Degradation of a GABAA Receptor Epilepsy Mutation That Inserts an Aspartate in the M3 Transmembrane Segment of the α1 Subunit

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A GABAA receptor α1 subunit epilepsy mutation (α1(A322D)) introduces a negatively charged aspartate residue into the hydrophobic M3 transmembrane domain of the α1 subunit. We reported previously that heterologous expression of α1(A322D)β2γ2 receptors in mammalian cells resulted in reduced total and surface α1 subunit protein. Here we demonstrate the mechanism of this reduction. Total α1(A322D) subunit protein was reduced relative to wild type protein by a similar amount when expressed alone (86 ± 6%) or when coexpressed with β2 and γ2S subunits (78 ± 6%), indicating an expression reduction prior to subunit oligomerization. In α1β2γ2S receptors, endoglycosidase H deglycosylated only 26 ± 5% of α1 subunits, consistent with substantial protein maturation, but in α1(A322D)β2γ2S receptors, endoglycosidase H deglycosylated 91 ± 4% of α1(A322D) subunits, consistent with failure of protein maturation. To determine the cellular localization of wild type and mutant subunits, the α1 subunit was tagged with yellow (α1-YFP) or cyan (α1-CFP) fluorescent protein. Confocal microscopic imaging demonstrated that 36 ± 4% of α1-YFPβ2γ2 but only 5 ± 1% α1(A322D)-YFPβ2γ2 colocalized with the plasma membrane, whereas the majority of the remaining receptors colocalized with the endoplasmic reticulum (55 ± 4% α1-YFPβ2γ2S, 86 ± 3% α1(A322D)-YFP). Heterozygous expression of α1-CFPβ2γ2S and α1(A322D)-YFPβ2γ2S or α1-YFPβ2γ2S and α1(A322D)-CFPβ2γ2S receptors showed that membrane GABAA receptors contained primarily wild type α1 subunits. These data demonstrate that the A322D mutation reduces α1 subunit expression after translation, but before assembly, resulting in endoplasmic reticulum-associated degradation and membrane α1 subunits that are almost exclusively wild type subunits.

GABAA receptors are pentameric ligand-gated chloride ion channels that are the major inhibitory neurotransmitter receptors in the mammalian central nervous system (1). The five subunits arise from seven subunit families that contain multiple subtypes and assemble in a limited number of subunit combinations, with the most prevalent consisting of two α1 subunits, two β2 subunits, and one γ2 subunit (2–5). Each subunit contains four hydrophobic segments (M1–M4) that are homologous to the four membrane spanning helices of the Torpedo marmorata nicotinic acetylcholine receptor (AChR) subunits whose three-dimensional structure has been determined to 4 Å (6).

A nonconserved missense mutation in the GABAA receptor α1 subunit gene (GABRA1, α1(A322D)) that codes for an aspartate in place of an alanine at position 7 of the M3 transmembrane segment is present in a form of autosomal dominant juvenile myoclonic epilepsy (7), an idiopathic generalized epilepsy syndrome that accounts for ~10% of all cases of epilepsy (8). When expressed in heterologous cells, this mutation affects both the function and expression of GABAA receptors. Expression of the α1(A322D) subunit with β2 and γ2 subunits (“homozygous expression”) reduced peak currents by ~90%, substantially altered whole cell current kinetics, and reduced mean single channel open times (7, 9, 10). We recently reported that the α1(A322D) mutation reduced α1 subunit expression by 94%, and that it produced asymmetrical, subunit position-dependent reduction of heterozygous receptor currents and α1 subunit protein expression (10). Heterozygous receptors constructed from concatamers with the α1(A322D) subunit positioned between two β2 subunits had 35% of peak current amplitudes and 70% of protein expression relative to wild type receptors, whereas heterozygous receptors with α1(A322D) positioned between β2 and γ2 subunits had 1% of peak current amplitude and 51% of protein expression of wild type receptors. To our knowledge, this is the first naturally occurring missense mutation that reduces expression of a ligand-gated ion channel subunit.

Because α1(A322D) substantially reduced the amount of α1 subunit, it is likely that it is this reduction in total α1 subunit expression, and not the alteration of GABAA receptor current kinetics, that is the predominant mechanism by which this mutation causes disinhibition and epilepsy. Here we determined the mechanism by which this single missense mutation reduced α1 subunit expression.

MATERIALS AND METHODS

Expression of recombinant GABAA Receptors—pcDNA3.1 plasmids containing cDNAs that encode human α1, β2S, and γ2S GABAA receptor subunits were a gift from Dr. Mathew Jones (University of Wisconsin, Madison, WI). α1-YFP and α1-CFP cDNAs were constructed by first inserting HpaI and SacII restriction sites between the codons encoding amino acids four and five of the mature α1 subunit. DNA encoding the fluorescent protein (FP) from the corresponding γ2S-FP subunit (11) was removed by HpaI and SacII digestion and then ligated into the α1

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§ The abbreviations used are: GABA, γ-aminobutyric acid; AChR, nicotinic acetylcholine receptor; GABA receptor, γ-aminobutyric acid receptor type; M3, transmembrane domain 3; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FP, fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IDV, integrated density volume; endo-H, endoglycosidase H; PNGaseF, peptide N-glycosidase-F; WT, wild type; AFU, arbitrary fluorescence units.
**GABA<sub>A</sub> α1(A322D) Mutation Causes ERAD**

subunit-containing plasmid between the codons encoding amino acids four and five of the mature subunit. Cycle 3 GFP-tagged α1 subunit was constructed by performing a blunt ligation of the α1 subunit cDNA into cycle 3 GFP-containing pcDNA3.1 plasmid (Invitrogen). The α1(A322D) mutation was made using the QuikChange site-directed mutagenesis kit (Stratagene). All cDNA sequences were confirmed by DNA sequencing.

Human embryonic kidney cells (HEK293T) were a gift from P. Connelly (COR Therapeutics, San Francisco, CA). Cells were grown at 37 °C in 5% CO<sub>2</sub>, 95% air using Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 100 IU/ml streptomycin and penicillin (Invitrogen). Cells were transfected using the FuGENE 6 transfection reagent (Roche Diagnostics, 2.7 μl/μg of DNA). For the Western blot experiments, the cells were transfected in 6-cm dishes (Corning, Corning, NY) using 2:2:2 (IDV, pixel intensity normalized to the loading control (GAPDH or actin). For the ER colocalization experiments, the cells were also transfected with 30 ng of a cyan fluorescent protein-tagged endoplasmic reticulum marker (CFP-ER, BD Biosciences).

