Complex Role of Collybistin and Gephyrin in GABA<sub>A</sub> Receptor Clustering**

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Gephyrin and collybistin are key components of GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) clustering. Nonetheless, resolving the molecular interactions between the plethora of GABA<sub>A</sub>R subunits and these clustering proteins is a significant challenge. We report a direct interaction of GABA<sub>A</sub>R α2 and α3 subunit intracellular M3–M4 domain (but not α1, α4, α5, α6, β1–3, or γ1–3) with gephyrin. Curiously, GABA<sub>A</sub>R α2, but not α3, binds to both gephyrin and collybistin using overlapping sites. The reciprocal binding sites on gephyrin for collybistin and GABA<sub>A</sub>R α2 also overlap at the start of the gephyrin E domain. This suggests that although GABA<sub>A</sub>R α3 interacts with gephyrin, GABA<sub>A</sub>R α2, collybistin, and gephyrin form a trimeric complex. In support of this proposal, tri-hybrid interactions between GABA<sub>A</sub>R α2 and collybistin or GABA<sub>A</sub>R α2 and gephyrin are strengthened in the presence of gephyrin or collybistin, respectively. Collybistin and gephyrin also compete for binding to GABA<sub>A</sub>R α2 in co-immunoprecipitation experiments and co-localize in transfected cells in both intracellular and submembrane aggregates. Interestingly, GABA<sub>A</sub>R α2 is capable of “activating” collybistin isoforms harboring the regulatory SH3 domain, enabling targeting of gephyrin to the submembrane aggregates. The GABA<sub>A</sub>R α2-collybistin interaction was disrupted by a pathogenic mutation in the collybistin SH3 domain (p.G55A) that causes X-linked intellectual disability and seizures. We confirm the intimate association of collybistin with the GABA<sub>A</sub>R α2 subunit in vivo using a novel collybistin antibody. Taken together, these data suggest that GABA<sub>A</sub>R α2 can trigger the synaptic clustering machinery via dual interactions with collybistin and gephyrin.
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**A** Western blot showing expression of GAL4 DNA binding domain-tagged GABA<sub>A</sub>R α subunit baits in the yeast strain Y190 detected using an anti-HA antibody. wt, wild type. B, GABA<sub>A</sub>R α subunit baits tested for interactions with full-length gephyrin, N-terminally truncated gephyrin (Geph(305–736)), and collybistin (CB3<sub>SH3</sub>α). LacZ freeze-fracture assays demonstrate that GABA<sub>A</sub>R α2 and α3 baits interact with gephyrin preys and that gephyrin residues 305–736 are sufficient for this interaction. Note that collybistin only interacts with the GABA<sub>A</sub>R α2 bait. See also supplemental Fig. 1 for experiments with GABA<sub>A</sub>R β and γ subunit baits.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs and Site-directed Mutagenesis**—GABA<sub>A</sub>R α2 subunit and gephyrin and collybistin cDNAs were amplified from rat spinal cord or human whole brain first-strand cDNA (Clontech) using Pfx DNA polymerase (Invitrogen) and were cloned into the yeast two- or three-hybrid vectors (pYTH15, pYTH16, or pACT2) (ref. 45) or the mammalian expression vectors pRK5myc, pRK5FLAG, pHcRed1-C1, or pEGFP-C1 (Clontech). Cloning resulted in an in-frame fusion of the GAL4 DNA binding domain (vector pYTH16), GAL4 activation domain (vector pACT2), HcRed, EGFP, myc, or FLAG epitope tags to the N termini of all expressed proteins with the exception of pRK5-GABA<sub>A</sub>R α2-FLAG, where a C-terminal FLAG tag was engineered. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene), and all constructs were verified by DNA sequencing.

**Yeast Two-hybrid Assays**—The yeast strain Y190 was co-transformed with pYTH16-GABA<sub>A</sub>R α, β, and γ or pYTH16-GlyR β subunit intracellular M3–M4 regions or pYTH16-gephyrin bait constructs together with pACT2-collybistin or pACT2-gephyrin prey constructs. pYTH16-GlyR β, pYTH16-gephyrin, pACT2-gephyrin deletions, alanine block mutants, and pACT2-collybistin constructs were described previously (3, 36). Additional pACT2-gephyrin mutants, pYTH16-GABA<sub>A</sub>R α baits, pYTH16-GABA<sub>A</sub>R α2 deletion mutants, and pYTH16-GABA<sub>A</sub>R α2/α1 chimeras were generated during this study. Transformations were plated on selective dropout media (either −LWH +30 mM 3-amino-1,2,4-triazole or −LW). After incubation at 30 °C for 3–6 days, LacZ reporter gene assays were performed as described (3).

