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A $GABA_A$ Receptor Mutation Linked to Human Epilepsy (γ_2R43Q) Impairs Cell Surface Expression of $\alpha\beta\gamma$ Receptors*

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Feyza Sancar‡ and Cynthia Czajkowski‡§¶

From the §Department of Physiology and ‡Neuroscience Training Program, University of Wisconsin, Madison, Wisconsin 53706

A mutation in the γ_2 subunit of the γ-aminobutyric acid (GABA) type A receptor (GABAR), which changes an arginine to a glutamine at position 43 (R43Q), is linked to familial idiopathic epilepsies. We used radioligand binding, immunoblotting, and immunofluorescence techniques to examine the properties of wild-type $\alpha_1\beta_2\gamma_2$ and mutant $\alpha_1\beta_2\gamma_2$R43Q GABARs expressed in HEK 293 cells. The γ_2R43Q mutation had no affect on the binding affinity of the benzodiazepine flunitrazepam. However, in cells expressing $\alpha_1\beta_2\gamma_2$R43Q GABARs, the number of binding sites for [^{3}H]flunitrazepam relative to wild-type receptors was decreased 75%. Using surface protein biotinylation, affinity purification, and immunoblotting, we demonstrated that expression of cell surface $\alpha_1\beta_2\gamma_2$R43Q GABARs was decreased. Surface immunostaining of HEK 293 cells expressing $\alpha_1\beta_2\gamma_2$R43Q GABARs confirmed that surface expression of the γ_2R43Q subunit was reduced. These data demonstrate that the γ_2R43Q mutation impairs expression of cell surface GABARs. A deficit in surface GABAR expression would reduce synaptic inhibition and result in neuronal hyperexcitability, which could explain why families possessing the γ_2R43Q subunit have epilepsy.

Idiopathic partial and generalized epilepsies are the most common forms of heritable seizure disorders, accounting for 40% of all epilepsies (1). Idiopathic epilepsies are those seizure disorders that are not preceded by or concomitant with trauma or other disorders but are due mainly to genetic factors. Because of the ubiquitous role of the neurotransmitter GABA[1] in mediating cortical inhibition, deficits in GABAergic transmission have long been surmised to play a role in the pathogenesis of epilepsy. Recently, several mutations in the $GABA_A$ receptor (GABAR) have been identified through the use of genetic linkage analysis in families with idiopathic generalized epilepsies.

GABARs are heteropentameric ligand gated chloride channels that mediate fast synaptic inhibition in the brain. The receptors are assembled from a number of different subunits and subunit isoforms, including α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, π, and θ (2, 3). The majority of GABARs in the brain are believed to consist of 2 α, 2 β, and 1 γ subunits (4, 5). The inclusion of a γ subunit confers benzodiazepine (BZD) sensitivity to GABARs (6) and also influences receptor expression, synaptic targeting, and trafficking (7, 8). To date, four mutations in the γ_2 subunit of the GABAR (missense mutations R43Q and K289M, the nonsense mutation Q351X, and the anomalous splice-donor site mutation IVS6 + 2T → G) and one mutation in the α_1 subunit of the receptor (missense mutation A322D) have been associated with idiopathic epileptic syndromes (9–13).

Several groups have explored the effects of the γ_2R43Q mutation on GABAR function. Using recombinant GABAR expression in *Xenopus laevis* oocytes and two-electrode voltage clamp recording techniques, no differences in GABA EC_{50} values between wild-type (WT)$\alpha_1\beta_2\gamma_2$ and mutant $\alpha_1\beta_2\gamma_2$R43Q receptors were observed (13). However, the ability of the BZDs flunitrazepam (FNZM) and diazepam to potentiate GABA-activated current was reduced in mutant receptors (13, 14). Recombinant receptor expression in HEK 293 cells combined with patch-clamping and rapid drug application techniques demonstrated that the γ_2R43Q mutation slowed GABAR deactivation and speeded desensitization (14). In contrast, another study using expression in HEK 293 cells and similar recording techniques reported no differences in receptor kinetics or diazepam potentiation of GABA current between mutant and WT receptors but instead observed reductions in maximal GABA-induced current in cells expressing mutant receptors (15).

In the present study, we used radioligand binding, immunoblotting, and immunofluorescence techniques to clarify and further characterize the properties of WT $\alpha_1\beta_2\gamma_2$ and mutant $\alpha_1\beta_2\gamma_2$R43Q $GABA_A$ receptors expressed in HEK 293 cells. Several facets of GABAR function were examined, including receptor cell surface expression, receptor pharmacology, and functional coupling between the GABA and BZD binding sites. Here, we report that the γ_2R43Q mutation reduces surface expression of $\alpha_1\beta_2\gamma_2$R43Q receptors while leaving the pharmacology and functional coupling between GABA and BZD binding sites of the receptor intact.

EXPERIMENTAL PROCEDURES

Transient Expression in HEK 293 Cells—Wild-type α_1, β_2, γ_{2s}, and mutant γ_2R43Q subunit cDNAs in pcDNA3.1 encoding for human GABA receptor protein were obtained from Dr. Steve Petrou (University of Melbourne, Victoria, Australia). Subunit cDNAs were verified by double-stranded DNA sequencing. HEK 293 cells were grown as described previously (16). Cells were co-transfected at ~70% confluency with α_1, β_2, γ_2, and mutant γ_2R43Q subunit cDNAs and the vector pAdvantage (Promega) at a ratio of 1:1:1:1.5 (4 μg of DNA per subunit and 6 μg of pAdvantage per dish) using a standard $CaHPO_4$ precipitation method (17).

