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The Effect of Acetic Acid-Induced Pain in Parkinson's Disease Model in Zebrafish

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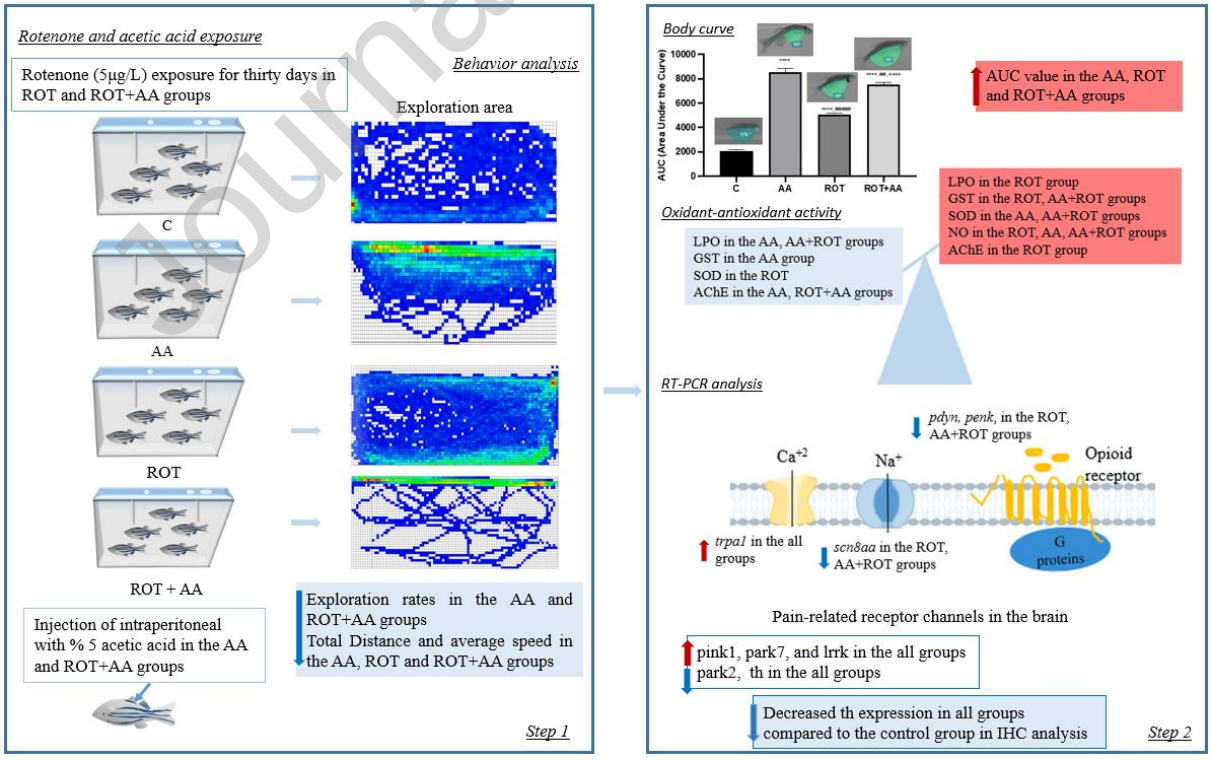
Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease caused by the degeneration of dopaminergic neurons and the accumulation of Lewy bodies. Pain is one of the most common non-motor symptoms in PD, but the molecular mechanism of pain in PD is not fully understood, which prevents early diagnosis of PD. We aimed to determine the changes in opioidergic pathways when external pain is inflicted by inducing pain intraperitoneally in zebrafish, for which we generated a rotenone-induced PD model. After behavioural analyses in control(C), acetic acid (AA), rotenone (ROT), and rotenone+ acetic

acid (ROT+AA) groups, catecholamine levels in brain tissue were determined by LC-MS/MS, expression of opioid peptides and their receptors by RT-PCR, expression of tyrosine hydroxylase by immunohistochemical method, and analyses of oxidant-antioxidant parameters by spectrophotometric methods. In the ROT group, distance travelled, average speed, and brain dopamine levels decreased, while LPO (lipid peroxidation) and NO (nitric oxide) increased as indicators of oxidative damage, and the SOD activity decreased. The mRNA expression of *lrrk*, *pink1*, and *park7* genes associated with PD increased, while the mRNA expression of *park2* decreased. This indicates that rotenone exposure is a suitable means to induce PD in zebrafish. The fact that body curvature was higher in the AA group than in the ROT and ROT+AA groups, as well as the decreased expression of *penka*, *pdyn*, and ion channels associated with the perception of peripheral pain in the ROT+AA group, suggest that mechanisms associated with pain are impaired in the rotenone-induced PD model in zebrafish.

Key Words: pain, behaviour, opioids, Parkinson disease, zebrafish

Graphical Abstract



Introduction

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with or resembling actual or potential tissue damage” (Raja et al., 2020). There have been numerous theories of pain for centuries, and many studies have been conducted on the occurrence and relief of pain (Free, 2002).

The opioid system controls pain, reward, and addictive behaviour. Opioids are endogenous and exogenous and are widely used to treat pain. Opioids exert their pharmacological effects through the three opioid receptors, mu, delta, and kappa (*oprm*, *oprd*, and *oprk* genes, respectively), which belong to the G protein-coupled receptor (GPCR) superfamily (Hasani and Bruchas, 2011). These opioid receptors in the brain are activated by a family of endogenous opioid peptides, including enkephalins, dynorphins, and endorphins released by neurons.

Morphine, an exogenous opioid, was discovered in 1804 by Friedrich Wilhelm Adam Sertürner and first isolated from the plant Papaver somniferum. Morphine enters cells by binding to opioid receptors and exerts its effects in living organisms. Because of their structural and functional similarities to morphine, endogenous opioids are also referred to as "morphine-like molecules" that have analgesic effects similar to morphine (Balch and Trescot, 2010).

Parkinson's Disease (PD) is the second most common neurodegenerative disease in which dopamine can no longer be produced due to the breakdown of dopaminergic neurons in the brain. PD has two distinct symptom groups, motor and non-motor. The non-motor symptoms may precede the motor symptoms in PD and are very important because of the impairment of quality of life in the advanced stages of the disease. The pathophysiology of non-motor symptoms is not fully understood, and dysfunctions of both the dopaminergic and non-dopaminergic systems are known to contribute to their development (Ahlskog, 2005). Pain is known to be one of the most common non-motor symptoms in PD (Aarsland et al., 2000; Findley et al., 2003). In individuals with PD, the pain symptom may occur long before the motor symptoms. Therefore, pain is considered an important symptom in the diagnosis of PD (Pages et al., 2008; Wasner and Deuschl, 2012).

Drugs such as levodopa are used to reduce symptoms in PD, but their mechanism of action has not been fully clarified. Although the decrease in pain threshold and tolerance in PD is associated with the loss of dopamine in the basal ganglia, the fact that the symptoms do not completely improve with dopaminergic drug (levodopa) suggests that non-dopaminergic

mechanisms are also effective in the emergence of pain symptoms (Brefel-Courbon et al., 2005; Schapira et al., 2017).

