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TRAFFICKING-DEFICIENT MUTANT *GABRG2* SUBUNIT AMOUNT MAY MODIFY EPILEPSY PHENOTYPE

Jing-Qiong Kang, MD, PhD¹, Wangzhen Shen, MD¹, and Robert L. Macdonald, MD, PhD^{1,2,3}

¹Department of Neurology, Vanderbilt University Medical Center, Nashville, TN 37212

²Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN 37212

³Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37212

Abstract

Objective—Genetic epilepsies and many other human genetic diseases display phenotypic heterogeneity, often for unknown reasons. Disease severity associated with nonsense mutations is dependent partially on mutation gene location and resulting efficiency of nonsense mediated mRNA decay (NMD) to eliminate potentially toxic proteins. Nonsense mutations in the last exon do not activate NMD, thus producing truncated proteins. We compared the protein metabolism and the impact on channel biogenesis, function and cellular homeostasis of truncated $\alpha 2$ subunits produced by *GABRG2* nonsense mutations associated with epilepsy of different severities and by a nonsense mutation in the last exon unassociated with epilepsy.

Methods—GABA_A receptor subunits were coexpressed in nonneuronal cells and neurons. NMD was studied using minigenes that support NMD. Protein degradation rates were determined using ³⁵S radiolabeling pulse chase. Channel function was determined by whole cell recordings while subunits trafficking and cellular toxicity were determined using flow cytometry, immunoblotting and immunohistochemistry.

Results—Although all *GABRG2* nonsense mutations resulted in loss of $\alpha 2$ subunit surface expression, the truncated subunits had different degradation rates and stabilities, suppression of wildtype subunit biogenesis and function, amounts of conjugation with polyubiquitin and endoplasmic reticulum stress levels.

Interpretation—We compared molecular phenotypes of *GABRG2* nonsense mutations. The findings suggest that despite the common loss of mutant allele function, each mutation produced different intracellular levels of trafficking-deficient subunits. The concentration-dependent suppression of wildtype channel function and cellular disturbance resulting from differences in mutant subunit metabolism may contribute to associated epilepsy severities and by implication to phenotypic heterogeneity in many inherited human diseases.

Keywords

GABA_A receptors; epilepsy phenotype; $\alpha 2$ subunit truncation mutation; loss of function; protein degradation; ER stress

Introduction

Mutations in GABA_A receptor $\alpha 1$, $\alpha 3$ and $\alpha 2$ subunits are associated with genetic epilepsy syndromes that vary from the relatively benign childhood absence epilepsy (CAE) syndrome, which remits with age, to more severe epilepsy syndromes associated with intellectual disability such as Dravet Syndrome^{1–5}. Among these mutations, those in $\alpha 2$ subunits are most frequently associated with epilepsy. This is not surprising given the wide expression and critical roles of $\alpha 2$ subunits in receptor composition⁶, channel properties⁷ and receptor targeting to and maintenance of synapses^{8,9}.

W429X is a recently reported nonsense mutation in the last exon of *GABRG2* and in the M3-M4 cytoplasmic loop of $\alpha 2$ subunits (Figure 1A, B) that is associated with febrile seizures and generalized tonic-clonic seizures¹⁰. Q390X is another nonsense mutation in a location similar to that of W429X (Figure 1A, B), but that is associated with epilepsies with a more severe phenotype including Dravet syndrome¹¹. We reported that the Q390X mutation did not activate nonsense-mediated mRNA decay (NMD), and that nonfunctional, but stable, mutant $\alpha 2$ (Q390X) subunits were produced and retained in the endoplasmic reticulum (ER). The $\alpha 2$ (Q390X) subunits had dominant negative effects on the biogenesis and trafficking of wildtype subunits¹² and were more prone to form intracellular high molecular weight complexes than were wildtype $\alpha 2$ subunits¹³. The molecular pathophysiology of the W429X mutation is unknown, and it is not clear why the phenotype produced by the W429X mutation is milder than that produced by the Q390X mutation since neither mutation should activate NMD and should produce similar truncated $\alpha 2$ subunits. It is also unknown why loss of one $\alpha 2$ allele function in heterozygous *GABRG2* knockout mice with no mutant $\alpha 2$ subunit protein detected only displayed hyperanxiety and were reportedly seizure free¹⁴.

To explore this, we expressed multiple truncated $\alpha 2$ subunits produced by nonsense mutations in *GABRG2* exons other than the last exon (Q40X, R136X, G273X, N297X, R323X and V360X) and in the last *GABRG2* exon (Q390X, W429X, K440X and W461X) to determine if truncated $\alpha 2$ subunits of varying lengths had similar or different effects on receptor biogenesis or functional properties. We found that none of the truncated $\alpha 2$ subunits had significant surface expression and thus all were nonfunctional. However, when we compared three representative truncations in the last *GABRG2* exon (Q390X, W429X, W461X), the truncated $\alpha 2$ subunits differed in stability and degradation rates, amounts of subunit aggregation and extent of suppression of the function of wildtype partnering subunits despite similar mRNA amounts. Thus the effects produced by these three truncated $\alpha 2$ subunits on GABAergic inhibition should differ, and the severity of the resultant epilepsy syndromes should be correspondingly different that should not depend on NMD efficiency since they are all in the last exon.

