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Pain-associated opioidergic, ion channel, inflammatory and oxidative stress responses in Zebrafish epilepsy model

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Epilepsy patients experience symptoms such as anxiety, depression, and increased pain sensitivity accompanied by pain during the preictal and ictal phases; however, the relationship between epileptic seizures and pain remains unclear. We aimed to investigate the effects of pain on the opioidergic system, ion channels, inflammation, and oxidative stress responses in a PTZ-induced zebrafish epilepsy model, as well as the effects of valproic acid on these mechanisms in this study. To examine epilepsy–pain interactions in the brain, zebrafish were divided into five groups to examine the changes in the brain caused by the interaction between epilepsy and pain: control (C), pentylenetetrazol (PTZ), acetic acid (AA), PTZ+AA, and PTZ+AA+VPA. After behavioral analysis, we determined the expressions of *cox1, il6, trpa1, scn8aa, scn1a, zasic 1.3, penka, pdyn, oprk1, and oprl1* using RT-PCR. Oxidant-antioxidant parameters and acetylcholinesterase activity were determined spectrophotometrically. Neurotransmitter levels were measured by LC-MS/MS, and GABRA1 and POMC proteins expressions were determined by Western blot. Total distance, exploration rate, and average speed significantly decreased in all groups. PTZ caused increased NO and LPO levels, decreased SOD activity and increased *cox1* and *il6* expressions. The expression of *scn8aa* decreased in the PTZ and AA groups, *trpa1* and *zasic1.3* expression increased in the AA and PTZ+AA groups. The expressions of the *pdyn*, *oprk1*, and *oprl1* genes increased in the AA group, decreased in the PTZ and PTZ+AA groups. Additionally, GABRA1 protein expression decreased in the PTZ, AA, and PTZ+AA groups, POMC protein expression showed a significant increase in all groups. In all groups, decreased dopamine, norepinephrine, serotonin and acetylcholinesterase indicate a disruption in neurotransmitter balance. This results indicate that the application of acetic acid in the PTZ-induced epilepsy model disrupts pain response mechanisms and neuromotor activities, increases pain perception during seizures, and VPA treatment reversed most of these effects.

Keywords: Pain, opioids, oxidative stress, valproic acid, epilepsy, zebrafish

Introduction

Epilepsy represents one of the most common neurological conditions worldwide, which manifests with recurring seizure episodes that trigger various symptoms, such as alterations in cognitive/affective patterns, motor function deficiencies, and attention deficits. Furthermore, it has been noted that among patients with epilepsy, episodes of pain are a common developing comorbid complication in the interictal period, displaying increased susceptibility to pain stimuli[1]. Nonetheless, despite the limited understanding of connections between epilepsy and pain, current evidence suggests that there are possibly major neurological associations between the two conditions[2,3]. Therefore, studies concerning the role of epilepsy in modulating pain thresholds and related mechanism processes might provide opportunities for upgrading management of epilepsy.

Pain syndromes are common in epilepsy, taking many forms, including but not limited to, headache from migraine, fibromyalgia, and neuropathic pain, as well as chronic pains[4]. Notably, there are interictal changes in pain thresholds, with suggestions that

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between seizures, tolerance to pain, as well as sensitivity to pain, has been shown to increase[1]. Consequently, it becomes critical to discern how, in epilepsy, and in patients with this condition, seizures affect tolerance to, as well as sensitivity to, pain. Seizure-related pains in epilepsy patients also take many different types, including but not limited to, hemi corporeal pains, cephalalgia, as well as abdominal pains[5]. These seizure-related pains provide clues to the underlying pathophysiology and underline the need to clarify how seizures and nociception interact.

Oxidative stress and inflammation are important pathophysiological processes involved in the interaction between epilepsy and pain. In cases of epilepsy, there can be oxidative stress that brings about an inflammatory response due to harmful effects on structures of a cell through free reactive radicals[6]. In epilepsy, enhanced inflammatory reactions can increase susceptibility to pain, which can contribute to the modulation of seizure occurrence and intensity. Changes in pain threshold based on inflammatory reactions can modulate the sensitivity of sodium channels and ion channels, which are critical in the pain transmission pathway and affect the seizure-related pain threshold[7]. Anomaly in sodium channels can increase neuronal hyperexcitability, thereby providing a mechanism for comorbid pain and seizure development.

The endogenous opioid system also plays a role in understanding epilepsy and pain. Families of opioid peptides, including endorphins, enkephalins, and dynorphins, provide a major mechanism for analgesia. These peptides are released through opioid receptors, including μ-opioid receptors (MOR), δ-opioid receptors (DOR), and κ-opioid receptors (KOR), which affect analgesia in response to nociceptive stimuli[8]. Endogenous opioids also provide a mechanism for regulating epilepsy seizure development. Some studies suggest that opioids can lead to a decreased rate of both seizure recurrence and intensity. An example of this involves nociceptin (NOP), which, although not part of the opioid family of receptors, plays a role in modulating pain in conjunction with epilepsy[9]. Alongside, the genes involved in opioid peptide synthesis, namely proenkephalin (penk) and prodynorphin (pdyn), are proposed to contribute to both epileptogenesis and pain regulation[10,11].

Studies on the neurobiological associations that exist between epilepsy and pain are focused on how neuronal hyperexcitability and neurotransmission modulation contribute to both conditions. Glutamate, the principal excitatory neurotransmitter in the central nervous system, plays a critical role in the pathophysiology of epileptic seizures. An excess release of glutamate has been shown to cause neuronal hyperexcitability, which in turn brings about seizure episodes[12]. In the complex relation between pain and epilepsy, glutamine, as a glutamate precursor, can affect pain processing and be involved in the regulatory processes of the neural circuits of pain in the CNS. Gamma-Aminobutyric Acid (GABA) represents a major inhibitory neurotransmitter that supports overall brain functions and remains a major target in anti-epileptic therapy. The dysfunction of the GABA system in epilepsy, along with neuronal excitability and seizure episodes, also affects pain processing[13]. Serotonin, on the other hand, influences nociception as well as seizure initiation and severity. Further, dysfunction of the serotonin system has been shown to increase pain thresholds as well as decreased seizure thresholds[14]. The serotonin receptor has a crucial role in the neural circuits associated with seizure and/or pain, and alterations in this system provide potential therapeutic strategies for treating both conditions, that of pain and epilepsy.

The aim of this study was to investigate the molecular mechanisms of pain in a PTZ-induced zebrafish model of epilepsy and to clarify how a superimposed nociceptive challenge with acetic acid modifies seizure-related changes. We hypothesized that acetic acid nociception superimposed on PTZ-induced seizures would aggravate disturbances in endogenous opioid signaling (*pdyn, penka, oprk1, oprl1*), pain and seizure related ion channels (*scn1a, scn8aa, zasic1.3, trpa1*), the oxidant–antioxidant balance and neuroinflammation (*cox1, il6*), and that valproic acid would attenuate these alterations. To test this, locomotor behavior, the body curvature pain index, oxidant–antioxidant parameters, gene expression, GABRA1 and POMC protein levels, and brain neurotransmitter content were evaluated.

Materials and Methods

Animals and Treatments

AB/AB strain adult zebrafish were housed in a ZebTEC mini aquarium rack system (Tecniplast, Italy) at a temperature of 27 ± 1 °C under a 14/10 hour light/dark cycle. All procedures were approved by the Marmara University Institutional Animal Care

and Use Committee (Approval Number: 68.2020.mar). Anesthesia was achieved using a wet ice-cold plate before intraperitoneal injections. The number of fish needed to yield statistically significant results was calculated through the power analysis procedure, utilizing the G*Power 3.1 software. The significance level was set at ($\alpha = 0.05$), the statistical power and the effect size were set at ($\beta = 0.8$, $f = 0.4$). Calculations showed that the number of fish needed in the experimental groups to yield a statistically significant sample was 14. The fish were randomly divided into five groups, and each group had 15 fish. Fish in the pentylenetetrazole (PTZ) group were injected with 170 mg/kg PTZ (Sigma- USA, CAS no: 54-95-5) and treatment group to 100 mg/kg VPA (Sigma- USA, CAS no: 1069-66-5), diluted in embryo medium[15]. Fish in the acetic acid (AA) group, at 5%, were dissolved in physiological saline. The doses of PTZ, AA, and VPA were selected according to the previously reported studies[16-21].

All chemicals were injected intraperitoneally with a Hamilton microinjector after the fish were anesthetized on a wet ice plate for ~30 s. VPA was injected 15 min before PTZ, and AA was injected simultaneously with PTZ. After a 30–60 s interval (kept short to avoid missing early seizure onset), locomotor activity was recorded for 10 min. Fish were then anesthetized on a wet ice plate and sacrificed, and brain tissues were promptly harvested for RNA extraction and biochemical analysis.

