ABSTRACT

SUSAN KLODNICKI
Pediatric Seizures in Larval Zebrafish
Under the Direction of James Lauderdale and Andrew Sornborger

About 55,000 cases of pediatric epilepsy are diagnosed annually. Although there are seizure treatments for adults, there are no known effective treatments for childhood seizures. The reason is not well known and is the cause for our research. Study of neural mechanisms at a young age is necessary to provide knowledge that will allow other researchers to develop more effective drugs for childhood epilepsy. Epileptic episodes are characterized by recurrent unprovoked seizures which result from widespread abnormal brain activity with characteristic discharge patterns. The larval zebrafish is a useful organism that allows us to visualize seizures in a developing vertebrate using confocal imaging. These seizures have a similar pattern to that of children. Zebrafish transgenic for a calcium indicator can be used to monitor neural activity induced by chemoconvulsants. In imaging data, action potentials are sensed as calcium changes, and brain images are collected for further analysis. Using computational algebraic methods, individual seizures and neural pathways are detected, estimated and visualized. Prior to 5 days post fertilization (dpf) waves are characterized by short durations (33 sec/wave). After 5 dpf, cyclical wave patterns emerge characterized by a series of short duration waves followed by a single long-duration wave (9 min/wave). These results show that the pattern and duration of seizure-induced neural activity change as a function of brain development. This work provides a better understanding of possible mechanisms involved in pediatric seizures that may lead to improved epilepsy treatment.

INDEX WORDS: Epilepsy, Larval Zebrafish, Confocal Imaging, Calcium Indicator, Neural Activity, Calcium Changes, Brain Development, Pediatric Seizures

PEDIATRIC SEIZURES IN LARVAL ZEBRAFISH

by

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CHAPTER 1
INTRODUCTION

According to the National Institute of Neurological Disorders and Stroke, epilepsy is defined as abnormal activity of neurons in the brain. Two causes of epilepsy are abnormal neuronal wiring and imbalance of neurotransmitters (NTs). NT imbalance can be either excitatory, where seizure would result from too much excitation, or inhibitory, where seizure would result from lack of inhibition. Currently, several effective antiepileptic drug treatments exist for adult epilepsy. However, these treatments are highly ineffective in children. This indicates that a fundamental, unknown difference exists between pediatric and adult seizures. Our research is geared toward more fully understanding the pediatric seizure in comparison to the adult seizure in order to shed light on more effective treatments for children.

The Model Organism

The study of pediatric seizures requires a model organism that is both easy to use and similar to humans in brain mechanisms. The first logical organism is the mouse. Although useful in many laboratory experiments, the mouse is not the best choice for confocal imaging experiments because its size prevents effective microscope imaging. *Danio rerio* (zebrafish) was the model organism used in all experiments in this research project. The zebrafish is a relatively simple vertebrate that is an ideal organism for our experiments. Its anatomical structure has been widely studied and is well understood. Embryonic development occurs inside a transparent egg (over the first 72 hours), which allows for easy observation. Fish reach sexual maturity in 3-4 months. This short generation time allows us to raise new adults to breed frequently. In addition,

female fish are able to spawn every 2-3 days, laying clutches of up to around two-hundred eggs. This characteristic is very useful for imaging experiments that require fish which are 3 to 10 days post fertilization (dpf). We also needed a model organism that could display brain activity *in vivo* on the cellular level during imaging experiments (Srinivasamoorthy, Keith, Sornborger, & Lauderdale, submitted). Larval zebrafish have permeable skin that allows uptake of chemicals in bath exposure (gills have not yet developed), thus allowing us to use a non-invasive imaging protocol to collect data. These qualities make the zebrafish the most effective organism to use.

In addition to technical requirements, our model organism must possess a brain organization similarity to humans. This is true in zebrafish. In the larval zebrafish brain, olfactory bulbs are well developed, the diencephalon contains adult-like subdivisions, and the optic tectum begins to develop a layered organization at 5 dpf. We know that regulatory genes controlling zebrafish development such as *hedgehog* have similar functions in higher vertebrates although the similarity to human brain development still remains unclear. It is also known that GABA, glutamate, and acetylcholine receptors are highly conserved across species, indicating that neural mechanisms are similar in zebrafish and humans (Scott C. Baraban, 2006). Thus, it is useful to study the movement of neural impulses through the larval zebrafish brain during seizure activity and to compare these data to human pediatric seizures.

Zebrafish Seizure and Pentylenetetrazol (PTZ)

We induce seizure by using the chemoconvulsant PTZ, a GABA(A) receptor antagonist. Suppressing GABA(A) activity results in decreased inhibition and more rapid firing of neurons that cause convulsions.

Behavioral studies demonstrate that larval zebrafish exhibit defined stages of seizure when exposed to a chemoconvulsant such as PTZ (Scott C. Baraban, 2006). In Stage I, fish show increased swim activity. Instead of flitting around the dish occasionally, the fish move more frequently. Stage II is characterized by a rapid circling around the outer rim of the dish. Once fish lose posture and undergo full head-to-tail convulsions in one place, they have entered Stage III . Figure 1 shows normal movement of a larval zebrafish in embryo medium (control). Blue tracks the movement of fish over the span of two minutes. Figure 2 is typical Stage I and II seizure behavior and shows the movement of fish ten minutes after exposure to 15 mM PTZ.

