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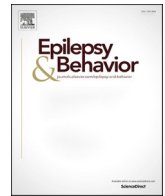
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# Effects of baicalin pre-treatment on pentylenetetrazole-induced seizures: Insights from zebrafish larvae locomotor behavior and neuronal calcium imaging

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## ABSTRACT

Natural compounds are increasingly being studied for their potential neuroprotective effects against inflammatory neurological diseases. Epilepsy is a common neurological disease associated with inflammatory processes, and around 30% of people with epilepsy do not respond to traditional treatments. Some flavonoids, when taken along with antiseizure medications can help reduce the likelihood of drug-resistant epilepsy. Baicalin, a plant-based compound, has been shown to possess pharmacological properties such as anti-inflammatory, neuroprotective, anticonvulsant, and antioxidant activities. In this study, we tested the effect of baicalin on an established model of pharmacologically induced seizure in zebrafish using measures of both locomotor behavior and calcium imaging of neuronal activity. The results of our study showed that, at the tested concentration, and contrary to other studies in rodents, baicalin did not have an anti-seizure effect in zebrafish larvae. However, given its known properties, other concentrations and approaches should be explored to determine if it could potentially have other beneficial effects, either alone or when administered in combination with classic anti-seizure medications.

## 1. Introduction

Epilepsy is a neurological disease that affects around 1 % of people worldwide. It is characterized by spontaneous and recurrent seizures due to the synchronous overexcitation of large brain neuron populations [1–4]. This excessive electrical activity can lead to odd sensations or involuntary movements of different parts of the body [2]. Although several antiseizure medications (ASMs) have been developed in recent decades, they are ineffective in at least one-third of people with epilepsy [1,5]; moreover, drug-resistant epilepsy (DRE) is prevalent in children but is also described in adults [6]. However, preclinical studies have

shown that flavonoids have a stronger therapeutic efficacy for the treatment of epileptic disorders. In particular, certain flavonoids can also be taken together with some ASMs to reduce drug resistance in DRE patients [7].

Natural compounds are increasingly being explored as potential neuroprotective agents against inflammatory neurological diseases. According to Traditional Chinese Medicine (TCM), natural compounds can be used to treat epilepsy due to their functions of calming effect on the liver, promotion of blood circulation, fever reduction, orifice opening, and nourishing properties [8]. Studies on mice and rats have revealed the anticonvulsant effects of vitexin, quercetin, wogonin, and

**Abbreviations:** ASMs, antiseizure medications; DRE, drug-resistant epilepsy; TCM, Traditional Chinese Medicine; BAI, baicalin; PTZ, pentylenetetrazole; PTU, N-Phenylthiourea; VPA, valproic acid sodium salt; WT, wild-type; DPF, days post fertilization, VMR, visual motor response; HPF, hours post-fertilization; ROI, region of interest; OSLP, *Orthosiphon stamineus* proteins; BBB, blood-brain barrier.

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hesperidin [4]. Similarly, studies on luteolin, apigenin, and baicalin have demonstrated a reduction in the number of seizures in rodent epilepsy models [7]. Thus, the potential efficacy of natural drugs with lower toxicity and fewer side effects has attracted increasing attention for epilepsy treatments. Baicalin is among these components.

Baicalin (C<sub>21</sub>H<sub>18</sub>O<sub>11</sub>; 5,6,7-trihydroxyflavone 7-O-beta-D-glucuronide or baicalein 7-O-D-glucuronic acid or 7-D-glucuronic acid-5,6-dihydroxyflavone, BAI) is the main active component found in the roots of *Scutellaria baicalensis*. This compound has several pharmacological properties, including anti-inflammatory, neuroprotective, anticonvulsant, and antioxidant effects [9]. Studies have reported that it also has anxiolytic activity by acting on GABA and glutamate acid in rat or mice brains [4,10–14]. BAI exhibits preferential accumulation in the hippocampus, thalamus, and striatum, despite its very limited oral delivery [15–18]. In this study, we aimed to test the effect of BAI in a pharmacologically induced seizure model in zebrafish.

The zebrafish (*Danio rerio*) has become increasingly popular in epilepsy research, especially in phenotype-based drug screening and discovery. One example of the translational potential of this model is the study of Baraban and collaborators, who identified clemizole, a novel ASM currently undergoing human trials. This compound was uncovered through drug screening using a zebrafish epilepsy model [19]. Furthermore, a mathematical analysis of electrical seizures in mice, zebrafish, and humans has concluded that the underlying rules governing the initiation and termination of seizures are universal [20].

The use of zebrafish as an animal model for epilepsy studies was first proposed by Baraban and collaborators in 2005. They demonstrated that epileptic seizures can be induced in zebrafish larvae by exposure to pentylentetrazole (PTZ), by which the animals exhibited behavioral, electrographic, and molecular changes similar to those found in other animal models such as rodents [21].

