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The Juvenile Myoclonic Epilepsy $GABA_A$ Receptor $\alpha 1$ Subunit Mutation A322D Produces Asymmetrical, Subunit Position-Dependent Reduction of Heterozygous Receptor Currents and $\alpha 1$ Subunit Protein Expression

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Individuals with autosomal dominant juvenile myoclonic epilepsy are heterozygous for a $GABA_A$ receptor $\alpha 1$ subunit mutation ($\alpha 1$A322D). $GABA_A$ receptor $\alpha\beta\gamma$ subunits are arranged around the pore in a β-α-β-α-γ sequence (counterclockwise from the synaptic cleft). Therefore, each $\alpha 1$ subunit has different adjacent subunits, and heterozygous expression of $\alpha 1$(A322D), β, and γ subunits could produce receptors with four different subunit arrangements: β-$\alpha 1$-β-$\alpha 1$-γ (wild type); β-$\alpha 1$(A322D)-β-$\alpha 1$-γ ($Het_{\beta\alpha\beta}$); β-$\alpha 1$-β-$\alpha 1$(A322D)-γ ($Het_{\beta\alpha\gamma}$); β-$\alpha 1$(A322D)-β-$\alpha 1$(A322D)-γ (homozygous). Expression of a 1:1 mixture of wild-type and $\alpha 1$(A322D) subunits with $\beta 2$S and $\gamma 2$S subunits (heterozygous transfection) produced smaller currents than wild type and much larger currents than homozygous mutant transfections. Western blot and biotinylation assays demonstrated that the amount of total and surface $\alpha 1$ subunit from heterozygous transfections was also intermediate between those of wild-type and homozygous mutant transfections. $\alpha 1$(A322D) mutations were then made in covalently tethered triplet ($\gamma 2$S-$\beta 2$S-$\alpha 1$) and tandem ($\beta 2$S-$\alpha 1$) concatamers to target selectively $\alpha 1$(A322D) to each of the asymmetric $\alpha 1$ subunits. Coexpression of mutant and wild-type concatamers resulted in expression of either $Het_{\beta\alpha\beta}$ or $Het_{\beta\alpha\gamma}$ receptors. $Het_{\beta\alpha\beta}$ currents were smaller than wild type and much larger than $Het_{\beta\alpha\gamma}$ and homozygous currents. Furthermore, $Het_{\beta\alpha\beta}$ transfections contained less β-α concatamer than wild type but more than both $Het_{\beta\alpha\gamma}$ and homozygous mutant transfections. Thus, whole-cell currents and protein expression of heterozygous $\alpha 1$(A322D)$\beta 2$S$\gamma 2$S receptors depended on the position of the mutant $\alpha 1$ subunit, and $GABA_A$ receptor currents in heterozygous individuals likely result primarily from wild-type and $Het_{\beta\alpha\beta}$ receptors with little contribution from $Het_{\beta\alpha\gamma}$ and homozygous receptors.

Key words: **juvenile myoclonic epilepsy; chloride ion channel; $GABA_A$ receptors; mutant; myoclonus; patch clamp; protein; concatamer**

Introduction

Juvenile myoclonic epilepsy (JME) is a generalized epilepsy syndrome that accounts for 5–10% epilepsy patients and is characterized by myoclonic, tonic-clonic, and absence seizures as well as typical electroencephalographic findings (Genton and Gelisse, 2001). Recently, a missense mutation (A322D) in the $GABA_A$ receptor $\alpha 1$ subunit gene (GABRA1) was found in all family members who were affected with an autosomal dominant form of JME (ADJME) (Cossette et al., 2002).

$GABA_A$ receptors, the major inhibitory neurotransmitter receptors in the mammalian CNS, are pentameric ligand-gated chloride ion channels. The five subunits arise from seven subunit families that contain multiple subtypes ($\alpha 1$–6, $\beta 1$–3, $\gamma 1$–3, $\delta 1$, $\epsilon 1$, $\pi 1$, $\theta 1$). $GABA_A$ receptors assemble in a limited number of subunit combinations, with the most prevalent combination consisting of two $\alpha 1$ subunits, two $\beta 2$ subunits, and one $\gamma 2$ subunit (McKernan and Whiting, 1996). The arrangement of each subunit around the pore ($\beta 2$-$\alpha 1$-$\beta 2$-$\alpha 1$-$\gamma 2$; counterclockwise when viewed extracellularly) has been determined (Tretter et al., 1997; Baumann et al., 2001, 2002). We designated the α subunit between the β subunits, the $\beta\alpha\beta$ α subunit, and the α subunit between the β and γ subunits, the $\beta\alpha\gamma$ α subunit.

All patients with ADJME were heterozygous for the $\alpha 1$(A322D) mutation and, therefore, had one wild-type and one mutant $\alpha 1$ subunit allele. These patients could express four different $\alpha 1\beta\gamma$ $GABA_A$ receptor phenotypes: wild-type $\alpha 1\beta\gamma$, heterozygous $\beta\alpha\beta$ $\alpha 1$(A322D)$\beta\gamma$ ($Het_{\beta\alpha\beta}$), heterozygous $\beta\alpha\gamma$ $\alpha 1$(A322D)$\beta\gamma$ ($Het_{\beta\alpha\gamma}$), and homozygous $\alpha 1$(A322D)$\beta\gamma$. Previous studies demonstrated that homozygous $\alpha 1$(A322D)$\beta\gamma$ receptors had ~10% of the peak current and an ~200-fold higher GABA EC_{50} value compared with wild-type $\alpha 1\beta\gamma$ receptors; however, no unique heterozygous response was identified (Cossette et al., 2002). Because patients with ADJME were heterozygous, not homozygous, for the $\alpha 1$(A322D) mutation, it raises the questions what is the abnormal heterozygous $\alpha 1$(A322D)$\beta\gamma$ receptor phenotype and do the mutations have a positional effect

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on receptor assembly, transport to the cell surface, and receptor function? To characterize the properties of heterozygous α1(A322D) $GABA_A$ receptors, we transfected HEK293T cells with wild-type β2S and γ2S subunits and wild-type α1 subunit, α1(A322D) subunit, or a 1:1 mixture (heterozygous expression) of wild-type α1 and α1(A322D) subunits and measured their peak currents and current kinetics after rapid, saturating GABA application. We then measured their levels of α1 subunit protein by using Western blot and biotinylation assays.

Next, we characterized the same properties of Het$\beta\alpha\beta$ and Het$_{\beta\alpha\gamma}$ receptors by individually mutating the α1 subunit in tethered triplet [γ2S-β2-α1(A322D)] and tandem [β2-α1(A322D)] constructs. Because functional ion channels only form from association of one triplet (γ2S-β2-α1) and one tandem (β2-α1) construct (Baumann et al., 2002), expression of mutated triplet [γ2S-β2-α1(A322D)] with wild-type tandem (β2-α1) constructs forced assembly of Het$_{\beta\alpha\beta}$ receptors, whereas expression of wild-type triplet (γ2S-β2-α1) with mutated tandem [β2-α1(A322D)] constructs forced assembly of Het$_{\beta\alpha\gamma}$ receptors.

