Mechanisms Involved in the Reduction of GABA<sub>A</sub> Receptor α1-Subunit Expression Caused by the Epilepsy Mutation A322D in the Trafficking-competent Receptor*  

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A mutation in the α1-subunit (A322D) of GABA<sub>A</sub>Rs is responsible for juvenile myoclonic epilepsy in a large Canadian family. Previous work has identified that this mutant affects the cell expression and function of recombinant GABA<sub>A</sub>Rs, expressed in HEK293 cells. Here we have extended these observations by showing that the mutation promotes association with the endoplasmic reticulum chaperone calnexin and accelerates the degradation rate of the subunits ~2.5-fold. We also find that the mutation causes the subunit to be degraded largely by a lysosomal-dependent process. Furthermore, we find that the mutation results in receptors that are inserted into the plasma membrane but are more rapidly endocytosed by a dynamin and caveolin1-dependent mechanism. These results suggest that the mutant subunit can form functional receptors, but that these have a shorter lifetime on the plasma membrane.

The γ-aminobutyric acid type A receptor (GABA<sub>A</sub>R) is a ligand-gated chloride channel that is the predominant mediator of fast synaptic inhibition in the brain. The GABA<sub>A</sub>R is a heteropentameric protein complex that can be assembled from seven subunit classes with multiple members and splice variants: α(1–6), β(1–3), γ(1–3), δ, ε, π, θ(1,2). The most common receptor found in the brain contains the α<sub>1</sub>-, β<sub>2</sub>-, and γ<sub>2</sub>-subunits (3, 4). GABA<sub>A</sub>R subunits are synthesized, differentially N-glycosylated, and oligomerized within the endoplasmic reticulum (ER) to form a functional receptor complex that then traffics to the cell surface (1, 5–7).

Roughly 0.4–1% of the population suffers from various forms of idiopathic-generalized epilepsy (2, 5, 8) any of which appear to have significant inheritability of this disorder. Previously, we studied the effects of a point mutation that was identified in a large French Canadian family that had heritable juvenile myoclonic epilepsy (8). The mutation in the α1-subunit of the GABA<sub>A</sub>R, α1(A322D) resulted in a substantial reduction in GABA currents recorded in HEK293 cells. This reduction in GABA responsiveness could be due to one or more dysfunctions caused by the mutation, including impairment in α1-subunit synthesis, altered receptor assembly, reduced trafficking to the cell surface, increased lability at the cell surface, or a reduction in function of the mutant-containing receptors.

Other studies have also identified several alterations in the function of expressed GABA<sub>A</sub>Rs containing the A322D mutation (9–13). For example, the mutation causes a reduction in mean channel open time, an increase in the rate of deactivation, and slows desensitization, but does not affect single channel conductance (9). In addition, this mutation has been found to reduce the surface expression of GABA<sub>A</sub>Rs, by increasing the endoplasmic reticulum-associated degradation (ERAD) of the α1(A322D)-subunit (12). Building upon our preliminary results (Bradley et al., 33) we found that the mutation impairs the surface expression of α1-subunits in HEK293 cells, transfected with β2-subunits, and that there is a substantial reduction in the total expression of α1-subunits. We also confirm recent evidence that the reduction in total expression is due to enhanced degradation and establish the difference in half-lives between the mutant and wild-type subunits assembled with β2-γ2-subunits. While we confirm the role of the ubiquitin proteosome complex in the degradation of both native and mutant subunits, our data suggest that lysosomal proteases are also involved in this process and that the role of this degradation pathway is enhanced for the mutant receptor. In addition, we identify another factor that affects the total expression of mutant α1-containing receptors. We show that blocking endocytosis via a dynamin-dependent and lipid raft-dependent mechanism results in a greater accumulation of mutant compared with wild-type GABA<sub>A</sub>Rs, suggesting that the mutant receptor is less stably expressed on the plasma membrane. Enhanced receptor endocytosis is a novel mechanism by which an epilepsy mutation may affect the function of GABA<sub>A</sub>Rs.

