A proline-rich motif in the large intracellular loop of the glycine receptor α1 subunit interacts with the Pleckstrin homology domain of collybistin

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ABSTRACT

Introduction: The inhibitory glycine receptor (GlyR), a mediator of fast synaptic inhibition, is located and held at neuronal synapses through the anchoring proteins gephyrin and collybistin. Stable localization of neurotransmitter receptors is essential for synaptic function. In case of GlyRs, only beta subunits were known until now to mediate synaptic anchoring.

Objectives: We identified a poly-proline II helix (PPII) in position 365–373 of the intra-cellular TM3-4 loop of the human GlyRα1 subunit as a novel potential synaptic anchoring site. The potential role of the PPII helix as synaptic anchoring site was tested.

Methods: Glycine receptors and collybistin variants were generated and recombinantly expressed in HEK293 cells and cultured neurons. Receptor function was assessed using patch-clamp electrophysiology, protein-protein interaction was studied using co-immuno-precipitation and pulldown experiments.

Results: Recombinantly expressed collybistin bound to isolated GlyRα1 TM3-4 loops in GST-pulldown assays. When the five proline residues P365A, P366A, P367A, P369A, P373A (GlyRα1P1-5A) located in the GlyRα1-PPII helix were replaced by alanines, the PPII secondary structure was disrupted. Recombinant GlyRα1P366L, identified in a hyperekplexia patient, is also disrupting the PPII helix, and caused reduced collybistin binding.
Introduction

The inhibitory glycine receptor (GlyR), a pentameric ion channel belonging to the Cys-loop receptor family is predominantly expressed in mammalian spinal cord and brain stem [1,2]. Each GlyR subunit is characterized by a large extracellular domain, four transmembrane domains (TM1-4) and a large TM3-4 loop followed by a short extracellular C-terminus. GlyR alpha subunits (x1-4) can form functional, homomeric channels, while the beta subunit is associated with intracellular anchoring [1,2]. The GlyR beta subunit was shown to bind gephyrin, a synaptic protein that anchors GlyRs and GABAARs to the subsynaptic cytoskeleton and mediates tethers of glycine [3–6] and GABA, [7–10] receptors at postsynaptic membranes. Since GlyR α subunits failed to bind recombinant gephyrin, it was concluded that the beta subunit is necessary for successful clustering [3,7,11,12]. Screening approaches to identify gephyrin-binding proteins revealed the brain-specific GDP/GTP exchange factor collybistin as a gephyrin binding partner [4,13–15]. Gephyrin was shown in vitro to bind simultaneously to both collybistin and the GlyR β-subunit binding motif [16], however direct interactions between gephyrin and GlyR could not be detected by biotin-streptavidin (not shown). In this study, we demonstrate that the gephyrin content of inhibitory postsynapses [14,23,24] can be increased by expression and functionalized with an engineered V5 tag. Genetic and biochemical approaches were used to identify gephyrin-binding proteins. Screening of full-length GlyRs and GABAARs to the subsynaptic cytoskeleton and mediate tethers of glycine [3–6] and GABA, [7–10] receptors at postsynaptic membranes. Since GlyR α subunits failed to bind recombinant gephyrin, it was concluded that the beta subunit is necessary for successful clustering [3,7,11,12]. Screening approaches to identify gephyrin-binding proteins revealed the brain-specific GDP/GTP exchange factor collybistin as a gephyrin binding partner [4,13–15]. Gephyrin was shown in vitro to bind simultaneously to both collybistin and the GlyR β-subunit binding motif [16], however direct interactions between gephyrin and GlyR alpha subunits have not been observed [14], although mutations in the GlyRβ1 subunit were shown to affect receptor surface expression [17,18].

Subunit dependent GABAAR binding to gephyrin is weak to intermediate, while the interaction between gephyrin and GlyRβ was shown to be strong [11,19]. Formation of complexes between GABAAR α2 subunits and collybistin was reported [20], and the role of gephyrin in the stabilization of GABAergic and glycinergic synapses is well documented [3,7,10,21]. A critical interaction partner of gephyrin at inhibitory synapses is the GTP/GDP exchange factor collybistin [14,15,22]. A recent study defines a collybistin-based network of protein interactions that controls the gephyrin content of inhibitory postsynapses [14,23,24]. There, collybistin can adopt open-active or closed-inactive conformations to act as a switchable adaptor that links gephyrin to plasma membrane phosphoinositides [23,24]. This function of collybistin is regulated by binding of the adhesion protein neuroligin-2, which stabilizes the open-active conformation of collybistin at the postsynaptic plasma membrane [24].

A polyproline II (PPII)-helix in the GlyRβ1 subunit TM3-4 loop was discovered by secondary structure analysis using circular dichroism (CD) spectroscopy of recombinant [25] and recombinant [26] GlyRs. While GlyRz3 subunits lack the PPII motif, GlyRβ as well as α2 subunits both contain a polyproline motif of related sequence at this position. PPII conformations consisting of a homopolymer of proline residues were first discovered in 1968 [27]. They resemble single-stranded collagen triple helices, comprising 4-8 proline, hydroxyproline and glycine residues. The PPII helix structure is characterized by the lack of any intra- or intermolecular hydrogen bonds that are present in α-helices and β-sheets [28]. In general, PPII helical structures play an essential role in mediating protein–protein interactions [29–31]. Binding partners of PPII helices include SH3 [32], and EVH-1 domains [31,33].

Here, we show that a PPII helix in the glycine receptor α1 intracellular loop interacts with collybistin. An isolated TM3-4 polypeptide is sufficient to pull down the heterologously expressed collybistin splice variants 1 (SH3+) and 2 (SH3-) indicating that this interaction is not mediated via the SH3 domain. Replacement of five neighbouring proline residues (P365A, P366A, P367A, P369A, P373A) by alanine, generating the GlyRα1P365A/P369A/P373A mutant, interfered with collybistin binding. Immunoprecipitation experiments from HEK293 cells expressing GFP-CB2 or an isolated GFP-CB-PH domain confirmed GlyRα1 – collybistin association, suggesting the C-terminal PH domain as critical binding site. Notably, expression of full length GlyRα1, carrying the GlyRα1P1-5A group mutation did not affect subcellular localization or functional GlyR properties. Our data suggest that the PPII motif may play a relevant role in synaptic anchoring of α1 GlyRs.