Materials and Methods

Expression of recombinant $GABA_A$ receptors. The cDNAs encoding human α1, β2S, and γ2S subunits in pcDNA3.1 plasmid were a gift from Dr. Matthew Jones (University of Wisconsin, Madison, WI). The construction of the cDNAs encoding tethered rat subunits (γ2S-β2-α1) and (β2-α1) in **pCMV** vector has been described (Baumann et al., 2002) and were kindly provided to us by Dr. Erwin Sigel (University of Bern, Bern, Switzerland). Here, the γ2S-β2-α1 and β2-α1 constructs will be abbreviated as γ-β-α and β-α, respectively. α1(A322D) point mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing. The details of cell culture and $GABA_A$ receptor expression have been described previously (Angelotti et al., 1993). Human embryonic kidney cells (HEK293T; a gift from P. Connely, COR Therapeutics, San Francisco, CA) were grown in DMEM (Invitrogen, Bethesda, MD) with 10% FBS (Invitrogen) and 100 IU/ml each of penicillin and streptomycin (Invitrogen) at 37°C in 5% CO_2/95% air. Cells were transfected in 6 cm culture dishes (Corning Glass Works, Corning, NY) using a modified calcium phosphate method. For the untethered experiments, cells were cotransfected with 4 μg:4 μg:4 μg:2 μg (α1:β2S:γ2S:pHook) (Invitrogen, Carlsbad, CA), which is used for immunomagnetic separation (Greenfield et al., 1997). For the tethered experiments, to express γ-β-α and β:α in 1:1 molar ratios, cells were transfected with 4.8 μg:4 μg:2 μg (γ-β-α:β-α:pHook). Four hours after DNA addition, the cells were shocked with 15% glycerol in *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline for 30 sec. Approximately 20 hr after transfection, cells were trypsinized, centrifuged (400 × *g*), and incubated with hapten-coated magnetic beads at 37°C for 30 min to allow the pHook antibody of the positively transfected cells to bind to the beads. The bound cells were then isolated using a magnetic stand and plated on 35 mm culture dishes.

Electrophysiology experiments. Electrophysiological experiments were performed 20–36 hr after plating on 35 mm dishes. The external recording solution consisted of the following (in mM): 142 NaCl, 8 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4 (osmolarity, 323–330 mOsm). The intrapipette solution contained (in mM): 153 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES, and 2 MgATP, pH 7.3 (osmolarity, 300–316 mOsm). This combination of external and intrapipette solutions produced a chloride equilibrium potential (E_{Cl}) of approximately zero. Recording pipettes were pulled from borosilicate capillary glass (Fisher, Pittsburgh, PA) on a Sutter P-2000 micropipette electrode puller (Sutter Instrument, San Rafael, CA). $GABA_A$ receptor currents were recorded using a lifted whole-cell patch-clamp technique with cells clamped at −20 mV during the recordings. Recordings were obtained from lifted whole cells rather than macropatches, because it allowed measurement of larger currents and avoided the cytoskeletal disruption of detached membrane patches. Lifting the cell from the surface of the dish improved solution exchange time and allowed better resolution of fast (<20 msec) components of desensitization compared with whole-cell recordings when the cell is attached to the dish (Bianchi and Macdonald, 2002; Hinkle et al., 2003). Signals were processed using a List EPC-7 amplifier (List Electronics, Darmstadt, Germany) in voltage-clamp mode, sampled at 10 kHz, and recorded on a computer. GABA was applied to the cells (via gravity) using a rapid perfusion system consisting of pulled multibarrel square glass (final size, 200–300 μm) connected to a Perfusion Fast-Step (Warner Instrument, Hamden, CT) (Hinkle et al., 2003). 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 ≤1.0 msec; it is assumed that the exchange time for a whole cell is significantly greater. Because the cells adjust their position within the solution flow after being detached from the dish surface, 1 mM GABA was applied for 400 msec every 30 sec until reproducible current traces were obtained; the first of the reproducible traces was used for analysis of current kinetics.

Current amplitudes and rapid kinetic properties were measured on a computer using Clampfit 9.0 (Axon Instruments, Foster City, CA). Only cells with ≥0.5 GΩ seals were included in the analyses. For the untethered experiments, ~15% of the cells lacked a fast component of desensitization; these were not included in the analyses. All the tethered $GABA_A$ receptors had a fast component of desensitization. Activation was measured as the 10–90% rise time from baseline to peak current. The desensitization and deactivation time courses were fit using the Levenberg–Marquardt least squares method to the equation $\Sigma a_n exp^{(-t/\tau n)}$, where *n* is the number of exponential components, *a* is the amplitude of that component, *t* is time (milliseconds), and τ is the desensitization time constant. Time constants were constrained to be positive.

It has been shown that γ subunit-containing $GABA_A$ receptors have at least four components of desensitization (Bianchi and Macdonald, 2002) and that their desensitization time course after 400 msec applications of 1 mM GABA can typically be best fit by two exponential functions, whereas the desensitization time course after 6 sec GABA applications can be best fit by three exponential functions. Therefore, we fit the desensitization and deactivation time courses of 400 msec GABA applications to two exponential components and the desensitization time course of 6000 msec GABA applications to three exponential components. The weighted deactivation time, τ_{weight}, was calculated $[a_1/(a_1 + a_2) \times \tau_1 + a_2/(a_1 + a_2) \times \tau_2]$.

Concentration–response curves were obtained from the peak $GABA_A$ receptor currents evoked by increasing GABA concentrations. The EC_{50} value was calculated by fitting the peak currents to the equation: $I = I_{max}/[1 + (EC_{50}/(GABA))^{Hill\ Slope}]$, where *I* is the peak current at a given GABA concentration and I_{max} is the peak current at the maximal GABA concentration.

Immunoblots. Western blots were performed essentially as described previously (Baumann et al., 2001; Hahn et al., 2003). Transfected cells were solubilized for 30 min in 20 mM Tris, 20 mM EGTA, 1 mM DTT, 1 mM benzamidine, 100 μM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1% Triton X-100, and either with or without 0.5% SDS. Protein concentrations were measured using the Micro BCA Protein assay (Pierce, Rockford, IL). The lysate was centrifuged at 14,000 × *g* for 30 min, and the supernatant was fractionated by 7.5% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were incubated with a monoclonal antibody against either the $GABA_A$ receptor α1 subunit (20 μg/ml, clone BD24; Chemicon, Temecula, CA) or β2/β3 subunit (1 μg/ml, clone 62-3G1; Upstate, Charlottesville, VA) and then were incubated with a HRP-coupled anti-mouse secondary antibody (1:6000 dilution; Jackson ImmunoResearch, West Grove, PA). $GABA_A$ receptor α1 subunits and β-α concatamers were then visualized with a chemiluminescent detection system (Bio-Rad, Hercules, CA) using x-ray film (Pierce). The x-ray film was scanned into a computer, and the immunopositive bands were quantified using the software Quantity One 4.5.0 (Bio-Rad). The rectangular volume (pixel intensity times square millimeter) of each band was normalized to the volume of the wild-type band.