**Immunoblot—**The Western blot protocol has been described (10). Transfected cells were lysed in modified radioimmunoprecipitation solution (RIPA, 20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.25% deoxycholate) that contained one pellet of Complete Mini<sup>TM</sup> protease inhibitor (Roche Diagnostics) per 10 ml. The lysates were centrifuged at 10,000 × g for 30 min. Lysates were fractionated by SDS-PAGE at the acrylamide concentrations given in the figure legends. After SDS-PAGE, the proteins were electrotransferred to polyvinylidene fluoride membranes (Millipore Inc.). All primary antibodies were monoclonal and were purchased from Chemicon Inc. (Temecula, CA). In experiments in which the cells were transfected with untagged α1 and α1(A322D) subunits, the membranes were first incubated with an antibody against the α1 subunit (5 μg/ml, clone BD24) in addition to an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 0.1 μg/ml, clone 6C5), which was used to control for the amount of protein loaded on the gel. In experiments in which the cells were transfected with FP-tagged α1 subunit, the membranes were incubated with a monoclonal antibody to green fluorescent protein (1:2500) as well as an antibody to β-actin (clone C4, 1 μg/ml). After incubation with the primary antibody, all immunoblots were incubated with horseradish peroxidase-coupled goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, 1:6000 dilution) and then visualized with a chemiluminescence detection system (Amersham Biosciences) using a digital imager (Alpha Innotech, San Leandro, CA). The integrated density volume (IDV, pixel intensity × mm<sup>2</sup>) of each band was calculated using the Alpha Innotech software. Background IDVs were obtained from regions adjacent to the bands of interest and subtracted from the total IDV. All protein bands were normalized to the loading control (GAPDH or actin).

**Endoglycosidase Digestion—**Protein lysates were prepared as described above and their protein concentrations were measured using the Micro BCA Protein Assay<sup>TM</sup> (Pierce). Endoglycosidase H (endo-H) and peptide N-glycosidase-F (PNGaseF) were obtained from Sigma. Endo-H and PNGaseF digestion of nicotine AChR δ-subunit has been described (13). Both endoglycosidase digestions were performed for 3 h at 37 °C. Endo-H digestions were performed in 50 mM sodium citrate, pH 5.5, 1% Triton X-100, 0.1% SDS, 50 mM β-mercaptoethanol with 0.2 units/ml endo-H. PNGaseF digestions were performed in 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 50 mM β-mercaptoethanol with 0.2 units/ml PNGaseF. The reactions were terminated by addition of Laemmli sample buffer, and the reaction products were detected by SDS-PAGE and Western blot as described above.

**Confocal Microscopy and Image Analysis—**Approximately 24 h before the confocal microscopy experiments, the transfected cells were plated in collagen-coated 35-mm glass-bottom dishes (MatTek, Ashland, MA). The dishes were then coded, and thus the microscopy experiments were performed in a single-blinded fashion. Immediately before imaging, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM 4-64, 0.3 μg/ml, Invitrogen) was added to the culture media to stain the cells’ plasma membranes without staining the ER (14). Cells were chosen at random and were imaged using a Zeiss 500 META confocal microscope with a ×40, 1.3 numerical aperture Pan neofluor objective. For all cells except those transfected with α1-CFP, the pinhole of all channels was adjusted so that the images were obtained as a single 2-μm slice through the middle of the cell. To collect more emitted light from the cells that were transfected with α1-CFP, the pinhole of all channels was adjusted so that the images were obtained as a single 3-μm slice through the middle of the cell. Excitation wavelengths were 455, 514, and 543 nm for CFP, YFP, and FM 4-64, respectively. For each excitation wavelength, the laser power was adjusted as necessary to utilize the full dynamic range of the detector for each respective fluorophore. CFP emission was detected with a 475–525-nm band pass filter, and FM 4-64 emission was detected using a 560-long pass filter. YFP emission was spectrally separated from CFP and FM 4-64 by reflecting the emitted light off a NFT545 dichroic mirror and then filtering it through a 530–600-band pass filter. The digital images were obtained with 2.8 times scanning zoom and 8 bit, 512 × 512 pixel resolution. All images presented in the figures are unprocessed.

The confocal image files were coded and processed in a single-blind fashion using the ImageJ software (National Institute of Health, Bethesda, MD). Background FP fluorescence for each image was obtained from cells that stained with FM 4-64 but did not express FP. Background CFP-ER fluorescence was defined as the CFP fluorescence in the nucleus. The background fluorescence was subtracted from the images at the 99th percentile. For cells cotransfected with CFP-ER, membrane YFP fluorescence was defined as the YFP outside the boundary of CFP-ER and FM 4-64 staining. For cells not cotransfected with CFP-ER, membrane FP fluorescence was defined as the FP outside the inner portion of FM 4-64 staining. ER YFP fluorescence was defined as intracellular YFP that colocalized with ER fluorescence. The integrated fluorescence from each subcellular region was calculated by summing all the background-subtracted fluorescence values from each pixel in that area.

**Fluorescence Spectroscopy—**Lysates of cells expressing β2 and γ2S subunits and wild type, heterozygous, or homozygous cycle 3 GPP-tagged α1 subunit (α1-GFP) were prepared as described above. Cycle 3 GFP was used to tag the α1 subunit for fluorescence spectroscopy rather than enhanced fluorescence proteins (CFP or YFP) because of its high fluorescence efficiency (15); it was not used for microscopy because its absorbance maximum is in the ultraviolet region of the spectrum. Aliquots (200 μl) of the lysates were placed in microtiter plates, and the fluorescence was determined in a Flexstation<sup>TM</sup> fluorescence spectrometer (Molecular Devices, Sunnyvale, CA) with an excitation of 395 nm and emission of 507 nm. Background fluorescence was defined as the
fluorescence from lysates from untransfected cells; the background was subtracted from the fluorescence of the experimental lysates.

Electrophysiology—Electrophysiological recordings were performed 48 h after transfection. The intrapipette solution contained (in mM): 153 KCl, 1 MgCl₂, 5 EGTA, 10 HEPES, and 2 MgATP (pH 7.3, osmolarity = 305–310 mOsm). The external recording solution consisted of (in mM): 142 NaCl, 8 KCl, 6 MgCl₂, 1 CaCl₂, 10 glucose, 10 HEPES (pH 7.4, osmolarity = 319–325 mOsm). Recording pipettes were pulled on a Sutter P-2000 micropipette electrode puller (Sutter Instrument Co., San Rafael, CA) from borosilicate capillary glass (Fisher). GABA was applied to the cells via gravity using a system consisting of pulled four-barrel square glass (200–300 µm) connected to a Perfusion Fast-Step (Warner Instrument Corp., Hamden, CT). The solution exchange time was determined by stepping a 10% dilute external solution across the open electrode tip to measure a liquid junction current; the 10–90% rise times for solution exchange were consistently ≤0.4 ms. GABA₆ receptor currents were recorded in voltage clamp mode with cells clamped at −50 mV using a lifted whole cell patch-clamp technique (16) using an Axon 200B amplifier (Molecular Devices). The signals were sampled at 10 kHz and written to a computer hard drive.

