Sustained Glutamate Receptor Activation Downregulates GABA\textsubscript{B} Receptors by Shifting the Balance from Recycling to Lysosomal Degradation

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Metabotropic GABA\textsubscript{B} receptors are abundantly expressed at glutamatergic synapses where they control excitability of the synapse. Here we tested the hypothesis that glutamatergic neurotransmission may feed back to regulate GABA\textsubscript{B} receptors. We found that application of glutamate to cultured cortical neurons led to rapid downregulation of GABA\textsubscript{B} receptors via lysosomal degradation. This effect was mimicked by selective activation of AMPA receptors and further accelerated by co-activation of group I metabotropic glutamate receptors. Inhibition of NMDA receptors, blockade of L-type Ca\textsuperscript{2+} channels and removal of extracellular Ca\textsuperscript{2+} prevented glutamate-induced downregulation of GABA\textsubscript{B} receptors, indicating that Ca\textsuperscript{2+}-influx plays a critical role. We further established that glutamate-induced downregulation depends on the internalization of GABA\textsubscript{B} receptors. Glutamate did not affect the rate of GABA\textsubscript{B} receptor endocytosis but led to reduced recycling of the receptors back to the plasma membrane. Blockade of lysosomal activity rescued receptor recycling, indicating that glutamate redirects GABA\textsubscript{B} receptors from the recycling to the degradation pathway. In conclusion, the data indicate that sustained activation of AMPA receptors downregulates GABA\textsubscript{B} receptors by sorting endocytosed GABA\textsubscript{B} receptors preferentially to lysosomes for degradation on expense of recycling. This mechanism may relieve glutamatergic synapses from GABA\textsubscript{B} receptor-mediated inhibition resulting in increased synaptic excitability.

In the mature mammalian nervous system excitatory neurotransmission mediated by glutamate is balanced by the inhibitory actions of GABA. Slow, prolonged inhibitory neurotransmission is largely mediated by metabotropic GABA\textsubscript{B} receptors, which are widely expressed in the central nervous system and play an important role in many neurological disorders such as epilepsy, anxiety, depression, addiction and chronic pain (for a review, see Ref. (1)). Functional GABA\textsubscript{B} receptors are heterodimers composed of the two subunits GABA\textsubscript{B1} and GABA\textsubscript{B2} and expressed in brain as two receptors subtypes (GABA\textsubscript{B(1a,2)} and GABA\textsubscript{B(1b,2)}) based on alternative promoter usage of GABA\textsubscript{B1}. GABA\textsubscript{B} receptors are either localized at postsynaptic sites where they induce slow inhibitory postsynaptic potentials by activating K\textsuperscript{+} channels or at presynaptic sites where they suppress neurotransmitter release by inhibiting Ca\textsuperscript{2+} channels (for a review, see Ref. (1)).

GABA\textsubscript{B} receptors are not only localized at GABAergic synapses but are also abundantly expressed at glutamatergic synapses (2-5). At these synapses they are activated by GABA spillover derived from sustained GABAergic neurotransmission (6) and control glutamatergic activity at pre- and postsynaptic sites (7-11). There is accumulating evidence that sustained activation of glutamate receptors in turn may affect GABA\textsubscript{B} receptors. Treatment of cultured cortical neurons with glutamate resulted in the loss of cell surface GABA\textsubscript{B} receptors (12) and NMDA-induced excitotoxicity in hippocampal slices led to increased GABA\textsubscript{B1} and decreased GABA\textsubscript{B2} expression levels (13). In addition, it was reported that glutamate slows the rate of GABA\textsubscript{B} receptor endocytosis in cultured hippocampal neurons (14). These findings suggest that sustained glutamatergic activity may regulate the availability of GABA\textsubscript{B} receptors, which in turn is expected to
affect excitability of glutamatergic synapses. However, the mechanisms involved in this putative regulation of GABA\textsubscript{B} receptors by glutamate remain to be established.

Previously, we had shown that endocytosed GABA\textsubscript{B} receptors are predominantly recycled back to the plasma membrane and are eventually degraded in lysosomes (15). Recycling and degradation of GABA\textsubscript{B} receptors needs to be precisely balanced to maintain the required number of receptors at the cell surface. On the other hand, shifting the balance towards degradation would be a powerful mechanism to downregulate GABA\textsubscript{B} receptors. Here we tested the hypothesis that sustained activation of glutamate receptors might affect GABA\textsubscript{B} receptor levels by regulating their rate of recycling and degradation. We found that treating cultured cortical neurons with glutamate resulted in a rapid downregulation of GABA\textsubscript{B} receptors. The glutamate-induced downregulation was triggered by sustained activation of AMPA receptors, depended on Ca\textsuperscript{2+} influx through NMDA receptors as well as L-type Ca\textsuperscript{2+} channels and finally resulted in reduced constitutive recycling and increased lysosomal degradation of GABA\textsubscript{B} receptors.

**EXPERIMENTAL PROCEDURES**

**Antibodies** – The following primary antibodies were used: rabbit GABA\textsubscript{B1a,b} directed against the C-terminus of GABA\textsubscript{B1} (affinity-purified, 1:500 for In Cell Western assay, immunofluorescence and Western blotting; for specificity see (16); custom-made by GenScript), guinea pig GABA\textsubscript{B2} (1:1000 for immunofluorescence and 1:2000 for Western blotting, Chemicon International), rabbit GABA\textsubscript{B2,F(ab')\textsubscript{2}} directed against the N-terminus of GABA\textsubscript{B2} (1:50 for internalization and recycling assays; for specificity see (17); custom-made by GenScript), mouse actin (1:1000 for In Cell Western assay and 1:80,000 for Western blotting, Chemicon International), rabbit GABA\textsubscript{A} receptor directed against the N-terminus of the \(\alpha1\) subunit (affinity purified, 1:100 for In Cell Western assay (18)), mouse PSD95 (1:500 for immunofluorescence, Affinity Bioreagents), rabbit PSD95 (1:2000 for immunofluorescence), guinea pig vGlut1 (1:10,000 for immunofluorescence, Chemicon International), rabbit vGlut1 (1:10,000 for immunofluorescence, Synaptic Systems).