Cell surface biotinylation. Live, transfected cells were washed with PBS (pH 7.4; Invitrogen) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then

incubated with sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin, 1.5 mg/ml; Pierce) for 1 hr at 4°C. The sulfo-NHS-SS-biotin was quenched with PBS containing 0.1 mM glycine. The cells were washed and then lysed as described above and incubated with immobilized strepavidin (Pierce) for 1 hr at room temperature. The biotinylated proteins were eluted from the strepavidin by incubation with Laemmli sample buffer at 70°C and fractionated on Western blots as described above.

Data analysis. Values are reported as means ± SEM. Statistical significance was evaluated using the Student's unpaired *t* test (GraphPad Prism; Graph Pad, San Diego, CA), with Welch's correction used if the variances were unequal (Bartlett's test; GraphPad Prism). Determinations of whether data deviated from Gaussian distributions were made using the Kolmogorov–Smirnov test (GraphPad Prism).

Results

Transient kinetic properties of heterozygous α1(A322D)βS2γ2 currents were similar to wild-type α1βS2γ2S but differed from those of homozygous α1(A322D)βS2γ2S currents

Whole-cell currents were recorded from lifted HEK293T cells transfected with wild-type β2S and γ2S subunits and wild-type α1 subunit (Fig. 1*A*, wild-type transfection), a 1:1 mixture of wild-type and mutant α1 subunits (Fig. 1*B*, heterozygous transfection), or mutant α1(A322D) subunit (Fig. 1*C*, homozygous transfection). The heterozygous transfection would be predicted to contain a binomial mixture of receptors with wild-type, homozygous mutant $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ receptors, and thus peak currents and current kinetics would be predicted to be the sum of currents obtained separately from each of these receptors. Cells were exposed to 1 mM GABA, approximating the peak GABA concentration at a synapse, for 400 msec. Previous studies have shown that neurons with endogenous $GABA_A$ receptors (Mellor and Randall, 1997; Zhu and Vicini, 1997) as well as cells expressing recombinant $GABA_A$ receptors (Gingrich and Burkat, 1998; Haas and Macdonald, 1999; Bianchi and Macdonald, 2002; Scheller and Forman, 2002) with α, β, and γ subunits have fast (<20 msec), intermediate (~200 msec), slow (~ 1000 msec), and ultraslow (~10,000 msec) components of desensitization when GABA is applied for several seconds. With the briefer (400 msec) GABA applications used in this study, only the fast and intermediate phases of desensitization could be resolved.

The transient kinetic properties of wild-type (Fig. 1*A*) and heterozygous α1(A322D)βS2γ2S (Fig. 1*B*) currents were similar to each other but differed substantially from those of homozygous α1(A322D)βS2γ2S currents (Fig. 1*C*; Table 1). The 10–90% activation times of heterozygous (4.5 ± 0.5 msec) receptor currents were significantly faster than those of homozygous (53.9 ± 4.7 msec) α1(A322D)βS2γ2S currents ($p < 0.001$) but were not significantly different from wild-type (4.0 ± 0.8 msec) currents. Heterozygous receptor currents differed from homozygous α1(A322D)βS2γ2S currents, but not wild-type currents, by having both fast and intermediate phases of desensitization. Heterozygous currents had faster deactivation times (147 ± 10 msec) than wild-type currents (267 ± 50 msec) but had slower deactivation times than homozygous currents (31 ± 5 msec). The slower activation, lack of fast and intermediate desensitization, and faster deactivation of homozygous receptor currents were also present when tested with 10 mM GABA (data not shown).

Six-second applications of 1 or 10 mM GABA demonstrated that heterozygous currents had a slow component of desensitization with a time constant (2462 ± 45 msec) that was similar to those of wild-type (3856 ± 1092 msec; $p > 0.05$) and homozygous mutant (3463 ± 632 msec; $p > 0.05$) currents (supplementary Table 1; available at www.jneurosci.org). Because homozygous mutant currents lacked fast and intermediate components of desensitization, their component of slow desensitization (70 ± 3%) was greater than wild-type (38 ± 2%) or heterozygous (35 ± 6%) currents ($p < 0.001$), but their component of total desensitization (70 ± 3%) was less than heterozygous (87 ± 2%) or wild-type (82 ± 3%) currents ($p < 0.01$).

Peak amplitudes of heterozygous α1(A322D)βS2γ2S currents were intermediate between wild-type and homozygous α1(A322D)βS2γ2S currents

It was previously reported that maximal whole-cell peak currents from HEK293 cells transfected with homozygous α1(A322D)β2Sγ2L receptors were ~10% of those of wild-type α1β2Sγ2L receptors and that heterozygous α1(A322D) receptor peak currents resembled either wild-type or homozygous α1(A322D)βS2γ2S currents without an intermediate response (Cossette et al., 2002). We determined the mean maximal whole-cell peak currents from cells after heterozygous transfections and compared them with currents obtained from cells after wild-type and homozygous α1(A322D)βS2γ2S mutant transfections (Fig. 1*D*). We found that heterozygous α1(A322D) peak currents (1404 ± 271 pA) were significantly smaller than wild-type peak currents (2820 ± 551 pA; $p < 0.05$) but substantially larger than homozygous α1(A322D) peak currents (161 ± 62 pA; $p < 0.001$). The peak homozygous α1(A322D) currents were 6% that of wild-type currents, similar to a previous report (Cossette et al., 2002).

The GABA EC_{50} values of homozygous and heterozygous α1(A322D) currents were higher than wild-type α1β2Sγ2S currents

We determined the GABA EC_{50} values for the whole-cell currents from cells obtained from wild-type, heterozygous, and homozygous transfections (Fig. 1*E*). Although the GABA EC_{50} values for cells from heterozygous transfections (22 ± 3 μM) was almost twice that of wild-type receptors (13 ± 3 μM), the difference did not reach significance ($p = 0.06$; $n = 5$). As reported previously (Cossette et al., 2002), homozygous mutant receptors had a significantly higher GABA EC_{50} values (197 ± 66 μM) than wild-type receptors (13 ± 3 μM; $p < 0.001$). Cells from the wild-type and heterozygous transfection conditions had maximal currents at GABA concentrations ≤1 mM, the presumed synaptic GABA concentration and the concentration of GABA that was used in this study to measure both peak currents and rapid current kinetics. The mean Hill coefficient of the homozygous mutant receptor currents (0.8 ± 0.1) was smaller than wild-type receptor currents ($p < 0.05$), and the mean Hill coefficient of the cells from the heterozygous transfections (1.1 ± 0.2) was between wild-type and homozygous mutant receptor currents, but these differences were not statistically significant ($p > 0.05$).

