Characterisation and automated quantification of induced seizure-related behaviours in *Xenopus laevis* tadpoles

Running/Abbreviated title: Behavioural seizures in *Xenopus* tadpoles

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Abstract

Epilepsy, a clinical diagnosis characterized by paroxysmal episodes known as seizures, affects 1% of people worldwide. An estimated 30% of patients continue to have seizures even on medication, and adverse effects are common. Safe and patient-specific treatment is vital and can be achieved by the development of rapid pre-clinical models of for identified epilepsy genes. Epilepsy can result from either brain injury or gene mutations, and can also be induced chemically. *Xenopus laevis* tadpoles could be a useful model for confirmation of variants of unknown significance found in epilepsy patients, and for drug re-purposing screens that could eventually lead to benefits for patients. Here, we characterise and quantify seizure-related behaviours in *X. laevis* tadpoles arrayed in 24-well plates. To provoke acute seizure behaviours, tadpoles were chemically induced with either pentylenetetrazole (PTZ) or 4-aminopyridine (4-AP). To test the capacity to adapt this method for drug testing, we also exposed induced tadpoles to the anti-seizure drug valproate (VPA). Four induced seizure-like behaviours were described and manually quantified, and two of these (darting, circling) could be accurately detected automatically, using the video analysis software TopScan. Additionally, we recorded swimming trajectories and mean swimming velocity. Automatic detection showed that either PTZ or 4-AP induced darting behaviour and increased mean swimming velocity compared to untreated controls. Both parameters were significantly reduced in the presence of VPA. In particular, darting behaviour was a shown to be a sensitive measure of epileptic seizure activity. While we could not automatically detect the full range of seizure behaviours, this method shows promise for future studies, since *X. laevis* is a well-characterised and genetically tractable model organism.

Keywords: Epilepsy, seizure behaviour, African Clawed Frog, *Xenopus laevis*, TopScan

Introduction

Epilepsy is a common neurological condition characterised by recurrent seizures, which are transient episodes of abnormal excessive brain activity (Fisher et al., 2005). Currently, 50 million people are estimated to have epilepsy and five million people are diagnosed with epilepsy each year (Fiest et al., 2017, Fisher et al., 2014). Epilepsy can be classed as either acquired (e.g. following injury or trauma) or genetic in origin. Genetic epilepsy incidence peaks within the first year of life (Camfield and Camfield, 2015). Children under 5 years of age have the highest age-specific incidences (~60 per 100,000) (Symonds et al., 2021). Developmental and epileptic encephalopathies (DEE) are the most severe forms of childhood epilepsy with frequent severe epileptiform activity and developmental slowing (McTague et al., 2016). DEE increases the risk of premature mortality and patients who survive have significant lifelong disabilities (Palmer et al., 2021). Patients are normally treated with one or more anti-epileptic drugs (AEDs) which aim to reduce the frequency of seizures. However, AEDs are not always sufficient and at best they only suppress seizure activity, with the underlying developmental defect untreated. Safe and effective patient-specific approaches are required for the treatment of DEE, ideally targeting the specific molecular pathways that are affected by specific gene mutations (Loscher, 2020).

Recent advances in molecular genetics have led to the discovery of more than 140 epilepsy-associated genes or loci (Perucca et al., 2020, Epi et al., 2013). Within each defective gene there are unique pathogenic variants, and phenotypic variation is common (Begemann et al., 2021, Indelicato and Boesch, 2021, Sadleir et al., 2017). This combination of a large number of genes and variants within genes means developing pre-clinical seizure models is a huge challenge in terms of time, cost and efficient results. Being able to develop models that can be used to both validate potential variants as pathogenic and quickly assess conventional or repurposed drugs for their ability to reduce seizure frequency will help in developing drugs against genetic epilepsy.

To date, various animal models of epilepsy/epileptic seizures have been established and characterized (Grone and Baraban, 2015, Loscher, 2017). Genetic and pharmacological rodent models of epilepsy have greatly contributed on the genetics of epilepsy and strongly improved our understanding of the pathophysiology of this neurological disorder (Grone and Baraban, 2015). However, every model has its benefits and its limitations (Kandratavicius et al., 2014) and functional studies of the growing number of epilepsy-causing genes and

variants is failing to keep pace with gene discovery. Recent approaches take advantage of the smaller, more fecund aquatic vertebrate models *Danio rerio* (Zebrafish) and *Xenopus laevis* (South African clawed frog), both of which are already well-established models for behavioural neuroscience and are additionally amenable to genetic manipulation (Hortopan et al., 2010, Pratt and Khakhalin, 2013, Adamson et al., 2018, Vaz et al., 2019, Willsey et al., 2022).

Xenopus are prolific breeders, and their brain development is very well characterised (Exner and Willsey, 2021). The development from eggs to pre-feeding, transparent tadpoles with well-developed brains takes place in a week and can be controlled by manipulating temperature (Nieuwkoop and Faber, 1994). The *X. laevis* genome has been sequenced and is well annotated (Session et al., 2016) and the Xenbase database supports research into genetic disease (Nenni et al., 2019). Further, *Xenopus* are highly amenable to manipulation such as CRISPR/Cas9 editing (Horb et al., 2019, Naert et al., 2020) or expression of exogenous proteins via mRNA injections (Gurdon et al., 1974). These unique properties make *X. laevis* tadpoles a viable model organism for the functional study of genetic epilepsy and epileptic seizures, as well as for pre-clinical drug testing.

