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Targeted knockout of GABA receptor gamma 2 subunit provokes transient light-induced reflex seizures in zebrafish larvae

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SUMMARY STATEMENT

Novel *in vivo* genetic model of idiopathic epilepsy in zebrafish (gabrg2-/-): a new model to study ictogenesis and a convenient genetic tool for drug screening.

ABSTRACT

Epilepsy is the most common primary neurological disorder characterized by the chronic tendency of a patient to experience epileptic seizures, which are abnormal body movements or cognitive states that result from excessive, hypersynchronous brain activity. Epilepsy has been found to have numerous etiologies and whilst about two thirds of epilepsies were classically considered idiopathic, a majority of those is now believed to be of genetic origin. Mutations in genes involved in GABA-mediated inhibitory neurotransmission have been associated with a broad range of epilepsy syndromes. Mutations in the GABA-A receptor gamma 2 subunit gene (*GABRG2*), for example, have been associated with absence epilepsy and febrile seizures in humans. Several rodent models of *GABRG2* loss-of-function depict clinical features of the disease, however, alternative genetic models more amenable for the study of ictogenesis and for high-throughput screening purposes are still needed. In this context, we generated a *gabrg2* knock-out zebrafish model (R23X) that displayed light/dark-induced reflex seizures. Through high-resolution *in vivo* calcium imaging of the brain, we showed that this phenotype is associated with widespread increases in neuronal activity that can be effectively alleviated by the anti-epileptic drug valproic acid. Moreover these seizures only occur at the larval stages but disappear after one week of age. Interestingly, our whole transcriptome analysis showed that gabrg2-KO does not alter the expression of genes in the larval brain. As a result, gabrg2-/- zebrafish is a novel in vivo genetic model of early epilepsies that opens new doors to investigate ictogenesis and for further drug-screening assays.

INTRODUCTION

Epilepsy is a neurological disorder with abnormal, hypersynchronous electrical brain activity causing abnormal movements or cognitive states (Paudel et al., 2019; Vezzani et al., 2016). Epilepsy affects more than 50 million individuals all over the world causing mortality, disability, social and behavioural stigma (Moshé et al., 2015). Although more than 25 Anti-Epileptic Drugs (AEDs) are available to patients, treatment response is often unpredictable, and approximately one-third of patients fails to gain complete seizure control with pharmacotherapy alone (Chen et al., 2018). Epilepsy can arise from a range of causes associated with different prognoses and treatment outcomes, including metabolic disorders, brain lesions, and autoimmune causes, as well as 'idiopathic' cases (Sirven, 2015; Thomas and Berkovic, 2014). However, both (1) in severe epilepsies of early childhood and infancy (McTague et al., 2016)(in particular the early infantile epileptic encephalopathies, EIEE), and (2) in 'idiopathic' generalised epilepsy, the role of genetic mutations is now increasingly recognised (Gardiner, 2005). Consistently, in a recent update of the nomenclature the 'idiopathic' generalised epilepsy syndromes have even been relabelled *genetic* generalised epilepsies (GGE). Neurotransmitters are the chemical agents that carry signals across the synapses in the brain and gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain (Olsen and Li, 2012). It binds to $GABA_A$ receptors that act as inhibitory ligand-gated ion channels consisting of five subunits surrounding a central chloride ion pore (Macdonald and Olsen, 1994; Miller et al., 2017; Olsen and Sieghart, 2008; Sigel and Steinmann, 2012). The majority of $GABA_A$ receptors in the brain are composed of two alpha (α) subunits, two beta (β) subunits and one gamma (γ) or delta (δ) subunit (Baumann et al., 2002) although diverse stoichiometries have also been reported *in vitro* (Patel et al., 2014). Both GGEs and EIEEs have been linked with mutations in genes encoding $GABA_A$ receptor subunits including *GABRA1*, *GABRB3*, *GABRG2* and *GABRD* (Butler et al., 2018; Cossette et al., 2012; Hirose, 2014; Macdonald et al., 2010; Macdonald et al., 2006; Moller et al., 2017; Reid et al., 2013; Shen et al., 2017; Sperk et al., 2004).

In particular, numerous mutations in *GABRG2* gene have been reported to cause epilepsy, about half of which interrupt normal protein translation (i.e nonsense, splice-site or genomic deletion) (Boillot et al., 2015). Several missense mutations were also reported and are expected to disrupt normal GABA receptor function by reducing its hyperpolarizing effect. Mutations in *GABRG2* have been most strongly associated with childhood absence epilepsy (a GGE syndrome) and febrile seizures (Baulac et al., 2001; Wallace et al., 2001). Further studies on larger cohorts of epileptic patients confirmed an association of *GABRG2* polymorphisms with GGE more broadly (Chou et al., 2007). Some studies demonstrated *in vitro* that *GABRG2* mutations cause a range of functional defects, RNA alteration, defects in the stability of the resultant protein, trafficking abnormalities, and kinetic defects of the ion channel (Huang et al., 2012; Johnston et al., 2014; Kang et al., 2015). Recently, several mouse models of *GABRG2* loss-of-function have been generated. In particular a heterozygous knock-in mouse model bearing the *GABRG2* Q390X mutation has been generated and displays reduced cortical inhibition associated with epilepsy phenotypes (Kang et al., 2015; Reid et al., 2013; Tan et al., 2007). The comparison of two mouse models with *GABRG2* loss-of-function mutations (*GABRG2*$^{+/Q390X}$ Knockin (KI) and *GABRG2*$^{+/-}$ Knockout (KO)) highlighted different severity in seizure intensity and memory defects. While KI mice exhibited severe seizures and behavioural comorbidities as well as neurodegeneration, *GABRG2*-KO mice display mild absence epilepsy caused by functional haploinsufficiency (Warner et al., 2016). These examples illustrate the complexity of phenotype-genotype relationships in animal models of epileptic syndromes associated with different mutations within the same gene.

In the last decade, zebrafish has emerged as a leading model to investigate biological questions in development and physiology, particularly to decipher basic developmental mechanisms of the central nervous system (Blader and Strahle, 2000; Schmidt et al., 2013). One reason for the popularity of zebrafish is its external fertilization as it makes the embryo suitable for observations from the earliest stages of development. Moreover, this is very

convenient for genetic manipulation from the very first cell stage in particular for CRISPR/Cas9 genome editing (Hwang et al., 2013). Furthermore, the transparency of the embryo allows one to follow *in vivo* organogenesis, in particular the observation of central nervous system structures with a single-cell resolution. As a vertebrate, zebrafish has a high homology to the human genome, with over 80% conservation of disease-causing genes (Howe et al., 2013), and a complex yet easily accessible central nervous system, which makes it relevant for the study of human diseases. Indeed, many CNS-related disorders have been successfully modeled in the past such as amyotrophic lateral sclerosis (Patten et al., 2017), hereditary spastic paraplegia (Gan-Or et al., 2016), autism spectrum disorder (Liu et al., 2018; Marin-Valencia et al., 2018) and epilepsy (Baraban et al., 2013). In particular, zebrafish epilepsy models reproduce several phenotypic features of the disorders and are also very favourable for drug discovery since it can be used in a high-throughput fashion as many early motor assays give easily interpretable read-outs for evaluating drug efficacy (Stewart et al., 2014). A recent review showed that zebrafish models of epilepsy are more reliable in terms of clinical relevance and pharmacological features than their mammalian counterparts (Griffin et al., 2018).

Thus, in this study we aimed to establish and characterize a new *in vivo* genetic model of epilepsy caused by mutations in the *GABRG2* gene. Thus, we generated a novel *gabrg2*-KO zebrafish model and showed that homozygous larvae undergo light/dark-reflex seizures. We confirmed the neuronal basis for the observed hyperactivity by monitoring calcium levels *in vivo* after light stimulus. Interestingly, the epileptic phenotype of *gabrg2*-/- larvae was only observed during a specific developmental time window (from day 3 to day 6 post-fertilization). We also confirmed that a canonical anti-epileptic drug (valproic acid) alleviates seizure activity, thus validating the epileptic nature of the neuronal hyperactivity phenotype. Surprisingly, our transcriptomic analysis of the mutant brain suggests that GABRG2 loss-of-function does not alter the general brain transcriptome. This study brings a new *in vivo* genetic model of epilepsy

that is convenient for further studying the mechanisms of epileptogenesis/ictogenesis and for evaluating novel therapeutic agents for this particular genetic epilepsy.

RESULTS

Knock-out of *gabrg2* by CRISPR/CAS9 leads to a decrease in larval survival.

The zebrafish genome carries a single ortholog of the human *GABRG2* gene (*gabrg2*: ENSDARG00000053665) and it encodes a protein sharing 82.6% of identity with the human GABRG2 protein (Fig. S1). There are four possible transcripts from the *gabrg2* zebrafish genomic locus on chromosome 2, that are composed of 8 to 10 exons depending of the alternative splicing events. In order to induce a complete loss-of-function, we targeted the beginning of exon 1 that is common to all isoforms (Fig. 1A). We successfully isolated a founder fish transmitting a +5-nucleotide insertion (+TG+GAT) that is predicted to lead to a premature stop codon at position 23 of the protein sequence (Fig. 1A-B). This abrogates the translation of the GABA receptor subunit in its N-terminal GABA binding domain thus presumably leading to a shortened protein lacking all functional domains. After incrossing heterozygous *gabrg2*+/-, we followed the survival of all three genotypes among the offspring (WT: +/+, HT: +/-, HM: -/-) and noticed a decreased survival rate of *gabrg2*-/- larvae specifically from 10 days post-fertilization (dpf) compared to their siblings (Fig. 1C). By 30 dpf, the survival of *gabrg2*-/- is reduced by half but the remaining survivors are able to reach adulthood and mate. Of note is that there are no particular morphological differences between homozygous mutants and their siblings at any age.

Gabrg2-/- zebrafish larvae are hypoactive and display light/dark-reflex seizures.

