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Lateral Diffusion of the $GABA_B$ Receptor Is Regulated by the $GABA_{B2}$ C Terminus*[S]

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$GABA_B$ (γ-aminobutyric acid, type B) is a heterodimeric G-protein-coupled receptor. The $GABA_{B1}$ subunit, which contains an endoplasmic reticulum retention sequence, is only transported to the cell surface when it is associated with the $GABA_{B2}$ subunit. Fluorescence recovery after photobleaching studies in transfected COS-7 cells and hippocampal neurons revealed that $GABA_{B2}$ diffuses slowly within the plasma membrane whether expressed alone or with the $GABA_{B1}$ subunit. Treatment of cells with brefeldin A revealed that $GABA_{B2}$ moves freely within the endoplasmic reticulum, suggesting that slow movement of $GABA_{B2}$ is a result of its plasma membrane insertion. Disruption of the cytoskeleton did not affect the mobility of $GABA_{B2}$, indicating that its restricted diffusion is not due to direct interactions with actin or tubulin. To determine whether the C terminus of $GABA_{B2}$ regulates its diffusion, this region of the subunit was attached to the lymphocyte membrane protein, CD2, which then exhibited a slower rate of lateral diffusion. Furthermore, co-expression of a cytoplasmically expressed soluble form of the $GABA_{B2}$ C terminus increased movement of the $GABA_{B2}$ subunit. We constructed forms of $GABA_{B2}$ with various C-terminal truncations. Truncation of $GABA_{B2}$ after residue 862, but not residue 886, caused a dramatic increase in its mobility, suggesting that the region between these two residues is critical for restricting $GABA_{B2}$ diffusion. Finally, we investigated whether activation of $GABA_B$ might modulate its movement. Treatment of COS-7 cells with the $GABA_B$ receptor agonist baclofen significantly increased its mobile fraction. These data show that the restricted movement of $GABA_B$ at the cell surface is regulated by a region within its C terminus.

$GABA_B$ receptors are metabotropic receptors for the inhibitory neurotransmitter γ aminobutyric acid (GABA).[2] Pre- and post-synaptic $GABA_B$ receptors are coupled to inhibitory G-proteins and can regulate neurotransmission via several mechanisms, including modulation of adenylyl cyclase (1), inhibition of voltage-gated Ca^{2+} channels (2), and modulation of K^+ channels (3, 4). Formation of a functional receptor requires the heterodimerization of two subunits, $GABA_{B1}$ and $GABA_{B2}$ (5). Previous work has demonstrated that the stable assembly of these subunits occurs, to some extent, via association of coiled-coil domains within their C termini (6). The subunits appear to serve different functions within the fully formed receptor. $GABA_{B1}$ contains the agonist binding site on its large extracellular N terminus, and the affinity of this site for agonists is increased following heterodimerization with $GABA_{B2}$ (7). The $GABA_{B2}$ subunit contains intracellular loops that couple the receptor to the G-protein (8–10).

Heterodimerization of the $GABA_B$ subunits is important not only for proper receptor function but also for forward trafficking of the receptor to the cell surface (5, 7). In the absence of $GABA_{B2}$, the $GABA_{B1}$ subunit is retained within the endoplasmic reticulum due to the presence of a C-terminal RSRR retention motif on its C terminus. The interaction of $GABA_{B2}$ with $GABA_{B1}$ apparently masks this motif, allowing the fully formed receptor to traffic to the cell surface, where it may be targeted to the synapse.

The number of neurotransmitter receptors within post-synaptic membranes is dependent not only on insertion of new receptors but also on lateral diffusion of extrasynaptic receptors into the synaptic compartment (11). Regulation of receptor movement within the plasma membrane is therefore likely to be important for plasticity at individual synapses. At excitatory synapses, lateral diffusion of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors has been suggested to regulate synaptic strength following either long-term potentiation or long-term depression (12–14). Furthermore, *N*-methyl-D-aspartate receptors readily exchange between synaptic and extrasynaptic compartments via movement within the plasma membrane (15). However, the behavior of inhibitory neurotransmitter receptors is less well understood. The dynamics of the glycine receptor (16) and the ionotropic $GABA_A$ receptor (17) have recently been examined. Extrasynaptically, both receptors diffuse freely, but within the synaptic compartment interactions with the synaptic scaffolding protein gephyrin significantly slow their movement. To date, however, membrane dynamics of the $GABA_B$ receptor have yet to be investigated.

In the present study we explored the movement of the $GABA_B$ receptor within the plasma membrane. We found lateral diffusion of $GABA_B$ at the cell surface to be slow, due to restricted mobility of the $GABA_{B2}$ subunit. Disruption of the cytoskeleton did not affect $GABA_{B2}$ diffusion; however, truncation of the $GABA_{B2}$ C-terminal region allowed $GABA_{B2}$ to diffuse more rapidly. We show, therefore, that $GABA_B$ exhibits

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: GABA, γ-aminobutyric acid; FRAP, fluorescence recovery after photobleaching; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; HA, hemagglutinin; PBS, phosphate-buffered saline AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid.

