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The desensitization pathway of GABA_A receptors, one subunit at a time

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Abstract

GABA_A receptors mediate most inhibitory synaptic transmission in the brain of vertebrates. Following GABA binding and fast activation, these receptors undergo a slower desensitization, whose conformational pathway remains largely elusive. To explore the mechanism of desensitization, we used concatemeric $\alpha 1\beta 2\gamma 2$ GABA_A receptors to selectively introduce gain-of-desensitization mutations one subunit at a time. A library of twenty-six mutant combinations was generated and their bi-exponential macroscopic desensitization rates measured. Introducing mutations at the different subunits shows a strongly asymmetric pattern with a key contribution of the $\gamma 2$ subunit, and combining mutations results in marked synergistic effects indicating a non-concerted mechanism. Kinetic modelling indeed suggests a pathway where subunits move independently, the desensitization of two subunits being required to occlude the pore. Our work thus hints towards a very diverse and labile conformational landscape during desensitization, with potential implications in physiology and pharmacology.

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Abbreviations: ECD, extracellular domain; GABA, γ -aminobutyric acid; GABA_AR, type A γ -aminobutyric acid receptor; pLGIC, pentameric ligand-gated ion channel; MWC, Monod-Wyman-Changeux; nAChR, nicotinic acetylcholine receptor; TEVC, two-electrode voltage clamp; TMD, transmembrane domain.

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Author contributions

M.G. designed the study; M.G. and N.B. performed molecular biology and TEVC recordings; M.G. analysed the data and performed the kinetic modelling; all authors discussed results; M.G. and P-J.C. wrote the manuscript; P-J.C. and M.G. acquired funding.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical compliance

We used oocytes from *Xenopus laevis*. Ovaries were obtained from CRB Xenopes (UMS 3387, CNRS / Université de Rennes, Rennes, France). No animals were handled by the authors.

For the supply of *Xenopus laevis* ovaries, authorization was granted to CRB Xenopes by the Direction Départementale de la Cohésion et de la Protection des Populations, Santé et Protection Animales et Végétales, at the Préfecture d'Ille-et-Vilaine, under the number A 35-238-42.

INTRODUCTION

GABA_A receptors (GABA_ARs) are the main inhibitory synaptic receptors in the forebrain of vertebrates, and are involved in key physiological and pathological processes such as memory, epilepsy, anxiety, sedation. This is well-illustrated by their medical significance, since the most prevalent GABA_ARs are the target of the widely used benzodiazepine class of drugs¹.

GABA_ARs belong to the pentameric Ligand-Gated Ion Channel (pLGIC) superfamily, which also comprises the anionic glycine receptor, as well as the excitatory 5HT₃ serotonin receptors and the nicotinic acetylcholine receptors (nAChRs)². Upon binding of their agonist, the transmembrane pore of pLGICs quickly opens to enable the selective flow of permeant ions across the plasma membrane, thereby affecting cell excitability. However, during sustained binding of the agonist, most pLGICs will gradually enter a shut-state refractory to activation, called the desensitized state, thereby preventing excessive activation³. The exact roles of desensitization *in vivo* are still debated, but potentially include the reduction of responses during high-frequency release of neurotransmitter⁴, the prolongation of synaptic currents due to a contribution of the recovery from desensitization⁵, as well as the modulation of extra-synaptic receptors subjected to tonic activation by low ambient concentrations of neurotransmitters⁶.

Recent functional and structural studies, mostly performed on anionic pLGICs, provide compelling evidence for a “dual-gate” model, in which the transmembrane domain (TMD) of pLGICs contains both an activation gate, located in the upper half of the channel, and a desensitization gate, located at the intracellular end of the channel^{3,7-11}. Structural work on homopentameric receptors always showed symmetrical structures^{7,9,11}, while the recent structures of the heteromeric GABA_A receptor show important asymmetric features within the extracellular domain (ECD)¹⁰, but still a strong pseudo-symmetrical organization of the TMD.

The current view of the dual-gate model thus supports that resting, active and desensitized states are essentially symmetrical at the level of the TMD, desensitization involving, in the lower part of the channel, a movement of all subunits to occlude the permeation pathway. However, desensitization is a multiphasic process, since the sustained application of agonist elicits currents that desensitize with several distinct decay time constants, which are usually portrayed by the existence of “fast” and “slow” desensitized states (noted D_{fast} and D_{slow} below, respectively)^{3,12–15}. The structural rearrangements underlying these distinct desensitization components remain elusive. In particular, it is currently unknown whether subunits rearrange in a concerted manner, with D_{fast} and D_{slow} reflecting distinct states at the single-subunit level, or whether individual subunits can rearrange independently with distinct time courses. The first scheme would predict that pLGICs only visit pseudo-symmetrical states during desensitization, while the latter scheme would imply that desensitization involves asymmetrical states.

To examine the contribution of individual subunits to this process, we herein introduced gain-of-desensitization mutations in each individual subunit, both one by one and in combinations, and assessed their interplay during desensitization. We selected mutations nearby the desensitization gate, which were previously found to specifically alter the desensitization kinetics and amplitude, without significant alteration of the upstream activation process. Since the stereotypical synaptic GABA_ARs are composed of two α , two β and one γ subunits^{16,17}, targeting a single α or β subunit within the pentamer is out of reach using classical site-directed mutagenesis approaches. To circumvent this problem, we used a concatemeric construct, whereby all five subunits are linked together by polyglutamine linkers. Owing to the fixed organization of subunits within this concatemer, we could introduce and combine gain-of-desensitization mutations in a defined manner, ensuring the perfect homogeneity of the resulting recombinant GABA_ARs populations. We generated a library of 26 combinations

of mutated subunits, recorded their macroscopic desensitization kinetics, and analyzed the data by Markov-chain kinetics simulations.

RESULTS

A pentameric concatemer recapitulates the biphasic desensitization profile of the GABA_AR reconstituted from loose subunits

To force the subunit arrangement, we used a concatemer previously described¹⁸. It consists of $\beta 2$ - $\alpha 1$ - $\beta 2$ - $\alpha 1$ - $\gamma 2$ subunits fused together with polyglutamine linkers that are 15- to 20-residues long. When assembled in the counter-clockwise orientation as seen from the extracellular space, it shows a canonical organization with two GABA binding sites at the $\beta 2$ - $\alpha 1$ interfaces and one benzodiazepine site at the $\alpha 1$ - $\gamma 2$ interface (Figure 1A). In contrast, in the clockwise orientation, the concatemer would carry a single GABA binding site and no benzodiazepine binding site. This orientation, if it occurs, should therefore yield minimal, if any, GABA-gated currents and no potentiation by benzodiazepine. We previously showed that expression of the concatemer in oocytes yields robust GABA-elicited currents with an apparent affinity for GABA similar and a potentiation by benzodiazepine indistinguishable from that of GABA_A receptors expressed from loose subunits¹⁸. This shows that the counter-clockwise assembly largely dominates the electrophysiological response. It also suggests that the linkers connecting the concatemer's subunits do not constrain the conformational dynamics of the ECD, since this would impact on the pharmacology of extracellular ligands.

To record desensitization kinetics at the best possible temporal resolution using Two-Electrode Voltage Clamp (TEVC) recordings of *Xenopus laevis* oocytes, we minimized the dead volume of our set-up and applied GABA at a supersaturating concentration (10 mM). This boosted the rise of GABA concentration in the recording chamber, enabling us to

optimize the onset of the electrophysiological responses in the 20-25 ms timescale (corresponding to 20%-80% current rise times). As discussed in a previous publication, TEVC recordings of *Xenopus laevis* oocytes are well-suited to the study of desensitization of pLGICs owing to the robustness of the approach, which contrasts with the very high inter- and intra-cellular variability when recording desensitization with patch-clamp methods³. Recordings of the wild-type concatemer show robust currents, which display desensitization kinetics and amplitudes similar to that of the conventional $\alpha 1\beta 2\gamma 2$ GABA_ARs assembled from unconnected subunits (Figure 1B&C; Supplementary Table 1; see ref⁸), further arguing that the constraints provided by the linkers do not affect the conformational changes at play during desensitization. Desensitization shows two well-separated components that are perfectly resolved by our procedure, a fast ($\tau_{\text{fast}} = 4.8 \pm 1.2$ s) and a slow one ($\tau_{\text{slow}} = 24.4 \pm 7.8$ s). The amplitude of the former carries about a third of the total desensitization amplitude, both fast and slow rates together yielded a weighted desensitization time constant (τ_w) of about 18 s. The residual current remaining after one minute of GABA application accounted for about 10% of the peak current (Figure 1B&C; Supplementary Table 1).

Single desensitizing mutations show contrasting phenotypes depending on their location within the pentamer

For gain-of-desensitization mutations in $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits, we chose the valine mutation at the 5' position of the third transmembrane segment (M3), namely $\alpha 1^{\text{N307V}}$ on $\alpha 1$ subunits (SU2 and SU4), $\beta 2^{\text{N303V}}$ on $\beta 2$ subunits (SU1 and SU3) and $\gamma 2^{\text{H318V}}$ on the single $\gamma 2$ subunit (SU5) (Figure 1D-F) - this prime notation, akin the one largely used for the M2 segment, starts at the cytoplasmic end of the M3 segment¹⁹. Indeed, on GABA_ARs composed of loose subunits, we previously showed that these mutations markedly speed up the onset of desensitization of $\alpha 1\beta 2\gamma 2$ GABA_ARs⁸. We also showed that mutations in this region of the

transmembrane domain do not alter significantly the concentration-response curve of the GABA-elicited peak currents measured before the onset of desensitization. This indicates only a weak effect of the mutations on the resting-to-active state transition, and a major effect on the active-to-desensitized state transition.

Mutations were introduced one at a time on the concatemer. We define C^{WT} as the wild-type concatemer, C^i the concatemer with a single M3-5' valine mutation on subunit number i , and C^{ij} the concatemer where subunits i and j are both mutated, up to C^{12345} where all subunits are mutated (Figure 2A).

For the single mutations, C^1 ($SU1 = \beta2$) and C^2 ($SU2 = \alpha1$) display desensitization kinetics similar to that of C^{WT} , while constructs C^3 , C^4 and C^5 displayed robust gain-of-desensitization phenotypes (Figure 2; Supplementary Figure 1 & Supplementary Table 1), yielding weighted desensitization rates of 6.2, 3.4 and 3.3 s, respectively, as compared to 18 s for C^{WT} . The three mutations accelerate fast desensitization by about 2-fold and slow desensitization by about 3-fold ($\tau_{fast} = 2.7, 2.9$ and 2.9 s; $\tau_{slow} = 7.1, 7.3$ and 7.2 s for C^3, C^4 and C^5 , respectively). C^4 and C^5 in addition increase the relative amplitude of the fast component ($\%A_{fast} = 20.0\%, 86.7\%$ and 86.3% for C^3, C^4 and C^5 , respectively), explaining their stronger effect. Of note, the C^5 construct displays an identical desensitization phenotype compared to the single mutant $\alpha1\beta2\gamma2^{H318V}$ expressed from unconnected subunits⁸, which is consistent with the assumption that our concatemeric design does not affect the desensitization properties of GABA_ARs, even in the context of receptors harbouring M3-5' mutations. This is not surprising, given that the linkers are located in the extracellular part, and cannot interact directly with the M3-5' residues located at the intracellular end of the pore.

It is noteworthy that the mutations are located at the cytoplasmic end of the TMD, with the side-chain of the mutated residue facing the M1-M2 linker of the neighbouring subunit (Figure 1F). Therefore, C^1, C^2, C^3, C^4 and C^5 are mutated at $\beta2-\alpha1, \alpha1-\beta2, \beta2-\alpha1, \alpha1-\gamma2$ and

$\gamma 2$ - $\beta 2$ interfaces, respectively. The different mutations being introduced at different interfaces, it was expected that they display different phenotypes. However, the difference between C¹ and C³ is surprising, since they both correspond to mutations at the $\beta 2$ - $\alpha 1$ interface, showing virtually identical microenvironment. This indicates that the effect of the single mutations not only depends on the nature of the mutated interface, but also on the particular position of the mutated subunit within the pentamer.

Combining mutated subunits increases desensitization kinetics and reveals synergistic effects

To investigate the functional interaction between mutations at the various interfaces, we built an extensive library of twenty-six cDNAs including concatemers comprising two mutations (ten different constructs), three mutations (six different constructs), four mutations (four different constructs) or five mutations (one single construct, C¹²³⁴⁵), and assessed their desensitization profile as described above (Figure 2; Supplementary Figure 1 & Supplementary Table 1).

Recordings confirmed the modest effect of SU1 and SU2 mutations, which produce small effects when performed on concatemers with background mutations at other subunits (0.8 to 1.8-fold decrease in τ_w for SU1 and 1.1 to 2.8-fold for SU2, among 9 background mutated concatemers for both). They also confirm the intermediate effect of SU3 (2 to 6.3-fold decrease in τ_w among 10 background mutated concatemers), and the marked effect of SU4 and SU5 (effect of 5 to 16-fold among 9 and 10 mutated concatemers for SU4 and SU5, respectively; Supplementary Figure 42).

In all cases, combining gain-of-desensitization mutations together adds up to increase desensitization kinetics. For instance, the double mutant C⁴⁵ displays a fast desensitization

component ($\tau_{\text{fast}} = 180$ ms) 26-fold faster than C^{WT} , accounting almost entirely for the overall desensitization ($\%A_{\text{fast}} = 98.8\%$), and a barely measurable steady-state current ($\%I_{\text{res}} = 0.8\%$). Such phenotype is further strengthened by mutating SU3: C^{345} desensitizes with an even faster desensitization component in the 70 ms timescale. Mutating all five subunits gave a slightly more profound phenotype, with a fast desensitization component of 40 ms (see construct C^{12345} ; Figure 2D & E; Supplementary Table 1). Of note, for constructs akin C^{345} and C^{12345} , the fast component is so fast that we probably miss a sizeable fraction of the peak current, thereby overestimating the amplitude of the slow desensitization component and the measurement of the relative steady-state current. Also, the steady-state current values and the amplitudes of the slow desensitization components are barely measurable for constructs displaying the fastest and most complete desensitization, rendering the related values ($\%I_{\text{res}}$ and τ_{slow}) unreliable.

To investigate the additivity of the effect of the various mutations, we first compared the effect of individual mutations on the weighted desensitization kinetics of different concatemers with background mutations (Supplementary Figure 42). While this analysis is crude, the series of double mutants already suggests some level of inter-subunit coupling. Indeed, while the SU1 mutation barely affects the desensitization of C^{WT} , it increases the weighted desensitization kinetics of C^2 by 75%, thereby hinting towards a coupling between SU1 and SU2. More strikingly, SU4 mutation speeds up desensitization about 5-fold on both C^{WT} , C^1 , C^2 and C^3 backgrounds, while it increases the weighted desensitization kinetics of C^5 by 15-fold, clearly hinting towards synergistic effects of SU4 and SU5 mutations.

Second, we compared the desensitization profiles of C^{34} and C^{35} . Since mutating SU4 or SU5 yields identical desensitization phenotypes (Figure 2C-E; Supplementary Table 1), C^{34} and C^{35} should yield identical phenotypes if the effects of mutations were additive. Our data contradict such hypothesis, since both desensitization components of C^{35} are faster than the

ones of C³⁴, resulting in a 55% faster weighted desensitization rate (Figure 2C-E; Supplementary Table 1). Thus, the effects of mutating the M3-5' residues are non-additive, especially for SU3 and SU4 or SU3 and SU5 subunit combinations.

The conformational pathway of desensitization involves asymmetrical and non-concerted quaternary motions: implementation of a general model

The present analysis unravels two key features governing the desensitization kinetics.

First, the markedly different effects observed upon mutation of SU1 and SU3, which both involve homologous mutations that are located in identical micro-environments, show that strongly asymmetrical motions are involved in the desensitization pathway. Since SU3 mutation has a strong effect on desensitization, the structural reorganization at this interface appears to be a limiting process. In contrast, mutation in SU1 has very weak effect, suggesting either a small structural reorganization at this level, or, more likely, that the structural reorganization would not be rate limiting (see Discussion).

Second, the marked non-additive nature of the mutations, as discussed above, is not compatible with a concerted mechanism. Indeed, in such a scheme, the effect of mutations should directly translate their impact on the free energy landscape of the receptor, and should thus be additive.

As an illustration, we attempted to fit the whole set of data with a concerted model, in which the receptors can only visit a handful of pseudo-symmetrical conformations that include a fast and a slow desensitized state (Supplementary Figure 23A-E). Here and throughout the manuscript, each model was built as a Markov-chain kinetic scheme and the whole-cell currents activated by a supersaturating concentration of GABA were simulated using the software QUB²⁰ (Supplementary Table 2). However, adjusting the parameters to correctly fit

the desensitization of C^{WT} , C^4 and C^5 , did not account for their synergistic effect since the simulated C^{45} τ_{fast} and τ_w values are respectively 4.7 and 4.1-fold higher than the values observed experimentally (Supplementary Figure 23F-G).

To implement the asymmetric and non-concerted properties, we turned to a radically different scheme in which all subunits can desensitize independently from the other subunits (Figure 3). In this model, each subunit can enter its desensitized conformation while the other subunits are either in their open or desensitized conformations. For simplicity, we decided to implement only the desensitization of SU3, SU4 and SU5, since these subunits are by far the main contributors to the phenotypes in the dataset. This enabled us to reduce the model to ten different states, rather than thirty-four distinct states involving all subunits. We also strongly simplified the activation transition whereby the resting receptor (R state) binds the agonist (AR state) and subsequently open (AO state). The model thus does not account for unliganded receptors openings (O state) that rarely occur at wild-type $\alpha 1\beta 2\gamma 2$ GABA_ARs, with a spontaneous open probability as low as 10^{-5} in the absence of agonist²¹, nor does it include the binding of two GABA molecules: we only considered the gating equilibrium for fully occupied receptors, as we work with supersaturating concentrations of GABA. From the AO state, either SU3, SU4 or SU5 can desensitize, to produce AD₃, AD₄ or AD₅ states, respectively. From these, the receptor can be further driven into states where two subunits are desensitized, e.g. desensitization of SU5 from the AD₄ state leads to the AD₄₅ state, where both SU4 and SU5 are desensitized. Finally, in that instance, SU3 could also desensitize to yield the AD₃₄₅ state, in which all three subunits are desensitized.

