Gephyrin-mediated γ-Aminobutyric Acid Type A and Glycine Receptor Clustering Relies on a Common Binding Site

Hans-Michael Maric‡, Jayanta Mukherjee§, Verena Tretter‡, Stephen J. Moss‡, and Hermann Schindelin‡1

Background: Gephyrin clusters inhibitory GABA\textsubscript{A} and glycine receptors at postsynaptic sites.

Results: GABA\textsubscript{A} and glycine receptor binding to gephyrin is a mutually exclusive process relying on distantly related sequence motifs.

Conclusion: Clustering of GABA\textsubscript{A} and glycine receptors is mediated by a shared binding site on gephyrin.

Significance: Gephyrin-dependent synaptic clustering of chloride-permeable ligand channels at synaptic sites relies on an evolutionarily conserved mechanism.

Gephyrin is the major protein determinant for the clustering of inhibitory neurotransmitter receptors. Earlier analyses revealed that gephyrin tightly binds to residues 398–410 of the glycine receptor β subunit (GlyR β) and, as demonstrated only recently, also interacts with GABA\textsubscript{A} receptors (GABA\textsubscript{A}Rs) containing the α\textsubscript{1}, α\textsubscript{2}, and α\textsubscript{3} subunits. Here, we dissect the molecular basis underlying the interactions between gephyrin and GABA\textsubscript{A}Rs containing these α-subunits and compare them to the crystal structure of the gephyrin-GlyR β complex. Biophysical and biochemical assays revealed that, in contrast to its tight interaction with GlyR β, gephyrin only loosely interacts with GABA\textsubscript{A}R α\textsubscript{2}, whereas it has an intermediate affinity for the GABA\textsubscript{A}R α\textsubscript{1} and α\textsubscript{3} subunits. Despite the wide variation in affinities and the low overall sequence homology among the identified receptor subunits, competition assays confirmed the receptor-gephyrin interaction to be a mutually exclusive process. Selected gephyrin point mutants that critically weaken complex formation with GlyR β also abolished the GABA\textsubscript{A}R α\textsubscript{1} and α\textsubscript{3} interactions. Additionally, we identified a common binding motif with two conserved aromatic residues that are central for gephyrin binding. Consistent with the biochemical data, mutations of the corresponding residues within the cytoplasmic domain of α\textsubscript{2} subunit-containing GABA\textsubscript{A}Rs attenuated clustering of these receptors at postsynaptic sites in hippocampal neurons. Taken together, our experiments provide key insights regarding similarities and differences in the complex formation between gephyrin and GABA\textsubscript{A}Rs compared with GlyRs and, hence, the accumulation of these receptors at postsynaptic sites.

Gephyrin was initially discovered by co-purification with glycine receptors (GlyRs)\textsuperscript{2} (1) and was found to accumulate these receptors at postsynaptic sites by simultaneous binding of the GlyR β subunit (2–4) and elements of the cytoskeleton (5, 6). Gephyrin is a modular protein composed of an N-terminal domain (GephG, residues 1–181) followed by a presumably unstructured linker (residues 182–317) and a C-terminal domain (GephE, residues 318–736) that mediates interactions with the GlyR. The crystal structure of GephE in complex with a peptide derived from the large cytoplasmic loop between transmembrane helices 3 and 4 (TM3–4) of the GlyR β subunit (7) defined the interactions in atomic detail. GephE forms a dimer, and residues 398–410 of the GlyR β subunit engage in critical interactions at the dimer interface; however, they primarily interact with only one monomer. Specifically, the interaction of Phe-330 and Asp-327 in GephE with Phe-398, Ser-399, and Ile-400 of the GlyR β-subunit was found to be a major contributor to the overall binding strength. Concurrently the F398A mutation significantly weakened the gephyrin-GlyR β complex as verified by pulldown assays, isothermal titration calorimetry (ITC), and transient expression in HEK 293 cells (7).

