RESEARCH ARTICLE

Abnormal expression of GABA\textsubscript{A} receptor subunits and hypomotility upon loss of gabra1 in zebrafish

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ABSTRACT

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 GABRA1 gene (c.875C>T). 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 subunits that compose the GABA\textsubscript{A}R have been previously associated with human disease. To understand the mechanisms by which GABRA1 regulates brain development, we developed a zebrafish model of gabra1 deficiency. gabra1 expression is restricted to the nervous system and behavioral analysis of morpholino injected larvae suggests that the knockdown of gabra1 results in hypoactivity and defects in the expression of other subunits of the GABA\textsubscript{A}R. Expression of the human 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.

KEY WORDS: Development, Zebrafish, Genetics, GABRA1, Locomotion

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, 2012).

In 2013, the Undiagnosed Disease Network was founded and includes seven clinical sites across the United States of America, 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 Undiagnosed Disease Network operating, 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 non-verbal 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) subunit of the multi-subunit gamma-aminobutyric acid receptor (GABA\textsubscript{A}R). The GABA\textsubscript{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 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 swim. This hypomotility was accompanied by distinct changes in the expression of the major subunits of the GABA\textsubscript{A}R, including decreased expression of β2 and γ2 transcripts. Despite this decrease in the expression of unique GABA\textsubscript{A}R subunits, morphants continued to respond to treatment with pentylenetetrazol (PTZ), a potent antagonist of the GABA\textsubscript{A}R, indicating that morphants continue to produce an active GABA\textsubscript{A}R even in the absence of adequate gabra1 expression.

RESULTS

Subject

The subject initially presented to medical care at 3 months of age with infantile spasms that evolved into Lennox-Gastaut syndrome. Seizure activity included a light-sensitive myoclonic epilepsy and generalized

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tonic-clonic seizures. The seizures were treated with adrenocorticotropic hormone, multiple antiepileptic medications and a ketogenic diet, although seizure activity continued to occur daily.

His clinical course was also marked by 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, ALD7H41, ARX, ATP6AP2, CDKL3, 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 patient and these were considered for further analysis. Parental WES data were 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 SCN1B, FNIP1, TTN, OTOG, and FA14.

Additional evaluation of each candidate gene according to the criteria described in the Materials and Methods section identified two 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

Fig. 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 cystolic 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.
work (Monesson-Olson et al., 2018; Samarut et al., 2018). Hindbrain regions (Fig. 2), consistent with previously published expression of GABRA1 mRNA at 1 DPF (Fig. 2). Over the course of development the expression of 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).

Expression patterns of the zebrafish ortholog of GABRA1

In order to understand the mechanisms by which GABRA1 regulates development, we used the zebrafish (Danio rerio) as a model organism. We first confirmed the spatial and temporal expression patterns of zebrafish gabra1 using whole-mount in situ hybridization (WISH). We performed WISH at 1, 2, and 3 days post fertilization (DPF). gabra1 expression was localized to the developing nervous system at each time point with the broadest expression at 1 DPF (Fig. 2). Over the course of development the expression of 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).

Gabra1 regulates zebrafish larval motility

Mutations in GABRA1 have been associated with epileptic phenotypes (Cossette et al., 2002; Maljevic 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 et al., 2018). Consequently, we developed a protocol using the Zebrabox behavioral unit to monitor swim speed and total distance swim in larvae injected with anti-sense morpholinos that inhibit either the translation of 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 swim. As shown in Fig. 3, the tbMO was associated with a statistically significant (P<0.001) reduction in total swim speed (Fig. 3A) and decreased total distance swim (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. S1). Importantly, injection of an equivalent concentration of sMO induced a hypomotility phenotype (Fig. S2B,C; P=0.0263). These results are consistent with the phenotype present in mutants injected with the translational blocking morpholino. Importantly, we validated the effects of injection of sMO on mRNA splicing using RT-PCR. As shown in Fig. S2A, injection of the gabra1 targeting sMO induced abnormal alternative splicing relative to injection with RC morpholinos (note double band observed using RT-PCR). RT PCR analysis confirmed a near 50% reduction in wild-type gabra1 (Fig. S2A).

