Taylor & Francis
Taylor & Francis Group

Toxicology Mechanisms and Methods

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/itxm20

Morphine ameliorates pentylenetetrazole-induced locomotor pattern in zebrafish embryos; mechanism involving regulation of opioid receptors, suppression of oxidative stress, and inflammation in epileptogenesis

Fümet Duygu Üstündağ, İsmail Ünal, Ünsal Veli Üstündağ, Derya Cansız, Merih Beler, A. Ata Alturfan, Pınar Mega Tiber & Ebru Emekli-Alturfan

To cite this article: Fümet Duygu Üstündağ, İsmail Ünal, Ünsal Veli Üstündağ, Derya Cansız, Merih Beler, A. Ata Alturfan, Pınar Mega Tiber & Ebru Emekli-Alturfan (2023) Morphine ameliorates pentylenetetrazole-induced locomotor pattern in zebrafish embryos; mechanism involving regulation of opioid receptors, suppression of oxidative stress, and inflammation in epileptogenesis, Toxicology Mechanisms and Methods, 33:2, 151-160, DOI: 10.1080/15376516.2022.2105182

To link to this article: https://doi.org/10.1080/15376516.2022.2105182

Published online: 31 Jul 2022.

TOXICOLOGY MECHANISMS AND METHODS
2023, VOL. 33, NO. 2, 151–160
https://doi.org/10.1080/15376516.2022.2105182

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Morphine ameliorates pentylenetetrazole-induced locomotor pattern in zebrafish embryos; mechanism involving regulation of opioid receptors, suppression of oxidative stress, and inflammation in epileptogenesis

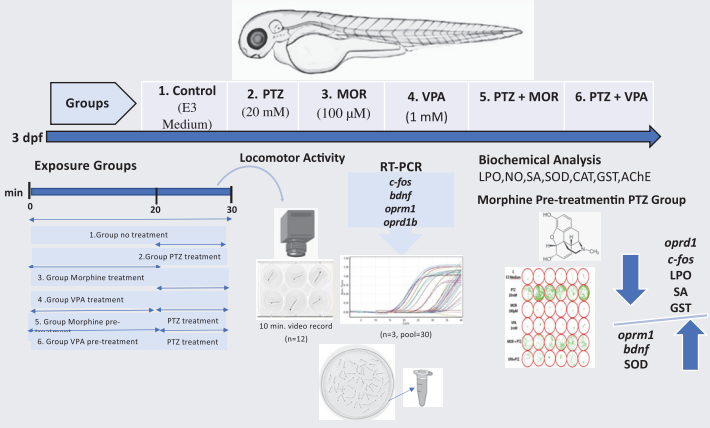
Fümet Duygu Üstündağ[a], İsmail Ünal[b], Ünsal Veli Üstündağ[c], Derya Cansız[c], Merih Beler[b], A. Ata Alturfan[d], Pınar Mega Tiber[e] and Ebru Emekli-Alturfan[f]

[a]Department of Biophysics, Institute of Health Sciences, Marmara University, Istanbul, Turkey; [b]Department of Biochemistry, Institute of Health Sciences, Marmara University, Istanbul, Turkey; [c]Faculty of Medicine, Medical Biochemistry, Istanbul Medipol University, Istanbul, Turkey; [d]Department of Biochemistry, Faculty of Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey; [e]Department of Biophysics, Faculty of Medicine, Marmara University, Istanbul, Turkey; [f]Department of Basic Medical Sciences, Faculty of Dentistry, Marmara University, Istanbul, Turkey

ABSTRACT

Zebrafish (*Danio rerio*) is becoming an increasingly important model in epilepsy research. Pentylenetetrazole (PTZ) is a convulsant agent that induces epileptic seizure-like state in zebrafish and zebrafish embryos and is most commonly used in antiepileptic drug discovery research to evaluate seizure mechanisms. Classical antiepileptic drugs, such as valproic acid (VPA) reduce PTZ-induced epileptiform activities. Opioid system has been suggested to play a role in epileptogenesis. The aim of our study is to determine the effects of morphine in PTZ-induced epilepsy model in zebrafish embryos by evaluating locomotor activity and parameters related to oxidant-antioxidant status, inflammation, and cholinergic system as well as markers of neuronal activity *c-fos*, *bdnf*, and opioid receptors. Zebrafish embryos at 72 hpf were exposed to PTZ (20 mM), VPA (1 mM), and Morphine (MOR) (100 µM). MOR and VPA pretreated groups were treated with either MOR (MOR + PTZ) or VPA (VPA + PTZ) for 20 min before PTZ expoure. Locomotor activity was quantified as total distance moved (mm), average speed (mm/sec) and exploration rate (%) and analyzed using ToxTrac tracking programme. Oxidant-antioxidant system parameters, acetylcholinesterase activity, and sialic acid leves were evaluated using spectrophotometric methods. The expression of *c-fos*, *bdnf*, *oprm1*, and *oprd1* were evaluated by RT-PCR. MOR pretreatment ameliorated PTZ-induced locomotor pattern as evidenced by improved average speed, exploration rate and distance traveled. We report the restoration of inflammatory and oxidant-antioxidant system parameters, *c-fos*, *bdnf*, and opioid receptor *oprm1* as the possible mechanisms involved in the ameliorative effect of MOR against PTZ-induced epileptogenic process in zebrafish embryos.

GRAPHICAL ABSTRACT



ARTICLE HISTORY

Received 16 March 2022
Revised 24 June 2022
Accepted 19 July 2022

KEYWORDS

Pentylenetetrazole; valproic acid; morphine; oxidant-antioxidant; zebrafish embryos

CONTACT Ebru Emekli-Alturfan ebruemekli@yahoo.com, eiemekli@marmara.edu.tr Department of Basic Medical Sciences, Faculty of Dentistry, Marmara University, Istanbul, Turkey

Introduction

Epilepsies are prevalent neurological disorders that affect over 50 million individuals globally, with a frequency of 1–2% (Beghi 2020). At least one novel antiepileptic medication has been reported to be approved for clinical use per year (Loacker et al. 2007). On the other hand, the effectiveness of antiepileptics is debatable, since only two-thirds of individuals with epilepsy are seizure-free after pharmacological therapy and the range and severity of adverse effects remain considerable (Kwan and Sander 2004).

One of the earliest proconvulsant medications used in animal models to elicit seizure activity was pentylenetetrazole (PTZ) (Shimada and Yamagata, 2018). PTZ is suggested to cause seizures by interfering with GABAergic neurotransmission which is a quick inhibitory synaptic transmission regulated through the interaction of γ-aminobutyric acid (GABA) with the ionotropic and the metabotropic membrane receptors (Bormann 2000). On the other hand, Valproate (VPA), currently one of the most widely prescribed antiepileptic drugs in the world, promotes the inhibitory action of GABA through both pre-synaptic and post-synaptic pathways, increasing synaptic GABA availability and enhancing GABA-mediated responses (Rogawski and Löscher 2004).

The PTZ seizure test in mice has been one of the most commonly utilized models for antiseizure development over the last decades, and it's typically predictive of drugs that may prevent seizures in humans (Shimada and Yamagata 2018). In recent years, zebrafish have become well-recognized animal models for a variety of central nervous system (CNS) disorders. They are used for both clarifying the origins of these illnesses and the sequencing of events leading up to their development, as well as for high-throughput *in vivo* drug screening (Gawel et al. 2020). Zebrafish embryos/larvae can also be used in chemoconvulsant-based epilepsy models (Baraban et al. 2005; Baraban et al. 2007).

