The invertebrate glutamate-gated chloride-selective receptors (GluClRs) are ion channels serving as targets for ivermectin (IVM), a broad-spectrum anthelmintic drug used to treat human parasitic diseases like river blindness and lymphatic filariasis. The native GluClR is a heteropentamer consisting of α and β subunit types, with yet unknown subunit stoichiometry and arrangement. Based on the recent crystal structure of a homomeric GluClR, we introduced mutations at the intersubunit interfaces where Glu (the neurotransmitter) binds. By electrophysiological characterization of these mutants, we found heteromeric assemblies with two equivalent Glu-binding sites at α/β intersubunit interfaces, where the GluClβ and GluClα subunits, respectively, contribute the “principal” and “complementary” components of the putative Glu-binding pockets. We identified a mutation in the IVM-binding site (far away from the Glu-binding sites), which significantly increased the sensitivity of the heteromeric mutant receptor to both Glu and IVM, and improved the receptor subunits’ cooperativity. We further customized this heteromeric GluClR mutant as a receptor having a third GluCl-binding site at an α/α intersubunit interface. Altogether, our data unveil heteromeric GluClR assemblies having three α and two β subunits arranged in a counterclockwise β-α-β-α-α fashion, as viewed from the extracellular side, with either two or three Glu-binding site interfaces.

Significance

Cys-loop receptors (CLRs) are transmembrane ion channels activated by neurotransmitters to mediate chemoelectric excitation or inhibition throughout the nervous system. Hence, CLRs play a key role in our day-to-day life, from coordination of motions to cognition. Impairment of CLRs’ activity leads to various pathological conditions. The CLR studied here is a glutamate-gated chloride-selective receptor (GluClR). GluClRs are unique to invertebrates, yet they are pharmacologically important because they serve as targets for ivermectin, an anthelmintic drug used to treat humans suffering from filarial diseases. This study provides better understanding of the subunit arrangement and stoichiometry of Glu-binding sites in GluClRs.

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GLUTamate receptors (GluClRs) are inherently responsive to IVM but not to Glu. CHO cells transfected with the GluClβWT subunit alone showed very weak responses to 10 mM Glu (less than 230 pA in eight cells; Fig. S2A), in line with results obtained in HEK cells (45). No responses to 500 nM IVM in CHO cells transfected with the GluClβWT subunit alone were observed (10 cells), in agreement with the same observations in HEK cells (18, 22). In contrast to these differential responses, cells cotransfected with both GluClαWT and GluClβWT subunits displayed robust responses to 1.5 mM Glu (EC50 concentration) and 500 nM IVM (Fig. S2C). We therefore deduce that robust responses to Glu and IVM (independently) in a cell cotransfected with the GluClαWT and microchimeric GluClβ subunits (Fig. S2 D–F) reflect the function of heteromeric GluClαβR complexes. This deduction also applies for the site-specific mutants discussed further below.

Fig. 2 shows representative current traces elicited by increasing Glu concentrations (Fig. 2A) and the corresponding dose–response curves (Fig. 2B) for the heteromeric WT and microchimeric GluClRs. The Glu-EC50 values specified in Table S1 indicate that the apparent affinities of the GluClαWT/βmicrochimeric receptors for Glu were very close to the apparent affinity of the GluClαWT/βWT receptor. The Hill coefficients of all four receptors (Table S1) were >1, indicating their activation with positive cooperativity. Note that the Glu-EC50 and the Hill coefficient determined here for the GluClαWT/βWT receptor (Table S1) are very close to those values determined in *Xenopus* oocytes [Glu-EC50 = 1.36 ± 0.05 mM and Hill coefficient (nH) = 1.7 ± 0.1] (13). Remarkably, Glu readily activates the GluClαWT/βmicrochimeric receptor, all of whose LigBD’s coupling loops are of the GluClα subunit (Fig. 2 and Table S1). We thus conclude that the β1β2, Cys, and β8β9 loops of the GluClα subunit are inherently capable of coupling Glu binding to pore gating, with no need for IVM prebinding.

Fig. 1. Structural characteristics of GluClRs. (A) Two neighboring subunits of the homopentameric GluClCryst [Protein Data Bank (PDB) ID code 3RIF] are shown from the side in light and dark gray colors. Wide gray horizontal lines mark the putative membrane borders. The four coupling loops are colored as shown in C and the upper row of D. Glu and IVM are shown as space-filling models with carbon, oxygen, and nitrogen atoms colored in yellow, red, and blue, respectively. They are bound at the α(+)α(−) intersubunit interface far away from each other: Glu in the extracellular LigBD and IVM in the upper part of the pore-domain periphery, between M1 (of the light gray subunit) and M3 (of the dark gray subunit). Note that in Cys-loop receptors, the principal and complementary faces of a neurotransmitter-binding pocket are formed by the (+) and (−) sides of two adjacent subunits, respectively. (B) Top view of the GluClCryst pentamer showing five identical subunits, which are colored differently to highlight the intersubunit interfaces located between the (+) and (−) sides. (C) Space-filling models of residues belonging to the coupling loops, which create an extensive bond network at the interface between the LigBD and the ion-channel pore domain. (D) Schemes of GluClR subunits used in this study. The M1–M4 transmembrane segments are numbered 1–4. Different colors reflect differences in amino acid sequences (Fig. S1).

CHO cells transfected with the GluClαWT subunit alone showed very weak responses to 10 mM Glu (135 ± 27 pA in eight cells; mean ± SEM), but responded well to 500 nM IVM (Fig. S2B; 14 cells). This observation is in line with the findings of Frazier et al. (45), who reported that HEK cells expressing GluClα homomers are responsive to IVM but not to Glu.

