PENTYLENETETRAZOLE INDUCED CHANGES IN ZEBRAFISH BEHAVIOR, NEURAL ACTIVITY AND C-FOS EXPRESSION

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Abstract—Rodent seizure models have significantly contributed to our basic understanding of epilepsy. However, medically intractable forms of epilepsy persist and the fundamental mechanisms underlying this disease remain unclear. Here we show that seizures can be elicited in a simple vertebrate system e.g. zebrafish larvae (*Danio rerio*). Exposure to a common convulsant agent (pentylenetetrazole, PTZ) induced a stereotyped and concentration-dependent sequence of behavioral changes culminating in clonus-like convulsions. Extracellular recordings from fish optic tectum revealed ictal and interictal-like electrographic discharges after application of PTZ, which could be blocked by tetrodotoxin or glutamate receptor antagonists. Epileptiform discharges were suppressed by commonly used antiepileptic drugs, valproate and diazepam, in a concentration-dependent manner. Upregulation of *c-fos* expression was also observed in CNS structures of zebrafish exposed to PTZ. Taken together, these results demonstrate that chemically-induced seizures in zebrafish exhibit behavioral, electrographic, and molecular changes that would be expected from a rodent seizure model. Therefore, zebrafish larvae represent a powerful new system to study the underlying basis of seizure generation, epilepsy and epileptogenesis. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

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Epilepsy, a common neurological disorder marked by the occurrence of spontaneous seizures, afflicts nearly 50 million people worldwide. Proposed mechanisms to explain how seizures occur incorporate multiple levels of analysis ranging from behavioral (e.g. defined seizure stages), to electrophysiological (e.g. electrical events contributing to a seizure episode) to molecular (e.g. alterations in gene expression). To date, the vast majority of epilepsy research has been performed in rodents (Mello et al., 1993; Lothman et al., 1989; Buhl et al., 1996; Jacobs et al., 1999; Galvan et al., 2000; Sperber et al., 1991; Lukasiuk et al.,

2003), with additional insights derived from analysis of human tissue obtained during surgical resection for intractable epilepsy (Prince and Wong, 1981; Schwartzkroin and Knowles, 1984; Andre et al., 2004). Although recent studies incorporate mutant mice exhibiting a spontaneous seizure phenotype (Noebels, 2001), our greater understanding of epilepsy largely derives from animal models in which seizures were induced (e.g. kindling, kainic acid, pentylenetetrazole [PTZ], pilocarpine, etc.). Among the recent insights gained from studying induced seizure models, we know that status epilepticus leads to changes in postsynaptic GABA receptor expression (Brooks-Kayal et al., 1998) and hilar neurogenesis (Parent et al., 1997), excitatory circuits are necessary for seizure propagation (Khalilov et al., 2003) and synaptic re-organization contributes to a process of epileptogenesis (Sutula et al., 1988). These, and a vast literature of additional findings, have significantly contributed to our understanding of epilepsy and directly led to the discovery of novel antiepileptic drugs (AEDs). Despite these advances, many patients still suffer with intractable forms of epilepsy and there remains much to be learned about how seizures are generated.

To further our understanding of basic mechanisms underlying epileptogenesis and develop novel treatments requires additional animal model research. One strategy would be the development and characterization of new animal models, specifically those that are amenable to rapid drug screening and genetic manipulation. Zebrafish, *Danio rerio,* are small freshwater teleosts rapidly emerging as an important model organism in genetics and developmental neurobiology. Similar to invertebrate models, *Drosophila* and *C. elegans, D. rerio* can produce hundreds of offspring and large colonies can be established in a relatively short time frame. As a vertebrate, zebrafish genes share a 70–80% homology to those of humans (Dooley and Zon, 2000) making identification of human orthologues of zebrafish genes straightforward. Although zebrafish enjoy widespread use in biomedicine (e.g. Shin and Fishman, 2002), their usefulness in studying neurological disorders remains unexplored.

To provide new insights into epilepsy and to "borrow" from research benefits currently being realized in genetics and development, we propose to establish a simple vertebrate model of induced seizures. We recognize that the validity of animal models derives from their success in reproducing essential aspects of the human condition (Sarkisian, 2001) e.g. abnormal electrical discharge in a CNS structure and stereotyped behaviors. Accepted rodent induced seizure models exhibit well-documented seizure-like behaviors (Racine, 1972), ictal- and interictal-like

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electrical activity (Lothman et al., 1981; Clifford et al., 1987; Jensen et al., 1991), and c-Fos expression in brain regions participating in seizure generation (Morgan et al., 1987; Dragunow and Robertson, 1987). Here, we hypothesize that zebrafish larvae (postembryonic day 7 fish) possess the brain structure necessary for development of complex seizure activity. To demonstrate the general usefulness of such a model we describe behavioral, electrophysiological and molecular changes that occur in zebrafish larvae exposed to a common convulsant agent.

EXPERIMENTAL PROCEDURES

Animals and maintenance

Zebrafish of the TL strain were maintained according to standard procedures (Westerfield, 1995). All experiments conformed to UCSF and AAALAC guidelines on the ethical use of animals. All animal care procedures were reviewed and approved by the UCSF Institutional Animal Care and Use Committee and were designed to minimize the number of animals used and their suffering. For all experiments zebrafish larvae were maintained in a "normal bathing medium" consisting of 0.03% Instant Ocean (Aquarium Systems, Inc., Mentor, OH, USA) in deionized water.

Behavioral monitoring

Zebrafish were placed individually in 96-well Falcon culture dishes. Each well contained approximately 50 μl Ringer's solution and one 7 days postfertilization (dpf) zebrafish larvae. For analysis of seizure stages, swimming behavior was monitored using a high-resolution Sony Digital Handycam video camera (DCR-VX1000). Recording sessions (40–60 min) were stored on videocassette and later scored by an investigator blind to the status of the fish. For locomotion analysis, swimming behavior was monitored using a Hamamatsu C-2400 CCD camera (Hamamatsu, Japan) and EthoVision 3.0 locomotion tracking software (Noldus Information Technology, Inc., Leesburg, VA, USA).

