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Analysis of $GABA_A$ receptor activation by combinations of agonists acting at the same or distinct binding sites

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Running title: Activation of the $GABA_A$ receptor by agonist combinations

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Abbreviations: 3α5αP, 5α-pregnan-3α-ol-20-one (allopregnanolone); 3α5βP, 5β-pregnan-3α-ol-20-one (pregnanolone); c_X, ratio of the equilibrium dissociation constant of the open receptor to that of the closed receptor; *ent*-3α5βP, enantiomer of 5β-pregnan-3α-ol-20-one; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; K_X, equilibrium dissociation constant of the closed receptor; P4S, piperidine-4-sulfonic acid; P_{open}, open probability of the receptor; $P_{open,const}$, open probability of a constitutively active receptor

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ABSTRACT

Under both physiological and clinical conditions the $GABA_A$ receptors are exposed to multiple agonists, including the transmitter GABA, endogenous or exogenous neuroactive steroids, and various GABAergic anesthetic and sedative drugs. The functional output of the receptor reflects the interplay among all active agents. We have investigated the activation of the concatemeric α1β2γ2L $GABA_A$ receptor by combinations of agonists. Simulations of receptor activity using the co-agonist model demonstrate that the response amplitude in the presence of agonist combinations is highly dependent on whether the paired agonists interact with the same or distinct sites. The experimental data for receptor activation by agonist combinations were in agreement with the established views of the overlap of binding sites for several pairs of orthosteric (GABA, β-alanine, piperidine-4-sulfonic acid) and/or allosteric agents (propofol, pentobarbital, several neuroactive steroids). Conversely, the degree of potentiation when two GABAergic agents are coapplied can be used to determine whether the compounds act by binding to the same or distinct sites. We show that common interaction sites mediate the actions of a 5α- and a 5β-reduced neuroactive steroid, and a natural and an enantiomeric steroid. Furthermore, the results indicate that the anesthetics propofol and pentobarbital interact with partially shared binding sites. We propose that the findings may be used to predict the efficacy of drug mixtures in combination therapy and thus have potential clinical relevance.

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INTRODUCTION

The γ-aminobutyric acid type A ($GABA_A$) receptor is a transmitter-gated ion channel and a key component in regulating the excitatory-inhibitory balance in the brain. The binding of the transmitter GABA to the two orthosteric binding sites in the extracellular domain of the receptor leads to opening of an anion-selective ion channel, thereby contributing to cellular inhibition (Bouzat, 2012; Chua and Chebib, 2017). Besides GABA, numerous endogenous and exogenous compounds including many neurosteroids and volatile and intravenous anesthetics can activate the receptor (Olsen, 2018; Sieghart, 2015). Coapplication of two (or more) GABAergic agents typically results in potentiation of the current response. Direct activation of the $GABA_A$ receptor and potentiation of transmitter-activated receptors underlie the clinical actions of GABAergic anesthetics.

The degree or magnitude of potentiation, and by extension the clinical efficacy of an anesthetic drug, depends on multiple factors. One such factor is whether the two GABAergic agents in a combination interact with the same site(s). Coapplication of two agonists acting at the same sites can result in potentiation because of "concentration additivity", i.e., an increase in the effective concentration of the ligand. However, the exact nature of modulation depends on the efficacies and concentrations of each compound. For example, coapplication of a low-efficacy orthosteric agonist, such as piperidine-4-sulfonic acid (P4S), enhances the peak response to GABA when the concentrations of both agonists are low. At higher concentrations, P4S displaces GABA from the orthosteric binding sites and the response amplitude becomes limited by the gating efficacy of P4S. Coapplication of multiple allosteric agents that act through the same sites, for example different species of structurally-related neuroactive steroids, can be expected to perform analogously.

Agonist combinations where the individual agents interact with distinct sites produce potentiation via "energetic additivity". In this instance, one agonist acts to independently reduce the free energy difference to be overcome by the other. This process is exemplified by coapplication of an allosteric agonist with an orthosteric agonist, e.g., coapplication of propofol with GABA. The peak response to

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the combination of GABA + propofol can be accurately predicted based on energetic additivity of the effects of each individual agent (Akk et al., 2018; Ruesch et al., 2012; Shin et al., 2018). Coapplication of two allosteric agonists that interact with distinct sites would be mechanistically similar, resulting in additivity of free energies provided by each agonist towards stabilization of the open state (Shin et al., 2017).

Here, we have analyzed the activation of the concatemeric α1β2γ2L $GABA_A$ receptor by combinations of orthosteric and/or allosteric agents, using the co-agonist concerted transition model (Akk et al., 2018; Forman, 2012; Monod et al., 1965) to. In this model (Fig. 1), the receptor can exist in two states, resting and active, that have different affinities for the agonist. When the receptor transitions from one state to the other, the properties of all sites change. Receptor activation by a given agonist can be fully characterized by four parameters: basal activity of the receptor in the absence of agonist, affinity of the resting receptor to the agonist, affinity of the active receptor to the agonist, and the number of binding sites for the agonist. The effect of coapplication of a second agonist interacting with distinct sites can be considered to modify basal activity with no specific effect on receptor interaction with the principal agonist. Coapplication of a second agonist interacting with the same sites as the principal agonist can be considered as a simple competitive interaction.

The overall goal of the study was to compare the magnitude of potentiation for combinations of GABAergic compounds that act through the same or distinct sites. We show that the functional response to an agonist combination is a computable value and that it depends on the extent of overlap between the sites for the individual agents. Conversely, we propose that the response amplitude in the presence of an agonist combination can be used to determine whether the compounds interact with the same or distinct sites.

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MATERIALS AND METHODS

Receptor expression

The $GABA_A$ receptors were expressed in *Xenopus* oocytes. Harvesting of oocytes was conducted under the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal protocol was approved by the Animal Studies Committee of Washington University in St. Louis (Approval No. 20170071).

The receptors comprised concatemeric β2-α1-γ2L (βαγ) and β2-α1 (βα) constructs. The design and properties of the receptors have been described previously (Akk et al., 2018; Bracamontes et al., 2011; Bracamontes and Steinbach, 2009). Receptors formed of βαγ and βα constructs without further mutations are referred to as wild-type concatemeric receptors. Constructs containing the α1(L263S) or β2(Y143W+M286W) mutations were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The coding region was fully sequenced prior to use. The cDNAs in the pcDNA3 vector were linearized with Xba I (NEB Labs, Ipswich, MA) and the cRNAs generated using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with a total of 12 ng cRNA in a 1:1 ratio for the concatemeric constructs and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) with supplements (2.5 mM Na pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin) at 16 ^{o}C for 1-3 days prior to conducting electrophysiological recordings.

Electrophysiology

The recordings were done using standard two-electrode voltage clamp. The oocytes were clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT) was perfused with ND96 at 5-8 ml/min. Solutions were gravity-applied from 30-ml glass syringes with glass luer slips via

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Teflon tubing. A typical experiment consisted of recording of a 10-20-s baseline, followed by a drug application for 20-60 s, and bath (ND96) application until full recovery. Solutions were switched manually. The concentration-response relationships were determined by exposing each oocyte to a full range of agonist concentrations (6-9 concentration points). Due to the low gating efficacy of neuroactive steroids, the concentration-response relationships for alfaxalone, 3α5αP, 3α5βP, and *ent*-3α5βP were conducted in the presence of a low concentration of GABA. The properties of etiocholanolone and alfaxalone were also investigated on a receptor containing the gain-of-function α1(L263S) mutation (Chang and Weiss, 1999).

The current responses were amplified with an Axoclamp 900A (Molecular Devices, Sunnyvale, CA) or OC-725C amplifier (Warner Instruments, Hamden, CT), digitized with a Digidata 1320 or 1200 series digitizer (Molecular Devices), and stored using pClamp (Molecular Devices). The current traces were analyzed using Clampfit (Molecular Devices) to determine the peak amplitude.

Data analysis

The current amplitudes were converted to units of open probability by matching the relative peak responses against a scale ranging from the open probability (P_{open}) of 0 to 1 (Eaton et al., 2016; Forman and Stewart, 2012). Wild-type concatemeric receptors in the absence of agonist exhibit minuscule constitutive activity ($P_{open,const}$ = 0.00011) (Akk et al., 2018); therefore, the holding current in the absence of agonists was considered to have P_{open} of 0. The current level corresponding to P_{open} of 1 was estimated by exposing the receptors to the combination of saturating GABA plus 100 μM pentobarbital (Ziemba and Forman, 2016).

In receptors containing the gain-of-function mutations, the current level corresponding to P_{open} of 0 was estimated by exposing the oocytes to 100-500 μM of the channel blocker picrotoxin. In these receptors, no increase in peak amplitude was observed during coapplication of pentobarbital with saturating GABA. Accordingly, the mutant receptors were considered to have a P_{open} indistinguishable

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from 1 in the presence of saturating GABA alone. The open probability of the constitutively active mutant receptors ($P_{open,const}$) was calculated as $I_{picrotoxin}$ / ($I_{picrotoxin}$ - I_{GABA}) where $I_{picrotoxin}$ is the current amplitude during the application of picrotoxin and I_{GABA} is the current amplitude in the presence of saturating GABA.

We note that this approach for estimating the P_{open} values can lead to potential errors. One source of error is incomplete blockade of constitutive activity in the presence of picrotoxin that may result in overestimation of the holding current associated with zero activity. Desensitization, particularly in the presence of saturating GABA and a potentiator, may result in underestimated peak amplitude. This, however, is not a major concern because the majority of experiments were conducted at low concentrations of agonists, where desensitization is reduced.

