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FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

γ-Aminobutyric acid receptor alpha 1 subunit loss of function causes genetic generalized epilepsy by impairing inhibitory network neurodevelopment

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Summary

Objective: In humans, mutations of the γ-aminobutyric acid receptor subunit 1 (*GABRA1*) cause either mild or severe generalized epilepsy. Although these epilepsy-causing mutations have been shown to disrupt the receptor activity in vitro, their in vivo consequences on brain development and activity are not known. Here, we aim at unraveling the epileptogenesis mechanisms of *GABRA1* loss of function.

Methods: We generated a *gabra1*$^{-/-}$ zebrafish mutant line displaying highly penetrant epileptic seizures. We sought to identify the underlying molecular mechanisms through unbiased whole transcriptomic assay of *gabra1*$^{-/-}$ larval brains.

Results: Interestingly, mutant fish show fully penetrant seizures at juvenile stages that accurately mimic tonic–clonic generalized seizures observed in patients. Moreover, highly penetrant seizures can be induced by light stimulation, thus providing us with the first zebrafish model in which evident epileptic seizures can be induced by nonchemical agents. Our transcriptomic assay identified misregulated genes in several pathways essential for correct brain development. More specifically, we show that the early development of the brain inhibitory network is specifically affected. Although the number of GABAergic neurons is not altered, we observed a drastic reduction in the number of inhibitory synapses and a decreased complexity of the GABAergic network. This is consistent with the disruption in expression of many genes involved in axon guidance and synapse formation.

Significance: Together with the role of GABA in neurodevelopment, our data identify a novel aspect of epileptogenesis, suggesting that the substratum of GABRA1-deficiency epilepsy is a consequence of early brain neurodevelopmental defects, in particular at the level of inhibitory network wiring.

KEYWORDS
animal model, antiepileptic screening, GABA receptor, neurodevelopment, zebrafish

1 | INTRODUCTION

In the past decades, much effort has been deployed in trying to unravel the genetic origins of idiopathic forms of epilepsy, which represent more than two-thirds of all epilepsies.[1] Among the causative genes identified in patients, mutations in several γ-aminobutyric acid (GABA) receptor subunits (alpha1, alpha6, beta2, beta3, gamma2, and delta) have been characterized in patients suffering from epilepsy.[2,3] In particular, mutations in the GABA type A receptor ($GABAR_A$) subunit alpha 1 (*GABRA1*) have been identified as causative of juvenile myoclonic epilepsies,[4] idiopathic generalized epilepsies,[3,5] and more recently, Dravet syndrome.[6] GABA is the main inhibitory neurotransmitter in the adult brain, and it acts by binding to pentameric receptors and ligand-gated chloride channels whose activation leads to hyperpolarization of neurons.[7] The identification of several epilepsy-causing mutations in genes encoding GABA receptor subunits and associated proteins was taken as direct evidence linking defective GABA inhibition with neuronal overexcitation.[4,8] However, GABA signaling has been shown also to display key regulatory functions during neurogenesis and early brain development.[9,10] At early developmental stages, due to a high intracellular chloride level, GABA is able to depolarize neuronal cells. Many studies showed that this early depolarizing effect of GABA regulates a broad range of processes from formation and maturation of neuronal circuits,[9] in particular, early GABAergic synaptogenesis.[11]

Therefore, it is likely that epilepsy-causing mutations impairing *GABRA1* function not only impact the excitatory/inhibitory balance in the brain, but may also disrupt early brain development and/or the maturation of specific neuronal networks. However, in the context of epilepsy, the effects of GABA receptor loss of function on embryo brain development and their relevance to the disease are still poorly studied in vivo. In mice, deletion of $GABA_A$ receptor α1 subunits results in the loss of half of all $GABA_A$ receptors in the brain.[12] Interestingly, the homozygous knockout (KO) mouse only displays a tremor phenotype,[13] suggesting that mammalian models only display a limited relevance to the disease and that alternative genetic models of *GABRA1*-KO are urgently needed.

Here, we generated a zebrafish model of *GABRA1* loss of function displaying fully penetrant generalized seizures at juvenile stages, which accurately mimic those observed in patients. This behavior is associated with an increase in the neuronal activity that we quantified in vivo using a calcium-dependent fluorescent reporter line. RNA-sequencing analysis of *gabra1*$^{-/-}$ larval brains identified a marked down-regulation of genes encoding inhibitory synapse components as well as proteins involved in axon guidance. Consistently, immunocytochemical analysis of *gabra1*$^{-/-}$ embryos revealed a marked decrease in the accumulation of GABA synapse markers. Altogether, our results reveal in vivo the neurodevelopmental defects that are likely to be causative factors for *GABRA1* loss-of-function epileptogenesis.

Key Points

- *Gabra1* loss of function causes epileptic seizures that can be triggered by light in zebrafish
- Seizures of *gabra1*$^{-/-}$ can be alleviated by canonical antiepileptics such as clonazepam but not carbamazepine
- *Gabra1*$^{-/-}$ embryos are suitable for high-throughput antiepileptic drug screening
- *Gabra1* loss of function causes broad changes in the larval brain transcriptome
- *Gabra1* specifically regulates the development of the inhibitory neuronal network that is impaired in *gabra1*$^{-/-}$ brains

2 | MATERIALS AND METHODS

2.1 | Fish husbandry and fish lines

Wild-type *Danio rerio* were reared at 28.5°C, kept under a 12-hour dark, 12-hour light cycle, and staged as described previously.[14] All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and conducted at the Research Center of the University of Montreal Hospital Center. The NeuroD: GCaMP6F transgenic line is a gift from Claire Wyart.[15]

2.2 | Whole mount in situ hybridization and probe cloning

A specific 854-bp *gabra1* probe was cloned within the $pCS2^+$ vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, California). Whole mount in situ hybridization of zebrafish embryos was performed as described previously.[16]

2.3 | sgRNA and Cas9 preparation and microinjection

A gRNA sequence was designed using the online tool CRISPRscan (protospacer adjacent motif site in parentheses): GCCGTTGTGGAAGAACGTGT(CGG). Synthesis of gRNA and Cas9 mRNA as well as embryo microinjection was performed as described previously.[16]

2.4 | Fish tracking and seizure triggering

A DanioVision (Noldus, Wageningen, The Netherlands) setup was used as a lightproof recording chamber with an infrared camera. 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.

2.5 | GCaMP6F monitoring and fluorescence quantification

Larvae were immobilized dorsal side up in 1% low-melting agarose and placed under a spinning disk confocal microscope. The embedded fish was kept in complete darkness for at least 30 minutes. Seizure was triggered by switching on the laser, and fluorescence was recorded on a single focal plan for 60 seconds at a rate of 2.5 images per second. 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.

2.6 | Antiepileptic drug treatment

All solutions were prepared extemporaneously as follow: 100 mmol/L stock solution in dimethylsulfoxide of carbamazepine (Teva Pharmaceutical Industries, Petah Tikva, Israel), 1.47 mol/L stock solution in water of levetiracetam (AURO, Woodbridge, Canada), 0.5 mg/mL stock solution in dimethylsulfoxide of clonazepam (Mylan, Canonsburg, Pennsylvania), and 1 mol/L stock solution in water of valproic acid (Sigma, St Louis, Missosuri). The antiepileptic drug (AED) solution was dissolved in fish water to reach the final concentration.

