Targeting neurotrophic factors and their receptors, but not cholinesterase or neurotransmitter, in the neurotoxicity of TDCPP in Chinese rare minnow adults (*Gobiocypris rarus*)

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ABSTRACT

Organophosphate flame retardants (OPFRs) have been detected at high concentrations in various environmental and biotic samples, but little is known about their toxicity. In this study, the potential neurotoxicity of three OPFRs (TCEP, TDCPP, and TPP) and Chlorpyrifos (CPF, an organophosphate pesticide) were compared in Chinese rare minnow using an acute toxicity test and a 21-day fish assay. The acute test demonstrated significant inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) by CPF. Although significant AChE inhibition at high concentration of TPP was also observed, none of the OPFRs had effects similar to CPF on these enzymes, indicating that their acute toxicities to Chinese rare minnow may be unrelated to cholinesterase inhibition. In addition, the 21-day fish assay with TDCPP demonstrated no significant effects on cholinesterase activities or neurotransmitter levels. Nonetheless, this OPFR exhibited widespread effects on the neurotrophic factors and their receptors (e.g., *ntf3*, *ntkr1*, *ntkr2*, *ngfr*, and *fgf2*, *fgf11*, *fgf22*, *fgfr4*), indicating that TDCPP or other OPFRs may elicit neurological effects by targeting neurotrophic factors and their receptors in Chinese rare minnow.

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1. Introduction

The phasing out of commercial brominated flame retardants such as penta- and octa-brominated diphenyl ethers (PBDEs) has resulted in a gradual increase in the production and use of organophosphate flame retardants (OPFRs), a major replacement for brominated flame-retardants (Stapleton et al., 2009; Wang et al., 2015). OPFRs have been detected at high concentrations in various environmental samples, including household dust, indoor air, drinking water, and sediment (Reemtsma et al., 2008; Cao et al., 2012; van der Veen and de Boer, 2012; Li et al., 2014), as well as biotic samples, including fishes, mussels, birds, and human breast milk (Sundkvist et al., 2010; Kim et al., 2014). Among the OPFRs, tris

(2-chloroethyl) phosphate (TCEP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) have been detected in numerous environmental samples (Dishaw et al., 2014). For example, TCEP has been detected in surface water, wastewater treatment plants, oceans and drinking water at ng/l to µg/l concentrations (Ren et al., 2008), and TCEP is found in sewage treatment plants in Europe at concentrations of several hundred ng/l (Reemtsma et al., 2006). In addition, TDCPP has been detected in surface water of the Ruhr river at a maximum concentration of 50 ng/l (Andresen et al., 2004), and a high concentration of TDCPP (up to 3 µg/l) has been detected in effluents from sewage treatment plants (Marklund et al., 2005). Moreover, TPP was one of the most frequently detected compounds in fishes and mussels from Swedish lakes and coastal areas, at concentrations ranging from 21 to 180 ng/g lipid weight (Sundkvist et al., 2010).

Unfortunately, there are very limited toxicity and health data available for OPFRs (Dishaw et al., 2011, 2014). Although little is known about OPFR toxicity, recent studies have shown that

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exposure to OPFRs has the potential to cause carcinogenic changes (Freudenthal and Henrich, 2000), oxidative stress (Dishaw et al., 2011), endocrine disruption (Liu et al., 2012; Wang et al., 2013), and neurological effects (Dishaw et al., 2011; Wang et al., 2015) in different organisms. Despite the limited information, a few studies have described the neurotoxicity of OPFRs (Dishaw et al., 2011, 2014; Ta et al., 2014; Wang et al., 2015). Treatment with OPFRs (e.g., TDCPP) resulted in mitotic inhibition and reduced cell numbers during neurodifferentiation (Dishaw et al., 2011) and also decreased the expression levels of nervous system-related genes and proteins (e.g., GAP43, tubulin and NF-H) in PC12 cells (Ta et al., 2014). OPFR exposure was also able to reduce neurotransmitter levels (e.g., dopamine and serotonin in female zebrafish), reduce the expression of nervous system developmental genes (e.g., *mbp* and *syn2a*) (Wang et al., 2015) and alter neurobehavioral responses (decrease in larval swimming activity) (Dishaw et al., 2014) in zebrafish. Although OPFRs are known to cause developmental neurotoxicity (Dishaw et al., 2014; Wang et al., 2015), the mechanism of OPFR neurotoxicity in adult organisms remains unclear.

