

Hyperglycosylation and Reduced GABA Currents of Mutated *GABRB3* Polypeptide in Remitting Childhood Absence Epilepsy

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Childhood absence epilepsy (CAE) accounts for 10% to 12% of epilepsy in children under 16 years of age. We screened for mutations in the GABA_A receptor (GABAR) β 3 subunit gene (*GABRB3*) in 48 probands and families with remitting CAE. We found that four out of 48 families (8%) had mutations in *GABRB3*. One heterozygous missense mutation (P11S) in exon 1a segregated with four CAE-affected persons in one multiplex, two-generation Mexican family. P11S was also found in a singleton from Mexico. Another heterozygous missense mutation (S15F) was present in a singleton from Honduras. An exon 2 heterozygous missense mutation (G32R) was present in two CAE-affected persons and two persons affected with EEG-recorded spike and/or sharp wave in a two-generation Honduran family. All mutations were absent in 630 controls. We studied functions and possible pathogenicity by expressing mutations in HeLa cells with the use of Western blots and an in vitro translation and translocation system. Expression levels did not differ from those of controls, but all mutations showed hyperglycosylation in the in vitro translation and translocation system with canine microsomes. Functional analysis of human GABA_A receptors (α 1 β 3-v2 γ 2S, α 1 β 3-v2[P11S] γ 2S, α 1 β 3-v2[S15F] γ 2S, and α 1 β 3-v2[G32R] γ 2S) transiently expressed in HEK293T cells with the use of rapid agonist application showed that each amino acid transversion in the β 3-v2 subunit (P11S, S15F, and G32R) reduced GABA-evoked current density from whole cells. Mutated β 3 subunit protein could thus cause absence seizures through a gain in glycosylation of mutated exon 1a and exon 2, affecting maturation and trafficking of GABAR from endoplasmic reticulum to cell surface and resulting in reduced GABA-evoked currents.

Introduction

Childhood absence epilepsy (CAE [ECA1 (MIM 600131), ECA2 (MIM 607681), ECA3 (MIM 607682), ECA4 (MIM 611136)]),^{1–4} a common idiopathic generalized epilepsy (EIG [MIM 600669]), accounts for 10% to 12% of epilepsy in children under 16 years of age according to prospective community-based epidemiologic studies.^{5,6} Absence is characterized by frequent brief loss of consciousness lasting 3 to 10 s and occurring up to about 200 attacks per day. The electroencephalograph shows bilateral, symmetrical, synchronous, 3–4 Hz spike-and-wave bursts during absence seizures.⁷ CAE appears more frequently in girls,^{8,9} and when present as the sole phenotype, CAE has better prognosis¹⁰ and remits in 95% of cases in Loiseau's report.¹¹ Historically, a strong genetic contribution to the spike-wave traits of CAE has been supported by 74% concordance for monozygotic twins and 27% concordance for dizygotic twins.¹² Metrakos and Metrakos showed that siblings and offspring had (a) 50% risk of inheriting the 3Hz spike-wave trait, (b) 35% risk of expressing the EEG trait in their

lifetime, and (c) 12% risk for tonic-clonic seizures and 8% risk for absences.^{13–15} These family and twin studies support the concept of a major gene interacting with additional genetic and environmental factors in CAE.

In 1999, Feucht et al.¹⁶ used a Monte Carlo version of the multiallele Transmission Disequilibrium Test to show possible association between *GABRB3* (MIM 137192) and CAE in 50 Austrians. Urak et al.¹⁷ then replicated significant association between 45 CAE patients and 13 SNPs located between the exon 1a promoter and the beginning of intron 3 within *GABRB3*. Reporter-gene assays in NT2 cells (human neuronal-like cell lines) showed lower transcriptional activity of the disease-associated *GABRB3* promoter haplotype. We screened for mutations in *GABRB3* in families ascertained through a proband with remitting pyknoleptic CAE because of the findings in the above studies by Feucht et al.¹⁶ and Urak et al.,¹⁷ because typical and atypical absence attacks are present in Angelman syndrome patients whose chromosome 15q11–13 deletion includes *GABRB3*,¹⁸ and because heterozygous and homozygous null mutants for *GABRB3* in mice show absence-like

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attacks with EEG characteristics and pharmacological responses similar to human absence seizures.^{19,20}

Subjects and Methods

Family Material

We ascertained 48 families, through probands with remitting pyknoleptic childhood absence epilepsy, from Mexico City and Hermosillo, Mexico; Tegucigalpa, Honduras; and San Miguel, El Salvador (See Table 1 for inclusion and exclusion criteria). Initial diagnosis was made by neurologists at the study sites of the international consortium, GENESS (Genetic Epilepsy Studies) and then validated by M.T.M. and A.V.D.E. The diagnosis in probands and affected family members was based on the guidelines of the Commission on Classification of the International League Against Epilepsy.²¹ Each responsible person (parent or adult patient) in each family signed an informed-consent form that was approved by the Human Subject Protection Committee at the David Geffen School of Medicine at the University of California, Los Angeles; or by the National Institute of Neurology and Neurosurgery in Mexico City; or by the Secretary of Health of El Salvador; or by the Research Unit at the School of Medical Sciences at the National Autonomous University of Honduras. We obtained blood from 416 healthy Hispanic blood donors from Mexico. We also obtained blood samples from 190 healthy blood donors residing in Honduras and 24 individuals considered as "married-ins" belonging to Honduras families that had been recruited for genetic studies of childhood absence epilepsy and juvenile myoclonic epilepsy. Genomic DNA was extracted from EDTA-treated blood samples with the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) and the Wizard Genomic DNA Purification System (Promega, Madison, WI).

Linkage Analysis

We first used the computer-simulation method of Ott^{22,23} to assess the strength of genetic material for linkage in families M120, HMO10, H12, and H08, with the assumption that a dominant disease with 50% penetrance was present. We then genotyped eight microsatellite markers located in the vicinity of the *UBE3A*, *GABRB3*, $\alpha 5$ subunit gene (*GABRA5*), and $\gamma 3$ subunit gene (*GABRG3*) cluster on chromosome 15q11.2-12. These markers were selected from the OMIM database and according to the report of Glatt et al.^{24,25} Two-point linkage analyses were performed with the MLINK and ILINK programs of the LINKAGE software package, version 5.21, under the assumption of autosomal-dominant inheritance with 50% penetrance, with the frequency of the disease allele at 0.001, phenocopy and gene-mutation rates of 1%. The allele frequencies were estimated with the use of the Centre d'Etude du Polymorphisme Humain (CEPH) database.