Secondary antibodies were coupled either to Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch), IRDye\textsuperscript{®}680 (1:800, LI-COR Biosciences), IRDye\textsuperscript{®}800CW (1:200, LI-COR Biosciences) or horseradish peroxidase (1:5000, Jackson ImmunoResearch).

**Drugs** – \(\omega\)-Agatoxin TK (0.5 \(\mu\)M, Tocris Bioscience), AMPA (100 \(\mu\)M, Tocris Bioscience), bafilomycin A1 (0.5 \(\mu\)M, Sigma-Aldrich), chloroquine (100 \(\mu\)M, Sigma-Aldrich), CNQX (20 \(\mu\)M, Tocris Bioscience), \(\omega\)-conotoxin GVIA (3 \(\mu\)M, Sigma-Aldrich), \(\omega\)-conotoxin MVIIC (2 \(\mu\)M, Tocris Bioscience), D-AP5 (50 \(\mu\)M, Tocris Bioscience), DCG IV (100 \(\mu\)M, Tocris Bioscience), dynasore (100 \(\mu\)M, Sigma-Aldrich), glycine (10 \(\mu\)M, Sigma-Aldrich), glutamate (50 \(\mu\)M, Sigma-Aldrich), L-AP4 (100 \(\mu\)M, Tocris Bioscience), leupeptin (100 \(\mu\)M, Sigma-Aldrich), MG 132 (10 \(\mu\)M, Sigma-Aldrich), monensin (50 \(\mu\)M, Sigma-Aldrich), nifedipine (100 \(\mu\)M, Tocris Bioscience), NASPM (10-250 \(\mu\)M, Sigma-Aldrich), NMDA (100 \(\mu\)M, Tocris Bioscience), tADA (200 \(\mu\)M, Sigma-Aldrich), UBEI-45 (50 \(\mu\)M, Biogenova).

**Cell Culture** – Primary neuronal cultures of cerebral cortex were prepared from E18 embryos of time-pregnant Wistar rats as described previously (15). Neurons were plated to a density of about 30,000 cells onto poly-L lysine coated 96-well plates or 60,000 cells onto poly-L lysine coated coverslips, respectively. Neurons were kept in culture at 37 °C and 5% CO\textsubscript{2} for 11 to 21 days. The vast majority of experiments were performed with cultures kept for 11-16 days in vitro and a few experiments were done with older cultures (up to 21 days in culture). Since the results derived from the older cultures were similar to those obtained from younger cultures the data were pooled.

**Western Blotting** – Western blot experiments using GABA\textsubscript{B1a,b} and GABA\textsubscript{B2} antibodies were performed with cortical neurons grown on 6 cm culture dishes (about 2.5 \(\times\) 10\textsuperscript{6} cells per dish) as described previously (15).

**Immunocytochemistry and Confocal Laser Scanning Microscopy** – Double labeling immunocytochemistry on cortical neurons cultured on coverslips was performed as described previously (15,19). Incubation with primary antibodies diluted to the appropriate concentration
in PBS containing 10% normal goat serum was done for 1 h at room temperature. After embedding in fluorescence mounting medium (DakoCyto) neurons were analyzed by confocal laser scanning microscopy (LSM510 Meta; Zeiss) using a 100x plan apochromat oil differential interference contrast objective (1.4 NA). Images were acquired at a resolution of 1024 x 1024 pixels in the sequential mode. For each neuron 5 optical sections spaced by 0.38 µm were taken. Images were processed using Imaris (version 4.2.0 and 5.7.2, Bitplane, Zurich, Switzerland).

For colocalization studies on dendrites of cortical neurons, raw confocal images were smoothed using the Edge Preserving Filter (filter width 0.636 µm) and then further processed by setting threshold cutoffs for each image individually to only visualize significant signals above background. Individual thresholds cutoffs accounted for differences in background staining among distinct neurons and dendrites. A colocalization channel was built (colocalization intensity 255, constant value) and GABA\text{B}\text{a} receptor and synaptic marker protein clusters (>15 pixels) as well as their colocalization were counted within 10 µm of the dendrite beginning at a distance of 10 µm from the soma. In addition, the width of the dendrite was measured to calculate the area analyzed.

Quantification of fluorescence intensities was done using the Image J software (http://www.macbiophotonics.ca/imagej/index.htm). For the analysis of GABA\text{B}\text{a} receptor expression the soma excluding the nucleus of stained neurons was carefully outlined and the integrated density of the fluorescence signals measured. For quantification of cell surface GABA\text{B}\text{a} receptors the integrated density of the fluorescence signals derived from the surface of living stained neurons was determined. Background fluorescence was determined for each image and subtracted. In Cell Western Assay – Cortical neurons grown in 96-well plates were rapidly cooled to 4°C, washed twice with ice-cold PBS and fixed with 4% paraformaldehyde (PFA) for 10 min. Subsequently, cells were permeabilized four times for 5 min with 0.1% Triton-X-100 (Sigma-Aldrich) in PBS and then incubated with 5% non-fat dry milk in PBS for 2 h at room temperature to block nonspecific antibody binding sites. Cultures were then incubated for 24-48 h simultaneously with rabbit GABA\text{B}\text{a},b and mouse actin antibodies (in PBS, 5% non-fat dry milk). Non-specific GABA\text{B}\text{a},b antibody binding was assessed in parallel cultures by competition using the peptide-antigen (10 µg/ml). Subsequently, neurons were extensively washed with 0.1% Tween 20 (Sigma-Aldrich) in PBS and incubated with the appropriate secondary antibodies (IRDye680 goat anti-mouse; IRDye800CW donkey anti-rabbit) in PBS containing 5% non-fat dry milk for 60 min at room temperature protected from light. After washing the neurons with 0.1% Tween 20 in PBS and once with PBS the buffer was completely removed and the fluorescence was measured with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Data evaluation: Integrated fluorescence intensities for non-specific GABA\text{B}\text{a},b signals (as assessed by competition with the peptide antigen) were determined in multiple wells, averaged and subtracted from GABA\text{B}\text{a},b signals. Obtained specific GABA\text{B}\text{a},b signals were then normalized to the actin signal determined in parallel to account for potential cell loss during washing steps or unequal plating of neurons.

