Mapping a molecular link between allosteric inhibition and activation of the glycine receptor

Paul S Miller¹, Maya Topf² & Trevor G Smart¹

Cys-loop ligand-gated ion channels mediate rapid neurotransmission throughout the central nervous system. They possess agonist recognition sites and allosteric sites where modulators regulate ion channel function. Using strychnine-sensitive glycine receptors, we identified a scaffold of hydrophobic residues enabling allosteric communication between glycine-agonist binding loops A and D, and the Zn^{2+} -inhibition site. Mutating these hydrophobic residues disrupted Zn^{2+} inhibition, generating novel Zn^{2+} -activated receptors and spontaneous channel activity. Homology modeling and electrophysiology revealed that these phenomena are caused by disruption to three residues on the '-' loop face of the Zn^{2+} -inhibition site, and to D84 and D86, on a neighboring β 3 strand, forming a Zn^{2+} -activation site. We provide a new view for the activation of a Cys-loop receptor where, following agonist binding, the hydrophobic core and interfacial loops reorganize in a concerted fashion to induce downstream gating.

The Cys-loop ligand-gated ion channel superfamily includes the nicotinic acetylcholine receptor (nAChR), γ-aminobutyric acid type A receptor (GABAAR), glycine receptor (GlyR) and serotonin type 3 receptor (5HT₃R). Each subunit of these pentamers contains three domains: a ligand binding extracellular domain (ECD), formed by a sandwich of two β-sheets; a four α-helical membrane-spanning domain; and an intracellular region of unspecified quaternary structure¹. The interior of the ECD is hydrophobic^{2,3}, and, as for most globular proteins, it is considered to be an entropic stabilizer of protein folding⁴. Given the presumed stability of this hydrophobic core and its location between two sheets of rigid β-strands, it is usually regarded as a relatively inflexible structure. Thus, after agonistinduced activation, the core would move, if at all, as a rigid body⁵. Accordingly, it would be the agonist binding loops A–F, supported by the surrounding rigid β-strands, that would undergo a conformational change upon agonist binding to trigger rigid body movement and downstream channel opening^{2,6-11}. An alternative view, based on structural and modeling data, suggests that substantial portions of the inner and outer β-sheets of the ECD shift their orientation relative to one another upon receptor activation^{1,12}. Given the location of the hydrophobic core between the inner and outer β-sheets of each ECD, it would then be expected that the core would reorganize, rather than move as a rigid body, to facilitate the reorientation of the β -sheets^{13–17}. This movement may induce separation of important charge interactions along neighboring receptor subunit interfaces, allowing the ECDs to twist and induce downstream channel opening^{8,16,18,19}.

An ideal model system to investigate the role of the hydrophobic core in Cys-loop receptor activation is that involving Zn²⁺ inhibition of GlyR. These receptors readily form homomers that are modulated by

the physiological cation Zn²⁺ in a biphasic fashion. Zn²⁺ can be found in nanomolar concentrations in external medium and is also packaged into vesicles and released at synapses in sufficient amounts to endogenously modulate GlyRs, with low micromolar concentrations potentiating submaximal glycine responses and higher concentrations causing inhibition $^{20-22}$. Two Zn^{2+} binding sites have been identified: the potentiation site is contained solely on the outer β-sheet of the ECD²³, whereas the inhibition site spans neighboring subunits on the inner β -sheet of the ECDs^{24–26} (Fig. 1a–c). Inhibition by Zn²⁺ of GlyR function involves the stabilization of charge interactions between neighboring subunit ECD interfaces, thereby hindering their movement. This supports the notion that charge separation of neighboring ECD interfaces is necessary for receptor activation and that agonist binding must transduce a signal near to the Zn²⁺-inhibition site to evoke a conformational change in this area, leading to receptor activation. As the hydrophobic core is located between the glycine binding site and the Zn2+-inhibition site, identifying the molecular requirements for Zn²⁺ inhibition will elucidate the roles of the hydrophobic core and the subunit ECD interface in receptor function.

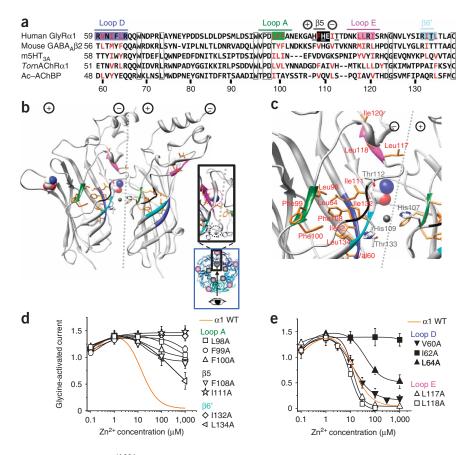
Here we demonstrate that a cluster of residues forming a scaffold across the hydrophobic core are crucial for Zn^{2+} inhibition and spontaneous opening of the human GlyR ion channel. Spontaneous opening was attributed to the apparent flexibility of a loop on the '–' face of the Zn^{2+} -inhibition site, which is exquisitely sensitive to the molecular composition of the hydrophobic core. Disruption of this loop and the discovery of previously uncharacterized elements in a neighboring $\beta 3$ strand that are also important for receptor activation demonstrate that charge redistribution at the ECD inner subunit interface is a key component of GlyR activation.

Received 10 April; accepted 27 August; published online 21 September 2008; doi:10.1038/nsmb.1492



¹Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK. ²School of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, UK. Correspondence should be addressed to T.G.S. (t.smart@ucl.ac.uk).

Figure 1 Hydrophobic determinants of Zn²⁺ inhibition. (a) Partial protein alignment of Cys-loop receptor ECDs showing GlyR $\alpha 1$ hydrophobic residues that are examined (red), positions where hydrophobicity is retained in other Cys-loop receptors (red), conserved residues (boxed), and the GlyR Zn2+-inhibition binding site residues (underlined). Hydrophobic motifs are labeled and color coded in accordance with b and c. (b,c) The GlyR $\alpha 1$ ECD homology model based on TornAChRα₁ (ref. 1). Clustered hydrophobic residues (orange) connect the Zn²⁺inhibition site at the inner-subunit interface (black and light blue motifs-note, the black motif is not a β -strand in the model but is referred to as such in main text for clarity²) to glycine binding loops A (green), D (dark blue) and E (mauve) of the outer-subunit interface. Zn²⁺ inhibition–site residues His107. His109. Thr112 and Thr133 are in gray (nitrogen atoms are blue and oxygens are red). Dotted line depicts the interface with '+' and '-' subunit sides²⁸. Glycine, displayed in CPK, spacefill, was fitted manually in accordance with ref. 35; Zn²⁺, fitted manually in gray spacefill. Exact side chain orientations presented here are speculative, based on best alignment and modeling estimations. Inset, the blue box shows a pentamer plan view showing the viewing angle (arrow) for the main picture and revealing the separation between the Zn²⁺ binding site (gray circle) and glycine binding site (mauve circle); the black box shows and enlarged plan view of the interface shown. (\mathbf{d} , \mathbf{e}) Zn^{2+} -concentration response curves for modulation of EC₅₀ glycine



currents on wild-type (WT; dashed line) and alanine-substituted receptors. $\alpha 1^{1120A}$ did not traffic to the cell surface, so no recordings were made (**Supplementary Fig. 2**). Curves were fitted with the Hill equation. All points are means \pm s.e.m. (n = 3-6).

RESULTS

Hydrophobic residues are required for Zn²⁺ inhibition

A GlyR homology model was constructed to guide our site-directed mutagenesis studies into receptor activation. The GlyR protein sequence was first aligned with other Cys-loop receptors. This revealed that the region encompassing the GlyR Zn²⁺-inhibition site is not conserved across any of the Cys-loop receptors, for which atomicresolution ECD templates are available, and, furthermore, that this region contains a two to three amino acid insertion. Structural alignment of three ECD template structures (conotoxin-bound Aplysia californica acetylcholine binding protein (Ac-AChBP²⁷); Torpedo (Tor) nAChR α_1 (ref. 1); and mouse nAChR α_1 subunit¹⁴), revealed substantial structural variation at the two loops of the '+' and '-' face²⁸ flanking the β5 strand (nomenclature of ref. 2; Supplementary Fig. 1 online), which corresponds to the Zn²⁺-inhibition site. Using the variable loop regions as insertion points for the extra residues in our GlyR alignment (Fig. 1a) allowed us to generate a GlyR homology model (MODELLER-9.2 (ref. 29); based on the TornAChR α₁ template) with His107 and His109 exposed at the subunit interface²⁵ and Phe108 solvent accessible³⁰, in accordance with published data.

