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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 γ2 subunit mRNA in a position that codes for the first amino acid of the mutant subunit. We determined the effects of the mutation on γ2 subunit mRNA and protein synthesis and degradation, as well as on α1β2γ2 $GABA_A$ receptor assembly, trafficking and surface expression in HEK cells. Using bacterial artificial chromosome (BAC) constructs, we found that γ2(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 γ2(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 the 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 γ2 subunits was partially restored, and surface biotinylation and whole cell recording experiments suggested that rescued γ2 subunits could corporate into functional, surface $GABA_A$ receptors, indicating a possible direction for future therapy.

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 γ2 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 γ2 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

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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 γ2 and γ2(Q40X) subunit protein and the properties of $GABA_A$ receptors that were assembled with coexpression of α1, β2 and γ2 or γ2(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 γ2 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 γ2(Q40X) subunits. We demonstrated that gentamicin partially restored the expression of full-length γ2 subunits, and that the rescued γ2 subunits assembled with α1β2 subunits to form functional α1β2γ2 $GABA_A$ receptors.

Materials and methods

Expression vectors

The coding sequences of human α1, β2 and γ2S $GABA_A$ 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 γ2S(Q40X) and γ2S(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 residues of the mature peptide of both wildtype and mutant γ2S 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% CO_2, 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 h 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 γ2 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 γ2S 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 h 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