Using this general model, we progressively tuned the kinetic and functional parameters to best fit the dataset.

Model I, in which desensitization of a single subunit shuts the channel, shows anti-synergistic behaviour

We first postulated that the receptor is functionally desensitized, i.e. non-conducting, as soon as one subunit is desensitized, with only the AO state allowing the passage of ions (Figure 4A).

In model I, the desensitization and recovery rates (δ^+ and δ^-) for each subunit do not depend on the state of the other subunits. For simplicity, the parameters for SU4 and SU5 are set equal, since C⁴ and C⁵ display similar phenotypes. Thus, only four parameters (δ^+ , δ^- , δ_3^+ and δ_3^-) are used to constrain the desensitization of C^{WT}, i.e. exactly the number of independent numerical constraints provided by the experimental data (τ_{fast} , τ_{slow} , %A_{fast}, %I_{res}). We also assumed that mutating subunit i simply increases its desensitization rate by a ratio c_i^+ (Figure 4B).

For each set of parameters, we performed kinetic simulations using QUB (Figure 4 and Supplementary Table 3). Data are then analyzed using bi-exponential fitting of each virtual recording. In every simulation, we included all combinations of SU3, SU4 and SU5 mutants, from C^{WT} to C³⁴⁵.

In simulation “**a**”, we set up the parameters to reproduce C^{WT} and single mutant concatemers (Figure 4C, D & F and Supplementary Table 3). However, these parameters largely underestimate the kinetics of the fast desensitization component for the double mutant C⁴⁵: simulation **a** predicts a value of 1.76 s for the τ_{fast} of C⁴⁵, i.e. 10-fold slower than the experimental value. In simulation “**b**”, we used the same parameters for C^{WT}, and set up the c_i^+ ratios to reproduce the C⁴⁵ phenotype (Figure 4C, E & F and Supplementary Table 3). In that situation, we now largely overestimate the kinetics of the fast desensitization component for the single mutants C⁴ and C⁵: simulation **b** predicts a value of 0.34 s for the τ_{fast} of both C⁴

and C^5 , i.e. an order of magnitude faster than the experimental values. In this particular example, it is striking that model I actually predicts *anti-synergistic* effects when mutating SU4 and SU5, with the fast desensitization kinetics of both the single and double mutants being similar (Figure 4F).

Model I is thus incompatible with the dataset, and the reason is actually straightforward: if one desensitized subunit is enough to shut the pore, there should be a limiting fast subunit, whose mutation should have a strong effect on the kinetics of the fast desensitization component. This is not what we observe experimentally: the single mutant concatemers with the strongest phenotypes, C^4 and C^5 , only display 40% increases in τ_{fast} (see above).

Model II, in which at least two desensitized subunits are required to shut the pore, accounts for the synergy between SU4 and SU5 mutations

We consequently modified the kinetic model to incorporate a key hypothesis: namely, that functional desensitization of the channel involves the rearrangement of at least two subunits, i.e. that AO, AD₃, AD₄ and AD₅ do conduct ions, while AD₃₄, AD₄₅, AD₃₅ and AD₃₄₅ do not (model II, Figure 5A).

To simulate responses with steady-state currents consistent with experimental values, we also allowed mutations to increase the rates for desensitization recovery of the mutated subunits (Figure 5B; Supplementary Table 3). Indeed, not enabling this increase in recovery rates yields largely overestimated steady-state desensitization levels (Supplementary Figure 4; see the residual currents in panel F). Using this model II, we could perfectly account for the fast desensitization rate of C^4 , C^5 and C^{45} (Figure 5C-E). When SU4 is mutated, SU5 desensitization still provides a limiting step for functional desensitization, acting as a brake, while in C^{45} both “brakes” are relieved, enabling the channel to desensitize with fast kinetics,

thereby generating a synergistic effect. This serves as a gentle reminder for studies using mutant-cycle analysis: it is indeed possible to have a strong functional coupling between non-interacting residues located far apart in a receptor's structure, if their motions are not concerted.

While model II accounts for the main features of the dataset, we further refined it to precisely fit some desensitization kinetics. Indeed, simulation of C^3 shows a mono-exponential process with $\%A_{\text{fast}} = 100\%$ (Figure 5D & F), and an overestimated residual current (Figure 5G; Supplementary Figure 35). To circumvent this issue, we assumed that mutating SU3 increases the desensitization and recovery rates of SU4 (model II- β ; Supplementary Figure 46). From a structural point of view, such hypothesis seems plausible: the M3-5' residue mutated in SU3 is located at the interface with SU4 (Figure 1A&D-F), potentially interfering with conformational rearrangements of SU4. Using this model II- β , we could correctly simulate C^3 with two components for desensitization, (Supplementary Figure 57A & B and Supplementary Table 3).

Still, for C^{WT} and C^3 , model II- β produces an overestimation of both the fast component amplitude and the residual current (Supplementary Figure 57C&D). Increasing the desensitization equilibrium constant (δ^+/δ^-) for SU4 and SU5 would reduce the amount of residual current, but would also lead to an increase in $\%A_{\text{fast}}$ further out of the experimental range. Moreover, the rates of the slow desensitization components and the amplitudes of the fast components are both underestimated for C^4 and C^5 , as well as for multiple mutant combinations (Supplementary Figure 57B & C).

Model III: adding inter-subunit coupling provides the best fit to experimental data

We finally improved the model by adding a degree of structural coupling between adjacent subunits. We postulated that desensitization of a particular subunit would favour desensitization of its neighbouring subunits. We thus incorporated coupling constants between subunit pairs in model II- β , and performed iterative kinetic modelling to minimize the divergences with electrophysiological data. The best fit was achieved assuming that, first, desensitization of SU4 decreases the recovery rate of SU5 by $\epsilon=10$ -fold - and vice versa, and second that desensitization of SU4 increases the desensitization rate of SU3 by $\gamma=100$ -fold – and vice versa (Figure 6A, Supplementary Table 3). Apart from these couplings, model III retains all features from model II- β (Figure 6A&B). Of note, we don't need to include any effect of SU4 or SU5 mutation on the recovery from desensitization (i.e. $c_4^- = c_5^- = 1$; Supplementary Table 3).

As shown in Figure 7, model III largely accounts for experimental data, with experimental traces and simulated responses overlaying well (Figure 7A-H), including for the wild-type situation. The kinetics of the fast desensitization component, which are the most reliable experimental constraints in the dataset, are particularly well simulated (Figure 7I). The amplitudes of the fast component are overall in good agreement with the data, even though they are significantly underestimated for constructs C⁵, C³⁴ and C³⁵ (Figure 7K), while slow desensitization rates and steady-state currents are also underestimated for C⁴⁵ and C³⁴⁵ (Figure 7J & L). Those minor discrepancies might reflect the contribution of SU1 and/or SU2 to the receptors' desensitization, or even additional effects of the mutations (see Discussion below). Measurement errors on residual currents and slow desensitization kinetics might also occur when recording strongly desensitizing constructs.

Altogether, the whole set of data is consistent with a non-concerted model for pLGICs GABA_ARs' desensitization, characterized by three main features: 1) subunits can rearrange one at a time during desensitization, the multiple temporal components of desensitization

reflecting the existence of intermediate asymmetrical desensitized states; 2) rearrangements of adjacent subunits during desensitization are nonetheless partially coupled; and 3) the desensitization of at least two subunits is required to shut the pore, i.e. to lead to functional desensitization.

DISCUSSION

To illustrate the main features of our model of wild-type $\alpha 1\beta 2\gamma 2$ GABA_ARs desensitization, we show in Figure 8A the time-dependence of the various desensitized states' occupancies during desensitization. Since SU4 and SU5 desensitize the fastest, the receptors in the active state will transit first through a pre-desensitized open-pore state, in which either SU4 or SU5 is desensitized (Supplementary Figure 68). Functional desensitization, i.e. loss of electrophysiological response, subsequently occurs upon desensitization of the second fast subunit to yield the AD₄₅ state (Figure 8A). The final step along the desensitization pathway would correspond to the desensitization of SU3, resulting in the slow component of desensitization, i.e. the entry in the AD₃₄₅ state (Figure 8A). Like in all kinetic schemes where the slow- and fast-desensitized states are connected, this final step slowly depletes receptors from the fast-desensitized pool, which in turn displaces the overall population away from active conformations. We can thus extract the kinetically favoured pathway and provide a schematic structural depiction of the movements of the M2 helices during desensitization, as shown in Figure 8B. Interestingly, the requirement for two desensitized subunits to occlude the pore provides a framework to interpret results at $\alpha 7$ nAChRs, whose desensitization is blocked by PNU-120596. Indeed, it was shown that at least four $\alpha 7$ subunits need to be bound by PNU-120596 in order to block desensitization, meaning that as soon as two subunits are unbound, the receptors can undergo functional desensitization²². We thus suggest that our kinetic scheme may be extended to the entire pLGIC family.

The whole set of data points to the $\gamma 2$ subunit as a major determinant of the desensitization of $\alpha 1\beta 2\gamma 2$ GABA_ARs. Interestingly, the TMD of the $\gamma 2$ subunit appears highly flexible in detergent conditions, its TMD collapsing within the pore when $\alpha 1\beta 3\gamma 2$ GABA_ARs are solubilized in decylmaltoside neopentylglycol²³ or n-dodecyl- β -D-maltopyranoside^{24,25}. The addition of lipids stabilizes the $\gamma 2$ TMD in a more physiologically relevant conformation²³, but it still remains highly mobile and necessitates nanodiscs to be well resolved¹⁷. While the lack of the M3-M4 intracellular loop might impact the structures solved in detergent, it is tempting to speculate that the dynamic nature of the $\gamma 2$ TMD during desensitization is a functional counterpart of this biochemical instability. It is also interesting to note that the $\gamma 2$ subunit contains a phosphorylation site at a serine located at the intracellular end of the M3 segment, namely S327²⁶. This residue is located in an intracellular cassette modulating the desensitization properties of inhibitory pLGICs⁸, only eight residues downstream of the M3-5' residues that we have targeted in the current study. One could thus easily imagine that phosphorylation of $\gamma 2$ -S327 provides a mean to modulate the desensitization of $\gamma 2$ -containing GABA_ARs. Interestingly, a recent study shows that the desensitization of $\gamma 2$ -containing GABA_ARs promotes a form of long-term potentiation at inhibitory synapses by increasing the phosphorylation of $\gamma 2$ -S327²⁷, in line with such hypothesis. Last but not least, the prominent role of the $\gamma 2$ subunit in shaping the desensitization of $\alpha 1\beta 2\gamma 2$ GABA_ARs makes it an interesting target for pharmacological modulation of these receptors. Modulating desensitization should barely affect basic synaptic signalling, which could thus lead to fairly safe compounds with a large therapeutic window. Targeting the $\gamma 2$ subunit specifically, in a desensitization locus with divergent sequences among pLGICs such as the intracellular end of the M3 segment, should also provide an easy mean to achieve subtype selectivity. The current γ -selective pharmacology is embodied by the widely used class of benzodiazepines. Unfortunately, benzodiazepines modulate $\alpha\beta\gamma$ GABA_ARs likely by affecting a preactivation

step, upstream from the ion channel opening¹⁸. They impact the overall conformational equilibrium of the extracellular domain, as their binding at the α - γ extracellular interface affects indiscriminately both GABA binding sites¹⁸, but most probably don't affect the desensitization process *per se*. Neurosteroids, which act at the transmembrane level and likely modulate desensitization³, would be more promising, although their binding sites have currently been delineated for α and β subunits^{28,29}.

The apparent lack of effect on desensitization when mutating SU1 or SU2 alone is another striking feature of the dataset. A first hypothesis might be that these subunits don't desensitize during the one-minute-long GABA application from our protocol. This is unlikely: in that case, mutating SU1 and/or SU2 should not affect the fast desensitization of concatemers harbouring mutations on other subunits. However, mutating SU1 and SU2 leads to an almost 2-fold increase in the fast desensitization kinetics of C³⁴⁵ (Figure 2; Supplementary Table 1). An alternative hypothesis would then be that SU1 and/or SU2 display very fast desensitization, but with a desensitization equilibrium largely displaced towards their open conformation (" $\delta^+ / \delta^- \ll 1$ "), thereby barely contributing to the macroscopic course of desensitization. Such desensitization equilibrium would minimally affect the size of currents, nor the apparent affinity for the agonist. In that event, it is conceivable that SU1 and/or SU2 mutations effects could be revealed on a mutant background owing to inter-subunit coupling. This potential impact of inter-subunit coupling involving SU1 or SU2 might also explain why our kinetic simulations slightly differ from experiments for certain mutants – for example leading to an increased weight of the fast desensitization component (%A_{fast}) of C³⁴ and C³⁵ as compared with our simulations. There are of course other potential explanations for this discrepancy. For example, the intersubunit couplings themselves might be affected by mutations, with the SU3-SU4 coupling (γ) and the SU4-SU5 coupling (ϵ) being decreased by the SU3 and SU4 mutations, respectively. Our dataset unfortunately provides too little

constraint to build a comprehensive scheme for these hypotheses ~~contribution of SU1 and SU2~~, and prevents their inclusion in our kinetic model. It is also worth stressing again that the experimental components driven by the slow component (I_{res} , τ_{slow}) are difficult to measure reliably for strongly desensitizing mutants. In those cases, it is near impossible to fully discard the contribution of endogenous currents, or even the contribution of a tiny conductance from fully desensitized channels, as has been suggested for AMPA receptors³⁰. One should thus be careful when interpreting such measurements – our most reliable measurements being the τ_{fast} values. It is noteworthy that our experimental design allows for a 20-80% rise times in the 20-25 ms timescale. Therefore, very-fast desensitizing mutants may already desensitize during the onset of activation, compromising the accurate measurement of their fast desensitization kinetics. Yet using a simplified model, we evaluate that the system allows for an accurate measurement of τ_{fast} down to the ~ 25 ms timescale (Supplementary Figure 9), supporting that τ_{fast} has been correctly evaluated for all constructs used here.

Our non-concerted asymmetrical model provides a clear departure from a classical view in which D_{fast} and D_{slow} states are fundamentally different. It raises the possibility that these states are identical at the single-subunit level, with D_{fast} only reflecting asymmetrical intermediates, mainly AD_{45} , along the desensitization process. Such scheme might appear surprising given the widely accepted concerted nature of pLGICs gating, as described for the muscle-type nAChR³¹. However, the analysis and concepts in favour of a concerted gating of pLGICs, like the MWC model framed more than half a century ago³², have largely focused on biochemical and electrophysiological data obtained under gating equilibrium conditions such as concentration-response curves^{21,31}. In the case of desensitization, the events are slow enough that intermediate events are directly detectable, namely the D_{fast} state(s). If one could record the activation kinetics with sufficient temporal precision, it is likely that proper data fitting would also require the use of non-concerted asymmetric rearrangements. This is

actually hinted by the prime model of muscle-type nAChR activation, in which conformational changes can affect independently either of the two ACh binding sites³³, as well as by rate-equilibrium free energy relationship analyses arguing for non-concerted rearrangements of M2 helices during nAChR activation³⁴. Moreover, molecular dynamics studies also pinpoint the cytoplasmic end of the pore as a locus for asymmetric conformations at the μ s timescale: the five -2' residues are often distributed in a non-symmetrical fashion during simulations of the open state of the zebrafish α 1 Glycine receptor^{35,36}. Of note, channels and receptors from other families are also known to rely on asymmetric gating. This is the case of the prokaryotic magnesium channel CorA, whose active state actually stems from an asymmetric conformation as reported by cryo-electron microscopy³⁷. This is also the case for NMDA receptors, for which the cryo-electron microscopy of tri-heteromeric GluN1/GluN2A/GluN2B receptors reveals an asymmetric organisation³⁸.

The exact structural underpinnings of desensitization remain however ill-defined, in particular since the current structures have been obtained for presumable resting and desensitized conformations so far^{10,17}. In the absence of an active conformation, one can only speculate on the precise molecular events occurring during the active to desensitized transition.

METHODS

Molecular biology. The GABA_A concatemeric $\alpha 1\beta 2\gamma 2$ construct was previously described¹⁸, based on the concatenation of mouse GABA_A subunits. Briefly, the five subunits were subcloned in the order $\beta 2-\alpha 1-\beta 2-\alpha 1-\gamma 2$ into a low copy number vector pRK5, retaining the peptide signal of the first subunit only. We used the short splice variant of the $\gamma 2$ subunit, $\gamma 2S$. All five subunits are flanked by unique restriction sites to allow the subcloning of mutated subunits, and separated by 15-20 residues long polyglutamine linkers, depending on the length of the C-terminus end of the subunit preceding the linker. The construct thus shows the arrangement ClaI- $\beta 2$ -20Q-AgeI- $\alpha 1$ -15G-SalI- $\beta 2$ -20Q-NheI- $\alpha 1$ -15Q- $\gamma 2S$ -Stop-HindIII. Site-directed mutagenesis was performed on individual subunits as previously described⁸. Owing to the unique restriction sites, mutated subunits were then sequentially subcloned in the concatemer to yield the desired combinations of mutated subunits. We finally sequenced the resulting mutated concatemers to check for the incorporation of the desired mutated subunits. We could not use primers annealing anywhere in $\alpha 1$ or $\beta 2$ for sequencing, as both subunits are present as duplicates in the concatemer. Instead, we sequenced SU1-4 subunits with primers annealing at their 5' DNA extremity, centered on the sequence of the unique restriction site preceding the following subunit. Such reverse primers enable the sequencing of the 5' end of the subunits' DNA, coding for their C-terminus once translated.

