Received: 22 November 2016 | Revised: 17 January 2017 | Accepted: 18 January 2017

DOI 10.1002/syn.21961

RESEARCH ARTICLE

WILEY SYNAPSE

Anticonvulsant effect of sodium cyclamate and propylparaben on pentylenetetrazol-induced seizures in zebrafish

Antonella Pisera-Fuster[1]* | Sofía Otero[1]* | Alan Talevi[2] | Luis Bruno-Blanch[2] | Ramón Bernabeu[1]

[1]Instituto de Fisiología y Biofísica (IFIBIO-Houssay), Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

[2]Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

Correspondence
Ramón Bernabeu, Institute of Physiology and Biophysics (IFIBIO-Houssay), School of Medicine, University of Buenos Aires, Paraguay 2155, Buenos Aires C1121ABG, Argentina.
E-mail: rbernabeu@fmed.uba.ar

Abstract

Screening for novel anticonvulsant drugs requires appropriate animal seizure models. Zebrafish provide small, accessible, and cost-efficient preclinical models applicable to high-throughput small molecule screening. Based on previous results in rodents, we have here examined the effects of artificial sweetener sodium cyclamate and antimicrobial agent sodium propylparaben on a model of pentylenetetrazole (PTZ)-induced seizures in zebrafish. Sodium cyclamate reduced the bursts of hyperactivity, the spasms, increased the latency to spasms, and the latency to seizure, while propylparaben increased the latency to spasms. The results show the potential of zebrafish to detect novel anticonvulsant compounds while they also demonstrate the ability of two commonly ingested chemical compounds to modify the seizure threshold when were administrated at low concentration.

KEYWORDS

animal behavior, anticonvulsant drugs, seizures, zebrafish

1 | INTRODUCTION

The term epilepsy describes a heterogeneous group of neurological disorders characterized by recurrent spontaneous seizures, hyperactivity of brain structures, and alteration in the balance between excitatory and inhibitory neurotransmission pathways (Bakvis et al., 2009; Mehta, Dham, Lazar, Narayanswamy, & Prasad et al., 1994; Tunca et al., 2000). A diversity of antiepileptic drugs (AEDs) are available, although they are ineffective in up to one-third of the patients and present severe side effects (Mula & Sander, 2007; Frye et al., 2013). Animal models of acutely evoked seizures in which the convulsions are induced by chemical (Sadek et al., 2016a; Sadek & Stark, 2016b) or electrical stimuli, including the maximal electroshock seizure (MES) test and the subcutaneous pentylenetetrazol (sc PTZ) test are among the most widely used models in primary AED screening (Löscher, Hönack, Fassbender, & Nolting, 1991; Löscher, 2011). PTZ may have multiple mechanisms of action, but inhibition of the type A gamma amino butyric acid ($GABA_A$) receptors has been suggested as the primary one (Kalueff, 2007). PTZ is widely used to induce seizures in rodents and other species (Akula, Dhir, & Kulkarni et al., 2009; Carmody & Brennan, 2010; Prigol, Brüning, Godoi, Nogueira, & Zeni, 2009). In rodents, exposure to PTZ induces a concentration-dependent sequence of stereotyped behavioral changes which culminates in strong generalized tonic–clonic seizures.

Zebrafish (*Danio rerio*) has become extensively popular in preclinical research, sharing extensive genetic and physiological homology with other vertebrate species (Brittijn et al., 2009; Egan et al., 2009). They exhibit well characterized behavioral and physiological phenotypes, and they are capable of producing a large number of offspring in a short time-period (Aleström, Holter, & Nourizadeh-Lillabadi, 2006; Blaser & Gerlai, 2006; Spence, Gerlach, Lawrence, & Smith, 2008). This specie exhibits several anatomic similarities and a high genetic homology with mice and humans (Barbazuk et al., 2000). Moreover, zebrafish present a tight junction-based blood–brain barrier similar to higher vertebrates (Eliceiri, Gonzalez, & Baird, 2011; Jeong et al., 2008), which makes this model an attractive organism for screening Central Nervous System (CNS)-target drug candidates. Considering the necessity for new experimental models to study seizure and epilepsy at behavioral and pharmacological level (Stables et al., 2002; Baraban, 2007), zebrafish may represent a reasonable source of such models (Cachat et al., 2010; Wong et al., 2010).

*These authors made equal contributions to these studies.

The availability of both larval and adult zebrafish enables the investigation of a wide spectrum of epilepsy-related phenomena from young to adult (e. g., childhood and adult epilepsies); to the moment, there is no single animal model of epilepsy that can fully represent this disease. Zebrafish is a vertebrate model organism with tremendous potential for modeling acute seizures and epilepsies (Baraban, Taylor, Castro, & Baier, 2005; Baraban, 2007; Berghmans, Hunt, Roach, & Goldsmith, 2007; Winter et al., 2008). Recent studies have indeed reported seizure-like behavior in larval and adult zebrafish (Baraban, Taylor, Castro, & Baier, 2005; Berghmans et al., 2007; Goldsmith, 2004; Langheinrich, 2003). Concentration-dependent effects of PTZ on adult zebrafish have already been characterized using a seizure score (Mussulini et al., 2013; Gupta Khobragade, & Shingatgeri, 2014). The brain PTZ levels in adult zebrafish immersed into the chemoconvulsant solution at 5 or 10 mM were equivalent to those described for the mouse model. PTZ brain levels denote a concentration exposure-dependency, presenting an interaction between concentration and time of exposure (Mussulini et al., 2013).

In contrast with rodent models of epilepsy, which frequently require invasive procedures to administer the candidate drug, pharmacologic testing in zebrafish can be performed for different time exposures without any aversive intervention, since the drug can be dissolved in the tank water.

We have previously reported the anticonvulsant effects of non-nutritive sweetener sodium cyclamate (SC) and antimicrobial agent propylparaben (PP), which were identified through computer-guided virtual screening (Talevi, Bellera, Castro, & Bruno-Blanch, 2007; Talevi, Enrique, & Bruno-Blanch, 2012; Di Ianni et al., 2015). Here, we have exposed adult zebrafish to the chemoconvulsant drug PTZ, examining their responses using a detailed behavioral analysis, and we have explored the anticonvulsant effects of SC and sodium PP in this model to verify if their protective effects could be observed in nonrodent vertebrate species. Considering that many currently approved AEDs have shown other beneficial effects on other psychiatric and neurological disorders (Ettinger & Argoff, 2007) we have also used zebrafish as a behavioral model to assess possible alternative medical uses of both substances (namely, anxiolytic and antidepressant activity).

2 | MATERIALS AND METHODS

2.1 | Reagents

PTZ was purchased from Siegfried (Zofingen, Switzerland). Sodium Valproate (VPA) and SC were purchased from Sigma Aldrich (Steinheim, Germany). PP was a gentle gift from the Pharmaceutical Technology lab, Faculty of Exact Sciences, University of La Plata, La Plata, Argentina.

