Structural Link between γ-Aminobutyric Acid Type A
(GABA\textsubscript{A}) Receptor Agonist Binding Site and Inner β-Sheet
Governs Channel Activation and Allosteric Drug Modulation

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Rapid opening and closing of pentameric ligand-gated ion channels (pLGICs) regulate information flow throughout the brain. For pLGICs, it is postulated that neurotransmitter-induced movements in the extracellular inner β-sheet trigger channel activation. Homology modeling reveals that the β4-β5 linker physically connects the neurotransmitter binding site to the inner β-sheet. Inserting 1, 2, 4, and 8 glycines in this region of the GABA\textsubscript{A} receptor β-subunit progressively decreases GABA activation and converts the competitive antagonist SR-95531 into a partial agonist, demonstrating that this linker is a key element whose length and flexibility are optimized for efficient signal propagation. Insertions in the α- and γ-subunits have little effect on GABA or SR-95531 actions, suggesting that asymmetric motions in the extracellular domain power pLGIC gating. The effects of insertions on allosteric modulator actions, pentobarbital, and benzodiazepines, have different subunit dependences, indicating that modulator-induced signaling is distinct from agonist gating.

Electrochemical signaling in the CNS depends on ligand-gated ion channels (LGICs). These proteins couple the binding of neurotransmitter to the rapid opening of an integral ion-conducting pore. The “Cys-loop” LGIC family of receptors comprises pentameric proteins (pLGICs) that include nicotinic acetylcholine receptors (nAChRs), glycine receptors (GlyRs), GABA type A receptors (GABA\textsubscript{A}Rs), and serotonin type-3 receptors. Although a structural picture of pLGICs is rapidly emerging from the 4 Å resolution cryo-EM structure of the Torpedo nAChR (1), the crystal structures of the extracellular binding domain of the nAChR α-subunit (2) liganded and unliganded acetylcholine-binding proteins (AChBP), which are homologs of the extracellular binding domain (3, 4), the crystal structures of full-length prokaryotic pLGIC homologs from Erwinia chrysanthemi (ELIC) and Gloeobacter violaceus (GLIC) (5–7), as well as the recent crystal structure of a related invertebrate pLGIC (8), our understanding of the structural elements and protein movements that couple neurotransmitter binding to channel gating is still under debate. For receptors in this superfamily, the neurotransmitter binding site is located in the extracellular N-terminal domain between adjacent subunits formed by at least six noncontiguous protein regions (loops A–F), whereas the channel gate is located 50 Å away in the trans-membrane region (9). Neurotransmitter binding is believed to trigger structural changes at the binding site that are propagated as a conformational wave to the channel gate (10). The secondary structure of the extracellular domain is predominantly composed of 10 β-strands arranged in two sheets, inner and outer, that form a β-sandwich (see Fig. 1). Three flexible loops (β4-β5, β6-β7, and β8-β9 linkers) link the inner β-sheet to the outer sheet.

One model of receptor activation based on nAChR structural studies suggests that agonist binding in the extracellular domain induces a clockwise rotation of the extracellular inner β-sheet in two out of five subunits, which triggers movements in the trans-membrane helices that result in channel gating (11). Comparison of the recent ELIC and GLIC bacterial channel structures (closed and open, respectively) suggests that channel activation is accompanied by an anti-clockwise concerted twist of each extracellular β-sandwich domain (6, 7, 12). In homomeric glycine receptors, receptor activation is believed to occur via a reorganization of the extracellular β-sandwich hydrophobic core and the negative subunit interface loops (13). In the GABA\textsubscript{A}R, the β-subunit β4-β5 linker links the loop A region of the GABA binding site to the inner β-sheet (see Fig. 1) and is in an ideal position to propagate initial binding site movements to gating movements in the channel domain. Consistent
with this idea, molecular dynamic simulations of the nAChR identified motions in the H9252/4-H9252/5 linker that were correlated with motions in loops 2, 7, and M2-M3 near the extracellular mouth of the channel (14), and mutations in the nAChR H9251- subunit H9252/4-H9252/5 linker alter channel gating (15, 16). Based on the crystal structures of the nAChR H9251-subunit extracellular domain, AChBP, GLIC, ELIC, and the invertebrate glutamate-activated chloride channel (2, 3, 5–8), this linker region spans each subunit and is relatively unstructured. Here, we inserted glycine residues in the H9252/4-H9252/5 linkers of the GABAAR H9251-, H9252-, and H9253-subunits to alter their length and flexibility and examined the effects on GABA-mediated channel activation and pento- barbital (PB)-mediated channel activation and on benzodiazepine (BZD) modulation of GABA responses. The absence of a H9252-carbon allows glycine to access energetically prohibited protein dihedral angles (17).