Due to their structural similarity to organophosphate pesticides (OPs) such as Chlorpyrifos (CPF), several studies have suggested that OPFRs may also have the potential to cause neurological effects similar to those of OPs (Dishaw et al., 2011, 2014; Wang et al., 2015). For example, TDCPP exhibited neurological effects (e.g., alterations in neurodifferentiation) similar to those of CPF in PC12 cells (Dishaw et al., 2011). Previous studies have reported that the acute toxicity of OPs primarily occurs via inhibition of the various forms of cholinesterase, such as AChE (Terry, 2012). Therefore, cholinergic markers (especially AChE activity) have been widely used as biomarkers in determining the neurotoxicity of OPs (Wang et al., 2015). However, previous studies have also reported that the mechanism of the chronic neurological toxicity of OPs cannot be solely related to cholinesterase inhibition (Slotkin et al., 2008; Terry, 2012). Accordingly, several non-cholinesterase targets of OPs have been reported, such as neurotrophic factors and their receptors as well as the axonal transport process (Terry et al., 2007; Slotkin et al., 2008; Terry, 2012), and due to similarities in the effects of TDCPP and CPF in PC12 cells, neurotrophic factors may also be targeted by OPFRs (Dishaw et al., 2011). The neurotrophin and fibroblast growth factor (FGF) families are two primary groups of neurotrophic factors and are known to play critical roles in neural development and damage/repair processes (Slotkin et al., 2007, 2008; Pomeroy-Black and Ehrlich, 2012). Previously, several studies have evaluated the effects of OPs on the neurotrophin and FGF families (Terry et al., 2007; Slotkin et al., 2007, 2008; Pomeroy-Black and Ehrlich, 2012). For example, exposure to OPs altered levels of the phosphorylated forms of neurotrophin receptors (Terry et al., 2007; Pomeroy-Black and Ehrlich, 2012) and caused significant activation of their related intracellular signaling pathways (Pomeroy-Black and Ehrlich, 2012). In addition, Slotkin's study found that two OPs (CPF and diazinon) differentially regulate members of the FGF gene family (Slotkin et al., 2007). Although OPs exhibit significant effects on different families of neurotrophic factors, to our knowledge, the impacts of OPFRs on these neurotrophic factors have not yet been reported.

The purpose of the present study was to compare the potential neurotoxicity of three OPFRs (TDCPP, TCEP, and TPP) with the insecticide CPF in Chinese rare minnow (*Gobiocypris rarus*) using an acute toxicity test and a 21-day fish assay. Chinese rare minnows, which are distributed in the upstream region of the Yangtze River and in the Sichuan Province of China, are considered to be an appropriate species for assessing chemical toxicity due to their small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Zha et al., 2007; Li et al., 2009; Yuan et al., 2013). Based on their structural similarity to OPs, OPFRs

may have similar neural effects (Dishaw et al., 2011). Similar to OPs, OPFRs may target the cholinesterase or neurotransmitter systems. Therefore, several potential targets, including the activities of AChE and BChE and concentrations of two neurotransmitters (ACh and serotonin), were identified. In addition, several non-cholinesterase targets for OPs have been reported, such as neurotrophic factors and their receptors (Slotkin et al., 2008), thus, OPFRs may elicit effects on these neurotrophic factors, similar to OPs.

2. Materials and methods

2.1. Chemicals

Reagents were purchased from the following sources: TCEP (purity 98%), TDCPP (purity 96%) and TPP (purity 99%) from Adamas-beta (Adamas-beta, Switzerland); CPF (purity 99.5%) and acetone from Sigma-Aldrich (Sigma, USA). Stock solutions of TDCPP, TCEP, TPP and CPF were prepared by dilution in acetone. The final acetone concentration in the water was less than 0.01%.

2.2. Test fish and culture conditions

The brood stock of the rare minnows was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg CaCO₃/l; temperature 25 ± 1 °C) with a photoperiod of 16:8 h (light: dark) and has been used for testing chemicals in our laboratory for more than 10 years (Zha et al., 2007; Li et al., 2009). The fish were fed a commercial food pellet (Trea, Germany) at a rate of 0.1% body weight per day and were also provided with newly hatched brine shrimp (*Artemia nauplii*) twice daily.

