Annu. Rev. Pharmacol. Toxicol. 2001. 41:23–51

ANESTHETICS AND ION CHANNELS: Molecular Models and Sites of Action*

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Key Words general anesthetics, ligand-gated ion channels, recombinant receptors, electrophysiology, molecular modeling

■ **Abstract** The mechanisms of general anesthesia in the central nervous system are finally yielding to molecular examination. As a result of research during the past several decades, a group of ligand-gated ion channels have emerged as plausible targets for general anesthetics. Molecular biology techniques have greatly accelerated attempts to classify ligand-gated ion channel sensitivity to general anesthetics, and have identified the sites of receptor subunits critical for anesthetic modulation using chimeric and mutated receptors. The experimental data have facilitated the construction of tenable molecular models for anesthetic binding sites, which in turn allows structural predictions to be tested. In vivo significance of a putative anesthetic target can now be examined by targeted gene manipulations in mice. In this review, we summarize from a molecular perspective recent advances in our understanding of mechanisms of action of general anesthetics on ligand-gated ion channels.

INTRODUCTION

General anesthetics are some of the most widely used and important therapeutic agents. However, despite over a century of research, the molecular mechanisms of general anesthesia in the central nervous system remain elusive. As a result of research during the past several decades, there has been a transition from initial notions of nonspecific actions of general anesthetics on membrane lipids to the

belief that their primary sites of action are neuronal proteins. Although many neuronal proteins can be affected by general anesthetics, a consensus has emerged that a group of ligand-gated ion channels, which are important for neuronal function, are particularly sensitive to general anesthetics (1–4). This review is restricted to discussion of ligand-gated ion channels.[1]

Ligand-gated ion channels include γ-aminobutyric acid type A ($GABA_A$), glycine, nicotinic acetylcholine (nACh), and 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptors, along with α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-, kainate-, and *N*-methyl-D-aspartate (NMDA)–selective glutamate receptors. $GABA_A$, glycine, nACh, and $5\text{-}HT_3$ receptors form part of an evolutionarily related ligand-gated ion channel gene superfamily (5), whereas glutamate receptors are thought to belong to a distinct ion channel class. Members of a ligand-gated ion channel superfamily have a basic transmembrane topology, with a large N-terminal extracellular domain, four putative transmembrane segments (TM1–TM4), a heterogeneous intracellular loop between TM3 and TM4, and a short extracellular C-terminal domain. Residues within the extracellular domain form the agonist-binding domain, whereas amino acid residues within TM2 line the ion channel pore. Native receptors are composed of pentameric arrangements of individual receptor subunits (3). On the other hand, glutamate receptors have three transmembrane segments (M1, M3, and M4) plus a cytoplasm-facing re-entrant membrane loop (M2) that lines the ion channel pore. Thus, the N-terminal domain is located extracellularly, and the C-terminal domain, intracellularly. Subunit stoichiometry of native glutamate receptors is controversial between tetrameric and pentameric structures (6).

General anesthesia is a behavioral state that requires critical degrees of immobility, amnesia, hypnosis/unconsciousness, analgesia, and muscle relaxation. Thus, key questions include the following:

1. Sensitivity: Which ligand-gated ion channels are sufficiently sensitive to clinically relevant concentrations of general anesthetics? How important is subunit composition for anesthetic sensitivity?
2. Mechanism: What is the molecular mechanism by which general anesthetics affect the function of ligand-gated ion channels?
3. In vivo importance: Which ligand-gated ion channels determine specific behavioral actions of general anesthetics? Is the responsible target common to a wide variety of general anesthetics or specific for certain agents?

[1]Abbreviations: ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; F6, 1,2-dichlorohexafluorocyclobutane; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine; MAC, minimum alveolar concentration; MPPB, 1-methyl-5-phenyl-5-propyl barbiturate; nACh, nicotinic acetylcholine; NMDA, *N*-methyl-D-aspartate; TM, transmembrane segment.

