

Chronic Treatment with Paraquat Induces Brain Injury, Changes in Antioxidant Defenses System, and Modulates Behavioral Functions in Zebrafish

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Abstract Paraquat (PQ) administration consists in a chemical model that mimics phenotypes observed in Parkinson's disease (PD), due to its ability to induce changes in dopaminergic system and oxidative stress. The aim of this study was to evaluate the actions of PQ in behavioral functions of adult zebrafish and its influence on oxidative stress biomarkers in brain samples. PQ (20 mg/kg) was administered intraperitoneally with six injections for 16 days (one injection every 3 days). PQ-treated group showed a significant decrease in the time spent in the bottom section and a shorter latency to enter the top area in the novel tank test. Moreover, PQ-exposed fish showed a significant decrease in the number and duration of risk assessment episodes in the light–dark test, as well as an increase in the agonistic behavior in the mirror-induced aggression (MIA) test. PQ induced brain damage by decreasing mitochondrial viability. Concerning the antioxidant defense system, PQ increased catalase (CAT) and glutathione peroxidase (GPx) activities, as well as the non-protein sulfhydryl content (NPSH), but did not change ROS formation and decreased lipid peroxidation. We demonstrate, for the first time, that PQ induces an increase in aggressive behavior, alters non-motor patterns associated to defensive behaviors, and changes redox parameters in zebrafish brain. Overall, our findings may serve as useful tools to investigate the

interaction between behavioral and neurochemical impairments triggered by PQ administration in zebrafish.

Keywords Paraquat · Zebrafish · Aggression · Anxiety-like behavior · Chronic exposure

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; PQ) is an herbicide commonly used in agriculture presenting a potential toxicity in several species, including humans [1–4]. This molecule acts mainly in dopaminergic neurons (DN), due to its specificity with the same neutral amino acid transporter used by L-valine and L-dopa [5]. Additionally, DN are sensitive to oxidative stress triggered by superoxide anions through redox cycling induced by PQ [6]. PQ is absorbed as paraquat dication (PQ^{2+}) and crosses the inner mitochondrial membrane by a potential-dependent carrier [7]. In the mitochondrial matrix, PQ^{2+} is reduced to PQ radical monocation (PQ^+) by the complex I of the electron transport chain (ETC) in mammals. The PQ^+ radical reacts rapidly with oxygen to generate the anion superoxide ($O_2^{\cdot-}$), which undergoes non-enzymatic dismutation to form hydrogen peroxide (H_2O_2). In the presence of ferrous iron (Fe^{2+}), the hydroxyl radical ($\cdot OH$) may be formed by Fenton's reaction, which is highly reactive to biomolecules [8].

The interactions between PQ and DN have been used for modelling Parkinson's disease (PD) in animal species [8]. Several studies associate the neurodegenerative disease development with redox imbalance, since the brain is highly vulnerable to oxidative stress due to its high O_2 consumption, its modest antioxidant defenses, and its lipid-rich constitution [9–11]. Therefore, the redox imbalance triggered by PQ, due

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to mitochondrial dysfunction, may be associated with neuronal disorder and behavioral impairments [9]. In rats chronically treated with PQ, significant alterations in anxiety-like phenotypes [12, 13], loss of olfactory discrimination, locomotor and motor deficits were observed [14]. The responses of mice in the light–dark box are accompanied by significantly high levels of intracellular reactive oxygen species (ROS) in blood cells [15].

More recently, Bortolotto et al. [16] and Bretaud et al. [17] proposed a model that mimics some phenotypes observed in PD for adult zebrafish (*Danio rerio*) exposed chronically to PQ. This species is a prominent animal model used in neuroscience studies, presenting evolutionary conserved brain functions and well-characterized neurotransmitter systems [18]. In addition, other features such as low-cost, easy maintenance, and abundant offspring [19–24] make zebrafish an alternative vertebrate system complementing the existent rodent approaches. This species is a suitable organism to neurobehavioral studies due to its well-described behavior patterns [19]. However, behavioral parameters such as aggression and anxiety-like phenotypes have not been fully elucidated in this model of PQ exposure, as well as the response of antioxidant defense system and mitochondrial viability in the brain. The investigation of such parameters would straightforward the construct validation of a PQ model in zebrafish. Therefore, the goal of the present study was to investigate the effects of PQ exposure in motor and non-motor patterns, as well as to assess the influence of PQ on neurochemical parameters related to oxidative stress in brain samples of zebrafish.

Materials and Methods

Animals

We used adult zebrafish (4–6 month-old) of short fin (SF) wild-type phenotype of both sexes. The fish were obtained from a commercial supplier (Hobby Aquários, RS, Brazil) weighing 0.5 ± 0.1 g and measuring 3.0 ± 1.0 cm in length. The fish were maintained in aerated and temperature-regulated water (27 ± 1 °C) in 40 L aquaria under constant mechanical, biological, and chemical filtration at a density of three animals per liter. Illumination was provided by ceiling-mounted fluorescent light tubes set under a light/dark photoperiod cycle of 14/10 h (lights on at 7:00 a.m.). All animals used in this study were experimentally naïve and fed with alcon BASICTM flakes (Alcon, Brazil) twice daily. Before treatment, the fish were acclimated for 15 days in an aquarium of 35 L with partitions filled with non-chlorinated water treated with AquaSafeTM (Tetra, VA, USA). This aquarium allowed the maintenance of 16 animals individually separated in the same aerated and heated home-tank water. Moreover, the fish were able to maintain direct visual contact with others

in order to minimize the stress of isolation. Animal experimentation in this study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 046/2014.