A rising amount of research suggests that the opioid system may play a role in epileptogenesis. High dosages of short-acting opioids, such as morphine (MOR) was found to cause myoclonus and seizures as the most prevalent and serious adverse effects (Fichna et al. 2013; Woodward et al. 2017). Furthermore, alterations in opioid receptor shape and function have been linked to epilepsy (Burtscher and Schwarzer 2017). A modulatory impact of opioids on neuronal excitability and neuroexcitation as a probable adverse effect of the opioid medication has also been identified (Saboory et al. 2007). On the other hand, there is no study examining and comparing the effects of MOR and VPA in the model of PTZ-induced epilepsy.

Due to their great genetic and physiologic similarity with humans, zebrafish larvae/embryos bring numerous advantages as an *in vivo* screening platform for drug development (Peterson and Macrae 2012). A single adult zebrafish mating pair may produce up to 200 offspring each week, which grow fast *ex utero*. Substances can be directly introduced to the surrounding media and absorbed by larvae via the gastrointestinal system, skin, or gills. Zebrafish embryos can survive in small spaces due to their small size, and so only need microgram quantities of substance for every test (Afrikanova et al. 2013). The zebrafish embryo has been employed in a rising number of efficacy and safety tests of drugs. In terms of efficacy, the zebrafish embryo model has shown to be a potent new system for researching the fundamental causes of disorders like epilepsy (Baraban et al. 2005; Afrikanova et al. 2013). The zebrafish larva/embryo PTZ seizure assay has been demonstrated to be comparable to human epilepsy and may be utilized to screen anticonvulsant medications at a medium/high-throughput level using the startle response of zebrafish larvae/embryo (Baraban et al. 2005; Baraban et al. 2007).

Accordingly, the aim of our study is to determine the effects of MOR in the PTZ-induced epilepsy model zebrafish embryos in comparison with VPA through locomotor activity and parameters related to oxidant-antioxidant status, cholinergic system, and inflammation as well as c-fos, bdnf, and opioid receptors.

Methods

Zebrafish

Zebrafish (Strain AB/AB) were housed in a Zebtec, Tecniplast, Italy aquarium system at a temperature of 27 ± 1 °C under a 14/10 h light/dark cycle. The pH of the system water is between 6.9 and 7.2. Reverse osmosis water containing 0.018 mg L^{-1} Instant Ocean™ salt was used for all experiments. Fish were fed with commercial flake fish chow and live artemia twice daily. After natural laying, fertilized eggs were collected and selected for experimental use according to their development and morphology as described in Westerfield (2000) using a stereomicroscope (Zeiss Discovery V8, Germany). No ethical approval was required for the protocols implemented as specified by the Council of Europe (1986), Directive 86/609/EEC, as the zebrafish embryos used were not older than 5 days.

Drugs and treatment

PTZ (≥99% pure; CAS no: 54-95-5) and VPA sodium salt (98% pure, CAS no: 1069-66-5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Morphine hydrochloride (98% pure) was purchased from Merck, Darmstadt, Germany. They were all analytical grades with the highest purity available. All compounds were dissolved in embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 150 μM KH_2PO_4, 50 μM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$, pH 7.2) (Westerfield 2000).

To establish accurate dose–response associations for the investigated chemicals, toxicity experiments with zebrafish embryos require a sufficient number of healthy and freshly fertilized embryos for each exposure and control treatment. To get a sufficient amount for the RNA pool (50 embryos/pool; 3 biological replicates for each group) and biochemical analyses (50 embryos/pool; 3 biological replicates for each group), we prepared as six biological replicates of pools of zebrafish embryos at 72 h post-fertilization (hpf), (50 embryos/pool) for each group. Each group of zebrafish embryos were placed in glass petri dishes (60 × 15 mm) with

10 ml of embryo medium. Embryos were exposed to final concentrations of 20 mM PTZ; 100 μM MOR; 1 mM VPA, 20 mM PTZ + 100 μM MOR; 20 mM PTZ + 1 mM VPA. The control group was exposed to the embryo medium.

The dose of PTZ was chosen based on the previously reported dose of PTZ inducing seizures exhibiting many of the features of epilepsy, including tonic-clonic-like convulsions (Baxandale et al. 2012). The doses of MOR were selected according to our previous study and the previously calculated EC_{50} (concentration required to produce abnormalities in 50% of embryos) (Ali et al. 2014; Cansız et al. 2021). VPA dose was selected according to the previously reported EC_{50} for VPA (Baraban et al. 2005).

Video tracking and locomotor activity

For behavioral activity, at 3 dpf zebrafish embryos were transferred to a 6-well plate with 1 larvae per well and each experimental replicate was comprised of 12 larvae per treatment group. Baseline behavioral activity of zebrafish embryos was recorded for 5 min., then movements were recorded over the next 10 min following the addition of chemical treatment. Treatment embryos with 20 mM PTZ were observed scores seizure-like behavior from stages 1 to 3 (Baraban et al. 2005). Scores: 1—increased swim behavior/hyperactivity; 2—at least two episodes of 'whirlpool-like' swimming pattern in which fish swim rapidly in circles; 3—tonic-clonic seizure-like convulsions and loss of posture (Baraban et al. 2005; Baxandale et al. 2012). Embryos exposed to MOR and VPA pretreatment for 20 min were also recorded for 10 min. After the 20th min, the embryos were taken into the PTZ solution and the epilepsy activities were video recorded for 10 min (Figure 1). MOR and VPA decreased the scores seizure and rapid swim behavior of embryos (Figure 2(A)).

Behavior activity was quantified as the total distance moved (mm), average speed (mm/s), and exploration rate (%). These data were analyzed using the ToxTrac (Windows Program optimized for tracking) and exported to Excel (Microsoft) for statistical analyses with Graphpad Prism 9.1.

Biochemical parameters

At 72 hpf, zebrafish embryos were used for the biochemical analyses and prepared as replicate pools (50 embryos/pool; three biological replicates for each group). For each pool, embryos were homogenized in physiological saline and centrifuged briefly.

Determination of total protein

The total protein level was determined by the Lowry method. In this method, proteins are first reduced with phosphomolybdic-phosphotungstic acid reagent (foline reagent) after reacting with copper ions in an alkaline environment. The intensity of the blue color formed is directly proportional to the protein concentration and is measured with a spectrophotometer at 500 nm. The results of all parameters are expressed per protein (Lowry et al. 1951)

Determination of lipid peroxidation (LPO)

LPO levels were defined using the Yagi method. The absorbance of the pinkish color formed as a result of the reaction between malondialdehyde (MDA), a product of LPO, and thiobarbituric acid (TBA) was measured by spectrophotometer. The extinction coefficient of 1.56.105 $M^{-1}cm^{-1}$ was used and LPO was expressed as nmol MDA/mg protein in MDA equivalents (Yagi 1984).

Determination of nitric oxide (NO)

Nitric oxide (NO) levels were determined using the Miranda method. In the Miranda method, nitrate is reduced to nitrite with vanadium (III) chloride. Then, nitrite was formed using an acidic environment and the sulfonyl amide reacted with N-(1-Naphthyl) ethylenediamine dihydrochloride to produce the complex diazonium compound. The colored solution formed at 540 nm was measured by spectrophotometer and the results were calculated as nmol NO/mg protein (Miranda et al. 2001)

Determination of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by the Mylorie method. In the Mylorie method, the effect of riboflavin-sensitized photo-oxidation of SOD, o-dianisidine, is enhanced. Superoxide activity is produced by illuminating the reaction mixture containing O-dianisidine dihydrochloride and riboflavin with the light of a fluorescent lamp. Oxidation of O-dianicidin is sensitized by riboflavin and enhanced by SOD, and the increase is linearly dependent on SOD concentration. Absorbances at 0 and 8 min of illumination were measured at 460 nm using a spectrophotometer and net absorbances were calculated. Results were expressed as U/mg protein (Mylorie et al. 1986).

Determination of catalase (CAT) activity

The catalase (CAT) activities of the supernatants were determined by the modified method of Aebi, which is based on the conversion of hydrogen peroxide (H_2O_2) into the water by the action of the CAT enzyme. This conversion was observed as a decrease in absorbance measured at 240 nm, and the CAT activity of the samples was given as U/mg protein (Aebi 1984).