α1(A322D) reduced the amount of total and surface α1 subunit

To determine the effect of the α1(A322D) mutation on the expression of the α1 subunit, HEK293T cells were transfected with wild-type β2 and γ2 subunits and either wild-type α1 subunit (WT), a 1:1 mixture of wild-type and α1(A322D) subunits (Het), α1(A322D) subunit (Hom), or only the β2 and γ2 subunits but with no α1 (βγ) subunits, and Western blot analyses were performed using a monoclonal antibody targeted against the N terminus of the α1 subunit (Fig. 2*A*). In these blots of whole-cell lysates (20 μg of protein), the only specific immunoreactive band was present at 50 kDa, the size of the α1 subunit. The bands were

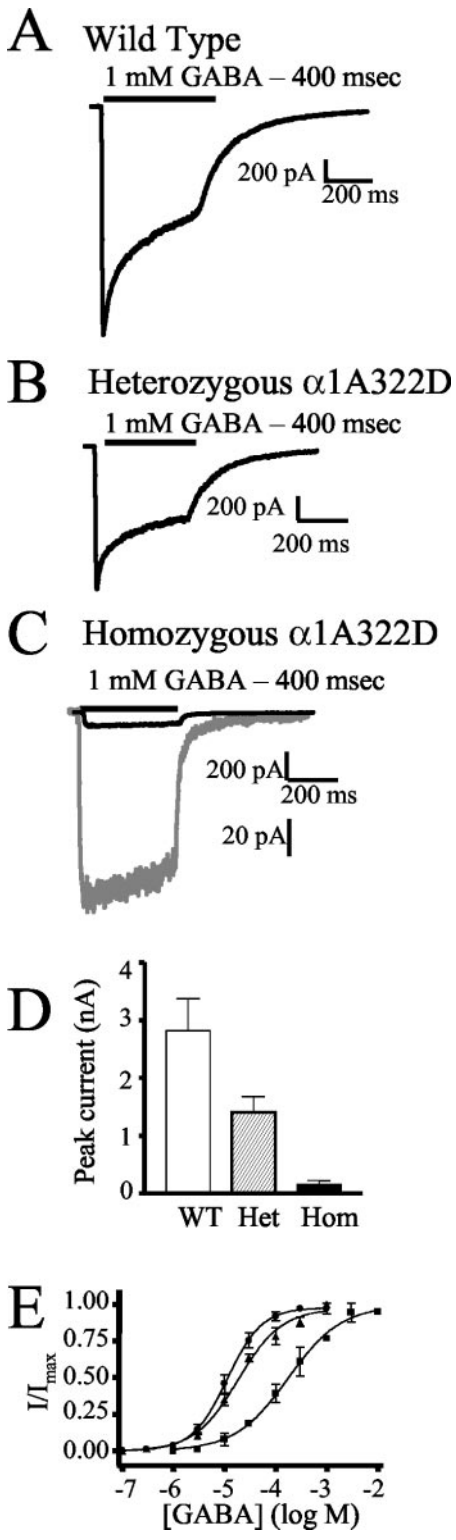

Figure 1. Whole-cell currents of untethered $GABA_A$ receptors. Representative current traces obtained from wild-type (*A*), heterozygous (*B*), or homozygous (*C*) mutant transfections after a 400 msec step into 1 mM GABA. The black trace in *C* is depicted on the same voltage scale (top scale bar) as the traces in *A* and *B*, but the gray trace is plotted in an expanded scale (bottom scale bar) to display the time course of the current kinetics. *D*, The mean peak currents are depicted ± SEM. Peak currents from heterozygous transfections ($n = 16$) were significantly larger than homozygous mutant peak currents ($n = 5$; $p < 0.001$) and significantly smaller than wild-type peak currents ($n = 17$; $p < 0.05$). *E*, The GABA EC_{50} values for wild-type (●), heterozygous (▲), and homozygous (■) mutant transfections were determined by 6 sec, rapid-perfusion, whole-cell response in increasing GABA concentrations. Cells were voltage clamped at −20 mV. Currents were normalized to the maximal peak current for each cell, and the EC_{50} values were calculated and fit as described in Materials and Methods.

quantified, and the band volumes (intensity times square millimeter) were normalized to the wild-type band for each experiment. The heterozygous α1(A322D) transfection had reduced α1 subunit relative to wild-type receptor transfection (56 ± 13% wild-type expression; $n = 4$) but contained more α1 subunit than the homozygous mutant transfection (6 ± 4% wild-type expression; $n = 5$).

To determine the effect of the α1(A322D) mutation on surface expression of receptors, transfected HEK293T cells were incubated with sulfo-NHS-SS-biotin, and the biotinylated protein was analyzed by Western blots (Fig. 2*B*). Heterozygous α1(A322D) transfections had reduced surface α1 subunit relative to wild-type transfections (22 ± 10% wild type; $n = 3$) and more surface α1 subunit than homozygous mutant transfections (3 ± 2% wild-type expression; $n = 3$).

These results differ from those previously reported, which showed similar α1 subunit protein expression in wild-type and homozygous mutant receptors (Cossette et al., 2002). Although it was unlikely that a single missense mutation in the M3 domain reduced antibody recognition at the N terminus on Western blots, we repeated the experiments using the same polyclonal antibody used in the previous studies and found that none of the immunoreactive bands were specific (data not shown). The previous studies (Cossette et al., 2002) also used high concentrations of denaturing detergents that could increase detection of mutant α1 subunits if they were sequestered in subcellular compartments that were not solubilized with nondenaturing detergents. Therefore, we repeated the Western blots using 0.5% SDS and found results identical to those depicted in Figure 2*A*.

Tethered heterozygous α1(A322D)β2Sγ2S receptor expression

The electrophysiological properties of both wild-type and homozygous mutant receptors could be definitively characterized in the previous sections because transfection with only wild-type α1, β2, and γ2 subunits produced only wild-type receptors and transfection of only α1(A322D), β2, and γ2 subunits produced only homozygous mutant receptors. In contrast, the properties of $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ receptors could not be determined definitively because heterozygous transfection with a 1:1 mixture of wild-type α1 and mutant α1(A322D) subunits (heterozygous expression) could produce receptors with all four possible wild-type and mutant α1 subunit combinations (w/w, wt/βαβ, wt/βαγ, $Het_{\beta\alpha\beta}$/βαγ). To determine the properties of $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ receptors, we forced their assembly using γ-β-α and β-α constructs. Transfection of mutant γ-β-α(A322D) triplet concatamers with wild-type β-α tandem concatamers produced $Het_{\beta\alpha\beta}$ receptors, and transfection of the wild-type γ-β-α triplet concatamer with the mutant β-α(A322D) tandem concatamer produced $Het_{\beta\alpha\gamma}$ receptors.