Materials and methods

Constructs and mutagenesis

Single-nucleotide exchanges were introduced by PCR-mediated site-directed mutagenesis using an overlap extension PCR approach. Mutagenesis primers (MWG, Ebersberg, Germany) contained nucleotides encoding for specific amino acid exchanges. PCR products were set up as previously described [34]. For GlyRα1 mutations GlyRα1P1-5A and α1P366G [18], PCR products were inserted into the plasmid pRK5 using EcoRI and PstI restriction sites. For bacterial expression and purification of GlyRα1 TM3-4 loop constructs, PCR products were inserted into the plasmid pET30a (Novagen, Darmstadt, Germany) using BamHI and HindIII restriction sites. All clones were verified by DNA sequencing (MWG, Ebersberg, Germany). The cDNA of HA-GlyRα1 fusion protein was generated by insertion of annealed oligonucleotides including a Bsp1407I overhang into pcIS–GlyRα1. The cDNAs encoding full-length collybistin II or the collybistin PH-domain were amplified and subcloned as Xhol fragment or EcoRI –Xhol fragment, respectively (pAcGFP-C vector, Clontech, Mountain View, CA, USA).

Expression and purification of fusion proteins in E. coli

After transformation and expresssion-on of GlyRα1 TM3-4 loop constructs, BL21 cells (Novagen, Darmstadt, Germany) were harvested by centrifugation, resuspended in 50 mM Tris-HCl, 2.5 mM EDTA, pH 7.4, treated with lysozyme (0.1 mg/mL, 30 min, 0 °C) and sonicated on ice. Sonification was repeated and the supernatant collected for purification. Native purification was performed using a Ni-agarose column by washing and eluting with increasing imidazole concentrations, followed by size exclusion chromatography. Successful purification was verified on Coomassie stained SDS PAGE gel electrophoresis.

CD Spectroscopy

Purified intracellular GlyR domains, expressed in E. coli were buffered in 10 mM K-Pi pH 7.4 and subjected to CD analysis. Measurements were performed on a JASCO-J810 spectrometer (Jasco, Gross-Umstadt, Germany) in a 0.1 cm analytical cell chamber. All spectra were baseline corrected by subtracting buffer runs. Eight individual scans were taken at 22 °C in a range of 320 to 185 nm, with a 0.5 nm step size and averaged. Protein concentrations were determined by measuring the absorbance at 280 nm using the equation c = A280 nm/(L ε L). The path length L was 1 cm,
the extinction coefficients were calculated from the protein sequence [26]. While α-helical structure elements lead to distinct CD spectra with negative maxima at 222 nm and 208 nm, spectra obtained from β-sheets reveal a broad negative maximum at 215 nm [35]. The left-handed type II polyproline (PPII) helix yields spectra with an intense negative band at 204 nm [35–37].

**Cell Culture, transfection and membrane preparation**

HEK293 cells were grown in 10 cm tissue culture Petri dishes in Minimum Essential Medium (MEM, Sigma, Deisenhofen, Germany) supplemented with 10% FBS (Invitrogen, Karlsruhe, Germany) and Penicillin/Streptomycin. Cells were plated on poly-lysine treated glass coverslips in 6 cm dishes. For Western blot analysis, cells were prepared in 10 cm dishes. Transfection was performed 1 day after cell passage using the Ca2+ phosphate method. 10 μg plasmid DNA, 10 μg GFP vector, 430 μl H2O and 50 μl CaCl2 (2.5 M) were mixed and 500 μl HBS-buffer (50 mM HEPES, 12 mM Glucose, 10 mM KCl, 280 mM NaCl, 1.5 mM Na2HPO4 × 12 H2O, pH 6.95) was added drop wise. After a 20 min incubation period, the transfection mixture was added to the cells. Cells were washed 1 day later with MEM full medium and harvested 24–48 h later. Briefly, cells were taken in ice cold PBS buffer, centrifuged (10 min, 2000 g, 4°C) and pellets were taken in buffer H (20 mM K-phosphate pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% TritonX-100, 3% bovine serum albumin (BSA)). Primary antibodies were applied for 60 min. Secondary antibodies were incubated for 30 min in the dark. After washing, proteins labelled by the secondary antibody were detected on a Storm 860 Fluoromager (Molecular Dynamics, Krefeld, Germany). Primary antibodies: monoclonal mouse anti GlyR mAB4a (hybridoma cells supernatant 1:1); secondary antibody: goat anti mouse IgG-Cy3 (1:200) (Dianova, Hamburg, Germany).

**Immunocytochemistry on HEK293 cells**

All steps were performed at room temperature. Fixation: coverslips containing transfected HEK293 cells were transferred into a 24 well plate containing 500 μl medium per well. The medium was exchanged by a 4% paraformaldehyde-PBS solution. After a 10 min incubation period, cells were washed twice with cold PBS buffer. Staining of surface proteins: cells were blocked with PBS buffer including 5% BSA for 30 min; intracellular staining: cells were blocked with PBS buffer. Staining of surface proteins: cells were blocked with PBS buffer including 5% BSA and 0.1% Triton for 30 min. Primary antibodies were labelled by the secondary antibody and detected on a Storm 860 Fluoromager (Molecular Dynamics, Krefeld, Germany). Primary antibodies: monoclonal mouse anti GlyR mAB4a (hybridoma cells supernatant 1:1); secondary antibody: goat anti mouse IgG-Cy3 (1:200) (Dianova, Hamburg, Germany).

**Hippocampal cultures**

Hippocampal neurons were prepared from mouse embryos of the CD1 strain at stage E18 (male and female embryos were taken) and grown in neurobasal medium (Thermo Fischer, Darmstadt, Germany) containing 5 ml of L-glutamine (200 mM) and B27 supplement (Thermo Fischer, Darmstadt, Germany) with an exchange of 50% medium after 7 days in culture. Experiments were authorized by the local veterinary authority and Committee on the Ethics of Animal Experiments (Regierung von Unterfranken).

Hippocampal neurons were infected with 1 μg lentiviral low copy vector FUVa1-GFP (provided by R. Blum) containing the cDNA of GlyRx1 wildtype or GlyRx1F386EC after seeding and cultured for 14 days (days in vitro = DIV). At DIV15 live cell stainings of surface GlyRs were performed using an α1-specific mAB2b antibody (recognizes a native epitope in the N-terminus of GlyRx1 (residues 1–10 of mature protein; cat. no.146111, SYSY, Göttingen, Germany) for 2 hrs at 4 °C. After 20 min of fixation (4% paraformaldehyde, 4% sucrose) cells were blocked and permeabilized with 5% goat serum and 0.1% Triton for 30 min. Endogenous collybistin was stained overnight with the rabbit anti-collybistin antibody at 4 °C. Subsequently, cells were washed and incubated with goat-anti-mouse-Cy3 and goat-anti-rabbit Cy5 secondary antibodies (Dianova, Hamburg, Germany) for 2 hrs at 21 °C. Before mounting of the coverslips with mowiol, nuclear staining using DAPI was performed.

