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Activation of PPAR Can Protect Zebrafish Embryos from PFOA-Induced Abnormal Neural Development and Impaired Motor Function

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Simple Summary

Perfluorooctanoic acid is a persistent environmental pollutant that can enter aquatic ecosystems, but its effects on the developing nervous system remain incompletely understood. We exposed zebrafish to PFOA during early development and examined their movement, responses to touch, brain tissue, and the expression of genes involved in nerve function and inflammation. Exposed fish swam shorter distances and responded more slowly to touch. These changes were accompanied by signs of increased brain inflammation and cell death, together with reduced activity of an enzyme involved in nerve signaling. We also tested rosiglitazone and bezafibrate, two drugs that act on cellular proteins involved in metabolism and inflammation. Co-treatment was associated with improvements in touch responses and several measures of brain injury. These findings help explain how this pollutant may affect aquatic animals during early development and suggest possible directions for further research on protection against its harmful effects. Further studies are needed to clarify the underlying biological mechanisms and determine whether these findings apply to other species or exposure conditions.

Abstract

Perfluorooctanoic acid (PFOA) is a persistent environmental pollutant that can be detected in air, dust, rivers, soil, and human plasma. Exposure to PFOA has been associated with neurotoxicity, developmental toxicity, and changes in locomotor activity in aquatic organisms. However, the mechanisms underlying PFOA-induced neurotoxicity and motor function impairment remain poorly understood. In this study, newly fertilized zebrafish embryos were exposed to PFOA, resulting in motor dysfunction characterized by reduced movement distance and lower sensitivity to touch stimuli. Hematoxylin and eosin staining, immunofluorescence, and acridine orange staining revealed changes in brain tissue, increased cell death-associated signals, and signs of neuroinflammation. Quantitative PCR analysis showed altered expression of genes related to acetylcholinesterase, neuronal development, neurotransmitter transport, apoptosis, and inflammation. In addition, PFOA exposure significantly downregulated peroxisome proliferator-activated receptor gamma (PPARγ)-related genes in zebrafish embryos. Co-treatment with the PPARγ agonist rosiglitazone or the pan-PPAR agonist bezafibrate partially alleviated PFOA-induced motor impairment and several indicators

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of neural injury. These findings suggest that PPAR-related pathways may be involved in the response to PFOA exposure and provide a basis for further investigation of PFOA-induced neurotoxicity and potential protective mechanisms.

Keywords: PFOA; neurotoxicity; neuroinflammation; apoptosis; acetylcholinesterase; PPAR agonists

1. Introduction

Per- and polyfluoroalkyl substances (PFAS), including perfluorooctanoic acid (PFOA), have been widely used in industrial and household applications since the 1940s [1,2]. The strong carbon–fluorine bonds in PFOA contribute to its environmental persistence [3], PFOA is listed under the Stockholm Convention because of concerns about its persistence and potential adverse effects [4]. PFOA has been detected in groundwater at concentrations of up to 6.57 μg/mL [5]. Studies have also reported PFOA concentrations ranging from 1 to 10 ng/mL in human plasma [3,6]. Prolonged PFOA exposure may be associated with adverse health outcomes, including hypercholesterolemia, kidney cancer, and testicular cancer [7]. However, the effects of PFOA exposure on the human nervous system remain poorly understood. Therefore, studying the effects of PFOA on the zebrafish nervous system may provide insights into its potential effects on the human nervous system.

Environmental toxicology studies have revealed that exposure to PFOA can cause neurotoxicity, developmental toxicity, immunotoxicity, disturbances in lipid metabolism, and changes in motor activity, with a 50% lethal concentration of 300 μg/mL in zebrafish [1,8–10]. For example, PFOA exposure affects zebrafish embryonic heart development, increases heart rate, reduces motor activity, and downregulates the neurotoxicity marker gene *ache* [11]. Moreover, PFOA exposure has been found to repress hepatic secretion of very low-density lipoprotein (VLDL) and cholesterol, while also disturbing peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element-binding protein (SREBP) homeostasis, resulting in lipid metabolism disorders in zebrafish [9,12]. Furthermore, PFOA exposure has been shown to hinder neural cell proliferation and differentiation in zebrafish embryos, consequently impacting the development of dopaminergic neurons and potentially contributing to subsequent neurobehavioral abnormalities [13].

Peroxisome proliferator-activated receptor (PPAR), a fatty acid-activated nuclear receptor, includes three subtypes, namely PPARα, PPARβ (also known as PPARδ), and PPARγ [14,15], each of which has a distinct expression pattern in tissues and cells such as the kidney, liver, heart, skeletal muscle, adipocytes, gastrointestinal tract, macrophages, and brain [16]. These subtypes are important regulators of fatty acid metabolism, lipid synthesis, inflammation, and oxidative stress responses, and are involved in cancer [15]. The detection of PPARγ transcripts in the mouse brain [17] has prompted research into the potential role of PPAR in neurodevelopment, neuroprotection and suppression of neuroinflammation [18–20]. PPARα modulates glutamate balance and influences dopaminergic transmission via glutamatergic and cholinergic neurons in the brain [21]. In the brain, the PPARγ gene is expressed primarily in neurons and astrocytes; PPARγ agonists have potent anti-inflammatory, neuroprotective and anti-addictive effects and show promise in the treatment of Alzheimer's disease and other neurodegenerative conditions [22].

Despite extensive research on PFOA's impact on lipid metabolism in aquatic organisms [9], a significant gap remains in our understanding of how PFOA induces neurobehavioral defects. This study aimed to address this gap by investigating PFOA-induced neurotoxicity and motor dysfunction in zebrafish embryos and exploring the

underlying mechanisms. The findings may enhance our understanding of the environmental impact of PFOA and shed light on the potential role of PPAR in neuroprotection and inflammation regulation.

2. Materials and Methods

2.1. Zebrafish Rearing and Embryo Collection

Wild-type AB strain zebrafish were obtained from the China Zebrafish Resource Center, and the *sox10:eGFP* transgenic zebrafish were provided by Huazhong Agricultural University. Adult zebrafish of the AB strain and *sox10:eGFP* transgenic strain were housed in a recirculating filtration system, with a water pH of 7.5 ± 0.2 and a temperature of. 28.5 °C. Zebrafish were fed Artemia three times a day (morning, noon, and evening). On the night before embryo collection, two male and two female zebrafish were placed in the same breeding tank, separated by a divider, and kept overnight in the dark. The next morning, the divider was removed for breeding. Fertilized embryos were collected within two hours after natural spawning and incubated in a constant-temperature incubator at 28 ± 0.5 °C.

2.2. Chemicals, Reagents, and Antibodies

Perfluorooctanoic acid (PFOA, 171468, 5 g) was purchased from Sigma-Aldrich (Shanghai, China). Anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody and FITC-conjugated goat anti-rabbit IgG were obtained from ABclonal (Wuhan, China). Rosiglitazone and bezafibrate were provided by MedChemExpress (MCE, Monmouth Junction, NJ, USA).

2.3. Embryo Treatment and Observation

In each experimental replicate, 50 normally developing zebrafish embryos (≤2 hpf) were placed in each 60-mm Petri dish for each exposure group and the control group. PFOA (Sigma-Aldrich Shanghai, China)solutions at nominal concentrations of 0.01, 0.1, 1, and 10 μg/mL were prepared using water collected from the recirculating filtration system described in Section 2.1. The control embryos were maintained in the same system water without added PFOA (0 μg/mL). All groups were incubated at 28 ± 0.5 °C until 96 hpf. Zebrafish embryos exposed to different concentrations of PFOA were used for motor behavior assessment and neurotoxicity evaluation. For subsequent rescue experiments, the exposure group received the addition of rosiglitazone (20 μM) and bezafibrate (2 μM) to the solution before treatment. During the exposure, dead embryos were removed daily, and the solution was not changed. The cumulative hatching rate at 24 hpf, 48 hpf, 72 hpf and 96 hpf were calculated for each Petri dish as follows: hatching rate (%) = (number of embryos hatched by 48 hpf / initial number of embryos) × 100. All embryos used in the experiments were from the same batch and allocated to the exposure and control groups. Each experiment was repeated three times, and zebrafish phenotypes were captured using an optical microscope (SMZ1270, Nikon Corporation, Tokyo, Japan) at specific developmental stages.

