymis (mg)	
	Control	CB	Control	CB	Control	CB
5 wk	32.6 ± 2.3	33.2 ± 2.3	116.0 ± 12.0	112.1 ± 14.9	32.8 ± 7.4	28.1 ± 2.8
10 wk	41.3 ± 2.9	41.2 ± 2.8	147.9 ± 23.6	149.8 ± 19.0	55.6 ± 5.9	58.0 ± 6.2
15 wk	46.8 ± 3.9	45.3 ± 3.0	156.6 ± 28.7	150.5 ± 14.3	67.3 ± 8.8	65.7 ± 5.9

Note: Data are presented as mean ± SD; n = 16.

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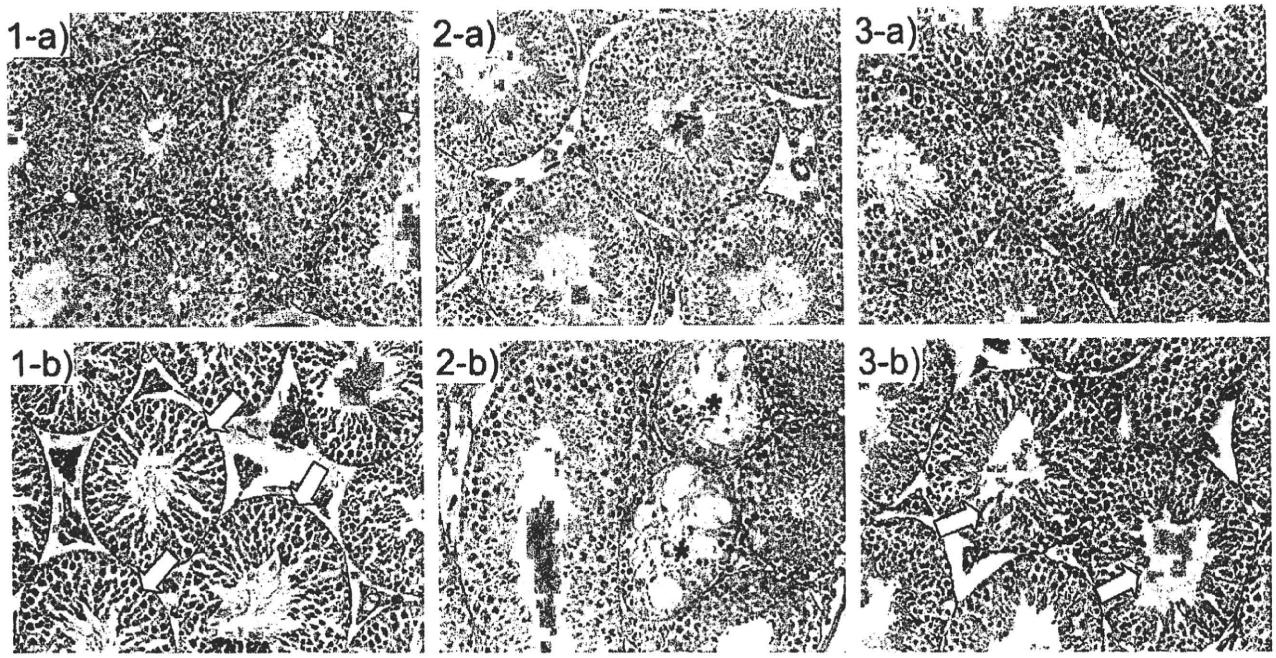
FIGURE 1

Testicular tissue of male offspring exposed to carbon nanoparticles (CB) as fetuses. (1) 5-week-old mouse; (2) 10-week-old mouse; (3) 15-week-old mouse; (a) control; (b) CB group. ×200 magnification. At all three ages, seminiferous epithelial damage (*arrows*) was seen in the CB group. Vacuolation was seen with some seminiferous tubules (*asterisks*).



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assess sperm motility. With each sample, about 800–1,000 spermatozoa were analyzed.

Daily Sperm Production

As a lysis buffer for DSP measurement, 8.8 g/L sodium chloride and 200 mL/L 0.02% eosin-Y were used. The testis was homogenized in 1 mL lysis buffer and stored at 4 °C. The number of cells (spermatozoa) in the solution was counted to calculate DSP using the following formula:

sperm count/mL = testicular sperm count
testicular sperm count/4.84* = sperm produced/day
(sperm produced/day)/testis weight = DSP

where 4.84 is a coefficient for calculating sperm production in mice.

Serum Testosterone Measurement

Serum T was measured using the Testosterone ELISA Test Kit (Endocrine Technologies, Newark, NJ).

Histologic Analysis

For histologic analysis, the testis was fixed in Bouin solution, embedded in paraffin, sliced thin, stained using hematoxylin and eosin (HE), and observed under a light microscope. The ratios of seminiferous tubule degeneration and multinucleated giant cells in seminiferous tubules were determined.

Statistical Analysis

Using KyPlot version 5 (Kyens Lab, Tokyo, Japan), Welch *t* test was used for statistical analysis. A P value of $<.05$ was considered to represent statistical significance.

RESULTS

Effects of CB Administration on Maternal and Fetal Growth

The effects of CB administration on dams were determined by comparing gestation length, litter size, fertility, and gender ratio between the two groups. No significant differences were seen in gestation length, litter size, fertility. or gender ratio (Table 1).

Effects of fetal CB Exposure on Body, Testis and Epididymis Weights

There were no significant differences in body and organ weight between the CB and control groups at the ages of 5, 10, and 15 weeks (Table 2).

FIGURE 2

Percentage of degenerated seminiferous tubules in cross-sections of carbon nanoparticles (CB)–treated and control mice. Estimation of testicular damage was conducted by counting the number of tubular cross-sections and determining the percentage of total degenerated tubules in three cross-sections per testis. Mean ± SD (n = 8). ***$p < .001$ vs. control.

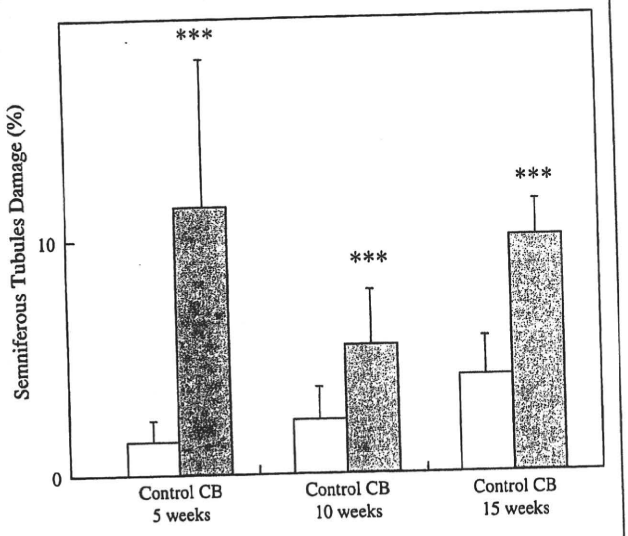

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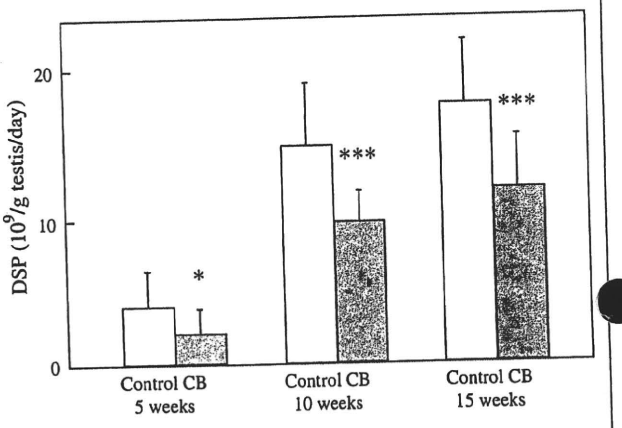
FIGURE 3

Effects of fetal carbon nanoparticle (CB) exposure on spermatogenesis. Right testis sample was weighed and seminiferous tubules released from the tunica albuginea into a saline solution in a homogenizer tube. Tissue was homogenized and diluted to the desired volume, and a sample of the diluted suspension was taken to count sperm heads or sperm cells in a hemocytometer chamber. Daily sperm production (DSP) was calculated as described in Materials and Methods. Mean ± SD (n = 16). *$P < .05$; ***$P < .001$; vs. control.



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Histologic Changes in the Testes of Mice Exposed to CB in Fetal Period

The testes of male mice exposed to CB as fetuses exhibited the vacuolation of seminiferous tubules and low cellular adhesion of seminiferous epithelia (Fig. 1). Compared with the control group, the ratio of seminiferous tubule damage in the CB group was significantly higher (5-week-old mice: 10% [$P < .001$]; 10-week-old mice: 3.3% [$P < .001$]; and 15-week-old mice: 5.8% [$P < .001$]; Fig. 2).

