Morphological analyses of sex differentiation in mouse midbrain. And the effects of fetal and neonatal exposure to three typical environmental chemicals with different…

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Graduate School of Agricultural Science,
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博士論文

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マウス中脳の性分化および環境中微量化学物質複合曝露の中脳ドーパミン作動性神経核への作用

July, 2009

Department of Bioresource Science,

Graduate School of Agricultural Science, Kobe University

神戸大学大学院農学研究科　資源生命科学専攻

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Preface

Dramatic development of scientific technology has provided us with numerous benefits of industrial products. The safety of materials of those products has been assessed by many toxicity tests using adult animals. Recently, it has been noticed that some chemicals derived from industrial products are hormonally active and can disturb endocrine and reproductive systems (Markey, *et al*., 2002) even though lower than no-observed-adverse-effect level (NOAEL) determined by the toxicological tests when they are exposed to during fetal and neonatal period. Such chemicals including phenols, phthalates, dioxins, polychlorinated biphenyls (PCBs), and heavy metals are known as 'endocrine disruptors (EDs)', and probably transfer from the mother to the fetus through the placenta and lactation (Mori, 2001). It is currently known that fetus, infants, and children may be more susceptible than adults to such chemicals, and their exposures to EDs in the environment are often greater than those for adults (Goldman and Koduru, 2000).

Because sex differentiation of mammal brain is classically approved to depend on sex steroid hormone secreted from testes (MacLusky and Naftolin, 1981), perinatal exposure to EDs is thought to disturb the function related to sexual dimorphism of brain and/or behavior. Some clinical data indicate the sex differences in the midbrain, suggesting the relation of sex steroid hormones and/or sex chromosomal genes to mesencephalic development and differentiation. The prevalence of both Parkinson's disease (Baldereschi, *et al*., 2000; Bower, *et al*., 1999, 2000; Diamond, *et al*., 1990), which is characterized by the selective loss of dopaminergic neurons from the substantia nigra (SN) and attention-deficit/hyperactivity-disorder (ADHD) (Arnold, 1996), which is associated with midbrain dopaminergic system has been dominated by males. Sexual dimorphism is found in the tyrosine hydroxylase (TH) activity of SN and in the exploratory behavior of both BALB/cJ and CBA/J inbred mouse strains (Vadasz, *et al*., 1985).

More recently, it has also become evident that the influence of EDs on developmental

brain is observed not only in brain sexual differentiation but also in wide range of brain function associated to motor activity (Ishido, *et al*., 2004a; b; 2005; Masuo, *et al*., 2004, 2007), learning and memories (Kobayashi, *et al*., 2002; Watanabe, *et al*., 2003), and intelligence quotient (Jacobson and Jacobson, 1996; Patandin, *et al*., 1999).

Our living environment contains a number of xenobiotics in various concentrations, growing a question whether exposure to mixtures of low-dose EDs having different action mechanisms affects neurodevelopment differently than exposure to EDs individually. However, there are few reports, especially *in vivo*, that assess the health risks of exposure to low doses of multiple environmental chemicals. Because developmental midbrains of female and male mice are poorly understood in morphology, I firstly studied the age-related changes of midbrain of female and male mice using histological and immuno-histoplanimetrical technique to elucidate whether dopaminergic nuclei (A9, A10, and A8) show developmental changes postnataly. Second, I studied the effects of fetal and neonatal exposure to typical EDs such as bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane), di-(2-ethylhexyl)-phthalate (DEHP), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the midbrain dopaminergic nuclei of mice and compare the effects between sole and mixed administration.

Chapter I

Morphological analyses of sex differentiation in C3H mouse midbrain

Introduction

Sex differentiation of the mammalian brain is influenced mainly by perinatal testicular steroid hormones (MacLusky and Naftolin, 1981). In males, androgen (or its metabolite estrogen) initiates a program of male-specific development in many brain regions. In females, on the other hand, neural development traces female-specific processes caused by a low level of gonadal steroid hormone. These mechanisms result in morphological sexual dimorphism, which controls sexually dimorphic behaviors and functions (Gorski, *et al*., 1978). Sex steroid hormones are associated with the constitution of several sexual dimorphisms via estrogen receptor (ER) (Kuhnemann, *et al*., 1994). Moreover, the sexual differentiation of dopaminergic neurons within the preoptic region of the diencephalon depends on ER (Simerly, *et al*., 1997).

However, other sex differentiation processes have been found to depend on the sex chromosomal gene directly rather than on gonadal steroid hormones in the midbrain. The cell cultures dissociated from female rat mesencephalon on embryonic day 14 (ED 14; before exposure to the gonadal steroid hormone shower) contain higher numbers of dopaminergic neurons than male cultures, and hormonal treatment does not erase this differentiation (Beyer, *et al*., 1991). The sex-determining region of the Y chromosome (*Sry*) is transcribed in the hypothalamus, mesencephalon, and testis in adult (3-month-old) male but not in adult female mice (Lahr, *et al*., 1995). *Sry* transcripts were also found in the male diencephalon, midbrain, and cortex in the postnatal day (d) period between 14 and 90 (Mayer, *et al*., 2000). These reports indicate that *Sry* has a crucial role in regulating the sex differentiation of the mammalian central nervous system (CNS). The sex difference in the mesencephalon is also found in Parkinson's disease, a progressive neurodegenerative disease characterized by the selective loss of dopaminergic neurons from the substantia nigra (SN) and the concomitant appearance of motor disturbances including tremors, rigidity, and slowness of movement (akinesia). Many studies have indicated that the prevalence of Parkinson's disease is 1.36-3.7

times higher in men than in women (Baldereschi, *et al*., 2000; Bower, *et al*., 1999, 2000; Diamond, *et al*., 1990). Dewing, *et al*. (2003) detected at least seven murine genes that show differential expression between female and male mouse developing brains at ED 10.5 before any gonadal hormone influence. For these reasons, the midbrain likely has sex differences of some kind. In fact, sexual dimorphism was found in the tyrosine hydroxylase (TH) activity of SN and in the exploratory behavior of both BALB/cJ and CBA/J inbred mouse strains (Vadasz, *et al*., 1985).

Despite these many demonstrations of sex differences in the midbrain, research also indicates that there is no sex difference within mesencephalic dopaminergic nuclei. Lieb, *et al*. (1996) investigated the numbers of dopaminergic neurons in the mesencephalon of male and female CBA/J mice between ED 13 and 90 d, but they could not find statistically significant sex differences in the numbers of dopaminergic neurons at any time point. In addition, there is a genetic control of the neuron number (including midbrain dopaminergic neurons) within inbred strains of mice (Baker, *et al*., 1980; Zaborszky and Vadasz, 2001). Presently, however, I still lack detailed information about sex and/or genetic strain differences within the midbrain.

There are other reasons why mesencephalic dopaminergic neurons are of interest: midbrain dopaminergic nuclei, which play crucial roles in cognition, emotion, and behavior, are also significant in clinical medicine because their functional disorder causes various neurodegenerative diseases, such as schizophrenia (Meltzer, *et al*., 1976), Parkinson's disease (Hornykiewicz, 1979), substance abuse (Koob, 1999), and disturbances in the control of motor activity and learning (Beninger, 1983), attention (Yamaguchi, *et al*., 1998), and other behaviors including attention-deficit hyperactivity disorder (Faraone and Biedermann, 1998).

Mesencephalic dopaminergic nuclei are distributed in the A8, A9, and A10 areas (Dahlstroem and Fuxe, 1964). A8 contains the retrorubral field (RRF); A9 consists of the SN pars compacta (SNc), SN pars reticulata (SNr), and SN pars lateralis (SNl); and A10 is

composed of the ventral tegmental area (VTA), parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), and central linear nucleus (CLi). Neurons in the A9 area have projective fibers toward the striatum and form the nigrostriatal dopaminergic system, which promotes the extrapyramidal function. The dopaminergic neurons within A10 project to the subcortical and cortical limbic areas as well as to the prefrontal cortex, while A8 neurons provide dopaminergic input to both the striatum and the limbic areas. A dopaminergic neuronal deficit within the A10 area contributes to drug addiction and is thought to be a point of action by schizophrenia-curative drugs (Nemeroff and Bisette, 1988).

In the present study, male and female C3H mice, whose midbrain is poorly understood, were examined at 3 d (neonatal period), 21 d (pre-puberty), and 11 week (wk) of age (post-puberty) to elucidate the sex differences and age-related changes within the midbrain of this inbred strain during sexual maturation.

Materials and methods

Animals: Female and male mice of the C3H/He strain were purchased from Japan SLC, Inc. (Shizuoka, Japan) for breeding stock. The animals used for the experiments were bred in a laboratory at the Department of Animal Science at Kobe University, under a 12 hr light/dark cycle at 21-24°C and 40-60% humidity. A laboratory diet (Lab MR Stock; Nosan Corp., Yokohama, Japan) and filtered water were available *ad libitum*. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of Kobe University. Nineteen mice were divided into three age groups—3 d (n = 5), 21 d (n = 8), and 11 wk (n = 6)—in each sex.

Tissue preparation: I gave careful attention to each experimental treatment in order to maintain the equality of all brain samples. At the time of necropsy, mice were deeply anesthetized with diethyl ether and were subcutaneously injected with heparin (1 U/g body weight) to avoid blood coagulation. After 10 min, the mice were transcardially perfused with 0.1 M ice-cold phosphate buffer (PB), pH 7.4, including 3.4 mM EDTA, followed by perfusion with ice-cold 4% paraformaldehyde in PB. The brains were post-fixed with the same fixative overnight at 4°C, dehydrated through a graded series of ethanol followed by xylene, and embedded in paraffin. Coronal serial sections 10 μm in thickness were then cut, and each section was mounted on a glass slide precoated with silane (Matsunami, Osaka, Japan).

Cresyl fast violet staining: A series of sections was made by collection at 80-100 μm intervals. After deparaffinization and rehydration, the sections were stained with 0.1% cresyl fast violet solution, which visualizes Nissl's substance for 10 min. They were then placed in distilled water (DW) and a graded series of ethanol for appropriate bleaching of dye, dehydrated with ethanol, cleared by xylene, and coverslipped with Eukitt. Each section was

morphologically categorized by using a guide (Paxinos and Franklin, 2001) (Fig. I-1).

Immunohistochemistry: The immunoreactivity of TH was applied to visualize dopaminergic neurons. Deparaffinized tissue sections were analyzed using a Histofine Mouse Stain Kit (Nichirei Bioscience, Tokyo, Japan) for monoclonal antibody in the mouse tissues following the manufacturer's instructions. After immersion in 100% methanol including 3% H_2O_2 for 30 min at room temperature (RT) to quench endogenous peroxidase activity, the sections were incubated with the primary antibody against TH (MAB-318; Chemicon International, Temecula CA, USA) diluted 1:1,200 in phosphate-buffered saline at 4°C overnight. Immunoreactivity was then detected by incubation with 3,3'-diaminobenzidine solution (Dako, Glostrup, Denmark) containing 0.03% H_2O_2. The sections were counterstained lightly with 0.1% cresyl fast violet solution for 1 min. Finally, they were placed in DW and a graded series of ethanol, dehydrated with absolute ethanol, cleared by xylene, and coverslipped with Eukitt. The distribution of each dopaminergic nucleus (A8, A9, and A10) within the midbrain was classified morphologically (Fig. I-1). To compare the staining intensity across different sections, each semi-serial section was captured by a digital still camera (DS-L1, Nikon, Tokyo, Japan) connected to a light microscope. The background staining intensity was manually adjusted to the regions lacking TH and Nissl staining in each microphotograph. Then the immunoexpression within the perikarya of each dopaminergic nucleus (A8, A9, and A10) was evaluated (data not shown).

Histoplanimetrical analysis: The coronal semi-serial sections stained immunohistochemically were observed from the caudal level of the diencephalon to the rostral level of the pons (Fig. I-1) at magnifications of ×200 using an Olympus light microscope. The first sampling tissue section for counting was taken when the most rostral TH-immunoreactive (TH-ir) cells were observed in the midbrain unilaterally (right side), and

all the following sections were taken at intervals of 80-100 μm from the previous one. Then only neurons containing perikarya clearly stained for TH within the right side of the A8, A9, and A10 areas were manually and separately counted through the entire midbrain, and the total (A8 + A9 + A10) number of neurons was calculated.

Statistical analysis: Unpaired t-test (StatView version 5.0; SAS Institute, Cary, NC, USA) was applied to determine the statistical differences in the TH-ir cell numbers by sex and age. Analyses were considered statistically significant at $p < 0.05$.

Results

The age-related changes found in both cresyl fast violet and immunostained sections are shown in Figs. I-2 and I-3. In general, all of the neurons I investigated were multipolar, which are most typical in the CNS, and the perikarya and cellular nuclei increased slightly in size as the mice developed. The volume of nuclei also increased concomitantly with brain development. In the cresyl fast violet-stained sections, the nuclei composed of round or ovoid cells with short dendrites were arcuate hypothalamic nuclei (Arc), interpeduncular nuclei (IP), PN, CLi, and pontine nuclei (Pn). On the other hand, nuclei whose perikarya (with comparatively long dendrites) appeared stellate or pyramidal were SNc, SNl, RRF, and VTA. Extremely large cells were distributed within oculomotor nuclei (3N) and red nuclei (Rn) in both sexes. These findings were common between the sexes. The findings observed in TH-immunostained sections of both sexes were as follows: TH-ir cells contained deeply stained perikarya and round unstained nuclei. SNc seemed to consist of the largest TH-ir cells in the midbrain dopaminergic nuclei (Fig. I-3). CLi and PN appeared to contain comparatively small TH-ir cells, and their immunostained fibers were comparatively short (Fig. I-3 D-I). The regions in which TH-ir cells projected comparatively long fibers were observed in SNc, SNl, and RRF (Fig. I-3). The cell bodies were pyramidal or spindled within SNc, SNl, and VTA (Fig. I-3A-F) but were round and ovoid within CLi and PN (Fig. I-3 D-I).

The immunohistochemical comparison between the sexes is shown in Fig. I-4. Although the greatest individual differences were observed at 3 d, the differences did not appear to depend on sex (Fig. I-4 A-F). Nor was any sexual dimorphism more apparent than individual differences in 21 d mice (Fig. I-4 G-L). In the 11 wk mouse midbrain, female VTA appeared to contain higher immunoreactivity of TH than male VTA (Fig. I-4 M-R). This sex difference seemed to contribute to the significant increases in A10 TH-ir cells at 3 d, 21 d, and 11 wk.