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 swim (P=0.000161) and the overall swim speed (P=0.036384) (Fig. 4A, B; tbMO relative to RC). Injection of GABRA1 encoding mRNA had no significant effect on speed or distance at a concentration of 1000 pg/embryo (Fig. 4A, B; mRNA and RC). The co-injection of the tbMO and GABRA1 encoding mRNA at 1000 pg/embryo restored the total distance swim 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 1000 pg 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 SDM 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 1000 pg/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 GABAARs consist of two $\alpha_1$, two $\beta_2$, and one $\gamma_2$ subunit (Sigel and Steinmann, 2011). Knockdown of gabra1 causes hypomotility. (A) Total swim speed of larvae injected with RC morpholinos or tbMO morpholinos was determined using Zebrabox technology at 5 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. All experiments were performed in biological duplicate or triplicate and statistical analysis was performed using a standard two-tailed t-test. Error bars represent standard error of the mean of independent experiments.

Fig. 3. Knockdown of gabra1 causes hypomotility. (A) Total swim speed of larvae injected with RC morpholinos or tbMO morpholinos was determined using Zebrabox technology at 5 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. All experiments were performed in biological duplicate or triplicate and statistical analysis was performed using a standard two-tailed t-test. Error bars represent standard error of the mean of independent experiments.

Fig. 4. Ineffective restoration of hypomotility by co-injection of the c.875C>T variant. (A) Total swim speed of larvae injected with RC morpholinos, tbMO morpholinos, GABRA1 encoding mRNA (1000 pg/embryo), RC with GABRA1 mRNA (RC+), or tbMO with GABRA1 mRNA (tbMO+) was determined using Zebrabox technology at 5 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. *$P=0.03684$ and **$P=0.000161$ and ***$P=0.00301689$. Representative images of larval swim patterns are depicted above panel A and B. (C) Total distance swam of larvae injected with RC, tbMO, GABA1 c.875C>T (SDM) encoding mRNA, or tbMO with GABA1 c.875C>T encoding mRNA (tbMO+SDM) was determined at 5 DPF. Total number of animals is indicated in the graph.◊$P=0.0153$. All experiments were performed in biological triplicate and statistical analysis was performed using a standard two-tailed t-test. Error bars represent standard error of the mean of independent experiments.
We hypothesized that the knockdown of gabra1, which encodes the α1 subunit, would alter the subunit composition of the GABA<sub>α</sub>R. To begin to test this, we analyzed the expression of the genes that encode the β2 and γ2 subunits. As shown in Fig. 5A, knockdown of gabra1 caused a decrease in the expression of gabrb2 (β2) and gabrg2 (γ2). We next measured the expression of other alpha subunits in gabra1 morphants. As shown in Fig. 5A, injection of the tbMO was associated with increased expression of gabrb1a and gabrb6b, but only gabrb6b was statistically significant across biological triplicates. A similar expression pattern of gabrb1a and gabrb6b was observed upon injection of the sMO (Fig. S2D), with both genes demonstrating a statistically significant increase in expression. We did not detect a statistical change in the expression of any other α subunit across either the tbMO or the sMO (Fig. 5A; Fig. S2D).

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<sub>α</sub>R. Non-subunit across either the tbMO or the sMO (Fig. 5A; Fig. S2D).

Of these subunits, mutations in GABRA1 (Cossette et al., 2002; Kodera et al., 2016; 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 showed bursts of generalized spike and wave at 2.5 Hz with multiple foci of epileptiform activity. He presented with features of generalized tonic-clonic 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 fifth 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 three 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 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 Gabral (mouse) affect GABA$_A_R$ function, but the results have been difficult to interpret, as the deletion of Gabral (mouse) causes strain and sex specific phenotypes (Arazi et al., 2012). Due to these strain differences, additional systems have been developed including a zebrafish harboring a mutation in the gabral gene. Interestingly, mouse models of Gabral deletion are viable, but the homozygous deletion of gabral 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 gabral function in fish at 7–10 weeks post fertilization (Samarut et al., 2018).

