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Clinical commentary

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A novel *SCN1A* mutation in a cytoplasmic loop in intractable juvenile myoclonic epilepsy without febrile seizures

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ABSTRACT – Generalised (genetic) epilepsy with febrile seizures plus (GEFS+) is a familial epilepsy syndrome with various phenotypes. The majority of individuals with GEFS+ have generalised seizure types, in addition to febrile seizures (FS) or febrile seizures plus (FS+), defined as either continued FS after 6 years of age or afebrile seizures following FS. A 27-year-old man with no history of FS/FS+ experienced intractable generalised convulsive seizures. The patient's father had a history of similar seizures during puberty and the patient's siblings had only FS. No individual in the family had both generalised seizures and FS/FS+, although GEFS+ might be considered to be present in the family. Analysis of *SCN1A*, a sodium channel gene, revealed a novel mutation (c.3250A>T [S1084C]) in the cytoplasmic loop 2 of *SCN1A* in both the patient and his father. Most previously reported *SCN1A* mutations in GEFS+ patients are located in the conserved homologous domains of *SCN1A*, whereas mutations in the cytoplasmic loops are very rare. *SCN1A* gene analysis is not commonly performed in subjects with generalised seizures without FS. *SCN1A* mutation may be a clinically-useful genetic marker in order to distinguish GEFS+ patients from those with classic idiopathic generalised epilepsy, even if they present an atypical clinical picture.

Key words: *SCN1A* gene, novel mutation, intractable, IGE, JME, GEFS+

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Idiopathic generalised epilepsy (IGE) is classified into classic IGE and the recently proposed "generalised (genetic) epilepsy with febrile seizures plus" (GEFS+). By definition, GEFS+ is a familial epilepsy syndrome and is diagnosed on the basis of more than one individual within a family with a history of manifestations associated with the GEFS+ spectrum. The GEFS+ spectrum consists mostly of generalised seizures commonly combined with FS or febrile seizures plus (FS+) in

the same individual. FS+ is defined as either continued FS after the age of 6 years or afebrile seizures following FS (Scheffer & Berkovic, 1997). Various point mutations in the sodium channel voltage-gated type I alpha subunit (SCN1A; MIM 182389, GenBank AB093548) are the genetic cause of certain GEFS+ phenotypes. The GEFS+ spectrum can range from benign phenotypes, such as febrile seizures (FS), to severe epileptic encephalopathies, such as Dravet syndrome.

We present a patient whose initial diagnosis was believed to be juvenile myoclonic epilepsy (JME). The patient did not have FS/FS+, but his two siblings had only FS. His father and paternal aunt had generalised convulsive seizures alone without FS/FS+. No family member had both epilepsy and FS. However, the possibility of the presence of GEFS+ within the family was considered because the proband's seizures were very poorly controlled, in contrast to classic IGE. Genetic analysis of the *SCN1A* gene showed that the patient and his father had a novel point mutation in the cytoplasmic loop. Thus, their diagnosis as a family was considered to be GEFS+. With the exception of Dravet syndrome, the presence of *SCN1A* missense mutations in patients without any characteristics of the GEFS+ spectrum is very rare. This family may demonstrate the clinical divergence of patients with *SCN1A* missense mutations as well as the clinical variation within the GEFS+ spectrum.

Case study

A 27-year-old Japanese man with no history of FS was regularly treated for frequent generalised tonic-clonic seizures (GTCSs) and presumed myoclonic seizures, in combination. The first GTCS occurred at 14 years of age. The seizures often occurred while he was watching television, with flickering images, suggesting photo-sensitivity. Valproate (VPA) was effective and the seizures diminished within days. At the age of 17 years, GTCSs reappeared and he developed other repetitive myoclonic jerks in his arms, which occurred mostly in the morning. When carbamazepine (CBZ) was administrated, his seizure control worsened and CBZ was discontinued. Myoclonic jerks occurred almost every day, and the GTCS frequency gradually increased to more than one a month, when he was aged 27 years and visited our hospital. Based on his medical history, his diagnosis was believed to be JME. He was receiving VPA and clobazam (CLB) upon admission. Blood tests were normal and brain MRI showed neither atrophy nor any structural abnormality. His intelligence quotient scores were within the average range. An EEG showed paroxysmal generalised polyspike-and-wave complexes with myoclonic seizures, one of which evolved into a GTCS during a routine EEG examination. Abnormally enhanced, somatosensory, early cortical evoked potentials, in response to median nerve stimulation (giant SEPs), were not elicited.

