

## ACCELERATED COMMUNICATION

# The Actions of Drug Combinations on the GABA<sub>A</sub> Receptor Manifest as Curvilinear Isoboles of Additivity

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### ABSTRACT

Drug interactions are often analyzed in terms of isobolograms. In the isobologram, the line connecting the axial points corresponding to the concentrations of two different drugs that produce an effect of the same magnitude is termed an isobole of additivity. Although the isobole of additivity can be a straight line in some special cases, previous work has proposed that it is curvilinear when the two drugs differ in their maximal effects or Hill slopes. Modulators of transmitter-gated ion channels have a wide range of maximal effects as well as Hill slopes, suggesting that the isoboles for drug actions on ion channel function are not linear. In this study, we have conducted an analysis of direct activation and potentiation of the human  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub>

receptor to demonstrate that: 1) curvilinear isoboles of additivity are predicted by a concerted transition model where the binding of each GABAergic drug additively and independently reduces the free energy of the open receptor compared with the closed receptor; and 2) experimental data for receptor activation using the agonist pair of GABA and propofol or potentiation of responses to a low concentration of GABA by the drug pair of alfaxalone and propofol agree very well with predictions. The approach assuming independent energetic contributions from GABAergic drugs enables, at least for the drug combinations tested, a straightforward method to accurately predict functional responses to any combination of concentrations.

### Introduction

Administration of two (or more) drugs that produce similar physiologic effects can be a powerful way to lower drug dosage requirements while maintaining the intended functional effect (Hendrickx et al., 2008). Characterization of the effectiveness of drug combinations is often conducted using isobolographic analysis (Loewe, 1953; Fouquier and Guedj, 2015; Tallarida, 2016). In classic two-drug isobolograms, the doses of the two drugs that separately produce a functional effect of the same magnitude are plotted as axial points on a two-dimensional Cartesian graph. The two points, with coordinates of  $(x, 0)$  and  $(0, y)$ , are connected with a line known as the isobole of additivity, which corresponds to additive dose pairs and separates subadditive (antagonistic) from superadditive (synergistic) dose combinations (Loewe, 1953; Geary, 2013).

It has been shown that the isobole of additivity is linear when the two drugs exhibit a constant effect ratio (i.e., the concentration-response curves for the two drugs are superimposable by a shift along the abscissa; Grabovsky and Tallarida, 2004; Geary, 2013; Tallarida, 2016). This, however, is not the case for many ion channel modulators, which often differ in their maximal effects or numbers of interaction sites. In these cases, the isoboles can be curvilinear (Grabovsky and Tallarida, 2004) and may not even be uniquely defined (Tallarida, 2006; Geary, 2013), making classic isobolographic analysis an unreliable approach to defining synergy.

Here, we have conducted an isobolographic analysis of direct activation and potentiation of the  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor by pairs of GABAergic drugs. We employed GABA and propofol, separately and in combination, to activate receptors. To examine potentiation, we activated receptors with a low concentration of GABA, and potentiated with propofol and the steroid alfaxalone, separately and in combination. We predicted the isoboles using a coagonist concerted transition model (Monod et al., 1965; Forman, 2012). In this model, each agonist independently contributes to the energy

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**ABBREVIATIONS:**  $c_{ALF}$ , ratio of the affinity of the receptor to alfaxalone when the channel is open to that when the channel is closed;  $c_{GABA}$ , ratio of the affinity of the receptor to GABA when the channel is open to that when the channel is closed;  $c_{PROP}$ , ratio of the affinity of the receptor to propofol when the channel is open to that when the channel is closed;  $K_{ALF}$ , affinity of the receptor to alfaxalone when the channel is closed;  $K_{GABA}$ , affinity of the receptor to GABA when the channel is closed;  $K_{PROP}$ , affinity of the receptor to propofol when the channel is closed;  $L_0$ , gating equilibrium constant for unliganded receptors; MWC, Monod-Wyman-Changeux;  $P_o$ , open probability;  $P_o^{est}$ , estimated open probability.

stabilizing the open-channel state, with no interactions between agonists (Forman, 2012). The predicted isoboles have strongly curvilinear shapes. The results from electrophysiological experiments confirm the predictions.

## Materials and Methods

### Receptors, Expression, and Two-Electrode Voltage Clamp.

The experiments were conducted on human  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptors. The cDNAs were subcloned into the pcDNA3 vector in the T7 orientation, and linearized by digestion with XbaI (NEB Laboratories, Ipswich, MA). The complementary RNAs were produced using mMessage mMachine (Ambion, Austin, TX).

The receptors were expressed in *Xenopus laevis* oocytes. Oocyte harvests were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. The protocol was approved by the Animal Studies Committee of Washington University in St. Louis (Approval 20140150). Oocytes are a widely used expression system that enables functional studies of receptors of defined composition to provide specific and reliable pharmacological information.

Oocytes were injected with a total of 7 ng of complementary RNA in a final volume of 20 nl of distilled H<sub>2</sub>O at a ratio of 1:1:5 ( $\alpha$ : $\beta$ : $\gamma$ ). After injection, the oocytes were incubated in ND96 buffer with supplements (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 2.5 mM Na pyruvate, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml gentamycin, and 5 mM HEPES, pH 7.4) at 16°C, and used in electrophysiological recordings within 24–48 h.

All experiments were conducted using standard two-electrode voltage clamp. The oocytes were clamped at  $-60$  mV. The RC-1Z chamber (Warner Instruments, Hamden, CT) was perfused continuously with a bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4). Solutions were gravity applied from 30-ml glass syringes. Current responses were amplified with an OC-725C amplifier (Warner Instruments), filtered at 40 Hz, digitized with a Digidata 1200 series digitizer (Molecular Devices, Sunnyvale, CA) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were subsequently analyzed with Clampfit (Molecular Devices) to determine the maximal amplitude of current response. Concentration-response relationships for activation were determined by exposing an oocyte to increasing concentrations of GABA or propofol. The durations of drug applications were typically 20–40 s, which was aimed at reaching the peak response without unnecessary further exposure to the drug. Each drug application was followed by a 2–3 min washout in bath solution.

