The Availability of Surface GABA_B Receptors Is Independent of γ-Aminobutyric Acid but Controlled by Glutamate in Central Neurons

The efficacy of synaptic transmission depends on the availability of ionotropic and metabotropic neurotransmitter receptors at the plasma membrane, but the contribution of the endocytic and recycling pathways in the regulation of γ-aminobutyric acid type B (GABA_B) receptors remains controversial. To understand the mechanisms that regulate the abundance of GABA_B receptors, we have studied their turnover combining surface biotin labeling and a microscopic immunoendocytosis assay in hippocampal and cortical neurons. We report that internalization of GABA_B receptors is agonist-independent. We also demonstrate that receptors endocytose in the cell body and dendrites but not in axons. Additionally, we show that GABA_B receptors endocytose as heterodimers via clathrin- and dynamin-1-dependent mechanisms and that they recycle to the plasma membrane after endocytosis. More importantly, we show that glutamate decreases the levels of cell surface receptors in a manner dependent on an intact proteasome pathway. These observations indicate that glutamate and not GABA controls the abundance of surface GABA_B receptors in central neurons, consistent with their enrichment at glutamatergic synapses.

The efficacy of synaptic transmission depends on the mechanisms of intracellular trafficking, which modify the availability of neurotransmitter receptors (1). At the molecular level, the trafficking of glutamate receptors and ionotropic GABA receptors has been extensively studied (2, 3). For example, differential trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors modifies synaptic strength and influences experience-dependent plasticity in vivo (4). However, the molecular mechanisms that govern the trafficking of metabotropic GABA_B receptors (GABA_BRs) remain less clear. GABA_BRs mediate the slow component of synaptic inhibition by acting on pre- and postsynaptic targets (5, 6). They have been implicated in epilepsy, anxiety, stress, sleep disorders, nociception, depression, and cognition (7). They also represent attractive targets for the treatment of withdrawal symptoms from drugs of addiction such as cocaine (8). GABA_BRs are heteromers composed of two subunits, namely GABA_B1R and GABA_B2R. GABA_B1R contains an endoplasmic reticulum retention motif in the intracellular C-terminal domain (9, 10). The endoplasmic reticulum retention sequence is masked upon assembly with GABA_B2R, which results in the appearance of the functional receptor at the plasma membrane. GABA_B1R contains the ligand-binding site, whereas G protein signaling is exclusively mediated by GABA_B2R (11). GABA_BRs are located in dendrites and axons, but it has been difficult to establish what determines their pre- versus postsynaptic localization. Recent evidence suggests that the extracellular domains of the GABA_B1R and -1b isoforms, which differ in two sushi domains (12), may specify axonal/dendritic targeting (13). Unexpectedly, postsynaptic GABA_BRs are enriched at glutamatergic synapses, frequently adjacent to the postsynaptic density in the CA1 region of the hippocampus and Purkinje cells of the cerebellum, thus constituting perisynaptic receptors (14–16). They are also found adjacent to presynaptic release sites of glutamatergic and gabaergic synapses (15).

The existence of a classical endocytic pathway for GABA_BRs has been the cause of a recent controversy. Whereas most reports agree that receptors do not internalize in response to agonist (17–21), at least two studies suggest the existence of agonist-induced internalization (22, 23). Likewise, although several studies argue that constitutive receptor endocytosis occurs via clathrin-depend-ent mechanisms (20–23), others suggest the existence a nonclassical mechanism (18, 19). These discrepancies are likely to originate from the different experimental systems utilized, the different methodology, and the varying times of observation.

Here we characterize the turnover of GABA_BRs in cultured cortical and hippocampal neurons combining surface biotin labeling and a microscopic immunoendocytosis assay. We
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report that internalization of GABA<sub>B</sub>Rs is agonist-independent and that the mechanism depends on clathrin and dynamin-1. We also demonstrate that receptors endocytose as heterodimers in the cell body and dendrites but not in axons, and that they recycle to the plasma membrane. More importantly, we show that membrane availability is regulated by glutamate in a proteasome-dependent manner. These results support the notion that GABA<sub>B</sub>R function is intimately linked to glutamatergic neurotransmission in the brain (15).

