Occupation of Either Site for the Neurosteroid Allopregnanolone Potentiates the Opening of the GABA$_{\alpha}$ Receptor Induced from Either Transmitter Binding Site

John Bracamontes, Megan McCollum, Caroline Esch, Ping Li, Jason Ann, Joe Henry Steinbach, and Gustav Akk

Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri

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ABSTRACT

Potentiating neuroactive steroids are potent and efficacious modulators of the GABA$_{\alpha}$ receptor that act by allosterically enhancing channel activation elicited by GABA. Steroids interact with the membrane-spanning domains of the $\alpha$ subunits of the receptor, whereas GABA binds to pockets in the interfaces between $\beta$ and $\alpha$ subunits. Steroid interaction with a single site is known to be sufficient to produce potentiation, but it is not clear whether effects within the same $\beta$-$\alpha$ pair mediate potentiation. Here, we have investigated whether the sites for GABA and steroids are functionally linked (i.e., whether the occupancy of a steroid site selectively affects activation elicited by GABA binding to the transmitter binding site within the same $\beta$-$\alpha$ pair). For that, we used receptors formed of mutated concatenated subunits to selectively eliminate one of the two GABA sites and one of the two steroid sites. The data demonstrate that receptors containing a single functional GABA site are potentiated by the neurosteroid allopregnanolone regardless of whether the steroid interacts with the $\alpha$ subunit from the same or the other $\beta$-$\alpha$ pair. We conclude that steroids potentiate the opening of the GABA$_{\alpha}$ receptor induced by either agonist binding site.

Introduction

Neuroactive steroids can act as powerful anesthetics, anticonvulsants, and neuroprotectants. The GABA$_{\alpha}$ receptor, the major target of many exogenous neuroactive steroids, binds synaptically released or ambient GABA, resulting in the activation of an ion-selective channel. Interaction of steroid with the receptor enhances channel open probability through specific changes in channel open and closed times, resulting in increased flow of Cl$^-$ ions through the cell membrane (Akk et al., 2010).

The GABA$_{\alpha}$ receptor is a pentameric protein. The major class of mammalian synaptic receptors consists of two $\alpha_1$ subunits, two $\beta_2$ subunits, and a single $\gamma_2$ subunit (McKernan and Whitington, 1996). The highly homologous subunits are organized pseudosymmetrically around the central channel. The arrangement of subunits around the central pore is $\beta\gamma\beta\alpha$, counterclockwise when viewed from the outside of the cell (Baumann et al., 2002). The extracellular domain of the receptor contains two binding sites for the transmitter at the $\beta$-$\alpha$ subunit interfaces (Kash et al., 2004). Potentiation by neuroactive steroids results from the interaction of steroid with the membrane-spanning domains of the two $\alpha$ subunits. Although the exact structural determinants of steroid binding are unknown, the actions of potentiating steroids are strongly reduced or eliminated by mutations to specific residues in the M1 and M4 transmembrane domains in the $\alpha_1$ subunit (Hosie et al., 2006; Akk et al., 2008; Li et al., 2009). Specifically, the $\alpha 1Q241L$ mutation abolishes potentiation by the steroid allopregnanolone (Hosie et al., 2006; Akk et al., 2008). The GABA$_{\alpha}$ receptor contains two $\alpha$ subunits and, presumably, two binding sites for steroids. Recent work using mutated concatameric subunits has demonstrated that receptors containing a single intact steroid site retain the ability to be potentiated by steroids (Akk et al., 2009; Bracamontes and Steinbach, 2009).

The $\alpha$ subunit is involved in binding both GABA and steroid. We sought to determine whether the binding of steroid selectively facilitates channel opening via occupancy of the transmitter binding site within the same $\beta$-$\alpha$ pair. In this hypothesis, steroid binding modifies GABA binding or signal transduction within the same $\beta$-$\alpha$ pair. An alternative hypothesis is that steroid actions are nonspecific with regard to which transmitter binding site is occupied and that the ste-

ABBREVIATIONS: PCR, polymerase chain reaction; lmt, low melting temperature; PBS, phosphate-buffered saline.
roid acts through a global change in receptor conformation or channel gating.

For this purpose, we used concatenated GABA_\alpha receptors, allowing us to selectively mutate one of the two \(\alpha\) and \(\beta\) subunits present in the receptor. The steroid (allopregnanolone) and agonist (GABA) binding were selectively disrupted by the \(\alpha1\) (Q241L) and \(\beta2\) (Y205S) mutations, respectively, introduced to one of the two \(\beta-\alpha\) pairs. The data indicate that the steroid effect is not selective, and that steroid binding to one \(\alpha\) subunit essentially equally well potentiates activity from receptors binding GABA to the opposing as well as the same \(\beta-\alpha\) pair.

