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Agonist Gating and Isoflurane Potentiation in the Human γ-Aminobutyric Acid Type A Receptor Determined by the Volume of a Second Transmembrane Domain Residue

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ABSTRACT

γ-Aminobutyric acid type A ($GABA_A$)receptors are targets for allosteric modulation by general anesthetics. Mutation of Ser270 within the second transmembrane domain of the $GABA_A$ receptor α subunit can ablate the modulation of the receptor by the anesthetic ether isoflurane. To investigate further the function of this critical amino acid residue, we made multiple amino acid substitutions at Ser270 and analyzed the concentration-dependent gating by GABA and regulation by isoflurane in each mutant receptor. There is a strong negative correlation between the EC_{50} for GABA and the molecular volume of the amino acid residue at position 270. Replacement of Ser by large residues such as His and Trp produced a shift of the GABA concentration-response curve to the left, whereas replacement of Ser with Gly had the opposite effect. There also was a strong negative association between the molecular volume of the amino acid residue at 270 and the degree of enhancement of submaximal GABA responses by isoflurane. These results indicate the significance of the amino acid at position α270 in gating of the $GABA_A$ receptor. In addition, the data on isoflurane are consistent with the existence of a cavity of finite size in the region of α270 that may be filled by the anesthetic molecule or by the side chain of a larger residue at α270. The introduction of isoflurane, or of a large residue, into this cavity may stabilize the open state of the $GABA_A$ receptor relative to the closed state.

The major family of receptors for γ-aminobutyric acid (GABA), the $GABA_A$ receptors, are members of the "gene superfamily" of "Cys-loop" ligand-gated ion channels (Ortells and Lunt, 1995). The $GABA_A$ receptor is a pentameric complex of protein subunits surrounding a central Cl^--permeable pore (Bormann et al., 1987). Native $GABA_A$ receptors usually contain α, β, and γ subunits (McKernan and Whiting, 1996) in a 2:2:1 stoichiometry (Chang et al., 1996; Tretter et al., 1997). The existence of six α subunit isoforms enables considerable anatomical and functional diversity of $GABA_A$ receptors (Fritschy and Mohler, 1995; Sieghart, 1995; Nusser at al., 1996). In particular, the α subunit isoform may influence agonist potency (Levitan et al., 1988), agonist efficacy (Ebert et al., 1994), regulation by benzodiazepines (Wafford et al., 1992), and channel kinetics (Tia et al., 1996; Lavoie et al., 1997). Many general anesthetics are allosteric modulators of the $GABA_A$ receptor (Franks and Lieb, 1994; Harris et al., 1995), and mutation of a critical Ser residue within the second transmembrane domain (TM2) of the $GABA_A$ receptor α subunit can ablate or mask the modulation of the receptor by the anesthetic ethers enflurane (Mihic et al., 1997) and isoflurane (Krasowski et al., 1998). Results obtained with the $GABA_A$ receptor α2 S270I (Mihic et al., 1997) and S270H mutants (Krasowski et al., 1998) strongly suggested an important role for α Ser270 in the regulation of $GABA_A$ receptor function by these anesthetics. Because the side chains of Ile and His are both physically larger and Ile is considerably more hydrophobic than is Ser, we sought to determine the effects of other amino acid substitutions at this position. The multiple point mutants were then used to investigate whether size, hydrophilicity, or some other physical parameter of the residue at this position was relevant to the gating behavior and anesthetic modulation of the $GABA_A$ receptor.

Materials and Methods

Site-Directed Mutagenesis. To create the mutant series at $GABA_A$ receptor α2 Ser270, mutations were introduced into the

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ABBREVIATIONS: GABA, γ-aminobutyric acid; Glu, glutamate; HEK, human embryonic kidney; α2(S270X)β1, receptor harboring a mutation in the α subunit at Ser270; AChR, nicotinic acetylcholine receptor; TM1, -2, -3, first, second, third transmembrane domain.

cDNA encoding the human $GABA_A$ receptor $\alpha 2$ subunit at bases 890 to 892, with simultaneous loss of a *Dde*I restriction site. Oligonucleotides, 24 to 30 bases in length, were obtained from Operon Technologies (Alameda, CA) and 5′-phosphorylated using polynucleotide kinase, and used to create mutations using the unique site-elimination method (Deng and Nickoloff, 1992) (USE kit; Pharmacia Biotechnology Inc., Piscataway, NJ), a two-primer method in which a unique *Ssp*I site in the expression vector pCIS2 was mutated concurrently to an alternate restriction site (*Eco*RV or *Mlu*I). *Ssp*I digestion then was used to select in favor of mutants, and clones were screened for the appearance of the desired mutation by digestion with *Dde*I. A few additional mutations were created, at Thr268, Leu269, and Ile271 in $GABA_A$ receptor $\beta 1$, and at Ser265 in $GABA_A$ receptor $\beta 1$, using a *Pfu* polymerase/*DpnI* selection method (QuikChange; Stratagene, La Jolla, CA). All restriction enzymes and polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). The sequences of all cDNA inserts were confirmed throughout by double-stranded sequencing (Sequenase 2.0; U.S. Biochemical Corp., Cleveland, OH), using appropriate oligonucleotide sequencing primers, and are available for inspection on request.

Cell Culture and Transfection. Wild-type or mutant receptor $GABA_A$ receptor cDNAs were expressed via the vector pCIS2, which contains one copy of the strong promoter from cytomegalovirus and a polyadenylation sequence from simian virus 40. These constructs were used to transfect human embryonic kidney (HEK)293 cells (American Type Culture Collection, Rockville, MD), as described previously (Pritchett et al., 1988). HEK293 cells were maintained in culture on glass coverslips; cells were passaged weekly by trypsin treatment up to 15 times before being discarded and replaced with early passage cells. Each coverslip of cells was transfected using the $CaPO_4$ precipitation technique (Okayama and Chen, 1987). One to five micrograms of each cDNA was used per coverslip; the cDNA was in contact with the cells for 24 h in an atmosphere containing 3% CO_2 before being removed and replaced with fresh culture medium in an atmosphere of 5% CO_2.