EXPERIMENTAL PROCEDURES

Reagents—Glutamate, baclofen, GABA, saclofen, and MG132 were purchased from Sigma.

Plasmids—The constructs containing MYC-GABA<sub>B</sub>R1, HA-GABA<sub>B</sub>R2, and FLAG-GABA<sub>B</sub>R2 in pRK5 have been described previously and contain their epitope tags on the extracellular N-terminal domains (9, 17). EGFP-wt-dynamin-1, EGFP-K44A-dynamin-1, EGFP-wt-dynamin-2, and EGFP-K44A-dynamin-2 were kindly provided by M. McNiven. EGFP-wt-Rab11 was kindly provided by F. Bronfman.

Antibodies—GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 antibodies have been described previously (24). Anti-MYC antibodies were purchased from Sigma. HA antibodies were purchased from Roche Applied Science. The secondary anti-mouse and anti-rabbit antibodies conjugated to Texas Red (TR), fluorescein isothiocyanate (FITC), cyanine (Cy5), and horseradish peroxidase were purchased from Jackson ImmunoResearch (West Grove, PA).

Animals—Adult pregnant female Sprague-Dawley rats were purchased from the Central Animal Facility at Universidad Católica de Chile and killed by asphyxia in a CO2 chamber according to the Guide for Care and Use of Laboratory Animals.

Neuronal Cultures and Transfection—Primary cultures of cortical and hippocampal neurons were obtained from E18 rats and transfected by calcium phosphate as reported earlier (25, 26).

Biotinylation—Labeling of surface proteins for steady-state and internalization assays were performed as reported previously in 5 div cortical neurons (19).

Antibody Feeding—14–21 div rat hippocampal neurons grown on poly-l-lysine-coated coverslips were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2. Live cells were washed twice with phosphate-buffered saline and incubated for 15 min in media containing MYC and/or HA antibodies. Neurons were left untreated or stimulated with 100 μM baclofen or 100 μM GABA for 60 min. Control neurons were kept at 4 °C. Treated cells were returned to the 37 °C incubator for 60 min. Cells were then fixed and processed for immunofluorescence. The surface GABA<sub>B</sub>R1 and/or GABA<sub>B</sub>R2 pools were detected with MYC, HA, and secondary antibodies prior to permeabilization. The internalized receptor pool was detected using secondary antibodies after permeabilization. For experiments using low K<sup>+</sup>, cells were incubated in hypotonic medium (Dulbecco’s modified Eagle’s medium diluted 1:1 with water) for 5 min at 37 °C, followed by isotonic KCl-free medium for 30 min at 37 °C prior to immunoenodcetosis. Control treatments were carried out in isotonic KCl medium. Images were acquired with a Zeiss LSM-5 Pascal 5 Axiovert 200 confocal microscope and a Plan-Apochromat 63x/1.4 Oil differential interference contrast objective, using the LSM 5 3.2 image capture and analysis software. Raw confocal images were deconvolved by Huygens Scripting software (Scientific Volume Imaging, Hilversum, Netherlands). Quantification of internalization was performed using the MetaMorph software. The amount of internalized receptor was expressed as the percent of the internalized intensity (internalized intensity of one channel excluding the surface intensity of both channels) relative to the total intensity (internalized intensity of one channel plus the surface intensity of both channels).

RESULTS

GABA<sub>B</sub>Rs Endocytose in Neurons—We used microscopy and biochemistry to determine whether GABA<sub>B</sub>R endocytosis occurs in cultured hippocampal and cortical neurons, two model systems widely used to study neurotransmitter receptor trafficking (27), and which express abundant GABA<sub>B</sub>Rs (19, 28). GABA<sub>B</sub>Rs were transfected into hippocampal neurons, and the fate of surface receptors was subsequently followed by immunoendocytosis. Abundant GABA<sub>B</sub>R1 concentrated at the plasma membrane after transfection, and a significant proportion accumulated in intracellular compartments after 60 min of endocytosis (Fig. 1, A and B, arrows). The accumulation occurred in vesicle-like structures that located throughout the cell body and often converged to a perinuclear compartment. Stimulation with baclofen, a GABA<sub>B</sub> agonist, or GABA for 60 min failed to increase the intracellular buildup of GABA<sub>B</sub>R1 (Fig. 1, C and D, arrows, and E).

