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Proteasomal Degradation of γ-Aminobutyric Acid$_B$ Receptors Is Mediated by the Interaction of the GABA$_{B2}$ C Terminus with the Proteasomal ATPase Rtp6 and Regulated by Neuronal Activity*

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Background: The expression level of GABA$_B$ receptors is controlled by proteasomal degradation.
Results: Proteasomal degradation of GABA$_B$ receptors is mediated by interaction with Rpt6 and modulated by neuronal activity.
Conclusion: The level of neuronal activity regulates via proteasomal degradation the ER pool of GABA$_B$ receptor competent for forward trafficking.
Significance: This mechanism might contribute to homeostatic neuronal plasticity.

Regulation of cell surface expression of neurotransmitter receptors is crucial for determining synaptic strength and plasticity, but the underlying mechanisms are not well understood. We previously showed that proteasomal degradation of GABA$_B$ receptors via the endoplasmic reticulum (ER)-associated protein degradation (ERAD) machinery determines the number of cell surface GABA$_B$ receptors and thereby GABA$_B$ receptor-mediated neuronal inhibition. Here, we show that proteasomal degradation of GABA$_B$ receptors requires the interaction of the GABA$_{B2}$ C terminus with the proteasomal AAA-ATPase Rpt6. A mutant of Rpt6 lacking ATPase activity prevented degradation of GABA$_B$ receptors but not the removal of Lys^{48}-linked ubiquitin from GABA$_{B2}$. Blocking ERAD activity diminished the interaction of Rtp6 with GABA$_B$ receptors resulting in increased total as well as cell surface expression of GABA$_B$ receptors. Modulating neuronal activity affected proteasomal activity and correspondingly the interaction level of Rpt6 with GABA$_{B2}$. This resulted in altered cell surface expression of the receptors. Thus, neuronal activity-dependent proteasomal degradation of GABA$_B$ receptors by the ERAD machinery is a potent mechanism regulating the number of GABA$_B$ receptors available for signaling and is expected to contribute to homeostatic neuronal plasticity.

A prominent characteristic of neuronal plasticity is the regulation of the number of neurotransmitter receptors available for signaling (1). The operative process can include regulation of protein synthesis, cell surface trafficking, endocytotic removal from the plasma membrane, or degradation of the receptors. It is now well recognized that protein degradation via the ubiquitin-proteasome system plays a key role in synaptic plasticity (2–4). For instance, pharmacological modulation of neuronal activity in cultured hippocampal neurons induced a remodeling of postsynaptic proteins, which was dependent on proteasome-mediated protein degradation (5). On the receptor level, chronic elevation of neuronal activity has been shown to down-regulate AMPA receptors (6) and NMDA receptors (5, 7) in a proteasome-dependent manner as a homeostatic response.

The most well established role for proteasomal degradation of membrane receptors is the quality control of newly synthesized receptors in the endoplasmic reticulum (ER).[2] Folding and assembly of receptors is a rather inefficient process frequently resulting in incorrectly folded and misassembled proteins. Defective receptor proteins are Lys^{48}-linked polyubiquitinated, exported from the ER membrane, and degraded by proteasomes. This process is executed by a multiprotein machinery called ER-associated protein degradation (ERAD) (8). In addition to its quality control function, ERAD may also be involved in the activity-dependent regulation of neurotransmitter receptors. For instance, the level of functional GABA$_A$ receptors in the plasma membrane has been shown to be down-regulated after suppression of neuronal activity or blocking L-type voltage-gated Ca^{2+} channels by a mechanism that involved ubiquitination of the GABA$_A$ receptor β3 subunit and proteasomes (9, 10). Pulse-chase experiments in combination with inhibiting ER-Golgi transport indicated that newly synthetized GABA$_A$ receptors in the ER were degraded most likely by ERAD.

The excitability of neurons is controlled, among others, by the G protein-coupled GABA$_B$ receptors. GABA$_B$ receptors are heterodimers composed of the two subunits GABA$_{B1}$ and GABA$_{B2}$. They mediate slow inhibitory neurotransmission by activating K^+ channels and inhibiting Ca^{2+} channels (11). We recently showed that a fraction of GABA$_B$ receptors is Lys^{48}-

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[2] The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; GABA, γ-aminobutyric acid; PLA, proximity ligation assay; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; ANOVA, analysis of variance.

linked ubiquitinated at $Lys^{767/771}$ located in the C-terminal domain of the $GABA_{B2}$ subunit and constitutively degraded by ERAD (12). This mechanism controls the pool of assembled $GABA_B$ receptors in the ER destined for forward trafficking to the plasma membrane and consequently determines the level of $GABA_B$ receptor-mediated inhibition. In this study, we addressed the unresolved questions of which proteasomal component interacts with $GABA_B$ receptors and whether ERAD-mediated degradation of $GABA_B$ receptors is regulated by changes in neuronal activity.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit $GABA_{B1a,b}$ (13, 14) directed against the C terminus of $GABA_{B1}$ (affinity-purified, 1.8 μg/ml for immunofluorescence), rabbit $GABA_{B2N}$ (13, 14) directed against the N terminus of $GABA_{B2}$ (affinity-purified, 2.6 μg/ml for whole cell ELISA and total as well as cell surface immunofluorescence staining, 13 μg/ml for *in situ* PLA), guinea pig $GABA_{B2}$ (1:1000 for Western blotting, 1:250 for *in situ* PLA, Chemicon Intl.), mouse Rpt6 (clone p45–110, 1:1000 for immunofluorescence using HEK293 cells and 1:50 using neurons, 1:50 for Western blotting, 1:20 for *in situ* PLA, Enzo), rabbit ubiquitin Lys^{48}-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore), rabbit ubiquitin Lys^{63}-specific (clone Apu3, 1:50 for *in situ* PLA; Millipore), mouse actin (1:1000 for whole cell ELISA, Chemicon International), mouse HA (1:500 for immunofluorescence, 1:200 for *in situ* PLA, Santa Cruz Biotechnology). Secondary antibodies were labeled with either horseradish peroxidase (1:5000, Jackson ImmunoResearch Laboratories), Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch Laboratories), IRDye680 (1:400, Li-COR Biosciences), or IRDye800CW (1:400, Li-COR Biosciences).

Drugs—The following chemicals were used for this study: 2 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, Tocris Bioscience), 50 μM D-AP5 (Tocris Bioscience), 5 μM eeyarestatin I (Chembridge), 10 μM MG132 (Sigma-Aldrich), 20 μM picrotoxin (Tocris Bioscience), and 50 μM pyrenebutyric acid (Sigma-Aldrich).

Plasmids—Rat $GABA_{B(1a)}$ (15), rat $GABA_{B2}$ (16), rat $GABA_{B2T749}$ (17) ($GABA_B$ plasmids were provided by Dr. B. Bettler, University of Basle and Dr. K. Kaupmann, Novartis, Basle), rat $GABA_{B2}$(RR) (12), human HA-Rpt6 and human HA-$Rtp6^{K196M}$ (18) (gift from Dr. G. Swarup, Council of Scientific and Industrial Research, India), mouse p45/Rpt6 (gift from Dr. Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, University of Strasbourg), pGBT9PheS (gift from Dr. Gerald Radziwill and Dr. Karin Moelling, University of Zurich).

Yeast Two-hybrid Assay—The sequence encoding the last 12 C-terminal amino acids of rat $GABA_{B2}$ was introduced into the pGBT9PheS vector (19) and used for screening a human brain cDNA library (Clontech) with the yeast two-hybrid system using standard techniques.

Culture and Transfection of HEK 293 Cells—HEK (human embryonic kidney) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (PAA Laboratories) and penicillin/streptomycin (PAA Laboratories). Plasmids were introduced into HEK 293 cells using the polyethylenimine method according to the jet-PEI protocol (Polyplus Transfection).

