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Research Report

Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish

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ABSTRACT

Larval zebrafish (*Danio rerio*) have recently been suggested as a high-throughput experimental model of epilepsy-related pathogenetic states. Here we use *adult* zebrafish to study behavioral symptoms associated with drug-evoked seizures. Experimental epilepsy-like states were evoked in zebrafish by exposure for 20 min to three chemoconvulsant drugs: caffeine (250 mg/L; 1.3 mM), pentylenetetrazole (1.5 g/L; 11.0 mM) and picrotoxin (100 mg/L; 0.17 mM). Fish behavior was analyzed using manual and video-tracking methods (Noldus Ethovision XT7). Compared to their respective controls, all three drug-treated groups showed robust seizure-like responses (hyperactivity bouts, spasms, circular and corkscrew swimming) accompanied by elevated whole-body cortisol levels (assessed by ELISA). In contrast, control fish did not display seizure-like behaviors and had significantly lower cortisol levels. Paralleling behavioral and endocrine phenotypes observed in clinical and rodent studies, our data implicates adult zebrafish as an emerging experimental model for epilepsy research.

1. Introduction

Epilepsy is a common debilitating neurological disorder characterized by recurrent seizures, pathological brain hyperactivity and imbalances in excitatory/inhibitory neurotransmission (Bakvis et al., 2009; Mehta et al., 1994; Tunca et al., 2000). Endocrine dysregulation, such as pathological activation of the hypothalamic–pituitary–adrenal (HPA) axis, have also been associated with epilepsy pathogenesis (Arida et al., 2009; Mazarati et al., 2009; Taher et al., 2005). However, the exact pathobiological mechanisms of epilepsy remain poorly understood (Aroniadou-Anderjaska et al., 2008; Lowenstein, 1996). Rodents have been widely used to model seizures, due to their homology to humans and ability to display key epilepsy-related phenotypes (Baraban, 2007; Frankel, 1999). However, the need to better understand the neurobiological and genetic mechanisms of epilepsy pathogenesis requires novel approaches and experimental models of seizure-like states.

Zebrafish (*Danio rerio*) are becoming increasingly popular in biomedical research, sharing substantial genetic and physio-

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logical homology with other vertebrate species (Brittijn et al., 2009; Egan et al., 2009). Zebrafish exhibit robust behavioral and physiological phenotypes, and have a rapid reproductive cycle, capable of producing a large number of offspring (Alestrom et al., 2006; Gerlai et al., 2006; Spence et al., 2008). Recently, epilepsy-like behavior has been reported in *larval* zebrafish (Baraban et al., 2005), which are widely used in biomedical research for high-throughput screening of various drugs (Berghmans et al., 2007; Goldsmith, 2004; Langheinrich, 2003). Mounting evidence emphasizes the growing interest in using zebrafish-based models to study neurological disorders, such as epilepsy (Baraban et al., 2007, 2005; Berghmans et al., 2007; Winter et al., 2008). However, several limitations of larval models can exist, including an underdeveloped neural system, small body size and basic locomotor responses (Ingham, 2009; Kari et al., 2007; Muller and van Leeuwen, 2004; Penberthy et al., 2002; Stewart et al., 2010). Thus, when assessing certain behavioral measurements (such as the endpoints used in this study), utilizing *adult* zebrafish models may represent another promising approach.

The present study sought to explore the utility of adult zebrafish as potential models of seizure research. Here, we exposed adult zebrafish to several typical chemoconvulsant drugs and examined their behavioral and endocrine (cortisol) responses. Three different agents used in this study – caffeine, pentylenetetrazole (PTZ), and picrotoxin – were chosen for their ability to evoke seizures in various animal models. Caffeine is a nonselective antagonist of adenosine receptors, able to induce seizures at high doses in both humans (Iyadurai and Chung, 2007; Mortelmans et al., 2008; Rudolph and Knudsen, 2010) and animal models (Shen et al., 2009; Zagnoni and Albano, 2002). PTZ is a blocker of gamma amino butyric acid (GABA) A receptor channel, widely used to induce seizures in rodents and other species (Akula et al., 2009; Carmody and Brennan, 2009; Prigol et al., 2009). It has also been shown to induce seizures in larval zebrafish (Baraban et al., 2005; Goldsmith et al., 2007; Naumann et al., 2010; Tiedeken and Ramsdell, 2009). Picrotoxin, another noncompetitive GABA-A blocker, has long been shown to induce seizures in various experimental models (Reza et al., 2009; Saito and Tokunaga, 1967; Vazquez-Lopez et al., 2006). Data from three typical convulsants were included in this study, using them as selected "reference" drug probes, as frequently done in experimental epilepsy studies in mice and rats (Borekci et al., 2010; Keogh et al., 2005; Pericic et al., 2005; Sturman and Freeman, 1992; Vellucci and Webster, 1984).