Expressing GABA_ARs in *Xenopus laevis* oocytes. Ovaries from *Xenopus laevis* were obtained from CRB Xenopes in Rennes. Free oocytes were obtained by incubating segments of ovary in collagenase type 1 (Sigma) dissolved in a Ca²⁺-free OR2 solution, which contained (mM): 85 NaCl, 5 HEPES, 1 MgCl₂, pH adjusted to 7.6 with KOH. After 2-4 hrs exposure to collagenase I, defolliculated oocytes were washed several times with OR2, and thereafter maintained in a Barth's solution containing (mM): 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.4 NaHCO₃, 10 HEPES, pH adjusted to 7.6 with NaOH. Single

oocytes were injected with 27.6 nl of concatemeric GABA_AR cDNAs (nuclear injection) at a concentration of 30 ng/μl. Oocytes were incubated at 17°C in Barth's solution devoid of serum or antibiotics.

Two-electrode voltage clamp recording. Oocytes expressing pentameric concatemers were recorded 2-4 days after injection. They were superfused with a solution containing (mM): 100 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH adjusted to 7.4 with NaOH. Solution flowed at an approximate speed of 12 mL/min. Currents were recorded using a Warner OC-725C amplifier, a Digidata 1550A interface and pCLAMP 10 (Molecular Devices). Currents were digitized at 500 Hz and filtered at 100 Hz (30-60 Hz used for display purposes). Oocytes were voltage-clamped at -60 mV and experiments conducted at room temperature. Desensitising currents were induced by 1 min applications of 10 mM GABA. 20-80% current rise times of 20-25 ms were achieved for C^{WT}.

Data analysis. The extent of desensitisation was determined as $(1 - I_{\text{res}}/I_{\text{peak}})$, where I_{peak} is the peak current and I_{res} the residual current remaining at the end of the agonist application.

Weighted decay time constants for desensitisation were determined by fitting the desensitising phase with two exponential components (pCLAMP 10), as given by the following equation: $\tau_w = \%A_{\text{fast}} * \tau_{\text{fast}} + (1 - \%A_{\text{fast}}) * \tau_{\text{slow}}$. All data values are means \pm standard deviation.

Drugs and chemicals. All compounds were purchased from Sigma. GABA was prepared as a 1 M stock solution in recording solution. Aliquots were stored at -20°C.

Kinetic modelling. We used QUB²⁰ to build Markov-chain kinetic models. Each simulation contained 10,000 – 30,000 channels. The binding and gating rate constants are broadly consistent with previously published values for GABA_ARs³⁹. For each model, we performed iterative rounds of kinetic simulations by adjusting manually the set of parameters. Binding

and gating constants being fixed, Model I (Figure 4), Model II (Figure 5), Model II- β (Supplementary Figure 46) and the concerted model (Supplementary Figure 23) only contain four parameters for the wild-type receptors (δ^+ , δ^- , δ_3^+ and δ_3^- for Models I, II and II- β ; fast and slow desensitization rates and their recovery counterparts for the concerted model). This equates to the number of independent experimental measurements related to the two desensitization components (τ_{fast} , τ_{slow} , $\%A_{fast}$, $\%I_{res}$). We could thus be confident that, once we have a set of parameters accounting for the wild-type data, the model has a good predictive value. We used ballpark figures to build the initial set of parameters, already having in mind that the fast desensitization component might be mostly carried by subunits 4 and 5 (due to the C^{45} phenotype). For example, taking into account only the two pathways linking the AO state to the AD_{45} state in Model I, the fast desensitization kinetics could be approximated with $\tau_{fast} \sim 2.(\delta^+ + \delta^-)$, while the amplitude of the fast component would yield estimates for the ratio $D = \delta^+ / \delta^-$ approximated with the equation $A_{fast} / I_{peak} \sim 2.D$, resulting in a D value approximated by ~ 0.2 , as well as δ^+ and δ^- values of $\sim 0.2 \text{ s}^{-1}$ and 1 s^{-1} , respectively. In Model II, τ_{fast} is in the order $2.(\delta^+ / \delta^-) . \delta^+$, while the ratio D is constrained by the fast component amplitude (A_{fast}) with the following approximation : $A_{fast} / I_{peak} \sim D^2 / (1 + 2.D)$. Such approximation yields $D \sim 0.9$ and $\delta^+ \sim 0.14 \text{ s}^{-1}$. Mutation-induced changes in those parameters (c_3^+ , c_3^- , c_4^+ , c_4^- , c_5^+ , c_5^-) for models Models I, II were then adjusted manually to account for the effects of individual mutants (C^3 , C^4 , and C^5), Model II- β requiring the additional adjustment of c_{34}^+ , c_{34}^- for the effect of SU3 mutation. The effects of mutations in the concerted model (Supplementary Figure 23) led to four mutation-related parameters (γ_f , ϵ_f , γ_s and ϵ_s) for each individual mutant C^3 , C^4 , and C^5 . In all cases, mutation-related parameters derived from individual mutants were then combined to predict the effect of combining and mutations in constructs C^{34} , C^{35} , C^{45} and C^{345} . The quality of the fit was merely assessed by visual inspection of the bar graphs illustrating the predictions for τ_{fast} , τ_{slow} , $\%A_{fast}$ and I_{res} . It

may thus be possible to obtain better fits to the data. For Model III, we generated a series of wild-type models with values for coupling constants (γ and ε) in the 1-1000 range (1, 10, 100 and 1000). We next manually adjusted the mutation-induced changes as described above for Model II- β .

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FIGURE LEGENDS

Figure 1. The wild-type $\alpha 1\beta 2\gamma 2$ pentameric GABA_AR concatemer. **A**, Schematic top view of the concatemer. The two $\beta 2/\alpha 1$ ECD interfaces (SU1/SU2 and SU3/SU4) harbour the two GABA-binding sites, while the $\alpha 1/\gamma 2$ ECD interface (SU4/SU5) contains the benzodiazepine-binding site. **B**, Representative TEVC recording of a *Xenopus laevis* oocyte expressing the wild-type concatemer, C^{WT}. **C**, Depiction of the experimental values used to quantify desensitization: τ_{fast} and τ_{slow} are the time constants of fast and slow desensitization components, respectively; %A_{fast} is the relative amplitude of the fast component; %I_{res} is the relative residual current after 1 min of 10 mM GABA application. Of note, the weighted desensitization time constant can be defined as $\tau_w = \%A_{fast} * \tau_{fast} + (1 - \%A_{fast}) * \tau_{slow}$. **D**, Cryo-EM structure of the $\alpha 1\beta 3\gamma 2$ GABA_AR (pdb 6I53¹⁷), as seen from the extracellular space. The $\beta 2$ and $\beta 3$ GABA_A subunits are highly homologous, and both display an asparagine residue at the M3-5' position. Note the central pore, lined by the M2 helices of the five subunits, forming the transmembrane channel. **E**, Sequence alignment of the M3 segment of various pLGIC subunits. All sequences are the mouse orthologs, except GLIC (*Gloeobacter violaceus*), as well as the $\alpha 4$ and $\beta 2$ nAChR subunits (human). The M3-5' residues, mutated in the present study, are highlighted (grey box; bold characters for GABA_A subunits). **F**, Enlarged view of the $\alpha 1\beta 3\gamma 2$ GABA_AR structure highlighting the location of the M3-5' residue at the M2/M3 transmembrane interface as seen from the side of the channel, facing the M1-M2 linker of the adjacent subunit.

Figure 2. Desensitization kinetics of $\alpha 1\beta 2\gamma 2$ concatemers harbouring combinations of M3-5' valine mutations. **A**, Schematic top views of the C³ (left), C⁴⁵ (middle) and C¹²³⁴⁵

(right) concatemers. **B-D**, Representative TEVC recording of *Xenopus laevis* oocytes expressing the indicated concatemers. Note the change in timescale for recordings in panel **D**. **E**, Plot indicating the fast (red squares), slow (blue squares) and weighted (black diamonds) desensitization time constants for the indicated concatemers. Error bars are standard deviations. See Supplementary Figure 1 for individual data points and Supplementary Table 1 for numerical values.

Figure 3. General scheme for the simulation of desensitization: an asymmetric non-concerted model. The first part in the kinetic scheme is the binding of the agonist A to the resting state R, which favours the opening of the channel (AO state) with a gating efficacy $E = \beta/\alpha$. Of note, unliganded openings do exist but are not taken into account for our kinetic modelling as they barely contribute to the electrophysiological response (see main text). We also only include one binding event, even though $\alpha 1\beta 2\gamma 2$ GABA_ARs contain two binding sites whose occupation is required for substantial activation. Upon channel opening, the receptor can then transit from a fully activated AO state to states where only one subunit enters its desensitized conformation (AD₃, AD₄ and AD₅). From these states, a second subunit can also desensitize, before the final step leading to the state in which all subunits are desensitized.

Figure 4. Model I: only the fully open state is conducting, and subunits move independently during desensitization. **a**, We assume in this model that a single desensitized subunit is enough to shut the pore of the channel, leading to functional desensitization. Moreover, subunits SU₃, SU₄ and SU₅ can undergo a desensitization rearrangement independent of the other subunits. Thus, desensitization rates (δ_3^+ for SU₃, δ^+ for SU₄ and SU₅) and recovery rates (δ_3^- for SU₃, δ^- for SU₄ and SU₅) do not depend on the conformation

of the neighbouring subunits. **B**, Effect of M3-5' valine mutations in Model I. Mutations are hypothesized to specifically increase the desensitization rates of the mutated subunits, without altering any other parameter. **C-E**, Representative currents for C^{WT} (panel **C**), C^4 (panel **D**) and C^{45} (panel **E**), in black, are compared to the outcome of two distinct simulations. In simulation *a* (red), the mutation-induced increase in the desensitization rates of SU4 and SU5 is adjusted so that the simulation of single mutants C^4 and C^5 broadly fits the experimental data, as seen in panel **D**. In simulation *b* (blue), the mutation-induced increase in the desensitization rates of SU4 and SU5 is adjusted so that the simulation of the double mutant C^{45} accounts for the experimental data, as seen in panel **E**. **F**, Bar graph summarizing the experimental data vs the predicted effects of SU4 and/or SU5 mutations on the kinetics of the fast desensitization component in simulations *a* and *b*. For panels **C-F**, note that parameters from simulation *a* fail at describing the data for the double mutant C^{45} , while parameters from simulation *b* largely overestimate the effect of single mutants. See Supplementary Table 3 for the numerical values of parameters.

Figure 5. Model II: Two desensitized subunits are required to occlude the pore. **A**, Model II builds upon Model I by adding one key hypothesis: receptors with only one subunit in its desensitized conformation are still conducting, and desensitization occurs when at least two subunits are desensitized. Thus, states AD_3 , AD_4 and AD_5 are open states from a functional point of view. **B**, In Model II, mutation of a subunit can affect both its desensitization and recovery, as shown here with an example in which both SU4 and SU5 are mutated (construct C^{45}): c_4^+ and c_5^+ reflect the increase in desensitization rates, c_4^- and c_5^- reflecting the increase in recovery rates. **C**, Simulated currents for C^{WT} , C^4 and C^{45} . **D**, Representative currents for C^{WT} and C^3 in black, are compared to their simulation counterparts in red. **E-G**, Bar graphs summarizing the experimental data (in black) vs the simulations (in red) for the indicated

concatemers on the kinetics (panel *E*) and the amplitude (panel *F*) of the fast desensitization component as well as the residual current after a 1 min long application of 10 mM GABA (panel *G*). Note that the results for the C⁵ construct are not displayed, since the experimental data are almost identical to that of C⁴ (see Figure 2) and since the simulations for C⁴ and C⁵ are identical (see Supplementary Table 3). See Supplementary Figure 25 for all simulation results, and Supplementary Table 3 for the numerical values of parameters.

Figure 6. Model III introduces inter-subunit coupling during desensitization. *A*, For the wild-type receptors, Model III builds upon Model II by adding some coupling between adjacent subunits during desensitization. On the one hand, desensitization of SU3 accelerates the desensitization of SU4 by a factor γ , and reciprocally. On the other hand, desensitization of SU4 slows the recovery of SU5 by a factor ϵ , and reciprocally. *B*, For mutated concatemers, Model III incorporates the additional hypothesis that the mutation of SU3 also affects the desensitization of SU4 by increasing both its desensitization and recovery rates, by ratios c_{34}^+ and c_{34}^- , respectively.

Figure 7. Model III simulations are broadly consistent with experimental data. *A-H*, Representative currents for the indicated constructs, in black, are overlaid with their simulation counterparts in red. Note the changes in timescales. *I-L*, Bar graphs summarizing the experimental data (in black) vs the simulations (in red) for the indicated concatemers on the kinetics of the fast (panel *I*) and slow (panel *J*) desensitization components, the relative amplitude of the fast component (panel *K*) and the residual current after a 1 min long application of 10 mM GABA (panel *L*). See Supplementary Table 3 for the numerical values of parameters.

Figure 8. States occupancies predictions and structural depiction of Model III. *A*, The overall population of wild-type receptors in an active conformation is compared to the relative occupancies of the various desensitized states. As depicted by the red box, the early phase of desensitization is carried by the AD₄₅ state. On longer time scales (blue box), slow desensitization is largely embodied by the entry in the AD₃₄₅ state. The analysis of states occupancies was performed with QuB simulations. *B*, In this simplified depiction of model III, we extracted the kinetically favoured pathway for the desensitization of wild-type $\alpha 1\beta 2\gamma 2$ GABA_ARs. Upon agonist binding, the receptor is transiently stabilized in a fully open pseudo-symmetrical conformation. The two first subunits to rearrange during desensitization are the $\alpha 1$ and the $\gamma 2$ subunits involved in the binding of benzodiazepines, namely SU4 and SU5 in our concatemers. While one desensitized subunit is not enough to occlude the pore, fast desensitization corresponds to the rearrangement of both SU4 and SU5 subunits, which are coupled. Slow desensitization is then driven by the slower rearrangement of the SU3 subunit, i.e. the $\beta 2$ subunit opposite to the $\gamma 2$ subunit.

