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Zebrafish as a model for studying genetic aspects of epilepsy

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Despite a long tradition of using rats and mice to model epilepsy, several aspects of rodent biology limit their use in large-scale genetic and therapeutic drug screening programs. Neuroscientists interested in vertebrate development and diseases have recently turned to zebrafish (*Danio rerio*) to overcome these limitations. Zebrafish can be studied at all stages of development and several methods are available for the manipulation of genes in zebrafish. In addition, developing zebrafish larvae can efficiently equilibrate drugs placed in the bathing medium. Taking advantage of these features and adapting electrophysiological recording methods to an agar-immobilized zebrafish preparation, we describe here our efforts to model seizure disorders in zebrafish. We also describe the initial results of a large-scale mutagenesis screen to identify gene mutation(s) that confer seizure resistance. Although the adaptation of zebrafish to epilepsy research is in its early stages, these studies highlight the rapid progress that can be made using this simple vertebrate species.

Epilepsy is a common neurological disorder marked by abnormal electrical discharges in the brain (seizures) and is typically manifested by sudden brief episodes of altered or diminished consciousness, involuntary movements, or convulsions. Although acquired forms of epilepsy are common, genetic factors also play a significant role. Recent progress in human genetics has provided evidence that single gene mutations resulting in ion channel dysfunction, neurotransmitter receptor alterations or brain malformations predispose an individual to seizure activity. Mutant mice, including seizure-prone strains that emerged spontaneously through selective breeding and targeted mutations created by transgenic or homologous recombination techniques, have contributed to the identification of an additional 80 or more epilepsy genes (Noebels, 2003).

If gene mutations can lead to epilepsy, it is also possible that there are gene mutations that modify the disease in a protective fashion. This concept is based, in part, on the following observations: (1) rodents exhibit strain differences in their sensitivity to common convulsant agents, with some animals showing very little epilepsy-related hippocampal pathology (Freund et al., 1987; Schauwecker and Steward, 1997; Ferraro et al., 1998; Steward et al., 1999); (2) evidence that febrile seizure episodes lead to acquired epilepsy in some children but not others (Nelson and Ellenberg, 1978; Knudsen, 2000); and (3) observations of delayed seizure onset in select gene knockout mice (Kokaia et al., 1995; Hiroi et al., 1998). Although theoretically possible, the identification of a gene mutation(s) that confers 'protection' against epilepsy-related pathologies, or 'resistance' to the consequences of seizures, presents a number of practical problems. Foremost among them is the necessity for a large sample population. The mammalian epilepsy models (primarily rodent) that are available require a considerable cost for daily maintenance, making them unsuitable for the large-scale genetic screens needed to systematically identify rare genes. Human studies rely on isolated families manifesting specific symptoms of the disease, and large families of 'normal' individuals with no history of seizure activity are not readily available for genetic testing. By contrast, zebrafish offer a model system that is ideally suited for large-scale mutagenesis screening. *Danio rerio* (zebrafish) are a small vertebrate species that that can be maintained in captivity at a relatively low cost and effort (Westerfield, 2000). They can be easily subjected to chemical mutagens and large numbers of mutant zebrafish, which can produce hundreds of offspring from a single spawning, can be generated quickly (Granato and Nusslein-Volhard, 1996). Given these advantages, zebrafish hold tremendous clinical potential for the identification of disease-causing and disease-modifying genes (Dooley and Zon, 2000; Shin and Fishman, 2002).

Genetics of epilepsy

Because the excitability of individual neurons depends on a variety of membrane-bound ion channels, it is not surprising that ion channel mutations are implicated as a cause of epilepsy in humans and experimental models. Progress in the positional cloning of human epilepsy genes associated with idiopathic epilepsies (e.g. those of unknown origin) suggests that ion channel mutations can result directly in enhanced seizure susceptibility. Mutations in two voltage-gated potassium channel genes (*KCNQ2* and *KCNQ3*) are responsible for benign familial neonatal convulsions (BFNC), an inherited form of epilepsy in the neonate (Biervert et al., 1998; Singh et al., 1998). A mutation in the voltage-gated sodium channel β1 gene (*SCN1B*) confers susceptibility to febrile seizures and generalized epilepsies generalized epilepsy with febrile seizures plus (GEFS+) in a large Australian family (Wallace et al., 1998); additional voltage-activated sodium (Na_v) channel mutations also result in a GEFS+ phenotype (Escayg et al., 2001; Sugawara et al., 2001). Calcium and γ-aminobutyric acid type A ($GABA_A$) receptor mutations have also been identified in humans (Noebels et al., 1990; Jouvenceau et al., 2001; Harkin et