The results of this study demonstrate robust, chemoconvulsant-induced seizure-like behavior in adult zebrafish. Paralleling the phenotypes reported in larvae, as well as in clinical and rodent studies, our data implicates the potential of *adult* zebrafish as an emerging experimental model relevant to epilepsy.

2. Results

Zebrafish exposure to caffeine reduced the number of transitions to top and increased freezing behavior, but did not affect the time spent in top and erratic movements (Fig. 1). Caffeine also reduced distance traveled and velocity but did not affect meandering and turn angle. Unlike controls, caffeine-treated zebrafish exhibited robust seizure-like behavior, including hyperactivity bursts, spasms, corkscrew swimming, and circular swimming (Fig. 1). In addition, hyperactivity was observed in 64±13% of experimental fish (vs. 21±12% controls, $P<0.06$, trend), spasms were observed in 50±14% (vs. 0±0% controls, $P<0.05$), while corkscrew and/or circular swimming were noted in 14±10% (vs. 0±0% controls, NS). The average cumulative seizure score was predictably higher in the caffeine-exposed cohort (1.43±0.25 vs. 0.21±0.11 controls, $P<0.005$). Finally, caffeine-evoked seizures were accompanied by a significant increase in cortisol, compared to the control fish (Fig. 1).

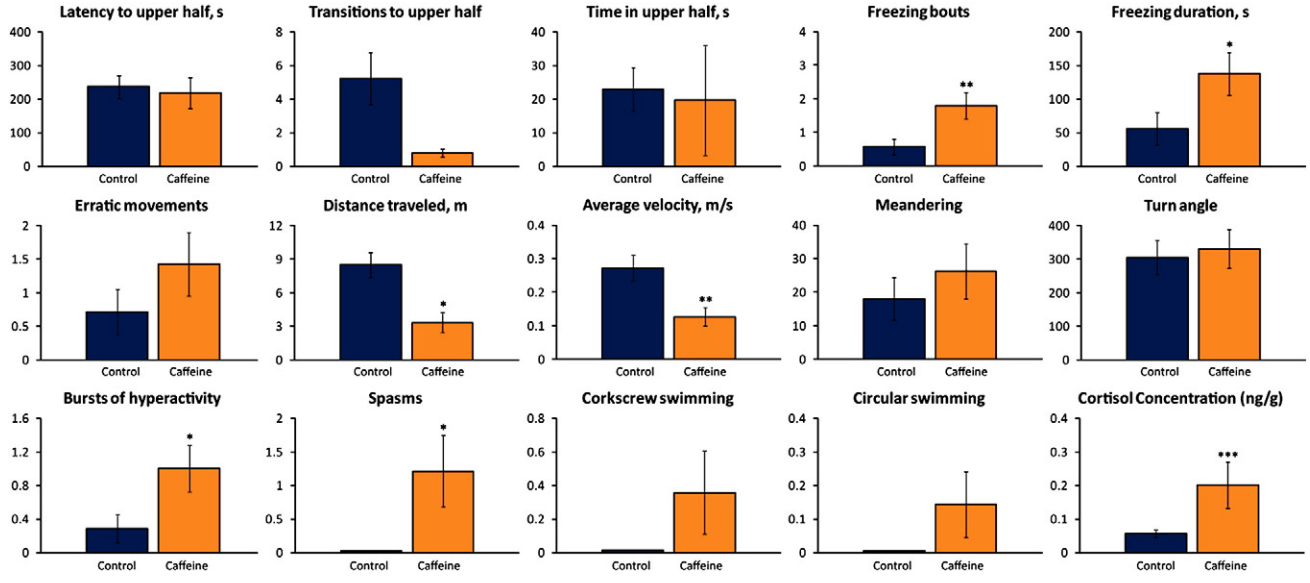

