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High-Throughput Behavioral Assay to Investigate Seizure Sensitivity in Zebrafish Implicates ZFHX3 in Epilepsy

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Abstract

Epilepsy, which affects ~1% of the population, is caused by abnormal synchronous neural activity in the central nervous system (CNS). While there is a significant genetic contribution to epilepsy, the underlying causes for the majority of genetic cases remain unknown. The NIH Undiagnosed Diseases Project (UDP) utilized exome sequencing to identify genetic variants in patients affected by various conditions with undefined etiology, including epilepsy. Confirming the functional relevance of the candidate genes identified by exome sequencing in a timely manner is crucial to translating exome data into clinically useful information. To this end, we developed a high throughput version of a seizure-sensitivity assay in zebrafish (*Danio rerio*) to rapidly evaluate candidate genes found by exome sequencing. We developed open access software, SEIZR (Studying Epilepsy In Zebrafish using R), to efficiently analyze the data. SEIZR was validated by disrupting function of a known epilepsy gene, *prickle 1*. Next, using SEIZR, we analyzed a candidate gene from the UDP screen (*Zinc Finger Homeobox 3*, *ZFHX3*), and showed that reduced *zfhx3* function in zebrafish results in a significant hyperactive response to the convulsant drug pentylenetetrazol (PTZ). We find that *zfhx3* shows strong expression in the CNS during neurogenesis including in the pallium, thalamus, tegmentum, reticular formation, and medulla oblongata—all regions which have roles in motor control and coordination. Our findings in the zebrafish confirm human ZFHX3 is a strong candidate for further neurological studies. We offer SEIZR to other researchers as a tool to rapidly and efficiently analyze large behavioral data sets.

Keywords

epilepsy; central nervous system; *zfhx3*; tracking data analysis; neurogenesis; development

Introduction:

Epilepsy is characterized by abnormally excessive or synchronous neural activity in the central nervous system (CNS) resulting in episodic seizure events (Fisher et al., 2005). Unfortunately, current therapies have varying efficacies in different patients depending on the seizure types (<http://www.ninds.nih.gov/disorders/epilepsy/epilepsy.htm>). Epilepsy affects ~1% of the general population and 70% of these identified cases have no known cause and are presumed to be genetic (Begley et al., 2000; Heron, Scheffer, Berkovic, Dibbens, & Mulley, 2007). Consequently, there is a clear need to identify the genetic factors associated with epilepsy in the general population, especially to develop more effective treatment options (Heron et al., 2007; Myers & Mefford, 2015; Scheffer, 2014; Vadlamudi et al., 2014).

Next generation sequencing technologies have revolutionized the field of genetics. The NIH Undiagnosed Diseases Program (UDP) was established to take advantage of this technology to collect exome sequencing data from patients affected by various genetic conditions with no identified cause, including epilepsy (Gahl et al., 2012; Gahl et al., 2016; Gahl & Tifft, 2011; Tifft & Adams, 2014). This has allowed for the identification of several genetic variants potentially implicated in disease. However, validation of identified variants remains challenging as these variants are rare (often present in only one patient). Direct functional evaluation of identified variants and their role in disease in tractable animal models can address this challenge but current models are not well-suited for high through-put analyses.

Development of rapid high-throughput methods to analyze candidate genes implicated by exome sequencing is a critical next step for aiding patients who have genetic variants of these genes. To this end, we sought to develop a high-throughput behavioral assay using the zebrafish (*Danio rerio*) to rapidly and efficiently evaluate the contribution of candidate genes in epilepsy. The zebrafish has several traits making it ideal for high-throughput studies including large clutch sizes, transparent embryos, and rapid development. Furthermore, when exposed to convulsant drugs, zebrafish show seizure-like responses, by both electrophysiological and behavioral criteria (Hortopan, Dinday, & Baraban, 2010; Teng et al., 2010; Teng et al., 2011). Using behavioral assays, work from our group has confirmed the role of the *prickle 1* (*pk1*) and *2* (*pk2*), genes that are associated with seizures in humans (Bassuk et al., 2008; Hirotaka Tao et al., 2009). Specifically in *pk1* and *pk2* morphant larvae (larvae treated with antisense morpholino oligoes, MOs), we observed hyperactive motility in response to the seizure-inducing drug, pentylenetetrazol (PTZ) (Mei et al., 2014; Mei, Wu, Bassuk, & Slusarski, 2013); such a sensitized response to PTZ implies that normal levels of Pk1 and Pk2 activity directly or indirectly mitigate the risk of epileptic seizures and loss of function enhances the risk.

In the current study, we optimized this behavioral assay for high-throughput analysis, in order to facilitate evaluation of candidate genes selected from the NIH UDP. This analysis uses the motility tracking software of the ZebraBox (Viewpoint Life Sciences) to quantify the distance moved (in cm) of larvae in 96-well plates. This assay generates 120 megabytes of data in spreadsheets with more than 700,000 rows in 24 columns for each assay. In order to efficiently analyze these data sets and reduce the possibility of human error while

handling the data sets, we developed software called Studying Epilepsy In Zebrafish using R (SEIZR) that will automatically process the raw data and return manageable tables for further analysis. In addition, we utilize antisense mediated knockdown to titrate gene activity, as many of the candidates in the UDP have essential roles in development and knockouts cause early defects that prevent analysis of neural function. High-throughput motility based tracking in combination with SEIZR processing of large-scale datasets, allowed for the rapid and efficient analysis of candidate genes for seizure sensitivity in zebrafish knockdown larvae. Here we discuss the findings for one of the UDP candidate genes, Zinc Finger Homeobox 3 (*ZFHX3*) (also known as *ATBFI*), and offer SEIZR as a tool to other investigators for automated behavior analysis of genetic and pharmacological manipulations in zebrafish.

