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The *GABRG2* Nonsense Mutation, Q40X, Associated with Dravet Syndrome Activated NMD and Generated a Truncated Subunit That was Partially Rescued by aminoglycoside-Induced Stop Codon Read-through

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Abstract

The *GABRG2* nonsense mutation, Q40X, is associated with the severe epilepsy syndrome, Dravet syndrome, and is predicted to generate a premature translation-termination codon (PTC) in the GABA_A receptor $\gamma 2$ subunit mRNA in a position that codes for the first amino acid of the mutant subunit. We determined the effects of the mutation on $\gamma 2$ subunit mRNA and protein synthesis and degradation, as well as on $\alpha 1\beta 2\gamma 2$ GABA_A receptor assembly, trafficking and surface expression in HEK cells. Using bacterial artificial chromosome (BAC) constructs, we found that $\gamma 2(Q40X)$ subunit mRNA was degraded by nonsense mediated mRNA decay (NMD). Undegraded mutant mRNA was translated to a truncated peptide, likely the signal peptide, which was cleaved further. We also found that mutant $\gamma 2(Q40X)$ subunits did not assemble into functional receptors, thus decreasing GABA-evoked current amplitudes. The *GABRG2(Q40X)* mutation is one of several epilepsy-associated nonsense mutations that have the potential to be rescued by reading through the PTC, thus restoring full-length protein translation. As a first approach, we investigated use of the aminoglycoside, gentamicin, to rescue translation of intact mutant subunits by inducing mRNA read-through. In the presence of gentamicin, synthesis of full length $\gamma 2$ subunits was partially restored, and surface biotinylation and whole cell recording experiments suggested that rescued $\gamma 2$ subunits could incorporate into functional, surface GABA_A receptors, indicating a possible direction for future therapy.

Keywords

GABA_A receptors; Dravet syndrome; generalized epilepsy; $\gamma 2$ subunit; *GABRG2(Q40X)* mutation; loss of function; gentamicin

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Introduction

Epilepsy is a common neurological disorder that affects about 1% of the world's population (Sander, 2003). Epilepsy syndromes are usually either symptomatic and due to a known brain injury or idiopathic and not due to brain injury. Idiopathic genetic epilepsy syndromes (IGES) comprise ~30% of all cases and can vary in severity from the mild childhood absence epilepsy syndrome to the severe Dravet syndrome (Reid et al., 2009; Steinlein, 2004). While many IGES are benign, Dravet syndrome is not. It is associated with myoclonic and generalized tonic-clonic seizures that begin at an early age, frequent episodes of status epilepticus and progressive intellectual decline, and it is resistant to a wide range of antiepileptic drugs. About one half of Dravet syndrome-associated mutations are nonsense mutations in genes such as voltage-gated sodium channels that create premature translation-termination codons (PTCs), and thus, truncated subunit proteins (De Jonghe, 2011). Although rare, nonsense mutations in GABA_A receptor subunit genes have been identified also in Dravet syndrome patients (Harkin et al., 2002). *GABRG2(Q40X)* is a nonsense mutation located in GABA_A receptor $\gamma 2$ subunits that has been associated with Dravet syndrome (Kanaumi et al., 2004).

GABA_A receptors are heteropentameric chloride ion channels that mediate the majority of inhibitory neurotransmission in the CNS. The receptor complex is composed of five subunits from nineteen different genes, and the main synaptic receptors are composed of two α subunits, two β subunits and one $\gamma 2$ subunit. Out of the fifteen *GABR* epilepsy-associated mutations or variants, seven are in *GABRG2*, and these mutations have been shown to decrease channel function by altering receptor biogenesis or channel function (Macdonald and Kang, 2009). The *GABRG2(Q40X)* mutation was shown to impair GABA_A receptor channel function and to form granules in neurons (Kanaumi et al., 2004). However, the effects of this mutation on GABA_A receptor function are unknown.

Current therapies for the devastating epilepsies produced by truncation mutations are symptomatic and relatively ineffective. One potential treatment would be to rescue the nonsense mutation by drug-induced read-through. Aminoglycosides such as G-418 and gentamicin partially restore the expression and function of full-length proteins by inducing PTC read-through (Brooks et al., 2006; Linde et al., 2007b). A drug designed to specifically induce ribosomes to read through stop codons generated by PTCs (Ataluren®) is currently under Phase 3 clinical trial to treat cystic fibrosis patients carrying PTCs in the gene *CFTR*, further confirming the clinical feasibility of this strategy (Goodier and Mayer, 2009; Welch et al., 2007). Because the dramatic loss of function produced by subunit truncation mutations likely contributes to the pathogenesis of Dravet syndrome, the read-through strategy presents a potential approach to treat epilepsies associated with PTCs.

To explore the effects of the *GABRG2(Q40X)* mutation, we studied the transcription of wildtype and mutant *GABRG2* mRNA, the translation of $\gamma 2$ and $\gamma 2(Q40X)$ subunit protein and the properties of GABA_A receptors that were assembled with coexpression of $\alpha 1$, $\beta 2$ and $\gamma 2$ or $\gamma 2(Q40X)$ subunits in HEK 293T cells. We found that the Q40X mutation engaged the cellular quality control machinery to activate nonsense mediated mRNA decay (NMD) to decrease mutant mRNA levels and produced a truncated signal peptide that was not incorporated into functional receptors. Restoring expression of the full-length wildtype $\gamma 2$ subunit by read-through should be able to rescue the subunit truncation caused by the Q40X mutation. To evaluate the plausibility of aminoglycoside-induced read-through of an epilepsy-associated PTC, we determined whether gentamicin could rescue mutant $\gamma 2(Q40X)$ subunits. We demonstrated that gentamicin partially restored the expression of full-length $\gamma 2$ subunits, and that the rescued $\gamma 2$ subunits assembled with $\alpha 1\beta 2$ subunits to form functional $\alpha 1\beta 2\gamma 2$ GABA_A receptors.

