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The thyroid-disrupting effects of long-term perfluorononanoate exposure on zebrafish (*Danio rerio*)

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Abstract Concentrations of perfluorononanoate (PFNA) suggest an obvious increase in the environment, wildlife, and humans. However, the potential toxicity of PFNA still remains to be fully elucidated. Our present work is directed toward evaluating specific thyroid endpoints, and studying the long-term and the trans-generational effects of PFNA on zebrafish. Zebrafish (*Danio rerio*) were exposed to different concentrations of PFNA (0, 0.05, 0.1, 0.5, and 1 mg/l) from their early life stages (F_0, 23 day post-fertilization dpf), and the exposure period lasted for 180 days. At the end of the exposure period, thyroid follicle histology and plasma thyroid hormone levels in male zebrafish were evaluated as direct endpoints for the specific thyroid toxicities, while gene expression relative to the hypothalamus–pituitary–thyroid axis was also investigated to study the underlying mechanisms. In addition, offspring embryos (F_1) from the PFNA exposure parental zebrafish was reared in water either without PFNA or under continual exposure to PFNA for an additional 180 days to investigate effects of multi-generational exposures on the circulating T_3 levels and thyroid-associated gene expression. Our results demonstrate significantly elevated plasma T_3 levels were observed in both F_0 and F_1 adults, as well as PFNA-induced histological changes in the thyroid follicles of F_0 male zebrafish. In the liver, the abundance of gene transcript encoding the protein transthyretin (TTR) was significantly induced, while the expression of UDP-glucuronosyltransferases in F_0 adult males was inhibited. The induced thyroid-disrupting effects also demonstrated a trans-generational effect that was reflected by altered gene expression related to thyroid hormone (TH) synthesis and metabolism in F_1 larvae. Our results provide the first evidence for the thyroid-disrupting effects of long-term PFNA exposure in zebrafish.

Keywords Perfluorononanoate · Zebrafish · Thyroid-disrupting effects · Long-term · Trans-generational exposure

Introduction

Perfluoroalkyl acids (PFAAs) are a class of chemicals that consist of a C–F backbone and a terminal charged moiety. Their wide application in industry and common consumer products over the past several decades has resulted in persistent and widespread pollution, and ascribed these chemicals to a new kind of persistent organic pollutants (POPs) (Giesy and Kannan 2001; Prevedouros et al. 2006). Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) have been the two primary PFAA components. However, as the manufacturing emission of PFOA and PFOS dropped, perfluorononanoate (PFNA), a perfluorinated alkyl acid containing nine carbon atoms, demonstrated an obvious increase in concentrations in the environment and in the tissues of humans and wildlife (Andersen et al. 2008; Calafat et al. 2007). Meanwhile, concerns about the possible health impacts of PFNA exposure have arisen.

Apart from the general toxic effects of PFAAs, several studies on PFOA/PFOS have also revealed their

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endocrine-disrupting properties. In mammals, for example, PFAA exposure can affect testosterone and estradiol levels in rats (Biegel et al. 1995; Jensen and Leffers 2008). Furthermore, studies conducted on rats, mice, and monkeys have revealed that in vivo PFOA/PFOS exposure consistently leads to reduced plasma levels of thyroid hormones (TH) (Lau et al. 2003; Luebker et al. 2002; Seacat et al. 2002). In teleost fish, PFOS/PFOA exposure can alter circulating-sex-steroid-hormone levels, induce hepatic vitellogenin (VTG) expression in mature males, and even lead to bisexual gonads (Oakes et al. 2005; Wei et al. 2007; Du et al. 2009). However, the effects of PFAAs on the thyroid system in teleosts have not yet been reported (Shi et al. 2009).

TH plays important roles in fish development and metabolism, and disruption of the thyroid system may lead to decreased fitness by altering behaviors or physiological pathways key to survival and the reproduction (Power et al. 2001; Tyler et al. 1998). Currently, there have been increasing reports of the disruption of thyroid systems by environmental contaminants (Miller et al. 2009; Zoeller 2005). Because the class of PFAA chemicals is persistent in the environment, it would be beneficial to employ a long-term or even trans-generational exposure to explore the potential thyroid-disrupting effects. Thus, the goal of our present study was to investigate the thyroid-disrupting effects in zebrafish after long-term PFNA exposure. Thyroid histological changes and plasma TH levels were assessed. To reveal the potential modes of action, gene transcriptional expression of multiple physiological steps within the hypothalamic–pituitary–thyroid (HPT) axis were investigated, including genes involved in TH biosynthesis, transport, metabolism, and utilization. Moreover, the trans-generational effect was assessed, and impacts of PFNA exposure on plasma T_3 levels of two consecutive generations were also analyzed to provide further insight into the thyroid disrupting effects of PFNA.

