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Susceptibility of larval zebrafish to the seizurogenic activity of GABA type A receptor antagonists

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ABSTRACT

Previous studies demonstrated that pentylenetetrazole (PTZ), a GABA type A receptor ($GABA_AR$) antagonist, elicits seizure-like phenotypes in larval zebrafish (*Danio rerio*). Here, we determined whether the $GABA_AR$ antagonists, tetramethylenedisulfotetramine (TETS) and picrotoxin (PTX), both listed as credible chemical threat agents, similarly trigger seizures in zebrafish larvae. Larvae of three, routinely used laboratory zebrafish lines, Tropical 5D, NHGRI and Tupfel long fin, were exposed to varying concentrations of PTZ (used as a positive control), PTX or TETS for 20 min at 5 days post fertilization (dpf). Acute exposure to PTZ, PTX or TETS triggered seizure behavior in the absence of morbidity or mortality. While the concentration-effect relationship for seizure behavior was similar across zebrafish lines for each $GABA_AR$ antagonist, significantly less TETS was required to trigger seizures relative to PTX or PTZ. Recordings of extracellular field potentials in the optic tectum of 5 dpf Tropical 5D zebrafish confirmed that all three $GABA_AR$ antagonists elicited extracellular spiking patterns consistent with seizure activity, although the pattern varied between chemicals. Post-exposure treatment with the $GABA_AR$ positive allosteric modulators (PAMs), diazepam, midazolam or allopregnanolone, attenuated seizure behavior and activity but did not completely normalize electrical field recordings in the optic tectum. These data are consistent with observations of seizure responses in mammalian models exposed to these same $GABA_AR$ antagonists and PAMs, further validating larval zebrafish as a higher throughput-screening platform for anti-seizure therapeutics, and demonstrating its appropriateness for identifying improved countermeasures for TETS and other convulsant chemical threat agents that trigger seizures via $GABA_AR$ antagonism.

1. Introduction

The $GABA_AR$ antagonists, tetramethylenedisulfotetramine (TETS) and picrotoxin (PTX), are considered credible chemical threat agents that pose a serious health threat to military personnel and civilian populations (Jett and Spriggs, 2018). TETS, a tricyclodecane originally developed as a rodenticide, is banned from production worldwide because of its potent toxicity and biological persistence; however, it is easily synthesized and widely available on the black market [reviewed by (Banks et al., 2014; Laukova et al., 2019b; Whitlow et al., 2005)]. Acute intoxication with TETS can trigger seizures that rapidly progress to life-threatening *status epilepticus* in humans (Li et al., 2012; Whitlow et al., 2005; Wu and Sun, 2004; Zhang et al., 2011) and in rodent models (Laukova et al., 2019a; Shakarjian et al., 2012; Zolkowska et al., 2012). Clinical reports indicate significant morbidity amongst human survivors of TETS-induced *status epilepticus*, including cognitive dysfunction, affective disorders and spontaneous recurrent seizures (Li et al., 2012; Lu et al., 2008; Whitlow et al., 2005; Zhang et al., 2011). PTX is a natural product isolated from plants of the moonseed genus (*Menispermacea*) that can also be readily obtained (Palmer and Casida, 1988). PTX is widely used as a pharmacological tool and as a chemical convulsant (De Deyn et al., 1992). Both TETS and PTX are presumed to

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induce seizures via $GABA_AR$ inhibition (Laukova et al., 2019b).

There is currently no standard medical countermeasure for acute intoxication with $GABA_AR$ antagonists, and treatment is largely supportive (Croddy, 2004). Benzodiazepines are the standard-of-care for treating acute seizures and *status epilepticus*, and when administered within minutes of seizure initiation, these drugs can terminate chemical-induced seizures (McDonough et al., 2000). However, their efficacy decreases with sustained seizure activity because of the rapid internalization of synaptic $GABA_AR$ (Kapur et al., 1989; Walton and Treiman, 1988). Consistent with downregulation of their key pharmacologic target, clinical reports suggest that TETS-induced seizures recur following successful initial treatment with benzodiazepines, and these recurrent seizures are resistant to further administration of these drugs (Anderson et al., 1997; Croddy, 2004; Laukova et al., 2019b; Whitlow et al., 2005). These clinical observations, together with the likelihood of delayed treatment of civilians following accidental, suicidal or terrorist-related exposures, underscore the urgent need for more effective medical countermeasures for terminating seizures triggered by $GABA_AR$ antagonists.

Efficient screening platforms that recapitulate key characteristics of the human condition are critical for identifying and evaluating therapeutic candidates for chemical-induced seizures (Jett and Yeung, 2010). Larval zebrafish (*Danio rerio*), a small freshwater teleost, are a well-established platform for medium-high throughput screens for drug-induced seizure liability and antiseizure efficacy (Baraban et al., 2005; Baxendale et al., 2012; Koseki et al., 2014). Zebrafish express the full complement of $GABA_A$ and $GABA_B$ receptor genes, as well as $GABA_AR$-associated protein encoding genes. Zebrafish express a single homolog of the α1, α3, α4, α5, β1, β2, β3, β4, ρ1, γ1, γ2, γ3, and δ subunits, and two homologs for the α2 (LOC100150704 and LOC556056), α6 (gabrb6a and gabra6b), ρ2 (gabrr2a and gabrr2b), ρ3 (gabrr3a and gabrr3b), and π (gabrp and gabrz) subunits (Monesson-Olson et al., 2018). Expression of $GABA_AR$ subunits is expected to be similar across multiple zebrafish strains; however, one study identified differences in the gene encoding the β1 $GABA_AR$ subunit in short fin zebrafish vs. the AB zebrafish (Pan et al., 2012), neither of which were used in this study. The average percentage of identical residues between encoded proteins for the GABA receptor genes in humans vs. zebrafish is 81 % (range 60–98 %) (Klee et al., 2012).

In response to relevant genetic or chemical manipulations, zebrafish larvae exhibit behavioral phenotypes and brain electrical activity that are similar to human and rodent seizures (Baraban et al., 2005; Grone et al., 2017). Exposure of larval zebrafish to pentylenetetrazole (PTZ), a chemical convulsant that, like PTX and TETS, induces seizures via $GABA_AR$ antagonism, triggers a stereotypic hyperactive swimming pattern that culminates in clonic-like seizures, and generates seizure-like electroencephalographic (EEG) profiles (Afrikanova et al., 2013; Baraban et al., 2005). PTZ-induced locomotor behavior in larval zebrafish is used extensively in genetic (Chege et al., 2012; Hortopan et al., 2010; Zdebik et al., 2013) and pharmacologic (Afrikanova et al., 2013; Baxendale et al., 2012; Copmans et al., 2018; Gupta et al., 2014; Koseki et al., 2014; Moradi-Afrapoli et al., 2017) screens for antiseizure activity. Similarly, PTX has been used previously for anti-seizure/anti-epileptic drug discovery in the zebrafish (Wong et al., 2010; Yang et al., 2017).

To date, however, few studies have evaluated larval zebrafish as a model of acute intoxication with seizurogenic chemical threat agents. Therefore, we tested the hypothesis that acute exposure to PTX or TETS triggers seizures in zebrafish larvae. We employed three strains of wildtype zebrafish routinely used in the laboratory (Tropical 5D, Tupfel long fin and NHGRI) to evaluate the effects of PTX and TETS on seizure-like behavior and electrographic activity, and to screen the efficacy of $GABA_AR$ positive allosteric modulators (PAMs) in mitigating seizure phenotypes induced by these chemical convulsants. Our data demonstrate that wildtype zebrafish larvae recapitulate effects reported for TETS and PTX-induced seizures in mammalian models, thereby supporting the use of larval zebrafish as a screening platform for identifying improved countermeasures for chemical threat agents that trigger seizures via $GABA_AR$ antagonism.

2. Materials and methods

2.1. Fish husbandry

Fish husbandry, spawning and all experiments using fish were performed in accordance with protocols approved by the University of California-Davis (UC Davis) Institutional Animal Care and Use Committee. Adult Tropical 5D zebrafish were obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University (Corvallis, OR); adult NHGRI and Tupfel long fin zebrafish, from the Zebrafish International Resource Center (ZIRC, Eugene, OR). The Tropical 5D zebrafish line is well established to be representative of the diversity within laboratory zebrafish lines, and it is ideally suited for examining the influence of genetic diversity on toxicity responses (Balik-Meisner et al., 2018). Specifically, this line of outbred fish is reported to have more single nucleotide polymorphisms (SNPs) than any other laboratory zebrafish line, including the Tupfel long fin and NHGRI line (Balik-Meisner et al., 2018). The NHGRI line, derived from one mating pair of TAB-5 (a Tubingen (TU) and AB cross) is a healthy and fecund inbred fish line that is routinely used for molecular and genetic studies in zebrafish (LaFave et al., 2014). The Tupfel long fin is a fish line that is not extensively used in toxicity studies, but is used in studies of seizures (Baraban et al., 2005) and behavior (Gorissen et al., 2015). There is limited information regarding the diversity of the Tupfel long fin line; however, studies have shown that compared to the AB line, the Tupfel long fin line has lower basal cortisol levels and expression of stress-related genes, and, thus, performs significantly different in a number of behavioral paradigms as 5 day old larvae (van den Bos et al., 2017v) and as adults (Gorissen et al., 2015). Our search of the literature yielded no specific information regarding differences in $GABA_AR$ expression or responses to $GABA_AR$ antagonists between these three strains. Collectively, these three fish lines are representative of the diversity of zebrafish lines routinely used to assess the outcomes of interest in this study.

Following acclimatization, fish from each line were mated, and subsequent generations of all three lines of wildtype zebrafish were raised at UC Davis under standard aquaculture laboratory conditions (Westerfield, 2000b) with 14 h light (~850 lx), 10 h dark cycles (Harper and Lawrence, 2011) in a standalone aquatic flow-through system (Aquaneering, San Diego, CA) at a density of 8 fish per liter. Water used in the system (referred to as “system fish water”) was deionized water that was further purified by reverse osmosis (Reverse Osmosis System Model AAA-1005, Applied Membranes Inc., CA). System fish water was maintained at 28.5 ± 0.5 °C, and supplemented with 20 g/L $NaHCO_3$ to maintain a pH of 7.5 ± 0.3 and 40 g/L sea salt solution (Instant Ocean) to maintain conductivity at 700 ± 100 μS. Adult fish were fed twice daily with commercially available GEMMA Micro 500 (Skretting, Salt Lake City, UT). Embryos obtained by natural group spawning overnight were transferred to petri dishes containing system fish water and kept in a light cycle-controlled (14 h light, 10 h dark) incubator at 28.5 °C until removed for experimental manipulation. During this incubation period, lights were turned on at 10:00 AM daily, and all behavioral and EEG experiments were conducted during the hours between 12:00 and 19:00. Fish were randomly chosen for chemical exposures.