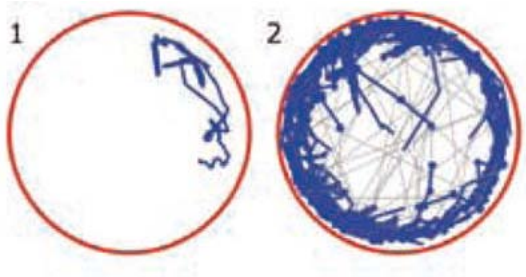

Figure 1: Larval Zebrafish Movement in Embryo Medium

Figure 2: Seizure Movement of Larval Zebrafish Exposed to PTZ

(S. C. Baraban et al., 2007)

Cameleon

We need a way to see the neural activity in a Stage III seizing zebrafish. At the cellular level, calcium events occur during neuronal signal transmission. As an action potential reaches the end of an axon, voltage-gated Ca^{2+} channels open and calcium influx into the presynaptic terminal causes release of a neurotransmitter (NT) into the synapse. The uptake of NTs by receptors located on the post-synaptic neuron can lead to an excitatory or inhibitory membrane

potential (Lodish, 2008). GABA, a NT responsible for an inhibitory post-synaptic potential, is the primary NT affected by PTZ. Tracking the calcium activity is one way to measure neural activity of a seizure (Tao, Lauderdale, & Sornborger, submitted).

We track this calcium using transgenic larval zebrafish that express cameleon, a calcium indicator protein. Cameleon is composed of two fluorescent proteins, CFP and YFP (see Figure 3). Under normal conditions, energy is absorbed and emitted from CFP at 442 nm and 480 nm, respectively. When excess calcium is present, the calcium binds to CaM, a subcomponent of the cameleon protein, causing a conformational change that allows Förster resonance energy transfer (FRET) to occur from CFP to YFP. Energy is emitted from YFP at a wavelength of 530 nm. Thus, changes in ratio of YFP:CFP emission indicate calcium events (McCombs & Palmer, 2008). We detect widespread Ca^{2+} events upon exposure to PTZ, which we interpret as seizure (Srinivasamoorthy, et al., submitted).

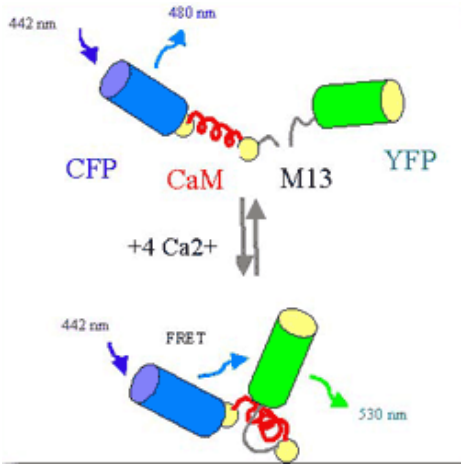

Figure 3

Adapted from http://www.stanford.edu/group/moerner/slides/cameleon-1.gif

Cameleon Conformation Changes with Calcium Exposure

CHAPTER 2
MATERIALS & METHODS

Hypothesis

Larval zebrafish exposed to PTZ will exhibit different seizure patterns as they develop (3dpf-10 dpf).

Breeding

Zebrafish are maintained in the University of Georgia Zebrafish Facility following standard procedures (Westerfield, 2000). Breeding fish are separated out in the evening, and eggs are collected the next morning. The eggs are transferred to a dish with egg water and put in a 28.5°C incubator until fish reach the necessary age for experiments (3dpf-10dpf).

The cam-transgenic zebrafish we use for experiments appear bright green under a green fluorescent protein (GFP) filter (see Figure 4).

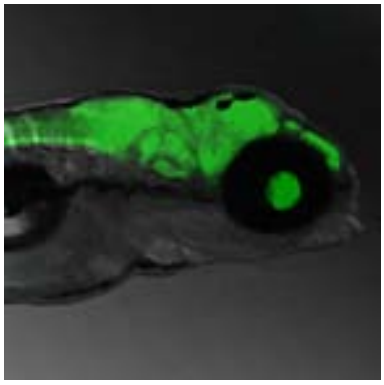

Figure 4

Adapted from http://www.devbio.uga.edu/gallery/images/zebrafish2.jpg

Fluorescent Cam-transgenic Zebrafish

Materials

15 mM PTZ

0.5 mM pancuronium

Egg water

1% agarose gel

p35 petri dish

Pipettes

Confocal microscope

Protocol

Set-Up

- Larval fish fed off nutrients from the yolk prior to experiments. Once fish reached preferred developmental stage, putative homozygous and heterozygous cam-transgenic fish were segregated using a GFP-filtered microscope.
- Experimental fish were bathed in 15mM PTZ (in egg water solution) until catatonic seizure was reached, typically 15-45 minutes.
- Fish were transferred to a 0.5 mM pancuronium (see Paralytic Compound section) solution (in egg water) until motionless, typically 3-15 minutes.
- Throughout experimental set-up fish were monitored periodically for heartbeat and blood flow.
- Once paralyzed, fish were embedded and immobilized in a small drop of 1% agarose gel and mounted, either laterally or dorsally, on the surface of a small petri dish.

- Fish were transferred to Leica TCS SP5 microscope (inverted) or Zeiss LSM 510 Meta confocal microscope (upright). Egg water was added to dish.

Confocal Imaging

- Fish were imaged under 10x objective with fluorescence excitation using the 458 nm line of argon laser. Frames were collected at 1 frame per second for one hour at a scale of 128 x 128 pixels. Emission was imaged at 485±20 nm (CFP) and 561±37 nm (YFP).

Changes from Previous Protocols

Over the past two years changes have been made to our experimental protocol in order to streamline the procedure into a more effective, efficient method of gathering neural data from larval zebrafish. The following are major changes that have improved experimental methods.