The numbers of TH-ir neurons within midbrain dopaminergic nuclei (A8, A9, A10, and total) and the statistical outline of this study are summarized in Figs. I-5 and I-6, respectively.

There were no significant sex differences in the number of TH-ir neurons when they were tested between age-matched groups. However, when the increment in TH-ir cell numbers was examined between 3 and 21 d and between 21 d and 11 wk, the time period in which the numbers significantly increased differed between the sexes (Figs. I-5 and I-6); the numbers of female A9, A10, and total TH-ir neurons increased significantly between 3 and 21 d (Figs. I-5B, C, D, and I-6); and the numbers of female A8, A10, and male A9 TH-ir neurons increased significantly between 21 d and 11 wk (Figs. I-5A, B, C and I-6). In the A8 area, the numbers of TH-ir neurons increased significantly from 21 d to 11 wk in females only, while neither sex showed significant increases in the numbers of TH-ir neurons between 3 and 21 d (Figs. I-5A and I-6). The numbers of TH-ir neurons within the A9 area reached to adult level before 21 d in female mice, whereas in males the numbers did between 21 d and 11 wk. Among females, the numbers of A10 TH-ir neurons increased significantly from 3 to 21 d and from 21 d to 11 wk, whereas among males the number did not significantly change except between 3 d and 11 wk (Figs. I-5 A-D and I-6). The numbers of TH-ir neurons within the female A8 area seemed to reach to adult level after than in males.

Discussion

The present study demonstrates the postnatal profiles of age-related changes within the midbrain of the female and male C3H mouse. The rationale for using this strain in this study is, although several other reports have shown the data related to both female and male midbrain dopaminergic nuclei by using strains CBA/J (Lieb, *et al*., 1996), BALB/c (Ivanova and Beyer, 2003), C57BL/6J (Hamre, *et al*., 1999), and ddY (Tando, *et al*., 2007), there is little information about C3H mice, which are extensively used as experimental animals. The profiles in this study could provide a framework for further investigations of the C3H mouse midbrain.

The VTA of female mice at 11 wk contained higher levels of TH-ir activity than that of males in the immunohistochemical analysis. While the number of TH-ir cells did not significantly differ between the sexes, there was a female-dominant tendency in TH-ir cell numbers within A10 ($p = 0.073$). However, two other immunostaining groups—that at 3 d and that at 21 d—did not show any sex differences. The numbers of TH-ir cells within the A8, A9, and A10 areas peaked at 11 wk in both female and male mice. Some studies have shown decreased TH-ir cell numbers within the midbrain through the ontogenetic period. Tepper, *et al*. (1994) reported a decrease in TH-ir profile counts in the rat SNc between 1 and 14 d, which they interpreted as cell loss. Lieb, *et al*. (1996) also demonstrated a decrease in profile density but an increase in total numbers of TH-ir cells. However, in the present study, I did not detect a decrease in TH-ir cell numbers at any time point. This discrepancy may be attributable to differences in species and strains between our study and the previous ones. Alternatively, the number of TH-ir neurons may decrease at time points other than 3 d, 21 d, and 11 wk. The numbers of dopaminergic neurons in A8, A9, and A10 did not significantly differ between females and males at any time point investigated, supporting the report of Lieb, *et al*. (1996). However, the time period in which the dopaminergic neuron number significantly increased differed between the sexes. This means that the development rate of

each dopaminergic nucleus within the midbrain differs between sexes. This, in turn, has two implications. First, statistically significant sex differences in the numbers of TH-ir neurons may be found at time points other than 3 d, 21 d, and 11 wk. Second, sex differences may exist within the midbrain and may reflect the growth rate in dopaminergic neuron numbers. The brain of the 11 wk mouse is mature enough to be unsusceptible to sex steroid hormones, whereas the brain at 3 and 21 d may be affected by gonadal secretions in male mice, which may cause a sex difference in which the growth rate in the number of TH-ir neurons increases.

Many reports have demonstrated functional sex differences within the midbrain dopaminergic system affected by sex steroid hormones through the intermediary of AR and/or ERs. Both ERα- and ERβ-ir were expressed in the SNc and VTA of young adult female mice (Mitra, *et al*., 2003). There are double-immunoreactive neurons of AR and TH within the SN, VTA, and RRF of rat (Kritzer, 1997); these neurons project to either the amygdala or the nucleus accumbens (Creutz and Kritzer, 2004). ERβ-ir nuclei were found only in neurons, more specifically, within subsets of both dopaminergic and nondopaminergic neurons in the dorsal VTA, the PBP, the SNl, and the RRF (Creutz and Kritzer, 2002). Studies suggesting functional sex differences within the midbrain have also been reported. Estrogen acts as a neuroprotective agent differentially between the sexes in neurodegenerative rats administered 6-hydroxydopamine (6-OHDA). Estrogen replacement therapy reduces the lesion size in females but increases it in males (Murray, *et al*., 2003). Analysis of dopaminergic cell loss within A9 after injury by 6-OHDA administration shows that males are more susceptible to toxicity; female rats have significantly less dopaminergic cell loss and respond to 6-OHDA with a significantly higher degree of behavioral recovery after the injury (Tamas, *et al*., 2005). A9 lesions significantly deplete neostriatal dopamine, which is highly correlated with mount latency, mount rate, ejaculation latency, and ejaculation frequency in males (Brackett, *et al*., 1986). A10 dopaminergic neurons, which are sensitive to gonadotropin-releasing hormone,

are related to the regulation of lordosis in female rats (Sirinathsinghji, *et al*., 1986). These reports strongly suggest that the midbrain dopaminergic system includes sex differences underlying the pathogenic mechanism as well as functional sexual dimorphisms. Given that there are sexual dimorphisms in AR and/or ERs, the sensitivity of gonadal secretion will be very different between the sexes. In the present study, the sex differences observed in the maturation rate likely reflect functional sex differences within the midbrain dopaminergic system involving cognition, emotion, and behavior.

Chromosome Y genes may also affect the sex-specific development and the functions of the midbrain dopaminergic system. One of the genes presumed to cause the male-specific increment pattern of TH-ir neurons is *Sry*, which is expressed specifically in the brains of adult male mice (Dewing, *et al*., 2003; Mayer, *et al*., 2000). The transcript product of the gene is specifically detected in the TH-ir neurons within the SN of the adult male rodent and involves the regulation of mesencephalic TH (Dewing, *et al*., 2006). An experiment using cell cultures dissociated from midbrains of female and male mice also indicates *Sry*'s involvement in the regulation of dopaminergic neurons (Carruth, *et al*., 2002). The lack of a statistically significant increase in the numbers of TH-ir neurons from 3 to 21 d in males may reflect individual differences in the complicated mechanisms regulated by male-specific factors, such as neonatal testicular secretion and chromosome Y genes. In females, the absence of male-specific factors might induce 'uniformly' developing patterns of TH-ir neurons that showed statistical significance.

In conclusion, I detected age-related changes within the midbrain of female and male C3H mice and showed sex-specific development patterns in the numbers of TH-ir neurons there, suggesting that the midbrain dopaminergic system may be sexually dimorphic in the maturation process. These differences may reflect the direct regulation of *Sry* or the multiple effects of both *Sry* and sex steroid hormones. I have not yet determined clearly which factor forms sexual dimorphism in the midbrain. To elucidate the mechanism underlying female and

male development within midbrain dopaminergic nuclei, I need to perform further experiments applying gonadectomy and the administration of sex steroid hormones. I also have to determine the meaning of the sex-specific increment patterns of TH-ir cells. In light of the fact that there is genetic variation in midbrain dopaminergic neuron numbers, the influence levels of hormonal and/or genetic factors may differ according to the inbred strain.

Summary

While it is commonly hypothesized that sexual differentiation in the mammalian brain is initiated mainly by gonadal sex steroids, recent evidence has suggested that dopaminergic neurons within the rodent midbrain have sex differences independent of gonadal secretions. More recently, it has been reported that *Sry* (the sex-determining region of the Y chromosome) is directly involved in this difference. The possibility of sexual dimorphism in the mouse midbrain needs to be elucidated. In the present study, the midbrain of C3H mice, which are little understood, were investigated histologically and immuno-histoplanimetrically to reveal sexual and developmental differences. The female ventral tegmental area appeared to contain higher immunoreactivity to tyrosine hydroxylase (TH) than that of males at 11 weeks of age, whereas general histological differences between the sexes were not clearly found. The TH-immunoreactive (TH-ir) neurons within the A8, A9, and A10 mesencephalic areas were examined separately. The time period in which TH-ir cell numbers significantly increased differed between the sexes, indicating that the growth rate of midbrain dopaminergic nuclei also differs and that the midbrain dopaminergic system may trace different sexual maturation processes between the sexes. These differences between female and male may reflect the direct regulation by *Sry* or the multiple effects of both *Sry* and sex steroids. Further experiments are needed to determine which factor forms this difference in the growth pattern in the numbers of TH-ir neurons.

Figures and Tables

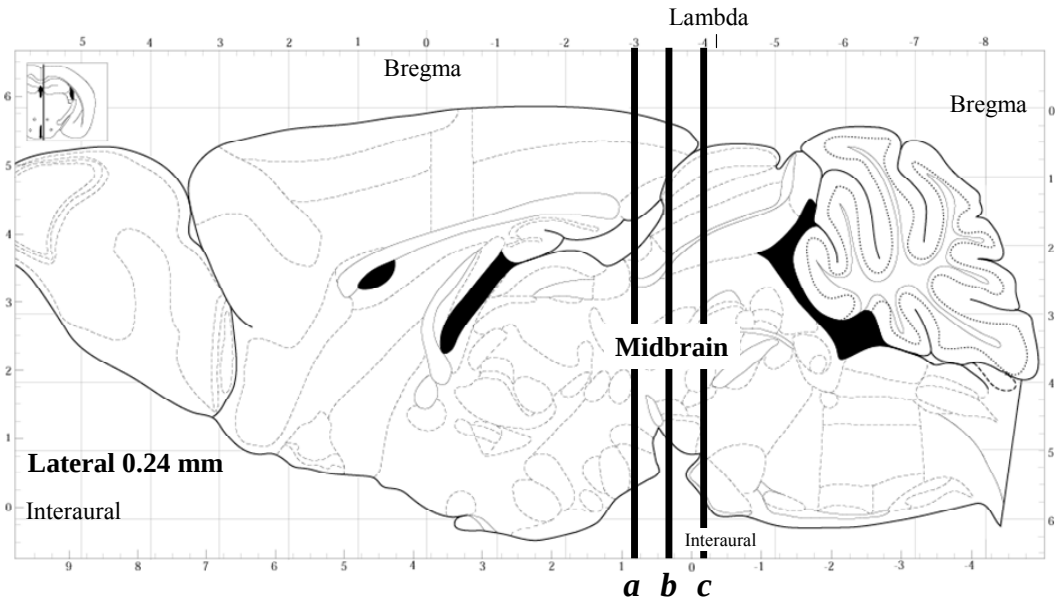


Fig. I-1 Sagittal section of the mouse brain referred from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001). The transverse lines of *a*, *b*, and *c* correspond to the rostral, intermediate, and caudal parts of the midbrain, respectively. The lines show brief coordinates of the following Figs. Line *a* corresponds to coronal photomicrographs of Fig. I-2, Fig. I-3A-C, and Fig. I-4A, D, G, J, M, P. Line *b* corresponds to coronal photomicrographs of Fig. I-3 D-F and Fig. I-4B, E, H, K, N, Q. Line *c* corresponds to coronal photomicrographs of Fig. I-3 G-L and Fig. I-4C, F, I, L, O, R.

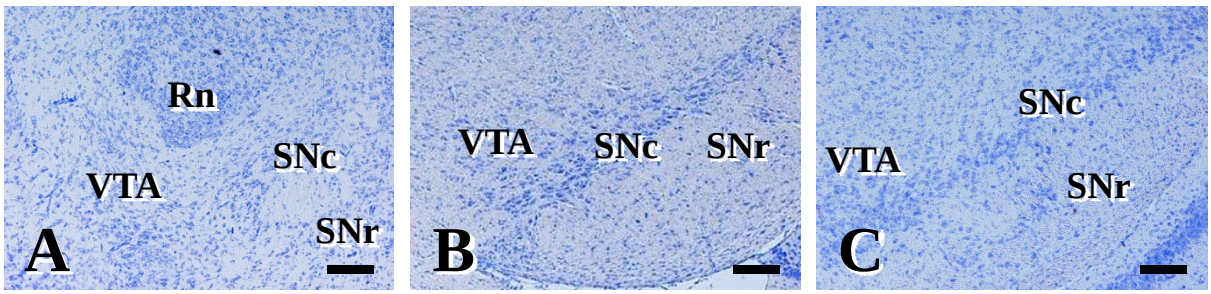


Fig. I-2 Age-related changes of rostral midbrain in cresyl fast violet-stained sections corresponding to the transverse line *a* in Fig. I-1. Coronal photomicrographs show changes at 3 d (A), 21 d (B), and 11 wk (C). The size of perikarya and cellular nuclei constituting each neuronal nucleus increased slightly, as did the contents of neuronal nuclei in parallel with brain development. Bar = 100 μm.