2.2. Chemical exposures

At 24 h post fertilization (hpf), healthy zebrafish embryos were collected and placed 1 fish per well in 96-well polystyrene plates (Falcon, Corning, New York) with 50 μL of embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 150 μM KH_2PO_4, 50 μM

Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$, pH 7.2) (Westerfield, 2000a). In order to minimize any embryo medium evaporation during incubation, the 96-well plates were covered with Parafilm M plastic membrane (Sigma Aldrich, St. Louis, MO). To assess the seizurogenic activity of chemical convulsants, zebrafish larvae at 5 days post fertilization (dpf) were randomly selected for exposure to pentylenetetrazole (PTZ, ≥ 99 % pure; Sigma-Aldridge, St. Louis, MO), picrotoxin (PTX, ≥ 98 % pure, Sigma-Aldridge) or TETS (≥ 97 % pure, synthesized as previously described in Zolkawska et al., 2012). PTZ was directly diluted in EM to prepare 2X stocks, whereas TETS and PTX were initially dissolved in DMSO (Sigma-Aldrich), and then diluted in EM to prepare 2X stocks, 50 µL of which were added directly to 96-well plates containing fish in 50 µL of EM to yield final water concentrations indicated in the figures. The final DMSO concentration in the wells was ≤ 1 %. EM alone or EM containing 1 % DMSO were used as vehicle controls for PTZ or PTX/TETS, respectively. For studies assessing the effects of antiseizure agents, 50 µL of EM containing chemical convulsants at 3x concentration were added to 5 dpf zebrafish larvae in 96 well plates containing 1 fish in 50 µL EM per well. After 20 min, 50 µL of 3x stock of the antiseizure agent was added per well, bringing the final volume up to 150 µL in each well. Diazepam (DZP, ≥ 98 % pure, Sigma-Aldridge), midazolam (MDZ, ≥ 98 % pure, Hospira, Lake Forrest, IL) or allopregnanolone (ALLO, ≥ 99 % pure, custom synthesized by SAFC Pharmaceuticals, Madison, WI) were diluted directly in EM. Addition of these drugs was confirmed to not change the pH of the EM at any of the concentrations tested.

2.3. Quantification of morbidity and mortality

At 24 h post fertilization (hpf), zebrafish embryos were transferred to 96-well plates (1 fish per well) and maintained in EM. At 5 dpf, zebrafish larvae were exposed to varying concentrations of PTZ, PTX or TETS. Larvae were inspected for morphological defects and lethality at 1 and 24 h post-exposure by an investigator blinded to experimental conditions using a dissecting scope (Olympus, SZ61) at 6.7X magnification. Morphological defects were scored using the following criteria: Grade 0 – no apparent morphological abnormalities; Grade 1 – mild defects in gross morphology, with slight axial deformation; Grade 2 – pronounced axial deformation (high curvature) and/or decreased trunk length; Grade 3 – loss of body posture, sinking and death.

2.4. Scoring of seizure behavior

At 5 dpf, larvae were acutely exposed to increasing concentrations of PTZ, PTX or TETS. During the 20 min following addition of chemical convulsant, individual larvae were observed under a dissecting scope (Olympus, SZ61) at 6.7X magnification by an investigator blinded to experimental conditions who scored behavior using a previously published seizure behavior scoring system (Baraban et al., 2005). This system scores seizure-like behavior from stages 0–3 based on the following criteria: 0 – no difference in swim behavior compared to controls; 1 – increased swim behavior/hyperactivity; 2 – at least two episodes of 'whirlpool-like" swimming pattern in which fish swim rapidly in circles; 3 – clonic seizure-like convulsions and loss of posture and/or death (see Table 1).

2.5. Electrophysiological recording

Electrophysiological recordings were performed as previously described (Baraban et al., 2005). Briefly, 5 dpf zebrafish larvae were exposed to 2 µM *D*-tubocurarine (≥ 97 % pure, Sigma-Aldridge) in EM for 10 min and immobilized in 1.5 % (w/v) low melting temperature agarose (Lonza, Basel, Switzerland) in EM. Using a stereomicroscope at 10X magnification, a glass microelectrode (5 MΩ) filled with 2 M KCl in deionized water was placed in the optic tectum. Extracellular electrical activity was recorded using an AM-systems model 3000 extracellular amplifier (AM-systems, Washington). Voltage records were low pass filtered at 1 kHz, high pass filtered at 0.1 Hz, digitized at 5 − 10 kHz using a Digidata 2300 analog-to-digital interface and stored on a PC running pClamp software (Molecular Devices, San Jose, California). To assess the effects of $GABA_AR$ antagonists, 20 min recordings from eight randomly selected individual 5 dpf larvae from ≥ 3 separate spawning events were collected for each experimental condition. To assess the effects of antiseizure agents, baseline tectal field potentials were recorded for 5 min, larvae were exposed to 10 mM PTZ, 0.4 mM PTX or 4 µM TETS for 20 min prior to adding DZP, MDZ or ALLO and recording for an additional 20 min. Epileptiform activity was analyzed using Clampfit software (Molecular Devices) and the frequency of epileptiform discharges (bursts/min) was averaged for the last 10 min of each 20 min epoch. The threshold for an epileptiform discharge was set at three times the background noise (peak amplitude) and > 100 ms duration.

2.6. Automated analyses of locomotor behavior

Locomotor behavior of 5 dpf larvae was quantified using the DanioVision system (Noldus, Leesburg, VA). Zebrafish larvae in 96-well plates (1 fish per well) were allowed to habituate to the chamber for 5 min prior to addition of test chemical(s). Throughout testing, the DanioVision testing chamber was maintained at 28.5 °C to minimize variance in movement due to changes in temperature, and the light level in the chamber was set to 1800 − 2000 lx. Locomotor behavior, quantified as the total distance moved, was recorded using an infrared sensitive camera. These data were analyzed using the EthoVisionXT software (Noldus), and exported to Excel (Microsoft) and Prism (Graphpad 6.01) for statistical analyses.

2.7. Quantification of TETS concentrations by ELISA

DMSO (0.01 % v/v final) or TETS (0.4, 4, or 40 µM final) were spiked into 2 ml EM containing thirty 5 dpf larvae. Following a 20 min incubation period, the exposure water was collected and stored at − 20 °C until analyzed by ELISA. Extraction methods and ELISA were performed as previously described (Barnych et al., 2017; Vasylieva et al., 2017).

2.8. LC/MS analyses to determine DZP, MDZ and ALLO uptake into larval zebrafish

At 24 hpf, embryos were placed into individual wells of 96-well plates containing 50 µL of EM. At 5 dpf, larvae were exposed to DZP, MDZ or ALLO at 3 µM for 60 min. At the end of the exposure, larvae were euthanized by placing the plate into a -20 °C freezer for 10 − 20 min. Following euthanasia, 1 ml of exposure water was transferred to a 1.5 ml glass vial for LC/MS analysis. The remaining exposure water was removed and the larvae were rinsed with EM two times and then placed onto Sylgard with their lateral side facing up. Following a brief drying period (~ 10 min), an incision was made from the liver through the back portion of the hind-brain. Heads, which were examined to ensure no contamination by the yolk sac, and bodies minus the heads were separately pooled, and weighed for downstream analytical analysis. These pooled samples, which weighed on average 3 − 8 mg were suspended in acetonitrile (HPLC, Thermo Fisher Scientific, Waltham, MA, USA) in a 1.5 mL Eppendorf tube. Twelve stainless steel balls of 1.6 mm diameter (Next Advance, Troy, NY) were added and the samples homogenized thoroughly at 1500 rpm for 2 × 30 s using a 2010 Geno/Grinder™ homogenizer (SPEX™ SamplePrep., NJ, USA). The samples were then centrifuged for 5 min at 8,608 x g, the supernatant was collected, evaporated under a constant flow of air using a PIERCE Reacti-Vap™ III evaporator (PIERCE, Il, USA), and reconstituted with 100 µL acetonitrile and used for LC/MS analysis.

Table 1
A comparison of rodent vs. zebrafish seizure scoring systems[a].

Scoring of Rodent Seizure Behavior	Rodent Seizure Stage	Scoring of Zebrafish Seizure Behavior[a]	Zebrafish Seizure Stage
Normal behavior (grooming, walking, etc.)	0	Normal Swimming Behavior	0
Freezing	1	N/A	N/A
Head nodding, isolated twitches, orofacial seizures, hyperlocomotion	2	Hyperlocomotion	1
Clonic seizures	3	Circular swimming (cork screw swimming), rapid movement	2
Tonic seizures	4	Tonic seizure, loss of body posture and sinking	3
Death	5	Death	3

[a] Adapted from Baraban et al. (2005).

To quantify MDZ, a 2.5 mM stock solution of MDZ was prepared by dissolving 3.62 mg midazolam hydrochloride in 4.0 mL acetonitrile. Working standard solutions were obtained by diluting the stock solution with acetonitrile. LC/MS analysis was performed with a Waters Acquity UPLC (Waters, New York NY) equipped with an Acquity UPLC BEH 1.7 µm C-18 2.1 × 50 mm column (Waters), heated to 25 °C, interfaced to a TSQ Quantum Access Max mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, MA, USA). A gradient elution starting with 90 % mobile phase A and 10 % mobile phase B for 1 min was gradually taken to 20 % mobile phase A and 80 % mobile phase B at 2.0 min and was kept at this ratio until 3.0 min. It was then taken back to 90 % mobile phase A and 10 % mobile phase B at 3.10 min. Total run time was 5.0 min at a flow rate of 200 µL/min using 0.1 % formic acid in water as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. The injection volume was 3.0 µL. Under these conditions, MDZ had a retention time of 2.58 min. Using a heated electrospray ionization (HESI-II) source in positive ion mode, capillary temperature 221 °C, vaporizer temperature 400 °C, spray voltage 3500, sheath gas pressure (N_2) 35.0 units, auxiliary gas pressure (N_2) 5.0 units & ion sweep gas pressure (N_2) 2.0 units, MDZ was analyzed by the selective reaction monitoring (SRM) transition of its positively charged quasi-molecular ion 326.08 (M + 1)+ into product ions of 223.05, 249.04 & 291.05 *m/z*. A 9-point calibration curve from 25 nM to 10 µM was used for quantification. All reference and analysis samples were run in triplicate.

To quantify DZP, a 5.0 mM stock solution of DZP was prepared by dissolving 3.20 mg diazepam in 2.248 mL acetonitrile. Using the same instrumentation and liquid chromatography conditions as for MDZ, diazepam eluted at 3.07 min. Using the same HESI-II source in positive ion mode, capillary temperature 221 °C, vaporizer temperature: 400 °C, spray voltage 3000, sheath gas pressure (N_2) 20.0 units, auxiliary gas pressure (N_2) 5.0 units and ion sweep gas pressure (N_2) 2.0 units, DZP was analyzed by the SRM transition of its positively charged quasi-molecular ion 285.08 (M + 1)+ into product ions of 227.99, 193.03 & 154.04 *m/z*. An 8-point calibration curve from 25 nM to 5 µM was used for quantification.

To quantify ALLO, a 5.0 mM stock solution of ALLO was prepared by dissolving 4.10 mg ALLO in 2.575 mL acetonitrile. Using the same instrumentation as described above, a gradient elution starting with 95 % mobile phase A and 5 % mobile phase B for 1 min was gradually taken to 5 % mobile phase A and 95 % mobile phase B at 2.0 min and was kept at this ratio until 4.0 min. It was then taken back to 95 % mobile phase A and 5 % mobile phase B at 4.10 min. Total run time was 6.0 min at a flow rate of 250 µL/min using 0.1 % formic acid in water as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. The injection volume was 8.0 µl. Under these conditions, ALLO had a retention time of 3.08 min. Using the atmospheric pressure chemical ionization (APCI) source in positive ion mode, capillary temperature 290 °C, vaporizer temperature 425 °C, discharge current 4.0, sheath gas pressure (N_2) 15 units and auxiliary gas pressure (N_2) 5 units, ALLO was analyzed by the SRM transition of its positively charged quasi-molecular ion 319.19 (M + 1)+ into product ion of 283.10 *m/z*. A 6-point calibration curve from 100 nM to 5 µM was used for quantification.