MOLECULAR ACTION OF ANESTHETICS ON LIGAND-GATED ION CHANNELS

Relevant Anesthetic Concentrations

For a target to have relevance to anesthesia, it must be sensitive to clinical concentrations of general anesthetics. Minimum alveolar concentration (MAC) conventionally refers to the concentration of inhaled anesthetic that produces immobility, the lack of movement in response to a noxious stimulus, in 50% of subjects studied (7). The use of immobility as an anesthetic endpoint is helpful in that, for most general anesthetics, anesthetic concentrations two- to fourfold above the MAC cause deleterious side effects (1). Thus, anesthetic concentrations severalfold greater than the MAC define the upper boundary of the concentration range that is clinically relevant.

The Hill coefficient value of concentration-effect relationships for inhaled anesthetic MAC is generally large—6 to 20 (8). Some would argue that this is related to anesthetic mechanisms (9), but others argue that the steepness results from small population variations (8). The important point we have to keep in mind, however, is the categorical (move/no move) nature of the MAC measurement. Specifically, the slope of dose-response curves becomes steep when the data are categorically processed. The slope of categorical responses for each individual does not depend on the Hill value of the underlying dose-response relationship, but depends on the interval of concentrations examined, and it could be infinitely steep (Figure 1). This steep slope for the individual categorical response would be a basis of the steep slope of a population dose-response relationship (for MAC determination). Thus, the steep slope of MAC response may not necessarily be related to mechanisms of anesthetic actions on targets.

The issue of clinically relevant concentrations for intravenous anesthetics is considerably more complicated than that for volatile anesthetics because of limited pharmacokinetic data adequately collected for a defined endpoint of anesthesia and because of the difficulty in ascertaining steady-state free aqueous anesthetic concentrations in the brain (1). Relevant anesthetic concentrations of propofol and barbiturates have been carefully estimated (1). As for other intravenous anesthetics, plausible concentrations for producing immobility estimated from available data are shown by Krasowski and Harrison (3).

General Anesthetic Actions on Recombinant Ligand-Gated Ion Channels

The advent of cloning and expression techniques has greatly accelerated and facilitated attempts to classify ligand-gated ion channel sensitivity to general anesthetics. Molecular biology techniques have the advantage of identifying the sites of receptor subunits critical for anesthetic modulation. Because sensitivity to general anesthetics varies considerably, sometimes even among closely related receptors,

