

Developmental disorders of the brain can be caused by PCBs; low doses of hydroxy-PCBs disrupt thyroid hormone-dependent dendrite formation from Purkinje neurons in culture.

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Introduction

Exposure to some environmental chemicals during the perinatal period causes developmental disorders of the brain. Cognitive impairment and hyperactivity in infants were reported in Taiwan, known as Yu-cheng (1) incidents caused by the accidental contamination of polychlorinated biphenyls (PCBs). Together with recent experimental data, Kuroda proposes a hypothesis that spatio-temporal disruptions of developing neuronal circuits by PCB exposure can cause the comorbidity of learning disorders (LD), attention deficit hyperactivity disorder (ADHD) and autism with the co-exposure to other environmental chemicals(2).

PCBs and hydroxylated PCBs (OH-PCBs) have similar chemical structures to thyroid hormones (TH), thyroxine (T₄) and triiodothyronine (T₃). TH deficiency in the perinatal period causes cretinism children with severe cognitive and mental retardation. In primate model, Rice demonstrates that postnatal exposure to PCBs can dramatically influence later behavioral function (3). Epidemiological studies also indicate the possible developmental neurotoxicity of PCBs (4) accumulated in human bodies (5). However, the precise underlying mechanisms and which types of PCB or OH-PCB with such effects have yet to be elucidated..

It is important to establish a simple, reproducible, and sensitive *in vitro* assay for determining the effects of PCBs and OH-PCBs on the development of the central nervous system(2). . Recently Iwasaki et al. (6) established a reporter assay system and disclosed that low doses of PCBs potentially interfere TH-dependent gene expressions. This is the first demonstration that PCBs and OH-PCBs directly

affect TH-receptor (TR)-mediated gene expressions crucial to the brain development, through unique mechanism(7).

We also have demonstrated TH-dependent development of Purkinje neurons *in vitro* using a serum-free chemically defined medium. The degree of dendritic development of Purkinje cells is TH dose-dependent and exhibits high sensitivity in the pM order(8). Therefore, in the present study, we examined the effects of OH-PCBs on the development of cultured mouse Purkinje cells in the presence or absence of TH.

Methods and Materials

Cerebellar cultures BALB/C mice were decapitated under diethylether anesthesia on the day one of birth. All experiments conformed to the Guidelines for Animal Experimentation at Tokyo Metropolitan Institute for Neuroscience on the ethical use of animals, and all efforts were made to minimize the number of animals used and their suffering. Details of the culture methods have been described previously (8). One day later, T4, T3, and/or other reagents were added to the wells of the chamber slides. Mixed cerebellar cells were cultivated in the presence of 7% CO₂ for 14-21 days. During cultivation, one-half of the medium was changed with a fresh medium at 3~4-day intervals.

Culture media The basic serum-free medium is a modification of a medium developed by Furuya et al. (9).

PCBs and OH-PCBs: Two types of OH-PCB, 4(OH)-2',3,3',4',5'pentaCB and 4(OH)-2',3,3',5,5',6'-hexaCB were purchased from AccuStandard Chemicals (New Haven, CT, USA). They are present as 4(OH)-pentaCB (106) and 4(OH)-hexaCB (165), respectively according to the PCB numbering scheme.

Immunocytochemistry Purkinje cells were visualized by immunostaining with a rabbit polyclonal (Chemicon) or mouse monoclonal (CL-300, SIGMA) antibody against calbindin-D_{28K}. For the staining of glial cells, other neurons and synapses, rabbit anti-glial fibrillary acidic protein (GFAP, Dako), mouse monoclonal anti-microtubule-associated protein-2 (MAP-2, Boehringer Mannheim Biochemica), and rabbit anti-synapsin I (Chemicon) antibodies were used, respectively. Cell cultures were fixed, double-stained with the anti-calbindin antibody and one of the others (anti-GFAP, anti-MAP-2 or anti-synapsin I antibody). Next, the cultures were incubated with biotinylated anti-mouse IgG (Amersham) followed with FITC-labeled anti-rabbit IgG (Chemicon), and streptoavidin-conjugated Texas Red (Amersham). The double-stained cultures were observed under a Zeiss Axiovert 135 fluorescence microscope.