He had a positive family history of both generalised convulsive seizures and typical FS, but neither of these occurred in combination in the same individual. His father and paternal aunt showed a similar phenotype; they had only generalised convulsive seizures. On the other hand, his two sisters had a history of only typical FS (*figure 1*). None of the family members had both epilepsy and FS. There was an autosomal dominant genetic trait of epileptogenicity in the family; thus, the possibility of GEFS+ within the family was considered. In addition, contrary to typical JME, the proband's seizures were very poorly controlled.

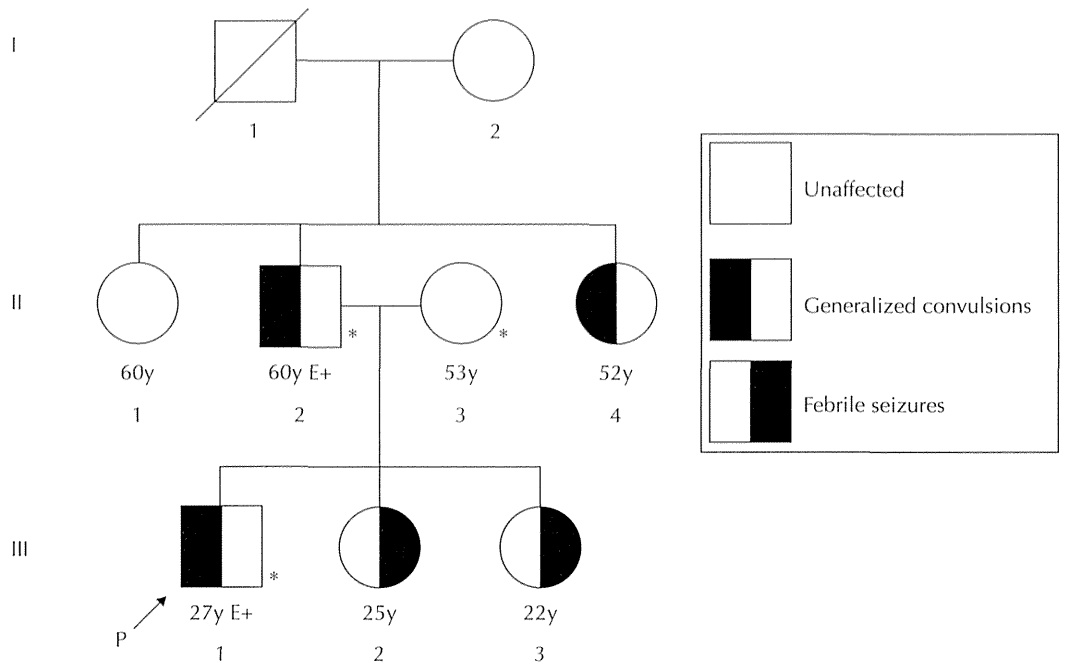
Written informed consent for genetic analysis was obtained from the patient and his parents, but not from the patient's sisters. The sisters previously had FS but currently have no symptoms and are of childbearing age. The family did not wish the sisters to undergo gene testing. Molecular screening was carried out by direct sequencing of the 26 exons of *SCN1A* using DNA from blood cells. A heterozygous missense mutation at c.3250A>T (S1084C) in the *SCN1A* gene of the patient and his father were identified. S1084C is located in the cytoplasmic loop 2, which links homologous domains II and III (*figure 2A*). The putative impact of the amino acid substitution resulting from this gene mutation on the structure and function of the protein was assessed with PolyPhen-2 (prediction of functional effects of human nsSNPs; available at http://genetics.bwh.harvard.edu/pph/). This substitution was considered likely to be damaging, with a score of 0.991 (sensitivity: 0.71; specificity: 0.97).

GEFS+ was considered to be present within the family. Since the father had a history of treatment with phenobarbital (PB) since the age of 20 years and his seizures were well controlled, we chose PB as an add-on AED for our patient; his seizure frequency and myoclonic jerks decreased remarkably afterwards.

Discussion

We identified a novel *SCN1A* mutation (S1084C), located in the cytoplasmic loop 2, in a patient with clinically intractable JME (*figure 2B*). The family exhibited a familial classic IGE-like epilepsy syndrome which should be added to the spectrum of large phenotypic variation associated with *SCN1A* mutation.