It is crucial to understand the molecular mechanisms that trigger epileptic seizures and develop new drugs to suppress them. BAI has been tested in many studies with rodents and has shown anxiolytic, neuroprotective, and antiseizure effects [4,22,23]. However, there are only a few studies on its effects in the zebrafish model [24]. In this research, we investigated the potential anti-seizure effects of BAI in zebrafish larvae by using an established PTZ-induced seizure model in zebrafish to analyze locomotor behavior and neuronal Ca<sup>2+</sup> imaging. This is the first study to investigate the use of BAI as a potential ASM in a zebrafish larvae-seizure model.

## 2. Materials and methods

All animal experiments were conducted at the French National Institute of Health and Medical Research (Inserm) UMR 1141 in Paris. The experimental procedures were carried out in compliance with the European Community Council directive addressing the protection of animals employed for scientific purposes.

### 2.1. Chemicals

N-Phenylthiourea (PTU ≥ 98 %, CAS No. 103–85-5), Pancuronium bromide (CAS No. 15500–66-0), Pentylentetrazole (PTZ, CAS No. 54–95-5), Baicalin (BAI 95 %, CAS No. 21967–41-9) and Valproic acid sodium salt (VPA 98 %, CAS No. 1069–66-5) were purchased from Sigma-Aldrich (St Louis, Missouri, USA) and low melt agarose (CAS. No. 35–2010) was purchased from PeqLab.

### 2.2. Fish husbandry and zebrafish lines

Zebrafish (*Danio rerio*) were kept at 26–28 °C in a 14 h light/10 h dark cycle. Embryos were collected by natural spawning and raised in E3 solution at 28.5 °C. Wild-type (WT) AB fish were used to do the locomotor activity test and Tg[HuC:GCaMP5G] transgenic line to do calcium imaging test.

### 2.3. Zebrafish embryo survival assay

Zebrafish embryos (n = 30/group) were placed in a 6-well plate and treated with five different concentrations of baicalin (1, 2.5, 5, 7.5, or 10 μM). The medium was renewed every day, and the mortality was observed every 24 h until 5 days post fertilization (dpf).

### 2.4. Behavioral assessment

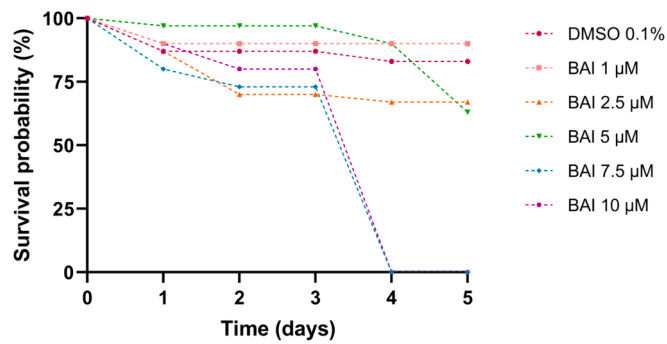
Locomotor behavior was monitored with an automated video tracking device (Zebrafish<sup>®</sup>, ViewPoint). To test the neuroprotective effect of baicalin, different protocols were performed. At 4 dpf WT larvae were placed in a 6-well plate and treated with 7.5 μM baicalin or with 0.1 % DMSO (ctrl negative) for 24 h. At 5 dpf, larvae were treated with baicalin or 1 mM VPA (ctrl positive) for 1 h, washed twice with E3 medium, placed individually in the wells of a 96-well plate (final volume: 200 μl) and their activity was measured according to the described protocols. For both protocols: 30 min of acclimatization was performed with 100 % backlight. 5 mM PTZ was then added for the epileptic seizure groups. Protocol 1: 30 min of video tracking with light off. Protocol 2: visual motor response (VMR) assay. The light protocol was as follows: one cycle of 20 min duration light ON (100 % backlight), 10 min duration light OFF (0 % light intensity), 20 min duration light ON and 10 min duration light OFF. The total duration of the experiment was 60 min. Activity during the experiments was measured in mm/s.