Effects of Fetal CB Exposure on Sperm Production

The DSP was significantly decreased in the CB treated group at all three ages (5-week-old mice: 47% [$P < .001$]; 10-week-old mice: 34% [$P < .001$]; and 15-week-old mice: 32% [$P < .001$]; Fig. 3). On the other hand, there were no significant changes in sperm cytomorphology.

Effects of CB on Serum Testosterone

The CB tended to increase serum T at ages of 10 and 15 weeks after birth. However, there were no significant changes in serum T between the control and CB groups at all three ages (Fig. 4).

DISCUSSION

Our previous study investigated the effects of CB on male reproductive function; however, the effects of fetal CB exposure on male reproductive function in offspring had not been identified. In the present study, we investigated that CB administered in utero effects on the reproductive function of male offspring.

Fetal CB exposure significantly reduced DSP in male offspring. When CB was administered to adult mice, DSP decreased significantly (11). Therefore, CB reduces DSP through both fetal exposure and exposure during adulthood. Furthermore, it has been reported that fetal exposure to diesel exhaust (DE) lowers the DSP of male offspring (5). Diesel exhaust consists of various components, including DEP. The carbon nanoparticles used in the present study are made of carbon, which is the basic structure of DEP. Therefore, fetal DE exposure may lower DSP in male offspring due to particulate matters in DE, particularly CB. However, in the present study, it was not clear whether CB lowered DSP by altering the maternal environment or by directly affecting fetuses. In the future, it will be necessary to determine the effects of CB on both dams and offspring.

In the testis of male offspring, intercellular adhesions of seminiferous epithelia and seminiferous tubules damage were observed. The low cellular adhesion of seminiferous epithelia may indicate reduced adhesion of Sertoli and spermatogenic cells. Because Sertoli cells supply nutrients and send signals for cellular differentiation to spermatogenic cells (12), weak adhesion of Sertoli and spermatogenic cells

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ORGAN TOXICITY AND MECHANISMS

Perinatal exposure to diesel exhaust affects gene expression in mouse cerebrum

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Abstract Many environmental toxins alter reproductive function and affect the central nervous system (CNS). Gonadal steroid hormones cause differentiation of neurons and affect brain function and behavior during the perinatal period, and the CNS is thought to be particularly susceptible to toxic insult during this period. It was, therefore, hypothesized that inhalation of diesel exhaust (DE) during the fetal or suckling period would disrupt the sexual differentiation of brain function in mice, and the effects of exposure to DE during the perinatal period on sexual differentiation related gene expression of the brain were investigated. In the fetal period exposure group, pregnant ICR mice were exposed to DE from 1.5 days post-coitum (dpc) until 16 dpc. In the neonatal period exposure group, dams and their offspring were exposed to DE from the day of birth [postnatal day (PND)-0] until PND-16. Then, the cerebrums of males and females at PND-2, -5, and -16 from both groups were analyzed for expression level of mRNA encoding stress-related proteins [cytochrome P450 1A1 (CYP1A1), heme oxygenase-1 (HO-1)] and steroid hormone receptors [estrogen receptor alpha (ER alpha), estrogen receptor beta (ER beta), androgen receptor (AR)]. Expression levels of ER alpha and ER beta mRNA were increased in the cerebrum of newborns in the DE exposure groups as well as mRNA for CYP1A1 and HO-1. Results indicate that perinatal exposure to DE during the critical period of sexual differentiation of the brain may affect endocrine function.

Keywords Diesel exhaust · Fetal · Neonatal · Cerebrum · mRNA · ICR · Mice

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Introduction

Air pollution is one form of environmental pollution that affects humans, and death due to environmental pollution was first recognized during the nineteenth and early twentieth century in Great Britain. Contaminants come from both stationary and mobile sources. The most common, suspended particulate matter (SPM) containing diesel exhaust particulate (DEP) and nitrogen oxide (NOx), are from mobile sources such as automobiles and pose a serious problem in large cities. DEP is an important air pollutant. Takano et al. (2002) found that intratracheal instillation of DEP caused a dose-dependent increase in expression level of both cytochrome P450 1A1 (CYP1A1) mRNA and protein in lung tissue.

There have been several reports on the effects of DE on the reproductive system. Watanabe and Oonuki (1999) reported that serum levels of testosterone and estradiol were significantly higher in animals exposed to total DE and filtered exhaust than in controls, and that sperm production and testicular hyaluronidase activity were significantly lower in exhaust-exposed groups. Tsukue et al. (2002) also reported that toxic substances in DE might cause abnormal delivery in mice, and that DE exposure in females could affect the growth and sexual maturation of their offspring. Even low levels of DE reduced expression of genes essential to normal male gonadal development, including Müllerian inhibitory substance (MIS) and steroidogenic factor-1 (Ad4BP/SF-1), and may affect development of the male gonads (Yoshida et al. 2002).

The effects on the brain of various chemical substances have been studied (Gore 2001; Ferguson 2002). It is thought that, in general, if a chemical substance cannot cross the blood–brain barrier, the deleterious effect may be somewhat mitigated. However, during fetal development, the blood–brain barrier is immature, and therefore, it may be possible for substances that can cross the placenta and/or are secreted into milk to pass into the brain of an embryo or newborn (Dalgaard et al. 2001; Oh et al. 2008). Many substances that disrupt endocrine function are similar structurally to steroids, and may directly influence gene expression through interaction with steroid receptors, and DE has been reported to contain substances with estrogenic (such as 4-nitrophenol and 4,6-dimethyldibenzothiophene), anti-estrogenic, and anti-androgenic (such as 3-methyl-4-nitrophenol) activities (Furuta et al. 2004; Mori et al. 2002; Taneda et al. 2002; Kizu et al. 2003; Li et al. 2006a, b). During embryonic and postnatal development, steroid hormones play an important role in nerve specialization during cerebral functional development, sexual differentiation of the brain, and behavior development (Weiss 1997, 2002). In male mice, androgen secreted from sperm during the critical period of perinatal development irreversibly masculinizes the brain. The estrogen interacts with an estrogen receptor (ER), and the nervous system is thus masculinized through expression of specific target genes (Zoeller et al. 2002). Therefore, tight regulation of steroids and their receptors is very important for specialization of the central nervous system (CNS), including sexual differentiation of the brain, during the perinatal period.

Thyroid hormone also plays an important role in the development of the CNS during the perinatal stage. Congenital hypothyroidism leads to cretinism, an irreversible brain development disorder (Jacobson and Jacobson 1996). It has been suggested that exposure to endocrine disrupting chemicals, such as polychlorinated biphenyl (PCB), can affect brain development in a manner similar to that of decreased thyroid activity (Winneke et al. 2002). In the nervous system, thyroid hormone interacts with TR to alter expression of various genes important for brain development including brain-derived neurotrophic factor (BDNF), neurogranin, and other neurotrophic factors and transfer factors (Iñiguez et al. 1993; Koibuchi et al. 2001; Calzà et al. 1997). BDNF participates in growth, specialization, and survival of nerve cells. Neurogranin is a nervous system-specific calmodulin binding protein, as is a protein kinase II, which regulates phosphorylation and oxidization (Li et al. 2003).

Oberdörster et al. (2004) reported that the CNS can be targeted by airborne solid ultrafine particles and that the most likely mechanism is through deposits on the olfactory mucosa of the nasopharyngeal region of the respiratory tract, and subsequent translocation via the olfactory nerve. Sugamata et al. (2006) have also reported that the brain of newborn mice was affected by maternal DE exposure, and that granular perithelial cells (scavenger cells surrounding cerebral vessels) showed signs of apoptosis. These reports suggest that DEP acts not only through the respiratory organs but also on the brain, and affects the CNS.

Although DE is known to have adverse effects on humans, the detail of how DE influences the cerebrum is not known. To investigate the effects of DE exposure on the development and sexual differentiation of the cerebrum during the perinatal stage, this study examined the expression of steroid hormone-related and thyroid hormone-related genes in the brains of newborn mice exposed to DE. Moreover, expression of mRNA from stress-related genes in the brain was measured to evaluate if the toxic effects of DE had reached the brain.

Materials and methods

Animals

Mice used in the two experiments were derived from pregnant Slc: ICR mice [plug positive, the day of plug was checked as 0 day postcoitum (dpc)], the strain commonly used in DE inhalation experiments. The total number of 100 mice was divided into four groups per experiment and placed in wire cages of separate inhalation chambers at the National Institute for Environmental Studies (NIES) in Tsukuba, Japan. The concentrations of DE that mice were exposed to were as follows: 0.3, 1.0, or 3.0 mg DEP/m^3, for 12 h (10:00 p.m.–10:00 a.m.) per day, 7 days per week. The control group was exposed to clean air in a chamber of the same design. Mice were given free access to a commercial stock diet (CE-2, CLEA Japan Inc., Japan) and water. The number of offspring was regulated to five males and five females at PND-5. Offspring were weaned at PND-21, and then kept in a clean animal room at NIES.