Radioligand Binding—Cells were harvested 48 h after transfection, membrane homogenates were prepared, and homologous competition binding experiments were performed as described in Boileau *et al.* (16). In brief, membrane homogenates (100 μg) were incubated at room temperature for 1 h with sub-K_d concentrations of radioligand ([^{3}H]muscimol (28.1 Ci/mmol) or [^{3}H]FNZM (84.5 Ci/mmol), Perkin-Elmer Life Sciences) in the absence or presence of 7 different concen-

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¶ To whom correspondence should be addressed: Dept. of Physiology, Rm. 197 MSC, University of Wisconsin, 1300 University Ave., Madison WI 53706. Tel.: 608-265-5863; Fax: 608-265-5512; E-mail: czajkowski@physiology.wisc.edu.

[1] The abbreviations used are: GABA, γ-aminobutyric acid; $GABA_A$, GABA, type A; GABAR, $GABA_A$ receptor; BZD, benzodiazepine; WT, wild-type; FNZM, flunitrazepam; PBS, phosphate-buffered saline.

trations of corresponding unlabeled ligand (ranging from 0.1 nM-10 μM) in a final volume of 250 μl. Specific binding was defined as ^{3}H-drug bound in the absence of displacing ligand minus the amount bound in the presence of displacing ligand (10 μM). Data were fit by non-linear regression to a one-site competition curve defined by the equation $y = B_{max}/(1+ (x/IC_{50}))$, where y is bound ^{3}H ligand in dpm, B_{max} is maximal binding, x is the concentration of displacing ligand, and IC_{50} is the concentration of unlabeled ligand that inhibits 50% of ^{3}H ligand binding (Prism, GraphPAD Software, San Diego, CA). Because radioactive and non-radioactive ligands were chemically identical, K_i values were calculated using a simplified Cheng-Prusoff/Chou equation: $K_i = IC_{50} - [L^*]$, where K_i refers to the equilibrium dissociation constant of the radioligand and $[L^*]$ refers to the concentration of radioactive ligand used (Prism). In Table I, B_{max} values for FNZM were determined in two ways. In three independent experiments, both B_{max} and K_i values for FNZM were determined from eight-point homologous competition assays and curve fitting. In five other experiments, the specific binding of [^{3}H]FNZM to membrane homogenates was measured using only one concentration of [^{3}H]FNZM. The B_{max} values were then calculated using the following equation, Bound = $((B_{max} \times [L])/([L] + K_i))$, where Bound is the measured specific [^{3}H]FNZM binding in dpm, $[L]$ is the concentration of [^{3}H]FNZM used in the experiment, and K_i is the apparent binding affinity of FNZM, which we previously established from the original three competition binding assays. Non-radioactive FNZM was obtained as a gift from Dr. Sepinwall (Hoffman-La Roche, Nutley, NJ). Muscimol was obtained from RBI/Sigma.

To measure GABA potentiation of [^{3}H]FNZM binding, membrane homogenates (100 μg) were incubated for 20 min at 37 °C followed by 40 min at room temperature with 3 nM (WT) or 6 nM (R43Q) [^{3}H]FNZM in the presence of seven different GABA concentrations ranging from 30 nm-100 μM (final volume 250 μl) and then filtered as described above. Data were fit using non-linear regression analysis to a sigmoidal dose response curve described by the equation $Y = Min + (Max - Min)/(1 + 10\hat{}[(logEC50 - X)*n])$, where Max is maximal potentiation, Min is potentiation at lowest [GABA] tested, EC_{50} is half-maximal potentiation, X is the logarithm of GABA concentration, and n is the Hill coefficient (Prism). Potentiation was calculated for each GABA concentration as follows: $p = (dpm_{GABA}/dpm_{control}) - 1$, where dpm_{GABA} is the specific [^{3}H]FNZM bound in the presence of GABA, and $dpm_{control}$ is the specific [^{3}H]FNZM bound in the absence of GABA.

Affinity Purification of Surface Receptors—Twenty-four to 48 h post-transfection, intact cells were washed with ice-cold PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl, and 14 mM Na_2HPO_4, pH 7.1) and incubated with the non-membrane permeable biotinylation reagent NHS-LC (or SS)-Biotin (0.3–0.5 mg/ml in PBS, Pierce) for 30 min at 4 °C to label surface membrane proteins. To quench the reaction, cells were incubated with 10 mM glycine in PBS (10 min at room temperature). Sulfhydryl groups were blocked by incubating the cells with 5 mM *N*-ethylmaleimide in PBS (15 min at room temperature). Cells were solubilized (overnight at 4 °C) in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5) supplemented with protease inhibitors (0.5 mg/ml Pefabloc, 1 μg/ml pepstatin, 1 μg/ml leupeptin, Roche Applied Science) and 5 mM *N*-ethylmaleimide. Lysates were cleared by centrifugation (16,000 $\times$ *g*, 10 min at 4 °C) and lysate protein concentration was determined using a BCA protein assay (Pierce). A sample of each lysate (equal protein concentrations) was prepared for SDS-PAGE and Western blot analysis by adding 2$\times$ Laemmli sample buffer (LSB) + 6 M Urea + 100 mM dithiothreitol. Biotinylated surface proteins were affinity purified from the remaining cell lysates by incubating (1 h at 4 °C, rotating) with 100 μl of Immobilized Neutravidin-conjugated agarose bead slurry (Pierce). The samples were then centrifuged (16,000 $\times$ *g*, 10 min at 4 °C), and the beads were washed (six times) with buffer (0.1 or 0.5% Triton X-100, 50 mM Tris-Cl, 150 mM NaCl, and 5 mM EDTA, pH 7.5). Surface proteins were eluted from the beads with 200 μl of LSB/urea buffer (6% SDS, 20% glycerol, 100 mM dithiothreitol, and 6 M urea, pH 6.8).