The current response data in units of open probability were analyzed in the framework of the co-agonist concerted transition model (Fig. 1A). The experimental concentration-response curves were fit to Eq. 1 describing the state function of the receptor:

$$P_{open} = \frac{1}{1+L\times\left[\frac{1+[X]/K_X}{1+[X]/(K_X c_X)}\right]^{N_X}} \quad \text{Eq. 1}$$

where X is an agonist, K_X is the equilibrium dissociation constant for X in the closed receptor, c_X is the ratio of the equilibrium dissociation constant for X in the open receptor to K_X, and N_X is the number of binding sites for X. The number of binding sites was constrained to 2 for GABA, P4S and β-alanine (Amin and Weiss, 1993; Jones et al., 1998; Krogsgaard-Larsen et al., 1980), 6 for propofol (Shin et al., 2018), 2 for pentobarbital (Ziemba and Forman, 2016), and 2 for all steroids (Bracamontes et al., 2011; Hosie et al., 2006). The parameter L is a measure of background activity. For wild-type concatemeric receptors in the absence of additional agonists, L, calculated as (1-$P_{open,const}$) / $P_{open,const}$, was held at 8000 (Akk et al., 2018). To analyze the steroid concentration-response data recorded in the wild-type concatemeric receptor in the presence of a low concentration of GABA, L was

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constrained to (1-$P_{open,GABA}$) / $P_{open,GABA}$. For receptors containing the α1(L263S) or β2(Y143W+M286W) mutations L was estimated experimentally as (1- $P_{open,const}$) / $P_{open,const}$. Curve-fitting was carried out using Origin v. 7.5 (OriginLab, Northhampton, MA) on averaged data obtained from at least 5 cells.

Experimental and predicted responses to agonist combinations

In experiments involving measurements of responses to agonist combinations, the cells were first exposed to each agonist separately, followed by the application of the combination. Additionally, each cell was exposed to 3 mM GABA + 100 μM pentobarbital (wild-type concatemeric receptors), 10 μM GABA (receptors containing the α1(L263S) mutation), or 300 μM GABA (receptors containing the β2(Y143W+M286W) mutations), that generated a response with P_{open} that was considered to be indistinguishable from 1 (Chang and Weiss, 1999; Shin et al., 2018; Ziemba and Forman, 2016). Activation of the wild-type concatemeric receptor by steroid combinations was recorded in the presence of a low concentration of GABA. In this case, the cells were initially exposed to GABA and combinations of GABA plus a single steroid. This was followed by application of GABA plus both steroids.

The predicted peak responses to agonist combinations were calculated using three models. First, a prediction was made assuming energetic additivity, i.e., that each agonist in the combination interacts with a distinct set of binding sites. The activation scheme for two agonists interacting with distinct sites is given in Fig. 1B. To calculate the predicted peak responses, we employed Eq. 1 using K_X and c_X for the primary agonist and constrained L to the value calculated from the direct activating effect of the potentiator as (1-$P_{open,potentiator}$) / $P_{open,potentiator}$. There are no objective criteria to designate one agonist in the pair as primary and the other as potentiator. In combinations that involved GABA as one of the agonists, we named GABA as primary. In other cases, we arbitrarily assigned one agonist as primary.

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Second, predictions were made assuming that the paired agonists compete for common binding sites (Fig. 1C). The predicted peak responses in this model were calculated using Eq. 2:

$$P_{open} = \frac{1}{1+L\times\left[\frac{1+[X]/K_X+[Y]/K_Y}{1+[X]/(K_X c_X)+[Y]/(K_Y c_Y)}\right]^N} \quad \text{Eq. 2}$$

where X and Y are the two agonists, N is the number of shared sites, K_X and K_Y are the equilibrium dissociation constants for X and Y in the closed receptor, and c_X and c_Y are the ratios of the equilibrium dissociation constants for X and Y in the open receptor to K_X and K_Y, respectively. We note that this approach could only be employed when the number of binding sites was the same for each agonist in the pair. For example, this approach was not used when analyzing interactions of GABA (N_{GABA} = 2) with propofol ($N_{Propofol}$ = 6).

We also explored a situation where where one agonist interacts with a subset of binding sites available to the other compound. In this case, the interaction is a mixture of competition and energetic additivity. To test this scenario, the response predictions were made using Eq. 3:

$$P_{open} = \frac{1}{1+L\times\left[\frac{1+[X]/K_{X,I}+[Y]/K_{Y,I}}{1+[X]/(K_{X,I}c_{X,I})+[Y]/(K_{Y,I}c_{Y,I})}\right]^{N_I}\left[\frac{1+[X]/K_{X,II}}{1+[X]/(K_{X,II}c_{X,II})}\right]^{N_{II}}} \quad \text{Eq. 3}$$

In this model, agonist X binds to class I and class II sites whereas agonist Y binds only to class I sites. The terms in Eq. 3 are as defined earlier.

In cases where the predictions could be made using the distinct (Eq. 1) and same site (Eq. 2) models, the observed values of P_{open} were compared to predicted P_{open} by calculating the log likelihood ratio (LLR) as follows (Burnham et al., 2011):

$$LLR = -\left(\frac{n}{2}\right)\ln\left(\frac{RSS_{Model\ 1}}{n}\right)+\left(\frac{n}{2}\right)\ln\left(\frac{RSS_{Model\ 2}}{n}\right) \quad \text{Eq. 4}$$

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where n is the number of cells for the condition, RSS is the residual sum of squares, and Model 1 and Model 2 describe models assuming distinct and same sites, respectively, for the paired agonists. The likelihood ratio ($LR=e^{LLR}$) is reported in Tables 2 and 3 as a gauge of how much one model is more likely than the other to describe the data. Additionally, we report in Table 2 and 3 the values of the parameter Δ calculated as:

$$\Delta = n \ln\left(\frac{RSS_{Model\ 1}}{n}\right) - n \ln\left(\frac{RSS_{Model\ 2}}{n}\right) \quad \text{Eq. 5}$$

where Model 1 stands for the model with lower likelihood and Model 2 for the model with higher likelihood. The value of Δ thus calculated is interpretable with regard to empirical support for a model. Models with a Δ of up to 2 are considered to have substantial support, models with a Δ of 4-7 considerably less support, and those with Δ of >10 essentially no empirical support (Burnham and Anderson, 2004).

In cases where the paired agonists were expected to act through a different number of distinct binding sites (GABA + propofol, alfaxalone + propofol) and only a prediction for P_{open} for the model with distinct sites could be made, the predicted and observed P_{open} values were compared using the paired t-test. The results are reported in text.

Materials and chemicals

The inorganic salts used in ND96, GABA, β-alanine, P4S, pentobarbital, and picrotoxin were purchased from Sigma-Aldrich (St. Louis, MO). Propofol was purchased from MP Biomedicals (Solon, OH). The steroids (alfaxalone, 3α5αP, 3α5βP, and etiocholanolone) were bought from Sigma-Aldrich or Tocris (Bio-Techne, Minneapolis, MN). The enantiomer of 3α5βP was synthesized as described previously (Nilsson et al., 1998).

The stock solution of GABA was made in ND96 at 500 mM, stored in aliquots at -20°C, and diluted as needed on the day of experiment. The stock solutions of β-alanine and P4S were made on

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the day of experiment in ND96 at 100 mM and 5 mM, respectively, and further diluted immediately before experiment. Stock solutions of propofol (200 mM in DMSO) and pentobarbital (5 mM in bath solution) were stored at room temperature. The steroids were dissolved in DMSO at 10-50 mM and stored at room temperature. The agonist solutions were pH-adjusted when needed.

The highest final DMSO concentration in working solutions was 0.1%. We have previously found that DMSO at up to 0.5% is without effect on holding current or peak amplitude of the response to an EC_{50} concentration of GABA from oocytes expressing the closely-related α1β3γ2L receptors (Germann et al., 2016).

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RESULTS

$GABA_A$ receptor activation by orthosteric and allosteric agonists

We commenced by examining activation of the concatemeric $GABA_A$ receptor by several orthosteric (P4S and β-alanine) and allosteric (pentobarbital, and the steroids alfaxalone, 3α5αP, 3α5βP, *ent*-3α5βP, and etiocholanolone) activators. Receptor function was recorded at 6-9 concentration points from at least 5 cells for each agonist.

The wild-type $GABA_A$ receptor is only weakly activated by neuroactive steroids. To obtain robust current responses, the properties of steroids were studied in the presence of ~EC_{10} GABA and/or in receptors containing the gain-of-function α1(L263S) mutation. Sample current traces are given in Fig. 2.

The activation properties of each agent were determined by fitting Eq. 1 to the concentration-response data. The concentration-response curves are shown in Fig. 3, and the fitting results are summarized in Table 1.

Coapplication of an allosteric agonist with the transmitter GABA

Coapplication of an allosteric agonist, such as propofol or pentobarbital, enhances the peak current response to GABA. In the co-agonist model, description of receptor activity in the presence of an agonist combination does not require that there is specific interaction between the agonists; the potentiating effect is explained by each active compound independently and additively contributing free energy to stabilization of the open state (Ruesch et al., 2012; Shin et al., 2018; Ziemba and Forman, 2016). Potentiation can also be viewed as the change in receptor activation by the primary agonist due to reduction in L (increase in background activity) resulting from the direct activating effect of the potentiator.

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To illustrate receptor potentiation by a combination of GABA and an allosteric agonist, we coapplied propofol or pentobarbital with GABA. The experimental peak responses to the combination were compared with the predicted peak responses, which were calculated assuming independent and additive energetic contributions by each agonist (Eq. 1). Each cell was exposed to low concentrations of GABA, propofol (or pentobarbital), and the combination of GABA with propofol (or pentobarbital). The cells were also exposed to the combination of saturating (3 mM) GABA + 100 μM pentobarbital to generate a response with the estimated P_{open} of 1 (Ziemba and Forman, 2016), which was used as the reference response to which the responses to single agonists and agonist combinations from that cell were compared.

The application of 1.5 μM GABA generated a response with a P_{open} of 0.013 ± 0.007 (n = 9 cells). In the same set of cells, the application of 15 μM propofol generated a response with a P_{open} of 0.011 ± 0.004. The combination of GABA with propofol produced a response that had a P_{open} of 0.47 ± 0.19.