In recent years, zebrafish has been shown to be a suitable organism to generate neurotoxin-induced PD models (Unal and Emekli-Alturfan, 2019; Unal et al., 2020). The zebrafish is also a well-established animal model for studying pain behavior and pain monitoring (Costa et al., 2019; Deakin et al., 2019). Although dopaminergic, serotonergic, and cholinergic pathways are known to be associated with pain, current treatments for pain relief are not sufficient, and the pathways that are effective in the generation and perception of pain remain to be explored.

The aim of our study is to investigate the mechanisms associated with pain as an important non-motor symptom in PD by inducing acute pain intraperitoneally with acetic acid in a neurotoxin-induced PD model using zebrafish as a model organism.

Materials and Methods

Animals and treatment

Animal experiments were performed in accordance with the Council of the European Communities Directive of November 24, 1986 (86/609/EEC) (Louhimies, 2002). The Institutional Animal Care and Use Committee of Istanbul Medipol University approved all the procedures used in the study (38828770-604.01.01-E-62957). Adult zebrafish (Danio rerio) of the wild type AB /AB strain (10-12 months) were housed in an aquarium rack system (ZebTEC, Tecniplast, Italy) at 27–28 ± 1°C and a light/dark cycle of 14/10 hours.

Zebrafish (n: 60, weighing 0.350–0.500 g) were randomly divided into four groups using the GraphPad Prism calculator (https://www.graphpad. com/quickcalcs/randomize1/): control (vehicle) group (C), acetic acid group (AA), rotenone group (ROT), and rotenone acetic acid group (ROT +AA). G*Power 3.1. software (https://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/) was used to calculate the sample size for One-Way ANOVA with four groups. Using the significance level ($\alpha = 0.05$), power = 0.9, and effect size f = 0.8, the minimum number of fish required to obtain statistically significant results was estimated, and sample sizes were set as (n) = 15 fish/group. Male and female fish were included in a 50:50 male: female ratio per group, as Costa et al. (2019) found no differences in body curvature indexes between male and female fish after injection of 5.0% acetic acid. The control group included healthy fish treated with the vehicle.

The water in the tanks of control and rotenone-treated groups was changed every 48 h to ensure constant uptake by the zebrafish. Zebrafish were fed commercial fish food (Tetramine) twice a day. Tetramine content is minimum 11% crude oil, 51% crude protein, 2.3% calcium, 1.5% phosphorus, maximum 15% ash, 3% crude fibre and 6.5% moisture.

ROT and ROT +AA groups were exposed to fish water by adding rotenone (5µg/L) for thirty days. 5 µg/L rotenone (Sigma, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO) (Sigma, USA). The amount of rotenone was administered according to our previous studies (Cansız et al., 2021; Unal et al., 2023; Yurtsever et al., 2020). At the end of 30 days, the ROT+AA and AA groups were treated with 5% acetic acid (Costa et al., 2019). Acetic acid (5% acetic acid: saline) was administered by intraperitoneal injection with a Hamilton syringe as 5 µl per gram.

At the end of 30 days, locomotor activities were determined and the fish were anaesthetized and euthanized by decapitation, followed by rapid removal of the brain for RNA extraction, biochemical analysis, and immunohistochemical analysis. Researchers performing the treatments were unaware of group assignment to ensure that all fish in the experiment were handled, monitored, and treated in the same manner. The bioanalytical and behavioural portions of the experiments, as well as the data analysis, were performed by blinded experimenters.

Behavior Analysis

After exposure to acetic acid, the fish were recorded on camera for behavioural analysis in a special tank (21cm x 9 cm x 11 cm) for 20 minutes and then the fish were anaesthetized using ice water and sacrificed. The camera recordings of the zebrafish were evaluated to analyse the explored areas, total distance, exploration rate, and average speed during the 20 minutes in the tank. Average speed and total distance travelled by fish were analysed using the Tox-Track programme, which detects and tracks animals in enclosed arenas (Rodriguez et al., 2018). For the exploration rate, a partition of 25 images on the y-axis and 60 images on the x-axis was created using the tracking software Tox-Track, and the ratio of zebrafish activity in vertical and horizontal areas of the tank were quantified and analysed (Nadig et al., 2020).

Writhing Response

Body curvature was measured every 30 seconds for the first 6 minutes after acetic acid was injected into the fish (Costa et al., 2019). Body curvature was measured using Kinovea 8.15

software. Three points were selected to estimate the body curvature of the fish: a frontal point (at the front of the head), a central point (in the middle of the body), and a posterior point (at the caudal fin) (Fig. 1). The results obtained with the program Kinovea were subtracted from 180º to calculate a value representing a body curvature index.

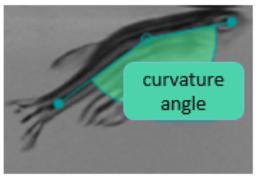


Fig. 1. Angular body curvature

Biochemical Analysis

Brain tissues were collected from anesthetized fish, and tissues from four fish were pooled for each group for each biological replicate, and three technical replicates were performed for each biological replicate. Tissues were homogenized in physiological saline to produce 10% (w/v) homogenates. After centrifuging briefly, the supernatant was separated and used to determine biochemical parameters. Total protein content was measured by the method of Lowry, and results were expressed per protein (Lowry et al., 1951). The Yagi's method was used to measure malondialdehyde (MDA) levels as thiobarbituric acid reactive substances as the end product of lipid peroxidation (Yagi, 1994). The method of Miranda was used to measure nitric oxide (NO) levels (Miranda et al., 2001). Superoxide dismutase (SOD) activity was measured by the ability of SOD activity to increase the effect of photooxidation of o-dianisidine sensitized by riboflavin, and the results were expressed in U/mg protein (Mylroie et al., 1986). Glutathione *S*-Transferase (GST) catalyzes the conjugation of GSH, and GST activity was determined using a spectrophotometer at 340 nm (Habig and Jakoby, 1981). Acetylcholine esterase (AChE) activity is based on the hydrolysis of acetylcholine to choline and acetic acid. AChE activity was measured using a spectrophotometer at 405 nm (Ellman et al., 1961).

RT-PCR Analysis

Rneasy Mini Kit and Qiacube (Qiagen, Germany) were used to isolate RNA from zebrafish. Single-stranded cDNA was synthesized from 1 μg of total RNA using RT^2 Profiler PCR Arrays (Qiagen). DNA Master SYBR Green kit (Qiagen, Germany) was used for PCR analyses. Quantitative RT-PCR was used for gene expression analyses using the Rotor Gene-Q Light Cycler (Qiagen, Germany). β-actin was used as the housekeeping gene, and the delta-

delta Ct method was used to calculate relative transcript levels by normalizing the values to the housekeeping gene (Livak and Schmittgen, 2001). For each biological replicate, three technical replicates of each RT-PCR reaction were performed. Primers used for gene expression analysis are given in Table 1.