To probe the link between Zn²⁺ inhibition and GlyR activation, hydrophobic residues positioned between the Zn²⁺-inhibition site, defined by His107, His109, Thr112 and Thr133 (refs. 24,26,31), and the three closest agonist binding loops (A, D and E; **Fig. 1b,c**), were substituted with alanine. Substituted GlyRs were expressed in human embryonic kidney (HEK) cells, and their sensitivities to Zn²⁺ potentiation and inhibition were assessed by whole-cell recording of

glycine-evoked responses (EC50) in the presence of increasing concentrations of Zn²⁺. Substituting residues at the Zn²⁺-inhibition site '-' face (β5 F108A, I111A; β6' I132A, L134A) and the loop A face (L98A, F99A, F100A) substantially reduced sensitivity to Zn²⁺ inhibition compared to the wild type, whereas Zn²⁺ potentiation remained the same (Fig. 1d,e). Substitutions in agonist binding loop D (V60A, I62A, L64A) also reduced Zn²⁺ inhibition, with I62A causing ablation. This residue is orientated in the GlyR model toward other residues required for Zn²⁺ inhibition from loop A (Leu98, Phe99, Phe100) and those from β5 and β6' adjoining the Zn²⁺-inhibition site (Phe108, Ile111, Ile132, Leu134). By contrast, substituting distally located hydrophobic residues in agonist binding loop E (L117A and L118A; Fig. 1e and Table 1; I120A did not traffic to the cell surface (Supplementary Fig. 2 online) and other hydrophobic residues located away from loops A and D and B5 and B6' (Supplementary Fig. 3 and Supplementary Table 1 online) did not disrupt Zn²⁺ inhibition. All the substituted receptors with attenuated Zn²⁺ inhibition retained glycine EC₅₀s within ten-fold of the wild-type (**Table 1**) and comparable maximal glycine currents (Imax) and Hill slopes, with the exception of $\alpha 1^{\text{F99A}}$ ($I_{\text{max}} = 3.9 \pm 0.4 \text{ nA}$; wild-type GlyR $I_{\text{max}} = 6.8 \pm 0.5 \text{ nA}; P < 0.01$).

Removing Zn2+ inhibition creates Zn2+-activated GlyRs

Although Zn^{2+} does not activate wild-type GABA_AR or GlyRs, it generated inward currents at GlyRs with impaired Zn^{2+} inhibition, that is, those with alanine substitutions in the glycine binding loops A and D and Zn^{2+} -inhibition binding strands $\beta 5$ and $\beta 6'$ (L98A, F99A,



Table 1 Sensitivities to glycine and Zn²⁺ for wild-type and mutant GlyRs

	Glycine				Zn ²⁺				
					Inhibition		Activation		
	EC ₅₀ (μM)	η_{H}	I _{max} (nA)	N	IC ₅₀ (μM)	EC ₅₀ (μM)	η_{H}	Relative efficacy (% glycine I_{max})	N
α1 wild type	35 ± 5	2.7 ± 0.2	6.8 ± 0.5	6	15 ± 2	None	_	_	4
Agonist binding loop A									
$\alpha 1^{L98A}$	35 ± 6	1.9 ± 0.3	6.4 ± 0.5	6	>1,000	0.26 ± 0.08	1.1 ± 0.2	49 ± 5	4
$\alpha 1^{F99A}$	250 ± 40	2.3 ± 0.3	3.9 ± 0.4	4	>1,000	0.13 ± 0.03	1.1 ± 0.1	86 ± 13	5
$\alpha 1^{\text{F100A}}$	120 ± 20	1.8 ± 0.2	4.5 ± 0.7	5	>1,000	9.2 ± 0.6	0.5 ± 0.1	7 ± 4	4
Zn ²⁺ binding site: β5 strand									
α1 ^{F108A}	15 ± 3	2.3 ± 0.1	4.5 ± 0.5	5	>1,000	2.3 ± 0.6	0.7 ± 0.1	25 ± 6	6
$\alpha 1^{1111A}$	39 ± 4	1.3 ± 0.2	4.6 ± 0.8	4	>1,000	470 ± 130	0.7 ± 0.2	83 ± 3	3
Zn ²⁺ binding site: β6' strand									
α1 ^{1132A}	95 ± 15	2.7 ± 0.4	6.1 ± 0.7	6	>1,000	140 ± 30	1.2 ± 0.1	72 ± 5	5
$\alpha 1^{L134A}$	58 ± 3	3.0 ± 0.4	6.0 ± 0.3	7	>1,000	0.06 ± 0.01	1.3 ± 0.1	38 ± 9	5
Agonist binding loop D									
α1 ^{V60A}	91 ± 15	3.5 ± 0.8	6.5 ± 1.3	4	10.1 ± 1.26	None	_	_	4
$\alpha 1^{162A}$	240 ± 7	2.5 ± 0.1	7.6 ± 0.7	3	>1,000	>1,000	_	3 ± 1	4
$\alpha 1^{L64A}$	10 ± 3	2 ± 0.6	5.6 ± 0.8	4	70 ± 9	1.5 ± 1.3	1.1 ± 0.4	10 ± 3	3
Agonist binding loop E									
α1 ^{L117A}	4200 ± 100	1.67 ± 0.1	4.4 ± 0.7	8	13.3 ± 3.3	None	_	_	3
α1 ^{L118A}	1060 ± 110	3.00 ± 0.2	6.3 ± 0.7	3	10.3 ± 0.36	None	_	_	3

Glycine-activation, Zn²⁺-modulation and Zn²⁺-activation data from wild-type receptors and from GlyRs carrying alanine substitutions of hydrophobic residues at positions running from the Zn^{2+} -inhibition site to nearby agonist binding loops, expressed in HEK cells. η_H is the Hill slope.

F100A, F108A, I111A, I132A, L134A, I62A and I64A; Fig. 2a). The Zn²⁺-activated currents reversed close to the Cl⁻ equilibrium potential $(E_{\rm Cl})$ (0.4 ± 1.4 mV, n = 5), and the current-voltage relationships were comparable to those for glycine-activated Cl⁻ currents at wild-type GlyRs (Fig. 2b). Zn²⁺-concentration response curves revealed that the potency and relative efficacy (maximal Zn²⁺ response as a percentage of maximal glycine response in the same cell) varied substantially between the substituted receptors (Fig. 2c and Table 1). $\alpha 1^{L134\text{A}}$

showed the highest sensitivity to Zn^{2+} (EC₅₀ = 0.06 \pm 0.01 μM , n = 5), whereas $\alpha 1^{\text{F99A}}$ showed the highest relative efficacy (86 ± 13%, n = 5). By contrast, $\alpha 1^{162A}$ supported only $3 \pm 1\%$ maximal activation with 1 mM Zn²⁺ (Fig. 2c and Table 1). Entirely consistent with Zn²⁺ activating the substituted GlyRs, the anion-selective channel blocker cyanotriphenylborate (CTB, 20 μM) abolished the Zn²⁺-activated currents (Fig. 2d). Furthermore, both strychnine, a selective GlyR competitive antagonist, and picrotoxin (PTX), a GABAAR and GlyR

1.0

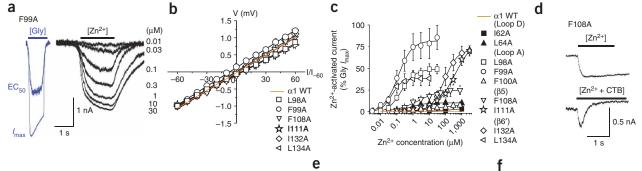
0.75

α1 WT

L98A Gly

1 98A 7n²





1.0

0.75

0.5

Figure 2 Direct Zn²⁺ activation of substituted GlyRs. (a) Representative glycine currents (blue) in the absence of Zn²⁺ (EC₅₀ 30 μ M; I_{max} 1,000 μ M) and Zn²⁺ currents (black) in the absence of glycine (0.01–30 $\mu\text{M})$ for GlyR $\alpha 1^{F99\text{A}}.$ (b) Zn^{2+} current-voltage (I-V) relationships (normalized to the current recorded at -60 mV and fitted by linear regression) for the six most efficacious ZAGs, whose EC₅₀ Zn²⁺ responses were large enough to be recorded reliably, and the glycine I-V for the wildtype (WT) receptor. (c) Zn2+ activation-response curves for alanine-substituted GlyRs; Zn2+ maxima are normalized to glycine maxima (10 mM) in the same cell. (d) Representative

