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BRIEF REPORT

Hydroxylated polychlorinated biphenyls may affect the thyroid hormone-induced brain development during metamorphosis of *Xenopus laevis* by disturbing the expression of matrix metalloproteinases

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Abstract

Background Thyroid hormones are primarily responsible for the brain development in perinatal mammals. However, this process can be inhibited by external factors such as environmental chemicals. Perinatal mammals are viviparous, which makes direct fetal examination difficult.

Methods We used metamorphic amphibians, which exhibit many similarities to perinatal mammals, as an experimental system. Therefore, using metamorphic amphibians, we characterized the gene expression of matrix metalloproteinases, which play an important role in brain development.

Results The expression of many matrix metalloproteinases (*mmps*) was characteristically induced during metamorphosis. We also found that the expression of many *mmps* was induced by T_3 and markedly inhibited by hydroxylated polychlorinated biphenyls (PCBs).

Conclusion Overall, our findings suggest that hydroxylated PCBs disrupt normal brain development by disturbing the gene expression of *mmps*.

Keywords Hydroxylated polychlorinated biphenyls · Thyroid hormone · Matrix metalloproteinase · Brain development

Abbreviations

ANOVA	Analysis of variance
DMSO	Dimethyl sulfoxide
EDCs	Endocrine-disrupting chemicals
FETAX	Frog embryo teratogenesis assay *Xenopus*
MMPs	Matrix metalloproteinases
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
T_3	3,3′,5-Triiodo-L-thyronine
THs	Thyroid hormones

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Introduction

Maternal exposure to environmental chemicals during the perinatal period disrupts the thyroid system and inhibits normal brain development [1, 2]. Exposure to endocrine-disrupting chemicals (EDCs) in the mother leads to developmental disorder-like symptoms, including abnormal behaviors, such as attention-deficit/hyperactivity disorder, in children [3, 4]. Although mammalian experimental models have the advantage of establishing several experimental systems to verify social behavior, molecular analyses of mammalian species are difficult because the uterus surrounds the fetus. Moreover, the direct effects of EDCs on the fetus are difficult to examine because chemical substances are altered by the maternal drug-metabolizing systems [5]. Therefore, the development of an effective experimental system is important to easily model the development of the perinatal mammalian brain.

Amphibian metamorphosis, induced by thyroid hormones (THs), causes drastic changes in larvae [6]. During metamorphosis, TH levels transiently increase, and signals are

transduced via the regulation of gene expression by hormone nuclear receptors. Metamorphosis has been suggested to correspond with the perinatal period in mammals [7], making amphibians a suitable experimental model. Treatment of *Xenopus laevis* larvae with THs during pre-metamorphosis induces brain development, but it has been reported that brain development is inhibited by disrupting the expression of multiple TH-responsive genes by bisphenols [8, 9]. We have previously found that hydroxylated polychlorinated biphenyls (PCBs) inhibit metamorphosis and disrupt the expression of TH-responsive genes, including matrix metalloproteinases (MMPs) [10], which play important roles in extracellular matrix remodeling and tissue homeostasis. MMPs are involved in various physiological and pathological processes, such as wound healing, angiogenesis, tissue repair, and cancer progression [11, 12]. MMPs play important roles in amphibian metamorphosis [13]. These studies highlight the importance of examining the effects of hydroxylated PCBs on TH-induced MMP expression in metamorphic amphibian brains. Thus, the present study aimed to examine the effect of hydroxylated PCBs on changes in *mmp* gene expression induced by TH during metamorphosis. We found that the expression of many *mmps* was induced by 3,3′,5-triiodo-L-thyronine (T_3) and that the induction was markedly inhibited by hydroxylated PCBs.

Materials and methods

Reagents and animals

For this study, T_3 (approximately 98% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The hydroxylated PCBs (4-OH-PCB106 and 4-OH-PCB159) were obtained from AccuStandard (New Haven, CT, USA). All other chemicals used in this study were of the highest grade available and purchased from Wako (Osaka, Japan). T_3 and 4-OH-PCBs were dissolved in dimethyl sulfoxide (DMSO) and diluted with frog embryo teratogenesis assay *Xenopus* (FETAX) buffer to create a < 1.0% (v/v) solvent.