Materials and Methods

Expression vectors with GABA_A receptor subunits

The cDNAs encoding human $\alpha 1$, $\alpha 2$, $\alpha 3$ and FLAG- and HA-tagged GABA_A receptor subunits (e.g. $\alpha 2^{\text{FLAG}}$, $\alpha 1^{\text{FLAG}}$ or $\alpha 2^{\text{HA}}$) and $\alpha 2$ subunit minigenes in pcDNA(3.1) vector with Cytomegalovirus (CMV) promoter were as described previously^{12,13}. HA-tagged polyubiquitin (ubiquitin^{HA}) was kindly provided by Dr. Robert Coffey (Vanderbilt University Medical Center) and is the same as described in the previous study¹⁵. All the truncation mutations were generated with the method as previously described¹². The short form of the $\alpha 2$ subunit was used in this study, and numbering of $\alpha 2$ subunit amino acids was based on the immature peptide that includes the 39 amino acids of the signal peptide.

Cell culture and transfection

HEK293T and human L929 cells were cultured with the same condition as described¹². The mouse L929 cells was kindly provided by Dr. Terry Dermody (Vanderbilt University Medical Center) and maintained in culture medium as previously described¹⁶. Rat cortical neurons were prepared as previously described¹³.

Electrophysiology, confocal microscopy, [³⁵S] radiolabeling metabolic pulse-chase assays and flow cytometry

Experiments with lifted whole cell recordings, confocal microscopy, [³⁵S] radiolabeling metabolic pulse-chase assays and flow cytometry were carried out based on the protocols from previous studies^{13,17}.

Mice

The *GABRG2* knockout (KO) mouse line^{14,18} was kindly provided by Dr. Luscher (Penn State Univ) and the *GABRG2(Q390X)* knockin (KI) mouse line was generated by University Connecticut Health Center. The mice used in this study were in a C57BL/129 mixed background.

Data analysis

Macroscopic currents were low pass filtered at 2 kHz, digitized at 10 kHz, and analyzed using the pClamp9 software suite (Axon Instruments, Union City, CA). Numerical data were expressed as mean ± SEM. Proteins were quantified by ChemiImager AlphaEaseFC software and data were normalized to either wildtype subunit proteins or loading controls. Pulse-chase experiments were quantified by using Quantity One software (Bio-Rad, Hercules, CA). Fluorescence intensities from confocal microscopy experiments were determined using MetaMorph imaging software and the measurements were modified from previous description^{13,17}. Statistical significance, using Student's unpaired t test (GraphPad Prism, La Jolla, CA), was taken as $p < 0.05$.

Results

Loss of function is a common phenomenon for all nonsense mutations in GABA_A receptor $\gamma 2$ subunits

Nonsense mutations in early exons usually result in mRNA degradation by NMD and thus minimal intracellular truncated protein. Nonsense mutations in the last exon or 50–55 nt from the last exon-exon junction do not activate NMD and often produce nonfunctional, truncated proteins that are retained in the ER. Thus independent of mutation location the truncated proteins produced by nonsense mutations have minimal cell surface expression. We compared the surface expression of multiple epilepsy-related (Q40X, R136X, Q390X, W429X) and epilepsy-unrelated (G273X, N297X, R323X, V360X, K440X and W461X) *GABRG2* nonsense mutations in early and last exons that produce truncation of GABA_A receptor $\gamma 2$ subunits (Figure 1A, B). Using flow cytometry we determined the surface expression of the wildtype and mutant $\gamma 2$ subunits in HEK293T cells cotransfected with $\alpha 1$, $\gamma 2$ and $\gamma 2$ subunit cDNAs (Figure 1C). Compared with wildtype $\gamma 2$ subunits, all of the nonsense mutations resulted in significant loss of surface $\gamma 2$ subunit expression due to ER retention (Figure 1C, D), consistent with loss of function as a common defect among all the $\gamma 2$ subunit truncation mutations.

Similar amounts of mRNA, but different steady-state total protein levels, were present with $\gamma 2(Q390X)$, $\gamma 2(W429X)$ and $\gamma 2(W461X)$ subunit due to different protein degradation rates

We focused on three mutations in the last GABRG2 exon that should produce truncated $\gamma 2$ subunits (Figure 2A). We expressed NMD-sensitive wildtype and mutant $\gamma 2$ subunit minigenes as in a previous study¹² (Figure 2B), but none of them activated NMD as evidenced by similar amount of mRNA (Figure 2C). Surprisingly however, different amounts of mutant proteins were detected in both HEK293T cells (1 for $\gamma 2$, 2.31 ± 0.14 for $\gamma 2(Q390X)$ and 0.76 ± 0.06 for $\gamma 2(W429X)$ subunits) and in rat cortical neurons (1 for $\gamma 2^{FLAG}$, 2.79 ± 0.18 for $\gamma 2(Q390X)^{FLAG}$ and 0.89 ± 0.25 for $\gamma 2(W429X)^{FLAG}$ subunits in neurons) (Figure 2D–F). Also unexpectedly, $\gamma 2(W461X)$ subunits were almost undetectable in both HEK293T cells and neurons.