To confirm that only pentameric $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ receptors were formed and that there were no substantial currents arising from β-α tetramers, γ-β-α hexamers or pentamers formed from concatamer breakdown, HEK293T cells were transfected with twice the usual amount of (8.0 μg) wild-type β-α construct without the γ-β-α construct, and no current was detected ($n = 3$). Similarly, when cells were transfected with twice the usual amount (9.6 μg) of wild-type γ-β-α cDNA without the β-α construct, three cells had no current and two cells had very small peak currents of 32 and 33 pA. In contrast, all cells transfected with a 1:1 molar ratio of γ-β-α and β-α cDNAs produced much larger currents (mean, 967 ± 279 pA). This demonstrated that in HEK293T cells as in *Xenopus* oocytes (Baumann et al., 2001,

Table 1. Current kinetic properties of untethered $GABA_A$ receptors

Construct (*n*)	Activation 10–90% (msec)	Desensitization					Deactivation τ_{weight} (msec)
		τ1 (msec)	τ2 (msec)	%A1	%A2	%C	
WT (5)	4.0 ± 0.8	19.5 ± 0.9	232.5 ± 32.9	17 ± 3	29 ± 3	54 ± 5	267 ± 50
Het (5)	4.5 ± 0.5	14.8 ± 1.1	181.2 ± 14.6	20 ± 1	35 ± 1	46 ± 2	147 ± 10[a]
Hom (5,3)	53.9 ± 4.7[a,b]	NA	NA	07[a,b]	07[a,b]	1007[a,b]	31 ± 5[a,b]

Current kinetic parameters were obtained from wild-type (WT), heterozygous mutant (Het), and homozygous mutant (Hom) transfections during a 400 msec step into 1 mM GABA. Activation time, weighted deactivation time (τ_{weight}), and the desensitization fast (τ1) and intermediate (τ2) time constants as well as the percentage components of fast (%A1), intermediate (%A2), and residual (%C) desensitization were calculated as described in Materials and Methods. Each of the five cells expressing homozygous mutant receptors included in the analysis lacked fast and intermediate components of desensitization. Because only three of the cells expressing homozygous mutant receptors had peak currents >100 pA, only currents from these cells were fit for activation and deactivation times, each of which were significantly different from both wild-type and heterozygous currents. The deactivation of heterozygous receptor currents was significantly faster than that of wild-type currents, but there was no difference in current activation or desensitization.

[a]Statistically different from wild type.

[b]Statistically different from heterozygous.

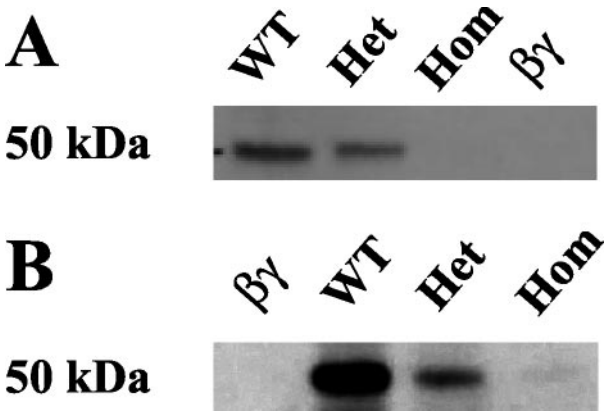

Figure 2. Western blot analysis of total and surface α1 receptor protein. HEK293T cells were transfected with wild-type β2 and γ2 subunits and either wild-type α1 subunit (WT), a 1:1 mixture of wild-type and α1(A322D) subunit (Het), α1(A322D) subunit (Hom), or only β- and γ- subunits but no α1 subunit ($\beta\gamma$). *A*, Western blots of whole-cell lysates (20 μg of protein) demonstrated a specific immunoreactive band at 50 kDa. *B*, Transfected HEK293T cells were incubated with sulfo-NHS-SS-biotin, and the biotinylated proteins were analyzed by Western blot.

2002), expression of exactly one triplet and one tandem construct is required to produce functional $GABA_A$ receptors.

The transient kinetic properties of tethered $Het_{\beta\alpha\gamma}$ currents differ from those of $Het_{\beta\alpha\beta}$ and wild-type currents

Representative current traces for tethered wild-type, $Het_{\beta\alpha\beta}$, and $Het_{\beta\alpha\gamma}$ currents after 400 msec applications of 1 mM GABA for are presented in Figure 3. Currents were obtained from all cells expressing wild-type (Fig. 3*A*) or $Het_{\beta\alpha\beta}$ (Fig. 3*B*) receptors but from only three of the eight cells expressing $Het_{\beta\alpha\gamma}$ receptors (Fig. 3*C*). Both the wild-type and $Het_{\beta\alpha\beta}$ currents had fast and intermediate components of desensitization. The low current amplitudes of the $Het_{\beta\alpha\gamma}$ receptors prohibited analyses of their current kinetics, and thus it is not known whether these currents differed from currents recorded from cells transfected with only wild-type γ-β-α subunits.

Peak amplitudes of tethered $Het_{\beta\alpha\gamma}$, $Het_{\beta\alpha\beta}$, and wild-type currents differed

We determined the mean peak currents of tethered wild-type, $Het_{\beta\alpha\beta}$, and $Het_{\beta\alpha\gamma}$ receptors after 400 msec applications of 1 mM GABA (Fig. 3*D*). The mean peak current of $Het_{\beta\alpha\beta}$ receptors (335 ± 87 pA) was smaller than that of wild-type receptors (967 ± 279 pA; $p < 0.05$). $Het_{\beta\alpha\gamma}$ receptors had significantly smaller peak currents (9 ± 5 pA) than either wild-type (967 ± 279 pA) or $Het_{\beta\alpha\beta}$ (335 ± 87 pA) receptors ($p < 0.01$). The variability of the peak currents from the wild-type tethered receptors (coefficient of variation, 115%) was larger than that of wild-type untethered receptors (coefficient of variation, 80%), a finding similar to that obtained in *Xenopus* oocytes (Baumann et al., 2002), which may reflect inefficient assemblies and thus more cell-to-cell variability of expression of the tethered constructs. Homozygous tethered receptors were expressed by cotransfecting the mutant triplet γ-β-α(A322D) construct with the mutant tandem β-α(A322D) construct, but no currents were obtained, a result that was not surprising considering the very small $Het_{\beta\alpha\gamma}$ peak currents.

