The C loop at the orthosteric binding site is critically involved in GABA\textsubscript{A} receptor gating

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HIGHLIGHTS

- Macroscopic and single-channel analysis revealed the effects of a C loop mutation.
- The C loop controls receptor gating, including preactivation, desensitization, and opening.
- The C loop mutation alters flurazepam sensitivity by affecting the preactivation transition.
- The C loop mutation distorts its structure, displacing the tip from the binding site.
- The C loop is critically involved in the binding and gating of GABA\textsubscript{A}Rs.

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ABSTRACT

GABA\textsubscript{A} receptors (GABA\textsubscript{ARs}) play a crucial role in mammalian adult brain inhibition. The dysfunction of GABAergic drive is related to such disorders as epilepsy, schizophrenia, and depression. Substantial progress has recently been made in describing the static structure of GABA\textsubscript{ARs}, but the molecular mechanisms that underlie the activation process remain elusive. The C loop of the GABA\textsubscript{AR} structure shows the largest movement upon ligand binding to the orthosteric binding site, a phenomenon that is referred to as “capping.” The C loop is known to be involved in agonist binding, but its role in the gating of Cys-loop receptors is still debated. Herein, we investigated this issue by analyzing the impact of a β\textsubscript{2}F200 residue mutation of the C loop on gating properties of α\textsubscript{1}β\textsubscript{2}γ\textsubscript{2} GABA\textsubscript{AR}s. Extensive analyses and the modeling of current responses to saturating agonist application demonstrated that this mutation strongly affected preactivation, opening, closing and desensitization, i.e. all considered gating steps. Single-channel analysis revealed that the β\textsubscript{2}F200 mutation slowed all shut time components, and open times were shortened. Model fitting of these single-channel data further confirmed that the β\textsubscript{2}F200 mutation strongly affected all of the gating characteristics. We also found that this mutation altered receptor sensitivity to the benzodiazepine flurazepam, which was attributable to a change in preactivation kinetics. In silico analysis indicated that the β\textsubscript{2}F200 mutation resulted in distortion of the C loop structure, causing the movement of its tip from the binding site. Altogether, we provide the first evidence that C loop critically controls GABA\textsubscript{AR} gating.

1. Introduction

γ-Aminobutyric acid type A receptors (GABA\textsubscript{AR}s) are pentameric ligand-gated ion channels (pLGICs) that are permeable to anions and mediate inhibition in the adult brain (Farrant and Nusser, 2005; Mody and Pearce, 2004). GABA\textsubscript{A} receptors are also a member of the Cys-loop receptor family together with nicotinic acetylcholine receptors (nACRs), glycine receptors (GlyRs), and 5-hydroxytryptamine type 3 receptors (5-HT\textsubscript{3}Rs; Cederholm et al., 2009; Miller and Smart, 2010; Thompson et al., 2010). The balance between inhibition and excitation is critical for proper functioning of the central nervous system. The dysfunction of GABAergic drive may lead to epilepsy, anxiety disorders,
2. Materials and methods

2.1. Cell culture and expression of recombinant GABA<sub>A</sub> receptors

All of the experiments were performed with the human embryonic kidney 293 (HEK293) cell line (European Collection of Authenticated Cell Culture). The cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The cells were replated on poly-o-lysine (1 μg/ml)-coated coverslips (Carl Roth) 24 h before transfection. The cells were transiently transfected using FuGENE HD (Promega) at a 1:3 FuGENE HD:DNA ratio with an adenoviral vector (plasmid) with the promoter for cytomegalovirus (pCMV) that contained cDNA of GABA<sub>A</sub>R subunits. The α<sub>2</sub>/β<sub>2</sub>γ<sub>2</sub> subunits were mixed in a 1:1:3 ratio (0.5:0.5:1.5 μg) in transfection solution, together with 0.5 μg human cluster of differentiation 4 (CD4) or enhanced green fluorescent protein (EGFP)-encoding plasmid. This subunit ratio was maintained for WT and mutated receptors because mutation of the β<sub>2</sub>F200 residue that, together with in silico docking and structural considerations, clearly indicated that this mutation affects both binding and gating transitions. Qualitative changes in the modulatory effect of the benzodiazepine flurazepam (FLU) on mutants relative to the wildtype (WT) receptor provided further evidence of the impact of the mutation on the preactivation transition. Altogether, we showed that the C loop is critically involved in both the binding and gating of GABA<sub>A</sub>Rs.

2.2. Electrophysiological recordings

Recordings were performed 24–48 h after transfection. All of the electrophysiological data were recorded using the patch-clamp technique.
technique. Currents were low-pass-filtered at 10 kHz and recorded at a holding potential of ~40 mV using an Axopatch 200B amplifier (Molecular Devices) and acquired using a Digidata 1550A acquisition card (Molecular Devices). For signal acquisition, pClamp 10.7 software (Molecular Devices) was used. Pipettes were pulled from borosilicate glass (outer diameter, 1.5 mm; inner diameter, 1.0 mm; Hilgenberg) using a P-97 horizontal puller (Sutter Instruments) and filled with intracellular solution that contained 137 mM KCl, 1 mM CaCl₂, 2 mM ATP-Mg, 2 mM MgCl₂, 10 mM K-gluconate, 11 mM EGTA, and 10 mM HEPES, with the pH adjusted to 7.2 with KOH. The pipette resistance ranged from 3 to 5 MΩ. Standard Ringer’s solution was used as the external saline, which contained 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 20 mM glucose, with the pH adjusted to 7.2 with NaOH. The osmolarity of solutions that contained > 10 mM of agonist was adjusted with glucose up to ~320 mOsm as described previously (Szczot et al., 2014). The solutions were supplied using an ultrafast perfusion system. Theta-glass capillaries (Hilgenberg) were mounted on a piezoelectric-driven translator (Physik Instrumente) as an ultrafast perfusion system. Theta-glass capillaries (Hilgenberg) were previously (Szczot et al., 2014). The solutions were supplied using an ultrafast perfusion system. Theta-glass capillaries (Hilgenberg) were previously described in more detail by Jonas (1995) and our group (Mozrzymas et al., 2003a, 2003b, 2007; Szczot et al., 2014). The solutions were simultaneously supplied to the two channels of the theta-glass by a high-precision SP220Z syringe pump (World Precision Instruments). The open tip solution exchange time ranged from 150 to 250 µs, depending on the size of the theta-glass and speed of flux.

Single-channel recordings and analyses that were applied herein are described in more detail in our recent paper (Kisiel et al., 2018). Recordings were performed in the cell-attached configuration at a pipette potential of 100 mV using an Axopatch 200B amplifier (Molecular Devices). The signals were first filtered at 10 kHz with a low-pass Bessel filter (mounted on the amplifier) and digitized at 100 kHz using a Digidata 1550B acquisition card and Clampex 10.7 software (Molecular Devices). Pipettes were prepared from thick-wall (outer diameter, 1.5 mm; inner diameter, 0.87 mm), filamented borosilicate glass (Hilgenberg) using a P-1000 horizontal puller (Sutter Instruments), and their resistance (with intrapipette Ringer’s solution) was in the range of 5–12 MΩ. The pipettes were coated with Sylgard (Dow Corning) to reduce noise and fire-polished. Extracellular (and intrapipette) saline differed from the saline solution that was used for the macroscopic current recordings and consisted of 102.7 mM NaCl, 20 mM Na-gluconate, 2 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES (Carl Roth), 20 mM TEA-Cl, 14 mM D(+)-glucose, and 15 mM sucrose (Carl Roth), dissolved in deionized water with the pH adjusted to 7.4 by 2 M NaOH. In the experiments in which a high GABA concentration (100 mM) was applied, a low-chloride solution was used instead to maintain osmolarity of ~320 mOsm: 70 mM NaCl, 10 mM Na-gluconate, 2 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES (Carl Roth), 20 mM TEA-Cl, and 14 mM D(+)-glucose. To reduce noise, the level of the extracellular solution was kept at a minimal possible level (1 ml in the 35 mm diameter recording dish; Nunc), and traces were selected for further analysis if the patches had a seal resistance > 10 GΩ. All of the electrophysiological recordings were conducted at room temperature (20–23 °C). All of the chemicals were purchased from Sigma-Aldrich Merck unless stated otherwise.