In general, *SCN1A* mutations in GEFS+ patients are present in one of the four conserved homologous domains, whereas mutations in the loop region are comparatively less common (Zuberi *et al.*, 2011). Other mutations have been identified in loop 2 (41 mutations; 14 of which are missense mutations), however,



	Age (years)		Clinical manifestation		Clinical results		
	Current	Onset of Symptoms	Symptoms	AED	EEG	MRI	SCNIA analysis
II-2	60	15	GC	PB	N/A	N/A	S1084C
II-4	52	15	GC	N/A	N/A	N/A	N/A
III-1	27	14	GC, Myoclonic seizures	VPA, CLB	Polyspikes-and-wave complex	Normal	S1084C
III-2	25	<1	FS (5 times until 6 years old)	None	N/A	N/A	N/A
III-3	22	1	FS (once)	None	N/A	N/A	N/A

Figure 1. The pedigree and clinical features of the GEFS+ family.
Arrow: the patient; number under each symbol: age; asterisk: subject examined; E+: positive evaluation of gene analysis.
GC: generalised convulsions; FS: febrile seizures; N/A: not available; PB: phenobarbital; VPA: valproate; CLB: clobazam.
The patient's two siblings had only FS. Subject III-2 had five FS and III-3 a single FS. The patient, his father, and paternal aunt had only generalised convulsions.

most of them cause Dravet syndrome (*SCN1A* Variant Database; available at http://www.molgen.vib-ua.be/SCN1AMutations/). With regards to Nav 1.1 function, a cytoplasmic loop could be functionally less important relative to the homologous domains. The cytoplasmic loops could possibly act as phosphorylation sites. Point mutations in the loops might cause altered sodium channel ($Na_v1.1$) function and neuronal hyperexcitability (Smith and Goldin, 1997). Loop 2 is also important as an ankyrin G binding site, which is essential for targeting sodium channels to the axon initial segment and nodes of Ranvier (Rasband, 2010). The S1084C mutation is very close to the ankyrin G binding site and may interfere with its functions, moreover, we confirmed that S1084C may potentially result in damaging functional abnormalities using PolyPhen-2. Among the 24 previously reported *SCN1A* mutations in GEFS+ patients, identified from the *SCN1A* infobase (available at http://www.scn1a.info/), only one other missense mutation in a loop region has been reported. Escayg *et al.* (2001) identified a W1204R missense mutation in *SCN1A*, located in the cytoplasmic loop 2, similar to our case. In their study, a German GEFS+ family was reported, in which most of the members