To predict the peak response to GABA + propofol, we first calculated the modified L (see Materials and Methods) from the direct activating response to propofol (modified L=(1-0.011)/0.011=89.9). We then calculated, using Eq. 1, the response to GABA employing the modified L, and the K_{GABA} and c_{GABA} values given in Table 1. This approach produced a predicted P_{open} of 0.48 ± 0.24 (mean ± S.D. for predictions made for each of the 9 cells individually) for the combination. The experimental (0.47 ± 0.19) and predicted (0.48 ± 0.24) P_{open} values are not different (p=0.78; paired t-test).

Conversely, we calculated the value for modified L for the response to GABA (modified L=(1-0.013)/0.013=75.9) and then determined the response to propofol employing the modified L of 75.9, and the $K_{Propofol}$ and $c_{Propofol}$ values in Table 1. It is not crucial whether receptor activation by GABA is estimated on the background of propofol-elicited activity or activation by propofol is estimated on the background of GABA-elicited activity. In the co-agonist model, either compound can be considered to enhance the background activity upon which the response to the other agonist is measured. As expected, both approaches produced identical results (predicted P_{open} = 0.48). From these

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experiments we infer that the actions of GABA and propofol can be described through energetic additivity, and that the two agonists act on the $GABA_A$ receptor through distinct binding sites. Both inferences are in agreement with prior reports (O'Shea et al., 2000; Ruesch et al., 2012; Shin et al., 2018). We did not test the model in which GABA (N_{GABA}=2) and propofol ($N_{Propofol}$=6) share some of the binding sites.

In this analysis, the nominal concentrations of agonists were adjusted for each oocyte to account for day-to-day variability and to reflect the actual, observed peak amplitudes. This was done by matching the experimental peak amplitude with the concentration-response data given in Fig. 3 and Table 1. Thus, the prediction of the response to an agonist combination is based on the responses to the individual agonists at their observed P_{open} values rather than at their nominal concentrations. In this experiment, the mean adjusted concentrations were 2.2 ± 0.8 μM for GABA (nominal concentration 1.5 μM) and 9.4 ± 2.1 μM for propofol (nominal concentration 15 μM). The reasons for variability are not fully clear to us but may include errors in preparation of solutions, differences in levels of endogenous modulators, and/or slow rundown or hysteresis in the concentration-response measurements. The mean adjusted concentrations for each agonist are provided in Table 2.

Coapplication of pentobarbital (P_{open} = 0.013 ± 0.013; n = 6) with GABA (P_{open} = 0.021 ± 0.016) generated a response with the mean peak P_{open} of 0.63 ± 0.11. The predicted P_{open} for the combination, assuming distinct binding sites, was 0.53 ± 0.25. Both GABA and pentobarbital were postulated to bind to two sites, so the predicted P_{open} could also be calculated using a model in which GABA and pentobarbital interact with the same sites (Eq. 2; 0.052 ± 0.043). The comparative ability of the two models to describe the observed response to the combined application was assessed by computing the likelihood ratio (see Materials and Methods). As shown in Table 2, the distinct site model was estimated to be about 1209-fold more likely.

Coapplication of 1 μM alfaxalone, that by itself generated a response with the mean P_{open} of 0.0007 ± 0.0005 (n = 6), with GABA (P_{open} = 0.087 ± 0.021) produced a response with the mean peak P_{open} of 0.43 ± 0.19. The predicted P_{open} for the pair assuming independent sites for GABA and

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alfaxalone was 0.33 ± 0.19, and assuming that the same sites mediate the actions of the two agonists 0.060 ± 0.017. In this case the likelihood ratio was about 39-fold for the ability of the distinct site model over the same site model to describe the observations. We infer that GABA and pentobarbital, and GABA and alfaxalone act independently and energetically additively to stabilize the open channel. These findings are in agreement with prior reports (Shin et al., 2017; Ziemba and Forman, 2016). The data are summarized in Fig. 4 and Table 2.

Coapplication of an orthosteric agonist with the transmitter GABA

The data above showed that combination of an allosteric agonist with GABA results in potentiation of the current response. Receptor behavior is fundamentally different when two agonists acting at the orthosteric sites are coapplied. In particular, the effect of coapplication depends on whether the paired compounds have similar or different gating efficacies, and on the concentration of each agent.

Using Eq. 2, we modeled the effect of coapplication of GABA with the low-efficacy orthosteric agonist P4S. The simulations were done at four concentrations of GABA, selected to elicit responses with P_{open} of 0.05, 0.1, 0.2, or 0.5. The underlying assumption was that GABA and P4S act at the same sites (Krogsgaard-Larsen et al., 1980). The simulations (Fig. 5A) show that at low concentrations of GABA, coapplication with P4S generates a larger response than when either agonist is applied alone. As the concentration of P4S is increased and P4S outcompetes GABA at the transmitter binding site, the P_{open} of the response to the combination approaches that of saturating P4S. At higher transmitter concentrations, when the response to GABA is greater than the response to saturating P4S, the latter acts as a competitive inhibitor at all concentrations (Fig. 5A).

GABA and β-alanine have similar maximal P_{open} (Fig. 3, Table 1). Coapplication of β-alanine is predicted to lead to potentiation of GABA-activated receptors (Fig. 5B). At saturating concentrations of β-alanine, the response to GABA + β-alanine reaches the maximal open probability for β-alanine.

We tested experimentally receptor activation in the presence of the agonist pairs of GABA + P4S

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and GABA + β-alanine. Coapplication of P4S (P_{open} = 0.049 ± 0.009; n = 5) with GABA (P_{open} = 0.034 ± 0.004) resulted in a response with the mean peak P_{open} of 0.059 ± 0.010. Coapplication of β-alanine (P_{open} = 0.024 ± 0.004; n = 5 cells) with GABA (P_{open} = 0.021 ± 0.002) generated a response with the mean peak P_{open} of 0.054 ± 0.011. The predictions for the combinations were done using two models. In the model in which each agonist interacts with distinct sites (Eq. 1), the predicted P_{open} for GABA + P4S and GABA + β-alanine were 0.93 ± 0.02 and 0.80 ± 0.04, respectively. In the model assuming that the agonists bind to the same set of sites (Eq. 2), the predicted P_{open} was 0.085 ± 0.009 for GABA + P4S, and 0.074 ± 0.010 for GABA + β-alanine. Thus, the assumption of shared binding sites resulted in predicted P_{open} values close to the experimental values. The likelihood ratios indicated that the same site model was >10^6 times more likely to describe the data. A summary of the data is provided in Fig. 4 and Table 2.

Coapplication of two allosteric agonists that interact with the same binding sites

Receptor behavior is similar in principle when two allosteric agonists that interact with the same sites are coapplied. We tested receptor activation by several combinations of structurally related steroids, for which it was assumed that the same sites mediate their effects on the $GABA_A$ receptor (Hosie et al., 2006; Miller et al., 2017). The experiments were conducted in the presence of a low concentration of GABA or on receptors containing the gain-of-function α1(L263S) mutation. Both approaches increase background activity enabling studies of weak activators such as neuroactive steroids (Akk et al., 2018).

The steroid pair of alfaxalone + 3α5αP was tested on the wild-type concatemeric receptor in the presence of 2 μM GABA. Application of GABA elicited a response with the mean P_{open} of 0.012 ± 0.005 (n = 6). Coapplication of 0.5 μM alfaxalone with GABA generated a response with the mean P_{open} of 0.032 ± 0.016. When GABA was combined with 0.2 μM 3α5αP the mean P_{open} was 0.034 ± 0.014. Coapplication of both steroids with GABA generated a response with the mean P_{open} of 0.053 ±

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0.022. The predicted P_{open} of the response to GABA + alfaxalone + 3α5αP using a model with two shared binding sites (Eq. 2) for the steroids is 0.053 ± 0.024. Using a model with distinct sites for alfaxalone and 3α5αP, the predicted P_{open} was 0.09 ± 0.04. The likelihood ratio indicated that the same site model was about 40,000-fold more likely. We infer that alfaxalone and 3α5αP interact with the same sites.

We next examined potentiation of GABA-activated receptors by the combination of 3α5αP + 3α5βP. The peak responses in the presence of GABA and 0.3 μM 3α5αP or 3α5βP had mean P_{open} of 0.052 ± 0.011 (n = 6) and 0.044 ± 0.010, respectively. The mean P_{open} in the presence of GABA + 3α5αP + 3α5βP was 0.074 ± 0.013. The predicted P_{open} assuming shared sites was 0.071 ± 0.014. For comparison, the predicted P_{open} assuming unique sites for 3α5αP and 3α5βP was 0.18 ± 0.06. The likelihood ratio indicated that the same site model was $>10^6$ times more likely. Our conclusion that 3α5αP and 3α5βP interact with the same sites is in agreement with prior data (Hosie et al., 2006; Miller et al., 2017).

We also examined potentiation of GABA-activated receptors by the combination of the natural steroid 3α5βP and its enantiomer (*ent*-3α5βP). Coapplication of 0.3 μM 3α5βP with GABA increased the P_{open} from 0.025 ± 0.005 (n = 6) to 0.10 ± 0.02. In the presence of GABA + *ent*-3α5βP, the P_{open} was 0.12 ± 0.03, and in the presence of GABA + 3α5βP + *ent*-3α5βP 0.17 ± 0.03. The predicted P_{open} was 0.16 ± 0.03 using the model in which 3α5βP and *ent*-3α5βP bind to the same sites, and 0.36 ± 0.08 with the model with unique sites for the two steroids. The likelihood ratio indicated that the same site model was $>10^6$ times more likely. We infer that 3α5βP and *ent*-3α5βP act on the $GABA_A$ receptor through the same sites.