**Fig. 2.** Glu-activation properties of WT and microchimeric GluClRs. (A) Representative Glu-elicted currents measured in cells cotransfected with the indicated subunits. In all cases, Glu was applied for 3 s. Glu concentrations: 0.1 mM, 0.3 mM, 0.6 mM, 1 mM, 3 mM, 10 mM, and 30 mM in the upper row and 0.1 mM, 0.3 mM, 0.6 mM, 1 mM, 10 mM, and 30 mM in the lower row. Measurements were performed at +60 mV. (B) Dose-response curves plotted for responses measured in cells cotransfected with the GluClαWT subunit and the GluClβWT subunits indicated in the figure. Curves were fitted to the averaged data points with a nonlinear regression using the Hill equation (Eq. 1) (r² > 0.99). Error bars correspond to SEM.
Contribution of the GluClα Subunit (−) Side to Glu Accommodation in Heteromeric GluClRs Mutants. The aforementioned observations brought us to the recognition that a thorough study of how the GluClα subunit contributes to Glu binding in heteromeric GluClRs is necessary. Therefore, we first introduced mutations in the (−) side of the GluClα subunit based on the crystal structure of the homomeric GluClαC (R. R. (22)) ([the (−) and (+) subunit sides are defined in Fig. 1 A and B]). This structure indicates that the β-guanidino groups of α(−)R98 and α(−)R117 are at an appropriate distance to form ion pairing with the α- and γ-carbonyl groups of Glu, respectively (Fig. 3D). A mutation that eliminated the charge and drastically reduced the side-chain size of α(−)R117, but kept hydrophilicity at this position (i.e., R→S), did not provide a functional GluClαR117S/βWT receptor. We therefore replaced the two Arg (one at a time) with a more conservative and bulkier hydrophobic residue, Asn or Gln, which can function as hydrogen bond donor (or acceptor) with no capability to form salt bridges. A mutant having an αR98N substitution (GluClαR98N/βWT receptor) provided robust responses, but very high Glu concentrations were necessary to reach saturation (Fig. 3A, traces (Right) and brownish dose–response curve (Left)). Note that to dissolve Glu, it was titrated with equimolar concentrations of NaOH; therefore, we did not change the Nernst potential for Cl during Glu application. However, the osmolarity and negative charge of the external solution drastically increased during the application of high Glu concentrations (for 0.6 s). Even so, we assume that these factors did not affect the responses, as discussed in SI Text, section 1, in conjunction with Fig. S3.

In the case of the GluClαR117N/βWT receptor, the current responses did not allow us to analyze the dose–response relation reliably because they were very low (~300 pA at 1 M Glu) and did not reach saturation, unlike in the case of the GluClαR98N/βWT receptor. In contrast, introducing Q at position α(−)N117, which has a longer side chain than N, created a responsive GluClαR117Q/βWT receptor that enabled us to determine its Glu-EC50 and Hill coefficient (Fig. 3A and Table S1). The crystal structure also indicates that α(−)S182 forms a hydrogen bond with the γ-carbonyl group of Glu (23) (Fig. 3D). Preventing this hydrogen bonding in the heteromeric GluClαS182A/βWT receptor produced an effect similar to the effect observed with the α(−)R98N and α(−)R117Q substitutions (Fig. 3A and Table S1). The drastic effects exerted by mutations in the GluClα (−) side raised the question of whether mutations at the homologous positions in GluClβ would exert the same effects.

Contribution of the GluClβ Subunit to Glu Accommodation in Heteromeric GluClRs Mutants. Sequence alignments (17, 23) indicate that the GluClβ subunit has identical residues at positions homologous to GluCl α(−)R98, α(−)R117, and α(−)S182. These residues are β(−)R66, β(−)R85, and β(−)S152, respectively (Fig. 3E). A 3D homology model built here for the α(+)β(−) intersubunit interface (SI Materials and Methods and Fig. S4) predicts that these three β-subunit residues are sufficiently close to interact with Glu (Fig. 3E). However, heteromeric mutant receptors assembled as the αWT subunit, together with β(−)R66N, β(−)R85N, or β(−)S152A, did not display the drastic increase in Glu-EC50 typical of the homologous α-subunit mutants (Fig. 3B and Table S1). Most recently, Daeffler et al. (22) published a study where they investigated homomeric GluClRs carrying a βT283S mutation in the pore-lining segments (see sequence with entry code Q17328 in the UniProtKB database). The latter mutation, per se, caused a dramatic improvement in the response to Glu (70-fold decrease in Glu-EC50). Interestingly, when the βT283S mutation was combined with a β(−)S152A mutation (no. 126 in ref. 22), the Glu-EC50 relatively increased by 590-fold (22). Clearly, if the β-subunit (−) side were to contribute the “complementary” Glu-binding components in our heteromeric GluClαWT/βS152A receptor, we would have observed a much larger rightward shift of...
the dose–response curve (complementary is defined in Fig. 1). Taken
the results of the previous and current sections together, we infer
that in the recombinant heteromeric receptors studied here, the
GluCl(–) side, rather than the GluCl(+) side, contributes the
complementary components to Glu binding. Hence, we hypothe-
sized that the GluCl(+) side contributes the “principal” Glu-bind-
ing components in heteromeric GluClRs (principal is defined in
Fig. 1).

