SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein expression and sample preparation

The protein was expressed using the Novagen pET-31b(+) system (Novagen, Milwaukee, WI) in *E. coli* BL21(DE3)pLysS competent cells (Novagen). M9 minimum medium was used for protein expression with $(^{15}NH_4)_2SO_4$ and [U-^{13}C] glucose as the sole source of nitrogen and carbon for ^{15}N-labeling and ^{15}N ,^{13}C-double labeling. To assist in chemical shift assignment, specific ^{15}N labeling of alanine, phenylalanine, leucine, isoleucine, and valine were performed using a previously reported method (Tong et al., 2008). The fusion protein was purified on staggered His-Bind chromatography columns (Novagen). The cleavage of the hGlyR-α1 TM domain from the fusion protein was achieved using the standard protocol (Skopp and Lane, 1988). Final purification for the hGlyR-α1 TM domain was carried out using reverse-phase HPLC with a C4 column (Vydac, Hesperia, CA). NMR samples were prepared as described previously (Ma et al., 2005; Tang et al., 2002). Aliquots of the hGlyR-α1 TM domain (4 mM) dissolved in trifluoroethanol (TFE) were titrated into a 200 mM solution of LPPG micelles (10 mM sodium phosphate buffer, pH 5.8) to a protein-to-LPPG ratio of ~1:200. Distilled H_2O was added to reach the water-to-TFE ratio of 16:1 by volume. The sample was vigorously mixed, rapidly frozen in liquid N_2, and lyophilized overnight at –80°C to remove all solvents, particularly TFE. The lyophilized sample was rehydrated in deionized H_2O with 5% D_2O for NMR field lock. NMR samples for structure determination typically had a protein concentration of ~500 μM with a protein-to-LPPG ratio of ~1:200 (pH 5.8).

For site-directed paramagnetic spin labeling, the wild-type hGlyR-α1 TM (with one cysteine, C290) and two single-cysteine mutants (C290S/S296C and C290S/S308C) were prepared as described in the literature with minor modifications (Battiste and Wagner, 2000). Cysteine was reduced using dithiothreitol (DTT) at a 10-fold molar excess for 30 minutes at room temperature. Excess DTT was removed by dialysis overnight in 10 mM sodium phosphate buffer at pH 5.8. [1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl]-methanethiosulfonate (MTSL) was added from a concentrated stock in acetonitrile to a MTSL-to-protein molar ratio of 5:1 and incubated overnight at room temperature before removing the free MTSL by dialysis in a 10 mM sodium phosphate buffer.

For functional measurements, LUVs were prepared using the same method reported previously (Tang et al., 1999). Briefly, phosphatidylcholine (PC) and phosphatidylglycerol (PG) in a 3:1 molar ratio were dissolved and mixed in chloroform. The mixture was divided equally into three portions for use in: control samples without the protein, samples with the protein, and samples with the same amount of protein and picrotoxin (PTX, Tocris Bioscience, Ellisville, MO). The mixtures were dried into thin films under a stream of N_2 gas and left under vacuum overnight to completely remove the organic solvents. The dried films were rehydrated with one volume of 5 mM Tris-HCl buffer at pH 7.2 (buffer A) and four volumes of 0.5 M KCl in buffer A. After vigorous vortexing and brief sonication, the samples were subjected to two cycles of freeze and thaw alternating between –80°C and room temperature, respectively. Immediately before the MIT experiments, the vesicles were expanded by adding buffer A to reach a final total lipid concentration of 25 mM, a KCl concentration of 200 mM, a protein concentration of 26 μM and a picrotoxin concentration of 1 mM.

For EM measurements, serial dilutions, using a buffer containing 10 mM HEPES and 50 mM KNO_3, were made from a stock solution in the same buffer with a protein concentration of 54 μM and a protein-to-LPPG ratio of 1:50. 5 μl of diluted samples were deposited onto a glow-discharged carbon foil grid, blotted with filter paper, and stained with 2% uranyl acetate.