Materials and Methods

The experiments were conducted on wild-type and mutated rat concatameric GABA_\alpha receptors. The receptors consisted of a triple \(\beta2-\alpha1-\gamma2L\) (\(\beta\gamma\)) construct and a \(\beta2-\alpha1\) (\(\beta\alpha\)) tandem construct (Fig. 1). To eliminate GABA binding, the Y205S mutation (Amin and Weiss, 1993) was introduced to one or both \(\beta\) subunits. To eliminate steroid interaction with the receptor, the Q241L mutation (Hosie et al., 2006; Akk et al., 2008) was introduced to one or both \(\alpha\) subunits. The \(\alpha\) (Q241L) and \(\beta\) (Y205S) mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The mutated clones were fully sequenced to verify that only the desired mutation(s) had been produced.

The \(\beta\alpha\) and \(\beta\gamma\) (Q241L) concatamers have been reported previously (Bracamontes and Steinbach, 2009). The \(\beta\) (Y205S) construct was made by subcloning a ClaI/AflI fragment from the \(\beta\) (Y205S) clone into the \(\beta\) construct. The \(\beta\) (Y205S) construct was constructed by subcloning the ClaI/AflI fragment from the \(\beta\) (Y205S) clone into \(\beta\) (Q241L).

The triple construct \(\beta\gamma\) was generated with the use of PCR overlap extension (Ho et al., 1989), using the \(\gamma2L\) single subunit and the \(\beta\) construct as templates. The \(\beta\) construct was amplified with a forward oligonucleotide complementary to an internal sequence of the \(\alpha\) subunit and a reverse oligonucleotide complementary to the 3' end of the \(\alpha\) coding region with additional sequence at the 3' end to form part of the linker between the \(\alpha\) and \(\gamma\) subunits. The \(\gamma2L\) subunit was amplified with the SP6 reverse oligonucleotide, and a forward oligonucleotide complementary to the 5' coding region of the mature protein that excluded the signal peptide with additional sequence at the 5' end encoding part of the linker sequence that overlaps the \(\alpha\) oligo by 25 nucleotides. Both PCR products were purified by electrophoresis using a low melting temperature (Imt) agarose gel. PCR bands were excised from the gel and purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). These purified PCR fragments were combined along with the previously used \(\alpha1\) forward and SP6 reverse oligonucleotides for overlap PCR, resulting in a partial \(\alpha1-\gamma2L\) concatamer. This product was purified on an Imt gel as described above. The \(\beta\) and concatamer and the purified PCR product were digested with EcoNI (internal to \(\alpha\)) and XbaI (in pcDNA3) and subsequently purified on an Imt gel. The PCR fragment was ligated to the digested \(\beta\) concatamer forming a \(\beta\gamma\) concatamer. The triple concatamer was subsequently verified by sequencing. The \(\beta\) (Y205S) construct as template. The \(\beta\) (Y205S)\(\gamma\) construct was made as described above, using the \(\beta\) (Y205S)\(\alpha\) (Q241L)\(\gamma\) construct and \(\beta\gamma\), respectively.

The amino acid sequence of the \(\beta-\alpha\) linker in the triple concatamers \(\beta\) (Y205S)\(\alpha\) (Q241L)\(\gamma\) and \(\beta\) (Y205S)\(\gamma\) and in all \(\beta\) tandems is Q5A3PTGQ2AQA3PA2Q5. The \(\beta-\alpha\) linker sequence in the triple concatamers \(\beta\gamma\) and \(\beta\) (Q241L)\(\gamma\) is Q5A3PGAQAGP.A.QQ5, and the nucleotide sequence includes an FseI restriction site. The \(\alpha-\gamma\) linker sequence in all triple concatamers is Q5A3PGQQAQA3PQ5, and the nucleotide sequence includes a PinAI restriction site.

The receptors were expressed in Xenopus laevis oocytes. The cDNAs for the receptor subunits were subcloned into the pcDNA3 expression vector in the T7 orientation. The cDNA was linearized by XbaI (New England Biolabs, Ipswich, MA) digestion, and the cDNA was produced using Message Machine (Ambion, Austin, TX). The oocytes were injected with 7 to 14 ng of cDNA per construct in a final volume of 20 to 60 nl and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2, 1 mM MgCl_2, 2.5 mM sodium pyruvate, and 5 mM HEPES, pH 7.4) at 16°C for 2 to 3 days before recording.