2.3. Exposure and experimental design

Five-month-old healthy Chinese rare minnows (*n* = 525) and offspring from the same pair of brood stock were used in this experiment. The body weights and lengths were 0.58 ± 0.13 g and 38.83 ± 2.2 mm, respectively. Similar to CPF, acute and chronic exposure to OPFRs may lead to different levels of neurotoxicity; therefore, two assays, an acute (96 h) toxicity test and a 21-day fish assay, were performed in this study. After the acclimation period, the fish were either acutely exposed (96 h) to the OPFRs and CPF or exposed at a low dose (21 days). During both exposure experiments, the water temperature was maintained at 25 ± 1 °C with a pH of 7.0 ± 0.2. The fish were fed twice a day with newly hatched brine shrimp, and the exposure water was renewed every day. When referring to OPFR or CPF exposure, the nominal concentrations are used throughout the manuscript.

In the acute test, fish (*n* = 225) were randomly distributed into five groups: TCEP (1.25, 2.5, 5 mg/l), TDCPP (0.75, 1.5, 3 mg/l), TPP (0.5, 1, 2 mg/l), CPF (5, 10, 20 µg/l) and the control group, acetone served as the negative control in the experiments. The exposure concentrations were set based on the 96-h LC₅₀ (lethal concentration) for each compound, which was determined by a test conducted prior to the exposure experiment (Table S2). After the 96-h exposure, the remaining fish were sacrificed, and the brains were excised for measurement of AChE and BChE activities.

In the 21-day fish assay, approximately 10% of the acute toxicity concentration was selected for TDCPP and CPF. A total of 300 fish were randomly distributed into five groups: the control group, 40 µg/l TDCPP exposure group, 200 µg/l TDCPP exposure group, 0.4 µg/l CPF exposure group, or 2 µg/l CPF exposure group. After 21 days of exposure, the fish were sacrificed, and their tissues were excised for measurements of enzyme activity, neurotransmitter concentration, and gene expression.

2.4. Cloning of cDNA fragments of the neurotrophin and FGF family genes by RT-PCR

Total RNA was isolated from the brains of Chinese rare minnows using the SV Total RNA Isolation System following the manufacturer's protocol (Promega, USA). The RNA samples were dissolved in ribonuclease-free water and stored at -80°C until reverse-transcriptase polymerase chain reaction (RT-PCR) was performed. The protocol was performed according to a previous report from our laboratory (Li et al., 2009). The reaction mixtures containing 10 μl of total RNA, 2 μl (0.05 $\mu\text{g}/\mu\text{l}$) of Oligo (dT)₁₅, and diethylpyrocarbonate-treated water (to a total volume of 12 μl) were heated to 70°C for 5 min and then quickly chilled on ice. After cooling, 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl_2 , 2 mM deoxynucleotide triphosphate (10 mM each), 40 units of RNasin (RNAase inhibitor; Promega), and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, USA) were added in a total volume of 25 μl and incubated for 1 h at 42°C .

To obtain cDNA fragments of the neurotrophin (ntf3, ntf7, ntrk2, ntrk3, and ngfr) and the FGF (fgf2, fgf11, fgf16, fgf17, fgf20, fgf22, fgfr1, fgfr2, fgfr3, and fgfr4) family genes, primer pairs were designed based on highly conserved regions according to known sequences from other fish species or based on the zebrafish sequences available in GenBank (Table S1). Each 50 μl DNA amplification reaction contained PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl_2), 200 μmol of dNTPs, 20 pmol of each gene-specific primer, 1 μl of a 20-fold dilution of total cDNA, and 2.5 U Taq DNA polymerase (Invitrogen, USA). The thermocycler program involved a denaturation step at 94°C (8 min); 35 cycles of 94°C (30 s), 55°C (30 s), and 72°C (30 s); and a final elongation step at 72°C (10 min). The PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands of the expected size were excised and purified using the Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa, Japan). The isolated fragments were inserted into the pMD 19-T vector (TaKaRa, Japan), and transformed into *E. coli* (DH5 α). The partial cDNAs were sequenced and compared to sequences available in GenBank using BLAST.