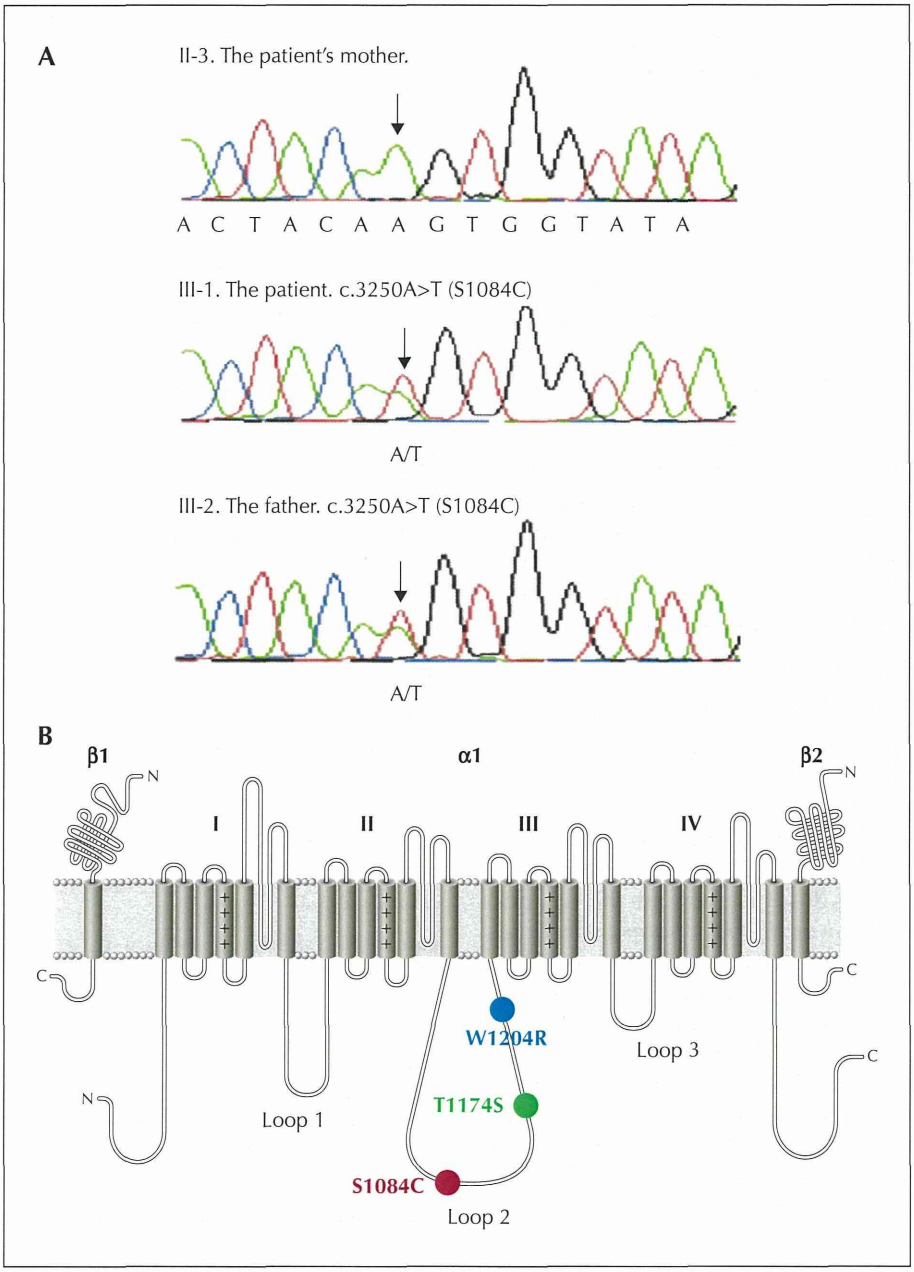


Figure 2. (A) The novel mutation in *SCN1A* genomic DNA. The patient and his father had a heterozygous mutation consisting of an adenine-to-thymine substitution within codon 3250 (S1084C). (B) Schematic diagram of voltage-gated sodium channel alpha and beta subunits. The position of the novel mutation S1084C (red circle) and previously reported mutations are depicted; T1174S (green circle) and W1204C (blue circle). The four homologous domains in the alpha subunit are labelled I-IV, with each domain containing six transmembrane segments. These mutations were in the cytoplasmic loop 2, linking the homologous domains II and III.
Figure modified with permission from Hirose *et al.* (2002).

demonstrated the characteristics associated with the GEFS+ spectrum, however, curiously, the proband's initial diagnosis was also JME and he did not have FS/FS+. In the context of studies to date, the mutation T1174S is of particular interest among those identified in loop 2, and is implicated in different phenotypes including JME and familial hemiplegic migraine (Escayg *et al.*, 2001; Gargus and Tournay, 2007; Yordanova *et al.*, 2011; Cestele *et al.*, 2013). The functional effect of T1174S was studied by expressing the protein in cell lines and it was hypothesized that a loss of function is the cause of epileptogenicity (Cestele *et al.*, 2013).

It is noteworthy that two other missense mutations in loop 2 (W1204R and T1174S) have been previously

reported to be associated with a JME phenotype, and these were furthermore identified in patients with different ethnic origins. Thus, in addition to these two mutations, we have identified a similar mutation in loop 2 in this GEFS+ family. Further data is required in order to evaluate the association between these phenotypes and loop 2 missense mutations.

Since the *SCN1A* mutation alters $Na_v1.1$ activity, AEDs that block sodium channels (e.g. CBZ, oxycarbamazepine, phenytoin, lamotrigine) should theoretically be avoided in *SCN1A*-related syndromes. On the other hand, VPA, stripentol, levetiracetam, and topiramate reportedly provide good control for these syndromes (Delgado-Escueta and Bourgeois, 2008). Thus, the genetic diagnosis provided us with information that was useful in choosing an appropriate add-on AED, avoiding arbitrary AEDs that could aggravate the patient's seizures. In our case, PB, which the patient's father was taking, with good seizure control, was also greatly effective. PB does not modulate sodium channel function and, thus, it is reported to be effective for *SCN1A*-related seizure disorders (Miller and Sotero de Menezes, 1993).