Introduction of Peptides into HEK 293 Cells—Small synthetic peptides were introduced into HEK 293 cells as described in Ref. 20. A synthetic peptide comprising the last 14 C-terminal amino acids of $GABA_{B2}$ with seven additional arginines for rendering it cell-permeable was generated (RRRRRRR-RHVPPSFRVMVSGL, GenScript). A peptide containing the same amino acids but in a random sequence was used as a control (RRRRRRR-RLGPHVRMFVSSVP, GenScript). Both peptides were biotinylated at their N terminus to permit detection via DyLight649-conjugated steptavidin (Jackson ImmunoResearch Laboratories). Twenty-four hours after transfection with $GABA_B$ receptor and Rpt6 plasmids, the HEK 293 cells were washed with PBS and incubated for 5 min with 50 μM pyrenebutyric acid in PBS. Then, the peptide was added (final concentration of 10 μM) and incubated for 15 min, followed by washing the cells two times with PBS. After the addition of fresh culture medium, the cells were incubated for an additional 24 h at 37 °C/5% CO_2 and used for immunofluorescence experiments.

Culture and Transfection of Cortical Neurons—Primary neuronal cultures of cerebral cortex were prepared from embryonic day 18 embryos of Wistar rats as detailed previously (13, 14). Neurons were used after 12 to 17 days in culture. Neurons were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) and CombiMag (OZ Biosciences) exactly as described in Ref. 21.

Proteasome Activity Assay—Neurons cultured in 96-well plates were incubated for 12 h with either 20 μM picrotoxin or 10 μM CNQX, 20 μM D-AP5 followed by determination of proteasome activity using the Proteasome Glo chymotrypsin-like cell-based assay (Promega) according to the manufacturer's instructions.

Whole Cell ELISA—Whole cell ELISA was exactly done as described previously (12, 14). Neurons cultured in 96-well plates were treated with the indicated drugs for 12 h at 37 °C and 5% CO_2. For determining total expression of $GABA_B$ receptors, the neurons were fixed, permeabilized, and incubated simultaneously with antibodies directed against $GABA_{B2}$ and actin. The fluorescence signals were quantified using the Odyssey imaging system. $GABA_{B2}$ signals were normalized to the actin signal determined in parallel.

For cell surface staining, living neurons were incubated with $GABA_{B2N}$ antibodies for 2 h at 4 °C. For normalization, the cell-permeable nuclear marker DRAQ5 (1:2000, Biostatus Ltd.) was used.

Immunoprecipitation and Western Blotting—$GABA_B$ receptors were immunoprecipitated from 0.5% deoxycholate extracts of rat brain membranes followed by Western blotting for the detection of $GABA_{B2}$ and Rpt6 as described previously (13).

Immunocytochemistry and Confocal Laser Scanning Microscopy—Double-labeling immunocytochemistry on HEK 293 cells and cortical neurons was done exactly as described previously (13, 14). Images of cells were taken by confocal laser scanning microscopy (LSM510 Meta, Zeiss, 100× plan apochromat oil differential interference contrast objectitve, 1.4 numerical

aperture, or LSM700, Zeiss, 40× plan apochromat oil differential interference contrast objective, 1.4 numerical aperture) at a resolution of 1024 × 1024 pixels in the sequential mode. Quantification of fluorescence signals and image processing was done as described in Ref. 14.

In Situ Proximity Ligation Assay (in Situ PLA)—The *in situ* PLA technology enables the microscopic detection of protein-protein interactions and posttranslational modifications of proteins in cells *in situ* (22, 23). The target proteins are detected using two primary antibodies raised in different species and a corresponding pair of oligonucleotide labeled species-specific secondary antibodies (PLA probes). Only when the two primary antibodies bound to their target proteins are located in very close proximity (<30 nm), specific oligonucleotides can hybridize to the PLA probes enabling a rolling cycle amplification reaction that generates a long DNA strand to which specific fluorophore-labeled oligonucleotides are hybridized. The signal from each pair of PLA probes generates an individual fluorescent spot detectable by fluorescence microscopy.

In this study, *in situ* PLA was employed for detecting the interaction of GABA$_B$ receptors with Rpt6 (using mouse Rpt6 1:20 and rabbit GABA$_{B2N}$ 13 μg/ml μg/ml), HA-tagged Rpt6 with GABA$_B$ receptors (using 1:200 mouse HA and 13 μg/ml rabbit GABA$_{B2N}$), as well as for detecting Lys^{48}-linked (rabbit ubiquitin Lys^{48}-specific (1:50) and guinea pig GABA$_{B2}$ (1:250)) and Lys^{63}-linked (rabbit ubiquitin Lys^{63}-specific (1:50) and guinea pig GABA$_{B2}$ (1:250)) ubiquitination of GABA$_B$ receptors. 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.

In situ PLA was performed using the Duolink *in situ* kit (Olink Bioscience) according to the manufacturer's instructions. Briefly, cortical neurons grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed again with PBS, and permeabilized for 10 min with 0.2% Triton X-100 in PBS. After washing again with PBS, the neurons were incubated with the appropriate pair of primary antibodies (diluted in PBS containing 3% BSA) overnight at 4 °C. Thereafter, the cells were washed four times for 5 min with PBS, followed by incubation with the PLA probes (PLA probe anti-mouse minus and PLA probe anti-rabbit plus or PLA probe anti-guinea pig plus, all diluted 1:5 in 3% BSA/PBS) for 1 h at 37 °C. After washing the cells two times for 5 min with PBS, the ligation solution diluted 1:5 in water was added to the neurons and incubated in a preheated humidity chamber for 1 h at 37 °C. The neurons were then washed two times for 5 min with 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20. Finally, the amplification solution containing the fluorescently labeled oligonucleotides diluted 1:5 in water along with secondary antibodies for the determination of the GABA$_{B2}$ expression levels was added to the neurons and incubated in a preheated humidity chamber for 100 min at 37 °C. Subsequently, the neurons were washed two times with 0.2 M Tris, pH 7.4, 0.1 M NaCl, and once with 0.002 M Tris, 0.001 M NaCl for 1 min in the dark at room temperature and mounted on microscope slides with Dako fluorescent mounting medium. Stained neurons were immediately analyzed by laser scanning confocal microscopy (LSM 510 Meta, Zeiss, 100× plan apochromat oil differential interference contrast objective, 1.4 numerical aperture). Five optical sections were taken with a distance of 0.3 μm and a resolution of 1024 × 1024 pixels.

Quantification was done by counting the PLA spots within the soma of the neurons using MacBiophotonics ImageJ software (version 1.41n). First, the area of the soma and the integrated fluorescence intensity of the GABA$_{B2}$ signal were determined, and then the PLA spots were counted. PLA signals were normalized to the GABA$_{B2}$ signal and the area of the soma.

RESULTS

GABA$_{B2}$ Interacts with the Proteasomal AAA-ATPase Rpt6—We recently showed that the expression of total and cell surface GABA$_B$ receptors is regulated by proteasomes via the ERAD (12). To identify proteins that might be involved in proteasomal degradation of GABA$_B$ receptors, we screened a brain cDNA library with a sequence comprising the last 12 C-terminal amino acids of GABA$_{B2}$ for interacting proteins using the yeast two-hybrid assay. One of the eight putative GABA$_B$ receptor-interacting proteins detected with this system, the AAA-ATPase Rtp6/Sug1/p45 (hereafter named Rpt6), was related to protein degradation. Rpt6 is a component of the 19S regulatory particle of the proteasome and has been implicated in recruiting proteins to proteasomes for degradation (24–29). We verified the interaction of Rpt6 with native GABA$_B$ receptors by their co-immunoprecipitation from rat brain extracts (Fig. 1*A*) and by *in situ* PLA in cultured neurons (Fig. 1*B*). Moreover, inhibition of proteasomal activity for 30 min with MG132 considerably increased the interaction of Rpt6 with GABA$_{B2}$ (156 ± 19% of control, Fig. 1*B*), suggesting that the receptors are no longer degraded and remained bound to Rpt6. This finding was further corroborated by colocalization studies. Blocking proteasomal activity with MG132 for 30 min resulted in a small increase of GABA$_{B2}$ clusters (120 ± 8% of control, Fig. 1*C*), whereas Rpt6 clusters remained unchanged. However, the co-localization of GABA$_{B2}$ clusters with Rpt6 clusters was considerably increased (193 ± 12% of control, Fig. 1*C*). These results suggest that interaction of Rpt6 with GABA$_{B2}$ mediates proteasomal degradation of GABA$_B$ receptors.

