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Endoplasmic Reticulum-associated Degradation Controls Cell Surface Expression of γ-Aminobutyric Acid, Type B Receptors*

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Background: The amount of cell surface $GABA_B$ receptors determines the strength of $GABA_B$-mediated inhibition of neuronal excitability.
Results: $GABA_B$ receptors are Lys^{48}-linked polyubiquitinated and degraded by proteasomes via ERAD.
Conclusion: ERAD constitutively degrades $GABA_B$ receptors and thereby determines the number of functional receptors available for signaling.
Significance: Modulation of ERAD activity may be a mechanism to adjust the level of functional $GABA_B$ receptors.

Metabotropic $GABA_B$ receptors are crucial for controlling the excitability of neurons by mediating slow inhibition in the CNS. The strength of receptor signaling depends on the number of cell surface receptors, which is thought to be regulated by trafficking and degradation mechanisms. Although the mechanisms of $GABA_B$ receptor trafficking are studied to some extent, it is currently unclear whether receptor degradation actively controls the number of $GABA_B$ receptors available for signaling. Here we tested the hypothesis that proteasomal degradation contributes to the regulation of $GABA_B$ receptor expression levels. Blocking proteasomal activity in cultured cortical neurons considerably enhanced total and cell surface expression of $GABA_B$ receptors, indicating the constitutive degradation of the receptors by proteasomes. Proteasomal degradation required Lys^{48}-linked polyubiquitination of lysines 767/771 in the C-terminal domain of the $GABA_{B2}$ subunit. Inactivation of these ubiquitination sites increased receptor levels and $GABA_B$ receptor signaling in neurons. Proteasomal degradation was mediated by endoplasmic reticulum-associated degradation (ERAD) as shown by the accumulation of receptors in the endoplasmic reticulum upon inhibition of proteasomes, by the increase of receptor levels, as well as receptor signaling upon blocking ERAD function, and by the interaction of $GABA_B$ receptors with the essential ERAD components Hrd1 and p97. In conclusion, the data support a model in which the fraction of $GABA_B$ receptors available for plasma membrane trafficking is regulated by degradation via the ERAD machinery. Thus, modulation of ERAD activity by changes in physiological conditions may represent a mechanism to adjust receptor numbers and thereby signaling strength.

The signaling strength of neurotransmitter receptors is significantly controlled by the number of receptors in the plasma membrane. Protein synthesis, cell surface trafficking, endocytotic removal from the plasma membrane, and degradation of the receptors need to be precisely balanced to maintain an appropriate level of cell surface receptors. These mechanisms thus provide means for adapting receptor numbers in response to plastic changes in neurons. There is accumulating evidence that regulated protein degradation via the ubiquitin-proteasome system plays an important integrative role in synaptic plasticity (1–3). Proteasomal degradation at the endoplasmic reticulum (ER)[3] is crucial for the quality control of newly synthesized receptors. Incorrectly folded and misassembled receptor proteins are efficiently eliminated from the endoplasmic reticulum via the ER-associated degradation (ERAD) (4). Defective receptor proteins are polyubiquitinated, exported from the ER membrane and degraded by proteasomes in the cytoplasm. There is evidence that ERAD may also be involved in the regulation of the number of functional receptors in response to physiological stimuli. Prolonged activation of IP_3 receptors, which release Ca^{2+} from the ER, down-regulates the expression of the receptors in ER membranes via ERAD-dependent proteasomal degradation (5). This is thought to be a homeostatic response to counterbalance excessive accumulation of Ca^{2+} in the cytoplasm. However, it is currently unclear whether the ERAD machinery contributes to the regulation of the cell surface density of neurotransmitter receptors.

$GABA_B$ receptors are G protein-coupled receptors assembled from the two subunits $GABA_{B1}$ and $GABA_{B2}$. They mediate slow inhibitory neurotransmission in the CNS and are thought to be involved in a variety of neurological disorders (6). It is meanwhile well established that $GABA_B$ receptors are

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[3] The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; PLA, proximity ligation assay; sPSC, spontaneous postsynaptic current; ANOVA, analysis of variance; PDI, protein-disulfide isomerase.

endocytosed from the plasma membrane via the classical dynamin- and clathrin-dependent pathway and are eventually degraded in lysosomes (7). Lysosomal targeting appears to be mediated by the ESCRT (endosomal sorting complex required for transport) machinery (8) that sorts mono- and Lys^{63}-linked polyubiquitinated proteins to lysosomes (9). It is currently unclear whether proteasomal degradation contributes to the regulation of GABA$_B$ receptors available for signal transduction. Therefore, we tested in this study the hypothesis that cell surface levels of GABA$_B$ receptors might be controlled by proteasomal degradation.

EXPERIMENTAL PROCEDURES

Antibodies—The following primary antibodies were used: rabbit GABA$_{B1a,b}$ (10, 11) directed against the C terminus of GABA$_{B1}$ (affinity-purified, 1:500 for in-cell Western assay and immunofluorescence), rabbit GABA$_{B2N}$ (10, 11) directed against the N terminus of GABA$_{B2}$ (affinity-purified, 1:250 for in-cell Western assay and immunofluorescence, 1:50 for *in situ* PLA), guinea pig GABA$_{B2}$ (1:1,000 for immunofluorescence in neurons and 1:4,000 in HEK 293 cells, 1:1,000 for Western blotting; Chemicon International), mouse PDI (1:1,000 for immunofluorescence; Santa Cruz Biotechnology), mouse ubiquitin (P4D1, 1:50 for Western blotting; Santa Cruz Biotechnology), mouse ubiquitin Lys^{48}-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore), mouse VCP (p97) (1:50 for *in situ* PLA, 3E8DC11; Abcam), mouse actin (1:1,000 for in-cell Western assay; Chemicon International), mouse HA (1:500 for immunofluorescence; Santa Cruz Biotechnology), and rabbit SYVN1/Hrd1 (1:50 for *in situ* PLA; Bioss). Secondary antibodies were coupled either to horseradish peroxidase (1:5,000; Jackson ImmunoResearch), Alexa Fluor 488 (1:1,000; Invitrogen), Cy-3 (1:500; Jackson ImmunoResearch), IRDye680 (1:400; LI-COR Biosciences), or IRDye800CW (1:400; LI-COR Biosciences).

Drugs—The following drugs were used: baclofen (50 μM; Tocris Bioscience), betulinic acid (20 μg/ml; Sigma-Aldrich), bicucullin (4 μM; Tocris Bioscience), Eeyarestatin I (5 μM; Chembridge), 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile (CNQX 2 μM; Tocris Bioscience), lactacystin (50 μM; Sigma-Aldrich), MG132 (10 μM; Sigma-Aldrich), pyrenebutyric acid (50 μM; Sigma-Aldrich), SMI-UPS14 (5 μM; BostonBiochem), and tetrodotoxin (0.5 μM; Tocris Bioscience).