Fig. 1 – Behavioral and cortisol responses to caffeine-evoked seizures (250 mg/L, 20-min pretreatment) in adult zebrafish tested in a 6-min observation tank. *P<0.05, **P<0.01, ***P<0.005, U-test.

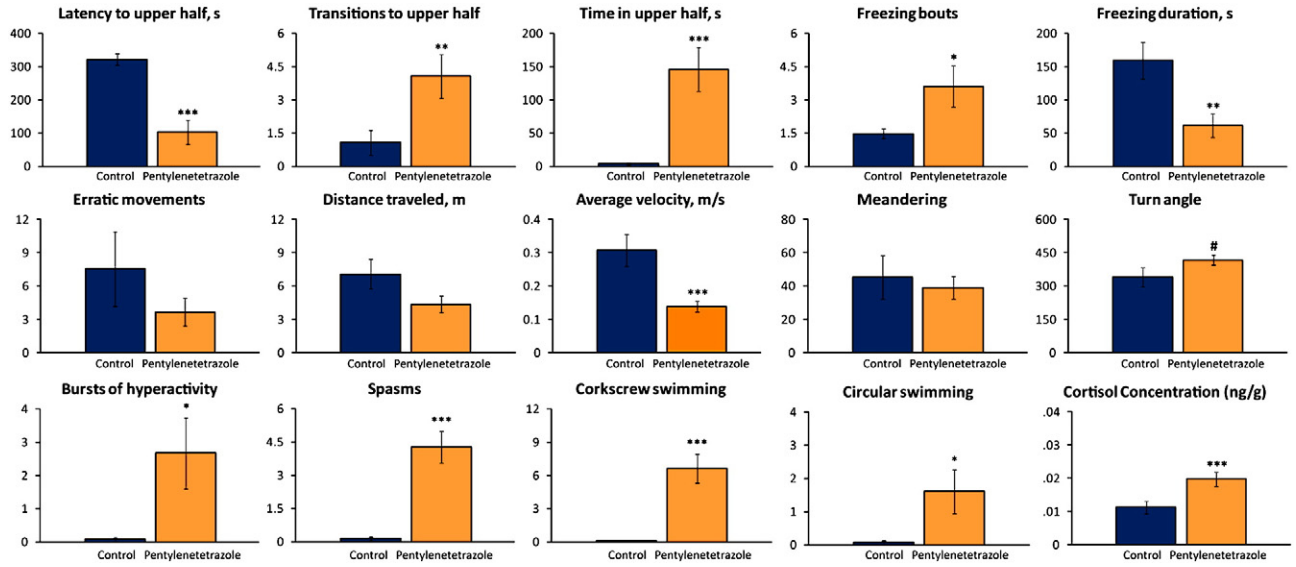

Fig. 2 – Behavioral and cortisol responses to pentylenetetrazole-evoked seizures (1.5 g/L, 20-min pretreatment) in adult zebrafish (n=12–15 in each group) tested in a 6-min observation tank. *P<0.05, **P<0.01, ***P<0.005, # P=0.05–0.1 (trend), U-test.

As shown in Fig. 2, acute PTZ treatment produced mixed effects including behavioral activation (more entries and time spent in top, reduced freezing duration) and inhibition (more freezing bouts and lower velocity). PTZ did not affect distance traveled or meandering, and tended to increase the turn angle. Notably increased seizure-like behaviors, including hyperactivity, circular swimming, spasms, and corkscrew swimming were also observed. Compared to control fish, hyperactivity was observed in 46±13% of PTZ-treated fish (vs. 7±7%, NS), while spasms were recorded in 93±7% (vs. 13±9%, P<0.05), corkscrew swimming in 80±11 (vs. 7±7%, P<0.05), and circular swimming in 47±10 (vs. 7±7% controls, P<0.06, trend) fish. The cumulative seizure score was 2.7±0.3 for experimental fish (vs. 0.6±0.3 for controls, P<0.001), and cortisol levels were significantly increased in PTZ-treated zebrafish (Fig. 2).