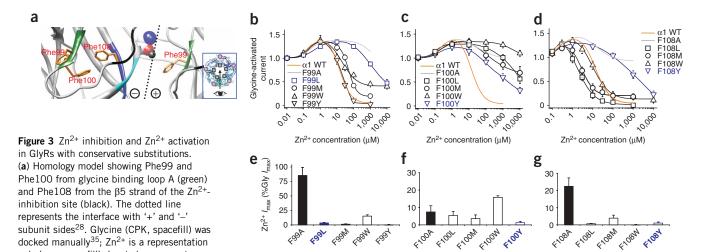
Agonist-activated current Agonist-activated current 0.25 0.25 0 0 0.001 0.01 0.1 100 10 100 10 1.000 Strychnine concentration (µM) PTX concentration (µM)

 α 1F108A Zn²⁺ I_{max} (100 μ M) current, co-applied with and without 20 μ M CTB. Inhibition greater than 100% is due to additional background leak (discussed later in Fig. 6). (e,f) $\alpha 1^{L_{98A}}$ concentration-inhibition curves for strychnine and picrotoxin, respectively, on glycine- or Zn²⁺-activated EC₅₀ currents (n = 3-6). Wild-type receptor activation sensitivities to the antagonists are shown in orange.

α1 WT

L98A Gly

1 98A 7n2



plan viewing angle (arrow), with the Zn²⁺ binding site (gray spot) and the glycine binding site (mauve spot). (**b**–**d**) Zn²⁺ concentration-response curves for modulation of EC₅₀ glycine currents from GlyRs with conservative hydrophobic substitutions at positions Phe99 (**b**), Phe100 (**c**) and Phe108 (**d**). For comparison, wild-type (WT) inhibition profiles (orange) and alanine-substituted receptor profiles (gray) are included. The same set of substituted GlyRs were assessed for maximal (1 mM) Zn²⁺-activated currents (I_{max}), normalized to glycine I_{max} (10 mM) in the same cell: Phe99 (**e**), Phe100 (**f**) and Phe108 (**g**). Alanine-substituted receptor Zn²⁺ I_{max} (black bars) is shown for comparison. Note that F99L, F100Y and F108Y showed substantially attenuated Zn²⁺ inhibition (navy blue lines) but almost no Zn²⁺ activation (navy blue bars; <2% glycine I_{max}) (n=3–6).

allosteric blocker, also inhibited Zn^{2+} activation (**Fig. 2e,f**). This prompted the classification of these substituted GlyRs as Zn^{2+} -activated GlyRs (ZAGs).

Hydrophobicity and sensitivity to Zn²⁺ inhibition

only (gray spacefill). Inset shows a pentamer

To investigate the ZAGs further, we substituted Phe99 and Phe100 of loop A and Phe108 of $\beta 5$ (Fig. 3a) individually with either tyrosine or tryptophan (both aromatic like phenylalanine) or leucine or methionine (aliphatic). Although many substitutions reduced Zn^{2+} inhibition (Fig. 3b–d), only some generated ZAG behavior (Fig. 3e–g). Of the three phenylalanine residues, we found that Phe100 is the most crucial for maintaining wild-type sensitivity to Zn^{2+} inhibition; however, there was no correlation between the properties of the substituting residue and the disruption to Zn^{2+} inhibition (Fig. 3b–d and Supplementary Table 2 online), suggesting that each position has unique chemical and physical requirements.

Glycine EC₅₀s for $\alpha 1$ Phe100- and Phe108-substituted receptors remained within two-fold of those of the wild type with the exceptions of $\alpha 1^{F100Y}$ and $\alpha 1^{F108W}$ (which increased 15-fold and 25-fold, respectively; P<0.05; **Supplementary Table 2**). For Phe99-substituted receptors, glycine EC₅₀s were significantly increased for $\alpha 1^{F99L}$, $\alpha 1^{F99W}$ and $\alpha 1^{F99Y}$ (3–30-fold; P<0.05), but surprisingly the $\alpha 1^{F99M}$ receptor was six-fold more sensitive to glycine (wild-type EC₅₀ = 35 ± 5 μ M; $\alpha 1^{F99M}$ EC₅₀ = 5.5 ± 0.5 μ M, n=4–6; P<0.05), suggesting an important role for this residue in determining glycine binding. Notably, the GlyR model positions Phe99 facing into the glycine binding site (**Fig. 1b,c**).

Zn²⁺ activation originates from a novel binding site

The switch from Zn^{2+} inhibition to activation in ZAGs could have arisen if the function of an existing modulatory Zn^{2+} binding site was altered, enabling activation in response to Zn^{2+} binding. However,

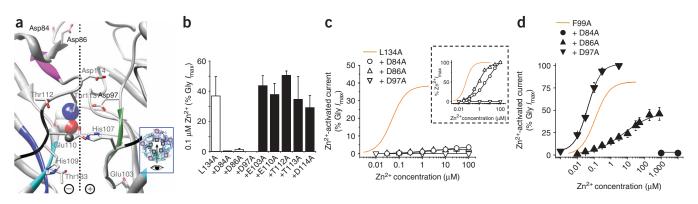


Figure 4 Identifying potential residues for the Zn²⁺-activation binding site in ZAGs. (a) Homology model showing potential Zn²⁺-coordinating residues at the subunit interface around the His107/His109/Thr112/Thr133 Zn²⁺-inhibition site. Side chains are shown in gray, nitrogens in blue and oxygens in red. The dotted line indicates the interface with '+' and '-' sides²⁸. Glycine (CPK, spacefill) was docked manually³⁵; Zn²⁺ is a representation only (gray spacefill). Inset shows the viewing angle (arrow), with Zn²⁺ (gray spot) and glycine binding sites (mauve spot). (b) 0.1 μM Zn²⁺ activation of alanine-substituted α 1^{L134A}-background GlyRs, expressed as a percentage Gly/_{max} in the same cell. (c) Zn²⁺ activation-concentration response curves for α 1^{L134A}, D84A, α 1^{L134A}, D86A and α 1^{L134A}, D97A receptors normalized to the glycine I_{max} and also normalized to the Zn²⁺ I_{max} (inset). (d) Zn²⁺-activation, Asp97 is not (n = 3–6).



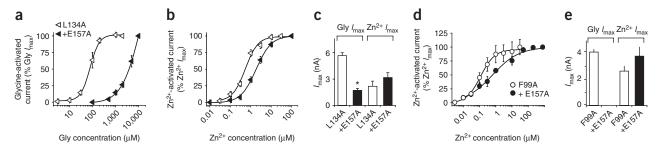


Figure 5 Zn²⁺ activation of GlyRs lacking a high-affinity glycine binding site. (a,b) Glycine (a) and Zn²⁺ (b) concentration-response curves on an α 1^{L134A} background with an extra mutation in the glycine binding site (E157A). (c) Maximum currents evoked by glycine (10 mM) and Zn²⁺ (1 mM) for α1^{L134A} and α1^{L134A, E157A}. Incorporating E157A on an α1^{F99A} background ablated activation by concentrations of glycine up to 10 mM, but induced only a modest, three-fold decrease in sensitivity to Zn^{2+} activation (d) and no reduction in Zn^{2+} I_{max} (1 mM) (e) (n=3-4).

substitution of Zn²⁺ binding residues with non-Zn²⁺-coordinating alanines at either the Zn²⁺-inhibition or Zn²⁺-potentiation sites revealed that neither site was required for Zn2+ activation (Supplementary Fig. 4 online). Given that Zn2+ inhibition was severely compromised in ZAGs, we reasoned that regions bordering the inhibition site may have become structurally perturbed, sufficient to form a new Zn²⁺-activation site.