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al., 2002). In mice, three autosomal recessive models of absence epilepsy (tottering, lethargic and stargazer) arise from mutations in genes encoding three types of calcium channel subunits (Noebels et al., 1990; (Fletcher et al., 1996; Burgess et al., 1997). These findings provide compelling evidence that gain-of-function (*SCN1B*) or loss-of-function (*KCNQ2/3*) mutations lead to neuronal hyperexcitability and/or epilepsy. Interestingly, homologs for approximately 85% of the known epilepsy genes can be found in zebrafish. Given these similarities, and the wide variety of techniques available to modify gene expression in zebrafish [e.g. transgenesis, mutagenesis, TILLING (targeting induced local lesions in genomes), morpholino antisense oligonucleotides, RNA interference (RNAi), etc.], it is entirely possible that zebrafish models can be generated for all single gene mutations causing epilepsy.

Zebrafish as a model system

Zebrafish are now widely recognized as an extremely valuable model system with proven utility in the analysis of brain development and function (Fetcho, 2007; Holder and Xu, 2008; Veldman and Lin, 2008). As simple vertebrates, they share many similarities with commonly used laboratory species such as rats or mice, and are closer in the phylogenetic tree to humans than either nematodes (*Caenorhabditis elegans*) or fruit flies (*Drosophila melanogaster*). The features of zebrafish biology that make this a particularly attractive genetic and molecular experimental model include the rapid generation times (3-4 months) and the ability to generate clutch sizes of 50-200 embryos from a pair of adult zebrafish almost every week. Larval life begins around 72 hours post-fertilization (when the fish are nearly freely swimming) and proceeds over a period of approximately 27 days. The developing zebrafish is transparent and the larval brain shows signs of an everted telencephalic organization by as early as 5 days post-fertilization (dpf), with an externally lying ventricular region and well-developed telencephalic hemispheres. In the larval zebrafish brain, all of the major adult subdivisions are present and the optic tectum, the largest midbrain structure in zebrafish, begins to take on a layered 'cortical' organization at 5 dpf. Cell types that are necessary for the generation of abnormal excitatory discharge within a network (e.g. excitatory glutamatergic neurons, inhibitory GABAergic interneurons and astrocytes) are present and functional in the immature zebrafish (Kawai et al., 2001; Baraban et al., 2005; Szobota et al., 2007; Delgado and Schmachtenberg, 2008; Klooster et al., 2009). From a logistical perspective, zebrafish grow to only 3-4 centimeters, and aquatic systems allow for relatively low-cost maintenance of large colonies of adult wild-type and mutant fish. Owing to these advantages, several human diseases including, but not limited to, cancer (Amatruda and Patton, 2008), cardiovascular disease (Dahme et al., 2009), leukemia (Payne and Look, 2009), malignant melanoma and hypothyroidism (Lieschke and Currie, 2007) have been successfully modeled in zebrafish.

A survival assay to identify seizure-resistant mutants

Although many seizure susceptibility genes are known (for a review, see Noebels, 2003), a systematic search for genes conferring 'seizure resistance' has not, to our knowledge, been attempted. Before such studies could be performed, we needed to establish and validate a model to induce seizures in zebrafish (Fig. 1A). Using behavioral, molecular, pharmacological and electrophysiological techniques, we demonstrated that seizures in immature zebrafish that are exposed to a common convulsant drug (pentylenetrazole, PTZ) reproduce many of the crucial aspects of mammalian seizures (Baraban et al., 2005). Perhaps foremost among these, are extracellular field recording data from the optic tectum (or forebrain) of agar-immobilized zebrafish, showing distinct brief-duration, high-frequency 'interictal'-like activity interrupted by large-amplitude and long-duration multi-spike 'ictal'-like discharges (Fig. 1B). Given that abnormal electrical discharges originating in a central nervous system structure are the hallmark feature of epilepsy, these electroencephalogram (EEG)-like recordings in immature zebrafish highlight an important recapitulation of the human condition. Moreover, it is well established that the sporadic electrical manifestations that are typical of a seizure appear, at least in their main qualitative aspects, to be rather stereotyped across species and across different experimental modes of seizure induction (Jasper et al., 1969; Pitkänen et al., 2006). To further demonstrate that immature zebrafish exhibit the crucial and stereotyped features