In order to characterize behavioural phenotypes associated with *gabrg2*-KO, we monitored the locomotor swimming pattern of 5 dpf *gabrg2*-/- larvae and their siblings during 2-hour dark and 2-hour light cycles using an automated recording chamber. Interestingly, we noticed a

generalized hypoactivity of *gabrg2*-/- larvae compared to their siblings both in dark and light periods although the difference is only statistically significant during the light phase (Fig. 2A). However upon light stimulus, we observed that *gabrg2*-/- larvae display a stereotypical whirlpool behaviour characterized by uncontrolled movements. This behaviour is reminiscent to the increased swimming activity and whole-body convulsions induced by exposure of zebrafish larvae to convulsing drugs (Baraban et al., 2005) (Fig. 2B and Movie S1). Interestingly, we observed the same behaviour upon sudden darkness (Fig. 2C). Consistently, we showed that the maximum acceleration of *gabrg2*-/- larvae upon light or dark stimuli is significantly higher than their wildtype siblings (Fig. 2B-C, lower panels). Of note is that *gabrg2*+/- siblings show no behavioural difference compared to their wild-type (+/+) siblings (data not shown).

We previously showed that *gabra1*-/- larvae (mutant for another subunit of GABA receptors) display light-induced brain hyperactivity associated with an increased startling swimming acceleration on light stimulation after a period of darkness (Samarut et al., 2018). Thus, we decided to compare the behaviour of *gabrg2*-/- and *gabra1*-/- larvae under the same experimental settings. Remarkably, we observed no difference in the general swimming activity of *gabra1*-/- larvae compared to wildtype siblings thus suggesting that the hypoactivity observed in *gabrg2*-/- larvae is specific to this genetic condition. Moreover, although in *gabrg2*-/- larvae an increased activity can be triggered by both light or dark stimuli, for *gabra1*-/- larvae this was only observed when switching from dark to light (and not the reverse). Of note is that at that stage (5 dpf), the hyperactivity phenotype is much stronger in *gabrg2*-/- than in *gabra1*-/- larvae (compare traces in Fig. 2B-C). Moreover, we did not notice any hyperactivity phenotype in response to an increase in temperature (Fig. S2).

Altogether, our result show that *gabrg2*-/- display a stereotypical whirlpool behaviour (motor hyperactivity, movie S1) upon changes in environmental light exposure. The measured hyperactivity is much more pronounced than in *gabra1*-/- larva of the same age. This

phenotype is reminiscent of seizure-like behaviours observed in acute models of epilepsy in zebrafish larvae upon exposure to pro-convulsant drug.

The light/dark-triggered hyperactivity of *gabrg2-/-* larvae is associated with abnormally increased neuronal activity

We next wanted to confirm that the observed hyperactivity phenotype has a neuronal correlate. To do so, we measured neuronal activity in *gabrg2*+/+ and -/- larval brains during light exposure using the [neurod:GCaMP6f] transgenic line expressing the genetically encoded fluorescent calcium indicator GCaMP6f neuronally (Chen et al., 2013). There was no difference in baseline brain activity of *gabrg2-/-* compared to their siblings (Fig. S3). However, upon sudden exposure to the imaging laser light (following a >30-minute period in complete darkness), we observed widely distributed, high amplitude activity across multiple brain areas, shown by confocal imaging (Fig. 3A) as well as by the quantification of GCaMP6f-driven fluorescence (Fig. 3B). Such a generalized and intense neuronal activity was never observed in *gabrg2*+/+ larvae that only display a modest increase of fluorescence upon laser light stimulation. The generalized brain activity in *gabrg2-/-* larvae lasted several seconds before coming back to basal activity level.

We replicated these findings and further delineated distributed patterns of neuronal hyperexcitability using single plane light-sheet imaging. This showed particularly high amplitude activity in the optic tectum in *gabrg2-/-* larvae compared to their wildtype siblings, where again none of the wild type larvae showed levels of fluorescence in the mutant range in the first few seconds after light exposure (Fig. 3C). Using non-negative matrix factorisation of regionally averaged fluorescence, we can show that light exposure causes multidimensional changes in fluorescence across multiple brain areas. This reveals a state-space separation of whole-brain activity following light stimulation in *gabrg2-/-* larvae not found in gabrg2+/+ larvae, indicating abnormal neuronal dynamics compared with wildtype siblings (Fig. 3E).

Altogether, our results suggest that gabrg2-/- larvae do not show abnormal basal neuronal activity but undergo a reflex hyperactivity triggered by light stimulus both at the behavioral and neuronal levels.

Gabrg2-/- light-induced reflex seizures can be alleviated by acute valproic acid exposure

In order to confirm the seizure nature of the neuronal hyperactivity observed in *gabrg2*-/-, we tested the ability of valproic acid (VPA), a canonical anti-epileptic drug, to rescue it. Remarkably, we showed that a 2-hour incubation with 200µM VPA completely abolishes the seizure both at the behavioural and neuronal levels (Fig. 4). Indeed, acute VPA exposure completely rescues the maximum acceleration value of *garbg2*-/- larvae to wild-type levels (Fig. 4A). Consistently, we did not observed a whirlpool behaviour (e.g indicative of seizure) upon light in any of the *gabrg2*-/- treated larvae (Fig. 4B). Although this acute VPA treatment is not inducing any lethargy in the larvae, we aimed to confirm that this acute VPA exposure is also preventing neuronal firing of *gabrg2*-/- larvae upon light. By confocal imaging and quantification of GCaMP6f-driven fluorescence from the optic tecta over time, we showed that VPA exposure completely abolishes the hyperexcitability of neuronal activity (Fig. 4C, D).

Reflex hyperexcitability caused by _gabrg2_-KO only occur at the larval stage.

So far we have shown that *gabrg2*-/- larvae undergo light or dark-triggered hyperactivity at 5 dpf. We then aimed to identify the earliest stage at which the embryos are subjected to these reflex phenotype. To do so, we monitored and compared the behaviour of *gabrg2*+/+ and *gabrg2*-/- larvae in response to light from 3 dpf until 11 dpf (Fig. 5A). Interestingly, we found that maximum acceleration following a light stimulus is already significantly increased at 3 dpf in *gabrg2*-/- compared to their siblings. This is associated with an increased swimming activity leading to a whirlpool behaviour reminiscent of seizures (Fig. 5A, B). This hyperactivity upon light stimulus is still observed at 4, 5 and 6 dpf but it becomes less penetrant at 7 dpf with no difference between wildtype and *gabrg2-/-* at 8 dpf. As a matter

of fact, it appears that reflex hyperactivity can be triggered by light/dark stimuli in *gabrg2*-/- larvae from 3 to 6 dpf only.

Interestingly, we previously showed that *gabra1*-/- larvae show increased neuronal activity in response to light from 4 dpf with continually worsening seizure phenotypes until the juvenile stage, eventually becoming lethal after 6 weeks of age (Samarut et al., 2018). In this work, we showed that *gabrg2*-/- larvae undergo a light/dark-triggered seizing phenotype during the larval stage only. Thus we wondered if this transient occurrence could be explained by a different temporal expression of GABRA1 and GABRG2 subunits. As a result, we checked expression of *gabra1* and *gabrg2* transcripts during development by reverse-transcripase polymerase chain reaction (RT-PCR), from the 8-cell stage (at which the zygotic transcription has not yet started) until 9 dpf (Fig. 5C). Interestingly, we found that *gabra1* transcript is detected among the maternal transcripts but starts to be expressed zygotically from 1 dpf with a more robust expression at 2 dpf that is sustained constantly over time from 3 dpf. However, *gabrg2* transcript only starts to be detectable from 8 hpf and increases over time until 5 dpf. We further note a decrease in the expression of *gabrg2* transcript from 6 dpf with a weaker expression at 9 dpf (Fig. 5C). As a result, *gabrg2* expression appears peak in expression at 5 dpf, whilst *gabra1* expression levels are sustained from 3 dpf. We further examined regional expression of both transcripts at larval stages expressed by whole-mount in situ hybridization and observed that they are both strongly expressed at 2, 3 and 4 dpf (Fig. 5D). We showed that at these stages, both *gabra1* and *gabrg2* transcripts are broadly expressed in the brain and that their regional expression is largely overlapping.

Gabrg2-KO does not impair larval brain transcriptome

Since we showed that gabrg2-/- larvae undergo reflex hyperexcitabiliy that is reminiscent

of seizures at larval stages, we wondered if these brain hyperactivity events were associated with changes in gene expression. Hence, we conducted a whole transcriptome sequencing analysis (RNAseq) from whole larval brains extracted from 5 dpf *gabrg2*+/+ and *gabrg2*-/-

larvae (Fig. S4A). Surprisingly, differential expression analysis only reported that a single gene was significantly upregulated in the brain of *gabrg2*-/- larvae compared to wild-type siblings (Fig. S4B, C). Although there are some differences in the raw expression of certain genes between +/+ and -/- samples (i.e *pvalb4*, *nkx6.3*, Table S1), our differential expression analysis show that these changes are not consistent between triplicates. Therefore, it is impossible to conclude if these changes are natural variations between samples or if they can be specifically attributed to the mutation. As a result, the Ras-related associated with diabetes gene (*rrad*, ENSDARG00000052011) was the only gene statistically upregulated by a fold change of 1.36 (logFoldChange 0.44, pvalue $7.09E^{-3}$, Table S1). Consistently, sample hierarchizing by clustering and principal component analysis also revealed no clear genotype-specific clustering nor distinguishable patterns between +/+ and -/- samples (Fig. S4D, E). We also confirmed the quality of cDNA library preparation (Fig. S4F) and that all triplicates of *gabrg2*+/+ and *gabrg2*-/- samples that have been sequenced only contained the wild-type or mutant sequence respectively thus validating the experimental procedure (Fig. S4G). Moreover, we checked the expression of genes that we previously identified as being dependant on GABA receptor signaling (*gabra1, gabrg2, gata3, kcc2, sema6d, cacna2d2a*) (Samarut et al., 2018) and confirmed by qRTPCR that their expression is not significantly affected upon gabrg2-KO (Table S2). Interestingly, the fact that the expression of the *gabrg2* transcript is not reduced in gabrg2-/- larvae suggest that the mutation does not induce the messenger RNA degration through non-sense mediation decay. As a result, it appears that *gabrg2*-KO does not lead to obvious differences in the expression of genes within the brain at the larval stage.