Homology Modeling and Secondary Structure Prediction—Multiple amino acid sequences were aligned using the ClustalW software (www.ebi.ac.uk/clustalw/). We identified amino acids of the M3 segments of the Torpedo marmorata AChR subunits that were homologous to the Ala-322 residue of the GABA₆ receptor α1 subunit in two steps. First, the following amino acid sequence were aligned in four groups: 1) Torpedo marmorata nAChR α, β, γ2, and δ subunits; 2) human GABA₆ receptor α1—6 subunits; 3) human GABA₆ receptor β1—3 subunits; and 4) human γ1—3 subunits. Next, the 16 sequences in these four groups were aligned together in conjunction with the human GABA₆ receptor δ-subunit, and the results of the 17-sequence alignment were visually compared with the small group analyses to make certain the sequences that were most similar with one another remained in alignment.

Identification and scoring of transmembrane helices were performed using the relevant ExPASy Proteomics tools (us.expasy.org/tools/) topology prediction algorithms (DAS, TopPred, TMpred, and TMHMM). The predictions were performed first on the native AChR sequences and then on the sequences in which the amino acid in the M3 domain homologous to the GABA₆ α1 subunit Ala-322 was changed to an aspartate. The scores from the topology prediction programs, which predicted a transmembrane helix for the 25-amino acid segments beginning at α1Tyr-277, β1Tyr-283, γ1Tyr-291, and δ1Tyr-286 (corresponding to the M3 transmembrane helices in the Torpedo marmorata electron diffraction data) for the sequences with or without the aspartate substitution, were compared.

Data Analysis—Values are reported as mean ± S.E. Statistical significance for the endoglycosidase, confocal, and fluorescence spectroscopy experiments were determined using the Student’s unpaired t test and the significance of expression differences in the Western blot experiments and the effect of aspartate substitution on topology prediction scores were determined using the paired t test (GraphPad, San Diego, CA).

RESULTS

α1(A322D) Subunit Expression Was Reduced With or Without Coexpression with β2 and γ2S Subunits—GABA₆ receptor subunits oligomerize in the ER via distinct pathways (17) utilizing specific intersubunit contacts (18–23), and unassembled subunits are degraded (24). Because expression of α1(A322D)β2γ2S receptors reduced currents and protein expression in a subunit position-dependent fashion (10), we hypothesized that α1(A322D) subunit mutation inhibited subunit oligomerization, thereby leading to mutant α subunit degradation. To test this hypothesis, we transfected cells with wild type α1 or heterozygous or homozygous α1(A322D) subunits and either wild type β2 and γ2S subunits (α1β2γ2S) or an equivalent amount of empty pcDNA3.1 plasmid (α1pcDNA) and performed Western blots on whole cell lysates (Fig. 1, A and B). The ratio of α1 subunit to GAPDH expression from whole cell lysates was determined by quantification of Western blots from whole cell lysates (Fig. 1, C and D). With transfection of both α1β2γ2S and α1pcDNA subunits, heterozygous α1 subunit expression was intermediate between those of wild type and the homozygous mutant α1 subunit expression (percentage of wild type expression of α1β2γ2S: n = 10, heterozygous 66 ± 6%, p = 0.045, homozygous 22 ± 6% p = 0.011; α1pcDNA n = 10: heterozygous 49 ± 6%, p = 0.005, homozygous 14 ± 6%, p = 0.003). There were no significant differences in the reduction of α1 subunit expression between cells transfected with α1β2γ2S subunits and those transfected with α1 subunits alone (heterozygous p = 0.061, homozygous p = 0.335). These results were unexpected because they indicated that the α1(A322D) mutation reduced α1 subunit protein levels prior to receptor assembly.

α1(A322D) Was Endoglycosidase H Sensitive—A reduction in α1(A322D) subunit expression prior to receptor assembly could result from decreased efficiency of transcription or translation or from increased post-translational degradation of the mutant α1(A322D) subunit, known as ER-associated degradation (ERAD). If α1(A322D) subunit transcription or translation were reduced, the post-translational processing of residual α1(A322D) subunit would be similar to that of the wild type subunit. In contrast, accelerated ERAD would result in the residual α1(A322D) subunits having ER- but not Golgi-associated processing.

The α1 subunit has two sites of N-linked glycosylation that reside on its extracellular N terminus (17, 25). Membrane proteins are N-linked glycosylated with high mannose carbohydrates co-translationally within the ER, but upon trafficking to the trans-Golgi the high mannose carbohydrates are replaced with low mannose carbohydrates (26). Digestion with endo-H removes high mannose N-linked carbohydrates, whereas digestion with PNGaseF removes all carbohydrates. Therefore,
endo-H sensitivity indicates a protein has not been trafficked at least as far as the trans-Golgi (27). To determine the glycosylation state of mutant α1(A322D) subunits, we digested cell lysates with endo-H or PNGaseF from cells transfected with wild type α1β2γ2S, heterozygous α1α1(A322D)β2γ2S, and homozygous α1(A322D)β2γ2S receptor subunits.

Undigested cell lysates and those digested with endo-H or PNGaseF were analyzed by Western blot (Fig. 2, A and B). Because the wild type α1 subunit expresses more efficiently than heterozygous and homozygous α1 subunits, protein was loaded in the ratio WT:Heterozygous: homozygous, 8:15:50 μg, to balance the amount of α1 subunit protein. Western blots demonstrated that the α1 subunits from undigested lysates migrated at 50 kDa, and those digested with PNGaseF migrated at 46 kDa. Endo-H digestions of homogenous α1 subunit migrated in a single band at 46 kDa, but those of WT and heterozygous subunits migrated in two bands at 48.4 (endo-H resistant) and 46 kDa (endo-H sensitive). The fraction of endo-H-resistant α1 to total α1 subunit was plotted.

FIGURE 2. A, lysates (2.5 mg/ml) from cells expressing wild type (WT), heterozygous (het), and homozygous (hom) receptors were left undigested (U) or digested with endo-H (E) or PNGaseF (F). Digestion products were fractionated via 12.5% SDS-PAGE and were loaded on the gel in the ratios 8:15:50, WT:heterozygous:homozygous, to balance the amount of the α1 subunit protein. Western blots demonstrated that the α1 subunits from undigested lysates migrated at 50 kDa, and those digested with PNGaseF migrated at 46 kDa. Endo-H digestions of homogenous α1 subunit migrated in a single band at 46 kDa, but those of WT and heterozygous subunits migrated in two bands at 48.4 (endo-H resistant) and 46 kDa (endo-H sensitive). B, the fraction of endo-H-resistant α1 to total α1 subunit was plotted.