Materials and methods

Fish husbandry and experimental design

Zebrafish (*Danio rerio*, Tubingen strain) were obtained from the Peking University Zebrafish Resource Center and maintained in our laboratory according to guidelines from THE ZEBRAFISH BOOK (http://zfin.org/zf_info/zfbook/zfbk.html). Simulated natural conditions were employed, consisting of a 16-h light:8-h dark photoperiod, water temperatures ranging from 24 to 26°C, and pH values of 8.1–8.3. PFNA was obtained from Sigma-Aldrich (CAS number 375-95-1, 97% purity; St. Louis, MI). Solvent-free stock solutions of PFNA were prepared by dissolving crystals in water by stirring. Two stock solutions of 10 and 100 mg/l were made to cover the desired range of target solution in the exposure water. Using a static-renewal procedure, a brood of zebrafish juveniles ($n = 250$), at the development stage of 23 days post fertilization (dpf), were randomly assigned to nominal concentrations of 0 (control), 0.05, 0.1, 0.5, and 1 mg/l PFNA, and 50 fish were used per treatment group. These fish referred as "F_0 generation" were exposed to PFNA for 180 days. During the experiment, the size of the tanks was adjusted according to the size of the fish, starting with 2-l tanks followed by 5-, 15-, and 30-l tanks as they grew larger. A small volume of water was siphoned from the bottom of the tank daily to remove debris. One-third of the water volume was removed and replaced with newly prepared PFNA water every other day.

The gender was phenotypically determined when the zebrafish became sexually mature (120 dpf). Males and females from the same treatment group were then separated into different aquaria while the exposure was continued. At the conclusion of the 180-day exposure, the zebrafish were anesthetized in ice water. Only adult males were recruited for the subsequent analysis, while the females were used for another experiment. Male fish were then bled following partial caudal severance, and the blood was collected using heparinized capillary tubules. Blood from 6 to 8 individuals were pooled in 1 mg/ml heparin-washed 200-μl EP tubes as one sample, and stored at 4°C for up to 4 h until the plasma was separated by centrifugation at 3,000 × g for 10 min. The plasma was stored at −80°C until analysis. Four males in each treatment were sampled for histological analysis, and the sampled fish were placed in Bouin's fixative following an abdominal incision. The livers and brains of the remaining fish were surgically removed, immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Prior to the conclusion of PFNA exposure for the F_0 zebrafish, males and females from the same dose group were paired for spawning. A subset of fertilized embryos from each group was transferred to a glass plate and cultured in water without PFNA for 5 days, and then the 5-dpf larvae in each group were pooled together, flash-frozen in liquid nitrogen, and stored at −80°C for subsequent RNA extraction. The other subsets of F_1 embryos were reared in the same PFNA concentrations as their parental F_0 zebrafish, and continued the exposure for another 180 days. The exposure process was the same as for the F_0 zebrafish, and blood was collected at the end of the experiment for plasma separation and analysis.

Plasma TH measurement

Plasma total T_3 and T_4 levels were detected using commercial kits from Beijing North Institute of Biological

Technology, China. Total T_3 and T_4 levels were determined by radioimmunoassay (RIA) according to the manufacturer's instructions. The RIAs for T_4 and T_3 were validated for the zebrafish samples by demonstrating parallelism between a series of diluted and spiked plasma samples in relation to a standard curve. The inter- and intra-assay coefficients of variation were <5 and <10%, respectively. Assay sensitivities were 3 and 0.2 ng/ml for T_4 and T_3, respectively.

Histological analysis

Whole fish were fixed in Bouin's solution at 4°C for 48 h, rinsed in tap water, and stored in 70% ethanol. The head of the fish was separated from the trunk region by incision and was embedded in blocks of paraffin. The thyroid histological procedures used are described in Patino et al. (2003). The sections (thickness, 4 μm) were processed and stained with hematoxylin and eosin.