Western Blotting—Protein samples were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (0.45 micron). Blots were incubated overnight at 4 °C in blocking buffer containing 2% nonfat milk in TTBS (500 mM NaCl, 20 mM Tris base, 0.05% Tween-20, pH 7.5). Blots were washed once in TTBS (5 min at room temperature) and incubated (1 h at room temperature) in γ_2-(1–33) or $\beta_{2/3}$ primary antibody at dilutions of 1:250 or 1: 100 in blocking buffer, respectively. The anti-γ_2-(1–33) rabbit polyclonal antibody was provided as a generous gift by Dr. Werner Sieghart (University Clinic for Psychiatry, Vienna, Austria) and the anti-$\beta_{2/3}$ mouse monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Following incubation in primary antibody, the blots were washed (3 $\times$ 10 min) with TTBS and then incubated (1 h at room temperature) in horseradish peroxidase-conjugated goat-anti-rabbit IgG (for anti-γ_2, Pierce) or horseradish peroxidase-conjugated goat-anti-mouse (for anti-β_2, Pierce) at a dilution of 1:10, 000 in blocking buffer. The blots were then washed (6 $\times$ 5 min) in TTBS and developed with either SuperSignal West Pico or West Femto chemiluminescent substrates (Pierce) for visualization of γ_2 and β_2 subunits, respectively. A background eliminator was applied to exposed films to remove excessive levels of background signal to better visualize the pertinent bands (Erase-It™ Background Eliminator, Pierce).

Immunofluorescence—Cells used for immunofluorescence were transfected without pAdvantage, incubated overnight at 31 °C (5% CO_2) and then re-plated on poly-D-lysine-coated glass coverslips and returned to the incubator for an additional 24 h at 37 °C. Cells were washed and fixed in 2% paraformaldehyde in PBS. For membrane permeabilization, cells were incubated with 0.1% Triton X-100 (v/v) in PBS for 10 min at room temperature. Cells were then washed three times with PBS, blocked for 15–20 min with PBS containing 20% goat serum, and incubated in anti-γ_2 antibody (diluted 1:500 in blocker) overnight at 4 °C; this was followed by three washes with PBS. Cells were then incubated for 1 h (at room temperature) with Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted 1:1000 in blocker. Cells were washed three times with PBS and mounted onto glass slides. In some cases, both permeabilized and intact cells were incubated in secondary antibody only to determine nonspecific labeling. Fluorescently labeled cells were imaged using a Bio-Rad MRC-1024 laser scanning confocal microscope with a $\times$40 lens. Gain settings were normalized to cells treated with secondary antibody only (such that no fluorescent signal was detected in these controls) and the same gain and laser power settings were used for subsequent analysis of WT and mutant receptor expressing cells labeled with both primary and secondary antibodies.

RESULTS

The Effect of γ_2R43Q on [^{3}H]Flunitrazepam Binding—Homologous competition binding experiments using FNZM were performed on membrane homogenates prepared from HEK 293 cells expressing $\alpha_1\beta_2\gamma_2$ WT and $\alpha_1\beta_2\gamma_2$R43Q GABARs (Fig. 1*A*). Both ligand apparent affinity (K_d) and total number of binding sites (B_{max}) were measured. The K_d for FNZM was not significantly different between WT and mutant receptors, suggesting that the γ_2R43Q mutation does not affect FNZM binding affinity (Table I). However, there was an ~4-fold (75%) decrease in the maximal number of specific [^{3}H]FNZM binding sites (B_{max}) for mutant receptors compared with WT receptors ($p < 0.01$, Table I). Because FNZM binding affinity was unaltered by the mutation and the mutation only decreased the total number of [^{3}H]FNZM binding sites, the data suggest that the γ_2R43Q mutation disrupts expression of $\alpha_1\beta_2\gamma_2$ receptors. These results were supported by the finding that the binding of the imidazobenzodiazepine [^{3}H]Ro15–1788 followed a similar trend, where the total number of binding sites was reduced in $\alpha_1\beta_2\gamma_2$R43Q expressing cells, whereas K_d values were comparable between WT and mutant receptors (data not shown).

The Effect of γ_2R43Q On [^{3}H]Muscimol Binding—In HEK 293 cells, the co-expression of α_1, β_2, and γ_2 subunits can result in the formation of both $\alpha\beta$ and $\alpha\beta\gamma$ GABARs (18). As FNZM binds only to GABARs containing a γ subunit, [^{3}H]FNZM binding reveals no clues as to whether the γ_2R43Q mutation affects GABA agonist binding and/or $\alpha\beta$ receptor expression. To address these questions, we measured the binding of [^{3}H]muscimol, a GABA binding site agonist, which binds to both $\alpha\beta$ and $\alpha\beta\gamma$ GABARs. The K_d for [^{3}H]muscimol binding was not significantly different between WT and mutant receptors, suggesting that the γ_2R43Q mutation does not affect the apparent binding affinity of muscimol (Fig. 1*B*, Table I). However, unlike our findings with [^{3}H]FNZM, the total number of specific muscimol binding sites (B_{max}) was comparable in membrane homogenates prepared from HEK 293 cells expressing $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$R43Q GABARs (Table I). Because muscimol K_d and B_{max} values were not altered by the mutation, the data suggest that

