reductions in testicular weight and daily sperm production (DSP) at a dose as low as 64 ng TCDD/kg body weight. Some of the animals showed reduced fertility at higher doses (400 and 1000 ng TCDD/kg body weight), suggesting that maternal TCDD exposure induces defects in sperm production and causes male infertility. Gray and colleagues (Gray et al., 1997) reported that maternal TCDD exposure (50, 200 or 800 ng TCDD/kg body weight) on GD15 induces changes in the reproductive system of male Long–Evans (LE) rats, in which they detected reductions in the epididymal and ejaculated sperm counts; however, neither testicular weight nor DSP was affected at any of the doses used. Faqi and co-workers (Faqi et al., 1998a) gave female Wistar rats an initial loading dose of 25, 60 or 300 ng TCDD/kg body weight at 2 weeks prior to mating, followed by a weekly maintenance dose of 5, 12 or 60 ng TCDD/kg body weight, and reported a slight decrease in DSP with no changes in testicular weight. In other studies, no reduction in testicular weight was observed following maternal exposure to low-dose TCDD (Wilker et al., 1996). We also used the experimental protocol of Mably et al. (1992a) and detected severe reduction of the ventral prostate by TCDD, but there were no changes in testicular weight or DSP after TCDD administration, even at the 800 ng/kg body weight dose (Ohsako et al., 2001). Taking all of these findings together, it is difficult to draw a conclusion concerning consistent dose–response relations between dioxins on sperm production.

In utero and lactational exposure to 1 μg TCDD/kg on GD15 resulted in reduction of the plasma testosterone level of male rat fetuses and lowered the magnitude of the testosterone surge in neonatal male rats (Mably et al., 1992b). Although it is still unclear how the neonatal testosterone surge influences the development of reproductive organs, such as the penis, prostate and testis, the reduction of testosterone production in fetal and neonatal life in maternally TCDD-exposed animals may contribute to developmental delay of the male reproductive system during puberty.

We intended to investigate the toxic effects of coplanar PCB congener, instead of TCDD, on mammalian spermatogenesis and steroidogenesis, because the level of dietary intake of coplanar PCB is estimated to be comparable to that of PCDDs and PCDFs (Safe, 1994). In the present study, we employed an organ culture of neonatal mouse testis and 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), a most potent coplanar PCB congener, having one-tenth the toxic potency of TCDD (Van den Berg et al., 1998), and studied whether PCB126 directly alters the activity of pre-spermatogenesis and somatic cell differentiation and gene expression levels in the testis. We used the neonatal mouse testis, since it is thought to be most susceptible to perinatal exposure to dioxin exposure. We demonstrate here that PCB126 did not directly affect proliferation of pre-spermatogenic cells and Sertoli cells in neonatal mouse testis, but directly down-regulated the level of expression of the P450scc gene and up-regulates expression of the P450c17 gene.

2. Materials and methods

2.1. Chemicals and materials

3,3′,4,4′,5-Pentachlorobiphenyl (IUPAC Classification, PCB126) was kindly provided by Dr. M. Morita at NIES, Tsukuba, Japan. Nucleopore filters were from Whatman (Clifton, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), calf serum (Lot #1027934), Amplification Grade Deoxyribonuclease I (DNase I), SuperScript™ II RNaseH-Reverse Transcriptase, Oligo (dT) 12-18 primer, and Urtla PURE™ Agarose were purchased from Life Technologies (Rockville, MD, USA). Antibiotics (penicillin plus streptomycin) and 5-bromo-2′-deoxyuridine (BrdU) were from Sigma (St. Louis, MO, USA). Peroxidase conjugated anti-BrdU mouse monoclonal antibody was from Becton Dickinson (San Jose, CA, USA). The in situ Cell Death Detection Kit-AP was obtained from Roche Molecular Biochemicals (Mannheim, Germany). The QIA prep RNA purification kit (RNeasy) was from QIAGEN GmbH (Hilden, Germany). TaKaRa Ex Taq™ polymerase with 10×Ex Taq™ Buffer, TaKaRa LA Taq™ polymerase with 2×GC Buffer I, II, and 10 mM dNTP mixture were from TaKaRa Biomedicals (Otsu, Japan).

2.2. Organ cultures

Pregnant ICR mice (Charles River, Tokyo, Japan) were housed under the conventional conditions in the vivarium at NIES. Neonatal male mice were killed by cervical dislocation immediately after birth (postnatal day 0; PND 0), and their testes were removed and immediately placed on a nucleopore filter (pore size: 0.1 μm, ϕ 25 mm) floating on DMEM containing 10% calf serum and 100 μg/ml antibiotics. The medium was supplemented with 0, 10, 100 or 1000 nM PCB126. The testes were incubated at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. After incubation for 48 h with medium containing PCB126, the fresh medium was added, and culture was continued for an additional 48 h. After addition of 50 μg/ml of BrdU, culture was continued for another 1 h. The same procedure as described above was carried out with PCB126-free medium, and the cultured testis samples were collected at 0, 6, 12, 24, 48, 96 and 192 h. Preliminary studies showed that the central area of the testicular tissue was subject to necrosis for 12 days of culture, but that nearly all the germ cells and Sertoli cells were found viable for 4 days of culture, and thus we selected 4-day culture for the current assay.

2.3. BrdU-labeling and TUNEL

The cultured testis was fixed with Camoy's solution and embedded in paraffin. Deparaffinized 5 μm-sections were incubated in 3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase and then immersed in 1 N HCl for 60 min to denature the genomic DNA. After rinsing, the specimens were treated with a blocking solution containing 1% bovine serum albumin and incubated with peroxidase labeled anti-BrdU mouse monoclonal antibody (1:50) for 1 h. After rinsing, 3,3′-diaminobenzidine solution was applied to the sections. BrdU-positive germ cells and Sertoli cells were counted, and the counts were divided by the numbers of all live germ cells and Sertoli cells, respectively.

The in situ Cell Death Detection Kit was used to detect apoptosis in cultured neonatal mouse testis. Deparaffinized sections were incubated with 20 μg/ml protease K for 15 min at 37 °C. The sections were then washed twice in 10 mM PBS (pH 7.4) and incubated with TUNEL reagents according to manufacturer's protocols.

2.4. Semiquantitative RT-PCR

The semiquantitative RT-PCR method used in this study was described previously (Ohsako et al., 2001). Total RNA from the cultured testis ($n=4$) was extracted with the QIA prep RNA purification kit. Eluted total RNA samples were treated with 1 unit of DNase I per 1 μg total RNA. Total RNA (20 μg) was reverse-transcribed in a 20 μl reaction volume with 200 units of SuperScript™ II reverse transcriptase and 0.5 μg of oligo (dT) 12–18 primer according to standard protocol of the supplier. The reverse-transcribed samples were subjected to measurement of the mRNA levels of hsp86, calnexin-t, protamine-2, androgen binding protein (ABP), cytochrome P450 1A1 (CYP1A1), cytochrome P450 side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase/17,20-lyase (P450c17), 3β-hydroxysteroid dehydrogenase-type I (3β-HSD), 17β-hydroxysteroid dehydrogenase type III (17β-HSD), cyclophilin, and G3PDH. The primer sequences and PCR product sizes are shown in Table 1.

For hsp86, calnexin-t, protamine-2, ABP, cyclophilin, G3PDH, P450scc, and 3β-HSD mRNA amplification, 0.5 μl of the resultant cDNA was amplified in a total volume of 25 μl of reaction mixture solution that consisted of 0.625 units of TaKaRa Ex Taq™ polymerase, 1×EX Taq™ buffer, 0.2 mM of each dNTP mixture, and 2 μM of specific primers. For CYP1A1 mRNA amplification, 0.5 μl of the resultant cDNA was amplified in a 25 μl reaction solution in the presence of 1.25 units of TaKaRa La Taq™ polymerase, 1×GC Buffer I, 0.4 mM of each dNTP mixture, and 4 μM of specific primer. For P450c17, and 17β-HSD mRNA amplification, 0.5 μl of the resultant cDNA was amplified in a 25 μl reaction solution in the presence of 1.25 units of TaKaRa La Taq™ polymerase, 1×GC Buffer II, 0.4 mM of each dNTP mixture, and 4 μM of each primer. Amplification was carried out in a thermalcycler, using 20 cycles for cyclophilin and G3PDH, 25 cycles for hsp86 and 3β-HSD, and 35 cycles for calnexin-t, protamine-2, ABP, P450scc, P450c17 and 17β-HSD. Cycling parameters were as follows: 95 °C, 30 s; 60 °C, 30 s; 70 °C, 45 s for hsp86, calnexin-t, protamine-2, ABP, cyclophilin, G3PDH; and 95 °C, 30 s; 57 °C, 30 s; 70 °C, 1 m for P450scc, P450CL7, 3β-HSD, 17β-HSD. The PCR products were separated on a 2% agarose gel. Relative amounts of target mRNA products were quantified by standardizing with the PCR product of cyclophilin or G3PDH using Scion Images software (Scion Corporation, Frederick, MD, USA). The PCR products were subcloned into pGEM-T Easy vectors and sequenced by the dideoxynucleotide chain termination method using the ABI Prism Big Dye terminator cycle sequencing kit (PE Biosystems, Foster City, CA, USA).

Table 1
Primers used for semiquantitative RT-PCR

Genes	Primer sequence (5′-3′)		Product size (bp)	GenBank accession No.
	Forward primer	Reverse primer		
Hsp86	AACCAATGGGTCGTGGAACAAAG	ACAGATCAAAAGGAGCGCGTCTT	512	M36830
Calnexin-t	TGCTAGCAGACACTGGTGATTTG	TGACTCTAGATGCCCGATCTCTC	1418	U08373
Protamine-2	GGTTCGCTACCGAATGA	CTTCGGGATCTTCTGCA	422	X07626
ABP	CTATGCCTGAGACAAATCTCTGC	ATAGAAAGGACTTCCATCTTTGC	660	NM011367
P450scc	CGCTCAGTGCTGGTCAAAG	GGTTGAGCATGGGGACACT	714	AF195119
P450c17	CTTGTGGGTCTCTTGCTGCTCAT	TCTTCAACCACGGGAATATGTCC	655	M64863
3β-HSD	ATGTTGGTGCAGGAGAAAGAACT	TGGAGAATTTGCCAGTAACACAC	595	M58567
17β-HSD	ACAACGTTGGAATGCTCCCCAGC	GGAATCGTTGAGCGGTGCTGCTA	492	U66827
CYP1A1	CTCATTGAGCATTGTCAGGACAG	TGGTCTGGTGAGCATCCTGGACA	734	NM009992
Cyclophilin	TGGAGATGAATCTGTAGGACGAG	TAGCACATCCATGCCCTCTAGAA	382	M60456
G3PDH	AGTGGAGATTGTTGCCATCAACGA	GGGAGTTGCTGTTGAAGTCGCAGGA	791	NM008084