Internalization Assay – Cortical neurons grown in 96-well plates were rapidly cooled to 4°C and washed with ice-cold buffer A (25 mM HEPES, pH 7.4, 119 mM NaCl, 5 mM KCl, 2 mM CaCl\text{2}, 2 mM MgCl\text{2}, 30 mM glucose). Cell surface GABA\text{B}\text{a} receptors were then labeled by incubation with GABA\text{B}\text{a},F(ab')\text{2} antibodies in buffer A containing 10% normal donkey serum for 60 min at 4°C. After extensive washing with buffer A, the neurons were incubated for 1.5 - 15 min at 37°C to permit internalization of the receptors in the absence or presence of glutamate (50 µM). Control cultures for determining the total cell surface GABA\text{B}\text{a} receptors were left at 4°C, a condition that is non-permissive for internalization. After washing with ice-cold buffer A, the cultures were incubated with IRDye800CW donkey anti-
rabbit secondary antibody in buffer A for 90 min at 4°C. To account for potential cell loss during washing steps or unequal plating of neurons, cultures were incubated in parallel with the cell-permeant nuclear stain DRAQ5 (1:1000, Biostatus Limited). After extensive washing, the buffer was completely removed and the fluorescence of dry cultures was measured with the Odyssey Infrared Imaging System. Non-specific GABA\(_B\)\(_2\)-F(ab')\(_2\) antibody signals were determined by competition with the peptide-antigen (10 µg/ml) in multiple wells. DRAQ5 signals were used for normalization. For data evaluation see In Cell Western.

**Recycling Assay** – Cell surface GABA\(_B\) receptors of cortical neurons grown in 96-well plates were labeled with GABA\(_B\)\(_2\)-F(ab')\(_2\) antibodies as described above for the internalization assay. For internalization of labeled receptors, cultures were incubated for 15 min at 37°C. Thereafter, the neurons were incubated for 60 min at 4°C with a secondary antibody (Alexa Fluor 488-conjugated, 1:50, Invitrogen) that is not detected by the infrared imaging system and is used to mask primary antibodies that labeled remaining cell surface GABA\(_B\) receptors. After washing, the neurons were again incubated for 30 min at 37°C to permit internalized antibody-labeled receptors to recycle back to the cell surface. Recycled receptors were then detected at the cell surface using IRDye800CW donkey anti-rabbit secondary antibodies and cultures were processed further as described above for the internalization assay.

**RESULTS**

*Sustained Activation of Glutamate Receptors Triggers Lysosomal Degradation of GABA\(_B\) Receptors* - To investigate whether modulation of neuronal activity regulates degradation of GABA\(_B\) receptors, cultured cortical neurons grown in 96-well plates were incubated for 90 min with agonists and antagonists of the main neurotransmitter systems and were analyzed for changes in GABA\(_B\) receptor expression using the In Cell Western assay. Among the drugs tested (not shown), only glutamate and AMPA led to a significant reduction of GABA\(_B\) receptor signals in cultured neurons with glutamate being more effective than AMPA (90 min glutamate: 46 ± 14%, AMPA: 64 ± 13% of control; Fig. 1A).

Immunofluorescence microscopy showed that levels of GABA\(_B_{1a}\) and GABA\(_B_{1b}\) subunits were downregulated to a similar extent by glutamate (30 min glutamate, GABA\(_B_{1a}\): 57 ± 20%, GABA\(_B_{1b}\): 53 ± 17% of control, n=33-48 neurons; Fig. 1B). Furthermore, Western blot experiments demonstrated that GABA\(_B_{1a}\) and GABA\(_B_{1b}\) receptor subtypes were equally affected by glutamate (90 min glutamate, GABA\(_B_{1a}\): 42 ± 6%, GABA\(_B_{1b}\): 43 ± 12% of control, mean ± SD, n=5-6 cultures, Fig. 1E). The glutamate-induced downregulation of GABA\(_B\) receptors was not due to reduced viability of neurons upon drug exposure since no significant cell death was observed within the time frame of the experiment (control: 0.4 ± 0.6 %, 90 min glutamate: 0.9 ± 0.9 %, mean ± SD, n=22 cultures; trypan blue dye exclusion assay). Glutamate-induced downregulation of GABA\(_B\) receptors was found to be dose-dependent with 5 µM glutamate triggering the half-maximal effect (Fig. 1C). Glutamate did not affect expression of GABA\(_A\) receptors, indicating that it specifically downregulates GABA\(_B\) receptors (Fig. 1D).