Mutation Analysis

We designed primers with the Primer 3 program to amplify the full region spanned from the 5' UTR region to exon 3. This span starts from 1.5 kb upstream of exon 1a to 62 bp downstream from exon 3. We also amplified all coding regions from exon 4 to exon 9, including splice sites, and the selected regions from intron 3 and 3' UTR. For PCR reactions, the AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) or Fast Taq polymerase (Roche, Indianapolis, IN) was used according to manufacturer's instructions. Both patient and healthy-control genomic-DNA samples served as templates for PCR amplifications. Each PCR

Table 1. Inclusion and Exclusion Criteria of Childhood Absence Epilepsy

Inclusion Criteria for Remitting Pyknoleptic Absence Epilepsy (Modified from ILAE*)

Age at onset between 2 and 12 years
Brief (3-20 seconds) and frequent (>10/day) absence seizures
EEG generalized high amplitude 2.5-3.5 Hz spike (maximum 3 spikes) and slow wave complexes lasting 3-20 seconds, spontaneously or on hyperventilation or on photic stimulation
Normal neurological state and development
Remission of absence seizure between 10 years and 18 years of age

Exclusion Criteria for Childhood Absence Epilepsy

Myoclonic jerks prior to or during active stage of absence
Symmetric synchronous or arrhythmic myoclonus of head, trunk or limb or a diagnosis of juvenile myoclonic epilepsy or a diagnosis of progressive myoclonus epilepsy
Progressive neurological deterioration

*International League Against Epilepsy Commission and Terminology 1989 Classification of Epilepsies and Epileptic Syndromes

When the proband is in late childhood or early adolescence still requiring treatment, we consider the family to have the remitting form of absence if an affected family member has remitting form of absence.

product of probands and controls was screened by heteroduplex analysis with the use of denaturing high-performance liquid chromatography (DHPLC WAVE, Transgenomic)^{26,27} or directly sequenced with the use of ABI 3700 capillary automated-sequencing system (Applied Biosystems, Foster City, CA). Suspected variants were subjected to PCR at least twice and confirmed by digestion or sequences of parents.

Expression Constructs and Mutagenesis

Preparation of *GABRB3* cDNAs for Expression

Full-length cDNA for human GABA_A receptor $\beta 3$ subunit in expression vector pCMV-SPORT 6 was obtained from American Type Culture Collection, Manassas, VA. We isolated the complete exon 1a and part of exon 2 by PCR, yielding one wild-type and two mutated versions of exon 1a from probands. We then cloned them into pCMV-SPORT after TOPO TA cloning (Invitrogen, Carlsbad, CA). The stop codon was removed from each transcript, which was inserted in the frame with the GFP coding sequence, in the vector pFP-N1 to produce three versions of C-terminal GFP-tagged *GABRB3*-exon 1a cDNA.

In Vitro Transcription, Translation, and Translocation

The isolated cDNA constructs from probands were cloned into pCMV-XL5 (Origene, Rockville, MD) after TOPO TA cloning (Invitrogen, Carlsbad, CA). A stop codon was introduced in exon 6 after amino acid Ile-206 by QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in order to express only the extracellular domain of the *GABRB3* protein. Each cRNA was transcribed with the use of the mMESSAGEMACHINE T7 kit (Ambion, Austin, TX).

Immunoblotting Analysis

HeLa cells were transfected with expression constructs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and homogenized 11-48 hr after transfection in hypotonic buffer (0.25 M sucrose, 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5) supplemented with a mixture of protease inhibitors (Roche, Indianapolis, IN). The lysate was centrifuged at 3300 g for 5 min to remove nuclei, and the supernatant was used as total cytosolic-plus-membrane

protein fraction according to Miyawaki²⁸ and Ganesh.²⁹ Protein samples were run on 10% Tris-HCl gels (BioRad, Hercules, CA) and transferred onto a nitrocellulose filter (BioRad, Hercules, CA) at 100 mA for one hour in transfer buffer (48 mM Tris base, 39 mM glycine, 0.037% [v/v] SDS [electrophoresis grade], 20% [v/v] methanol, pH 8.3). The filter was incubated in blocking solution (PBS + 0.05% Tween 20 pH 7.4) containing 5% nonfat dry milk powder for one hour at room temperature. The membrane was processed through sequential incubations with primary antibody (anti-GFP [Santa Cruz Biotechnology, Santa Cruz, CA], 1:200, dilution, monoclonal antibody against the GABA_A receptor β 3 subunit, bd17 [Chemicon, Temecula, CA], 1:500, and C20 [Santa Cruz Biotechnology, Santa Cruz, CA], 1:500) for one hour, then secondary antibodies were added at 1:3000 for one hour. Immunoreactive proteins on the filter were visualized by Typhoon software 9410. Immuno-quantitation was calculated by ImageQuant 5.2.

In Vitro Transcription, Translation, and Translocation

In vitro translation and translocation were performed with the use of the above plasmids in a coupled transcription and translocation rabbit reticulocyte lysate system or with the use of cRNAs in nuclease-treated rabbit reticulocyte lysate system (Promega, Madison, WI), with L-[³⁵S]methionine (GE Healthcare Bio-Sciences, Piscataway, NJ) in accordance with the manufacturer's protocol. Canine pancreatic microsomal membranes (1.8 μ l) (Promega, Madison, WI) were added directly to each reaction medium for translocation experiments. After incubation at 30°C for 60–90 min, aliquots of 3–10 μ l were diluted into 100 μ l of phosphate-buffered saline (PBS, pH 7.4) with 1 mM PMSF and kept on ice for 30 min. Microsomes were collected by centrifugation (20,000 \times g for one hour, 4°C). The pellets were rinsed twice with 100 μ l of PBS. Supernatant proteins or the reaction without microsomes were precipitated with 1000 μ l of acetone with 10% trichloroacetic acid at –20°C overnight, centrifuged, then dissolved in sample buffer (S). For cleaving of whole N-linked carbohydrates, each microsomal pellet was resuspended in H₂O and then treated with 100 units of PNGase F (NEB, Ipswich, MA) according to the manufacturer's instructions (37°C for 3 hr). Each pellet that was treated (D) and/or not treated (P) with sample buffer was analyzed by SDS-PAGE on 12% or 6%–18% Tris-HCl gels (BioRad, Hercules, CA) after denaturing at 65°C for five min with supernatant protein. After electrophoresis, gels were soaked in 50% methanol, 7% glacial acetic acid for 30 min and then 7% methanol, 7% glacial acetic acid and 2% glycerol for 15 min. Gels were dried and exposed to a Phosphorimager cassette plate. Molecular weight was ascertained by the commercial protein standard (Invitrogen, Carlsbad, CA) and the size of the supplemental control of canine microsomes. Immuno-quantitation was calculated by ImageQuant 5.2. For mutations in exon 1a, the proportion of each band density was compared with the wild-type.

