Cys-loop neurotransmitter-gated ion channels are vital for communication throughout the nervous system. Following activation, these receptors enter into a desensitized state in which the ion channel shuts even though the neurotransmitter molecules remain bound. To date, the molecular determinants underlying this most fundamental property of Cys-loop receptors have remained elusive. Here we present a generic mechanism for the desensitization of Cys-loop GABA<sub>A</sub> (GABA<sub>A</sub>Rs) and glycine receptors (GlyRs), which both mediate fast inhibitory synaptic transmission. Desensitization is regulated by interactions between the second and third transmembrane segments, which affect the ion channel lumen near its intracellular end. The GABA<sub>A</sub>R and GlyR pore blocker picrotoxin prevented desensitization, consistent with its deep channel-binding site overlapping a physical desensitization gate.
he presynaptic release of neurotransmitters is a prelude to their diffusion across the synaptic cleft and subsequent activation of postsynaptic ionotropic receptors, which are responsible for fast chemical neurotransmission. Agonist binding initiates the rapid opening of ligand-gated ion channels permitting a selective flow of ions through the pore. The sustained presence of the neurotransmitter will cause ligand-gated channels to transit from the active open-channel agonist-bound conformation to a desensitized shut-channel, agonist-bound state, thereby limiting current flow\(^2\). Receptor desensitization is a fundamental property of most ligand-gated ion channels and can have profound physiological consequences. These include the progressive reduction of postsynaptic current on repetitive synaptic neurotransmitter release\(^5\); the pre-desensitization of receptors by low ambient concentrations of neurotransmitter due to spillover from active neighbouring synapses\(^6\); and the slowing of synaptic current decays leading to their prolongation\(^7\).

In the central nervous system, neuronal activity results from a balance between excitation and inhibition. This is largely dictated by the activity of excitatory ionotropic glutamate receptors, and inhibition caused by GABA and glycine\(^7\) activating GABA\(_A\) (GABA\(_A\)Rs) and glycine receptors (GlyRs). These latter receptors belong to the superfamily of pentameric Cys-loop receptors that also comprises excitatory nicotinic acetylcholine (nAChRs) and serotonin receptors (5HT\(_3\)Rs)\(^8\). Differential desensitization kinetics of excitatory and inhibitory receptors has the potential to profoundly affect the spike firing profiles of neurons\(^9\) and thus neural network activity. At the molecular level, desensitization of AMPA and kainate classes of ionotropic GluRs is relatively well understood and involves structural rearrangements at the dimer interface between adjacent extracellular agonist-binding domains\(^8\)\(^\text{-}\)\(^11\). However, for the Cys-loop GABA\(_A\)Rs and GlyRs, although the structure–function studies have improved our understanding of how these receptors activate\(^7\)\(^\text{-}\)\(^12\), the molecular determinants underlying the process of desensitization have yet to be defined.

Here we show that for the inhibitory neurotransmitter GABA\(_A\) and glycine receptors, the mechanism of agonist-induced desensitization is regulated by residue interactions between the transmembrane domains of the receptor, resulting in a time-dependent constriction of the ion channel pore that reduces the agonist-activated membrane conductance. This constriction has the characteristics of a desensitization ‘gate’ that is discrete from the ion channel gate involved in ligand-gated channel opening.

**Results**

Desensitization profiles differ between GABA\(_A\) receptors. To locate the molecular components responsible for desensitization, we selected two GABA\(_A\)R isoforms that differ markedly in their determinants underlying the process of desensitization have yet to be defined. Here we show that for the inhibitory neurotransmitter GABA\(_A\) and glycine receptors, the mechanism of agonist-induced desensitization is regulated by residue interactions between the transmembrane domains of the receptor, resulting in a time-dependent constriction of the ion channel pore that reduces the agonist-activated membrane conductance. This constriction has the characteristics of a desensitization ‘gate’ that is discrete from the ion channel gate involved in ligand-gated channel opening.

**Internal end of M3 and M1–M2 linker control desensitization.** We refined our search using homomeric \(\rho_1\) receptors and sequentially replacing individual transmembrane segments, M1 to M3, with those from \(\rho_2\). These constructs yielded very small currents, precluding analysis; however, one chimera incorporating the intracellular end of M3, the M3–M4 intracellular linker and M4 of \(\rho_1\) into the \(\rho_2\) subunit (\(\rho_1\)346–M1), produced robust currents, which strongly desensitized (%Des = 94\% and \(\tau_W = 200\) ms, Fig. 1b).

