GABAB Receptor Association with the PDZ Scaffold Mupp1 Alters Receptor Stability and Function*

Srividya Balasubramanian, Sami R. Fam, and Randy A. Hall[1]
From the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

γ-Aminobutyric acid, type B (GABAB) receptors are heterodimeric G protein-coupled receptors that mediate slow inhibitory synaptic transmission in the central nervous system. To identify novel interacting partners that might regulate GABAB receptor (GABABR) functionality, we screened the GABABR2 carboxyl terminus against a recently created proteomic array of 96 distinct PDZ (PSD-95/Dlg/ZO-1 homology) domains. The screen identified three specific PDZ domains that exhibit interactions with GABABR2: Mupp1 PDZ13, PAPIN PDZ1, and Erbin PDZ. Biochemical analysis confirmed that full-length Mupp1 and PAPIN interact with GABABR2 in cells. Disruption of the GABABR2 interaction with PDZ scaffolds by a point mutation to the carboxyl terminus of the receptor dramatically decreased receptor stability and attenuated the duration of GABAB receptor signaling. The effects of mutating the GABABR2 carboxyl terminus on receptor stability and signaling were mimicked by small interference RNA knockdown of endogenous Mupp1. These findings reveal that GABAB receptor stability and signaling can be modulated via GABABR2 interactions with the PDZ scaffold protein Mupp1, which may contribute to cell-specific regulation of GABAB receptors in the central nervous system.

GABAB[2] receptors are G protein-coupled receptors responsible for mediating slow inhibitory synaptic transmission by the neurotransmitter GABA (1). They belong to G protein-coupled receptor Family C and bear a high degree of homology to other family members such as the metabotropic glutamate receptors, calcium receptor, and vomeronasal receptors. GABAB receptors are believed to be heterodimeric combinations of two G protein-coupled receptors, GABABR1 and GABABR2 (2–4). Heterodimerization of GABABR1 and GABABR2 is necessary for the proper trafficking of GABABR1 to the cell surface (5, 6). In the context of the heterodimer, GABABR1 is thought to bind the ligand (7), whereas GABABR2 is believed to be the primary G protein contact site (8–11).

Given that GABAB receptors are important therapeutic targets for a wide variety of diseases, including depression, anxiety, epilepsy, and drug addiction (12, 13), understanding GABAB receptor signaling and regulation is of significant clinical interest. The cloning of the GABAB receptors has advanced the study of the GABAB receptors substantially over the past decade. However, some discrepancies between the properties of native GABAB receptors and heterologously expressed recombinant receptors still remain. For example, GABAB receptors in native tissue undergo robust endocytosis and desensitization (14), whereas recombinant GABABR1/GABABR2 expressed in most heterologous cells neither internalize nor desensitize (14, 15). One possible explanation for such discrepancies is that GABAB receptor signaling and trafficking properties are highly dependent on cellular context. This implies that interaction with differentially expressed cellular proteins might modulate GABAB receptor function. Indeed, we previously reported that association of GABAB receptors with the GABAA receptor γ2S subunit confers agonist-mediated endocytosis on GABAB receptors expressed in heterologous cells (16). Furthermore, GABAB receptors have also been shown to be regulated by interactions with several other protein partners, including the transcription factors cAMP-response element-binding protein 2 and ATF4 (17, 18), the adaptor protein 14-3-3 (19), the RNA-binding protein Marlin-1 (20), and the coat protein I (21).

GABABR2 possesses a carboxyl-terminal motif (VSGL) that has the potential to interact with PDZ-domain containing scaffold proteins. PDZ (PSD-95/Discs-large/ZO-1) domains are 90-amino acid protein-protein interaction modules that recognize and bind to specialized motifs in the distal carboxyl termini of target proteins such as G protein-coupled receptors and ion channels (22). Multiple PDZ domains on the same PDZ protein can allow these proteins to act as scaffolds for the assembly of large protein complexes at the cell surface. In addition, PDZ proteins can play crucial roles in regulating the sorting, clustering, trafficking, signaling, and stability of proteins in multicellular organisms (23). More than 440 PDZ domains are predicted to exist in the human genome, of which more than a quarter are likely to be Class I PDZ domains based on the amino

[1] To whom correspondence should be addressed: Dept. of Pharmacology, Emory University School of Medicine, 5113 Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-3699; Fax: 404-727-0365; E-mail: rhall@pharm.emory.edu.

[2] The abbreviations used are: GABAB, γ-aminobutyric acid type B; GABAAR, GABA type A receptor; GABABR1, GABAB receptor 1; GABABR2, GABAB receptor 2; GBR1, GABABR1; GBR2, GABABR2; PDZ, PSD-95/Drosophila Discs Large/ZO1 homology; PSD, post synaptic density; ZO, Zona occludens; CREB, cAMP response element-binding protein; ATF, activating transcription factor; GST, glutathione S-transferase; CT, carboxyl terminus; Mupp1, multi PDZ domain protein 1; ERBIN, ErbB2 interacting protein; PAPIN, plakophilin-related *armadillo* repeat protein-interacting PDZ protein; ERK, extracellular signal-regulated kinase; HtrA, high temperature requirement; 5-HT2C, 5-hydroxytryptamine receptor type 2C; c-Kit, class III transmembrane tyrosine kinase receptor; TAPP1, tandem PH-domain-containing protein-1; NG2, protein new-glue 2 precursor; siRNA, small interference RNA; HA, hemagglutinin; GFP, green fluorescent protein; BSA, bovine serum albumin.

acid requirement for their binding partners. Class I PDZ proteins bind to the motif $(S/T)X\Phi$, where Φ represents a hydrophobic residue at the carboxyl terminus and X represents any amino acid. The $GABA_BR2$ carboxyl-terminal motif of VSGL thus conforms to the preferred binding motif for Class I PDZ domains and may therefore interact with PDZ proteins that could potentially regulate $GABA_B$ receptor function.

In this study, we screened a proteomic array consisting of known or putative Class I PDZ domains to identify PDZ proteins that might interact with $GABA_BR2$. We identified three PDZ proteins that interact with the $GABA_BR2$ carboxyl terminus: Mupp1, Erbin, and PAPIN. We further studied the interactions of these proteins with $GABA_BR2$ in cells and examined the roles of these interactions in regulating $GABA_B$ receptor signaling, trafficking, and stability.

MATERIALS AND METHODS

Construction of the PDZ Domain Proteomic Array—PDZ protein cDNA constructs were kindly donated by a large number of colleagues (24, 25). These cDNAs were used as templates to amplify by means of PCR the regions encoding various PDZ domains, which were ultimately subcloned into pET30A for fusion protein expression. PDZ domains were expressed as His- and S-tagged fusion proteins by using the vector pET30A (Novagen) and purified using ProBond nickel resin (Invitrogen).

Plasmids—Epitope-tagged (HA-, FLAG-, Myc-, and His-tagged) versions of human $GABA_BR1b$ and $GABA_BR2$ in the mammalian expression vector pcDNA3.1 were kindly provided by Fiona Marshall (GlaxoSmithKline). Myc-Mupp1 was kindly provided by Dr. Yoko Hamazaki (Kyoto University). GFP-PAPIN was kindly provided by Dr. Yutaka Hata (Tokyo Medical and Dental University). Myc-Erbin was kindly provided by Dr. Amy Lee (Emory University). FLAG-$GABA_BR2V938A$, FLAG-$GABA_BR2S939A$, and FLAG-$GABA_BR2L941A$ mutants were generated using a site-directed mutagenesis kit from Stratagene.