**The Concerted Transition Model: Data Analysis and Predicted Isobolograms.** Functional characterization of receptor activity was conducted in the Monod-Wyman-Changeux (MWC) allosteric model framework (Monod et al., 1965; Karlin, 1967; Chang and Weiss, 1999; Forman, 2012). MWC analysis uses open probability ( $P_o$ ) rather than raw amplitude values as the dependent variable. The conversion of raw concentration-response data and response amplitudes into units of  $P_o$  was accomplished by matching the relative peak responses against a scale ranging from an estimated  $P_o$  ( $P_o^{\text{est}}$ ) of 0–1. The term  $P_o^{\text{est}}$  rather than  $P_o$  is used to describe experimental data due to potential errors that are associated with this approach. The major source of potential error is an underestimated true peak amplitude in response to saturating GABA and propofol due to, for example, fast desensitization.

A current level corresponding to a  $P_o^{\text{est}}$  value of 1 was determined by activating the receptors with a saturating concentration (1 mM) of GABA in the presence of 50  $\mu$ M propofol. From this approach, the  $P_o^{\text{est}}$  of  $\alpha 1\beta 2\gamma 2L$  receptors in the presence of saturating GABA is  $0.83 \pm 0.03$  (mean  $\pm$  S.E.M.;  $n = 5$  cells), which is in agreement with previous single-channel and whole-cell studies (Steinbach and Akk, 2001; Lema and Auerbach, 2006; Keramidas and Harrison, 2010; Ruesch et al., 2012; Hernandez et al., 2017).

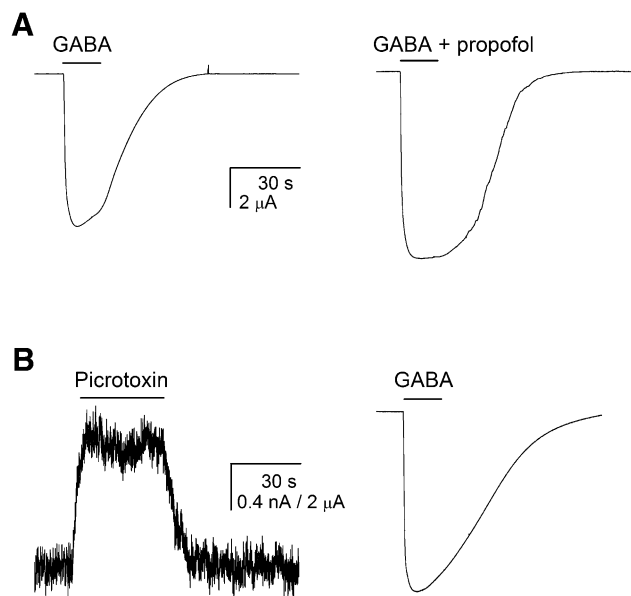
Spontaneous activity (i.e., current in the absence of an agonist) was determined by exposing the receptors to the channel blocker picrotoxin. The  $P_o$  of spontaneously active receptors ( $P_{o, \text{spont}}$ ) was calculated by comparing the amplitude of the response to 300  $\mu$ M picrotoxin, which is expected to produce a current level with  $P_o^{\text{est}}$  of 0, and the peak response to saturating GABA that produces a response with  $P_o^{\text{est}}$  of 0.83. Using this approach, we estimate that the  $P_{o, \text{spont}}$  value of the human  $\alpha 1\beta 2\gamma 2L$  receptor is  $0.00012 \pm 0.00001$  ( $n = 5$ ). This value is in the range of previous estimates for  $P_{o, \text{spont}}$  (Chang and Weiss, 1999; Ruesch et al., 2012; Ziemba and Forman, 2016). Sample traces are shown in Fig. 1.

In the MWC formalism, the probability of being open is given by the following equation (Chang and Weiss, 1999; Ruesch et al., 2012):

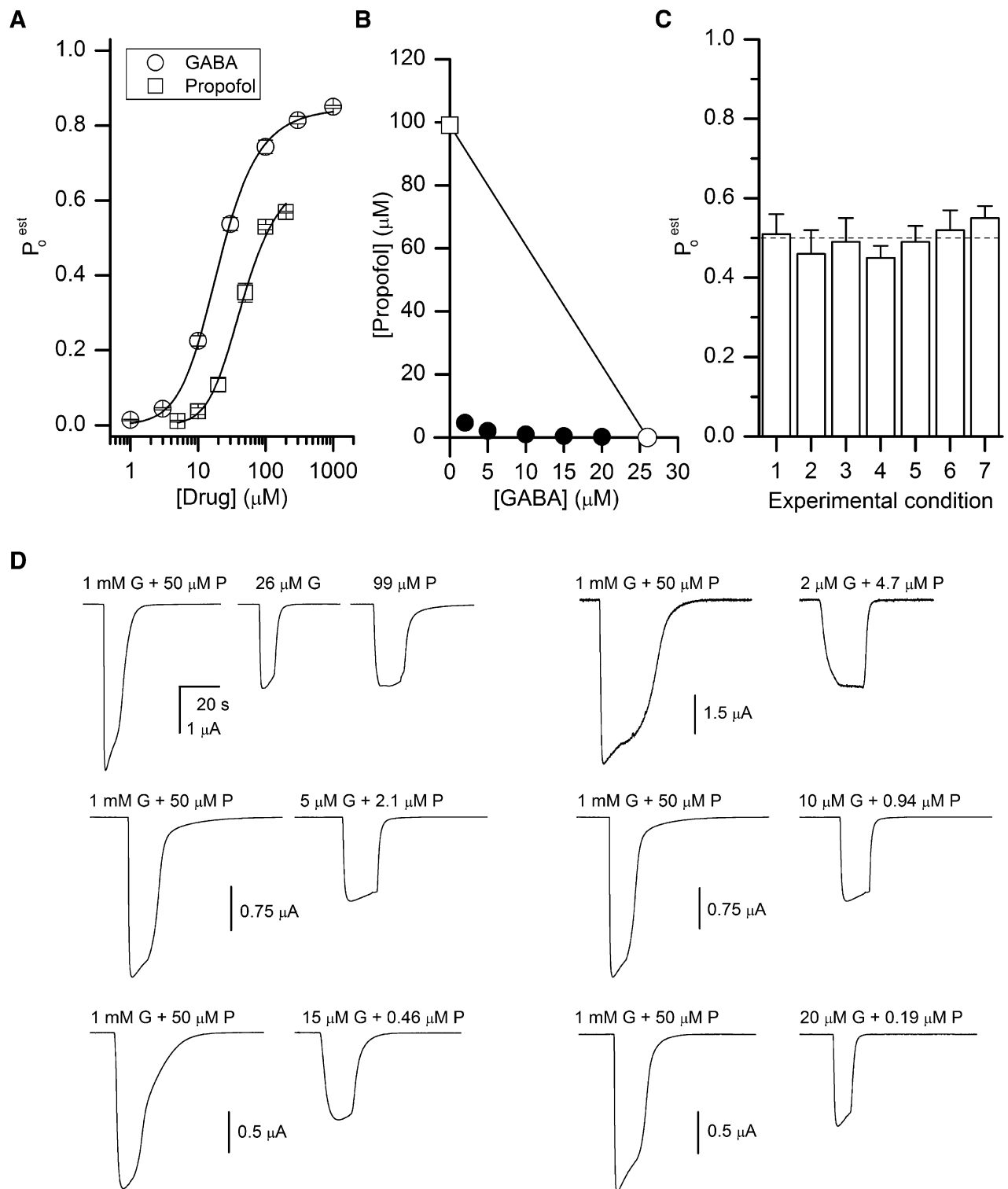
$$P_o = \frac{1}{1 + L_0 \times \left[ \frac{1 + [A]/K_A}{1 + [A]/c_A K_A} \right]^{N_A}} \quad (1)$$