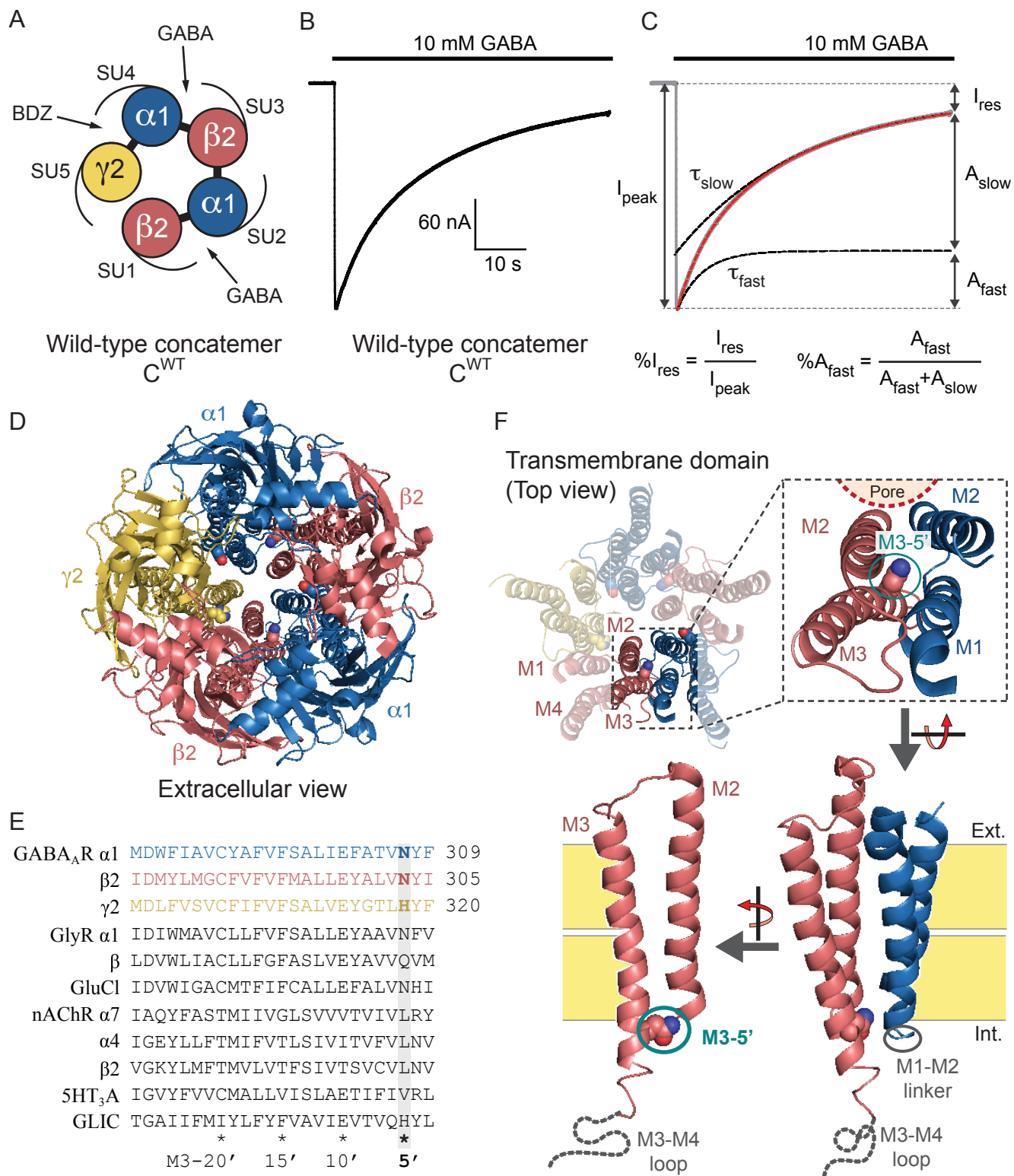


Figure 1

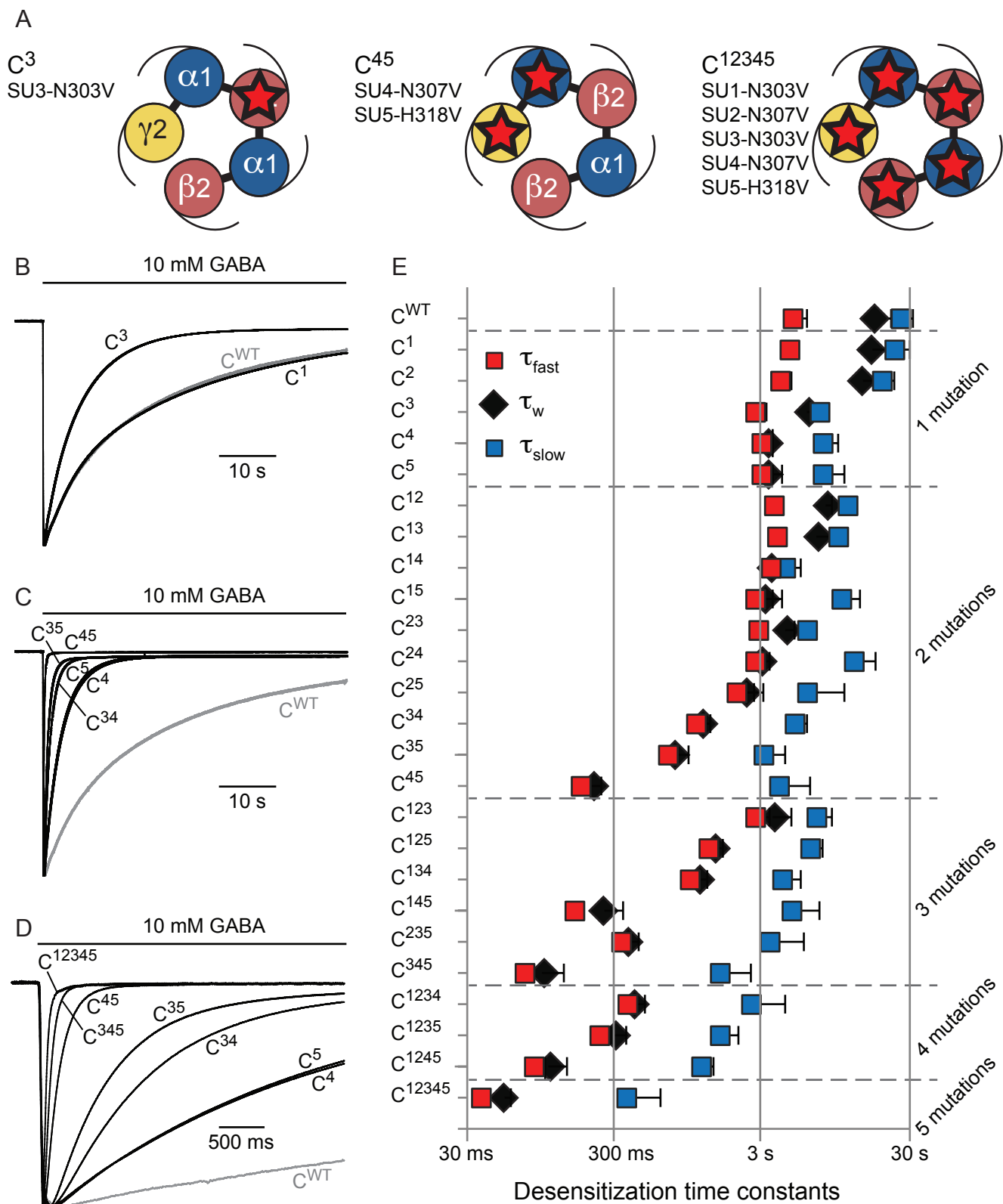


Figure 2

General asymmetrical non-concerted model

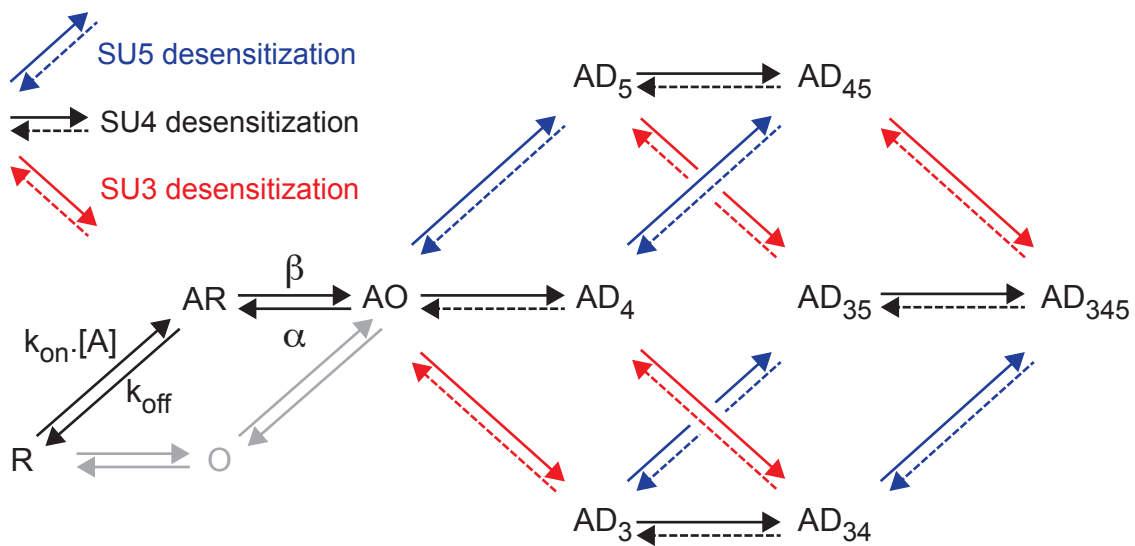
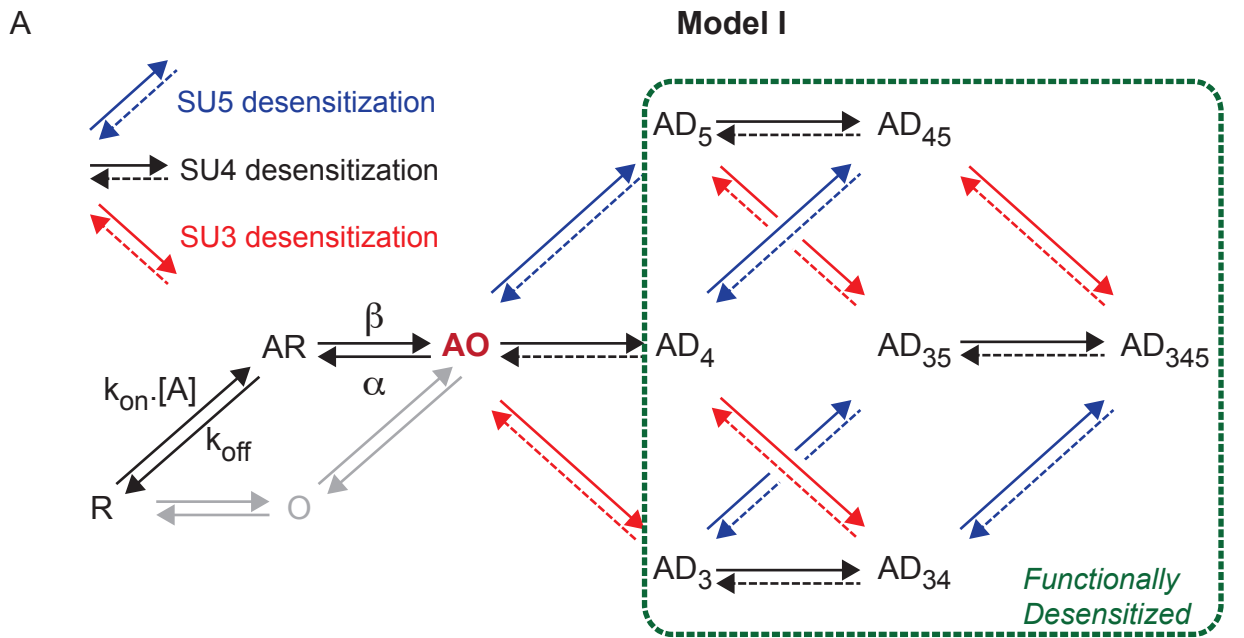
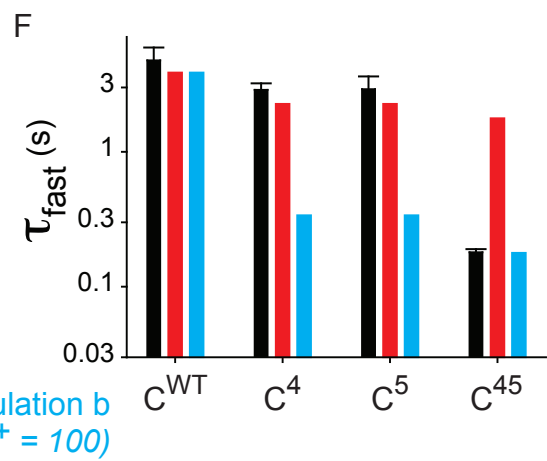
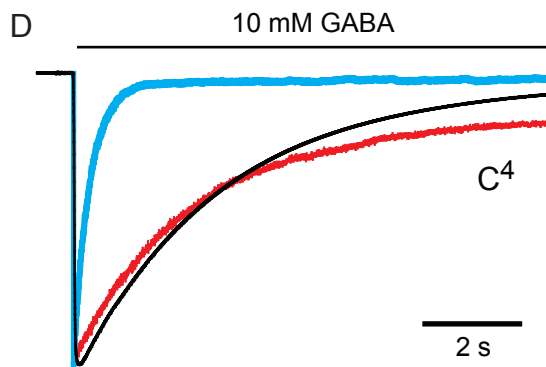
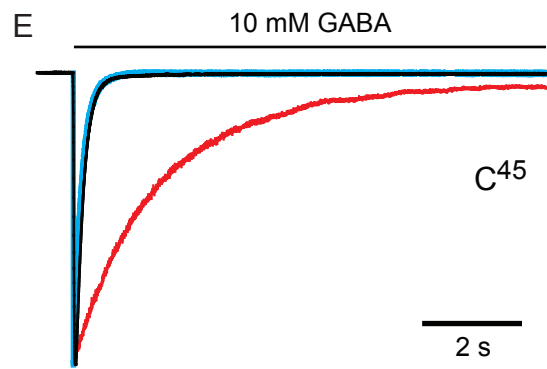
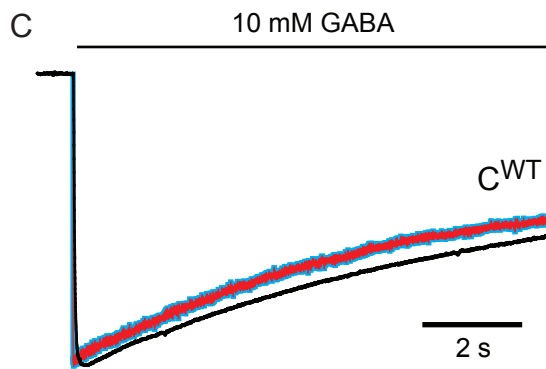


Figure 3



B

	wild-type	<i>SU3</i> mutant	<i>SU4</i> mutant	<i>SU5</i> mutant
SU3 desensitization rate	$\bar{\delta}_3^+$	$c_3^+ \cdot \bar{\delta}_3^+$	$\bar{\delta}_3^+$	$\bar{\delta}_3^+$
SU3 recovery rate	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$
SU4 desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_4^+ \cdot \bar{\delta}^+$	$\bar{\delta}^+$
SU4 recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$
SU5 desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_5^+ \cdot \bar{\delta}^+$
SU5 recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$



— Experiment — Simulation a ($c_4^+ = c_5^+ = 10$) — Simulation b ($c_4^+ = c_5^+ = 100$)

Figure 4

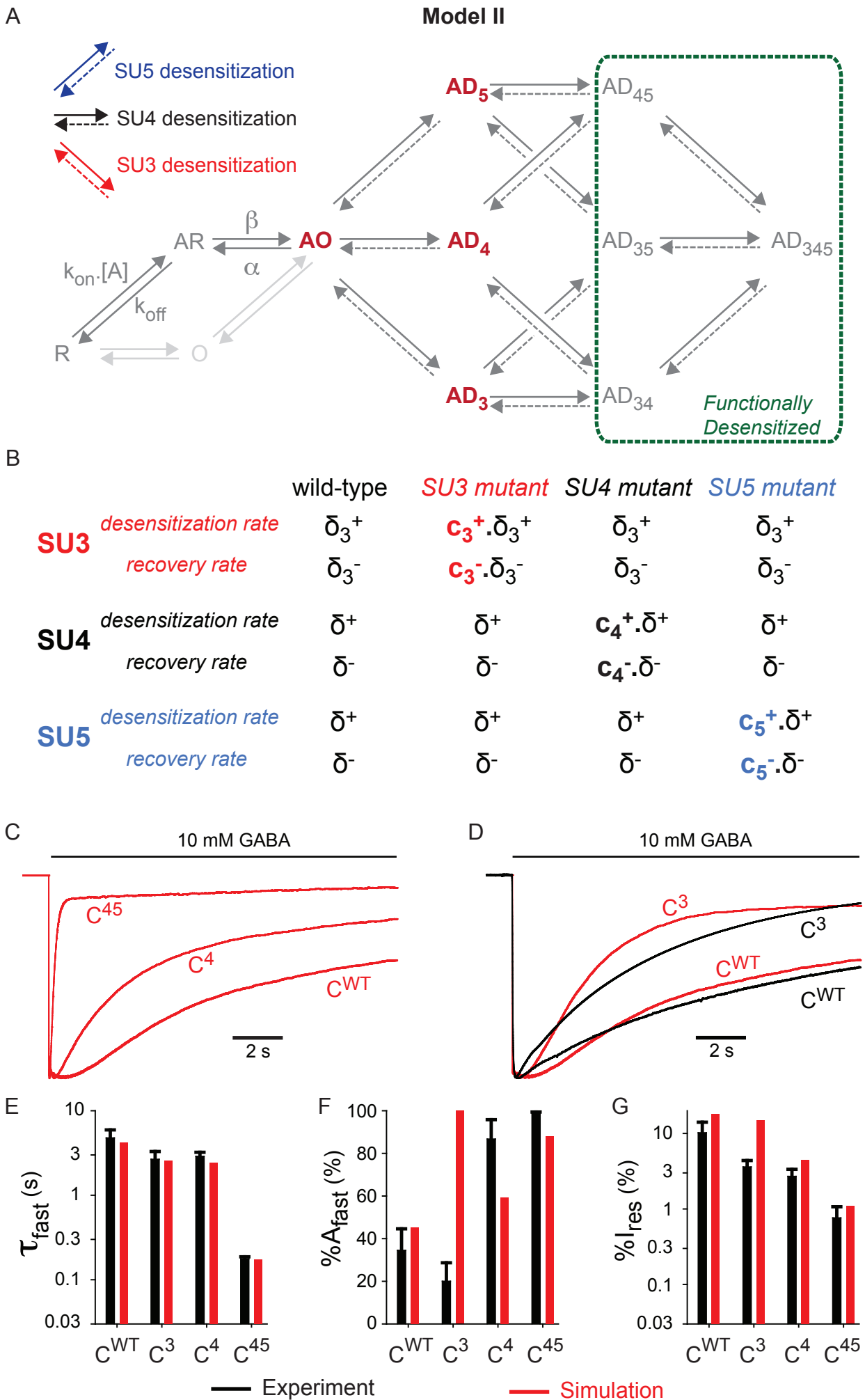
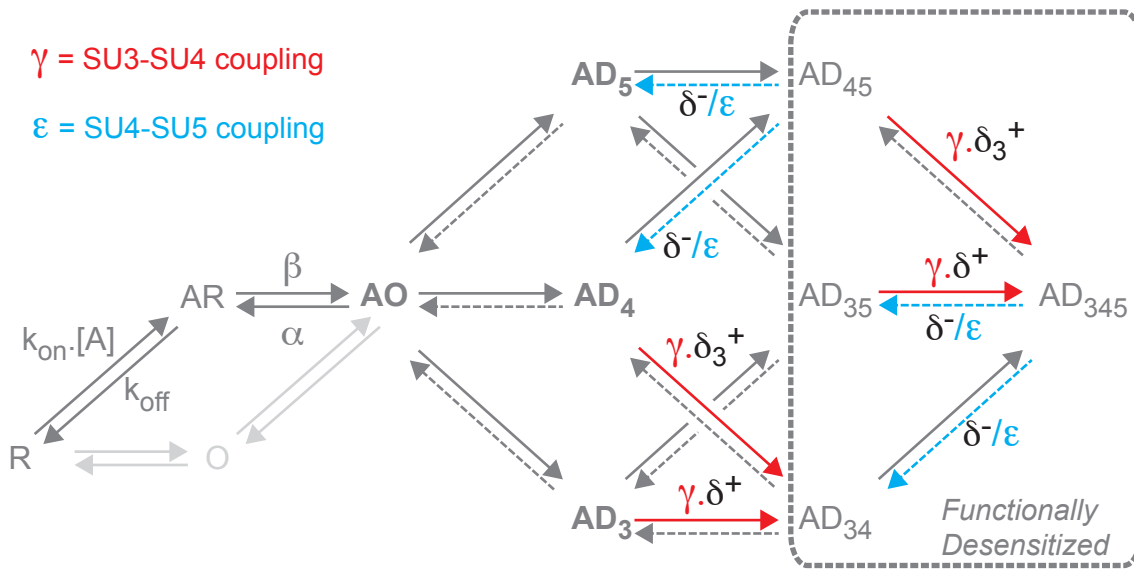


Figure 5

A

Model III



B

	wild-type	<i>SU3 mutant</i>	<i>SU4 mutant</i>	<i>SU5 mutant</i>
SU3	desensitization rate	$\bar{\delta}_3^+$	$c_3^+ \cdot \bar{\delta}_3^+$	$\bar{\delta}_3^+$
	recovery rate	$\bar{\delta}_3^-$	$c_3^- \cdot \bar{\delta}_3^-$	$\bar{\delta}_3^-$
SU4	desensitization rate	$\bar{\delta}^+$	$c_{34}^+ \cdot \bar{\delta}^+$	$c_4^+ \cdot \bar{\delta}^+$
	recovery rate	$\bar{\delta}^-$	$c_{34}^- \cdot \bar{\delta}^-$	$c_4^- \cdot \bar{\delta}^-$
SU5	desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_5^+ \cdot \bar{\delta}^+$
	recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$c_5^- \cdot \bar{\delta}^-$