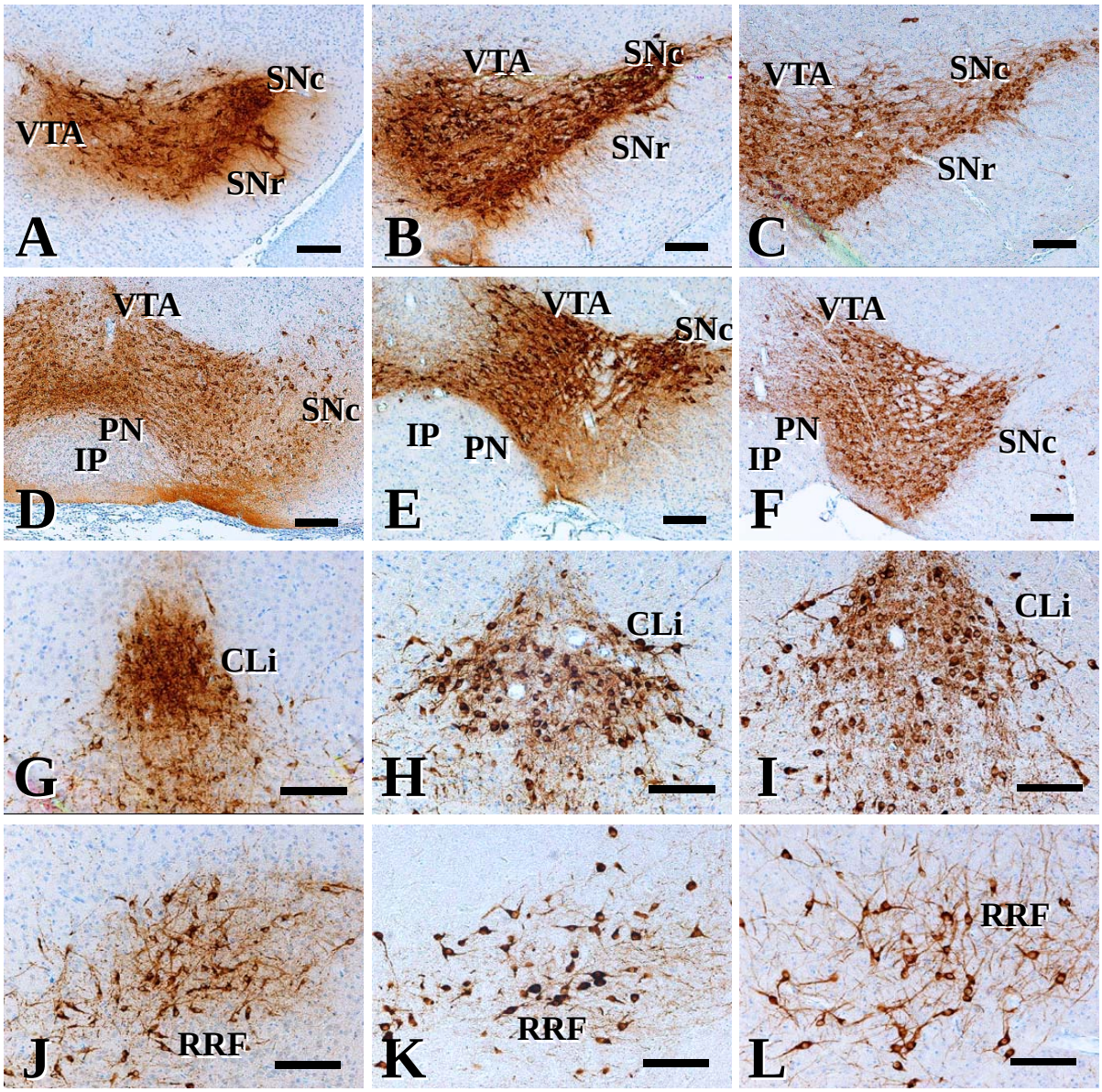


Fig. I-3 Age-related changes of dopaminergic nuclei within rostral (A-C), middle (D-F), and caudal (G-L) levels of midbrain containing TH-ir cells at the age of 3 d (A, D, G, and J), 21 d (B E, H, and K), and 11 wk (C, F, I, and L). TH-ir perikarya became slight larger, and the volumes of TH-ir nuclei increased in parallel with brain development. Bar = 100 μm.

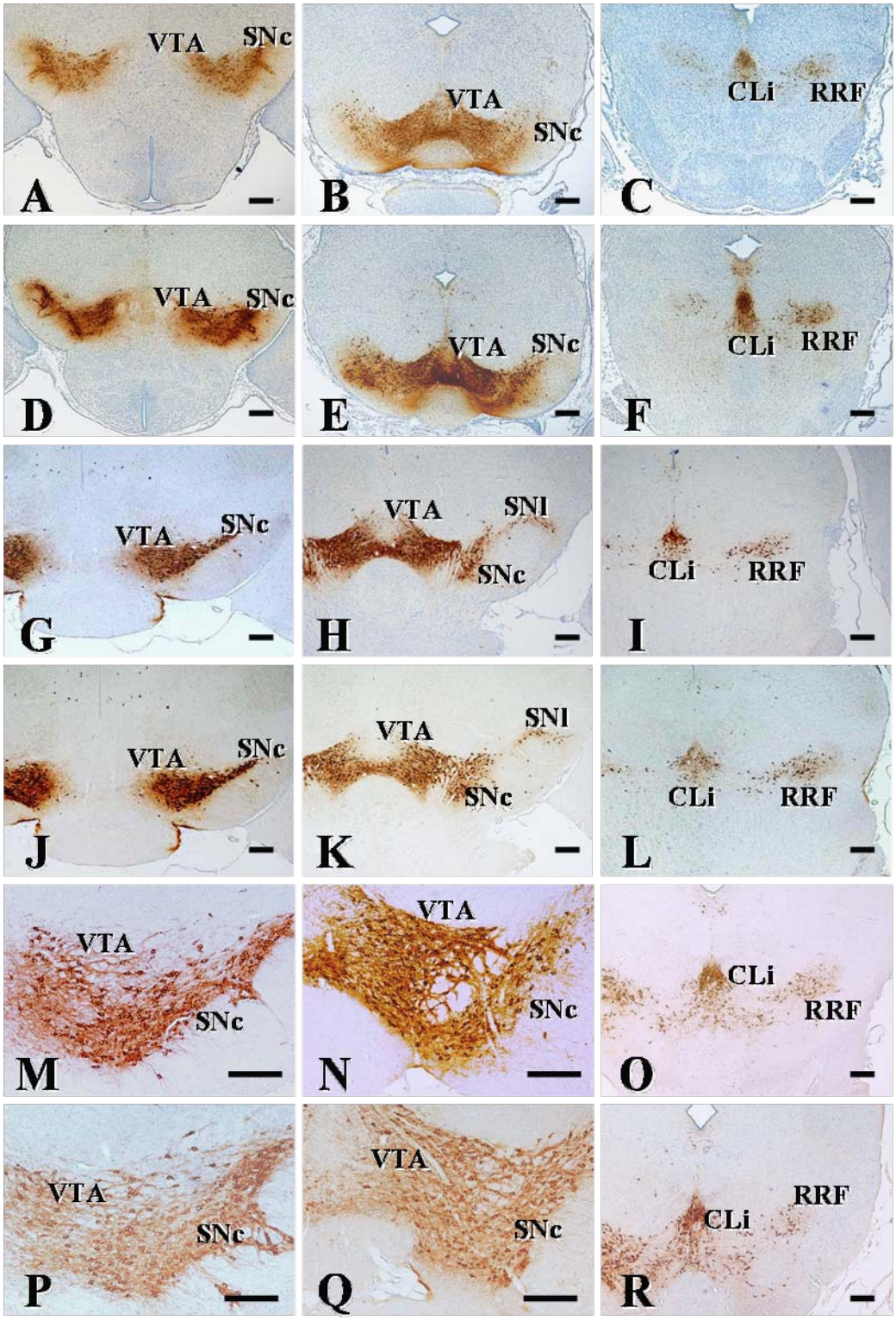


Fig. I-4 Immunohistochemical comparison between female (A-C, G-I, and M-O) and male (D-F, J-L, and P-R) mice at 3 d (A-F), 21 d (G-L), and 11 wk (M-R). Coronal Figs show rostral (A, D, G, J, M, and P), middle (B, E, H, K, N, and Q), and caudal (C, F, I, L, O, and R) levels of midbrain. Although individual differences were observed within the midbrain of 3d mice, sex differences appeared to be independent of the sex. Bar = 200 μm.

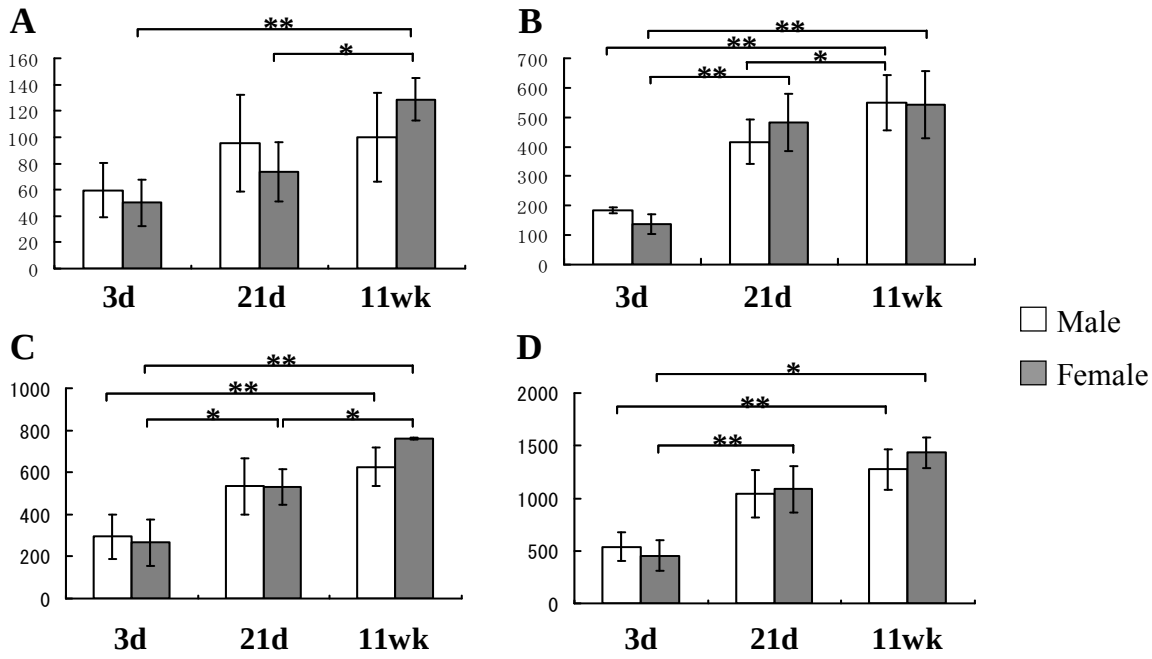


Fig. I-5　TH-ir neurons counted within semi-serial sections of A8 (A), A9 (B), A10 (C), and total (D) in female (solid column) and male (open column) 3 d, 21 d, and 11 wk mice. Significant differences were not detected in the number of TH-ir neurons between the sexes when I tested between age-matched groups, while the time period in which the number significantly increased differed between females and males. In a comparison between 3 d and 11 wk mice, all nuclei except for male A8 area significantly increased TH-ir neuron numbers. The A9 and A10 areas of both sexes and the female A8 area showed a significant increase of $p < 0.01$. Values are means ± SD. *: $p < 0.05$, **: $p < 0.01$.

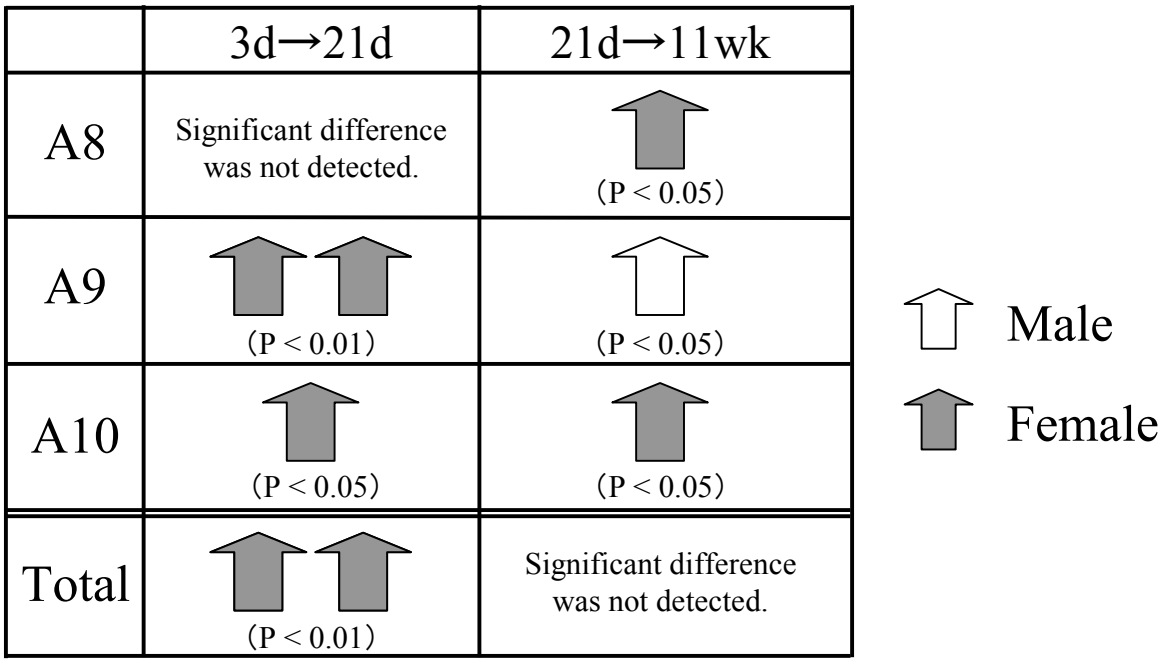


Fig. I-6　The statistical outline of this study. The significant increase of the numbers of TH-ir neurons was detected and its increment patterns differed between sexes. It is notable that females showed significant increases within all nuclei while males did so only within the A9 area.

Chapter II

Fetal and neonatal exposure to three typical environmental chemicals with different mechanisms of action: Mixed exposure to phenol, phthalate, and dioxin cancels the effects of sole exposure on mouse midbrain dopaminergic nuclei

Introduction

It has been known that many environmental chemicals derived from industrial products exert estrogen-like activity, and these are known as endocrine disruptors (EDs). A number of studies have recently focused on EDs' effects on living organisms. Because fetuses and neonates are highly sensitive to physiologically active agents (Warita, *et al*., 2008) and because such chemicals transfer from the mother to her offspring through the placenta and lactation (Mori, 2001; Kobayashi, *et al*., 2002; Myllymäki, *et al*., 2005), ED exposure pre- and neonatally can disturb development even though the amounts of exposure are lower than the no-observed-adverse-effect level (NOAEL) or the lowest-observed-adverse-effect level (LOAEL) determined by toxicological tests using adult animals. It has also recently become evident that EDs influence the neurodevelopment of a wide range of brain functions associated with motor activity (Ishido, *et al*., 2004a, b, 2005; Masuo, *et al*., 2004, 2007), learning, memory (Kobayashi, *et al*., 2002), and intelligence quotient (Jacobson and Jacobson, 1996).

