Proteomic Characterization of Inhibitory Synapses Using a Novel pHluorin-tagged γ-Aminobutyric Acid Receptor, Type A (GABA_A), α2 Subunit Knock-in Mouse*‡§

Yasuko Nakamura†, Danielle H. Morrow†, Amit Modgil‡, Deborah Huyghe†, Tarek Z. Deeb§, Michael J. Lumb§, Paul A. Davies†, and Stephen J. Moss†‡§

From the †Department of Neuroscience, Tufts University School of Medicine, Boston Massachusetts 02111 and the §Department of Neuroscience, Physiology and Pharmacology, University College, London WC1E 6BT, United Kingdom

The accumulation of γ-aminobutyric acid receptors (GABA_As) at the appropriate postsynaptic sites is critical for determining the efficacy of fast inhibitory neurotransmission. Although we know that the majority of synaptic GABA_A subtypes are assembled from α1–3, β, and γ subunits, our understanding of how neurons facilitate their targeting to and stabilization at inhibitory synapses is rudimentary. To address these issues, we have created knock-in mice in which the pH-sensitive green fluorescent protein (GFP) and the Myc epitope were introduced to the extracellular domain of the mature receptor α2 subunit (pHα2). Using immunoaffinity purification and mass spectroscopy, we identified a stable complex of 174 proteins that were associated with pH2, including other GABA_A subunits, and previously identified receptor-associated proteins such as gephyrin and collybistin. 149 of these proteins were novel GABA_A binding partners and included G-protein-coupled receptors and ion channel subunits, proteins that regulate trafficking and degradation, regulators of protein phosphorylation, GTPases, and a number of proteins that regulate their activity. Notably, members of the postsynaptic density family of proteins that are critical components of excitatory synapses were not associated with GABA_As. Crucially, we demonstrated for a subset of these novel proteins (including cullin1, ephxin, potassium channel tetramerization domain containing protein 12, mitofusin2, metabotropic glutamate receptor 5, p21-activated kinase 7, and Ras-related protein 5A) bind directly to the intracellular domains of GABA_As, validating our proteomic analysis. Thus, our experiments illustrate the complexity of the GABA_A proteome and enhance our understanding of the mechanisms neurons use to construct inhibitory synapses.

GABA_As are Cl⁻-permeable ligand-gated ion channels that mediate the majority of fast synaptic inhibition in the central nervous system (CNS) (1, 2). They are also of therapeutic significance as they are the sites of action for barbiturates, benzodiazepines, general anesthetics, and neuroactive steroids (3). Consistent with their critical roles in regulating neuronal excitability, deficits in the activity of GABA_As contribute to a plethora of neurological disorders ranging from anxiety to schizophrenia (4).

Structurally, GABA_As can be assembled from 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3). The majority of GABA_As are believed to be heteropentamers composed of two copies of a single α subunit, two copies of a single β subunit, and one copy of either γ or δ subunits (5, 6). GABA_As containing an α1–3 and γ are enriched at inhibitory synapses and mediate phasic inhibition, whereas those containing α4–6 and δ are found at extrasynaptic locales and mediate tonic inhibition (1, 2). Notably, subunit composition impacts the pharmacological and physiological properties of these varying receptor subtypes (1, 7, 8). Moreover, GABA_As containing unique subunit combinations are selectively targeted to distinct types of inhibitory synapses. However, our understanding of the cellular mechanisms that neurons utilize to regulate GABA_A accumulation at inhibitory synapses is rudimentary. Importantly, the processes that regulate inhibitory synaptogenesis are distinct to those used to build excitatory synapses, which are largely dependent upon PDZ domain-mediated protein-protein interactions (9).

To identify proteins that are relevant for inhibitory synaptogenesis and maintenance, we created knock-in mice in which the pH-sensitive green fluorescent protein (GFP) and the Myc epitope were introduced between amino acids 4 and 5 of the mature GABA_A α2 subunit (pHα2). Following purification on Myc and/or GFP matrices, GABA_A complexes were analyzed by mass spectrometry, and a stable complex of 174 interacting proteins was identified. Importantly, these included the GABA_A α1–5, β1–3, γ1–3, and δ subunits in addition to the previously identified GABA_A-associated proteins gephyrin (Gphn) and collybistin (Arhge9). However, 149 of these proteins were novel GABA_A binding partners G-protein-coupled receptors (GPCRs); ion channel subunits; regulators of membrane trafficking and protein stability; modulators of protein phosphorylation; GTPases; and related exchange factors. Sig-
nificantly, these interactions were confirmed using in vitro binding coupled with immunoprecipitation. Collectively, these results provide new insights into the components of the GABA<sub>R</sub> proteome.

**Experimental Procedures**

*Animals*—All animal protocols were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee of Tufts University.

*Antibodies and Expression Constructs*—The following antibodies were used for immunocytochemistry: C-terminal anti-α2 antibody was provided by Drs. V. Tretter and W. Sieghart (Medical University of Vienna); anti-gephyrin (1:1000, Synaptic Systems, catalog no. 147021); Alexa Fluor 568 and 647 secondaries (1:1000, Invitrogen). The following antibodies were used for Western blotting: anti-GABA<sub>R</sub> α2 (1:500, Phospho-Solutions, catalog no. 822-GA2C); anti-GABA<sub>R</sub> α4 (1:5000) antiserum was raised against the intracellular domain of this subunit (379–421), as described previously (10); anti-GABA<sub>R</sub> β3 (1:1000, Phospho-Solutions, catalog no. 863–GB3C and 1:1000, NeuroMab, catalog no. 75-149); anti-collybistin (1:500, Synaptic Systems, catalog no. 261–003); anti-cul1 (1:2500, Abcam, catalog no. AB75817); anti-ephexin (1:1000, provided by Dr. M. E. Greenberg, Harvard University); anti-GAPDH (1:5000, Santa Cruz Biotechnology, catalog no. SC25778); anti-gephyrin (1:1000, C13B11, Synaptic Systems, catalog no. 147111); anti-GFP (1:1000, Synaptic Systems, catalog no. 132002); anti-Mfn2 (0.5 μg/ml, Abcam, catalog no. 56889); anti-mGLUR5 (1:4000, Millipore, AB5765); anti-NR1 (1:1000, BD Biosciences); anti-PAK5 (1:1000, R&D Systems, catalog no. MAB4696); anti-Rab5 (1:1000, Abcam, catalog no. AB18211); anti-tubulin (1:10,000, Millipore, catalog no. 05661); and anti-HRP-conjugated secondary (1:10,000, Jackson ImmunoResearch, catalog nos. 715035150 and 715035152). The following constructs were used: GST fusion protein constructs encoding the large intracellular loop of GABA<sub>R</sub> subunits α1, α2, β3, and γ2 as described previously (11, 12). FLAG-ephexin was provided by M. E. Greenberg (Harvard University), as described previously (13). pH<sub>2</sub> and β3 constructs have been described previously (14, 15), respectively.

*Creation of Myc-pHluorin GABA<sub>R</sub> α2 Knock-in Mice*—pH<sub>2</sub>A mice were generated by homologous recombination in embryonic stem (ES) cells (129Sv/Pas ES cells). A targeting vector was constructed to insert the pHluorin and Myc tag into embryonic stem (ES) cells (129Sv/Pas ES cells). A targeting vector was electroporated into 129Sv ES cells, and clones were screened by PCR and Southern blot analysis. ES cell clones were then expanded and clones were screened by PCR and Southern blot analysis. ES cell clones were then expanded and selected for C57BL/6J blastocyst injections. The resulting chimeras were bred with wild type C57BL/6J mice. The neomycin selected for C57BL/6J blastocyst injections. The resulting chimeras were bred with wild type C57BL/6J mice. The neomycin selected for C57BL/6J blastocyst injections. The resulting chimera was bred with wild type C57BL/6J mice. The neomycin selected for C57BL/6J blastocyst injections. The resulting chimera was bred with wild type C57BL/6J mice. The neomycin...
Bound proteins were detected by Western blotting. For experiments using HEK293 cells, pre-cleared lysates were incubated with anti-FLAG conjugated beads (Sigma, catalog no. F3169) or GFP-Trap for 2 h and subsequently washed four times in lysis buffer. Bound proteins were detected by Western blotting. A minimum of three independent experiments were performed for all coIP experiments.

