Trafficking-Deficient Mutant GABRG2 Subunit Amount May Modify Epilepsy Phenotype

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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 γ2 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: γ-Aminobutyric acid type A receptor subunits were coexpressed in non-neuronal 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, and subunits trafficking and cellular toxicity were determined using flow cytometry, immunoblotting, and immunohistochemistry.

Results: Although all GABRG2 nonsense mutations resulted in loss of γ2 subunit surface expression, the truncated subunits had different degradation rates and stabilities, suppression of wild-type 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 wild-type 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.

Mutations in γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor α1, β3, and γ2 subunits are associated with genetic epilepsy syndromes that vary from the relatively benign childhood absence epilepsy 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 γ2 subunits are most frequently associated with epilepsy. This is not surprising, given the wide expression and critical roles of γ2 subunits in receptor composition,6 channel properties,7 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 γ2 subunits (Fig 1A, B) that is associated with febrile seizures and generalized tonic–clonic seizures.10 Q390X is another nonsense mutation in a location similar to that of W429X (see Fig 1A, B), but that is associated with epilepsies with a more severe phenotype, including Dravet syndrome.11 We reported that the Q390X mutation did not activate nonsense-mediated mRNA decay (NMD), and that nonfunctional, but stable, mutant γ2(Q390X) subunits were produced and retained in the endoplasmic reticulum (ER). The

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c2(Q390X) subunits had dominant negative effects on the biogenesis and trafficking of wild-type subunits and were more prone to form intracellular high molecular weight complexes than were wild-type γ2 subunits. 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, because neither mutation should activate NMD and they should produce similar truncated γ2 subunits. It is also unknown why loss of one γ2 allele function in heterozygous GABRG2 knockout mice with no mutant γ2 subunit protein detected only displayed hyperanxiety and the mice were reportedly seizure free.

To explore this, we expressed multiple truncated γ2 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 whether truncated γ2 subunits of varying lengths had similar or different effects on receptor biogenesis or functional properties. We found that none of the truncated γ2 subunits had significant surface expression and thus all were nonfunctional. However, when we compared 3 representative truncations in the last GABRG2 exon (Q390X, W429X, and W461X), the truncated γ2 subunits differed in stability and degradation rates, amounts of subunit aggregation, and extent of suppression of the function of wild-type partnering subunits despite similar mRNA amounts. Thus, the effects produced by these 3 truncated γ2 subunits on GABAergic inhibition should differ, and the severity of the resultant epilepsy syndromes should be correspondingly different, not depending on NMD efficiency because they are all in the last exon.

Materials and Methods

Expression Vectors with GABA<sub>A</sub> Receptor Subunits

The cDNAs encoding human α1, β2, γ2, and FLAG (DYKDDDDK) and HA (YPYDVPDYA)-tagged GABA<sub>A</sub> receptor subunits (eg, γ2FLAG, α1FLAG, or γ2314A) and γ2 subunit minigenes in pCDNA3.1 vector with cytomegalovirus promoter were as described previously. HA-tagged polyubiquitin (ubiquitinHA) was kindly provided by Dr Robert Coffey (Vanderbilt University Medical Center) and is the same as described in the previous study. All the truncation mutations were generated with the method as previously described. The short form of the γ2 subunit was used in this study, and numbering of γ2 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 described. The mouse l929 cells were kindly provided by Dr Terry Dermody (Vanderbilt University Medical Center) and maintained in culture medium as previously described. Rat cortical neurons were prepared as previously described.
Electrophysiology, Confocal Microscopy, \( ^{35} \text{S} \) Radiolabeling Metabolic Pulse Chase Assays, and Flow Cytometry

Experiments with lifted whole cell recordings, confocal microscopy, \( ^{35} \text{S} \) 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 Bernhard Lüscher (Penn State University), and the GABRG2(Q390X) knockin (KI) mouse line was generated by University Connecticut Health Center. The mice used in this study were of a C57BL/129 mixed background.