Materials and Methods

Expression vectors

The coding sequences of human $\alpha 1$, $\beta 2$ and $\gamma 2S$ GABA_A receptor subunits were cloned into pcDNA3.1 expression vectors (Invitrogen) as previously described (Gallagher et al., 2005). All subunit residues were numbered based on the immature peptide. The $\gamma 2S(Q40X)$ and $\gamma 2S(Q40X,TGA)$ subunit constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene). An HA epitope was inserted at a functionally silent site (between the 4th and 5th residue of the mature peptide of both wildtype and mutant $\gamma 2S$ subunit) to facilitate our experiments (Connolly et al., 1996). To detect the truncated protein generated by the mutation, we also inserted an HA epitope at the N terminus of the unprocessed subunit, while an FLAG epitope was inserted between the 4th and 5th residue of the mature peptide, using overlapping PCR.

The *GABRG2* BAC construct containing the Q40X mutation was generated using the BAC clone number RP11-1035I20 (BACPAC Resources; <http://bacpac.chori.org>), which contains the wildtype human *GABRG2* gene genomic sequence. The human chromosome sequence upstream of *GABRG2* translation start site was replaced with a CMV promoter, and the mutation was introduced by *galK* facilitated BAC recombineering (Warming et al., 2005). The oligonucleotide sequences for BAC recombineering are available upon request. A reporter gene containing an SV40 early promoter-driven eGFP was integrated to BACs using Cre (NEB) recombination (Wade-Martins et al., 2001). Thus, both wildtype and mutant *GABRG2* BACs contained the CMV promoter-driven *GABRG2* gene and an eGFP reporter gene driven by the SV40 early promoter.

Cell culture and transfection

Human embryonic kidney cells (HEK 293T) (ATCC, CRL-11268) were incubated at 37°C in humidified 5% CO₂, 95% air and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were transfected using the FuGENE 6 transfection reagent (Roche Applied Science) at a DNA:Transfection Reagent ratio of 1:3 according to the manufacturer's instructions. Eighteen to 20 hours after transfection, gentamicin (50 mg/ml, GIBCO) was added to the culture dish.

The NMD essential factor UPF1 or SMG6 was knocked down using siRNAs to block the NMD machinery. SilencerSelect® pre-designed and validated siRNA (Ambion, siRNA ID s11926) was transfected to cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's manual. Twenty-four hours later cells were transfected again with the wildtype or mutant BAC constructs and harvested two days later for RT-PCR.

RNA extraction, RT-PCR and Taqman real-time qPCR

Total RNAs from transfected HEK 293T cells were extracted by using the PerfectPure RNA Cultured Cell kit (5Prime) following the manufacturer's protocol and then reverse transcribed to cDNA using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). The transcribed cDNA was used then as the PCR template to identify $\gamma 2$ subunit transcripts using a forward primer located in exon 6 and a reverse primer located in exon 7. Taqman® probes detecting human *GABRG2* and *GAPDH* mRNA, 18S rRNA, or *eGFP* mRNA (part number 4331348 [Custom Taqman Gene Expression Assay Service]) were used to quantify the amount of transcribed cDNA. Samples were obtained in triplicate for each experiment, and the average threshold cycle (Ct) value for each sample was calculated by the Sequence Detection System v2.3 Standard Edition (Applied Biosystems). The average Ct values of *GABRG2* gene mRNA were normalized to the endogenous human

GAPDH mRNA, 18S rRNA or *eGFP* mRNA levels to compare the relative RNA abundance.

Western Blot, PNGase F digestion and surface biotinylation

After sonication, the whole cell lysates of transfected HEK cells were collected in modified RIPA buffer (Pierce) and 1% protease inhibitor mixture (Sigma). Collected samples were subjected to gel electrophoresis using NuPAGE® (Invitrogen) or TGX (BioRad) precast gel and then transferred to PVDF-FL membranes (Millipore).

Monoclonal anti-HA antibody (Covance or Cell signaling) and monoclonal anti-FLAG antibody (Sigma) were used to detect the epitope tag in γ 2S subunits. Anti-sodium potassium ATPase antibody (Abcam) was used as a loading control. After incubation with primary antibodies, IRDye® (LI-COR Biosciences) conjugated secondary antibody was used at 1:10,000 dilution, and the signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity value (IDV) of each specific band was calculated using the Odyssey 3.0 software (LI-COR Biosciences).

To remove all N-linked glycans, cell lysates were incubated with the enzyme PNGase F (NEBiolab) at 37°C for 3 hours following manufacturer's manual. Treated samples were then subjected to SDS-PAGE and Western blot.

Surface proteins were collected using surface biotinylation as described before (Lo et al., 2010). Transfected cells were biotinylated using the membrane-impermeable reagent sulf-HNS-SS-biotin (1 mg/ml, Thermo Scientific) at 4°C for 1 h. Cells were lysed after being quenched with 0.1 M glycine. The biotin-labeled plasma membrane proteins were pulled down by High Binding Capacity NeutrAvidin beads (Thermo Scientific Pierce) after centrifugation.

Flow cytometry

High throughput flow cytometry was performed to investigate the surface expression of GABA_A receptor subunits. Transfected cells were collected in phosphate-buffered saline containing 2% fetal bovine serum and 0.05% sodium azide as described before (Lo et al., 2008). Cell samples were incubated with an Alexa fluorophore (Invitrogen)-conjugated monoclonal anti- α 1 antibody (Millipore), monoclonal anti- β 2/ β 3 antibody (Millipore) or monoclonal anti-HA antibody (Covance), then fixed by 2% paraformaldehyde. The fluorescence signals were read on a BD Biosciences FACSCalibur system. Nonviable cells were excluded from study based on the previously determined forward and side scatter profiles. The fluorescence index of each experimental condition was subtracted by the fluorescence index of mock-transfect condition and then normalized to that of the control condition. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource, which is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