Table 1 Forward and reverse primers used in the study

Primers	**(forward/reverse)**
β actin	5′-AAGCAGGAGTACGATGAGTCTG-3′
	5′-GGTAAACGCTTCTGGAATGAC-3′
lrrk2	5′-CCCTAAACCGCAGAGTATCA-3′
	5′-ATTCATAGTCCACCGGTCTG-3′
park7	5′-GGCCGGTAAAAGAGCGTTAG-3′
	5′-ACCCATGAGTCCTCCACTA-3′
th	5′-ATCTGTACACGACCCACGCC-3′
	5′-TGCCACTGGCCTCAACTGAA-3′
pink1	5′-GGCAATGAAGATGATGTGGAAC-3′
	5′-GGTCGGCAGGACATCAGGA-3′
park2	5’GCGAGTGTGTCTGAGCTGAA-3’
	5’CACACTGGAACACCAGCACT-3’
pdyn	5′-GATGCGCGCAACAGATCTCC-3′
	5′-CGTCTGCGCCTGTATTGAGC-3′
penka	5′-CGTTTACCGGCTCCTCCGAC-3′
	5′-CAAGGCGATCCTCTTCGGTGA-3′
zasic1.3	5′-AGCATACGAAGTCGCTGGGT-3′
	5′-GTCATGCAGAGAAACAATTACCAGA-3′
cox1	5′-AGGGAGCTTCGACTTCAGCC-3′
	5′-TACCGCACCAGGTCGTGTTT-3′
scn8aa	5′-CCTCCCGAGTGGTCCAAACA-3′
	5′-TCAGCGCAGATACATTCCCC-3′
trpa1	5′-AGCCGATAAAGAAGTGCTCCCA-3′
	5′-CCGCTGCGTTTCCTTGCTTA-3′

Tyrosine hydroxylase immunohistochemistry

For immunohistochemistry analysis, zebrafish brain samples were fixed in 10% neutral buffered formalin, dehydrated in ascending ethanol series (70%, 90%, 96% and 100% ethanol), cleared in toluene, and embedded horizontally in paraffin. Four-µm-thick sections were taken from the diencephalic region of the brain tissue on the positively charged slides. After deparaffinization and rehydration, the sections were incubated with 3% H2O2 in methanol to inhibit endogenous peroxidase activity. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) in 200-W microwave. To eliminate nonspecific immunoreactivity, sections were treated with protein blocking solution (mouse- and rabbit-specific HRP/DAB micropolymer detection kit, ab236466, Abcam). Sections were incubated with mouse anti-TH primary antibody (1:50 dilution, Immunostar 22941) at +4 ^{0}C overnight. Following incubation of primary antibody, mouse specifying reagent (complement) and goat anti-rabbit HRP-conjugate were applied according to the manufacturer's protocol. Immunostaining was visualized using 3.30-diaminobenzidine tetrahydrochloride. The sections were counterstained with Mayer hematoxylin, dehydrated ethanol and finally mounted with entellan. All stained sections were photographed using a digital camera (Olympus DP72, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan). Finally, Fiji image program was used to calculate the density of immunoreactivity of TH.

LC/MSMS Analysis

The levels of dopamine, serotonin, 5-HIAA, DOPAC and norepinephrine in brain tissue were determined by LC-MS/MS method. For this purpose, the brain tissues removed at the end of the experiment were homogenized with saline and the homogenates were centrifuged at 13,000 g for 20 minutes. Measurements of neurotransmitter in homogenates filtered through a syringe filter were determined by LC-MS/MS method. For quantification of neurotransmitter by LC-MS/MS, the loading steps of the homogenate to the column were performed using the following procedure. First, the sample was filtered directly using a 0.45 µm nylon filter. Then, 950 µL of filtrate was transferred to a vial and 50 µL of internal standard was added, and finally 10 µL of sample was injected into the LC-MS/MS system (Agilent 6470 QQQ). LC-MS/MS Calibration Range* (ng/ml) 0.05-0.1-0.5-1.0-5.0 *Prepared using 0.9% NaCl solution.

Data analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). Normality of the distribution was tested using the Shapiro–Wilk test. To compare the

groups one-way ANOVA test was used which was followed by Tukey's multiple Comparison tests. The data obtained were given as the mean ± standard deviation. p-value less than 0.05 was regarded as significant.

Results

Results of body curvature measurement

In our study, we observed changes in the pain mechanism as a result of intraperitoneal acetic acid injection in the PD model induced by rotenone.

First, acetic acid elicited an abdominal constriction-like response determined by measuring a body curvature index. The changes in body curvature index over time are shown in Figure 1A. The area under the curve (AUC) values of the body curvature measurements are shown in Figure 1B. The AUC increased significantly in the AA, ROT and ROT +AA groups compared to the control group ($p < 0.0001$). The AUC decreased significantly in the ROT group compared to the AA group ($p < 0.0001$), while it increased significantly in the ROT +AA group compared to the ROT group ($p<0.0001$) (Figure 2).

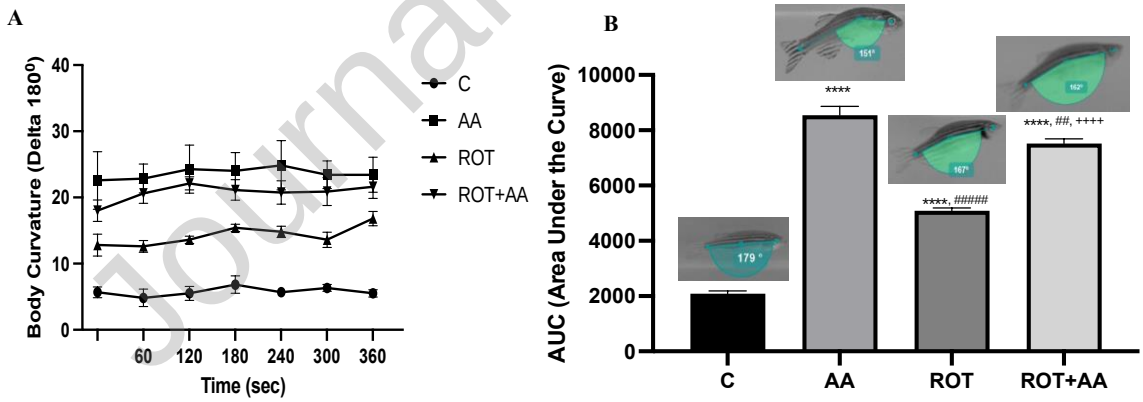


Fig. 2. Body curvature in zebrafish: A. Graph of changes in body curvature index over time. The effects of administered agents were assessed by measuring the body curvature index, which is a response like abdominal contraction. Three points were selected to determine the body curvature, namely an anterior point (in front of the head), a central point (in the middle of the body) and a posterior point (in the caudal fin), which were measured using the program Kinovea. The values obtained were subtracted from 180° and expressed as delta 180°. **B.** Temporal changes determined in the body curve index were expressed as area under the curve

(AUC). Differences between groups were determined by one-way analysis of variance One-Way ANOVA (factor: applied agents), and then pairwise comparisons between groups were evaluated using Tukey's multiple comparison test. **** $p<0.0001$ different from Control; #### $p<0.0001$ different from AA; ## $p<0.01$ different from control ++++ $p<0.0001$ different from ROT

Results of locomotor activity

The fish in the control group showed swimming behaviour in the whole area of the tank during the 20 minutes they spent in the tank. AA group showed swimming behaviour near the surface of the tank. The red-orange areas in the upper right and middle parts of the tank are the areas where the fish remained immobile. In addition, rapid tail movements and hyperlocomotion were observed in the first seconds after acetic acid exposure. Later, slowed swimming movements and immobility of the fish were observed. The ROT group spent more time near the bottom of the tank. The ROT group spent more time near the bottom of the tank. The ROT +AA group showed predominantly swimming behaviour near the surface of the tank (Fig. 3A).