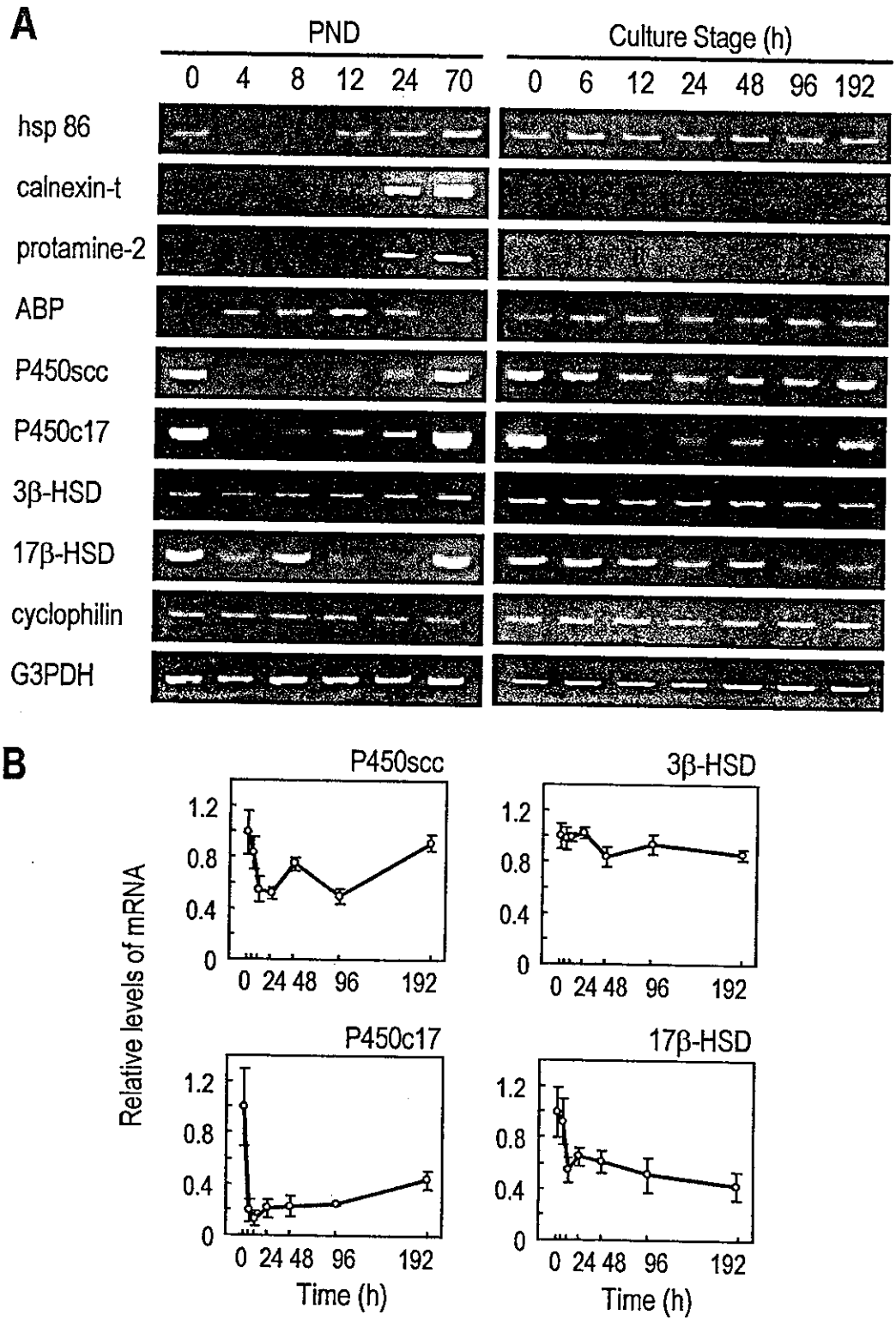

Fig. 1. Profiles of changes in gene expression with development and culture period in intact mouse testis and organ-cultured mouse testis, respectively. A series of intact mouse testes from PND 0 (neonate) to PND 70 (adulthood) and neonatal testes cultured for various times were subjected to semiquantitative RT-PCR for 10 genes (hsp86, calnexin-t, protamine-2, ABP, P450scc, P450c17, 3β-HSD, 17β-HSD, cyclophilin and G3PDH) as described in Materials and methods. (A) Comparison of the mRNA profiles between testes in vivo (left panels) and in vitro (right panels). Note that the expressed level of P450scc gene in neonatal mouse testis (in vivo) rapidly decreased on PND 4 and increased again on PND 70, whereas its mRNA level in the cultured testis was maintained at almost the same level throughout all stages of culture. (B) Relative levels of the four steroidogenic enzymes in cultured testis normalized to cyclophilin. The values expressed are means±S.E. of data for the total of three samples examined for each group. The levels of P450scc and 3β-HSD mRNAs in cultured testes remained stable for 8 days at the same level as in neonatal testis. The levels of P450c17 mRNA in the cultured testes rapidly decreased during the first 6 h.

2.5. Statistical analysis

Means of BrdU indices and band intensity in the semiquantitative RT-PCR analysis were statistically compared by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test as a post-hoc test by using StatView for Windows version 5.0 (SAS Institute, Cary, NC, USA). P values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Comparison of the mRNA expressions between in vivo and in vitro mouse testes

To characterize the organ-culture system used in this study, we determined the basal expression profiles of 10 genes (hsp86, calnexin-t, protamine-2, ABP, P450scc, P450c17, 3β-HSD, 17β-HSD, cyclophilin and G3PDH) in the testis of intact mice and the one cultured in vitro. The testis was collected from mice in vivo on PND 0 (neonate), 4, 8, 12, 24 and 70 (adulthood), or cultured in vitro for various times (0, 6, 12, 24,48, 96 and 192 h) in the established neonatal testicular organ-culture system with control medium containing 10% calf serum (Fig. 1).

Three markers, hsp86, calnexin-t and protamine-2, were used to evaluate germ cell development. Hsp86, a broad range germ cell marker (Lee, 1990), expressed from gonocytes to postmeiotic germ cells, was detected in all testes examined in vivo (Fig. 1A). Its mRNA levels were lower on PNDs 4 and 8 than on PND 0, and increased after PND 24. By contrast, the hsp86 mRNA levels in organ-culture neonatal testis were constant. The mRNA level of calnexin-t, an early meiotic phase germ cell marker (Ohsako et al., 1994), was detected on as early as PND 12 testis in vivo, while protamine-2, a haploid germ cell marker (Johnson et al., 1988), was detected as early as in PND 24 testis. However, none of these mRNAs was detected in organ-cultured testis (Fig. 1A). Androgen binding protein (ABP), used as a marker for quantitating Sertoli cells (Wang et al., 1989), was found to increase for the first 12 days in vivo and decrease thereafter (Fig. 1A). In cultured testis, however, ABP mRNA was constantly expressed from 6 to 192 h, and expressions of the housekeeping genes cyclophilin and G3PDH remained fairly constant in this culture system.

The steroidogenic enzymes P450scc, P450c17, 3β-HSD and 17β-HSD are responsible for testosterone synthesis in Leydig cells, and their mRNA levels were measured by semiquantitative RT-PCR. The mRNA levels of P450scc, P450c17, 3β-HSD and 17β-HSD detected in the neonatal testis on PND 0 were the same as in organ-cultured testis at 0 h (Fig. 1A). By contrast, hardly any constitutive expression of P450scc and P450c17 mRNA in the testis was detected on PND 4 in vivo, and expression gradually increased until PND 70 (Fig. 1A). Expression of P450scc mRNA in cultured testis was maintained at higher levels throughout all stages of culture (0–192 h) than PND 4 and 8-testes in vivo (Fig. 1A,B). By contrast, expression of P450c17 mRNA in cultured testis was barely detectable from 6 to 96 h, and had increased slightly at 192 h (Fig. 1B). Basal expression of 3β-HSD in vivo remained stable from PND 0 to PND 24, and had increased slightly on PND 70 (Fig. 1A). The 3β-HSD mRNA level in cultured testis also remained stable throughout the culture period (Fig. 1B). Basal expression of 17β-HSD mRNA in vivo was decreased during the first 24 days, with a slight increase on PND 8, and a greater increase on PND 70 (Fig. 1A) whereas, in cultured testis it gradually decreased until 192 h of culture (Fig. 1B).

3.2. Induction of CYP1A1 mRNA

Neonatal mouse testes (PND 0) were cultured for 48 h in medium containing 0, 10, 100 or 1000 nM PCB126. As

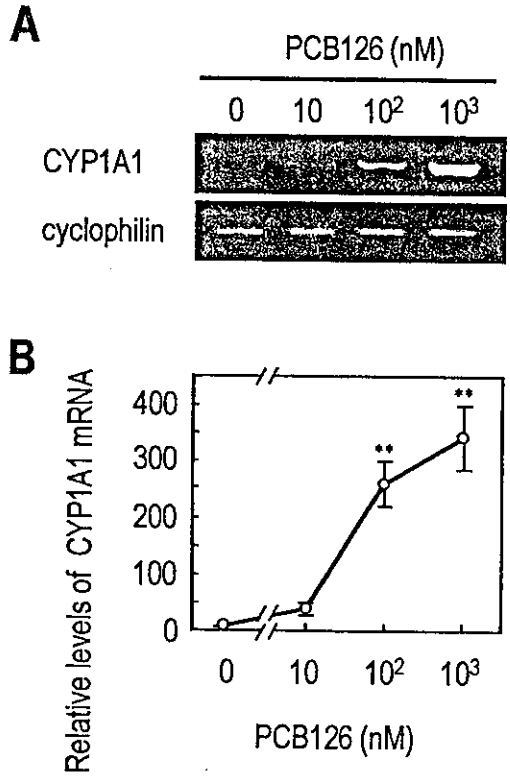

Fig. 2. Induction of CYP1A1 mRNA in cultured neonatal mouse testis by PCB126. RNA isolated from neonatal testis cultured in calf serum-supplemented medium containing 0–1000 nM PCB126 for 48 h. (A) Agarose gel electrophoresis pattern of PCR products for CYP1A1 and cyclophilin. (B) Levels of CYP1A1 mRNA relative to cyclophilin. The results are expressed as means ± S.E. of data from the total of four samples. Asterisks denote a significant difference from control levels (**$P < 0.01$) by ANOVA followed by Fisher's PLSD test.

the induction of CYP1A1, a marker of exposure or presence of dioxins, is usually observed 24 h post-administration of dioxin-like compounds, we adopted 48 h for an exposure period to investigate subsequent alteration in the testis. After further culture for 2 days, total RNA was isolated from these testes and analyzed for CYP1A1 mRNA expression by semiquantitative RT-PCR. No expression of CYP1A1 mRNA was detected in organ-culture controls (DMSO). However, the CYP1A1 mRNA level was significantly higher in cultured testis exposed to 100 or 1000 nM PCB126, compared to the control (Fig. 2).