1. Experimental fish were previously mounted straight from egg water into 1% agar that was placed on a coverslip. The mounted fish then went into an imaging chamber that was subsequently filled with 15 mM PTZ (or other chemoconvulsant) prior to start of imaging. The exposure to chemoconvulsant immediately prior to data collection resulted in a lag time of 45 minutes to 90 minutes before any calcium events were detected in data analysis. This meant we had to image for a solid three hours to get a significant amount of data during which the fish was exposed to surrounding chemicals and a high-energy laser beam. The agar provided a barrier through which PTZ had to diffuse before producing any kind of useful data.

   Under the guidance of Dr. Scott Baraban (University of California San Francisco), we began exposing zebrafish to PTZ prior to mounting. Fish are bathed in 3

mL 15mM PTZ until they reach Stage III seizure. Next, fish are transferred to a 0.5 mM pancuronium solution until immobile. This seizing but unmoving state allows us to image neural activity without interference from muscle movement that would also produce calcium events.

2. We quickly learned that motion can interfere with accurate neuronal data. Thus, a paralytic compound was needed to maintain immobility. We began by using curare (see Paralytic Compound section), a muscle relaxant that blocks nAChRs. The primary problem with curare was the harmful effect on larval fish. Most fish did not tolerate curare for longer than a couple hours.

    Pancuronium proved to be both less harsh on the fish and more efficient. Fish became immobile within the first 10 minutes exposed to 0.5 mM pancuronium. We ultimately stopped using curare as a paralytic and replaced it with pancuronium.

Paralytic Compounds

Curare

Curare (d-tubocurarine) is a competitive antagonist of nAChRs. We use curare as a skeletal muscle relaxant.

Pancuronium

Pancuronium is a steroid muscle relaxant that competes with ACh for nAChR binding sites.

CHAPTER 3
DATA ANALYSIS

Imaging data was saved and transferred to the analysis computer where each hour segment was converted to raw data and read into ImageJ. We used the SOARS (Statistical Optimization for the Analysis of Ratiometric Signals) method with MATLAB, a technical computing language. SOARS is an effective way to "denoise" the imaging data, allowing detection of small FRET signals produced by cameleon. SOARS includes standardization and subtraction of the two fluorescent wavelength signals (CFP and YFP), singular value decomposition, selection of statistically significant eigenimages, and reconstruction of the ratio of the denoised data (Fan et al., 2007).

Once data is denoised, we analyze the eigenimages and the corresponding CFP/YFP ratios produced from the data. Anticorrelation of CFP and YFP bands indicates a calcium event, interpreted as seizure (see Figure 5). The eigenvector shows the area of brain activity during this event. We can also combine together the statistically significant components of the data taken during imaging to make a movie of seizure movement through the brain.

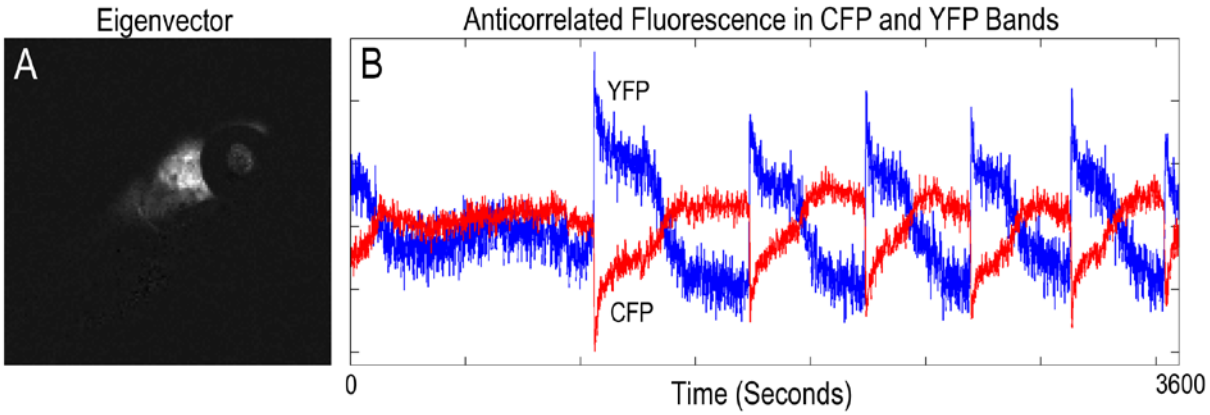

Figure 5

Eigenvector and YFP/CFP Bands of a 1 Hour Segment

The eigenvector in (A) shows a lateral mount of a larval zebrafish taken from an hour-long experiment. Bright areas map the location of Ca^{2+} activity. In (B), the red band represents CFP and the blue represents YFP. Anticorrelations shown in the ratio plot indicate Ca^{2+} activity.