3. Theory/calculation

3.1. Experimental design and analysis of macroscopic currents

Dose-response relationships for mutated receptors were obtained from currents that were evoked by a wide range of non-saturating [GABA] concentrations relative to responses that were elicited by saturating [GABA] concentrations on the same cell and fitted using the Hill equation:

$$EC_{50} = \frac{1}{1 + \left(\frac{[GABA]}{EC_{50}}\right)^{n_H}}$$

where \(EC_{50}\) is half-maximal concentration, and \(n_H\) is the Hill coefficient.

The saturating concentration of GABA was assessed for each mutant individually. For dose-response relationships, recordings were performed in both whole-cell (lifted cell mode) and excised-patch configurations. The effect of the benzodiazepine FLU was assessed at 3 µM, and this drug was present in the wash (pretreatment) and in the solution that contained saturating [GABA]. Kinetic analysis was performed exclusively for currents that were recorded in the excised-patch configuration. Two application protocols were used: long (500 ms) and short (1.5–3 ms). The current onset was assessed as the 10–90% rise time (RT). The macroscopic desensitization time course (current kinetics after agonist removal) was fitted with either a single exponential or a sum of two exponential components according to the following equation:

$$I(t) = \sum_{n=1}^{1} A_n e^{-t/\tau_n}$$

where \(A_n\) is the amplitude of the \(n\)-th component, and \(\tau_n\) is the respective time constant. The mean time constant (\(\tau_{des}\)) for \(f = 2\) was calculated as the following:

$$\tau_{des} = \%A_1 \tau_1 + \%A_2 \tau_2$$

where \(\%A_n\) is the percentage of the respective component (%\(A_1 + \%A_2 = 1\)). Deactivation kinetics were determined for both short applications (to minimize macroscopic desensitization) and after long (500 ms) applications when deactivation was observed after macroscopic desensitization onset.

The rapid macroscopic desensitization component was assessed by fitting the exponential function with a constant coefficient:

$$I(t) = Ae^{-t/\tau_{des}} + C$$

where \(A\) is the fast desensitization amplitude, \(\tau_{des}\) is the time constant, and \(C\) is the constant value that represents the stationary non-desensitizing current. To precisely describe the kinetics of the rapid desensitization component, the time window for fitting that ranged from 7 to 15 ms from the current peak was set for each fitting. The proper choice of the fitting range allowed to avoid the interference from slower desensitization components that typically have a time constant of roughly 100 ms (Jatczak-Śliwa et al., 2018; Jones and Westbrook, 1995; Szczot et al., 2014). The steady state-to-peak parameter (ss/peak) was used to describe the extent of fast desensitization (low ss/peak – large extent of desensitization), calculated as the following:

$$ss/peak = \frac{C}{A_{max}}$$

where \(C\) is the aforementioned constant value, and \(A_{max}\) is the peak amplitude.

Data presentation and the statistical analysis were performed using SigmaPlot 11.0 software (Systat Software) and Excel 2016 software (Microsoft). Data were analyzed in terms of distribution normality using the Shapiro-Wilk test and outlier identification using Grubb’s test. Significant differences between WT (control group) and each of the β₃F200 mutants were tested independently using Student’s t-test or the Mann-Whitney U test (when data did not pass the normality test). The use of this simple test was justified by the fact that all of the results were based on comparisons between only two groups at a time. Similarly, in the case of pharmacological considerations (Fig. 4), in which test and control recordings were performed on the same cell, the paired test was used, but only two groups were compared at a time. Values of \(p < 0.05\) were considered statistically significant.
3.2. Homology modeling and ligand docking

The sequence alignment of α1, β2, and γ2 GABAAR subunits and other pLGICs was performed in T-Coffee (Di Tommaso et al., 2011) and refined manually using Jalview (Waterhouse et al., 2009). Three “base WT” homology models of the α1β2γ2 GABAAR ECD were used in the present study. The first model was based on a homomeric β2 GABAAR structure (Miller and Aricescu, 2014), similar to Jatzczak-Sliwa et al. (2018). The other two models were based on A and B conformations of α1β2γ2 GABAARs that were described by Zhu et al. (2018). In the case of these latter two models (Zhu et al., 2018), only the missing residues were introduced because these templates depicted the same assembly type as the one that was investigated in the present study. The MODELLER Python package (Šali and Blundell, 1993) was used to construct these models using automodel class. In the next step, mutations (F200C, F200I, and F200Y) were introduced to each “base WT” model, and 20 new models of each type were constructed. From these 20 models, the best model was selected by best molpdf score, assessed by the MODELLER package. Thus, three “base WT” and three “base F200C,” “base F200I,” and “base F200Y” models of α1β2γ2 GABAARs were obtained. The GABA molecule was initially positioned in both LBs of each model according to its position in the structures from Zhu et al. (2018). The final binding positions and binding energy were determined by the AutoDock Vina (Trott and Olson, 2009) stiff and flexible fit docking methods. In the stiff method, only the GABA molecule was able to move within the LBS, whereas the local macromolecule’s structure was kept immobilized. In the flexible method, both GABA and the functional groups of residues that formed the site were mobile (with a fixed backbone). Because there were three “base” models for each receptor type (WT and mutants) and because GABA was docked to both LBs, the total number of six binding modes was assessed for each receptor type for both the stiff and flexible methods. C loop modeling was also performed using the MODELLER package. The same models (“base”) as those that were used for docking were rebuilt with the loopmodel class with constrained positions of all atoms, with the exception of residues that formed the C loop tip. A total of 100 new models for each “base” model were created (resulting in 300 “loop WT,” “loop F200C,” “loop F200I,” and “loop F200Y” models). DOPE energy scores of residues that formed the C loop were calculated using MODELLER evaluation functions, and all of the models were evaluated visually to exclude incorrect ones (e.g., having overlapping loops). Analysis of the results and visualizations were performed using VMD (Humphrey et al., 1996) and Python scripts.

3.3. Simulations and kinetic modeling of macroscopic currents

The kinetic modeling of macroscopic currents was performed using ChannelLab 2.0 software (Synaptosoft). No formal fitting to experimental traces was performed. An extensive trend analysis based on several scenarios for the values of the rate constants was instead considered. The final model rate constants were selected according to the best reproduction of experimentally observed current response parameters: RT, ss/peak ratio, τd+ns (long pulse), and τd−act (short pulse). Additionally, the rates were selected to reproduce the EG50 value and amplitude increase in the presence of FLU (for the F200C mutant). Information from docking studies (i.e., the assessment of binding affinity) and single-channel analysis (e.g., shortening of open times; see below) was also considered. The best reproduction of the experimental results was achieved for τd+ns (long pulse), ss/peak, and EC50. Slightly lower accuracy was achieved for reproduction of the RT and deactivation time constant, τd−act, in the case of quickly deactivating mutants. However, the relative changes relative to WT were properly reproduced. Lower accuracy in reproducing these current features could result from the limited speed of our application system. The kinetic modeling of macroscopic currents was performed for the F200C mutant only because this receptor presented the largest differences compared with WT.