**Semi-quantitative and Quantitative Yeast Three-hybrid Assays**—The yeast strain Y190 was co-transformed with pYTH16-GABA<sub>A</sub>R α2, pYTH16-GlyR β, or pYTH16-gephyrin bait constructs with either pACT2-CB3<sub>SH3</sub>α or pACT2-gephyrin P1 prey constructs. Transformations were plated on selective dropout media −LWM to induce expression of CB3<sub>SH3</sub>α or gephyrin E domain, or supplemented with 2 mM 1-methionine to suppress expression of these proteins.

**Quantitative Yeast Assays**—Cell pellets were resuspended in Z-buffer containing 40 mM β-mercaptoethanol followed by lysis in 0.1% (w/v) SDS and 0.1% (v/v) chloroform (46). All protein interactions were assayed in four to six independent experiments in triplicate. After addition of chlorophenol red-β-d-galactopyranoside the color change was recorded at 540 nm, and readings were adjusted for turbidity of the yeast suspension at 620 nm. The background signal (co-transfected pACT2 negative control) was subtracted from each co-transformation reading. Established yeast three-hybrid interactions (gephyrin-collybistin and gephyrin-gephyrin) were set as 100%. Statistically significant differences in the interaction strength of two proteins in the presence or absence of a third protein were determined using a Student’s two-tailed t test.

**Cell Culture, Immunocytochemistry, Confocal Microscopy, and Image Analysis**—HEK293 cells (ATCC CRL1573) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in 95% air and 5% CO<sub>2</sub>. Exponentially growing cells were transfected with constructs encoding epitope-tagged gephyrin, collybistin, and GABA<sub>A</sub>R α subunit constructs using Lipofectamine LTX reagent (Invitrogen). After 24 h cells were fixed in 4% (w/v) paraformaldehyde and stained with antibodies recognizing myc (Sigma) and FLAG (Sigma) and secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 488, Alexa Fluor 546, or Cy3 fluorochromes (Molecular Probes). Confocal microscopy was performed using a Zeiss LSM 510 META. All images were taken with a 63× objective. Fluorescence excited by the 488 nm, 543 nm, and 633 nm laser
lines of argon and helium/neon lasers was detected separately using only one laser at the time (multitrack function) and a combination of band pass filters (BP 505–530, BP 560–615), long pass (LP 560) filters, and meta function (649–798) dependent on the combination of fluorochromes.