Whole cell electrophysiology

Whole cell voltage-clamp recordings were performed at room temperature on lifted HEK293T cells 24-72 hrs after transfection with GABA_A receptor subunits as described previously (Hernandez et al., 2011). Successfully transfected cells were identified by the presence of GFP fluorescence (see Cell culture and transfection, above). Cells were bathed in an external solution containing 142 mM NaCl, 1 mM CaCl₂, 8 mM KCl, 6 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4, ~325 mOsM). Recording electrodes were pulled from thin-walled borosilicate capillary glass (World Precision Instruments, Sarasota, FL) using a P2000 laser electrode puller (Sutter Instruments, San Rafael, CA), fire-polished

with a microforge (Narishige, East Meadow, NY), and filled with an internal solution containing 153 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, 2 mM Mg²⁺-ATP (pH 7.3, ~300 mOsm). All patch electrodes had a resistance of 1–2 MΩ. The combination of internal and external solutions yielded a chloride reversal potential of ~ 0 mV, and cells were voltage-clamped at -20 mV using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). A rapid exchange system (open tip exchange times ~ 400 μs), composed of a four-barrel square pipette attached to a Perfusion Fast-Step (Warner Instruments Corporation, Hamden, CT) and controlled by Clampex 9.0 (Axon Instruments), was used to apply GABA to lifted whole cells. The channels were activated by 1 mM GABA for 4 s, followed by an extensive wash for 40 s, then blocked by 10 mM Zn²⁺ for 10 s. GABA (1 mM) was then applied for 4 s in the presence of 10 μM Zn²⁺. Peak current amplitudes after the Zn²⁺ application were normalized to those before the Zn²⁺ application to calculate the sensitivity to Zn²⁺ blockade. Diazepam sensitivity was determined by co-application of 1 μM diazepam with 2 μM GABA for 4 s. Peak currents before and after diazepam co-application were compared to determine the % enhancement by diazepam. All currents were low-pass filtered at 2 kHz, digitized at 5–10 kHz, and analyzed using the pCLAMP 9 software suite.

Data analysis

Numerical data were reported as mean ± S.E. Statistical differences were determined by one way analysis of variance or by pair wise *Student's t*-test.

Results

The γ2S subunit mutation, Q40X, decreased γ2S subunit transcripts

The nonsense mutation, Q40X, generated a PTC in the second exon of the nine exon *GABRG2* (Figure 1A). Since nonsense mutations located at least 50–55 nt upstream of an exon-exon junction activate NMD to degrade susceptible transcripts (Maquat, 2004), the mutant γ2S(Q40X) subunit mRNA level should be decreased. NMD efficiency is an inherent property of cells and varies among cell types (Linde et al., 2007a). In HEK 293T cells, the mRNA level of an NMD-competent construct was degraded by about 60% (Kang et al., 2009b). To determine whether mutant *GABRG2(Q40X)* mRNA was degraded by the NMD machinery, we expressed mutant or wildtype CMV promoter-driven *GABRG2* BACs in HEK293T cells with siRNAs against the NMD essential factor UPF1 or negative control siRNAs. Total RNA was extracted from transfected cells 36 hours after transfection, and mRNAs were reverse transcribed to cDNA. RT-PCR using primers flanking *GABRG2* 5' exon 6 and 3' exon 7 amplified a fragment from both wildtype and mutant BAC transfected cells (Figure 1B). Sequencing showed that the mutant BAC transcript contained the γ2S subunit containing a PTC at codon 40. The γ2S subunit mRNA levels were then quantified using real-time PCR with a probe targeting the *GABRG2* 5' exon 4 and 3' exon 5 border and normalized to GFP or GAPDH mRNA levels for each condition. The transcript levels from cells treated with siRNA against UPF1 were compared to those from cells treated with control siRNA. The γ2S subunit mRNA level in cells transfected with wildtype *GABRG2* BACs was not changed by UPF1 siRNA (1.03 ± 0.08 fold, n = 6) after UPF1 knock down (Figure 1C). The mutant γ2S(Q40X) subunit mRNA level in cells transfected with mutant *GABRG2(Q40X)* BACs, however, was increased by UPF1 siRNA (1.94 ± 0.23 fold, n = 6, *p* < 0.05) (Figure 1C). Thus, blocking NMD rescued the mutant γ2S(Q40X) subunit mRNA, but did not alter wildtype γ2S subunit mRNA levels. A similar trend was observed in cells transfected with siRNAs against the NMD essential factor SMG6 (data not shown).

The $\gamma 2S$ subunit mutation, Q40X, generated a truncated peptide

Since not all mutant mRNA was degraded by NMD, we studied the protein generated by the mutant $\gamma 2S(Q40X)$ subunit cDNA. The Q40X nonsense mutation is located in the 40th residue of the immature $\gamma 2S$ subunit, which is the first residue of the predicted mature subunit (Pritchett et al., 1989). Thus, this mutation is predicted to generate a truncated protein encoding the 39 amino acid $\gamma 2$ subunit signal peptide. To explore this prediction, we inserted an HA-tag at the N terminus of the immature $\gamma 2S$ subunit cDNA and a FLAG-tag between the 4th and 5th residue of the mature $\gamma 2S$ subunit cDNA, generating a double tagged SP^{HA}- $\gamma 2S^{FLAG}$ subunit (Figure 2A). Signal peptides are composed typically of a positively charged 'N domain', a hydrophobic 'H domain' and a slightly polar 'C domain' (Hegde and Bernstein, 2006; Tuteja, 2005). The additional HA tag at the N terminus of the immature $\gamma 2S$ subunit did not significantly affect the hydrophobicity pattern of the signal peptide calculated *in silico* using the ProtScale software (Gasteiger E., 2005) (Figure 2B). Insertion of an epitope in the N domain should not change signal peptide topology or function (Eichler et al., 2004; Kang and Schnetkamp, 2003).

We expressed wildtype $\gamma 2S^{HA}$, mutant SP^{HA}- $\gamma 2S(Q40X)^{FLAG}$ or wildtype SP^{HA}- $\gamma 2S^{FLAG}$ subunits in HEK293T cells and ran Western blots for HA- or FLAG-tagged proteins (Figure 2C). In cells transfected with $\gamma 2S^{HA}$ subunits, a large band was detected by anti-HA antibody at about 44 kDa, and as expected, no signal was detected by anti-FLAG antibody (Figure 2C, lane 2). In cells transfected with SP^{HA}- $\gamma 2S^{FLAG}$ subunits, a large band around 44 kDa was detected by anti-FLAG antibody and a small band around 7.5 kDa was detected by anti-HA antibody (Figure 2C, lane 4). The size of the higher molecular mass FLAG-band was consistent with mature, glycosylated $\gamma 2S$ subunits (Kang et al., 2009a), and the size of the lower molecular mass HA-band was consistent with the predicted signal peptide. In contrast, in cells transfected with mutant SP^{HA}- $\gamma 2S(Q40X)^{FLAG}$ subunits, no FLAG-specific signal was detected (Figure 2C, lane 3), indicating that synthesis of full length $\gamma 2S$ subunits was abolished by the *GABRG2(Q40X)* mutation. Interestingly, two different small peptides around/below 7.5 kDa were detected by anti-HA antibody in the mutant SP^{HA}- $\gamma 2S(Q40X)^{FLAG}$ subunit transfected cells (Figure 2C, lane 3), which may have been caused by a further cleavage of the signal peptide by signal peptide peptidase (Xia and Wolfe, 2003).