3.4. Single-channel analysis

Single-channel kinetic analysis and modeling were performed using SCAN, EKDIST, and HJCFIT software (DCprogs pack), which was kindly provided to our group by David Colquhoun. Similar to the macroscopic current analysis, the single-channel experiments were assumed to be performed at saturating [GABA] concentrations. For the F200C mutant, 100 mM GABA saturation was incomplete by a small margin. This problem is discussed further in Section 5.2. Traces that contained at least 10000 events were considered for the analysis. Signals were stored as Axon Binary File (.abf) files and filtered to obtain a signal-to-noise ratio of at least 15. The final cutoff frequency (fc) was calculated as the following:

\[ \text{fc} = \frac{1}{fa} - \frac{1}{fd} \]

where fa is the analog filter frequency (typically 10 kHz), and fd is the digital frequency (offline filtering with 8-pole low-pass Bessel filter by pClamp software). The sampling frequency, fs, was reduced to \( fs = \frac{10 \cdot fc}{\text{fs}} \). Recordings that showed multilevel openings were excluded from the analysis. The recordings revealed different modes of activity (Kisiel et al., 2018; Lema and Auerbach, 2006) that clearly differed in open probability. In the present study, only the predominant activity mode was included in the analysis. The criteria that were used to select this predominant mode are described in detail in Kisiel et al., 2018. Only clusters were included in our analysis, which were extracted visually and exported individually to separate files. Recordings that were selected in this way were then idealized by a time-course fitting procedure and saved with SCAN software as *.scn files and then used to generate distributions (with EKDIST) of shut and open time periods and fit them with the sum of exponentials (in figures; P% = relative areas, \( \tau = \text{time constants} \)). Time resolution was identified separately for each recording within the range of 40-80 μs for open and shut times, and these values were then used in the model simulations. As explained in our recent study (Kisiel et al., 2018), the longest shut time components were not considered informative because of their low percentage and high variability and because they could be affected by the activity of more than one channel in the patch. Thus, although each time shut times distributions for WT receptors were fitted with four exponentials, the statistics is presented for three fastest components. To define bursts, critical time (tcrit) was determined, based on the analysis of the shut times distributions, which typically consisted of four components for WT and F200Y receptors and three components for F200C and F200I mutations. Bursts were then defined by tcrit, calculated based on EKDIST software, using Jackson’s criterion (Jackson et al., 1983) that was applied to the third and fourth components of the shut times distributions for WT and F200Y or for the second and third components of the shut times distributions for F200I and F200C mutations. Bursts that were detected using this method consisted of several events. For this reason, even if at least 10000 events were present in the analyzed trace, they were typically not sufficiently numerous to build a burst duration distribution that could be reliably fitted. Instead, the mean burst duration was calculated as the arithmetic mean.

Kinetic modeling based on single-channel data (stored in *.scn files) was performed using HJCFIT software (DCWinpros, provided by David Colquhoun), which is based on the maximum likelihood method that enabled optimization of the rate constants in the model. To verify the model predictions, dwell times distributions that were generated by the model (at experimental and 0 μs resolutions) were confronted with those that were obtained experimentally. For recordings that were performed for WT receptors, we compared the present results (Tables 1 and 2) with our previous data (Kisiel et al., 2018) and found that they were very consistent. For the mutants, we considered that the modeling was consistent if the distribution parameters that were obtained from...
the model fitting (P and \(\tau\); Tables 1 and 2) reproduced significant changes between the same parameters in the experimental distributions when comparing WT and specific mutants (see Section 4.7).

4. Results

4.1. Sequence analysis of C loop in pLGICs and choice of mutations

The C loop connects \(\beta\)-strands 9 and 10 and is a structural motif that is well conserved in each member of the pLGIC family. In the \(\beta_2\) subunit of the GABA\(_A\)R, the C loop starts at position F200 and ends at Y205. The latter residue is well conserved in pLGICs (Fig. 1). In our hands, GABA\(_A\)R\(_s\) with mutations at this position were non-functional. Although F200 is less conserved than Y205, it is conserved in all \(\beta\), \(\tau\), and \(\delta\) GABA\(_A\)R subunits (which form the principal side of the LBS). Interestingly, in all \(\alpha\)- and \(\gamma\)-type subunits that do not form the principal side of the LBS, phenylalanine is replaced by other residues (Fig. 1), indicating its role in ligand binding. A similar scheme is observed in nAChRs (tyrosine at a position that is homologous to F200 in principal subunits and a non-aromatic residue in complementary subunits), GLIC, ELIC, and GlyRs (aromatic residue at a position that is homologous to F200; Fig. 1). Considering these observations, the following scheme emerges. Phenylalanine or tyrosine starts or ends the C loop in pLGIC subunits, forming the principal side of the LBS. This indicates the key involvement of F200- and Y205-homologous residues in the activation of pLGICs. In the present study, we examined the role of an F200 residue mutation in \(\alpha_1\beta_2\gamma_2\) GABA\(_A\)Rs. The following mutations were selected: \(\beta_2\)F200C (F200C) because this mutation is expected to induce substantial disturbances in the structure of the C loop, \(\beta_2\)F200I (F200I) to validate the effect of aromatic ring removal and thus modification of the “aromatic box” structure of the LBS, and \(\beta_2\)F200Y (F200Y) to examine the effects of a slight modification of LBS architecture by the addition of a hydroxyl group while maintaining the aromatic group.

4.2. Impact of \(\beta_2\)F200 mutation on macroscopic GABAergic currents

To assess the impact of mutations at the \(\beta_2\)F200 residue on receptor responsiveness to GABA, dose-response relationships were determined (Fig. 2a). As expected, Cys and Ile substitutions resulted in a substantial rightward shift of the dose-response relationship that was in qualitative agreement with previous reports (Tran et al., 2011; Wagner and Czajkowski, 2001). Note that the dashed line in Fig. 2a shows the dose-response relationship function for WT receptors that was recently determined by our group under the same experimental conditions (Brodzki et al., 2016). Interestingly, in the case of the F200Y mutant, the dose-response function was shifted in the opposite direction relative to WT.

**Fig. 2. Mutations of \(\beta_2\)F200 residue affect the dose-response relationship and kinetics of currents that are evoked by saturating [GABA] concentrations.** (a) Dose-response relationships normalized to maximum current amplitudes that were evoked by saturating [GABA] (F200Y: 10 mM; F200I: 30 mM; F200C: 200 mM) and fitted with the Hill equation. The dose-response curve for WT (black dashed line) was recently assessed by our group (Brodzki et al. (2016); EC\(_{50}\) = 0.04; Hill coefficient \(n_h = 0.67\). EC\(_{50}\) values for respective mutants are shown in the inset. Hill coefficient for F200Y (gray squares): \(n_h = 0.76\). Hill coefficient for F200I (dark red circles): \(n_h = 1.04\). Hill coefficient for F200C (orange triangles): \(n_h = 0.99\). (b) Examples of typical normalized responses to 500 ms application of a saturating [GABA] concentration for each mutant and for WT. Note the marked differences in the current time course between WT and the mutants, indicating marked alterations of receptor gating.
to WT, although the extent of this shift was markedly less than with F200I and F200C.