Electrophysiology. The coverslips were transferred, between 24 and 72 h after removal of the cDNA, to a large chamber (60 ml) and perfused continuously (20 ml/min) with extracellular medium. Recordings from HEK293 cells were made using the whole-cell patch-clamp technique, as described previously (Koltchine et al., 1996). Patch pipettes contained: 145 mM *N*-methyl-D-glucamine hydrochloride, 5 mM dipotassium ATP, 1.1 mM EGTA, 2 mM $MgCl_2$, 5 mM HEPES/KOH, 0.1 mM $CaCl_2$ (pH 7.2). Pipette resistance was 4 to 5 MΩ. The extracellular medium contained: 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM D-glucose, 10 mM HEPES/NaOH (pH 7.4). HEK293 cells were voltage-clamped at −60 mV. In addition to the continuous slow bath perfusion, drugs and solutions were applied rapidly to the cell by local perfusion using a motor-driven solution exchange device (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA). Laminar flow was achieved by driving all solutions at identical flow rates via a multichannel infusion pump (Stoelting; Wood Dale, IL). Loss of the anesthetic isoflurane using this perfusion device has been measured using gas chromatography and represents only 5 to 10% of the total applied drug concentration (M. D. Krasowski, unpublished observations). The solution changer was driven by protocols in the acquisition program pCLAMP5 (Axon Instruments, Foster City, CA), as described previously (Koltchine et al., 1996). Responses were digitized (TL-1-125 interface; Axon Instruments) using pCLAMP5 (Axon Instruments). Numerical data are presented throughout as mean ± S.E. ATP was obtained from Calbiochem and isoflurane from Ohmeda (Madison, WI); all other chemicals were obtained from Sigma (St. Louis, MO).

Data Analysis, Curve Fitting, and Determination of Parameters of Dose–Response Curves. Control GABA concentration-response data were expressed as a fraction of the maximal response

TABLE 1

A summary of the characteristics of GABA responses in the $\alpha 2(S270X)\beta 1$ series of $GABA_A$ receptors

EC_{50} concentration, Hill coefficient (n_H), and I_{max} are given for each receptor as mean ± S.E. for N cells determined, using the methods described. Statistical significance was assessed using a Student's *t* test.

$GABA_A$-R	EC_{50} (μM)	N	n_H	I_{max} (pA)
$\alpha 2\beta 1$ WT	8.6 ± 0.8	9	1.9 ± 0.2	455 ± 76
$\alpha 2(S270A)\beta 1$	15.5 ± 2.2*	4	1.6 ± 0.2	168 ± 36
$\alpha 2(S270C)\beta 1$	4.8 ± 0.4*	5	1.4 ± 0.2	168 ± 23
$\alpha 2(S270E)\beta 1$	3.0 ± 0.3**	7	1.5 ± 0.1	197 ± 28
$\alpha 2(S270F)\beta 1$	1.3 ± 0.3**	7	1.6 ± 0.1	219 ± 20
$\alpha 2(S270G)\beta 1$	27.3 ± 1.9**	4	1.3 ± 0.2*	146 ± 16
$\alpha 2(S270H)\beta 1$	2.4 ± 0.5**	9	1.5 ± 0.1	322 ± 40
$\alpha 2(S270I)\beta 1$	1.3 ± 0.2**	6	2.0 ± 0.4	289 ± 49
$\alpha 2(S270R)\beta 1$	1.1 ± 0.2**	5	1.9 ± 0.4	352 ± 60
$\alpha 2(S270T)\beta 1$	7.2 ± 1.5	6	1.5 ± 0.1	442 ± 62
$\alpha 2(S270W)\beta 1$	0.9 ± 0.3**	5	1.0 ± 0.2*	347 ± 85
$\alpha 2(S270Y)\beta 1$	0.9 ± 0.2**	4	1.2 ± 0.1*	332 ± 86

* $p < .01$ compared with the control group.
** $p < .001$ compared with the control group.