The use of *Xenopus* tadpoles to model acute seizures (induced by chemicals) has been previously described, and a series of stereotypical behaviours were classified (Bell et al., 2011, Hewapathirane et al., 2008). The most severe behaviour involves a strong contraction of trunk muscles on one side of the tadpole such that its head and tail connect briefly. These movements are known as C-shaped contractions due to the characteristic extreme posture (Hewapathirane et al., 2008). Recently, CRISPR/Cas9-mediated knockdown in *Xenopus* tadpoles was used to confirm two *de novo* human NEUROD2 variants as causative for DEE-75 (Sega et al., 2019). Importantly, Sega and colleagues described both abnormal swimming and the spontaneous occurrence of C-shaped contractions (CSC) as seizure-related behaviour, showing that chronic epilepsy behaviours can also be phenotyped in this model (Sega et al., 2019). However, automated detection of these behaviours, which may be important if these models are to be useful in closing the gap between diagnosis and treatments, has not yet been described. Here, we evaluate the potential for a popular software for mammalian behaviour analysis, TopScan, to detect seizure-like behaviours in *X. laevis* tadpoles. Two acute seizure-inducing drugs were applied to tadpoles arrayed in 24-well plates in the presence or absence of anti-seizure drug valproic acid (VPA), and the resulting behaviours both manually and automatically tallied. While we could not reliably automatically detect the C-shaped

contractions, other seizure-associated behavioural parameters could be automatically detected. Automatic behaviour tracking therefore adds to the toolkit that makes *X. laevis* tadpoles a promising model for rapid phenotyping of models of chronic epilepsy in future.

2 Materials and methods

2.1 Husbandry and production of *Xenopus laevis* eggs and embryos

Xenopus laevis adult males and females are maintained in standard aquarium conditions with 12/12h light dark cycles at 18 ºC in carbon filtered tap water at the Zoology Department, University of Otago. The colony has been closed since its establishment in 2004 and is now in the F2 generation. Adult female *X. laevis* were induced to lay eggs by injecting with 500 IU per 75 g body weight of human chorionic gonadotrophin (Chorulon) into the dorsal lymph sac, 16 hours before eggs were required. They were placed in a dark incubator overnight in pairs in small holding tanks containing filtered tap water. After egg laying commenced, each female was placed in 1 L of 1 x Marc's modified ringer's solution ((MMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5mM HEPES, 0.1 mM EDTA pH 8.0) to prevent activation. Eggs were collected hourly and fertilised using fresh testes from a euthanised adult *X. laevis* male. Egg jelly was removed immediately following embryo rotation (15-20 minutes), using a 2% solution of L-Cysteine (pH 7.9) and the resulting de-jellied embryos were rinsed three times in MMR. Embryos were raised in small batches in 0.1 x MMR in 10 cm diameter petri dishes with no antibiotics and staged according to Nieuwkoop and Faber's staging series (Nieuwkoop and Faber, 1994). All procedures were approved by the University of Otago's Animal ethics committee under AUP 19/01.

2.2 Preparation and delivery of the seizure-inducing chemicals pentylenetetrazole (PTZ) and 4- aminopyridine (4-AP), and the anti-seizure chemical valproic acid (VPA)

All chemicals were freshly prepared before every scheduled experiment. The concentration of these chemicals used in this study was based on previously published information for *Xenopus* tadpoles (Hewapathirane et al., 2008). PTZ (Sigma Aldrich, USA) was prepared as a 100 mM stock solution. Individual tadpoles were placed in 1.2 ml of 5 mM PTZ in 0.1x MMR. Similarly, 5 mM and 10 mM of VPA (Sigma Aldrich, USA) were prepared from 100 mM stock solution. 0.5 mM of 4-AP (Sigma Aldrich, USA) was prepared from 10 mM stock solution.

2.3 Recording of stage 47 tadpole behaviour in 24-well plates

Individual tadpoles were transferred to each well of a 24-well culture plate (Cellstar, Greiner bio-one) (Fig. 1) using a cut down, wide bore glass pipette. The wells were filled with 1.2 ml of 0.1 x MMR, with or without chemicals, so that the tadpoles were submerged. The tadpoles were recorded using a Panasonic DMC-FZ1000 camera set in movie mode at 25 frames per second. Frames were captured at 1280x 720 pixels at 10Mbps. Plates were illuminated from below using white LED light source (Neewer 176 ultra bright ϕ5 LED, K5600) and a white perspex diffuser, and the camera was fixed above the well plate with a tripod (Figure 1). Where stated, PTZ, 4-AP and/or VPA were added to wells just prior to the start of video recording. Thirty minutes of video was captured in MP4 format and converted into MPEG2 format for the analysis using Adobe Media Encoder CC 2019 software.

2.4 Analysis of videos using TopScan (CleverSys).

To analyse seizure behaviour in tadpoles, the TopScan-TopView behaviour analysing system software (CleverSys Inc., USA) was used. Modules *Locoscan*, *DrugAbuse* and *Circling* were used to quantify seizure-related behaviour in tadpoles. In TopScan, the images of tadpoles were subtracted to create a clear background with just the 24-well plate. Arenas were defined as individual tadpole wells, and were created by using *Arena Design* toolbar. This enables simultaneous analysis of each individual tadpole's behaviour. For quantification of distance travelled and velocity, the well diameter, which is 17 mm, was entered as 17 cm, and parameters based on distance travelled were corrected *post hoc* by dividing by 10. Event rules and output parameters were then set up to detect and quantify the behaviours of tadpoles such as darting, circling, velocity and movement trajectories to enable phenotyping. A step-by-step procedure of using TopScan software to generate behavioural data from recorded video can be found in supplementary file 1.