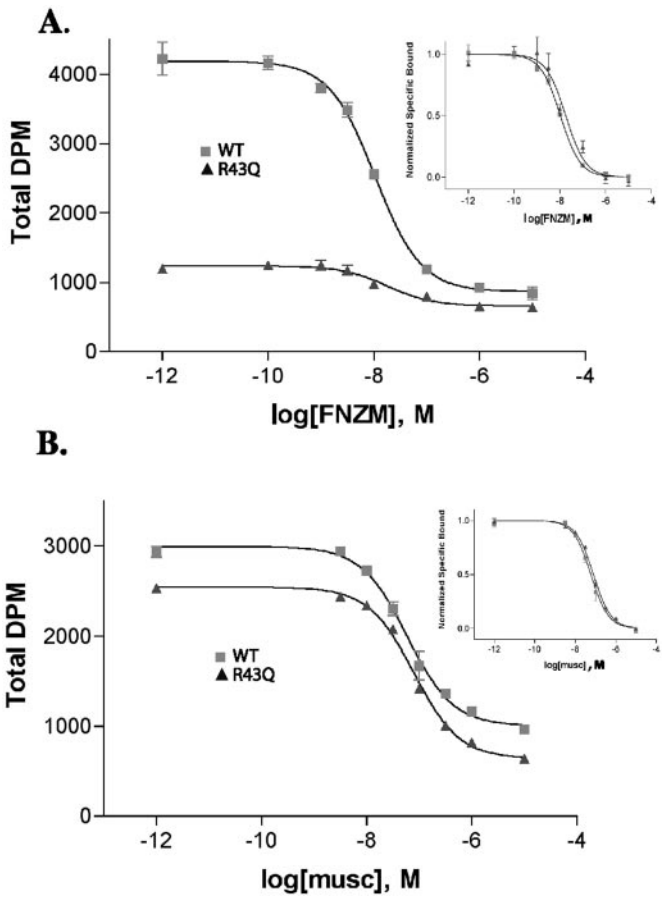

FIG. 1. **γ_2R43Q decreases [^{3}H]FNZM binding and has no effect on [^{3}H]muscimol binding.** *A*, displacement of [^{3}H]FNZM binding (*DPM*) by unlabeled FNZM in membranes prepared from HEK 293 cells expressing WT $\alpha_1\beta_2\gamma_2$ (▫) and $\alpha_1\beta_2\gamma_2$R43Q (▲) GABARs. *B*, displacement of [3H]muscimol (*musc*) binding by unlabeled muscimol. The data are from single representative experiments where each point is the mean ± S.E. of triplicate measurements. Data were fit by non-linear regression analysis as described under "Experimental Procedures." K_i and B_{max} values are summarized in Table I. *Insets*, plots of the same data after normalizing to specific [3H] ligand bound in the absence of unlabeled ligand. (Note: In some cases, the error is smaller than the symbol-size.)

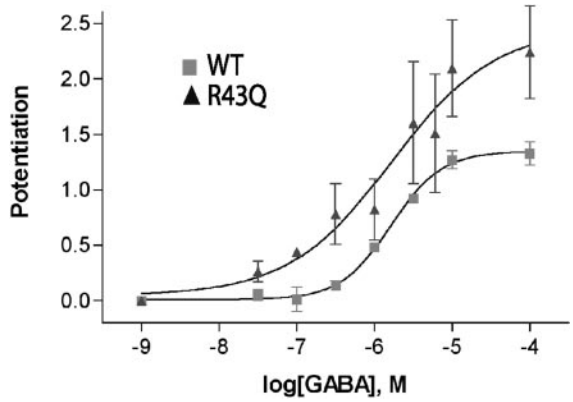

FIG. 2. **GABA and benzodiazepine binding sites are functionally coupled in $\alpha_1\beta_2\gamma_2$R43Q receptors.** GABA potentiation of [^{3}H]FNZM binding in membranes prepared from HEK 293 cells expressing WT $\alpha_1\beta_2\gamma_2$ (▫) and $\alpha_1\beta_2\gamma_2$R43Q (▲) receptors. Each point is the mean ± S.E. from three (WT) or four (*R43Q*) experiments. Potentiation was calculated for each GABA concentration as follows: $p = (dpm_{GABA}/dpm_{control}) - 1$, where dpm_{GABA} is the specific [^{3}H]FNZM bound in the presence of GABA and $dpm_{control}$ is the specific [^{3}H]FNZM bound in the absence of GABA. Data were fit using non-linear regression analysis as described under "Experimental Procedures." Maximal potentiation and GABA EC_{50} values are reported in Table II.

the γ_2R43Q mutation selectively decreases the number of BZD binding sites while leaving the number and apparent affinity of the GABA binding sites unperturbed.

Allosteric Coupling of the GABA and BZD Binding Sites—Although the decrease in the number of [^{3}H]FNZM binding sites suggests that the γ_2R43Q mutation disrupts $\alpha_1\beta_2\gamma_2$ GABAR expression, expression was not completely abolished because some [^{3}H]FNZM binding was still detected in membrane homogenates prepared from cells expressing mutant receptors (Fig. 1*A*). Thus, it is possible that the mutation alters not only expression but also the function of $\alpha_1\beta_2\gamma_2$R43Q GABARs. Although the K_d values for muscimol and flunitrazepam were unaltered by the mutation, we speculated that the mutation could disrupt cross-talk (*i.e.* coupling) between the BZD and GABA binding sites. To address this possibility, we assessed the ability of GABA to potentiate [^{3}H]FNZM binding to membrane homogenates prepared from HEK 293 cells expressing WT and mutant receptors. In this experimental paradigm, only the receptors containing a γ_2 subunit are monitored because $\alpha\beta$ GABARs do not bind BZDs. Fig. 2 plots the potentiation of specific [^{3}H]FNZM binding as a function of GABA concentration. In mutant and WT receptors, GABA maximally potentiated FNZM binding 2.4- and 1.4-fold, respectively (Table II). Furthermore, the concentrations of GABA needed to elicit half-maximal potentiation of [^{3}H]FNZM binding (EC_{50}) for WT and mutant receptors were equivalent (Table II). These findings indicate first that the R43Q mutation does not alter the apparent affinity for GABA and second that the mutation leaves the allosteric coupling between the BZD and GABA binding sites intact.