2.5. Measuring AChE and BChE activities

The fish were euthanized, and their brains were removed after the acute (the remaining fish) or 21-day low-dose exposure. Four or five brains were pooled for use as a single replicate for each treatment (with 3 replicates per treatment). The brains were

weighed, homogenized on ice in 100 mM phosphate buffer at a pH of 7.4 (1/3, W/V), and centrifuged at 3000 g for 10 min at 4°C ; the supernatants were used to determine enzyme activities. The activity of the AChE or BChE enzyme was measured using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Enzymatic activity was determined by measuring the increase in the extinction at 412 nm in a spectrophotometer (Thermo Fisher, USA). Enzyme activity is expressed in $\mu\text{mol}/\text{min}/\text{mg}$ of protein for AChE and in $\text{nmol}/\text{min}/\text{mg}$ of protein for BChE. The protein content of the supernatant was determined using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China).

2.6. Determining the neurotransmitter concentration

For the determination of ACh concentrations in Chinese rare minnow brains after 21 days of low-dose exposure, four or five brains were pooled for use as a single replicate. The brains were weighed, homogenized on ice in lysis buffer (1/9, W/V) (Nanjing Jiancheng Bioengineering Institute, China), and centrifuged at 3000 g for 10 min at 4°C . The supernatants were used to determine the ACh concentration using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. The optical density of the supernatant was measured at 550 nm in a spectrophotometer (Thermo Fisher, USA), and the ACh concentration was calculated from a freshly prepared ACh calibration curve. The results are expressed in terms of μg ACh per mg of protein.

To determine the plasma serotonin levels in the samples, the roots of the caudal fin of the fish were cut to collect blood into a heparinized microcapillary tube. The blood samples were centrifuged at 3000 g for 20 min to prepare the plasma samples. A volume of 20 μl of plasma was used as one replicate to determine serotonin levels, and each treatment was performed in triplicate using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China). The levels were calculated from a freshly prepared serotonin calibration curve. The results are expressed in terms of ng/l plasma.

2.7. Determining the neurotrophin and the FGF family gene expression levels

To determine the effects on neurotrophin and FGF family gene transcription in Chinese rare minnows after 21 days of TDCPP or CPF exposure, total RNA was isolated from brains, and cDNA was

Table 1
Primer sequences used for quantification of the neurotrophin and the FGF family genes expression by real-time PCR in rare minnow.

Gene	Sequence	(5' → 3')	Product size (bp)	Genbank accession no.
β -actin	F: CTATGTTGGTGACGAGGCTCA	R: CCCAGTTGGTGACAATACCG	82	EF370390.1
ngf	F: CAAACACCCAGAACCTCC	R: GTTATCATCAACAGCTCCAA	84	KP178114
bdnf	F: AGAAAGAGTAACGGCGGCTC	R: AGTTGCTTGAGGTGGAAGGG	168	KP178115
ntf3	F: ACAGAAGCAGGACGAGGGT	R: TCACGGCTGGTAGAAGG	101	KP728105
ntf7	F: TTTTGCTGCTCGGTGCTG	R: TGCCCATGAGGTCGTTGA	116	KP728106
ntrk1	F: CTGGTGCTCTCCCATTTGC	R: GTGTAGCGGTTTCGTTT	90	EE397822.1
ntrk2	F: CTCGGTGGAAATCTGCTC	R: TGTCTCTGGCTGAATGCTA	108	KP728108
ntrk3	F: AAACACGGAGACCTCAACA	R: CAGCATCTGAGACAAACCC	111	KP728109
ngfr	F: CATCCCATCTACACCTCC	R: ATTGGCTCCTTGCTTATT	105	KP728107
fgf2	F: GACCGACGACTGTTTGA	R: TGGTACTAGAGCCGACTTAT	157	KP728095
fgf11	F: TGCTCTGGATGGCGACTA	R: ACGGCACAAAGGACGAAA	81	KP728096
fgf16	F: ATCCACGCCTCGAATGCT	R: CGGAACAGACAGGACCAC	85	KP728098
fgf17	F: GCCTCTCGGTTTCATACA	R: AACACTTTCGCCAGACTTTAT	102	KP728097
fgf20	F: TGGTGCAAGGCACGAGAA	R: TCCAGATAACAATCCGCTA	110	KP728099
fgf22	F: TGACAGATTTGATGGCGACA	R: TGGCACCCGTAGGAAGAAT	82	KP728100
fgfr1	F: ATAAGGAGATGGAGGTCTGTC	R: CAGAGTGATGGGAGTTCCGA	101	KP728101
fgfr2	F: CTCAAATACTCCCGAAGG	R: GACGGTGGCTGCTCAAGAT	195	KP728102
fgfr3	F: AATGTCAGAGTTTCGTTTG	R: CTGTATGTGCTGGTGGAG	122	KP728103
fgfr4	F: CATATTGTTCTCCACCAC	R: GAGGCATCAAGTTACGAC	99	KP728104