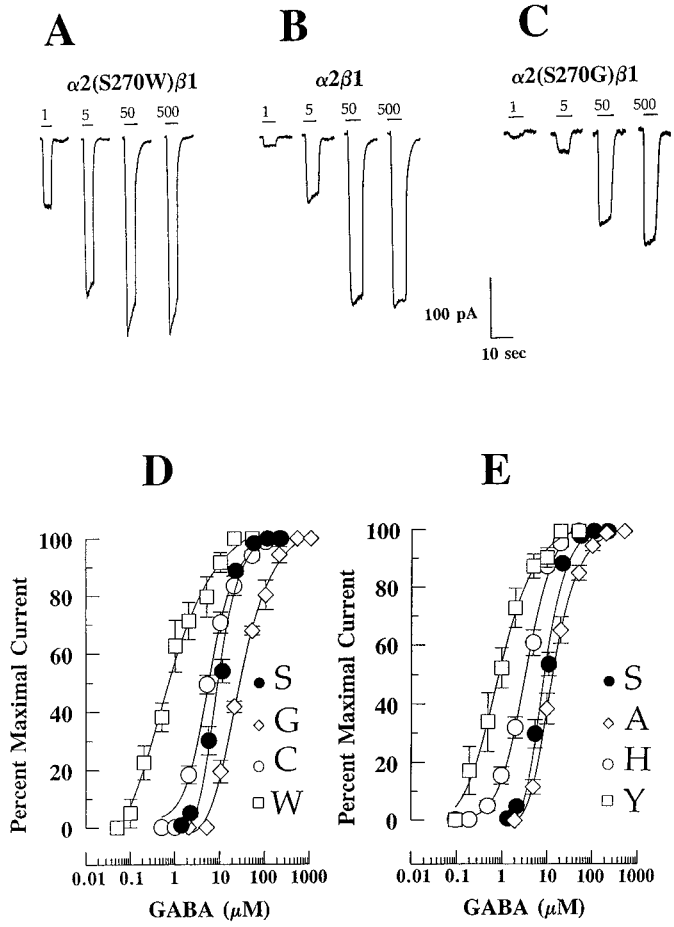

Fig. 1. HEK293 cells coexpressing mutant $\alpha 2(S270X)$ and wild-type $\beta 1$ $GABA_A$ receptor subunits form functional GABA-activated Cl^- channels. A–C, representative currents elicited by 2-s applications of 1, 5, 50, and 500 μM GABA recorded from cells expressing receptors made up of $\alpha 2(S270W)\beta 1$ (A), wild-type $\alpha 2\beta 1$ (B), and $\alpha 2(S270G)\beta 1$ subunits (C). Notice the modest current activated by 5 μM GABA when α2 Ser270 is mutated to Gly, whereas the same concentration of GABA activates near-maximal current in receptors in which Ser270 is mutated to Trp. The bars above the current traces indicate the period of GABA application and are labeled with the GABA concentration in μM. D, concentration-response curves for GABA in wild-type (S), and $\alpha 2(S270G)\beta 1$, $\alpha 2(S270C)\beta 1$, and $\alpha 2(S270W)\beta 1$ mutant $GABA_A$ receptors. E, concentration-response curves for GABA in wild-type (S), and $\alpha 2(S270A)\beta 1$, $\alpha 2(S270H)\beta 1$, and $\alpha 2(S270Y)\beta 1$ mutant $GABA_A$ receptors.

to GABA in each cell, allowing normalized data from different cells to be combined. Pooled data were fitted, using a weighted sum of least-squares method, to a Hill equation of the form:

$$\frac{I}{I_{max}} = 100 \times \frac{[GABA]^n}{[GABA]^n + EC_{50}{}^n} \quad (1)$$

where I is the whole-cell current amplitude expressed as a percentage of the current maximal peak current (I_{max},) [GABA] is the GABA concentration, EC_{50} is the GABA concentration eliciting a current equal to half of I_{max}, and n is the Hill coefficient. Percentage potentiation of a submaximal GABA response by the anesthetic isoflurane was then calculated as the percentage increase above the control (EC_{20}) response to GABA in the presence of anesthetic. Statistical comparisons were made, and significance was assessed using Student's t test.

Results

Eleven mutant GABA$_A$ receptors harboring a mutation in the α subunit at Ser270 [α2(S270X)β1] were studied by whole-cell voltage clamp, after transient expression in HEK-293 cells. First, reproducible whole-cell Cl^- currents activated by 50 nM to 1 mM GABA were recorded in the absence of other drugs. In all mutant receptors, the maximal response amplitude was between 30 and 100% of that in the wild-type GABA$_A$ receptor (Table 1). Figure 1A shows that in the wild-type receptor, 5 μM GABA recruited a current that was ~30% of the maximal response activated by 500 μM GABA. When Ser270 was mutated to Trp, the same concentration of GABA elicited a near-maximal response; conversely, when Ser270 was mutated to Ala, only 2% of the maximum current was obtained at 5 μM GABA.

Full concentration-response curves for GABA were determined in cells expressing the wild-type GABA$_A$ receptor and the α2(S270X)β1 receptor mutants. The currents activated by at least seven concentrations of GABA were expressed as a fraction of the maximal GABA response, and these normalized data were fitted by a Hill equation (see *Methods*, equation 1). Mutation of Ser270 to smaller amino acid residues (Ala and Gly) produced a rightward shift in the GABA concentration-response curve; conversely, mutating Ser270 to larger amino acid residues such as His and Tyr caused a leftward shift in the curves (Fig. 1B). Concentration-response curves also were constructed for wild-type and mutant α2β1 and α2β1γ2s GABA$_A$ receptors (Table 2). As described previously (Draguhn et al., 1990; Smart et al., 1991; Horenstein and Akabas, 1998), the coexpression of the γ2s subunit along with the $\alpha\beta$ subunits was detected by, and associated with, changes in zinc sensitivity of the resulting GABA$_A$ receptor. The inclusion of the γ2s subunit had little or no effect on the GABA EC_{50} in receptors containing either wild-type or mutant α2 subunits (Fig. 2). Therefore, we suggest that the data from α2(S270X)β1 GABA$_A$ receptors also apply to α2(S270X)β1γ2 GABA$_A$ receptors. The isoflurane experiments described below were not repeated in the presence of the γ2 subunit, because this subunit is not required for modulation by isoflurane (Harrison et al., 1993; Krasowski et al., 1998).

The effects of Trp mutations at positions adjacent to α2 Ser270 and at the β1 homolog Ser265 also were determined. The α2(T268W) mutant expressed poorly or not at all (typically <50 pA GABA current). The α2(I271W) and α2(L269W) mutations had little effect on the GABA concentration-response relationship. Mutation of Ser265 to Trp in the β1 subunit reduced the GABA EC_{50}, but less so than for the α2(S270W) mutant (Table 2). Coexpressing α2(S270W) and β1(S265W) mutant subunits produced a receptor with a similar GABA EC_{50}, but weak expression.

