GABA<sub>B</sub> Receptor Association with the PDZ Scaffold Muppl
Alters Receptor Stability and Function*

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γ-Aminobutyric acid, type B (GABA<sub>B</sub>) 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 GABA<sub>B</sub> receptor (GABA<sub>B</sub>R) functionality, we screened the GABA<sub>B</sub>R2 carboxy 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 GABA<sub>B</sub>R2: Muppl PDZ13, PAPIN PDZ1, and Erbin PDZ. Biochemical analysis confirmed that full-length Muppl and PAPIN interact with GABA<sub>B</sub>R2 in cells. Disruption of the GABA<sub>B</sub>R2 interaction with PDZ scaffolds by a point mutation to the carboxy terminus of the receptor dramatically decreased receptor stability and attenuated the duration of GABA<sub>B</sub> receptor signaling. The effects of mutating the GABA<sub>B</sub>R2 carboxy terminus on receptor stability and signaling were mimicked by small interference RNA knockdown of endogenous Muppl. These findings reveal that GABA<sub>B</sub> receptor stability and signaling can be modulated via GABA<sub>B</sub>R2 interactions with the PDZ scaffold protein Muppl, which may contribute to cell-specific regulation of GABA<sub>B</sub> receptors in the central nervous system.

GABA<sub>B</sub> 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. GABA<sub>B</sub> receptors are believed to be heterodimeric combinations of two G protein-coupled receptors, GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 (2–4). Heterodimerization of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 is necessary for the proper trafficking of GABA<sub>B</sub>R1 to the cell surface (5, 6). In the context of the heterodimer, GABA<sub>B</sub>R1 is thought to bind the ligand (7), whereas GABA<sub>B</sub>R2 is believed to be the primary G protein contact site (8–11).

Given that GABA<sub>B</sub> receptors are important therapeutic targets for a wide variety of diseases, including depression, anxiety, epilepsy, and drug addiction (12, 13), understanding GABA<sub>B</sub> receptor signaling and regulation is of significant clinical interest. The cloning of the GABA<sub>B</sub> receptors has advanced the study of the GABA<sub>B</sub> Receptors substantially over the past decade. However, some discrepancies between the properties of native GABA<sub>B</sub> receptors and heterologously expressed recombinant receptors still remain. For example, GABA<sub>B</sub> receptors in native tissue undergo robust endocytosis and desensitization (14), whereas recombinant GABA<sub>B</sub>R1/GABA<sub>B</sub>R2 expressed in most heterologous cells neither internalize nor desensitize (14, 15). One possible explanation for such discrepancies is that GABA<sub>B</sub> receptor signaling and trafficking properties are highly dependent on cellular context. This implies that interaction with differentially expressed cellular proteins might modulate GABA<sub>B</sub> receptor function. Indeed, we previously reported that association of GABA<sub>B</sub> receptors with the GABA<sub>B</sub> receptor γ2S subunit confers agonist-mediated endocytosis on GABA<sub>B</sub> receptors expressed in heterologous cells (16). Furthermore, GABA<sub>B</sub> 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).

GABA<sub>B</sub>R2 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...
acid requirement for their binding partners. Class I PDZ proteins bind to the motif (S/T)XΦ, where Φ represents a hydrophobic residue at the carboxyl terminus and X represents any amino acid. The GABA<sub>B</sub><sub>2</sub> 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<sub>B</sub> 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<sub>B</sub><sub>2</sub>. We identified three PDZ proteins that interact with the GABA<sub>B</sub><sub>2</sub> carboxyl terminus: Mupp1, Erbin, and PAPIN. We further studied the interactions of these proteins with GABA<sub>B</sub><sub>2</sub> in cells and examined the roles of these interactions in regulating GABA<sub>B</sub> 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<sub>B</sub><sub>1b</sub> and GABA<sub>B</sub><sub>2</sub> 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<sub>B</sub><sub>2</sub>R2V938A, FLAG-GABA<sub>B</sub><sub>2</sub>R2S939A, and FLAG-GABA<sub>B</sub><sub>2</sub>R2L941A 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 well and incubated with three distinct PDZ domains fusion proteins (Mupp1 PDZ13, Erbin PDZ, and Papin PDZ1) in a pull-down experiment (6). Western blot analysis with anti-GABA2 antibody revealed that wild-type GABA2 interacts robustly with all three fusion proteins. The serine and valine GABA2 mutants exhibited only partial binding to the fusion proteins, whereas the GABA2 leucine mutant showed markedly decreased binding to all three fusion proteins. The data shown are representative of three independent experiments.