Standard two-electrode voltage clamp was used to record the currents. Both voltage and current electrodes were patch-clamp electrodes filled with 3 M KCl and had resistances of 0.5 to 1.5 MΩ. The oocytes were typically clamped at −60 mV. The chamber (RC-1Z; Warner Instruments, Hamden, CT) was perfused continuously at approximately 5 ml/min. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2, and 10 mM HEPES, pH 7.4) was perfused through all test applications.

Solutions were switched by pClamp using a Warner Instruments VC-8T valve controller. Solutions were applied from glass reservoirs via metal or Teflon tubing to reduce adsorption. A typical drug application protocol was to expose an oocyte to bath solution for 10 s, followed by a 20-s drug (GABA, pentobarbital, allopregnanolone) application and a switch back to bath solution. The washout period between successive drug applications was 1 to 3 min.

The current responses were amplified with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digitizer 1230 series digitizer (Molecular Devices) at a 100-Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were analyzed with Clampfit (Molecular Devices). The GABA concentration-response curves were fitted using the program NFT (The University of Texas Medical Branch at Galveston, Galveston, TX). Statistical analyses were carried out using Excel (Microsoft Corp., Redmond, WA).

Western blotting was conducted on extracts from X. laevis oocytes injected with various combinations of cRNA for concatameric constructs. The oocytes were injected with 10 ng of mRNA per construct. After 2 days of incubation at 15.8°C in ND96, the oocytes were put in a 1.5-ml microfuge tube and washed once with 0.5 ml of PBS. The PBS was removed and the oocytes were washed with 0.5 ml of PBS plus a protease inhibitor cocktail (P8465; Sigma-Aldrich, St. Louis, MO). The PBS was again aspirated and then 10 μl of lysis buffer (10 mM HEPES, pH 8.0, 100 mM NaCl, and 10 mM EDTA plus the protease inhibitor cocktail) per oocyte were added to the microfuge tube. The oocytes were homogenized by pipetting through a syringe.

Fig. 1. Concatameric receptors. A, the concatameric constructs were generated by linking the rat \(\beta2\) and \(\alpha\) subunits, carboxyl to amino termini, via a 23-amino acid residue long linker (see Materials and Methods for the sequence). The \(\gamma2L\) subunit was then linked to the carboxyl terminus of the \(\beta-\alpha\) construct via a 26-amino acid residue long linker. B, view of the organization of the GABA_\alpha receptor from the extracellular side. The GABA binding sites (G) are located at the \(\beta-\alpha\) subunit interfaces. The sites were disrupted by introducing the Y205S mutation to one or both \(\beta\) subunits. The binding sites for potentiating steroids are located within the \(\alpha\) subunits (S). Steroid actions were disrupted by introducing the Q241L mutation to one or both \(\alpha\) subunits.
neural. The homogenate was spun for 5 min at 1000 rpm, 4°C, and the supernatant was removed and spun again under identical conditions. Triton X-100 was added to the supernatant to a final concentration of 2% after which the mixture was rotated for 30 min at 4°C. The mixture was then spun at 14,000 rpm for 10 min at 4°C. The supernatant was added to 20 μl of FLAG agarose beads (Sigma-Aldrich, St. Louis, MO), and enough lysis buffer was added to dilute the concentration of Triton to 1% for an overnight immunoprecipitation reaction. The next day, the agarose beads were washed, and 5 μl of lysis buffer per oocyte were added to the washed agarose beads along with an equal volume of 2X Laemmli buffer. The solution was boiled for 5 min and spun at 2000 rpm to pellet the agarose beads. Fifty microliters of each sample were then loaded onto a precast 4 to 15% gradient Tris-glycine polyacrylamide gel (Bio-Rad, Hercules CA) and electrophoresed. The gel was then transferred to a nitrocellulose Hybond-ECL membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The membrane was preblocked in 100% Odyssey block solution (LI-COR Biosciences, Lincoln NE) at room temperature for 1 h, followed by overnight incubation at 4°C in a solution of 50% Odyssey block solution: 50% phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.3) containing 0.2% Tween 20 (Thermo Fisher Scientific, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody. The primary antibody was raised to the cytoplasmic region of the α₁ subunit (1:500 dilution; Santa Cruz Biotechnology, Waltham, MA) with primary antibody.