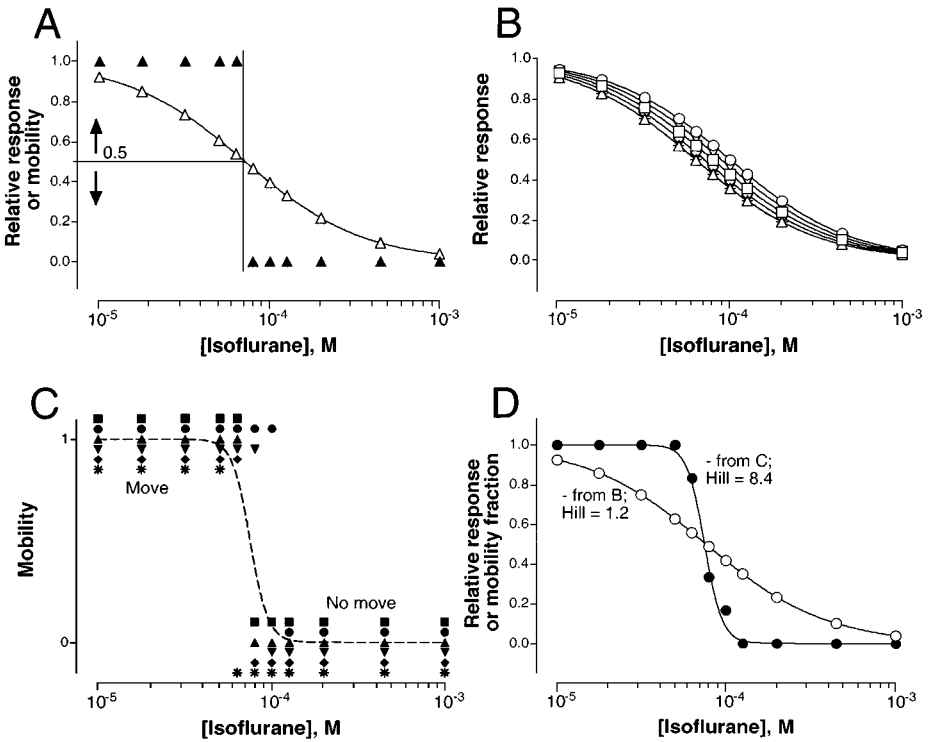

Figure 1 Schematic simulation showing that the slope of categorical responses could become steep independent of the Hill coefficient value of underlying dose-response relationships. (*A*) Isoflurane dose-inhibition relationships for nACh receptors expressed in a single oocyte (*open triangle*) and categorical responses [move (1)/no move (0)] for a single oocyte (or a patient) processed based on the dose-inhibition curve of an oocyte, assuming that relative responses $\geq$0.5 produce movement and relative responses $<$0.5 produce no movement (*filled triangle*). (*B*) Isoflurane dose-inhibition relationships for six oocytes (Hill coefficient values are $\sim$1.25). (*C*) Categorical responses of six oocytes (or six patients) as a function of isoflurane concentrations processed based on isoflurane dose-inhibition curves for respective six oocytes in panel *B*. (*D*) Population dose-response relationships for isoflurane inhibition of six oocytes (*open circle*, from panel *B*) and for categorical responses of six oocytes (or six patients) (*filled circle*, from panel *C*). Note that the slope of resultant population categorical responses is much steeper than the original slope of isoflurane dose-inhibition curves.

chimeric receptors can be used to delimit regions of subunits essential for anesthetic actions, after which site-directed mutagenesis can be used to pinpoint the critical residues.

Table 1 and Table 2 summarize effects of volatile and intravenous anesthetics on ligand-gated ion channels of defined subunit composition with a rough estimate of the change in receptor function produced by a given concentration of the anesthetic. Each class of anesthetics exhibits a characteristic pattern of action

on ligand-gated ion channels as follows. (*a*) Volatile anesthetics show prominent potentiation of $GABA_A$ and glycine receptor function, and strong inhibition of neuronal nACh receptors. (*b*) Barbiturates markedly potentiate $GABA_A$ receptors and inhibit nACh, AMPA, and kainate receptors. (*c*) Propofol and etomidate are relatively selective for $GABA_A$ receptors. (*d*) Steroid anesthetics potentiate $GABA_A$ receptors and inhibit neuronal nACh receptors. (*e*) Ketamine does not affect $GABA_A$ receptors but inhibits NMDA receptors potently and neuronal nACh receptors with lower potency. Thus, it is only $GABA_A$ receptors that are sensitive to every volatile and intravenous anesthetic (except ketamine) at clinically relevant concentrations.

Differences in anesthetic sensitivity between homologous subunits allow the following conclusions: (*a*) Enhancement of $GABA_A$ receptor function by volatile anesthetics does not require any specific subunit and is similar for a wide variety of different subunit combinations, but $GABA_C$ ρ_1 receptors are inhibited by volatile anesthetics (10). (*b*) Etomidate sensitivity of $GABA_A$ receptors is different between β subunits, and receptors containing β_2 or β_3, but not β_1, subunits are highly sensitive to etomidate (11, 12). (*c*) $GABA_A$ receptors are generally sensitive to intravenous anesthetics, whereas glycine receptors are much less sensitive to intravenous anesthetics (13, 14). (*d*) Neuronal heteromeric nACh receptors, especially those composed of β_2 subunits, are very sensitive to volatile anesthetics, whereas homomeric α_7 and muscle nACh receptors are relatively insensitive to volatile anesthetics (15–17). (*e*) AMPA receptors (GluR1–4 subunits) are slightly inhibited by volatile anesthetics, whereas kainate receptors (GluR5–7 and KA-1,2 subunits) are potentiated by volatile anesthetics (18). (*f*) AMPA receptors containing GluR2 subunits are more sensitive to barbiturates than those without GluR2 subunits (19). Some critical amino acid residues have been identified by using these differences in subunit sensitivity to anesthetics (see below).