To corroborate these observations, we carried out experiments of surface biotinylation and internalization for endogenous GABA<sub>B</sub>Rs in cultured cortical neurons. As expected GABA<sub>B</sub>R1 was efficiently detected at the cell surface, and a significant fraction accumulated in intracellular stores under basal conditions (Fig. 1F, lanes 1–3). However, the intracellular accumulation of GABA<sub>B</sub>R1 was not stimulated by baclofen or GABA, in agreement with our microscopic observations (Fig. 1F, lanes 4 and 5). A quantitative analysis of multiple experiments confirmed these observations (Fig. 1G). Saclofen, a competitive antagonist for GABA<sub>B</sub>Rs, had no effect on the levels of internalized receptors (Fig. 1H). Consistent with these results the steady-state abundance of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 at the cell surface was not significantly modified by baclofen or GABA (supplemental Fig. 1, A and B). Taken together these results indicate that a proportion of GABA<sub>B</sub> receptors undergo agonist-independent endocytosis in neurons.

GABA<sub>B</sub>Rs Endocytose in the Cell Body and Dendrites but Not in Axons—To determine whether GABA<sub>B</sub>R endocytosis differs in pre- and postsynaptic compartments, we carried out the same immunoendocytosis analysis in dendrites and axons. GABA<sub>B</sub>R1 was expressed abundantly at the cell surface of dendrites (Fig. 2A). More importantly, under basal conditions a significant proportion of GABA<sub>B</sub>R1 endocytosed constitutively in vesicle-like structures that accumulated along the dendritic shaft (Fig. 2, A and B, arrows). GABA<sub>B</sub>R1 was also abundant at the surface of the axon, but no intracellular accumulation was visible throughout the length of the axonal shaft, as indicated by the absence of exclusively red vesicle-like structures (Fig. 2, D and E, arrows). This was not because of the resolution limit of
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FIGURE 1. GABAB<sub>R</sub>Rs endocytosis in neurons. A–D, 14 div hippocampal neurons were transfected with MYC-GABAB<sub>R1</sub>A and HA-GABAB<sub>R2</sub> and processed for immunendocytosis. Live cells were incubated with MYC antibodies for 15 min, washed, and left nonstimulated (A and B) or treated with 100 μM baclofen (C) or 100 μM GABA (D). Neurons were kept at 4 °C (A) or returned to the 37 °C incubator for 60 min (B–D). Cells were then processed for immunofluorescence. The surface receptor pool was detected with MYC- and FITC-conjugated secondary antibodies prior to permeabilization (red channel). The internalized receptor pool was detected using TR-conjugated secondary antibodies after permeabilization (green channel). The merged images are shown on the right. Scale bar in D (5 μm) applies to A–D. E, multiple images were quantified, and the amount of receptor internalization was expressed as the % of the internalized intensity relative to the total intensity (each bar in the plot represents the average value ± S.E. of 11–16 individual neurons). NS, not significant; p < 0.05. F, 5 div rat cortical neurons were incubated with 1 mg/ml biotin for 15 min and washed. Control dishes were kept at 4 °C (surface and cleaved; Cleav, lanes 1 and 2), and remaining dishes were incubated at 37 °C for 60 min (lanes 3–5) in the absence (nonstimulated, Non-s, lane 3) or presence of 100 μM baclofen (Baco, lane 4) or 100 μM GABA (lane 5). The surface biotin remaining after the incubation periods was left uncleaved (lane 1) or removed by cleaving with glutathione (lanes 2–5). Cells were lysed, precipitated with Sepharose-Neutravidin beads, and samples separated by SDS-PAGE. ImmunobLOTS of biotin-associated proteins were performed with GABAB<sub>R1</sub> antibodies (top panel). Total GABAB<sub>R1</sub> in the lysate was also detected (bottom panel). ImmunobLOTS were visualized by chemiluminescence. WB, Western blot. G, multiple experiments were analyzed by densitometry (each bar in the plot represents the average value ± S.E. of 4–5 independent experiments). H, amount of GABAB<sub>R1</sub> internalized in the presence of 1 μM saclofen was compared with a nonstimulated condition (each bar in the plot represents the average value ± S.E. of four independent experiments).

our technique as dendrites of small diameter also showed clear punctate accumulation of endocytosed GABA<sub>R</sub>R1 (Fig. 2C, arrow).