Results

Concatameric Constructs Are Not Degraded. We created concatameric GABA<sub>3</sub> receptors made of rat α₁, β₂, and γ₂L subunits. The concatameric constructs (Fig. 1) were generated, amino- to carboxyl-terminal, in the β-α-γ (βγ) and β-α configuration (βα). In some experiments, one or both β subunits contained the β₂(Y205S) mutation, and one or both α subunits contained the α₁(Q241L) mutation.

Proteolysis after expression can lead to partial or full degradation of the concatamers, potentially resulting in breakup of the constructs into single free subunits. If the degraded constructs retain the ability to assemble into functional receptors, the interpretation of the results presented below would be complicated. We performed Western blots of proteins extracted from injected oocytes (Fig. 2) to confirm that subunit concatamers are not appreciably degraded after expression in oocytes. Proteins were immunoprecipitated with antibody to a FLAG epitope placed near the amino terminus of the β₂ subunit, and transfers were probed with antibody to the cytoplasmic region of the α₁ subunit. The blots indicate that assembled receptors (i.e., receptors containing the β₂ subunit) do not include detectable lower molecular mass material reacting with the anti-α₁ antibody. Free α₁ subunit migrates at ~50 kDa, whereas the duplex concatameric constructs (βα) migrate at ~120 kDa, comparable with results obtained previously [110–140 kD; (Bau-
(Table 1). The GABA EC\textsubscript{50} was shifted to 156 μM in the receptor containing the mutation in both constructs. The β(Y205S) mutation has been shown to eliminate channel gating by GABA in receptors consisting of free subunits (Amin and Weiss, 1993). When the β(Y205S) mutation was introduced to both the β\textgamma and βα constructs, the receptors did not respond to application of up to 3 mM GABA (<5 nA; Table 1). Functional expression of the β(Y205S)\textgamma-β(Y205S)α receptors was demonstrated by current responses to pentobarbital (Fig. 4E). In four oocytes exposed to 2 mM pentobarbital, the maximal peak response was 280 nA, and the tail response upon the removal of the drug reached 700 nA. The peak response in the presence of a high concentration of pentobarbital, the maximal peak response was 280 nA, and the tail results from rapid unblocking of the channel. The peak of the tail response for receptors containing unmutated concatamers was 3 μA, suggesting that the total number of receptors was not greatly reduced by the presence of β(Y205S) in both concatamers. Receptors containing a single β(Y205S) mutation responded to 1 mM GABA with peak responses of up to 2.5 μA. The presence of the mutation had a relatively small effect on the GABA concentration-response properties. The EC\textsubscript{50} values were at 32 μM (no shift) or 95 μM (a 2.9-fold effect) when the β(Y205S) mutation was in the β\textgamma or βα construct, respectively. However, the mutation reduced the Hill coefficient from 2.0 in the wild-type concatameric receptor to 1.1 (mutation in the β\textgamma construct) or 1.4 (mutation in βα). At the concentrations of transmitter used here (<10 mM), the concentration-response curves were well characterized with a single component (Fig. 3).

### Potentiation of Receptors Activated by GABA

We examined allopregnanolone-mediated potentiation of concatameric receptors activated by GABA. The concentrations

**Table 1**

<table>
<thead>
<tr>
<th>Receptor Configuration</th>
<th>Maximal Response</th>
<th>EC\textsubscript{50} μM</th>
<th>nH</th>
<th>No. of Cells</th>
<th>Range of Currents at Maximal GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβγ</td>
<td>1.0 ± 0.03</td>
<td>4.6 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td>7</td>
<td>675–1780</td>
</tr>
<tr>
<td>βγ-βα</td>
<td>1.0 ± 0.03</td>
<td>3.2 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td>5</td>
<td>4364–8075</td>
</tr>
<tr>
<td>βγ-βα*</td>
<td>1.0 ± 0.02</td>
<td>9.5 ± 0.6</td>
<td>1.4 ± 0.1</td>
<td>6</td>
<td>827–2443</td>
</tr>
<tr>
<td>βγ-βα*</td>
<td>1.0 ± 0.02</td>
<td>7.6 ± 0.3</td>
<td>1.9 ± 0.1</td>
<td>5</td>
<td>1831–5133</td>
</tr>
<tr>
<td>βα<em>γ-βα</em></td>
<td>1.0 ± 0.02</td>
<td>78 ± 4</td>
<td>1.6 ± 0.1</td>
<td>5</td>
<td>1171–4003</td>
</tr>
<tr>
<td>βα<em>γ-βα</em></td>
<td>1.0 ± 0.02</td>
<td>53 ± 4</td>
<td>1.3 ± 0.1</td>
<td>5</td>
<td>3730–4592</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.1 ± 0.3</td>
<td>94 ± 23</td>
<td>1.0 ± 0.2</td>
<td>4</td>
<td>129–211</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.1 ± 0.03</td>
<td>156 ± 12</td>
<td>1.4 ± 0.1</td>
<td>5</td>
<td>487–1212</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.1 ± 0.01</td>
<td>126 ± 5</td>
<td>1.2 ± 0.05</td>
<td>6</td>
<td>92–263</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.0 ± 0.02</td>
<td>32 ± 2</td>
<td>1.1 ± 0.1</td>
<td>4</td>
<td>277–2823</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.0 ± 0.02</td>
<td>140 ± 11</td>
<td>1.2 ± 0.1</td>
<td>7</td>
<td>35–142</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.0 ± 0.02</td>
<td>24 ± 6</td>
<td>1.0 ± 0.2</td>
<td>5</td>
<td>460–3585</td>
</tr>
<tr>
<td>βα*γ-βα</td>
<td>1.2 ± 0.02</td>
<td>187 ± 13</td>
<td>1.1 ± 0.1</td>
<td>5</td>
<td>50–148</td>
</tr>
</tbody>
</table>