The Effect of γ_2R43Q on Cell Surface Expression of γ_2 and β_2 Subunits—Whereas the radioligand binding data suggest a selective decrease in γ_2 subunit-containing receptors, the data reflect binding to membrane homogenates that contain assembled and partially assembled intracellular receptors as well as fully assembled cell surface receptors. To directly determine the effect of γ_2R43Q on surface GABAR expression, we compared total and surface expression of γ_2 and β_2 subunits by Western blot analysis. Surface proteins of intact cells were labeled with the NHS-biotin and affinity-purified using avidin-conjugated agarose beads. When probed with anti-γ and anti-β GABAR antibodies, blots of surface protein from cells expressing WT $\alpha_1\beta_2\gamma_2$ receptors displayed significant amounts of γ_2 and β_2 subunit immunoreactivity (Fig. 3). Expression of $\alpha_1\beta_2\gamma_2$R43Q receptors decreased surface β_2 subunit immunoreactivity to 42 ± 15.7% of WT surface β_2 labeling (as measured by densitometry, $n = 3$) and almost completely abolished the surface γ_2 subunit signal to 2.7% ± 1.3% of WT surface γ_2 labeling ($n = 6$, Fig. 3). Western blots of total protein lysates (cell surface and intracellular proteins) showed no significant differences in β_2 or γ_2 subunit immunoreactivities between cells expressing WT and mutant receptors. These findings suggest that although the γ_2R43Q mutation does not alter the total amount of γ_2 subunit proteins produced within the cell, it does cause a significant decrease in the fraction of the total γ_2 subunit protein pool expressed on the cell surface. Whereas a decrease in surface β_2 signal was observed from cells expressing $\alpha_1\beta_2\gamma_2$R43Q receptors compared with WT receptors (58 ± 15.7%), the decrease in signal was significantly less ($p < 0.05$, Mann-Whitney) than the decrease seen in γ_2 subunit reactivity (97 ± 1.3%) and suggests that $\alpha_1\beta_2$ pentameric receptors are being formed and expressed on the cell surface (see "Discussion").

To verify the loss of γ_2-containing surface receptors, cell surface expression of WT and mutant γ_2 subunits was assayed

TABLE I

FNZM and muscimol K_i and B_{max} values for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$R43Q GABARs

Values are mean ± S.E. for N number of experiments (see "Experimental Procedures"). Statistical differences between WT and mutant logK_i and B_{max} values were determined using the Wilcoxon matched pairs test of significance, with the exception of logK_i for FNZM, where differences were determined by the paired t-test of significance since $N < 4$.

	Flunitrazepam				Muscimol			
	K_i	N	B_{max}	N	K_i	N	B_{max}	N
	nM		*fmol/mg*		*nM*		*fmol/mg*	
$\alpha_1\beta_2\gamma_{2s}$	7.03 ± 2.5	3	424.5 ± 53.6	8	61.8 ± 28.5	4	654.4 ± 85.5	4
$\alpha_1\beta_2\gamma_2$R43Q	13.35 ± 1.8	3	108 ± 15.8[a]	8	109.4 ± 44.2	4	859.1 ± 104.2	4

[a] Significantly different from $\alpha_1\beta_2\gamma_2$ ($p < 0.01$).

TABLE II

Potentiation of [^{3}H]FNZM binding by GABA

Values shown are the mean ± S.E. for N number of experiments. EC_{50} is the GABA concentration needed to reach half-maximal potentiation of [^{3}H]FNZM binding. Max potentiation refers to potentiation of [^{3}H]FNZM binding at the highest concentrations of GABA tested (top plateau of the dose-response curve). No statistical differences between WT and mutant values were measured using the non-parametric Mann-Whitney (unpaired) test of significance.

	EC_{50}	N	Max potentiation	N
	μM			
$\alpha_1\beta_2\gamma_{2s}$	1.63 ± 0.28	3	1.35 ± 0.11	3
$\alpha_1\beta_2\gamma_2$R43Q	1.65 ± 0.92	4	2.43 ± 0.36	4

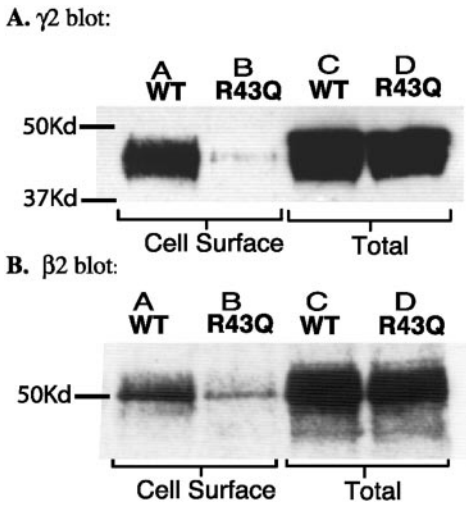

FIG. 3. **γ_2R43Q disrupts surface expression of GABAR γ_2 and, to a lesser extent, β_2 subunits.** *A*, representative Western blot from cells expressing WT $\alpha_1\beta_2\gamma_2$ (*lanes A* and *C*), and $\alpha_1\beta_2\gamma_2$R43Q (*lanes B* and *D*) GABARs probed with anti-γ_2 antibody. Similar results were obtained in six experiments. *B*, representative Western blot from cells expressing WT $\alpha_1\beta_2\gamma_2$ (*lanes A* and *C*) and $\alpha_1\beta_2\gamma_2$R43Q (*lanes B* and *D*) GABARs probed with anti-β_2 antibody. The experiment was performed three times with similar results. *Cell Surface* represents affinity-purified cell surface protein. *Total* represents samples of equal protein concentration from whole cell lysates (surface + intracellular protein). In the above blots, as determined by densitometry, the γ_2R43Q mutation decreased γ_2 surface immunoreactivity 97.5% as compared with wild-type transfections and decreased β_2 surface immunoreactivity 69%. Thus, the γ_2R43Q mutation decreases γ_2 surface immunoreactivity to a greater extent than β_2 surface immunoreactivity.