In this report, we demonstrated that morpholino-mediated knockdown of gabral in zebrafish leads to hypomotility in the presence and absence of light. We performed our studies at 5 DPF, during the larval stage, prior to the onset of feeding or sexual dimorphism, but after swim bladder formation. Gabral 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 gabral results in hypomotility, albeit at a later stage in development, which 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 gabral, which may be 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 gabral 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 wild-type GABRA1 restored the total distance travelled 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 gabral causes abnormal expression of other subunits of the GABA$_A_R$. Despite changes in the expression of various GABA$_A_R$ subunits, morphant animals continue to respond to PTZ stimulus. PTZ is a potent antagonist of the GABA$_A_R$ and treatment of wild-type 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 6 subunits of the GABA$_A_R$. Other subunits did not demonstrate consistent changes in expression across multiple morpholinos or biological replicates. Interestingly, mutations in GABRA6, which encodes the 6 subunit 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 gabral or the upregulation of gabra6. Future studies analyzing the function of 6 and other alpha subunits in gabral mutant animals are needed.

The gene expression changes we observe are strongly supported by previous conclusions in mice with mutations in the Gabral gene (Arazi et al., 2015; Zhou et al., 2015). Recent work in zebrafish has demonstrated that the homozygous nonsense mutation of gabral 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 subunits may compensate for the loss of gabral, ultimately producing unique compositions of the GABA$_A_R$. The function of these receptors is unknown. However, it is conceivable that the production of GABA$_A_R$ with unique subunit compositions in incorrect regions of the brain might underlie the impaired synapse formation observed in zebrafish harboring germline mutations in the gabral 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 gabral in zebrafish. Behaviorally, morphant animals present with hypomotility at 5 DPF measured by reduced swim speed and total distance travelled. These deficits coincide with significant changes in the expression of GABA$_A_R$ subunits and cannot be restored by the de novo c.875C>T allele. Although a zebrafish harboring a mutation in the gabral 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 gabral was lethal (Samarut et al., 2018). Our behavioral study is the first to our knowledge that comprehensively characterizes the phenotype of gabral 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 wild type or Tufpel Long Fin wild-type adults. Fish were maintained at The University of Texas at El Paso according to the Institutional Animal Care and Use committee (IACUC) guidelines. 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, USA) in D.I. water at 28°C.

**WES and data analysis**

High quality, unmapped, 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, USA). Whole exome sequencing was performed using the service provided by Beijing Genomics Institute (Cambridge, MA, USA). Details of data analysis were similar to the procedure as previously described (Ep4K 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 (Li and Durbin, 2009).
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 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 that had homoyzgotes 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 (forward: 5′-GCTGTGAT-AGGGTTAGGAGGTG-3′, reverse: 5′GCTATCAACGGCATTGTGAAG-3′) using 1X GoTaq (Promega, Madison, WI, USA) with a final primer concentration of 0.2 µM. Reaction parameters for PCR include an initial cycle at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, finishing with extension at 72°C for 5 min. 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.