Results:

Behavioral Assay:

At 3 days post-fertilization (dpf), zebrafish larvae have developed the basic neural networks required to swim and interact with their environment (Jontes, Buchanan, & Smith, 2000). The movement of the larvae can be observed using an infrared camera in an automated video tracking device. We utilize the Viewpoint ZebraBox to analyze up to 96 individuals simultaneously in a 96 well plate (Figure 1A). The Zebbralab software records multiple parameters of the movement of each individual larva. One commonly used property for seizure analysis is the total distance moved by the larvae, which is represented as small distance (slower than 10mm/sec) and large distance (10mm/sec and faster), denoted, respectively, by the green and red lines in Figure 1B and C. At 3dpf, wild-type (WT) larvae are typically quiet with little total movement (Video 1, Figure 1B). When the same larva shown in Video 1 is exposed to PTZ, the animal displays seizure-like behavior with hyperactive, jerky, swimming (Video 2, Figure 1C). Figure 1 D is a plot of the average activity, over one-minute intervals, for each larval group, to illustrate changes in total distance moved, before and after reagent addition. For our analysis, we establish a baseline of activity prior to treatment. Larval movement generally ranges between 0–15 cm/min (Figure 1D). After a thirty-minute baseline is collected, PTZ or control media is added and movement is monitored for an additional thirty minutes. In contrast to the control treated set (blue), the PTZ treated set (green) shows a robust increase in distance moved (Figure 1D).

SEIZR Software Development:

While tracking systems facilitate behavioral assays, investigators need to manually extract the data relevant to their analyses. As mentioned, tracking each animal in a 96 well plate (every second for the total sixty-minute duration of the experiment) generates 120 megabytes of data, resulting in spreadsheets with more than 700,000 rows and 24 columns. Manual extraction of the relevant data from the large spreadsheets was time consuming and set up the potential for error in extraction. In order to efficiently analyze the tracking data and reduce the possibility of human error, we used R programming and created SEIZR (Studying Epilepsy In Zebrafish using R (R Core Team, 2015). SEIZR was programed to extract the relevant columns of data from the spreadsheets. In addition, the Shiny application (R Studio) was used to create a web-based graphical user interface for SEIZR that supports

user-friendly access. In this interface, raw tracking data can be uploaded (Figure 2A). Users have several options to select from for data grouping and output. The program groups larvae by row. The sample plate in Figure 2A shows rows A and B (group 1), C and D (group 2), E and F (group 3), and G and H (group 4). For data output, users can set a minimum distance (cm) that each larva must move in order to be included in the data analysis (Figure 2A). SEIZR calculates the total distance moved by each larva for the entire assay (before and after PTZ), and if the distance is lower than the user defined threshold, the well is excluded from analysis. This minimum distance parameter is a filter to exclude empty wells and larvae that are inactive over the entire hour. For our assays, we screen larvae for normal morphology, touch response, and swimming behavior prior to tracking. Our threshold is set at 0.1cm. SEIZR will calculate the total distance moved for each larva by adding all of the small and large movements over each thirty-minute period (before and after PTZ) and return the data in an organized and easy-to-read table (Figure 2B). SEIZR will also calculate the average distance moved and standard error of each user-defined group for the thirty minutes before and thirty minutes after PTZ exposure (Figure 2C shows data corresponding to groups selected in Figure 2A). SEIZR was developed to allow investigators to analyze large data sets and is presently hosted publicly online at the following URL: https://tyson-fuller.shinyapps.io/SEIZR_Tracking_App/. It is important to note that SEIZR was designed to upload and analyze data sets created with the Viewpoint Zebralab Tracking software. However, there are many tools available to track larval activity. All of these allow the user to set various parameters according to their respective assays. The utility of SEIZR is the ability to take the spreadsheets returned and extract the relevant data, grouping them into the appropriate experimental sets. In order to maintain user-friendly functionality of SEIZR the program must be used in combination with the Viewpoint Zebralab Tracking software and the data are returned to the user in thirty-minute intervals regardless of the time bins used to record the raw data. However, the source code is available upon request. This makes it possible for users with data sets collected from different software or those who would like to view the data according to other parameters such as different time intervals to make edits to the existing code to optimize the program for their respective analyses.

SEIZR Software Validation:

To validate the SEIZR software, we first determined a PTZ dose-response curve for uninjected, age-matched WT larvae. Larval activity naturally fluctuates from minute to minute; therefore, we combine data from 30 minutes of movement for statistical analysis. With WT larvae, we observe increased activity with PTZ dose (Figure 3A, Spearman rank correlation of $r=0.551$, $p<0.0001$). Consistent with our previously published work (Mei et al., 2013), the 5 mM PTZ dose showed the most consistent response (smallest variation between larvae) and, therefore, this dose was used for further analyses.