To examine this hypothesis, we mutated residues β(+)F122,
β(+)T229, and β(+)Y232 that might contact Glu, as predicted by
sequence alignments (17, 23) and our 3D homology model (Fig.
3D). We then coexpressed the mutated β subunits (one at a
time), together with the αWT subunit, and found that they
shifted the dose–response curves rightward (Fig. 3C). Table S1
shows the extent of increase in the Glu-EC50 values, with the
most prominent shift in the GluClWT/βT229N and GluClαWT/
βT229W receptors (by approximately eightfold compared with
the GluClαWT/βWT receptor). We infer that the GluCl(+) side in
the heteromeric assemblies generated here contributes the
principal Glu-binding components. Daefler et al. (22)
added to the homomeric GluClT283S receptor a β(+)T229A
mutation (no. 203 in ref. 22), which increased the Glu-EC50 to
a much larger extent than observed here for the heteromeric
GluClαWT/βT229N or GluClαWT/βT229W receptor. This differ-
cence can be attributed to the nature of the replacing amino
acids. In the current study, we did not wish to change the
chemical properties of the amino acids too much. This approach
was adopted because the GluClαWT/βWT receptor inherently
displays low affinity for Glu, which would probably make a
dramatic increase in Glu-EC50 difficult to probe. Hence, we kept the
capability of the replacing residues at position β(+)T229 to act as
hydrogen bond donors (Asn, Trp) or a hydrogen bond acceptor
(Asm). We expected that the greater size of the replacing residues
would interfere with, but not abolish, Glu accommodation.
This expectation emerged because position β(+)229 is located on loop
C, which caps the putative Glu-binding pocket but, on the other
hand, is considered to be flexible and mobile (46) (Fig. 3 D and E).
As to the β(+)Y232S substitution, we probably eliminated a cation-
pi interaction that was recently suggested to be formed in a
homomeric GluClRI, between the β(+)Y232 aromatic ring and the
ω-amino nitrogen of Glu (22). Still, one cannot exclude hydrogen bonding between the hydroxyl group of the Ser
we introduced at this position [β(+)232] and the ω-amino ni-
trogen of Glu, which could explain the moderate effect of the
β(+)Y232S mutation.

Stoichiometry of the Glu-Binding Sites in a Heteromeric WT GluClα/β.
The results presented in the previous sections suggest that a
β(+)α(−) intersubunit interface is involved in Glu accommoda-
tion; so, how many such functional interfaces exist per hetero-
pentamer? The various single-site mutant receptors discussed so
far share with the GluClαWT/βWT receptor Hill coefficients
smaller than 2 but clearly larger than 1 (Table S1). This property
suggests that there is more than one Glu-binding site per hetero-
pentamer. To determine the number of functional sites and
their microscopic equilibrium dissociation constants for Glu
binding in the heteromeric GluClαWT/βWT receptor, we used
an allosteric model based on the Monod–Wyman–Changeux
(MWC) theory (47), as applied also by Karlin (48) to the nicotinic
ACh receptor (nAChR) (reviewed in refs. 49 and 50). Because
the GluClαWT/βWT receptor displays very slow and weak desensiti-
ization, we simplified the allosteric model by focusing on two
major states as previously performed for weakly or nondesensitizing
Cys-loop receptors such as: homomeric α7-nAChR mutants (51),
homomeric α7-5HT3R chimeras (52), and heteromeric GABA
receptors (53, 54). If the GluClαWT/βWT receptor has two equiva-
 lent (identical) Glu-binding sites, then Scheme I describes its
MWC allosteric activation mechanism as follows:

\[
\begin{align*}
R & \xrightarrow{K_{d,R}} AR \\
R* & \xrightarrow{K_{d,R*}} AR* \\
L & \xrightarrow{K_{d,L}} L_{c} \\
L_{c} & \xrightarrow{K_{d,L_{c}}} L_{c}^{2} \\
& \xrightarrow{A_{2}R} A_{2}R* \\
& \xrightarrow{A_{2}R*} A_{2}R* \\
\end{align*}
\]

where R and R* are resting (closed) and active (open) receptor
conformational states, respectively; A is an agonist molecule (Glu)
that can complex with the receptor; K_{d,R} and K_{d,R*} are the
microscopic equilibrium dissociation constants for agonist binding
to the closed and open receptor states, respectively; and L is the
equilibrium constant of the two receptor states (closed and open)
in the absence of ligands. L is calculated by R/R* based on quan-
titative determinations, as follows.

Unoccupied R* corresponds to spontaneously open channels.
Spontaneous activity (I_{spont}) was measured as the fraction of the
leak current that could be blocked by picrotoxin, an ion-channel
pore blocker of GluClRs (55) (e.g., Fig. 3F, indicated by “a”;
explained in SI Text, section 2). Unoccupied R is estimated
based on the current elicited by saturating Glu concentrations
[maximal current response (I_{max})]. That is, I_{max} represents
the activatable receptor population, which is at rest in the absence
of Glu (Fig. 3F, indicated by “b”). However, I_{max} might not rep-
resent all of the activatable channels because not all of them are
necessarily open at saturating Glu concentrations. Therefore, we
determined the maximum open probability (P_{o, max}) of the
ion channel by single-channel recordings at a saturating Glu
concentration (Fig. 3 G and H) and then calculated R by I_{max}/P_{o, max}.
Thus, \( L = (I_{max}/P_{o, max})/(I_{spont}) \). Experimental P_{o, max} and L
values of three receptors are specified in Table S2 (footnotes).
I_{spont} and P_{o, max} (0.64) were also used to normalize the dose-
response data points of the GluClαWT/βWT receptor to esti-
mate its open probability (P_{o, open}) at varying Glu concentrations
by [(I + I_{spont})/(I_{max} + I_{spont})]P_{o, max} (Fig. 3F). Then, to assess the
applicability of Scheme I to the WT receptor activation mode, a
curve was fitted to the normalized data points using an MWC
allosteric model with two equivalent Glu-binding sites (n = 2)
and the experimental mean L value (85) (Fig. 3F, dashed black
curve and Eq. 2). Table S2 provides the resulting K_{d} values (in
bold). At very high Glu concentrations, the theoretical maximum
open probability P_{o, max} = 1/(1 + c^L), where c = K_{d,R}/K_{d,R*}
(54). So, when n = 2, the theoretical P_{o, max} = 0.65 for the
GluClαWT/βWT receptor, which closely predicts the experi-
mental P_{o, max} (0.64). In contrast, fitting curves using an MWC
model with other n values (one or equivalent three, four, or five
Glu-binding sites; Eq. 2) resulted in a theoretical P_{o, max} \geq 0.68
(Table S2). Moreover, analysis of the second-order Akaike
information criterion difference (AΔICc) (56) (SI Materials and
Methods) selected the allosteric model with n = 2 as the most
suitable MWC model for curve fitting in the GluClαWT/βWT
receptor case (Table S2). Hence, we infer that the GluClαWT/
βWT receptor has two functional equivalent Glu-binding sites.
Taken together with the results shown in Fig. 3 A–C, we suggest
that these two Glu-binding sites likely lie at two β(+)α(−)
intersubunit interfaces (Fig. 3F). Although one cannot absolutely
exclude the possibility of changes in the stoichiometry due to
mutations, we argue that such a change is unlikely to occur
(SI Text, section 3, Fig. S5, and Table S3).