As a second method to determine the effect of α1(A322D) on the expression of the α1-YFP subunit, we performed Western blots using an anti-GFP antibody on whole cell lysates from cells transfected with wild type, heterozygous, and homozygous α1-YFP subunits, with independent processing of these subunits, 42, not 64%, of heterozygous α1 subunit should have been endo-H sensitive. This suggests that other processes (such as protein degradation) may alter the relative ratios of wild type α1 and α1(A322D) subunits. Another alternative is that this assay lacks the resolution and statistical power to detect a difference in the extent of glycosylation between wild type and heterozygous receptors.

α1-YFPβ2γ2S and α1(A322D)-YFPβ2γ2S Receptors Were Functional—To further characterize the subcellular localization of wild type and mutant α1 subunits, we constructed YFP-tagged wild type (α1-YFP) and mutant (α1(A322D)-YFP) subunits. Electrophysiological analysis of N-terminal fluorescent-tagged γ2-subunit-containing GABA A receptors has been performed in fibroblasts (11, 28), and electrophysiological analysis of GABA A receptors containing α1 subunits tagged with GFP at the C terminus has been done in oocytes (29). We evaluated GABA-evoked currents in cells transfected with GABA A receptors containing wild type or mutant α1 subunits tagged with YFP at the N terminus. Currents were evoked from both wild type α1-YFPβ2γ2S (n = 7) and homozygous α1(A322D)-YFPβ2γ2S (n = 5) receptors upon application of 1 mM GABA (Fig. 3). Wild type α1-YFPβ2γ2S receptors, but not the homozygous α1(A322D)-YFPβ2γ2S receptors, had fast phases of GABA-evoked desensitization, and thus the current kinetics were similar to those of the respective non-FP-tagged receptors (9, 10). Thus, adding a fluorescent protein at the N terminus of the GABA A receptor α1 subunit did not substantially change the properties of wild type or α1(A322D) mutant α1 subunit currents evoked by 1 mM GABA.

α1-YFP and α1(A322D)-YFP Subunit-containing Receptors Had Similar Relative Expression Levels, but Different Processing Than Non-α1-YFP-tagged Receptors—To determine whether α1(A322D) altered expression of α1-FP subunits, we performed fluorescence spectroscopy on whole cell lysates from cells transfected with β2 and γ2S subunits and wild type, heterozygous, and cycle 3 GFP-tagged-α1 subunits. Measuring the fluorescence intensity of a whole cell lysate rather than from individual cells during microscopy gives the mean fluorescence of the sample and avoids cell-to-cell variability that would be obtained during microscopy. Wild type α1-GFPβ2γ2S receptor fluorescence (18,093 ± 1096, arbitrary fluorescence units, AFU) was greater than heterozygous α1-GFPα1(A322D)-GFPβ2γ2S receptor fluorescence (10,870 ± 626 AFU), and heterozygous α1-GFPα1(A322D)-GFPβ2γ2S receptor fluorescence was greater than homozygous α1(A322D)-GFPβ2γ2S receptor fluorescence (3,922 ± 1100 AFU, n = 4, p < 0.001, Fig. 4A). Thus, A322D reduced α1-GFP fluorescence to a similar extent as it reduced protein expression of non-FP-tagged α1 subunit (Fig. 1).
were two specific immunoreactive bands at 64 and 72 kDa \((n = 6)\). For homozygous \(\alpha1(322D)\)-YFP\(\beta2\gamma2\)S receptors, there was only one specific immunoreactive band at 64 kDa.

The bands were quantified, and for each lane, the IDVs were divided by the IDV of actin, which served as a control for the amount of protein loaded in each lane of the gel (Fig. 4, C–E). For \(\alpha1\)-YFP\(\beta2\)\(\gamma2\)S receptors, the total IDV:actin ratio of the two immunoreactive bands for the wild type receptors (normalized to 100%) was greater than those of heterozygous and homozygous receptors (heterozygous 57 ± 6%, \(p = 0.038\), homozygous 46 ± 4%, \(p = 0.037\)) (Fig. 4C). A similar trend was observed for cells transfected with the \(\alpha1\)-YFP subunit alone (heterozygous 92 ± 12%, \(p = 0.377\), homozygous 56 ± 5.8%, \(p = 0.030\)) (Fig. 4D), although the difference between wild type and heterozygous was not statistically significant. Thus, for cells transfected with \(\alpha1\)-YFP subunit alone or cotransfected with \(\alpha1\)-YFP\(\beta2\gamma2\)S subunits, wild type \(\alpha1\)-YFP subunit expression was greater than heterozygous \(\alpha1\)-YFP\(\alpha1(322D)\)-YFP subunit expression, and heterozygous \(\alpha1\)-YFP\(\alpha1(322D)\)-YFP subunit expression was greater than homozygous \(\alpha1(322D)\)-YFP subunit expression. These results were in agreement with those obtained with non-YFP-tagged receptors (Fig. 1). However, for both cells transfected with \(\alpha1\)-YFP\(\beta2\gamma2\)S subunits as well as those transfected with \(\alpha1\)-YFP subunit alone, the fraction of homozygous \(\alpha1(322D)\)-YFP subunit to wild type \(\alpha1\)-YFP subunit was greater than that for the non-YFP-tagged receptors.

The amounts of expression of the 72- and 64-kDa bands were quantified individually (Fig. 4, E and F). Because distinct 72-kDa bands could not be visually identified for homozygous receptors, we quantified the IDV from the region of the homozygous lanes that corresponded to the 72-kDa bands from the wild type lanes. The 64-kDa band from wild type \(\alpha1\)-YFP\(\beta2\gamma2\)S receptors was smaller (38 ± 3.0% of wild type total) than the 72-kDa band (62 ± 3.0% of wild type total), but for the heterozygous \(\alpha1\)-YFP\(\alpha1(322D)\)-YFP\(\beta2\gamma2\)S and homozygous \(\alpha1(322D)\)-YFP\(\beta2\gamma2\)S receptors, the 64-kDa bands (heterozygous 32 ± 3.3%; homozygous 37 ± 4.6% wild type total) were larger than the 72-kDa bands (heterozygous 25 ± 3.4% \(p = 0.009\); homozygous 8.7 ± 1.8%, \(p = 0.015\) wild type total) (Fig. 4E). For the cells in which the \(\alpha1\)-YFP subunit was transfected alone, expression of the wild type 64-kDa band (48 ± 3.2% total wild type) was smaller than the 72-kDa band (52 ± 3.2% wild type), but expression of the heterozygous and homozygous 64-kDa band (heterozygous 55 ± 7.7%, homozygous 40 ± 4.6% total wild type) was larger than the 72-kDa band (heterozygous 38 ± 5.5%, \(p = 0.026\); homozygous 16 ± 2.7%, \(p = 0.011\)) (Fig. 4F).
The difference in both the total α1-YFP subunit expression as well as the ratios of the 64- to 72-kDa bands among the different transfection states could be attributed exclusively to differences in the expression of the 72-kDa band. The 72-kDa band in heterozygous α1-YFPα1(1(A322D))β2γ2 receptors was 40 ± 4% (p < 0.001) of the wild type 72-kDa band, whereas the 72-kDa band from homozygous α1(1(A322D)) was 15 ± 3% (p < 0.001) of the wild type 72-kDa band. For cells transfected with α1-YFP subunit alone, the heterozygous α1-YFPα1(1(A322D)) subunit 72-kDa band was 63 ± 13% (p = 0.005) of the wild-type 72-kDa band, and the homozygous α1(1(A322D))-YFP subunit kDa band was 26 ± 5.8% (p < 0.0001) of the wild type 72-kDa band. In contrast, there were no significant differences among the wild type, heterozygous, and homozygous 64-kDa bands from either α1β3γ2S receptors (heterozygous = 79 ± 11%, p = 0.770; homozygous 86 ± 8%, p = 0.888) or from α1-YFP subunit expressed alone (heterozygous 108 ± 22%, p = 0.014; homozygous 83 ± 9.6%, p = 0.214).