Glycine insertions in the H9252-subunit (H9252Gly)3 reduced GABA binding and GABA-induced channel gating, and surprisingly, converted the competitive antagonist SR-95531 into a partial agonist, demonstrating that this linker is a key structural element whose length and flexibility are optimized for transducing GABA binding to efficient channel gating. Insertions in the α- and γ-subunits had little to no effect on GABA or SR-95531 actions, supporting the idea that asymmetric subunit motions in the extracellular domain help power GABA-mediated gating. Moreover, the effects of the glycine insertions on PB activation and BZD modulation were much smaller and had different subunit dependences, indicating that the structural mechanisms underlying GABA, PB, and BZD actions are distinct.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression in Oocytes—Rat cDNAs encoding α1-, β2-, and γ2S-subunits of the GABAAR, subcloned into the pGH19 vector (18), were utilized in this study. Mutant receptors with 1-, 2-, 4-, and 8-glycine insertions (Gly1, Gly2, Gly4, and Gly8) indicate mutant receptors with 1-, 2-, 4-, and 8-glycine insertions.

3 Throughout this study, αGly represents glycine insertions in the α-subunit; βGly represents glycine insertions in the β-subunit; and γGly represents glycine insertions in the γ-subunit. In addition, Gly1, Gly2, Gly4, and Gly8 indicate mutant receptors with 1-, 2-, 4-, and 8-glycine insertions.
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described previously (19) and recorded from 2–14 days after injection.

Oocyte Electrophysiology—Oocytes were voltage-clamped at −80 mV in a 200-μl chamber and continuously perfused with ND96 at 10 ml/min, and data were acquired as described previously (20). Stock solutions of 1 mM GABA, 10 mM SR-95531 (Sigma-Aldrich), and 10 mM flurazepam (FZ) (Research Biochemicals, Natick, CA), were prepared in ND96, stored at −20 °C, and thawed once before use. Stock solutions of 30 or 50 mM PB (Research Biochemicals, Natick, CA) were prepared fresh on the day of the experiment.

Concentration Response and Data Analysis—Concentration response analyses were performed as described previously (21). For GABA concentration response experiments, each test concentration was preceded by a low nondesensitizing PB concentration to correct for the drift in PB-induced current during the experiment. Currents induced by each test concentration were normalized to the corresponding nondesensitizing concentration before curve fitting. In the second method, the PB-induced currents were not normalized to a low, nondesensitizing PB concentration. The relief of channel block upon drug wash yields a characteristic tail current. For PB concentration-response curves, PB current amplitudes at high micromolar concentrations were measured using the tail currents.

SR-95531 IC_{50} experiments were performed as described previously (22). Oocytes expressing WT or mutant receptors were measured using two methods. In the first method, each test PB concentration was preceded by a low nondesensitizing PB concentration to correct for any drift in PB-induced current during the experiment. Responses to each test PB concentration were then normalized to its corresponding low nondesensitizing concentration, prior to curve fitting. In the second method, the PB-induced currents were not normalized to a low, nondesensitizing PB concentration. The curve fits and calculated values obtained using the two methods were not different, and thus, the data were pooled for statistical analysis. At high micromolar concentrations and above, PB blocks GABA_{α}R current responses. The relief of channel block upon drug wash yields a characteristic tail current. For PB concentration-response curves, PB current amplitudes at high micromolar concentrations were measured using the tail currents.

SR-95531 IC_{50} experiments were performed as described previously (22). Oocytes expressing WT or mutant receptors were challenged with EC_{50} GABA concentration (except for experiments involving βGly2, -4, and -8, which were performed at 30 mM GABA) followed by co-application of the same concentration of GABA and a test concentration of SR-95531. GABA-induced currents in the presence of increasing SR-95531 concentrations were then normalized to the GABA response in the absence of SR-95531 before curve fitting.

FZ potentiation experiments were performed at EC_{4–8} GABA. Potentiation is defined as (I_{GABA + BZD} / I_{GABA}) − 1, where I_{GABA + BZD} is the GABA-mediated current in the presence of flurazepam and I_{GABA} is the GABA-mediated current in the absence of flurazepam. Because GABA EC_{50} values could not be reliably measured for βGly2-4-8-containing receptors, they were not tested for flurazepam potentiation.