Mutation in the IVM-Binding Pocket Gives Rise to a Third Glu-Binding
Site. During our research, we identified a mutation in the putative
IVM-binding site (αL279W; position α(−)L218 in GluClαWT) that
decreased the Glu-EC50 of the GluClαWT/βWT receptor by ~25-fold, compared with the GluClαWT/βWT receptor.
Fig. 4. Subunit stoichiometry and arrangement in heteromeric GluClα/βR mutants. (A, Left) Glu dose–response curves plotted for the activation of receptors consisting of the indicated subunits. Curves were fitted as described in Fig. 2B ($r^2 > 0.99$). Error bars correspond to SEM. (A, Right) Representative current traces evoked by applying increasing Glu concentrations on cells cotransfected with the indicated receptor subunits. Glu concentrations: 0.01 mM, 0.02 mM, 0.03 mM, 0.06 mM, 0.1 mM, 0.2 mM, and 0.3 mM (for the αL279W/βWT receptor) and 3 mM, 10 mM, 30 mM, 100 mM, 300 mM, and 600 mM [for the α(L279W, T258N)/βT229N receptor]. Currents were measured at +60 mV. (B) Three-dimensional models of the IVM-binding pocket at the α+/α− intersubunit interface. (Left) Side chain of the native αL279, which does not interact with IVM. (Right) Side chain of the substituting Trp, which potentially forms multiple van der Waals interactions with the lactone backbone of IVM. Side chains are shown as gray (carbon atoms) and blue (nitrogen) spheres. IVM is shown as yellow (carbon atoms) and red (oxygen atoms) spheres. Hydrogen atoms were removed for better viewing. The PDB 3RIF structure was used for generating the αL279W mutant and the pictures. (C and D) Representative current traces elicited by saturating concentrations of Glu in cells cotransfected with the indicated subunits (0.5 mM and 30 mM Glu in C and D, respectively). (Insets) Magnifications for the effect of picrotoxin (PTX; 200 μM) are shown. (E and F) Representative single-channel currents recorded in cell-attached patches from cells cotransfected with the indicated receptor subunits. The pipette solution included saturating Glu concentrations (0.5 mM and 30 mM Glu in E and F, respectively). The voltage command was −90 mV. The closed and open state levels are indicated by c and o, respectively. $P_{\text{open}}$-max values are 0.86 and 0.60 for the receptors indicated in E and F, respectively. On the right side of each current trace shown are curves fitted to event histograms plotted as described in Fig. 3H; they provide mean amplitudes of 2.1 pA and 2.2 pA for the open states of the receptors indicated in E and F, respectively. Mean channel open times ($\tau_o$) are indicated inside the rightmost panels. (G and H) Estimated $P_{\text{open}}$ plotted as a function of varying Glu concentrations. Red and orange lines correspond to the Hill curves. Other curves were plotted based on an MWC allosteric model using Eq. 2 for cases with either two ($n = 2$) or three ($n = 3$) equivalent Glu-binding sites or, alternatively, Eq. 3 for a case with two equivalent and a third distinct Glu-binding sites ($n = 2, m = 1$). (G and H, Right) Plausible subunit arrangements with intersubunit Glu-binding sites (black triangles), as viewed from the extracellular side.
This mutation increased the Hill coefficient to 2.6, suggesting that the number of occupiable Glu-binding sites in the receptor mutant is probably not less than three. Intrigued by this possibility, we initially examined an MWC allosteric model with either two or three equivalent Glu-binding sites. To this end, we determined the values of $P_{\text{open, max}}$, $P_{\text{open}}$, and $L$ for the GluCl(L279W)/\beta WT receptor [Fig. 4 C and E and Table S2 (footnotes)] and estimated its $P_{\text{open}}$ at varying Glu concentrations, as all described above for the GluClWT/\beta WT receptor. Then, a curve was fitted to the normalized dose–response data points using an MWC allosteric model with two equivalent Glu-binding sites ($n = 2$) and the experimental mean $L$ value (81) (Fig. 4G, salmon-colored curve and Eq. 2). The resulting $K_d$ values (Table S2, same line of “2,” 0”) were applied to calculate the theoretical $P_{\text{open, max}}$ by $1/(1 + c^2L) = 0.98$, which turned out to be much higher than the experimental $P_{\text{open}}$ (0.86). Extrapolating the salmon-colored curve in Fig. 4G (model with $n = 2$) until the theoretical $P_{\text{open, max}}$ is reached indicates a strong deviation of this curve from the Hill plot at high Glu concentrations. Alternatively, a curve was fitted to the normalized dose–response data points using an MWC allosteric model with three equivalent Glu-binding sites ($n = 3$) and the same $L$ value (81) (Fig. 4G, cyan-colored curve and Eq. 2). The resulting $K_d$ values (Table S2, same line of “3,” 0”) were used to calculate the theoretical $P_{\text{open, max}}$ by $1/(1 + c^2L) = 0.96$, which is also much higher than the experimental $P_{\text{open}}$ (0.86). Extrapolation of the cyan-colored curve in Fig. 4G (model with $n = 3$) until the theoretical $P_{\text{open, max}}$ is reached indicates a strong deviation of this curve from the Hill plot at high Glu concentrations. Curve fitting with other values for $n$ (one, or equivalent four or five Glu-binding sites) resulted in a theoretical $P_{\text{open, max}} \geq 0.95$ (Table S2). We therefore applied an MWC allosteric model with two equivalent and a third distinct Glu-binding sites ($n = 2, m = 1$), using the same $L$ value (81) (Fig. 4G, dashed black curve and Eq. 3). In this case, $K_d$ and $K_d^{\text{WT}}$ characterize the two equivalent Glu-binding sites in the closed and open states, respectively; and $K_d^{\text{WT}}$ characterizes the third Glu-binding site in the closed and open states, respectively [Table S2 (in bold)]. Scheme II describes the MWC allosteric mechanism that corresponds to the GluCl(L279W)/\beta WT receptor:

\[
\begin{align*}
A R &\quad \quad A R^{*} \\
K_d^{\text{WT}} &\quad \quad K_d^{\text{WT}}^{*} \\
K_d^{\text{WT}} &\quad \quad K_d^{\text{WT}}^{*} \\
A_1 R &\quad \quad A_1 R^{*} \\
K_d^{\text{WT}}^{*} &\quad \quad K_d^{\text{WT}}^{*} \\
R &\quad \quad R^{*} \\
A_2 R &\quad \quad A_2 R^{*} \\
K_d^{\text{WT}}^{*} &\quad \quad K_d^{\text{WT}}^{*} \\
A_3 R &\quad \quad A_3 R^{*} \\
K_d^{\text{WT}}^{*} &\quad \quad K_d^{\text{WT}}^{*}
\end{align*}
\]

where $c = K_d^{\text{WT}}/K_d^{\text{WT}}$ and $c' = K_d^{\text{WT}}/K_d^{\text{WT}}$. In this case, the theoretical $P_{\text{open, max}}^{\text{WT}} = 1/(1 + c^2L) = 1/(1 + c'^2L) = 0.89$, which is much closer to the experimental $P_{\text{open}}^{\text{WT}}$ (0.86) than to the theoretical $P_{\text{open, max}}^{\text{WT}}$ (0.86). Hence, if the fifth subunit is GluCl\beta, then it will give rise to $\alpha(+)\beta(-)$ and $\beta(+)/\beta(-)$ intersubunit interfaces (envisioned in Fig. 3J); however, based on the aforementioned results, the GluCl\beta(-) side is less likely to contribute to Glu binding. If the fifth subunit is GluCl, then it will give rise to $\alpha(+)\alpha(-)$ and $\alpha(+)\beta(-)$ intersubunit interfaces (envisioned in Fig. 4G, Right); so, the $\alpha(+)\alpha(-)$ intersubunit interface remains a reasonable candidate to form the third Glu-binding site. However, this working hypothesis required further experimental investigation. Because the GluCl\alpha(-) side was inferred to line the two Glu-binding pockets (Fig. 3 and main text), we introduced an $\alpha(+)T225N$ mutation (in loop C), in addition to the $\alpha$L279W mutation. The homologous mutation $[\beta(+)T229N]$ in the GluClWT/\beta T229N receptor was shown to increase the Glu-EC_{50} by approximately eightfold, compared with the GluClWT/\beta WT receptor (Fig. 3C and Table S1; presented again in Fig. 4A in gray for convenience). Hence, an $\alpha(+)T225N$ mutation was anticipated to affect a potential $\alpha(+)\alpha(-)$ intersubunit Glu-binding site, without directly interfering with Glu binding at the two $\beta(+)\alpha(-)$ sites. Fig. 4A shows that the dose–response curve of the GluCl(L279W,T225N)/\beta WT receptor is significantly shifted to the right relative to the curve of the GluCl(L279W)/\beta WT receptor, with an ~57-fold increase in the Glu-EC_{50}, and a decrease of the Hill coefficient to $n_H = 1.6$ (Table S1). These macroscopic properties resemble the properties displayed by the GluClWT/\beta WT receptor, which has two equivalent Glu-binding sites.