DISCUSSION

In this work, we introduce a novel zebrafish model of a human genetic epilepsy: We show that zebrafish carrying a knockout of the *gabrg2* gene – orthologous to the human epilepsy gene *GABRG2* – show inducible, short lived whole body convulsions associated with wide-spread increases in brain activity. This activity is confined to the larval stages (consistent with the developmental expression profile of the *gabrg2* gene) and whilst reducing overall survival, gabrg2-/- fish can reach adulthood.

The hypermotor phenotype is associated with widespread increase in brain activity and can be rescued with anti-epileptic medication, indicating that the phenotype is epileptic in nature, even though we did not observe spontaneous seizure-like events in these fish (Fig. S3). In human patients, epileptic seizures triggered by specific sensory events, environmental factors or cognitive states are considered 'reflex seizures' (Panayiotopoulos, 2005a). Whilst the majority of patients with reflex seizures will also have unprovoked seizures, there is a subgroup of patients with 'reflex epilepsy' who only ever experience seizures in response to provoking stimuli (Okudan and Ozkara, 2018). Furthermore, in humans abnormal brain dynamics in response to photic stimulation (i.e. a photoparoxysmal response on EEG recording) are both highly heritable, and associated with a increased risk of developing epilepsy (Tauer et al., 2005). In fact, case reports identify photosensitivity as a potential phenotypic feature of *GABRG2*-related epilepsy in humans. Our novel *gabrg2-/-* model may therefore offer insights into the genotype-phenotype relationship between abnormal GABA signalling and brain hyperexcitability, which at its extremes causes epileptic seizures like the ones observed in our model.

Surprisingly, although *GABRG2* mutations have often been associated with febrile seizures (Boillot et al., 2015; Hirose, 2014), we did not observe any particular effect of increasing the temperature of the bathing water (up to 31ºC for 15 minutes, Fig. S2). Yet, epileptic seizures can be triggered by transient hyperthermia in zebrafish larvae and have been shown to be evocative of febrile seizures in humans (Hunt, Hortopan et al. 2012). This highlights the fact that particular aspects of the epileptic phenotype could be due to specific

mutations in the gene. In other words, some specificity of the epileptic syndrome may be due to the level of haploinsufficiency that is driven by a specific mutation. In this context, a complete loss-of-function as we induced here might therefore not mimic the broad spectrum of the epileptic syndromes associated with all the mutations in one gene. This emphasizes the idea that epilepsies should not be classified solely based on the affected gene, since different mutations in the same gene could have drastic different effects (i.e different types of seizures, manifestations or triggers). Thus, our findings confirm the fact that each genetic background associated with epilepsy should be considered uniquely in terms of epileptogenesis, ictogenesis and therefore treatment responsiveness.

Another interesting aspect of the phenotype we are describing is the fact that it is limited to the larval stage. Indeed, whilst survival overall is reduced, *gabrg2*-/- fish can reach adulthood and 'outgrow' their seizures. In fact, in human patients on the severe end of the phenotypic spectrum of *GABRG2*-related epilepsies, seizures start very early in life. In one recent cohort of EIEE patients, mean age of onset was 7.7 months (range 1 day – 12 months) (Shen et al., 2017). Many of the milder phenotypes also start in childhood, and for patients with the most strongly associated clinical phenotypes - childhood absence epilepsy (a GGE syndrome), or febrile seizures (Boillot et al., 2015; Kang and Macdonald, 2016) – seizures typically remit by the end of puberty. In fact, transient epilepsies are the most common epilepsy phenotypes in childhood, largely due to the prevalence of benign childhood epilepsies / seizure susceptibility syndromes (Panayiotopoulos, 1993). One of the most common human benign epileptic syndromes are Rolandic seizures (RS) or Benign childhood epilepsy with centrotemporal spikes (Herranz, 2002) and Panayiotopoulos syndrome (PS) or Early onset benign childhood occipital epilepsy (Loiseau and Duche, 1992). In RS and PS, seizures start between 1-14 years and patients are seizure-free after 2-4 years of onset (RS) and 1-2 years (PS) onset and could not need antiepileptic drugs (Panayiotopoulos, 2005b). Thus, about 27% of patients with PS manifest only one seizure in their life (Panayiotopoulos, 2005b). At this juncture our novel line therefore captures further aspects of the natural history of the human disease. Indeed, the transient reflex seizures phenotype of *gabrg2*-/- fish, confined to the larval

stage, is in stark contrast to existing zebrafish models of epilepsy (Griffin et al., 2018; Samarut et al., 2018).

Our results showing that *gabrg2* transcript is maximally expressed at 3, 4 and 5 dpf could explain why *gabrg2*-/- only display the seizure phenotype at these stages. Yet in the normally developing larvae, *gabrg2* expression is still detectable at later stages (i.e 8-9 dpf) whilst the hyperactivity phenotype remit at that stage. Taken together with the distinct developmental profile in *gabra1* expression, this observation suggests that the transient time window during which a seizing phenotype can be induced in *gabrg2*-/- larvae reveal a particularly sensitive developmental period. $GABA_A$ receptor subunit expression is dynamically regulated throughout the lifespan, resulting in different stoichiometry of $GABA_A$ receptor subunit composition at different times of development and/or in different regions of the brain. Thus, the lack of one specific subunit at a specific time at a specific place could lead to hyperexcitability and to an epilepsy phenotype. Yet at a different stage of development that subunit may be less crucial and have less of an observable effect on brain function.

Surprisingly, although we previsouly showed that *gabra1*-KO leads to defects of neurodevelopment, our present work suggests that *gabrg2*-KO does not. Indeed, 460 genes were found to be differentially expressed in the brain of 5 dpf *gabra1*-/- mutants (Samarut et al., 2018). Specifically, we showed that the inhibitory network development was impaired in *gabra1*-/- larvae and that this neurodevelopmental component of the pathology might play an important role in the excitation/inhibition imbalance of the mutant brain throughout lifespan. Our present work shows almost no difference at the transcriptomic level in *gabrg2*-/- mutants, suggesting that although being expressed at early stages of development, the gamma 2 subunit does not seem to play a crucial role in broader GABA-mediated neurodevelopmental processes, at least at the transcriptomic level. This is consistent with the fact that *gabrg2*-/- fish are only undergoing seizing events at the larval stages and not later in life and also with the fact that the homozygotes can reach adulthood (unlike *gabra1*-/- (Samarut et al., 2018)). It is now well admitted that GABA signalling regulates many aspects of neurodevelopment including synaptogenesis. Interestingly, our present work brings one more piece of complexity

by suggesting that the different GABA receptor subunits do not influence the same processes meditated by GABA signalling.

Lastly, our work brings a new *in vivo* genetic model of idiopathic epilepsy that is convenient for a broad range of scientific applications. Indeed, the fact that the hyperactivity phenotype can be triggered by light or dark stimuli greatly improves the standardisation of the seizure assays thus potentially facilitating further drug-screening applications. Moreover, this new genetic model will open new doors of investigation, in particular if studied in parallel to the *gabra1*-/- zebrafish mutant (Samarut et al., 2018). Indeed, such studies would be important to decipher the specific role of each GABA receptor subunit at the cellular, molecular and electrophysiological levels.

MATERIALS AND METHODS

Fish husbandry and fish lines

Wild-type Danio rerio were raised at 28.5°C, kept under a 12-hour dark, 12-hour light cycle, and staged as described in (Swaminathan et al., 2018a). All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and conducted at the Centre de recherche du rentre hospitalier de l'Université de Montréal (CRCHUM) and approved by the Comité institutionnel de protection des animaux of CRCHUM (approval # N15018PMDz). All experiments were performed on sexually undifferentiated zebrafish larvae. Humane endpoints were in place during the study and all animals were monitored and assessed daily for well-being as per guidelines established by the Canadian Council of Animal Care committee at the CRCHUM. Behavioral signs of poor health in adult animals necessitating euthanasia included an inability to feed and swim. Physical abnormalities were also monitored daily and adult animals displaying a distended abdomen, skin ulcerations/wounds and skeletal deformities were euthanasia immediately by anesthetic overdose and rapid chilling.The NeuroD: GCaMP6F transgenic line is a gift from Claire Wyart.

Whole mount in situ hybridization and probe cloning

A specific 911-bp *gabrg2* probe was amplified from total RNAs extracted from 1-day old embryos using the following primers: F_5'_ATTTTTCCTCTAAATGCCTGAACGCGATG_3'; R_5'_ CGTTGTGATTCCCAAGGAGGTTCG_3'. The PCR fragment was then cloned within the pCS2+ vector using the TOPO TA cloning kit (Invitrogen). Whole mount in situ hybridization using gabra1 probe on zebrafish embryos was performed as described previously (Samarut et al., 2016).

sgRNA and Cas9 preparation and microinjection

A gRNA sequence was designed using the online tool CRISPRscan to target the following early coding sequence of *gabrg2* gene (ENSDART00000075740.4)(protospacer adjacent motif site in parentheses): GAACGCGATGGCCATCCCCG(CGG). Synthesis of gRNA and Cas9 mRNA as well as embryo microinjection was performed as described previously (Samarut et al., 2016).

