Title: Abnormal expression of GABA<sub>A</sub> receptor sub-units and hypomotility upon loss of *gabra1* in zebrafish.

Running title: *gabra1* function in development.

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We used whole exome sequencing (WES) to determine the genetic etiology of a patient with a multi-system disorder characterized by a seizure phenotype. WES identified a heterozygous de novo missense mutation in the \textit{GABRA1} gene (c.875C>T). \textit{GABRA1} encodes the alpha subunit of the Gamma-Aminobutyric Acid receptor A (GABA\textsubscript{A}R). The GABA\textsubscript{A}R is a ligand gated ion channel that mediates the fast inhibitory signals of the nervous system and mutations in the sub-units that compose the GABA\textsubscript{A}R have been previously associated with human disease. To understand the mechanisms by which \textit{GABRA1} regulates brain development, we developed a zebrafish model of \textit{gabra1} deficiency. \textit{gabra1} expression is restricted to the nervous system and behavioral analysis of morpholino injected larvae suggests that the knockdown of \textit{gabra1} results in hypoactivity and defects in the expression of other sub-units of the GABA\textsubscript{A}R. Expression the human \textit{GABRA1} protein in morphants partially restored the hypomotility phenotype. In contrast, the expression of the c.875C>T variant did not restore these behavioral deficits. Collectively, these results represent a functional approach to understand the mechanisms by which loss of function alleles cause disease.
INTRODUCTION

Rare disorders affect 4-8% of the global population (Boycott et al., 2013) and approximately 80% of these disorders are predicted to have a genetic etiology (Bick et al., 2019). In recent years, whole exome sequencing (WES) emerged as a diagnostic tool for patients with rare disorders of unknown origin (Sawyer et al., 2015; Tetreault et al., 2015). The success of WES has provided a unique window of opportunity to identify disease related genes in humans and it is predicted that gene identification of rare disorders has the potential to contribute to our knowledge of other, more complex genetic disorders (Danielsson et al., 2014; Koboldt et al., 2013). Most importantly, studies of rare disorders have demonstrated that WES can be successful with very few subjects and/or using a trio based approach (Gilissen et al., 2011; Gilissen et al., 2012).

In 2013, the Undiagnosed Disease Network (UDN) was founded and includes seven clinical sites across the nation, a coordinating center, two DNA sequencing centers, a model organism screening center, a metabolomics core, and a central biorepository (Macnamara et al., 2019). Within the first 4 years of the UDN, the sequencing centers identified 956 genes associated with human disease, 375 of them have not previously been associated with disease (Wangler et al., 2017). This is a staggering number, as it suggests that nearly 1/3 of the genes identified are of unknown function. These data strongly support the need for in vivo functional analysis of gene function.

Here we describe the identification of a putative disease variant and perform in vivo functional analysis of gene function using genetic loss of function. We describe a patient who presented with a severe seizure disorder, intellectual disability, cardiac arrhythmia, and nonverbal speech. We identified a heterozygous de novo missense mutation in the GABRA1 gene (c.875C>T), which resulted in a single amino acid substitution in one of the three known transmembrane domains (p.Thr292Ile). GABRA1 is located on chromosome 5 and encodes the alpha (α) sub-unit of the multi-sub-unit gamma-aminobutyric acid receptor (GABA_A,R). The GABA_A,R is the primary inhibitory receptor of the central nervous system and the c.875C>T variant was previously associated with epileptic phenotypes in the Epi4K consortium (Epi4K Consortium et al., 2013). Although mutations in GABRA1 have been associated with disease, the molecular and cellular mechanisms by which GABRA1 regulates neural development are not completely understood. Consequently, we performed functional analysis in the developing zebrafish embryo.
Zebrafish are a cost-effective model organism and nearly 75% of their genome is conserved with humans (Ackermann and Paw, 2003; Reyes-Nava, Nayeli G. et al., 2018). Additionally, they are highly amenable to genetic manipulation. To ascertain the function of $GABRA1$ during development and behavior, we performed morpholino mediated knockdown of the zebrafish ortholog of $GABRA1$. We analyzed the behavioral and molecular consequences associated with knockdown of $gabra1$. Morphants exhibited hypomotility as indicated by swim speed and total distance swam. This hypomotility was accompanied by distinct changes in the expression of the major sub-units of the $GABA_{A}R$, including decreased expression of $\beta2$ and $\gamma2$ transcripts. Despite this decrease in the expression of unique $GABA_{A}R$ sub-units, morphants continued to respond to treatment with pentylenetetrazol (PTZ), a potent antagonist of the $GABA_{A}R$, indicating that morphants continue to produce an active $GABA_{A}R$ even in the absence of adequate $gabra1$ expression.