Nonlinear regression analysis for GABA, PB, FZ, and SR-95531 concentration response experiments was performed using the GraphPad (San Diego, CA) Prism 4 software. GABA and PB concentration responses were fit to the following equation: I = I_{max} / (1 + EC_{50} / [A])^{n}, where I is the peak response (including the tail current for PB) to a given GABA or PB concentration, I_{max} is the current amplitude (including the tail current for PB) of the maximal GABA− or PB−evoked current, EC_{50} is the concentration of GABA or PB that produces a half-maximal response, [A] is the agonist concentration, and n is the Hill coefficient. For SR-95531 competition experiments, inhibition was calculated as I_{GABA + SR-95531} / I_{GABA}. Data were fit to the following equation: inhibition = 1 − 1 / (1 + (IC_{50} / [Ant]))^{n}, where IC_{50} is the concentration of antagonist that blocks half of I_{GABA}; [Ant] is the concentra-
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Summary of GABA and SR-95531 concentration response data

Data are mean ± S.E. of at least two different batches of oocytes. Mutant/WT (mut/WT) EC<sub>50</sub> or IC<sub>50</sub> ratios were calculated. SR-95531 experiments were done at EC<sub>50</sub> GABA for WT, αGly, βGly1, and γGly insertions and at 30 mM GABA for βGly2, 4, and 8 insertions. *, values are significantly different from WT, p < 0.05 (one-way ANOVA).

| Receptor | GABA (EC<sub>50</sub> μM) | n<sub>hit</sub> | mut/WT | n | SR-95531 (IC<sub>50</sub> nM) | SR-95531 (K<sub>i</sub>) | n<sub>hit</sub> | mut/WT | IC<sub>50</sub> | n |
|----------|-----------------|-------|--------|---|-----------------|-------------|-------|--------|--------|---|
| WT       | 14.1 ± 2.2      | 1.4 ± 0.2 | 1       | 6  | 158 ± 22        | 76 ± 11     | −1.1 ± 0.1 | 1  | 3      |
| αGly1    | 28.8 ± 3.5      | 1.2 ± 0.1 | 2       | 3  | 272 ± 27        | 133 ± 13*   | −1.1 ± 0.1 | 2  | 3      |
| αGly2    | 27.2 ± 5.4      | 1.3 ± 0.2 | 2       | 4  | 142 ± 17        | 70 ± 9      | −1.1 ± 0.1 | 1  | 5      |
| αGly4    | 155 ± 70<sup>##</sup> | 0.9 ± 0.1 | 11      | 3  | 338 ± 23        | 187 ± 13*   | −1.2 ± 0.1 | 2  | 3      |
| αGly8    | 31.0 ± 2.1<sup>##</sup> | 1.2 ± 0.1 | 2       | 4  | 270 ± 45        | 116 ± 19    | −1.0 ± 0.1 | 2  | 3      |
| βGly1    | 370 ± 90<sup>##</sup> | 0.6 ± 0.1* | 26      | 8  | 1390 ± 350      | 767 ± 192*  | −1.0 ± 0.1 | 9  | 3      |
| βGly2    | >150 mM         | >10,000 | 10      | 7500 ± 1400 | 1.0 ± 0.1 | 47            | 3      |
| βGly4    | >150 mM         | >10,000 | 3       | 31,000 ± 11,300 | 1.0 ± 0.1 | 200          | 3      |
| βGly8    | >1 s            | >50,000 | 3       | 13,300 ± 3100 | 1.1 ± 0.2 | 85            | 4      |
| γGly1    | 41.5 ± 3.3<sup>##</sup> | 1.2 ± 0.1 | 3       | 3  | 195 ± 7        | 100 ± 3     | −1.3 ± 0.1 | 1  | 3      |
| γGly2    | 43.7 ± 12.6<sup>##</sup> | 1.2 ± 0.2 | 3       | 3  | 252 ± 18       | 118 ± 8     | −1.2 ± 0.1 | 2  | 3      |
| γGly4    | 27.1 ± 10       | 1.4 ± 0.2 | 2       | 4  | 180 ± 13       | 76 ± 2.5    | −1.2 ± 0.1 | 1  | 3      |
| γGly8    | 43.0 ± 4<sup>##</sup> | 1.1 ± 0.1 | 3       | 3  | 234 ± 18       | 117 ± 9     | −1.3 ± 0.1 | 2  | 3      |

Current Rise Time and Maximal Current Amplitude Ratio Analysis—10–90% apparent rise times for GABA-induced currents at saturating GABA concentrations (10 mM for WT and 100 mM for βGly1) were calculated using a built-in feature in the WinWCP data acquisition software (provided by J. Dempster, Univ. of Strathclyde, Glasgow, UK). Data from n > 3 oocytes from at least two different batches were pooled for statistical analysis.

