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Parkinson's disease-like motor and non-motor symptoms in rotenone-treated zebrafish

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ABSTRACT

The pesticide rotenone is widely used to produce Parkinson's disease (PD)-like symptoms in rodents, but few studies have examined whether rotenone-treated zebrafish can serve as an animal model of PD. Here, we report that 4 weeks of rotenone treatment induced motor and non-motor PD-like symptoms in adult zebrafish. Compared with control fish, rotenone-treated fish spent less time swimming at a fast speed, indicating a deficit in motor function. In the light-dark box test, rotenone-treated fish exhibited longer latencies to enter the dark compartment and spent more time in the light compartment, reflecting anxiety- and depression-like behavior. Furthermore, rotenone-treated fish showed less of an olfactory preference for amino acid, indicating olfactory dysfunction. These behavioral symptoms were associated with decreased levels of dopamine in the brains of rotenone-treated fish. Taken together, these results suggest that rotenone-treated zebrafish are a suitable model of PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting 1.7% of the aged population (Zhang et al., 2005). The main manifestations of PD can be classified into motor and non-motor symptoms. Although motor symptoms are often the most pronounced, non-motor symptoms such as sleep disorders, depression, and olfactory dysfunction are closely related to the daily quality of life of PD patients (Li et al., 2010) and are increasingly receiving attention from neurologists (Pfeiffer, 2016). However, most research on non-motor PD symptoms is limited to clinical studies due to the lack of an appropriate animal model.

Zebrafish are vertebrate animals that are widely used in the study of neurological disorders and cardiovascular disease (Kalueff et al., 2014). The popularity of zebrafish as a research model can be explained by several reasons, such as a low cost of breeding, short period of reproduction, and physiological homology to human beings. Moreover, their amenability to high-throughput screening makes zebrafish suitable for wide applications in drug development.

Rotenone is a pesticide commonly used in farming and fishing. In 2000, a group of researchers found that chronic exposure to rotenone is associated with a risk of developing PD (Betarbet et al., 2000). Rotenone administration reproduces several PD-like behavioral features in rats, such as hypokinesia and rigidity. Moreover, fibrillar cytoplasmic inclusions containing α-synuclein and ubiquitin are also observed after rotenone treatment. Rotenone inhibits the function of mitochondrial complex I and results in the loss of dopaminergic neurons, with its specific toxicity to dopaminergic neurons depending on dopamine (DA) metabolism and redistribution from vesicles to the cytosol (Watabe and Nakaki, 2007).

Although rotenone has been found to induce PD-like symptoms in several species, such as rats, mice, and drosophila (Liu et al., 2015; Coulom and Birman 2004; Betarbet et al., 2000), only one study has examined the effects of this toxin in zebrafish (Bretaud et al., 2004). In this previous study, no obvious motor dysfunction was observed in adult zebrafish after rotenone treatment, and the

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researchers partially explained this negative result by stating that rotenone is unable to access the brain due to the blood-brain barrier. However, it is well accepted that this toxin is lipid-soluble and able to penetrate into the brain parenchyma and cell membrane (Betarbet et al., 2000). Therefore, we carefully examined the effects of rotenone in zebrafish while also assessing non-motor symptoms that may precede motor symptoms. We found that rotenone-induced mild deficits in motor ability, anxiety- and depression-like behavior, and olfactory dysfunction in zebrafish, which may be associated with decreased DA levels.

2. Materials and methods

2.1. Animals and treatment

Wild-type male zebrafish (*Danio rerio*, 5–7 months old) were bred in a recirculating water system at 28.5 °C and kept on a 14-h light/10-h dark cycle (Wang et al., 2016). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Soochow University. Zebrafish were randomly divided into two groups; one group was treated with vehicle and the other was treated with 2 μg/L rotenone (Sigma, USA; dissolved in distilled water) for 4 weeks (Bretaud et al., 2004). The water in the tanks was changed daily. Zebrafish were placed in the test room 1 h before behavioral testing to adapt to the environment.

2.2. Locomotor activity

Locomotor activity was monitored in a 2-L tank (18 × 9 × 7 cm). A tower system was used to capture a video recording of each fish for 5 min. Swimming activity was categorized into three speeds: fast (>20 mm/s), slow (>2 mm/s and <20 mm/s), and freezing (<2 mm/s). Movement distance and duration at each speed were calculated using software (Viewpoint Life Sciences, France).