NMR spectroscopy

For backbone chemical shift assignment, the following spectra were collected: HNCO (1024x40x64) with ^{13}C spectral width of 12ppm, HNCA and HN(CO)CA (1024x40x80) with ^{13}C spectral widths of 32 ppm, and HNCACB and CBCA(CO)NH (1024x40x80) with ^{13}C spectral widths of 75ppm. HCCH-TOCSY was collected for side chain assignment. To obtain the distance restraints, ^{15}N- and ^{13}C-edited NOESY (1024x60x248) were acquired with spectral windows of 27 ppm and 80 ppm for the ^{15}N and ^{13}C dimensions, respectively. Mixing times were 150 ms for both ^{15}N- and ^{13}C-edited 3D NOESY. Unless otherwise specified, spectral windows for ^{1}H and ^{15}N dimensions were 12 ppm and 26 ppm, respectively. ^{15}N-edited 3D NOESY spectra were acquired for the samples with selectively ^{15}N-labeled Ala, Phe, Leu, Ile, or Val. Longitudinal (R_1) and transverse (R_2) ^{15}N relaxation rate constants and ^{15}N-{^{1}H} heteronuclear NOE were measured at 40°C on a Bruker Avance 700 MHz spectrometer. R_1 was determined using 9 delay values: 10, 100, 200, 400, 600, 800, 1000, 1500 and 2800 ms. The R_2 experiment also used 9 delay values: 16, 32, 64, 96, 128, 160, 240, 320 and 480 ms. For the steady-state ^{15}N-{^{1}H} NOE measurement, a train of 120 high-power pulses separated by 5 ms for the duration of 3 s was used for proton saturation and data was collected with and without proton saturation in an interleaved fashion. Temperature effects on chemical shift were measured using a series of ^{1}H-^{15}N HSQC experiments collected at 35, 40, 45, and 50°C on a Bruker 900 MHz spectrometer. DSS was used as an internal reference for ^{1}H chemical shift, with ^{15}N and ^{13}C chemical shifts indirectly referenced (Wishart et al., 1995). Topspin and NMRPipe (Delaglio et al., 1995) were used to process NMR data. Sparky was used for resonance assignment (Goddard and Kneller).

The MIT experiments (Hinton et al., 1994; Tang et al., 1999) were performed at 30°C using a 4-mm MAS probe on a Bruker Avance 600 MHz spectrometer. In the absence of the chloride shift reagent, only one peak was observed and manually set to 0 ppm. In the presence of ~20 mM $Co(gly)_3^-$, the extra-vesicle resonance ($^{35}Cl_{out}$) was separated from the intra-vesicle resonance ($^{35}Cl_{in}$) by ~25 ppm. A pair of hard 90° pulses, separated by the reciprocal of twice the chemical shift difference, was used to selectively invert the $^{35}Cl_{out}$ resonance while returning the $^{35}Cl_{in}$ magnetization back to the Z direction. The inversion-recovery time, t, varied from 10 μs to 0.5 ms, followed by a third 90° read pulse. Typically, 14 inversion-recovery times were used in each MIT experiment. The recycle delay was set to at least 10 times the T_1 value of the ^{35}Cl signals. The Mnova NMR program (Mestrelab Research, Escondido, CA) was used to measure the MIT peak intensities by spectral deconvolution. Influx and efflux rates were calculated using a two-site exchange mode, the details of which are described below under the section title: *NMR spectroscopy for measuring Cl^- flux across the hGlyR-α1 TM channel.*

Electron microscopy

The uranyl acetate stained samples were examined at 200 kV with a TF20 electron microscope (FEI, Hillsboro, OR). Images were recorded on a 4Kx4K Gatan CCD camera (Gatan, Inc., Warrendale, PA) at a nominal magnification of 50,000x and underfocus values ranging from 1.5 to 3.0 μm. The CCD images were processed using EMAN image analysis software (Ludtke et al., 1999). Specifically, the particles were boxed manually with 72 x 72 pixels (2.14 Å/pixel), normalized, and combined in one raw image stack file. A total of 526 individual particle images were initially picked. These raw images were band pass-filtered and iteratively aligned to each other. About 210 good particle images were selected. The aligned raw projection images were classified and averaged within each class (Ludtke et al., 1999).