These data demonstrate that similar to the non-YFP-tagged α1 subunits, the α1(1(A322D)) mutation reduced expression of α1-FP subunits. It had been reported recently that Western blots of whole cell lysates from cells expressing GABA<sub>A</sub> receptors containing α1(1(A322D))-GFP subunits demonstrated the presence of the α1(1(A322D))-GFP subunit. From this, it was concluded that α1(1(A322D)) does not preclude α1 subunit expression (30). Here we also show that α1(1(A322D))-YFP is expressed. However, our quantification of the Western blots demonstrated that its expression was reduced, a result confirmed by measurement of α1(1(A322D))-GFP fluorescence.

The addition of YFP to the N terminus of the α1 subunit did change some aspects of its processing. First, α1(1(A322D))-YFP expression relative to wild type expression (46 ± 4%) was greater than that of the non-FP-tagged receptors (22 ± 6%, Fig. 1). Second, on Western blot, α1-YFP ran as bands of two different molecular weights. One possibility is that the presence of the large YFP epitope tag on the N terminus interfered with N-glycosylation of the subunit.

α1-YFP, but Not α1(1(A322D))-YFP, Is N-Glycosylated—In contrast to the non-YFP-tagged α1 subunits that migrated in one band (Fig. 1), the wild type and heterozygous α1-YFP subunits migrated in two bands when expressed alone or with β2 and γ2S subunits. To determine whether the two α1-YFP subunit bands represented differentially glycosylated forms of α1-YFP subunit, we digested wild type α1-YFPβ2γ2S receptors and α1-YFP subunits with endo–H and PNGaseF (Fig. 5). For both α1-YFPβ2γ2S receptors (n = 11) and the α1-YFP subunit alone (n = 5), PNGaseF digestion resulted in a single specific immunoreactive band (anti-GFP antibody) that ran at 64 kDa. This demonstrated that the 72-kDa band was a glycosylated form of the 64-kDa band. PNGaseF digestion (not shown) of heterozygous α1-YFPα1(1(A322D))β2γ2S receptors (n = 8) and α1-YFPα1(1(A322D)) subunits (n = 3) also resulted in a single specific immunoreactive band at 64 kDa. PNGaseF digestion of homozygous α1(1(A322D))-YFPβ2γ2S receptors (n = 7) did not shift the α1-YFP subunit apparent molecular weight (not shown).

Endo–H digestion products of wild type α1-YFPβ2γ2S receptors (n = 4) ran as two bands with apparent molecular masses of 72 and 64 kDa, thus demonstrating that these receptors contained low mannose glycosylation and were thus processed in the trans-Golgi. In contrast, endo–H digestion of cell lysates transfected with α1-YFP subunits alone (n = 4) ran as a single band at 64 kDa, indicating maturation arrest in the ER.

Interestingly, although non-YFP-tagged α1 and α1(1(A322D)) subunits had substantially different fractions of endo–H-sensitive N-linked glycosylation, each was 100% glycosylated (Fig. 2). In contrast, only 85% of wild type α1-YFP and 15% of α1(1(A322D))-YFP subunits were glycosylated. This highlights the fact that even though fluorescently tagged α1 subunits form GABA<sub>A</sub> receptors with similar functional properties as native receptors (Fig. 3), and that the A322D mutation causes qualitatively similar reductions in α1-YFP subunits as non-fluorescently tagged α1 subunits (Fig. 4), there do exist some differences in post-translational glycosylation.

**Assembled, but Not Unassembled, α1-YFP Subunits Localized to the Membrane**—It has been shown that 9E10-tagged α1 subunit homomers do not traffic to the plasma membrane, but, instead, are sequestered in the ER (17) and degraded (24). Likewise, α1 subunit homomers tagged with GFP at the C terminus remain in the ER of oocytes (29). We tested the ability of α1-YFP subunits to traffic to cell membranes with and without coexpression of β2 and γ2S subunits. Cells were transfected with CFP-ER, YFP–α1 subunit, and either β2 and γ2S subunits or an equivalent amount of pcDNA3.1. The cells were imaged by confocal microscopy (Fig. 6A). Although the ER extends to the plasma membrane in these cells, dual labeling of the ER and membrane compartments allowed reliable separation of the ER component from the membrane component. Thus, the fraction of α-YFP subunit colocalized to the membrane and ER could be quantified (Fig. 6B). For receptors transfected with α1-YFP subunit and empty pcDNA3.1, 80 ± 3% of the α1-YFP subunit colocalized to the ER and 6.5 ± 0.8% colocalized to the membrane (n = 11). In contrast, for receptors transfected with α1-YFP, β2, and γ2S subunits, 55 ± 4% of the α1-YFP subunit colocalized in the ER (p < 0.001) and 36 ± 4% colocalized with the plasma membrane (p < 0.001, n = 13). Thus, assembled, but not unassembled, α1-YFP subunits were localized to the membrane.

**The α1(1(A322D))-YFP Subunit Localizes to the ER**—To determine the subcellular localization of α1(1(A322D))-YFP subunits, we transfected cells with CFP-ER and either wild type α1-YFPβ2γ2S, heterozygous α1-YFPα1(1(A322D))-YFPβ2γ2S, or homozygous α1(1(A322D))-YFPβ2γ2S receptors. Visual analysis of confocal images of these cells demonstrated that the majority of the α1-YFP subunit for wild type, heterozygous, and homozygous receptors colocalized with the ER (Fig. 7A). Only wild type and heterozygous cells had identifiable α-YFP subunit colocalized with the membrane. There were no distinct intracellular inclusions of α-YFP subunits for any of the transfection states.