Table 1 Engine system specifications

Engine system	
Type and duty	4JG2-type, light duty
Displacement	3.059 L
Dilution tunnel	
Diameter	300 mm
Length	8,141 cm
Temp.	35–36°C
Dilution rate	2:1 (dilution, air 4 m^3:exhaust gas 2 m^3)
Fuel	
Oil	Standard light oil (Shin-Nippon Sekiyu)
Sulfur contents	<20 ppm

This study was conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, and was approved by the Animal Care and Use Committee of the NIES.

Fetal period exposure group

Forty mice used in the experiment were acquired on the first day of pregnancy, placed in separate inhalation chambers, and exposed to DE from 2 dpc (beginning at 10:00 p.m.) until 16 dpc to evaluate the effects of constant exposure periods in all groups.

Neonatal period exposure group

Sixty mice used in the experiment were acquired as pregnant mice at 14 dpc, and then housed in a clean animal room. Births were checked everyday at 2:00 p.m. Dams and their offspring were transferred to the DE exposure chamber on postnatal day (PND)-0, then from 10:00 p.m. that day, mice were exposed to DE until PND-16 to evaluate the effects of constant exposure periods in all groups.

Generation of diesel exhaust

The mice were exposed to DE at NIES. DE was generated by a 3,059 cc, four-cylinder diesel engine (4JG2-type, Isuzu Motors Limited, Japan) (Table 1). The engine was operated on standard diesel fuel at a speed of 1,500 rpm under a torque of 10 kg m by a feedback control system run by a microcomputer. The engine exhaust was introduced into a dilution tunnel 300 mm in diameter and 8.14 m in length. Therefore, the exhaust was mixed with temperature-controlled clean air. The mean diameter of the particle size was estimated to be 0.3–0.4 μm. The weight of the filters, flow volume, and the measurement time were confirmed, and the concentration of DEP in the chamber was calculated (Table 2). Filter measurements were used for calibration of particle counter. There was a good relationship between the particle counts and filter measurement. Therefore, particle counters were monitored to control the particle concentration (particle mass weight). The diluted exhaust was then delivered directly into the animal exposure chamber (volume 2.25 m^3) at different concentrations.

RNA preparation

On PND-2, -5, and -16, offspring were weighed and placed under deep ether anesthesia (Fujimoto et al. 2005) by the intracardiac method immediately after the exposure period. Mice were decapitated, and the brains dissected and weighed. Brain samples for analysis of gene expression were divided into the cerebrum (containing the hypothalamus) and cerebellum (containing the medulla oblongata), which were each weighed separately. Samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Total RNA from the cerebrum was extracted using ISOGEN (a commercially available solution of phenol and guanidine isothiocyanate, Nippon Gene Co. Ltd., Japan) according to the manufacturer's protocol, and each sample was homogenized in a fixed volume of ISOGEN with an HG 30 homogenizer (Hitachi Ltd., Japan). The concentration and purity of total RNA were determined by measurement of the optical density at 260 and 280 nm on a UV spectrophotometer. Total RNA was diluted to working concentration with double-distilled water (DDW).

DNase treatment and reverse transcription

Total RNA dissolved in 16 μl DDW, 2 μl RQ1 DNase 10× reaction buffer (Promega Corporation, WI, USA), 0.5 μl

Table 2 Exposure chamber components

Component	Control	0.3 mg DEP/m^3	1.0 mg DEP/m^3	3.0 mg DEP/m^3
Particles (mg/m^3)	0.004 ± 0.00002	0.300 ± 0.011	1.000 ± 0.032	2.992 ± 0.048
Gaseous				
NO_2 (ppm)	0.119 ± 0.009	1.339 ± 0.088	4.615 ± 0.357	10.523 ± 0.920
NO (ppm)	0.060 ± 0.006	3.243 ± 0.296	11.748 ± 1.183	27.488 ± 2.004
SO_2 (ppm)	0.024 ± 0.001	0.075 ± 0.003	0.207 ± 0.013	0.391 ± 0.042
CO_2 (ppm)	651.06 ± 65.09	1,880.61 ± 115.07	4,922.25 ± 243.80	9,718.31 ± 556.48
CO (ppm)	0.000 ± 0.000	0.858 ± 0.214	8.585 ± 0.928	24.457 ± 2.316

Data are mean ± SE of components present in the chamber

ppm parts per million

Rnasin RNase inhibitor (40 U/μl, Promega), and 1 μl RQ1 Rnase-free DNase (1 U/μl, Promega) was incubated at 37°C for 30 min. DNase was inactivated by addition of 1 μl RQ1 DNase Stop Solution (Promega) and 22 μl DDW. Total RNA was denatured at 65°C for 10 min and then chilled on ice for 5 min. Reverse transcription of total RNA into cDNA was performed with 2 μl M-MLV reverse transcriptase (Invitrogen Corporation, USA), 20 μl total RNA, 10 μl of random primers (40 ng/ml, Takara Bio Inc., Japan), 20 μl 5× First strand Buffer (Invitrogen), and 25 μl polymerase chain reaction (PCR) nucleotide mix (each dNTP 2 mM, Roche Diagnostics, USA) at 37°C for 60 min. cDNA was stored at −20°C after incubation at 95°C for 5 min.

Quantification of mRNA expression

Quantitative analysis of specific mRNA expression was performed with the ABI PRISM® 7700, CA, USA, sequence detection system, as per the manufacturer's instructions. Primers and TaqMan probes were designed (Primer Express software; Perkin-Elmer) to amplify ER alpha, ER beta, androgen receptor (AR), cytochrome P450 aromatase (aromatase), CYP1A1, heme oxygenase-1 (HO-1), metallothionein-1 (MT-1), thyroid hormone receptor (TR) alpha, BDNF, and neurogranin (Table 3), and were not disclosed by the manufacturer. The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a ubiquitously expressed housekeeping gene, was included as an internal marker of mRNA integrity, and as a control for normalization of mRNA expression. The probes were labeled with 6-carboxy-fluorescein (FAM), and the fluorescence was quenched with 6-carboxy-tetramethyl-rhodamine (TAMRA). PCR amplification was performed in a 96-well optical plate with caps and a 25 μl final reaction mixture consisting of 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA), 2 μM TaqMan fluorescent probe (Applied Biosystems), 3 μM each primer (Espec Oligo Service Corp., Japan), and cDNA. Amplification conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each gene expression was calculated by the mean of each of the duplicated samples. The relative intensities were normalized to an endogenous control gene (GAPDH) and were represented as mean ± standard deviation (SD).

Statistics

All values are expressed as mean ± SD. The statistical significance of differences between exposed groups and the control group was assessed by the Dunnett test. A P value of less than 0.05 was considered significant.

Results

Litter size, body weight (BW), and brain weight

Litter size was not significantly different in comparison with the control group for both exposure groups (Table 4). Tables 5 and 6 show the results for BW and brain weight from males and females.

In the fetal period exposure group, BW in males was not significantly different in the DE exposure group in comparison to the control group, but BW in females was lower at PND-5 in the 0.3 and 3.0 mg DEP/m^3 groups than in the control group ($P < 0.05$ for both). In the neonatal period exposure group, BW was higher in males at PND-5 in the 0.3 mg DEP/m^3 group, males at PND-16 in the 0.3 or 1.0 mg DEP/m^3 group, and females at PND-5 in the 3.0 mg DEP/m^3 group, and at PND-16 in the 0.3 mg DEP/m^3 group than in the respective control groups ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively).

Brain weight in the fetal period exposure group was lower in males at PND-5 in the 0.3 mg DEP/m^3 group ($P < 0.05$) than in the control group. In the neonatal period exposure group, brain weight was lower in females at PND-2 in the 1.0 mg DEP/m^3 group ($P < 0.05$) but higher in males at PND-16 in the 0.3 mg DEP/m^3 group, and females at PND-16 in the 0.3 and 1.0 mg DEP/m^3 groups ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively) than in the respective control groups.

MRNA expression in the brain of offspring (fetal exposure)

To investigate the effect of fetal DE exposure on expression of genes related to brain development and differentiation, the offspring of pregnant mice exposed to DE from 2 to 16 dpc were studied. Tables 7 and 8 show the changes in mRNA expression from the fetal period exposure in males and females.

Expression level of ER alpha mRNA was significantly higher in males on PND-5 in the 3.0 mg DEP/m^3 group ($P < 0.01$) than in the control group. ER alpha mRNA level was significantly higher in females on PND-2 in the 1.0 and 3.0 mg DEP/m^3 groups, and on PND-16 in the 0.3 and 1.0 mg DEP/m^3 groups ($P < 0.001$, $P < 0.001$, $P < 0.05$, and $P < 0.05$, respectively) than in the respective control groups.