We had previously shown that GABA\(_B\) receptors are degraded in lysosomes (15,19). Therefore we tested the hypothesis that activation of glutamate receptors triggers lysosomal degradation of GABA\(_B\) receptors. Indeed, blocking lysosomal proteases with leupeptin resulted in a decreased glutamate-induced downregulation of GABA\(_B\) receptors, as shown by Western blot analysis (GABA\(_B_{1a}\): glutamate: 42 ± 6%, glutamate + leupeptin: 79 ± 18%; GABA\(_B_{1b}\): glutamate: 43 ± 12%, glutamate + leupeptin: 71 ± 16%; GABA\(_B_{2}\): glutamate: 65 ± 10%, glutamate + leupeptin: 105 ± 19% of control; mean ± SD, n= 5-6 cultures, Fig. 1E). Surprisingly, also the proteasome inhibitor MG132 inhibited glutamate-induced downregulation of GABA\(_B\) receptors (GABA\(_B_{1a}\): 73 ± 8%; GABA\(_B_{1b}\): 70 ± 19%; GABA\(_B_{2}\): 103 ± 29% of control; mean ± SD, n= 5-6 cultures, Fig. 1E). This finding ostensibly suggests that GABA\(_B\) receptors may also be degraded by proteasomes. However, experiments with proteasome inhibitors are difficult to interpret since blocking proteasomal activity rapidly results in the depletion of free ubiquitin. Ubiquitin not only tags proteins for proteasomal degradation but also serves as sorting signal to lysosomes for many proteins. If the proteasome directly contributes to
the degradation of GABA<sub>B</sub> receptors it is expected that simultaneous inhibition of proteasomes and lysosomes would block GABA<sub>B</sub> receptor downregulation to a greater extent than inhibition of each degradation pathway alone. However, incubation of neurons simultaneously with leupeptin and MG132 did not result in an increased inhibition of glutamate-induced GABA<sub>B</sub> receptor degradation (Fig. 1F). Therefore, it is unlikely that the proteasome directly contributes to the degradation of GABA<sub>B</sub> receptors. Instead, this finding suggests that ubiquitin may serve as lysosomal targeting signal for endocytosed GABA<sub>B</sub> receptors. Blocking ubiquitination using the ubiquitin-activating enzyme (E1) inhibitor UBEI-45 partially inhibited glutamate-induced downregulation of GABA<sub>B</sub> receptors (Fig. 1F) indicating that ubiquitination is a critical factor in this process.

Glutamate-Induced Downregulation of GABA<sub>B</sub> Receptors is Mediated by AMPA Receptors and Accelerated by Type I Metabotropic Glutamate Receptors – Since AMPA largely mimicks the effect of glutamate (Fig. 1A), AMPA receptors appear to primarily trigger the downregulation of GABA<sub>B</sub> receptors. However, although the AMPA receptor antagonist CNQX fully inhibited AMPA-induced downregulation of GABA<sub>B</sub> receptors it only partially reversed glutamate-induced downregulation (Fig. 2A). This result indicates that other glutamate receptors than AMPA receptors may contribute to this effect. Surprisingly, the selective NMDA receptor antagonist D-AP5 completely reversed glutamate-induced downregulation of GABA<sub>B</sub> receptors (Fig. 2A) although application of NMDA/glycine did not affect glutamate-induced downregulation of GABA<sub>B</sub> receptors at the levels observed after glutamate treatment, while tADA was ineffective to downregulate GABA<sub>B</sub> receptors in the absence of AMPA (Fig. 2D). In contrast, coactivation of either group II mGluRs with DCG-IV or group III mGluRs with L-AP4 did not enhance AMPA-induced downregulation of GABA<sub>B</sub> receptors. Indeed, coapplication of the selective mGluR1/5 agonist tADA with AMPA decreased GABA<sub>B</sub> receptors to the levels observed after glutamate treatment, while tADA was ineffective to downregulate GABA<sub>B</sub> receptors in the absence of AMPA (Fig. 2E). These results indicate that the sustained activation of AMPA and NMDA receptors is required for the downregulation of GABA<sub>B</sub> receptors, which is accelerated by additional activation of mGluR1/5.

Glutamate-Induced Downregulation Depends on Ca<sup>2+</sup> Influx Through L-Type Voltage-Gated Ca<sup>2+</sup> Channels – So far our data indicate that glutamate-induced downregulation of GABA<sub>B</sub> receptors involves activation of AMPA and NMDA receptors which both are permeable for Ca<sup>2+</sup>. Therefore, we hypothesized that Ca<sup>2+</sup>-influx may be required for this effect. As expected, removal of extracellular Ca<sup>2+</sup> using EGTA completely inhibited glutamate and AMPA-induced downregulation of GABA<sub>B</sub> receptors (Fig. 3A). Next we tested whether Ca<sup>2+</sup>-influx through Ca<sup>2+</sup>-permeable AMPA receptors (AMPA receptors lacking GluR2) is required for downregulation of GABA<sub>B</sub> receptors. NASPM, a selective antagonist for Ca<sup>2+</sup>-permeable AMPA receptors, did not affect downregulation of GABA<sub>B</sub> receptors up to 100 µM (Fig. 3B).
However, at 250 µM NASPM inhibited glutamate-induced downregulation of GABA<sub>B</sub> receptors (not shown). At this high concentration NASPM has been reported also blocking Ca<sup>2+</sup>-impermeable AMPA receptors (20). Thus, our results suggest that AMPA receptors containing the GluR2 subunit, which are impermeable to Ca<sup>2+</sup>, mediate the downregulation of GABA<sub>B</sub> receptors.

Since AMPA receptors activate voltage-dependent Ca<sup>2+</sup> channels we tested whether Ca<sup>2+</sup>-influx through these channels is required for downregulation of GABA<sub>B</sub> receptors. Inhibition of L-type Ca<sup>2+</sup> channels with nifedipine completely prevented downregulation of GABA<sub>B</sub> receptors. In contrast, blocking P/Q-type and N-Type Ca<sup>2+</sup> channels with ω-conotoxin MVIIC or inhibiting selectively P/Q-type or N-type Ca<sup>2+</sup> channels with ω-agatoxin TK and ω-conotoxin GVIA, respectively, was without effect (Fig. 3B). These observations suggest that glutamate-induced downregulation of GABA<sub>B</sub> receptors depends on the activity of L-type Ca<sup>2+</sup> channels.