Surprisingly, incorporating just M4 from the \(\rho_2\) subunit did not affect the desensitizing phenotype of \(\rho_1\) (\(\rho_1\)433–\(\rho_2\)). We therefore targeted M3 in \(\rho_1\) and replaced six contiguous residues at the carboxyl-terminal (C-terminal; intracellular) end of M3 with the homologous residues from \(\rho_1\). This produced a receptor (\(\rho_1\)21[\(\Delta\)postM3]) that desensitized profoundly within one second following activation by GABA (Fig. 1b). Within this six amino-acid cassette, substituting just one residue, \(\rho_1\)T349K, was sufficient to confer a rapid desensitizing profile on \(\rho_1\) receptors (Fig. 1b).

Interestingly, on the \(\rho_1\)21[\(\Delta\)postM3] background, incorporating the M1–M2 intracellular linker from the \(\beta_2\) subunit considerably reduced desensitization towards that for wild-type \(\rho_1\) (chimera \(\rho_1\)B2[M1–M2 link] + \(\Delta\)postM3). Such a reversion was not observed after incorporating the \(\rho_1\) M1–M2 linker (Supplementary Fig. 4). These data indicate that the intracellular end of M3 and the M1–M2 linker of \(\beta_2\) modulate desensitization, conceivably via an intersubunit (\(\alpha\)–\(\beta\)) interaction, which is consistent with the close proximity of \(\alpha_1\) M3 C-terminal end to the M1–M2 linker of an adjacent \(\beta_2\) subunit, as seen in our structural models (see below).

We subsequently examined this potential interaction in \(\beta_1\)2B2GABA\(_A\)Rs by first introducing the M1–M2 linker of \(\rho_1\) into \(\beta_2\) subunits (\(\rho_2\)\(\beta_1\)(M1–M2 link)). Co-expressing this chimera with wild-type \(\beta_1\) produced receptors that, unexpectedly, desensitized almost completely at a rate 50-fold faster than wild-type \(\beta_1\) receptors (Fig. 1c). Moreover, exchanging eight residues from the intracellular end of M3 in \(\rho_1\) and \(\beta_2\) with those from \(\rho_2\) resulted in a modest three-fold increase in the desensitization rate for \(\rho_1\)\(\beta_1\)(\(\Delta\)postM3)\(\beta_2\)\(\rho_1\)(\(\Delta\)postM3) (Fig. 1c). However, on this background, reintroducing the M1–M2 linker of \(\rho_1\) into \(\beta_2\) supported large GABA currents with limited desensitization, comparable to the profile for wild-type \(\rho_1\) (Fig. 1a, see Table 1 for all time constants and extents of desensitization data and Supplementary Table 1 for GABA current amplitudes), thereby locating the main determinants of desensitization to the transmembrane and/or intracellular regions of the receptor.
Table 1 | Weighted decay time constant for desensitization ($\tau_w$) and extent of desensitization (% Des) of wild-type and mutant GABA$_A$ and glycine receptor constructs.

| Construct | $\tau_w$ (s) | % Des | n | Construct | $\tau_w$ (s) | % Des | n |
|-----------|-------------|-------|---|-----------|-------------|-------|---|
| $\alpha_1\beta_2$ wt | 17.4 ± 3.6 | 86.4 ± 2.6 | 17 | $\alpha_1\beta_2^V$ | 2.5 ± 0.8 | 97.5 ± 0.8 | 5 |
| $\rho_1$ wt | 23.3 ± 7.9 | 19 ± 13 | 13 | $\alpha_1\beta_2^V$ | 1.3 ± 0.4 | 93.5 ± 12 | 4 |
| $\rho_1\alpha_{260}$ | 8.1 ± 0.5 | 90.7 ± 0.3 | 3 | $\alpha_1\beta_2^V$ | 0.78 ± 0.20 | 99.0 ± 0.4 | 6 |
| $\rho_1\alpha_{346}$ | 0.2 ± 0.04 | 93.6 ± 2.4 | 6 | $\alpha_1\beta_2^V$ | 0.75 ± 0.06 | 97.4 ± 0.5 | 5 |
| $\rho_1\alpha_{343}$ | 28.2 ± 2.6 | 11 ± 4 | 3 | $\alpha_1\beta_2^V$ | 11.4 ± 1.0 | 90.8 ± 0.4 | 4 |
| $\beta_2$ | 1.0 ± 0.4 | 72.2 ± 4.2 | 9 | $\alpha_1\beta_2^M$ | 1.2 ± 0.4 | 97.9 ± 0.9 | 4 |
| $\rho_1\alpha_{343}$ | 5.0 ± 3.7 | 58.6 ± 8.0 | 9 | $\alpha_1\beta_2^M_3$ | 0.19 ± 0.03 | 99.6 ± 0.1 | 7 |
| $\beta_2$ | 10.5 ± 3.3 | 25.3 ± 8.6 | 10 | $\alpha_1\beta_2^M_3$ | 4.4 ± 0.1 | 97.9 ± 0.4 | 4 |
| $\beta_2$ | 22.9 ± 4.0 | 24.3 ± 10.9 | 4 | $\alpha_1\beta_2^M_2$ | 0.41 ± 0.12 | 87.1 ± 4.1 | 4 |
| $\beta_2$ | 15.5 ± 3.2 | 79.8 ± 4.5 | 8 | $\alpha_1\beta_2^M_2$ | 17.4 ± 2.5 | 98.7 ± 0.2 | 7 |

% Des, % desensitization; wt, wild type.
Values are means ± s.d., n is the number of cells recorded for each construct.