Electrophysiology

To obtain stable physiological recordings zebrafish larvae (7 dpf) were immobilized in a low-melting temperature agarose. Agarose was prepared fresh at a concentration of 1.2% in low-strength phosphate-buffered saline. Zebrafish were placed in agarose so that the dorsal aspect of the fish was exposed to the agarose gel surface and was accessible for electrode placement. Using this approach, zebrafish were completely immobilized in an agar block and anesthetic agents, with their potential for interfering with synaptic function and convulsant activity, were not necessary. After embedding, fish were placed on the upright stage of an Olympus or Leica microscope and perfused with normal Ringer's medium. Under direct visual guidance, a glass microelectrode (approximately 1 μm tip diameter, 2–7 MΩ) was placed in the optic tectum, the largest midbrain structure in the zebrafish CNS. Electrodes were filled with 2 M NaCl and electrical activity recorded using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Voltage records were low-pass filtered at 1 kHz (-3 dB; eight-pole Bessel), high-pass filtered at 0.1–0.2 Hz, digitized at 5–10 kHz using a Digidata 1300 A/D interface, and stored on a PC computer running pClamp software (Axon).

Reverse transcription–polymerase chain reaction (RT-PCR) and whole-mount *in situ* hybridization

RT-PCR and whole-mount *in situ* hybridization was performed against *c-fos* mRNA. To obtain the zebrafish c-Fos genomic se-

quence, the cDNA sequence (accession number BC065466) was used to BLAST the zebrafish genome (http://www.sanger.ac.uk/Projects/D_rerio/). For RT-PCR, 7 dpf larvae were exposed to PTZ then total RNA was isolated from the whole larvae using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was quantified by spectrophotometry and all samples were adjusted to 400 ng/μl. cDNA was synthesized using an oligo dT primer and SuperScript III reverse transcriptase (Invitrogen). cDNA was PCR amplified with forward primer: 5′-AACTGTCACGGCGATCTCTT and reverse primer: 5′-GCAGGCATGTATGGTTCAGA and run on a 1.5% agarose gel. DNA primers were designed to amplify a PCR product that spans two small introns to distinguish between genomic DNA (1234 bp) and cDNA (1030 bp).

For whole-mount *in situ* hybridization, DNA primers (forward primer: 5′-AACTGTCACGGCGATCTCTT; reverse primer: 5′-CTTGCAGATGGGTTTGTGTG) were used to PCR amplify a 767 bp fragment of c-Fos from genomic DNA. This product was gel purified with QiaexII (Qiagen, Valencia, CA, USA) and cloned into pCRII-TOPO (Invitrogen). DIG-labeled sense and anti-sense probes were generated using T7 and Sp6 RNA polymerase, respectively, according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). Unincorporated nucleotides were removed with a G-50 column (Roche). Seven dpf zebrafish larvae were unexposed or exposed to PTZ for 1 h, fixed in 4% paraformaldehyde/PBS overnight at 4 °C, dehydrated in methanol, and stored at -20 °C. Larvae were raised in 0.003% phenylthiourea to inhibit melanin production. Hybridization was performed as described (Schulte-Merker, 2002).

RESULTS

Behavioral seizure activity in larval zebrafish

It is well established that seizures induced in rodents are associated with distinct motor behaviors (Racine, 1972). If zebrafish are to be used for epilepsy research, it is first necessary to establish and define a sequence of seizure-like behaviors. To induce seizures, PTZ (2.5–15 mM) a common convulsant agent, was added to normal bathing medium. Control experiments demonstrated that zebrafish larvae aged 7 dpf swim, infrequently, in small dart-like steps when placed in normal bathing medium. PTZ added to the bathing medium reliably elicited distinct seizure-like behaviors in a concentration-dependent manner ($n=98$). Initially fish were observed to dramatically increase their swim activity (Stage I) this was followed by a rapid "whirlpool-like" circling swim behavior (Stage II) and culminated with a series of brief clonus-like convulsions leading to a loss of posture, e.g. fish falls to one side and remains immobile for 1–3 s (Stage III). None of these motor behaviors was observed in fish exposed to normal bathing medium for observation periods up to 10 h. The latencies to first sign of Stage I, II or III seizure activity exhibited a concentration-dependent profile, e.g. at higher PTZ concentrations the latency to a given seizure stage was shorter than that measured at lower drug concentrations (Fig. 1A). Lower concentrations of PTZ evoked only Stage I and Stage II seizure behavior, but nearly 75% of all fish exhibited at least one clonus-like convulsion (Stage III) in the presence of high concentrations (15 mM; Fig. 1B).