Co-immunoprecipitation and Western Blotting—HEK293 cells were transfected as above and harvested 48 h post-transfection, lysed in a solution containing 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA (pH 8), 0.25% (v/v) Nonidet P-40/complete protease inhibitor mixture (Roche Applied Science) and homogenized using a tissue grinder. Following centrifugation (4 °C, 15 min, 16,000 × g), 1 ml of cell lysate containing ~700 µg of protein was added to 40 µl of anti-FLAG M2 affinity gel (Sigma) and incubated overnight at 4 °C. The affinity gel was subjected to centrifugation (4 °C, 3 min, 100 × g) followed by two washes in 50 mM NaCl, 50 mM Tris, 0.1% (v/v) Triton X-100, two washes in 150 mM NaCl, PBS, 0.1% (v/v) Triton X-100, and two washes in PBS, 0.1% (v/v) Triton X-100. The FLAG fusion proteins were eluted with 150 ng of 3 × FLAG peptide (Sigma) for 30 min at room temperature. The eluates were analyzed by SDS-PAGE and immunoblotting. Approximately 10 µg of protein was loaded into 4–12% (w/v) BisTris precast gels (Invitrogen). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and nonspecific binding blocked with 5% (w/v) skimmed milk in PBS plus 0.1% (v/v) Tween 20 or with 20% (v/v) horse serum in PBS. Anti-myc antibody (Sigma) was used at a 1:2,000 dilution, and anti-FLAG antibody (Sigma) was used at a 1:3,000 dilution at 4 °C overnight. For detection, a HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) was used at a 1:2,000 dilution together with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunohistochemistry, Microscopy, and Image Analysis—Polyclonal antiserum against collybistin were generated using the peptide sequence CFWQNFSRLTFKK by Multiple Peptide Systems (NeoMPS, San Diego, CA). This peptide was purified to 97% purity by HPLC, coupled to keyhole limpet hemocyanin via an N-terminal cysteine and used to immunize three New Zealand White rabbits using standard procedures. This generated two high affinity collybistin antibodies, as assessed by ELISA results against the source peptide (B2652 pre-bleed 50, 3rd bleed 103,400; B2653 pre-bleed 50; 136,200). One of these antibodies (B2653) was affinity-purified (pABCB3; 0.98 mg/ml, titer 123,800). Immunohistochemistry on rat retina was performed using primary antibodies recognizing GABA<sub>A</sub>R α subunits (a generous gift from Prof. Jean-Marc Fritschy), GlyRs (mAb4a), collybistin, and gephyrin (mAb7a) as previously described (47, 48). Briefly, adult rats were anesthetized with intraperitoneal ketamine-xylazine 1:1 (0.1 ml/kg) and decapitated. The posterior eyecups were fixed for 30 min in formaldehyde (4% (w/v) in 0.1 M phosphate buffer (pH 7.4)). The eyecups were then rinsed in phosphate buffer and cryoprotected in sucrose (10, 20, and 30% (w/v) in phosphate buffer). The retina was dissected and sectioned vertically with a cryostat. Details about the double immunofluorescence procedure are provided in Ref. 49). The sections were analyzed with a confocal microscope (Zeiss LSM5 Pascal), using a
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Sequential acquisition system (multitrack mode). For analysis of co-localization, confocal images were segmented manually and processed with the co-localization module of the software Imaris (Bitplane, Zurich, Switzerland). Single- and double-labeled clusters were then quantified by a particle detection algorithm using Image J (National Institutes of Health).

RESULTS

Selective GABA<sub>A</sub>R α Subunit Interactions with Gephyrin and Collybistin—We utilized the yeast two-hybrid system to test for direct interactions between M3–M4 intracellular loops of the GABA<sub>A</sub>R α1–6, β1–3, γ1–3 subunits with gephyrin and collybistin. Although all bait proteins were expressed in yeast (Fig. 1A and supplemental Fig. 1), only the GABA<sub>A</sub>R α2 and α3 baits interacted with full-length gephyrin as assessed by <sup>LacZ</sup> freeze-fracture assays (Fig. 1B and supplemental Fig. 1). A fragment of gephyrin containing part of the C domain (collybistin binding motif) plus the entire E domain (Geph305–736) was sufficient for the interaction with GABA<sub>A</sub>R α2 or α3 baits (Fig. 1B). Curiously, the GABA<sub>A</sub>R α2 bait (but not the α3 bait) also interacted with a collybistin prey (CB3SH3<sub>G55A</sub>; Fig. 1B). Using deletion and domain swap mutations (Fig. 2A), we mapped the binding sites for gephyrin and collybistin in the GABA<sub>A</sub>R α2 M3–M4 loop. Deletion of a 10-amino acid motif in this region (G55A, lacking AYAVA-VANYA) abolished the interaction of the GABA<sub>A</sub>R α2 subunit with both gephyrin and collybistin (Fig. 2A). However, inserting this motif into the GABA<sub>A</sub>R α1 bait (α2SΔ lacking AYAVA-VANYA) abolished the interaction of the GABA<sub>A</sub>R α1 subunit with both gephyrin and collybistin (Fig. 2A, B and C). However, inserting this motif into the GABA<sub>A</sub>R α2 subunit with both gephyrin and collybistin (Fig. 2, B and C). However, inserting this motif into the GABA<sub>A</sub>R α2 subunit with both gephyrin and collybistin (Fig. 2, B and C). Hence, the binding sites for gephyrin and collybistin on GABA<sub>A</sub>R α2 are distinct but over-
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GABA\textsubscript{A}R \(\alpha2\) (Fig. 4, E–L). This effect was not observed for the equivalent HcRed-GABA\textsubscript{A}R \(\alpha1\) or \(\alpha3\) fusion proteins. Taken together, these results suggest that GABA\textsubscript{A}R \(\alpha2\) binds to the collybistin SH3 domain via a non-PXXP motif (AYAVAVANYA) and that this interaction is disrupted by a known pathogenic mutation in ARHGEF9.