2.9. Statistical analysis

Data were analyzed using GraphPad (Version 6.01, Graphpad Software Inc., La Jolla, CA) and SAS software (Version 9.4, SAS Institute, Inc., Cary, NC). A two parameter log-logistic model was used to estimate the dose response curves for each $GABA_AR$ antagonist and each zebrafish line. Latency to a seizure behavior score of 2 and duration of seizures were transformed using the natural logarithm prior to analysis to better meet the underlying assumption of constant variance across groups; latency was shifted by 10 and duration was shifted by 0.1 prior to transformation to handle observed zeroes. Distance moved was square root transformed. These outcomes were then analyzed using a one-way ANOVA and if an overall group difference was detected, pairwise comparisons of experimental groups versus the control were conducted. To determine if there were differences between zebrafish lines, a general linear model was used to analyze data for a given $GABA_AR$ antagonist; these models included terms for concentration, zebrafish line and the interaction. To identify significant differences across these models and the one-way ANOVA for the three $GABA_AR$ antagonists for a given outcome, while accounting for the multiple comparisons, Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) was used; while raw p-values are presented, only those results that survived this correction are shown. For natural logarithm transformed outcomes, the exponentiated coefficients provide percentage differences between groups. Electrophysiological data were analyzed by one-way ANOVA; if an overall group effect was identified, Dunnett's multiple comparison test was used to identify statistically significant differences between experimental groups. To assess the effects of antiseizure agents on locomotor behavior, a mixed effects regression model was fit using three time periods of observation (0 − 20 min, 20 − 40 min and 40 − 60 min) as repeated measures. Terms of interest in these models included time period, the antiseizure agent, and the concentration of the antiseizure agent, as well as corresponding interactions. Specific contrasts were constructed to compare each antiseizure agent at a given concentration and time period and tested using a Wald test. Similar contrasts were constructed to compare different concentrations of an antiseizure agent to control larvae exposed to only the chemical convulsant or to vehicle control larvae exposed to only DMSO. As in earlier models, FDR was used to account for the multiple comparisons being made for locomotor behavior; while raw p-values are presented, only those results that survived this correction are shown. Assumptions of all models were assessed and were met by the data.

3. Results

3.1. $GABA_AR$ antagonists cause concentration- and exposure duration-dependent dysmorphology and lethality

To identify sublethal exposures of $GABA_AR$ antagonists for

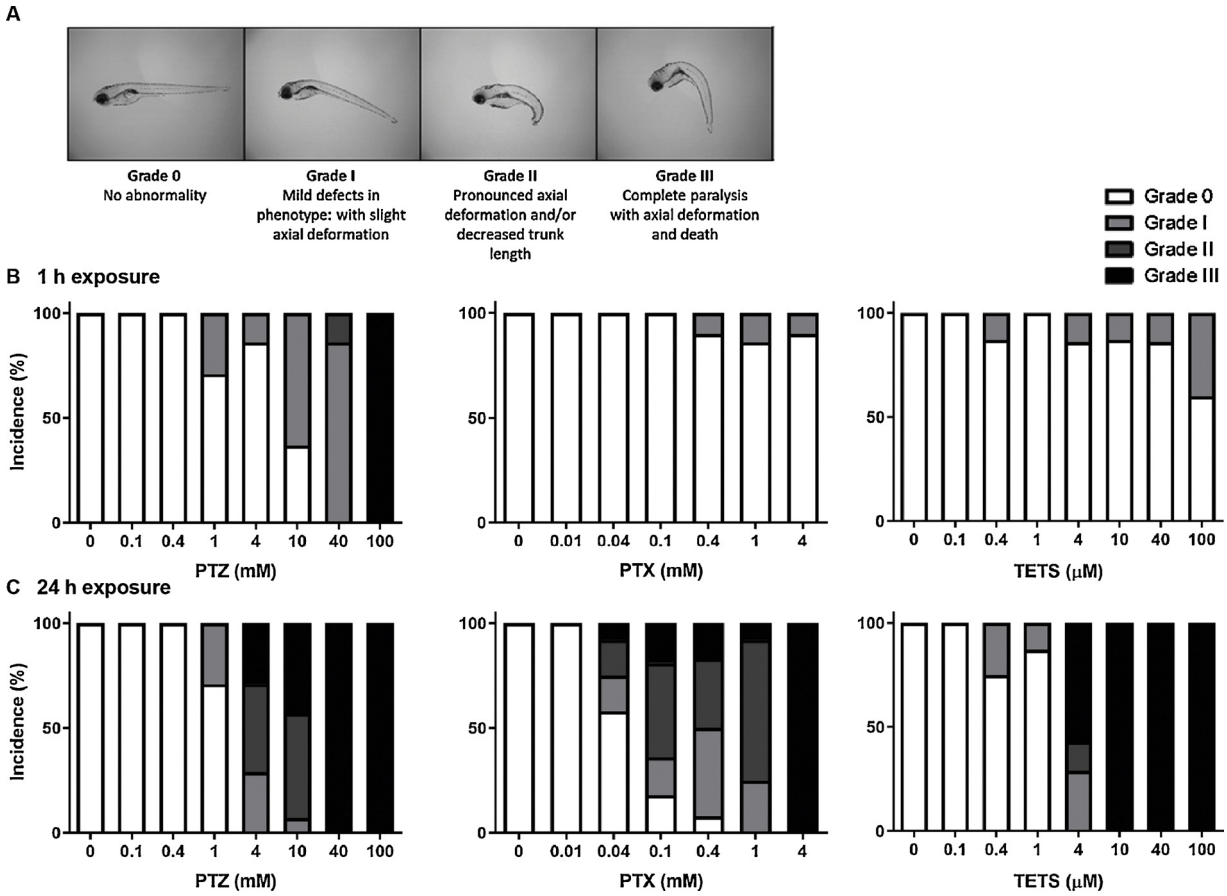


Fig. 1. $GABA_AR$ antagonists cause concentration-dependent morphological defects and lethality. At 5 dpf, Tropical 5D larvae were exposed to convulsant chemicals for 1 or 24 h. At the end of the exposure period, larvae were evaluated for morphological defects and lethality. (A) Representative examples of morphologies evaluated using the following grading system to score morbidity and mortality: Grade 0 - no overt morphological abnormality; Grade 1 - mild defects in gross morphology, with slight axial deformation; Grade 2 - pronounced axial deformation (high curvature) and/or decreased trunk length; Grade 3 – marked axial deformation and/or death. Quantitative analyses of the incidence of morphological defects after 1 h (B) or 24 h (C) continuous exposure to $GABA_AR$ antagonists (n = 24 per group from ≥ 3 independent spawning events).

subsequent studies of seizure behavior, 5 dpf Tropical 5D zebrafish larvae were exposed to vehicle or increasing concentrations of PTZ, PTX and TETS for 1 and 24 h. At the end of these exposure periods, larvae were scored for morphological defects and death (Fig. 1A). No dysmorphologies or lethality were observed in vehicle controls at either time point (Fig. 1). After a 1 h exposure, PTZ elicited the most toxicity, causing Grade I morphological defects that increased in incidence from < 25 % to > 90 % of the exposed population as PTZ concentrations increased from 1 mM to 40 mM (Fig. 1B). At 40 mM PTZ, Grade II morphological defects were observed in ~10 % of the PTZ-exposed larvae; and at 100 mM, PTZ caused 100 % lethality (Fig. 1B). In contrast, only Grade I morphological defects were observed in larvae exposed for 1 h to PTX (0.4–4 mM) or TETS (0.4–100 μM). With the exception of TETS at 100 μM, which caused Grade I morphological defects in ~40 % of exposed fish, the incidence of affected larvae was ≤ 10 % across the range of PTX and TETS concentrations that were tested (Fig. 1B).

Increasing the exposure duration to 24 h increased the incidence and severity of toxicity in response to all three chemical convulsants (Fig. 1C). The lowest concentration that elicited Grade I morphological defects after a 24 h exposure was 1 mM for PTZ, 0.04 mM for PTX, and 0.4 μM for TETS. Grade II morphological defects were observed in zebrafish larvae exposed to PTZ at 4–10 mM, PTX at 0.04–1 mM, or TETS at 4 μM. The lowest concentration that elicited lethality was 4 mM for PTZ, 0.04 mM for PTX and 4 μM for TETS, and 100 % lethality was observed at PTZ concentrations ≥ 40 mM, PTX at 4 mM and TETS at ≥ 10 μM (Fig. 1C). Based on these data, exposures for all subsequent studies of seizure-like phenotypes were limited to a maximum of 1 h, and only larvae with no morphological abnormalities or overt signs of toxicity that also showed normal escape response to touch were used for testing.

3.2. $GABA_AR$ antagonists elicit seizure-like behavior in 5 dpf larvae of 3 different lines of wildtype zebrafish

PTZ has previously been shown to elicit seizure-like phenotypes in zebrafish larvae (Baraban et al., 2005; Baxendale et al., 2012; Cassar et al., 2017; Shao et al., 2018; Yang et al., 2017). Thus, we used PTZ as a positive control for our experiments. Tropical 5D, NHGRI and Tupfel long fin zebrafish were exposed at 5 dpf to varying concentrations of PTZ (0.1–100 mM), PTX (0.01–4 mM) or TETS (0.1–100 μM). Seizure behavior was scored throughout a 20-min exposure period using previously described criteria (Baraban et al., 2005) (also see Table 1). All three $GABA_AR$ antagonists triggered seizure-like behavior in all three zebrafish lines (Fig. 2). For each $GABA_AR$ antagonist, the seizure score increased in a concentration-dependent manner, and the concentration-effect relationship for any given $GABA_AR$ antagonist was similar across all three zebrafish lines. Interestingly, while both PTZ and PTX elicited seizure behavior scores of "3" at the highest concentrations tested, 100 mM and 4 mM, respectively, TETS did not elicit seizure behavior scores of "3" at any of the concentrations tested. Of note, the TETS effect did not increase at concentrations between 1 and 40 μM,

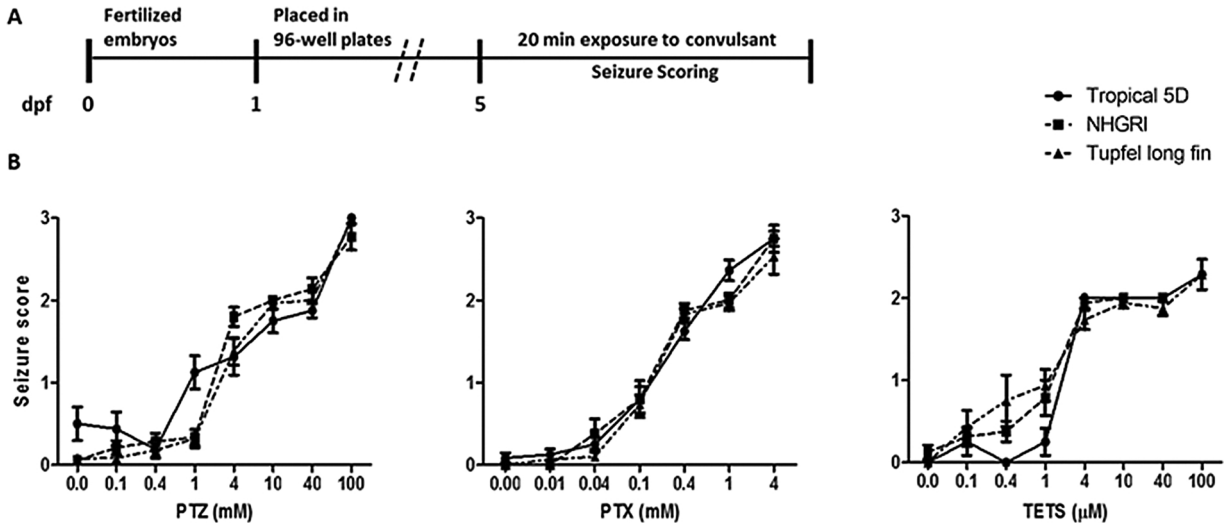
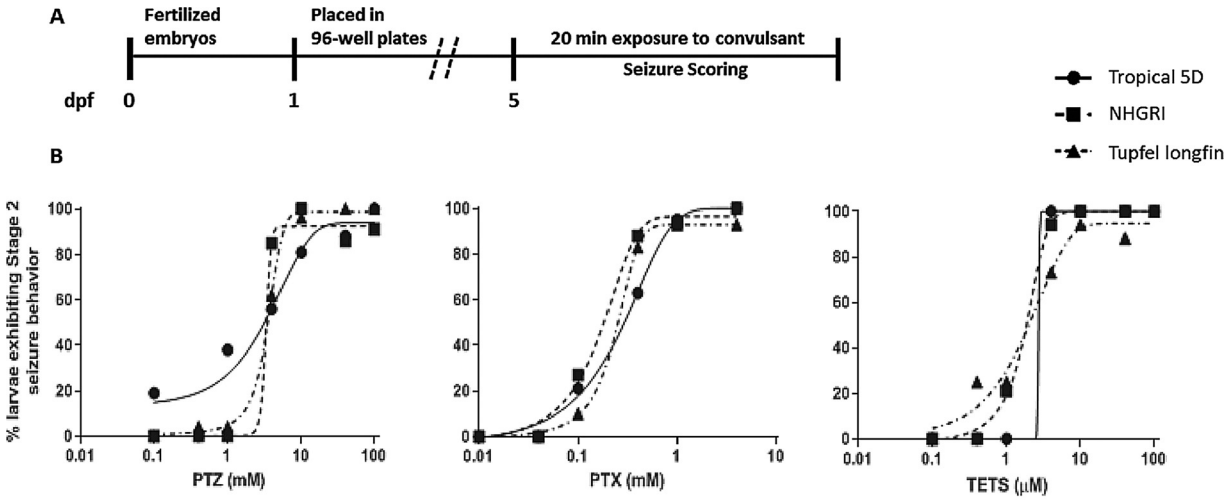