Fish tracking and seizure triggering

A DanioVision (Noldus, Wageningen, The Netherlands) setup was used as a lightproof recording chamber with an infrared camera. Light-induced seizures were induced after 1 hour spent in darkness and by switching on the built-in cold-white light-emitting diode light for at least 1 minute. Ethovision XT12 (Noldus) was used for analyzing the distance swam and maximum acceleration as well as to extract swimming tracks.

Antiepileptic drug treatment

A one molar solution of valproic acid (Sigma, St Louis, Missosuri) was prepared extemporaneously in water and was subsequently dissolved in fish water to reach the final concentration.

GCaMP6f monitoring and fluorescence quantification

For imaging experiments all larvae were raised in danieau solution (69% NaCL2 + 24% HEPES buffer + 4% Ca(NO3)2 + 2% MgSO4 + 1% KCL pH 7) diluted at 1:50 in deionised water, with 1-phenyly-2-thiourea (PTU) to prevent pigment formation thus maximising optical accessibility (Karlsson et al., 2001) (ref provided in comment).

For confocal imaging 6 dpf larvae were immobilized dorsal in 1% low-melting agarose and mounted dorsal side up under a spinning disk confocal microscope. The embedded fish was kept in complete darkness for at least 30 minutes. The seizure was triggered by switching on the fluorescence excitation laser. Single-plane images were acquired at 2.5 Hz for 60 seconds. Fluorescence quantification from the optic tecta was performed using the mean gray value measurement from an 8-bit image (ImageJ, NIH, Bethesda, Maryland). All gray values were normalized against a resting basal mean gray value.

For light sheet imaging non-anaesthetised larvae at 6dpf were immobilised in 2.5% low-melting point agarose (Sigma-Aldrich) prepared in Danieau solution and mounted dorsal side up on a raised glass platform that was placed in a custom-made Danieau-filled chamber. The embedded fish was kept in complete darkness for 45 minutes. The seizure was triggered by switching on the fluorescence excitation laser from a custom light sheet microscope (microscope details are reported in (Rosch et al., 2018). Single-plane images were acquired at 40Hz for 5 minutes. Gross anatomical regions were segmented manually and regional fluorescence average traces were extracted from the first 30 seconds of the recording, and normalised to the baseline level achieved during the last 30 seconds of the 5 minute recording window. Here we report normalised ΔF/F values for the regional averages.

Constrained least squares non-negative matrix factorisation over a $r * t$ matrix (r – number of regions, t – time samples, where each entry represents a fluorescence value at the time t in region r) was run to identify a set of three time varying distributed fluorescence factors to explain the maximum variance in the data.

RT-PCR

Reverse transcription was performed from 1µg of total RNA using the superscript VILO reverse transcription mix (Invitrogen). PCR was performed on 1 µL of cDNA using regular GeneDirex Taq polymerase (FroggaBio) using the following gene-specific primers gabrg2_F: GGATCAACAAGGATGCAGTG, gabrg2_R: GAAAAGAGCCGCAGGAGAG; gabra1_F: TCGAGCCATCCTGATTTTTC, gabra1_R: TCAGCCTTTCATCCTTCCAG; polr2d_F: AACGCAAAGTGGGAGATGTG, polr2d_R: TGTAACTCCTCATCCTCGAACC.

Transcriptomic assay, differential expression assay and pathway analysis

Briefly, 5 dpf larvae were anesthetized in 0.04mg/ml tricaine in cold calcium-free Ringer's. The larvae was pinned laterally and the brain was gently extracted manually using Dumont#5 fine forceps. The dissection was performed in cold calcium-free Ringer's. Extracted brains were immediately flash-frozen in liquid nitrogen for further extraction. Total RNA was extracted from whole brains of 5 dpf gabrg2 -/- and +/+ larvae using picopure RNA extraction kit (Thermo Fisher Scientific) following the manufacturer's standard protocol. Quality of total RNA was assessed with the BioAnalyzer Nano (Agilent) and all samples had a RIN above 9. Library preparation and sequencing was done at the Institute for Research in Immunology and Cancer's Platform (University of Montreal) as described by (Swaminathan et al., 2018b). Sequencing was performed with the Illumina NextSeq500 using the SBS Reagent Kit v3 (80 cycles, paired-end) with 1.6 nM of the pooled library. Cluster density was targeted at around 800k clusters/mm2. The quality of the reads from the paired-end 80bp sequencing on NextSeq500 was correct for all samples with a number of reads between 171M and 192M and an average of 87% of them passing the quality filter. Sequences were trimmed for sequencing adapters and low quality 3' bases using Trimmomatic version 0.35 (Bolger et al., 2014) and aligned to the reference genome version GRCz10 (gene annotation from Ensembl version 87) using STAR version 2.5.1b STAR (Dobin et al., 2013). On average, 92% of the reads could be successfully mapped onto the genome using STAR allowing an average coverage of 130X. Gene expression were obtained both as readcount directly from STAR as well as computed

using RSEM (Li and Dewey, 2011) in order to obtain transcript level expression. - Differential gene expression analysis was assessed by DeSeq2 package using R software. Differential gene expression was filtered on a False Discovery Rate (or adjusted p value > 0.05).

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COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

Conceptualization: ES; Methodology: ES, ML ; Formal analysis: ES, ML, UK, RER, DRWB, BOAB; Investigation: ES, ML, UK, RER, DRWB, BOAB; Writing - original draft: UK, ES; Writing - review & editing: ES, UK, RER, DRWB, BOAB; Supervision: ES, MPM, PC; Project administration: ES

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DATA AVAILABILITY

RNA-seq data have been deposited in NCBI Sequence Read Archive GEO (GSE134951)

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Figures

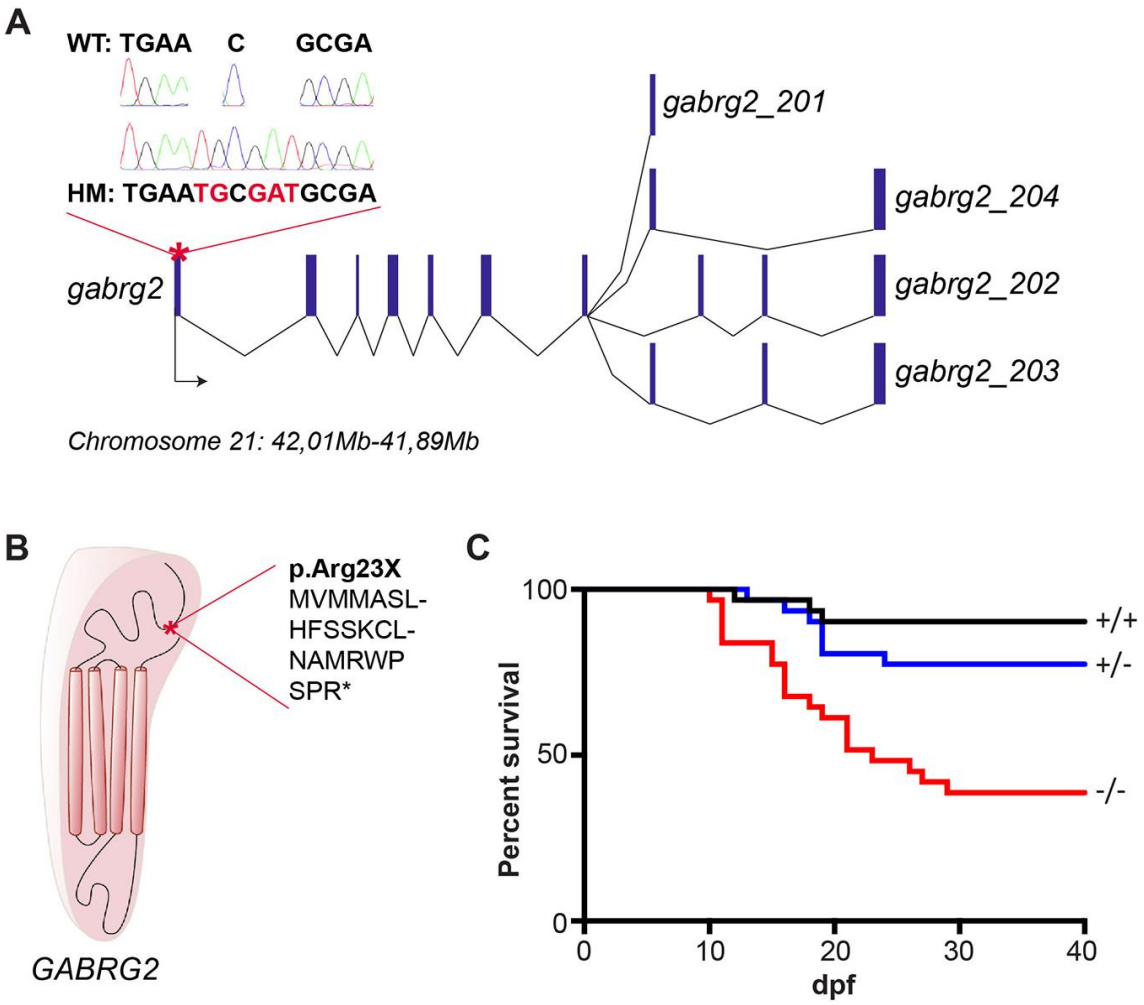


Figure 1: *gabrg2*-KO (R23X) reduces survival of homozygous larvae. (A) Scheme of the genomic organization of *gabrg2* zebrafish gene on chromosome 21 with the four transcribed transcripts composed of 8 to 10 exons. The CRISPR/Cas9 targeted region in exon 1 is indicated by a red asterisk and the +5-nucleotide insertion has been confirmed by Sanger sequencing (chromatograms). (B) Schematic representation of GABRG2 subunit proteic domains with the premature stop codon indicated by a red asterisk. Of note is that the mutation affect the N-terminal GABA binding domain of the protein thus preventing the translation of the subsequent transmembrane domains. (C) Survival assays of embryos obtained from an incross between gabrg2+/- fish. All three genotypes were determined by High-Resolution-

Melting genotyping at 5 days and the survival of each group has been followed for 40 days. Of note is that gabrg2-KO induces a premature death of gabrg2-/- larvae with only 40% of survival from 30 days post-fertilization (dpf).