SCN1A analyses have been widely conducted in patients with FS/FS+ and those with intractable childhood epilepsy (Hirose *et al.*, 2013). However, testing in patients with classic IGE is not yet commonly performed. JME is a comparatively benign form of classic IGE, but about 15% of JME cases are reported to have persisting seizures despite adequate therapy and healthy lifestyles (Gelisse *et al.*, 2001). Some individuals with GEFS+, in the broad sense, might be misclassified with the more common syndromes of classic IGE. In certain cases of intractable classic IGE, when the family history of generalised seizures or FS is present, it would be useful to consider *SCN1A* gene mutation as well as the differential diagnosis of GEFS+. *SCN1A* mutation may be a clinically useful genetic marker for detecting GEFS+ patients among classic IGE cases, even if they present an atypical clinical picture. □

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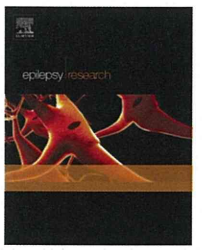

Association of nonsense mutation in *GABRG2* with abnormal trafficking of $GABA_A$ receptors in severe epilepsy

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Summary Mutations in *GABRG2*, which encodes the γ2 subunit of $GABA_A$ receptors, can cause both genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome. Most *GABRG2* truncating mutations associated with Dravet syndrome result in premature termination codons

Abbreviations: GEFS+, genetic epilepsy with febrile seizures plus; PTCs, premature termination codons; NMD, nonsense-mediated decay.

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Trafficking;
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Unfolded protein response

(PTCs) and are stably translated into mutant proteins with potential dominant-negative effects. This study involved search for mutations in candidate genes for Dravet syndrome, namely *SCN1A*, *2A*, *1B*, *2B*, *GABRA1*, *B2*, and *G2*. A heterozygous nonsense mutation (c.118C>T, p.Q40X) in *GABRG2* was identified in dizygotic twin girls with Dravet syndrome and their apparently healthy father. Electrophysiological studies with the reconstituted $GABA_A$ receptors in HEK cells showed reduced GABA-induced currents when mutated γ2 DNA was cotransfected with wild-type α1 and β2 subunits. In this case, immunohistochemistry using antibodies to the α1 and γ2 subunits of $GABA_A$ receptor showed granular staining in the soma. In addition, microinjection of mutated γ2 subunit cDNA into HEK cells severely inhibited intracellular trafficking of $GABA_A$ receptor subunits α1 and β2, and retention of these proteins in the endoplasmic reticulum. The mutated γ2 subunit-expressing neurons also showed impaired axonal transport of the α1 and β2 subunits. Our findings suggested that different phenotypes of epilepsy, e.g., GEFS+ and Dravet syndrome (which share similar abnormalities in causative genes) are likely due to impaired axonal transport associated with the dominant-negative effects of *GABRG2*.

Introduction

Epilepsy is associated with various gene mutations. However, the exact molecular mechanisms underlying the pleomorphic phenotypes of this disease remain unclear. There are two major epilepsy phenotypes associated with mutations in the $GABA_A$ receptor. The first is genetic epilepsy with febrile seizures plus (GEFS+). Individuals with GEFS+ have a missense mutation in the gene encoding the γ2 subunit of the $GABA_A$ receptor, *GABRG2* (Baulac et al., 2001). The second phenotype is Dravet syndrome, which is a malignant epilepsy condition characterized by refractory seizures and psychomotor developmental arrest.

Compared with Dravet syndrome, GEFS+ is relatively benign. However, these seemingly different syndromes are considered part of a single disease spectrum as mutations detected in both syndromes (Mulley et al., 2005; Singh et al., 2001) lie on the same genes, namely *GABRG2* (Baulac et al., 2001; Harkin et al., 2002), the gene encoding the α1 subunit of the neuronal voltage-gated sodium channel, *SCN1A* (Abou-Khalil et al., 2001; Claes et al., 2001, 2003; Escayg et al., 2000, 2001; Fujiwara et al., 2003; Fukuma et al., 2004; Gennaro et al., 2003; Kimura et al., 2005; Nabbout et al., 2003; Ohmori et al., 2002; Sugawara et al., 2001, 2002; Wallace et al., 2003), and the gene encoding the α2 subunit of the neuronal voltage-gated sodium channel, *SCN2A* (Shi et al., 2009; Kamiya et al., 2004; Sugawara et al., 2001). Most of the mutations initially identified in Dravet syndrome were truncation mutations (Claes et al., 2001; Sugawara et al., 2002). On the other hand, mutations identified in GEFS+ were exclusively missense mutations (Abou-Khalil et al., 2001; Escayg et al., 2000; Sugawara et al., 2001). Based on these studies and the more malignant nature of Dravet syndrome, it has been since postulated that mutations found in Dravet syndrome are associated with the more severe phenotypes due to a more significant genetic loss compared to the usually milder effects of missense mutations. Intriguingly, subsequent analysis revealed that missense mutations are also associated with Dravet syndrome (Fujiwara et al., 2003; Fukuma et al., 2004; Gennaro et al., 2003; Ohmori et al., 2002).

