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Alterations in gene expression of glutamate receptors and exocytosis-related factors by a hydroxylated-polychlorinated biphenyl in the developing rat brain

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ABSTRACT

Polychlorinated biphenyls (PCBs), major environmental hormonally active agents, are metabolized into hydroxylated PCBs in the liver to facilitate excretion. Some of hydroxylated PCBs also have potencies disturbing endogenous hormonal activities at least *in vitro*. Hormonal activities of hydroxylated PCBs raise a possibility of their interfering with normal brain development which is strictly regulated by endogenous hormones. We investigated whether and how prenatal exposure to a congener of hydroxylated PCBs (4-OH-2′,3,3′,4′,5′-penta CB; 4-OH-PCB106) having activities to disrupt thyroid hormone-dependent signals *in vitro* could perturb normal gene expression in the developing brain *in vivo*. Pregnant rats were exposed to 4-OH-PCB106 subcutaneously at the dose of 1.0 mg/(kg day) from day 7 of gestation to postnatal day 1. Then three brain regions (cerebral cortex, hippocampus and striatum) were obtained from offspring on postnatal day 1 and subjected to further gene expression analyses. Comprehensive analyses of mRNA expression by oligo DNA microarrays and subsequent validations by quantitative RT-PCR revealed that prenatal exposure to 4-OH-PCB106 affected mRNA expression of glutamate receptors as well as that of thyroid hormone-responsive genes in region-specific manners. Concomitantly 4-OH-PCB106 exposure increased mRNA expression of genes related to exocytosis in the three brain regions. These results raise the possibility that prenatal exposure to some hydroxylated PCBs with thyroid hormone-disrupting potencies leads to abnormal brain development via perturbations on the expression of genes involved in glutamatergic neurotransmission.

1. Introduction

Polychlorinated biphenyls (PCBs) are known as worldwide environmental contaminants and are found in human and many wildlife species. PCBs undergo bioconcentration through the food chain due to their high chemical stability and lipophilicity (Patandin et al., 1999; Winneke et al., 2002) and have been considered and studied as endocrine disrupting chemicals (Barter and Klaassen, 1994; Brouwer, 1989; Byrne et al., 1987; Goldey et al., 1995; Gray et al., 1993; Meerts et al., 2002; Morse et al., 1993; Van Birgelen et al., 1994a,b; van den Berg et al., 1988).

In addition, recent *in vitro* studies revealed that hydroxylated PCBs, intermediate metabolites of PCBs produced by first-phase metabolism in the liver, also have abilities to disrupt the gene transcription which depends on endogenous hormones such as thyroid hormone (Bansal et al., 2005; Iwasaki et al., 2002; Miyazaki et al., 2004; You et al., 2006), and can inhibit the thyroid hormone-dependent dendritic arborizations of cultured cerebellar Purkinje cells (Kimura-Kuroda et al., 2005). Among endogenous hormones, thyroid hormone is an essential hormone for normal brain development (Rice and Barone, 2000). Congenital hypothyroidism causes cretinism (Bernal, 2002; Porterfield and Hendrich, 1993) with mental retardation (Carranza et al., 2006) and insufficient thyroid hormone signal during the critical period is proposed as one of the environmental factors responsible for attention deficit/hyper activity disorder (ADHD) (Negishi et al., 2005; Vermiglio et al., 2004).

Unfortunately, human fetuses seem to be exposed to hydroxylated PCBs (Park et al., 2007; Sandau et al., 2002) as well as parent PCBs (Bjerregaard and Hansen, 2000; Korrick et al., 2000; Sala et al., 2001) through placentas of contaminated mothers. These contexts increase concerns about the effect of exposure to hydroxylated PCBs on brain development, because brain development is strictly regulated by endogenous hormones such as thyroid hormone. Therefore, prenatal exposure to hydroxylated PCBs during the period corre-

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sponding to critical period of thyroid hormone action may perturb thyroid hormone-dependent brain development. In the present study, we investigated whether and how prenatal *in utero* exposure to a congener of hydroxylated PCBs (4-OH-2′,3,3′,4′,5′-penta CB (4-OH-PCB106)), which is known to have thyroid hormone-disrupting activities at the molecular level (Miyazaki et al., 2004; You et al., 2006), could interfere with gene expression in the developing brain *in vivo*. In the present study, we did not use oral gavages but osmotic pumps subcutaneously implanted in mother at the dose of 1.0 mg/(kg day) from gestational day 7 until postnatal day 1, because this chemical is metabolized intermediates rather than direct contaminant from environment via foods or water.