2.4. Motor Activity Test and Touch Response

At 24 hpf, the frequency of spontaneous body contractions of unhatched zebrafish embryos within the chorion in the control group and various exposure concentrations was recorded using an optical microscope (SMZ1270, Nikon Corporation, Japan) for 1 min, with 10 zebrafish embryos randomly selected for each group. EthoVision XT 11 behavior tracking software (Noldus Information Technology Inc., Leesburg, VA, USA) was used to track and analyze the movement behavior of zebrafish larvae. Six randomly selected zebrafish embryos from each treatment group (96 hpf) were placed in a 24-well plate after being rinsed three times with water from the recirculating system to ensure normal

swimming. After a ten-minute acclimation, the zebrafish embryos were subjected to spontaneous locomotor activity was recorded for 15 min. The touch response experiment was recorded using high-speed video and analyzed using QuickTime Player software (version 10.4, Apple Inc., Cupertino, CA, USA) to obtain frames at different time points. The above experiments were conducted in three replicates, and three batches of zebrafish were used for each repetition.

2.5. Cryosectioning, H&E Staining, and Immunofluorescence

At 96 hpf, larvae from the control group, 0.1 μg/mL PFOA-exposed group, and two rescue groups with the addition of rosiglitazone and bezafibrate (n = 20 per group) were collected and fixed overnight with 4% paraformaldehyde (PFA) at 4 °C. Subsequently, the larvae were immersed in 30% sucrose in PBS for cryoprotection at room temperature for approximately 4 h. The larvae were then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and frozen sections of specific developmental tissues were obtained at a thickness of 8 μm using a cryostat (Leica Biosystems, Deer Park, IL, USA). After air-drying for 30 min at 37 °C, the sections were used for H&E staining and immunofluorescence. Hematoxylin and Eosin staining (H&E staining) was carried out following previously reported methods [23], and high-resolution images of the phenotypes were captured using a microscope (Nikon, Japan). For immunofluorescence, primary antibodies against Iba1 (1:200 dilution) and phospho-histone H3 (ph3, 1:500 dilution) were used, along with a secondary antibody conjugated with Alexa Fluor 488 (1:1000 dilution). The cell nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI). High-resolution images of immunofluorescence were obtained using a fluorescence microscope (Nikon, Japan). Each experiment was repeated three times.

2.6. Total RNA Extraction and Qpcr

Total RNA was extracted pooled embryos from each group (n = 45) using Trizol reagent (Life Technologies, Carlsbad, CA, USA). RNA quality was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Madison, WI, USA), and RNA integrity was evaluated by agarose gel electrophoresis. Subsequently, cDNA was synthesized using the PrimeScript™ RT reagent kit (with gDNA eraser) (Takara Bio Inc., Shiga, Japan). Quantitative PCR was performed using TB Green™ Premix Ex Taq™ II (Takara, Japan) on the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression was calculated using the $2^{\Delta\Delta Ct}$ method, with β-actin as the internal control. The specificity of all primers was tested before use, and the primer sequences, gene IDs, and amplicon lengths are listed in Table S1. Each experiment was repeated three times.

2.7. Quantitative Analysis of PFOA Using HPLC/MS/MS

Quantitative analysis of PFOA in embryos and solutions was performed using an Agilent 1260 High Performance Liquid Chromatograph coupled with an Agilent 6420A Triple Quadrupole Mass Spectrometer (HPLC/MS/MS, 1260-6420A, Agilent Technologies, Santa Clara, CA, USA) at Nanchang Kangxuhe Biotechnology Co., Ltd. Nanchang, China. The perfluorooctanoic acid standard (PFOA, 335-67-1, Dr. Ehrenstorfer GmbH, Augsburg-Inningen, Germany) was prepared as a 1000 ng/mL stock solution in a methanol-water mixture (10:8, *v*:*v*) and stored temporarily at 4 °C temporarily. A series of standard solutions with concentrations of 5, 10, 50, 100 and 500 were freshly prepared before use by diluting the stock solution with the methanol-water mixture (10:8, *v*:*v*) freshly before use. For sample preparation, 180 μL of pre-cooled methanol-water mixture (10:8, *v*:*v*) was added to the embryo samples, followed by 15 min of low-temperature ultrasonic disruption using an ultrasonic cleaner (KQ3200DA, Kunshan, China). Then, 300

μL of methanol and 280 μL of experimental water (Watsons, Hong Kong, China) were added, and the mixture was vortexed for 1 min to fully dissolve the target compound. Subsequently, 400 μL of dichloromethane was added to remove lipids and other lipophilic impurities. The mixture was subjected to 10-min ultrasonic extraction. After centrifugation at 4 °C and 10,000 rpm for 10 min, the supernatant was collected, filtered through a 0.22 μm membrane, and stored at −20 °C for analysis.

2.8. Micro-Injection

The coding sequences (CDS) of *ache* and *slc25a38a* were cloned and ligated into the pXT7 plasmid vector. Capped mRNA was synthesized in vitro from the pXT7-*ache* and pXT7-*slc25a38a* plasmids using the mMESSAGE mMACHINE™ T7 kit (Thermo Fisher, USA), under RNase-free conditions. The *ache* and *slc25a38a* mRNA was subsequently injected into embryos before the two-cell stage.

2.9. AO Staining

At 96 hpf, zebrafish larvae (n = 30) were incubated in 10 mL of acridine orange (AO) staining solution for 10 min in the dark. The staining solution was prepared by diluting the AO stock solution (1000×; C0233S, Beyotime, Shanghai, China) 1:1000 in the supplied assay buffer to obtain a 1× working solution. The larvae were then imaged using the green fluorescence channel of a fluorescence microscope (Nikon, Japan).

2.10. Ache Activity Assay

AChE activity was measured using an acetylcholinesterase (AChE) activity assay kit (ML077325, Mlbio, Shanghai, China) for each group of zebrafish embryos (n = 200). Zebrafish embryos at 96 h post-fertilization (hpf) were first weighed (mg). The samples were mixed with Reagent 1 at a 1:10 (weight/volume) ratio, homogenized at 4 °C, and then centrifuged at 8000× *g* for 10 min at 4 °C. The supernatant was collected for analysis. Before measurement, the microplate reader and Reagent 2 were pre-warmed for 30 min, and the reader was set to a wavelength of 412 nm with distilled water used for zeroing. A 96-well plate was then prepared, and 20 μL of each sample supernatant, 160 μL of Reagent 2, 10 μL of Reagent 3, and 10 μL of Reagent 4 were added sequentially and mixed thoroughly. Absorbance changes were measured within 3 min at 412 nm, with the absorbance at 10 s recorded as A1 and the absorbance at 190 s recorded as A2. The change in enzyme activity was calculated as $\Delta A = A2 - A1$. AChE activity (U/mg protein) = $245 * \Delta A$ / Weight

2.11. Ache Content Assay

The AChE content in pooled zebrafish embryos from each group (n = 200) was measured using the Fish Acetylcholinesterase (AChE) ELISA Kit (H529-1-1, NJJCBIO, Nanjing, China). Zebrafish embryos at 96 h post-fertilization (hpf) were first weighed, and the samples were homogenized in PBS at a ratio of 1 g:9 mL. The homogenate was centrifuged at 3000 rpm for 20 min at 4 °C, and the supernatant was collected for analysis. Subsequently, the reagents were added and incubated according to the manufacturer's instructions. Finally, the AChE content in the samples was calculated using a standard curve generated from the optical density (OD) values of the standards.

2.12. Statistical Analysis

All data are presented as mean ± SEM. The assumptions for one-way ANOVA were assessed. Normality of distribution was evaluated using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. All datasets met both assumptions ($p > 0.05$); therefore, one-way ANOVA followed by Dunnett's multiple comparison test was used. GraphPad Prism version 9.0 was used for data visualization.

Statistical significance between groups was indicated by *p*-values, with $p < 0.01$ and $p < 0.05$ denoted as ** and *, respectively. The specific *p*-values, F-value, and degrees of freedom (DFn and DFd) are presented in Table S2.