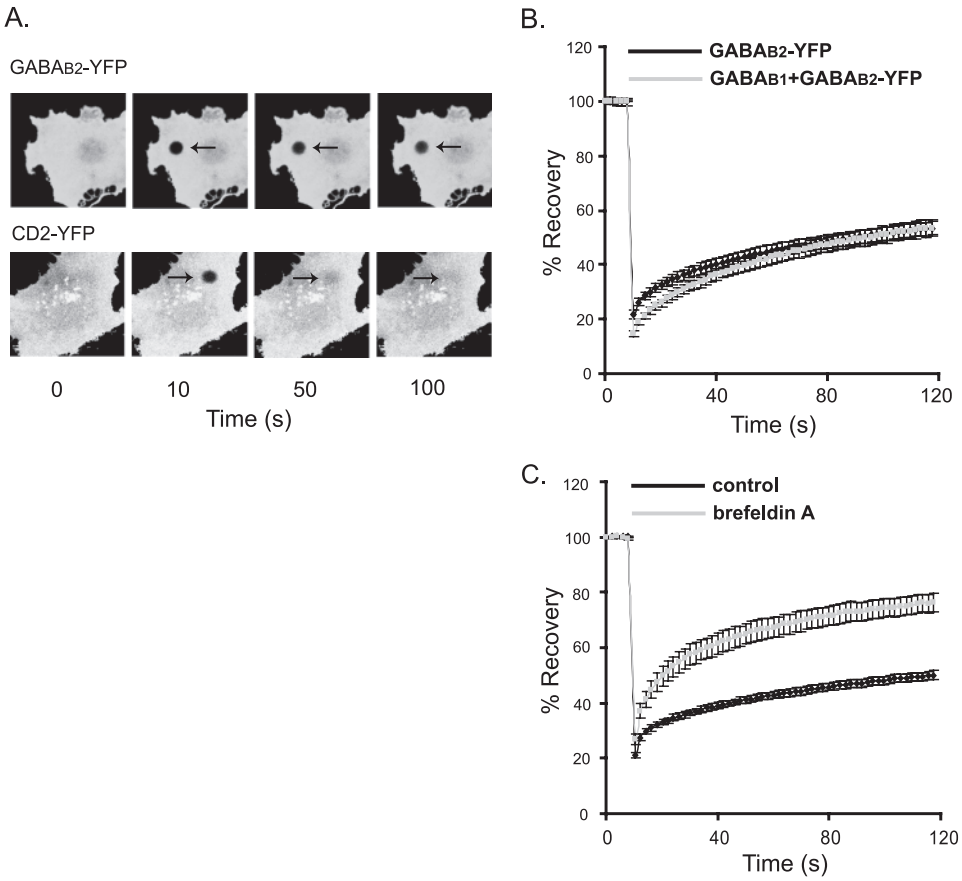

FIGURE 1. **GABA$_B$ diffuses slowly at the cell surface.** *A*, COS-7 cells were transfected with either GABA$_{B2}$-YFP (*top*) or CD2-YFP (*bottom*). A circular region (*arrow*) was bleached with a high intensity laser, and recovery of the YFP signal was imaged over time. *B*, diffusion of GABA$_{B2}$-YFP into the bleached region is very slow, whether expressed alone or together with the GABA$_{1b}$ subunit. *C*, COS-7 cells expressing GABA$_{B2}$-YFP were treated with brefeldin A, which causes retention of proteins in the endoplasmic reticulum. Within the endoplasmic reticulum GABA$_{B2}$-YFP diffused rapidly, indicating that GABA$_{B2}$ only exhibits restricted movement within the plasma membrane.

distinct cell surface dynamics that can be regulated by a region within the C terminus of the GABA$_{B2}$ subunit.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-7 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37 °C, 5% CO_2. For experiments, cells were seeded onto glass coverslips and transfected 24 h later using JetPEI (Autogen Bioclear, Calne, UK) according to the manufacturer's instructions. Cells were incubated for 48 h post-transfection before FRAP (fluorescence recovery after photobleaching) analysis. Primary neuron cultures were prepared from E18 Sprague-Dawley rat embryos. Briefly, dissected hippocampi were mechanically dissociated in Hanks' balanced salt solution lacking calcium and magnesium, supplemented with 1 mM pyruvate and 10 mM HEPES. Cells were plated immediately onto glass coverslips coated with poly-D-lysine (5 μg/ml) and cultured in Neurobasal medium containing 2% B-27 serum-free supplement, 0.5 mM L-glutamine, 25 μM glutamate, 0.05% gentamicin. To restrict the proliferation of non-neuronal cells, after 4 days the medium were changed to Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, 3 μM cytosine arabinofuranoside, 0.05% gentamicin. Neurons were transfected with Lipofectamine (Invitrogen) 5 days *in vitro* according to the manufacturer's instructions and imaged 48 h later. Approximately 1–3% of cells were transfected per coverslip; 8–10 dendrites from distinct cells were analyzed in each independent experiment.

Plasmids—HA-tagged GABA$_{B2}$ and Myc-tagged GABA$_{B1b}$ were obtained from GlaxoSmithKline, as were the GABA$_{B1b}$-YFP and GABA$_{B2}$-YFP and their CFP derivatives. All GABA$_{B2}$ truncations and chimeras were generated by PCR from the HA-tagged construct in pCDNA3.1(-). The truncated forms of GABA$_{B2}$ were produced by excising the EcoRV/HindIII fragment from the HA-tagged GABA$_{B2}$ in pCDNA3.1(-). The different regions of interest were obtained using polymerase chain reaction (PCR) to amplify them using the common forward primer 5′-GCA GGA CGG GAT ATC TCC ATC CGC CCT CTC C-3′ that covers the EcoRV site positioned in the third extracellular loop of GABA$_{B2}$. The reverse primers covered the C terminus from amino acids 920, 886, 862, or 841 and contained codons at these positions that permitted ligation in-frame into eYFP-N1 (Clontech). These were as follows: GABA$_{B2}$Δ920, 5′-GCT AAG CTT GAC GCA GGG GCT GAC ACA GCT GGC-3′; GABA$_{B2}$Δ886, 5′-GCT AAG CTT TGG GAG AGT TTA TAT CTT CTA TAC G-3′; GABA$_{B2}$ Δ862, 5′-TGT GTT CCA CTG AAG CTT GGG ATT TTG ATC GAG-3′; GABA$_{B2}$Δ841, 5′-GCA AGC TTT CCC AGG TTG AGG ATG TCA TTG AGC-3′. The PCR products were then ligated into the EcoRV/HindIII-digested HA-tagged GABA$_{B2}$ to produce the protein of interest. For production of the YFP-tagged truncated forms of GABA$_{B2}$ the plasmids containing the ligated PCR products were excised from pCDNA3.1 using NheI and HindIII, and this fragment was then ligated into NheI/HindIII-digested eYFP-N1.