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4 The abbreviations used are: GABA<sub>A</sub>R, γ-aminobutyric acid type A receptor; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; UPS, ubiquitin-proteasome system; cav1, caveolin-1; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium.
**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Transfections**—HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma). Cells were grown to 70% confluence and transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. Unless otherwise stated, cells were transfected for 18–20 h at 37 °C with wild-type rat FLAG epitope-tagged α1 or α1(A322D) with or without rat β2, γ2, or γ2-GFP, or co-transfected with 1.5–3 µg GFP (Invitrogen), as previously described (8) in a 2:2:1-µg ratio for the full receptor complex. Dynamin 1 wild-type and K44E dominant negative constructs (14) and caveolin 1-N-terminal GFP plasmid (15) were cotransfected with the GABA\(_A\)R subunits at 1.5–3 µg. Transfection efficiency was assessed after each transfection for GFP signal and was typically 85–90% of total cells after visual inspection.

**Cell ELISA**—HEK293 cells were transfected with wild-type or mutant α1-FLAG constructs in a 2:1 ratio with β2 constructs, to limit the number of receptors reaching the surface, plus GFP as described above. Transiently transfected cells were then split and equally plated onto poly-D-lysine (PDL)-coated 12-well dishes and incubated overnight at 37 °C. Cell ELISAs were performed as previously described (16) at room temperature. Briefly, transfected cells were washed with Dulbecco’s phosphate-buffered saline (D-PBS; Invitrogen) and fixed for 10 min with 4% paraformaldehyde and 4% sucrose in PBS. Half the wells from each plate were permeabilized for 3 min with 0.1% Triton X-100 (Sigma) to determine total expression. They were then washed three times with D-PBS, and then all cells were blocked for at least 1 h in 2% goat serum (GS). After blocking, the cells were incubated with either mouse anti-FLAG (Sigma) or polyclonal rabbit anti-α1 (Upstate) at a 1:500 dilution in 2% GS for 1 h on an orbital shaker. The cells were washed three times with D-PBS and then incubated with horseradish peroxidase secondary antibodies (1:500; Amersham Biosciences). Labeled cells were incubated with the chromagen substrate o-phenylenediamine (Sigma). The reaction was stopped according to wild-type level color development with 3 N HCl, and the chromagen product absorbances were read at 492 nm. One control well from each condition was incubated with only secondary antibodies to determine background levels and used as the reference well for subsequent analysis.

**Coimmunoprecipitation Assay**—We performed the coimmunoprecipitation (coIP) assays as previously described (17) with minor modifications. Briefly, for analysis of the proportion of calnexin associated with the wild-type and mutant α1-subunits, we lysed the transfected cells with modified radioimmunoprecipitation assay buffer containing a protease inhibitor mixture. To coIP proteins associated with the α1-subunit, we used either 10 µg of FLAG antibody preconjugated to protein A-Sepharose beads for 2 h at room temperature (Amersham Biosciences) or anti-FLAG M2 agarose affinity gel (40 µl; Sigma). Antibody-bound beads were incubated with 0.5 mg of total lysate, diluted to 0.5 mg/ml with D-PBS, and immunoprecipitated for 2–4 h, rotating at 4 °C. SDS-PAGE, immunoblotting, and band intensity quantification was carried out as described above. Membranes were probed for the α1-subunits and then reprobed with a polyclonal rabbit calnexin antibody (Stressgen; 1:1,000) or GFP (Chemicon; 1:1,000). After obtaining signal intensity values from all the bands, the proportion of calnexin signal relative to α1 intensity within each sample was then assessed.

**Metabolic Pulse-chase Assays**—20–24-h post-transfection with α1 or α1β2γ2, the transfection media was replaced with 5 ml of fresh 5% fetal bovine serum/DMEM for 1–2 h. This and all subsequent incubations were carried out at 37 °C. Medium was replaced again with 4 ml of methionine-free DMEM (Invitrogen) for a period of 30–45 min. Expressing proteins were radio-labeled with 100 µCi/ml [\(^{35}\)S]methionine (PerkinElmer) for 30 min, washed three times with D-PBS, and the medium was changed to chase medium containing 100 µg/ml cycloheximide (Sigma) in DMEM to block further protein synthesis for 0, 60, 120, 240, or 480 min. At the end of the chase times, cells were washed three times with ice-cold D-PBS and lysed in PC lysis buffer (0.5% SDS, 1% Nonidet P-40, 5 mM EGTA, 5 mM EDTA in PBS with a protease inhibitor mixture). GABA\(_A\)R α1-subunits were immunoprecipitated (IP) from radiolabeled lysates (1 mg/ml) with anti-FLAG M2-agarose affinity gel (40 µl) and incubated rotating at 4 °C for 2–4 h (Sigma). IPs were eluted from the beads with 2× Laemmli Buffer (Bio-Rad) and loaded on a 12% acrylamide midi-gel (Hoeffer). These gels were run at 4 °C overnight or until the dye front band had reached the bot-
in 65% perchloric acid and hydrogen peroxide. The next day, scintillation mixture (Amersham Biosciences) was added to the dissolved samples, mixed on a vortexer for 30 s, and allowed to sit at room temperature for 1 h. The samples were then quantified for radioactivity in counts per minute (cpm). Background levels were subtracted for each band, and the normalized cpm for each time point was averaged, fitted to a single exponential function, and then plotted to determine the overall rate of degradation.