In addition to the small signal peptide, a clear, but faint, band with a higher molecular mass was also detected from SP^{HA}- $\gamma 2S^{FLAG}$ transfected cells using an anti-HA antibody (Figure 2C, lane 4). Its molecular mass was similar to that of immature $\gamma 2S$ subunits containing signal peptides. To determine molecular masses of $\gamma 2S$ and SP^{HA}- $\gamma 2S^{FLAG}$ subunits more accurately, we removed all of their glycans by PNGase F digestion (Figure 2D). After glycan removal, the size of HA-tagged $\gamma 2S^{HA}$ subunits was shifted from about 45 kDa to about 37 kDa, consistent with a mature, glycosylated subunit. In contrast, the size of HA-tagged SP^{HA}- $\gamma 2S^{FLAG}$ subunits was unchanged by glycan removal and remained at about 42 kDa, consistent with an immature, unglycosylated subunit. The 5 kDa difference in molecular mass of the two subunits after PNGase F treatment was consistent with the molecular mass of the signal peptide. Thus, SP^{HA}- $\gamma 2S^{FLAG}$ subunits produced an HA-tagged immature subunit in addition to the HA-tagged signal peptide and FLAG-tagged mature subunit. Mutant SP^{HA}- $\gamma 2S(Q40X)^{FLAG}$ subunits, however, only produced an HA-tagged signal peptide that was subjected to further cleavage. These results demonstrated that the $\gamma 2S$ subunit mutation, Q40X, disrupted translation of mature $\gamma 2S$ subunits and generated a truncated protein composed of the signal peptide.

The $\gamma 2S$ subunit mutation, Q40X, disrupted the membrane insertion of $\gamma 2S$ subunits and changed the composition of GABA_A receptors

To explore the effects of the *GABRG2(Q40X)* mutation on receptor assembly and channel function, we created HA-tagged $\gamma 2S(Q40X)^{HA}$ subunits with the HA-tag inserted between the 4th and 5th residue of the mature $\gamma 2S(Q40X)$ subunits. We then cotransfected HEK 293T cells with $\alpha 1$, $\beta 2$ and $\gamma 2S^{HA}$ or $\gamma 2S(Q40X)^{HA}$ subunits. Surface levels of different GABA_A receptor subunits were detected by flow cytometry (Figure 3A). The fluorescence indices of each subunit under different experimental conditions were normalized to those obtained with cotransfection of $\alpha 1\beta 2\gamma 2S^{HA}$ subunits. Cotransfection of either $\alpha 1\beta 2$ or $\alpha 1\beta 2\gamma 2$ subunits can produce functional GABA_A receptors on the cell surface (Angelotti and Macdonald, 1993; Connolly et al., 1996). Binary $\alpha\beta$ receptors are likely composed of two α and three β subunits while ternary $\alpha\beta\gamma$ receptors are likely composed of two α , two β and one γ subunits (Baumann et al., 2002; Tretter et al., 1997). Our flow cytometry analysis revealed a significant relative increase of surface $\beta 2$ subunit levels with cotransfection of $\alpha 1\beta 2$ subunits compared to cotransfection of $\alpha 1\beta 2\gamma 2S^{HA}$ subunits ($\alpha 1\beta 2$: 2.14 ± 0.23 ; $\alpha 1\beta 2\gamma 2S^{HA}$: 1.00; $n = 7$) with no change in the relative amount of surface $\alpha 1$ subunits ($\alpha 1\beta 2$: 0.92 ± 0.05 ; $\alpha 1\beta 2\gamma 2S^{HA}$: 1.00; $n = 7$) (Figure 3A). In the presence of the Q40X mutation, no surface $\gamma 2S(Q40X)^{HA}$ signal was detected by anti-HA antibody (Figure 3A), consistent with finding that synthesis of the full-length $\gamma 2S(Q40X)$ subunits was disrupted by the mutation (Figure 2C). With cotransfection of $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits, surface $\alpha 1$ subunit levels were similar to those obtained with cotransfection of $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2S^{HA}$ subunits ($\alpha 1\beta 2$: 0.92 ± 0.05 ; $\alpha 1\beta 2\gamma 2S^{HA}$: 1.00; $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$: 0.91 ± 0.03 ; $n = 7$). However, with cotransfection of $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits, surface $\beta 2$ levels were increased significantly compared to those obtained with cotransfection of $\alpha 1\beta 2\gamma 2S^{HA}$ subunits, reaching the levels of $\alpha 1\beta 2$ receptors ($\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$: 1.99 ± 0.20 ; $n = 7$; $p < 0.05$) (Figure 3A). We also evaluated the total cell expression of the receptor subunits (Figure 3B). The total levels of $\alpha 1$ and $\beta 2$ subunits with cotransfection of $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits were also similar to those obtained with cotransfection of $\alpha 1\beta 2$ subunits. These data indicated that mutant $\gamma 2S(Q40X)$ subunits did not incorporate into surface receptors, and thus GABA_A receptors assembled in the presence of mutant $\gamma 2S(Q40X)$ subunits were binary $\alpha\beta$ receptors.