Gaseous Anesthetics

The anesthetic properties of gasses such as nitrous oxide and xenon have long been central to molecular theories of anesthesia that seek to explain how these simple and small molecules are able to produce anesthesia. Recent studies suggest that $GABA_A$ receptor currents are only weakly potentiated by nitrous oxide (20–22; but see 23) and are little affected by xenon (22, 24, 25), which contrasts clearly with volatile and intravenous anesthetics (Table 3). Glycine receptors are moderately potentiated by nitrous oxide and xenon at clinical concentrations (22, 26), whereas $GABA_C$ ρ_1 receptors are slightly inhibited by nitrous oxide (22). In contrast to $GABA_A$ receptors, nitrous oxide and xenon have marked inhibitory effects on NMDA receptors (20–22, 24, 25). AMPA and kainate receptors are also inhibited by nitrous oxide, though by a smaller degree than NMDA receptors (21, 22), whereas they are little affected by xenon (24, 25). The 5-HT_3 receptor is slightly inhibited by nitrous oxide, which is opposite to the effects of volatile anesthetics

TABLE 1 Effects of volatile anesthetics on ligand-gated ion channels of defined subunit composition[a]

		Volatile Anesthetics				
Receptor	**Subunit**	**Halothane**	**Isoflurane**	**Enflurane**	**Sevoflurane**	**F3**
Glycine	$\alpha 1$	+++/1 (13, 106)	+++/1 (106)	+++/1 (13, 106)	++/1 (106)	+++/1 (13)
	$\alpha 2$		+++/3 (135)			
$GABA_A$	$\alpha 1\beta 1$		+++/1 (136)	+++/1.5 (28)		
	$\alpha 1\beta 1\gamma 2$	+++/1 (137)	+++/1 (136, 137)	+++/3 (138)	+++/1 (137)	
	$\alpha 1\beta 1\delta$		+++/1 (136)			
	$\alpha 1\beta 2$			+++/2 (115)		++/1 (115)
	$\alpha 1\beta 1\gamma 2$	++/1[b]	+++/1[b]	+++/2 (115)		+++/1 (115)
	$\alpha 2\beta 1$		+++/2 (31)			
	$\alpha 2\beta 1\gamma 2$		+++/6 (135)			
	$\alpha 2\,\gamma 2$		+++/3 (135)			
$GABA_C$	$\rho 1$	0/2 (10)	−/4 (10)	−/2.5 (10)		
nACh	$\alpha 1\beta 1\gamma\delta$	0/1 (17)	0/1 (17)	−/1 (119)	0/1 (17)	
	$\alpha 2\beta 2$		− − −/1 (17)			
	$\alpha 2\beta 4$	− − −/1 (17)	− −/1 (17, 117)			− −/1 (117)
	$\alpha 3\beta 2$		− − −/1 (17)			
	$\alpha 3\beta 4$		− −/1 (17, 117)			−/1 (117)

(*Continued*)

TABLE 1 (*Continued*) Effects of volatile anesthetics on ligand-gated ion channels of defined subunit composition[a]