On an $\alpha 1^{L134A}$ ZAG background (most Zn²⁺-sensitive ZAG), potential Zn²⁺-coordinating residues neighboring the Zn²⁺-inhibition site were substituted with alanine and assessed for activation by 0.1 μ M Zn²⁺ (EC₇₀ for α 1^{L134A}; **Fig. 4a,b**). Of these substitutions, Asp84, Asp86 (strand β3) and Asp97 (loop A) virtually abolished Zn^{2+} activation from 38 \pm 9% (α 1^{L134A}) to 5 \pm 1% (α 1^{L134A} D84A) and 2.4 \pm 1.2% ($\alpha 1^{L134A}$ D86A), with no detectable activation for $\alpha 1^{L134A~D97A}$ (Fig. 4c). The Zn^{2+} $EC_{50}s$ for $\alpha 1^{L134A~D84A}$ and α1^{L134A} D86A were increased 120-fold and 15-fold, respectively (Fig. 4c, inset), whereas glycine EC50s were shifted only two-fold (Supplementary Table 3 online). Notably, using a wild-type receptor background, the substitutions D84A, D86A or D97A caused only

inhibiting leak currents for $\alpha 1^{F99A}$ and $\alpha 1^{L134A}$ ZAGs (c) and

EC₅₀ glycine-activated current (d). The orange line indicates

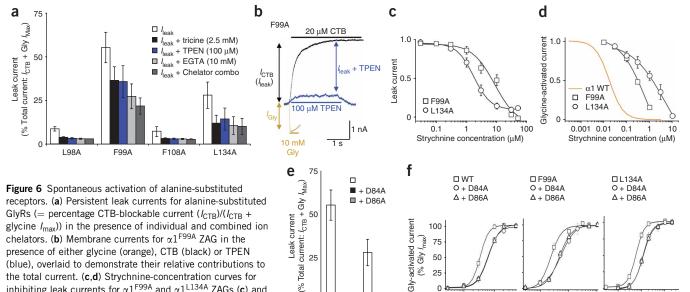
modest (<two-fold) changes in GlyR sensitivity to Zn²⁺ inhibition and potentiation (Supplementary Fig. 5 and Supplementary Table 4 online).

According to the GlyR homology model (Fig. 4a), Asp84 and Asp86 are positioned approximately 17 Å from Asp97, too far for the three residues to coordinate a single Zn²⁺ ion³². Furthermore, Asp97, which is conserved across the Cys-loop receptor family, probably supports loop B via the carboxyl side chain³³, precluding its involvement in Zn²⁺ binding. To establish the importance of Asp84, Asp86 and Asp97 for Zn²⁺ activation, alanine substitutions were also made on an alternative ZAG background, α1^{F99A} (the most efficacious ZAG). Substituting Asp84 or Asp86 again substantially reduced the sensitivity to Zn²⁺ activation, but substituting Asp97 was ineffective (Fig. 4d). Thus, the role of Asp97 in Zn²⁺ activation is more complex than can be explained by just its direct binding of Zn²⁺.

Zn²⁺-activation site is not a potentiation site

As reagents and water are ubiquitously contaminated with glycine (\sim 50 nM³⁴), it is feasible that Asp84 and Asp86 may actually form





the strychnine-concentration inhibition curve for wild-type GlyRs, which are notably more sensitive as the agonist binding loops A and D are not perturbed. (e) Persistent leak current is absent from receptors with alanine substitutions at Asp84 or Asp86 on $\alpha 1^{F99A}$ and $\alpha 1^{L134A}$ backgrounds. (f) Glycine sensitivity is modestly reduced in alanine-substituted Asp84 or Asp86 receptors, as compared to wild type, $\alpha 1^{F99A}$ and $\alpha 1^{L134A}$ backgrounds (n = 3-6).

F99A

L134A

%

10

100

100

1,000

100 1,000



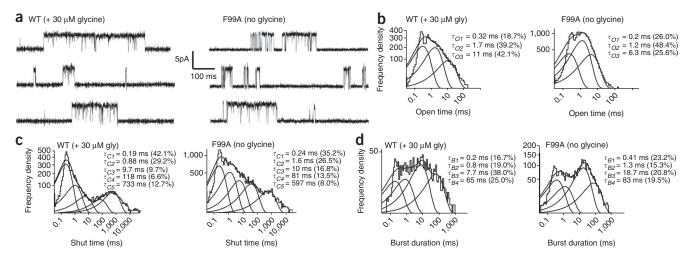


Figure 7 Spontaneous channel activation mimics agonist-induced activation. (a) Single channel currents from cell-attached recordings of HEK cells expressing either wild-type (WT) $\alpha 1$ GlyRs or GlyR $\alpha 1^{F99A}$, at a pipette potential +60 mV. Burst activity was recorded in the presence of glycine (EC₃₀) for wild type and in its absence for spontaneously activated $\alpha 1^{F99A}$. TPEN (100 μ M) was present in the pipette solution to remove contaminating Zn²⁺. (b–d) Dwell-time distributions for open times (b), shut times (c) and burst durations (d).

part of a (second) Zn2+-potentiation site, rather than an activation site. Occupancy of this site by Zn²⁺ would then enhance the receptor's sensitivity to glycine, allowing activation by low contaminating glycine concentrations. To address this, an α1^{L134A} ZAG background was used with an extra mutation, E157A on glycine binding loop B35, to produce a receptor with a 50-fold-reduced sensitivity to glycine. Using this mutant, the threshold concentration for glycine was now $> 100 \mu M$ (Fig. 5a) and 2,000-fold higher than the predicted level of glycine contamination. Nevertheless, the $\alpha 1^{L134}$ E157A ZAG showed only a modest three-fold reduced sensitivity to Zn2+ activation (Fig. 5b) and retained comparable maximal responses to Zn²⁺ (**Fig. 5c**). Using an alternative F99A background, $\alpha 1^{\overline{F99}A}$ E157A showed identical Zn²⁺ sensitivity to α1^{F99A}, despite being insensitive up to 10 mM glycine (Fig. 5d,e). Thus, in the absence of glycine-mediated activation, Zn²⁺ activation is still apparent, suggesting that it originates from a pure activation site, not an additional Zn²⁺-potentiation site.

ZAGs show spontaneous channel activity

HEK cells expressing the most sensitive ZAGs, $\alpha 1^{\text{L98A}}$, $\alpha 1^{\text{F99A}}$, $\alpha 1^{\text{F108A}}$ and $\alpha 1^{\text{L134A}}$, all showed sizable (0.5–3 nA) leak currents. A minor component was caused by Zn²⁺ contamination of the external solution (\sim 200 nM³⁶) activating the ZAGs, as this was reduced by the Zn²⁺ chelators, tricine (2.5 mM) or *N*,*N*,*N'*,*N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN (100 μM); **Fig. 6a,b**). The remaining component depended on spontaneous GlyR channel activity, because it was abolished by CTB (20 μM) to less than 50 pA standing current (considered full abolition of receptor-mediated leak). Strychnine also attenuated the leak, by 100% for ZAG $\alpha 1^{\text{F99A}}$ and by 90 ± 4% for $\alpha 1^{\text{L134A}}$. Strychnine was ten-fold less potent in inhibiting the leak current compared to glycine-activated currents (**Fig. 6c,d**).

Notably, Asp84 and Asp86, which are important for Zn^{2+} activation, were also required for spontaneous activation, with $\alpha 1^{F99A\ D84A}$, $\alpha 1^{F99A\ D86A}$, $\alpha 1^{L134A\ D84A}$, and $\alpha 1^{L134A\ D86A}$ failing to show spontaneous channel activity (**Fig. 6e**). Furthermore, Asp84 and Asp86 also influenced glycine-induced receptor activation, as alanine substitutions induced a modest but consistent two-fold reduction in glycine sensitivity in wild-type, $\alpha 1^{F99A}$ and $\alpha 1^{L134A}$ backgrounds

(Fig. 6f and Supplementary Table 4). A double-substituted receptor, $\alpha 1^{D84A~D86A}$, was nonfunctional.