To quantify specific aspects of the induced seizure activity, further behavioral analysis was performed with a high-speed video tracking system (Noldus et al., 2001). For these experiments zebrafish larvae were monitored
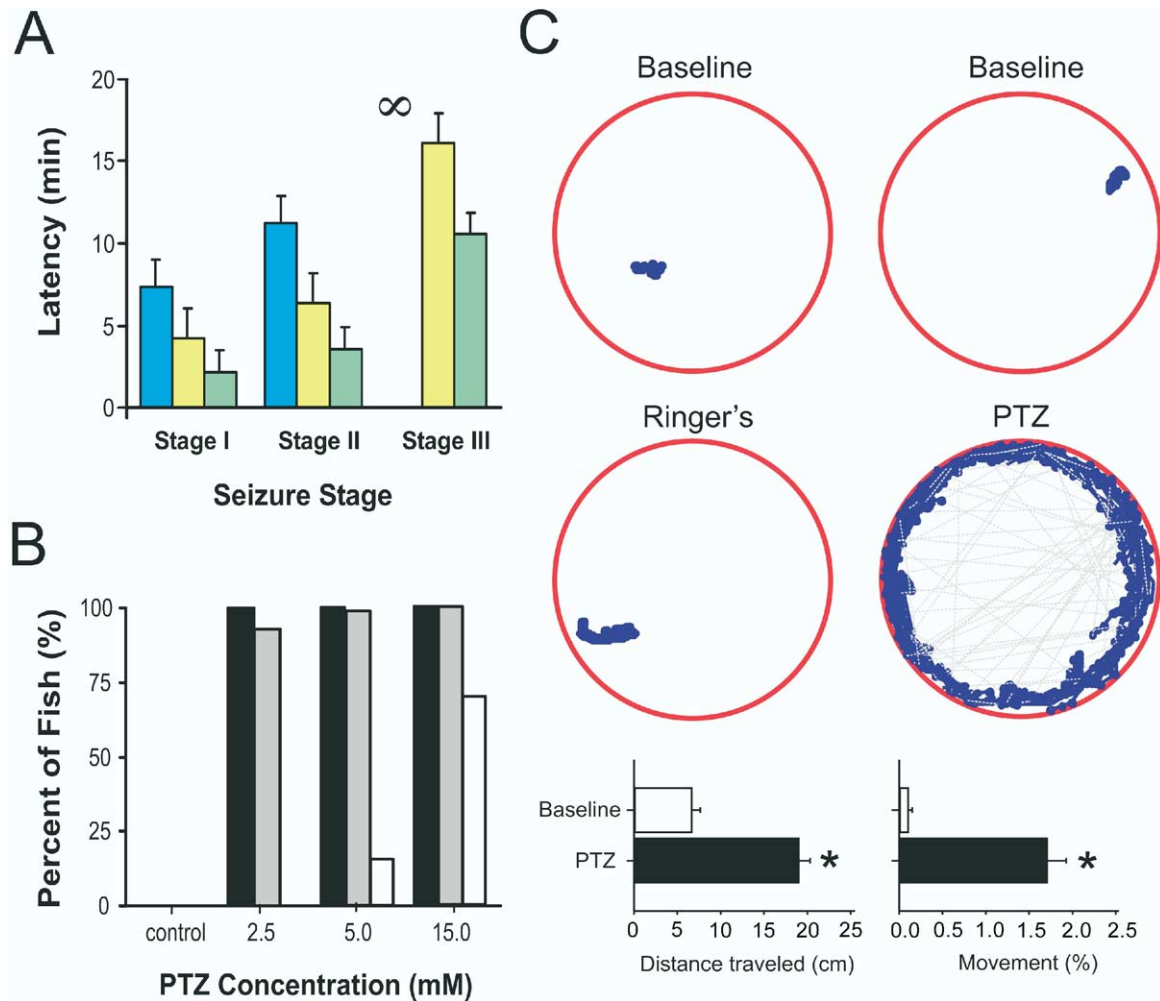
Fig. 1. Behavioral seizure scoring in zebrafish larvae. (A) Plot of the latency to first sign of seizure behavior for three different stages of behavior vs. PTZ concentration ($n=96$ fish per drug). Note that PTZ evokes seizure-like behaviors in a concentration-dependent manner. Fish were placed in a 96-well Falcon dish, videotaped and scored by an experimenter blind to the status of the animal. Infinity symbol (in A) denotes that no fish reached Stage III seizure behavior at this PTZ concentration. Plots (in A) are means±S.E.M. PTZ concentrations are as follows: 2.5 mM (blue), 5 mM (yellow), 15 mM (green). (B) Plot of the percentage of zebrafish larvae exposed to a given concentration of PTZ vs. the seizure stage reached ($n=96$ fish per drug concentration). Stage I (black bar), Stage II (gray bar), Stage III (open bar). (C) Sample locomotion tracking plots are shown for individual zebrafish in normal bathing medium (baseline) and 10 min after exposure to fresh Ringer's or 15 mM PTZ. Blue dots indicate movement; dashed lines indicate rapid convulsive seizure activity (this fish exhibited >30 convulsive episodes). Plots were obtained from recording epochs 2 min in duration. Bar plots (bottom) are shown for the total distance traveled and percent of time fish spent moving in a user-defined arena. Plots (in C) are means±S.E.M; baseline recording (open bars); 15 mM PTZ (closed bars).

using a CCD camera and locomotion tracking software. Baseline recordings (2 min) were obtained from fish exposed to normal bathing medium. Baseline recording epochs were characterized by a general lack of movement (Fig. 1C), as indicated above. Solution was then replaced with medium containing 15 mM PTZ and a second recording epoch (2 min) was obtained after a 10 min exposure. Locomotion tracking plots indicate a pattern of activity consistent with zebrafish undergoing repeated episodes of convulsive (Stage III) type activity (Fig. 1C, PTZ; $n=36$); seizure-like behavior was confirmed by simultaneous monitoring of the recording epoch on a video monitor. Brief clonus-like convulsive episodes accompanied by rapid movement across the well (indicated by dashed lines in plot) were followed by brief post-ictal loss of posture. For

comparison, a solution change to fresh bathing medium did not result in a change in locomotion pattern (Fig. 1C, Ringer's; $n=6$). To analyze locomotion plots we measured the total distance traveled (in cm) and movement (as a percentage of time moving) for each recording epoch. PTZ-induced seizures were associated with a significant increase in both parameters in comparison with baseline, pre-convulsant recording epochs (Fig. 1C).