3. Results

3.1. PFOA Induces Motor Ability Impairment in Zebrafish

In this study, we exposed zebrafish embryos to different concentrations of PFOA (0.01, 0.1, 1, and 10 μg/mL) and recorded and analyzed the hatching rate of embryos at 24 hpf, 48 hpf, 72 hpf and 96 hpf (Figure 1C). Moreover, we monitored the mortality rate of zebrafish in the control group and exposure groups every 24 h (Figure 1B). Phenotypic images were captured using a camera (Figure 1A). No significant abnormal phenotype was observed in both the control group and exposure groups. Data analysis also revealed no significant difference in mortality rate and hatching rate (Figure 1B,C).

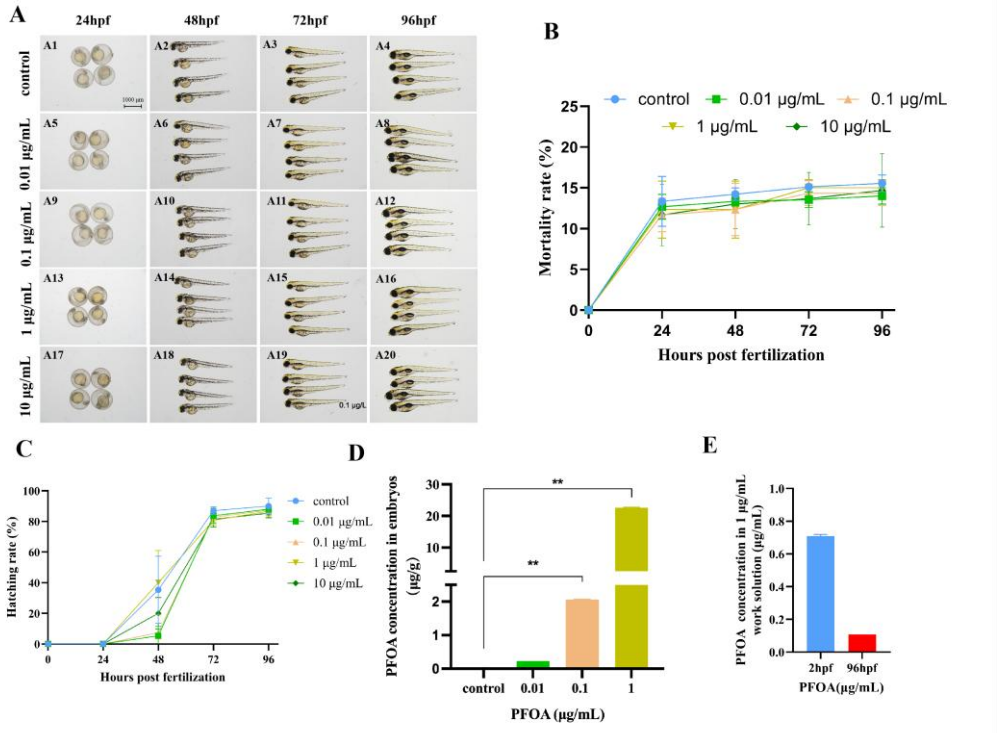

Figure 1. Effects of PFOA Exposure on Zebrafish Development. (**A**) Lateral views of zebrafish embryos at 24, 48, 72, and 96 h post-fertilization (hpf) exposed to different concentrations of PFOA. (**B**) Mortality rate of zebrafish embryos exposed to different concentrations of PFOA from 0 hpf to 96 hpf, calculated as the percentage of embryos that had hatched by 48 hpf relative to the initial number of embryos. (**C**) Hatching rate of zebrafish embryos exposed to different concentrations of PFOA at 24 hpf, 48 hpf, 72 hpf and 96 hpf. (**D**) PFOA mass fraction in zebrafish embryos exposed to different concentrations of PFOA at 96 hpf measured by wet weight. (**E**) PFOA mass fraction in the exposure solution (1 μg/mL) at 0 and 96 hpf. Statistical significance: **, $p < 0.01$.

At 96 hpf, the PFOA concentration was measured in the control group and exposure groups at the concentrations of 0.01, 0.1, and 1 μg/mL, which was 0, 0.2356, 2.0644, and 22.6129 μg/g wet weight, respectively (Figure 1D and Table S3). The PFOA concentration in the 1 μg/mL exposure group was also measured at 2 and 96 hpf, which was 0.709 and 0.108 μg/mL, respectively (Figure 1E and Table S4), indicating that the developing larvae absorbed PFOA during the developmental process.

Subsequently, we randomly selected zebrafish from the control, 0.01, 0.1, and 1 μg/mL exposure groups for motor behavior tests. At 24 hpf, we recorded and analyzed the spontaneous movement frequency of zebrafish within one minute (Figure 2A). As a result, there was no significant difference in spontaneous movement frequency among groups at 24 hpf. However, in the touch response test on zebrafish embryos at 96 hpf (Figures 2B and S1), the control group and the 0.01 μg/mL exposure group exhibited faster responses (Figures 2B and S1A,B), while the 0.1 and 1 μg/mL exposure groups showed slower responses (Figures 2B and S1C,D), and some individuals even remained immobile after several touches.

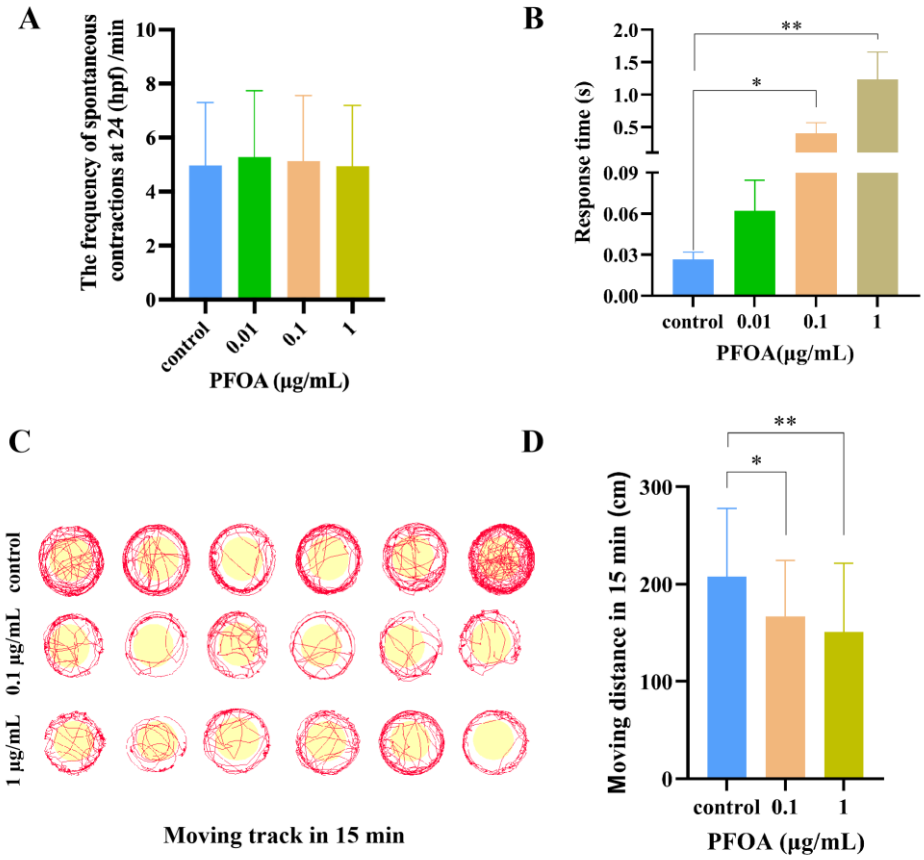

Figure 2. Effects of PFOA exposure on zebrafish embryonic motor behavior. (**A**) Spontaneous movement frequency of zebrafish embryos at 24 hpf at different exposure concentrations. (**B**) Response time of zebrafish at 96 hpf. Movement trajectories (**C**) and movement distance (**D**) of zebrafish at 96 hpf at different exposure concentrations. **, $p < 0.01$; *, $p < 0.05$.

Based on the initial behavioral observations, the 0.1 and 1 μg/mL PFOA concentrations were selected for movement tracking and subsequent assessments of brain tissue, apoptosis, and neuroinflammation. Furthermore, we tracked the movement trajectories of zebrafish from the control, 0.1, and 1 μg/mL exposure groups at 96 hpf (Figure 2C). The results showed that compared with the control group, both exposure groups displayed shorter movement distances (Figure 2D).