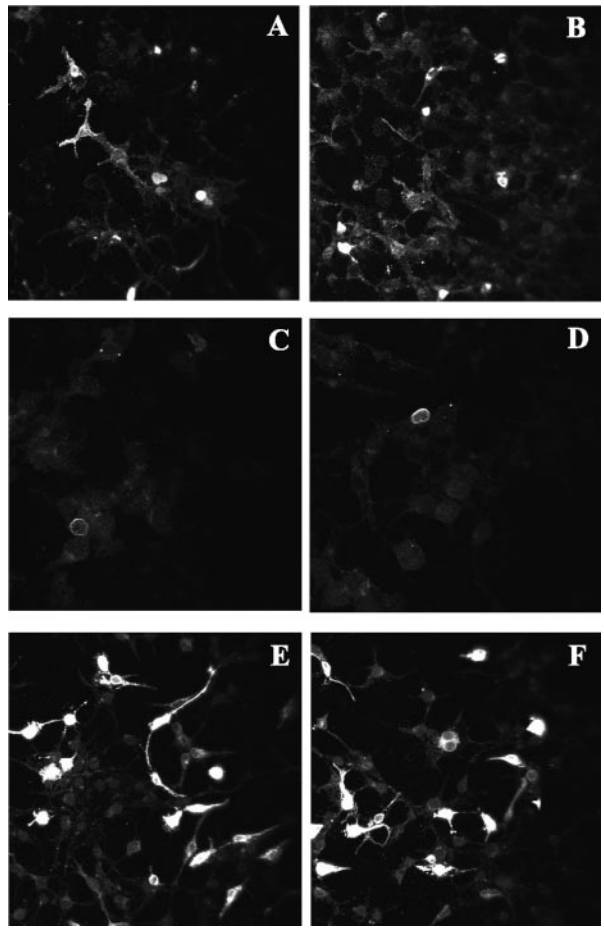

FIG. 4. **The γ_2R43Q mutation inhibits surface expression of the γ_2 subunit.** γ_2 immunostaining of intact HEK 293 cells expressing WT $\alpha_1\beta_2\gamma_2$ (*A* and *B*) and$\alpha_1\beta_2\gamma_2$R43Q (*C* and *D*) GABARs reveal a large decrease in γ_2R43Q surface labeling. Immunostaining of permeabilized HEK 293 cells expressing WT $\alpha_1\beta_2\gamma_2$ (*E*) and $\alpha_1\beta_2\gamma_2$R43Q receptors (*F*) indicate that γ_2R43Q is expressed and localized intracellularly. Images are from one representative experiment repeated three times with similar results. All *panels* contain similar numbers of cells (as visualized in bright field).

by immunostaining intact cells with an anti-γ subunit antibody and a fluorescently tagged secondary antibody. Cells expressing WT $\alpha_1\beta_2\gamma_2$ receptors displayed robust γ_2 subunit labeling on the cell surface (Fig. 4, *A* and *B*, are two representative 40× fields depicting γ_2 staining of intact HEK cells expressing WT receptors). In cells expressing $\alpha_1\beta_2\gamma_2$R43Q receptors, the intensity of the γ_2 subunit cell surface staining as well as the number of cells labeled were dramatically reduced relative to WT-expressing cells, confirming that the γ_2R43Q mutation caused a reduction in γ_2 subunit-containing GABARs expressed on the cell surface (Fig. 4, *C* and *D*, are two representative 40× fields illustrating γ_2 staining of intact cells expressing mutant $\alpha_1\beta_2\gamma_2$R43Q receptors, where the total number of cells in each *panel* under bright field are comparable with the number of cells in Fig. 4, *A* and *B*). In permeabilized cells expressing either WT (Fig. 4*E*) or mutant $\alpha_1\beta_2\gamma_2$R43Q receptors (Fig. 4*F*), intense perinuclear fluorescent staining of the γ_2 subunit was observed indicating that the mutant γ_2R43Q subunit was being produced in similar quantities as WT γ_2 subunit. Perinuclear staining was seen in permeabilized cells expressing either WT or mutant receptors, which is consistent with an endoplasmic reticulum localization of the γ_2 subunit.