Gephyrin was also shown to co-localize with γ-aminobutyric acid type A receptors (GABA\textsubscript{A}Rs) harboring the α\textsubscript{1}–3, β\textsubscript{2}/3, or γ\textsubscript{2} subunits (8, 9). Conflicting results have been published, however, on the role gephyrin plays in GABA\textsubscript{A}R clustering. Whereas gephyrin knock-out and antisense RNA knockdowns in mice completely abolished all GlyR clusters, GABA\textsubscript{A}R clustering could still be observed, albeit depending on their subunit composition, at reduced levels. In particular GABA\textsubscript{A}R\textsubscript{1}Rs harboring the α\textsubscript{2} or γ\textsubscript{2} subunit showed a significantly reduced level of clustering (10–14). Conversely, gephyrin clustering itself was shown to depend on GABA\textsubscript{A}R\textsubscript{1}Rs containing either the α\textsubscript{1}, α\textsubscript{3}, or γ\textsubscript{2} subunits (15–20), and a recent analysis of GABA\textsubscript{A}R α\textsubscript{2}...
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knock-out mice demonstrated a differential subcellular effect on gephyrin and GABA<sub>R</sub> a1 clustering (21). Additionally, both GlyRs and GABA<sub>R</sub>s can co-exist within single postsynaptic densities. Mixed glycineergic/GABAergic inhibitory synapses have been functionally identified in motoneurons of the hypoglossal nucleus (22) and abducens nucleus (23), in interneurons of the spinal cord (24, 25), and the lateral superior olive (26). Which developmental role mixed inhibitory synapses play and how receptor co-clustering is mediated on the molecular level remains to be determined.

Recent studies demonstrated direct interactions of GABA<sub>R</sub> α1, α2, and α3 subunits with gephyrin (27–29), possibly explaining the mutual dependence of GABA<sub>R</sub>αs and gephyrin and GlyRs for postsynaptic accumulation. Interestingly, alanine-scanning mutagenesis identified residues 325–334 of gephyrin to be essential for GABA<sub>R</sub>α3 (28) and residues 325–343 to be essential for GABA<sub>R</sub>α2 (30) interaction and hence suggest overlapping binding sites for these GABA<sub>R</sub> subtypes and the GlyR β-subunit. Here, we study in detail how the GABA<sub>R</sub>α1, α2, and α3 subunits mediate the proposed gephyrin interactions at the molecular level and compare it to the gephyrin-GlyR β interaction.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Gephyrin variants were expressed and purified as described earlier (7). The large cytoplasmic loops between TM3–4 of the GABA<sub>R</sub>α1, α2, and α3 subunits were PCR-amplified and inserted into the Ncol/NotI sites of the pETM-11 vector. Sequence numbers given in the text refer to the mature receptors without the signal sequence. Mutations were generated with the QuikChange site-directed mutagenesis kit (Stratagene). GABA<sub>R</sub>α1, α2, and α3 loop variants were expressed in Escherichia coli BL21 cells (Stratagene, CA) as His<sub>6</sub>-tagged proteins. Cells were grown in LB medium at 30 °C, and protein expression was induced after the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 18 h at 30 °C. Cells were harvested by centrifugation (4000 × g), resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol), and passed through a cell disruptor (Constant Systems), and cell debris was removed by centrifugation (70,000 × g). All proteins were initially purified using 5 ml of HisTrap FF crude columns according to the instructions of the manufacturer (GE Healthcare). Protein-containing fractions were collected, concentrated, and applied to a 26/60 Superdex 200 size exclusion column (Amersham Biosciences) equilibrated with buffer (10 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1 mM β-mercaptoethanol). Pure fractions were pooled, concentrated to 1–100 mg/ml, flash-frozen in 0.5 ml aliquots, and stored at −80 °C.

**Isothermal Titration Calorimetry**—Before all ITC experiments, protein samples were extensively dialyzed against 10 mM Tris–HCl, pH 8.0, 250 mM NaCl, 1 mM β-mercaptoethanol at 4 °C overnight followed by filtration and degassing. To analyze the gephyrin-receptor subtype interaction, 300 μl of a solution containing 180–1640 μM concentrations of the recombinant GABA<sub>R</sub>α1, α2, and α3 subtype intracellular loops, the respective variants, or the synthesized GlyR β-subunit-derived peptide (DFSIVGLPRDFEL, Genscript) were titrated as the ligand into the 1.5-ml sample cell containing 9–100 μM GephE, GephG with linker, or full-length gephyrin (P2 splice variant). In each experiment a volume of 10–15 μl of ligand was added at a time with a total number of 20–30 injections, resulting in a final molar ratio of ligand to protein of 4.5:1. All experiments were performed using a VP-ITC instrument (MicroCal, Northampton, MA) at 25 °C. Buffer-to-buffer titrations were carried out as described above, so that the heat produced by injection, mixing, and dilution could be subtracted before curve-fitting. The binding enthalpy was directly measured, whereas the dissociation constant (K<sub>D</sub>) and stoichiometry (N) were obtained by data analysis using the ORIGIN software, assuming a single site binding model. Binding parameters from singly performed measurements are given with standard derivations resulting from curve-fitting in ORIGIN. Measurements conducted several times are given as mean values and the resulting standard deviation (supplemental Table S2).