Quantification of the YFP distribution (Fig. 7B) demonstrated that the fraction of total α1-YFP subunit colocalized with the membrane was 36 ± 4% for wild type (n = 13), 20 ± 3% for heterozygous (n = 12, p = 0.003), and 4.8 ± 0.6% for homozygous (n = 10, p = 0.003) receptors. In contrast, the percentage of YFP fluorescence colocalized with the ER was least for wild type (55 ± 4%), intermediate for heterozygous (70 ±
3%, \( n = 12, p = 0.008 \), and most for homozygous (86 ± 2%, \( n = 10, p = 0.001 \)) receptors. There were no differences in the percentage of \( \alpha_1 \)-YFP subunit that was intracellular, but outside the ER (\( p > 0.70 \)).

Membrane \( \alpha_1 \)-YFP Subunits with Heterozygous Expression Are Derived from Wild Type \( \alpha_1 \)-YFP, but Not Mutant \( \alpha_1(\text{A322D}) \)-YFP, Subunits—Individuals with autosomal dominant juvenile myoclonic epilepsy are heterozygous for the \( \alpha_1(\text{A322D}) \) mutation (7), and thus it was of interest to determine the relative amounts of wild type \( \alpha_1 \) and mutant \( \alpha_1(\text{A322D}) \) subunit that were trafficked to the plasma membrane with heterozygous expression. We constructed CFP-tagged wild type (\( \alpha_1 \)-CFP) and mutant (\( \alpha_1(\text{A322D}) \)-CFP) subunits. Cells that were heterozygous for the \( \alpha_1(\text{A322D}) \) subunit mutation were formed by cotransfecting either wild type \( \alpha_1 \)-YFP and mutant \( \alpha_1(\text{A322D}) \)-YFP subunits or wild type \( \alpha_1 \)-CFP and mutant \( \alpha_1(\text{A322D}) \)-YFP subunits. Visual analysis of confocal microscopic images demonstrated that with

![Diagram](image-url)
FIGURE 8. A, cells were transfected with either $\alpha_1$-YFP or $\alpha_1$-CFP and their plasma membranes were labeled with FM 4-64. YFP fluorescence is colored green and CFP fluorescence is colored blue. Imaging of the wild type $\alpha_1$-FP subunit is labeled WT and imaging of the $\alpha_1$-(AD)-FP subunit is labeled AD. $\alpha_1$-CFP that colocalized with FM 4-64 is colored purple and $\alpha_1$-YFP that colocalized with FM 4-64 is colored yellow. B, the fraction of each $\alpha_1$-FP that colocalized with the plasma membrane marker was graphed.

Both $\alpha_1$-YFP and $\alpha_1$-CFP colocalized with FM 4-64, indicating that the majority of wild type $\alpha_1$-FP subunits are associated with the plasma membrane. The total expression of wild type $\alpha_1$-FP subunits in $\alpha_1$-YFP and $\alpha_1$-CFP was nearly identical (2.2-fold), although there was a 2.3-fold higher expression of $\alpha_1$-CFP. With heterozygous expression, the fraction of total wild type $\alpha_1$-FP subunits on the surface was 2.3-fold higher than the fraction of total mutant $\alpha_1$-(AD)-FP subunits on the cell surface (Fig. 8B). Multiplying the differences in expression (2.2-fold) with the differences in subcellular distribution (2.3-fold), we estimate that in heterozygous cells, the molar ratio of wild type $\alpha_1$-FP subunit to $\alpha_1$-(AD)-FP on the cell surface is $\sim$5:1.

With both heterozygous $\alpha_1$-(AD)-CYP and $\alpha_1$-CFP, $\alpha_1$-YFP was used to label the ER. YFP fluorescence is colored green and CFP fluorescence is colored blue. Using, when possible, both membrane and ER markers in cells such as HEK293T cells, whose ER is diffusely distributed and thus abuts the membrane. These results demonstrate that in the cells transfected with heterozygous $\alpha_1$ and $\alpha_1$-(AD)-FP subunits, the majority of the mutant protein remained sequestered in the ER and only wild type $\alpha_1$ subunit formed $\alpha_1$-(AD)-FP receptors at the membrane surface, a result consistent with the nearly identical current kinetic profiles of wild type and heterozygous $\alpha_1$-FP subunits (10).

DISCUSSION

We previously demonstrated that the $\alpha_1$-(AD)-FP mutation reduces $\alpha_1$ subunit expression in a subunit position-dependent manner. Here, we report that the trafficking of the mutant $\alpha_1$-(AD)-FP subunit is altered between translation and oligomerization within the ER. Previously identified $\alpha_1$-FP receptor epilepsy mutations altered the function of $\alpha_1$-FP receptors at the membrane surface, a result consistent with the nearly identical current kinetic profiles of wild type and heterozygous $\alpha_1$-FP subunits (31, 32). However, it is possible that the membrane-trafficking step of $\alpha_1$-(AD)-FP is altered in these cells.


\[ \text{GABA}_\alpha \text{a1(A322D) Mutations Causes ERAD} \]

\( \alpha1(\text{A322D}) \) Alters \( \alpha1 \) Subunit Trafficking after Translation but Before Receptor Assembly—We previously asserted that it was unlikely that the GCC to GAC mutation in the GABRA1 mutant subunit gene reduced \( \alpha1 \) subunit expression by disrupting transcription or translation. Because GAC codons 11 of the 23 aspartates in the wild type \( \alpha1 \) subunit, a codon bias against GAC is improbable. Here, we demonstrate that post-translational trafficking is altered with this mutation; the mutant \( \alpha1(\text{A322D}) \) subunit has only immature N-linked glycosylation that is characteristic of ER-associated glycosylation. In addition, confocal microscopy demonstrated that essentially all of the \( \alpha1(\text{A322D}) \)-YFP subunit was sequestered in the ER, whereas a substantial portion of wild type \( \alpha1 \)-YFP subunit was trafficked to the cell membrane. These trafficking differences between \( \alpha1 \) and \( \alpha1(\text{A322D}) \) subunits cannot be explained by reduced transcription or translation. However, the possibility of reduced translation in addition to altered trafficking cannot be excluded.