Degradation Assay—Transfections were performed as above for 6 h prior to treatment. The cells were washed and allowed to recover in fresh 10% fetal bovine serum/DMEM for 2 h and then treated with or without (controls) water soluble lactacystine (BioMol International; 10 or 15 μM) or leupeptin (Sigma; 25 or 50 μM). Cells were lysed and assessed by Western blotting as described earlier.

Statistical Analysis—We performed a two-tailed, Student t-tests to assess statistical significance and set significance at p < 0.05. All quantifications are expressed as the means ± S.E.

RESULTS

The Effects of the α1(A322D) Mutation on the Cell Surface and Total Expression of Recombinant GABA<sub>A</sub>Rs in HEK293 Cells—To address cellular trafficking or stability changes resulting from the point mutation, we first asked whether the mutation changed the surface expression of GABA<sub>A</sub>Rs. We compared the surface and total levels of the wild-type and mutant receptors, using recombinant N-terminal FLAG epitope-tagged α1β2 and α1(A322D)β2 constructs (8). In all cells imaged, the wild-type receptor was readily detected on the membrane surface as well as intracellularly, as determined following permeabilization (Fig. 1A). In contrast, the mutant receptor showed pronounced intracellular staining, but little or no surface labeling when exposure times were optimized for wild-type surface expression. To quantify this reduced surface expression effect we used a colorimetric cell ELISA approach. Cell surface expression of wild-type GABA<sub>A</sub>R (α<sub>1</sub>β<sub>2</sub>) averaged 44 ± 8% of the total cell expression.
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In contrast, cell surface expression of mutant GABA<sub>A</sub>R (α1(A322D)β<sub>2</sub>)-transfected cells was only 13 ± 3% of total cell expression (Fig. 1B; n = 3, p < 0.05). The cell ELISA also revealed a significant decrease in the total expression of the mutant receptor (to 44 ± 1% of control; n = 3; p < 0.05; Fig. 1C).

To more readily quantify the effects of the mutation on total GABA<sub>A</sub>R expression we performed Western blots. For these experiments we wanted to compare the effects of the mutation on the α1-subunit alone and on the most common trafficking competent receptor, which comprises α1β2γ2. We performed immunoprecipitation with anti-FLAG to pull-down α1 wild-type and mutant subunits and immunoblotted to confirm incorporation of the γ2-subunit into the full receptor complex (Fig. 1D). The A322D mutation caused a reduction in the expression in both the single α1-subunit (73 ± 1% of control; n = 5, p < 0.01; Fig. 1E) and the α1β2γ2 assembly (46 ± 7% of control; n = 6, p < 0.01; Fig. 1F). Interestingly, the mutation causes a more dramatic effect on the expression of the assembled receptor than on the isolated α1-subunit (p < 0.01).

The A322D Mutation Causes Enhanced Association with the ER Chaperone Calnexin—The reduction in total mutant GABA<sub>A</sub>R expression could be due to reduced synthesis. However, if the mutation caused a misfolding of the subunit then the mutant subunit or receptor assembly could be targeted for ERAD. One way to test for this is to assess its ability to bind to the chaperone calnexin, which is known to bind GABA<sub>A</sub>R subunits in the ER via their N-terminal glycosylation moieties (19). Prolonged association with calnexin and other ER chaperones retains misfolded proteins in the ER and tags them for subsequent degradation (20–22). Consistent with an increase in targeting for ERAD, we found a ~3-fold increase in association of the mutant receptor compared with the wild-type receptor (Fig. 2A). However, the total amount of calnexin remained the same (Fig. 2B). A similar difference was observed with α1β2 (Fig. 2C), as well as with the α1, or α1γ2 combinations (data not shown). These results suggest that the mutation causes a protein misfolding, which leads to calnexin-dependent targeting to ERAD.