**RESULTS**

**Subject**

The subject initially presented to care at three months of age with infantile spasms that evolved into Lennox-Gastaut syndrome. Seizure activity included a light-sensitive myoclonic epilepsy and generalized tonic clonic seizures. The seizures were treated with adrenocorticotropic hormone (ACTH), multiple antiepileptic medications, and ketogenic diet, although seizure activity continued to occur daily.

His clinical course was also marked for hypotonia, visual impairment, developmental delay and bilateral neuromuscular hip dysplasia. He had a Torsades de pointes cardiac arrest during an acute illness, with normal cardiac function outside of the acute event. Laboratory findings included lactic acidosis (peak serum lactate 4.18, ref range 0.5-2.0 mM) and metabolic acidosis. Multiple diagnoses were suggested based on his clinical history including a channelopathy and primary mitochondrial dysfunction. In previous clinical testing, the patient was negative for mutations in a panel of genes including $ADSL$, $ALDH7A1$, $ARX$, $ATP6AP2$, $CDKL5$, $CLN3$, $CLN5$, $CLN6$, $CLN8$, $CNTNAP2$, $CTSD$, $FOXG1$, $GABRG2$, $GAMT$, $KCNQ2$, $KCNQ3$, $MECP2$, $MFSD8$, $NRXN1$, $PCDH19$, $PNKP$, $PNPO$, $POLG$, $PPT1$, $SCN1A$, $SCN1B$, $SCN2A$, $SLC25A22$, $SLC2A1$, $SLC9A6$, $SPTAN1$, $STXBP1$, $TCF4$, $TPP1$, $TSC1$, $TSC2$, $UBE3A$, and $ZEB2$. In order to investigate the underlying genetic etiology for his complex medical history, the subject and his parents were enrolled into a research protocol approved by the Colorado Multiple Institutional Review Board (COMIRB #07-0386).
Whole-Exome Sequencing

WES was performed on a male subject and his unaffected parents to obtain over 70X coverage of targeted exons in each sample (Table S1). A large number of variants (106,737 variants) were detected in the patient after applying appropriate quality measures as described in Table S2. Our downstream analyses focused on nonsynonymous coding variants, coding InDels (insertions/deletions <50 bp), and variants affecting splice-sites as they are more likely to have a functional impact on the gene product and hence more likely to be pathogenic (9,631 variants). Common variants with minor allele frequency (MAF) greater than 1% in dbSNP137 were filtered out. Our analysis identified 1,846 rare variants in the patient and these were considered for further analysis. Parental WES data was used to detect the pathogenic variant under various inheritance models including dominant (de novo mutations) and recessive (compound heterozygous, homozygous, and X-linked hemizygous mutations) models. This resulted in identification of seven candidate genes (Table S2). These included de novo variants in CACNA1C, GABRA1, and compound heterozygous variants in SCNN1B, FNIP1, TTN, OTOG, and FAT4.

Additional evaluation of each candidate gene according to the criteria described in MATERIAL AND METHODS section identified 2 top priority candidate genes, including the de novo variant in GABRA1 under a dominant model and compound heterozygous variants in TTN under a recessive model (Table S2). TTN encodes Titin, a sarcomeric protein involved in the assembly of cardiac and skeletal muscle. The second candidate gene, GABRA1 has been associated with early infantile epileptic encephalopathy (EIEE19; MIM: 615744) and juvenile myoclonic epilepsy (EJM4, EJM5; MIM: 611136) and therefore, became the primary putative candidate gene based on clinical phenotype. Both parents had normal alleles but the subject had a heterozygous missense variant in GABRA1 (NM_000806.5:c.875C>T, NP_000797.2:p.Thr292Ile) that results in a change in protein sequence (Fig. 1A). Sanger sequencing also confirmed that the variant is de novo (Fig. 1B) and mostly likely the result of a germline mutation. Amino acid Thr292 is highly conserved evolutionarily between multiple vertebral species (Fig. 1C) according to several conservation algorithms (PhyloP: 7.66; PhastCons: 1; GERP: 5.8). Notably, multiple mutation prediction algorithms predict this variant to be deleterious (CADD: 33; PolyPhen2 = probably damaging (1); PROVEAN = deleterious (-5.34); SIFT = damaging (0); MutationTaster: disease causing (0.99999)). Amino acids from position 279 to 300 of GABRA1 form a functionally important transmembrane helix domain (TM2) that is critical for overall functionality (Fig. 1D). The significance of p.Thr292Ile variant in the
subject is further supported by previous studies, which have established that de novo mutations in the first three transmembrane domains (TM1, TM2, and TM3) are associated with neurological and epileptic conditions (Kodera et al., 2016). Most importantly, the c.875C>T heterozygous variant has been reported by the Epi4K consortium (Epi4K Consortium et al., 2013). Collectively, these data provide strong evidence that the heterozygous c.875C>T missense variant in \textit{GABRA1} is likely pathogenic.