2. Materials and methods

2.1. Animals and treatments

Male and female F344/N rats were purchased from SLC (Shizuoka, Japan) and maintained under controlled temperature (24 ± 1 °C) and humidity (55 ± 5%) on a 12-h light (09:00–21:00 h):12-h dark (21:00–09:00 h) cycle. Food and water were freely available. After acclimatization for 1 week, female rats were placed with males. Vaginal smears were examined daily and sperm-positive smear determined gestational day (GD) 0. After detection of sperm-positive smear, the pregnant dams were housed individually and were randomly assigned to an exposure condition (Fig. 1). Nine dams were exposed to hydroxylated PCB (4-OH-2′,3,3′,4′,5′-penta CB (4-OH-PCB106); chemical purity >98%, AccuStandard Inc., New Haven, CT) dissolved in vehicle consisting of N,N-dimethylacetamide (Wako Pure Chemical Industries, Ltd., Japan) and polyethylene glycol (1:1) (Wako Pure Chemical Industries, Ltd.) via subcutaneously implanted osmotic pump (DURECT Corporation, Palo Alto, CA) at the dose of 1.0 mg/(kg day) from GD 7 until postnatal day (PND) 1. Ten dams were exposed to vehicle only. Then, whole brain tissue was dissected from male offspring at PND1 and separated anatomically into three regions (the cerebral cortex, the hippocampus, and the striatum). These samples were subjected to total RNA preparation. This study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, University of Tokyo. All animals were treated with humanity.

2.2. Total RNA preparation

Total RNA of the cerebral cortex, the hippocampus and the striatum was prepared using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Quality of individual total RNA was checked by BioAnalyzer (Agilent Technologies, Palo Alto, CA). For real-time PCR, total RNA in each region and groups were individually used ($n = 6$; 1–2 pups/litter, 5–6 litter).

2.3. Fluorescent labeling of cRNA and Hybridization condition

For microarray analysis, equal amount of individual total RNA samples were mixed within each region and group. These mixed samples (2 μg total RNA in each) were considered as average total RNA of each group. These mixed samples were used only for microarray analysis. The mixed RNA was reverse transcribed with RNA Transcript SureLABEL Core kit (Takara, Shiga, Japan), which was followed by transcription into promoter-tagged second strand cDNA for linear amplification. During linear amplification, 5 mM aminoallyl (aa)-UTP (Applied Biosystems, CA) was incorporated into the cRNA, which is subsequently coupled with Cy3 (4-OH-PCB106-exposed group) or Cy5 (control group). Dye-labeled cRNA was purified and concentrated by RNeasy MinElute cleanup kit (QIAGEN Science, Maryland). Finally, Cy3- (4-OH-PCB106-exposed group) and Cy5- (control group) labeled cRNA (1 μg in each) were mixed, fragmented by RNA Fragmentation Reagents (Applied Biosystems), and hybridized to oligonucleotide microarray (Rat V2 Oligo Microarray Kit, Agilent Technologies), according to the operations recommended by the manufacturer.

2.4. Microarray data acquisition and data normalization

After hybridization, microarrays were scanned by DNAscope™IV (GeneFocus Biomedical Photometrics Inc., Canada). The fluorescent signals of gene-specific spots were quantified after background correction by ImaGene 5.5 (Biodiscovery, CA) and normalized using the global normalization (Chen et al., 2003; Workman et al., 2002).

2.5. Functional annotation

We used GoCharts of Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov) (Dennis et al., 2003) to obtain molecular functional annotation.