3.2. PFOA Induces Apoptosis in Zebrafish Brain Cells and Triggers Neuroinflammation (NI)

To investigate the effects of PFOA exposure on zebrafish neurodevelopment, we exposed *sox10:eGFP* transgenic zebrafish to PFOA at concentrations of 0.1 and 1 μg/mL (Figure 3A). Although no significant change was observed at 48 hpf (Figure 3(A1–A3)), zebrafish from the 1 μg/mL exposure group exhibited an abnormal fluorescence

phenotype at 96 hpf (Figure 3(A6)). The H&E staining results (Figure 3B) revealed that at 96 hpf, the exposure group (represented by 0.1 μg/mL) exhibited a significant reduction in cell density compared to the control group (Figure 3(B1,B2), red arrows indicate a decreased number of cells). To investigate the underlying causes of brain cell sparsity, immunofluorescence was employed to assess the expression of the cell proliferation marker PH3 in both the control group and the exposure group (0.1 μg/mL) (Figures 3(C1–C6)). The results indicated no significant difference in PH3 expression between the two groups. However, acridine orange (AO) staining (Figure 3D) revealed a marked increase in apoptotic signals in the brain of the exposure group (0.1 μg/mL) (Figure 3(D2), red arrows). Furthermore, qPCR analysis (Figure 3E) showed a significant upregulation of apoptosis-related genes, including *bax*, *caspase3*, and *caspase9*, in the exposure group in the 0.1 μg/mL exposure group compared with the control group.

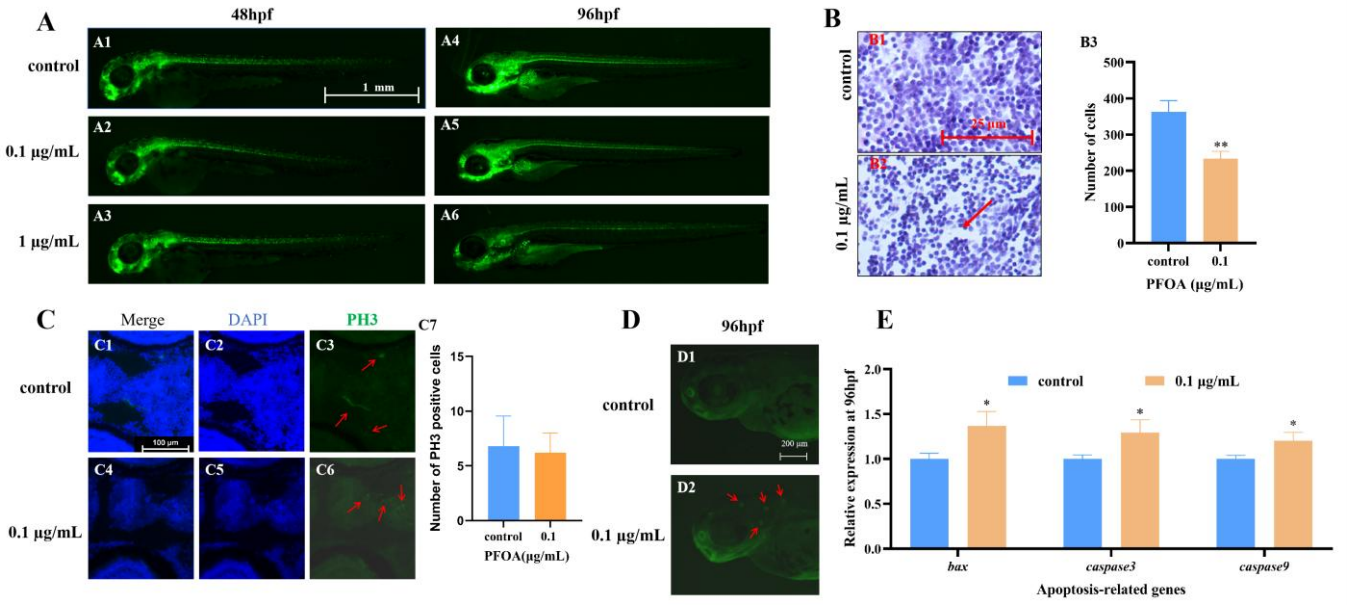

Figure 3. PFOA exposure induces apoptosis in brain cells. (**A**) Representative phenotypic images of *sox10*:eGFP zebrafish embryos in the control and exposure groups at 48 and 96 h post-fertilization. (**B**) Hematoxylin and eosin (H&E) staining analysis of brain cross-sections in zebrafish embryos from the control and PFOA exposure groups at 96 h post-fertilization, with red arrows indicating a reduction in cell number. (**C1–C6**) Representative immunofluorescence (IF) images of brain sections from PFOA-treated zebrafish embryos at 96 h post-fertilization, showing PH3 expression in green, with red arrows indicating increased PH3 protein expression. (**C7**) The number of PH3-positive cells. (**D**) Acridine orange (AO) staining results of zebrafish embryos in the control and PFOA exposure groups at 96 h post-fertilization, with red arrows indicating significant apoptotic signals. (**E**) Expression of genes associated with apoptosis (*bax*, *caspase3*, *caspase9*). **, $p < 0.01$; *, $p < 0.05$.

Additionally, immunofluorescence was used to detect the protein expression of the neuroinflammation marker Iba1 in the control and exposure groups (0.1 μg/mL PFOA exposure). The results showed that the exposure group had a significant increase in the protein expression of Iba1 relative to the control group (Figure 4A, red arrows indicate increased expression of Iba1 protein). qPCR results (Figure 4B) indicate a significant upregulation in the expression levels of neuroinflammation-related genes *il1β* and *mmp9* compared to the control group.

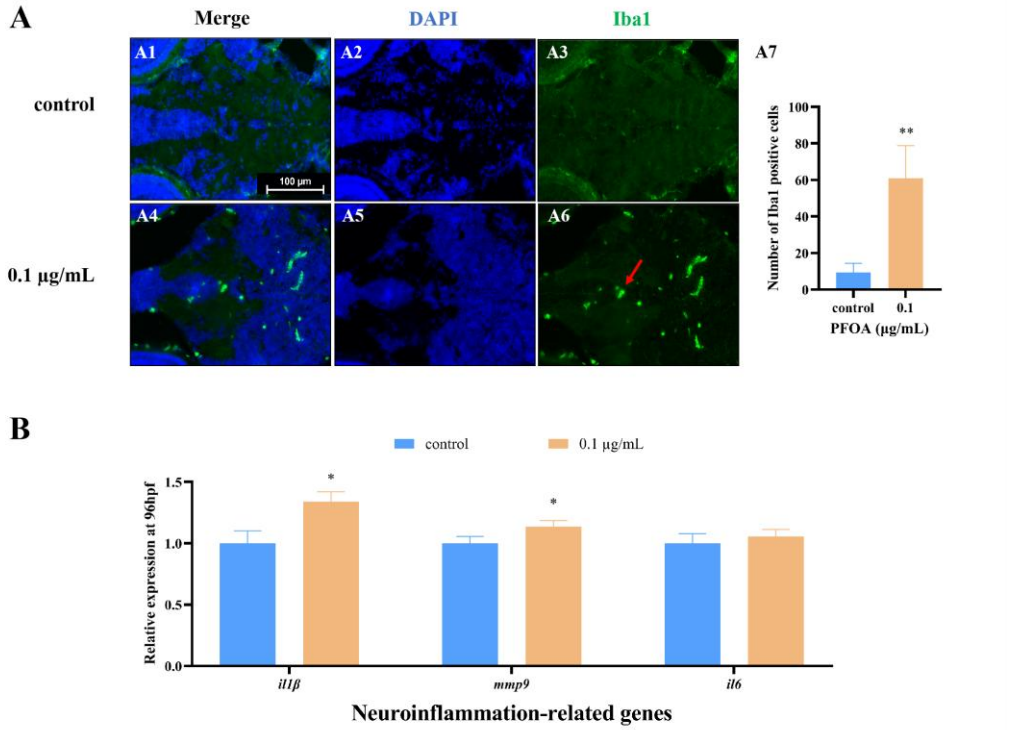

Figure 4. PFOA exposure induces neuroinflammation in zebrafish embryos. (**A1–A6**) Representative immunofluorescence (IF) images of brain sections from PFOA-treated zebrafish embryos at 96 h post-fertilization (hpf), with Iba1 expression shown in green; red arrows indicate increased Iba1 protein expression. (**A7**) The number of Iba1-positive cells. (**B**) Expression of genes associated with neuroinflammation (*il1b*, *mmp9*, *il6*). **, $p < 0.01$; *, $p < 0.05$.