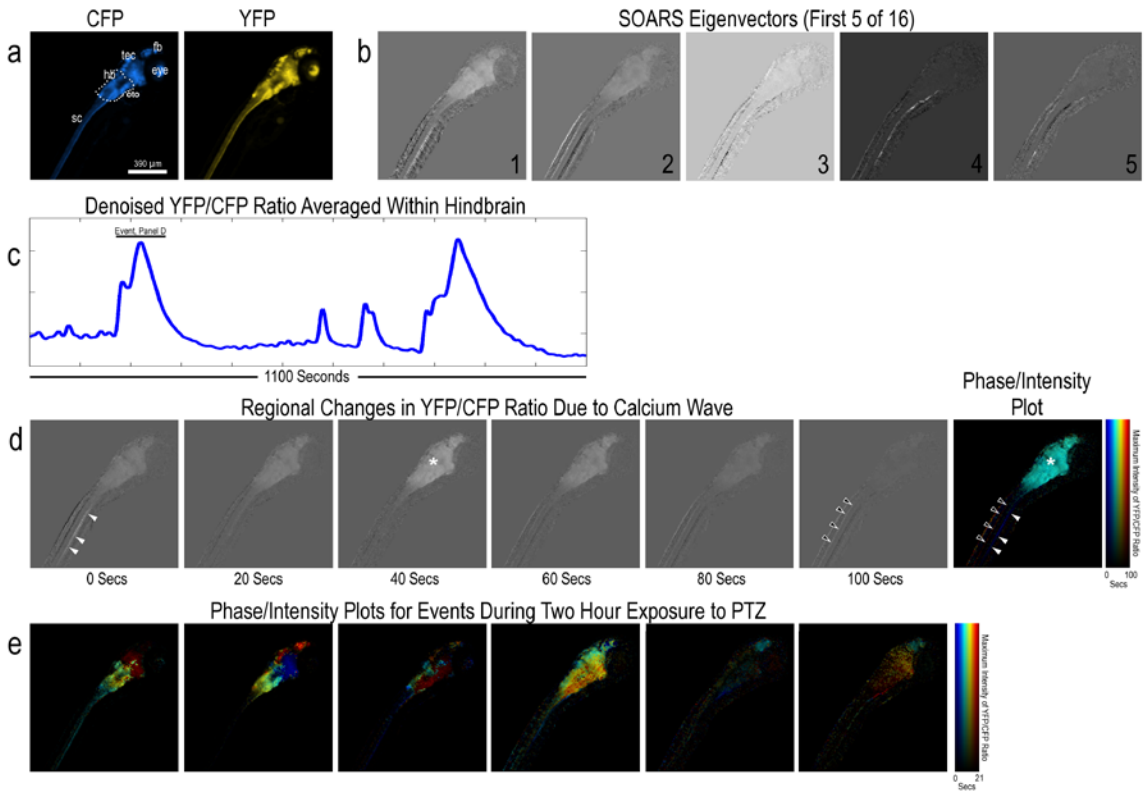

Figure 6 (Fan, et al., 2007)

Ratiometric Imaging Data of 7 dpf Ca^{2+} Wave Events from PTZ

Figure 6 shows ratiometric imaging data of Ca^{2+} wave events in 7 dpf cam-transgenic zebrafish upon exposure to PTZ. (a) includes the CFP and YFP images from the imaging dataset. The larval fish is mounted laterally with the spinal cord in the lower left corner and the brain in the upper right. (sc: spinal cord, hb: hindbrain, tec: tectum, fb: forebrain)
(b) shows results from a SOARS analysis. These eigenvectors show part of the changing ratiometric signal detected by the SOARS method. (c) is the averaged, denoised (post-analysis) data extracted from an ROI (region of interest) of the hindbrain. Two large-amplitude and two small-amplitude Ca^{2+} events are depicted in this plot over the course of 1100 seconds. (d) shows frames from a movie of the first large 100 second Ca^{2+} event shown in (c). Light pixels indicate Ca^{2+} concentrations above the baseline whereas dark pixels indicate Ca^{2+} concentrations below the baseline. The last frame shows a multi-colored phase-intensity plot that indicates the spatio-temporal movement of the 100 second calcium event. This particular event begins in axonal tracts in the spinal cord marked by the white arrowheads, travels to the hindbrain and tectum, then travels back to a different set of axonal tracts in the spinal cord marked by black arrowheads. (e) includes six other phase-intensity plots of Ca^{2+} wave events that occurred during the two hour imaging experiment. A variety of propagation patterns are clearly visible (Fan, et al., 2007).

CHAPTER 4
RESULTS

PTZ Activity

The plots in Figure 7 (B and C) clearly show changes in YFP/CFP ratio in 7 dpf fish exposed to 15 mM PTZ over 60 minutes. As seen in Fig 7(C), PTZ neural activity is characterized by a large-amplitude spike followed by a refractory period and several small spikes of higher frequency. This pattern appears to recur throughout the imaging time. The three images of the fish show where data was taken to obtain these plots.

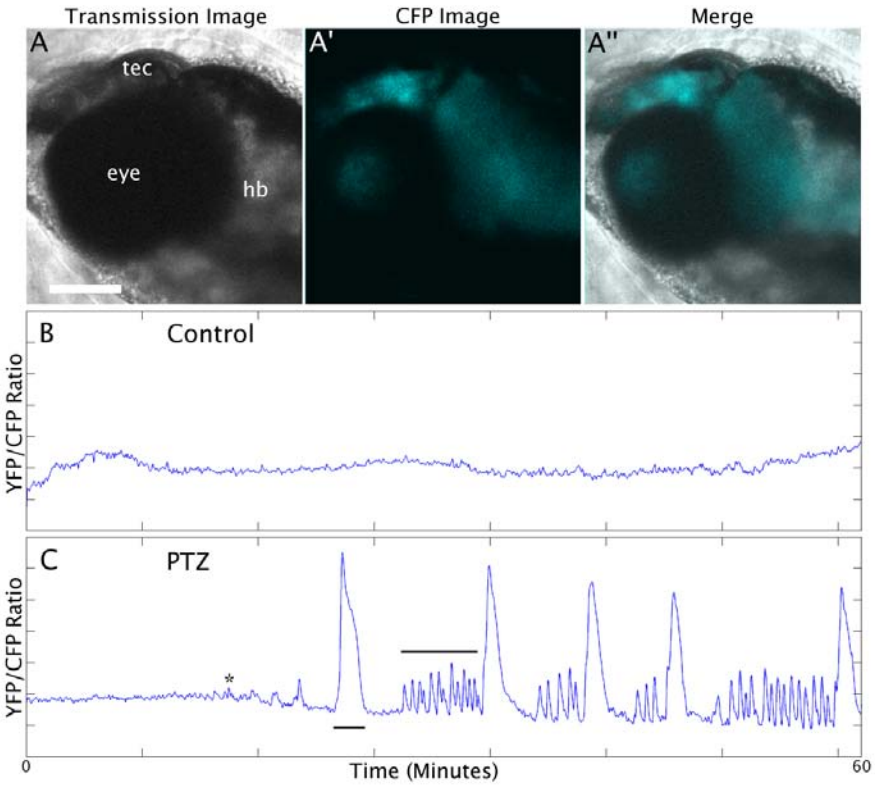

Figure 7 (Srinivasamoorthy, et al., submitted)