		Volatile Anesthetics				
Receptor	**Subunit**	**Halothane**	**Isoflurane**	**Enflurane**	**Sevoflurane**	**F3**
	$\alpha4\beta2$	− − −/1 (17)	− − −/1 (16, 17, 117)	− − −/1[b]	− −/1 (17)	− −/1 /(117)
	$\alpha4\beta4$	− −/1 (17)	− −/1 (17)	−−/1[b]		
	$\alpha7$	0/1 (15)	0/1,2 (15),(16)			
5-HT_3	5-HT_{3A}	+/1 (15, 116)	+/1 (15, 116)	+/1[b]		++/1/(116)
AMPA	GluR1		0/2 (18)	−/2 (18)		0/2 (18)
	GluR1/GluR2	−/2[b]	−/2[b]			
	GluR2/GluR3		0/2 (18)	−/2 (18)		0/2 (18)
	GluR3	−/2 (18)	0/2 (18)	−/2 (18)		−/2 (18)
Kainate	GluR5	+++/8 (44)	+++/7 (44)	++/3 (44)		+/1 (44)
	GluR6	++/2 (18)	+/2 (18)	++/2 (18)		++/2 (18)
	GluR6/KA2	++/2[b]	+/2[b]			
NMDA	NR1/NR2A	0/2[b]	−/2[b]			

[a]The first symbol for each entry denotes the amount of potentiation of receptor function: +, 20–50%; ++, 50–100%; +++, >100%. Amount of inhibition of receptor function: −, 20–50%; − −, 50–80%; − − −, 80–100%. The second symbol denotes the drug concentration used as a multiple of the anesthetic EC_{50} concentration (1, 3). Whenever possible, data obtained with concentrations near anesthetic were selected for presentation. The numbers in parentheses refer to the reference number. Anesthetic EC_{50} concentrations were taken as halothane 0.25 mM; isoflurane 0.3 mM; enflurane 0.6 mM; 1-chloro-1,2,2-trifluorocyclobutane (F3) 0.8 mM.

[b]T Yamakura & RA Harris, unpublished data.

TABLE 2 Effects of intravenous anesthetics on ligand-gated ion channels of defined subunit composition[a]

		Intravenous Anesthetics				
Receptor	**Subunit**	**Barbiturates**	**Propofol**	**Etomidate**	**Steroid Anesthetics**	**Ketamine**
Glycine	$\alpha1$	+/2 (13)	+/1.5 (13)	+/1 (14)	+/1 (13)	0/10 (133)
	$\alpha1\beta$	0/1 (14)	+/1 (14)	0/1 (14)	0/1 (14)	
$GABA_A$	$\alpha1\beta1$	+++/0.5 (139, 140)	+++/5 (141)		+++/1 (142, 143)	
	$\alpha1\beta1\gamma1$	+++/0.5 (139)				
	$\alpha1\beta1\gamma2$	+++/1 (14, 144, 145)	+++/1 (14)	+,+++/1 (146) (11, 14)	+++/1 (14, 142–144, 147)	
	$\alpha1\beta2$		+++/5 (148)			
	$\alpha1\beta2\gamma2$	+++/1 (145)	+++/5 (148)	+++/1 (11, 146)	+++/1 (146, 147)	0/10 /(133, 149)
	$\alpha1\beta3\gamma2$	+++/0.5,1 (150),(145)	+++/1 (150)	+++/1 (146)	++/1 (146)	
	$\alpha1\gamma2$		+++/5 (141)			
	$\alpha2\beta1$	+++/1 (31, 151)	+++/1 (28, 31, 152)	+++/2 (31)	+,+++/1 (143),(31, 153)	
	$\alpha2\beta2\gamma2$		+++/3 (152)	+++/1 (11)	+,+++/1 (143), (147)	
	$\alpha2\beta2$		+++/5 (148)			
	$\alpha2\beta2\gamma2$	+++/1 (145)	+++/5 (148)	+++/1 (11)		
	$\alpha3\beta1$				++/1 (143)	
	$\alpha3\beta1\gamma2$	+++/1 (154)	+++/1 (154)	+++/1 (11, 154)	+++/1 (143, 147, 154)	
	$\alpha3\beta2\gamma2$	+++/1 (145)		+++/1 (11)		
	$\alpha5\beta1$		+++/5 (148)			
	$\alpha5\beta1\gamma2$		+++/5 (148)			
	$\alpha5\beta2\gamma2$	+++/1 (145)				
	$\alpha6\beta1\gamma2$			+/1 (11)	+++/1 (147)	
	$\alpha6\beta2\gamma2$	+++/1 (145)		+++/1 (11)		
	$\alpha6\beta3\gamma2$	+++/0.5 (37, 150)	0,++/1 (150), (37)	+++/1 (12)	+++/1 (37)	
	$\beta1$	+++/1 (141)	+++/5 (141)		+++/1 (142)	
$GABA_C$	$\rho1$	0/4 (10, 155)	0/2 (10)	0/2[b]	0/0.2 (10)	0/10[b]
nACh	$\alpha1\beta1\gamma\delta$	− −/1 (97, 156)	0/1 (17)	0/1 (157)	0/1 (157)	−/1 (157)