The effect of Rpt6 on GABA$_B$ receptors was analyzed in detail using coexpression experiments in HEK 293 cells. HEK 293 cells overexpressing Rpt6 displayed reduced levels of total (GABA$_{B1}$, 56 ± 3%; GABA$_{B2}$, 49 ± 3% of control; Fig. 2*A*) as well as cell surface GABA$_B$ receptors (GABA$_{B1}$, 38 ± 5%; GABA$_{B2}$, 47 ± 5% of control; Fig. 2*B*), indicating that Rpt6 mediates degradation of GABA$_B$ receptors. Coexpression of Rpt6 with individual GABA$_B$ receptor subunits reduced the expression level of GABA$_{B2}$ (60 ± 4% of control, Fig. 2*C*) but did not affect the level of GABA$_{B1}$ (Fig. 2*D*), demonstrating that Rpt6 specifically interacts with GABA$_{B2}$ to down-regulate GABA$_B$ receptors. This notion was further substantiated by the finding that the expression level of a C-terminal truncated version of GABA$_{B2}$ (GABA$_{B2}$(T749)), which does not contain the Rpt6 interaction site, was not affected by coexpression with Rpt6 (Fig. 2*E*).

Recently, we showed that proteasomal degradation of GABA$_B$ receptors requires Lys^{48}-linked ubiquitination of the

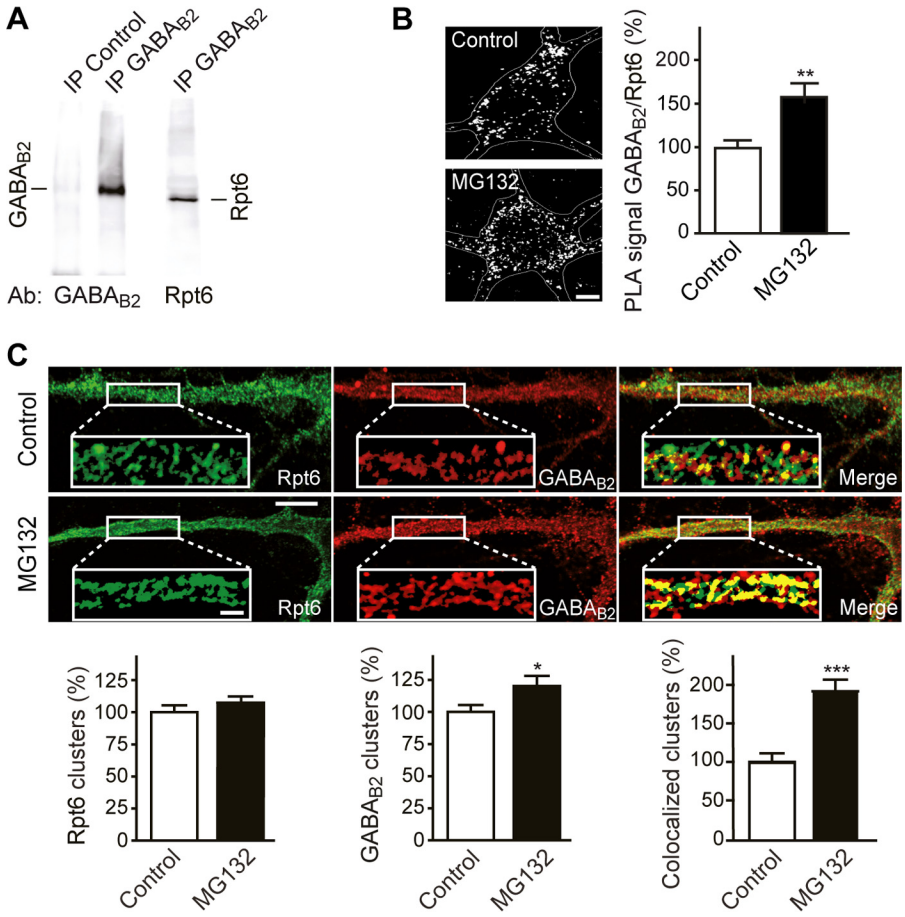

FIGURE 1. **GABA$_B$ receptors interact with the proteasomal AAA-ATPase Rpt6.** *A*, coimmunoprecipitation of GABA$_B$ receptors with Rpt6. GABA$_B$ receptors were immunoprecipitated from 0.5% deoxycholate extracts prepared from rat brain membranes using GABA$_{B2N}$ antibodies coupled to protein A-agarose. The extensively washed immunoprecipitate was subjected to Western blotting for detection of GABA$_{B2}$ and Rpt6. Specificity of the immunoprecipitation was verified by using protein A beads conjugated to non-immune antibodies (control). *IP*, immunoprecipitate; *Ab*, antibody. *B*, enhanced GABA$_{B2}$/Rpt6 interaction after proteasome inhibition detected by *in situ* PLA (*left*, *white dots*). Neurons were incubated for 30 min with the proteasome inhibitor MG132 (10 μM), followed by *in situ* PLA using antibodies directed against GABA$_{B2}$ and Rpt6. The images depict the PLA signals in the soma and proximal dendrites (outlined in *white*). *Right*: quantification of *in situ* PLA signals in the soma (means ± S.E., 30–36 neurons from three experiments). *Scale bar*, 5 μm. **, $p < 0.001$, *t* test. *C*, increased colocalization of GABA$_{B2}$ and Rpt6 after blocking proteasomal activity in proximal dendrites. Neurons were incubated for 30 min with the proteasome inhibitor MG132 (10 μM) and stained with antibodies directed against GABA$_{B2}$ (*red*) and Rpt6 (*green*). *Yellow clusters* in the merged image indicate the colocalization of GABA$_{B2}$ and Rpt6 (*scale bars*, 5 μm (*top*) and 1 μm (*insets*)). *Bottom*: quantification of GABA$_{B2}$ and Rpt6 colocalization after proteasome inhibition (means ± S.E., 20–24 neurons from two experiments). *, $p < 0.05$; ***, $p < 0.0001$; *t* test.

GABA$_{B2}$ C-terminal domain at Lys$^{767/771}$ (12). To analyze whether Rpt6-mediated down-regulation depends on ubiquitination of GABA$_{B2}$, we expressed Rpt6 together with a mutant of GABA$_{B2}$ in which Lys$^{767/771}$ were exchanged for arginines (GABA$_{B2}$(RR)) to prevent ubiquitination at these sites (12). As expected, GABA$_{B2}$(RR) was resistant to Rpt6-mediated down-regulation, verifying that Rpt6 is involved in proteasomal degradation of Lys^{48}-linked ubiquitinated GABA$_B$ receptors (Fig. 2*F*).

Finally, to prove that Rtp6-mediated down-regulation of GABA$_B$ receptors in fact depends on interaction of GABA$_{B2}$ with Rtp6, we used a synthetic peptide (R2C-Pep) comprising the last 14 C-terminal amino acids of GABA$_{B2}$ to disrupt the GABA$_{B2}$/Rpt6 interaction. R2C-Pep inhibited the down-regulation of GABA$_{B2}$ by Rpt6 (108 ± 6% of control, Fig. 3), whereas a control peptide (R2r-Pep, random order of the same amino acids) had no significant effect (80 ± 4% of control, Fig. 3). These findings indicate that proteasomal degradation of GABA$_B$ receptors depends on ubiquitination of GABA$_{B2}$ and is mediated by the interaction of the GABA$_{B2}$ C terminus with Rpt6.