To determine how mutant $\gamma 2S(Q40X)$ subunits affected GABA_A receptor function, we used a rapid exchange system to apply 1 mM GABA for 4s to lifted HEK293T cells coexpressing $\alpha 1\beta 2$, $\alpha 1\beta 2\gamma 2S^{HA}$, or $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits (Figure 3C). Peak current amplitude recorded from cells coexpressing $\alpha 1\beta 2$ subunits was 1351 ± 158 pA ($n = 9$), approximately 33% of currents recorded from cells coexpressing $\alpha 1\beta 2\gamma 2S^{HA}$ subunits (4106 ± 156 pA, $n = 15$, $p < 0.001$) (Figure 3C, left traces), a difference consistent with previously reported data (Angelotti and Macdonald, 1993; Angelotti et al., 1993; Gingrich and Burkat, 1998). Peak current amplitude from cells coexpressing $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits was also decreased significantly (1778 ± 232 pA, $n = 18$) to about 43% of that recorded from cells coexpressing $\alpha 1\beta 2\gamma 2S^{HA}$ subunits ($p < 0.001$), but not different from that obtained from cells coexpressing only $\alpha 1\beta 2$ subunits ($p > 0.05$). Furthermore, currents recorded from cells containing $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits were substantially more sensitive to Zn^{2+} inhibition than currents recorded from cells containing $\alpha 1\beta 2\gamma 2S^{HA}$ subunits. Currents evoked by 1 mM GABA from cells coexpressing $\alpha 1\beta 2$, $\alpha 1\beta 2\gamma 2S^{HA}$ or $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits were inhibited to different extents by coapplication of 10 μM Zn^{2+} (Figure 3C, right traces). The fractional Zn^{2+} inhibition of currents evoked from cells coexpressing $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits was significantly higher than inhibition of currents from cells coexpressing $\alpha 1\beta 2\gamma 2S^{HA}$ subunits ($93 \pm 1\%$, $n = 18$; $9 \pm 2\%$, $n = 15$, respectively, $p < 0.001$) but similar to inhibition of currents evoked from cells containing $\alpha 1\beta 2$ subunits ($94 \pm 1\%$, $n = 17$, $p > 0.05$). Because the sensitivity of GABA_A receptors to Zn^{2+} inhibition

depends on subunit composition, these results also suggested that mutant $\gamma 2S(Q40X)$ subunits were not incorporated into ternary $\alpha 1\beta 2\gamma 2S(Q40X)$ receptors, thus leading to expression primarily of binary $\alpha 1\beta 2$ receptors on the cell surface.

Full-length $\gamma 2S(Q40X)$ subunits were partially rescued by gentamicin-induced stop codon read-through

The Q40X mutation generated a PTC in *GABRG2* and failure to produce functional, full-length $\gamma 2S$ subunits likely contributes to its epilepsy pathogenesis. Aminoglycosides, such as G-418 and gentamicin, can promote partial read-through of PTCs, thus partially rescuing the synthesis of functional, full-length subunits (Howard et al., 1996; Zingman et al., 2007). Therefore, we determined to what extent gentamicin could rescue the *GABRG2(Q40X)* mutation. The read-through efficiency of gentamicin depends on the nature of the stop codon as well as the surrounding nucleotides, with the TGA stop codon being most efficiently bypassed (Martoglio, 2003). To maximize read-through efficiency, we replaced the original TAG stop codon with the TGA stop codon (Figure 4A) and then transfected $\gamma 2S(Q40X,TGA)^{HA}$ subunit cDNA into HEK cells. Eighteen hours after transfection, varying concentrations of gentamicin were added to the culture media. Forty-eight hours later, the transfected cells were collected, and amounts of full length $\gamma 2S^{HA}$ subunit translated from the mutant $\gamma 2S(Q40X,TGA)^{HA}$ subunit mRNA was evaluated by Western blot with anti-HA antibody (Figure 4B).

In the absence of gentamicin treatment, mature, full-length, HA-tagged $\gamma 2S$ subunits were detected from wildtype transfected cells (Figure 4B, lane 9), but mature, full-length, HA-tagged $\gamma 2S(Q40X,TGA)^{HA}$ subunits were not detected from mutant transfected cells (Figure 4B, lane 1). After addition of gentamicin, we were able to detect an HA-tagged protein band of the same size as the wildtype $\gamma 2S^{HA}$ subunit in cells transfected with $\gamma 2S(Q40X,TGA)^{HA}$ subunits (Figure 4B, lanes 2-6). No HA signal was detected from mock transfected cells in the presence or absence of gentamicin (Figure 4B, lanes 7-8), indicating that the rescue was specific and that expression of full length $\gamma 2S$ subunits was partially restored from $\gamma 2S(Q40X,TGA)^{HA}$ transfected cells. Compared to non-treated wildtype $\gamma 2S^{HA}$ subunit transfected cells, the rescue efficiency of $\gamma 2S(Q40X,TGA)^{HA}$ subunits was gentamicin concentration-dependent (Figure 4D, filled circles), reaching as high as $6.2 \pm 0.7\%$ at a concentration of 2 mg/ml gentamicin ($n = 7$), which is comparable to previous reports (Baker and Parker, 2004; Martoglio, 2003). We also evaluated the read-through efficiency of $\gamma 2S(Q40X)^{HA}$ subunits whose mRNA contained the native TAG stop codon. We found that a smaller, but still substantial, amount of full-length $\gamma 2S^{HA}$ subunit ($2.5 \pm 0.2\%$, $n = 5$) was rescued (Figure 4C) in a gentamicin concentration-dependent fashion (Figure 4D, filled squares).

Gentamicin-rescued $\gamma 2S$ subunits were trafficked to the cell surface

A functional $\gamma 2S$ subunit will oligomerize with partnering α and β subunits to form pentameric $\alpha\beta\gamma 2S$ receptors that are trafficked to the cell surface. To determine whether the $\gamma 2S$ subunits rescued by gentamicin were functional, we evaluated their surface expression. We cotransfected HEK 293T cells with $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits, and after forty-eight hours of gentamicin treatment (1 mg/ml), surface protein was collected through surface biotinylation and blotted by anti-HA antibody. We found that after gentamicin treatment a small, but significant, amount of HA-signal was detected on the cell surface with a molecular mass similar to that of wildtype $\gamma 2S^{HA}$ subunits (Figure 5A, lane 2 versus 4). HA-signal was not found in non-biotinylated samples, indicating that the detected HA-signal was not caused by artifact introduced during experiments (Figure 5A, lane 3).

To exclude the possibility that the HA-signal we detected through surface biotinylation was due to membrane destruction after gentamicin treatment, we also blotted for the cytoplasmic marker GAPDH. Then we compared the HA/GAPDH ratio between total samples and surface samples. Although a little GAPDH signal was found in surface samples, it was much lower than that obtained from total samples. After gentamicin treatment, the HA/GAPDH ratio of surface samples from mutant transfected cells was more than 200 times higher compared to the HA/GAPDH ratio of total samples (data not shown). This result indicated that the HA signal detected through surface biotinylation was not caused by cytoplasmic contamination and that the rescued $\gamma 2S$ subunits were expressed on the cell surface.