### 2.5. Calcium imaging

Calcium activity was recorded using Zeiss LSM 880 laser scanning confocal microscope. Briefly: at 19 h post fertilization (hpf), 0.003 % PTU was added to inhibit pigmentation in Tg[HuC:GCaMP5G] embryos. At 4 dpf, larvae were screened and those exhibiting the same level of fluorescence were selected, placed in a 6-well plate, and treated with 7.5 μM baicalin or with 0.1 % DMSO for 24 h. At 5 dpf, larvae were paralyzed using 300 μM PB during 5 min, embedded in 100 μl of 2 % low-melting agarose in the center of a 35 mm glass-bottomed dish, then covered with 200 μL of E3 solution containing 300 μM PB. Larvae were placed in the incubator of the Zeiss LSM 880 laser scanning confocal microscope at 28 °C for 15 min for acclimatization, and then placed horizontally with a 20x/1.0 objective. ZEN-black (ZEISS) software was configured as follows; 1- imaging setup: a laser scanning confocal microscope (LSM) was selected. The applied imaging channel was for enhanced green fluorescent protein (EGFP) with emission range of 492–60 nm, along was incorporated a multi-beam splitter (MBS 488) for an optimal signal separation. 2- acquisition parameters: acquisitions were performed in frame scan mode with a frame size of 512x512 pixels. A line step of 2, speed set at 9, a depth of 8 bit and bidirectional scanning was employed. 3- channel configuration: an argon 488 nm laser with an intensity of 4.5 % was used as excitation source. The pinhole aperture was set at 61.5 (1 Airy Unit). For the EGFP channel, the gain master was set to 687, with a digital offset of 0 and a digital gain of 1. 4- time series imaging: a time series of 6000 cycle was acquired with a temporal interval of 0 ms between frames. The duration for capturing one frame was set to 157.29 ms. For the groups CTRL and BAI, calcium activity was recorded for 15 min, then 5 mM PTZ was added, and after 15 min calcium activity was recorded for an additional 15 min. The total exposure time to PTZ was 30 min. The fluorescence intensity of each frame was measured using a homemade macro on ImageJ 1.53v (<https://imagej.nih.gov/ij/>).

### 2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California USA, <https://www.graphpad.com>). Datasets were analyzed by one-way or two-way ANOVA followed by Bonferroni post hoc test.  $P < 0.05$  was



**Fig. 1.** The survival rate (%). Survival of zebrafish embryos and larvae exposed to different concentration of baicalin (1, 2.5, 5, 7.5 or 10  $\mu$ M) over a period of 5 days. The medium was renewed every day.

applied for statistical significance. All graphs show mean values  $\pm$  standard error of the mean (Mean  $\pm$  SEM).

### 3. Results

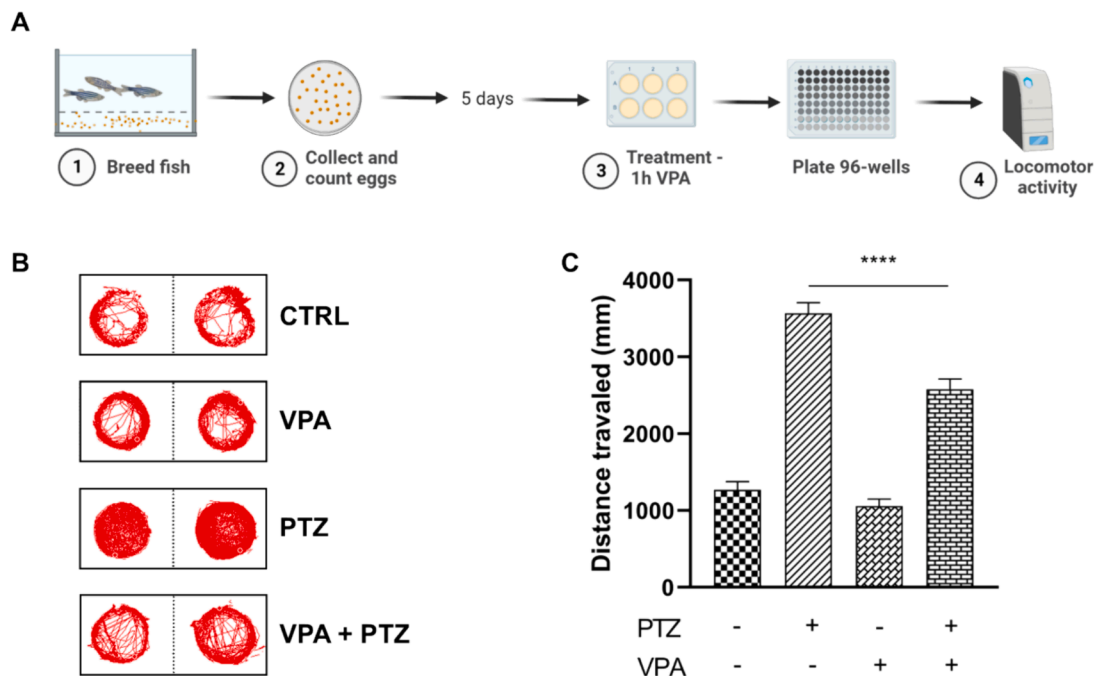
#### 3.1. Effects of BAI on the survival rate of zebrafish embryos

To assess the potential toxicity of BAI, zebrafish embryos were exposed to increasing concentrations of BAI, ranging from 1, 2.5, 5, 7.5, or 10  $\mu$ M from 6 h post-fertilization (hpf) onward up to 120 hpf. Our findings indicated that almost all concentrations of BAI had an impact on the survival rate of the embryos. Therefore, the concentration of 7.5  $\mu$ M was chosen since it resulted in a 75 % survival rate at 3 dpf as shown in Fig. 1.