**Glutamate Affects Recycling but not Internalization of GABA<sub>B</sub> Receptors** – One potential mechanism that may contribute to the downregulation of GABA<sub>B</sub> receptors might involve an increased rate of GABA<sub>B</sub> receptor endocytosis. We therefore tested first whether downregulation of GABA<sub>B</sub> receptors depends on the internalization of the receptors. We had previously shown that GABA<sub>B</sub> receptors internalize via the classical dynamin- and clathrin-dependent pathway (19). As expected, inhibition of dynamin with dynasore completely blocked the glutamate-induced downregulation of GABA<sub>B</sub> receptors, indicating that endocytosis of receptors from the plasma membrane is required for the downregulation of GABA<sub>B</sub> receptors (Fig. 4A). However, application of glutamate did not affect the rate of GABA<sub>B</sub> receptor internalization (control: t<sub>1/2</sub>=1.6 ± 0.7 min, glutamate: t<sub>1/2</sub>=1.5 ± 0.6 min; Fig. 4B).

Since GABA<sub>B</sub> receptors are constitutively recycled back to the plasma membrane (15) glutamate may instead interfere with recycling of the receptors. Indeed, treatment of neurons with glutamate significantly reduced recycling of internalized receptors back to the cell surface (Fig. 4C). The reduced recycling was reversed by blocking lysosomal activity with leupeptin, indicating that receptors are preferentially sorted to lysosomes after glutamate application without direct inhibition of the recycling pathway (Fig. 4C). This result was confirmed by directly interfering with receptor recycling using monensin, which blocks fusion of intracellular vesicles with the plasma membrane. Application of monensin resulted in the downregulation of cell surface GABA<sub>B</sub> receptors to the same extent as observed after glutamate treatment (Fig. 4D). However, the downregulation elicited by glutamate but not the downregulation induced by monensin was rescued by various blockers of lysosomal activity (leupeptin, chloroquine, bafilomycin A1, Fig. 4D). This result indicates that sustained glutamate application shifts the balance between recycling and degradation towards receptor degradation.

**Glutamate Induces the Downregulation of Synapse-Associated GABA<sub>B</sub> Receptors** – To analyze whether GABA<sub>B</sub> receptors localized at glutamatergic synapses are downregulated we performed immunofluorescence colocalization experiments using antibodies directed against GABA<sub>B</sub>1 (Fig. 5A) or GABA<sub>B</sub>2 (Fig. 5B) and antibodies against glutamatergic postsynaptic sites (PSD95) or glutamatergic presynaptic sites (vGlut1), respectively. Treatment of neurons for 30 min with glutamate resulted in a dramatic loss (about 50%) of GABA<sub>B</sub>1 and GABA<sub>B</sub>2 clusters associated with either PSD95 (Fig. 5A1, B1) or vGlut1 (Fig. 5A2, B2). GABA<sub>B</sub> receptor clusters not associated with pre- or postsynaptic marker proteins (which also include intracellular receptors) were found to be reduced to a lesser extent (about 30%, Fig. 5A3, B3). The total number of PSD95 (Fig. 5A4, B4) and vGlut1 clusters (Fig. 5A5, B5) was not affected by glutamate. These results suggest that GABA<sub>B</sub> receptors associated with synapses may be preferentially downregulated.

**DISCUSSION**

GABA<sub>B</sub> receptors are abundantly localized at glutamatergic synapses to regulate excitatory neurotransmission. In this study we tested the hypothesis that glutamatergic activity may in turn feed back to regulate GABA<sub>B</sub> receptors. Such a mechanism had been suggested by Vargas et al. (12) based on their finding that treating neurons with glutamate resulted in a reduction of plasma membrane GABA<sub>B</sub> receptors. Our results indicate that sustained activation of AMPA receptors in
cultured cortical neurons leads to a rapid and
dramatic downregulation of GABA_B receptors by
shifting the balance of constitutive recycling and
lysosomal degradation towards degradation.

Treatment of neurons with glutamate led to a
rapid decrease of the levels of both GABA_B
receptor subtypes, GABA_B(1a,2) and GABA_B(1b,2),
expressed in neurons. Immunofluorescence
analysis showed a slightly stronger loss of GABA_B
receptor clusters colocalized with marker proteins
for glutamatergic synapses than not colocalizing
GABA_B receptor clusters, suggesting that GABA_B
receptors associated with synapses might be
preferentially downregulated. The limited
resolution of this method permits however no
conclusion on whether pre- or postsynaptic
GABA_B receptors are affected to different extent.

Glutamate activates a variety of distinct receptors,
such as AMPA receptors, NMDA receptors and
mGluRs1/5, which all mediate or contribute to
downregulation of GABA_B receptors. We found
that selective activation of AMPA receptors
mimicked the effect of glutamate but was less
efficient in downregulating GABA_B receptors, i. e.
activating selectively AMPA receptors required
more time to reach the same level of
downregulation than after glutamate treatment.
Simultaneous activation of mGluRs1/5 (group I
mGluRs) accelerated AMPA-induced
downregulation of GABA_B receptors to the level
of glutamate-induced downregulation, whereas
coaactivation of group II or group III mGluRs had
no effect. Group I mGluRs are predominantly
localized postsynaptically and generally promote
cell excitability (21). Thus, activation of group I
mGluRs in addition to AMPA receptors may
further increase excitation of the neuron
accelerating downregulation of GABA_B receptors
and explain the difference in time course of
GABA_B receptor downregulation by glutamate and
AMPA (Fig. 2B). In addition to AMPA receptors
and mGluRs1/5 also NMDA receptors play a
critical role. Although treatment of neurons with
NMDA and glycine (or D-serine) did not induce
downregulation of GABA_B receptors, blocking
NMDA receptors with D-AP5 completely blocked
the effects of glutamate (Fig. 2A) and AMPA (not
shown). Basal AMPA receptor activity in
unstimulated cultures may be too low to permit
sufficient activation of NMDA receptors (which
are blocked by Mg^{2+} at resting potential) by
administration of NMDA and glycine to induce
downregulation of GABA_B receptors. Thus, our
data indicate that the minimum requirement for
 glutamate-induced downregulation of GABA_B
receptors is the sustained activation of AMPA and
NMDA receptors.