The public concern seems to have been focused on EDs' effects on brain function concomitantly with the recent increase in neuropsychiatric disorders, including autism, learning disabilities, and attention-deficit/hyperactivity disorder (ADHD), which is characterized by symptoms including attention deficit, impulsivity, and hyperactivity in childhood and often persists into adulthood (Kidd, 2000). ADHD can be attenuated by psychostimulant drugs (methamphetamine or methylphenidate) known to enhance extracellular dopamine concentrations (Shaywitz, *et al*., 1976b; Heffner and Seiden, 1982). The rats 6-hydroxydopamine intracisternally injected during the neonatal period (Shaywitz, *et al*., 1976a,b) and congenic wiggling rats (Masuo, *et al*., 2007), both described as good models for ADHD, contain low levels of tyrosine hydroxylase (TH)-immunoreactivity within midbrain dopaminergic nuclei, suggesting the association between the midbrain dopaminergic system and the pathogenesis of this neurodevelopmental disorder.

Environmental chemicals such as phenols, phthalates, and the dioxin-like compounds shown below are typical EDs that have become ubiquitous in developed countries and have been suspected of disturbing normal brain development. Bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl) propane), used in polycarbonate plastic products including baby bottles (Brede, *et al*., 2003), has been studied for its estrogenic activity for over 50 years (Dodds and Lawson, 1936). Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate among phthalate esters, which have been widely used in polyvinyl chloride products such as vinyl flooring, wall coverings, food containers, cosmetics, medical products, and infant toys. It is well known that DEHP exerts an anti-androgenic effect by inhibiting fetal testicular testosterone production (Gray, *et al*., 1999). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be the most potent and is the most studied (Van den Berg, *et al*., 2006) of the dioxin-like compounds, which include polychlorinated dibenzo-p-dioxins, dibenzofurans, biphenyls (PCBs), and related chemicals; it belongs to a family of toxic agents sharing a common mechanism of toxic action mediated by aryl hydrocarbon receptor (AhR) (Myllymäki, *et al*., 2005). Humans are generally exposed to such compounds, which are found in food, drinking water, soil, dust, smoke, and air.

The three chemicals BPA, DEHP, and TCDD are good for our examination by virtue of the abundance of toxicological findings that they disrupt a wide range of brain functions and exert disrupting effects through different mechanisms of action.

On the most practical level, I think the problem is that our living environment contains a number of xenobiotics in various concentrations. However, there are few reports, especially *in vivo*, that assess the health risks of exposure to low doses of multiple environmental chemicals. Therefore, the main purposes of this study are: (i) to elucidate whether or not fetal and neonatal exposure to lower doses of EDs than NOAEL or LOAEL injected through the practical (transplacental and oral) route disturbs the development of midbrain dopaminergic neurons related to cognition, affection, behavior and, especially, ADHD; (ii) to give a first

answer to the growing question about the influence of exposure to mixtures (BPA + DEHP + TCDD) of low-dose EDs. The present experiments applied the quantitative immunohistochemical technique to TH-immunoreactive (ir) and Fos-ir neurons as markers of brain dopamine and neuronal activity, respectively, within the midbrain dopaminergic nuclei (A9, A10, and A8) at the ages of 2, 4, and 6 weeks (wk), in order to compare the difference in effects between sole and mixed administrations.

Materials and methods

Chemicals: BPA and TCDD were purchased from Wako Pure Chemical Industries (Osaka, Japan); DEHP from Sigma Chemical (St. Louis, MO, USA); and laboratory-grade sesame oil from Kanto Chemical (Tokyo, Japan). These chemicals are summarized in Table 1.

Animals: ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) for breeding stock. The animals used for experiments were bred in a laboratory at the Department of Kobe University, with a 12 hr light/dark cycle at 21-24°C and 40-60% humidity. Diet (Lab MR Stock: Nosan, Yokohama, Japan) and filtered water were available *ad libitum*. This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 16-8-06) and carried out according to the Kobe University Animal Experimentation Regulations. Twenty-seven female mice in proestrus were mated 1:1 with males overnight, and females that had a vaginal plug the following morning were designated as being at gestational day (GD) 0. The litter sizes of each dams and the dosing regime are summarized in Table 1 and Fig. 1, respectively. In order to minimize maternal possible effect, I used the male pups born from 4-7 dams per each treatment groups and excluded pups showing extremely large and/or small body weights from the examination. Laboratory-grade sesame oil was the vehicle for the dosing solutions. To asses the effect of sole exposure to each ED, 5-6 pregnant mice and their male pups were orally administered 5 mg/kg body weight/day of BPA or 1 mg/kg/day of DEHP from GD 8 to 17 and from postnatal day (PD) 3 to 7, respectively. Considering the extended biological half-life of TCDD (Koshakji, *et al*., 1984), 5 pregnant mice were treated with a single oral injection of 8 ng/kg of TCDD. To assess the effect of multiple-ED exposure, BPA (5 mg/kg/day, GD 8-17), DEHP (1 mg/kg/day, GD 8-17), and TCDD (8 ng/kg, GD 8) were coadministered to the 4 pregnant mice, and BPA (5 mg/kg/day) and DEHP (1 mg/kg/day) were coadministered to their male pups from PD 3 to 7. The volumes of oral administration were adjusted to 3.3 ml/kg body weight of sesame oil. As

control groups, 7 dams and their male pups were treated with an equivalent volume of the vehicle (Fig. 1). The pups were weaned at 3 wk of age.

Tissue preparation: The mice that had been exposed to the chemicals during the *in utero* and neonatal periods were used at 2, 4, and 6 wk after birth for this study. At the time of necropsy, the mice were deeply anesthetized with diethyl ether and were intraperitoneally injected with heparin (1 U/g body weight) to avoid blood coagulation. Ten minutes after injection, the mice were transcardially perfused by 0.1 M ice-cold phosphate buffer (PB), followed by perfusion of ice-cold 4% paraformaldehyde in PB. The brains were then excised, weighed, and postfixed with the same fixative overnight at 4°C, dehydrated through a graded series of ethanol followed by xylene, and embedded in paraffin. Then coronal semi-serial sections 10 μm thick were cut, and each tissue section was mounted on a glass slide precoated with silane (Matsunami, Osaka, Japan).

Immunohistochemistry: A series of semi-serial sections was made by collecting tissue at 100 μm intervals (one of every 10 tissue sections). This method is appropriate from a quantitative standpoint in case the neurons are counted. After deparaffinization and hydration, the sections were immersed in 100% methanol containing 3% H_2O_2 for 30 min at room temperature (RT) to quench the endogenous peroxidase activity, and were autoclaved at 121°C for 20 min by immersion in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. The sections were incubated with 1% normal goat serum dissolved in 10 mM phosphate-buffered saline (PBS) for 30 min at RT for protein blocking and then were incubated with the rabbit polyclonal antibody against Fos (Ab-2, Calbiochem, Darmstadt, Germany) diluted 1:50 in PBS for 1 hr at 37°C and overnight at 4°C. After washing with PBS, the sections were reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in Tris-HCl buffer (EnVision Plus, DAKO, Glostrup, Denmark) for 30 min at RT. Immunoreactivity was

then detected by incubation with 3,3'-diaminobenzidine solution (EnVision$^+$ kit/HRP [DAB], DAKO) containing 0.03% H_2O_2. After immersion in 3% H_2O_2 for 30 min at RT to eliminate peroxidase activity, Fos-immunostained sections were washed with PBS and incubated with blocking reagent A (Histofine Simplestain system, Nichirei Bioscience, Tokyo, Japan) and the mouse monoclonal antibody against TH (MAB-318, Chemicon International, Temecula, CA, USA) diluted 1:1,200 in PBS for 1 hr at 37°C. After washing with PBS, the sections were incubated with blocking reagent B (Nichirei) for 30 min at RT and Histofine MAX-PO (M) (Nichirei) for 1 hr at RT. The sections were then washed with PBS and the immunoreactivity was detected by incubation with VECTOR SG Peroxidase substrate kit (SK-4700, Vector Laboratories, Burlingame, CA, USA). The sections were counterstained lightly with 0.1% cresyl fast violet solution for 1 min. Finally, they were placed in distilled water and a graded series of ethanols, dehydrated with absolute ethanol, cleared by xylene, and coverslipped with Eukitt (Sigma Chemical).

Immunohistoplanimetry: Coronal sections stained immunohistochemically were observed from the caudal level of the diencephalon to the rostral level of the pons under a light microscope (Nikon, Tokyo, Japan) (Fig. II-2). Each section was anatomically categorized by using a guide (Paxinos and Franklin, 2001).

The intensity of TH-immunoreactivity within midbrain dopaminergic nuclei is an index of the biosynthetic activity of dopamine, because TH is the rate-limiting enzyme of catecholamine biosynthesis. Each semi-serial section was captured by a digital still camera (DS-L1; Nikon) connected to a light microscope. To compare the staining intensity across different sections, the background staining intensity was manually adjusted to the regions unstained by both TH and cresyl fast violet in each microphotograph. All of the immunostained semi-serial sections were observed, and the immunoexpression intensity level within the perikarya was documented in percentages that were defined as 100% in the control

groups. The data were expressed in the tabular form: -, 0%; ±, 0-20%; +, 20-50%; ++, 50-80%; +++, 80-100% intensity compared to controls (Table 4).

The TH- and Fos-ir neurons were counted in order to assess organic changes within midbrain dopaminergic nuclei. The first sampling tissue section for counting was taken when the most rostral TH-ir or Fos-ir cells were observed in the midbrain unilaterally (right side), and each following section was taken at an interval of 100 μm from the previous one. Then only neurons containing perikarya clearly stained for TH and cell nuclei clearly stained for Fos within the right side of the A9, A10, and A8 areas were counted separately through the entire midbrain. The total number of neurons from A9-A10-A8 was then calculated.

The total number of TH-ir perikarya was estimated by using the following equation (Zuccolilli, *et al*., 1994):

$$N = \frac{d}{n.s.} \sum_{i=1}^{n} x$$

Where, N = total estimated number of TH-ir perikarya; d = length (μm) of the rostrocaudal axis of the midbrain being assessed; n = number of noncontiguous slices counted per midbrain; s = thickness of the section (10 μm); x = number of perikarya counted per noncontiguous slice assessed. Therefore, n represents approximately one-tenth of the total number of slices obtained from each midbrain.

Statistical analysis: The data on the numbers of TH- and Fos-ir neurons, and the weights of bodies and brains, were analyzed by one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests to determine individual differences, performed with StatView software for Windows (version 5.0; SAS Institute Inc., Cary, USA). The results were considered significant when the *p* value was less than 0.05.

Results

Body and brain weights: The body and brain weights at each time point investigated are summarized in Table 3. Here, I confirmed that fetal and neonatal exposure to EDs influenced the growth of both the body and the brain. All of the sole administration groups showed significantly lower body weight than vehicle-treated groups (controls) at 2 wk ($p < 0.01$), and their brain weight was also significantly lower than that of controls at 6 wk (sole administration groups of BPA and DEHP, $p < 0.05$; the TCDD group, $p < 0.01$). Only mice treated with mixed (BPA + DEHP + TCDD) chemicals (mixture groups) showed significantly lower brain weight than controls at 2 wk. The mice treated with DEHP alone (DEHP groups) did not recover their body weight to the control level, in contrast to the mice in the mixture groups, which maintained control level of body weight throughout the experimental periods. All ED-treated groups showed significantly higher relative weights of the brains than controls at 2 wk (sole groups, $p < 0.01$; mixture groups, $p < 0.05$).

Immunohistochemical findings: The distributions of TH-immunoreactivity were granularly detected in perikarya of the neurons and were also observed within axons and dendrites within the mouse midbrains. The nuclei of TH-ir neurons were round or ovoid, and immunoreactivity was not detected there (Fig. II-3A). The immunohistochemical findings of TH-ir neurons were well supported in a reference (Tanida, *et al.*, 2009). The Fos-immunoreactivity was detected in round or ovoid cell nuclei within A9, A10, and A8 neuronal nuclei (Fig. II-3B). Comparatively large numbers of Fos-ir neurons were detected within the A9 (SNc and SNl) and A10 areas. The neurons stained for both Fos and TH (double-ir neurons) were hardly detected within the midbrain dopaminergic nuclei of any group. A few of them were detected within the A9 or A10 areas. The photomicrograph is present in Fig. II-3C.

The intensity of TH-immunoreactivity: Table 4 and Figs. II-4-6 summarize the intensity of TH-immunoreactivity and typical photomicrographs of TH-ir neurons within midbrain dopaminergic nuclei at each time point investigated. Figs II-4, II-5, and II-6 correspond to the transverse lines of a, b, and c of Fig. II-2, respectively. In general, sole administration groups show lower intensity of TH-immunoreactivity compared to control group at least at 6 wk of age whereas mixture groups maintained the intensity to control level throughout the experimental period. At 4 wk of age, all the administration groups show control level of the intensity. BPA treatment seems to result in the most severe effect on the intensity level among the administration groups. DEHP group show slightly lower intensity than controls at 2 and 6 wk within A9 and A8 whereas A10 of DEHP group show weak intensity compared to controls at 6 wk of age. TCDD groups show slightly lower intensity of the immunoreactivity at 6 wk of age.

Numbers of TH-ir neurons: The numbers of TH-ir neurons at each time point investigated are summarized in Fig. II-7. The results here also show the different effects of chemicals among BPA, DEHP, and TCDD as well as between sole and mixed administrations. Significantly lower numbers of TH-ir neurons were detected between sole administration groups and age-matched controls within the A9 area at 4 (BPA, $p < 0.05$) and 6 wk (BPA, $p < 0.01$; DEHP, $p < 0.05$; TCDD, $p < 0.01$) and/or within the A10 area at 2 (BPA, $p < 0.05$) and 4 wk (BPA, $p < 0.05$; DEHP, $p < 0.05$). Only the A10 area of the TCDD group at 2 wk contained significantly greater numbers of TH-ir neurons ($p < 0.01$). All the sole administration groups showed significantly lower numbers of total TH-ir neurons at 6 wk compared to controls. Interestingly, the mixture groups did not show statistical significance compared to the controls at any age investigated (Table 5).

Numbers of Fos-ir neurons: The numbers of Fos-ir neurons are summarized in Fig. II-8.