ER beta mRNA level was significantly higher in males on PND-5 in the 1.0 mg DEP/m^3 group ($P < 0.05$), and in females on PND-2 in the 1.0 mg DEP/m^3 group ($P < 0.05$) than in the respective control groups.

AR mRNA level was significantly lower in females on PND-5 in the 0.3 mg DEP/m^3 group ($P < 0.05$) than in the control group, but there was no significant difference in expression level in males. Aromatase mRNA level was significantly higher in males on PND-5 in the 3.0 mg DEP/m^3

Table 3 Sequence of PCR primers for GAPDH and endocrine-related genes

Gene	Sequence (5′–3′)	Length (mer)
GAPDH		
Forward primer	TGCACCACCAACTGCTTAG	19
Reverse primer	GGATGCAGGGATGATGTTC	19
Probe	CAGAAGACTGTGGATGGCCCCTC	23
ER alpha		
Forward primer	CCAGCAGTAACGAGAAAGGAAAC	23
Reverse primer	TCATTGCACACGGCACAGTA	20
Probe	TGATCATGGAGTCTGCCAAGGAGACTCG	28
ER beta		
Forward primer	AGTCCGCCTCTTGGAAAGCT	20
Reverse primer	ACTTCCCCTCATCCCTGTCC	20
Probe	CCCACCATCAGCACCTCCATCCA	23
AR		
Forward primer	CCAGATGGCGGTCATTCAGT	20
Reverse primer	GAAGGACCGCCAACCCA	17
Probe	TTCCTGGATGGGACTGATGGTATTTGCC	28
Aromatase		
Forward primer	CTGTACTTCATGTTACTTCTCGTCGC	26
Reverse primer	TCGATCTTTATGTCTCTGTCACCC	24
Probe	ATCCAGAGGTCGAAGCAGCAATCCTG	28
CYP1A1		
Forward primer	CATTCATCCTTCGTCCCCTTC	21
Reverse primer	CACTGGTTCACAAAGACACAGCA	23
Probe	CCCACAGCACCACAAGAGATACAAGTCTG	29
HO-1		
Forward primer	GGTGATGGAGCGTCCACAG	19
Reverse primer	TGGTGGCCTCCTTCAAGG	18
Probe	CGACAGCATGCCCCAGGATTTGTC	24
MT-1		
Forward primer	CTCCACCGGCGGCTC	15
Reverse primer	CGGCGCCTTTGCAGAC	16
Probe	CTGCTCCAAATGTGCCCAGGGCT	24
TR alpha		
Forward primer	GGCAGCCATTGGAAACAGA	19
Reverse primer	CCGGCATGGAGACAATAGGT	20
Probe	AATTCCTGCCGGATGACATTGGCC	24
BDNF		
Forward primer	CGTGACAACAATGTGACTCCACT	23
Reverse primer	TGCAACCGAAGTATGAAATAACCA	24
Probe	TCAGGGTCCACACAAAGCTCTCGGAT	26
Neurogranin		
Forward primer	GACCCTCAACACCGGCAAT	19
Reverse primer	AATATCGTCGTCTGGCTTGGA	21
Probe	AGGCGCTCTCCGTGCAGCAGTC	22

GAPDH glyceraldehyde-3-phosphate dehydrogenase, *ER* estrogen receptor, *AR* androgen receptor, *CYP1A1* cytochrome P450 1A1, *HO-1* heme oxygenase-1, *MT-1* metallothionein-1, *TR* thyroid hormone receptor, *BDNF* brain-derived neurotrophic factor

group ($P < 0.05$), and in females on PND-2 in the 3.0 mg DEP/m^3 group ($P < 0.001$) than in the respective control group.

TR alpha mRNA level was significantly higher in males on PND-5 in the 0.3 and 1.0 mg DEP/m^3 groups ($P < 0.05$ for both) and in females on PND-2 in the

Table 4 Litter size of dams at birth in each group

Group	*n*	Control	*n*	0.3 mg DEP/m^3	*n*	1.0 mg DEP/m^3	*n*	3.0 mg DEP/m^3
Fetal exposure	8	11.8 ± 2.2	8	11.1 ± 4.9	6	12.2 ± 2.1	5	12.2 ± 1.9
Neonatal exposure	15	13.7 ± 2.9	15	13.3 ± 3.1	15	14.5 ± 2.3	15	13.1 ± 2.4

Data are mean ± SD

Table 5 BW of mice exposed to diesel exhaust during perinatal development

	Control	0.3 mg DEP/m^3	1.0 mg DEP/m^3	3.0 mg DEP/m^3
Fetal exposure				
Male, *n*	11–14	11–13	12–14	11–13
PND-2	3.06 ± 1.06	3.04 ± 0.74	3.27 ± 1.23	2.97 ± 0.89
PND-5	4.10 ± 0.42	3.70 ± 0.59	4.07 ± 0.71	4.18 ± 1.50
PND-16	8.95 ± 3.18	10.17 ± 1.19	8.79 ± 1.72	8.55 ± 2.02
Female, *n*	10–12	11–13	12–14	11–13
PND-2	2.32 ± 0.15	2.24 ± 0.28	2.38 ± 0.36	2.20 ± 0.16
PND-5	4.17 ± 0.45	3.48 ± 0.57*	3.87 ± 0.82	3.49 ± 0.45*
PND-16	9.25 ± 2.29	9.91 ± 1.29	9.07 ± 1.82	8.17 ± 1.67
Neonatal exposure				
Male, *n*	15–16	14–16	14–16	16
PND-2	2.36 ± 0.27	2.44 ± 0.26	2.27 ± 0.31	2.37 ± 0.20
PND-5	3.76 ± 0.53	3.88 ± 0.66*	3.64 ± 0.52	3.72 ± 0.35
PND-16	7.67 ± 1.26	9.58 ± 2.08**	9.43 ± 1.19**	8.52 ± 1.55
Female, *n*	15–17	14–17	13–16	16–17
PND-2	2.35 ± 0.21	2.30 ± 0.25	2.19 ± 0.31	2.21 ± 0.19
PND-5	3.34 ± 0.54	3.77 ± 0.63	3.53 ± 0.49	3.47 ± 0.31*
PND-16	8.06 ± 1.23	9.60 ± 2.02*	9.34 ± 1.32	8.94 ± 1.54

Data (g) are mean ± SD
BW body weight, *PND* postnatal day
* $P < 0.05$, ** $P < 0.01$ vs. control by Dunnett test

Table 6 Brain weight of offspring exposed to diesel exhaust during the perinatal period

	Control	0.3 mg DEP/m^3	1.0 mg DEP/m^3	3.0 mg DEP/m^3
Fetal exposure				
Male, *n*	11–14	11–13	12–14	11–13
PND-2	173.7 ± 51.5	166.9 ± 37.8	179.3 ± 50.4	161.0 ± 40.2
PND-5	222.6 ± 15.1	202.2 ± 22.6*	223.3 ± 22.9	208.5 ± 18.8
PND-16	400.4 ± 45.2	421.1 ± 27.8	400.6 ± 32.7	403.5 ± 25.3
Female, *n*	10–12	11–13	12–14	11–13
PND-2	128.6 ± 8.2	123.9 ± 13.3	132.2 ± 11.7	126.1 ± 6.9
PND-5	217.9 ± 20.3	200.4 ± 18.5	212.0 ± 25.7	207.5 ± 14.2
PND-16	410.2 ± 26.7	408.2 ± 16.9	402.0 ± 19.9	390.4 ± 24.5
Neonatal exposure				
Male, *n*	15–16	14–16	14–16	16
PND-2	137.5 ± 12.8	135.5 ± 10.0	134.3 ± 10.9	138.0 ± 6.9
PND-5	215.9 ± 16.9	207.1 ± 17.6	210.7 ± 16.3	217.3 ± 13.7
PND-16	398.6 ± 20.6	424.0 ± 22.8**	417.4 ± 18.0	409.3 ± 23.3
Female, *n*	15–17	14–17	13–16	16–17
PND-2	136.6 ± 8.1	132.8 ± 9.7	128.2 ± 9.1*	133.0 ± 7.6
PND-5	205.6 ± 17.6	210.6 ± 18.9	212.0 ± 25.2	208.3 ± 12.3
PND-16	390.9 ± 28.0	414.4 ± 24.0*	418.4 ± 17.8**	408.1 ± 23.8

Data (mg) are mean ± SD
PND postnatal day
* $P < 0.05$, ** $P < 0.01$ vs. control by Dunnett test

Table 7 Effect of fetal exposure to diesel exhaust on mRNA expression in male mice