Mapping the GABA\textsubscript{A}R \(\alpha2\) binding site on gephyrin revealed that although the GABA\textsubscript{A}R \(\alpha2\) bait interacted with the Geph305–736 prey, shorter deletion constructs (Geph323–736, Geph336–736, Geph305–643, Geph305–674, and Geph305–704) did not mediate this interaction. Because similar findings were previously reported for collybistin binding to gephyrin (3) we used alanine scanning mutagenesis to locate the GABA\textsubscript{A}R \(\alpha2\) binding site on gephyrin to a 19-amino acid stretch in the N-terminal part of the gephyrin E domain (SMDKAFITVLEMTVPVLGTE; Fig. 5). Although 5 alanine block mutants (A5–A8) disrupted GABA\textsubscript{A}R \(\alpha2\)-gephyrin interactions, they did not overlap GlyR \(\beta\)-gephyrin interactions (Fig. 5). This newly identified GABA\textsubscript{A}R \(\alpha2\) binding site overlaps the previously determined collybistin binding site (PFPLTSMDKA) (3) by 5 amino acids.

Synergistic Interactions among GABA\textsubscript{A}R \(\alpha2\), Collybistin, and Gephyrin—To assess the possibility that GABA\textsubscript{A}R \(\alpha2\), collybistin, and gephyrin form a multimeric complex, we used the yeast tri-hybrid system to measure changes in apparent interaction strength between interacting proteins in the presence of a third polypeptide expressed under the control of an inducible methionine promoter (Fig. 6, A and B). For example, although the GABA\textsubscript{A}R \(\alpha2\)-gephyrin P1 bait-prey interaction is weaker in yeast strain SDY151 in LacZ freeze-fracture assays (Fig. 6C), this interaction is dramatically strengthened by inducing expression of collybistin (CB3\textsubscript{SH3}). Quantitative assays demonstrated that this effect was statistically significant and not observed for either GABA\textsubscript{A}R \(\alpha3\) or GlyR \(\beta\) bait (Fig. 6C, and E).

Similarly, induction of the gephyrin fragment Geph305–736 strengthened collybistin-GABA\textsubscript{A}R \(\alpha2\) but not collybistin-GABA\textsubscript{A}R \(\alpha3\) interactions (Fig. 6D and F). Note that the GlyR \(\beta\)-collybistin interaction mediated by induction of Geph305–736 (Fig. 6D) is also a genuine tri-hybrid interaction because these two proteins occupy separate binding sites on gephyrin (3, 29). We also confirmed the interaction of GABA\textsubscript{A}R \(\alpha2\) with gephyrin P1 and collybistin by co-immunoprecipitation of a full-length FLAG-tagged GABA\textsubscript{A}R \(\alpha2\) subunit with myc-CB3\textsubscript{SH3} and/or myc-gephyrin from HEK293 cells (Fig. 7A). Interestingly, GABA\textsubscript{A}R \(\alpha2\) preferentially co-immunoprecipitates myc-CB3\textsubscript{SH3} rather than myc-gephyrin when all three overlapping. It is also noteworthy that GABA\textsubscript{A}R \(\alpha2\)-gephyrin and \(\alpha3\)-gephyrin interactions are substantially weaker in LacZ freeze-fracture assays than the well characterized GlyR \(\beta\)-gephyrin interaction (Fig. 2B).