3.3. Effects of PFOA on Zebrafish Neuronal Development, Neurotransmission, and PPAR

The AChE content (Figure 5A) and enzymatic activity (Figure 5B) were measured across all groups (control, 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL). The results showed a significant decrease in both AChE content and enzymatic activity in all exposure groups (0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) compared to the control group qPCR was performed to determine the expression of neurotoxicity marker (*ache*) (Figure 5C), neurotransmitter transporter genes (*slc25a38a*, *slc6a18*, *slc6a19.1*, *slc6a19.2*, *slc6a19b*, *slc6a2*, and *slc6a6b*) (Figure 5F), GABAergic neuronal-related genes (*gad2* and *gad1b*) (Figure 5D), dopaminergic neuronal marker (*otpa*) (Figure 5G), and motor neuron markers (*insm1a*, *isl1*, and *sox2*) (Figure 5G) in different groups (control, 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) at 96 hpf. The results showed that the expression of *ache* was downregulated in all exposure groups relative to that in the control group (Figure 5C). Among the neurotransmitter transporter genes, *slc6a18*, *slc6a19.2*, and *slc6a19b* were downregulated in the 0.01 μg/mL exposure group, while all genes showed downregulated expression in the 0.1 μg/mL exposure group. In the 1 μg/mL exposure group, the *slc25a38a*, *slc6a18*, *slc6a19.1*, and *slc6a19b* genes were downregulated (Figure 5F). In terms of GABAergic neuronal-related genes, *gad2* was significantly upregulated in the 0.01 μg/mL and 1 μg/mL exposure groups, and *gad1b* was significantly upregulated in the 1 μg/mL exposure group (Figure 5D). Among the dopaminergic neuronal markers, the *otpa* gene was significantly downregulated in all tested exposure groups (Figure 5G). In terms of motor neuron markers, *insm1a* and *sox2* were downregulated in the 0.01 μg/mL and 0.1 μg/mL exposure groups, and *sox2* was downregulated in the 1 μg/mL exposure group (Figure 5G). Generally, the motor neuron-related genes exhibited a downregulation trend in the exposure groups. Since the discovery of PPARγ, its function and mechanism have been

extensively studied, and it has been found to play certain roles in neuroprotection, neural repair, and inflammation regulation [24]. Based on the above results (Figure 5A–D,F,G), we determined the expression of PPARγ-related genes in the 0.1 μg/mL exposure group (Figure 5E). The qPCR results showed that the PPARγ-related genes (*cd36* and *pparg*) were significantly downregulated by PFOA exposure (Figure 5E).

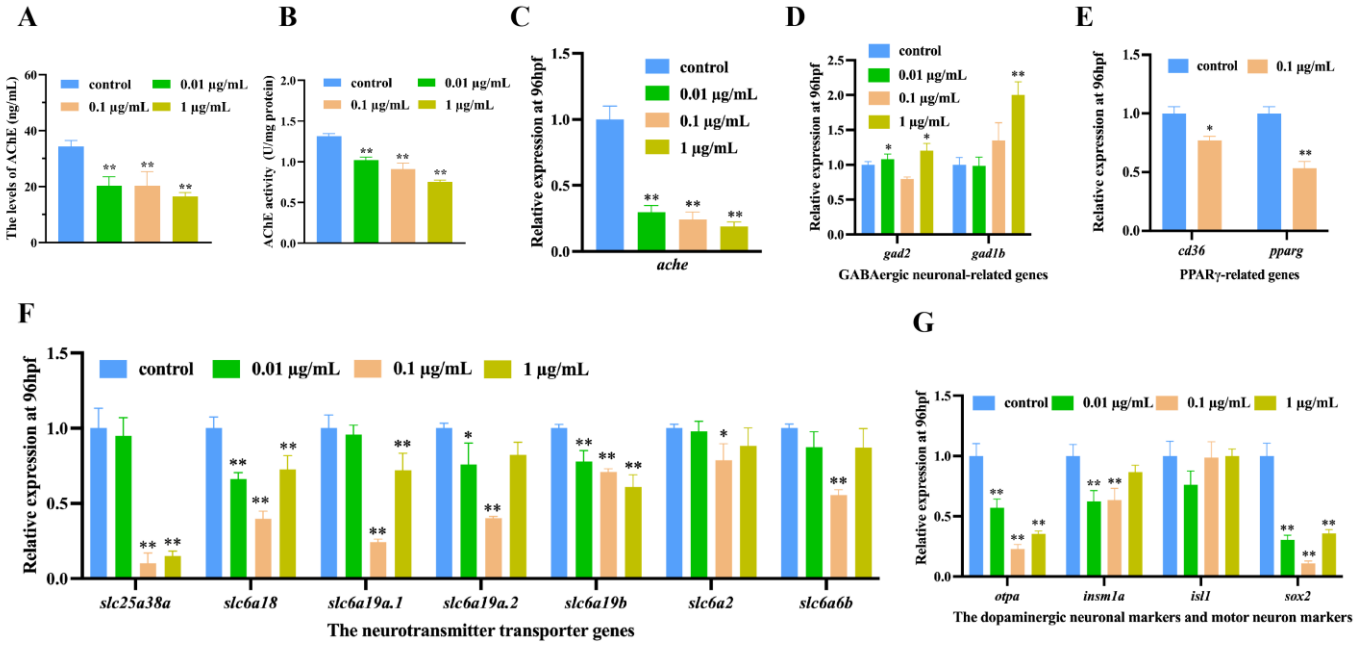

Figure 5. Effects of PFOA exposure on neuronal-related genes in zebrafish embryos at 96 h post-fertilization. (**A**) AChE content levels. (**B**) AChE activity levels. (**C**) Expression of the *ache* gene (neurotoxicity marker). (**D**) Expression of the *gad1b* and *gad2* genes (related to GABAergic neurons). (**E**) Expression of the *cd36* and *pparg (pparγ)* genes (related to PPARγ). (**F**) Expression of the *slc6a6b*, *slc25a38a*, *slc6a19a.1*, *slc6a19b*, *slc6a19a.2*, *slc6a18*, and *slc6a2* genes (related to neurotransmitter transporter protein activity). (**G**) Expression of the *sox2*, *isl1*, *otpa*, and *insm1a* genes (related to motor neuron markers). **, $p < 0.01$; *, $p < 0.05$.

3.4. Activation of PPAR Rescues the Motor and Neural Impairment Caused by PFOA

To further elucidate the mechanisms driving PFOA-induced motor dysfunction, we synthesized *ache* and *slc25a38a* mRNA, which was subsequently injected into zebrafish embryos prior to 0.1 μg/mL PFOA. Despite mRNA injection, the embryos subjected to PFOA treatment continued to exhibit significantly impaired motor responses in the touch response assay (Figure S3A), with significantly prolonged response times compared with the control group (Figure S3B).

Considering the significant downregulation of PPARγ-related genes (Figure 5E), we assessed whether the PPAR agonists rosiglitazone and bezafibrate could alleviate PFOA-induced motor and neural impairments to rescue the motor and neural impairments caused by PFOA. Zebrafish from each group were selected for locomotor and touch response tests. The results showed that after the addition of PPAR agonists, representative movement trajectories are shown in Figure 6A., and their movement distances were restored to the level of the control group (Figure 6B). In the touch sensitivity test, the co-treatment groups showed significantly shorter response times than the PFOA exposure group (Figures 6C and S2).