For maximum PB versus maximum GABA-induced current ratio determinations, each oocyte (WT or βGly1) was first challenged with maximum GABA concentration (10 mM for WT and 100 mM for βGly1) and then allowed to recover completely by washing sufficiently to remove any trace remnants of GABA from previous exposure, which was followed by application of maximum PB concentration (10 mM for WT and 30 mM for βGly1). GABA- and PB-induced currents at saturating concentrations were then measured, and ratios (maximum PB/maximum GABA) were determined by dividing the maximum PB current amplitude by maximum GABA current amplitude for each experiment. Ratiometric data from n > 3 oocytes from at least two different batches were pooled for statistical analysis. PB current amplitudes at saturating concentrations were measured using tail currents.

Statistical Analysis—LogEC<sub>50</sub> values for GABA, PB, FZ, potentiation, and LogK<sub>i</sub> for SR-95531 concentration response were analyzed using one-way ANOVA followed by a post hoc Dunnett’s test to determine the level of significance between wild-type (WT) and mutant receptors at an α-level of 0.05. The Dunnett’s test compares group means and is used to identify samples whose means are significantly different from the mean of a reference group, in our case the WT sample. 10–90% rise times in response to maximal GABA concentration and ratios of maximum PB versus maximum GABA current amplitudes for oocytes expressing WT and mutant GABA<sub>4</sub>Rs were analyzed using unpaired two-tailed Student’s t test. All data reported are mean ± S.E. unless noted otherwise.

Structural Modeling—Homology modeling was performed as described previously (20). Briefly, we modeled the GABA<sub>4</sub>R extracellular domain after the AChBP (3) structure and modeled its trans-membrane domain after the structure of the nAChR trans-membrane domain (at a resolution of 4 Å) solved by Miyazawa et al. (11) (Protein Data Bank (PDB) code 1OED).

![FIGURE 3. βGly1 reduces GABA efficacy and slows GABA rise times. A, representative current traces from WT- or βGly1-expressing oocytes in response to sequential applications of saturating concentrations of GABA and PB are depicted. Concentrations of GABA and PB (in mM) used were 10 and 10 for WT and 100 and 30 for βGly1 receptors. Bars represent mean ± S.E. of maximum PB maximum GABA current ratios from (n) experiments for WT and αβGly1 receptors. B, representative saturating GABA current traces from WT- and βGly1-expressing oocytes are peak normalized to highlight the slowing in GABA 10–90% rise time for βGly1 mutant receptors. Bars represent mean ± S.E. of 10–90% rise times (in seconds) from (n) experiments for WT and αβGly1 receptors. * values are significantly different from WT, p < 0.05 (one-way ANOVA).](image-url)
Subsequently, the two structures were docked, and global energy minimizations were undertaken followed by examining the protein for gross structural distortions. The GABAAR model images were developed using PyMOL (Schrödinger, LLC, New York).

RESULTS

Effects of Glycine Insertions on GABA Actions—Gly1, Gly2, Gly4, or Gly8 were inserted after position Lys-103 in the β-subunit (BK103) and after aligned positions in the α- (αK105) and γ- (γK118) (Fig. 1, A and B) subunits to evaluate how increasing the length and flexibility of the linker regions in the α-, β-, and γ-subunits would affect GABAAR function. Oocytes were injected with mutant and wild-type αβγ subunit cRNAs to form αβγ receptors and functionally characterized using two-electrode voltage clamp. All of the mutant subunits as assembled into functional receptors that responded to GABA. In general, the αβγ Gly and γβγ Gly insertions had minimal effects on GABA EC50 values (4-fold) as compared with WT (14.1 ± 2.2 μM), except for αβγ Gly4 that had an 11-fold increase in GABA EC50 (Fig. 2, Table 1). The αβγ Gly and γβγ Gly insertions had no effects on the Hill slopes for GABA activation. The maximal GABA-activated currents elicited from receptors containing the αβγ Gly or γβγ Gly insertions ranged from 3.5 to 11 μA and did not significantly differ from WT (9.8 ± 1 μA; n = 6).