The trapping of unassembled GABA\( _\alpha \) receptor subunits in the ER identified that the \( \alpha1(\text{A322D}) \) mutation occurred before subunit oligomerization. It has been shown that GABA\( _\alpha \) receptors require at least an \( \alpha \) and a \( \beta \) subunit to assemble into pentamers; homomeric GABA\( _\alpha \) receptor subunits are subject to ERAD (17, 24, 29). We extended these studies by using confocal microscopy to show that unassembled \( \alpha1 \)-YFP subunits are also retained in the ER (Fig. 6), and that unassembled \( \alpha1(\text{A322D}) \)-YFP subunits lack Golgi-associated glycosylation (Fig. 4). Because the \( \alpha1(\text{A322D}) \) mutation reduced \( \alpha1 \) subunit expression to approximatively the same extent in the absence of cotransfected \( \beta2 \) and \( \gamma2 \) subunits as in \( \alpha1 \beta2 \gamma2 \) receptors (Fig. 1), it is clear that this mutation reduces \( \alpha1 \) subunit expression prior to subunit oligomerization. Whereas it is possible that the \( \alpha1(\text{A322D}) \) mutation may also affect other trafficking processes for the small fraction of \( \alpha1(\text{A322D}) \) subunits that escape ERAD, oligomerize, and traffic to the cell surface, the bulk of \( \alpha1(\text{A322D}) \) subunit expression reduction can be explained by ERAD alone.

Membrane Expression of Heterozygous Receptors Is Composed of Wild Type \( \alpha1 \) Subunits—By making heterozygous transfections using dually labeled \( \alpha1 \)-YFP/\( \alpha1(\text{A322D}) \)-CFP/\( \beta2 \)YFP constructs, we observed that the plasma membrane expression of \( \alpha1 \)-CFP/\( \alpha1(\text{A322D}) \)-YFP/\( \beta2 \)YFP receptors, we determined the plasma membrane expression of \( \alpha1 \)-CFP/\( \alpha1(\text{A322D}) \)-YFP/\( \beta2 \)YFP receptors resulted almost exclusively from wild type \( \alpha1 \)-FP subunits and not from \( \alpha1(\text{A322D}) \)-FP subunits. In one respect this result was expected given that the \( \alpha1(\text{A322D}) \) mutation reduced \( \alpha1 \) subunit expression after translation but before oligomerization and transport out of the ER. However, this result was also surprising given that we previously showed that heterozygous receptors with the \( \alpha1(\text{A322D}) \) subunit positioned between the two \( \beta2 \) subunits (Het\( _{\text{a1b2}} \)) produce relatively large GABA-evoked peak currents (335 ± 87 pA) (10), a result that implied that substantial the \( \alpha1(\text{A322D}) \) subunit trafficked to the plasma membrane in heterozygous receptors. One explanation for this apparent discrepancy was that even though Het\( _{\text{a1b2}} \) produced relatively large GABA-evoked currents, they were still only 35% of wild type receptor currents. Therefore, one would expect that approximately only 25% of the \( \alpha1 \) subunit on the cell surface contained the mutant \( \alpha1(\text{A322D}) \) subunit, a result that could be consistent with our confocal microscopy studies (5–17%). A second explanation for this apparent contradiction is that the construction of Het\( _{\text{a1b2}} \) and Het\( _{\text{a1b2}} \) receptors were forced using subunit concatamers. The mechanisms that target untagged \( \alpha1(\text{A322D}) \) subunits for degradation might not apply to larger concatameric subunits. Finally, it is possible that the \( \gamma \beta(\text{A322D}) \) concatamer may fold more efficiently than the \( \alpha1(\text{A322D}) \) monomeric subunit and thus not even activate ERAD mechanisms.

\( \alpha1(\text{A322D}) \) Subunit Misfolding May Cause ERAD—We hypothesize that the mechanism by which the \( \alpha1(\text{A322D}) \) expression was by misfolding and ERAD (35, 36). Could a single point mutation cause misfolding of the majority of translated \( \alpha1 \) protein? It has been shown in model peptides that at neutral or high pH, a single aspartate positioned near the center of a transmembrane helix would disrupt the helix to allow the aspartate to reside near the aqueous surface (37). Therefore, at physiological pH, it may be expected that a substantial number of \( \alpha1(\text{A322D}) \) M3 segments would fail to form stable transmembrane helices after translation. These misfolded subunits could then be targeted for degradation via ERAD.

The Torpedo AChR three-dimensional structure has been determined and each of its four subunit M3 segments have been demonstrated to be a helical (6). Because the primary structure of the Torpedo marmorata AChR four subunits are homologous to GABA\( _\alpha \) receptor subunits, we determined the effect on predicted helix formation of placing an aspartate position 7 of the M3 helices of the Torpedo AChR \( \alpha, \beta, \gamma \), and \( \delta \) subunits. Alignment of the GABA\( _\alpha \) receptor subunits and Torpedo AChR subunits demonstrated that the Torpedo \( \alpha \)-helix, \( \beta \)-helix, \( \gamma \)-helix, and \( \delta \)-helix residues (numbering from the mature protein) were homologous to the GABA\( _\alpha \), \( \alpha1(\text{A322D}) \) subunit M3 segment sequences are shown here. This alignment demonstrated that the \( \alpha1(\text{A322D}) \) subunit M3 segment destabilizes, but does not preclude, formation of a transmembrane helix, a hypothesis that needs to be verified empirically.

The reduction of confidence was most significant when using the TopPred algorithm (\( p < 0.001 \)), a method that is based upon the clustering of hydrophobic residues. The reduction of confidence of forming a transmembrane helix was also significant using the TMHMM (\( p = 0.005 \)) and DAS (\( p = 0.02 \)), but not the THMM (\( p = 0.1 \)) algorithms. These theoretical analyses suggest that the substitution of an aspartate at position 7 of an M3 segment destabilizes, but does not preclude, formation of a transmembrane helix, a hypothesis that needs to be verified empirically.

We hypothesize that the misfolding and subsequent elimination of the mutant \( \alpha1(\text{A322D}) \) subunit in ADJME is similar to the misfolding and elimination of the cystic fibrosis transmembrane regulator protein (38, 39). The majority of the cases of cystic fibrosis are caused by a deletion mutation, \( \Delta \)Phe-508, in its cytoplasmic loop. Like the \( \alpha1(\text{A322D}) \) mutation, the cystic fibrosis transmembrane regulator \( \Delta \)Phe-508 retains high mannose core glycosylated and sequestered in
the ER. Cystic fibrosis transmembrane regulator ΔPhe-508 has been shown to misfold and undergo rapid ERAD via the 26 S proteasome.

It should be emphasized that our studies were performed in fibroblasts and not in neurons. Although protein trafficking in neurons differs from that in fibroblasts, we suggest that because the trafficking error occurs before oligomerization, it is likely that in neurons the mutant α1(A322D) subunit would also undergo ERAD. However, it is also possible that overexpression of the α1(A322D) subunit (either in fibroblasts or cultured neurons) may overwhelm protein folding mechanisms and that increased ERAD of the α1(A322D) subunit protein would not occur to such an extent in a more physiologic expression system. It would be of substantial interest to create this mutation in a transgenic animal. We predict that in a heterozygous α1(A322D) knock-in mouse, the wild type α1 subunit would likely be trafficked to the cell surface and the mutant α1(A322D) subunit would undergo ERAD, producing a mouse that, like the heterozygous α1 knock-out mouse, would have fewer total brain GABA<sub>A</sub> receptors (40–42).