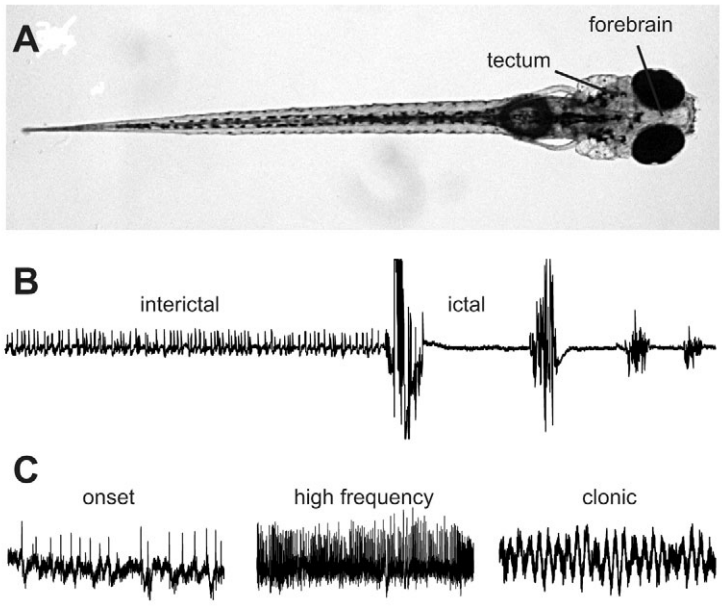

Fig. 1. Acute seizure activity in zebrafish. (A) Zebrafish larvae at 7 dpf. (B) Representative extracellular field recording obtained from the optic tectum of a 7-dpf zebrafish larvae exposed to 15 mM PTZ. Note the presence of fast, small-amplitude 'interictal'-like activity, and a burst-suppression sequence marked by the occurrence of multi-spike, large-amplitude 'ictal'-like activity. (C) Representative single-unit recording obtained from an individual cell in the optic tectum of a 7-dpf zebrafish larvae exposed to 15 mM PTZ. Note the initial presence of increased firing activity (onset), followed by high-frequency firing and a period of clonic activity.

Table 1. Scoring system for seizure behaviors in immature zebrafish

Stage	Behavior
Stage 0	No behavioral alteration; very little swim activity (Racine Stage 0*)
Stage I	Zebrafish larvae show a general increase in swim activity
Stage II	Characterized by a rapid 'whirlpool-like' circling around the outer edge of the well (Racine Stage 6*)
Stage III	Brief head-to-tail 'convulsions' followed by a loss of posture, i.e. the fish floating on its side. Stage III-type convulsions are characterized by very rapid movements across the dish, lasting between 1 and 3 seconds (Racine Stage 8*)

*Racine et al., 1972.

of an epileptic seizure, we obtained single-unit recordings during PTZ exposure. As shown in Fig. 1C, an increase in cell firing is observed at seizure onset, which is followed by high-frequency burst firing and, finally, waves of 'clonic' activity. These recordings bear a striking similarity to those obtained in an acute seizure focus from monkey cortex (Schmidt et al., 1959) or within regions identified as epileptic in the human cortex (Calvin et al., 1973; Wyler et al., 1982).

As a tool for high-throughput screening, we then designed a simple PTZ 'survival' assay. The principle underlying our assay is that acute exposure to a high concentration of PTZ (15 mM) reliably elicits distinct electrographic and behavioral seizures in immature zebrafish (at ages between 3 and 8 dpf), and continuous PTZ exposure results in uncontrolled status-like epileptic activity, ultimately resulting in fatality. This assay is based on clinical observations that uncontrolled status epilepticus is invariably fatal (Simon, 1985). We hypothesized that, at least some mutant zebrafish surviving a PTZ assay at 7 dpf have a gene mutation(s) regulating a mechanism to resist the effects of prolonged seizure activity. To identify these mutants in a genome-wide forward-genetic screen, we used a widely accepted and proven method, namely ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis, to introduce random point mutations in zebrafish. In the search for 'seizure-resistant' mutants using our PTZ survival assay, we screened more than 500,000 F_3 larvae from an ENU colony of nearly 2000 F_2 mutagenized fish (Muto et al., 2005; Baraban et al., 2007). Our screen initially identified 27 putative mutant families on a Tubingen line (TL) background strain; additional PTZ tests, out-crossing, and re-test assays using picrotoxin or bicuculline were performed to reduce this number to six. Secondary screening using behavioral and electrophysiology assays allowed us to prioritize these six mutants for further study. Mutant s334 (designated as the *colbert* mutant) was ranked as the highest priority based on physiology data demonstrating an inability to generate 'ictal'-like, long-duration epileptiform discharges in response to PTZ or 4-aminopyridine exposure, and seizure behavior that, on our scoring system, only reached Stage II, which is characterized by rapid whirlpool-like circling in the PTZ locomotion tracking assay (Table 1) (Baraban et al., 2007).