2.6. Real-time quantitative fluorescence-based PCR

Expression levels of each gene were quantified by fluorescence-based real-time PCR using Smart Cycler System (Cepheid, CA) with SYBR Premix ExTaq (Takara). The individual cDNA templates ($n = 6$; 1–2 pups/litter, 5–6 litter) were synthesized from 3 μg each of total-RNA using SuperScript II (Invitrogen). Cyclophilin-A (Ppia) was used as a housekeeping gene based on its independency from thyroid hormone status (Munoz et al., 1991). PCR primers were designed using Oligo 6.0 primer analysis software (Molecular Biology Insights, CO), with one of the pair of primers selected within the microarray probe sequence if possible. PCR primers were as follows: Ppia, 5′-GCAAGACCAGCAAGAAGATCACC-3′ and 5′-CTTCAGTGAGAGCAGAGATTACAG-3′; neurogranine (RC3/Nrgn), 5′-GACTTCCCTACTGTGTTTGTGAG-3′ and 5′-CTACGCCACGACGAAGCCAGC-3′; myelin basic protein (MBP), 5′-CACAGAGACACGGGCATCCTTG-3′ and 5′-CTTGGGTCCTCTGCGACTTCTG-3′; hairless (Hr), 5′-GCTGACCCCTCCCCTCATGG-3′ and 5′-GCAGTTGAGATACACAGAGGAAG-3′; Reelin (Reln), 5′-GGTGGATGTTTCCCTCACTGTG-3′ and 5′-GTGCAGCCTTCTGTTTCAGGAC-3′, thyroid hormone receptor alpha (THRα), 5′-CCACTCAAGTGTCACCTCCTTC-3′ and 5′-GCACTGGGCTACAGACATGATTC-3′; thyroid hormone receptor beta (THRβ), 5′-CTCTGTAGAGGCTCACCTTTCAG-3′ and 5′-GCCTACCGTTCTTGTTCCTTCC-3′; VAMP1, 5′-GAGGTGAAGTGTCTGAGAAGTTAG-3′ and 5′-CAACTATTCTTGCTTCCCTCCTG-3′; VAMP2, 5′-GGAACCAAGGAGATCTAAACTGTG-3′ and 5′-GGAAGGCAGCGGACACTGGG-3′; Stx1b2, 5′-GATCATCATTTGCTGTGTGGTGC-3′ and 5′-GGTATTGCTCCCGATGTGTGG-3′; SNAP25, 5′-GACACCCAGAATCGCCAGATTG-3′ and 5′-CAGATTTAACCACTTCCCAGCATC-3′; NR1, 5′-GGTCCTCCAAAGACACGAGCAC-3′ and 5′-CGTCTCAGCTCTCCCTATGACG-3′; NR2A, 5′-GAGATGTCCCTCGGACCCTTAC-3′ and 5′-CTCTGAAATATACACATCACTGTGG-3′; NR2B, 5′-CCTTGTCACCAATAAGCCAGTGG-3′ and 5′-CATGTCCATTGCTGGAGCCATTG-3′; NR2C, 5′-CTGTCTGCCGTCACCCTCCAC-3′ and 5′-GCACCCCACTGTCCCTGTAGC-3′; NR3A, 5′-GTCTGCTATGCCCTTCTGTTTGG-3′ and 5′-CTCACCTACCATGACAGCAGCC-3′; NR3B 5′-CTCACCTACCATGACAGCAGCC-3′ and, 5′-GTTTGTCCTGCTGTGCCTCGGG-3′.

2.7. Statistical analyses

Effect of 4-OH-PCB106 exposure on maternal body weights were analyzed by two-way repeated-measures analysis of variance (ANOVA) and that on the umber of total infants and male infants and body weight of male infants were analyzed by student's *t*-test. Effect of 4-OH-PCB106 exposure on mRNA expression of each gene in each region was analyzed independently by student's *t*-test. Normal mRNA expression levels of each gene were compared among three regions by analysis of variance (ANOVA), which was followed by Tukey–Kramer multiple comparisons. The percentage of affected genes in functional categories indicated by DAVID was statistically tested by statistical test for the population proportion, where the null hypothesis

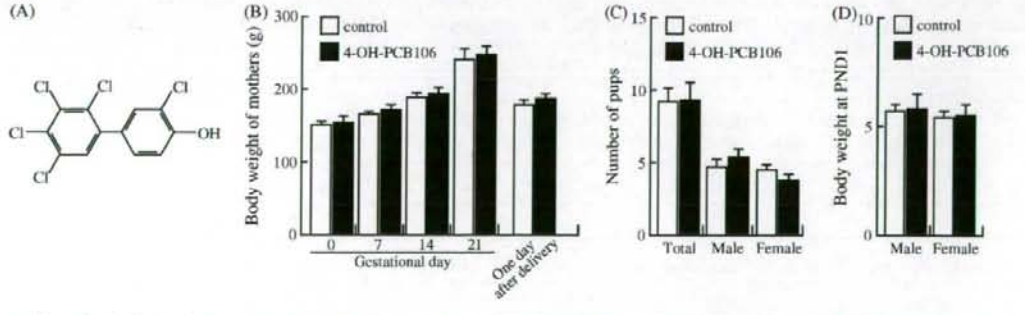

Fig. 1. Structural formula of 4-OH-2′,3,3′,4′,5′-penta CB (4-OH-PCB106) (A). Maternal body weight (B), the number of total, male, and female infants delivered (C), and body weights of infants at postnatal day 1 (PND1) were not affected by 4-OH-PCB106 exposure (D). Data is expressed as mean + S.D.

was that the percentage was larger than 21.93% (2918 (the number of genes affected by hydroxylated PCB exposure at least in one region)/13,304 (the number of analyzable genes in the microarray used in the present study)). All statistical rejection level was set at 0.05.

3. Results

3.1. General toxicity of exposure to hydroxylated PCB

Under the present experimental conditions, exposure to 4-OH-2′,3,3′,4′,5′-penta CB (4-OH-PCB106, Fig. 1A) had no significant effect on the body weight of mothers as well as the number and body weight of male offspring (Fig. 1).