**Confocal microscopy, image acquisition and analysis on Hippocampal Neurons**

An inverted Olympus IX81 microscope equipped with an Olympus FV1000 confocal laser scanning system, a FVD10 SPD spectral detector and diode lasers of 495 nm (Alexa488) and 550 nm (Cy3) (Olympus, Tokyo, Japan) was used to acquire confocal images. To take images an Olympus UPLSAPO 60x (oil, numerical aperture: 1.35) objective was used. The whole cell collybistin signal intensity was analyzed from infected cells (GFP positive) and discriminated between cell soma and neurite using the Open View software [38]. The mAb2 channel was used as a mask (cluster) and the collybistin signal intensity was determined within the mAb2b clusters. All immunofluorescence analysis is shown as means ± standard errors of the mean (SEM). Calculated signal intensities were compared using ANOVA with a probability of error of p < 0.05 considered significant. The images were further developed and organized by Adobe Photoshop (Adobe, San Jose, CA, USA) or ImageJ (1.51/Fiji (https://imagej.net/Imagej)).

**Electrophysiological recordings and data analysis**

HEK293 cells were transfected 2–4 days prior to electrophysiological recordings. Current responses were measured at room temperature (21–23 °C) at a holding potential of −50 mV. Whole-cell recordings were performed using a HEKA EPC10 amplifier (HEKA Electronics, Lambrecht, Germany) controlled by Pulse software (HEKA Electronics). Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Berlin, Germany) using a Sutter P-97 horizontal puller (Sutter, Novato, CA). Solutions were applied using an Octaflow system (NPI electronics, Tamm, Germany), where cells were bathed in a laminar flow of buffer, giving a time resolution for solution exchange and re-equilibration of about 100 ms. The external buffer consisted of 135 mM NaCl, 5.5 mM KCl, 2 mM CaCl2, 1.0 mM MgCl2, and 10 mM Heps (pH 7.4, NaOH); the internal buffer was 140 mM CsCl, 1.0 mM CaCl2, 2.0 mM MgCl2, 5.0 mM EGTA, and 10 mM Heps (pH 7.2, CsOH). Using a nonlinear algorithm in Microcal Origin (Additive,
Friedrichsdorf, Germany), dose-response data were fitted to the Hill equation:

\[
\text{EC}_{50} = \frac{\text{IC}_{50} \cdot \text{I}_{\text{sat}}}{1 + \left(\frac{\text{I}_{\text{sat}}}{\text{EC}_{50}}\right)^n}
\]

where \(\text{IC}_{50}\) is the current amplitude at a given glycine concentration, \(\text{I}_{\text{sat}}\) is the current amplitude at saturating concentrations of glycine, \(\text{EC}_{50}\) is the glycine concentration at half-maximal current responses, and \(n\) is the Hill coefficient. In all experiments \(\text{EC}_{50}\) values were determined for each individual cell from a non-linear fit of dose response data to the logistic equation (above). Differences between \(\text{EC}_{50}\) values recorded for wildtype and GlyR1 (P1-SA) mutant receptor or co-expression of wildtype and collybistin were compared using one-way ANOVA with \(p \leq 0.05\) taken as significant. Significance levels are indicated with \(p\)- and \(F\)-values. An unweighted average \(\pm\) standard error (SEM) was calculated from all individual \(\text{EC}_{50}\) values, without considering the fitting errors.

**Fusion constructs and GST Pulldown assays**

HEK293 cells were transfected with HA-tagged collybistin I or II cDNAs [14] using the calcium phosphate precipitation method. 16 hrs after transfection cells were washed with PBS and harvested in 1 ml PBS supplemented with 1% Triton X-100 and 1 mM PMSF. After 30 min incubation on ice, the lysate was centrifuged at 11,000 \(\times\) g for 5 min and the supernatant was retained. GST-fusion proteins of the isolated GlyR1 wildtype or mutant GlyR1 (P1-SA) TM3-4 loop were recombiantly expressed in E. coli BL21 cells (New England Biolabs Inc. Ipswich, MA). Cells were pelleted and lysed by sonification in protein extraction buffer (400 mM NaCl, 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\)) and subsequently incubated with glutathione sepharose beads (Thermo Scientific, Dreieich, Germany) for 3 hrs. Beads were washed 3 times with washing buffer for 5 min and the supernatant was retained. GST-fusion proteins were transfected onto PVDF membranes using the wet blot method. Membranes were blocked with rabbit anti-HA antibody (Sigma-Aldrich, Taufkirchen, Germany) at 4°C in milk/TBST. Secondary antibody goat anti-HA antibody 1:500, cat. no. sc-8034, both from Santa Cruz Biotechnology, Santa Cruz, CA, USA. For statistical analysis collybistin signals were normalized to GlyR1 a cat. no. 146003, both from Synaptic Systems, Göttingen, Germany) and 50 \(\mu\)l of protein A-sepharose beads (1:1, GE Healthcare, Freiburg, Germany) was incubated for 1 h at room temperature in milk/TBST. After incubation with ECL substrate, signals were detected by Chemolab ECL detection system (Intas GmbH, Göttingen, Germany).

**Co-immunoprecipitation**

**GlyR1 – CB2; GlyR1-β – CB2 – Gephyrin**

HEK293 cells were grown on 10-cm dishes and used 48 hrs after transfection. Cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR (P1-SA)). (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control. For co-immunoprecipitation experiments, HEK293 cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR1 (P1-SA)), (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control. For co-immunoprecipitation experiments, HEK293 cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR1 (P1-SA)), (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control. For co-immunoprecipitation experiments, HEK293 cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR1 (P1-SA)), (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control. For co-immunoprecipitation experiments, HEK293 cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR1 (P1-SA)), (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control. For co-immunoprecipitation experiments, HEK293 cells were transfected with (i) GlyR1 together with collybistin I (CB1) or collybistin II (CB2) in a ratio of 1:1, (ii) GlyR1 with GlyR1, CB2, and gephyrin in a ratio of 1:2:2:2 (or mutant GlyR1 (P1-SA)), (iii) GlyR1 was instead of GlyR1 wildtype; same ratio transfected (Fig. 5C). GST transfusions were used as mock control.
Results

**GlyR α1 subunits carrying a proline to alanine group mutation in the PPII helix of the TM3-4 loop lose PPII helix conformation but retain cell surface membrane expression**

A polyproline II (PPII) helix within the GlyR α1 TM3-4 loop (Fig. 1A) has been demonstrated via CD spectroscopy [26,28,30]. Within this loop the motif 365PPPAPSK573 is thought to mediate its secondary structure (Fig. 1B). To investigate the influence of the PPII helix on receptor targeting and localization at the cell surface, proline residues located between amino acid positions 365 and 373 were systematically substituted to alanine. Mutant GlyR α1P1-5A is characterized by the simultaneous exchange of five proline to alanine residues within the helix motif: P365A, P366A, P367A, P369A and P373A (Fig. 1B).

