



Central Nervous System Immediate Early Gene Expression Patterns in Zebrafish Models of Epilepsy

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Central Nervous System Immediate Early Gene
Expression Patterns in Zebrafish Models of Epilepsy

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Abstract

Zebrafish are a powerful model for studying neurodevelopmental disease, and more recently epilepsy. The characteristics of seizures in zebrafish have until now included largely descriptive abnormalities in swim patterns and electrophysiology in larval zebrafish. A quantitative method of characterization that capitalizes on the high-throughput potential of zebrafish is analysis of immediate early gene (IEG) transcription. IEGs are transiently and rapidly activated in response to stimuli such as calcium influx in neurons. In order to better understand the abnormal expression patterns of IEGs due to seizure in a zebrafish model, we investigated transcriptional activity of the six IEGs, *cfos*, *npas4*, *nr4a1*, *bdnf*, *cebp β* , and *homer1a*, in both a chemical and genetic model of epilepsy. This study found that a number of commonly used qPCR loading control are unsuitable for pentylenetetrazole (PTZ) models of zebrafish epilepsy because they are altered with exposure to PTZ. However, *mglobulin* remained static across all tested drug exposures, making it an appropriate control. Abnormal IEG expression from PTZ exposure generally recapitulated the data found in the mouse models, but expression was also greatly induced by conventional chemicals controls. These data suggest that IEGs tested can reveal a transcriptional PTZ effect in zebrafish, but that the effect cannot be attributed solely to an increase in neuronal activity. Finally, differential IEG expression in a genetic model of zebrafish epilepsy was explored by generating an *osgep* KO zebrafish. IEGs tested in this study however did not show a statistically significant difference between *osgep*^{+/+} *osgep*^{-/-}, and control.

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Chapter I.

Introduction

Zebrafish Models of Epilepsy

Epilepsy is a common neurological disorder characterized by recurrent seizures. Underlying etiologies are heterogeneous and include both genetic and environmental factors. Genetic epilepsies typically disrupt whole networks within the central nervous system (CNS) (Poduri & Lowenstein, 2011), which often require *in vivo* studies to model disease states. Conventional epilepsy models, such as rodent, are expensive to generate and time-consuming to study. Zebrafish (*Danio rerio*) are an attractive alternative epilepsy model because of a high genetic homology to humans, large clutch sizes, inexpensive husbandry costs, and the ease with which their small, transparent larvae allow for morphological phenotyping (Hensley, Hassenplug, McPhail, & Leung, 2012). Furthermore, zebrafish readily absorb compounds, facilitating noninvasive exposures of proconvulsant and anti-epileptic drugs (AED). These characteristics create a powerful model capable of affordable high-throughput screens. However, seizure characterization in zebrafish is a relatively new field, and further classification is needed. (Hortopan, Dinday, & Baraban, 2010).

One method for detecting seizure-like activity in zebrafish is electrophysiology. Electrophysiology is currently used to detect seizure-like discharges in a variety of

genetic (Zabinyakov et al., 2017), chemical (Duy, Berberoglu, Beattie, & Hall, 2017), and electrical stimulation (Shin, Field, Stucky, Furgurson, & Johnson, 2017) zebrafish models of epilepsy. However, the technique is time-consuming, skill-intensive, and impractical for large screens. Further, normative data at various developmental stages have yet to be catalogued systematically, and there are no accepted parameters for differentiating normal from abnormal patterns.

Behavioral paradigms have been established as a high-throughput method for detecting abnormal zebrafish seizure-like activity. The characterization of behavioral seizure in zebrafish is based on a pentylenetetrazole (PTZ), chemically induced seizure model in larvae (Baraban, Taylor, Castro, & Baier, 2005). PTZ is a proconvulsant whose mechanism of action was first identified in rat as a GABA_A agonist. Competitive binding of $\alpha 1$, $\beta 2$, $\gamma 2$ allosteric sites (Huang et al., 2001) leads to GABA inhibition. GABA is the primary vertebrate CNS inhibitory neurotransmitter (Jessen, Mirsky, Dennison, & Burnstock, 1979). Therefore, competitive PTZ GABA_A binding leads to a loss of neuronal inhibition and seizure-like excitation. Highly conserved GABA_A subunits in zebrafish suggests a similar mechanism for convulsion in zebrafish models (Delgado & Schmachtenberg, 2008).

Larval zebrafish convulsion from PTZ exposure has been characterized by distinct seizure stages and a stereotyped swimming behavior (Baraban et al., 2005). This paradigm has since become important for the characterization of zebrafish epilepsy models (Turrini et al., 2017) (Zabinyakov et al., 2017), despite its limitations as an indirect measure of abnormal CNS electrical activity and potential for lack of specificity without electrophysiological corroboration. Seizure-like swimming behavior after PTZ

exposure is categorized into three stages. Stage I includes abnormal agitated swimming, stage II; a rapid “whirlpool-like” swimming, and stage III; a period of immobility and the inability to right posture (Figure 1), often occurring directly after stage II behavior. Evidence of stereotyped behavior as a suitable indicator of larval seizure is supported by abnormal electrical activity detected via electrophysiology of fish exposed to PTZ (Baraban et al., 2005). Additionally, PTZ-induced seizure-like electrical activity detected by electrophysiology is attenuated by subsequently GABAergic AED exposure (Baraban et al., 2005) and exposure to other anti-epileptic compounds (Lin, 2018). PTZ-exposed zebrafish respond to a wide variety of AEDs, while mammalian PTZ models of epilepsy have only been shown to respond to AEDs acting on GABA (Gupta, Khobragade, & Shingatgeri, 2014). These findings suggest possible additional mechanisms for PTZ-induced convulsions that must be considered in experimental paradigms.

Genetic models of epilepsy-related genes in zebrafish do not necessarily present behaviorally with the same stereotyped manner of PTZ models. Due to the heterogeneity of comorbid disease phenotypes (e.g., abnormal cognitive development) and the possibility of variation in seizure types between models representing different mechanisms, behavior could vary widely across models. Comorbid dysregulation of eye development, metabolism, and skeletal musculature can lead to an almost complete loss of fish movement, as seen in an STXBP1 KO zebrafish, regardless of seizure-like electrical activity (Grone, 2016). Conversely, zebrafish can spontaneously mimic the seizure-like swimming behavior as a startle response, creating stage II type false positives. Because of these confounds, seizure behavior alone is an insufficient indication of seizure in a zebrafish model.

Zebrafish Paralytics and Anesthesia

Curare

Curare is a paralytic that has been shown to be a nicotinic acetylcholine receptor (nAChR) antagonist in BC3H-1 embryonic mouse cells. Curare competitively binds nAChRs NMJ, effectively blocking skeletal muscle stimulation from peripheral neuron acetylcholine (ACh) (Tyagi et al., 2007). Although curare has not been directly studied in a zebrafish NMJ model, high zebrafish and mouse nAChR homology predict similar mechanistic response. Furthermore, curare is commonly used to eliminate movement artifact in electrophysiology recordings of PTZ-induced seizure models (Turrini et al., 2017; Zdebik et al., 2013).

Ms-222

Tricaine mesylate (ms-222) is a commonly used amino amide anesthetic shown to inhibit both voltage-gated sodium (Na_v) and calcium (Ca_v) channel subunits in mammalian models (Scholz et al, 2002). Although the precise mechanism of action has not been fully elucidated in zebrafish, highly conserved Na_v and Ca_v channels and anesthetic drug effects suggest a similar mechanism of action. Additionally, ms-222 is commonly used in the zebrafish field for anesthesia as well as euthanasia at higher concentrations (Harper & Lawrence; 2011).

Immediate Early Genes

IEGs are a group of highly conserved molecules transiently and rapidly activated in response to stimuli (Sun & Lin, 2016). Stimuli, such as growth factors, neurotransmitters, and calcium influx initiate an intracellular cascade, which activates poised RNA polymerase II (pol II) and subsequent transcription of IEGs (Saha et al., 2011). IEG transcription can be robust and near-instantaneous because of a pol II stall on transcription start sites (Figure 2). IEGs are generally transcription factors or recruit proteins for stimuli response. Most IEGs are localized across a variety of cell types and respond to a multitude of stimuli (Sun & Lin, 2016). However, highly specified IEGs exist and will be discussed in further detail.

IEGs can be categorized into two major classes: early transcription factors and effectors. Early genes are the first transcriptional response to an extracellular stimulus and are transcribed directly from poised pol II. Effectors are late responders and modify cellular behavior as a result of early IEG activation. IEGs are categorized according to the timing of peak temporal mRNA expression following stimulation. Fast IEG expression occurs within 30 minutes, and slower IEGs peak after 60 minutes of initial initiation. However, in neurons, the definition of an IEG is not as clearly defined. Some fast IEGs can be activated by already expressed slower IEGs due to ongoing basal activity. Strict definitions of IEGs would disqualify neuronal genes because effectors can activate early transcription factors, but because this biology is unique, contemporary definitions largely include both early and effector genes in neurons (Sun & Lin, 2016).

Seizure activity has been widely reported to have a causal relationship with IEG expression (Baraban et al., 2005). An IEG response occurs in neurons as a result of

excitation and depolarization. This phenomenon was first observed in a generalized clonic motor seizure, electrical stimulation, kindling mouse model. *In situ* hybridization revealed an increase in *c-Fos*, *c-Jun*, and NGFI-A in the neurons of the hippocampus (Labiner et al., 1993; Risse, Jooss, Neuberg, Bruller, & Muller, 1989). Since then, a variety of IEGs have been found to respond to CNS activity across a multitude of models (Sun & Lin, 2016).

c-Fos

C-fos is an early IEG and proto-oncogene subunit of the activator protein (AP1) complex (Rauscher, Voulalas, Franza, & Curran, 1988). AP1 is a transcription factor comprised of both *Fos* and *c-Jun* family heterodimer subunits. The AP1 complex contains a cis-regulatory binding element, which promotes transcriptional activation of target genes (Risse et al., 1989). *C-Fos* became a seizure activity marker in mouse when immunohistochemistry revealed neuronal localization in a noxious stimulation model and was thought to be limited to certain neuronal types as high levels were also present in non-stimulated mice (Bullitt, 1990). However, more recent studies of *c-Fos* have identified expression in a variety of non-CNS specific cell types as well as transcriptional responses to a variety of stimuli. Up-regulation of *c-Fos* has been shown in cellular proliferation (Chakraborty & Roy, 2016), differentiation (He, 2017), and survival (Suda et al., 2014).

C-fos is the conventional molecular marker of zebrafish seizure (Baxendale et al., 2012; Mussulini et al., 2013) largely due to the PTZ studies of Baraban et al (Baraban et al., 2005). However, this work may have limited utility in genetic models, which will

later be discussed at a greater length. Additionally, *c-fos* expression increases in skeletal muscle in response to increased swimming (Baxendale et al., 2012), which may confound quantitative analysis of neuronal activity. The identification of additional IEG markers of seizure activity may allow for increased specificity and precision in the interpretation of IEG data in genetic models.

Npas4

Npas4 is an early IEG transcription factor responsible for coordinating the formation of GABA-releasing synapses (Lin et al., 2008). *Npas4* is a highly neuron-specific IEG, selectively induced by Ca²⁺ influx in cultured mouse hippocampal neurons. Importantly, other neurotrophic factors do not appear to lead to increase in *Npas4* expression (Lin et al., 2008), making it one of the only known IEGs to respond solely to one stimulus. In mice, the majority of *Npas4* expression occurs in neurons, but recent evidence suggests an additional role for *Npas4* in calcium signaling of pancreatic beta cells (Speckmann, Sabatini, Nian, Smith, & Lynn, 2016).

Neuronal *npas4* expression increases in response to PTZ exposure in larval zebrafish can be detected by *in situ* hybridization experiments (Baxendale et al., 2012). Additionally, quantitative polymerase chain reaction (qPCR) experiments of adult zebrafish exposed to PTZ showed a quantitative increase in expression (Klaric, 2014). However, no expression pattern studies using reliable reference genes in larval fish have been conducted. Emphasis is placed on full characterization of this IEG because of its high specificity for neuronal activity and potential utility in genetic epilepsy models.