Circular dichroism (CD)

The CD spectra in the wavelength range of 187-280 nm were acquired at room temperature using a cuvette with a 1 mm path length on a Jasco CD spectrometer (model J-810, Jasco Co., Japan). The wavelength step was 1 nm with an averaging time of 1 s. Two scans were acquired for each CD

measurement. After solvent subtraction, the spectra were analyzed using the Web-based CD analysis software DICHROWEB (Lobley et al., 2002).

NMR spectroscopy for measuring Cl⁻ flux across the hGlyR-α1 TM channels

A two-site exchange model was used to derive the unidirectional flux rate constants from the MIT experiments. With the approximation that the longitudinal relaxation time, T_1, is similar for the intra- and extra-vesicle magnetizations, it has been shown (Tang et al., 1999) that the time dependence of the uninverted magnetization, I, and inverted magnetization, S, on the inversion recovery time are given by:

$$I = I_0\left[1 - \frac{2k_e}{k_i + k_e}e^{-(t/T_1)}(1 - e^{-(k_i + k_e)t})\right] \quad (1)$$

$$S = S_0\left[1 - \frac{2}{k_i + k_e}e^{-(t/T_1)}(k_e + k_i e^{-(k_i + k_e)t})\right] \quad (2)$$

where I_0 and S_0 are the spectral intensities of a fully relaxed spectrum, and k_i and k_e are influx and efflux rate constant. The thermal equilibrium condition is satisfied by $k_i S_0 = k_e I_0$. T_1 was measured using the conventional inversion-recovery method. The k_i and k_e values can be determined by non-linear least square fitting of the MIT data using Eq. (1) and Eq. (2).

Structure calculations

The upper and lower bound distance restraints were derived from NOESY and PRE data. Backbone dihedral angles were predicted from chemical shift values using the semi-empirical method implemented in TALOS (Cornilescu et al., 1999). Hydrogen bonding restraints were generated for those residues whose H-D exchange was in the slow category (absolute temperature slope < 4.5 ppb/K) (Baxter and Williamson, 1997) and in addition whose CSI and NOE restraints indicated a helical secondary structure. Restraints used in structure calculations are summarized in Table 1 and Figure S3. The 76 backbone dihedral angle restraints from the available chemical shift data of Cα, Cβ, C, N, H, and Hα. The long-range distance restraints for accurate tertiary structure determination were derived from PRE experiments (Battiste and Wagner, 2000), in addition to the unambiguous inter-domain NOESY cross peaks. We made three separate spin labeling positions along the length of the TM3 domain at C290, S296C, and S308C. A simultaneous C290S mutation was made in the latter two cases, so that only one spin labeling position is present in each mutant. Distance restraints from PRE were generated using the established method (Battiste and Wagner, 2000). The excellent correlation between the corresponding distances calculated from PRE and from the averaged NMR structure is plotted in Figure S4.

<u>Monomer Calculation</u>

One hundred (100) random structures were generated and annealed using Cyana-3.0 (Guntert et al., 1997; Herrmann et al., 2002). Structures were annealed using 2,000,000 steps and an annealing schedule modified slightly from the default annealing schedule in Cyana-3.0. Modifications to the annealing schedule include using a quadratic (vs. a quartic) cooling profile in the second annealing stage. With the large number of steps, decreasing the temperature more slowly in the second annealing stage improves the annealing. By default, the van der Waals (VDW) penalty is initially divided by four and restored to its default value at the end of the second annealing stage. In the modified algorithm the VDW penalty was initially divided by 4, then multiplied by 2 halfway through the second annealing stage, and restored to its default value at the end of the second annealing stage. This change was made to

prevent structures from entering into minima with bad VDW contacts. Of the 100 structures calculated, the 32 structures with lowest target functions were refined. Refinement was performed using Cyana-3.0 and the annealing schedule outlined in Table S1. Radii and weights were not adjusted at any point in the refinement schedule and parameters not reported below are identical to those in Cyana's default annealing algorithm. Two rounds of refinement were performed. The first round of refinement used an input temperature of 0.3 target function units per degree of freedom and 2,000,000 steps. The second used an initial temperature of 0.03 target function units per degree of freedom and 1,000,000 steps. From the 32 refined structures the 15 with lowest target function were obtained and reported.