Clinically relevant concentrations of anesthetics such as isoflurane (0.2–0.7 mM) enhance submaximal responses in neuronal GABA$_A$ receptors (Jones et al., 1992). This enhancement is associated with a parallel leftward shift of the GABA concentration-response curve for the wild-type GABA$_A$ receptor (Fig. 3A), consistent with an increase in the apparent affinity of GABA in the presence of the anesthetic. This leftward shift in the GABA concentration-response curve is strikingly similar to that observed on mutagenesis of Ser270 to Trp (Fig. 3B). Potentiation of receptor function by isoflurane was assessed by using an EC_{20} concentration of GABA appropriate for the receptor under study. Figure 3C shows the modulation by isoflurane of responses to a submaximal (EC_{20}) concentration of GABA in cells expressing wild-type α2β1, mutant α2(S270A)β1, and mutant α2(S270W)β1 GABA$_A$ receptors. In the α2(S270A) receptor mutant, the magnitude of potentiation by isoflurane is similar to that seen in the wild-type GABA$_A$ receptor, whereas in the α2(S270Y) mutant, isoflurane does not enhance GABA currents. Table 3 shows the percentage modulation by 0.5 and 1 mM isoflurane of GABA-activated currents in the 11

TABLE 2

A summary of GABA response data for the α2(S270X)β1γ2 series of GABA$_A$ receptors and for mutant GABA$_A$ receptors of the form α2*β1, in which mutations are made in residues neighboring Ser270

EC_{50} concentration, Hill coefficient (n_H), and I_{max} *are given for each receptor as mean ± S.E. for N cells determined, using the methods described. Statistical significance was assessed using Student's t* test.

GABA$_A$-R	EC_{50} (μM)	N	n_H	I_{max} (pA)
α2β1 WT	8.6 ± 0.8	9	1.9 ± 0.2	455 ± 76
α2(S270A)β1	15.5 ± 2.2*	4	1.6 ± 0.2	168 ± 36
α2(S270W)β1	0.9 ± 0.3**	5	1.0 ± 0.2*	347 ± 85
α2β1γ2 WT	12.0 ± 1.3	5	1.7 ± 0.2	779 ± 453
α2(S270A)β1γ2	19.0 ± 2.7*	4	1.9 ± 0.1	360 ± 99
α2(S270W)β1γ2	1.7 ± 0.9**	4	1.2 ± 0.3	214 ± 28
α2β1 WT	8.6 ± 0.8	9	1.9 ± 0.2	455 ± 76
α2(L269W)β1	9.3 ± 1.0	5	1.3 ± 0.1*	216 ± 27
α2(I271W)β1	8.9 ± 0.9	5	1.5 ± 0.2	163 ± 17
α2β1(S265W)	2.4 ± 0.7**	5	1.2 ± 0.1*	348 ± 36
α2(S270W)β1(S265W)	3.5 ± 0.5**	5	1.5 ± 0.3	137 ± 13

* $p < .01$ compared with the control group.
** $p < .001$ compared with the control group.

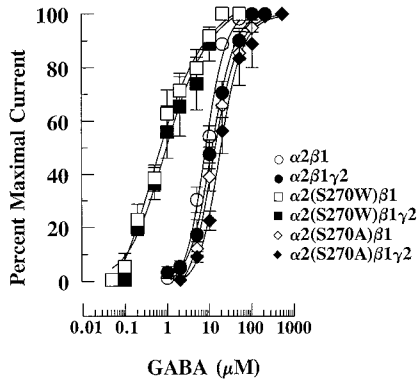

Fig. 2. Inclusion of the γ subunit does not alter significantly the GABA concentration-response curve in GABA$_A$ receptors consisting of α2(S270X)β1.

α2(S270X)β1 mutant receptors. The largest potentiation was observed in the wild-type; α2(S270A)β1 showed identical potentiation. Mutation of Ser270 to Gly, Cys, or Thr resulted in modest anesthetic potentiation; Phe, Ile, His, and larger substitutions rendered the receptor insensitive to clinically relevant concentrations of isoflurane. An extensive study of the concentration-effect curve for GABA potentiation by isoflurane was made in three mutant receptors: α2(S270C)β1, α2(S270Y)β1, and α2(S270W)β1, and compared with the results obtained in the wild-type receptor (Fig. 3D). These data suggest that the effects of Cys and Thr mutations result from a decrease in efficacy for potentiation rather than from a change in the affinity of the receptor for isoflurane. The mutant receptors α2(L269W)β1 and α2(I271W)β1 showed near-normal potentiation by isoflurane (data not shown).

Discussion

Gating of Receptor Mutants by GABA. We conclude from our data that substitution at α2 Ser270 did not compromise ion channel function, consistent with the notion that these point mutations do not induce large changes in secondary structure, at least in the region of the pore. No mutation at Ser270 completely abolished gating by GABA, consistent with the idea that the structure of the agonist binding site also was not disrupted dramatically. The mutations