To further validate the behavior assay and gene manipulation techniques used in conjunction with SEIZR, we tested a previously validated epilepsy gene, *pk1a* (Bassuk et al., 2008; Mei et al., 2013; H. Tao et al., 2011). We performed the behavior assay on *pk1a* morphant larvae along with age-matched, uninjected controls. We analyzed the data with SEIZR and demonstrated that SEIZR recognized the *pk1a* morphants as being seizure-sensitized (Figure

3B, $p=0.027$) in agreement with previously published findings (Mei et al., 2013). Thus, SEIZR efficiently and accurately analyzes differences in larval activity between groups.

Zfhx3 Knockdown Sensitizes Zebrafish to PTZ Treatment: We next use the approach to evaluate a disease candidate gene. *ZFHX3* was identified by the NIH UDP in one patient who carried two missense mutations, one of each allele (<https://www.genome.gov/27544402/the-undiagnosed-diseases-program/>). This patient showed initial focal seizures that progressed to multifocal and generalized seizures. Independently, two additional *de novo*, *ZFHX3* variants were found by whole-exome sequencing in two patients suffering from epileptic encephalopathies (Epi & Epilepsy Phenome/Genome, 2013). Zebrafish *Zfhx3* is 63% identical to human *ZFHX3* and the amino acids corresponding to the UDP patient's mutations are conserved. Mouse knockouts of *Zfhx3* are embryonic lethal as homozygous and heterozygous deletion results in pre-weaning mortality (Sun et al., 2012). This is consistent with a human population-genetic analysis, which showed that *ZFHX3* alleles containing missense or nonsense mutations are strongly underrepresented in the general population. This is a striking result considering the large size of the *ZFHX3* protein (3838 amino acids), indicating that such mutations are likely to be heterozygous lethal in humans (<http://exac.broadinstitute.org/gene/ENSG00000140836>). In order to allow development to a stage in which one can evaluate neural function, a targeted/conditional and/or titrated manipulation of *ZFHX3* activity is needed. To model this, we utilized gene knockdown (KD) with two independent MOs, one that targets splicing (*zfhx3* sb) and a second that blocks translation (*zfhx3* met) of endogenous *zfhx3* mRNAs in the zebrafish. The *zfhx3* sb MO targets the intron 2-exon 3 splice acceptor site. The resultant product skips the 232 base pair exon 3 and leads to a frame shift and early termination (Figure 4A). For analysis of swimming behavior in morphant larvae, we selected a low dose KD of *zfhx3* that shows normal morphology and touch response (Figure 4B) as well as normal transcript along with the altered transcript (Figure 4A). Consistently before PTZ, *zfhx3* morphant larval swimming activity is the same as age-matched WT (Figure 4C, before PTZ). In contrast, after PTZ exposure, the *zfhx3* morphant larvae showed a hyperactive response compared to WT (Figure 4C, after PTZ). In addition, we find the same seizure-sensitized response with the second MO targeting the translation start site. *zfhx3* morphant larval swimming activity is the same as age-matched WT and negative control MO injected larvae (Figure 5A) and a hyperactive response of the *zfhx3* morphant larvae is observed after PTZ compared to WT and control MO injected larvae (5A, after PTZ). Taken together, these results indicate that reduced *Zfhx3* function results in seizure sensitivity.

The standard approaches used to analyze seizure-sensitivity have typically been limited by sample size and/or computational effort. The high-throughput pipeline that we have developed allows for multiple replicates for each experimental condition. While we control for the time of day, replicate experiments are performed on different days, necessitating proper evaluation of day-by-day (plate-by-plate) variability. To this end, we used a formal assessment of statistical interaction through linear modeling – to see whether there was evidence that the effects of treatment differed among the replications represented by plate. Where there was evidence of significant statistical treatment-by-plate interaction [i.e., the *zfhx3* met analyses], a meta-analytic approach was taken utilizing a random effects model

(DerSimonian & Laird, 1986). In this approach, the forest plots show the mean differences (black squares) and 95% confidence intervals between treatment groups for each experimental assay (plate) (Figure 5 B-F; detailed numerical results in Supplemental Figure 1 A-C); they also graphically illustrate heterogeneity among these assays. Evaluation of the significance of treatment effects is based on all available data, and also provides a 95% confidence interval for the combined estimate (gray diamond) and a formal statistical assessment of heterogeneity (Borenstein, Hedges, Higgins, & Rothstein, 2009). We note that square root transformation of the data was required to meet model requirements of normality and homoscedasticity; all forest plots are on the transformed scale, but point estimates of the overall treatment effects and their 95% confidence limits were back-transformed to the original distance scale in cm.

A comparison of uninjected embryos to control MO injected embryos provides no evidence that distance traveled differed significantly between uninjected wild type vs. control MO larvae ($p=0.999$) (Figure 5B and Supplemental Figure 1A). The 95% confidence interval between the plates is centered on 0cm distance moved (Figure 5B, gray diamond). This indicates that, after adjusting for the intrinsic heterogeneity between experimental replicates and evaluating with all of our available data, there is no significant difference in distance moved between WT and control MO larvae after reagent addition.

Similar analyses for *pk1a* MO and *zhfx3* sb MO do not show any plate-by-plate variation. The forest plots (Figure 5 C and D) demonstrate the consistency of treatment differences across plates, corroborated by the lack of significant statistical (plate-by-treatment) interaction, which made it possible to use the conventional linear modeling approach.