Since most of the nonsense mutations are located in the 5′ end of *SCN1A*, the mutant transcripts go through a nonsense-mediated decay (NMD) pathway (Holbrook et al., 2004). Thus, NMD processing underlies the distinct phenotypes resulting from truncation mutations and, further, may explain the variety of phenotypes associated with different mutations in the same gene. However, the evidence that GEFS+ and Dravet syndrome occupy the same spectrum of disorders or are allelic variants is not compelling.

In this study, we identified a nonsense mutation (c.118C>T, p.Q40X) in the *GABRG2* gene in individuals with Dravet syndrome. To specify the molecular mechanisms underlying the phenotypes of GEFS+ and Dravet syndrome resulting from allelic *GABRG2* truncation mutations, we focused on intracellular trafficking since several mutants are retained in the endoplasmic reticulum (ER) (Hirose, 2006; Gallagher et al., 2005; Hales et al., 2005; Harkin et al., 2002; Kang and Macdonald, 2004; Macdonald et al., 2004).

Materials and methods

Ethics standards

The study methodologies conformed to the standards set by the Declaration of Helsinki; and the study methodologies were approved by the Ethics Review Committees of Fukuoka University. The parents of patient and her sibling provided signed informed consent with the understanding before the study.

Patients

We studied a Japanese family, including dizygotic twin girls with the epilepsy phenotype of Dravet syndrome. The twins had experienced seizures from 2 months of age, and one of the twins died in bed at 3 years and 5 months of age. The twins had a novel nonsense mutation of the gene encoding the $GABA_A$ receptor γ2 subunit. We found the same mutation in their father, who did not have any seizure episodes. In contrast, their mother did not have the mutation, but had several seizure episodes in early childhood.

Genetic analysis

Genomic DNAs were prepared from ethylene diamine tetra acetic acid (EDTA)-treated whole blood samples using a QIAamp DNA Blood kit (Qiagen, Hilden, Germany). All exons and their flanking intronic splice sites of genes encoding major subunits of sodium channels (*SCN1A*, *SCN2A*, *SCN1B* and *SCN2B*) and $GABA_A$ receptors (*GABRA1*, *GABRB2* and *GABRG2*) were screened for abnormalities using a direct sequencing method with an automatic sequencer as described previously (Fukuma et al., 2004; Hirose et al., 1999, 2000; Ishii et al., 2009). Details of the PCR conditions and the primers used are available upon request. Reference sequences of mRNA were based on information available from RefSeq (accession numbers): Human *SCN1A*, NM006920; Human *SCN2A*, NM021007; Human *SCN1B*, NM001037; Human *SCN2B*, NM004588; Human *GABRA1*, NM000806; Human *GABRB2*, NM021911; Human *GABRG2*, NM19890; and Rat *Gabrg2*, NM183327.

Recombinant constructs

Full-length Rattus α1, β2, and γ2 subunit cDNAs containing either the *myc* epitope tags (between amino acids 4 and 5 of the mature polypeptide) have been described previously (Connolly et al., 1996) and shown to be functionally silent with respect to receptor pharmacology and physiology. The cDNAs were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and pIRES-EGFP (Clontech, Mountain View, CA). The mutant expression construct γ2 (p.Q40X) was generated in each construct using the QuickChange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). Clones were validated by direct double-strand DNA sequencing on an ABI3100 sequencer using DyePrimer chemistry (Applied Biosystems, Foster City, CA).

Tissue culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM; Bio-Whitaker, Walkersville, MD) supplemented with 10% fetal bovine serum and transfected using FuGene 6 transfection reagents (Roche Applied Science, Indianapolis, IN). Cells were incubated for 24 h at 37 °C in a humidified incubator containing 5% CO_2.