3.2. Effect of prenatal hydroxylated PCB exposure on mRNA expression of thyroid hormone receptor genes and the known thyroid hormone-regulated genes

Since the congener of hydroxylated PCB used in this study has been shown to exert thyroid hormone-disrupting activity *in vitro* (Iwasaki et al., 2002; You et al., 2006), we first evaluated the effect of prenatal exposure to 4-OH-PCB106 on the mRNA expression of thyroid hormone receptors α (THRα) and β (THRβ), as well as neurogranin (RC3/Nrgn), myelin basic protein (MBP), hairless (Hr) and reelin (Reln) in the cerebral cortex, hippocampus and striatum (Fig. 2). RC3/Nrgn, MBP and Hr have thyroid hormone-responsive element (TRE), and Reln is a well-known gene up-regulated by thyroid hormone (Alvarez-Dolado et al., 1999; Engelhard and Christiano, 2004; Farsetti et al., 1991; Martinez de Arrieta et al., 1999). In the cerebral cortex, 4-OH-PCB106 exposure resulted in an increase of THRα mRNA (Fig. 2A), and decreases of THRβ, MBP and Hr mRNA (Fig. 2B, D and E). In the hippocampus, only an increase of THRα mRNA was observed by 4-OH-PCB106 exposure (Fig. 2A). In the striatum, 4-OH-PCB106 exposure induced an increase of THRα mRNA (Fig. 2A) and a decrease of MBP mRNA (Fig. 2D). When normal mRNA expression levels of these genes in neonates of control group were compared among the three regions, mRNA expression of both THRα and THRβ in the striatum was significantly lower than that in the other regions (Fig. 2A and B). With regard to thyroid hormone-responsive genes, mRNA expression level of RC3/Nrgn was significantly lower in hippocampus and striatum compared with the cerebral cortex (Fig. 2C), while that of MBP was highest in the striatum and decreased progressively in the cerebral cortex and hippocampus (Fig. 2D).

3.3. Overall view of transcriptomic alterations by 4-OH-PCB106 exposure

High-density microarrays were used for comprehensive screening of alterations in gene expressions in the brain by 4-OH-PCB106 exposure *in vivo*. Genes showing more than 2-fold increase or decrease of mRNA expression were considered as those affected by 4-OH-PCB106 exposure. Numbers of affected genes among 13,304 genes on the microarray were 1659, 504 and 1285 in the cerebral cortex, hippocampus and striatum, respectively, and a total of 2918 genes were affected at least in one region. We subsequently assigned these 2918 genes to the categories based on the biological process annotation using DAVID (Dennis et al., 2003) and calculated the percentages of affected genes in each functional category. Indeed, 128 functional categories were chosen as those significantly ($p < 0.05$) affected by 4-OH-PCB106 exposure (Supplementary data 1). Among these functional categories, we focused on, considering the percentage of affected genes, those containing genes involved in neurotransmitter transport and release ("neurotransmitter transport": 60.0%, "secretory pathway": 56.5%) for further analyses. From the functional category of "neurotransmitter transport", we selected genes encoding receptor subunits for the six major neurotransmitters (glutamate, gamma-aminobutyric acid (GABA), acetylcholine, serotonin, dopamine and noradrenaline) (Supplementary data 2). For each neurotransmitter in each brain region, average (*Rave*) and variance (*Rvar*) of logarithmic expression ratios, $\log_2$(4-OH-PCB106-exposed/control), among all the receptor subunit genes were calculated (Table 1). Similarly calculated values for cytoskeleton and ribosomal protein genes were used as internal standard factors. Gene expression of the receptors of major neurotransmitters except for adrenaline were highly sus-

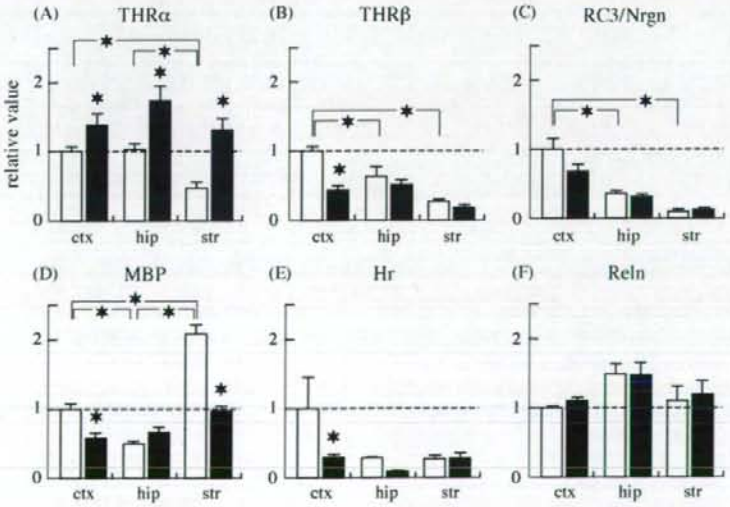

Fig. 2. Effects of prenatal 4-OH-PCB106 exposure on mRNA expression of thyroid hormone receptors (THRs) and thyroid hormone-responsive genes in the neonatal brain. Expression levels of mRNA for THRα (A), THRβ (B), neurogranin (RC3/Nrgn) (C), myelin basic protein (MBP) (D), hairless (Hr) (E) and reelin (Reln) (F) in the cerebral cortex (ctx), hippocampus (hip) and striatum (str) of male control (open bars) and 4-OH-PCB106 exposed (filled bars) neonates at PND 1 were quantified by real-time PCR. Each mRNA expression level is expressed relative to the value of the cerebral cortex of control neonates, which is indicated by a dashed line (mean ± S.E.M., $n = 6$; 1–2 pups/litter, 5–6 litter). *$p < 0.05$ between indicated pairs.