Gentamicin-rescued $\gamma 2S$ subunits were functional

We then evaluated assembly of $\alpha 1\beta 2\gamma 2S(Q40X)$ receptors after gentamicin treatment by studying Zn^{2+} sensitivity of GABA-evoked currents to distinguish $\alpha\beta$ from $\alpha\beta\gamma$ receptor currents. In the absence of gentamicin, currents recorded from cells containing $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits were substantially sensitive to Zn^{2+} inhibition (Figure 3B), consistent with assembly of only $\alpha 1\beta 2$ receptors. In contrast, after 24 h gentamicin treatment, the fractional Zn^{2+} inhibition of currents recorded from treated cells containing $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits was significantly smaller than those recorded from untreated cells (Figure 5B,C) ($79 \pm 1\%$, $n = 19$, treated, $93 \pm 1\%$, $n = 18$, untreated; $p < 0.001$). This appearance of Zn^{2+} insensitive currents indicates the existence of $\alpha\beta\gamma$ receptors on the cell surface. We also determined the diazepam sensitivity of $\alpha 1\beta 2\gamma 2S(Q40X)$ receptors since γ subunits are required for potentiation of GABA_A receptor currents by diazepam (Angelotti et al., 1993; Gunther et al., 1995). In the absence of gentamicin, currents recorded from cells containing $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits were not potentiated by diazepam application ($1.9 \pm 1.9\%$, $n = 5$) (Figure 5B,C), consistent with the insensitivity to diazepam potentiation of $\alpha\beta$ receptors (Angelotti et al., 1993). In contrast, after gentamicin treatment, the peak current amplitudes recorded from cells containing $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits was significantly enhanced (Figure 5B,C) ($302 \pm 55\%$, $n = 11$, treated; $2 \pm 2\%$, $n = 5$, untreated; $p < 0.05$). Taken together, these results suggested that gentamicin caused read-through of some of the $\gamma 2S(Q40X)$ subunit transcripts to produce full length $\gamma 2S$ subunits, and that the rescued full length $\gamma 2S$ subunits were assembled with $\alpha 1$ and $\beta 2$ subunits to form functional $\alpha 1\beta 2\gamma 2S$ receptors on the cell surface.

Discussion

The GABRG2 mutation, Q40X, may induce epilepsy through haploinsufficiency

The *GABRG2(Q40X)* mutation was identified from heterozygous dizygotic twin sisters with Dravet Syndrome (Kanaumi et al., 2004). We investigated the effects of this mutation on the assembly, trafficking and function of receptors in HEK cells cotransfected with $\alpha 1\beta 2\gamma 2S(Q40X)$ subunits. Q40X is a mutation that produces a PTC in exon 2 of *GABRG2* genomic DNA. Using BAC constructs containing this mutation, we found that mutant $\gamma 2S$ subunit mRNA levels were increased significantly after we knocked down the NMD factor UPF1 or SMG6, indicating that the mutant mRNA was degraded by NMD. NMD is a cellular surveillance mechanism that reduces expression of truncated products by degrading nonsense mutation-containing mRNA during translation (Baker and Parker, 2004). It was shown that NMD could reduce the level of a PTC-containing transcript to 20% in the brain, although the regional specificity was not addressed (Contet et al., 2007). If NMD destroys the mutant mRNA completely, heterozygous patients carrying one mutant *GABRG2(Q40X)* allele would suffer from *GABRG2* haploinsufficiency. However, not all mutant transcripts will be degraded, and NMD efficiency was shown to vary among different cell types (Linde et al., 2007a). Thus, we also characterized the mutant protein generated by this mutation.

Q40 is the first residue of the predicted mature $\gamma 2$ subunit. Therefore, production of a truncated protein composed only of the signal peptide would be predicted. To investigate this small peptide, we generated double tagged SP^{HA}- $\gamma 2$ S(Q40X)^{FLAG} subunits. We found that synthesis of full-length $\gamma 2$ subunit protein was abolished by this mutation and production of the signal peptide was increased. Surprisingly, the signal peptide generated by SP^{HA}- $\gamma 2$ S(Q40X)^{FLAG} subunits was further cleaved (Figure 2B), probably through signal peptide peptidase (Martoglio, 2003; Xia and Wolfe, 2003). Our strategy successfully demonstrated the signal peptide processing products of $\gamma 2$ subunits, providing a method to study other signal peptide related mutations. Our strategy also revealed an additional outcome of the Q40X mutation. It is possible that the signal peptide peptidase cleavage site was better exposed in the truncated $\gamma 2$ (Q40X) subunits, resulting in further cleavage. Although quite limited, a few studies have indicated that in addition to membrane targeting, signal peptide fragments could interact with signaling molecules (Martoglio et al., 1997) or be processed as antigenic epitopes (El Hage et al., 2008). Whether or not the novel cleavage pattern of the $\gamma 2$ (Q40X) subunit signal peptide contributes to the epilepsy pathogenesis requires more detailed study.

To further explore how the truncated $\gamma 2$ (Q40X) subunits affected receptor assembly, we compared GABA_A receptors formed by coexpression of $\alpha 1\beta 2\gamma 2$ S or $\alpha 1\beta 2\gamma 2$ S(Q40X) subunits. Both flow cytometry and whole cell recordings showed that mutant $\gamma 2$ (Q40X) subunits did not incorporate into functional ternary $\alpha 1\beta 2\gamma 2$ S(Q40X) receptors. Instead, binary $\alpha 1\beta 2$ receptors were formed that conducted much smaller currents. Therefore, *GABRG2*(Q40X) is likely a non-functional allele, and this mutation could cause haploinsufficiency of $\gamma 2$ subunits in patients. $\gamma 2$ subunits are widely distributed in the brain (Pirker et al., 2000), and homozygous $\gamma 2$ knockout mice died within a few days after birth (Gunther et al., 1995). Although seizures have not been reported from heterozygous $\gamma 2^{+/-}$ knockout mice, heterozygous $\gamma 2^{R82Q/+}$ knock-in mice carrying one mutant *GABRG2* allele developed absence epilepsy (Tan et al., 2007). Several epilepsy-associated *GABRG2* mutations have been identified in GEFS+ families (Macdonald and Kang, 2009). Hence, loss of one functional *GABRG2* allele in patients carrying the *GABRG2*(Q40X) mutation combined with other unidentified modifier genes is likely responsible for development of the Dravet syndrome phenotype.