PTZ Causes Neural Activity

The PTZ fish was not given PTZ until the start of imaging, at t = 0. (A) shows a lateral view of the fish with dorsal side up and anterior to the left. (A') is an image with visible cameleon cyan fluorescence. (A'') merges the two. (B) plots YFP/CFP ratio in an hour of imaging data from a 7 dpf fish in egg water. Plot (C) shows the pattern of ratio change in a 7 dpf fish exposed to 15 mM PTZ. (Srinivasamoorthy, et al., submitted).

Spatio-temporal Movement of Calcium Activity

Figure 8 shows a large amplitude event exhibited by a 7 dpf fish dorsally mounted (anterior brain in bottom right corner).

Fig 8(A) is an imaging sequence of calcium changes over the course of the seizure. The seizure begins at about 10 seconds with high calcium in the optic tectum and forebrain and then proceeds to move into the hindbrain while Ca^{2+} concentrations decrease in the tectum (at 40 seconds). Ca^{2+} concentrations decrease in the optic tectum and edges of the hindbrain and continue to decrease below baseline (150 and 230 seconds). Calcium returns to baseline at 500 seconds.

Fig 8(B) shows how the first 21 seconds of the seizure moved through the brain. Ca^{2+} concentrations peaked in a front-to-back pattern, starting in the forebrain at about 10 seconds and moving to the hindbrain.

Fig 8(C) shows normalized YFP/CFP ratios in the thalamus, optic tectum, cerebellar plate, and medulla oblongata. Maximal concentration occurred in the optic tectum, thalamus, cerebellar plate and medulla oblongata sequentially. (Srinivasamoorthy, et al., submitted).

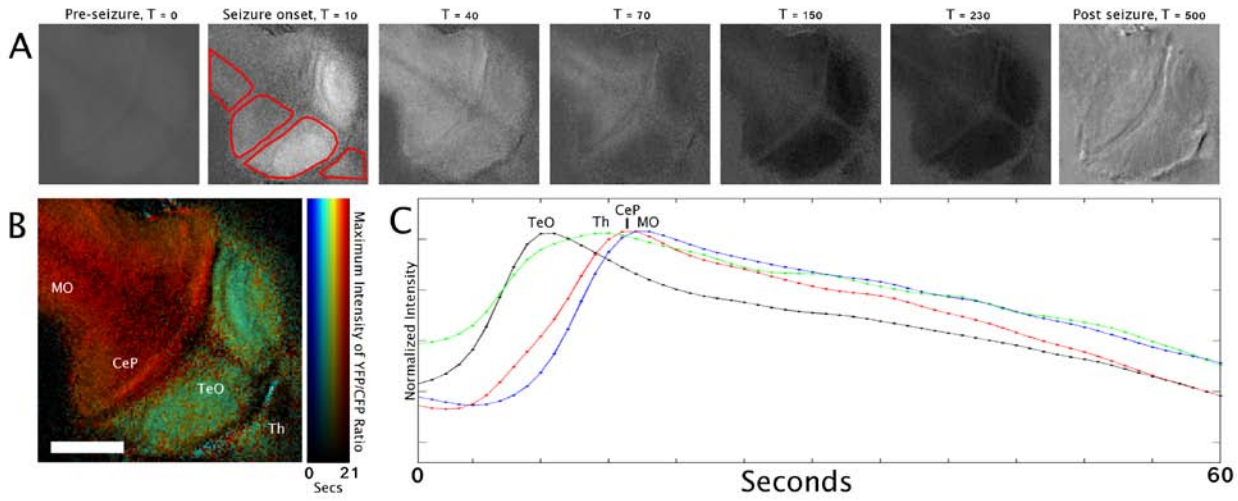

Figure 8 (Srinivasamoorthy, et al., submitted)

Spatio-temporal Movement of Calcium Activity in a 7 dpf Fish

This imaging data is taken from a 7 dpf fish exposed to 15 mM PTZ. (A) is a series of images that show calcium events during 500 seconds of a seizure. Specific ROIs are highlighted in the second image. Darker pixels indicate lower calcium than baseline and lighter pixels indicate increased calcium. (B) is a phase-intensity plot showing areas of maximal Ca^{2+} concentrations. Color shows maximum ratio while pixel intensity shows amplitude of maximum (see color key). (C) shows a plot of the seizure in (B) out to 60 seconds. Colors indicate different regions in the brain (labeled on figure). [Th: thalamus, TeO: optic tectum, CeP: cerebellar plate, MO: medulla oblongata]

Seizure Propagation

Through imaging data, we have found that seizures move in different ways. Most calcium events recorded in larval zebrafish are uniform (occur throughout brain at one time). However, many events propagate either rostrally or caudally. All bursting events, observed as the large spikes in CFP/YFP ratio, are propagating events. Figure 9 shows the spatio-temporal differences between propagating and uniform Ca^{2+} events.