To investigate whether spontaneous activity mimics agonist-induced activity, we recorded single-channel currents in cell-attached mode (pipette potential +60 mV) for wild-type channels activated by glycine (20 $\mu M;~EC_{30})$ and for spontaneously gating $\alpha 1^{F99A}$ ZAGs without glycine. TPEN (100 $\mu M)$ was present throughout to remove any activation by contaminating Zn²+. The single-channel currents for each receptor population were comparable at 4–5 pA, with estimated conductances of $\sim\!60~pS^{37}$ (Fig. 7a). The corresponding open-time distributions were best fit by three Gaussian components with similar

Table 2 Single-channel analyses for wild-type and F99A mutant GlyRs

	α1 wild type (+30	μM glycine)	α1 ^{F99A} (no glycine)			
Open times	τ_{O} (ms)	Area (%)	τ_{O} (ms)	Area (%)		
1	0.28 ± 0.029	36 ± 11	0.4 ± 0.1	36.9 ± 9.3		
2	1.5 ± 0.2	39 ± 3	1.7 ± 0.3	41.9 ± 4.0		
3	8.9 ± 2.1	26 ± 10	5.2 ± 0.8	21.2 ± 7.2		
Closed times	τ_C (ms)	Area (%)	$\tau_{\mathcal{C}}$ (ms)	Area (%)		
1	0.22 ± 0.03	44 ± 16	0.26 ± 0.026	37 ± 2		
2	0.90 ± 0.24	35 ± 15	1.6 ± 0.17 *	30 ± 3		
3	4.3 ± 1.6	8 ± 1	10.0 ± 2.9	18 ± 1*		
4	109 ± 6.2	10 ± 2	89 ± 39	12 ± 1		
5	$2,200 \pm 1,000$	4 ± 1	$1,200 \pm 400$	3 ± 3		
Burst durations	τ_B (ms)	Area (%)	τ_B (ms)	Area (%)		
1	0.3 ± 0.06	28 ± 7	0.3 ± 0.06	26 ± 2		
2	1.4 ± 0.6	22 ± 4	1.4 ± 0.1	22 ± 8		
3	6.9 ± 2.2	35 ± 5	17.6 ± 0.8 *	32 ± 8		
4	63.4 ± 14.8	19 ± 7	77.1 ± 10.7	20 ± 1		
Amplitude (pA)	4.5 ± 0	.7	4.1 ± 0.6			
P_{O}	0.9 ± 0.0	03	0.53 ± 0.07			

Data from cell-attached single-channel recordings made from $\alpha 1$ wild-type or $\alpha 1^{F99A}$ receptors expressed in HEK 293 cells. Includes average durations of open, closed and burst time constants and areas of exponential components that fitted the distributions, single channel amplitudes, P_0 for openings within bursts (n=3). *Significant variations between the two receptor populations (P<0.05).

mean time constants and relative areas (P>0.05; **Fig. 7b** and **Table 2**). The shut-time distributions required five Gaussian components, giving similar time constants (τ) for both receptors, with the exception of the shut-time constants $\tau_{\rm C2}$ and $\tau_{\rm C3}$, which were two-fold higher for $\alpha 1^{\rm F99A}$ receptors (**Fig. 7c** and **Table 2**). These changes will contribute to the lower open probability ($P_{\rm O}$) for clusters of openings at $\alpha 1^{\rm F99A}$ (0.53 \pm 0.07) compared to wild-type (0.9 \pm 0.03, n=3) channels. With regard to the burst-duration distributions, four Gaussian components were required with comparable time constants and relative areas, except for $\tau_{\rm B3}$, which was two-fold longer for $\alpha 1^{\rm F99A}$ (**Fig. 7d**).

The '-' face affects Zn²⁺ activation and spontaneous activity

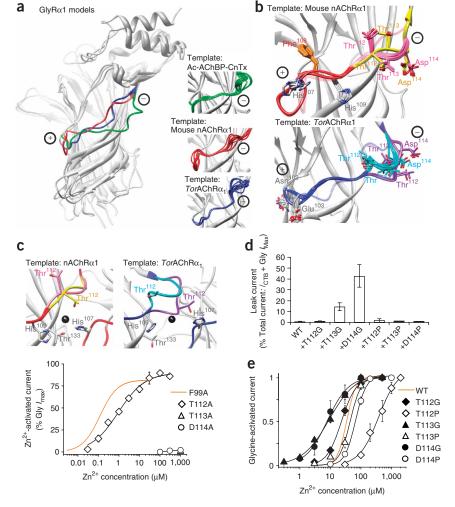
Conceivably, Zn²⁺ activation and spontaneous channel activity may arise if the substitutions of hydrophobic residues exert a common conformational effect on a region that undergoes crucial movement during channel gating. As the Zn²⁺-inhibition site is perturbed in ZAGs, it is the ideal region to examine for conformational flexibility. The top ten GlyR homology models (lowest distant-dependent

atomic statistical potential (DOPE)³⁸ score from 100 models run in MODELLER-9.2) based on three related template structures, conotoxin-bound Ac-AChBP, *Tor*nAChR α_1 and mouse nAChR α_1 (refs. 1,14,27), showed much greater structural variability at the '+' and '–' loop faces surrounding the β 5 strand of the Zn²⁺-inhibition site, compared to other more rigid β -strands and across the structure as a whole (**Fig. 8a**, insets, and **Supplementary Table 5** online). By using DOPE loop modeling to optimize the structures of the GlyR '+' and '–' face loops, using *Tor*nAChR α_1 and mouse nAChR α_1 as templates, the ten best conformations for the '+' loops were all comparable (**Fig. 8b**), whereas, for the '–' face loops, variable conformations were equally favored with residues showing multiple orientations at this location (**Fig. 8b** and **Supplementary Table 6** online).

To corroborate the modeling data, polar residues Thr112, Thr113 and Asp114 on the apex of the '–' face, the point of greatest variability between DOPE loop-fitted structures, were individually substituted with alanine and examined for Zn²⁺ activation and spontaneous activity. Although T112A yielded a highly sensitive and efficacious

Figure 8 Zn²⁺ and spontaneous channel activation originates via the $\mathrm{Zn}^{2+}\text{-inhibition}$ site '-' loop face. (a) Combinatorial extension structural alignments⁵⁵ of GlyR α1 homology models based on three predicted 'closed' conformation templates. Note the structural variability at the Zn^{2+} -inhibition site '+' and '-' faces between the conotoxin (CnTx)-bound Ac-AChBP²⁷ (green), mouse nAChR α1 (ref. 14; red) and TornAChR α_1 (ref. 1; blue) templates. Insets, overlays of the ten lowest-energy models for each template reveal particular uncertainty over the fitting of the '-' face but less uncertainty for the '+' face. (b) Ten lowest-energy loop conformations resulting from DOPE loop refinement³⁸ of the '+' and '-' Zn²⁺-inhibition site loops starting from the best GlyR $\alpha 1$ model based on two different templates. This revealed one favorable conformation for the '+' face loop for each template-derived model, with only minor deviations in the peptide backbone (red) and side chain positions between models. In contrast, numerous peptide backbone conformations were equally preferred for the '-' face loop (pink or yellow for mouse nAChR α1; aquamarine or purple for $\textit{Tor}\, nAChR \ \alpha_1)$ with clear divergence in side chain positions. Original model backbone conformations before loop refinement are shown in translucent red (mouse nAChR α 1) and translucent blue (TornAChR α_1). (c) Zn²⁺ activation-response curves from GlyRs with potential Zn²⁺-coordinating residues on the '+' and '-' inhibition site faces substituted with alanines; Zn^{2+} maxima are normalized to glycine maximal current (10 mM) in the same cell. Only $\alpha \mathbf{1}^{T112A}$ showed highly sensitive and efficacious ZAG activity. ZAG $\alpha 1^{F99A}$ (orange line; originally from Fig. 2c) is included for comparison. Insets, DOPE loop refinement of GlyR α1 models from

mouse nAChR $\alpha 1$ and TornAChR α_1 templates



show how alternative favorable conformations on the '-' face loop could have a substantive impact on the organization of Thr112 in the Zn²⁺ binding site. Zn²⁺ was fitted manually (dark gray, spacefill). (**d**) Significant spontaneous activity, measured as persistent leak currents ($I_{CTB}/(I_{CTB} + \text{glycine } I_{max})$) in the presence of 100 μ M TPEN was observed for GlyR α 1^{T113G} and α 1^{D114G}, where '-' loop flexibility was increased, but not for GlyR α 1^{T113P} and α 1^{D114P}, where flexibility was reduced. (**e**) Glycine concentration-response curves for receptors with glycine or proline substitutions at positions 112, 113 or 114 in the '-' loop. Glycine increased agonist sensitivity compared to proline substitution at the equivalent position. Glycine curve for wild-type receptors included for comparison (orange line; n=3-7).

gdu

ZAG (Fig. 8c), no single alanine substitution generated a spontaneously active receptor (data not shown). We altered the apex flexibility of the '-' face by individually substituting Thr112, Thr113 and Asp114 with either glycine to increase or proline to reduce backbone flexibility^{39,40}. Whereas the proline-substituted receptors lacked spontaneous activity, two glycine-substituted receptors, $\alpha 1^{\text{T113G}}$ and $\alpha 1^{\text{D114G}}$, showed 15 \pm 4% and 43 \pm 10% (n=4–6) spontaneous activity, respectively (Fig. 8d). Furthermore, there was a three-fold increase in glycine sensitivity for $\alpha 1^{T113G}$ (EC₅₀ = 9 ± 2 μM) and $\alpha 1^{\mathrm{D114G}}$ (EC₅₀ = 10 \pm 3 μM) compared to wild type (EC₅₀ = 35 \pm 5 μ M; n = 4–6), whereas the proline-substituted receptors all showed reduced sensitivities to glycine (Fig. 8e). Thus, increasing the flexibility of the '-' face around Thr112-Asp114 increased the propensity of GlyR to open spontaneously and in response to agonist binding, whereas decreasing flexibility by proline substitution had the opposite effect.