where  $[A]$  is the concentration of agonist A,  $K_A$  is the closed receptor equilibrium dissociation constant for the agonist, and  $N_A$  corresponds to the number of agonist binding sites (constrained to 2 for GABA, 5 for propofol).  $c_A$  is a measure of gating efficacy, expressed as the ratio of the open receptor dissociation constant to the closed receptor dissociation constant.  $L_0$  is the gating equilibrium constant for unliganded receptors, which is calculated as the ratio of the fraction of receptors with closed channels to the fraction with open channels. From  $P_{o, \text{spont}}$  data, we calculate an  $L_0$  of  $8409 \pm 610$  ( $n = 5$ ). Throughout the analysis,  $L_0$  was constrained to 9000. The concentration-response relationship for  $P_o^{\text{est}}$  was fitted with eq. 1 using constrained values for  $L_0$  and  $N_A$  to provide estimates for  $K_A$  and  $c_A$ .

When two agonists (A and B) that do not bind to the same site are present at the same time, the state function of the receptor is as follows:



**Fig. 1.** Determination of receptor  $P_o$ . (A) The  $P_o$  for  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptors activated by saturating GABA was determined by comparing peak responses to 1 mM GABA (left trace) and to 1 mM GABA + 50  $\mu$ M propofol (right trace). The combination of GABA + propofol is expected to generate a response with a  $P_o$  value indistinguishable from 1 (Ruesch et al., 2012). Both traces are from the same cell. (B) The  $P_o$  value of receptors in the absence of agonist (i.e.,  $P_{o, \text{spont}}$ ) was determined by comparing the effect of 300  $\mu$ M picrotoxin on holding current (left trace) to the peak response to saturating GABA (right trace). Exposure to 300  $\mu$ M picrotoxin is expected to result in a current level with a  $P_o$  value of 0, whereas the peak response to saturating GABA has a  $P_o^{\text{est}}$  value of 0.83. Both traces are from the same cell.



**Fig. 2.** Activation of the  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor by GABA, propofol, or combinations of GABA and propofol. (A) Concentration-response relationships for activation by GABA or propofol. The data show mean  $\pm$  S.E.M. from seven (GABA) or five (propofol) cells. The  $P_o^{est}$  value was determined as described in *Materials and Methods*. The curves were generated by fitting pooled data to eq. 1. The best-fit parameters are  $K_{GABA} = 35 \mu M$  and  $c_{GABA} = 0.0045$  for GABA ( $R^2$  of the fit 0.9997), and  $K_{Prop} = 19 \mu M$  and  $c_{Prop} = 0.139$  for propofol ( $R^2 = 0.9956$ ). (B) An isobologram for receptor activation by GABA or propofol, calculated to produce a response with a  $P_o$  value of 0.5. The two axial points (open symbols) are connected with a straight line. The filled circles show GABA and propofol concentration combinations predicted to produce responses with a  $P_o$  value of 0.5. (C) Summary of electrophysiological recordings. The graph shows response ratios (mean  $\pm$  S.E.M. from five to nine cells) vs. 1 mM GABA + 50  $\mu M$  propofol for 1) 26  $\mu M$  GABA ( $0.51 \pm 0.05$ ;  $P = 0.9$  for comparison with the expected ratio of 0.50), 2) 99  $\mu M$  propofol ( $0.46 \pm 0.06$ ;  $P = 0.52$ ), 3) 2  $\mu M$  GABA + 4.7  $\mu M$  propofol ( $0.49 \pm 0.06$ ;  $P = 0.9$ ), 4) 5  $\mu M$  GABA + 2.1  $\mu M$  propofol ( $0.45 \pm 0.03$ ;  $P = 0.20$ ), 5) 10  $\mu M$  GABA + 0.94  $\mu M$  propofol ( $0.49 \pm 0.04$ ;  $P = 0.76$ ), 6) 15  $\mu M$  GABA + 0.46  $\mu M$  propofol ( $0.52 \pm 0.05$ ;  $P = 0.67$ ), or 7) 20  $\mu M$  GABA + 0.19  $\mu M$  propofol ( $0.55 \pm 0.03$ ;  $P = 0.20$ ). The dashed line shows the expected response of 0.5. (D) Sample current traces showing responses to 1 mM GABA (G) + 50  $\mu M$  propofol (P) expected to produce a  $P_o$  value of 1, and 26  $\mu M$  GABA, 99  $\mu M$  propofol, or various combinations of GABA + propofol expected to produce a  $P_o$  value of 0.5. The traces in each set are from the same cell.