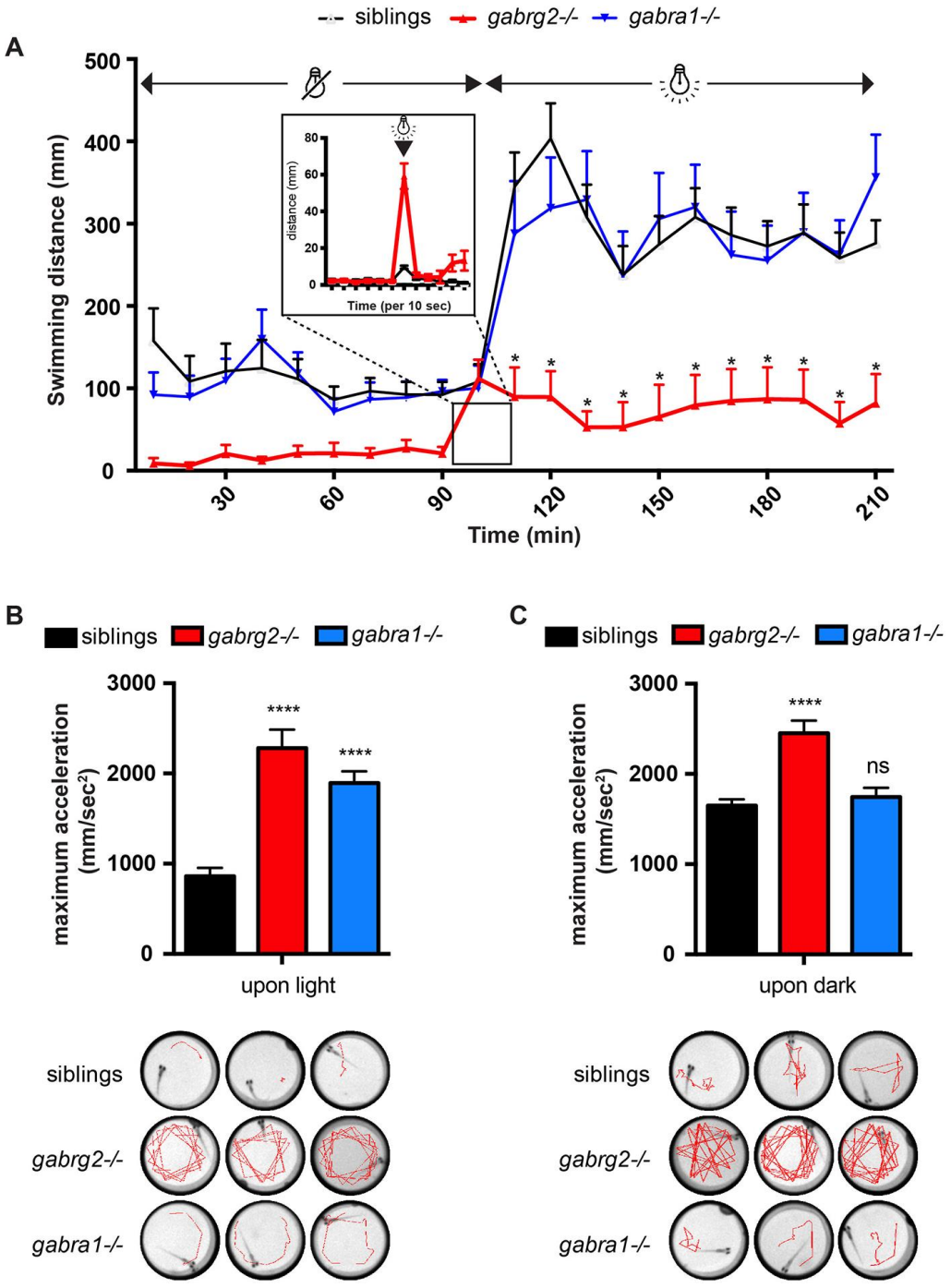


Figure 2: Light- and dark-triggered hyperactivity in *gabrg2-/-* larvae. (A) Four-hour swim assay (2-hour dark followed by a 2-hour light period) shows that *gabrg2-/-* larvae (5 dpf) are hypoactive compared to their siblings. Interestingly, *gabra1-/-* larvae do not show this hypoactivity phenotype at that stage. (* pvalue < 0.05, 2-way ANOVA). A higher temporal resolution is shown at the light stimulus event. It reveals the sudden hyperactivity of gabrg2-/-

larvae compared to siblings. (B) Quantification of the maximum acceleration value over 30 seconds following light (B) or dark (C) stimuli. The corresponding representative swimming tracks of three larvae per genotype is shown in the lower panel (tracks represent 15 seconds following photic stimulus). (**** pvalue < 0.0001, unpaired t-test)

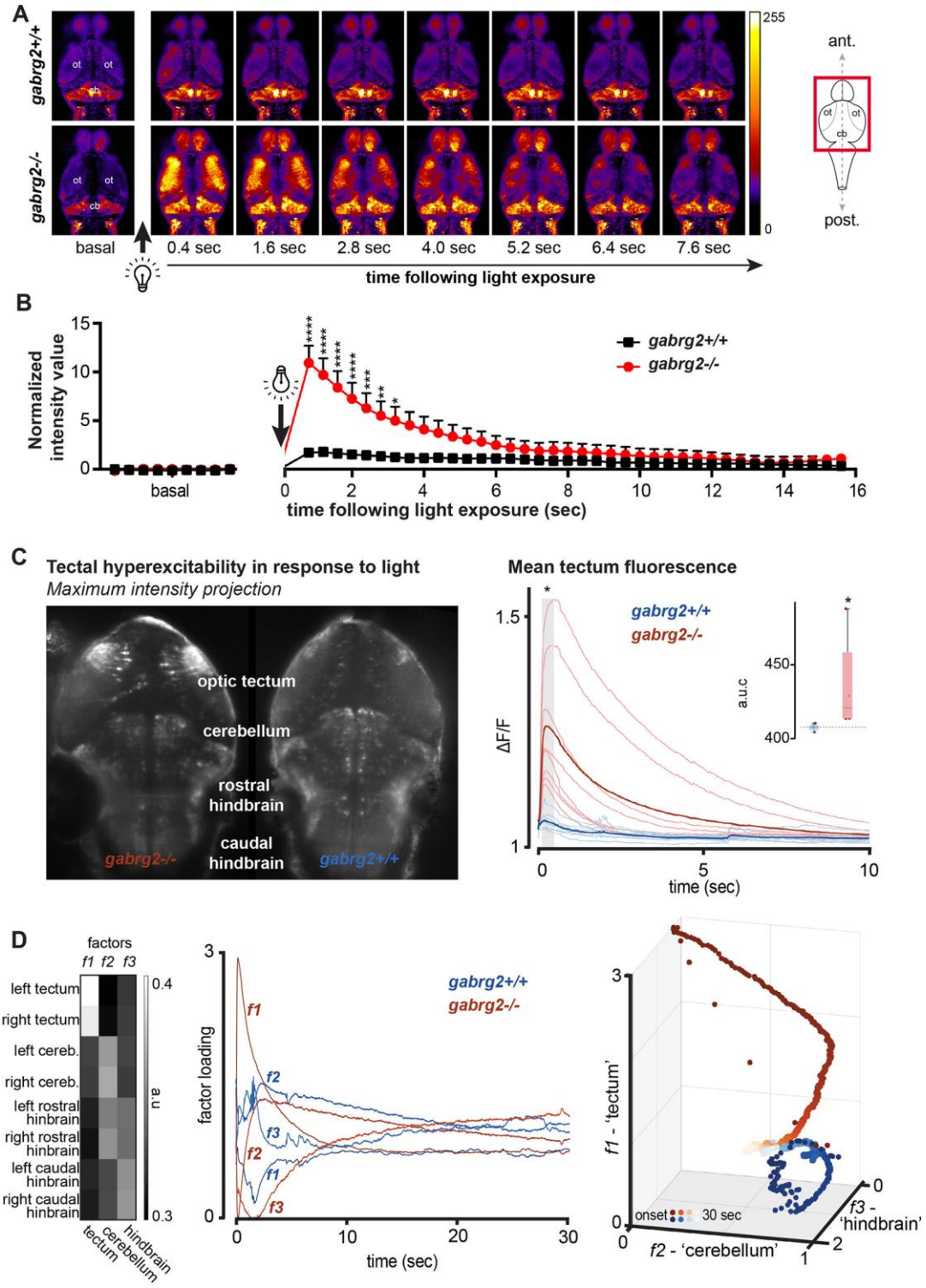


Figure 3: Calcium imaging reveals broad neuronal activity triggered by light in *gabrg2-l-* larvae. (A) *Gabrg2*+/− and [NeuroD:GCaMP6f] +/− were intercrossed, and neuronal activity was monitored under a confocal microscope (2.5 Hz) at 5 dpf. After a period of >30 minutes

in complete darkness, switching on the laser induced a broad neuronal activity in both optic tecta in *gabrg2*-/- (n = 8) compared to gabrg2+/+ siblings (n = 7). *Ot: optic tectum, cb: cerrebellum* (**** pvalue < 0.0001, *** pvalue < 0.001, ** pvalue < 0.01, * pvalue <0.05, 2-way ANOVA) (B) Fluorescence quantification in the optic tecta before and after laser exposure shows a significant increase of fluorescence in gabrg2-/- larvae compared to wild-type siblings. Each point corresponds to the relative quantification of fluorescence from a frame with 400-millisecond exposure. (C) Single plane light sheet microscopy revealed marked hyperexcitability in the tectum in *gabrg2*-/- fish. The left shows a maximum intensity projection of representative knock-out and wild type larval fish recordings. The optic tectum shows marked bilateral increase in fluorescence in the knock-out, with a much smaller response in the wild type fish. The right shows ΔF/F normalised fluorescence intensity for 10 seconds after switching on visible light, with an early difference in the average amplitude of wildtype and knock-out tectal fluorescence. Inset shows area under the curve of the fluorescence traces from onset to 10 seconds. (D) Shows a non-negative matrix factorisation of regionally averaged fluorescence traces. The factor weights show distinct response patterns for bilateral tectum, bilateral cerebellum/rostral hindbrain and bilateral hindbrain regions (left), with distinct temporal profiles of the loading of these three factors (middle plot). In three-dimensional factorised state-space, there is a clear separation of wild-type and *gabrg2*-/- responses early after being exposed to visible light.