Each group consisted of 15 fish. Locomotor activity and the body-curvature pain index were assessed in 6 fish per group (n = 6), whereas all biochemical assays (MDA, NO, SOD, GST, AChE), RT-qPCR, Western blotting and LC-MS/MS were performed on three biological replicates per group (n = 3). Because a single adult zebrafish brain yields insufficient tissue and protein for the full assay panel, each replicate pooled the brains of three different fish, with the three pools per group derived from non-overlapping fish (n = 3 independent biological replicates). Each parameter was measured in triplicate.

The abdominal constriction, writhing-like behavior

The Image J program was used to measure of zebrafish body curvature indexes after acetic acid injection. The abdominal narrowing, writhing-like behavior was measured as previously described[19,20,22]. In short, frontal photographs were taken every 30 seconds during the 6 minutes and analyzed using ImageJ software. Three points, the anterior part (anterior to the head), central part (middle, anal, and dorsal fins), and posterior part (dorsal fin) of the fish, were chosen for the measurement of body curvature (Fig. 1c). Differences in results were subtracted from 180° to yield body curvature index values.

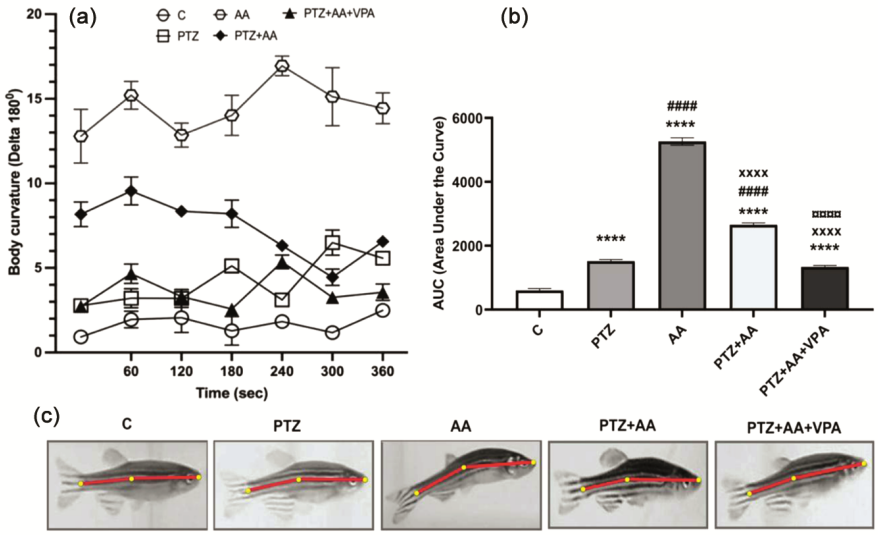

Fig. 1 — Time-dependent changes in the body-curvature index, area under the curve (AUC), and representative phenotype images across treatment groups. (a) Time course of the body-curvature index, calculated as the angle of abdominal constriction after PTZ, AA and VPA treatment. (b) AUC of the body-curvature index. (c) Zebrafish angular body curvature, measured using anterior, central and posterior reference points. Data are mean ± SD. Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc test. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

Behavior Analyses

A glass tank (21 cm x 9 cm x 11 cm) was used to determine locomotor activities.Video recording for each fish swimming pattern was taken daily by individuals, under the same time and same conditions, for 10 minutes after 30 seconds to 1 minute post-injection. The camera was placed 30 cm away from the 21 cm long side of the tank. Using tracking software (Tox-Track), a section consisting of 25 squares on the y-axis and 60 squares on the x-axis was created. The average speed of the fish, the total distance they traveled and the exploration rate were analyzed[23,24].

Biochemical Analyses

The fish were decapitated on a wet ice plate, and the whole brain was rapidly removed under a microscope. For each group, the brains of three fish were pooled in physiological saline to prepare 10% (w/v) homogenates. Pooling was necessary because the small mass of a single zebrafish brain does not provide sufficient protein for the complete spectrophotometric panel. Each pool constituted one biological replicate, and three independent pools per group were analysed, so that n = 3 corresponds to three biologically independent replicates [authors: please confirm the total number of fish used per group for the pooled samples]. Homogenates were centrifuged at 3000 rpm for 10 min, and the supernatant was used for the biochemical assays. Protein concentration was determined by the Lowry method, and all biochemical parameters were normalized to protein content[25]. The principle of this test is based on the measurement of the colored complex formed by the reduction of the foline reagent by Cu^{+2} ions in the alkali protein solution in a spectrophotometer at 500 nm. The level of malondialdehyde (MDA), the end product of LPO, was determined using the thiobarbituric acid reagent according to the Yagi method[26]. The results were expressed as nmol MDA/mg protein, using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$. Nitric oxide (NO) levels were determined using the Miranda method. The Miranda method reduces nitrate to nitrite with vanadium (III) chloride, and the results were calculated as nmol NO/mg protein[27]. Superoxide dismutase (SOD) activity was determined by the Mylorie method. In the Mylorie method, the effect of riboflavin-sensitized photooxidation of SOD, o-dianisidine, is enhanced. Results were expressed as U/mg protein[28]. The glutathione-S-transferase activity was determined through the spectrophotometric determination at 340 nm of the absorbance of the product formed by the conjugation of GSH and 1-chloro-2,4-dinitrobenzene (CDNB)[29]. Acetylcholinesterase (AChE) activity in the supernatants was determined using the Ellman (1961) method[30].

Reverse transcription (cDNA synthesis) and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit and QIAcube (Qiagen, Germany). Each sample was a pool of three brains and three independent pools were processed per group (n = 3 biologically independent replicates). RT^2 Profiler PCR Arrays (Qiagen, Germany) were used to synthesise single-stranded cDNA. DNA Master SYBR Green kit (Qiagen, Germany) was used for the PCRs. The Qiagen RotorGene-Q Light Cycler instrument was used to determine the genes of primer sequences are given in (Table 1). Relative transcript levels were evaluated by using the Delta–Delta CT (ΔΔCT) method through the normalisation of the values to the housekeeping gene, β-actin[31].

Western Blot

GABRA1 and POMC proteins were evaluated by semiquantitative Western blotting. Tissue samples

Table 1 — Forward and reverse primer sequences used in this study.

Primers	(forward/reverse)
β actin	5′-AAGCAGGAGTACGATGAGTCTG-3′
	5′-GGTAAACGCTTCTGGAATGAC-3′
cox1	5′-AGGGAGCTTCGACTTCAGCC-3′
	5′-TACCGCACCAGGTCGTGTTT-3′
il6	5′-GGCATTTGAAGGGGTCAGGA-3′
	5′-TCAGGACGCTGTAGATTCGC-3′
trpa1	5′-AGCCGATAAAGAAGTGCTCCCA-3′
	5′-CCGCTGCGTTTCCTTGCTTA-3′
scn8aa	5′-CCTCCCGAGTGGTCCAAACA-3′
	5′-TCAGCGCAGATACATTCCCC-3′
scn1a	5′-TCCGCTTGGATCACTGCAT-3′
	5′-AGACTTTGAGGTCCTGGCGG-3′
zasic1.3	5′-AGCATACGAAGTCGCTGGGT-3′
	5′-GTCATGCAGAGAAACAATTACCAGA-3′
pdyn	5′-GATGCGCGCAACAGATCTCC-3′
	5′-CGTCTGCGCCTGTATTGAGC-3′
penka	5′- CGTTTACCGGCTCCTCCGAC-3′
	5′-CAAGGCGATCCTCTTCGGTGA-3′
oprk1	5′-CTGCCGTTTACTCCGTGGTG-3′
	5′-ATGGCCAAGAGTTCAGCAGGT-3′
oprl1	5′-ATTGGTGTTCCGGCGATGGT-3′
	5′-TGGGAGAACTAAGATGCACTCG-3′

were collected using Qiagen Tissuelyser (Qiagen) with 200 µl of 1XRIPA (Chem/Cruz, SC-24948A) buffer containing protease inhibitor (Protease Inhibitor Cocktail, Chem/Cruz) and 10 mM phosphatase inhibitor sodium fluoride (Santa Cruz, SC-24988B), homogenized. Protein quantification was performed with the Take3™ Multi-Volume Plate Reader (Synergy H1, BioTek Instruments Inc., Vermont, USA). 30 µg of protein was loaded onto 10% SDS-PAGE and electrophoresed, then transferred onto a gel to nitrocellulose membranes. After incubating, the membranes were treated with primary and secondary antibodies. β-actin (GT5512-GTX629630, GeneTex) and POMC (ACTH 1-24) (B427: sc-57021, Santa Cruz) are mouse-produced monoclonal antibodies. GABRA1(GTX125728, GeneTex) is a polyclonal antibody produced in rabbits. Imaging was performed using Chemiluminescent HRP Substrate (Western Bright ECL-Advansta, K-12045-D50) after horseradish peroxidase-conjugated secondary antibody interaction. Band intensities were determined with the Chemiluminescence Imaging System (Celvin S, BioStep). While analyzing protein expressions, the relative evaluation according to the level of β-actin, one of the housekeeping genes in the cell, was taken as a basis.