2.2 | Animals and housing

3-month old adult zebrafish (*Danio rerio*) were obtained from a local commercial supplier and maintained following standard procedures (Westerfield, 2007). They were kept in a 100-L tank with a constant 14 to 10 hr light/dark cycle at 28 ± 2°C and fed twice a day with *Arthemia sp.* and dry food. All fish used in this study were experimentally naive and were given at least 14 days to acclimatize to the laboratory facility. All animal work was carried out with approval from the University of Buenos Aires Research Ethics Committee. Care was taken to minimize the numbers of animals used in this experiment in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id = 1357).

2.3 | Experimental apparatus

The experimental apparatus was designed to evaluate the zebrafish movements with precision (Figure 1A) and consisted of: a heating plaque set at 28 ± 2°C; fish tank 1 and fish tank 2 (13 cm height × 7 cm width × 20 cm length), with three of their faces covered with white paper and containing 1.5 L of system water each; a fluorescent light tube (18 W) placed 35 cm behind the fish tanks and; a video camera (Microsoft LifeCam VX-700) placed 60 cm from the testing tanks and connected to a computer.

2.4 | Effect of potential AEDs on PTZ-induced seizures

We have investigated the effects of three anticonvulsants on a PTZ-induced seizure model in adult zebrafish: a classical AED, VPA, and two potential AEDs whose anticonvulsant effects in rodent models have previously been identified in our lab: SC and PP (Figure 2). SC has shown protective effects in MES test (30 mg/kg, mice) and sc PTZ test (abolishment of myoclonic and tonic–clonic seizures at 30 and 100 mg/kg and increased latencies to seizures at 30 mg/kg, mice) (Di Ianni et al., 2015 and refs therein). PP has shown protective effects in MES test (Talevi et al., 2007) and its neuroprotective effects following status epilepticus have been the object of an international patent application (PCT/IB2014/064484).

2.4.1 | Procedure

For AED acute exposure assays (Figure 1B), zebrafish were first brought individually from their home tank and placed for 20 min in tank 1 filled with system water to allow habituation to the new environment (test tank). They were then transferred to the tank 2 (behavioral testing tank) containing tank water (control group, Ctr), 830 mg/L (6 mM) PTZ (PTZ group), 830 mg/L PTZ plus 50 mg/L (300 μM) VPA (VPA group), 830 mg/L PTZ plus 1 μg/L (4.9 μM) SC (SC group) and 830 mg/L PTZ plus 9 μg/L (49 μM) PP (PP group). Each group consisted of 10 to 12 animals. VPA concentration was determined as previously reported by Gupta et al. (2014). PTZ test concentration and treatment time were selected based on our own data, with a wide range of concentrations tested (from 2 to 10 mM of PTZ). Among the PTZ concentrations tested, the one that caused seizure-like behavior without causing death was 830 mg/L (6 mM), which is in good agreement with previous studies (Gupta et al., 2014). SC and PP concentrations were determined during the setup conditions, where different concentrations of SC and PP were evaluated at behavioral level. SC was tested at 1, 5, 10, and 20 μg/L, and PP was tested at 5, 9, and 15

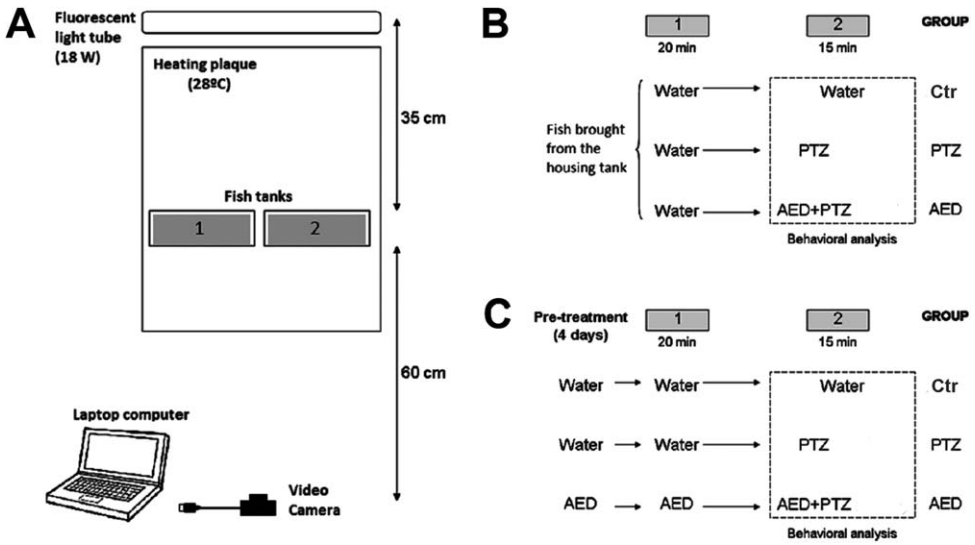

FIGURE 1 Schematic representation of the experimental conditions and procedures. (a): Experimental apparatus. (b): Schedules used to evaluate the behavioral effect of acute exposure of the potential antiepileptic drugs. (c): Schedules used to test the 4-day pretreatment before the behavioral analysis of the potential AEDs

μg/L mixed with PTZ (830 mg/L), where 1 μg/L of SC and 9 μg/L PP was finally used based on the behavioral results (Figure 3).

For 4-day AED continued exposure assays (Figure 1C), two groups of fish were placed for 4 days in 19 cm height × 22.5 cm width × 30 cm length tanks containing 1 μg/L SC (SC group), 9 μg/L PP (PP group), or saline (SAL group). On the fifth day fish were transferred (in a container filled with the solution in which they were already immersed) and kept for 15 minutes in tank 1 containing tank water (Ctr and PTZ groups), 1 μg/L SC (SC group) or 9 μg/L PP (PP group). Fish were then transferred to tank 2 (behavioral testing) containing tank water (Ctr group), 830 mg/L PTZ (PTZ group), 830 mg/L PTZ plus 1 μg/L SC (SC group) or 830 mg/L PTZ plus 9 μg/L PP (PP group).

2.4.2 | Behavioral analysis

The behavioral testing took place between 10:00 and 16:00 hr to ensure consistency and reduce potential variation in behavior due to the circadian rhythm. The behavior of each fish was recorded for a maximum of 15 min. Videos were then manually analyzed by trained blind observers. The duration of the following seizure-related endpoints was recorded by the observers: erratic movements, bursts of hyperactivity, and spasms. Erratic movements were defined as sharp changes in direction and/or velocity, which were represented as rapid darting behaviors (Wong et al., 2010). Hyperactivity was defined as prolonged (>3 s) periods of sharp changes in direction and/or velocity, which differ in duration from erratic movements. Spasms were recorded as sudden twitches or small jerks of the body that may or may not result in propulsion. Latency to spasms (s) and latency to seizure (s) were also measured.