2.5 **Treatment groups**

To establish seizure-related behaviour in *Xenopus* tadpoles, two seizure-inducing chemicals (PTZ and 4-AP) and one anti-seizure chemical (VPA) were included in this study. Untreated and a VPA-only treated control group were also included. Tadpole treatment groups included in this study are as follows: Untreated, 5 mM VPA, 5 mM PTZ, 5 mM PTZ and 5 mM VPA, 0.5 mM 4-AP, 0.5 mM 4-AP and 5 mM VPA, and 0.5 mM 4-AP and 10

mM VPA. All tadpoles were at stage 47 at the time of analysis, and data for each group was merged from multiple sibships and videos.

2.6 Data analysis

Ethograms were constructed from manually curated data from 10 tadpoles for each group using a Fruchterman–Reingold algorithm and generated using qgraph package v1.9.2 (Epskamp et al., 2012). Automatically generated behavioural data from different tadpole sibships was merged for automated analysis. Graphs and statistical analyses were calculated using Prism v9.1.2. Comparison between all treatment group means was performed using unpaired t-tests (for two groups) or 1-way ANOVA with Tukey's post hoc multiple comparison test for all means (three or more groups). Data were presented as mean ± standard error of the mean (SEM) and the statistical significance threshold was set to $p < 0.05$.

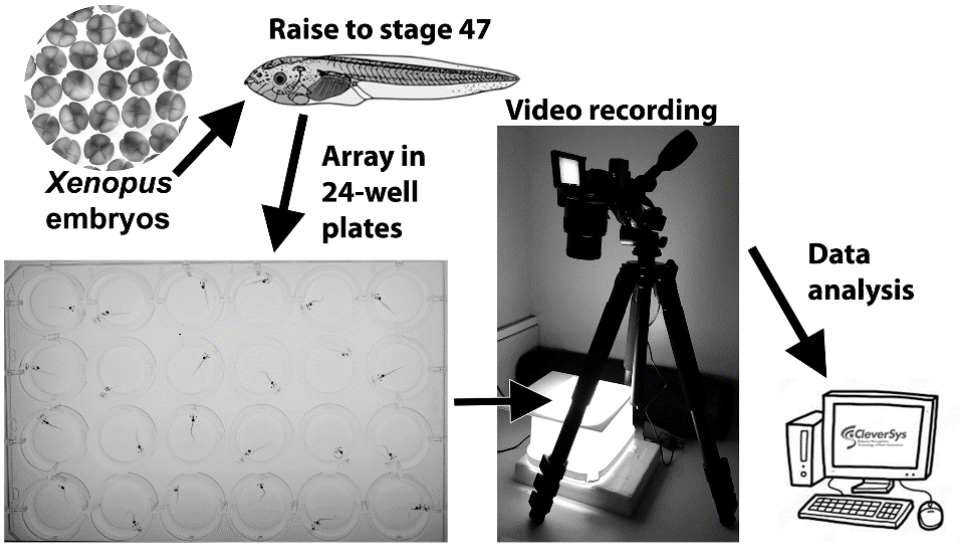

Figure 1: Pipeline for behavioural analysis of tadpoles induced to acute seizure activity with PTZ or 4-AP. *X. laevis* wild type embryos were raised to stage 47, at which point they will progress no further without feeding. Each tadpole was placed in its own well in a 24-well plate and behaviour recorded for 30 minutes. Videos were analysed offline using TopScan software.

3.0 Results

3.1 Distinct behaviours are elicited by the acute seizure inducing chemicals PTZ and 4-AP.

In rodent models, different seizure behaviours are elicited with different seizure-inducing chemicals. PTZ is associated with tonic-clonic seizures and status epilepticus, and functions via blockade of GABA-A receptors (Loscher, 2011). 4-AP acts as a potassium channel blocker, which causes increased neuronal excitability and release of glutamate, leading to status epilepticus (Traynelis and Dingledine, 1988, Kobayashi et al., 2008). To determine how the tadpole model responds to these drugs, 30 minutes of video data for ten tadpoles that were either untreated or exposed to PTZ or 4-AP were examined manually. Untreated stage 47 tadpoles (controls) were nominally active, usually remaining with the head facing the well side, slowly drifting in the well, or swimming slowly for a brief time. In contrast, tadpoles treated with the acute seizure-inducing drugs PTZ or 4-AP showed increased levels of activity, manifesting as distinct swimming behaviours which were not seen in untreated tadpoles (Table 1, Fig. 2A). Characteristic "C-shaped contractions" (CSC) were consistently observed, as previously described (Hewapathirane et al., 2008). In CSC, the trunk muscles on one side undergo rapid and strong contractions, and as a result the tail bends so far to one side that the tip touches the tadpole's head (Figure 2A). However, this was not the dominant or only behaviour elicited: tadpoles treated with 5 mM PTZ only spent 10% of their time in CSC, and for 0.5 mM 4-AP-treated tadpoles it was 13% (Table 1). In the remaining time, tadpoles were either immobile (like untreated controls), or exhibiting one of three distinct behaviours (Fig. 2A). These were termed "uncoordinated tail bends" (UTB), where the tadpole head remained still at the side of the well but the tail was rapidly beating from side to side, "circling" (rapid swimming around the well perimeter), and "darting" (rapid swimming with frequent changes in direction). UTB was the dominant behaviour in 4-

Table 1: Percentage of time spent in each behavioural state, for 10 tadpoles in 24-well plates over 30 minutes.