Picrotoxin reduced freezing duration and did not affect distance traveled, freezing bouts, erratic movements, velocity meandering, or turn angle (Fig. 3). Compared to control fish, the drug-treated group displayed slightly increased hyperactivity, and significantly more spasms, as well corkscrew swimming and circular swimming. Overall, hyperactivity was recorded in 50±15% of picrotoxin-exposed zebrafish (vs. 25±13% controls, NS), spasms 83.3±11% (vs. 0±0% controls, P<0.001), corkscrew swimming 75±13% (vs.8±8% controls, P<0.01), circular swimming 67±14% (vs. 0±0% controls, P<0.01). The cumulative seizure score was 2.8±0.3 in experimental fish vs. 0.3±0.1 in controls (P<0.001). Picrotoxin also produced a significant increase in cortisol levels following pharmacogenic seizures (Fig. 3). Finally, all three agents tested here evoked marked alterations in locomotory traces of

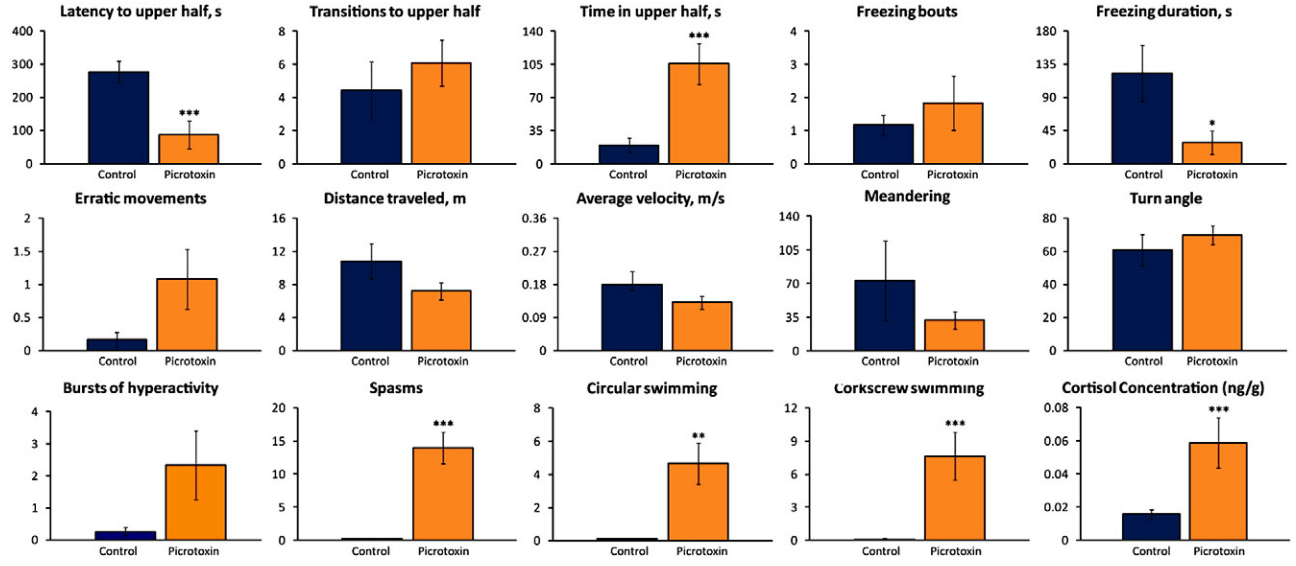

Fig. 3 – Behavioral and cortisol responses to picrotoxin-evoked seizures (100 mg/L, 20-min pretreatment) in adult zebrafish (n=12–15 in each group) tested in a 6-min observation tank. *P<0.05, **P<0.01, ***P<0.005, U-test.