Based on the linear modeling approach, on average, the distance traveled by *pk1a* morphants after PTZ exposure was 11.70 cm greater than that of uninjected larvae (95% CI: 0.07 – 43.340), a significant ($p=0.027$) difference. Similarly, on average, distance traveled by *zhfx3* sb morphants was 4.38 cm greater than that of uninjected larvae (95% CI: 0.58 – 11.72 cm), a significant ($p=0.0021$) difference. The mean distance traveled by *pk1a* morphants after PTZ exposure was 11.70 cm greater than that of uninjected larvae (95% CI: 0.07 – 43.34 cm) (Figure 5C) and that of *zhfx3* sb morphants was 4.38 cm greater than that of uninjected larvae (95% CI: 0.58 – 11.72 cm) (Figure 5D).

In contrast, the meta-analytic approach was applied to the series of experiments focusing on the *zhfx3* met MO because of the detected and highly significant ($p<0.0001$) statistical interaction and plate-to-plate heterogeneity. In the case of *zhfx3* met morphants, there was strong evidence that distance traveled was significantly greater in morphant larvae than in uninjected larvae ($p=0.002$, Figure 5E and Supplemental Figure 1B), on average 14.09 cm more. Similarly, distance traveled was greater in *zhfx3* met morphants than in control MO injected larvae ($p=0.026$, Figure 5F and Supplemental Figure 1C), on average 18.58 cm more. This approach ensured accurate experimental comparisons in a high-throughput mode (43– 262 larvae per experimental condition) and provides confidence for analysis of experiments that span over different days. The meta-analysis both provides insight into experimental heterogeneity and, while acknowledging it, provides consensus via an overall assessment of treatment effect that utilizes all available information.

Notable features in each of these series of experiments involving *pk1a* MO, *zfhx3 sb*, *zfhx3 met*, and controls, are the similar sizes of the boxes in the forest plots, indicating similar weighting of results from the different replicates, and the uniformity of the length of the confidence limits. Given the quite uniform sample sizes across treatment groups within each replicate, this implies very similar variability within plate.

***Zfhx3* Expression Pattern:** We characterized the expression domains of *zfhx3* in order to gain insight into how *zfhx3* manipulation may contribute to seizure sensitivity. Specifically, we characterized *zfhx3* expression using whole mount *in situ* hybridization techniques in 1–5 dpf zebrafish. The *zfhx3* transcript shows dynamic expression patterns in regions of the CNS (Figure 6). In 1 dpf embryos, there is low level *zfhx3* expression in the forebrain, midbrain and hindbrain (Figure 6A, F and K). By 2 dpf, there is increased *zfhx3* expression in the brain and spinal cord (Figure 6B and G). Spinal cord expression is diminished by 3 dpf while brain expression is maintained (Figure 6C and H). At 4 dpf, brain expression resolves into discrete areas (Figure 6D and I) while the signal is greatly reduced by 5 dpf (Figure 6E and J). Overall, the *zfhx3* expression pattern is coincident with tissues undergoing neurulation at critical developmental time points.

To characterize *zfhx3* expression in more detail, we evaluated transverse brain sections of the 2 and 3 dpf larvae after hybridization with *zfhx3* antisense probe (Figure 7) or with a sense control probe (Supplemental Figure 2). At 2 dpf, there is robust *zfhx3* expression in the pallium (Figure 7A), optic tectum and thalamus (Figure 7B), tegmentum and hypothalamus (Figure 7C), and medulla oblongata (Figure 7D). Strong expression is also observed in the lining of the telencephalic and rhombencephalic ventricles (Figure 7A and C). Expression in these regions at this developmental stage supports a role for *zfhx3* in neural differentiation (Kishimoto et al., 2011; Zupanc, Hinsch, & Gage, 2005). At 3 dpf, *zfhx3* expression persists in the pallium (Figure 7E), optic tectum (Figure 7F), thalamus (Figure 7F), tegmentum (Figure 7G), and medulla oblongata (Figure 7H). Expression is also observed in the eyes, specifically in the retinal ganglion cell layer (GCL), optic nerve, and optic chiasm (Fig 7F). There is also strong *zfhx3* expression along nerve fibers/axon tracts throughout the brain including the reticular formation (Figure 7D and H). The overlapping expression domains of *zfhx3* in the brain of 2 dpf and 3 dpf zebrafish indicate the regions that are most likely to be affected by *zfhx3* knockdown. Many of these areas have roles in motor coordination and are conserved functionally between zebrafish and mammals including the pallium, thalamus, hypothalamus, tegmentum, reticular formation, and medulla oblongata (Babin, Goizet, & Raldua, 2014; Ganz et al., 2014; Moens & Prince, 2002; T. Mueller, 2012; Sankrithi & O'Malley, 2010).

Discussion:

Using a well-characterized behavioral assay coupled with our newly developed analysis software, SEIZR, we efficiently evaluated candidate epilepsy genes from the UDP study for seizure sensitivity (ZFHX3, this paper, and 14 other loci, data not shown). SEIZR optimized the analysis of the generated behavioral data sets, greatly reducing the time required to evaluate the potential roles of the identified candidates in disease. We make SEIZR publicly available, offering it as a new tool for rapid behavior analysis of experimentally manipulated

zebrafish larvae (e.g. morphants or drug-treated larvae), to more efficiently translate biological data, especially exome sequencing data, into clinical benefits for patients suffering from diseases such as epilepsy.

