Prolonged activation of NMDA receptors promotes dephosphorylation and alters postendocytic sorting of GABA<sub>B</sub> receptors

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Slow and persistent synaptic inhibition is mediated by metabotropic GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs). GABA<sub>B</sub>Rs are responsible for the modulation of neurotransmitter release from presynaptic terminals and for hyperpolarization at postsynaptic sites. Postsynaptic GABA<sub>B</sub>Rs are predominantly found on dendritic spines, adjacent to excitatory synapses, but the control of their plasma membrane availability is still controversial. Here, we explore the role of glutamate receptor activation in regulating the function and surface availability of GABA<sub>B</sub>Rs in central neurons. We demonstrate that prolonged activation of NMDA receptors (NMDA-Rs) leads to endocytosis, a diversion from a recycling route, and subsequent lysosomal degradation of GABA<sub>B</sub>Rs. These sorting events are paralleled by a reduction in GABA<sub>B</sub>R-dependent activation of inwardly rectifying K<sup>+</sup> channel currents. Postendocytic sorting is critically dependent on phosphorylation of serine 783 (S783) within the GABA<sub>B</sub>R2 subunit, an established substrate of AMP-dependent protein kinase (AMPK). NMDA-R activation leads to a rapid increase in phosphorylation of S783, followed by a slower dephosphorylation, which results from the activity of AMPK and protein phosphatase 2A, respectively. Agonist activation of GABA<sub>B</sub>Rs counters the effects of NMDA. Thus, NMDA-R activation alters the phosphorylation state of S783 and acts as a molecular switch to decrease the abundance of GABA<sub>B</sub>Rs at the neuronal plasma membrane. Such a mechanism may be of significance during synaptic plasticity or pathological conditions, such as ischemia or epilepsy, which lead to prolonged activation of glutamate receptors.

Results

Activation of NMDA-Rs Triggers GABA<sub>B</sub>R Endocytosis. We measured the steady-state abundance of endogenous GABA<sub>B</sub>Rs by surface biotinylation. Glutamate produced a biphasic response in the cell surface levels of GABA<sub>B</sub>Rs. Short exposure to glutamate (1–5 min) increased the abundance of both GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits at the plasma membrane, whereas longer treatments (10–30 min) triggered a pronounced and steady decrease of surface receptors (Fig. 1). Neurons were treated with NMDA to determine directly whether activation of NMDA-Rs was sufficient to promote recycling of GABA<sub>B</sub>Rs via a non-conventional extracellular signal, uncovering an added interplay between excitation and inhibition in central neurons.

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The availability of neurotransmitter receptors, a major determinant of synaptic efficacy, is regulated by coordinated mechanisms of intracellular trafficking that deliver newly synthesized receptors to the plasma membrane and remove them for storage, recycling, or degradation (1). The molecular mechanisms controlling the availability of GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs), which are central players in the modulation of excitatory and inhibitory synaptic activity, are unclear.

GABA<sub>B</sub>Rs mediate slow and prolonged inhibitory synaptic signals (2, 3). Consistent with these roles, modifications in the function of GABA<sub>B</sub>Rs are implicated in epilepsy, anxiety, stress, sleep disorders, nociception, depression, cognition, and addictive mechanisms to drugs of abuse (3–7). GABA<sub>B</sub>Rs are members of the G-protein-coupled receptor (GPCR) superfamily and are obligatory heteromers composed of two related subunits, namely GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 (3, 8). GABA<sub>B</sub>R1 binds agonist with high affinity, whereas GABA<sub>B</sub>R2 mediates coupling to Gα (9, 10). GABA<sub>B</sub>Rs are located in GABA-ergic and glutamatergic pre- and postsynaptic terminals, but their distribution does not coincide with the active zone, postsynaptic density, or inhibitory postsynaptic specializations. Rather, they are perisynaptic receptors activated by GABA spillover (3, 11). Stimulation of GABA<sub>B</sub>Rs decreases the levels of cAMP, inhibits neurotransmitter release from presynaptic terminals, and hyperpolarizes postsynaptic neurons (2).