Analysis of Purkinje cell development; The area of dendritic arborization was quantified using a charge-coupled device (CCD) camera (SenSys) and MetaMorph imaging system (Universal Imaging) from at least 10 cells per sample. The anti-calbindin antibody-immunoreactive area of dendritic branches of Purkinje cells was measured using this system. The relative dendritic area (%) was calculated from these data as 100% without T4 and any reagent.

Results and Discussion

As previously reported (8), T4 markedly promoted the dendritic arborization of Purkinje cells, compared with the control culture without T4 (Figs. 1 and 2). The Purkinje cells in the control medium (Fig. 1A) showed poor or no dendritic growth, while those in the medium with T4 (Figs. 1B) apparently showed large treelike elaborate dendrites characterized by the presence of a main thick primary shaft and several secondary shafts with repeatedly bifurcating branches. In the absence of T4, the addition of either 4(OH)-pentaCB (106) or 4(OH)-hexaCB (165) to the cerebellar culture did not affect the dendritic development of Purkinje cells (Figs. 1A, 1C, 2A and 2B). But in the presence of THs, their addition caused an abnormal development of Purkinje cell dendrites. At 50 pM or higher concentrations, the Purkinje cell dendrites cultured in the medium containing OH-PCBs appeared to have poor growth and shrank (Fig. 1D); furthermore, dendritic area of Purkinje cells significantly decreased (Figs. 2A and 2B). Even at 5 pM, 4(OH)-hexaCB (165) disturbed the dendritic extensions, while 4(OH)-hexaCB (165) showed rather significant affects ($p < 0.001$) at 50 pM, 5 or 50 nM.