Fos-ir neurons were sparsely distributed within the nuclei compared to TH-ir neurons throughout the experimental period. I observed that the mean number of Fos-ir perikarya were increased from 2 to 4 wk and decreased from 4 to 6 wk in all of the groups investigated. A9 of BPA and TCDD groups tended to contain greater numbers of Fos-ir neurons than controls at 2 wk ($p = 0.054$) and 6 wk ($p = 0.070$), respectively. A8 of the DEHP group showed a significantly greater number of Fos-ir neurons at 4 wk ($p < 0.05$) than controls. The total numbers of Fos-ir neurons tended to larger within BPA group at 2 wk ($p = 0.073$) and DEHP group at 6 wk ($p = 0.076$), respectively. I failed to detect a statistically significant influence in the numbers of Fos-ir neurons between any of the other administration groups and the age-matched controls (Table 5).

Discussion

The present study has demonstrated the influences of *in utero* and neonatal exposure to environmental chemicals such as BPA, DEHP, TCDD, and their mixture on neurodevelopment, especially on the development of midbrain dopaminergic nuclei. The NOAEL of BPA or DEHP is determined as 50 or 3.7 mg/kg/day, respectively, by various *in vivo* toxicity studies, including reproductive toxicity tests (Kubo, *et al*., 2003; Poon, *et al*., 1997). The LOAEL of TCDD is judged to be 200 ng/kg (Gray, *et al*., 1997a, b). All of these chemicals were injected below their NOAEL or LOAEL in order to investigate the effect of low-dose administration in the present study. The vulnerability of the developing brain to chemicals reportedly depends on the duration of exposure to them (Jacobson 1991; Rice and Barone 2000). I used both transplacental and oral administration during the fetal and neonatal periods, when the murine nervous system develops, in order to investigate the influence of practical exposure routes on brain development.

The calculated body and brain weights suggest that sole exposure to BPA, DEHP, or TCDD during fetal and neonatal periods disturbs not only body growth but also brain development, even at the lower doses of their NOAEL or LOAEL. Moreover, the weight changes in the mixture groups demonstrate the different effects that exposure to mixed EDs has on body and brain development compared to sole exposure. Immunohistochemical analyses suggest that each chemical by itself affects development of the midbrain dopaminergic nuclei in the case that mammals, including humans, are exposed to them during fetal and neonatal periods. However, I was unable to detect either an additive or a synergistic effect of *in utero* and neonatal exposure to mixed EDs on the intensity of TH-immunoreactivity or on the numbers of TH-ir and/or Fos-ir neurons within midbrain dopaminergic nuclei. This is presumably because of the antagonistic effect through *in vivo* mechanisms, including the responses by both the organ and intracellular signaling.

Some studies have reported the effect of perinatal exposure to EDs by using motor

activity tests; exposure to several EDs (87 nmol/rat) including BPA, *p*-octylphenol, nonylphenol, dibutylphthalate, dicyclohexylphthalate, and DEHP in the neonatal period disrupts neurodevelopment related to hyperactivity concomitantly with a lower level of TH-ir within midbrain dopaminergic nuclei in the juvenile period (Ishido, *et al*., 2004a, b, 2005). Our results at least reveal that environmental chemicals such as phenols, phthalates, and dioxins below NOAEL or LOAEL can cause the loss of midbrain dopaminergic neurons and/or the decrease of TH-biosynthetic activity related to motor activity in case of exposure during the fetal and neonatal periods. This implies that EDs contribute to the incidence of neurodevelopmental disorders such as ADHD.

Fos is an immediate early gene and is used for various neuroanatomical studies as an immunohistochemical marker of neuronal activation (Hoffman, *et al*., 1993; Valjent, *et al*., 2004). DEHP-treated mice (A8, 4 wk) activated Fos within physiological levels independent of stress reaction, suggesting that fetal and neonatal exposure to EDs induces chronic or long-lasting alterations of not only TH but also Fos activation. Because the mice were bred without any extra stimuli, the significantly high activation of Fos in the normal condition suggests the dopaminergic nuclei within midbrain have become sensitive to the physiological stress. I hypothesize that the threshold level of Fos expression within the nuclei was significantly reduced by the *in utero* and neonatal exposure to DEHP in the mice treated with the chemicals. The patterns of Fos activation and/or TH repression in this study may be involved in the processes of reward and addiction.

Thyroid hormones, which play crucial roles in the normal brain development of both humans and experimental animals (Zoeller, 2005), are likely to be associated with dopamine expression within the midbrain. Genetically hypothyroid mouse had significantly fewer TH-ir neurons within both SN and VTA (Kincaid, 2001). Moreover, dopamine was absent in the SN and VTA of Nurr1-null mice. These mice deplete a transcription factor belonging to a gene family that includes receptors for thyroid hormone consistent with absent TH, L-aromatic

amino acid decarboxylase, and other dopaminergic neuron markers (Castillo, *et al*., 1998). In addition, some chemicals disrupt thyroid hormone-dependent neuronal development (Kimura-Kuroda, *et al*., 2007; Nakamura, *et al*., 2006); Because BPA can bind to the thyroid hormone receptor (TR) and inhibit the thyroid hormone's ability to regulate gene expression (Moriyama, *et al*., 2002), BPA's inhibitory effects on normal brain development may disturb TH expression of dopaminergic neurons via thyroid hormone-dependent gene regulation.

Some studies have reported possible antagonistic effects of phthalates on the thyroid gland *in vivo* and on thyroid tissue *in vitro* (Poon, *et al*., 1997; Sugiyama, *et al*., 2005; Pereira, *et al*., 2007). TCDD is also thought to affect thyroid hormone-related systems because animals exposed to high concentrations of it showed morphological abnormalities of thyroid tissue and reduced blood levels of thyroxine (Portier, 2002; Sewall, *et al*., 1995; Weber, *et al*., 1995). Members of the TCDD group, except for BPA and DEHP, increase the cellular responses to triiodothyronine by hyperactivating TR-mediated gene expression in HeLaTR cells (Yamada-Okabe, *et al*., 2004). The thyroid hormone receptor/retinoblastoma-interacting protein 230 interacts directly with AhR nuclear translocator (ARNT) and is essential for TCDD-mediated transcriptional responses (Beischlag, *et al*., 2004). Moreover, the disruption of thyroid hormone is mediated entirely via AhR (Nishimura, *et al*., 2005). These findings may reflect the different temporal pattern in the number of TH-ir neurons in the TCDD group compared to the BPA and DEHP groups (Fig. II-7A). The different effect of TCDD, compared to BPA or DEHP, on thyroid hormone-mediated gene expression may inhibit the numbers of TH-ir neurons in mixture groups in case TH expresses via the TR-related signaling pathway.

In contrast to the additive or synergistic effect of a mixture of EDs (Hotchkiss, *et al*., 2004), mixtures including phenols, plasticizers, and dioxins have negative effects (Takano, *et al*., 2006; Krűger, *et al*., 2008). Krűger, *et al*. (2008) investigated the effects of various EDs *in vitro* using luciferase reporter gene assay and observed an antagonistic effect on

TCDD-induced AhR transactivity caused by a mixture of several phenols and phthalates. In addition, the metabolite of DEHP, mono-(2-ethylhexyl) phthalate, was reported to induce the gene expression of AhR in granulose cells (Lovekamp-Swan, *et al*., 2003). This suggests that DEHP may activate AhR via its metabolite. The AhR-overexpressed murine neuroblastoma Neuro2a cells expressed TH mRNA (Akahoshi, *et al*., 2006). AhR, ARNT, and ARNT2 mRNAs are widely distributed throughout the brain and brainstem, including SN and VTA in murine (Petersen, *et al*., 2000). Although there is a discrepancy in cell lines or between *in vivo* and *in vitro*, our results for the mixture groups--in which neither a lower intensity of TH nor significantly lower numbers of TH-ir neurons compared with controls--may reflect the complicated AhR pathway, AhR-related factors, and TR-mediated gene expression. There might be interferential action in the cell signaling pathways related to AhR and TH.

In summary, I have demonstrated that *in utero* and neonatal exposure to EDs such as low doses of BPA, DEHP, or TCDD alone resulted in immunohistochemical and presumably functional changes in midbrain dopaminergic nuclei of mice, whereas such changes did not appear when the animals were exposed to mixtures of the same chemicals, hypothetically because of counteraction caused by thyroid hormones and/or aryl hydrocarbon receptor-related mechanisms. From the comparison data between brain weights of the mixture groups and those of controls (Table 3), this canceling effect is just one apparent response. These results suggest that the practical effects of exposure to multiple EDs that are ubiquitous in the environment are very unique and elusive, since the effects of EDs on neurodevelopment may apparently disappear, leading us to propose a new facet of responses to pollution from combined EDs. The combined effect of all the chemicals present in the human body must be considered in future risk assessments for human health.

Summary

A major question is whether exposure to mixtures of low-dose endocrine disruptors (EDs) having different action mechanisms affects neurodevelopment differently than exposure to EDs individually. I therefore investigated the effects of fetal and neonatal exposure to three typical EDs--bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl) propane), di-(2-ethylhexyl)-phthalate (DEHP), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)--on the midbrain dopaminergic system associated with functions--including motor activity, emotion, and cognition--affected by neuropsychiatric diseases such as attention-deficit/hyperactivity disorder. ICR mouse dams and their pups were orally treated with BPA (5 mg/kg/day), DEHP (1 mg/kg/day), or TCDD (8 ng/kg) individually, or with mixtures thereof, to compare the effects between sole and mixed administration. I analyzed tyrosine hydroxylase (TH)- and Fos-immunoreactive (ir) neurons as markers of dopamine and neuronal activation, respectively. The numbers of TH- and/or Fos-ir neurons and the intensity of TH-immunoreactivity within midbrain dopaminergic nuclei (A9, A10, and A8) of each sole administration group significantly differed from controls at 2, 4, and 6 weeks of age. In contrast, no significant differences were detected in the mixture groups, suggesting counteractions among those chemicals. These results indicate that ED mixtures as pollution have unique and elusive effects. Thyroid hormones and/or aryl hydrocarbon receptor-related mechanisms may be responsible for this counteraction.

Figures and Tables

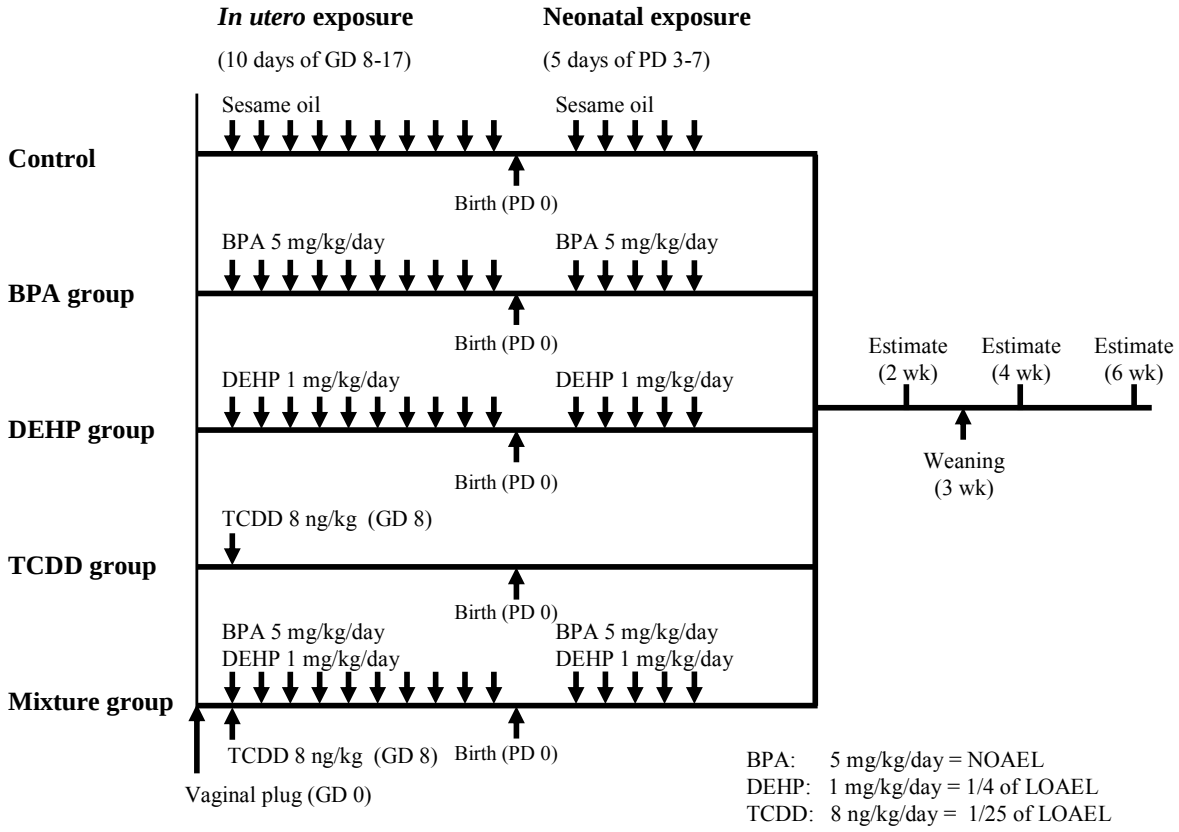


Fig. II-1. The pattern diagrams showing the dosing regime of our test: ICR dams were orally injected with BPA, DEHP, TCDD, mixture (BPA + DEHP + TCDD), or vehicle. BPA, DEHP, or vehicle was administered daily during GD 8-17 and PD 3-7, whereas TCDD was singly administered at GD 8. The pups were decapitated at 2, 4, and 6 wk of age and used for our study.