Nr4a1

Nr4a1 is an early IEG and transcription factor, highly expressed in neurons and macrophages (Pei, Castrillo, & Tontonoz, 2006), that responds to inflammation (X. M. Li et al., 2015). Studies in cultured J774 mouse cells and *Nr4a1*-deficient mice exhibit systemic inflammation and pro-inflammatory macrophage polarization (X. M. Li et al., 2015). Cancer cell culture models have also shown that *Nr4a1* plays a role in apoptosis and cell cycle regulation (Ke et al., 2004; Honkaniemi & Sharp, 1999). Importantly, studies in chemogenic and kindling-induced mouse models have demonstrated up-regulation of *Nr4a1* in the cortex, hippocampus, and amygdala (Akiyama, Ishikawa, & Saito, 2008). However, such quantitative *nr4a1* seizure expression patterns have not been elucidated in zebrafish.

BDNF

Bdnf is a neurotrophic growth factor and effector IEG involved in neuronal differentiation (Louhivuori et al., 2011; Y. Zhang, Moheban, Conway, Bhattacharyya, & Segal, 2000) and synapse formation (Yang et al., 2009; Bamji, Rico, Kimes, & Reichardt, 2006). Neuronal depolarization-dependent expression has been shown in mouse (Ogier et al., 2007) as well as in zebrafish (Baxendale et al., 2012). Whole-mount *in situ* of PTZ-induced larval zebrafish seizure models revealed *bdnf* up-regulation in telencephalon and diencephalon (Baxendale et al., 2012). However, quantitative studies suitable for high-throughput analysis have yet to be conducted. Expression is largely limited to the peripheral nervous system (PNS) and the CNS, but some evidence suggests an activity-

dependent expression pattern in muscle cells of mouse (Cuppini et al., 2007). Increased motor activity associated with zebrafish seizure and larval PTZ models necessitates proper movement controls to minimize expression ‘noise’ from cell types other than neurons.

C/EBP β

C/ebp β is a leucine zipper family transcription factor and effector IEG with a CCAAT motif binding affinity. *C/ebp β* has a similar cell type expression pattern and stimuli response as *c-Fos* because it belongs to the same superfamily of transcription factors including c-jun and c-myc (Guo, Li, & Tang, 2015). *C/ebp β* has largely been implicated as an immune and inflammatory response, IL-6 signaling mediator IEG (Screpanti et al., 1995; Hirai, Yokota, Tamura, Sato, & Maekawa, 2015). *C/ebp β* is also essential for cellular differentiation, including non-steady-state hematopoiesis (Hirai et al., 2015) and adipogenesis (Y. Y. Zhang et al., 2011; Guo et al., 2012), as well as for apoptosis regulation (Gade, Roy, Li, Nallar, & Kalvakolanu, 2008; Marchildon, Fu, Lala-Tabbert, & Wiper-Bergeron, 2016; Pena-Altamira, Polazzi, Moretto, Lauriola, & Monti, 2014). Rat models highlight the importance of *C/ebp β* in CNS development and plasticity (Kfoury & Kapatos, 2009). Quantitative PCR of brain tissue of an electroconvulsive seizure (ECS) rat model reveal a downregulation of *C/ebp β* present in the CNS after seizures (Chen et al., 2004). *C/ebp β* has been found in the brains of zebrafish (Lyssimachou et al., 2015), but its specific response to seizure activity has yet to be determined.

Homer1a

Homer family genes encode for a group of neuronal scaffolding proteins that form a mesh capable of binding ligands to the post-synaptic density (Worley et al., 2007; Hayashi et al., 2009). The *Homer1a* subtype is an effector IEG and is involved in transient breakdown of the homer mesh. *Homer1a* is upregulated in induced seizure models of mouse (Morioka, Kato, Fueta, & Sugiyama, 2001) and rat (Klugmann et al., 2005), and in cultured rodent hippocampal neurons (Y. Li, Krogh, & Thayer, 2012). However, *homer1a* expression patterns have yet to be studied in depth in the zebrafish model. *Homer1a* does have activity-dependent expression patterns in skeletal muscle of mouse (Baxendale et al., 2012), so properly controlling artifact from movement during any seizure model will be essential.

qPCR Standardization

Best practice for qPCR experiments has been outlined (Schmittgen & Livak, 2008), but the technique had not been well characterized in a larval zebrafish model of PTZ-induced seizure. Crucial to proper qPCR experiments is a stable reference gene across all experimental conditions. This is because the cycle threshold (ct) is normalized across conditions using data derived from reference genes. Ct is defined by the number of PCR cycles it takes within an experiment to detect a florescent signal above a give threshold. The cycle threshold of experimental (CTE) parameter is controlled internally by normalization from the cycle threshold control (CTC) to obtain a double delta ct (McCurley & Callard, 2008). For instance, levels of IEG variation between a PTZ

exposed and vehicle fish would be established by a vehicle cycle threshold (ct) double delta ct internal control.

A reference gene is typically used to normalize cDNA within a qPCR sample. Accurate normalization is crucial for comparison across experimental conditions (McCulloch, Ashwell, O'Nan, & Mente, 2012; Vandesompele et al., 2002) to account for variable quantities of transcript relative to all tested samples. It is crucial that reference gene expression remains constant between samples in order that it can serve as an appropriate control (Eisenberg & Levanon, 2013; Chervoneva et al., 2010). A recent meta-analysis reveals that reference gene expression stability is rarely assessed (15%) before conducting qPCR assays (Chapman & Waldenstrom, 2015) despite how critical it can be to experimental success. Because most studies use a standard *gapdh* or *β actin1* loading control, it can be difficult to find reference genes suitable for specialized paradigms. Acceptable qPCR reference gene standards have been established within PTZ rodent epilepsy models (Stamova et al., 2009) and within various zebrafish cell types and developmental stages (Chapman & Waldenstrom, 2015). To our knowledge however, no standard PTZ-induced zebrafish model loading control has been established for qPCR analysis. Before relative transcriptional states of PTZ-induced models of zebrafish epilepsy can be studied, suitable loading controls must be established.

In a survey of 100 zebrafish qPCR reference genes *β actin1* and *gapdh* were found to be the most common (McCurley & Callard, 2008). *Gapdh* is typically known for its catabolic role in glycolysis (Ravichandran, Seres, Moriguchi, Thomas, & Johnston, 1994), but also has a role in a large variety of cellular processes. Many known epilepsy disorders have been shown to be the result of altered metabolic processes (Wang,

Pascual, & De Vivo, 1993) including disruption of glycolysis in a *Scn1a* Dravet syndrome zebrafish model (Kumar et al., 2016). Additionally, *gapdh* kinase activity has been implicated in a cortical rat neuron epilepsy model due to disruption of GABA_A phosphorylation (Laschet et al., 2007). Taken together this suggests *gapdh* should be examined as a stable qPCR reference gene for any epilepsy model despite its common usage.

βactin1 is a highly conserved protein responsible for cell motility and stability (Wu et al., 2011). *βactin1* has been previously used as a qPCR loading control in a PTZ zebrafish seizure model of differential *bdnf* expression (Pollard & Cooper, 1986). The difficulties age matching zebrafish within a clutch (Singleman & Holtzman, 2014) and differential temporal neuronal expression during development (Barrallo et al., 1999) may predispose to variable expression in experimental groups.

Elfa, *tuba1* and *mglobulin* will be compared for suitable reference gene similarly to *gapdh* and *βactin1* in this study (Table 2). A 2008 qPCR chemical exposure study in larval zebrafish revealed the most stable reference gene to be *elfa* and *tuba1* after chemical exposure. *Elfa* is involved in translation and *tuba1* is a subunit in the cytoskeleton, while *mglobulin* is part of the major histocompatibility complex (McCurley & Callard, 2008).

Zebrafish epilepsy reference gene stability has been studied in drug exposure models other than PTZ. Zebrafish age-matched between 2 and 120hrs post fertilization were segregated into unexposed or exposed, vehicle injection of dimethyl sulfoxide (DMSO) vs. ethanol (EtOH) or chemical exposure with either 17β-estradiol (E2), testosterone (T), ICI 182,780 (ICI), β-naphthaflavone (BNF), or tetrachlorodibenzo-p-dioxin

(TCDD). *Gapdh*, *βactin1*, and *mglobulin* were shown to have differential expression between unexposed or vehicle control fish and exposed populations. Importantly, ICI exposure had been previously shown to affect the expression of the *cyp19a1b* protein in larval fish. *Cyp19a1b* normalization to *βactin1* and *gapdh*, which were downregulated by ICI exposure, showed no difference between unexposed *cyp19a1b* and ICI exposed *cyp19a1b* transcript levels. A 2-fold *cyp19a1b* difference between ICI exposed and unexposed fish was observed with an *elfa* and *tubal* *cyp19a1b* normalization. Interestingly, there was an observed downregulation of *elfa* and *gapdh* between unexposed and vehicle injected DMSO and EtOH fish populations (McCurley & Callard, 2008).

OSGEP

OSGEP is a tRNA N⁶-adenosine threonylcarbamoyltransferase subunit of the highly conserved KEOPS complex. The KEOPS complex regulates translation efficiency by OSGEP-mediated t6A modification of tRNA (Srinivasan et al., 2011), gene transcription (Kisseleva-Romanova et al., 2006), and telomere integrity (Downey et al., 2006). Mutations in four of the five KEOPS subunits, including OSGEP, have been implicated in human Galloway–Mowat syndrome (GAMOS) (Braun et al., 2017), an autosomal recessive disorder. Patients present with a range of phenotypes including nystagmus, renal failure, microcephaly, and epilepsy (Colin et al., 2014; Jinks et al., 2015; Vodopiutz et al., 2015). A recent report characterized a zebrafish GAMOS phenocopy in a CRISPR-generated OSGEP knockout (KO). Larval *osgep* KO fish were

shown to have decrease brain size and increased neuronal apoptosis (Braun et al., 2017) as well as seizures (unpublished data).

Research Aims, Goals, and Hypothesis

Detection of seizure-induced IEG activation would capitalize on the high-throughput potential of the zebrafish model without the hinderance of the behavioral analysis limitations. This molecular paradigm has been illustrated with the IEG *c-fos* (Baraban et al., 2005) (Y. Zhang et al., 2017) but not to date with other IEGs. Several questions remain in terms of the validity of IEG assessment as a marker of seizure propensity in the zebrafish system. Quantitative analysis is commonly conducted via qPCR, but valid reference gene studies have yet to been conducted in zebrafish. *C-Fos* is also expressed in a variety of cell types, and proper controls have not been tested to elucidate the degree to which *c-fos* expression induced by seizure activity is due exclusively to CNS activity. Furthermore, the heterogeneity of seizure models demands a variety of markers to detect abnormal electrical activity. A comprehensive characterization of the response of six IEGs, *cfos*, *npas4*, *nr4a1*, *bdnf*, *c/ebp*, and *homer1a*, to known seizure-inducing stimuli will further develop a molecular paradigm for seizure detection (Table 1). Elucidating the characterization of this high-throughput method will be conducted by addressing three specific aims.

Specific Aim 1 is to establish a standard reference gene for a PTZ-induced model of zebrafish epilepsy. In order to achieve this aim, a reference gene will need to be identified that remains transcriptionally constant between an unexposed and a PTZ-induced zebrafish seizure model. Additionally, Specific Aim 1 will identify a reference

gene that remains constant in zebrafish exposed with curare and ms-222. Curare and ms-222 are standard chemical exposures in larval zebrafish behavior and electrophysiology experiments, and establishing proper reference genes is essential for any subsequent qPCR seizure validation. A stable reference gene exposed with all three compounds is also essential for conducting Specific Aim 2.