The availability and function of GPCRs are typically controlled by mechanisms such as agonist-induced desensitization and internalization, followed by recycling or degradation (12, 13). In the case of GABA<sub>B</sub>Rs, the role that agonist-dependent desensitization plays in controlling signaling and cell surface availability remains controversial (14), but phosphorylation by GPCR-dependent kinases and arrestin binding have been excluded (15, 16). On the contrary, constitutive endocytosis in neurons has been reported consistently (16–22). Electron microscopy studies have demonstrated that GABA<sub>B</sub>Rs are enriched in the vicinity of glutamatergic synapses (23–27), but the role of glutamate receptors in controlling the efficacy of GABA<sub>B</sub>Rs signaling remains largely unexplored.

Here, we investigate the molecular mechanisms underlying the control of GABA<sub>B</sub>R cell surface availability and effector coupling by glutamate receptor activity. Our study demonstrates that glutamate, acting through NMDA receptors (NMDA-Rs), activates AMP-dependent protein kinase (AMPK) and protein phosphatase 2A (PP2A), resulting in transient changes to the phosphorylation state of the GABA<sub>B</sub>R2 subunit on serine 783 (S783). Phosphorylation changes alter the fate of endocytosed GABA<sub>B</sub>Rs, which diverge from a recycling to a lysosomal degradation route, attenuating GABA<sub>B</sub>R function. Our observations provide a unique mechanism to regulate the functional availability of GABA<sub>B</sub>Rs via a non-conventional extracellular signal, uncovering an added interplay between excitation and inhibition in central neurons.
Receptor disappearance. Activation of NMDA-Rs mimicked the effect of glutamate but triggered a faster disappearance of GABA\textsubscript{R}Rs (Fig. 1B). A23187, a calcium ionophore that potentiates the response to NMDA, also markedly reduced surface GABA\textsubscript{R}Rs (Fig. S1). To evaluate if glutamate affects functional GABA\textsubscript{R}Rs under physiological conditions, we used whole-cell patch-clamp recording of hippocampal neurons and applied a 15-s pulse of 1 mM glutamate to reproduce synaptic concentrations of the excitatory transmitter. Glutamate caused a significant reduction in the baclofen-induced GABA\textsubscript{R}R-dependent activation of inwardly rectifying K\textsuperscript{+} currents (Fig. 1C and D). This reduction was relatively rapid (onset within 2 min after glutamate application) and was maintained for a further 7 min before slowly recovering in amplitude after 10 min (Fig. 1D). MK801 (100 nM), a selective NMDA-R antagonist, abolished the regulatory effect of glutamate, and baclofen-activated currents remained at control levels (Figs. 1C and D). Glutamate acted directly via NMDA-Rs to modulate the GABA\textsubscript{R}R-activated current, because the application of 1 mM glutamate to a cell line expressing GABA\textsubscript{R}R1a and GABA\textsubscript{R}R2, with inwardly rectifying K\textsuperscript{+} channels Kir3.1 and Kir3.2, did not affect their activity following the addition of 10 \mu M GABA (Control I\textsubscript{K} = 100%; + 1 mM glutamate = 95.3 ± 7%; n = 6). Taken together, these observations suggest that activation of NMDA-Rs plays a significant role in the plasticity of cell surface GABA\textsubscript{R}Rs.