To quantify the effect of the $\alpha(+)T225N$ mutation, we determined the values of $P_{\text{open}}$, $P_{\text{open}}^{\text{max}}$, $P_{\text{open, max}}$, and $L$ for the GluCl(L279W,T225N)/\beta WT receptor [Fig. 4 D and F and Table S2 (footnotes)] and estimated its $P_{\text{open}}$ at varying Glu concentrations, as all described above for the GluClWT/\beta WT receptor. Then, a curve was fitted to the normalized dose–response data points using an MWC allosteric model with two equivalent Glu-binding sites ($n = 2$) and the experimental mean $L$ value (203) (Fig. 4H, dashed black curve and Eq. 2). The resulting $K_d$ values are provided in Table S2 (in bold). The theoretical and experimental maximum open probabilities were found to be equal (0.60), whereas other values for $n$ (one, or equivalent three, four, or five Glu-binding sites) resulted in higher theoretical $P_{\text{open, max}}$ values (Table S2). In addition, the analysis of the $\Delta$AICc selected the allosteric model with $n = 2$ as the most suitable MWC model for curve fitting in the GluCl(L279W,T225N)/\beta WT receptor case (Table S2). Hence, the results imply that this double-mutant receptor lost the third Glu-binding site, and its remaining two equivalent Glu-binding sites display slightly lower affinity and potency than the GluClWT/\beta WT receptor [Table S2 (in bold)]. Provided that the mutations have not changed the subunit stoichiometry (as argued in SI Text, section 3), the two Glu-binding sites of the GluCl(L279W,T225N)/\beta WT receptor likely lie at $\beta(+)\alpha(-)$ intersubunit interfaces (Fig. 4H, Right). As discussed above, the GluCl\beta(-) side is less likely to contribute to Glu binding, and so is an $\alpha(+)\beta(-)$ intersubunit interface. We therefore infer that the $\alpha(+)T225N$ mutation is likely located at an $\alpha(+)\alpha(-)$ intersubunit interface. Taken together, our results suggest that in the GluCl(L279W)/\beta WT receptor, an $\alpha(+)\alpha(-)$ intersubunit interface likely forms a third Glu-binding site (Fig. 4G, Right), whereas Glu binding to this interface is impaired by adding the $\alpha(+)T228N$ mutation (Fig. 4H, Right).
The Glu-binding affinity of the two equivalent β(+)/α(−) interfaces relative to the GluClαL279W/βWT receptor [Table S2 (in bold)]. We suggest that the mutation in the (+) side of the plausible α(+)/α(−) Glu-binding site interface could allosterically affect the other Glu-binding site interfaces. Combining all three mutations to produce a GluClαL279W,αT258N,αL279W/βWT receptor shifted the dose–response curve by 455-fold rightward relative to the GluClαL279W/βWT receptor (Fig. 4A and Table S1). This rightward shift is larger by ~90-fold than the fivefold rightward shift observed for the GluClαL279W/βT229N receptor, which suggests that also in the triple mutant, the α(+)/α(−) intersubunit interface has a strong allosteric relationship with the β(+)/α(−) Glu-binding site interfaces. Notably, the Hill coefficient decreased from nH = 2.6 in the GluClαL279W/βWT receptor to nH = 1.5 in the GluClαL279W/βT229N receptor (Table S1), suggesting that the β(+)/T229N mutation exerts a reciprocal allosteric effect on the third α(+)/α(−) intersubunit interface.

**Effect of the αL279W Mutation on the Responsiveness of the Heteromeric GluClαL279W/βWT Receptor to IVM.** The crystal structure of the homomeric GluClαL279W/βWT indicates that the backbone carbonyl oxygen of αL279 (L218 in GluClαcryst,R) forms a hydrogen bond with hydroxyl O13-H of IVM, whereas the αL279 side chain does not interact with IVM (23) (Fig. 4B, Left). Three-dimensional homology modeling predicts that a Trp side chain introduced at position α279 might form multiple contacts with IVM (Fig. 4B, Right). If so, how might this mutation affect the responsiveness of the GluClαL279W/βWT receptor to IVM? To answer this question, we had to determine the IVM EC50 for the WT and mutant receptors. However, unlike the fully reversible responses to Glu, after activation by IVM, the response could not be reproduced by reapplication of IVM even when the first IVM application was followed by a long-term wash (up to 30 min). Other groups also observed this phenomenon when the wash was applied for several minutes (13) or an hour (18). Hence, to quantify the effect of the mutation, we first used the methodology of Lester and coworkers (18) to determine the time constant of conductance development following IVM application. To this end, voltage ramps were carried out during the application of various IVM concentrations, with each application in a different cell. Fig. 5A shows an example of such an experiment. Superimposition of the output currents of the voltage ramps shows a sharp increase in slopes that reflects the robust IVM-induced conductance and a clear leftward shift (decrease) of the reversal potential that occurs mainly after the conductance reached its maximum (Fig. 5B). The shift of the reversal potential indicates a change in the Nernst potential for Cl−.
and in the electrochemical driving force acting on Cl– ions. The chloride conductance is defined by the slope of the current–voltage (I/V) relations extracted from the output currents of the voltage ramps, and could be determined at several membrane voltage spans. Fig. S6A shows the slope conductance determined between –75 mV and –65 mV, around the reversal potential, and between +10 mV and +20 mV, as a function of time. The rise time of the conductance increment was found to be similar for all of the three aforementioned voltage spans (Fig. S6A). Notably, during the applications of high IVM concentrations, the conductance rise was followed by a decrease in the conductance to a steady state in all voltage spans and in both the WT and indicated mutant receptors (Fig. S6A). Because the current decay under high IVM concentrations was faster at –65 mV than at +20 mV (Fig. S6B), and because the exponential fits of the conductance rise time were very similar at the different membrane voltage spans, we chose to analyze the conductance development further between +10 mV and +20 mV. Fig. 5 C and D shows the development of the conductance under the application of different IVM concentrations in different representative cells.