GABA<sub>R</sub>Rs Endocytosis as Dimers—GABA<sub>R</sub>Rs are heteromeric at the plasma membrane (29), but the fate of the heteromer during endocytosis has received little attention. Thus, to determine whether GABA<sub>R</sub>R1 and GABA<sub>R</sub>R2 subunits are endocytosed as monomers or heteromers, we simultaneously labeled GABA<sub>R</sub>R1 and GABA<sub>R</sub>R2 prior to internalization. Abundant GABA<sub>R</sub>R1 accumulated at the cell surface indicating that heteromeric receptors effectively reached the plasma membrane (Fig. 3A, left panel). Both GABA<sub>R</sub>R1 and GABA<sub>R</sub>R2 endocytosed efficiently after double labeling (Fig. 3A, central panels). Interestingly, the great majority of endocytosed GABA<sub>R</sub>R1 and GABA<sub>R</sub>R2 subunits co-localized in round vesicle-like structures in the cell body (Fig. 3A, right panel, arrow). Identical observations were obtained in dendrites (Fig. 3B, arrow). As predicted, when GABA<sub>R</sub>R1 and GABA<sub>R</sub>R2 were detected at the somatic or dendritic cell surface, they showed a high degree of co-localization (Fig. 3C, arrows). These results clearly indicate that the dimeric structure of functional GABA<sub>R</sub>Rs is maintained during endocytosis.

GABA<sub>R</sub>R Endocytosis Is Clathrin- and Dynamin-dependent—Next we determined whether endocytosis occurred via classical clathrin- and dynamin-dependent mechanisms. First, neurons were exposed to low K<sup>+</sup>, an established treatment that inhibits clathrin-dependent endocytosis (30). Under control conditions GABA<sub>R</sub>Rs endocytosed constitutively, and a significant accumulation of internalized receptors was observed after 60 min (Fig. 4A, top panels). Importantly, GABA<sub>R</sub>R endocytosis was strongly inhibited by low K<sup>+</sup> (Fig. 4A, bottom panels, and B). Interestingly, occasional intracellular structures were still visible near the cell surface (Fig. 4A, bottom panels, arrows). A similar inhibition was obtained with hypertonic sucrose, another treatment that inhibits clathrin-depend-
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FIGURE 2. GABA<sub>B</sub>Rs endocytosis in dendrites but not in axons. A–E, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for constitutive immunoeendocytosis. Live cells were incubated with MYC antibodies for 15 min and kept at 4 °C or returned to the 37 °C incubator for 60 min. The surface GABA<sub>B</sub>R1 pool was detected with MYC- and FITC-conjugated secondary antibodies prior to permeabilization (green channel). The internalized GABA<sub>B</sub>R1 pool was detected using TR-conjugated secondary antibodies after permeabilization (red channel). Merged images are shown on the right. Surface and internalized GABA<sub>B</sub>R1 pools were detected in large diameter dendrites (A and B) small diameter dendrites (C) and axons (D and E). MAP2 was used to discriminate between axons and dendrites (not shown). Scale bar in B (5 µm) applies to A and B. Scale bar in E (5 µm) applies to C–E.