So far, the intracellular second messenger
pathway(s) linking AMPA and NMDA receptor
activation to GABA_B receptor downregulation
remains to be established. Because removal of
extracellular Ca^{2+} by EGTA and inhibition of L-
type, but not P/Q and N-type, Ca^{2+} channels
blocked GABA_B receptor downregulation it is
very likely that influx and intracellular
accumulation of Ca^{2+} is one component of the
intracellular signaling cascade. However, our first
attempts to identify further components involved
were unsuccessful so far. Activation or inhibition
of major classical protein kinases such as protein
kinase A, protein kinase C, Ca^{2+}-calmodulin-
dependent kinase II (CaMKII) or blocking the
Ca^{2+}-dependent phosphatase calcineurin failed to
affect glutamate-induced downregulation of
GABA_B receptors (not shown). Thus, further work
is required to dissect the intracellular signaling
pathway(s) involved.

Endocytosis and sorting mechanisms critically
determine the availability of cell surface receptors
for signal transduction. We therefore hypothesized
that sustained activation of glutamate receptors
might affect endocytosis of GABA_B receptors.
Blocking endocytosis with the dynamin inhibitor
dynasore completely prevented downregulation of
GABA_B receptors. This result implies that constant
internalization of cell surface GABA_B receptors is
required for downregulation. Since GABA_B
receptors are rapidly endocytosed and
constitutively recycled back to the plasma
membrane in neurons (12,15) downregulation of
receptors can principally be achieved by either
increasing the rate of internalization or affecting
recycling. Our results indicate that the
internalization rate of GABA_B receptors remained
unaffected upon glutamate administration. This is
in contrast to a previous report indicating that
 glutamate administration to hippocampal neurons
slowed the internalization rate of GABA_B
receptors tagged with the α-bungarotoxin binding
site (14). The reason for this discrepancy is
currently unknown but may be caused by the
different assay systems used (e.g. endogenous
versus transfected receptors). However, while glutamate did not impair endocytosis of GABA_B receptors it affected the extent of their recycling in our experimental settings. Impaired receptor recycling may shift the balance between recycling and degradation of the receptors in favor of degradation as recently shown for the transferrin receptor (22). However, this appears not to be the underlying mechanism for the glutamate-induced downregulation of GABA_B receptors. Although directly blocking recycling with monensin resulted in a similar extent of GABA_B receptor downregulation as glutamate treatment, inhibition of lysosomal activity prevented glutamate-induced but not monensin-induced downregulation of cell surface receptors. This result suggests that glutamate treatment does not directly block recycling of the receptors but affects the balance of sorting endocytosed GABA_B receptors to recycling endosomes and lysosomes.

Regarding the involved degradation pathways we found that glutamate-induced downregulation was inhibited by blocking lysosomal as well as proteasomal activity by leupeptin and MG 132, respectively. In principle, this observation may suggest that the major protein degradation systems, lysosomes and proteasomes, are involved in downregulation of GABA_B receptors. However, it is rather unlikely that the proteasome is directly involved in glutamate-induced degradation of the receptors since the blocking effects of leupeptin and MG 132 did not add up. Inhibition of proteasomes rapidly results in the depletion of free ubiquitin, which is also required as a lysosomal targeting signal for many proteins. Therefore, inhibition of proteasomes with MG 132 most likely interferes with ubiquitin-dependent sorting to lysosomes. This interpretation is supported by the observation that sorting of GABA_B receptors to lysosomes appears to involve the ESCRT (endosomal sorting complex required for transport) machinery (23) that targets mono- and Lys63-linked poly-ubiquitinated proteins for degradation to lysosomes (24). Our finding that interfering with protein ubiquitination by blocking the ubiquitin-activating enzyme (E1) reduces glutamate-induced downregulation of GABA_B receptors documents the importance of ubiquitination in this process. However, it is currently unclear at what level ubiquitination may be involved. Ubiquitination may operate at different levels such as endocytosis, sorting and/or lysosomal targeting and may require direct ubiquitination of the receptors or of proteins involved in these processes or both. This issue needs to be delineated in further studies.

Based on our observations, we propose that sustained activation of AMPA receptors triggers the opening of NMDA receptors and L-type Ca^{2+} channels which leads to an intracellular accumulation of Ca^{2+}. An as yet to be established, most likely Ca^{2+}-dependent, signaling pathway shifts the balance between constitutive recycling and lysosomal degradation of GABA_B receptors towards degradation without affecting the rate of receptor internalization. Since all components required for glutamate-induced downregulation of GABA_B receptors are mainly colocalized postsynaptically (25,26) we propose that glutamate may preferentially downregulate GABA_B receptors associated with postsynaptic sites. In particular the fact that N-type and P/Q-type Ca^{2+} channels, which are predominantly associated with presynaptic sites (25,26), appear not to be involved in downregulating GABA_B receptors supports this hypothesis.

The physiological relevance of glutamate-induced downregulation of GABA_B receptors remains to be determined. Two obvious scenarios can be envisioned: 1) under pathophysiological condition, excessive release of glutamate leads to over-stimulation of AMPA and NMDA receptors and eventually to neuronal death (27). In this context glutamate induced downregulation of GABA_B receptors will result in reduced inhibition of glutamatergic synapses, which is expected to further promote excitotoxicity. 2) Under normal physiological conditions, GABA_B receptors associated with glutamatergic synapses control excitability of the synapse and thereby activity of NMDA receptors (28,29). Sustained stimulation of individual glutamatergic synapses will downregulate its associated GABA_B receptors and relieves the synapse from inhibition. This is expected to result in locally increased synaptic excitability.