Tadpoles of *X. laevis* obtained by injecting adult frogs with human chorionic gonadotropin (ASKA Pharmaceuticals, Tokyo, Japan) were reared in dechlorinated tap water under natural lighting and fed Sera Micron (Heinsberg, Germany) every other day. The animals were classified according to the developmental stages outlined by Nieuwkoop and Faber [14]. Short-term exposure experiments were performed as previously described to examine the effects of 4-OH-PCBs on *mmp* gene expression levels [10]. Three premetamorphic tadpoles (stages NF53–NF54) were randomly transferred into 1-L glass beakers for each treatment group containing 500 mL of FETAX buffer [16]. The tadpoles were exposed to the solvent alone or 500 nM 4-OH-PCBs in the absence or presence of 1 nM T_3 for four days, anesthetized in 0.02% 3-aminobenzoic acid ethyl ester. The isolated brains were immediately frozen in liquid nitrogen and stored at − 85 °C until RNA extraction. Each experiment was repeated at least thrice using tadpoles from different sets of adults. The figure presents the results of one representative experiment out of the three.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from frozen tadpole brains using an SV Total RNA Isolation System (Promega, Madison, WI, USA). After treating the RNA samples with reverse transcriptase (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA, USA), the specific RNA transcript levels were estimated via RT-qPCR using the Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) and the Thermal Cycler Dice Real Time System Single TP850 (TaKaRa Bio, Shiga, Japan) with a specific primer set (200 nM each), summarized in Table 1, using the following protocol: 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min as described [16]. The relative transcript levels were quantified using the comparative Cq method [17]. Each PCR was performed in triplicate to control for variations. To standardize each experiment, the amount of gene transcript was divided by the amount of glyceraldehyde 3-phosphate dehydrogenase RNA in each sample.

Statistical analyses

All data are presented as mean ± standard error of the mean. Differences among groups were analyzed using one-way analysis of variance with Fisher's least significant difference test for multiple comparisons using Microsoft Excel 2003 Data Analysis Software (SSRI, Tokyo, Japan). Statistical significance was set at $p < 0.05$.

Results and discussion

Several *mmp* genes have been identified in *X. laevis*, which are divided into five subfamilies based on their domain structure: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs [18]. These *mmp* genes play important roles in various biological processes, including embryonic development, tissue remodeling, and immune responses in *X. laevis*.

Table 1 Primers for RT-qPCR

Gene	Synonym	Accession	Sense	Antisense
RT-qPCR				
mmp8.l	*mmp1*	NM_001087049	5'-GGAAACAAGGTGCAAGCAGTCT-3' (1338fw)	5'-TGCCGATTGCCATTGAAGA-3' (1402rv)
mmp7.l	*mmp7a*	NM_001086213	5'-CAGCACCTGCAAGGGTCAT-3' (1203fw)	5'-GGCCAGCGGCTTCTACACTA-3' (1262rv)
mmp9.1.s	*mmp9*	NM_001086503	5'-CATGGGTAGGGTGGGAGTTTT-3' (44fw)	5'-GAACGGAGTGACCCTTGGAA-3' (105rv)
mmp9.2.l	*mmp9th*	NM_001097836	5'-ATGCCCAGCCCATTAAGCTA-3' (2426fw)	5'-CTGGACCATGACATCAGAAGTGA-3' (2490rv)
mmp11.l	*mmp11*	NM_001086342	5'-GGAGGACGCTGGGACAAGA-3' (480fw)	5'-TTAGCTGCCATGGGAAACG-3' (540rv)
mmp13l.s	*mmp13*	NM_001100931	5'-GGATAGGGCCATCAAAAAAGC-3' (414fw)	5'-CGGAGCCTGGTGAAGTTCA-3' (482rv)
mmp14.l	*mmp14*	NM_001091009	5'-GAGTCTGGCTACCCGAAATCC-3' (1723fw)	5'-TTCCGGCTGTCGGACAGT-3' (1788rv)
mmp13.s	*mmp26*	NM_001086405	5'-GGTTGGACTCAGATACCATGACAA-3' (233fw)	5'-CGCAACATCGGGCATTC-3' (294rv)

Primer sets with similar sequences of transcripts transcribed from the S-subgenome- and L-subgenomes were used to detect both transcripts

Stage-dependent expression of *mmps* in metamorphosing *X. laevis* brain

Here, the transcript levels of all *mmp* genes (*mmp8.l*, *mmp7.l*, and *mmp9.1.* s, *mmp9.2.l*, *mmp11.l*, *mmp13l.s*, *mmp14.l*, and *mmp13.s*) were significantly higher in the brains of NF62 tadpoles than in those of NF60 tadpoles, suggesting the important role of *mmps* in the brains of tadpoles during metamorphosis (Fig. 1). In addition, the transcript levels of almost all *mmps* were higher at Stage 58 than at Stage 56 (Fig. 1). The plasma concentration of T_3 was too low to be detected at NF56, increased at NF57–58, and peaked at NF60–62 [19]. Moreover, TH receptor beta (TRβ) transcript levels are higher at NF58 than at NF56, reaching a peak at NF60–62 [20]. These results indicate that the TRβ transcript levels in the brain change according to the T_3 concentration, suggesting its potential role as a transcription factor. Several *mmps* are early TH response genes with a functional TH response element (TRE) in their regulatory regions [21, 22]. In this study, all tested *mmps* had (putative) TRE(s) in their flanking upstream region (approximately 1 kbp; data not shown), suggesting their direct regulation by TRβ.