The expression and function of mutant $\gamma 2$ (Q40X) subunits were partially rescued by gentamicin *in vitro*

Out of the seven epilepsy-associated mutations identified in *GABRG2*, four generated PTCs (Macdonald and Kang, 2009), and out of mutations identified from Dravet Syndrome patients, 50% were nonsense mutations (De Jonghe, 2011). Aminoglycosides, including G418 and gentamicin, promote read-through of PTCs by disturbing stop codon recognition during translation. *In vitro*, in animals *in vivo* and in preclinical studies in humans, successful rescue of the mutant phenotype has been reported for several different disease models (Linde and Kerem, 2008; Malik et al., 2010; Zingman et al., 2007). In our study, we observed that full length $\gamma 2$ S subunits were rescued from both $\gamma 2$ S(Q40X, TGA) subunits containing an optimized PTC and $\gamma 2$ S(Q40X) subunits containing the native PTC TAG, suggesting that this strategy could be applied to partially compensate for nonsense mutations. Furthermore, the rescued $\gamma 2$ subunits were trafficked to the cell surface and were incorporated into functional receptors, which is promising for future therapy.

Aminoglycoside-induced read-through has been used primarily in recessive genetic disorders where protein expression is almost null. However, this therapeutic approach may also work in autosomal dominant disorders (Kellermayer et al., 2006), including epilepsy. It is possible that a small amount of rescued $\gamma 2$ subunits during a critical time period could benefit patients substantially. GABA acts as a trophic factor during neural development (Ge

et al., 2006; Owens and Kriegstein, 2002; Wu et al., 2012) and disrupting postsynaptic $\gamma 2$ subunit clusters decreased presynaptic GABAergic innervation (Li et al., 2005). Study of heterozygous $\gamma 2^{R82Q/+}$ mice revealed that GABA_A receptor dysfunction during development increased seizure threshold in adulthood (Chiu et al., 2008). Thus lack of functional GABA_A receptors during development may cause reduction of GABAergic neurons, further contributing to the decreased inhibitory tone in adult brain. If neuronal inhibitory tone could be increased in patients carrying mutations such as Q40X before synaptogenesis is complete, it is possible that only a small amount of rescued $\gamma 2$ subunits could ameliorate the developmental deficits and decreased seizure susceptibility in later life. On the other hand, perhaps full rescue of mutant $\gamma 2$ subunits is not needed to compensate for the haploinsufficiency. Our *in vitro* data showed that 75% of $\gamma 2$ subunits were still expressed on the cell surface when only half amount of $\gamma 2$ subunit cDNA was transfected with $\alpha 1$ and $\beta 2$ subunit cDNAs at 1:1:0.5 ratio and had about 63% of GABA-evoked current compared to cells expressing $\alpha 1\beta 2\gamma 2$ subunit cDNAs at 1:1:1 ratio (Kang et al., 2009a). According to the 2:2:1 stoichiometry ratio of $\alpha\beta\gamma$ receptors, with expression of $\alpha\beta\gamma 2$ subunits mRNA in a 1:1:1 ratio, $\gamma 2$ subunits may be in excess. *In vivo* studies in heterozygous $\gamma 2^{+/-}$ knockout mice also showed 25% reduction of $\alpha\beta\gamma$ receptors (Crestani et al., 1999). If that also holds true in patients carrying a haplo-insufficient *GABRG2* allele such as *GABRG2(Q40X)*, less than 50% of $\gamma 2$ subunits would be required to restore the normal function of $\gamma 2$ subunits. Furthermore, mutations like Q390X in $\gamma 2$ subunits display a dominant negative effect to impair trafficking of wildtype subunits (Kang et al., 2009a). Read-through of $\gamma 2(Q390X)$ subunits could not only increase surface $\gamma 2$ subunits translated from mutant $\gamma 2(Q390X)$ subunits, but also increase trafficking of $\gamma 2$ subunits translated from wildtype $\gamma 2$ subunits as well as partnering α and β subunits. Therefore, it would be interesting to evaluate read-through of *GABRG2(Q390X)* subunit mRNA. Besides, as mutations in neuronal sodium channel SCN1A account for approximately 70% of all Dravet patients, it will be worthwhile to study how the chemical read-through approach could rescue SCN1A-associated nonsense mutations.

Long term use of aminoglycosides could cause nephrotoxicity and ototoxicity (Kaufman, 1999). With treatment using a high concentration of gentamicin (2 mg/ml), our cells also exhibited lower survival rates (data not shown). Although gentamicin has been tested in patients with cystic fibrosis (Wilschanski et al., 2003) and Duchenne muscular dystrophy (Malik et al., 2010) carrying PTCs, it is necessary to explore other less toxic drugs. PTC124 (Ataluren®) is a nonaminoglycoside compound with superior read-through efficacy and lower toxicity (Du et al., 2008; Welch et al., 2007). A phase II prospective trial showed that PTC124 administration reduced abnormalities in cystic fibrosis patients (Kerem et al., 2008). Another strategy is to use suppressor tRNA (Buvoli et al., 2000; Temple et al., 1982). However, both transfection and read-through efficiency of suppressor tRNA is not high, and high level of suppressor tRNA was shown to be toxic to cells (Buvoli et al., 2000; Kiselev et al., 2002). Recently, pseudouridylation has been suggested to target a specific nonsense codon into sense codon, but the rescue efficiency of this method is similar to that of aminoglycosides (Karijolich and Yu, 2011). Compounds with better efficacy and therapeutic window could be identified in future and our work shows a possible direction for epilepsy therapy.

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Highlights

- Dravet syndrome-associated mutation *GABRG2(Q40X)* decreased $\gamma 2$ subunit mRNA levels.
- Undegraded mutant mRNA was translated to truncated $\gamma 2$ subunits that were cleaved.
- Mutant $\gamma 2(Q40X)$ subunits were not assembled into functional GABA_A receptors.
- Aminoglycosides partially rescued wildtype $\gamma 2$ subunit expression from mutant mRNA.
- Rescued $\gamma 2$ subunits had the same expression and function as wildtype $\gamma 2$ subunits.