Plasmids—The following cDNAs in the appropriate expression vectors were used: GABA$_{B(1a)}$ (12) (pcDNA1), GABA$_{B2}$ (13) (pcI) (pcDNA1, GABA$_B$ plasmids were kindly provided by Dr. B. Bettler (University of Basle) and Dr. K. Kaupmann (Novartis, Basle)), ubiquitin and ubiquitin (K48R) (14) (pRK5-HA; Addgene plasmids 17604 and 17608), VCP/p97-EGFP and VCP/p97(DKO)-EGFP (15) (pEGFP-N1; Addgene plasmids 23971 and 23974).

Mutation of GABA$_{B2}$—Lysines 767 and 771 in GABA$_{B2}$ were mutated to arginines using the QuikChange II XL site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

Culture and Transfection of Cortical Neurons—Primary neuronal cultures of cerebral cortex were prepared from day 18 embryos of time-pregnant Wistar rats as described previously (10, 11). Neurons were kept in culture for 12–17 days before being used. Neurons were transfected with plasmid DNA using magnetofection as detailed by Buerli *et al.* (16).

Culture and Transfection of HEK 293 Cells—HEK 293 cells were cultured in minimum essential medium (Invitrogen) containing 10% fetal calf serum (Invitrogen), 2 mM glutamine (Invitrogen), and 4% gentamicin (Invitrogen). HEK 293 cells were transfected with plasmids using the calcium phosphate precipitation method.

Proteasome Activity Assay—Neurons cultured in 96-well plates were incubated for 12 h with either 10 μM MG132, 50 μM lactacystin, or 20 μM betulinic acid followed by determination of proteasome activity using the Proteasome Glo Chymotrypsin-like cell-based assay (Promega) according to the manufacturer's instructions.

Immunoprecipitation and Western Blotting—Immunoprecipitation of GABA$_B$ receptors from deoxycholate extracts of rat brain membranes and Western blotting for the detection of GABA$_{B2}$ and ubiquitin was done as described previously (10, 17).

Immunocytochemistry and Confocal Laser Scanning Microscopy—Double labeling immunocytochemistry was performed with cortical neurons cultured on coverslips as described previously (10, 11, 17). Neurons were analyzed by confocal laser scanning microscopy (LSM510 Meta; Zeiss, 100× plan apochromat oil differential interference contrast objective, 1.4 NA) at a resolution of 1,024 × 1,024 pixels in the sequential mode. Quantification of fluorescence signals and image processing was done as detailed in Ref. 11. Images shown represent a single optical layer.

In-cell Western Assay—The in-cell Western assay was exactly done as in Ref. 11. Neurons cultured in 96-well plates were treated with the drug to be tested for the indicated time at 37 °C and 5% CO_2. After fixation and permeabilization, the neurons were incubated simultaneously with GABA$_B$ receptor and actin antibodies. Nonspecific GABA$_B$ receptor antibody binding was determined in parallel cultures by competition using the respective peptide-antigen (10 μg/ml). After incubation with the appropriate secondary antibodies, the fluorescence was measured with the Odyssey infrared imaging system (LI-COR Biosciences). Specific GABA$_B$ signals were normalized to the actin signal determined in parallel.

In Situ Proximity Ligation Assay (PLA)—The *in situ* PLA technology is a highly sensitive antibody-based method for the microscopic detection of protein-protein interactions and post-translational protein modifications in cultured cells and tissue section (18, 19). For *in situ* PLA, we used Duolink PLA probes and detection reagents according to the manufacturer's instructions (Olink Bioscience). The specificity of the PLA signal was validated for each pair of antibodies in HEK 293 cell expressing or not expressing GABA$_B$ receptors. In addition, in neurons, omitting one of the primary antibodies did not generate PLA signals.

For signal quantification, cells were imaged for GABA$_B$ receptor expression and PLA signals by confocal microscopy (LSM510 Meta; Zeiss, 100× plan apochromat oil differential interference contrast objective, 1.4 NA, resolution 1,024 × 1,024 pixels, sequential mode). GABA$_B$ receptor fluorescence intensities, PLA spots, and the cell area were quantified using

ImageJ. PLA signals were normalized to the $GABA_B$ receptor signal and the cell area.

Electrophysiology—Cortical neurons at 13–15 days *in vitro* were recorded in the whole cell voltage clamp configuration at room temperature. Total spontaneous postsynaptic currents (sPSCs) were recorded at a holding potential of −60 mV. Baclofen-evoked potassium currents were elicited using a 10-s pulse of 50 μM baclofen at −90 mV. Patch electrodes were filled with 120 mM CsCl/KCl, 10 mM EGTA, 10 mM HEPES (pH 7.4), 4 mM $MgCl_2$, 0.5 mM GTP, and 2 mM ATP. Spontaneous PSCs recordings were performed using intracellular CsCl, whereas the potassium currents were recorded using an intracellular solution containing KCl. The external solution contained 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), and 10 mM glucose. Potassium currents were recorded in the presence of tetrodotoxin (0.5 μM), 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile (CNQX, 2 μM), and bicuculline (4 μM). To enhance the amplitude of the baclofen-evoked currents, the potassium concentration of the extracellular solution was increased to 30 mM, and the sodium concentration was reduced to 120 mM (to keep osmolarity constant) before the application of the $GABA_B$ agonist. All the synaptic events displaying amplitudes above the background noise (5–12 pA) were identified and analyzed off-line using MiniAnalysis 6.0.7 software (Synaptosoft). Mean amplitudes and frequency values were obtained from 1 min of epoch recordings on each experimental condition and normalized to the control condition of the individual neuron.

Statistical Analysis—The data are presented as means ± S.E. The statistical analysis of data were performed with the GraphPad Prism 5 software. Unpaired *t* test was used for comparing two conditions and one-way ANOVA followed by Dunnett's post hoc test for analysis of multiple conditions. The level of significance and the *n* values are indicated in the figure legends. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The Expression Level of $GABA_B$ Receptors Is Controlled by Proteasomal Degradation—It is currently unknown whether the ubiquitin-proteasome system contributes to the regulation of $GABA_B$ receptor expression levels in neurons. To gain evidence for a potential degradation of $GABA_B$ receptors by proteasomes, we treated cultured cortical neurons for 12 h with the proteasome inhibitors MG132 or lactacystin and determined the $GABA_{B1}$ and $GABA_{B2}$ protein expression levels. Under these conditions, MG132 and lactacystin decreased proteasomal activity to 31 ± 2 and 17 ± 1% of untreated controls, respectively (Fig. 1*A*). Both drug treatments increased total $GABA_B$ receptor expression levels (MG132: $GABA_{B1}$, 131 ± 2%; $GABA_{B2}$, 143 ± 5%; lactacystin: $GABA_{B1}$, 142 ± 4%; $GABA_{B2}$, 147 ± 2% of control; Fig. 1*B*), suggesting that under basal conditions $GABA_B$ receptors were constitutively degraded to a certain extent by proteasomes.