Finally, we tested direct activation of the βα(L263S)γ + βα(L263S) receptor by the steroid combination alfaxalone + etiocholanolone. Previous studies of single-channel kinetics had suggested that etiocholanolone did not interact with all sites occupied by a more efficacious steroid, such as 3α5βP or alfaxalone (Li et al., 2007). This experiment was conducted in the absence of GABA. The gain-of-function mutation increases unliganded activity and enables studies of weak agonists

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($P_{open,const}$ = 0.11; (Akk et al., 2018)).

Exposure of the mutant receptor to alfaxalone or etiocholanolone produced responses with the mean peak P_{open} of 0.18 ± 0.02 (n = 6) or 0.15 ± 0.01, respectively. Coapplication of alfaxalone and etiocholanolone generated a response with the mean P_{open} of 0.19 ± 0.01. The mean P_{open} predicted from the model with two shared sites for alfaxalone and etiocholanolone was 0.19 ± 0.01. Using a model with distinct binding sites for alfaxalone and etiocholanolone, the predicted P_{open} was 0.23 ± 0.03. The likelihood ratio indicated that the same site model was about 2000-fold more likely. The data are summarized in Fig. 6 and Table 2.

In sum, the actions of all tested steroid combinations (alfaxalone + 3α5αP, 3α5αP + 3α5βP, 3α5βP + *ent*-3α5βP, and alfaxalone + etiocholanolone) were best accounted for by a model where the paired steroids interacted with the same binding sites.

Coapplication of two allosteric agonists that interact with distinct binding sites

In the presence of two allosteric agonists with distinct, i.e., unshared binding sites, receptor behavior is similar to the situation where an allosteric agonist is coapplied with GABA. In this situation, either agonist can be considered to independently increase background activity and thereby promote activation by the other agonist.

We first examined receptor activation in the presence of alfaxalone and propofol, which are expected to act on the $GABA_A$ receptor through distinct sites (Nourmahnad et al., 2016) with N_{ALF}=2 and $N_{Propofol}$=6. The experiments were conducted both in the absence and presence of GABA. In the absence of GABA, the mean P_{open} was 0.0006 ± 0.0001 (n = 6) for alfaxalone and 0.10 ± 0.03 for propofol. Coapplication of alfaxalone with propofol generated a peak response with a P_{open} of 0.38 ± 0.03. Assuming independent actions of the two agonists, the predicted average open probability is 0.33 ± 0.10 (*p*=0.15; paired t-test).

The mean P_{open} in the presence of 3 μM GABA + 0.2 μM alfaxalone was 0.18 ± 0.09 (n = 6).

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Coapplication of 2 μM propofol with GABA + alfaxalone increased the mean P_{open} to 0.31 ± 0.12. Assuming independent actions of GABA, alfaxalone and propofol (Eq. 1), the predicted P_{open} for the triple drug combination is 0.37 ± 0.17 (p=0.09; paired t-test). Thus the data obtained for the alfaxalone + propofol combination support the previous finding of distinct sites for alfaxalone and propofol (Nourmahnad et al., 2016).

Analogously, we probed the effect of the combination of alfaxalone + pentobarbital. Exposure to alfaxalone elicited a peak response with the mean P_{open} of 0.0006 ± 0.0001 (n = 6). Exposure to pentobarbital generated a mean P_{open} of 0.057 ± 0.025. Coapplication of alfaxalone with pentobarbital produced responses with the mean P_{open} of 0.35 ± 0.09. The predicted P_{open} is 0.21 ± 0.10 assuming different sites for alfaxalone and pentobarbital, and 0.042 ± 0.018 assuming that the same sites mediate the actions. In this case, the likelihood ratio is 16 for the distinct sites over the same sites model.

The application of 3 μM GABA + 0.2 μM alfaxalone elicited a response with the mean P_{open} of 0.12 ± 0.04 (n = 7). Coapplication of 25 μM pentobarbital with GABA + alfaxalone generated a response with the mean P_{open} of 0.30 ± 0.13. Using the model with distinct binding sites for GABA, alfaxalone and pentobarbital, the predicted P_{open} for the triple drug combination was 0.28 ± 0.08. The mean P_{open} predicted using a model in which alfaxalone and pentobarbital interact with the same sites is 0.23 ± 0.07. The likelihood ratio is 228 for the distinct sites over the same sites model. A summary of the the data is provided in Fig. 6 and Table 2.

Coapplication of two allosteric agonists that interact with partially shared binding sites

In the models described above the two paired agonists were assumed to share all or none of the binding sites. As shown through modeling and experimental data, the two situations are associated with different levels of potentiation during coapplication. An extension of these models is one where one of the compounds interacts with a subset of the binding sites available to the other. In this

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mechanism, the effect is a mix of the agonists binding to distinct sites (energetic additivity) and competition (concentration additivity) at the shared sites.

We hypothesized that such a model describes the interaction between propofol and pentobarbital on the $GABA_A$ receptor. Photolabeling studies have shown that propofol and a barbiturate analogue bind with high affinity to overlapping sites at the α-β and γ-β interfaces near the β(M227) residue (Chiara et al., 2013). In addition, propofol binds to the β-α interface with high affinity (Jayakar et al., 2014; Nourmahnad et al., 2016). Thus, photolabeling experiments suggest that the αβγ $GABA_A$ receptor contains two common sites for propofol and pentobarbital in addition to distinct sites to which propofol binds.

To test this hypothesis, we exposed cells expressing wild-type concatemeric receptors to propofol, pentobarbital, or the combination of the two. In 7 cells, the mean P_{open} in the presence of propofol was 0.023 ± 0.015. The mean P_{open} in the presence of pentobarbital was 0.023 ± 0.019. Coapplication of the two drugs generated a peak response with the mean P_{open} of 0.29 ± 0.17.

The experimental data were compared with predicted P_{open} values calculated using two approaches. First, as the null hypothesis, we assumed that propofol and pentobarbital interact with distinct sets of sites and that the actions of the drugs are governed by energetic additivity. Such a model (Eq. 1) predicts a mean P_{open} of 0.67 ± 0.25 for propofol + pentobarbital. In the second approach (Eq. 3), we constrained the total number of propofol binding sites to 6 (Shin et al., 2018) and varied the number of sites shared with pentobarbital from 1 to 3. In this case, the activation curve for pentobarbital was fit with $N_{Pentobarbital}$ constrained to 1, 2 or 3 to obtain the appropriate values for K and c for pentobarbital. The predicted mean P_{open} for propofol + pentobarbital were 0.44 ± 0.26, 0.26 ± 0.18, and 0.18 ± 0.12 for 1, 2 and 3 shared sites, respectively. We infer that the combination of a total of 6 sites for propofol, of which 2 alternatively can bind pentobarbital adequately describes activation of the wild-type concatemeric receptor. The likelihood ratio indicated that this model of partially shared sites was $>10^5$ times more likely than the model with no shared sites.

Introduction of the β2(Y143W) and β2(M286W) mutations has been shown to reduce the number

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of functional binding sites for propofol so that concatemeric receptors containing the two mutations in each of the β subunits (a quadruple-mutant receptor) effectively retain only two propofol binding sites (Shin et al., 2018). These mutations are located in the β subunit or at the β-α interface where they are expected to disrupt the actions of propofol (Eaton et al., 2015; Franks, 2015; Shin et al., 2018). A change in the number of propofol binding sites can be expected to alter receptor activation by the propofol + pentobarbital combination in a predictable manner.

We tested activation of the β(Y143W+M286W)αγ + β(Y143W+M286W)α receptor by 5 μM propofol, 15 μM pentobarbital, and the combination of the two drugs. The mean P_{open} was 0.27 ± 0.06 (n = 6 cells) in the presence of propofol, and 0.27 ± 0.04 in the presence of pentobarbital. When propofol was coapplied with pentobarbital, the mean P_{open} was 0.36 ± 0.06. An activation model (Eq. 2) with 2 common binding sites for propofol and pentobarbital predicted a P_{open} of 0.37 ± 0.06. In contrast, a model where propofol and pentobarbital interact with distinct sites predicted a P_{open} of 0.49 ± 0.10 for the drug combination. The likelihood ratio indicated that the model in which two sites could bind either propofol or pentobarbital was $>10^4$ times more likely than the model in which the sites were distinct. The data are summarized in Table 3.

The predicted isobolograms for agonist combinations

The data shown above demonstrate that receptor activity in the presence of various agonist combinations can be markedly different when the compounds bind to the same vs. distinct binding sites. Specifically, the potentiating effect resulting from the addition of a second GABAergic drug is greater when the two drugs bind to distinct sites. This can have clinical implications when combination therapies are considered.

We have simulated isobolograms for situations where the transmitter is combined with another orthosteric agonist or an allosteric drug, and when two allosteric drugs are combined. The results (Fig. 7) demonstrate that the combination of two orthosteric agonists results in strict concentration

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additivity illustrated by the linear isobole of additivity. In contrast, the combination of GABA with the allosteric drug propofol generates a curvilinear isobole. This effect is mediated by energetic additivity that manifests as apparent synergy (Shin et al., 2017). Coapplication of propofol and pentobarbital also produces a curvilinear isobole, however, the curvature is less pronounced due to partial overlap of binding sites.

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DISCUSSION

The goal of this study was to compare activation of the $GABA_A$ receptor by various combinations of orthosteric and allosteric agonists. We were motivated by the fact that native $GABA_A$ receptors under physiological and clinical conditions can be exposed to multiple GABAergic drugs whose net action and interactions are not well understood. We examined receptor activity in the presence of combinations of orthosteric agonists (GABA + β-alanine, GABA + P4S), an orthosteric agonist + an allosteric agonist (GABA + propofol, GABA + steroid), and combinations of allosteric agents (steroid + steroid, propofol + pentobarbital). The experimental data were analyzed and compared with predictions made using variations of the co-agonist activation model. The major finding, in agreement with simulations based on the model, is that the degree of potentiation resulting from combining GABAergic compounds depends on whether the individual agonists bind to the same or distinct sites. Conversely, we propose that receptor activity in the presence of agonist combinations can be used to determine whether the compounds interact with the same or distinct sites.