**Hippocampal Slice Preparation for Electrophysiology Recordings**—Coronal slices were prepared from male WT and pHα2 animals (8–10 weeks old). Isoflurane-anesthetized mice were decapitated, and brains were rapidly removed and put in an ice-cold cutting solution (126 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 2 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM glucose, 1.5 mM sodium pyruvate, and 3 mM kynurenic acid). 310-μm slices cut with a vibratome VT1000S (Leica Microsystems, St Louis, MO) were transferred to an incubation chamber filled with warmed (31 °C) oxygenated artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM glucose, 1.5 mM sodium pyruvate, 1 mM glutamine, 3 mM kynurenic acid, and 5 μM GABA) and bubbled with 95% O₂ to 5% CO₂. Slices were allowed to recover for 1 h before recording.

**Electrophysiology Recordings**—After recovery, slices were transferred to a submerged recording chamber on the stage of an upright microscope (Nikon FN-1) with a ×40 water immersion objective equipped with DIC/IR optics. Slices were gravity superfused with ACSF solution throughout experimentation and perfused at a rate of 2 ml/min with oxygenated (O₂/CO₂ 95:5%) ACSF at 32 °C. Adequate O₂ tension and pH 7.3–7.4 values were maintained by continuously bubbling the media with 95% O₂, 5% CO₂. Currents were recorded from the dentate gyrus granule cells (DGGCs) in coronal hippocampal slices. Patch pipettes (5–7 MΩ) were pulled from borosilicate glass (World Precision Instruments) and filled with intracellular solution (140 mM CsCl, 1 mM MgCl₂, 0.1 M EGTA, 10 mM HEPES, 2 mM Mg-ATP, 4 mM NaCl, and 0.3 M Na-GTP, pH 7.25). A 5-min period for stabilization after obtaining the whole-cell recording configuration was allowed before currents were recorded using an Axopatch 200B amplifier (Molecular Devices), low pass-filtered at 2 kHz, digitized at 20 kHz (Digidata 1440A; Molecular Devices), and stored for off-line analysis. The holding potential was −60 mV for all recordings.

**Electrophysiology Analysis**—Tonic current measurements were measured from an all-points histogram that was plotted for a 10-s period before and during picrotoxin application. A Gaussian fit to these points gave the mean current amplitude, and the difference between these two values was considered to be the tonic current and normalized to cell capacitance (pA/pF). Throughout the course of the experiment, series resistance and whole-cell capacitance were continually monitored and compensated. If series resistance increased by >20%, recordings were eliminated from the data analysis. Statistical significance was determined using Student’s t test. Spontaneous IPSCs (sIPSCs) were analyzed using the mini-analysis software (version 5.6.4; Synaptosoft, Decatur, GA). sIPSCs were recorded for a minimum of 5 min. To detect sIPSCs, the minimum threshold detection was set to three times the value of baseline noise signal. The recording trace was visually inspected, and only sIPSC events with a stable baseline, sharp rising phase, and single peak were used to negate artifacts due to event summation. Only recordings with a minimum of 100 events fitting these criteria were analyzed. 8–10 cells were recorded from three animals of each genotype. Amplitude, decay, and frequency distributions of sIPSCs were examined by constructing all-point cumulative probability distributions and compared using the Mann-Whitney test and Kolmogorov-Smirnov test. Values of p < 0.05 were considered significant.

**Large Scale Immunoprecipitation for Mass Spectrometry Analysis**—Hippocampus and cortex of age-matched (8–10 weeks) and sex-matched WT and pHα2 mice (seven animals each) were prepared as above. Lysates were filtered and pre-cleared with agarose beads conjugated to IgG overnight. For tandem IPs, pre-cleared lysates were incubated with Myc antibody overnight. Sepharose beads were added and incubated at 4 °C for 4 h. These beads were washed (three times at 400 g, 2 min, 4 °C), and the proteins were eluted off beads with 200 μg/ml c-Myc peptide (Alpha Diagnostics) in lysis buffer. The eluate was incubated with GFP-Trap for 1 h, followed by four washes (2500 × g, 2 min, 4 °C) in lysis buffer. Gels were run and stained with silver stain (Sigma), and gel bands of interest from pHα2 and the corresponding regions from WT mice were excised. For single IPs, pre-cleared lysates were incubated with GFP-Trap for 2 h, followed by four washes in lysis buffer (2500 × g, 2 min, 4 °C). Gels were run and stained with colloidal Coomassie (18). Each gel lane (for pHα2 or WT IP) was cut into five pieces and sent to Taplin Mass Spectrometry Facility (Harvard University) for proteomic analysis.

**Mass Spectrometry Analysis**—Trypsin digestion, liquid chromatography-tandem mass spectrometry (LC-MS/MS), and MS/MS spectra search in a mouse database (Uniprot) using the Sequest 28 analysis program was performed by Taplin Mass Spectrometry Facility (Harvard University). Peptide matches were considered true matches for ΔCN scores (Δ correlation) >0.2 and XCorr values (cross-correlation) of greater than 2, 3, 4 for +1, +2, +3, +4 charged peptides, respectively (supplemental Tables 1 and 2). A particular protein would only be considered present if at least five such high quality peptides were detected. Three independent mass spectrometry experiments were performed. Proteins identified in pHα2 mice were compared with those found in WT animals to control for nonspecific binding of proteins. Proteins found at similar levels to a list of nonspecific binding proteins often found in mass spectrometry experiments were removed (19). For tandem IP experiments, proteins were identified by a minimum of seven peptides. Peptides found in WT control IPs were removed from the final list of proteins displayed in Table 1. For GFP-Trap IPs, proteins listed in Tables 2–7 have been identified by a minimum of five peptide, or were at least 3-fold enriched in the pHα2 compared with WT IPs. Furthermore, these peptides were present in all three experiments. Proteins in Tables 2–7 were manually organized into broad functional categories from information through a GeneCards, HUGO gene nomenclature committee, and the literature.

**Glutathione S-transferase (GST) Production and Pulldown Assay**—GST fusion proteins expressed in *Escherichia coli* BL21 were induced (0.2 mM isopropyl 1-thio-β-D-galactopyranoside, 2 h), pelleted, and resuspended in buffer A (10 mM Tris-Cl, pH 7.5).
After sonication, 2.5 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 20% glycerol), and the lysate was spun down. Supernatants containing GST fusion proteins were immobilized on pre-swollen glutathione-agarose beads (Sigma). Beads were washed five times with buffer B and kept frozen until use.

Hippocampal and cortical lysates (prepared as above) from male WT mice were pre-cleared with GST alone. These samples were then incubated with GST tagged to various GABAAR subunits immobilized on glutathione-agarose beads overnight. Beads were washed three times (400 g, 2 min, 4 °C), and bound proteins were detected by immunoblotting. A minimum of three independent GST experiments was performed for each protein studied.

**Results**

Creation of a pHluorin/Myc-tagged GABAAR α2 Subunit Knock-in Mouse—To date, our understanding of the mechanisms responsible for the formation and maintenance of inhibitory synapses has been limited. These issues are confounded by the structural diversity of GABAARs and technical limitations such as the paucity of high affinity subunit-selective antibodies. To overcome these limitations, mice were created in which pHluorin, a pH-sensitive GFP, and the Myc epitope (EQKLISEEDL, Fig. 1, A and E) were introduced into the GABAARα2 subunit. These reporters were introduced into exon 3 of the GABAARα2 subunit gene between the codons encoding amino acids 4 and 5 of the mature protein (pH2).

pH2 homozygotes were viable, bred normally, and did not exhibit any overt phenotypes. In addition, Nissl staining did not reveal any gross morphological changes in the hippocampal anatomy of pH2 mice. Scale bar, 500 μm. E. DNA and protein sequence of N-terminal segment of pH2 knock-in mouse. pHluorin (green, italics) and Myc (red, underline) reporters are depicted.