To determine whether the disappearance of surface GABA\textsubscript{R}Rs was the result of internalization, we first examined whether phenylarsine oxide (PAO) affected their removal. PAO is a widely used inhibitor of clathrin-mediated endocytosis (28, 29) that completely blocks the endocytosis of GABA\textsubscript{R}Rs (Fig. S2). Surface levels of GABA\textsubscript{R}Rs were reduced by glutamate, an effect prevented by PAO (Fig. 1E). Because no differences were observed for GABA\textsubscript{R}R1 and GABA\textsubscript{R}R2, both subunits were suitable to explore the effect of glutamate. We used an immunoelectronoendocytosis assay to visualize directly the effect of NMDA-R activation on GABA\textsubscript{R}Rs by following the GABA\textsubscript{R}R1 subunit. GABA\textsubscript{R}Rs were efficiently detected at the neuronal plasma membrane and concentrated in intracellular structures after constitutive internalization. These structures accumulated throughout the cell body and frequently displayed perinuclear localization (Fig. 1F Upper, arrowhead). In contrast, after glutamate stimulation, the majority of internalized GABA\textsubscript{R}Rs remained spatially restricted in the proximity of the plasma membrane (Fig. 1F Lower, arrowhead). Combined, these findings provide direct evidence that activation of NMDA-Rs decreases GABA\textsubscript{R}R function by triggering their internalization and altering their intracellular distribution after endocytosis.

Activation of NMDA-Rs Diverts GABA\textsubscript{R}Rs from a Recycling Route.

After internalization, GABA\textsubscript{R}Rs recycle to the plasma membrane, a mechanism that is accelerated by receptor agonist (21, 22). To determine whether glutamate stimulation interferes with recycling, we combined immunoelectronoendocytosis with detection of Rab11, a marker for recycling endosomes (30). After endocytosis, GABA\textsubscript{R}Rs localized significantly with Rab11 (Fig. 2A Upper, arrowhead). By contrast, after glutamate stimulation, internalized GABA\textsubscript{R}Rs were restricted to the proximity of the plasma membrane, dissociated from Rab11-positive compartments (Fig. 2A Lower, arrowhead).

Fig. 1. (A) Cortical neurons were left untreated or exposed to 20 \mu M glutamate for 0–30 min. Biotinylated (surface) and total proteins were visualized by immunoblotting with GABA\textsubscript{R}R2 (R2) and GABA\textsubscript{R}R1 (R1) antibodies. (Lower) Immunoblots for each condition were analyzed by densitometry. **P < 0.01. (B) Surface expression of GABA\textsubscript{R}R2 was evaluated as above after exposure to 20 \mu M NMDA for 0–10 min. (Lower) Immunoblots were quantified as above. *P < 0.05; **P < 0.01. (C) Whole-cell recordings from cultured hippocampal neurons with 10 \mu M baclofen-activated K\textsuperscript{+} currents before (Left) and after (Right) glutamate receptor activation (1 mM, 15 s) in the absence (Upper) and presence (Lower) of 100 nM MK801 at 7, 10, 13, and 19 min. Baclofen responses were obtained in a high K\textsuperscript{+} Krebs’ solution supplemented with AP-5 (20 \mu M), CNQX (10 \mu M), TTX (500 nM), and Bicuculline (25 \mu M). (D) Baclofen-activated currents normalized to the first response at t = 0 min were recorded as control responses (▪) and responses interposed with glutamate (○) or glutamate and MK801 applications (▲). All points represent the mean ± SEM (n = 5–12 cells; *P < 0.05). (E) Cortical neurons were left untreated or exposed to 20 \mu M glutamate, 20 \mu M PAO, or 20 \mu M glutamate plus 20 \mu M PAO for 30 min. Biotinylated (surface) and total proteins were visualized by immunoblotting with GABA\textsubscript{R}R2 and GABA\textsubscript{R}R1 antibodies. (F) Hippocampal neurons were transfected with MYC-GABA\textsubscript{R}R1a and HA-GABA\textsubscript{R}R2 and processed for immunoendocytosis with MYC antibodies. Cells were left untreated (Upper) or exposed to 20 \mu M glutamate for 30 min (Lower). Surface receptors were detected with MYC- and Texas Red-conjugated secondary antibodies (red channel). Internalized receptors were detected using FITC-conjugated secondary antibodies (green channel). (Right) Merged images are shown. (Scale bar: 5 \mu m.)
To determine whether postglutamate internalized GABA\(_{\text{A}}\)Rs were targeted for degradation, immunoenocytosis was performed after pretreating the neurons with lysosomal blockers. The redistribution of internalized GABA\(_{\text{A}}\)Rs by glutamate was markedly inhibited following lysosomal blockade. These results indicate that lysosomal blockade forces internalized GABA\(_{\text{A}}\)Rs to continue along the recycling pathway.