The plasmid containing CD2 fused to the C-terminal tail of mGluR1a previously described (18) was digested with BamHI and NotI to remove the mGluR1a sequence. YFP (Clontech) was amplified by PCR with the primers 5′-GAC TCA GAT CTC GAG CTA AGC TTC GAA TTC-3′ and 5′-GAT CTA GAG TCG CGG CCG CTT TAC TTG TAC-3′ containing 5′-BglII and 3′-NotI sites. The PCR product was gel-purified, digested with the appropriate enzymes, and ligated into the digested CD2 plasmid to give the construct CD2-YFP. This in turn was digested with XhoI and BamHI and ligated with the PCR product obtained from amplifying the C-terminal tail of the GABAB$_{B2}$ subunit using the primers 5′-GTG CCG AAG

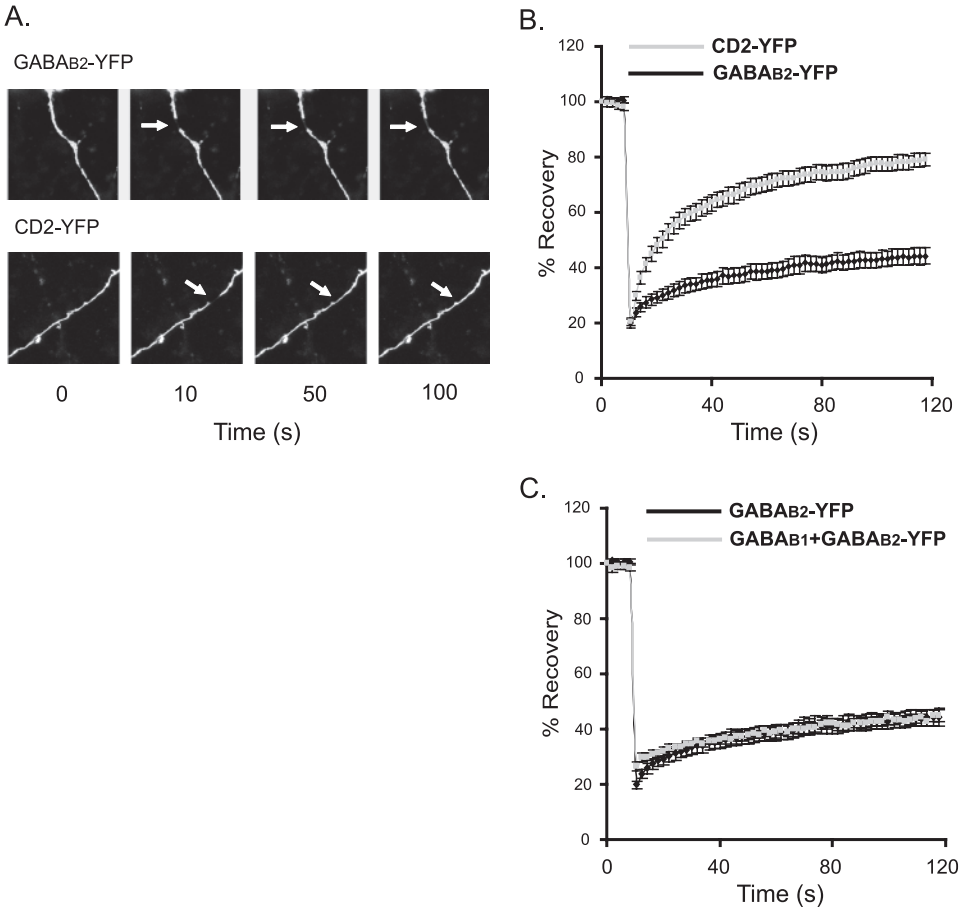

FIGURE 2. **GABA$_{B2}$ diffuses slowly in hippocampal neurons.** *A* and *B*, cultured rat hippocampal neurons were transfected with either GABA$_{B2}$-YFP or CD2-YFP. Protein diffusion was monitored in dendrites using FRAP analysis. GABA$_{B2}$-YFP diffused slowly compared with diffusion of the control protein CD2-YFP. *C*, co-expression of GABA$_{B2}$-YFP with GABA$_{B1}$ did not alter the rate of diffusion of GABA$_{B2}$-YFP. Values represent means ± S.E.

CTC GAG ACC CTG AGA ACA AAC-3′ and 5′-CCA CGG ATC CAG GCC CGA GAC CAT GAC TCG-3′, after digestion with the same enzymes, to give CD2-R2-YFP. This contained the N-terminal and transmembrane domain of CD2 followed by the C terminus of the GABA$_{B2}$ subunit and then YFP. To give a soluble form of the GABA$_{B2}$ subunit C terminus that could be detected in cells, the C terminus of the subunit was amplified by PCR using the primers 5′-CTC ATC ACC CTG AGA TCT AAC CCA GAT GCA GC-3′ and 5′-CGT ATC TAG ATT ACA GGC CCG AGA CCA TGA CTC G-3′. The product was digested with BglII and XbaI and the product inserted into similarly digested pECFP-C1 (Clontech). All PCR reactions were carried out using the proofreading KOD polymerase (Invitrogen) and the conditions recommended by the manufacturer. DNA alterations to all constructs were verified by DNA sequencing.

FRAP—We subjected transfected COS-7 cells or hippocampal neurons to FRAP analysis to assess the lateral diffusion of expressed proteins (19). Cells expressing YFP-tagged proteins were maintained at 37 °C and imaged on a Zeiss LSM510 inverted confocal microscope with a ×40 oil objective. Regions of interest (ROIs) in COS-7 cells were circular with a diameter of 7 μm; in hippocampal neurons, the ROIs were 7-μm lengths of dendritic processes. ROIs were scanned for 5 cycles with an Argon 514 laser at 1% maximal power to determine initial fluorescence intensity before being bleached by 15 cycles at 90% maximal laser power. The fluorescence intensity of the whole cell was captured for 2 min at 1% laser power; recovery is presented as percentage of original fluorescence, corrected for any bleaching due to repetitive scanning. To examine the effects of brefeldin A (5 μg/ml; Sigma-Aldrich), baclofen (100 μM; Sigma), lactrunculin (5 μM; Sigma), or colchicine (5 μM; Sigma), the cells were incubated with the appropriate compound at 37 °C for 1 h prior to FRAP analysis. For antagonist treatments, cells were incubated with the GABA$_B$ antagonist CGP-54626 (4.2 μM; Tocris Cookson, Ellisville, MO) for 10 min and then co-treated with antagonist (4.2 μM) and baclofen (100 μM) for 1 h prior to FRAP analysis. For treatment with antagonist only, cells were incubated with 4.2 μM CGP-54626 for 1 h.

For diffusion analysis of HA-tagged proteins, we incubated COS-7 cells expressing these constructs with an anti-HA antibody (kindly provided by GlaxoSmithKline) conjugated with the fluorophore Alexa 488 (Alexa Fluor 488 Protein Labeling kit; Molecular Probes, Eugene, OR) for 20 min at 37 °C. The cells were washed twice with Hanks' balanced salt solution and then imaged as described above, except using an Argon 488 laser instead of the 514 laser.