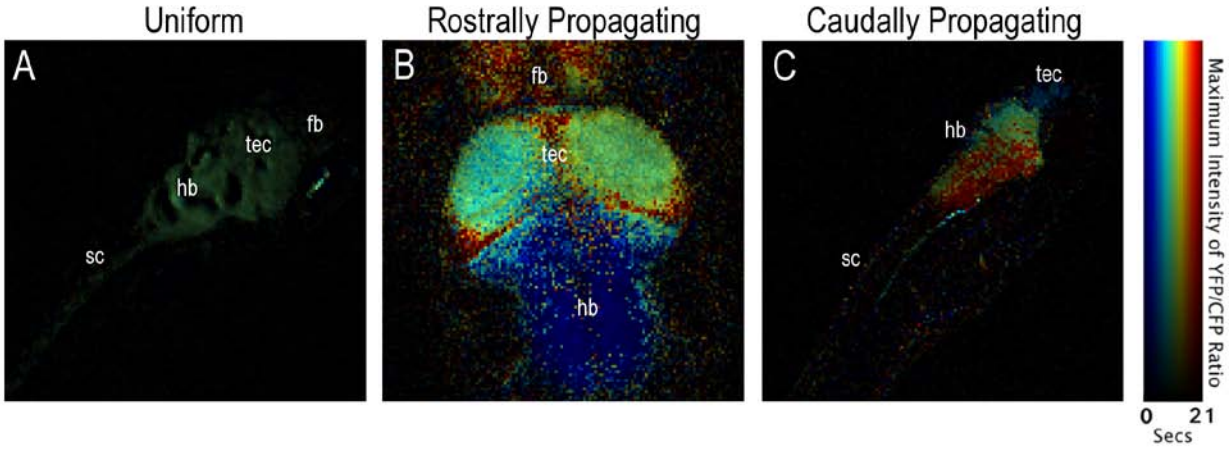

Figure 9

Seizure Propagation Patterns

(A) shows the intensity plot of a uniform seizure in a lateral zebrafish. (B) shows a rostrally moving seizure that moves from hindbrain to forebrain over 21 seconds. (C) shows a caudally moving seizure that starts in the tectum and moves through the hindbrain and spinal cord.

Calcium Dynamics

Large amplitude Ca^{2+} events, or bursting events, typically propagate either caudally or rostrally. Figure 10 shows phase intensity images of seizure dynamics in time windows (21 seconds) around Ca^{2+} maxima that occurred during large-amplitude Ca^{2+} events. Most large-amplitude events that we have analyzed move from the hindbrain through the tectum (rostrally). Fig 10(A), (B), (C) and (D) all show rostrally moving seizures in fish at different developmental stages. Each image shows a 21-second segment of seizure taken from 15 mM PTZ experiments. (Srinivasamoorthy, et al., submitted).

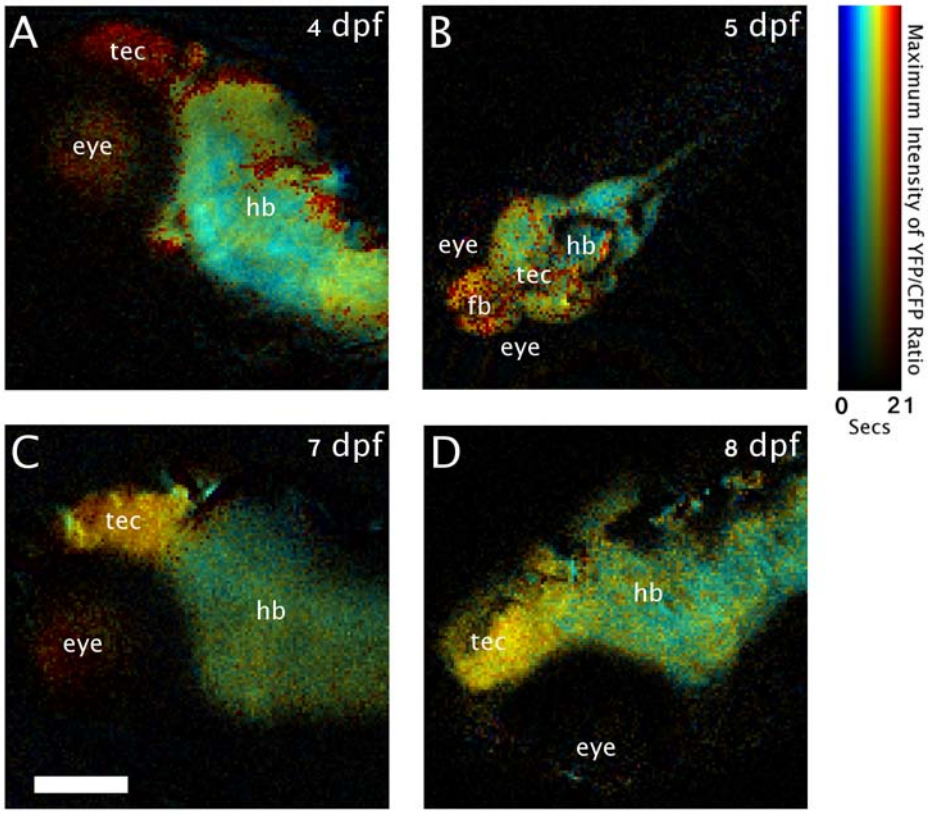

Figure 10 (Srinivasamoorthy, et al., submitted)

Calcium Dynamics in Larval Zebrafish

(A), (C), and (D) are lateral zebrafish mounts with the anterior to the left. The forebrain is not included in the plane of focus. Image (B) is a dorsal view of the brain with the anterior to the left. (A) shows a Ca^{2+} event in a 4 dpf fish that starts in the ventral hindbrain, moves dorsally, then propagates to the tectum. (B) shows a 5 dpf Ca^{2+} event where the wave began in the spinal cord and hindbrain, moved first to the optic tectum, and then to the edges of the tectum and forebrain. (C) shows a 7 dpf wave that propagated to the tectum from a uniform initiation in the hindbrain. (D) shows a Ca^{2+} wave in an 8 dpf fish that began unevenly in the hindbrain and moved to the tectum.