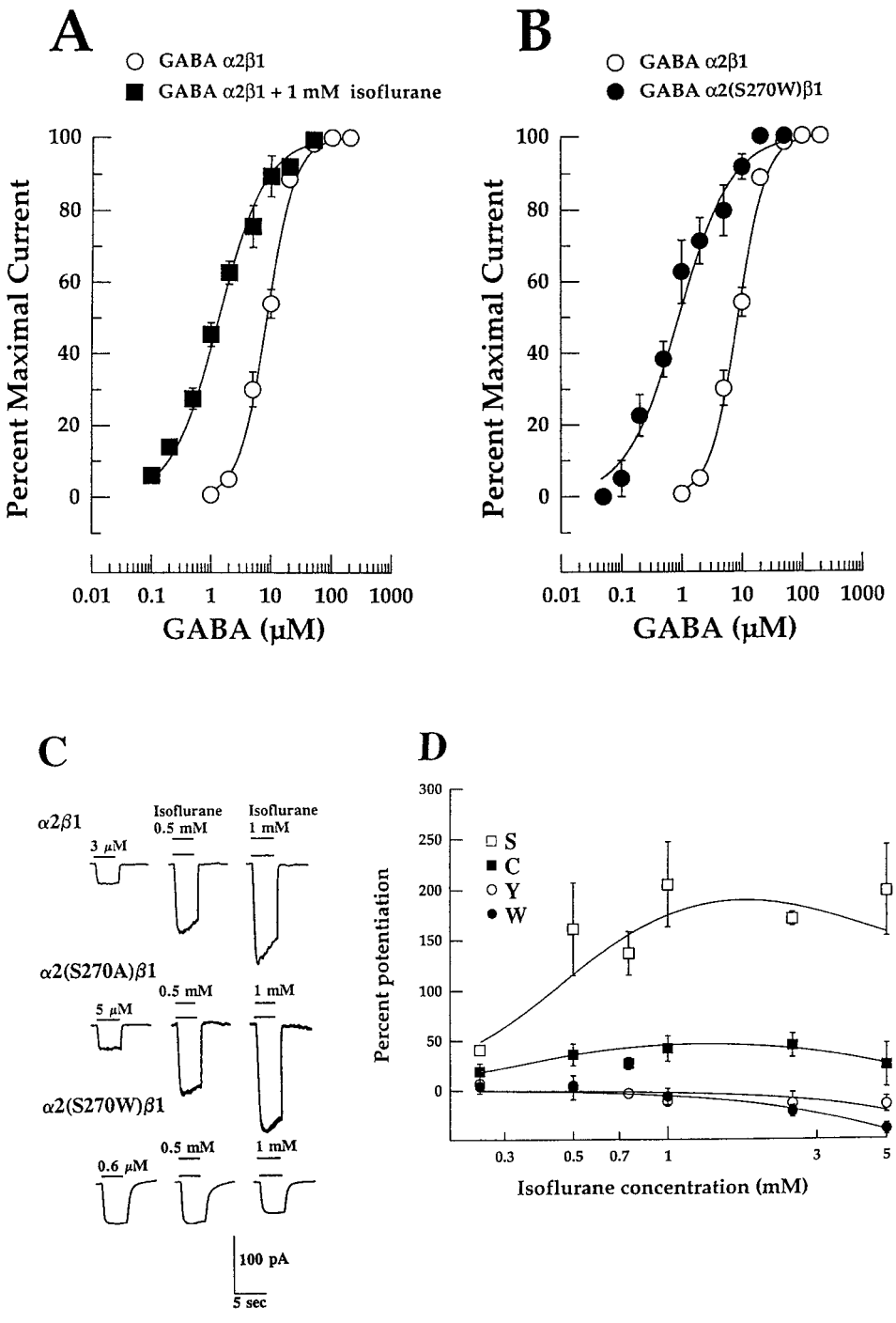

Fig. 3. Trp mutagenesis at α Ser270 mimics the effect of isoflurane on $GABA_A$ receptor function. A, leftward shift of the GABA concentration-response curve for the wild-type $\alpha2\beta1$ $GABA_A$ receptor in the presence of 1 mM isoflurane. B, leftward shift of the GABA concentration-response curve for the α2(S270W)β1 $GABA_A$ receptor relative to the wild-type $\alpha2\beta1$ $GABA_A$ receptor. C, allosteric potentiation of submaximal (EC_{20}) GABA responses occurs in the α2(S270X)β1 series of $GABA_A$ receptors when the α270 substituent is small (Ser, Ala), but not when it is larger (Tyr). D, concentration-response curves for isoflurane potentiation of an EC_{20} concentration of GABA in wild-type (S), and mutant α2(S270C)β1, α2(S270Y)β1, and α2(S270W)β1 mutant $GABA_A$ receptors.

α2(L269W) and α2(I271W) had little effect on receptor function, suggesting again no large changes in secondary structure in this region. These results highlight the significance of the α Ser270 residue. In studies of mutations in the highly homologous and presumably structurally related glycine receptor, it appears that mutations in the nearby TM2 to TM3 linker region alter the transduction mechanism between agonist binding and channel gating, but do not affect ligand binding per se (Lynch et al., 1997).

The data shown in Fig. 2 and Table 1 clearly show the apparent affinity for GABA of these mutant GABA$_A$ receptors to be dependent on the specific amino acid residue at position 270 on the α2 subunit. To determine whether this was attributable to a specific physical property of each amino acid, we plotted the GABA EC_{50} against the volume of the amino acid side chain (Fig. 4A), amino acid polarity (Fig. 4B), hydrophobicity (Fig. 4C), or hydrophilicity (Fig. 4D). A strong negative correlation ($r = -0.94$) was observed between the volume of the amino acid residue/side chain present at α270 and the GABA EC_{50} value (Fig. 4A). However, no statistically significant correlation was found between GABA EC_{50} and amino acid polarity, hydrophobicity, or hydrophilicity. The striking association shown in Fig. 4A might reflect an increase in the stability of the open state, relative to the closed state, of the GABA-operated ion channel as the size of the side chain at α270 increases. A similar phenomenon with respect to side chain volume was noted in the nicotinic acetylcholine receptor (nAChR) with restricted series of mutations of a cysteine residue in TM1 of the γ subunit (Lo et al., 1991). In mutational studies of the GABA$_A$ receptor α1 Leu264 residue (Chang et al., 1996), a leftward shift in the GABA concentration-response curve was produced by substitution of Leu with smaller residues such as Ser. This also was ascribed to an effect on channel gating, rather than to alterations in the affinity of agonist binding. In any case, this specific hypothesis can be evaluated in the future with single-channel recordings in selected receptor mutants. A fine example of this is the recent analysis of mutants involving

TABLE 3

A summary of the effects of 0.5 and 1 mM isoflurane on the α2(S270X)β1 series of GABA$_A$ receptors

Percent potentiation of EC_{20} GABA responses by 0.5 and 1.0 mM isoflurane are given for each receptor as mean ± S.E. for N cells determined, using the methods described. Statistical significance was assessed using a Student's t test.

