Chapter 24

Assessing Epilepsy-Related Behavioral Phenotypes in Adult Zebrafish

Daniel Desmond, Evan Kyzar, Siddharth Gaikwad, Jeremy Green, Russell Riehl, Andrew Roth, Adam Michael Stewart, and Allan V. Kalueff

Abstract

Over the past decades, zebrafish have been presented as a novel and valuable tool for modeling complex human diseases. Epilepsy is a serious brain disorder with multiple genetic and environmental causes. Our poor understanding of its pathogenesis requires novel paradigms and model organism for translational experimental epilepsy research. Seizure-like behavior has already been studied in both larval and adult zebrafish models, including genetically modified strains and convulsant drugs. This protocol describes how to quantify seizure-like behavioral phenotypes commonly observed in adult zebrafish models of epilepsy.

Key words: Epilepsy, Seizures, Locomotion, Disease models

1. Introduction

The zebrafish (*Danio rerio*) is a useful model organism for studying complex human pathology (1–5). Their high fecundity and low cost (compared to other popular animals, such as mice and rats) make this aquatic species a simple, cost-effective and high-throughput model for genetic research (2), drug discovery (3), and disease modeling (6).

Epilepsy is a common neurological disorder caused by pathological over-excitation in the brain, with a graded characteristic behavioral (seizures) and neurophysiological (EEG spikes) responses (7). In animals, epilepsy has long been modeled is various rodent paradigms, including genetically modified or convulsant-exposed animals (Table 1). Revealing striking similarities with rodent models of experimental epilepsy, recent studies have successfully modeled seizures in larval and adult zebrafish (Tables 1–4, also see (8)).

Allan V. Kalueff and Adam Michael Stewart (eds.), *Zebrafish Protocols for Neurobehavioral Research*, Neuromethods, vol. 66,
DOI 10.1007/978-1-61779-597-8_24, © Springer Science+Business Media New York 2012

t1.1 **Table 1**
t1.2 **Examples of rodent and zebrafish studies using similar experimental**
t1.3 **models of epilepsy**

t1.4 t1.5 Experimental models of epilepsy	Rodent studies	Zebrafish studies (see Table 2 for details)
t1.6 PTZ-evoked seizures	(19, 20)	(9)
t1.7 Kainate-evoked seizures	(21)	(8)
t1.8 Picrotoxin-evoked seizures	(22)	(13)
t1.9 Caffeine-evoked seizures	(23)	(13)
t1.10 RDX-evoked seizures	(24)	(12)

t2.1 **Table 2**
t2.2 **Examples of recent studies of epilepsy in larval and adult zebrafish**
t2.3 **(PTZ—pentylenetetrazole)**

t2.4 Study (see Table 3 for details)	Drugs/doses	References
t2.5 *Larval pharmacological models*		
t2.6 Seizure behavioral and c-fos assays	2.5–15 mM PTZ[a]	(9)
t2.7 Screening for seizure liability	0.0625–1 mM (25 compounds)[a]	(25)
t2.8 Seizure behavioral assays	200 μM kainate and 10 mM PTZ	(17)
t2.9 *Larval genetic models*		
t2.10 A large-scale mutagenesis screen	15 mM PTZ[a]	(4)
t2.11 Spontaneous seizures in a *mind bomb* mutant	–	(26)
t2.12 Knockdown of zebrafish Lgi1a gene	2.5 mM PTZ[a]	(27)
t2.13 *Adult pharmacological models*		
t2.14 Seizure behavioral assays	1–8 mg/kg kainate[b]	(8)
t2.15 Electrophysiological recordings	15 mM PTZ	(28)
t2.16 t2.17 Seizure behavioral, cortisol, and c-fos assays	250 mg/L caffeine, 11 mM PTZ, 0.17 mM picrotoxin[a]	(13)
t2.18 Seizure behavioral, cortisol, and c-fos assays	1 mM RDX[a]	(12)

t2.19 [a]Drug administered systemically, via water immersion
t2.20 [b]Intraperitoneal injection