M2 and M3 interface strongly affects GABA$_A$R desensitization.

While the identified cassette critically modulates desensitization, it is not the sole determinant. Indeed, exchanging the entire transmembrane portion of M3 for $\alpha_1$ and $\beta_2$ with $\rho_1$, virtually eliminated desensitization when these two chimeric subunits were co-expressed ($\alpha_1\beta_2^M_2$, Supplementary Fig. 5). We therefore compared the primary sequences for M3, and noted that the residue homologous to V338 in $\alpha_1$ was minimally affected. For wild-type receptors (Fig. 3), indicating the gating efficacy of the homologous residue homologous to V338 in $\alpha_1$. Conservatively exchanging these residues for valines ($\alpha_1\beta_2^V$, $\beta_2$), produced receptors that desensitized 2.5-fold faster than the wild type, while mutation to bulky hydrophobic valines ($\alpha_1\beta_2^V$) increased the desensitization rate by 14-fold (Fig. 2a) without changing the GABA concentration–response curve (Fig. 3).

We next assessed the importance of the highly conserved M3 valine ($\alpha_1\beta_2^V$), which is located one $\alpha$-helical turn above $\alpha_1$ (Supplementary Fig. 6). Together these residues effectively straddle the $\alpha$-helix (G258), in M2 (Fig. 2b). Significantly, their mutation to leucines in $\alpha_1\beta_2^M_2$ strongly disrupted desensitization, further suggesting that rearrangements to the lower part of the M2/M3 transmembrane interface underlies the process of receptor desensitization (Fig. 2b).

Replacing this conserved glycine with neutral alanine in $\alpha_1$ and the $\beta_2$ subunits ($\alpha_1\beta_2^G$) produced receptors that desensitized 2.5-fold faster than the wild type, while mutation to bulky hydrophobic valines ($\alpha_1\beta_2^V$) increased the desensitization rate by 14-fold (Fig. 2a) without changing the GABA concentration–response curve (Fig. 3).

Exploring this interface further, we examined the consequences of mutating $\alpha_1\beta_2$ and $\alpha_1\beta_3$. Valine 251 is located at the –3 position in intracellular M2, bordering the M1–M2 linker, and facing M3 at the level of $\alpha_1\beta_2$ (Fig. 2a). Notably, this was achieved with very little change in the GABA concentration–response curve for $\alpha_1\beta_2^{251V}$ compared with that for wild-type receptors (Fig. 3), indicating the gating efficacy was minimally affected.

To understand how M3 residues could affect receptor function, we constructed a three-dimensional (3D) model of the $\alpha_1\beta_2$ GABA$_A$R (Fig. 2a) based on the structure of the C. elegans glutamate-gated Cl$^-$ channel (GlucI) in the open conformation14, which shares high sequence homology with GABA$_A$Rs and GlyRs (Supplementary Fig. 6). In this model, the side chain of the M3 leucine ($\alpha_1\beta_2$) is oriented towards M2 near the 4' position (Fig. 2b; Supplementary Fig. 7), and therefore any conformational changes to M3 may be detected in M2 by a proximal glycine residue ($\alpha_1\beta_2^G$) that is highly conserved in all GABA$_A$Rs and GlyRs (Supplementary Fig. 6).
Figure 1 | Intracellular end of M3 and the M1–M2 linker control desensitization of GABAARs. (a–c) Peak-scaled membrane currents elicited by 10 mM GABA (black line), showing the desensitization phase for the indicated receptor constructs (left column). The recovery phase is omitted for clarity. See Table 1 for values of $\tau_w$ and extent of desensitization (% Des) in all figures. The subunit chimeras are depicted by a colour code: green (a1), red (b2) and grey (p1) (right column). Numbering refers to the position of the interface between the two subunits in the chimera. TM/M, transmembrane domain, INT, intracellular loops.

To investigate the importance of some of the key residues at the M2–M3 interface, and to ensure that the use of Xenopus oocytes was not altering the desensitizing profiles of the GABA-activated currents, we explored the impact of several receptor mutants when expressed in HEK cells. As observed with the oocyte-based experiments, the expression of a1V251F/b2 generated currents that desensitized faster, by ~10-fold compared with those for the a1b2 wild type (Supplementary Fig. 8, Supplementary Table 3). Again, similar current profiles were evident by co-expressing the nearby residues, a1N307V with the homologous b2N303V, in HEK cells giving a sevenfold increase in the desensitization rate (Supplementary Fig. 8, Supplementary Table 3).