The exploration rates decreased significantly in the AA and ROT+AA groups compared to the control group ($p<0.001$ and $p<0.0001$ respectively). The exploration rate in the ROT group was significantly higher than in the AA group ($p<0.0001$). The exploration rate decreased significantly in the ROT+AA group compared to the ROT group ($p<0.0001$) (Fig. 3B).

The total distance decreased significantly in the AA, ROT and ROT+AA groups compared with the control group ($p<0.0001$). In the ROT group total distance was significantly higher than in the AA group ($p<0.0001$) and acetic acid treatment resulted in significantly lower total distance in the ROT group ($p<0.0001$) (Fig. 3C).

The average speed decreased significantly in the AA, ROT and ROT+AA groups compared to the control group ($p<0.0001$). The average speed in the ROT group was found to be significantly higher than in the AA group ($p<0.0001$). On the other hand, treatment with acetic acid in the ROT group significantly decreased the average speed ($p<0.05$) (Fig. 3D).

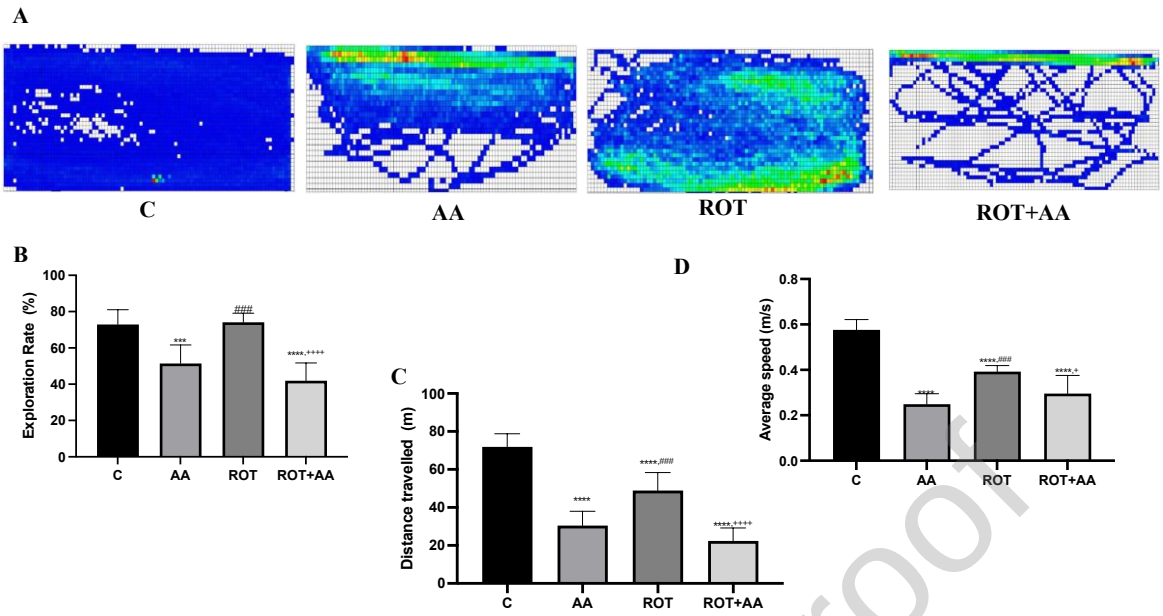


Fig. 3. A. **Explored Areas**, Scorings were performed from 1 to 5 for areas where fish spend time; blue, green, yellow, orange, and red, respectively (1-5). **B. Exploration Rate, C. Total Distance, D. Average Speed.** Differences between groups were determined by one-way analysis of variance One-Way ANOVA (factor: applied agents), and then pairwise comparisons between groups were evaluated using Tukey's multiple comparison test. ****p<0.0001, ***p<0.001 different from control; $^{\#\#\#\#}$p<0.0001 different from AA group; $^{+}$p<0.05 and $^{++++}$p<0.0001 different from ROT group

Results of biochemical parameters

Oxidative stress has a vital role in the destruction of dopaminergic neurons in PD, leading to nitrative and oxidative damage to critical cellular components. To determine the oxidant/antioxidant status in the brain, the levels of lipid peroxidation (LPO), NO, and the activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione S-transferase (GST) were determined.

Compared to the control group, LPO significantly increased in the ROT group ($p < 0.01$), while it significantly decreased in the AA and ROT +AA groups ($p<0.0001$ and $p<0.01$ respectively) (Fig. 4A).

SOD activities decreased significantly in the ROT group ($p < 0.0001$) and increased significantly in the AA and ROT +AA groups compared with the control group ($p < 0.05$ and $p < 0.001$, respectively). On the other hand, acetic acid treatment significantly increased the activity of SOD in the ROT group($p<0.0001$) (Fig. 4B).

Nitric oxide (NO) is a free radical and a neuronal messenger molecule that plays a role in central pain sensitization (Freire et al., 2009). Research conducted in humans and in experimental models of PD has supported the contribution of NO to oxidative stress, inflammation, and impairment of mitochondrial function (Jiménez-Jiménez et al., 2016). To investigate the role of NO in the pain mechanism of PD, we examined the levels of NO in the brain and found a significant increase in the AA, ROT and ROT +AA groups compared to the control group ($p < 0.0001$, $p < 0.05$ and $p < 0.00001$, respectively). Acetic acid treatment significantly increased NO values in the ROT group ($p < 0.01$) (Fig. 4C).

GSTs have vital importance in detoxification. We determined GST activity in brain as the detoxification of environmental chemicals including rotenone and xenobiotics are associated to PD (Ozdamar and Can-Eke, 2013). Compared to the control group, GST activity increased significantly in the ROT group, whereas a significant decrease was observed in the AA group ($p<0.001$). In the ROT group, acetic acid treatment led to significantly higher GST activity ($p<0.0001$) (Fig. 4D).

Cholinergic dysfunction is important in the pathophysiology of many PD, and AChE inhibitors have been shown to be successful in treating individuals with PD, who exhibit cognitive impairment (Pagano et al., 2014). In addition, cholinergic deficiency is considered an early sign of persistent pain and cognitive impairment (Eldufani and Blaise, 2019). AChE activity significantly decreased in both the AA and ROT +AA groups ($p < 0.001$), while a significant increase was observed in the ROT group ($p < 0.001$). Moreover, treatment with acetic acid in the ROT group resulted in a significant decrease in AChE activity ($p < 0.0001$) (Fig. 4E). In the ROT group, the increased AChE activity suggests decreased acetylcholine secretion, which is associated with the progression of PD.