Table 2

The detailed information of the neurotrophin and the FGF family genes identified in rare minnow.

Gene name	Genbank access. No.	Description	% Identity of zebrafish
Neurotrophin			
ntf3	KP728105	neurotrophin 3	75%
ntf7	KP728106	neurotrophin 7	78%
ntrk2 (TrkB)	KP728108	neurotrophic tyrosine kinase, receptor, type 2	98%
ntrk3 (TrkC)	KP728109	neurotrophic tyrosine kinase, receptor, type 3	98%
ngfr (p75 ^{NTR})	KP728107	nerve growth factor receptor	96%
FGF family			
Fgf2	KP728095	fibroblast growth factor 2	89%
Fgf11	KP728096	fibroblast growth factor 11	98%
Fgf16	KP728098	fibroblast growth factor 16	100%
Fgf17	KP728097	fibroblast growth factor 17	94%
Fgf20	KP728099	fibroblast growth factor 20	96%
Fgf22	KP728100	fibroblast growth factor 22	96%
Fgfr1	KP728101	fibroblast growth factor receptor 1	88%
Fgfr2	KP728102	fibroblast growth factor receptor 2	99%
Fgfr3	KP728103	fibroblast growth factor receptor 3	98%
Fgfr4	KP728104	fibroblast growth factor receptor 4	97%

"% identity of zebrafish" indicated percent identities of the novel identified partial rare minnow predicted amino acid sequences with the sequences of zebrafish available in GenBank. Percent identities were obtained from NCBI Blastp.

synthesized according to the methods described above. Real-time quantitative PCR was performed using an ABI 7500 real-time quantitative PCR system (Life Technologies, USA) according to a previously described method (Chen et al., 2014). The primer pairs used for real-time PCR are shown in Table 1, and β -actin was used as an endogenous control. The results were analyzed using the delta–delta Ct method (Schmittgen and Livak, 2008), and the experiments were performed in triplicate.

2.8. Statistical analysis

Statistical analyses were performed with SPSS (version 13.0) and OriginPro (version 8.0). All quantitative data are expressed as the mean $\pm$ S. E. of the mean (S.E.M.). The differences in cholinesterase

activities and neurotransmitter concentrations between the CPF- and OPFR-treated groups and the control as well as the differences in gene expression levels between the means of the control and the CPF- or TDCPP-treated groups were evaluated using one-way ANOVA ($p < 0.05$) followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Cloning and analysis of cDNA fragments of the neurotrophin and FGF family genes

Partial cDNAs of five neurotrophin family genes (ntf3, ntf7, ntrk2, ntrk3, and ngfr) and ten FGF family genes (fgf2, fgf11, fgf16,

Fig. 1. AChE and BChE activities in rare minnow brain after 96 h of acute exposure to the OPFRs and CPF. The values presented are the means $\pm$ SEM. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters.

Fig. 2. Brain AChE activity (A), Brain BChE activity (B), Brain ACh concentration (C), and Plasma Serotonin content (D) in rare minnow after 21 days of exposure to the TDCPP (40 and 200 μg/l) and CPF (0.4 and 2 μg/l). The values presented are the mean ± SEM. A significant difference among the groups of $p < 0.05$ (ANOVA) is indicated by differences in the letters.

Fig. 3. Relative expressions of the neurotrophin family genes in the rare minnow brain following 21 days of exposure to CPF (0.4 and 2 μg/l) and TDCPP (40 and 200 μg/l). The bars represent the mRNA expression levels in the exposed fish relative to the control fish. The values presented are the mean ± SEM. Asterisks (*) denotes significant differences compared with the control ($p < 0.05$; $n = 6$).

fgf17, fgf20, fgf22, fgfr1, fgfr2, fgfr3, and fgfr4) were isolated and cloned from Chinese rare minnow brain tissues. The partial fragments were then sequenced and submitted to GenBank. Detailed information about these genes is shown in Table 2. The predicted

amino acid sequences of these genes from Chinese rare minnow share a high pair-wise identity with the zebrafish sequences available in GenBank (Table 2).