TABLE 1

Summary of data for activation by GABA, propofol, and combinations of the two agonists

The first column shows the concentrations of GABA and/or propofol (Prop) applied while the second identifies the data sets shown in Figs. 2C and 4A. The column headed  $\Delta P_o$  gives the mean  $\pm$  S.E.M. (number of cells) for the measured  $P_o$  produced by that agonist or combination of agonists, expressed as the difference from the basal  $P_o$  value (0.00012).  $P$  vs.  $\Delta P_o$  predicted gives the  $P$  value (one-sample  $t$  test) for the difference between the measured and predicted differences (in each case, the predicted response is 0.49988). The column headed  $\Delta$ Sum gives the arithmetic sum of the predicted responses to the given concentrations of agonists applied separately (e.g.,  $P_o$  of 2  $\mu$ M GABA applied alone plus  $P_o$  of 4.65  $\mu$ M propofol applied alone) in terms of the difference from the basal  $P_o$  value. The final column ( $P$  to  $\Delta$ Sum) gives the  $P$  value for the difference between the measured response and the predicted sum.

Agonists Used	Set	$\Delta P_o$	$P$ vs. $\Delta P_o$ Predicted	$\Delta$ Sum	$P$ vs. $\Delta$ Sum
26 $\mu$ M GABA	1	$0.51 \pm 0.05$ (5)	0.9	0.50	0.9
99 $\mu$ M Prop	2	$0.46 \pm 0.06$ (5)	0.5	0.50	0.5
2 $\mu$ M GABA + 4.65 $\mu$ M Prop	3	$0.49 \pm 0.06$ (6)	0.9	0.02	0.001
5 $\mu$ M GABA + 2.1 $\mu$ M Prop	4	$0.45 \pm 0.03$ (5)	0.2	0.08	<0.001
10 $\mu$ M GABA + 0.94 $\mu$ M Prop	5	$0.49 \pm 0.04$ (7)	0.8	0.22	<0.001
15 $\mu$ M GABA + 0.46 $\mu$ M Prop	6	$0.52 \pm 0.05$ (7)	0.7	0.34	0.01
20 $\mu$ M GABA + 0.19 $\mu$ M Prop	7	$0.55 \pm 0.03$ (7)	0.2	0.42	0.006

$$P_o = \frac{1}{1 + L_o \times \left[ \frac{1 + [A]/K_A}{1 + [A]/c_A K_A} \right]^{N_A} \left[ \frac{1 + [B]/K_B}{1 + [B]/c_B K_B} \right]^{N_B}} \quad (2)$$

College Station, TX). Curve-fitting results are shown as the best-fit parameter  $\pm$  S.E.

Inspection of eq. 2 indicates that the effect of agonist B on activation by agonist A can be expressed through a new parameter  $L^*$ , as follows:

$$L^* = L_o \times \left[ \frac{1 + [B]/K_B}{1 + [B]/c_B K_B} \right]^{N_B} \quad (3)$$

In essence, the presence of drug B changes the level of basal gating.

Binding and gating parameters for alfaxalone were determined from  $P_o^{\text{est}}$  data obtained in experiments where receptors activated by 5  $\mu$ M GABA were potentiated by alfaxalone. This approach was necessary because alfaxalone is such an inefficacious agonist that the direct approach employing eq. 1 was unreliable due to the small responses in direct activation by the steroid. The parameters for activation by alfaxalone were then determined by fitting a modified version of eq. 1, as follows:

$$P_o = \frac{1}{1 + L^* \times \left[ \frac{1 + [ALF]/K_{ALF}}{1 + [ALF]/c_{ALF} K_{ALF}} \right]^{N_{ALF}}} \quad (4)$$

The value for  $L^*$  was directly determined from the response to 5  $\mu$ M GABA alone, according to  $(1 - P_{o, 5 \mu\text{M GABA}})/P_{o, 5 \mu\text{M GABA}}$  ( $L^* = 16.25$ ;  $P_{o, 5 \mu\text{M GABA}} = 0.058$ ). The number of binding sites for alfaxalone ( $N_{ALF}$ ) was held at 2 (Hosie et al., 2006; Bracamontes et al., 2011).

Using eq. 1, we first calculated the concentrations of GABA or propofol alone that would produce a  $P_o$  value of 0.5. We then calculated the concentrations of propofol necessary to elicit this response when combined with arbitrarily selected GABA concentrations of 2, 5, 10, 15, or 20  $\mu$ M. In each case, a value for  $L^*$  was calculated for a specific concentration of GABA, then the concentration of propofol required to produce  $P_o = 0.5$  estimated using eqs. 2 and 3.

Effects of two potentiating drugs were predicted in a similar fashion. We measured the response to 5  $\mu$ M GABA alone, then calculated the concentrations of alfaxalone and propofol that separately would potentiate the response to GABA to  $P_o = 0.5$ . We then predicted the concentrations of propofol that when combined with alfaxalone at concentrations of 0.2, 0.5, 1, or 1.5  $\mu$ M would potentiate the response to 5  $\mu$ M GABA to a  $P_o$  value of 0.5.

These calculations resulted in highly curvilinear isoboles of additivity. To test the predicted isobolograms experimentally, we exposed oocytes to the drugs separately or in combination at the predicted concentrations, as well as to the combination of 1 mM GABA + 50  $\mu$ M propofol to estimate the response corresponding to  $P_o^{\text{est}} = 1$ .