Structural studies of the PPII helix required pure protein samples of the isolated TM3-4 loop. Expression of the wildtype or mutant hs α1 TM3-4 loop polypeptide in E. coli cells and subsequent purification using a Ni-agarose column and size exclusion chromatography yielded pure protein samples, as visualized by SDS PAGE Coomassie Blue staining (Fig. 1C). Purified TM3-4 loops of mutant and wildtype receptor were subjected to CD analysis. Wildtype spectra displayed a minimum at 205 and a shoulder at 225 nm, typical for structures containing β-sheets and PPII helices (Fig. 1D), and consistent with previous reports [28,36]. The GlyR α1P1-5A mutant, in contrast, had the minimum shifted to 208 nm, and the shoulder in the 225–228 nm region became more pronounced, presenting as a new minimum. These differences are in agreement with a loss of the PPII helical conformation and an increase of the α-helical content of the protein.

We then examined plasma membrane targeting of the full length GlyR group mutant GlyR α1P1-5A upon expression in HEK293 cells, using Western blot analysis (Fig. 2A). Here, the mutant receptor was present in the membrane fractions enriched for the plasma membrane. Immunocytochemistry also confirmed the delivery of mutant receptors to the cell surface similar to the wildtype (Fig. 2B), suggesting normal cell surface delivery of GlyR α1P1-5A mutant receptors.

GlyR α1 subunits carrying a proline to alanine group mutation in the PPII helix of the TM3-4 loop display normal glycinergic currents

To study the role of the mutant GlyR α1P1-5A on receptor function, electrophysiological experiments were performed on homomeric full length GlyRs carrying the wildtype GlyR α1 subunit, as compared to GlyRs carrying the α1 group mutant GlyR α1P1-5A (Fig. 1B). To this end HEK293 cells were transfected with the respective constructs and current responses analysed at increasing glycine concentrations of 10–1000 μM. GlyR α1 wildtype receptors

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Fig. 1. Localization and characterization of the GlyR α1 PPII helix. (A) Model of GlyR α1 with its predicted PPII helix in the TM3-4 loop (adapted from [44]). (B) Sequence of wildtype GlyR in comparison with group mutant at position 365–373. (C) SDS-PAGE of GlyR α1 as well as GlyR α1P1-5A mutant TM3-4 loops after over-expression in E. coli cells and purification via Ni-NTA column and gel filtration chromatography. Lane 1: α1 wildtype TM3-4 loop; lane 2: GlyR α1P1-5A TM3-4 loop; lane 3: protein standard. Position of TM3-4 loop and 12 kDa is indicated. (D) CD spectra of α1-wt (solid line) and GlyR α1P1-5A (dashed line). All spectra were measured in 10 mM K-phosphate, pH 7.4 in a 1 cm cuvette at 22 °C. Eight single spectra were summed and the reference spectrum (10 mM K-phosphate, pH 7.4) was subtracted.
showed EC_{50} values of 38.4 ± 3.4 μM (n = 20), while receptors carrying the GlyR_{1P1-5A} group revealed EC_{50} values of 56.9 ± 4.4 μM (n = 9) (Fig. 3A-B, D). These data show that exchanging all proline residues to alanine in the intracellular SH3 domain was not sufficient to influence channel functions of homomeric α1 GlyRs. Although the increase of EC_{50} was statistically significant (p = 0.016), a physiologically active mutant channel was retained. We therefore compared the dependency of current responses to the transmembrane voltage for both subunit species at saturating concentrations of glycine (2 mM) in the range of −60 mV to +60 mV (Fig. 3C). Both, wildtype and mutant receptors displayed a linear I-V relationship, as expected for inhibitory GlyRs. These data suggest that ion conductance of the GlyR channel is independent of the polyproline motif located in the GlyR_{1} subunit TM3-4 loop.

Proline residues in the SH3 helix of an isolated GlyR_{1} TM3-4 loop are critical for interaction with collybistin

The SH3 motif of the GlyR belongs to the class II type of polyproline 2 helices. Some SH3 helices have been described to preferentially interact with SH3 domains of specific target proteins [30,39,40]. We therefore applied GST assays to select for different candidate SH3 domains containing class II PPII helices that have been described in the literature and may be potential GlyR-interacting domains. In addition, a search for potential SH3 domains that would be sufficient for GlyR_{1} collybistin binding would be an alternative way to anchor homomeric glycine receptors. Next, we investigated the binding of full length GlyR_{1} wildtype using immunoprecipitation upon HEK293 cell expression. Co-expression experiments of GlyR_{1} and CB1 or CB2 displayed strong collybistin signals detected in Western blot analysis, confirming the interaction of GlyR_{1} and collybistin variants CB1 and CB2 (Fig. 4C). GAPDH was used as a loading control.

We then tested whether the C-terminal collybistin PH domain would be sufficient for GlyR_{1}-collybistin binding. Therefore, we heterologously co-expressed full-length GlyR_{1} with either GFP-CB2 or an isolated CB2-PH domain fused to GFP. HA specific antibodies led to co-IP of full length CB2. In addition, an isolated CB2-PH domain fusion protein also revealed interaction with the full-length wildtype HA-GlyR_{1} subunit (Fig. 4D), suggesting PH domain residues as critical mediators of GlyR_{1} binding.

The influence of collybistin on glycineric currents was tested in patch-clamp experiments. To this end, GlyR_{1} subunits were co-expressed with either CB1 or CB2 in HEK293 cells. Whole cell recordings displayed no significant differences of half maximum currents. EC_{50} values were 34.8 ± 3.0 μM and 31.5 ± 2.2 μM for cells co-expressing CB1 or CB2, respectively, as compared to 38.4 ± 3.4 μM in wildtype controls in the absence of collybistin (Fig. 4E).

Finally, we compared a pathological GlyR_{1} mutant, GlyR_{1}^{P166L}, which had been identified in a hyperekplexia patient [18] to the wildtype receptor. Immunocytochemistry experiments on hippocampal neurons (DIV15) revealed co-assembly of GlyR_{1} and collybistin, which was reduced in mutant GlyR_{1}^{P166L} receptors (Fig. 5A). Collybistin intensities adjusted to a GlyR_{1} (mAb2b) mask were determined for whole cells and differentiated between signal in neurites and soma (Fig. 5B). While no significant difference was detected between wildtype and mutant receptors in neurites, differences in whole cell signal and more dominant

Fig. 2. Expression and cellular distribution of GlyR_{1} wildtype and GlyR_{1P1-5A} mutant subunits. (A) Western blot analysis of GlyR_{1} subunits. 20 μg of membrane preparation was loaded per lane, primary antibody was mAb4a supernatant. Lane 1: α1 wildtype; lane 2: GlyR_{1P1-5A}; lane 3: protein standard; 48 kDa is indicated. (B) Immunofluorescence: HEK293 cells were transfected on cover slides and treated for immunocytochemistry with mAb4a and goat anti mouse Cy3. For surface expression no Triton X-100 was added. To detect the intracellular protein distribution, the cell membrane was permeabilized with Triton X-100. Controls: untransfected cells or GlyR_{1} transfected cells treated with secondary antibody only are shown. Pictures were taken at 400× magnification. Scale bar indicates 10 μm.