Figure 6

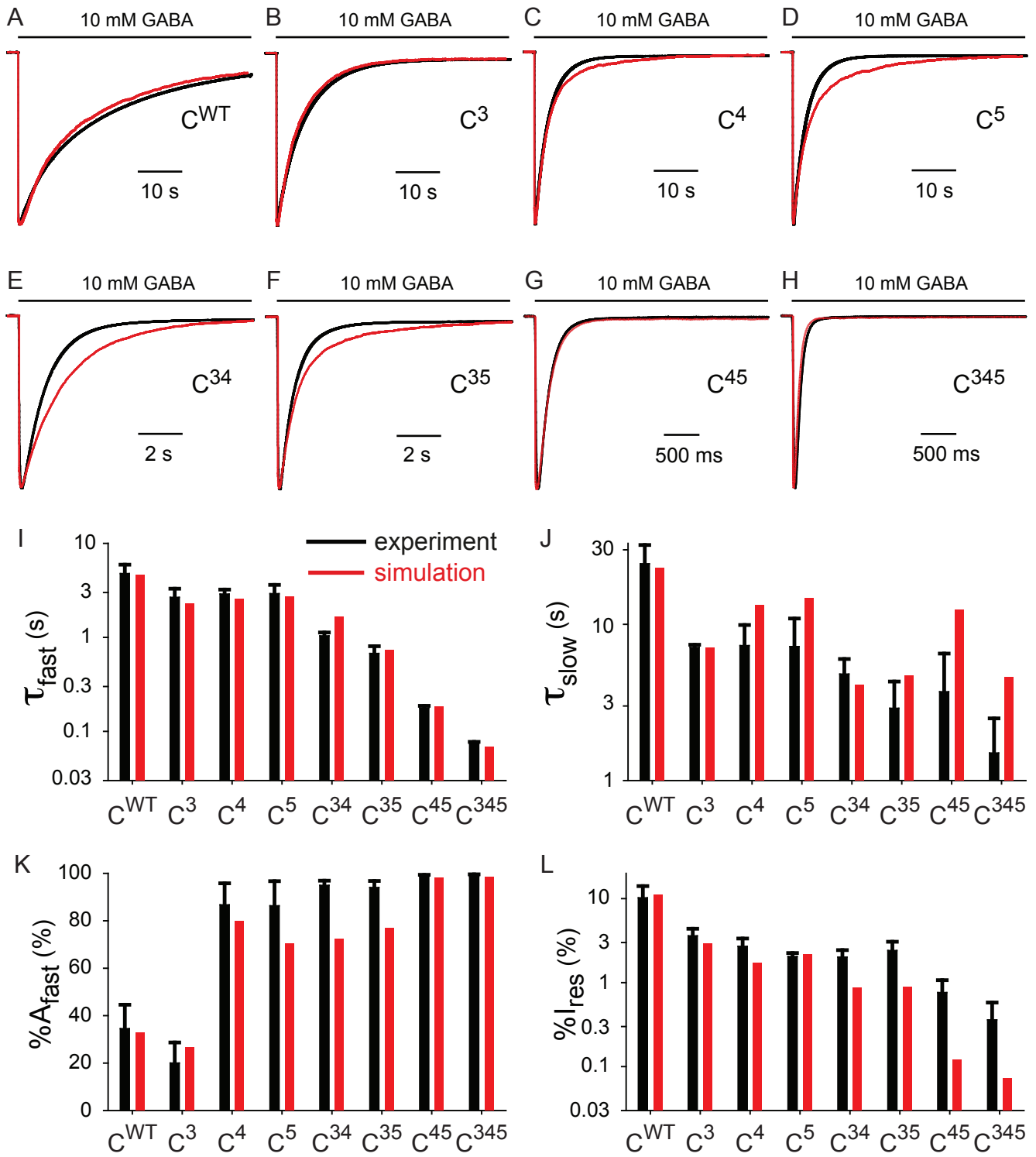


Figure 7

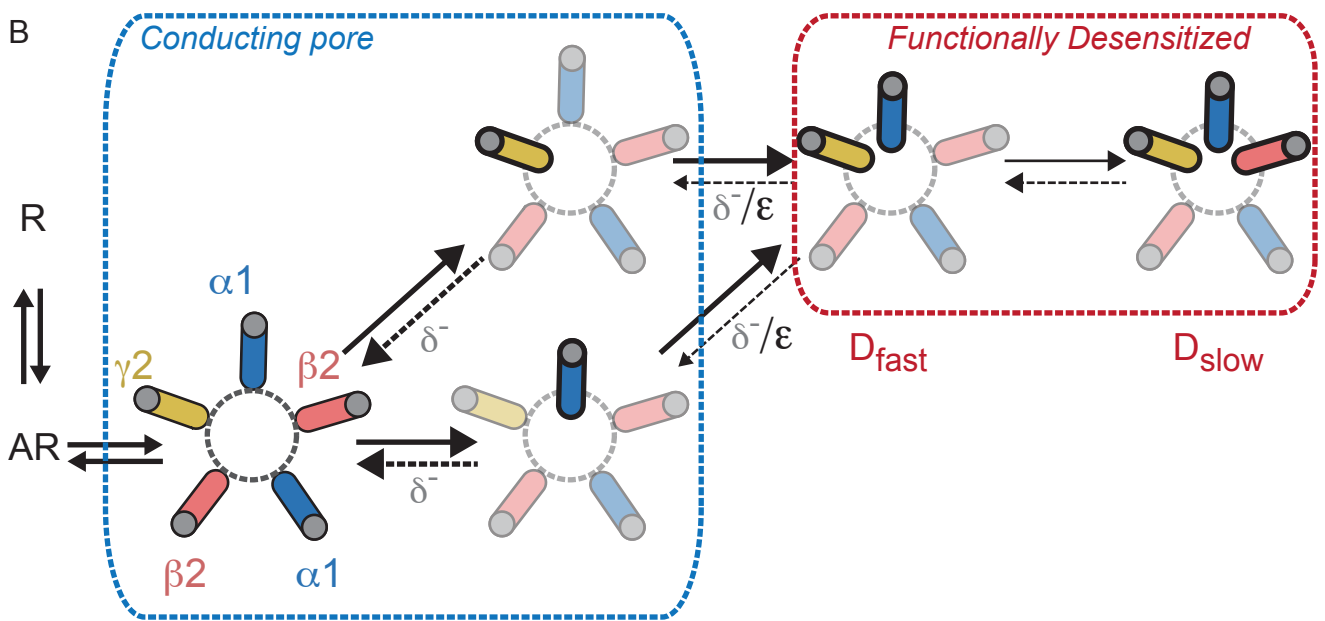
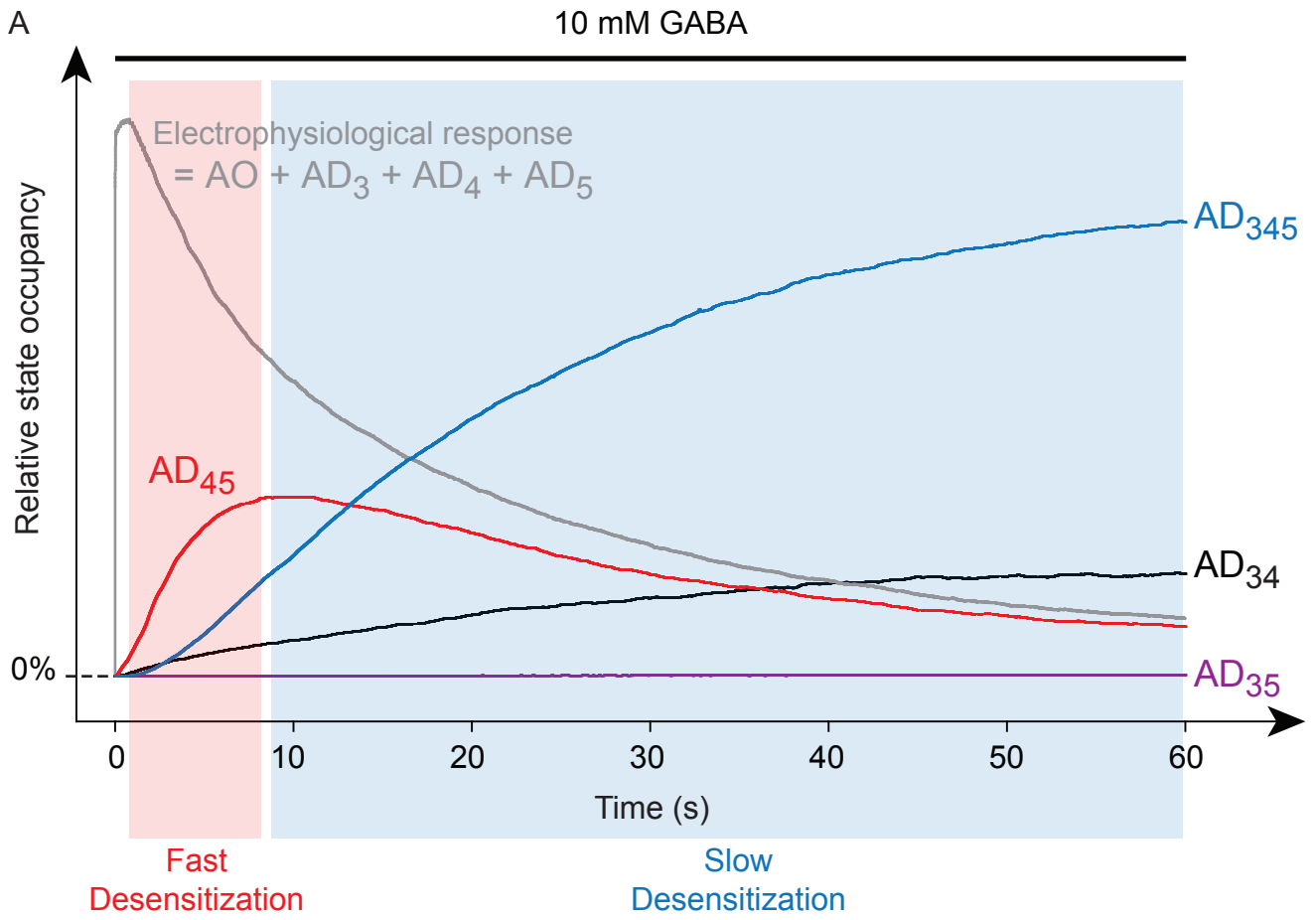


Figure 8

The desensitization pathway of GABA_A receptors, one subunit at a time

Marc Gielen, Nathalie Barilone & Pierre-Jean Corringer

Supplementary Information

SUPPLEMENTARY DISCUSSION

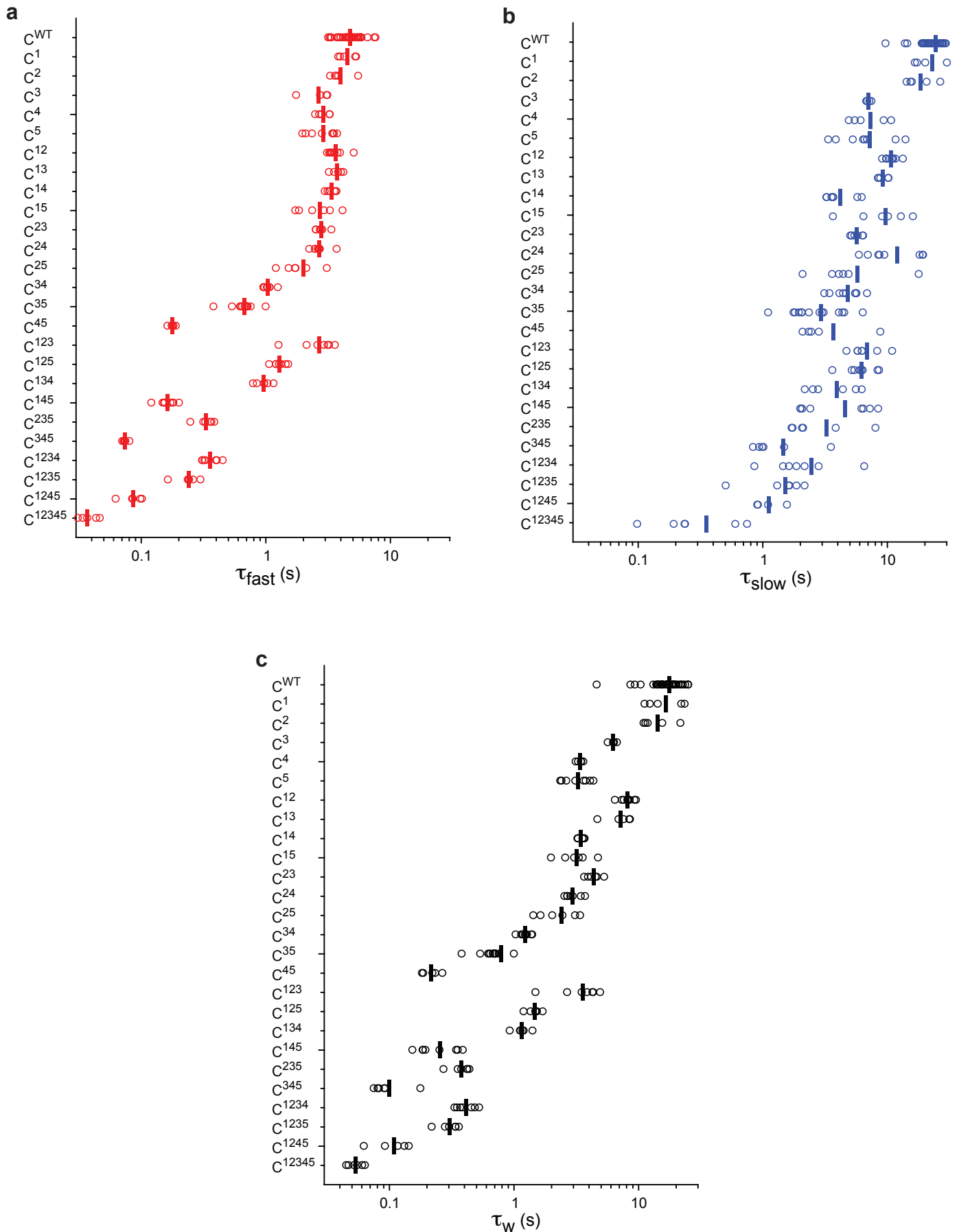
Model III: limitations and implications for single-channel recordings

We have to acknowledge here two main shortfalls of our model. First, no unliganded desensitized state is present, although these are known to exist and contribute to the recovery from desensitization upon removal of the agonist¹. Our kinetic model thus cannot be used to model recovery from desensitization. Second, at the single channel level, our model makes a strong prediction when the channel transits from the open state to a more stable pre-desensitized state still capable of conducting ions. Indeed, in the open state, single-channel openings on the ms timescale are separated by brief shut times reflecting sojourns in resting or pre-active states², giving rise to a maximum open probability of about 70%-80% for $\alpha 1\beta 2\gamma 2$ GABA_ARs³. However, once the channel enters a pre-desensitized state in our final scheme – i.e., AD₃, AD₄ or AD₅ -, the predicted lifetime of such state should produce uninterrupted single-channel openings of several seconds for wild-type receptors. Such openings are not observed in single-channel recordings. It is likely that, when one subunit enters its desensitized conformation, the other subunits retain the ability to visit their resting conformation, thereby providing a way to maintain an open probability well below unity. Still, one could expect a strong influence of the receptor's desensitization on its single-channel gating efficacy under equilibrium conditions, e.g. in cell-attached patch-clamp recordings. It is thus striking that a recent study highlighted such phenomenon at $\alpha 1$ glycine receptors (GlyRs)⁴. Indeed, Ivica *et al.* showed that deletion of the intracellular domain (ICD) of homomeric human $\alpha 1$ GlyRs largely increases their efficacy of gating. At the same time, the ICD is known to affect the desensitization of $\alpha 1$ GlyRs⁵⁻⁷, and the study by Ivica *et al.* displays single-channel clusters that seem largely lengthened by the ICD deletion, suggesting that their ICD-deleted construct shows reduced levels of desensitization (comparing the

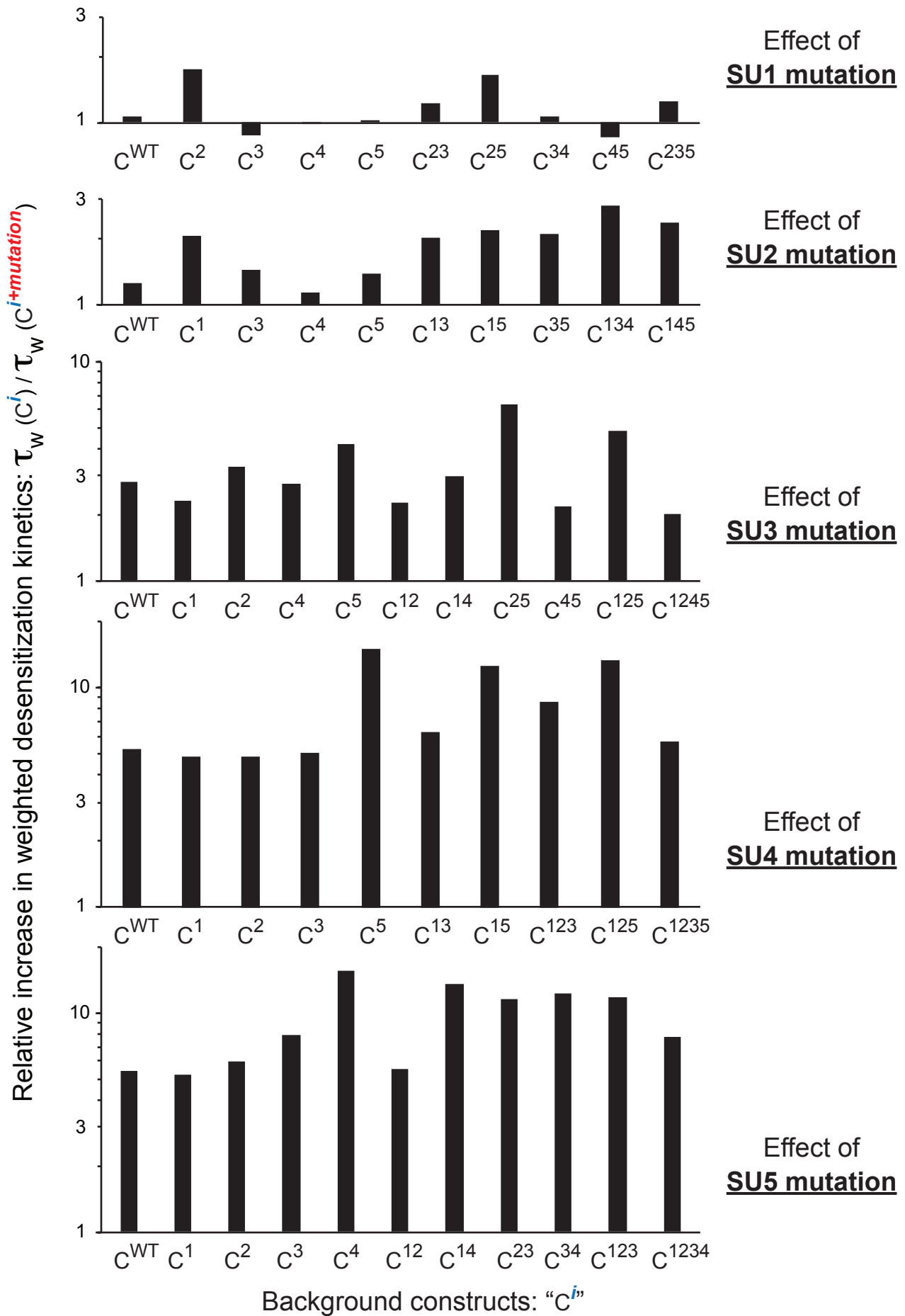
glycine and alanine recordings from panels from Figure 2B and Figure 3B in ref⁴). It is thus tempting to speculate that the ICD-deleted GlyRs constructs from the study by Ivica *et al.* show a greater stability of their pre-desensitized states with one single subunit being desensitized, resulting in both lengthened single-channel clusters and higher cluster open probability under equilibrium conditions.