DISCUSSION

This study identifies a scaffold of hydrophobic residues in GlyR that functionally link glycine binding loops A and D with the Zn²+ binding $\beta 5$ and $\beta 6'$ strands of the Zn²+-inhibition site. Exchanging the hydrophobic residues, but not others outside the scaffold, initiated spontaneous channel opening, severely attenuated Zn²+ inhibition and enabled Zn²+ to act as a novel activator of GlyRs. This suggests that the hydrophobic scaffold is pivotally involved in receptor activation by stabilizing one or more closed GlyR conformations. This is achieved by regulating the '–' loop face of the Zn²+-inhibition site, as specific substitutions of polar residues in the '–' face produced receptors with the same properties to those generated by alanine substitutions in the hydrophobic scaffold.

Structurally linking two discrete ligand binding sites

The current view of Cys-loop receptor activation is that agonist binding at the interface between subunits induces a rearrangement of interacting residues, allowing the ECDs to twist relative to one another. The newly orientated loops at the bases of the ECDs then promote rearrangement of opposing transmembrane domains to open the channel^{6,16,18,41–44}. By binding to its interfacial inhibitory site on GlyRs, Zn²⁺ stabilizes subunit interfaces, preventing the ECDs from twisting and initiating activation. It is therefore plausible that by distorting the Zn²⁺ binding '-' loop interface we will not only ablate Zn²⁺ inhibition, but also enable spontaneous channel activity, particularly if the distortion mimics the conformation that occurs in the activated receptor state. Thus, the attenuation of Zn²⁺ inhibition and appearance of spontaneous channel activation are intrinsically linked. The extent to which both properties are seen in mutated receptors will depend on the degree to which each substitution perturbs the '-' loop away from a closed and toward an activated conformation.

Notably, the molecular pathway by which Zn^{2+} causes inhibition is entirely different to that for Zn^{2+} potentiation at GlyRs. The potentiation site resides close to the Cys-loop, where it may interact directly with Thr151 to facilitate channel gating²³. This negates the need for any interaction with the hydrophobic scaffold identified here, explaining why Zn^{2+} potentiation was unaffected in this study.

The molecular pathway identified here is the first to be described in a Cys-loop ligand-gated ion channel that functionally connects two distinct binding sites, linking the agonist binding site to downstream activation. The importance of the hydrophobic scaffold in mediating GlyR activation is emphasized by the common kinetics of spontaneously active $\alpha 1^{F99A}$ and agonist-activated wild-type GlyRs. Specifically, for $\alpha 1^{F99A}$, it is the alanine substitution that artificially perturbs

loop A to induce activation, whereas, for wild-type GlyRs, it is presumably agonist binding that similarly perturbs loop A to cause activation. Although a crucial role for loop A in directing receptor activation is evident for $GABA_ARs^{45}$ and $nAChRs^{46}$, loop C, possibly via transmission of a conformational change along the outside of the ECDs (β 7, 9 and 10 strands), has also been suggested to mediate activation upon agonist binding^{8,10}. Our data do not preclude this scenario, but advocate loop A as an important contributor to the conformational wave that precedes channel opening⁴⁷.

At the glycine binding site, Phe99 seems ideally positioned to directly influence the receptor's sensitivity to glycine, possibly via a cation- π interaction⁴⁸. The action of Phe99 to induce GlyR activation in response to agonist binding may then be mediated via the hydrophobic scaffold and subsequent '-' loop face of the Zn²+inhibition site. Indeed, Phe99 probably does this via Leu98 and Phe100, which are predicted to face, opposite to Phe99, into the hydrophobic scaffold toward the residues supporting the '-' loop face. Such an interaction with Phe99 would explain why Phe100 could also influence the receptor's sensitivity to glycine (**Supplementary Fig. 6** and **Supplementary Table 2** online). The ability of Phe99 to influence important residues within the hydrophobic scaffold may explain why it produces the most efficacious ZAG and the most spontaneously active receptors when substituted with alanine.

From the perspective of the polar residues at the Zn²⁺ site's '-' loop face, substituting Thr112 or Ile111 produced a receptor that was insensitive to Zn²⁺ inhibition (see also refs. 25,31) and capable of Zn²⁺ activation. Ile111 faces into the core, in close proximity with the other residues that constitute the hydrophobic scaffold. Thus, Thr112, via Ile111, is ideally located to act as a relay following perturbation of the hydrophobic scaffold. Sequential substitution of Thr113 and Asp114 within the '-' loop by glycine, but not by alanine or proline, also yielded spontaneously active receptors. These residues must also be ideally located to respond to perturbations of the hydrophobic scaffold, with increased loop flexibility enabling the receptor to shift to an activated state, whereas imposed rigidity (for example, proline insertion or Zn²⁺ binding, which stabilizes this region) hinders receptor activation.

β5 loop movement during GlyR activation

Although we propose that the '-' loop face undergoes a conformational change to facilitate receptor activation, comparative structural evidence does not, so far, support this idea. Overlaying crystal structures of Aplysia californica AChBP bound to either α-conotoxin PnIA ('inactive conformation') or HEPES ('active conformation'²⁷) does not reveal any variation around the corresponding '-' loop face region in the GlyR model (Supplementary Fig. 7 online). Furthermore, structurally aligning TornAChR α_1 and α_2 subunits (presumed closed conformation) compared to β , δ and γ subunits (presumed open) for the pentamer reveals only a small degree of variability around the corresponding '-' loop face region (Supplementary Fig. 8 online). Of course, as static structures, it is possible that neither the HEPES-bound AChBP nor βδγ TorAChR subunits represent fully activated receptors. Alternatively, they might undergo different conformational rearrangements after activation compared to GlyRs. Simulation studies on nAChRs also do not support movements in the '-' face region⁴⁹, although the nanosecond timescales for these studies are as yet too short to encompass all conformational rearrangements in pentameric Cys-loop receptors.

Despite the caveats, the '-' loop face of the GlyR Zn²⁺-inhibition site was predicted to adopt numerous conformations and side chain orientations with the potential to influence receptor function.

Moreover, previous functional studies support a role for the '-' loop face in the GlyR-activation process: Thr112 is important in determining partial agonist efficacy⁵⁰; it is also accessible to cysteine-scanning mutagenesis, resulting in dynamic disruption to agonist-evoked responses³⁰; and Zn²⁺ binds between subunits at the '-' loop face to stabilize the GlyR closed conformation. These data suggest that this interface is mobile during receptor activation^{25,31}.

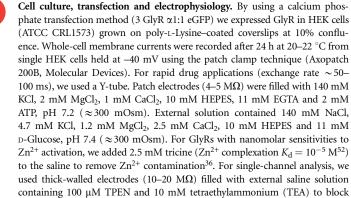
Creating a Zn2+-activation site

The Zn^{2^+} -activation site was localized to Asp84 or Asp86 on strand $\beta 3$, directly above the '–' loop face. Structural perturbation of the '–' loop face may therefore have a knock-on effect on the $\beta 3$ region, allowing Asp84 or Asp86 to form a previously undescribed Zn^{2^+} binding site that aids movement of the subunit interfaces, rather than hinders them, thereby inducing activation. This provides further evidence that charge dispersal at subunit interfaces has an important role in regulating Cys–loop receptor excitability 16,19,46,51 and also indicates that a dynamic interaction occurs between the '–' loop face and the $\beta 3$ strand to facilitate activation of wild-type receptors. The variable potency and efficacy of Zn^{2^+} at different ZAGs further indicates that hydrophobic residues within the scaffold differentially affect the '–' loop face and consequently the juxtaposed $\beta 3$ strand, so determining the efficiency with which Asp84 or Asp86 forms a new Zn^{2^+} -activation site.