β\text*, β(Y205S); α, α(Q241L); G, intact GABA site; S, intact steroid site; N.D., not determined.

**Fig. 3. GABA concentration-response curves of the concatameric receptors.** A, receptors containing the wild-type βγ construct in combination with four variants of the βα construct. The dashed line gives the GABA concentration-response curve for receptors containing free α1, β2, and γ2L subunits (from Akk et al., 2011). B, receptors containing the βα(Q241L)γ construct in combination with four variants of the βα construct. C, receptors containing the β(Y205S)\textgamma construct in combination with two variants of the βα construct. No responses to GABA were observed from β(Y205S)\textgamma + β(Y205S)α or β(Y205S)\textgamma + β(Y205S)α(Q241L) receptors. D, receptors containing the β(Y205S)α(Q241L)γ construct in combination with two variants of the βα construct. No responses to GABA were observed from β(Y205S)α(Q241L)γ + β(Y205S)α or β(Y205S)α(Q241L)γ + β(Y205S)α(Q241L) receptors. The correspondence between symbols and receptor types are given separately in each panel. The asterisk stands for the presence of mutation (in the β subunit, Y205S; in the α subunit, Q241L). The results from fitting are presented in Table 1.
Steroid Potentiation of Monoliganded GABA<sub>α</sub> Receptors

of GABA used in these experiments were selected on the basis of the concentration-response data presented in Fig. 3 to elicit approximately 20 to 30% of the maximal response. These are concentrations at which most of our previous work on steroid potentiation has been conducted (Akk et al., 2005, 2009; Li et al., 2007), thus offering the most material for comparison. Furthermore, the peak GABA currents were strongly reduced for several mutant combinations, probably as a result of the combined effects of the occupation of a single transmitter binding site (Baumann et al., 2003; Baur and Sigel, 2005) and the reduction in channel open probability that results from the α(Q241L) mutation (Akk et al., 2008). In these cases, the use of lower fractional GABA concentrations would have resulted in unreliable estimates for control currents.

The effect of 1 μM allopregnanolone was examined. This concentration elicits a maximal potentiating effect in wild-type receptors composed of free subunits (Li et al., 2007) as well as concatameric receptors containing wild-type subunits or receptors containing a single α1(Q241L) mutation (Akk et al., 2009; Bracamontes and Steinbach, 2009). The effect of steroid is given as multiples of the control peak response (1 means no effect).

Coapplication of 1 μM allopregnanolone with 15 to 20 μM GABA (EC<sub>22</sub>) potentiated the peak response by 4.2 ± 1.6-fold in the wild-type βγ+α receptor (Table 2; Fig. 4A). For comparison, allopregnanolone potentiates receptors containing free α1β2γ2 subunits by 2.9 ± 0.5-fold (Table 2). In agreement with previous data (Akk et al., 2009; Bracamontes and Steinbach, 2009), the presence of a single α1(Q241L) mutation allowed potentiation (2.2-fold for βγβα(Q241L) and 3.4-fold for βα(Q241L)γβα). We note that the GABA