MMPs are important in normal brain development. For example, myelination of the corpus callosum in *MMP-9* and/or *MMP-12* null mice is defective at postnatal days 7–14 compared to that in wild-type mice, suggesting that these MMPs participate in myelinogenesis [23]. MMP-2 is expressed in the developing cerebellum and regulates granule cell proliferation by affecting cell cycle dynamics in the cerebellum of postnatal day 3 mouse pups [24]. Metamorphosing amphibian tadpoles and perinatal mammals share several common features such as a transient increase in TH concentration during brain development. In addition, *mmp* expression was regulated by TH. These findings revealed similar changes in *mmp* expression in both species because of the important roles of MMPs in brain development and amphibian metamorphosis, suggesting that metamorphic amphibians are suitable model experimental systems for perinatal mammals.

Effects of 4-OH-PCBs on TH-induced mmp expression in pre-metamorphic *X. laevis* brain

The transcript levels of all *mmps* were significantly increased after T_3 treatment (Fig. 2). Furthermore, co-treatment with 4-OH-PCBs and T_3 inhibited the increase in the expression of almost all *mmp*s. Notably, 4-OH-PCB159 did not inhibit T_3-induced increase in *mmp14.l* expression. These results suggested that T_3 induces *mmp* expression, whereas 4-OH-PCBs inhibit T_3-induced *mmp* expression in metamorphic amphibian brains.

In mammals, the components and structure of the extracellular matrix change dynamically during brain development [25]. MMP plays an important role in determining brain plasticity by affecting the extracellular matrix during development [26]. In our previous study, Gene Ontology enrichment analysis of genes whose expression fluctuated in a TH-dependent manner in the brains of metamorphic amphibians and genes whose expression fluctuated by 4-OH-PCBs revealed the enrichment of terms such as brain development, cell differentiation and migration [10]. Metamorphosis has been suggested to occur during the perinatal period of mammalian brain development. Therefore, our