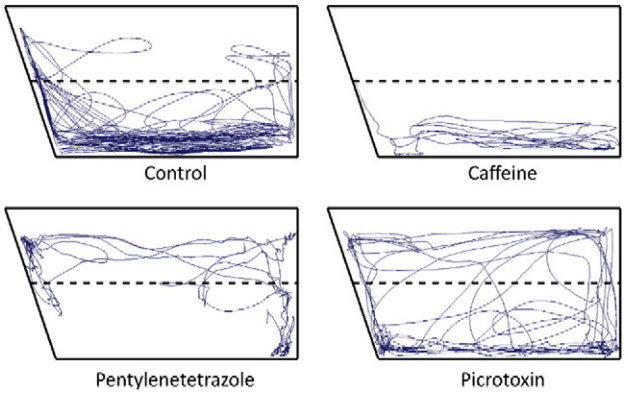

Fig. 4 – Representative computer-generated behavioral traces produced by caffeine, pentylenetetrazole and picrotoxin exposure in adult zebrafish tested in a 6-min observation tank (note the disorganized swim patterns resulting from convulsants' exposure, compared to the control fish).

zebrafish swimming, demonstrating reduced locomotion and less smooth swimming patterns (Fig. 4).

3. Discussion

This study represents the first comprehensive evaluation of chemoconvulsant-induced seizure behavior in adult zebrafish. It combined several different methodological approaches, including manual and video-assisted observation of zebrafish locomotion and seizures, as well as an endocrine assay to measure cortisol levels. Overall, all three convulsant drugs evoked similar behavioral profiles in zebrafish, including reduced normal swimming activity, accompanied by an increased frequency of hyperactivity episodes, spasms, and clonic/tonic-related behaviors, such as corkscrew and circular swimming (Figs. 1–3). Representative swim traces (Fig. 4) further illustrate this phenotype, showing a striking difference between spastic swimming patterns in all three experimental groups, compared to smooth swimming in the control fish.

In addition to behavioral alterations, pharmacogenic seizure-like activity in the present study was accompanied by an endocrine marker, whole-body cortisol levels, which was significantly elevated by all three drugs (Figs. 1–3). Consistent with this finding, endocrine dysfunctions have long been associated with epilepsy pathogenesis (Galimberti et al., 2005; Mehta et al., 1994; Pohlmann-Eden et al., 1993; Tunca et al., 2000; Zhang and Liu, 2008). Animal data generally parallel clinical observations that link epilepsy to glucocorticoids (Kumar et al., 2007; Mazarati et al., 2009; Schridde and van Luijtelaar, 2004; Talmi et al., 1995). Several mechanisms may underlie the link between epilepsy and glucocorticoids (Roberts et al., 1993). For example, the GABA-ergic system modulates corticosterone release in rats (Mikkelsen et al., 2008), whereas anti-epileptic and GABA-mimetic drugs inhibit glucocorticoid signaling (Basta-Kaim et al., 2007). Since corticosteroid receptors mediate PTZ-evoked seizures in mice (Roberts et al., 1993) and given our data on cortisol-elevating effects of GABA blockers PTZ and picrotoxin (Figs. 2 and 3), it is possible that a similar modulatory mechanism exists in zebrafish, underlying the convulsant-induced elevation of cortisol observed here.

Another explanation for this phenotype may be the overall increase in stress (evoked by chemoconvulsants) that triggers cortisol release independently from seizures. For example, sub-convulsant doses of picrotoxin reduce motor activity and elevate anxiety and corticosterone in rats (Stankevicius et al., 2008), while PTZ exerts similar effects in monkeys (Palit et al., 1998). Although sub-convulsant doses of caffeine (Egan et al., 2009), PTZ (Wong et al., 2009) and picrotoxin (own unpublished observations) may evoke anxiety in zebrafish, with higher (convulsant) doses used here, the reduction in latency to the top and freezing duration in PTZ- and picrotoxin-treated fish (Figs. 2 and 3) seems to resemble the hyperactivity common in animal models of epilepsy (Akula et al., 2009; Sarkisian, 2001), including larval zebrafish (Baraban et al., 2007; Baraban et al., 2005; Berghmans et al., 2007; Parent, 2006).

