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# The Apparent Voltage Dependence of $GABA_A$ Receptor Activation and Modulation Is Inversely Related to Channel Open Probability[S]

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## ABSTRACT

The GABA type A receptor ($GABA_AR$) is expressed ubiquitously throughout the brain and is a target for many therapeutic agents, including general anesthetics and benzodiazepines, which enhance receptor function by increasing the open probability ($P_o$) of the ion channel. It is commonplace for in vitro studies of receptor pharmacological characteristics to use negative membrane holding potentials to mimic the resting potential of neurons and symmetrical chloride to eliminate Goldman rectification, which results in chloride flow in the opposite direction, compared with in vivo conditions. This critical difference is usually overlooked because the $GABA_AR$ has been reported to behave as an ohmic pore, but our results show that the current-voltage relationship is nonlinear with respect to $P_o$. Specifically, we found that currents were outwardly rectifying at low $P_o$ and linear at high $P_o$. We confirmed the correlation between $P_o$ and rectification with a partial agonist, piperidine-4-sulfonic acid, and a gating-impaired mutation, $\alpha$1(L277A); both exhibited enhanced outward rectification. Furthermore, this correlation was independent of Goldman rectification and persisted under altered chloride gradient conditions, which suggests that rectification is linked to the direction of chloride flux. Finally, our results showed that the degree of potentiation by general anesthetics (etomidate, propofol, and isoflurane) was greater at negative membrane potentials. Traditional in vitro experiments thus overestimate the action of positive allosteric modulators of the $GABA_AR$. Our results show that the direction of the driving force on the permeant ion, as well as $P_o$, must be considered together for a complete understanding of drug actions on ligand-gated ion channels.

## Introduction

GABA type A receptors ($GABA_ARs$) are the primary sites of action for benzodiazepines and are one of several critical binding sites for general anesthetic drugs. Currently, there is a consensus that all such drugs exert their in vivo effects by initially enhancing $GABA_AR$ function. This manifests itself through an increase in the amplitude or duration of postsynaptic inhibition and an increase in the open probability ($P_o$) of the ion channel.

Under most common physiological conditions, the $GABA_AR$ protein allows the permeation of anions into a neuron via a synaptic lumen, a transmembrane pore, and a cytoplasmic lumen. These structures are defined by residues in the extracellular domain, residues in the second transmembrane domain, and an incompletely understood array of residues in the intracellular loop domain, in all five receptor subunits.

It has become commonplace in recent years to study the pharmacological features of the receptor by using a symmetrical chloride gradient, to avoid Goldman rectification (Goldman, 1943) and a negative holding potential and to mimic the resting membrane potential of a neuron. This method results in a stable assay system in which the actions of different drugs on receptor function can be measured accurately. However, this setting differs from the receptor's native setting in that GABA activation results in chloride efflux from the cell, rather than influx. The rationale for accepting such a functional difference stems from the fact that the ion channel has been reported to be a simple ohmic pore, conducting anions equally well in both directions across the membrane (Bormann, 1988; Angelotti and Macdonald, 1993). It has been reported that the ion channel exhibits varying degrees of rectification (Krishek and Smart, 2001; Pytel et al., 2006; Pavlov et al., 2009), which may not be attributable simply to asymmetry in the chloride concentration across the membrane, as predicted by the constant field equation (Goldman,

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**ABBREVIATIONS:** $GABA_AR$, GABA type A receptor; $P_o$, open probability; IV, current-voltage; RI, rectification index; P4S, piperidine-4-sulfonic acid; $P_{Cl}$, permeability of chloride.

1943; Hodgkin and Katz, 1949; Barker and Harrison, 1988; Hille, 2001), but may be attributable to the subunit stoichiometry of the receptor (Boileau et al., 2003) or the presence of neuromodulators (Shen et al., 2007).

In light of these differences, we chose to study the current-voltage (IV) relationship of the $GABA_AR$ with full and partial agonists, in the presence of positive allosteric modulators and with receptors harboring mutations that hinder channel gating. We found that low-$P_o$ conditions were associated with currents that exhibited outward rectification and, similarly, increasing $P_o$ resulted in linearization of the IV relationship. Our results also showed that the degree of receptor enhancement by therapeutic agents was voltage dependent; as the membrane potential increased and as it tended toward generating current flow in the physiological direction, the amount of enhancement was reduced.

## Materials and Methods

**Mutagenesis and Cell Culture.** Wild-type $GABA_AR$ cDNAs for the human $\alpha$1, $\beta$2, and $\gamma$2s subunits were expressed via the pCIS2 vector with a cytomegalovirus promoter in HEK293 cells (American Type Culture Collection, Manassas, VA). The $\alpha$1(L277A) mutation was created by using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and was confirmed through sequencing (Eurofins MWG Operon, Huntsville, AL). HEK293 cells were maintained at 37°C and 5% $CO_2$, in Eagle's minimal essential medium supplemented with 5% HyClone fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 40 $\mu$M L-glutamine, 100 U/ml penicillin, and 0.1 mM streptomycin. Once confluent, cells were passaged with trypsin treatment, up to 20 times. Seventy-two hours after being plated onto poly-D-lysine-coated glass coverslips, HEK293 cells were cotransfected with 2.5 $\mu$g of cDNA for each of the $GABA_AR$ $\alpha$1, $\beta$2, and $\gamma$2s subunits and adeno-associated virus-green fluorescent protein, through a calcium phosphate method (a total of 10 $\mu$g of DNA) (Chen and Okayama, 1987). By comparing the inhibitory potency of zinc (Trudell et al., 2000) acting on receptors generated with transfection ratios of 1:1:0, 1:1:1, and 1:1:10 for the $\alpha$1, $\beta$2, and $\gamma$2s subunits ($IC_{50}$ values of 88.9, 1597, and 1861 $\mu$M, respectively), we concluded that this transfection protocol resulted in a predominantly homogeneous population containing >92% $\alpha\beta\gamma$ receptors. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