Data analyses were performed using Igor Pro 5.05 software (Wavemetrics, Lake Oswego, OR) with FRAP plug-in written by K. Miura (EMBL Heidelberg, Germany). Data are presented as means ± S.E., representing at least three independent experiments/group, each group containing a minimum of 10 cells. For the region of interest of each cell, the fluorescence recovery curve was best fitted with a double exponential function with Phair normalization (20). The mobile fraction and time to half-maximal recovery ($t_{½}$ maximal) was calculated from each curve.

Immunocytochemistry—Following the transfection period, COS-7 cells were treated for 1 h at 37 °C with either lactrunculin, to block actin polymerization, or colchicine, to block tubulin polymerization. After treatment, cells were washed once with PBS, fixed for 5 min at in 4% paraformaldehyde at room temperature, and then washed twice with PBS and twice with Tris-saline. To detect intracellular proteins, cells were permeabilized with 0.2% Triton X-100 for 5 min. Cells were incubated for 1 h at room temperature in blocking solution containing 1% bovine serum albumin and 1% normal goat serum. To visualize actin, cells were incubated in PBS containing fluorescein isothiocyanate-labeled phalloidin (Molecular Probes) for 1 h, washed with PBS, and mounted on slides. For tubulin detection,

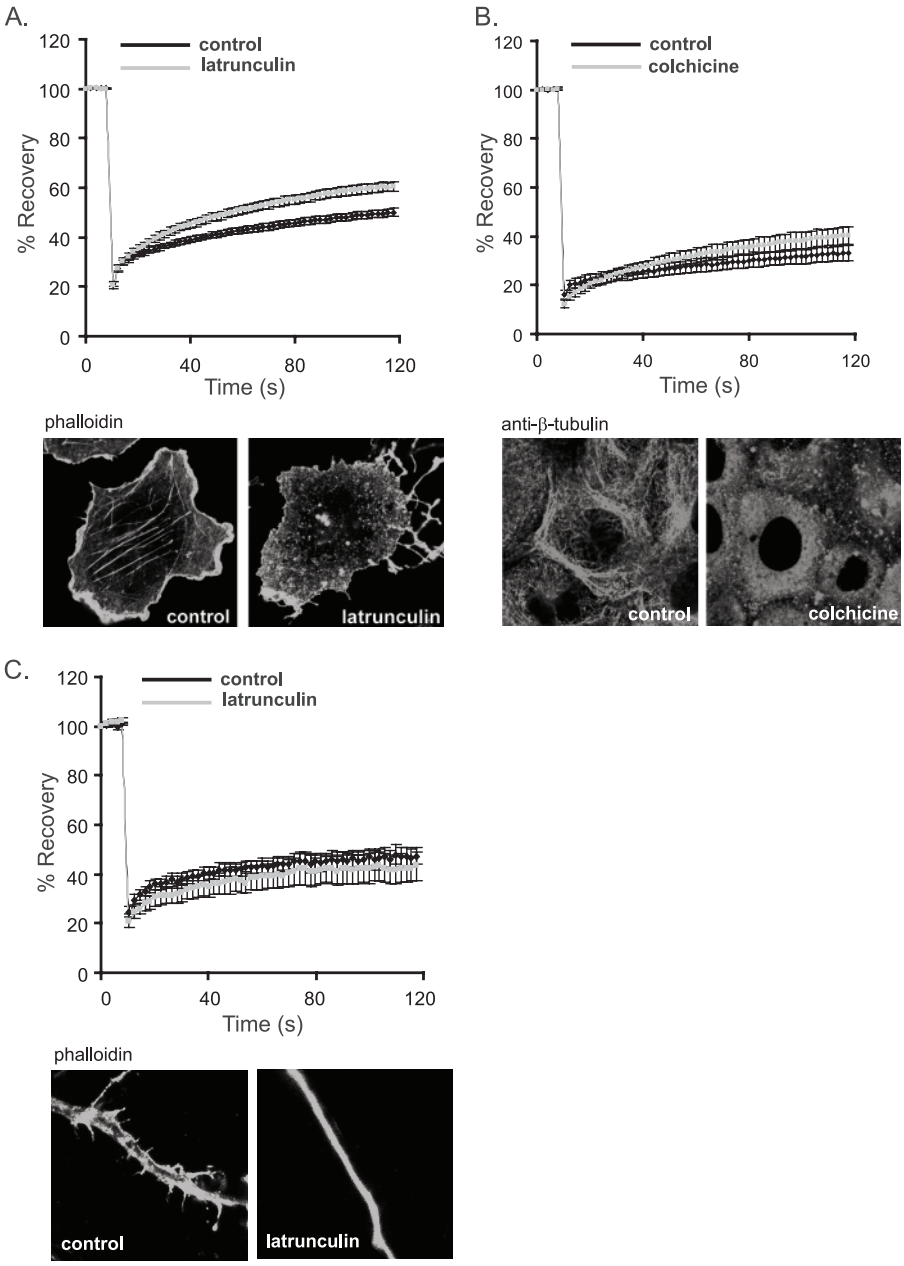

FIGURE 3. *A*, *top*, in COS-7 cells, latrunculin treatment does not significantly increase the recovery rate of GABA$_{B2}$-YFP compared with recovery in untreated cells. *Bottom*, phalloidin staining reveals that latrunculin treatment disrupts actin polymerization on COS-7 cells. *B*, *top*, treatment of COS-7 cells with colchicine did not alter GABA$_{B2}$-YFP diffusion. *Bottom*, colchicine treatment disrupts tubulin polymerization, as visualized using an antibody directed against β-tubulin. *C*, *top*, in hippocampal neurons, latrunculin treatment did not affect the diffusion rate of GABA$_{B2}$-YFP, although the treatment did disrupt dendritic actin filaments as visualized by phalloidin staining (*bottom*). Values represent means ± S.E.

the cells were incubated for 2 h at room temperature in PBS containing mouse anti-β-tubulin (1:1000; Sigma), washed three times with PBS, and incubated for 1 h with anti-mouse ALEXA 546 (Molecular Probes). After three washes with PBS, the coverslips were mounted and viewed with a Zeiss LSM510 inverted confocal microscope with a ×40 oil objective.