FIGURE 3. GABA<sub>B</sub>Rs endocytosis as dimers. A and B, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for constitutive immunoeendocytosis using MYC and HA antibodies simultaneously. The surface GABA<sub>B</sub>R1 pool was detected with MYC and Cy5 secondary antibodies prior to permeabilization (blue channel). The internalized GABA<sub>B</sub>R1 pool was detected using FITC-conjugated secondary antibodies after permeabilization (green channel). The internalized GABA<sub>B</sub>R2 pool was detected using TR-conjugated secondary antibodies after permeabilization (red channel). Merged images are shown on the right. A, cell body; B, dendrite. C, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for immunofluorescence using MYC and HA antibodies in the absence of permeabilization to detect surface receptors. A representative cell body (square panels) and dendrite (right panels) are shown. Scale bar in C soma (5 µm) applies to somas in A and C. Scale bar in C dendrites (3 µm) applies to dendrites in B and C.
However, we were not able to detect a significant increase in the accumulation of intracellular GABA$_\beta$Rs after glutamate treatment even in the presence of lysosomal inhibitors (not shown). These results suggest that glutamate controls the surface levels of GABA$_\beta$Rs and that it stimulates their degradation through a lysosome-independent mechanism.

To determine the fate of cell surface GABA$_\beta$Rs after glutamate treatment, neurons were incubated with MG132, an established inhibitor of the proteasome pathway. Incubation with MG132 alone did not change the steady-state cell surface levels of GABA$_\beta$Rs (supplemental Fig. 2). However, MG132 completely blocked the glutamate-mediated disappearance of GABA$_\beta$Rs (Fig. 8, A–D). These results indicate that glutamate stimulates the degradation of GABA$_\beta$Rs via the proteasome. Importantly, MG132 also caused a significant reduction of the endocytosed pool of GABA$_\beta$Rs (Fig. 8D). These observations indicate that the agonist-independent internalization and the glutamate-mediated disappearance of receptors are blocked by MG132, suggesting that both pathways share an initial step of internalization from the plasma membrane.

**DISCUSSION**

In this study we contribute to clarifying the controversy concerning GABA$_\beta$R endocytosis in central neurons. We show that heteromeric GABA$_\beta$Rs internalize basally, in an agonist-independent fashion, and that endocytosis is dependent on clathrin and dynamin-1. Our findings also establish the fate of internalized receptors by showing that after endocytosis GABA$_\beta$Rs recycle back to the plasma membrane. Importantly, they demonstrate that glutamate significantly alters the steady-state levels of GABA$_\beta$Rs by causing them to disappear from the plasma membrane. Finally, they demonstrate that there is a strong dependence between disappearance of surface receptors and proteasome activity. In view of the enrichment of GABA$_\beta$Rs at glutamatergic synapses, this constitutes a relevant mechanism for controlling the availability of GABA$_\beta$Rs in neurons.

**Agonist-dependent or -independent Endocytosis of GABA$_\beta$Rs in Neurons**—Several studies have addressed the endocytosis of GABA$_\beta$Rs (17–23). Two reports have shown that GABA$_\beta$R internalization is agonist-independent in neurons (18, 19), and at least one suggests the existence of agonist-induced internalization (23). The discrepancy between these findings may originate in the different systems used. Although the studies by Perroy et al. (18) and Fairfax et al. (19) center on cerebellar, cortical, and hippocampal neurons, the work by Laffray et al. (23) used a co-culture model of dorsal root ganglion and spinal cord. However, because GABA$_\beta$Rs display a unique molecular composition throughout the nervous system, this difference is unlikely to explain the discrepancy. Thus, alternative explanations need to be considered. These include trace amounts of GABA in the media and quantification procedures. Our results strongly support an agonist-independent internalization of GABA$_\beta$Rs in neurons taking into account their physiological context (cortical and hippocampal neurons), no free GABA in the internalization solutions, and an appropriate quantification methodology (biotinylation). Furthermore, they indicate that the GABA$_\beta$R antagonist saclofen has no effect on constitutive endocytosis corroborating that internalization is not the result of receptor activation by trace levels of GABA. Our study confirms that the pool of GABA$_\beta$Rs that accumulates in intracellular compartments in neurons is small compared with cell lines (19, 21).