As shown in the example traces in Fig. 2b, distinct mutations of the β2F200 residue profoundly affected the time course of currents that were elicited by saturating GABA concentrations, clearly indicating that this mutation may strongly affect receptor gating. Therefore, we investigated this issue by performing a detailed analysis of the current time course using different protocols. To ensure the highest fidelity of these recordings and analysis, they were performed exclusively in the outside-out configuration at which the fastest agonist exchange speed could be achieved. In the analysis that is presented below, all of the comparisons were performed relative to the values that were obtained for WT receptors. Mutation of the β2F200 residue clearly slowed the current onset, with the strongest effect for the cysteine mutant (WT: 0.45 ± 0.02 ms, n = 8; F200Y: 0.9 ± 0.1 ms, n = 7, p < 0.001; F200I: 1.07 ± 0.17 ms, n = 6, p < 0.001; F200C: 1.26 ± 0.08 ms, n = 8, p < 0.001; Fig. 3a and b). Next, we analyzed the rapid component of macroscopic desensitization and found that the mutation slowed the time constant, τdesens (Fig. 3c and d). Again, the strongest effect was observed for F200C (WT: 1.77 ± 0.12 ms, n = 7; F200Y: 1.91 ± 0.28 ms, n = 7, p > 0.05; F200I: 3.1 ± 0.43 ms, n = 7, p = 0.008; F200C: 4.17 ± 0.32 ms, n = 9, p < 0.001). However, no difference in τdesens was found between WT and the tyrosine mutant. Moreover, for the cysteine mutant, the extent of desensitization was markedly reduced (i.e., the ss/peak significantly increased; Fig. 3e), whereas no effect was found for F200I or F200Y (WT: 0.36 ± 0.04, n = 9; F200Y: 0.29 ± 0.03, n = 7, p > 0.05; F200I: 0.37 ± 0.03, n = 7, p > 0.05; F200C: 0.47 ± 0.02, n = 9, p = 0.042; Fig. 3e). The time course of current responses to the short application of a high GABA concentration is believed to qualitatively mirror kinetic features of synaptic currents (Jones and Westbrook, 1995). Therefore, we examined currents that were elicited by 1.5–3 ms applications of saturating GABA. As shown in Fig. 3f and g, β2F200 mutations affected deactivation kinetics, and this effect was particularly strong for the F200C and F200I mutants, indicated by the mean deactivation time constant, τdesact (WT: 59.43 ± 10.1 ms, n = 4; F200Y: 17.84 ± 4.52 ms, n = 5, p = 0.005; F200I: 5.96 ± 2.13 ms, n = 4, p = 0.002; F200C: 5.56 ± 1.23 ms, n = 4, p = 0.002; Fig. 3f and g).

4.3. β2F200 mutation affects receptor sensitivity to flurazepam

Recent studies provided extensive evidence that benzodiazepines upregulate GABA_A activity by affecting receptor gating (Downing et al., 2005; Gielen et al., 2012; Mercik et al., 2007). This modulatory effect was previously ascribed primarily to an enhancement of binding (Krampff et al., 1998; Lavoie and Twyman, 1996). The observation that benzodiazepines enhance the amplitudes of WT receptor-mediated responses to saturating partial agonists or currents that are elicited by saturating full agonists mediated by mutants, indicated that the major modulatory mechanism of benzodiazepines involves upregulation of the flipping (preactivation) transition. For instance, in our recent study (Jatczak-Śliwa et al., 2018), such a FLU-induced higher amplitude of
GABA-evoked responses provided key evidence that mutation of the α1F64 residue primarily affected the flipping (preactivation) transition. Thus, we tested the sensitivity of the β2F200 mutants to FLU, expecting that such sensitivity would reflect the impact of the mutations on receptor gating. Notably, among the mutants that were studied, F200Y exhibited a kinetic phenotype that was most similar to WT (Fig. 3), and the effect of FLU was comparable to WT (relative amplitude: 0.73 ± 0.07, n = 4, p = 0.018; comparison between amplitudes with and without FLU; Fig. 4a and b). This was comparable to the effect of FLU on WT that was described by Mercik et al. (2007) and Mozrzymas et al. (2007). The F200C mutant had the most pronounced alterations of kinetic features, and the effect of FLU on amplitude was inverted relative to WT (relative amplitude: 1.16 ± 0.04, n = 6, p = 0.023; Fig. 4a and b). The F200I mutant exhibited an intermediate effect of FLU (relative amplitude: 1.06 ± 0.05, n = 7, p > 0.05; Fig. 4a and b).

To further explore the impact of FLU on the mutants, we analyzed the way in which FLU affects the time course of current responses to saturating [GABA]. For the F200I and F200C mutants, FLU significantly accelerated the onset kinetics, whereas it did not change for the F200Y mutant (relative values for F200Y: 1.07 ± 0.03, n = 4, p > 0.05; relative values for F200I: 0.81 ± 0.05, n = 6, p = 0.027; relative values for F200C: 0.83 ± 0.02, n = 6, p = 0.008; Fig. 4a, c). Macroscopic desensitization was significantly accelerated by FLU for F200C and F200I. For F200Y, however, this effect was negligible (relative desensitization time constant (τdesens) values after a short pulse for the mutants. (f) Corresponding statistics for relative mean deactivation time constant (τdeact) values after a long (500 ms) pulse for this mutant. (g) Corresponding statistics for relative mean deactivation time constant (τdeact) values after a short pulse for the mutants. (h) Corresponding statistics for relative mean deactivation time constant (τdeact) values after a long pulse. Asterisks indicate a statistically significant difference between [GABA] + FLU and the control.

Fig. 5. Docking modeling indicates that the affinity for GABA is reduced for the F200I and F200C mutants but not for the F200Y mutant. (a) Best binding modes (determined by the flexible docking method) of the GABA molecule in WT and mutated receptor models based on the structure from Zhu et al. (2018). WT model (gray). F200Y mutant (blue). F200I (yellow). F200C (orange). For the mutants, only the GABA molecule and residues that formed the binding site are presented. The β2F200 residue, respective mutants, and GABA molecule are shown in bold stick representation. (b) Average affinities of GABA binding that were determined using the stiff and flexible docking methods. Note a significant reduction of affinity for the F200I and F200C mutants, which was mainly attributable to disturbances of low-energy “aromatic box” interactions that are conserved in the F200Y mutant. Asterisks indicate a statistically significant difference between groups (indicated by bars).
A structural analysis of C loop conformation suggests that F200C and F200I mutations induce changes in C loop structure. (a-d) Example alternative C loop folds of the F200C mutant. In each picture, the “base” F200C mutant homology model that was based on the GABA_A receptor structure from Zhu et al. (2018) is shown in gray (DOPE energy score = −0.713). Alternative folds of the C loop are shown in orange (backbone not constrained by experimental WT structure): (a) outward fold (−0.704), (b) inward fold and cysteine functional group twist (−0.708), (c) similar to b, but the cysteine orientation is conserved (−0.717), and (d) more pronounced disturbance in the structure but with less favorable energy (−0.667). (e) Same fold as in a but aligned to the F200C mutant binding mode from the docking studies. Note that the C loop tip is moved away from the LBS center, which could impair ligand binding and possibly gating transitions. (f, g) Example energy profiles of C loop residues for alternative folds. (f) Energy of WT models: WT “base” model (based on GABA_A structure from Zhu et al., 2018) (thick black line) and energies of subsequent models with unconstrained C loop structure (thin orange lines). (g) Same as f but for the F200C mutant. Note that in contrast to WT (f), the F200C mutant (g) has more alternative folds with energy that is similar or lower relative to the “base” model. (h) Average energies of C loop alternative folds for each mutant and WT relative to their “base” models. Each mutant showed more energetically favorable alternative folds than WT, but their number was the smallest for F200Y among the mutants.