Cell Culture, Transfection, and Immunomagnetic Selection

HEK293T fibroblasts were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (all cell-culture products from GIBCO, Carlsbad, CA) and incubated at 37°C with 5% CO₂/95% air. GABA_A receptor subunit cDNA was inserted into a pcDNA3.1 (α 1 and γ 2) or pCMV (β 3) promoter, and point mutations were generated with the use of a Quikchange kit (Stratagene, La Jolla, CA). For transfection, 0.3 μ g of each subunit cDNA (α 1, β 3, γ 2S) was cotransfected with 1 μ g of cDNA for the pHook antigen (Invitrogen, Carlsbad, CA) for selection. Twenty-four to

thirty-six hours after transfection, positively transfected cells were selected with the use of ferromagnetic beads in a protocol described previously.³⁰ After selection, cells were plated onto 35 mm dishes for electrophysiological recording the following day.

Electrophysiological Recording

Single cells were chosen for recording on the basis of the presence of two or more beads from the selection process. The external recording solution consisted of (in mM): NaCl 142, KCl 8, MgCl₂ 6, CaCl₂ 1, HEPES 10, glucose 10, pH 7.4 and 318–328 mOsm. The internal recording solution consisted of (in mM): KCl 153, MgCl₂ 1, MgATP 2, HEPES 10, EGTA 5, pH 7.3 and 305–312 mOsm (all reagents from Sigma-Aldrich, St. Louis, MO). Recording solutions were designed such that the Cl[–] reversal potential was 0 mV.

Whole-cell currents were low-pass filtered at 2kHz and recorded with the use of an Axopatch 200B Amplifier (Molecular Devices, Foster City, CA) and a Digidata 1332A (Molecular Devices) with pClamp9.1 software. Borosilicate recording pipettes (World Precision Instruments, Philadelphia, PA) were pulled with a Sutter P-2000 micropipette puller (Sutter Instruments, Novato, CA) and fire-polished with a Micro Forge (Narishige, Tokyo, Japan) to a resistance of 0.9 M Ω –1.5 M Ω . Once a seal was obtained on the cell and the membrane was perforated (whole-cell voltage clamp), the capacitance of the cell was recorded from the lab-bench tool bar in pClamp9.1 (Molecular Devices). The cells were lifted off the dish and placed in front of a glass multibarrel connected to a Piezo stepper used for rapid application of GABA. Perfusion from the barrel consisted of either external solution or external solution containing 1 mM GABA (Sigma). Open-tip exchange times between lumen of the barrel were determined by perfusion of low- and high-electrolyte solutions and were consistently less than 1 ms. For analysis of current density, the pipette capacitance (13 pF) was subtracted from the cell capacitance and the peak current amplitude was normalized to the capacitance of the cell. All data was compared with the use of a Student's paired t test, with Welch's correction when variances were significantly different, and plotted with the use of GraphPad Software (San Diego, CA).

In Silico Analysis

The genome information and homology searches were explored with the use of the UCSC genome browser (May 2006 assembly) and the NCBI website. GC percentage and GC-island prediction were performed by EMBL-EBI tools (EMBOSS CpGPlot/CpGReport/Isochore). The transmembrane helices were analyzed by SOSUI and TMHMM, version 2.0. The signal-peptide prediction was performed by the SignalP 3.0 server, and Signal CF. N-glycosylation and O-glycosylation sites were analyzed with NetNGlyc 1.0 and YinOYang, respectively. The secondary-structure analysis was performed by GOR IV secondary-structure-prediction method. The prediction of functional effect was analyzed by the Polyphen program.

Statistical Analysis

The Fisher exact χ^2 test was used in comparison of the prevalence of mutations in patients with remitting CAE versus that in controls. The results of image densities were calculated and compared by the use of a chi-square goodness-of-fit test.

Results

Phenotypes

Figure 1 illustrates the pedigrees of four families (M120, HMO10, H12, and H08) that have mutations in *GABRB3*.

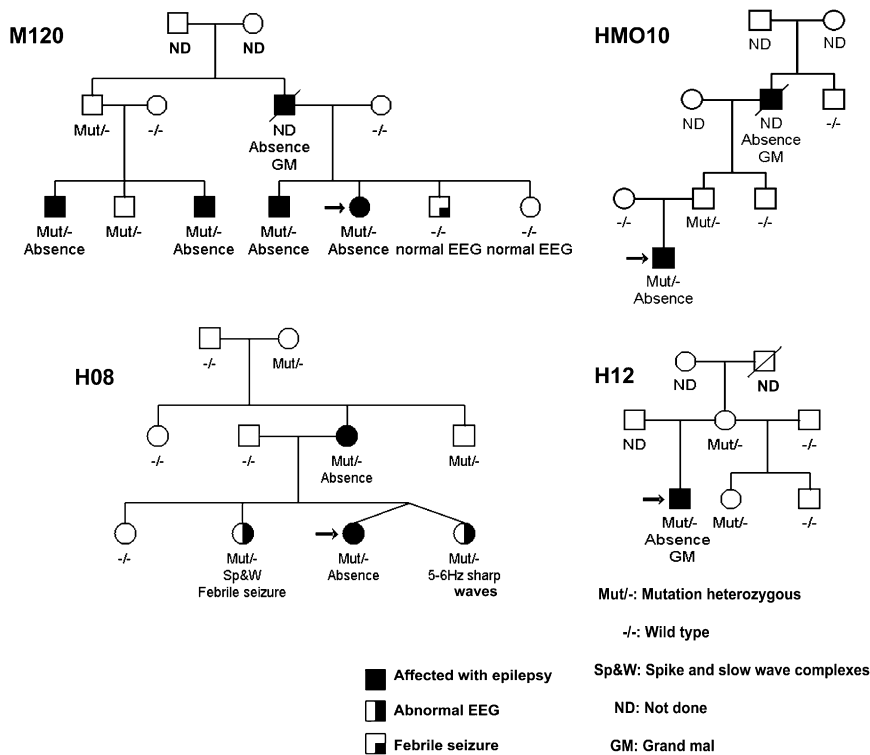


Figure 1. Four Families with *GABRB3* Mutations

Each family number is placed beside each pedigree. Black circles or squares represent epilepsy affected females or males. Asymptomatic persons who have EEG 3 Hz diffuse bilateral spike wave complexes or 5 to 6 Hz sharp waves are represented by half black circles or squares.

whereas the asymptomatic twin sister of the proband had epileptiform EEG bifrontal 5–6 Hz sharp waves.

Genotypes

We observed three heterozygous missense mutations, namely, P11S and S15F in exon 1a and G32R in exon 2 (Figure 2). The P11S mutation in exon 1a segregated with four CAE-affected members of family M120. The deceased father of the proband of family M120 was not tested for mutations. The same P11S mutation was

found in the proband and his clinically unaffected father in family HMO10. We considered family HMO10 as a singleton because we were unable to screen his CAE-affected

Table 2 summarizes the clinical characteristics of absence seizures in probands. Table 3 identifies nonproband family members who carry *GABRB3* mutations and are affected with absences. Table 3 also lists family members with *GABRB3* mutations who are not affected by epilepsy or absences.