Larval zebrafish have traditionally been used to demonstrate physiological and developmental mechanisms of brain function (4, 5, 9, 10). They present several advantages for research, including transparency, small size (to be visualized in a 96-well plate) (5), convenient mode of drug delivery via immersion (10), free locomotion, functionality of most organ systems within 3–5 days post fertilization (11), as well as the ability to inject proteins, DNA or RNA to modify gene expression (5). While epilepsy-like phenotypes have been modeled in larvae using both electrophysiological

Table 3
Summary of relevant endpoints in adult zebrafish models of epilepsy (see Fig. 1 for graphic examples and Table 4 for cross-species comparisons)

Phenotypes	Definition	Comments
Behavioral endpoints[a]		
Hyperactivity (*latency to onset, frequency, and duration measures*)		
Hyperactivity bursts	Episodes of abnormally fast erratic-like swimming, often followed by bouts of immobility-like freezing	Reflects hyperlocomotion—increased motor activity during the early stages of seizures
Distance traveled	Total distance (m) traveled during the test time	Reflects hyperlocomotion—increased motor activity during the early stages of seizures
Velocity	Average velocity (m/s) during the test time	Reflects hyperlocomotion—increased motor activity during the early stages of seizures
Erratic turning	Rapid turning of the zebrafish head in uncoordinated, unplanned fashion	Reflects erratic movements, typical for early stages of seizures
Twitching	Rapid movements of zebrafish body	Reflects mild neurological deficits associated with seizures
Convulsions and associated behaviors (*latency to onset, frequency, and duration measures*)		
Cork-screw swimming	Spiral uncoordinated swimming with high speed	Reflects significant neurological deficits associated with seizures
Circular swimming	Repetitive swimming in a circular direction	Reflects significant neurological deficits associated with seizures
Abnormal body position	The contortion of the fish body, swimming with bent body	Reflects uninstructed response of a zebrafish peripheral nervous system to seizure
Loss of body posture, paralysis and death (*latency to onset, frequency, and duration measures*)		
Loss of posture	Loss of dorso-ventral balance	Reflects major neurological deficits associated with seizures
Immobility (due to posture control loss)[b]	Cessation of movement except for continued respiratory and ocular motion	Reflects major neurological deficits associated with seizures
Mortality (%)	Percent of fish not surviving the treatment	Represents a terminal state of severe epilepsy
Death latency	Latency to death	Represents a terminal state of severe epilepsy
Physiological endpoints[c]		
Brain electric activity	Frequency and duration of epileptiform-like burst discharges	Electrophysiological recordings directly measure epilepsy-like activity in the brain
c-fos gene expression	Brain *c-fos* gene expression level vs. controls	The expression of early proto-oncogene *c-fos* correlates with neuronal excitation, and is elevated during seizures
Cortisol levels	Whole-body cortisol levels assessed by standard endocrine (e.g., ELISA) assays	Endocrine deficits (e.g., elevated glucocorticoids) are common in epilepsy, and may represent a novel phenotypes related to this disorder

[a]Based on classification developed by (8) and (17), with modifications (see (13) for details)
[b]This index can be similar to freezing behavior mentioned earlier, but is persistent and not followed by bursts of active locomotion/hyperactivity
[c]Assessing zebrafish physiological endpoints are not discussed in this chapter; see (9) and (13) for details

[AU1]

t4.1 **Table 4**

t4.2 **An example of seizure scoring system that can be used in zebrafish models**

t4.3 Rodent seizure-like responses	Zebrafish seizure-like responses
t4.4 No aberrant response (normal swimming)	No aberrant response (normal swimming)
t4.5 Initial freezing	Initial freezing with hyperventilation
t4.6 t4.7 Head nodding, isolated twitches and oro-facial seizures, hyperlocomotion	Hyperlocomotion
t4.8 t4.9 t4.10 t4.11 t4.12 Clonic seizures (rhythmic contractions of forelimbs and/or hind-limbs)	Circular and/or spiral swimming, rapid movements from left to right (erratic movements), abnormal spasms-like muscular contractions, rapid whole-body clonic-like convulsions
t4.13 t4.14 t4.15 Tonic seizures (rigid extension of the fore- and/or hind-limbs) with or without posture loss	Tonic seizures with rigid extension of the body, loss of body posture, sinking to the bottom of the tank, spasms for several minutes
t4.16 t4.17 t4.18 Death (the lack of heart beating upon manual check)	Death (total immobility with the lack of eye/gill movements for several minutes upon visual inspection)

t4.19 t4.20 Note the similarity between the epilepsy-like phenotypes in rodents (based on Racine's score (19, 20, 29), with modifications) and zebrafish (based on (8), with modifications)