$Het_{\beta\alpha\beta}$ currents had larger components of fast desensitization than wild-type currents

Activation, desensitization, and deactivation current kinetics were measured in lifted cells by applying 1 mM GABA for 400 msec to the tethered $GABA_A$ receptors. All of the tethered wild-type and $Het_{\beta\alpha\beta}$ currents had both a fast and an intermediate component of desensitization. The $Het_{\beta\alpha\beta}$ currents had a larger component of fast desensitization (40 ± 1%) than wild-type currents (30 ± 1%; $p < 0.001$) (Fig. 3*A*,*B*). The $Het_{\beta\alpha\beta}$ currents also had a greater total component of desensitization (65 ± 2%) than wild type (51 ± 3%; $p < 0.05$). The time constants of the fast (τ1) and intermediate (τ2) components of desensitization, the component of intermediate desensitization (%A2), and the activation and deactivation times did not significantly differ between wild-type and $Het_{\beta\alpha\beta}$ currents (Table 2). There was no significant difference in the slow component of desensitization as measured with 6 sec GABA applications (supplementary Table 2).

Tethered wild-type and $Het_{\beta\alpha\beta}$ receptor GABA concentration–response curves were similar

We determined the GABA concentration–response curves for wild-type and $Het_{\beta\alpha\beta}$ tethered receptors (Fig. 3*E*). There was no significant difference between the EC_{50} values or Hill coefficients of wild-type (42 ± 0.4 μM; 1.3 ± 0.07) and $Het_{\beta\alpha\beta}$ (32 ± 0.4 μM; 1.2 ± 0.10) receptors ($p > 0.05$). Similar to the untethered $GABA_A$ receptor, both wild-type and $Het_{\beta\alpha\beta}$ receptors had maximal currents at GABA concentrations <1 mM, the GABA concentration that was used in this study to measure both peak currents and rapid current kinetics. The GABA EC_{50} value of wild-type tethered receptors was somewhat lower than that measured previously (177 μM) in *Xenopus* oocytes (Baumann et al., 2002).

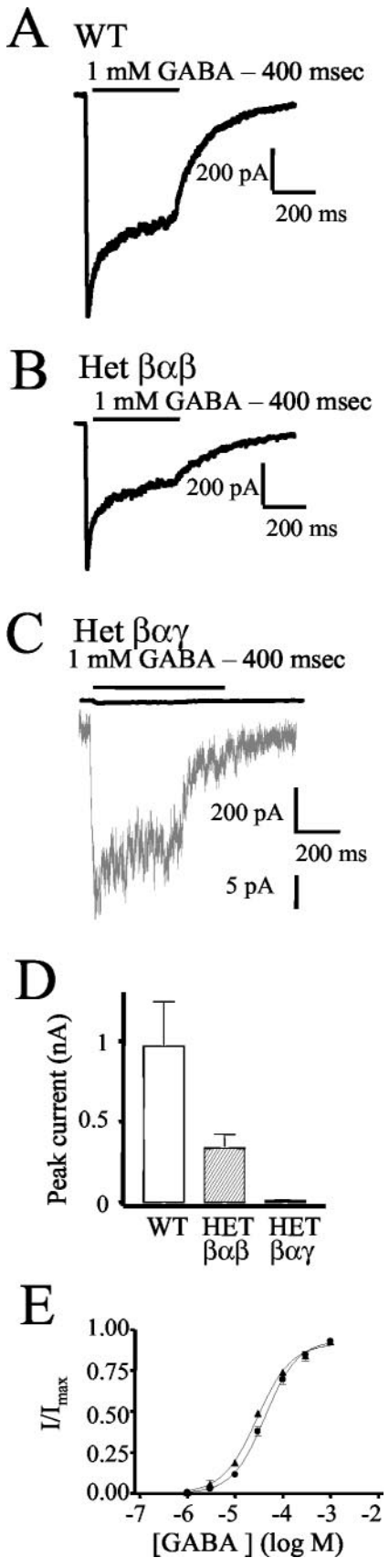

Figure 3. Whole-cell currents of tethered $GABA_A$ receptors. *A–C*, Representative current traces obtained from wild type (*A*; WT), $Het_{\beta\alpha\beta}$ (*B*) or $Het_{\beta\alpha\gamma}$ (*C*) receptors after a 400 msec step into 1 mM GABA. The black trace in *C* is depicted on the same voltage scale (top scale bar) as the traces in *A* and *B*, but the gray trace is plotted in an expanded scale (bottom scale bar) to display the time course of the current kinetics. *D*, The mean peak currents are depicted ± SEM. $Het_{\beta\alpha\gamma}$ peak currents ($n = 8$) were significantly smaller than both WT ($n = 20$) and $Het_{\beta\alpha\beta}$ ($n = 17$) currents ($p < 0.01$). Mean $Het_{\beta\alpha\beta}$ currents were smaller than WT currents ($p < 0.05$). $Het_{\beta\alpha\beta}$ currents had a greater component of fast desensitization and total desensitization than wild-type tethered $GABA_A$ receptor currents. There was no significant change in the component of intermediate desensitization or in the desensitization time constants (Table 2). *E*, The GABA EC_{50} values for tethered wild-type (●) and $Het_{\beta\alpha\beta}$ (▲) receptors were determined by 1 sec, rapid-perfusion, whole-cell response in increasing GABA concentrations. Cells were voltage clamped at −20 mV. Currents were normalized to the maximal peak current for each cell, and the EC_{50} values were calculated and fit as described in Materials and Methods. There was no significant difference between the EC_{50} value of wild-type (42 ± 0.4 μM) and $Het_{\beta\alpha\beta}$ receptors (32 ± 0.4 μM; $p > 0.05$).

α1(A322D) reduced protein expression of the β-α construct

We performed Western blots to determine how the α1(A322D) mutation affected protein expression of the tethered constructs. No specific immunoreactive peptides were detected using the monoclonal antibody targeted against the N terminus of the α1 subunit that had been used in the Western blots of the untethered subunits. This result was not surprising because it had been asserted that the anti-α1 antibody requires a free N terminus (Baumann et al., 2001). Therefore, the Western blots of the tethered $GABA_A$ receptor were probed with a monoclonal antibody directed against the β2 and β3 $GABA_A$ receptor subunits. Lysates (30 μg protein) from untransfected HEK293T cells (UT) or HEK293T cells transfected with tethered wild-type (WT), $Het_{\beta\alpha\beta}$ ($\beta\alpha\beta$), $Het_{\beta\alpha\gamma}$ ($\beta\alpha\gamma$), or homozygous (Hom) $GABA_A$ receptors were analyzed by Western blots and probed with the monoclonal antibody targeted against the β2 subunit. The only specific immunoreactive protein was present at 100 kDa, the theoretical molecular weight of the tethered β-α tandem construct. In each of the five Western blot experiments (Fig. 4), both visual and quantitative analyses demonstrated that $Het_{\beta\alpha\beta}$ transfections had lower expression of the β-α construct than wild-type transfections (71 ± 9% relative to wild type) but higher expression of the β-α construct than $Het_{\beta\alpha\gamma}$ transfections (51 ± 16% relative to wild type). However, because there was substantial variance in the quantification of the β-α band from the five experiments, the difference between the mean quantified β-α band from the $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ transfections was not statistically significant ($p = 0.32$). The tethered homozygous mutant receptors had even more reduced expression (36 ± 14% relative to wild type; $n = 4$) of the β-α concatamer.