Intact ATPase Activity of Rpt6 Is Required for Proteasomal Degradation of GABA$_B$ Receptors—All six proteasomal AAA-ATPases, including Rpt6, are involved in substrate recognition, unfolding, and translocation of proteins into the barrel-shaped destruction chamber of the 20S proteasome (30). To test whether ATPase activity of Rpt6 is required for degradation of GABA$_B$ receptors in their native environment, we transfected neurons with either EGFP (control), Rtp6, or a mutant of Rtp6 (Rpt6(DN)), which lacks ATPase activity (mutation of Lys^{196} to Met) (18) and tested for total and cell surface expression of GABA$_B$ receptors (Fig. 4). Unlike overexpression of Rtp6 in HEK 293 cells, transfection of neurons with Rtp6 did not reduce total (Fig. 4*A*) or cell surface (Fig. 4*B*) expression of GABA$_B$ receptors. This might be due to a lower level of overexpression in neurons, a saturation of proteasomes with Rpt6 in neurons or to the different cellular environment. However, transfection of neurons with Rpt6(DN) significantly increased both total

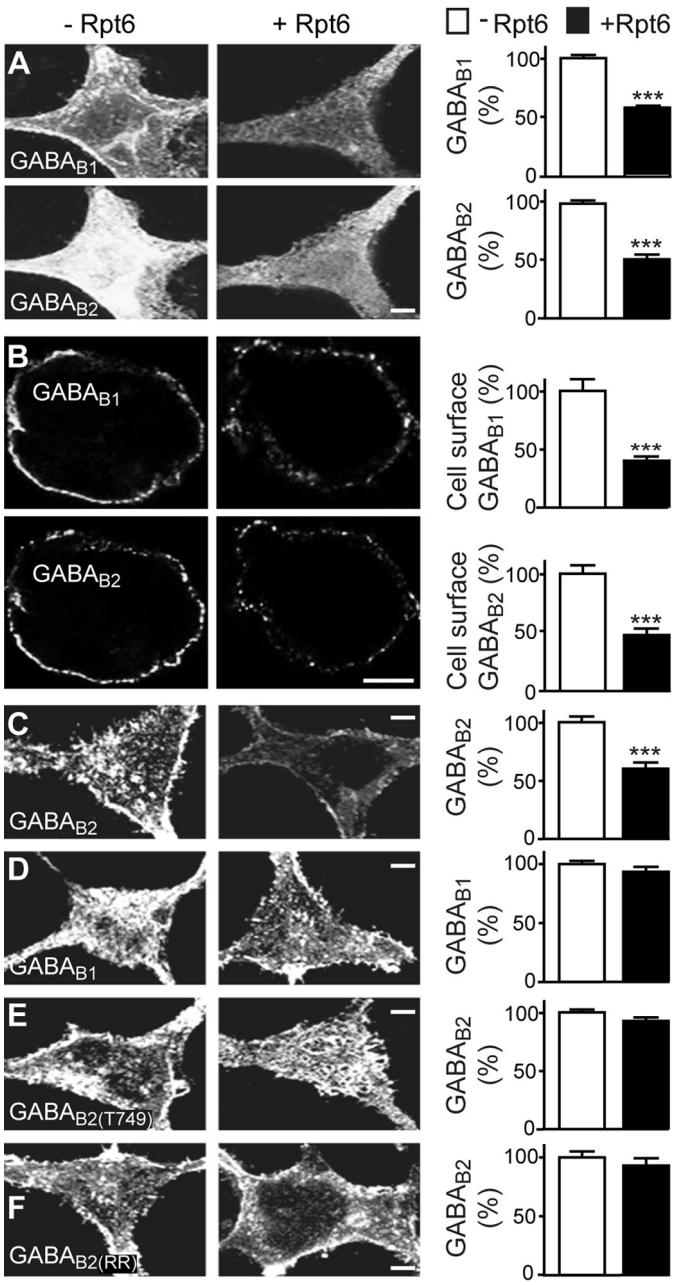

FIGURE 2. **Rpt6 mediates proteasomal degradation via interaction with the C terminus of GABA$_{B2}$.** HEK 293 cells were transfected with plasmids containing GABA$_{B1a}$ and GABA$_{B2}$ (*A* and *B*), GABA$_{B2}$ or GABA$_{B1a}$ alone (*C* and *D*), a truncated version of GABA$_{B2}$ (GABA$_{B2}$T749) lacking the intracellular located C-terminal domain (*E*), or GABA$_{B2}$(RR), a GABA$_{B2}$ mutant with inactivated Lys^{48}-linked ubiquitination sites required for proteasomal degradation (*F*) without (*left panels*, −Rpt6) or with Rpt6 plasmid (*right panels*, +Rpt6). Forty-eight hours after transfection, the cells were stained for the indicated GABA$_B$ receptor subunit (*left*, representative images) and Rpt6 (not shown). *Right panels*: quantification of GABA$_B$ receptor staining levels (means ± S.E., 23–120 cells from 2–4 experiments). ***, $p < 0.0001$; *t* test. *Scale bar*, 10 μm.

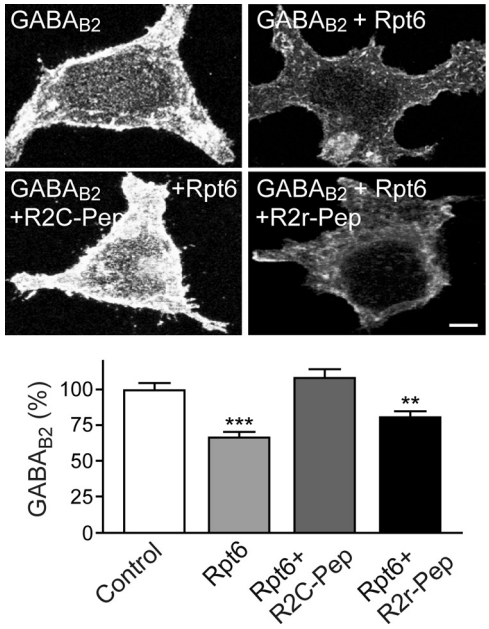

FIGURE 3. **A peptide comprising the last 14 C-terminal amino acids of GABA$_{B2}$ blocked the Rpt6-mediated down-regulation of GABA$_{B2}$.** Twenty-four hours after transfection of HEK 293 cells with plasmids containing GABA$_{B2}$ or GABA$_{B2}$ and Rpt6 (+Rpt6) a peptide comprising the last 14 C-terminal amino acids of GABA$_{B2}$ (R2C-Pep) or a peptide containing the same amino acids but in a random sequence (R2r-Pep) was added. After additional 24 h, the cells were stained for GABA$_{B2}$, Rpt6 (not shown), and the peptide (not shown). *Top*: representative images (*scale bar*, 10 μm). *Bottom*: quantification of total GABA$_{B2}$ staining. GABA$_{B2}$ staining in HEK 293 cells only expressing GABA$_{B2}$ (GABA$_{B2}$) was taken as control (means ± S.E., 90–110 cells from three experiments). **, $p < 0.01$; ***, $p < 0.001$; one way ANOVA, Dunnett's post hoc test.

(150 ± 7% of control, Fig. 4*A*) and cell surface (157 ± 8% of control, Fig. 4*A*) GABA$_B$ receptors. This finding indicates that ATPase activity of Rtp6 is required for constitutive proteasomal degradation of GABA$_B$ receptors.

To test whether the loss of proteasomal degradation of GABA$_B$ receptors in neurons expressing Rpt6(DN) was based on the impaired ATPase activity or on a potential inability of GABA$_{B2}$ to interact with the mutant Rtp6, we transfected neurons with either Rtp6 (control) or Rpt6(DN) and tested for interaction with GABA$_{B2}$ using *in situ* PLA. The number of interactions were similar (statistically not different) in neurons expressing wild type Rtp6 or Rpt6(DN) (Fig. 4*C*), demonstrating that the reduced proteasomal degradation for GABA$_B$ receptors in neurons expressing Rpt6(DN) was caused by the impaired ATPase activity of Rpt6(DN).