In contrast, βGly1 increased GABA EC50 26-fold and significantly reduced the Hill slope, whereas βGly2, -4, or -8 increased GABA EC50 more than 10,000-fold (Fig. 2, Table 1).

| Glycine Insertion | EC50 (μM) | Hill Slope |
|-------------------|----------|------------|
| Gly1              | 22.3     | 0.9        |
| Gly2              | 1.1      | 1.0        |
| Gly4              | 2.3      | 1.0        |
| Gly8              | 2.0      | 1.0        |
| WT                | 14.1     | 1.0        |

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| Subunit | Glycine Insertion |
|---------|-------------------|
| αβγ     | Gly1, Gly2, Gly4, Gly8 |
GABA EC\textsubscript{50} values for βGly2, -4, or -8 could only be estimated because the high concentrations of GABA (>300 mM) needed to reach maximal current responses changed the extracellular solution osmolarity and could not be used. The maximal GABA current amplitudes for βGly1 receptors were significantly smaller (1.28 ± 0.1 µA; n = 14) as compared with WT receptors (9.8 ± 1 µA; n = 6). To determine whether βGly1 was affecting GABA efficacy and/or receptor expression, we measured and compared currents induced by a saturating GABA concentration with those induced by a saturating PB concentration in the same oocyte. For WT receptors, saturating concentrations of PB and GABA elicited currents similar in magnitude (∆I\textsubscript{PB max} / ∆I\textsubscript{GABA max} ratio = 1.03 ± 0.07, Fig. 3A). In oocytes expressing βGly1 receptors, the currents elicited by saturating concentrations of GABA were 4-fold smaller than currents induced by saturating concentrations of PB, indicating a reduction in GABA efficacy (Fig. 3A). We also measured apparent 10–90% current rise times at saturating GABA concentrations for βGly1 and WT receptors. Although current onset is limited by the slow solution-exchange times when recording from oocytes (300 ms), differences in apparent current rise times between WT and mutant receptors would imply changes in channel gating. GABA apparent rise times for βGly1 receptors were significantly slower than WT receptors (0.35 ± 0.02 s for WT versus 1.5 ± 0.2 s for βGly1, Fig. 3B), suggesting that insertion of a single glycine in the β4–β5 linker of the β-subunit reduced channel opening.

Effects of Glycine Insertions on GABAA (SR-95531) Actions—We also examined the effect the glycine insertions had on the ability of the competitive antagonist SR-95531 to inhibit GABA-activated currents. αGly and γGly insertions caused less than 3-fold changes in SR-95531 K\textsubscript{I} as compared with WT (76 ± 11 nM) (Fig. 4, Table 1). Larger increases in SR-95531 K\textsubscript{I} were observed for the βGly insertions. Insertion of a single glycine resulted in an ~10-fold increase in SR-95531 K\textsubscript{I} (Table 1). Because the GABA EC\textsubscript{50} values were too right-shifted to be precisely determined for βGly2, -4, and -8 receptors (Table 1), we approximated the -fold changes in SR-95531 K\textsubscript{I} using the Cheng-Prusoff equation (K\textsubscript{I} = IC\textsubscript{50} (1 + ([A]/EC\textsubscript{50})), where A is the concentration of GABA used in the experiment, IC\textsubscript{50} is the GABA concentration that elicits half-maximal response, and IC\textsubscript{50} is the concentration of SR-95531 that inhibits 50% of the GABA-induced current. The equation predicts that at [A] << EC\textsubscript{50}, K\textsubscript{I} approaches IC\textsubscript{50} and at [A] = EC\textsubscript{50}, K\textsubscript{I} = (IC\textsubscript{50})/2. The SR-95531 inhibition experiments for βGly2, -4, and -8 used 30 mM GABA, which from curve-fitting estimates is below the GABA EC\textsubscript{50} (Table 1). Thus, for βGly2 receptors, we estimate that the SR-95531 K\textsubscript{I} is between 3750 nM (using 30 mM as an upper limit for GABA EC\textsubscript{50}) and 7500 nM (assuming that 30 mM is << EC\textsubscript{50}, an ~50–100-fold increase in K\textsubscript{I} as compared with WT. The estimated -fold increases in SR-95531 K\textsubscript{I} for βGly4 and -8 receptors were even larger (100–400-fold).