It is important to note that although this project was limited to 'resistance', it is not

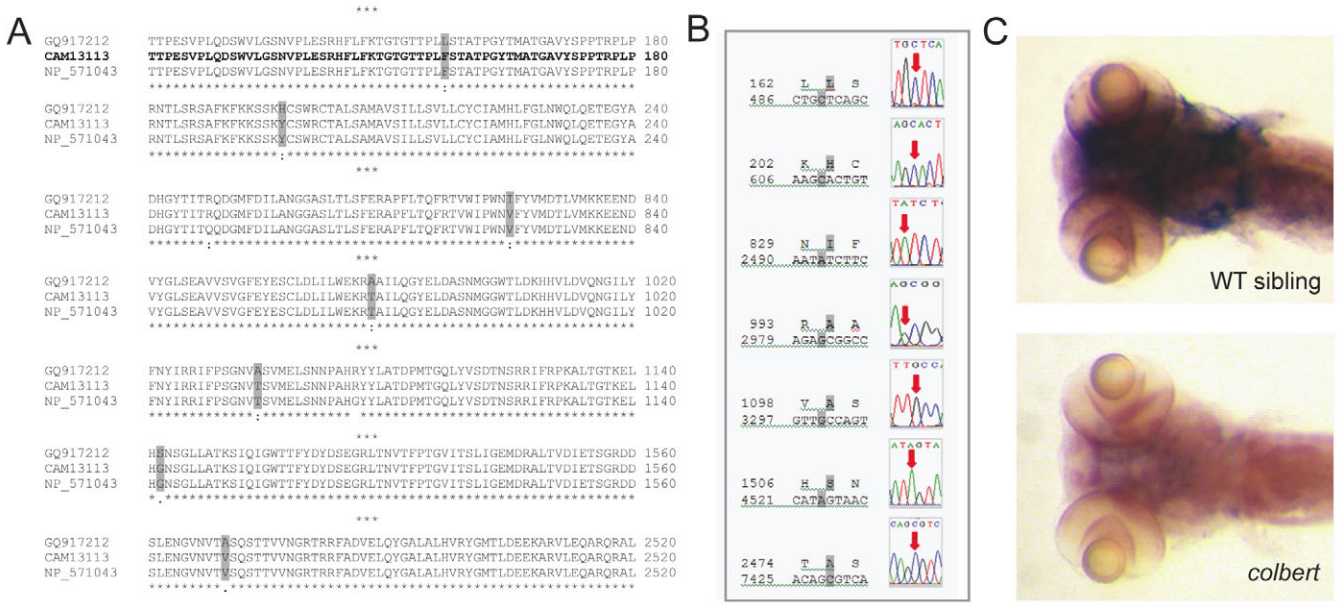

Fig. 2. Identification of a candidate gene for seizure resistance. (A) Alignment of the protein sequences for the *Danio rerio odz3* genes, GQ917212 (*colbert* mutant), CAM13113 (which is identical to the sequence we have found for the wild-type control group) and NP_571043 (CLUSTALW) (Higgins et al., 1996), indicating the amino acid substitution. (B) The relative positions of the point mutations in the nucleotide sequence are indicated; these mutations were caused by the substitution of thymine (T) to cytosine (C) at cDNA position 489, T to C at 609, guanine (G) to adenine (A) at 2493, A to G at 2982, A to G at 3300, G to A at 4524, and T to C at 7429 (GenBank accession GQ917212, 8521 base pairs). The fluorescent 'peaks' of the mutations using dye-terminator chemistry are also shown on the right. (C) Whole-mount in situ hybridization showing the localization and visualization of the *odz3* gene in zebrafish at 7 dpf. Note the downregulation of expression in the *colbert* mutant compared with an age-matched wild-type control.

hard to imagine that our approach could be used to discover mutations that result in photosensitive epilepsies, audiogenic epilepsies, or even pharmacological insensitivity to anticonvulsant medications. Another approach would be to use the ENU mutagenesis screening strategy to identify mutants that are seizure prone, perhaps by testing thresholds to convulsant drugs. Because many epilepsy patients often present missense mutations in susceptibility genes, the identification of hypomorphic alleles in a mutagenesis screen would be clinically relevant. Moreover, this approach would overcome the potential of overlooking an epilepsy gene that was also a homozygous null lethal mutation.