One of the genes we identified as seizure-sensitizing is Zinc Finger Homeobox 3, *ZFHX3*. Human *ZFHX3* encodes a large protein (3703 amino acids) that contains 4 homeobox domains and 23 zinc finger domains (Fortini, Lai, & Rubin, 1991; Ido, Miura, & Tamaoki, 1994; Jung et al., 2005; Miura et al., 1995). The gene was first identified as a DNA binding factor (Morinaga et al., 1991) that interacts with an AT-rich element upstream of α -fetoprotein promoter (AFP) to suppress gene transcription. The highly orthologous *Drosophila Zfh2*, is expressed strongly in the CNS and binds to an AT-rich element upstream of the DOPA decarboxylase gene (*Ddc*) as well as to a regulatory element of the opsin gene (Fortini et al., 1991; Ido et al., 1994; Jung et al., 2005; Miura et al., 1995).

Expression of the mouse, rat, and human *ZFHX3* genes *in vitro* and *in vivo* increases during neuronal differentiation in parallel with other neural markers (Ido et al., 1994; Jung et al., 2005; Miura et al., 1995). In cell lines, mouse and human *ZFHX3* expression is significantly increased after treatment with retinoic acid to induce neural differentiation (Ido et al., 1994; Miura et al., 1995). Furthermore, northern blot analysis of prenatal, postnatal, and adult mice brains showed high expression during embryonic development, but expression is very low in adult brain (Ido et al., 1994; Miura et al., 1995). *ZFHX3* is found abundantly in the cytoplasm of cells in the ventricular and sub-ventricular zones of embryonic rats, but localization of *ZFHX3* in the differentiating field is almost exclusively in the nucleus (Jung et al., 2005). Localization of *ZFHX3* to the nucleus in cell lines is shown to induce cell cycle arrest and *ZFHX3* is known to suppress nestin and activate neurod1, further demonstrating its role in neural differentiation (Jung et al., 2005). The expression pattern of *zfhx3* in the developing zebrafish CNS supports a conserved role for *zfhx3* in neurogenesis. Specifically, in 2 dpf brain sections we demonstrate localized expression of *zfhx3* in the telencephalic ventricle and rhombencephalic ventricle, regions which contain neural precursor cells (Kishimoto et al., 2011; Zupanc et al., 2005). Sections through the brains of 2 and 3 dpf larvae further reveal expression in regions associated with motor control including the pallium, thalamus, hypothalamus, tegmentum, reticular formation, and medulla oblongata (Babin et al., 2014; Ganz et al., 2014; Moens & Prince, 2002; T. Mueller, 2012; Sankrithi & O'Malley, 2010). Altered activity of *Zfhx3* in any of these regions could disrupt neural development, and in this manner, may contribute to epilepsy.

Sections of whole mount in situ 3dpf larvae show strong expression in the GCL of the retina and in the optic tectum, as well as the nerve fibers/axon tracts that connect the various regions of the brain. Since *zfhx3* is a transcription factor, its transcript is unlikely to be observed in the axons of neurons, raising the possibility that *zfhx3* is expressed in oligodendrocytes precursor cells (OPCs) which show overlapping expression domains (Brosamle & Halpern, 2002; Preston & Macklin, 2015; Schebesta & Serluca, 2009; Sidik & Talbot, 2015). Once OPCs reach their target axons they may differentiate immediately or wait until much later stages to differentiate into mature oligodendrocytes (highly specialized glial cells) which help stabilize axon tracts and increase conduction speed by wrapping the tracts in myelin sheaths (Brosamle & Halpern, 2002; Preston & Macklin, 2015; Sidik &

Talbot, 2015; Young et al., 2013). While by 2 dpf, genetic markers for mature oligodendrocytes can be detected in the hindbrain and nascent myelin can be detected by 3–4 dpf in larval zebrafish (Brosamle & Halpern, 2002; Preston & Macklin, 2015; Sidik & Talbot, 2015), expression of *zfhx3* is decreased in regions that have likely undergone oligodendrocyte maturation. Therefore, *Zfhx3* may play a role in OPC migration or differentiation. Disruption of this process would result in improper neural signal transduction through loss of axonal tract stability or lowered signal conductance contributing to epilepsy.

ZFHX3 has also been shown to regulate genes involved in tissue repair and protection against oxidative stress (Zhang et al., 2012). Of interest, ZFHX3 is activated as a result of ATM (serine-threonine kinase gene) and cAMP-Responsive Element Binding protein (CREB) activation in the nucleus, and ZFHX3 is shown to upregulate several adhesion molecules as well as Platelet Derived Growth Factor Receptor β (PDGFRB). The PDGF pathway has been implicated in protecting neurons from oxidative stress (T. S. Kim et al., 2010). Disruption of this pathway through altered ZFHX3 activity could reduce neural integrity, suggesting another way in which ZFHX3 may be implicated in epilepsy. The role of ZFHX3 in inhibiting cell proliferation appears to not be limited to neural cells. ZFHX3 is a tumor suppressor, and somatic inactivating mutations, deletions, and downregulation of ZFHX3 have been associated with several cancers including prostate cancer, breast cancer, and hepatocellular carcinoma (Dong et al., 2010; Kataoka et al., 2001; C. J. Kim et al., 2008; Sun et al., 2005).

Given that ZFHX3 is an essential gene, a titrated knockdown model was needed to evaluate the requirement of ZFHX3 in neural function. While the large size of the *Zfhx3* protein (3838 amino acids) limits our ability to test for rescue, our finding that *zfhx3* morphants, from two independent MOs, are sensitized to PTZ exposure validate ZFHX3 as a likely candidate in epilepsy. While we demonstrate the role of *Zfhx3* in seizure sensitivity in the context of reduced function, the potential impact of *Zfhx3* through gain of function variants is also worth exploring.