Fig. 4. Relative expressions of the FGF family genes in the rare minnow brain following 21 days of exposure to CPF (0.4 and 2 µg/l) and TDCPP (40 and 200 µg/l). The bars represent the mRNA expression levels in the exposed fish relative to the control fish. The values presented are the mean $\pm$ SEM. Asterisks (*) denotes significant differences compared with the control ($p < 0.05$; $n = 6$).

3.2. AChE and BChE activities in Chinese rare minnow brains after exposure to CPF and three OPFRs for 96 h

AChE and BChE activities were determined in Chinese rare minnow brains after 96 h of acute exposure to TCEP, TDCPP, TPP, and CPF. The AChE activities were significantly reduced by 85.4%, 87%, and 93.1% in the 5, 10, and 20 µg/l CPF-treated groups, respectively (Fig. 1, $p < 0.05$). In addition, a significant inhibition of BChE activity (reduced by 83.8%) was also observed in the 20 µg/l CPF-treated group (Fig. 1, $p < 0.05$). AChE and BChE activities were not significantly influenced by the OPFR treatments, except for a significant decrease in AChE activity (reduced by 50.2%) in the 2 mg/l TPP group; however, the extent of inhibition was greatly reduced compared to that of the CPF-treated groups (Fig. 1, $p < 0.05$).

3.3. AChE and BChE activities and neurotransmitter concentrations after 21 days of exposure to CPF or TDCPP

Brain AChE and BChE activities, ACh concentrations, and plasma serotonin levels were measured after 21 days of exposure to TDCPP (40 and 200 µg/l) and CPF (0.4 and 2 µg/l). In the 2 µg/l CPF treatment group, brain AChE activities were significantly reduced (Fig. 2, $p < 0.05$). Moreover, a decrease in plasma serotonin levels and an increase in brain ACh levels were also observed in the 2 µg/l CPF treatment group (Fig. 2). However, neither cholinesterase activity nor the neurotransmitter level was significantly altered after 21 days of exposure to TDCPP.

3.4. Neurotrophin family gene mRNA expression after 21 days of exposure to CPF or TDCPP

The mRNA expression levels of eight neurotrophin family genes (ngf, bdnf, ntf3, ntf7, ntrk1, ntrk2, ntrk3, and ngfr) were determined in Chinese rare minnow brain tissues after 21 days of exposure to TDCPP (40 and 200 µg/l) and CPF (0.4 and 2 µg/l). The expression of

ntf7 and ntrk1 mRNA was significantly decreased after the 2 µg/l CPF treatment (Fig. 3, $p < 0.05$), and transcripts of the ntf3, ntrk1, ntrk2, and ngfr genes were significantly decreased after the 200 µg/l TDCPP treatment (Fig. 3, $p < 0.05$).

3.5. FGF family gene mRNA expression levels after 21 days of exposure to CPF or TDCPP

The transcriptional levels of ten FGF family genes were also determined after 21 days of exposure to TDCPP (40 and 200 µg/l) and CPF (0.4 and 2 µg/l), and no significant transcriptional effects on these genes in the Chinese rare minnow brain were observed after CPF treatments (Fig. 4, $p < 0.05$). However, significant suppression of fgf2, fgf11, fgf22, and fgfr4 mRNA expression levels was observed after the 200 µg/l TDCPP treatment (Fig. 4, $p < 0.05$), and a significant increase in fgf17 transcripts was also observed after the 200 µg/l TDCPP treatment (Fig. 4, $p < 0.05$).

4. Discussion

In the present study, fifteen previously unreported neurotrophic factor genes were cloned from Chinese rare minnow brains, and the predicted amino acid sequences share high similarity with those from zebrafish (Table 2). Compared to mammals, relatively little is known about neurotrophin and fibroblast growth factor family genes in fish (except for zebrafish, data from GenBank). Thus, the identification of these genes is very important for elucidating their functions in Chinese rare minnows.