The exponential fits of the conductance rise time (e.g., Fig. 5 C and D, orange curves) provide the time constant of conductance development (τ), whose reciprocal (1/τ) increased linearly with the increase in IVM concentration (Fig. 5E). Because IVM does not readily dissociate from the receptor (13, 18) and the number of possible intermediate IVM-bound closed states is not known, the simplest possible kinetic model that could describe the activation mechanism by IVM would be one in which the channel opens when IVM binds and closes after a very long time when IVM dissociates. Scheme III describes this kinetic model:

$$\text{IVM} + R \xrightarrow{k_f} \text{IVM}\cdot R^* \xrightarrow{k_b} \text{IVM}$$

where R is the unoccupied closed receptor, IVM\cdot R* is the IVM-bound open receptor, and $1/\tau = k_t [\text{IVM}] + k_c$. The slope of the curves in Fig. 5E corresponds to the IVM association rate constant (k forward, k_t). The IVM dissociation rate constant (k backward, k_b) is the extrapolated intercept of the linear curve with the y axis in Fig. 5E. The apparent K_d would be $k_b/k_t$, giving $73 \times 10^{-9}$ M for IVM binding to the GluClRβ WT receptor ($k_b = 5.3 \times 10^{-5}$ s⁻¹ and $k_t = 7.3 \times 10^{2}$ s⁻¹ M⁻¹). In contrast, the apparent K_d for IVM binding to the GluClL279W/β WT receptor was $9.7 \times 10^{-9}$ M ($k_b = 3.4 \times 10^{-5}$ s⁻¹ and $k_t = 3.5 \times 10^{2}$ s⁻¹ M⁻¹), which indicates that the affinity of the mutant receptor for IVM is 7.5-fold higher than the affinity of the WT receptor for IVM. Note that because no experiments revealed that IVM could be washed out of the receptor (13, 18), the $k_b$ values are expected to be on the order of $<10^{-4}$ s⁻¹. However, the values here were found to be on the order of $10^{-2}$ s⁻¹, implying that IVM should be removable. We therefore cannot exclude the possibility that after opening of the GluClR ion channel by IVM, a subsequent conformational change leads to trapping of IVM between the transmembrane helices irreversibly.

Discussion

To determine unequivocally the subunit stoichiometry and arrangement in native GluClRβRs, high-resolution X-ray crystallography of heteropentameric receptors purified from the organisms that naturally express them is necessary. To the best of our knowledge, such a determination is yet out of reach. Hence, an alternative methodology must be considered. In Cys-loop receptors, the neurotransmitter-binding pockets lie at the interface between adjacent subunits (1–9). One could therefore use site-specific mutagenesis and biophysical characterization of activation mechanisms in recombinant receptors to find the types of subunits that line the agonist-binding pockets. By working with recombinant receptors, however, one cannot exclude the possibility that the ratio of subunit cDNA transfected, the type of the expressing cell, or a mutation might influence the receptor’s subunit composition (e.g., 45, 57, 58). We nevertheless argue that the specific mutations we introduced are less likely to change the subunit stoichiometry of the recombinant receptors studied here (see SI Text, section 3).

In various Cys-loop receptors, the β1β2, Cys, and β8β9 loops were shown to play a key role in transducing the agonist-binding energy into ion-channel gating force (35–44). Here, we first demonstrated that although the homomeric GluClRβ is not responsive to Glu, the β1β2, Cys, and β8β9 loops of the GluClR subunit are fully capable of coupling Glu binding to channel gating in a heteromeric GluClRβ microchimera that has the sequences of the α-subunit loops. Subsequently, we undertook to identify the intersubunit interfaces involved in Glu accommodation by heteromeric GluClRβRs. Taking advantage of the crystal structure of a truncated homomeric GluClRα WT R as a template, we built a 3D homology model for the GluClβ subunit. Then, based on the two structures, we introduced single-site mutations in the (∓) side of either the GluClR subunit or the GluClβ subunit at positions carrying residues that putatively interact with Glu. Characterization of the effects of these mutations on the receptor function allowed us to suggest that in the heteromeric GluClRβRs studied here, the (∓) side of the α subunit, rather than the (∓) side of the β subunit, contributes complementary components to Glu binding. Single-site mutations and functional analysis of heteromeric GluClRβRs carrying mutations in the (+) side of the β subunit imply that this side contributes principal components to Glu binding.

When considering the GluClRβ WT receptor in terms of the MWC allosteric mechanism, we infer that a maximum of two equivalent binding sites can be occupied by Glu [Fig. 3F, Scheme I, and Table S2 (in bold)]. Provided that the aforementioned single-site mutations introduced at the intersubunit interfaces have not changed the subunit stoichiometry (as argued in SI Text, section 3), Glu binding likely takes place at two β(+)α(–) intersubunit interfaces. Hence, one can envision a subunit arrangement as illustrated in Fig. 3F for a recombinant GluClRβ WT receptor expressed in CHO cells, with no information regarding the type of the fifth subunit.

When considering the GluClL279F/β WT receptor in terms of the MWC allosteric mechanism, we infer that Glu can occupy three sites (Fig. 4G and Scheme II). These sites are (i) two equivalent Glu-binding sites that are likely located at β(+)α(–) intersubunit interfaces and display considerably higher affinity for Glu than their homologous binding sites in the GluClR WT/β WT receptor and (ii) a third distinct site with slightly lower Glu-binding affinity, in both the resting (closed) and active (open) receptor states [Table S2 (in bold)]. We argue that the third Glu-binding site is formed between two adjacent α subunits; the arguments for that conclusion are as follows:

i) In CHO cells, a WT GluClβ subunit does not assemble into a homopentamer capable of responding to Glu or IVM, which indicates that the β subunit has difficulties in creating Glu-binding β(+)β(–) intersubunit interfaces (Fig. S2A and Table S1).

ii) In the heteromeric GluClRβRs studied here, three single-site mutations in the β(–) side did not lead to drastic effects on the receptor activation by Glu, unlike the case of the same mutations introduced at the homologous positions in the α(–) side.

iii) The homomeric GluClL279F/β WT receptor responds to very high Glu concentrations (Fig. S7), indicating the capability of an α(+)α(–) intersubunit interface to accommodate Glu (with no need for IVM prebinding).
iv) Adding a mutation in loop C (+ side) of the αL279W subunit gave rise to an αL279WT,C258N)/βWT receptor that lost the third Glu-binding site (Fig. 4H), whereas the remaining two equivalent Glu-binding sites displayed microscopic equilibrium dissociation constants slightly higher than the microscopic equilibrium dissociation constants of the GluClCmT/Pj WT receptor [Table S2 (in bold)].