Since expression of the three mutant subunits resulted in markedly different levels of intracellular subunit proteins, we determined the degradation and synthesis rates of the mutant subunits using the [³⁵S] methionine radio-labeling pulse-chase technique. We found the half life of $\gamma 2(W429X)$ subunits was 91.7 min while that of wildtype $\gamma 2$ subunits was 106.5 min (Figure 3A, B). The half life of $\gamma 2(Q390X)^{FLAG}$ subunits has been previously reported to be ~4 hr¹³. The synthesis rates of wildtype $\gamma 2$ and mutant $\gamma 2(W429X)$ and $\gamma 2(Q390X)^{FLAG}$ ¹³ subunits were the same (Figure 3C, D). The differences in degradation rates among subunits ($\gamma 2(W461X)^{FLAG} \gg \gamma 2(W429X)^{FLAG} > \gamma 2^{FLAG} \gg \gamma 2(Q390X)^{FLAG}$) subunits thus resulted in different steady-state amounts of high molecular mass $\gamma 2$ subunit complexes as well as single subunits ($\gamma 2(Q390X)^{FLAG} \gg \gamma 2^{FLAG} > \gamma 2(W429X)^{FLAG} \gg \gamma 2(W461X)^{FLAG}$ subunits) (Figure 3E, F).

The epilepsy truncation mutations had reduced peak currents due to expression of $\alpha 1\beta 2$ rather than $\alpha 1\beta 2\gamma 2$ receptors

We compared the peak current amplitude and zinc sensitivity of currents recorded from cells coexpressing $\gamma 1$ and $\gamma 2$ subunits with $\gamma 2$, $\gamma 2(Q390X)$ or $\gamma 2(W429X)$ subunits or with mixed $\gamma 2/\gamma 2(W429X)$ subunits. The peak current from cells expressing mixed $\gamma 2/\gamma 2(W429X)$ subunits (1937 ± 656.9 , $n = 13$) was larger than from those expressing $\gamma 2(W429X)$ (876.9 ± 311.3 , $n = 10$) or $\gamma 1 \gamma 2 \gamma 2(Q390X)$ (102.2 ± 35.1 , $n = 8$) subunits but was smaller than recorded from cells expressing wildtype $\gamma 2$ subunits (4832 ± 1437 , $n = 7$) (Figure 4A, B). Compared to currents from cells expressing wildtype $\gamma 2$ subunits, currents recorded from cells expressing mutant $\gamma 2$ subunits had enhanced zinc sensitivity, suggesting surface expression of $\gamma 1 \gamma 2$ receptors (Figures 1D; 4C, D).

When coexpressed with $\alpha 1$ and $\beta 2$ subunits, truncated mutant $\gamma 2$ subunits had primarily immature glycosylation and were not trafficked to the cell surface

We determined glycosylation of wildtype and mutant $\gamma 2$ subunits with Endo-H or PNGase F treatment when coexpressed with $\gamma 1$ and $\gamma 2$ subunits¹². The majority of wildtype $\gamma 2^{HA}$ subunits (0.93 ± 0.07 , $n = 4$) were insensitive to Endo-H digestion, consistent with mature glycosylation and trafficking to and beyond the Golgi apparatus. In contrast, mutant $\gamma 2(W429X)^{HA}$ (0.07 ± 0.03 , $n = 4$) and $\gamma 2(Q390X)^{HA}$ (0.03 ± 0.01 , $n = 4$) subunits had minimal resistance to Endo-H treatment (Figure 5B), consistent with retention in the ER and failure to forward traffic. The portion of $\gamma 2(W429X)^{HA}$ subunits resistant to Endo-H digestion was greater than that of $\gamma 2(Q390X)^{HA}$ subunits. Consistent with the glycosylation patterns, wildtype $\gamma 2$ subunits had substantial surface expression but mutant $\gamma 2$ subunits all had minimal surface expression (3.5% - 8.8% of wildtype subunit levels) (Figure 5C, D).

Truncated $\gamma 2$ subunits reduced total expression and maturation of wildtype $\alpha 1$ and $\beta 2$ subunits in a concentration-dependent manner