Our model makes another prediction regarding single-channel recordings, which would provide strikingly different estimation of desensitization kinetics under equilibrium versus non-equilibrium conditions. Indeed, with cell-attached recordings performed in the continued presence of the agonist, some desensitized channels would spontaneously recover and visit a conducting state, amenable to electrophysiological detection. Given our set of parameters, the kinetically favoured pathway would be for a receptor in a AD_{345} state to transit to the AD_{34} state and then to the conducting pre-desensitized AD_3 state. From there, the favoured transition would be to re-enter the AD_{34} state with a microscopic rate of $\gamma.\delta^+$, i.e. 28 s^{-1} , resulting in a cluster duration of about 36 ms. On the contrary, a receptor recorded in non-equilibrium conditions, such as performed in fast-perfusion experiments in the outside-out configuration or in whole-cell experiments, would start off in the resting state R, transit to the agonist-bound AR state, and then finally open (AO state). The electrophysiological current would be detectable for the entire duration from the entry to the open state to the exit in a fully desensitized state. In this case, the favoured routes would either be AO to AD_4 to AD_{45} or AO to AD_5 to AD_{45} . The apparent corresponding macroscopic rate would roughly equate to $2.(\delta^+/\delta^-).\delta^+$, i.e. about 0.25 s^{-1} , resulting in a cluster duration of about 4 s. Thus, our model suggests that cell-attached patch-clamp recordings might not be predictive of the desensitization properties of $\alpha 1\beta 2\gamma 2$ GABA_ARs driven by a sudden rise in agonist concentration, as observed during phasic synaptic release. Of note, a recent study on NMDA receptors has shown the existence of two distinct preactivated / primed states by analysing the

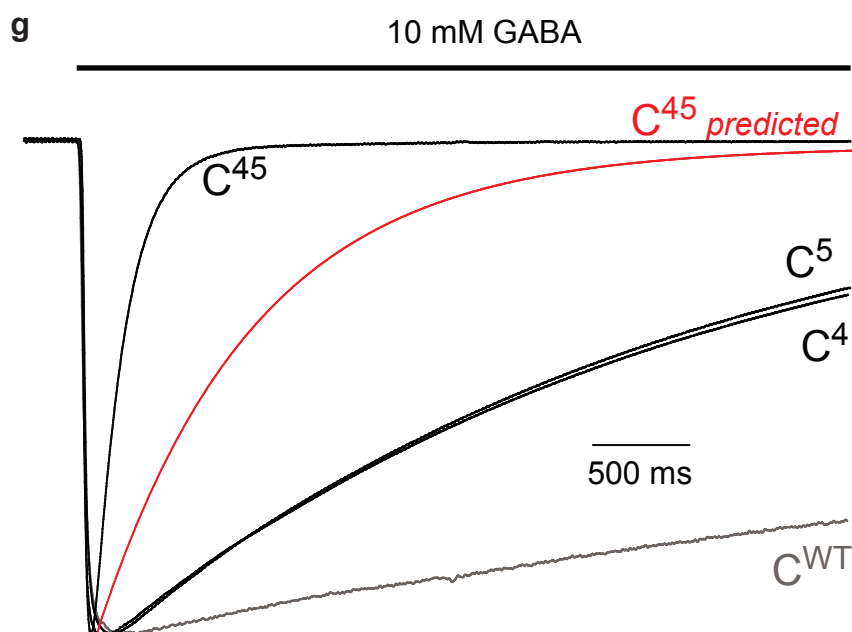
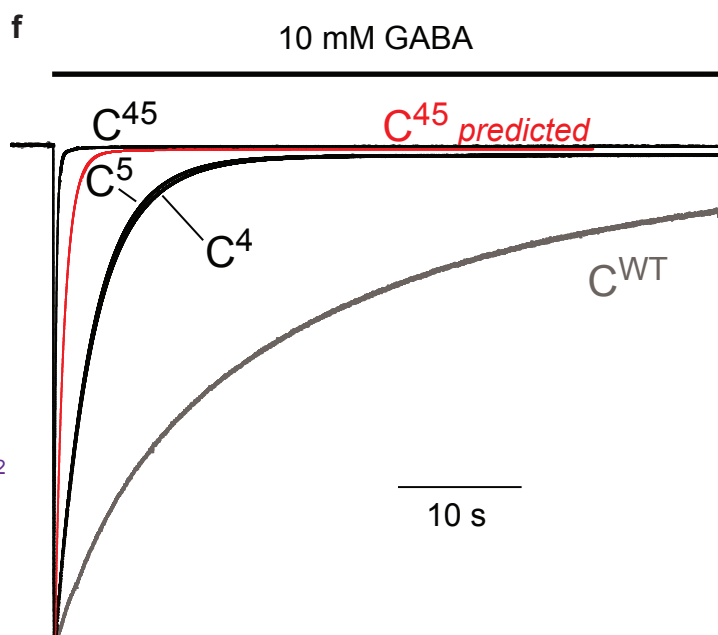
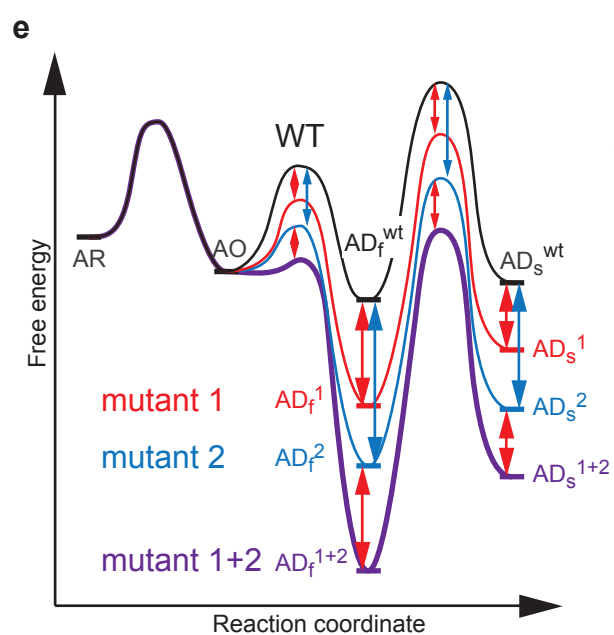
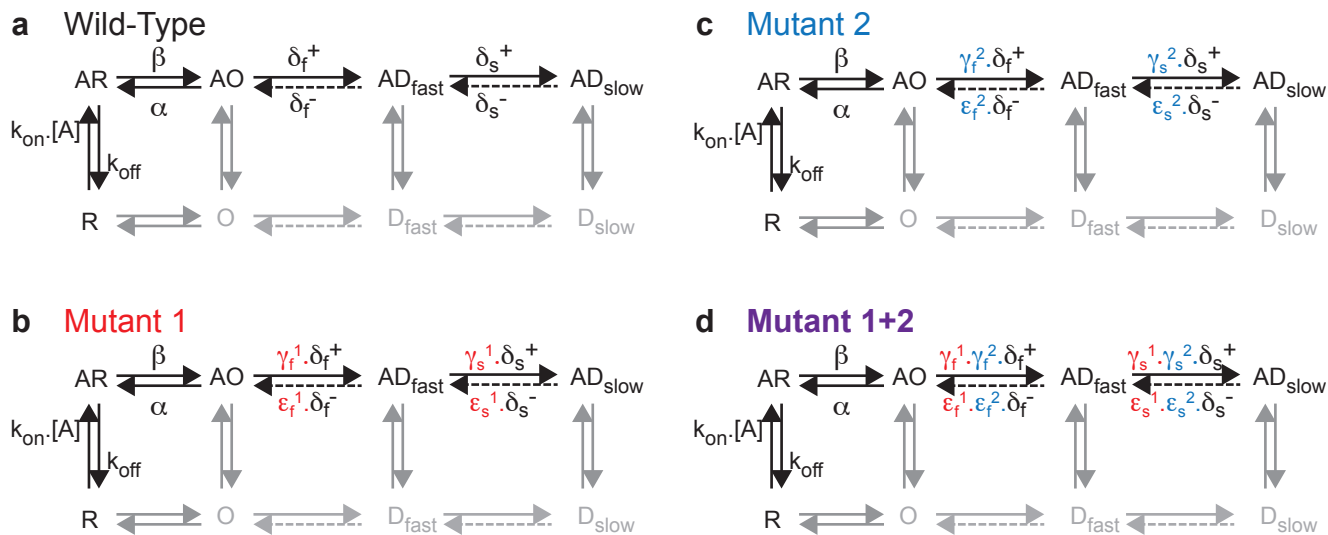
opening latency (“time to open”) in single-channel recordings, which were obtained with fast-perfusion of outside-out patches containing only one channel⁸. This study highlights the importance to study ligand-gated ion channels under non-equilibrium conditions, in order to gain insights in their gating pathways.



Supplementary Figure 1. Individual data points for desensitization decay time constants. **a-c**, Individual data points and means are shown as circles bars, respectively. **a**, Data for fast desensitization time constants (red). **b**, Data for slow desensitization time constants (blue). **c**, Data for weighted desensitization time constants (black). See Supplementary Table 1 for the numerical mean values, the number of cells and the number of independent experiments.



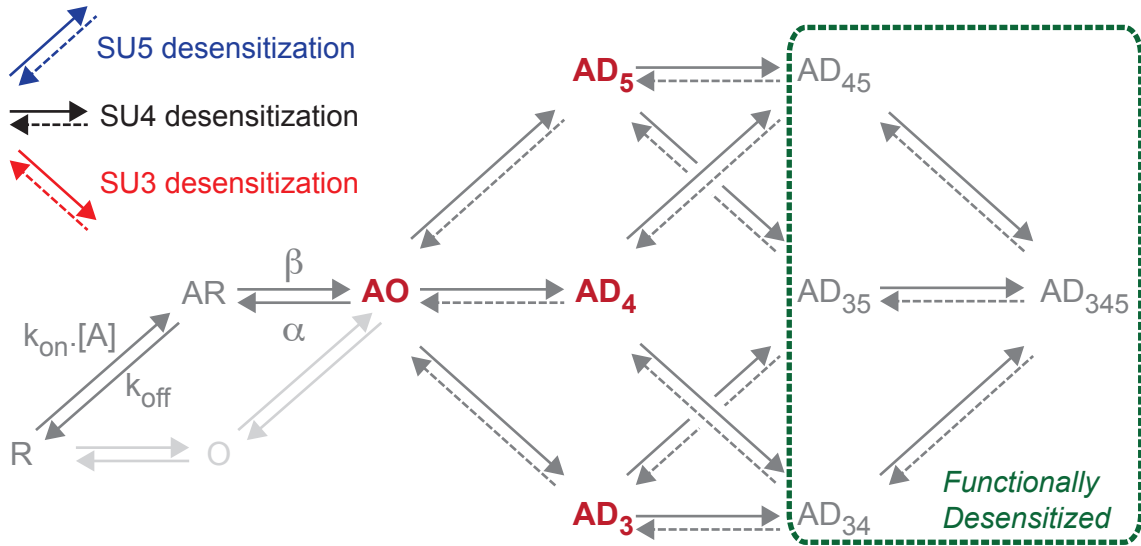
Supplementary Figure 2. Relative effect of M3-5' mutations on the weighted desensitization kinetics of a series of background mutated concatemers. Each bar plot corresponds to the relative effect of the M3-5' mutation of the indicated subunit on the weighted desensitization rate of the background constructs. Values were calculated as the ratio between corresponding mean τ_w values.



Supplementary Figure 3. A concerted model only including pseudo-symmetrical states cannot account for the synergistic effects of SU4 and SU5 M3-5' valine mutations. **a-d**, Simplified concerted kinetic schemes used to model wild-type (panel **a**), single mutant (panels **b** & **c**) and double mutant (panel **d**) concatemers. While unliganded openings are possible at all ligand-gated receptors, consistent with a Monod-Wyman-Changeux model of allosteric proteins, they are exceedingly rare at wild-type $\alpha 1\beta 2\gamma 2$ GABA_ARs (see Main Text), and we therefore decided not to include them in our kinetic modelling. In the absence of direct interaction between the mutated residues, mutations are expected to have additive effects (see Main Text) as described in panel **d**. **e**, Putative free energy diagram translating the kinetic schemes from panels **a-d**. **f-g**, Representative currents (in black) from C^{WT}, C⁴, C⁵ and C⁴⁵ constructs are overlaid with the simulated C⁴⁵ response (in red) as predicted from the concerted model adjusted to account for the wild-type and single mutant constructs (C^{WT}, C⁴ and C⁵). Note that the concerted model largely underestimates the desensitization kinetics of the double mutant C⁴⁵.

a

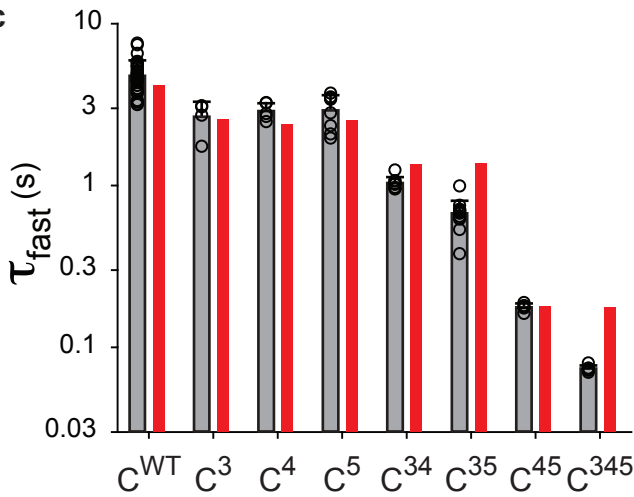
Model II'



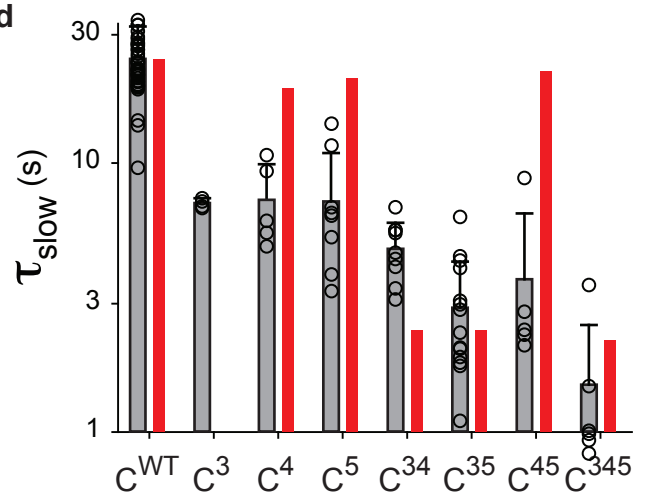
b

	wild-type	<i>SU3</i> mutant	<i>SU4</i> mutant	<i>SU5</i> mutant
SU3 desensitization rate	$\bar{\delta}_3^+$	$c_3^+ \cdot \bar{\delta}_3^+$	$\bar{\delta}_3^+$	$\bar{\delta}_3^+$
SU3 recovery rate	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$	$\bar{\delta}_3^-$
SU4 desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_4^+ \cdot \bar{\delta}^+$	$\bar{\delta}^+$
SU4 recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$
SU5 desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_5^+ \cdot \bar{\delta}^+$
SU5 recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$	$\bar{\delta}^-$