**FIGURE 1. Construction of pHluorin-Myc-tagged GABAAR α2 mouse.** A, a schematic representation of pHluorin-Myc tagged at the N terminus of the GABAARα2 subunit. B, illustrations of the targeting vector and the targeted α2 subunit gene with addition of pHluorin-myc into exon 3. C, genotyping for wild type (+/−), heterozygotes (+/+), and pH2 (+/+ ) mice using primers flanking pHluorin. D, cresyl violet staining of hippocampus shows there are no gross morphological changes in the hippocampal anatomy of pH2 mice. Scale bar, 500 μm. E, DNA and protein sequence of N-terminal segment of pH2 knock-in mouse. pHluorin (green, italics) and Myc (red, underline) reporters are depicted.
sion of the pHα2 subunit, immunoblotting was utilized with α2 subunit antibodies. In accordance with the addition of pHluorin, the molecular mass of the α2 subunit was increased by ~30 kDa in extracts prepared from pHα2 homozygotes compared with WT (Fig. 2A). However, there were no significant differences in the total expression levels of the GABAARs α4 and β3.
subunit, GAPDH, gephyrin, NMDA receptor NR1 subunit, and tubulin in pH2 mice compared with wild type animals (Fig. 2B; p > 0.05).

Plasma membrane accumulation of the α2 subunit is dependent upon oligomerization with receptor β subunits (1, 2, 21). To test whether pH2 subunits are associated with endogenous receptor β subunits, detergent-solubilized brain extracts were subjected to immunoprecipitation with Myc or GFP antibodies. As measured by immunoblotting, the α2 and β3 subunits were detected to immunoprecipitate with Myc or GFP antibodies from pH2 but not WT brains (Fig. 3A and B). Molecular, genetic, and biochemical approaches suggest that the multifunctional protein gephyrin and the GDP-GTP exchange factor collybistin play important roles in determining the synaptic accumulation of GABAAR subunits (1, 2, 21). Consistent with this, both of these proteins were detected to immunoprecipitate with Myc/GFP antibodies from pH2 but not WT brain extracts. Thus, in mouse brain pH2 assembles with endogenous GABAAR subunits and is associated with gephyrin and collybistin.

**pH2 Subunits Are Targeted to Functional Inhibitory Synapses**—In the brain, GABAARs containing α2 subunits are highly concentrated at inhibitory synapses (1, 2, 23, 24). To assess whether this synaptic targeting also occurs in pH2 mice, 18 days in vitro hippocampal cultures produced from these mice were stained with α2 and gephyrin antibodies and imaged by confocal microscopy. Endogenous green fluorescence colocalized with GABAAR α2 subunit immunoreactivity (Fig. 3C; p < 0.001) at gephyrin-positive postsynaptic inhibitory specializations (Fig. 3C; p < 0.005).

Next, we compared the properties of phasic and tonic inhibition in the dentate gyrus granule cells (DGGCs) of WT and pH2 mice (Fig. 4). Examination of sIPSCs revealed that there was no significant difference in the amplitude (Fig. 4B; WT 68.7 ± 1.6 pA, n = 8; pH2 67.3 ± 2.0 pA, n = 8, p = 0.06), frequency (Fig. 4C; WT 2.7 ± 0.4 Hz, n = 8; pH2 2.8 ± 0.2 Hz, n = 8, p > 0.99), and decay time (Fig. 4D; WT 4.6 ± 0.1 ms, n = 8; pH2 4.9 ± 0.1 ms, n = 8, p = 0.82) between genotypes. Similarly, the tonic current amplitude (Fig. 4F; WT 30.7 ± 0.7 pA, n = 9; pH2 21.0 ± 6.0 pA, n = 10, p = 0.34) and current density (Fig. 4G; WT 1.3 ± 0.3 pA/pF; n = 10; pH2 0.8 ± 0.2 pA/pF n = 9, p = 0.18) were comparable between WT and pH2 mice.

Collectively, these data suggest that GABAARs containing pH2 subunits are targeted to inhibitory synapses, and their incorporation at these subcellular specializations does not have an impact on GABAergic inhibition.

**Isolation of GABAARs from the Brains of pH2 Mice Using Two-step Tandem Affinity Purification**—To assess which proteins associate with GABAAR subunits in the brain, a two-step immunoaffinity purification protocol was performed. First, hippocampi and cortices from age/sex-matched WT and pH2 mice were solubilized and exposed to Myc antibody followed by G-Sepharose beads. After extensive washes, bound material was eluted with Myc peptide and exposed to immobilized GFP-Trap beads. Bound material was subsequently eluted using 2% SDS and subjected to SDS-PAGE followed by silver staining. Bands that were present in the pH2 lane and the adjacent lane from WT mice were excised and subjected to LC-MS/MS (Fig. 5). Three independent purifications were performed for both WT controls and pH2 animals. Table 1 shows a list of the proteins identified by MS analysis that associate with pH2. Proteins listed were identified by a minimum of two-step Tandem Affinity Purification—

![Image](158x538 to 191x694)

![Image](195x538 to 228x694)
there was some contamination between bands, the majority of GFP and /H9251 2 subunit peptides were identified in the major silver-stained product at /H11011 80 kDa. Atp1a1 was found at the 100-kDa region, /H9251 4 subunit at the 65-kDa region, and the rest were found in the 50–55-kDa region of the gel. Collectively, these results suggest that pH /H9251 2 is capable of assembling with the /H9253 2 and multiple /H9251 and /H9252 subunit isoforms in the brain.

GFP-Trap Purification of GABAARs Reveals Their Association with Known Binding Partners—To increase the probability of identifying proteins that are associated with the pH /H9251 2-containing GABAARs, a single purification with GFP-Trap was used. Lysates from hippocampi and cortices of age- and sex-matched WT and pH /H9251 2 mice were incubated with GFP-Trap beads. These samples were then subjected to SDS-PAGE followed by Coomassie staining (Fig. 6). The single step purification method led to an increased yield of protein compared with the tandem purification as indicated by the increased number of peptides identified and greater protein coverage for GABAAR /H9251 2 (Figs. 5B and 6B; GFP/myc IP 8.4%, GFP IP 43%). Three independent purifications were performed, and proteins identified by LC-MS/MS in all three experiments and found to be at least 3-fold enriched in the pH /H9251 2 samples are listed in Tables 2–7 and supplemental Table 2.

Identification of Novel Components of the GABAAR Proteome Using GFP-Trap Purification—In addition to known interacting proteins as detailed in Table 2, 149 novel binding partners for GABAARs were identified in material purified from pH /H9251 2 animals. For brevity, these proteins were divided into five

---

**TABLE 1**
Proteins identified with pH /H9251 2 identified using tandem myc/GFP-Trap purification

| Gene symbol | Reference Name | Total peptide WT | pH /H9251 2 |
|-------------|----------------|-----------------|-------------|
| Atp1a1      | Na⁺ /K⁺-transporting ATPase subunit α1 | 0 | 34 |
| Gabra1      | GABA R, subunit α1 | 0 | 9 |
| Gabra2      | GABA R, subunit α2 | 0 | 15 |
| Gabra3      | GABA R, subunit α3 | 0 | 8 |
| Gabra4      | GABA R, subunit α4 | 0 | 14 |
| Gabra5      | GABA R, subunit α5 | 0 | 11 |
| Gabrb1      | GABA R, subunit β1 | 0 | 23 |
| Gabrb2      | GABA R, subunit β2 | 0 | 17 |
| Gabrb3      | GABA R, subunit β3 | 0 | 40 |
| Gabrg2      | GABA R, subunit γ2 | 0 | 10 |