\[ \tau_{\text{desens}} \text{ for F200Y: } 0.94 \pm 0.07, n = 4, p > 0.05; \text{ relative } \tau_{\text{desens}} \text{ for F200I: } 0.78 \pm 0.06, n = 7, p = 0.024; \text{ relative } \tau_{\text{desens}} \text{ for F200C: } 0.75 \pm 0.05, n = 6, p = 0.023; \text{ Fig. 4d and e). Flurazepam had no significant effect on the extent of desensitization (relative ss/peak for F200Y: } 0.95 \pm 0.02, n = 7; \text{ relative ss/peak for F200C: } 0.92 \pm 0.04, n = 6, p > 0.05, \text{ data not shown). Finally, the effect of FLU on deactivation kinetics was examined for currents that were mediated by the mutants. When applying a short (1.5–3 ms) GABA pulse, we observed a trend toward a FLU-induced slowing of deactivation kinetics that was statistically significant for F200Y and F200C (relative \( \tau_{\text{deact}} \) for F200Y: 1.48 ± 0.08, \( n = 4, p = 0.018 \); relative \( \tau_{\text{deact}} \) for F200I: 1.12 ± 0.04, \( n = 4, p > 0.05 \); relative \( \tau_{\text{deact}} \) for F200C: 1.22 ± 0.04, \( n = 3, p = 0.02 \)). Moreover, FLU significantly prolonged the deactivation time course that was observed after long (500 ms) GABA application (relative \( \tau_{\text{deact}} \) for F200Y: 1.53 ± 0.11, \( n = 6, p = 0.031 \); relative \( \tau_{\text{deact}} \) for F200I: 1.36 ± 0.1, \( n = 3, p < 0.001 \); relative \( \tau_{\text{deact}} \) for F200C: 1.3 ± 0.08, \( n = 6, p = 0.003 \); Fig. 4f–h).

4.4. Interactions between C loop and GABA molecule and impact of mutations on receptor structure

To provide further insights into the molecular mechanisms whereby the mutations affected receptor kinetics, including the dose-response relationship, GABA docking to structural models was investigated. The selected binding modes are presented in Fig. 5a, and their affinities are presented in Fig. 5b. Independent of the method used (i.e., stiff or flexible), each mutation led to a decrease in binding affinity, with the exception of F200Y, in which the difference relative to WT did not reach significance (Fig. 5b). The similar affinities for WT and the F2000Y mutant was unsurprising because conservation of the aromatic ring in F200Y was expected to largely maintain the network of cation-π interactions with a ligand molecule (Fig. 5a). However, for the F200Y mutant, a leftward shift of the dose-response relationship was observed (Fig. 2a), which unlikely results from an increase in affinity. Moreover, as indicated by Colquhoun (1998), alterations of gating could also result in a shift of the dose-response relationship. As we present below, modeling of the experimental data indicated that for the tyrosine mutant, a lower EC_{50} might be attributable to an increase in the flipping gating rate. In contrast to F200Y, GABA docking for F200C and F200I predicted significantly lower binding affinity compared with WT, which is compatible with the rightward shift of the dose-response relationships for these mutants (Fig. 2a). Additionally, deactivation was strongly accelerated for these mutants, which could reflect less efficient ligand binding compared with WT. However, an additional consideration in these docking studies was that the backbone conformation of the receptor was kept immobile and was the same as in the experimental structural template that was used to build the WT and mutant models. Thus, this type of docking approach would be unable to reveal possible effects of mutations on general structure of the C loop, which is known to be highly mobile. To address the issue of structural changes in the ligand-free C loop that were induced by the mutations, an additional approach was applied. In contrast to docking, the C loop tip was allowed to fold freely according to the most favorable energy. Example alternative structures of the F200C mutant C loop are presented in Fig. 6a–d. Fig. 6e further presents visualization in the context of the LBS. The thick black line in Fig. 6f and g represents the energy profile for the part of the protein at the LBS that contains the C loop region (residues 190–210) in the reference structure. In the case of WT and the mutants, most of the alternative C loop folds (thin orange lines in Fig. 6f and g) had higher energy than in the case of backbone preservation from the structural template (thick black line in Fig. 6f and g). For WT (Fig. 6f), almost none of the models had more favorable energies than the reference structure, and similar results were obtained for the tyrosine mutant. In contrast, a fraction of the alternative fold models of F200C (Fig. 6g) and F200I exhibited energies that were more favorable than the reference backbone fold. This means that these mutations may favor structural changes in this loop relative to the template.
Furthermore, in most cases, this alternative fold reflected C loop tip movement away from the LBS, but some folds also exhibited inward C loop movement (Fig. 6a–d). In both cases, this would lead to a reduction of binding affinities below those that were estimated in the docking studies.

Altogether, the results of the structural modeling of WT receptors and the mutants (Fig. 6h) indicated that both the binding mode and C loop structure of F200Y were most similar to WT. The F200C and F200I mutants may exhibit disruptions of C loop conformation that could explain the increase in EC50 values and changes in current response kinetics. Importantly, the F200I mutant exhibited a smaller decrease in binding affinity than F200C, which appears to be compatible with a 10-fold lower EC50 value in the case of F200I.

4.5. Model simulations for macroscopic currents

To provide a mechanistic interpretation of the profound kinetic changes that were observed in our macroscopic recordings and that were induced by the β2F200 residue mutation, model fitting was considered. The F200Y mutation resulted in a phenotype that was similar to WT, but the F200I and F200C mutations clearly altered receptor kinetics, and the impact of the F200C mutation was considerably larger. We thus conducted model fitting for the cysteine mutant. We used the framework of the model that is presented in Fig. 7a, which was previously considered by our group in our recent report (Szczot et al., 2014). Notably, this model is markedly simplified with regard to the one that was used for single-channel modeling (Model 1, Fig. 10a) because it contains only one open state and one desensitized state. As reported by Colquhoun and Lape (2012), the modeling of macroscopic currents is much more vulnerable to overparametrization than the single-channel analysis. Indeed, in our macroscopic modeling that was based on extended models that were used in single-channel modeling, making a unique interpretation was difficult in terms of the reliable optimization of rate constants. We thus considered a minimum requirement model, in which each gating feature is represented by one branch of the model.

The rate constants of the model were selected to best reproduce the effect of the F200C mutation. For this mutant, a very strong rightward shift of the dose-response function was observed (Fig. 2a). Although a shift of the dose-response relationship can principally occur because of a change in affinity or gating (especially efficacy; Colquhoun, 1998), strong modification of the binding properties was required to reproduce the effect of FLU for the F200C mutation (see Fig. 4) was best reproduced by an increase in the flip rate (δ) up to the level of WT and by increasing desensitization rates. (e–e) Simulated current responses. (c) Normalized response to a long pulse of a saturating GABA concentration. Notice prolonged current onset, slower desensitization, and a higher ss/peak ratio for the F200C mutant and their “rescue” toward the levels of WT after FLU treatment. (d) The same responses as in c but not normalized, showing the reproduction of the effect of FLU on current amplitude. (e) Response to short GABA application under control conditions (black) and in the presence of FLU (gray). Notice a markedly slower deactivation time course for WT and the prolongation of deactivation by FLU for the F200C mutant.