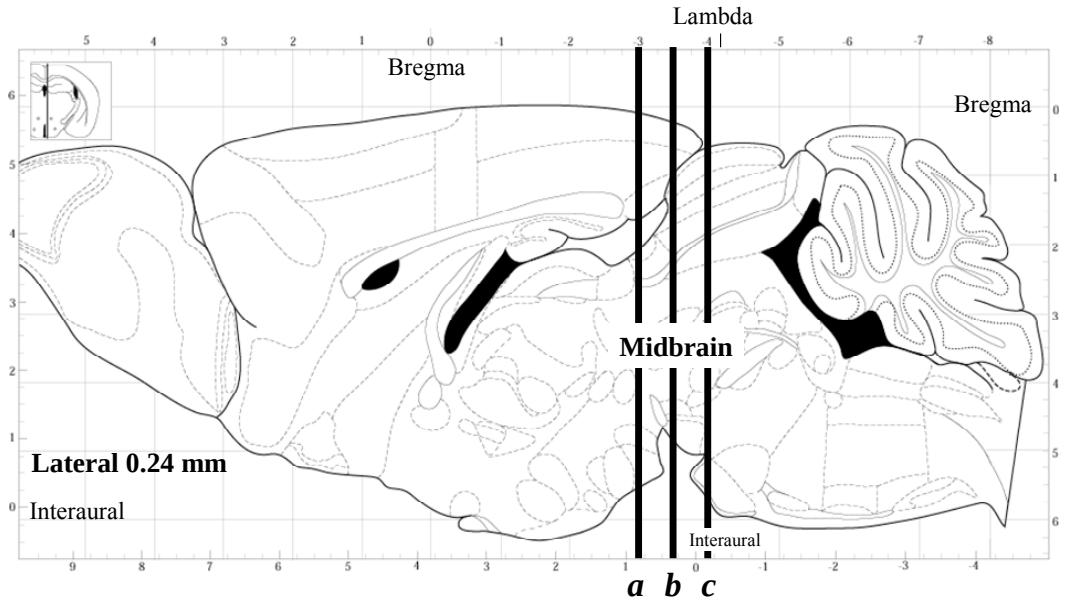


Fig. II-2. Sagittal section of the mouse brain from Paxinos and Franklin (2001). The transverse lines of *a*, *b*, and *c* show brief coordinates of the following Figs, which correspond to the rostral (Fig. II-4), intermediate (Fig. II-5), and caudal (Fig. II-6) parts of the midbrain, respectively.

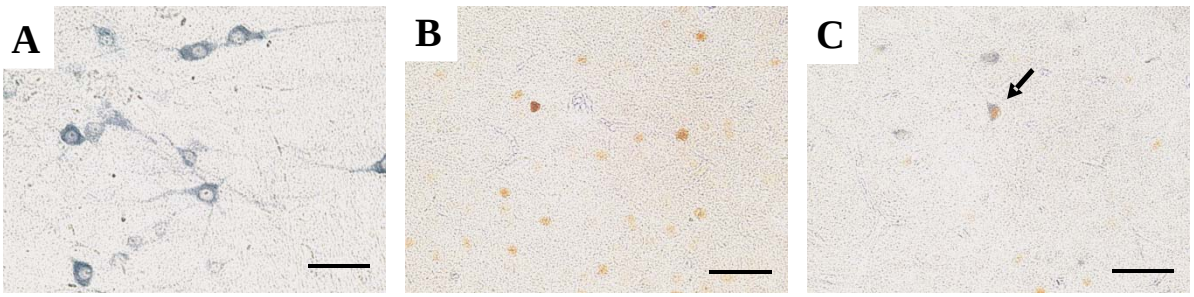


Fig. II-3. TH-ir (A), Fos-ir (B), and Double-ir (C) neurons stained immunohistochemically. The TH-ir neuron contains a deeply stained cell body, axon, dendrite, and nucleolus, whereas its nucleus except for the nucleolus is not stained (A). The nucleus of the Fos-ir neuron is deeply stained (B). The double-ir neuron (arrow) contains a blue-stained perikaryon and a brown-stained nucleus, showing the characteristics of both TH-ir and Fos-ir neuron (C). Bar = 100 μm.

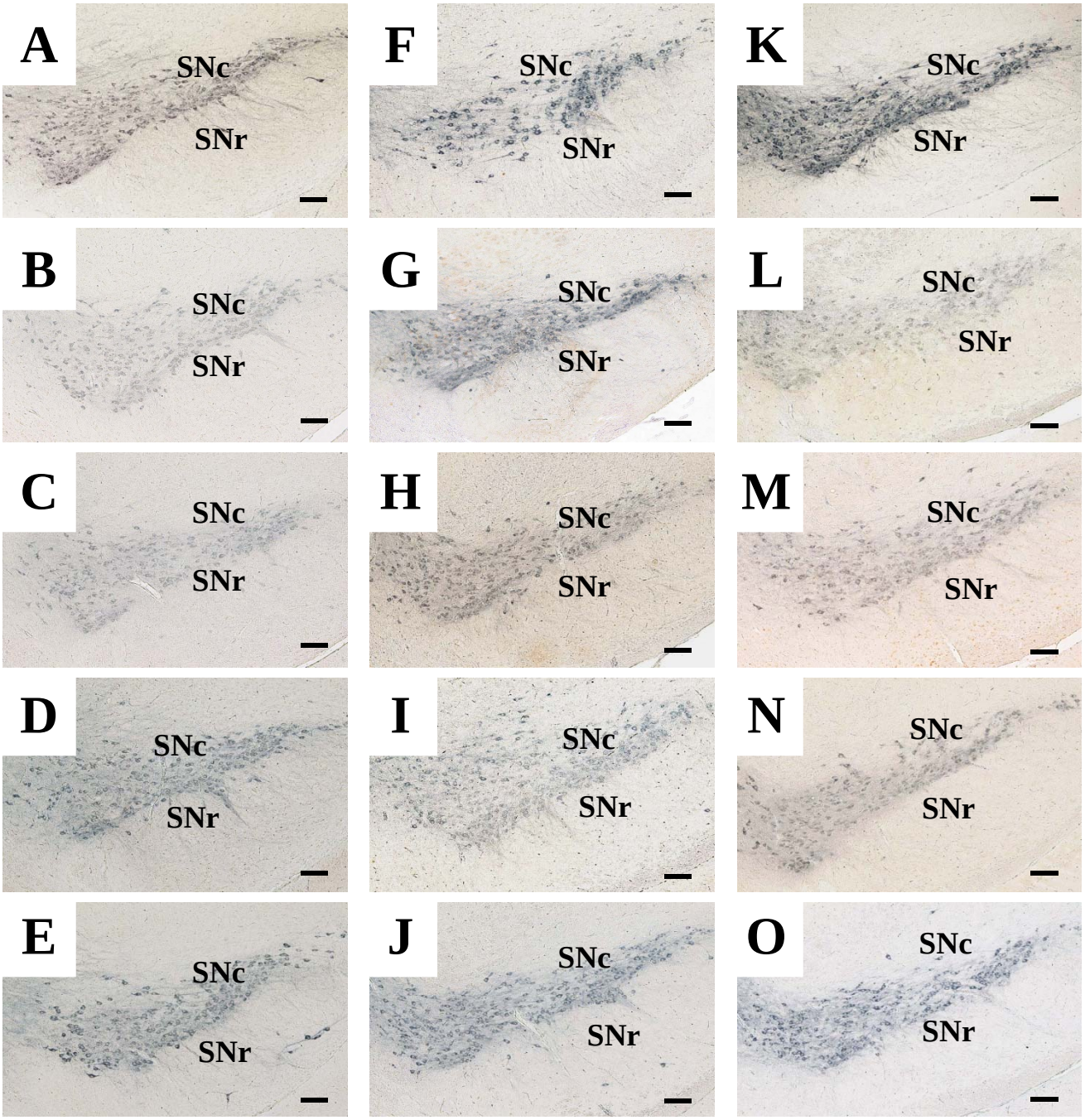


Fig. II-4. TH-ir neurons within A9 area of 2- (A-E), 4- (F-J), and 6-wk-old (K-O) mice corresponding to line *a* in the sagittal diagram (Fig. II-2). Each photomicrograph shows the vehicle (A, F, K), BPA (B, G, L), DEHP (C, H, M), TCDD (D, I, N), and mixture (E, J, O) group. Note that the TH-ir intensity of BPA groups seems to be quite lower than those of the controls at 2 and 6 wk. Slightly lower intensity of TH-immunoreactivity than controls was detected in the DEHP groups at 2 and 6 wk and in the TCDD group at 6 wk. Bar = 100 μm.

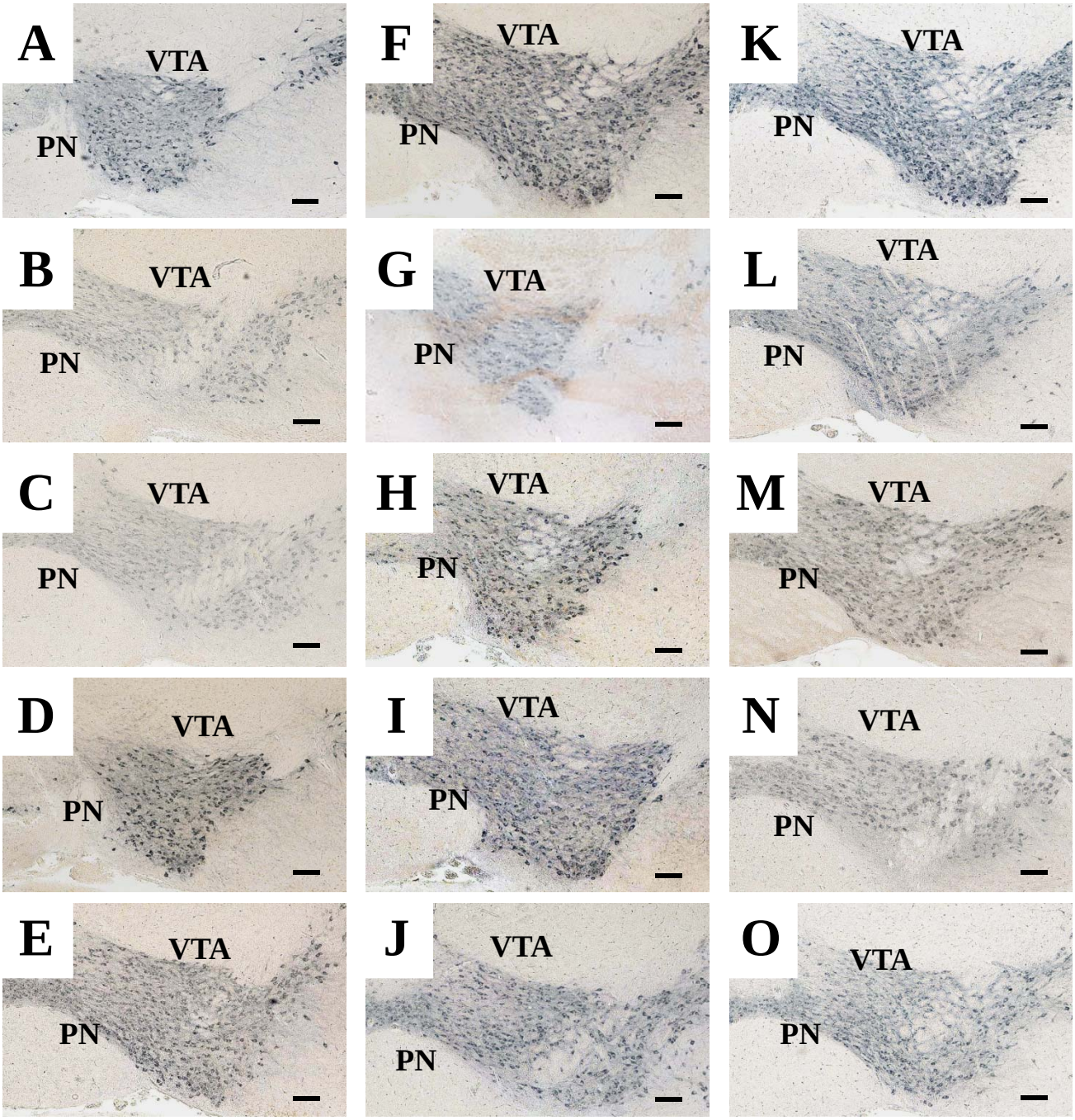


Fig. II-5. TH-ir neurons within the A10 area of 2- (A-E), 4- (F-J), and 6-wk-old (K-O) mice corresponding to line *b* in the sagittal diagram (Fig. II-2). Each photomicrograph shows controls (A, F, K), BPA (B, G, L), DEHP (C, H, M), TCDD (D, I, N), and the mixture (E, J, O) group. The BPA group and the DEHP group showed much lower or slightly lower intensity of TH-immunoreactivity compared to controls at 2 wk, respectively. All of the single-administration groups showed quite weak intensity of TH-immunoreactivity compared to controls at 6 wk. The mixture groups exhibited the same intensity level as controls throughout the investigated time points. Bar = 100 μm.

