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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.**

Key words: *Danio rerio*, epilepsy, extracellular, c-Fos, interictal, ictal.

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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Abbreviations: AED, antiepileptic drug; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; dpf, days postfertilization; IEG, immediate early gene; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazole; RT-PCR, reverse transcription–polymerase chain reaction; VPA, valproic acid.

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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 sequence, 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

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