\textbf{Expression Patterns of the zebrafish ortholog of \textit{GABRA1}.}

In order to understand the mechanisms by which \textit{GABRA1} regulates development, we used the zebrafish (\textit{Danio rerio}) as a model organism. We first confirmed the spatial and temporal expression patterns of zebrafish \textit{gabra1} using whole-mount in situ hybridization (WISH). We performed WISH at 1, 2, and 3 days post fertilization (DPF). \textit{gabra1} expression was localized to the developing nervous system at each time points with the broadest expression at 1 DPF (Fig. 2). Over the course of development the expression of \textit{gabra1} became more restricted to the midbrain-hindbrain regions (Fig. 2) consistent with previously published work (Monesson-Olson et al., 2018; Samarut et al., 2018).

\textbf{Gabra1 regulates zebrafish larval motility.}

Mutations in \textit{GABRA1} have been associated with epileptic phenotypes (Cossette et al., 2002; Maljevic Snezana et al., 2006; Lachance-Touchette et al., 2011; Kodera et al., 2016; Farnaes et al., 2017; Nolan and Fink, 2018) and behavioral assays to monitor seizure like behaviors in zebrafish have emerged (Baraban et al., 2005; Reyes-Nava, Nayeli G. et al., 2018). Consequently, we developed a protocol using the Zebrabox behavioral unit to monitor swim speed and total distance swam in larvae injected with anti-sense morpholinos that inhibit either the translation of \textit{gabra1} or mRNA splicing. Embryos were injected at the single cell stage with randomized control morpholinos (RC), translational targeting morpholinos (tbMO), or mRNA disrupting morpholinos (sMO) and raised to 5 DPF. Larvae were monitored according to the protocol described in the MATERIALS AND METHODS for swim speed and total distance swam. As shown in Figure 3, the tbMO was associated with a statistically significant (p<0.001) reduction in total swim speed (Fig. 3A) and decreased total distance swam (Fig. 3B) consistent with a hypomotility phenotype. The decrease in speed and distance was observed in both light and dark conditions at 5 DPF (Fig. 3C). Importantly, injection of an equivalent concentration of
sMO induced a hypomotility phenotype (Fig. S1B,C; p=0.0263). These results are consistent with the phenotype present in morphants injected with the translational blocking morpholino. Importantly, we validated the effects of injection of sMO on mRNA splicing, which demonstrated a near 50% reduction in wildtype gabra1 (Fig. S1A).

Next, we sought to restore the hypomotility phenotype in morphants (tbMO) by co-injection of GABRA1 encoding mRNA. Embryos were injected at the single cell stage with RC morpholinos, tbMO morpholinos, GABRA1 mRNA, or a combination of GABRA1 mRNA with RC or tbMO morpholinos. Injection of the tbMO caused a statistically significant decrease in the total distance swam (p= 0.000161) and the overall swim speed (p=0.036384) (Fig.4A,B; RC relative to tbMO). Injection of GABRA1 encoding mRNA had no significant effect on speed or distance at a concentration of 1000pg/embryo (Fig. 4A,B; mRNA and RC+). The co-injection of the tbMO and GABRA1 encoding mRNA at 1000pg/embryo restored the total distance swam to normal levels (p= 0.003010689), but was not sufficient to restore the deficits in overall speed to control levels (Fig. 4A,B). Thus, co-injection of 1000pg of GABRA1 encoding mRNA with the tbMO produced a partial rescue of the observed phenotype. Injection of GABRA1 mRNA at higher concentrations was accompanied by some degree of toxicity (cardiac edema and death) and therefore, additional rescue experiments with higher concentrations could not be attempted.

The c.875C>T GABRA1 variant does not restore the hypomotility phenotype in morphants.

The functional consequences of the c.875C>T variant are currently unknown. Therefore, we asked whether expression of the c.875C>T variant was sufficient to restore the hypomotility induced by knockdown of gabra1. Embryos were injected at the single cell stage with RC morpholinos, tbMO morpholinos, GABRA1 c.875C>T mRNA (SDM), or a combination of GABRA1 mRNA with tbMO morpholinos. Consistent with previous experiments, injection of the tbMO morpholino caused a significant reduction (p=0.0153) in the total distance swam relative to embryos injected with the RC (Fig. 4C). Interestingly, the co-injection of the mRNA encoding the c.875C>T (SDM) and the tbMO was unable to restore the total distance traveled to control levels (Fig. 4C). Importantly, the injection of the GABRA1 c.875C>T variant (SDM) at 1000pg/embryo had no significant effects on the total distance swam (Fig. 4C).