Table S1: Cyana Refinement Annealing Schedule

	Steps[1]	Starting T[2]	Ending T	Tau[3]	VDW[4]
Minimization	100	N/A	N/A	N/A	N/A
Pre-Equilibration	0.02N	T	T	1.0	10
Equilibration 1	0.02N	0.9T	0.9T	1.0	10
Equilibration 2	0.02N	0.855T	0.855T	1.0	10
First Cooling	0.36N	0.855T	0.154T	10.0	10
Second Cooling	0.44N	0.154T	0.0086T	10.0	5
Final Cooling	0.16N	0.0086T	0	1.0	20
Final MD	4000	0	0	1.0	20
Minimization	1000	N/A	N/A	N/A	N/A

[1]N is total number of steps. Pre-Equilibration and Final MD are not included when adding up to N. [2]T is the input temperature. Temperatures visited in between the starting and ending temperatures result from a linear interpolation between starting and ending temperatures. [3]Tau is the correlation time controlling coupling to the temperature bath. [4]VDW column shows the number of steps after which VDW forces are evaluated.

Pentamer Calculation

The calculation of the pentamer structure was developed based on the symmetric arrangement of monomer subunits in a pentameric configuration. The intra-subunit NMR restraints were copied five times. We defined five groups of symmetric contacts within Cyana from which Cyana automatically generated 3996 angle identity restraints and 100,000 symmetric distance restraints. From the EM images we obtain a restraint on the radial distance of each monomer subunit from the pore. Tilting angles for TM2 with respect to the channel pore were obtained from RDC measurements in low-q bicelles (Canlas et al., 2008). The average angle between TM2 and the channel pore (13±1°) is within the range of angle values for proteins in the same superfamily of Cys-loop receptors: the α1 nAChR (12°) (Montal and Opella, 2002), the α4 nAChR (12±1° and 16±1° for α4 and α4β2 respectively) (Cui et al., 2010), and the $GABA_A$ receptor (15±2°)(Kandasamy et al., 2009). The angles all fall in the range of 11° to 17°. We considered the possibility that the presence of TM1 and TM4 may produce changes to the TM2 tilting angle and used a ±5° error term for this angle in the pentamer structure calculation (see the section on *Rotational Error*). To generate Cyana distance restraints based on the EM and RDC restraints, a model of the pentamer was constructed using MATLAB® (2010a, The MathWorks, Inc., Natick, Massachusetts). Details for the construction of the model and generation of Cyana restraints are described below.

Atoms in the Model

Residues present in the model coming from TM2 correspond to the residues in TM2 over which

the RDC data was reported (residues 253 to 265) (Canlas et al., 2008). Residues in TM1 (226 to 241), TM3 (290 to 302) and TM4 (402 to 414) were added to the model. The RMSD for backbone atoms listed was less than 0.5 Å. Coordinates for backbone atoms of the listed residues were extracted from the structure bearing the minimum RMSD to all other structures in the bundle and imported into MatLab. The imported coordinates and corresponding atoms define the monomer subunit used in construction of the pentamer model.

Pentamer Construction

The pentamer model was constructed by creating vertices for a pentagon inscribed on a unit circle lying in the membrane plane and centered at (0, 0, 0). The pentagon coordinates were multiplied by the radius obtained from the EM data. The radius was the distance from the center of the EM image to the peak in electron density after circular averaging of the EM images. Hydrogen and nitrogen coordinates for residues in TM2 were oriented according to the RDC constraints. The monomer was centered according to its center of density, moved to one of the vertices, and TM2 was oriented to face the pore. The center of density was calculated for the bundle of 15 monomer structures using the VolMap tool in VMD. The pentamer was created by moving subunits to each remaining vertex and applying a 72° rotation for each consecutive movement around the pentagon.