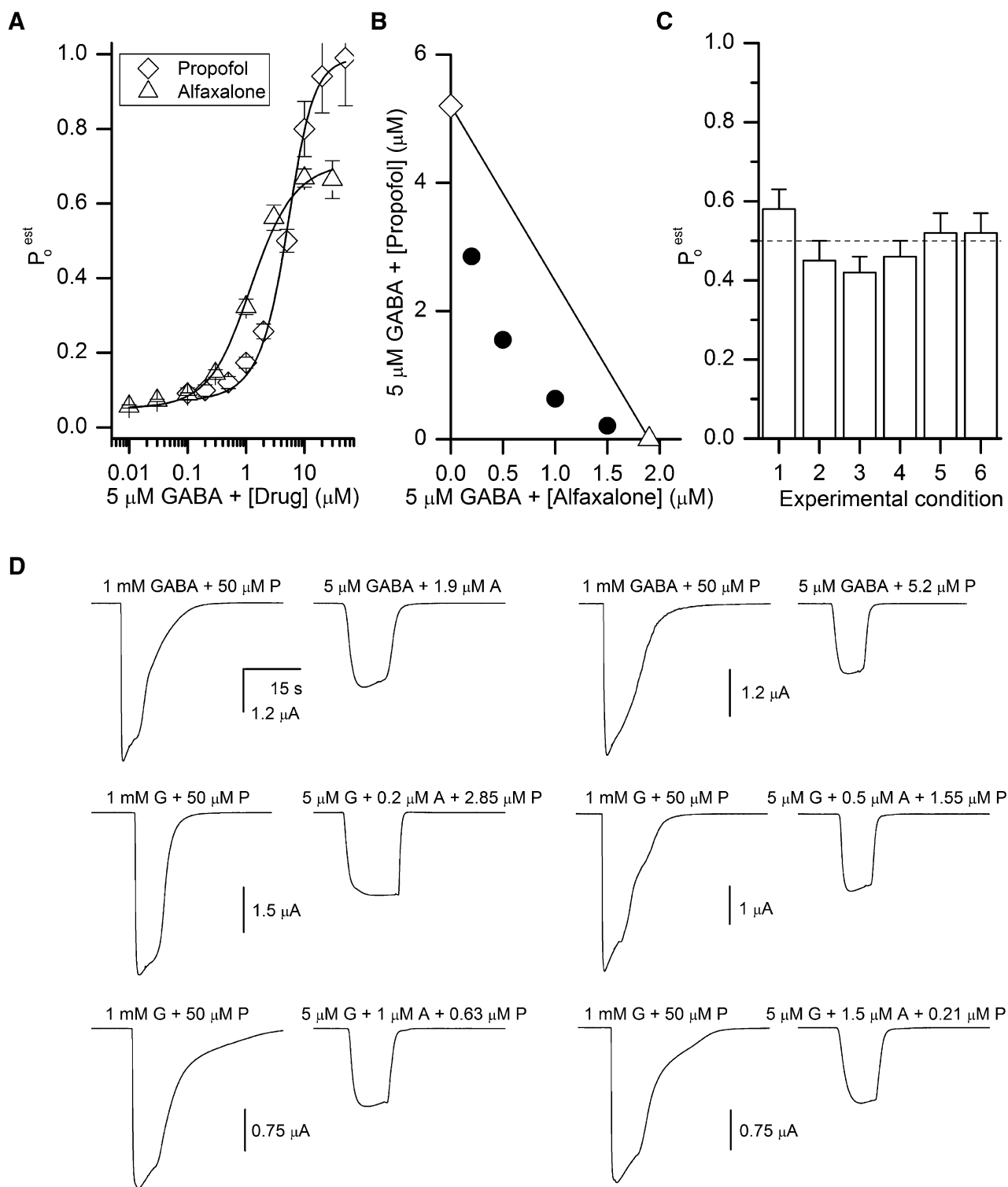
Curve fitting was performed using Origin version 7.5 (Originlab Corp., Northampton, MA). Pairwise statistical comparison was conducted using Student's  $t$  test. Statistical analyses were performed using Excel (Microsoft, Redmond, WA) or Stata/IC (StataCorp LP,

## Results

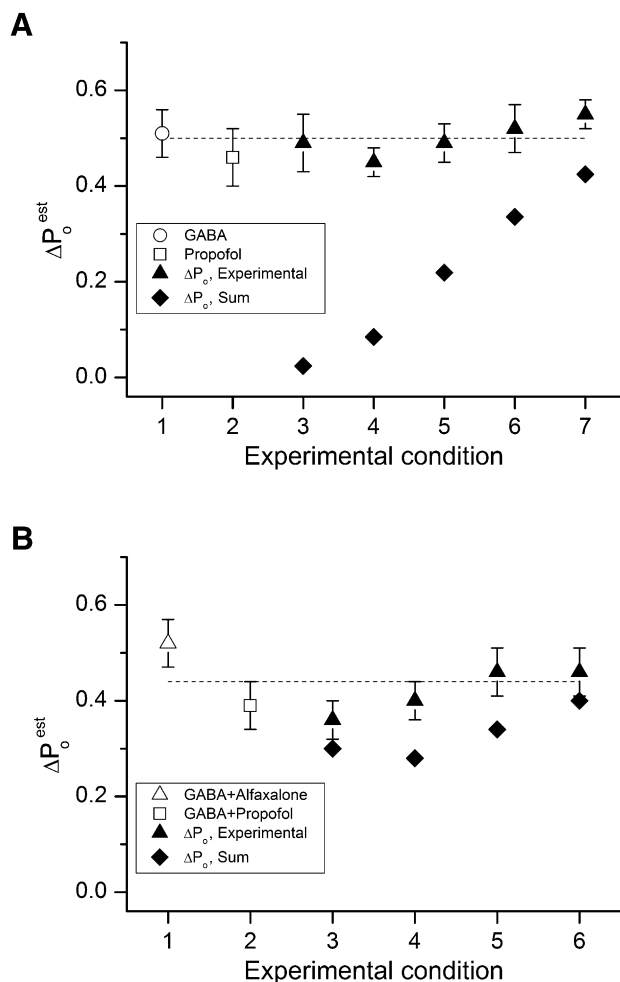
### GABA<sub>A</sub> Receptor Activation by GABA, Propofol, or Combinations of GABA and Propofol.

Human  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptors were expressed in oocytes and exposed to 1–1000  $\mu$ M GABA or 5–500  $\mu$ M propofol. The raw current responses were converted to units of  $P_o$ . For that, the concentration-response data were normalized to the peak response obtained from the same set of cells in the presence of 1 mM GABA + 50  $\mu$ M propofol. This drug combination elicits a current response that is >10% larger than the response to 1 mM GABA alone and that we have assigned a  $P_o^{\text{est}}$  value of 1. Curve fitting was performed on data pooled from at least five cells under each condition using eq. 1. The gating equilibrium constant for unliganded receptors ( $L_o$ ) was held at 9000 (see *Materials and Methods*). For receptors activated by GABA, we estimate a  $K_{GABA}$  (affinity of the receptor to GABA when the channel is closed) value of  $35 \pm 2$   $\mu$ M and a  $c_{GABA}$  (ratio of the affinity of the receptor to GABA when the channel is open to that when the channel is closed) value of  $0.0045 \pm 0.0001$ . The number of binding sites for GABA was held at 2 (Amin and Weiss, 1993; Baumann et al., 2003). For propofol, we estimate a  $K_{Prop}$  (affinity of the receptor to propofol when the channel is closed) value of  $19 \pm 2$   $\mu$ M and  $c_{Prop}$  (ratio of the affinity of the receptor to propofol when the channel is open to that when the channel is closed) of  $0.139 \pm 0.003$  when the number of binding sites is fixed at 5. The actual number of propofol binding sites is not well established. Photolabeling studies have indicated that there are two classes of sites with two copies of each class of sites per ternary receptor, giving a total of at least four sites (Yip et al., 2013; Jayakar et al., 2014). Previous functional studies have proposed two to five sites per receptor (Ruesch et al., 2012; Eaton et al., 2016; Maldifassi et al., 2016; Nourmahad et al., 2016). The data and fits are shown in Fig. 2A. From the  $K$  and  $c$  values, we calculate that the concentration of GABA that would produce a response with a  $P_o$  value of 0.5 is 26  $\mu$ M, and the concentration of propofol that would produce a  $P_o$  response of 0.5 is 99  $\mu$ M (open symbols in Fig. 2B).