Data Analysis

Macroscopic currents were low pass filtered at 2kHz, digitized at 10kHz, and analyzed using the pClamp9 software suite (Axon Instruments, Union City, CA). Numerical data were expressed as mean ± standard error of the mean. Proteins were quantified by ChemiImager AlphaEaseFC software, and data were normalized to either wild-type subunit proteins or loading controls. Pulse chase experiments were quantified by using Quantity 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 wild-type and mutant γ2 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 γ2(Q390X) subunit, but present in that of the γ2(W429X) subunit. The segment contains charged residues (bold), palmitoylation sites (pink), tyrosine phosphorylation sites (green), and 4 residues of the γ-aminobutyric acid type A receptor-binding protein (GABARAP) binding sequences (underlined). (B, C) γ2 Subunit intron 8 minigenes were constructed by including intron 8 between exons 8 and 9 in γ2 subunit cDNA constructs and were expressed in HEK293T cells. For reverse transcriptase polymerase chain reaction (RT-PCR), primers in flanking exons 7 and 9 (arrows) were used. (D, E) HEK293T cells or rat cortical neurons were cotransfected with α1, \( \beta_2 \), and \( \gamma_2 \), or γ2(W429X), or γ2(W461X) subunits, or γ2\( ^{\text{FLAG}} \), γ2(Q390X)\( ^{\text{FLAG}} \), γ2(W429X)\( ^{\text{FLAG}} \), or γ2(W461X)\( ^{\text{FLAG}} \) subunits alone or the empty vector pcDNA (M) and then maintained in culture for 2 or 8 days. Total γ2 or immunoprecipitated γ2\( ^{\text{FLAG}} \) subunits were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted by anti-γ2 (D) or anti-FLAG (E) antibody. LC = loading control. Individual wild-type (wt) γ2 or γ2\( ^{\text{FLAG}} \) subunits were predicted to be 55kDa and migrated at about 50kDa, and individual native or FLAG-tagged truncated mutant γ2(Q390X), γ2(W429X), and γ2(W461X) subunits migrated at lower molecular masses predicted to be about 40kDa, 44.8kDa, and 49kDa, respectively. (F) Total mutant subunit band integrated density values (IDVs) were normalized to the wild-type γ2 or γ2\( ^{\text{FLAG}} \) subunits (\( p < 0.05 \) vs wt; \( || \) \( p < 0.001 \) vs Q390X; \( p < 0.001 \) vs W461X).
One software (Bio-Rad, Hercules, CA). Fluorescence intensities from confocal microscopy experiments were determined using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA), and the measurements were modified from previous description.13,17 Statistical significance, using Student unpaired t test (Prism; GraphPad Software, 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 \( \gamma2 \) 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 to 55 nucleotides from the last exon–exon junction do not activate NMD and often produce non-functional, 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, and 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 \( \gamma2 \) subunits (see Fig 1A, B). Using
flow cytometry, we determined the surface expression of the wild-type and mutant γ2 subunits in HEK293T cells cotransfected with α1, β2, and γ2 subunit cDNAs (see Fig 1C). Compared with wild-type γ2 subunits, all of the nonsense mutations resulted in significant loss of surface γ2 subunit expression due to ER retention (see Fig 1C, D), consistent with loss of function as a common defect among all the γ2 subunit truncation mutations.

**Similar Amounts of mRNA, but Different Steady-State Total Protein Levels, Were Present with γ2(Q390X), γ2(W429X), and γ2(W461X) Subunits due to Different Protein Degradation Rates**

We focused on 3 mutations in the last GABRG2 exon that should produce truncated γ2 subunits (Fig 2). We expressed NMD-sensitive wild-type and mutant γ2 subunit minigenes as in a previous study, but none of them activated NMD as evidenced by similar amounts of mRNA. Surprisingly, however, different amounts of mutant proteins were detected in both HEK293T cells (1 for γ2, 2.31 ± 0.14 for γ2[Q390X], and 0.76 ± 0.06 for γ2[W429X] subunits) and in rat cortical neurons (1 for γ2[FLAG], 2.79 ± 0.18 for γ2[Q390X][FLAG], and 0.89 ± 0.25 for γ2[W429X][FLAG] subunits in neurons. Also unexpectedly, γ2(W461X) subunits were almost undetectable in both HEK293T cells and neurons.

Because expression of the 3 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 radiolabeling pulse chase technique. We found the half-life of γ2(W429X) subunits was 91.7 minutes, whereas that of wild-type γ2 subunits was 106.5 minutes (Fig 3A, B). The half-life of γ2(Q390X)[FLAG] subunits has been previously reported to be ~4 hours. The synthesis rates of wild-type γ2 and mutant γ2(W429X) and γ2(Q390X)[FLAG] subunits were the same (see Fig 3C, D). The differences in degradation rates among subunits (γ2[W461X][FLAG] > γ2[W429X][FLAG] > γ2[Q390X][FLAG]) thus resulted in different steady-state amounts of high molecular mass γ2 subunit complexes as well as single subunits (γ2[Q390X][FLAG] > γ2[W429X][FLAG] > γ2[W461X][FLAG]; see Fig 3E, F).