Since the mutant subunits were primarily retained in the ER, we determined their impact on the biogenesis of wildtype subunits. Mutant $\gamma 2(Q390X)$ and $\gamma 2(W429X)$, but not $\gamma 2(W461X)$, subunits reduced total $\alpha 1$ subunit expression in both transfected HEK293T cells and mouse cortical neurons in cell culture (Figure 6A, B). A similar $\alpha 1$ subunit reduction was observed in the *GABRG2(Q390X)* KI, but not in the *GABRG2* KO, mouse brains (Figure 6C, D). We also determined the glycosylation pattern of $\alpha 1$ subunits in the presence of mutant $\gamma 2$ subunits. Compared with the wildtype subunits, the $\alpha 1$ subunits had reduced mature glycosylation when coexpressed with $\gamma 2(Q390X)$ and $\gamma 2(W429X)$, but not with $\gamma 2(W461X)$, subunits (Figure 6E, F), consistent with a dominant negative effect of the mutant $\gamma 2$ subunits. The differentially reduced surface expression and glycosylation maturation of $\alpha 1$ subunits was likely due to a differential oligomerization of $\alpha 1$ and mutant $\gamma 2$ subunits (Figure 6G, H). [^{35}S] radiolabeling pulse chase indicated that substantial degradation of $\alpha 1$ subunits occurred within 1 hr of onset of synthesis (Figure 6I, J). The differential $\alpha 1$ subunit suppression by mutant $\gamma 2$ subunits was likely due to the different steady-state levels of each mutant subunit (Figure 2D,E,F), with higher steady-state amounts of the mutant $\gamma 2$ subunits resulting in lower surface $\alpha 1$ subunit expression (Figure 7A, B). This notion was further supported by the fact that increasing mutant $\gamma 2$ subunits progressively diminished the surface and total expression of mutant more extensively than wildtype $\alpha 1$ subunits (Figure 7C1, C2).

Mutant $\gamma 2$ subunits had different amounts of high molecular mass protein complexes that were conjugated with ubiquitin

Our previous work demonstrated that $\gamma 2(Q390X)$ subunits were subject to ubiquitin-proteasome degradation^{19,20}. We demonstrated that in the cells coexpressing $\gamma 2^{FLAG}$ subunits with ubiquitin^{HA}, mutant $\gamma 2$ subunits were differentially conjugated with polyubiquitin when pulled down with HA beads (Figure 8A, B). There was a denser high molecular mass protein complex for mutant $\gamma 2(Q390X)^{FLAG}$ subunits (2.96 ± 0.21 , $n = 5$) than for wildtype (taken as 1, $n = 5$), $\gamma 2(W429X)^{FLAG}$ (0.87 ± 0.15 , $n = 5$) or $\gamma 2(W461X)^{FLAG}$ subunits (0.53 ± 0.03 , $n = 5$) (Figure 8B) while the total amount of polyubiquitin was unchanged in each condition (Figure 8C, D). Mutant $\gamma 2(Q390X)^{FLAG}$ and $\gamma 2(W429X)^{FLAG}$ subunits also had increased conjugation with polyubiquitin in the neuronal somata (Figure 8E, F).

Mutant $\gamma 2$ subunits protein triggered ER stress in a concentration-dependent manner

Accumulation of mutant protein in the ER could cause ER stress and trigger the unfolded protein response (UPR)²¹. Thus we determined the expression of the ER stress hallmark growth arrest- and DNA damage-inducible gene 153 (GADD153), also known as C/EBP homologous protein (CHOP), in mouse or human L929 cells (Figure 9A, C) and in neurons (Figure 9D, E). We demonstrated that mutant $\gamma 2$ subunits differentially upregulated GADD153 expression (wt 1.11 ± 0.10 , $n = 6$; $\gamma 2(Q390X)$ (1.60 ± 0.13 , $n = 7$; $\gamma 2(W429X)$ (1.43 ± 0.06 , $n = 6$; $\gamma 2(W461X)$ (1.22 ± 0.10 , $n = 5$) (Figure 9B). With increasing expression of the mutant subunits, the GADD153 expression levels were progressively increased (Figure 9C). Consistent with the findings in mouse and human L929 cells, mutant $\gamma 2(Q390X)$ and $\gamma 2(W429X)$ subunits also upregulated expression of GADD153 in neurons (Figure 9D, E).

Discussion

We propose that stability of trafficking-deficient mutant $\gamma 2$ subunits may be a phenotype modifier in the associated genetic epilepsies. With nonsense mutations, the presence of

different amounts of nonfunctional mutant protein among patients due to differential NMD efficiency has been associated with variation of disease phenotypes in both CNS and non CNS diseases^{22,23}. In both cases, patients with mutations that escaped NMD had more severe phenotypes. Our present study suggested that the degradation rate of the trafficking-deficient mutant subunits and the resultant ER resident truncated and trafficking-deficient subunits could be another disease phenotype modifier in addition to the efficiency of NMD, disease modifying genes²⁴ and genetic backgrounds. This may at least partially explain why the 2(Q390X) subunit mutation is associated with a more severe epilepsy phenotype¹¹ than the 2(W429X) subunit mutation¹⁰ and why heterozygous *GABRG2* deletion mice with no mutant truncated 2 subunits displayed only hyperanxiety¹⁴ or simple absence seizures²⁵.