2.7 | Transcriptomic assay, differential expression assay, and pathway analysis

Two independent batches of 5 days postfertilization (dpf) +/+ and −/− larvae were dissected to extract the whole brains. Total RNA was extracted using a PicoPure RNA extraction kit (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's standard protocol. For each sample, RNA extraction was made from five whole brains. Quality of total RNA was assessed with a Nano bioanalyzer (Agilent Technologies, Santa Clara, California), and all samples had an RNA integrity number > 9. Library preparation was done with a TruSeq RNA Library Prep Kit (Illumina, San Diego, California) as previously described.[17] Sequencing was performed using an Illumina Hiseq 2000 as described previously.[17] Between 67 and 80 million reads were generated for each sample. About 86% of high-quality reads were mapped onto the zv9 version of the zebrafish genome (ensemble release 77) using TopHat version 2.0.10 (Johns Hopkins University, Baltimore, Maryland).

Differential gene expression analysis was assessed with the DeSeq2 package using R software. Differential gene expression was filtered on a false discovery rate > 0.05. Pathway analysis was performed using DAVID bioinformatics resources.

2.8 | Real-time quantitative polymerase chain reaction and real-time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) primers were designed using the Universal Probe Library tool from Roche (Basel, Switzerland). Reverse transcription was performed from 500 ng of total RNA using the superscript VILO RTmix (Invitrogen). qPCR was performed on 2 μL of 1:10-diluted cDNA using SYBR Green (Roche) on a LightCycler 480. The *Polr2d* gene (ENSDART00000108718) was used as a reference gene for ddCt quantification.

2.9 | Immunohistochemistry and GAD65/67 quantification

Embryos were fixed as previously described.[17] Embryos were cut into 20-μm-thick sections on cryostat, mounted on SuperFrost slides, and stored at −80°C. Immunohistochemistry was performed as previously described[17,18] with anti-GAD65/67 rabbit polyclonal antibodies (1:500, ab11070; Abcam, Cambridge, Massachusetts), antigephyrin (ab185930, rabbit polyclonal, 1:100, Abcam), or anti–acetylated-tubulin (1:1000, Sigma). Sections were analyzed using a Leica (Wetzlar, Germany) TCS SP8 confocal scanning system. Images were analyzed in Imaris 7.6.4 (Bitplane, Zurich, Switzerland). MeasurementPro's Filaments (Bitplane), which automatically detect filament-like structures, were used to outline and quantify the dendrites.

3 | RESULTS

3.1 | *Gabra1*$^{-/-}$ loss of function by CRISPR KO leads to premature death

A single copy of the *gabra1* gene can be found in the zebrafish genome (ENSDARG00000068989), and it shows >83% overall predicted amino acid identity with the human protein (Figure 1A). In particular, the GABA binding site and the transmembrane domain show 95% and

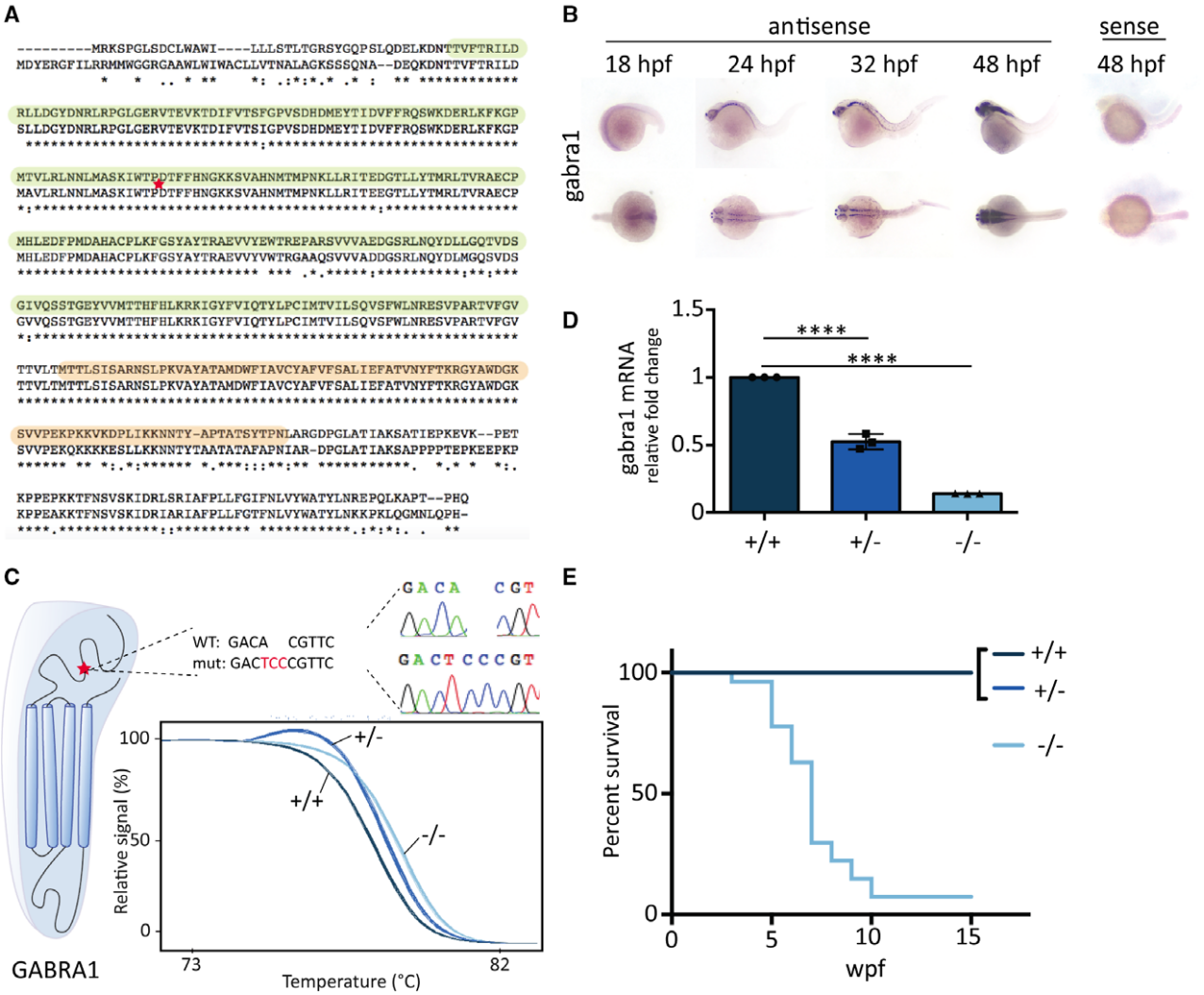

FIGURE 1 *GABRA1* knockout leads to premature death in zebrafish. A, Human (top, ENST00000393943.9) and zebrafish (ENSDART00000100000.4) GABRA1 protein sequences were aligned using Clustal W algorithm, and amino acid identity is indicated by asterisks. Colons and dots indicate conservation between amino acid of strongly or weakly similar properties, respectively. The γ-aminobutyric acid (GABA) binding and transmembrane domains are highlighted in green and orange, respectively. The CRISPR-targeted site is indicated with a red star. B, Whole mount in situ hybridization against *gabra1* mRNA showed a faint expression in the central nervous system at 18 hours postfertilization (hpf) that increased in the developing brain at 24 and 32 hpf. The expression was broad, strong, and restricted to the brain at 48 hpf. C, CRISPR mutagenesis of the *GABRA1* GABA-binding domain coding sequence led to the A>T+CC mutation (mut). The mutation was confirmed by Sanger sequencing. WT, wild-type. D, Real-time quantitative polymerase chain reaction analysis of RNAs from 5 days postfertilization larvae (in triplicate with n = 7) shows a significant decrease of *gabra1* mRNA expression in $gabra1^{+/-}$ (52.52% of WT ± 0.0327) and $gabra1^{-/-}$ (14.04% of WT ± 0.00058) larvae when compared to their WT siblings (one-way analysis of variance and Tukey multiple comparison test; $^{****}P < 0.0001$). E, The progeny of $gabra1^{+/-}$ individuals was genotyped by tail-clipping at 1 week postfertilization (wpf), raised separately according to their genotypes, and the survival of each class ($^{+/+}$, $^{+/-}$, $^{-/-}$; n = 8) was monitored every week until 15 weeks of age

88% identities, respectively, when compared to the human protein. Whole mount in situ hybridization revealed that *gabra1* transcripts accumulated in the central nervous system from 18 hours postfertilization onward, with a strong expression observed throughout the brain and spinal cord at 48 hours postfertilization (Figure 1B). These results suggest a robust evolutionary conservation of GABA receptor A function, thus supporting the pertinence of investigating *GABRA1* function in relation with epilepsy in zebrafish.