2.3. Light/dark box test

The light/dark box test was performed as previously described (Gebauer et al., 2011). The tank (18 × 9 × 7 cm) was separated into equally sized dark and light compartments with a plastic barrier. The barrier was lifted up 1 cm to allow fish to swim between compartments, and the water level was kept at a depth of 3 cm. Fish were individually placed into the light compartment and allowed to swim freely for 6 min, during which a video recording was obtained using the tower system. Latency to enter the dark compartment, time spent in the light compartment, and the number of crossings between compartments were calculated using software provided by the manufacturer.

2.4. Olfactory preference test

Olfactory preference for a mixture of amino acids was assessed according to a previously described method (Koide et al., 2009). Briefly, fish were individually placed in a tank (18 × 9 × 7 cm) and video recorded for 16 min using the tower system. After a 6-min pre-stimulation, 0.6 ml amino acid mixture (Cys and Met, 0.1 mM) was delivered into a corner of the tank at a rate of 1.5 ml/min. Time spent in the amino acid (T_A) and non-amino acid side (T_C) was analyzed, and swim paths were tracked using software. Preference index (PI) for each minute was calculated as: $PI = (T_A - T_C)/(T_A + T_C)$.

2.5. Neurotransmitter measurement

To determine brain levels of neurotransmitters, fish were anesthetized on ice, and brains were quickly removed. After homogenization with 0.4 M perchloric acid, homogenates were centrifuged at 13,000*g* for 20 min, and supernatants were collected. Supernatants were then filtered with a 0.22-μm syringe filter and subjected to measurement of DA, dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), 5-hydroxy indole acetic acid (5-HIAA), and norepinephrine (NE) levels using high performance liquid chromatography (HPLC) coupled with an electrochemical detector (Antec, Netherlands). The final results were calculated using standard curves and expressed as ng/mg of tissue.

2.6. Western blotting

Tissue homogenates were prepared using lysis buffer with protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were sonicated and centrifuged at 13,200*g* for 15 min at 4 °C. Protein concentration was measured by BCA assay. Next, protein lysates (30 μg) were separated by 12% Tris-glycine gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat dry milk in 0.1% Tris buffered saline/Tween 20 and incubated with primary antibodies against tyrosine hydroxylase (TH, 1:5000; Sigma, St. Louis, MO, USA) at 4 °C overnight. Membranes were then incubated with HRP-conjugated secondary antibodies (1:1000; Cell Signaling Technology, Danvers, MA, USA) for 1 h. Finally, protein bands were visualized using ECL chemiluminescence (Thermo, West Chester, PA, USA) and analyzed with ImageJ software (National Institutes of Health, USA).

2.7. Real-time polymerase chain reaction (PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, Camarillo, CA, USA) following the manufacturer's manual, and reverse transcription was performed using a reverse transcription kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR reactions were performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). qPCR primers were as follows: *PINK1* (forward: 5′-GGCAATGAAGATGATGTGGAAC-3′, reverse: 5′-TTGTGGGCATGAAGGAACTAAC-3′), *PARKIN* (forward: 5′-GAGGAGTTTCACGAGGGTCC-3′, reverse: 5′-TGAGTGGTTTTGGTGATGGTC-3′), *DJ-1* (forward: 5′-CCTGCTGTGAAAGAGGTGTTG-3′, reverse: 5′-ACTGTGCTGCCATATGCAATAC-3′), *LRRK2* (forward: 5′-ACTCGGATTAAGTTCCACCAGA-3′, reverse: 5′-CAGTGAGGGTTGATGGTCTGTA-3′), *alpha-SNCA* (forward: 5′-ATGCAC TGAAGAAGGGATTCTC-3′, reverse: 5′-AGATTTGCCTGGTCAGTTGTTT-3′), and *ACTIN* (forward: 5′-GGCATCACACCTTCTACAATGA-3′, reverse: 5′-TACGACCAGAAGCGTACAGAGA-3′). The final results were normalized to *ACTIN*.

2.8. Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Graphpad Prism 5.0. Group differences were tested using Student's *t*-tests, and *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Rotenone impaired motor ability and down-regulated expression of TH

Bradykinesia, defined as a decrement in movement speed or amplitude, is one of the most prominent clinical manifestations of PD (Postuma et al., 2015). Our locomotor activity test showed no obvious differences between rotenone-treated and control fish in the duration of freezing or distance traveled during freezing (Fig. 1A and B). Likewise, rotenone-treated and control fish showed