Table 1
High-density microarray analysis of neurotransmitter receptor genes affected by 4-OH-PCB106 exposure.

Category	Total[a]	No. of affected genes				Rave[b]	Rvar[c]
		Cerebral cortex	Hippocampus	Striatum	All regions		
Neurotransmitter receptors							
Glutamate receptors	27	3	6	2	9	−0.052	1.3
GABA receptors	18	3	1	1	5	−0.071	0.354
Acetylcholine receptors	16	0	1	5	6	0.248	0.451
Serotonin receptors	9	1	3	2	5	0.039	1.027
Dopamine receptors	6	2	1	1	3	0.045	0.976
Adrenaline receptors	8	0	0	0	0	-[d]	-
Cytoskeleton and ribosomal proteins							
Tubulin	13	1	0	0	1	0.044	0.293
Microfilament and actin filament	22	3	1	1	5	0.053	0.449
Ribosomal proteins	51	2	0	1	3	0.025	0.208

[a] Number of receptor genes on the high-desnsity microarray.
[b] Average of expression ratios expressed in logarithmic form (of base 2) in each category.
[c] Variance of expression ratios in each category calculated by the formula $\Sigma(Rave - Ri)^2/N$, in which Ri is each gene expression ratio expressed in logarithmic form (of base 2), and N is the number of analyzable genes in each category.
[d] Not defined.

ceptible to 4-OH-PCB106 exposure, while that of the cytoskeletal and ribosomal proteins were less affected (Table 1). Among the receptors, glutamate receptor family showed the largest variance of mRNA expression ratios ($Rvar = 1.300$), suggesting that glutamate receptor family responded most sensitively to 4-OH-PCB106 exposure whether it was an increase or a decrease in expression (Table 1).

3.4. Alterations in mRNA expression of glutamate receptors by 4-OH-PCB106 exposure

Based on overall screening, we tried to validate mRNA expression levels of glutamate receptors (N-methyl-D-aspartate (NMDA)-type (NRs) and metabotropic type (mGluRs)) quantitatively by real-time PCR. In the cerebral cortex, 4-OH-PCB106 exposure decreased mRNA expression of NR2B (Fig. 3C) and mGluR7 (Fig. 3M). In the hippocampus, 4-OH-PCB106 exposure decreased mRNA of NR2A (Fig. 3B), NR2C (Fig. 3D), NR3A (Fig. 3E), mGluR1 (Fig. 3G), mGluR2 (Fig. 3H) and mGluR5-8 (Fig. 3K–N) significantly and that of other mGluRs slightly. On the other hand, in the striatum, 4-OH-PCB106 exposure increased mRNA expression of NR2C (Fig. 3D), NR3B (Fig. 3F) and mGluR6 (Fig. 3L). In control neonates, there were some gene-specific and/or region-specific expression patterns of glutamate receptors (Fig. 3).

3.5. Up-regulation of exocytosis-related genes by 4-OH-PCB106 exposure

Next, we quantified mRNA expression of genes associated with neurotransmitter release (VAMP1, VAMP2, SNAP25 and Stx1b2) using real-time PCR (Fig. 4) because microarray analyses indicated the alteration of mRNA expression of factors related to "secretory pathway" as well as "neurotransmitter transport". In the cerebral cortex, 4-OH-PCB106 exposure increased mRNA expression of VAMP1 (Fig. 4A) and VAMP2 (Fig. 4B) significantly. In the hippocampus and striatum, 4-OH-PCB106 exposure increased mRNA expression of all four genes examined (Figs. 4A–D). There were significant region-specificities in the mRNA expression levels of these genes in control neonates. The expression level of VAMP1 mRNA was highest in the hippocampus and decreased the cerebral cortex and striatum in this order (Fig. 4A). VAMP2 (Fig. 4B) and Stx1b2 mRNA (Fig. 4C) expression showed the highest level in the cerebral cortex among the three regions. SNAP25 mRNA expression level was lowest in the striatum among the three regions (Fig. 4D).

4. Discussion

In the present study, we demonstrated that prenatal exposure to 4-OH-PCB106 caused significant alterations in mRNA expression of genes involved in glutamatergic neurotransmission in the developing brain.