### Summary of Pentobarbital concentration response data

| Receptor | PB (EC\textsubscript{50} µM) | n\textsubscript{p} | mut/WT | n |
|----------|-----------------|--------|---------|---|
| WT       | 231 ± 10        | 2.4 ± 0.3 | 1       | 3 |
| αGly1    | 288 ± 84        | 2.5 ± 0.1 | 1       | 3 |
| αGly2    | 385 ± 56        | 2.6 ± 0.2 | 2       | 4 |
| αGly4    | 720 ± 74        | 1.3 ± 0.1 | 3       | 3 |
| αGly8    | 484 ± 100*      | 1.5 ± 0.2 | 2       | 4 |
| βGly1    | 1910 ± 320*     | 1.2 ± 0.1 | 8       | 8 |
| βGly2    | 3020 ± 510*     | 2.1 ± 0.2 | 13      | 5 |
| βGly4    | 1840 ± 320*     | 2.1 ± 0.2 | 8       | 5 |
| βGly8    | 2500 ± 200*     | 2.5 ± 0.1 | 11      | 3 |
| γGly1    | 1280 ± 60*      | 2.9 ± 0.2 | 6       | 5 |
| γGly2    | 2230 ± 310*     | 2.4 ± 0.2 | 10      | 3 |
| γGly4    | 1390 ± 50*      | 2.5 ± 0.1 | 6       | 3 |
| γGly8    | 1570 ± 150*     | 2.0 ± 0.3 | 7       | 4 |

Table 2.
Interestingly, even at high concentrations, SR-95531 only inhibited 70% of the GABA-induced current for βGly2-, -4-, and -8-containing receptors as compared with 100% inhibition seen for WT and βGly1 receptors (Fig. 4). To investigate the mechanism underlying the partial inhibition, we applied SR-95531 in the absence of GABA. At concentrations >30 μM, SR-95531 directly gated βGly2-, -4-, and -8-containing receptors (Fig. 4, middle inset, data not shown for βGly4 and -8). These concentrations of SR-95531 never elicited currents from WT- or βGly1-containing receptors. Thus, for βGly2-, -4-, and -8 receptors, SR-95531 behaved as a weak partial agonist.

**Effects of Glycine Insertions on PB Actions**—PB is an allosteric modulator of the GABA<sub>α</sub>R that binds at a site distinct from GABA (25). At high concentrations, PB can directly open the channel. The single channel conductances of GABA<sub>α</sub>Rs activated by PB and GABA are similar (26, 27), suggesting that the receptor open-state channel structures induced by their binding are similar (28). To test whether disrupting the linker regions in the α-, β-, and γ-subunits affected PB activation, we measured PB concentration responses from wild-type and mutant receptors. Glycine insertions in both the β-subunits and the γ-subunits significantly increased PB EC<sub>50</sub> values by ~10-fold as compared with WT receptors (231 ± 10 μM) (Fig. 5, Table 2). The αGly insertions altered PB EC<sub>50</sub> = 3-fold (Fig. 5, Table 2). The mean maximal currents elicited by PB for all the mutant receptors ranged from 3.3 to 12.4 μA and did not significantly differ from WT (9.1 ± 1.3 μA; n = 12), indicating that none of the glycine insertions in any of the subunits altered GABA<sub>α</sub>R surface expression.

**Effects of Glycine Insertions on FZ Actions**—BZDs modulate GABA responses by binding at a site formed at the interface between the extracellular N-terminal regions of the α- and γ-subunits (29–31) (Fig. 1, A and B). At subsaturating concentrations of GABA, positive BZD modulators increase GABA-induced current. To examine whether the linker regions were involved in mediating BZD-positive allosteric modulation, we tested the effects the Gly insertions had on the ability of the BZD-positive modulator FZ to potentiate GABA currents. For WT GABA<sub>α</sub>Rs, FZ maximally potentiated EC<sub>4–8</sub> GABA-induced current with a potentiation value of 2.49 ± 0.18 and an EC<sub>50</sub> of 468 ± 94 nM (Fig. 6, Table 3). All of the αGly and the βGly1 insertions reduced FZ potentiation of EC<sub>4–8</sub> GABA-mediated current responses (50–60%) without changing the FZ EC<sub>50</sub> (Fig. 6, Table 3), suggesting that these regions are important for mediating BZD efficacy. Because the magnitude of the maximal BZD potentiation of I<sub>GABA</sub> measured is highly dependent upon the effective GABA concentration being applied (32), FZ potentiation of GABA currents for βGly2-, -4-, and -8-containing receptors were not measured because their GABA EC<sub>50</sub> values could not be precisely determined. The γGly insertions not only reduced FZ potentiation of GABA currents (40–50% for γGly4 and γGly8) but also increased FZ EC<sub>50</sub> values ~4-fold (Fig. 6, Table 3).