Fig. 7. Model simulations of macroscopic currents reveal that the F200C mutation affects both ligand binding and gating of the receptor. (a) Kinetic model topology (“flipped Jones-Westbrook model”) from Szczot et al. (2014) that was used to model macroscopic currents for WT and the F200C mutant. (b) Rate constants for WT receptors and the F200C and F200C mutants in the presence of FLU. The F200C mutation strongly impaired binding by reducing the kon rate and increasing the unbinding rate (koff). Gating transitions were also affected: transition into flipped state (δ), opening/closing (β and α), and desensitization (d and r). The effect of FLU for the F200C mutation (see Fig. 4) was best reproduced by an increase in the flip rate (δ) up to the level of WT and by increasing desensitization rates. (c–e) Simulated current responses.
both the dose-response relationship shift and modifications of the time course of macroscopic currents that were evoked by non-saturating [GABA] and mediated by the mutant. This was unsurprising because this mutation involves a key residue at the orthosteric LBS. As described above, the extent of impact of the mutation on receptor binding properties was assessed using docking and C loop structure modeling. Both of these aspects were involved in weakening of the LBS. The major difficulty in model fitting was that each current kinetic feature (e.g., current onset or macroscopic desensitization) may depend on all of the rate constants in the kinetic model (Colquhoun, 1998; Mozrzymas et al., 2003). To reproduce the effect of the F200C mutation on current onset kinetics, a reduction of flipping and desensitization rates was required. A decrease in the flipping rate, δ, was also needed (together with the weakening of binding characteristics) to reproduce a robust increase in the EC50 value for the F200C mutant. Another key observation was a marked slowing of macroscopic desensitization (Fig. 3c and d). This effect could be fairly reproduced by reducing the desensitization rate, d, which was also compatible with the reduction of the current onset rate. Additionally, a reduction of the δ rate constant enabled us to reproduce rapid deactivation kinetics in the case of the F200C mutant. Slower macroscopic desensitization could be alternatively reproduced by a decrease in the closing rate, α. However, this scenario would predict slow deactivation for currents that are mediated by the mutant, in contrast to our experimental observations. Most importantly, it would contradict the single-channel data that consistently revealed a shortening of open time (Table 2) and hence the increase in α rates. Simulated responses for WT and F200C are presented in Fig. 7c–e, together with tables of the respective rate constants (Fig. 7b).

Another line of evidence that was useful for deciphering the mechanism of action of the F200C mutation on receptor gating was the effect of FLU which, in contrast to WT receptors, increased the amplitudes of current responses that were mediated by the F200C mutant (Fig. 4a and b). Importantly, as suggested by the aforementioned kinetic modeling, this mutation markedly reduced the flipping rate, δ, and its upregulation was necessary to reproduce the FLU-induced increase in current amplitude. Importantly, the reduction of this rate constant by the mutation was a prerequisite to reproduce the observed effect of FLU on amplitude for the F200C mutant. As discussed in our recent paper (Jatczak-Śliwa et al., 2018), the flipping rate, δ, for WT receptors is so fast that its further acceleration by FLU does not increase the open probability. Thus, the lack of the FLU-induced increase in the amplitude of current responses that are mediated by the F200I mutant (i.e., no effect on amplitude) and F200Y mutant (i.e., lower amplitude) is interpreted as a consequence of a weaker effect of these mutations on the flipping rate and, hence, closer similarity to the phenotype that is observed for WT receptors. The effect of FLU on the flipping rate, δ, was crucial for reproducing kinetic changes that were induced by this compound (Fig. 4); therefore, other gating rate constants had to be modified. To reproduce the effect of FLU on macroscopic desensitization (Fig. 4d, e), δ and r had to be increased to values that were closer to WT receptors (see Fig. 7f for transition rates and Fig. 7c–e for simulated responses). The upregulation of desensitization by FLU is consistent with our previous study of a different mutant (α1F64, Jatczak-Śliwa et al., 2018). Moreover, under conditions of a rapid flipping rate, δ, an increase in the desensitization rate, d, led to a reduction of the current amplitude that was consistently described previously and also in the present study for the F200Y mutant. Altogether, our macroscopic analysis indicated that the F200C mutation dramatically affected binding and altered all of the considered gating features, including preactivation, opening/closing, and desensitization.

4.6. Impact of β2F200 mutation on single-channel activity

Single-channel recordings were performed in the cell-attached...
configuration using GABA concentrations that sought to saturate the receptors. For WT, F200Y, and F200I receptors, 10 and 30 mM GABA, respectively, were clearly sufficient to ensure saturation. However, as shown by the macroscopic recordings, a GABA concentration of 100 mM missed saturation for F200C by a small margin (12%). Short-lasting macroscopic recordings could be performed under conditions of a very high [GABA] concentration (even 200 mM, which reached saturation), but long-lasting single-channel recordings showed greater instability that resulted in a progressive increase in noise, much faster patch loss, and alterations of single-channel activity (e.g., a random drift in contributions from various modes). Thus, under these conditions, we were unable to reach an appropriate number of transitions (~10000), which reduced the reliability of our analysis. We decided to complete single-channel recordings at 10 mM (WT) and 100 mM (F200C mutant). In Section 5.2 below, we argue that a minor offset from saturation for the F200C mutant was unlikely to significantly affect our conclusions.

Both WT receptors and all of the considered β2F200 mutants presented clear cluster activity that could be easily identified visually for the dominant mode of activity (Fig. 8), and the analysis was limited to clusters of the dominant mode (in WT and β2F200 mutants) as described previously (Kisieli et al., 2018). Although activity of the mutants took the form of clusters (similar to WT), their single-channel activity presented marked differences from WT (Fig. 8). The analysis of the open and shut times distributions indeed confirmed a profound impact of the mutations on receptor kinetics. As explained in Section 3.4, for the F200C mutants, the three shortest shut time components could be reliably described; therefore, comparisons with WT were made for the three shortest components. As shown in the example distributions in Fig. 8 and Table 1, the cysteine mutation resulted in a several-fold slowdown of all three shut time constants, and the percentage of the fastest component was reduced in the F200C mutant by nearly half (Table 1). The weighted average of shut times was 0.3 ms ± 0.06 ms for WT (n = 5) and 1.37 ± 0.2 ms for F200C (n = 5; p < 0.001), thus confirming a profound change that was induced by this mutation. The F200C mutation also strongly affected receptor opening (Fig. 8). Indeed, both time constants were shortened relative to WT by at least half (Table 2), and the weighted average of open times was 1.9 ± 0.17 ms for WT and 0.6 ms ± 0.08 ms for F200C (n = 5; p < 0.001). For the F200I mutant, both the shut and open times distributions were also strongly altered relative to WT, but the differences were smaller than for the F200C mutant (Tables 1 and 2). Again, all three of the considered shut time constants significantly slowed, and the weighted average of shut times was 0.69 ± 0.06 ms (n = 6; p = 0.004) (Table 1). In the case of open times for the F200I mutant, both time constants were shortened relative to WT, and the weighted average of open times was 0.98 ± 0.08 ms (n = 6; p < 0.001), which was significantly shorter than in WT. Both the F200C and F200I mutations decreased Popen (Fig. 9c), but changes in the burst length reached statistical significance only for F200C (18.31 ± 3.09 ms, n = 5, p = 0.013; Fig. 9b) compared with WT (73.86 ± 17.26 ms, n = 5; burst length for F200I: 41.94 ± 6.62 ms, n = 6, p = 0.096). Thus, the F200I mutation produced a kinetic phenotype that was between WT and F200C, which is compatible with the macroscopic recordings (Figs. 2 and 3). The F200Y mutation resulted in a phenotype that was very similar to WT (Figs. 8 and 10). Surprisingly, the distribution of shut and open times revealed significant changes in their percentages (Tables 1 and 2). However, for the F200Y mutant, the weighted average for shut times (0.36 ± 0.05 ms) and open times (1.78 ± 0.13 ms, n = 4) was not significantly different from WT (p = 0.47 and p = 0.42, respectively).
the β2F200 mutants, only one desensitized state was needed (Model 2, Kisiel et al., 2018) was considered (Model 1, Fig. 10a). However, for WT receptors, the model with two open and two desensitized states resulted in a reduction of fit reliability because one of the de-