**Native Gel Electrophoresis**—Protein samples were mixed with OrangeG dye (Carl Roth) and 10% glycerol. 5-μl samples containing 5–100 μM concentrations of the respective protein were applied to 0.8% NEE0 ultra quality agarose (Carl Roth) gels containing 50 mM Tris/glycine, pH 8.4, buffer. Electrophoresis was conducted at 4 °C and terminated when the dye front was leaving the gel. Gels were stained for 10 min in 5% CH<sub>3</sub>COOH, 10% C<sub>2</sub>H<sub>5</sub>OH, and 0.005% Coomassie Brilliant Blue R250 (Carl Roth) and destained at least 1 day in 5% CH<sub>3</sub>COOH and 10% C<sub>2</sub>H<sub>5</sub>OH.

**Pulldown Assays**—N-terminally biotinylated GABA<sub>R</sub>α1 (KNNTYAPTATSYTPN)-, α3 (KNTTFNIVGTTPYTPN)-, and GlyR β (FSIVGLPRDFEL)-derived peptides were synthesized by PANATecs. Because of excessive hydrophobicity, the corresponding GABA<sub>R</sub>α2-derived peptide (QNNAYAVAVANY-APN) could not be synthesized. The biotinylated peptides were coupled to streptavidin beads and incubated with GephE (50 μM) in 10 mM Tris/HCl, pH 8.0, 250 mM NaCl, and 1 mM β-mercaptoethanol for 1 h. After three washing steps with the same buffer, the beads were boiled with Laemmli buffer containing 10% SDS, and the supernatant was applied to an SDS-PAGE.

**cDNA Constructs and Cell Culture**—The full-length GABA<sub>R</sub>α2 subunit (rat) used for the live imaging was modified with an N-terminal pHisGor as described previously (27). Double mutations were introduced using sequential PCR with appropriate sets of primers. Hippocampal neurons were prepared and nucleofected as described previously from E18 rat embryos of either sex (31, 32). All imaging experiments were performed using 18–21 days in vitro hippocampal cultures.

**Antibodies, Immunocytochemistry, and Image Analysis**—The GFP antibody (mouse monoclonal) was purchased from Roche Diagnostics, and the VIAAT (rabbit polyclonal) antibody was a generous gift from Dr. Bruno Gasnier (CNRS).

For immunostaining, cultures were lightly fixed, incubated with anti-GFP antibody followed by permeabilization with 0.4% Triton X-100, and incubated with the VIAAT antibody and subsequently with fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

Images were acquired using a Nikon Eclipse Ti series confocal microscope with a 60× objective (NA = 1.4) and analyzed by the MetaMorph software (Molecular Devices, Downingtown, PA).
RESULTS

Comparative Binding Studies on the GABA\(_R\) \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) Gephyrin Interactions—After the initial observation that gephyrin directly interacts with \(\alpha_2\) subunit-containing GABA\(_R\)s (27), an interaction of gephyrin with the \(\alpha_1\) (29) and \(\alpha_3\) subunits (28) has been described involving the large cytoplasmic TM3–4 loop.

We cloned, expressed, and purified the corresponding loops of the GABA\(_R\) \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) subunits and investigated their complex formation with GephE on native gels. Unlike GABA\(_R\) \(\alpha_1\) and \(\alpha_3\), which fully shifted GephE already at a molar ratio of 3 to 1 (Fig. 1A), only a 10-fold stoichiometric excess of GABA\(_R\) \(\alpha_2\) was sufficient to complex all GephE under the experimental conditions (Fig. 1B), suggesting a significantly lower affinity of \(\alpha_2\) for gephyrin compared with \(\alpha_1\) and \(\alpha_3\). To analyze gephyrin interactions with GABA\(_R\) \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) in more detail, we determined their binding parameters under similar experimental conditions by ITC (Fig. 1C). The GABA\(_R\) \(\alpha_3\) subunit intracellular loop displayed the strongest interaction with GephE (\(K_D = 5.3 \pm 1.5 \mu M\)) followed by the GABA\(_R\) \(\alpha_1\) subunit intracellular loop (\(K_D = 17 \pm 11 \mu M\)). Under the same experimental conditions, an interaction between the \(\alpha_2\) subunit of GABA\(_R\) and gephyrin could not be detected by ITC. This finding is in line with our native gel experiments, which together suggest that GABA\(_R\) \(\alpha_2\) and gephyrin form a rather loose complex.