The simulated and experimental data indicate that the degree of potentiation is greater when agonists interacting with distinct sites are combined than when agonists interacting with the same sites are combined. We have used the terms shared or common sites when a site can be occupied by either agent in the pair, and unique or distinct sites when only one agent in the pair can bind to a given site. Combination of agonists interacting with distinct sites underlies classic potentiation that is observed when, for example, propofol or a neuroactive steroid is combined with GABA. Receptor activity in the presence of such combinations can be predicted by summing energetic contributions of the individual agents. Classic potentiation manifests as synergy in isobolographic analysis of the effects of drug combinations (Shin et al., 2017). In contrast, the amplitude of the response to a combination of agonists interacting with the same sites depends on the relative efficacies and concentrations of the individual agents but is rarely larger than the sum of responses to either drug alone. Importantly, the response amplitude to agonist combination in all cases is a computable value.

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This approach can be used to assess whether two (or more) agents in a combination act through the same or distinct sites, as the responses predicted by models with different degrees of overlap of binding sites are in most cases well separated. By comparing the experimental and predicted responses we show that the same sites mediate the actions of a 5α-reduced steroid (3α5αP) and a 5β-reduced steroid (3α5βP), that is in agreement with previous structural and mutational data (Hosie et al., 2006; Miller et al., 2017). In addition, we show that a natural steroid (3α5βP) and its enantiomer (*ent*-3α5βP), and the weak steroid etiocholanolone (Li et al., 2007) and the strong steroid alfaxalone (Cao et al., 2018) act through the same binding sites. Finally, we propose that propofol and pentobarbital interact with partially shared sites. The electrophysiological data are best described by the receptor containing six sites for propofol (Shin et al., 2018), two of which can alternatively bind pentobarbital. Elimination of the four unique propofol sites through mutagenesis produced a receptor whose activity in the presence of propofol + pentobarbital was best described by the drugs competing for two common sites. These findings provide a functional confirmation to previous photolabeling data indicating shared binding sites for these anesthetics (Jayakar et al., 2014).

The concerted transition model (Fig. 1) postulates that all binding sites for a given agonist possess identical properties, i.e., K and c values. Within experimental error this has largely been proven true, that is apparent affinities within a factor of 2, when tested with mutations introduced to individual binding sites for GABA (Baumann et al., 2003), the steroid 3α5αP (Bracamontes et al., 2011), or propofol (Shin et al., 2018), although not for etomidate (Maldifassi et al., 2016). Anesthetic agents, including barbiturates, have been shown to interact with varying affinities at different intersubunit interfaces in photolabeling studies (Chiara et al., 2013; Jayakar et al., 2014), raising a possibility that the number of functionally apparent binding sites is dependent on the concentration of the drug. One implication of this, and a caveat to the data and conclusions in the present study, is that the apparent effect of coapplication of GABAergic anesthetics can be dependent on the concentrations of individual drugs.

The findings have potential clinical relevance as they can predict the efficacy of drug

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combinations in combination therapy. For example, propofol is predicted to be highly synergistic when combined with neuroactive steroids because the two classes of drugs interact with distinct sites. On the other hand, the combination of propofol with pentobarbital is expected to show less synergy, due to partial overlap between the binding sites. It has been shown previously that coapplication of the neuroactive steroid alfaxalone enhances the GABAergic effects of etomidate (Li et al., 2014). In the present work we find that the endogenous neurosteroid 3α5αP is a low efficacy agonist that interacts with the same sites as alfaxalone. Based on our analysis, we would predict that an increase in the concentration of endogenous 3α5αP would enhance the effects of etomidate in the absence of exogenous alfaxalone, but would actually reduce the ability of co-administered alfaxalone to enhance the sedative effect of etomidate. The extent of this effect would, of course, depend on the precise relationship between open probability of the $GABA_A$ receptor and behavior, which is not well understood at present.

In sum, we have shown that the co-agonist activation model predicts widely different magnitude of potentiation depending on whether the individual agonists in a combination interact with the same or distinct sites. The experimental data confirm previous views on the overlap of binding sites for specific GABAergic agents including several orthosteric agonists, intravenous anesthetics and steroids. We provide functional evidence that 5α- and 5β-reduced steroids, and natural and enantiomeric steroids interact with the same sites on the $GABA_A$ receptor, and support for the notion that propofol and barbiturates share some of their binding sites. We also propose that the approach can be exploited to determine whether a novel drug shares a binding site with a known drug in a combination.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Steinbach, Akk

Conducted experiments: Shin, Germann

Contributed new reagents or analytical tools: Covey

Performed data analysis: Shin, Germann, Steinbach, Akk

Wrote or contributed to the writing of the manuscript: Shin, Germann, Covey, Steinbach, Akk

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Footnotes

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Legends for Figures

Figure 1. The state diagram of the activation model. The receptor is exposed to a single agonist X (**A**), or to the combination of agonists X and Y that interact with distinct sites (**B**) or the same sites (**C**). Note that the front plane in panel B is the scheme in panel A, and that panel C is a subset of states shown in B (missing states indicated by gray color). The inactive states (R) are depicted on the bottom plane and active states (A) on the top plane. The equilibrium between the states is determined by the constants given next to the arrows. K_X is the equilibrium dissociation constant of the inactive receptor and c_XK_X is the equilibrium dissociation constant of the active receptor. L (=A/R) describes the equilibrium between the inactive and active states. Note that in panel B two inactive states (YRX and Y_2RX) are hidden and in panel C YRX is hidden.

Figure 2. Sample current traces in the presence of orthosteric or allosteric agonists, or combinations of agonists. (**A**) The wild-type concatemeric receptors were activated by 30 μM GABA, 100 μM propofol, 100 μM P4S, 3 mM β-alanine, or 1 mM pentobarbital. The concentrations were selected to generate approximately half-maximal responses for the given agonist. The amplitudes of the current responses are given in units of open probability for easier comparison of gating efficacy between the agonists. (**B**) The wild-type concatemeric receptors were activated by a low concentration of GABA (4-8 μM) in the absence (left trace in each pair) and presence of a steroid (1 μM alfaxalone, 0.3 μM 3α5αP, 0.3 μM 3α5βP, or 1 μM *ent*-3α5βP). The calibration bars apply to all traces in panel B. (**C**) The concatemeric βα(L263S)γ + βα(L263S) receptors were activated by 1 μM alfaxalone or 5 μM etiocholanolone. Note that these recordings were conducted in the absence of added GABA.

Figure 3. Activation properties of GABAergic agonists. Estimated open probability (P_{open}) of the concatemeric β2α1γ2L + β2α1 $GABA_A$ receptor is given as a function of concentration of GABA,

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propofol (PRO), P4S, β-alanine (β-Ala), pentobarbital (PEB), or the steroids alfaxalone (ALF), 3α5αP, 3α5βP, *ent*-3α5βP, and etiocholanolone (Etio). The data points and error bars show mean ± S.D. from 5-8 cells. The curves were generated by fitting Eq. 1 to the P_{open} data (see Materials and Methods). The fitted values of K and c are provided in Table 1. The data for GABA (dashed line) and propofol (dotted line) are from prior reports (Akk et al., 2018; Shin et al., 2018). The effects of alfaxalone, 3α5αP, 3α5βP and *ent*-3α5βP on the wild-type concatemeric receptor were obtained in the presence of a low concentration of GABA that generated a background response with P_{open} of ~0.1 in wild-type concatemeric receptors. Introduction of the α1(L263S) mutation increases the constitutive open probability and mimics the presence of GABA. A receptor containing the α1(L263S) mutation in both concatemeric constructs was used to determine the activation properties of alfaxalone and etiocholanolone.

Figure 4. Coapplication of GABA with an allosteric or orthosteric agonist. The experimental and predicted P_{open} are given for combinations of GABA with propofol (PRO), pentobarbital (PEB), alfaxalone (ALF), P4S, or β-alanine (β-Ala). The open circles show experimental data from each cell separately. The open triangles and squares show predictions based on models assuming distinct or same sites for the paired compounds. The filled symbols and error bars shown mean ± S.D. for each condition. The data and results of statistical analyses are summarized in Table 2. Prediction with same site model was not done for the GABA + PRO combination because of a difference in the number of postulated binding sites.

Figure 5. Predicted responses to coapplication of two orthosteric agonists. The effects of coapplication of GABA with a low-efficacy agonist, P4S (**A**) or a high-efficacy agonist, β-alanine (**B**) on receptor open probability. The concentration-response data for P4S and β-alanine in the absence of GABA are shown as open circles. The concentration of GABA was held at 5.0 μM, 7.6 μM, 12.2 μM, or 29.8 μM, to elicit responses with a P_{open} of 0.05, 0.1, 0.2, and 0.5, respectively (filled circles,

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bottom to top in the graphs). The simulations were done using Eq. 2 and the parameters provided in Table 1. The approach assumes that two shared sites mediate the actions of GABA and P4S, and GABA and β-alanine.

Figure 6. Coapplication of allosteric agonists. The experimental and predicted P_{open} are given for the steroid combinations of alfaxalone (ALF) + 3α5αP, 3α5αP + 3α5βP, 3α5βP + ent-3α5βP, alfaxalone + etiocholanolone (Etio), and the combinations of alfaxalone with propofol (PRO) or pentobarbital (PEB). The open circles show experimental data from each cell separately. The open triangles and squares show predictions based on models assuming distinct or same sites for the paired compounds. The filled symbols and error bars shown mean ± S.D. for each condition. The experimental conditions, data, and results of statistical analyses are summarized in Table 2. Prediction with same site model was not done for the ALF + PRO combination because of a difference in the number of postulated binding sites.