Electrographic seizure activity in larval zebrafish

Seizures, whether induced or spontaneous, are defined by the occurrence of abnormal electrical discharge (Lennox and Lennox, 1960; Penfield and Jasper, 1954). To determine whether PTZ-evoked zebrafish seizures exhibit an
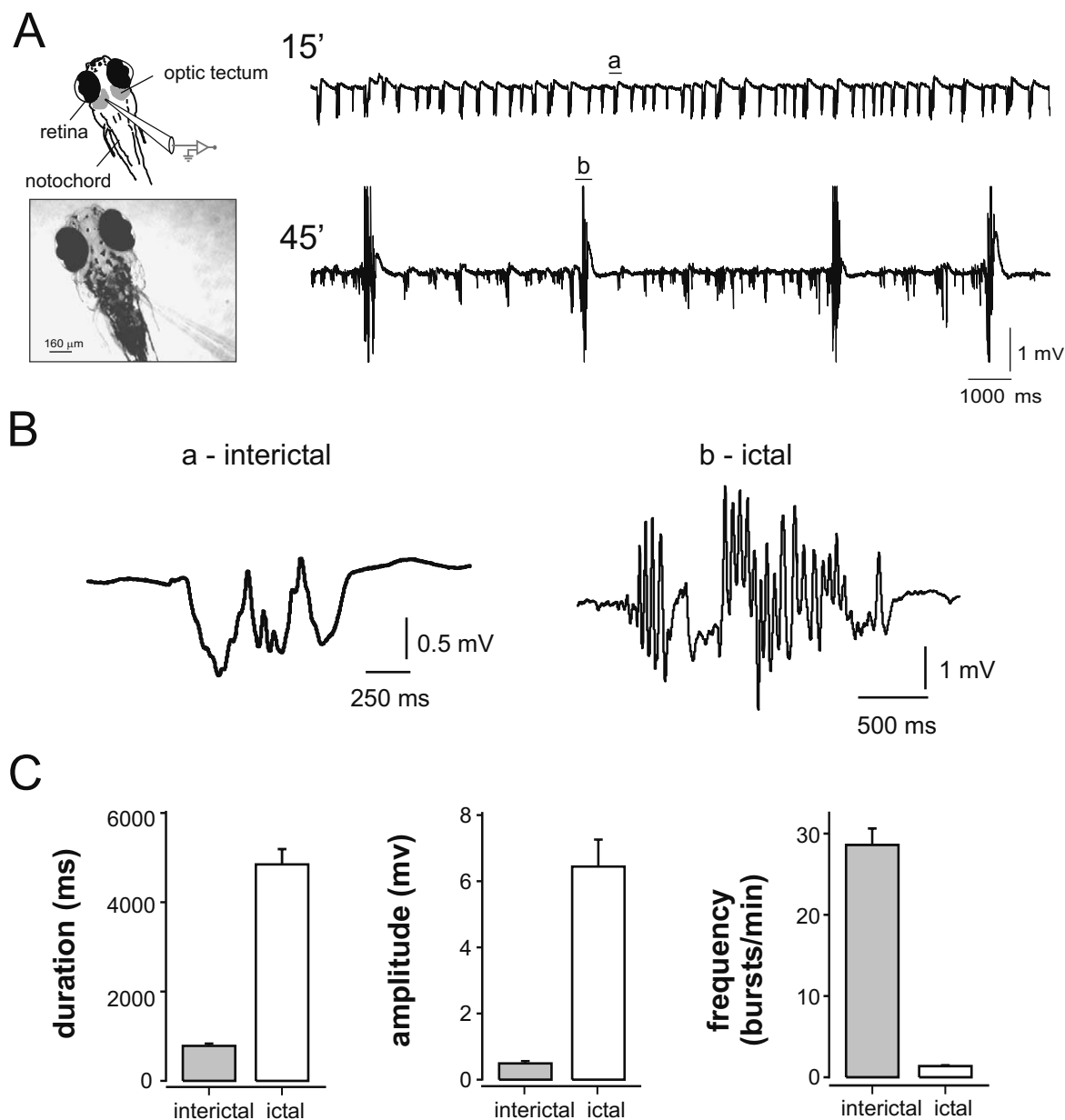
Fig. 2. Epileptiform-like electrographic activity in zebrafish larvae. (A) Schematic of the configuration used to obtain tectal field recordings from agar-embedded zebrafish larvae. Top left: schematic showing the location of the optic tectum, and other relevant brain structures as well as the position of a glass microelectrode. Bottom left: a frame-grabber image showing the placement of an electrode in the zebrafish optic tectum. Top right: Representative field recording from a zebrafish larvae exposed to 15 mM PTZ for 15 min. Bottom right: a second trace of field recording from the same fish after 45 min of PTZ exposure is also shown. Note the presence of interictal- and ictal-like epileptiform discharges. (B) An isolated "interictal-like" burst at a faster time resolution as indicated by the letter "a" (in A); an isolated "ictal-like" burst (b in panel A) is also shown. (C) Bar graphs of the cumulative data obtained from all wild-type fish exposed to 15 mM PTZ. In each plot we measured 10 individual interictal or three to five ictal bursts for each fish and data are presented as mean±S.E.M.

electrographic component *in vivo* electrophysiology experiments were performed. Fish immobilized in agar at 7 dpf were exposed to normal bathing medium containing 15 mM PTZ e.g. a concentration shown to reliably induce clonus-like seizure behavior (see Fig. 1). Electrical activity was monitored with a field electrode placed under visual guidance in the optic tectum (Fig. 2A). In all fish (*n*=97), 15 mM PTZ induced spontaneous epileptiform discharges which were initially brief, small in amplitude and occurred at a relatively high frequency (10–30 min exposure). These brief, small-amplitude discharges appear similar to interictal bursts recorded in tissue from rodents or humans (Gutnick et al., 1982; Prince et al., 1983; Traynelis and Dingledine, 1988). With continued exposure to 15 mM PTZ (45–80 min) a pattern of epileptiform discharge developed that incorporated brief interictal-like bursts with larger, long duration events (Fig. 2B, C). Large amplitude (>4 mV) events occurred at a low frequency and appear similar to

ictal bursts seen in mammalian nervous systems; these events were followed by a brief period of reduced electrical activity i.e. post-ictal depression. Epileptiform events were not observed during baseline recording periods prior to PTZ exposure and never occurred in control fish bathed in normal bathing medium for periods up to 90 min ($n=15$; data not shown). Latencies to observed seizure-like activity were longer in electrophysiology experiments (compare Figs. 1 and 2) and likely reflect the slower incorporation of drug into zebrafish larvae embedded in an agar block versus fish freely swimming in PTZ.

Pharmacology of PTZ-induced seizures

If chemically evoked epileptiform discharge in the fish is similar to that observed in mammals, then we can expect to modulate this activity with agents that block synaptic transmission (Traynelis and Dingledine, 1988; Swann et al., 1993). To test this hypothesis, pharmacology experiments were performed on wild-type agar-embedded fish (7 dpf). Fish were initially bathed in medium containing 15 mM PTZ until a stable pattern of epileptiform activity developed (45–60 min exposure); antagonists were then applied and activity observed for up to 1 h. Addition of 5 μM tetrodotoxin (to block Na^+-dependent action potentials) to bathing medium containing 15 mM PTZ abolished interictal and ictal forms of epileptiform discharge within 45 min ($n=3$; Fig. 3A, B). Next we tested agents that block excitatory, glutamate-mediated synaptic transmission: kynurenate (a non-specific blocker of postsynaptic glutamate receptors) and a "cocktail" containing 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)/APV (non-*N*-methyl-D-aspartate [NMDA] and NMDA glutamate receptor blockers, respectively). Kynurenate (2.5 mM; $n=4$) and 20 μM CNQX/50 μM APV ($n=4$) significantly reduced PTZ-evoked epileptiform discharge activity in all zebrafish (Fig. 3A, B). Baclofen (50 μM; $n=3$; Fig. 3B), a GABA-B receptor agonist, potentiated epileptiform discharges.