γ2 subunit M2 and M3 affects a1b2γ2 GABAAR desensitization. To initially simplify our structural approach to exploring desensitization, we used a1b2 GABAARs. However, native synaptic GABAAR receptors will contain a single copy of the γ2 subunit. To probe desensitization in the γβγ receptor, we examined the effect of mutations at the intracellular end of the γ2 subunit. We selected γ2V262 and γ2H318 for mutation since these are homologous to a1V251 and a1N307, respectively, which have a profound effect on desensitization of γβ receptors (Supplementary Fig. 6). While co-expressing wild-type a1, b2 and γ2L subunits produced receptors that desensitized similarly (% Des = 80%, $\tau_w = 15.5$ s) to wild-type a1b2, mutating γ2L V262F and γ2H318V increased the rate of desensitization by 12- and 7-fold respectively, and strongly increased the extent of desensitization (% Des = 98.7 and 96.3, respectively; Fig. 4a). These mutations did not generally perturb receptor function as GABA potency was minimally affected at a1b2γ2L V262F and a1b2γ2L H318V compared with wild-type a1b2γ2 L GABAARs (Supplementary Fig. 9), suggesting that receptor gating was unaffected. Moreover, we also examined

**Figure 2** Mutating the intracellular end of the M2/M3 interface regulates desensitization of a1b2 GABAARs. (a) Left: 3D model of an a1b2 GABAAR based on GluCl template. Right in a–c: membrane currents induced by 10 mM GABA. (b) Left: enlarged side view of the intracellular end of the ion channel showing transmembrane domains M1–M3 for a1 (green) and b2 (red) subunits and the positions of various labelled residues. (c) Left: enlarged plan view of the ion channel. Note, in b,c, M4 segments were omitted for clarity.
another key pharmacological facet of γ2-containing GABA\textsubscript{A}Rs involving modulation by benzodiazepines\textsuperscript{16}. Diazepam-induced potentiation of low concentration (EC\textsubscript{1/2}, 1–3 μM) GABA currents at wild-type α1β2γ2L, and mutant α1β2γ2L\textsuperscript{H318V} and α1β2γ2L\textsuperscript{H318V} receptors, remained unaltered (2.53 ± 0.20-fold (α1β2γ2L); 2.14 ± 0.16-fold (α1β2γ2L\textsuperscript{H318V}); and 2.51 ± 0.05-fold (α1β2γ2L\textsuperscript{H318V}), n = 3), again suggesting that the mutations have neither affected gating, nor assembly, nor the folding of the receptors. It is important to note that, since there is only one copy of the γ2 subunit per α1β2γ2L receptor pentamer, there is also only one copy of the γ2 subunit mutation, further emphasizing the importance of the cytoplasmic end of the M2/M3 interface on the desensitization of Cys-loop receptors.

A similar mechanism accounts for the desensitization of GlyRs. If the identified residues in GABA\textsubscript{A}Rs form a part of the generic mechanism for receptor desensitization at inhibitory Cys-loop receptors, then we should be able to replicate the effects in GlyRs. Applying saturating concentrations (10 mM) of glycine to GlyR α1 homomeric receptors (GlyR\textsubscript{α1}) evoked peak currents that desensitized by ~72% with a τ\textsubscript{w} of 11 s (Table 1, Fig. 4b,c). As mutations to the M1–M2 linker of GlyR\textsubscript{α1} have been reported to strongly affect desensitization\textsuperscript{13,17}, we focused on the intracellular end of M3, as we had done with the GABA\textsubscript{A}Rs, and exchanged six consecutive residues with those from ρ1. This chimera (GlyR\textsubscript{α1}ρ\textsubscript{1(M6-posM3)}) desensitized by ~98% (τ\textsubscript{w} = 0.9 s, Fig. 4b), establishing the intracellular end of M3 in GlyRs as a key component for desensitization.