To our knowledge, this is the first paper to demonstrate the role of ZFHX3 in seizures and epilepsy in an *in vivo* animal model. We find strong, distinct expression of *zfhx3* in the CNS during neural development and demonstrate that knockdown of *zfhx3* activity in zebrafish larvae sensitizes them to PTZ exposure. While the exact mechanisms that lead to seizure sensitivity after ZFHX3 manipulation remain unclear, our findings suggest a role in OPC differentiation and offer ZFHX3 as a strong candidate for further studies, especially to better understand its role in seizures and potentially identify new pathways that can be targeted for therapy.

Methods:

Animal Care:

Zebrafish were maintained in standard conditions under the approval of the University of Iowa IACUC. Natural spawning was used to collect embryos and embryos were raised

between 28.5°C and 30°C with no more than 50 embryos per 100mm plate. Embryo plates were cleared of debris and dead daily and water was added to the plates as needed.

Microinjection:

At the 1–2 cell stage of development, embryos were injected with 0.3–5ng of antisense morpholino oligonucleotide directed against each gene of interest or control MO.

Morpholinos were ordered from gene tools. Control:

CCTCTTACCTCAGTTACAATTATA, Zfhx3 met:

CACAGCTTTCCATGCTGATGCCTAC, Zfhx3 sb:

ATGCTGTAGTGATAGAAGGAAGGTT, Pk1a met:

CAGCTCCATCACTAACACCCCCTCA.

Behavioral Assay:

At 3 days post fertilization (dpf), morphologically normal larvae (morphant, control and uninjected WT) were evaluated for motility using a touch response. Larvae with normal swimming behavior were loaded individually in a 96 well plate with a fixed volume of 150µl of egg water. Experimental and control larvae were separated by rows in the plate. Plates were placed in the Zebrabox with the temperature set at 28°C and the lights off and larvae were allowed to acclimate for thirty minutes prior to analysis. After acclimation, larval activity was recorded using the motility tracking software for thirty minutes to determine baseline activity levels for each plate. During this run, powdered PTZ (Sigma-Aldrich (P6500), St Louis, MO) was dissolved into egg water to a final concentration of 20mM PTZ, in order to make a fresh working stock for each experiment. After the baseline activity run, 50µl of the 20mM PTZ stock was added to each well for a final volume of 200µl of 5mM PTZ in egg water in each well. Larval activity was then recorded using the motility tracking software for another thirty minutes to determine larval responses to PTZ. Tracking data for each animal was collected every second for the duration of the experiment (1 hour), which allowed us to quantify the distance moved (in cm) of individual larva before and after drug treatment. Each experimental assay was performed at the same relative time of day.

An infrared light constantly illuminates the Zebrabox, allowing the camera to record movement independent of light. The detection sensitivity level in the Zebralab capture software was set at 23 to focus on the head of each larva. Movement detection thresholds were set at 10.0mm/sec and 0.1mm/sec for the small/large threshold and the inactive/small threshold respectively. Activities below 0.1, from 0.1 to 10, and above 10 were designated by the software as ‘inactive’, ‘small movement’ (green lines in Fig 1B&C) and ‘large movement’ (red lines in Fig 1B&C), respectively. The Zebrabox camera is capable of recording at 60 frames per second. For quantification, total swimming distances for each 30 minute interval (before and after PTZ exposure), including small and large movements, were calculated.

In situ Hybridization and RTPCR:

Whole mount *in situ* hybridization was performed according to protocols already described (Thisse et al., 1993). Embryos were fixed at stages from 1–5 dpf in 4% paraformaldehyde in 1× PBS. Digoxigenin (DIG) -UTP labeled RNA riboprobes were made from linearized

constructs using the MAXIscrip in vitro transcription kit (Ambion, Life Technologies, Carlsbad, CA) and DIG RNA labeling Mix (Roche). Hybridized embryos were imaged at 50x and 100x magnification for all stages. After *in situ* staining, 2 and 3 dpf larvae were infiltrated in 15% sucrose and 30% sucrose and in OCT (optimal cutting temperature medium, Fisher Healthcare, Scigen Scientific, Gardena, CA) overnight at 4°C. Then, embryos were aligned and embedded in OCT, frozen and sectioned at -21°C. Sections (12 µm) were mounted on glass slides and left to dry overnight. Sections were rehydrated with 1× PBS and cover slipped with Aqua-Mount (Lerner Laboratories). Sections were imaged at 20× magnification. Labeled Tissues were identified using an embryonic zebrafish brain atlas (Thomas Mueller & Wullmann, 2016).

Total RNA from 2dpf *zfhx3* sb MO injected and age-matched uninjected zebrafish embryos was extracted and reverse transcribed into cDNA using SMART MMLV Reverse Transcriptase (Clontech). PCR using primers 5'-GACTCCCACCAATGCAAGTT-3' and 5'-TCTTTGGTTTGGTCCTCCTG-3' for *zfhx3* with Choice Taq DNA polymerase (Denville Scientific Inc.) was performed and products were ran on a 2% agarose gel.

Statistical Methods:

The Spearman rank correlation test was used to assess whether there was a dose-response relationship between activity (distance traveled in cm) and increasing PTZ dose.