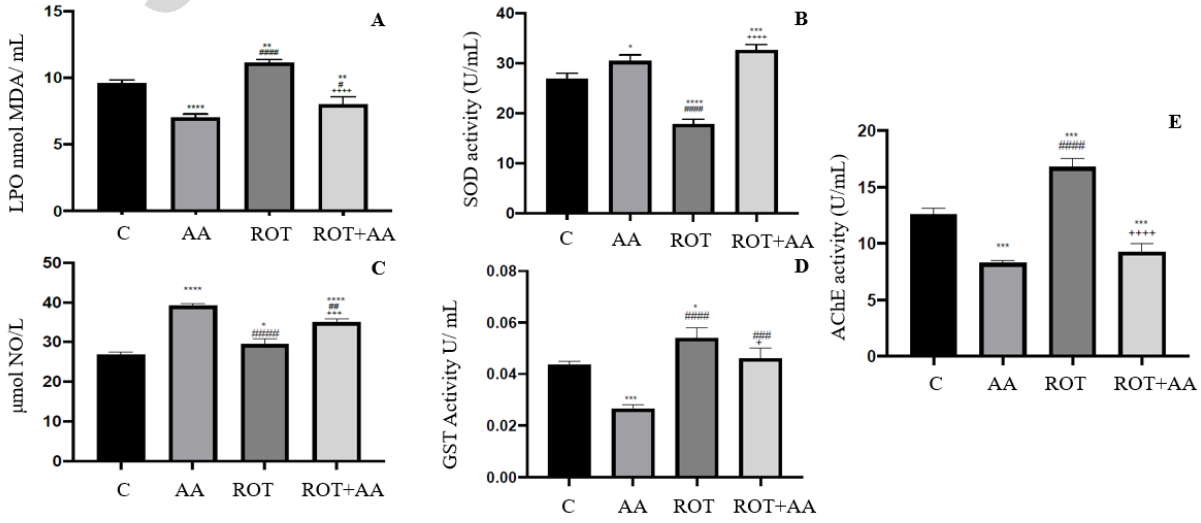


Fig. 4. A. Lipid Peroxidation levels of the groups. B. Superoxide Dismutase activities of the groups. C. Nitric Oxide levels of the groups D. Glutathione *S*-Transferase (GST) activity of the groups. E. Acetylcholinesterase activity (AChE) of the groups. Data are expressed as mean ± SD from the three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 different from C group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#\#}$ p<0.0001 different from AA group; $^{+}$ p<0.05, $^{+++}$ p<0.001, $^{++++}$ p<0.0001 different from ROT group. C: Control; AA: Acetic Acid; ROT: Rotenone; ROT+AA: Rotenone+Acetic Acid, MDA: Malondialdehyde.

Results of Gene expression Analysis

The mRNA expression levels of *penka* and *pdyn* were upregulated in the AA group and downregulated in the ROT and ROT +AA groups. Rotenone treatment decreased the mRNA expression levels of *penka* and *pdyn* compared with the AA group ($p < 0.001$). Acetic acid treatment in the ROT group decreased the mRNA expression levels of *penka* compared to the ROT group ($p < 0.05$) (Fig. 5A).

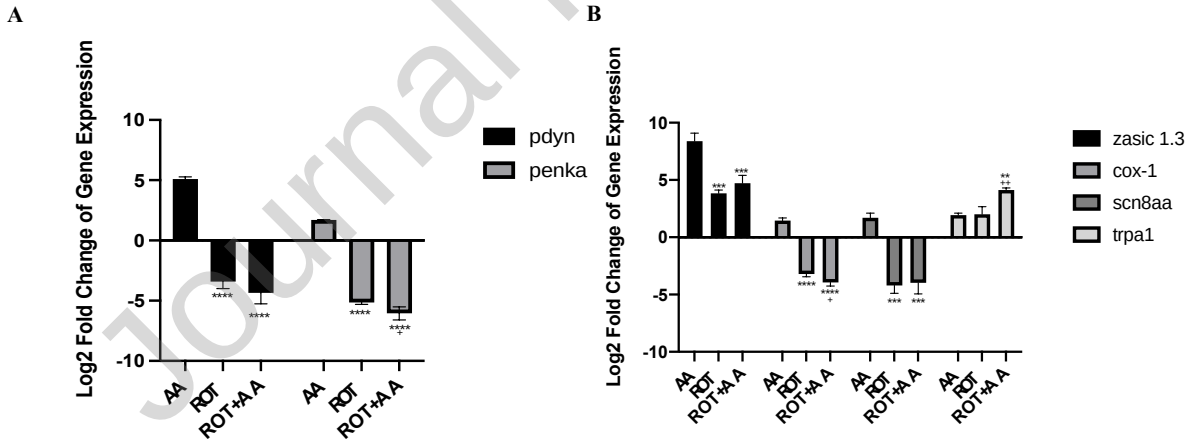


Fig. 5. Bar graph presentation of the log2 fold change of A. *pdyn* and *penka* transcripts and **B**. *zasic 1.3, trpa1, scn8aa* and *cox-1* transcripts quantified by RT-PCR. All RT-PCR results are normalized to *β-actin,* the house keeping gene and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented mean ± SD. **** p<0.0001; *** p<0.001, ** p<0.01, * p<0,05 significantly different than the AA; $^{+}$ p<0.05, $^{++}$ p<0.01 significantly different than the ROT; SD: standard deviation.

While *zasic1.3* mRNA expressions were upregulated in the AA, ROT and ROT +AA groups, *zasic1.3* mRNA expression levels decreased significantly in the ROT and ROT +AA groups compared to the AA group ($p < 0.001$). trpa1 mRNA expressions were upregulated in the AA, ROT and ROT +AA groups, and acetic acid treatment in the ROT group significantly increased *trpa1* mRNA expression compared to the ROT and AA groups ($p < 0.01$) (Fig. 5B).

While the mRNA expressions of *scn8aa* and *cox-1* were downregulated in the ROT and ROT +AA groups, acetic acid treatment increased their expressions. In addition, acetic acid treatment resulted in decreased *cox-1* mRNA expression in the ROT group compared with the ROT group ($p < 0.001$ and $p < 0.0001$, respectively) (Fig. 5B).

Significant changes were observed in the expression of PD -related genes. The mRNA expression levels of *pink1*, *lrrk* and *park7* were upregulated in the AA, ROT and ROT + AA groups, and their expression significantly increased in the ROT and ROT + AA groups compared to the AA group. In addition, acetic acid treatment significantly increased the expressions of *park7* and *lrrk* in the ROT group compared with the AA group. (Figure 6A and 6B).

The expressions of *park2* were downregulated in the AA, ROT and ROT+AA groups. The mRNA expression of *park2* decreased in the ROT and ROT+AA groups compared to the AA group ($p<0.0001$), and acetic acid treatment in the ROT group decreased the mRNA expression of *park2* (Fig. 6A).

The mRNA expressions of *th* were downregulated in the AA, ROT and ROT+AA groups, while the mRNA expressions of *th* were significantly higher in the ROT group than in the AA group ($p<0.05$) (Fig. 6B).