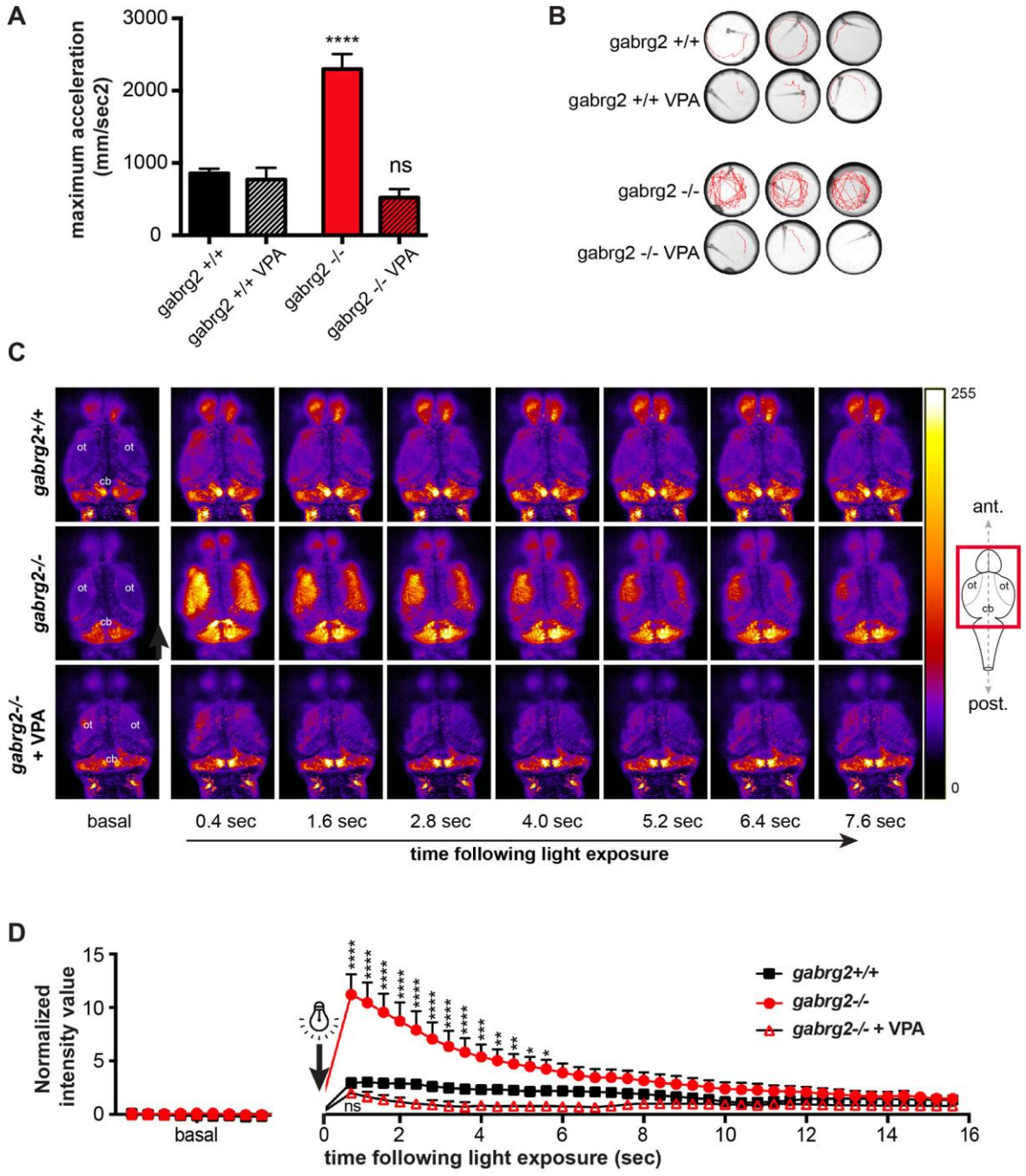


Figure 4: Valproic acid (VPA) treatment alleviates the movement phenotype and neuronal hyperexcitability of gabrg2-/- larvae. (A) Quantification of the maximum acceleration upon light stimulus before and after an acute VPA treatment of 200µM for 2 hours showing the alleviating effect of VPA. (**** pvalue < 0.0001, unpaired t-test) (B) Representative swimming tracks (15 seconds following photic stimulus) showing that VPA

exposure prevent the whirlpool behaviour of gabrg2-/- (5 dpf). (C, D) Calcium imaging and fluorescence quantification from gabrg2-KO; NeuroD:GCaMP6f larvae (5 dpf) showing that the neuronal hyperexcitability is relieved after VPA treatment (200µM for 2 hours) *Ot: optic tectum, cb: cerebellum.* (**** pvalue < 0.0001, *** pvalue < 0.001, ** pvalue < 0.01, * pvalue <0.05, 2-way ANOVA)

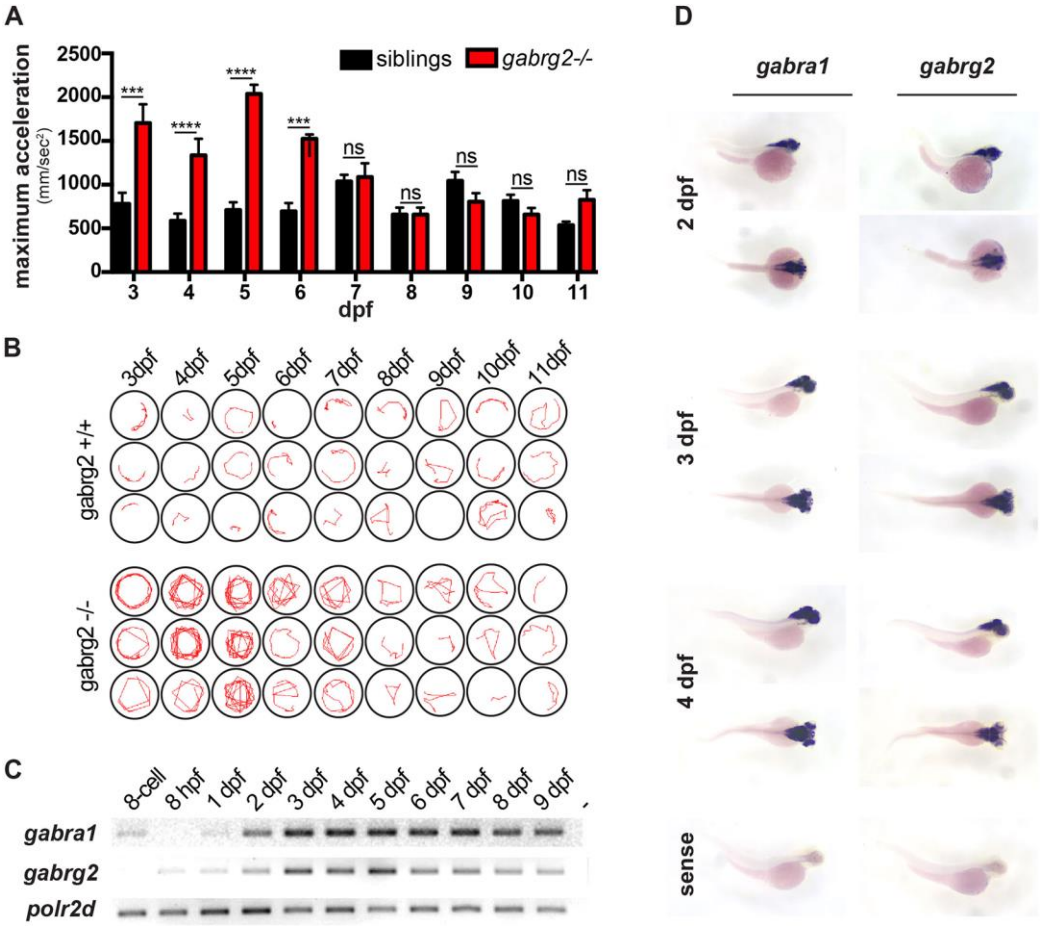


Figure 5: The photic reflex seizures of gabrg2-/- fish are transient during early larval stage. (A) Quantification of the maximum acceleration upon light stimulus of gabrg2-/- and +/+ siblings at different age (from 3 to 11 dpf). A significant difference is observed at 3, 4, 5 and 6 dpf only. (**** pvalue < 0.0001, *** pvalue < 0.001, unpaired t-test). (B) Representative swimming tracks (15 seconds following photic stimulus) of gabrg2+/+ versus -/- larvae at different age showing that the stereotypical whirlpool behaviour is not observable from 8 dpf. Of note is that the phenotype is the strongest and the more penetrant at 5 dpf. (C) RT-PCR for *gabra1* and *gabrg2* transcripts over wild-type zebrafish development. *Polrd2d* is used as a house-keeping gene. (D) Whole mount in situ hybridization on whole mount embryos using an anti-sense probe against *gabra1* or *gabrg2* transcripts. Both gene are strongly expressed in the brain and their expression is largely overlapping. (A sense probe is shown as a negative control).