**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, 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% CO2 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 culture 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,
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, 0.25 mM glutamine, 25 mM KCl, 0.025 mM glutamic acid, and 25 mM KCl. The neurons were plated onto poly-D-lysine-coated culture slides (Fisher) at a density of 10^5 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 GABAB receptors was verified using a lumino-meter-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-GABABR1 antibody (Chemicon), anti-GABABR2 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-GABABR2 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). 

**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-[35S]methionine (Amersham Biosciences) was added to each plate and incubated for another 30 min. The radioactive
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 shown in blue. G–I, in COS-7 cells co-transfected with Myc-tagged Mupp1, FLAG-tagged GABABR2, and HA-tagged GABA\(_{\text{B}}\)R1, Mupp1 (Rhodamine Red) was found predominantly on the plasma membrane. These data are representative of four or five independent experiments each.

**FIGURE 3.** GABA\(_{\text{B}}\) 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\(_{\text{B}}\) 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\(_{\text{B}}\)R2, and HA-tagged GABA\(_{\text{B}}\)R1, Mupp1 (Rhodamine Red) was found predominantly on the plasma membrane. These data are representative of four or five independent experiments each.

**RESULTS**

**Screening of a PDZ Proteomic Array with the GABA\(_{\text{B}}\)R2 Carboxyl Terminus**—To identify PDZ domain-containing proteins that might associate with the GABA\(_{\text{B}}\)R2 carboxy terminus (GABA\(_{\text{B}}\)R2-CT), we first created a GST fusion protein corresponding to the last 35 amino acids of GABA\(_{\text{B}}\)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\(_{\text{B}}\)R2-CT-GST fusion protein. GABA\(_{\text{B}}\)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-PDZ16.

**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.
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<sub>B</sub>R2 and various full-length GABA<sub>B</sub>R2 carboxyl terminus mutants. The amino acids Val-938, Ser-939, and Leu-941 of the GABA<sub>B</sub>R2 carboxyl-terminal motif (VSGL) were sequentially mutated to alanine. Lysates from COS-7 cells transfected with wild-type GABA<sub>B</sub>R2 or one of the three GABA<sub>B</sub>R2 mutants were separately incubated with the three PDZ domains (Muppl-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<sub>B</sub>R2 was observed (Fig. 1). Alanine mutations at the Ser-939 and Val-938 positions of the GABA<sub>B</sub>R2 PDZ-binding motif partially inhibited GABA<sub>B</sub>R2 binding with all the three PDZ proteins. Strikingly, mutation of the GABA<sub>B</sub>R2 terminal leucine (Leu-941) to alanine strongly reduced the interaction with the PDZ proteins. These results confirm that full-length GABA<sub>B</sub>R2 associates with PDZ domains from Muppl, PAPIN, and Erbin and also elucidate key residues on GABA<sub>B</sub>R2 that mediate the interaction with PDZ proteins.