Binding Site Mapping of the GABA\(_R\) \(\alpha_1\) and \(\alpha_3\) and GlyR \(\beta\) Gephyrin Interaction—The binding affinities between full-length gephyrin, different gephyrin domains, and selected GABA\(_R\) \(\alpha_1\) and \(\alpha_3\) variants were further investigated via ITC (Fig. 1) and pulldown assays (supplemental Fig. S1) as summarized in Table 1. Similar to what we observed earlier for the interaction between the GlyR \(\beta\) subunit and gephyrin, titration of full-length gephyrin with GABA\(_R\) \(\alpha_1\) yielded similar binding parameters as the titration of GephE alone (Fig. 1C). Conversely, titration with GephG and the largest part of the linker region showed no detectable interaction (data not shown), thus confirming that the binding site resides in the E domain (Table 1A).

After our deletion variant mapping we studied binding of the identified minimum peptides of \(\alpha_1\) (residues 334–348, Table 1B), and \(\alpha_3\) (residues 364–378, Table 1D), which were determined to be crucial for binding and exhibit only limited homology. ITC analysis of the GlyR \(\beta\) derived peptide containing just the critical 14 amino acids visible in the crystal structure (referred to as GlyR short) displayed a significantly lower binding affinity (\(K_D = 4.9 \pm 0.4 \mu M\)) (Fig. 1C and Table 2) compared with the 49-residue construct (referred to as GlyR long) used for the structural studies (7). GlyR long, in addition to binding more tightly, also necessitated the use of a two-site binding model for data analysis resulting in dissociation constants of 0.14 ± 0.1 and 7.7 ± 0.1 \(\mu M\) for the high and low affinity binding sites, respectively (Table 2). Similar to the GlyR \(\beta\) loop, shortening of the GABA\(_R\) \(\alpha_1\) and \(\alpha_3\) loops lowered their affinity significantly; hence, the \(\alpha_1\)- and \(\alpha_3\)-derived minimum peptides could not be studied by ITC (data not shown). Instead we performed streptavidin pulldown experiments with the corresponding peptides covalently linked to biotin (supplemental Fig. S1). The pulldown data demonstrated that the residues contained within these peptides are sufficient to mediate a specific interaction with GephE with their relative binding strengths mirroring the full-length loops (supplemental Fig. S1 and Table 1, B and D).
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Binding of Gephyrin to GABA<sub>R</sub> α1 and α3, and GlyR β Is Mutually Exclusive—On the basis of the mapping experiments described here and published recently (28, 29), our binding studies identified the core motifs in the GABA<sub>R</sub> α1 and α3 and GlyR β subunits, which directly interact with GephE. The wide range of affinities and the moderate overall homology observed here suggested different binding mechanisms for the respective receptor subunits. Concurrently recent studies (28, 30) did not identify individual residues within the core motifs that play a key role in binding to gephyrin. To address this issue, we conducted ITC competition experiments between GABA<sub>R</sub> α1 and GlyR β (Fig. 2), which exhibit the lowest overall identity in their gephyrin-interacting region. Should GABA<sub>R</sub> α1, α2, and α3 and GlyR β share a single binding site on gephyrin, we expected receptor binding by gephyrin to occur in a mutually exclusive fashion. Strikingly, our experiments revealed that pre-equilibration of gephyrin with a GlyR β-derived peptide containing only the 14-residue core motif was sufficient to significantly weaken and ultimately abolish the GABA<sub>R</sub> α1 gephyrin interaction depending on the molar ratio of both loops (Fig. 2). This experiment can be rationalized as follows; upon pre-equilibration of GephE with GlyR β (Fig. 2, B–D) fewer unoccupied GABA<sub>R</sub> α1 binding sites are available. Although the GABA<sub>R</sub> α1 loop at high concentrations will eventually replace the GlyR β peptide, this will not be accompanied by a significant heat release because both binding events are exothermic reactions (Fig. 1C) and, hence, cancel each other out. Accordingly, the characteristic heat signature of the GABA<sub>R</sub> α1 GephE titration (Fig. 2A) is significantly weakened upon applying a 3-fold molar excess of GlyR β peptide (Fig. 2D). Vice versa GABA<sub>R</sub> α1 also interferes with GlyR β binding to GephE (supplemental Fig. S2), and taken together this indicates that GABA<sub>R</sub> α1 and GlyR β compete for a single binding site located in the gephyrin E domain.