Prolonged inhibition of proteasomes depletes the pool of free ubiquitin (20, 21), which might also affect ubiquitin-dependent processes unrelated to proteasomal degradation. There is some evidence that $GABA_B$ receptors are sorted to lysosomes via the ubiquitin-dependent ESCRT (endosomal sorting complex required for transport) machinery (8). Hence, prolonged inhibition of proteasomes might indirectly compromise lysosomal degradation of the receptors. However, an indirect contribution of lysosomal degradation could be ruled out. Pharmacologically increasing proteasome activity by treating cortical neurons for 12 h with the proteasome activator betulinic acid (22), which enhanced proteasomal activity to 143 ± 17% of control (Fig. 1*A*), significantly decreased $GABA_B$ receptor levels ($GABA_{B1}$, 69 ± 2%; $GABA_{B2}$, 64 ± 3% of control; Fig. 1*C*).

It has recently been shown that inhibition of the proteasome-associated deubiquitinating enzyme USP14 enhanced the degradation of proteasome substrates (23). Inhibition of USP14 by incubation of cortical neurons with SMI-USP14 (small molecule inhibitor of USP14) strongly reduced $GABA_B$ receptor levels ($GABA_{B1}$, 49 ± 4%; $GABA_{B2}$, 29 ± 2% of control; Fig. 1*D*), further supporting the view that $GABA_B$ receptors are degraded by proteasomes.

Finally, we assessed the functional consequences of decreased $GABA_B$ receptor levels after enhancing proteasomal activity with betulinic acid by measuring spontaneous synaptic activity in electrophysiological experiments. Activation of $GABA_B$ receptors with the selective agonist baclofen considerably decreased the amplitude as well as the frequency of sPSCs to 43 ± 4 and 56 ± 7%, respectively (Fig. 1*E*). Treatment of cultures for 12 h with betulinic acid diminished baclofen-induced inhibition of sPSCs (amplitude, from 43 ± 4 to 90 ± 12% of control; frequency, from 56 ± 7 to 94 ± 15% of control; Fig. 1*E*), supporting the hypothesis that enhanced proteasomal activity leads to reduced levels of functional $GABA_B$ receptors available for neuronal inhibition.

$GABA_B$ Receptors Undergo Lys^{48}-linked Polyubiquitination—Lys^{48}-linked polyubiquitination of proteins serves as a signal for proteasomal degradation. Consistent with polyubiquitination, $GABA_B$ receptors immunoprecipitated from deoxycholate extracts of crude rat brain membranes exhibited on Western blots ubiquitin immunoreactivity in the high molecular range (Fig. 2*A*). This suggests that $GABA_B$ receptors are ubiquitinated under basal conditions to a certain extent. Likewise, using the *in situ* PLA, we found that $GABA_B$ receptors in cultured cortical neurons display Lys^{48}-linked polyubiquitination, which was considerably increased upon inhibition of proteasomal activity with MG 132 (172 ± 11% of control; Fig. 2*B*). This indicates the accumulation of Lys^{48}-linked polyubiquitinated $GABA_B$ receptors destined for proteasomal degradation.

Next we tested whether preventing Lys^{48}-linked polyubiquitination affects $GABA_B$ receptor levels. Overexpression in neurons of a Lys^{48} chain elongation-defective ubiquitin mutant, in which lysine 48 had been exchanged for an arginine (Ub(K48R)), considerably increased the level of $GABA_B$ receptors ($GABA_{B1}$, 166 ± 7%; $GABA_{B2}$, 140 ± 7% of control; Fig. 2*C*). This finding corroborates a Lys^{48}-linked polyubiquitin-mediated proteasomal degradation of $GABA_B$ receptors.

The C-terminal Domain of $GABA_{B2}$ Contains a Major Lys^{48}-linked Polyubiquitination Site—An *in silico* analysis predicted two lysines in the C-terminal domain of $GABA_{B2}$ at positions 767 and 771 as likely candidates for ubiquitination. We inactivated these potential ubiquitination sites by exchanging

