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Perfluorooctane sulfonate induced neurotoxicity responses associated with neural genes expression, neurotransmitter levels and acetylcholinesterase activity in planarians *Dugesia japonica*

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HIGHLIGHTS

- The neurotoxicity of PFOS on biochemical and molecular levels was assessed.
- PFOS exposure altered nerve fiber density and brain branches.
- Expression of neurodevelopmental related genes were altered.
- Neurotransmitters levels and the acitivity of AChE were altered.

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ABSTRACT

As a persistent and widespread toxic organic pollutant in the environment, perfluorooctane sulfonate (PFOS) has the potential to cause great harm to wildlife. In our study, the effects of PFOS on neurodevelopment gene expression, neurotransmitter content, neuronal morphology, acetylcholinesterase (AChE) activity were examined, and the potential neurotoxicity mechanisms of PFOS were also investigated in planarians, *Dugesia japonica*. Using quantitative real-time PCR analysis, five neurodevelopmental related genes were measured, among which, *DjotxA*, *DjotxB*, *DjFoxD*, and *DjFoxG* were found to be down-regulated, while *Djnlg* was found to be up-regulated, following exposure to PFOS for 10 days compared with control groups. In addition, the neurotransmitters including dopamine, serotonin, and γ-aminobutyricacid as well as the acitivity of AChE were altered by PFOS exposure. Furthermore, PFOS exposure altered brain morphology as well as smaller cephalic ganglia which displayed reduced nerve fiber density decreased brain branches compared to controls. Our results demonstrate that neurotransmission was disturbed after exposure to PFOS and that exposure to this pollutant can cause neurotoxic defects. Results from this study provide valuable information regarding the neuro- and ecological toxicity of PFOS in aquatic animals and aquatic environments.

1. Introduction

Perfluoroalkyl substances (PFASs) are a group of manmade synthetic fluorinated compounds where hydrogen atoms in carbon hydrogen bonds are replaced with fluorine atoms (Li et al., 2017). Due to their combined water and oil repellency, PFASs are widely found in industrial and commercial products, such as surfactants, fire retardant, nonstick coatings, textile and food packaging, paints and varnishes (Wang et al., 2015; Liu et al., 2016). Perfluorooctane sulfonate (PFOS) is one of the most prevalently used PFASs, and is the end degradation product of many PFASs (So et al., 2006). Due to the carbon fluoride bonds (Butenhoff et al., 2004), PFOS is highly stable and has been widely detected in the environment, wildlife and humans (Naile et al., 2010; Zhao et al., 2012). As a result of the persistence and bioaccumulation, PFOS was listed as a common persistent organic pollutant (POP) in the Stockholm Convention in 2009 (Geneva: Stockholm Convention Secretariat, 2009).

PFOS has reportedly been detected in serum, liver, umbilical cord blood, milk and neural tissue of humans (Chang et al., 2009; Miralles-Marco and Harrad, 2015), with the elimination half-life of PFOS in serum found to be approximately 4.8 years (Olsen and Zobel, 2007). PFOS-induced toxicity includes oxidative stress,

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apoptosis, estrogenic-related reproductive toxicity, metabolic dysfunction, hepatotoxicity, lung injury, thyroid and gonad histological alterations, neurotoxicity, developmental toxicity, and immune abnormalities (Chen et al., 2012, 2016; Louis et al., 2015). Although PFOS has been included in the POP forbidden list, the production and consumption of PFOS and related chemicals in China has increased due to the heavy demands in recent years, and the total emission of PFOS equivalents in China was 70 t in 2010 (Xie et al., 2013; Fu et al., 2015).

Planarians are representative of the phylum Platyhelminthes and possess a central nervous system (CNS) with bilateral symmetry and three germ layers. Due to an abundant population of adult somatic pluripotent stem cells known as neoblasts, planarians has remarkable tissue regeneration capabilities (Scimone et al., 2014). The CNS of freshwater planarians exhibits cephalization, consisting of a bi-lobed cephalic ganglion and ventral nerve cords, and despite its simplicity, the overall structure of neurons, and the fact that dendritic spines and neurotransmitters were present, pointed to many similarities with vertebrates, including humans (Cebrià, 2007). Furthermore, as 95% of *D. japonica* CNS related genes are homologous with humans (Mineta et al., 2003), and the fact that planarians were widely distributed, easily bred, and impressively sensitive to low concentrations of environmental toxins in the water (Ofoegbu et al., 2016; Yuan et al., 2016a) make planarian a promising model to study the molecular genetics of regeneration and neurodevelopmental toxicity, as well as assess direct and indirect effects of contaminants and water quality (Stevens et al., 2015; Rodrigues et al., 2016).