Fig. 2. $GABA_AR$ antagonists trigger seizures across three different lines of wildtype zebrafish larvae. (A) Larvae from three different commonly used lines of wildtype zebrafish (Tropical 5D, NHGRI and Tupfel long fin) were placed in 96-well plates at 24 hpf. At 5 dpf, larvae were exposed to increasing concentrations of PTZ, PTX or TETS for 20 min. Seizure behavior was scored throughout the 20 min exposure period using the scoring system described in Table 1. (B) Seizure scores in zebrafish larvae exposed to PTZ (left panel), PTX (middle panel), and TETS (right panel) reveal similar concentration-response curves between wildtype fish lines for each $GABA_AR$ antagonist tested. Data are presented as mean ± S.D. (n = 24 per group from ≥ 3 independent spawning events).



C

Endpoint	Convulsant	Tropical 5D	NHGRI	Tupfel long-fin
CC_{50}[a]	PTZ (mM)	2.42 (0.96, 3.89)	3.30 (2.02, 4.58)	3.09 (2.23, 3.95)
	PTX (mM)	0.26 (0.18, 0.34)	0.18 (0.10, 0.25)	0.25 (0.18, 0.32)
	TETS (μM)	1.99 (0, 79.58)	1.58 (1.02, 2.14)	1.92 (0.81, 3.04)
CC_{90}[b]	PTZ (mM)	34.71 (0, 72.4)	15.55 (5.98, 25.33)	7.95 (4.83, 11.06)
	PTX (mM)	0.81 (0.42, 1.20)	0.52 (0.21, 0.84)	0.71 (0.42, 1.01)
	TETS (μM)	2.20 (0, 90.76)	3.15 (1.45, 4.85)	15.88 (0.92, 30.84)

a. CC_{50}= Concentration that produces seizure behavior scores of 2 in 50% of exposed larvae.
b. CC_{90}= Concentration that produces seizure behavior scores of 2 in 90% of exposed larvae.

Fig. 3. TETS is a potent chemical convulsant in zebrafish larvae. (A) Tropical 5D, NHGRI and Tupfel long fin zebrafish larvae were placed in 96-well plates at 24 hpf. At 5 dpf, larval zebrafish were exposed to increasing concentrations of PTZ, PTX and TETS for 20 min. (B) Percentage of animals within each zebrafish line that exhibited a seizure score of 2 following exposure to increasing concentrations of each $GABA_AR$ antagonist (n = 16–28 per group from ≥ 3 independent spawning events). (C) CC_{50} and CC_{90}, $GABA_AR$ antagonist concentrations at which 50 % or 90 % of 5 dpf zebrafish exhibit a seizure score of 2. Data presented as the estimated concentration and corresponding 95 % confidence interval.

suggesting that the effect may have plateaued, although there appears to be a further slight increase in the behavioral response at 100 μM, perhaps indicating a biphasic response. However, no experiments were conducted at TETS concentrations > 100 μM due to safety concerns regarding the use of this highly toxic compound.

To compare the concentration-dependency of PTZ vs. PTX vs. TETS in triggering seizure-like behavior in 5 dpf zebrafish larvae, we determined the concentration of each chemical convulsant that produced a seizure behavior score of "2" in 50 % (CC_{50}) and 90 % (CC_{90}) of the exposed larvae (Fig. 3). The CC_{50} and CC_{90} values were similar across the three fish lines for each $GABA_AR$ antagonist. However, significantly lower concentrations of TETS were required compared to PTX and PTZ.

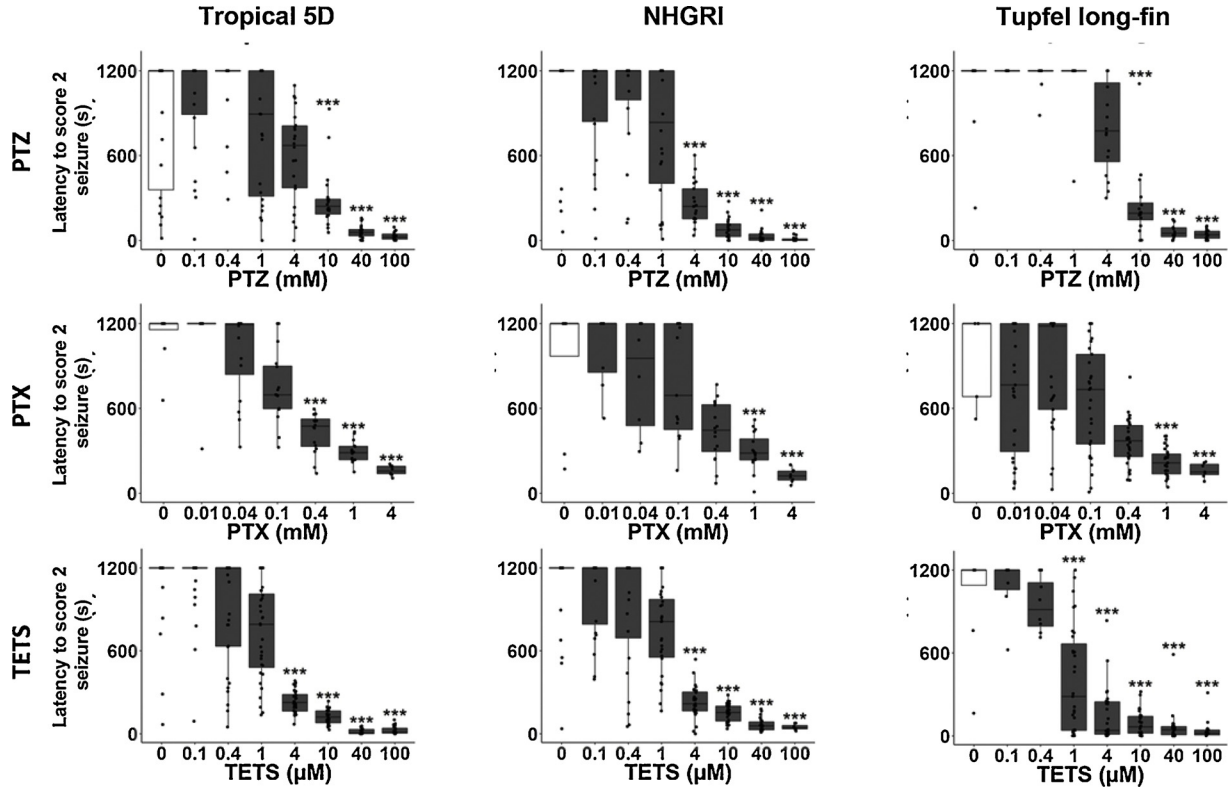


Fig. 4. The latency to a seizure score of 2 decreases with increasing concentrations of $GABA_AR$ antagonists. Latency to the first sign of behavior consistent with a seizure score of 2 that persisted at least 0.2 s was recorded in Tropical 5D, NHGRI and Tupfel long fin zebrafish larvae exposed to varying concentrations of PTZ, PTX or TETS. For all three convulsant chemicals, latency was significantly reduced at higher concentrations. Data are presented as box plots, in which dots represent individual data points; the horizontal line in the box, the median; the ends of the box, the interquartile range (IQR), and the vertical lines extending to the last observation within 1.5 IQR of the ends of the box (n = 5–32 per group from ≥ 3 independent spawning events). Although we emphasize the groups with the largest difference from vehicle control in the figure, a few other differences remained significant after FDR correction. Tropical 5D larvae exposed to 0.1 mM PTX ($p = 0.006$) or 1 μM TETS ($p = 0.02$) had lower latency than vehicle controls as did NHGRI larvae exposed to 0.4 mM PTX ($p = 0.009$) or 1 μM TETS ($p = 0.01$) and Tupfel long fin larvae exposed to 0.4 mM PTX ($p = 0.007$). ***Significantly different from vehicle control (C, 0 μM) at $p < 0.001$.

In 5 dpf Tropical 5D, NHGRI and Tupfel long fin larvae, the latency to the first sign of seizure behavior scored as "2" decreased significantly with increasing concentrations of PTZ, [Tropical 5D: $F(7, 168) = 46.95$, $p < 0.001$; NHGRI: $F(7, 163) = 85.00$, $p < 0.001$; Tupfel long fin: $F(7, 113) = 73.83$, $p < 0.001$], PTX [Tropical 5D: $F(6, 81) = 43.64$, $p < 0.001$; NHGRI: $F(6, 72) = 13.06$, $p < 0.001$; Tupfel long fin: $F(6, 153) = 10.55$, $p < 0.001$] or TETS [Tropical 5D: $F(7, 221) = 187.55$, $p < 0.001$; NHGRI: $F(7, 197) = 85.97$, $p < 0.001$; Tupfel long fin: $F(7, 157) = 21.23$, $p < 0.001$] (Fig. 4). The concentration-effect relationship pattern for any given chemical convulsant was generally consistent across the three wildtype lines, although some significant differences in susceptibility were observed between lines exposed to PTZ [$F(14, 444) = 2.75$, $p < 0.001$] and TETS [$F(14, 575) = 6.18$, $p < 0.001$]. The ratio of latency in vehicle control relative to NHGRI larvae exposed to PTZ at 4 mM was 2.6 times that in the Tupfel long fin larvae [$\exp(\beta) = 2.6$, 95 % CI = (1.2, 5.7), $p = 0.01$], while the ratio of latency in vehicle control relative to Tropical 5D larvae exposed to 10 mM PTZ was 60 % lower than in Tupfel long fin [$\exp(\beta) = 0.4$, 95 % CI = (0.2, 0.8), $p = 0.01$]. The ratio of the latency in vehicle control relative to larvae exposed to TETS at 1 or 4 μM, was smaller for Tropical 5D [1 μM: $\exp(\beta) = 0.3$, 95 % CI = (0.1, 0.6), $p = 0.002$; 4 μM: $\exp(\beta) = 0.3$, 95 % CI = (0.2, 0.7), $p = 0.004$] and for NHGRI [1 μM: $\exp(\beta) = 0.3$, 95 % CI = (0.1, 0.6), $p = 0.002$; 4 μM: $\exp(\beta) = 0.4$, 95 % CI = (0.2, 0.9), $p = 0.02$] than for Tupfel long fin.