Cholinesterase activities (e.g., AChE) are widely used as biomarkers for assessing the presence of neurotoxicants such as OPs in aquatic environments (Whitehead et al., 2005; Wang et al., 2015). In this study, the activities of both AChE and BChE were significantly inhibited in Chinese rare minnow brains after 96 h of exposure to CPF. Previously, CPF was found to significantly inhibit AChE or BChE activity in various fish species, such as common carp (*Cyprinus carpio*) (Halappa and David, 2009), zebrafish (Yen et al.,

2011), and mosquito fish (*Gambusia affinis*) (Kavitha and Rao, 2008). It is generally believed that the acute toxicity mechanisms of CPF occur primarily through the inhibition of the various forms of cholinesterase, leading to excessive peripheral and central cholinergic activity (Terry, 2012). Therefore, it is clear that the acute toxicity of CPF in Chinese rare minnow can primarily be attributed to the inhibitory actions on cholinesterase activity.

In contrast, acute exposure to the OPFRs had no significant effects on cholinesterase activities. Although treatment with 2 mg/l TPP significantly inhibited AChE activity, the level of inhibition was much reduced compared to that in the CPF-treated groups (Fig. 1). Previous studies have reported that OPFRs were not potent AChE inhibitors (Dishaw et al., 2014), and exhibited no effects on the AChE activity in zebrafish (Wang et al., 2015), in agreement with our results for Chinese rare minnow. However, in the present study, the exposure concentrations of TDCPP and TPP were set at or near the lethal concentrations, with no significant effects on cholinesterase activities, indicating that the mechanism of acute toxicity of TDCPP or TPP in Chinese rare minnow may be unrelated to cholinesterase inhibition.

Neurotransmitter systems have emerged as important targets for assessing neurochemical, behavioral and toxicological phenotypes in zebrafish (Rico et al., 2011). In this study, 21 days of exposure to CPF significantly reduced brain AChE activity in the 2 µg/l CPF treatment group, and a decrease in the plasma serotonin level and an increase in the brain ACh level were also observed (Fig. 2). Conversely, neither cholinesterase activity nor the neurotransmitter level was significantly altered after 21 days of exposure to TDCPP. Consistent with our findings, previous studies have shown that the cholinesterase and neurotransmitter systems are targeted by OPs, including CPF (Slotkin and Seidler, 2008; Lima et al., 2011). In addition, long-term exposure to TDCPP resulted in no effects on AChE activities in zebrafish, indicating that TDCPP is not a typical neurotoxin, unlike OPs (Wang et al., 2015).

Neurotrophic factors control neural cell differentiation and the assembly of neural circuits (Slotkin et al., 2008). Chronic exposure to OPs (e.g., CPF) at doses that produce no overt signs of acute toxicity or cholinesterase inhibition could elicit neurological effects by targeting neurotrophic factors and their receptors in rats (Terry et al., 2007, 2011). Due to their structural similarities to OPs, OPFRs are also likely to target neurotrophic factors (Dishaw et al., 2011). However, the effects of OPFRs on neurotrophic factors have not been reported to date. Therefore, the transcriptional effects on eighteen neurotrophic factor genes (eight from the neurotrophin family and ten from the FGF family) were determined in Chinese rare minnow brains after 21 days of exposure to CPF and TDCPP.

For the neurotrophin family genes, a significant decrease in *ntf7* and *ntkr1* transcripts was observed after exposure to 2 µg/l CPF. Moreover, the transcripts of *ntf3*, *ntkr1*, *ntkr2* and *ngfr* genes were significantly decreased after 21 days of low-dose TDCPP treatments (Fig. 3). Previously, exposure to low doses of OPs (including CPF) showed widespread effects on the *ntf* and *ntkr* genes, but much less significant effects on the *bdnf* and *ngf* genes (Slotkin et al., 2008). For example, low-dose CPF exposure altered the expression of *ntf3*, *ntf5*, *ntkr2* and *ntkr3* genes (Slotkin et al., 2008) and the concentration of *ntkr1* protein in rats (Terry et al., 2007). However, no significant effects on *ngf* or *bdnf* were reported (Slotkin et al., 2008). Moreover, high doses CPF or other OPs produced only modest effects on *bdnf* and *ngf* mRNA expression or protein concentrations in rats or chickens (Betancourt et al., 2006; Pomeroy-Black et al., 2007; Slotkin et al., 2008). These results are consistent with our findings in Chinese rare minnow brains, in which low doses of CPF and TDCPP elicited effects on *ntf* and *ntkr* genes but had no significant effects on *ngf* or *bdnf* gene, indicating that the effects of OPs on neurotrophins are selective for certain members

(e.g., *ntf3* and *ntkr1*) (Slotkin et al., 2008).