Linear modeling was used to assess the effects of treatment and plate on the primary outcome, distance traveled in cm. Residual analyses and plots were used to assess the validity of model assumptions of normality (via the Shapiro-Wilk test) and variance homogeneity. Statistical interaction, i.e., whether treatment effects differed by plate, were specifically evaluated. The square root transformation of the primary outcome was found to be a normalizing transformation that also stabilized variance, resulting in satisfactory conformance with model requirements. Point estimate of treatment effects and their associated 95% confidence intervals were back-transformed to the original distance scale in cm. In the random effects model (DerSimonian & Laird, 1986) it is assumed that mean treatment differences may differ by plate, and this meta-analysis model provides a combined estimate of the mean difference in treatment effect; the variability associated with that estimate includes a random, within-plate component, as well as a component associated with plate-to-plate variability. This approach makes possible evaluation of the significance of treatment effects based on all available data, and also provides a 95% confidence interval for the combined estimate, assessments of heterogeneity, and graphical representation of results in the form of forest plots (Borenstein et al., 2009). In the case of the *pk1a* and *zfhx3 sb* analyses, there was no evidence of statistical interaction (i.e., variation in treatment effect by plate), and the linear modeling approach was the primary means of analysis, providing assessment of treatment group differences while adjusting for plate effects; in these two instances, the forest plots serve to illustrate the consistency of treatment differences among experimental replicates. In the case of the *zfhx3 met* experiments, significant statistical interaction was found, and the meta-analysis was the primary approach for evaluating and describing treatment differences and characterizing plate-to-plate variation.

Meta-analyses were conducted and graphics were created using the Comprehensive Meta-Analysis software, version 2.2.064 (Biostat, Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Representative image of 96 well plate containing one 3 dpf larvae in each well. A') Blown up image inside square from A to show larval size in comparison to wells. B & C) Representative image of the swimming track of a single larva, generated from the Viewpoint Zebralab Tracking software for thirty seconds before (B) and after (C) PTZ exposure. Green and red lines indicate movements slower and faster than 10mm/s respectively. D) Line graph comparing average distance moved (cm) per minute for two groups of larvae. Data from two experimental replicates were combined. Data are shown over thirty minutes under normal conditions followed by thirty minutes after stimulus. Green line indicates larvae exposed to 5 mM PTZ after baseline recording (n=48). Blue line indicates larvae exposed to 0 mM PTZ (equal volume egg water added to wells) after baseline activity (n=37).

Figure 2.

A) SEIZR interface allowing users to upload raw data, select the threshold larvae must move (cm) to be included in analysis, and select experimental groups by rows on plate. B) Representative tables SEIZR returns showing the total distance (cm) moved by larvae in wells 1–5 before and after PTZ exposure. C) Representative table showing the average distance (cm) moved as well as the standard error of the average for user-defined groups of larvae before and after PTZ exposure (Data shown for groups corresponding to selection in Fig 2A).

Figure 3.

A) Box and whisker plot showing distance moved (cm) by uninjected WT larvae groups before and after PTZ exposure. Groups of larvae were treated with 0 mM, 1 mM, 3 mM, and 5 mM PTZ demonstrating an increasingly hyperactive response with increasing PTZ dose (0 mM n=52, 1 mM n=60, 3 mM n=64, and 5 mM n=69), based upon the Spearman rank correlation ($r=0.551$, $p<0.0001$). B) Box and whisker plot showing distance moved (cm) by *pk1a* morphant (n=43) and age-matched uninjected larvae (n=47) before and after 5 mM PTZ exposure. Statistical interaction was assessed through linear modeling. *pk1a* morphants show a significantly hyperactive response to PTZ exposure relative to uninjected larvae ($p=0.027$), after adjustment was made for plate effects. The bottom and top of boxes

represent the 1st and 3rd quartile while the horizontal lines represent the medians for each data set. Whiskers represent the 5th and 95th percentile.

Figure 4.

A) Schematic of the zebrafish *zfhx3* genomic region showing exons 2, 3, and 4 and the splice junction targeted by the *zfhx3* sb MO. Blue arrows indicate primer used for RT-PCR. The red line indicates the splice defect observed in *zfhx3* sb morphants. RNA was isolated from 2 dpf uninjected and from 2dpf larvae injected with 0.3ng of MO. Gel photo of the RT-PCR product amplified uninjected, left, larva. and *zfhx3* sb morphants, right. Arrow indicates the altered *zfhx3* transcript. B) Representative image showing 3 dpf uninjected wild-type larva (top) and 3 dpf *zfhx3* morphant larva (bottom). *zfhx3* met morphant (up to

4.5ng) at 3 dpf are morphologically similar to age-matched uninjected wild-type larvae. C) Box and whisker plot showing distance moved (cm) by *zfhx3* sb MO injected (n=131) and age-matched uninjected larvae (n=130) before and after 5 mM PTZ exposure. Statistical interaction was assessed through linear modeling. *zfhx3* sb morphants show a significantly hyperactive response to PTZ exposure relative to uninjected larvae (p=0.0021), after adjustment was made for plate effects. The bottom and top of boxes represent the 1st and 3rd quartile while the horizontal lines represent the medians for each data set. Whiskers represent the 5th and 95th percentile.

Figure 5.