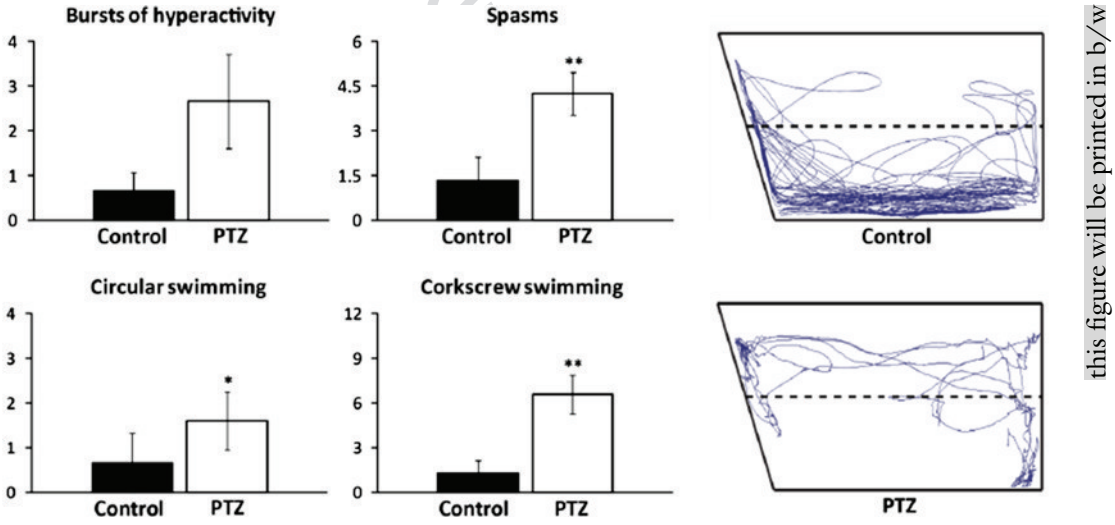

Fig. 1. Typical examples of seizure-like behaviors induced by acute exposure to 11 mM pentylenetetrazole (PTZ) and recorded in adult zebrafish in the observation tank for 6 min (based on (13), with modifications). Representative traces were video-recorded and visualized using Noldus Ethovision software. *$P<0.05$, **$P<0.005$, *U*-test vs. control fish ($n=12$–15 per group).

techniques and behavioral observations (4), several limitations of this model include difficulties to detect seizures due to small object size and somewhat under-developed neural, endocrine, and motor systems (10, 12).

Adult zebrafish are also used as an effective model for investigating brain disorders (see previous chapters of this book), including epilepsy (see Tables 2 and 3 for a detailed summary). Some characteristic behaviors valuable for assessing seizure-like phenotypes in adult zebrafish include erratic, spasm-like, circular, and cork-screw swimming (Table 3, Fig. 1). The utility of these behavioral phenotypes is further enhanced with the advent of video-recording technology, thereby maximizing detection accuracy while allowing for an un-biased automated and high-throughput quantification.

Finally, it is important to recognize that epilepsy and seizures are inter-related, but not identical, biological phenomena. For example, some forms of epilepsy may be observed without seizures, whereas some seizures can be unrelated to epilepsy. While this aspect deserves further studies in various paradigms, the present chapter will focus on modeling seizure-related behaviors in adult zebrafish, eschewing electrophysiological recordings of brain activity and other physiological markers (comprehensively evaluated in (9) and (13), see Table 3).

2. Materials

2.1. Animals

Animals (e.g., *short-fin* wild type zebrafish) can be obtained from a local commercial distributor or raised in house. Adult fish (e.g., ~5–8 months old, of both sexes, ~50:50%) can be housed in groups of 20–25 fish per 40-L tank, filled with filtered system water maintained at 25–27°C. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 14:10-h cycle (e.g., on: 6:00 h; off: 20:00 h) according to the standards of zebrafish care. All fish must be experimentally naïve, and can be fed twice daily (e.g., Tetramin Tropical Flakes, Petco Inc., San Diego, CA). Animal experiments must be approved by IACUC, and adhere to National and Institutional guidelines and regulations.