The amount of time that 5 dpf zebrafish larvae exhibited seizure behavior scored as "2" (e.g., the duration of stage 2 seizures) generally increased as concentrations of the $GABA_AR$ antagonists increased [PTX: Tropical 5D: $F(6, 81) = 133.36$, $p < 0.001$; NHGRI: $F(6, 70) = 78.92$, $p < 0.001$; Tupfel long fin: $F(6, 269) = 237.12$, $p < 0.001$; PTZ: Tropical 5D: $F(7, 174) = 155.83$, $p < 0.001$; NHGRI: $F(7, 167) = 172.21$, $p < 0.001$; Tupfel long fin: $F(7, 113) = 127.47$, $p < 0.001$; TETS: Tropical 5D: $F(7, 221) = 347.73$, $p < 0.001$; NHGRI: $F(7, 198) = 320.67$, $p < 0.001$; Tupfel long fin: $F(7, 161) = 32.5$, $p < 0.001$] (Fig. 5). The concentration-effect relationship pattern for any given $GABA_AR$ antagonist was consistently non-monotonic across all three zebrafish lines; however, some significant differences between the lines were observed [PTZ: $F(14, 454) = 9.83$, $p < 0.001$; PTX: $F(12, 420) = 2.7$, $p = 0.002$; TETS: $F(14,580) = 7.51$, $p < 0.001$]. Amongst PTZ-exposed larvae, at 4 mM, the ratio of duration relative to vehicle control was almost 10 times greater for NHGRI than for Tupfel long fin [$\exp(\beta) = 9.9$, 95 % CI = (4.4, 22.3), $p < 0.001$]; however, the ratio was smaller for 100 mM relative to vehicle control for Tropical 5D [$\exp(\beta) = 0.4$, 95 % CI = (0.2, 0.9), $p = 0.02$] and NHGRI [$\exp(\beta) = 0.3$, 95 % CI = (0.1, 0.6), $p = 0.001$] compared to Tupfel long fin. For larvae exposed to PTX at 0.1 mM, the ratio relative to vehicle control was 4 times larger for Tropical 5D than for Tupfel long fin [$\exp(\beta) = 4.2$, 95 % CI = (1.5, 11.5), $p = 0.006$]. Amongst TETS-exposed larvae, the ratio of duration at 1 μM relative to vehicle controls was smaller for Tropical 5D than for Tupfel long fin [$\exp(\beta) = 0.2$, 95 % CI = (0.1, 0.5), $p < 0.001$], whereas at 10 μM TETS, the ratio was greater for Tropical 5D [$\exp(\beta) = 2.9$, 95 % CI = (1.2, 7.3), $p = 0.02$] and NHGRI [$\exp(\beta) = 3.1$, 95 % CI = (1.3, 7.7), $p = 0.01$] than for Tupfel long fin; the ratio was also greater for larvae exposed to 40 μM TETS for Tropical 5D compared to Tupfel long fin [$\exp(\beta) = 3.6$, 95 % CI = (1.4, 9.3), $p = 0.007$].

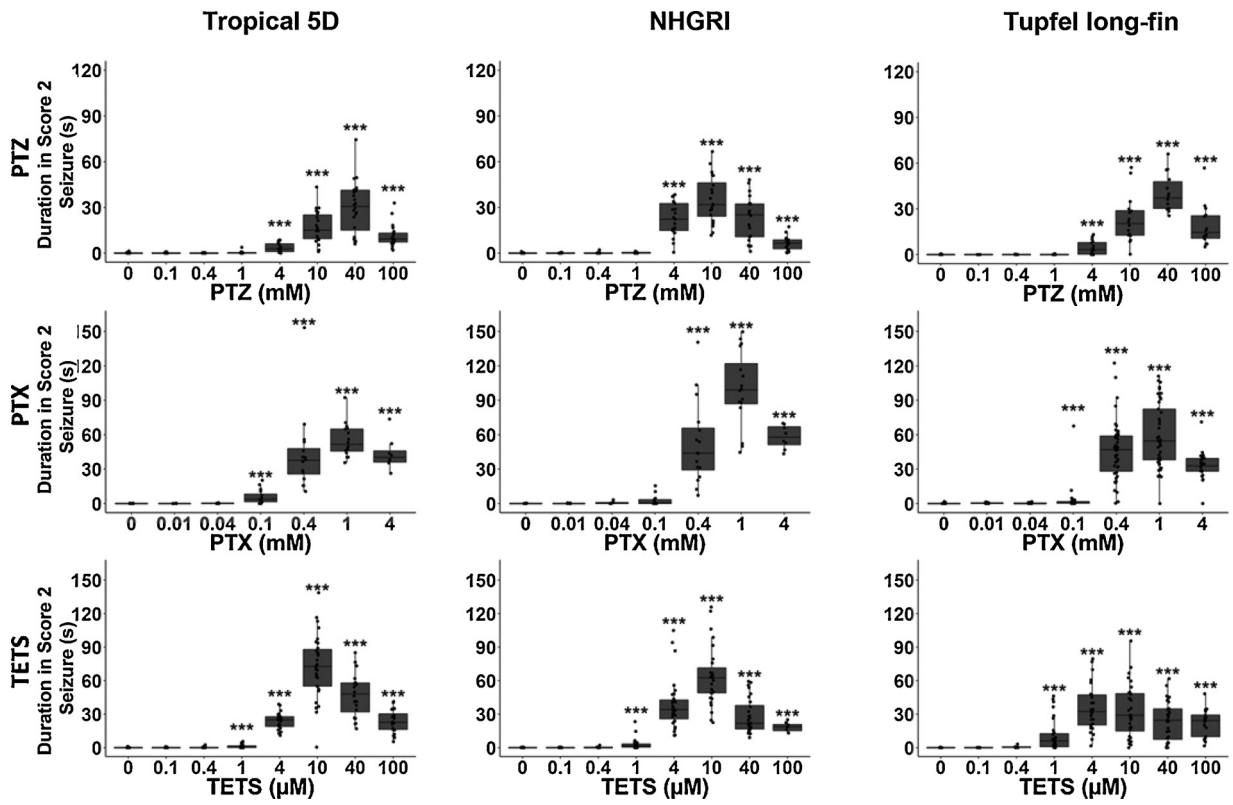


Fig. 5. The duration of GABA$_A$R antagonist-induced seizure behavior scored as 2 exhibits a non-monotonic concentration-response relationship. Total time that fish exhibited behavior consistent with a seizure score of 2 during 20 min of exposure to varying concentrations of PTZ, PTX or TETS was recorded for 5 dpf Tropical 5D, NHGRI and Tupfel long fin zebrafish. Data are presented as box plots, in which dots represent individual data points; the horizontal line in the box, the median; the ends of the box, the interquartile range, and the vertical lines extending to the last observation within 1.5 IQR of the ends of the box (n = 6–47 per group from ≥ 3 independent spawning events). Although we emphasize the groups with the largest difference from vehicle control in the figure, a few other differences remained significant after FDR correction, all for the NHGRI larvae. In particular, the NHGRI larvae exposed to 1 mM of PTZ (p = 0.007), 0.1 mM of PTX (p = 0.002), or 0.4 μM TETS (p = 0.01) had longer seizures than vehicle control. ***Significantly different from vehicle control (0 μM) at $p < 0.001$.

3.3. GABA$_A$R antagonists evoke epileptiform-like electrographic activity in 5 dpf zebrafish larvae

To determine whether concentrations of PTZ, PTX and TETS that elicited seizure-like behavior in zebrafish larvae also triggered seizure-like electrographic activity in their brain, we recorded extracellular field potentials in the optic tectum of 5 dpf Tropical 5D zebrafish larvae. Larvae were immobilized with the neuromuscular junction blocking agent *D*-tubocurarine, embedded in agar and superfused with chemical convulsants (Fig. 6A). Epileptiform activity was not observed in vehicle control larvae or during baseline recordings prior to the addition of chemical convulsant. However, an epileptiform-like discharge, evident as a significant increase in burst frequency (Fig. 6C), was consistently observed during the 20 min following exposure to PTZ, PTX or TETS (Fig. 6B). Epileptiform events following exposure to these GABA$_A$R antagonists were initially brief and occurred sporadically but became longer in duration and more uniform with time. Interestingly, each GABA$_A$R antagonist triggered a unique pattern of electrographic activity (Fig. 6B).

We next determined whether post-exposure administration of GABA$_A$R PAMs attenuated the aberrant electrographic activity. Specifically, we tested GABA$_A$R PAMs shown clinically or preclinically to mitigate seizures, specifically, the benzodiazepines, DZP and MDZ, and a neurosteroid, ALLO (Figueiredo et al., 2018; Joshi and Kapur, 2019; Laukova et al., 2019b; Meldrum and Rogawski, 2007; Vaitkevicius et al., 2017; Zolkowska et al., 2018). DZP, MDZ and ALLO concentrations were chosen based on the lowest concentrations that most consistently attenuated seizure behaviors in the GABA$_A$R seizure model (described in detail below). Analysis of variance confirmed a significant effect of GABA$_A$R PAMs on GABA$_A$R antagonist-induced burst frequency [F (3, 10) = 6.213, p = 0.01]. Post hoc analysis confirmed that DZP and MDZ, but not ALLO, significantly ($p \leq 0.05$) attenuated PTZ- and TETS-induced electrographic seizure activity; however, none of the GABA$_A$R PAMs had a significant effect on PTX-induced seizure activity (Fig. 7). Importantly, none of the GABA$_A$R PAMs completely abolished epileptic discharges induced by PTZ or TETS (Fig. 7).

3.4. Automated analyses of locomotor activity in larval zebrafish acutely exposed to GABA$_A$R antagonists

The assays used to this point to assess the effects of PTX and TETS on seizure phenotypes in larval zebrafish are time-consuming and not feasible for screening large numbers of compounds for seizure effects. Thus, we next assessed an automated version of the PTZ-induced locomotor behavior assays in which distance moved is a validated proxy for seizure severity (Afrikanova et al., 2013; Baraban et al., 2005; Koseki et al., 2014). Exposure to PTZ [Tropical 5D: F (7, 111) = 27.25, $p < 0.001$; NHGRI: F (7, 165) = 96.27, $p < 0.001$; Tupfel long fin: F (7, 110) = 59.8, $p < 0.001$], PTX [Tropical 5D: F (6, 79) = 99.49, $p < 0.001$; NHGRI: F (6, 70) = 90.03, $p < 0.001$; Tupfel long fin: F (6, 266) = 202.22, $p < 0.001$] or TETS [Tropical 5D: F (7, 116) = 77.72, $p < 0.001$; NHGRI: F (7, 198) = 243.25, $p < 0.001$; Tupfel long fin: F (7, 91) = 72.28, $p < 0.001$] significantly increased distance moved in 5 dpf larvae (Fig. 8). Generally, a non-monotonic concentration-dependent effect on locomotor activity was observed for all three GABA$_A$R antagonists. For any given chemical convulsant, the concentration-effect relationship was generally similar across the three wildtype zebrafish lines, although a few statistically significant