Next, based on the observations that GABA\(_{\text{A}}\)R activation induces postsynaptic hyperpolarization and accelerates recycling, we hypothesized that baclofen might counteract the NMDA-R-dependent degradation signal. To test this prediction, we measured the abundance of cell surface GABA\(_{\text{A}}\)Rs after coincident stimulation of glutamate and baclofen for 30 min. Under such conditions, baclofen significantly reduced the disappearance of GABA\(_{\text{A}}\)Rs after glutamate stimulation (Fig. 2C). Together, these observations demonstrate that glutamate receptor activation alters the endocytic route of GABA\(_{\text{A}}\)Rs and produces a diversion from a recycling route to a degradation route. Furthermore, they indicate that activation of GABA\(_{\text{A}}\)Rs is sufficient to evade the NMDA-R–triggered degradation switch partially, which is possibly attributable to reduced activation of NMDA-Rs at hyperpolarized potentials.

**GABA\(_{\text{A}}\)R Sorting After NMDA-R Activation Is Controlled by AMPK Activation and Phosphorylation of S783 in GABA\(_{\text{A}}\)R2.** Multiple phosphorylation sites regulate the function and surface stability of GABA\(_{\text{A}}\)Rs (17, 31). To explore whether phosphorylation plays a role in the glutamate-mediated removal of GABA\(_{\text{A}}\)Rs, we examined known phosphoacceptor sites. Phosphorylation of S783 in GABA\(_{\text{A}}\)R2 was significantly augmented by intracellular Ca\(^{2+}\) levels and by KCl-induced depolarization, two biochemical models of increased neuronal activity (Fig. 3A and B). Glutamate also enhanced phosphorylation of S783 (Fig. 3C). To determine if NMDA-Rs were involved, phosphorylation of S783 was examined after treatment with specific glutamate receptor agonists and antagonists. Phosphorylation was rapidly stimulated by NMDA and robustly inhibited by AP5, a selective NMDA-R antagonist (Fig. 3D and E). Importantly, the time course of S783 phosphorylation triggered by NMDA-R activation paralleled the trafficking behavior of cell surface GABA\(_{\text{A}}\)Rs, showing an early rise in phosphorylation followed by steady dephosphorylation after 5 min of treatment.

Next, we investigated the signaling mechanisms involved in the phosphorylation of S783 after NMDA-R activation. Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx were necessary to increase S783 phosphorylation (Fig. S3A). This led us to examine the role of AMPK, a metabolic sensor activated by low ATP/AMP ratios and high Ca\(^{2+}\) levels (32), which is responsible for phosphorylating S783 (31). Phosphorylation of S783 was stimulated by a constitutive form of AMPK in the absence or presence of glutamate and was blocked by a dominant negative AMPK (Fig. 3F). In agreement with these findings, phosphorylation of S783 was prevented by STO-690, a selective inhibitor of the upstream kinase CaM KK (Fig. S3B).

To demonstrate that neuronal activity and NMDA-R activation stimulate AMPK, we measured phosphorylation of threonine 172 (T172) in AMPK as an indicator of kinase activity. In accordance with the data presented above, AMPK was activated by A23187, by KCl, by glutamate, and by NMDA (Fig. S4). Thus, NMDA-Rs activate AMPK, resulting in phosphorylation of GABA\(_{\text{A}}\)R2 at S783.