	Control	0.3 mg DEP/m^3 (% of control)	1.0 mg DEP/m^3 (% of control)	3.0 mg DEP/m^3 (% of control)
PND-2				
Stress-related genes				
CYP1A1	344.7 ± 94.5	344.0 ± 72.1 (99.8)	540.6 ± 112.9* (156.8)	442.9 ± 155.0 (128.5)
HO-1	8,265.0 ± 1,462.3	8,763.3 ± 362.1 (106.0)	7,302.0 ± 633.5 (88.3)	7,746.0 ± 487.8 (93.7)
MT-1	16,381.5 ± 5,721.3	22,677.0 ± 3,962.0 (138.4)	22,382.5 ± 1,363.6 (136.6)	29,635.8 ± 2,966.4*** (180.9)
Steroid hormone-related genes				
ER alpha	2,470.8 ± 212.8	2,431.5 ± 394.4 (98.4)	2,411.6 ± 376.5 (97.6)	2,601.9 ± 140.2 (105.3)
ER beta	113.6 ± 42.1	133.6 ± 40.2 (117.6)	170.4 ± 107.0 (150.0)	160.6 ± 29.7 (141.4)
AR	934.2 ± 138.4	885.2 ± 180.9 (94.8)	1,004.8 ± 172.7 (107.6)	1,033.8 ± 74.3 (110.7)
Aromatase	263.3 ± 58.0	254.1 ± 63.7 (96.5)	303.0 ± 17.7 (115.1)	263.7 ± 60.0 (100.1)
Thyroid hormone-related genes				
TR	823,346.2 ± 109,306.6	913,060.2 ± 70,529.2 (110.9)	852,494.8 ± 46,700.4 (103.5)	884,287.0 ± 62,586.6 (107.4)
BDNF	485.1 ± 108.1	465.1 ± 78.5 (95.9)	511.7 ± 40.1 (105.5)	542.9 ± 89.6 (111.9)
Neurogranin	414,853.5 ± 88,432.4	448,480.6 ± 24,465.7 (108.1)	481,988.5 ± 95,225.0 (116.2)	397,786.0 ± 61,276.7 (95.9)
PND-5				
Stress-related genes				
CYP1A1	681.1 ± 203.2	692.6 ± 187.8 (101.7)	796.3 ± 131.7 (116.9)	659.5 ± 384.2 (96.8)
HO-1	16,726.3 ± 2,229.9	19,084.8 ± 1,198.9 (114.1)	18,822.6 ± 2,208.7 (112.5)	16,230.2 ± 619.9 (97.0)
MT-1	65,275.5 ± 13,139.1	70,320.6 ± 8,613.0 (107.7)	71,611.1 ± 17,441.5 (109.7)	54,590.8 ± 2,940.0 (83.6)
Steroid hormone-related genes				
ER alpha	1,095.8 ± 193.5	1,193.6 ± 152.9 (108.9)	1,216.2 ± 182.9 (111.0)	1,550.5 ± 160.8** (141.5)
ER beta	224.4 ± 54.8	245.5 ± 49.8 (109.4)	330.3 ± 88.8* (147.2)	316.4 ± 42.1 (141.0)
AR	1,475.3 ± 168.3	1,403.1 ± 278.7 (95.1)	1,450.2 ± 150.6 (98.3)	1,588.7 ± 90.6 (107.7)
Aromatase	177.6 ± 14.0	189.5 ± 20.2 (106.7)	193.3 ± 27.2 (108.8)	241.4 ± 59.2* (135.9)
Thyroid hormone-related genes				
TR	793,220.3 ± 82,645.1	954,284.7 ± 44,844.1* (120.3)	905,240.8 ± 153,561.3* (114.1)	939,081.0 ± 72,187.1 (118.4)
BDNF	723.2 ± 110.9	750.0 ± 97.6 (103.7)	794.7 ± 104.1 (109.9)	751.6 ± 68.3 (103.9)
Neurogranin	717,516.6 ± 48,708.4	625,142.2 ± 96,993.5 (87.1)	708,550.8 ± 81,215.7 (98.8)	755,930.3 ± 86,692.2 (105.4)
PND-16				
Stress-related genes				
CYP1A1	122.4 ± 26.8	130.5 ± 26.9 (106.6)	100.9 ± 37.7 (82.4)	133.3 ± 51.4 (108.9)
HO-1	6,952.1 ± 749.5	7,619.8 ± 1,142.0 (109.6)	8,529.6 ± 1,164.8 (122.7)	8,609.9 ± 1,036.6 (123.8)
MT-1	255,778.3 ± 81,960.8	136,123.0 ± 32,776.6* (53.2)	184,818.9 ± 69,828.6 (72.3)	159,244.5 ± 43,695.8 (62.3)
Steroid hormone-related genes				
ER alpha	663.6 ± 81.4	586.1 ± 82.4 (88.3)	717.1 ± 67.5 (108.1)	594.8 ± 101.9 (89.6)
ER beta	101.9 ± 22.2	102.1 ± 21.7 (100.2)	116.3 ± 32.0 (114.2)	119.2 ± 21.7 (117.0)
AR	4,292.0 ± 434.3	4,369.7 ± 626.9 (101.8)	4,670.1 ± 402.6 (108.8)	4,319.4 ± 352.2 (100.6)
Aromatase	95.2 ± 14.6	83.9 ± 7.1 (88.1)	103.3 ± 13.9 (108.5)	94.0 ± 17.2 (98.7)
Thyroid hormone-related genes				
TR	441,183.8 ± 100,455.7	407,878.9 ± 17,390.7 (92.5)	450,240.0 ± 32,763.8 (102.1)	540,065.7 ± 119,801.4 (122.4)
BDNF	1,441.4 ± 204.4	1,460.9 ± 151.9 (101.4)	1,579.5 ± 233.2 (109.6)	1,506.3 ± 236.2 (104.5)
Neurogranin	1,230,501.6 ± 111,725.3	1,323,928.6 ± 45,117.1 (95.9)	1,459,844.3 ± 164,212.7 (118.6)	1,342,119.9 ± 160,356.4 (109.1)

Data ($\times 10^6$) show the ratio of target mRNA expression to GAPDH mRNA expression (% of control), and are mean ± SD for 4–6 mice per group

PND postnatal day

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control by Dunnett test

Table 8 Effect of fetal exposure to diesel exhaust on mRNA expression in female mice