To generate zebrafish embryos with loss of *gabra1* function, we targeted the GABA binding domain with a specific guide RNA for CRISPR/CAS9 mutagenesis (Figure 1C). We selected a positive founder carrying frameshifting mutation leading to a premature stop codon at (extracellular) position 150. We confirmed the genomic

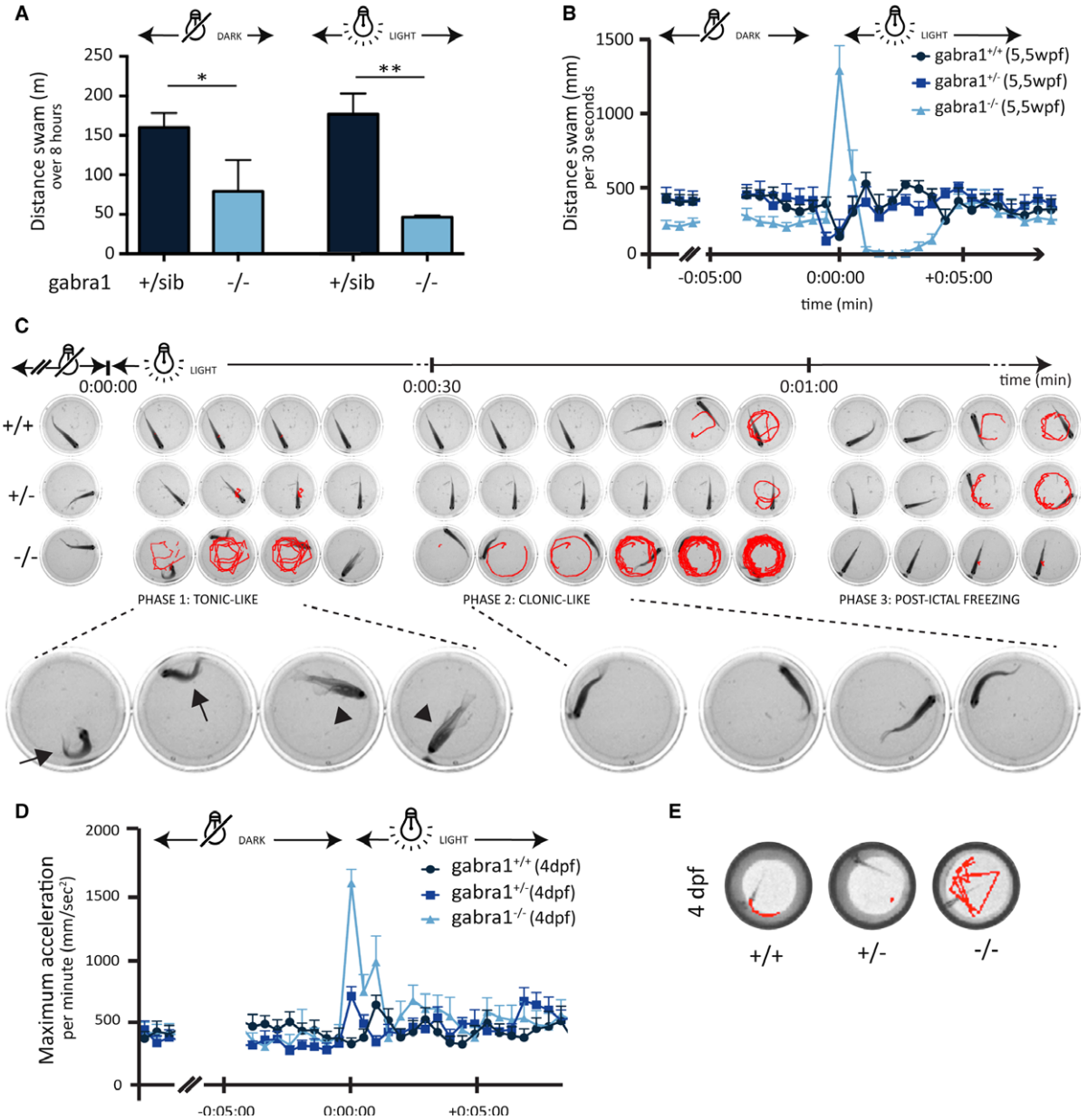

FIGURE 2 *Gabra1*$^{-/-}$ fish undergo evident generalized seizures upon light. A, Distance swam over 8 hours in the dark (left) or light (right) by 5 weeks postfertilization (wpf) *gabra1*$^{+/-}$ or $^{-/-}$ juveniles revealed significant hypoactivity of *gabra1*$^{-/-}$ compared to their heterozygous siblings (sib) in both light (*t* test, $P < 0.03$) and dark cycles (*t* test, $P < 0.003$). B, Measurements of the distance swam per 30-second period after light was turned on revealed a strong increase of 5.5 wpf *gabra1*$^{-/-}$ motility just after light was turned on and followed by a period of immobility. C, Video frames of juvenile (5-6 wpf) *gabra1*$^{+/+}$ (top), $^{+/-}$ (middle), and $^{-/-}$ (bottom) siblings upon light exposure. Immediately after light, *gabra1*$^{-/-}$ fish underwent a first tonic-like phase characterized by arching of the body (arrows), uncontrolled twitching (as seen with the red tracks), and loss of posture (arrowheads). After this first phase, which lasted a few seconds, *gabra1*$^{-/-}$ fish underwent a second, clonic-like phase, during which they underwent rapid and uncontrolled movements, fast muscle contractions, and whole-body convulsions leading to a whirlpool swimming pattern (tracks in red). Lastly, after about 1 minute of seizure, they entered a third, postictal phase, during which they stayed immobile and breathed heavily for 3-10 minutes. D, E, Maximum acceleration upon light of 4 days postfertilization (dpf) *gabra1* embryos (n = 96) showing an increased startle response to light of *gabra1*$^{-/-}$ specifically. Tracks of 3 seconds following light exposure show a stereotyped pattern of $^{+/+}$ (left), $^{+/-}$ (middle), and $^{-/-}$ (right) light response

sequence by Sanger sequencing and high-resolution melting assay (Figure 1C). Using real-time qPCR, we observed that *gabra1* transcript accumulation was reduced by almost 50% in *gabra1*$^{+/-}$ embryos and by >85% in homozygotes, the likely result of nonsense-mediated mRNA decay. We therefore predicted the mutation to lead to full loss of *GABRA1*

function in homozygotes. Although *gabra1*$^{-/-}$ embryos developed and grew normally like their wild-type siblings, almost all homozygotes died prematurely between 7 and 10 weeks postfertilization (wpf; Figure 1E).