Comparison of the Identified GABA<sub>R</sub> α1, α2, and α3 and GlyR β Motifs Reveals Common Elements—On the basis of the finding of exclusive binding of the receptors to gephyrin, we

![TABLE 1](image1)

| No. | Gephyrin          | Receptor          | Affinity |
|-----|-------------------|-------------------|----------|
| A   | 1–734 (full-length) GABA<sub>R</sub> α1 307–393 (TM3-TM4) | + + +       |          |
|     | 1–303 (G domain + Linker) GABA<sub>R</sub> α1 307–393 (TM3-TM4) | + +         |          |
| B   | 318–734 (E domain) GABA<sub>R</sub> α1 307–393 (TM3-TM4) | + ++        |          |
|     | E domain GABA<sub>R</sub> α1 Δ333–348 | --          |          |
|     | E domain GABA<sub>R</sub> α1 334–348 | +           |          |
| C   | E domain GABA<sub>R</sub> α1 307–393 (TM3-TM4) | + ++        |          |
| D   | D domain GABA<sub>R</sub> α3 332–429 (TM3-TM4) | + +         |          |
|     | E domain GABA<sub>R</sub> α3 Δ369–378 | --          |          |
|     | E domain GABA<sub>R</sub> α3 364–378 | +           |          |
| E   | E domain GABA<sub>R</sub> α3 GlyR β 398–411 | ++          |          |
|     | E domain GABA<sub>R</sub> α3 GlyR β 387–426 (TM3-TM4) | ++/++       |          |

![FIGURE 2](image2)

**FIGURE 2. ITC competition assay.** Shown is the heat signature of 10 μM GephE titrated with 200 μM GABA<sub>R</sub> α1 loop A in the absence and in the presence of 3 μM GlyR β (B), 6 μM GlyR β (C), and 30 μM GlyR β (D) peptide. Increasing amounts of GlyR β significantly reduce the heat released resulting from GephE-GABA<sub>R</sub> α1 binding.

![TABLE 2](image3)

| Receptor      | Binding motif | K<sub>D</sub> | Receptor      | Binding motif | K<sub>D</sub> |
|---------------|---------------|--------------|---------------|---------------|--------------|
| GlyR β (long) | 398EAVGSLP405 | 2.0 ± 0.2    | GABA<sub>R</sub> α1 | 340APTATS347 | 0.1        |
| GABA<sub>R</sub> α1 | 340APTATS347 | 17 ± 11      | GlyR β (long) | 398AVGSLP405 | 13 ± 0.1    |
| GABA<sub>R</sub> α2 | 339YAFAVANY346 | ND          | GlyR β (long) | 340AIVGTPY375 | ND          |
| GABA<sub>R</sub> α3 | 368NYIVVT375 | ND          | GlyR β (long) | 398AVGSLP405 | 13 ± 0.1    |
| GlyR β (short) | 398EAVGSLP405 | 5.3 ± 1.5    | GlyR β (long) | 340AIVGTPY375 | 2.7 ± 0.7   |
| GlyR β (long) | 398EAVGSLP405 | 0.14 ± 0.17   | GlyR β (long) | 340AIVGTPY375 | ND          |

<sup>a</sup> Residues 398–411 (FSIVGSLPRDFELS).  
<sup>b</sup> Residues 378–426 (VGETRCKVKCV5KSLR5NDESIVGSLPRDFELSNVYDCGYKPIEVNNGL).
investigated the GABA<sub>A</sub>R α1, α2, and α3 binding motifs and also the gephyrin-interacting region of the GlyR β subunit for common sequence features. Each gephyrin subunit is composed of four subdomains (I-IV) of which subdomains III and IV are important for the interaction with the GlyR (Fig. 3, A and B). Several elements, summarized in Fig. 3C, appear noteworthy; our earlier structural analysis defined that Phe-398 of the GlyR β loop is critical for the GABA<sub>A</sub>R α3 subtype and moderately in α1 and α2, form a hydrophobic core motif marked in red. The tyrosine residue positioned in the C-terminal half of the motif (green) is conserved among the GABA<sub>A</sub>R subtypes but not in GlyR β. The binding sites are divided into an N-terminal region (motif 1) and a C-terminal part (motif 2). Elements a, b, and c are conserved among all receptor subtypes and together form motif 1. Motif 2 relies on a conserved tyrosine residue (element d), which is conserved among GABA<sub>A</sub>R subtypes but not in the GlyR β subunit.