Experimental Groups

After the acclimation period, the fish were separated into two experimental groups: PQ-treated group (PQ) and untreated group (control, CTL). CTL group was treated with 0.9 % saline solution. PQ group was treated with 20 mg/kg of PQ (Methyl viologen dichloride hydrate; Sigma[®]) diluted in 0.9 % saline solution.

Drug Administration

PQ and saline were administrated intraperitoneally with one injection every 3 days for 16 days totalizing six injections as described previously by Bortolotto et al. [16]. For each injection, a total volume of 10 µL was applied, corresponding to concentration of 20 mg/kg PQ for each injection. Before each injection, the zebrafish were anesthetized with 0.1 g/L tricaine (3-amino benzoic acid ethylester; Fluka). The fish were kept in an aquarium with partitions as described above during the treatment period.

Behavioral Measurements

The behavioral tests were performed 24 hours after the last injection between 10:00 a.m. and 4:00 p.m. The apparatuses were filled with non-chlorinated water (27 ± 1 °C), and the experimental procedures were performed on a stable surface with all environmental distractions kept to a minimum. The behavioral activities of zebrafish were recorded for a single session of 300 s except for the novel tank test (experiment 1, 360 s). The swimming location of zebrafish was recorded using a webcam connected to a laptop at a rate of 30 frames/s using appropriate video-tracking software (ANY-mazeTM, Stoelting CO, USA). The animals were further removed from the test tanks and euthanized as described below.

Experiment 1: Novel Tank Test

Locomotor and exploratory activities were analyzed in the novel tank test, which may reflect habituation to novelty stress [20, 25, 26]. After the treatment period, zebrafish ($n=21$ per group) were individually placed in a novel apparatus filled with 2 L water and their swimming behavior was recorded. The apparatus was virtually divided in three horizontal sections (bottom, middle, and top) to assess the vertical exploration by the following endpoints: number of entries and time

spent (s) in the bottom area, latency (s) to enter the top, number of entries and time spent (s) in the top area. Distance travelled (m), maximum speed (m/s), and absolute turn angle ($^{\circ}$) were used to measure locomotor and motor patterns. For measuring of fear/anxiety-related behaviors, we determined the number and duration (s) of freezing bouts as well as the number and duration (s) of erratic movements. Freezing was computed when zebrafish were immobile presenting increased opercular movements for at least 2 s. Erratic movements were defined as abrupt changes in swimming direction with constant rapid darting behavior [27, 28]. Both endpoints are associated to fear/anxiety-like behaviors [28] and were manually assessed by two trained researchers (inter-rater reliability >0.85).

Experiment 2: Light–Dark Test

The light–dark test was carried out based in the method described by Maximino et al. [27]. The test apparatus consisted in tank ($15 \times 10 \times 25$ cm, height \times depth \times length) divided into two equally sized dark and lit areas and filled with 2 L water. Each animal was placed initially at the lit area, and during a 5-min trial, the number of entries and time spent (s) in the lit area, number and duration (s) of freezing bouts, latency (s) to enter the dark area, and number of risk assessments events were measured. Risk assessments were defined as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline) associated to a fast return to the dark compartment [27, 28]. The test was carried out using $n=20$ per group.

Experiment 3: Mirror-Induced Aggression Test

The aggression test was performed using the inclined mirror-induced aggression task (MIA) [29]. Fish ($n=25$ per group) were individually netted into a small experimental tank ($15 \times 10 \times 25$ cm, height \times depth \times length). The apparatus was filled with 2 L water. A mirror was placed inclined at 22.5° to the back wall of the tank so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. Fish were able to explore both compartments for 5 min and the following behaviors were determined: number of entries and time spent (s) in each area (A1, A2, A3, and A4) (Fig. 3c) and number and duration (s) of attacks. Entry to A1 segment indicated preference for proximity to the “opponent” whereas entry to A4 segment implied avoidance. In addition, the time in which fish presented aggressive display, or attack behavior, was also measured and analyzed as potential aggression. Aggressive display was defined as a posture during in which the fish erects its dorsal, caudal, pectoral, and anal fins, usually associated with undulating body movements and

attacks. Attack behavior is a characteristic short bout of fast swimming directed towards the opponent when fish open the mouth and bite the image [29].

Biochemical Parameters

Tissue Preparation

Twenty-four hours after the last injection, zebrafish were anesthetized with 0.25 g/L tricaine [30] and euthanized by punching the spinal cord behind the opercula and the brains were dissected out in ice. The brains were further washed with 150 mM saline solution, packed in microtubes, and kept at -80°C for posteriors assays.