REFERENCES
FOOTNOTES
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The abbreviations used are: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; GABA_B, γ-aminobutyric acid type B; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; PSD95, postsynaptic density protein 95; vGlut1, vesicular glutamate transporter 1.

FIGURE LEGENDS

FIGURE 1. Sustained activation of glutamate receptors induces the downregulation of GABA_B receptors via lysosomal degradation.

A) Downregulation of GABA_B receptors is triggered by glutamate and AMPA. Primary cortical neurons were treated either with 50 μM glutamate, 100 μM AMPA or 100 μM NMDA + 10 μM glycine for 90 min and tested for GABA_B receptor levels using the In Cell Western assay and antibodies directed against the C-terminus of GABA_B1. Mean ± SD; n=20-40 cultures from 3-5 preparations, ***= p<0.001, one way ANOVA, Bonferroni post test.

B) Glutamate downregulates GABA_B1 and GABA_B2 as verified by immunocytochemistry. For immunofluorescence analysis, cells were treated with 50 μM glutamate for 30 min. This incubation time resulted in sufficient remaining fluorescence signals in the glutamate treated cultures enabling a reliable quantification. After fixation and permeabilization neurons were stained with antibodies directed against GABA_B1 and GABA_B2, respectively. Representative confocal images. B') Quantification of fluorescence signals. Scale bar represents 10 µm. Mean ± SD; n=33-48 neurons from 3 preparations, ***= p<0.0001, t-test.

C) Dose-dependency of glutamate-induced downregulation of GABA_B receptors. Neurons were treated with increasing concentrations of glutamate for 90 min and analyzed for GABA_B receptor levels using the In Cell Western assay and GABA_B antibodies. A concentration of 5 μM glutamate led to half-maximal downregulation of GABA_B receptors. Mean ± SD; n=20 cultures from 3 preparations.

D) Glutamate does not affect expression levels of GABA_A receptors. Neurons were subjected to 50 μM glutamate for 90 min and analyzed for GABA_B and GABA_A receptor levels using the In Cell Western assay and antibodies directed against GABA_B1 and the GABA_A receptor α1 subunit, respectively. Mean ± SD; n=40-60 cultures from 5 preparations, ***= p<0.0001, n.s.: p=0.8, t-test.

E) Glutamate-induced downregulation is prevented by lysosome and proteasome inhibitors. Neurons were treated with 50 μM glutamate in the presence or absence of the lysosome blocker leupeptin (100 μM) or the proteasome blocker MG 132 (10 μM), solubilized and analyzed for GABA_B receptor levels by Western blotting using GABA_B1 and GABA_B2 antibodies. GABA_B1a and GABA_B1b isoforms as well as GABA_B2 subunits were downregulated after glutamate treatment. Downregulation was blocked by the lysosomes inhibitor leupeptin and by the proteasome inhibitor MG132. Bands present in the middle of the blot were observed at varying intensities and most likely represents degradation products of GABA_B receptors. The experiment shown was repeated once with three cultures for each condition. E') Quantification of the Western blots. Mean ± SD; n=46 cultures, n.s. = p>0.05, *=p<0.05, **=p<0.01 ***= p<0.001, one way ANOVA, Dunnett’s post test.

F) The proteasome is most likely not directly involved in downregulating GABA_B receptors. Neurons were treated for 90 min with 50 μM glutamate in the presence or absence of the lysosome blocker leupeptin (100 μM), the proteasome blocker MG 132 (10 μM) or a combination of both. In addition, the involvement of ubiquitination was tested by treating neurons with the ubiquitin-activating enzyme (E1) inhibitor UBEI-45 (50 μM). GABA_B receptor levels were determined using the In Cell Western assay and GABA_B1 antibodies. Since the effects of leupeptin and MG132 did not add-up it is unlikely that GABA_B
receptors are degraded by proteasomes. Mean ± SD; n=40-48 cultures from 4 preparations, n.s.= p>0.05, **=p<0.01 ***= p<0.001, one way ANOVA, Bonferoni post test.

FIGURE 2. Glutamate-induced downregulation of GABA<sub>B</sub> receptors is mediated by AMPA receptors and accelerated by type I mGluRs.
A) Glutamate-induced downregulation of GABA<sub>B</sub> receptors is partially reversed by the AMPA antagonist CNQX and fully reversed by the NMDA antagonist D-AP5. Cells were treated for 90 min either with 50 μM glutamate, 100 μM AMPA, 20 μM CNQX, 50 μM D-AP5 or with the indicated combinations and tested for GABA<sub>B</sub> receptor levels using the In Cell Western assay and antibodies directed against the C-terminus of GABA<sub>B</sub>1. Mean ± SD; n=27-44 cultures from 3-5 preparations, **=p<0.01, ***= p<0.001, one way ANOVA, Bonferoni post test.

B) Selective activation of AMPA receptors mimics the effect of glutamate but is less efficient in downregulating GABA<sub>B</sub> receptors. Neurons were incubated with 50 μM glutamate or 100 μM AMPA and were analyzed for GABA<sub>B</sub> receptor levels at different time points (10 min to 6 h) using the In Cell Western assay. Data were fitted to one phase exponential decay: glutamate, t₁/₂=25 ± 13 min; AMPA, t₁/₂=49 ± 17 min. Mean ± SD; n=12 cultures from 3 preparations.

C-E) Combined activation of AMPA and mGluR group I receptors (D), but not AMPA and NMDA (C) or mGluR group II or III receptors (E), mimic glutamate induced downregulation of GABA<sub>B</sub> receptors. Neurons were incubated for 90 min with either 50 μM glutamate, 100 μM AMPA, 100 μM NMDA + 10 μM glycine, 200 μM tADA (mGluR1/5 agonist), 100 μM L-AP4 (mGluR4/6/7/8 agonist) or 100 μM DCGIV (mGluR2/3 agonist) alone or with the indicated combinations. Mean ± SD; C: n=24 cultures from 3 preparations, D: n=21 cultures from 3 preparations, D: n=32 cultures from 4 preparations. n.s.=p>0.05, **=p<0.01, ***= p<0.001, one way ANOVA, Bonferoni post test (C and D) or Dunnett post test (E).