WISH 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 (Electron Microscopy Sciences, PA, USA) for 1 h at room temperature (RT). Embryos were dehydrated using a methanol: PBS gradient and stored in 100% methanol overnight at −20°C. Embryos were rehydrated using PBS: methanol gradient, washed in PBS with 0.1% Tween 20 and permeabilized with proteinase K (10 µg/ml) for the time indicated by Thisse and Thisse (2008). Permeabilized embryos were pre-hybridized in hybridization buffer (HB) [50% deionized formamide (Thermo Fisher Scientific, Waltham, MA, USA), 5× SSC (Thermo Fisher Scientific), 0.1% Tween 20 (Thermo Fisher Scientific), 50 µg/ml heparin (Sigma-Aldrich, St Louis, MO, USA), 500 µg ml−1 of RNase-free tRNA (Sigma-Aldrich), 1 M citric acid (Thermo Fisher Scientific)] (460 µl for 50 ml of HB) for 4 h at RT, and incubated with anti-DIG Fab fragments (1:10,000) (Sigma-Aldrich) overnight at 4°C. Samples were developed with BM purple AP substrate (Sigma-Aldrich) and images were collected with a Zeiss Discovery Stereo Microscope fitted with Zen Software. The gabral probe was created using primers specific to the endogenous cDNA sequence (gabral ISH forward: 5′-TAAGCTGCGCTTCTCTCCTCT-3′, gabral ISH reverse: 5′-GAGAATCTGCTCTCAGGAG-3′) targeting gabral were designed. The efficiency of knockdown for the smO was performed with primers flanking the target site (forward: GAGACGTCCTCTGAGTGTGA and reverse: GCAGAGTCCGTCCTCGTGTG). 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, USA). The c.875C→T GABRA1 variant was created from the original vector obtained from TransOMIC Technologies using the QuickChange II Site-Directed Mutagenesis Kit (Thermo Fisher Scientific) with forward (TAACAACCTGTGCTCATCATGACAATTTGAG) and reverse primers (GAGTTAACAGTACGACTCTGGTCCAAAT). In vitro RNA was synthesized using the mMessage Machine kit (Thermo Fisher Scientific). 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 (Thermo Fisher Scientific). Reverse transcription was performed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) 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 (Thermo Fisher Scientific) based primer pairs for each gene analyzed are as follows: gabra2a forward: GATGGCTACGACACAGGCT, gabra2a reverse: TGTCCTACGCTGCTGGAAGAAAA, gabra3 forward: GCTGAGT-TGGGAGCTATG, gabra3 reverse: GAGAGCTGATGCTTCTTTGG, gabra4 forward: GACTGCGATTGAACCCACTT, gabra4 reverse: ATC-CAAGTGCGATCTGTGG, gabra5 forward: CATGACAACCAACCAAA-CAAAGC, gabra5 reverse: GAGGGCCCTTTTGGCTATTTA, gabrataba forward: TCCGGTACACCTTCTTCTT, gabrataba reverse: CCTCGGACTTTTTGCCAGTG, gabrabb forward: CGGGAGGATGCTGAAGAAC-AC, gabrabb reverse: GGGGAAAGGATGCGTGAGTA, gabb2 forward: CCGGCAACATTCTTCTTCA, gabrb2 reverse: TCTCGATCCAGT-GTGCAG, gabrsg2 forward: ACACAAATAGGAGGCTTG, gabrsg2 reverse: AGCTGCGCTTCCACTGTAT. Analysis performed using 2−ΔΔct. 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 Zebrobox (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 min divided into 5 min intervals of dark/light/dark conditions. For light exposure, the Zebrobox produces 8000 Lux of light at 550 nM at the maximum setting. We ran experiments at 100% light power for 5 min and then removed the light stimulus. All larvae were acclimated to the dish and housing conditions for 1 h prior to analysis. A baseline measure of activity was performed for 5 min in the dark prior to the onset of light stimulus. An additional measure of activity was performed post-light stimulus for 5 min. Settings for the program include a threshold of 16 distance traveled in large and small movements (smldist+lardist)/[total distance traveled (mm) and total swim speed (mm/s) {swim speed=[total distance traveled (mm) + total swim speed (mm/s)]/total time traveled}. We ran experiments at 100% light power for 5 min and then removed the light stimulus. All larvae were acclimated to the dish and housing conditions for 1 h prior to analysis. A baseline measure of activity was performed for 5 min in the dark prior to the onset of light stimulus. An additional measure of activity was performed post-light stimulus for 5 min. Settings for the program include a threshold of 16 distance traveled in large and small movements (smldist+lardist)/[total distance traveled (mm) + total swim speed (mm/s)]/total time traveled}. All experiments were performed in biological triplicate. For PTZ treatment, PTZ (10 mM) 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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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.R.-N., H.-G.Y., A.M.Q.; Methodology: N.R.-N., A.M.Q.; Validation: H.-C.R., A.M.Q.; Formal analysis: N.R.-N., H.-G.Y., A.M.Q.; Investigation: N.R.-N., C.R.C., A.M.Q.; Resources: A.M.Q.; Data curation: N.R.-N., H.-C.Y., A.M.Q.; Writing – original draft: N.R.-N., C.R.C., A.M.Q.; Writing – review & editing: N.R.-N., C.R.C., A.M.Q.; Visualization: N.R.-N., H.-C.Y.; Supervision: N.R.-N., C.R.C., A.M.Q.; Project administration: N.R.-N., C.R.C., A.M.Q.; Funding acquisition: N.R.-N., C.R.C., A.M.Q.
C.R.C., T.H.S., A.M.Q.: Writing - original draft: N.R.-N., H.-C.Y., C.R.C., T.H.S., A.M.Q.; Writing - review & editing: N.R.-N., T.H.S., A.M.Q.; Supervision: A.M.Q.; Project administration: C.R.C., T.H.S., A.M.Q.; Funding acquisition: A.M.Q.

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Data availability
All reagents are available upon request from the corresponding author.

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