Mitochondrial Viability Assay

To evaluate the mitochondrial viability in zebrafish CNS ($n=6$ per group), whole brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC). Because PQ administration caused visually scattered mitochondrial injuries, the formazan produced from TTC by mitochondrial activities was spectrophotometrically quantified [31]. This protocol has already been standardized for zebrafish brain samples and was performed in accordance to previous reports [31, 32]. Brains were singly immersed in vials containing 1 mL of 2 % TTC in phosphate-buffered saline (pH 7.4) at 37°C for 40 min, protected from light. Next, the TTC solution was removed and 10 % formalin in phosphate-buffered solution (pH 7.4) was used to stop the reaction. The brains were visualized in a stereomicroscope in order to verify whether different brain areas of zebrafish (e.g., telencephalon, optic tectum, and cerebellum) were marked with formazan product. For spectrophotometric analysis, the brains were further dried at 40°C for 2 h and, then, the tissues were weighed. Afterwards, the brains were transferred to 96-well plates and immersed in 200 μL of dimethylsulfoxide. The plates were kept under constant agitation for 4 h in order to elute the formazan produced in brain tissue from TTC reaction. The absorbance of the supernatant was read at 490 nm in a microplate reader. Data were reported in absorbance per tissue dry weight (g) and normalized as percentage of control.

Lipid Peroxidation Estimation Assay

Lipid peroxidation was estimated by thiobarbituric acid-reactive substance (TBARS) production [33]. Two zebrafish brains were homogenized in 0.3 mL phosphate buffer saline (PBS) pH 7.4, containing the following: NaCl (137 mM), Na_2HPO_4 (10.1 mM), and KH_2PO_4 (1.76 mM). The homogenate ($n=6$ per group) was initially centrifuged ($700g$ for 5 min, 4°C). Then, supernatant samples (80–100 μg protein) were mixed with 600 μL of 15 % trichloroacetic acid (TCA)

and further centrifuged (10,000g, 10 min). Supernatants (100 μ L) were mixed with 100 μ L of 0.67 % thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2- thiol) and heated at 100 °C for 30 min. TBARS levels were determined by the absorbance at 532 nm using malondialdehyde (MDA) as standard. Results were expressed as nmol MDA/mg of protein.

ROS Levels

The ROS levels were measured using the fluorescent dye 2,7-dichlorofluorescein-diacetate (DCFDA) as described by Ali et al. [34]. A whole zebrafish brain ($n=6$ per group) was homogenized in 50 mM Tris HCl (pH 7.5) buffer and centrifuged (3000g, 10 min at 4 °C). Supernatants (300–500 μ g of protein) were mixed with 0.1 mM 2',7'-dichlorofluorescein-diacetate (DCFH-DA). ROS levels were determined by fluorescence at emission (570 nm) and excitation (545 nm) using dichlorofluorescein (DCF) as standard. Results were expressed as μ mol DCF/mg protein.

Antioxidant Enzymes

Antioxidant enzyme measurements were performed using six independent experiments per group ($n=6$). Five brains were pooled and homogenized in 0.5 mL of 20 mM potassium phosphate buffer, pH 7.5. Samples were further centrifuged at 10,000g for 10 min at 4 °C. Catalase (CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm by ultraviolet spectrophotometry [35]. The reaction mixture consisted by 2 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M), and 0.02 mL homogenate (20–30 μ g protein). The results were expressed as nmol/min/mg of protein. Brain glutathione peroxidase (GPx) activity was measured by ultraviolet spectrophotometry following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase [36]. The assay mixture consisted of potassium phosphate buffer (100 mM, pH 7.0), 1 mM NaN_3 , 1 mM reduced glutathione (GSH), 0.15 mM NADPH, and homogenized tissue (5 μ L, ranging from 40–60 μ g protein). The reaction was started by adding 30 μ L of 0.4 mM H_2O_2 with a final volume of 300 μ L. The specific activity was determined in a microplate reader using the extinction coefficient of 6.22 mM/cm and expressed as nmol/min/mg of protein. Glutathione S-transferase (GST) activity was analyzed according to Habig et al. [37]. The assay mixture contained 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in ethanol, 10 mM GSH, 20 mM potassium phosphate buffer (pH 6.5), and 50 μ L of the tissue homogenates (40–60 μ g protein). Enzyme activity was calculated from the changes in absorbance at 340 nm using a molar extinction coefficient of 9.6 mM/cm. One unit GST activity was defined as the amount of enzyme required to catalyze the conjugate on of 1 mol CDNB with GSH/min at 25 °C [37]. The activity was

expressed as μ mol GS-DNB/min/mg protein. GS-DNB is defined as μ mol GST complexed with 1-(S-glutathionyl)-2,4-dinitrobenzene.