To explore further whether epileptiform events in zebrafish resemble activity reported in mammals, we examined AED responses. Standard AEDs with known effects on rodent PTZ-evoked seizures were tested. Again, fish were initially bathed in 15 mM PTZ until a stable pattern of epileptiform activity developed; AEDs were then applied and activity observed for up to 1 h. Addition of carbamazepine (100–200 μM) or ethosuximide (5–10 mM) to the PTZ solution had little effect on epileptiform discharge (Fig. 4A, B). Phenytoin (100 μM), an anticonvulsant which prolongs Na^+ channel inactivation, reduced burst discharge amplitude by approximately 50% whereas phenobarbital (100 μM), an AED with $GABA_A$ receptor potentiation actions, decreased amplitude by approximately 60% (Fig. 4B). Rodent pharmacology studies indicate that valproic acid (VPA) and benzodiazepines are the most effective AEDs to inhibit PTZ-evoked seizures (Krall et al., 1978). As predicted, saturating concentrations of VPA (5 mM) or diazepam (100 μM) produced the most potent anticonvulsant effects in fish, dramatically reducing epileptiform discharge in all trials (Fig. 4B). Clonazepam at 100 μM, another benzodiazepine AED, also significantly re-

duced epileptiform activity ($n=3$); 100 μM nifedepine (a Ca^{2+} channel blocker), however, had little effect ($n=4$; data not shown). Further analysis indicated that VPA produced a concentration-dependent decrease in PTZ-evoked epileptiform burst discharge amplitude (Fig. 4C); the estimated EC_{50} for VPA was approximately 1 mM. Diazepam (DZPM) evoked a concentration-dependent reduction in burst discharge amplitude with an estimated EC_{50} of approximately 5 μM. We also tested the control solution (bathing medium plus 10% methanol) that was used to dissolve DZPM and this solution had no effect on burst amplitude (Fig. 4C). None of the AEDs tested was lethal as we could visualize a strong heartbeat and pulsing blood flow at the conclusion of each experiment.

Seizure-induced changes in gene expression

Seizures induced in rodents result in expression of immediate early genes (IEGs) in brain regions corresponding to sites of seizure initiation (Morgan et al., 1987; Dragunow and Robertson, 1987). To determine whether PTZ-evoked seizures alter IEG expression, *c-fos* mRNA was measured. First, RT-PCR was used to demonstrate an increased temporal expression of *c-fos* mRNA during following PTZ exposure. Zebrafish larvae were exposed to 15 mM PTZ for 0, 15, 30, and 60 min then total RNA from the whole larvae was isolated. Following normalization of RNA concentrations, cDNA was synthesized and serial dilutions were amplified by PCR. Using this semi-quantitative approach, *c-fos* mRNA was shown to be up-regulated by approximately three-fold after 15 min PTZ exposure and nearly 30-fold after 30 and 60 min exposure (Fig. 5A). Next, whole-mount *in situ* hybridization was performed to examine the spatial expression of *c-fos* mRNA following chemically evoked seizures. In untreated zebrafish larvae ($n=20$), very low levels of *c-fos* mRNA were detected (Fig. 5B). After a 1 h treatment with 15 mM PTZ, zebrafish ($n=20$) displayed a substantial induction of *c-fos* mRNA, particularly in the optic tectum and cerebellum (Fig. 5C).