| Open time | $P_1$ | $t_1$ [ms] | $P_2$ | $t_2$ [ms] | $T_{open}$ [ms] |
|-----------|-------|------------|-------|------------|----------------|
| WT        | 38.20 ± 10.13 | 0.75 ± 0.20 | 71.90 ± 0.98 | 2.67 ± 0.2 | 1.98 ± 0.17 |
|           | (46.95 ± 10.23) | (0.90 ± 0.18) | (53.05 ± 10.23) | (2.39 ± 0.23) | (1.69 ± 0.13) |
|           | [61.76 ± 9.09] | [0.76 ± 0.15] | [38.24 ± 8.09] | [2.10 ± 0.28] | [1.24 ± 0.11] |
| F200Y     | 53.43 ± 7.34* | 0.94 ± 0.20 | **46.58 ± 7.34*** | 2.70 ± 0.23 | 1.78 ± 0.13 |
|           | (54.66 ± 3.95) | (0.95 ± 0.12) | (45.33 ± 3.95) | (2.71 ± 0.37) | (1.77 ± 0.21) |
|           | [72.11 ± 0.07] | [0.74 ± 0.07] | [27.89 ± 5.26] | [2.37 ± 0.29] | [1.22 ± 0.12] |
| F200I     | 44.65 ± 7.83 | 0.53 ± 0.09 | 55.35 ± 7.83 | 1.31 ± 0.10* | 0.98 ± 0.08* |
|           | (59.74 ± 6.82) | (0.65 ± 0.09) | (40.26 ± 6.82) | (1.27 ± 0.17)* | (0.93 ± 0.07)* |
|           | [67.93 ± 6.40] | [0.62 ± 0.07] | [32.07 ± 6.40] | [1.28 ± 0.13]* | [0.82 ± 0.06]* |
| F200C     | 67.32 ± 7.82* | 0.43 ± 0.06 | **32.67 ± 7.82*** | 1.07 ± 0.08* | 0.63 ± 0.08* |
|           | (60.41 ± 12.96) | (0.41 ± 0.06)* | (39.59 ± 12.96) | (1.13 ± 0.07)* | (0.73 ± 0.13)* |
|           | [68.29 ± 12.05] | [0.37 ± 0.05]* | [31.71 ± 12.05] | [1.03 ± 0.05]* | [0.59 ± 0.06]* |

5. Discussion

5.1. Fundamental role of the C loop in the GABA<sub>A</sub> receptor activation process

The major finding of the present study was that mutation of the key aromatic residue of the C loop, F200, at the principal β2 subunit gave rise to dramatic impairments in binding and also caused profound alterations of receptor gating properties. A weakening of binding is unsurprising because this residue is a key constituent of the “aromatic box” at the LBS. Our in silico docking studies clearly indicated that the F200C mutation decreased the affinity of the orthosteric LBS. Standard docking (with a fixed backbone) alone was insufficient to reproduce the extent of the reduction in binding affinity that was needed to reproduce the experimental data. However, modeling the impact of this mutation on the C loop structure provided further evidence of such a robust decrease in binding affinity. Docking indicated a lower impact of the F200I mutation on affinity than for F200C (Fig. 5b), but the effects of both mutations on the structure of the C loop were similar. Both F200C and F200I are proposed to cause deformation of the C loop backbone by changing the position of its tip, thus impairing the ability of the receptor to “catch” the ligand (Fig. 6e). Moreover, after ligand binding and C loop stabilization, the docking studies predicted that the interactions between the agonist and the receptor structure were more effective for the F200I mutant than for the F200C mutant, which was consistent with a lower EC<sub>50</sub> value for the F200I mutant. A major role for the C loop in defining the affinity of Cys-loop receptors was previously proposed by other authors (Pless and Lynch, 2009; Purohit and Auerbach, 2013; Wagner and Czajkowski, 2001). Our most novel and surprising finding was the pronounced impact of the β2F200 mutation on gating properties of the GABA<sub>A</sub>R. Importantly, this mutation affected all aspects of receptor gating, including flipping (preactivation), efficacy (opening and closing), and desensitization. These changes in gating properties have been consistently supported by several lines of evidence: (i) robust alterations of the time course of currents that are elicited by saturating [GABA] (Figs. 2 and 3) and (ii) dramatic changes in single-channel features, reflected by the fact that nearly all open and shut time constants in distributions of shut and open dwell times were altered by this mutation. All of the time constants in the shut times distributions showed a few-fold increase compared with WT (Table 1).
Kinetic rate constants that describe cluster activity for WT and βF200 mutants. Rate constants were determined for a saturating concentration of GABA with Model 1 (WT, F200Y) and Model 2 (F200C, F200I) (Fig. 10). Significant changes in rate constants relative to WT are marked in bold and with an asterisk (*). For each considered case (WT, mutation), the data were obtained from at least four patches.

Table 3

| Kinetic rate constants (ms⁻¹) | WT      | F200Y   | F200I   | F200C   |
|-------------------------------|---------|---------|---------|---------|
| $\delta_2$                   | 5.17 ± 0.61 | 9.60 ± 1.16* | 3.20 ± 0.45* | 2.60 ± 0.13* |
| $\gamma_2$                   | 3.42 ± 0.68 | 5.96 ± 0.87* | 3.60 ± 1.04 | 3.89 ± 0.61 |
| $\alpha_2$                   | 1.50 ± 0.28 | 1.38 ± 0.13 | 1.70 ± 0.18 | 2.88 ± 0.50* |
| $\beta_2$                    | 6.31 ± 1.13 | 6.13 ± 0.65 | 3.21 ± 0.61* | 2.13 ± 0.16* |
| $\alpha_2'$                  | 0.51 ± 0.06 | 0.45 ± 0.07 | 0.82 ± 0.09 | 0.97 ± 0.06* |
| $\beta_2'$                   | 4.35 ± 1.11 | 2.4 ± 0.5 | 1.47 ± 0.36 | 1.32 ± 0.71* |
| $\delta_3$                   | 0.92 ± 0.25 | 1.14 ± 0.16 | 0.42 ± 0.15 | 1.01 ± 0.39 |
| $r_2$                        | 0.96 ± 0.17 | 1.67 ± 0.33 | 0.39 ± 0.12 | 0.39 ± 0.06* |
| $d_2'$                       | 0.34 ± 0.10 | 0.17 ± 0.04 | -          | -          |
| $r_2'$                       | 0.21 ± 0.07 | 0.07 (fixed) | -          | -          |