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**TABLE 2**

Steroid modulation of the concatameric receptors activated by GABA

The table shows the effect of coapplication of 1 μM allopregnanolone with a submaximal concentration of GABA. Given are the effect of steroid (mean ± S.D.) in the presence of a low concentration of GABA as the ratio of the response in the presence of 1 μM allopregnanolone to the response of that concentration of GABA alone and saturating GABA (fourth column). The first row (αγ) gives the results obtained from receptors containing free α, β2, and γ2L subunits. Receptors containing two mutated β subunits showed negligible currents in the presence of GABA. Accordingly, steroid potentiation of GABA-elicited currents was not examined.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Configuration</th>
<th>Potentiation by Allopregnanolone</th>
<th>Fractional GABA Response</th>
<th>No of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>αγ</td>
<td>GS/GS</td>
<td>2.9 ± 0.5</td>
<td>0.26 ± 0.03</td>
<td>7</td>
</tr>
<tr>
<td>βαγ-βα</td>
<td>GS/GS</td>
<td>4.2 ± 1.6</td>
<td>0.22 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>GS−/−</td>
<td>10.4 ± 2.6</td>
<td>0.23 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>GS/G−/−</td>
<td>2.2 ± 0.3</td>
<td>0.27 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/GS</td>
<td>4.4 ± 0.5</td>
<td>0.23 ± 0.04</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>2.7 ± 0.3</td>
<td>0.28 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>1.0 ± 0.1</td>
<td>0.24 ± 0.15</td>
<td>4</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>1.2 ± 0.2</td>
<td>0.27 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>4.9 ± 0.6</td>
<td>0.21 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>2.5 ± 0.6</td>
<td>0.29 ± 0.04</td>
<td>6</td>
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<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>N.D.</td>
<td>N.D.</td>
<td>5</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>N.D.</td>
<td>N.D.</td>
<td>6</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>4.2 ± 2.0</td>
<td>0.26 ± 0.08</td>
<td>3</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>1.1 ± 0.1</td>
<td>0.26 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>N.D.</td>
<td>N.D.</td>
<td>4</td>
</tr>
<tr>
<td>βαγ-βα*</td>
<td>G−/−</td>
<td>N.D.</td>
<td>N.D.</td>
<td>4</td>
</tr>
</tbody>
</table>

β<sup>+</sup>, β(Y205S); α<sup>+</sup>, α(Q241L); G, intact GABA site; S, intact steroid site; N.D., not determined.
fractional responses at which the steroid effects were measured differed slightly (Table 2). Hence, the comparative potentiating effects should be judged with care because they depend on the level of baseline activation. Receptors containing two mutated α subunits were not potentiated by allopregnanolone (1.0 ± 0.1 of control; Table 2; Fig. 4D).

Receptors containing the β(Y205S) mutation in a single concatameric construct were strongly potentiated by steroid. When the mutation was in the βαγ construct, 1 μM allopregnanolone potentiated the peak response to 4.9-fold of control. When the mutation was in βα, the effect was 10.4-fold (Table 2). These relatively large values for potentiation are an indication that the β(Y205S) mutation depresses the maximal open probability in the presence of GABA, thus allowing modulation to levels above the maximal current seen with GABA. This kind of effect has been described previously for δ subunit-containing receptors, which have an intrinsically low maximal open probability when activated by GABA (Bianchi and Macdonald, 2003). Receptors containing two β(Y205S) mutations demonstrated no currents in the presence of up to 3 mM GABA. Accordingly, steroid potentiation of GABA responses from these receptors could not be studied.

Combinations of α and β subunit mutations could be divided into two classes. In the first combination, the mutations are within the same β-α pair, thus eliminating one of the GABA binding sites as well as the steroid site associated with the same pair. This leaves the other β-α pair intact to bind both GABA and steroid. In the second combination, the mutations are made in opposite β-α pairs, and the intact GABA and steroid sites lie within different β-α pairs.

Receptors containing the GABA and steroid site mutations in the same β-α pairs (βαγ + β(Y205S)α(Q241L) and β(Y205S)α(Q241L)γ + βα) were potentiated by allopregnanolone. The effect was 4.2-fold when the mutations were in the βαγ construct, and 4.4-fold when the mutations were in βα (Table 2).

Receptors containing the β and α subunit mutations in opposite β-α pairs were also potentiated by steroid. The potentiating effect of 1 μM allopregnanolone was 2.7-fold in βα(Q241L)γ + β(Y205S)α and 2.5-fold in β(Y205S)αγ + βα(Q241L) receptors (Table 2; Fig. 4, B and C). From here, we infer that steroid modulation does not occur by an effect confined to a single α subunit.