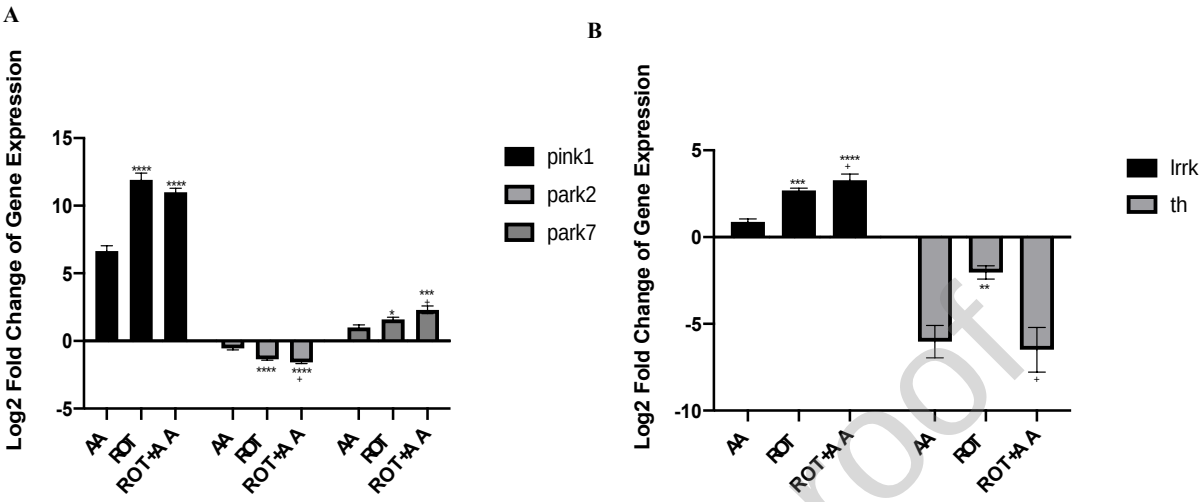


Fig. 6. Bar graph presentation of the log2 fold change of **A**. *pink1*, *park2*, and *park7* transcripts and **B**. *lrrk* and *th* quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the house keeping gene and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± SD. **** $p<0.0001$; ** $p<0.01$; * $p<0.05$ different from AA group; $^{+}$ $p<0.05$ different from the ROT group; SD: standard deviation.

Tyrosine hydroxylase immunohistochemistry results

Tyrosine hydroxylase (TH) immunoreactivity in brain tissue samples was observed as brown color in both neurons and fibers. In the control group, TH- positive neurons and fibers were observed in the diencephalon region. In the AA and ROT groups, a slight decrease in immunoreactivity of the TH-positive neurons and a significant decrease in immunoreactivity of the TH-positive fibers in the diencephalon region were observed. In the ROT +AA group, a significant decrease in immunoreactivity of the TH-positive neurons (arrows) and fibers (arrowheads) was observed in and near the posterior tuberculum in the diencephalon region (Fig. 7.A-H).

The results of the integrated density of TH immunohistochemical staining performed in zebrafish brain are shown in Fig. 7.I. There was a significant decrease in TH expression in the diencephalon region of the zebrafish brain in the AA, ROT, and ROT+AA groups compared

to the control ($p<0.0001$). The TH expression in the ROT group was significantly higher than that of AA group ($p<0.01$) (Fig. 7I).

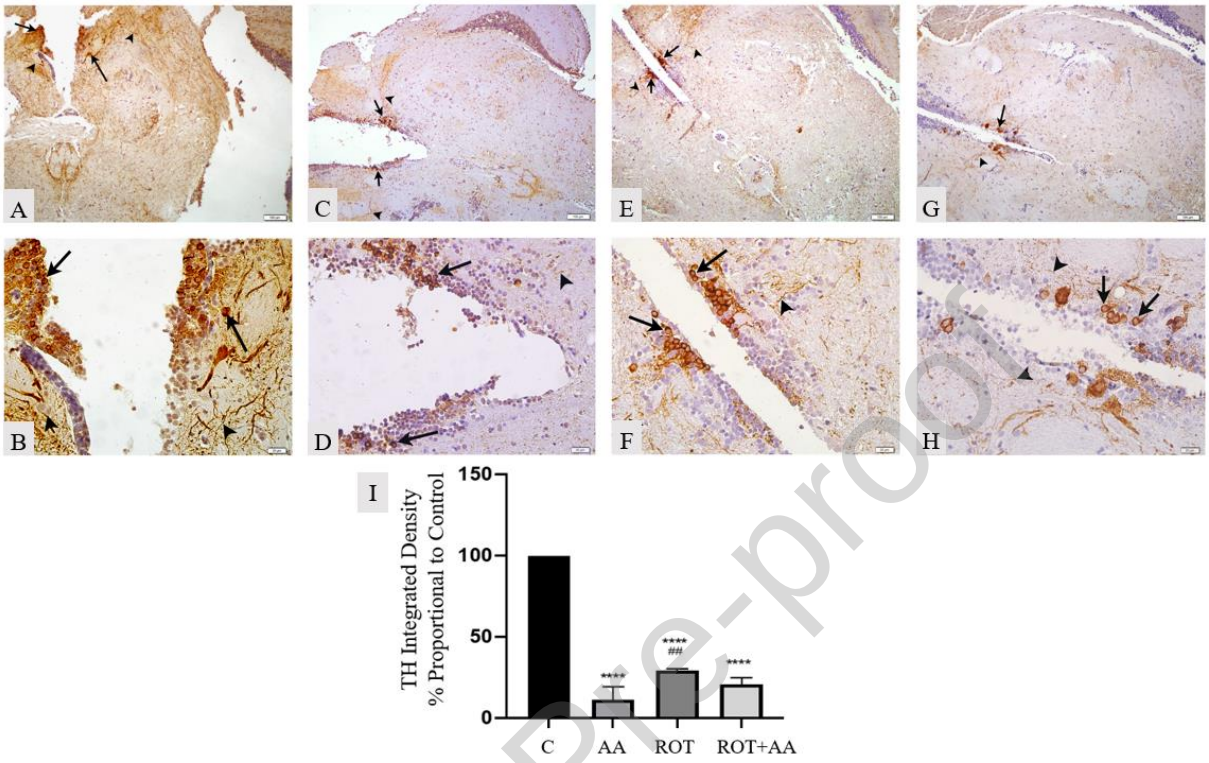


Fig. 7. A-H. Representative light micrographs of Tyrosine hydroxylase (TH) - immunostained zebrafish brain tissues. TH-positive neurons (arrows) and processes (arrowheads) in the posterior tuberculum and in near of the posterior tuberculum in the diencephalon region. I. The graph of TH immunostaining integrated density ****$p<0.0001$ Different from control; ##$p<0.01$ different from AA, Original magnifications are x100 (A, C, E, G) and x400 (B, D, F, H) (A-B: Control group; C-D: AA group; E-F: ROT group; G-C: ROT+AA group)

LC/MSMS Analysis

Dopamine levels decreased significantly in AA, ROT and ROT+AA groups compared to the control group ($p<0.0001$, $p<0.05$ and $p<0.01$, respectively). In the AA group, dopamine levels were significantly lower compared to the ROT and ROT+AA groups ($p<0.0001$). While Dopac levels increased in the AA group compared to the control group, they decreased significantly in the ROT and ROT+AA groups ($p<0.05$, $p<0.001$ and $p<0.0001$, respectively). Dopac levels decreased significantly in the ROT and ROT+AA group compared to the control and AA groups. Compared to the control group, the AA group had significantly increased

norepinephrine levels ($p<0.001$), while norepinephrine levels significantly decreased in the ROT group ($p<0.05$). A significant decrease was observed in the ROT and ROT+AA groups compared to the AA group ($p<0.0001$ and $p<0.01$, respectively).