We then examined the effect of single mutations towards the cytoplasmic end of M2 and M3. GlyR\textsubscript{α1}V294\textsuperscript{L} is the homologous M3 leucine to GABA\textsubscript{A}R\textsubscript{α1}N305. Although the mutant GlyR\textsubscript{α1}V294\textsuperscript{L} did not affect desensitization (Table 1), mutating the highly conserved M2 glycine in GlyR\textsubscript{α1}G254\textsuperscript{V}, significantly increased desensitization (% Des = 99.4%, τ\textsubscript{w} = 0.8 s, Fig. 4c) while minimally affecting the apparent affinity for glycine (Supplementary Fig. 2b). Furthermore, mutating the M3 V294 (GlyR\textsubscript{α1}V294\textsuperscript{L}), homologous to GABA\textsubscript{A}R\textsubscript{α1}N305 (M3), which are homologous to GABA\textsubscript{A}R\textsubscript{α1}V294\textsuperscript{L} and GlyR\textsubscript{α1}N305\textsuperscript{V}, in accord with our GABA\textsubscript{A}R data, the mutant GlyR\textsubscript{α1}A249\textsuperscript{F} and GlyR\textsubscript{α1}A249\textsuperscript{F} displayed near-complete desensitization at rates 13- and 60-fold higher, respectively, than wild-type GlyR\textsubscript{α1} (Fig. 4b). This is consistent with our results for α1β2 GABA\textsubscript{A}Rs, and indicates that similar determinants and therefore mechanisms of desensitization apply to both GABA\textsubscript{A}Rs and GlyRs, involving structural rearrangements at the cytoplasmic end of the M2/M3 interface.

Locating the desensitization gate using a channel blocker. To understand how the identified residues regulate the desensitization in GABA\textsubscript{A}Rs and GlyRs, we compared the crystal structure of GluCl, with those of ELIC and GLIC, the prokaryotic homologues of Cys-loop receptors in putative shut and open conformations, respectively\textsuperscript{18–20}. While receptor activation involves an increase of the pore diameter between the 9’ and 20’ positions, there is a corresponding constriction of the pore at its cytoplasmic end, between –3’ and 4’ (Fig. 5a)\textsuperscript{14,18}. We hypothesized that desensitization could be an extension of the conformational rearrangements that occur during the activation process, which is consistent with desensitization proceeding from the open state of the channel\textsuperscript{21,22}. This implies that desensitization would involve a gate that is located proximal to the cytoplasmic end of the channel.

To corroborate our desensitization gate model for inhibitory Cys-loop receptors, we used the pore blocker, picrotoxin (PTX), since its binding site is generally considered to be located deep in the pore (between –2’ and 2’) precisely where the pore constricts during the activation process (Fig. 5a)\textsuperscript{14,23}. After applying a low GABA concentration (0.3 μM) to α1β2 receptors, enabling the resting (shut) state(s) of the receptor to remain significantly populated, we co-applied a saturating concentration (50 μM) of PTX, which blocked the GABA current. Subsequent washout of PTX while still in GABA revealed an extremely slow dissociation of the pore blocker (Fig. 5b), consistent with it being trapped in a resting (shut) state of the channel\textsuperscript{23}. Repeating this protocol now with a saturating GABA concentration (10 mM) to deliberately
drive receptors into the desensitized state, the apparent dissociation of PTX was much faster (Fig. 5c). This implies that PTX is not trapped in a desensitized state and that the desensitization gate must be distinct from the activation gate (Model 1, Fig. 6a).

Two simple schemes may explain the fast dissociation of PTX in the presence of saturating GABA. First, PTX binding/unbinding could be the same to open and desensitized receptor states. However, when GABA is saturating, the peak current after washing out PTX is clearly larger than the steady-state current before its application, which is not consistent with equal binding/unbinding to both states (Model 2, Fig. 6b). Second, PTX binding could be prevented in the desensitized state (Model 3, Fig. 6c). The recovery of current following PTX removal could then reflect two distinct processes: PTX dissociation from its binding site and subsequent receptor desensitization. For this scheme, we would expect the recovery current to exhibit a peak followed by a plateau. However, such behaviour is not obvious from our recordings with the GABA\(_A\)R (Fig. 5c), probably because PTX dissociation is much slower than the rate of desensitization of GABA\(_A\)Rs (Fig. 6c, Supplementary Table 2).

To gain a clearer view as to where PTX is binding in relation to our proposed desensitization locus, we examined GlyRs, where PTX dissociation is notably much faster\(^{24,25}\). During saturating glycine concentrations, PTX blocked the plateau current (Fig. 7a). Washing out PTX now revealed a clear peak-plateau current profile as expected theoretically, indicating that PTX had blocked the receptor in an open conformation, a profile that is accurately predicted only by Model 3 (Fig. 7b–d). These observations are consistent with a mechanism whereby the PTX-binding site spatially overlaps the desensitization gate. Furthermore, as desensitization leads to an increase in the agonist occupancy, such a model could account for the negative allosteric interaction between the receptor agonist and PTX\(^{24,25}\).