Non-protein thiols (NPSH)

Two zebrafish brains were pooled per sample and homogenized in 250 μ L Tris HCl 50 mM (pH 7.5) and followed by centrifugation at 700 \times g for 5 min at 4 °C. An aliquot of supernatant (100 μ L) was further mixed with 100 μ L of 10 % trichloroacetic acid (TCA) and centrifuged (3000g for 10 min at 4 °C). Supernatants (60–80 μ g protein) were mixed with DTNB (0.01 M dissolved in ethanol) and the intense yellow color developed was measured at 412 nm after 1 h [38]. Results were expressed as nmol SH/mg of protein ($n=6$).

Protein Determination

Protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm [39].

Statistics

Normality of data and homogeneity of the variances were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Data were expressed as mean \pm standard error of the mean (S.E.M.) and analyzed by unpaired Student's *t* test. The significance level was set at $p\leq 0.05$.

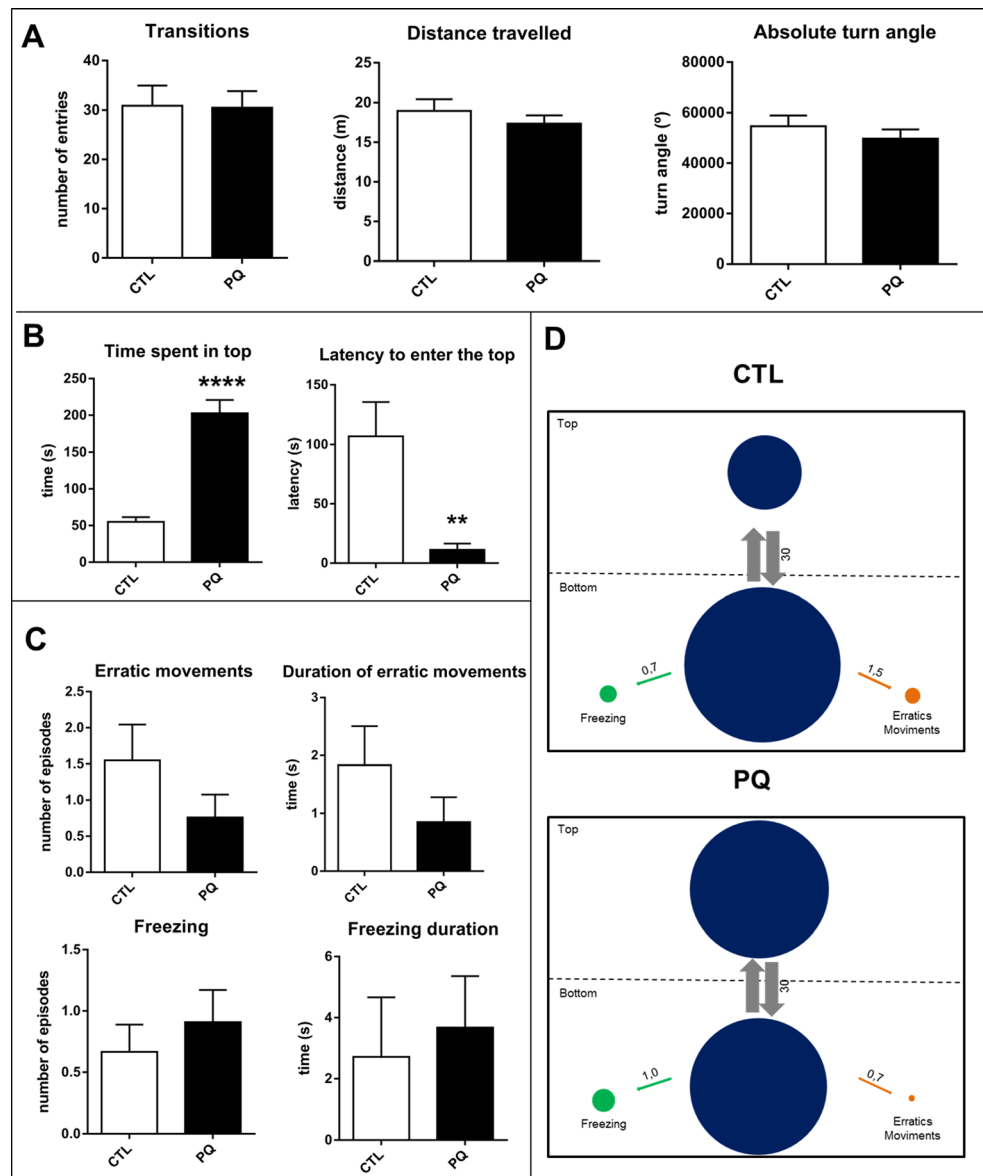
Results

In the novel tank test (Fig. 1), PQ did not significantly alter total distance travelled, absolute turn angle, and transitions to top area in comparison to untreated group (Fig. 1a). A significant decrease in the latency to enter the top ($t_{0.05;40}=3.303$, $p=0.0020$) was observed in fish exposed to PQ, which also spent more time in the upper area of the test tank ($t_{0.05;40}=6.795$, $p<0.0001$) (Fig. 1b) and less time in the bottom third of the aquarium ($t_{0.05;40}=2.925$, $p=0.0056$) (data not shown). Moreover, zebrafish exposed to PQ did not present significant changes in the number and duration of freezing and erratic movements (Fig. 1c).