The two homeobox genes of *DjotxA* and *B*, in the planarians are closely related to the Drosophila and vertebrate orthodenticle homeobox genes, which are known to be involved in rostral head development (including olfactory, auditory, and visual systems) and photoreceptor development in both fruit flies and vertebrates (Acampora et al., 2000; Arendt et al., 2008; Larsen et al., 2009). *Djnlg* (a noggin-like gene) expression was observed in the proximal region of the brain branch structures in planarians, and was the first noggin homologue identified in invertebrates by the planarian Expressed Sequence Tag (EST) project (Ogawa et al., 2002). Noggin genes are well-known antagonists of bone morphogenetic protein signaling pathway and play a major role in axis formation and neural differentiation (Molina et al., 2009). Planarians possess mammalian-like neurotransmitter systems, including major excitatory and inhibitory neurotransmitters (dopamine, DA; serotonin, 5-HT; γ-aminobutyricacid, GABA), second messengers, respective receptors and genes encoding enzymes that synthesize these neurotransmitters (Nishimura et al., 2011; Wu et al., 2015). Acetylcholinesterase (AChE) is responsible for the recycling of the neurotransmitter acetylcholine, it is also involved in neural transmission, neuronal development and the regeneration of nerves (Shahidi et al., 2008; Khan et al., 2012).

The aim of the present studies were to determine the PFOS-induced neurotoxicity using the freshwater planarians *D. japonica* as an animal assay. Five neural genes (*DjotxA, DjotxB, DjFoxD*, *DjFoxG* and *Djnlg*) were selected, and their expression levels were monitored by quantitative real-time PCR. In parallel, the expression levels of three neurotransmitters (DA, 5-HT and GABA) were analyzed by ELISA. The effects of PFOS on neural morphology were also analyzed by immunofluorescence. Finally, changes in AChE induced by PFOS were also examined in *D. japonica*.

2. Materials and methods

2.1. Animals and chemicals

Planarians were obtained from stream in a fountain of Boshan, China, and cultivated in containers with Lushan fountain in an incubator for more than 2 weeks. Organic pig liver were used for breeding planarians once a week and cleaned daily. Planarians were subsequently starved for 5–7 days to create a balanced metabolic state before experiments (Nishimura et al., 2011; Hagstrom et al., 2017). Sigma-Aldrich (St Louis, MO, USA) provided the experimental PFOS (purity>99%). The PFOS solution was prepared and then diluted into different concentration by the previous method (Yuan et al., 2014). Other chemicals used in this study were all analytical grade. Ten intact planarians (approximately 1 cm in length) were randomly selected and exposed to PFOS solutions with 0, 0.5, 1, 5 and 10 mg/L at each time point with three replicates for each treatment concentration, respectively. All solutions were changed daily.

2.2. Quantitative real-time PCR(qPCR) analysis

Differential expression of neural genes (*DjotxA*, *DjotxB*, *DjFoxD*, *DjFoxG*, *Djnlg*) in planarians were analyzed using *q*PCR after exposure to PFOS (0, 0.5, 1, 5 and 10 mg/L) for 1 d, 4 d and 10 d. Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA) and then was reverse transcribed into cDNA. The mRNA levels of each gene were compared with controls using the *D. japonica β-actin* (*Djβ-actin*) gene as a reference gene in order to normalize RNA input (Yuan et al., 2010). The primer sequences are provided in Table 1. Reactions were carried out on the 7500 Real-Time PCR System (Applied Biosystems, CA, USA) using the Faststart Universal SYBR Green Master Mix (Roche, Mannheim, Germany) following the manufacturer protocol and PCRs were performed in triplicate by previous method (Yuan et al., 2010).

2.3. Measurement of DA, 5-HT and GABA levels

To study the effects of PFOS on the levels of DA, 5-HT and GABA of treated *D. japonica*, planarians were sacraficed and homogenized in PBS buffer (pH 7.4) after PFOS exposure for 1d, 4d and 10d (0, 0.5, 1, 5 and 10 mg/L), respectively. Homogenates were centrifuged at 12,000g for 20 min at 4 °C and then supernatants were used to analyze neurotransmitter levels using a standard enzyme reader at 450 nm. The levels of DA, 5-HT and GABA were measured using Elisa Assay Kits for DA, 5-HT and GABA (Nanjing Jiancheng Bioengineering institute, Nanjing, China), respectively according to manufacturer's instructions.

2.4. Immunofluorescence

After treatments with PFOS solution of different concentrations (0, 0.5, 1, 5 and 10 mg/L) for 7 d, planarian was treated according to the method previously reported (Yuan et al., 2016b).

Table 1
Primer sequences of genes used for qPCR analysis in this study.

Gene name	Primer sequences (5′-3′)
DjotxA	FP: GATCAACTTACCTGAATC
	RP: TCGAGAAGTAGATCCGAG
DjotxB	FP: CCAGAATCACGTAGTCAG
	RP: GGTTGCTCGACTATCTAG
DjFoxD	FP: CAACAGAATCTGGTTACG
	RP: CTCGTCTTGCATCATGTG
DjFoxG	FP: TTGGATGGTAGATCCTGC
	RP: ATGGATCTGATGGATGTG
Djnlg	FP: CAGAGAATCGTGTTCATG
	RP: ATGTCGTCGAAGTCTTCG
Djβ-actin	FP: GGATGATGAGATGCGATGTTG
	RP: ATGCCAGGTCCAGATTCGTCA