There was no obvious effect of *in utero* 4-OH-PCB106 exposure at the dose of 1.0 mg/(kg day) on the delivery results and body weight of male offspring. In addition, we could not reveal abnormalities in the systemic thyroid hormone status when we measured plasma T4 of offspring (data not shown). To the best of our knowledge, there is no information about the effect of *in vivo* exposure to 4-OH-PCB106. Although subcutaneous administration at the dose of 1.0 mg/(kg day) would induce high circulation level of this congener, which is not physiologically relevant, the present results revealed that this congener had little general toxicity even at this dose. In fact, circulation level of sum of major hydroxylated PCBs in human was lower than 0.001 mg/kg (Park et al., 2007). Further dose response studies should be performed to assess human risk. About the route of exposure (subcutaneously implanted pump), as far as hydroxylated metabolites of PCBs are concerned, subcutaneous exposure would be more relevant than oral exposure, because hydroxylated PCBs are thought to be produced mainly in the liver from their parent PCBs rather than they enter the body directly from environment. It is notable that gene expression in brain was perturbed by *in utero* exposure without apparent general toxicity both in mothers and infants. Here we selected subcutaneous exposure. Based on the previous reports referring to thyroid hormone-disrupting activities of the same congener at the molecular level *in vitro* (Kimura-Kuroda et al., 2005; Miyazaki et al., 2004; You et al., 2006), we first assessed gene expression profiles of thyroid hormone-related factors in *in vivo* developing brain. Increased mRNA expression of THRα in all three regions would indicate a compensatory response to reduced thyroid hormone-dependent signals caused by anti-thyroid hormone-like activity of 4-OH-PCB106. Despite this compensatory response, 4-OH-PCB106 exposure decreased the mRNA expression of two representative thyroid hormone-responsive genes (MBP and Hr) in the cerebral cortex and one (MBP) in the striatum. These results suggest that 4-OH-PCB106 exerted anti-thyroid hormone-like activity in the developing brain and that there was a difference in sensitivity to 4-OH-PCB106 exposure among the three brain regions.

However, it may be difficult to simply attribute the decreased mRNA expression of thyroid hormone-responsive genes only to the anti-thyroid hormone-like activity of 4-OH-PCB106. Exposure