Targeted knockout of GABA receptor gamma 2 subunit provokes transient light-induced reflex seizures in zebrafish larvae

SUPPLEMENTAL FIGURES

Figure S1: Protein sequence alignment between human (*Homo sapiens*) and zebrafish (*Danio rerio*) GABRG2 proteins. The coverage is 99.4% between both sequences with a percentage of identify of 82.6%. (human sequence: AAD50273.1, zebrafish sequence: NP_001243179.1).

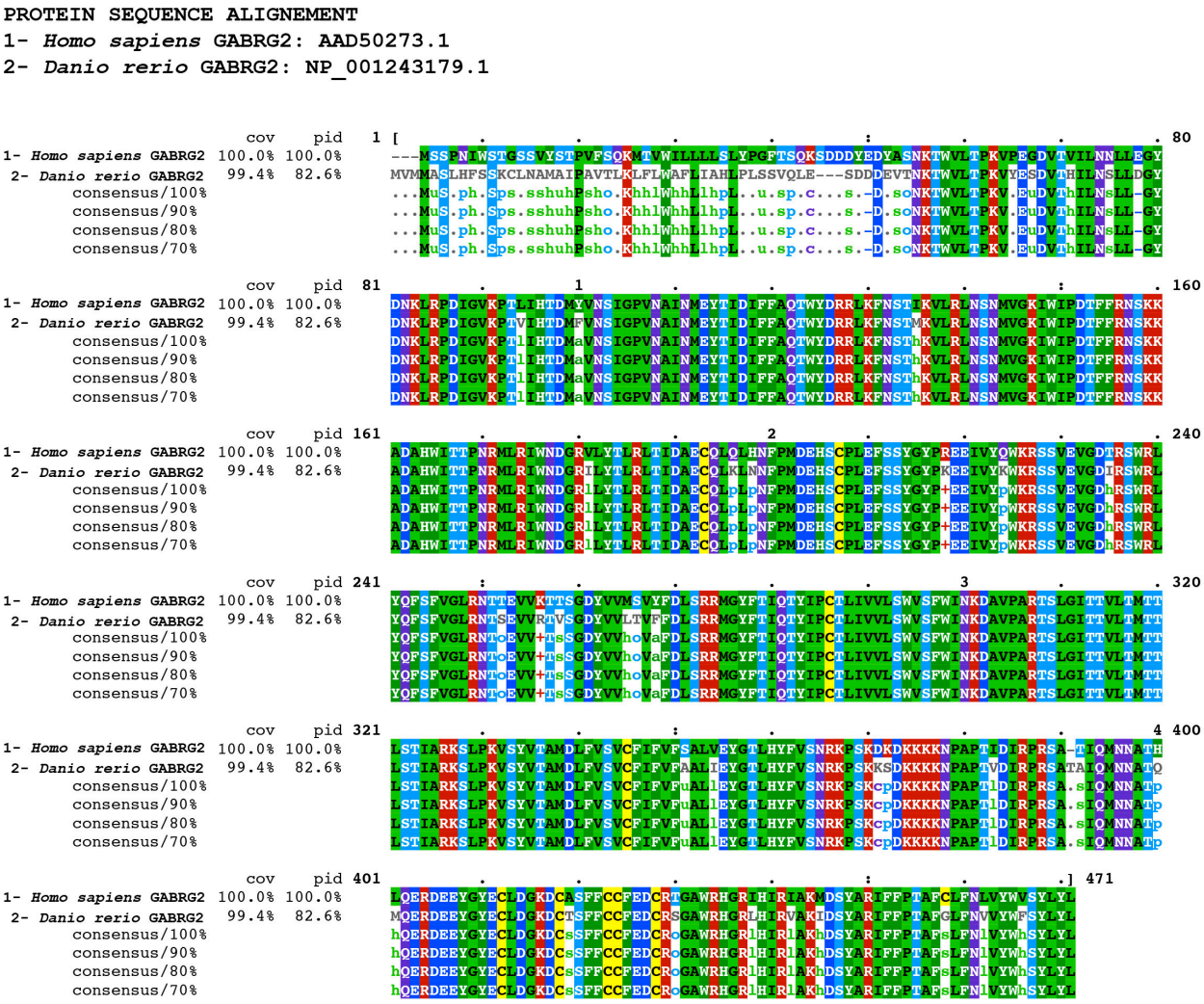
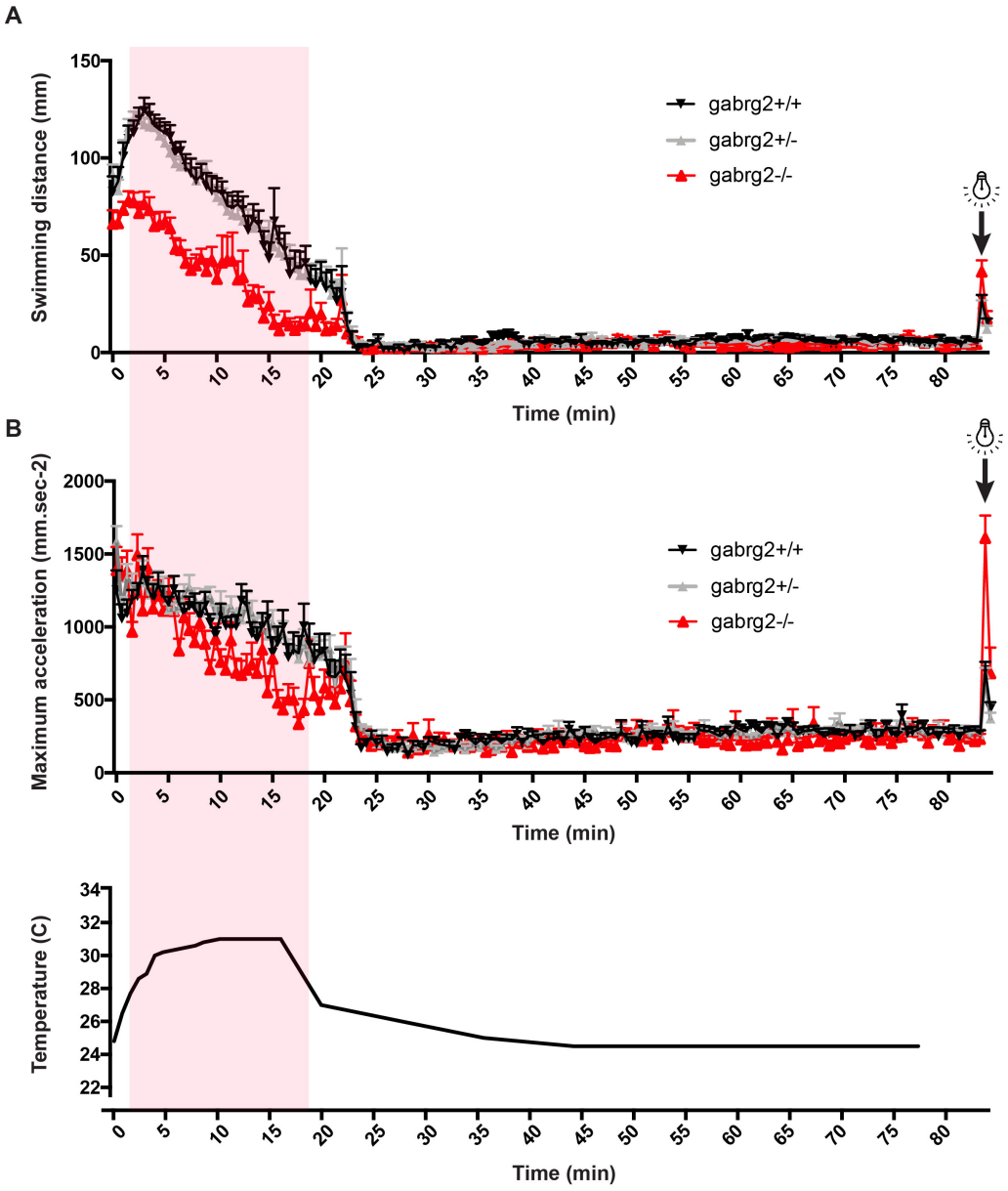


Figure S2: Temperature increase does not induce any specific behavioural changes in gabrg2-/- larvae. (A) Swimming distance and (B) maximum acceleration of 6 dpf gabrg2+/+, +/- and -/- larve have been monitored during a transient temperature increase (+7C over 15 minutes). No specific behaviour is noticed among *gabrg2*-/- population except the general hypoactivity compared to sublings described previously. In particular, we did not notice any drastic increase



of swimming activity that would be informative of seizure as it is the case with a light stimulus (arrow).

Figure S3: No difference of basal neuronal activity between gabrg2+/+ and -/- larvae. *Gabrg2*+/− and [NeuroD:GCaMP6f] +/− were intercrossed, and neuronal activity was monitored under a confocal microscope (2.5 Hz) at 5 dpf. After the neuronal hyperactivity triggered by the LASER stimulus, we recorded the basal activity for 10 minutes.