LC/MS-MS analysis

In the brain tissue, the levels of GABA (gamma-aminobutyric acid), glutamate (Glu), glutamic acid, dopamine, serotonin, and norepinephrine were determined by the LC-MS/MS method. The brain tissues were separated from the fish under anesthesia. After homogenization with saline, quantitative determination of GABA (gamma-aminobutyric acid), glutamate (Glu), and glutamic acid parameters in homogenates was performed using the Jasem Quantitative Amino Acid LC-MS/MS analysis kit (Sem Laboratory Devices Marketing Industry, Istanbul, Turkey). Quantitative determination of dopamine, serotonin (5-hydroxytryptamine, 5-HT), and norepinephrine (NE) parameters was performed using the Jasem Biogenic Amines LC-MS/MS analysis kit (Sem Laboratory Devices Marketing Industry, Istanbul, Turkey). Steps were taken to follow the analysis kit sample preparation protocols. 950 µL of extract was transferred to an HPLC vial, and 50 µL of biogenic amine IS mixture was added and vortexed for 5 seconds. It was then injected into the LC-MS/MS system. Sample analyses were performed on a 6470 tandem mass spectrometer (6470A, Agilent Technologies, Santa Clara, CA, USA) connected to the Agilent HPLC system. Agilent MassHunter Data Acquisition software was used to operate the system, and MassHunter Quantitative Analysis and Qualitative Analysis software were used for quantitative and qualitative operations, respectively.

Statistical Analysis

GraphPad Prism 9.0 was used for statistical analysis. Data distribution was assessed using the Shapiro-Wilk normality test. Group differences were evaluated by one-way ANOVA followed by Tukey's HSD post-hoc test for all pairwise comparisons. Tukey's HSD inherently controls the familywise Type I error rate, thereby addressing the multiple comparison problem. Variance homogeneity across groups was verified using the Brown-Forsythe test ($P > 0.05$), and all groups featured equal sample sizes. Results are expressed as mean ± standard deviation (SD), with statistical significance set at $P \leq 0.05$.

Results

Body curvature measurement

In this study, in the PTZ+AA group, AUC significantly increased compared to the PTZ group ($P<0.0001$) and it significantly decreased compared to the AA group ($P<0.0001$) (Fig. 1b). The AUC, a quantitative indicator of body curvature index, increased in all groups compared to the control group ($P_{ANOVA}<0.0001$), (Fig. 1a,b). With VPA treatment, AUC values significantly decreased in the PTZ+AA+VPA group compared to the AA and PTZ+AA groups ($P<0.0001$), (Fig.1b). Acetic acid caused a writhing-like response (body curvature index). Time-dependent changes in body curvature index and AUC measurements are given in Fig. 1a and 1b; representative phenotype images illustrating the body curvature (anterior, central and posterior reference points) are shown in Fig. 1c.

Locomotor Patterns

The control animals performed swimming patterns in the entire region for the duration of the 10-minute in the tank; however, abnormal and circular locomotor movements, hyperlocomotion, and spontaneously triggered tonic-clonic seizures were observed in the PTZ group. In the AA group, fish exhibited rapid tail movements, hyper locomotion, and swimming behavior near the upper surface of the tank (Fig. 2a).

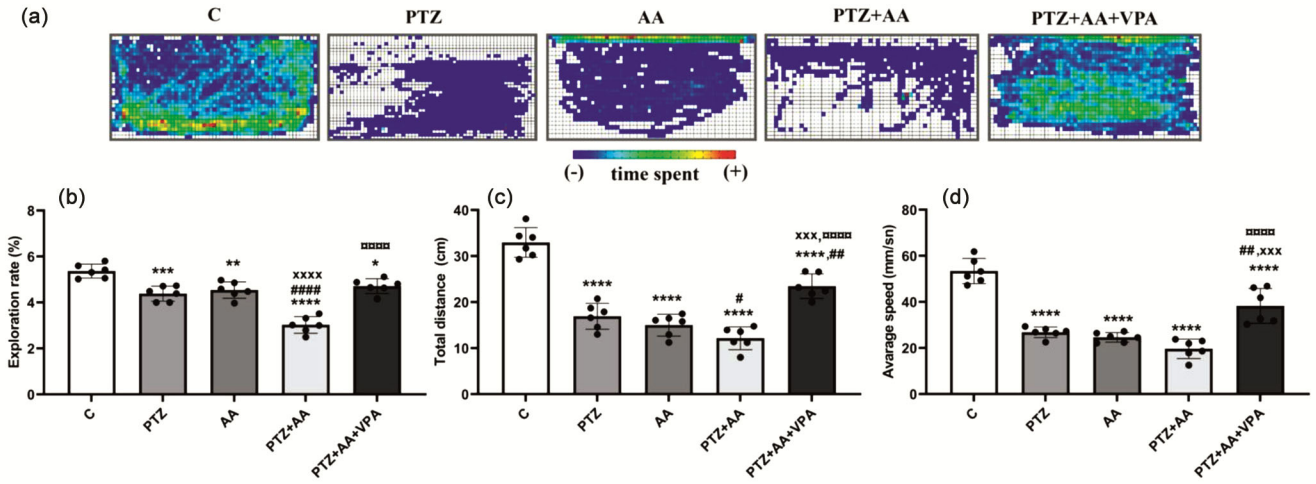

Fig. 2 — (a) Representative images of the tank areas explored by the fish over 10 min, showing time spent in each zone (blue (−) to red (+)). Locomotor pattern of the groups expressed as exploration rate (b), total distance (c) and average speed (d). Data are mean ± SD (n = 6). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc test. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

Total distance and average speed were significantly reduced in all exposure groups compared to the control group (P<0.0001), (Fig. 2c & Fig. 2d). Fish in the PTZ+AA group spent more time at the upper surface of the aquarium similar to the AA group and exhibited seizure-like swimming behaviors similar to the PTZ group (Fig. 2a). In the PTZ+AA+VPA group, improvements in swimming movements induced by PTZ and AA were observed (Fig. 2a). The exploration rate was significantly decreased in the PTZ+AA group compared to the control (P<0,0001), PTZ (P<0,0001) and AA (P<0,0001) groups (Fig. 2b). VPA treatment in the PTZ+AA+VPA group significantly increased the average speed and total distance compared to the PTZ (respectively, P= 0.0028; P= 0,0028), AA (respectively, P=0.0004; P<0,0001) and PTZ+AA groups (respectively P<0.0001; P<0,0001), (Fig. 2b, Fig. 2c & Fig. 2d). Moreover, the exploration rate was also increased in the PTZ+AA+VPA group compared to the PTZ+AA group (P<0,0001).

Biochemical Parameters

Malondialdehyde (MDA), a byproduct of lipid peroxidation, is an organic compound that damages cell membranes and serves as a marker of oxidative stress. MDA levels significantly decreased in the AA (P<0,0001) and PTZ+AA (P<0,0001) groups compared to the control group (P_{ANOVA}<0.0001), while significant increases were observed in the PTZ (P=0.0033) and PTZ+AA+VPA (P<0.0001) groups. VPA treatment significantly increased MDA levels in the PTZ+AA+VPA group compared to the AA and PTZ+AA groups (P<0.0001) (Fig 3a & 3b).

NO levels were significantly increased in the PTZ (P=0.0026) group compared to the control group (P_{ANOVA}<0.0001), while no significant difference was found in the AA (P=0.0846), PTZ+AA (P=0,6145) and PTZ+AA+VPA (P=0,9917) groups.

In this study, SOD activity significantly decreased (P_{ANOVA}<0.0001) compared to the control group in the PTZ (P=0,0090), PTZ+AA (P=0,0075) groups, whereas it increased in the AA (P=0,0141) and PTZ+AA+VPA (P<0,0001) groups. Glutathione S-transferase (GST) activity significantly increased in all groups (P_{ANOVA}<0.0001) except the PTZ+AA group. VPA treatment significantly increased SOD and GST activity in the PTZ+AA+VPA group (P<0.0001) compared to the PTZ+AA groups (Fig. 3c & 3d).