**Whole-Cell Recordings.** Wild-type ($\alpha1\beta2\gamma2s$) and mutant $GABA_A$ receptors were characterized in whole-cell voltage-clamp electrophysiological assays at room temperature (22°C), 36 to 72 h after transfection. Patch pipettes were fabricated from thin-walled borosilicate glass (o.d., 1.5 mm; i.d., 1.12 mm; World Precision Instruments, Sarasota, FL), by using a horizontal puller (P-97; Sutter Instrument Co., Novato, CA), to a resistance of 2 to 5 M$\Omega$. The compositions of intracellular and extracellular solutions are presented in Table 1. All experiments were carried out in standard solutions (I1/E1) unless noted otherwise. Two 10-channel infusion pumps (KD Scientific, Holliston, MA) and a rapid solution exchanger (Bio-Logic, Claix, France) were used to apply agonists and modulators, dissolved in the appropriate extracellular solution, at a rate of 1.0 ml/min. Leak-subtracted whole-cell currents were recorded by using a MultiClamp 700B amplifier, digitally filtered at 100 Hz by using MultiClamp Commander software, and digitized at 200 Hz by using a DigiData 1322A interface; the solution changer was driven by protocols written in pClamp 9.2 (all from Molecular Devices, Sunnyvale, CA). To generate the IV relationship, a voltage ramp from −60 mV to +60 mV and back to −60 mV (0.24 mV/ms, for a total ramp duration of 0.5 s) was applied within the plateau response to each drug and in the baseline washout period (Fig. 1). The baseline ramp was subtracted, to control for the basal response of the membrane. Each sweep was then normalized with respect to its response at −60 mV, and the up-and-down component of each ramp was averaged.

TABLE 1
Compositions of intracellular and extracellular electrophysiological saline solutions containing standard and low chloride concentrations
The concentration of each reagent is listed for the four electrophysiological saline solutions used in this study, together with the final concentration of acid or base used to adjust intracellular solutions to a pH of 7.2 and extracellular solutions to a pH of 7.4. Also shown are the final concentrations of monovalent anions and cations for each solution. Intracellular and extracellular solutions were adjusted to a final osmolarity of 315 and 325 mOsM, respectively, with sucrose or deionized water as necessary.

| | Concentration | | | |
|---|---|---|---|---|
| | I1 | I2 | E1 | E2 |
| | *mM* | | | |
| Solute | | | | |
| NaCl | | | 160 | 5 |
| KCl | 120 | 68 | 3 | 3 |
| Sodium gluconate | | | | 155 |
| Potassium gluconate | | 48 | | |
| $MgCl_2$ | 2 | 2 | 1 | 1 |
| $CaCl_2$ | | | 1.5 | 1.5 |
| HEPES | 10 | 10 | 10 | 10 |
| EGTA | 10 | 10 | | |
| D-Glucose | | | 6 | 6 |
| pH correction | | | | |
| HCl | | | 0.2 | 0.2 |
| NaOH | 12.5 | 12.5 | | |
| Final ion composition | | | | |
| $Cl^-$ | 124 | 72 | 168 | 13 |
| $Na^+ + K^+$ | 133 | 129 | 163 | 163 |

I, intracellular; E, extracellular; 1, standard chloride concentration; 2, low chloride concentration.

**Analysis.** Analysis of recordings was performed by using MATLAB (MathWorks, Natick, MA). Peak currents ($I$) from agonist exposures with concentrations spread over 3.5 logarithmic decades were fit to the Hill equation (eq. 1), for determination of the apparent affinity ($EC_{50}$), current magnitude ($I_{max}$), and Hill coefficient ($n_H$).

$$I = \frac{I_{max} \times [A]^{n_H}}{[A]^{n_H} + EC_{50}{}^{n_H}} \quad (1)$$

Theoretical reversal potentials for individual ions were calculated by using the Nernst equation (eq. 2), with room temperature ($T$ = 295 K), valence ($z$) of −1 for anions, and the Faraday and molar gas constants ($F$ and $R$, respectively). The subscripts i and o denote concentrations inside and outside the cell, respectively. Experimentally determined reversal potential ($E_{rev}$) values were calculated from membrane potential ramp data through interpolation of the membrane potential at $I$ = 0. Liquid junction potentials between each intracellular solution and extracellular saline were calculated with Clampex 9.0 (Molecular Devices) and were corrected a posteriori.

$$E_{rev} = \frac{RT}{F} \times \ln([Cl]_i/[Cl]_o) \quad (2)$$

Rectification was defined by using a quantitative metric, the rectification index (RI) (eq. 3). RI = 0 represents an ohmic response, whereas RI < 0 indicates outward rectification and RI > 0 indicates inward rectification.

$$RI = \frac{I_a}{I_b} \quad (3)$$

when $I_a = E_{rev} - 30$ mV and $I_b = E_{rev} + 30$ mV. Rectification generated by asymmetric concentrations of the permeant ion was predicted by using the Goldman-Hodgkin-Katz constant field equa-

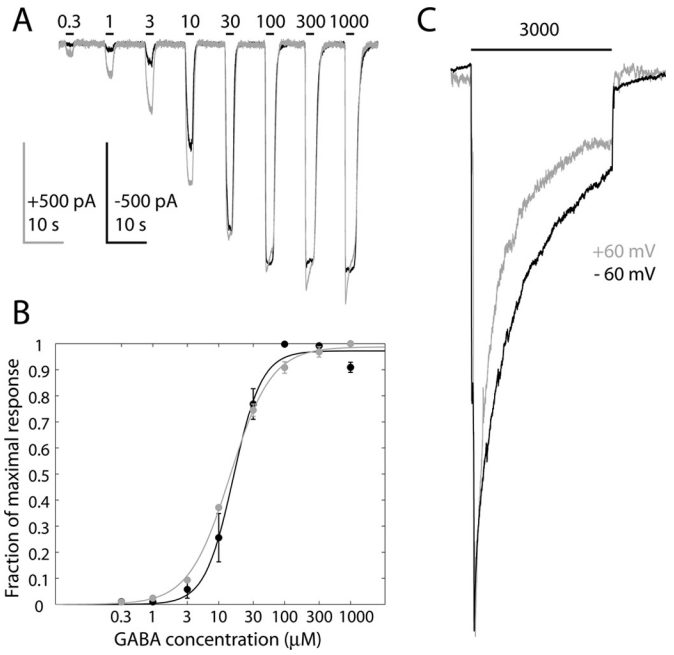


**Fig. 1.** Effects of inversion of the membrane potential from −60 to +60 mV on GABA concentration-response function, response magnitude, and accelerated desensitization. A, representative traces from the same cell gathered at holding potentials of +60 mV (gray) and −60 mV (black) in standard solutions (I1/E1; see Table 1). Bars indicate the duration of GABA application and are labeled with the concentration applied (in micromolar). Outward currents elicited at +60 mV are shown inversed, to highlight differences in kinetics. B, Hill equation fit of normalized peak currents at +60 mV (gray), compared with −60 mV (black). Data points depict mean ± S.E.M. ($n$ = 3). C, averaged currents elicited by a 10-s application of 3000 μM GABA to macroscopic outside-out patches held at +60 mV (gray) or −60 mV (black) ($n$ = 10). Currents are shown normalized with respect to the peak value, to highlight the increased speed of desensitization at the positive membrane potential.