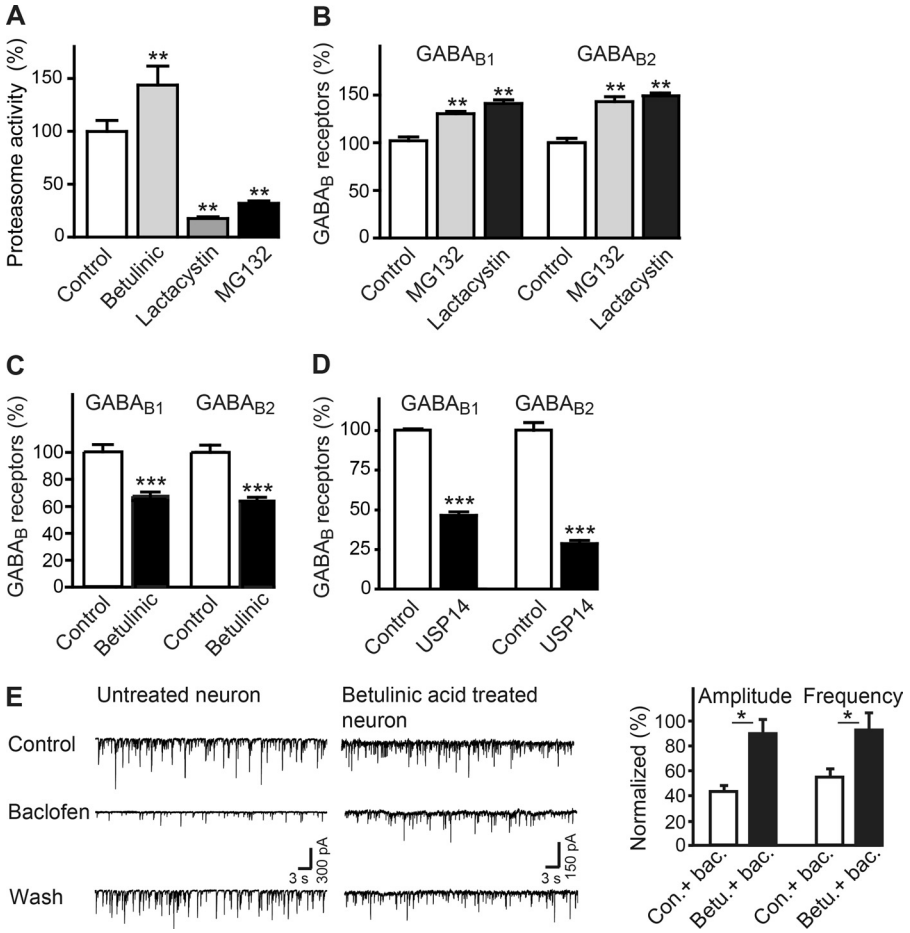

FIGURE 1. **The expression level of GABA$_B$ receptors is controlled by proteasomes.** *A*, verification of drug effects on proteasome activity. Neurons were incubated for 12 h with the indicated drugs and tested for proteasome activity ($n = 12$ cultures) **, $p < 0.01$, ANOVA. *B*, blocking proteasome activity increased the level of GABA$_B$ receptors. Neurons were incubated for 12 h with drugs, and GABA$_B$ receptor levels were determined using the in-cell Western assay. Untreated neurons served as a control ($n = 40$ cultures). **, $p < 0.01$; ***, $p < 0.001$, ANOVA. *C*, enhancing proteasome activity decreased the level of GABA$_B$ receptors. Neurons were incubated for 12 h with betulinic acid, followed by determination of GABA$_B$ receptor levels using the in-cell Western assay ($n = 40$ cultures). ***, $p < 0.0001$, *t* test. *D*, inhibition of the deubiquitinating enzyme USP14 decreased the expression level of GABA$_B$ receptors. Neurons were incubated for 12 h with SMI-USP14 (USP14) and tested for GABA$_{B1}$ and GABA$_{B2}$ levels using the in-cell Western assay ($n = 20–27$ cultures). ***, $p < 0.0001$, *t* test. *E*, enhancing proteasome activity diminished baclofen-induced inhibition of sPSCs. *Left panel*, representative current traces showing sPSCs recorded from untreated cultured cortical neurons or from neurons treated for 12 h with betulinic acid. *Right panel*, normalized amplitude and frequency values of the sPSCs. Mean amplitudes and frequency values were normalized to the control condition of the individual neuron. *Con.*, control; *bac.*, baclofen; *Betu.*, betulinic acid ($n = 6$). *, $p < 0.05$, *t* test.

both lysines for arginines (GABA$_{B2}$(RR)) (Fig. 3*A*). Upon transfection into HEK 293 cells, GABA$_{B2}$(RR) displayed reduced Lys^{48}-linked polyubiquitination (61 ± 6% of wild type; Fig. 3*B*), indicating that Lys$^{767/771}$ is a main site for Lys^{48}-linked polyubiquitination in GABA$_{B2}$.

We then tested whether GABA$_{B1}$ is also a target for Lys^{48}-linked polyubiquitination. However, HEK 293 cells transfected with GABA$_{B1}$ showed only marginal GABA$_{B1}$/Lys^{48}-linked ubiquitination PLA signals as compared with HEK 293 cells expressing GABA$_{B1}$ and GABA$_{B2}$ (12 ± 6%; Fig. 3*C*). In line with this finding, co-expression of GABA$_{B1}$ with GABA$_{B2}$(RR) yielded a similar reduction in GABA$_B$ receptor/Lys^{48}-linked polyubiquitination signals (56 ± 8%; Fig. 3*C*) as observed for GABA$_{B2}$(RR) alone (61 ± 6%; Fig. 3*B*). Thus, GABA$_{B2}$ appears to be the main target for Lys^{48}-linked polyubiquitination of GABA$_B$ receptors.

Overexpressing GABA$_{B2}$(RR) in cultured neurons increased GABA$_B$ receptor levels to a similar level as observed after chronic proteasome inhibition (GABA$_{B1}$, 152 ± 15%; GABA$_{B2}$, 146 ± 9% of control; Fig. 3*D*). This suggests that Lys$^{767/771}$ in GABA$_{B2}$ is the major Lys^{48}-linked polyubiquitination site required for proteasomal degradation of GABA$_B$ receptors.

The functional consequence of the increased GABA$_{B2}$ cell surface density after transfecting neurons with GABA$_{B2}$(RR) was analyzed by measuring baclofen-induced K^+ currents using whole cell patch clamp recordings. Transfection of GABA$_{B2}$(RR) in neurons resulted in 2.8 ± 0.6-fold increased K^+ channel current amplitudes after activation of GABA$_B$ receptors with baclofen as compared with neurons transfected with wild type GABA$_{B2}$ (Fig. 3*E*). Thus, preventing proteasomal degradation of GABA$_{B2}$ by overexpression of GABA$_{B2}$(RR) increased the number of functional cell surface GABA$_B$ receptors available for signaling.

Cell Surface Expression of GABA$_B$ Receptors Is Regulated by ERAD—The most likely mechanism for proteasomal degradation of GABA$_B$ receptors is the ERAD. If GABA$_B$ receptors are degraded by ERAD, inhibition of proteasomal activity should result in an accumulation of GABA$_B$ receptors in the ER.