Specific Aim 2 is to elucidate the degree to which early and late zebrafish IEG expression change is exclusively the result of abnormal neuronal electrical activity in a PTZ-induced model (vs. muscle-related activity that results from movement during seizures). Exposure with the paralytic curare is predicted to attenuate any IEG expression associated with movement. Exposure with the anesthetic ms-222 is predicted to be a suitable negative control and decrease IEG expression associated with neuronal activity. Drug exposures should help gain an understanding of IEG expression patterns from various stimuli and cell types and elucidate which IEGs specifically respond exclusively to neuronal activity.

The purpose of Specific Aim 3 is to elucidate differential IEG expressions in a genetic model of zebrafish *osgep*-related seizures. Rapid induction and degradation of early mRNA markers makes it difficult to ascertain increased transcription levels in genetic models with sporadic seizure activity as opposed to sustained and easily timed seizure activity observed in induced PTZ models. Characterizing inducer IEG expression in genetic seizure models could help investigators detect seizure activity because of the long-lasting increase in transcription. By evaluating a genetic zebrafish seizure model Specific Aim 3 will help define changes in IEG expression, particularly inducer IEG

expression, as a biomarker for seizure activity that may be relevant to a number of genetic zebrafish models of epilepsy

Prior to experimentation, it is predicted that once proper controls are established, immediate early genes will have a significant increase in expression in response to induced abnormal electrical activity in zebrafish, and that *npas4* will have the most robust and seizure specific response in a PTZ model. It is also predicted that late IEGs will be a better indication of seizure activity in genetic models. Characterization of zebrafish IEG seizure response should provide a rapid, straightforward, and inexpensive method for detecting abnormal electrical activity in genetic models of epilepsy in zebrafish.

Chapter II.

Materials and Methods

Animal Husbandry

TAB strain vehicle zebrafish were maintained according to standard procedures (Harper & Lawrence; 2011). Groups of sexually mature zebrafish were separated overnight in breeding cages at a 3:1 male to female ratio. Embryos were collected and kept in 100mL sterile “fish water” consisting of 60 mg/L “Instant Ocean” (ThermoFisher, Carlsbad, MA) filtered through .20uM nylon membrane. Developing larvae were grown at 28C at embryo density of <100 zebrafish per 100mL. Fish water was cleaned daily and clutches with <70% survivability up to 5 dpf were discarded. All experiments conformed to Boston Children’s Hospital (BCH) animal welfare standards and aimed to minimize animals used and maximize quality of life; all research was conducted with approval from the BCH Institutional Animal Care and Use Committee.

DanioVision Behavior Tracking

Video recordings of 5dpf larval zebrafish were collected using a DanioVision™ (Noldus, Wageningen, The Netherlands) tracking system, and movement parameter quantification was garnered from EthoVision XT® (Noldus) software. Stage I agitated

swimming-seizure behavior (Baraban, 2005) was defined by statistically significant difference between experimental groups parameters of “average velocity” and “total distance moved” over an entire trail of either 30 or 60 minutes. Automated counting of Stage 2 “whirlpool swimming events” (Baraban, 2005) was collected using a Gaussian mixture model (support vector machine, SVM) classifier algorithm. Behavior data for algorithm classification was trained with 5 second time bins due to stage II event duration occurring in less than 5 seconds. The SVM classifier was trained by manual identification of stage II seizure events within 5 second time bins in 1 hour, 15mM PTZ exposed trials. The classifier determined a maximum threshold for stage II seizure events given the parameters “velocity max” and “distance traveled” within 5 second bins.

Zebrafish Drug Exposure Assay

5dpf TAB larvae were pipetted individually into 96-well plates in “fish water” (Harper & Lawrence; 2011). Larvae were exposed with anesthesia in the form of ms-222 (*Sigma-Aldrich*, St. Louis, MO) to a final concentration of 4mM, and/or paralytic tubocurarine (*Sigma-Aldrich*) to a final concentration of 5uM, or “fish water” vehicle, and incubated for 15 minutes. After 15-minute incubation, PTZ (*Sigma-Aldrich*) was added to a final concentration of 5mM and incubated for 10 minutes. All wells were normalized with “fish water” to a final volume of 200ul per well. Experimental workflow is diagrammed in Figure 3, and individual experimental groups are outlined in Table 3. Larvae were then incubated for the specified time period for the corresponding experimental group in DanioVision™ (Noldus) and immediately flash frozen with liquid nitrogen. Frozen larvae were exposed with RNAlater –ICE (ThermoFisher) and stored

overnight in -20C according to standard kit specifications. After 24 hours -20 RNA later incubation larval fish were kept in -80C for long term storage.

OSGEP CRISPR Generation

TAB zebrafish embryos were generated and kept by standard methods. (Harper & Lawrence; 2011). Embryos were injected between the 1 and 2-cell stages of development with synthetic CRISPR RNA guides (Synthego, Menlo Park, CA) and purified Cas9 protein (ThermoFisher). Guide design was taken from Braun et al., 2016 and has no suspected off-target effects. Predicted cutting efficiency was between 95-97%. Injected larvae were kept in petri dishes at 28 degrees C in 100ml “fish water” until 6dpf. 7dpf larvae were transferred and reared in 2.8 L tanks with a maximum capacity of 30 fish per tank for 2-3 months. Sexually mature F0 fish were outcrossed to vehicle TAB fish. Generated F1 outcrosses were raised for 2 months in 2.8 L tanks with a maximum capacity of 30 fish per tank. Adult F1 fish were fin-clipped using standard methods (Harper & Lawrence; 2011), and DNA was isolated by 20min incubation in 50ul of 50mM NaOH at 95C followed by 5ul of 1M Tris/HCl pH8. Clipped fish were kept in .8L isolation tanks while awaiting genotype confirmation by PCR (Genewiz, Cambridge, MA) of the *osgep* guide region. Fish found to have the have a 10-base pair (bp) deletion in exon 2 (Figure 10) were pooled and bred to sexual maturity for F2 experimentation.

qPCR

RNA was isolated from 5dpf larval zebrafish under sterile conditions according to the specifications of the RNA/DNA extraction kit (Qiagen, Valencia, CA). Pools of 10 stabilized larvae from chemically exposed experiments or individuals from OSGEP larvae were transferred to sterile microcentrifuge tubes. Chemical and mechanical lysis was conducted in Qiagen-provided lysis buffer. Mechanical lysis of each sample pool included motorized mortar and pestle followed by repeated trituration with a 50mm syringe. Endogenous DNA was removed by Qiagen DNA filter column. DNA of OSGEP larvae was extracted for genotyping via Sanger (Genewiz). cDNA was generated according to standard a superscript III kit (ThermoFisher). Oligo dt was used for amplifying larval mRNA. cDNA was quantified using nanodrop and discarded if 260/280 > 2.0 (Schmittegen & Livak, 2008). QPCR was conducted using high ROX from a Kappa SYBR fast qPCR kit (ThermoFisher) and an Applied Biosystems instrument. Primer amplification efficiency was calculated using an assumption free linear regression model (Moorman et al.; 2003) for low quantity RNA. Primer amplification efficiencies were calculated to all be within 10% of 2 of each other and of a doubling per cycle (Schmittegen & Livak, 2008). Groups were run in triplicate to control for potential pipetting error. *Mglobulin* was used as a loading control due to the smallest difference in ct value between drug exposures. Statistical significance threshold for two-tailed t-tests was p-value < 0.05. Statistical significance was determined by either paired or unpaired t-test between ct and CTE groups. Fold changes will be calculated between ct and CTE groups.

Electrophysiology

5dpf larval zebrafish were embedded into 2% low melting point agarose in sterile “fish water” to eliminate movement artifact without the use of analgesic or paralytic. Embedded fish were perfused with artificial CSF throughout the recording process. A glass microelectrode was placed in the telencephalon, the largest forebrain region in the zebrafish CNS. Electrodes were backloaded with 2 M NaCl prior to placement. Electrical activity was recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Voltage records were low-pass filtered at 1 kHz (3dB; eight-pole Bessel), high-pass filtered at 0.1–0.2 Hz, digitized at 5–10 kHz using a Digidata 1440A interface, and stored on a PC computer running pClamp software (Axon).

Chapter III.

Results

Drug-Exposed Larvae Behavior

Larval movement detected by 60-minute behavioral tracking yielded results consistent with findings in the literature. 5mM PTZ-exposed fish had a significant increase in both total distance moved (Figure 4a) and average velocity (Figure 4b) compared to vehicle unexposed; consistent with type I seizure activity. Additionally, a highly significant increase in type II seizure activity was detected by the classifier in PTZ exposed fish compared to unexposed control (Figure 5). Nearly all movement was blocked by fish exposed with curare and ms-222 for the entirety of the 60 minute trial, including fish exposed with both curare, ms-222, and PTZ (Figure 4a/ 4b).

Drug Exposure Reference Genes

Drug exposure effect on reference gene expression varied for *β actin*, *gapdh*, and *elfa*. A preliminary investigation for a suitable loading control for drug-exposed larval zebrafish showed a significant **decrease** in *β actin* expression when exposed to 5mM PTZ for 30 minutes (Figure 6). *β actin* is commonly used as a qPCR loading control for zebrafish expression studies, which prompted an investigation into other commonly used

qPCR reference genes in larval zebrafish. PTZ exposure caused a significant **increase** in relative mRNA levels of *elfa* and *gapdh*. Furthermore, ms-222 exposure greatly **increased** relative mRNA in *elfa* and *gapdh*, while curare exposure caused a highly significant **decrease** in both reference genes (Figure 6). No significant difference of mRNA levels of *mglobulin* or *tuba1* was detected between unexposed controls and any of the three drug exposures. Additionally, *mglobulin* values were more consistent across all drug exposures and control compared to *tuba1*; *mglobulin* has a more robust mRNA expression in larval zebrafish.

Drug Exposure

30 minute PTZ exposure significantly **increased** all assayed IEG's compared to unexposed control with the exception of *nr4a1* (Figure 7a). The IEG with the largest difference between PTZ exposure and unexposed control was *c-fos*; with a 28-fold increase (Table 3) in a 30-minute trial compared to the vehicle. In the 60-minute trial only *c-fos* increased compared to vehicle (1.8-fold) (Table 3) while all other IEGs remained statistically unchanged (Figure 7b). Interestingly, only an early IEG increased expression in the 60-minute trial with a significantly reduced increase compared to control, but none of the inducer IEGs were upregulated.

Despite the paralytic and anesthetic effects of curare and ms-222, **all** IEG expression was significantly increased by drug exposures compared to unexposed control (Figure 8, Figure 9). In fact, all curare-exposed fish showed higher IEG expression change than PTZ-exposed fish when both were compared to vehicle controls. For example, 30 minute curare increased *nr4a1* 25.61-fold compared to vehicle while the

same conditions of PTZ exposure caused an increase of 1.14-fold (Table 3). Similarly, almost all ms-222 exposed larvae had a greater increase in all IEGs than PTZ exposed larvae with the exception of *npas4*. PTZ-exposed larvae had a 4.86-fold increase of *npas4* compared to unexposed control, while ms-222 exposure increased *npas4* by 5.61-fold (Table 3). All other IEGs of ms-222 exposed larvae had a greater increase than that of PTZ exposed larvae. For example, *c-fos* had a 28.72-fold PTZ increase and a 29.03-fold ms-222 increase (Table 3) compared to vehicle. This data suggests that curare and ms-222 drug treatment effects IEG expression independent of the behavioral effects they produce.