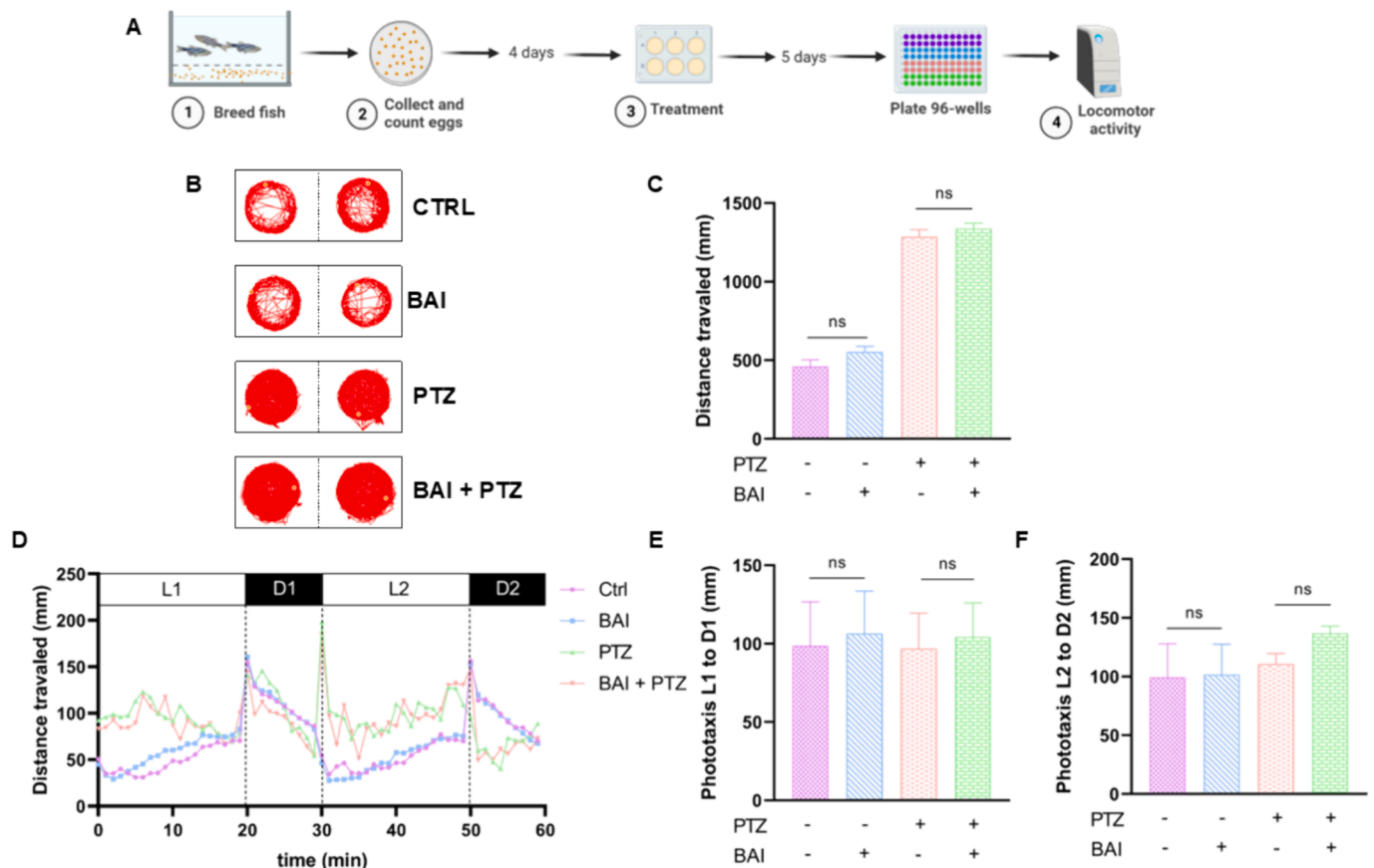
#### 3.2. Behavioral assessment

To evaluate the anticonvulsant effects of baicalin on PTZ-treated zebrafish larvae, a behavioral assessment was performed using the ZebraLab<sup>®</sup> (ViewPoint), a high-speed video tracking system. The movements of larvae were monitored with a camera and locomotion-tracking software, the ZebraLab (ViewPoint). The results showed that the PTZ-induced seizures led to a significant increase in traveled distance and movements of larvae as described in Baraban *et al.*, 2005. Moreover, we confirmed the anticonvulsant effect of VPA as a positive control (Fig. 2). We showed that animals treated with VPA had a strong reduction of PTZ-induced movement (Fig. 2b,c), in line with the findings published by Baraban *et al.* (2005) [21] and Shaw *et al.* (2022) [25].