tion (eq. 4) (Goldman, 1943; Hodgkin and Katz, 1949), where $\varphi$ represents $RT/F$ for simplicity.

$$I_{Cl} = pz^2 \frac{EF^2}{RT} \frac{[Cl]_i - [Cl]_o e^{-zE/\varphi}}{1 - e^{-zE/\varphi}} \quad (4)$$

Relative permeabilities for gluconate ($P_G/P_{Cl}$) in ion replacement solutions were calculated by using the Goldman-Hodgkin-Katz voltage equation (eq. 5) (Goldman, 1943; Hodgkin and Katz, 1949).

$$E_{Cl} = \frac{RT}{F} \times \ln\left(\frac{\frac{P_G}{P_{Cl}}[G]_i + [Cl]_i}{\frac{P_G}{P_{Cl}}[G]_o + [Cl]_o}\right) \quad (5)$$

**Statistical Analysis.** All data metrics were compared with those for wild-type α1β2γ2s receptors with the Student's $t$ test in MATLAB (MathWorks). Multicomponent measurements were compared by using analyses of variance with Tukey's and Dunnett's post hoc analyses. Significance was held at $p < 0.05$ for all metrics.

## Results

**Membrane Potential Affects the Magnitude and Shape of GABAergic Currents.** We observed striking differences in the α1β2γ2s GABA$_A$R concentration-response relationship when the direction of currents was reversed by changing the membrane holding potential (Fig. 1). Outward currents showed increased current magnitude at low GABA concentrations (0.3–10 μM) and enhanced desensitization at high GABA concentrations (100–1000 μM), compared with inward currents (Fig. 1A). It is noteworthy that although the apparent affinity for GABA remained independent of membrane potential ($EC_{50}$ values of 44.7 ± 1.1 μM and 51.2 ± 8.5 μM determined at +60 and −60 mV, respectively), the concentration-response relationship of outward currents had a significantly decreased Hill coefficient, compared with inward currents elicited from the same cell ($n_H$ values of 1.4 ± 0.03 and 2.1 ± 0.09 determined at +60 and −60 mV, respectively; mean ± S.E.M., $n$ = 3; Student's $t$ test) (Fig. 1B). Changes in the Hill coefficient can have several causes; alterations in receptor cooperativity, changes in modal activation, and ion channel desensitization can all manifest as differences in $n_H$ values (Jones and Westbrook, 1995). To test the latter factor, we measured the time course of current relaxation in the presence of 3 mM GABA in excised, outside-out, macroscopic patches. The data shown in Fig. 1C demonstrated a significant increase in the speed of desensitization with holding at +60 mV, compared with −60 mV, as indicated by weighted time constants for desensitization of −1.1 ± 0.02 and −0.56 ± 0.01 $s^{-1}$, respectively ($n$ = 10; Student's $t$ test, $p < 0.05$).

**IV Relationships Were Outwardly Rectifying at Low GABA Concentrations.** To better understand the changes in GABAergic currents at different voltages, we ramped the membrane potential from −60 mV to +60 mV and back, to determine IV relationships for α1β2γ2s receptors across the full concentration range for responses to GABA (0.3–1000 μM). First, we found that ramp application did not affect our ability to measure the GABA concentration-response relationship (at −60 mV, $EC_{50}$ = 48.7 ± 7.48 μM, $n_H$ = 1.94 ± 0.07, and $I_{max}$ = −3234 ± 261.4 pA; $n$ = 34) (Fig. 2C). Second, IV relationships were identical when they were determined from ramp data or from data obtained by stepping the membrane potential to a different level for each trace (data not shown).

IV relationships determined with the ramp protocol for α1β2γ2s receptors revealed a significant change in rectification with regard to the degree of channel activation by GABA (Fig. 2). RI values were determined at each GABA concentration; responses to 0.3 to 30 μM GABA showed outward rectification and were associated with RI values of <1, whereas RI values of ~1 were observed for the more-ohmic responses to 100 to 1000 μM GABA (Table 2). These two discrete populations, from currents elicited by low and high concentrations of GABA, had RI values that were significantly different from one another (Tukey's post hoc test, $p <$ 0.05) (Table 2). In light of the correlation between channel activation and rectification, we constructed a rectification profile for α1β2γ2s receptors (Fig. 2D). When RI values for these responses were plotted against the degree of receptor activation, we observed a linear correlation ($R^2$ = 0.99), with a slope of 0.37 ± 0.03 and an intercept of 0.67 ± 0.0 ($n$ = 34).

**Receptor Potentiation Is Voltage Dependent.** To test whether reduction of channel rectification was related to membrane potential or current amplitude, we investigated the effects of increases in receptor function by using positive allosteric modulators. We determined the IV relationship for GABA responses in the presence of intravenous and inhaled general anesthetics (Fig. 3A). The positive allosteric modulators etomidate, propofol, and isoflurane enhanced current magnitude by 162 ± 15, 148 ± 31, and 137 ± 28%, respec-