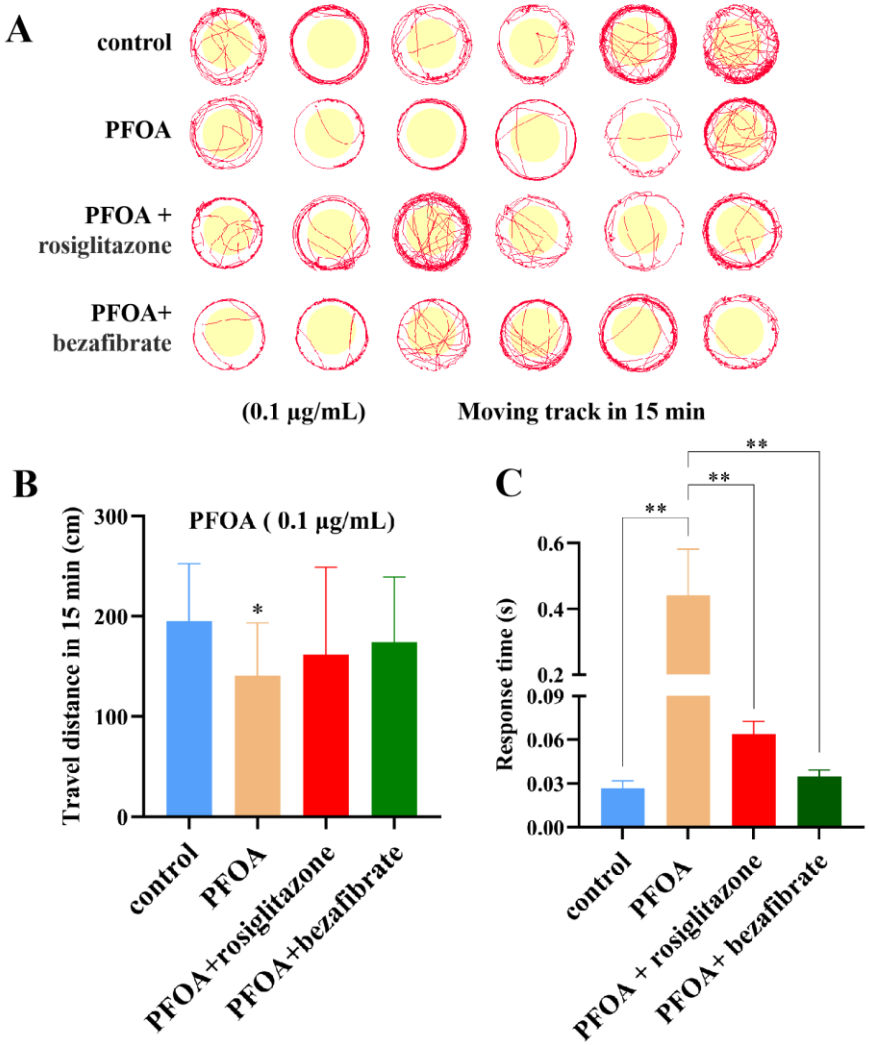

Figure 6. Effects of PPAR agonists on the motor behavior impairment induced by PFOA exposure in zebrafish embryos at 96 hpf. Movement trajectories (**A**) and movement distances (**B**) of zebrafish from the control group, exposure group (0.1 μg/mL), exposure group supplemented with rosiglitazone (0.1 μg/mL + rosiglitazone), and bezafibrate (0.1 μg/mL + bezafibrate). (**C**) Response time of zebrafish at 96 hpf. **, $p < 0.01$; *, $p < 0.05$.

Immunofluorescence analysis revealed that the number of Iba1-positive cells was significantly higher in the 0.1 μg/mL PFOA exposure group than in the control group (indicated by red arrows; Figure 7(A6,A13)). However, in the exposure group supplemented with rosiglitazone (0.1 μg/mL + rosiglitazone) or bezafibrate (0.1 μg/mL + bezafibrate), the numbers of Iba1-positive cells in the rosiglitazone and bezafibrate co-treatment groups were not significantly different from that in the control group (Figure 7(A9,A12,A13)). Furthermore, H&E staining results revealed that the number of sparse cells in the exposure group supplemented with rosiglitazone and bezafibrate was significantly reduced compared with that of the exposure group (Figure 7B). AO staining revealed an increase in AO-positive signals within the brain tissue of the 0.1 μg/mL exposure group compared to the control group (Figure 7(C2), red arrows). However, in the exposure groups co-treated with rosiglitazone (0.1 μg/mL + rosiglitazone) or bezafibrate (0.1 μg/mL + bezafibrate), AO-positive signals were less prominent in the two co-treatment groups than in the PFOA exposure group (Figures 7(C3,C4)). qPCR analysis (Figure 7D) demonstrated

that, relative to the 0.1 μg/mL exposure group, the expression levels of the apoptosis-related gene *caspase3* were significantly reduced in both the rosiglitazone and bezafibrate co-treatment groups. Additionally, the expression levels of the neuroinflammation-related gene *il1b* and the apoptosis-related gene *bax* were significantly downregulated in the rosiglitazone co-treatment group (0.1 μg/mL + rosiglitazone).

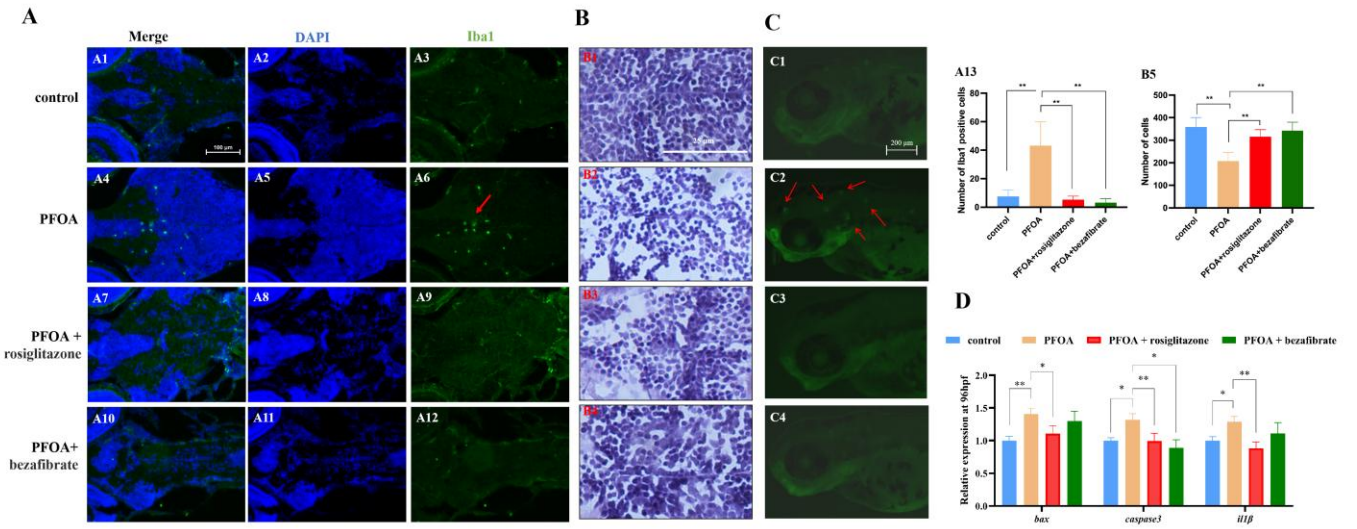

Figure 7. Effects of PPAR agonists on PFOA-induced neuroinflammation and brain damage in zebrafish embryos at 96 hpf. (**A1–A12**) Representative immunofluorescence images of brain sections from the control group and exposure groups (0.1 μg/mL, 0.1 μg/mL + rosiglitazone, and 0.1 μg/mL + bezafibrate) in zebrafish embryos at 96 hpf, with Iba1 protein expression shown in green. Red arrows indicate increased expression of Iba1 protein. (**A13**) The number of Iba1 positive cells. (**B1–B4**) H&E staining results of brain cross-sections from the control group and exposure groups (0.1 μg/mL, 0.1 μg/mL + rosiglitazone, and 0.1 μg/mL + bezafibrate) in zebrafish embryos at 96 hpf. (**B5**) The number of cells of H&E staining. (**C1–C4**) Acridine orange (AO) staining of zebrafish embryos at 96 h post-fertilization from the control group and exposure groups (0.1 μg/mL, 0.1 μg/mL + rosiglitazone, and 0.1 μg/mL + bezafibrate). Apoptotic signals are shown in green, with red arrows indicating prominent apoptotic signals. D Expression of genes associated with neuroinflammation and apoptosis (*bax*, *caspase3*, *il1b*). **, $p < 0.01$; *, $p < 0.05$.