Notably, HEK293 cell pulldowns using mutant GlyR_{1P1-5A} TM3-4 loop (compare with Fig. 1B) abolished binding of both collybistin variants, indicating a critical involvement of the GlyR_{1} polyproline helix. In spite of endogenous expression of gephyrin in HEK293 cell, cells are unlikely to endogenously express a GlyR_{1} subunit. Our data therefore suggest that GlyR_{1}-collybistin interaction may occur in addition to GlyR_{Δβγ}–gephyrin interactions or may be an alternative way to anchor homomeric glycine receptors.
in soma were detected (**p < 0.01), indicating that exchange of only one proline residue in the PPII helix motif into the bulky leucine is sufficient to weaken the binding between GlyRα1 and collybistin (Fig. 5B). In addition, co-immunoprecipitation experiments were performed on wildtype GlyRα1 and GlyRα1P366L receptors. Co-expression of GlyRα1 and CB2 revealed strong CB signals in Western blot analysis upon immunoprecipitation with mAb2b, which was only slightly increased in co-expression experiments with GlyRα1, GlyRβ, CB2 and gephyrin (Fig. 5C-D). When co-expression experiments were performed on GlyRα1P366L receptors, the relative signal adjusted to input was reduced to 0.6 for the mutant compared to 1.1 for wildtype GlyRα1. Co-expression of GlyRα1, GlyRβ, CB2 and gephyrin resulted in a reduction of similar extent of relative signal intensity for the GlyRα1P366L mutant compared to wildtype (Fig. 5D).

In summary, we conclude that the PPII motif located in the GlyRα1 TM3-4 loop mediates interaction with the guanine nucleotide exchange factor collybistin. Mutagenesis in the PPII motif neither alters GlyR surface targeting nor receptor function, but appears to affect synaptic anchoring of GlyRα1 subunits.

Discussion

The large intracellular loop of the inhibitory GlyR contains numerous motifs for intracellular interaction [2,4,17,18,42–45] and modulation of receptor function [7,17,34,46,47]. Here, we investigated the function of a proline-rich motif, SPSPPAPPSKSP373, which forms a PPII helix that is located in the intracellular TM3-4 loop of the human GlyRα1 subunit. Proline residues of this motif were replaced by alanine, resulting in the group mutant P365A, P366A, P367A, P369A and P373A, termed GlyRα1P1-5A. The isolated TM3-4 loop of wildtype GlyRα1 displayed robust binding to the anchoring protein collybistin, binding both splice isoforms, collybistin I SH3+ and collybistin II SH3-, equally well. Thus, binding between collybistin and the GlyRα1 subunit was independent of the N-terminal SH3 domain, while other SH3 domains, notably that of syndapin, do bind to the PPII helix motif [18]. Here, the C-terminal PH domain of collybistin was shown to mediate GlyRα1 binding via the PPII helix, since disruption of the PPII helix motif in the group mutant GlyRα1P1-5A, or a hyperekplexia mutation GlyRα1P366L [18] abolished collybistin binding, While indirect binding through one or several linker proteins cannot be ruled out completely, our data strongly suggest that a specific interaction between the PPII motif and the PH domain of collybistin is present. Our data suggest a direct interaction between GlyRα1 subunits and collybistin.