3.3. Effect of PCB126 on the proliferation of the germ cell and Sertoli cells

To investigate the effects of PCB126 on the mitotic activity of germ cells (gonocytes) and Sertoli cells in mouse neonatal cultured testis, neonatal testes ($n=12$) were individually cultured in the presence of various concentrations of PCB126 and labeled with BrdU at the end of culture. In the presence of PCB126, the labeling indices of the germ cells remained fairly constant at all the PCB126 concentrations used, whereas in the Sertoli cells they tended to decrease slightly in the presence of PCB126, although the decrease was not statistically significant (Fig. 3). We also investigated whether PCB126 induced apoptosis in organ-cultured testes by applying the TUNEL method to the same specimens. However, almost no TUNEL-positive germ cells or Sertoli cells were found in either the control or the PCB126-exposed testes (data not shown).

Expression of the germ cell marker hsp86 and that of the Sertoli cell marker ABP were examined by semi-quantitative RT-PCR. Expression of hsp86 mRNA was unchanged in the presence of PCB126 (Fig. 4), whereas

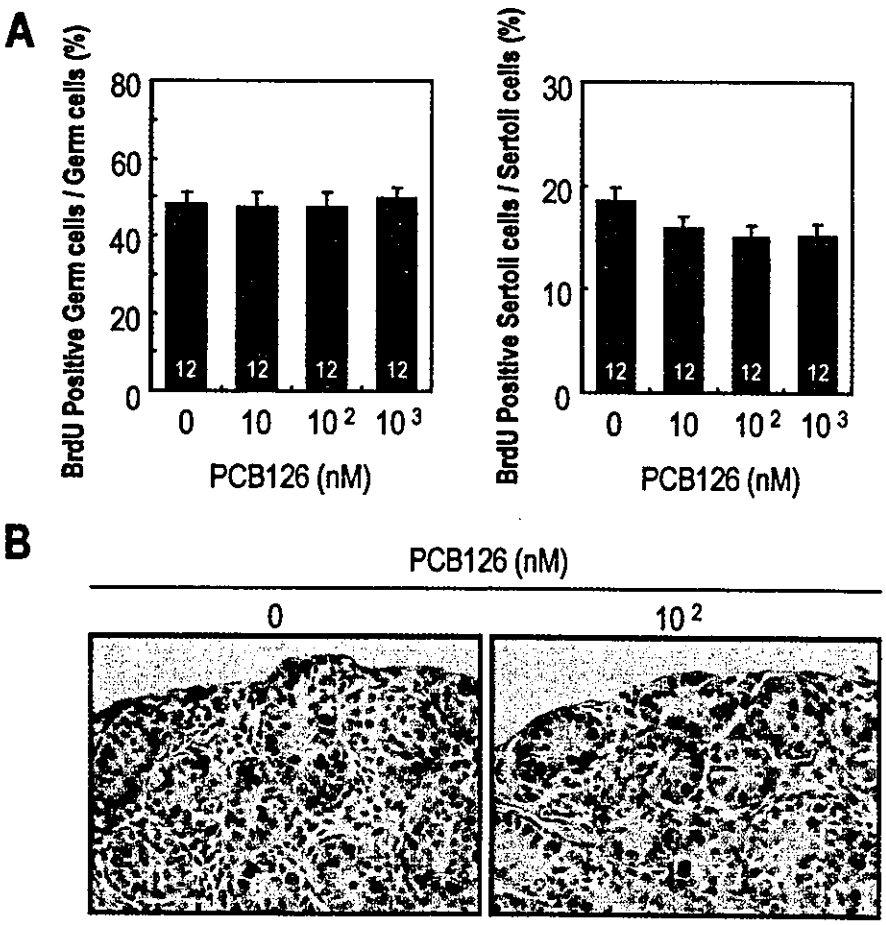

Fig. 3. Effect of PCB126 on the proliferation of germ cells and Sertoli cells in neonatal cultured testis. The neonatal mouse testis was cultured in calf serum-supplemented medium containing 0–1000 nM PCB126 for 48 h as described in Section 2. (A) Frequency of BrdU-labeled germ cells and Sertoli cells in organ-cultured testes. The values expressed are means±S.E. for a total of 12 samples examined for each group. No dose-dependent change in proliferation was observed in either cell type. (B) BrdU immunohistochemistry of the testis. Representative photographs of control testis and 100 nM PCB126-exposed testis are shown. The nuclei in brown represent BrdU-positive cells. All sections were counterstained with hematoxylin. Magnification ×200.

expression of ABP mRNA decreased slightly in the presence of 1000 nM PCB126, although not significantly (Fig. 4).

3.4. Effect of PCB126 on expression of steroidogenic enzyme mRNA

The mRNA levels of 3β-HSD and 17β-HSD were unchanged by any doses of PCB126 (Fig. 5). However, P450scc mRNA levels of the testes exposed to all the concentrations of PCB126 were significantly lower than that in the control ($P<0.01$), and the reduction appeared to be dose dependent (Fig. 5). By contrast, the P450c17 mRNA level in 1000 nM PCB126-treated testis was significantly higher than in the control ($P<0.01$).

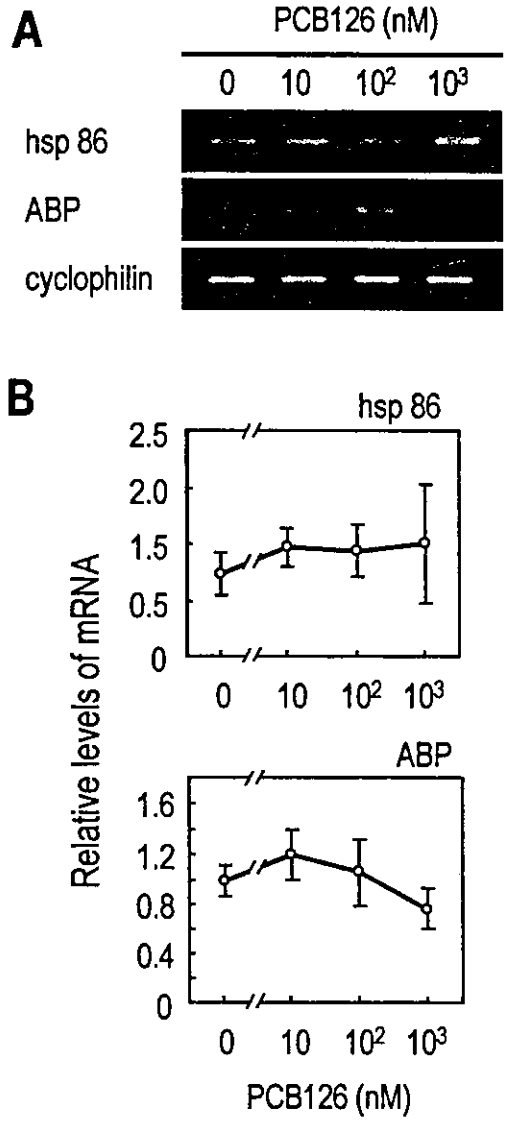

Fig. 4. Semiquantitative RT-PCR analysis of the effect of PCB126 on hsp86 and ABP mRNA levels in cultured neonatal mouse testis. RNA isolated from neonatal testis cultured for 48 h in calf serum-supplemented medium containing 0–1000 nM PCB126. (A) Agarose gel electrophoretic pattern of PCR products for hsp86 and ABP and cyclophilin. (B) Levels of hsp86 and ABP mRNA relative to cyclophilin. The results are expressed as means±S.E. of data from a total of four samples. No dose-dependent changes in hsp86 and ABP mRNA were observed.

4. Discussion

4.1. Characteristics of the mouse testes in the organ-culture system used in this study

This study is the first to elucidate the gene expression profiles for spermatogenesis and steroidogenesis in mouse testis from birth through adulthood and to compare them with the gene expression pattern in the established organ-culture system. In mouse testis, hsp86 mRNA is expressed only in the germ cell (Lee, 1990). Immunohistochemical analysis with neonatal rat testis has revealed that hsp86 protein is highly expressed in the cytoplasm of gonocytes and proliferating spermatogonia (Ohsako et al., 1995). In the present study, we found reduced expression of hsp86 mRNA in the testis on PNDs 4 and 8 compared to PND 0 (Fig. 1A). This phenomenon may reflect an increase in the number of somatic cells, especially Sertoli cells, compared to the number of germ cells, resulting in a lower germ cell ratio during the first 8 days after birth (Nagano et al., 2000). This speculation is also supported by the finding that ABP, a secretory protein specifically produced by Sertoli cells (Wang et al., 1989), increased for the first 12 days (Fig. 1A). The level of hsp86 mRNA expressed in the testicular organ culture system was found to be fairly constant throughout the culture period, suggesting that the germ cell/somatic cell ratio was unchanged. The other germ cell markers, calnexin-t, expressed in pachytene stage spermatocytes or later (Ohsako et al., 1994), and protamine-2, expressed in haploid germ cells (Johnson et al., 1988), were not detected in this culture system during 8 days of culture, showing that germ cells did not progress further into meiosis. In neonatal mouse testis in vivo, gonocytes start to reproliferate on PND 1.5 (Nagano et al., 2000), and the developed spermatogonia enter the first cycle of meiosis on PND 8 (Fig. 1A), when calnexin-t is detected. Therefore, our findings suggest that the gonocytes in this culture system did not proceed to the meiosis. BrdU-labeling indices at 4 days of culture were the same levels as those found in mouse testis on PND 4 in vivo (Nagano et al., 2000). The proliferative activity of the germ cells at 4 days of culture in this organ culture system seems to be very similar to that of the testis on PND 4 in vivo.