The AChE activity assay results (Figure 8A) demonstrated that AChE activity in the 0.1 μg/mL exposure group was significantly lower than in the control group. However, compared to the 0.1 μg/mL exposure group, AChE activity significantly increased in the rosiglitazone and bezafibrate co-treatment groups. qPCR analysis revealed that the expression of selected genes was significantly affected by PFOA exposure, including the neurotoxicity marker gene (*ache*) (Figure 8B), dopamine neuronal marker gene (*otpa*), motor neuron marker gene (*sox2*), and neurotransmitter transport marker genes (*slc25a38a*, *slc6a18*, *slc6a19.1*, and *slc6a19.2*) (Figure 8C). Compared with the exposure group (0.1 μg/mL), the addition of bezafibrate significantly upregulated the expression of *ache*, *slc25a38a*, *slc6a18*, *slc6a19.1*, and *slc6a19.2*, whereas rosiglitazone co-treatment significantly upregulated the expression of *ache*, *otpa*, *sox2*, *slc25a38a*, and *slc6a19.1*.

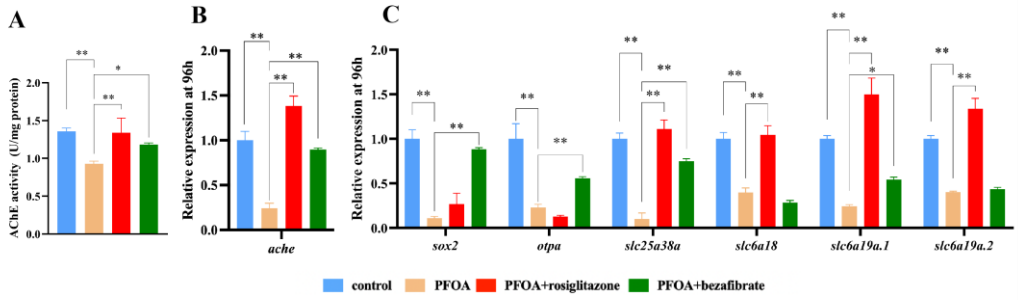

Figure 8. Effects of PPAR agonists (rosiglitazone and bezafibrate) on the expression of neuro-related genes in zebrafish embryos exposed to PFOA at 96 hpf. (**A**) AChE activity levels. (**B**) Expression of the neurotoxicity marker gene *ache*. (**C**) Expression of *sox2* (motor neuron) and *otpa* genes (associated with dopamine neurons), *slc25a38a*, *slc6a18*, *slc6a19.1*, and *slc6a19.2* (associated with neurotransmitter transport activity). **, $p < 0.01$; *, $p < 0.05$.

Although PFOA is generally considered an agonist of the PPAR pathway, most research has been focused on its effects on lipid homeostasis, lipid metabolism, and carcinogenesis [25]. Currently, there is a significant research gap regarding the functional impact of PFOA on the PPAR pathway related to neuroprotection, apoptosis, neural repair, and inflammation.

4. Discussion

4.1. PFOA Induces Impairment on Zebrafish Embryonic Motor and Neurological Functions

PFOA is widely distributed in the environment due to its historical use and resistance to natural degradation, leading to its bioaccumulation in ecosystems [1]. Consequently, human exposure to PFOA is widespread, and PFOA has been detected in blood, serum, and breast milk [12,26,27]. Therefore, it is particularly important to monitor the biotoxicity caused by PFOA. Numerous studies have demonstrated the potential neurotoxicity of PFOA [28]. For example, PFOA exposure during zebrafish development can increase heart rate, impair motor activity, and affect dopaminergic neuronal development and function, potentially contributing to later neurobehavioral abnormalities [13]. In this study, PFOA exposure impaired motor function in zebrafish, as indicated by reduced movement distance and delayed responses to touch. Impaired motor function is one of the typical characteristics of neurotoxicity [29]. Previous studies have suggested that shortened movement distances and delayed touch responses in zebrafish may indicate damage to brain and spinal cord neurons during zebrafish development [30]. These behavioral changes were accompanied by altered acetylcholinesterase (AChE) activity and reduced expression levels of genes related to neural development and neurotransmitter transport [31].

Zebrafish embryos exhibit high sensitivity to exogenous toxins during development [32]. The *sox10* gene plays a critical role in neural crest cell maintenance, migration, and differentiation, and is expressed in dorsal neural tubes, neural glial cells, and immature neurons [33,34], contributing to the formation of the peripheral nervous system [35]. At 96 hpf, an abnormal fluorescence phenotype was observed in *sox10*:eGFP zebrafish exposed to 1 μg/mL PFOA (Figure 3A). *Sox10*-expressing populations include glial lineages but are not limited to glial cells [36]. This phenotype may therefore indicate altered development or distribution of *sox10*-expressing cells. Previous research has also linked early-life PFOA exposure to altered neural development and reduced locomotor activity in zebrafish [13]. However, further cell-specific and quantitative analyses are needed to identify the affected cells and determine whether these changes contribute to

motor impairment. Glial cells play important roles in zebrafish central nervous system (CNS) development by regulating water balance, modulating extracellular neurotransmitter levels, and participating in synapse formation and pruning [31,37,38]. As neurotransmission and synaptic activity are involved in zebrafish locomotion, changes affecting glial cells may contribute to motor impairment; however, further studies are needed to determine whether this occurred following PFOA exposure.

H&E staining showed a significant reduction in the number of cells in the brain of the PFOA exposure group, accompanied by increased AO-positive signals. qPCR analysis also revealed significant upregulation of the apoptosis-related genes *bax*, *caspase3*, and *caspase9*. BAX is involved in the mitochondrial apoptotic pathway, which can activate Caspase-9 and downstream Caspase-3 [39–41]. Activated *caspase-3* then participates in the execution of apoptosis [42]. Together, these findings suggest that apoptosis-related pathways may be involved in PFOA-associated brain injury, although further studies are needed to confirm the mechanisms and identify the affected cell types.

Immunofluorescence showed an increased number of Iba1-positive cells in the brains of PFOA-exposed zebrafish. Iba1 is a marker of microglia, which participate in neural circuit development and respond to inflammation and tissue injury [43,44]. Depending on the context, microglial responses may contribute to tissue protection or injury [45,46]. The expression of the inflammation-related genes *il1b* and *mmp9* was also significantly upregulated following PFOA exposure. IL-1β is involved in inflammatory signaling and has been associated with central nervous system injury and inflammation [47,48]. MMP-9 can contribute to the degradation of the parenchymal basement membrane, a protective structure in the central nervous system, potentially facilitating inflammation [49,50]. Together, these changes suggest that inflammatory responses may contribute to PFOA-associated brain injury.

The nervous system regulates locomotion and responses to external stimuli in zebrafish [51]. In our study, larvae exposed to 0.1 μg/mL PFOA showed inflammatory changes and signs of apoptosis in the brain at 96 hpf. They also swam shorter distances and responded more slowly to touch than controls. Similarly, a previous study reported reduced locomotor activity and altered expression of dopamine-related genes in zebrafish larvae after 7 days of PFOA exposure [13]. Our findings suggest that neuroinflammation and apoptosis may contribute to PFOA-induced motor impairment during early development.

4.2. PFOA Impairs Zebrafish Embryonic Neurodevelopment

Acetylcholinesterase (AChE) is widely used as a biomarker in aquatic toxicology [52]. It plays a key role in cholinergic neurotransmission and has also been linked to apoptosis and cell cycle regulation [53]. Exposure to mercury or lead can reduce AChE activity in the zebrafish brain [54]. In zebrafish larvae exposed to triclosan, reduced AChE activity was accompanied by abnormal motor neuron innervation and impaired touch responses [55]. In our study, AChE content, AChE activity, and *ache* expression were significantly lower than in controls at all three PFOA concentrations tested (0.01, 0.1, and 1 μg/mL). These findings suggest that altered cholinergic signaling may contribute to the motor impairment observed in PFOA-exposed larvae.