Mapping Binding Sites for GABA\textsubscript{A}R \(\alpha2\) on Gephyrin and Collybistin—Interestingly, the GABA\textsubscript{A}R \(\alpha2\) bait did not interact with a prey for an alternatively spliced form of collybistin that lacks the N-terminal SH3 domain (CB3\textsubscript{SH3}–; Fig. 2C), although this prey did interact with gephyrin. We subsequently verified that a collybistin SH3 domain prey was capable of interacting with GABA\textsubscript{A}R \(\alpha2\), \(\alpha1\) in \(\alpha1\), and La2 in \(\alpha1\) baits, but not the \(\alpha2\) construct lacking the collybistin binding motif, \(\alpha2\)S\(\Delta\) (Fig. 3). These interactions were also disrupted by inserting a pathogenic mutation (p.G55S) (3) into the CB3\textsubscript{SH3} or SH3 domain preys (Fig. 3). To confirm that this interaction also occurs in mammalian cells, we expressed HcRed1-tagged GABA\textsubscript{A}R \(\alpha1\) and \(\alpha2\) M3–M4 domain fusion proteins with myc-tagged collybistin isoforms (myc-CB3\textsubscript{SH3}− and myc-CB3\textsubscript{SH3}+) in HEK293 cells. As previously observed for GlyR \(\alpha1\) and \(\alpha3\) intracellular loops (28) the HcRed-GABA\textsubscript{A}R \(\alpha1\), \(\alpha2\), and \(\alpha3\) fusion proteins show nuclear localization, due to cryptic nuclear localization sequences in the M3–M4 domain. This localization was supported by PSORTII analysis that predicts that the isolated GABA\textsubscript{A}R \(\alpha1\)–3 M3–M4 domains should localize to the nucleus (calculated probabilities: \(\alpha1\), 74%; \(\alpha2\), 70%; \(\alpha3\), 61%). Despite this unusual subcellular compartmentalization, co-expression leads to redistribution of myc-CB3\textsubscript{SH3}+ (but not myc-CB3\textsubscript{SH3}−) to nuclear aggregates of HcRed-
proteins are co-expressed (Fig. 7B), although this effect may reflect the ready availability of cytoplasmic myc-tagged collybistin versus myc-gephyrin, which typically occurs in large cytoplasmic aggregates when expressed in HEK293 cells (2, 3).

GABA<sub>A</sub>R α2 Activates Collybistin-mediated Submembrane Gephyrin Clustering—To assess whether GABA<sub>A</sub>R α2 interactions with the collybistin SH3 domain can activate gephyrin submembrane aggregation, we used a cellular model of clustering (2, 3). As previously described, collybistin isoforms lacking the SH3 domain (myc-CB2SH3/H92512 or myc-CB3 SH3/H92512) were able to cluster EGFP-gephyrin in submembrane compartments (Fig. 8, A–H). Although in previous experiments HcRed-GABA<sub>A</sub>R<sub>/H9251</sub>2 fusion protein targeted to the cell nucleus (Fig. 4, E–L), on co-expression with EGFP-gephyrin and myc-collybistin isoforms lacking the SH3 domain (e.g. myc-CB2SH3/H92512), HcRed-GABA<sub>A</sub>R<sub>/H9251</sub>2 redistributed to cytoplasmic and submembrane gephyrin/collybistin aggregates (Fig. 8, E–H). This effect is likely to be mediated by GABA<sub>A</sub>R α2-gephyrin interactions because it was not seen for HcRed (Fig. 8, A–D). Co-expression of SH3 containing collybistin isoforms (e.g. myc-CB2SH3/H92512) with EGFP-gephyrin does not normally lead to submembrane gephyrin accumulation (2, 3). However, co-expression of HcRed-GABA<sub>A</sub>R α2 was able to activate collybistin-mediated gephyrin clustering: all three proteins co-localized in both submembrane and intracellular aggregates.

Collybistin Preferentially Co-localizes with GABA<sub>A</sub>R α2 in Vivo—One major barrier to understanding the function of collybistin in vivo has been the lack of collybistin antibodies that function in immunostaining experiments. To overcome this issue, we generated a polyclonal antibody against a peptide epitope at the collybistin CB3 C terminus (FWQNFSRLTPFKK) because this is one of two commonly expressed alternatively splice variants: CB2 and CB3 (3). This antiserum specif-
These observations support a preferential association of the proteomic techniques we found that the GABAAR/H9251 all GlyR retina. Double labeling with mAb4a (Fig. 9 recognizes the predominant collybistin isoform(s) expressed in the visualized with collybistin, whereas 92% of collybistin puncta co-localize with gephyrin (Fig. 9), which localizes to the EGFP-gephyrin/myc-collybistin (CB2SH3) aggregates upon triple expression. Note that this co-localization is likely to be mediated by the GABAAR α2-gephyrin interaction. I–L, co-expression of collybistin containing a SH3 domain (CB3SH3), EGFP-gephyrin and HcRed-GABAAR α2 leads to co-localization of all three proteins in submembrane aggregates and intracellular aggregates. Note that collybistin isoforms containing the SH3 domain are usually incapable of targeting gephyrin to the submembrane compartment (3, 6). Scale bars: 10 μm.