DISCUSSION

The key finding of this study is that PTZ, a common convulsant, reliably elicited seizures in zebrafish larvae. Previous studies demonstrated the usefulness of rodent PTZ models for AED discovery (Ferrendelli et al., 1989; Shank et al., 1994; Loscher, 2002) and analysis of seizure-generating mechanisms (Mirski and Ferrendelli, 1986; Barkai et al., 1994; Psarropoulou et al., 1994). Our observations indicate that in a simple genetically tractable vertebrate PTZ-induced seizures result in behavioral, electrographic and IEG expression changes that closely resemble those reported in rodents. Furthermore, chemically induced seizures in zebrafish can be inhibited in a concentration-dependent fashion by common AEDs. Importantly, we found that both seizure-inducing and seizure-preventing agents could be bath-applied to intact organisms, while they were being held in small volumes of liquid. This feature may enable high-throughput screens for the systematic discovery of chemical or genetic modifiers of epilepsy.
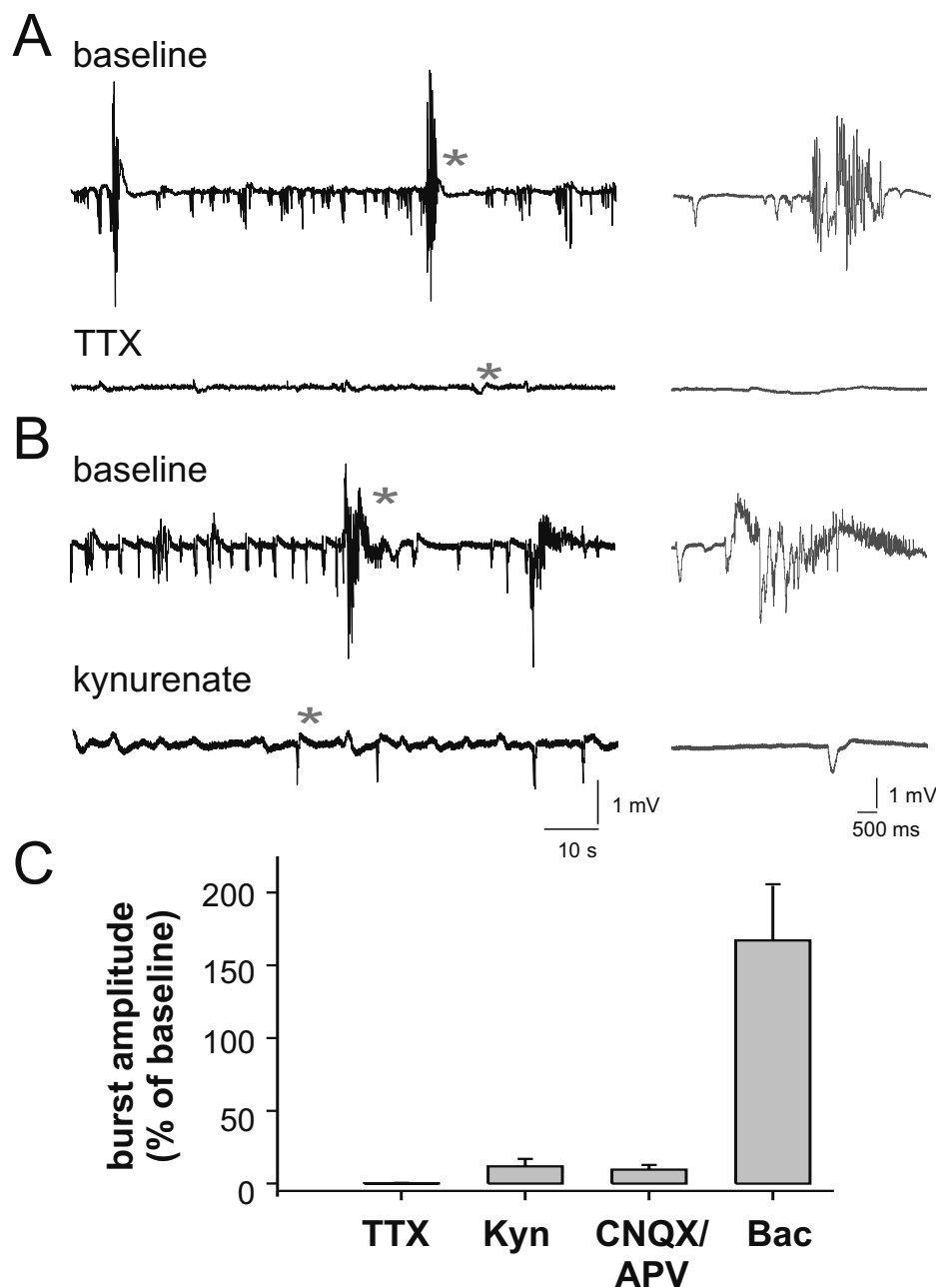
Fig. 3. Synaptic pharmacology of electrographic seizure activity evoked in zebrafish larvae. (A) Representative tectal field recordings obtained from a wild-type zebrafish bathed in normal Ringer's medium plus 15 mM PTZ (baseline) and 45 min after application of tetrodotoxin to block presynaptic action potential generation (TTX). Top trace is a 1 min recording epoch and the trace at right is an isolated ictal-like burst discharge (asterisk). (B) Similar set of traces for application of a non-specific glutamate receptor antagonist (kynurenate). (C) Bar graph of ictal burst amplitude following application of a synaptic transmission modulator: Bac, baclofen (*n*=3); Kyn, kynurenate (*n*=4); TTX, tetrodotoxin (*n*=3). Drug effects are plotted as the percentage of baseline response obtained after 45 min of drug perfusion (e.g. % of baseline). Data are presented as mean±S.E.M.

Our combined results suggest that zebrafish larvae serve as a novel model system for epilepsy research.

A critical feature of any animal model of epilepsy, and a "gold standard" in clinical diagnosis of this disorder, is the presence of abnormal electrical discharges in a CNS. As demonstrated here, for the first time, PTZ-induced epileptiform burst discharges recorded in zebrafish optic tectum appear remarkably similar to those reported in tissue slices or electrocorticography recordings from rodents. For ex-

ample, acute hippocampal slices exposed to PTZ (2–10 mM) exhibit spontaneous interictal-like burst discharges (20–70 ms in duration) at a frequency of six to 12 per min (Oliver et al., 1977; Piredda et al., 1986; Leweke et al., 1990). In zebrafish exposed to 15 mM PTZ, we initially observed interictal-like discharges (approximately 1000 ms duration) at a frequency of 27–30 per min. Note that the slightly higher drug concentrations used in fish presumably reflect differences in drug diffusion across a cut tissue slice
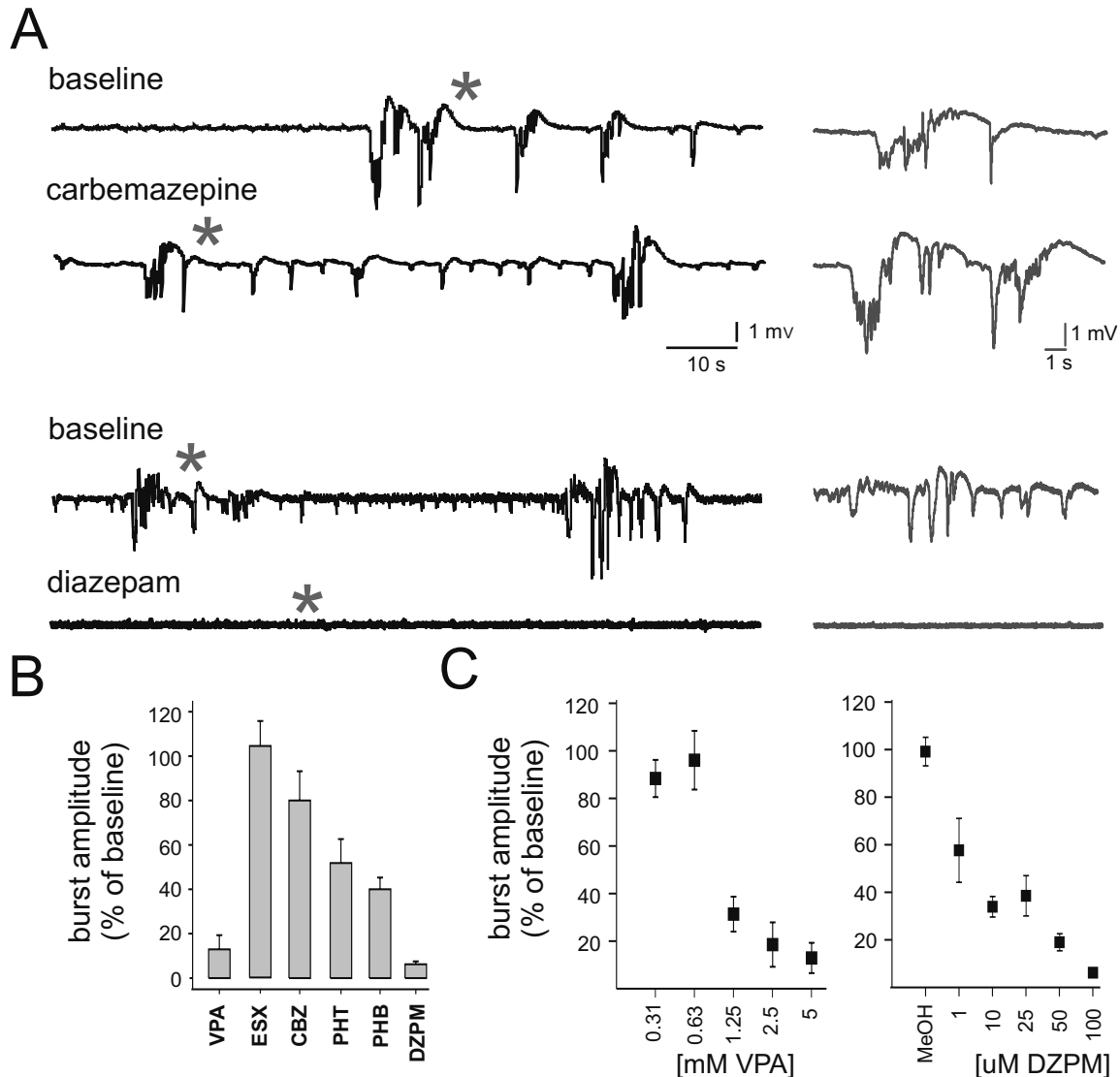
Fig. 4. AED pharmacology of electrographic seizure activity evoked in zebrafish larvae. (A) Representative tectal field recordings obtained from a wild-type zebrafish bathed in normal Ringer's medium plus 15 mM PTZ (baseline) and 45 min after application of an AED, carbamazepine (top traces) or diazepam (bottom traces). In both sets of recordings the top traces are 1 min recording epochs and the traces at right indicate isolated ictal-like burst discharge (asterisk). (B) Bar graph of ictal burst amplitude following application of a synaptic transmission modulator: CBZ, carbamazepine (*n*=4); DZPM, diazepam (*n*=3); ESX, ethosuximide (*n*=7); PHB, phenobarbital (*n*=5); PHT, phenytoin (*n*=4); VPA (*n*=6). Drug effects are plotted as the percentage of baseline response obtained after 45 min of drug perfusion. Data are presented as mean±S.E.M. (C) Plots of the concentration-dependent inhibition of burst amplitude for VPA and diazepam (DZPM). Three to five fish were used for each drug concentration tested.