Overlay Assays—To assess the binding of receptor carboxyl-terminal GST fusion proteins to the PDZ domain array, the purified PDZ domain fusion proteins were spotted at 1 µg per bin onto Nytran SuperCharge 96-grid nylon membranes (Schleicher & Schuell). The membranes were allowed to dry overnight and then blocked in "blot buffer" (2% nonfat dry milk/0.1% Tween 20/50 mM NaCl/10 mM HEPES, pH 7.4) for 1 h at room temperature. GST-$GABA_BR2$ carboxyl terminus (CT) was prepared via PCR amplification of the region encoding the last 35 amino acids of rat $GABA_BR2$ and subcloned into the pGEX-4T1 vector (Amersham Biosciences) using EcoR1 and XhoI restriction enzymes. Overlays with $GABA_BR2$-CT fusion protein (100 nM in blot buffer) were then performed by using a previously described technique (24, 25).

Fusion Protein Pull-down Assays—Hexahistidine-tagged PDZ domain fusion proteins were grown in *Escherichia coli* and purified on ProBond nickel resin (Invitrogen). Aliquots of the fusion protein on beads were blocked for 30 min with 1 ml of a 3% "BSA buffer" (10 mM HEPES, 50 mM NaCl, 0.1% Tween 20, 3% BSA) at 4 °C. Solubilized lysates from transfected COS-7 cells were then incubated with the beads end-over-end at 4 °C for 2 h. Following three washes with 1 ml of BSA buffer, the proteins were eluted off of the beads with sample buffer,
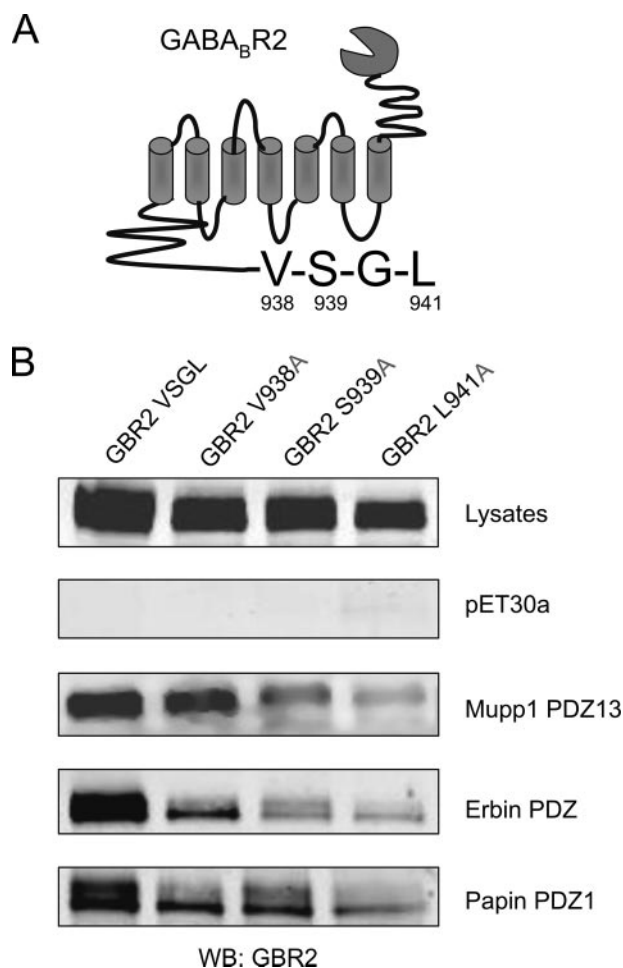

FIGURE 1. **$GABA_BR2$ binds to Mupp1, Erbin, and PAPIN PDZ domains via the carboxyl-terminal VSGL motif.** The amino acids Val-938, Ser-939, and Leu-941 on the CT of $GABA_BR2$ (*A*) were sequentially mutated to alanine, and wild-type GBR2-VSGL as well as the three mutants, GBR2-V938A, GBR2-S939A, and GBR2-L941A, were expressed in COS-7 cells. The cell lysates were then incubated with three distinct PDZ domains fusion proteins (Mupp1 PDZ13, Erbin, and Papin PDZ1) in a pull-down experiment (*B*). Western blot analysis with anti-GBR2 antibody revealed that wild-type GBR2 interacts robustly with all three fusion proteins. The serine and valine GBR2 mutants exhibited only partial binding to the fusion proteins, whereas the GBR2 leucine mutant showed markedly decreased binding to all the three fusion proteins. The data shown are representative of three independent experiments.

resolved via SDS-PAGE, and analyzed via Western blot using appropriate antibodies.

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from Invitrogen. COS-7 and HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO_2 incubator. For heterologous expression of receptors, 2–4 µg of cDNA was mixed with 15 µl of Lipofectamine 2000 (Invitrogen) and added to 5 ml of serum-free medium in 10-cm tissue cultures plates containing cells at 80–90% confluency. Following overnight incubation, the medium was replaced with 12 ml of complete media, and the cells were harvested 24 h later.

Cerebellar Granule Neuron Culture—Primary cultures of cerebellar granule neurons were obtained from 7-day-old Sprague-Dawley rats. Isolated cerebella were stripped of meninges, minced by mild trituration with a Pasteur pipette,
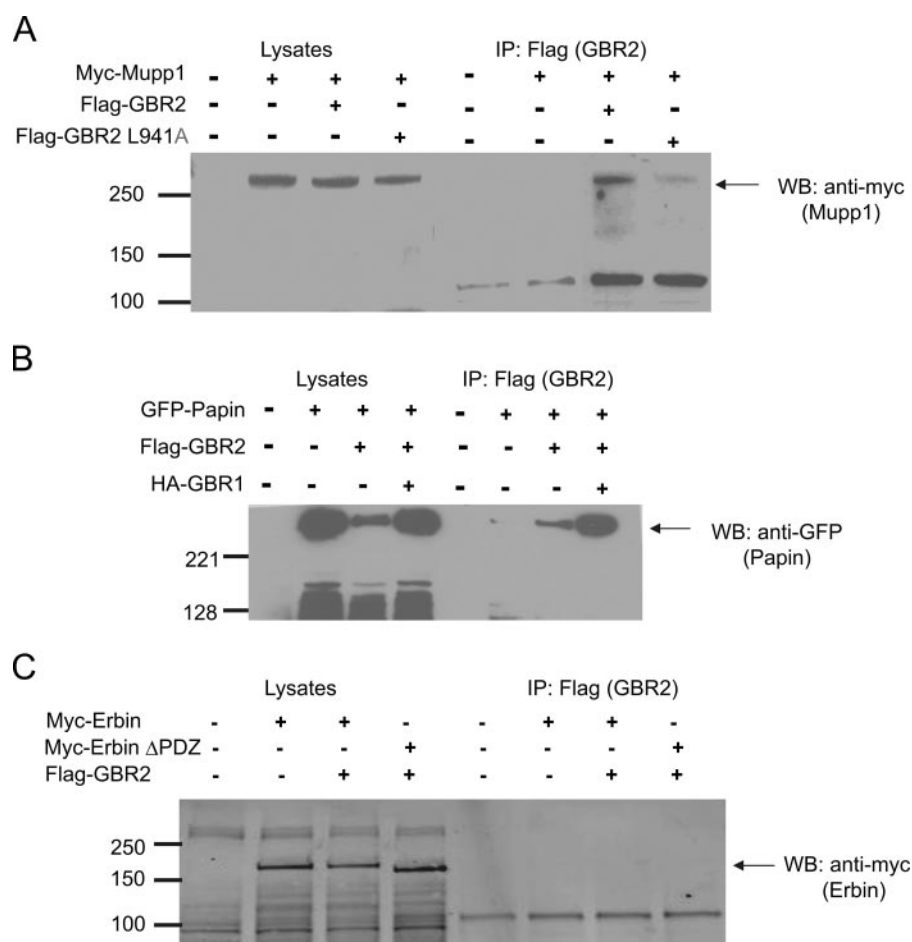
GABA_B Receptor Regulation by Mupp1