(*Continued*)

TABLE 2 (*Continued*) Effects of intravenous anesthetics on ligand-gated ion channels of defined subunit composition[a]

		Intravenous Anesthetics				
Receptor	**Subunit**	**Barbiturates**	**Propofol**	**Etomidate**	**Steroid Anesthetics**	**Ketamine**
	$\alpha 2\beta 2$					0/1 (133)
	$\alpha 2\beta 4$					−/1 (133)
	$\alpha 3\beta 2$					0/1 (133)
	$\alpha 3\beta 4$					− −/1 /(133)
	$\alpha 4\beta 2$	− −/1 (97)	0,−/1 (16), (17)	0/2[b]	−/1[b]	0/1 (133)
	$\alpha 4\beta 4$	−/1 (149)	0/1[b]	0/1 (149)	−/1[b]	−,− − −/1 (133), (149)
	$\alpha 7$	−/1 (97)	0/2 (16)			
5-HT_3	5-HT_{3A}	0/2[b]	0/1 (116)	0/2[b]	0/2[b]	0/3 (133)
AMPA	GluR1	0/1 (19, 45)	0/50 (158)			
	GluR1/GluR2	−,− −/1 (19), (45)	0/50 (158)	0/2[b]	0/2[b]	0/10[b]
	GluR1/GluR3	0/1 (19)				
	GluR2/GluR3	−/1 (19)				
	GluR3	0/1,2 (19), (18)	0/30 (2)			
Kainate	GluR5	0/2 (44)				
	GluR6	−/2 (18)	+/30 (2)			
	GluR6/KA2	−/2[b]	0/50 (158)	0/2[b]	0/2[b]	0/10[b]
NMDA	NR1/NR2A	0/2[b]		0/2[b]	0/2[b]	− − −/1 /(48)
	NR1/NR2B		0/50 (158)			− − −/1 (48)
	NR1/NR2C		0/50 (158)			− − −/1 (48)
	NR1/NR2D					− − −/1 (48)

[a]The first symbol for each entry denotes the amount of potentiation of receptor function: +, 20–50%;, ++, 50–100%;, +++, >100%. Amount of inhibition of receptor function: −, 20–50%;, − −, 50–80%;, − − −, 80–100%. The second symbol denotes the drug concentration used as a multiple of the clinically relevant anesthetic concentration (1, 3). Whenever possible, data obtained with concentrations near anesthetic were selected for presentation. The numbers in parentheses refer to the reference number. Anesthetic concentrations were taken as pentobarbital 50 μM, thiopental 25 μM, and methohexital 25 μM for barbiturates; propofol 1 μM; etomidate 5 μM; alphaxalone 5 μM, and 3α-hydroxy-5α-pregnan-20-one (DHP) 0.3 μM for steroid anesthetics; ketamine 10 μM.