A third Glu-binding site located at an α(+)/β(−) intersubunit interface requires that the fifth subunit would be a GluClα subunit. We therefore suggest that the subunits of the recombiant heteromeric GluClβRαRs studied here assemble in an antickwise β-α-β-α-α fashion, as viewed from the extracellular side (Fig. 4G and H, Right). Notably, previous studies show that expressing the heteromeric αL4Iβ4 nACHRs under conditions that favor an α4β2c4 stoichiometry (three α4 and two β2 subunits) results in a receptor having two α(4+)β2(−) interfaces with high agonist sensitivity and a third binding site at the α(4+α4(−)) interface that displays low agonist sensitivity (59–61).

As discussed in Results, the function of heteromeric receptors containing the αL279W mutation, together with a Thr→Asn substitution in loop C of the α subunit, β subunit, or both subunits, suggests that the two intersubunit interface types, α(+)/β(−) and β(+)α(−), likely affect each other allosterically. Possible structural reasons for this mutual allosteric influence are provided in SI Text, section 4. Interestingly, an allosteric relationship between different extracellular intersubunit interfaces was proposed for the heteromeric αL272Gβ8A receptor (62). In the latter case, conformational movements induced by benzodiazepine binding at the α/γ extracellular interface were suggested to propagate across the α subunit to the β/α GABAβA site (63).

In the GluClCmR, L218 (αL279) in the full-length subunit used here) is part of the IVM-binding pocket located between M1 and M3 of adjacent subunits (23) (Fig. 4B, Left). The clear increase in the affinity of the GluClCmT/Pj WT receptor for IVM (Fig. 5E, 7.5-fold) implies that the IVM-binding pockets of the heteromeric receptor are homologous to the IVM-binding pockets of the homomeric GluClCmR. The structural mechanism underlying the effect of the αL279W mutation in the IVM-binding site is not clear. However, the microscopic equilibrium dissociation constants for Glu binding determined here imply that the conformational change induced by this mutation in the IVM-binding pocket propagates to the Glu-binding pockets and affects their affinity for Glu. It is not known whether Glu and IVM induce the same conformational change in the coupling loops. In the heteromeric αL2β2/GABA receptor, for example, it was demonstrated that positive benzodiazepine modulators induce movements in loop F (β8β9 loop) of the γ2 subunit near the transmembrane channel domain (63). Such movements were not triggered by the binding of GABA, the allosteric modulator pentobarbital, or the inverse agonist methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (63).

In conclusion, our study provides evidence that the C. elegans heteromeric GluClR contains three α subunits and two β subunits arranged in an antickwise β-α-β-α-α fashion, as viewed from the extracellular side, with two Glu-binding sites located at the β(+)α(−) intersubunit interfaces. The α(+)β(−) intersubunit interface creates a third “dormant” Glu-binding site that becomes functional upon a conformational change induced by a mutation in the IVM-binding pocket.

Materials and Methods

Additional experimental procedures and data analyses are described in SI Materials and Methods.

Data analysis and mathematical modeling were performed using the Clampfit 10 program implemented in pClamp 10, and GraphPad Prism software.

Dose–response curves were fitted to the data points by a nonlinear regression using the Hill equation (Eq. 1):

\[
\frac{J}{J_{\text{max}}} = \frac{1}{1+10^{(EC_{50} - \log \text{Glu}_{\text{con}})}}
\]

where \(J\) is the current response, \(J_{\text{max}}\) is the maximal current response, \(EC_{50}\) is the agonist effective concentration that elicits 50% of the maximal current response, [Glu] is the concentration of Glu, and \(n_h\) is the Hill coefficient.

For the allosteric modeling, Eq. 2 was used:

\[
P_{\text{open}} = \frac{1}{1 + L + \left(\frac{1 + \text{Glu}_{\text{K}_{\text{d,R}}} + \text{Glu}_{\text{K}_{\text{d,R}}}}{1 + \text{Glu}_{\text{K}_{\text{d,R}}} + \text{Glu}_{\text{K}_{\text{d,R}}}}\right)^n}.
\]

where \(P_{\text{open}}\) is the open probability estimated at varying Glu concentrations (4c) (main text) [Glu] is the concentration of the agonist (Glu) for which there are \(n\) equivalent binding sites, each with a microscopic equilibrium dissociation constant of \(K_{\text{d,R}}\) in the resting (closed) state and \(K_{\text{d,R}}\) in the open (active) state. \(L\) is the equilibrium constant of the two states in the absence of ligands. The \(L\) values were determined by functional experiments, as described in the main text.

For a receptor phenotype that does not behave as a receptor having only \(n\) equivalent Glu-binding sites, Eq. 3 [cf. Karlin (48)] was used:

\[
P_{\text{open}} = \frac{1}{1 + L + \left(\frac{1 + \text{Glu}_{\text{K}_{\text{d,R}}} + \text{Glu}_{\text{K}_{\text{d,R}}}}{1 + \text{Glu}_{\text{K}_{\text{d,R}}} + \text{Glu}_{\text{K}_{\text{d,R}}}}\right)^n}.
\]

where \(n\) is the number of sites that Glu binds with microscopic equilibrium dissociation constants, \(K_{\text{d,R}}\) in the closed state and \(K_{\text{d,R}}\) in the open state.

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