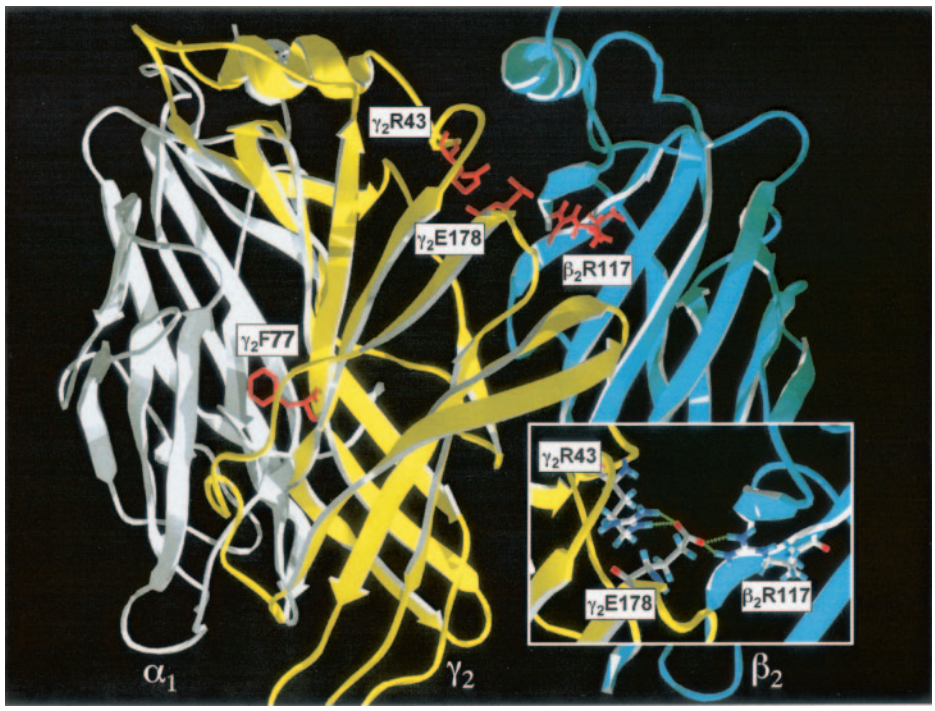

FIG. 5. **Model of the extracellular $\alpha_1/\gamma_2/\beta_2$ subunit interfaces of the GABAR.** The α_1 subunit is *white*, the γ_2 subunit is *yellow*, and the β_2 subunit is *blue*. Several residues are highlighted; γ_2F77 is located at the BZD binding site (α_1/γ_2 interface) whereas γ_2R43 is located at the γ_2/β_2 subunit interface. *Inset*, putative salt bridge interactions (*green dashed lines*) between γ_2R43, γ_2E178, and β_2R117.

DISCUSSION

The goal of this study was to evaluate the functional effect of the GABAR γ_2R43Q mutation linked to human epilepsy. We demonstrate that the main effect of the γ_2R43Q mutation is to reduce surface expression of $\alpha_1\beta_2\gamma_2$R43Q receptors while leaving the pharmacology and functional coupling between GABA and BZD binding sites of the receptor intact. As discussed below, our data both extend previous findings and help clarify the inconsistencies in the reported electrophysiological effects of this mutation.

Using two electrode voltage clamp recording techniques and expression in *Xenopus laevis* oocytes, Wallace *et al.* (13) reported no differences in GABA evoked current amplitude or GABA EC_{50} but observed a complete abolition in the ability of diazepam to potentiate GABA current in mutant receptors compared with wild-type receptors and suggested that the mutation disrupted BZD binding. Although the observed inability of diazepam to potentiate GABA current in mutant receptors may suggest an alteration in BZD binding (*i.e.* altered BZD binding affinity), it could also result from the uncoupling of GABA and BZD binding sites resulting in a loss of BZD modulation of GABA-activated current or from a reduction in the relative expression of $\alpha_1\beta_2\gamma_2$ containing GABARs as compared with $\alpha_1\beta_2$ receptors, which are not modulated by BZDs but are still activated by GABA. Our results indicate that the γ_2R43Q mutation disrupts expression of γ_2 containing GABARs as evidenced by the decrease in cell surface amounts of γ_2 subunit (Figs. 3 and 4) and the 75% reduction in the total number of binding sites for the BZD [^{3}H]FNZM in cells expressing mutant receptors (Fig. 1). Moreover, for the $\alpha_1\beta_2\gamma_2$R43Q receptors that were expressed, the binding affinity for [^{3}H]FNZM and the ability of GABA to potentiate [^{3}H]FNZM binding were similar to wild-type $\alpha_1\beta_2\gamma_2$ receptor values (Figs. 1 and 3). Thus, it is unlikely that the loss of BZD sensitivity observed by Wallace *et al.* (13) and the decrease in BZD sensitivity observed by Bowser *et al.* (14) are caused by alterations in BZD binding affinity or the functional coupling between the BZD and GABA binding sites but may instead be the result of a reduction in the relative expression of $\alpha_1\beta_2\gamma_2$R43Q containing GABARs as compared with $\alpha_1\beta_2$ receptors. Alternatively, the differences between our data and those of Wallace *et al.* (13) may be caused by different expression systems (HEK 293 cells *versus* oocytes).

Interestingly, in cells expressing $\alpha_1\beta_2\gamma_2$R43Q receptors, we observed a decrement in the level of β_2 subunit expressed on the cell surface in addition to the almost complete loss of surface γ_2 subunit (Fig. 3*B*). This observation raises the intriguing possibility that the γ_2R43Q mutation has a dominant negative effect on surface GABAR expression in that it may hamper the expression of $\alpha_1\beta_2$ GABARs as well as $\alpha_1\beta_2\gamma_2$ receptors. Although our binding data demonstrate that the total number of muscimol binding sites is not affected by the γ_2R43Q mutation (Fig. 1), the data reflect binding to membrane homogenates that contain assembled and partially assembled intracellular receptors as well as fully assembled cell surface receptors. Intracellular and incompletely assembled receptors containing α and β subunits bind muscimol with similar high affinity (~10 nM) as fully assembled pentameric receptors (19). As such, any small decrement in cell surface expression could be compensated by muscimol binding to a larger pool of intracellular assembly intermediates, hence explaining the comparable levels of muscimol binding between membranes prepared from cells expressing mutant and WT receptors. This possibility is supported by the finding that in the absence of γ subunits, α and β subunits have a greater propensity to form assembly intermediates (19, 20).