**DISCUSSION**

Here, by altering the length and flexibility of the β-subunit β4-β5 linker, we demonstrate that this linker plays a critical role in mediating agonist-induced GABA<sub>α</sub>R functional responses. Moreover, agonists and allosteric modulators react uniquely toward insertions in the linker in the α-, β-, and γ-subunits of the GABA<sub>α</sub>R, suggesting that the allosteric trajectories underlying their actions are distinct. Overall, our data identify the β4-β5 linker as a key structural element in the GABA<sub>α</sub>R that shapes the energetic landscape associated with channel activation and drug modulation.

**β-Subunit Linker Region Couples GABA Binding and Gating**—Glycine insertions in the β-subunit linker region, which physically connects the GABA binding site (loop A) to the extracellular domain inner β-sheet, increased GABA EC<sub>50</sub> more than 10,000-fold, whereas insertions in the α- and γ-subunits had little to no effect on GABA EC<sub>50</sub> (Fig. 7A, Table 1). These data are consistent with structural and molecular dynamic studies (1, 3, 4, 33–35) in the pLGIC family that indicate asymmetric subunit motions in the extracellular domain help power agonist-mediated gating. Recently, photochemical cleavage of the α-subunit GABA<sub>α</sub>R linker was shown to disrupt GABA activation (36). In this study, the cleavage site was located near the β/α interface close to loop E of the GABA binding site (at αM113, see Fig. 1). We speculate that proteolysis at this site likely altered the structure of the GABA binding site, which resulted in the loss of GABA-mediated functional responses observed. Here, the α-subunit glycine insertions are located near the α/γ and α/β interfaces (at non-GABA binding site interfaces). Surprisingly, inserting even up to 8 glycine residues in the α- and γ-subunits was tolerated, indicating that the length and/or flexibility of the linker in these subunits is not critical for GABA-mediated current responses. In the crystal structures of the nAChR α-subunit extracellular domain, AChBP, GLIC, ELIC, and the invertebrate glutamate-activated chloride channel (2, 3, 5–8), the linker region is relatively

### Summary of Flurazepam Concentration Response Data

| Receptor | FZ (EC<sub>50</sub> nM) | Potentiation (P) | n<sub>10</sub> | WT/mut EC<sub>50</sub> | mut/WT (P) | n |
|----------|-----------------|-----------------|-----------|-----------------|----------|---|
| WT       | 2.56 ± 0.16     | 1.6 ± 0.1       | 1         | 1               | 1        | 12|
| αGly1    | 9.70 ± 0.64     | 1.2 ± 0.1       | 1         | 1               | 0.4      | 6 |
| αGly2    | 1.23 ± 0.06     | 1.8 ± 0.2       | 1         | 0.5             | 4        |   |
| αGly4    | 0.86 ± 0.03     | 1.2 ± 0.2       | 1         | 0.3             | 4        |   |
| αGly8    | 1.17 ± 0.13     | 1.5 ± 0.5       | 1         | 0.5             | 4        |   |
| βGly1    | 1.08 ± 0.14     | 1.5 ± 0.1       | 1         | 0.4             | 4        |   |
| γGly1    | 2.27 ± 0.1      | 1.5 ± 0.1       | 1         | 0.9             | 5        |   |
| γGly2    | 2.53 ± 0.14     | 1.2 ± 0.1       | 2.8       | 1               | 4        |   |
| γGly4    | 1.53 ± 0.07     | 1.4 ± 0.1       | 4.7       | 0.6             | 4        |   |
| γGly8    | 1.47 ± 0.06     | 1.3 ± 0.1       | 3.3       | 0.6             | 3        |   |
unstructured and located on the surface of each subunit facing the extracellular channel vestibule, which likely allows the glycine insertions to be accommodated without large perturbations in the overall folding and structure of the β-sandwich cores of the subunits. Unstructured loop regions may impart flexibility that is essential for protein function.