---

**FIGURE 6.** Single-step purification to isolate pH /H9251 2 complexes. Detergent-solubilized hippocampal and cortical lysates from WT and pH /H9251 2 mice were immunoprecipitated with GFP antibodies and subjected to SDS-PAGE and colloidal Coomassie staining (A). Each gel lane was cut into five pieces and pooled for mass spectrometry analysis. Protein coverage of GABAAR α2 subunit (blue, underline) identified by MS analysis (B). Example of MS/MS spectrum for tryptic peptide identified as GABAAR /H9251 2 is shown (C). The sequence of the identified peptide is indicated.
non-specific binding of proteins. Proteins listed have appeared in all three experiments, have been identified by a minimum of five peptides, and there is a 3-fold difference between peptides found in pH compared with WT IPs.

| Gene symbol | Reference | Name | Total peptide |
|-------------|-----------|------|---------------|
| Arhgefl9    | GFP_Aequorea | Green fluorescent protein | 2 | 855 |
| Gabbr2      | ARHG9_MOUSE | Cdc42 guanine nucleotide exchange factor 9, collybistin | 1 | 62 |
| Gabbr2      | GABR2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, 2 | 2 | 16 |
| Gabbr2      | GABR2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, α1 | 10 | 501 |
| Gabbr2      | GABR2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, α2 | 5 | 341 |
| Gabbr4      | GRB4_MOUSE | γ-Aminobutyric acid (GABA) A receptor, α4 | 1 | 369 |
| Gabbr5      | GRB5_MOUSE | γ-Aminobutyric acid (GABA) A receptor, α5 | 3 | 146 |
| Gabbr6      | GRBB1_MOUSE | γ-Aminobutyric acid (GABA) A receptor, β1 | 7 | 481 |
| Gabbr6      | GRBB2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, β2 | 6 | 293 |
| Gabbr6      | GRBB3_MOUSE | γ-Aminobutyric acid (GABA) A receptor, β3 | 7 | 422 |
| Gabbr6      | GRBRD_MOUSE | γ-Aminobutyric acid (GABA) A receptor, δ | 0 | 80 |
| Gabbr6      | GBRG1_MOUSE | γ-Aminobutyric acid (GABA) A receptor, δ | 0 | 112 |
| Gabbr6      | Q3UVW2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, γ2 | 0 | 9 |
| Gabbr6      | GBRG2_MOUSE | γ-Aminobutyric acid (GABA) A receptor, γ2 | 1 | 198 |
| Gabbr3      | GBRG3_MOUSE | γ-Aminobutyric acid (GABA) A receptor, γ3 | 0 | 56 |
| Gprb        | GLRB_MOUSE | Glycine receptor β | 0 | 6 |
| Gphn        | GPHN_MOUSE | Gephyrin | 5 | 140 |
| Nlg1        | NLGN1_MOUSE | Neurologin 1 | 2 | 59 |
| Nlg1        | NLGN2_MOUSE | Neurologin 2 | 0 | 117 |
| Nlg1        | NLGN3_MOUSE | Neurologin 3 | 4 | 33 |
| Nlg1        | NLGN4_MOUSE | Neurologin 4 | 0 | 6 |
| Prkacb       | KAPCB_MOUSE | Protein kinase, cAMP-dependent, β catalytic subunit | 1 | 7 |
| Prkca        | KPCA_MOUSE | Protein kinase C, α | 15 | 55 |
| Prkg         | KPCG_MOUSE | Protein kinase C, γ | 27 | 95 |

groups based on literature searches of their presumed functions: 1) G-protein coupled receptors (GPCRs), ion channels, and transporters (Table 3); 2) regulators of protein trafficking, stability, and cytoskeletal anchoring (Table 4); 3) regulators of GTP exchange and protein phosphorylation (Table 5); 4) miscellaneous enzymes (Table 6); and 5) miscellaneous proteins (Table 7). These various binding partners presumably act sequentially to control receptor assembly, forward trafficking in the secretory pathway, trafficking to and stabilization at inhibitory synapses, receptor endocytosis, and endocytic sorting followed by lysosomal or proteosomal degradation.

**Cullin1, Ephexin, KCTD12, Mitofusin2, mGluR5, PAK5/7, and Rab5 Bind to the Intracellular Loop of Specific GABA_A Subunits**—To confirm our MS findings, we examined the binding of selected hits to the intracellular domains of GABA_A subunits. Our initial studies focused on the GPCR mGluR5 (Grm5), the kinase PAK5/7 (Pk7), the GTPases mitofusin2 (Mfn2), and Rab5, the RhG guanine nucleotide exchange factor ephexin (Ngef) and regulator of ubiquitination cullin1 (Cul1) (Tables 3–5). These proteins were chosen for their range in the total number of peptides identified by MS analysis as follows: from a lower number of peptides (e.g. mGluR5; 0 peptides WT
and 6 peptides pH2) to protein identified by a larger number of peptides (e.g. ephexin; 5 peptides WT and 37 peptides pH2). In addition, GPCRs and the respective activities have all been previously implicated in regulating GABA<sub>R</sub> membrane trafficking (1). Furthermore, we also assessed the interaction of KCTD12 (Table 7), an auxiliary subunit of GABA<sub>R</sub>S previously implicated in regulating GABA<sub>R</sub> signaling and G-protein activation (33). For these experiments, purified GST fusion pro-

### Table 4

| Gene symbol | Reference | Name | Total peptide |
|-------------|-----------|------|---------------|
| Adam22      | ADA22_MOUSE | ADAM metalloprotease domain 22 | 0 9 |
| Add3        | ADDG_MOUSE | Adducin 3 | 4 17 |
| Afg3L       | AFG3_MOUSE | AFG3-like AAA ATPase 2 | 8 42 |
| Cal1        | CUL1_MOUSE | Cullin 1 | 2 22 |
| Cal2        | CUL2_MOUSE | Cullin 2 | 3 17 |
| Cal3        | CUL3_MOUSE | Cullin 3 | 5 21 |
| Dcaf8       | DCAF8_MOUSE | DDB1- and CUL4-associated factor 8 | 0 7 |
| Ddb1        | DDB1_MOUSE | Damage-specific DNA-binding protein 1 | 1 10 |
| Dnaa1       | DNJ1 MOUSE | Dnaa1 heat shock protein family (Hsp40) member A1 | 2 11 |
| Dyncl2      | DC1L2_MOUSE | Dyncl2, cytoplasmic 1, intermediate chain 2 | 3 13 |
| Epn1        | EPNI1_MOUSE | Epsin 1 | 0 8 |
| Erlin1      | ERNL1_MOUSE | Endoplasmic reticulum lipid raft-associated 1 | 3 13 |
| Exoc3       | EXOC3_MOUSE | Exocyst complex component 3 | 1 18 |
| Exoc7       | EXOC7_MOUSE | Exocyst complex component 7 | 1 28 |
| Exoc8       | EXOC8_MOUSE | Exocyst complex component 8 | 2 13 |
| Hook3       | HOOK3_MOUSE | Hook microtubule-tethering protein 3 | 1 8 |
| Ipo9        | IPO9_MOUSE | Importin 9 | 2 10 |
| Kbtbd7      | G5E8C2_MOUSE | Kelch repeat and BTB (POZ) domain containing 7 | 0 13 |
| Kif5a       | KIF5A_MOUSE | Kinesin family member 3A | 7 26 |
| Lrc7        | LRR7_MOUSE | Leucine-rich repeat-containing 7 | 1 7 |
| Mag3        | MAG3_MOUSE | Membrane-associated guanylate kinase, WW, and PDZ domain containing 3 | 0 14 |
| Mapre2      | MARE2_MOUSE | Microtubule-associated protein RP/EB family member 2 | 0 10 |
| Nap1        | SNA1A_MOUSE | NSF attachment protein a | 5 22 |
| Nap2        | SNA1B_MOUSE | NSF attachment protein b | 2 15 |
| Nefl        | NFM_MOUSE | Neurofilament, light polypeptide | 3 11 |
| Ngly1       | NGLY1_MOUSE | N-Glycanase 1 | 0 10 |
| On9         | OS9_MOUSE | Osteosarcoma-amplified 9, endoplasmic reticulum lectin | 0 8 |
| Pomp9       | PSM9D1_MOUSE | Proteosome 26S subunit, non-ATFase 9 | 2 9 |
| Scamp3      | SCAM3_MOUSE | Secretory carrier membrane protein 3 | 0 8 |
| Sec23b      | SC23B_MOUSE | Sec23 homolog B, COPII coat complex component | 0 9 |
| Sqt1m1      | SQSTM1_MOUSE | Sequestosome 1 | 1 8 |
| Sv2a        | SV2A_MOUSE | Synaptic vesicle glycoprotein 2A | 15 64 |
| Sv2b        | SV2B_MOUSE | Synaptic vesicle glycoprotein 2B | 6 35 |
| Trim32      | TRIM32_MOUSE | Tripartite motif containing 32 | 7 24 |
| Uch1        | UCH1_MOUSE | Ubiquitin C-terminal hydrolase L1 | 7 22 |
| Usp9        | USP9_MOUSE | Ubiquitin-specific peptidase 9, X-linked | 2 12 |
| Vps35       | VPS35_MOUSE | VPS35 retromer complex component | 21 69 |
| Vps52       | VPS52_MOUSE | VPS25 GARP complex subunit | 2 21 |