Quantitative real-time PCR assays

Total RNA was extracted from liver and brain samples of F_0 zebrafish, and from homogenate of F_1 larvae by using the MiniPrep RNeasy Kit (Qiagen, Inc., Valencia, CA). One microgram of total RNA was reverse-transcribed with an oligo-$(dT)_{15}$ primer and M-MuLV transcriptase as described by the manufacturer (Promega, Madison, WI). Quantitative real-time PCR was performed on a Stratagene Mx3000p qPCR system (Stratagene, Cedar Creek, TX). The 25-μl reaction mixture contained 12.5 μl of 2× SYBR Premix Ex Taq (Takara, Dalian, China), forward and reverse primers (0.1 μM each), 0.5 μl of ROX reference Dye II, nuclease-free water, and 3 μl of cDNA template. The gene-specific primers (Supplementary data, Table S1) were designed with the Primer Premier 5.0 software. The geNorm VBA applet for Microsoft Excel (http://medgen.ugent.be/genorm/) was used to assess the expressional stability of candidate house-keeping genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; hypoxanthine guanine phosphoribosyl transferase, HPRT; β-actin, Actin; elongation factor 1a, EF1a), and the most stable one was chosen as an internal control for normalization in the subsequent PCR quantification. Each sample run in triplicate in the qPCR assays. The amplification efficiencies between the target genes and the reference gene were regulated to differ by less than 5%. The amplification protocol was as follows: 95°C for 10 s followed by 40 cycles of 94°C for 5 s, 55°C for 15 s, and 72°C for 10 s. Expression values of each target gene was defined relative to the reference gene, and plotted on a scale relative to expression levels in the control treatment group.

Statistical analyses

All statistical analyses were performed using SPSS v13.0 software (SPSS, Inc., Chicago, IL). The data are presented as means with standard errors of the mean (mean ± SE). Results were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc pairwise statistical analyses. A probability of $p < 0.05$ was considered statistically significant.

Results

Thyroid histopathology

Like other teleost fishes, zebrafish thyroid follicles were found in the cross sections of the head, where the posterior portion of the eyes overlapped with the anterior portion of the gill arches (Fig. 1a). In the control fish, follicles of normal size were well filled with colloid and had flat epithelia (Fig. 1b). Abnormally enlarged follicles with flat epithelium could be observed in the 0.05 mg/l group (Fig. 1c). Hypertrophy of follicular epithelium and hyperplasia of follicle cells were observed in the 0.1 and 0.5 mg/l PFNA groups (Fig. 1d, e). A reduction in the cross-sectional area of the colloid could be observed in the 1 mg/l group (Fig. 1f).

Plasma thyroid hormones

Analysis of TH in plasma samples showed significantly increased T_3 levels in F_0 adult males from the 0.05, 0.1, and 1 mg/l PFNA groups ($p < 0.05$), while T_4 levels were not significantly different between the control and PFNA groups. Although the elevation of T_3 levels in the 0.5 mg/l group was not significant, this group demonstrated the highest TH levels (Table 1). Plasma T_3 levels were also analyzed in F_1 adult, and the results demonstrated that T_3 levels for both males and females were significantly elevated in all PFNA treatment groups (Fig. 2).

Gene transcripts in the liver

Stable transcription of the internal control was vital for the validity of the qRT-PCR examination. Among the four house-keeping genes, HPRT1 proved to be the most stable (M value, HPRT1 0.959, ACTIN 0.985, EF1α 1.053, GAPDH 1.525) and was used for the qRT-PCR examination. The transcriptional expression of genes responsible for TH transport, metabolic conversion, and function were investigated in the livers of F_0 males. Transthyretin (TTR) was significantly up-regulated in the 0.1, 0.5, and 1 mg/l groups ($p < 0.05$). There were two isoforms of deiodinases