3.2 | *Gabra1*$^{-/-}$ juvenile fish exhibit seizures upon light exposure

Because *GABRA1* mutations have been reported to induce juvenile myoclonic epilepsy,[3,4] we monitored the swimming behavior of 5 wpf juvenile fish during an 8-hour dark, 8-hour light cycle, using an automated recording chamber. Interestingly, we noticed that *gabra1*$^{-/-}$ larvae were hypoactive (Figure 2A), but more importantly, we noticed a drastic increase in the distance swam by *gabra1*$^{-/-}$ juveniles at the exact time the light was switched on, followed by a period of complete inactivity during the next few minutes (Figure 1B). Careful observation indicated that all *gabra1*$^{-/-}$ juveniles underwent intense seizures in the seconds immediately after the lights were turned on (Figure 2C and Video S1). Interestingly, the seizures were characterized by an initial phase with a series of short but intense convulsions, which were followed a brief collapse, a loss of swimming posture, and repetitive jerks of the jaws. Then, in the following minute, the fish underwent a second phase with rapid uncontrolled movements, fast muscle contractions, and whole-body convulsions, leading to rapid and intense swimming. Specifically, we observed "whirlpool" behavior, with fish swimming rapidly in circles, a pattern reminiscent of what is observed following treatment with proconvulsant drugs such as pentylenetetrazol.[19] We also witnessed seizures triggered by light in larger tanks in which mutant fish are swimming uncontrollably throughout the water tank (Video S2). After this two-step seizure that is evocative of human tonic/clonic episodes, *gabra1*$^{-/-}$ fish entered a third phase of freezing during which they exhibited heavy gill breathing and complete immobility for a few minutes.

As fully penetrant seizures were observed from 5 wpf onward, we sought to determine the earliest stage at which *gabra1*$^{-/-}$ zebrafish show epileptic features. Therefore, we analyzed the response to light and swimming behavior of embryos and found that *gabra1*$^{-/-}$ zebrafish larvae displayed increased swimming activity in response to light as early as 4 dpf (Figure 2D and 2E). Before that age, +/+ and −/− embryos depict the same normal level of light-induced startle response (asterisks in Figure S1).

To confirm that the phenotype observed does not correspond only to a hypermotility but rather to a genuine epileptic behavior, we monitored the temporal and regional neuronal activity following light exposure in *gabra1*$^{+/+}$ versus *gabra1*$^{-/-}$ larvae (Figure 3A and 3B). To do so, we crossed our *gabra1* mutant line with the NeuroD:GCaMP6f transgenic line that expressed a calcium-dependent fluorescent protein in the neuronal population.[15,20] We triggered the seizure by switching on the laser after incubating the larvae for at least 30 minutes in complete darkness, and we observed an immediate burst of neuronal activity in both optic tecta of −/− larvae. This activity is generalized throughout the optic tecta and is intense compared to the slight neuronal activity observed in siblings in response to the laser light (Figure 3A). Interestingly, exposure to valproic acid (VPA) rescues the *gabra1*$^{-/-}$ light-induced neuronal activity to the wild-type level (Figure 3A). Quantification of the fluorescent signal from the optic tecta showed a significant increase in the neuronal activity of −/− larvae immediately after laser exposure and lasting for a few seconds (Figure 3B). Such a generalized and synchronized burst of neuronal activity in both optic tecta is never observed in sibling larvae even after light exposure, and it is fully rescued with exposure to a potent antiepileptic agent (VPA). Thus, it confirms that the observed phenotype in *gabra1*$^{-/-}$ larvae is associated with intense and generalized neuronal activity.

Until 2 to 3 wpf, *gabra1*$^{-/-}$ larvae only underwent tonic-like seizures in response to light, without the subsequent clonic-like whirlpool behavior. However, even at these early stages, the seizures in response to light stimulus were evident (Video S3). Interestingly, we noticed that the penetrance of the whirlpool phenotype increased with age, with almost all embryos showing whirlpool swimming from 6 wpf onward (Figure 3C). Although we did not notice any specific sensitivity of *gabra1* mutants to sound, temperature, or sleep deprivation, we noticed an increase in spontaneous generalized seizures upon stressful events (eg, tank cleaning and introduction of a net into the tank) in fish older than 7 wpf. This suggests that rather than being specifically triggered by light, *gabra1*$^{-/-}$ epileptic seizures can be provoked with specific starling stimuli (including light). Lastly, we witnessed death following generalized seizures in *gabra1*$^{-/-}$ juveniles from 4 wpf onward, with a predominant occurrence between 6 and 7 wpf (Figure 3D, Video S4). Occurring immediately after a seizure, this is evocative of sudden unexpected death in epilepsy and is consistent with the premature death observed in the homozygous population from 6 wpf (Figure 1E).

3.3 | Differential attenuation of seizures by known AEDs

We then tested the ability of known AEDs to rescue the seizures. Following a 1-day incubation with different AEDs, we triggered a seizure with light and monitored both tonic-like phase (by measuring the motion tracks of the startle response immediately upon light exposure, left panels) and clonic-like phase (by quantifying the distance swam upon light exposure, graphs on right panels;