2.2. Reagents and Equipment

- Experimentally naïve adult zebrafish (as in Sect. 2.1)
- Standard observation tanks to assess seizure-like responses (e.g., 1.5-L trapezoidal tank 15 height × 28 top × 23 bottom × 7 cm width; Aquatic Habitats, Apopka, FL)
- Treatments (e.g., convulsant drugs, see below) to evoke seizures

- Exposure beakers (e.g., plastic 3-L containers) for drug pretreatment
- Trained observers (inter-rater reliability >85%, determined by Spearman correlation)
- Web-camera and video-tracking system (similar to those previously described in different chapters of this book)

3. Experimental Setup and Typical Results

Most studies using adult zebrafish involve simple behavioral observations following a specific experimental manipulation, such as acute exposure to a convulsant drug. Commonly used epileptogenic agents include pentylenetetrazole (PTZ), picrotoxin, caffeine and kainate (Tables 1 and 2), all known to promote seizures at high convulsant doses in humans and rodents. Due to its ability to evoke prominent generalized seizures in various species, PTZ can be recommended as a "reference" standard convulsant agent for pilot studies. A continuum of typical behaviors reflecting epilepsy-like states is briefly summarized in Table 4.

Evoked by various convulsant drugs, seizure-related endpoints include swimming in an erratic manner, cork-screw (spiral) and circular swimming, rapid twitching, spasms, bent body, immobility or freezing, loss of posture control, and death (see Tables 3 and 4 for a comprehensive catalogue). These behaviors can be assessed by both manual observation and video-recording in terms of (1) latency to onset, (2) frequency, (3) duration, and (4) occurrence (% of animals displaying the respective phenotype). If using a seizure scoring system (e.g., (9) or (8)), an average score for each group can be used as an additional index of epilepsy severity. The seizure scoring system used can be flexible, depending on the goals of the study. For example, a global analysis of robust phenotypes may utilize a relatively simple scoring system (e.g., 0—normal swimming, 1—hyperactivity, 2—clonic-like swimming, 3—tonic-like swimming) (9). A more complex scoring system may be used for detailed analyses of seizure responses, ranging between 0 (no seizures) and 5 (death), as shown in Table 4 (e.g., a score of 4 will be recorded for surviving fish with tonic seizures, and a score of 5 for the fish showing seizures, but not surviving the treatment). Finally, ED_{50} may be calculated for all these endpoints, similar to standard approaches traditionally used in toxicology research.

3.1. Procedure

- Expose individual zebrafish to a convulsant drug (experimental group) or vehicle (control group) for a specific period of time (e.g., 5–20 min) in the pretreatment beaker. If testing anticonvulsant drugs in zebrafish models, an additional pretreatment

procedure may be needed to administer these drugs prior to applying a convulsant agent (to evoke seizures).

- Place the fish in the observation tank, and observe their seizure-related behavioral responses (Tables 3 and 4) manually and using video-recording, for 5 min. Remove fish from the tank when finished, and analyze data, to generate diagrams and visualize representative traces (see Fig. 1 for examples).
- If necessary, additional (physiological) endpoints can be assessed (Table 3). For example, brain c-fos expression or whole-brain cortisol levels can be assayed, as specified in (9) and (14, 15), respectively.

3.2. Statistical Analysis

The nonparametric Wilcoxon–Mann–Whitney *U*-test can be used for comparing two groups (parametric Student's *t*-test may be used for data distributed normally). For more than two groups, apply analysis of variance (ANOVA), followed by an appropriate post hoc test (e.g., Tukey, Dunn, Newman–Keuls, or Dunnet test) (15).