### Table 5

| Gene symbol | Reference | Name | Total peptide |
|-------------|-----------|------|---------------|
| Adrbk1      | ARRBK1_MOUSE | Adrenergic, β receptor kinase 1 | 4 27 |
| Argef3      | BIG3_MOUSE | ARGEF family member 3 | 1 11 |
| At1l        | ATIA1_MOUSE | Atlantis GTPase 1 | 1 8 |
| Dnm1l       | DNM1L_MOUSE | Dynamin 1-like | 17 61 |
| Elm1        | ELMO1_MOUSE | Engulfment and cell motility 1 | 0 6 |
| Gd1         | GN1L1_MOUSE | Guanine nucleotide-binding protein-like 1 | 4 15 |
| Gpsm1       | GPSM1_MOUSE | G-protein signaling modulator 1 | 1 14 |
| Iqsec3      | IQEC3_MOUSE | IQ motif and Sec7 domain 3 | 0 13 |
| Lpp4        | LPP4_MOUSE | Phospholipid phosphatase-related 4 | 8 67 |
| Mn2         | MFN2_MOUSE | Mitofusin 2 | 8 32 |
| Nedd4l      | NED4L_MOUSE | Nuclear precursor cell expressed, developmentally down-regulated 4-like, E3 ubiquitin protein ligase | 1 7 |
| Ngef        | NGEF_MOUSE | Neuronal guanine nucleotide exchange factor | 5 37 |
| Opal1       | OPAL1_MOUSE | Optic atrophy 1 (autosomal dominant) | 10 32 |
| Pak7        | PK7_MOUSE | P21 protein (Cdc42/Rac)-activated kinase 7 | 0 6 |
| Pmm1e       | PPME1_MOUSE | Protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup>-dependent 1E | 1 7 |
| Ppdrd       | PTRD_MOUSE | Protein-tyrosine phosphatase, receptor type D | 6 32 |
| Ppt3        | PPT3_MOUSE | Protein-tyrosine phosphatase, receptor type S | 6 29 |
| Rab14       | RAB14_MOUSE | Rab14, member RAS oncogene family | 8 31 |
| Rab1b       | RAB1B_MOUSE | RAB1B, member RAS oncogene family | 1 8 |
| Rab33b      | RAB33B_MOUSE | RAB33B, member RAS oncogene family | 2 11 |
| Rab5a       | RAB5A_MOUSE | RAB5A, member RAS oncogene family | 1 11 |
| Rab5b       | RAB5B_MOUSE | RAB5B, member RAS oncogene family | 0 7 |
| Rhot1       | ROI1_MOUSE | Ras homolog family member T1, Miro1 | 4 25 |
| Ric8a       | RIC8A_MOUSE | RIC8 guanine nucleotide exchange factor A | 2 10 |
| Tic1d15      | TBC1D15_MOUSE | TBC1 domain family member 15 | 1 8 |
| Tic1d17      | TBC1D17_MOUSE | TBC1 domain family member 17 | 2 9 |
teins encoding the intracellular domains of the receptor α1, α2, β3, and γ2 subunits were exposed to detergent-solubilized brain extracts from WT mice, and bound material was subjected to immunoblotting. Cullin1, a component of an E3 ubiquitin ligase complex (34), bound to GST-β3 and γ2 compared with GST alone (Fig. 7A; β3 p < 0.05, γ2 p < 0.05) as did KCTD12 (Fig. 7C; β3 p < 0.05, γ2 p < 0.05). Likewise, mito-

fusin2, a GTPase localized at the outer mitochondrial membrane (35), bound β3 and γ2 (Fig. 7D; β3 p < 0.001, γ2 p < 0.0001). The GTPase Rab5 is found at endosomes, phagosomes, caveosomes, and the plasma membrane (36) and has been shown to colocalize with the GABA<sub>A</sub>R β3 subunit (37). Consistent with these results, Rab5 bound GST-β3 and γ2 (Fig. 7G; β3 p < 0.0001, γ2 p < 0.05). In contrast to this, PAK5/7, a poorly

### TABLE 6

Miscellaneous enzyme activities associated with pH2 identified using GFP-Trap purification

| Gene symbol | Reference | Name | Total peptide |
|-------------|-----------|------|---------------|
| Acetylglutamate synthase | P324_MOUSE | Acs6 | 14 |
| Acs3 | Acs3_MOUSE | Acs3 | 16 |
| Acs4 | Acs4_MOUSE | Acs4 | 16 |
| Acs2 | Acs2_MOUSE | Acs2 | 16 |
| Adprh | Adprh_MOUSE | Adprh | 16 |
| Aldh18a1 | Aldh18a1_MOUSE | Aldh18a1 | 16 |
| Ca2 | CAH2_MOUSE | CAH2 | 16 |
| Capn2 | Can2_MOUSE | Capn2 | 16 |
| Cds2 | Cds2_MOUSE | Cds2 | 16 |
| Cpi1a | Cpi1a_MOUSE | Cpi1a | 16 |
| Dpp3 | Dpp3_MOUSE | Dpp3 | 16 |
| Ech1 | Ech1_MOUSE | Ech1 | 16 |
| Ecl1 | Ecl1_MOUSE | Ecl1 | 16 |
| Glpt1 | Glpt1_MOUSE | Glpt1 | 16 |
| Gata1 | Gata1_MOUSE | Gata1 | 16 |
| Gucyl1a2 | Gvq1a2_MOUSE | Gucyl1a2 | 16 |
| Hsd17b8 | Hsd17b8_MOUSE | Hsd17b8 | 16 |
| Mps1 | Mps1_MOUSE | Mps1 | 16 |
| Ndufs1 | Ndufs1_MOUSE | Ndufs1 | 16 |
| Ndufs3 | Ndufs3_MOUSE | Ndufs3 | 16 |
| Pank4 | Pank4_MOUSE | Pank4 | 16 |
| Ppil | Ppil_MOUSE | Ppil | 16 |
| Pld1 | Pld1_MOUSE | Pld1 | 16 |
| Rpn2 | Rpn2_MOUSE | Rpn2 | 16 |
| Srr | Srr_MOUSE | Srr | 16 |
| Tars | Tars_MOUSE | Tars | 16 |