**FIGURE 4: Currents recorded from cells coexpressing α1 and β2 with truncated γ2 subunits had reduced peak current amplitudes and were more sensitive to zinc inhibition. (A) γ-Aminobutyric acid type A (GABA_A) receptor currents were obtained with application of 1mM GABA for 6 seconds from HEK293T cells coexpressing α1 and β2 subunits with wild-type γ2 (1:1:1 cDNA ratio; black trace), mixed γ2[α1β2γ2] (1:1:0.5:0.5 cDNA ratio; gray trace with star), mutant γ2(W429X) (1:1:1 cDNA ratio, green trace), or mutant γ2(Q390X) (1:1:1 cDNA ratio, silver trace) subunits. (B) The amplitudes of GABA_A receptor currents from A were plotted. Values were mean ± standard error of the mean (n = 7–13 patches from 6 different transfections; *p < 0.05, **p < 0.01 vs wild type [wt]; †p < 0.05 vs Q390X). (C) GABA_A receptor currents were obtained with application of 1mM GABA for 6 seconds or with coapplication of 1mM GABA and 10μM zinc after preapplication of 10μM zinc for 6 seconds. (D) The percentage reduction of peak amplitudes of GABA_A receptor currents with coapplication of GABA and zinc compared with application of 1mM GABA alone were plotted (*p < 0.05, **p < 0.001 vs wt). [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]**
The Epilepsy Truncation Mutations Had Reduced Peak Currents due to Expression of α1β2 rather than α1β2γ2 Receptors

We compared the peak current amplitude and zinc sensitivity of currents recorded from cells coexpressing α1 and β2 subunits with γ2, γ2(Q390X), or γ2(W429X) subunits or with mixed γ2/γ2(W429X) subunits. The peak current from cells expressing mixed γ2/γ2(W429X) subunits (1,937 ± 656.9 pA, n = 13) was larger than from those expressing γ2(W429X) (876.9 ± 311.3 pA, n = 10) or α1β2γ2(Q390X) (102.2 ± 35.1 pA, n = 8) subunits but was smaller than those recorded from cells expressing wild-type γ2 subunits (4,832 ± 1437 pA, n = 7; Fig 4A, B). Compared to currents from cells expressing wild-type γ2 subunits, currents recorded from cells expressing mutant γ2 subunits had enhanced zinc sensitivity, suggesting surface expression of α1β2 receptors (see Figs 1D; 4C, D).

When Coexpressed with α1 and β2 Subunits, Truncated Mutant γ2 Subunits Had Primarily Immature Glycosylation and Were Not Trafficked to the Cell Surface

We determined glycosylation of wild-type and mutant γ2 subunits with Endo-H or PNGase F treatment when coexpressed with α1 and β2 subunits.12 The majority of wild-type γ2HA 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 γ2(W429X)HA (0.07 ± 0.03, n = 4) and γ2(Q390X)HA (0.03 ± 0.01, n = 4) subunits had minimal resistance to Endo-H treatment (Fig 5B), consistent with retention in the ER and failure to forward traffic. The portion of γ2(W429X)HA subunits resistant to Endo-H digestion was greater than that of γ2(Q390X)HA subunits. Consistent with the glycosylation patterns, wild-type γ2 subunits had substantial surface expression, but mutant γ2 subunits all had minimal surface expression (3.5–8.8% of wild-type subunit levels; see Fig 5C, D).

Truncated γ2 Subunits Reduced Total Expression and Maturation of Wild-Type α1 and β2 Subunits in a Concentration-Dependent Manner

Because the mutant subunits were primarily retained in the ER, we determined their impact on the biogenesis of wild-type γ2 subunits (4,832 ± 1437 pA, n = 7; Fig 4A, B). Compared to currents from cells expressing wild-type γ2 subunits, currents recorded from cells expressing mutant γ2 subunits had enhanced zinc sensitivity, suggesting surface expression of α1β2 receptors (see Figs 1D; 4C, D).