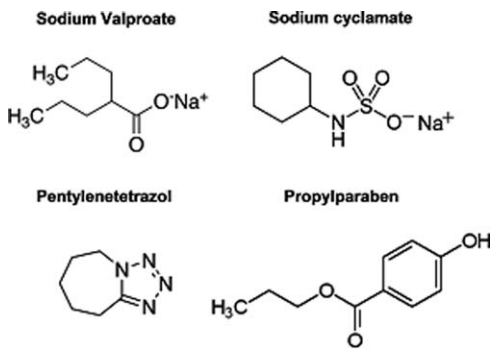

FIGURE 2 Molecular structures of VPA, PTZ, SC, and PP

To examine possible AEDs side effects or additional benefits, video tracking software (Ethovision XT7, Noldus Information Technology, Netherlands) was used in addition to direct observation, studying possible anxiety/depression-like behaviors of Ctr, SC, and PP groups (acute and 4-day exposure fish) (Stewart et al., 2011; Egan et al., 2009; Levin, Bencan, & Cerutti, 2007; Nguyen, Stewart, & Kalueff, 2014). Using the software, two zones were determined by tracing a horizontal line in the middle of the tank: top zone and bottom zone. The following end points were measured: total distance travelled (cm), average velocity (cm/s), and total time spent in the top zone of the tank (s). Movement pattern analysis is a promising approach for zebrafish behavioral phenotyping using trajectory based on individual locomotion parameters (i. e., velocity or distance swum) to extract local locomotion features, likely to be useful for the dissection of zebrafish movement-related pathologies, such as, depression and seizure. Moreover, increased top exploration with reduced bottom dwelling is an excellent neurophenotyping approach which reveals robust behavioral differences between controls versus anxiolytic zebrafish cohorts (Cachat et al., 2010; Nguyen et al., 2014).

2.5 | Statistics

All data were expressed as mean ± SEM and analyzed using the non-parametrical Wilcoxon-Mann–Whitney *U* test to compare the control groups (Ctr, VPA, and PTZ) with experimental groups (SC and PP, acute

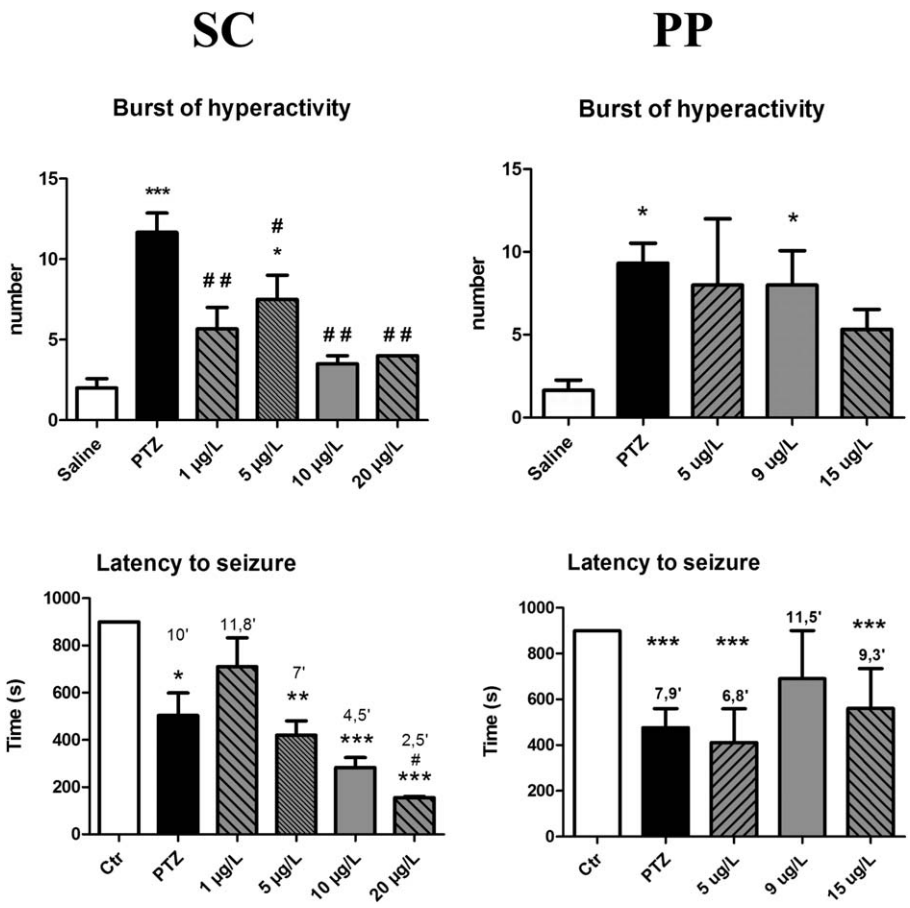

FIGURE 3 Behavioral analysis of SC and PP at different doses (indicated as μg/L). Zebrafish were habituated for 20 min in tank 1 (see Figure 1A) and then moved to tank 2 (behavioral tank) for 15 min maximum where the burst of hyperactivity and the latency to seizure were quantified. The experimental groups were: drug-free system water (Ctr), 830 mg/L PTZ, 830 mg/L PTZ plus 1, 5, 10, and 20 μg/L SC, and 830 mg/L PTZ plus 5, 9, and 15 μg/L PP. Data are expressed as mean ± SEM and were analyzed using the nonparametrical Wilcoxon-Mann–Whitney *U* test. Asterisks (*) indicate significant difference with Ctr ($^{*}p < .05$, $^{**}p < .001$, $^{***}p < .0001$), numerals (#) indicate significant difference with PTZ ($^{\#}p < .05$, $^{\#\#}p < .001$, $^{\#\#\#}p < .0001$) ($n = 8$–9 in each group)

and 4-days continued exposure). Significance was set at $p < .05$ in all experiments.

3 | RESULTS

Based in previous reports, the optimal concentration of PTZ to induce seizure in zebrafish was determined. Preliminary studies were carried out using 2, 4, 6, and 10 mM of PTZ (data not shown). After detailed analysis 6 mM (830 mg/L) was chosen based on its consistent efficacy to induce all the behaviors to evaluate seizures in our zebrafish model, without causing death; previous reports also used this concentration (Mussulini et al., 2013; Gupta et al., 2014).

3.1 | Behavioral analysis of SC and PP at different concentrations

Considering our previous results in mice using SC, we determined the concentration of SC testing four doses by quantification of two behavioral parameters associated with seizure-induced by PTZ, such as, burst of hyperactivity and latency to seizure (see Section 3.2). Pharmacological studies in zebrafish offer the advantage that exposure and systemic levels of the drug can be continuous and stable. In fact, the concentration of a drug in fish tissues after a while (sec to min), for diffusible substances can be considered equal to its concentration in the tank water (Faillace & Bernabeu, 2016).