Fish exposed to multiple drugs **increased** expression of all IEGs compared to control, but drug effects were **not** necessarily compounded with multiple drug exposures (Figure 8, Figure 9). Expression of all IEGs increased with 30 minute curare exposure, but all early IEGs and *c/ebpβ* increased further when exposed with curare and PTZ. *Bdnf* and *homer1a* actually significantly **decreased** expression when exposed with curare and PTZ compared to only exposure with curare. The differences in these experimental groups could be indicative of PTZ movement artifact corrected by curare and properly normalized with by curare treated larvae. Early IEGs in curare- and PTZ-exposed larvae were increased compared to just curare-treated larvae, while late IEGs were not. This is exactly what is expected of IEG variability due solely to neuronal activity with the absence of effects from muscle activation. For example, the difference between the *homer1a* fold change between 30-minute PTZ exposure (3.06) and the fold change of 30-minute curare PTZ exposure (.63) may be the neuron-specific IEG response. Furthermore, all targets decreased IEG expression when exposed to curare, ms-222, and

PTZ compared to exposure to only curare and PTZ (Table 3). These data suggest that, as expected, if all samples are normalized to curare, ms-222 seems to greatly reduce PTZ-induced IEG increase. Taken together, these data suggest that compounded drug exposures generally reveal the expected IEG variability due to drug effects, but when properly normalized the ‘noise’ of individual drug treatment is minimized. However, due to the unexpected IEG increase of curare and ms-222 larvae, further studies will need to be conducted to see if this conclusion is valid.

OSGEP

All *osgep*-generated fish had a deletion of 10bp in exon 2, resulting in a frameshift and an early stop codon. The mutant protein was reduced from 335 amino acids to 62 (Figure 10). Behavioral analysis showed no statistical difference in the average velocity or total distance traveled in a 1-hour trial of 5dpf larvae between *osgep*^{+/+}, *osgep*^{+/-}, *osgep*^{-/-} (Figure 11). Similarly, there was no increase in seizure-like events in either the *osgep*^{+/-} or the *osgep*^{-/-} detected by the classifier algorithm (Figure 12). However, electrophysiology of *osgep*^{-/-} larvae show increased high frequency activity (Figure 13), signifying abnormal electrical activity in KO larvae despite a lack of discernable behavioral phenotype.

Surprisingly, all individual IEGs from both the *osgep*^{+/-} and *osgep*^{-/-} had a trend toward **reduced** expression compared *osgep*^{+/+} with the exception of *c/ebpβ* (Table 3), but no statistical difference was observed (Figure 14). Similarly, multivariate analysis of the combined decrease of all IEGs is statically insignificant between the vehicle in both the *osgep*^{+/-} and *osgep*^{-/-} groups.

Chapter IV.

Discussion

Significance of Results

Reference Gene Instability

Unstable reference gene expression due to drug exposure has implications for the reliability of the magnitude of variable IEG expression found in previous studies. In this study *elfa* significantly increased in response to PTZ exposure compared to unexposed control. A previous study showed a decrease in *c-fos* expression in PTZ exposed larvae when treated with indomethacin. However, quantitation was conducted via qPCR and utilized *elfa* as a reference gene ([Barbalho](#), 2016). The effect to which PTZ activity is attenuated by indomethacin is likely much greater than reported in Barbalho et al. because the increased PTZ-induced *elfa* expression would reduce its difference with PTZ exposed *c-fos* compare to the unexposed control. Similarly, relative concentration of *npas4* has been previously measured in PTZ-exposed adult zebrafish with a *β actin* loading control (Klaric, 2014). Because *β actin* decreases in response to PTZ exposure, the effects of PTZ exposure on *npas4* expression in zebrafish is likely exaggerated. Adult fish in the Klaric et al experiment were exposed to 3.3mM PTZ for 20 minutes and labeled severe if they entered stage III seizure behavior. In this study larval fish were

exposed to 5mM PTZ for 30 minutes, which is sufficient for stage III seizure response in the literature and in our laboratory. Severely affected fish from Klaric et al. showed an 11-fold difference in *npas4* expression while this study showed a 5-fold difference.

PTZ IEG Effect

The difference in IEG expression in this study due to PTZ exposure was similar, but did not greatly vary from what we expected based on previous studies. *C-fos* and *npas4* increased with short PTZ exposure as previously seen in reports from Baraban et al. and Klaric et al., respectively. All early IEGs were subsequently reduced in a 60-minute trial, as seen in studies of PTZ exposed isolated mouse neurons (Lacar, 2018). Both *Bdnf* (Ogier et al., 2007) and *Homer1a* (Morioka, Kato, Fueta, & Sugiyama, 2001) increased transcription in prolonged exposures, as previously seen in PTZ mouse models. *C/ebpβ* expectedly decreased, as shown in an ECS rat model (Chen et al., 2004). The general reproduction of what has been previously shown corroborates the results of this study and exemplifies the prediction that these highly conserved genes have similar dynamics between rodent and zebrafish when exposed to PTZ.

However, some IEGs had unexpected dynamics in response to PTZ. *Nr4a1* did not significantly increase with 30-minute PTZ exposure in larval zebrafish as it has been shown to in mouse (Akiyama, Ishikawa, & Saito, 2008). The difference between these two species could be due to a difference in dynamics in response between the two models. For instance, it is possible that *nr4a1* has a very fast transcriptional increase in response to PTZ that is cleared in under 30 minutes in zebrafish, or, that *nr4a1* acts a very late effector in zebrafish whose response would be seen much later than

1 hour after stimuli. Alternatively, it is possible that *nr4a1* does not respond to neuronal activity in zebrafish at all. More qPCR flash freeze time points would help to elucidate *nr4a1* dynamics. Similarly, inducer targets significantly increasing with a 30-minute PTZ exposure. The reason for the large increase within an “early” IEG time point for these inducers could be due to a response to the drug as a stimuli rather than the increase in neuronal activity as the stimuli. As with the unexpected increase in all IEGs due to curare treatment, PTZ on its own may increase inducer transcription quickly and neuronal stimuli response may come later.

Curare and Ms-222 IEG Effect

The purpose of curare in this study was to control for IEG expression caused by increased motor activity from PTZ exposure, while ms-222 was meant to act as a neuronal activity negative control. Quantitative PCR of the IEGs in this study have not been conducted in larval zebrafish exposed to these two drugs. To our surprise, both drugs greatly **increased** expression of **all** IEGs (Figure 8, Figure 9). Many of combined drug exposures seemed to have the predicted effects, such as *c-fos*. PTZ-exposed *c-fos* had a fold change of 28.72 compared to unexposed control, while *c-fos* curare/PTZ and *c-fos* curare/ms-222/PTZ had fold changes of 1.73 and 1.5 respectively when normalized to curare exposure. This could have indicated that movement artifact is greatly reduced by curare exposure and ms-222 further reduces the induction of *c-fos* expression due to PTZ. However, because both curare and ms-222 cause dramatic increases *c-fos* expression individually and some other targets do not follow this logical pattern, it is likely that the movement artifact and neuronal inhibition control aspects of these drug exposures are lost

in the noise of the drug-specific effects. This idea will be further discussed in the drug exposure section of the study limitations. Our finding, that curare and ms-222 are not suitable to control for possible muscle-induced activity in a PTZ model of zebrafish epilepsy, is important for future experiments in which zebrafish are exposed to these commonly used drugs downstream of immediate early gene investigation. Immobilization of larvae with curare for electrophysiology followed by IEG characterization will likely result in an excess of variability in the data and is therefore not recommended based on our results.

Osgep IEG

The lack of statistical significance of the *osgep* IEG data, despite positive indications of seizure-like activity from electrophysiology data in *osgep* mutant animals, suggests that the IEGs assayed here are not suitable correlates for the abnormal electrical activity found in this genetic model. These effects could be the result of assayed IEG dysregulation in other organs due to disease state. It is possible that IEGs are upregulated in the brain but that effect is lost due to a stronger down regulation effect in the kidney, another organ chiefly affected by *osgep* mutation. It is unlikely that the effect is due to a difference in behavior because the behavioral tracking results were not statistically significant. However, the fish behavior could be different in a manner that is not captured by either the velocity or total distance parameters that we have assayed. Most likely, the difference can be attributed to sampling issues and the the ‘randomness’ of capturing isolated, brief, sporadic seizure-like events. If only a single fish has a seizure among all of the fish pooled for IEG analysis, averaging will limit the ability to detect an increase.

The increased variance due to outliers in the *osgep*^{+/-} fish found in our data supports this “seizure event” hypothesis. For instance, the variance of *npas4 osgep*^{+/-} is 3.26 while the variance of *osgep*^{+/+} is .42. The large variance may well be due to one individual fish (or a small number) with seizure-like activity coupled with other without seizures displaying less activity. Additionally, even though the general decrease in expression of all IEGs combined was not statistically significant, if more IEGs were added, a combination of multiple IEGs could be a suitable “toolbox” capable of discerning subtle alterations of abnormal electrical activity.

Study Limitations

Drug Exposure

A limitation of the chemical model of zebrafish epilepsy is, according to our data, the surprising effects that non-proconvulsant drug exposure have on the expression of IEGs in the zebrafish model. Curare was utilized in this study to control for excessive movement in a PTZ-induced epilepsy model in zebrafish while ms-222 was used as a negative control. The increase in all IEG expression when exposed to both curare and ms-222 appears to be a reaction to drug exposure independent of subsequent behavior or altered neuronal electrical activity. It is likely that a decrease in movement and neuronal firing are causing a decrease in expression of the IEGs assayed in this study, but that increase is lost in the noise of non-neuronal or non-movement related effects. Although, the drugs in this study are considered the gold standard for chemical manipulation in the zebrafish epilepsy field their transcriptional effects are largely

unknown. It is conceivable that zebrafish have a unique reaction to ms-222 at lower concentrations that add noise from IEG fluctuation in the heart. Because of the large differences in IEG expression between vehicle and ms-222 or curare exposure it is likely that the drug exposure directly initiates IEG expression in a wide variety of cell types in the larval zebrafish adding noise to the data. It is also possible that the IEGs tested in this study are particularly sensitive to the effects of ms-222 and curare.

CRISPR Confounds

A limitation to the proposed genetic model of epilepsy is the complex nature of disease states in an *in vivo* model. GAMOS is characterized by disruption of multiple organs including the eyes, kidney and brain. RNA from whole larval lysates reflect IEG fluctuations from disrupted organs as well as abnormal electrical activity. Using the “toolbox” developed in this study, decreased IEG expression cannot be attributed to epileptic activity alone. Disease complexity of GAMOS is representative of many epilepsy-related disorders, despite the disease’s heterogeneity. Other studies using a zebrafish models of epilepsy that implement the IEG toolbox characterized here will likely have similar confounds. Any claims about whole lysates of translational models will therefore be limited to differential IEG expression as a whole to the disease states and nonspecific to excitability.

Future Directions

A major surprise from this study was the unexpected increase in IEG expression due to the ‘control’ drugs, curare and ms-222. These drugs induced IEG expression independent of their paralytic and anesthetic effects, consequently exacerbating the ‘noise’ they were intended to eliminate. It is possible that these drugs are uniquely responsible for the increase in IEG expression seen in past reports. Other paralytics such as pancuronium bromide and α -bungarotoxin (Baraban & Lee, 2013) have been used for immobilizing larval zebrafish for electrophysiology. However, pancuronium bromide has been shown to cause a small decrease in neuronal activity in larval zebrafish (Lauderdale, 2013). Similarly, benzocaine has been used as larval zebrafish anesthetic for electrophysiology. (Allen & Marcotti, 2016) However, because these drugs are chemically and mechanistically similar to curare and ms-222, it is quite possible that they will produce a similar increase in IEG expression, but those experiments have yet to be conducted.

The unexpected increase in IEG expression due to curare and ms-222 illustrates the importance of elucidating the mechanism of IEG fluctuations in PTZ models of zebrafish epilepsy. Curare and ms-222 likely activate IEG expression independent of neuronal activity and the extent to which non-neuronal activity plays a role in IEG increase in a PTZ-induced model is still unknown. A possible way to better isolate IEG increase in a PTZ model is to compare whole body lysates to dissected brains. This type of experiment does present technical difficulties because of the low RNA yield

from isolated larvae zebrafish brains. Additionally, it is possible that PTZ drug exposure increases expression of IEGs in the brain independent of increased neuronal activity. A comparison of other commonly use proconvulsant, such as 4-aminopyridine (Taylor, 2007) and linopirdine (Baraban, 2007), rescued with known AEDs in larvae zebrafish, could help elucidate the role of IEG expression due to increased neuronal activity. As we have observed with more detailed movement pattern analysis different patterns of movement in larval zebrafish when they are exposed to different pro-convulsants (unpublished data, Jeremy Ullmann and Hien Nguyen), analysis of different IEG expression patterns may yield correlations that will be useful when evaluating genetic models for abnormal seizure-like activity.