4. Notes

- Detecting effective convulsant doses for a drug in zebrafish studies can be a challenging task. To identify a suitable dose range for a pilot study, consult published literature or search online the Zebrafish Neurophenome Project (ZNP) Database (see chapter by Zapolsky in this book) for various convulsants tested in zebrafish models. For example, if a laboratory plans to test a novel compound and does not know its effective doses (since it has not yet been tested in adult zebrafish), examine the literature for this drug in larval models (if any) and use a similar (or higher) doses for a pilot study in adult zebrafish. Reduce the dose if it appears to be toxic or lethal. Moreover, the knowledge of the basic pharmacology of various drugs may also be useful. For example, knowing an effective convulsant dose of drug A (e.g., 11 mM PTZ) in zebrafish and its relative potency compared to another drug B (e.g., picrotoxin >> PTZ), it is likely that significantly lower doses of drug B can induce seizures in pilot studies in fish (as was confirmed using 0.17 mM in a recent study (13)).
- In addition to seizure-induced hyperactivity per se, zebrafish may display altered locomotion, for example, showing more erratic behavior due to high baseline anxiety, "transfer" anxiety, or fear evoked by external startling stimuli. To avoid startling the fish, all sounds and movements produced by the investigators in the experimental room should be kept to an absolute minimum during the testing. Consider using blinds

that block visual stimuli from the observation tank area. To minimize transfer anxiety/stress, ensure that animals had sufficient time to acclimate to the testing room prior to testing. Other factors, such as differences in water temperature or excessive net stress can markedly affect locomotion, either reducing it (freezing) or evoking erratic behavior and bursts of hyperactivity, which all can be misinterpreted as seizure-like responses. If using highly anxious animals, consider a different strain of zebrafish for the experiment. To identify a suitable zebrafish strain, consult recently published literature or search online the ZNP Database for strain differences in zebrafish behavior and activity.

- As already mentioned, some specific behaviors, such as circling swimming, are commonly seen during experimental epilepsy in zebrafish models. Note, however, that similar phenotypes may also be evoked by some drugs independent of seizures. For example, glutamatergic drugs, such as ketamine and MK801, evoke circling behaviors in zebrafish (16) without causing seizures, and even have antiepileptic effects in some zebrafish models (8). Therefore, a complex analysis of multiple endpoints is needed, before a conclusion is made about the ability of a certain drug to modulate seizures. Electrophysiological validation will also be needed, to avoid incorrect interpretation of results.
- With the complexity of phenotypes associated with human ictal pathology, interpreting epilepsy-like responses in zebrafish may be a challenging task. Tables 3 and 4 provide a useful framework for different types of seizure-like behavior observed in zebrafish. However, as the number of convulsant agents or genetic mutations screened in zebrafish continue to grow, it is possible that some rare, less common phenotypes (e.g., unique head-shake motions observed in larvae following kainate exposure (17)) may also be observed in zebrafish epilepsy models. Carefully examine unusual behaviors observed in your models, and try to interpret them in an unbiased manner. As already mentioned, a more thorough electrophysiological validation will help make correct interpretation of the results after the initial behavioral screening.
- Note that some convulsant drugs (e.g., strychnine or RDX) may have poor solubility in water. If using water immersion to administer the drug, use a solvent (e.g., 3 mL of 100% dimethyl sulfoxide, DMSO) to dissolve the drugs, prior to diluting the solution with water to obtain the 3-L exposure mix. Accordingly, control zebrafish should be exposed to water containing 0.1% DMSO. Note that at this concentration DMSO does not evoke any abnormal seizure-like responses, and therefore can be used as vehicle control for such studies.

Alternatively, consider intraperitoneal (i.p.) injection for such drugs (see (18) for methodological details). This route may also be useful to mimic rodent models, since various convulsant drugs are usually given to them by i.p. injections (8).

5. Conclusion

Animal models continue to serve as invaluable tool for studying human disease physiology and pathology. The utility of zebrafish as a model for epilepsy research is growing rapidly, and promises to continue, as traditional models are being complemented with high-throughput zebrafish models. With continued addition of chemical, biochemical, and genetic manipulations, coupled with data-dense behavior analysis, further applications of larval and adult zebrafish models in experimental epilepsy research will improve our understanding of this disorder, also fostering the development of new antiepileptic therapies.

Acknowledgments

The study was supported by Tulane University Intramural funds, Zebrafish Neuroscience Research Consortium (ZNRC), LA Board of Regents P-Fund and Tulane University Synergy grant to AVK.

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