The A322D Mutation Enhances the Degradation Rate of GABA<sub>A</sub>Rs—To quantify the effects of the mutation on the degradation rate of the α1β2γ2 complex (Fig. 3) we performed pulse (30 min of [35S]methionine) chase (for 1, 2, 4, 8 h) exper-
iments with 100 μM cycloheximide in the chase medium to block further protein synthesis. Isotope counts for the A322D containing α1-subunit did not significantly differ from wild-type counts at time point 0 (70 ± 10% of control, p > 0.05). However, the mutation had a dramatic effect on the half-life of the α1-subunit; reducing the half-life from 267 to 77 min (when fitted to a single exponential function from 0 to 4 h). At the later time point, lower molecular weight bands were also more prominent. These were sensitive to tunicamycin and represent non-glycosylated and mono-glycosylated forms of the α1-subunit (data not shown). These results demonstrate directly an increased degradation of the α1-subunit when assembled that is caused by the mutation and further suggests an enrichment of a pool of lower molecular weight α1 receptors because of this mutation.

Pathways Involved in the Degradation of Wild-type and Mutant α1-Subunits—Abnormal proteins that are destined for degradation are often targeted to the ubiquitin-proteasome system (UPS). To determine whether the UPS is preferentially involved in the elimination of mutant subunits, we used the proteosome inhibitor lactacystin. Cells were transfected for 6 h and incubated for 18 h with either 10 or 15 μM of the inhibitor. This treatment had no effect on cell viability, as assessed visually, but significantly increased the total expression levels of both the wild-type and mutant GABA_ARs (Fig. 4A). There was a trend for a larger effect on the mutant subunit (62 ± 15%; n = 6 versus 36 ± 3%; n = 6), suggesting that mutant subunits may be targeted to the UPS more than wild-type subunits, but this difference did not reach statistical significance.

An alternative route for protein degradation is via the lysosomal pathway. To investigate the possible role of this pathway, we used the lysosomal protease inhibitor leupeptin. We found that when cells were incubated with leupeptin (25 or 50 μM) for 18 h, there was a significant increase in the total levels of mutant receptors (30 ± 12%; n = 6), but no significant difference in the total levels of wild-type receptor (4 ± 6%; n = 6; Fig. 4B). Thus, mutant-containing receptors seem to be preferentially targeted for lysosomal degradation.

Blocking Endocytosis Enhances the Total Expression of Mutant GABA_ARs—While these experiments, and those of other studies (10–12), clearly demonstrate an increase in the degradation of mutant α1-subunits and mutant GABA_A receptors, the possibility remains that the mutation causes other deficits. One possibility is that mutant receptors are expressed on the plasma membrane but are less stable, because of enhanced endocytosis. To test for this, we inhibited endocytosis using a dominant negative construct of dynamin 1 (K44E) and compared this to cells transfected with wild-type dynamin 1, which does not alter endocytosis (14, 23). This treatment resulted in a small but significant increase in the total expression of wild-type α1β2γ2, which is consistent with a stabilization of GABA_A receptors on the plasma membrane due to block of dynamin-dependent endocytosis. In contrast to the wild type, there was a much larger increase in the expression of the mutant α1β2γ2 in cells expressing the dominant negative (Fig. 5A). The difference between the effect of the dominant negative on wild type (8 ± 2%; n = 5) and mutant receptors (52 ± 16%; n = 5) was statistically significant (p < 0.05), suggesting that mutant receptors are preferentially endocytosed relative to wild-type receptors by a dynamin-dependent pathway.