The expression of gabrb2 and gabrg2 are decreased in gabra1 morphants.
Previous studies suggest that approximately 60% of all GABA_A Rs consist of two α1, two β2, and one γ2 subunits (Sigel and Steinmann, 2012). We hypothesized that the knockdown of gabra1, which encodes the α1 sub-unit would alter the sub-unit composition of the GABA_A R. To begin to test this, we analyzed the expression of the genes that encode the β2 and γ2 sub-units. As shown in Figure 5A, knockdown of gabra1 caused a decrease in the expression of gabrb2 (β2) and gabrg2 (γ2). We next measured the expression of other alpha sub-units in gabra1 morphants. As shown in Figure 5A, injection of the tbMO was associated with increased expression of gabra6a and gabra6b, but only gabra6b was statistically significant across biological triplicates. A similar expression pattern of gabra6a and gabra6b was observed upon injection of the sMO (Fig. S1D), with both genes demonstrating a statistically significant increase in expression. We did not detect a statistical change in the expression of any other α sub-unit across either the tbMO or the sMO (Figs 5A, S1D).

We sought to build upon these data by determining whether morphant larvae had an intact receptor capable of responding to pentylenetetrazol (PTZ), an antagonist of the GABA_A R. Non-injected wildtype embryos treated with 10mM PTZ exhibit short convulsions and a whirlpool swimming behavior with a 2-fold increase in swim speed (p = 6.74E-06) and an approximate 6-fold increase in total distance swam (p = 2.14E-05) (Fig. 5B,C). These phenotypes were consistently observed in larvae injected with RC morpholinos as the RC morpholino had no affect on larval behavior or their response to PTZ. Interestingly, gabra1 morphants (tbMO) responded normally to PTZ according to both distance and speed measurements (Fig. 5B,C). Collectively, these data demonstrate that knockdown of gabra1 alters the expression of unique GABA_A R subunits, although, morphants continue to respond to PTZ treatment.

**DISCUSSION**

We have identified an individual presenting with multi-system disorder carrying a de novo missense variant in the GABRA1 gene (NM_000806.5:c.875C>T, NP_000797.2:p.Thr292Ile). The GABRA1 gene encodes the α1 sub-unit of the GABA_A R, which mediates the fast inhibitory synapses of the nervous system. GABA_A Rs are pentameric and can be composed of different combinations of the following components: six α subunits, three β subunits, three γ subunits, three ρ subunits, one ε, δ, θ, or π subunits. Of these sub-units, mutations in GABRA1 (Cossette et al., 2002, 1; Kodera et al., 2016, 1; Macdonald and Gallagher, 2015; von Deimling et al., 2017), GABRA6 (Hernandez et al., 2011), GABRB2
(Macdonald and Gallagher, 2015), GABRB3 (DeLorey et al., 1998; von Deimling et al., 2017), GABRG2 (von Deimling et al., 2017), and GABRD (Macdonald and Gallagher, 2015) have been associated with epileptic phenotypes (reviewed in (Hirose, 2014)). Most importantly, in a recent international collaboration (Epi4K Consortium), the heterozygous de novo p.Thr292Ile variant we describe here was identified in a male patient diagnosed with infantile spasms (Epi4K Consortium et al., 2013). The individual studied in the Epi4K study had febrile seizures at the age of 1-month and at 15-months of age, his electroencephalogram (EEG) showed bursts of generalized spike and wave (GSW) at 2.5 Hz with multiple foci of epileptiform activity. He presented with features of generalized tonic clonic (GTC) and myoclonic seizures. He was developmentally delayed, hypotonic, and did not speak at 18-months of age with additional features that include esotropia, poor vision, abnormal electroretinogram, and a head circumference at 5th percentile. The subject reported here was diagnosed with seizure disorder, intellectual disability, vision loss, and was non-verbal; phenotypes consistent with the previously identified case. Additionally, the p.Thr292Ile variant is present in one of the 3 transmembrane domains of the GABRA1 protein and these domains have been associated with epileptic phenotypes (Kodera et al., 2016). Collectively, these data strongly suggest that the heterozygous mutation p.Thr292Ile causes a complex disorder characterized by a severe seizures. This is supported by the fact that there are at least two subjects with overlapping phenotypes harboring this variant.

It is not yet known how mutations in the GABRA1 transmembrane domain result in seizure like phenotypes. Genetic knockout mice have been developed to understand how mutations in Gabra1 (mouse) affect GABA\textsubscript{A}R function, but the results have been difficult to interpret, as the deletion of Gabra1 (mouse) causes strain and sex specific phenotypes (Arain et al., 2012). Due to these strain differences, additional systems have been developed including a zebrafish harboring a mutation in the gabra1 gene. Interestingly, mouse models of Gabra1 deletion are viable, but the homozygous deletion of gabra1 in fish is lethal (Samarut et al., 2018). Despite this lethality, mutant zebrafish survive to several weeks post fertilization, which has allowed for the characterization of gabra1 function in fish at 7-10 weeks post fertilization (Samarut et al., 2018).