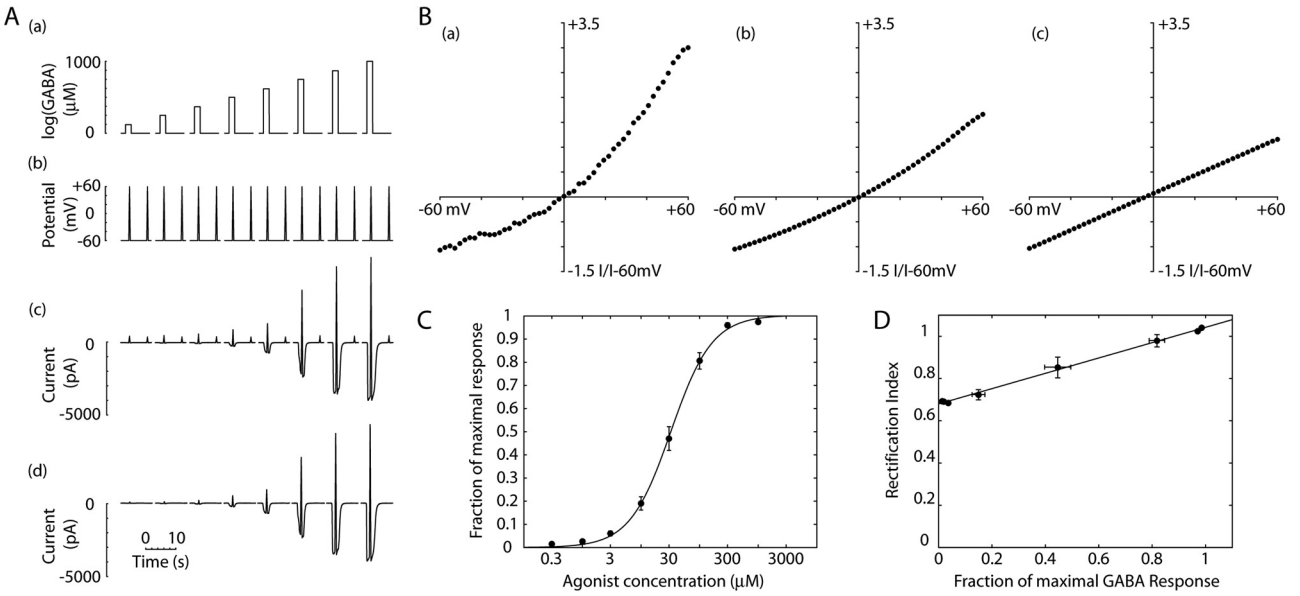


**Fig. 2.** Rectification profile for $\alpha1\beta2\gamma2s$ receptors. A, data acquisition scheme depicting the application of GABA (a) and the membrane holding potential (b). The membrane was initially held at −60 mV and then a voltage ramp, from −60 mV to +60 mV and back to −60 mV, was applied during the application of GABA and afterward in the baseline washout period. The baseline ramp was subsequently subtracted from both time points. Each sweep was then normalized with respect to its response at −60 mV, and the up-and-down component of each ramp was averaged to generate the IV relationship. Representative whole-cell current traces for the $\alpha1\beta2\gamma2s$ receptor determined in standard solutions (I1/E1; see Table 1) are shown before (c) and after (d) leak ramp subtraction. B, IV relationships for currents elicited with 3 μM (a), 30 μM (b), and 300 μM (c) GABA. Data points depict means ($n$ = 34). C and D, Hill equation fit of normalized peak currents at −60 mV (C) and rectification profile (D) for $\alpha1\beta2\gamma2s$ $GABA_A$Rs. Peak currents normalized with respect to the maximal response to GABA were plotted against RI values, and the data points were best fit with a straight line, $y = mx + b$. The slope ($m$) and intercept ($b$) of the fit were then used to compare experimental conditions. Data points depict mean ± S.E.M. ($n$ = 34).

TABLE 2

Rectification profile of GABA-elicited currents for $\alpha1\beta2\gamma2s$ receptors

RI values were plotted against the degree of activation ($I/I_{max}$) to generate a rectification profile. Tukey's post hoc test was used to compare all RI values with one another, with significance held at $P < 0.05$ for differences from 3 and 1000 μM GABA. Values are mean ± S.E.M. ($n$ = 34).

| | GABA | | | | | |
|---|---|---|---|---|---|---|
| | 3 μM | 10 μM | 30 μM | 100 μm | 300 μM | 1000 μM |
| RI | 0.68 ± 0.06* | 0.72 ± 0.04* | 0.85 ± 0.04 | 0.98 ± 0.02† | 1.02 ± 0.01† | 1.04 ± 0.01† |
| $I/I_{max}$ | 0.04 ± 0.01 | 0.15 ± 0.02 | 0.45 ± 0.05 | 0.82 ± 0.03 | 0.97 ± 0.01 | 0.99 ± 0.01 |

* Different from 1000 μM GABA.
† Different from 3 μM GABA.

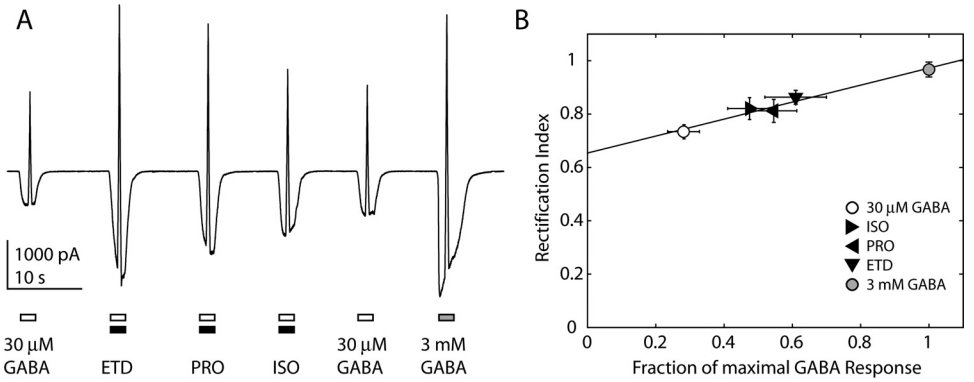


**Fig. 3.** Linear relationship between rectification and the degree of activation for $\alpha1\beta2\gamma2s$ receptors. A, representative trace elicited by 30 μM GABA plus coapplication of 1 μM etomidate (ETD), 2 μM propofol (PRO), and 330 μM isoflurane (ISO) in standard solutions (I1/E1; see Table 1). Peak currents were normalized with respect to the maximal response of each cell to 3 mM GABA. B, rectification profile for the positive modulators isoflurane (▶), etomidate (▼), and propofol (◀), as well as GABA at 30 μM (○) and 3 mM (◎). Data points depict mean ± S.E.M. ($n$ = 8).

tively, at a holding potential of −60 mV and by 66 ± 8, 81 ± 11, and 70 ± 14%, respectively, at a holding potential of +60 mV. We found that all three drugs reduced the amount of outward rectification (Fig. 3). In addition, we found that the effect on rectification increased with potentiation and was voltage dependent (Fig. 4). The rectification profile for these positive allosteric modulators had a slope of 0.37 ± 0.03 and an intercept of 0.62 ± 0.01, values that were not statistically different from those for GABA ($n$ = 8; Student's $t$ test, $p > 0.05$) (Fig. 3B).