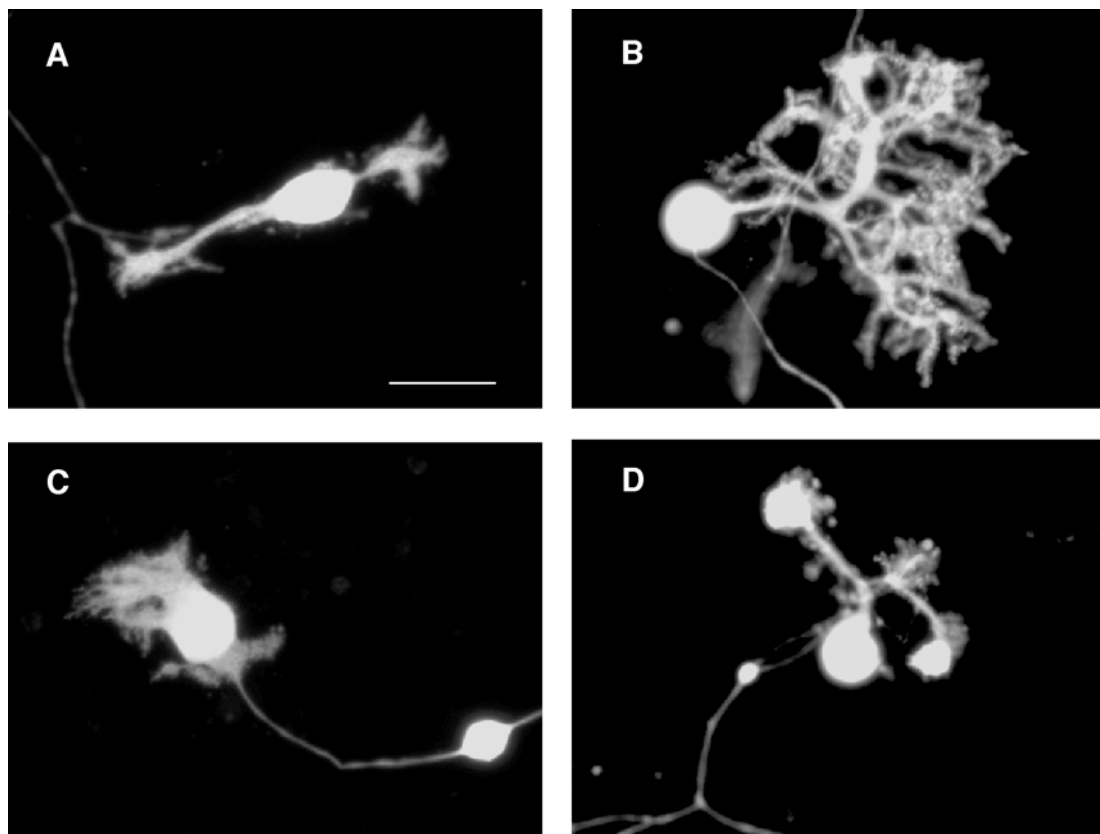


Fig. Effects of OH-PCB on the T4-dependent development of Purkinje cell dendrites. The Purkinje cells were stained with anti-calbindin antibodies. A and C were in the absence of T4. B and D were in the presence of T4 (5 nM). 4(OH)-pentaCB (106) (50 pM) was added to cultures shown in C and D.

The number of surviving Purkinje cells was about 50-150 cells/well at each concentration of reagents, and did not show a significant difference, but slightly decreased at a concentration of 50 and 500 nM compared with the control (data not shown) in the presence or absence of T4.

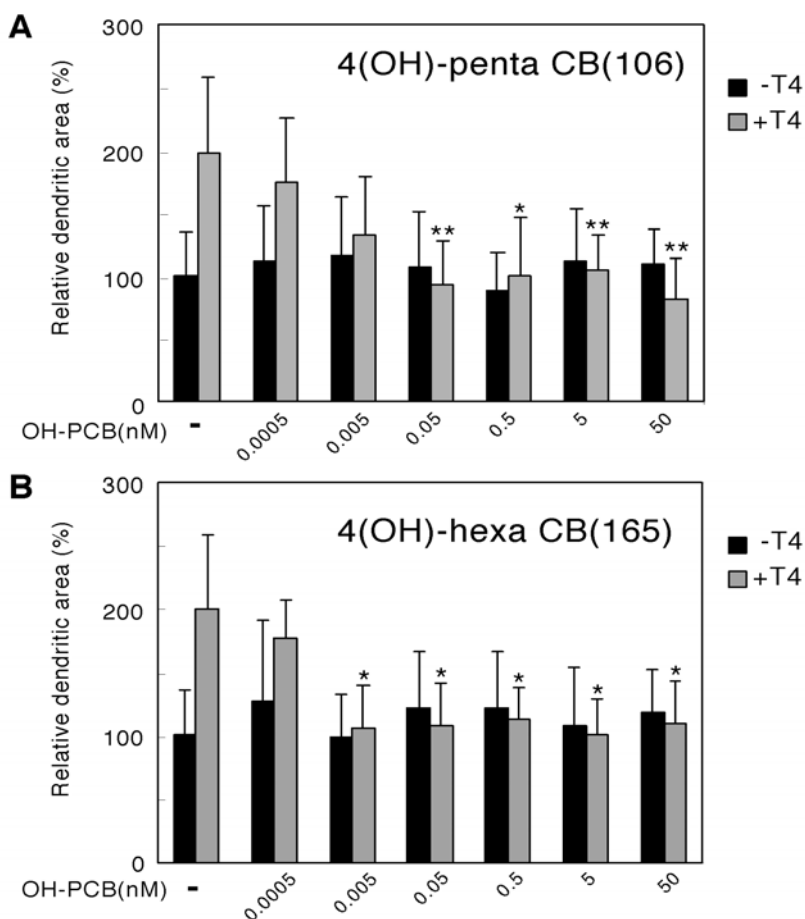


Fig. 3 OH-PCB inhibited T4-dependent extension of Purkinje cell dendrites. OH-PCB was added to the culture at concentrations 5 pM–50 nM. $n=10$ determinations using two wells for each experiment. *: $P<0.01$, **: $P<0.001$. P values were vs. 0 nM OH-PCB as the control. Bars indicate standard deviations.