**Muppl and PAPIN, but Not Erbin, Associate with GABA<sub>B</sub> Receptors in Cells**—We next examined whether GABA<sub>B</sub>R2 can interact with full-length versions of the various PDZ proteins in a cellular environment. Myc-tagged Muppl was expressed alone or in the presence of either wild-type FLAG-tagged GABA<sub>B</sub>R2 or FLAG-tagged GABA<sub>B</sub>R2 L941A mutant in COS-7 cells (Fig. 2A). When FLAG-tagged GABA<sub>B</sub>R2 was immunoprecipitated, robust co-immunoprecipitation of Muppl was observed from the cell lysates expressing wild-type GABA<sub>B</sub>R2 and Muppl. However, Muppl co-immunoprecipitation from cell lysates expressing the GABA<sub>B</sub>R2 L941A mutant and Muppl was much weaker. Similarly, GFP-tagged PAPIN co-immunoprecipitated with GABA<sub>B</sub>R2 from cells expressing GABA<sub>B</sub>R2 or GABA<sub>B</sub>R2/R1 (Fig. 2B). We also expressed either Myc-tagged Erbin or Myc-tagged Erbin lacking the PDZ domain (Myc-ErbinΔPDZ) in the presence or absence of FLAG-tagged GABA<sub>B</sub>R2 in COS-7 cells (Fig. 2C). However, immunoprecipitation of GABA<sub>B</sub>R2 from these cell lysates did not yield any detectable co-immunoprecipitation of Erbin. These results demonstrate that full-length Muppl and PAPIN, but not Erbin, physically associate with GABA<sub>B</sub> receptors in transfected COS-7 cells.
Mupp1 Co-localizes with GABA\textsubscript{B}R2 in Neurons and Transfected Cells—GABA\textsubscript{B}R2, 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\textsubscript{B}R2 and Mupp1 in cultured cerebellar granule neurons (Fig. 3, A–C) and cortical neurons (data not shown) via immunohistochemistry using specific GABA\textsubscript{B}R2 and Mupp1 primary antibodies and differentially tagged fluorescent secondary antibodies. We observed a significant overlap in the distribution patterns of GABA\textsubscript{B}R2 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\textsubscript{B}R2 and Mupp1 in transfected cells. COS-7 cells were transfected with either Myc-tagged Mupp1 alone or Myc-tagged Mupp1 plus FLAG-tagged GABA\textsubscript{B}R2. Double immunofluorescence was performed with monoclonal anti-Myc and polyclonal anti-GABA\textsubscript{B}R2 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\textsubscript{B}R2 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\textsubscript{B}R2 can alter the subcellular distribution of Mupp1.

Mutation of the PDZ-binding Motif Decreases GABA\textsubscript{B} Receptor Stability—Transfection of the GABA\textsubscript{B}R2 L941A mutant into COS-7 cells resulted in consistently low expression of this mutant compared with wild-type GABA\textsubscript{B}R2 as assessed by Western blot (Fig. 4A). Additionally, quantification of the plasma membrane expression of GABA\textsubscript{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\textsubscript{B}R2 (Fig. 4B). Thus, we postulated that the stability of GABA\textsubscript{B}R2 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\textsubscript{B}R2 and the GABA\textsubscript{B}R2 L941A mutant using pulse-chase metabolic labeling. COS-7 cells were transfected with GABA\textsubscript{B}R2 alone, GABA\textsubscript{B}R1 plus GABA\textsubscript{B}R2, or GABA\textsubscript{B}R1 plus the GABA\textsubscript{B}R2 L941A mutant. The cells were then metabolically labeled with \textsuperscript{35}S\textsuperscript{1}methionine and chased with cold methionine for various periods of time (Fig. 4C). The half-life of wild-type GABA\textsubscript{B}R2 alone measured by this method was found to be 6 h, whereas the half-life of wild-type GABA\textsubscript{B}R2 co-expressed with GABA\textsubscript{B}R1 was found to be significantly increased to 13.2 h. Interestingly, the half-life of the GABA\textsubscript{B}R2 L941A mutant co-expressed with GABA\textsubscript{B}R1 was only 5.2 h (Fig. 4D). These results indicate that disruption of the interaction between GABA\textsubscript{B} receptors and PDZ scaffold proteins decreases the stability of the receptors.