	Untreated	PTZ Treated	4-AP Treated
Immobile	100%	27%	14%
Circling	0%	25%	23%
Darting	0%	23%	23%
Uncoordinated tail bends (UTB)	0%	15%	27%
C-shaped contractions (CSC)	0%	10%	13%

AP treated tadpoles, and PTZ treated tadpoles were more likely to be immobile or circling (Table 1 and Fig. 2).

The frequency of transitions between one behaviour type and another was also recorded and used to construct an ethogram (Figure 2B). 4-AP-treated tadpoles were most active, and also spent more time in CSC and UTB behaviours than PTZ-treated tadpoles. In PTZ-treated tadpoles, immobile state was followed by circling behaviour 57% of the time, and circling was most often followed by darting (42%) UTB were followed by immobility 45% of the time and by CSC 14% of the time. (Fig. 2C). 4-AP-treated tadpoles did not follow the same pattern as PTZ-treated tadpoles, with darting and circling behaviour frequently alternating with UTB.

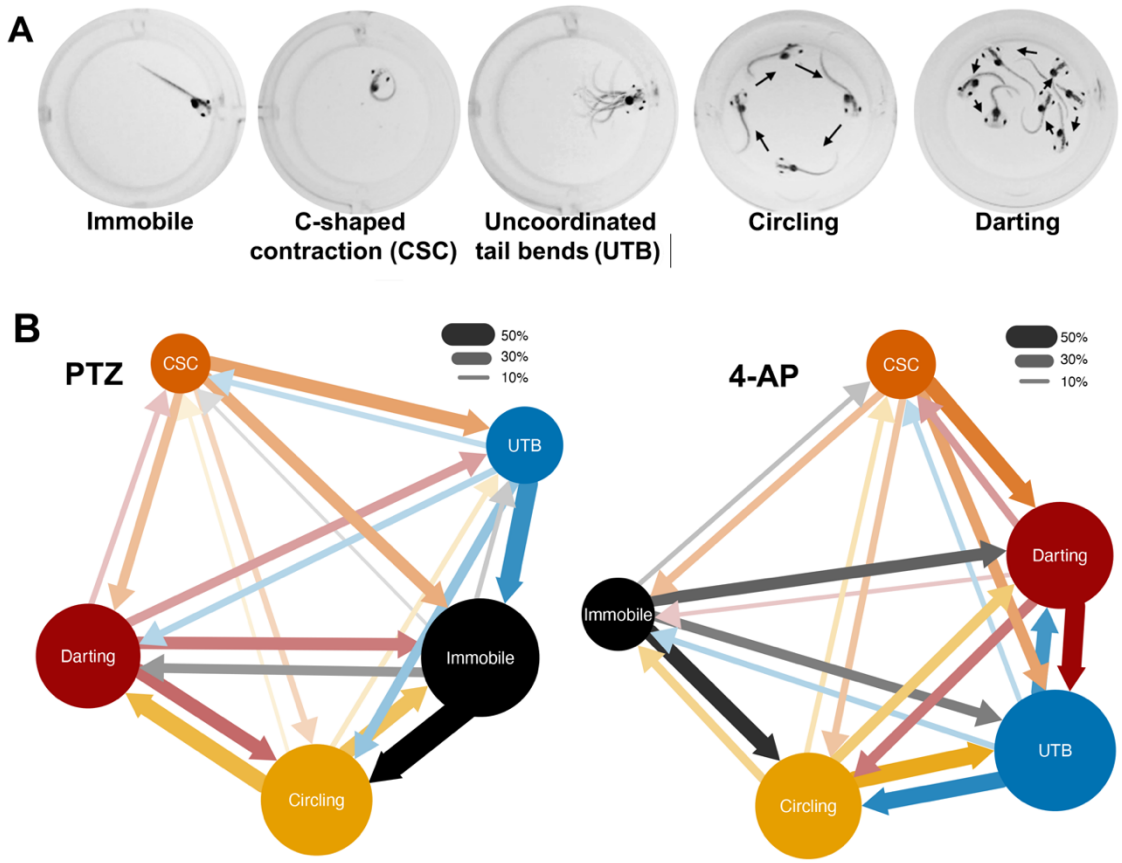

Figure 2: PTZ and 4-AP induce different patterns of swimming behaviour. (A) Typical behaviours of PTZ- and 4-AP-treated tadpoles. Multiple consecutive frames are overlayed for UTB, Circling and Darting, with arrows indicating the sequence of frames. (B) Ethograms created by manual analysis of 10 tadpoles treated with either 5 mM PTZ or 0.5 mM 4-AP to induce acute seizures. Circle diameter represents the percentage of time spent in each behaviour (see Table 1). Transitions between different behaviours are represented by arrows. Thicker arrows and closer circles indicates transitions between the two behaviours are more frequent. Data can be found in Table 1 and the supplemental file 2.

3.2 Automated behavioural analysis of video recordings with TopScan can be used to track movement and to detect circling and darting behaviour.