We also conducted control experiments in which one of the β-α pairs contained the GABA site mutation and both steroid sites in the two α subunits were mutated. As expected, no potentiation was detected in βα(Q241L)γ + β(Y205S)α(Q241L) (1.2-fold) or β(Y205S)α(Q241L)γ + βα(Q241L) receptors (1.1-fold; Table 2).

**Potentiation of Receptors Activated by Pentobarbital.** Receptors containing two β(Y205S) mutations could not be tested for potentiation of currents elicited by GABA because of very small responses. However, such receptors remain responsive to the allosteric activator pentobarbital (Amin and Weiss, 1993). Accordingly, to verify that the β(Y205S) mutation does not interfere with steroid actions when it is present in both β subunits, we tested steroid-mediated potentiation of currents elicited by submaximal concentrations of pentobarbital.

To quantify the potentiating effect of allopregnanolone, we initially exposed the receptors to a low concentration (100–400 μM) of pentobarbital. This concentration was selected to produce approximately 15 to 25% of the tail response observed in the presence of 2 mM pentobarbital. In the next step, 1 μM allopregnanolone was coapplied with the low concentration of pentobarbital.

Receptors lacking mutations to the steroid site [i.e., β(Y205S)αγ and β(Y205S)α receptors] were potentiated by allopregnanolone with an average effect of 6.9-fold (Fig. 4E; Table 3). The introduction of a single α(Q241L) mutation to the double β(Y205S) mutant has little effect on potentiation by allopregnanolone. When the α(Q241L) mutation is in the βαγ, the effect of allopregnanolone is 6.6-fold, and when the α(Q241L) mutation is in the βα construct, the effect of steroid is 4.8-fold. When both α subunits contain the mutation, no potentiation of pentobarbital-elicited currents is observed. The results are summarized in Table 3. From these experiments, we conclude that the β(Y205S) mutations do not interfere with the ability of allopregnanolone to produce channel potentiation.

**Discussion**

We present results from a study on the interactions between the primary orthosteric transmitter binding sites and the allosteric binding sites for potentiating neurosteroids in the α1β2γ2L GABA_A receptor. We employed a set of concatenated subunits in which each of the two transmitter sites and each of the two steroid sites was selectively disrupted by the β(Y205S) and α(Q241L) point mutations, respectively. We confirm previous data demonstrating that a single intact steroid site is sufficient to confer sensitivity to the actions of potentiating steroids. Furthermore, we show that receptors containing a single intact GABA binding site can be activated by GABA and potentiated by the neurosteroid allopregnanolone and that potentiation can be mediated by steroid interactions with its site within the same β-α pair that mediates receptor activation as well as the opposite β-α pair.

We examined the potentiation of responses to pentobarbital in the absence of functional GABA-binding sites. In this case, it also appears that the presence of a single steroid-binding site confers full ability to potentiate activation by an

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**TABLE 3**

Steroid modulation of concatamer receptors with no intact GABA-binding sites, activated by pentobarbital

The table shows the effect of coapplication of 1 μM allopregnanolone with a submaximal concentration of pentobarbital. Given are the effect of steroid (mean ± S.D.) in the presence of a low concentration (100–400 μM) of pentobarbital (1 stands for no effect) (third column) and the ratio of the peak response to a low concentration of pentobarbital and the tail current after the application of 2 mM pentobarbital (fourth column).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Configuration</th>
<th>Potentiation by Allopregnanolone</th>
<th>Fractional Pentobarbital Response</th>
<th>No. of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>βαγβαγβαγ</td>
<td>–S–S</td>
<td>6.9 ± 1.7</td>
<td>0.20 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>βαγβαγβαγ*</td>
<td>–S–S</td>
<td>4.8 ± 1.2</td>
<td>0.25 ± 0.10</td>
<td>5</td>
</tr>
<tr>
<td>βαγβαγβαγ*</td>
<td>–S–S</td>
<td>6.6 ± 1.5</td>
<td>0.14 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>βαγβαγβαγ*</td>
<td>–S–S</td>
<td>1.2 ± 0.3</td>
<td>0.21 ± 0.06</td>
<td>4</td>
</tr>
</tbody>
</table>

βαγβαγβαγ; *αγβαγβαγ; S, intact steroid site.
agonist that does not bind to the GABA-binding site and that also apparently induces somewhat different conformational changes in the extracellular domain of the receptor (Muroi et al., 2009; Akk et al., 2011).