**GABA$_\beta$R Endocytosis Is Clathrin- and Dynamin-1-dependent in Neurons**—The clathrin dependence shown here is in agreement with other findings in neurons and the role of dynamin-1 expands previous findings regarding the generic participation of dynamins in GABA$_\beta$R endocytosis in cell lines (21, 23, 34). Although dynamin-1 is preferentially presynaptic, it has been found in pre- and postsynaptic sites (35). Our studies do not uncover a preferential function of dynamin-1 on pre- or postsynaptic GABA$_\beta$Rs, and more experiments are needed to address this issue, which may have significant physiological implications (13).

**Recycling**—We have shown the recycling of GABA$_\beta$Rs to the plasma membrane after accumulation in Rab11 positive endosomes. Recycling in neurons has been indirectly examined before using pharmacological blockers (23). As the authors correctly indicate, monensin inhibits the recycling of internalized proteins back to the plasma membrane. However, monensin is an ion-selective ionophore that neutralizes the acidification of compartments and may have notorious effects on the Golgi apparatus, endosomes, and lysosomes (36). To avoid these unrelated effects, we directly demonstrated the reinsertion of GABA$_\beta$Rs at the plasma membrane of hippocampal neurons. Unexpectedly, our data disagree with findings in HEK-293 cells (21). At present we cannot explain this discrepancy, but the turnover of GABA$_\beta$Rs is likely to contain additional steps and checkpoints in neurons that have been uncovered in our analyses (34).
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**FIGURE 5.** GABA<sub>B</sub> endocytosis is dynamin-1-dependent. A and B, neurons were transfected with MYC-GABA<sub>B</sub>R1a, HA-GABA<sub>B</sub>R2, and EGFP-WT-dynamin-1 or EGFP-K44A-dynamin-1 and processed for constitutive immunofluorescence. Live cells were incubated with MYC antibodies for 15 min and kept at 4 °C or returned to the 37 °C incubator for 60 min. Cells were then processed for immunofluorescence. The surface GABA<sub>B</sub>R1 pool was detected with MYC- and Cy5-conjugated secondary antibodies prior to permeabilization (blue channel). The internalized GABA<sub>B</sub>R1 pool was detected using TR-conjugated secondary antibodies after permeabilization (red channel). Merged images are shown on the right. Cell bodies are shown on top panels, and dendrites are shown below. Scale bars, soma, 5 μm; dendrite, 3 μm. B–D, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for immunofluorescence. Live cells were incubated with MYC antibodies for 15 min, washed, and returned to the 37 °C incubator for 90 min (B). After endocytosis receptors remaining at the plasma membrane were removed by acid-stripping (C). We returned neurons for a second cycle to the 37 °C incubator for 60 min to allow the reappearance of surface receptors after acid stripping (D). After treatment cells were fixed and processed for immunofluorescence. The internalized receptor pool was detected using TR-conjugated secondary antibodies after permeabilization (red channel). The surface receptor pool was detected with MYC- and FITC-conjugated secondary antibodies prior to permeabilization (green channel). Merged images are shown on the right. Scale bar in D (5 μm) applies to B–D.

**FIGURE 6.** GABA<sub>B</sub>Rs recycle to the plasma membrane from Rab11 positive endosomes. A, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for immunofluorescence using MYC antibodies. The surface GABA<sub>B</sub>R1 pool was detected with MYC and Cy5-conjugated secondary antibodies prior to permeabilization (blue channel). Merged images are shown on the right. Cell bodies are shown on top panels, and dendrites are shown below. Scale bars, soma, 5 μm; dendrite, 3 μm. B–D, 14 div hippocampal neurons were transfected with MYC-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R2 and processed for immunofluorescence. Live cells were incubated with MYC antibodies for 15 min, washed, and returned to the 37 °C incubator for 90 min (B). After endocytosis receptors remaining at the plasma membrane were removed by acid-stripping (C). We returned neurons for a second cycle to the 37 °C incubator for 60 min to allow the reappearance of surface receptors after acid stripping (D). After treatment cells were fixed and processed for immunofluorescence. The internalized receptor pool was detected using TR-conjugated secondary antibodies after permeabilization (red channel). The surface receptor pool was detected with MYC- and FITC-conjugated secondary antibodies prior to permeabilization (green channel). Merged images are shown on the right. Scale bar in D (5 μm) applies to B–D.