FIGURE 2. **Co-immunoprecipitation of PDZ proteins and GABA_B receptors.** *A,* Myc-tagged Mupp1 was expressed in the presence and absence of FLAG-tagged wild-type GBR2 or FLAG-tagged GBR2 L941A mutant in COS-7 cells. GBR2 was immunoprecipitated with anti-FLAG resin, and the immunoprecipitates were probed with anti-Myc antibodies. Western blot analysis revealed robust co-immunoprecipitation of Mupp1 with wild-type GBR2 and markedly decreased co-immunoprecipitation with the GBR2 L941A mutant. *B,* GFP-tagged PAPIN was expressed in the presence and absence of GBR2 and GBR1. When GBR2 was immunoprecipitated with anti-FLAG resin and the immunoprecipitates were probed with anti-GFP antibodies, PAPIN was found to be co-immunoprecipitated with both GBR2 alone and GBR1/GBR2. *C,* COS-7 cells were transfected with Myc-Erbin or Myc-ErbinΔPDZ in the presence and absence of GABA_B receptors. GBR2 was immunoprecipitated with anti-FLAG resin, and the immunoprecipitates were probed with anti-Myc antibodies. Neither Erbin nor ErbinΔPDZ was found to co-immunoprecipitate with GBR2. These experiments show that Mupp1 and PAPIN, but not Erbin, interact with GABA_B receptors in cells. These data are representative of at least four independent experiments each.

nometer-based surface expression assay as described previously (16). Purified proteins, cell extracts, and/or immunoprecipitated samples were separated by SDS-PAGE, blotted onto nitrocellulose, and detected with appropriate antibodies as described previously (16).

Antibodies—The primary antibodies utilized were M2 monoclonal anti-FLAG antibody (Sigma), horseradish peroxidase-coupled 12CA5 anti-HA antibody (Roche Applied Science), monoclonal anti-c-*myc* 9E10 antibody (Sigma), anti-GABA_BR1 antibody, anti-GABA_BR2 antibody (Chemicon), anti-Mupp1 antibody (Upstate Biotechnology), and anti-GFP antibody (BD Biosciences).

Double Immunofluorescence Microscopy—Cerebellar granule neurons or transfected COS-7 cells were plated in culture slides, fixed with 4% paraformaldehyde, and permeabilized with buffer containing 2% bovine serum albumin and 1% Triton X-100 in phosphate-buffered saline for 30 min at room temperature. The cells were then incubated with anti-GABA_BR2 antibody (Chemicon) plus either monoclonal anti-Mupp1 (Upstate) or anti-c-*myc* 9E10 antibody (Sigma) for 1 h at room temperature. After three washes (5 min) with buffer, the cells were incubated with a Rhodamine Red-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories)

and treated with trypsin for 15 min at 37 °C. Granule cells were then dissociated by three successive trituration and sedimentation steps in DNase-containing Neurobasal media, centrifuged, and resuspended in Neurobasal medium containing 10% heat-inactivated fetal bovine serum, B-27 serum-free supplement, 0.5 mM glutamine, 25 μM glutamic acid, and 25 mM KCl. The neurons were plated onto poly-D-lysine-coated culture slides (Fisher) at a density of ~0.25 × 10^6 cells/well and incubated at 37 °C in a 5% CO₂/95% humidity atmosphere. Cytosine arabinoside (10 μM) was added after 18–24 h to inhibit replication of non-neuronal cells.

Immunoprecipitation, Surface Expression Assay, and Western Blotting—Co-immunoprecipitation of full-length proteins from COS-7 cells was performed using appropriate primary antibodies and methods described previously (16). Monoclonal anti-FLAG M2 antibody resin (Sigma) was the primary antibody used to immunoprecipitate epitope-tagged proteins. Surface expression of GABA_B receptors was verified using a lumi-

for 30 min at room temperature. After three washes (5 min) with buffer, 4′,6-diamidino-2-phenylindole was used to label the nucleus. After one wash with phosphate-buffered saline, coverslips were mounted, and Rhodamine Red-labeled Mupp1 and fluorescein isothiocyanate-labeled GABA_BR2 were visualized with a Zeiss LSM-510 laser confocal microscope. Multiple control experiments, utilizing either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody, revealed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in the cells was specific.

Pulse-Chase Analysis—Transiently transfected COS-7 cells were split into 60-mm tissue culture plates. Approximately 40 h after transfection, the cells were washed with sterile phosphate-buffered saline and incubated for 30 min in methionine-free Dulbecco's modified Eagle's medium (BIOSOURCE). 60 μCi of Redivue L-[^{35}S]methionine (Amersham Biosciences) was added to each plate and incubated for another 30 min. The radioactive

GBR2 MUPP1 MERGE

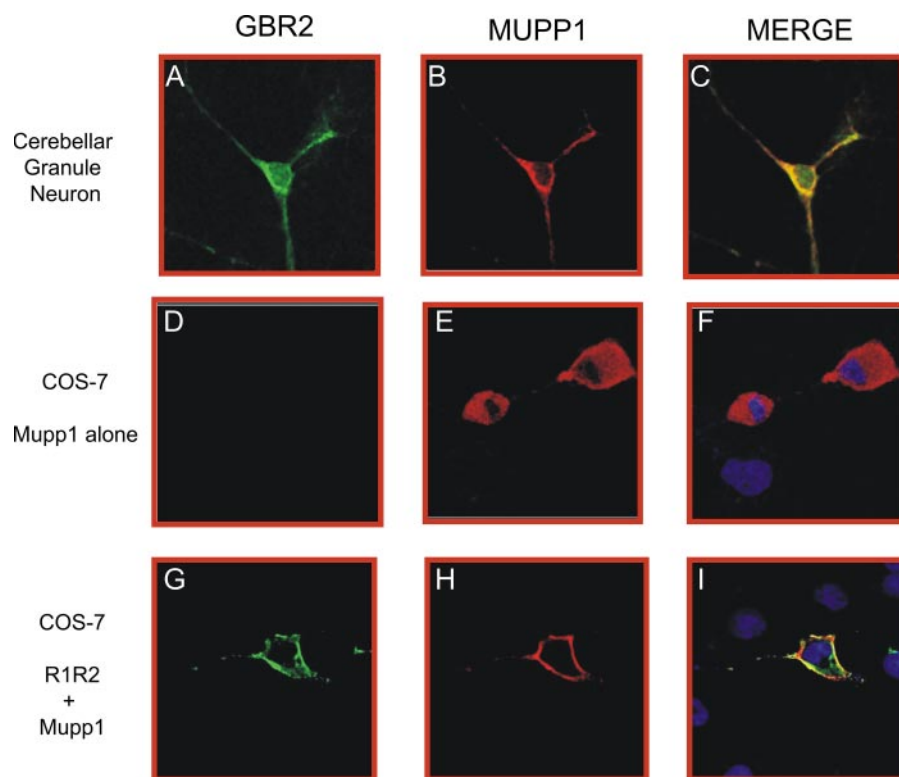
FIGURE 3. **GABA$_B$ receptors and Mupp1 exhibit overlapping patterns of expression in cultured cerebellar granule neurons and transfected cells.** *A–C*, cerebellar granule neurons were fixed and labeled with appropriate antibodies. GABA$_B$ receptors (fluorescein isothiocyanate) and Mupp1 (Rhodamine Red) were co-localized on the plasma membrane of these neurons along the cell bodies and cell processes. These data are representative of two independent experiments. *D–F*, COS-7 cells were transfected with Myc-tagged Mupp1 alone and fixed. Labeling with anti-Myc primary antibody and Rhodamine Red conjugated secondary antibody showed diffuse intracellular Mupp1 staining. Cell nuclei are labeled via 4′,6-diamidino-2-phenylindole and shown in blue. *G–I*, in COS-7 cells co-transfected with Myc-tagged Mupp1, FLAG-tagged GABA$_B$R2, and HA-tagged GABA$_B$R1, Mupp1 (Rhodamine Red) was found predominantly on the plasma membrane. These data are representative of four or five independent experiments each.