Brain AChE activity decreased in the PTZ (P=0.007), PTZ+AA and PTZ+AA+VPA (P<0.0001) groups compared to the control group (P_{ANOVA}<0.0001). VPA treatment significantly increased brain AChE activity in the PTZ+AA+VPA group (P=0.0048) compared to the PTZ+AA group (Fig. 3e).

RT-PCR Gene Analysis

***Changes in Gene Expression Related to Epilepsy, Inflammation, and* Pain**

COX-1 promotes the production of prostaglandins, which lead to inflammation and pain[32], while IL-6 (interleukin-6), an inflammatory cytokine, enhances

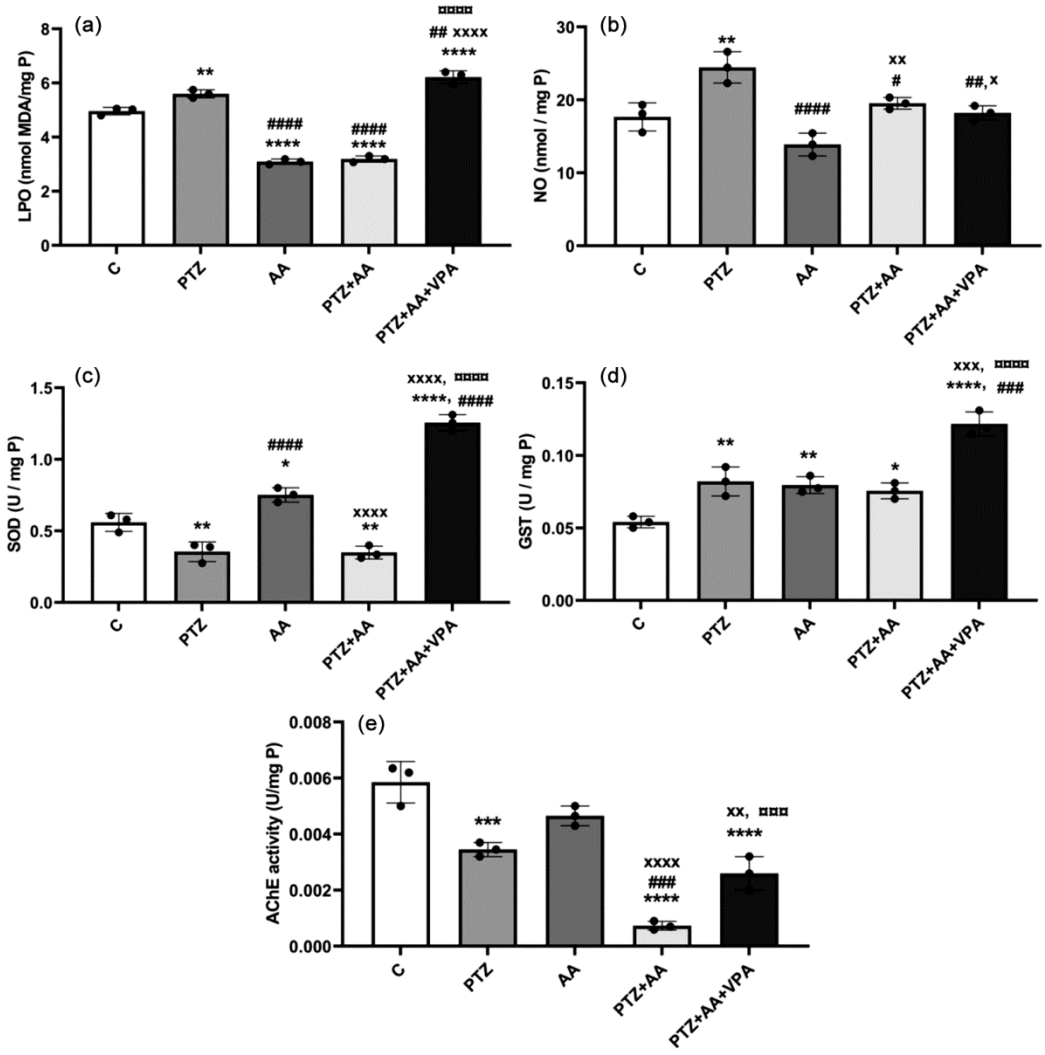

Fig. 3 — Biochemical analysis of oxidative stress: (a) LPO levels, (b) NO levels, (c) SOD activity, (d) GST activity and (e) AChE activity of the groups. Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc test. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = P < 0.05, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid; LPO: lipid peroxidation; MDA: malondialdehyde.

this process[33]. TRPA1, which responds to temperature and mechanical stimuli associated with pain and inflammation[34], can amplify the effects of these molecules[35]. The expression of *cox1* and *il6* in the PTZ (P_{cox1}<0.0001, P_{il6}<0.0001), AA (P_{cox1}<0.0001), PTZ+AA (P_{cox1}<0.0001, P_{il6}=0.0029) and PTZ+AA+VPA (P_{cox1}<0.0001, p_{il6}=0.0080) groups was significantly higher compared to the control group (respectivly, ($P_{ANOVAcox1}$<0.0001); ($P_{ANOVAil6}$<0.0001)), while VPA treatment significantly reduced *cox1* expression in the PTZ+AA+VPA groups (P_{cox1}<0.0001) (Fig. 4a & 4b). Additionally, the expression of *trpa1* was significantly increased in the AA (P_{trpa1}=0.0440), PTZ+AA groups (P_{trpa1}<0.0001) compared to the control group (P_{ANOVA}<0.0001), whereas it decreased in the PTZ group (P_{trpa1}=0.0259). VPA treatment led to a decrease in expression in the PTZ+AA+VPA (P_{trpa1}<0.0001) group compared to the PTZ+AA group (Fig.4c).

Changes in Gene Expression Related to Sodium Ion Channels and Pain Response

The *scn8aa* and *scn1a* genes encode sodium channels that regulate neuronal electrical conduction. Mutations in these genes are associated with increased epileptic seizures and pain. *scn8aa* expression was significantly lower in the PTZ (P_{scn8aa}=0.002) and AA (P_{scn8aa}=0.0062) groups compared to the control group

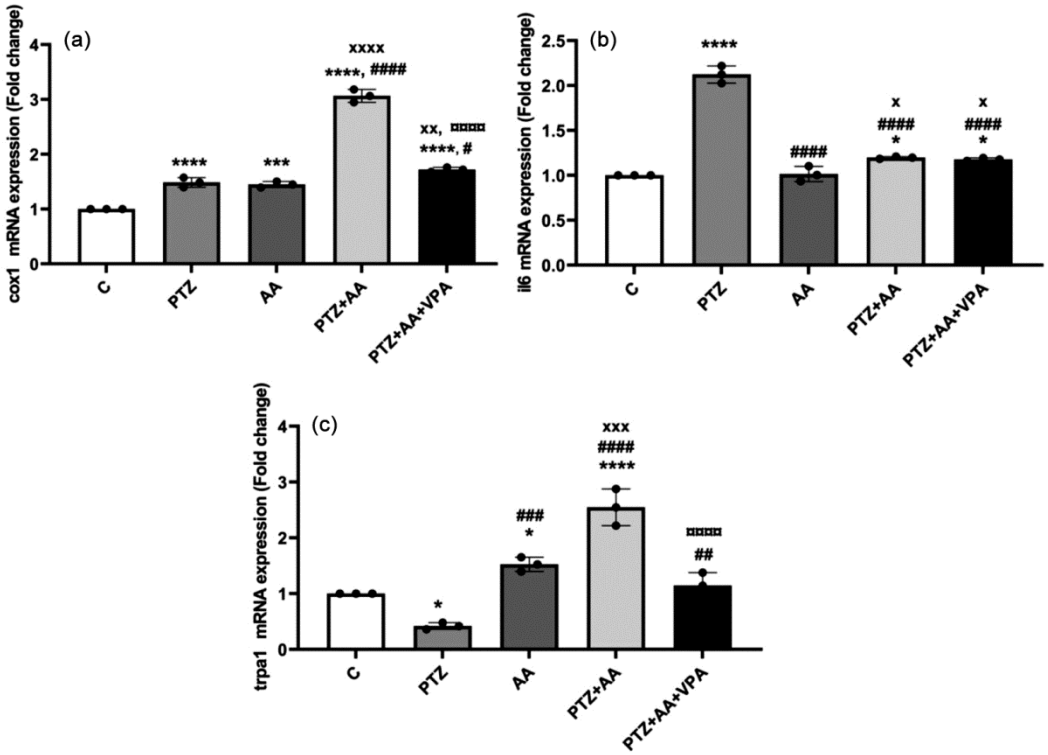

Fig. 4 — Fold change in brain *cox1* (a) *il6*, (b) and *trpa1*, (c) expression. Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

(P_{ANOVA}<0.0001) but was increased with VPA treatment in the PTZ+AA+VPA groups (P_{scn8aa}<0.0001), (Fig. 5a). *scn1a* expression was upregulated (P_{ANOVA}<0.0001) with VPA treatment in the PTZ+AA+VPA groups (P_{scn1a}<0.0001), (Fig. 5b).