Determination of Glutathione-S-transferase (GST)

The determination of Glutathione-S-transferase activity was performed through the spectrophotometric determination at 340 nm of the absorbance of the product formed by the conjugation of GSH and 1-chloro-2,4-dinitro-benzene (CDNB) (Habig et al. 1974).

Determination of sialic acid (SA)

SA levels were determined by Warren's (1959) thiobarbituric acid method. After incubation of homogenates with 0.1 N H_2SO_4 at 80 °C for 1 h, hydrolyzate was used for analysis. SA is oxidized with sodium periodate in concentrated phosphoric acid, and the product of periodate oxidation is combined with thiobarbituric acid, and then the resulting chromophore is extracted in cyclohexanone. Absorbance was measured using spectrophotometer at 549 nm wavelength and results were expressed as mg SA/g protein.

Determination of acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) activity in the supernatants was determined using the method of Ellman (Ellman et al. 1961). In the Elmann method, thiocholine is produced by the action of acetylcholinesterase, which produces a yellow color with 5,50-dithiobis (2-nitrobenzoic acid). The intensity of the yellow product color is proportional to the enzyme activity in the sample and was measured using spectrophotometer at 412 nm.

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

Each embryos groups were used for RNA isolation at the end of the treatment. Rneasy Mini Kit and Qiacube (Qiagen, Hilden, Germany) were used according to the instructions of the manufacturer. A single-stranded cDNA was produced from 1 μg of total RNA using RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany). DNA Master SYBR Green kit (Qiagen, Hilden, Germany) was used to perform RT-PCRs. Beta-actin was used as the housekeeping gene. Relative levels of transcription were calculated using the ΔΔCT method based on the normalization of the values using the housekeeping gene (Livak and Schmittgen 2001). The primers used are c-fos, bdnf, oprm1, oprd1b, and housekeeping gene β-actin.

Statistical analysis

The effect of PTZ, MOR, and VPA treatments on zebrafish embryos were assessed using One-way analysis of variance (ANOVA), considering the locomotor activity parameters, LPO, NO, SOD, GST, CAT, SA, and AchE, and the gene expressions as the response variables and the treatments as the explanatory variable. Tukey's multiple comparison test was used as *post-hoc* test to compare the effects of VPA and MOR pretreatments on PTZ-exposed embryos. Statistical analysis was done using GraphPad Prism 9. $p < 0.05$ was considered as significant.

Results

Results of locomotor activities

Feeding, social, and defensive actions of zebrafish are all influenced by locomotor behavior (Colwill and Creton 2011). When animals are in an anxious condition, thigmotaxis refers to the habit of avoiding the center of an area and staying or moving in close proximity to the edges of an unfamiliar environment. This behavior is a confirmed anxiety indicator that has evolved in a variety of animals, including fish, rats, and humans. In our study, PTZ-exposed zebrafish embryos showed altered locomotor behavior and, indications of agitation within a few seconds of coming into touch with the proconvulsant. The embryos swam around the outside of the well indicating thigmotaxis, and exhibiting Stage I seizure-like activity, characterized by the altered locomotor activity occurring in a matter of seconds to minutes, defined by a series of events that begin with rapid movements around the periphery of the well as previously reported (Baraban et al. 2005). This was followed by 'whirlpool'-like movements, a brief break, and then swimming in a rapid, jerky way, with intermittent body stiffening and loss of posture (Stage II). These occurrences are similar to mammalian tonic and clonic seizure stages II and III (Berghmans et al. 2007). In our study, the control group presented normal swimming traces, however, abnormal swimming patterns were observed in the PTZ-exposed group which was ameliorated by MOR and VPA pretreatments (Figure 1(A)). PTZ-exposed group had significantly increased average speed and although the effect observed in VPA group is more, both MOR and VPA administration decreased average speed in the PTZ-exposed groups (Figure 1(B)) ($p < 0.05$ and $p < 0.0001$, respectively). PTZ administration also increased total distance (Figure 1(C)) and exploration rate (Figure 1(D)) and MOR and VPA had similar effects by decreasing both parameters.

Results of biochemical analysis

Results of LPO, NO, SOD, CAT, and GST analysis

PTZ administration caused oxidative stress as evidenced by increased LPO and NO levels ($p < 0.01$ and $p < 0.05$, respectively) (Figure 1). SOD and CAT activities decreased in the PTZ-treated group ($p < 0.05$ and $p < 0.01$, respectively) (Figure 2). Both MOR and VPA pretreatments in PTZ-exposed embryos decreased LPO levels significantly ($p < 0.05$ and $p < 0.0001$, respectively) (Figure 1(A)).

Both MOR and VPA treatments improved SOD activities in the PTZ-exposed groups ($p < 0.0001$) but did not change CAT activities (Figures 3(A,B)). By conjugating GSH to a hydrophobic substrate, GST detoxifies endobiotic and xenobiotic substances (Öztetik 2008). In our study, PTZ treatment increased GST activities significantly ($p < 0.05$) and MOR treatment in the PTZ group reduced and normalized this effect ($p < 0.01$) (Figure 4(A)).

Results of SA and AChE analysis

SA residues are connected to glycans and may play a role in a variety of biological processes, including cell-to-cell communication. SA may reduce immunogenicity by affecting inflammatory and immunological responses in cells (Rodrigues and Macauley 2018). Both PTZ exposure and VPA alone led to significant elevations in the SA levels ($p < 0.05$ and $p < 0.0001$, respectively). On the other hand, both MOR and VPA significantly decreased and normalized SA levels in

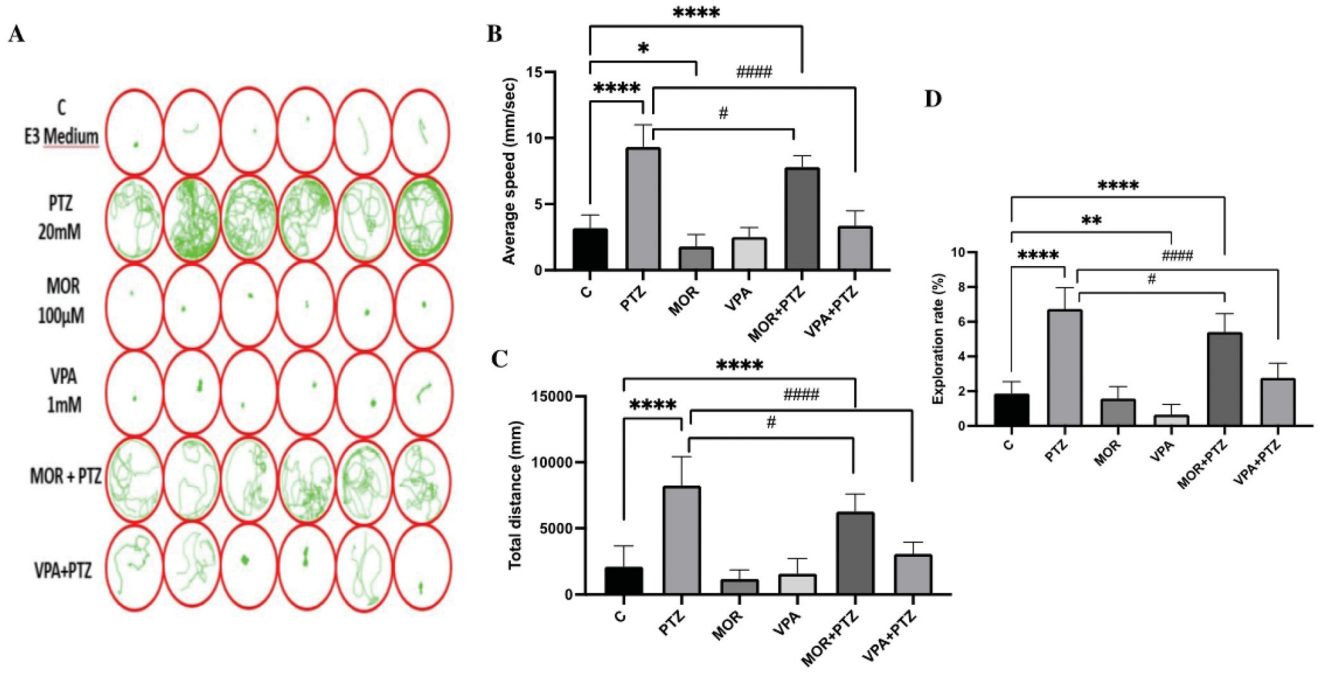


Figure 1. (A) Swimming patterns of the groups (B) Average speed (mm/s), (C) Total distance (mm), and (D) Exploration rate (%) of the embryos in the groups. Data are expressed as mean ± *SD*, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to the Control group; $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; MOR: morphine; VPA: valproate; *SD*: standard deviation.