The use of concatenated subunits is an effective way to constrain the stoichiometry and order of subunits. Furthermore, it allows a selective introduction of mutations to one of the two α or β subunits present in most GABA_{α} receptors. We previously created and characterized concatameric receptors containing γ2L-β2-α1 (γβα) and β2-α1 (βα) constructs (Akk et al., 2009; Bracamontes and Steinbach, 2009). To avoid potential issues with constraining the amino terminus of the β subunit, the present work was conducted on β2-α1-γ2L (βγα) + β2-α1 (βα) receptors. We find that receptors containing the γβα triple construct have a GABA concentration-response curve shifted to higher agonist concentrations compared with βαγ-containing receptors or receptors consisting of α1, β2, γ2L free subunits, potentially reflecting reduced flexibility of the aminoterminal of the β subunit. In other work, Baumann et al. (2002) found that the GABA EC_{50} estimates for βγα + βα receptors were the closest to the unlinked receptor. The activation curves for γβα + βα or αβα + γβ receptors were further right-shifted. It is thus clear that the linkage of subunits quantitatively affects receptor function. However, the majority of the macroscopic and single-channel biophysical and pharmacological properties remain unaltered in concatameric receptors (Baumann et al., 2002; Boileau et al., 2005; Akk et al., 2009), and the approach remains a powerful tool for selective and focused manipulation of the receptor structure.

A previous study (Baumann et al., 2003), which used concatameric αβα + γβ receptors and in which the β(Y205S) mutation was selectively introduced to one of the constructs, found that the GABA EC_{50} was right-shifted by ~2-fold when the mutation was in the γβ construct [a configuration similar to βγα + β(Y205S)α in the present study]. When the β(Y205S) mutation was introduced to the αβα construct (a configuration similar to the mutation being in the βγγ construct), the GABA EC_{50} was shifted by 5-fold to higher concentrations (Baumann et al., 2003). It was proposed that these values reflect activation due to occupation of the unmutated site, whereas a second component, with estimated EC_{50} values at 1 to 10 μM, arises from the occupation of the mutated GABA binding site. From these data, Baumann et al. (2003) estimated that the β-α pair flanked by the γ and β subunits (defined as site 1) has a 3-fold lower affinity to GABA than the β-α pair flanked by the α and γ subunits (site 2). Our data show the opposite relationship between the position of the mutation and receptor activation by GABA. Introduction of the β(Y205S) mutation to the βα construct (analogous to a mutated site 1) leads to a larger shift in the GABA concentration-response curve than when the mutation is introduced to the βγγ construct. The underlying reason for the difference is unclear to us.

Our data demonstrating that receptors containing intact transmitter and steroid sites within different β-α subunit pairs can be poteniated by allopregnanolone indicate that the conformational changes induced by the occupation of either steroid-binding site are equivalently transduced to both transmitter-binding sites. Alternatively, the occupation of a steroid site may lead to general conformational changes in the gate domain that, in turn, stabilize the open channel state of the receptor. The present work does not distinguish between the two possibilities. In any case, there seems to be no selective linkage between a steroid site and a transmitter binding site. The present study focused on potentiation by the endogenous neurosteroid allopregnanolone. It is probable that other steroids (e.g., pregnanolone, tetrahydrodeoxycorticosterone) the actions of which depend on the αIQ241 residue demonstrate behavior qualitatively similar to that observed in the present study.

There have been relatively few previous studies of linkage between allosteric modulator sites and agonist sites. A previous study examining the linkage between the site for benzodiazepines positioned at the γ-α subunit interface and the transmitter binding sites found that the occupation of the benzodiazepine site similarly affected channel opening induced by the occupation of either GABA site (Baur and Sigel, 2005).

The neuronal-type nicotinic α4β2 receptor is potentiated by 17β-estradiol, with a critical set of amino acids at the extreme carboxyl-terminal end of the α4 subunit (Paradiso et al., 2001; Curtis et al., 2002). A recent study has found that these amino acids can be placed on either a transmitter-binding or the structural subunit to confer potentiation (Jin and Steinbach, 2011). In combination with the present results, these findings suggest that allosteric potentiators act globally on Cys-loop receptors to enhance the probability of being open, rather than preferentially through “preferred” partner subunits.

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Authorship Contributions

Participated in research design: Bracamontes, Steinbach, and Akk.

Conducted experiments: Bracamontes, McCollum, Esch, Li, Ann, and Akk.

Performed data analysis: McCollum, Ann, and Akk.

Wrote or contributed to the writing of the manuscript: Steinbach and Akk.

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**Address correspondence to:** Gustav Akk, Dept. of Anesthesiology, Washington University, Campus Box 8054, 660 S. Euclid Ave., St. Louis, MO 63110.

E-mail: akk@morpheus.wustl.edu