GABA$_A$-R	Percent potentiation by isoflurane			
	0.5 mM	N	1.0 mM	N
$\alpha2\beta1$ WT	161 ± 46	6	205 ± 42	6
α2(S270A)β1	162 ± 20	4	236 ± 31	4
α2(S270C)β1	36 ± 11*	6	42 ± 13*	6
α2(S270E)β1	11 ± 11*	7	17 ± 9*	7
α2(S270F)β1	−0.4 ± 0.6*	6	−13 ± 5*	6
α2(S270G)β1	14 ± 8*	6	45 ± 11*	7
α2(S270H)β1	−8 ± 2*	4	−5 ± 2*	4
α2(S270I)β1	2 ± 6*	8	2 ± 5*	6
α2(S270R)β1	−5 ± 14*	6	−12 ± 18*	5
α2(S270T)β1	47 ± 6*	4	67 ± 15*	4
α2(S270W)β1	2 ± 16*	4	−24 ± 21*	4
α2(S270Y)β1	3 ± 12*	5	−11 ± 5*	5

* $p < .01$ compared with the control group.

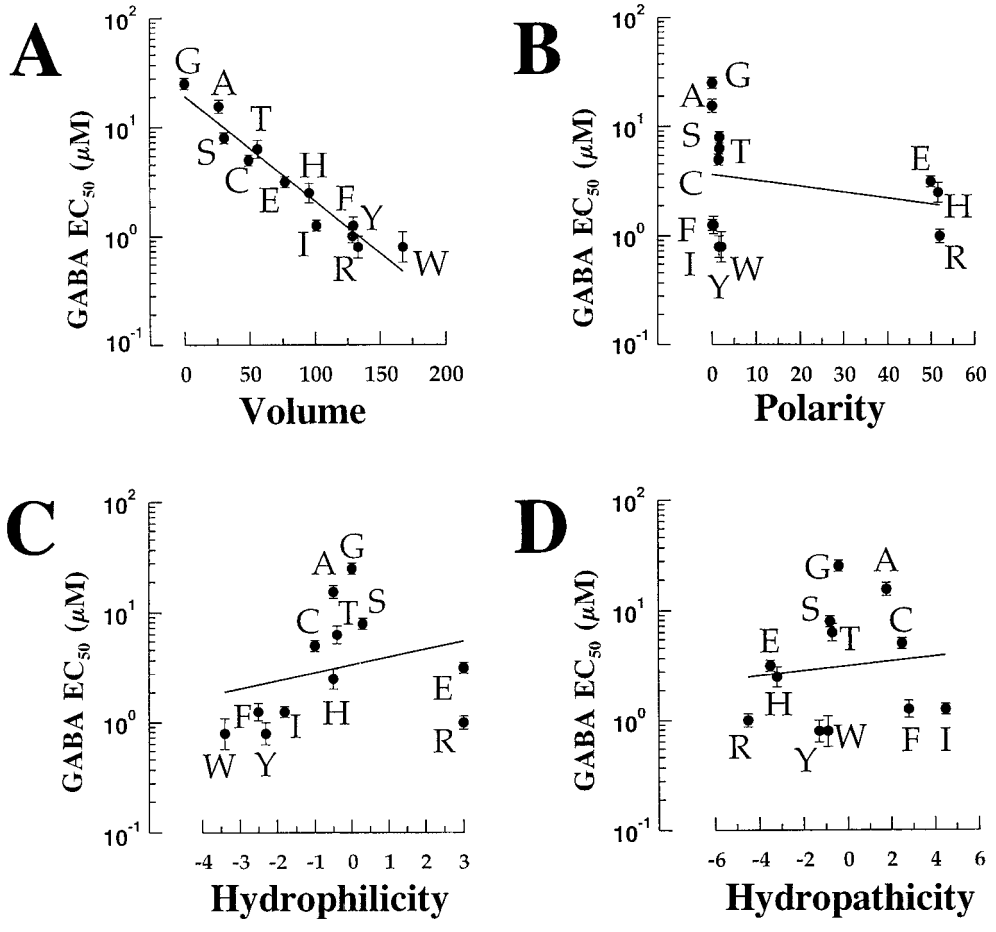

Fig. 4. The EC_{50} for GABA within the series of α2(S270X)β1 mutant GABA$_A$ receptors is correlated inversely with the molecular volume of the amino acid at position 270 on the GABA$_A$-R α2 subunit. The correlation of the physical properties of the amino acid at position 270 with GABA EC_{50} values obtained from Hill equation fits to GABA concentration-response data (see *Materials and Methods*, Fig. 3, and Table 1) showed (A) a strong negative correlation with molecular volume ($T = 9.25$, $p < .0005$, $r = -0.95$), whereas no significant correlations were found with (B) side-chain polarity ($T = 0.78$, $p > .22$, $r = -0.24$), (C) hydrophilicity ($T = 0.91$, $p > .18$, $r = 0.28$), or (D) hydropathicity ($T = 0.37$, $p > .36$, $r = 0.11$). Physical parameters are from Harpaz et al. (1994), Hopp and Woods (1981), Kyte and Doolittle (1982), and Zimmerman et al. (1968).

substitution of a Val residue in TM3 of the nAChR α1 subunit (Wang et al., 1999).

Regulation of Receptor Mutants by Isoflurane. When the $GABA_A$ receptor α2 Ser270 mutants were tested for modulation by isoflurane, we noted that there appeared to be a limiting volume for the side chains, such that amino acid residues with small side chains, such as Ser or Ala, permitted isoflurane modulation, whereas larger side chains such as Cys or Thr allowed only weak modulatory effects of isoflurane (Fig. 5). Many potential explanations exist for the data for the α2 Ser270 mutants and the effects of isoflurane on the wild-type and mutant $GABA_A$ receptors. Without structural information on this part of the receptor molecule, such ideas necessarily must remain speculative. One possibility is that residue α2 Ser270 is involved in structural rearrangements that accompany gating, and that isoflurane, like GABA, binds elsewhere on the receptor molecule to influence the transduction of agonist binding into channel opening. We prefer at present to speculate that the wild-type receptor, with Ser present at α270, contains one or more cavities of finite size within the $GABA_A$ receptor protein, possibly contained entirely within a single α subunit. Mutation of α2 Ser270 to very large residues, such as Phe, Tyr, or Trp (side chain volumes 130, 133, or 168 $Å^3$, respectively; Harpaz et al., 1994), may stabilize the open state of the channel relative to the closed state by occlusion of this cavity. Similarly, the cavity may be filled partially by an isoflurane molecule (molecular volume 140$Å^3$; J. Trudell, personal communication), stabilizing the open state in similar manner. The existence, although not the location, of such cavities in ion channel proteins has been inferred from studies of the "cutoff" in activity in the *N*-alcohol series as chain length increases. Not only do different ligand-gated ion channels show different alcohol cutoffs (Li et al., 1994; Peoples and Weight, 1995; Mihic and Harris, 1996), suggesting cavities of discrete size in each receptor molecule, but the alcohol cutoff actually can be manipulated by mutagenesis within TM2 and TM3 (Wick et al., 1998).