RESULTS

GABA$_B$ Diffuses Slowly within the Plasma Membrane, as Measured by FRAP Analysis—To determine the diffusional mobility of GABA$_{B2}$ at the cell surface, we performed FRAP experiments on COS-7 cells transiently transfected with a YFP-tagged GABA$_{B2}$ construct. We monitored subunit movement within the plasma membrane by focusing the laser excitation at the cell surface. Fluorescent proteins within a defined region of the cell were photobleached by high intensity laser, and the diffusion of unbleached proteins into the bleached region was monitored for 2 min. GABA$_{B2}$ diffused very slowly at the cell surface compared with the diffusion rate of YFP-tagged CD2, a membrane-targeted protein known to freely move within the plasma membrane (Fig. 1*A*). To examine the mobility of the GABA$_B$ receptor, cells were co-transfected with GABA$_{B1b}$-CFP and GABA$_{B2}$-YFP. In the absence of GABA$_{B2}$, GABA$_{B1b}$ did not reach the cell surface (data not shown). Movement of the GABA$_{B1b}$-GABA$_{B2}$ heterodimer was similar to the diffusion rate of GABA$_{B2}$ (Fig. 1*B*), suggesting that the restricted movement of GABA$_B$ is limited by the diffusion of the GABA$_{B2}$ subunit. Slow diffusion of GABA$_{B2}$ is specific to the plasma membrane, because retention of GABA$_{B2}$ in the endoplasmic reticulum following brefeldin A treatment increased its diffusion rate (Fig. 1*C*). To confirm that the YFP tag was not affecting subunit diffusion, we also analyzed movement of HA-GABA$_{B2}$ by live labeling of surface receptors with fluorescein isothiocyanate-labeled antibodies directed against HA (supplemental Fig. S1). No difference in mobility was detected between YFP-tagged GABA$_{B2}$ and HA-tagged GABA$_{B2}$.

Diffusion of GABA$_{B2}$ was also restricted in plasma membrane of cultured rat hippocampal neurons. As expected, transfection of primary neurons using Lipofectamine yielded transfection rates of ~1–3%. FRAP analysis of neurons transiently transfected with either GABA$_{B2}$-YFP or CD2-YFP revealed that movement of GABA$_{B2}$ was constrained within dendrites relative to movement of CD2-YFP (Figs. 2, *A* and *B*). The mobility of GABA$_{B2}$-YFP was not affected by co-expression with GABA$_{B1}$ (Fig. 2*C*).

Disruption of the Cytoskeleton Does Not Significantly Affect GABA$_{B2}$ Movement—Previous studies investigating receptor movement suggest that receptors may interact directly or indi-

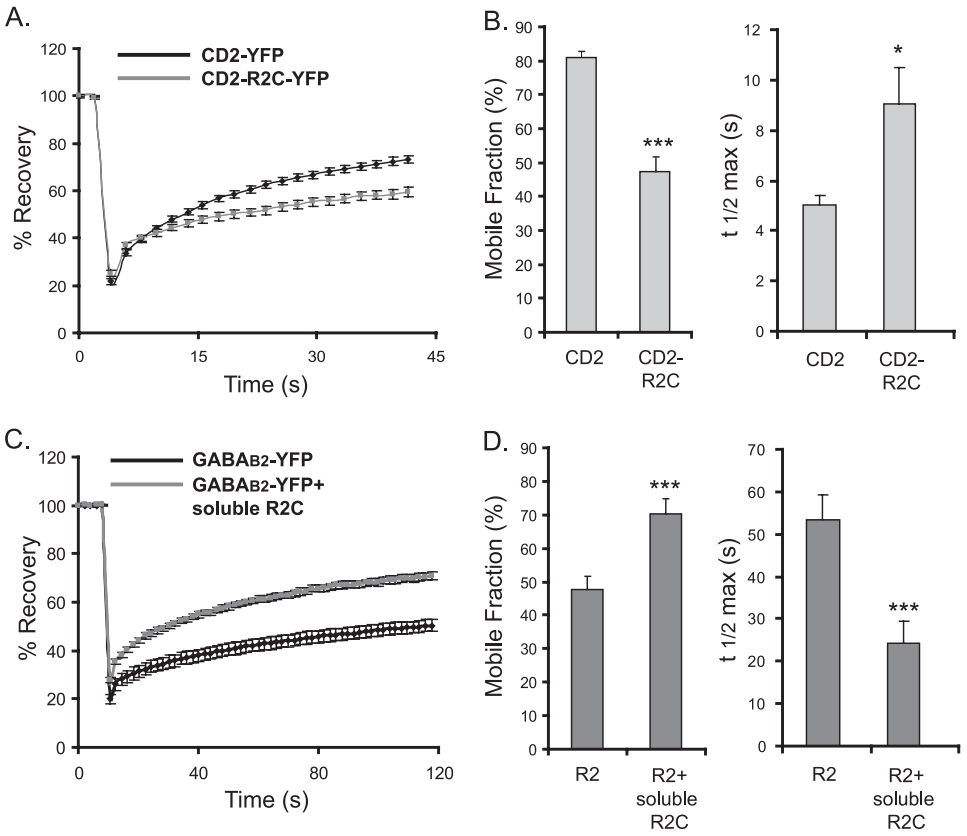

FIGURE 4. **The C terminus of GABA$_{B2}$ regulates protein diffusion.** *A* and *B*, addition of the GABA$_{B2}$ C-terminal to CD2-YFP significantly slows diffusion of this protein in the plasma membrane of COS-7 cells, as indicated by a decrease in the mobile fraction and an increase in time to half-maximal recovery. *C* and *D*, co-expression of a soluble GABA$_{B2}$ C-terminal construct with GABA$_{B2}$-YFP significantly increases diffusion of the subunit, evidenced by an increase in the mobile fraction and a decrease in time to half-maximal recovery. *R2C*, GABA$_{B2}$ C terminus; *R2*, GABA$_{B2}$-YFP. Values represent means ± S.E. *, $p < 0.05$; ***, $p < 0.001$ versus control.

rectly with the cytoskeleton. The AMPA receptor binds actin directly (21–23), whereas in the inhibitory synapse GABA$_A$ interacts with the anchoring protein gephyrin (16, 17). To determine whether slow diffusion of the GABA$_B$ receptor might be due to interaction with the cytoskeleton, we transiently transfected COS-7 cells with GABA$_{B2}$-YFP and treated them with either latrunculin (5 μM, 1 h), to inhibit actin polymerization, or colchicine (5 μM, 1 h), to inhibit tubulin polymerization. Using FRAP analysis, we found that neither latrunculin (Fig. 3*A*) nor colchicine (Fig. 3*B*) treatment substantially affected GABA$_{B2}$ lateral diffusion within the plasma membrane. Furthermore, lactrunculin treatment did not affect diffusion of GABAB2-YFP in the dendrites of hippocampal neurons (Fig. 3*C*). These results suggest that slow movement of GABA$_{B2}$ is unlikely due to direct interaction between the subunit and the cytoskeleton.