### TABLE 7

Miscellaneous proteins associated with pH2 identified using GFP-Trap purification

| Gene symbol | Reference | Name | Total peptide |
|-------------|-----------|------|---------------|
| Apol1 | Apol1_MOUSE | Apol1 | 16 |
| Armc10 | Armc10_MOUSE | Armc10 | 16 |
| Avl9 | Avl9_MOUSE | Avl9 | 16 |
| Bcl2l13 | Bcl2l13_MOUSE | Bcl2l13 | 16 |
| Chchd6 | Chchd6_MOUSE | Chchd6 | 16 |
| Cia5 | Cia5_MOUSE | Cia5 | 16 |
| Cyc1 | Cyc1_MOUSE | Cyc1 | 16 |
| Efb2 | Efb2_MOUSE | Efb2 | 16 |
| Fam49a | Fam49a_MOUSE | Fam49a | 16 |
| Fam49b | Fam49b_MOUSE | Fam49b | 16 |
| Fathg1 | Fathg1_MOUSE | Fathg1 | 16 |
| Hbs1l | Hbs1l_MOUSE | Hbs1l | 16 |
| Hmt | Hmt_MOUSE | Hmt | 16 |
| Kctd12 | Kctd12_MOUSE | Kctd12 | 16 |
| Lhpl4 | Lhpl4_MOUSE | Lhpl4 | 16 |
| Lr2 | Lr2_MOUSE | Lr2 | 16 |
| Mag | Mag_MOUSE | Mag | 16 |
| Nbea | Nbea_MOUSE | Nbea | 16 |
| Pgrmc1 | Pgrmc1_MOUSE | Pgrmc1 | 16 |
| Pd | Pd_MOUSE | Pd | 16 |
| Phb | Phb_MOUSE | Phb | 16 |
| Pdx1 | Pdx1_MOUSE | Pdx1 | 16 |
| Ppp2 | Ppp2_MOUSE | Ppp2 | 16 |
| Sam50 | Sam50_MOUSE | Sam50 | 16 |
| Ssh7a | Ssh7a_MOUSE | Ssh7a | 16 |
| Tmem132b | Tmem132b_MOUSE | Tmem132b | 16 |
| Ywhab | Ywhab_MOUSE | Ywhab | 16 |
| Ywhag | Ywhag_MOUSE | Ywhag | 16 |
| Ywhaz | Ywhaz_MOUSE | Ywhaz | 16 |
| Zer1 | Zer1_MOUSE | Zer1 | 16 |
FIGURE 7. Cullin1, ephexin, KCTD12, mitofusin2, mGluR5, PAKS7/7 and Rab5 bind the intracellular loop of specific GABA_A Rs. Detergent-solubilized hippocampal and cortical lysates from WT mice were incubated with GST or GST tagged to the large intracellular loop of various GABA_AR subunits. Bound proteins including Cull1 (A), ephexin (B), KCTD12 (C), Mfn2 (D), mGluR5 (E), PAKS7/7 (F) and Rab5 (G) were detected by immunoblotting. The upper panels show representative immunoblots; the lower panels show Ponceau staining depicting the relative amounts of GST utilized. Graphs show pooled quantification of immunoblots. *, p < 0.05; **, p < 0.01; ***, p < 0.0001 compared with GST alone and #, p < 0.05; ##, p < 0.001; ###, p < 0.0001 compared with other subunits, analysis of variance with Games-Howell post hoc test (due to differences in variance), n = 3–8. Data are means ± S.E.

The potential interaction of ephexin with GABA_AR subunits was of particular interest because ephexin belongs to the same family of GDP-GTP exchange factors (GEFs) as collybistin, a molecule that plays a key role in determining the formation of hippocampal inhibitory synapses (22, 41). To further corroborate our findings in pHα2 mice, we expressed FLAG-ephexin, pHα2, and β3 in HEK293 cells. Reciprocal immunoprecipitation with FLAG and GFP antibodies revealed the robust association of ephexin with GABA_AR Rs in HEK293 cells (Fig. 8A).

Together, these studies demonstrate that proteins identified by mass spectroscopy can be validated in the brain and in expression systems.

Discussion

Inhibitory fast synaptic transmission is critically dependent upon the accumulation and stabilization of selected GABA_AR subtypes at inhibitory postsynaptic specializations. To further elucidate the processes neurons utilize to regulate the synaptic accumulation of these critical ligand-gated ion channels, we have created mice in which the α2 subunit is modified with pHluorin and Myc reporters by targeting the respective gene using homologous recombination. These reporters were introduced between residues 4 and 5 of the mature subunit. pHα2 homozygotes were viable and did not exhibit any overt phenotypes but exhibited endogenous fluorescence at inhibitory synapses. Moreover, the properties of shPSCs and tonic currents, the unitary events that underlie phasic and tonic inhibitory synaptic transmission, were similar between genotypes. Importantly, gephyrin and collybistin, which were previously reported to associate with GABA_AR, could be shown to coimmunoprecipitate in brain lysates, highlighting the necessity for the tagged protein to enable high-affinity purifications.

Consensus opinion suggests that the α1–3 subunits are components of synaptic GABA_ARs and that the anxiolytic and sedative properties of benzodiazepines are mediated by specific receptor subtypes containing individual α subunit isoforms. Therefore, we assessed which receptor subunits associate with pHα2 using tandem purification on Myc and GFP antibodies followed by LC-MS/MS. This approach revealed that the pHα2 subunit copurified with α1, α3, α4, α5, β1–3, and γ2 subunits. Using GFP-Trap alone, we further detected association with the γ1, γ3, and δ subunits. Although these results are not quantitative and do not discriminate between surface and intracel-
ular populations, our results do suggest the existence of multiple receptor subtypes with mixed α and/or β subunits, supporting previous observations of the coexistence of different α subunits in a single receptor complex (42–46). Consistent with our results, previous studies to identify proteins associated with the GABAAR α5 subunit through MS analysis exclusively identified other GABAAR subunits, including α1–3, α5, β1–3, and γ2 (47). A more recent investigation into the proteins associated with the GABAAR α1 subunit isolated 18 associated proteins via MS analysis, more than half of which were other GABAAR subunits (48), further supporting the possibility of a more heterogeneous population of receptors than originally predicted (5, 49). It is important to note that some of these subunit interactions may represent “non-productive” or non-functional receptor assembly intermediates that are not present on the plasma membrane (1, 2, 25, 50). Because GABAARs are a major target for pharmacological agents such as benzodiazepines, barbiturate, neurosteroids, and general anesthetics (3), the heterogeneity of these receptors may have major implications in the design of subunit-selective drugs for therapeutic use.

In addition to receptor subunits, we also isolated the known GABAAR binding partners gephyrin, collybistin, PKC, PKA, and GABAAR2. To the best of our knowledge, this is the first time that these respective protein-protein interactions have been simultaneously demonstrated for GABAARs in their native environment. The use of a single GFP-Trap protein purification yielded a 174-multiprotein complex comprising 149 novel protein components that copurified with pHα2 compared with material isolated from WT mice. Novel components of the GABAAR complex include other receptors, proteins required for trafficking, ubiquitination/degradation, GTPases and their regulators, cytoskeletal components, and a host of enzymes. Significantly, the PSD95 family of proteins, which is enriched in excitatory synapses (32), was absent from these purifications.

As an initial means of assessing the significance of our MS experiments, we tested the interaction of selected proteins from brain extracts with GST fusion proteins encoding the intracellular domains of GABAAR subunits. Our studies focused on mGluR5, PAK5/7, mitofusin2, Rab5, ephexin, and cullin1 due to the availability of suitable antibodies. All of the proteins bound to the intracellular domains of the receptor α1, α2, β, or γ2 subunits, confirming the veracity of our GFP-Trap purifications.