The mRNA expression of four steroidogenic enzymes (P450scc, P450c17, 3β-HSD, 17β-HSD) is essential for biosynthesis of testosterone by Leydig cells, and conversion of cholesterol to pregnenolone by mitochondrial cytochrome P450scc in the testis is the rate-limiting enzymatic step in steroidogenesis (Stocco and Clark,

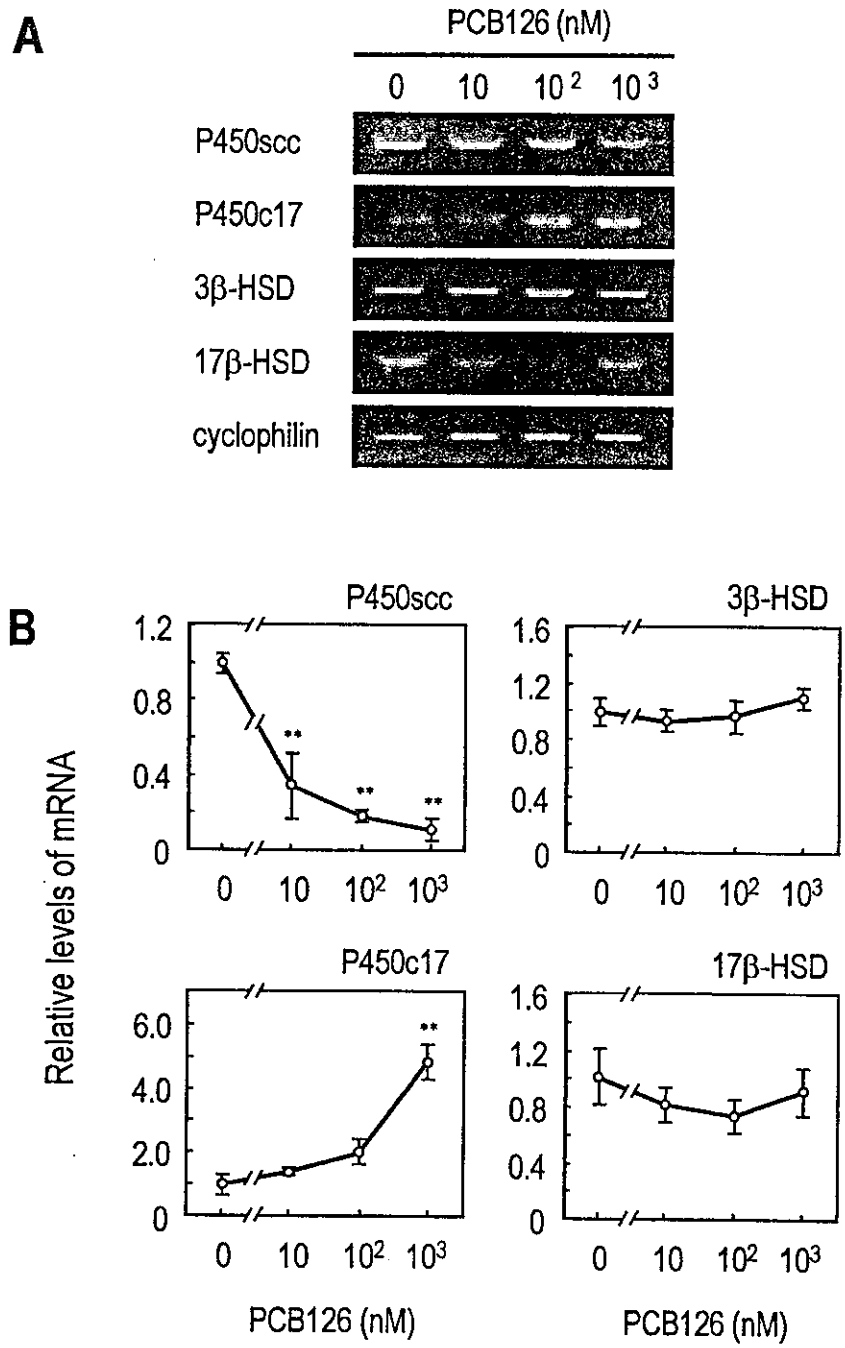

Fig. 5. Semiquantitative RT-PCR analysis of the effect of PCB126 on steroidogenic enzyme mRNA levels in the neonatal mouse cultured testis. RNA isolated from neonatal testis cultured in calf serum-supplemented medium containing 0–1000 nM PCB126 for 48 h. (A) Agarose gel electrophoretic pattern of PCR products for P450scc, P450c17, 3β-HSD, 17β-HSD and cyclophilin. (B) Levels of the four steroidogenic enzyme mRNAs relative to cyclophilin. The results are expressed as means ± S.E. for a total of four samples. Asterisks denote a significant difference from control levels (**$P < 0.01$) by ANOVA followed by Fisher's PLSD test. Note that P450scc mRNA was increased by PCB126 in a dose-dependent manner whereas P450c17 mRNA was induced by PCB126 at 1000 nM. No dose-dependent changes in 3β-HSD and 17β-HSD mRNA were observed.

1996). The primer sequences used for amplification of 3β-HSD and 17β-HSD in the present study were derived from those of 3β-HSD type I and 17β-HSD type III, respectively, which are the most dominant HSD isoforms in the testis (Baker et al., 1997, 1999). Although 3β-HSD in the intact testis was constant at all ages, we found that the mRNA levels of P450scc and P450c17 were relatively high on PND 0, but rapidly decreased during the first 4 days, and then continued to a gradually increase towards the maturity on PND 70. In fact, the serum testosterone concentration in the male rodents dramatically and transiently increases between 0

and 2 h after birth, subsequently rapidly decreases (Rhoda et al., 1984; Corbier et al., 1978), and increase again during puberty (Miyachi et al., 1973). The mRNA expression levels of steroidogenic enzymes P450scc and P450c17 in this study seemed to reflect this testosterone production profile from birth through adulthood. In contrast, the expression of P450scc mRNA in cultured testis remained at higher levels for 8 days than on PND 4–8 testes in vivo. It is therefore concluded that at least the expression of P450scc mRNA was maintained in this established organ-cultured testis. This may be attributable to contamination of the calf serum used in preparing the medium, by bovine gonadotropins, which could stimulate the signal transduction pathway for P450scc transcription.

4.2. PCB126 induces expression of CYP1A1 mRNA in cultured neonatal mouse testis

Expression of the CYP1A1 gene by exposure to dioxins occurs via an AhR-mediated mechanism (Poland and Knutson, 1982; Safe, 1986; Whilock, 1990; Denison, 1991), and induction of this xenobiotic enzyme is considered as one of the most sensitive biomarkers of exposure to dioxins (Safe, 1994; Hooper and Clark, 1995). PCB126 was assigned a rating of 0.1 for Toxic Equivalent Factor (Van den Berg et al., 1998), and can, for example, induce CYP1A1 enzyme activity expressed as ethoxyresorufin-*O*-deethylase (EROD) in the liver (DeVito et al., 2000). The present study demonstrated marked induction of CYP1A1 mRNA by the PCB126 in the neonatal testis cultured in vitro, indicating this coplanar PCB has relatively strong TCDD-like activity in the testis. The data suggested that PCB126 can act directly on neonatal testis, probably via AhR in the testis. However, TCDD has been reported not to induce CYP1A1 mRNA in adult rat testis (Roman et al., 1998b), which was confirmed in our preliminary experiment with male rats. Further studies are needed to clarify the striking difference in CYP1A1 mRNA expression between cultured mouse neonatal testis and adult rat testis. The reason for this difference may be a developmental stage-specific difference in CYP1A1 expression in the testis or an interspecies difference in CYP1A family genes.

4.3. PCB126 did not alter the mitotic activity of germ cells and Sertoli cells in neonatal mouse testis in vitro

PCB126, at all the doses used in the present study, did not alter the BrdU-labeling indices of the germ cells or Sertoli cells in the organ-cultured testes. There were no changes in the number of germ cells or Sertoli cells, based on the mRNA levels of hsp86 and ABP, respectively, that were used as biomarkers for these cells (Fig. 4). These data, the BrdU-labeling indices, as well as the semiquantitative RT-PCR results, suggested that PCB126 did not affect the proliferation activity of either cell type, namely pre-spermatogenic cells (gonocytes) or Sertoli cells, in neonatal testis. No TUNEL-positive germ cells or Sertoli cells were detected in the control or PCB126-treated testes. In view of the report that no population of apoptotic cells is present in neonatal mouse testis (Wang et al., 1998), these results suggest that PCB126 does not directly induce apoptosis or impair proliferation of testicular pre-spennatogenic cells or Sertoli cells during the neonatal stages.

It is still a matter of controversy whether maternal exposure to TCDD affects spermatogenesis in the offspring. Mably et al. (1992a) reported that perinatal exposure to a low TCDD dose significantly reduced testis weight and DSP, whereas Gray et al. (1997) failed to find them. On the other hand, Faqi et al. (1998a) found a slight decrease of DSP with no changes in testicular weight by administering female rats an initial loading dose 2 weeks prior to mating, followed by a weekly maintenance dose. However, they also reported that a single oral dose of 10 μg PCB126/kg on GD15 did not induce any decrease in testicular weight or sperm counts (Faqi et al., 1998b). In our previous study, male offspring of Holtzman rats given a single oral dose of TCDD on GD15 did not show any alterations of testicular development or the sperm production (Ohsako et al., 2001). The present results obtained by using the neonatal mouse testicular organ culture system suggest that dioxins do not directly affect the proliferation of testicular pre-spermatogenic cells or Sertoli cells during the neonatal life of male mice. This finding may partially support the above-cited earlier observations on the apparent absence of effects of perinatal exposure to dioxins on testicular weight and DSP in the rat testis.

4.4. PCB126 affects expression of steroidogenic enzyme mRNAs in neonatal mouse testis

It has not been elucidated whether dioxins directly affect neonatal testicular steroidogenesis in terms of steroidogenic enzyme mRNA levels. In the present study, semiquantitative RT-PCR analysis demonstrated that exposure to PCB126 significantly down-regulated the P450scc mRNA level in cultured neonatal mouse testis, but up-regulated P450CL7 mRNA level. On the other hand, RT-PCR analysis of cultured granulosa cells from prepubertal and mature female rats showed a significant reduction in the expression of cytochrome P450 aromatase mRNA levels in the presence of TCDD (Dasmahapatra et al., 2000). In fact, genomic sequence analysis suggested that the 5′-flanking region of P450 aromatase gene contains a xenobiotic responsive element (XRE)-like enhancer element in the promoter region or exons (Hickey et al., 1990). Dioxin–AhR

complex may therefore directly modulate gene expression of P450 aromatase. However, our preliminary study using semiquantitative RT-PCR failed to detect P450 aromatase mRNA levels in intact mouse testis on PND 70 (data not shown), and it remains to be studied whether the gene expression of P450 aromatase is directly affected by dioxin treatment.

There have been many reports that dioxins affect the activity of the P450 steroidogenic enzyme in adult animals. Recently, Andric and co-workers (2000) reported that systemic administration of the PCB mixture Askarel, which contains coplanar PCB, has a mode of action completely different from that of the compound administered locally into the testis. In brief, a single intraperitoneal injection of Askarel inhibited P450c17 activity and markedly decreased serum testosterone levels whereas a single intratesticular injection of this compound stimulated P450c17 activity and significantly increased serum testosterone levels. This observation suggests that when administered systemically, PCBs affected the endocrine status of the pituitary–testis axis, perhaps by altering gonadotropin secretion, and indirectly decreased testicular testosterone production. Direct administration of PCBs, however, increased the enzymatic activity of P450c17. The increased level of P450c17 mRNA in 1000 nM PCB126-exposed testis (Fig. 5) suggests that PCB126 directly increased the transcriptional activity of the P450c17 gene.

A number of studies have demonstrated that dioxins reduce P450scc activity in steroidogenic organs, including the testis. In rats, TCDD reduced intratesticular testosterone content and inhibited the pregnenolone formation, which was considered to be due to a reduction in the P450scc activity and/or impairment of mobilization of cholesterol to P450scc enzyme in the testis (Kleeman et al., 1990; Moore et al., 1991). Similarly, injection of PCB mixture containing PCB126, in vivo, strongly inhibited the activity of mitochondrial P450scc in the bull testis (Machala et al., 1998), and TCDD significantly reduced the P450scc mRNA level in cultured granulosa cells from prepubertal female rats (Dasmahapatra et al., 2000). To our knowledge, the present study is the first to demonstrate that coplanar PCB directly down-regulates P450scc gene expression in neonatal mouse testis. This could influence testosterone production in the testis. In fact, Peterson and co-workers have reported that the postnatal testosterone peak of male rat offspring born from dams administered 1.0 μg TCDD/kg body weight on GD15 was delayed until 4 h after birth, and that the amplitude of the peak was significantly reduced to 53% compared with controls (Mably et al., 1992b). The above observations concerning alteration of the neonatal testosterone surge by dioxins, in terms of direct inhibition of P450scc mRNA expression, are thought to be explained at least partially by the present observations.