We demonstrated that all nonsense *GABRG2* mutations share a common defect, loss of the mutant allele function. In addition, the truncated 2 subunits also had a dominant negative effect to reduce to different extents (2(Q390X) > 2(W429X) > 2(W461X)) the surface expression of 1 and 2 subunits and wildtype 2 subunits^{12,26}. The different extents of dominant negative suppression by the truncated subunits were due to subunit-dependent steady-state protein levels and degradation rates (2(390X) $t_{1/2}$ > 2(W429X) $t_{1/2}$ > 2(W461X) $t_{1/2}$). It is unclear why the truncated 2 subunits had different protein stabilities. Increased hydrophobicity or exposure of hydrophobic residues is associated with protein aggregation and lower solubility²⁷, and the additional 39 residues present in the 2(W429X) subunit (Figure 2A2) relative to the 2(390X) subunit are relatively hydrophilic, which may explain, at least in part, the reduced amount of 2(429X) subunit aggregation. It is worth noting that the reduced peak current, wildtype subunit expression and glycosylation maturation among mutations was only ~20%. Given that only hyperanxiety¹⁴ and absence seizure phenotypes were observed in heterozygous *GABRG2* KO mice²⁵, that patients with the loss-of-function *GABRG2*(Q390X) mutation had DS and that there was only a 20–30% more reduction of current and wildtype subunit expression in the *GABRG2*(Q390X) “heterozygous” condition, this small difference at the molecular level may be enough to alter clinical phenotype severity. Similar observations have been made in other diseases such as Marfan’s syndrome²⁸. Some Marfan’s syndrome mutations produce 7–10% of the wildtype transcript level, which results in a severe phenotype due to suppression of wildtype protein function by the mutant protein. In contrast, other mutations activated more efficient NMD resulting in lower transcript levels and produced only subclinical phenotypes. However, the altered magnitude may vary regionally and temporally. Future work with KI mice will further elucidate the correlation of molecular and behavioral phenotypes among these mutations.

The different amounts of each of the different mutant 2 subunits will result in their oligomerization with different amounts of wildtype subunits inside the ER. Mutant 2(Q390X) subunits had the most, 2(W429X) subunits had intermediate and 2(W461X) subunits had the least oligomerization with 1 subunits. The less stable mutant 2 subunits were degraded more rapidly and formed fewer stable oligomers with wildtype subunits, and thus were less effective in preventing wildtype subunits from forming functional receptors that trafficked to the cell surface.

Similarly, the polyubiquitin in cells expressing mutant 2(Q390X) subunits was more overloaded than in cells expressing mutant 2(W429X) or 2(W461X) subunits. Ubiquitin is not only critical for protein disposal, it is also essential for many cellular events including DNA repair, cell differentiation and apoptosis, transcription as well as stress tolerance, synaptic plasticity and receptor endocytosis^{29–32}. Dysfunction of ubiquitin has been established to contribute to pathogenesis of many misfolded or aggregated protein related diseases including Parkinson’s disease, amyotrophic lateral sclerosis and Huntington’s disease^{33,34}. In these diseases, chronic overloading of ubiquitin by misfolded or aggregated

protein causes impaired synaptic function, plasticity and eventually synaptic loss and neuronal death³⁵.

Each mutant $\gamma 2$ subunit also induced different levels of ER stress as evidenced by the upregulation of the ER stress marker GADD153. The more ER-retained $\gamma 2$ subunits, the more ER stress. It is established that ER accumulation of mutant protein can disturb cellular homeostasis and trigger the UPR³⁶. The truncated $\gamma 2$ subunits oligomerized with wildtype $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits, thus trapping them in the ER. Consequently, both mutant and trapped wildtype subunits could trigger the UPR. Sustained ER stress would impose a negative impact on cells resulting in cellular dysfunction, injury and death. Misfolded proteins impose a concentration-dependent fitness cost in yeast and trigger the UPR independent of loss of protein function³⁷, suggesting similar costs in various types of cells that could contribute to many human diseases. In addition to triggering the UPR in the ER, misfolded proteins in the cytosol provoke a heat-shock response, oxidative stress, altered membrane integrity and reduced growth rate³⁸. It is likely that ER retention of mutant protein contributes to the pathogenesis of many inherited degenerative diseases³⁹ and conventionally nondegenerative diseases like genetic epilepsies.

In summary, we found that the amount of ER-trapped truncated $\gamma 2$ subunits modulates wildtype channel function and cellular homeostasis. The loss of $\gamma 2$ subunit function could alter seizure threshold and give rise to the core phenotype such as febrile seizures. The mutant subunit-dependent suppression of biogenesis and function of wildtype partnering subunits and disturbance of cellular homeostasis including differential conjugation of polyubiquitin, activation of ER stress and many undefined cellular signaling pathways could contribute to phenotypic variation.