Zebrafish were exposed to tank 1 for 20 min (Figure 1B) and then exposed to tank 2 filled with no drug (Ctr), PTZ alone (830 mg/L), and SC at 1, 5, 10, and 20 μg/L. Figure 3 (left graphs, SC) shows lower number of burst of hyperactivity at 1, 10, and 20 μg/L of SC compared to PTZ group ($p < .01$). Moreover, 1 μg/L showed the higher latency to seizure (11.8 min) compared with the other experimental groups. Next we performed the same type of characterization for PP evaluating the antiepileptic effect of the drug (Figure 3, right graphs, PP). The number of burst of hyperactivity were significant different from control only for PTZ and 9 μg/L but all the values were around eight bursts. When the latency to seizure was analyzed 9 μg/L showed the higher value (11.5 min) compared to the other experimental groups. It is important to remark that other behavioral parameters were measured, such as,

erratic movements, spasm, freezing bouts, corkscrews swimming (data not shown), and the doses of 1 μg/L for SC and 9 μg/L for PP were selected considering first the burst of hyperactivity and the latency to seizure, but also taken into account the analysis from the other parameters mentioned.

3.2 | Behavioral analysis of SC and PP on PTZ-induced seizures

Once the concentration of both AEDs was fixed, we evaluated the effect of SC at 1 μg/L and PP at 9 μg/L on PTZ-induced seizure by quantification of several behavioral parameters. Adult zebrafish were exposed to a novel tank 1 for 20 min (to habituate the zebrafish to the new environment), and then fish were exposed to tank 2 for a maximum of 15 min to PTZ alone, VPA alone, PTZ + SC, and PTZ + CC (see Figure 1B). Zebrafish behavior was recorded from tank 2 to later analyze the different behavioral parameters described in Methods section. The first parameter measured was erratic movements. An erratic movement is a complex behavior characterized by sharp changes in direction or velocity and repeated rapid darting. It is commonly observed in adult zebrafish in tanks, and this parameter helps to evaluate the normal behavior of fish. The increase in erratic movements is usually evoked by acute light stressors or reflects a general baseline of anxiety/fear state (Kalueff et al., 2013).

All the groups exhibited erratic movements (Figure 4). VPA, SC (acute exposure), and PP (4-day exposure) groups showed a significant decrease in time-periods of erratic movements compared to PTZ ($p < .01$, $p < .05$, and $p < .01$, respectively). SC (acute) and PP (4-day exposure) groups showed no significant differences in this parameter compared to control fish. PP (acute) erratic movements were significantly increased compared to VPA ($p < .001$ and $p < .05$, respectively), but without significant changes related to PTZ group. When the number of erratic movements were quantified, VPA and SC (acute and 4 days) groups showed significant differences with control group ($p < .01$, .05, and .001, respectively). 4 days SC group of animals was significant different from PTZ group ($p < .001$).

Burst of hyperactivity (time and number) and spasm (time and number) were then quantified to assess seizure-related behavior (Figure 4). All groups exhibited significant differences in the bursts of hyperactivity and spasms compared to Ctr group ($p < .001$) when the time duration of the burst or spasms were quantified. Ctr group showed no bursts of hyperactivity or spasms.

Considering the bursts, only SC group (acute exposure) displayed a significant decrease in the time of bursts of hyperactivity in comparison to the PTZ group ($p < .01$). Moreover, SC acute was the only treatment that showed a significant decrease ($p < .05$) in the number of bursts compared to PTZ group and also was the only group that showed no significant differences with control. SC (4-day exposure) and PP (acute and 4-day exposure) showed significant increases in the number of burst compared to control ($p < .01$, $p < .001$, and $p < .01$, respectively).

Regarding spasms (time), while SC and PP (4-day exposure) showed statistically significant shorter periods of spasms compared to control group ($p < .01$), SC (4-days) showed also significant differences with VPA group ($p < .05$). The number of spasm was evaluated and all groups showed significant differences with control group ($p < .01$ and $p < .05$). PTZ and VPA groups presented higher significant differences ($p < .01$) than SC and PP ($p < .05$) respect to control values.

Next, the latency to spasms was analyzed to better characterize the effect of SC and PP on PTZ-induced spasms. All groups showed significant lower latencies to spasms compared to control group ($p < .01$) and higher latencies compared to PTZ group ($p < .01$). Moreover, SC (acute and 4-day exposure) showed statistically significant longer latencies to spasms ($p < .01$) than PP (acute and 4-day exposure) ($p < .05$) all compared to PTZ group.

In addition the latencies to seizure were evaluated. SC (acute) and PP (4-day exposure) decreased the latency to seizure ($p < .05$ and $p < .01$, respectively) when compared to control animals. SC 4-days exposure was the only group significant different from PTZ, showing latencies close to controls (13.1 min). Table 1 shows, for each treatment, the percentage of individuals that reached seizures before 15 min of PTZ exposure.

3.3 | Behavioral analysis of SC and PP alone

To determine if the drugs per se induce some changes in the previously measured behavioral parameters, we determined the effect of SC or PP alone (acute and 4-day exposure).

Three controls were performed: control (ctr), PTZ alone, and VPA alone (Figure 5). All groups, except VPA, presented statistically significant longer periods of erratic movements than saline control (Ctr). Furthermore, VPA and PP (4-day exposure) showed significant differences with PTZ group ($p < .001$ and $p < .01$, respectively), displaying shorter periods of erratic movements. SC (acute), PP (acute), and PP (4-day exposure) showed significant longer periods of erratic movements than VPA ($p < .01$, $p < .001$, and $p < .05$, respectively). Except Ctr group, all groups presented bursts of hyperactivity. All acute treatments (VPA, SC, and PP) presented statistically shorter bursts of hyperactivity than the PTZ group ($p < .001$, $p < .01$, and $p < .05$, respectively). 4-day exposure to SC and PP showed no significant differences in comparison with PTZ group, but a significant difference compared to VPA ($p < .05$). Neither Ctr nor SC or PP (both after a 4-day exposure) presented spasms. PTZ, VPA, SC (acute), and PP (acute) all presented spasms of different times. All the treatments showed a significant reduction in the duration of the spasms compared to PTZ ($p < .001$). SC and 4-day exposure PP presented significant differences with VPA group ($p < .001$).