Manual counting of behaviours from video recordings, while informative, is time-consuming and not scalable to high throughput applications like drug testing. To determine if we could automate this process for drug testing, a pilot study with N=10 tadpoles was conducted. As we could not reliably detect CSC or UTB with TopScan, we compared manually counted CSC to automated analysis of tadpole swimming using TopScan software (Fig. 3). PTZ and 4-AP have been previously shown to induce CSC in stage 47 *Xenopus* tadpoles (Hewapathirane , 2008). Manually counted data confirmed that PTZ-induced tadpoles had an average of 13.4±5.2 CSC during the 30-min recording period, compared to 9.6±2.8 for the cohort treated with both 5 mM VPA and PTZ (Fig. 3A). 4-AP-treated tadpoles had 43.3±7.8 CSC and this was significantly more than groups with 4-AP plus either 5mM VPA (8.4±8.0) or 10mM VPA (7.0±5.70). Both VPA treatment groups had <20% of the CSC seen in PTZ only controls (Fig. 3B).

The same videos were also analysed with TopScan to determine mean swimming velocity (cm/s) and the percentage of time spent circling or darting. Of the automatically scored behaviours, VPA only resulted in a significant reduction in the percentage of time tadpoles spent darting (Fig. 3A,B).

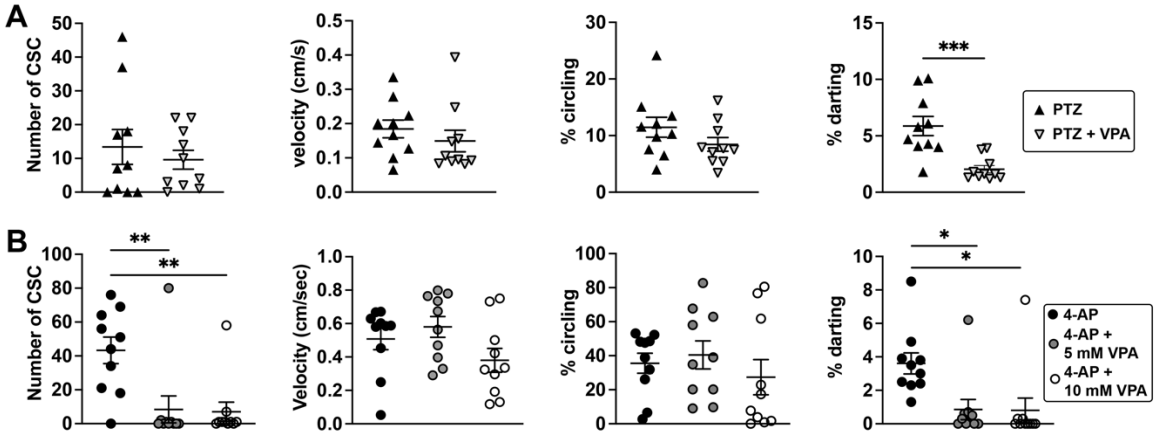

Figure 3: The anti-seizure drug VPA reduces 4-AP-induced C-shape contractions (CSC) and 4-AP- and PTZ-induced darting behaviour. Scatterplots showing manually counted CSC, mean velocity of tadpoles (cm/s), percentage of time spent circling and percentage of time spent darting. Black line indicates the mean and error bars are standard error of the mean (sem) (A) Tadpoles treated with 5 mM PTZ with or without 5 mM VPA, N=10 for each group, compared using unpaired t-test. (B) Tadpoles treated with 4-AP, with or without VPA at two concentrations , 5 and 10mM. Statistical analysis was by 1-way ANOVA with Tukey's post hoc test of all means. N=10 tadpoles, each group. Statistically different groups are indicated by asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data can be found in the supplemental file 2.

3.3 High-throughput automated analysis confirms the efficacy of VPA in reducing seizure-related activity.

Having shown the potential of automated behavioural analysis to quantify the effect of seizure-inducing chemicals PTZ and 4-AP, at least for darting, we increased the number of tadpoles in the analysis. We also included untreated tadpoles (controls) and tadpoles treated with 5mM VPA in this analysis (Fig. 4). As CSC could not be reliably detected by TopScan, we sought to use the other abnormal behaviours to assess the effectiveness of anti-seizure drugs, using VPA as a benchmark. Movement trajectories (tracks) of individual tadpoles confirmed the increased activity of PTZ and 4-AP treated tadpoles (Fig. 4A). Further, the co-administration of VPA reduced the excessive movement of the tadpoles to control levels. Notably, 4-AP-treated tadpoles were still more active than controls even in the presence of 10 mM VPA (Fig. 4A).

All three measured parameters: mean velocity, percentage of time circling and percentage of time darting, were significantly increased in either 5 mM PTZ- or 0.5 mM 4-AP-treated tadpoles (Fig. 4B). Circling and darting behaviour was rarely seen in control treatment groups (< 0.2% and <0.1% respectively). PTZ-induced behaviours were all significantly lower in the cohort with co-administered 5 mM VPA. Mean velocity was 3.3-fold lower, the percentage of time spent circling was 5.7-fold lower, and the percentage of time darting was 2.9-fold lower in the presence of VPA (Fig. 4B).

Rapid swimming induced by 4-AP, seen as velocity and circling measures, gave mixed results with 5 mM VPA, but tadpoles co-treated with 4-AP and the higher dose of 10 mM showed a significant reduction in both behaviours (2.2-fold less time circling, 1.5-fold lower mean velocity). 4-AP-induced darting was not seen when VPA was present at either concentration (Fig. 4B).

3.4 VPA does not affect the latency to first seizure related event induced by PTZ, but delays the onset of 4-AP induced events.