In this report, we demonstrate that morpholino mediated knockdown of gabra1 in zebrafish leads to hypomotility in the presence and absence of light. We perform our studies at 5 DPF, during the larval stage, prior to the onset of feeding or sexual dimorphism, but after swim bladder formation. gabra1 morphants consistently demonstrated with reduced swim speed and reduced overall distance travelled relative to control. These data are consistent with Samarut et.
al., who demonstrated that mutation of *gabra1* results in hypomotility, albeit at a later stage in development that would be equivalent to a juvenile onset (Samarut et al., 2018). In contrast to Samarut et. al., we did not observe overt indications of myoclonic seizures at any time point in our protocol. For example, within the first minute of light exposure, Samarut and colleagues observed intense seizures characterized by convulsions, uncontrolled movements, and whirlpool swim behavior. This phenotype was not observed in morphant animals (data not shown). This can likely be attributed to the fact that our study is performed using a knockdown of *gabra1*, which maybe more consistent with the heterozygous phenotypes reported by Samarut and colleagues at 4 DPF. To address the function of the c.875C>T *GABRA1* variant, we performed restoration experiments in which this variant was co-injected with *gabra1* targeting morpholinos. Co-injection of mRNA encoding the c.875C>T variant did not restore the hypomotility phenotype present in morphants, whereas co-injection of wildtype *GABRA1* restored the total distance travels to control levels. These data suggest that the c.875C>T variant is a loss of function allele, however, future studies characterizing the function of this variant are warranted. Should this allele be a loss of function allele, morpholino mediated knockdown is an alternative approach towards understanding the mechanisms by which the c.875C>T allele causes disease.

We further demonstrate that knockdown of *gabra1* causes abnormal expression of other sub-units of the GABA\(_A\)R. Despite changes in the expression of various GABA\(_A\)R sub-units, morphant animals continue to respond to PTZ stimulus. PTZ is a potent antagonist of the GABA\(_A\)R and treatment of wildtype larvae with PTZ induces a myoclonic seizure (Afrikanova et al., 2013; Baraban et al., 2005) because PTZ binds directly to the GABA\(_A\)R resulting in disinhibition (Huang et al., 2001). The continued response of morphants to PTZ, suggests that these embryos maintain the ability to produce some form of the GABA\(_A\)R. Consistent with this hypothesis, we observed increased expression of *gabra6a* and *gabra6b* mRNA, which encode the two zebrafish \(\alpha6\) sub-units of the GABA\(_A\)R. Other sub-units did not demonstrate consistent changes in expression across multiple morpholinos or biological replicates. Interestingly, mutations in *GABRA6*, which encodes the \(\alpha6\) sub-unit are associated with disease (Hernandez et al., 2011). Thus, it is unclear whether the hypomotility phenotype observed is the direct result of a lack of *gabra1* or the up-regulation of *gabra6*. Future studies analyzing the function of \(\alpha6\) and other alpha sub-units in *gabra1* mutant animals are needed.

The gene expression changes we observe are strongly supported by previous conclusions in mice with mutations in the *Gabra1* gene (Arain et al., 2015; Zhou et al., 2015). Recent work in zebrafish has demonstrated that the homozygous nonsense mutation of *gabra1*,
does not disrupt overall brain structure or the total number of GABAergic cells, but does
influence the brain transcriptome (Samarut et al., 2018). Collectively, these data raise the
possibility that other alpha sub-units may compensate for the loss of gabra1, ultimately
producing unique compositions of the GABA<sub>a</sub>R. The function of these receptors is unknown. But
it is conceivable that the production of GABA<sub>a</sub>R with unique sub-unit composition in incorrect
regions of the brain might underlie the impaired synapse formation observed in zebrafish
harboring germline mutations in the gabra1 gene (Samarut et al., 2018).