3.4 | Anxiety/depression-like behavior evaluation of SC and PP

We decided to evaluate anxiety and depression-like behaviors for two reasons: first, evaluate if SC and PP present some side effects at the doses used in this work, and second: verify if these drugs present, as was proposed for other AEDs, other effects on some neurological disorders, in this case, anxiety and depression-like behaviors. Anxiety was measured because several antiepileptic drugs are anxiolytic, such as

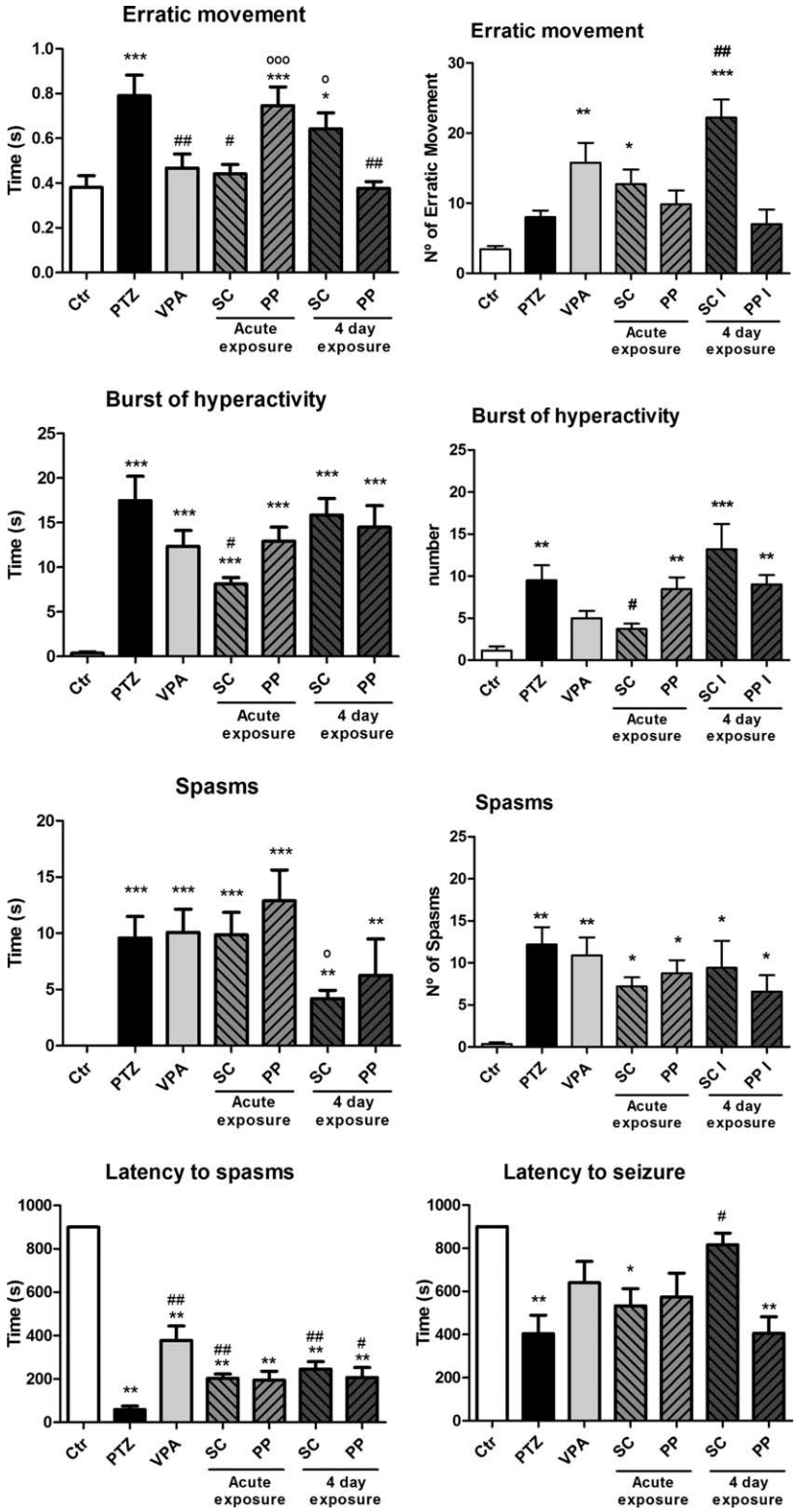

FIGURE 4 Behavioral endpoints measured in adult zebrafish during an exposure of maximum 15 min in behavioral tank (tank 2, Figure 1A). The experimental groups were: drug-free system water (Ctr), 830 mg/L PTZ, 830 mg/L PTZ plus 50 mg/L VPA, 830 mg/L PTZ plus 1 μg/L SC after acute and continued (4-day) exposure, and 830 mg/L PTZ plus 9 μg/L PP after acute and continued (4-day) exposure. Data are expressed as mean ± SEM and were analyzed using the nonparametrical Wilcoxon-Mann–Whitney *U* test. Asterisks (*) indicate significant difference with Ctr ($^{*}p < .05$, $^{**}p < .001$, $^{***}p < .0001$), numerals (#) indicate significant difference with PTZ ($^{\#}p < .05$, $^{\#\#}p < .001$, $^{\#\#\#}p < .0001$) and circles (°) indicate significant difference with VPA ($^{\circ}p < .05$, $^{\circ\circ}p < .001$, $^{\circ\circ\circ}p < .0001$) ($n = 10–12$ in each group)

TABLE 1 The percentage of individuals that reached seizure in each of the treatment performed

Treatment	Individuals with seizures (%)
Ctr	0
PTZ	100
PTZ+VPA	60
PTZ+SC	
Acute	54
4 day	63
PTZ+PP	
Acute	60
4 day	72

The experimental groups were: control (Ctr), pentylentetrazol (PTZ), pentylentetrazol plus valproate (PTZ + VPA), pentylentetrazol plus sodium cyclamate (PTZ + SC), and pentylentetrazol plus sodium propylparaben (PTZ + PP) ($n = 10–12$ per group).

$GABA_A$ receptor agonist (valproate and benzodiazepines). Anxiety (anxiety-like) behavior was defined as a complex behavior evoked by dangerous or potentially dangerous environment/stimuli. Includes reduced exploration, and typically manifests in geotaxis (diving), thigmotaxis, and an increased time in the bottom of the tank (Kalueff et al., 2013). Depression-like behaviors were analyzed since some antiepileptic drugs contribute to the termination of a seizure but promote postictal depression (Bernard, 2015). In zebrafish depressive-like behaviors were associated with a reduction in distance moved and velocity (Kalueff et al., 2013). Our idea was to evaluate if these drugs induce per se induce some behavioral alterations observed with other antiepileptic drugs, and taken into account that some approved AEDs have shown beneficial effects on other psychiatric and neurological disorders (Ettinger & Argoff, 2007).

Considering the previously cited antecedents, we evaluated the time in the top of the tank, the velocity and distance swum. No significant differences between the treatment groups (SC and PP, acute and 4-day exposure) and the control group in any of the endpoints analyzed were found (Figure 6) suggesting that both drugs (at the concentrations tested in this work) have not anxiety and depression-like effects.

4 | DISCUSSION

Recent studies have demonstrated that PTZ elicits seizures in adult zebrafish. Such studies have included electrophysiology, immunolocalization and behavioral analysis (Stewart et al., 2012; Pineda, Beattie, & Hall, 2011; Wong et al., 2010) but only lately a detailed behavioral analysis of PTZ induced seizures in adult zebrafish was performed (Mussulini et al., 2013; Gupta et al., 2014). Here we observed that SC reduced some behavioral seizure-related parameters induced by PTZ in adult zebrafish (burst of hyperactivity, spasms, and latency to induce spasms and seizure, whereas PP showed only a longer latency to induce spasms.