**Similar Interactions Are Central to Complex Formation between GABA<sub>A</sub>R α1, α2, and α3 and GlyR β with Gephyrin—** Based on the mutually exclusive binding and the identified features shared among the receptor motifs, we assumed that Gephyrin binding to the N-terminal motif of the GABA<sub>A</sub>R α1, α2, and α3 binding sites is mechanistically very similar to the interaction of the homologous residues in the GlyR β subunit. To test this and to explore the molecular basis of this phenomenon, we analyzed the effect on GABA<sub>B</sub> binding of two critical gephyrin point mutants identified earlier. The mutation P713E in gephyrin was shown to completely abolish the tight GlyR β binding (7), possibly by introducing its bulky and negatively charged side chain into the receptor binding pocket formed by subdomains III, IV, and V (Fig. 3A). A less severe change is represented by the gephyrin mutant F330A,
which nevertheless significantly weakens GlyR β binding, most likely by diminishing the contribution of the hydrophobic interactions mediated by the hydrophobic core of the GlyR β loop and subdomain III of GephE. The binding of both mutants was analyzed by ITC for the GABAAR α1 and α3 subunits, and interestingly, both impaired binding so strongly that it could no longer be assessed by ITC (Fig. 4, A and B). The effect of the P713E variant supports the hypothesis that the binding site for the GlyR β subunit overlaps with that of the GABAAR α1 and α3 subunits in the vicinity of Pro-713. The impaired binding of the F330A variant likewise demonstrates overlap of the different binding sites in the vicinity of this residue but also argues that interactions with the GABAAR α1 and α3 subunits are driven by a similar contribution of the hydrophobic core region (Fig. 3C, a and b). The corresponding residues interacting with Phe-330 are strongly conserved among the GABAAR α1, α2, and α3 subunits and to some extent also in GABAAR α2 and α3 subunits. Following the idea of a similar binding mechanism, we identified several residues corresponding to GlyR residues that were identified earlier to be key contributors to the complex formation with GephE. Based on an alignment of the identified motifs, we predicted that Phe-369 of GABAAR α3 and Tyr-340 of GABAAR α1 are positioned in an analogous fashion as Phe-398 of GlyR α3. In line with the >100-fold reduction in binding...
strength for GlyR β F398A, binding was no longer detectable by ITC (Fig. 4, C and D) and native gel electrophoresis (Fig. 4, E and F) for the GABAAR α3 F369A and GABAAR α1 Y340A variants. In an analogous fashion we proposed a similar role for GABAAR α3 Ile-371 as compared with GlyR β Ile-400, which critically weakened the gephyrin interaction upon alanine mutation. Not surprisingly, the GABAAR α3 I371A mutant was also significantly impaired in interacting with GephE (Fig. 4D).

Puzzled by our finding of a very weak GABAAR α2 affinity for GephE despite the high homology to the GABAAR α1 and α3 subunits, we carefully compared the identified binding regions, also in light of the GephE-GlyR β complex. This analysis focused our attention on Gly-374, which points toward subdomain IV of GephE in the GlyR β-GephE complex structure and is strictly conserved in GABAAR α3 (Gly-373) and type-conserved in GABAAR α1 (Ala-344) but not conserved in GABAAR α2, where it is replaced by a bulkier residue (Val-343) (Fig. 3C, c). In line with our suggestion of similar binding modes, we proposed this residue to repel the receptor loop by sterically clashing with subdomain IV of GephE (Fig. 3A), thus possibly explaining our observation of a rather weak GephE affinity as compared with GABAAR α1 and α3. We replaced the corresponding residue in GABAAR α3 with valine (G373V), and subsequent ITC analysis (Fig. 4D) revealed a significantly reduced affinity. Although we could not reconstitute binding to the level of the GABAAR α1 or α3 subtype, native gel electrophoresis of the corresponding GABAAR α2 mutant (V343G) visualizes an increased gephyrin affinity (supplemental Fig. S3). Taken together this indicates that this residue could at least be partially responsible for the observed weak GephE GABAAR α2 affinity.

**Hydrophobic Interactions Mediated by Aromatic Residues Are Critical for GABAAR α1 and α3 GephE Complex Formation**—After our mutational analysis we wanted to dissect the relative contributions to the overall binding strength of the conserved N-terminal hydrophobic motif (Fig. 3C, a and b) and the C-terminal parts (Fig. 3C, d) of the respective GABAAR α1 and α3 motifs. Given that ITC is not sensitive enough to display any residual binding for the respective mutants, we instead used confocal imaging was performed on hippocampal neurons at ~21 days in vitro expressing pITα2 WT and pITα2 DM after cells were lightly fixed and stained with the respective antibodies. Surface staining with a GFP antibody revealed a significant loss of GABAARs clusters per 30 μm (11.7 ± 0.6 versus 4.6 ± 0.4, n = 30 neurons, pITα2 WT and pITα2 DM, respectively, unpaired t-test; p ≤ 0.001) (Fig. 5B). These clusters are primarily synaptic as they are either co-localized or opposed to VIAAT-positive puncta. Moreover, cells expressing mutant pITα2 subunit are positive for VIAAT puncta, suggesting that they are innervated by inhibitory presynaptic terminals.