The significant changes in GABA\(_{\text{A}}\)R2 phosphorylation led us to investigate whether S783 regulates receptor removal. HEK293 cells were transfected with WT GABA\(_{\text{A}}\)R1 and GABA\(_{\text{A}}\)R2 subunits or with GABA\(_{\text{A}}\)R1 and GABA\(_{\text{A}}\)R2 containing a serine-to-alanine substitution at position 783 (S783A). Ca\(^{2+}\) influx was used as a stimulus in these cells lacking NMDA-Rs. A23187 promoted the disappearance of recombinant receptors from the plasma membrane, but removal was significantly attenuated in receptors containing the S783A mutated subunit (Fig. 3G). Cell surface levels of heterodimers containing S783A were lower than those of WT, suggesting that dephosphorylation favors degradation, thus reducing the availability of plasma membrane GABA\(_{\text{A}}\)Rs.

We used immunoenocytosis to visualize directly the fate of internalized receptors containing mutated phosphorylation sites. The majority of WT receptors accumulated throughout the cell body after constitutive endocytosis and glutamate produced a marked redistribution of the internalized receptors (Fig. 4A). In contrast, internalized receptors containing S783A were spatially restricted to the proximity of the plasma membrane even before glutamate treatment, mimicking the endocytic pattern of postglutamate WT receptors (Fig. 4A, arrowheads). Additionally, glutamate did not affect the pattern of internalized receptors containing S783A (Fig. 4A Lower). These data imply that transient changes in S783 phosphorylation regulate the postendocytic sorting of GABA\(_{\text{A}}\)Rs after NMDA-R activation. Because S783A is a dephosphomimetic substitution, these results suggest that dephosphorylation of S783 is involved in diverting from the recycling route. Thus, we explored the role of dephosphorylation on the surface availability of GABA\(_{\text{A}}\)Rs. Surface levels of GABA\(_{\text{A}}\)Rs were significantly enhanced by okadaic acid (OA) at a concentration that efficiently inhibits PP2A but not PP1 (Fig. 4B). This effect was not caused by increased GABA\(_{\text{A}}\)R exocytosis, because OA treatment had no effect on the insertion of de novo synthesized receptors (Fig. 4C). Consistent with these data, phosphorylation of S783 in GABA\(_{\text{A}}\)R2 and T172 in AMPK was stimulated by OA (Fig. 4D). More importantly, reduction of GABA\(_{\text{A}}\)Rs surface levels required PP2A activity (Fig. 4E). In addition, dephosphorylation of GABA\(_{\text{A}}\)Rs after 30 min of glutamate treatment was blocked by OA and not by PP1.
cyclosporin A, consistent with the role of PP2A in receptor dephosphorylation (Fig. 4F). In agreement with these observations, PP2A associated with GABA_B2R1 in cortical neurons (Fig. S5). These observations indicate that PP2A-mediated dephosphorylation is necessary for reduction of cell surface GABA_B2Rs.

**Discussion**

**Dynamic Balance Between Recycling and Degradation Controls the Abundance of GABA_B2Rs Through a Phosphorylation Switch.** Our observations are consistent with an integrated intracellular trafficking model in which activation of NMDA-Rs and GABA_B2Rs controls the balance between endocytosis, recycling, and degradation of GABA_B2Rs in neurons (Fig. S6). Briefly, GABA_B2Rs internalize and recycle in a constitutive manner. Activation of NMDA-Rs raises intracellular Ca^{2+}, activating AMPK and promoting a transient increase in cell surface GABA_B2Rs through S783 phosphorylation. Prolonged NMDA-R stimulation triggers the endocytosis of GABA_B2Rs (by a mechanism not uncovered in this study) and the activation of PP2A. PP2A favors dephosphorylation of S783 in GABA_B2R2 and diversion of the endocytosed pool from recycling to degradation. Combined, these events downstream of NMDA-Rs effectively decrease the availability of cell surface receptors. GABA_B2R activation overcomes the glutamate effect, possibly by hyperpolarizing the cell membrane, thereby preventing persistent depolarization and favoring recycling over degradation. However, concurrent GABA_B2R activation can also attenuate the Ca^{2+} signal induced by NMDA-R activation without affecting postsynaptic currents. This effect proceeds via GABA_B2R down-regulation of adenylylate cyclase activity and consequence attenuation of cAMP-dependent protein kinase activity, which reduces the NMDA-R-induced Ca^{2+} signal (33).