Investigation of IEG expression in zebrafish epilepsy other than drug-induced models would help better elucidate variable transcription from abnormal electrical activity. Established genetic models of zebrafish epilepsy such as *Scn1ab* (Baraban, 2013) and *kcnq3* (Chege, 2011) have not been tested for variable IEG expression. *Scn1a* is a sodium channel shown to cause spontaneous abnormal electrical discharges in a chemically mutated zebrafish model (Baraban 2013). *Scn1a* mutants also exhibited behavioral phenotype similar to fish exposed with PTZ. A *Kcnq3* morpholino fish also showed abnormal bursts of electrical discharges (Chege, 2011). Both of these zebrafish epilepsy models are channelopathies with minimal comorbidities noted in the experimental models, which may provide better models for investigation of exclusively abnormal electrical activity effecting IEG expression. Heat-induced zebrafish seizures (modeling the human condition of febrile seizures) could also provide a better look at IEG transcription without the confounds of drug-induced seizure models.

Electrophysiology of zebrafish heated to 45°C show long-duration bursts of electrical discharges (Hunt, 2013), but a detailed investigation of IEG expression after heat-induced seizure events has yet to be conducted.

The IEGs chosen for this study were thought to be a good indicator of seizure-like activity based on previously described investigation in larval zebrafish and specificity of stimuli. An expansion of the IEG “toolbox” could better identify genes more highly correlated to transcriptional fluctuation and abnormal electrical activity. For example, *Arc/Arg3.1* is upregulated in the dendrites of glutamatergic neurons in the forebrain of an electroconvulsive mouse model. (Tzingounis, 2006). Additionally, a PTZ kindling mouse model of *Arc/Arg3.1* showed a marked increase in expression in the CA1 region of the hippocampus and the dentate gyrus (Szyndler, 2013)

Conclusion

Well established loading controls for qPCR in drug-exposed models of zebrafish seizures have not been previously well characterized. Our data indicate that *Mglobulin* and *tuba1* are the controls best suited for stability between unexposed, PTZ, curare and ms-222 exposure. Established controls such as *βactin* and *gapdh* fluctuated with these commonly used drug exposures. Furthermore, curare and ms-222 caused IEG increases in larval zebrafish in opposition to the behaviors and neuronal inhibition the drugs elicit. PTZ exposure causes all IEG expression to rapidly increase and then decline with prolonged exposure. One overall aim of this project was to identify independent measures of abnormal electrical activity that could be used in the future evaluation of genetic models of zebrafish epilepsy. We piloted this approach using the *osgep* KO model since

seizures are seen in a human condition associated with homozygous recessive mutation (loss of function). In the *osgep* zebrafish model, we had observed abnormal electrophysiological activity despite no observed behavioral seizure phenotype. Our IEG results in this model showed a trend in the direction of what was observed with prolonged PTZ exposure, with decreased fold change, but the results were not statistically significant. Further investigation of IEG response will be required to better elucidate the highly variable expression patterns of abnormal zebrafish neuronal activity to a sufficient degree that it will be a useful screen for abnormal seizure-related activity in genetic models.

Appendix.

Gene	Function	Design
Tuba1	Cytoskeleton	F) CCTGCTGGGAACTGTATTGT
		R) TCAATGAGTTCCTTGCCAAT
Gapdh	Glycolysis Enzyme	F) GTGGAGTCTACTGGTGTCTTC
		R) GTGCAGGAGGCATTGCTTACA
Elfa	Translation	F) CTTCTCAGGCTGACTGTGC
		R) CCGCTAGCATTACCCTCC
βactin1	Cytoskeleton	F) CGGTGGATCCTGCGAATTA
		R)TGACAGGTTATGAAGCAAAACAAC
Mglobulin	Histocompatibility complex	F) GCCTTCACCCCAGAGAAAGG
		R) GCGGTTGGGATTTACATGTTG

Table 1. Reference Genes Probes. Q-PCR reference gene probe design and related function (Amy T McCurley and Gloria V Callard 2008).

Table 2. Immediate Early Gene Probes

Gene	IEG Type	Function	Design
c-Fos	Early	Transcription factor subunit of the AP1 complex.	F) AACTGTCACGGCGATCTCTT
			R) GCAGGCATGTATGGTTCAGA
Npas4	Early	Neuronal transcription factor exclusively induced by Ca ²⁺ .	F) AGCCAAGTCTGCCCTTCTTCT
			R) TGCTGTGCTAAAAGCGAGATCT
NR4a1	Early	Transcription factor highly expressed in neurons and macrophages.	F) CGGTTTCTCTGCTTTCTTGG
			R) GCATTTGATTTGCACCATTG
Cebp	Inducer	Transcription factor with a CCAAT motif binding affinity.	F) CGACTTTCTCTCTGAGGGGAACA
			R) CAGCGAGATGTAGTTCTTGAGTTCT
BDNF	Inducer	Neurotrophic growth factor largely associated with synapse formation.	F) TCGAAGGACGTTGACCTGTATG
			R) TGGCGGCATCCAGGTAGT
Homer1a	Inducer	Involved in the transient breakdown of mesh scaffolding in the post synaptic density.	F) CGCCGTGGGATCTATTTCAGT
			R) TTATGAGCAGCGGCATTCTG

Table 2. Immediate Early Gene Probes. Primer design for qPCR as they relate to immediate early genes and their corresponding peak activation time (Sun & Lin, 2016).

CTC	Treatment	cFos	Npas4	Nr4a1	BDNF	Cebp	Homer1a
Untreated Control	30min Curare	141.82	7.75	25.61	14.85	63.71	10.09
	30min ms-222	29.03	4.86	2.27	3.51	5.90	1.73
	30min PTZ	28.72	5.61	1.14	3.06	4.74	2.31
	60min PTZ	1.80	1.50	0.80	1.08	0.88	1.17
Curare	30min Curare PTZ	1.73	1.13	1.47	0.36	1.23	0.63
	30min Curare Tricaine PTZ	1.50	0.52	0.89	0.42	0.60	0.51
Osgp +/-	OSGEP +/-	0.36	0.20	0.57	0.32	0.37	0.55
	OSGEP -/-	0.63	0.18	0.33	0.70	0.34	0.50

Table 3. Condition Fold Changes. Conditions for qPCR experiments and accompanying fold changes of each IEG. n = 4 pools of 10 larval zebrafish for all 30 minute test conditions and all OSGEP +/- conditions; n = 6 for 60 minute PTZ treatment group and the untreated control. All samples were run in triplicate and averaged to avoid pipetting error. CTC= Control Test Condition (normalization group for treatment condition)

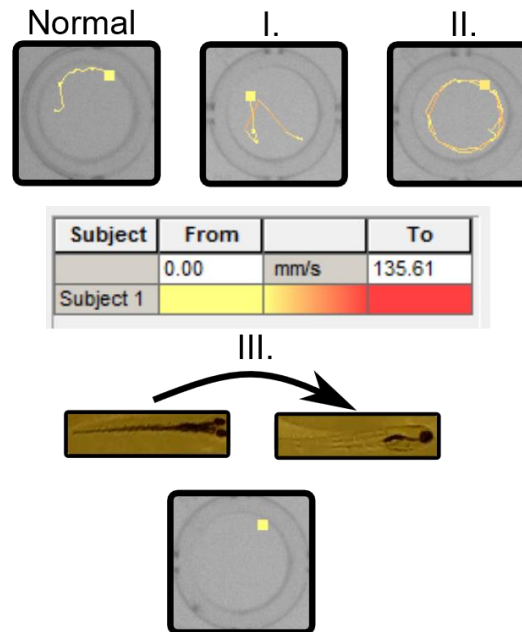


Figure 1. Seizure Stages. Zebrafish larvae seize in 3 distinct stages in response to PTZ treatment. Untreated larvae typically move in short, quick bursts known as “beat and glide” movement. Stage I behavior is characterized by an increased frequency and intensity of the normal beat and glide movement. Stage II behavior is marked by events of rapid “whirlpooling” around the outside of a well. Stage III behavior is characterized by events of short convulsions followed by an inability to right posture; often preceded by a stage II event (Baraban 2005). Figure 1a. Depicts normal, stage I and Stage II behavior captured by a DanioVision within a five second time period. Figure 1b. Depicts Stage III convulsion captured by a DanioVision within a 5 second time period as well as an image of a 5dpf larvae unable to right its posture.

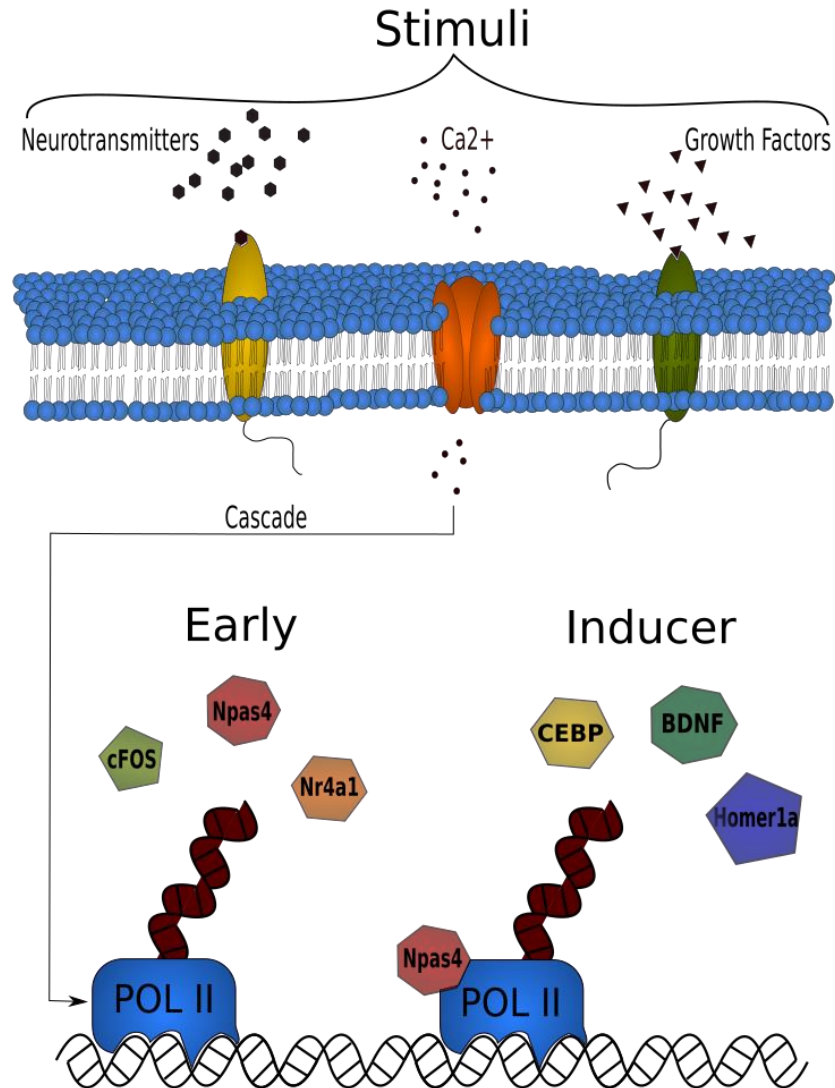


Figure 2. IEG Pathway. Various extracellular stimuli produce an intracellular cascade which activate early IEG's. Early IEG's are transcription factors for inactive pol II poised on DNA start sites. After early IEG binding pol II is activated and transcribes inducer IEG's (Sun & Lin, 2016).