**Outward Rectification Is Enhanced at Low $P_o$.** Because of the strong correlation between RI values and current magnitudes, we hypothesized that outward rectification would be more pronounced at low $P_o$. To test this hypothesis, we measured the IV relationships of two systems known to exhibit reduced agonist efficacy and thus low $P_o$. First, we

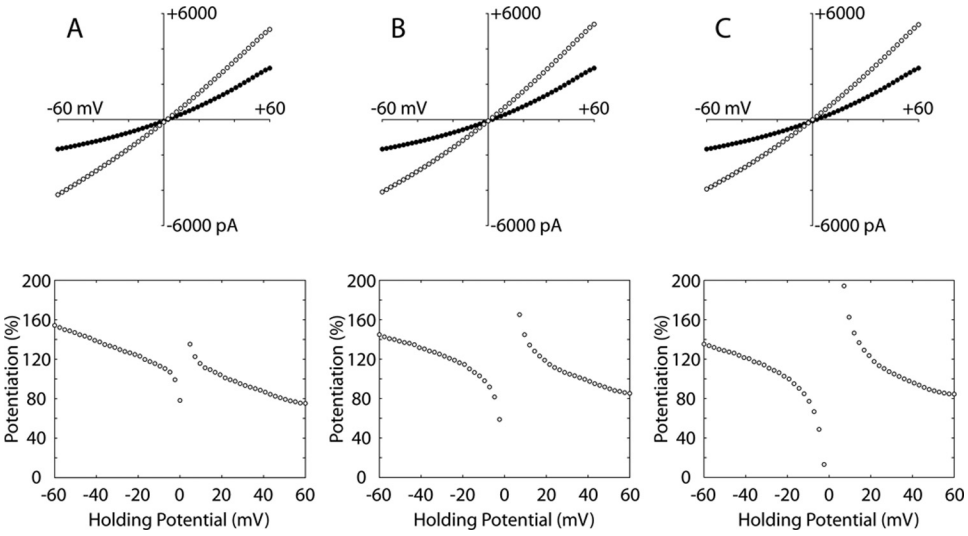


**Fig. 4.** Variations in the degree of potentiation by positive allosteric modulators with respect to membrane potential. IV relationships for 30 $\mu$M GABA (●) and 30 $\mu$M GABA potentiated by 1 $\mu$M etomidate (A), 2 $\mu$M propofol (B), and 330 $\mu$M isoflurane (C) (○) were studied in standard solutions (I1/E1; see Table 1). The degree of potentiation for each modulator became asymptotic at the reversal potential and decreased as the membrane potential became more positive.

characterized the IV relationship for receptors containing the $\alpha$1(L277A) mutation, which has been shown to yield impaired gating, resulting in reduced $P_o$ (Colquhoun, 1998; O'Shea and Harrison, 2000; Hille, 2001). The $\alpha$1(L277A)$\beta$2$\gamma$2s receptor exhibited significantly decreased GABA apparent affinity, with an $EC_{50}$ of 281 ± 43.6 $\mu$M ($n$ = 8; Student's $t$ test, $p <$ 0.05) (Fig. 5E); there were no changes in $n_H$ (1.34 ± 0.14) or $I_{max}$ (−1908 ± 620.9 pA), compared with the $\alpha$1$\beta$2$\gamma$2s receptor. The $\alpha$1(L277A) mutation enhanced outward rectification across the receptor's entire response to GABA. Current responses activated by 10 mM GABA in cells expressing $\alpha$1(L277A)-containing receptors had a RI value of 0.58 ± 0.06 (Fig. 5C). When the RI values of responses were plotted against the degree of activation, we found that the $\alpha$1(L277A) mutation significantly altered the rectification profile, with a slope of 0.01 ± 0.10 (Student's $t$ test, $p < 0.05$); however, the intercept of 0.71 ± 0.07 was not statistically different from that for $\alpha$1$\beta$2$\gamma$2s (Fig. 5F).

Second, we chose to test our $P_o$-rectification hypothesis by using the partial agonist piperidine-4-sulfonic acid (P4S), which has decreased efficacy, compared with the full agonist GABA, with $\alpha$1$\beta$2$\gamma$2s, to construct a rectification profile at