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REFERENCES

1. Macdonald, R. L., and Olsen, R. W. (1994) Annu. Rev. Neurosci. 17, 569–602
2. McKernan, R. M., and Whiting, P. J. (1996) Trends Neurosci. 19, 139–143
3. Tretter, V., Eliga, N., Fuchs, K., and Sieghart, W. (1997) J. Neurosci. 17, 2728–2737
4. Baumann, S. W., Baur, R., and Sigel, E. (2002) J. Biol. Chem. 277, 36275–36280
5. Baumann, S. W., Baur, R., and Sigel, E. (2002) J. Biol. Chem. 277, 46020–46025
6. Miyazawa, A., Fujishoshi, Y., and Unwin, N. (2003) Nature 424, 949–955
7. Posnette, P., Liu, L., Bristoeis, K., Dong, H., Lortie, A., Vanasse, M., Saint-Hilaire, J. M., Carman, L., Verner, A., Lu, W. Y., Wang, Y. T., and Rouleau, G. A. (2002) Nat. Genet. 31, 184–189
8. Genton, P., and Gelisse, P. (2001) Arch. Neurol. 58, 1487–1490
9. Fisher, J. L. (2004) Neuropharmacology 46, 629–637
10. Gallagher, M. I., Song, L., Arain, F., and Macdonald, R. L. (2004) J. Neurosci. 24, 5570–5578
11. Kang, J., and Macdonald, R. L. (2004) J. Neurosci. 24, 8672–8677
12. Greenfield, L. J., Jr., Sun, F., Neelands, T. R., Burgard, E. C., Donnelly, J. L., and Macdonald, R. L. (1997) Neuropharmacology 36, 63–73
13. Chiara, D. C., and Cohen, J. B. (1997) J. Biol. Chem. 272, 32940–32950
14. Bolte, S., Brown, S., and Satat-Jeuneumaire, B. (2004) J. Cell Sci. 117, 943–954
15. Cramer, A., Whitehorn, E. A., Tate, E., and Sternner, W. P. (1996) Nat. Biotechnol. 14, 315–319
16. Hinkle, D. J., Bianchi, M. T., and Macdonald, R. L. (2003) BioTechniques 35, 472–474,
476
17. Connolly, C. N., Krishek, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1996) J. Biol. Chem. 271, 89–96
18. Klausberger, T., Sarto, I., Eliga, N., Fuchs, K., Furtrumiller, R., Mayer, B., Huck, S., and Sieghart, W. (2001) J. Neurosci. 21, 9124–9133
19. Klausberger, T., Eliga, N., Fuchs, K., Fuchs, T., Ebert, V., Sarto, I., and Sieghart, W. (2001) J. Biol. Chem. 276, 16024–16032
20. Klausberger, T., Fuchs, K., Mayer, B., Eliga, N., and Sieghart, W. (2000) J. Biol. Chem. 275, 8921–8928
21. Bollan, K., King, D., Robertson, L. A., Brown, K., Taylor, P. M., Moss, S. J., and Connolly, C. N. (2003) J. Biol. Chem. 278, 4747–4755
22. Taylor, P. M., Thomas, P., Gorrie, G. H., Connolly, C. N., Smart, T. G., and Moss, S. J. (1999) J. Neurosci. 19, 6290–6271
23. Taylor, P. M., Connolly, C. N., Kittler, J. T., Gorrie, G. H., Hosie, A., Smart, T. G., and Moss, S. J. (2000) J. Neurosci. 20, 1297–1306
24. Gorrie, G. H., Vallis, Y., Stephenson, A., Whitfield, J., Browning, B., Smart, T. G., and Moss, S. J. (1997) J. Neurosci. 17, 6587–6596
25. Buller, A. L., Hastings, G. A., Kirkness, E. F., and Fraser, C. M. (1994) Mol. Pharmacol. 46, 858–865
26. Helenius, A., and Aebi, M. (2004) Annu. Rev. Biochem. 73, 1019–1049
27. Matsuda, S., Hanner, R., Matsuda, K., Yamada, N., Tubbs, T., and Yuzaki, M. (2004) Eur. J. Neurosci. 19, 1683–1690
28. Kittler, J. T., Wang, J., Connolly, C. N., Vicini, S., Smart, T. G., and Moss, S. J. (2000) Mol. Cell. Neurosci. 16, 440–452
29. Connor, J. X., Boule, A. J., and Czajkowski, C. (1998) J. Biol. Chem. 273, 28906–28911
30. Krampl, K., Maljevic, S., Cossette, P., Ziegler, E., Rouleau, G. A., Kerche, H., and Buller, J. (2005) Eur. J. Neurosci. 22, 10–20
31. Bianchi, M. T., Song, L., Zhang, H., and Macdonald, R. L. (2002) J. Neurosci. 22, 5321–5327
32. Dibbets, L. M., Feng, H. J., Richards, M. C., Harkin, L. A., Hodgson, B. L., Scott, D., Jenkins, M., Petrou, S., Sutherland, G. R., Scheffer, E. E., Berkovic, S. F., Macdonald, R. L., and Mulley, J. C. (2004) Hum. Mol. Genet. 13, 1315–1319
33. Sancar, F., and Czajkowski, C. (2004) J. Biol. Chem. 279, 47034–47039
34. Hales, T. T., Tang, H., Bollan, K. A., Johnson, S. J., King, D. P., McDonald, N. A., Cheng, A., and Connolly, C. N. (2005) Mol. Cell. Neurosci. 29, 120–127
35. Hampton, R. Y. (2002) Curr. Opin. Cell Biol. 14, 476–482
36. Hirsch, C., Jarosch, E., Sommer, T., and Woll, D. H. (2004) Biochim. Biophys. Acta 1695, 215–223
37. Caputto, G. A., and London, E. (2004) Biochimica Biophysica Acta 43, 8794 – 8806
38. Gregersen, N., Bross, P., Jorgensen, M. M., Corydon, T. J., and Andresen, B. S. (2000) J. Inherit. Metab. Dis. 23, 441–447
39. Amaral, M. D. (2004) J. Mol. Neurosci. 23, 41–48
40. Krlica, J., Korpi, E. R., O’Buckley, T. K., Homanics, G. E., and Morrow, A. L. (2002) J. Pharmacol. Exp. Ther. 302, 1037–1045
41. Krlica, J., O’Buckley, T. K., Kusti, R. T., Hodge, C. W., Homanics, G. E., and Morrow, A. L. (2002) Neuropharmacology 43, 685–694
42. Vicini, S., Ferguson, C., Pybyloewski, K., Krlica, J., Morrow, A. L., and Homanics, G. E. (2001) J. Neurosci. 21, 3009–3016