FIGURE 8. GABAAR α2 activates collybistin-mediated submembrane aggregation of gephyrin. A–D, confocal microscopy showing expression of myc-tagged collybistin, EGFP gephyrin, and HcRed. Co-expression of collybistin without the SH3 domain with EGFP-gephyrin results in submembrane gephyrin aggregates (3); however, HcRed does not co-localize to these aggregates. E–H, HcRed-GABAAR α2 M3-M4 fusion protein localizes to the EGFP-gephyrin/myc-collybistin (CB2SH3) aggregates upon triple expression. Notably, 60% of gephyrin clusters co-localize with HcRed, whereas 92% of collybistin puncta co-localize with gephyrin, suggesting that our antiserum recognizes the predominant collybistin isoform(s) expressed in the retina. Double labeling with mAb4a (Fig. 9B), which recognizes all GlyR α subunits (10), demonstrated that collybistin is not associated with GlyRs in the retina. This is consistent with previous findings in collybistin knock-out mice, where normal postsynaptic aggregation of gephyrin and GlyRs was observed at glycinergic synapses (25). By contrast, there was a prominent co-localization of collybistin with GABAAR α2 (75%; Fig. 9, C and D) and more limited co-localization with GABAAR α1 (31%; Fig. 9, C and E) and GABAAR α3 (27%; Fig. 9, C and F). These observations support a preferential association of the collybistin-gephyrin complex with GABAAR α2 containing the α2 subunit.

DISCUSSION

The aim of this study was to determine the molecular basis of the clustering of GABAARs by gephyrin and collybistin. Using proteomic techniques we found that the GABAAR α2 and α3 subunits harbor binding sites for gephyrin, whereas GABAAR α2 also binds to the RhoGEF collybistin. For GABAAR α2, these interactions are mediated by two overlapping binding sites in the intracellular M3–M4 domain, the collybistin SH3 domain and a motif at the start of the gephyrin E domain that is distinct from the GlyR β binding site (29). This suggests that gephyrin is capable of binding GlyRs and GABAARs simultaneously, providing a molecular explanation for the co-localization of different inhibitory receptors under presynaptic terminals releasing distinct neurotransmitters. Our data also provide for gephyrin-dependent and gephyrin-independent modes of GABAAR clustering. GABAARs containing α2 and α3 subunits are typically postsynaptic receptors, whereas receptors containing the GABAAR α4 and α6 subunits play an important role in tonic inhibition and are located preferentially at extrasynaptic sites (30), consistent with a lack of interaction with gephyrin in our assays. Similarly, the majority of GABAARs containing the α5 subunit do not form clusters that co-localize with gephyrin.

One major unexpected finding of our study was the association of GABAAR α2 with the RhoGEF collybistin in multiple in vitro assays and retina sections. GABAAR α2 associates with collybistin via a distinct alanine-rich non-PXXP motif (34, 35) that is not conserved in other GABAAR subunits (7, 9), perhaps hinting at a more complex regulatory mechanism, e.g. subtle post-translational modifications or GABAARs containing more than one α subunit.

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Interestingly, an extended version of this motif (GSVMIQNNAYAVAVANYA) was required for GABAAR α2 and gephyrin interactions with gephyrin. Tretter et al. (24) previously suggested that the collybistin binding site described here (AYAVAVANYA) was sufficient for the interaction between the GABAAR α2 and gephyrin, but this assumption was based on targeting experiments in primary neuronal cell cultures. Thus, the collybistin-binding AYAVAVANYA motif is likely to be sufficient for targeting GABAAR α2 to postsynaptic sites where both collybistin and gephyrin are present and therefore does not contradict our observations. Consistent with this view, the postsynaptic localization of GABAAR fusion proteins harboring the collybistin binding motif (AYAVAVANYA) was equivalent to that of constructs expressing the complete M3–M4 motif.
intracellular GABA$_A$R $\alpha_2$ domain (24). It is also relevant that the collybistin SH3 domain binding site on GABA$_A$R $\alpha_2$ is not a typical PXXP motif like that recently described for neuroligin 2 (36) but is mediated via a non-PXXP motif. This allows simultaneous interactions of both neuroligin 2 and GABA$_A$R $\alpha_2$ with collybistin and is also consistent with recent data suggesting a preferential co-localization of neuroligin 2 with GABAergic synapses (37). Like neuroligin 2, GABA$_A$R $\alpha_2$ was also able to activate submembrane gephyrin clustering by SH3 domain containing collybistin isoforms in cellular assays. By inference, GABA$_A$R $\alpha_2$ could also act as a signal for triggering synaptic accumulation of collybistin and gephyrin and subsequent assembly of inhibitory postsynaptic densities. Although the nature of this activation is speculative at present, perhaps binding of peptide ligand to the SH3 domain relieves autoinhibition of collybistin in terms of RhoGEF domain activation of the small GTPase Cdc42 (38, 39) or enables interactions of the pleckstrin homology domain with trafficking proteins or phosphoinositides (40).