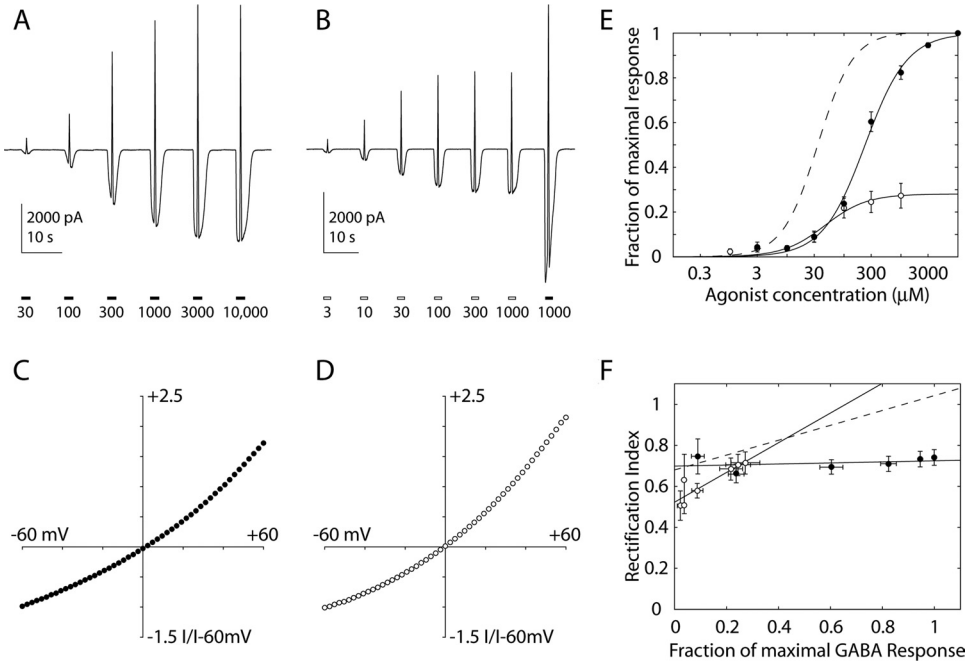


**Fig. 5.** Enhancement of outward rectification at low $P_o$. A, representative whole-cell current trace for the $\alpha$1(L277A)$\beta$2$\gamma$2s receptor in standard solutions (I1/E1; see Table 1). Bars indicate the duration of GABA application and are labeled with the concentration (in micromolar). B, representative currents elicited by the partial agonist P4S (□) and the maximal concentration of GABA (■) for the $\alpha$1$\beta$2$\gamma$2s receptor in standard solutions (I1/E1; see Table 1). Bars depict the application of agonist and are labeled with the concentration (in micromolar). C and D, IV relationships determined with maximally effective concentrations of 10 mM GABA for $\alpha$1(L277A)$\beta$2$\gamma$2s ($n$ = 8) (C) and 1 mM P4S for $\alpha$1$\beta$2$\gamma$2s ($n$ = 7) (D). E, Hill equation fit of normalized peak currents, showing the significant shift in GABA apparent affinity caused by the gating mutation (●) and the decreased efficacy of P4S (○). Dashed line, Hill fit for $\alpha$1$\beta$2$\gamma$2s GABAergic currents. F, enhanced outward rectification observed with $\alpha$1(L277A)$\beta$2$\gamma$2s (●) and P4S (○), with specific changes in the rectification profile of GABA$_A$Rs. The $\alpha$1(L277A) mutation showed rectification independent of the degree of channel activation, as indicated by a profile slope near 0, whereas the partial agonist significantly increased the slope and decreased the intercept. Dashed line, rectification profile for $\alpha$1$\beta$2$\gamma$2s GABAergic IV relationships.

low $P_o$ (Mortensen et al., 2004). P4S decreased $I_{max}$ by ~70%, compared with GABA, but was active within the same concentration range, with an apparent affinity of 41.8 ± 6.85 μM (Fig. 5E). There was significantly greater outward rectification with 1 mM P4S (RI = 0.71 ± 0.05) than with the same concentration of GABA (RI = 1.02 ± 0.01, $n = 7$; Student's $t$ test, $p < 0.05$) (Fig. 5D). Furthermore, the slope of the rectification profile for P4S, 0.85 ± 0.24, was significantly greater than that for GABA (Student's $t$ test, $p < 0.05$) (Fig. 5D).

**Outward Rectification at Low $P_o$ Is Independent of the Chloride Gradient.** To test whether current rectification through $GABA_A$Rs was dependent on $P_o$ or was related to the chemical force on permeant ions, we compared IV relationships in symmetrical chloride solution (I1/E1) and in solutions with decreased intracellular (I2) and extracellular (E2) chloride levels (see Table 1 for composition of solutions). In experiments using solutions with decreased extracellular chloride concentrations (I1/E2), the inward driving force on the permeant ion was decreased and the experimental reversal potential was shifted in a positive direction, as would be predicted for an anion pore (Table 2; Fig. 6). The reversal potential in I1/E2 was ~20 mV shifted from the theoretical $E_{Cl}$ value, which suggests a relative permeability of gluconate of 0.18 ± 0.02, in agreement with previously reported values (Jensen et al., 2005). As predicted by the constant field equation (Goldman rectification), results showed that a decrease in the intracellular concentration of chloride (I2/E1) specifically enhanced outward rectification (Fig. 6). This resulted in a significantly decreased intercept for the rectification profile, compared with the symmetrical chloride condition (Student's $t$ test, $p < 0.05$; $n = 8$) (Table 2). It is noteworthy that the correlation between rectification and the degree of channel activation persisted in altered ionic gradients, as shown by the slope values for each rectification profile, which were not statistically different from one another (Student's $t$ test, $p > 0.05$) (Table 2).

## Discussion

In this study, we demonstrated that the outward rectification of currents flowing through $GABA_A$R ion channels is inversely related to the channel open probability and occurs independently of Goldman rectification. As a result, the potentiating effects of positive modulators are markedly less when chloride ions are flowing into the cell. Therefore, the $GABA_A$R ion channel does not behave as a simple ohmic pore.

We established IV relationships for $\alpha1\beta2\gamma2$s receptors under several different conditions and found that rectification of current flow was inversely related to the degree of channel activation. When the magnitude of the current was increased through the application of a greater concentration of GABA or a positive allosteric modulator, the degree of current rectification was reduced; responses became more ohmic (Figs. 2 and 3). This did not occur when current amplitude was increased with simple changes in the electrical driving force on the permeant ion, which suggests that rectification is linked to the direction of chloride flux and is not caused by a direct action of membrane potential on the ion channel protein (as occurs with voltage-gated ion channels).

The link between the degree of channel activation and the amount of rectification was further emphasized in experiments in which we hindered channel-gating by using a par-