The concatamer immunoblots also demonstrated that there was no immunoreactive protein at 50 kDa, and thus the linker between the β and α subunits in the tandem construct remained intact. Furthermore, there was also no specifically labeled band at 150 kDa, the theoretical molecular weight of the γ-β-α triplet concatamers, suggesting that the monoclonal antibody targeted against the N terminus of the β2 subunit required a free N terminus similar to the antibody against the α1 subunit N terminus. Unfortunately, there are no commercially available monoclonal antibodies targeted against the γ2 subunit that would immunoreact with the triplet (γ-β-α) construct. Incubation of Western blots of tethered or untethered subunits with polyclonal antibodies directed against the N terminus did not reveal any specifically labeled protein.

Discussion

Genetic mutation is an important etiological factor in many idiopathic epilepsy syndromes (Annegers et al., 1996). To date, 13 genes have been identified in which mutations are directly associated with human epilepsy (Scheffer and Berkovic, 2003). Be-

Table 2. Current kinetic properties of tethered $GABA_A$ receptors

Construct (*n*)	Activation 10–90% (msec)	Desensitization τ1 (msec)	τ2 (msec)	%A1	%A2	%C	Deactivation τ_{weight} (msec)
WT (6)	4.8 ± 0.6	14.9 ± 2.3	178 ± 22	30 ± 1	21 ± 2	49 ± 3	224 ± 27
$Het_{\beta\alpha\beta}$ (6)	6.7 ± 1.0	15.7 ± 1.9	252 ± 27	40 ± 1[a]	25 ± 2	35 ± 2[a]	239 ± 15

Current kinetic parameters were obtained from tethered wild-type (WT) and $Het_{\beta\alpha\beta}$ receptors during a 400 msec step into 1 mM GABA. Activation time, weighted deactivation time (τ_{weight}), and the desensitization fast (τ_1) and intermediate (τ_2) time constants as well as the percentage components of fast (%A1), intermediate (%A2), and residual (%C) desensitization were calculated as described in Materials and Methods. The $Het_{\beta\alpha\beta}$ receptors had significantly larger components of fast desensitization and smaller components of undesensitized receptors compared with wild type.

[a]Statistically different from wild type.

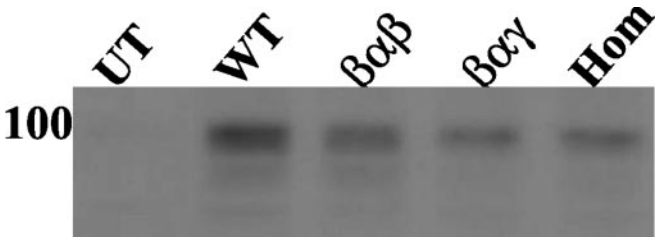

Figure 4. Western blot analysis of tethered α1(A322D) $GABA_A$ receptors. Lysates (30 μg of protein) from untransfected HEK293T cells (UT) or HEK293T cells transfected with tethered wild-type (WT), $Het_{\beta\alpha\beta}$ ($\beta\alpha\beta$), $Het_{\beta\alpha\gamma}$ ($\beta\alpha\gamma$), or homozygous mutant (Hom) $GABA_A$ receptors were analyzed by Western blots and probed with a monoclonal antibody targeted against the β2 subunit.

cause each of these genes is inherited in an autosomal dominant pattern, characterizing $GABA_A$ receptor properties with these mutations in a heterozygous expression system is necessary to determine how mutations of these genes cause disease. We characterized the ADJME α1(A322D) mutation in a standard heterozygous expression system, in which all four possible $GABA_A$ receptor α1 subunit combinations are likely expressed, as well in a tethered system, in which each position of the α1 subunit in the $GABA_A$ receptor could be specifically targeted. Finally, we demonstrated the technical validity of expressing tethered $GABA_A$ receptor subunits in a mammalian expression system without degradation of the tether as evidenced by the lack of significant currents when either concatamers was expressed alone as well as by using Western blots, which demonstrated the integrity of the tethered β-α subunits.

The most prominent consequence of α1(A322D) is the reduction of peak current resulting from decreased α1 subunit expression

The Western blot assays demonstrated that the most important means by which the A322D mutation decreased the mean peak currents in heterozygously and homozygously transfected HEK293T cells was by reducing total and surface α1 subunit. However, it should be emphasized that it is not known whether this inhibition is also present in neurons expressing endogenous mutant α1 subunit-containing receptors. The mechanism by which α1(A322D) reduced α1 protein expression is unknown. It is unlikely that the GCC to GAC mutation reduced the efficiency of translation. In the wild-type α1 subunit, GAC codes 11 of the 23 aspartates, and thus a codon bias against GAC is improbable.

It is possible that α1(A322D) reduced α1 by inhibiting correct $GABA_A$ receptor folding and assembly. Our finding that the $Het_{\beta\alpha\beta}$ transfections contained less β-α concatamer than wild-type tethered transfections is consistent with this conclusion. Although the α1(A322D) mutation in the $Het_{\beta\alpha\beta}$ transfections is in the γ-β-α concatamer, we detected a reduction of the β-α concatamer. Because $GABA_A$ receptors are assembled in the endoplasmic reticulum and incorrectly assembled receptors are degraded (for review, see Barnes, 2000), if γ-β-α(A322D) reduced the amount of correctly assembled pentamers, both γ-β-α(A322D) and β-α would be reduced.

Although many assembly studies identified amino acids at the N termini of the α, β, and γ subunits that are important for receptor assembly (Taylor et al., 1999, 2000; Klausberger et al., 2000, 2001a,b; Sarto et al., 2002a,b), recent studies suggest that other domains may also be important. A post-M3 truncation mutant of the γ2 subunit caused γ2 retention in the endoplasmic reticulum (Harkin et al., 2002). A motif in the M1 transmembrane domain of the nicotinic ACh receptor (AChR), a ligand-gated ion channel that is homologous to the $GABA_A$ receptor, is critical for targeting unfolded or misfolded receptors for endoplasmic reticulum degradation (Wang et al., 2002). Furthermore, tryptophan scanning mutagenesis of the AChR αM3 transmembrane domain demonstrated that mutation of the interior-facing M3 amino acids, including αI283, the homologous residue to GABRA1 α1(A322D), substantially reduced surface expression (Guzman et al., 2003).