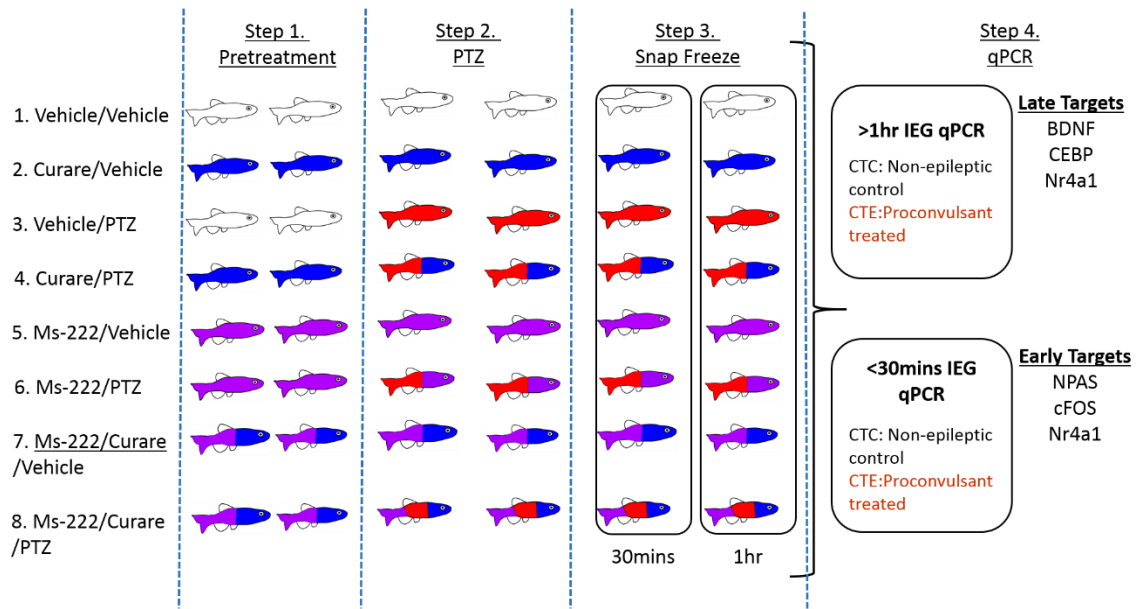


Figure 3. Drug Treatment Assay. The drug treatment assay is divided into four stages. During stage 1 larvae are plated in 96 well plates incubated with anesthetic or paralytic and incubated for 10 minutes. During stage 2 larvae are treated with PTZ incubated for 10 minutes and then place in the DanioVision. At stage 3 larvae are flash frozen in liquid nitrogen at either 30 minutes or 60 minutes. QPCR of IEG targets is conducted at Stage 4. White = Vehicle ; Blue = Curare ; Purple = Ms-222 ; Red = PTZ.

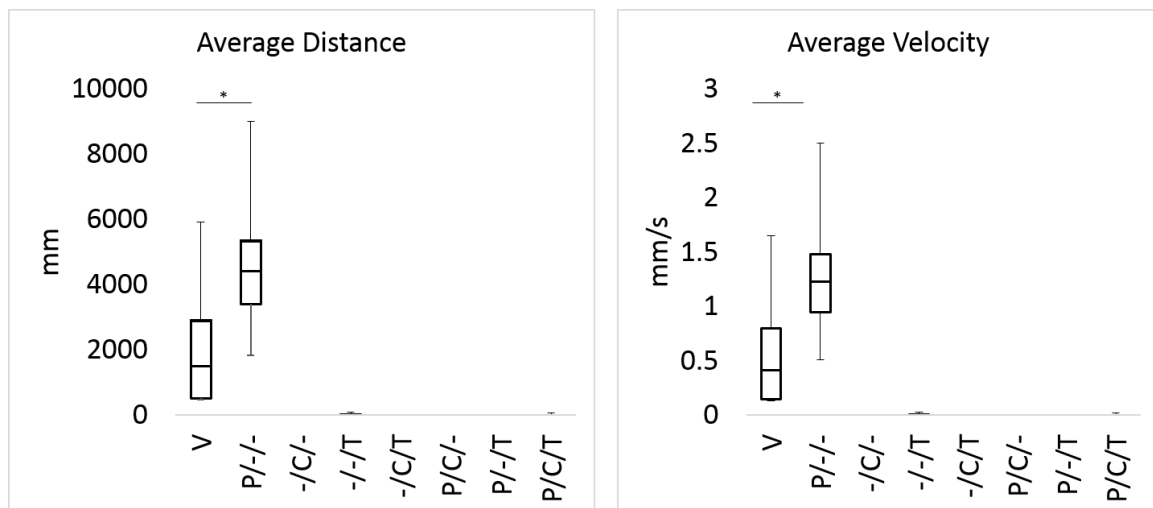


Figure 4. Chemically Treated Behavior. Movement parameters increase in response to proconvulsant and cease in fish treated with both paralytic and anesthesia in a one hour experiment. Average distance traveled is the average of the total distances an individual fish traveled during an entire trial. Average velocity is the average of the average velocity of an individual fish during an entire trial. $n = 96$ for each drug treatment group. All conditions are significantly difference from untreated vehicle by t -test $p < .05$. V = Vehicle ; P/-/- = PTZ ; -/C/- ; -/-/T = Ms-222 ; -/C/T = Curare/Ms-222 ; P/C/- = PTZ/Curare ; P/-/T = PTZ/Ms-222 ; P/C/T = PTZ/Curare/Ms-222.

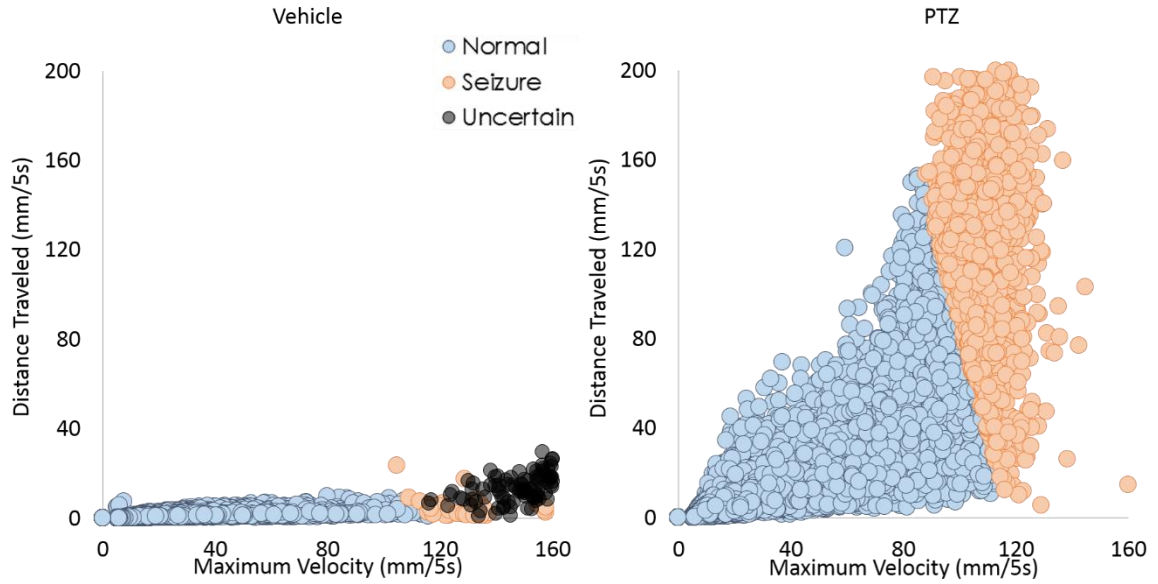


Figure 5. Chemically Treated Seizure Classifier. PTZ treatment greatly increases the number of stage II seizure events called by the Seizure Classifier Algorithm. Each data point is a 5 second time bin of larval behavior. “Distance traveled” is defined as the total mm a larvae traveled in 5 seconds and “maximum velocity” is the highest velocity a larvae reached within a 5 second time bin. Bins that cross the Stage II threshold as determined by the classifier algorithm are considered seizure. $n = 96$ 5 dpf larvae during a 60 minute trial.

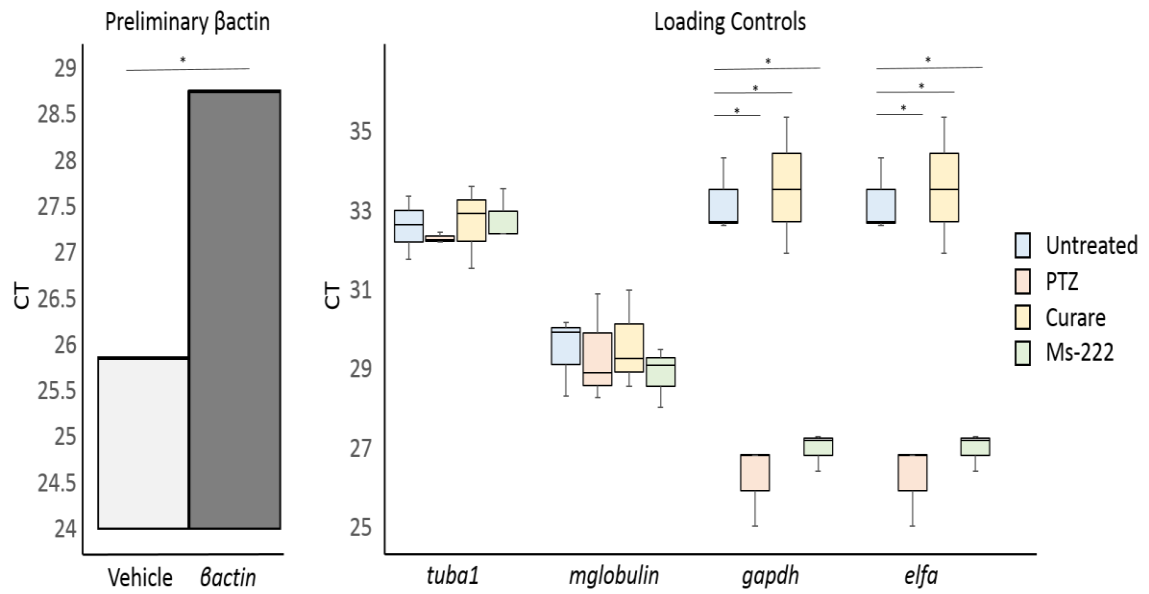


Figure 6. Reference Gene Stability. Preliminary qPCR of Beta Actin in PTZ treated larval zebrafish has a significantly higher ct value than vehicle control. Tuba1 and mglobulin remain transcriptionally constant across all drug treatments while gapdh and elfa vary. All drug treatments were conducted for 30 minutes. All samples concentrations were normalized and run in triplicate. $n = 6$ vehicle- β actin1 and PTZ- β actin1; $n = 4$ for all samples in 6b. Drug treated conditions are significantly different from untreated vehicle by t-test $p < .05$.