The C Terminus of GABA$_{B2}$ Regulates Its Membrane Diffusion—Because recent studies suggest that the C terminus of GABA$_{B2}$ might contain interaction regions important for trafficking (24) and stabilization (25, 26) of the fully formed GABA$_B$ receptor, we next investigated whether the C-terminal region of GABA$_{B2}$ might also be involved in regulating diffusion of the subunit at the cell surface. To determine whether the presence of the GABA$_{B2}$ C terminus is sufficient to slow diffusion of a surface protein, we ligated the GABA$_{B2}$ C terminus (R2C) to the C terminus of CD2 to generate CD2-R2C-YFP. FRAP analysis of COS-7 cells expressing either this construct or CD2-YFP revealed that the mobile fraction of CD2-R2C-YFP was significantly ($p < 0.001$) smaller compared with the mobile fraction of CD2-YFP (52.82 ± 2.63 *versus* 80.63% ± 1.43) (Fig. 4, *A* and *B*). Conversely, CD2-R2C-YFP took significantly ($p < 0.05$) more time to reach half-maximal ($t_{½}$ maximal) recovery than CD2-YFP (9.04 ± 1.42 *versus* 4.99 s ± 0.41 s) (Fig. 4*B*). CD2-R2C-YFP also diffused significantly more slowly than CD2-YFP in the dendrites of cultured hippocampal neurons (supplemental Fig. S2). Next, we constructed a soluble version of the GABA$_{B2}$ C terminus. Co-expression of COS-7 cells with GABA$_{B2}$-YFP and soluble R2C significantly ($p < 0.001$) increased the mobile fraction of GABA$_{B2}$-YFP relative to GABA$_{B2}$-YFP expressed alone (72.01 ± 3.14 *versus* 47.53% ± 3.88) and decreased $t_{½}$ maximal recovery (24.28 ± 5.01 *versus* 53.23 s ± 5.96 s) (Fig. 4, *C* and *D*). These data suggest that the GABA$_{B2}$ C terminus regulates diffusion of GABA$_B$ at the cell surface.

Lateral Diffusion of GABA$_{B2}$ Is Regulated by a 24-Amino Acid Region within the C Terminus—To determine which region of the GABA$_{B2}$ C terminus might be involved in regulating its lateral diffusion rate, we constructed a series of C-terminal-truncated forms of GABA$_{B2}$ with truncations at residues 841, 862, 886, or 920 (Fig. 5*A*). We then transfected COS-7 cells with one of the following constructs: GABA$_{B2}$-YFP, Δ920-YFP, Δ886-YFP, Δ862-YFP, or Δ841-YFP. Although plasma membrane diffusion rates of Δ920-YFP and Δ886-YFP were similar to the diffusion of GABA$_{B2}$-YFP, both Δ862-YFP and Δ841-YFP exhibited faster movement as measured by FRAP analysis than GABA$_{B2}$-YFP (Fig. 5*B*). The mobile fraction of Δ862-YFP was significantly ($p < 0.01$) higher compared with the mobile fractions of either GABA$_{B2}$-YFP or Δ886-YFP (67.91 ± 7.74 *versus* 43.24% ± 3.21 or 43.15% ± 4.39) (Fig. 5*C*). Correspondingly, the $t_{½}$ maximal recovery of Δ862-YFP was also significantly reduced relative to recovery of either GABA$_{B2}$-YFP or Δ886-YFP (7.80 ± 0.55 *versus* 47.47 ± 4.69 or 52.89 s ± 4.69 s) (Fig. 5*C*). Co-expression of GABA$_{B1}$-YFP with Δ841, compared with co-expression with GABA$_{B2}$, significantly increased its mobile fraction (71.62 ± 3.23 *versus* 48.83% ± 2.72) and decreased its $t_{½}$ maximal recovery (15.6 ± 4.32 *versus* 31.15 s ± 4.26 s) (Fig. 5*D*). These data suggest that diffusion of GABA$_{B1}$ is therefore regulated by the GABA$_{B2}$ subunit. Thus, the region of the GABA$_{B2}$ C terminus between amino acids 862– 886 may be important for regulating mobility of GABA$_B$ at the cell surface.