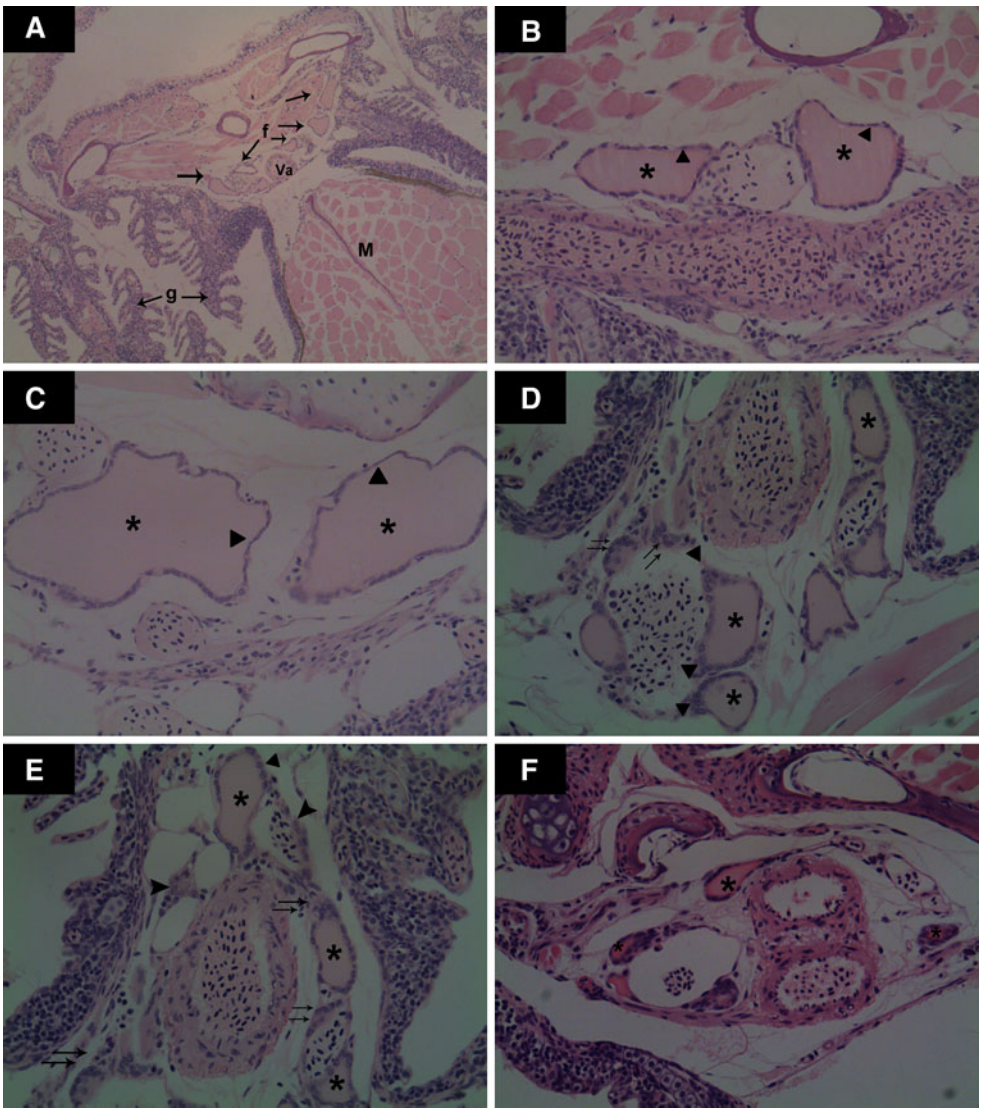

Fig. 1 Photomicrographs of thyroid follicle sections of zebrafish after a 180-day exposure to PFNA. **a** The cross section of fish head area around the ventral aorta (×100); **b** thyroid follicles in the control group (×400); **c** follicles in fish exposed to 0.05 mg/l PFNA showed abnormally enlarged follicles (×400); **d** and **e**, thyroid follicles in fish exposed to 0.1 and 0.5 mg/l PFNA respectively: hypertrophy of follicular epithelium was observed, hyperplasia was recognized as the presence of follicle cell masses associated with the follicles or as small clusters in the extravascular space around blood and secondary circulation vessels (×400); **f** a reduction in the cross-sectional area of the colloid could be observed in the 1 mg/l PFNA group (×400). *Asterisks*, colloid; *arrowheads*, follicular epithelium; *thin double arrows*, the proliferation of follicle cell clusters in extravascular space. *f* thyroid follicle, *g* gill, *Va* ventral aorta, *M* muscle

Table 1 Plasma thyroid hormone levels in F_0 adult males after exposure to PFNA for 180 days

Group	T_3 (ng/ml)	T_4 (ng/ml)	$T_3 + T_4$ (ng/ml)
Control	2.26 ± 0.21	14.88 ± 2.08	17.13 ± 2.09
0.05 mg/l	5.52 ± 1.17*	16.53 ± 0.07	22.05 ± 1.17
0.1 mg/l	7.39 ± 0.49*	18.49 ± 1.35	25.88 ± 1.38*
0.5 mg/l	4.85 ± 1.42	22.04 ± 4.24	26.89 ± 3.43*
1 mg/l	6.87 ± 0.38*	15.78 ± 1.56	22.64 ± 1.43

Asterisks indicate significant difference from the control ($n = 3$, $p < 0.05$)

(Dio1 and Dio2) identified in zebrafish. The expression of Dio1 was not significantly altered after PFNA exposure, while inhibited expression of Dio2 was only observed in the 0.05 and 1 mg/l PFNA groups. PFNA exposure did not affect the expression of TH receptor (TRα), but significantly reduced the expression of TRβ in the 1 mg/l PFNA group ($p < 0.05$). Males exposed to 0.05, 0.1, and 1 mg/l PFNA exhibited significant down-regulation of Ugt1A5 as compared to the control, while expression of Ugt2A1 was significantly down-regulated in all PFNA groups ($p < 0.05$) (Fig. 3a). The expression of four isoforms of