ASICs are proton-gated members of the degenerin/epithelial Na^+ channel family and the expression of *zasic1.3*, the most important pH sensor for pain, was significantly reduced in the PTZ ($P_{zasic1.3}$=0.0001) group compared to the control group (P_{ANOVA}<0.0001), but significantly increased in the AA ($P_{zasic1.3}$<0,0001) and PTZ+AA groups ($P_{zasic1.3}$<0.0001) (Fig 5c).

Changes in Gene Expression Related to the Opioid System

The *pdyn* and *penk* genes produce opioid peptides, dynorphin and enkephalin, which interact with each other and modulate their effects on pain, stress, and emotional regulation processes. The expression of *pdyn* and *penka* decreased in the PTZ (p_{pdyn}<0.0001, P_{penka}<0.0001) compared to the control group ($P_{ANOVApdyn}$<0.0001); $P_{ANOVApenka}$<0.0001), (Fig. 6a & Fig. 6b). In the AA group, *pdyn* expression significantly increased (P_{pdyn}<0.0001), while *penka* expression showed no significant change (P_{penka}=0.7686). *pdyn* expression significantly decreased in the PTZ+AA group (P_{pdyn} = 0.0067), while *penka* expression no significant change (P_{penka} = 0,0536). With VPA treatment, *pdyn* gene expression increased in the PTZ+AA+VPA (P_{pdyn}=0.082) group compared to the PTZ+AA group. As for *penka* gene expression levels, a significant increase was observed in the PTZ+AA+VPA group (P_{penka}<0.0001) following VPA treatment.

The expression of *oprl1* and *oprk1* increased in the AA (P_{oprl1}=0.0064, P_{oprk1} <0,0001) but decreased in the PTZ (P_{oprl1}<0.0001, P_{oprk1}<0,0001) group compared to the control group ($P_{ANOVAoprl1}$<0.0001; $P_{ANOVAoprk1}$<0.0001), (Fig. 6c & Fig. 6d). On the other hand, the expression of *oprl1* significantly decreased in the PTZ+AA group (P_{oprl1}<0,0001) compared to the control group. Furthermore, with VPA treatment, *oprl1* and *oprk1* expression levels increased in the PTZ+AA+VPA group (P_{oprl1}<0.0001, P_{oprk1}<0,0001).

Western Blot Analysis

The protein expressions of GABRA1 and POMC were measured by comparing their band densities to

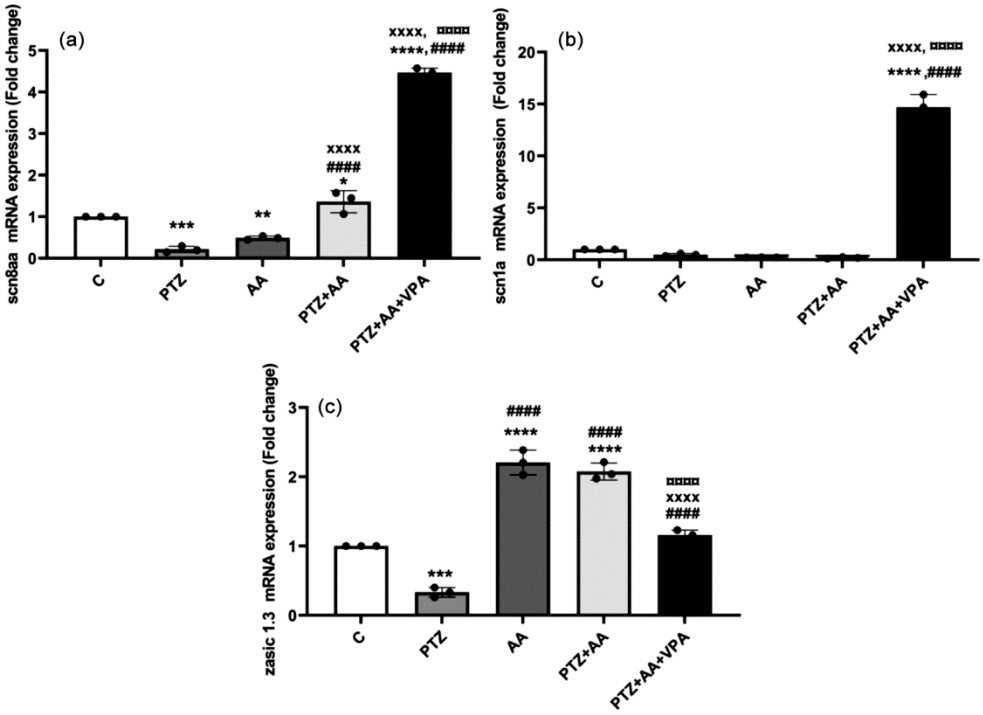

Fig. 5 — Fold change in brain *scn8aa* (a), *scn1a*, (b) *zasic1.3*, and (c) expression. Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

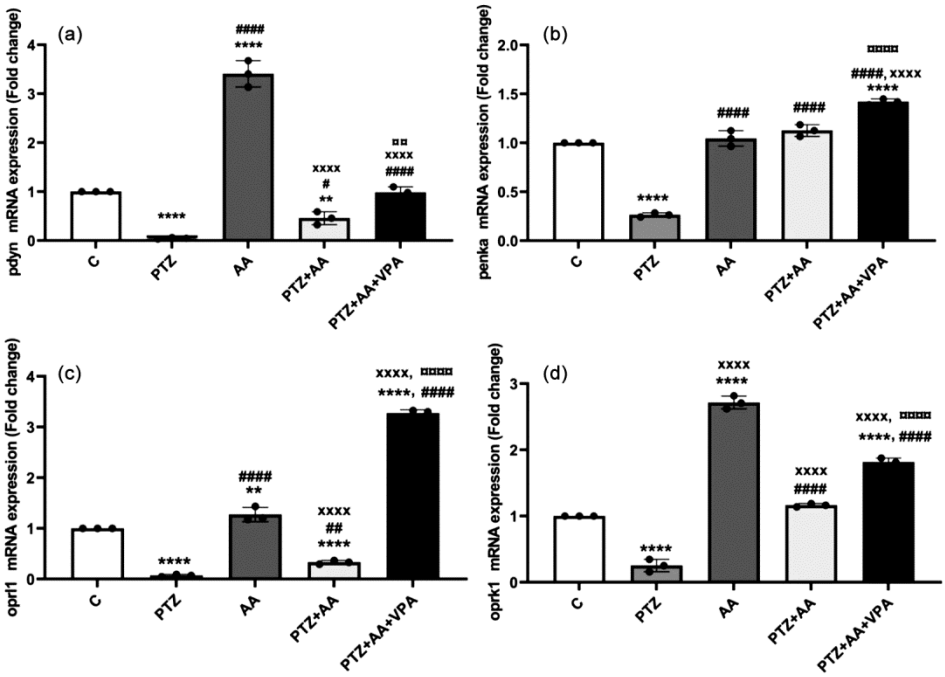

Fig. 6 — The Fold change in brain *pdyn*, (a) *penka*, (b) *oprl1*, (c) *oprk1*, and (d) expression. Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