Absences with eyelid myoclonias that were sensitive to photic stimulation started at 5 and 11 years of age, respectively, in probands of families M120 and H12. The proband of family M120 only had absences and never developed grand mal seizures. On the other hand, five separate grand mal tonic-clonic seizures appeared at 12 years of age in the proband of family H12. Absences without eyelid myoclonias rarely appeared at 2 years of age in the proband of family HMO10. However, they increased to more than 30 absence attacks a day between 4 and 6 years of age. Rare sudden atonic falls appeared in early childhood, and grand mal or myoclonic seizures never developed. The proband of family H08 had absence without eyelid myoclonias as the sole phenotype, and absences started at 5 years of age. Absences and accompanying seizures disappeared after 12 years of age in all four probands.

Three probands of families M120, HMO10, and H08 remain without seizures and without treatment. Absences remained suppressed by valproate in the 18-year-old proband of family H12. When affected by seizures, family members most commonly had absences seizures. The father of the proband in family M120 and the grandfather of the proband in family HMO10 both had grand mal tonic-clonic seizures in addition to absences. A clinically asymptomatic sister of the proband in family H08 had epileptiform EEG 3–4 Hz polyspike wave complexes,

Table 2. Clinical Characteristics of Pyknoleptic^a Absences and Associated Seizure in Probands of Families with *GABRB3* Mutations

Family	Present Age (Yrs) and Years of Remission	Onset (Yrs)	Clinical Semiology
M120	30 (18 yrs w/o treatment, w/o seizures)	5	Staring with eyelid myoclonias ^b as eyeballs roll up. No grand mal seizure (GM).
HMO10	14 (2 yrs w/o treatment, w/o absence or atonic seizures)	2	Staring with 3 Hz eye blinks as eyeballs roll up, Rarely absences Absences appears at 2 yrs of age, increased frequency (more than 20 attacks per day) between 4 and 6 yrs. Rare episodes of atonic seizures with flaccid limbs and vomiting. No GM.
H12	18 (no seizures and GM seizures for 2 yrs but still on treatment)	11	Staring with eyelid myoclonia ^b triggered by sunlight. GM at 12 yrs.
H08	15 (5 yrs w/o treatment, w/o absence seizures)	7	Staring as eyeballs roll up triggered by light, No GM.

^a Pyknoleptic means more than one absence seizure per day, often 20 to 200 seizures per day.

^b Eyelid myoclonia consists of very rapid blinking and flickers of the eyelids as the eyes deviate upwards.

Table 3. Family Members with Mutation and Absence

Family Number	Family Members with Mutation	Absence-Affected Members with Mutation	Family Members Affected with Absence	Family Members with Mutation but No Epilepsy	Nucleotide Change	Effect on Protein
M120	6	4	proband, brother of proband, two paternal male cousins	father of cousin; one brother of two affected paternal cousins	c.31C→T	Pro11Ser
HM010	2	1	proband	father of proband	c.31C→T	Pro11Ser
H12	3	1	proband	mother of proband; half-sister of proband (with different father)	c.44C→T	Ser15Phe
H08	5	2	proband, mother of proband	maternal grandmother of proband and two sisters ^a of proband	c.962G→A	Gly32Arg

^a Two sisters with EEG abnormalities; one sister has 2–4 Hz diffuse spike- and slow-wave complexes and febrile convulsions, and the other sister has 5–6 Hz fronto-central sharp waves.

grandfather for mutations. Theoretically, family HM010 would be considered multigenerational if we had had access to the CAE-affected grandfather. Families M120 and HM010 are not related and reside in two separate cities of Mexico, namely, Mexico City and Hermosillo, and have different family names across three generations. Both have the heterozygous c.31C → T mutation in exon 1a at position 31 from the start codon.

The S15F mutation in exon 1a is present in the proband, his asymptomatic mother, and his asymptomatic half brother in family H12 from Honduras. The G32R mutation in exon 2 segregated in four individuals who were clinically symptomatic or clinically asymptomatic with the EEG trait only; members of two generations in family H08, also from Honduras. P11S, S15F, and G32R mutations were not found in 630 healthy controls from Mexico and Honduras.

Linkage Analysis

Based on quadratic interpolation, the average simulated pooled LOD score was 1.9414 (SD 0.8848) and the maximum simulated pooled LOD score was 3.6339. The pooled

maximum two point LOD score for all 4 families (M-120, H08, HMO10 and H12) was 1.022 for *GABRB3*, 2.019 for D15S1002 and 2.305 for 85CA at theta = 0 m = f. 85CA lies in 5'UTR of *GABRB3* (see Figure 3 and Table 4). We obtained 0.351 for D15S122 and –3.918 for D15S1021.

In Silico Analysis

GABRB3, located on chromosome 15q11.2-q12, spans almost 230 kb (UCSC Genome Browser, March 2006). The mRNA of *GABRB3* consists of nine exons. Two alternative first exons, exon 1a and exon 1, encode the signal peptides of *GABRB3*.³¹ Exon 1a to exon 3 spans a 1.4 kb genomic region (GenBank accession number L04311) and contains a GC-rich (55%–80%) region with high content of CpG islands. The P11S, S15F, and G32R missense mutations reside in evolutionarily conserved amino acid sequences of exon 1a and exon 2 (Figure 4A). All missense mutations are predicted to have the same cleavage site as the wild-type, cleaved between Gly22 and Ser23 amino acids as predicted by software programs Signal P 3.1 and Signal CF (Figure 4B). The G32R missense mutation is

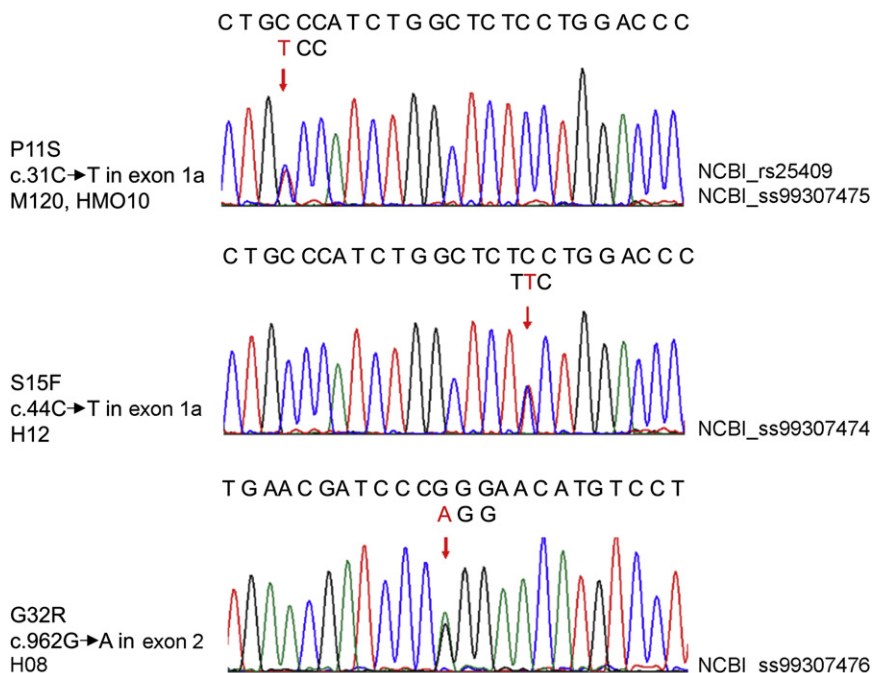


Figure 2. cDNA Sequencing in Each Proband
Each arrow shows the location of the mutation. The upper sequence represents wild-type. The lower triplet above the arrow represents the mutated code.