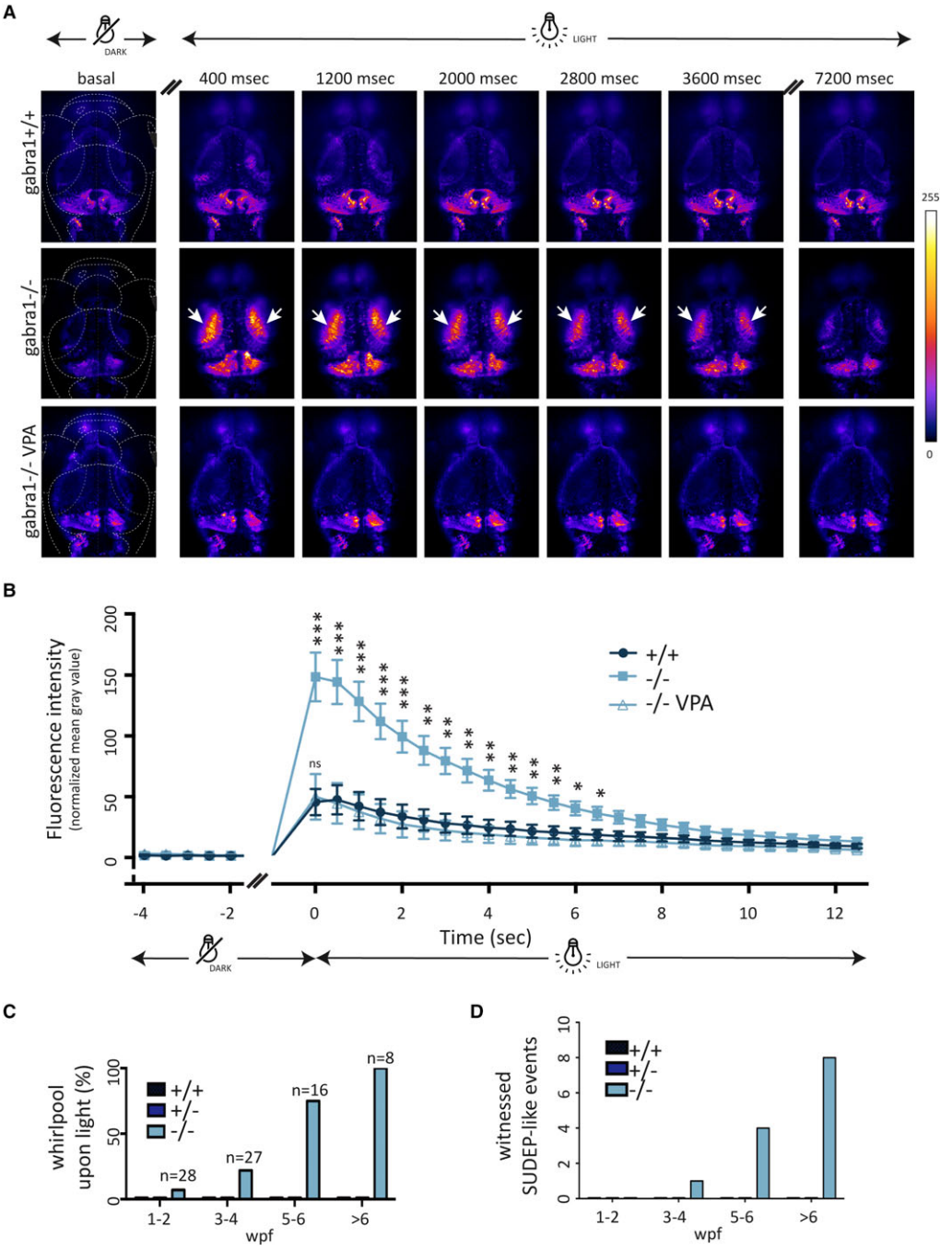

FIGURE 3 Generalized neuronal activity in *gabra1*$^{-/-}$ following light exposure and sudden death following generalized seizures. A, *Gabra1*$^{+/-}$ and NeuroD:GCaMP6f +/− were intercrossed, and neuronal activity was monitored under a confocal microscope at 8 days postfertilization. After a period of 30 minutes in complete darkness, switching on the laser induced a broad neuronal activity in both optic tecta in *gabra1*$^{-/-}$ (n = 12) compared to *gabra1*$^{+/+}$ siblings (n = 10) that lasted for few seconds (arrows). The intense neuronal activity of −/− larvae was rescued to wild-type (WT) level after exposure to valproic acid (VPA; 50 μmol/L, n = 7). B, Fluorescence quantification in the optic tecta before and after laser exposure shows a significant increase of fluorescence in −/− larvae compared to +/+ siblings. Each point corresponds to the relative quantification of fluorescence from a frame with 400-millisecond exposure. VPA treatment fully rescues the fluorescence back to the WT level (Student *t* test: **P* < 0.05, ** *P* < 0.01, ****P* < 0.005). C, The whirlpool onset (ie, clonic-like phase) increased with age, with a full penetrance at 6 weeks postfertilization (wpf) onward. D, Sudden unexpected death in epilepsy (SUDEP) events were quantified and occurred from 4 wpf onward only in *gabra1*$^{-/-}$ mutants. Of note is that data shown here correspond to the actual SUDEP events witnessed, but it is very likely that it occurs in many more fish

Figure 4). We found that VPA and clonazepam completely abolished both phases of the seizures (Figure 4A and 4B, left panels). Interestingly, calcium imaging showed that VPA exposure rescues brain activity back to the same level as wild type (Figure 3B), thus indicating that the positive effect of VPA is not due to oversedation. Interestingly, we found that levetiracetam and carbamazepine had a mild effect on the first phase of the seizure (as shown by seizure tracks, left panels in Figure 4A-D) but only rescued the clonic-like phase, suggesting that their effect on *gabra1*$^{-/-}$ generalized seizure is milder (Figure 4C and 4D, right panels). This mild effect of carbamazepine is consistent with previous studies in an *scn1a* mutant zebrafish model.[21]

We next examined whether larvae, rather than juvenile fish, could be used for drug screening so that more biological replicates could be used in a shorter time. Thus, we tested the effects of the same four AEDs on young larvae. Interestingly, overnight treatment with VPA, clonazepam, or levetiracetam fully abolished this phenotype, as shown by either quantification of acceleration (Figure 4E) or visual inspection of the tracking records (Figure 4F). In contrast, carbamazepine treatment was unable to fully alleviate the swimming behavior of *gabra*$^{-/-}$ larvae, although the acceleration values were significantly reduced. These results accurately matched those observed with juvenile fish (compare Figure 4A-D to Figure 4E and 4F), thus demonstrating that the *gabra1* line is a convenient tool for AED screening as early as 4 dpf.

3.4 | *Gabra1* loss of function does not modify brain structure but alters developmental brain transcriptome

Because *gabra1*$^{-/-}$ mutants display highly penetrant epileptic seizures, we investigated whether they could depict a defect in brain structure and/or neuronal content that may explain their hyperexcitability. To do so, we checked the main brain neuronal fibers by immunolabeling using an