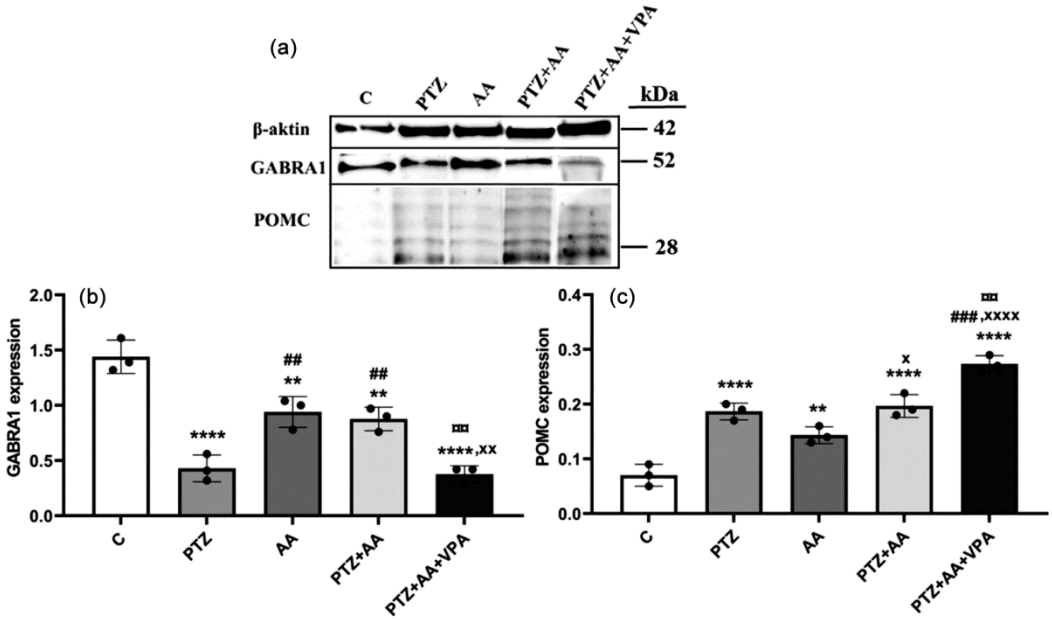

Fig. 7 — Representative protein expression and densitometric analysis of GABRA1 and POMC across treatment groups. (A) Representative western blot images of β-actin (42 kDa), GABRA1 (52 kDa) and POMC (28 kDa). (B) Densitometric analysis of GABRA1 expression normalized to β-actin. (C) Densitometric analysis of POMC expression normalized to β-actin. Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc test. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = P < 0.05, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

the housekeeping protein beta-actin and quantified using the western blot analysis method (Fig.7a). GABRA1 protein expression decreased in the PTZ (P<0.0001), AA (P=0.0037) and PTZ+AA groups (P=0.0015) compared to the control group (P_{ANOVA}<0,0001). GABRA1 protein levels decreased in the PTZ+AA+VPA group (P=0.0037) compared to the PTZ+AA group (Fig. 7b). POMC protein expression significantly increased in all exposure groups compared to the control group (P_{ANOVA}<0.0001). With VPA treatment, POMC protein expression increased in the PTZ+AA+VPA group (P=0.0023) compared to the PTZ+AA group (Fig. 7c).

Neurotransmitter Analysis Using LC-MS/MS

To evaluate the chemical changes in the brain following exposure, we conducted a molecular analysis of GABA, Glutamate, Glutamine, Dopamine, Norepinephrine, and Serotonin levels in zebrafish brain tissues using LC/MS-MS. GABA levels significantly increased in the PTZ, AA, PTZ+AA+VPA (P<0.0001) groups compared to the control group (P_{ANOVA}<0.0001), but decreased significantly in the PTZ+AA group (P<0.0001) (Fig. 8a). Glutamate levels significantly decreased in the PTZ (P<0.0001) and PTZ+AA groups (P<0.0001), but increased in the AA group (P<0.0001) compared to the control group (P_{ANOVA}<0.0001). After VPA treatment, glutamate levels increased significantly in the PTZ+AA+VPA group (P<0.0001), (Fig. 8b). Glutamine levels significantly increased in all exposure groups compared to the control group (P_{ANOVA}<0.0001). In the groups treated with VPA a significant increased was noted in the PTZ+AA+VPA group (P<0.0001) as shown in (Fig. 8c).

Dopamine (DA), Norepinephrine (NE), and Serotonin (5-HT) levels significantly decreased in all exposure groups compared to the control group (P_{ANOVA}<0.0001), (Fig. 8d, Fig. 8e, Fig. 8f). Additionally, there was no significant change in DA and NE levels in the groups treated with VPA. However, brain 5-HT levels significantly decreased in the PTZ+AA+VPA (P<0.0001) group with VPA treatment.

Discussion

Shared mechanisms between neuropathic pain and epilepsy suggest a potential role for antiseizure drugs in pain management. Here, we investigated the behavioral, oxidative, inflammatory, and

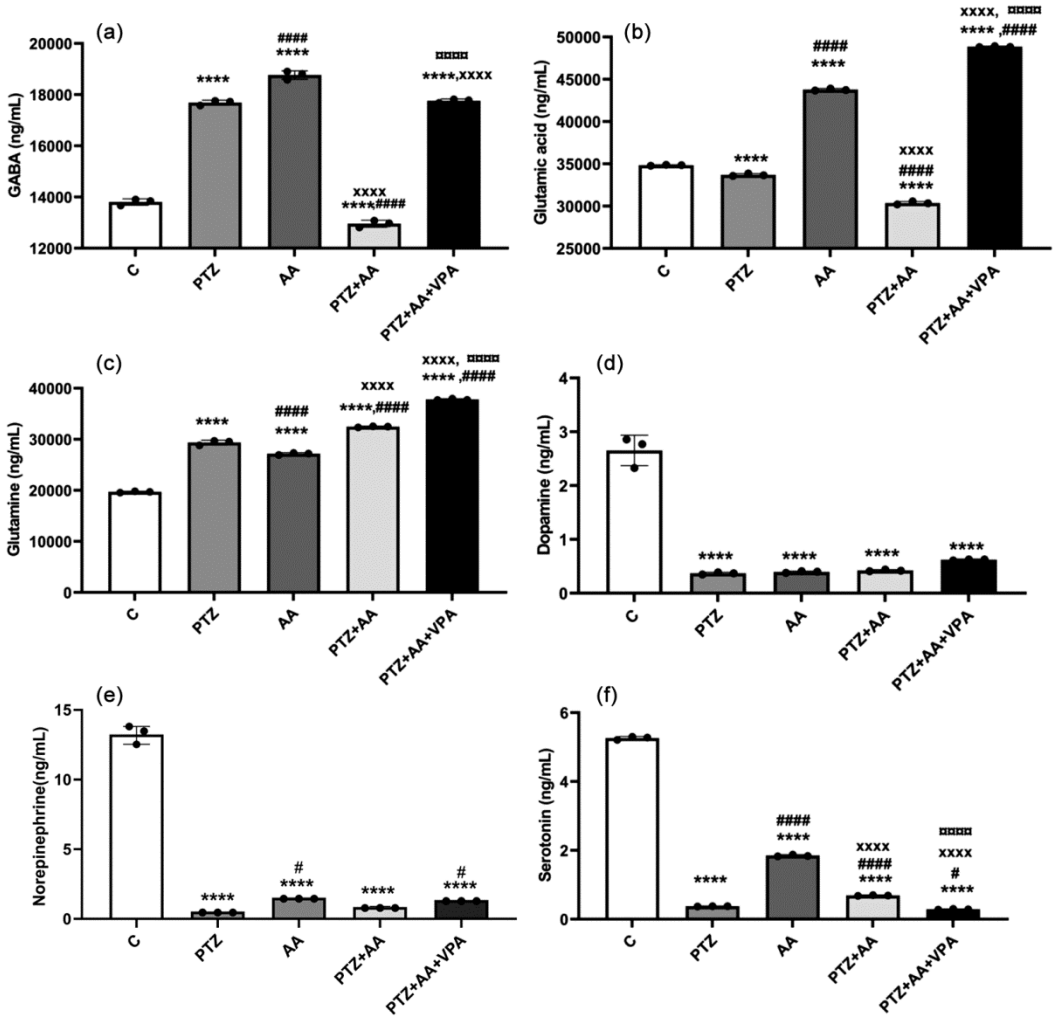

Fig. 8 — Neurotransmitter analysis in the zebrafish brain: concentrations of GABA (a) glutamate, (b) glutamine, (c) dopamine (DA), (d) norepinephrine (NE), (e) serotonin (5-HT), and (f). Data are mean ± SD (n = 3). Significance was assessed by one-way ANOVA with Tukey's multiple-comparison post-hoc test. Symbols denote significant differences versus control (*), PTZ (#), AA (x) and PTZ+AA (¤); the number of symbols indicates the significance level (one to four symbols = $P < 0.05$, < 0.01, < 0.001 and < 0.0001, respectively). C: control; PTZ: pentylenetetrazole; AA: acetic acid; VPA: valproic acid.

neuromolecular effects of valproic acid in a PTZ/acetic acid-induced adult zebrafish model. VPA treatment improved pain-related body curvature index, disrupted neuromotor activity, and seizures observed in the epileptic pain group (distance traveled, exploration rate, and average velocity) in this study. PTZ-induced seizures led to oxidative stress with increased NO and LPO and decreased SOD activity. It also triggered an inflammatory response with increased expression of *cox-1*, *ıl-6*, and *trpa1*. Furthermore, *scn1a* expression was upregulated in the AA-induced pain and PTZ+AA groups, whereas the decrease in *scn8aa* expression in the PTZ and AA groups would also indicate of increased pain transmission. Opioid system genes *pdyn, oprk1*, and *oprl1*, were increased in the AA group but decreased in both PTZ and PTZ+AA groups. A marked reduction in dopaminergic, norepinephrinergic, and serotoninergic neurotransmitter content in the brain, as well as decreased acetylcholinesterase activity in all groups, suggests a disruption in the neurotransmitter balance.