and an *in vivo* preparation, and it is further possible that an intact zebrafish allows for generation of more robust (e.g. longer duration/higher frequency) seizure activity than can be obtained *in vitro*. Indeed, ongoing exposure to PTZ elicited epileptiform activity with prominent interictal- and ictal-like components (see Fig. 2A) suggesting that intact agar-immobilized zebrafish larvae are a suitable system to study the complex molecular/cellular events associated with generation (or propagation) of seizures.

Further evidence that PTZ-induced seizures in zebrafish are similar to those reported in rodents can be derived from our pharmacology studies. From the wealth of rodent data available it is well established that valproate and benzodiazepines (e.g. diazepam, clonazepam, fluraz-epam, nitrazepam and oxazepam) effectively inhibit PTZ-induced seizures and other AEDs are less effective (Oliver et al., 1977; Krall et al., 1978; Swinyard et al., 1986; Piredda et al., 1986). Similarly, our data show that valproate and diazepam effectively abolished zebrafish seizure activity in a concentration-dependent fashion whereas phenytoin, ethosuximide, carbamazepine and phenobarbital were less effective. Because pharmacokinetic data on AED distribution in zebrafish larvae are not currently available (a limitation of this system), all AEDs and other drug concentrations used in this study were chosen empirically (and are loosely based on EC_{50} values reported in rodent studies). Because PTZ is thought to act via blockade of GABAergic systems, in particular inhibition
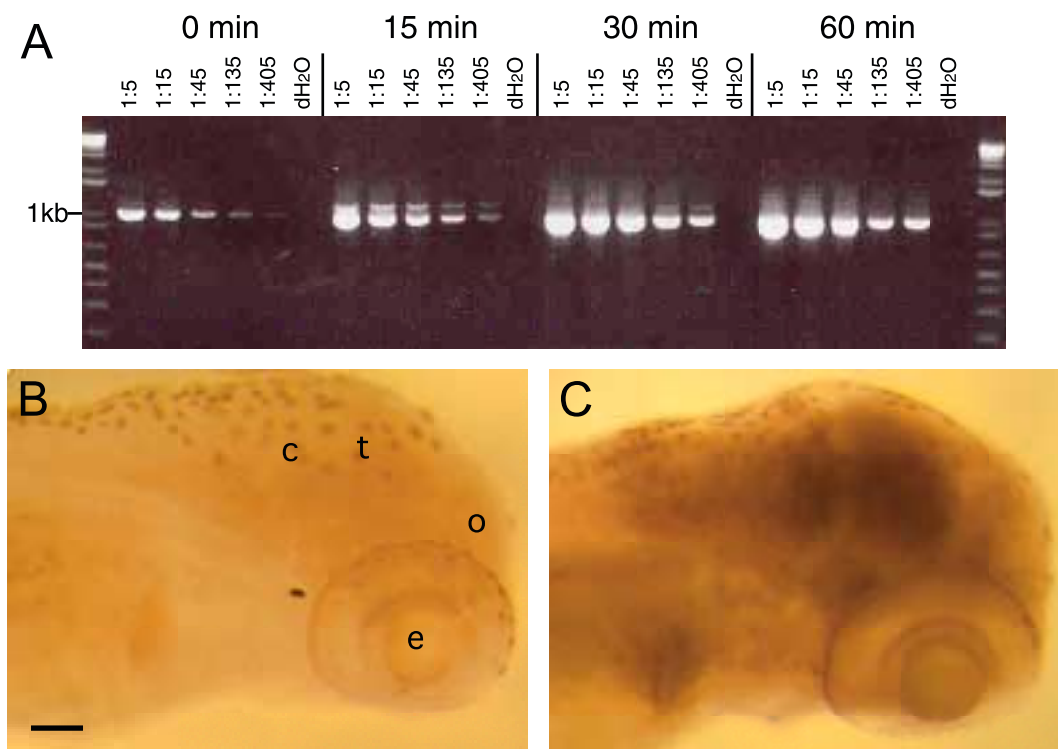
Fig. 5. Seizure-induced *c-fos* expression in zebrafish larvae. (A) RT-PCR was performed on zebrafish larvae (7 dpf) that were untreated (0 min) or treated for 15, 30, and 60 min with 15 mM PTZ. Serial dilutions of cDNA at each time point were amplified by PCR to show a semi-quantitative increase in *c-fos* mRNA expression over time. (B and C) Whole-mount *in situ* hybridization was used to show increased *c-fos* mRNA expression within the brain after exposure to PTZ. Note the low *c-fos* expression in untreated larvae (B) versus the high *c-fos* expression, particularly in the optic tectum and cerebellum, in larvae treated for 1 h in 15 mM PTZ (C). c, cerebellum; e, eye; o, olfactory bulb; t, optic tectum. Scale bar=50 μm.