It has been observed that the relative lack of significant improvement in efficacy achieved by third generation antiepileptic agents compared with classic AEDs might be related to the fact that most of the clinical AEDs have been identified through the same screening approaches (MES and scPTZ seizure models in rodents) (Löscher, 2011). Thus, new screening models are necessary to find novel therapeutic solutions to epilepsy. Beside their potential bias to select compounds with similar pharmacological profile, available rodent seizure models are expensive and time-consuming.

New species have been proposed as emerging useful models to screen drugs or mutations related to epilepsy (Baraban, 2007; Stewart et al., 2012). Invertebrates have provided novel approaches to epilepsy (Baraban, 2007), but it is difficult to interpret seizure in a model without a complex nervous system. Zebrafish offer a perfect combination of physiological and behavioral complexity, while being suitable for medium- and high-throughput screening (Stewart et al., 2012; Cachat et al., 2010; Wong et al., 2010).

Exposure of zebrafish larvae to PTZ demonstrated that this organism is highly susceptible to pharmacological induction of epileptic-like

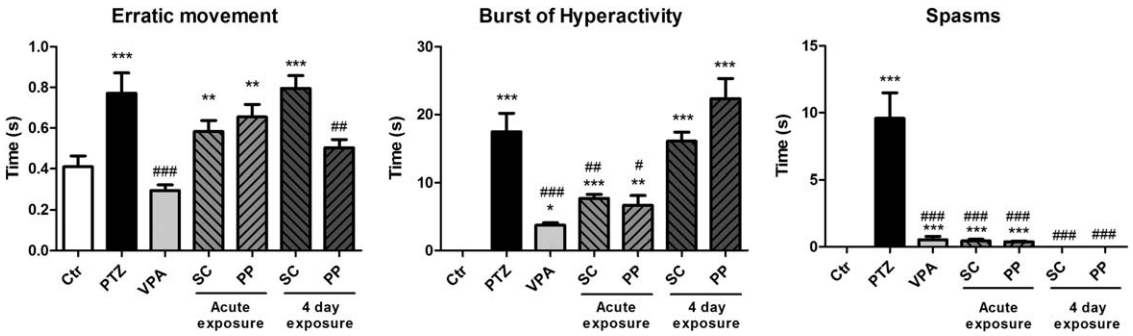

FIGURE 5 Behavioral endpoints measured in adult zebrafish during a 15 min exposure in behavioral tank. The experimental groups were: drug-free water (Ctr), 1 μg/L SC after acute and continued (4-day) exposure, and 9 μg/L PP after acute and continued (4-day) exposure. Data are expressed as mean ± SEM and were analyzed using the nonparametrical Wilcoxon-Mann–Whitney *U* test. Asterisks (*) indicate significant difference with Ctr ($^{**}p < .001$, $^{***}p < .0001$), numerals (#) indicate significant intergroup difference ($^{\#}p < .05$, $^{\#\#}p < .001$, $^{\#\#\#}p < .0001$) ($n = 8–9$ in each group)

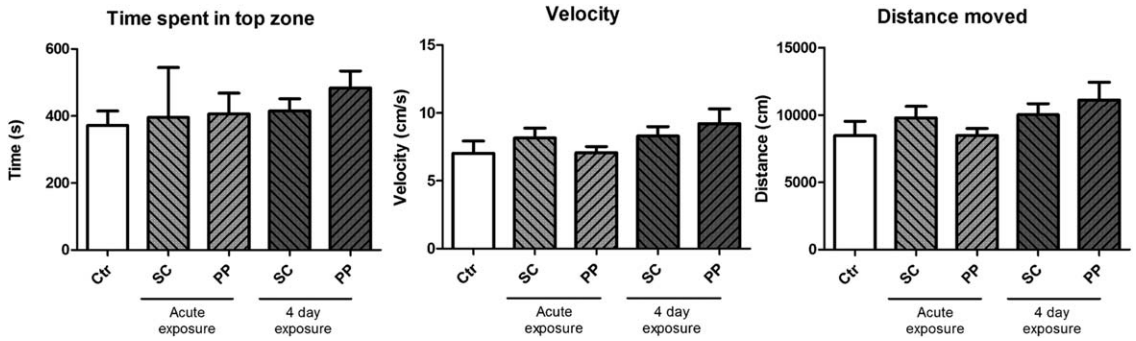

FIGURE 6 Behavioral analysis of time spent in the top zone (as a measure of anxiety), velocity and distance moved (as a measure of anxiety-like and depression-like behaviors, for details see the text) of adult zebrafish during 15 min exposure in the behavioral tank. The experimental groups were: drug-free water (Ctr), 1 μg/L SC after acute and continued (4-day) exposure, and 9 μg/L PP after acute and continued (4-day) exposure. Data are expressed as mean ± SEM and were analyzed using the nonparametrical Wilcoxon-Mann–Whitney *U* test ($n = 8–9$ in each group)

seizures (Baraban, Taylor, Castro, & Baier, 2005). Treating zebrafish larvae or adults with PTZ induces epileptiform discharges, together with vigorous convulsive behaviors and c-fos expression in the brain (Baraban, Taylor, Castro, & Baier, 2005; Mussulini et al., 2013; Afrikanova et al., 2013). To the moment, PTZ is thus the most widely used and validated proconvulsant agent in zebrafish.

Considering that the general setup was changed in relation to previous studies (Wong et al., 2010; Mussulini et al., 2013; Gupta et al., 2014), different concentrations of PTZ were evaluated to determine the optimal concentration to quantify the chosen behavioral parameters. 10 mM PTZ, in our conditions, induced immobilization of the fish with the subsequent fall to the bottom of the tank in 3 to 4 min, as had previously been observed in another experimental setup conditions (Mussulini et al., 2013; Gupta et al., 2014). Previous reports showed that the different phases of seizures were elicited in zebrafish after 15-min exposure to 6 mM PTZ (Gupta et al., 2014). Our preliminary data suggested that 6 mM was the optimal concentration of PTZ to measure seizure, producing the seizure about 10 min after exposure without causing death in any fish. This latency is adequate because it is possible to evaluate how the behavior of the fish changes during those 10 min allowing appropriate characterization of drug treatments.

This study extends the earlier work described previously (Mussulini et al., 2013; Gupta et al., 2014), assessing a specific range of behaviors in adult zebrafish to determine seizure-related activity. Moreover, we completed this study by screening the effect of two commercially available food additives after acute and 4-day continued exposure. Adult zebrafish have already been used in previous works to evaluate some classical drug with anticonvulsive effects, like diazepam and gabapentin (Mussulini et al., 2013; Gupta et al., 2014), and classical AEDs have also been tested in larval zebrafish.