Solute carrier (SLC) proteins transport amino acids, ions, and other solutes across membranes [56,57]. One mitochondrial carrier, SLC25A38, is involved in heme biosynthesis [58]. In zebrafish embryos, combined knockdown of *slc25a38a* and *slc25a38b* reduced hemoglobin levels [59]. In our study, *slc25a38a* expression was significantly lower in the 0.1 and 1 μg/mL PFOA groups than in controls. Whether this change affected heme synthesis in exposed larvae remains unclear. The SLC6 family includes transporters for neurotransmitters, amino acids, and osmolytes [56]. At 0.1 μg/mL PFOA, the expression of all six SLC6 genes examined was significantly lower than in controls (Figure 5F).

Coordinated neurotransmission is essential for normal swimming and escape responses in zebrafish [60,61]. The lower transcript levels coincided with shorter swimming distances and delayed touch responses, although their contribution to these behavioral changes remains unclear.

GABA is a major inhibitory neurotransmitter in the brain [62]. GABAergic and glutamatergic signaling help regulate escape responses and rhythmic swimming in developing zebrafish [61]. The genes *gad1b* and *gad2* encode glutamate decarboxylases that synthesize GABA from glutamate [63,64]. In our study, *gad2* expression showed a non-monotonic pattern across the tested PFOA concentrations, with a lower mean value at 0.1 μg/mL than in the control group. In contrast, *gad1b* showed an overall increasing trend. These different patterns may reflect differences in gene regulation or experimental variability; however, their underlying causes remain unclear. Further studies are needed to determine whether these expression changes are associated with alterations in GABAergic neuron development or function. In zebrafish, *otpa* contributes to the development of dopaminergic neurons in the ventral diencephalon [65–67]. Delayed differentiation of dopaminergic neurons has also been associated with reduced spontaneous movement in zebrafish larvae [68]. In our study, *otpa* expression was significantly lower than in controls at all three PFOA concentrations tested (0.01, 0.1, and 1 μg/mL), including 0.01 μg/mL, at which touch responses resembled those of controls.

The gene *insm1a* is expressed in primary motor neurons, and its loss impairs spinal motor neuron differentiation in zebrafish [60,69]. The transcription factor *sox2* also contributes to central nervous system development [70]. In zebrafish, *sox2* knockout disrupts motor axon development and prevents normal tail-flick and swim-up behaviors [71]. In our study, expression of both *insm1a* and *sox2* was lower at 0.01 and 0.1 μg/mL PFOA, whereas only *sox2* showed lower expression at 1 μg/mL. These changes in motor neuron-related gene expression may contribute to the shorter swimming distances observed in PFOA-exposed larvae.

4.3. PFOA Exposure Affects Zebrafish Motor Function Through the PPAR Signaling Pathway

CD36 is an 88-kDa transmembrane glycoprotein of the class B scavenger receptor family. It is expressed in several cell types, including adipocytes and macrophages [72], and contributes to metabolic and immune regulation [73]. PPARγ is a ligand-activated nuclear receptor that regulates cellular homeostasis [74]. It is expressed in adipose tissue and immune cells, among other sites, and regulates genes involved in lipid and glucose metabolism, adipogenesis, and inflammation [16,75]. In macrophages, hexarelin, a peptide that binds CD36, enhances the transcriptional activity of PPARγ [76]. We selected a nominal PFOA concentration of 0.1 μg/mL for the rescue experiments because larvae exposed to this concentration swam shorter distances and responded more slowly to touch than controls. This concentration also falls within the range reported in surface waters near a fluoropolymer manufacturing facility [77]. Previous work has shown that PFOA exposure alters lipid metabolism in zebrafish [9]. In our study, both *cd36* and *pparg* expression were significantly lower in larvae exposed to 0.1 μg/mL PFOA than in controls (Figure 5E). PPARγ signaling has also been implicated in the regulation of microglial responses in experimental models of Alzheimer's disease [78,79]. These observations prompted us to test whether PPAR agonists could alleviate the neural and behavioral effects of PFOA exposure.

Neuroinflammation has been implicated in conditions such as Alzheimer's disease, Parkinson's disease, and depression [80–82]. Inflammatory mediators released by glial and infiltrating immune cells can contribute to neuronal injury and apoptosis [83–85]. Microglia monitor the central nervous system for signs of injury [86], and their responses can promote inflammation or support tissue repair, depending on the stimulus [87–89].

The effects of PPARγ on microglia appear to depend on the experimental context. In a mouse model of traumatic brain injury, phillyrin reduced neuroinflammation and blood–brain barrier damage through a mechanism involving PPARγ signaling [90]. In lipopolysaccharide-stimulated microglia, a PPARγ antagonist also reduced the expression of pro-inflammatory markers [91]. In our study, larvae exposed to 0.1 μg/mL PFOA showed significantly lower *pparg* expression and higher *il1b* and *mmp9* expression than controls. These changes coincided with shorter swimming distances and delayed touch responses. Together, these findings suggest that altered PPARγ signaling may be involved in the inflammatory response to PFOA. Whether changes in microglial function contribute to the observed motor impairment remains unclear. Because PFOA exposure reduced *ache* and *slc25a38a* expression, we tested whether injecting either mRNA before exposure to 0.1 μg/mL PFOA could improve the touch response. At 96 hpf, response times remained longer than in controls in both mRNA-injected groups (Figure S3). We then tested rosiglitazone, a PPARγ agonist, and bezafibrate, a pan-PPAR agonist, in separate co-treatment groups [92,93]. Compared with PFOA exposure alone, both treatments shortened touch-response times and increased AChE activity (Figures 6C and 8A). Brain sections from both groups showed fewer Iba1-positive cells and higher cell counts on H&E staining. AO-positive signals were also less prominent (Figure 7A–C). The transcriptional responses differed by gene. Both treatments reduced *caspase3* expression and increased ache and *slc25a38a* expression relative to PFOA exposure alone. Rosiglitazone also reduced *bax* and *il1b* expression (Figures 7D and 8B,C). Together with the lower *cd36* and *pparg* expression observed after PFOA exposure, these findings suggest that PPAR signaling may contribute to the protective effects observed.

Several questions remain. Cell-specific analyses of dopaminergic, motor, and GABAergic neurons, together with measurements of neurotransmitter levels, could help identify the changes underlying the behavioral deficits. Assessing microglial morphology and activation state could clarify the inflammatory response, while direct measurements of PPARγ activity could help establish the receptor's role in the effects of the two drugs. Finally, measuring hemoglobin levels could clarify whether reduced *slc25a38a* expression has hematological consequences.

5. Conclusions

This study demonstrates that PFOA exposure induces motor dysfunction in zebrafish embryos, including reduced movement distance and delayed responses to touch stimuli. PFOA exposure was associated with changes in PPARγ-related signaling, increased neuroinflammation and apoptosis, and altered expression of genes involved in neural function. Furthermore, co-treatment with the PPARγ agonist rosiglitazone or the pan-PPAR agonist bezafibrate partially alleviated PFOA-induced neurotoxicity and motor impairment and improved the expression of several neural function-related genes. These findings suggest that PPAR-related pathways may be involved in the response to PFOA exposure. Further studies are needed to clarify the underlying mechanisms and assess the relevance of these findings to other aquatic organisms and exposure conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci13121290/s1, Figure S1: Touch sensitivity responses of zebrafish embryos exposed to different PFOA concentrations; Figure S2: Touch sensitivity responses of zebrafish embryos following PFOA exposure and co-treatment with rosiglitazone or bezafibrate; Figure S3: Touch sensitivity responses, response times, and gene expression following mRNA injection and PFOA exposure; Table S1: Primers used in this study; Table S2: F-values, degrees of freedom, and *p*-values for the statistical analyses; Table S3: PFOA concentrations in zebrafish

embryos; Table S4: PFOA concentrations in the exposure solution; Table S5: *p*-values for comparisons between experimental groups.

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Institutional Review Board Statement: The animal study was reviewed and approved by the Laboratory Animal Welfare Ethics Committee of Nanchang University and the Institutional Animal Care and Use Committee of Shenyang Agricultural University (approval number: SNLL25031012 approval date 10 March 2025).

Informed Consent Statement: Not applicable. This study used institution-owned laboratory zebrafish and did not involve client-owned animals.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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