To further test surface stability of mutant containing GABA_ARs, we also examined caveolin 1-dependent endocytosis, another major form of endocytosis in HEK293 cells, which occurs only in the lipid raft domains (24). We therefore performed experiments using a dominant negative construct of caveolin-1 (cav1) containing GFP tagged to the N terminus (Cav DN), which has previously been demonstrated to block endocytosis of SV-40 virus via caveolae formation in cav1-containing cells (22). This treatment also increased the total expression of both wild-type and mutant receptors (Fig. 5B), but again had a proportionally larger effect on the expression of the mutant form (20 ± 10%; n = 6 and 82 ± 30%; n = 6, respectively; p < 0.05). Taken together, these results suggest that the mutant receptor is targeted to the plasma membrane, but is considerably less stable after insertion.
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FIGURE 5. Blocking endocytosis enhances the total expression of α1(A322D)-containing receptors in HEK293 cells expressing the α1/β2γ2 constructs. A, co-expression of a dynamin dominant negative (K44E) or a dynamin (Dyn) control with either wild-type or mutant GABA<sub>A</sub>Rs. K44E increased the expression of mutant α1-subunits more than wild-type ones (n = 5). B, co-expression of a Cav1 dominant negative (Cav DN) or a GFP control with either wild-type or mutant GABA<sub>A</sub>Rs (n = 6). The graphs plot the levels expressed in each condition relative to wild-type GABA<sub>A</sub>R-expressing HEK293 cells cotransfected with Dyn (A) or GFP (B). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

DISCUSSION

In the present study, we have produced further evidence that the α1(A322D) point mutation leads to the production of a GABA<sub>A</sub>R subunit that is less stable than its native counterpart. Because homomeric α1 receptors do not reach the cell surface (19), it can be concluded that receptors containing the mutant subunit can be assembled and targeted to the plasma membrane. The reduction in the number of receptors in the plasma membrane could be because fewer subunits are assembled and inserted and/or because the expressed receptor is less stable on the membrane surface.

By using a pulse-chase protocol we have been able to quantify the difference in degradation rates of the mutant and wild-type receptors. The increase in degradation rates can, at least in part, account for the reduction in total expression of the mutant receptors. This decrease in the receptor reserve could account for the reduction in function. Indeed, such a mechanism has been suggested to account for the reduction in GABA sensitivity in HEK293 cells expressing the mutant subunits (10–12). However, the present study has revealed an additional effect of the mutation that profoundly affects the surface stability of the GABA<sub>A</sub>R. The stability of the mutant receptor is greatly enhanced by treatments which prevent either dynamin-dependent or caveolin1-dependent endocytosis. These results suggest that the mutant receptors that escape ERAD are expressed on the membrane surface, but are less stable due to enhanced endocytosis.

Changes in Total Expression of the α1(A322D) Mutant—In our initial study, the preliminary analysis using Western blotting of total cell lysates did not reveal an obvious alteration in the steady-state levels of GABA<sub>A</sub>Rs after transfection of α1(A322D) with β2- and γ2-subunits (8). However, in a more vigorously quantitative analysis, we reported a significant reduction of the mutant receptor (33) as have other studies (10–12). Further analysis in the present study confirms that the mutation results in a substantial reduction in the total amount of receptor.

Protein Degradation Is Increased by the α1(A322D) Mutation—Misfolding rates for wild-type GABA<sub>A</sub>R subunits are very high (6, 25), and so mechanisms exist to degrade errant subunits via ERAD. Given that the α1(A322D) mutation introduces a negative charge into the middle of the third transmembrane domain of the α1-subunit, it would seem plausible that the mutation results in increased misfolding rates of the subunit via ERAD. Consistent with this idea, we found an increased association of calnexin with the mutant compared with the wild-type receptors. Calnexin binds to GABA<sub>A</sub>R subunits within the ER (19), where it retains misfolded subunits (20–22). Thus, the increase in association with calnexin suggests that a high proportion of mutant subunits are retained within the ER and subsequently targeted for degradation.

Indeed, enhanced ERAD and an increase in the degradation rate of the mutant α1-subunit expressed alone has been reported (12). We have confirmed that there is an enhanced degradation of mutant α1-subunits using pulse-chase experiments with the trafficking competent receptor. In our experiments, however, there were some quantitative differences from the previous report. In particular, we observed a much longer half-life for the mutant α1-subunit of 77 min, compared with an estimated 23 min in the previous study. This is unlikely to be due to a difference between the isolated subunit and the full receptor, because our pulse-chase experiments with the α1-subunit alone yielded similar results. One possible explanation for the difference was that we included a protein synthesis inhibitor in the chase medium to prevent synthesis of new unlabeled subunits, which might otherwise interfere with the efficiency of the colP of radiolabeled α1-subunits. By extending the chase time of our experiments to 8 h we were also able to...
directly measure the half-life of the native α1β2γ2 combination, which was 267 min. Therefore, the mutation increased the rate of the α1-subunit degradation by ~3.5-fold.