We tested the sensitivity of the fitting results to the imposed value of  $L_o$ . The  $K_{GABA}$  and  $K_{Prop}$  values were minimally affected when  $L_o$  varied between 1000 and 50,000. An increase



**Fig. 3.** Potentiation of GABA-activated receptors by alfaxalone, propofol, or combinations of alfaxalone and propofol. (A) Concentration-response relationships for potentiation of receptors activated by 5  $\mu$ M GABA by propofol or alfaxalone. The data show the mean  $\pm$  S.E.M. from five cells.  $P_o^{est}$  was determined as described in *Materials and Methods*. The curves were generated by fitting pooled data to eq. 3 with a modified  $L_0$  ( $L^*$ ) reflecting basal activity in the presence of 5  $\mu$ M GABA. The best-fit parameters are  $K_{P_{prop}} = 21 \mu$ M and  $C_{P_{prop}} = 0.21$  for propofol ( $R^2 = 0.9966$ ), and  $K_{ALF} = 1.75 \mu$ M and  $C_{ALF} = 0.147$  for alfaxalone ( $R^2 = 0.9955$ ). (B) An isobologram for potentiation of GABA-activated receptors by alfaxalone or propofol, calculated to produce a response with a  $P_o$  value of 0.5. The two axial points (open symbols) are connected with a straight line. The filled circles show alfaxalone and propofol concentration combinations predicted to produce responses with a  $P_o$  of 0.5. (C) Summary of electrophysiological recordings. The graph shows response ratios (mean  $\pm$  S.E.M. from six cells) vs. 1 mM GABA + 50  $\mu$ M propofol for 1) 5  $\mu$ M GABA + 1.9  $\mu$ M alfaxalone ( $0.58 \pm 0.05$ ;  $P = 0.18$  for comparison with the expected ratio of 0.50), 2) GABA + 5.2  $\mu$ M propofol ( $0.45 \pm 0.05$ ;  $P = 0.41$ ), 3) GABA + 0.2  $\mu$ M alfaxalone + 2.85  $\mu$ M propofol ( $0.42 \pm 0.04$ ;  $P = 0.09$ ), 4) GABA + 0.5  $\mu$ M alfaxalone + 1.55  $\mu$ M propofol ( $0.46 \pm 0.04$ ;  $P = 0.42$ ), 5) 5  $\mu$ M GABA + 1  $\mu$ M alfaxalone + 0.63  $\mu$ M propofol ( $0.52 \pm 0.05$ ;  $P = 0.7$ ), and 6) 5  $\mu$ M GABA + 1.5  $\mu$ M alfaxalone + 0.21  $\mu$ M propofol ( $0.52 \pm 0.05$ ;  $P = 0.7$ ). The dashed line shows the expected response of 0.5. (D) Sample current traces showing responses to 1 mM GABA (G) + 50  $\mu$ M propofol (P) expected to produce a  $P_o$  value of 1, and 5  $\mu$ M GABA + 1.9  $\mu$ M alfaxalone (A), 5  $\mu$ M GABA + 5.2  $\mu$ M propofol, or various combinations of 5  $\mu$ M GABA + alfaxalone + propofol expected to produce a  $P_o$  value of 0.5. The traces in each set are from the same cell.



**Fig. 4.** Summary of the data. (A) Calculated and experimentally determined  $P_o$  responses for GABA, propofol, and GABA + propofol: 1) 26  $\mu\text{M}$  GABA, 2) 99  $\mu\text{M}$  propofol, 3) 2  $\mu\text{M}$  GABA + 4.7  $\mu\text{M}$  propofol, 4) 5  $\mu\text{M}$  GABA + 2.1  $\mu\text{M}$  propofol, 5) 10  $\mu\text{M}$  GABA + 0.94  $\mu\text{M}$  propofol, 6) 15  $\mu\text{M}$  GABA + 0.46  $\mu\text{M}$  propofol, and 7) 20  $\mu\text{M}$  GABA + 0.19  $\mu\text{M}$  propofol. The graph shows the effects of agonists and agonist combinations on receptor  $P_o$  ( $\Delta P_o$  calculated as experimentally determined  $P_o$  - basal  $\Delta P_o$  of 0.00012). The symbols show the effect on receptor  $P_o$  for GABA alone (open circle), propofol alone (open square), and for combinations of GABA + propofol (filled triangles). The filled diamonds give the arithmetic sums of  $\Delta P_o$  for predicted responses to the given concentrations of agonists applied separately. (B) Calculated and experimentally determined  $P_o$  responses for GABA in combination with alfaxalone, propofol, and alfaxalone + propofol: 1) 5  $\mu\text{M}$  GABA + 1.9  $\mu\text{M}$  alfaxalone, 2) GABA + 5.2  $\mu\text{M}$  propofol, 3) GABA + 0.2  $\mu\text{M}$  alfaxalone + 2.85  $\mu\text{M}$  propofol, 4) GABA + 0.5  $\mu\text{M}$  alfaxalone + 1.55  $\mu\text{M}$  propofol, 5) 5  $\mu\text{M}$  GABA + 1  $\mu\text{M}$  alfaxalone + 0.63  $\mu\text{M}$  propofol, and 6) 5  $\mu\text{M}$  GABA + 1.5  $\mu\text{M}$  alfaxalone + 0.21  $\mu\text{M}$  propofol. The symbols show  $P_o$  values for GABA + alfaxalone (open triangle), GABA + propofol (open square), and for combinations of GABA + alfaxalone + propofol (filled triangles). The filled diamonds give the arithmetic sums of  $\Delta P_o$  for predicted potentiating effects of each potentiator in the combination applied separately.

in  $L_0$  was associated with a decrease in fitted  $c$  values, reflecting the larger energetic contribution by the ligand required to offset the increased standard enthalpy,  $\Delta H$ , of the gating equilibrium. However, manipulations in imposed  $L_0$  did not affect the calculated agonist concentrations eliciting responses with a  $P_o$  value of 0.5, which were 24–26  $\mu\text{M}$  and 97–101  $\mu\text{M}$ , respectively, for GABA and propofol.