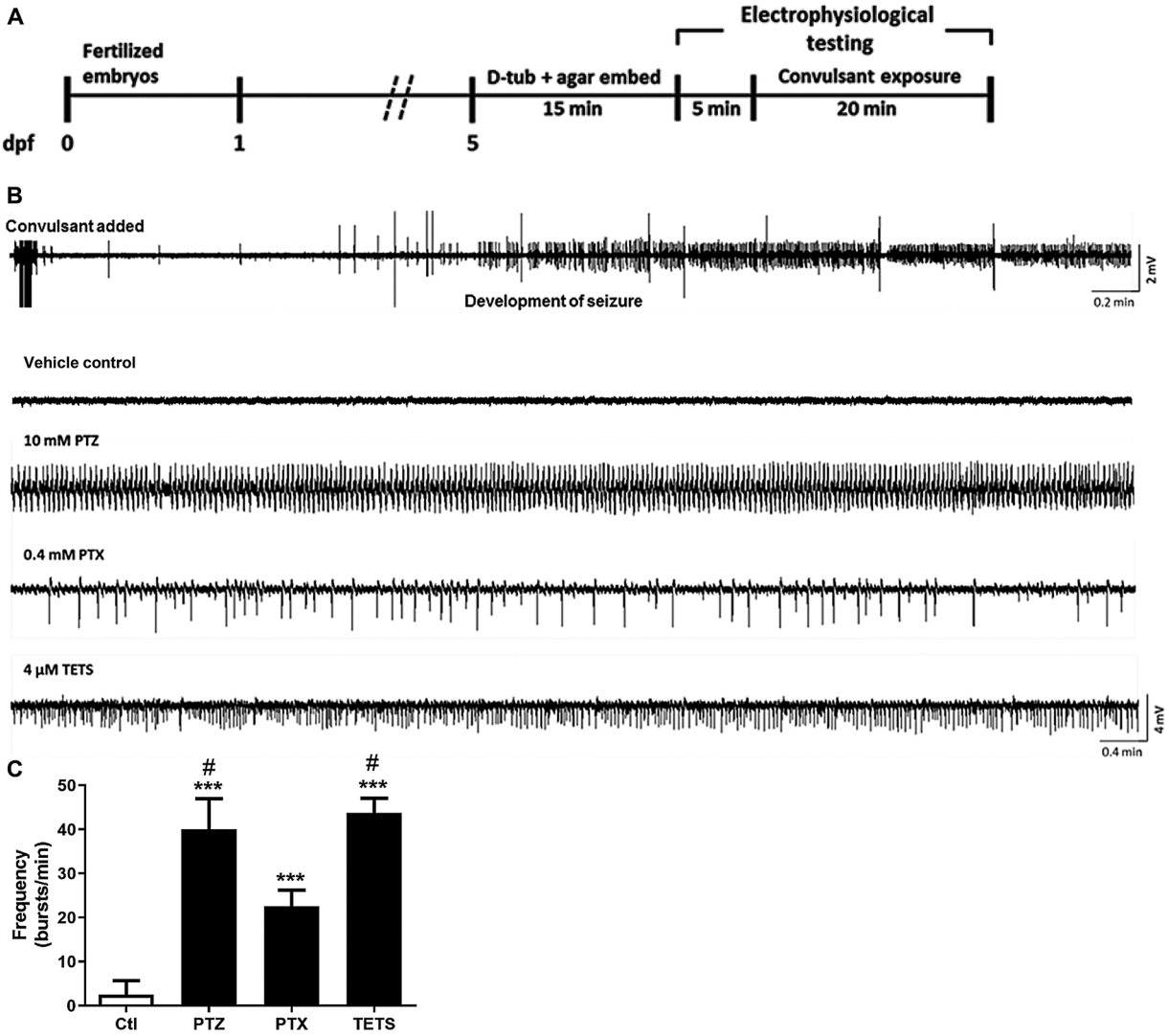


Fig. 6. $GABA_AR$ antagonists evoke epileptiform-like electrographic activity in larval zebrafish. (A) For electrophysiological assessments, 5 dpf Tropical 5D zebrafish larvae were individually exposed to D-tubocurarine (D-tub) for 10 min then embedded in 1.5 % agar. Field recordings from the forebrain were collected for 20 min in the presence of $GABA_AR$ antagonists. (B) Representative tectal field recordings from vehicle control zebrafish larvae (no seizure development) and from zebrafish larvae exposed to a chemical convulsant. (C) Burst frequency was averaged for the last 10 min of recording (n = 5–8 per group from ≥ 3 independent spawning events). Data are presented as mean ± SD. ***Significantly different from vehicle control (0 μM) at $p < 0.001$. #Significantly different from PTX (0.4 NM) at $p < 0.001$.

differences between line were identified [PTZ: $F(14, 386) = 8.34$, $p < 0.001$; PTX: $F(12, 415) = 2.62$, $p = 0.002$]. In larvae exposed to PTZ at 1 [β = 13.8, 95 % CI = (4.6, 23.0), $p = 0.003$], 4 [β = 27.7, 95 % CI = (18.4, 37.0), $p < 0.001$] or 10 [β = 17.1, 95 % CI = (7.8, 26.4), $p < 0.001$] mM, the difference compared to vehicle controls was larger for NHGRI than for Tupfel long fin. However, at 100 mM, the difference compared to vehicle controls was less for Tropical 5D relative to Tupfel long fin [β = -11.2, 95 % CI = (-21.1, -1.3), $p = 0.03$]. In larvae exposed to PTX at 0.1 mM, the difference compared to vehicle controls was larger for Tropical 5D than for Tupfel long fin [β = 13.3, 95 % CI = (4.0, 22.6), $p = 0.005$]; at 1 mM, the difference from vehicle controls was greater for NHGRI than for Tupfel long fin [β = 11.1, 95 % CI = (1.4, 20.8), $p = 0.03$], while at 4 mM, the difference from vehicle controls was larger for NHGRI than for Tupfel long fin [β = 12.5, 95 % CI = (1.5, 23.5), $p = 0.03$]. In larvae exposed to TETS, there were no significant differences between lines.

We next determined whether $GABA_AR$ PAMs attenuated the effects of $GABA_AR$ antagonists on locomotor activity in Tropical 5D larvae. For these studies, we used the lowest concentration of each chemical convulsant that significantly ($p < 0.001$) increased distance moved in the locomotor activity assay (10 mM PTZ, 0.4 mM PTX and 4 μM TETS). $GABA_AR$ PAMs were added 20 min later, and distance moved was measured for 60 min. Post-exposure administration of MDZ, DZP or ALLO at a variety of concentrations significantly reduced the locomotor activity ($p < 0.001$) induced by exposure to PTZ, PTX or TETS (Fig. 9); many of these reductions brought locomotor activity to levels similar to or lower than that of vehicle control larvae exposed only to DMSO ($p < 0.001$). This was especially true for ALLO, suggesting that the anticonvulsant effects of ALLO may be mediated in part by sedative effects. In larvae exposed to PTZ or TETS, ALLO caused a greater decrease in distance moved at any of the three time periods of observation than did either DZP or MDZ at all concentrations tested ($p < 0.001$) but the lowest (0.1 μM). There was not a consistent difference in the effect of ALLO vs. DZP or MDZ at a concentration of 3 μM in the TETS-exposed larvae at the three time periods; ALLO caused a greater decrease in distance moved in the first period compared to DZP ($p < 0.001$) and compared to DZP ($p = 0.002$) or MDZ ($p = 0.02$) in the third period (Fig. 9). In PTX-exposed larvae, ALLO treatment was also more effective in reducing distance moved relative to DZP and MDZ treatment at all concentrations ($p < 0.001$) at all three time

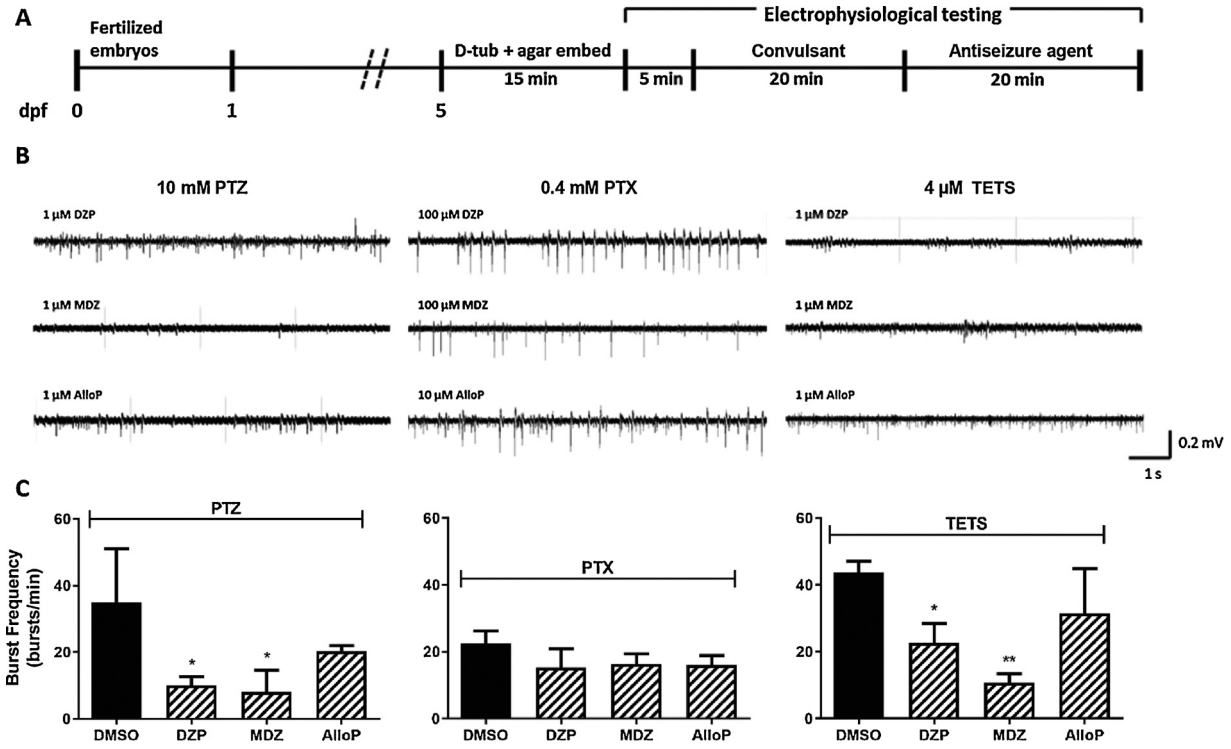


Fig. 7. Post-exposure exposure to benzodiazepines attenuates PTX and TETS, but not PTX-induced epileptic activity in vivo. (A) For electrophysiological assessments, 5 dpf Tropical 5D zebrafish larvae were exposed to D-tubocurarine (D-tub) and then embedded in 1.5 % low melting agarose. After recording baseline tectal field potentials for 5 min, larvae were exposed to 10 mM PTZ, 0.4 mM PTX or 4 μM TETS for 20 min prior to adding diazepam (DZP), midazolam (MDZ) or allopregnanolone (ALLO) and recording for an additional 20 min. (B) Representative electrophysiological tracings after application of antiseizure agents (1 μM DZP, MDZ and ALLO for larvae exposed to PTZ or TETS; 100 μM DZP/MDZ pr 10 μM ALLO for larvae exposed to PTX). (C) Burst frequency was averaged for the last 10 min of each 20 min recording period. *Significantly different from $GABA_AR$ antagonist-induced electrographic activity in the absence of antiseizure agent (black bars) at $p < 0.05$; **$p < 0.01$. Data are presented as mean ± S.D. (n = 3–4 per experimental condition from ≥ 3 independent spawning events).

periods (Fig. 9).

3.5. Disposition of TETS and $GABA_AR$ PAMs in the larval zebrafish model

While there are previous reports of PTZ and PTX effects on locomotor behavior in larval zebrafish (Baraban et al., 2005; Yang et al., 2017), to our knowledge, this is the first report of the effects of TETS on larval zebrafish. Therefore, we quantified TETS in fish water using a TETS ELISA (Vasylieva et al., 2017). These analyses indicated that at 20 min after addition of TETS, the actual concentrations of TETS in the water were 1.5 %, 80 % and 64 % of the nominal water concentrations of 0.4, 4, and 40 μM, respectively (Table 2), which verifies that TETS persists in exposure water throughout the 20 min incubation period, but presumably precipitates at the higher concentrations.