	Control	0.3 mg DEP/m^3 (% of control)	1.0 mg DEP/m^3 (% of control)	3.0 mg DEP/m^3 (% of control)
PND-2				
Stress-related genes				
CYP1A1	219.0 ± 38.8	232.8 ± 49.7 (106.3)	261.7 ± 41.9 (119.5)	418.9 ± 144.9** (191.3)
HO-1	15,738.1 ± 1,977.2	17,877.2 ± 2,504.4 (113.6)	17,542.2 ± 1,599.2 (111.5)	16,412.2 ± 542.7 (104.3)
MT-1	31,028.8 ± 7,652.4	39,881.3 ± 17,679.5 (128.5)	30,451.5 ± 4,241.1 (98.1)	30,904.9 ± 9,383.9 (99.6)
Steroid hormone-related genes				
ER alpha	3,898.3 ± 397.4	4,484.7 ± 820.6 (115.0)	6,116.5 ± 353.2*** (156.9)	6,317.7 ± 538.7*** (162.1)
ER beta	338.3 ± 51.7	366.5 ± 104.1 (108.3)	526.2 ± 74.4* (155.5)	400.8 ± 86.7 (118.5)
AR	1,074.0 ± 191.0	853.3 ± 124.6 (79.5)	1,115.0 ± 226.5 (103.8)	1,172.7 ± 115.3 (109.2)
Aromatase	271.2 ± 26.2	283.4 ± 49.9 (104.5)	326.6 ± 46.5 (120.4)	382.5 ± 20.0*** (141.0)
Thyroid hormone-related genes				
TR	804,699.2 ± 86,465.6	815,917.1 ± 80,711.9 (101.4)	870,431.8 ± 107,996.5 (108.2)	975,733.1 ± 108,108.7* (121.3)
BDNF	588.1 ± 88.3	510.2 ± 75.9 (86.7)	663.7 ± 70.1 (112.9)	558.3 ± 110.5 (94.9)
Neurogranin	409,322.4 ± 43,936.1	327,992.6 ± 73,619.1* (80.1)	404,171.2 ± 39,637.2 (98.7)	412,240.9 ± 23,310.1 (100.7)
PND-5				
Stress-related genes				
CYP1A1	789.2 ± 186.6	759.0 ± 209.8 (96.2)	817.0 ± 132.5 (103.5)	700.5 ± 162.4 (88.8)
HO-1	15,069.1 ± 1,773.4	18,273.2 ± 3,314.8 (121.3)	21,505.9 ± 2,470.5** (142.7)	19,051.7 ± 2,032.2 (126.4)
MT-1	64,761.9 ± 10,567.3	74,465.8 ± 20,783.7 (115.0)	68,817.1 ± 11,263.5 (106.3)	48,374.7 ± 7,004.8 (74.7)
Steroid hormone-related genes				
ER alpha	1,916.8 ± 79.0	1,484.1 ± 451.1 (77.4)	1,506.0 ± 259.3 (78.6)	1,628.9 ± 156.0 (85.0)
ER beta	342.8 ± 65.5	351.4 ± 137.7 (102.5)	398.6 ± 61.7 (116.3)	432.1 ± 73.4 (126.0)
AR	2,415.9 ± 576.1	1,388.8 ± 509.7* (57.5)	2,113.3 ± 191.8 (87.5)	2,224.0 ± 576.0 (92.1)
Aromatase	196.4 ± 37.5	175.9 ± 83.4 (89.6)	235.5 ± 61.0 (119.9)	216.4 ± 32.5 (110.2)
Thyroid hormone-related genes				
TR	932,094.7 ± 65,248.6	998,970.2 ± 56,753.5 (107.2)	935,784.2 ± 89,997.2 (100.4)	1,010,202.0 ± 89,313.6 (108.4)
BDNF	869.8 ± 80.8	567.7 ± 175.7 (65.3)	819.1 ± 99.5 (94.2)	812.6 ± 90.2 (93.4)
Neurogranin	1,061,606.8 ± 112,803.0	765,232.9 ± 57,230.8*** (72.1)	907,460.0 ± 49,564.2* (85.5)	920,943.8 ± 85,226.5* (86.7)
PND-16				
Stress-related genes				
CYP1A1	79.2 ± 25.4	125.4 ± 64.4 (158.3)	121.4 ± 24.6 (153.3)	96.2 ± 31.3 (121.5)
HO-1	8,102.0 ± 1,000.5	8,893.0 ± 698.1 (109.8)	9,262.7 ± 1,316.4 (114.3)	8,776.5 ± 917.5 (108.3)
MT-1	279,368.6 ± 35,986.8	321,502.7 ± 105,701.5 (115.1)	332,180.7 ± 220,793.2 (118.9)	218,320.4 ± 19,590.9 (78.1)
Steroid hormone-related genes				
ER alpha	624.2 ± 31.7	753.6 ± 81.1* (120.7)	771.6 ± 67.4* (123.6)	675.0 ± 88.3 (108.1)
ER beta	125.7 ± 13.5	139.8 ± 19.0 (111.1)	147.2 ± 35.7 (117.1)	180.8 ± 33.1 (143.8)
AR	4,458.4 ± 250.1	4,900.1 ± 906.6 (109.9)	4,764.0 ± 700.2 (106.9)	4,966.5 ± 381.0 (111.4)
Aromatase	82.0 ± 15.4	88.8 ± 16.1 (108.3)	91.4 ± 18.2 (111.5)	99.5 ± 4.9 (121.4)
Thyroid hormone-related genes				
TR	403,229.1 ± 63,534.6	473,411.2 ± 70,557.5 (117.4)	443,068.4 ± 40,105.0 (109.9)	401,998.8 ± 16,478.9 (99.7)
BDNF	1,201.2 ± 199.9	1,596.1 ± 250.8 (132.9)	1,453.4 ± 281.9 (121.0)	1,529.4 ± 261.2 (127.3)
Neurogranin	1,077,675.1 ± 86,493.6	1,154,996.6 ± 112,244.4 (107.2)	1,154,830.5 ± 100,892.3 (107.2)	1,305,482.0 ± 489,353.2 (121.1)

Data ($\times 10^6$) show the ratio of target mRNA expression to GAPDH mRNA expression (% of control), and are mean ± SD for 4–6 mice per group

PND postnatal day

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control by Dunnett test

3.0 mg DEP/m^3 group ($P < 0.05$) than in the control group.

BDNF mRNA level did not exhibit a significant difference in expression between males and females, but in females there was a tendency toward decreased expression on PND-5 in DE exposed groups. Neurogranin mRNA level in males was not significantly different, however, in females was significantly lower on PND-2 in the 0.3 mg DEP/m^3 group, and on PND-5 in the 0.3, 1.0 and 3.0 mg DEP/m^3 groups ($P < 0.05$, $P < 0.001$, $P < 0.05$, and $P < 0.05$, respectively) than in the respective control groups.

CYP1A1 mRNA level was significantly higher in males on PND-2 in the 1.0 mg DEP/m^3 group ($P < 0.05$), and in females on PND-2 in the 3.0 mg DEP/m^3 group ($P < 0.01$) than in the respective control group.

HO-1 mRNA level in males did not differ significantly between groups, but in females was significantly higher on PND-5 in the 0.3 mg DEP/m^3 group ($P < 0.01$) than in the control group.

MT-1 mRNA level was significantly higher in males on PND-2 in the 3.0 mg DEP/m^3 group ($P < 0.001$), and significantly lower on PND-16 in the 0.3 mg DEP/m^3 group ($P < 0.05$) than in the control group. There was no significant difference in the expression of MT-1 in females.

MRNA expression in the brain of offspring (neonatal exposure)

Tables 9 and 10 show the changes in mRNA expression in the neonatal period exposure for males and females.

Expression level of ER alpha mRNA was significantly higher in males on PND-2 in the 3.0 mg DEP/m^3 group ($P < 0.05$), and in females on PND-2 in the 3.0 mg DEP/m^3 group, on PND-5 in the 0.3 mg DEP/m^3 group, and on PND-16 in the 1.0 mg DEP/m^3 group ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) than in the respective control groups.

ER beta mRNA level was significantly higher in males on PND-2 in the 3.0 mg DEP/m^3 group ($P < 0.05$) than in the control group. There was no significant difference in expression level of ER beta mRNA level in females exposed to DE. There was no significant difference in expression level of AR or aromatase mRNA in either males or females exposed to DE. There was no significant difference in expression level of TR alpha or BDNF mRNA in males exposed to DE. TR alpha mRNA level was significantly lower in females on PND-5 in the 1.0 mg DEP/m^3 group ($P < 0.001$), however, BDNF mRNA level was significantly higher on PND-2 in the 1.0 mg DEP/m^3 group ($P < 0.01$) than in the respective control groups.

Neurogranin mRNA level was significantly higher in males on PND-16 in the 1.0 mg DEP/m^3 group ($P < 0.05$) and in females on PND-16 in the 0.3, 1.0, and 3.0 mg DEP/m^3 groups ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) than in the respective control groups.

CYP1A1 mRNA level was significantly higher in males on PND-5 in the 1.0 mg DEP/m^3 group ($P < 0.05$), and in females on PND-5 in the 3.0 mg DEP/m^3 group ($P < 0.05$) than in the respective control groups.

HO-1 mRNA level was significantly higher in males on PND-2 in the 0.3 and 3.0 mg DEP/m^3 groups, on PND-5 in the 1.0 and 3.0 mg DEP/m^3 groups, and on PND-16 in the 3.0 mg DEP/m^3 group ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) than in the respective control groups. HO-1 mRNA level was slightly increased in the other exposure groups as compared with the control group. A significant difference in expression was not observed for HO-1 mRNA level in females.

MT-1 mRNA level was significantly lower in males on PND-5 in the 3.0 mg DEP/m^3 group ($P < 0.05$), and significantly lower in females on PND-2 in the 1.0 and 3.0 mg DEP/m^3 groups ($P < 0.05$ and $P < 0.01$, respectively) than in the respective control groups.

Discussion

In the present study, the effect of fetal and neonatal DE exposure on development of the cerebrum and expression of brain-related genes was examined in newborn mice. These results could indicate a novel finding that perinatal exposure to DE during the critical period of sexual differentiation of the brain may affect endocrine function. There are various chemical substances in DE that may affect growth, and it is thought that different concentrations of DE may have different effects or different components. DE had been reported to contain substances with estrogenic, anti-estrogenic, and anti-androgenic activities (Furuta et al. 2004; Mori et al. 2002; Taneda et al. 2002; Kizu et al. 2003). The pro- and anti-steroidogenic effects were examined through analysis of the expression of steroid hormone-related genes.

Neonatal DE exposure tended to increase BW of newborns, whereas fetal exposure tended to decrease BW. The perinatal period is a time of remarkable growth, and it is possible that some of the components present in DE directly or indirectly affect BW for the exposure periods tested. There are many reports concerning this change in BW (Tsukue et al. 2002; Watanabe and Kurita 2001; Watanabe and Oonuki 1999), but the experimental results are not consistent. Watanabe and Kurita (2001) reported that females were exposed to DE from day 7 (day of impregnation = day 0) until day 20 of pregnancy, after which the mean of BW was greater in exhaust-exposed female fetuses than in that of the control group. However, they did not refer what substances in DE caused the effects.