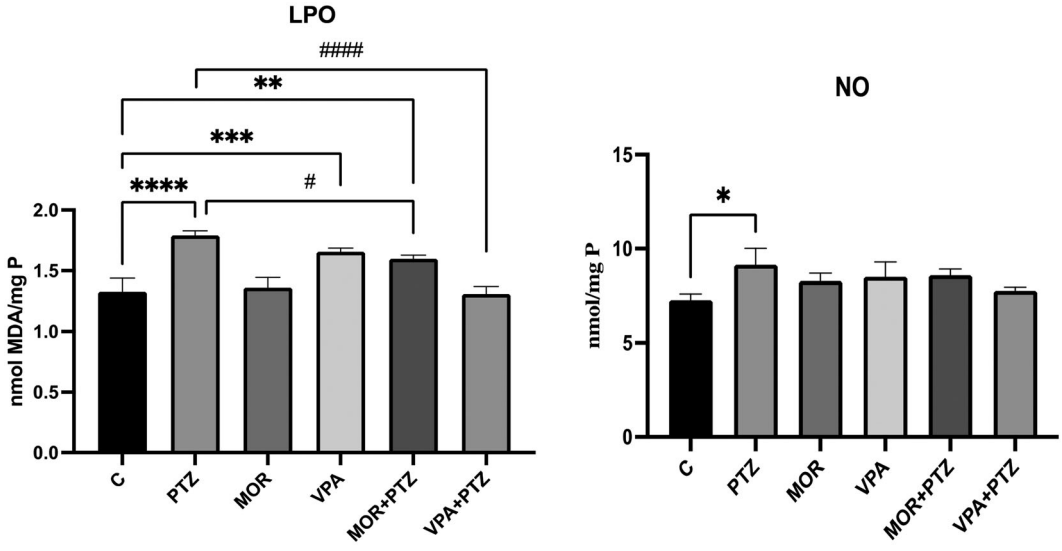


Figure 2. (A) Malondialdehyde (MDA) levels as an index of lipid peroxidation (LPO); (B) Nitric Oxide (NO) levels of the groups. Data are expressed as mean ± *SD* from the three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to the Control group; $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; Mor: morphine; VPA: valproate; *SD*: standard deviation.

the PTZ groups ($p < 0.0001$) (Figure 4(B)). AChE hydrolyzes acetylcholine (ACh) into acetyl-CoA and choline, thereby shutting off cholinergic transmission.

In our study, PTZ exposure decreased AChE activity ($p < 0.01$) however, it was ameliorated by VPA pretreatments in the PTZ-group ($p < 0.01$) (Figure 4(C)).

Results of gene expression analysis

Expression of *c-fos* increased significantly in the PTZ-exposed group ($p < 0.0001$), which was ameliorated both by MOR and VPA treatments ($p < 0.0001$) (Figure 5(A)). *bdnf* expressions significantly increased in the PTZ exposed group and MOR treatment alone also led to increased *bdnf* expressions. Moreover, in the PTZ groups, both MOR and VPA treatments led to higher *bdnf* expressions ($p < 0.01$ and $p < 0.001$, respectively) (Figure 5(B)). The opioid receptor gene *oprd1* decreased significantly in all groups and in the PTZ groups both MOR and VPA treatments led to reduced *oprd1* expressions (Figure 6(A)). *oprm1* expression also decreased in the PTZ exposed group however in the PTZ groups both MOR and VPA treatments led to significantly increased *oprd1* expressions ($p < 0.0001$) (Figure 6(B)).

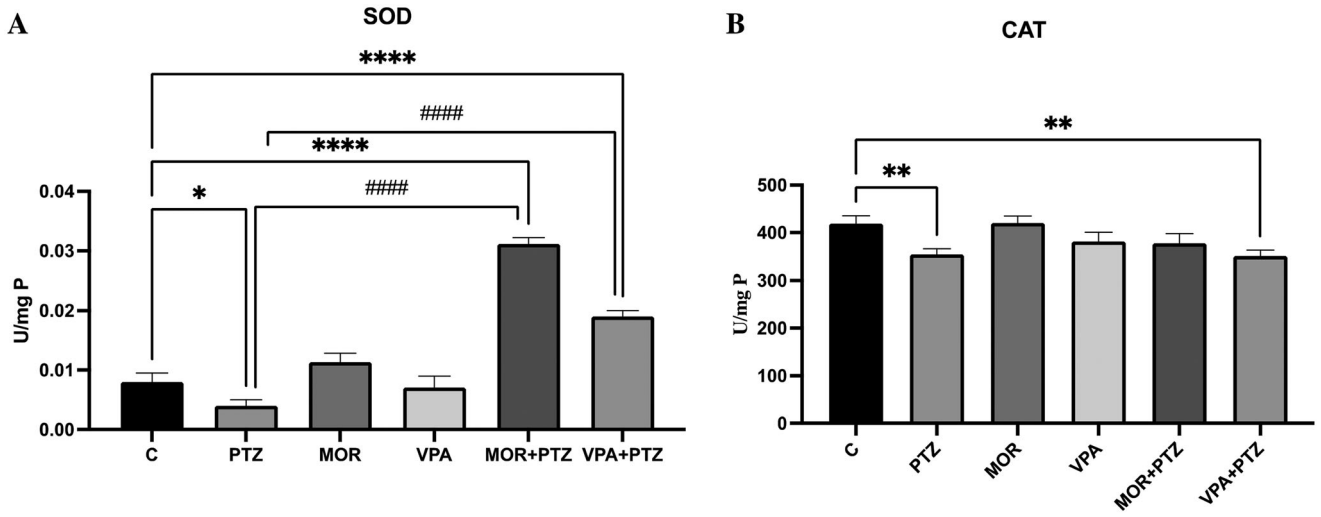


Figure 3. (A) Superoxide dismutase and (B) Catalase activities of the groups. Data are expressed as mean ± *SD* from the three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to the Control group; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; Mor: morphine; VPA: valproate; *SD*: standard deviation.