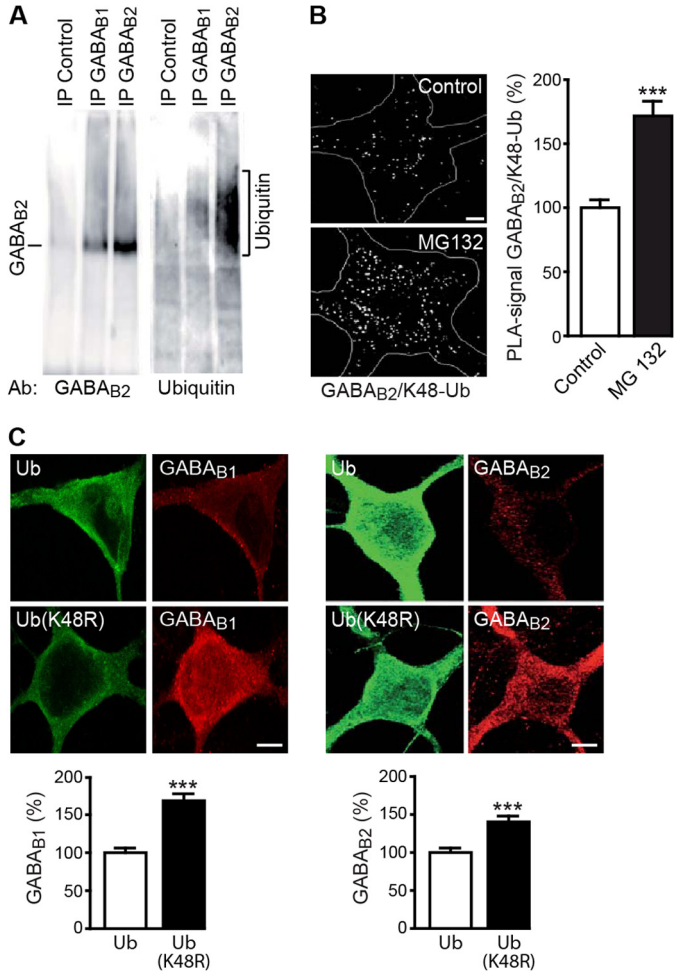

FIGURE 2. **Lys^{48}-linked ubiquitination controls the expression level of GABA$_B$ receptors.** *A*, demonstration of ubiquitination of native GABA$_B$ receptors. GABA$_B$ receptors were immunoprecipitated from deoxycholate extracts of rat brain membranes using either GABA$_{B1a,b}$ or GABA$_{B2N}$ antibodies. The immunoprecipitate was subjected to Western blotting for detection of GABA$_{B2}$ and ubiquitin. The high molecular smear detected with the ubiquitin antibody is typical for polyubiquitinated proteins. The ubiquitin signal in the GABA$_{B1}$ immunoprecipitate was considerably weaker than in the GABA$_{B2}$ immunoprecipitate because the GABA$_{B1a,b}$ antibody beads were less efficient in precipitating GABA$_B$ receptors than the GABA$_{B2}$ antibody beads. Specificity of the immunoprecipitation was verified with nonimmune antibodies (control). *IP*, immunoprecipitate; *Ab*, antibody. *B*, inhibition of proteasomes enhanced Lys^{48}-linked polyubiquitination of GABA$_B$ receptors. Neurons were incubated for 12 h in the absence (control) or presence of MG132 and processed for *in situ* PLA using antibodies directed against GABA$_{B2}$ and Lys^{48}-linked polyubiquitin to detect Lys^{48}-linked polyubiquitinated GABA$_B$ receptors (*white dots* in images, *left panel*). *Right panel*, quantification of *in situ* PLA signals ($n = 30$ neurons). ***, $p < 0.0001$, *t* test. *Scale bar*, 5 μm. *C*, overexpression in neurons of a Lys^{48} chain elongation defective ubiquitin mutant upregulated GABA$_B$ receptors. Neurons were transfected with plasmids containing HA-tagged ubiquitin (*Ub*) or HA-tagged mutant ubiquitin (Ub(K48R)). Neurons were stained for GABA$_{B1}$ or GABA$_{B2}$ (*red*) and Ub (*green*). *Top panels*, representative images. *Bottom panels*, quantification of total GABA$_{B1}$ and GABA$_{B2}$ levels in neurons expressing Ub or Ub(K48R) ($n = 28–40$ neurons). ***, $p < 0.0001$, *t* test. *Scale bar*, 10 μm.

Indeed, blocking proteasomal activity in neurons for 12 h with MG132 increased the number of GABA$_{B2}$ clusters (136 ± 6% of control) as well as the clusters co-localizing with a marker protein for the ER (protein-disulfide isomerase (PDI), 133 ± 7% of control; Fig. 4*A*).

To further establish the role of ERAD in regulating cellular GABA$_B$ receptor levels, we tested the effect of directly inhibit-

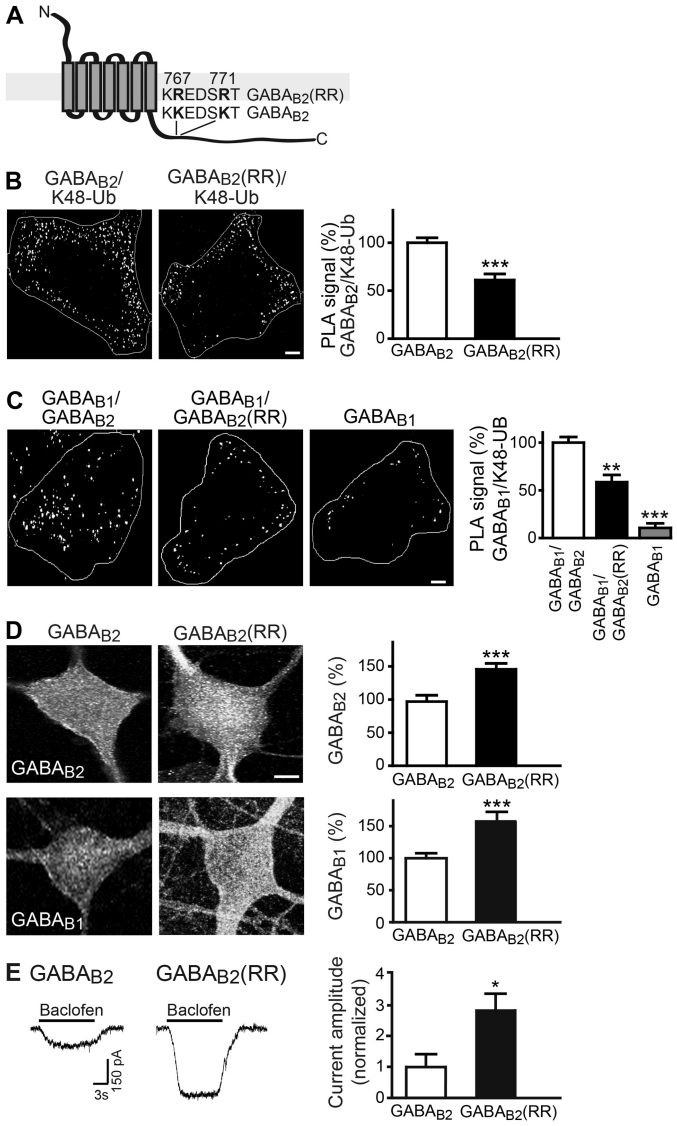

FIGURE 3. **The C-terminal domain of GABA$_{B2}$ contains a major Lys^{48}-linked ubiquitination site.** *A*, scheme depicting the location of mutated lysines in GABA$_{B2}$. *B*, decreased Lys^{48}-linked polyubiquitination of a GABA$_{B2}$ mutant in which lysines 767 and 771 had been changed to arginines (GABA$_{B2}$(RR)). HEK 293 cells were transfected with plasmids containing either GABA$_{B2}$ or GABA$_{B2}$(RR) together with HA-Ub plasmid. Cells were analyzed by *in situ* PLA using antibodies directed against GABA$_{B2}$ and Lys^{48}-linked polyubiquitin to detect Lys^{48}-linked polyubiquitinated GABA$_{B2}$ (*left panel*, *white dots*). *Scale bar*, 5 μm. *Right panel*, quantification of PLA signals ($n = 30$ cells). ***, $p < 0.0001$, *t* test. *C*, GABA$_{B2}$ is the main target for Lys^{48}-linked polyubiquitination. HEK 293 cells were transfected with plasmids containing cDNA for ubiquitin and either GABA$_{B1}$ alone, GABA$_{B1}$ and wild type GABA$_{B2}$, or GABA$_{B1}$ and GABA$_{B2}$(RR). Cells were analyzed by *in situ* PLA to detect Lys^{48}-linked polyubiquitinated GABA$_B$ receptors (*left panel*, *white dots*). *Scale bar*, 5 μm. *Right panel*, quantification of PLA signals ($n = 25–30$ cells). **, $p < 0.001$; ***, $p < 0.0001$, ANOVA. *D*, increased GABA$_B$ receptor expression levels in neurons overexpressing GABA$_{B2}$(RR). Neurons were co-transfected with plasmids containing GFP and GABA$_{B2}$ or GFP and GABA$_{B2}$(RR) and stained for GABA$_{B2}$ (*left*, *upper panels*) or GABA$_{B1}$ (*left*, *lower panels*). *Right panel*, quantification of fluorescence signals ($n = 27$ (GABA$_{B1}$) and 40 (GABA$_{B2}$) neurons). ***, $p = 0.0003$, *t* test. *Scale bar*, 10 μm. *E*, overexpression of GABA$_{B2}$(RR) in neurons increased GABA$_B$ receptor-mediated K$^+$ currents. *Left panel*, representative traces of baclofen-induced K$^+$ currents recorded in neurons transfected with wild type GABA$_{B2}$ or GABA$_{B2}$(RR). *Right panel*, normalized K$^+$ current amplitudes. Current amplitude of GABA$_{B2}$(RR) transfected neurons were normalized to the mean of current amplitudes recorded from GABA$_{B2}$ transfected neurons ($n = 16$ for GABA$_{B2}$ and $n = 18$ for GABA$_{B2}$(RR)). *, $p < 0.05$, *t* test.