The isolated TM3-4 loops of GlyRα1 wildtype and GlyRα1P1-5A revealed distinct changes of secondary structure, as determined by CD spectroscopy. Spectra of the wildtype agreed with the presence of a PPII helix, while that of the GlyRα1P1-5A mutant was consistent with a loss of the PPII helix, and an increase in α-helical structure. Complete replacement of all prolines by alanine seems to destroy the PPII structure, but replacement of a single residue
Fig. 4. Interaction between collybistin and the GlyR α1 TM3-4 loop. (A) Schematic structure of collybistin variants 1–3. (B) Binding studies between α1-GlyR and collybistin I–II using GST pull down assay upon HEK293 cell transfection with CB1 SH+-HA and CB2 SH--HA. GST-fusion proteins of the isolated GlyR α1 wildtype or GlyR α1P1-5A TM3-4 loop were recombinantly expressed in E. coli BL21 cells, coupled to glutathione-sepharose beads and finally incubated with HEK293 cell lysates. Results are shown after Western Blot analysis. Lane 1: input, lane 2: GST control, lane 3: α1-wt TM3-4 loop, lane 4: GlyR α1P1-5A TM3-4 loop. C. Co-immunoprecipitation of glycine receptors and collybistin splice variants. The GlyR α1-specific antibody mAb2b was used for co-precipitation. Lane 1: GlyR α1 + CB1 (1:1); lane 2: GlyR α1 + CB2 (1:1); lane 3: GlyR α1 + GlyR β + CB2 + gephyrin (1:2:2:2); lane 4: untransfected cells; lane 5: GFP = mock control (left panel). Collybistin was detected at the appropriate molecular weight of 60 kDa (precipitated with GlyR α1 – see IP, upper panel and input control expression of collybistin - see input second panel; gephyrin at 93 kDa, and GAP-DH at 32 kDa. The observed shift in molecular weight between CB1 and CB2 is due to the presence of the SH3 domain in CB1 but not CB2. Right panel: Quantification of the relative collybistin expression normalized to GAPDH. At least 4 independent experiments have been performed and were used for quantification analysis. (D) Co-immunoprecipitation of GFP-CB2 or GFP-PH domain together with pCIS-HA-GlyR α1. Beads were coupled with mouse anti-HA antibody or mouse IgG, HEK293 cell lysates were incubated with antibody coupled beads (see methods). Samples were subjected to SDS PAGE and Western Blotting. Lane 1: input; lane 2: IgG control; lane 3: IP HA tag. Left panel: GFP-CB2; right panel: GFP-PH domain. (E) Electrophysiological data after co-expression of GlyR α1 with CB1 or CB2. Left panel: EC50 curve of GlyR α1 (solid squares, solid line); GlyR α1 co-expressed with CB1 (open circle, dashed line) and GlyR α1 co-expressed with CB2 (open triangle, dotted line). Right panel: comparison of GlyR α1 and co-transfection with CB1 and CB2. Differences were not significant (p > 0.05, one-way ANOVA).
Fig. 5. Endogenous collybistin colocalizes with GlyRα1 in primary murine neurons. (A) Hippocampal neurons were infected with a lentivirus encoding either GlyRα1 wildtype or a pathological GlyRα1P366L variant carrying a mutation in the PPII helix. At DIV15, cells were co-stained for GlyRα1 (mAb2b, 1:500, red) and endogenous collybistin (polyclonal rabbit anti-collybistin antibody, 1:500, green). Note, infected cells were controlled by bidirectional GFP expression. Hence, collybistin was stained with the secondary goat-anti-rabbit Cy5 antibody and is shown in false color. Nuclear DAPI staining is shown in blue. White bar in left overview panels refers to 50 μm, white bar in zoomed pictures (second to fourth lane) refers to 10 μm. (B) Quantification of the collybistin intensities determined in mAb2b (GlyRα1) clusters. Mean intensities are shown for whole cells, neurites and soma. P-values to represent level of significance are indicated **p < 0.01, n.s. = non-significant. (C) Co-immunoprecipitation of the GlyRα1 wildtype and the pathological GlyRα1P366L variant with collybistin following overexpression in HEK293 cells. Both variants were expressed with CB2 (ratio 1:1) only to detect direct interaction and co-transfected with the GlyRβ subunit and gephyrin (ratio 1:2:2:2) to detect if these structural GlyR complex proteins promote interaction with collybistin. Precipitated collybistin is shown at the appropriate molecular weight of 60 kDa in the upper panel, collybistin input second panel, GAP-DH (32 kDa) input control third panel, gephyrin (93 kDa) expression lower panel. Note, although the collybistin expression is similar (input), collybistin precipitated more efficiently in the presence of GlyRβ and gephyrin (abbreviation Geph). (D) Quantification of relative collybistin expression in the presence of collybistin only or together with GlyRβ and gephyrin. Four independent experiments have been performed and were used for analysis; n.s. non-significant.
(as found in the rat α1 sequence) may be tolerated. The loss of the observed interaction between the polyproline helix and collybistin in the GlyR1P1-5A mutant indicates that the prolines in the region 365–373 play an important role for the interaction between GlyR1x1 and the binding partner collybistin. The interaction between the pleckstrin homology (PH) domain [48–50] of collybistin and the GlyR1 PPII helix – initially surprising – could be rationalized from the protein structures. The PH domain of collybistin shows structural similarity to Enabled/VASP Homology 1 (EHV-1) domains, which are known to bind to PPII domains. Here, the backbone conformation is defined by the PPII helix, while the specific surface, formed by proline side chains is required for the binding to EHV1 [31,33]. The sequence identity between the PH domain of collybistin and human EHV-1 is only ~8%, yet the PPII-binding regions of both proteins overlap very well, to a RMSD of 2.2 Å, as evident from a comparison between the PDB structures of rat collybistin [51] (PDB file 2DFK) and a murine EHV-1 domain [33] (PDB file 1EVE).

In structural and functional tests, GlyR1P1-5A mutant receptors were expressed, targeted to the cell surface and showed wildtype-like GlyR currents. Half maximum concentrations were slightly but significantly increased in the mutant receptor compared to wildtype. Large variations (up to 10-fold) of recombinant wildtype GlyR EC50 values have been described previously [52,53] and were compatible with regular “wildtype” function of the receptor. Thus, the 1.5-fold increase in EC50 of the GlyR1P1-5A mutant is not expected to be physiologically relevant. After co-expression of wildtype GlyR1x1 and CB1 or CB2 we observed a slight decrease in the EC50 of glycine, which was not statistically significant. Taken together, we conclude that the GlyR1P1-5A mutant receptor is active and that absence or presence of collybistin I or II has no relevant influence on wildtype channel activity of the receptor.

To date, GlyRs are known to cluster via β-subunits that directly interact with the sub-synaptic scaffolding protein gephyrin at inhibitory postsynaptic sites [3,5–7,11,12,54]. Since we tested the binding mediated by the α1 subunit alone, we mostly expressed homomeric α1 receptors (all experiments in Figs. 2–4 were in the absence of GlyRβ expression), thus avoiding possible interactions of the GlyRβ subunit with gephyrin in the recombinant system. Homomeric α1 GlyRs have never been reported to interact with gephyrin, in fact, absence of this interaction has been suggested [14]. It is presently unknown whether (and how) homomeric α1 glycine receptors could attach and cluster at postsynaptic plasma membranes. Expression of homomeric GlyR1x1 or α2 subunits in neurons is mostly unknown, as also demonstrated by electrophysiological investigation of neuronal glycine receptor channels, where only conductance levels of αβ GlyRs were observed [2].

Conductance levels consistent with homomeric α1 or α2 GlyRs have seldom been reported, suggesting that these are not expressed in significant amounts. There is, however, sporadic evidence that homomeric GlyRs are expressed in non-somatic locations on presynaptic nerve terminals of central neurons [2,55]. A study performed on neurons of the rat supraoptic nucleus showed a differential distribution of heteromeric and homomeric GlyRs, where former are distributed exclusively on the soma and dendrites and homomeric GlyRs are found exclusively on distal axonal regions [56]. A recent study showed that α1 subunits can also affect the GlyR–gephyrin interaction despite the absence of direct binding [57].

Our data demonstrate that the intracellular TM3–4 loop of GlyR1x1 subunit is able to bind collybistin in GST pull down assays and suggest that the α1 subunit itself may be able to mediate membrane attachment. Since binding of CB1 or CB2 to the GlyR1P1-5A TM3–4 loop was absent in HEK293 cells, we conclude that the PPII motif mediates this interaction. To validate the finding of pulldown experiments, we performed immunoprecipitation studies of the GlyR1x1 and collybistin or the isolated CB-PH domain. Our results confirmed the binding of GlyR1x1 to CB1 and CB2 as well as the isolated PH domain, indicating that the PH domain mediates the observed interaction. Our data extend the present ensemble of glycine receptor – scaffolding proteins interaction, suggesting alternative collybistin binding determinants in different GlyR subunits.

Interaction between collybistin and gephyrin [13,14,16,23] occurs near the N-terminus of collybistin, in a linker region between SH3 domain and the DH domain [16] while GABAAR binding sites are located inside the N-terminal SH3 domain [20]. Hence, in contrast to the GlyR1x1 subunit, the GABAAR interaction requires the presence of the SH3 domain. Interactions of collybistin with both, gephyrin or GABAAR are independent of the PH domain which contains the binding site of the here discovered GlyR1x1–CB interaction. Interestingly, Harvey and colleagues reported that gephyrin clustering in recombinant systems and cultured neurons required both, CB-gephyrin interactions and an intact collybistin PH domain [13]. Also, in hippocampus, substitution of residues in the PH domains of CB2 abolished clustering of gephyrin [58]. Thus, the PH domain of collybistin plays an important role in synaptic clustering.