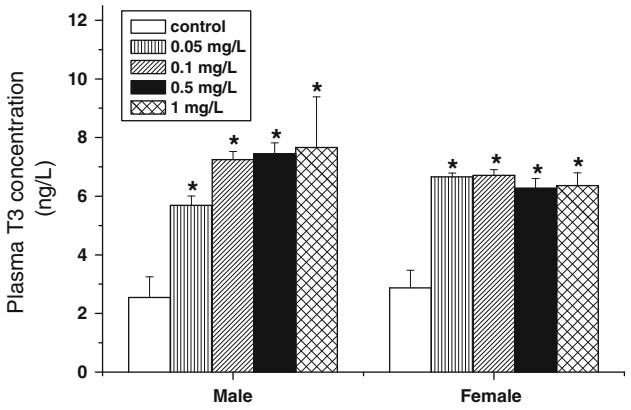

Fig. 2 Effects of PFNA exposure for two consecutive generations on plasma T_3 content of F_1 adults. Significance is indicated by * for $p < 0.05$ as compared to the control

sulfuryl transferase in the liver (SUL ST1, ST4, ST5, and ST6) was also examined. Down-regulation of ST1, ST5, and ST6 was observed in the highest PFNA group (1 mg/l), with no significant difference observed for ST4 (Supplementary data, Fig. S1).

Gene transcripts in the brain

Transcripts for TRα and TRβ, glycoprotein hormone α-subunit (GPHα), thyrotropin β-subunit (TSHβ), and the TH-responsive transcription factor basic transcription element-binding protein (BTEB) in the brain were studied (Fig. 3b). TRα transcription was down-regulated in the 0.1 and 0.5 mg/l treatment groups, while TRβ was down-regulated in the 0.1 mg/l group ($p < 0.05$). No significant alteration in the transcription of TSHβ, GPHα, and BTEB was observed in any of the PFNA treatment groups.

Gene transcripts in F_1 larvae

We further analyzed expression of the genes encoding the sodium/iodide symporter (solute carrier family 5 member 5, slc5a5), thyroperoxidase (TPO), thyroglobulin (TG), and cathepsin b (Ctsb) in the 5-dpf F_1 larvae. The transcription of slc5a5 was significantly up-regulated in the 0.1 and 0.5 mg/l PFNA groups, and the expression of TPO was significantly increased in the 0.05, 0.1, and 0.5 mg/l groups as compared to the control ($p < 0.05$). No significant difference was observed in the transcription of TG between the control and PFNA treatment groups. With regard to gene transcription of Cstb, only the 1 mg/l group showed significantly up-regulated expression (Fig. 4a). The expression of TRα was induced in the 0.05, 0.1, and 1 mg/l groups, and TRβ was induced in all of the PFNA treatment groups ($p < 0.05$). Parental exposure to 0.05, 0.5, and 1 mg/l PFNA significantly induced the expression of TTR ($p < 0.05$) in the F_1 larvae. Ugt2A1 was up-regulated in the 0.1, 0.5, and 1 mg/l PFNA groups; in contrast, the expression of Ugt 1A5 was down-regulated in the 0.05, 0.1, and 1 mg/l groups ($p < 0.05$) (Fig. 4b).