To control for possible negative effects of mutations on receptor assembly or surface trafficking, we examined the ability of the pITα2 WT and pITα2 DM subunit to gain access to the plasma membrane on co-expression with the β3 subunit in HEK 293 cells. Transfected cells were surface-labeled (without permeabilization) with GFP antibodies. Fig. 5C shows that in the presence of the β3 subunit, both pITα2 WT and pITα2 DM subunits gain access to the surface membrane to a similar extent. This process seems specific as we observed minimal surface trafficking when expressing pITα2 subunit alone (data not shown). Collectively these experiments suggest that both tyrosine residues are critical for regulating the accumulation of GABAARs α2 and α1 subunits at inhibitory postsynaptic sites via a direct interaction with gephyrin.

**DISCUSSION**

Gephyrin has been implicated for quite some time in the anchoring of GABAARs; however, a direct interaction between this protein and specific receptor subunits has only been demonstrated recently for the α1, α2, and α3 subunits (27–29). Following up on these studies we investigated the interaction between the full-length intracellular TM 3–4 loops of the GABAAR α1, α2, and α3 subunits and the gephyrin E domain and observed that the affinities varied considerably. Moderately tight interactions that could be described by a one-site binding model were observed for the GABAAR α1 (K_D = 17 ± 11 μM) and α3 (K_D = 5.3 ± 1.5 μM) subunits, whereas the GephE interaction with GABAAR α2 was too weak to be analyzed by ITC.

Clustering of α2-containing GABAARs in Hippocampal Neurons Critically Depends on Two Conserved Aromatic Residues—Our in vitro data obtained from the native gel electrophoresis and ITC experiments identified two conserved aromatic residues in the α1 and α3 subunits of GABAARs and GlyR β subunit (a and d in Fig. 3C) to be critical for direct and exclusive binding to gephyrin. Earlier studies demonstrated that the region containing these two aromatic residues is crucial in vivo for gephyrin-mediated clustering of GABAAR subtypes α1 (29) α2 (27), α3 (28), and GlyR β (4), and our earlier studies demonstrated that two aromatic residues (Phe-398 and Phe408 together with Ile-400) are critical for GlyR β clustering by gephyrin in HEK 293 cells (7). Therefore, we tested whether the aromatic residues, Tyr-339 and Tyr-346, are also crucial for clustering of α2 containing GABAARs. We mutated both residues to alanine and compared (Fig. 5A) the synaptic accumulation of GABAARs between wild-type and the α2 subunit double mutant (DM), both modified with N-terminal pHluorin reporters (pITα2 WT and pITα2 DM (Y339A/Y346A), respectively).
Concurrently, the analysis via native PAGEs revealed the same order of relative binding strengths for the receptor loops, and this ranking is maintained at different pH values (pH 7–9) and salt concentrations ranging from 50 to 250 mM (data not shown). The measured moderate GABA<sub>R</sub> α1 and α3 affinities match the binding constants determined for the interactions between the C-terminal tails of excitatory NMDA and AMPA receptors and PDZ (post synaptic density protein (PSD95)) domain-containing scaffolding proteins that play a critical role in the formation of the postsynaptic density at excitatory synapses and are characterized by $K_D$ values varying between 1 and 50 μM (33).

In contrast, the much weaker gephyrin-GABA<sub>R</sub> α2 affinity seems not sufficient for receptor anchoring and instead suggests that additional binding partners are involved, or that affinity is enhanced by posttranslational modifications. Interestingly, it was recently shown that GABA<sub>R</sub> α2 but not α3 forms a tripartite complex with collybistin (30), a brain-specific Cdc42-activating GDP-GTP exchange factor that promotes submembrane clustering of gephyrin and is essential for the postsynaptic localization of gephyrin and GABA<sub>R</sub>s.