SC (an artificial sweetener) showed moderate anticonvulsant activities in mice (Talevi et al., 2012, Di Ianni et al., 2015) in both scPTZ and MES tests, whereas PP (a food preservative) displayed an anticovulsant effect using the MES test in mice (Talevi et al., 2007). We decided then to evaluate them in an alternative seizure model such as adult zebrafish, quantifying the effects of these two compounds which present the additional benefit that they are used by human beings daily without apparent side effects at the acceptable daily intake.

The concentration of SC and PP was determined and 1 μg/L of SC and 9 μg/L of PP was used considering the data principally from burst and latencies behaviors and the lowest concentration of both drugs with antiepileptic effects.

In zebrafish, SC reduces the erratic movements showing similar values than controls, indicating an effect of this drug on fish behaviors induced by PTZ. By reducing the bursts of hyperactivity and increasing the latency to spasms (acutely and 4-day exposure) and the latency to seizure (after 4-day exposure) this compound showed an effect in a range of behaviors associated with seizure. PP effects were observed only in the latency to spasms (4-day exposure) without changes (compared with PTZ group) in the spasms duration.

Interestingly, when the previously described behavioral parameters were evaluated following the exposure to the drugs alone, the burst of hyperactivity were reduced in the acute treatment of SC and PP, and the time of spasms was significantly reduced for both drugs at both experimental conditions (acute and 4-days treatment), showing the strongest effect in 4-day exposure fish. These results reaffirm the previously reported anticonvulsant effects of SC and PP (mice). These drugs, at the concentrations used in this work, not only display anticonvulsant effects in seizure models of zebrafish, but also show no behavioral effects when administered alone.

To further evaluate the effects of these two compounds, anxiety- and depression-like behaviors in zebrafish were measured. No effect was observed in any experimental condition for any of the drugs, suggesting that both are innocuous (at the concentration tested) when are administrated alone. Moreover, no parallel sedation and toxicity effects were observed.

Taken together all the results, we can suggest that both drugs present potential actions as AEDs, and could be considered as important tools for future investigations and translational research, considering the massive use of these products by the population.

5 | CONCLUSION

Our results corroborate the utility of the zebrafish PTZ-induced seizure model in the screening of novel anticonvulsant drug candidates. In comparison with the widely accepted rodent models of seizure, zebrafish provide higher throughput while simultaneously reducing the incidence of invasive, stressful drug administration procedures (typically, intraperitoneal administration) used in rodent models, which could possibly introduce more variability to the test results. It is interesting to note that both SC and PP, which have already been shown to display moderate anticonvulsant effects in rodents, also show seizure protection effects in zebrafish. Since these substances have a long history of use as food additives and/or medication ingredients, they have previously undergone safety assessment (e. g., chronic or long-term exposure effects), which could be advantageous to expedite the exploitation of their potential medical use.

Finally, we would like to underline that AEDs are frequently prescribed off-label and repurposed for other medical uses, and zebrafish could be used as a medium to high-throughput screening model for the early and fast detection of other potentially useful pharmacological effects of AEDs on behavior, for example, anxiolytic or mood-stabilizing activity, as well as adverse reactions (e. g., depressant effect). Here, we have explored the possible anxiolytic and antidepressant effects of SC and PP, without positive results.

REFERENCES

Afrikanova, T., Serruys, A. S., Buenafe, O. E., Clinckers, R., Smolders, I., de Witte, P. A., … Esguerra, C. V. (2013). Validation of the zebrafish pentylenetetrazol seizure model: Locomotor versus electrographic responses to antiepileptic drugs. *PLoS One*, *8*(1), e54166.

Akula, K., Dhir, A., & Kulkarni, S. (2009). Effect of various antiepileptic drugs in a pentylenetetrazol-induced seizure model in mice. *Methods and Findings in Experimental and Clinical Pharmacology*, *31*(7), 423–432.

Aleström, P., Holter, J. L., & Nourizadeh-Lillabadi, R. (2006). Zebrafish in functional genomics and aquatic biomedicine. *Trends in Biotechnology*, *24*(1), 15–21.

Bakvis, P., Roelofs, K., Kuyk, J., Edelbroek, P. M., Swinkels, W. A., & Spinhoven, P. (2009). Trauma, stress, and preconscious threat processing in patients with psychogenic nonepileptic seizures. *Epilepsia*, *50*(5), 1001–1011.

Baraban, S. C., Taylor, M. R., Castro, P. A., & Baier, H. (2005). Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience*, *131*(3), 759–768.

Baraban, S. C. (2007). Emerging epilepsy models: insights from mice, flies, worms and fish. *Current Opinion in Neurology*, *20*(2), 164–168.

Barbazuk, W. B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J. A., McPherson, J. D., Johnson, S. L. 2000. The syntenic relationship of the zebrafish and human genomes. *Genome Research*. *10*:1351–1358.

Bernard, C. (2015). Spreading depression: Epilepsy's wave of death. *Science Translational Medicine*, *7*, 282fs14.

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 Research*, *75*(1), 18–28.

Blaser, R., & Gerlai, R. (2006). Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. *Behavior Research Methods*, *38*(3), 456–469.

Brittijn, S. A., Duivesteijn, S. J., Belmamoune, M., Bertens, L. F., Bitter, W., de Bruijn, J. D., … Richardson, M. K. (2009). Zebrafish development and regeneration: New tools for biomedical research. *International Journal of Developmental Biology*, *53*(5–6), 835–850.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., … Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocol*, *5*, 1786–1799.

Carmody, S., & Brennan, L. (2010). Effects of pentylenetetrazole-induced seizures on metabolomic profiles of rat brain. *Neurochemistry International*, *56*(2), 340–344.

Di Ianni, M. E., Del Valle, M. E., Enrique, A. V., Rosella, M. A., Bruno, F., Bruno-Blanch, L. E., & Talevi, A. (2015). Computer-aided identification of anticonvulsant effect of natural nonnutritive sweeteners Stevioside and Rebaudioside A. *Assay and Drug Development Technology*, *13*(6), 313–318.

Egan, R., Bergner, C., Hart, P., Cachat, J., Canavello, P., … Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioral Brain Research*, *205*(1), 38–44.

Eliceiri, B. P., Gonzalez, A. M., & Baird, A. (2011). Zebrafish model of the blood-brain barrier: morphological and permeability studies. *Methods in Molecular Biology*, *686*, 371–378.

Ettinger, A. B., & Argoff, C. E. (2007). Use of antiepileptic drugs for non-epileptic conditions: psychiatric disorders and chronic pain. *Neurotherapeutics*, *4*, 75–83.

Faillace, M. P., & Bernabeu, R. (2016). Conditioned place preference and behavioral analysis to evaluate nicotine reinforcement properties in zebrafish. In M. Li (Ed.), *Nicotinic acetylcholine receptor technologies*. *Neuromethods* (Vol. *117*, pp. 51–64). Heidelberg, Germany: Springer Protocols, Humana Press.