Calcium Imaging—The Ca^{2+}-sensitive fluorophore fura-2AM (Molecular Probes) was used for ratiometric Ca^{2+} imaging in COS-7 cells. All fluorescence measurements were made from subconfluent areas of the dishes so that individual cells could be readily identified. After transfection in 100-mm plates, cells were split onto coverslips immersed in 0.5 ml of media in 24-well plates and grown for 1–2 days. Before imaging, coverslips were incubated at room temperature for 30 min in extracellular recording solution composed of 150 mM NaCl/10 mM Hepes/3 mM KCl/2 mM CaCl$_2$/2 mM MgCl$_2$/5.5 mM glucose, pH 7.3, 325 mosM. Extracellular recording solution was supplemented with pluronic acid (0.001%) and fura-2 AM (2 μM). Subsequently, coverslips were thoroughly rinsed with extracellular solution lacking fura-2AM and BSA and mounted onto the microscope stage for imaging. Intensity images of 510 nm emission wavelengths were taken at 340 and 380 nm excitation wavelengths, and the two resulting images were taken from individual cells for ratio calculations. Imaging work-bench 2.2.1 (Axon Instruments, Union City, CA) was used for acquisition of intensity images and conversion to ratios. Baclofen (100 μM) was dissolved in extracellular recording solution and applied by bath perfusion.

Mupp1 siRNA—A Mupp1 siRNA construct (identification no. 107246) was purchased from Ambion along with control siRNA. Approximately 6 h after transfection with appropriate plasmids, cells were transfected for 36–48 h with 100 nM of either Mupp1 siRNA or control siRNA using TransIT-Quest transfection reagent from Mirus.

RESULTS

Screening of a PDZ Proteomic Array with the GABA$_B$R2 Carboxyl Terminus and Elucidation of the Structural Determinants of GABA$_B$R2 Binding to PDZ Proteins—To identify PDZ domain-containing proteins that might associate with the GABA$_B$R2 carboxyl terminus (GABA$_B$R2-CT), we first created a GST fusion protein corresponding to the last 35 amino acids of GABA$_B$R2, which possesses the putative PDZ binding motif VSGL. We next screened a previously reported (24, 25) proteomic array containing 96 distinct Class I PDZ domains for interactions with the GABA$_B$R2-CT-GST fusion protein. GABA$_B$R2-CT did not detectably associate with the vast majority of PDZ domains on the array but did specifically interact with three PDZ domains: Mupp1-PDZ13, PAPIN-PDZ1, and Erbin-

media was removed; the cells were washed with sterile phosphate-buffered saline and then chased with Dulbecco's modified Eagle's medium supplemented with 3 mM cold L-methionine (Sigma) for various time periods. Cells were harvested at 0-, 1-, 2-, 4-, 8-, 12-, 24-, and 48-h time points and frozen at −80 °C. The cells were solubilized, adjusted for protein concentration, and immunoprecipitated using anti-FLAG resin. The immunoprecipitates were run on an SDS-PAGE gel, dried, and exposed to a phosphor screen for 1 week. The autoradiographic images were obtained with a phosphorimaging device (Typhoon) and analyzed with ImageQuaNT and GraphPad prism software. Within each experiment, the values of GABA$_B$R2 expression at the zero time point were considered as 100%, and then other time point values were normalized as a percentage of this starting value. The averaged data were subjected to nonlinear regression curve fitting (one phase exponential decay) to determine the protein half-life values.

ERK Activation Assay—Transfected HEK-293 cells were plated in 35-mm tissue culture plates at 80% confluency and serum starved overnight the day before the assay. The cells were stimulated with 200 μM baclofen for specified time periods, rinsed with ice-cold phosphate-buffered saline/Ca^{2+}, and lysed in 80 μl of sample buffer. The cell lysates were run on SDS-PAGE gels and then analyzed via Western blotting with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies (Cell Signaling).
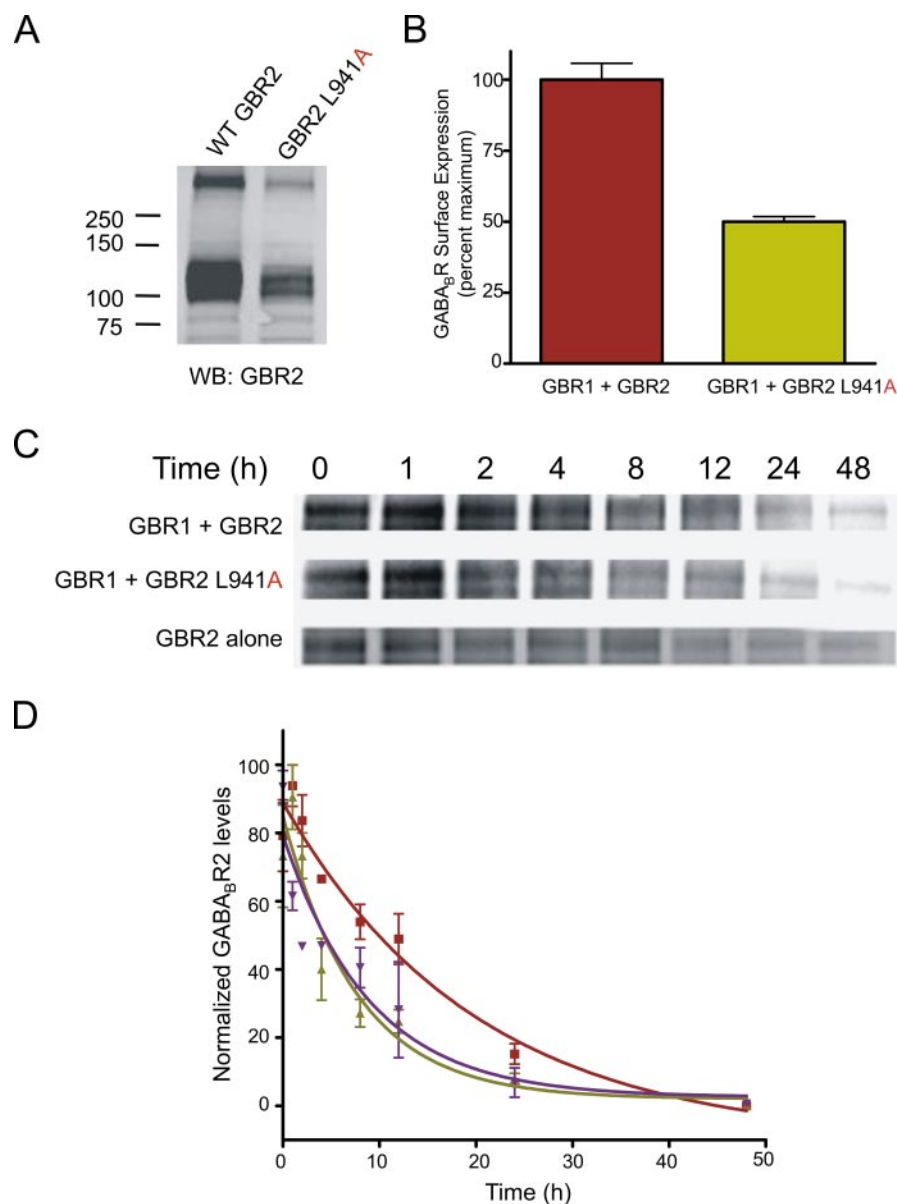
FIGURE 4. **Mutation of the GABA$_B$R2 PDZ-binding motif decreases GABA$_B$ receptor expression.** *A*, cell lysates from COS-7 cells expressing wild-type GABA$_B$R2 or GABA$_B$R2 L941A mutant were analyzed by SDS-PAGE. Western blot analysis with anti-GBR2 antibody showed that the mutant receptor exhibited markedly decreased total expression compared with the wild-type receptor. *B*, COS-7 cells were transfected with GABA$_B$R1 plus either wild-type GABA$_B$R2 or the GABA$_B$R2 L941A mutant. The surface expression of these receptors was assessed by a luminometer-based cell surface assay. Mutant GABA$_B$ receptors (*green*) exhibited reduced expression on the cell surface compared with the wild-type GABA$_B$ receptors (*red*). *C*, pulse-chase metabolic labeling of transfected COS-7 cells expressing wild-type GABA$_B$ (*GBR1 + GBR2*) receptors, PDZ mutant GABA$_B$ receptors (*GBR1 + GBR2 L941A*), or wild-type GABA$_B$R2 (*GBR2*) alone. Cell lysates were analyzed by GBR2 immunoprecipitation and SDS-PAGE, and autoradiographic images were obtained. *D*, a graph of the kinetic degradation plots of GBR2 protein levels from three separate experiments was generated from the autoradiographs. *Points* and *error bars* represent means and standard error values from the three different experiments. The half-life of wild-type GABA$_B$ receptors (*red squares*) was 13.2 h, whereas the half-life of PDZ mutant GABA$_B$ receptors (*green triangles*) was markedly reduced to 5.2 h (significantly different from wild-type, $p < 0.05$). Wild-type GBR2 (*purple inverted triangles*) expressed alone in the absence of GBR1 exhibited half-life of 6.0 h.