In addition to enhanced degradation, the mutation also increased the proportion of unglycosylated α1-subunits observed over time. The reduction of glycosylated α1(A322D) subunits is most likely due to the increased targeting of these subunits to the ER degradation pathway. This is consistent with the mutation causing misfolding of the α1-subunit and its targeting for ER degradation via prolonged calnexin binding (21, 22).

Pathways Involved in the Degradation of Wild-type and Mutant α1 GABA_A Rs—We have confirmed that the ubiquitin proteosome complex is involved in the degradation of both native (26) and mutant subunits (12). However, proportionally there was not a significantly greater degradation of mutant compared with wild-type subunits by this pathway when expressed as the full receptor. However, our data did reveal a much larger degradation of mutant compared with wild-type receptors via the lysosome pathway. It has been suggested that GABA_A Rs that are endocytosed from the plasma membrane can be reinserted if blocked from degradation by a leupeptin-sensitive route (27). Therefore, our data are consistent with an increase in degradation of mutant receptors, which have been trafficked to and removed from the plasma membrane. This observation prompted us to look at the role of endocytosis directly.

Trafficking of GABA_A Rs Is Altered by the α1(A322D) Mutation—Clathrin-mediated endocytosis is important for constitutive endocytosis of GABA_A Rs (28). By the use of dominant negative constructs of dynamin, it has been shown that this GTPase, which is involved in the budding off of internalizing clathrin coats (29), is important in GABA_A R internalization (30). In the present study we found that interfering with dynamin, using expression of the K44E mutant, had only a small effect on the total levels of wild-type α1-subunits. This presumably reflects the relative stability of GABA_A Rs expressed on the surface of HEK293 cells. In contrast, the dynamin mutant had a pronounced effect on the total levels of mutant α1-subunits, suggesting that receptors containing this mutant subunit are more susceptible to dynamin-dependent endocytosis relative to wild-type receptors. On the basis of these observations, we suggest that mutant-containing receptors are being inserted into the plasma membrane but then readily endocytosed and broken down. In contrast wild-type receptors are expressed and stabilized on the plasma membrane, and this fraction contributes significantly to the total levels of receptors measured in the Western analysis.

However, dynamin is involved not only in clathrin-dependent endocytosis but also in clathrin-independent, cholesterol-dependent endocytosis from lipid rafts (31). Dynamin-dependent raft pathways involve the endocytosis of caveolae, which are enriched in the protein cav-1. This protein is essential for the formation of caveolae, and so interference with its function can be used to prevent caveolae/raft-dependent endocytosis. Because GABA_A Rs have been reported to be resident in the lipid raft fractions of neurons (32) we also investigated the role of this form of endocytosis. Our findings closely resembled our observations with the dynamin dominant negative, suggesting that endocytosis of mutant GABA_A Rs from HEK293 cells is at least in part mediated by a dynamin-dependent, caveolin1-dependent mechanism.

Mechanisms Contributing to the Reduction in GABA_A R Function in the α1(A322D) Mutant—Our initial report showed that the α1(A322D) mutation caused a reduction in sensitivity to GABA (8). Broadly speaking, two different mechanisms have been proposed to explain this effect; a reduction in channel function (9, 13) and a reduction in the formation of trafficking competent receptors, which are instead targeted for ERAD (10–12). Based on this latter mechanism, it was proposed that the primary deficit in a heterozygous situation is a reduction in the number of wild-type receptors (11). In the present study, we have identified another error in the handling of the mutant receptor. We find that receptor complexes, which escape ERAD, are available for insertion into the plasma membrane but are considerably less stable after insertion than the wild-type receptor. This endocytosis of the mutant receptor occurs by both dynamin-dependent and caveolin1-dependent processes. These data suggest that processes that regulate the rate of receptor endocytosis can act as an additional quality control mechanism. However, because mutant α1(A322D)-containing receptors are clearly inserted into the plasma membrane, they will contribute to the total GABA current in heterozygotes. Their shorter half-life in the plasma membrane and altered electrical properties will both contribute to the reduction in GABA current. A major challenge for the future will be to determine the relative importance of these different effects of the mutation for the epilepsy phenotype in humans.

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