Electrophysiological studies

cDNA constructs for rat $GABA_A$ receptor subunits were transferred to the eukaryotic expression vector, pcDNA3 (Invitrogen). cDNA constructs for CD8 in the expression vector piH3 were provided by B. Seed (Massachusetts General Hospital, MA). HEK293 cells (Japanese Collection of Research Bioresources, Japan) were cultured in DMEM (Gibco, Tokyo, Japan) supplemented with 7% fetal bovine serum (Gibco), 7% horse serum (Sigma, St. Louis, MO), L-glutamine (Gibco, USA), and gentamicin (Boehringer Mannheim, Mannheim, Germany), in a humidified atmosphere containing 5% CO_2 and 95% air, at 37 °C. The cells were passaged twice a week and used at 8 ± 16 passages (mean ± SD). The cells were plated at 1×10^5 cells/35-mm culture dish containing poly-l-lysine (Sigma) and collagen (Vitrogen100, USA)-coated coverslips, the day before transfection. HEK293 cells were transfected with cDNAs (total 1.0 g for all subtypes and 0.1 g of CD8) using Lipofectamine (Gibco), and the conventional whole-cell patch clamp recordings were obtained at 36–72 h after transfection. Cells highly expressing the $GABA_A$ receptor channels were identified under a microscope using the bead-labeling technique (Jurman et al., 1994), with beads covalently coupled antibody to CD8 (Dynabeads, Invitrogen, Carlsbad, CA). The pipette solution contained (in mM): CsCl 140, $MgCl_2$ 2, EGTA 5, and HEPES 10 (pH 7.2 with CsOH). The pipette resistance was approximately 5 MΩ. The series resistance (2–8 MΩ) and the cell capacitance (12–80 pF) were compensated up to 80%. In all cases, data were obtained from isolated single cells. All currents were filtered at 1 kHz and measured with an Axopatch 200 A amplifier (Axon Instruments, Palo Alto, CA). Data obtained were then sampled at 5 kHz and stored using pClamp ver. 9 (Axon Instruments). The external solution contained (in mM): NaCl 150, KCl 5.0, $MgCl_2$ 2.0, $CaCl_2$ 2.0, D-glucose 10, and HEPES 10 (pH 7.4 with NaOH). The bath solution was perfused at 1 ± 2 ml/min from a separate perfusion line and removed from the bath with an aspirator. Each drug was applied through polyethylene Y-tube (equilibration time <20 ms) by gravity feed (Ueno et al., 1997). GABA was applied at an interval of 4 min to avoid running down of the current responses. All experiments were performed at room temperature (20–25 °C).

Immunohistochemistry

Patient brain obtained at autopsy was fixed in formalin and embedded in paraffin. Control brain specimens (temporal lobe, basal ganglia, and thalamus) were obtained from a 3-year-old boy who died from acute lymphocytic leukemia. Sections of formalin-fixed and paraffin-embedded tissues were cut at 4 μm thickness and immunohistochemically stained using the streptavidin–biotin method with antibodies against $GABA_A$ receptor α1 and β2 subunits. The sections were deparaffinized in xylene and then rehydrated in ethanol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol and then antigen retrieval was achieved using microwave irradiation. After cooling to room temperature, the sections were washed thoroughly in Tris-buffered saline (TBS), and then incubated overnight at 4 °C with anti-$GABA_A$ receptor α1 antibody (dilution 1:100, Upstate Biotechnology, New York, NY) and overnight at 4 °C with $GABA_A$ receptor γ2 antibody (dilution, 1:200, Alpha Diagnostic International Inc., TX). After three washes in TBS, biotinylated secondary antibodies and peroxidase-conjugated streptavidin [Simplestain MAX-PO (MULTI), Nichirei, Tokyo] were applied to the sections (the former at 4 °C overnight and the latter at room temperature for >2 h). After three washes in TBS, the immunoproducts were visualized using diaminobenzidine (Nichirei), and the sections counterstained with hematoxylin.

Microinjection

HEK293T cells were cultured on a coverslip, and microinjection was performed using a semi-automated microinjection

system (InjectMan; Eppendorf, Hamburg, Germany). For nuclear injections, plasmids were used at 100 mg/ml.

Immunostaining of HEK293T cells

HEK293T cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 7 min. Cells were washed and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min, and finally rinsed three times with 0.1% bovine serum albumin (BSA) in PBS. Fixed cells were incubated with the primary antibody diluted in PBS with 0.1% BSA at appropriate concentrations for 40 min at 37 °C. The antibodies used included anti-myc 10 mg/ml and rabbit monoclonal PDI (dilution 1:1000; Affinity Bioreagents, Golden, CO). This incubation was followed by three washes with 0.1% BSA in PBS and another incubation with Alexa Fluor goat anti-mouse or anti-rabbit antibody (dilution 1:1000; Molecular Probes, Eugene, OR) for 40 min at 37 °C. ProLong Gold Antifade Kit (Molecular Probes) was used to mount sections onto glass slides according to the instructions supplied by the manufacturer. Fluorescently labeled cells were visualized by standard fluorescence microscopy and confocal microscopy (model 510 META, Carl Zeiss, Jena, Germany).