COS-7 cells transfected with wild-type GABA$_B$R2 or one of the three GABA$_B$R2 mutants were separately incubated with the three PDZ domains (Mupp1-PDZ13, Erbin-PDZ, and Papin-PDZ1) expressed as hexahistidine-tagged fusion proteins and adsorbed to nickel resin. A robust association of all the three PDZ fusion proteins with the wild-type GABA$_B$R2 was observed (Fig. 1). Alanine mutations at the Ser-939 and Val-938 positions of the GABA$_B$R2 PDZ-binding motif partially inhibited GABA$_B$R2 binding with all the three PDZ proteins. Strikingly, mutation of the GABA$_B$R2 terminal leucine (Leu-941) to alanine strongly reduced the interaction with the PDZ proteins. These results confirm that full-length GABA$_B$R2 associates with PDZ domains from Mupp1, PAPIN, and Erbin and also elucidate key residues on GABA$_B$R2 that mediate the interaction with PDZ proteins.

Mupp1 and PAPIN, but Not Erbin, Associate with GABA$_B$ Receptors in Cells—We next examined whether GABA$_B$R2 can interact with full-length versions of the various PDZ proteins in a cellular environment. Myc-tagged Mupp1 was expressed alone or in the presence of either wild-type FLAG-tagged GABA$_B$R2 or FLAG-tagged GABA$_B$R2 L941A mutant in COS-7 cells (Fig. 2A). When FLAG-tagged GABA$_B$R2 was immunoprecipitated, robust co-immunoprecipitation of Mupp1 was observed from the cell lysates expressing wild-type GABA$_B$R2 and Mupp1. However, Mupp1 co-immunoprecipitation from cell lysates expressing the GABA$_B$R2 L941A mutant and Mupp1 was much weaker. Similarly, GFP-tagged PAPIN co-immunoprecipitated with GABA$_B$R2 from cells expressing GABA$_B$R2 or GABA$_B$R2/GABA$_B$R1 (Fig. 2B). We also expressed either Myc-tagged Erbin or Myc-tagged

PDZ (data not shown). The results from the proteomic array screens were confirmed via a second independent technique in pull-down experiments examining PDZ interactions with both wild-type full-length GABA$_B$R2 and various full-length GABA$_B$R2 carboxyl terminus mutants. The amino acids Val-938, Ser-939, and Leu-941 of the GABA$_B$R2 carboxyl-terminal motif (VSGL) were sequentially mutated to alanine. Lysates from

Erbin lacking the PDZ domain (Myc-ErbinΔPDZ) in the presence or absence of FLAG-tagged GABA$_B$R2 in COS-7 cells (Fig. 2C). However, immunoprecipitation of GABA$_B$R2 from these cell lysates did not yield any detectable co-immunoprecipitation of Erbin. These results demonstrate that full-length Mupp1 and PAPIN, but not Erbin, physically associate with GABA$_B$ receptors in transfected COS-7 cells.
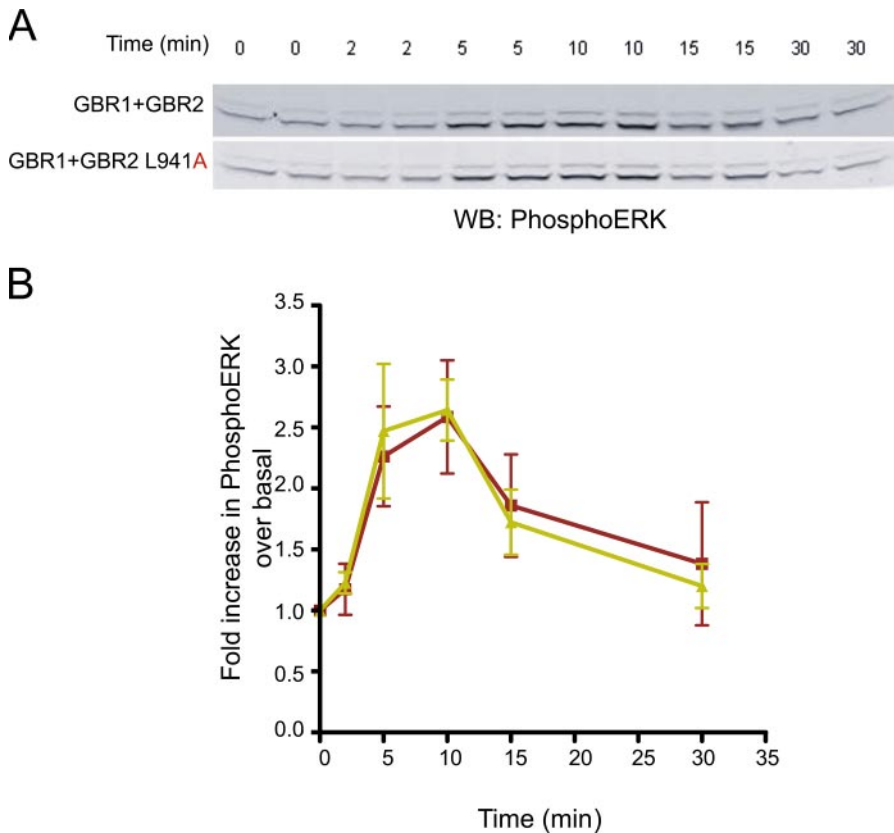
$GABA_B$ Receptor Regulation by Mupp1

A

B

FIGURE 5. **Mutation of the PDZ-binding motif of GABA_B receptors does not alter GABA_B receptor-mediated ERK activation.** *A*, HEK-293 cells expressing wild-type GABA_B receptors (*GBR1 + GBR2*) or PDZ mutant GABA_B receptors (*GBR1 + GBR2 L941A*) were stimulated with 200 μM baclofen for 2, 5, 10, 15, and 30 min, and ERK activation was measured using anti-phospho-ERK antibodies. *B*, quantification of three independent experiments examining GABA_B receptor activation of ERK. Mutation of the GABA_BR2 PDZ-binding motif did not alter either the total phospho-ERK levels or the time course of ERK activation (*red squares*, wild-type GABA_B receptors; *green triangles*, PDZ mutant).