The general activation mechanism presented here for GlyR is in accordance with the hydrophobic scaffold and '–' loop face dynamically responding to agonist binding. This provides a new vista on Cys–loop receptor activation, whereby during activation the reorganization of the hydrophobic scaffold and '–' face facilitates the realignment of the inner and outer β -sheets relative to one another 12,13,16,18 . This then initiates movement of subunit interfaces, which is subsequently transmitted to the transmembrane domains for receptor activation 42 .

METHODS

cDNA constructs and mutagenesis. We used human GlyR α 1L cDNA constructs, and mutant cDNAs were prepared using the Stratagene Quikchange kit. Mutated cDNAs were sequenced using an ABI sequencer.



Data acquisition and analysis. Membrane currents were filtered using a highpass Bessel filter at 3 kHz (-36 dB per octave) and series resistance compensation was routinely achieved up to 70%. Data were recorded in 20-s epochs directly to a Pentium IV, 3.5-GHz computer into Clampex 8.0 via a Digidata 1322A (Axon instruments) sampling at 200-µs intervals. Because of Zn^{2+} activation in many of the receptors, Zn^{2+} -inhibition profiles were measured by prolonged (4-s) co-application of Zn^{2+} with glycine, with response measure-

endogenous potassium channel activity. Single-channel recordings were made

in cell-attached mode at +60 mV pipette potential.

ments being taken at the 4-s time point (to allow Zn^{2+} inhibition sufficient time to reach equilibrium²⁶). We analyzed the digitized membrane-current records offline using Axoscope 8.2. The concentration-response relationships for glycine and Zn^{2+} were fitted with modified Hill equations as previously described²⁶.

For the single-channel data analyses, stored prefiltered (2.7-kHz Bessel) single-channel data were digitized at 33 kHz before analysis. A fixed time resolution based on the dead time of the system was set at 80 μ s. We analyzed the single-channel current amplitudes by fitting Gaussian components to the amplitude distributions to determine the mean single-channel current, s.d. and the total area of the component using a nonlinear least-squares fitting routine. Single-channel conductances were calculated from the mean unitary current and the difference between the patch potential and glycine response reversal potential. The patch potential was estimated in cell-attached recordings by estimating the cell-membrane potential.

All open and shut durations were measured with a 50% threshold cursor applied to the main single-channel current amplitude (WinEDR v2.8.9). The duration of events that were included in the analysis was not less than 200 µs before fitting the dwell-time histograms. Frequency distributions were constructed from the measured individual open and shut durations and analyzed by fitting a mixture of exponentials, defined by equation (1):

$$y(t) = \sum_{i=1}^{n} (A_i/\tau_i) \cdot \exp(-t/\tau)$$
 (1)

where A_i represents the area of the *i*th component to the distribution and τ_i represents the respective time constant. The areas, time constants and standard errors of the individual components of the distribution were determined. The burst-duration analysis required the determination of a critical shut time $(\tau_{\rm crit})^{53}$ determined between the shut-time constants, $\tau_{\rm C3}$ and $\tau_{\rm C4}$, by solving equation (2):

$$\exp(-t_{\text{crit}}/\tau_{\text{C3}}) = 1 - \exp(-t_{\text{crit}}/\tau_{\text{C4}}) \tag{2}$$

Channel-open probability ($P_{\rm O}$) was calculated as the percentage of time that the channel spent in the open state within a cluster. All statistical comparisons used an unpaired t-test, and P < 0.05 was considered significant.

Homology and loop modeling. We used ClustalW⁵⁴ to produce protein sequence alignments. Aplysia californica acetylcholine binding protein, Ac-AChBP (PDB 2BR8, the conotoxin-bound form²⁷), TornAChR α₁βδα₂γ (PDB 2BG9)1 and mouse nAChR α1 (PDB 2QC1)14 were used for the combinatorial extension structural alignment method⁵⁵, which helped identify divergent regions in the GlyR $\alpha 1$ model. The final alignment reflected both alignment strategies. The TornAChR α_1 subunit was selected as the final template structure to guide the homology modeling of the GlyR α subunit, as it has only two fewer residues around the β5 strand (the GlyR Zn²⁺inhibition site), compared to three fewer residues for Ac-AChBP; also, the structure of TornAChR α_1 was determined as part of a pentamer, whereas mouse nAChR α1 was crystallized as a nonphysiological monomer with several artificial point mutations¹⁴. The TornAChR α₁ pentamer was built by overlaying a second α_1 subunit over the α_2 subunit and then using Chimera⁵⁶ to build a five-fold symmetrical pentamer. Using MODELLER-9.2 (ref. 29), 100 human GlyR α1 models were generated with cysteine bridges added into the agonist binding loop C (C198-C209) for the principle TornAChR α₁ pentamer template and also for the Ac-AChBP conotoxin-bound pentamer and the mouse nAChR \alpha1 monomer. Side chain configurations were generated using SCWRL3 (ref. 57). Fifty loops were generated using DOPE loop modeling in Modeller 9.2, for each loop before ('+') and after ('-') the β5 strand for each of the subunit templates ('+' loop residues 102-NEKGAH-107; '-' loop residues 110-EITTDN-115). Models were evaluated using MolProbity⁵⁸ and gave good general agreement with each other. Uncertainty regarding the short β5 strand, ascribed as a β-strand in Ac-AChBP (PDB 1UW6) and nAChR α1 (PDB 2QC1) but not in TornAChR α₁ (PDB 2BG9), was considered unimportant, as it had little effect on side chain positioning, and a PSIPRED⁵⁹ secondary-structure prediction of the GlyR sequence gave low confidence for the presence of a β-strand, suggesting that neither template was more likely than the other to be correct. All three-dimensional images were prepared and rendered using Chimera⁵⁶.



Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Counsil, the Biotechnology and Biological Sciences Research Council and the Wellcome Trust. We thank A. Hosie, P. Thomas and M. Wilkins for helpful comments and H. Da Silva for technical assistance.