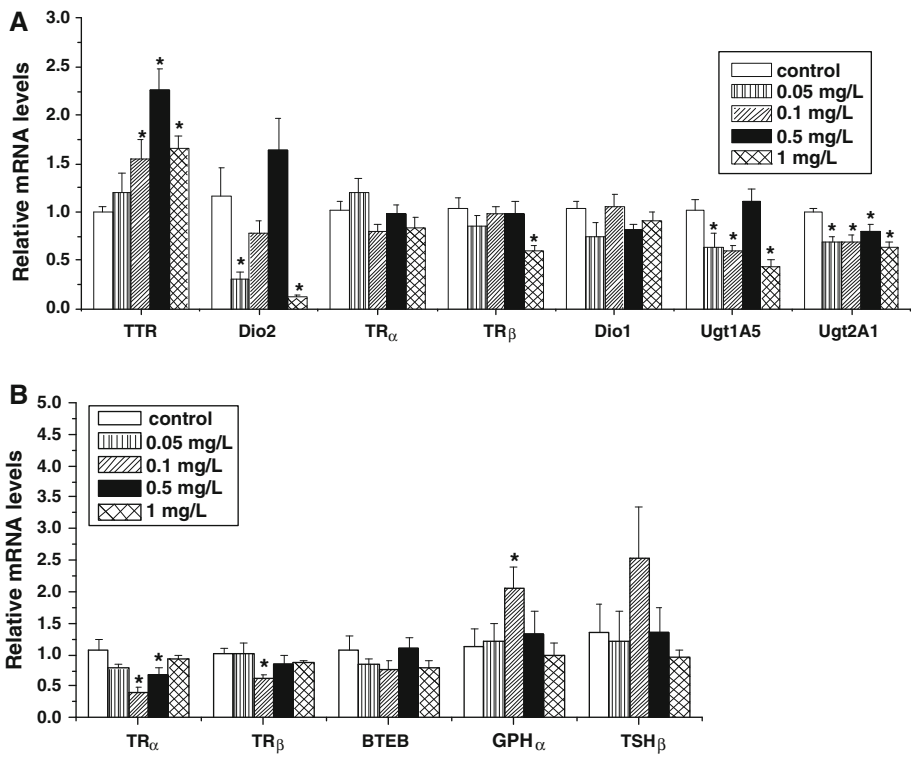

Fig. 3 Real-time PCR analysis of mRNA expression in the liver and brain of F_0 males after PFNA exposure. **a** Hepatic mRNA expression levels of TTR, Dio2, TRα, TRβ, Dio1, Ugt1A5, and Ugt2A1 in control and PFNA-exposed males; **b** mRNA expression levels of TRα, TRβ, BTEB, GPHα, and TSHβ in the brain of control and PFNA-exposed male zebrafish. The values represent relative mRNA levels as compared to the control group. Significance is indicated by * for $p < 0.05$ as compared to the control

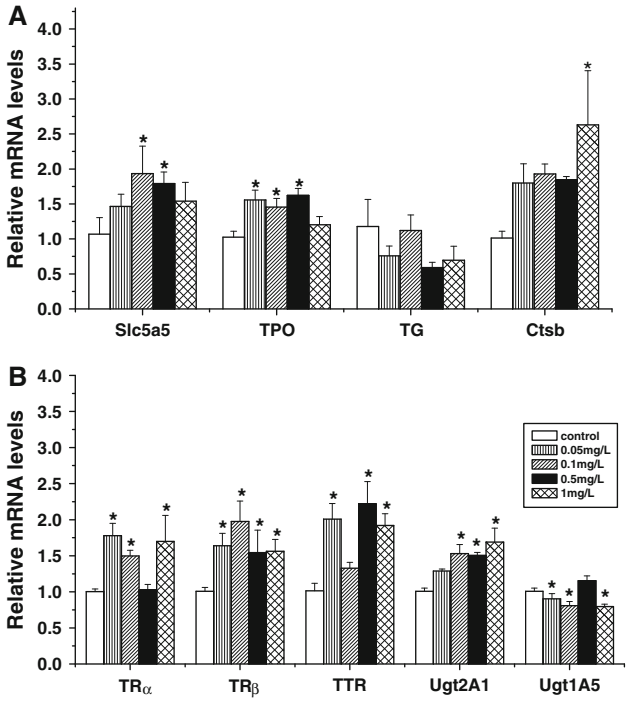

Fig. 4 Real-time PCR analysis of mRNA expression of Slc5a5, TPO, TG, Ctsba, TRα, TRβ, Ugt1A5, and Ugt2A1 in 5-dpf F_1 larvae. The values represent relative mRNA levels as compared to the control group. Significance is indicated by * for $p < 0.05$ as compared to the control

Discussion

The persistent presence of PFNA in aquatic environments and the potential disturbance of these chemicals on the endocrine system could influence the normal physiology of teleosts. Therefore, it would be informative to evaluate the potential thyroid-disrupting effects of chronic exposure to PFNA. In the present study, we employed a multigenerational PFNA exposure procedure with zebrafish to evaluate the potential thyroid-disrupting effects of chronic exposure to PFNA. In this test, changes in plasma TH levels and thyroid follicle histology were used as direct endpoints to assess potential thyroid-disrupting effects. Moreover, gene transcription within the HPT axis was assessed to facilitate understanding of the potential mode of action of PFNA on the thyroid signaling system.

Thyroid histopathology and circulating TH levels are often used as indicators of thyroid disorders (Brown et al. 2004). Recently, approximately 116 environmental compounds have been identified as suspected disruptors of thyroid function (Howdeshell 2002; Schnitzler et al. 2008). Impacts of PFAA chemicals on teleost thyroid were not clear due to the limited information available. In the present study, we simultaneously employed two thyroid endpoints to evaluate the effects of PFNA on the thyroid. Significantly elevated plasma T_3 levels were observed after long-term PFNA exposure in both F_0 and F_1 adults. Thyroid histological changes also demonstrated an apparent PFNA thyroid toxicity. Alterations in the thyroid histology, such as the size of the follicle colloid lumen, the hypertrophy of follicular epithelium, and hyperplasia of follicle cells observed in the F_0 males after long-term PFNA exposure indicated a hyperstimulation of the follicles. This histopathological evidence is consistent with the significantly elevated plasma levels of TH in fish. All of evidences indicated that PFNA exposure triggers a hyperstimulation of TH synthesis/secretion. The combined analysis of both histological and hormonal biomarkers in the present study allowed for a better estimation of the thyroid function status.