During the experiment, the locomotor behavior of the larvae was analyzed by analyzing their swimming behavior. Three different pre-treatment periods with BAI were used: 24 h (Fig. 3c), 12 h (Fig. 4e), and 1 h (Fig. 4c) prior to PTZ-induced seizure treatment, however, no significant differences were observed between groups treated at the different times. The VMR test was conducted to measure the swimming ability of larvae and their responsiveness to light stimulation, integrating sensory and motor responses. In this test, the animals were exposed to BAI for 24 h. During the test, the larvae's response to light-switch stimulation was evaluated for 60 min, and the distance covered was recorded in real-time minute-by-minute. The phototactic response was then evaluated by measuring the L1 to D1 and L2 to D2 light/dark transition. The results showed that all groups increased their swimming activity when they moved from L (light)1 to D (dark)1, but the groups with PTZ decreased their activity during phase L2 to D2 (Fig. 3d). However, when analyzed one minute before, during, and one minute after the light/dark transition, there was no significant difference observed between the treated groups (Fig. 3 e,f).



**Fig. 2.** Locomotor activity. (A) Shows the swim test used to evaluate the locomotor activity of zebrafish larvae at 5 days post-fertilization following treatments with VPA and PTZ. The experiment involved treating the larvae with 1 mM VPA ( $n = 44$ ) or 0.1 % DMSO ( $n = 44$ ) for 60 min, and then PTZ to induce seizures (Graphic created with BioRender.com). (B) The distance traveled by the larvae during the 30 min was tracked and plotted by the software ZebraLab, and (C) the quantified results were then presented in a bar plot with in the y axis the distance traveled in mm and x axis the different treatments.  $n =$  number of larvae. Error bars on all graphs represent the standard error of the mean (SEM). \*\*\*\*,  $p < 0.0001$ . Comparisons were performed using One-way ANOVA followed by Bonferroni multiple comparisons post-test.



**Fig. 3.** Locomotor activity and VMR. (A) Experimental setup used to analyze the locomotor activity in zebrafish (5 dpf). Larvae (4 dpf) were treated with 7.5  $\mu$ M BAI ( $n = 48$ ) or 0.1 % DMSO ( $n = 48$ ) for 24 h prior to PTZ exposure (Graphic created with [BioRender.com](#)). (B) Tracking plots illustrating the distance traveled for 30 min. (C) Quantification of the distance traveled for 30 min. (D) Phototactic response for 60 min. (E) Subtracting the swimming distances covered in 1 min before and 1 min after the L1-D1 light/dark transition. (F) Subtracting the swimming distances covered in 1 min before and 1 min after the L2 – D2 transition.  $n =$  number of larvae. Error bars on all graphs represent the standard error of the mean (SEM). Comparisons were performed using One-way or two-way ANOVA followed by Bonferroni multiple comparisons post-test.

### 3.3. *In vivo* calcium imaging

Confocal microscopy was used to observe *in vivo* calcium neuronal activity in zebrafish larvae treated with BAI and PTZ. We used the transgenic line Tg[HuC:GCaMP5G], which encodes a calcium-dependent fluorescent protein expressed under the control of the pan-neuronal HuC promoter. To record calcium activity during epileptic seizures, we image the optic tectum of 5 dpf larvae that were paralyzed with pancuronium bromide and immobilized with agarose, before the addition of PTZ. As shown in Fig. 5c, we extracted and processed pixel fluorescence signals from the optic tectum region of interest (ROI) for analysis using ImageJ. We characterized the calcium neuronal activity by calculating amplitude and the number of peaks. The results showed a similar calcium activity in the groups treated with PTZ and BAI + PTZ (Fig. 5e-f). Although we did not conduct any specific analysis to examine seizure propagation, according to the movies (Movies 3,4), we can observe that it occurs from anterior-to-posterior. We can also see the basal calcium neuronal activities in the CTRL and BAI groups (Movies 1,2).

## 4. Discussion

Epilepsy is a common neurological disease associated with inflammatory processes and a rate of refractoriness to current medications of around 30 %. Many studies have indicated that BAI could affect the functions of immune cells, inhibit the production of inflammatory factors, and increase the anti-inflammatory effects [26]. Based on this, we