Acute seizures induced by PTZ in adult zebrafish have been demonstrated in previous studies[36,37]. Choo *et al.* was observed that zebrafish pre-treated with VPA before receiving 170 mg/kg PTZ injections exhibited regular swimming movements compared to the PTZ group[18]. In our study, zebrafish subjected to acute i.p. 170 mg/kg PTZ exhibited anxiety-like behavior and seizure-like movements, as previously

described in the literature[17,38,39], The reduction in traveled distance, exploration rate, and average speed suggests that the impairment in motor activity is associated with epilepsy. Furthermore, in previous studies, 100 mg/kg VPA treatment has been shown to reduce PTZ-induced acute seizures, bring the swimming behavior of zebrafish closer to normal and improve these parameters[18,40].

Acute exposure to 5% acetic acid resulted in decreased locomotor activity, which is consistent with pain-related behavioral responses reported in previous studies[19,20,41,42]. Adedara *et al*, as well as Cansız et al., showed that in acetic acid-induced zebrafish pain models, the distance traveled and absolute turn angle were reduced, while time spent on the upper surface of the tank was increased, with body curvature being a pain indicator[22,43]. In the present study, acetic acid injection caused zebrafish in the AA group to spend more time in the upper region of the tank, remain immobile, and exhibit body curvature and contraction behaviors, whereas acetic acid administration in the PTZ group resulted in significant reductions in body curvature, swimming pattern, and locomotor parameters (exploration rate, distance traveled, and swimming speed). These findings suggest that the combined effects of epilepsy and pain impair neuromotor function and increase pain sensitivity. Furthermore, VPA pretreatment significantly ameliorated these behavioral abnormalities, while epilepsy-associated motor dysfunction may be further exacerbated by oxidative stress-induced neuronal damage.

NO levels are a significant cause of neuroinflammation in epilepsy[44,45]. Watanabe *et al.* reported that NO levels in the brain increase fivefold after PTZ exposure, which may affect the seizure threshold[44]. Peroxynitrite ($ONOO^-$) radicals and ROS causes an increase in malondialdehyde (MDA) levels, which is a product of lipid peroxidation. Similarly, in our study, as well as studies by Guna et al. and Oktay *et al*. in rat models of PTZ epilepsy, MDA levels significantly increased in the PTZ group, while SOD activity decreased[46,47]. NO and LPO levels increased in the PTZ group, while NO levels remained unchanged in the acetic acid and acetic acid-treated PTZ groups, and LPO levels decreased. Moreover, acetic acid administration increased SOD enzyme and GST activities, suggesting the activation of a neuroprotective mechanism and can be explained by the anti-inflammatory effects of acetic acid. Our interpretation is supported by the findings of Tain *et al*. have reported that acetic acid, as a short-chain fatty acid, has anti-inflammatory effects on brain cells[48]. Although VPA is used as an anticonvulsant in seizure treatment, it is known to have toxicity[49]. Jafarian *et al*. demonstrated in rats that valproic acid (VPA) induces the formation of reactive oxygen species (ROS) in hepatic tissue[50]. Turkyılmaz *et al*. and Oktay *et al.* Shown that VPA exposure decreased SOD activity in rats[47,51]. In our study, VPA treatment led to a significant increase in LPO levels, while SOD and GST activities also increased. These results indicate that the antioxidant mechanism is activated in reaction to oxidative stress induced by VPA administration. Furthermore, the absence of significant changes in NO levels in the VPA-treated groups suggests that peroxynitrite formation did not reach toxic levels and that antioxidant defense was preserved.

Oxidative stress-mediated neuroinflammation contributes to nociceptor sensitization through the COX-1 pathway[52]. In this study, cox-1 expression was upregulated in epilepsy, pain, and epilepsy with pain conditions, whereas VPA attenuated this inflammatory response. During epileptic seizures, increased cox-1 activity and elevated il-6 levels may enhance prostaglandin production, thereby increasing pain perception. During epileptic seizures, increased cox-1 activity and elevated il-6 levels may enhance prostaglandin production, thereby increasing pain perception[53-55]. In our study, *il6* expression in brain tissue showed an increase in both epilepsy and in models of epileptic pain. Yan et al. has been shown that *il6* over-expression reduces the threshold of action potential in Na^+ channels, a result of hyperexcitability of neurons in rat epilepsy model[56].

Changes in *trpa1* expression in cationic ion channels, which take place in epilepsy, have been found to modulate epilepsy-induced seizure activity[57,58] as well as affect pain perception due to injury[59]. A decreased expression of trpa1 in epilepsy has been found in our study. Also, a high expression of *trpa1* in both the pain group as well as in the epileptic-pain group supports this result. Taken together, it seems that decreased expression of *trpa1* in this epilepsy model may represent an adaptive, potentially neuroprotective response that limits TRPA1-mediated Ca^{2+} influx; because expression was measured at the transcript level only, this interpretation remains correlative. Additionally,

TRPA1 channels also present a drug target for various migraine-inducing medications that have been shown to suppress channel function[60]. Furthermore, in the epileptic-pain group, VPA suppressed this enhanced trpa1 expression, a change that is consistent with, but does not by itself demonstrate, an antinociceptive effect of VPA. Moreover, overactivation of sodium channels such as s*cn1a* and *scn8a* through the TRPA1 channel during episodes of epilepsy can lead to a decrease in pain tolerance. Increased sensitivity to the TRPV1 channel can make nociceptor activation easier, resulting in enhanced pain intensity. In addition, *scn1a* and *scn8a* mutations can also lead to increased pain thresholds.

Epilepsy and pain share ion channel dysfunction and neuronal hyperexcitability. Altered pain perception in scn8a-related disorders underscores their close association. In case a decrease in Nav.1.6 expression, encoded by the *scn8aa* gene, has been reported in trigeminal neuralgia[61] and in diabetic neuropathy in humans[62]. Defects in sodium channel subunits in zebrafish lead to insensitivity (hyposensitivity) in pain perception[63]. In present study, *scn8aa* expression decreased in the epilepsy and pain groups but increased in the epileptic pain group. VPA treatment, *scn8aa* expression increased in the epileptic pain group. This increase suggests that VPA may reduce pain by stabilizing neuronal excitability. The reduced scn8aa (Nav1.6) expression observed in the PTZ and AA groups is consistent with the decreased Nav1.6 levels reported in human neuropathic pain conditions and may indicate reduced excitability of nociceptive neurons. In contrast, the elevated scn8aa expression detected in the epilepsy–pain group may reflect a compensatory response triggered by the combined effects of seizure activity and nociceptive stimulation. However, since Nav1.6 was assessed only at the mRNA level, functional studies are required to confirm these interpretations.

Ievglevskyi *et al*. demonstrated that blocking ASIC conductivity in the hippocampal CA1 cells, in response to kainate-induced seizure-like activity, has a significant anti-seizure effect[64]. Furthermore, inflammation and tissue damage have been shown to increase ASIC mRNA expression levels in dorsal root ganglion (DRG) neurons, and ASICs have been inhibited by cannabinoids and nonsteroidal anti-inflammatory drugs[65]. In this study, the increased expression of *zasic1.3* in the pain and epileptic pain groups is associated with inflammation, and the reduced expression of *zasic1.3* with VPA treatment suggests that VPA may reduce pain.

POMC, PENK, and PDYN encode endogenous opioid peptides involved in pain modulation. Increased penk and pdyn expression has been associated with neuropathic pain[66]. It is also thought that Enkephalins, by regulating mitochondrial functions under hypoxic conditions, reduce reactive oxygen species and play a neuroprotective role[67]. In the acute epilepsy model induced by PTZ[68] did not detect any differences in *penk* expression in the hippocampus, neocortex, or prefrontal cortex. *penk* expression increased in rats with 24-hour PTZ administration[69]. Contrarily, in this study, a significant decrease in *penk* expression was evidenced in the PTZ group, suggesting that seizure episodes can suppress the opioid system by means of neuroinflammatory and stress-mediated pathways. Furthermore, a substantial increase in *penk* expression in the pain group suggests that a protective pathway has been activated in response to nociception. As calcium ions regulate neuronal excitability and calcium ion imbalance is thought to trigger epilepsy, PENK has been linked to calcium ion channels[70]. In our study inhibition of *penk* expression may link that these altered calcium levels in this study. The fact that VPA administration leads to a significant increase in *penk* expression suggests that it can increase pain tolerance and pain sensitivity, and these findings suggest that VPA may alleviate epilepsy-associated pain by upregulating penk and oprd1 expression. Consistent with the reported anticonvulsant role of pdyn[71], VPA also increased pdyn expression in the epileptic pain group, further supporting its antinociceptive effect.