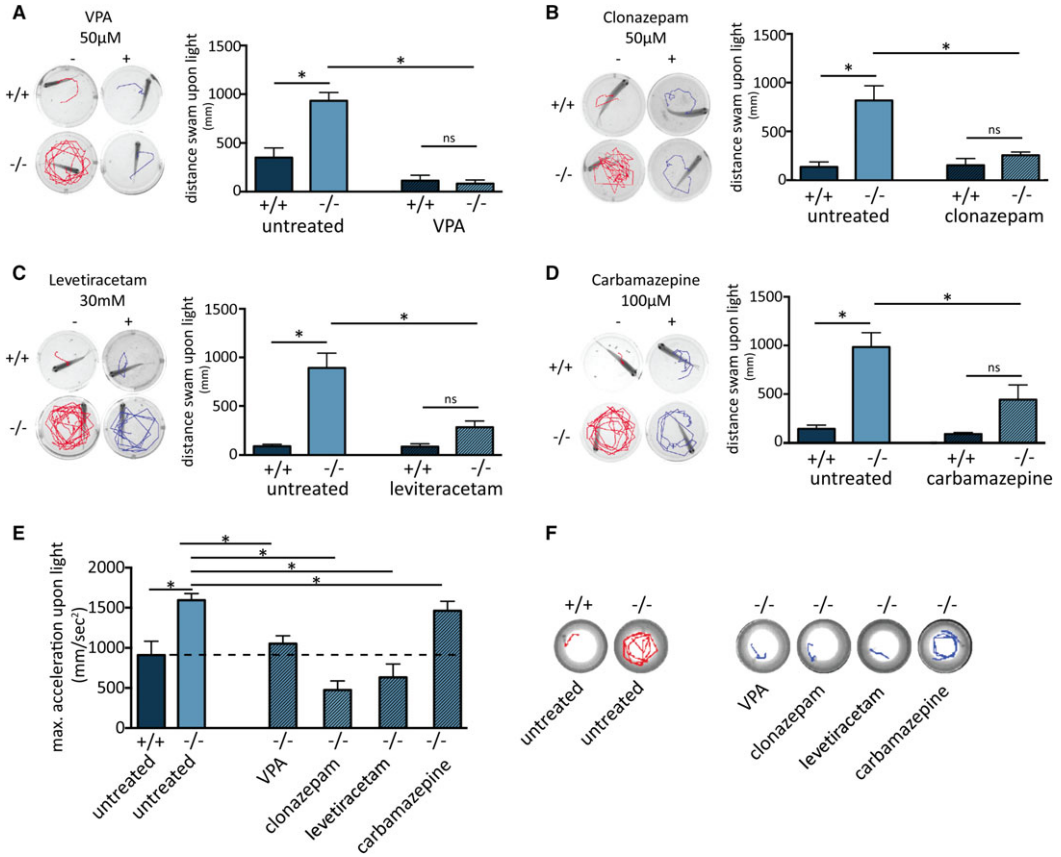

FIGURE 4 Differential attenuation of seizures by known antiepileptic drugs (AEDs). Five to 6 weeks postfertilization wild-type ($^{+/+}$, n = 5) or homozygous ($^{-/-}$, n = 5) fish were incubated overnight with valproic acid (VPA; A), clonazepam (B), levetiracetam (C), or carbamazepine (D). Both steps of the light-triggered seizure were analyzed before and after the treatment using the track of single fish for 3 seconds following light being switched on (left panels), as well as by quantifying the distance swam during the minute following light being turned on (right panels). E, The increased acceleration of 4 days postfertilization (dpf) *gabra1*$^{-/-}$ embryos was differentially alleviated by the four tested AEDs (n = 22 per assay). F, Stereotyped tracks of 4 dpf untreated *gabra1*$^{-/-}$ embryos and mutant embryos treated with the four AEDs. All drug treatments were performed overnight with a final concentration of either 50 µmol/L VPA, 50 µmol/L clonazepam, 30 mmol/L levetiracetam, or 100 µmol/L carbamazepine. ns, not significant. *P value <0.05

antibody against acetylated tubulin (Figure 5A). No major loss of neuron fibers was observable even at closer zooms in the optic tectum, which is a highly innervated region, especially by inhibitory interneurons (right panel in Figure 4A). Moreover, we noticed no major difference in the brain structure as revealed by cresyl violet staining (Figure 5B, upper panel) and no specific change in the neuronal content after immunolabeling with anti-HuC antibody (Figure 5B, lower panel). These results indicate that the main brain structures and neuronal population are not obviously affected by *GABRA1* loss of function. However, in an attempt to determine molecular defects, we dissected whole brains from *gabra1*$^{-/-}$ embryos and their wild-type siblings and extracted total RNA for deep sequencing (Figure 5C). Interestingly, transcriptome analysis identified 460 genes that were either up-regulated (251 genes) or down-regulated (209 genes) in *gabra1*$^{-/-}$ embryos when compared to their wild-type siblings (Figure 5C-E, Table S1). The down- and up-regulation of some genes of interest were confirmed by real-time qPCR analyses (Figure S3). By performing gene clustering and pathway analysis, and also using DAVID,[22] we were able to identify the following clusters: ribosomal subunits (65 genes), embryo development (21 genes), neurogenesis (50 genes), synapse function/activity (36 genes), GABA network (10 genes), and cell-cell interaction (15 genes). Finally, we found many genes already related to epilepsy (23 genes) or other neurological disorders (39 genes; Figure 5D and Tables S1 and S2).

In particular, we found many genes involved in axon guidance to be misregulated in *gabra1*$^{-/-}$ brains (Figure S4A, Table S2). Moreover, the expression of many genes involved in synaptic vesicle docking, priming, and endocytosis was altered in mutant brains (Figures S4B and 5). Altogether, these results strongly suggest that *GABRA1* loss of function induces transcriptional defects in a large number of genes involved in early brain development.

More specifically, our transcriptomic analysis pointed to a specific down-regulation of many genes involved in GABAergic synapse function (Figure S4D). Specifically, 3 GABA receptor subunits (besides GABRA1) were significantly down-regulated in *gabra1*$^{-/-}$ mutants. Also, neurexophilin 2a (*nxph2a*) and neuroligins 2 and 4 (*nlgn2*, *nlgn4*) were down-regulated in mutant brains and these genes have been shown to be important inducers and maintainers of GABA synapses.[23,24] Moreover, we noticed a down-regulation of the motor kinesin 5 (*KIF5B*) as well as its associated kinesin light chain 1a (*KLC1A*), both involved in the trafficking of $GABA_A$ receptors at the postsynaptic membrane.[25,26] Our dataset also showed that the *slc6a11b* gene encoding GABA transporter 3 (*GAT3*), which is expressed in glial cells surrounding GABA synapses, was also down-regulated. Altogether, these results show that *gabra1* loss of function induced specific transcriptome down-regulation in components of synaptic GABA signaling in the larval brain.

3.5 | *Gabra1*$^{-/-}$ embryos exhibit markedly decreased inhibitory synapses throughout the brain

Although we showed previously that the brain structure is not altered by *GABRA1* loss of function (Figure 5A and 5B), we sought for subtler consequences, specifically in the inhibitory neuronal network as our transcriptomic data suggested. Thus, we crossed the *gabra1* line with the dlx5/6:GFP transgenic line[27] in which GABAergic interneurons are fluorescently labeled in the brain. The distribution and number of the GABAergic cell population are similar when comparing *gabra1*$^{-/-}$ and sibling larval brains (Figure 6A and 6B). However, because our transcriptomic data specifically identified members of the inhibitory synapses (Figure S4D), we analyzed the accumulation of glutamate decarboxylase (GAD65/67), the enzymes involved in GABA synthesis in presynaptic buttons of inhibitory synapses, using an anti-GAD65/67 antibody. Immunostaining of larval brain sections from *gabra1*$^{+/+}$ and *gabra1*$^{-/-}$ embryos showed that accumulation of GAD65/67 proteins was markedly decreased in mutants (Figure 6C-G). Interestingly, this reduction was seen throughout the brain, including the telencephalon, optic tectum, hindbrain, and anterior spinal cord. Quantification of GAD65/67-labeled neurofilaments showed a decrease of >50% in −/− larval brains compared to +/+ siblings (Figure 6G). Upon performing three-dimensional reconstructions of representative GAD65/67^{+} punctae (Figure 6H, Videos S5 and S6), we observed that the number of GAD65/67-containing structures was markedly reduced in *gabra1*$^{-/-}$ brains, suggesting a reduction in GABAergic presynaptic signaling. To confirm this observation, we investigated the expression of gephyrin, another marker of inhibitory synapses, as it anchors postsynaptic GABA receptors to the cytoskeleton.[28] Consistently, we noticed a decrease in the number of gephyrin-positive clusters in *gabra1*$^{-/-}$ brains compared to wild-type siblings (Figure 6I).

Altogether, these results indicate that, although *GABRA1* loss of function does not affect the overall development of the inhibitory cell population, GABRA1 is required for the formation of GABAergic synaptic buttons as well as for the establishment of the complex branching of inhibitory synaptic network throughout the brain.