FIGURE 3. Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels is necessary for glutamate-induced downregulation of GABA<sub>B</sub> receptors.
A) Removal of extracellular Ca<sup>2+</sup> using EGTA completely inhibited glutamate and AMPA-induced downregulation of GABA<sub>B</sub> receptors. Cells were treated with either 50 μM glutamate, 100 μM AMPA or 5 mM EGTA or with the indicated combinations for 90 min and tested for GABA<sub>B</sub> receptor levels using the In Cell Western assay. Mean ± SD; n=25-32 cultures from 4 preparations, ***= p<0.001, one way ANOVA, Bonferoni post test.

B) Inhibition of L-type Ca<sup>2+</sup> channels but not P/Q-type and N-type channels prevents downregulation of GABA<sub>B</sub> receptors. Neurons were incubated for 90 min either with 50 μM glutamate, 50 μM glutamate +100 μM NASPM (blocker of Ca<sup>2+</sup>-permeable AMPA receptors) +100 μM nifedipine (L-type Ca<sup>2+</sup> channel blocker), +2 μM ω-conotoxin MVIIC (P/Q and N-type Ca<sup>2+</sup> channel blocker), +0.5 μM ω-Agatoxin TK (P/Q-type Ca<sup>2+</sup> channel blocker) or +3 μM ω-conotoxin GVIA (N-type Ca<sup>2+</sup> channel blocker) and subjected to the In Cell Western assay for determination of GABA<sub>B</sub> receptor levels. Mean ± SD, n=16-32 cultures from 2-4 preparations, n.s.=p>0.05, ***= p<0.001, one way ANOVA, Bonferoni post test.

FIGURE 4. Glutamate affects recycling but not internalization of GABA<sub>B</sub> receptors.
A) Inhibition of dynamin with dynasore completely blocks the glutamate-induced downregulation of GABA<sub>B</sub> receptors. Neurons were treated either with 50 μM glutamate, 100 μM dynasore or with 50 μM glutamate + 100 μM dynasore for 90 min and subjected to the In Cell Western assay for determination of GABA<sub>B</sub> receptor levels. Mean ± SD, n=40 cultures from 5 preparations, ***= p<0.001, one way ANOVA, Bonferoni post test.

B) Glutamate did not affect the rate of GABA<sub>B</sub> receptor internalization. Cell surface receptors of neurons were labeled at 4 °C for 60 min with Fab fragments of an antibody directed against the N-terminus of GABA<sub>B</sub>2. Neurons were then incubated for 1.5 - 15 min at 37 °C in the presence or absence of 50 μM
glutamate followed by determination of cell surface GABA_B receptor levels. Mean ± SD; glutamate: n=18 cultures, 3 preparations; AMPA: n=12 cultures, 2 preparations.

C) Glutamate reduces recycling of internalized receptors back to the cell surface. Cell surface receptors of neurons were labeled as described above and neurons were incubated for 15 min at 37 °C in the presence or absence of 50 μM glutamate, 100 μM leupeptin or 50 μM glutamate + 100 μM leupeptin to allow internalization of receptors. After masking remaining antibody-tagged cell surface receptors with secondary antibodies not detected by the imaging system neurons were incubated again for 30 min at 37°C to allow internalized receptors to recycle back to the plasma membrane. Recycled receptors were detected using an appropriate labeled secondary antibody for 90 min at 4 °C. Mean ± SEM, n=30-94 cultures from 4-9 preparations, ***= p<0.001, one way ANOVA, Bonferoni post test.

D) Glutamate shifts the balance of GABA_B receptor recycling and lysosomal degradation towards degradation without blocking recycling. Neurons were incubated for 1 h with 100 μM leupeptin, 100 μM chloroquine or 0.5 μM bafilomycin A1 before inducing downregulation of GABA_B receptors by 50 μM glutamate or 50 μM monensin (blocks recycling by preventing fusion of intracellular vesicles with the plasma membrane) for 90 min. Living neurons were subsequently incubated with an antibody directed against the N-terminus of GABA_B2 to label cell surface receptors and further processed for immunocytochemistry. Cell surface fluorescence signals of individual neurons were quantified. Control: no treatment. Mean ± SEM, n=31-81 neurons derived from two to four independent experiments, n.s. = p>0.05, **=p<0.01, one way ANOVA, Dunnett’s post test.

FIGURE 5. **GABA_B receptors associated with synapses are downregulated by activation of glutamate receptors.** Neurons were incubated in the presence or absence of 50 μM glutamate for 30 min, fixed and subjected to double labeling immunocytochemistry using antibodies directed against GABA_B1 (A, green) or GABA_B2 (B, green) and antibodies against glutamatergic postsynaptic sites (PSD95, red) or glutamatergic presynaptic sites (vGlut1, red), respectively. Large images depict overviews of immunoreactivities in dendrites of lower magnification (scale bars: 5 μm) and the small images below show sections of dendrites after being processed for counting of clusters at high magnification (see Experimental Procedures for details; scale bars: 1 μm). In the absence of glutamate GABA_B clusters frequently colocalized (yellow, arrowheads) with either pre- or postsynaptic markers. Glutamate treatment resulted in a significant loss of GABA_B1 and GABA_B2 clusters associated with either PSD95 (A1, B1) or vGlut1 (A2, B2). GABA_B receptor clusters not associated with pre- or postsynaptic marker proteins (which also include intracellular receptors) were found to be reduced to a lesser extent (A3, B3). The number of PSD95 (A4, B4) and vGlut1 (A5, B5) clusters was not affected by glutamate. Mean ± SEM, n=24 neurons derived from two independent experiments. **=p<0.01, ***= p<0.001, t-test.
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