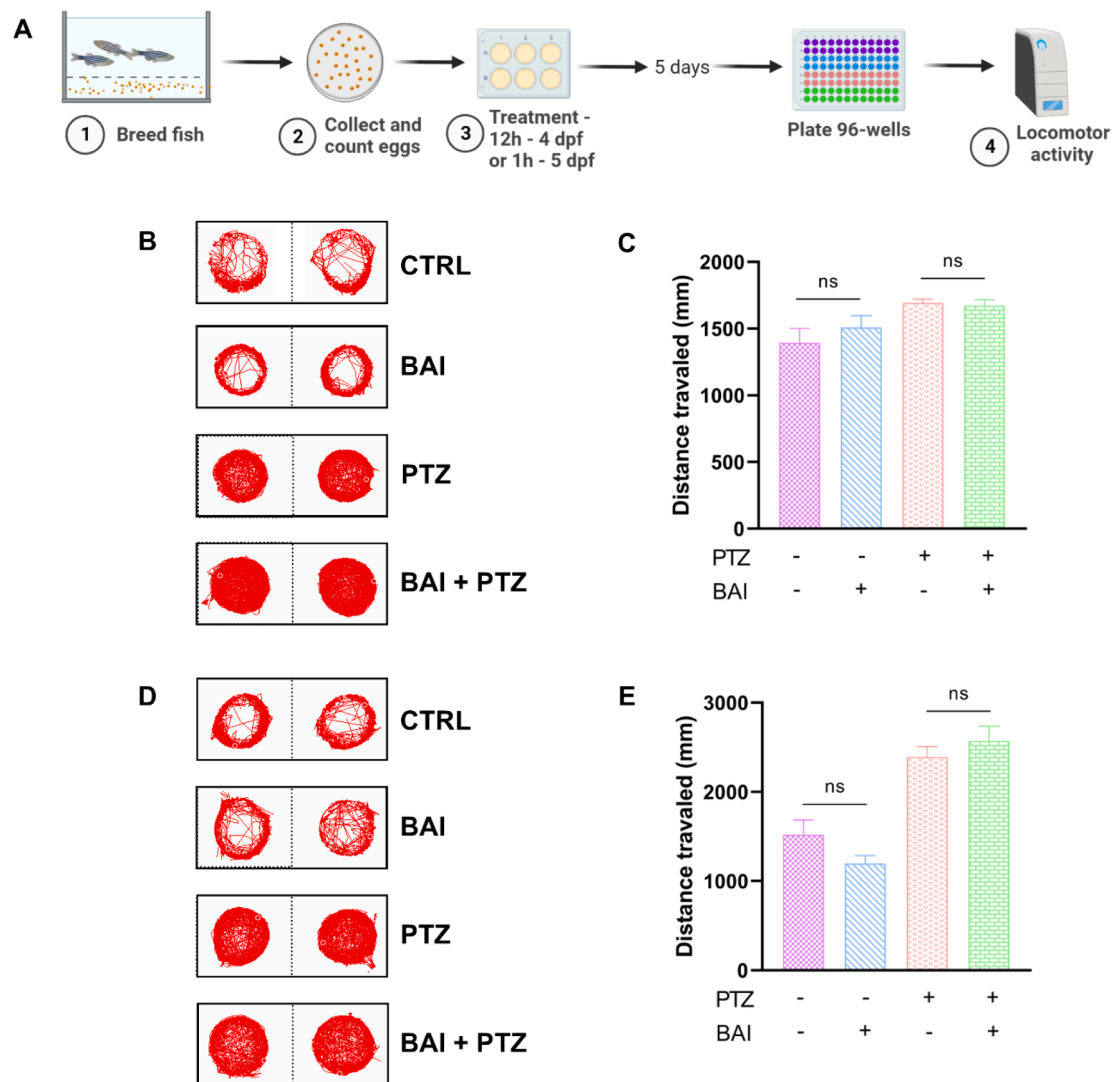
conducted a study to investigate the potential neuroprotective effects of BAI in the zebrafish larvae PTZ-seizure model. In this study, we tested the effect of BAI using a well-established epilepsy seizure model followed by locomotor behavior assessment and *in vivo* calcium imaging.

Studies using rats and mice as models led to a large disparity of results regarding the effects of BAI as a potential neuroprotective and anticonvulsive drug [10,13,14,23]. This disparity can be due to the species and ages of the animal models, differences in the drug dosage and epilepsy models, and manner of treatment [23].

Chung *et al.* (2020) investigated *Orthosiphon stamineus* proteins (OSLP) as a potential anticonvulsive component due to the plant's high concentration of BAI. Their locomotion test data were similar to ours obtained in larvae despite reporting the plant's neuroprotective factor in the PTZ-seizure adult zebrafish model. The total distance covered by the control group and the OSLP group was the same, as was that of the PTZ and OSLP + PTZ groups [24].

The findings reported by Wang *et al.* (2008) and Yang *et al.* (2021) in their studies with rats align closely with our results. In the picrotoxin-induced seizure, BAI did not significantly alter the motor incoordination effect in the rotarod test [10], and in the low concentration of BAI in the kindling PTZ model, the total distance was not significantly altered [13]. These findings contradict the study presented by Li *et al.* (2022), who reported a difference using low and high concentrations of BAI [14].