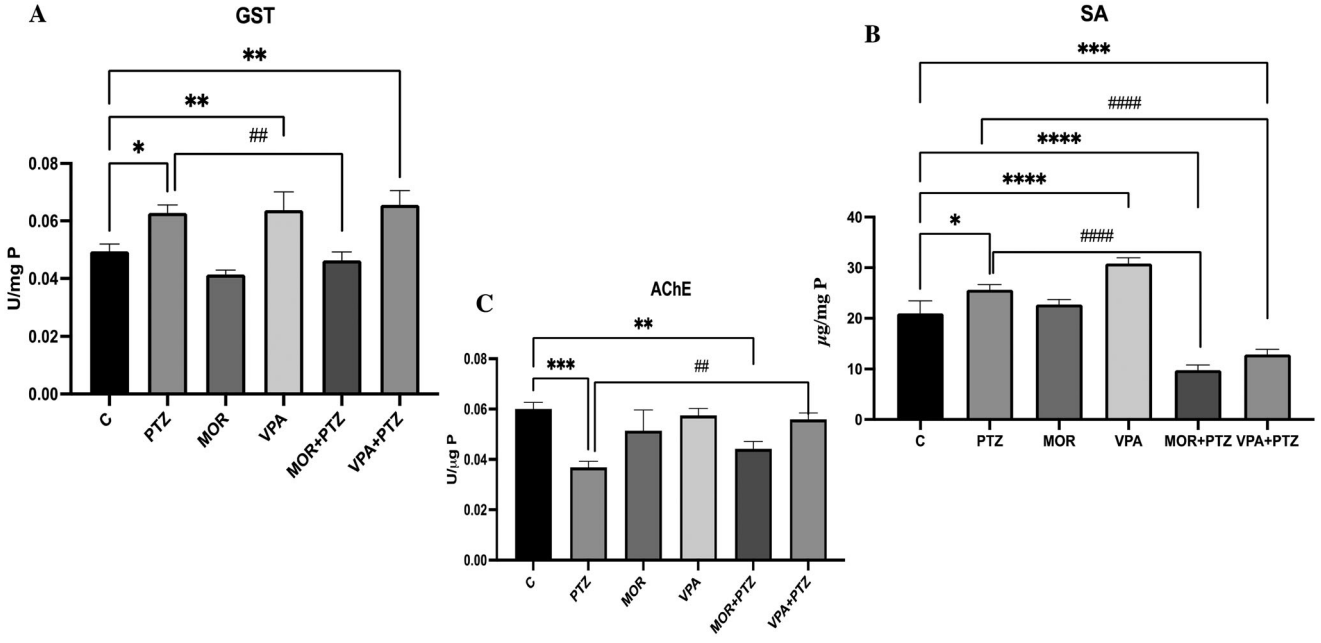


Figure 4. (A) Glutathione S-Transferase activities (GST); (B) Sialic acid (SA) levels and (C) Acetylcholinesterase activities of the groups. Data are expressed as mean ± *SD* from the three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to the Control group; $^{\#\#}p < 0.01$; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; Mor: morphine; VPA: valproate; *SD*: standard deviation.

Discussion

The opioid system has been suggested to affect epileptogenesis through the modulation of neuronal excitability and neuroexcitation (Woodward et al. 2017; Burtscher and Schwarzer 2017). In the present study, we compared the effects of morphine and VPA in PTZ-induced epilepsy model in zebrafish embryos. According to the results of our study, altered locomotor behavior, indications of agitation, and 'whirlpool'-like movements were observed similar to mammalian seizure stages II and III in PTZ-exposed zebrafish embryos. When the locomotor activity was evaluated in terms of average speed, exploration rate, and total distance, it was observed that morphine pretreatment had an ameliorative effect comparable to VPA in PTZ-exposed zebrafish embryos. Moreover, abnormal swimming patterns observed in the PTZ-exposed group were ameliorated by morphine and VPA pretreatments.

As a GABA receptor antagonist, PTZ has been widely utilized in animal models to cause seizures. The principal effect of a PTZ-induced seizure is to lower GABA levels (Erdtmann-Vourliotis et al. 1998). PTZ-induced epilepsy model is characterized by progressive excitability changes and a significant imbalance in excitatory-inhibitory neurotransmission (Zhu et al. 2016). High increases in Ca^{++} and K^{+} intracellular currents, as well as glutamate release in post-synaptic terminals, are triggered by each chemical or electrical stimulus (Bymaster et al. 2003). Together, these activities cause a significant bioenergetics malfunction, resulting in free radical overproduction and activation of caspase-mediated cell

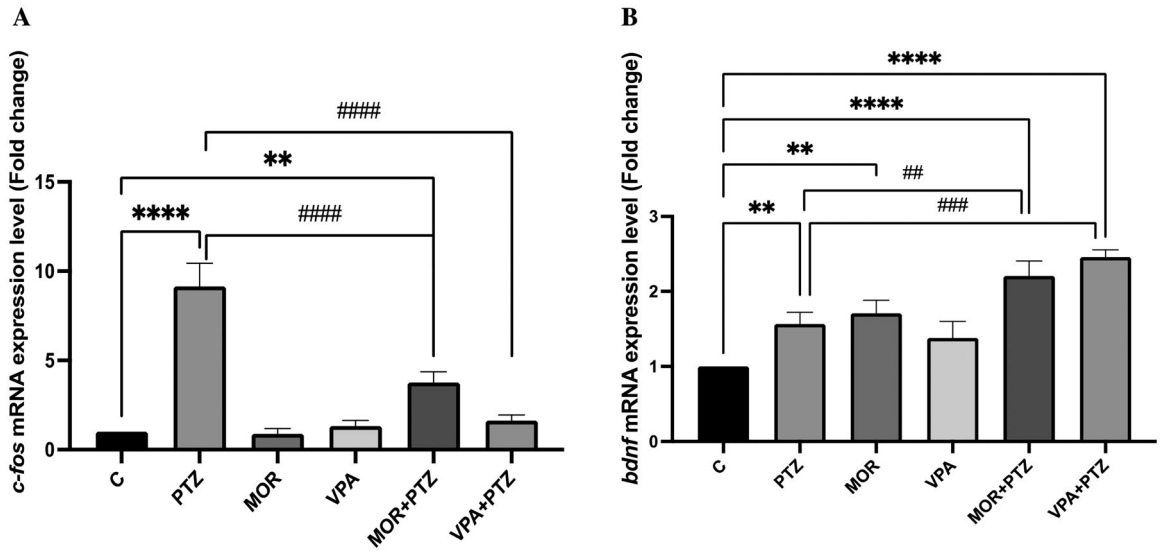


Figure 5. Bar graph presentation of the fold change of (A) *c-fos* (B) *bdnf* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± *SD*. $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to the Control group; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; Mor: morphine; VPA: valproate; *SD*: standard deviation.

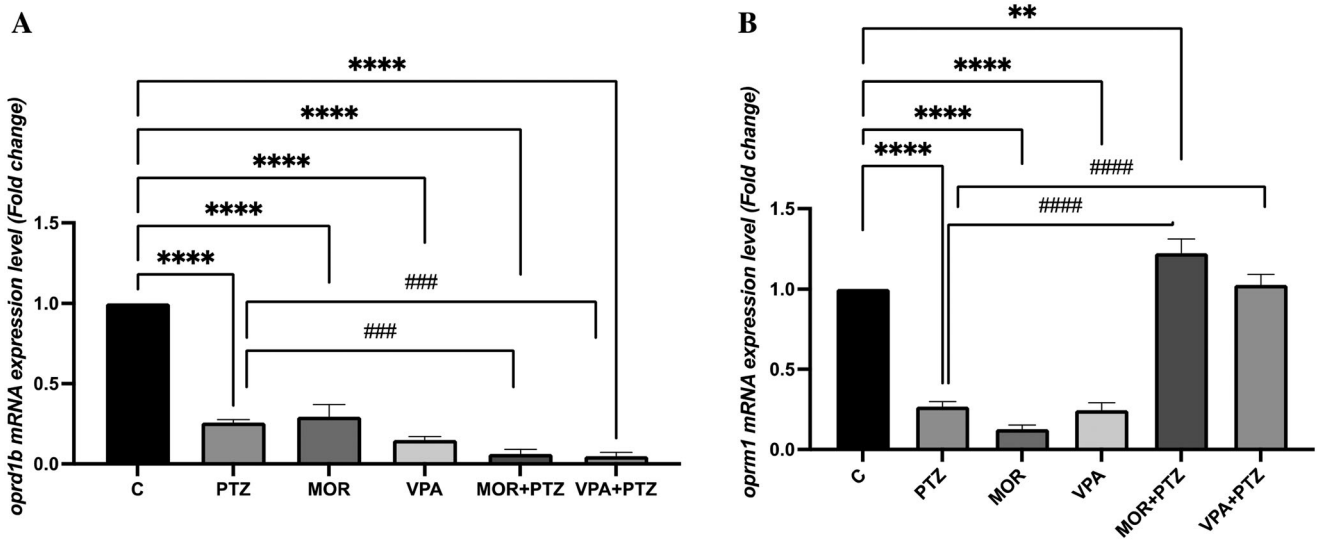


Figure 6. Bar graph presentation of the fold change of (A) *oprd1* (B) *oprm1* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± *SD*. $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to the Control group; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ compared to the PTZ group, C: control; PTZ: pentylenetetrazole; Mor: morphine; VPA: valproate; *SD*: standard deviation.