Mupp1 Co-localizes with GABA_BR2 in Neurons and Transfected Cells—GABA_BR2, Mupp1, and PAPIN have been reported to exhibit overlapping distributions in various regions of the brain (26–28), but it is not known if these proteins are expressed in the same cells. Therefore, we examined the subcellular distributions of GABA_BR2 and Mupp1 in cultured cerebellar granule neurons (Fig. 3, *A–C*) and cortical neurons (data not shown) via immunohistochemistry using specific GABA_BR2 and Mupp1 primary antibodies and differentially tagged fluorescent secondary antibodies. We observed a significant overlap in the distribution patterns of GABA_BR2 and Mupp1 on the plasma membrane of the cell bodies and processes of these neurons, suggesting that these two proteins are present together in the same subcellular domains of the same cells. Comparable studies examining PAPIN were not possible due to the lack of a specific anti-PAPIN antibody. Next, we used fluorescence immunohistochemistry to study the subcellular distribution of GABA_BR2 and Mupp1 in transfected cells. COS-7 cells were transfected with either Myc-tagged Mupp1 alone or Myc-tagged Mupp1 plus FLAG-tagged GABA_BR2. Double immunofluorescence was performed with monoclonal anti-Myc and polyclonal anti-GABA_BR2 antibodies. In contrast to the prominent expression in the plasma membrane that was seen in neurons, Mupp1 expressed alone in COS-7 cells was distributed diffusely throughout the cytoplasm with little or no

plasma membrane localization (Fig. 3, *D–F*). Interestingly, upon co-expression with GABA_BR2 in COS-7 cells, Mupp1 displayed a predominantly plasma membrane localization similar to its native subcellular distribution in neurons (Fig. 3, *G–I*). These findings suggest that association with GABA_BR2 can alter the subcellular distribution of Mupp1.

Mutation of the PDZ-binding Motif Decreases GABA_B Receptor Stability—Transfection of the GABA_BR2 L941A mutant into COS-7 cells resulted in consistently low expression of this mutant compared with wild-type GABA_BR2 as assessed by Western blot (Fig. 4*A*). Additionally, quantification of the plasma membrane expression of GABA_B receptors using a luminometer-based cell surface expression assay yielded evidence for a striking decrease in surface expression of the L941A mutant relative to wild-type GABA_BR2 (Fig. 4*B*). Thus, we postulated that the stability of GABA_BR2 might be affected by the L941A mutation that disrupts the PDZ-binding motif. To test this hypothesis, we compared the half-lives of wild-type GABA_BR2 and the GABA_BR2 L941A mutant using pulse-chase metabolic labeling. COS-7 cells were transfected with GABA_BR2 alone, GABA_BR1 plus GABA_BR2, or GABA_BR1 plus the GABA_BR2 L941A mutant. The cells were then metabolically labeled with L-[³⁵S]methionine and chased with cold methionine for various periods of time (Fig. 4*C*). The half-life of wild-type GABA_BR2 alone measured by this method was found to be 6 h, whereas the half-life of wild-type GABA_BR2 co-expressed with GABA_BR1 was found to be significantly increased to 13.2 h. Interestingly, the half-life of the GABA_BR2 L941A mutant co-expressed with GABA_BR1 was only 5.2 h (Fig. 4*D*). These results indicate that disruption of the interaction between GABA_B receptors and PDZ scaffold proteins decreases the stability of the receptors.

Mutation of the GABA_BR2 PDZ-binding Motif Alters GABA_B Receptor Signaling—We next addressed the role of PDZ interactions on the functional activity of GABA_B receptors. We have previously shown that GABA_B receptors are capable of activating the ERK1/2 pathway (16). We therefore examined the ability of transfected GABA_BR1 plus GABA_BR2, or transfected GABA_BR1 plus the GABA_BR2 L941A mutant, to activate the ERK1/2 pathway in HEK-293 cells following agonist stimulation. Activation of both wild-type GABA_B receptors and PDZ-mutant GABA_B receptors resulted in a similar -fold increase in phospho-ERK levels (Fig. 5*A*). In addition, the time course of
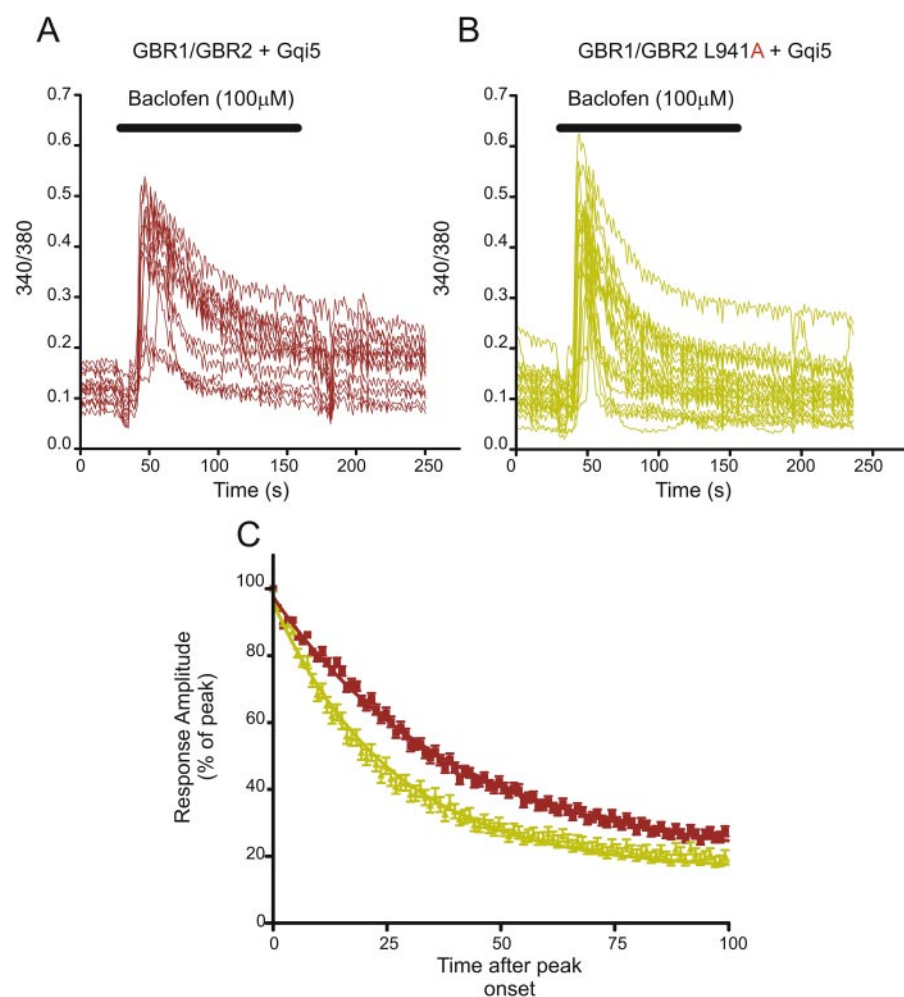
FIGURE 6. **Mutation of the GABA_BR2 PDZ-binding motif decreases the duration of GABA_B receptor signaling.** Individual traces show records of Δ340/380 fura-2 fluorescence in a field of COS-7 cells transfected with wild-type GABA_B receptors (*GBR1 + GBR2*) (*A*) or PDZ mutant GABA_B receptors (*GBR1 + GBR2 L941A*) (*B*). Baclofen (100 μM) was applied as indicated by the *black bar*. Traces of responsive cells in each field are shown in *red* (wild-type) and *green* (PDZ mutant), respectively. Data are representative of three independent experiments for each condition. *C*, responses evoked by applying baclofen (100 μM) were plotted as a function of time elapsed following the onset of peak amplitude. Response amplitudes were normalized to the peak amplitude of each response. Peak amplitudes were not significantly different between wild-type and PDZ mutant receptors, and variations in peak amplitudes from experiment to experiment did not correlate with changes in decay times. However, the decay times were significantly different for wild-type GABA_B receptors (*red squares*, n = 88, 27.8 ± 0.7 s) *versus* the PDZ mutant receptors (*green triangles*, n = 68, 18.2 ± 0.6 s). The *lines* represent the best single exponential fit for each data set.

transfected with wild-type GABA_B receptors plus G_{qi5} or PDZ mutant GABA_B receptors plus G_{qi5} resulted in transient increases in intracellular Ca^{2+} (Fig. 6, *A* and *B*). The mean ± S.E. Δ340/380 evoked by baclofen in wild-type GABA_B receptors was 0.31 ± 0.01 as compared with 0.30 ± 0.01 for PDZ mutant GABA_B receptors. However, while the magnitude of the response amplitudes was similar between the wild-type *versus* mutant receptors, the duration of the responses initiated by PDZ mutant GABA_B receptors was much shorter than those initiated by wild-type receptors. Quantification of the decay of the amplitudes of the Ca^{2+} responses as a function of time after the onset of peak amplitude (Fig. 6*C*) revealed that the responses of the PDZ mutant GABA_B receptors decayed much faster (18.2 ± 0.6 s) than the responses of the wild-type GABA_B receptors (27.8 ± 0.7 s) ($p < 0.0001$). These data demonstrate that interaction with PDZ scaffolds influences the kinetics of GABA_B receptor signaling.