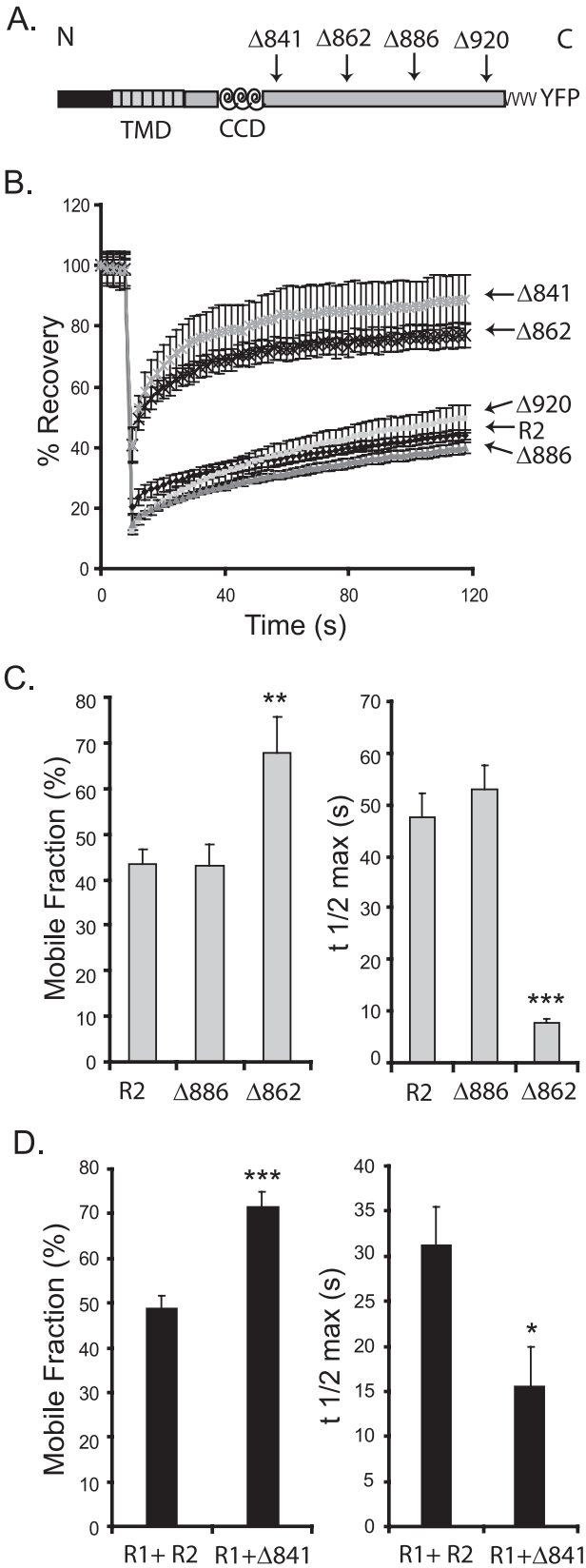

FIGURE 5. **The region of GABA$_{B2}$ between residues 862 and 886 regulates mobility of the subunit.** *A*, schematic diagram of the GABA$_{B2}$ subunit constructs. Truncations of the subunit were made at the indicated amino acid residues. The YFP tag was placed at the C terminus of each construct. *B*, Δ862-YFP and Δ841-YFP diffused rapidly within the plasma membrane of COS-7 cells compared with diffusion of R2-YFP, Δ920-YFP, and Δ886-YFP. *C*, the Δ862-YFP construct is significantly more mobile than either Δ886-YFP or R2-YFP as it exhibits a larger mobile fraction and a shorter time to half-maximal recovery. *D*, GABA$_{B1}$-YFP displays a significantly higher mobile fraction and significantly lower time to half-maximal recovery when co-expressed with Δ841 than when co-expressed with GABA$_{B2}$. *N*, N terminus; *C*, C terminus; *CCD*, coiled-coil domain; *TMD*, seven-transmembrane domain; *R2*, GABA$_{B2}$. Values represent means ± S.E.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* R2 and Δ886 groups.

Agonist Binding Increases Lateral Diffusion of GABA$_{B2}$—To determine whether lateral diffusion of GABA$_{B2}$ could be dynamically regulated, we treated COS-7 cells co-transfected with GABA$_{B1b}$-CFP and GABA$_{B2}$-YFP with the GABA$_B$ agonist baclofen. After 1 h of baclofen treatment (100 μM), cells were subjected to FRAP analysis of GABA$_{B2}$-YFP. Treatment with baclofen increased lateral diffusion of GABA$_{B2}$-YFP (Fig. 6*A*) and significantly ($p < 0.001$) increased its mobile fraction relative to untreated controls (63.49 ± 6.92 *versus* 39.62% ± 2.47) (Fig. 6*B*). Co-treatment of cells with the GABA$_B$ antagonist CGP-54626 (4.2 μM) prevented the effect of baclofen on the mobile fraction (35.69% ± 4.77; $p < 0.01$); treatment with the antagonist alone had no effect on the mobile fraction relative to the control group (Fig. 6*B*). These results suggest that, although GABA$_{B2}$ movement is restricted under basal conditions, stimulation of the receptor might alter its membrane dynamics.

DISCUSSION

In the present study, we examined lateral diffusion of the GABA$_B$ receptor within the plasma membrane using FRAP analysis of fluorescently tagged GABA$_{B2}$ subunits. We found that the GABA$_B$ receptor and the GABA$_{B2}$ subunit diffuse slowly at the cell surface in both hippocampal neurons and heterologous cells. Our data suggest that the restricted diffusion of the receptor is regulated by a region within the C terminus of the GABA$_{B2}$ subunit.

The diffusion characteristics of GABA$_B$ differ from those reported for other neurotransmitter receptors. We consistently found GABA$_B$ to exhibit a mobile fraction of ~40%. By contrast, AMPA receptors are reported to have a mobile fraction of >80% both in COS-7 cells (27) and in extrasynaptic regions of neurons (13). Similarly, the GABA$_A$ receptor has been shown to diffuse rapidly within the extrasynaptic membrane (28). Rapid diffusion is not restricted to receptor ion channels, as the G-protein-coupled serotonin 5-HT1a (29) and dopamine D1 (30) receptors also move freely in extrasynaptic membrane. Taken together, the data indicate that GABA$_B$ receptors exhibit distinctive diffusion dynamics.

To assess the regulation of GABA$_B$ diffusion, we examined the effect of disrupting the cytoskeleton on receptor movement. We observed that the restricted diffusion of GABA$_{B2}$ was not altered following latrunculin or colchicine treatment. In contrast, disruption of microtubules increases the diffusion rate of glycine receptors (28) and disturbance of the actin cytoskeleton by latrunculin has been shown to disrupt AMPA clustering due to interaction between the GluR1 subunit and the actin complex (21–23). Our data indicate that direct interactions between GABA$_{B2}$ and either actin or tubulin are unlikely to be responsible for anchoring GABA$_B$ at the cell surface. Similarly, diffusion of the GABA$_A$ receptor appears to be unaffected by latrunculin treatment (31) and diffusion of the