We provide strong evidence that heterozygous de novo mutation of GABRA1 is
associated with a multi-system disorder characterized by severe seizures. We further
characterized the developmental and behavioral defects associated with knockdown of gabra1
in zebrafish. Behaviorally, morphant animals present with hypomotility at 5 DPF measured by
reduced swim speed and total distance traveled. These deficits coincide with significant
changes in the expression of GABA<sub>a</sub>R sub-units and cannot be restored by the de novo
c.875C>T allele. Although a zebrafish harboring a mutation in the gabra1 gene has recently
been created, detailed behavioral analysis was performed at the juvenile stage (weeks post
fertilization). Here we complement previous studies using a morpholino mediated knockdown
approach, as the homozygous deletion of gabra1 was lethal (Samarut et al., 2018). Our
behavioral study is the first to our knowledge that comprehensively characterizes the phenotype
of gabra1 deletion during early development (DPF as opposed to weeks post fertilization). We
observed hypomotility consistent with previous studies in zebrafish and our study likely informs
about specific types of mutations, those of which result in loss of function alleles. Importantly,
our restoration experiments with the c.875C>T allele suggest that this allele is in fact a loss of
function allele. Thus, morpholino mediated studies might provide insight into the mechanisms by
which loss of function alleles cause disease.

**MATERIALS AND METHODS**

**Animal Husbandry**

For all experiments, embryos were obtained by crossing AB wildtype or Tupfel Long Fin
wildtype. Fish were maintained at The University of Texas at El Paso according to the
Institutional Animal Care and Use committee (IACUC) guidelines (Protocol Number 811689-5).
They were maintained and bred in groups of two females and two to four males. The collected
zebrafish embryos were kept in egg water consisting of 0.03% Instant Ocean (Aquaneering,
San Diego, CA) in D.I. water at 28°C.
Whole-Exome Sequencing and Data Analysis

High quality, unamplified, and unfragmented genomic DNA (A260/A280 ≥ 1.8 and A260/A230 ≥ 1.9) was extracted from whole blood obtained from the subject and his parents using the Puregene Blood kit from Qiagen (Valencia, CA). Whole exome sequencing was performed using the service provided by Beijing Genomics Institute (Cambridge, MA). Details of data analysis were similar to the procedure as previously described (Epi4K Consortium et al., 2013). Approximately 78 to 168 million, 100 bp, paired-end reads (>70X) were obtained and mapped to the reference human genome (GRCh37/hg19) using Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009, 200) (summarized in Table S1). Variants were determined by the utilities in the SAMtools (Li et al., 2009) and further annotated with SeattleSeq. Filtering and the test of inheritance model was performed using tools available in Galaxy (Goecks et al., 2010). Variants were filtered against dbSNP build 137, 1000 Genomes (November 23, 2010 release version), Exome Variant Server (EVS, ESP6500SI-V2) and Exome Aggregation Consortium ExAC browser (version 0.3). Rare variants were identified as a variant with a minor allele frequency (MAF) less than 1% using dbSNP137. The sequence data from the family was then used to test for causal variants under different inheritance models, including dominant (de novo mutations) and recessive (compound heterozygous, homozygous, and X-linked hemizygous mutations) models. In the dominant model, variants found in any database (dbSNP, 1000 Genomes, EVS, ExAC) were removed from the top candidacy list. In the recessive model, autosomal variants which had homozygotes found in the databases, such as EVS and ExAC, (or variants on chrX or chrY with hemizygotes in databases) were deleted from the top candidacy list.

Sanger Sequencing Verification

Sanger sequencing was used to validate the variant described. Briefly, primers were used to amplify the PCR product (fwd 5’-GCTGTFATAGGGTGGAGGTG-3’, rev 5’GCTATCAACGCCATTGTGAA-3’) using 1X GoTaq Green (Promega, Madison, WI) with a final primer concentration of 0.2uM. Reaction parameters for PCR include an initial cycle at 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 second, and 72°C for 1 minute, finishing with extension at 72°C for 5 minutes. Amplified PCR products were sequenced using the PCR primers as sequencing primers. Variations detected in GABRA1 were assigned using cDNA accession number NM_000806.5.

Whole mount in situ hybridization and Injections
WISH was performed as previously described (Thisse and Thisse, 2008). Embryos were harvested at 1, 2, and 3 DPF and fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, PA) for 1 hour at room temperature (RT). Embryos were dehydrated using a methanol: PBS gradient and stored in 100% methanol overnight in -20°C. Embryos were rehydrated using PBS: Methanol gradient, washed in PBS with 0.1% Tween 20 and permeabilized with proteinase K (10μg/ml) for the time indicated by Thisse and Thisse (Thisse and Thisse, 2008). Permeabilized embryos were prehybridized in hybridization buffer (HB) (50% deionized formamide (Fisher, Waltham, MA), 5X SSC (Fisher, Waltham, MA), 0.1% Tween 20 (Fisher, Waltham, MA), 50μg ml⁻¹ heparin (Sigma, St. Louis, MO), 500μg ml⁻¹ of RNase-free tRNA (Sigma, St. Louis, MO), 1M citric acid (Fisher, Waltham, MA) (460μl for 50ml of HB) for 2-4 hours and then incubated overnight in fresh HB with probe (gabra1 100ng) at 70°C. Samples were washed according to protocol, blocked in 2% sheep serum (Sigma, St. Louis, MO), 2 mg ml⁻¹ bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 2-4 hours at RT, and incubated with anti-DIG Fab fragments (1:10,000) (Sigma, St. Louis, MO) overnight at 4°C. Samples were developed with BM purple AP substrate (Sigma, St. Louis, MO) and images were collected with a Zeiss Discovery Stereo Microscope fitted with Zen Software. The gabra1 probe was created using primers specific to the endogenous cDNA sequence (gabra1 ISH fwd 5' - TAAGCTGCGCTCTTCTCCTC-3', gabra1 ISH rev 5'-GCAGAGTCCCTTCCTCTGTG-3').