Proteins destined for proteasomal degradation are usually tagged with Lys^{48}-linked polyubiquitin. After binding to the proteasome, the protein is deubiquitinated by Rpn11 present in the 19 S regulatory particle, unfolded by the proteasomal AAA-ATPases located at the base of the 19 S regulatory particle and thread into the 20 S proteasome for degradation (31). Because Rpn11 activity is unlikely to be affected in neurons transfected with Rpt6(DN), we expected GABA$_B$ receptors bound to Rpt6(DN) to be deubiquitinated, although they cannot be translocated into the degradation chamber of the 20S proteasome. Using *in situ* PLA, we indeed detected a strongly reduced level of Lys^{48}-linked ubiquitinated GABA$_B$ receptors in neurons expressing Rpt6(DN) (39 ± 3% of control, Fig. 5*A*). In contrast, Lys^{63}-linked ubiquitination of GABA$_B$ receptors, which is not involved in proteasomal degradation, was not affected in Rpt6(DN) expressing neurons (Fig. 5*B*). These experiments indicate that GABA$_B$ receptors bound to Rtp6 are deubiquinated but cannot be degraded if the ATPase activity of Rtp6 is impaired.

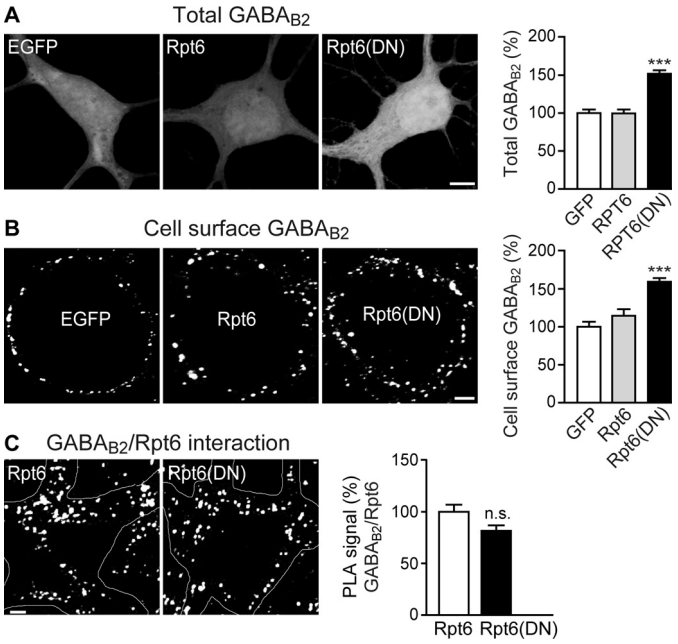

FIGURE 4. **Intact ATPase activity of Rtp6 is required for degradation of GABA$_B$ receptors.** *A* and *B*, inactivation of ATPase activity of Rpt6 increased GABA$_B$ receptor expression levels. Neurons were transfected with either wild type Rpt6 or a non-functional mutant of Rpt6 (Rpt6(DN)) lacking ATPase activity. After 48 h, neurons were tested for total (*A*) and cell surface (*B*) expression of GABA$_{B2}$ by immunofluorescence staining. *Left*: representative images (*scale bar*, 5 μm). *Right*: quantification of immunofluorescence signals (means ± S.E., 30 neurons from three experiments). ***, $p < 0.0001$; one-way ANOVA, Dunnett's post hoc test. *C*, the GABA$_{B2}$/Rpt6 interaction is independent of Rpt6 ATPase activity. Neurons transfected with either wild type Rpt6 or Rpt6(DN) were tested for interaction of GABA$_{B2}$ with Rpt6 by *in situ* PLA. *Left*: representative images depicting PLA signals (*white dots*; *scale bar*, 5 μm). *Right*: quantification of *in situ* PLA signals (means ± S.E., 23 neurons from three preparations). *n.s.*, not significant; $p > 0.05$; *t* test.

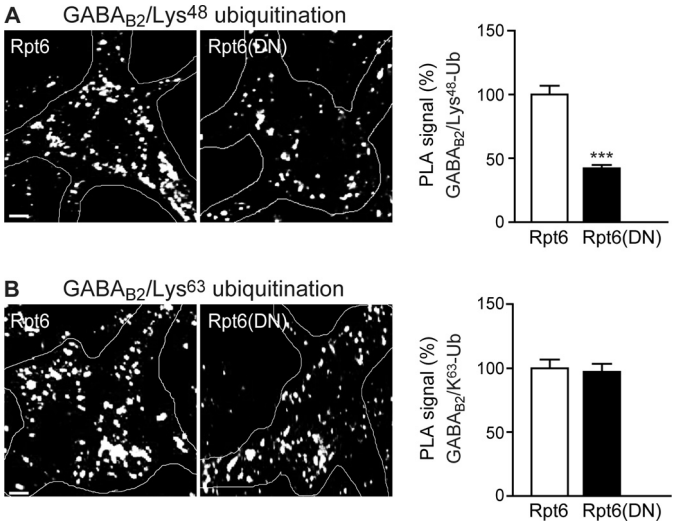

FIGURE 5. **Inactivation of Rtp6 reduced the fraction of Lys^{48}-linked ubiquitinated GABA$_B$ receptors.** Neurons were transfected with either wild type Rpt6 or a non-functional mutant of Rpt6 (Rpt6(DN)) lacking ATPase activity. After 48 h, neurons were fixed, permeabilized, and tested for Lys^{48} (*A*) and Lys^{63}-linked ubiquitination (*Ub*; *B*) of GABA$_B$ receptors using *in situ* PLA. *Left*: representative images depicting the PLA signals (*white dots*; *scale bar*, 5 μm). *Right*: quantification of *in situ* PLA signals (means ± S.E., 30 (*A*) and 24 (*B*) neurons from three preparations). ***, $p < 0.0001$; *t* test.

Interaction of GABA$_{B2}$ with Rtp6 Is Reduced upon Blocking ERAD—We previously showed that GABA$_B$ receptors are constitutively degraded by proteasomes via the ERAD machinery

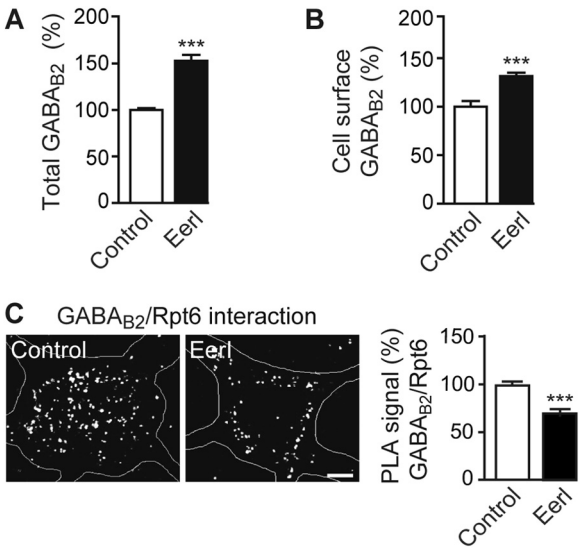

FIGURE 6. **Blocking ERAD reduced GABA$_{B2}$/Rpt6 interaction and increased GABA$_B$ receptor expression.** *A* and *B*, blocking the ERAD pathway increased the level of GABA$_B$ receptors. Neurons grown in 96-well plates were incubated for 12 h with the ERAD inhibitor eeyarestatin I (*Eerl*, 5 μM). Total (*A*) and cell surface (*B*) GABA$_B$ receptor levels were determined using the whole cell ELISA and GABA$_{B2}$ antibodies. *Control*, cultures not treated with eeyarestatin I (means ± S.E., 30 cultures from three preparations). ***, $p < 0.0001$; *t* test. *C*, diminished GABA$_{B2}$/Rpt6 interaction upon inhibition of ERAD. Neurons were incubated for 12 h with the ERAD inhibitor eeyarestatin I (*Eerl*, 5 μM) and analyzed for interaction of GABA$_{B2}$ with Rpt6 using *in situ* PLA. *Left*: representative images depicting PLA signals (*white dots*; *scale bar*, 5 μm). *Right*: quantification of *in situ* PLA signals (means ± S.E., 28 neurons from three preparations). ***, $p < 0.0001$; *t* test.