Additionally, open times were dramatically shortened for the F200C and F200I mutations. However, the effect of the F200Y mutation was qualitatively different from F200I and F200C. The dose-response relationship for the F200Y mutant was shifted to the left, in contrast to F200I and F200C. The F200Y mutation did not increase receptor affinity. We propose that this shift might be attributable to an increase in flipping rate. Similarly, the changes in the time course of macroscopic currents and distributions of single-channel events that were caused by the F200I and F200C mutations would be highly unlikely to be observed if these mutations solely modified binding features of this receptor. Further supporting the robust impact of the F200C mutation on receptor gating, specifically flipping, was the observation that FLU increased the amplitude of currents that were mediated by the F200C mutants, whereas the opposite effect was observed for WT receptors (Mercik et al., 2007; Mozrzymas et al., 2007) and the F200Y mutant, which presented the closest similarity to WT among the mutants that were considered herein. Such a potentiation of current amplitude by FLU requires that the flipping rate is substantially decreased (also see Jatczak-Śliwa et al., 2018, where the mechanism of action of FLU was specifically addressed). Interestingly, a similar scenario for a potentiating effect of FLU on responses to saturating GABA was recently described by our group for the α1F64 mutation (at the LBS on the D loop) (Szczot et al., 2014). Thus, despite different localization of the C and D loops on different subunits and the distinct structural features of these loops (i.e., the C loop is largely unstructured, whereas the D loop is a part of a rigid β sheet), their mutation leads to robust modification of the flipping (preactivation) process. The impacts of the β2F200 and α1F64 mutations were clearly distinct. Mutating the β2F200 residue resulted in large changes in all gating features, whereas the α1F64 mutation affected primarily preactivation. Thus, key residues that are located at the orthosteric LBS are likely strongly involved in receptor gating.

Our major conclusions about the impact of the C loop on GABAR gating qualitatively differed from the conclusions that were drawn by Purohit and Auerbach (2013), who performed an elegant single-channel analysis of the AChR and found that even radical manipulation of this loop affected binding rather than gating. This discrepancy most likely resulted from the fact that in distinct Cys-loop receptors (GABAAR vs. AChRs), the role of the C loop might be different. One example of such different roles that are played by homologous elements in different Cys-loop receptors is that mutation of the α1F64 residue resulted primarily in preactivation impairment in the GABAR (Szczot et al., 2014), whereas the homologous residue in α7 AChR homomers was implicated in rapid desensitization (Gay et al., 2008). However, Mukhtasimova et al. (2009) analyzed activity of the AChR receptor (similar to Purohit and Auerbach, 2013) and reported a causal link between C loop capping and receptor priming (preactivation). Pless and Lynch (2009) applied
voltage-clamp fluorometry and electrophysiology and identified structural changes that were associated with closed flipping transitions in GlyRs. They did not support any clear involvement of C loop movement in encoding efficacy transitions, but these authors did not exclude alternative scenarios, such as an interaction between the C loop and the D and E loops via bound agonist, thus affecting a signal transfer within the macromolecule.

One issue is whether the 100 mM concentration of GABA that missed saturation by a small margin (12%) affected our conclusions that were derived from single-channel recordings. Importantly, the macroscopic and single-channel investigations led to similar conclusions, in which all aspects of gating were affected by the β2F200 mutation. Notably, our single-channel analysis was restricted to three components in the shut times distributions, which most likely represent features of the fully bound receptor. For WT GABAARs, lowering [GABA] from a saturation concentration (10 mM) to 1 mM did not affect the distributions of these three shut time components, and lowering the concentration further (30 μM) did not affect the time constants, reducing only the percentages of the fastest ones (data not shown). We expected a similar pattern for the F200C mutant. Additionally, the open times distribution (Fig. 8) clearly indicated that the vast majority of opening events that comprised the distribution were fully bound. Singly bound or spontaneous openings are very characteristic because of their very brief duration (“needle”-like events). The open times distributions that are illustrated in Fig. 8 show that the contribution from not fully bound receptors is negligible at the GABA concentration of 100 mM. Lastly, the macroscopic recordings indicated that responses to 100 and 200 mM GABA did not show any clear differences in time course, indicating that when activated, the kinetic behavior of the receptor at these concentrations was very similar. Altogether, our macroscopic and single-channel analyses consistently indicated a robust effect of the β2F200 mutation on receptor gating.

5.2. Possible mechanistic scenarios of the impact of the C loop on GABA<sub>A</sub> receptor gating

The important role of the C loop in ligand binding is well established. From a structural perspective, the critical contribution of β2F200 and β2Y205 residues is emphasized in the context of “armchair box” formation of the LBS that separates the orthosteric LBS from bulk solution. However, the structural mechanisms that underlie the role of this loop in receptor gating remain largely unknown. Our studies provide solid evidence of the involvement of the C loop in GABA<sub>A</sub>R gating but do not provide any direct mechanistic or structural explanations for this role. However, some likely, albeit speculative, scenarios can be suggested for subsequent studies. The C loop connects the β9 and β10 strands. The β9 strand is beyond the LBS and is connected via the so-called loop 9 with the β8 strand, which is located on the opposite side of the subunit that contributes to the interface with a preceding subunit (α<sub>i</sub> or γ<sub>i</sub>). Notably, both loop 9 and the β9 strand are known to be involved in channel gating (Hanson and Czajkowski, 2011; Williams et al., 2010). Thus, the β8 strand is in a position that is readily susceptible to changes that occur at the C loop and participates in the transduction of anticalcineal signaling which, in turn, may contribute to rotation of the extracellular domain (ECD) that is an important step in receptor activation. The concept of the ECD twist was recently described by Masulis et al. (2019) for GABA<sub>A</sub>Rs and previously for AChRs (Gupta et al., 2016) and GLIC (Sauguet et al., 2014). Backbone deformation of the C loop through the mutations that were imposed herein likely alters possible coupling of the β9 strand–loop 9–β8 strand–preceding subunit, thus affecting intersubunit interactions and receptor gating. In addition to the possible impact of the C loop on lateral intersubunit interactions within the GABA<sub>A</sub>R macromolecule, this loop is connected to the transmembrane domain (TMD) via a rigid β10 and short β10–M1 helix linker. This structural arrangement facilitates mechanical signal transfer from the LBS toward the receptor gate. Deformation of the C loop may weaken the “stiff” β-strand connection between the LBS and ECD-TMD interface, which is known to play a critical role in receptor activation (e.g., Cederholm et al., 2009) that in turn affects the receptor gating process. In summary, a profound impact of the C loop mutation on GABA<sub>A</sub>R gating may result from interference with intersubunit interactions within the ECD (“horizontal” path) or the hindrance of “vertical” ECD-TMD interactions within a single receptor (principal) subunit.

5.3. Conclusions

In conclusion, based on extensive experimental data, we provide evidence that the C loop of the GABA<sub>A</sub>R that is located at the LBS in the ECD contributes to almost all stages of receptor activation, effectively shaping all aspects of receptor gating. Interestingly, mutation of the C loop, particularly the F200C mutation, also affected receptor pharmacology, altering sensitivity of the receptor to the benzodiazepine FLU. The present findings may be particularly helpful for elucidating mechanisms of the pharmacological modulation of GABA<sub>A</sub>Rs and designing new drugs.

Authorship contribution statement

K.T. participated in performing the experiments, data analysis, and writing the paper. P.T.K. performed the experiments, data analysis, and model simulations for single-channel activity and contributed to writing the paper. M.A.M. performed the in silico analysis to enable the choice of mutants, homology modeling, ligand docking, C loop structure modeling, and kinetic simulations for macroscopic currents and contributed to writing the paper. A.D. participated in performing some of the experiments and data analysis. J.W.M. conceived the project, procured financial support, supervised project realization, participated in designing the experiments, data analysis, and model simulations, and wrote and edited the final version of the manuscript.

Declaration of competing interest

None.

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