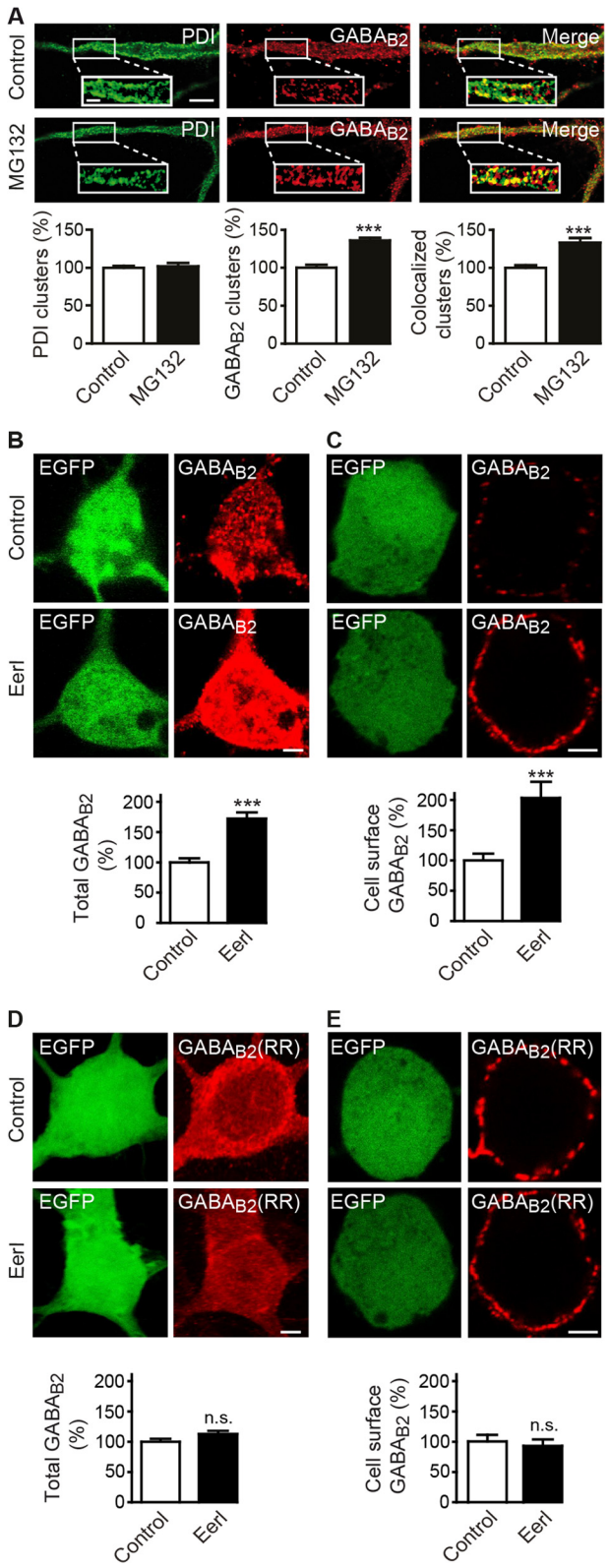

FIGURE 4. **GABA$_B$ receptors are degraded via the ERAD pathway.** *A*, blocking proteasomal activity increased the number of GABA$_{B2}$ clusters co-localized with the ER marker protein PDI. Neurons were incubated for 12 h with MG132 and stained for GABA$_{B2}$ (*red*) and PDI (*green*). The *yellow* clusters in the merged image indicate the co-localization of GABA$_{B2}$ and PDI. *Scale bars*, 5 μm (1 μm for *insets*). *Lower panels*, quantification revealed enhanced co-localization of GABA$_{B2}$ and PDI after proteasome inhibition. Control refers to the number of clusters in neurons not treated with MG132 ($n = 25–30$ neurons). ***, $p < 0.0001$, *t* test. *B–E*, blocking the ERAD pathway increased the level of GABA$_B$ receptors. Neurons were transfected with plasmids containing EGFP (for detection of transfected neurons) and either wild type GABA$_{B2}$ (*B* and *C*) or GABA$_{B2}$(RR) (*D* and *E*). After 48 h, the cultures were incubated for 12 h with or without (controls) the ERAD blocker Eeyarestatin I (EerI). Total (*B* and *D*) and cell surface (*C* and *E*) GABA$_B$ receptor levels were determined immunocytochemically using GABA$_{B2}$ antibodies (*red*, *upper panels*). *Scale bars*, 10 μm. *Lower panels*, quantification of GABA$_{B2}$ fluorescence signals ($n = 28–30$ neurons). ***, $p < 0.0001$. *n.s.*, $p > 0.05$, *t* test.

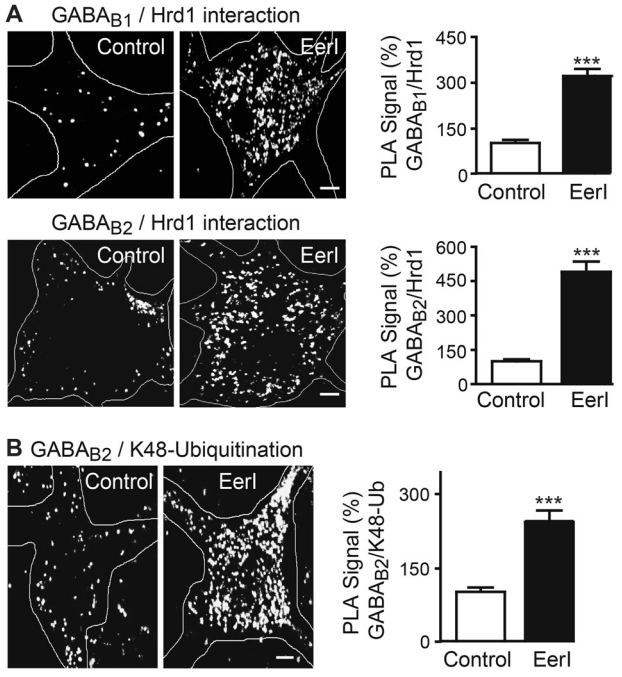

FIGURE 5. **GABA$_B$ receptors interact with the ERAD E3 ligase Hrd1.** *A*, demonstration of the interaction of GABA$_B$ receptors with the ERAD E3 ligase Hrd1 using *in situ* PLA with Hrd1 and GABA$_{B2}$ antibodies (*left*, *upper panels*) or GABA$_{B1}$ antibodies in cortical neurons (*left*, *lower panels*). Treatment of neurons for 12 h with EerI strongly increased the number of interactions. *Right panel*, quantification of *in situ* PLA signals ($n = 21–27$ neurons). ***, $p < 0.0001$, *t* test. *Scale bar*, 5 μm. *B*, inhibition of ERAD induced the accumulation of Lys^{48}-linked polyubiquitinated GABA$_B$ receptors. Neurons were incubated with EerI for 12 h and analyzed for Lys^{48}-linked ubiquitination using *in situ* PLA (*white dots* in representative images). *Scale bar*, 5 μm. *Right panel*, quantification of *in situ* PLA signals ($n = 32$ cells). ***, $p < 0.0001$, *t* test.