Clearly, future studies on adult zebrafish can further validate this species as an animal model of epilepsy. For example, electrophysiological and neurochemical analyses in the brain (as common biomarkers of epilepsy) may be applied to parallel behavioral and endocrine findings reported here. Our pilot study of c-fos gene expression in zebrafish brains showed a significant 70% elevation in this marker of neuronal activation following PTZ-evoked seizures (own unpublished data). Screening a wide range of doses of various convulsant drugs in such models may provide valuable insights into their complex neuropharmacological profiles. Anti-convulsant treatment in conjunction with chemoconvulsant exposure may be useful in further understanding the physiological mechanism of endocrine regulation in this experimental model. Additionally, various zebrafish mutants, and other pathogenetic factors (e.g., neurotoxicity or brain aging) associated with epilepsy, may be studied using our approach. The use of several cameras with subsequent data integration enables a 3D reconstruction of zebrafish locomotion (e.g., (Stewart et al., 2010)), which may further increase the density of behavioral data generated in this model. Finally, other imaging methods may prove useful in the visualization of zebrafish seizure-like activity (Naumann et al., 2010).

Importantly, *adult* zebrafish models complement the existing *larval* models of epilepsy. As already mentioned, the advantage of larval models is their high-throughput nature, which can be used for fast screening of numerous pro- and anti-convulsant compounds. The main advantage of adult zebrafish, as shown here, is the opportunity to target a wider spectrum of endophenotypes related to epilepsy (Figs. 1–4). Collectively, our results implicate adult zebrafish as an emerging experimental model of seizures.

4. Experimental procedures

4.1. *Animals and housing*

A total of 86 adult (5–7 month-old; ≈50:50 male:female ratio) wild type, short-fin zebrafish were used in this study. The animals were obtained from a local commercial distributor (50 Fathoms, Metairie, LA) and were given at least 20 days to acclimate to the animal facility. The fish were housed in groups

of approximately 30 fish per 40-L tank, filled with deionized water that was maintained at≈25 °C and pH=7.0–8.0. Illumination (1000–1100 lx) was provided by ceiling-mounted fluorescent light tubes on a 12-h cycle (on: 6:00, off: 18:00), according to the zebrafish standard of care (Westerfield, 2007). All animals used in this study were experimentally naïve and were fed Tetramin Tropical Flakes (Tetra USA, VA) twice daily.

4.2. *Drugs and experimental manipulations*

To evoke experimental seizures, the animals (n=12–15 in each group) were individually exposed to 250 mg/L (1.3 mM) caffeine, 100 mg/L (0.17 mM) picrotoxin, or 1.5 g/L (11.0 mM) PTZ for 20 min in a 3 L plastic beaker. One fish was exposed to the treatment beaker at a time. All drugs used for this study were obtained from Sigma-Aldrich (St. Louis, MO) and are readily soluble in water. Each experimental group was treated with only one of the three drugs, and none of the animals were retested in this study. Control fish tanks were exposed to equivalent volumes of drug-free filtered facility (system) water, drawn from the vivarium and tailored to the health of the fish. Drug dose and pre-treatment time for each drug were selected based on our own pilot data, with a wide range of concentrations tested (data not shown), as well as based on previous studies of behavioral effects of these drugs (Egan et al., 2009; Wong et al., 2009), and the known ratio between behavioral and pro-convulsant doses of these drugs. A *single* non-lethal effective dose for each drug was used here to evoke seizure-related behavior, as the use of a single "reference" active dose is a commonly used approach in experimental epilepsy research in mice (Dengiz and Bakrici, 2009; Kondziella et al., 2002; Signorini et al., 1997) and rats (Acharya and Katyare, 2006; Nyitrai et al., 2002). Behavioral testing was performed using a standard observation tank, representing a 1.5-L trapezoidal tank (15.2 height×7.1 width×27.9 top×22.5 cm bottom length; Aquatic Habitats, Apopka, FL) maximally filled with aquarium water. The observation tanks rested on a level, stable surface and were divided into two equal horizontal portions, pre-marked by a line on the exterior.