death pathways (Agarwal et al. 2011; de Souza et al. 2019). In the present study, PTZ exposure led to increased NO levels. NO interacts with ROS to produce peroxynitrite which is a powerful cell death inducer (Borutaite et al. 2000). Similar to our results, De Souza et al. (2019), reported that PTZ-kindling increased nitrite levels in all of the brain locations in mice. The oxidative damage to membrane lipids causes LPO. LPO was found to be higher in rats subjected to PTZ in previous investigations (Mazhar et al. 2017; de Souza et al. 2019). Our data support these findings since the PTZ-exposed zebrafish embryos had higher MDA levels. The reduction in MDA levels was maintained by both morphine and VPA pretreatments.

Brain-derived neurotrophic factor (BDNF) and c-Fos have been demonstrated to enhance epileptogenesis and are used as indicators of neuronal activity (Malhi et al. 2014). In our study, both PTZ and morphine exposures led to increased *bdnf* expressions. Regional excitability is largely mediated by seizure-induced plasticity. Epileptogenesis is known to upregulate BDNF, making it a supportive element for this process (Wang et al. 2012). BDNF, on the other hand, is a key regulator of neuronal survival and development, with implications for the pathophysiology of depression, anxiety, and other neuropsychiatric disorders (Mitre et al. 2017). Accordingly, PTZ-kindled mice have been shown to present a significant increase in BDNF levels when compared to the control group (De Souza et al. 2019). In the present study, both morphine and VPA pretreatments in the PTZ-exposed groups led to higher *bdnf* expressions when compared with the PTZ group. VPA is a widely-used anti-epileptic and

mood-stabilizing drug, and fetal VPA exposure in humans leads to behavioral and intellectual disorders, such as autism. Similar to our results, Almeida et al. (2014) showed that VPA administration increased both BDNF mRNA and protein levels in the fetal mouse brain and suggested that as a regulator of neurogenesis, an aberrant increase in BDNF expression in the fetal brain may lead to VPA-induced cognitive disorders through the alteration of brain development.

PTZ has been shown to enhance c-Fos expression, which is an immediate early gene and sign of increased neuronal activity, in a variety of brain areas in previous research (Szyndler et al. 2009). However, in PTZ-induced seizure mice, the hippocampus is most commonly studied as a representative brain area (Chen et al. 2011). Li et al. showed that PTZ substantially elevated c-Fos expression in the dentate gyrus, CA1, CA3, and CA4 of the hippocampus by immunohistochemical investigation (2014). In their study, similar to our results VPA significantly inhibited PTZ-induced *c-fos* expression. We observed that morphine inhibited PTZ-induced *c-fos* increase with a similar effect to VPA.

In our study, PTZ exposure led to decreased AChE activity which was ameliorated in the VPA pretreated PTZ-group. Cholinergic disruption has been linked to epilepsy and cholinergic transmission has been associated with anxiety and depression-like reactions (Wang et al. 2021). AChE hydrolyzes acetylcholine (ACh) in the brain, converting it to acetyl-CoA and choline and thereby ending cholinergic transmission (Mineur and Picciotto 2010). Our results are in line with the previous report significantly decreased AChE activity in PTZ-treated mice (Anesti et al. 2020). Similarly, a single dose of PTZ caused a significantly reduced AChE activity with a significant increase in ACh content in specific regions of brain including the cerebral cortex, cerebellum, and hippocampus (Visweswari et al. 2010). Decreased AChE activity in different brain regions during seizures have been suggested to regulate ACh levels in epileptic and seizure models (Freitas et al. 2006). In our study, morphine pretreatment did not affect AChE activity, however, decreased AChE activity due to PTZ was ameliorated by VPA pretreatments.

We determined the levels of SA in the epileptogenesis induced by PTZ and the effect of morphine on this process. Increased SA levels in the PTZ-exposed group indicated increased inflammation and morphine acted similar to VPA by ameliorating this effect by lowering SA acid levels. Inflammation may have a role in the evolution of spontaneous and recurrent seizures. As a result of pro-convulsant occurrences, immune cells are stimulated, pro-inflammatory cytokines are released, neuronal excitation and blood-brain barrier malfunction are elicited, and seizures are generated or recurred (Meng and Yao 2020).

The opioid receptor belongs to the opioid family of G-protein-coupled receptors. *oprm1* and *oprd1* are opioid receptor genes that may influence morphine analgesia variability (Pathan and Williams 2012). Our study showed that *oprd1* and *oprm1* expressions decreased in the PTZ-exposed embryos. Decreased expressions of opioid receptors might be the result of increased opioid peptide release due to PTZ exposure. Following PTZ injection, increased opioid peptide release has been implicated in seizure arrest processes. PTZ-induction raises opioid peptides in a variety of brain locations and causes the release of peptides in the striatum (Kilinc and Gunes 2019). Studies show that electrical kindling affects mu and delta opioid receptors (Grecksch et al. 2004). Systemic injection of PTZ reduces retention of a one-trial step-through inhibitory avoidance test, which is reversed by the opiate antagonist naltrexone (Kilinc and Gunes 2019).

Rocha et al. (1996) reported lower mu and delta opioid receptor levels as a result of increased exposure to opioid peptides due to PTZ treatment. They concluded that PTZ exposure is related to an imbalance between the excitatory and inhibitory systems that is evident early during the epileptogenic process.

Oprd1 is homologous to human OPRD1 (opioid receptor delta 1). It has enkephalin and morphine receptor activity. It's a part of the opioid receptor signaling pathway. It is found in the central nervous system, notochord, prechordal plate, swim bladder bud, and trunk, among other places (Arévalo et al. 2018). Nielsen et al. (2017) reported that genetic polymorphisms in the Comt and Oprm1 genes, regardless of gender, as well as Oprd1 in males, may impact morphine analgesia variability in experimental pain models. *oprm1* is a mu-opioid receptor-homologous gene in the zebrafish (Barrallo et al. 2000) has comparable pharmacological features to mu opioid receptor in mammals (de Velasco et al. 2009). The distribution of the *oprm1* gene and protein has been shown in larval zebrafish (Bretaud et al. 2007; Sanchez-Simon and Rodriguez 2008). Morphine binds to mu opioid receptor, the delta, and the kappa opioid receptors. On the other hand, it binds to MOR, to exhibit its analgesic pharmacological effects (Pathan and Williams 2012). Sivalingam et al. (2020) investigated the effect of acute morphine exposure on the expressions of *oprm1*, *cfos*, and *npas4a* in brain by *in situ* hybridization and real-time PCR and reported increased oprm1 and npas4a mRNA levels in dorsal and ventral telencephalon, preoptic area, and in the hypothalamus but decreased signals in dorsal habenula.