5. Summary

This is the first study to demonstrate direct effects of PCB126, a most potent dioxin-like coplanar PCB congener, on neonatal testis in an organ culture system. The results show that PCB126 does not directly affect proliferation of pre-spermatogenic cells and Sertoli cells in neonatal mouse testis, but that PCB126 directly down-regulates the level of expression of the P450scc gene and up-regulates expression of the P450c17 gene. The organ culture system described in this study will provide a very useful tool for examining the direct effects of potentially toxic chemicals on the mammalian testis.

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Review Article

Developmental Neurotoxicity of Dioxin and Its Related Compounds

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Abstract: This manuscript reviewed the neurotoxicity of dioxins and related compounds with an emphasis on maternal exposure. The brain during developmental period is thought to be highly sensitive to dioxin and its related compounds that affect a broad range of brain functions from the advanced brain function to the reproduction-controlling function, even at low doses. It is suggested that dioxins exhibit endocrine-disrupting action on the gonadal and thyroid hormone axes, as well as the 'neural-disrupting action' on neural transmission and neural network formation. From behavioral toxicological studies as well as studies on the underlying mechanisms of dioxins' toxicity, dioxins affect some specific functions in particular regions or cells of the brain at critical windows during the developmental period.

Key words: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), Polychrorinated biphenyl (PCB), Brain development, Neuron, Glial cell

Introduction

Halogenated dioxins and dibenzofurans are produced as byproducts mainly in burning processes as well as contaminants during the production of a certain type of herbicides. Among 75 polychlorinated dibenzodioxin (PCDD) isomers and 135 polychlorinated dibenzofuran (PCDF) isomars and 209 polychlorinatel biphenyl (PCB) isomers, the large family of coplanar polychlorinated biphenyls and highly chlorinated PCDD and PCDF isomers tend to persist in the environment without degradation. They are concentrated in the biota through food chains and taken into the human body mainly via food. Dioxins exhibit a wide spectrum of toxicities such as oncogenesis, reproductive toxicity, and immunosuppression[1–6]. Since dioxins in the blood circulation are distributed systemically, the brain could be a target of dioxins after penetrating the blood brain barrier.

Furthermore, perinatal (*in utero* and lactational) exposure to dioxins has been reported to affect the development of offspring even when the exposure level is too low to induce the toxicity in their mother. When developmental neurotoxicity has appeared as a result of maternal exposure, it is often difficult to identify the causal relationship between the exposure and the effects. For neurotoxicity, unlike lethality and oncogenesis, there are no clear-cut endpoints, because most of the brain function itself remains to be defined yet, in addition, individual differences in the response are relatively large. And yet, such apparently unidentifiable effects of chemicals are now drawing most attention and of greatest concern both socially and scientifically.

In this article, we reviewed the effects of exposure to dioxins on the brain, mainly focusing upon low-dose exposure effects during brain development, with some special references to subtle effects observed in animal experiments.

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We also summarized the currently available information on the effects of dioxins on the central nervous system, specifically effects on the advanced brain function, principally in the cerebral neocortex, and the reproductive function-controlling brain function, principally in the hypothalamus.

Dioxins and Related Compounds

Dioxin and its related compounds include PCBs, PCDDs, and PCDFs. Each of these compounds comprises a pair of benzene rings bound together via two (for PCDDs) or one (for PCDFs) oxygen bond or via one carbon bond (for PCBs). Theoretically, there can be 75 isomers of PCDDs, 135 of PCDFs, and 209 of PCBs.

Different congeners of dioxins exhibit different toxicities. The toxic equivalency factor (TEF) of each dioxin has been determined compared to the toxicity of the most toxic dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (TEF = 1)[7]. TEF has been determined for 29 compounds: 7 isomers of PCDDs and 10 isomers of PCDFs which have chlorine at the "2, 3, 7, 8" positions, and 12 isomers of PCBs which have a planar structure (coplanar PCBs); these congeners are assumed to possess TCDD-like toxicities, and we first describes the TCDD-like toxicity.

The lethal dose of TCDD varies widely depending on recipient animal species and strain. For the rat, oral LD_{50} values are on the order of several tens of micrograms per kg body weight or more, while there is a 60-times difference between Long-Evans rats (50 μg/kg b.w.) and Han/Wistar rats (3000 μg/kg b.w.)[8]. The adverse effects such as the inhibition of ovulation and effects on the thyroid hormone system are often observed in adult animals when several micrograms per kg body weight (as body burden), as described later. On the other hand, when dams are exposed to low levels of dioxins that do not cause effects in themselves, developmental anomalies such as the cleft palate and the hydronephrosis are caused in their offspring, indicating that the animals during development are extremely sensitive to dioxins.

As described in details below, the exposure to TCDD at a dose of 1 μg/kg or less, the level of which does not cause serious developmental anomalies, has been reported to affect the brain of offspring, as described in the next chapter. The term "low-dose exposure" as used herein refers to an exposure at a level below 1 μg/kg.

Dioxins show extremely high potential for retention in the body. The half-life of TCDD is estimated to be approximately 7 to 10 years for humans and 12–24 days for the rat, mouse and hamster[9]. Accordingly, when a single oral dose of TCDD is given to pregnant rats, it is transferred to their offspring via transplacental and lactational routes just after exposure until weaning[10, 11]. This observation was confirmed by our study on the TCDD amounts determined by high-resolution gas chromatography/mass-spectroscopy: TCDD was transferred dose-dependently to the brain tissue of pups born from Long-Evans dam receiving an oral dose of TCDD at 200 or 800 ng/kg b.w. on day 15 of gestation (approximately 10 pg/g or 22 pg/g, respectively, on PND (postnatal day) 5)[12].

Adverse Developmental Effects on Reproductive Functions

Effects on the hypothalamo-pituitary-gonadal axis and reproductive organ

Reproductive functions such as ovulation, pregnancy and lactation in females and spermatogenesis in males are all regulated by interactions between pituitary hormone and peripheral reproductive organs, and secretion of the pituitary hormone being governed by the hypothalamus. Previousely, Mably *et al.* (1992) have reported that fetal plasma testosterone (T) concentrations decreased when Holtzman rat dams received 1 μg TCDD/kg on gestational day (GD) 15, indicating that maternal exposure to dioxins decreased the circulating testosterone of offspring[13]. However, the subsequent report from the same laboratory showed that no such effects were observed on GD 18, GD 20, and 2 hours after birth[14]. It has also been reported that TCDD did not affect plasma testosterone when LH analogs and hCG (human chorionic gonadotrophin) were treated to promote testosterone synthesis[14], and that no effects were observed in plasma testosterone concentrations at 6 hours after birth and LH-induced testosterone secretion in Long-Evans offspring[15]. On the other hand, administration of TCDD in the fetal stage was suggested to facilitate testosterone synthesis, based on the results that TCDD (1 μg/kg to dams by gavage, on GD13, in Han/Wistar rats) increased the fetal pituitary LH secretion and T surge on GD 19.5[16]. From these findings together, it is thought that TCDD does not seriously affect fetal testosterone levels.

Chaffin *et al.* (1996) conducted *in utero* and lactational exposure to TCDD (1 μg/kg to dams by gavage, on GD15, in Holtzman rats), and reported that the estrogen receptor (ER) mRNA content increased in the hypothalamus, uterus and ovaries but decreased in the pituitary, and that DNA binding increased in the uterus and decreased in the hypothalamus[17]. In this experiment, ovarian weight was increased whereas uterine weight remained unchanged[17].

These results suggest that dioxins may have variable effects with tissue specificity, rather than exhibiting suppressive or stimulatory action on the entire HPG axis.

Regarding male reproductive organs, TCDD administration exhibited toxic actions depending on the developmental stage, in addition to the tissue specific actions. Ohsako *et al.* (2001) extensively examined the effects of TCDD on all male reproductive organs, and reported that the most conspicuous effects of TCDD were observed in the shortening of anogenital distance (AGD) and the decrease in ventral prostate weight with no effects on testes, and that the increase in 5α-reductase II mRNA and the decrease in androgen receptor mRNA were observed specifically in the ventral prostate[18]. Because this effect on male reproductive organs does not involve a significant difference in plasma testosterone and LH contents, and because a significant effect was observed even in very low-dose exposure (50 ng/kg given to dams on GD 15)[18], which suggests the notion that low levels of TCDD affect particularly the reproductive organ, rather than an effect on the hypothalamo-pituitary axis, and that the development of the ventral prostate and the external genitalia is very susceptible to TCDD. Additionally, it is indicated that there is a critical window in the late fetal stage because these effects did not occur when a considerable amount of TCDD was administered at GD18 and PND2 (1 μg/kg to dams by gavage to dams and i.p. to offspring, respectively)[19]. Peterson and coworker has also reported that TCDD affects male reproductive organ without inhibition of androgen production in Holtzman rat[20,21], and that *in utero* TCDD exposure alone inhibited development of prostate lobe in C57BL/6 mice[22].

Taken together, it is suggested that TCDD does not exhibit estrogenic or anti-estrogenic action, but affects specific tissues in the HPG axis at a particular window of the developmental period, and that those developmental adverse effects may occur clearly in reproductive organs, rather than in the hypothalamus. These endocrine disrupting actions of dioxins are thought to be explained by the toxicological profile mediated by the aryl hydrocarbon receptor (AhR) system as described below.

Do dioxins affect the brain sexual differentiation?

The period from the late gestational to just after birth in rats corresponds to the critical period of the brain sexual differentiation, the time when the sex difference of the brain is determined. During this period, androgen exposure masculinizes and de-feminize the brain, and in contrast, the brain is feminized and de-masculinized when no such exposure occurred[23,24]. As a result, the sex-specific patterns of sexual behavior appears as the most conspicuous behavioral sex difference in adulthood; males exhibit masculine sexual behavior such as the mount, intromissive and ejaculatory behavior, whereas females exhibit feminine sexual behavior such as lordosis[25]. The expression of feminine type sexual behavior in male animals indicates the inhibition of de-feminization of the male brain, and the reduction in masculine sexual behavior indicates the disturbance of the brain masculinization.