FIGURE 5: Truncated γ2 subunits had minimal surface expression due to endoplasmic reticulum retention. (A) HEK293T cells were cotransfected with α1 and β2 subunits and γ2HA (wt), γ2(Q390X)HA (390), or γ2(W429X)HA (429) subunits. The cells were treated with lactacystin (lac; 10μM) for 6 hours before harvest. Total lysates of the HEK293T cells were undigested (U) or digested (H) with Endo-H or PNGase-F (F) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with anti-HA antibody. ATPase = adenosine triphosphatase. (B) The protein bands insensitive to Endo-H were quantified and presented as a fraction of their total undigested bands (50kDa + 100kDa). (C) The flow cytometry histograms depict surface HA levels detected with HA-Alexa 647 with coexpression of γ2HA (wt), γ2(Q390X)HA (390), or γ2(W429X)HA (429) subunits or empty vector (α1β2 control) with α1 and β2 subunits. (D) The relative fluorescence intensities of HA signals from cells expressing the mutant γ2HA subunits or empty vector were normalized to those from wild-type γ2HA subunits. In B and D, ***p<0.001 vs wt; p<0.001 vs Q390X; p<0.05 vs W461X. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]
FIGURE 6: Total α1 subunit levels differed when coexpressed with truncated γ2 subunits due to oligomerization with γ2 subunits and endoplasmic reticulum retention. (A) Total lysates of HEK293T cells or rat cortical neurons expressing human α1 and β2 and γ2 subunits were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted by anti-α1 (BD24) antibody. (B) Total α1 subunit protein integrated density values (IDVs) were normalized to the α1 subunit of wild-type receptors. (C) Total lysates from cerebral cortex (cor), cerebellum (cere), hippocampus (hip), and thalamus (thal) from newborn heterozygous (het) GABRG2 knockout (KO) and GABRG2(Q390X) knockin (KI) mice and their wild-type littermates (wt) were analyzed by SDS-PAGE and immunoblotted by antimouse α1 antibody. LC = loading control. (D) Total α1 subunit protein IDVs from both KO and KI mice were normalized to the α1 subunit of their wild-type littermates (n=4). (E) Total lysates of HEK cells expressing α1β2γ2 receptors were either undigested (U) or digested (H) with Endo-H and analyzed by SDS-PAGE. (F) Total undigested α1 subunit protein IDVs were normalized to their internal control Na+/K+ adenosine triphosphatase IDVs (n=5). The fractions of Endo-H–digested α1 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 α1FLAG and γ2HA subunits were treated with lactacystin (lac; 10 μM) for 6 hours and brefeldin A (0.5 μg/ml) 36 hours before harvest. The subunits were pulled down with α1FLAG subunits and immunoblotted for γ2HA and α1FLAG subunits. In G, IB = immunoblotting; IP = immunoprecipitation. (H) The ratios of the total amount of mutant γ2HA to α1FLAG subunits were normalized to the ratio of wild-type γ2HA to α1FLAG subunits. (I) HEK293T cells containing 35S methionine radio-labeled wild-type or mutant α1FLAG/γ2;2 subunits were chased and analyzed by SDS-PAGE. (J) The radioactivity of the α1FLAG subunits was normalized to α1FLAG subunits with coexpression of α1FLAG/γ2;2 for each chase time. In B, F, H, J (*p<0.05, **p<0.01 vs wt; †p<0.05, ††p<0.01 vs Q390X; p<0.05, p<0.01, p<0.001 vs W461X).
wild-type subunits. Mutant γ2(Q390X) and γ2(W429X) but not γ2(W461X) subunits reduced total α1 subunit expression in both transfected HEK293T cells and mouse cortical neurons in cell culture (Fig 6). A similar α1 subunit reduction was observed in the GABRG2(Q390X) KI but not in the GABRG2 KO mouse brains. We also determined the glycosylation pattern of α1 subunits in the presence of mutant γ2 subunits. Compared with the wild-type subunits, the α1 subunits had reduced mature glycosylation when coexpressed with γ2(Q390X) and γ2(W429X) but not with γ2(W461X) subunits, consistent with a dominant negative effect of the mutant γ2 subunits. The differentially reduced surface expression and glycosylation maturation of α1 subunits was likely due to a
FIGURE 8: Polyubiquitin was conjugated with different amounts of truncated γ2 subunits in different mutations. (A–D) Total lysates from HEK293T cells expressing HA-tagged polyubiquitin (ubiquitinHA) and γ2FLAG subunits at a cDNA ratio of 1:3 were immunoprecipitated with HA beads and immunoblotted with anti-FLAG antibody (A), and ubiquitinHA from A (C, left panel) or from total lysates of cells expressing empty vector (con) or ubiquitinHA (C, right panel) was detected with anti-HA antibody. The γ2FLAG subunits that were pulled down with ubiquitinHA in the mutant condition (γ2FLAG/ubiquitinHA) were normalized to the wild-type (wt) condition. (B) The γ2FLAG subunits were measured from about 75 to 250kDa (***p < 0.01 vs Q390X; *p < 0.05 vs W461X). The total ubiquitinHA integrated density values (IDVs) in C (left panel) were measured from about 25 to 250 kDa. (D) The ubiquitinHA IDVs were normalized to the ubiquitinHA IDVs when coexpressed with wt γ2FLAG subunits. (E) Rat cortical neurons coexpressing ubiquitinHA and γ2FLAG subunits were permeabilized and labeled with polyclonal anti-FLAG antibody and monoclonal anti-HA antibodies and visualized with fluorescein isothiocyanate rabbit immunoglobulin G (IgG) and Cy5 mouse IgG. (F) The ratio of γ2FLAG over the ubiquitinHA 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). In A and C, IB = immunoblotting; IP = immunoprecipitation. In E, DIC = differential interference contrast.
differential oligomerization of α1 and mutant γ2 subunits. [35S] Radiolabeling pulse chase indicated that substantial degradation of α1 subunits occurred within 1 hour of onset of synthesis. The differential α1 subunit suppression by mutant γ2 subunits was likely due to the different steady-state levels of each mutant subunit (see Fig 2D–F), with higher steady-state amounts of the mutant γ2 subunits resulting in lower surface α1 subunit expression (Fig 7A, B). This notion was further supported by the finding that increasing mutant γ2 subunits progressively diminished the surface and total expression of wild-type α1 subunits (see Fig 7C1, C2).