Table 9 Effect of neonatal exposure to diesel exhaust on mRNA expression in male mice

	Control	0.3 mg DEP/m^3 (% of control)	1.0 mg DEP/m^3 (% of control)	3.0 mg DEP/m^3 (% of control)
PND-2				
Stress-related genes				
CYP1A1	201.0 ± 36.7	223.3 ± 26.9 (111.1)	199.4 ± 24.5 (99.2)	243.0 ± 59.9 (120.9)
HO-1	6,412.6 ± 853.6	7,851.1 ± 213.3** (122.4)	7,009.2 ± 680.4 (109.3)	8,399.3 ± 564.5*** (131.0)
MT-1	15,344.5 ± 3,921.0	19,087.7 ± 2,077.7 (124.4)	18,553.6 ± 4,768.6 (120.9)	19,641.2 ± 1,735.8 (128.0)
Steroid hormone-related genes				
ER alpha	2,649.2 ± 314.4	2,754.2 ± 387.8 (104.0)	3,074.0 ± 206.9 (116.0)	3,212.2 ± 334.2* (121.3)
ER beta	92.5 ± 28.1	96.2 ±24.3 (104.0)	118.9 ± 35.9 (128.5)	140.2±25.8*(151.5)
AR	1,058.4 ± 103.5	1,014.5 ± 186.8 (95.9)	1,078.7 ± 85.0 (101.9)	1,101.1 ± 195.0 (104.0)
Aromatase	235.7 ± 20.8	258.8 ± 45.7 (109.8)	278.8 ± 21.3 (118.3)	282.0 ± 48.3 (119.7)
Thyroid hormone-related genes				
TR	836,854.2 ± 106,794.7	936,360.1 ± 32,631.6 (111.9)	903,715.8 ± 66,427.0 (108.0)	941,379.8 ± 66,428.9 (112.5)
BDNF	548.0 ± 80.0	551.1 ± 55.6 (100.6)	582.0 ± 49.7 (106.2)	651.5 ± 112.5 (118.9)
Neurogranin	420,505.0 ± 52,879.3	423,555.3 ± 53,251.3 (100.7)	412,312.6 ± 37,258.5 (98.1)	463,679.5 ± 39,063.7 (110.3)
PND-5				
Stress-related genes				
CYP1A1	530.5 ± 38.4	476.2 ± 112.7 (89.8)	662.2 ± 81.6* (124.8)	542.9 ± 23.1 (102.3)
HO-1	6,066.8 ± 1,174.2	6,674.4 ± 559.4 (110.0)	8,165.5 ± 694.2** (134.6)	7,684.8 ± 773.2* (126.7)
MT-1	33,911.6 ± 6,136.5	27,305.4 ± 3,545.1 (80.5)	30,266.2 ± 6,126.9 (89.3)	23,274.3 ± 6,082.1* (68.6)
Steroid hormone-related genes				
ER alpha	2,656.2 ± 544.9	3,046.5 ± 331.8 (114.7)	2,872.6 ± 362.4 (108.1)	3,288.5 ± 466.2 (123.8)
ER beta	136.1 ± 27.1	105.0 ± 30.3 (77.1)	129.5 ± 12.0 (95.1)	138.5 ± 46.2 (101.8)
AR	1,417.2 ± 400.9	1,331.9 ± 125.6 (94.0)	1,472.3 ± 194.3 (104.0)	1,572.9 ± 208.5 (111.1)
Aromatase	136.1 ± 27.1	105.0 ± 30.3 (77.1)	129.5 ± 12.0 (95.1)	138.5 ± 46.2 (101.8)
Thyroid hormone-related genes				
TR	762,454.8 ± 145,996.5	717,414.9 ± 62,281.6 (94.1)	722,014.2 ± 98,438.0 (94.7)	718,272.8 ± 71,306.1 (94.2)
BDNF	788.3 ± 145.8	796.4 ± 56.0 (101.0)	862.3 ± 165.8 (109.4)	834.9 ± 107.6 (105.9)
Neurogranin	622,640.6 ± 103,411.8	644,352.5 ± 30,584.7 (103.5)	632,066.8 ± 41,476.1 (101.5)	638,372.6 ± 50,480.3 (102.5)
PND-16				
Stress-related genes				
CYP1A1	40.1 ± 5.4	31.9 ± 10.9 (79.6)	73.0 ± 43.6 (182.1)	52.8 ± 23.0 (131.7)
HO-1	4,748.5 ± 282.8	5,273.4 ± 448.4 (111.1)	5,411.3 ± 523.8 (114.0)	5,684.0 ± 509.8* (119.7)
MT-1	84,304.5 ± 34,977.1	112,003.7 ± 18,225.5 (132.9)	63,848.7 ± 12,811.1 (75.7)	78,326.6 ± 16,552.9 (92.9)
Steroid hormone-related genes				
ER alpha	1,742.6 ± 159.1	1,794.5 ± 249.3 (103.0)	2,106.6 ± 337.3 (120.9)	1,947.1 ± 109.7 (111.7)
ER beta	56.2 ± 9.9	45.6 ± 6.4 (81.0)	73.8 ± 15.0 (131.3)	72.9 ± 11.6 (129.7)
AR	4,898.2 ± 1,268.3	4,900.4 ± 835.5 (100.0)	5,015.7 ± 1,110.2 (102.4)	4,994.5 ± 573.2 (102.0)
Aromatase	84.3 ± 19.5	87.2 ± 18.1 (103.5)	99.9 ± 25.7 (118.6)	83.6 ± 12.2 (99.2)
Thyroid hormone-related genes				
TR	356,789.0 ± 41,626.1	378,557.2 ± 44,897.8 (106.1)	419,573.1 ± 55,096.4 (117.6)	400,165.2 ± 13,577.8 (112.2)
BDNF	2,209.8 ± 262.0	2,349.8 ± 209.7 (106.3)	2,400.5 ± 275.1 (108.6)	2,178.5 ± 197.4 (98.6)
Neurogranin	1,002,279.9 ± 79,492.1	1,136,901.4 ± 120,942.9 (113.4)	1,189,367.8 ± 94,646.2* (118.7)	1,107,322.1 ± 47,042.1 (110.5)

Data ($\times 10^6$) show the ratio of target mRNA expression to GAPDH mRNA expression (% of control), and are mean ± SD for five mice per group

PND postnatal day

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control by Dunnett test

Table 10 Effect of neonatal exposure to diesel exhaust on mRNA expression in female mice