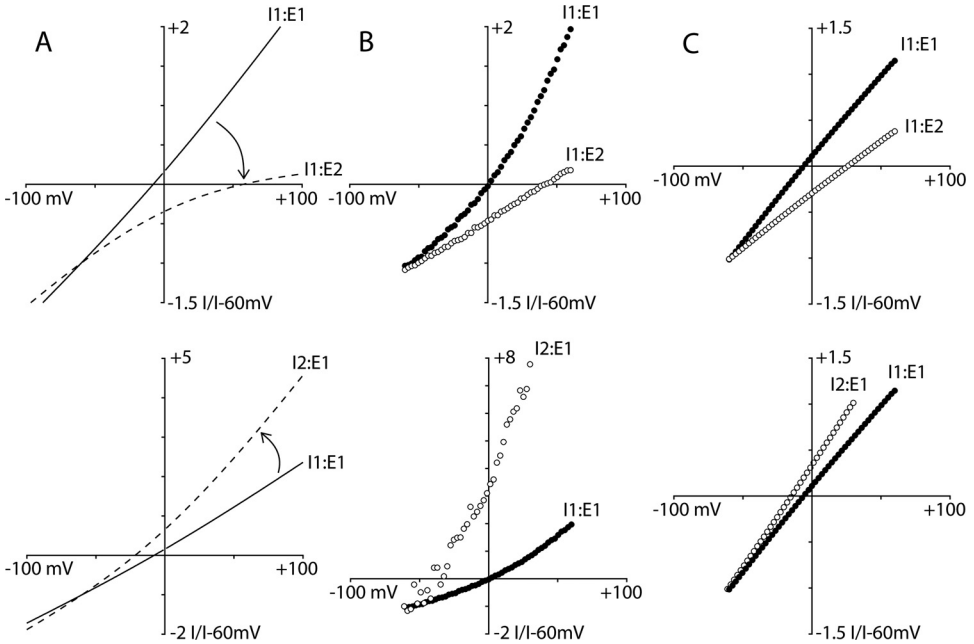


**Fig. 6.** Predicted and measured IV relationships of $\alpha1\beta2\gamma2$s receptors under chloride gradient conditions. A, theoretical IV relationships in ion replacement solutions, as predicted by the constant field equation. The IV relationship in symmetrical chloride (I1/E1; see Table 1) is predicted to be ohmic (solid line). Rectification is predicted to occur when the chemical driving force on the permeant ion is asymmetric (dashed line). For example, decreasing the extracellular concentration of chloride (E2) caused a rightward shift in $E_{Cl}$ and induced inward rectification (top); conversely, decreasing the intracellular concentration of chloride (I2) caused a leftward shift in $E_{Cl}$ and enhanced outward rectification (bottom) (see Table 1 for composition of solutions). B and C, experimentally determined IV relationships for $\alpha1\beta2\gamma2$s generated from currents elicited with 10 μM (B) and 1 mM (C) GABA in ion replacement solutions, with altered reversal potential and RI values. Outward rectification was enhanced, as predicted for decreased intracellular chloride concentrations; however, the degree of rectification was greater than predicted at low $P_o$. In addition, the linearization of the IV relationship at maximally effective concentrations of GABA produced less rectification than was predicted for both conditions.

tial agonist to activate the channel and by introducing a well-characterized, deleterious, gating mutation. In both cases, impaired channel gating, and thus decreased $P_o$, was associated with increased outward rectification (Fig. 5). Taken together, all of these results demonstrate a robust link between $GABA_AR$ channel $P_o$ and current rectification.

Upon GABA application, the channel quickly transitions from the closed state to the open state as the receptor binds its ligand and the ion channel gate is opened. At low $P_o$, the more frequent presence of gating elements within the pore may hinder ion permeation directionally, generating an asymmetry similar to that observed in the low intracellular chloride condition (I2/E1) (see Table 1 for solution composition). At high $P_o$, the ion channel behaved as a simple ohmic pore, with a linear relationship between the magnitude of current and the electrical driving force contributed by the membrane potential. With prolonged GABA application, the open channel transitions to a nonconducting desensitized state. We observed faster desensitization of currents elicited by maximally effective concentrations of GABA when chloride flux was inward (Fig. 1). Therefore, it is tempting to speculate that the asymmetry established by the primary channel gate remains at high $P_o$ but is masked by the enhanced desensitization of outward currents, and this serves to linearize the IV relationship. If this is the case, then the closed-open and open-desensitized transitions must be mediated by different structures within the ion channel. If the primary channel gate and the desensitization gate were the same structure, then the decrease in $P_o$ caused by desensitization would also correspond with an increase in outward rectification and not the linear IV relationship that we observed. It is noteworthy that many therapeutic agents modulate channel function by directly influencing the probability that the receptor would occupy one conformational state over another, and our results show that this state dependence, and hence drug action, is inherently voltage dependent.

To determine whether the rectification of currents was linked to the membrane potential or the direction of chloride flux, we manipulated the chloride gradient to shift the reversal potential and determined the effect on current rectification (Fig. 6; Supplemental Fig. 1). The constant field equation predicts that rectification would occur under ionic gradient conditions when the chemical driving force on the permeant ion is much greater in one direction (Goldman, 1943; Hodgkin and Katz, 1949); more specifically, for $GABA_ARs$ conducting anions, outward rectification at negative reversal potentials, a linear response under symmetrical ion conditions, and inward rectification at positive reversal potentials are predicted (Fig. 6). This Goldman rectification was shown to occur for inhibitory postsynaptic currents recorded from cultured hippocampal neurons in asymmetrical (and physiological) chloride solutions (Barker and Harrison, 1988).

Our results showed that decreasing the extracellular concentration of chloride (I1/E2) (see Table 1 for solution composition) caused a rightward shift in $E_{Cl}$ and the IV relationship was more inwardly rectifying, as predicted by the constant field equation (Fig. 6). Likewise, decreasing the intracellular concentration of chloride (I2/E1) (see Table 1 for solution composition) shifted $E_{Cl}$ to the left and induced outward rectification that was greater than that under symmetrical chloride conditions (Fig. 6). During experimentation, the degree of rectification under all three chloride gradient conditions was greater than that predicted for low-$P_o$ conditions. Conversely, the linear response of the IV relationship at high $P_o$ was less rectifying than predicted. It is noteworthy that the relationship between $P_o$ and rectification (the slope of the rectification profile) persisted independent of Goldman rectification (Table 3; Supplemental Fig. 1). Therefore, our results show that, for $GABA_ARs$, the curvature of the IV relationship at low $P_o$ is attributable to the direction of chloride permeation and is not simply voltage dependent.

From the constant field equation, it is clear that only two variables can account for outward rectification: the permeability of chloride ($P_{Cl}$) and the concentration of chloride. If we assume that permeability is constant, then the local extracellular concentration of chloride at the pore entrance must be greater than the bulk solute concentration to account for the enhanced inward drive on the ion at low $P_o$. Bormann (1988) showed that increasing chloride concentration equally on both sides of the membrane increased channel conductance by increasing the number of charge carriers available. With this in mind, it is easy to envision how gating elements could restrict anion access to parts of the pore, which would result in charge asymmetry and thus rectification. Alternatively, if the inward permeability of chloride was greater than the outward permeability, then the magnitude of outward currents would be enhanced, which would result in outward rectification. Calculation of chloride permeability from experimental current values, with the assumption that the chloride concentration would remain constant, predicted that $P_{Cl}$ would vary with changes in membrane potential (Fig. 7). $P_{Cl}$ increased as the membrane potential became more positive at low $P_o$, whereas $P_{Cl}$ was more constant at high $P_o$. Tangents to the $P_{Cl}$-potential relationship at ±60 mV revealed a striking disconnect (Fig. 7). $P_{Cl}$ not only was strongly voltage-dependent but also seemed to be tending toward different inward and outward limits, which emphasizes the asymmetry of chloride permeation. Our results show that the $GABA_AR$ does not behave as a simple ohmic pore. To predict receptor behavior and specific drug actions