We further validated some of the MS results by demonstrating that mGluR5, KCTD12, and ephexin coIP with pHα2 from brain lysates. We are particularly interested in ephexin due to some similarities with collybistin. Collybistin is a member of the Dbl family of GEFs necessary for the proper clustering of gephyrin and gephyrin-dependent GABAARs (41). Like collybistin, ephexin also belongs to the Dbl family of GEFs and therefore has a similar domain structure to collybistin. Studies on ephexin have described its role in axon guidance in retina ganglion cells (13) and dispersal of synaptic acetylcholine receptor clusters in the neuromuscular junction through its capacity to activate Rho family GTPases (51). Numerous regulators of the actin cytoskeleton such as the Rho family GTPases have been demonstrated to be critical for synapse remodeling at excitatory synapses (52). In addition, similar roles for the regulation of the actin cytoskeleton at inhibitory GABAergic synapses have only more recently begun to emerge (53). Although how ephexin, other GTPases, and GTPase regulators identified here may affect GABAARs remains to be seen, it is tantalizing to speculate that they may have similarly important roles at inhibitory synapses.

Typical contaminants such as highly abundant proteins (e.g. actin, tubulin, and ribosomal proteins) and proteins that bind...
unfolded proteins (e.g., heat shock proteins) are commonly found in affinity-purified protein preparations (54). Our use of proper WT controls removed many of these contaminants. Furthermore, the requirement for the detection of proteins from three different experiments unveiled protein binding partners that may weakly but stably form a complex with pH2. Thus, potential pH2-associated proteins cannot readily be discarded due to a low number of total peptides discovered. Indeed, although only six peptides were identified for mGluR5, we demonstrated that it was robustly coimmunoprecipitated with pH2 (Fig. 8A).

Previously described GABA_A-R-associated proteins have been demonstrated to be essential for regulatory processes crucial for GABA_A-R function (1, 2, 55). The characterization of the protein components that form the inhibitory synaptic complex described here have wide-ranging ramifications for the understanding of GABA_A-R activity and trafficking and therefore its role in synaptic transmission and plasticity. The vast majority of proteins purified here are novel putatively GABA_A-R-associated proteins, indicating that the inhibitory synapse is likely to be far more complex than initially appreciated. Thus, the challenge still remains to elucidate the effects of these associations on GABA_A-Rs. Considering the crucial role of GABA_A-R in brain function, it is of fundamental importance to ascertain the underpinning mechanisms that govern these receptors thereby clarifying its role in CNS health and disease.

Author Contributions—Y. N. conducted most of the experiments, analyzed the results, and co-wrote paper. D. H. M. performed PCRs to sequence the mouse and collybistin coIPs, produced GSTs, and provided technical assistance. A. M. performed electrophysiology experiments. D. H. produced GSTs and performed some GST experiments. T. Z. D. performed some electrophysiological experiments. P. A. D. and S. J. M. conceived and coordinated the study and wrote the paper with Y. N. M. L. created the pH2 mouse. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—The FLAG-ephrin construct and ephrin antibody were the generous gifts from Prof. Michael Greenberg (Harvard University). The C-terminal anti-α2 antibody was provided by Dr. Verena Tretter and Prof. Werner Sieghart (Medical University of Vienna).

References
1. Jacob, T. C., Moss, S. J., and Jurd, R. (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat. Rev. Neurosci. 9, 331–343
2. Luscher, B., Fuchs, T., and Kilpatrick, C. L. (2011) GABA(A) receptor trafficking-mediated plasticity of inhibitory synapses. Neuron 70, 385–409
3. Sieghart, W. (2015) Allosteric modulation of GABA(A) receptors via multiple drug-binding sites. Adv. Pharmacol. 72, 53–96
4. Rudolph, U., and Möhler, H. (2014) GABA(A) receptor subtypes: therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. Annu. Rev. Pharmacol. Toxicol. 54, 483–507
5. Olsen, R. W., and Sieghart, W. (2008) International Union of Pharmacology. LXX. Subtypes of γ-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol. Rev. 60, 243–260
6. Patel, B., Mortensen, M., and Smart, T. G. (2014) Stoichiometry of δ subunit containing GABA(A) receptors. Br. J. Pharmacol. 171, 985–994
7. Verdoorn, T. A., Draguhn, A., Ymer, S., Seeburg, P. H., and Sakmann, B. (1990) Functional properties of recombinant rat GABAA receptors depend upon subunit composition. Neuron 4, 919–928
8. Rudolph, U., and Knoflach, F. (2011) Beyond classical benzodiazepines: novel therapeutic potential of GABA(A) receptor subtypes. Nat. Rev. Drug Discov. 10, 685–697
9. Feng, W., and Zhang, M. (2009) Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. Nat. Rev. Neurosci. 10, 87–99
10. Hörttagl, H., Tasan, R. O., Wieseltäher, A., Kirchmair, E., Sieghart, W., and Sperk, G. (2013) Patterns of mRNA and protein expression for 12 GABA(A) receptor subunits in the mouse brain. Neuroscience 236, 345–372
11. Brandon, N. J., Jovanovic, J. N., Colledge, M., Kittler, J. T., Brandon, J. M., Scott, J. D., and Moss, S. J. (2003) A-kinase anchoring protein 79/150 facilitates the phosphorylation of GABA(A) receptors by cAMP-dependent protein kinase via selective interaction with receptor β subunits. Mol. Cell. Neurosci. 22, 87–97
12. Tretter, V., Jacob, T. C., Mukherjee, J., Fritschi, J. M., Pangalos, M. N., and Moss, S. J. (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor α2 subunits to gephyrin. J. Neurosci. 28, 1356–1365
13. Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A., and Greenberg, M. E. (2001) EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephrin. Cell 105, 233–244
14. Jacob, T. C., Michels, G., Silayeva, L., Haydon, J., Succol, F., and Moss, S. J. (2012) Benzodiazepine treatment induces subtype-specific changes in GABA(A) receptor trafficking and decreases synaptic inhibition. Proc. Natl. Acad. Sci. U.S.A. 109, 18595–18600
15. Abramian, A. M., Comenencia-Ortiz, E., Vithlani, M., Trett, E. V., Sieghart, W., Davies, P. A., and Moss, S. J. (2010) Protein kinase C phosphorylation regulates membrane insertion of GABA(A) receptor subtypes that mediate tonic inhibition. J. Biol. Chem. 285, 41795–41805
16. Bolte, S., and Cordelières, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232
17. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to Image: 25 years of image analysis. Nat. Methods 9, 671–675
18. Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carnemolla, B., Orcchi, P., Zardi, L., and Righetti, P. G. (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327–1333
19. Mellacheruvu, D., Wright, Z., Couzens, A. L., Lambert, J. P., St-Denis, N. A., Li, T., Miteva, Y. V., Hauri, S., Sardiu, M. E., Low, T. Y., Halim, V. A., Bagshaw, R. D., Hubner, N. C., Al-Hakim, A., Bouchard, A., et al. (2013) The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. Nat. Methods 10, 730–736
20. Jacob, T. C., Bogdanov, Y. D., Magnus, C., Saliba, R. S., Kittler, J. T., Haydon, P. G., and Moss, S. J. (2005) Gephyrin regulates the cell surface dynamics of synaptic GABA(A) receptors. J. Neurosci. 25, 10469–10478
21. Connolly, C. N., Wooltorton, J. R., Smart, T. G., and Moss, S. J. (1996) Subcellular localization of γ-aminobutyric acid type A receptors is determined by receptor β subunits. Proc. Natl. Acad. Sci. U.S.A. 93, 9899–9904
22. Saiepour, L., Fuchs, C., Patrizi, A., Sassoé-Pognetto, M., Harvey, B. J., and Harvey, K. (2010) Complex role of collybistin and gephyrin in GABA receptor clustering. J. Biol. Chem. 285, 29623–29631
23. Essrich, C., Lorez, M., Benson, J. A., Fritschi, J. M., and Lüscher, B. (1998) Postsynaptic clustering of major GABA(A) receptor subtypes requires the γ2 subunit and gephyrin. Nat. Neurosci. 1, 563–571
24. Kneussel, M., Brandstätter, J. H., Laube, B., Stahl, S., Müller, U., and Betz, H. (1999) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. J. Neurosci. 19, 9289–9297
25. Connolly, C. N., Krishkev, B. J., McDonald, B. J., Smart, T. G., and Moss, S. J. (1996) Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. J. Biol. Chem. 271, 89–96
26. Mukherjee, J., Kretschmannova, K., Gouzer, G., Maric, H. M., Ramsden, S., Tretter, V., Harvey, K., Davies, P. A., Triller, A., Schindelin, H., and...
Moss, S. J. (2011) The residence time of GABA(A)Rs at inhibitory synapses is determined by direct binding of the receptor α1 subunit to gephyrin. *J. Neurosci.* **31**, 14677–14687

