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In utero and lactational dioxin exposure induces *Sema3b* and *Sema3g* gene expression in the developing mouse brain

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ABSTRACT

In the developing mammalian brain, neural network formation is regulated by complex signaling cascades. *In utero* and lactational dioxin exposure is known to induce higher brain function abnormalities and dendritic growth disruption in rodents. However, it is unclear whether perinatal dioxin exposure affects the expression of genes involved in neural network formation. Therefore, we investigated changes in gene expression in the brain regions of developing mice born to dams administered 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dose: 0, 0.6, or 3.0 μg/kg) on gestational day 12.5. Quantitative RT-PCR showed that TCDD exposure induced *Ahrr* expression in the cerebral cortex, hippocampus, and olfactory bulb of 3-day-old mice. Gene microarray analysis indicated that the mRNA expression levels of *Sema3b* and *Sema3g*, which encode proteins that are known to control axonal projections, were elevated in the olfactory bulb of TCDD-exposed mice, and the induction of these genes was observed during a 2-week postnatal period. Increased *Sema3g* expression was also observed in the brain but not in the kidney, liver, lung, and spleen of TCDD-exposed neonatal mice. These results indicate that the *Sema3b* and *Sema3g* genes are sensitive to brain-specific induction by dioxin exposure, which may disrupt neural network formation in the mammalian nervous system, thereby leading to abnormal higher brain function in adulthood.

1. Introduction

The development of the mammalian nervous system involves numerous pivotal processes, including proliferation, neurite outgrowth, and synaptogenesis, which leads to the establishment of the neural network that supports higher brain function [1]. In particular, the neural connectivity between brain regions, i.e., hippocampus, cerebral cortex, amygdala, and olfactory bulb, is associated with learning, memory, and fear behavior [2–4]. Neural network formation is regulated by signaling molecules, such as semaphorins and neurotrophins, in the developing mammalian brain [5]. Semaphorins are classified into eight classes, among which classes 1–2, 3–7, and V are expressed in invertebrates, vertebrates, and viruses, respectively [6]. Among them, the Class 3 semaphorin in vertebrates is a secreted type, and comprises Sema3A to Sema3G [7,8], and plays a role in orientating the projection of axons and dendrites. For example, attraction and repulsion by Sema3B are critical for the positioning of a commissural projection in the mammalian nervous system [9–11]. Sema3G is expressed in the vertebrate brain during development, and it exhibits a chemorepellent activity in sympathetic axons [12,13].

Dioxin is a ubiquitous environmental contaminant, and is known to exert a variety of toxicities such as reproductive toxicity, immunotoxicity, carcinogenicity and teratogenicity [14]. Adult rodent offspring born to dams exposed to low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic congener in the group of dioxin congeners [15], exhibit cognitive and behavioral abnormalities, such as impaired spatial, reversal, and paired associate learning and memory, as well as anxiety and altered social behavior [16–22]. Additionally, *in utero* and lactational TCDD exposure induces abnormal dendritic morphology in the mouse

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hippocampus and amygdala [23]. These studies strongly suggest that perinatal TCDD exposure disrupts formation of the neural network required for higher brain function in adulthood. TCDD can bind to the aryl hydrocarbon receptor (AhR), a cytosolic transcription factor present in the cells of various organs, including the brain [24,25]. The TCDD-AhR complex translocates to the nucleus and enhances the transcription of AhR-target genes, such as *AhR repressor* (*Ahrr*), *Cyp1a1*, and *Cyp1b1* [26]. Excessive expression of these genes may disrupt signaling cascades via the relevant proteins, but the molecular mechanisms responsible for the neurodevelopmental toxicity of TCDD remain elusive. Interestingly, TCDD exposure disrupts the expression pattern of *sema3a* mRNA in the developing fish brain [27]; therefore, the Class 3 semaphorins could be upregulated in a liganded AhR-dependent manner in mammals.

To elucidate the molecular mechanisms responsible for TCDD toxicity in the central nervous system, we used a quantitative RT-PCR method to investigate the expression levels of AhR-target genes in various brain regions, such as cerebral cortex, hippocampus, and olfactory bulb in mice that were perinatally exposed to TCDD. Little information is available regarding changes in the expression levels of various genes involved in neural network formation; therefore, we determined the mRNA expression levels in TCDD-exposed mice by gene microarray analysis and compared the gene expression levels in several organs to detect the possibility of organ-specific TCDD toxicity.

2. Materials and methods

2.1. Reagents and chemicals

TCDD (purity >99.5%) was purchased from Cambridge Isotope Laboratory (Andover, MA, USA). Corn oil and *n*-nonane were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. The manufacturers of the other reagents and instruments used in this study are described in the following sections.