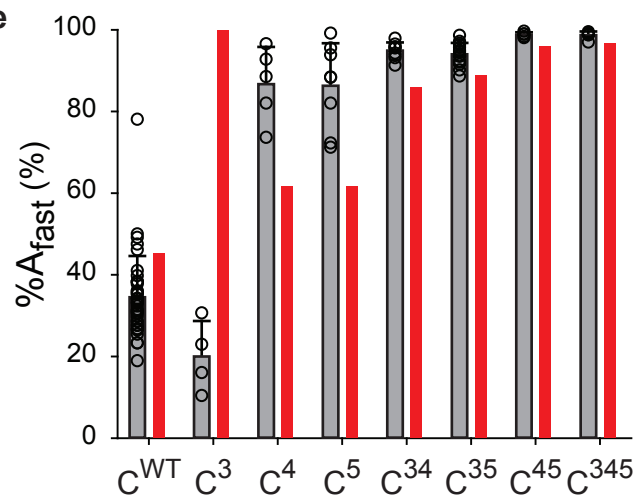
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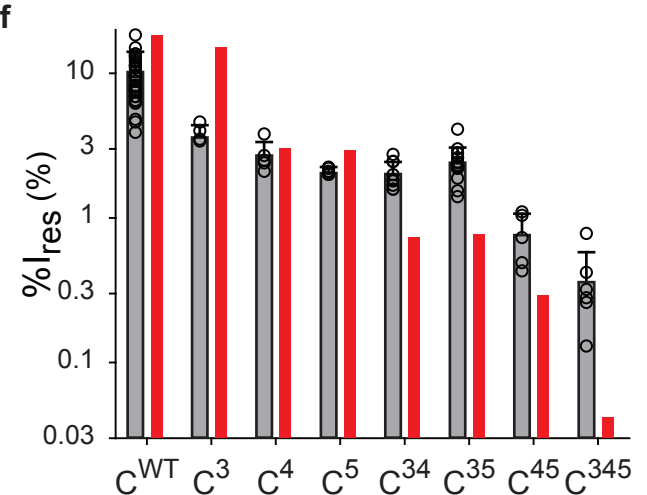
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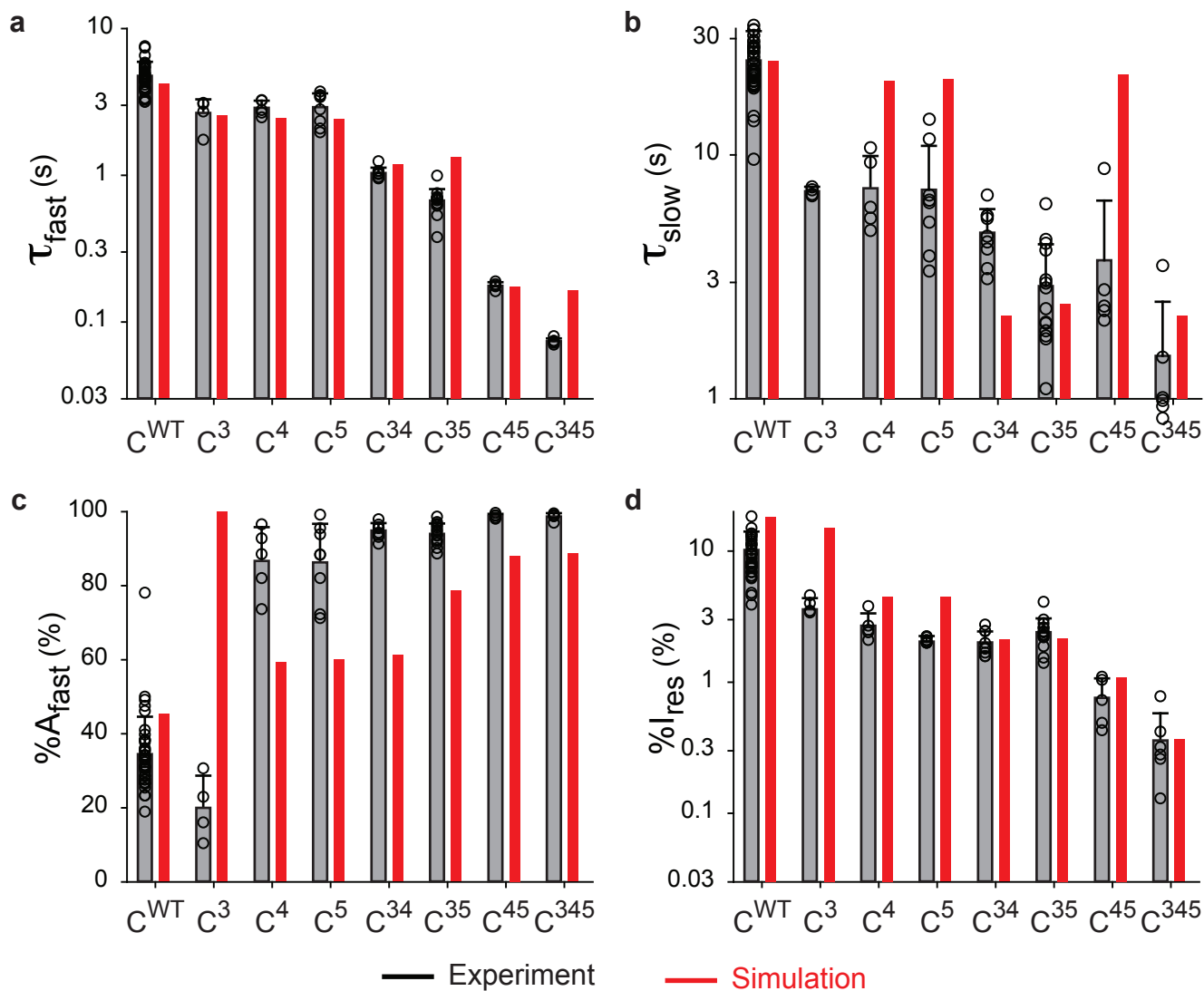
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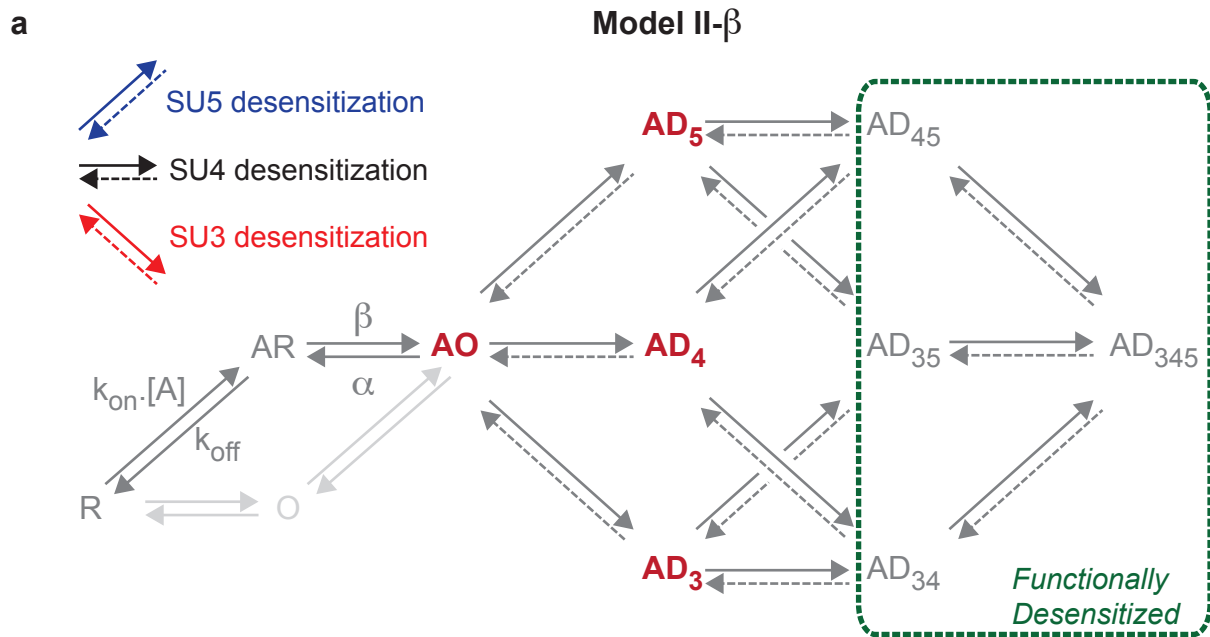


Supplementary Figure 4. Model II': underestimated values for the residual current in the absence of mutant-induced effects on desensitization recovery. **a**, For wild-type receptors, Model II and Model II' are strictly identical (see Main Figure 5). **b**, For mutated concatemers, Model II' incorporates the hypothesis that the mutations do not affect desensitization recovery rates. **c-f**, Bar graphs summarizing the experimental data (in black) vs the simulations (in red) for the indicated concatemers on the kinetics of the fast (panel **a**) and slow (panel **b**) desensitization components, the relative amplitude of the fast component (panel **c**) and the residual current after a 1 min long application of 10 mM GABA (panel **d**). Note that all simulated multiple mutants display largely reduced residual current as compared with experimental data. Experimental data are shown as means (bar graphs) and standard deviations (error bars), with individual data points indicated as circles. See Supplementary Table 1 for numerical values, number of cells and number of independent experiments.



Model II

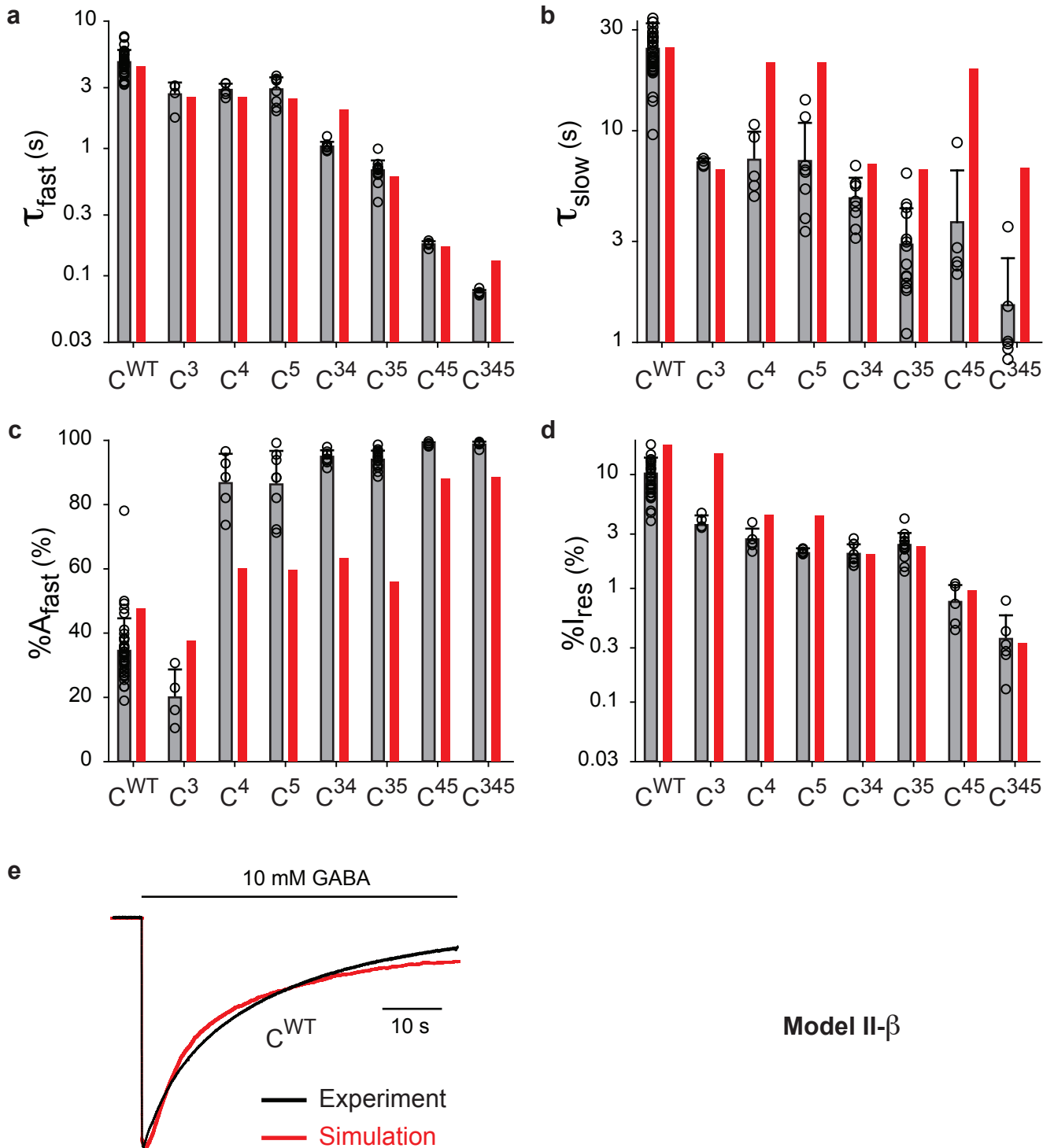
Supplementary Figure 5. Simulations from Model II. a-d, Bar graphs summarizing the experimental data (in black) vs the simulations (in red) for the indicated concatemers on the kinetics of the fast (panel a) and slow (panel b) desensitization components, the relative amplitude of the fast component (panel c) and the residual current after a 1 min long application of 10 mM GABA (panel d). See Supplementary Table 3 for the numerical values of parameters. Experimental data are shown as means (bar graphs) and standard deviations (error bars), with individual data points indicated as circles. See Supplementary Table 1 for numerical values, number of cells and number of independent experiments.



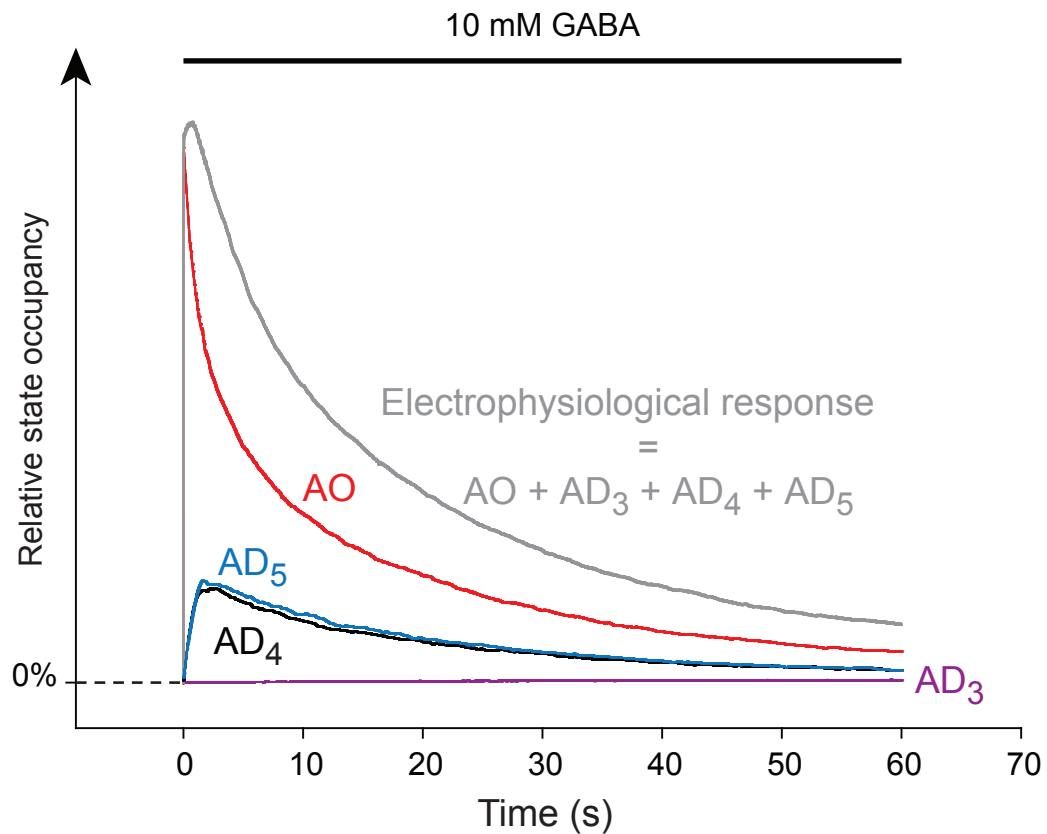
b

	wild-type	<i>SU3 mutant</i>	<i>SU4 mutant</i>	<i>SU5 mutant</i>
SU3	desensitization rate	$\bar{\delta}_3^+$	$c_3^+ \cdot \bar{\delta}_3^+$	$\bar{\delta}_3^+$
	recovery rate	$\bar{\delta}_3^-$	$c_3^- \cdot \bar{\delta}_3^-$	$\bar{\delta}_3^-$
SU4	desensitization rate	$\bar{\delta}^+$	$c_{34}^+ \cdot \bar{\delta}^+$	$\bar{\delta}^+$
	recovery rate	$\bar{\delta}^-$	$c_{34}^- \cdot \bar{\delta}^-$	$\bar{\delta}^-$
SU5	desensitization rate	$\bar{\delta}^+$	$\bar{\delta}^+$	$c_5^+ \cdot \bar{\delta}^+$
	recovery rate	$\bar{\delta}^-$	$\bar{\delta}^-$	$c_5^- \cdot \bar{\delta}^-$