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Figure 1. All GABRG2 nonsense mutations produce nonfunctional $\gamma 2$ subunits with minimal surface expression

(A) Schematic presentation of human *GABRG2* nonsense mutations in exons 1–8 that are sensitive to nonsense-mediated mRNA decay (NMD) or in exon 9 (the last exon) that are insensitive to NMD. (B) Schematic presentation of $\gamma 2$ subunit topology and the locations of multiple subunit truncation mutations. The blue dots represent relative locations of the $\gamma 2$ subunit mutations unrelated to epilepsy, and the red dots indicate mutations associated with epilepsy. (C) When coexpressed with $\alpha 1$ and $\beta 2$ subunits, the relative surface level of each truncated $\gamma 2$ subunit was normalized to that obtained with wildtype $\gamma 2$ subunit. The data were pooled from multiple experiments. (D) The schematic presentation illustrates that

when coexpressed with $\alpha 1$ and $\alpha 2$ subunits, the wildtype $\alpha 2$ subunits in (C) were present on the cell surface as $\alpha 1 \alpha 2 \alpha 2$ receptors while only $\alpha 1 \alpha 2$ receptors were formed and present on the cell surface in the presence of the truncated $\alpha 2$ subunits that were retained intracellularly.

Figure 2. GABRG2 subunits with different truncation mutations in the last exon had similar mRNA levels but different steady-state protein levels

(A1) Schematic topologies of wildtype and mutant γ_2 subunits are presented. The dots represent the mutation locations, Q390X (red dot), W429X (yellow dot) and W461X (pink dot). (A2) These 39 amino acids were absent in the M3-M4 loop of the γ_2 (Q390X) subunit, but present in that of the γ_2 (W429X) subunit. The segment contains charged residues (bold), palmitoylation sites (pink), tyrosine phosphorylation sites (green) and four residues of the GABA_A receptor binding protein (GABARAP) binding sequences (underlined). (B, C) γ_2 subunit intron 8 minigenes were constructed by including intron 8 between exons 8 and 9 in γ_2 subunit cDNA constructs and were expressed in HEK293T cells. For PCR, primers in

flanking exons 7 and 9 (arrows) were used. **(D–E)** HEK293T cells or rat cortical neurons were cotransfected with 1, 2 and 2, 2(Q390X), 2(W429X) or 2(W461X) subunits or 2^{FLAG}, 2(Q390X)^{FLAG}, 2(W429X)^{FLAG} or 2(W461X)^{FLAG} subunits alone or the empty vector pcDNA (M) and then maintained in culture for 2 or 8 days. Total 2 or immunoprecipitated 2^{FLAG} subunits were fractionated by SDS-PAGE and immunoblotted by anti-2 (D) or anti-FLAG (E) antibody. LC stands for loading control. Individual wildtype 2 or 2^{FLAG} subunits were predicted to be 55 KDa and migrated at about 50 KDa, and individual native or FLAG-tagged truncated mutant 2(Q390X), 2(W429X) and 2(W461X) subunits migrated at lower molecular masses predicted to be about 40 KDa, 44.8 KDa and 49 KDa, respectively. **(F)** Total mutant subunit band IDVs were normalized to the wildtype g2 or g2^{FLAG} subunits (* < 0.05 vs wt; ††† p < 0.001 vs Q390X; §§§ p < 0.001 vs W461X).

Figure 3. The truncated g2 subunits had different protein degradation rates

(A, B) [^{35}S] pulse labeled untransfected cells (U) or cells transfected with wildtype γ_2^{FLAG} (W) or mutant $\gamma_2(\text{W429X})^{\text{FLAG}}$ (M) subunits were lysed, immunopurified using anti-FLAG beads and analyzed by SDS-PAGE. After 20 min of labeling, cells were chased for the indicated times. The percent radioactivity at each chase time point relative to the amount at time 0 for either wildtype or mutant subunits ($n = 5$) were plotted. (C, D) Cells expressing γ_2^{FLAG} or $\gamma_2(\text{W429X})^{\text{FLAG}}$ subunits were pulse-labeled, lysed and analyzed by SDS-PAGE after immunopurification. The percent radioactivity incorporated during subunit synthesis was plotted by normalizing to the value obtained at 20 min for either wildtype or mutant

subunits at each time point (n = 4). **(E, F)** Cells transfected with 2^{FLAG} (W), 2(W429X)^{FLAG} (429) or 2(Q390X)^{FLAG} (390) subunits were [³⁵S] pulse labeled for 20 min and then chased for 0, 1, 2 and 4 hr. Cycloheximide (CHX) (100 µg/ml) was added in the chase medium to block protein synthesis. The percent radioactivity at each time point relative to the amounts of radioactivity measured at time 0 was plotted for either wildtype or mutant subunits (n = 4). In A–F, both the 100 and 50 KDa bands were included. (In B and F, * < 0.05, **p < 0.01 vs wt; ††† p < 0.001 vs Q390X).