2.2. Animals and TCDD treatment

The animal experiment protocols used in this study were approved by the Animal Care and Use Committee of the University of Tokyo. Pregnant female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and housed in an animal facility at a temperature of 22°C–24 °C and humidity of 40%–60%, with a 12/12-h light-dark cycle (lights on from 08:00 to 20:00). Laboratory rodent chow (Lab MR Stock; Nosan, Yokohama, Japan) and distilled water were provided *ad libitum*. Pregnant female C57BL/6J mice were orally administered vehicle (corn oil containing 0.6% *n*-nonane) or TCDD in vehicle at a dose of 0, 0.6, or 3.0 μg/kg on gestational day 12.5 (i.e., the control, TC-0.6, and TC-3.0 groups, respectively). One male from 6 to 8 pups per dam was selected randomly to analyze the gene expression levels to minimize possible litter effects ($n = 4$–8 mice/group).

2.3. Quantitative RT-PCR

Male offspring were sacrificed by decapitation, and several fresh organs, i.e., the brain, kidney, liver, lung, and spleen on postnatal day (PND) 1; fresh brain regions, including the cerebellum, hypothalamus, and striatum on PND 7; cerebral cortex and hippocampus on PND 3 or 7; and olfactory bulb on PND 3, 7, or 14 were collected quickly and stored at −80 °C until analysis by quantitative RT-PCR or gene microarray [28]. The total RNA was isolated from each tissue using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The cDNA for a given mRNA was synthesized using oligo-dT and random hexamers with a Primescript RT reagent kit (Takara, Kusatsu, Japan). Gene expression levels were determined quantitatively using a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN, USA) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The primers for the genes are shown in Table 1. No-template controls were analyzed in every PCR to monitor cross-contamination. To verify the specificity of amplification, melting curve analyses of the products were performed for every PCR. The mRNA expression levels were calculated using the $\Delta\Delta C_t$ method and normalized against *Peptidylprolyl isomerase A* (*Ppia*) mRNA expression.

2.4. Gene microarray analysis

In gene microarray analysis, we used a 3D-Gene Mouse Oligo chip 24 k (Toray Industries Inc., Tokyo, Japan) to compare the gene expression levels in the olfactory bulb of 3-day-old mice from the control and TCDD-exposed groups. Total RNA pooled from seven, eight, and eight mice was obtained for the control, TC-0.6, and TC-3.0 groups, respectively. The Cy5-labeled RNA in the control group and Cy-3-labeled RNA in the TC-0.6 or TC-3.0 groups were hybridized. Hybridization signals were scanned using a ScanArray Express Scanner (PerkinElmer Life Sciences, Waltham, MA, USA) and processed by GenePixPro software (Molecular Devices, Sunnyvale, CA, USA). The signals detected for each gene in the TC-0.6 or TC-3.0 groups were normalized against those in the control group (Cy3/Cy5 ratios). The hybridization and signal scanning procedures were performed according to the supplier's protocols [29].

2.5. Statistical analysis

Gene expression was analyzed using the Student's *t*-test or one-way analysis of variance (ANOVA), followed by Dunnett's *post-hoc* test. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Ahrr, Cyp1a1, and Cyp1b1 mRNA levels in the developing brains of TCDD-exposed 3-day-old mice

To investigate the effects of TCDD exposure on the expression levels of the AhR target genes, we examined the expression of *Ahrr*, *Cyp1a1*, and *Cyp1b1* mRNA in the cerebral cortex, hippocampus, and olfactory bulb obtained from TCDD-exposed mice on PND 3. In all three regions, the *Ahrr* mRNA expression level in the TC-3.0 group increased significantly compared with that in the control group (Fig. 1A). There was a significant increase in the *Ahrr* expression level in the olfactory bulb, but not in the cerebral cortex or hippocampus, in the TC-0.6 group compared with that in the control group (Fig. 1A). There were no significant changes in the *Cyp1a1* mRNA expression levels in the olfactory bulb, cerebral cortex, and hippocampus between the control and TCDD-exposed groups (Fig. 1B). In the olfactory bulb, the *Cyp1b1* expression levels increased significantly in both the TC-0.6 and TC-3.0 groups

Table 1
Primer sequences used for quantitative RT-PCR.

Gene symbol	Forward primer	Reverse primer
Ahrr	5′-cagggcagacattgtggtta-3′	5′-ctccattgctctttcctgct-3′
Cyp1a1	5′-caccgtattctgccttggat-3 ′	5′-cagcatgtgaccaatgaagg-3′
Cyp1b1	5′-ggacaaggacggcttcatta-3′	5′-gcgaggatggagatgaagag-3′
Ppia	5′-tcatcctaaagcatacaggtcct-3′	5′-tttcaccttcccaaagaccaca-3′
Sema3b	5′-gagtgagaaccctgatgacg-3′	5 ′-catttgttgaccaagctccg-3′
Sema3g	5′-ctcaagagccaaggtgacat-3′	5′-tgaccaccctcagtagagac-3′

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