Serotonin play an important role in the sensory and emotional features of pain; secretion of serotonin in the forebrain is dependent on robust local dopaminergic neurotransmission (Mendlin et al., 1999). Serotonin levels also affect locomotor activity, but no significant difference was found between groups based on the results of LC-MS/MS analysis. While 5-HIAA levels significantly increased in the AA and ROT groups compared to the control ($p<0.0001$ and $p<0.01$ respectively), acetic acid treatment resulted in a significant decrease in 5-HIAA levels in the ROT group ($p<0.01$) (Fig. 8).

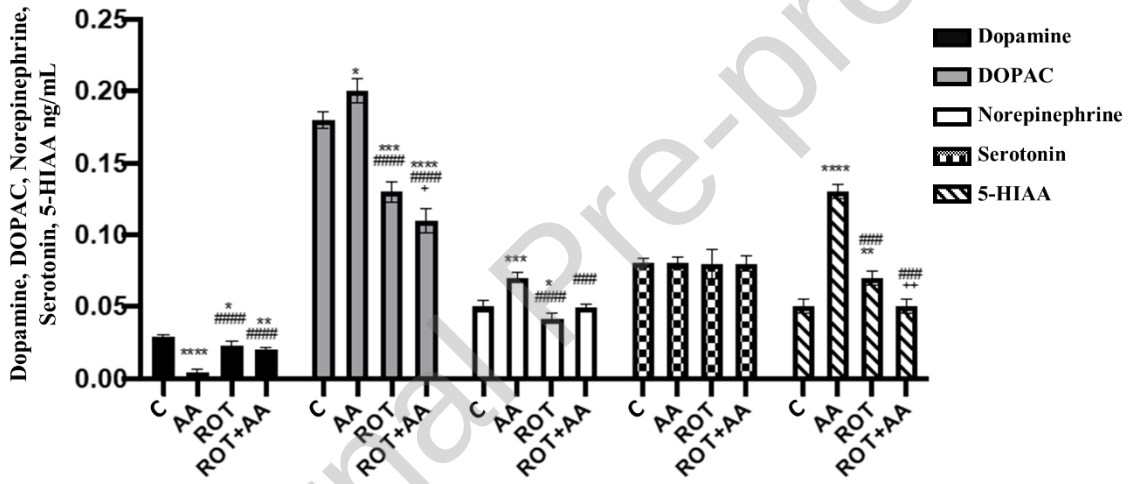


Fig. 8. Dopamine, Dopac, Norepinephrine, Serotonin and 5-HIAA levels of the groups
**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; *$p<0.05$ different from the C group; $^{\#\#\#\#}$ $p<0.0001$; $^{\#\#\#}$ $p<0.001$ different from group AA; $^{++}$ $p<0.01$ different from ROT group. A5-HIAA: 5-hydroxyindolacetic acid.

Discussion

We observed changes in the pain mechanism caused by intraperitoneally administered acetic acid in the PD model. In the neurotoxin induced PD model, we observed significant changes in behavioral parameters such as distance travelled, average speed, and decreased brain dopamine levels, as well as increased LPO and NO levels and decreased SOD activity as indicators of oxidative damage. In response to oxidative stress, there was an increase in the expression of the PD-related genes *lrrk, pink1,* and *park7* and a decrease in expression of *park2*, which is known to be inactivated by nitrosative, dopaminergic, and oxidative stress.

This study confirmed the relevance of the PD model for rotenone-induced zebrafish as demonstrated by both our team as well as other previous studies (Yurtsever et al., 2022; Ünal et al., 2019; Ünal et al., 2020; Sürmen et al., 2022).

The acetic acid-induced Writhing test is a widely used visceral pain model to screen potential analgesic (Kalueff et al., 2013). Consistent with our results, Costa et al. (2019) showed that acute intraperitoneal administration of 5% acetic acid to zebrafish significantly altered body curvature and the area under the curved area (Costa et al., 2019a; Deakin et al., 2019; Costa et al., 2019b).

Consistent with data of Costa et al. (2019) and Deakin et al. (2019), in our study, the AA group swam close to the water surface, which is also referred to as passive swimming, and they showed a response to trying to get out of the water. The zebrafish in the ROT group swam in a specific area at the bottom of the aquarium, and also remained still there. This phenomenon is called geotaxy and is usually observed when fish are exposed to toxic substances (Kalueff et al., 2013). ROT+AA group also showed a tendency to swim near the surface of the tank, indicating neurological impairment, and spent more time in the upper right and left corners of the upper surface of the tank (hypolocomotion) (Farrand et al., 2017; Aryal et al., 2019).

Locomotor activity is closely related to catecholamine levels. Because the dopaminergic system is present in both descending and ascending brain pathways, two different functions of the dopaminergic system have been hypothesised in the modulation of pain: pain-activatory and inhibitory. Moreover, the effect of dopaminergic pathways on pain modulation varies not only with dopamine receptor types, but also depending on the type of pain (Li et al., 2019). We hypothesise that this may be due to the differet pain types in the AA group (visceral pain) and rotenone-induced groups (visceral pain, other types of pain).

Rotenone, a neurotoxic agent, reduces electron flow to ubiquinone from Fe-S centres in complex I in mitochondria, reducing ATP production and NADH oxidation, and thus electrons leaking from complex I cause the formation of ROS. The increased production of ROS induced by rotenone leads to aggregation of α-synuclein, which in turn leads to neurodegeneration (Schuler, 2001; Sherer et al., 2001). Consistent with these data, in our study we found increased LPO and GST activity in response to increased oxidative damage in the ROT groups and increased expressions of *lrrk2* and *park7* induced by ROS. On the other hand, acetic acid treatment decreased LPO levels. Acetic acid belongs to short-chain fatty

acids (SCFAs), and beneficial effects of SCFAs on signalling pathways, involved in inflammation and lipid metabolism have been reported in recent years (He et al., 2020). Accordingly, Hu et al. (2020) reported that SCFAs have beneficial effects on the metabolism of pancreatic β-cells and can reduce oxidative stress resulting from diabetes (Hu et al., 2020). The increased SOD activity in the ROT+AA may be associated with the suppressive effect of acetic acid on the production of proinflammatory mediators.