(12). Blocking ERAD activity using the p97 inhibitor eeyarestatin I, which inhibits translocation of proteins from the ER membrane to the cytoplasm for proteasomal degradation (32, 33), increased both total (152 ± 6% of control, Fig. 6*A*) as well as cell surface GABA$_B$ receptors (129 ± 6% of control, Fig. 6*B*), confirming our previous findings. Because eeyarestatin I targets p97, preventing ER exit of ubiquitinated proteins and their interaction with the proteasomes located in the cytoplasm, we expected a diminished interaction of GABA$_B$ receptors with Rpt6. We employed *in situ* PLA to test whether the increase in GABA$_B$ receptors upon blocking p97 is associated with a decreased interaction of GABA$_{B2}$ with Rtp6. Consistent with the essential role of Rtp6 for proteasomal degradation of GABA$_B$ receptors inhibition of p97 with eeyarestatin I significantly reduced the GABA$_{B2}$/Rpt6 interaction (71 ± 4% of control, Fig. 6*C*).

Neuronal Activity Modulates GABA$_B$ Receptor Expression and GABA$_{B2}$/Rpt6 Interaction—Proteasomal activity has been reported to be regulated by the level of neuronal activity (34). Therefore, we tested whether changes in neuronal activity affects the amount of GABA$_B$ receptor. We pharmacologically manipulated the activity of cultured neurons and determined their GABA$_B$ receptor protein levels using GABA$_{B2}$ antibodies and whole cell ELISA. Treatment of neurons for 12 h with CNQX/D-AP5 to block excitatory synaptic transmission by inhibiting AMPA and NMDA receptors considerably increased total and cell surface GABA$_{B2}$ levels (total, 129 ± 4%; cell surface, 135 ± 4% of control; Fig. 7, *A* and *B*). In contrast, chronically blocking GABA$_A$ receptor activity with picrotoxin to elevate neuronal activity decreased total as well as cell surface

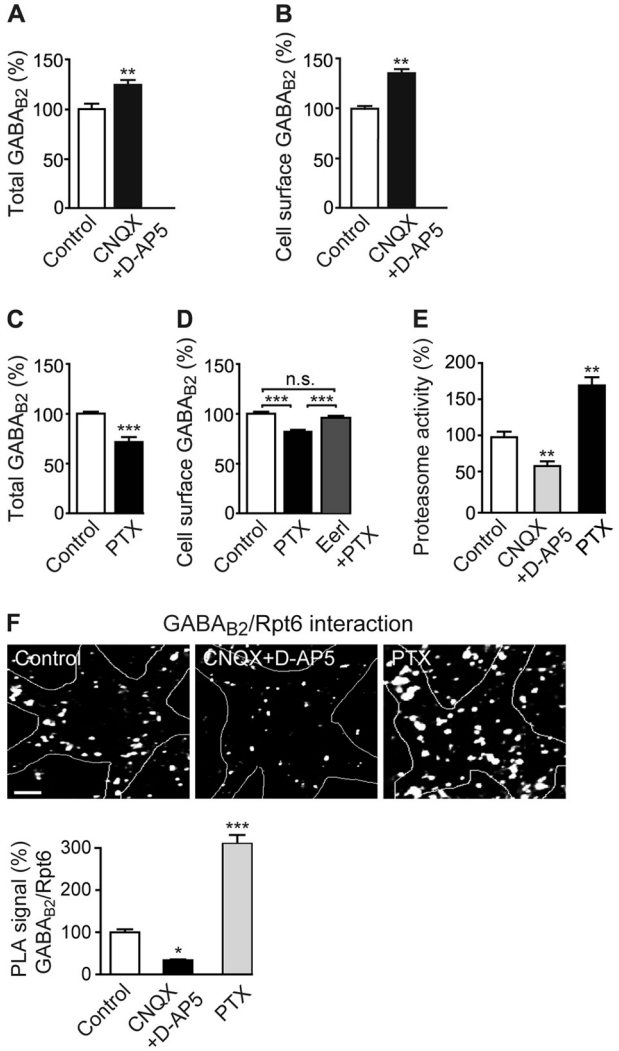

FIGURE 7. **The expression level of GABA$_B$ receptors and interaction with Rtp6 is controlled by neuronal activity.** *A–D*, chronic changes in neuronal activity modulate GABA$_B$ receptor expression. Neurons grown in 96-well plates were incubated for 12 h with 10 μM CNQX/20 μM D-AP5 (*A* and *B*) or with 20 μM picrotoxin (*PTX*; *C* and *D*) in the absence or presence of the ERAD inhibitor eeyarestatin I (*EerI*, 5 μM) followed by determination of total (*A* and *C*) and cell surface (*B* and *D*) GABA$_{B2}$ levels using the whole cell ELISA. Shown are means ± S.E. of 20–47 cultures from two to four experiments. *A–C*, **, $p < 0.01$; ***, $p < 0.0001$; *t* test; *D*, ***, $p < 0.0001$; *n.s.*, not significant ($p > 0.05$); one-way ANOVA, Bonferroni's multiple comparison post hoc test. *E*, chronic changes in neuronal activity modulate proteasome activity. Proteasome activity was determined in neurons grown in 96-well plates incubated for 12 h with 10 μM CNQX/20 μM D-AP5 or with 20 μM picrotoxin (*PTX*) using the Proteasome-Glo cell-based assay. Shown are means ± S.E. of 12 cultures from four preparations. **, $p < 0.01$, one-way ANOVA, Dunnett's post hoc test. *F*, chronic changes in neuronal activity modulate interaction of GABA$_{B2}$ with Rpt6. Neurons were incubated for 12 h with 10 μM CNQX/20 μM D-AP5 or with 20 μM picrotoxin followed by determination of the GABA$_{B2}$/Rpt6 interaction levels using *in situ* PLA. *Top*, representative images depicting the PLA signals (*white dots*; *scale bar*, 5 μm). *Bottom*, quantification of *in situ* PLA signals. Shown are means ± S.E. of 37 neurons from two experiments. *, $p < 0.05$; ***, $p < 0.0001$; one-way ANOVA, Dunnett's post hoc test.

GABA$_{B2}$ levels (total, 72 ± 7%; cell surface, 83 ± 1% of control; Fig. 7, *C* and *D*). The decrease in cell surface GABA$_{B2}$ was prevented by cotreatment with the ERAD inhibitor eeyarestatin I (97 ± 1% of control, Fig. 7*D*). This finding suggests that neuronal activity regulates GABA$_B$ receptor expression levels via modulating proteasomal activity associated with the ERAD machinery. Under our test conditions, blocking AMPA and NMDA receptors reduced proteasomal activity to 62 ± 6% of control neurons as determined with a luminogenic proteasome substrate (Fig. 7*E*). By contrast, blocking GABA$_A$ receptors with picrotoxin increased protasomal activity to 169 ± 11% of control neurons (Fig. 7*E*). These findings suggest that changes in neuronal excitation controls the expression level of GABA$_B$ receptors via proteasomal degradation.

If neuronal activity indeed regulates total and cell surface GABA$_B$ receptor expression via proteasomal degradation the interaction of GABA$_{B2}$ with Rtp6 should be concomitantly regulated. Therefore, we expected a reduced level of GABA$_B$ receptor/Rtp6 interaction after treating neuronal cultures for 12 h with CNQX plus D-AP5, conditions that lead to diminished proteasome activity. Indeed, blocking glutamate receptors considerably reduced the level of GABA$_{B2}$/Rpt6 interaction (39 ± 4% of control, Fig. 7*F*). In contrast, enhancing neuronal activity by blocking GABA$_A$ receptors, which enhanced proteasome activity, increased the number of GABA$_{B2}$/Rpt6 interactions (302 ± 22% of control, Fig. 7*F*). These findings suggest that neuronal activity modulates cell surface expression of GABA$_B$ receptors via changes in proteasomal activity, which is reflected by changes in the level of GABA$_{B2}$/Rpt6 interaction.