The regulation mechanisms involved in thyroid homeostasis are complex and may act at many levels in the thyroid system, including hormone synthesis, competitive binding to thyroid hormone binding proteins in blood, metabolic activation or inactivation, TH function through TH-responsive elements, and so on (Boas et al. 2006; Brown et al. 2004; Ishihara et al. 2003; Zoeller et al. 2007). In addition to the direct effects via these sites, indirect effects via the hypothalamus and pituitary are also possible (Blanton and Specker 2007; Lema et al. 2008). Thus, a gene transcript profile at multiple levels of the HPT axis was conducted to elucidate the thyroid-disrupting mechanism induced by PFNA; results of gene expression profile are summarized in Fig. 5.

TTR behaves as a thyroid hormone-binding protein, and the TTR in the bloodstream is principally secreted by the liver (Kawakami et al. 2006; Santos and Power 1999). Previous observations have indicated that TTR expression in fish could be regulated by circulating TH levels. Physiological factors that induce a reduction in circulating TH are usually coincident with decreases in TTR, while administration of T_3 and T_4 can significantly increase the concentration of circulating TTR (Morgado et al. 2007). Therefore, the increased expression of TTR in the liver of PFNA-exposed F_0 males could possibly be due to the PFNA-induced elevated plasma TH levels. On the other hand, as TTR is a TH transporter in fish, it may regulate the plasma levels of TH. The increased expression of TTR observed in the present study should have helped to restrain increase in TH levels induced by PFNA exposure, and in this way, it would be involved in the regulation of the thyroid axis. Moreover, due to the hydrophilic and lipophilic nature of PFAAs, the environmental behavior of this class of chemicals differs from that of the more lipophilic POPs. PFAAs have been found to accumulate in the liver and blood and are prone to bind serum proteins (Jones et al. 2003; Kannan et al. 2001). Recently, an in vitro study employing a radioligand binding assay also demonstrated

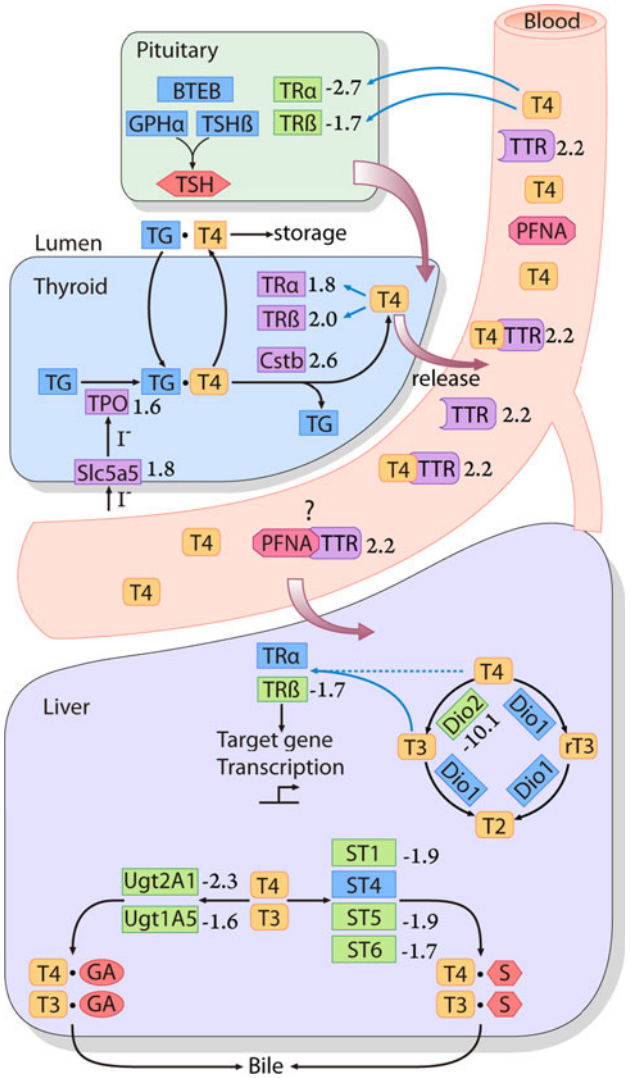

Fig. 5 Schematic presentation of PFNA disruption in gene expression involved in TH homeostasis (TH synthesis, circulation transport, activation and elimination, feedback regulation in pituitary). *Blue gene boxes* indicate unaltered transcriptional expression. Significantly increased expressions are indicated in *purple gene boxes*, and genes presented within green boxes indicated significantly down-regulated expression ($p < 0.05$). Data outside the *purple* or *green gene boxes* indicate the maximal fold-change in gene expression between the control and PFNA treatment groups

the capacity of PFAAs to compete with thyroxine for binding to the human TTR (Weiss et al. 2009). Thus, the increased TTR transcription observed in our study could also be interpreted as induction caused by PFNA competitive binding. This viewpoint could be supported by the consistently elevated TTR expression in both rats and zebrafish, despite the completely opposite alteration in TH levels in these two species.