A second consequence of α1(A322D) is altered channel gating

Although reduction of α1 is likely the most important factor in the diminution of whole-cell peak currents, α1(A322D) also increased the GABA EC_{50} value and altered whole-cell current kinetics. In addition, single-channel currents from homozygous expression of α1A294D (the homologous mutation in rat $GABA_A$ receptors) had substantially altered channel gating; mean open times were reduced from 2.23 to 0.54 msec, and the contribution of the longest open state was reduced from 14.8 to 0.8% (Fisher, 2004). These data demonstrate that α1(A322D) directly alters channel function. This conclusion is consistent with prior structure–function studies of ligand-gated ion channels. Electron microscopic images of the AChR identified a water-filled pocket between the M3 and M2 domains that is likely important for receptor function (Miyazawa et al., 2003). In the $GABA_A$ receptor, water-soluble probes of this pocket react with α1 subunit M3 residues near α1A322 only in the presence GABA, suggesting that the M3 domain undergoes an agonist-induced conformational change that is important in signal transduction (Williams and Akabas, 1999). In addition, M3 residues have been found to be important for $GABA_A$ receptor interaction by benzodiazepines, ethanol, propofol, and volatile anesthetics, thus demonstrating that this domain is important for $GABA_A$ receptor modulation (Krasowski and Harrison, 2000; Williams and Akabas, 2000, 2002; Jenkins et al., 2001). Therefore, it would be expected that a nonconservative amino acid substitution of an alanine with an aspartate might alter the function of the M3 domain.

The α1(A322D) mutation has asymmetrical effects at the two α1 subunits

It was of particular interest that the $Het_{\beta\alpha\gamma}$ receptors differed substantially from the $Het_{\beta\alpha\beta}$ receptors. Because the mutant α1

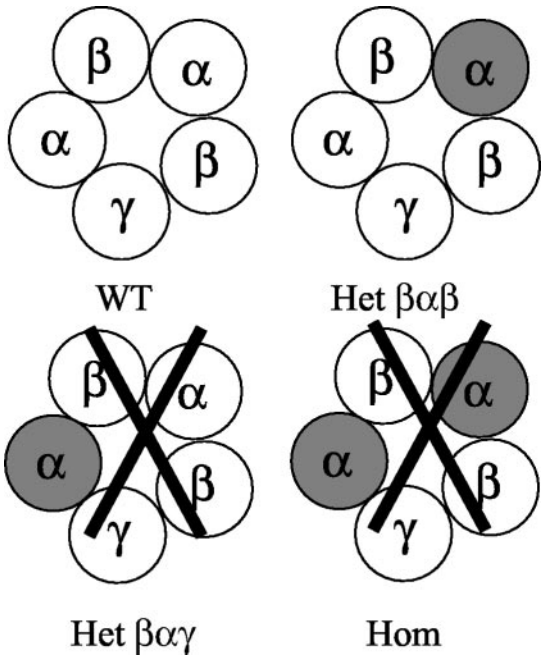

Figure 5. Subunit combinations in a heterozygous expression. There are four receptor phenotypes formed with heterozygous expression of wild-type (white) and mutant α1(A322D) (shaded) subunits. The $Het_{\beta\alpha\gamma}$ and homozygous mutant receptors produced <7% of the mean current of wild-type receptors. Therefore, the currents of the heterozygous receptors were determined by the wild-type (WT) and $Het_{\beta\alpha\beta}$ receptors.

subunit in $Het_{\beta\alpha\gamma}$ receptors is adjacent to the γ2S subunit, the large difference in peak currents between $Het_{\beta\alpha\beta}$ and $Het_{\beta\alpha\gamma}$ receptors may indicate an interaction is needed between α1 M3 and γ2S subunits to produce large currents and could explain the relatively small currents produced by $GABA_A$ receptors formed from receptors that consist of only α and β subunits and no γ subunit (Fisher and Macdonald, 1997).

It is possible that the α1A322D mutation affects assembly differently at the α1-γ2 interface than the α-β2 interface. Previous studies aimed at identifying assembly intermediates of $GABA_A$ receptors found α1-β3 and α1-γ2 subunit heterodimers in approximately equal proportions (Klausberger et al., 2001b). However, it is not known whether both these heterodimers are used equally in the formation of a mature pentamer. If the α1A322D subunit differentially affects the formation or utilization of the α1-β2 or α1-γ2 heterodimer intermediates, this mutation would have asymmetric consequences in formation of mutant receptors.

Because the mean peak currents of the homozygous mutant and $Het_{\beta\alpha\gamma}$ receptors were substantially smaller than those of wild type, neither of these receptor types would be expected to contribute significantly to the current kinetic profile with heterozygous expression; only the wild-type and $Het_{\beta\alpha\beta}$ receptors would be expected to contribute (Fig. 5). Therefore, it is not surprising that the rapid desensitization of the untethered heterozygous receptor currents did not reflect the lack of desensitization of the homozygous mutant receptors.

Our observation that neither $Het_{\beta\alpha\beta}$ nor $Het_{\beta\alpha\gamma}$ currents were the average of the wild-type and homozygous mutant currents has implications for understanding the pathophysiological basis of other diseases resulting from mutated ion channels. The epilepsy syndromes, benign familial neonatal seizures, and autosomal dominant nocturnal frontal lobe epilepsy are associated with mutations of voltage-gated potassium channels (Biervert et al., 1998; Singh et al., 1998; Dedek et al., 2001) and AChR channels (Steinlein et al., 1995, 1997; Hirose et al., 1999; Saenz et al., 1999; De Fusco et al., 2000; Phillips et al., 2000, 2001), respectively. Both of these ion channels are multimeric proteins, and in both cases, the mutated subunit is present in greater than one copy within the protein complex. Thus, the mutations in these epilepsy syndromes, like that in ADJME epilepsy, may produce asymmetrical heterozygous mutants, in which a mutation in each assembly position has a current that could not be predicted simply from an algebraic combination of wild-type and homozygous currents. Creating these epilepsy mutations in tethered constructs will help elucidate the physiology of each phenotype in these and other autosomal dominant diseases associated with ion channel mutations.

Once the whole-cell currents of the four phenotypes are individually characterized using tethered and untethered subunits, one must determine the whole-cell currents that result when the phenotypes are expressed together in a single cell. Cotransfecting a 1:1 ratio of wild-type α1 and α1(A322D) subunits with β2S and γ2S subunits would recapitulate the subunit expression expected for a heterozygote individual. If the α1(A322D) mutation induced reduction in α1 subunit-containing receptor expression and whole-cell currents in the transiently transfected HEK293T cells also occurs with endogenous mutant α1 subunit-containing neuronal receptors, one would expect that IPSCs would be reduced in heterozygous patients. This would result in reduced inhibition and an increased predisposition to seizures. In addition, the changes in current kinetics we observed would also be consistent to reduced IPSCs. Although the magnitude of their effect would be expected to be small, the rapid deactivation of the homozygous mutant and increased desensitization but unchanged deactivation of the heterozygous $Het_{\beta\alpha\beta}$ receptors would also contribute to shorter IPSC durations.

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