A decrease in the circulating testosterone level during perinatal period results in perturbation of brain sexual differentiation. When pregnant Holtzman rats were exposed to TCDD on GD15, their male offspring revealed decreased testosterone concentrations in the fetal stage, with manifestation of feminine type sexual behavior, inhibition of masculine sexual behavior, and induction of LH surge-like secretion after estrogen treatment[13]. However, because studies performed later failed to show a decrease in circulating testosterone concentrations[14–16] as described in the previous section, it is difficult to conclude in simple terms whether dioxins do affect brain sexual differentiation. Morphologically, no effects of exposure to TCDD have also been reported in the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in male Holtzman[26] and Long-Evans rats[12]. The SDN-POA volume is highly sensitive to perinatal testosterone exposure because the dose-dependent increase was observed by perinatal administration with testosterone[27,28]. In our separate experiment (M. Ikeda, unpublished), TCDD was found to affect the SDN-POA volume in Holtzman rat offspring, the same as the strain in which Mably *et al.* (1992)[13] used. Taken together, there is the possibility that effects of dioxins on brain sexual differentiation are not so potent as to be found in every single experiment, but that the resulting effects were influenced by difference in animal species or strain, even animal's conditions and other experimental conditions.

Neocortical dysfunction and altered sexual behavior of male rats

Perinatal exposure to a low dose of TCDD has been reported to affect expression of masculine sexual behavior in male rat offspring. These effects have been associated with inhibition of brain masculinization. However, various patterns of sexual behavior after exposure to TCDD were observed in each report. Mably *et al.* (1992) reported that maternal exposure to TCDD (0.064–1.0 μg/kg given to dams at GD15 in Holtzman rats) resulted in a dose-dependent increase in intromission latency, ejaculation latency, and postejaculatory interval in the male offspring[13]. In their

subsequent report, a significant effect was observed only in an increase of intromission latency in Holtzman rats[26]. On the other hand, Gray *et al.* (1995) found an increase in the number of mounts and ejaculation latency, but no other effects in Long-Evans rat (1.0 μg/kg given to dams at GD15)[29]. In Wistar rats, only mount latency and intromission latency were affected with no other effects[30]. Thus, it seems that those effects caused by TCDD do not always represent the suppression of sexual behavior.

Expression of sexual behavior can also be altered as a result of disruption of neural network formation, aside from the effects on brain sexual differentiation. We examined how perinatal exposure to TCDD affects the sexual behavior of male offspring in Long-Evans rats[12]. Exposure to TCDD (800 ng/kg given to dams on GD 15) altered the expression of male sexual behavior by reduction in the number of mounts and intromissions in both the gonadaly-intact offspring and the castrated+testosterone-treated offspring.

As to the copulation-induced activity-dependent mRNA expression, we found that the copulation induced the brain-derived neurotrophic factor (BDNF) mRNA in the frontal cortex and that the level of BDNF mRNA expression in TCDD-exposed offspring that had altered sexual behavior was lower than that in control offspring[12]. Thus, it is conceivable that TCDD do not inhibit but "alters" expression of sexual behavior, by inhibiting the activity-dependent function of the frontal cortex rather than by suppressing the brain masculinization.

Possible existence of "endocrine disruption"-independent action of dioxins on brain sexual differentiation

Endogenous testosterone during perinatal period plays a major role in determining the brain sex, and yet this stimulus caused by testosterone is merely the starting point of brain development for the sex specific function. As with the effects on male sexual behavior, there is a possibility that TCDD affects the development of sex specific function of the brain by disrupting the neural network formation, independent from the effects on the brain sexual differentiation. Mably *et al.* (1992) reported that perinatal exposure to TCDD resulted in manifestation of feminine type sexual behavior in male offspring[13]. Although this phenomenon suggests a possibility that TCDD inhibits de-femninization of the brain of male offspring, it proposes another possibility that TCDD inhibits the construction of brain mechanism which controls sexual behavior. It has been shown that the reason why male rat does not show feminine type sexual behavior, is that the male brain does not lack the mechanism for expression of feminine type sexual behavior, but the midbrain serotonergic neurons suppresses it[31, 32]. Notably, Dong *et al.* (2001) reported that TCDD increased apoptotic cells specifically in the midbrain in zebrafish[33], and Kuchiiwa *et al.* (2002) recently reported that the TCDD decreased serotonergic immunoreactive cells in the midbrain raphe nuclei of ddY mice offspring[34]. These facts suggest that TCDD can affect the expression of heterotypical sexual behavior independently from the effects on the brain sexual differentiation, by inhibition of the serotonergic system in the midbrain.

The LH surge-like secretion in TCDD-exposed male offspring can also be explained as the result of disruption of the neural network formation. Hays *et al.* (2002) reported that GABAergic neurons (GAD67 positive cells by *in situ* hybridization) in the rostral part of mPOA express AhR mRNAs at 3 days after birth, and that perinatal exposure to TCDD eliminated the sex difference in GABA mRNA expression[35], indicating that TCDD affects the GABAergic system via AhR in the mPOA in the brain development. Because the rostral part of mPOA is a site to be critical for the LH surge-generating system[36], TCDD may affect the development of the LH surge-generating system as the neural network-disturbing action mediated by AhR, rather than endocrine-disrupting action.

Adverse Developmental Effects on Advanced Brain Function

Effects of dioxins on learning performance

Dioxins had been thought to impair learning and memory formation, because the perinatal period is critical for the development of the brain in terms of the neural network formation, such as the neuronal migration, the axonal elongation and elimination, and the synaptic remodeling. However, it is very difficult to interpret the effects of dioxins at low-dose exposure on the advanced brain function. Shantz *et al.* (1996) reported that exposure to TCDD (0.25 or 1 μg TCDD/kg/day on GD10-16, SD rats) decreased the number of errors in an 8-arm radial arm maze (RAM) test in male offspring[37]. The decrease in errors in RAM test was confirmed by exposure to 0.1 μg TCDD/kg/day (on GD10-16, Sprague Dawley (SD) rats)[38]. A subsequent study was conducted by using both 12-arm RAM and 8-arm RAM showed that TCDD had a decreasing effect in the 8-arm RAM test, but no effects in the 12-arm RAM test[39]. In the Morris water maze, which examines spatial learning, TCDD had no effects[38]. How TCDD exposure caused these behavioral changes may be interpreted by the following five possibilities[39]. First, TCDD might improve learning and

memory formation at low doses. Second, TCDD might impair learning and memory formation at higher exposure levels as shown by lead; A low dose of exposure to lead can promote learning behavior, whereas a higher level of lead inhibit learning and memory formation, as Seo *et al.* (1999) pointed out[39]. Third, learning and memory formation might be improved as a secondary effect to the effects on sex difference of the brain, because those effects were observed predominantly in male rats. Forth, TCDD improves particularly the RAM performance. Finaly, this improvement in RAM performance represents a manifestation of memory defects.

The first possibility, the improvement of learning and memory formation, can be excluded, because no effects were found in Morris water maze and the 12-arm RAM test[38]. Regarding the second possibility that is the inhibition of learning and memory formation at higher doses, it is suggested that such effects may rather be observed conversely at lower exposure levels. In rhesus monkeys, it has been reported that learning performance was decreased in offspring born to dams receiving lower doses of TCDD (5 ppt or 25 ppt in diet from 7 months before pregnancy to weaning)[40], whereas a definite conclusion is difficult to be reached because of the small sample numbers. It has also been reported that perinatal exposure to TCDD in Hotlzman rats (60 ng TCDD/kg was given to dams on GD 18), produced a significant dose-related reduction in the number of earned opportunities to run, the lever response rate, and the total number of revolutions in the operant responding for motor reinforcement[41], suggesting that the "wish to do" exercise of offspring might be decreased.

Regarding the third possibility on the secondary effects of TCDD on sex-related differences in the brain, Weiss and coworkers recently presented a series of interesting reports. Hojo *et al.* (2002) reported that exposure to TCDD (60 ng/kg was given to dams on GD 8, in SD rats) eliminated the sex difference in the operant behavior responding[42]. While control males and females responded at high rate and low rate, respectively, TCDD exposure resulted in more responsive in females and less responsive in males. These effects of TCDD on the sex difference were confirmed in the operant responding for motor reinforcement[43]. Subsequent histological study has shown that TCDD also eliminated the sex difference in the thickness of the cerebral cortex[44]. Taken together, Weiss proposed that exposure to TCDD at extremely low doses might affect the sexual differentiation of non-reproductive behavior[45].

To evaluate the forth and fifth possibilities, 4) particular improvement of the RAM performance and 5) the representation of memory defects, only few reports are available. However, the reduction of errors should be considered as a toxic effect because even if performance has improved at a given exposure level, no further improvement is expected with increased exposure level. Previous studies reported that the impairment of memory formation by the hippocampal lesion induced improvement of test performance, as the results of the reduction of "waving" behavior[46, 47].

Regarding the effects of low-dose exposure to TCDD, it is unlikely that linear relationship be observed between the effects and exposure level, as with lethality. For example, the aforementioned report of Weiss and coworkers[42, 45] showed a remarkable effect of TCDD at a perinatal dose of 60 ng/kg b.w. without a significant change at a higher dose (180 ng/kg b.w.). The effects of low-dose exposure are thought to include behavioral changes that cannot be explained by the simple scheme, 'toxicity = dysfunction'. Toxicities associated with low-dose exposure are thought to develop conspicuously in some specific functions, rather than as general functional impairment. Because brain functions rely on interactions of numerous molecules and brain regions, rather than on solitary actions of particular molecules, combination of some behavioral tests and/or interdiciplinal studies at behavioral, histological and molecular levels may be able to confirm the effects on the brain function.

Effects of dioxins on the composition of NMDA receptor

Recently, we have reported that the perinatal exposure to TCDD (200 or 800 ng/kg was given to dams on GD 15, Long-Evans rats) altered the levels of glutamate NMDA type receptor subunit mRNAs expression in the neocortex and the hippocampus[48]. These changes at the molecular level may characterize the dioxin toxicities in advanced brain function. Exposure to TCDD caused a remarkable reduction in the NR2B subunit mRNA, and an increase in NR2A subunit mRNA[48]. Glutamic acid is a major excitatory transmitter; NMDA receptors, in particular, are known to play a key role in memory formation and maintenance. The observed reduction in NR2B mRNA indicates the impairment of memory formation, whereas the rise in NR2A indicates the enhancement of any function in memory formation. Thus, there is a possibility that the results in the aforementioned reports of RAM performance[37–39] show the characteristics of TCDD toxicity.

Possible Mechanisms and Actions of Dioxins

AhReceptor system

The manifestation of toxicities of dioxins can be explained, although not entirely, on a molecular basis by aryl hydrocarbon receptor (AhR) system. AhR, a transcription factor to which aromatic hydrocarbons including dioxins bind, is proven as a dioxin receptor[49-52]. Dioxins enter into cells and bind to AhR to form a heterodimer with the nuclear conjugation factor ARNT[53, 54], and then bind to xenobiotic responsive element (XRE) to regulate the expression of various genes such as the CYP1A1. The AhR system has several interesting characters. First, AhR is one of the ligand-dependent receptors which activate different pathways; for example, polycyclic hydrocarbons including TCDD induce the expression of CYP1A1, but aldehydes induce the expression of the Aldh3α1 gene. Each pathway has been suggested to have some cross-talks[55]. Second, AhR can exhibit a protein-protein interaction with other proteins such as the estrogen receptor[56, 57] or the hypoxia-inducible factor 1α (HIF-1α)[58] to alter gene expression. Thus, AhR may alter the transduction activity of genes, dependently on cell/tissue-specificity and on time-specificity in the developmental stage.