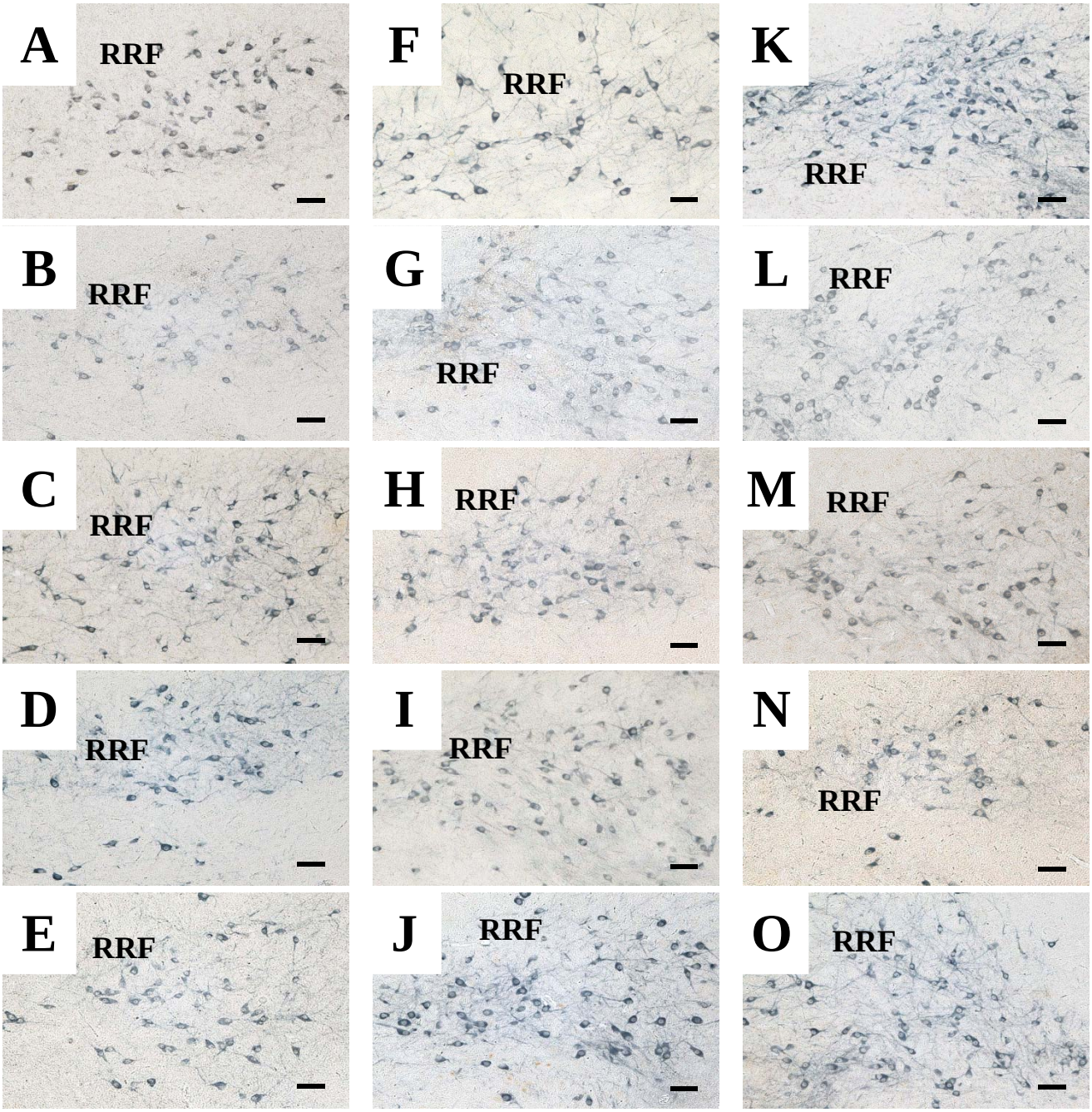


Fig. II-6. TH-ir neurons within the A8 area of 2- (A-E), 4- (F-J), and 6-wk-old (K-O) mice corresponding to the line *c* in the sagittal diagram (Fig. II-2). Each photomicrograph shows controls (A, F, K), BPA (B, G, L), DEHP (C, H, M), TCDD (D, I, N), and the mixture (E, J, O) groups. The BPA group and the DEHP group showed much lower or slightly lower intensity of TH-immunoreactivity, respectively, compared to controls at 2 wk. At 6 wk, the BPA and DEHP groups exhibited markedly weak intensity of TH-immunoreactivity, and the TCDD group showed slightly weak intensity of TH-immunoreactivity compared to controls. The intensity level as controls was seen in the mixture groups at all of the investigated time points. Bar = 100 μm.

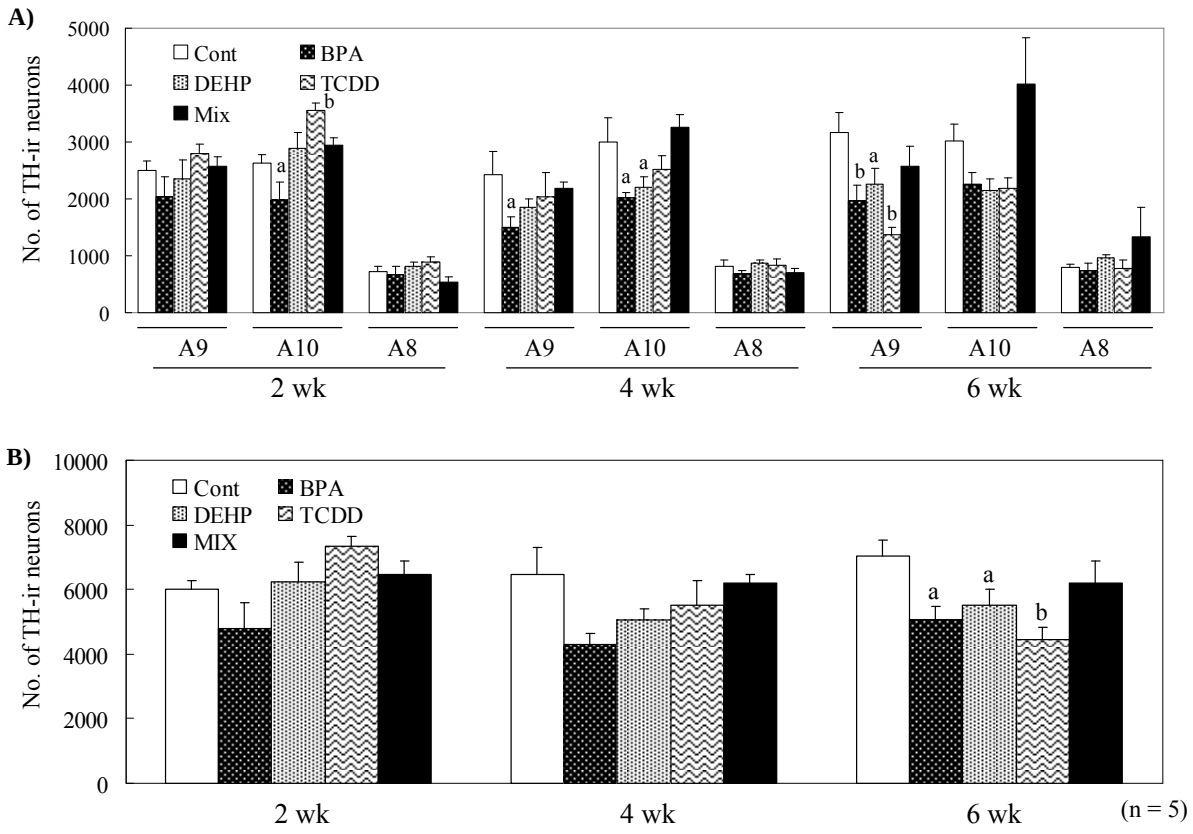


Fig. II-7. The number of TH-ir neurons within the A9, A10, and A8 areas (A) and the total (B) at each time point investigated. Significantly lower numbers of TH-ir neurons were detected within the A9 area at 4 wk for BPA treatment and within the A10 area at 2 wk in the BPA-treated group, at 4 wk for both the BPA and DEHP groups, and at 6 wk of all the sole administration groups compared to controls. The A10 area of the TCDD group contained significantly more TH-ir neurons compared to controls at 2 wk. Note that the mixture groups did not show significance at any weeks of age (A). Each singly administered group contained significantly fewer TH-ir neurons at 6 wk. In contrast, the mixture groups did not show significance at any time point investigated (B). Values are the means ± SE (n = 5 replicates). The data were analyzed using Bonferroni multiple comparison test. [a] $p < 0.05$; [b] $p < 0.01$ (significantly different from controls).

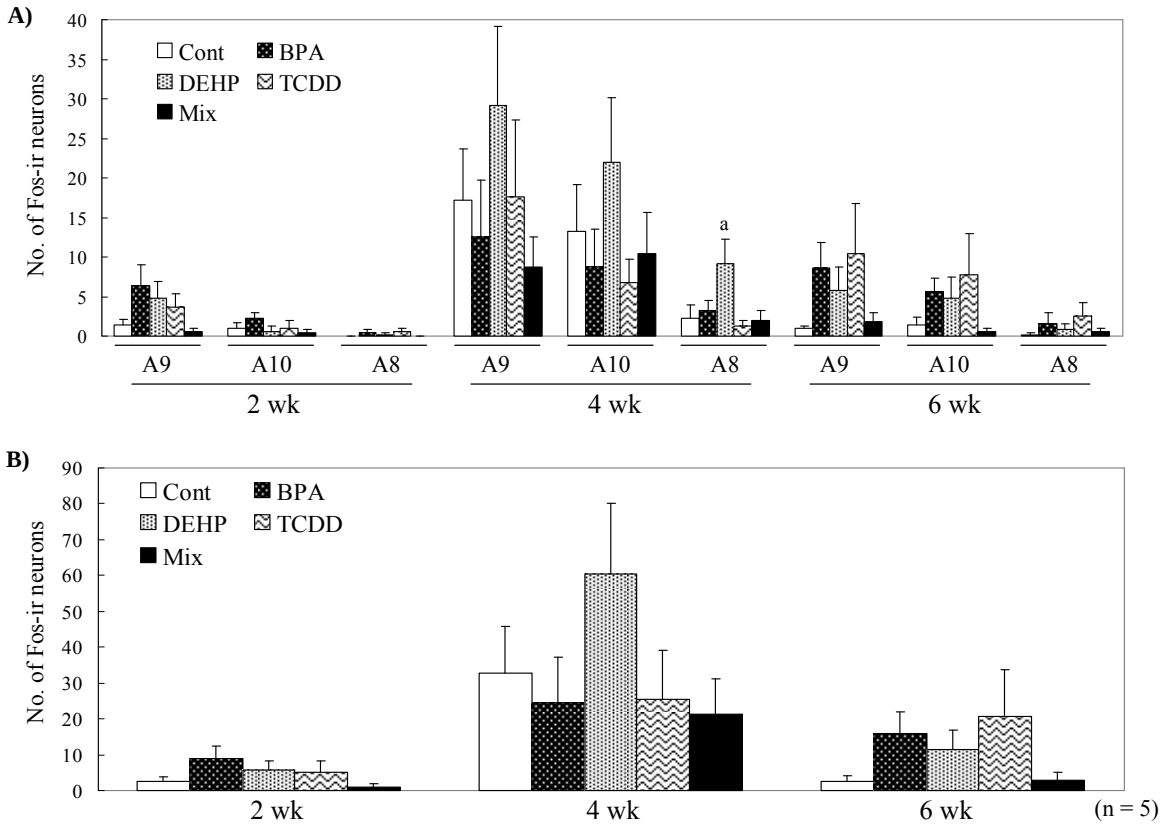


Fig. II-8. The number of Fos-ir neurons within A9, A10, and A10 (A) and the total (B) at each time point investigated. Significantly larger numbers of Fos-ir neurons were detected within the A8 area of the DEHP group at 4 wk compared to controls. None of the other groups showed significance compared to controls (A). None of administration groups show a significant difference in the total number of Fos-ir neurons compared to controls (B). Values are means ± SE (n = 5 replicates). The data were analyzed using Bonferroni's multiple comparison test. [a] $p < 0.05$; [b] $p < 0.01$ (significantly different from control group).

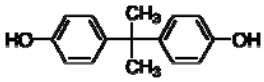
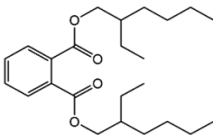
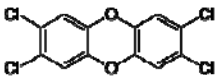
	Bisphenol A (BPA)	**DEHP**	**TCDD**
CAS RN.	80-05-7	117-81-7	1746-01-6
Chemical name	2,2-Bis-(4-hydroxyphenyl)-propane	Di-(2-ethylhexyl)-phthalate	2,3,7,8-Tetrachlorodibenzo -*p*-dioxin
Formula	$C_{15}H_{16}O_2$	$C_{24}H_{38}O_4$	$C_{12}H_4O_2C_{l4}$
Common usage	Polycarbonate plastic Pesticide adjuvant	Plasticizer Sealant	-
Chemical structure			

Table 1. Environmental chemicals treated in this work. The CAS RN., chemical name, formula, common usage, and chemical structure are presented.

Administration Group	Dam ID	Litter size		
		Female	Male	Total
Control	1101	6	2	8
	1102	7	6	13
	1104	9	10	19
	1105	6	8	14
	1106	4	6	10
	1107	8	5	13
	1108	6	4	10
	Total	46	41	87
BPA	2101	6	8	14
	2102	5	5	10
	2103	5	7	12
	2104	5	4	9
	2105	4	11	15
	2106	4	4	8
	Total	29	39	68
DEHP	3101	6	5	11
	3102	4	8	12
	3103	5	7	12
	3104	4	2	6
	3105	6	9	15
	Total	25	31	56
TCDD	4101	5	9	14
	4102	8	6	14
	4103	4	5	9
	4104	5	8	13
	4105	6	6	12
	Total	28	34	62
Mixture	5101	6	6	12
	5102	5	8	13
	5103	8	5	13
	5104	2	3	5
	Total	21	22	43

Table 2. The numbers of female, male, and total mice born from each dams. Seven (Control), 6 (BPA), 5 (DEHP), 5 (TCDD), and 4 (Mixture) dams were used for the experiment, respectively.

	Age (weeks)	Administration Group				
		Control	BPA	DEHP	TCDD	Mixture
No. of Examined Mice	2	7	9	6	6	6
	4	14	9	6	8	6
	6	14	7	7	6	6
Body Weight (g)	2	8.16 ± 0.18	7.13 ± 0.34**	7.07 ± 0.28**	6.73 ± 0.092**	7.88 ± 0.15
	4	23.86 ± 0.63	23.69 ± 0.78	21.63 ± 0.52*	21.85 ± 0.54*	24.52 ± 0.42
	6	33.49 ± 0.67	31.83 ± 0.70	31.36 ± 0.60*	32.32 ± 0.54	33.38 ± 0.86
Brain Weight (mg)	2	388 ± 6.72	377.67 ± 8.68	384.50 ± 5.25	385.17 ± 6.24	412.83 ± 5.56*
	4	456.93 ± 3.59	457.89 ± 7.96	450.83 ± 5.11	432.86 ± 5.58**	472.50 ± 5.38
	6	478.14 ± 4.41	461.14 ± 5.52*	458.43 ± 8.25*	456.33 ± 3.63**	470.33 ± 6.44
Brain Relative Weight (%)	2	4.76 ± 0.063	5.35 ± 0.17**	5.47 ± 0.16**	5.72 ± 0.061**	5.24 ± 0.11*
	4	1.93 ± 0.057	1.94 ± 0.039	2.09 ± 0.038*	1.99 ± 0.048	1.93 ± 0.033
	6	1.43 ± 0.023	1.45 ± 0.036	1.46 ± 0.021	1.41 ± 0.019	1.41 ± 0.019

Table 3. Body, brain, and brain relative weight at each time point investigated (mean ± SE). The data were analyzed using Bonferroni's multiple comparison test. *$p < 0.05$, **$p < 0.01$ (significantly different from controls).