In contrast to the moderately tight single site interaction between GABA<sub>R</sub> α1 and α3 subunits and gephyrin, earlier biochemical and biophysical analyses of GlyR β residues 387–426, which cover the central part of the TM 3–4 loop, suggested two binding sites with $K_D$ values of 0.14 ± 0.1 and 7.7 ± 0.1 μM, respectively (3). A subsequent co-crystal structure (7) revealed that residues 398–411 of the GlyR β subunit engage in close contacts with a binding site near the GephE dimer interface. In this study we also investigated the interaction between the core region (residues 398–411) of the GlyR β subunit and GephE by ITC and observed, in contrast to the 49 residue GlyR fragment, only a single binding site that displayed an affinity of 4.9 ± 0.4 μM. Because the triple alanine GlyR β mutation

**FIGURE 5.** Mutation of conserved tyrosine residues attenuates the clustering of α2-containing GABA<sub>R</sub>s at postsynaptic sites. Hippocampal neurons were nucleofected with α<sup>WT</sup> and α<sup>DM</sup> (Y339A/Y346A), lightly fixed (~21 days in vitro), stained with GFP antibody against extracellular pHluorin tag (red), and subsequently stained with presynaptic marker VIAAT (blue) in the presence of 0.04% Triton. **A**, a single plane confocal image shows the clustering pattern of α<sup>WT</sup> (upper panel) and α<sup>DM</sup> (lower panel). Note that although α<sup>WT</sup> can form mostly synaptic clusters, as demonstrated by a predominant co-localization with VIAAT puncta (blue, merge), the ability to form clusters is greatly attenuated for α<sup>DM</sup> as cluster number and intensity are both strongly reduced. For each panel, a higher magnification image of the boxed area is shown on the right, with arrows pointing to clusters (scale bar = 15 μm). **B**, quantification of the average number of clusters formed by α<sup>WT</sup> and α<sup>DM</sup>. Clusters that are opposed to VIAAT-positive puncta were counted along dendrites per 30 μm (n = 30 cells each, unpaired t test, p < 0.001). **C**, to test whether attenuation of the clusters formed by α<sup>DM</sup> is not due to compromised assembly or surface trafficking, α<sup>WT</sup> and α<sup>DM</sup> were expressed along with the γ2 subunit in HEK 293 cells. Cells were lightly fixed and surface-labeled with GFP antibody against extracellular pHluorin tag (red). Confocal images show that both α<sup>WT</sup> (left panel) and α<sup>DM</sup> (right panel) can access the surface membrane to a similar extent (scale bar = 15 μm).
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F398A/I400A/F408A is sufficient to completely abrogate complex formation in vitro and in vivo (7), we conclude that GlyR β residues 398–411 represent the core binding site, whereas residues 387–397 and 412–426 contain elements that enhance the affinity of this core motif. In a comparable manner, applying only the homologous core motifs of either the GABA<sub>A</sub>R α1 or α3 subunits resulted in a decreased affinity as compared with the respective full-length loops. Our native gel assays, ITC, and cell culture experiments, however, provide evidence that mutation of the conserved aromatic residues (Fig. 3C, a and d) is sufficient to completely abrogate GephE binding and, hence, strongly argue against the existence of other major gephyrin binding determinants in the remaining parts of the receptor loop. How this increase in affinity for the full-length loops can be explained on the molecular level, and how it is connected to the observed second binding site in the case of the GlyR remain open questions. Possibly there are structural rearrangements in either GephE or the intracellular loops.

Our binding studies with deletion variants described here and published earlier (28, 29) identified a moderately conserved either GephE or the intracellular loops. opened questions. Possibly there are structural rearrangements in GABA<sub>A</sub>R synaptically accumulation, we studied the respective alanine mutants. This finding strongly suggest that, in addition to GABA<sub>A</sub>R competitive binding between GABA<sub>A</sub>R and gephyrin complex formation and, hence, represents possible regulatory mechanism for the gephyrin GABA<sub>A</sub>R α1 interaction (34, 35).

Collectively our in vitro and in vivo studies on the pleiotropic effects of the mutants define two different hotspots of GABA<sub>A</sub>R recognition by gephyrin. The first is the N-terminal half of the ~15-residue core binding site. This region relies on interactions that are highly similar to the GlyR β subunit and involves hydrophobic contacts between the gephyrin Phe-330 and the respective aromatic residues in GABA<sub>A</sub>R α1, α2, and α3. This conserved interaction is also at least in part responsible for a mutually exclusive binding between the different GABA<sub>A</sub>R subtypes and the GlyR β subunit. The second is the C-terminal half of the core region. This region displays a conserved tyrosine residue in the GABA<sub>A</sub>R α1, α2, and α3 subunits; however, it is distinct from the important second aromatic residue of the GlyR β subunit. Further details of the gephyrin-GABA<sub>A</sub>R interaction, however, will have to await the structural characterization of a complex involving the E domain of gephyrin and a peptide derived from the α1, α2, or α3 subunits.

Acknowledgment—We thank Bodo Sander for contributions to the project.

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