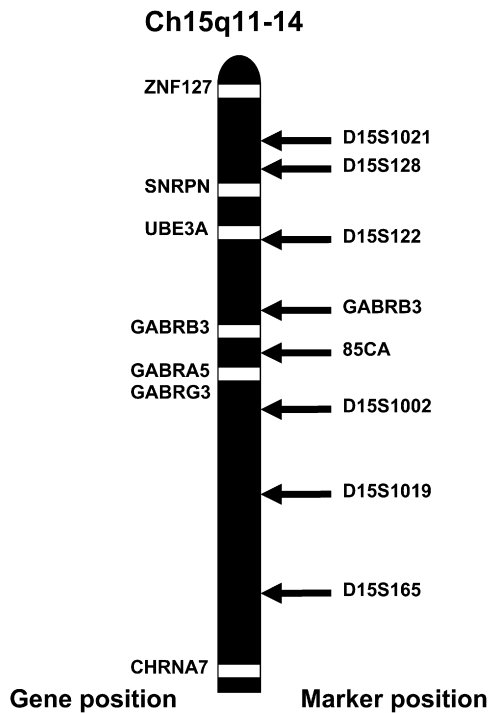


Figure 3. Gene and Marker Position on Chromosome 15q11-14
 Figure 3 shows the relative position of markers in chr. 15q11-14. *GABRB3* (marker) is about 60kb beyond the 3' terminus of *GABRB3*, and 85CA is about 50kb from exon 1a of *GABRB3*.^{24,25}

also predicted to have the same cleavage site as the wild-type pre-peptide, *GABRB3* protein isoform 1 precursor that is translated from the exon 1 mRNA, namely *GABRB3* transcript variant 1 (NM_000814). On the basis of the predicted cleavage site, we calculate the location of the G32R mutation in exon 2 to occur at position 10 from the N terminus of the mature polypeptide. This polypeptide is produced from the isoform 2 precursor translated by exon 1a mRNA, namely *GABRB3* transcript variant 2 (NM_021912). The secondary structure of all mutations can be predicted to show significant changes in secondary structure, according to the software GOR4 IV (Figure 4C).

The software programs NetNGlyc.1.0 and YinOYang predict with probability three N-glycosylation sites at asparagines and four O-glycosylation sites at serine and threonine to be located in the extracellular domain (Figure 5). Table 5 shows the potential score for the predicted N-glycosylation and O-glycosylation. There is no difference in potential for N-glycosylation between the wild-type and missense mutations in exon 1a. However, the G32R mutation has a slight but measurable lower potential at 33Asn for an N-glycosylation site and a higher potential for an O-glycosylation site at 23Ser (Table 5). In contrast, the S15F mutation has only a slightly lower potential than the wild-type and P11S for an O-glycosylation site at 23Ser.

The prediction for functional effects by mutations with the use of the Polyphen program is applicable for the G32R exon 2 mutation in the mature polypeptide and the P11S exon 1a mutation but not for S15F. G32R is pre-

Table 4. Summed Two-Point LOD Scores of Microsatellite Markers on Chromosome 15q11-14

Microsatellite Marker	θ				
	0	0.1	0.2	0.3	0.4
D15S1021	-3.919	-0.291	-0.104	-0.035	-0.008
D15S128	-0.084	-0.131	-0.104	-0.093	-0.044
D15S122	0.351	0.331	0.247	0.121	0.0054
<i>GABRB3</i>	1.022	0.822	0.583	0.325	0.096
85CA	2.306	1.78	1.233	0.688	0.219
D15S1002	2.019	1.535	1.038	0.554	0.16
D15S1019	0.712	0.554	0.384	0.211	0.064
D15S165	2.117	1.64	1.138	0.632	0.193

θ : recombination fraction; m = f.

dicted to have a damaging effect on function, with a PSIC score difference of 1.973 by *GABRB3* protein isoform 1 (NP_000805) and 1.78 by *GABRB3* protein isoform 2 (NP_068712). P11S is predicted to be benign, with a PSIC score difference of 1.053.

Expression Study: Immunoblotting Analysis

For functional analysis, we studied first the expression level of full-length *GABRB3*-GFP fusion protein after transfection into HeLa cells by immunoblotting. We used GFP primary antibody and beta3 primary antibody to determine whether mutations in exon 1a and exon 2 had any consequences for expression. No differences in expression levels were observed between the mutated constructs and the wild-type when they were harvested at earlier times; 11–12 hr after transfection (Figure 6). At expression times of > 24 hr, the amount of each protein was variable, probably because they were digested.

In Vitro Translation and Translocation

Next, we tested whether the observed mutations influenced the first steps of translation by using a cell-free in vitro translation and translocation system. In the absence of canine pancreatic microsomes, both wild-type and mutant cRNA (exon 1a to exon 6) were translated to *GABRB3* proteins of the same size, namely 30 kDa (Figure 7, wild-type lane S, supernatant protein). Such proteins corresponded to the preproteins of the $\beta 3$ subunit before cleavage and contain the signal peptides and the unglycosylated *GABRB3* protein. Canine pancreatic microsomal membranes (1.8 μ l) (Promega, Madison, WI) were then added directly to each reaction medium for translocation experiments. After one hour of centrifugation, pellets were separated from supernatant. The supernatant fluid of all samples did not contain *GABRB3* protein (not shown). The pellets, on the other hand, contained the *GABRB3* protein that had been translocated into the microsome (Figure 7—see translocated proteins represented as “P”). Translocation in the presence of 1.8 μ l of canine pancreatic microsomes precipitated all beta 3 subunit protein into the pellet. This implied that all signal peptides were oriented toward the membrane and that all *GABRB3* protein was

MCSGLELLLPIWLSWTLGTRGSEPRSVNDPCNMSFVKETVDKLLKGYDI
 RLRPDFGGPPVCVGMNIDIASIDMVSEVNMDYTLTMYFQQYWRDKRLAYS
 GIPLNLTLDNRVADQLWVWPDYFLNDKKSFFVHGVTVKNRMIRLHPDGTVL
 YGLRITTTAACMMDLRRYPLDEQNCTLEIESYG YTTDDIEFYWRGGDKAV
 TGVERI

N: N-linked glycosylation site
S T: O-linked glycosylation site

mutation in exon 2 might have a different cleavage site, no cleavage, or normal cleavage with additional O-glycosylation. In silico analysis predicts normal cleavage and gain of O-glycosylation at 23Ser. This observation further suggests that the G32R mutation was subjected to more degradation than the exon 1a mutations, perhaps due to misfolding of GABRB3 protein containing the G32R mutation.