The concept of a binding cavity occupied by isoflurane is supported further by the observation that the GABA concentration-response curves for the isoflurane-insensitive α2(S270Y), α2(S270F), and α2(S270W) mutants (Figs. 2, 4B) closely resemble the concentration-response curve for the wild-type $GABA_A$ receptor in the presence of 1 mM isoflurane (Fig. 4A). It also would explain why isoflurane does not potentiate GABA in the α2(S270Y), α2(S270F), and α2(S270W) mutants. When the cavity is partially filled by a small side group from α270, as in α2(S270C) ($\Delta = \pm 49$ $Å^3$), there is a small leftward shift of the GABA concentration-response curve relative to the wild-type, consistent with a modest stabilization of the open state of the channel. However, isoflurane (molecular volume of 140 $Å^3$) still can be accommodated within the remainder of the cavity, resulting in additional potentiation of receptor function; when the side chain volume is increased, for example, in α2(S270E) (77 $Å^3$) or α2(S270R) (129 $Å^3$), the combined volume of the isoflurane molecule together with the bulkier side chain is now 217 or 269 $Å^3$. Therefore, we estimate the volume of the hypothetical cavity as between 189 and 217 $Å^3$. By analogy with NMR studies of cavities in soluble proteins such as lysozyme (Eriksson et al., 1992), the rather small free energy of stabilization associated with the presence of a small molecule such as isoflurane, or of a bulky side chain, might result in part from hydrophobic interactions with the cavity lining, and/or partly from an entropic component caused by displacement of bound water molecules from within the cavity. Clearly, structural studies ultimately will be required to determine the involvement of α Ser270 in cavity formation and anesthetic binding. In this regard, it is worth noting that anesthetic (bromoform) binding within a protein cavity recently has been described in the 2.2 Å resolution X-ray structure of the firefly luciferase molecule (Franks et al., 1998).

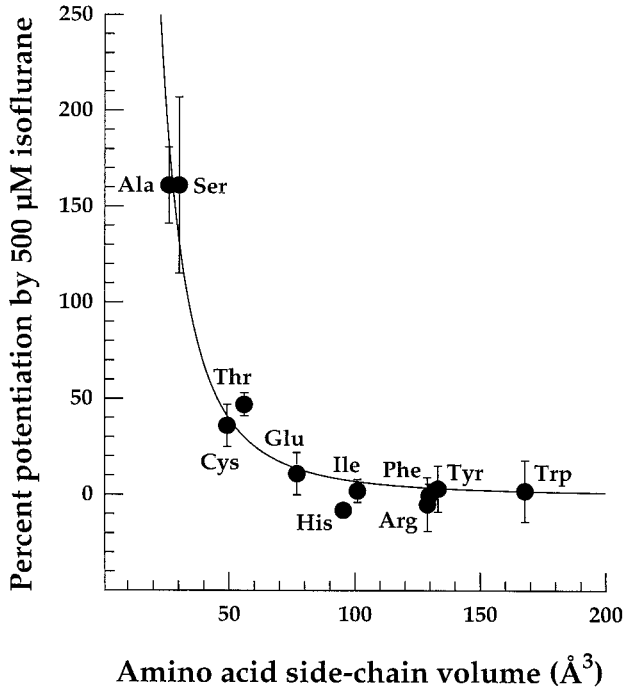

Fig. 5. Potentiation of submaximal GABA responses by isoflurane within the series of α2(S270X)β1 mutant $GABA_A$ receptors declines with increasing molecular volume of the amino acid at position α270. Each amino acid is represented by the usual three-letter abbreviation. The line represents a best fit to the data but has no theoretical significance.

Conclusion. Whatever the physical mechanisms involved in $GABA_A$ receptor modulation by anesthetics such as isoflurane, α270 certainly influences the behavior of the $GABA_A$ receptor ion channel. Although it is unlikely to participate directly in permeation, given an α-helical structure for TM2 (Xu and Akabas, 1996), Ser270 probably lies physically close to the ion channel and may play an important role in channel gating. Furthermore, this residue clearly determines the modulation of the $GABA_A$ receptor by isoflurane and may represent one determinant of an anesthetic binding cavity.

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References

Bormann J, Hamill OP and Sakmann B (1987) Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. *J Physiol (Lond)* **385:**243–286.

Chang Y, Wang R, Barot S and Weiss DS (1996) Stoichiometry of a recombinant GABA-A receptor. *J Neurosci* **16:**534–541.