Anticonvulsive drugs fail to work for a large percentage of epileptic patients. Drug-resistant epilepsy occurs when an epileptic patient fails to maintain seizure-free status after trying two anti-epileptic medicines (Sturgeon et al. 2021). Results of our study show that morphine treatment was as effective as VPA in ameliorating the locomotor pattern induced by PTZ in zebrafish embryos. Opioid receptors play a complicated role in seizures. Our findings support the view that morphine has an anticonvulsant effect at low dosages, but has the opposite effect at high ones (Saboory et al. 2007; Sturgeon et al. 2021). On the other hand, it should be noted that opioids have been known to exacerbate absence-like seizures, while antagonists prevent them. Moreover, opioids enhanced the spontaneous seizure activity in a dose-dependent manner in hippocampus *in vitro* (Saboory et al. 2007). Neuroexcitation is one of the most intriguing and harmful side effects of opioids and myoclonus and seizures have been recorded due to high dosages of morphine administration (Woodward et al. 2017). The mechanism of opioid-related neuroexcitation, and consequently its therapy,

is unknown. Morphine's proconvulsant effect may be mediated by the 3-glucuronide morphine metabolite or NO generated by constitutive nonspecific NO synthase, according to previous animal studies (Hemstapat et al. 2003; Saboory et al. 2007).

As a conclusion, at the concentration used in this study, considering the restoration of locomotor activity in morphine pretreated embryos, we suggest the improvement of inflammatory and oxidant-antioxidant system parameters, *c-fos*, *bdnf*, and opioid receptor *oprm1* as the possible mechanisms involved in the ameliorative effect of morphine against PTZ-induced epileptogenic process in zebrafish embryos.

Ethical approval

As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC.

Consent to participate

All the authors have agreed to authorship, read and approved the manuscript, and given consent to participate.

Consent for publication

All the authors have agreed to authorship, read and approved the manuscript, and given consent for publication.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Fümet Duygu Üstündağ, İsmail Ünal, Ünsal Veli Üstündağ, Derya Cansız, and Merih Beler. The first draft of the manuscript was written by Fümet Duygu Üstündağ and Ebru Emekli-Alturfan in consultation with A. Ata Alturfan and Pınar Mega Tiber. Ebru Emekli-Alturfan supervised the study. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

ORCID

Ebru Emekli-Alturfan http://orcid.org/0000-0003-2419-8587

Data availability statement

Data will be available on reasonable request.

References

Aebi H. 1984. Catalase *in vitro*. Methods Enzymol. 105:121–126.

Afrikanova T, Serruys AS, Buenafe OE, Clinckers R, Smolders I, de Witte PA, Crawford AD, Esguerra CV. 2013. Validation of the zebrafish pentylenetetrazol seizure model: locomotor versus electrographic responses to antiepileptic drugs. PLOS One. 8(1):e54166.

Agarwal NB, Agarwal NK, Mediratta PK, Sharma KK. 2011. Effect of lamotrigine, oxcarbazepine and topiramate on cognitive functions and oxidative stress in PTZ-kindled mice. Seizure. 20(3):257–262.

Ali S, Aalders J, Richardson MK. 2014. Teratological effects of apanel of sixty water-soluble toxicants on zebrafish development. Zebrafish. 11(2):129–141.

Almeida LE, Roby CD, Krueger BK. 2014. Increased BDNF expression in fetal brain in the valproic acid model of autism. Mol Cell Neurosci. 59: 57–62.

Anesti M, Stavropoulou N, Atsopardi K, Lamari FN, Panagopoulos NT, Margarity M. 2020. Effect of rutin on anxiety-like behavior and activity of acetylcholinesterase isoforms in specific brain regions of pentylenetetrazol-treated mice. Epilepsy Behav. 102:106632.

Arévalo JC, Hernández-Jiménez E, Jiménez-González A, Torres-Valle M, Iwasaki RS, López-Bellido R, Vicente-García C, Rodríguez RE. 2018. Generation and characterization of antibodies against opioid receptors from zebrafish. IJMS. 19(1):14.

Baraban SC, Dinday MT, Castro PA, Chege S, Guyenet S, Taylor MR. 2007. A largescale mutagenesis screen to identify seizure-resistant zebrafish. Epilepsia. 48(6):1151–1157.

Baraban SC, Taylor MR, Castro PA, Baier H. 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience. 131(3):759–768.

Barrallo A, Gonzalez-Sarmiento R, Alvar F, Rodriguez RE. 2000. ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*). Brain Res Mol Brain Res. 84(1–2):1–6.

Baxendale S, Holdsworth CJ, Meza Santoscoy PL, Harrison MR, Fox J, Parkin CA, Ingham PW, Cunliffe VT. 2012. Identification of compounds with anti-convulsant properties in a zebrafish model of epileptic seizures. Dis Model Mech. 5(6):773–784.

Beghi E. 2020. The epidemiology of epilepsy. Neuroepidemiology. 54(2): 185–191.

Berghmans S, Hunt J, Roach A, Goldsmith P. 2007. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res. 75(1):18–28.

Bormann J. 2000. The "ABC" of GABA receptors. Trends Pharmacol Sci. 21(1):16–19.

Borutaite V, Budriunaite A, Brown GC. 2000. Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. Biochim Biophys Acta. 1459(2–3):405–412.

Bretaud S, Li Q, Lockwood BL, Kobayashi K, Lin E, Guo S. 2007. A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. Neuroscience. 146(3):1109–1116.

Burtscher J, Schwarzer C. 2017. The opioid system in temporal lobe epilepsy: functional role and therapeutic potential. Front Mol Neurosci. 7(10):245.

Bymaster FP, Carter PA, Yamada M, Gomeza J, Wess J, Hamilton SE, Nathanson NM, McKinzie DL, Felder CC. 2003. Role of specific muscarinic receptor subtypes in cholinergic parasympathomimetic responses, *in vivo* phosphoinositide hydrolysis, and pilocarpine-induced seizure activity. Eur J Neurosci. 17(7):1403–1410.

Cansız D, Ustundag UV, Unal I, Alturfan AA, Emekli-Alturfan E. 2021. Morphine attenuates neurotoxic effects of MPTP in zebrafish embryos by regulating oxidant/antioxidant balance and acetylcholinesterase activity. Drug Chem Toxicol. :1–9. doi:10.1080/01480545.2021.1957558.

Chen CR, Tan R, Qu WM, Wu Z, Wang Y, Urade Y, Huang ZL. 2011. Magnolol, a major bioactive constituent of the bark of *Magnolia officinalis*, exerts antiepileptic effects via the GABA/benzodiazepine receptor complex in mice. Br J Pharmacol. 164(5):1534–1546.

Colwill RM, Creton R. 2011. Locomotor behaviors in zebrafish (*Danio rerio*) larvae. Behav Processes. 86(2):222–229.

de Souza AG, Chaves Filho AJM, Souza Oliveira JV, de Souza DAA, Lopes IS, de Carvalho MAJ, de Lima KA, Florenço Sousa FC, Mendes

Vasconcelos SM, Macedo D, et al. 2019. Prevention of pentylenetetrazole-induced kindling and behavioral comorbidities in mice by levetiracetam combined with the GLP-1 agonist liraglutide: Involvement of brain antioxidant and BDNF upregulating properties. Biomed Pharmacother. 109:429–439.

de Velasco EMF, Law PY, Rodriguez RE. 2009. Mu opioid receptor from the zebrafish exhibits functional characteristics as those of mammalian mu opioid receptor. Zebrafish. 6(3):259–268.

Ellman GL, Courtney K, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7(2):88–95. doi:10.1016/0006-2952(61)90145-9.

Erdtmann-Vourliotis M, Riechert U, Mayer P, Grecksch G, Höllt V. 1998. Pentylenetetrazole (PTZ)-induced c-Fos expression in the hippocampus of kindled rats is suppressed by concomitant treatment with naloxone. Brain Res. 792(2):299–308.