Seizure Patterns with PTZ Exposure

Figure 11 shows the spatio-temporal patterns of Ca^{2+} activity from PTZ exposure in a 3 dpf fish. Events 1-4 were representative of the early, low-frequency Ca^{2+} spikes characterizing this seizure sequence (see Fig 11(B)). Waves consistently began in the spinal cord and propagated back-to-front, ending in the forebrain.

Events 5-8, which occurred after 90 minutes, exhibited inconsistent dynamics. Some waves (5) propagated front-to-back, some waves (7,8) propagated back-to-front, and some waves (6) were uniform and did not propagate (Fig 11(C)).

Events 9-12, which occurred after two hours, exhibited a regular pattern that was distinct from other time frames. First a wave traveled from spinal cord to forebrain (9,11) and then a wave traveled from forebrain to spinal cord (10,12). (Srinivasamoorthy, et al., submitted).

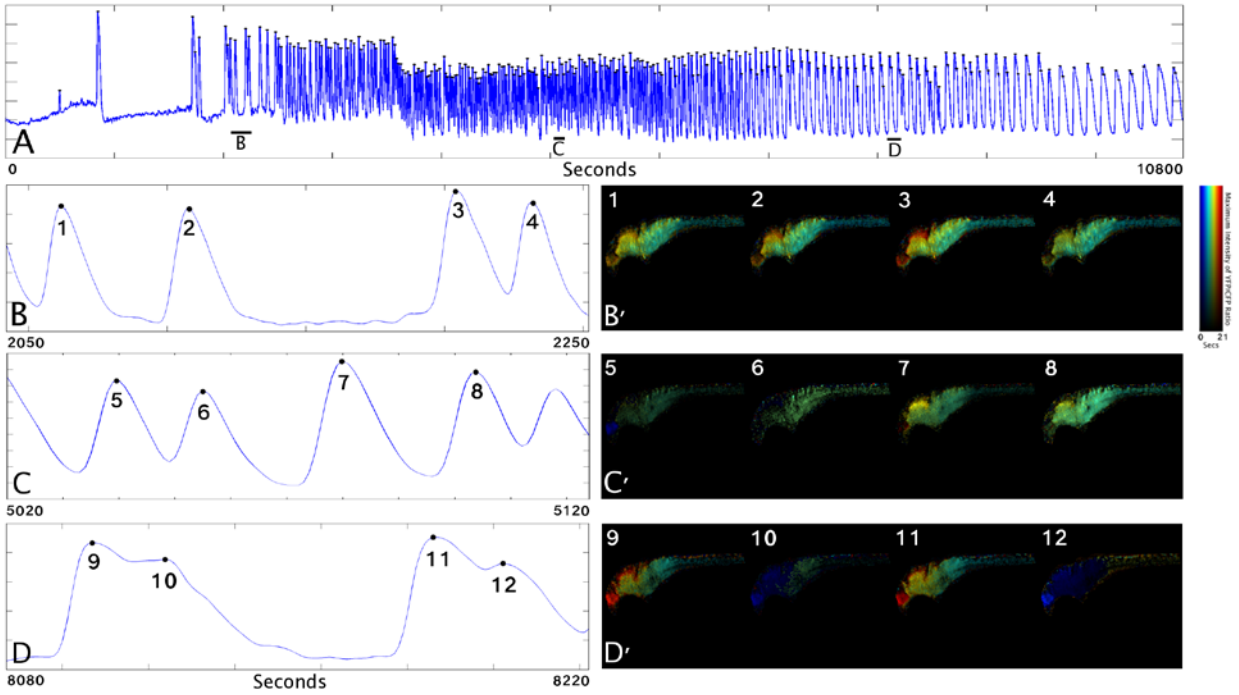

Figure 11 (Srinivasamoorthy, et al., submitted)

Ca^{2+} Activity from PTZ Exposure in a 3 dpf Zebrafish

Plot (A) is the average ratio from the entire brain region over the course of three hours. Bars denote time intervals in (B-D). Plot (B) shows four consecutive Ca^{2+} events from the first time interval in (A). Black dots mark the middle of the time interval (21 seconds in this case) during which the phase-intensity images were calculated. (B') includes the corresponding phase-intensity images of the (B) events. (C) and (C') show that events 5-8. (D) and (D') show events 9-12.

Seizure Activity and Development

Figure 12 shows a comparison of the dynamics of PTZ-induced Ca^{2+} activity as a function of developmental stage of the fish. The following observations were made about corresponding parts of Figure 12:

(A) At 2 dpf, seizure was characterized by low-frequency spikes.

(B) At 3 dpf, a higher frequency spike sequence began at about 30 minutes and lasted for the duration of the experiment.

(C) At 4 dpf, low frequency spikes gave way to a large event at 140 minutes, followed by larger spikes.

(D) At 5 dpf, regular large events began at about two hours.

(E) At 7 dpf, regular, high frequency spike trains interrupted large spikes.

(F) At 8 dpf, large, long-duration spikes were interspersed with higher frequency spike sequences.

These pattern differences indicate development in the zebrafish brain. (Srinivasamoorthy, et al., submitted).