4 | DISCUSSION

Here, we showed in vivo evidence that *GABRA1* loss of function impacts neurodevelopment, specifically inhibitory

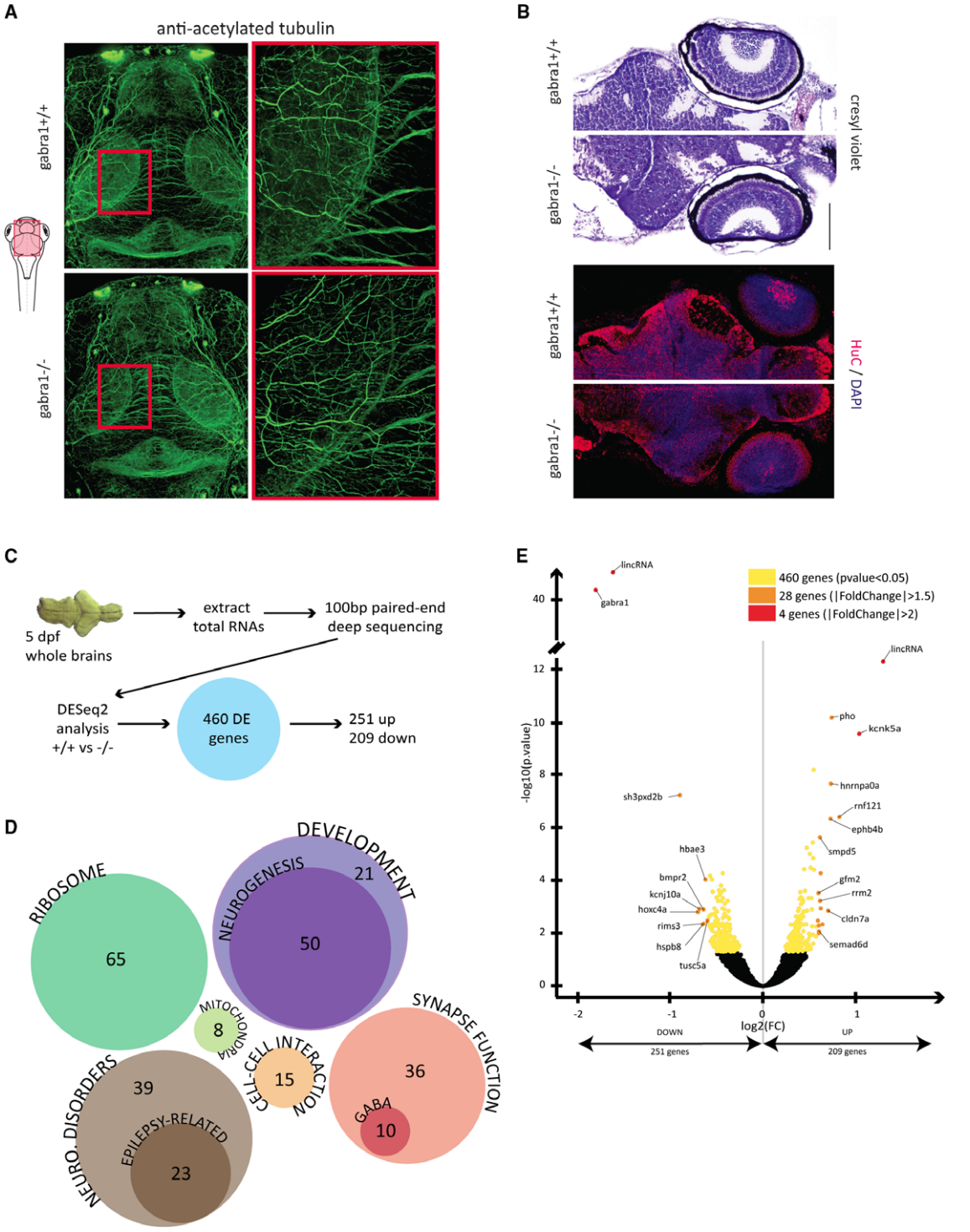

FIGURE 5 Whole transcriptome deep sequencing revealed 460 genes differentially expressed in *gabra1*$^{-/-}$ brains, although brain structure is not altered. A, Immunolabeling of 4 days postfertilization (dpf) whole *gabra1* embryos using an antiacetylated tubulin recognizing neuronal fibers. B, Longitudinal slices of 7 dpf *gabra1* larvae stained with cresyl violet (top) or immunostained against HuC (bottom), showing no major difference between mutants and siblings. DAPI, 4,6-diamidino-2-phenylindole. C, Whole brains from 5 dpf embryos were dissected, and total RNAs were extracted for subsequent deep sequencing. Using DESeq2, we identified 460 genes whose expression was significantly altered in homozygous brains. DE, differential expression. D, Gene clustering analysis allowed the classification of a list of genes according to their function. The full list is provided in Table S2. GABA, γ-aminobutyric acid. E, Volcano plot showing each individual gene plotted according to its log2 fold change (FC; x-axis) and the −log10 *P* value (y-axis). All differentially expressed genes with $P < 0.05$ are highlighted in yellow. Those whose absolute fold changes were >1.5 or >2 are shown in orange and red, respectively. A full list of genes is provided in Table S1

synaptic network wiring during embryogenesis. Thus, we confirm that this neurodevelopmental aspect of the disease is a main component of the epileptogenesis process and should be considered for further therapeutic strategies.

Interestingly, our unbiased transcriptomic analysis revealed gene families whose expression was altered in *gabra1*$^{-/-}$ brains. The main results from this analysis revealed that several key genes involved in axon guidance were misregulated in *gabra1*$^{-/-}$ brains. In particular, genes encoding protocadherins, ephrin/ephrin receptors, and semaphorins were either up- or down-regulated in mutant brains, suggesting that axon guidance and/or axonal branching as well as neural connectivity are subtly affected in mutants. This could lead to impaired synapse formation and function, as suggested by changes in the expression of several genes involved in synaptic vesicle trafficking (Figure S4B). Consistently, we observed that GABAergic projections were less arborized in mutant brains than in those of wild-type embryos, supporting possible axon guidance alterations. Interestingly, these changes in inhibitory synaptic connectivity seem to be independent from the development of the GABAergic neuron population, because the number of those cells is unchanged in *gabra1*$^{-/-}$ brains. Thus, a likely scenario is that *GABRA1* function is specifically required for the establishment of inhibitory synapses in already differentiated GABAergic neurons. More specifically, functional *GABRA1*-dependent GABA signaling may be essential during neurodevelopment for the correct temporal and regional expression of specific axon guidance cues such as semaphorins, plexins, and ephrins. Interestingly, semaphorins, ephrins, and plexins have already been shown to be essential players for the correct establishment of specific synapses throughout neurodevelopment but also postnatally. Moreover, mutations in some of these genes are known to cause epilepsy.[29–33] However, future work is needed to investigate the exact genetic and molecular mechanisms linking *GABRA1*-KO to the reduction of GABAergic synaptic connections.

Consistently, GABA signaling has already been shown to regulate GABAergic synaptogenesis during neurodevelopment. Different studies have shown that the integrity of GABA signaling is necessary for proper regulation of synaptogenesis during neurodevelopment.[34] In particular, postsynaptic GABAA receptors are able to initiate the formation and the maintenance of functional GABAergic synapses.[35] Because the alpha 1 subunit is known to be a major subunit composing the synaptic pull of GABA receptors, *GABRA1* loss of function is likely to disturb GABAergic synaptogenesis, which is consistent with our findings. Moreover, Oh et al[36] showed that exogenous local puffs of GABA in the developing mouse cortex are able to generate de novo GABAergic synaptogenesis, supporting the positive effect of GABA for the development of inhibitory synapses. Altogether, our in vivo results are consistent with previous work and connect the role of GABA in neurodevelopment with epilepsy pathogenicity.

Surprisingly, our transcriptomic assay also showed that the expression of many epilepsy-related genes is shifted to proepileptic levels (either over- or down-regulated) in *gabra1*$^{-/-}$ brains (Figure S4). In other words, genes for which loss of function causes epilepsy in patients (*dnm1a,*[37] *cntn2,*[38] *gabab1b*/*gabab2,*[2,39] *gabrg2,*[2,40] *kcc2* [*slc12a5b*],[41] *atp2b3,*[42] *kcnc1,*[43] *kcnj10,*[44] *gat3* [*slc6a11*][45] and *kif5*[46]) were significantly down-regulated in our dataset, whereas *cacna2d2a,* a gain of function of which causes epilepsy,[47] was up-regulated in *gabra1*$^{-/-}$ brains. Thus, these results show in vivo that, notwithstanding the down-regulation of *gabra1* itself, loss of *GABRA1* function significantly modifies the expression of a broad set of proepilepsy-related genes.