tenascin-M3, a candidate gene for seizure resistance

Candidate genes for suppressing seizure activity and/or protection from the pathological consequences of a seizure may include those encoding ion channels, neurotransmitter receptors, intracellular signaling components, gap junction proteins, transcription factors, and molecules involved in synaptogenesis or synaptic transmission. In fact, the list is probably much longer. Identification of such genes in zebrafish, especially those with human homologs, could provide molecular targets for the commercial development of new therapeutic compounds.

Although chemical mutagenesis using ENU is an efficient method, candidate genes have been identified for less than 10% of all mutants recovered in large-scale mutagenesis screens. As an alternative to laborious positional cloning methods, we used an insertional mutagenesis approach (Sivasubbu et al., 2007), in which an exogenous DNA serves as both a mutagen and a molecular tag for the gene whose disruption causes the phenotype. We carried out a complementation PTZ survival assay using the offspring from crosses between adult heterozygote *colbert* mutants and ~200 retroviral insertion fish (Znomics Inc.) (Gaiano et al., 1996). Using linker-mediated PCR analysis, a candidate gene with a neural gene expression profile was identified (Zgc:153720; *tenascin-M3*).

Tenascin-M3 is a novel protein similar to vertebrate odd Oz/ten-m (teneurin-3). The zebrafish homolog was initially discovered in a search for factors regulated by the LIM/homeodomain transcription factor Islet-3 (Mieda et al., 1999). Pools of putative homozygous *colbert* mutants and wild-type siblings were collected from PTZ survival assays and sequenced. Several point mutations were identified in independent s334/*colbert* lines from the ENU mutagenesis screen, including one (at cDNA position 489) that falls within a predicted promoter sequence (Fig. 2A,B). Whole-mount in situ hybridization results were consistent with an interpretation that one (or more) of these point mutations results in a significant downregulation of tenascin-M3 expression in *colbert* mutants (Fig. 2C). Tenascin is an extracellular matrix protein whose expression is upregulated in response to seizure activity (Ferhat et al., 1996; Nakic et al., 1996; Represa and Ben-Ari, 1997), and is thought to play a role in the formation of new recurrent excitatory synapses following a seizure (Represa and Ben-Ari, 1997). Our working hypothesis is that seizure activity in *colbert*-mutant zebrafish would not turn on tenascin expression and, therefore, new synaptogenesis would be prevented, making these mutants 'resistant' to the prolonged pathological consequences of PTZ, especially that associated with excess recurrent excitation.

Conclusions

This short Primer was designed to introduce the neuroscience community to the possibilities associated with studying epilepsy in zebrafish. Our principal findings, to date, include the establishment of an acute seizure model in immature zebrafish, the first large-scale epilepsy-relevant screen in zebrafish, and the identification of a candidate gene (*tenascin-M3*) for seizure resistance. The identification of small molecules that inhibit the activity of tenascin-M3, or gene therapy manipulation, could offer new treatment options to combat epileptogenesis in humans. Additional avenues of research that would take advantage of the unique features of zebrafish could include: imaging studies to identify the circuitry involved in generating seizure-like activity in a 'simple' vertebrate nervous system; the generation of zebrafish mutants mimicking single-gene mutations that cause human forms of epilepsy could be used for further analysis of the basic mechanisms underlying seizure genesis; or a variety of mutagenesis screens to identify additional epilepsy-related genes or genes that are responsible for resistance to available antiepileptic drugs. The studies we describe here are, admittedly, limited in scope, but it is our belief that further and more sophisticated use of this simple vertebrate species could lead to seminal discoveries in epilepsy.

Advantages of zebrafish as a model for epilepsy

- Zebrafish have known homologues for 85% of the recognized epilepsy genes found in humans, and show many neurological similarities to humans
- Zebrafish are highly amenable to genetic manipulation
- Zebrafish embryos absorb drugs directly from their bathing media
- Zebrafish are easy to maintain in large populations that are sufficient to screen for genes that protect against seizures, or that increase susceptibility to seizures
- From fertilization, it takes zebrafish 3-4 months to reach full maturity, and a parental pair can produce a clutch of offspring consisting of 50-200 embryos almost every week

COMPETING INTERESTS

The authors declare no competing financial interests.

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