Frye, R. E., Rossignol, D., Casanova, M. F., Brown, G. L., Martin, V., Edelson, S., … Adams, J. B. (2013). A review of traditional and novel treatments for seizures in autism spectrum disorder: Findings from a systematic review and expert panel. *Frontiers in Public Health*, *13*, 1–31.

Goldsmith, P. (2004). Zebrafish as a pharmacological tool: The how, why and when. *Current Opinion in Pharmacology*, *4*(5), 504–512.

Gupta, P., Khobragade, S. B., & Shingatgeri, V. M. (2014). Effect of various antiepileptic drugs in zebrafish PTZ-seizure model. *Indian Journal of Pharmaceutical Science*, *76*(2), 157–163.

Jeong, J. Y., Kwon, H. B., Ahn, J. C., Kang, D., Kwon, S. H., Park, J. A., & Kim, K. W. (2008). Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain Research Bulletin*, *75*(5), 619–628.

Kalueff, A. V. (2007). Mapping convulsants' binding to the GABA-A receptor chloride ionophore: A proposed model for channel binding sites. *Neurochemistry International*, *50*(1), 61–68.

Kalueff, A. V., Gebhardt, M., Stewart, A. M., Cachat, J. M., Brimmer, M., Chawla, J. S., … Schneider, H., Zebrafish Neuroscience Research Consortium. (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish*, *10*, 70–86.

Langheinrich, U. (2003). Zebrafish: A new model on the pharmaceutical catwalk. *Bioessays*, *25*(9), 904–912.

Levin, E. D., Bencan, Z., & Cerutti, D. T. (2007). Anxiolytic effects of nicotine in zebrafish. *Physiological Behavior*, *90*, 54–58.

Löscher, W., Hönack, D., Fassbender, C. P., & Nolting, B. (1991). The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. III. Pentylenetetrazole seizure models. *Epilepsy Research*, *8*(3), 171–189.

Löscher, W. (2011). Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure*, *20*(5), 359–368.

Mehta, S. R., Dham, S. K., Lazar, A. I., Narayanswamy, A. S., & Prasad, G. S. (1994). Prolactin and cortisol levels in seizure disorders. *Journal of Association of Physicians of India*, *42*(9), 709–712.

Mula, M., & Sander, J. W. (2007). Negative effects of antiepileptic drugs on mood in patients with epilepsy. *Drug Safety*, *30*(7), 555–567.

Mussulini, B. H., Leite, C. E., Zenki, K. C., Moro, L., Baggio, S., Rico, E. P., … de Oliveira, D. L. (2013). Seizures induced by pentylenetetrazole in the adult zebrafish: a detailed behavioral characterization. *PLoS One*, *8*(1), e54515.

Nguyen, M., Stewart, A. M., & Kalueff, A. V. (2014). Aquatic blues: modeling depression and antidepressant action in zebrafish. *Progress in Neuropsychopharmacology & Biological Psychiatry*, *55*, 26–39.

Pineda, R., Beattie, C. E., & Hall, C. W. (2011). Recording the adult zebrafish cerebral field potential during pentylenetetrazole seizures. *Journal of Neuroscience Methods*, *200*(1), 20–28.

Prigol, M., Brüning, C. A., Godoi, B., Nogueira, C. W., & Zeni, G. (2009). m-Trifluoromethyl-diphenyl diselenide attenuates pentylenetetrazole-induced seizures in mice by inhibiting GABA uptake in cerebral cortex slices. *Pharmacological Reports*, *61*(6), 1127–1133.

Sadek, B., Saad, A., Latacz, G., Kuder, K., Olejarz, A., Karcz, T., … Kiec-Kononowicz, K. (2016a). Nonimidazole-based histamine H3R antagonists with anticonvulsant activity in different seizure models in male rats. *Journal of Drug Design, Development and Therapy*, *10*, 3879–3898.

Sadek, B., & Stark, H. (2016b). Cherry-Picked ligands at histamine receptor subtypes. *Neuropharmacology*, *106*, 56–73.

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Review of Cambridge Philosophical Society*, *83*(1), 13–34.

Stables, J. P., Bertram, E. H., White, H. S., Coulter, D. A., Dichter, M. A., Jacobs, M. P., … Davis, M. (2002). Models for epilepsy and epileptogenesis: report from the NIH workshop, Bethesda, Maryland. *Epilepsia*, *43*(11), 1410–1420.

Stewart, A., Wu, N., Cachat, J., Hart, P., Gaikwad, S., Wong, K., … Kalueff, A. V. (2011). Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. *Progress in Neuropsychopharmacology and Biological Psychiatry*, *35*(6), 1421–1431.

Stewart, A. M., Desmond, D., Kyzar, E., Gaikwad, S., Roth, A., Riehl, R., … Kalueff, A. V. (2012). Perspectives of zebrafish models of epilepsy: What, how and where next?. *Brain Research Bulletin*, *87*(2-3), 135–143.

Talevi, A., Bellera, C. L., Castro, E. A., & Bruno-Blanch, L. E. (2007). A successful virtual screening application: prediction of anticonvulsant activity in MES test of widely used pharmaceutical and food preservatives methylparaben and propylparaben. *Journal of Computer Aided Molecular Design*, *21*(9), 527–538.

Talevi, A., Enrique, A. V., & Bruno-Blanch, L. E. (2012). Anticonvulsant activity of artificial sweeteners: a structural link between sweet-taste receptor T1R3 and brain glutamate receptors. *Bioorganic and Medical Chemistry Letters*, *22*(12), 4072–4074.

Tunca, Z., Ergene, U., Fidaner, H., Cimilli, C., Ozerdem, A., Alkin, T., & Aslan, B. U. (2000). Reevaluation of serum cortisol in conversion disorder with seizure (pseudoseizure). *Psychosomatics*, *41*(2), 152–153.

Westerfield, M. (2007). The zebrafish book, a guide for the laboratory use of zebrafish (danio rerio). Corvallis, Oregon: University of Oregon Press.

Winter, M. J., Redfern, W. S., Hayfield, A. J., Owen, S. F., Valentin, J. P., & Hutchinson, T. H. (2008). Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. *Journal of Pharmacological and Toxicological Methods*, *57*(3), 176–187.

Wong, K., Stewart, A., Gilder, T., Wu, N., Frank, K., Gaikwad, S., … Kalueff, A. V. (2010). Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish. *Brain Research*, *1348*, 209–215.

How to cite this article: Pisera-Fuster A, Otero S, Talevi A, Bruno-Blanch L, Bernabeu R. Anticonvulsant effect of sodium cyclamate and propylparaben on pentylenetetrazol-induced seizures in zebrafish. *Synapse*. 2017;71:e21961. https://doi.org/10.1002/syn.21961