Inflammatory, somatic, and visceral pain has been associated with NO (Abaclolu et al., 2000; Zhuo et al., 1993). NO may act as a potent inhibitor of LPO reaction by scavenging lipid peroxyl radicals. In addition, increased NO may also inhibit many potential activators of lipid peroxidation. Moreover, NO is thought to both induce and inhibit lipid peroxidation (Hogg, 1999). In our study, an increase in NO level was observed in the acetic acid-induced pain groups, which may be one reason for the decreased LPO level in these groups.

In the striatum, acetylcholine plays an important role in locomotor activity. Dopamine inhibits the release of acetylcholine, and locomotor activity is maintained by the balance between the dopaminergic system and the cholinergic system (Myslivecek, 2021). Experimental PD models have demonstrated decreased dopamine levels along with acetylcholine release and impaired locomotor activity (Rizzi and Tan, 2017).

Painful stimuli increase the release of acetylcholine to induce analgesia in animals (Eisenach et al., 1996). In the acetic acid-treated groups, the decrease in the activity of AChE, the enzyme responsible for the hydrolysis of acetylcholine, is an indicative of a decrease in acetylcholine release. In the rotenone-induced PD model, an increase in AChE activity and a decrease in dopamine release are compatible. AChE inhibitors are used to increase dopamine release in neurodegenerative diseases (Zhang et al., 2004).

NO has a mechanism of action that can cause changes in the tertiary structure of proteins by affecting the structure of disulfide bonds between cysteine residues (Stamler et al., 1997; Davis et al., 2001; Jaffrey et al. 2001). Because ASICs contain a large number of cysteine residues on their outer surfaces, changes in their structures can occur through the direct mechanism of action of NO (Ahern et al. 2002). Cadiou et al., demonstrated in their study that acid-mediated pain in humans is enhanced by NO-releasing substances (Cadiou et al. 2007).

The cation channel transient receptor potential ankyrin 1 (TRPA1) is a member of the TRP-channel superfamily (Nassini et al. 2014). TRPA1 has been shown to be sensitive to ROS and activated by ROS, and the most prominent feature of TRAP1 is its very pronounced

sensitivity to reactive products of oxidative and nitrosative stress (Stanford and Taylor-Clark, 2018). TRPA1 has been shown to act as a crucial sensor of tissue damage, contributing to pain perception, which is an important function of the nervous system (Obata et al. 2018). The increase in mRNA expression of *trpa1* in exposure groups in parallel with NO levels is consistent with these data.

Cox inhibitors have been reported to prevent the loss of dopaminergic neurons due to MPTP toxicity (Teismann and Ferger, 2001; Carrasco et al., 2005). Consistent with these data, rotenone exposure decreased *cox-1* mRNA expression but also decreased dopamine levels compared with the AA group. The increase in *cox-1* mRNA expression in the AA group is an indicator of pain generation. In rotenone groups, the decrease in *cox-1* mRNA expression indicates that the pain mechanism is affected by rotenone and not by the absence of pain.

Acid-sensing ion channels (ASICs) are excitatory receptors for extracellular H^+. Because synaptic vesicles in the central nervous system are acidic (pH 5.7), H^+ is released along with other transmitters. During inflammation, the extracellular H^+ concentration may also increase significantly. ASICs may therefore contribute to the perception of pain stimuli in the peripheral nervous system. Consistent with this information, in our study, the mRNA expression of *zasic1*.3 increased in the AA group, but less in the rotenone-treated groups than in the AA group.

Nav1.6 is encoded by the *scn8a* gene and is one of the main voltage-gated sodium channels in the central nervous system. Nav1.6 has also been associated with neuropathic pain in a number of studies. Silencing nav1.6a with morpholino in zebrafish embryos was found to reduce tactile sensitivity in morphant embryos (Pineda et al., 2005). Oin et al. (2017) showed that knockdown of the Nav1.6 gene alleviated mechanical behaviour in a model of local inflammation and neuropathic pain (Qin et al., 2017). In our study, *scn8aa* mRNA expressions and body curve index decreased in ROT groups, while they increased in AA group. Based on these data, we can conclude that the response to pain is attenuated in the rotenone-induced PD model.

It has been suggested that biomarker levels in CSF of PD patients change because dopaminergic signals affect opioid synthesis by promoting PDYN and reducing production of PENK (Gerfen et al., 1991). However, results from animal models suggest that extensive striatal dopamine withdrawal may be a necessary condition before PDYN and PENK levels in the brain can be significantly altered (Ziolkowska et al. 1995). Similar to our findings, another

study conducted in humans showed that both pENK and pDYN mRNA levels decreased in the putamen of older controls and older individuals affected by PD compared with young controls (Bäckman et al., 2007).

Guerrero et al. (2014) reported that endogenous dynorphin-derived opioids may have both antinociceptive and pronociceptive effects. Therefore, dynorphin may have multiple effects in modulating analgesic responses (Guerrero et al., 2013). In our study, the decrease in *pdyn* expression in rotenone-induced groups may indicate this property.

The decrease in mRNA expressions of *penk* and *pdyn* along with dopamine levels in the rotenone-treated groups may indicate that the interaction between the dopaminergic system and the opioidergic system is impaired.

Bissonnette et al. (2014) stated that overexpression of pENK has a protective effect against MPTP neurotoxicity by reducing dopamine consumption caused by MPTP exposure (Bissonnette et al., 2014). However, in our study, there was a decrease in mRNA expression of *penka* in rotenone-induced groups. The reason for this could be due to the differences in (i) the agent used, (ii) exposure times, (iii) the mode of exposure of the PD-inducing agent, and (iv) the site of exposure.

Conclusion

We aimed to determine how the molecular mechanisms associated with pain are regulated in PD and how acetic acid-induced pain model affects these mechanisms. The results of our study show that zebrafish are a suitable model for both neurotoxin-induced PD and pain studies, in agreement with other relevant studies in the literature. We have demonstrated for the first time the changes in dopaminergic, cholinergic and opioidergic pathways caused by the exposure of acetic acid as exogenous source of pain in the rotenone-induced PD model.

Based on our results, we can conclude that administration of acetic acid in the rotenone-induced PD model in zebrafish alters the mRNA expressions of acid-sensing ion channels and endogen opioid and disrupts mechanisms related to the pain response that play a role in the perception of peripheral pain through oxidative damage and neuroinflammation. Our studies further elucidate the altered molecular mechanisms of the pain in experimental PD model.

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Highlights

- Exposure to acetic acid in rotenone-induced PD model in zebrafish altered the mRNA expression of *pdyn*, *penka* genes.
- In PD, the levels of NO increased in parallel with the mRNA expression of *zasic1.3* in response to acetic acid-induced pain.
- Body curvature index decreased in response to acetic acid-induced pain in the rotenone-induced model PD compared with the acetic acid group, indicating impaired pain perception by rotenone exposure.

Credit author statement

Derya CANSIZ: Investigation, Methodology, Writing. **İsmail Ünal:** Investigation, Methodology. **Merih Beler:** Investigation, Methodology. **Ünsal V. Üstündağ:** Investigation. **Esin Ak:** Investigation Methodology. **Ebru Emekli-Alturfan:** Formal analysis, Resources, Writing - review & editing. **A. Ata Alturfan:** Review & editing, supervision

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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