Why a similar phenomenon is not observed for flunitrazepam binding can be explained by the finding that $\alpha\gamma$ assembly intermediates, though viable, possess a 100-fold lower affinity for flunitrazepam than fully formed $\alpha\beta\gamma$ pentamers (19). Our filtration binding assays would not detect the low affinity sites associated with assembly intermediates. Our assay preferentially detects high affinity flunitrazepam binding to pentameric receptors and thus the 75% decrease in flunitrazepam binding observed reflects a decrease in $\alpha\beta\gamma$ pentameric receptors. Whereas the mutant γ_2 subunit may still be able to assemble individually with α_1 or β_2 subunits (forming $\alpha_1\gamma_2$ or $\beta_2\gamma_2$ assembly intermediates), the γ_2 subunit mutation could prevent assembly and/or targeting of fully assembled pentameric receptor complexes, thus tagging or targeting both mutant γ and the associated α or β subunits for intracellular sequestration

and/or degradation. In this way, the γ_2 subunit could sequester some α_1 and β_2 subunits that would otherwise form "normal" and functional $\alpha_1\beta_2$ heteropentameric receptors that would be expressed on the cell surface.

Nevertheless, significant amounts of the β_2 subunit were detected in surface protein purified from cells expressing mutant receptors (Fig. 3*B*) indicating that fully formed $\alpha_1\beta_2$ pentameric receptors are being expressed on the cell surface. It is not likely that the β_2 subunits detected on the cell surface are incorporated into anything other than pentamers, as previous studies have shown that partially assembled receptor intermediates containing $\alpha\gamma$, $\alpha\beta$, or $\beta\gamma$ subunits are not expressed on the cell surface (19). In electrophysiological studies (14, 15), GABA-evoked currents were measured from HEK 293 cells co-expressing wild-type α, β, and mutant γ_2R43Q subunits, indicating expression of some β_2 subunit containing receptors on the cell surface. Our data, however, suggest a decrease in surface GABAR protein in cells expressing the mutant γ_2R43Q subunit compared with cells expressing WT subunits. This is consistent with results reported in Bianchi *et al.* (15), where HEK 293 cells expressing mutant γ_2R43Q subunits displayed reduced GABA current amplitudes.

A recent study by Bowser *et al.* (14), using rapid drug application and patch-clamp recordings of HEK 293 cells expressing mutant receptors found that $\alpha_1\beta_2\gamma_2$R43Q receptors displayed slowed deactivation, an increase in the fast component of desensitization, and an increase in paired-pulse desensitization, indicating that the mutation altered receptor kinetics. In contrast, a paper by Bianchi *et al.* (15), using a similar recording technique and cell expression system, reported no differences in macroscopic GABAR kinetics or diazepam potentiation of GABA current in $\alpha_1\beta_3\gamma_{2L}$R43Q receptors but observed reductions in peak GABA-induced currents instead. It is unclear why the γ_2R43Q mutation had different effects on GABAR current responses in the Bowser *et al.* (14) and Bianchi *et al.* (15) studies. One possible explanation for these conflicting findings is that different receptor subunit subtypes were expressed in the two studies. Regardless, our data, using biochemical approaches, directly demonstrate that one of the main effects of the γ_2R43Q mutation is to reduce cell surface $\alpha_1\beta_2\gamma_2$ receptor expression. However, because the equilibrium radioligand binding techniques used in this study cannot resolve alterations in receptor kinetics, it is certainly possible that the $\alpha_1\beta_2\gamma_2$R43Q receptors that are expressed have altered kinetic properties.

A homology model of the N-terminal region of the GABAR based on the crystal structure of the homologous acetylcholine binding protein (21) localizes γ_2R43 to the γ/β subunit interface (Fig. 5) and not the BZD binding site interface (α/γ). The γ_2R43Q mutation does not affect BZD or GABA apparent binding affinities, which supports the prediction that γ_2R43Q is not involved in forming the BZD or GABA binding pockets and also indicates that the mutation does not have an indirect allosteric effect on BZD or GABA apparent binding affinities. Our data demonstrate that γ_2R43 is important for efficient cell surface expression of GABARs. γ_2R43 is conserved not only in all GABAR subunit subtypes, but also in the subunits of other members of the ligand gated ion channel superfamily. The absolute conservation of this arginine points to a structural role. By mutating γ_2 Arg 43 to Glu, putative electrostatic interactions with a nearby negatively charged residue (γ_2E178) and a positively charged residue on the neighboring β_2 subunit (R117) may be disrupted (Fig. 5), thus preventing the stable or successful incorporation of the γ_2 subunit into a pentamer (22).

Although the idea that a single residue could have such an impact on cell surface expression may seem dubious, the concept is not unprecedented. Recently, a single residue (Ser-171) in the γ_2 subunit when mutated to glycine or cysteine has been found to completely abolish cell surface expression and assembly of $\alpha_2\beta_1\gamma_2$ GABARs (23). It is also equally possible that γ_2R43 is part of a larger assembly motif that when altered disrupts the assembly of the γ_2 subunit with either α_1 or β_2 subunits. Previous experiments have shown that residues within the N-terminal domain of the γ_2 subunit are critical for selective subunit assembly and subunit arrangement (24, 25). Although the underlying mechanism responsible for reduced cell surface expression of γ_2R43Q subunit containing GABARs is unclear (*e.g.* misfolding, reduced subunit assembly, reduced surface membrane receptor targeting, increased degradation), the γ_2 subunit is indispensable for the maintenance of GABARs at synapses (26). As such, the decrease in surface expression of γ_2 containing receptors by the γ_2R43Q mutation would likely blunt synaptic inhibitory neurotransmission, thereby contributing to neuronal hyperexcitability and hence the etiology of epilepsy.

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