	Control	0.3 mg DEP/m^3 (% of control)	1.0 mg DEP/m^3 (% of control)	3.0 mg DEP/m^3 (% of control)
PND-2				
Stress-related genes				
CYP1A1	238.9 ± 31.2	267.3 ± 27.5 (111.9)	285.9 ± 43.1 (119.7)	295.8 ± 55.9 (123.8)
HO-1	9,790.1 ± 862.5	10,003.6 ± 618.5 (102.2)	11,938.6 ± 3,081.5 (121.9)	10,592.0 ± 827.7 (108.2)
MT-1	29,474.6 ± 3,070.7	31,822.8 ± 3,774.9 (108.0)	23,058.6 ± 3,203.4* (78.2)	20,016.0 ± 3,530.5** (67.9)
Steroid hormone-related genes				
ER alpha	3,661.3 ± 180.2	3,659.0 ± 443.1 (99.9)	4,310.0 ± 565.6 (117.7)	4,540.4 ± 487.7* (124.0)
ER beta	106.6 ± 6.8	153.5 ± 37.4 (144.0)	136.8 ± 43.8 (128.3)	145.1 ± 21.1 (136.1)
AR	842.5 ± 180.9	769.1 ± 110.4 (91.3)	906.4 ± 260.6 (107.6)	822.7 ± 117.7 (97.6)
Aromatase	162.5 ± 29.2	167.1 ± 13.7 (102.9)	187.3 ± 29.2 (115.3)	161.9 ± 22.3 (99.6)
Thyroid hormone-related genes				
TR	814,022.9 ± 76,514.1	838,041.0 ± 29,636.3 (103.0)	976,755.5 ± 48,610.8 (120.0)	873,666.9 ± 50,611.9 (107.3)
BDNF	492.1 ± 67.2	524.7 ± 36.0 (106.6)	636.6 ± 44.0** (129.4)	527.2 ± 101.1 (107.1)
Neurogranin	431,984.3 ± 53,175.1	377,812.3 ± 65,690.1 (87.5)	417,247.4 ± 57,871.6 (96.6)	389,945.0 ± 24,657.7 (90.3)
PND-5				
Stress-related genes				
CYP1A1	223.2 ± 43.9	205.6 ± 7.5 (92.1)	302.5 ± 47.4 (135.6)	320.6 ± 99.5* (143.7)
HO-1	8,114.0 ± 778.2	8,426.2 ± 861.5 (103.8)	8,331.8 ± 1,368.3 (102.7)	9,609.2 ± 1,693.6 (118.4)
MT-1	20,238.4 ± 3,697.1	18,305.7 ± 2,274.8 (90.5)	16,740.7 ± 3,000.7 (82.7)	16,476.7 ± 2,999.1 (81.4)
Steroid hormone-related genes				
ER alpha	3,705.2 ± 483.1	5,331.2 ± 865.1** (143.9)	4,778.8 ± 772.3 (129.0)	4,708.4 ± 678.5 (127.1)
ER beta	130.8 ± 39.5	152.3 ± 53.4 (116.4)	143.6 ± 44.2 (109.8)	117.4 ± 44.1 (89.7)
AR	1,624.4 ± 65.1	1,918.6 ± 246.1 (118.1)	1,748.4 ± 273.0 (107.6)	1,955.5 ± 279.8 (120.4)
Aromatase	221.4 ± 19.3	246.4 ± 26.8 (111.3)	226.2 ± 33.4 (102.2)	210.0 ± 39.1 (94.8)
Thyroid hormone-related genes				
TR	864,256.1 ± 55,160.5	858,326.6 ± 88,580.9 (99.3)	843,896.1 ± 89,134.4*** (97.6)	826,926.5 ± 104,919.4 (95.7)
BDNF	970.9 ± 146.2	961.2 ± 123.5 (99.0)	989.2 ± 181.1 (101.9)	962.5 ± 62.6 (99.1)
Neurogranin	707,658.7 ± 81,254.2	793,518.8 ± 111,009.3 (112.1)	730,270.3 ± 104,866.6 (103.2)	767,827.7 ± 37,822.2 (108.5)
PND-16				
Stress-related genes				
CYP1A1	33.4 ± 11.8	28.2 ± 8.4 (84.5)	45.7 ± 15.2 (136.8)	32.1 ± 6.4 (96.2)
HO-1	3,634.8 ± 429.2	3,752.2 ± 230.3 (103.2)	3,806.8 ± 437.8 (104.7)	3,990.2 ± 294.9 (109.8)
MT-1	78,014.4 ± 27,395.2	85,966.9 ± 26,016.4 (110.2)	82,023.7 ± 12,013.7 (105.2)	71,978.7 ± 25,834.5 (92.3)
Steroid hormone-related genes				
ER alpha	1,595.5 ± 73.0	1,773.3 ± 179.8 (111.1)	2,062.6 ± 258.1** (129.3)	1,839.9 ± 186.9 (115.3)
ER beta	60.8 ± 8.3	60.5 ± 8.3 (99.5)	67.2 ± 7.1 (110.4)	71.5 ± 22.2 (117.5)
AR	4,362.4 ± 352.5	4,650.4 ± 853.0 (106.6)	5,488.6 ± 1,100.3 (125.8)	5,457.9 ± 661.6 (125.1)
Aromatase	87.4 ± 16.3	101.9 ± 18.0 (116.7)	105.2 ± 17.0 (120.4)	93.3 ± 17.9 (106.7)
Thyroid hormone-related genes				
TR	331,688.1 ± 53,746.3	355,758.3 ± 38,225.6 (107.3)	378,635.9 ± 37,156.2 (114.2)	368,671.8 ± 24,218.2 (111.2)
BDNF	1,247.3 ± 115.5	1,375.3 ± 222.2 (110.3)	1,488.2 ± 152.5 (119.3)	1,502.8 ± 280.7 (120.5)
Neurogranin	1,028,607.4 ± 87,359.9	1,161,839.8 ± 90,734.1* (113.0)	1,219,222.6 ± 61,525.2** (118.5)	1,233,878.8 ± 67,733.4** (120.0)

Data ($\times 10^6$) show the ratio of target mRNA expression to GAPDH mRNA expression (% of control), and are mean ± SD for 5 mice per group

PND postnatal day

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control by Dunnett test

To clarify these changes, further analyses, investigating the rate of weight change, are needed.

Expression of stress-related genes

DE contains not only gaseous compounds such as NOx, SOx, and carbon monoxide (CO), but also thousands of chemical substances, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals; these are known collectively as DEP. In mice, DEP produce oxygen radicals, such as superoxide and hydroxyl radicals (Sagai et al. 1996). CYP1A1, HO-1, and MT-1 are produced in response to cellular stressors such as PAHs, oxidization stress, and heavy metals (Takano et al. 2002; McCoubrey et al. 1997; Poss and Tonegawa 1997; Tortosa et al. 2008). Hougaard et al. (2008) reported that HO-1 has been measured on prenatal diesel exposure, although it was not measured in brain tissue. The expression level of these mRNAs was examined as an indicator of whether these components of DE might reach the brain and cause oxidative stress.

Perinatal DE exposure temporarily increased expression of CYP1A1 mRNA in the brains of newborn mice. Levels increased on PND-2 in fetal-exposed male and female offspring and on PND-5 in exposed male and female neonates. In particular, the expression of CYP1A1 mRNA in the female on PND-2 increased dose dependently. HO-1 mRNA levels showed a constant expression increase during the exposure period in neonatal-exposed males on PND-2, -5, and -16. However, no significant difference in expression was found for females during the neonatal period. Moreover, HO-1 expression was not increased in response to fetal DE exposure. Expression of MT-1 displayed a complicated pattern that varied by exposure group, sex, and observation time.

Cytochrome P450 (CYP) is a metabolic enzyme that participates in the metabolism and detoxification of various exogenous substances. There are several CYP subfamilies and expression of CYP1A1 is induced by PAH (Whitlock 1999). The usefulness of CYP1A1 as a biomarker of DEP exposure has been reported (Takano et al. 2002), and DEP induced expression of CYP1A1 mRNA in mouse testes has been found in our laboratory (unpublished data). Furthermore, the mechanism of crosstalk between the aryl hydrocarbon receptor (AhR) and ER has been elucidated (Ohtake et al. 2003), and it is possible that compounds in DEP act through AhR to disrupt function. In the present study, CYP1A1 mRNA level was increased on PND-5 in both male and female mice exposed to DE during the neonatal period, indicating that compounds in DE had reached the brain. In addition, both sexes showed a tendency toward increased expression of CYP1A1 mRNA in the brain on PND-2 after maternal exposure to DE. These findings suggest that some compounds in DE might reach the brain of the fetus through the placenta (Fujimoto et al. 2005), and continue to influence expression during the postnatal period. While the CYP1A1 mRNA increase suggested that compounds in DE could reach the brain, the expression pattern varied through individual difference, exposure period, and analysis time. More detailed analyses are required.

Expression of HO-1 resolved that heme is induced by various stressors (oxidization stress, heavy metal exposure, hypoxia, ultraviolet light), which increase the HO-1 mRNA transcription level (McCoubrey et al. 1997). It has been reported that HO-1 transgenic mice show increased tolerance to oxidization stress (Takeda et al. 2000a) and that tolerance to oxidization stress is decreased in HO-1 knockout mice (Poss and Tonegawa 1997). HO-1 is expressed in the brains of Alzheimer's disease patients (Takeda et al. 2000b), and its relation to this brain disorder has attracted attention. Organic compounds in DEP produce oxidization stress and induce expression of HO-1 (Hiura et al. 1999; Koike et al. 2002). In the present study, expression of HO-1 increased in the brains of males after neonatal DE exposure. This suggests that the male brain was subject to oxidation stress during neonatal DE exposure. The difference in HO-1 expression induction between male and female mice found in this study suggests that sensitivity to DE exposure differs between male and female newborn mice.

MT is important for mitigation of toxic heavy metals, accumulation of heavy metals, metabolic regulation of necessary metals, and free radical elimination. Expression of MT is induced by various heavy metals, hormones, catecholamines, cytokines, pesticides, and medicines. Expression of MT is also induced by physical stress, such as a restraint, oxidization stress, or irradiation, and it is therefore useful as a biomarker of environmental pollution. In the present study, MT levels increased in parallel with experimental and control groups with growth until PND-16, and it appears that MT is generated in the brain during the growth phase. However, MT mRNA level in females during neonatal exposure tended to decrease from DE exposure; thus, DE might affect MT production in brain. The pattern of MT expression after DE exposure during the perinatal period is complex, and no pattern could be discerned with regard to DE exposure.

This analysis of CYP1A1, HO-1, and MT-1 suggests that more detailed studies are required to evaluate the ability of DE to reach the brain. It appears that mainly PAH may access the brain during the perinatal period after DE exposure. In future studies, it will be necessary to investigate the levels of PAH that pass into the brain during DE exposure. PAH contained in DEP has both estrogenic and anti-estrogenic activities (Furuta et al. 2004; Mori et al. 2002; Taneda et al. 2002), and the results of the gene analyses in this study indicate that DE exposure during the perinatal period influences the steroid hormone system, especially the estrogen system in the brain.