Figure 7. Predicted isobolograms for agonist combinations. The combination of GABA with β-alanine (**A**) or P4S (**B**) produces linear isoboles of additivity, indicative of concentration additivity. The combination of GABA with the allosteric agonist propofol (**C**) produces a highly curvilinear isobole. The combination of pentobarbital and propofol (**D**) also generates a curvilinear isobole but with reduced curvature because of partial overlap between the binding sites for pentobarbital and propofol. The dashed lines in (C) and (D) show hypothetical linear isoboles that would be observed if the paired compounds interacted with the same sites on the receptor. The isobolograms were calculated for the target P_{open} of 0.15 in all panels.

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Table 1. Properties of GABAergic agonists.

Receptor	Agonist	K_X (μM)	c_X	N_X	Gating energy (kcal/mol)	$P_{open,max}$
wild-type	GABA*	72 ± 15	0.003 ± 0.000	2	-6.74	0.92
wild-type	P4S	38± 4	0.027 ± 0.000	2	-4.26	0.15
wild-type	β-alanine	6664 ± 2947	0.002 ± 0.001	2	-7.17	0.96
wild-type	propofol*	21 ± 3	0.222 ± 0.003	6	-5.33	0.51
wild-type	pentobarbital	1912 ± 1690	0.004 ± 0.002	2	-6.52	0.89
wild-type	alfaxalone[#]	2.3 ± 0.3	0.159 ± 0.009	2	-2.17	0.005
wild-type	3α5αP[#]	0.27 ± 0.07	0.233 ± 0.018	2	-1.72	0.002
wild-type	3α5βP[#]	0.45 ± 0.06	0.265 ± 0.010	2	-1.57	0.002
wild-type	*ent*-3α5βP[#]	2.4 ± 0.4	0.166 ± 0.012	2	-2.12	0.005
α1(L263S)	alfaxalone	3.0 ± 0.3	0.130 ± 0.006	2	-2.41	0.88
α1(L263S)	etiocholanolone	11.1 ± 1.5	0.685 ± 0.009	2	-0.45	0.21

A summary of the activation properties of the GABAergic agonists employed in the study. Wild-type is the ternary $GABA_A$ receptor consisting of βαγ + βα concatemeric constructs. The α1(L263S) is the receptor consisting of βα(L263S)γ and βα(L263S) concatemeric constructs. K_X is the equilibrium dissociation constant of the closed receptor for a given agonist. The parameter c_X gives the ratio of

the dissociation constants of the open receptor to that of the closed receptor. N_X is the number of binding sites for the agonist. Gating energy was calculated as $N_X RT \times \ln(c_X)$. The maximal predicted open probability ($P_{open,max}$) was calculated as $1/(1+Lc_X^N)$ with L held at 8000 for the wild-type receptor (Akk et al., 2018) and 8.1 for the mutant receptor (Shin et al., 2018). *The data for GABA and propofol are from prior reports (Akk et al., 2018; Shin et al., 2018). #The wild-type concatemeric receptor is only weakly activated by neuroactive steroids. Accordingly, the properties of the steroids alfaxalone, 3α5αP, 3α5βP, and *ent*-3α5βP were determined in the presence of a low concentration (~EC_{10}) of GABA.

Table 2. Effects of agonist combinations on open probability.

Agonist 1 (adj. conc.)	Agonist 2 (adj. conc)	$P_{open,agonist\ 1}$	$P_{open,agonist\ 2}$	$P_{open,agonist\ 1+agonist\ 2}$	Predicted P_{open} (same sites)	Predicted P_{open} (distinct sites)	LR	Δ
GABA (2.2 μM)	propofol (9.4 μM)	0.013 ± 0.007	0.011 ± 0.004	0.47 ± 0.19	N/A	0.48 ± 0.24	N/A	N/A
GABA (2.8 μM)	pentobarbital (67 μM)	0.021 ± 0.016	0.013 ± 0.013	0.63 ± 0.11	0.052 ± 0.043	0.53 ± 0.25	1209 ($8.3x10^{-4}$)	14
GABA (6.8 μM)	alfaxalone (0.97 μM)	0.083 ± 0.022	0.0008 ± 0.0005	0.36 ± 0.05	0.054 ± 0.011	0.35 ± 0.21	39 (0.026)	7
GABA (4.0 μM)	P4S (45 μM)	0.034 ± 0.004	0.049 ± 0.009	0.059 ± 0.010	0.085 ± 0.009	0.93 ± 0.02	$<10^{-6}$ ($>10^{6}$)	35
GABA (3.0 μM)	β-alanine (206 μM)	0.021 ± 0.002	0.024 ± 0.004	0.054 ± 0.011	0.074 ± 0.010	0.80 ± 0.04	$<10^{-6}$ ($>10^{6}$)	36
alfaxalone[#] (0.29 μM)	3α5αP[#] (0.07 μM)	0.032 ± 0.16	0.034 ± 0.014	0.053 ± 0.022	0.053 ± 0.024	0.09 ± 0.04	$2.5x10^{-5}$ (39883)	21
3α5αP[#] (0.17 μM)	3α5βP[#] (0.26 μM)	0.052 ± 0.011	0.044 ± 0.010	0.074 ± 0.013	0.071 ± 0.014	0.18 ± 0.06	$<10^{-6}$ ($>10^{6}$)	37
3α5βP[#] (0.28 μM)	*ent*-3α5βP[#] (0.80 μM)	0.10 ± 0.02	0.12 ± 0.03	0.17 ± 0.03	0.16 ± 0.03	0.36 ± 0.08	$<10^{-6}$ ($>10^{6}$)	30
alfaxalone[&] (0.15 μM)	etiocholanolone[&] (7.5 μM)	0.18 ± 0.02	0.15 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.23 ± 0.03	$4.7x10^{-4}$ (2134)	15

alfaxalone[#] (0.20 μM)	propofol[#] (1.2 μM)	0.18 ± 0.09	0.23 ± 0.12	0.31 ± 0.12	N/A	0.37 ± 0.17	N/A	N/A
alfaxalone (0.63 μM)	propofol (31 μM)	0.0006 ± 0.0001	0.10 ± 0.03	0.38 ± 0.03	N/A	0.33 ± 0.10	N/A	N/A
alfaxalone[#] (0.14 μM)	pentobarbital[#] (5.3 μM)	0.12 ± 0.04	0.19 ± 0.07	0.30 ± 0.13	0.23 ± 0.07	0.28 ± 0.08	16.4 (0.061)	6
alfaxalone (0.63 μM)	pentobarbital (172 μM)	0.0006 ± 0.0001	0.057 ± 0.025	0.35 ± 0.09	0.042 ± 0.018	0.21 ± 0.10	228 (0.0044)	11

The columns give the paired agonists (Agonist 1 and Agonist 2) and adjusted concentrations, open probabilities for each agonist separately ($P_{open,agonist\ 1}$ and $P_{open,agonist\ 2}$) or combined ($P_{open,\ agonist\ 1+agonist\ 2}$), the predicted P_{open} assuming the paired agonists act through the same or distinct sites. The column labeled LR shows the likelihood ratio ($LR=e^{LLR}$) that quantifies by how many times more likely is the model with distinct sites than the model with same sites to describe the data (and in parentheses the inverse of that likelihood ratio). The value of the parameter Δ (see Materials and Methods) indicates the empirical support for the lower ranked model. Values of Δ of 4-7 are considered to indicate little empirical support and values of >10 essentially no support (Burnham and Anderson, 2004). The data show mean ± S.D. from 5-9 cells at each condition. The nominal concentrations of agonists are provided in the text. The predicted P_{open} were not calculated for GABA + propofol and alfaxalone + propofol (N/A) using a model assuming the same sites because the number of binding sites is different for the agents in both pairs. [#]The experiments with alfaxalone + 3α5αP, 3α5αP + 3α5βP, and 3α5βP + ent-3α5βP, and sets of experiments with alfaxalone + propofol, and alfaxalone + pentobarbital were

conducted in the presence of a low concentration of GABA. [&]The experiments with alfaxalone + etiocholanolone were conducted on receptors containing the gain-of-function α1(L263S) mutation in both α subunits.

Table 3. Effects of combinations of propofol and pentobarbital on open probability.

Receptor	N_{PRO}	$N_{PRO\ or\ PEB}$	$P_{open,PRO}$	$P_{open,PEB}$	$P_{open,PRO+PB}$	Predicted P_{open} (shared sites)	Predicted P_{open} (distinct sites)
βαγ + βα	6	2	0.023 ± 0.015	0.023 ± 0.019	0.29 ± 0.17	0.26 ± 0.18	0.67 ± 0.25
β(Y143W+M286W)αγ + β(Y143W+M286W)α	2	2	0.27 ± 0.06	0.27 ± 0.04	0.36 ± 0.06	0.37 ± 0.06	0.49 ± 0.10

The columns give the type of receptor, the total number of propofol (PRO) sites, the number of sites that can bind either propofol or pentobarbital (PEB), the open probabilities in the presence of propofol, pentobarbital, or propofol + pentobarbital, the open probability calculated for the specified number of shared sites, and the open probability calculated assuming that propofol and pentobarbital bind to distinct sites. The number of cells was 7 for wild-type concatemers and 6 for the mutant receptor. The model with shared sites was more likely, by $>10^5$- and 10^4-fold respectively, than the model with distinct sites to describe the data for the wild-type concatemeric and mutant receptors. The Δ values (see Materials and Methods) for the lower ranked model were 27 and 19 for the wild-type and mutant concatemeric receptor, respectively.