Mutation of the GABA\textsubscript{B}R2 PDZ-binding Motif Alters GABA\textsubscript{B} Receptor Signaling—We next addressed the role of PDZ interactions on the functional activity of GABA\textsubscript{B} receptors. We have previously shown that GABA\textsubscript{B} receptors are capable of activating the ERK1/2 pathway (16). We therefore examined the ability of transfected GABA\textsubscript{B}R1 plus GABA\textsubscript{B}R2, or transfected GABA\textsubscript{B}R1 plus the GABA\textsubscript{B}R2 L941A mutant, to activate the ERK1/2 pathway in HEK-293 cells following agonist stimulation. Activation of both wild-type GABA\textsubscript{B} receptors and PDZ-mutant GABA\textsubscript{B} receptors resulted in a similar -fold increase in phospho-ERK levels (Fig. 5A). In addition, the time course of...
ERK activation was identical for the mutant versus wild-type receptors (Fig. 5B). These data reveal that mutation of the GABABR2 PDZ-binding motif does not render GABAB receptors non-functional. However, it is difficult to measure potentially subtle effects of mutation to the PDZ binding motif on the temporal kinetics of GABAB 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 Gq5 G proteins to allow for much greater temporal resolution. Gq5 is a chimeric G protein in which the last five amino acids of Goi have been replaced with the last five amino acids of Goq (29). This chimeric G protein can link Goq-coupled receptors such as GABAB receptors to Goq-activated intracellular Ca2+ mobilization, which can then be visualized using Ca2+-sensitive dyes such as Fura-2. Application of the GABAB receptor agonist baclofen (100 μM) to a field of cells transfected with wild-type GABAB receptors plus Gq5 or PDZ mutant GABAB receptors plus Gq5 resulted in transient increases in intracellular Ca2+ (Fig. 6, A and B). The mean ± S.E. Δ340/380 evoked by baclofen in wild-type GABAB receptors was 0.31 ± 0.01 as compared with 0.30 ± 0.01 for PDZ mutant GABAB 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 GABAB receptors was much shorter than those initiated by wild-type receptors. Quantification of the decay of the amplitudes of the Ca2+ responses as a function of time after the onset of peak amplitude (Fig. 6C) revealed that the responses of the PDZ mutant GABAB receptors decayed much faster (18.2 ± 0.6 s) than the responses of the wild-type GABAB receptors (27.8 ± 0.7 s) (p < 0.0001). These data demonstrate that interaction with PDZ scaffolds influences the kinetics of GABAB receptor signaling.

Association with Mupp1 Alters GABAB 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 GABAB receptors alters receptor function, we performed Ca2+ signaling experiments examining GABABR1/GABABR2 signaling in COS-7 cells in the absence of Mupp1. As shown in Fig. 7A, cells transfected with Mupp1 siRNA exhibited a significant decrease in the ability of our Mupp1 siRNA to knock down endogenous Mupp1. To determine whether the expression levels of transfected GABAB receptors was much shorter than those of endogenous GABAB receptors was much shorter than those of endogenous GABAB receptors plus Gqi5 or PDZ mutant GABAB receptors plus Gqi5 resulted in transient increases in intracellular Ca2+ (Fig. 6, A and B). The mean ± S.E. Δ340/380 evoked by baclofen in wild-type GABAB receptors was 0.31 ± 0.01 as compared with 0.30 ± 0.01 for PDZ mutant GABAB 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 GABAB receptors was much shorter than those initiated by wild-type receptors. Quantification of the decay of the amplitudes of the Ca2+ responses as a function of time after the onset of peak amplitude (Fig. 6C) revealed that the responses of the PDZ mutant GABAB receptors decayed much faster (18.2 ± 0.6 s) than the responses of the wild-type GABAB receptors (27.8 ± 0.7 s) (p < 0.0001). These data demonstrate that interaction with PDZ scaffolds influences the kinetics of GABAB receptor signaling.