ing ERAD. Treatment of neurons for 12 h with the ERAD inhibitor Eeyarestatin I (EerI) (24, 25) increased both total GABA$_{B2}$ (183 ± 15% of control; Fig. 4*B*) and cell surface levels of GABA$_{B2}$ (204 ± 32% of control; Fig. 4*C*). Overexpression of GABA$_{B2}$(RR), which lack the main Lys^{48}-linked polyubiquitination sites, did not further increase total (112 ± 7% of control; Fig. 4*D*) or cell surface GABA$_B$ receptor levels (93 ± 15% of control; Fig. 4*E*). These observations indicate that Lys^{48}-linked polyubiquitinated GABA$_B$ receptors are degraded by ERAD.

GABA$_B$ Receptors Interact with the ERAD E3 Ubiquitin Ligase Hrd1—Hrd1 is one prototypical ERAD E3 ubiquitin ligase responsible for Lys^{48}-linked polyubiquitination of ERAD substrates (26). Using *in situ* PLA, we further confirmed the potential degradation of GABA$_B$ receptors via ERAD by showing that GABA$_B$ receptors interact with Hrd1 (Fig. 5*A*). Inhibition of ERAD for 12 h with EerI increased the number of interactions (GABA$_{B2}$/Hrd1, 490 ± 45%; GABA$_{B1}$/Hrd1, 305 ± 18% of control; Fig. 5*A*), indicating the accumulation of GABA$_B$ receptors at this central ERAD multiprotein complex. In line with this observation, blocking ERAD function for 12 h with

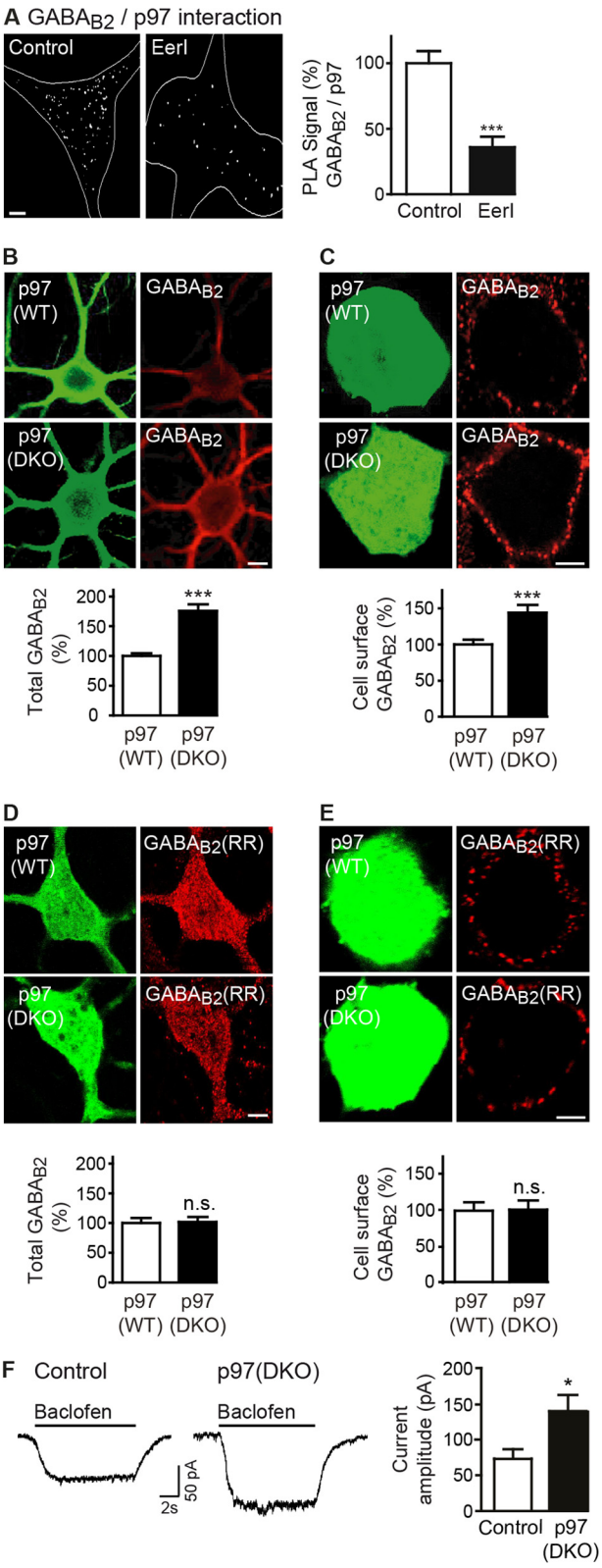

FIGURE 6. **GABA$_B$ receptors interact with the ERAD AAA-ATPase p97.** *A*, demonstration of the interaction of GABA$_B$ receptors with the ERAD AAA-ATPase p97 in cortical neurons using *in situ* PLA. Treatment of neurons for 12 h with the p97 inhibitor EerI strongly reduced the interaction. *Left panels*, quantification of *in situ* PLA signals (n = 18–21 neurons). ***, $p < 0.0001$, t test. *Scale bar*, 5 μm. *B* and *C*, disruption of ERAD function by overexpression of a dominant-negative mutant of p97 enhanced the level of total (*B*) and cell surface GABA$_{B2}$ receptors (*C*). Neurons were transfected with plasmids containing HA-tagged p97 or its dominant-negative mutant HA-tagged p97(DKO) and stained for GABA$_{B2}$ (*red*) and HA (*green*). *Scale bars*, 10 μm. *Lower panels*, quantification of GABA$_{B2}$ fluorescence signals (n = 40–50 neurons). ***, $p < 0.0001$, t test. *D* and *E*, overexpression of GABA$_{B2}$(RR) in neurons transfected with wild type p97 or p97(DKO) did not result in an additional increase of total (*D*) or cell surface (*E*) GABA$_{B2}$ levels. Neurons were transfected with plasmids containing GABA$_{B2}$(RR) and either HA-tagged p97 or its dominant-negative mutant HA-tagged p97(DKO) and stained for GABA$_{B2}$ (*red*) and HA (*green*). *Scale bars*, 10 μm. *Lower panels*, quantification of GABA$_{B2}$ fluorescence signals (n = 28–30 neurons). *n.s.*, $p > 0.05$, t test. *F*, disruption of ERAD by overexpression of p97(DKO) in neurons increased GABA$_B$ receptor-mediated K$^+$ currents. Neurons were transfected either with plasmids containing EGFP (control) or with plasmids containing p97(DKO). *Left panels*, representative traces of baclofen-induced K$^+$ currents. *Right panel*, K$^+$ current amplitudes (n = 10 for control and n = 8 for p97(DKO)). *, $p < 0.05$, t test.