In the light–dark test, no significant differences in transitions and in time spent in lit area were detected (Fig. 2a). Exposure to PQ induced a significant decrease in the number ($t_{0.05;38}=3.874$, $p=0.0004$) and duration ($t_{0.05;38}=3.214$, $p=0.0027$) of risk assessment episodes (Fig. 2b). The number and duration of freezing and erratic movements remained unchanged (Fig. 2b).

In the MIA test, PQ-exposed fish showed a significant increase in the number ($t_{0.05;48}=5.345$, $p<0.0001$), time

Fig. 1 Effects triggered by PQ in locomotion and exploration in the novel tank test in comparison to CTL. **a** Locomotor and motor activities represented by the transitions to the top third of the tank, distance travelled, and absolute turn angle. **b** Vertical exploration measured by time spent in the top area and latency to enter the top. **c** Number and duration of freezing bouts and erratic movements. **d** Representative ethograms based on frequencies and transitions between each individual behavioral activity. The *diameter of each circle* corresponds to the frequency of each individual behavioral activity, whereas the *arrow width and direction* reflect the frequency of transitions between these behaviors. Data are expressed as mean \pm SEM (** $p < 0.01$, **** $p < 0.0001$, CTL control, PQ paraquat)



($t_{0.05;48} = 3.907$, $p = 0.0003$), and average duration ($t_{0.05;48} = 5.875$, $p < 0.0001$) of aggressive episodes (Fig. 3a). In terms of exploration, animals exposed to PQ spent more time closer to the mirror in A1 section ($t_{0.05;48} = 2.976$, $p = 0.0046$) and less time in A4 section (farthest to the mirror) ($t_{0.05;48} = 3.727$, $p = 0.0005$). Additionally, CTL fish performed more entries in the A2 section when compared to PQ group ($t_{0.05;48} = 2.780$, $p = 0.0077$). When PQ-exposed animals were in the A1 section of the test apparatus, the number ($t_{0.05;48} = 3.708$, $p = 0.0005$) and duration ($t_{0.05;48} = 3.931$, $p = 0.0003$) of attack to the opponent image significantly increased in comparison to CTL. No significant alterations in aggression-related parameters were observed when fish were in the A2 section (Fig. 3c). The construction of representative ethograms confirmed the main alterations in behavioral profile of zebrafish in the novel tank (Fig. 1d), light–dark (Fig. 2c),

and in the MIA tests (Fig. 3d), showing a distinct behavioral pattern of PQ-treated group.

The biochemical parameters associated to oxidative stress and mitochondrial viability are shown in Fig. 4. PQ-treated fish showed a significant decrease in TBARS levels ($t_{0.05;10} = 7.129$, $p < 0.0001$) and an increase in GST activity ($t_{0.05;10} = 6.319$, $p < 0.0001$) (Fig. 4a). PQ group also presented a significant increase in CAT ($t_{0.05;10} = 4.210$, $p = 0.0018$) and GPx ($t_{0.05;10} = 3.651$, $p = 0.0045$) activities (Fig. 4b). The ROS formation did not significantly change in both groups and the NPSH content increased after chronic PQ administration ($t_{0.05;10} = 2.830$, $p = 0.0179$) (Fig. 4c). Considering mitochondrial function determined by TTC staining, no visual difference among brain regions was evident (data not shown). However, spectrophotometric quantification of formazan indicated a significant decrease in the