Regarding the FGF family, low doses of CPF-treated groups showed no significant effects on these FGF genes, which contradicts previous findings that CPF has widespread effects on FGF family genes in neonatal rat brains and PC12 cells (Slotkin et al., 2007, 2008). These differences may be partly due to the different experimental species, different exposure doses and methods of administration, and differences in the life stages between rats and Chinese rare minnows (Slotkin et al., 2007). Moreover, regionally selective (e.g., forebrain and brain stem) responses of FGF genes to OPs have been observed (Slotkin et al., 2007). In the present study, the response of certain FGF genes in a specific region of the Chinese rare minnow brain may be concealed due to the use of whole brains to evaluate transcriptional effects on FGF family genes following exposure to CPF. Nonetheless, low-dose TDCPP treatment elicited widespread effects on FGF family genes, and significant changes in *fgf2*, *fgf11*, *fgf22*, *fgfr4* and *fgf17* transcripts were observed (Fig. 4). Previously, alterations of similar genes of the FGF family, including *fgf2*, *fgf11*, *fgf22*, and *fgfr4*, by OPs were observed in rats (Slotkin et al., 2007), indicating that these FGF genes may play important roles in the neurotoxicity of organophosphates in Chinese rare minnow.

In the present study, TDCPP exposure resulted in no significant changes in cholinesterase activities or neurotransmitter concentrations, whereas TDCPP did alter the expression of neurotrophic factor genes. Given their structural similarities to OPs, it has been suggested that OPFRs have similar neural effects (Dishaw et al., 2011, 2014; Wang et al., 2015). Importantly, OPs can exert many neurological effects through mechanisms that are unrelated to acute toxicity via cholinesterase inhibition, and several non-cholinesterase targets for OPs, including neurotrophic factors, have been identified (Terry et al., 2007; Slotkin et al., 2008; Dishaw et al., 2011; Terry, 2012). Similarly, OPFRs may exert similar neural actions and elicit many neurological effects through mechanisms that are not based on cholinesterase inhibition (Dishaw et al., 2011). These notions are consistent with our findings in Chinese rare minnow, including the observation that TDCPP had widespread effects on neurotrophic factors without any cholinesterase inhibition. Moreover, it has been indicated that interactions of OPs with non-cholinesterase targets may contribute to the more delayed and persistent neurological effects observed following OP exposure, especially those associated with repeated exposure to levels that produce no overt signs of acute toxicity (Terry, 2012). For example, sub-threshold OP exposures (with no overt signs of cholinergic toxicity) can lead to protracted cognitive deficits, which may be related to persistent functional changes in brain neurotrophin (Terry et al., 2011). Because OPFRs and OPs may share similar neural actions, the widespread effects of TDCPP on neurotrophic factors in Chinese rare minnow brains may also lead to such delayed and persistent neurological effects due to the targeting of neurotrophic factors and their receptors (e.g., neurotrophins and their receptors), as well as their related intracellular signaling pathways (e.g., the MAPK and PI3K signaling pathways) (Slotkin et al., 2008; Terry et al., 2011; Pomeroy-Black and Ehrlich, 2012; Terry, 2012).

5. Conclusions

In summary, we cloned and sequenced fifteen previously unreported neurotrophic factor genes in Chinese rare minnow brains (five from the neurotrophin family and ten from the FGF family). CPF had significant effects on cholinesterase activities and neurotransmitter levels in both acute tests and low-dose tests, whereas none of the OPFRs showed any significant effects, with the exception of TPP, indicating that unlike OPs (e.g., CPF), OPFRs are not typical neurotoxins. However, TDCPP did have widespread effects

on neurotrophic factor genes, indicating that similar to OPs, TDCPP or other OPFRs may elicit neurological effects by targeting neurotrophic factors and their receptors in Chinese rare minnow.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2015.10.045>.

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