Discussion

The present view of desensitization at Cys-loop receptors is that the molecular determinants are most likely contained within the ECD\(^{26}\) or the ECD–TMD coupling interface\(^{27,28}\). Our study indicates that a different domain is involved whereby the cytoplasmic end of the ion channel forms a physical desensitization gate in inhibitory Cys-loop receptors. While this may initially appear contradictory to earlier studies, a role for residues in the ECD or the ECD–TMD interface in setting macroscopic desensitization can be entirely reconciled with our new view. First, in the event that channel opening precedes desensitization, a mutation affecting the efficacy of gating could ultimately affect the macroscopic rate of desensitization without actually modifying the microscopic rate of desensitization. Interestingly, the ECD–TMD coupling interface is critical in setting the efficacy of channel gating. Accordingly, exchanging residues in this region between nAChRs and 5HT\(_3\)Rs not only switches the desensitization properties of the receptors, but also their single-channel open lifetimes\(^{27}\).

Second, our proposal for a constriction (gate) of the channel lumen near its intracellular end during desensitization is likely to be accompanied by conformational rearrangements at the extracellular end that should be constrained by residues in the ECD–TMD coupling interface. Considering that M2 helices can exhibit rigid-body motion during gating\(^{12,29}\), we would predict that the pore may widen at its extracellular end during desensitization. This could be consistent with conformational rearrangements at the ECD–TMD interface of GABA\(_A\) receptors during desensitization, as reported by voltage-clamp fluorometry\(^{28}\). Moreover, such an effect has recently been proposed for GLIC, using electron paramagnetic resonance (EPR) spectroscopy to measure the distance between spin-labelled cysteines introduced along the ion channel pore\(^{30}\).

Interestingly, EPR predicts that desensitization might involve a mid-membrane gate with the intracellular end of the channel remaining mostly fixed. However, there are caveats to the interpretations of EPR data. First, in the narrowest part of the GLIC channel, spin labels might occlude the pore, thus acting as pore blockers, and as for PTX-bound GABA receptors, desensitization could conceivably be prevented by the labelling. Unfortunately, the irreversible inhibition by pore-blocking spin-labels would also render these receptors unsuitable for electrophysiological studies. Second, in the confined space of an ion channel pore, it is likely that the motion of the spin labels is also

Figure 5 | Promoting desensitization of GABA\(_A\)Rs causes fast dissociation of the pore blocker PTX. (a) Left: side-view cutaway section through the transmembrane domains showing two (\(\alpha1\)—green, \(\beta2\)—red) of the five M2 helices from our \(\alpha1\)\(\beta2\) GABA\(_A\)R model overlaid with the M2 helices of the PTX-bound GluCl structure (light blue; pdb 3RIS (ref. 14)). Note the deep location of the PTX-binding site between locations 2' and 3'. Right: predicted pore diameter plotted as a function of the distance along the pore axis for GluCl, GLIC (open; pdb 3EAM (ref. 18)) and ELIC (shut; pdb 2VL0 (ref. 19)). This is vertically aligned with the left panel schema for cross comparison. The dashed line reflects the hypothesis that desensitization is an extension of the mid-membrane gate with the intracellular end of the channel forming a physical desensitization gate in inhibitory Cys-loop receptors. While this may initially appear contradictory to earlier studies, a role for residues in the ECD or the ECD–TMD interface in setting macroscopic desensitization can be entirely reconciled with our new view. First, in the event that channel opening precedes desensitization, a mutation affecting the efficacy of gating could ultimately affect the macroscopic rate of desensitization without actually modifying the microscopic rate of desensitization. Interestingly, the ECD–TMD coupling interface is critical in setting the efficacy of channel gating. Accordingly, exchanging residues in this region between nAChRs and 5HT\(_3\)Rs not only switches the desensitization properties of the receptors, but also their single-channel open lifetimes\(^{27}\).

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Interestingly, EPR predicts that desensitization might involve a mid-membrane gate with the intracellular end of the channel remaining mostly fixed. However, there are caveats to the interpretations of EPR data. First, in the narrowest part of the GLIC channel, spin labels might occlude the pore, thus acting as pore blockers, and as for PTX-bound GABA receptors, desensitization could conceivably be prevented by the labelling. Unfortunately, the irreversible inhibition by pore-blocking spin-labels would also render these receptors unsuitable for electrophysiological studies. Second, in the confined space of an ion channel pore, it is likely that the motion of the spin labels is also
constrained. Thus, conformational changes around the spin labels could cause dynamic reorientation, making the extraction of distance information quite challenging\(^\text{a31}\). For these reasons, EPR spectroscopy could be confounding when studying conformational rearrangements in the constricted space of an ion channel.