TABLE 3

Rectification in ionic gradients

The intracellular and extracellular concentrations of chloride were varied to shift the reversal potential and to induce Goldman rectification. Decreasing the intracellular chloride concentration (I2/E1) was sufficient to enhance outward rectification significantly and to decrease the intercept of the rectification profile. Outward rectification at low $P_o$ persisted in all ion gradient conditions, as shown by the slopes of the rectification profiles.

| Solutions | $E_{Cl}$ | $E_{rev}$ | Slope | Intercept | $n$ |
|---|---|---|---|---|---|
| | *mV* | *mV* | | | |
| I1/E1 | −7.75 | −9.32 ± 0.55 | 0.37 ± 0.03 | 0.67 ± 0.04 | 34 |
| I1/E2 | +57.0 | 40.5 ± 4.53 | 0.43 ± 0.02 | 0.64 ± 0.04 | 6 |
| I2/E1 | −21.6 | −22.3 ± 2.31 | 0.39 ± 0.04 | 0.56 ± 0.05* | 8 |

* $P < 0.05$, compared with I1/E1, Student's $t$ test.

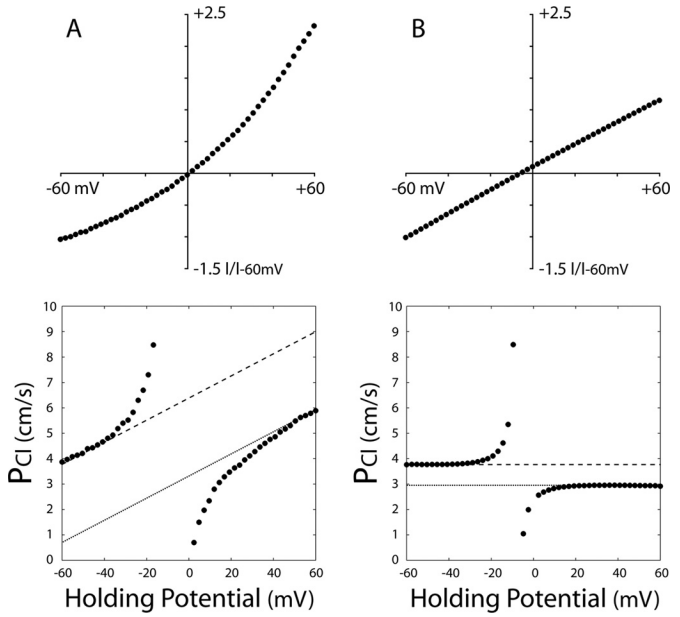


**Fig. 7.** Nonconstant chloride permeability at low open probability. The constant field equation was used to calculate the channel permeability to chloride from currents elicited with 10 $\mu$M (A) and 1 mM GABA (B). Mean IV relationships for $\alpha1\beta2\gamma2s$ receptors ($n$ = 34) were determined in standard solutions (I1/E1; see Table 1) (top) and were used to calculate $P_{Cl}$ values (bottom). Permeability was asymptotic around the reversal potential. Lines represent the trends of chloride efflux (dashed lines) and influx (dotted lines).

fully, we must consider the open probability of the channel and the direction of ion flow.

The $GABA_AR$ is the primary site of action for many clinically important therapeutic agents, including benzodiazepines and general anesthetics, which enhance channel activity by modulating $P_o$. Our results show an apparent voltage dependence of such allosteric modulators of the $GABA_AR$. The magnitude of potentiation by general anesthetics was greater at negative potentials and decreased as the membrane potential became more positive (Fig. 4). The magnitude of current potentiation by allosteric modulators is attenuated at positive membrane potentials because the IV relationship becomes linear as $P_o$ increases. Therefore, the degree of enhancement caused by positive modulators is blunted at positive potentials. Our findings suggest that experiments performed under common in vitro conditions (holding potential of −60 mV in symmetrical chloride solution) would overestimate the effects of positive allosteric modulators at positive potentials or when channel activation results in chloride influx. It is noteworthy that the potentiating effect of etomidate at −60 mV was 90% greater than that at +60 mV, whereas the effect of isoflurane was only 75% greater, which suggests a drug-specific effect. The action of $GABA_AR$ modulators was shown previously to be voltage dependent; benzodiazepines were shown to slow the rate of deactivation of GABAergic currents from cultured cerebellar granule cells at negative potentials but not at positive potentials (Mellor and Randall, 1998), inhibition of currents through $\alpha1\beta2\gamma2L$ receptors by amphiphiles such as SDS is greater at positive potentials (Chisari et al., 2010), and the neurosteroid allopregnanolone was shown to decrease the magnitude of outward currents but not inward currents for $\alpha4\beta2\delta$ receptors (Shen et al., 2007). Our results show that the magnitude of potentiation of $\alpha1\beta2\gamma2s$ receptors by intravenous and inhaled general anesthetics is dependent on the direction of chloride flux; etomidate, propofol, and isoflurane all selectively enhanced inward current magnitude.

In conclusion, our results showed that the apparent voltage dependence of the $\alpha1\beta2\gamma2s$ $GABA_AR$ is inversely related to channel open probability. The relative magnitude of outward currents was greater than the magnitude of inward currents under low-$P_o$ conditions, which resulted in outward rectification. At high $P_o$, desensitization of outward currents was enhanced, which resulted in linearization of the IV relationship. The different effects of gating and desensitization on the IV relationship suggest that these mechanisms are mediated by different channel structures. We also showed that the degree of receptor enhancement by therapeutic agents was voltage dependent; in particular, potentiation was greater at negative membrane potentials. Finally, our results showed that the apparent voltage dependence of channel activation and modulation was actually mediated by the direction of chloride flux. Therefore, for proper understanding of receptor activation and modulation, we must take both the channel open probability and the direction of ion flux into account.

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#### Authorship Contributions

*Participated in research design:* O'Toole and Jenkins.
*Conducted experiments:* O'Toole.
*Performed data analysis:* O'Toole.
*Wrote or contributed to the writing of the manuscript:* O'Toole and Jenkins.

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