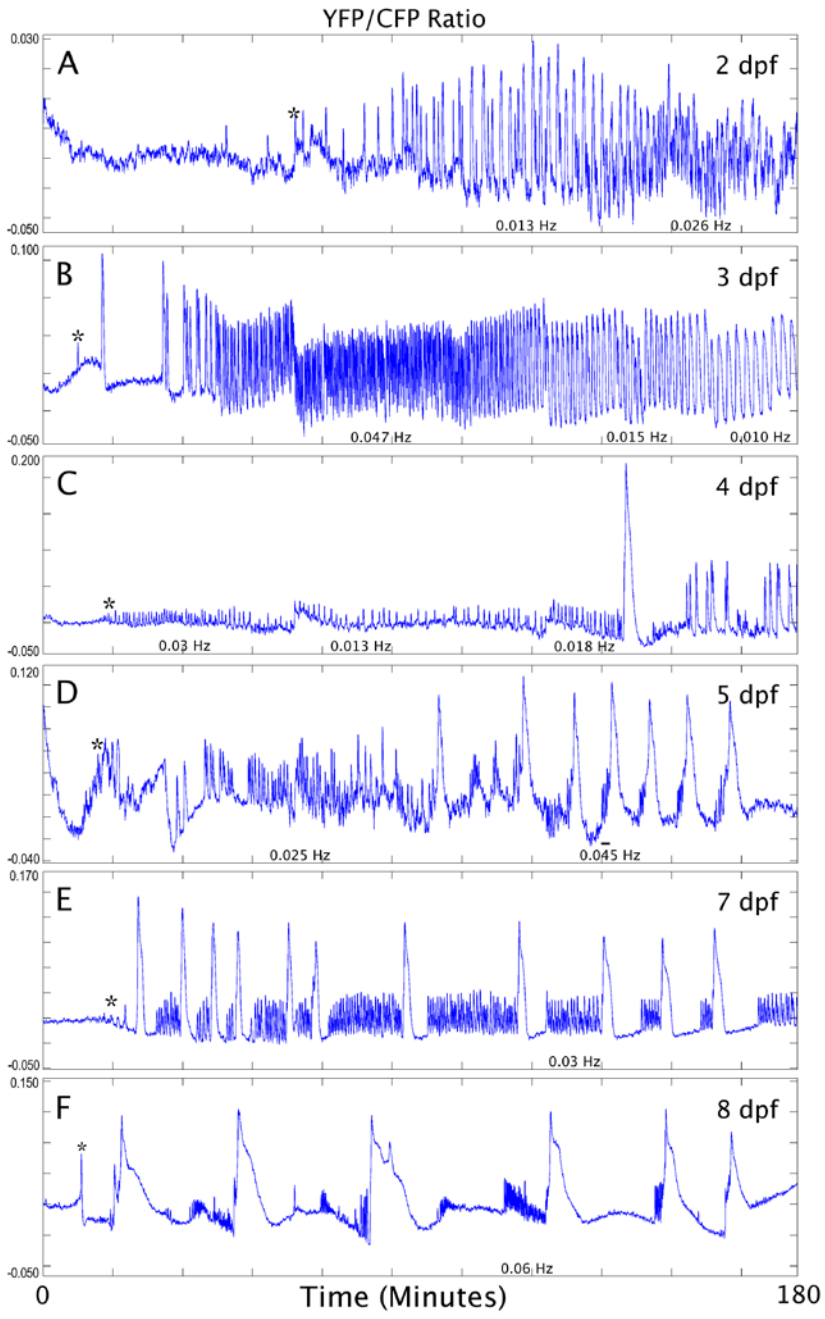

Figure 12 (Srinivasamoorthy, et al., submitted)

PTZ-induced Ca^{2+} Activity in Progressive Developmental Stages

PTZ was perfused continuously for three hours. Each plot shows deviations of the YFP/CFP ratio from background averaged over the brain region imaged. Plots were normalized to the same scale. Asterisks mark the first waves in the YFP/CFP ratio.

CHAPTER 5
DISCUSSION

Our results have shown that the larval zebrafish is a good model organism for epileptic seizure imaging experiments. The zebrafish's biological characteristics (size, transparency, breathing mechanism) allow us to image larval fish easily after exposure to PTZ. Analysis of calcium activity in imaging data allows us to study the mechanisms of pediatric seizure movement. Known conserved neurological mechanisms in the zebrafish make the findings of our experiments relevant to human pediatric seizures.

Secondly, we have learned about seizure propagation by using PTZ as a chemoconvulsant. PTZ produces Ca^{2+} events that are characteristic of seizure activity (Fig 7). By constructing ROIs and creating phase-intensity diagrams, the movement of seizure activity can be visually mapped by the location of Ca^{2+} waves in time (see Fig 8). This movement has three different basic propagation patterns (uniform, front-to-back, back-to-front) as well as much more complex propagation patterns. Finally, the characteristics of seizures (intensity, length, propagation) change as a function of PTZ exposure. The Ca^{2+} activity occurring during the first hour of a three hour experiment is usually very different from the activity during the third hour due to exposure time to PTZ (see Fig 11).

Ca^{2+} wave patterns change drastically at different developmental stages (2 dpf to 8 dpf). The change in seizure activity occurs during different developmental stages (see Fig 12). This supports our hypothesis that larval zebrafish exposed to PTZ will exhibit different seizure

patterns as they develop. Prior to 5 dpf, fish have shorter duration waves (33 sec) compared to those 5 dpf and older can exhibit much longer waves (up to 9 min). There is some developmental change in the zebrafish brain that produces these differences, though the structures or wiring that allow for this are unclear. Perhaps a change in wiring post-5 dpf causes the seizure change since it is known that at 5 dpf, fish have a fairly developed brain structure.

Further investigation of pediatric seizures in larval zebrafish would be useful to more fully understand mechanisms of seizure propagation. Another question to address in future research is at what developmental stage does the pediatric seizure become adult-like? This would allow for a more thorough study of the differences between adult and childhood seizures and perhaps identify some fundamental differences that would influence treatment development.

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