To determine whether significant differences in biological uptake contributed to differences in the antiseizure efficacy of $GABA_AR$ PAMs, we analyzed the water and total tissue concentrations of MDZ, DZP and ALLO by LC/MS in larval zebrafish heads and bodies (Table 3). Water concentrations of DZP, MDZ, and ALLO 60 min after addition of the $GABA_AR$ PAMs at 3 μM were 104 %, 110 % and 104 %, respectively. In two independently generated samples of larvae, we found that concentrations of MDZ, DZP and ALLO were ~2.5X, 1.5X and 1.4X higher in the heads than the bodies, respectively. Head concentrations of MDZ were ~1.7-fold higher than head concentrations of DZP, and in keeping with its higher lipophilicity, head concentrations of ALLO were 3.4X and 8.9X greater than head concentrations of MDZ and DZP, respectively.

4. Discussion

PTZ (Afrikanova et al., 2013; Baraban et al., 2005; Zdebik et al., 2013) and PTX (Cassar et al., 2017; Winter et al., 2008; Wong et al., 2010) have previously been demonstrated to elicit seizure-like behavior and EEG activity in larval zebrafish. Here, we confirm and extend these observations to demonstrate that the chemical threat agent TETS similarly induces seizure phenotypes in three, routinely used lines of zebrafish. Our major findings are: (1) acute exposure to PTZ, PTX, or TETS triggers seizure-like behavior and epileptiform-like electrographic activity in 5 dpf zebrafish larvae; (2) relative to PTZ and PTX, significantly less TETS is required to trigger seizure behavior; (3) post-exposure administration of $GABA_AR$ PAMs transiently mitigates seizure behavior and attenuates, but does not completely normalize, aberrant electrical activity; and (4) Tropical 5D, NHGRI, and Tupfel long fin larvae respond similarly to each $GABA_AR$ antagonist and $GABA_AR$ PAM.

While all three $GABA_AR$ antagonists induce seizure-like phenotypes in 5 dpf zebrafish, TETS triggered both seizure behavior and electrographic activity at significantly lower waterborne concentrations than either PTZ or PTX (Figs. 6 and 8). Absent tissue levels of these anticonvulsant chemicals in larval zebrafish, we cannot make any definitive statements regarding relative potencies, but these findings are consistent with observations of rodent models, in which TETS is 30- to 100-fold more potent as a convulsant than PTX following systemic administration (Duka et al., 1979; Lamanna and Hart, 1968; Shandra et al., 1996; Vogel, 2008; Zolkowska et al., 2012). The basis for the high in vivo seizurogenic potency of TETS is not understood. TETS is markedly more potent than PTZ, but not substantially more potent than PTX, in displacing the $GABA_AR$ ligand [^{35}S]t-butylbicyclophosphorothionate in rat brain membranes (Cole and Casida, 1986; Esser et al., 1991; Ratra et al., 2001; Squires et al., 1984). TETS and picrotoxin are similarly potent in inhibiting $GABA_AR$-mediated GABA-activated chloride ion uptake by membrane vesicles from rat cerebral cortex (Obata et al., 1988). These observations suggest that increased potency as a $GABA_AR$

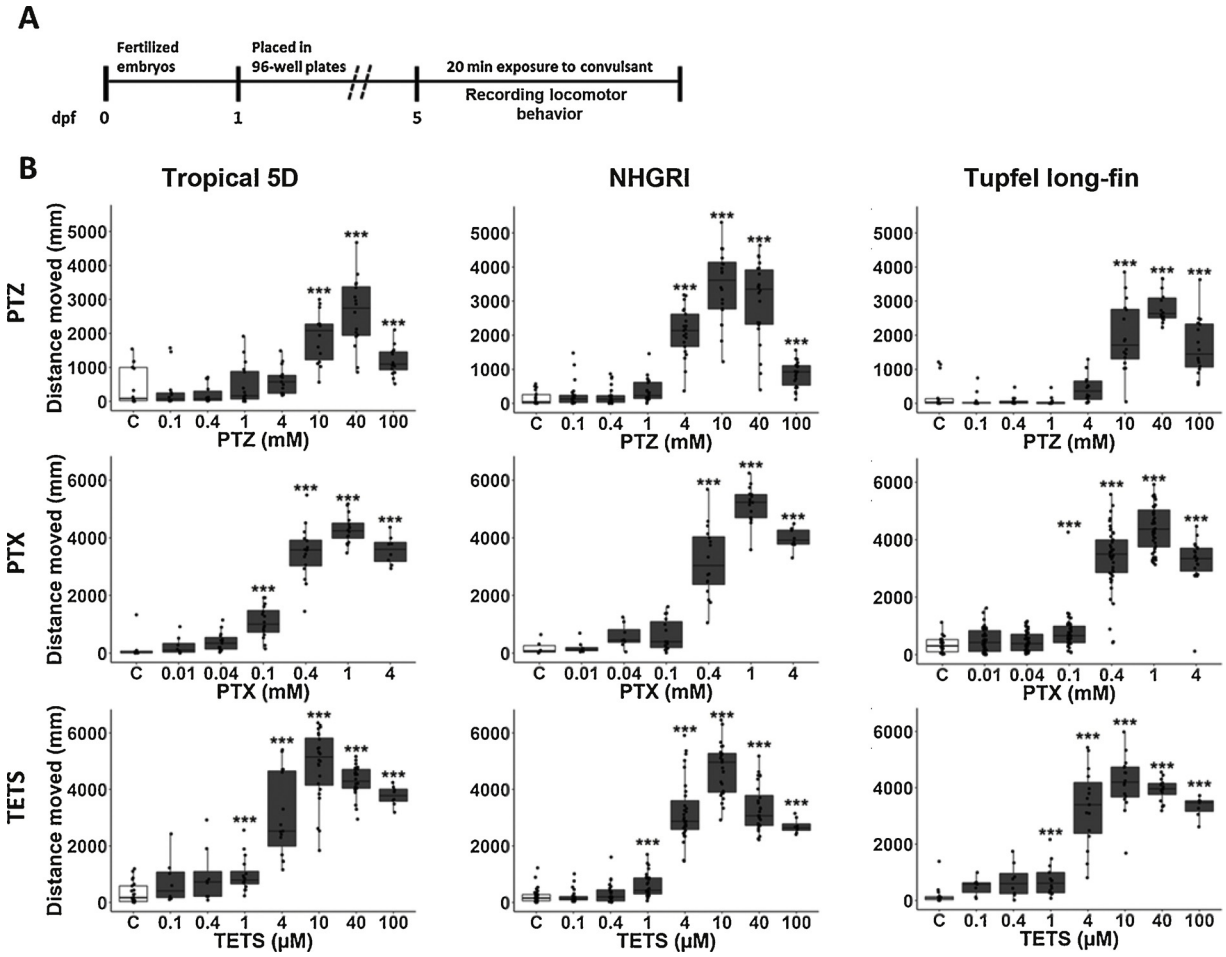


Fig. 8. Chemical convulsant-induced locomotor activity exhibits a non-monotonic concentration-effect relationship. (A) For automated assessments of locomotor behavior, Tropical 5D, NHGRI and Tupfel long fin zebrafish larvae were placed in 96-well plates at 24 hpf. At 5 dpf, larval zebrafish were exposed to varying concentrations of PTZ, PTX or TETS and locomotor behavior recorded for 20 min. (B) Locomotor behavior was quantified as the total distance moved over the 20 min recording period. Data are presented as box plots, in which dots represent individual data points; the horizontal line in the box, the median; the ends of the box, the interquartile range (IQR), and the vertical lines extending to the last observation within 1.5 IQR of the ends of the box (n = 6–47 per group from ≥ 3 independent spawning events). Although we emphasize the groups with the largest difference from vehicle control in the figure, a few other differences remained significant after FDR correction. In particular, Tropical 5D larvae exposed to 0.04 mM PTX (p = 0.02) or 0.4 μM TETS (p = 0.005) had more locomotor activity than vehicle controls as did NHGRI larvae exposed to 1 mM PTZ (p = 0.006) or 0.1 mM PTX (p = 0.01) and Tupfel long fin larvae exposed to 0.1 (p = 0.02) or 0.4 (p = 0.002) μM TETS. ***Significantly different from vehicle control (0 μM) at $p < 0.001$.

antagonist may contribute to the higher seizurogenic potency of TETS relative to PTZ, but not PTX. Other explanations include differential toxicokinetics, a possibility supported by the observation that TETS and PTX exhibit similar seizurogenic potency in rodents when administered intraventricularly (Zolkowska et al., 2012). These observations have prompted the hypothesis that TETS is concentrated in the brain via a transport mechanism (Zolkowska et al., 2012). However, testing this hypothesis is technically challenging because current methodologies for quantifying TETS are not sensitive enough to accurately measure TETS levels in small tissue samples of less than 10 mg (Vasylieva et al., 2017). Because of this limitation, we were unable to quantify TETS levels in zebrafish tissues in this study (data not shown).

Another distinctive feature of TETS vs. PTZ or PTX-induced seizures is the pattern of electrical discharges observed in the optic tectum of acutely intoxicated 5 dpf zebrafish (Fig. 6). The epileptiform-like extracellular field recordings we observed in zebrafish larvae exposed to PTX were similar to those of PTZ-exposed zebrafish (Afrikanova et al., 2013; Baraban et al., 2005). TETS also triggered seizure-like electrical activity in the optic tectum, but while the frequency of discharge between TETS and PTZ was similar, the pattern of high frequency electrical discharges triggered by TETS was distinctive from those generated by acute PTZ or PTX exposure. Like PTZ (Afrikanova et al., 2013; Baraban et al., 2005), PTX and TETS are thought to generate epileptiform activity via $GABA_AR$ blockade (Laukova et al., 2019b; Obata et al., 1988; Ratra et al., 2001; Zolkowska et al., 2012). However, in a recent study comparing the potency of TETS vs. PTX on synaptic vs. extrasynaptic $GABA_AR$ using whole-cell patch-clamp electrophysiology, we discovered that TETS and PTX exhibit significantly different $GABA_AR$ subunit selectivity (Pressly et al., 2018), which perhaps explains the generation of unique patterns of electrographic activity by these two chemical convulsants.