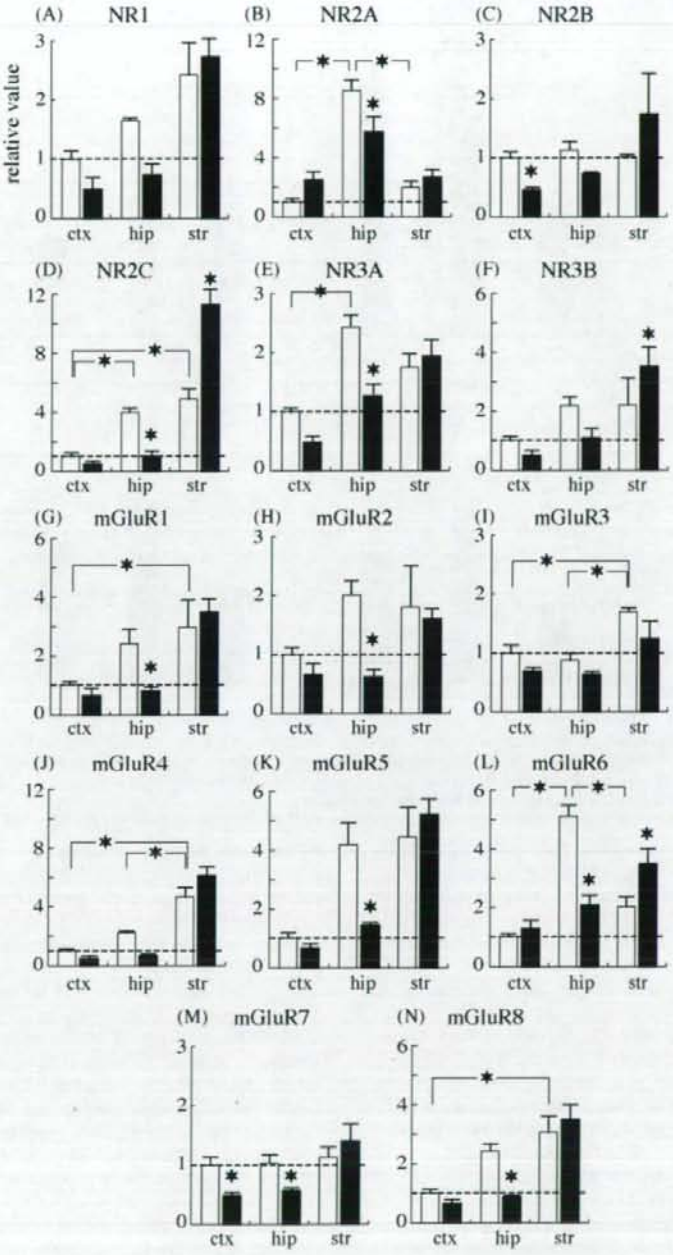

Fig. 3. Effect of prenatal 4-OH-PCB106 exposure on mRNA expression of glutamate receptors in the neonatal brain. Expression levels of mRNA for NMDA receptor subunits (NR1 (A), NR2A (B), NR2B (C), NR2C (D), NR3A (E), NR3B (F)) and metabotropic glutamate receptors (mGluR1 (G), mGluR2 (H), mGluR3 (I), mGluR4 (J), mGluR5 (K), mGluR6 (L), mGluR7 (M) and mGluR8 (N)) in the cerebral cortex (ctx), hippocampus (hip) and striatum (str) of male control (open bars) and 4-OH-PCB106 exposed (filled bars) neonates at PND 1 were quantified by real-time PCR. Each mRNA expression level is expressed relative to the value of the cerebral cortex of control neonates, which is indicated by a dashed line (mean ± S.E.M., $n = 6$; 1–2 pups/litter, 5–6 litter). *$p < 0.05$ between indicated pairs.

to 4-OH-PCB106 decreased mRNA expression of the other type of thyroid hormone receptor, THRβ, significantly in the cerebral cortex and slightly in the striatum, which suggests a causal relation with decreased expression of thyroid hormone-responsive genes in these two regions. That is, decreased THRβ caused reduction of mRNA expression of thyroid hormone-responsive genes. Direct inhibition of thyroid hormone-dependent signals by 4-OH-PCB106 at the molecular level is unlikely to be sufficient to explain any underlying mechanism of the reduction of THRβ mRNA, since the intranuclear regulatory system of thyroid hormone-dependent transcription is profoundly complicated. Indeed the two previous *in vitro* studies have reported that 4-OH-PCB106 can exert an "antago-

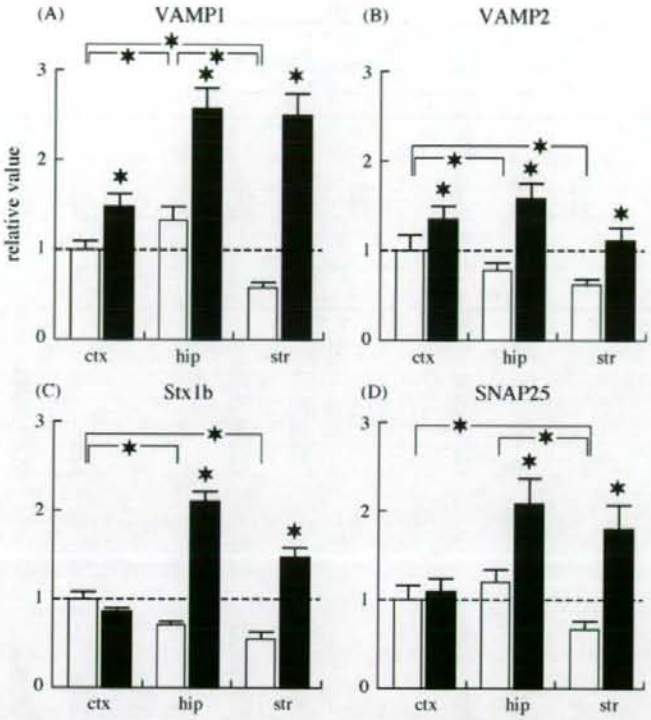

Fig. 4. Effect of prenatal 4-OH-PCB106 exposure on mRNA expression of exocytosis-related genes in the neonatal brain. Expression levels of mRNA for VAMP1 (A), VAMP2 (B), Stx1b2 (C) and SNAP25 (D) in the cerebral cortex (ctx), hippocampus (hip) and striatum (str) of male control (open bars) and 4-OH-PCB106 exposed (filled bars) neonates at PND 1 were quantified by real-time PCR. Each mRNA expression level is expressed relative to the value of the cerebral cortex of control neonates, which is indicated by a dashed line (mean ± S.E.M., $n = 6$; 1–2 pups/litter, 5–6 litter). $^*p < 0.05$ between indicated pairs.

nistic activity" by partially dissociating the THR/retinoid X receptor heterodimer complex from the thyroid hormone-responsive element (Iwasaki et al., 2002) and that this congener acts as a direct "agonist" on the THRβ1 (You et al., 2006). This discrepancy might be due to differences in methods of analyses as well as the cell lines used because it is well known that thyroid hormone signaling pathway is intricately prescribed by co-regulators (Iniguez et al., 1996; Ramos and Weiss, 2006). Although underlying mechanism remains unclear, in either case, 4-OH-PCB106 exposure would disrupt normal gene expression for factors involved in and/or relying on thyroid hormone-dependent signalling in the developing brain. The differences in sensitivity to 4-OH-PCB106 exposure among the three brain regions may also arise from differences in the expression of those thyroid hormone receptors and co-regulators.