In the pain group, *pdyn* expression increased with *oprk1* expression compared to the control group. On the other hand, decreased expression of *pdyn* and *oprk1* in the epilepsy and epileptic pain groups reflects a suppressed endogenous opioid system in these groups. Suppressed expression of these two genes supports a disrupted pathway in pain modulation and a decreased responsiveness to pain. Increased *oprk1* expression suggests activation of kappa-opioid receptors in response to painful stimuli, hence contributing to the regulation of pain perception. The increase the expression of *pdyn* and *oprk1* in response to VPA treatment suggests that VPAcontributes to the management of epileptic seizures by activating the endogenous opioid

system. The oprl1 gene, which encodes the nociceptin/orphanin FQ receptor, is involved in the modulation of seizures and pain[67]. In the present study, oprl1 expression decreased in the epilepsy and epileptic pain groups but increased following VPA treatment. Similarly, reduced penk expression in epilepsy may reflect impaired opioid-mediated pain modulation, whereas VPA-induced upregulation of penk suggests enhanced opioid signaling and a potential analgesic effect.

POMC, the precursor of ACTH and β-endorphin, is activated by stress and has neuroprotective and anti-inflammatory functions[71]. Consistent with this, POMC expression was significantly increased in the epilepsy and epileptic pain groups. This increase may reflect a compensatory response to seizure-induced neuronal stress and excitability[72-74]. ACTH has demonstrated anticonvulsant efficacy in both experimental and clinical studies. Nasiri et al. reported that ACTH administration reduced seizure episodes, while ACTH was also effective in drug-resistant generalized epilepsy[75]. Furthermore, seizure control was achieved within weeks using synthetic ACTH in combination with valproic acid[76], and post-seizure ACTH release has been shown to attenuate GABA-related excitotoxicity in the hippocampus[77]. ACTH over-expression in our study was found to be stimulated not only in epilepsy but also in painful conditions. VPA therapy in the epileptic pain group increased protein expression of POMC, which may point to stress due to a cumulative toxic effect of PTZ, AA, and VPA.

Choo et al. found that pentylenetetrazol treatment in zebrafish reduced gamma-aminobutyric acids and glutamic acids levels, which was determined by LC/MS-MS analysis of zebrafish brain. The discrepancy between the reduction in glutamate and the presence of seizures supports the idea that transamination reactions in the glutamate-glutamine cycle are reduced by PTZ[18]. However, in the pain group, gamma-aminobutyric acid, glutamic acid, and glutamine levels significantly increased compared to the control group. Furthermore, our result suggests that valproic acid has a role as a positive allosteric modulator of gamma-aminobutyric acid A receptors, increasing gamma-aminobutyric acid levels and modulating GABRA1 protein expression in epilepsy, acetic acid, and in the epileptic pain groups. The neuroprotective effect of VPA is related to the enhancement of GABAergic neurotransmission, a reduction in glutamate stimulation, and the weakening of neurogenic inflammation[78].

On the other hand, an increase in glutamate has been found in patients with migraines, but it is low in cases of back pain[79,80]. Peek and colleagues underlined the differences in both GABA and glutamate that exist in different types of pains, with a particularly marked increase in the case of migraines[81]. In the epileptic pain group, GABA and glutamine levels decreased compared to the control group, while glutamic acid levels increased. VPA treatment in the epileptic pain group significantly increased GABA, glutamic acid, and glutamine levels. Clinical studies have shown that glutamate levels increase in epileptic tissue during interictal periods and seizures[82-83]. GABA release is likely stimulated by the depolarizing effects of glutamate and/or aspartate on glutamate receptors via the depolarization of presynaptic GABAergic neurons. Thomas et al. 2005 reported that GABA levels increased before seizures in patients with temporal lobe epilepsy. In our study, we suggest that the increased GABA levels in the epilepsy and pain groups may be a response to stimulation, but prolonged exposure could lead to a decrease in GABA and an increase in glutamate.

Furthermore, dopamine, serotonin, and norepinephrine levels significantly decreased in all groups compared to the control group. Szot *et al.* (2004) found that a decrease in endogenous norepinephrine levels increased susceptibility to seizures and reported that norepinephrine has potent antiepileptic effects among neurotransmitters[84]. These findings suggest that increasing norepinephrine (NE) levels, which has widespread hormonal and stimulant effects in the body, reduces susceptibility to epileptic seizures. In this study, striking decreases in serotonin, dopamine, and norepinephrine levels in the epilepsy and pain groups, norepinephrine levels increased in the epileptic pain group after VPA administration. Before studies suggest that seizures and pain perception are regulated by neurotransmitters[85-87]. In addition, PTZ exposure led to a decrease in acetylcholinesterase (AChE) activity, consistent with the findings of decreased AChE activity in the Anesti 2020 studies[88]. A decreased level of acetylcholine reduces pain modulation and consequently enhances pain perception in relation to seizures[89,90]. VPA improved cognitive function by increasing acetylcholinesterase (AChE) activity among the group

with epileptic pain. These results show that VPA has potential use in both epilepsy and pain conditions, which can be achieved through reestablishing neurotransmitter balance.

The PTZ-induced epilepsy model employed in the present study reflects acute seizure activity and therefore does not fully recapitulate the chronic and progressive nature of epilepsy. Accordingly, several limitations should be considered when interpreting the findings. First, the acute PTZ paradigm captures only short-term seizure responses and cannot reproduce the long-term neuronal network remodeling and neuropathological alterations characteristic of chronic epilepsy. Second, the molecular analyses were conducted using a limited number of pooled biological replicates (n = 3), which may have reduced statistical power and constrained the sensitivity of multiple-comparison analyses. Third, seizure activity was assessed based on behavioral observations and was not validated by electrophysiological techniques such as electroencephalography (EEG) or field-potential recordings. Finally, the gene-expression data were evaluated exclusively at the mRNA level, and protein expression was assessed only for GABRA1 and POMC; the remaining targets were not corroborated by protein-expression analyses or functional assessments of ion-channel activity. Therefore, the observed molecular alterations should be interpreted as associative rather than causal. Future studies incorporating chronic epilepsy models, larger independent sample sizes, electrophysiological validation, and protein- and functional-level confirmation will be essential for a more comprehensive understanding of the mechanisms underlying the interaction between epilepsy and pain.

Conclusions

This study proposes a new model of pain that was formulated in conjunction with acute epilepsy in zebrafish. (VPA) attenuated PTZ-induced behavioural and neuromolecular alterations, supporting the potential usefulness of this model for further antiseizure studies. Findings of this study suggest that acetic acid challenge in conjunction with the PTZ-induced model of acute epilepsy interfered with pain regulatory pathways, as evidenced by changes in ion channels, endogenous opioids, oxidative stress, and neuroinflammatory components, whereas VPA showed anti-inflammatory and antinociceptive properties.Our study examines the molecular interaction of seizure-induced pain with the simultaneous induction of epilepsy and pain, providing insights into how these two conditions interact at the molecular level and how a commonly used antiepileptic drug like VPA affects this dynamic. These findings may help clarify how pain in epilepsy could be managed and which molecular pathways might be targeted in future work. By characterising the interaction between seizures and nociception in an accessible vertebrate model, the study provides a basis for further mechanistic and therapeutic investigation of epilepsy-related pain and adds to the limited literature in this field.

Ethics approval statement

The experimental procedures were approved by the Institutional Animal Care and Use Committee of Marmara University (68.2020.mar).

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Author Contributions

F.D.Ü: Conceptualization, Data curation, Formal analysis, Methodology, Funding acquisition, Writing – original draft – review & editing. İ.Ü: Methodology. Ü.V.Ü: Methodology, Writing – review & editing. D.C: Methodology. SKS: Methodology. MB: Methodology. E.E.A: Conceptualization, Data curation, Formal analysis, Supervision. P.M.T: Conceptualization, Data curation, Supervision, Funding acquisition, Writing – review & editing. All the authors have agreed for authorship, read and approved the manuscript, and given consent for publication.

Declaration of competing interest

All of the authors do not have financial interests or personal relationships to disclose.

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