There were several notable similarities between PTZ, PTX and TETS-induced seizures: all exhibited a classic concentration-dependent effect on the latency to seizure behavior (Fig. 4), which decreased with increasing concentrations, but a non-monotonic (inverted “U”-shaped) concentration-dependent effect on the duration of time larvae spent exhibiting seizure behavior scored as “2”, which is characterized by rapid movement and circular or corkscrew swimming (Fig. 5). While the biological basis for the non-monotonic concentration-effect relationship is unknown, two observations suggest that it is not likely due to concentration-dependent lethality. First, a 20 min exposure to TETS at any concentration did not elicit seizure behavior scores of “3”,

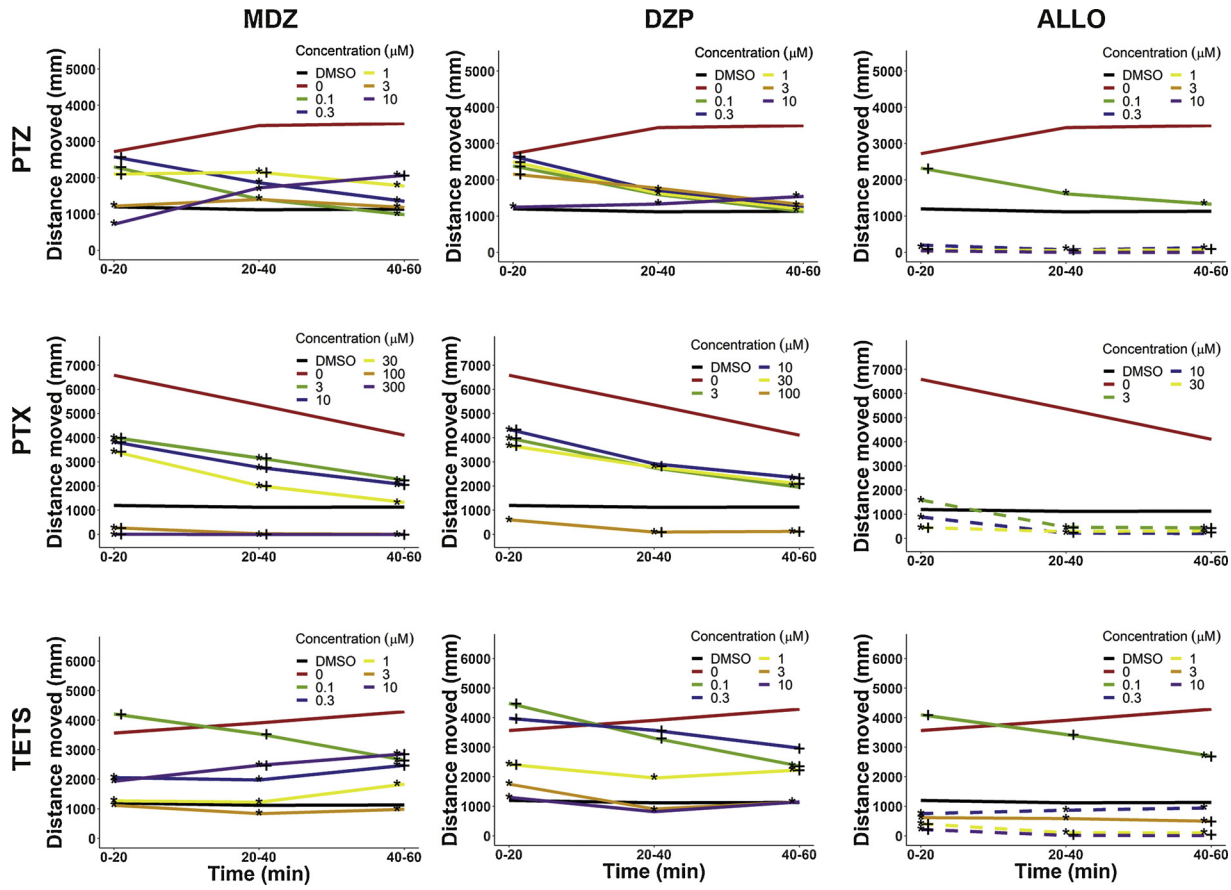


Fig. 9. Allopregnanolone suppresses $GABA_AR$ antagonist-induced behavioral seizures for up to 1 h. (A) For automated assessments of locomotor behavior, Tropical 5D zebrafish larvae were placed in 96-well plates at 24 hpf. At 5 dpf, larval zebrafish were exposed to 10 mM PTZ, 0.4 mM PTX or 4 μM TETS for 20 min prior to adding midazolam (MDZ), diazepam (DZP) or allopregnanolone (ALLO) and recording locomotor activity for an additional 60 min. (B) Locomotor behavior was quantified as the distance moved during the first, second and third 20 min blocks of the 60 min exposure to antiseizure agents. The black line in each graph indicates distance moved by fish exposed to vehicle (DMSO) in the absence of any chemical convulsant, while the red line indicates distance moved by fish exposed only to the chemical convulsant. Dashed lines in the ALLO plots indicate ALLO concentrations that are significantly different from the corresponding concentration in the other treatments. MDZ, DZP and ALLO significantly reduced the locomotor activity induced by exposure to PTZ, PTX, and TETS at most concentrations across the time period of observation ($p < 0.001$); most reductions resulted in levels similar to or lower than that of vehicle controls (DMSO), particularly for ALLO. The figure emphasizes the largest differences, relative to vehicle controls, but additional comparisons survived the FDR correction for multiple comparisons including larvae exposed to PTZ treated with 0.3 μM (period 2) and 10 μM (periods 1 and 2) of MDZ ($p < 0.01$), treated with DZP at all concentrations other than 10 μM during period 2 ($p < 0.02$), and treated with 0.1 μM of ALLO in the second period ($p = 0.005$); there were also differences in TETS exposed larvae treated with 0.3 μM (periods 1 and 2) or 3 μM (period 2) MDZ ($p < 0.03$), 1 or 10 μM (period 2) DZP ($p < 0.01$) or 3 μM ALLO (periods 1 and 2; $p < 0.01$). ALLO is significantly more effective in reducing distance moved ($p < 0.001$) in larvae exposed to PTZ compared to DZP and MDZ across all time blocks and all but the lowest concentration (0.1 μM). Similarly, all concentrations of ALLO are significantly more effective than DZP and MDZ in reducing locomotor activity induced by PTX ($p < 0.001$). ALLO also was significantly more effective than DZP and MDZ in reducing locomotor activity triggered by TETS at all concentrations except 0.1 μM ($p < 0.001$); ALLO caused a greater decrease in distance moved in the first period compared to DZP ($p < 0.001$) and compared to DZP ($p = 0.002$) or MDZ ($p = 0.02$) in the third period. * Significantly different from control larvae exposed only to the chemical convulsant ($p < 0.001$). + Significantly different from vehicle control larvae exposed only to DMSO ($p < 0.001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
TETS Concentrations in Exposure Water.

Compound	Nominal Water Concentration (μM)	Measured Water Concentration (μM)
DMSO	0	–
TETS	0.4	0.5 ± 0.08
	4	3.2 ± 0.78
	40	25.7 ± 3.7

Data presented as mean ± SD (n = 3 water samples per concentration).

identified as the loss of body posture, sinking, and/or death (Fig. 2). Second, we did not observe pronounced dysmorphologies or lethality in zebrafish larvae exposed for 1 h to PTX or TETS across the range of concentrations tested for seizure effects (Fig. 1). However, all three chemical convulsants caused significant, concentration-dependent axial deformation, decreased trunk length and death following a 24 h exposure. Similar dysmorphologies have been observed in larval zebrafish exposed to the organophosphate cholinesterase inhibitor, chlorpyrifos, which the authors proposed reflect rupture of muscle fibers following violent jerking of the trunk during seizures (Faria et al., 2015). Alternatively, it has been shown that neurons exposed to potent chemical convulsants, including TETS and the organophosphate, diisopropylfluorophosphate, exhibit persistently elevated intracellular Ca^{2+} levels (Cao et al., 2012; Deshpande et al., 2010), which are associated with decreased cell viability and death of neurons and muscle spindle fibers.

Analogous to rodent models, post-exposure administration of $GABA_AR$ PAMs to acutely intoxicated zebrafish larvae mitigated, but did not completely protect against, the seizurogenic effects of PTZ, PTX, and TETS (Fig. 9). All three $GABA_AR$ PAMs effectively reduced the locomotor activity induced by PTZ, PTX and TETS in a concentration-dependent manner. The highest concentrations of each $GABA_AR$ PAM

Table 3
Diazepam, Midazolam, and Allopregnanolone Concentrations in Fish and Water.

Compound	Nominal Water Concentration (μM)	Measured Water Concentration (μM)		Measured Head Concentration (μM)		Measured Body Concentration (μM)	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
DMSO	0	–	–	–	–	–	–
Diazepam	3	3.1	3.2	9.9	26.9	4.7	15.7
Midazolam	3	3.4	3.2	29.3	33.9	14.3	10.5
Allopregnanolone	3	3.1	3.2	90.7	123.7	83.5	67.7

Data presented as the mean (n = 2 water samples or 2 biological replicates, with 266–590 bodies or heads pooled in each biological replicate). Each biological replicate was analyzed in triplicate in the LC/MS assay.

reduced locomotor activity to levels below those observed in vehicle control larvae exposed only to DMSO ($p < 0.001$), suggesting a sedative effect as has been reported in rodent models (Kitajima et al., 2004). In general, compared to PTZ, higher $GABA_AR$ PAM concentrations were required to decrease PTX and TETS-induced locomotor behavior. Specifically, it was noted that MDZ concentrations < 1 μM, and DZP concentrations of < 3 μM were less effective in mitigating TETS effects on locomotor behavior compared to PTZ. Further, the benzodiazepine concentrations that mitigated increased locomotor activity following PTX intoxication were several-fold higher than the effective concentrations found for PTZ and TETS. At any given $GABA_AR$ PAM concentration, however, ALLO was generally more effective than either benzodiazepine in decreasing locomotor activity. This is also consistent with rodent data indicating that neuroactive steroids are more effective than DZP in protecting against chemical-induced *status epilepticus* (Gasior et al., 2000). The increased efficacy of ALLO in our model likely reflects pharmacodynamic and pharmacokinetic differences relative to benzodiazepines. While both benzodiazepines and neurosteroids positively modulate synaptic $GABA_AR$, which are internalized during *status epilepticus*, neurosteroids also act on extrasynaptic $GABA_AR$, which are not internalized (Herd et al., 2007; Kokate et al., 1994; Lambert et al., 2003; Reddy and Rogawski, 2012). However, we also observed that concentrations of ALLO in the larval zebrafish head were 3.5 and 6 times higher than MDZ and DZP, respectively, since zebrafish apparently concentrate ALLO more effectively out of the water (Table 3).

Post-exposure administration of benzodiazepines also attenuated the increased electrical burst frequency recorded in the optic tectum of larval zebrafish acutely intoxicated with PTZ and TETS; however, neither DZP nor MDZ completely normalized electrographic activity (Fig. 7). These observations are consistent with previous reports demonstrating that benzodiazepine treatment attenuated but did not normalize spike frequency in PTZ-exposed zebrafish larvae (Afrikanova et al., 2013; Baraban et al., 2005). Our observations are also in line with rodent data demonstrating that high dose DZP administered following acute intoxication with TETS prevented lethality (Shakarjian et al., 2012; Vito et al., 2014) but failed to normalize aberrant epileptic discharges (Shakarjian et al., 2012). Surprisingly, however, neither DZP nor MDZ significantly attenuated electrographic activity in larval zebrafish exposed to PTX, and ALLO had no significant effect on seizure activity induced by any $GABA_AR$ antagonist. The reason(s) for the discrepant efficacy of ALLO in mitigating convulsant effects on locomotor behavior vs. electrographic activity are not known. One possibility is differences in bioavailability associated with perfusion of $GABA_AR$ PAMs through the agarose in which larvae are embedded for electrophysiological recordings. Relative to DZP and MDZ, ALLO is larger and more lipophilic, and thus it is possible that insufficient amounts penetrated through the agarose to the zebrafish brain. Recent advances in EEG technology using multielectrode/multichannel arrays on larval zebrafish do not require zebrafish to be immobilized in agar (Cho et al., 2017; Hong et al., 2016; Meyer et al., 2016). If reliable, such approaches might be able to answer this question, as well as offer a non-invasive, higher throughput alternative to the single electrode recordings conducted in the current study.

In conclusion, our results demonstrate that the larval zebrafish exhibits relevant seizure phenotypes in response to acute intoxication with the chemical threat agents PTX and TETS that mirror seizure phenotypes observed in rodent models. Importantly, the seizure responses to both $GABA_AR$ antagonists and $GABA_AR$ PAMs were similar across three routinely used wildtype zebrafish lines, indicating the consistency and reliability of the phenotype. The genetic tractability of the zebrafish will enable future studies aimed at elucidating the mechanism(s) of action of various chemical threat agents, and its adaptability for higher-throughput screening assays will facilitate the identification of more effective medical countermeasures for chemical threat agents, including combinatorial therapies.

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CRediT authorship contribution statement

Suren B. Bandara: Conceptualization, Formal analysis, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Dennis R. Carty:** Investigation, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **Vikrant Singh:** Investigation, Methodology. **Danielle J. Harvey:** Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Natalia Vasylieva:** Investigation, Resources. **Brandon Pressly:** Investigation. **Heike Wulff:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing. **Pamela J. Lein:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare not conflict of interest.

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