Rotational Error

The error reported in the RDC experiments was 1° for rotations about the 1st and 2nd principle axes and 10° around the 3rd principle axis. The 1st and 2nd principle axes correspond to tilting within the membrane while the 3rd principle axis corresponds to a rotation about the monomer's principle axis. In this model a 5° rotational variance was allowed about each of the monomer axes. The 5° rotational variance was accounted for by applying rotations ranging from -5° to 5° about each axis with a step size of 1°. Coordinates resulting from these rotations were stored for each atom. The result is a cluster of 1331 (11x11x11) atom positions.

Atoms Restrained

For each TM domain restraints were created for atoms closest to the monomer center as well as farthest along the channel axis in both directions from the center of each TM domain were chosen to restrain. Atoms were also chosen at the midpoints along the channel axis, resulting in 5 atoms per TM domain for which restraints were imposed.

Upper and Lower Bound Limits

Self restraints were defined as restraints between identical atoms in different subunits. For example in TM1, restraints were calculated for the following combinations:

A1B1 B1C1 C1D1 D1E1
A1C1 B1D1 C1E1
A1D1 B1E1
A1E1

Where A1B1 = TM1 of chain A paired with TM1 of chain B, B1C1 = TM1 of chain B paired with TM1 of chain C, and so forth. These pairings were repeated for TM2, TM3, and TM4.

To determine the distance restraint to use in the Cyana calculation, pairwise distances were calculated between each atom position in each cluster. Where the cluster referred to is described under *Rotational Error*. The upper bound limit was the maximum distance calculated plus a 1 Å error term accounting for EM resolution. The lower bound limit was the minimum distance calculated minus 1 Å. For example, the distance restraint for atom (i) in the pair A1B1 is calculated as follows: The distance between atom (i) in TM1 of chain A and atom (i) in TM1 of chain B is calculated for each of the 1331

positions of atom (i) in the generated cluster. This results in a total of 1331 distances. The one to one correspondence of the number of atom positions stored in each cluster and the number of distances results from a symmetry assumption implicit in the model. Five-fold symmetry around the channel axis requires that any rotation applied to one subunit must necessarily be applied to all subunits. Therefore we only compute distances between points having the same applied rotations for each subunit. The upper bound limit was calculated by taking the maximum value of the 1331 distances plus 1 Å, while the lower bound limit was calculated by taking the minimum value of the 1331 distances minus 1 Å.

Restraints were also calculated between TM1 and TM3 in a similar fashion.

That is the restraints were as follows:

A1B3 B1C3 C1D3 D1E3		A3B1 B3C1 C3D1 D3E1
A1C3 B1D3 C1E3	&	A3C1 B3D1 C3E1
A1D3 B1E3		A3D1 B3E1
A1E3		A3E1

In this case, atoms in TM3 were necessarily different from atoms in TM1. However, the relative position of the atoms along the channel axis was fixed. In other words, the atom closest to the center in TM3 was paired with the atom closest to the center in TM1.

Calculation

Similar to the monomer calculation 100 random structures were generated and annealed using Cyana-3.0. Structures were annealed in 500,000 steps using the modified Cyana annealing schedule as described for the monomer calculation. An input temperature of 8.5 target function units per degree of freedom, rather than the default of 8.0 target function units per degree of freedom, was also used for the pentamer calculation. Of the 100 structures calculated, thirty-two were refined using the refinement schedule described in Table S1. Two rounds of refinement were also performed for the pentamer calculation. The first round of refinement used 500,000 steps and a temperature of 0.3 target function units per degree of freedom and the second round of refinement used 250,000 steps and a temperature 0.03 target function units per degree of freedom.

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