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Figure 1

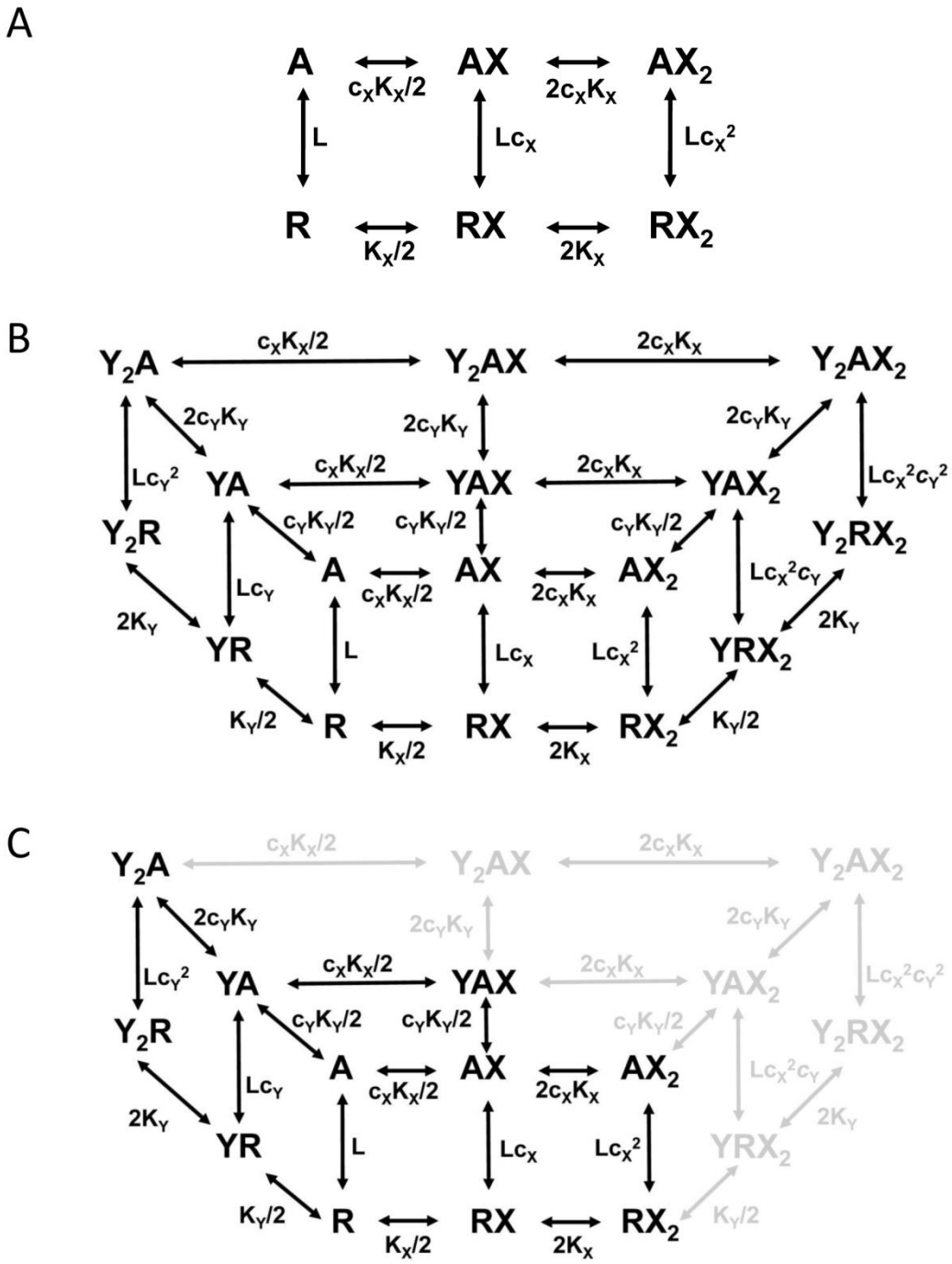

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Figure 2

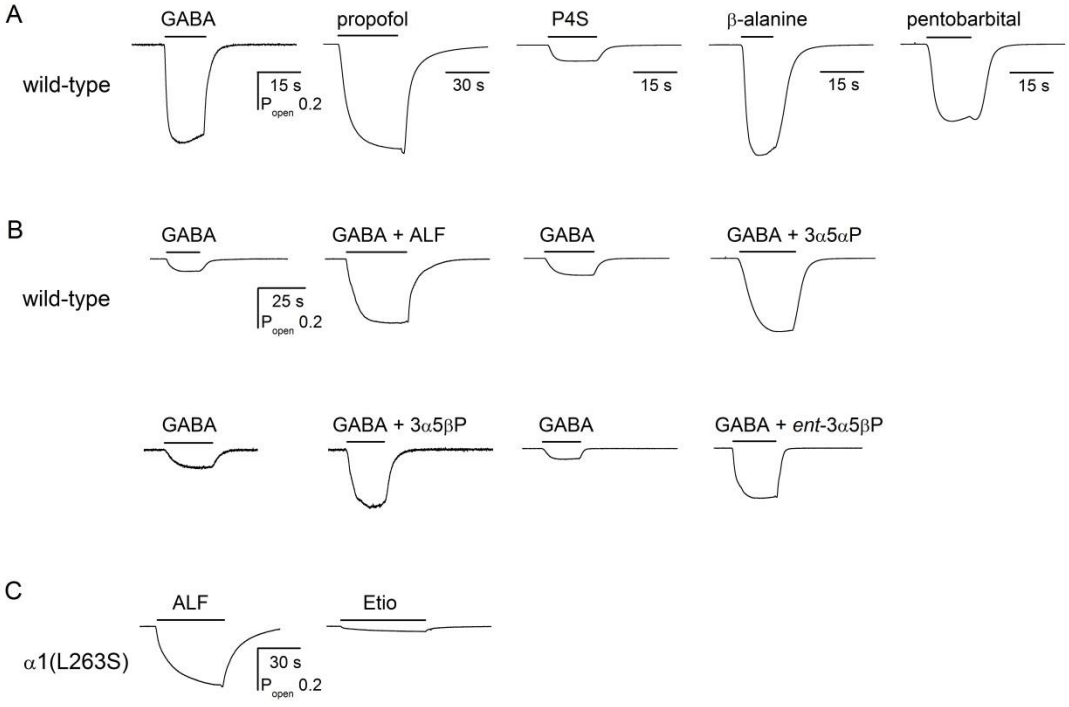

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Figure 3

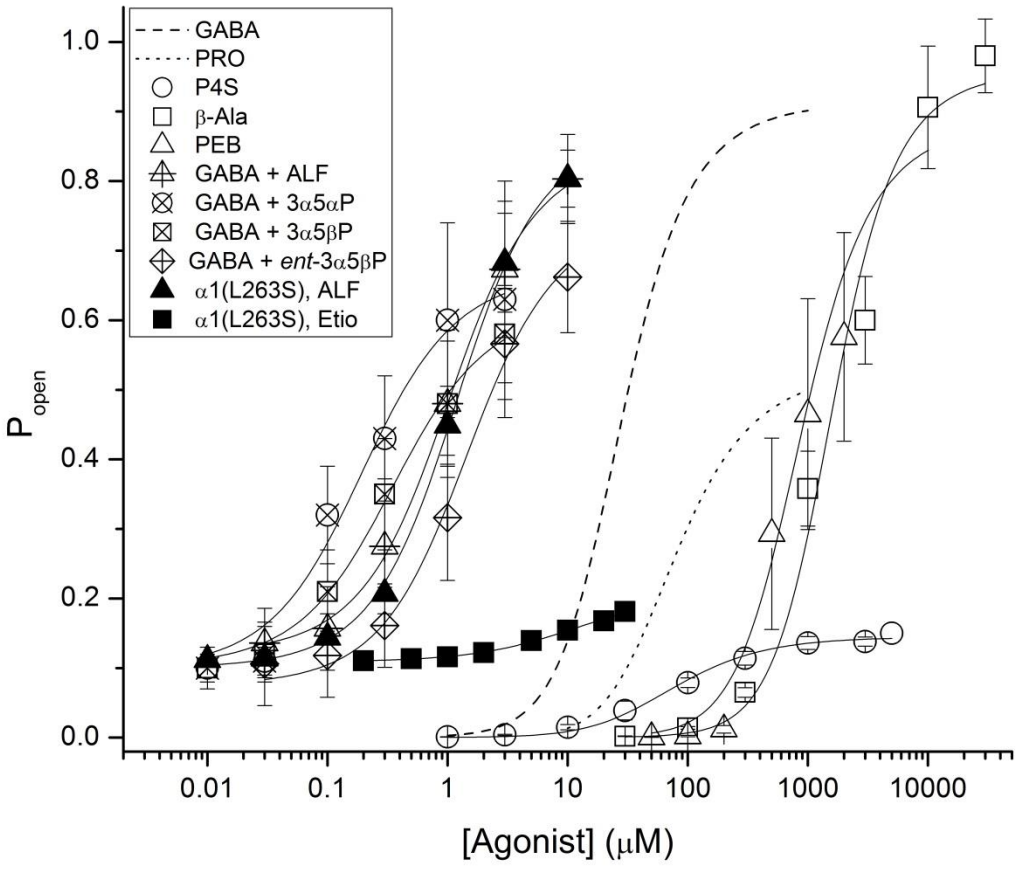

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Figure 4