Association with Mupp1 Alters GABA_B Receptor Function and Stability—Western blots with the specific anti-Mupp1 antibody revealed that COS-7 cells express a significant level of endogenous Mupp1. To determine whether the association of endogenous Mupp1 and GABA_B receptors alters receptor function, we performed Ca^{2+} signaling experiments examining GABA_BR1/GABA_BR2 signaling in COS-7 cells in the absence and presence of Mupp1 siRNA knockdown. We first verified the ability of our Mupp1 siRNA to knock down endogenous Mupp1. As shown in Fig. 7*A*, cells transfected with Mupp1 siRNA exhibited a nearly complete loss of detectable Mupp1 expression, whereas cells receiving the control siRNA exhibited Mupp1 expression equivalent to untransfected cells. To determine if the expression levels of transfected GABA_B receptors in these studies were comparable to endogenous levels of GABA_B receptor expression in CNS neurons, lysates were prepared from both mouse brain and the COS-7 cells transfected with GABA_BR1 and GABA_BR2. Equal amounts of protein were loaded on SDS-PAGE gels and analyzed via immunoblotting. These Western blotting analyses revealed that the expression levels of GABA_BR2 obtained by transient transfection of COS-7 cells were similar to the expression levels of endogenous GABA_BR2 in native brain tissue (Fig. 7*B*).

ERK activation was identical for the mutant *versus* wild-type receptors (Fig. 5*B*). These data reveal that mutation of the GABA_BR2 PDZ-binding motif does not render GABA_B receptors non-functional. However, it is difficult to measure potentially subtle effects of mutation to the PDZ binding motif on the temporal kinetics of GABA_B receptor signaling utilizing downstream read-outs such as ERK activation assays, in which the earliest time point measured is 2 min.

Thus, we next performed studies utilizing real-time calcium imaging and chimeric G_{qi5} G proteins to allow for much greater temporal resolution. G_{qi5} is a chimeric G protein in which the last five amino acids of $G\alpha_q$ have been replaced with the last five amino acids of $G\alpha_i$ (29). This chimeric G protein can link $G\alpha_i$-coupled receptors such as GABA_B receptors to $G\alpha_q$-activated intracellular Ca^{2+} mobilization, which can then be visualized using Ca^{2+}-sensitive dyes such as Fura-2. Application of the GABA_B receptor agonist baclofen (100 μM) to a field of cells
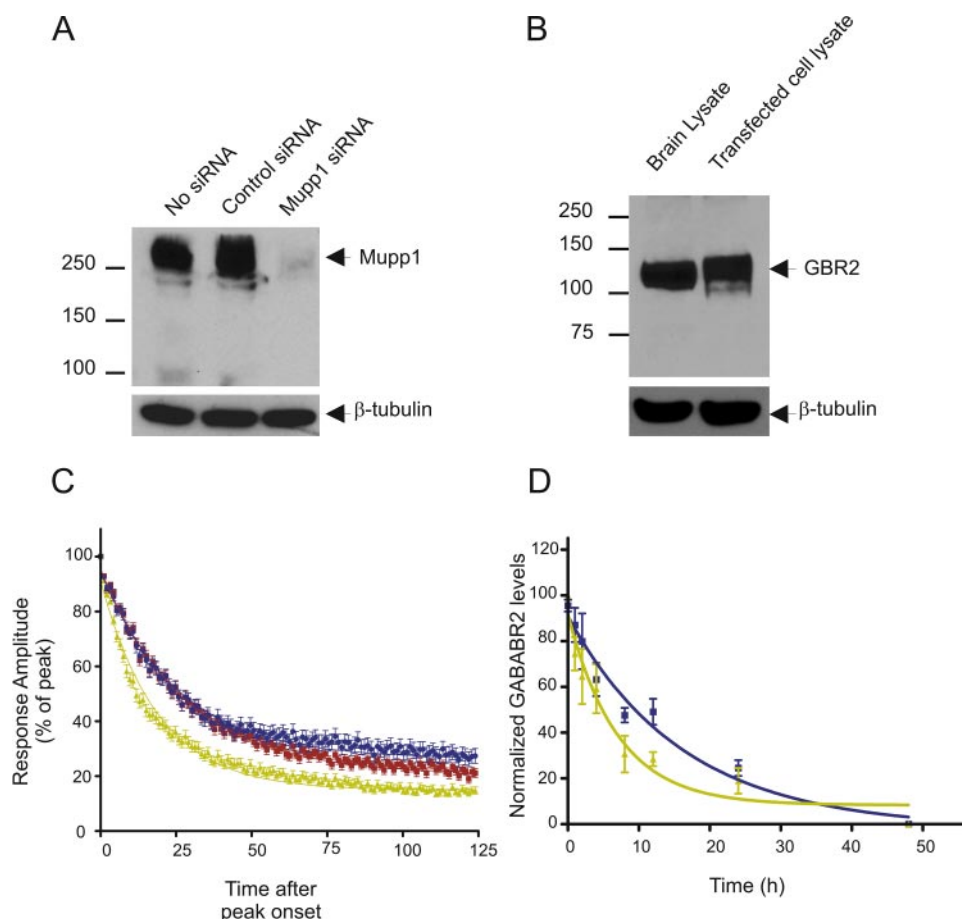
A

B

C

D

FIGURE 7. **Mupp1 prolongs the duration of GABAB receptor signaling.** *A*, COS-7 cells were treated with either no siRNA, control siRNA, or Mupp1 siRNA for 36 h. Cell lysates were harvested in sample buffer and probed with anti-Mupp1 antibody. Mupp1 siRNA almost completely knocked down the expression of endogenous Mupp1 in COS-7 cells (major band indicated by the *arrowhead*) as analyzed by Western blotting. *B*, brain lysates and lysates of COS-7 cell transfected with GBR1/GBR2 containing equal amounts of protein were run on SDS-PAGE gel. Western blot analyses with anti-GBR2 antibody revealed approximately equal levels of GABABR2 expression in the two lysates. Equal protein loading was confirmed by Western blotting for β-tubulin (*lower panel*). *C*, COS-7 cells were transfected with GBR1/GBR2 and G_{qi5} and then treated with no siRNA, control siRNA, or Mupp1 siRNA. Cells were loaded with fura-2AM and stimulated with baclofen. Responses evoked by applying baclofen (100 μM) were plotted as a function of time elapsed since the onset of peak amplitude, with the response amplitudes normalized to the peak amplitude of each response. Significant differences in the decay times between cells receiving no siRNA (19.3 $\pm$ 0.1 s, *red squares*, n = 48) and cells receiving Mupp1 siRNA (12.9 $\pm$ 0.9 s, *green triangles,* n = 66) were observed. Cells receiving control siRNA (17.7 $\pm$ 1.8 s, *blue circles*, n = 33) exhibited decay times that were not significantly different from cells receiving no siRNA. The *lines* represent the best single exponential fit for each data set. Data are representative of three independent experiments. *D*, COS-7 cells were transfected with GBR1/GBR2 and then treated with control siRNA or Mupp1 siRNA. A *graph* of the degradation of GBR2 protein levels measured using pulse-chase metabolic labeling shows that the half-life of GABAB receptors in cells treated with control siRNA (*blue squares*) was 11.3 h, whereas the half-life of GABAB receptors in cells treated with Mupp1 siRNA (*green triangles*) was significantly reduced to 4.9 h. Data are representative of four independent experiments.