In this study, we indicated that two types of OH-PCB, 4(OH)-pentaCB (106) and 4(OH)-hexaCB (165), suppress the TH-dependent development of Purkinje cell dendrites. The effective doses of OH-PCBs were as low as 50 pM. Recently using the same OH-PCB as 4(OH)-pentaCB (106) or 4(OH)-hexaCB (165), Iwasaki et al. (6) reported that low doses of PCBs can potentially interfere with TH receptor (TR)–mediated transactivation. The effective dose was 10^{-10} M, which is close to our results. Further they have just reported novel mechanisms of the PCBs' suppression on TR-mediated transcription (7). In the cerebellum, it was reported that TRs are expressed in most neurons, including Purkinje cells and granule cells

(10). In our results, the suppressed effects of OH-PCBs probably have depended on TR-mediated gene expressions. Furthermore, it was also reported that THs activate several astrocyte functions, which are important in the development of Purkinje cells, and not through TR-mediated gene expressions (11), because the actual existence of TRs has not yet been confirmed on the astrocytes. The OH-PCBs may affect these TH functions in the astrocytes via an unknown pathway, resulting in a rather higher sensitivity of dendritic development to OH-PCB obtained in our study than that by Iwasaki et al. (6).

THs play critical roles in the differentiation, growth, metabolism, and physiological function of virtually all tissues, particularly in the central nervous system via the regulation of fundamental gene expressions. Some target proteins of THs have been identified and many proteins may be regulated directly or indirectly by THs (12). Several reports indicated that the exposure to PCBs interferes with some proteins, PKC, RC3/neurogranin and myelin basic protein in the brain. These effects of PCBs may disturb the normal brain development via TH-dependent regulations.

In this study, we used 4(OH)-pentaCB (106) and 4(OH)-hexaCB (165), which are of the ortho-substituted, noncoplanar type and their inhibition of TR-dependent gene expressions was confirmed by Iwasaki et al (6). Many reports indicated that ortho-substituted, noncoplanar PCBs have neurotoxic effects in both *in vitro* and *in vivo* animal experiments. Kodavanti et al. indicated some differences in distributions of several types of PCBs in different brain regions (13). However further investigations are required to determine which type of PCB and OH-PCBs are neurotoxic.

PCBs and OH-PCBs are widespread and accumulate in animal and human bodies. Recently, Fangstrom et al. have indicated that 96-560 ng/g fat weight of OH-PCBs and 750-5900 ng/g of PCBs were detected in sera of pregnant Faroese women (14). In the umbilical cord plasma of Quebec neonates, 234-553 pg/g wet weight plasma of OH-PCBs were identified by Sandau et al. (5). Takasuga et al. have reported average 270 ng/g fat weight of total PCBs in sera of normal healthy Japanese people (15).

These concentrations of PCBs or OH-PCBs correspond to about several hundred pM- several nM orders, which could suppress dendritic development of Purkinje cells *in vitro*. The placental transfer of OH-PCBs and their effects on fetal TH homeostasis were observed in an experimental rat model (16). Our preliminary data showed some deficits in the performance of offspring monkeys in the **finger maze** learning test, born to mothers exposed to similar levels of PCBs and OH-PCBs (Negishi et al. ; this symposium), but **not** born to mothers exposed to even a

high dose of TCDD(300ng/kg body weight at first injection)(2). Considering recent increases of LD, ADHD and autism prevalence in US and Japan, to avoid the negative effects of PCBs and OH-PCBs on developing brain of our next generations, it is important to assess precise risks with more data, for which further experiments are required, especially extensive behavioral ones using primates.

References

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