Behavioral testing took place between 11:00 and 16:00 h, to ensure consistency and reduce potential variation in behavioral and endocrine responses. Following pre-treatment, the animals were transferred to observation tanks and their behaviors were recorded for 6 min by trained observers (inter-rater reliability >0.85) for two types of endpoints: traditional (non-seizure like) and seizure-related behaviors. The manually recorded endpoints, which have traditionally been used to describe zebrafish behavioral activity in novel tanks (Egan et al., 2009; Levin et al., 2007; Wong et al., 2009), included time spent (s) in the upper half/top of the tank, number of transitions to the top, number of erratic movements, and number and duration (s) of freezing bouts. Erratic movements were defined as sharp changes in direction and/or velocity, which were represented as rapid, darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for >2 s (Egan et al., 2009; Levin et al., 2007; Speedie and Gerlai, 2008). In addition to traditional locomotory endpoints, the frequency of the following seizure-related endpoints was recorded by the observers: bursts of hyperactivity, spasms, corkscrew swimming, and circular swimming. Hyperactivity was defined as prolonged (>3 s) periods of sharp changes in direction and/or velocity, different in duration from erratic movements. Spasms were recorded as sudden twitches or small jerks of the body that may or may not result in propulsion. Corkscrew swimming was defined as swimming in helical paths, and circular swimming episodes were recorded as rapid bouts of swimming in circles.

Seizure-related endpoints for the experimental and control groups were further evaluated using two additional scoring systems. First, the fish were assigned a score of 1 or 0 for each seizure-like phenotype (hyperactivity, spasm, corkscrew swimming, and circular swimming) based on whether the particular behavior was exhibited during the 6-min observation period, similar to those conducted in larval zebrafish and rodents (Baraban et al., 2005; Carmody and Brennan, 2009; Reza et al., 2009). The % of fish demonstrating the respective seizure-like phenotype was then calculated. Furthermore, cumulative seizure scores (on a scale of 0 to 4) were obtained for each fish's unique seizure-like behavior (as the sum of seizure scores obtained using a 0-or-1 system described above), in order to assess the spectrum of different seizure-like phenotypes displayed by each individual animal. The average cumulative seizure scores were calculated for each experimental cohort and were compared with their respective controls, providing a quantitative analysis of seizure severity similar to the Racine scale widely utilized in experimental rodent models of epilepsy (Racine, 1972; Racine, 1975), where greater values result in greater severity.

In addition to manual observation, we also used video-tracking tools (Ethovision XT7, Noldus Information Technology, Netherlands) to analyze zebrafish activity (Egan et al., 2009). Zebrafish swimming behavior was recorded with a webcam connected to a computer (side-view) and analyzed to calculate total distance traveled (m), average velocity (m/s), turn angle (°) and meandering (°/m), a quantification of turning relative to path length. In addition, traces were generated for each fish in order to visualize the patterns of their locomotion in the observation tank. These tracers were ranked from 1 to n by three trained observers on a consensus basis, and middle traces were selected as representative for the group (Fig. 4).

Immediately after testing, the animals were euthanized using 500 mg/L Tricaine (Sigma-Aldrich, MO). The cortisol analysis was performed based on a protocol developed in our laboratory using human salivary cortisol ELISA kit (Salimetrics LLC, PA) (Cachat et al., 2010; Egan et al., 2009). Cortisol data was calculated based on the absorbencies of standardized concentrations, and presented as relative concentrations per gram of body weight for each fish (Egan et al., 2009). All experimental protocols in this study were conducted in full compliance with ethical standards set forth by the NIH and Tulane University's Institutional Animal Care and Use Committee to minimize the pain and discomfort of our experimental animals.

4.3. *Statistical analysis*

All data was expressed as mean±S.E.M, and analyzed using the unpaired Wilcoxon–Mann–Whitney U-test to compare the control and single-dose experimental groups for each drug

(since normality of data was not examined here, the data were analyzed using a non-parametric U-test). Significance was set at $P<0.05$ in all experiments.

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