It is also important to note that a previous study showed that M1 and M2 were important in setting the desensitization properties of \(\gamma2\) and \(\delta\) subunit-containing GABA\(_A\) receptors\(^5\). While the molecular mechanisms were unclear, these results are interpretable in the light of our present study. The importance of M2 is clearly demonstrated by our mutagenesis data, and from our proposal regarding the location of a desensitization gate. Moreover, as there are extensive interactions between the TMDs, M1 would be expected to affect the conformation of both M2 and M3, and could also influence desensitization by affecting the M1–M2 linker. We should add a cautionary note to emphasize that perturbations to receptor structure can potentially have far reaching ramifications for predicting the consequences of mutagenesis to a functional outcome. For this reason, it is hard to predict the effects of residue substitution in the M2–M3 linker when manipulating the side-chain volume, charge and hydrophobicity. Nevertheless, structure–function studies of Cys-loop receptor activation, using similar methodologies to those employed here have proved to be very precise tools in identifying and attributing key parts of the receptor involved in function\(^1\text{2,29}\), as evidenced by recent corroborating data between EPR spectroscopy and the crystal structure of the apo form of GluCl\(^32\).

The molecular mechanisms underlying the desensitization of inhibitory Cys-loop receptors, which may conceivably extend to the entire Cys-loop family, appear strikingly different from the mechanisms responsible for desensitization of ionotropic GluRs. This is unsurprising, since from a structural point of view, Cys-loop receptors are quite distinct from ionotropic GluRs by forming pentamers with the ECD of each subunit interacting to form a ring, and the agonist binding sites located at interfaces between adjacent ECDs\(^7,33\). By contrast, ionotropic GluRs are tetramers harbouring discrete intrasubunit–binding sites\(^7,8\). Moreover, Cys-loop receptor subunits contain four trans-membrane segments, which differ from the three trans-membrane segments and one re-entrant P-loop topology of ionotropic GluR subunits.

The cytoplasmic end of the pore in Cys-loop receptors also contains the ion selectivity filter\(^34\). The conformational changes proposed to underlie desensitization here are reminiscent of the collapse of the selectivity filter that is considered responsible for slow inactivation of voltage-gated K\(^+\) and Na\(^+\) channels\(^35–37\).
Specific mutations near the P-loop of these voltage-gated channels profoundly impact on slow inactivation, in a similar manner to the effects of the mutations described in this study for Cys-loop receptor desensitization. Corroboration for the slow inactivation model of voltage-gated ion channels was obtained using pore-blockers, such as tetraethylammonium, which reduced slow inactivation of Shaker K⁺ channels by a 'foot-in-the-door' mechanism, essentially by binding near the selectivity filter to prevent its physical collapse. By analogy, we used the Cys-loop Cterminial channel blocker PTX to prevent GABA₂R and GlyR desensitization, which accorded with there being a spatial overlap between the desensitization gate and the PTX-binding site. 

In the GABA receptor, such as addiction, anxiety, depression, neurodegenerative diseases being unable to be activated by GABA, the crystallographic data further, our experimental results show that PTX actually prevents desensitization and the slow inactivation of voltage-gated K⁺ and Na⁺ channels, even though the structure and membrane topology of these tetrameric channels is completely different from pentameric Cys-loop receptors.

In this context, it is notable that a recent crystal structure for the GABA receptor β3 homomer has been solved. Although it is considered not to be a physiological form of the GABA₂R receptor, being unable to be activated by GABA, the crystallographic data also alludes to a constriction at the intracellular end of the ion channel pore as being representative of a desensitized state.

Our model suggests that the desensitization of Cys-loop receptors involves a rearrangement of the M2/M3 interface. This concept should accommodate the effects of modulators binding within the M1/M2/M3 intrasubunit cavity of nAChRs, which prevent desensitization. Our results therefore provide a rationale for the mechanism of action of these drugs, and could help in designing pharmaceutical compounds to modulate Cys-loop receptor function. This is likely to be beneficial since Cys-loop receptors are involved in many pathological conditions, such as addiction, anxiety, depression, neurodegenerative diseases and seizures.

Two-electrode voltage clamp and analysis. Xenopus laevis oocytes expressing recombinant receptors were prepared, injected with complementary DNAs (cDNAs; at 1–30 ng µl⁻¹), voltage-clamped and recorded as described previously. All compounds were purchased from Sigma. EDTA (10 μM) was added to the OR2 solution when recording from oocytes expressing GlyRs to chelate contaminating Zn²⁺, which potentiates GlyR function. Recordings were digitized at 500 Hz and filtered at 50 Hz. Desensitizing currents were induced by 1 min agonist applications. The design of our recording chamber and the use of supersaturating concentrations of agonist enabled us to elicit currents with 20–80% rise times of 22 and 15 ms for wild-type z1β2 GABA₂Rs and GlyRβ2, respectively. The extent of desensitization was determined as

\[ I_{peak} - I_{endata} \]

where \( I_{peak} \) is the agonist-induced peak current and \( I_{endata} \) the residual of this current remaining at the end of the agonist application. Weighted decay time constants for desensitization were determined by fitting the desensitizing phase with two or three exponential components (Clampfit ver 8). Experiments that assessed the time period for recovery of the receptor from desensitization were performed by first driving the receptor into a profound desensitized state (30 s exposure to millimolar GABA or glycine concentrations), then monitoring the recovery of peak currents (2 s exposures) repeated at 10, 20, 30, 45, 60, 90, 120, 300 and 600 s intervals after the initial desensitizing exposure to the agonist.