Deng WP and Nickoloff JA (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal Biochem* **200:**81.
Draguhn A, Verdoorn TA, Ewert M, Seeburg PH and Sakmann B (1990) Functional and molecular distinction between recombinant rat GABA$_A$ receptor subtypes by Zn^{2+}. *Neuron* **5:**781–788.
Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P and Kemp JA (1994) Molecular pharmacology of gamma-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. *Mol Pharmacol* **46:**957–963.
Eriksson AE, Baase WA, Zhang X-J, Heinz DW, Blaber M, Baldwin EP and Matthews BW (1992) Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect. *Science* **255:**178–183.
Franks NP and Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. *Nature* **367:**607–614.
Franks NP, Jenkins A, Conti E, Lieb WR and Brick P (1998) Structural basis for the inhibition of firefly luciferase by a general anesthetic. *Biophys J* **75:**2205–2211.
Fritschy J-M and Mohler H (1995) GABA$_A$ receptor heterogeneity in the adult rat brain: Differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359:**154–194.
Harpaz Y, Gerstein M and Chothia C (1994) Volume changes on protein folding. *Structure* **2:**641–649.
Harris RA, Mihic SJ, Dildy-Mayfield J and Machu TK (1995) Actions of anesthetics on ligand-gated ion channels: Role of receptor subunit composition. *FASEB J* **9:**1454–1462.
Harrison NL, Kugler JL, Jones MV, Greenblatt EP and Pritchett DB (1993) Positive modulation of human GABA$_A$ and glycine receptors by the inhalation anesthetic isoflurane. *Mol Pharmacol* **44:**628–632.
Hopp TP and Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* **78:**3824–3828.
Horenstein J and Akabas MH (1998) Location of a high affinity Zn^{2+} binding site in the channel of $\alpha1\beta1$ γ-aminobutyric acid$_A$ receptors. *Mol Pharmacol* **53:**870–877.
Jones MV, Brooks PA and Harrison NL (1992) Enhancement of gamma-aminobutyric acid-activated chloride currents in cultured rat hippocampal neurones by three volatile anaesthetics. *J Physiol (Lond)* **449:**279–293.
Koltchine VV, Ye Q, Finn SE and Harrison NL (1996) Chimeric GABA$_A$/glycine receptors: Expression and barbiturate pharmacology. *Neuropharmacology* **35:**1445–1456.
Krasowski MD, Koltchine VV, Rick CEM, Ye Q, Finn SE and Harrison NL (1998) Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid-A receptor distinct from that for isoflurane. *Mol Pharmacol* **53:**530–538.
Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157:**105–132.
Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB and Twyman RE (1997) Activation and deactivation rates of recombinant GABA$_A$ receptor channels are dependent on α-subunit isoform. *Biophys J* **73:**2518–2526.
Levitan ES, Blair LA, Dionne VE and Barnard EA (1988) Biophysical and pharmacological properties of cloned GABA$_A$ receptor subunits expressed in *Xenopus* oocytes. *Neuron* **1:**773–781.
Li C, Peoples RW and Weight FF (1994) Alcohol action on a neuronal membrane receptor: Evidence for a direct interaction with the receptor protein. *Proc Natl Acad Sci USA* **91:**8200–8204.
Lo DC, Pinkham JL and Stevens CF (1991) Role of a key cysteine residue in the gating of the acetylcholine receptor. *Neuron* **6:**31–40.
Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH and Schofield PR (1997) Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel. *EMBO J* **16:**110–120.
McKernan RM and Whiting PJ (1996) Which GABA$_A$ receptor subtypes really occur in the brain? *Trends Neurosci* **19:**139–143.
Mihic SJ and Harris RA (1996) Inhibition of ρ1 receptor GABAergic currents by alcohols and volatile anesthetics. *J Pharmacol Exp Ther* 270:987–991.
Mihic SJ, Ye Q, Wick M, Koltchine VV, Finn SE, Krasowski MD, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA and Harrison NL (1997) Sites of alcohol and volatile anaesthetic action on GABA$_A$ and glycine receptors. *Nature* **389:**385–389.
Nusser Z, Sieghart W, Benke D, Fritschy J-M and Somogyi P (1996) Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci USA* **93:**11939–11944.
Okayama H and Chen C (1987) High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:**2745–2751.
Ortells MO and Lunt GG (1995) Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends Neurosci* **18:**121–127.
Peoples RW and Weight FF (1995) Cutoff in potency implicates alcohol inhibition of *N*-methyl-D-aspartate receptors in alcohol intoxication. *Proc Natl Acad Sci USA* **92:**2825–2829.
Pritchett DB, Sontheimer H, Gorman CM, Kettenmann H, Seeburg PH and Schofield PR (1988) Transient expression shows ligand gating and allosteric potentiation of GABA$_A$ receptor subunits. *Science* **242:**1306–1308.
Sieghart W (1995) Structure and pharmacology of γ-aminobutyric acid$_A$ receptor subtypes. *Pharmacol Rev* **47:**181–234.
Smart TG, Moss SJ, Xie X and Huganir RL (1991) GABA$_A$ receptors are differentially sensitive to zinc: Dependence on subunit composition. *Br J Pharmacol* **103:**1837–1839.
Tia S, Wang JF, Kotchabhakdi N and Vicini S (1996) Distinct deactivation and desensitization kinetics of recombinant GABA$_A$ receptors. *Neuropharmacology* **35:**1375–1382.
Tretter V, Ehya N, Fuchs K and Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA$_A$ receptor subtype. *J Neurosci* **17:**2728–2737.
Wafford KA, Whiting PJ and Kemp JA (1992) Differences in the affinity and the efficacy of benzodiazepine receptor ligands on recombinant GABA$_A$ receptor subtypes. *Mol Pharmacol* **43:**240–244.
Wang HL, Milone M, Ohno K, Shen XM, Tsujino A, Batocchi AP, Tonali P, Brengman J, Engel AG and Sine SM (1999) Acetylcholine receptor M3 domain: Stereochemical and volume contributions to channel gating. *Nature Neurosci* **2:**226–233.
Wick M, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL and Harris RA (1998) Mutations of GABA and glycine receptors change alcohol cut-off: Evidence for an alcohol receptor? *Proc Natl Acad Sci USA* **95:**6504–6509.
Xu M and Akabas MH (1996) Identification of channel-lining residues in the M2 membrane-spanning segment of the GABA-A receptor α1 subunit. *J Gen Physiol* **107:**195–205.
Zimmerman JM, Eliezer N and Simha R (1968) The characterization of amino acid sequences in proteins by statistical methods. *J Theor Biol* **21:**170–201.

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