In summary, our data confirm in vivo the evidence for a developmental aspect of epileptogenesis. Interestingly, the neurodevelopmental defects of *GABRA1*-related epilepsy are an important aspect for the design of new treatment for epileptic patients. We propose that therapeutic strategies should not only aim at counterbalancing the activity of the mutant channel, but should be developed in combination with approaches to restore or at least compensate the developmental neurogenic defects we describe here. More research needs to be done to understand the exact neurodevelopmental abnormalities causing epilepsy and to restore them as early as possible rather than treat it symptomatically at later stages.

At this juncture, our *gabra1* mutant line is an advantageous model for future studies of epileptogenesis. Although several genetic models of epilepsy have already been generated in zebrafish, the epileptic phenotype is rarely as evident as it is in *gabra1*$^{-/-}$ fish described here. The seizures described in this study were evident in all of the *gabra1*$^{-/-}$ juvenile fish examined and could be triggered by light, a fully physiological and otherwise harmless stimulus. Interestingly, although we did not notice an effect of stroboscopic light exposure (6-60 Hz), future work could investigate in more detail the effect of conventional photic stimulation on seizure incidence in our model. We also have optimized our *gabra1*-KO mutant line as a powerful model to eventually perform large drug screens. AED efficacy can first be assessed in a standardized and high-throughput fashion with *gabra1*$^{-/-}$ larvae (4 dpf) and later confirmed with juvenile fish (5-6 wpf) that depict obvious generalized seizures, a funnel strategy that should help to narrow down positive hits more quickly and efficiently. As genetic generalized epilepsies account for about 15%-20% of all epilepsies,[1] our model should be useful in the future to screen new candidate antiepileptic molecules that are particularly needed in the context of refractory epilepsies.

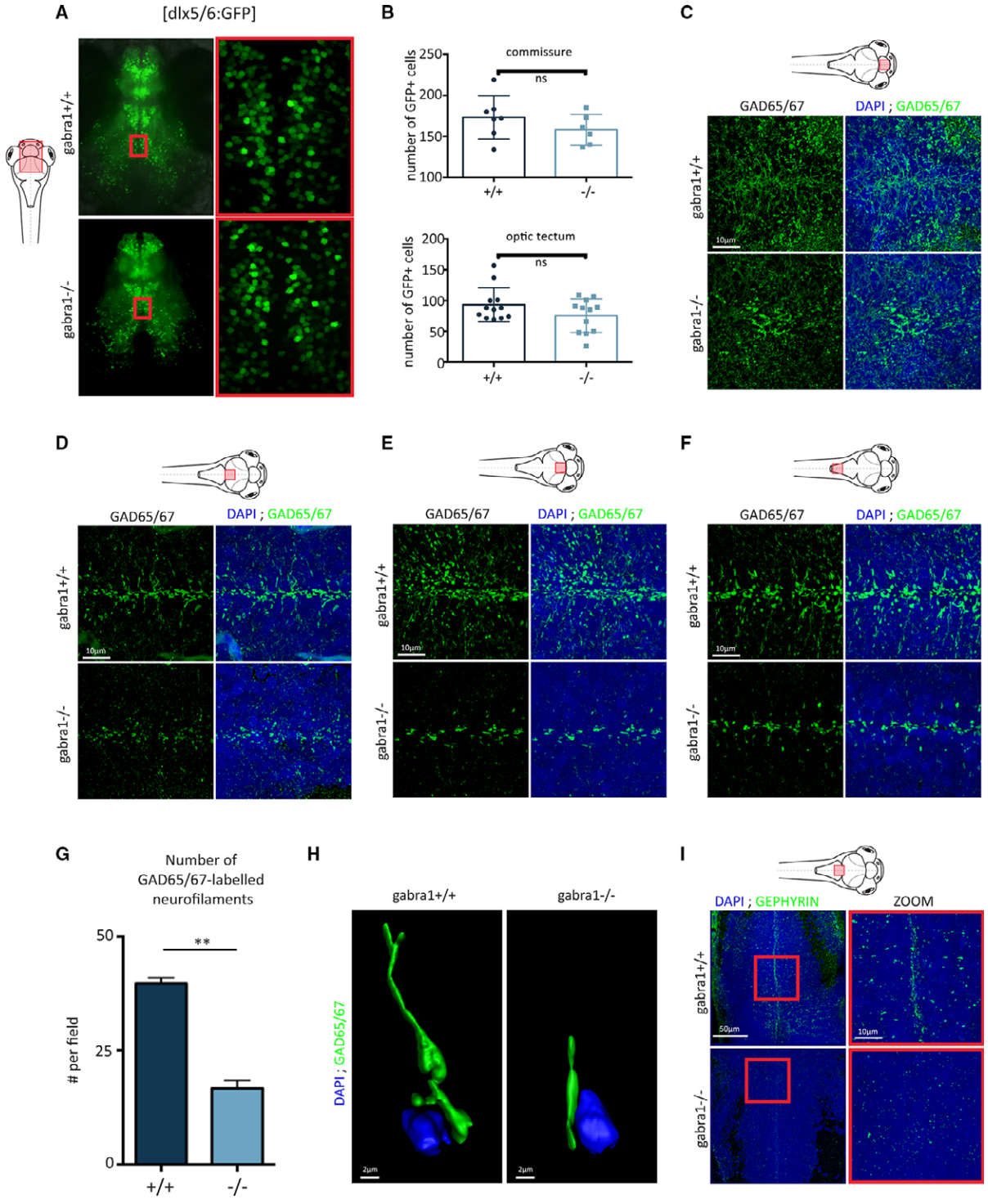

FIGURE 6 Reduction of inhibitory synaptic connectivity in *gabra1*$^{-/-}$ mutants. A, Confocal imaging of 8 days postfertilization (dpf) larval brains from *gabra1* × dlx5/6:GFP transgenic lines. B, Quantification of the number of green fluorescent protein (GFP)$^{+}$ cells at the commissure (top) or projecting to the optic tectum (bottom). ns, not significant. C-F, Fluorescent immunodetection of GAD65/67 (green) combined with 4,6-diamidino-2-phenylindole (DAPI) labeling on cryostat sections of different brain regions from 6 dpf *gabra1*$^{+/+}$ and *gabra1*$^{-/-}$ zebrafish embryos (scale bars = 10 µm): telencephalon (C), anterior part of optic tectum (D), posterior part of optic tectum (E), spinal cord (F). G, Quantification of GAD65/67-labeled neurofilaments (n = 3, Student *t* test: $^{**}P = 0.0019$). H, Imaris-reconstructed three-dimensional image with Gad65/67 shown in green and the nucleus (DAPI) shown in blue. Scale bar = 2 µm. I, Sagittal sections of 6 dpf *gabra1*$^{+/+}$ and *gabra1*$^{-/-}$ embryos hybridized with an antibody directed against postsynaptic density gephyrin (green) and nuclei stained with DAPI (blue; scale bar = 50 µm). Magnified views of the regions boxed in white and red rectangles are shown (scale bar = 10 µm). A decreased accumulation of gephyrin expression was observed in *gabra1*$^{-/-}$ mutants. For each imaging, the same field of observation is shown for +/+ and −/− larvae, thus avoiding regional bias

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

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