		2 wk	4 wk	6 wk
A9	Control	+++	+++	+++
	BPA	±	+++	±
	DEHP	++	+++	++
	TCDD	+++	+++	++
	Mixture	+++	+++	+++
A10	Control	+++	+++	+++
	BPA	±	+++	±
	DEHP	+++	+++	+
	TCDD	+++	+++	+
	Mixture	+++	+++	+++
A8	Control	+++	+++	+++
	BPA	+	+++	±
	DEHP	++	+++	++
	TCDD	+++	+++	++
	Mixture	+++	+++	+++

Table 4. The intensity of TH-immunoreactivity within A9, A10, and A8 of midbrain dopaminergic nuclei at each time point was investigated. The intensity was evaluated in immunohistochemical analysis by scoring: 0%; ±, 0-20%; +, 20-50%; ++, 50-80%; +++, 80-100% intensity compared to controls, respectively (n = 5 replicates).

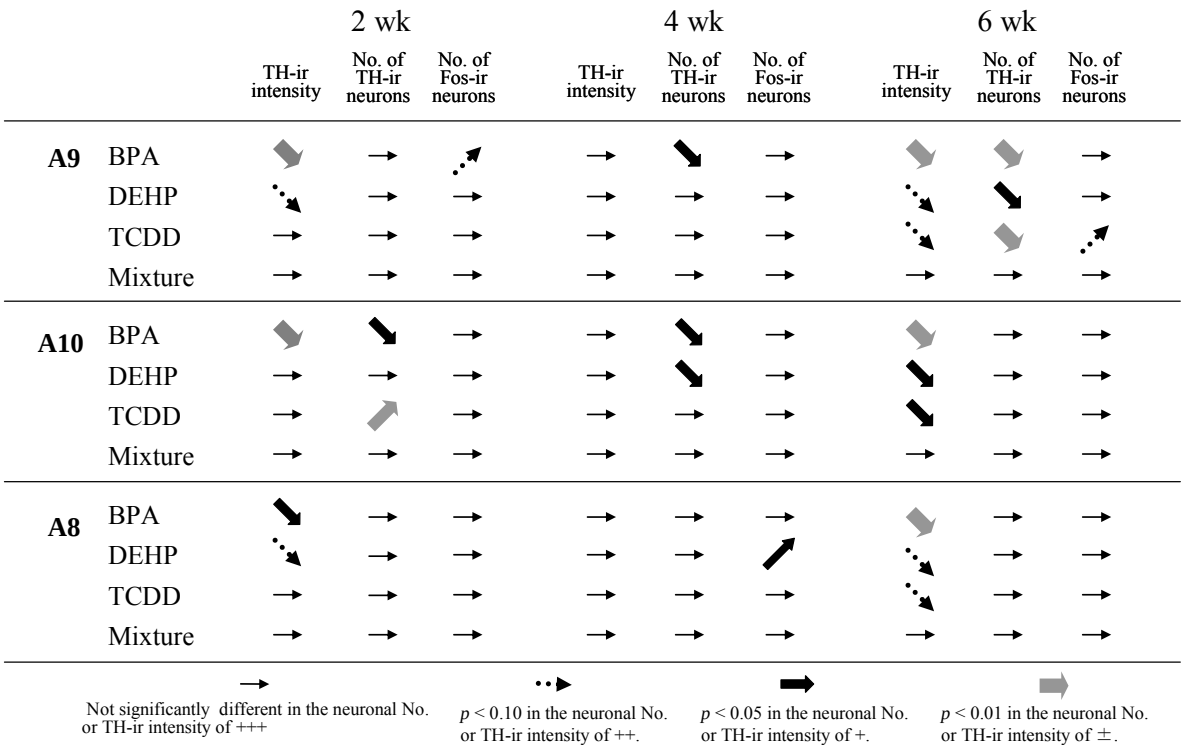


Table 5. The outline of this study. The arrows represent the statistical differences in the numbers of TH- and Fos-ir neurons or the level of TH-ir intensity compared to controls. Note that sole-administration groups showed the influence of perinatal exposure to EDs on the development of midbrain dopaminergic nuclei, whereas the mixture groups did not throughout the investigated ages in weeks (n = 5 replicates).

Conclusion

Originally, the public concern has been focused on the relation of endocrine disruptors (EDs) to wild animals since the publication of 'Our Stolen Future' (Colborn, *et al*., 1996) that indicates disruptions of their reproductive systems by EDs. Since then, numbers of reports has demonstrated the hormonal activity of environmental chemicals and has claimed the risk to development and differentiation of brains as well as reproductive function of mammals, including humans. Because the functions associating to midbrain dopaminergic system is sexually dimorphic in some clinical diseases such as Parkinson's disease and attention-deficit/hyperactivity-disorder (ADHD), the factors controlling the sex differentiation of midbrain dopaminergic system including sex steroid hormones and sex chromosomal genes may involve pathogenetic mechanisms of neuropsychiatric disorders, implying the possible involvement of the EDs in the disorders.

Chapter I demonstrates the postnatal profiles of age-related changes and sex-specific development patterns in the numbers of dopaminergic neurons within the midbrain of the female and male C3H mouse. The profiles in this study could provide a framework for further investigations of the C3H mouse midbrain. Importantly, the sex-specific developmental pattern of dopaminergic nuclei of the midbrain suggests that some sex steroid hormones-and/or sex chromosomal genes-related factors involves in its development and differentiation, supposing the vulnerability to the environmental chemicals. I hypothesize that those differences reflect the direct regulation of *Sry* (sex determining region of the Y chromosome) or the multiple effects of both *Sry* and sex steroid hormones [Tanida, *et al*., *J. Vet. Med. Sci.*, **71**, 855-863].

In our living environment, numerous chemicals ubiquitously exist in various concentrations. Even though some low-dose environmental chemicals were determined to be safe by toxicological tests, they may become synergistically-activated. However, few studies assess the risk of mixed exposure to environmental chemicals. In the Chapter II, I have demonstrated that fetal and neonatal exposure to typical EDs such as low doses of bisphenol

A (BPA), di-(2-ethylhexyl)-phthalate (DEHP), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alone resulted in irreversible changes of midbrain dopaminergic nuclei of mice in immunohistochemical and presumably functional level, whereas such changes did not appear when the animals were exposed to mixtures of the same chemicals, hypothetically because of counteraction caused by thyroid hormones and/or aryl hydrocarbon receptor-related mechanisms. I think that this canceling effect is just one apparent response. These results suggest that the practical effects of exposure to multiple EDs that are ubiquitous in the environment are very unique and elusive, leading us to propose a new facet of responses to pollution from combined EDs. The combined effect of all the chemicals present in the human body must be considered in future risk assessments for human health [Tanida, *et al*., *Toxicol.Lett*., **189**, 40-47].

Summary in Japanese

科学技術の急速な発展の中で合成された数多くの化学物質の中には，極めて微量でも内分泌攪乱作用を示すことにより生殖機能の破綻を引き起こすものがある。これらの多くはエストロゲン様の活性を有しており，種の絶滅を導く潜在的危険性をはらんでいることが指摘され，「内分泌攪乱化学物質（endocrine disruptors; EDs）」という概念が提唱された。哺乳動物の性特異的な脳の構造と機能は，胎生後期から新生子期に分泌される性ステロイドの有無に左右される脆弱性の上に成り立っていることから，性ステロイド様作用を示す外来化学物質は脳の性分化にも影響を及ぼすと懸念されている。更に近年，脳の性分化だけではなく，脳の発達や精神神経疾患にもこのような作用を有する化学物質が関わっているとする報告が相次いでなされている。

近年増加傾向にあるとされる注意欠陥多動性障害（attention-deficit/hyperactivity-disorder; ADHD）の罹患率は男児の方が女児より多く，EDsの胎子期曝露は中脳ドーパミン作動性ニューロンの減少および多動といった6-水酸化ドーパミンを投与したADHDモデルマウスと同様の症状を示す。中脳ドーパミン作動性ニューロンの選択的脱落を特徴とするパーキンソン病の罹患率もまた男性の方が女性より多く，いくつかの報告によりエストロゲン補充療法が閉経後の女性におけるパーキンソン病のリスクを著しく軽減し得ることが示されている。これらの報告は中脳ドーパミン作動性神経がエストロゲンを始めとする性ステロイドの制御を受けていることを非臨床・臨床の両面から示しているが，最近，脳において性ステロイド非依存的な性分化プロセスの存在を暗示する報告が相次いでおり，特に，中脳ドーパミン作動性神経においては*Sry*（sex determining region of the Y chromosome）等の性染色体上の遺伝子の直接的な作用によって性分化プロセスが調整されることが強く示唆されている。すなわち，中脳ドーパミン作動性神経の性分化および発達は，性ステロイドによる間接的な作用と遺伝子による直接的な作用の両方の制御を受けており，環境中微量化学物質がこれらの制御の中間因子に干渉していることが想定される。

EDsは食品，日用品，農薬，化粧品，医薬品，その他廃棄物等に含まれて様々な濃度で身の回りに存在し，複数の経路を介して複合的にヒトの体内に吸収されている。しかしなが

ら，外来化学物質が脳の発達過程を修飾することを示す報告のほとんどが単一の物質による影響に関するもので，実際の複合汚染を反映するものではない。

本研究では，認知機能，情動，行動に関与する中脳ドーパミン作動性神経の EDs への曝露が ADHD 等の精神神経疾患へのリスクを高める可能性があることを受け，この神経への EDs の胎子期・新生子期曝露の影響を解析し，単独投与と複合投与とで比較検討することで，低濃度複合汚染による性成熟過程への影響を解明することを試みた。

化学物質と中脳との関係を検証する基礎データとして，マウス中脳の性分化および発達について組織化学的および定量免疫組織化学的手法を用いて検証したところ，ドーパミン作動性神経核（A9，A10，A8）における性成熟過程におけるチロシン水酸化酵素発現（tyrosine hydroxylase-immunoreactive; TH-ir, ドーパミン産生ニューロンのマーカー）ニューロンの増加パターンが雌雄で異なることが明らかとなった。すなわち，TH-ir ニューロンの数が 3 日齢から 21 日齢にかけて雌の A9，A10，および合計（A9 + A10 + A8）で有意な増加を示し，21 日齢から 11 週齢にかけては雌において A8 で，雄においては A9 で，それぞれ有意に増加した。以上の結果は，脳の成長および性成熟過程において，認知，情動，および行動の制御に関連するドーパミン作動性神経に性差が存在することを示唆しており，上記のような内分泌攪乱作用を持つ物質が感受性の高い出生前後に曝露されると性分化や認知機能，情動行動を攪乱される可能性を示している。

続いて，日常生活において実際に曝露される機会が多く，作用点の異なる bisphenol A（BPA; 2,2-bis (4-hydroxyphenyl) propane），di-(2-ethylhexyl)-phthalate（DEHP），および 2,3,7,8-tetrachlorodibenzo-*p*-dioxin（TCDD）の 3 つの環境中微量化学物質を代表として投与し，マウスの胎子期および新生子期曝露における中脳ドーパミン作動性神経核（A9，A10，A8）への影響を，TH および Fos（神経活性のマーカー）を用いて定量免疫組織化学的に解析した。BPA はエストロゲン受容体結合能を有し，DEHP はアンドロゲン受容体を介さず，テストステロン生合成系を阻害することによって抗アンドロゲン作用を示す。TCDD はアリルハイドロカーボン受容体（aryl hydrocarbon receptor: AhR）結合型の化学物質であり，TCDD による神経毒性の発現機構はこの受容体を介するシグナル伝達路を経由すると考

えられている。尚，これら化学物質の投与量は無毒性量・最小毒性量以下の低用量とした。

その結果，TH 染色強度については，各単独投与群では 2 週齢および 6 週齢で対照群と比較して低下が認められたものの，複合投与群においてはこのような低下はいずれの週齢・神経核においても認められなかった。TH-ir ニューロン数については，BPA 投与群では 2 週齢の A10，4 週齢の A9 および A10，6 週齢の A10 において，また，DEHP 投与群では 4 週齢の A10，6 週齢の A9 において対照群よりも有意に少なかった。TCDD 投与群の TH-ir ニューロン数は，2 週齢の A10 では対照群よりも有意に多く，6 週齢の A9 では有意に少なかった。TH-ir ニューロン数の合計（A9 + A10 + A8）は，全ての単独投与群において，6 週齢で対照群と比べ有意な増加が認められた。Fos-ir ニューロン数は，4 週齢 DEHP 投与群の A8 において対照群と比べ有意に多かった。しかしながら，TH-ir，Fos-ir ニューロンのいずれの数についても，複合投与群においては対照群と比較した際にいずれの週齢・神経核でも有意差は認められなかった。すなわち，無毒性量あるいは最小毒性量以下の低用量であっても，胎子期・新生子期における環境中微量化学物質（BPA，DEHP，TCDD）への曝露は，中脳ドーパミン作動性神経核に免疫組織化学的なレベルでの変調を引き起こすことが明らかとなり，この神経に機能的な発達障害が起こり得ることが示唆された。更に，その変調は出生前後の曝露時期のみではなく，投与終了後の性成熟過程を通じて持続したことから，生理活性物質への感受性が非常に高い未成熟な神経の発達が環境中微量化学物質により不可逆的に影響されていることが強く示唆された。他の報告においても，不可逆的な悪影響を及ぼされる曝露時期は胎子期・新生子期のような未発達期であることが示されており，その時期および作用メカニズムを詳細に特定することが種々の発達障害を未然に防ぐ上でも重要であると考えられる。一方，複合曝露の場合，単独曝露時のような免疫組織化学的な変化は見受けられなかったが，対照群と比較した際の脳重量の有意な変化が 2 週齢では複合投与群でのみ認められたことから，神経発達への影響が化学物質の相互作用によって完全に打ち消された可能性は低いと考えられた。これは，環境中微量化学物質の複合曝露においては単独曝露とは異なる作用メカニズムが働いている，という複合汚染に対する新たな側面を示しており，「打ち消された」作用のメカニズムには，甲状腺ホルモン受

容体, AhR, および TH の相互作用が関与しているものと推察された。

今回の研究は, 中脳ドーパミン作動性神経核の性成熟過程における増加パターンの雌雄差, ならびに環境中微量化学物質の胎子期・新生子期複合投与による脳発達への影響を示した初めての成果である。また, 内分泌攪乱作用を持つ環境中微量化学物質の作用の複雑さ, 捉えどころの無さが如実に示された。単独の化学物質による影響に関する知見が蓄積してきた現段階においては, リスク評価に複合汚染を念頭に入れるべきであり, *in vivo*, *in vitro* 両面からの分子毒性遺伝学的手法を用いて, 複合効果のメカニズム解析およびリスク評価を包括的に行うことが期待される。

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