Despite the plethora of studies on the molecular basis of AhR-dependent toxicity of dioxins, few reports on the relationship between AhR and neurotoxicity are available. However, it has been shown that AhR protein was expressesed in the brain tissue[59]. Kainu *et al.* (1995) has shown that AhR is expressed in neurons and glial cells[60]. A recent study revealed the expression of CYP1A1, AhR, and ARNT in the olfactory bulb, hypothalamus, hippocampus, neocortex, substantia nigra, cerebellum, and pituitary, by a semi-quantitative assay by RT-PCR and western blotting[61]. Petersen *et al.* (2000) reported that the *in situ* hybridization signals for AhR and ARNT were observed in the arcuate nucleus (Arc), ventromedial hypothalamus (VMH), periventricular nucleus (PVN), suprachiasmatic nucleus (SCN), dorsal raphe nucleus, and median raphe nucleus in SD rats, and that the levels of ARNT2 mRNA expression were greater than that of ARNT mRNA[62]. It is thus considered that AhR and ARNT are expressed various areas of the brain. The autographical study revealed that highest concentrations of ^{14}C-TCDD were found in the hypothalamus and the pituitary after the exposure[63]. The expression of CYP1A1 mRNA and proteins were observed 1 day after the exposure to TCDD (10 μg/kg in SD rats) in all regions of the brain[61]. These studies indicate that dioxins can be transferred to the brain and affect immediately after exposure, via AhR system.

Neural and glial cell toxicity

TCDD has been shown to have acute effects on both neuron and glial cells. Regarding neurons, TCDD has been reported to induce intracellular αPKC with a subsequent increase in Ca^{2+} uptake in rat hippocampal primary cultured neurons[64]. Because this effect on calcium homeostasis was not eliminated by the block of protein synthesis by cychrohexymide administration, it is suggested that additional protein synthesis is required[64]. TCDD has also been reported to suppress excitatory postsynaptic potentials (EPSP) in rat ventral hippocampal slices[65]. It is suggested that the effect on EPSP is caused by calcium homeostasis abnormalities, because it was eliminated by nifedipine, a L-type calcium channel blocker[65].

Regarding glial cells, TCDD has been reported to affect the intracellular calcium homeostasis of glial cells[66]. Takanaga *et al.* (2000) reported that TCDD suppressed the differentiation of C6 glioma cells via AhR system[67]. It has also been reported that TCDD inhibited the astroglia-neuronal gap junctional communication in the hippocampal primary culture[68]. In rat hepatocytes, inhibition of gap junction has been suggested to be dependent on cAMP[69].

Oxidative stress

Dioxins are known to induce oxidative stress in the liver tissue. In the brain, a subchronic exposure to TCDD (0, 0.45, 1.5, 15, and 150 ng/kg/day at 5 days/week for 13 weeks) in B6C3F1 mice, showed a dose-dependent increase in bio-markers of oxidative stress, such as the superoxide anion, lipid peroxidation, and DNA-single-strand breaks in the brain tissue[70]. Bagchi *et al.* (2002) reported that a single oral dose of TCDD at a half level of LD_{50} increased oxidative stress in the brain[71].

The HIF1-α, which is induced by hypoxic stimulation, is known to form a heterodimer with ARNT, which binds to hypoxia-response element (HRE), and to activate VEGF (vascular endothelial growth factor) expression[72]. Because CYP1A1 expression is suppressed under hypoxic conditions[73, 74], dioxins are suggested to possibly be involved in oxidative stress perhaps by competing for ARNT with the HIF1-α.

AhR-independent action

It is thought that not all dioxin toxicities are mediated by AhR, while they are not limited to the nervous system. A typical AhR-independent action is fetal death caused by maternal exposure. Mimura *et al.* (1997) reported that

TCDD-induced fetal death occurred at higher incidence in AhR null mice than in wild-type (WT) mice[52]. Peters *et al.* (1999)[73] confirmed this phenomenon in another line of AhR null mice prepared by Fernandez-Salguero *et al.* (1995)[50]; that is, the incidence of morphological anomalies such as cleft palate induced by TCDD (25 μg TCDD/kg on GD10) was much lower in AhR-null mice than in wild-type mice, however, considerably increased resorptions were observed in AhR-null mice whereas no fetal death was observed in wild-type mice[73]. Thus, it is suggested that TCDD induces fetal death possibly via AhR-independent mechanism, and that its effects become more conspicuous in the absence of the AhR system. Although AhR-independent mechanism remains to be clarified in the nervous system, a possible action may arise from the studies on PCBs as described below.

Endocrine disruption

The function and development of the brain are prone to strong influence of steroid hormone. Dioxins have been suggested to act via an endocrine disrupting mechanism: This supposition is supported by the observation that a TCDD treatment (1 μg/kg/day TCDD for 13 weeks) to SD rats caused morphological changes in the ovary and uterus probably due to disturbance of estrus cycle[76], which suggests an anti-estrogen action. However, recent studies suggested that effects of TCDD do not represent a simple action as an estrogen-like substance or anti-estrogen-like substance.

There are conflicting reports on serum estradiol levels after exposure to TCDD, showing its decrease in monkeys[77] but no significant effect in rats[78]. Although TCDD (20 or 80 μg/kg) administration to 25-day-old female Long-Evans rats decreased hepatic and uterine estrogen receptor levels[79], TCDD did not compete with estrogen for binding to estrogen receptor (ER) or progesterone receptor, and estrogen was not found to compete with TCDD for the binding to AhR[79, 80]. Zacharewski *et al.* (1991) indicated that the decrease of ER levels by TCDD depends on AhR, based on the results that TCDD caused only a minimum decrease in the ER binding activity in the low-AhR cell line of Hepa 1c1c7, and did not affect it in the AhR-deficient mutant cell line of Hepa 1c1c7[81].

Recent *in vitro* study using procine follicular cells showed that TCDD increased estrogen production in theca cells but decreased progesterone and estrogen production in granulosa cells when they were cultured alone, and that TCDD decreased progesterone and estrogen production in these co-culture, indicating that TCDD affects cell-specific effect in ovarian follicular cells[82].

It has also been indicated that TCDD has cross-talks with the estrogen-induced biological reaction. TCDD suppressed estrogen-induced tPA (tissue type plasminogen activator) secretion even at low-doses that do not reduce ER mRNA in MCF-7 breast cancer cells[83]. Recently, Son and Rozman (2002) reported that TCDD induced PAI-1 (plasminogen activator inhibitor-1) in mouse hepatoma cell lines and that this effect is dependent on the AhR system because the effect was not observed in AhR-deficient Hepa1c1c7 mutant cells[84]. It has been shown that estrogen enhanced the TCDD-induced expression of CYP1A1 in the liver[85], and that TCDD-induced EROD (ethoxyresorufin-O-deethylase) activity in female SD rats during proestrus was especially high[86].

Thus, the endocrine disrupting action of dioxins has been suggested to represent not a simple direct agonistic or antagonistic action but complex actions such as 1) tissue and cell specific actions, 2) cross-talking actions at molecular levels.

Hypothalamo-pituitary-gonadal axis

Terranova and coworkers reported a series of remarkable studies about inhibitory effect of TCDD on ovulation. TCDD administration decreased the number of ova at ovulation in the hypophysectomized and gonadotropin-primed SD rats[87], and that TCDD also decreased it in the gonadotropin-primed immature rats[88]. Additionally, Petroff *et al.* (2000) reported that the treatment with GnRH (gonadotropin-releasing hormone) recovered the LH/FSH surge that was decreased by TCDD in the gonadotropin-primed immature rats[89], indicating that the LH/FSH secreting function and GnRH-sensing function in the pituitary is not affected by TCDD. Furthermore, the number of ova was not restored at a level to match the serum LH level, and GnRH-induced E2 (estradiol) and P4 (progesterone) levels were lower in TCDD-exposed animals than in controls[89]. Thus, TCDD is suggested to have apparently binary actions on the ovarian follicle maturation and hypothalamus.

Hypothalamo-pituitary-adrenal axis

The adverse effects caused by maternal exposure to dioxins, such as the disruption of brain sexual differentiation, immunosuppression, and endocrine disruption, can be caused by the maternal stress or the alteration of adrenocortical hormone during pregnancy. According to a large number of reports, low doses of dioxins have been thought not to affect the hypothalamo-pituitary-gonadal (HPA) axis in dams. The adverse effects of TCDD on HPA axis has been reported for serum corticosterone levels[90], the mitochondrial cholesterol levels induced by adrenocorticotropin (ACTH) in the adrenal primary culture[91] and ACTH concentration in the anterior pituitary primary cultures[92]. However, no

such effects were observed in plasma ACTH levels in SD rats treated at a TCDD dose of 15 μg/kg[93] and of 50 μg/kg[94]. Recently, Pitt *et al.* (2000) showed in an *ex vivo* perfusion system that TCDD did not affect the pituital ACTH and the adrenocortical hormone from the adrenal cortex[95]. Although a problem for differences in animal sensitivity remains to be ruled out, we could conclude that little evidence is available for the effect of dioxins on the HPA axis, at least *in vivo* and *ex vivo*.

Hypothalamo-pituitary-thyroid axis

Dioxins have been suggested to affect hypothalamo-pituitary-thyroid axis partly due to their similarity to thyroid hormone in chemical structure. It has been reported that maternal exposure to TCDD (100 ng/kg/day to dams, on GD10 to GD16) decreased serum T4 levels in offspring[96]. Nishimura *et al.* (2002) reported that serum T4 levels decreased significantly 7 days after the exposure to TCDD (2 μg/kg)[97]. Exposure to TCDD at dose of 4 μg/kg caused a more pronounced decrease in T4 levels and an increase in TSH (thyroid-stimulating hormone) secretion from the pituitary, which then resulted in the occurrence of thyroid hyperplasia. TCDD is shown to induce UGT1A6 (UDP-glucuronosyltransferase 1A6) in the liver perhaps via AhR, followed by glucuronate conjugation of T4, which in turn increases T4 excretion in bile, resulting in decreased blood T4 concentrations. Subsequently, the negative feedback mechanism was affected to cause TSH release from the pituitary, resulting in elevated serum TSH concentrations[97].

It is well known that the impairment of the thyroid homeostasis can affect the development of advanced brain function; T4 has recently been confirmed to promote the neural growth in mouse cerebellar Purkinje cells *in vitro*[98]. There is the possibility that the toxicities of dioxins in the brain development and growth may be attributable, at least in part, to a decreased level of T4 in the blood circulation. However, it has also been suggested that thyroid hormone is more affected by non-dioxin-like PCBs than by dioxins[99], as described below.