To test whether VPA affects the onset time of PTZ- and 4-AP- induced circling and darting behaviour, we recorded the time of the first darting or circling event for each tadpole in the study (Fig. 5). An increased time to the first seizure event in the presence of an anti-seizure drug may indicate elevated seizure threshold. In PTZ-treated tadpoles, the latency to first circling or darting event was unaffected by the presence of 5 mM VPA (Fig. 5A), but fewer tadpoles circled or darted (Table 2).

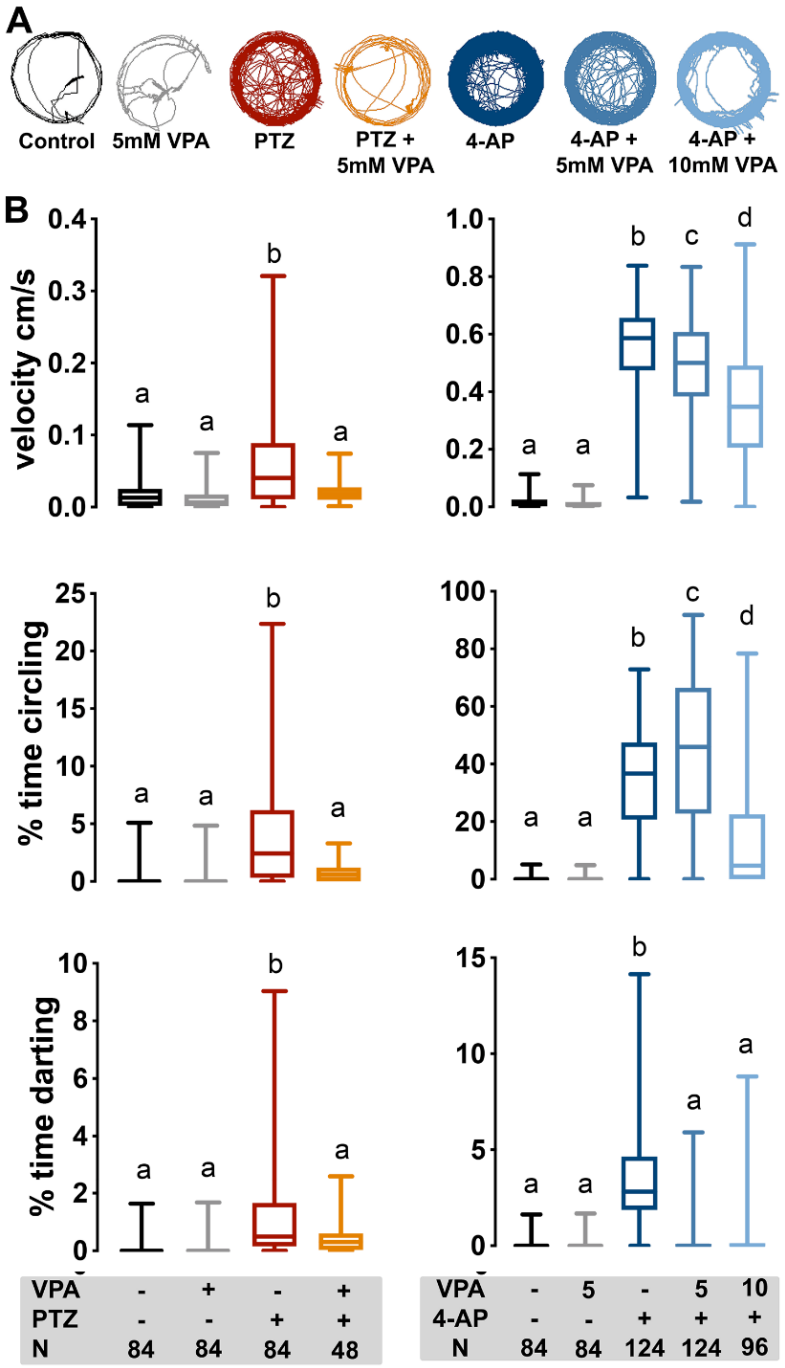

Figure 4 Automatic analysis shows that VPA reduces PTZ- and 4-AP-induced swimming behaviours (A) Representative examples of individual tadpole movement trajectories each treatment group, over the 30 minutes of the trial (treatment is colour-coded as in B). (B) Box plots of average tadpole swimming velocity (cm/sec), percentage of time spent swimming in rapid circles and percentage of time spent darting. Middle line indicates the median, upper and lower lines indicate the upper and lower quartile, and the whiskers indicate the minimum and maximum values. 1-way ANOVA with Tukey's post hoc multiple comparison test of all means was used to analyse each dataset. Different letters above bars indicate significant differences ($p<0.05$). N=number of tadpoles, VPA was used at 5 or 10 mM, PTZ at 5 mM and 4-AP at 0.5 mM). Raw data can be found in supplemental file 2.

All tadpoles exposed to 0.5 mM 4-AP alone had at least one circling event in the 30 minute period, but, in the cohorts co-treated with 5 mM VPA, 7% of tadpoles were recorded as having no circling events and this number rose 5 fold to 37% in the tadpoles co-treated with 10mM VPA (Table 2). Latency to first circling event was significantly different for each of the treatment groups, with 4-AP + 5mM VPA reducing the mean time to first event and 4-AP + 10mM VPA increasing it (Fig 5C). Co-treatment with VPA had a dose dependent effect on latency to first darting event, (Fig 5D). Darting events were not observed in 12% of tadpoles treated with 4-AP alone. Approximately 60% of 4-AP and VPA co-administrated tadpoles were completely free from darting behaviour during 30-minute testing period (Table 2).