In contrast to glycine receptors, direct interactions between GABAAR subunits α2 and α3 and collybistin are known [19,20,22,59,60]. Although GlyRs and GABAARs are structurally similar, both member being of the cysteine loop superfamily of ligand-gated ion channel [1,2], the collybistin binding motif ‘AYAYAVANYA’ of the GABAAR α2 subunit is situated in the TM3–4 loop upstream of the binding motif ‘PPPPASKSP’ of the glycine receptor. Collybistin binds the α1 glycine receptor via its PH domain (this study) while the GABAAR α2 receptor binds via its SH3 domain [20]. Thus, location and contributing domains of collybistin binding are different between glycine and GABAAR receptors.

It has been reported that GlyR clusters remained largely unaffected in collybistin knockout mice in contrast to GABAARs which were reduced following collybistin depletion [60]. Binding between gephyrin and GlyRβ subunits in neurons is much stronger than that of gephyrin and GABAAR α1, α2 or α3 subunits, which is reported to be moderate or even absent [11,19]. Thus, the rather weak binding of GABAAR receptors to gephyrin may require support via collybistin whereas GlyRβ-gephyrin interactions may be sufficient for effective synaptic clustering, independent of collybistin. Recently, subunit-specific clustering of glycine receptors was studied, showing that clustering of α1, but not α3 glycine receptors was sensitive to interleukin-1β and suggesting that alpha subunits alone can affect GlyR–gephyrin binding at synapses without need for β-subunits [57]. Collybistin expression is regulated by excitatory synaptic input [61] and driven by neuronal proteins such as neurologin-2 [23]. The direct interaction between GlyR1x1 subunits and collybistin described here may be relevant for the regulation of synaptic glycine receptor clustering.

Our data indicate a direct binding of collybistin to the GlyR1x1 subunit which would enable α1 homomers to cluster at synapses without involvement of the GlyRβ subunit. This adds to the variety of interactions that contribute to synaptic anchoring of glycine receptors. The role of the collybistin–GlyR1x1 interaction in synaptic clustering requires further investigation.

Author contributions

U.B. and K.W. performed mutagenesis and patch-clamp electrophysiology experiments, K.W. and R.E. planned and performed Y2H tests, Y.P. performed pulldown experiments, G.L. performed immunofluorescence and immunoprecipitation, H.S. performed
Compliance with Ethics Requirements

The work described here did not involve human probands or animals. All relevant ethical and scientific standards were observed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] Breitinger HG. Glycine Receptors. Chichester: eLS John Wiley & Sons Ltd; 2014.

[2] Lynch JW. Native glycine receptor subtypes and their physiological roles. Neuropharmacology 2009;56:303–9.

[3] Grunewald N, Jan A, Alvacho C, Kress V, Renner M, Triller A, et al. Sequencing the glycine receptor binding site of GlyBetaI tongue receptor stabilization at synapses. eNeuro. 2018;5:8.

[4] Kneussel M, Betz H. Receptors, gephyrin and gephyrin-associated proteins: novel insights into the assembly of inhibitory postsynaptic membrane specializations. J Physiol 2000;525(Pt 1):1–5.

[5] Oertel J, Melzer N, Becker CM. Recessive hyperekplexia mutations of the gephyrin gene result in altered gephyrin expression and clustering in mice. Hum Mol Genet 1999;8:1865–7.

[6] Prior P, Schmitt B, Grominghlo C, Prihila I, Multafaup C, Beyreuther K, et al. Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin link protein. Neuron 1992;2:1161–70.

[7] Breitinger U, Breitinger HG, Becker CM. Structure-function relationships of glycine and GABA receptors and their interplay with the scaffolding protein gephyrin. Front Mol Neurosci 2018;11:317.

[8] Kasaragod VB, Schindelin H. Heterogeneity of heteromeric GABA(A) receptors and receptor-anchoring properties of gephyrin. Front Mol Neurosci 2019;12:191.

[9] Kneussel M, Brandstätter JH, Laube B, Stahl S, Müller U, Betz H. Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. J Neurosci 1999;19:8288–97.

[10] Pizzarelle R, Grigoli M, Zacchì P, Petrin EM, Barberis A, Cattaneo A, et al. Tuning GABAergic inhibition: gephyrin molecular organization and functions. Neuroscience 2020;439:125–38.

[11] Maric HM, Mukihejje T, Tretter V, Moss SJ, Schindelin H. Glycine-mediated gamma-amino butyric acid type A and glycine receptor clustering relies on a common binding site. J Biol Chem 2011;286:42105–14.

[12] Meyer G, Kirsch J, Betz H, Langsjo C. Identiﬁcation of a glycine binding motif on the glycine receptor beta subunit. Neuron 1995;15:563–72.

[13] Harvey K, Duguid IC, Aldred M, Beatty S, Ward H, Keep NH, et al. The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. J Neurosci 2004;24:5816–26.

[14] Kins S, Betz H, Kirsch J. Collybistin, a newly identiﬁed brain-speciﬁc GEF, induces submembrane clustering of gephyrin. Nat Neurosci 2000;3:22–9.

[15] Papadopoulos T, Soykan T. The role of collybistin in gephyrin clustering at inhibitory synapses: facts and open questions. Front Cell Neurosci 2011;5:11.

[16] Gouazeue Y, Hermann A, Kins S, Fuhrmann JC, Betz H, Kneussel M. Identiﬁcation of a glycine-binding motif in the GDP/GTP exchange factor collybistin. Biochem Biophys Res Commun 2001;382:1452–55.

[17] Breitinger U, Bahnassawy LM, Janzen D, Roemer V, Becker CM, Villmann C, et al. PKA and PKC modulators affect ion channel function and internalization of recombinant Alpha1 and Alpha1-beta glycine receptors. Front Mol Neurosci 2018;11:154.

[18] Langhoff C, Schaefer N, Maric HM, Keramidas A, Zhang Y, Baumann P, et al. A novel glycine receptor variant with startle disease affects Syndapin I and glycineric inhibition. J Neurosci 2020;40:4954–69.

[19] Tretter V, Kerschner B, Milenkovic I, Ramsden SL, Ramrstorfer J, Saepour L, et al. Molecular basis of the gamma-aminobutyric acid A receptor alpha3 subunit interaction with the clustering protein gephyrin. J Biol Chem 2011;286:37702–11.