We then calculated the concentrations of GABA and propofol in mixtures of the two agonists that are predicted

to produce a response with a  $P_o$  value of 0.5. We chose values for GABA of 2, 5, 10, 15, and 20  $\mu\text{M}$  and then used eq. 2 to determine the concentrations of propofol that, when combined with the particular concentration of GABA, would generate a response with a  $P_o$  value of 0.5. These values are 4.65, 2.1, 0.94, 0.46, and 0.19  $\mu\text{M}$  propofol, respectively. In the isobologram graph (filled circles in Fig. 2B), the predicted pairs fall on a highly curvilinear plot.

To examine the agreement between the predictions and experimental data, we exposed oocytes expressing  $\alpha 1\beta 2\gamma 2\text{L}$  receptors to the drug mixtures predicted to generate a response with a  $P_o$  value of 0.5. As can be seen in Fig. 2C and Table 1, the mean responses are indistinguishable from the predicted value of 0.5. Sample current traces are shown in Fig. 2D.

**Potentiation of GABA-Activated Receptors by Alfaxalone, Propofol, or Combinations of Alfaxalone and Propofol.** We next examined potentiation of the  $\alpha 1\beta 2\gamma 2\text{L}$  receptor. The receptors were activated by 5  $\mu\text{M}$  GABA in the presence of alfaxalone, propofol, or combinations of alfaxalone and propofol. The raw current responses were converted to units of  $P_o$  as described above. We calculated the value for  $L^*$  from the response to 5  $\mu\text{M}$  GABA alone as 16.25 and assumed that there are two binding sites for alfaxalone (Hosie et al., 2006; Bracamontes et al., 2011). Employing eqs. 2–4, we estimate a  $K_{ALF}$  value of  $1.75 \pm 0.20 \mu\text{M}$  and a  $c_{ALF}$  value of  $0.147 \pm 0.005$  for alfaxalone, and a  $K_{PROP}$  value of  $21.0 \pm 5.8 \mu\text{M}$  and a  $c_{PROP}$  value of  $0.21 \pm 0.03$  for propofol. The concentration-response data are shown in Fig. 2A. The affinity and efficacy estimates for propofol are similar to those obtained in direct activation experiments, confirming the independent, additive actions of GABA and propofol. Alfaxalone is a weak activator of the  $\alpha 1\beta 2\gamma 2\text{L}$  receptors; determination of  $K_{ALF}$  and  $c_{ALF}$  from direct activation experiments was not technically feasible due to the low maximal  $P_o$  value ( $<0.004$  at 10  $\mu\text{M}$ ).

From the  $K$  and  $c$  estimates, we calculated the concentrations of alfaxalone, propofol, and several combinations of alfaxalone and propofol that when combined with 5  $\mu\text{M}$  GABA are expected to generate peak responses with a  $P_o$  value of 0.5. We estimated that 5  $\mu\text{M}$  GABA coapplied with 1.9  $\mu\text{M}$  alfaxalone or 5.2  $\mu\text{M}$  propofol will produce responses with a  $P_o$  value of 0.5 (open symbols in Fig. 3B). Similarly, we predicted that drug combinations of 5  $\mu\text{M}$  GABA + 0.2  $\mu\text{M}$  alfaxalone + 2.85  $\mu\text{M}$  propofol, 5  $\mu\text{M}$  GABA + 0.5  $\mu\text{M}$  alfaxalone + 1.55  $\mu\text{M}$  propofol, 5  $\mu\text{M}$  GABA + 1  $\mu\text{M}$  alfaxalone + 0.63  $\mu\text{M}$  propofol, and 5  $\mu\text{M}$  GABA + 1.5  $\mu\text{M}$  alfaxalone + 0.21  $\mu\text{M}$  propofol will generate responses with  $P_o$  of 0.5 (filled circles in Fig. 3B). While the predicted isobole is curvilinear, it is interesting to note that the degree of curvature of the isobologram in Fig. 3B is less than in Fig. 2B. Our simulations show that the curvature is strongly affected by the value of  $L_0$  or  $L^*$  ( $L_0 = 9000$  for the activation data in Fig. 2, while  $L^* = 16.25$  for the potentiation data in Fig. 3).

To verify these predictions, we compared peak amplitudes from oocytes expressing  $\alpha 1\beta 2\gamma 2\text{L}$  receptors exposed to the test drug mixtures and to the control response (1 mM GABA + 50  $\mu\text{M}$  propofol) with a  $P_o^{\text{est}}$  of 1. As can be seen in Fig. 3C the relative responses are indistinguishable from the predicted ratio of 0.5. Sample current traces are shown in Fig. 3D.

## Discussion

In this study, we conducted an isobolographic analysis of the human  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor to determine the concentrations of several GABAergic agents that, alone or in combination, produce a response with a  $P_o$  value of 0.5. The analysis was conducted in two experimental settings. We first studied the direct activation of the receptor by GABA, propofol, and combinations of GABA and propofol. In the second setting, we examined the potentiation of GABA-activated receptors by the steroid alfaxalone, propofol, and combinations of alfaxalone and propofol.

Deviation from a linear isobole of additivity is often interpreted as superadditivity or subadditivity of drug interactions (Loewe, 1953; Tallarida, 2016). We show here that simple addition of stabilization energy in a coagonist concerted transition model predicts strongly curvilinear isoboles, deep in the region classically assigned to synergistic interactions. The predicted deflection from linearity is confirmed by experimental data.