Larger Current Density from Cells Expressing Wild-Type β 3-v2 Transcript than from Cells Expressing β 3-v2(P11S), β 3-v2(S15F), or β 3-v2(G32R) Mutations

Cells were cotransfected with equivalent amounts of cDNA encoding for the α 1, γ 2S, and one of the β 3-v2, β 3-v2(P11S), β 3-v2(S15F), or β 3-v2(G32R) subunits. Saturating concentrations of GABA (1 mM) were applied to positively transfected cells for 4 s. Currents from α 1 β 3-v2 γ 2S (wild-type β 3 subunit with exon 1a) receptors and mutant α 1 β 3-v2(P11S), α 1 β 3-v2(S15F), and α 1 β 3-v2(G32R) receptors all had a fast rate of rise, substantial multiphasic desensitization, and fast deactivation upon removal of GABA (Figure 9A). α 1 β 3-v2 γ 2S receptors, however, had a mean current density (279.1 ± 34.0 ; $n = 36$) that was larger than the mean current densities of α 1 β 3-v2(P11S) γ 2S (118 ± 20.51 ; $n = 18$, $p < 0.001$), α 1 β 3-v2(S15F) γ 2S (134.1 ± 20.7 ; $n = 25$, $p < 0.001$), and α 1 β 3-v2(G32R) γ 2S receptors (174.7 ± 23.18 ; $n = 41$, $p < 0.05$) (Figure 9B). Peak-current amplitudes were reduced in each mutant condition as well (data not shown).

Discussion

We found three missense mutations of *GABRB3* (Pro11Ser, Ser15Phe, Gly32Arg) in four out of 48 (8%) CAE-affected patients with American Indian and Spanish European ancestry. Two mutations (P11S and S15F) reside in the alternative signal peptide, exon 1a of the *GABRB3* protein. A third mutation (G32R) is at amino acid 10 from the N terminus of the mature *GABRB3* protein, which in turn is made from beta 3 isoform 2 precursor. These mutations segregated in clinically and EEG affected individuals and in asymptomatic persons belonging to two generations of these four families. Vertical transmission of the *GABRB3* mutations in symptomatic and asymptomatic family members suggests a dominant trait with incomplete penetrance. We did not find the same mutations in 630 healthy ethnically and sex-matched controls.

Figure 5. Predicted Glycosylation Site in Exon 1a–Exon 6 of *GABRB3*

Bold letters without shadow show the amino acids to be displaced by mutations.

Numbers show locations of amino acids.

P11S is listed as rs25409 in the SNP database of the National Center for Biotechnology Information (NCBI), where it is recognized as a minor allele in two out of 157 persons with autism. We do not know if these two persons with P11S have absence epilepsy. Thirty-five percent to sixty-five percent of patients with autism spectrum disorder have epileptiform EEG abnormalities, and 10% to 30% have seizures including absences.^{32–35} The same NCBI database contains the results of a HapMap study in which P11S is not found in 60 European, 44 Han Chinese, 43 Japanese, and 59 SubSaharan African persons. We did not find the P11S mutation in 630 controls, in sharp contrast to two patients with CAE (Fisher's exact test: $p = 0.0049$). The S15F and G32R missense mutations are both previously unreported in the NCBI databases. We did not find them in 416 controls from Mexico or the 214 controls from Honduras.

The GABA_A receptor (GABA_R) is a heteropentameric-membrane glycoprotein that is composed of five subunits.^{36,37} The first half of the polypeptide, which is translated from sequences of exon 2 to exon 7, forms a hydrophilic glycosylated extracellular domain. Several parts of

Table 5. Predicted Glycosylation Sites and Each Potential

N-Glycosylation			
Sample Position	Wild-Type, P11S, S15F	G32R	
33 NMSF	0.5857	0.5140	
105 NLTL	0.7671	0.7671	
174 NCTL	0.5487	0.5487	
O-Glycosylation			
Sample Position	Wild-Type, P11S	S15F	G32R
23 S	0.5166	0.4891	0.5336
157T	0.4529	0.4529	0.4529
158T	0.5198	0.5198	0.5198
201T	0.5793	0.5793	0.5793

N-glycosylation is known to occur on Asparagines (N), which is located in the N-X-S/T stretch in which X is any amino acid except proline. The first column has the position number of the predicted glycosylation site in the amino acid sequences of exon 1a to exon 6 of *GABRB3* (see fig. 5). Columns 2 and 3 represent their potential scores, derived from the averaged output of nine neural networks in NetNGlyc 1.0 Server. Intracellular O-glycosylation is characterized by the addition of N-acetylglucosamine, in a beta anomeric linkage (O- β -GlcNAc), to Serine (S) and Threonine (T) residues in a protein. Each number shows each position number of the predicted O-glycosylation site. The YinOYang prediction server produces neural-network predictions for O- β -GlcNAc attachment sites, incorporating predicted phosphorylated sites.

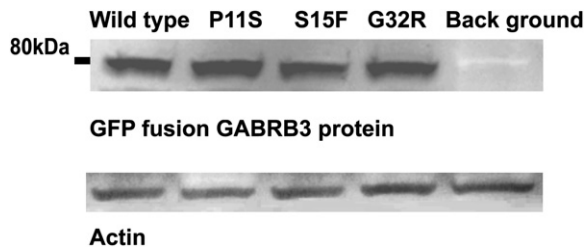


Figure 6. Western-Blot Analysis of Whole Cell Fractions Derived from HeLa Cell Expression 11 hr after Transfection

The GFP fusion GABRB3 protein was present at a slightly smaller molecular weight of 80kDa which was expected.

All expression levels of plasmids with mutated sequences were the same as the wild-type, even after normalization by Actin.

the extracellular domain are important for receptor function, including GABA and allosteric modulator binding sites,^{36,37} as well as assembly signals.³⁸ The last half of the polypeptide is translated from sequences of the remaining exon 7 to exon 9 and contains four hydrophobic sequences that form transmembrane domains. So far, the sites for binding GABA or allosteric modulators like benzodiazepines have been suggested to be somewhat removed from the N terminus in the extracellular domain, and binding sites for general anesthetic drugs are tentatively located in the transmembrane region.^{39–41} Thus, Pro11Ser and Ser15Phe mutations in the alternative signal peptide, exon 1a, and Gly32Arg mutation in exon 2 would not affect binding sites but instead influence protein maturation, topology, assembly, and subcellular localization of a GABAR.