Table 2: Percentage of tadpoles showing no circling or darting behaviour for each treatment group

Treatment group	Sample size	% with no circling	% with no darting
PTZ	84	15.1	15.5
PTZ and VPA	48	37.4	20.8
4-AP	124	0.0	12.1
4-AP and 5mM VPA	124	6.9	62.1
4-AP and 10mM VPA	96	37.1	60.4

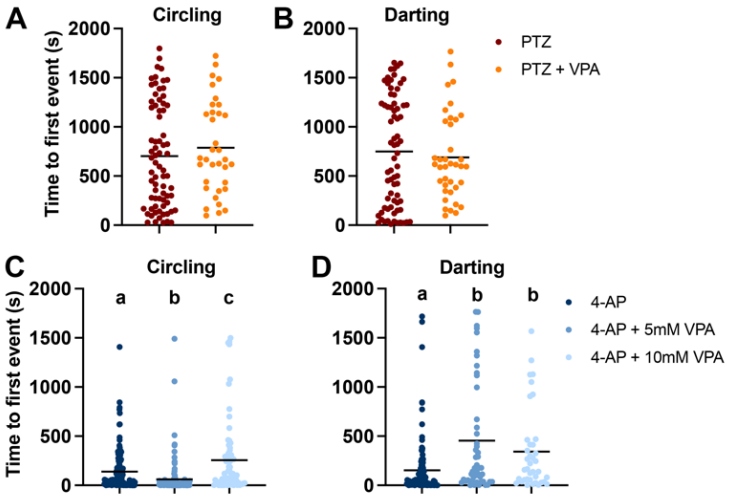

Figure. 5 Latency to first 4-AP induced circling or darting event is altered by the presence of anti-seizure drug VPA. Scatter plots showing the time of the first seizure related circling or darting event for each tadpole in the study that had at least one event in the 30 minutes. Black bar indicates mean. A, B) There was no difference in the mean time to first PTZ induced event in the presence of 5mM VPA (Unpaired t-test, N=73 PTZ circling, N=35 PTZ + VPA circling, N=71 PTZ darting, N= 38 PTZ + VPA darting. C, D) Mean onset of circling events was significantly different between 4-AP only groups and groups co-treated with VPA. 1-way ANOVA with Tukey's post-hoc analysis of all means, groups which are significantly different are indicated by a distinct lower case letter. N=124 4-AP circling, N=116 4-AP + 5mM VPA circling, N=69 4-AP + 10 mM VPA, N=109 4-AP darting, N=47 4-AP + 5 mM VPA darting, N=38 4-AP +10 mM darting. Raw data can be found in supplemental file 2.

4.0 Discussion

4.1 PTZ and 4-AP treated tadpoles elicit similar patterns of seizure-related behaviours

PTZ and 4-AP are widely used chemicals to induce acute seizures in various mammalian and non-mammalian animal model systems (Hewapathirane et al., 2008, Ellis et al., 2012, Cunliffe et al., 2015, Johan Arief et al., 2018). Depending upon the animal model system and concentration, PTZ and 4-AP induce seizures within few minutes and the epileptic effect can continue for hours. Studies have shown that both latency to seizure and seizure severity after PTZ treatment is dose-dependent (Kliouevа et al., 2001, Hewapathirane et al., 2008, Luttjohann et al., 2009, Pineda et al., 2011, Van Erum et al., 2020). PTZ induces acute seizures by antagonising GABA-A receptors, which reduces inhibitory GABA signalling (Loscher, 2011). 4-AP acts via a different mechanism, blocking potassium channels (Traynelis and Dingledine, 1988, Kobayashi et al., 2008), and is also proven to induce spontaneous seizure-like events in *X. laevis* and zebrafish larval models (Hewapathirane et al., 2008) (Winter et al., 2017).

While PTZ and 4-AP induced the same set of stereotypic behaviours, our ethogram revealed inducer-dependent distinct sequences of behavioural events (Fig. 2B). C-shaped contractions were detected for both groups, but could only be manually scored due to limitations with TopScan (primarily the low frame rate). CSC likely arise as a result of neuronal excitation spreading to the networks of the tadpole brain normally dedicated to the well characterised C-start kinetic response (Karin v and Wassersug, 2000). The other stereotypical behaviours we describe likely also arise from similar overstimulation of inherent motor pattern control networks in the tadpole brain. Hence, these behaviours are not unique to acute seizure models and we are detecting them due to their exaggerated or prolonged manifestations. In zebrafish larvae, a functional study comparing several acute seizure inducing chemicals showed that PTZ resulted in neural activity that was strongest in the rhomencephalon (hindbrain) and 4-AP the tectum (Winter et al., 2017).

4.2 Automated detection of seizure behaviours in tadpoles shows that darting is the most sensitive indicator of drug efficacy.