We conducted a study on locomotor activity using two different protocols. The first protocol evaluated the total distance covered in 30 min, with three different durations of pre-treatment of BAI. We



**Fig. 4.** Swim test to evaluate locomotor activity. (A) Experimental setup used to analyze the locomotor activity in zebrafish (5 dpf), larvae were treated with 7.5  $\mu$ M BAI ( $n = 24$ ) or 0.1 % DMSO ( $n = 24$ ) for 1 h (B,C) or 12 h (D,E) prior to PTZ-treatment (Graphic created with BioRender.com). (B, D) Tracking plots illustrating the distance traveled for 30 min. (C, E) Quantification of the distance traveled for 30 min.  $n =$  number of larvae. Error bars on all graphs represent standard error of the mean (SEM). Comparisons were performed using One-way ANOVA followed by Bonferroni multiple comparisons post-test.

attempted to observe an effect within 24 h of pre-treatment, but we did not see any significant results (Fig. 3). We then ran shorter treatments of 1 h, and 12 h to test the BAI degradation hypothesis, but still observed no effect (Fig. 4).

A limitation of our study was the inability to perform an absorption and distribution test for the compound. However, Zhang et al. (2015) investigated the distribution of BAI metabolites in rat tissues, and identified five derivatives, suggesting that they could cross the blood–brain barrier (BBB) and be responsible for the brain’s pharmacological responses to BAI [27,28]. It is important to note that the maturation of the BBB in zebrafish occurs between 3 and 10 dpf, and a not fully functional BBB could be more permeable [29]. Despite these favorable indications of BAI’s permeability to the BBB, we did not observe any action of BAI in 5 dpf larvae. This could be explained either by a reduced absorption of the compound during the exposure, or by a limited affinity for the protein targets involved at the age selected for the experimental assays [28]. Although we did not observe any significant behavioral results, our data showed reliability by presenting the same pattern of tracking and graphs, as well as a significant difference with the positive control group that supported the anticonvulsant effect of VPA.

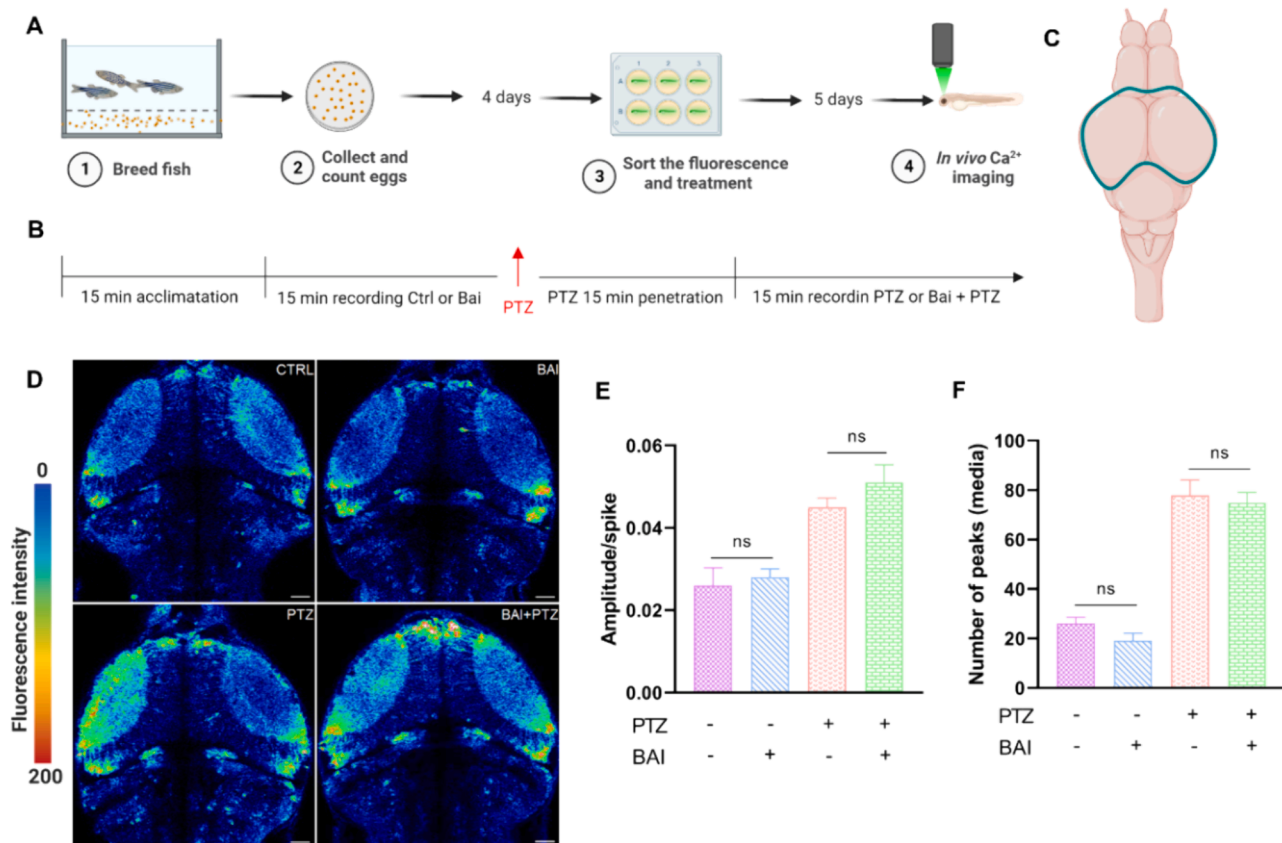
The second protocol for locomotor behavior was the VMR test, which