In addition, two mutations in the signal peptide of the $\beta 3$ -v2 subunit, P11S and S15F, caused reductions in GABA_A receptor current density when expressed as $\alpha 1\beta 3$ -v2(P11S) $\gamma 2S$ or $\alpha 1\beta 3$ -v2(S15F) $\gamma 2S$ receptors and compared to wild-type receptors ($\alpha 1\beta 3$ -v2 $\gamma 2S$). Similarly, cells expressing receptors containing a $\beta 3$ -v2 subunit mutation just beyond the signal peptide in exon 2, $\alpha 1\beta 3$ -v2(G32R) $\gamma 2S$, also had smaller GABA-evoked current den-

sity than did cells expressing wild-type receptors. This is important because as a ligand-gated, chloride-selective ion channel, the function of the GABA_A receptor is to provide the majority of synaptic inhibition in the central nervous system. GABAR expression and kinetic properties are determined by the subunit combination present in the receptor. Although there are numerous genes that encode for subunits and subunit subtypes of the GABA_A receptor ($\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , θ , and π), the majority of the GABA_A receptors in the central nervous system are composed of two α , two β , and a single γ or δ subunit.^{42,43} To date, several mutations have been identified in the $\alpha 1$, $\gamma 2$, and δ subunits of the GABA_A receptor associated with familial epilepsy syndromes. Although these families were classified under the generalized epilepsy with febrile seizures plus (GEFS+) (febrile and afebrile seizures) spectrum of epilepsy or juvenile myoclonic epilepsy, all had childhood absence seizures as a phenotype. These observations strongly suggest that GABAR might be the crucial pathogenic molecule for childhood absence epilepsy.⁴⁴ The majority of these published GABAR mutations also cause altered subunit trafficking and, subsequently, expression of the mutated subunit, and others alter the function of the ion channel.⁴⁵

Thus, several lines of evidence favor a pathogenic role for the P11S, S15F, and G32R mutations in absence seizures. First, all three mutations reside in evolutionarily conserved GC-rich regions and amino acid sequences of exon 1a and exon 2 (Figure 4A). Second, in silico analysis predicts the same cleavage sites for the wild-type as for P11S and S15F mutations in exon 1a. In silico analysis further predicts an alteration in secondary structure as a result of all mutations. More interestingly, both missense mutations in the signal peptide significantly increased N-glycosylation of the extracellular domain of the GABRB3 protein in actual experiments. G32R in the N terminus of the mature protein also increased in vitro glycosylation in actual experiments. N-glycosylation, conserved throughout evolution,^{46,47} is an essential modifier of protein folding

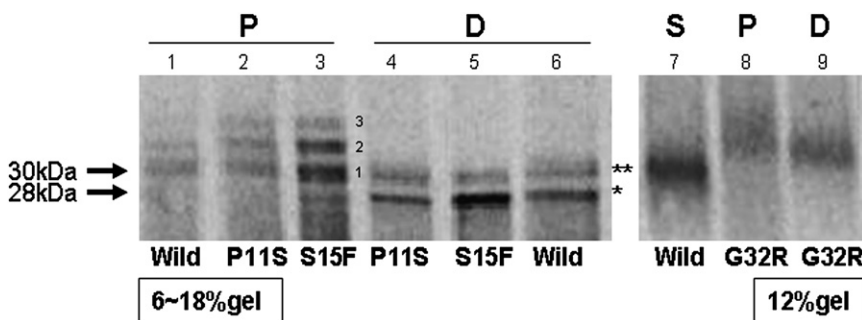


Figure 7. Increased Glycosylation of Mutated GABRB3 Protein

Products of in vitro translocation (P) and digestion (D) with N-glycosidase F, containing the exon 1a mutations P11S and S15F, were loaded on a 6%–18% gel (the left gel). Similar products containing the exon 2 mutation G32R were loaded on a 12% gel (the right gel). The supernatant protein (S) of only wild-type is shown (lane 7) in the 12% gel and is considered not to be translocated to microsomes and to pres-

ent the 30kDa GABRB3 including the signal peptide. The 30 kDa supernatant protein therefore consists of untranslocated “exon 1a to exon 6.” Translocated proteins (P) are shown in lanes 1–3 and 8. The molecular weight of band 1 was 30 kDa. Bands 2 and 3 of both P11S and S15F mutations in in vitro translocation (P) revealed clearly higher density than wild-type, suggesting increased glycosylation. After digestion with N-glycosidase F, two smaller sized bands, 28 kDa (*), and 30 kDa (**), appeared. The 30 kDa bands represent incompletely digested protein and 28 kDa bands represent completely digested protein. The translocated protein of G32R (P, lane 8) had only bands larger than 30kDa, also suggesting increased glycosylation. The band of G32R has higher molecular weight than the supernatant protein from the wild-type even after digestion.

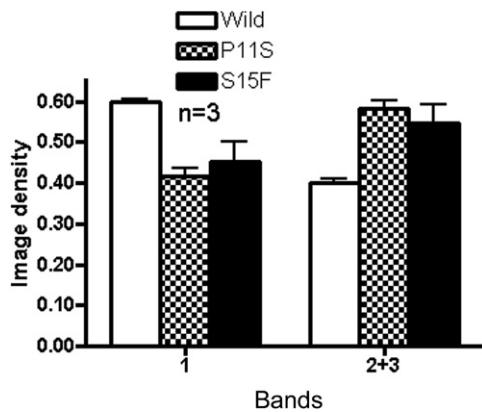


Figure 8. Quantitation of Density of Glycosylated Bands Comparing Wild-Type and Exon 1a Mutations Shown in Figure 7 Bands 2 and 3 are considered to be hyperglycosylated products. The image density of each band is the average of three experiments. Since bands 2 and 3 of one sample overlapped, the sum of bands 2 and 3 was compared. The proportion of the image density of band 1 and the sum of bands 2 and 3 were significantly different between the wild-type and mutations in exon 1a. (P11S, $p = 0.0004$, S15F, $p = 0.005$).

and transport; maintenance of cell structure; and protein adhesion, recognition, and cell-surface trafficking.^{47,48} Increased glycosylation can thus affect processing and subsequent assembly of GABA receptors,⁴⁹ possibly resulting in pathogenicity.⁵⁰

Third, mutations in the $\beta 3$ -v2 subunit of the GABA_A receptor in CAE show reduced $\alpha 1\beta 3$ -v2(P11S) $\gamma 2S$, $\alpha 1\beta 3$ -v2(S15F) $\gamma 2S$, and $\alpha 1\beta 3$ -v2(G32R) $\gamma 2S$ receptor currents.