Figure 1. Mutant mRNA was degraded by NMD

A. A schematic representation of the genomic structure of *GABRG2*. Vertical blue lines represent the exons composing the $\gamma 2S$ subunit cDNA. The Q40X mutation is located in exon 2. **B.** The $\gamma 2S$ transcript was identified in mutant *GABRG2(Q40X)* BAC transfected cells using RT-PCR. HEK 293T cells were treated with siRNA against the NMD factor UPF1 or with nonspecific siRNA and were then transfected with wildtype or mutant *GABRG2* BAC. A forward primer located in exon 6 and a reverse primer located in exon 7 of the $\gamma 2S$ subunit cDNA were used to amplify reverse transcribed cDNA from transfected

cells. **C.** The transcript level of the mutant *GABRG2(Q40X)* BAC was increased by NMD knock down (n = 6, mean $\pm$ SEM).

Figure 2. The GABRG2(Q40X) mutation generated a truncated peptide

A. To identify the protein generated by the *GABRG2(Q40X)* mutation, an HA tag was inserted into the N terminal signal peptide and a FLAG tag was inserted between the 4th and 5th residue of the mature γ 2S subunit protein to produce wildtype SP^{HA}- γ 2S^{FLAG} or mutant SP^{HA}- γ 2S(Q40X)^{FLAG} subunits. SP: signal peptide. SC: stop codon. **B.** The hydrophobicity patterns of γ 2S and SP^{HA}- γ 2S^{FLAG} subunit signal peptides were calculated using the online PScale program. The Y-axis represents scores calculated based on the hydrophobicity scale of different amino acids; the X-axis represents the numbering of each residue in the signal peptide sequence. **C.** The γ 2(Q40X) subunit mutation generated a truncated peptide. HEK

293T cells were transfected with wildtype $\gamma 2S^{HA}$, wildtype $SP^{HA}-\gamma 2S^{FLAG}$ or mutant $SP^{HA}-\gamma 2S(Q40X)^{FLAG}$ subunits. Cell lysates (10 μg) from wildtype $\gamma 2S^{HA}$ subunit transfected cells and cell lysates (50 μg) from wildtype $SP^{HA}-\gamma 2S^{FLAG}$ or mutant $SP^{HA}-\gamma 2S(Q40X)^{FLAG}$ subunit transfected cells were subjected to Western blot by anti-FLAG and anti-HA antibodies. ATPase levels were used as loading controls. **D.** Samples from cells transfected with $\gamma 2S^{HA}$ or $SP^{HA}-\gamma 2S^{FLAG}$ subunits were collected and treated with PNGase F to remove all glycans. F: PNGase F digestion; U: undigested control; M: protein loading marker. Figures are representative of 3 different experiments.

Figure 3. The mutant $\gamma 2S(Q40X)$ subunit was not expressed on the cell surface

A. and B. Wildtype $\alpha 1\beta 2$ or $\alpha 1\beta 2\gamma 2S^{HA}$ or mutant $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits were coexpressed in HEK293T cells. Surface and total level of each subunit were evaluated through flow cytometry. The fluorescence indices of each subunit under different experimental conditions were normalized to those obtained with cotransfection of $\alpha 1\beta 2\gamma 2S^{HA}$ subunits ($n = 7$, mean $\pm$ SEM). Group differences were analyzed by the one way ANOVA test. **C.** Sample traces of whole cell recordings of currents evoked by 1 mM GABA from cells expressing $\alpha 1\beta 2$, $\alpha 1\beta 2\gamma 2S^{HA}$ or $\alpha 1\beta 2\gamma 2S(Q40X)^{HA}$ subunits were obtained. After a 4.0 sec wash, the currents were recorded again with coapplication of 1 mM GABA and 10 μ M Zn^{2+} ($n > 9$).

Figure 4. Gentamicin partially restored expression of full length $\gamma 2S$ subunits by read-through of $\gamma 2S(Q40X)$ subunit mRNA

A. The original TAG stop codon was replaced by the TGA stop codon to maximize read-through efficiency. **B. and C.** Cells were transfected with $\gamma 2S^{HA}$ and $\gamma 2S(Q40X,TGA)^{HA}$ (B) or $\gamma 2S^{HA}$ and $\gamma 2S(Q40X)^{HA}$ (C) subunits and treated with different concentrations of gentamicin for 48 hours. Cell lysates (10 μ g) from wildtype $\gamma 2S^{HA}$ subunit transfected cells were loaded, while cell lysates (50 μ g) from mutant $\gamma 2S(Q40X,TGA)^{HA}$ or $\gamma 2S(Q40X)^{HA}$ subunits transfected cells were loaded. **D.** Band intensity of the $\gamma 2S^{HA}$ subunit was normalized to the ATPase signal and plotted against gentamicin concentration ($n = 7$ and 5 respectively, mean $\pm$ SEM).

Figure 5. Gentamicin increased surface expression of mutant $\gamma 2(Q40X)$ subunits and decreased Zn^{2+} sensitivity of mutant receptor currents

A. HEK 293T cells were cotransfected with $\alpha 1\beta 2\gamma 2S^{HA}$ or $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits. Cells were then treated with 1 mg/ml of gentamicin for 48 hours. Surface protein samples were collected through surface biotinylation and blotted by anti-HA, anti-ATPase and anti-GAPDH antibody. Cell lysates (0.5 mg and 1 mg) from cells expressing wildtype $\gamma 2S^{HA}$ or mutant $\gamma 2S(Q40X,TGA)^{HA}$ subunits were used to collect the surface fraction. Cell lysates (10 μ g and 50 μ g) from wildtype $\gamma 2S^{HA}$ or mutant $\gamma 2S(Q40X,TGA)^{HA}$ subunits transfected cells were loaded as total fraction. Samples not coated with biotin were also collected as controls. **B.** HEK 293T cells were cotransfected with $\alpha 1\beta 2\gamma 2S(Q40X,TGA)^{HA}$ subunits. Cells were treated then with 1 mg/ml of gentamicin for 24 hours, and whole cell currents in response to 1 mM or 2 μ M GABA then were recorded. The current amplitudes recorded in the presence of 10 μ M Zn^{2+} or 1 μ M diazepam were normalized to those recorded in the absence of Zn^{2+} or diazepam. The percentage of current amplitudes inhibited by Zn^{2+} (n = 19, mean $\pm$ SEM) or enhanced by diazepam (n = 11, mean $\pm$ SEM) was compared to that obtained from cells untreated with gentamicin (n = 18 and 5, respectively).