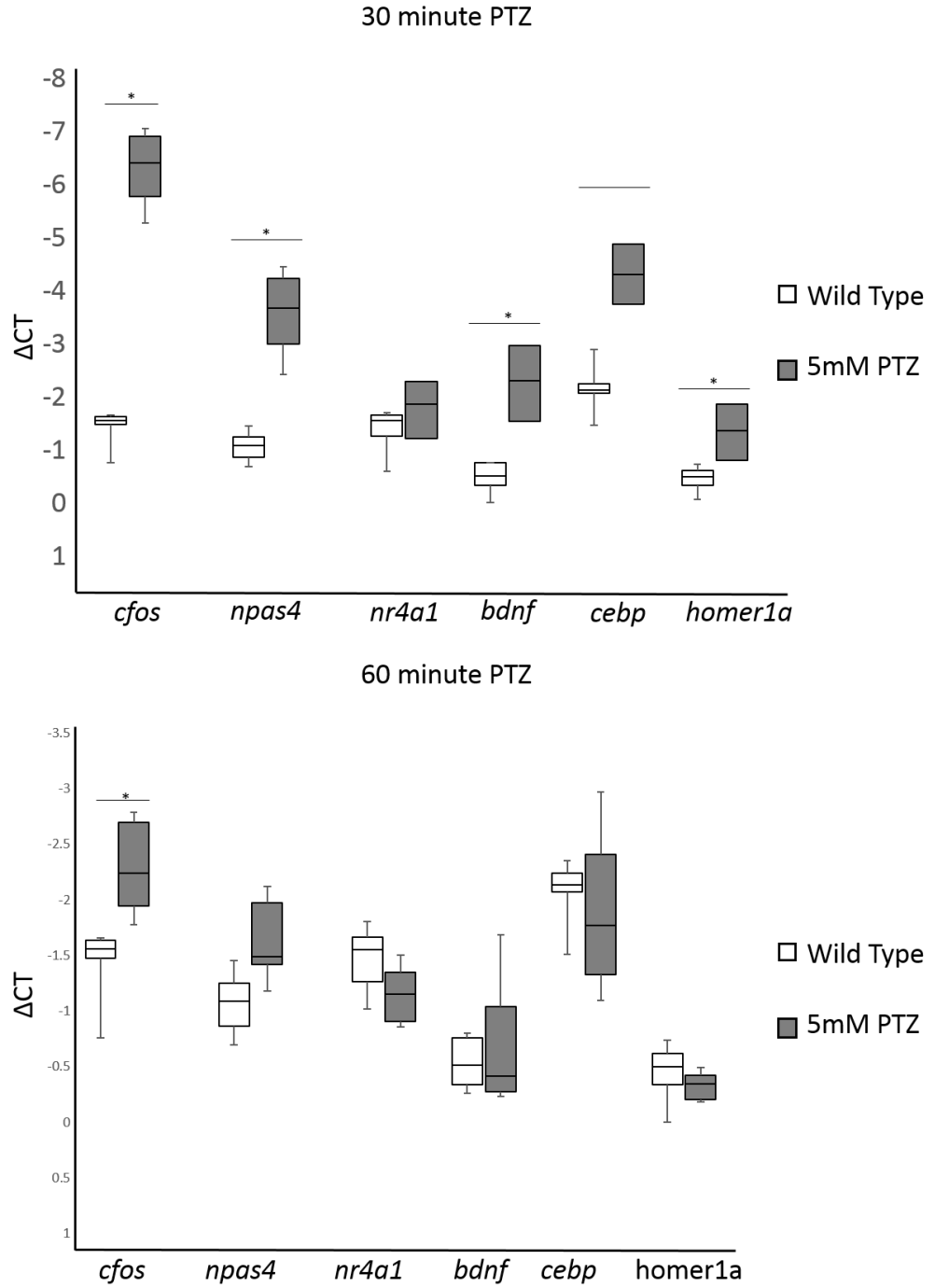


Figure 7. IEG qPCR PTZ Treatment. 30 minute PTZ exposure significantly increases gene expression of all tested IEG's excluding nr4a1. 60 minute PTZ exposure increases cfos expression while all other tested IEG's remain insignificantly changed. Delta CT values were obtained by mglobulin normalization. n = 4 pools of 10 larvae. Delta Ct was inverted to reflect the inverse relationship between ct value and quantity of RNA per sample. All samples were run in triplicate. PTZ significantly different from untreated vehicle by t-test $p < .05$.

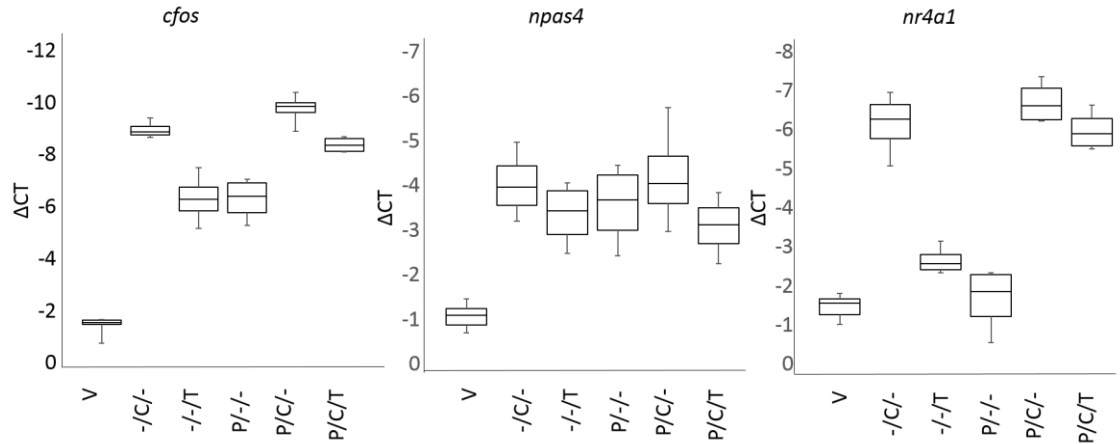


Figure 8. Early IEG Drug Treatments. All drug treatments increase gene expression of early IEGs compared to vehicle. Delta CT values were obtained by mglobulin normalization. $n = 4$ pools of 10 larvae. All samples were run in triplicate. Drug treated conditions are significantly different from untreated vehicle by t -test $p < .05$. Delta Ct was inverted to reflect the inverse relationship between ct value and quantity of RNA per sample. V = Vehicle ; P/-/- = PTZ ; -/C/- ; -/-/T = Ms-222 ; P/C/- = PTZ/Curare ; P/C/T = PTZ/Curare/Ms-222.

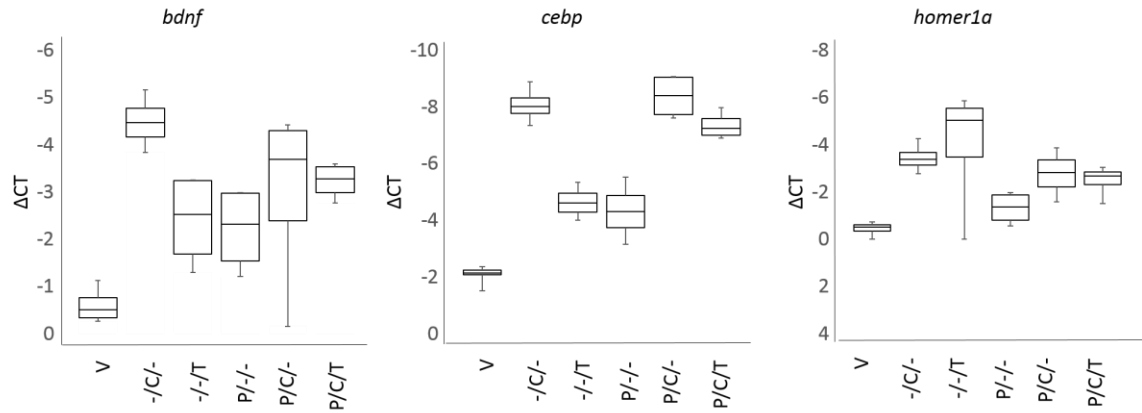


Figure 9. Late IEG Drug Treatments. All drug treatments increase gene expression of late IEGs compared to vehicle. Delta CT values were obtained by mglobulin normalization. $n = 4$ pools of 10 larvae. All samples were run in triplicate. Drug treated conditions are significantly different from untreated vehicle by t -test $p < .05$. Delta Ct was inverted to reflect the inverse relationship between ct value and quantity of RNA per sample. V = Vehicle ; P/-/- = PTZ ; -/C/- ; -/-/T = Ms-222 ; P/C/- = PTZ/Curare ; P/C/T = PTZ/Curare/Ms-222.

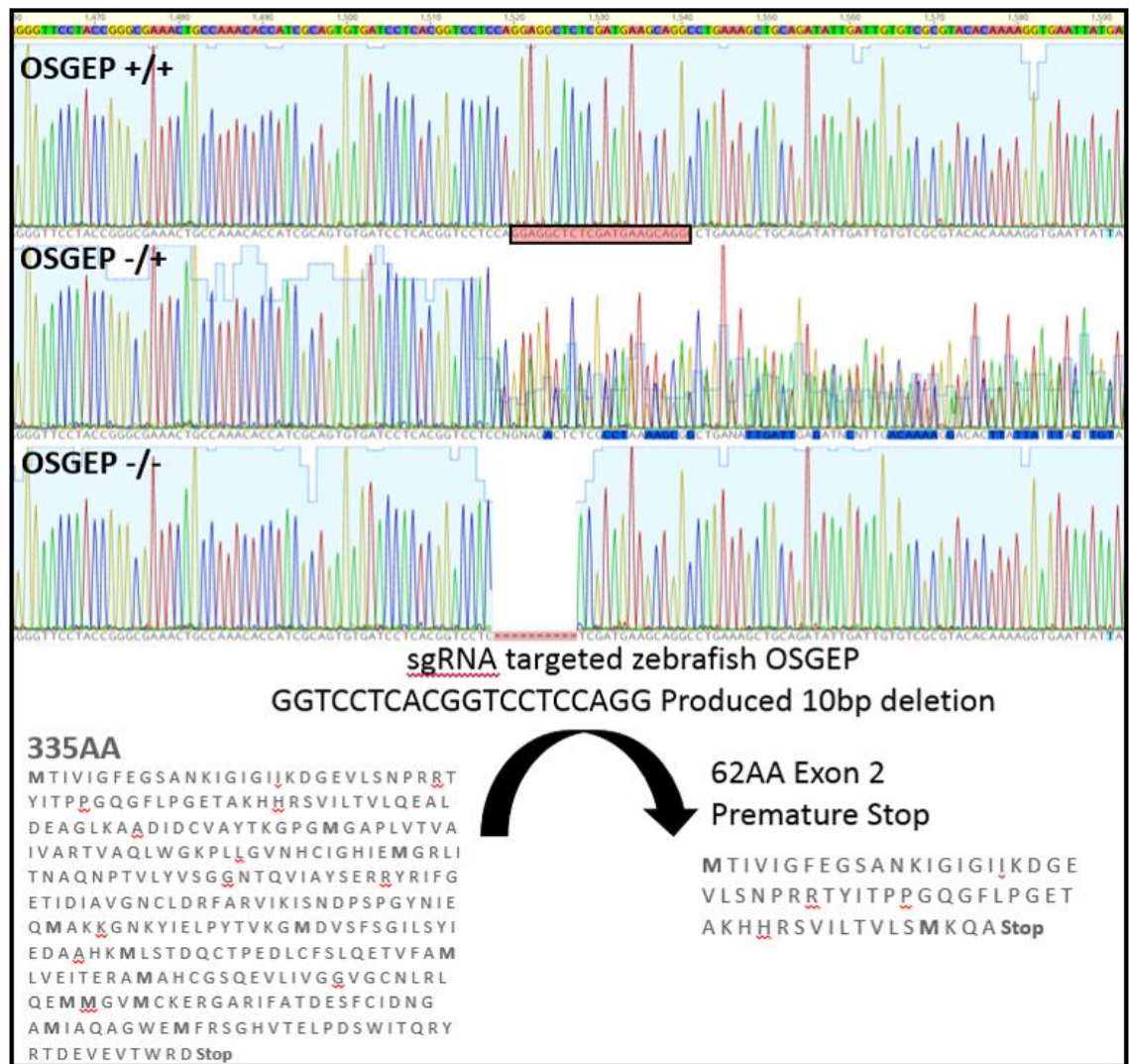


Figure 10. *Osgep* Knock Out. Schematic representation of CRISPR mediated *OSGEP* knockout. A 10 base pair deletion in exon 2 resulted in a premature stop codon. Resulting protein was reduced *OSGEP* from 335 amino acids to 62. Sanger traces were derived from F2 5dpf larval zebrafish.