The classic method for constructing an isobole of additivity is to transform the concentration of drug A into an equivalent concentration of drug B (denoted as B'), then to calculate the predicted effect of the combination of drugs by expressing the effect of the combination of A + B as B' + B using the empirical concentration-effect relationship for drug B (Grabovsky and Tallarida, 2004). One potential problem with this approach is that the direction of transformation (i.e., A to B' or B to A') can influence the curvature of isoboles when the two drugs have different Hill slopes (Lorenzo and Sanchez-Marin, 2006).

We calculated the isobole of additivity using an equation that describes a single concentration-effect relationship for all agonists (albeit with unique parameter values for each agent). Our approach relies on having a single relationship describing the activation of the receptor by multiple agonists, rather than utilizing empirical concentration-effect relationships for each agonist. The effect of each agonist is to change the relative free energies of the closed and open states in a fashion quantitatively described by the model. This approach assumes independent energetic contributions by all agonists (i.e., no interactions between agonists). Responses to any concentration of an agonist or a combination of multiple agonists can be predicted, once the affinity ( $K$ ) and efficacy ( $c$ ) values for the compounds are known. Furthermore, to predict an isobologram,  $K$  and  $c$  for just one of the agonists needs to be known. For the second agonist, only its functional response at the concentration of interest needs to be determined because that

determines the value for  $L^*$ . Our results apply to the agonist pair of GABA and propofol, and to the triple combination of GABA + alfaxalone + propofol. It remains to be determined whether these findings apply to other GABAergic drugs, particularly for combinations involving volatile anesthetics (Sebel et al., 2006; Jenkins et al., 2008).

Figure 4 and Tables 1 and 2 summarize the experimental observations in terms of the effects of the combinations of agents compared with the sum of the individual effects of the agents. The effects are expressed as the net change ( $\Delta P_o$ ) from the baseline or background activation present (no agent for data in Fig. 4A and Table 1, or 5  $\mu$ M GABA in Fig. 4B and Table 2). As can be seen for the activation by GABA, propofol, and combinations, the combinations produce a larger effect than the sum of the effects of GABA and propofol. It is interesting that the same model for activation may result in situations for which the effect of the combination is not significantly different from the sum of the individual effects. This situation arises when the basal level of activity of the receptor is relatively high, as can occur when a combination of drugs is used to potentiate the response to a low concentration of GABA (Fig. 4B).

Isobolographic analysis is widely used to describe the effects of combinations of GABAergic drugs. Deviation from a linear isobole of additivity, typically interpreted as synergy, has been observed in the modulation of GABA-activated  $\alpha 1\beta 2\gamma 2L$  receptors by the combination of propofol and flurazepam (Reynolds and Maitra, 1996), the enhancement of [<sup>3</sup>H]diazepam binding to cerebocortical synaptoneurosomes by the combination of GABA and pentobarbital, the enhancement of [<sup>3</sup>H]muscimol binding by pentobarbital and diazepam (DeLorey et al., 1993), and the production of loss-of-righting in rats by combinations of barbiturates and benzodiazepines (DeLorey et al., 1993). Superadditive anxiolytic effects have been found for certain concentration combinations of triazolam and pregnanolone and for clonazepam and ganaxalone in the elevated zero maze assay (Gunter et al., 2016). In patients, the combination of midazolam and propofol apparently synergistically produces a loss of response to command (McClune et al., 1992; Minto et al., 2000). Drug effects and interactions at the whole-organism level can depend on a variety of confounding factors, including ceiling effects or application cascades, and compensatory actions. The present study investigated a model system with a single target (the  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor) and an easily measured endpoint (a response with a  $P_o$  value of 0.5) to provide insight into a single step in the possible clinical effects of drugs acting on the GABA<sub>A</sub> receptor.

TABLE 2

Summary of data for potentiation of GABA-activated receptors by alfaxalone, propofol, and combinations

The first column shows the concentrations of GABA and of alfaxalone (ALF) and/or propofol (Prop) applied, whereas the second column identifies the data sets shown in Figs. 3C and 4B. The column headed  $\Delta P_o$  gives the mean  $\pm$  S.E.M. (number of cells) for the measured  $P_o$  value produced by that potentiator or combination of potentiators, expressed as the difference from the background  $P_o$  value in the presence of 5  $\mu$ M GABA (0.058).  $P$  vs.  $\Delta P_o$  predicted gives the  $P$  value (one-sample  $t$  test) for the difference between the measured and predicted differences (in each case the predicted response is 0.442). The column headed  $\Delta$ Sum gives the arithmetic sum of the predicted responses to the given concentration of potentiator or combinations of potentiators applied separately (e.g., 0.2  $\mu$ M alfaxalone applied alone plus 2.85  $\mu$ M propofol applied alone) in terms of the difference from the basal  $P_o$  value (0.058). The final column ( $P$  to  $\Delta$ Sum) gives the  $P$  value for the difference between the measured response and the predicted sum.

Drugs Used	Set	$\Delta P_o$	$P$ vs. $\Delta P_o$ Predicted	$\Delta$ Sum	$P$ vs. $\Delta$ Sum
5 $\mu$ M GABA + 1.9 $\mu$ M ALF	1	0.52 $\pm$ 0.05 (6)	0.2	0.44	0.2
5 $\mu$ M GABA + 5.2 $\mu$ M Prop	2	0.39 $\pm$ 0.05 (6)	0.9	0.44	0.4
5 $\mu$ M GABA + 0.2 $\mu$ M ALF + 2.85 $\mu$ M Prop	3	0.36 $\pm$ 0.04 (6)	0.6	0.30	0.2
5 $\mu$ M GABA + 0.5 $\mu$ M ALF + 1.55 $\mu$ M Prop	4	0.40 $\pm$ 0.04 (6)	0.7	0.28	0.04
5 $\mu$ M GABA + 1 $\mu$ M ALF + 0.63 $\mu$ M Prop	5	0.46 $\pm$ 0.05 (6)	0.2	0.34	0.05
5 $\mu$ M GABA + 1.5 $\mu$ M ALF + 0.21 $\mu$ M Prop	6	0.46 $\pm$ 0.05 (6)	0.2	0.40	0.2

## Authorship Contributions

Participated in research design: Steinbach, Akk.

Conducted experiments: Shin, Germann.

Performed data analysis: Shin, Germann, Steinbach, Akk.

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

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