The deiodination catalyzed by Dio2 in the endoplasmic reticulum of liver converted T_4 to the biologically more active T_3, and Dio2 may play a role in regulating plasma T_3 levels. The depressed expression of Dio2 observed in the 0.05 and 1 mg/l PFNA groups can be interpreted as its function in restraining the acutely increased plasma T_3 levels induced by PFNA exposure. Although the depressed expression of Dio2 in the present study did not demonstrate a dose–effect response, the effect was consistent with the phenomenon that hepatic Dio2 decreases during hyperthyroidism in all teleost species studied so far (Van der Geyten et al. 2001). Hence, the decreased Dio2 expression may be a negative feedback regulation in response to the increased T_3 levels. The action of TH also involves bioactive T_3 binding to nuclear receptors. TRs act as ligand-activated transcription factors by inducing or repressing the transcription of genes containing a thyroid response element. There have been two isoforms of TRs reported in zebrafish (TRα and TRβ). In our present study, neither of the two isoforms in the liver was sensitive to the TH changes induced by PFNA exposure.

The metabolic inactivation and elimination of TH mainly occurs in the liver through three different pathways: deiodination by Dio1, sulfate conjugation by sulfuryl transferases, and the glucuronide conjugation pathway by UDP-glycosyltransferases. Then the inactivated and more water-soluble conjugates are then excreted in bile (Brown et al. 2004). PFNA exposure did not affect the expression of Dio1, and its inhibition of the SULTs was also not apparent except in the highest dose group. However, the UDP-glycosyltransferase pathway was significantly inhibited, indicating the key role of glucuronidation via Ugt1A5 and Ugt2A1 in regulating TH elimination during PFNA exposure. The inhibited expression of SULTs and primarily Ugts would lead to reduced TH biliary elimination and may have accounted for the increased plasma TH levels observed in our experiment.

TH-regulated gene transcription in the brain of F_0 adults was not significantly changed. The transcription of TRα and TRβ was inhibited only in some treatment groups, while mRNA expression for TSHβ and GPHα, which encode the two protein subunits of the functional thyrotropin hormone, did not demonstrate obvious changes. The BTEB gene encodes a zinc-fingered transcription factor that binds GC-box domains to regulate TH-mediated gene transcription (Lema et al. 2009). The expression of BTEB was also unaltered. These results indicated that the elevated plasma TH levels had not induced the expected endocrine-negative feedback in the pituitary.

Genes involved in TH synthesis and those that were sensitively altered in the F_0 zebrafish were further studied in the F_1 larvae to investigate trans-generational effects. Slc5a5 is required in TH synthesis for the uptake of iodide from the blood into the thyroid follicles in which thyroperoxidase catalyzes the iodination of thyroglobin. Iodinated TG (also called TG-T_4) can be stored in the lumen of

thyroid follicles, and its release into blood as thyroid hormone involves proteolytic cleavage of TG-T_4 by proteases, including Ctsb (Alt et al. 2006). These changes in Slc5a5, TPO and Ctsb mRNA levels in F_1 larvae are consistent with the elevated T_3 levels observed in PFNA-treated F_0 adults. A significantly elevated expression of TRα and TRβ was observed, which was different from the imperceptible alteration or occasional depression observed in the F_0 livers and brains. TR mRNAs are clearly regulated by THs, but whether these genes are induced or repressed also depends on the tissue and receptor isoform (Lema et al. 2009). The expression of TTR, Ugt1A5, and Ugt2A1 was also sensitively and significantly altered in the F_1 larvae. The induced expression of Ugt2A1, TTR, and TRs observed in the F_1 larvae may be ascribed to the especially important role of TH in morphological development, which mediates the transition of zebrafish from embryos to larvae (Lema and Nevitt 2006; Tagawa and Aritaki 2005). Above all, the altered gene expression in the F_1 larvae noticeably reflected the trans-generational thyroid-disrupting effects of PFNA exposure.

In summary, our results provide the first evidence for the thyroid-disrupting effects of long-term PFNA exposure in a teleost species. PFNA may induce histopathological changes in the thyroid gland and significantly elevate zebrafish plasma T_3 levels in two consecutive generations. Gene expression profile studies further revealed the disrupting effects and the potential mode of action of PFNA on TH transport, metabolism, synthesis, and function, pointing out that the impact on the TH transport and glucuronide conjugation elimination may be the key targets of the PFNA-induced thyroid-disrupting effects.

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