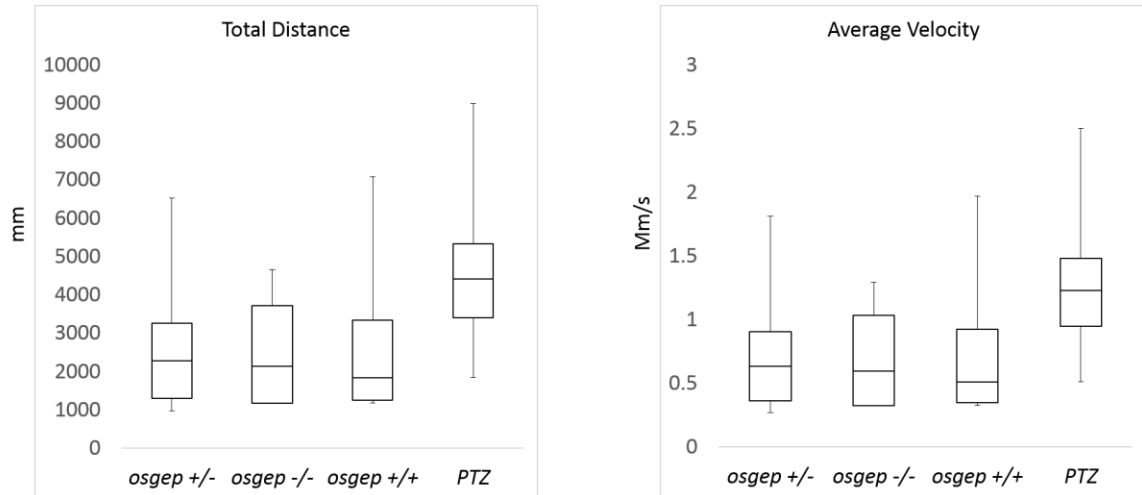


Figure 11. Osgep Behavior. Movement parameters are statistically unchanged between Osgep +/+, Osgep +/- and Osgep -/-. Figure 11a. Average distance traveled is the average of the total distances an individual fish traveled during an entire trial. Figure 11b. Average velocity is the average of the average velocity of an individual fish during an entire trial. $n = 96$ for each drug treatment group.

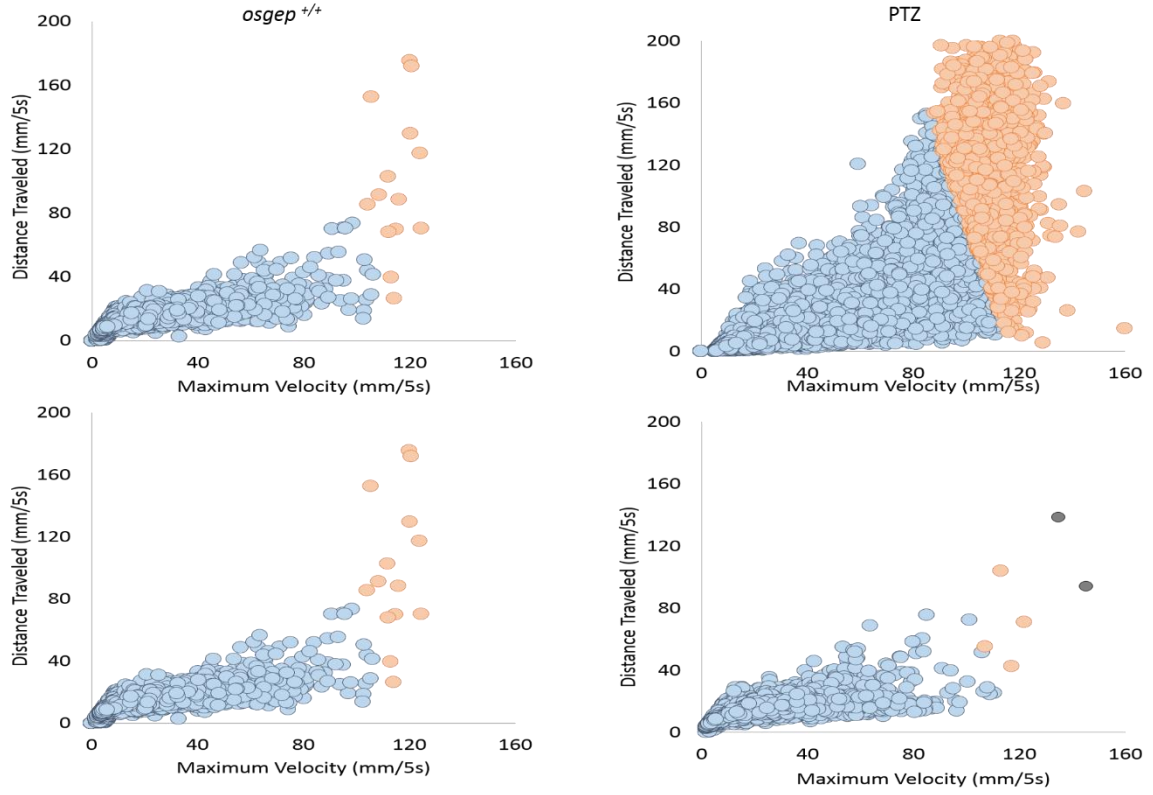


Figure 12. *Osgep* Seizure Classifier. Stage II seizure events called by the Seizure Classifier Algorithm are unchanged between *Osgep* +/+, *Osgep* +/- and *Osgep* -/-. Each data point is a 5 second time bin of larval behavior. “Distance traveled” is defined as the total mm a larvae traveled in 5 seconds and “maximum velocity” is the highest velocity a larvae reached within a 5 second time bin. Bins that cross the Stage II threshold as determined by the classifier algorithm are considered seizure. $n = 96$ PTZ treated, $n = 15$ *Osgep* +/+, $n = 55$ *Osgep* +/-, $n = 23$ *Osgep* -/-. All larvae were tested at 5dpf during a 60 minute trial.

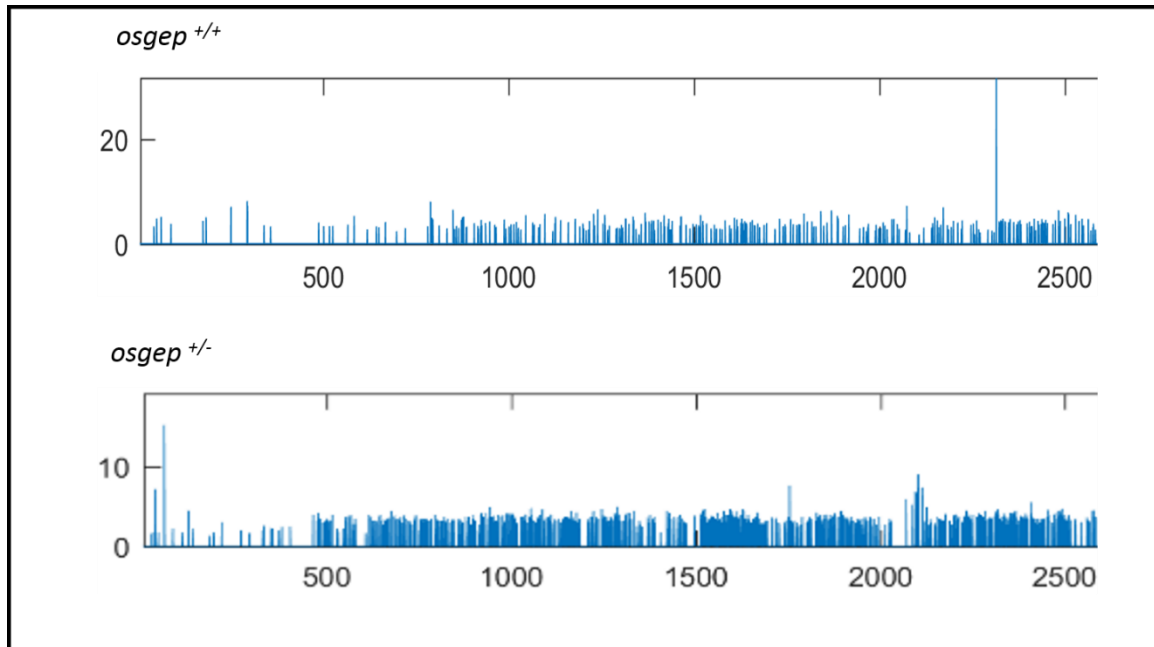


Figure 13. Osgep Electrophysiology. Osgep^{+/-} *exhibit an increase in high frequency spikes events compared to osgep*^{+/+}. *High frequency spikes are defined by > 5 amp au. Threshold.... Osgep*^{+/-} *n=3 and osgep*^{+/+} *n=4.*

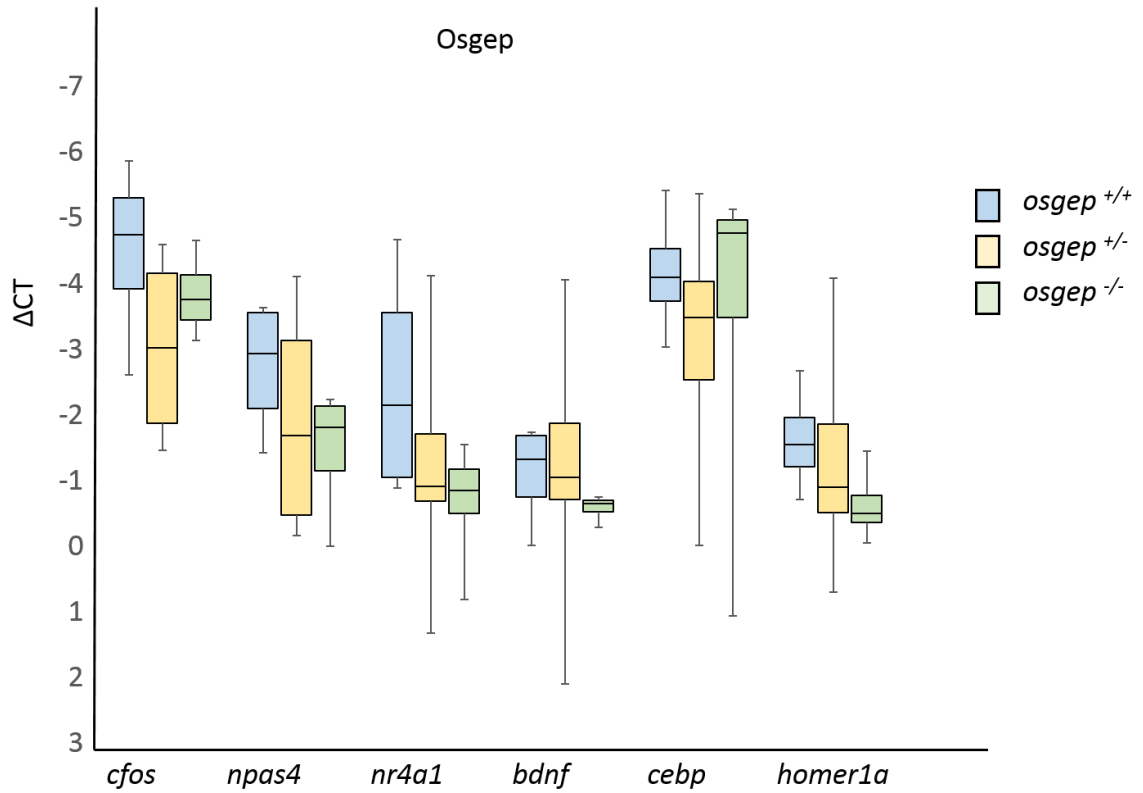


Figure 14. *Osgep* qPCR. Individual IEG expression are unchanged between *Osgep* ^{+/+}, *Osgep* ^{+/-} and *Osgep* ^{-/-} groups. Delta CT values were obtained by mglobulin normalization. *n* = 4 pools of 10 larvae. All samples were run in triplicate. Delta Ct was inverted to reflect the inverse relationship between ct value and quantity of RNA per sample. V = Vehicle ; P/-/- = PTZ ; -/C/- ; -/-/T = Ms-222 ; P/C/- = PTZ/Curare ; P/C/T = PTZ/Curare/Ms-222.

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