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**Heteromeric Endocytosis in Dendrites and Axons**—We did not detect internalization of GABA<sub>B</sub>Rs in axons despite the fact that receptors were abundantly expressed at the plasma membrane. The existence of a molecular machinery that regulates endocytosis for G protein-coupled receptors in the axon has been established. For example, mGluR5 is efficiently endocytosed in the axon of cultured hippocampal neurons (37). Therefore, we suggest that the axonal shaft is endocytosis-incompetent relative to GABA<sub>B</sub>Rs or that the rate/number of internalized receptors is significantly reduced compared with dendrites. Internalization in axon terminals remains to be explored. However, our data imply that if receptors are inter-
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FIGURE 7. Surface availability is decreased by glutamate. A, 5 div rat cortical neurons were left untreated (control) or exposed to 20 μM glutamate for 30 min. Neurons were then labeled with 1 mg/ml biotin. Cells were lysed, and biotinylated proteins were precipitated with Sepharose-Neutravidin beads and samples separated by SDS-PAGE. Immunoblots of surface-associated and total proteins were performed with GABA<sub>B</sub>R1 antibodies. B, immunoblots for surface GABA<sub>B</sub>R1 were analyzed by densitometry, and average values ± S.E. were plotted for each condition (each bar in the plot represents the average value ± S.E. of three independent experiments; *, p < 0.05). C and D, same as in A for GABA<sub>B</sub>R2. D, immunoblots for surface GABA<sub>B</sub>R2 were analyzed by densitometry, and average values ± S.E. were plotted for each condition (each bar in the plot represents the average value ± S.E. of four independent experiments; **, p < 0.005).

FIGURE 8. Glutamate-dependent loss of cell surface GABA<sub>B</sub>Rs is proteasome-dependent. A, 5 div rat cortical neurons were incubated with 1 mg/ml biotin and returned to 37 °C incubator in the absence or presence of 10, 20, or 200 μM MG132. B, immunoblots for surface GABA<sub>B</sub>R1 were analyzed by densitometry, and average values ± S.E. were plotted for each condition (control, Ctrl; glutamate, Glut). Each bar in the plot represents the average value ± S.E. of four independent experiments. Glutamate versus control; ***, p < 0.001; glutamate + MG132 versus control, not significant (NS), p = 0.225. C and D, same as above for GABA<sub>B</sub>R2 (each bar in the plot represents the average value ± S.E. of three independent experiments). Glutamate versus control; *, p < 0.05; glutamate + MG132 versus control, not significant (NS), p = 0.17). E, 5 div rat cortical neurons were incubated with 1 mg/ml biotin and returned to 37 °C incubator in the absence or presence of 10, 20, or 200 μM MG132 for 30 min. Biotin remaining after the incubation periods was removed by cleaving with glutathione. Cells were lysed, precipitated with Sepharose-Neutravidin beads, and samples separated by SDS-PAGE. Immunoblots of surface-associated proteins were performed with GABA<sub>B</sub>R1 antibodies (not shown). Total proteins were detected with GABA<sub>B</sub>R1 antibodies or tubulin antibodies (not shown).

The inhibition caused by MG132 raises the possibility that endocytosis of GABA<sub>B</sub>Rs is mediated by ubiquitination. This interpretation of our results is supported by the fact that MG132 depletes free ubiquitin thus preventing endocytosis of GABA<sub>B</sub>Rs from the plasma membrane.
ubiquitinated proteins (39). However, the exact mechanism behind the loss of cell surface receptors after glutamate exposure remains to be explored. We were unable to detect a buildup of intracellular GABA$_B$R$_2$s after glutamate treatment, even in the presence of lysosomal or proteasomal inhibitors, but the glutamate-mediated disappearance of steady-state cell surface GABA$_B$R$_2$s was strongly inhibited by the proteasomal inhibitor MG132. Taken together our results suggest that GABA$_B$R$_2$s endocytose basally and that glutamate causes a rapid redirection of the endocytosed pool to the proteasome for degradation.

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