For morpholino injections, a translational blocking morpholino (tbMO) (TCTTCCACCACACATCTCTCCCGA) and a splice site inhibiting morpholino (sMO) (ACACGCTCTGTGAGCAGAAATATT) targeting gabra1 were designed. The efficiency of knockdown for the sMO was performed with primers flanking the target site (Fwd: GACAGCCTCTCGATGGT and Rev: GCAGAGTCCCTTCCTCTGT). Each morpholino was injected independently at the single cell stage at a concentration of 1.6 ng/embryo. An equivalent concentration of randomized control morpholinos (25-N) was injected as a control. Final concentration of morpholino was determined empirically after an injection gradient was performed to determine optimal survival. For rescue experiments, the human GABRA1 complete open reading frame was purchased from TransOMIC Technologies (Huntsville, AL). The c.875C>T GABRA1 variant was created from the original vector obtained from TransOMIC Technologies using the QuikChange II Site-Directed Mutagenesis Kit (Fisher, Waltham, MA) with forward (TAACAACGTGTGCTCATGACAACATTGG) and reverse primers (GAGTTACAACAGTACGACTCGTGCTACCAAT). In vitro RNA was synthesized using the mMessage Machine kit (Fisher, Waltham, MA). The synthesized mRNA was injected at the
single cell stage alone or in conjunction with tbMO at the indicated concentrations in the figure
legends.

Quantitative Real Time PCR (QPCR)

Total RNA was isolated from brain homogenates obtained from embryos injected with
random control morpholinos or tbMO at 5 DPF using Trizol (Fisher, Waltham, MA). Reverse
transcription was performed using the Verso cDNA Synthesis Kit (Fisher, Waltham, MA) and
total RNA was normalized by concentration (ng) across all samples. PCR was performed in
technical triplicates for each sample using an Applied Biosystems StepOne Plus machine with
Applied Biosystems associated software. Sybr green (Fisher, Waltham, MA) based primer pairs
for each gene analyzed are as follows: gabra2a fwd GATGGCTACGACAACAGGCT, gabra2a
rev TGTCACATCGCTCGGAAAA, gabra3 fwd GCTGAAGTTCGGGAGCTATG, gabra3 rev
GGAGCGGTATGGCCTCTTTGC, gabra4 fwd GACTGCGATGTACCCCACTT, gabra4 rev
ATCCAGGTCCGAGTCTTGTTG, gabra5 fwd CATGACAACACCCGACGC, gabra5 rev
GGGAAGGATGCGTGGATA, gabra6a fwd TCGGTACCACATCTTTCTC, gabra6a rev
CCCTGAGCTTTCCAGAGTG, gabra6b fwd CGGAGGAGTGCTGAAGAAAC, gabra6b rev
GGGAAAGGATGCGTGAGTA, gabrb2 fwd CCCGACACCTATTTCTCTG, gabrb2 rev
TCTGATCTCAGTGTCAG, gabrg2 fwd ACACCAATAGGATGCTTCG, gabrg2 rev
AGCTGCGCTCCACTTGTAT. Analysis performed using 2ΔΔct. Statistical analysis of mRNA
expression was performed using a T-test. All QPCR was performed in biological duplicate or
triplicate using a pool of embryos (30-40) per time point.

Behavioral Analysis and Pentylenetetrazol treatment

Embryos injected with random control morpholinos, tbMO, sMO, GABRA1 mRNA,
GABRA1 (c.875C>T), or a combination as indicated in the figure legends were raised to 5 DPF.
Behavioral analysis was performed using the Zebrafish (ViewPoint Behavior Technology,
Montréal, Canada). Larvae were individually tracked for swim speed and total distance swam in
a 96 well plate. The behavioral protocol (adapted from (Afrikanova et al., 2013)) was a total of
15 minutes divided into 5 minute intervals of dark/light/dark conditions. All larvae were
acclimated to the dish and housing conditions for 1 hour prior to analysis. Settings for the
program include a threshold of 16 and integration period of 300 seconds. Data was measured
as total distance traveled (mm) and total swim speed (mm/sec) (Swim Speed= {Total distance
traveled in large and small movements) (SmlDist+Lardist)}/{Total duration spent by the animal in
small and large movements (smldur+lardur)). Statistical significance was determined according to a T-test. All experiments were performed in biological triplicate. For PTZ treatment, PTZ (10mM) was added directly to the 96 well plate following acclimation period. Final concentration of PTZ was determined from previously published results (Baraban et al., 2005; Afrikanova et al., 2013; Peng et al., 2016; Jin et al., 2018).
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Authors report no conflict of interest.
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DATA AVAILABILITY