The primary clinical diagnostic test for confirming epileptic seizures in humans is via analysis of electroencephalogram (EEG) data. However, the occurrence of abnormal behavioural movements is generally the first indicator of epilepsy and is an integral part of diagnosis (Fisher et al., 2017, Falco-Walter et al., 2018). In a pioneering study,

Hewapathirane et al (2008) described a series of progressive seizure-related behaviours (class I to V) using manual video analysis of *Xenopus laevis* tadpoles induced with epileptogenic drugs (Hewapathirane et al., 2008). Under normal conditions, tadpoles show slow swimming and brief pause-like behaviour which we also observed here, in both untreated and VPA only treated control groups of tadpoles. Tadpoles tend to display abnormal swimming behaviour such as increased locomotor activity/agitation (also termed as hyperlocomotion) as a sign or pattern of seizure progression. Circling and darting (detected here) best represent class I behaviour, defined as '*bouts of intermittent rapid swimming, involving swimming in tight circles or sporadic rapid swimming with abrupt changes in direction ("darting")'*. However there is also some overlap with class II and class III behaviours (class II: as class I but with intermittent immobility, class III: additional loss of postural control, e.g. tadpole is on its side or upside down). In contrast, UTB are most like class IV, and CSC are class V, representing the culmination of a generalised seizure (Hewapathirane et al., 2008). Therefore we have been able to automate detection of previously described class I-III behaviours, using "circling" and "darting" but not class IV or V. Time spent in both circling and darting behaviours was significantly higher in tadpoles treated with PTZ and 4-AP compared to control tadpoles, and treatment with VPA significantly reduced the number of circling and darting bouts (Fig. 4). We also used mean velocity as a measure of seizure-induced hyperactivity. This has not previously been used as a measure for seizure activity in tadpoles, but hyperlocomotion has been described in zebrafish larvae (Baraban et al., 2005, Afrikanova et al., 2013, Gawel et al., 2021). Together, velocity, darting and circling were useful indicators of seizure activity induced by either PTZ or 4-AP, but only when large cohorts were analysed. Automation of behaviours should therefore be useful in analysing the effects of variants of unknown significance in epilepsy causing genes.

Time spent engaged in darting behaviour was the most sensitive method of detecting reductions in acute seizure behaviours by the anti-seizure drug VPA. The broad anti-epileptic effect of VPA is due to a combination of effects that act to both increase inhibitory GABA signalling and decrease excitatory signals such as glutamate (Romoli et al., 2019). The latency, severity, nature and progression of seizure-related behaviour seen in this study is similar to the previous published studies in *Xenopus* tadpoles, zebrafish and rodents. For example, tonic/clonic and uncontrolled motor movements seen in mice (Van Erum et al., 2020), rats (Luttjohann et al., 2009), zebrafish (Afrikanova et al., 2013, Gawel et al., 2021)

and other models (Johan Arief et al., 2018) after PTZ can be correlated to increased velocity as well as time spent in darting and circling behaviour seen in this study.

4.3 *Xenopus* tadpole as a model organism for epilepsy, clinical significance and future directions

Several studies have been conducted in the past for the purpose of using zebrafish (*Danio rerio*), nematode worms (*Caenorhabditis elegans*) or fruit flies (*Drosophila melanogaster*) as a potential non-mammalian models for seizure or seizure-like activity (Hortopan et al., 2010, Cunliffe et al., 2015, Johan Arief et al., 2018, Afrikanova et al., 2013, Zhu et al., 2020, Gawel et al., 2021). The main idea is to establish a model which is less time-consuming, less expensive, robust and straight forward for high-throughput anti-epileptic drug discovery. With the exception of zebrafish, these studies quantified behavioural seizures or seizure progression just in terms of locomotion/movement, as we have done here. While this is a good first step, if non-mammalian models are to be useful for epilepsy research, seizure-related behaviours will need to be validated by identifying neural signatures of seizures (e.g., electrophysiological brain recordings (Hewapathirane et al., 2008) or seizure-induced changes in gene expression (Labiner et al., 1993, Ribak et al., 1997). In both zebrafish larvae, and *X. laevis* tadpoles, electrophysiological recording from the larval brain has been demonstrated (Diaz Verdugo et al., 2019, Baraban et al., 2013, Hewapathirane et al., 2008). Furthermore, this activity has been expressly linked to calcium signalling in both models (Hewapathirane et al., 2008, Diaz Verdugo et al., 2019). As per our current understanding, this is the first study to automatically quantify seizure-related behaviour in *Xenopus* tadpoles and should be useful to unlock the potential of *X. laevis* tadpoles as models of genetic epilepsy.

5.0 Concluding remarks

We quantified behaviours elicited in *X. laevis* tadpoles by the acute seizure-inducing chemicals PTZ and 4-AP and showed that these behaviours were eliminated or reduced by the anti-seizure drug VPA. To develop a new rapid pre-clinical *Xenopus* model for chronic epilepsy where a genetic basis is suspected, characterization and quantification of seizure-related behaviour will be crucial, as it provides a basis for triaging variants before undertaking more time-consuming electrophysiological experiments. We suggest that the automated behaviour analysis described here could be a useful first step in the development of much needed pre-clinical non-mammalian models of genetic epilepsy.

Data Availability Statement

A detailed methodology and a spreadsheet of all raw data and statistical testing results can be found in the supplementary information.

Supplementary file 1: detailed TopScan protocol and behavioural parameters

Supplementary file 2: Raw data and statistical analysis supporting the conclusions

Ethics Statement

All experiments were performed in accordance with ARRIVE guidelines. *Xenopus* embryos were produced using protocol approved by the University of Otago Animal Ethics Committee under AUP 19/01

Author Contributions

Conceptualization, methodology, investigation, SP, formal analysis, data curation: SP, CB, PC; Writing: SP, PS, CB; Supervision: PS and CB; Project administration and funding acquisition: CB.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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