We refer to the postendocytic sorting model as the glutamate switch model. Overall, it is similar to the control of AMPA receptor availability by activation of different glutamate receptors, which alter their endocytic fate (34). The implications of the model for the functional localization of GABA_B2Rs are not clear. For example, it is not known whether GABA_B2R exocytosis occurs at dedicated sites or randomly throughout the neuron and whether phosphorylation by AMPK or dephosphorylation by PP2A serves as a tag to redirect endocytozed populations from recycling endosomes to specific domains, such as synapses or perisympatic sites.

**Mechanisms Controlling Recycling and Degradation of GABA_B2Rs.** The involvement of lysosomes in GABA_B2R degradation has been suggested before (16, 20, 21). In addition, the connection between degradative and recycling routes is strongly supported by the fact that blocking recycling of GABA_B2Rs with monensin produced an increase in lysosomal degradation (21). These data are in agreement with our switch model. At the molecular level, a di-leucine motif in the C-terminal domain of GABA_B2R1 may act as a lysosomal degradation signal because its removal results in accumulation of GABA_B2R1 at the plasma membrane (35). This idea is consistent with di-leucine motifs playing multiple functions as export, endocytosis, recycling, or lysosomal targeting signals for GPCRs (36), but their role in GABA_B2R degradation has not been directly demonstrated.

A23187 and KCl produced slower GABA_B2R phosphorylation than direct activation of NMDA-Rs. One explanation for this discrepancy is that the physical proximity of NMDA-Rs and GABA_B2Rs in many synaptic regions favors local and rapid signaling between receptors. Alternatively, activation of phosphatases may be faster or more efficient after stimulation of NMDA-Rs, which are known to initiate a variety of second-messenger signaling cascades (37). Importantly, the time course of S783 phosphorylation triggered by NMDA-Rs paralleled the behavior of cell surface availability of GABA_B2Rs, showing an early rise followed by a steady decline. The specific postendocytic sorting consequence of NMDA-R activation and S783 phosphorylation suggests that different phosphorylation sites regulate distinct aspects of the biosynthetic pathway of GABA_B2Rs, a postulate that has been convincingly demonstrated for other neurotransmitter receptors (38, 39).
Glutamate, Hyperexcitability, and Neuronal Damage. High-resolution microscopy in the visual cortex, cerebellum, thalamus, and hippocampus has shown that GABA<sub>9</sub>Rs are enriched in the vicinity of excitatory synapses, supporting the participation of glutamate receptor activation in the regulation of GABA<sub>9</sub>R function (23–27). In agreement with this, we have recently shown that glutamate produces a rapid disappearance of GABA<sub>9</sub>R1 and GABA<sub>9</sub>R2 from the plasma membrane of primary neurons (22). Additionally, activation of NMDA-Rs causes a pronounced decrease in the total levels of GABA<sub>9</sub>R2 in organotypic slices of rat hippocampus (40). Thus, reduction in the abundance of GABA<sub>9</sub>Rs has been associated with NMDA-induced excitotoxicity, suggesting that hyperexcitability may include the elimination of an inhibitory component. Activation of kainate receptors also produces a decline in GABA<sub>9</sub>R levels after 24 h in adult mice, possibly contributing to temporal lobe epilepsy (41). Our present results demonstrate that GABA<sub>9</sub>R function is attenuated by brief pulses of glutamate, consequently decreasing inwardly rectifying K<sup>+</sup> currents and leading to reduced postsynaptic hyperpolarization. This attenuation is blocked by MK801-implicating NMDA-Rs in the regulation of functional cell surface GABA<sub>9</sub>Rs. Our data also indicate that persistent NMDA-R activity initiates a signaling cascade, including AMPK and PP2A, which effectively reduces the availability of GABA<sub>9</sub>Rs, possibly favoring hyperexcitability and neuronal damage. These observations are in agreement with those published earlier, which showed that increased phosphorylation of S783 reduces the attenuation of GABA<sub>9</sub>R-mediated responses during desensitization by promoting cell surface stability (31). They also agree with the initial increase in phosphorylation of S783 during transient ischemic injury (31). Additionally, they provide an integrated molecular explanation for the neuroprotective effect of GABA<sub>9</sub>R activity on NMDA-induced cell death (40).