of GABA-A receptor-mediated inhibitory postsynaptic potentials (Leweke et al., 1990), PTZ exposure (i.e. Metrazol test) is considered a model of acute, generalized clonic-type seizures (Ferrendelli et al., 1989; Meldrum, 2002). PTZ screening of anticonvulsants in the 1940s led to the discovery of trimethadione and subsequent work clarified the anticonvulsant properties of VPA and benzodiazepines (Krall et al., 1978a,b; Stone and Javid, 1979). Combining PTZ testing with the availability of genetically altered mice provided additional information regarding molecular pathways involved in generation (or termination) of seizures. A similar strategy using mutant, transgenic or morpholino-injected zebrafish (Stuart et al., 1990; Trowe et al., 1996; Baker et al., 1997; Brent and Drapeau, 2002) could be equally productive in uncovering novel antiepileptic targets.

In addition to exhibiting an AED pharmacological profile identical to rodents, zebrafish PTZ seizures are mediated by glutamatergic synaptic transmission (Velisek et al., 1990, 1991). For example, blocking presynaptic action potential generation (with TTX) or postsynaptic glutamate receptors (with kynurenate or CNQX/APV) abolished PTZ-induced bursting. These findings are consistent with the conclusion that zebrafish PTZ-induced seizures require excitatory synaptic neurotransmission whereas baclofen (enhanced bursting) data suggest that activation of GABA-B receptors is not necessary. That glutamatergic synaptic transmission is required for zebrafish seizures is

not surprising, given that glutamate is the primary excitatory neurotransmitter in all vertebrates. The complexity of induced electrical activity (i.e. interictal- and ictal-like components) observed in zebrafish, the requirement for postsynaptic glutamate receptors, and the genetic and chemical tractability of this organism suggest a wide range of studies aimed at analysis of seizure genesis are possible. Furthermore, because the zebrafish CNS is less complex (e.g. absence of a neocortex and fewer neurons) than that of a rodent, further analysis of evoked seizure events could provide important insights into the minimal cellular processes required for generation of complex epileptiform electrical discharge.

Advantages of a zebrafish seizure model are further highlighted by our behavioral studies. Using an observational approach we initially noted that zebrafish bath-exposed to PTZ progress through distinct stages starting with increased activity and culminating in clonus-like convulsions with loss of posture. The three seizure stages observed in zebrafish are reminiscent of behavioral seizure stages described in rodents (Racine, 1972), and like the widely used "Racine scale" provide a framework for analysis of epileptic behaviors in zebrafish. That these behaviors could be further analyzed using a high-speed locomotion tracking system allows an even greater level of behavioral quantification than is possible with investigator observation. For example, seizure severity (monitored as a distance-traveled or movement score) in wild-type or ge-

netically modified zebrafish larvae can be systematically quantified using this relatively simple approach.

A final advantage of our zebrafish model is that seizure-induced changes in gene expression can be monitored. Using RT-PCR and *in situ* hybridization techniques we demonstrated that PTZ-induced seizures up-regulated *c-fos* expression. Because IEG expression is a commonly used technique to identify brain regions participating in seizure generation and propagation (Morgan et al., 1987; Dragunow and Robertson, 1987; Simler et al., 1999), these types of data will allow us to map the zebrafish brain circuitry involved. As a starting point, our studies identified optic tectum and cerebellum as areas of significant seizure-induced *c-fos* expression. That tectal neurons are active also fits with our electrophysiological data (Fig. 2) and suggests an important role for this brain structure in seizure genesis. Future studies to analyze a wider spectrum of seizure-induced gene expression changes in PTZ-exposed zebrafish will further elucidate underlying cellular mechanisms. Investigators are cautioned, at this time, to interpret these data carefully as potential differences between zebrafish and human CNS function are not completely understood.

CONCLUSIONS

Genetically tractable model systems such as the bang-sensitive *Drosophila* mutants have been used to study genes that modify seizure susceptibility (Pavlidis and Tanouye, 1995; Kuebler and Tanouye, 2000; Kuebler et al., 2001). These studies utilize a stimulation-induced afterdischarge model and highlight roles for membrane-bound ion channels in seizure genesis. However, the limitation of this approach is that freely behaving seizure-like activity combined with evidence of abnormal electrical discharge in a CNS structure has not been demonstrated. In contrast, chemically induced seizures in zebrafish exhibit all behavioral, electrophysiological, and molecular components that would be expected from a rodent seizure model. More importantly, our model can easily be combined with a "forward-genetic" screening strategy (Nusslein-Volhard and Wieschaus, 1980; Malicki, 2000) to uncover genes that modify the development (or perhaps result in prevention) of epilepsy. An additional strategy could take advantage of the ease with which drugs can be bath-applied to intact zebrafish larvae. Zebrafish are also a model system of choice for "forward-chemical genetic" approaches (Specht and Shokat, 2002; Shin and Fishman, 2002) and our findings suggest that zebrafish could be used to screen AED candidates. Zebrafish epilepsy research based on the results described herein may lead to a better understanding of the molecular basis of epilepsy and epileptogenesis.

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