DISCUSSION

GABA$_B$ receptors are degraded by two distinct pathways. Cell surface GABA$_B$ receptors are constitutively internalized via the dynamin and clathrin-dependent pathway, recycled to the plasma membrane, and are eventually sorted to lysosomes for degradation (35). Newly synthetized and assembled GABA$_B$ receptors in the ER are constitutively degraded to a certain extent by proteasomes via the ERAD machinery (12). This mechanism controls the pool of new GABA$_B$ receptors destined for forward trafficking to the plasma membrane. In the present study, we show that the proteasomal AAA-ATPase Rpt6 interacts with the C terminus of GABA$_{B2}$, thereby mediating proteasomal degradation of GABA$_B$ receptors. Rpt6 is one of the six AAA-ATPases of the 19S regulatory particle of the proteasome (36). The six distinct AAA-ATPases located at the base of the 19S regulatory particle of the 26S proteasome recognize, unfold, and translocate the protein substrates into the 20S, protein-degrading core particle (31, 37). Initially, a yeast two-hybrid screening with a peptide comprising the last 12 C-terminal amino acids of GABA$_{B2}$ suggested that Rpt6 may interact with GABA$_{B2}$. Co-immunoprecipitation from rat brain extracts, colocalization in cultured neurons, and testing the association in neurons using *in situ* PLA verified the interaction of Rpt6 with GABA$_B$ receptors. This was further substantiated by experiments inhibiting proteasome activity, which increased the interaction and colocalization of Rpt6 with GABA$_B$ receptors. Finally, the interaction of Rpt6 with the C terminus of GABA$_{B2}$ was confirmed by peptide competition.

Experiments in HEK 293 cells support the view that Rpt6 is essential for proteasomal degradation of GABA$_B$ receptors. It had been previously reported that overexpression of Rpt6 in HEK 293 cells increase proteasome activity 3-fold, most likely due to a regulatory role of free Rpt6 on proteasome assembly and thereby activity (38). In line with this report, overexpression of Rpt6 in HEK 293 cells down-regulated total and cell

surface $GABA_B$ receptors. Down-regulation depended on the Rpt6/$GABA_{B2}$ interaction as well as on the Lys^{48}-linked ubiquitination of $Lys^{767/771}$ in $GABA_{B2}$. Because $Lys^{767/771}$ ubiquitination in $GABA_{B2}$ is a prerequisite for proteasomal degradation of $GABA_B$ receptors (12), these findings underline that the interaction of $GABA_{B2}$ with Rtp6 is required for proteasomal degradation of the receptors.

Although Rpt6 does not interact with $GABA_{B1}$, as indicated by the lack of its down-regulation when expressed in HEK 293 cells in the absence of $GABA_{B2}$, $GABA_{B1}$ and $GABA_{B2}$ were concomitantly reduced to the same extent. This strongly suggests that assembled $GABA_B$ receptor complexes are degraded by proteasomes and is in line with our previous observation that heterodimeric receptor complexes are degraded by the ERAD machinery and not single $GABA_B$ receptor subunits before being assembled in the ER (12). Our finding that blocking ERAD function increased total and cell surface $GABA_B$ expression and reduced the level of $GABA_{B2}$/Rpt6 interaction verifies that proteasomal degradation of $GABA_B$ receptors takes place at the ER.

Our data further indicate that proteasomal degradation of $GABA_B$ receptors in neurons require intact ATPase activity of Rpt6 because overexpression in neurons of a mutant Rpt6 lacking ATPase activity led to an increase of total as well as cell surface $GABA_B$ receptors. This finding provides further evidence that $GABA_B$ receptors are constitutively degraded to a certain extent by proteasomes (12). Lack of ATPase activity did not affect the interaction of Rpt6 with $GABA_{B2}$, suggesting that recruiting $GABA_B$ receptors to proteasomes is independent of Rpt6's ATPase activity.

Proteins targeted for proteasomal degradation are deubiquitinated before being degraded by the 20S proteasome. Lys^{48}-linked polyubiquitin of substrate proteins is bound by Rpn10 and released by the deubiquitinase activity of Rpn11 present in the 19S regulatory particle (31). Overexpression of mutant Rpt6 in neurons considerably reduced the fraction of Lys^{48}-linked ubiquitinated $GABA_{B2}$, although the receptors were not degraded and remained bound to Rpt6. This suggests that deubiquitination of $GABA_{B2}$ is independent of its degradation and takes place before or concomitant with its translocation into the degradation chamber of the 20S proteasome.

There is accumulating evidence that neuronal activity regulates proteasome-dependent protein degradation and, intriguingly, proteasome activity. It has recently been demonstrated that blockade of neuronal activity decrease proteasomal activity whereas enhancing neuronal activity increase proteasomal activity (34, 39, 40). The mechanism of enhancing proteasomal activity involves Ca^{2+} influx via NMDA receptors as well as L-type voltage-gated Ca^{2+} channels (34). This leads to the activation of CaMKII, which phosphorylates Rpt6 on Ser^{120} (34, 40, 41) to roughly double proteasomal activity (40, 41). Conversely, reduced neuronal activity decreases phosphorylation of Rpt6 (40), resulting in diminished proteasomal activity (34, 39). In view of this data, it was not surprising that blocking neuronal activity reduced the level of $GABA_{B2}$/Rpt6 interaction and up-regulated total as well as cell surface $GABA_B$ receptors. However, increasing neuronal activity by blocking $GABA_A$ receptors, which enhances proteasomal activity, increased the level $GABA_{B2}$/Rpt6 interaction, and consequently decreased the expression levels of $GABA_B$ receptors. This was reversed by pharmacologically blocking ERAD demonstrating that proteasomal degradation affects the pool of newly synthetized $GABA_B$ receptors present in the ER. These findings are in line with the view that the level of neuronal activity regulates proteasomal activity and thereby the pool of $GABA_B$ receptors available in the ER for trafficking to the plasma membrane.

In conclusion, our data support the hypothesis that cell surface trafficking of $GABA_B$ receptors is controlled by neuronal activity at the level of the ER by defining the amount of receptors present in the ER via regulated proteasomal degradation. This mechanism is expected to contribute to homeostatic synaptic plasticity.