Fourth, a deletion mutation in $\beta 3$ subunit of the GABA_A receptor is present in Angelman Syndrome, in which absence-like epilepsy is present.¹⁸

The fifth line of evidence supporting a pathogenic role for the P11S, S15F, and G32R mutations in absence seizures concerns *GABRB3* homozygous null mice that have absence-like episodes. *GABRB3* heterozygous null mice likewise show frequent absence-like arrests of movement with simultaneous theta bursts, suggesting an insufficient inhibition in the thalamocortical network.^{19,20,51} In *GABRB3*-deficient mice, ethosuximide stops seizures and CBZ aggravates seizures,⁵² the same pharmacological characteristics as those seen in human absence seizures. *GABRB3*, therefore, plays an important role in the thalamocortical network, which underlies absence seizures.^{51,53} Voltage-clamp recordings of reticular neurons and ventrobasal neurons of thalamic slices in *GABRB3* homozygous null mice show nearly abolished GABA-mediated inhibition in the reticular nucleus. GABA-mediated inhibition was unaffected in ventrobasal relay neurons. Oscillatory synchrony dramatically increases, showing that the recurrent inhibitory connections in the reticular nucleus, which are lost in the *GABRB3* null mice, actually result in desynchronization.⁵⁴

Why do some childhood absences remit, such as those in families M120, H12, and HMO10? The two mutations

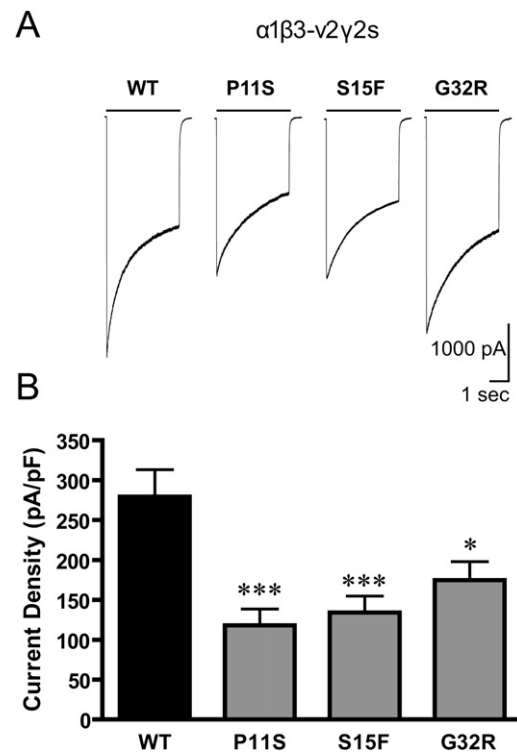


Figure 9. GABA-Evoked Currents of Mutations in Transfected HEK293 Cells

The current density from cells expressing the wild-type $\beta 3$ -v2 transcript was larger than in the cells expressing the $\beta 3$ -v2(P11S), $\beta 3$ -v2(S15F) or $\beta 3$ -v2(G32R) mutations.

The current density recorded from cells expressing GABA_A receptors with $\beta 3$ -v2 mutations associated with CAE was reduced. A. Representative traces of whole cell current elicited for 4 s with 1 mM GABA from cells expressing $\alpha 1\beta 3$ -v2 $\gamma 2S$ (WT), $\alpha 1\beta 3$ -v2(P11S) $\gamma 2S$, $\alpha 1\beta 3$ -v2(S15F) $\gamma 2S$ and $\alpha 1\beta 3$ -v2(G32R) $\gamma 2S$ receptors. B. Compared to cells expressing wild-type receptors ($n = 36$), the current densities of the cells expressing receptors containing the $\beta 3$ -v2 mutations P11S ($n = 18$), S15F ($n = 25$) and G32R ($n = 41$) were reduced (** $p < 0.001$, * $p < 0.05$).

(P11S and S15F) of these three families reside in exon 1a, located 543 base pairs upstream of exon 1. Exon 1a is richly expressed in whole fetal human brain, including the thalamus,³¹ whereas the adult brain contains a smaller amount of exon 1a. *GABRB3* protein is highly expressed in almost all brain regions at birth and stays constant in all regions except the thalamus.^{55–58} After birth, the *GABRB3* protein decreases rapidly in most thalamic nuclei but remains abundant in the reticular thalamic nucleus, where it is one of the main components of GABA_A.^{57,59} However, the alternative signal peptide coded by exon 1a is eliminated developmentally in some areas like the thalamus. Perhaps as exon 1a is eliminated developmentally, so are the absence seizures that correlate with mutations in exon 1a.

We only investigated 7 kb (the sum of the upper region from exon 1a, the full region from exon 1a to exon 3, all coding region from exon 4 to exon 9 and the part of intron 3 and 3' UTR) out of 230 kb for the full *GABRB3* gene, and

more mutations could be present in the other residues of *GABRB3*. Recently, the importance of epigenetic regulatory mechanisms for the expression of *GABRB3* has been emphasized,⁶⁰ in which a heritable change of *GABRB3*-gene expression could occur without a change in DNA sequence but with a change of DNA methylation on the CpG region. The mutations in *GABRB3* described here are associated with a gain in glycosylation of the $\beta 3$ subunit protein. Glycosylation of the $\beta 3$ subunit protein is known to change its maturation and alter overall GABAR trafficking to the cell surface from the endoplasmic reticulum. We suggest that the resulting hyperglycosylation and reduced current densities of the mutated $\beta 3$ subunit protein leads to absence seizures. Our results also allow us to hypothesize that mutated exon 1a leads to an abnormal isoform 2 precursor of *GABRB3* polipeptide during development. This in turn might explain the decrease or disappearance of absence seizures in adolescents and adults.

Supplemental Data

One figure is available at <http://www.ajhg.org/>.

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Web Resources

The URLs for data presented herein are as follows:

dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>

EMBL-EBI tools EMBOSS CpGPlot/CpGReport/Isochore, <http://www.ebi.ac.uk/emboss/cpgplot/>

GenBank, <http://www.ncbi.nih.gov/Genbank/>

GOR IV secondary structure prediction method, http://npsa-pbil.ibcp.fr/cgi/bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html

NCBI website, <http://www.ncbi.nlm.nih.gov/>

NetNGlyc 1.0, www.cbs.dtu.dk/services/NetNGlyc/

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

Polyphen program, <http://genetics.bwh.harvard.edu/pph/>

Primer 3 program, <http://frodo.wi.mit.edu/>

Signal CF, <http://chou.med.harvard.edu/bioinf/Signal-CF/>

SignalP 3.0, <http://cbs.dtu.dk/services/SignalP>

SOSUI, <http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>

TMHMM, version 2.0, <http://www.cbs.dtu.dk/services/TMHMM-2.0/>

UCSC Genome Browser, <http://genome.ucsc.edu/>

YinOYang, www.cbs.dtu.dk/services/YinOYang

Accession Numbers

The S15F and G32R missense mutations reported in this paper have been deposited in the NCBI databases under accession numbers NCBI_ss99307474 and NCBI_ss99307476, respectively.

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