Published online at http://www.nature.com/nsmb/

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

- Unwin, N. Refined structure of the nicotinic acetylcholine receptor at 4 resolution. J. Mol. Biol. 346, 967–989 (2005).
- Brejc, K. et al. Crystal structure of an Ach-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411, 269–276 (2001).
- Chen, Y., Reilly, K. & Chang, Y. Evolutionarily conserved allosteric network in the Cys loop family of ligand-gated ion channels revealed by statistical covariance analyses. J. Biol. Chem. 281, 18184–18192 (2006).
- Chandler, D. Interfaces and the driving force of hydrophobic assembly. Nature 437, 640–647 (2005).
- Corringer, P.J., Le, N.N. & Changeux, J.P. Nicotinic receptors at the amino acid level. *Annu. Rev. Pharmacol. Toxicol.* 40, 431–458 (2000).
- Lester, H.A., Dibas, M.I., Dahan, D.S., Leite, J.F. & Dougherty, D.A. Cys-loop receptors: new twists and turns. *Trends Neurosci.* 27, 329–336 (2004).
- Sine, S.M. & Engel, A.G. Recent advances in Cys-loop receptor structure and function. Nature 440, 448–455 (2006).
- Law, R.J., Henchman, R.H. & McCammon, J.A. A gating mechanism proposed from a simulation of a human α7 nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* 102, 6813–6818 (2005).
- Celie, P.H. et al. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. Neuron 41, 907–914 (2004).
- Lyford, L.K., Sproul, A.D., Eddins, D., McLaughlin, J.T. & Rosenberg, R.L. Agonistinduced conformational changes in the extracellular domain of α7 nicotinic acetylcholine receptors. *Mol. Pharmacol.* 64, 650–658 (2003).
- 11. McLaughlin, J.T., Fu, J., Sproul, A.D. & Rosenberg, R.L. Role of the outer β -sheet in divalent cation modulation of $\alpha 7$ nicotinic receptors. *Mol. Pharmacol.* **70**, 16–22 (2006).
- McLaughlin, J.T., Fu, J. & Rosenberg, R.L. Agonist-driven conformational changes in the inner β-sheet of α7 nicotinic receptors. Mol. Pharmacol. 71, 1312–1318 (2007).
- Purohit, P. & Auerbach, A. Acetylcholine receptor gating: movement in the α-subunit extracellular domain. *J. Gen. Physiol.* 130, 569–579 (2007).
- 14. Dellisanti, C.D., Yao, Y., Stroud, J.C., Wang, Z.Z. & Chen, L. Crystal structure of the extracellular domain of nAChR $\alpha 1$ bound to α -bungarotoxin at 1.94 resolution. *Nat. Neurosci.* **10**, 953–962 (2007).
- Chakrapani, S., Bailey, T.D. & Auerbach, A. Gating dynamics of the acetylcholine receptor extracellular domain. J. Gen. Physiol. 123, 341–356 (2004).
- 16. Unwin, N., Miyazawa, A., Li, J. & Fujiyoshi, Y. Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the α subunits. *J. Mol. Biol.* **319**, 1165–1176 (2002).
- 17. McLaughlin, J.T., Fu, J. & Rosenberg, R.L. Agonist-driven conformational changes in the inner β-sheet of α7 nicotinic receptors. *Mol. Pharmacol.* **71**, 1312–1318 (2007).
- Taly, A. et al. Implications of the quaternary twist allosteric model for the physiology and pathology of nicotinic acetylcholine receptors. Proc. Natl. Acad. Sci. USA 103, 16965–16970 (2006).
- Mukhtasimova, N. & Sine, S.M. An intersubunit trigger of channel gating in the muscle nicotinic receptor. J. Neurosci. 27, 4110–4119 (2007).
- Hirzel, K. et al. Hyperekplexia phenotype of glycine receptor α1 subunit mutant mice identifies Zn²⁺ as an essential endogenous modulator of glycinergic neurotransmission. Neuron 52, 679–690 (2006).
- Bloomenthal, A.B., Goldwater, E., Pritchett, D.B. & Harrison, N.L. Biphasic modulation of the strychnine-sensitive glycine receptor by Zn²⁺. *Mol. Pharmacol.* 46, 1156–1159 (1994).
- Laube, B. et al. Modulation by zinc ions of native rat and recombinant human inhibitory glycine receptors. J. Physiol. (Lond.) 483, 613–619 (1995).
- Miller, P.S., Da Silva, H.M. & Smart, T.G. Molecular basis for zinc potentiation at strychnine-sensitive glycine receptors. J. Biol. Chem. 280, 37877–37884 (2005).
- Harvey, R.J., Thomas, P., James, C.H., Wilderspin, A. & Smart, T.G. Identification of an inhibitory Zn²⁺ binding site on the human glycine receptor α1 subunit. *J. Physiol.* (Lond.) 520, 53–64 (1999).
- Nevin, S.T. et al. Insights into the structural basis for zinc inhibition of the glycine receptor. J. Biol. Chem. 278, 28985–28992 (2003).
- Miller, P.S., Beato, M., Harvey, R.J. & Smart, T.G. Molecular determinants of glycine receptor αβ subunit sensitivities to Zn²⁺-mediated inhibition. *J. Physiol.* 566, 657–670 (2005).
- Celie, P.H. et al. Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an α-conotoxin PnIA variant. Nat. Struct. Mol. Biol. 12, 582–588 (2005).

- Fu, D.X. & Sine, S.M. Asymmetric contribution of the conserved disulfide loop to subunit oligomerization and assembly of the nicotinic acetylcholine receptor. *J. Biol. Chem.* 271, 31479–31484 (1996).
- Sali, A. & Blundell, T.L. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815 (1993).
- Han, N.L., Haddrill, J.L. & Lynch, J.W. Characterization of a glycine receptor domain that controls the binding and gating mechanisms of the β-amino acid agonist, taurine. J. Neurochem. 79, 636–647 (2001).
- Laube, B., Kuhse, J. & Betz, H. Kinetic and mutational analysis of Zn²⁺ modulation of recombinant human inhibitory glycine receptors. *J. Physiol. (Lond.)* 522, 215–230 (2000).
- Auld, D.S. Zinc coordination sphere in biochemical zinc sites. *Biometals* 14, 271–313 (2001).
- Cashin, A.L., Torrice, M.M., McMenimen, K.A., Lester, H.A. & Dougherty, D.A. Chemical-scale studies on the role of a conserved aspartate in preorganizing the agonist binding site of the nicotinic acetylcholine receptor. *Biochemistry* 46, 630–639 (2007).
- Lerma, J., Zukin, R.S. & Bennett, M.V. Glycine decreases desensitization of N-methylp-aspartate (NMDA) receptors expressed in *Xenopus* oocytes and is required for NMDA responses. *Proc. Natl. Acad. Sci. USA* 87, 2354–2358 (1990).
- 35. Grudzinska, J. *et al.* The β subunit determines the ligand binding properties of synaptic glycine receptors. *Neuron* **45**, 727–739 (2005).
- Wilkins, M.E. & Smart, T.G. Redox modulation of GABAA receptors obscured by Zn²⁺ complexation. *Neuropharmacology* 43, 938–944 (2002).
- 37. Beato, M., Groot-Kormelink, P.J., Colquhoun, D. & Sivilotti, L.G. Openings of the rat recombinant α 1 homomeric glycine receptor as a function of the number of agonist molecules bound. *J. Gen. Physiol.* **119**, 443–466 (2002).
- 38. Shen, M.Y. & Sali, A. Statistical potential for assessment and prediction of protein structures. *Protein Sci.* **15**, 2507–2524 (2006).
- Creighton, T.E. The energetic ups and downs of protein folding. Nat. Struct. Biol. 1, 135–138 (1994).
- Yaron, A. & Naider, F. Proline-dependent structural and biological properties of peptides and proteins. Crit. Rev. Biochem. Mol. Biol. 28, 31–81 (1993).
- Mukhtasimova, N. & Sine, S.M. An intersubunit trigger of channel gating in the muscle nicotinic receptor. J. Neurosci. 27, 4110–4119 (2007).
- Kash, T.L., Jenkins, A., Kelley, J.C., Trudell, J.R. & Harrison, N.L. Coupling of agonist binding to channel gating in the GABA(A) receptor. *Nature* 421, 272–275 (2003).
- Lee, W.Y. & Sine, S.M. Principal pathway coupling agonist binding to channel gating in nicotinic receptors. *Nature* 438, 243–247 (2005).
- Lummis, S.C. et al. Cis-trans isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. Nature 438, 248–252 (2005).
- Boileau, A.J., Newell, J.G. & Czajkowski, C. GABA_A receptor β₂ Tyr97 and Leu99 line the GABA-binding site. Insights into mechanisms of agonist and antagonist actions. *J. Biol. Chem.* 277, 2931–2937 (2002).
- Chakrapani, S., Bailey, T.D. & Auerbach, A. The role of loop 5 in acetylcholine receptor channel gating. J. Gen. Physiol. 122, 521–539 (2003).
- Grosman, C., Zhou, M. & Auerbach, A. Mapping the conformational wave of acetylcholine receptor channel gating. *Nature* 403, 773–776 (2000).
- Padgett, C.L., Hanek, A.P., Lester, H.A., Dougherty, D.A. & Lummis, S.C. Unnatural amino acid mutagenesis of the GABA_A receptor binding site residues reveals a novel cation–π interaction between GABA and β₂Tyr97. J. Neurosci. 27, 886–892 (2007).
- 49. Henchman, R.H., Wang, H.L., Sine, S.M., Taylor, P. & McCammon, J.A. Ligand-induced conformational change in the $\alpha 7$ nicotinic receptor ligand binding domain. *Biophys. J.* **88**, 2564–2576 (2005).
- Schmieden, V., Kuhse, J. & Betz, H. A novel domain of the inhibitory glycine receptor determining antagonist efficacies: further evidence for partial agonism resulting from self-inhibition. *Mol. Pharmacol.* 56, 464–472 (1999).
- Chang, Y. & Weiss, D.S. Site-specific fluorescence reveals distinct structural changes with GABA receptor activation and antagonism. *Nat. Neurosci.* 5, 1163–1168 (2002).
- Paoletti, P., Ascher, P. & Neyton, J. High-affinity zinc inhibition of NMDA NR1–NR2A receptors. J. Neurosci. 17, 5711–5725 (1997).
- Colquhoun, D. & Sakmann, B. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J. Physiol. (Lond.)* 369, 501–557 (1985).
- Thompson, J.D., Higgins, D.G. & Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680 (1994).
- Shindyalov, I.N. & Bourne, P.E. Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng.* 11, 739–747 (1998)
- Pettersen, E.F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
- Canutescu, A.A., Shelenkov, A.A. & Dunbrack, R.L., Jr. A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci.* 12, 2001–2014 (2003).
- Davis, I.W. et al. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res. 35, W375

 –W383 (2007).
- Jones, D.T. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202 (1999).