**Mutant γ2 Subunits Had Different Amounts of High Molecular Mass Protein Complexes That Were Conjugated with Ubiquitin**

Our previous work demonstrated that γ2(Q390X) subunits were subject to ubiquitin–proteasome degradation.19,20 We demonstrated that in the cells coexpressing γ2FLAG subunits with ubiquitinHA, mutant γ2 subunits were differentially conjugated with polyubiquitin when pulled down with HA beads (Fig 8). There was a denser high molecular mass protein complex for mutant γ2(Q390X)FLAG subunits (2.96 ± 0.21, n = 5) than for wild-type (taken as 1, n = 5), γ2(W429X)FLAG (0.87 ± 0.15, n = 5), or γ2(W461X)FLAG subunits (0.53 ± 0.03, n = 5), whereas the total amount of polyubiquitin was unchanged in each condition. Mutant γ2(Q390X)FLAG and γ2(W429X)FLAG subunits also had increased conjugation with polyubiquitin in the neuronal somata.

**Mutant γ2 Subunit 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 CHOP (C/EBP homologous protein), in mouse or human L929

![Figure 9](image-url)

**Figure 9**: The truncated γ2 subunits upregulated expression of the endoplasmic reticulum stress hallmark GADD153 in a concentration-dependent manner. (A) Total lysates from mouse L929 cells expressing wild-type (wt) or mutant γ2 subunits were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis 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) Relative amounts of GADD153 fluorescence intensities vs control (wt). (D) Human L929 cells were transfected with α1β2 subunits with varying amounts of γ2 subunit cDNA, and total GADD153 fluorescence intensities (Fls) were analyzed by flow cytometry using anti-GADD153 antibody (1:100) conjugated with Alexa 647 immunoglobulin G (IgG; 1:500). The total amounts of cDNA (12 µg) were normalized to empty vector. Relative GADD153 expression was normalized to untreated control expression. Reduction of GADD153 in cells expressing large amounts of γ2(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 γ2FLAG subunits were permeabilized and labeled with rabbit anti-FLAG antibody and mouse anti-GADD153 antibodies and visualized with fluorescein isothiocyanate rabbit IgG and Cy5 mouse IgG. (E) Total GADD153 Fls in γ2FLAG-positive neurons was normalized to GADD153 Fl in neurons expressing wild-type γ2FLAG subunits (***p < 0.01, ****p < 0.001 vs wt; ††p < 0.01 vs Q390X).
cells and in neurons (Fig 9). We demonstrated that mutant γ2 subunits differentially upregulated GADD153 expression (wild type = 1.11 ± 0.10, n = 6; γ2(W429X) = 1.60 ± 0.13, n = 7; γ2(W429X) = 1.43 ± 0.06, n = 6; γ2(W461X) = 1.22 ± 0.10, n = 5). With increasing expression of the mutant subunits, the GADD153 expression levels were progressively increased. Consistent with the findings in mouse and human L929 cells, mutant γ2(Q390X) and γ2(W429X) subunits also upregulated expression of GADD153 in neurons.