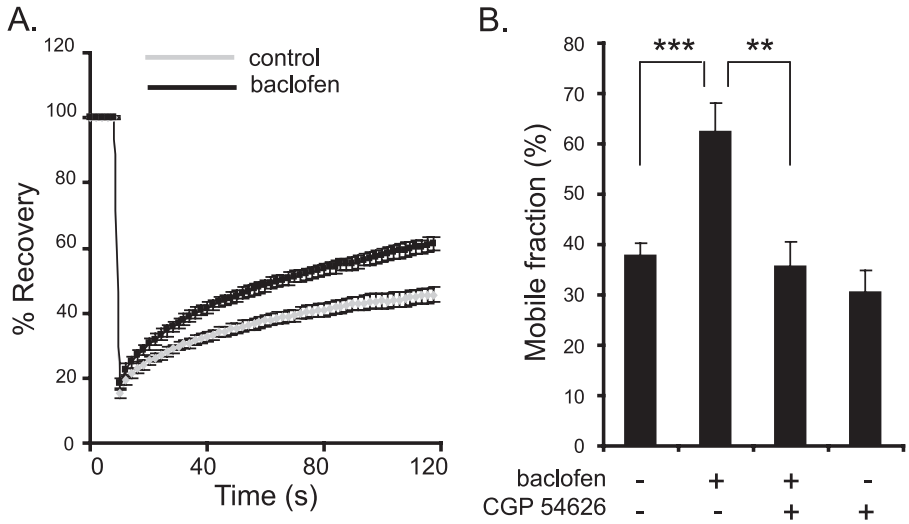

FIGURE 6. **Stimulation of the GABA$_B$ receptor increases its diffusion rate.** *A*, COS-7 cells transfected with GABA$_{B1b}$-CFP and GABA$_{B2}$-YFP were treated with the GABA$_B$ agonist baclofen. FRAP analysis of receptor movement was performed by bleaching the YFP signal and recording its recovery. *B*, baclofen treatment significantly increased the mobile fraction of the GABA$_B$ receptor. Co-treatment of cells with baclofen and the GABA$_B$ antagonist CGP-54626 prevented the baclofen-induced increase in the mobile fraction. Treatment of the cells with the antagonist alone had no effect. Values represent means ± S.E.; **, $p < 0.01$; ***, $p < 0.001$.

dopamine D1 receptor is unaffected by microtubule disruption (30). Diffusion of these receptors is likely regulated by interactions with intracellular proteins other than actin, as movement of GABA$_A$ is restricted when bound to gephyrin (16) and the D1 receptor is anchored via a direct interaction with the *N*-methyl-D-aspartate receptor (30). These findings suggested that GABA$_{B2}$ might possess an intracellular region capable of similar protein interactions.

We next investigated whether the C terminus of GABA$_{B2}$ might regulate its diffusion. Intracellular regions are likely to be involved in movement of receptor at the cell surface, as a recent study has demonstrated that an intracellular loop of GABA$_A$ regulates its diffusion (32). Previous studies have demonstrated that the C terminus of the GABA$_{B2}$ subunit contains several important regions, such as those involved in dimerization with GABA$_{B1}$ (6), G-protein binding (8–10), and mediating interactions with scaffolding proteins (33). In the present study we found that attachment of the GABA$_{B2}$ C terminus to a freely diffusing protein significantly slowed diffusion of that protein within the plasma membrane. Furthermore, when GABA$_{B2}$ was co-expressed with a soluble form of its C terminus, the diffusion rate of the subunit was increased. Taken together, these findings suggest that the C-terminal region of GABA$_{B2}$ does regulate its dynamics at the cell surface.

To determine whether a specific domain within the C terminus controls GABA$_{B2}$ diffusion, we constructed a series of truncated subunits. FRAP analysis of these constructs revealed that the region of the GABA$_{B2}$ C terminus between residues 862 and 886 is critical for restricting movement of the subunit at the cell surface, as removal of this region significantly increased its diffusion rate. Furthermore, our data on diffusion of GABA$_{B1}$-YFP indicate that the C terminus of GABA$_{B2}$ regulates movement of the heterodimerized receptor. MUPP1, a scaffolding protein, binds to a motif within the GABA$_{B2}$ C terminus (33); however, this interaction is unlikely to regulate GABA$_B$ diffusion because the motif (residues 938–941) is distal to the domain identified in the present study. In addition, truncation of the C-terminal region of GABA$_{B2}$ containing the MUPP1 binding motif did not affect GABA$_B$ diffusion. In the present study, we have identified a domain involved in regulation of GABA$_B$ movement, although the protein or proteins that might interact with GABA$_B$ at this site remain to be determined. Nevertheless, these data indicate interaction sites exist on the GABA$_{B2}$ C terminus that are important for trafficking, stability, and movement of GABA$_B$ at the cell surface.

We investigated whether stimulation of GABA$_B$ might affect receptor movement, as previous studies have demonstrated that movement of other metabotropic receptors, such as the serotonin 5-HT1a receptor, is greater following agonist stimulation (29, 34). We treated cells expressing GABA$_{B1}$ and GABA$_{B2}$ with the agonist baclofen and found that baclofen stimulation significantly increased its diffusion rate; the effect of baclofen was prevented by co-treatment with a GABA$_B$ antagonist. Agonist binding may induce a conformational change in the receptor that alters its interactions with intracellular scaffolding proteins. Our data suggest a mechanism by which release of neurotransmitter into the synapse might increase diffusion of nearby extrasynaptic receptors, thereby allowing movement into the post-synaptic density.

Regulation of receptor diffusion, and therefore receptor number, is crucial for maintaining synaptic strength. Recently, the dynamics of several neurotransmitter receptors, including AMPA, GABA$_A$, and glycine, have been described (13, 27, 28, 35). In the present study we have demonstrated for the first time that GABA$_B$ exhibits restricted movement within the plasma membrane; furthermore, we determined that diffusion of GABA$_B$ is regulated by a specific region within the C terminus of the GABA$_{B2}$ subunit. These findings provide insight into the regulation of GABA$_B$ receptor movement at the cell surface and are consistent with the hypothesis that regulated lateral diffusion is a mechanism by which the cell controls synaptic strength.

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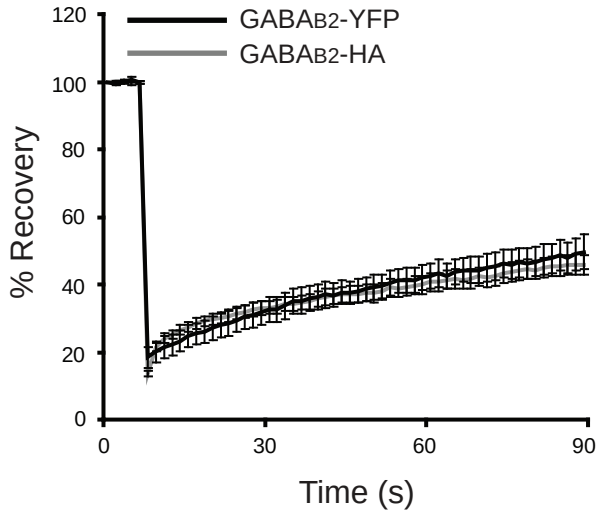
Suppl Figure 1



Suppl Figure 2