Figure 4. Currents recorded from cells coexpressing $\alpha 1$ and $\beta 2$ with truncated $\gamma 2$ subunits had reduced peak current amplitudes and were more sensitive to zinc inhibition
(A) GABA_A receptor currents were obtained with application of 1 mM GABA for 6 sec from HEK293T cells coexpressing $\alpha 1$ and $\beta 2$ subunits with wildtype $\gamma 2$ (1:1:1 cDNA ratio; black trace), mixed $\gamma 2$ / $\gamma 2$ (W429X) (1:1:0.5:0.5 cDNA ratio; gray trace with star), mutant $\gamma 2$ (W429X) (1:1:1 cDNA ratio, green trace) or mutant $\gamma 2$ (Q390X) (1:1:1 cDNA ratio, silver trace) subunits. **(B)** The amplitudes of GABA_A receptor currents from (A) were plotted. Values were mean $\pm$ SEM (n = 7–13 patches from 6 different transfections) (* p < 0.05; **p < 0.01 vs wt, †p < 0.05 vs Q390X). **(C)** GABA_A receptor currents were obtained with application of 1 mM GABA for 6 sec or with coapplication of 1 mM GABA and 10 μ M zinc

after preapplication of 10 μ M zinc for 6 sec. **(D)** The percent reduction of peak amplitudes of GABA_A receptor currents with coapplication of GABA and zinc compared with application of GABA alone were plotted. (* $p < 0.05$ *** $p < 0.001$ vs wt).

Figure 5. Truncated γ_2 subunits had minimal surface expression due to ER retention

(A) HEK293T cells were cotransfected with $\alpha 1$ and $\beta 2$ subunits and γ_2^{HA} (wt), γ_2^{HA} (Q390X) (390), or γ_2^{HA} (W429X) (429) subunits. The cells were treated with lactacystin (lac, 10 μ M) for 6 hr before harvest. Total lysates of the HEK293T cells were undigested (U) or digested (H) with Endo-H or PNGase-F (F) followed by SDS-PAGE and probed with anti-HA antibody. (B) The protein bands insensitive to Endo-H were quantified and presented as a fraction of their total undigested bands (50 kDa + 100 kDa). (C) The flow cytometry histograms depict surface HA levels detected with HA-Alexa 647 with coexpression of γ_2^{HA} , γ_2^{HA} / γ_2^{HA} (W429X), γ_2^{HA} (Q390X), γ_2^{HA} (W429X), or γ_2^{HA} (W461X) subunits or empty vector ($\alpha 1\beta 2$ control) with $\alpha 1$ and $\beta 2$ subunits. (D) The relative fluorescence intensities of HA signals from cells expressing the mutant γ_2^{HA} subunits or empty vector were normalized to those from wildtype γ_2^{HA} subunits. In B and D, *** p < 0.001 vs wt; †vs Q390X, § p < 0.05 vs W461X.

Figure 6. Total $\alpha 1$ subunit levels differed when coexpressed with truncated $\gamma 2$ subunits due to oligomerization with $\gamma 2$ subunits and ER retention

(A) Total lysates of HEK293T cells or rat cortical neurons expressing human $\alpha 1$ and $\gamma 2$ and $\beta 2$ subunits were analyzed by SDS-PAGE and immunoblotted by anti- $\alpha 1$ (BD24) antibody.

(B) Total $\alpha 1$ subunit protein IDVs were normalized to the $\alpha 1$ subunit of wildtype receptors.

(C) Total lysates from cerebral cortex (cor), cerebellum (cere), hippocampus (hip) and thalamus (thal) from new born heterozygous (het) *GABRG2* knockout (KO) and *GABRG2(Q390X)* knockin (KI) mice and their wildtype littermates (wt) were analyzed by SDS-PAGE and immunoblotted by anti- mouse $\alpha 1$ antibody. LC stands for loading control.

(D) Total $\alpha 1$ subunit protein IDVs from both KO and KI mice were normalized to the $\alpha 1$

subunit of their wildtype littermates ($n = 4$). **(E)** Total lysates of HEK cells expressing $\alpha_1\alpha_2\alpha_3$ receptors were either undigested (U) or digested (H) with Endo-H and analyzed by SDS-PAGE. **(F)** Total undigested α_1 subunit protein IDVs were normalized to their internal control Na^+/K^+ ATPase IDVs ($n = 5$). The fractions of Endo-H digested α_1 subunits were expressed as a ratio of the 48.4 KDa band (upper bands of H in E) over 48.4 plus 46 KDa bands (lower bands of H in E). **(G)** HEK293T cells expressing α_1^{FLAG} and α_2^{HA} subunits were treated with lactacystin (lac, 10 μM) for 6 hours and brefeldin A (0.5 $\mu\text{g/ml}$) 36 hours before harvest. The subunits were pulled down with α_1^{FLAG} subunits and immunoblotted for α_2^{HA} and α_1^{FLAG} subunits. **(H)** The ratios of the total amount of mutant α_2^{HA} to α_1^{FLAG} subunits were normalized to the ratio of wildtype α_2^{HA} to α_1^{FLAG} subunits. **(I)** HEK293T cells containing ^{35}S methionine radio-labeled wildtype or mutant $\alpha_1^{\text{FLAG}}\alpha_2\alpha_3$ subunits were chased and analyzed by SDS-PAGE. **(J)** The radioactivities of the α_1^{FLAG} subunits were normalized to α_1^{FLAG} subunits with coexpression of $\alpha_1^{\text{FLAG}}\alpha_2\alpha_3$ for each chase time. In B, F, H, J (* $p < 0.05$; *** $p < 0.001$ vs wt; † $p < 0.05$; †† $p < 0.01$ vs Q390X, § $p < 0.05$; §§ $p < 0.01$, §§§ $p < 0.001$ vs W461X).