Fichna J, Socała K, Nieoczym D, Gach K, Perlikowska R, Janecka A, Wlaź P. 2013. The mu-opioid receptor-selective peptide antagonists, antanal-1 and antanal-2, produce anticonvulsant effects in mice. Prog Neuropsychopharmacol Biol Psychiatry. 40:126–131.

Freitas RM, Sousa FCF, Viana GSB, Fonteles MMF. 2006. Acetylcholinesterase activities in hippocampus, frontal cortex and striatum of Wistar rats after pilocarpine-induced status epilepticus. Neurosci Lett. 399(1–2):76–78.

Gawel K, Langlois M, Martins T, van der Ent W, Tiraboschi E, Jacmin M, Crawford AD, Esguerra CV. 2020. Seizing the moment: zebrafish epilepsy models. Neurosci Biobehav Rev. 116:1–20.

Grecksch G, Becker A, Schroeder H, Kraus J, Loh H, Höllt V. 2004. Accelerated kindling development in mu-opioid receptor deficient mice. Naunyn Schmiedebergs Arch Pharmacol. 369(3):287–293.

Habig WH, Pabst MJ, Jakoby WB. 1974. Assays for differentiation of glutathione-S transferases. J Biol Chem. 249(22):7130–7139.

Hemstapat K, Monteith GR, Smith D, Smith MT. 2003. Morphine-3-glucuronide's neuro-excitatory effects are mediated via indirect activation of N-methyl-d-aspartic acid receptors: mechanistic studies in embryonic cultured hippocampal neurones. Anesth Analg. 97(2):494–505.

Kilinc E, Gunes H. 2019. Modulatory effects of neuropeptides on pentylenetetrazol-induced epileptic seizures and neuroinflammation in rats. Rev Assoc Med Bras. 65(9):1188–1192.

Kwan P, Sander JW. 2004. The natural history of epilepsy: an epidemiological view. J Neurol Neurosurg Psychiatry. 75(10):1376–1381.

Li B, Wang L, Sun Z, Zhou Y, Shao D, Zhao J, Song Y, Lv J, Dong X, Liu C, et al. 2014. The anticonvulsant effects of SR 57227 on pentylenetetrazole-induced seizure in mice. PLOS One. 9(4):e93158.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods. 25(4):402–408.

Loacker S, Sayyah M, Wittmann W, Herzog H, Schwarzer C. 2007. Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. Brain. 130(Pt 4):1017–1028.

Lowry O, Rosebrough N, Farr AL, Randall R. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193(1):265–275.

Malhi SM, Jawed H, Hanif F, Ashraf N, Zubair F, Siddiqui BS, Begum S, Kabir N, Simjee SU. 2014. Modulation of c-Fos and BDNF protein expression in pentylenetetrazole-kindled mice following the treatment with novel antiepileptic compound HHL-6. Biomed Res Int. 2014:1–9.

Mazhar F, Malhi S, Simjee S. 2017. Comparative studies on the effects of clinically used anticonvulsants on the oxidative stress biomarkers in pentylenetetrazole-induced kindling model of epileptogenesis in mice. J Basic Clin Physiol Pharmacol. 28(1):31–42.

Meng F, Yao L. 2020. The role of inflammation in epileptogenesis. Acta Epileptol. 2(1):15.

Mineur YS, Picciotto MR. 2010. Nicotine receptors and depression: revisiting and revising the cholinergic hypothesis. Trends Pharmacol Sci. 31(12):580–586.

Miranda KM, Espey MG, Wink DAA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 5(1):62–71.

Mitre M, Mariga A, Chao MV. 2017. Neurotrophin signalling: novel insights into mechanisms and pathophysiology. Clin Sci. 131(1):13–23.

Mylroie AA, Collins H, Umbles C, Kyle J. 1986. Erythrocyte SOD activity and other parameters of copper status in rats ingesting lead acetate. Toxicol Appl Pharmacol. 82(3):512–520.

Nielsen LM, Christrup LL, Sato H, Drewes AM, Olesen AE. 2017. Genetic influences of OPRM1, OPRD1 and COMT on morphine analgesia in a multi-modal, multi-tissue human experimental pain model. Basic Clin Pharmacol Toxicol. 121(1):6–12.

Öztetik E. 2008. A tale of plant glutathione S-transferases: since 1970. Bot Rev. 74(3):419–437.

Pathan H, Williams J. 2012. Basic opioid pharmacology: an update. Br J Pain. 6(1):11–16.

Peterson RT, MacRae CA. 2012. Systematic approaches to toxicology in the zebrafish. Annu Rev Pharmacol Toxicol. 52:433–453.

Rocha L, Briones M, Ackermann RF, Anton B, Maidment NT, Evans CJ, Engel J. 1996. Pentylenetetrazol-induced kindling: early involvement of excitatory and inhibitory systems. Epilepsy Res. 26(1):105–113.

Rodrigues E, Macauley MS. 2018. Hypersialylation in cancer: modulation of inflammation and therapeutic opportunities. Cancers. 10(6):207.

Rogawski MA, Löscher W. 2004. The neurobiology of antiepileptic drugs. Nat Rev Neurosci. 5(7):553–564.

Saboory E, Derchansky M, Ismaili M, Jahromi SS, Brull R, Carlen PL, El Beheiry H. 2007. Mechanisms of morphine enhancement of spontaneous seizure activity. Anesth Analg. 105(6):1729–1735.

Sanchez-Simon FM, Rodriguez RE. 2008. Developmental expression and distribution of opioid receptors in zebrafish. Neuroscience. 151(1):129–137.

Shimada T, Yamagata K. 2018. Pentylenetetrazole-induced kindling mouse model. J Vis Exp. 12(136):56573.

Sivalingam M, Ogawa S, Parhar IS. 2020. Mapping of morphine-induced OPRM1 gene expression pattern in the adult zebrafish brain. Front Neuroanat. 14:5.

Sturgeon ML, Langton R, Sharma S, Cornell RA, Glykys J, Bassuk AG. 2021. The opioid antagonist naltrexone decreases seizure-like activity in genetic and chemically induced epilepsy models. Epilepsia Open. 6(3):528–538.

Szyndler J, Maciejak P, Turzyńska D, Sobolewska A, Taracha E, Skórzewska A, Lehner M, Bidziński A, Hamed A, Wisłowska-Stanek A, et al. 2009. Mapping of c-Fos expression in the rat brain during the evolution of pentylenetetrazol-kindled seizures. Epilepsy Behav. 16(2):216–224.

Visweswari G, Prasad KS, Chetan PS, Lokanatha V, Rajendra W. 2010. Evaluation of the anticonvulsant effect of *Centella asiatica* (gotu kola) in pentylenetetrazol-induced seizures with respect to cholinergic neurotransmission. Epilepsy Behav. 17(3):332–335.

Wang C, Wu H, He F, Jing X, Liang Q, Heng G, Wang L, Gao G, Zhang H. 2012. Alleviation of ferric chloride-induced seizures and retarded behaviour in epileptic rats by cortical electrical stimulation treatment. J Int Med Res. 40(1):266–281.

Wang Y, Tan B, Wang Y, Chen Z. 2021. Cholinergic signaling, neural excitability, and epilepsy. Molecules. 26(8):2258.

Warren L. 1959. The thiobarbituric acid assay of sialic acids. J Biol Chem. 234(8):1971–1975.

Westerfield M. 2000. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Eugene (OR): University of Oregon Press.

Woodward OB, Naraen S, Naraen A. 2017. Opioid-induced myoclonus and hyperalgesia following a short course of low-dose oral morphine. Br J Pain. 11(1):32–35.

Yagi K. 1984. Assay for blood plasma or serum. Methods Enzymol. 105:328–331.

Zhu X, Dong J, Shen K, Bai Y, Chao J, Yao H. 2016. Neuronal nitric oxide synthase contributes to pentylenetetrazole-kindling-induced hippocampal neurogenesis. Brain Res Bull. 121:138–147.