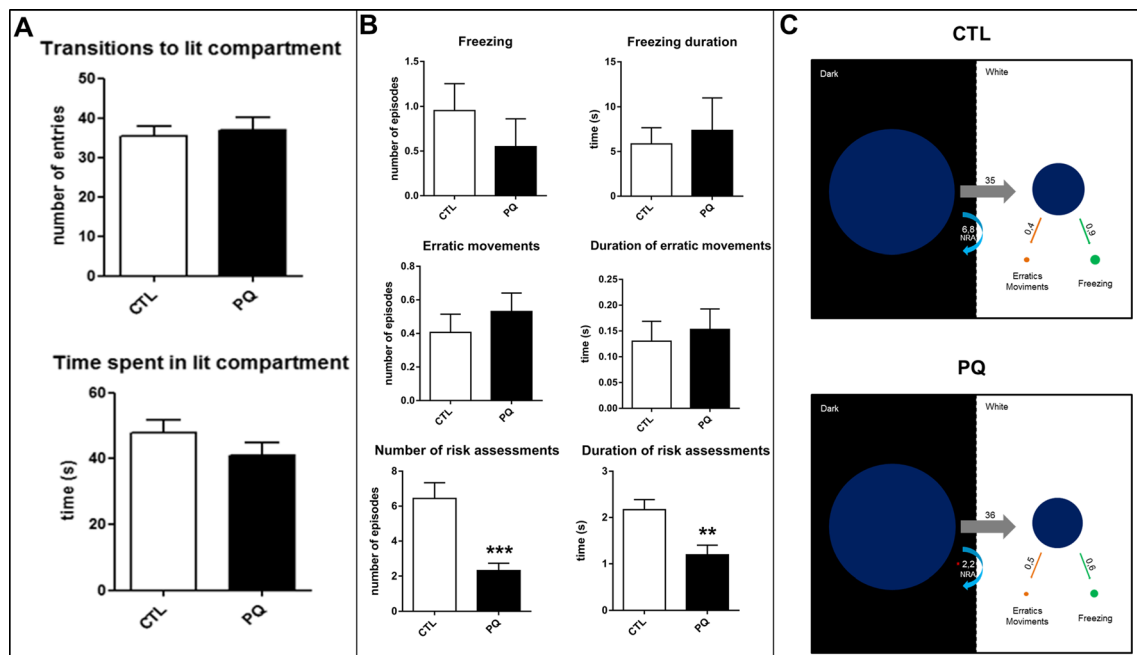


Fig. 2 Effects of PQ on behavioral pattern of zebrafish in the light–dark test. **a** Transitions and time spent in lit compartment. **b** Number and duration of freezing, erratic movements, and risk assessment episodes. **c** Representative ethograms based on frequencies and transitions between each individual behavioral activity. The diameter of each circle

corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. Data are expressed as mean \pm SEM (** $p < 0.01$, *** $p < 0.001$, CTL control, PQ paraquat)

mitochondrial viability of PQ group when compared to CTL ($t(0.05; 10) = 3.553, p = 0.0052$) (90.8 % of the absorbance of the control group) (Fig. 4c).

Discussion

The administration of PQ in experimental models has been associated with behavior changes and neurochemical impairments that closely resemble those observed in PD. Alterations in biochemical and neurochemical patterns are also observed in rodents, fish, and *Drosophila* [3–6, 8, 12–16, 40–44]. In the present study, the results clearly demonstrate, for the first time, that zebrafish exposed to repeated injections of PQ exhibit alterations in non-motor patterns by increasing aggression and decreased defensive behaviors. The analysis of the behavioral profile is an effective method to characterize the effects of different compounds on swimming activity of fish, predicting the potential action of a compound in the central nervous system (CNS) [20]. Bretau et al. [17] demonstrated that adult zebrafish exposed to 5 mg/L of PQ for 4 weeks by immersion did not show differences in some behavioral and neurochemical parameters, suggesting that a direct aquatic exposure may not induce significant alterations in brain homeostasis. However, when adult male zebrafish were treated with PQ (10 and 20 mg/kg) by intraperitoneal injection, according to model described by Bortolotto et al. [16], alterations in locomotor and neurochemical patterns may be

observed. Since alterations in non-motor behaviors may be usually detected in psychiatric disorders [45–47] and neurodegenerative disease models [48, 49], this model has an intrinsic translational relevance to behavioral phenomics research. The adult zebrafish presents a complex behavioral repertoire and exposure to stressors can evoke fear or anxiety-like phenotype that can be easily quantified by reduced vertical exploration, increased scototaxis, geotaxis, freezing, risk assessment episodes, and erratic movements [25]. We analyzed non-motor patterns associated with anxiety-like behaviors in the novel tank and in the light–dark tests. In the novel tank test, fish did not show alterations after PQ injections in locomotor patterns. However, PQ administration induced markedly changes in anxiety-like parameters, since time spent in the upper half increased and latency to enter the top area significantly decreased in the novel tank test. Moreover, a decrease in the number and duration of risk assessment episodes in PQ group related to CTL was observed in light–dark test,

Fig. 3 Agonistic behavior of CTRL and PQ-exposed zebrafish measured by the MIA test. **a** Number, time, and average duration of aggressive episodes. **b** Transition and time spent in the four sections of the apparatus. **c** Schematic representation of the apparatus sections in relation to mirror, number, and time of aggressive episodes in A1 and A2 sections. **d** Representative ethograms based on frequencies and transitions between each individual behavioral activity. The diameter of each circle corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. Data are expressed as mean \pm SEM and (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, CTL control, PQ paraquat)