All reagents are available upon request from the corresponding author.
NR synthesized hypothesis, performing behavioral and molecular experiments, *in situ* hybridization, and all morpholino injections. HY performed bioinformatics analysis and wrote portions of the manuscript. THS and CC supervised and managed patient IRB, genetic counseling, contributed to writing the manuscript, and provided patient assessment expertise. AMQ synthesized the hypothesis, performed data management and analysis, and wrote the manuscript.
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**FIGURE LEGENDS**

**Figure 1: Identification of Pathogenic Variants in the GABRA1 gene.**

(A) Depiction of a de novo missense variant c.875C>T (p.Thr292Ile) in the patient and his unaffected parents. (B) Partial chromatograms demonstrating Sanger Sequencing validation in the Proband. (C) Comparative analysis of the GABRA1 protein from multiple species. Thr292 (highlighted in red) and its neighboring amino acids are evolutionarily conserved. Protein sequences were obtained from NCBI Protein database or Ensembl. (D) Top: Annotation of the nine coding exons in the GABRA1 gene. Bottom: The GABRA1 protein includes an extracellular domain, a cytoplasmic domain and four transmembrane domains (TM1-4) (annotated by Universal Protein Resource, UniProt). Location of variant identified in the patient is indicated by arrows within TM2.

**Figure 2: gabra1 expression in the developing zebrafish.**

Whole mount in situ hybridization (WISH) was performed at 1, 2, 3 days post fertilization (DPF) with an anti-sense gabra1 probe. Arrows indicated the expression of gabra1 at each developmental stage.

**Figure 3: Knockdown of gabra1 causes hypomotility.**

(A) Total swim speed of larvae injected with random control morpholinos (RC) or translational targeting gabra1 morpholinos (tbMO) was determined using Zebrabox technology at 5 days post fertilization (DPF). Total number of embryos analyzed per group is depicted in the graph. *p<0.001. (B) The total distance swam was assessed at 5 DPF using Zebrabox technology. *p<0.001. Representative images of larval swim patterns are depicted above panel (A) and (B). (C) The total distance swam was assessed in gabra1 morphants and random control injected larvae at 5 DPF. Distance was calculated without light, after the onset of light for a 5 minute duration, and an additional period without light.

**Figure 4: Restoration of hypomotility by co-injection of human mRNA variants.**

(A) Total swim speed of larvae injected with random control morpholinos (RC), translational targeting gabra1 morpholinos (tbMO), GABRA1 encoding mRNA (1000pg/embryo), RC with GABRA1 mRNA (RC+), or tbMO with GABRA1 mRNA (tbMO+) was determined using Zebrabox technology at 5 days post fertilization (DPF). Total number of embryos analyzed per group is depicted in the graph. (B) The total distance swam was assessed at 5 DPF using Zebrabox
technology for each of the conditions in (A). *p=0.036384 and \#p=0.000161 and
\#\#p=0.003010689. Representative images of larval swim patterns are depicted above panel (A)
and (B). (C) Total distance swam of larvae injected with RC, tbMO, GABRA1 c.875C>T (SDM)
encoding mRNA, or tbMO with GABRA1 c.875C>T encoding mRNA (tbMO+SDM) was
determined at 5 DPF. Total number of animals is indicated in the graph. ◊ p=0.0153.

**Figure 5: Molecular and behavioral responses of gabra1 morphants.**

(A) Quantitative real time PCR (QPCR) was performed at 5 days post fertilization (DPF) to
measure the expression of each gene indicated. Total RNA was isolated from random control
injected embryos (RC) or translational blocking gabra1 morpholinos (tbMO). Error bars
represent standard deviation. Expression was measured in biological triplicate. \#p=0.0016,
\#\#p=0.003681, §p=0.009. (B) Total swim speed was assessed at 5 DPF in non-injected larvae,
larvae injected with RC, or larvae injected with MO treated with 10uM pentylenetetrazole (PTZ).
◊ p=6.74E-06, ◊◊ p=5.34E-07, ◊◊◊ p=1.019E-05. (C) Total distance travelled was assessed at 5
DPF in each of the groups described in (B). *2.14E-05. **p=3.92141E-05, ***p=0.0002636.