27. Hoon, M., Soyan, T., Falkenburger, B., Hammer, M., Patrizi, A., Schmidt, K. F., Sasoë-Pognetto, M., Löwel, S., Moser, T., Taschenberger, H., Brose, N., and Varoquaux, F. (2011) Neurologin-4 is localized to glycinergic postsynapses and regulates inhibition in the retina. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3053–3058

28. Poulopoulos, A., Aramuni, G., Meyer, G., Soyan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K., Jedlicka, P., Schwarzacher, S. W., Betz, H., Harvey, R. J., Brose, N., et al. (2009) Neurologin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* **63**, 628–642

29. Meyer, G., Kirsh, J., Betz, H., and Langosch, D. (1995) Identification of a gephyrin binding motif on the glycine receptor β subunit. *Neuron* **15**, 563–572

30. McDonald, B. J., and Moss, S. J. (1997) Conserved phosphorylation of the intracellular domains of GABA(A) receptor β2 and β3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase protein kinase C and Ca2+/calmodulin type II-dependent protein kinase. *Neuropharmacology* **36**, 1377–1385

31. Balasubramanian, S., Teissère, J. A., Raju, D. V., and Hall, R. A. (2004) Hetero-oligomerization between GABA(A) and GABA(B) receptors regulates GABA(A) receptor trafficking. *J. Biol. Chem.* **279**, 18840–18850

32. Sheng, M., and Kim, E. (2011) The postsynaptic organization of synapses. *Cold Spring Harb. Perspect. Biol.* **3**, a005678

33. Schwenk, J., Metz, M., Zolles, G., Turecek, F., Rüttimann, P., Fuchs, C., Harvey, K., Jedlicka, P., Schwarzacher, S. W., Betz, H., Harvey, R. J., Brose, N., et al. (2009) Neurologin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* **63**, 628–642

34. Petroski, M. D., and Deshaies, R. J. (2005) Function and regulation of the GTPase-activating activity toward GTPases of the Rab family. *EMBO J.* **24**, 465–473

35. Smith, K. R., Davenport, E. C., Wei, J., Pathania, M., Vaccaro, V., Fu, A. K., and Ip, N. Y. (2010) Ephexin1 is required for structural maturation and neurotransmission at the neuromuscular junction. *Neuron* **65**, 12407–12420

36. Stenmark, H. (2009) Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* **10**, 9–20

37. Smith, K. R., Muir, J., Rao, Y., Browarski, M., Gruenewald, M. C., Sheehan, D. F., Haucke, V., and Kittler, J. T. (2012) Stabilization of GABA(A) receptors at endocytic zones is mediated by an AP2 binding motif within the GABA(A) receptor β subunit. *J. Neurosci.* **32**, 2485–2498

38. Wells, C. M., and Jones, G. E. (2010) The emerging importance of group II mGluRs in synaptic remodeling. *Neuron* **65**, 421–432

39. Shi, L., Fu, A. K., and Ip, N. Y. (2010) Multiple roles of the Rho GEF ephexin1 in synapse remodeling. *Commun. Integr. Biol.* **3**, 622–624

40. Besheer, I., and Hodge, C. W. (2005) Pharmacological and anatomical evidence for an interaction between mGluR5- and GABA(A) receptors in the discriminative stimulus effects of ethanol. *Neuropsychopharmacology* **30**, 747–757

41. Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retiounskai, M., Harvey, R. J., Harvey, K., O’Sullivan, G. A., Laube, B., Hülsmann, S., Geiger, J. R., and Betz, H. (2007) Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. *EMBO J.* **26**, 3888–3899

42. del Río, J. C., Araujo, F., Ramos, B., Ruano, D., and Vitorica, J. (2001) Prevalence between different α subunits performing the benzodiazepine binding sites in native heterologous GABA(A) receptors containing the α2 subunit. *J. Neurochem.* **79**, 183–191

43. Benke, D., Fakitsas, P., Ruggeromosner, C., Michel, C., Rudolph, U., and Mohler, H. (2004) Analysis of the presence and abundance of GABA receptors containing different types of α subunits in murine brain using point-mutated α subunits. *J. Biol. Chem.* **279**, 43654–43660

44. Benke, D., Michel, C., and Mohler, H. (1997) GABA(A) receptors containing the α4-subunit: prevalence, distribution, pharmacology, and subunit architecture in situ. *J. Neurochem.* **69**, 806–814

45. Duggan, M. J., Pollard, S., and Stephenson, F. A. (1991) Immunofluorescence of GABA receptor α-subunit iso-oligomers. Demonstration of receptor populations containing α1 α2, α1 α3, and α2 α3 subunit pairs. *J. Biol. Chem.* **266**, 24778–24784

46. Pollard, S., Thompson, C. L., and Stephenson, F. A. (1995) Quantitative characterization of α6 and α1 α6 subunit-containing native γ-aminobutyric acid A receptors of adult rat cerebellum demonstrates two α subunits per receptor oligomer. *J. Biol. Chem.* **270**, 21285–21290

47. Fu, Y. H., Guzzo, A., Chiu, M. W., Taylor, P., Moran, M. F., Gurd, J. W., MacDonald, J. F., and Orser, B. A. (2009) Distinct properties of murine α5 γ-aminobutyric acid type A receptors revealed by biochemical fractionation and mass spectroscopy. *J. Neurosci.* **39**, 1737–1747

48. Heller, E. A., Zhang, W., Selimi, E., Earnheart, J. C., Šlimak, M. A., Santos-Torres, J., Ibañez-Tallon, I., Aoki, C., Chait, B. T., and Heintz, N. (2012) The biochemical anatomy of cortical inhibitory synapses. *PLoS ONE* **7**, e39572

49. Rudolph, U., and Mohler, H. (2004) Analysis of GABA receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu. Rev. Pharmacol. Toxicol.* **44**, 475–498

50. Gorrie, G. H., Valls, Y., Stephenson, A., Whitfield, J., Browning, B., Smart, T. G., and Moss, S. J. (1997) Assembly of GABA(A) receptors composed of α1 and β2 subunits in both cultured neurons and fibroblasts. *J. Neurosci.* **17**, 6587–6596

51. Shi, L., Butt, B., Ip, F. C., Dai, Y., Jiang, L., Yung, W. H., Greenberg, M. E., Fu, A. K., and Ip, N. Y. (2010) Ephexin1 is required for structural maturation and neurotransmission at the neuromuscular junction. *Neuron* **65**, 204–216

52. Tada, T., and Sheng, M. (2006) Molecular mechanisms of dendritic spine morphogenesis. *Curr. Opin. Neurobiol.* **16**, 95–101

53. Smith, K. R., Davenport, E. C., Wei, J., Li, X., Pathania, M., Vaccaro, V., Yan, Z., and Kittler, J. T. (2014) GIT1 and βPIX are essential for GABA(A) receptor synaptic stability and inhibitory neurotransmission. *Cell Rep.* **9**, 298–310

54. Gingras, A. C., Gstaiger, M., Raught, B., and Aebersold, R. (2007) Analysis of protein complexes using mass spectrometry. *Nat. Rev. Mol. Cell Biol.* **8**, 645–654

55. Charych, E. I., Liu, F., Moss, S. J., and Brandon, N. J. (2009) GABA(A) receptors and their associated proteins: implications in the etiology and treatment of schizophrenia and related disorders. *Neuropsychopharmacology* **37**, 481–495