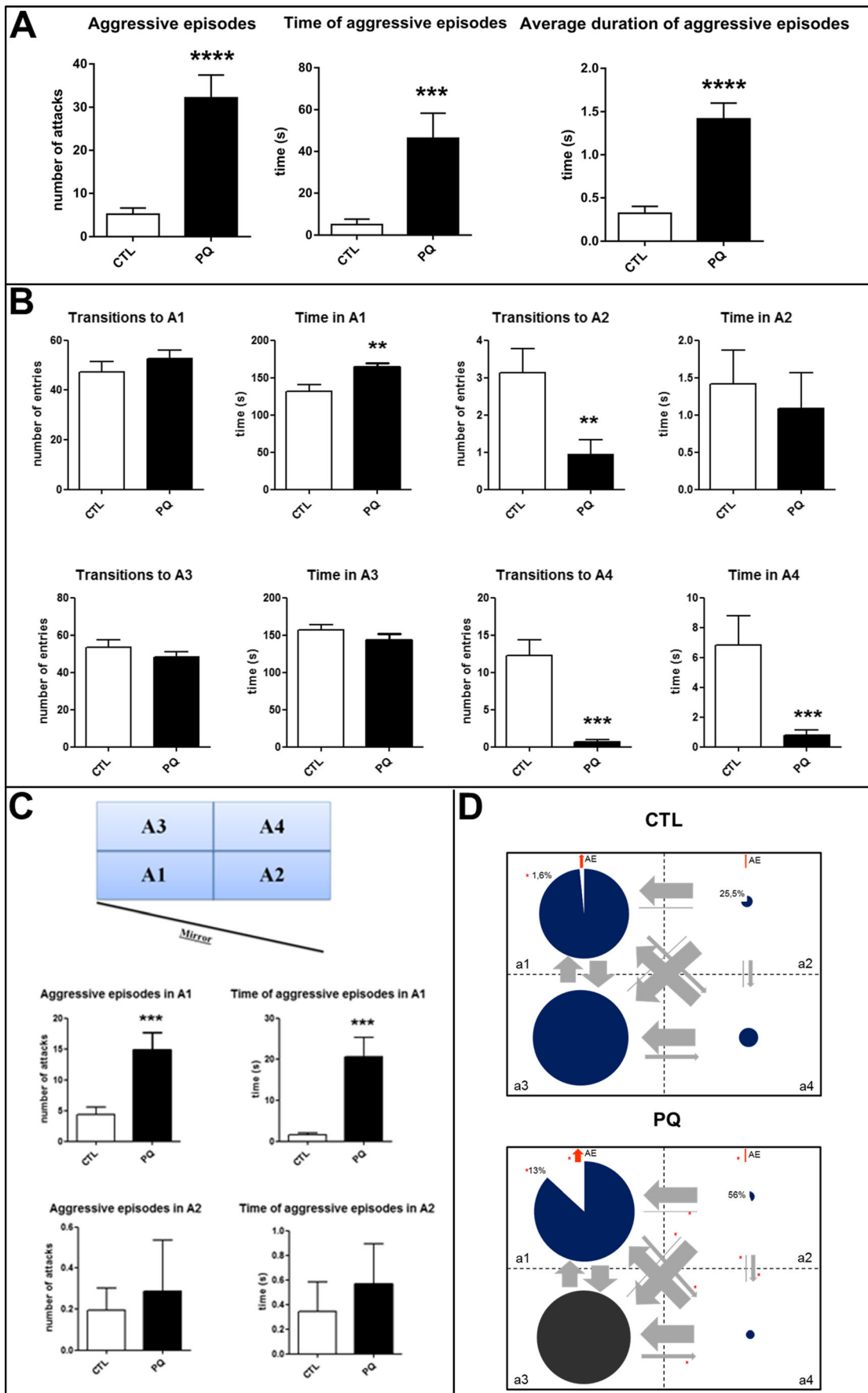
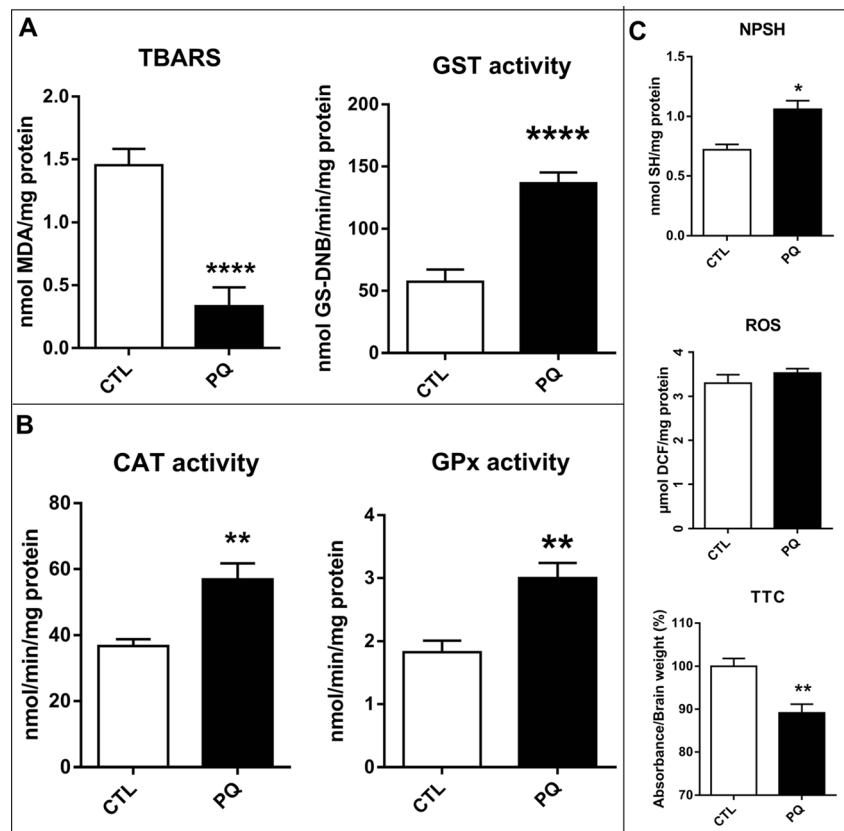