**Discussion**

We propose that stability of trafficking-deficient mutant γ2 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 central nervous system (CNS) and non-CNS diseases. 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 genes, and genetic backgrounds. This may at least partially explain why the γ2(Q390X) subunit mutation is associated with a more severe epilepsy phenotype than the γ2(W429X) subunit mutation, and why heterozygous GABRG2 deletion mice with no mutant truncated γ2 subunits displayed only hyperanxiety or simple absence seizures.

We demonstrated that all nonsense GABRG2 mutations share a common defect, loss of the mutant allele function. In addition, the truncated γ2 subunits also had a dominant negative effect to reduce to different extents (γ2(Q390X) > γ2(W429X) > γ2(W461X)) the surface expression of α1 and β2 subunits and wild-type γ2 subunits. The different extents of dominant negative suppression by the truncated subunits were due to subunit-dependent steady-state protein levels and degradation rates (γ2[390X] t1/2 > γ2[W429X] t1/2 > γ2[W461X] t1/2). It is unclear why the truncated γ2 subunits had different protein stabilities. Increased hydrophobicity or exposure of hydrophobic residues is associated with protein aggregation and lower solubility, and the additional 39 residues present in the γ2(W429X) subunit (see Fig 2A2) relative to the γ2(390X) subunit are relatively hydrophilic, which may explain, at least in part, the reduced amount of γ2(429X) subunit aggregation. It is worth noting that the reduced peak current, wild-type subunit expression, and glycosylation maturation among mutations was only ~20%. Given that only hyperanxiety and absence seizure phenotypes were observed in heterozygous GABRG2 KO mice, that patients with the loss-of-function GABRG2(Q390X) mutation had Dravet syndrome, and that there was only 20 to 30% more reduction of current and wild-type 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 syndrome. Some Marfan syndrome mutations produce 7 to 10% of the wild-type transcript level, which results in a severe phenotype due to suppression of wild-type 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 γ2 subunits will result in their oligomerization with different amounts of wild-type subunits inside the ER. Mutant γ2(Q390X) subunits had the most, γ2(W429X) subunits had intermediate, and γ2(W461X) subunits had the least oligomerization with α1 subunits. The less stable mutant γ2 subunits were degraded more rapidly and formed fewer stable oligomers with wild-type subunits, and thus were less effective in preventing wild-type subunits from forming functional receptors that trafficked to the cell surface.

Similarly, the polyubiquitin in cells expressing mutant γ2(Q390X) subunits was more overloaded than in cells expressing mutant γ2(W429X) or γ2(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. Dysfunction of ubiquitin has been established to contribute to pathogenesis of many misfolded or aggregated protein-related diseases, including Parkinson disease, amyotrophic lateral sclerosis, and Huntington disease. In these diseases, chronic overloading of ubiquitin by misfolded or aggregated protein causes impaired synaptic function, plasticity, and eventually synaptic loss and neuronal death.
and trigger the UPR.\textsuperscript{36} The truncated \(\gamma_2\) subunits oligomerized with wild-type \(\alpha_1, \beta_2,\) and \(\gamma_2\) subunits, thus trapping them in the ER. Consequently, both mutant and trapped wild-type 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 function,\textsuperscript{37} 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 rate.\textsuperscript{38} It is likely that ER retention of mutant protein contributes to the pathogenesis of many inherited degenerative diseases\textsuperscript{39} and conventionally nondegenerative diseases like genetic epilepsies.

In summary, we found that the amount of ER-trapped truncated \(\gamma_2\) subunits modulates wild-type 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 wild-type 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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Potential Conflicts of Interest

Nothing to report.

References

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