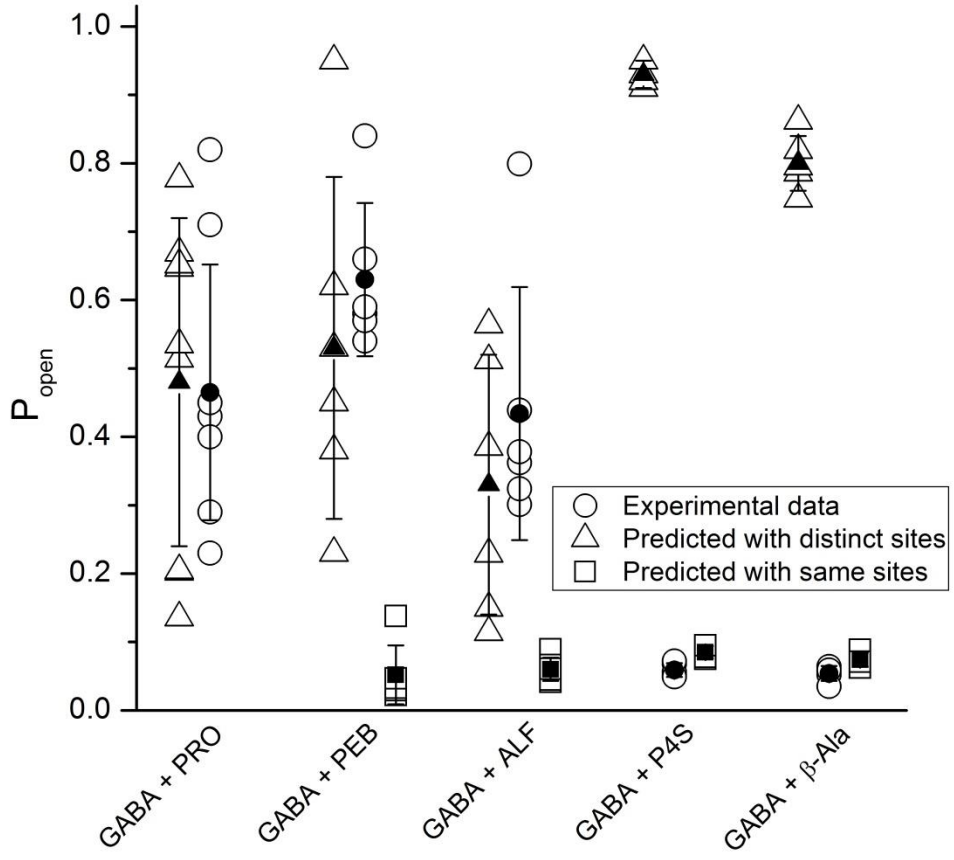

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Figure 5

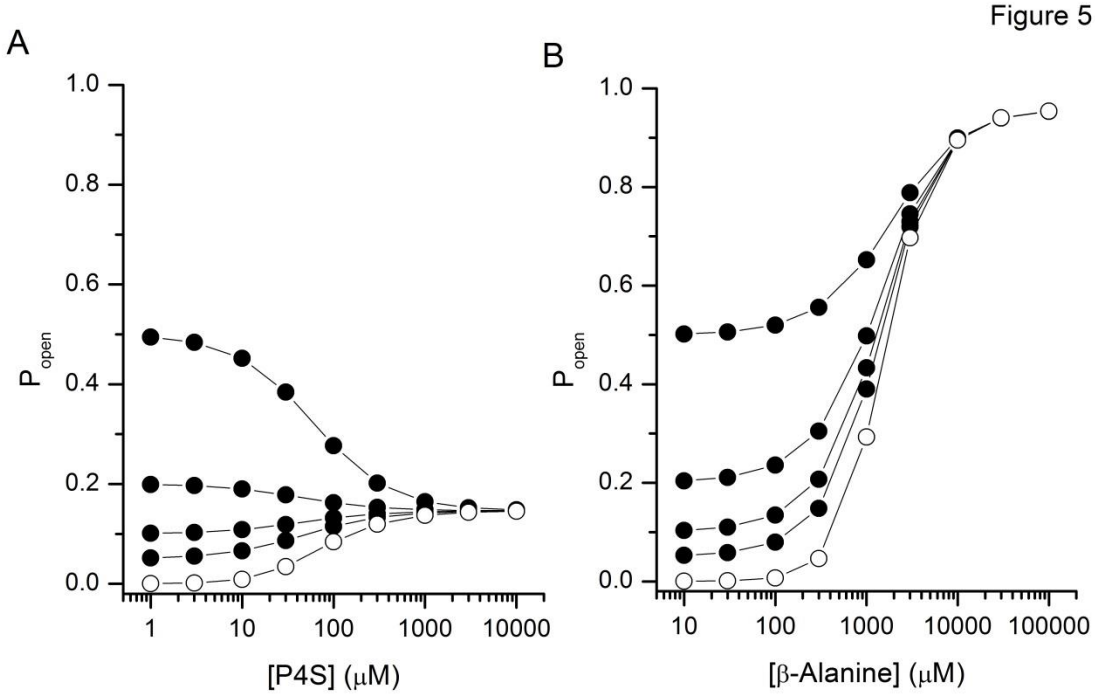

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Figure 6

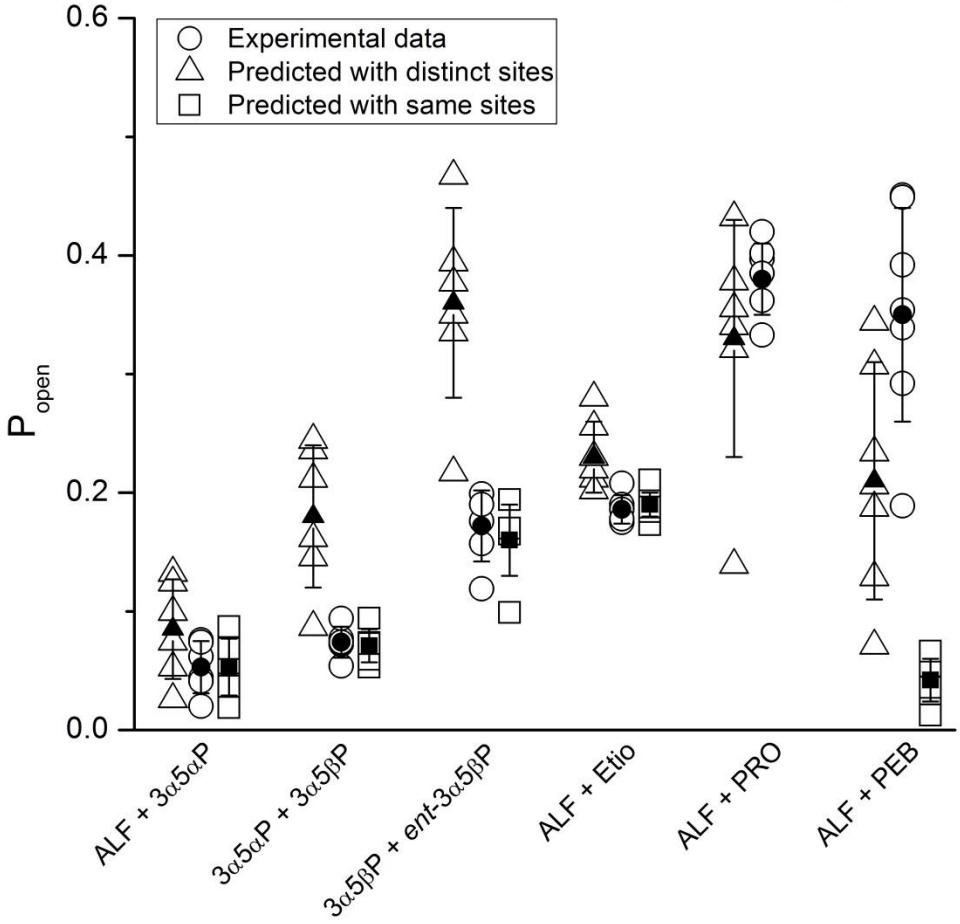

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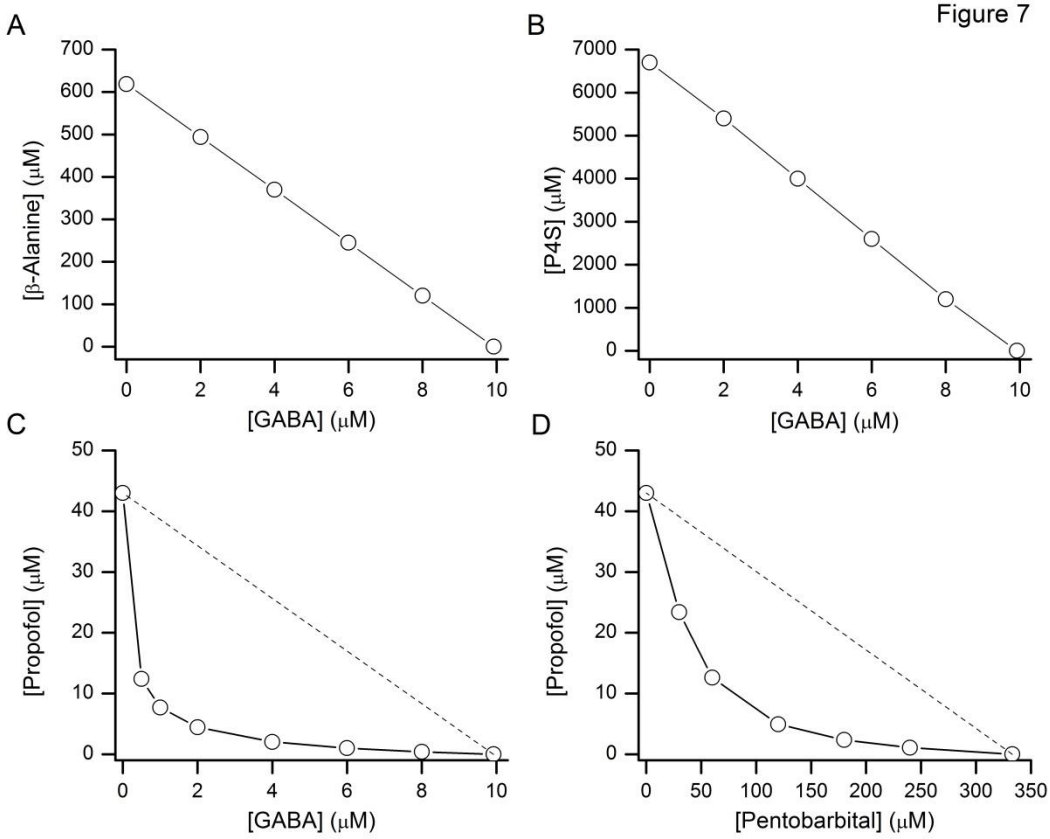
Figure 7



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