[20] Saepour L, Fuchs C, Patrizi A, Sasso-Poggetto M, Harvey RJ, Harvey K. Complex role of collybistin and gephyrin in GABA(A) receptor clustering. J Biol Chem 2010;285:29623–31.

[21] Pannazelli P, Gunn BC, Schlatter MC, Benke D, Tyagarajan SK, Scheffelle P, et al. Unique mechanisms of postsynaptic clustering of alpha3 and beta1 glycine receptors. J Biol Chem 2013;288:22456–68.

[22] Shi Q, Breitinger U, Breitinger HG, Bauer F, Fahmy K, Gocklenhammer D, Becker CM. Collybistin, a newly identified brain-specific GEF, determines the interaction of inhibitory postsynapses. EMBO J 2014;33:2113–33.

[23] Cascio M, Shenedi G, Grodzicki RL, Sigworth FJ, Fox RO. Functional reconstitution and characterization of recombinant human alpha1 glycine receptors. J Biol Chem 2001;276:20981–8.

[24] Breitinger U, Breitinger HG, Bauer F, Fahmy K, Gocklenhammer D, Becker CM. Conserved high affinity ligand binding and membrane association in the native and refolded extracellular domain of the human glycine receptor alpha1 subunit. J Biol Chem 2004;279:1627–36.

[25] Isemura T, Okabayashi H, Sakakibara S. Steric structure of L-proline oligopeptides. I. Infrared conformation spectra of the oligopeptides and poly-L-proline. Biopolymers 1968;6:307–21.

[26] Steerama N, Woody RW. Molecular dynamics simulations of polyproline conformations in water: A comparison of alpha, beta, and poly(pro) conformations. Proteins 1999;36:490–6.

[27] Kelly MA, Chellgren BW, Rucker AL, Troutman JM, Fried MG, Miller AF, et al. Host-guest study of left-handed polyproline II helix formation. Biochemistry 2001;40:14376–83.

[28] Adzhubej AA, Sterberg MG, Makarov AA. Polyproline-II helix in proteins: structure and function. J Mol Biol 2013;425:2100–32.

[29] Jones TP, Cowman A. Diversity of polyproline recognition by EVH1 domains. Front Biosci (Landmark Ed) 2009;14:833–46.

[30] Zwiebel K, Willmann MP, Sudol M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J 2000;14:231–41.

[31] Furhfeld KE, Lee DJ, Lin CP. Structural change of the enabled/VASP Homology 1Domain–Peptide Complex: A Key Component in the Spatial Control of Actin Assembly. Cell 1997;97:471–80.

[32] Breitinger HG, Villmann C, Melzer N, Renner J, Breitinger U, Schwarzinger S, et al. Novel regulatory site within the TM3-4 loop of human recombinant alpha3 glycine receptors determines channel gating and domain structure. J Biol Chem 2009;284:28624–33.

[33] Greenfield NJ. Methods to estimate the conformation of proteins and peptides from CD data. Trends Biochem Sci 2002;27:60–4.

[34] Mack L, Williams MP, Sudol M. The structural investigation of the binding of a herpesviral protein to the SH3 domain of tyrosine kinase. Biochemistry 2002;41:5120–30.

[35] Taffany ML, Krionis S, Nicholson MW. Conformation of poly-L-proline in an unordered conformation. Biopolymers 1968;6:1767–70.

[36] Tsurriel S, Geva R, Zamorano P, Dresbach T, Boeckers T, Gundelfinger ED, et al. PKA and PKC modulators affect ion channel function and internalization of the glycine receptor alpha1 subunit. J Biol Chem 2004;279:1627–36.

[37] Schwartz KE, Hoffmann S, Oetert J, Revesz K, Canals JM, Nissen M, et al. Mutations of recombinant Alpha1 and Alpha1-beta glycine receptors determine channel gating and domain structure. J Biol Chem 2009;284:28624–33.

[38] Greenfield NJ. Methods to estimate the conformation of proteins and peptides from CD data. Trends Biochem Sci 2002;27:60–4.
Naveenkumar N, Sowdhamini R, Srinivasan N. Specialized structural and functional roles of residues selectively conserved in subfamilies of the pleckstrin homology domain family. FEBS Open Bio 2019;9:1848–59.

Xiang S, Kim EY, Connelly JJ, Naissar N, Kirsch J, Winking J, et al. The crystal structure of Cdc42 in complex with collybistin II, a gephyrin-interacting guanine nucleotide exchange factor. J Mol Biol. 2006;359:35–46.

Breitinger U, Raaft KM, Breitinger HG. Glucose is a positive modulator for the activation of human recombinant glycine receptors. J Neurochem 2015;134:1055–66.

De Saint Jan D, David-Watine B, Korn H, Bregestovski P. Activation of human alpha1 and alpha2 homomeric glycine receptors by taurine and GABA. J Physiol 2001;535:741–55.

Maric HM, Kasaragod VB, Hausrat TJ, Kneussel M, Tretter V, Stromgaard K, et al. Molecular basis of the alternative recruitment of GABA(A) versus glycine receptors through gephyrin. Nat Commun 2014;5:5767.

Hruskova B, Trojanova J, Kulik A, Kralikova M, Pysanenko K, Bures Z, et al. Differential distribution of glycine receptor subtypes at the rat calyx of Held synapse. J Neurosci 2012;32:17012–24.

Deleuze C, Runquist M, Orcel H, Rabiel A, Dayanithi G, Alonso G, et al. Structural difference between heteromeric somatic and homomeric axonal glycine receptors in the hypothalino-neurohypophysial system. Neuroscience 2005;135:475–83.

Patrizio A, Renner M, Pizzarelli R, Triller A, Specht CG. Alpha subunit-dependent glycine receptor clustering and regulation of synaptic receptor numbers. Sci Rep 2017;7:10899.

Reddy-Alla S, Schmidt B, Birkenfeld J, Eulenburg V, Dutertre S, Bohringer C, et al. PH-domain-driven targeting of collybistin but not Cdc42 activation is required for synaptic gephyrin clustering. Eur J Neurosci 2010;31:1173–84.

Körber C, Richter A, Kaiser M, Schlicksupp A, Mukusch S, Kuner T, et al. Effects of distinct collybistin isoforms on the formation of GABAergic synapses in hippocampal neurons. Mol Cell Neurosci 2012;50:250–9.

Papadopoulos T, Korte M, Eulenburg V, Kubota H, Retoumskaia M, Harvey RJ, et al. Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. Embo J 2007;26:3888–99.

Horn ME, Nicoll RA. Somatostatin and parvalbumin inhibitory synapses onto hippocampal pyramidal neurons are regulated by distinct mechanisms. Proc Natl Acad Sci USA 2018;115:589–94.