Adverse Effects of Dioxin-Like PCBs and Non-Dioxin-Like PCBs in Developing Brain

Disruption of thyroxin function by PCB exposure

PCBs have been suggested to exhibit either different kinds of effects or more conspicuous actions in the brain as compared to TCDD. PCBs have been reported to reduce thyroid hormone concentrations[100, 101]. Seo *et al.* (1995) compared the effects of PCBs and TCDD on thyroid hormone, by administering TCDD (0.025 or 0.1 μg/kg/day), PCB 77 (3,3',4,4'-tetrachlorobiphenyl, 2 or 8 mg/kg/day, based on the 1997 WHO TEF of PCB 77 as 0.0001, which are equivalent to 0.2 or 0.8 μg TEQ/kg/day, respectively), or PCB126 (3,3',4,4',5-pentachlorobiphenyl, 0.25 or 1 μg/kg/day, based on TEF = 0.1, which is equivalent to 0.025 or 0.1 μg TEQ/kg/day, respectively), to SD rats on GD 10 to GD 16[96]. Although TCDD reduced serum T4, PCB77 had greater effects despite nearly the same exposure level based on TEQ[96].

Crofton and co-workers reported that exposure to Aroclor 1254 (4 and 8 mg/kg/day from GD 6 to weaning), a commercial PCB mixture on industrial scale in United States, which contains coplanar PCBs, reduced serum T4 levels with an auditory deficit at low-pitch frequency[102, 103]. This auditory deficit was confirmed by histological examination in that maternal exposure to Aroclor 1254 induced a loss of outer hair cells in the Corti organ, which has a role in the sound processing in the low-pitch frequency[104]. It has also been suggested by cross-fostering study that a decreased level of T4 by Aroclor 1254 may be attributable to lactational exposure[104]. Thus, PCBs have an adverse effect on thyroid function, via both AhR-dependent and independent actions.

As seemingly AhR-independent effects, PCB compounds and their hydroxylated metabolites have been suggested to compete with T4 for transthyretin and thyroid-binding globulin (TBG), both of which are proteins responsible for the transportation of T4. Zoeller *et al.* (2000) reported that administration of Aroclor 1254 (4 and 8 mg/kg/day from GD 6 to weaning, in SD rats) showed a marked increase in the expression of RC3/neurogranin that is mediated by a T3 receptor on PND15 despite the dramatically decreased blood T4 levels[105]. There are some hydroxylated PCBs with higher affinity for transthyretin and TBG compared to T4[106]. Iwasaki *et al.* (2002) showed by a reporter assay that the hydroxylated PCBs affected the TR (thyroid hormone receptor) /coactivator complex to inhibit T3-TR transactivation[107]. In contrast, Morse *et al.* (1996) suggested that the T3 level in the brain remained constant because the induction of brain type II thyroxine 5'-deiodinase activity compensates for decreases in brain T4 levels[108]. It is also indicated that the hydroxylated PCBs that inhibit T4-transthyretin binding in rodents do not inhibit T4-TBG binding in humans, suggesting that such PCBs exhibit differential effects on humans and rodents[109].

Disturbance of neural transmission by PCBs

In addition to the suppressive effects on thyroid hormone, PCBs have been suggested to directly disturb neural

transmission. Seegal *et al.* (1990) reported that Aroclor 1060, a PCB mixture, decreased dopamine contents in the striatum, substantia nigra, and hypothalamus in monkeys[110]. *In vitro* study revealed that each or combined exposure to PCB77 (1997 WHO TEF = 0.0001) and PCB 126 (TEF = 0.1) did not affect intracellular dopamine concentration in PC12 cells, whereas exposure to non-dioxin-like PCBs, PCB 28 (2,4,4'-trichlorobiphenyl, PCB 47 (2,2'4,4'-tetrachlorobiphenyl), or PCB 52 (2,2',5,5'-tetrachlorobiphenyl) affected it[110].

It has also been reported that PCBs affect the PKC translocation and on the Ca^{2+} buffering, all of which have been suggested to be associated with a structure-activity relationship, being independent from TEF[111, 112]. Very recently, Yang *et al.* (2003) indicates that the patterns of subcellular distributions of PKC isoforms following PCB exposure were PKC isozyme-specific and developmental stage-specific, based on the results that the maternal exposure to Aroclor 1254 (6 mg/kg/day, from GD 6 until weaning) caused decreases in cytosolic PKC-α, -γand -ε and increases in particulate PKC-α, -γand -ε in the cerebellum and the hippocampus, and that the effects were greatly significant at postnatal day 14[113].

Mariussen and Fonnum (2001) demonstrated that PCBs exhibited competitive inhibitory action on dopamine uptake in rat brain synaptic vesicles[114]. Moreover, analysis of the structure-activity correlation of PCBs indicated that only those PCBs having a chlorine substitution at the ortho-position suppressed dopamine uptake, and that the highest activity was possessed by penta-chlorinated and hexa-chlorinated PCBs[115]. Ortho-substitued congener PCB95 (2,2',3,5',6-pentachlorobiphenyl) into the cultured medium reduced long-term potentiation (LTP) in the hippocampal slices[116]. Gilbert *et al.* (2000) reported that maternal exposure to Aroclor 1254 (6 mg/kg/day from GD 6 to weaning, in Long-Evans rats) reduced LTP in the offspring hippocampal dentate gyrus[117]. Altmann *et al.* (1998) showed that maternal exposure to dioxin-like isomer PCB77 reduced LTP in the visual cortex, rather than in the hippocampus, whereas non-dioxin-like isomer PCB 47 did not affect it[118]. Hussain *et al.* (2000) reported that the tetanus stimulation-induced hippocampal LTP was significantly reduced by maternal exposure to non-dioxin-like isomer PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl, 1.25 mg/kg/day given from GD 3 to weaning)[119], indicating that non-dioxin-like PCB can affect hippocampal LTP. In the slice culture, LTP was also acutely suppressed by perfusion with 3 nM of PCB153[119], indicating that PCB153 suppresses the hippocampal LTP independently from thyroid actions. Thus, PCBs are thought to have direct and acute neurotoxic effects, being independent from TCDD-like toxicity.

Impairment of learning performance by PCBs

Some PCB isomers have also been reported to have stronger effects on learning performance than TCDD. It has been reported that maternal administration of dioxin-like isomer PCB 77 (TEF = 0.0001) (32 mg/kg/day, then 3.2 μg TEQ/kg/day, on GD10 to 16) to CD-1 mice induced the hyperactivity, reduction of front leg gripping, the disturbance in the vision replacement and disruption of the passive avoidance in their offspring[120], and that these behavioral changes are accompanied by the inhibition of synaptic formation in the nucleus accumbens[121] and by decrease in dopamine contents and its receptor binding in the striatum[122]. Eriksson *et al.* (1991) reported that postnatal exposure to PCB 77 (41 mg/kg, then 4.1 μg TEQ/kg) to male NMRI mice on PND10 affected locomotion in adulthood, i.e. the decrease in the initial stage of the test and the increase in the final stage of the test, suggesting that PCB inhibited habituation to the test environment[123]. Recently, Roegge *et al.* (2000) reported that maternal administration of Aroclor 1254 (6 mg/kg/day, given i.p.) to Long-Evans pregnant rats on GD 6 to weaning impaired spatial learning performance in male offspring; both the number of errors in working memory (number of re-entries into arms the animal once entered) and the number of errors in reference memory (number of entries into arms without pellets) increased in a 12-arm RAM test, whereas no effects were found in female offspring[124].

Rice *et al.* (1997, 1998, 1999) reported that exposure to a PCB mixture that resembled the human breast milk pattern of congeners, impaired the learning and memory performance such as the discrimination reversal and spatial delayed alternation performance in monkeys[125–127]. These behavioral effects can be characterized as follows. The monkeys exposed to PCBs were unable to apply a method to cope with the each task after learning its method and thus repeatedly made error. The impairment was also observed in a fixed interval schedule tasks in which the test animal is required to constitute its behavior in a short time. Taken together, Rice (2000) suggested that PCBs and other chemical substances which are found in our environment might serve as an inducer of ADHD (attention-deficit hyperactivity disorder), because above behavioral features are seen in ADHD in children[128].

Other experimental studies using rats also support her hypothesis. Administration of either PCB 77 (0.5 or 1.5 mg/kg/day, followed by 0.05 or 0.5 μg TEQ/kg/day), non-dioxin-like isomer PCB 47 (1.5 mg/kg/day), or their mixture (0.5 mg/kg of PCB 77 + 1.0 mg/kg of PCB 47) to dams

from GD 7 to 18, induced hyperactivity in offspring[129]. The exposure to PCB 77 also shortened the latency in the passive avoidance behavior and the latency in the haloperidol-induced catalepsy test, but PCB47 did not[129]. These findings suggest that PCBs exhibit toxicities different from the "dioxin-like toxicities" as described in the previous sections.

Future Directions

The effects of dioxins on the developing brain are attributable to toxicities on neural transmission and neural network formation, and those on the hypothalamo-pituitary-thyroid axis or the hypothalamo-pituitary-gonadal axis; i.e. 'the neural disruption' and 'the neuroendocrine/endocrine disrution'. The exposure to low doses of dioxins seems to affect more conspicuously the advanced brain function, rather than the reproduction-controlling function of the brain. This hypothesis is supported by the finding that changes in masculine sexual behavior by TCDD are caused by inhibition of the neocortical activity-dependent function[12].

Behavioral toxicological studies strongly suggest that exposure to dioxins cause a permanent or semi-permanent dysfunction in the developing brain. Sex differences in behavioral changes in non-reproductive function as pointed out by Weiss and coworkers[42, 43, 45], and behavioral changes in the RAM performance as reported by Shantz and coworkers[37-39] have been clearly observed with extremely low levels of exposure that is relevant to our exposure in the general environment. The neurotoxicity of dioxins in low-dose exposure can be elucidated in terms of their quality and features by further studies in these behavioral changes. To elucidate these behavioral changes, it is important to assess the molecular, cellular, histological and anatomical studies in the equivalent animal to behavioral studies.

From behavioral studies, exposure to PCBs are suggested to cause ADHD-linked behavioral abnormalities[125-127]. Although some behavioral toxicites of PCBs are attributable to the inhibition of thyroid hormone function, other toxicities are manifested by the inhibition of direct pathways of neural or neuronal transmission, such as the inhibition of dopamine uptake and the alteration of PKC translocation. These toxicities have been suggested not only with dioxin-like PCBs but also with non-dioxin-like PCBs, and can be viewed as 'non-dioxin-like toxicities' that are not mediated by the AhR system.

Dioxin emissions into the environment have been estimated to be decreased dramatically over the past several years, and the manufacture and use of PCBs in the open system have been banned since 1972. However, due to their persistency in nature, the greater amounts of these compounds remain in the environment. As described in this article, very low level of exposure to dioxins and related compounds as well as non-dioxin-like PCBs are a threat to particularly children. To establish and apply highly reliable scientific test methods to evaluate possible effects of very low doses of these chemicals on the brain remain to be done.

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