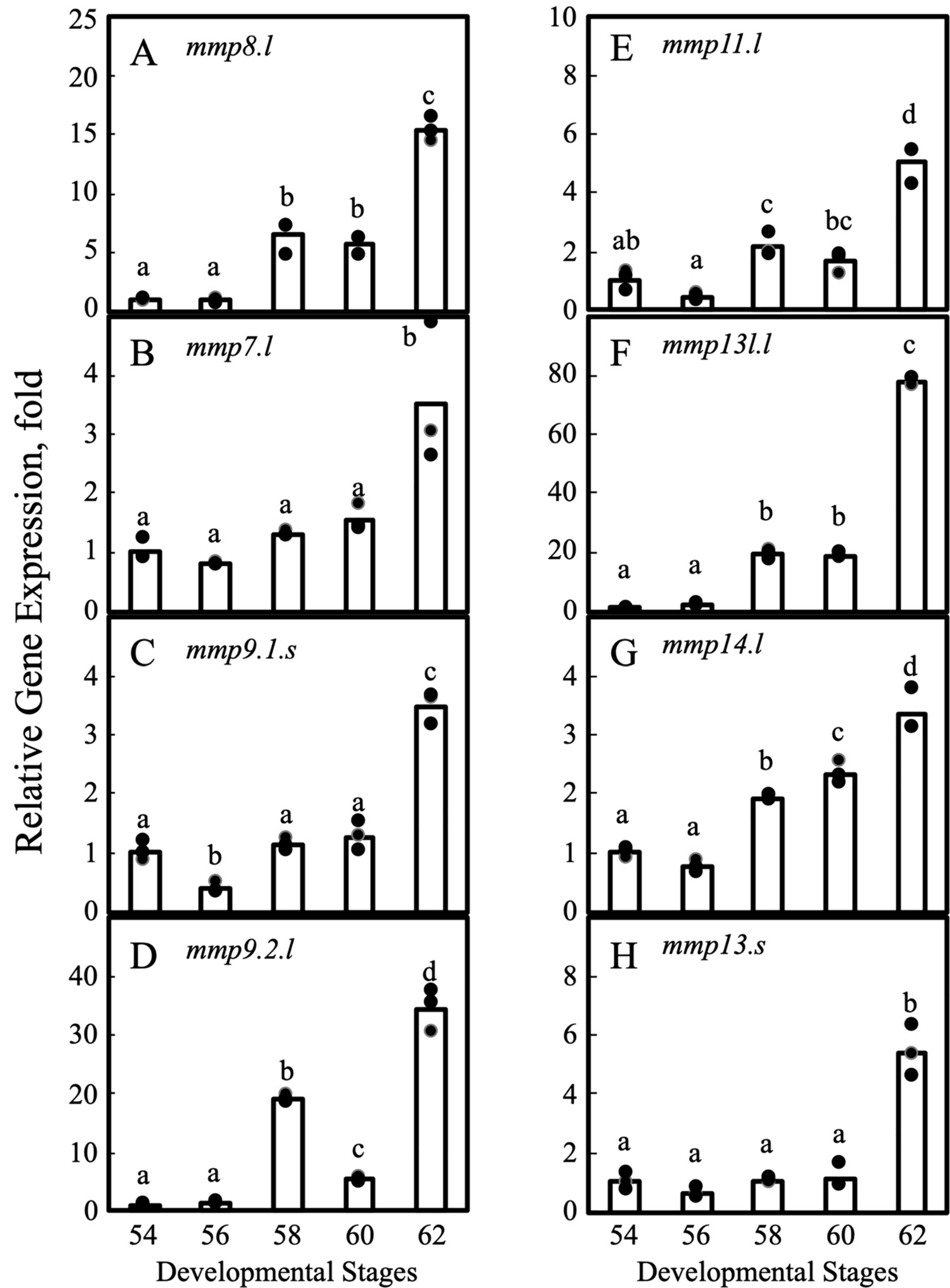

Fig. 1 Transcript levels of matrix metalloproteinase (*mmp*) genes in the brains of metamorphosing *Xenopus laevis* tadpoles. Total RNA was extracted from the tadpole brains at NF54, 56, 58, 60, and 62. Expression levels of several genes (*mmp8.l*: A; *mmp7.l*: B; *mmp9.1.s*: C; *mmp9.2.l*: D; *mmp11.l*: E; *mmp13l.s*: F; *mmp14.l*: G; *mmp13.l*: H) were analyzed using quantitative real-time PCR. The vertical axis represents the ratio of the *mmp* transcript levels in each sample to those in the NF54 sample as a magnitude of induction (fold) after normalization with the housekeeping gene levels, glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). All values are represented as the mean ± standard error of the mean of triplicate experiments (three tadpoles per group). NF is used to indicate the developmental stages outlined by Nieuwkoop and Faber. Different letters indicate significantly different means ($p < 0.05$; one-way ANOVA using Fisher's least significant difference test for multiple comparisons). The figure shows the results of one representative experiment out of three

finding that 4-OH-PCBs inhibited T_3-induced *mmp* expression during metamorphosis suggests that environmental chemicals inhibit normal brain development by disturbing extracellular matrix reconstruction.

Conclusion

These results suggest that normal brain development may be inhibited by environmental chemicals that disrupt T_3-dependent changes in *mmp* expression. However, in the