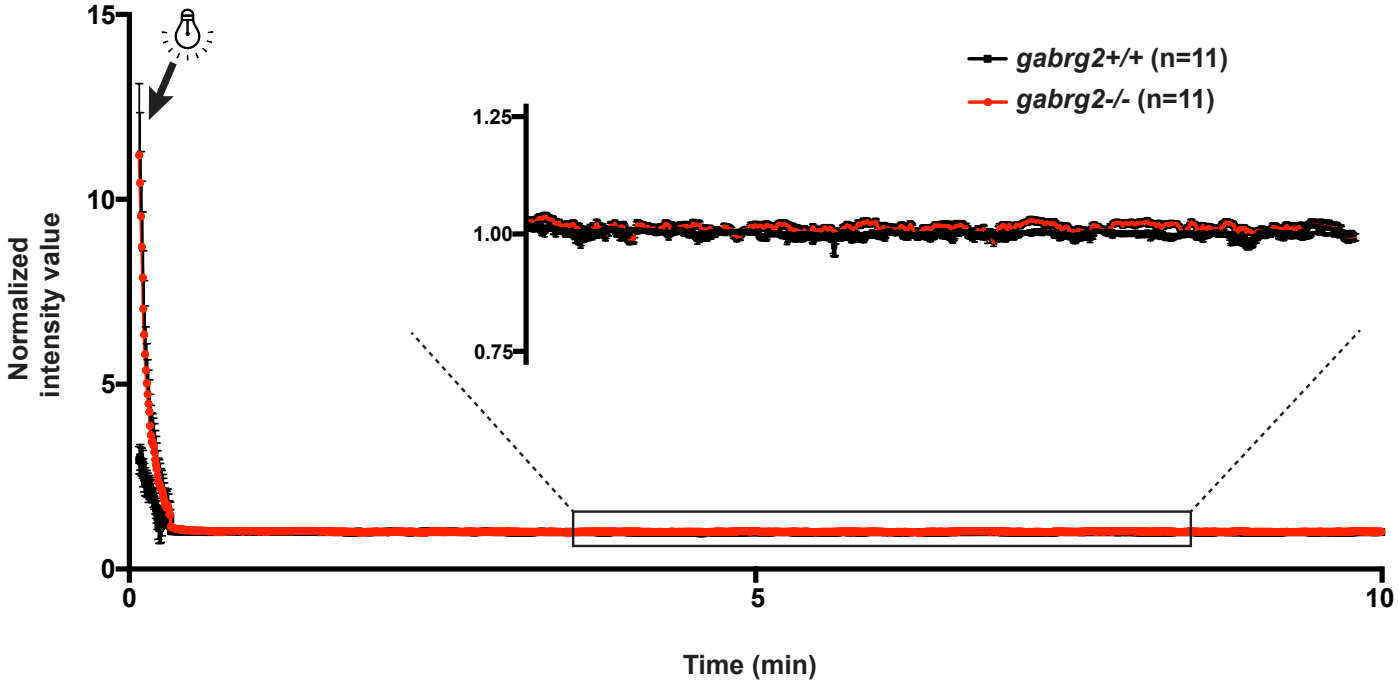
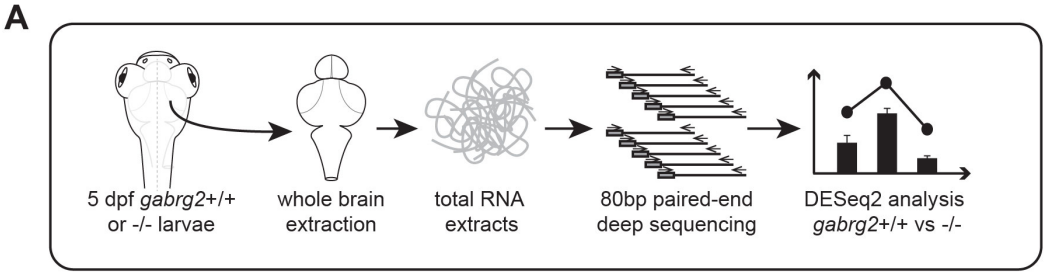
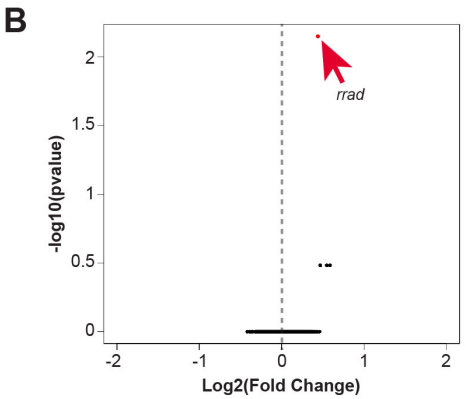
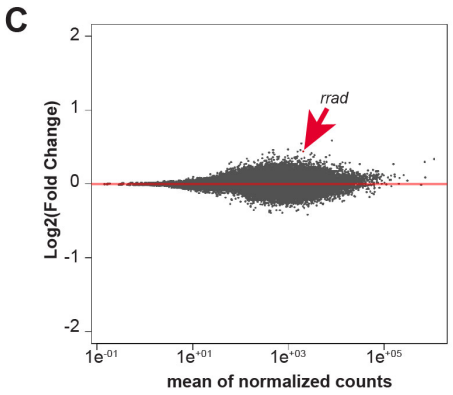
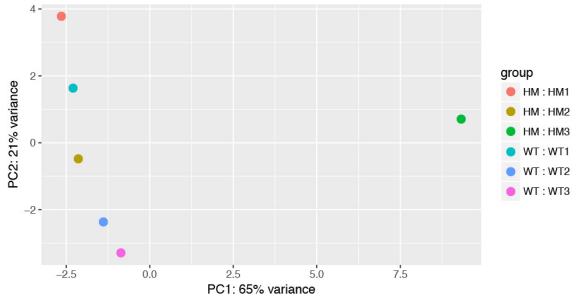


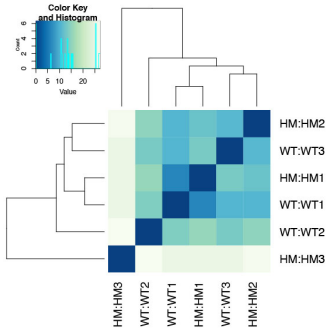
Figure S4: Whole transcriptome analysis of gabrg2-/- brains identified no major changes. (A) Experimental outline of the whole brain transcriptomic assay from 5 dpf larvae. (B) Volcano plot and MA plot (C) showing the unique significantly differentially expressed gene (*rrad:* logFoldChange 0.44, pvalue $7.09E^{-3}$, Table S1) highlighted in red (arrow). **Complementary clustering analysis of the transcriptomic data show no clustering per genotype.** (D) Principal component analysis displayed for the first two most significant components explaining the variability amon samples. (E) Hierarchy of samples based on normalized log readcounts produces the following. (F) Validation of cDNA library preparation and hit coverage of WT and mutant gabrg2 transcript in sequences samples.

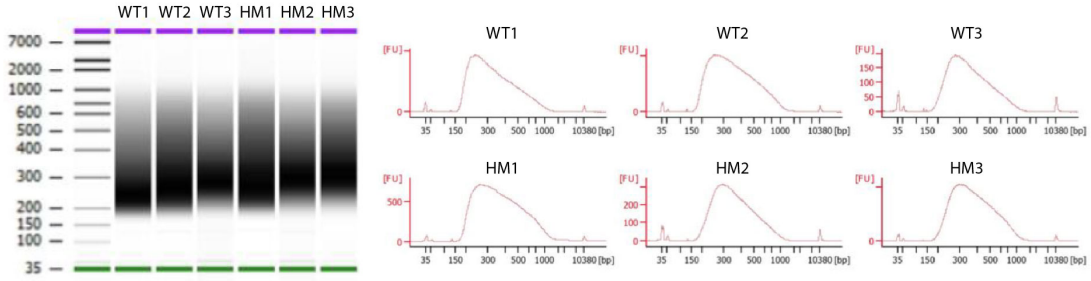












G

Alignment of exon 1 from gabrg2 gene in WT and HM samples

```
WT ATGGTGATGATGGCTTCTCTTCATTTTTCCTCTAAATGCCTGAA--C---GCGATGGCCATCCCCGCGGTGACACTCAAGCTCTTTCTGTGGGCTTTTCTAATCGCCCATCTTCCTCTGAG-------
HM ATGGTGATGATGGCTTCTCTTCATTTTTCCTCTAAATGCCTGAATGCGATGGCCATCCCCGCGGTGACACTCAAGCTCTTTCTGTGGGCTTTTCTAATCGCCCATCTTCCTCTGAG-------
```

Mutation coverage of the two variants in each samples

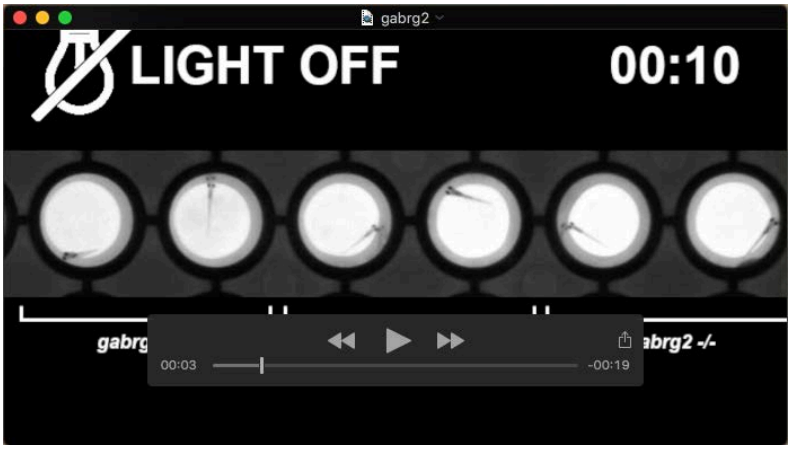
	Sample					
Variant	WT1	WT2	WT3	HM1	HM2	HM3
WT	53	87	74	5	0	0
Mutant	0	0	0	44	38	0

Table S1: Differential expression analysis from whole transcriptome sequencing.

https://www.dropbox.com/s/xq2snc594cxgt30/Table%20S1.xlsx?dl=0

Table S2: qPCR validation of GABA-related gene expression in gabrg2-/- larvae.

https://www.dropbox.com/s/x6sx5zx3li8rw6u/Table%20S2.xlsx?dl=0



Movie 1: Light-induced reflex seizures in gabrg2-/- at 6 days-post-fertilization. 6 dpf larvae were accommodated individualy in 96 wells for at least 30 minutes in a light-proof recording chamber (DanioVision, Noldus). The light was suddenly turned on (see countdown in movie).
https://www.dropbox.com/s/n6ltxskakv9pzfm/Movie%20S1.mp4?dl=0