After analyzing the expression levels of the GABAB receptors and endogenous Mupp1, we proceeded to assess GABAB receptor signaling through G_{qi5} in the presence *versus* absence of Mupp1 protein expression. Upon baclofen stimulation of COS-7 cells transfected with GABAB receptors and G_{qi5}, response amplitudes were unchanged by siRNA treatments (data not shown). However, the duration of Ca^{2+} responses in cells transfected with Mupp1 siRNA was much shorter (12.9 $\pm$ 0.9 s) compared with either cells transfected with control siRNA (17.7 $\pm$ 1.8 s) or cells receiving no siRNA (19.3 $\pm$ 0.1 s) (Fig. 7*C*). These findings, taken together with the results from the experiments shown in Fig. 6, suggest that interaction with

the PDZ scaffold protein Mupp1 enhances GABAB receptor signaling. Finally, we also examined the half-life of GABAB receptors in the presence and absence of Mupp1 protein expression. COS-7 cells transfected with GABABR1 plus GABABR2 were treated with either control siRNA or Mupp1 siRNA for ~36 h. Upon measuring the half-life of GABABR2 using pulse-chase metabolic labeling, a half-life of 11.3 h was observed for GABAB receptors treated with control siRNA, which is similar to the half-life observed for wild-type receptors in Fig. 4*D*. However, knockdown of endogenous Mupp1 by Mupp1 siRNA treatment dramatically reduced the half-life of the GABAB receptors to 4.9 h (Fig. 7*D*), an effect similar to the reduction in GABAB receptor half-life produced by the L941A mutation. These results indicate that association with Mupp1 enhances the stability of GABAB receptors.

DISCUSSION

We utilized a proteomic screen to identify three PDZ scaffold proteins, Mupp1, Erbin, and PAPIN, as novel GABABR2-interacting partners. These interactions were confirmed via fusion protein pull-down assays. Further characterization of cellular interactions between GABAB receptors and the three PDZ proteins via co-immunoprecipitation studies revealed that only Mupp1 and PAPIN physically associate with GABABR2 in cells. Moreover, Mupp1 was found to co-localize with GABABR2 on the plasma membrane of cerebellar granule neurons and to undergo a transloca-

tion from the cytoplasm of COS-7 cells to the plasma membrane upon co-transfection with GABABR2. These data suggest that Mupp1 is a specific binding partner of GABABR2, because out of 96 PDZ domains we found only three that bind well to GABABR2, only two that associate with full-length GABABR2 in cells, and only one (Mupp1) for which we could find evidence of regulation of GABAB receptor signaling and stability.

Our metabolic labeling and pulse-chase studies revealed that disruption of the interaction between GABAB receptors and PDZ proteins markedly decreases GABAB receptor stability. An identical decrease in stability was also produced by knockdown of endogenous Mupp1. Interestingly, PDZ interactions

have been shown to enhance the stability of various other target proteins. For example, disruption of the PDZ-interacting motif of aquaporin-4 increases the rate of aquaporin-4 degradation (30). Similarly, phosphatase and tensin homolog mutants deficient in PDZ binding have been reported to have reduced stability (31, 32), and PDZ proteins of the syntrophin family have been shown to enhance the stability of both α_{1D}-adrenergic receptors (33) and the transporter ABCA1 (34). The mechanisms underlying PDZ domain-mediated regulation of target protein stability are unclear. One possibility is that PDZ proteins may influence the trafficking of their partners such that the rate of targeting to lysosomal and/or proteasomal compartments is slowed. Another possibility is that PDZ domain associations may block proteolytic degradation of PDZ-binding partners. Relevant to this idea, members of the HtrA family of serine proteases have active sites that exhibit extensive homology with PDZ domains (35). It is not known if HtrA proteases play a role in the degradation of PDZ-interacting proteins, but if they do then it is conceivable that such protease-substrate interactions could be highly regulated by PDZ domain associations.

Our studies on GABA_B receptor signaling utilizing the chimeric G protein G_{qi5} revealed that disruption of GABA_BR2-PDZ interactions significantly decreased the duration of GABA_B receptor-induced responses. Similarly, we found that siRNA knockdown of endogenous Mupp1 markedly decreased the duration of GABA_B receptor-mediated signaling. These results provide evidence that association with Mupp1 enhances GABA_B receptor signaling, either through direct effects on G protein coupling or through alterations in receptor associations with other proteins. Thus, interactions with Mupp1 may result in a cell type-specific fine-tuning of GABA_B receptor signaling, with implications for various physiological phenomena in which GABA_B receptors are known to play important roles, including long term depression, epilepsy, neurotransmitter release, and neuroprotection (1, 36).

In addition to modifying receptor signaling and stability, Mupp1 interactions with GABA_B receptors might have other important physiological effects. For example, Mupp1 has been previously reported to interact with the serotonin 5-HT_{2C} receptor (37, 38). Mupp1 interacts with GABA_B receptors and 5-HT_{2C} receptors via different PDZ domains, the 13th and 10th PDZ domains, respectively. Because GABA_B receptors, 5-HT_{2C} receptors, and Mupp1 exhibit overlapping distribution in the brain (28, 39), it is possible that Mupp1 might physically link GABA_B and 5-HT_{2C} receptors together. Such interactions may serve to facilitate well known examples of cross-talk between GABA_B and serotonin receptors (40–42). Interestingly, the Mupp1 interaction with the 5-HT_{2C} receptor is known to be regulated by agonist-mediated receptor phosphorylation (43). Similar regulation of the Mupp1-GABA_B receptor interaction by agonist-promoted phosphorylation is possible and could further add to the complexity of GABA_B receptor regulation.

The GABA_B receptor agonist baclofen has been shown to be effective in reducing cravings for several addictive substances, including alcohol, by blocking the development of tolerance (44). Preliminary and preclinical evidence also suggests that baclofen ameliorates various aspects of alcohol addiction by reducing alcohol intake, reducing craving, and suppressing withdrawal symptoms (45–47). In this context, it is interesting to note that the Mupp1 gene has recently been identified as a quantitative trait gene in the loci characterized for alcohol and barbiturate dependence and withdrawal: specifically, lowered Mupp1 expression is genetically correlated with greater withdrawal susceptibility (48, 49). Thus, our finding that Mupp1, a protein linked in genetic studies to the development of drug and alcohol dependence, physically associates with GABA_B receptors, which are therapeutic targets for drug and alcohol addiction, represents an intriguing coincidence that may be worthy of future investigation. Additionally, it would be of interest to examine the role of Mupp1 in GABA_B receptor regulation of various cellular processes for which no connection to GABA_B receptors has previously been considered. Mupp1 has been reported to interact with a variety of disparate signaling proteins, including c-Kit (50), the tandem PH-domain-containing protein-1 TAPP1 (51), the proteoglycan NG2 (52), the tight junction protein claudin-8 (53, 54), the calcium/calmodulin-dependent protein kinase II (55), and other partners and, therefore, could potentially link GABA_B receptors to novel signaling cascades.

Therapeutics acting on GABA_B receptors are currently in development for the treatment of conditions such as epilepsy, drug addiction, drug withdrawal, depression, anxiety, and pain (12, 13). A thorough understanding of the factors that regulate GABA_B receptor signaling is vital to the eventual creation of such therapeutics. We have found in the studies reported here that GABA_B receptor functionality is modulated by interactions with the PDZ scaffold protein Mupp1. These findings provide insight into the mechanisms by which GABA_B receptor activity may be modulated in a cell-specific fashion and present a novel therapeutic target if small molecules can be developed to specifically disrupt receptor-PDZ interactions (56).

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