The nature and sequence of the gephyrin binding motif on GABA$_A$R $\alpha_2$ is distinct from that found in GlyR $\beta$ (16, 17), consistent with overlapping collybistin-GABA$_A$R $\alpha_2$ binding sites at the start of the gephyrin E domain and cooperative GABA$_A$R $\alpha_2$-collybistin-gephyrin interactions. The binding site for collybistin on gephyrin (3) was previously mapped to a 10-amino acid stretch (SMDKAFTV-LEMPTVLGE) that partially overlaps with the GABA$_A$R $\alpha_2$ binding site described here (SMDKAFTV-H9251). The close proximity of the collybistin/GABA$_A$R $\alpha_2$ binding sites on gephyrin and collybistin/gephyrin binding sites on GABA$_A$R $\alpha_2$ suggests an intimate relationship of these three proteins. Yeast tri-hybrid experiments confirmed a significant increase in the interaction strength between GABA$_A$R $\alpha_2$ and gephyrin in the presence of collybistin and GABA$_A$R $\alpha_2$ and collybistin in the presence of gephyrin. In addition, GABA$_A$R $\alpha_2$ exhibits a preference for collybistin rather than gephyrin in co-immunoprecipitation assays, suggesting a dynamic process of competition mediated by the GABA$_A$R $\alpha_2$ M3–M4 domain. Immunohistochemical analysis in retina, chosen because of the substantial separation of GABAergic and glycineric synapses (10), also support a selective function of collybistin at a subset of GABAergic (but not glycineric) synapses, predominantly those containing GABA$_A$R $\alpha_2$.

Collybistin knock-out mice suffer from increased anxiety and impaired spatial learning due to changes of GABAergic inhibition, network excitability, and synaptic plasticity (25–27). These results are mirrored in humans, where defects in ARHGEF9, encoding collybistin, give rise to a diverse range of symptoms linked to gephyrin and GABA$_A$R mislocalization, with intellectual disability as a common feature (3, 40, 41). Our proposed link between collybistin and GABA$_A$R $\alpha_2$ may help to explain why in collybistin knock-out mice, such a distinct loss of GABA$_A$R clustering was observed in parts of the hippocampus and basolateral amygdala, but no apparent effect on GlyR clustering was observed (25). Interestingly, the affected brain regions preferentially express the GABA$_A$R $\alpha_2$ subunit in rat retina.
ously been linked to anxiety (43, 44). The disruption of the GABA$_A$R $\alpha$2-collybistin interaction by a known mutation affecting the collybistin SH3 domain mutation (p.G55A) also illustrates the pathophysiological importance of our findings. We previously reported that the G55A mutation found in a male patient suffering from hyperekplexia, epilepsy, and intellectual disability led to the loss of GABA$_A$R$\alpha$2 at postsynaptic sites in primary neuronal cell cultures and dendritic accumulation of gephrin (3). We now know that the G55A missense mutation results in the loss of both neuroligin 2 and GABA$_A$R $\alpha$2 interactions, suggesting that this substitution completely perturbs the structure of the collybistin SH3 domain. Additional variable symptoms observed in patients harboring different collybistin mutations that are not easily explained by the loss of GABA$_A$R $\alpha$2 at postsynaptic sites are likely due to dominant negative effects of expressed but dysfunctional collybistin proteins, interfering with gephrin-mediated clustering of other GABA$_A$R and GlyR subtypes (3, 40). However, because GABA$_A$R $\alpha$2 shows such a distinct association and functional interplay with collybistin, it is highly likely that additional Rho-GEFs or unknown clustering factors mediate synaptic localization of gephrin and/or GABA$_A$R and GlyR subtypes.

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