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After analyzing the expression levels of the GABA<sub>B</sub> receptors and endogenous Mupp1, we proceeded to assess GABA<sub>B</sub> receptor signaling through G<sub>q5</sub> in the presence versus absence of Mupp1 protein expression. Upon baclofen stimulation of COS-7 cells transfected with GABA<sub>B</sub> receptors and G<sub>q5</sub>, response amplitudes were unchanged by siRNA treatments (data not shown). However, the duration of Ca<sup>2+</sup> responses in cells transfected with Mupp1 siRNA was much shorter (12.9 ± 0.9 s) compared with either cells transfected with control siRNA (17.7 ± 1.8 s) or cells receiving no siRNA (19.3 ± 0.1 s) (Fig. 7C). These findings, taken together with the results from the experiments shown in Fig. 6, suggest that interaction from the cytoplasm of COS-7 cells to the plasma membrane upon co-transfection with GABA<sub>B</sub>R2. These data suggest that Mupp1 is a specific binding partner of GABA<sub>B</sub>R2, because out of 96 PDZ domains we found only three that bind well to the PDZ scaffold protein Mupp1. Interestingly, PDZ interactions

**DISCUSSION**

We utilized a proteomic screen to identify three PDZ scaffold proteins, Mupp1, Erbin, and PAPIN, as novel GABA<sub>B</sub>R2-interacting partners. These interactions were confirmed via fusion protein pull-down assays. Further characterization of cellular interactions between GABA<sub>B</sub> receptors and the three PDZ proteins via co-immunoprecipitation studies revealed that only Mupp1 and PAPIN physically associate with GABA<sub>B</sub>R2 in cells. Moreover, Mupp1 was found to co-localize with GABA<sub>B</sub>R2 on the plasma membrane of cerebellar granule neurons and to undergo a transloca-
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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<sub>1D</sub>-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<sub>B</sub> receptor signaling utilizing the chimeric G protein G<sub>q5</sub> revealed that disruption of GABA<sub>B</sub>R2-PDZ interactions significantly decreased the duration of GABA<sub>B</sub> receptor-induced responses. Similarly, we found that siRNA knockdown of endogenous Mupp1 markedly decreased the duration of GABA<sub>B</sub> receptor-mediated signaling. These results provide evidence that association with Mupp1 enhances GABA<sub>B</sub> 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<sub>B</sub> receptor signaling, with implications for various physiological phenomena in which GABA<sub>B</sub> 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<sub>B</sub> receptors might have other important physiological effects. For example, Mupp1 has been previously reported to interact with the serotonin 5-HT<sub>2C</sub> receptor (37, 38). Mupp1 interacts with GABA<sub>B</sub> receptors and 5-HT<sub>2C</sub> receptors via different PDZ domains, the 13th and 10th PDZ domains, respectively. Because GABA<sub>B</sub> receptors, 5-HT<sub>2C</sub> receptors, and Mupp1 exhibit overlapping distribution in the brain (28, 39), it is possible that Mupp1 might physically link GABA<sub>B</sub> and 5-HT<sub>2C</sub> receptors together. Such interactions may serve to facilitate well known examples of cross-talk between GABA<sub>B</sub> and serotonin receptors (40–42). Interestingly, the Mupp1 interaction with the 5-HT<sub>2C</sub> receptor is known to be regulated by agonist-mediated receptor phosphorylation (43). Similar regulation of the Mupp1-GABA<sub>B</sub> receptor interaction by agonist-promoted phosphorylation is possible and could further add to the complexity of GABA<sub>B</sub> receptor regulation.

The GABA<sub>B</sub> 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<sub>B</sub> 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<sub>B</sub> receptor regulation of various cellular processes for which no connection to GABA<sub>B</sub> 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<sub>B</sub> receptors to novel signaling cascades.

Therapeutics acting on GABA<sub>B</sub> 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<sub>B</sub> receptor signaling is vital to the eventual creation of such therapeutics. We have found in the studies reported here that GABA<sub>B</sub> receptor functionality is modulated by interactions with the PDZ scaffold protein Mupp1. These findings provide insight into the mechanisms by which GABA<sub>B</sub> 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).

Acknowledgments—We thank Amanda Castleberry, Heide Oller, Hiroyuki Inuzuka, Erin Garcia, Irina Calin-Jageman, and Anthony Lau for helpful technical assistance and advice. We also thank Steve Traynells and Yue Feng for the use of their instruments and for advice.

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