evaluated the larvae’s ability to swim and respond to light stimulation by integrating sensory and motor responses [30]. Studies have shown that the optokinetic response becomes robust in larvae at 5 dpf and the sensory cells in the retina and deep-brain photoreceptors detect the changes in illumination [31,32].

Our data corroborates previous studies that the locomotor activity of wild-type zebrafish decreases in the light and increase in the dark period [22,30,33]. During the L2 to D2 transition, the PTZ group showed an increase in activity in the light phase compared to the dark phase, which is consistent with the data presented by Li et al. (2018) [22]. In the VMR test, groups containing BAI followed the same pattern as the CTRL or PTZ group.

To complement our behavioral assays, we tested the effect of BAI on neuronal firing using a zebrafish transgenic line Tg[HuC:GCaMP5G] calcium indicators. The variety of labeling methods and the small size are the main advantages of using the zebrafish for this technique, enabling *in vivo* imaging of many different structures in anesthetized or awake animals [34]. In parallel, a large neuronal cascade of calcium was demonstrated during interictal periods in the brains of patients with adult refractory epilepsy [35].

According to our data, there is an increase in neuronal calcium



**Fig. 5.** Effect of BAI *in vivo* calcium imaging. (A) Shows the experimental set-up for the *in vivo* calcium imaging. Tg[HuC:GCaMP5G] zebrafish larvae (4 dpf) were exposed to either 7.5  $\mu$ M BAI ( $n = 10$ ) or 0.1 % DMSO ( $n = 10$ ) for 24 h, and, at 5 dpf, calcium transients were recorded in brain neurons for 15 min. (B) Scheme of the *in vivo* calcium imaging. (C) Regions of interest (ROI) from the optic tectum (highlighted by blue line) were extracted to measure calcium activity (Graphics created with BioRender.com). (D) Fluorescence images of calcium activity during baseline and seizure activity, and the intensity of fluorescence was color-coded. (E) The amplitude/spike of peaks detected in the larvae and (F) the number of peaks detected.  $n =$  number of larvae. Scale bar = 30  $\mu$ m. The data was analyzed using One-way ANOVA followed by Bonferroni multiple comparisons post-test. The results are presented as mean  $\pm$  standard error of the mean (SEM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity after PTZ exposure (Fig. 5). Liu & Baraban (2019) showed the same data using PTZ [36], as well as Choo *et al* (2021) when using genetic lines (*gabra1* and *gabrg2*) to mimic febrile seizures [37]. Furthermore, data found by Liu *et al* (2021) and Brenet *et al* (2019) also showed an increase in neuronal calcium activity in genetic models of epilepsy that mimic Dravet syndrome [38,39].

Observing the dynamic activities of the neuronal network is crucial for understanding of seizure initiation and propagation [40]. Our study found evidence supporting the findings of Liu & Baraban (2019) regarding the propagation of seizure activity from anterior-to-posterior brain regions [36]. Although we observed a slight decrease in the peak number of the calcium activity in the animals treated with BAI, their statistical pattern closely resembled the CTRL or PTZ group.

Studies on the absorption and distribution of the compound must be carried out to better understand the molecule, as well as the approach that keeps the compound constant over time. This will allow us to standardize the effective dose and method of administration.

## 5. Conclusion

Our results demonstrated that BAI does not have an anti-seizure effect in zebrafish larvae in the behavioral test and neuronal calcium activity, at least in the PTZ model and at the concentration and developmental stage parameters delineated by our experimental protocol. However, this outcome enriches our understanding of BAI utilization in zebrafish models, shedding light on optimal dosing strategies and administration modalities. Given the well-known properties of BAI,

and the fact that the zebrafish is a model with many advantages for translational studies, different approaches and concentrations of BAI should be investigated to have a better understanding of its anticonvulsant effect.

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## CRediT authorship contribution statement

**Laís Cintra:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Constantin Yanicostas:** Writing – review & editing, Supervision. **Nadia Soussi-Yanicostas:** Writing – review & editing, Supervision, Resources, Project administration, Data curation, Conceptualization. **Claudia Vianna Maurer-Morelli:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yebeh.2024.109866>.

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