To resolve even faster channel kinetics, we have performed rapid-application perfusion experiments with outside-out patches pulled from Xenopus laevis oocytes expressing recombinant GABA₂Rs. Using the calcium phosphate precipitation method with cDNAs pre-mixed in a 1:1 ratio, we achieved 20–80% solution exchange times <100 μs. At wild-type z1β2 GABA₂Rs, a 3-s exposure of 10 mM GABA led to a current that desensitized almost completely and is described by three exponential components of ~10, 100 and 500 ms, consistent with published results obtained with recombinant receptors expressed in HEK cells.

Although the 10-ms component cannot be detected using whole-cell patch-clamp recordings, GABA- and glycine-mediated currents decay much faster than their respective membrane conductances in the continuous presence of the agonists. Since the currents were matched by the rates of change of internal Cl⁻ concentration, this observation led the authors to speculate that the fast decay of currents, initially thought to reflect desensitization, actually reflects a decrease in the driving force for Cl⁻ ions. This effect is likely to be present in most patch-clamp experiments performed on small cells or outside-out patches, especially under high series resistance conditions. The oocyte is therefore a reliable model system to study the Cys-loop receptor desensitization. Of note, the slow conductance decay rates measured in ref. 50 are consistent with the desensitization kinetics we measure in TEVC at wild-type receptors.

Furthermore, the almost complete extent of fast desensitization in most outside-out patches did not concur with the large currents obtained with TEVC after a few seconds of exposure to GABA. Interestingly, recent results demonstrate that, in whole-cell patch-clamp recordings, GABA- and glycine-mediated currents decay much faster than their respective membrane conductances in the continuous presence of the agonists. Since the currents were matched by the rates of change of internal Cl⁻ concentration, this observation led the authors to speculate that the fast decay of currents, initially thought to reflect desensitization, actually reflects a decrease in the driving force for Cl⁻ ions. This effect is likely to be present in most patch-clamp experiments performed on small cells or outside-out patches, especially under high series resistance conditions. The oocyte is therefore a reliable model system to study the Cys-loop receptor desensitization. Of note, the slow conductance decay rates measured in ref. 50 are consistent with the desensitization kinetics we measure in TEVC at wild-type receptors.

Finally, as mentioned above, we used outside-out patches pulled from oocytes that expressed mutants showing increased rates of desensitization under TEVC. Unfortunately, we could not resolve any current from patches containing z1β2p1 (M₁-M₂ link) or z1β2v²5β₂ receptors, even though the ‘parent’ oocytes exhibited strong functional expression (>5 μA peak GABA current). Moreover, using outside-out patches from oocytes expressing wild mutant z1β2 (G250A,R52G4,52), we rarely recorded currents, and only then of a few pA decaying fully within 100 ms. For all these reasons, we discarded the fast-perfusion experiments.

HEK whole-cell patch-clamp recording. HEK cells were transiently transfected using the calcium phosphate precipitation method with cDNAs pre-mixed in a 1:1 ratio, with 4 μg total cDNA applied to each per 22 mm coverlip. HEK cells were voltage-clamped and recorded as described previously. Recordings were digitized and filtered at 6.5 kHz. Desensitizing currents were induced by 8 s applications of GABA (3 mM). The use of a rapid U-tube perfusion system and the use of supersaturating concentrations of agonist enabled us to elicit currents with average 20–80% rise times of <8 ms for wild-type z1β2 GABA₂Rs. The extent of desensitization and weighted decay time constants were determined for TEVC, up to 6 s after the start of GABA application.

3D molecular modelling and illustration. Modeller (ver 9.7 (ref. 52)) was used to build 3D homology models of the z1β2 heteromeric GABA₂R based on the crystal structure of GluCl (pdb 3HRW (ref. 14)). Structures were visualized with Pymol; pore radii were calculated with MOLE.

Kinetic modelling. Channellab (ver 2, Synaptosoft, GA) was used to build the virtual recordings in Figs 6 and 7. The binding and gating rate constants are broadly consistent with previously published values for GABA₂Rs and GlyRs. The desensitization rates were chosen to account for the profile of our current
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Author contributions
M.G. performed the molecular biology and M.G. and P.T. performed and analysed the TEVC and the patch-clamp experiments. All authors discussed the results. M.G. and T.G.S. designed the study and all authors wrote the manuscript.

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