Comprehensive analyses by high-density oligoDNA microarrays led us to intensive analyses of genes for glutamate receptors and exocytotic factors at the transcriptional level. We found that prenatal exposure to 4-OH-PCB106 altered mRNA expression of a number of glutamate receptors. Glutamate receptors consist of two distinct families: ionotropic glutamate receptors (iGluRs) including NMDA receptors which are ligand-gated ion channels, and metabotropic glutamate receptors (mGluRs) which are coupled to the guanosine triphosphate (GTP)-binding protein. These glutamate receptors are expressed in the brain very early during ontogeny and play important roles in developmental processes such as proliferation of neural progenitor cells (Mochizuki et al., 2007), proper neuronal cell death (Segura Torres et al., 2006) and neurogenesis (Baskys et al., 2005). A functional NMDA receptor is a heteromeric tetramer of subunits and proper composition of subunits during development is important for its affinity (Turman et al., 1999) and stability (Groc et al., 2006). On the other hand, there are three groups of mGluRs comprising eight different subtypes that are either excitatory or inhibitory with regard to the action of glutamate (Nistri et al., 2006). Therefore, 4-OH-PCB106-induced decrease or increase of these glutamate receptors would result in perturbation of normal brain development and a previous report revealed that perinatal exposure to PCBs exerted disturbance effect on excitatory-inhibitory balance in the rat auditory cortex (Kenet et al., 2007). As found in the analyses of THRs and thyroid hormone-responsive genes, there were differences in 4-OH-PCB106-induced alteration of mRNA expression of glutamate receptors among the three brain regions. The numbers of subunits of NMDA receptors altered (either increase or decrease) by 4-OH-PCB106 exposure were one, three and two among the six examined subunits in the cerebral cortex, hippocampus and striatum, respectively. Similarly one, six and one among the eight mGluRs were altered by 4-OH-PCB106 exposure. It seems that, in the hippocampus, susceptibility to 4-OH-PCB106 exposure is highest among the three regions. These results might have some relation to decreasing dendrite length and spine density of hippocampal pyramidal neuron by PCBs developmental exposure (Lein et al., 2007). In addition, in the striatum, all alterations of glutamate receptor mRNA expression were up-regulation, whereas all of them in the cerebral cortex and hippocampus were down-regulation. These profound region-specificities towards 4-OH-PCB106 exposure make it difficult to refer to unified mechanistic insight into the cellular responses to 4-OH-PCB106 exposure leading to changes in mRNA expression. Different down-stream pathways undertaken by each subunit of NMDA receptor as well as distinct roles of mGluRs make it difficult to expect the exact functional outcome of these expression changes in the developing brain beyond "per-

turbations of glutamatergic neurotransmission". Although little is known about transcriptional regulation of each glutamate receptor by thyroid hormone, our preliminary data suggests that perinatal hypothyroidism induced by methimazole alters mRNA expression of some glutamate receptor subunits in neonatal rats (unpublished data).

In addition, mRNA expression analysis of exocytosis-related genes suggested that 4-OH-PCB106 exposure leads to a state of hyper exocytosis, which are shared in all three regions examined. It is likely that 4-OH-PCB106 exposure perturb not only cellular responses to glutamate but also its release.

Although we focused on glutamatergic neurotransmission system in the present study, it was unlikely that 4-OH-PCB106 exposure selectively and limitedly affected mRNA levels of genes related to glutamatergic neurotransmission, because overall screening by high-density microarray indicated that 4-OH-PCB106 exposure also affected diverse functional categories (as shown in Supplementary data 1). Categorical analyses indicated that 4-OH-PCB106 exposure also perturbed mRNA expression of the genes related to "cell differentiation (44.2%)", "cell death (38.3%)" and "neurogenesis (46.7%)". These genes are also important for brain development. Therefore, additional examinations in other categories might shed lights on diverse aspects of effect of 4-OH-PCB106 exposure on the developing brain.

We examined the effects of prenatal 4-OH-PCB106 exposure only on mRNA expression levels in the present study. It is certainly important to perform more detailed transcriptomic analyses and to examine neuronal functions regulated by glutamate as well as protein expression levels of gluramate receptors, composition of a NMDA receptor, and so on, in each brain region for understanding the mechanism and consequence of prenatal 4-OH-PCB106 exposure. However, this is the first report relating hydroxylated PCB exposure during development with possibilities of abnormal glutamatergic neurotransmission in the developing brain, and these results would be a guide for future studies investigating adverse effect of hydroxylated PCB exposure. Although we used only male infants in the present study, it is possible that female infants are also affected by prenatal hydroxylated PCB exposure, because thyroid hormone is essential for normal brain development also in females. However, it is very important and interesting to perform further experiments referring to sexual differences in the response to hydroxylated PCB exposure using both male and female animals.

The present results suggest that prenatal exposure to some of the hydroxylated PCBs would interfere with normal brain development by perturbing mRNA expression of genes important for glutamatergic neurotransmission.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2008.12.003.

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