Quantification of the localization of $GABA_A$ receptor

The quantification procedure was comprised of cDNA microinjection, staining, and cell counting, and this procedure was independently replicated three times for each $GABA_A$ receptor: WT $\alpha 1 myc \beta 2 \gamma 2$ (WT receptor), heterozygous $\alpha 1 myc \beta 2 \gamma 2 \gamma 2$ (Q40X, heterozygous receptor), and homozygous $\alpha 1 myc \beta 2 \gamma 2$ (Q40X, homozygous receptor). HEK293T cells were fixed at 4 or 18 h (short and long incubation, respectively) after the microinjection of cDNAs of the WT, heterozygous, or homozygous receptors, and labeled with secondary antibodies Alexa 488 (green) and Alexa 594 (red) (Molecular Probes) anti-*myc* and anti-PDI, respectively. Then, cells were mounted on glass slides with DAPI. The number of cells expressing the $\alpha 1$ subunit was quantified by counting nuclei stained with DAPI in cells that satisfied the conditions for *myc* (green) to match the cell morphology in the bright field. Some cells burst during and after the microinjection process; all remaining cells that were alive and stained with *myc*-tag were counted. For the WT receptor, the total number of cells counted was 66 (15, 21, and 30 in each of the three independent replications) at 4-h incubation, and 81 (40, 23, and 18) at 18-h incubation. For the heterozygous receptor, total number of cells counted was 47 (13, 22, and 12 in each of the three independent replications) at 4-h incubation, and 54 (19, 16, and 29) at 18-h incubation. For the homozygous receptor, the total number of cells counted was 114 (62, 15, and 37 in each of the three independent replications) at 4 h, and 137 (28, 47, and 62) at 18-h incubation. Colocalization with ER was determined based on overlapping *myc* and PDI staining. The colocalization with the plasma membrane was determined based on *myc* staining and the cell shape observed under the bright field. Cells that showed *myc* staining both on the membrane and in the ER were allocated to the membrane or ER colocalization based on the predominant staining location. Predominant location was based on the percentage of receptor distribution between the cell membrane and ER, and was determined relative to the total number of cells in both cases.

Culture of hippocampal neurons and transfection

Mouse hippocampal neurons were cultured as described previously with slight modifications (Brewer et al., 1993). Briefly, hippocampi from P1 pups were extirpated and placed into ice-cold Leibovitz L-15 (L-15) solution supplemented with 0.2 mg/ml BSA. These tissues were then digested with 0.4% DNase I and 0.25% trypsin in L-15/BSA for 15 min at 37 °C. The digested samples were then transferred to culture media (Neurobasal-A) supplemented with B-27, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite, followed by trituration by passing them through a Pasteur pipette until the disappearance of visible tissue fragments. We calculated the number of cells in the cell suspension and diluted it by adding an adequate amount of culture media. The cells were plated onto 22-mm × 22-mm coverslips precoated with L-polylysine and incubated in culture media at a density of ca. 250 cells/mm^2. Six days after plating, the cells were transfected using Lipofectamine LTX with either WT cDNA or the Q40X mutant construct of $GABA_A$ receptor γ subunit constructed in pEGFP-C1 (Clontech). The culture medium was changed on the next day and the cells were maintained for two more days.

Immunocytochemistry of cultured hippocampal neurons

For immunocytochemistry, the cultured neurons were briefly rinsed with prewarmed PBS twice, followed by fixation in 3.7% formaldehyde for 30 min at 37 °C. After fixation, the cells were rinsed 3 times with PBS, and then treated with 0.25% Triton X-100 for 10 min at room temperature (RT) to permeabilize the cell membranes. The cells were then treated with 2% skim milk in PBS for 30 min to attenuate non-specific binding. Subsequently, the cells were incubated overnight at 4 °C with primary antibodies, followed by three washes in PBS and blocking with 2% skim milk for 30 min at RT. The cells were then incubated with fluorophore-conjugated secondary antibody (Molecular Probes) for 2 h at RT. After thorough washing with PBS, the cells were stained with DAPI to visualize nuclei, and viewed under the confocal fluorescence microscope (FV-1000, Olympus, Tokyo).

Primary antibodies used to stain neurons

The following primary antibodies were used to detect and specify the localization of GABA receptors: anti-$GABA_A$ receptor $\beta 2$, 3-subunit antibody (Cat# MAB341, Millipore, Bedford, MA), anti-$GABA_A$ receptor antibody α-subunit (Cat# 06-868, Upstate Biotechnology), anti-$GABA_A$ receptor antibody $\gamma 2$-subunit (Cat# NB300-151, Novus, USA), anti-myc antibody (Clone 9E10, Sigma), anti-calnexin antibody (Cat# 610523, BD Transduction Laboratories, San Jose,