Fig. 4 PQ modulates biochemical parameters in zebrafish brain. **a** TBARS levels and GST activity. **b** CAT and GPx activity. **c** NPSH amount, ROS levels, and brain damage by measurement of formazan production (TTC protocol). Data are expressed as mean \pm SEM (* p < 0.05, ** p < 0.01, **** p < 0.0001, CTL control, PQ paraquat)



suggesting altered exploratory behavior. On contrary with the results previously reported by Bortolotto et al. [16], who showed a significant decrease in turn angle, transitions, and mean velocity after PQ injections (20 mg/kg), our data did not show alterations in the same locomotor patterns. We hypothesize that these differences could be explained, at least in part, by genotype differences and/or even by the random use of both sexes (male and female) in our experiments.

In the literature, rats treated with PQ (10 mg/kg) weekly for a month also presented a decrease in anxiety-like behavior after the first injection [16]. In rodents, alterations in anxiety-like behavior can be triggered by reduction of vesicular monoamine transporter that preceded depressive symptoms [49]. Other studies demonstrated a relation between depletion in serotonin levels with exaggerated aggression and decreased anxiety behaviors in human [50–52], monkey [53], and mice [54]. In our study, we observed similar results in these non-motor patterns. However, the data recorded are not sufficient to corroborate the hypothesis of possible effects of PQ treatment on serotonergic neurons. Additionally, PQ group showed a significant decrease in brain viability, suggesting that more studies are required to unravel the molecular mechanisms of anxiety-like behavior modulated by PQ.

In the mirror-induced aggression test, we verified that PQ-exposed fish spent more time closer to mirror and exhibited a significant increase in agonistic behavior, which may indicate

exacerbated aggression. These changes on behavioral functions strongly reinforce the idea that some phenotypes commonly observed in neurodegenerative disease models, such as anxiety-like behaviors and aggression, can be modulated in zebrafish chronically treated with PQ.

There is evidence that PQ impairs redox parameters [6–8], and, thus, we further investigated whether PQ-treated fish present changes on oxidative stress biomarkers. The significant increase in GST activity and decrease of TBARS levels observed could be related with the elimination of lipid peroxide by GST conjugated products. On the other hand, the increased GST activity can reduce glutathione (GSH) which could explain the elevation in NPSH levels of fish in response to oxidative damage induced by PQ. In addition, the analysis of GST superfamily in zebrafish revealed a great diversity of fish GSTs, showing 27 members in zebrafish [55]. The same authors reveal that analysis of GSTs superfamily in this species showed a conserved relationship with the human counterparts, offering a new perspective on evolution of GSTs in teleosts and other chordates [55]. In summary, there are differences in zebrafish GSTs expression and this fact could be involved in the increased GST activity in zebrafish treated with PQ. In mammals, the GST-pi isoform conjugates unsaturated aldehydes produced during the lipid peroxidation process and this regulation is coordinated with other antioxidant defenses. After the conjugation, the lipid peroxide by-

products may be eliminated from the cell by a GS-X pump transporter [56, 57]. Cytosolic, mitochondrial, and microsomal GST classes may act as important oxidative stress defenses, preventing lipid peroxidation [58]. Moreover, the increase in GPx and CAT activities with the significant increase in GST activity and NPSH levels suggest a compensatory mechanism in response to oxidant effects triggered by PQ, which are reported in the literature [2–4, 7]. The increase in antioxidants defenses represented by enzymatic and non-enzymatic antioxidants are probably involved in the protection against lipid peroxidation and ROS formation. We also observed that PQ induces a significant impairment in mitochondrial function using the TTC assay. Castello et al. [58] proposed that PQ^{2+} acts in complex III of the electron transport chain (ETC) promoting the increase in ROS. However, mitochondrial H_2O_2 production induced by PQ^{2+} in the brain is an early event that may further trigger other cellular events, such as nonspecific ETC inactivation, microgliosis, and NADPH oxidase activation. Thus, it is possible that the mitochondrial impairment detected could be one of the first effects triggered by PQ in the brain. Although we cannot properly assume a causal relationship between increased antioxidant responses/decreased lipid peroxidation and the behavioral effects so far, our data contribute to the construct validation of a chemically induced “PD-like model” by intraperitoneal PQ injections in zebrafish. Importantly, additional experiments are still required in order to understand the molecular mechanisms associated to the behavioral functions measured.

In conclusion, we showed that PQ modulates different behavior patterns in zebrafish, such as anxiety-like behaviors and aggression. Additionally, we also demonstrate that PQ enhances enzymatic antioxidant defenses, increases NPSH content, decreases lipid peroxidation and mitochondrial viability, but does not alter ROS levels. In this scenario, modulatory effects in GST activity could play a key role with peroxides detoxification. The behavioral alterations as well as the changes in redox parameters and mitochondrial function in zebrafish brain may serve as important tools to better understand the molecular mechanisms induced by chronic exposure to PQ in CNS.

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