A) Box and whisker plot showing distance moved (cm) by *zfhx3* met MO injected (n=167), control MO injected (ctrl) (n=168), and uninjected (WT) larvae (n=166) before and after 5 mM PTZ exposure. Data shown are from seven experimental replicates with age-matched larvae for each experimental condition on each plate. The bottom and top of boxes represent the 1st and 3rd quartile while the horizontal lines represent the medians for each data set. Whiskers represent the 5th and 95th percentile. B-F) Forest plots showing mean differences (black squares) and 95% confidence intervals between groups for each experimental assay

performed based on square-root transformed distance traveled following PTZ treatment (cm). The size of the square indicates the relative weighting of the results from a particular plate in the meta-analysis based upon precision; in these studies, sample sizes were quite similar across all treatments and plates, and the similarity of weights and lengths of the confidence intervals reflect the similarity of within-plate variability across the replicate experiments. In the forest plots, the gray diamond indicates the combined estimate of the treatment effect (and its 95% confidence interval), based upon data from all plates utilizing a random effects meta-analysis model. Estimates and confidence limits for treatment effects have been back-transformed below. In C and D, the meta-analysis provided no evidence of plate-to-plate heterogeneity in terms of treatment effect, corroborating the finding of no statistical plate-by-treatment interaction. In B, E and F, which correspond to the analyses in the *zfhx3* met series of experiments, there was strong evidence of such plate-to-plate heterogeneity ($p \leq 0.0001$).

B – Comparison of uninjected wild type vs. control MO. The data provide no evidence that distance traveled differed significantly between these two groups ($p=0.999$). The difference in the average distance traveled by control MO morphants and that traveled by uninjected larvae was 0.00 cm; 95% CI: -14.89 – 14.93, an interval containing the null value of zero difference in distance travelled.

*C – Comparison of *pk1a* met MO vs. uninjected wild type.* The data provide strong evidence that distance traveled was significantly greater in *pk1a* morphants than in uninjected wild type larvae ($p=0.027$). Back-transforming to the original scale gives a mean estimate of 11.70 cm greater distance traveled in *pk1a* morphants than in uninjected larvae (95% CI: 0.07– 43.34 cm). Estimates derived from linear modeling; forest plots included to show homogeneity of results across plates.

*D – Comparison of *zfhx3* sb vs. uninjected wild type.* The data provide strong evidence that distance traveled was significantly greater in *zfhx3* sb morphants than in uninjected wild-type larvae ($p=0.0021$). Back-transforming to the original scale gives a mean estimate of 4.38 cm greater distance traveled in *zfhx3* sb morphants than in uninjected larvae (95% CI: 0.58– 11.72 cm). Estimates derived from linear modeling; forest plots included to show homogeneity of results across plates.

*E – Comparison of *zfhx3* met vs. uninjected wild type.* The data provide strong evidence that distance traveled was significantly greater in *zfhx3* met morphants than in uninjected wild type larvae ($p=0.002$). On average, the distance traveled in *zfhx3* met morphants was 14.09 cm greater than in uninjected larvae (95% CI: 1.96 – 37.32 cm). Estimates derived via a random effects meta-analysis model.

*F – Comparison of *zfhx3* met vs. control MO.* The data provide evidence that distance traveled was significantly greater in *zfhx3* met morphants than in control MO morphants ($p=0.026$). On average, the distance travelled by *zfhx3* met morphants was 18.58 cm greater than that by the control MO morphants (95% CI: 0.26 – 65.79 cm). Estimates derived via a random effects meta-analysis model.

Figure 6.

Wild-type larva in situ hybridization with *zfh3* antisense and sense probes. A-E) Lateral view of 1 dpf (A), 2 dpf (B), 3 dpf (C), 4 dpf (D), and 5 dpf (E) embryos hybridized with antisense probe imaged at 50x magnification. Insets, lateral view of each time point hybridized with sense control probe. 2 dpf inset shows two images taken at 100x magnification merged to show full body. F-J) Dorsal view at 100x magnification of 1 dpf (F), 2 dpf (G), 3 dpf (H), 4 dpf (I), and 5 dpf (J) larva hybridized with antisense probe

showing *zfhx3* expression patterns in the developing brain. K) Ventral view at 100x magnification of 1 dpf embryo showing *zfhx3* expression in the developing forebrain.

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Figure 7.

A-D) Sections (12 μ m) through the brain (rostral to caudal) of 2 dpf larva after hybridization with *zfhx3* antisense probe. A) *zfhx3* expression is present in the pallium and the telencephalic ventricle. B) *zfhx3* expression is present in the optic tectum and thalamus. C) *zfhx3* expression is present in the rhombencephalic ventricle, tegmentum, and hypothalamus. D) *zfhx3* expression is present in the medulla oblongata and reticular formation. E-F) Sections through the brain and retina (rostral to caudal) of 3 dpf larva after hybridization with *zfhx3* antisense probe. E) *zfhx3* expression is present in the pallium. F)

zfhx3 expression is present in the thalamus, optic nerve, optic tectum, ganglion cell layer, and optic chiasm. G) *zfhx3* expression is present in the tegmentum. H) *zfhx3* expression is present in the medulla oblongata and the reticular formation. GCL, ganglion cell layer; H, hypothalamus; MO, medulla oblongata; ON, optic nerve; OptCh, optic chiasm; P, pallium; RF, reticular formation; RhVe, rhombencephalic ventricle; T, tegmentum; TeO, optic tectum; TeVe, telencephalic ventricle; Th, thalamus. All sections were imaged at 20x magnification.

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