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REFERENCES

1. Turrigiano, G. G. (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* **135,** 422–435
2. Cajigas, I. J., Will, T., and Schuman, E. M. (2010) Protein homeostasis and synaptic plasticity. *EMBO J.* **29,** 2746–2752
3. Hegde, A. N. (2010) The ubiquitin-proteasome pathway and synaptic plasticity. *Learn. Mem.* **17,** 314–327
4. Mabb, A. M., and Ehlers, M. D. (2010) Ubiquitination in postsynaptic function and plasticity. *Annu. Rev. Cell Dev. Biol.* **26,** 179–210
5. Ehlers, M. D. (2003) Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat. Neurosci.* **6,** 231–242
6. Fu, A. K., Hung, K. W., Fu, W. Y., Shen, C., Chen, Y., Xia, J., Lai, K. O., and Ip, N. Y. (2011) APC^{Cdh1} mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. *Nat. Neurosci.* **14,** 181–189
7. Kato, A., Rouach, N., Nicoll, R. A., and Bredt, D. S. (2005) Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 5600–5605
8. Vembar, S. S., and Brodsky, J. L. (2008) One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* **9,** 944–957
9. Saliba, R. S., Michels, G., Jacob, T. C., Pangalos, M. N., and Moss, S. J. (2007) Activity-dependent ubiquitination of $GABA_A$ receptors regulates their accumulation at synaptic sites. *J. Neurosci.* **27,** 13341–13351
10. Saliba, R. S., Gu, Z., Yan, Z., and Moss, S. J. (2009) Blocking L-type voltage-gated Ca^{2+} channels with dihydropyridines reduces γ-aminobutyric acid type A receptor expression and synaptic inhibition. *J. Biol. Chem.* **284,** 32544–32550
11. Bettler, B., Kaupmann, K., Mosbacher, J., and Gassmann, M. (2004) Molecular structure and physiological functions of $GABA_B$ receptors. *Physiol. Rev.* **84,** 835–867
12. Zemoura, K., Schenkel, M., Acuña, M. A., Yévenes, G. E., Zeilhofer, H. U., and Benke, D. (2013) Endoplasmic reticulum-associated degradation (ERAD) controls cell surface expression of γ-aminobutyric acid, type B receptors. *J. Biol. Chem.* **288,** 34897–34905
13. Grampp, T., Notz, V., Broll, I., Fischer, N., and Benke, D. (2008) Constitutive, agonist-accelerated, recycling and lysosomal degradation of $GABA_B$ receptors in cortical neurons. *Mol. Cell Neurosci.* **39,** 628–637
14. Maier, P. J., Marin, I., Grampp, T., Sommer, A., and Benke, D. (2010) Sustained glutamate receptor activation down-regulates $GABA_B$ receptors by shifting the balance from recycling to lysosomal degradation. *J. Biol. Chem.* **285,** 35606–35614

15. Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., and Bettler, B. (1997) Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. *Nature* **386,** 239–246
16. Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., and Bettler, B. (1998) $GABA_B$ receptor subtypes assemble into functional heteromeric complexes. *Nature* **396,** 683–687
17. Pagano, A., Rovelli, G., Mosbacher, J., Lohmann, T., Duthey, B., Stauffer, D., Ristig, D., Schuler, V., Meigel, I., Lampert, C., Stein, T., Prezeau, L., Blahos, J., Pin, J., Froestl, W., Kuhn, R., Heid, J., Kaupmann, K., and Bettler, B. (2001) C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of $GABA_B$ receptors. *J. Neurosci.* **21,** 1189–1202
18. Kumar, Y., Radha, V., and Swarup, G. (2010) Interaction with Sug1 enables Ipaf ubiquitination leading to caspase 8 activation and cell death. *Biochem. J.* **427,** 91–104
19. Schneider, S., Georgiev, O., Buchert, M., Adams, M. T., Moelling, K., and Hovens, C. M. (1997) An epitope tagged mammalian/prokaryotic expression vector with positive selection of cloned inserts. *Gene* **197,** 337–341
20. Takeuchi, T., Kosuge, M., Tadokoro, A., Sugiura, Y., Nishi, M., Kawata, M., Sakai, N., Matile, S., and Futaki, S. (2006) Direct and rapid cytosolic delivery using cell-penetrating peptides mediated by pyrenebutyrate. *ACS Chem. Biol.* **1,** 299–303
21. Buerli, T., Pellegrino, C., Baer, K., Lardi-Studler, B., Chudotvorova, I., Fritschy, J. M., Medina, I., and Fuhrer, C. (2007) Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nat. Protoc.* **2,** 3090–3101
22. Leuchowius, K. J., Jarvius, M., Wickström, M., Rickardson, L., Landegren, U., Larsson, R., Söderberg, O., Fryknäs, M., and Jarvius, J. (2010) High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell Proteomics* **9,** 178–183
23. Söderberg, O., Gullberg, M., Jarvius, M., Ridderstråle, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Methods* **3,** 995–1000
24. Su, K., Yang, X., Roos, M. D., Paterson, A. J., and Kudlow, J. E. (2000) Human Sug1/p45 is involved in the proteasome-dependent degradation of Sp1. *Biochem. J.* **348,** 281–289
25. Masuyama, H., and MacDonald, P. N. (1998) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. *J. Cell Biochem.* **71,** 429–440
26. Giannì, M., Bauer, A., Garattini, E., Chambon, P., and Rochette-Egly, C. (2002) Phosphorylation by p38MAPK and recruitment of SUG-1 are required for RA-induced RAR gamma degradation and transactivation. *EMBO J.* **21,** 3760–3769
27. Inoue, T., Kon, T., Ajima, R., Ohkura, R., Tani, M., Yokota, J., and Sutoh, K. (2006) MYO18B interacts with the proteasomal subunit Sug1 and is degraded by the ubiquitin-proteasome pathway. *Biochem. Biophys. Res. Commun.* **342,** 829–834
28. Wang, H., Jia, N., Fei, E., Wang, Z., Liu, C., Zhang, T., Fan, J., Wu, M., Chen, L., Nukina, N., Zhou, J., and Wang, G. (2007) p45, an ATPase subunit of the 19S proteasome, targets the polyglutamine disease protein ataxin-3 to the proteasome. *J. Neurochem.* **101,** 1651–1661
29. Rezvani, K., Baalman, K., Teng, Y., Mee, M. P., Dawson, S. P., Wang, H., De Biasi, M., and Mayer, R. J. (2012) Proteasomal degradation of the metabotropic glutamate receptor 1α is mediated by Homer-3 via the proteasomal S8 ATPase. *J. Neurochem.* **122,** 24–37
30. Sledź, P., Förster, F., and Baumeister, W. (2013) Allosteric effects in the regulation of 26S proteasome activities. *J. Mol. Biol.* **425,** 1415–1423
31. Sorokin, A. V., Kim, E. R., and Ovchinnikov, L. P. (2009) Proteasome system of protein degradation and processing. *Biochemistry* **74,** 1411–1442
32. Fiebiger, E., Hirsch, C., Vyas, J. M., Gordon, E., Ploegh, H. L., and Tortorella, D. (2004) Dissection of the dislocation pathway for type I membrane proteins with a new small molecule inhibitor, eeyarestatin. *Mol. Biol. Cell* **15,** 1635–1646
33. Wang, Q., Li, L., and Ye, Y. (2008) Inhibition of p97-dependent protein degradation by Eeyarestatin I. *J. Biol. Chem.* **283,** 7445–7454
34. Djakovic, S. N., Schwarz, L. A., Barylko, B., DeMartino, G. N., and Patrick, G. N. (2009) Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **284,** 26655–26665
35. Benke, D. (2010) Mechanisms of $GABA_B$ receptor exocytosis, endocytosis, and degradation. *Adv. Pharmacol.* **58,** 93–111
36. Rubin, D. M., Coux, O., Wefes, I., Hengartner, C., Young, R. A., Goldberg, A. L., and Finley, D. (1996) Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. *Nature* **379,** 655–657
37. Smith, D. M., Benaroudj, N., and Goldberg, A. (2006) Proteasomes and their associated ATPases: a destructive combination. *J. Struct. Biol.* **156,** 72–83
38. Wang, Y., Nie, J., Wang, Y., Zhang, L., Lu, K., Xing, G., Xie, P., He, F., and Zhang, L. (2012) CKIP-1 couples Smurf1 ubiquitin ligase with Rpt6 subunit of proteasome to promote substrate degradation. *EMBO Rep.* **13,** 1004–1011
39. Jakawich, S. K., Neely, R. M., Djakovic, S. N., Patrick, G. N., and Sutton, M. A. (2010) An essential postsynaptic role for the ubiquitin proteasome system in slow homeostatic synaptic plasticity in cultured hippocampal neurons. *Neuroscience* **171,** 1016–1031
40. Djakovic, S. N., Marquez-Lona, E. M., Jakawich, S. K., Wright, R., Chu, C., Sutton, M. A., and Patrick, G. N. (2012) Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. *J. Neurosci.* **32,** 5126–5131
41. Bingol, B., Wang, C. F., Arnott, D., Cheng, D., Peng, J., and Sheng, M. (2010) Autophosphorylated CaMKIIalpha acts as a scaffold to recruit proteasomes to dendritic spines. *Cell* **140,** 567–578