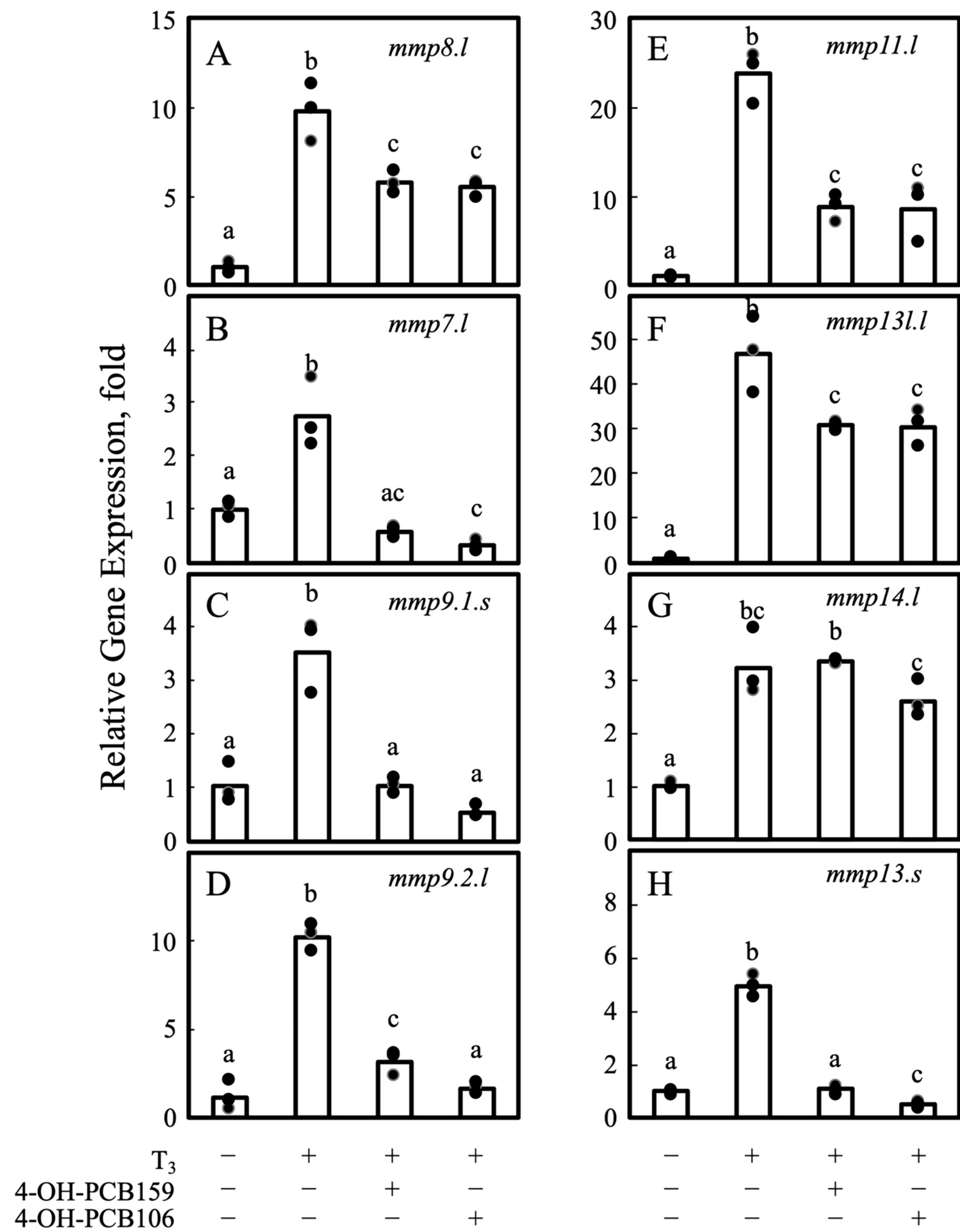

Fig. 2 Effects of hydroxylated PCBs on the T_3-induced increase in the transcript levels for *mmp* genes in the metamorphosing tadpole brain. Total RNA was extracted from the tadpoles after short-term (four days) exposure to hydroxylated PCBs. After treatment with the vehicle control, T_3 (1 nM), and 4-OH-PCB106 or 4-OH-PCB159 (500 nM), the expression levels of several genes (*mmp8.l*: A; *mmp7.l*: B; *mmp9.1.s*: C; *mmp9.2.l*: D; *mmp11.l*: E; *mmp13l.s*: F; *mmp14.l*: G; *mmp13.l*: H) were analyzed using quantitative real-time PCR. The vertical axis represents the ratio of the target gene transcript levels of T_3-treated samples to those of the T_3-untreated samples as a magnitude of induction (fold) after normalization with the housekeeping gene levels, *gapdh*. All values are represented as the mean ± standard error of the mean of triplicate experiments (three tadpoles per group). Different letters indicate significantly different means ($p < 0.05$; one-way ANOVA using Fisher's least significant difference test for multiple comparisons). The figure shows the results of one representative experiment out of three

present study, we did not examine the effects of hydroxylated PCBs on morphological and histological changes in the brain. Furthermore, the TH agonistic effects (s) of hydroxylated PCB have not been verified. By including these experiments in the future, it will be possible to examine the impact of hydroxylated PCBs on brain development in detail.

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Author contributions Material preparation, data collection and analysis, and the first draft of the manuscript was prepared by Akinori Ishihara.

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Data availability All data generated or analyzed during this study are included in this manuscript.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Research involving human/animal participants All breeding and experimental procedures were approved by the Shizuoka University Animal Experiment Committee (permits #2019F-10 and #2020F-11) under the International Guidelines on Welfare and Management of Animals (Ministry of the Environment).

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