Experimental Procedures

**Chemicals and Plasmids.** Glutamate, baclofen, GABA, PAO, leupeptin, bafilomycin, and OA were purchased from Sigma. NMDA, CNQX, and D-AP5 were purchased from Tocris Bioscience. Fluorescently conjugated α-Bgt and α-Bgt was purchased from Invitrogen. The constructs containing MYC-GABA<sub>9</sub>R1, HA-GABA<sub>9</sub>R2, FLAG-GABA<sub>9</sub>R2-S783A, and FLAG-GABA<sub>9</sub>R2 in pRK5 have been described previously and contain their epitope tags on the extra-}

**Antibodies.** GABA<sub>9</sub>R1 and GABA<sub>9</sub>R2 antibodies have been described previously (17, 31). Anti-phospho S783-GABA<sub>9</sub>R2 (p-S783) has been described (31). Anti-phospho T172-AMPK (p-T172) was purchased from Cell Signaling Technology. Actin, β-tubulin, anti-FLAG, and anti-MYC antibodies were purchased from Sigma. HA antibodies were purchased from Roche. The secondary anti-mouse and anti-
rabbit antibodies conjugated to Texas Red, FITC, cyanine, and HRP were purchased from Jackson Immuno Research Laboratories.

**Animals, Primary Neurons, and Transfections.** Adult pregnant female Sprague-Dawley rats were purchased from the Central Animal Facility at Universidad Católica de Chile and killed by means of asphyxia in a CO₂ chamber, according to the National Academy of Sciences’ guidelines for the care and use of laboratory animals. Primary cultures of cortical and hippocampal neurons were obtained from embryonic day 18 rats (45) and used at 5 and 14 days in vitro (div), respectively. Hippocampal neurons were transfected by calcium phosphate, as reported elsewhere (46).

**Biotinylation, GST Pull-Downs, Immunoprecipitations, and Phosphorylation.** Biotinylation of cortical neurons was conducted as reported elsewhere (16). Briefly, biotinylated proteins were precipitated with Neutravidin beads (Pierce Protein Research Products, Thermo Scientific), and samples were separated by SDS-PAGE. Surface and total proteins were visualized by immunoblotting. Actin or tubulin was used as an internal control (Fig. 57). GST pull-downs, immunoprecipitations, and detection of phosphorylated substrates were performed as described earlier and visualized by immunoblotting and chemiluminescence (17, 31). ImmunobLOTS were analyzed by densitometry. Graphical representations correspond to the mean ± SEM of at least three independent experiments. Significance was evaluated usingStudent’s t test (ns, nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001).

**Flow Cytometry.** HEK293 cells transfected with GABAβR1b-α-Bgt and HA-GABAβR2 were incubated with unlabeled α-Bgt for 60 min at 37 °C and subsequently with rhodamine-conjugated α-Bgt for increasing periods of time. Cells were subjected to flow cytometry, as described elsewhere (48).

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**Immunofluorescence, Immunoenedocytosis, and Image Processing.** Immunofluorescence, immunoenedocytosis, and image processing, including colocalization, were conducted in hippocampal neurons as described elsewhere (31).

**Patch-Clamp Recording and Analysis of Ligand-Activated Membrane Currents.** Recordings were conducted on 14-div hippocampal neurons as described previously (31).