If the linker in the β-subunit is involved in propagating GABA-triggered ligand binding site movements to the channel, one would predict that mutations in this region would affect ligand binding and channel gating. To evaluate whether the glycine insertions in the β-subunit altered the GABA binding site structure, we examined the ability of the competitive antagonist SR-95531 to inhibit GABA-gated current. βGly insertions significantly increased SR-95531 IC₅₀ (Fig. 7B, Table 1), suggesting that the orthosteric binding pocket was altered and that the shifts in GABA EC₅₀ observed with the glycine insertions are in part due to a change in GABA microscopic binding affinity. The proximity of the βGly insertions to loop A of the GABA binding site (37) and the >10,000-fold changes in GABA EC₅₀ measured also suggest that, at least in part, the insertions alter GABA binding (25). In support of this idea, in the related nAChR, deletion of residues in the β4–β5 linker in the α-subunit altered microscopic acetylcholine binding as well as inhibited channel gating (15).

The βGly insertions also affected channel gating. When the currents elicited from maximum PB versus maximum GABA were compared in the same oocyte, the GABA currents elicited from βGly1-containing receptors were ~4–5-fold smaller than the PB currents (Fig. 3A), indicating that βGly1 transformed GABA into a partial agonist and decreased GABA efficacy. Moreover, βGly1 increased the 10–90% apparent current rise times for maximum GABA (Fig. 3B), suggesting that βGly1 alters channel gating by decreasing the channel opening rate. In the GABAₐR, nAChR, and serotonin type-3 receptor, mutating residues in the β4–β5 linker near loop A increase unliganded channel opening (15, 16, 37, 38), indicating that this region influences channel gating. Moreover, agonist-mediated movements in the nAChR α-subunit β4–β5 linker have been observed (39). Thus, although a detailed understanding of the effects of the glycine insertions on GABA binding and channel gating will require higher resolution kinetic studies, the data provide strong evidence that the β-subunit β4–β5 linker is an important structural element involved in transducing GABA binding to channel gating.

A surprising observation seen with the βGly2–8 insertions was that the competitive antagonist SR-95531 was converted into a weak partial agonist and elicited ionic currents in the absence of GABA (Fig. 4). Because glycine does not have a β-carbon, it can adopt a large variety of conformations and impart a regional flexibility that may lead to a widening of the GABA binding pocket. This widening may allow the relatively bulkier SR-95531 to promote local movements in the GABA binding site (37).
binding pocket that trigger and stabilize an open channel protein conformation. Moreover, the binding site expansion would hinder GABA, a smaller molecule, from optimally establishing the necessary structural contacts and triggering the local movements required (i.e. binding site contraction) for receptor activation and would thus convert it into a partial agonist. Agonist affinity for the GABA binding site is linearly correlated to ligand size; about 17-fold (EC50) increase PB EC50 (Fig. 7A and B). We speculate that BZD-initiated movements in the BZD binding site are propagated directly through the α-subunit linker to the GABA binding site and that the αGly insertions hinder this propagation. BZD modulation of GABAPros was also altered by γGly insertions (Fig. 7, D and E), which increased BZD EC50 as well as decreased BZD efficacy (Fig. 7, D and E, Table 3). The γGly insertions are positioned near the γ/β interface (a nonbinding site interface). Interestingly, mutations of Arg-43 at this interface alter BZD binding (48) consistent with structural perturbations at this interface having long range effects on BZD actions. βGly1 also decreased BZD efficacy (Table 3, Fig. 7D). Some studies suggest that BZD agonists increase unliganded GABAPros channel opening (49–51). Because βGly1 appears to stabilize a closed state of the GABAPros by slowing channel opening (Fig. 3B), one might expect that βGly1 would decrease BZD efficacy. Overall, the glycin e insertions in the β-δ5 linkers of each of the subunits altered BZD actions, suggesting that BZD agonist-induced structural changes are transmitted via multiple pathways.

Linear free energy relationship analysis in the nAChR indicates that the β-δ5 linker moves early in the activation process (15) and likely transfers the energy imparted by the agonist to loop 2 of the coupling interface that then leads to receptor activation. Thus, we envision that increasing the length and flexibility of this linker in the GABAPros β-subunit decreases GABA activation presumably by accruing an energetic barrier, indicating that the structural dynamics of this linker are optimized for efficient signal propagation within the native protein.

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