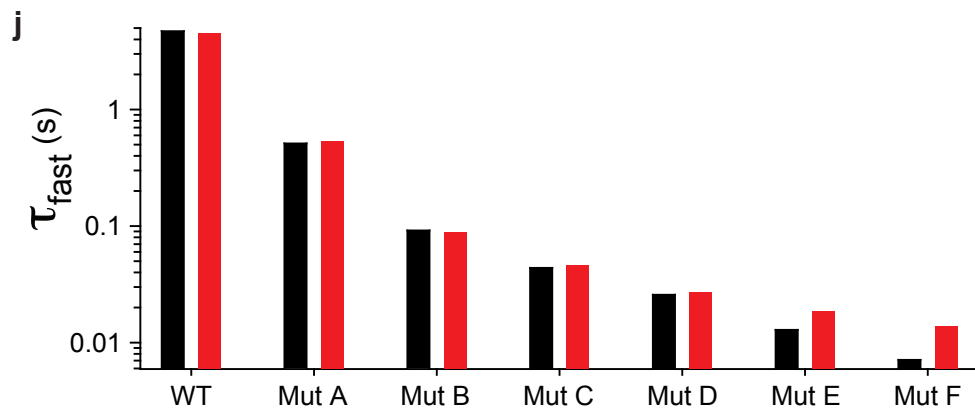
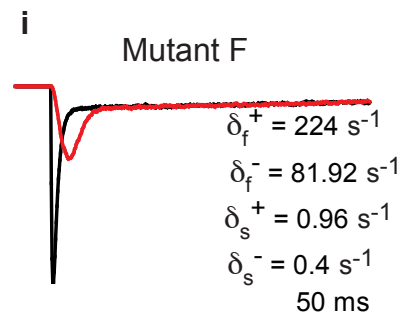
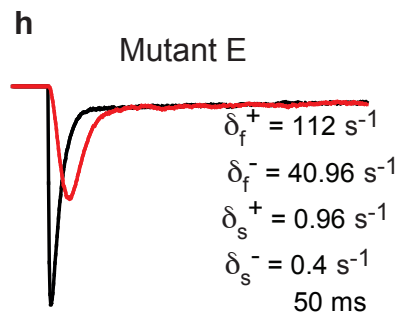
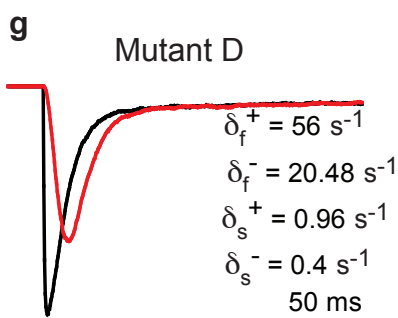
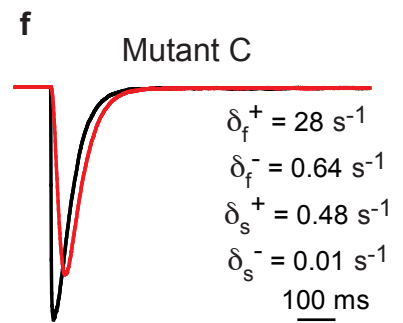
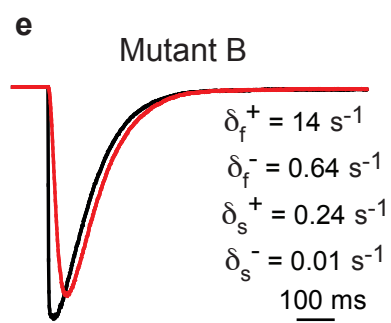
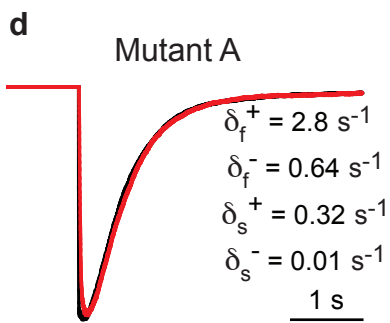
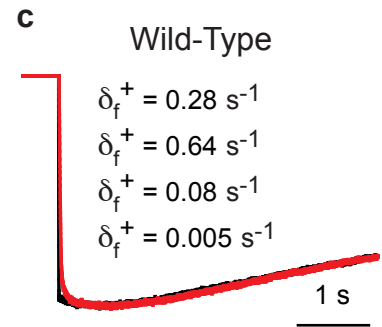
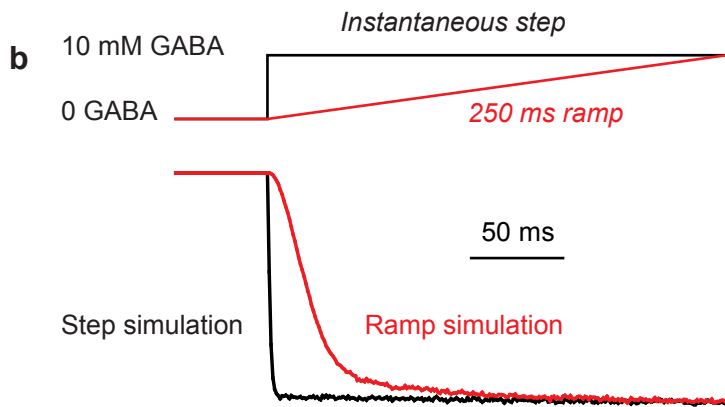
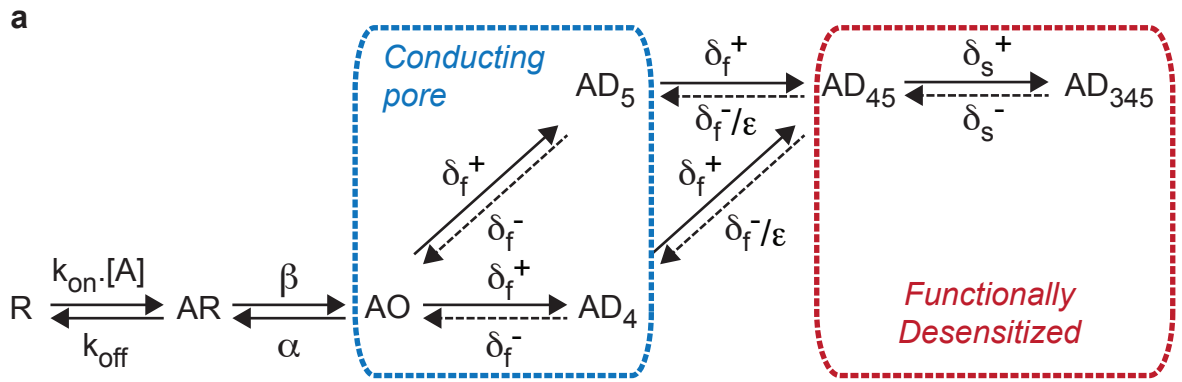
Supplementary Figure 6. Model II- β : mutation of SU3 directly impacts on SU4 desensitization. **a**, For wild-type receptors, Model II and Model II- β are strictly identical. **b**, For mutated concatemers, Model II- β incorporates the hypothesis that the mutation of SU3 also affects the desensitization of SU4 by increasing both its desensitization and recovery rates, by ratios c_{34}^+ and c_{34}^- , respectively.



Supplementary Figure 7. Simulations from Model II- β . **a-d**, Bar graphs summarizing the experimental data (in black) vs the simulations (in red) for the indicated concatemers on the kinetics of the fast (panel **a**) and slow (panel **b**) desensitization components, the relative amplitude of the fast component (panel **c**) and the residual current after a 1 min long application of 10 mM GABA (panel **d**). See Supplementary Table 3 for the numerical values of parameters. Experimental data are shown as means (bar graphs) and standard deviations (error bars), with individual data points indicated as circles. See Supplementary Table 1 for numerical values, number of cells and number of independent experiments. **e**, Overlay of a representative current for C^{WT} (in black) with the response simulated according to Model II- β (in red). Note that both the amplitude of the fast desensitization component and residual currents are overestimated in the simulation.



Supplementary Figure 8. Time-dependant changes in the occupancy of the receptor's states as predicted by model III. The relative occupancies of each state contributing to the electrophysiological currents, namely AO, AD₃, AD₄ and AD₅, are compared to the overall population of receptors in an active conformation (AO + AD₃ + AD₄ + AD₅), which directly translates the experimental recordings. The analysis of states occupancies was performed with QuB simulations.



Supplementary Figure 9. TEVC currents rise-time allow the measurement of fast desensitization kinetics down to the 25 ms timescale. **a**, Simplified kinetic scheme used to simulate receptors' desensitization, according to the kinetically favoured pathway from Main Figure 7. See Supplementary Table 3 for k_{on} , k_{off} , α and β numerical values. **b**, Simulating a gradual solution exchange with a 250 ms ramp protocol (red trace) yields a 20-80% current rise time of 23 ms, akin the experimentally achieved values, to be compared with a simulated instantaneous step solution exchange (black trace). **c-i**, Comparison between currents simulated with step (black traces) and ramp (red traces) application protocols, for a series of wild-type and virtual mutant receptors simulated with the indicated desensitization and recovery rates. Note the difference in peak current amplitude for Mutants C-F. **j**, Bar graph showing the time constants for the fast desensitization component of the virtual constructs simulated with step (black bars) and ramp (red bars). Note the discrepancy between the values from step and ramp protocols when desensitization occurs on a timescale faster than 20 ms: Mutant D displays τ_{fast} values of 25.7 ms and 27.1 ms with step and ramp protocols, respectively, while Mutant E shows τ_{fast} values of 12.8 ms and 18.7 ms with step and ramp protocols, respectively. The extreme case in the series, Mutant F, shows τ_{fast} values of 7.1 ms and 13.8 ms with step and ramp protocols, respectively.

Construct	SU1	SU2	SU3	SU4	SU5	I_{peak} (μA)	τ_{fast} (s)	τ_{slow} (s)	% A_{fast}	τ_w (s)	% I_{res}	# cells	# animals
C ^{WT}	WT	WT	WT	WT	WT	1.71 [1.05;2.8]	4.8 ± 1.2	24.4 ± 7.8	34.5% ± 10.1%	17.6 ± 5.1	10.2% ± 3.9%	n = 40	N = 17
C ¹	mut	WT	WT	WT	WT	3.24 [2.00;5.73]	4.5 ± 0.7	23.0 ± 7.0	35.9% ± 4.9%	16.6 ± 5.7	9.1% ± 3.4%	n = 5	N = 2
C ²	WT	mut	WT	WT	WT	3.35 [0.46;164]	4.0 ± 0.9	18.5 ± 5.1	29.9% ± 5.5%	14.2 ± 4.5	7.5% ± 2.9%	n = 5	N = 2
C ³	WT	WT	mut	WT	WT	1.42 [0.27;26]	2.7 ± 0.6	7.1 ± 0.3	20.0% ± 8.7%	6.2 ± 0.4	3.6% ± 0.8%	n = 4	N = 2
C ⁴	WT	WT	WT	mut	WT	1.18 [0.28;11]	2.9 ± 0.3	7.3 ± 2.6	86.7% ± 9.1%	3.4 ± 0.2	2.7% ± 0.7%	n = 5	N = 2
C ⁵	WT	WT	WT	WT	mut	0.86 [0.34;3.21]	2.9 ± 0.7	7.2 ± 3.7	86.3% ± 10.4%	3.3 ± 0.8	2.0% ± 0.2%	n = 8	N = 4
C ¹²	mut	mut	WT	WT	WT	0.91 [0.44;1.87]	3.6 ± 0.6	10.7 ± 1.2	35.8% ± 8.1%	8.1 ± 0.9	2.8% ± 0.2%	n = 10	N = 4
C ¹³	mut	WT	mut	WT	WT	1.21 [0.32;6.61]	3.7 ± 0.4	9.2 ± 0.9	36.9% ± 19.7%	7.2 ± 1.6	3.5% ± 0.5%	n = 5	N = 2
C ¹⁴	mut	WT	WT	mut	WT	1.68 [0.73;6.09]	3.3 ± 0.3	4.2 ± 1.2	60.8% ± 18.6%	3.4 ± 0.2	2.7% ± 1.0%	n = 7	N = 2
C ¹⁵	mut	WT	WT	WT	mut	2.05 [0.48;17.2]	2.7 ± 0.9	9.7 ± 4.4	83.4% ± 32.1%	3.2 ± 0.9	2.5% ± 0.6%	n = 6	N = 3
C ²³	WT	mut	mut	WT	WT	1.75 [0.53;18.9]	2.8 ± 0.3	5.7 ± 0.6	45.6% ± 18.2%	4.3 ± 0.6	3.3% ± 0.3%	n = 6	N = 2
C ²⁴	WT	mut	WT	mut	WT	3.09 [1.15;12.7]	2.7 ± 0.4	12.0 ± 5.8	95.7% ± 5.5%	3.0 ± 0.4	2.2% ± 0.6%	n = 8	N = 2
C ²⁵	WT	mut	WT	WT	mut	0.58 [0.33;1.13]	2.0 ± 0.7	5.7 ± 5.1	80.8% ± 25.0%	2.4 ± 0.7	1.9% ± 0.8%	n = 7	N = 3
C ³⁴	WT	WT	mut	mut	WT	0.60 [0.3;1.77]	1.0 ± 0.1	4.8 ± 1.2	94.9% ± 2.0%	1.2 ± 0.1	2.0% ± 0.4%	n = 9	N = 3
C ³⁵	WT	WT	mut	WT	mut	1.31 [0.68;2.81]	0.67 ± 0.13	2.9 ± 1.4	94.0% ± 2.8%	0.79 ± 0.15	2.4% ± 0.7%	n = 14	N = 6
C ⁴⁵	WT	WT	WT	mut	mut	2.64 [0.59;40.8]	0.18 ± 0.01	3.7 ± 2.8	98.8% ± 0.6%	0.22 ± 0.03	0.8% ± 0.3%	n = 5	N = 2
C ¹²³	mut	mut	mut	WT	WT	3.48 [1.62;9.94]	2.7 ± 0.8	6.8 ± 2.1	71.0% ± 20.1%	3.6 ± 1.2	3.6% ± 1.1%	n = 7	N = 2
C ¹²⁵	mut	mut	WT	WT	mut	0.30 [0.18;0.55]	1.3 ± 0.1	6.2 ± 1.6	96.1% ± 1.8%	1.5 ± 0.1	1.7% ± 0.4%	n = 8	N = 2
C ¹³⁴	mut	WT	mut	mut	WT	0.32 [0.14;0.69]	0.96 ± 0.13	3.9 ± 1.7	92.1% ± 3.5%	1.2 ± 0.2	2.5% ± 0.7%	n = 7	N = 2
C ¹⁴⁵	mut	WT	WT	mut	mut	0.43 [0.22;0.9]	0.16 ± 0.02	4.6 ± 2.7	98.0% ± 0.7%	0.26 ± 0.09	0.6% ± 0.3%	n = 8	N = 2
C ²³⁵	WT	mut	mut	WT	mut	1.26 [0.87;1.86]	0.33 ± 0.05	3.2 ± 2.5	97.8% ± 1.2%	0.38 ± 0.06	1.1% ± 0.4%	n = 6	N = 3
C ³⁴⁵	WT	WT	mut	mut	mut	1.61 [0.68;7.13]	0.07 ± 0.003	1.5 ± 1.0	98.7% ± 0.9%	0.10 ± 0.04	0.4% ± 0.2%	n = 6	N = 2
C ¹²³⁴	mut	mut	mut	mut	WT	0.86 [0.15;10.1]	0.36 ± 0.057	2.5 ± 1.9	96.0% ± 3.6%	0.41 ± 0.07	1.4% ± 0.4%	n = 7	N = 2
C ¹²³⁵	mut	mut	mut	WT	mut	1.19 [0.17;34.6]	0.24 ± 0.044	1.5 ± 0.6	93.4% ± 4.9%	0.31 ± 0.05	1.3% ± 1.2%	n = 6	N = 2
C ¹²⁴⁵	mut	mut	WT	mut	mut	0.35 [0.19;0.67]	0.09 ± 0.015	1.1 ± 0.3	97.4% ± 2.2%	0.11 ± 0.03	0.5% ± 0.2%	n = 5	N = 2
C ¹²³⁴⁵	mut	mut	mut	mut	mut	0.57 [0.28;1.37]	0.04 ± 0.007	0.4 ± 0.3	89.5% ± 10.7%	0.05 ± 0.01	0.5% ± 0.3%	n = 6	N = 2

SU1 mut & SU3 mut = β_2^{N303V} ; SU2 mut & SU4 mut = α_1^{N307V} ; SU5 mut = γ_2^{H318V}

Supplementary Table 1. Kinetics and extent of desensitization of the pentameric GABA_A concatemers used in the present study. Except for peak currents, all data are shown as means ± standard deviations. For each construct, n is the number of cells (*Xenopus laevis* oocytes) that we recorded, and N is the number of animals (i.e. number of independent *Xenopus* ovaries) that we used for all constructs - i.e. n cells recorded over N series of independent experiments. For peak currents, arithmetic means and 95% confidence interval are displayed, assuming log-normal distribution of currents and using the Cox method for confidence intervals⁹.

Parameter	Value	Unit
k_{on}	1.00E+05	$M^{-1}.s^{-1}$
k_{off}	100	s^{-1}
β	2000	s^{-1}
α	500	s^{-1}
δ_f^+	0.12	s^{-1}
δ_f^-	0.08	s^{-1}
δ_s^+	0.1	s^{-1}
δ_s^-	0.01	s^{-1}
γ_f^4	3.5	
ϵ_f^4	0.5	
γ_s^4	1	
ϵ_s^4	3	
γ_f^5	3.5	
ϵ_f^5	0.5	
γ_s^5	1	
ϵ_s^5	3	

Concerted model parameters

Supplementary Table 2. Numerical values used for the parameters from the concerted model in Supplementary Figure 2.

Parameter	Model Ia	Model Ib	Model II	Model II-β	Model III	Unit
k_{on}	1.0 E+05	1.0 E+05	1.0 E+05	1.0 E+05	1.0 E+05	$M^{-1}.s^{-1}$
k_{off}	100	100	100	100	100	s^{-1}
β	2000	2000	2000	2000	2000	s^{-1}
α	500	500	500	500	500	s^{-1}
δ^+	0.035	0.035	0.24	0.24	0.28	s^{-1}
δ^-	0.106	0.106	0.13	0.13	0.64	s^{-1}
δ_3^+	0.042	0.042	0.05	0.05	8.0 E-04	s^{-1}
δ_3^-	1.05 E-03	1.05 E-03	3.0 E-04	3.0 E-04	5.0 E-03	s^{-1}
γ	-	-	-	-	100	
ϵ	-	-	-	-	10	
c_3^+	4	10	10	3	3	
c_3^-	-	-	1	2	2	
c_{34}^+	-	-	-	4	4	
c_{34}^-	-	-	-	4	3	
c_4^+	10	100	30	30	20	
c_4^-	-	-	3	3	1	
c_5^+	10	100	30	30	60	
c_5^-	-	-	3	3	1	

Supplementary Table 3. Numerical values used for the parameters from the non concerted models I, II, II-β and III.

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