EerI considerably increased the level of Lys^{48}-linked polyubiquitinated GABA$_B$ receptors (242 ± 21% of control; Fig. 5*B*).

GABA$_B$ Receptors Interact with the Essential ERAD Component p97—The AAA-ATPase p97 is a central constituent of the ERAD machinery involved in the retrotranslocation of proteins to the cytoplasm for proteasomal degradation (27). Using *in situ* PLA, we found that GABA$_B$ receptors interact with p97 (Fig. 6*A*). This finding further demonstrates the ERAD-mediated degradation of GABA$_B$ receptors. Inhibition of p97 by EerI decreased the interaction of GABA$_{B2}$ with p97 (40 ± 8% of control; Fig. 6*A*), suggesting that the association is activity-dependent.

Inhibition of p97 function in neurons by overexpression of a dominant-negative mutant of p97 (p97[DKO]) considerably increased total (176 ± 11% of control; Fig. 6*B*) as well as cell surface GABA$_B$ receptor levels (143 ± 11% of control; Fig. 6*C*) as compared with neurons overexpressing wild type p97. Overexpressing in addition GABA$_{B2}$(RR) did not further increase total (wild type p97, 100 ± 6%; p97(DKO), 104 ± 6%; Fig. 6*D*) or cell surface GABA$_B$ receptor levels (wild type p97, 100 ± 12%; p97(DKO), 100 ± 11%; Fig. 6*E*), indicating that ubiquitination of GABA$_{B2}$ is required for being recognized by the ERAD machinery.

Whole cell patch clamp recordings finally verified that inhibition of ERAD function by overexpression of p97(DKO) increased the level of functional cell surface GABA$_B$ receptors (Fig. 6*F*). Neurons overexpressing p97(DKO) displayed considerably increased amplitudes of baclofen-induced K$^+$ currents (control, 72 ± 14 pA; p97(DKO), 139 ± 14 pA; Fig. 6*F*). These experiments show that GABA$_B$ receptors are degraded by ERAD, which affects the levels of total and cell surface GABA$_B$ receptors.

DISCUSSION

Mechanisms controlling the cell surface density of GABA$_B$ receptors are of pivotal importance for determining the level of GABA$_B$ receptor-mediated neuronal inhibition. Because GABA$_B$ receptors control glutamatergic neurotransmission (28), modulation of their cell surface density is presumed to significantly contribute to synaptic plasticity. However, the mechanisms that control cell surface expression of GABA$_B$ receptors are largely unknown. In the present study, we identified proteosomal degradation via the ER-resident ERAD machinery as a mechanism that determines cell surface expression of GABA$_B$ receptors.

Our data indicate that a fraction of GABA$_B$ receptors in the ER is constitutively Lys^{48}-linked polyubiqutinated and

degraded by the ERAD machinery. This conclusion is based on the observation that blocking proteasomal activity, inhibiting ERAD function, or interfering with $GABA_B$ receptor Lys^{48}-linked polyubiquitination increased the expression levels of $GABA_B$ receptors in neurons. Lysines 767/771 in the C-terminal domain of $GABA_{B2}$ appear to represent the main Lys^{48}-linked polyubiquitination sites required for proteasomal degradation because their mutational inactivation rendered $GABA_B$ receptors largely immune to degradation. It is currently unclear whether Lys^{48}-linked polyubiquitination of both lysines or only of Lys^{767} or Lys^{771} serves as a tag for proteasomal degradation. A recent proteomic study analyzing the ubiquitination state of rat brain synaptic proteins identified Lys^{771} in $GABA_{B2}$ as being ubiquitinated (29). This observation favors Lys^{771} as the main Lys^{48}-linked polyubiquitination site in $GABA_{B2}$.

There are several lines of evidence that in particular $GABA_B$ receptors residing in the ER are degraded by proteasomes via ERAD. First, upon blocking proteasomal activity, the receptors accumulated in the ER. Second, blocking ERAD function pharmacologically or by overexpressing a dominant-negative mutant of the AAA-ATPase p97, which mediates the retrotranslocation of proteins to the cytoplasm for proteasomal degradation (27), increased $GABA_B$ receptor levels. Third, $GABA_B$ receptors interacted with the ERAD proteins p97 and Hrd1. Hrd1 is the prototypical ERAD E3 ligase (26) and most likely one of the ubiquitin ligases that mediate ubiquitination of $GABA_B$ receptors because stalling proteasomal degradation considerably increased its interaction with $GABA_B$ receptors and the level of Lys^{48}-linked polyubiquitinated $GABA_B$ receptors.

In all cases tested, $GABA_{B1}$ and $GABA_{B2}$ were concomitantly up- or down-regulated to a similar extent, suggesting that assembled $GABA_B$ receptor complexes are degraded by ERAD. This notion is further strengthened by the finding that 1) inactivation of the ubiquitination sites in $GABA_{B2}$ increased the expression levels of $GABA_{B1}$ and $GABA_{B2}$ as well as $GABA_B$ receptor-activated K^+ current amplitudes, 2) that interfering with ERAD function increased $GABA_B$ receptor function (baclofen-induced K^+ currents), and 3) that both $GABA_{B1}$ and $GABA_{B2}$ generated *in situ* PLA signals with the ERAD E3 ubiquitin ligase Hrd1, although only Lys^{48}-linked polyubiquitination of $Lys^{767/771}$ in $GABA_{B2}$ appears to be required for proteasomal degradation of the receptors.

What might be the physiological implications of this mechanism? The most firmly established function of ERAD is the degradation of aberrant proteins in the ER (30). In addition, ERAD has been shown to rapidly degrade activated IP_3 receptors in the ER to prevent excessive elevation of cytosolic Ca^{2+} concentrations (5), indicating that ERAD may also contribute to the regulation of functional receptors. Because blocking ERAD increased the level of functional $GABA_B$ receptors, and ERAD appears to degrade assembled heterodimeric receptors, it is rather unlikely that the role of ERAD is simply the degradation of un- or misfolded $GABA_B$ receptor subunits. The constitutive degradation of $GABA_B$ receptors suggests that ERAD controls the amount of receptors available for cell surface trafficking. This view is supported by recent studies on the regulation of cell surface $GABA_A$ receptors. Chronic suppression of neuronal activity or inhibition of L-type voltage-gated calcium channels decreased the level of functional $GABA_A$ receptors in the neuronal plasma membrane by a mechanism dependent on the ubiquitination of the $GABA_A$ receptor β3-subunit and proteasome activity, most likely via the ERAD pathway (31, 32). These findings imply that regulation of ERAD activity is a potential mechanism to adjust the level of functional $GABA_B$ receptors to changing physiological condition. Our finding that modulation of proteasomal activity up- or down-regulates the level of functional $GABA_B$ receptors supports this view. Interestingly, the level of proteasomal activity correlates with the activity state of neurons (33). We therefore hypothesize that the amount of functional $GABA_B$ receptors inserted into the plasma membrane is regulated by neuronal activity via ERAD.

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