Figure 7. Surface levels of $\alpha 1$ subunits coexpressed with $\beta 2$ and $\gamma 2$ subunits were reduced in a trafficking-deficient $\beta 2$ subunit concentration-dependent manner

(A) The flow cytometry histograms depict surface $\alpha 1$ subunit expression levels on HEK293T cells coexpressing the wildtype or mutant $\alpha 1\beta 2\gamma 2^{\text{HA}}$ subunits. $\alpha 1$ subunits were detected by fluorescently conjugated anti-human $\alpha 1$ antibody (1-Alexa Fluor-647). (B) The relative fluorescence intensity of $\alpha 1$ subunit signals was normalized to the wildtype $\alpha 1\beta 2\gamma 2^{\text{HA}}$ receptors (wt) (** $p < 0.01$ vs wt; † $p < 0.05$ vs Q390X, § $p < 0.05$ vs W461X). (C) Human L929 cells were cotransfected with $\alpha 1$ subunits and wildtype or mutant $\beta 2$ subunits at 1:1:1, 1:1:2.5, 1:1:5, 1:1:10 cDNA ratios. The total amounts of cDNA (12 μg) were equalized by adding empty vector. Both surface (C1) and total (C2) $\alpha 1$ subunits were

labeled with α 1-Alexa Fluor-647. The relative fluorescence intensities of α 1 subunits were normalized to intensities obtained with the wildtype 1:1:1 condition, arbitrarily taken as 1.

Figure 8. Polyubiquitin was conjugated with different amounts of truncated 2 subunits in different mutations

(A–D) Total lysates from HEK293T cells expressing HA-tagged polyubiquitin (ubiquitin^{HA}) and γ 2^{FLAG} subunits at a cDNA ratio of 1:3 were immunoprecipitated with HA beads and immunoblotted with anti-FLAG antibody (A) while ubiquitin^{HA} from A (C, left panel) or from total lysates of cells expressing empty vector (con) or ubiquitin^{HA} (C, right panel) was detected with anti-HA antibody. The γ 2^{FLAG} subunits that were pulled down with ubiquitin^{HA} in the mutant condition (γ 2^{FLAG}/ubiquitin^{HA}) were normalized to the wildtype condition. The γ 2^{FLAG} subunits were measured from about 75 KDa to 250 KDa (B) (**p < 0.01 vs Q390X; †p < 0.05 vs W461X). The total ubiquitin^{HA} IDVs in C (left panel) were

measured from about 25 KDa to 250 KDa. The ubiquitin^{HA} IDVs were normalized to the ubiquitin^{HA} IDVs when coexpressed with wildtype 2^{FLAG} subunits (D). (E) Rat cortical neurons coexpressing ubiquitin^{HA} and 2^{FLAG} subunits were permeabilized and labeled with polyclonal anti-FLAG antibody and monoclonal anti-HA antibodies and visualized with FITC rabbit IgG and Cy5 mouse IgG. (F) The ratio of 2^{FLAG} over the ubiquitin^{HA} fluorescence from the somata or processes in the same neurons were quantified and normalized to wt levels. (*p < 0.05, *** p<0.001 vs wt; †††p < 0.001 vs Q390X; §§§p < 0.001 vs W461X).

Figure 9. The truncated $\beta 2$ subunits upregulated expression of the ER stress hallmark GADD153 in a concentration-dependent manner

(A) Total lysates from mouse L929 cells expressing wildtype or mutant $\beta 2$ subunits were analyzed by SDS-PAGE and immunoblotted with mouse monoclonal anti-GADD153 antibody. (B) The total amounts of endogenous GADD153 were normalized to untreated controls (con) (* $p < 0.05$ vs wt $n = 7$). (C) Human L929 cells were transfected with $\beta 1$ $\beta 2$ subunits with varying amounts of $\beta 2$ subunit cDNA, and total GADD153 fluorescence intensities (FI) were analyzed by flow cytometry using anti-GADD153 antibody (1:100) conjugated with Alexa 647 IgG (1:500). The total amounts of cDNA (12 μ g) were normalized to empty vector. Relative GADD153 expression was normalized to untreated

control expression. Reduction of GADD153 in cells expressing large amounts of $\Delta 2$ (Q390X) subunits (1:1:5–1:1:10) was due to loss of dead cells, which were excluded from the detected cell population. **(D)** Rat cortical neurons expressing $\Delta 2^{\text{FLAG}}$ subunits were permeabilized and labeled with rabbit anti-FLAG antibody and mouse anti-GADD153 antibodies and visualized with FITC rabbit IgG and Cy5 mouse IgG. **(E)** Total GADD153 FI in $\Delta 2^{\text{FLAG}}$ positive neurons was normalized to GADD153 FI in neurons expressing wildtype $\Delta 2^{\text{FLAG}}$ subunits (** $p < 0.01$, *** $p < 0.001$ vs wt, †† $p < 0.01$ vs Q390X).