[b]T Yamakura & RA Harris, unpublished data.

TABLE 3 Effects of gaseous anesthetics on ligand-gated ion channels[a]

		Gaseous Anesthetics	
Receptor	**Tissue/Subunit**	**Nitrous Oxide**	**Xenon**
Glycine	$\alpha 1$	+75,+33/1.1,0.6 (26), (22)	+50,+30/0.7,0.5 (26), (22)
$GABA_A$	Hippocampal neuron	+10,+15,+40/0.8 (20), (21), (23)	−8,+2/0.8,1.0 (24), (25)
	$\alpha 1\beta 2\gamma 2$	+17/0.6 (22)	+16/0.5 (22)
$GABA_C$	$\rho 1$	−13/0.6 (22)	
nACh	$\alpha 1\beta 1\gamma\delta$	−46/0.8 (27)	
	$\alpha 4\beta 2$	−39/0.6 (22)	−41/0.5 (22)
	$\alpha 4\beta 4$	−8/0.6 (22)	−13/0.5 (22)
5-HT_3	5-HT_{3A}	−13/0.6 (22)	
AMPA	Hippocampal neuron	−32/0.8 (21)	
	GluR1/GluR2	−20/0.6 (22)	
Kainate	GluR6/KA2	−18/0.6 (22)	
NMDA	Hippocampal neuron	−66,−49/0.8 (20), (21)	−60,−70/0.8 (24), (25)
	NR1/NR2A	−31/0.6 (22)	−34/0.5 (22)

[a]The first symbol for each entry denotes the percentage of potentiation (+) or inhibition (−) of receptor function. The second symbol denotes the drug concentration (partial pressure) in atmospheres. The numbers in parentheses refer to the reference number. Xenon is reported to have little apparent effect on non-NMDA (AMPA/kainate) receptors (24, 25).

and ethanol on this receptor (22). Muscle $\alpha_1\beta_1\gamma\delta$ nACh receptors are inhibited by nitrous oxide at clinical concentrations (27). Neuronal nACh receptors are inhibited by nitrous oxide and xenon, and sensitivity to gaseous anesthetics is different between $\alpha_4\beta_2$ and $\alpha_4\beta_4$ receptors, with $\alpha_4\beta_2$ receptors being more sensitive than $\alpha_4\beta_4$ receptors (22). Thus, nitrous oxide and xenon display a similar spectrum of receptor actions, but this spectrum is distinct from volatile and intravenous anesthetics. Based on their anesthetic sensitivity, NMDA receptors and nACh receptors composed of β_2 subunits are considered to be potential targets for nitrous oxide and xenon in the central nervous system.

MOLECULAR SITES OF ACTION OF ANESTHETICS

Critical Sites for Anesthetic Actions

Molecular biology techniques, such as construction of chimeric receptors and single amino acid mutations, have considerably facilitated the identification of regions/sites critical for anesthetic modulation. The use of a panel of glycine α_1/$GABA_C$ ρ_1 chimeric receptors and extensive site-directed mutagenesis of glycine receptor α_1 and GABA receptor α_1, α_2, β_1, and ρ_1 subunits has identified specific amino acid positions within TM2 and TM3 that are critical for potentiation of agonist-induced currents by volatile anesthetics and alcohols

(28–36). Agonist potentiation by propofol is also affected by a point mutation of these specific residues in TM2 (37) and TM3 (31) of the $GABA_A$ receptor β subunits.

Etomidate sensitivity of $GABA_A$ receptors is different between β subunits, and receptors containing β_2 or β_3, but not β_1, subunits are highly sensitive to etomidate (11, 12). Using $GABA_A$ β_1/β_2 chimeric receptors and site-directed mutagenesis, specific amino acid positions within TM2 of β subunits, which correspond to positions critical for volatile anesthetic and alcohol actions, are identified to be important for etomidate actions on $GABA_A$ receptors (12, 38, 39).

Pentobarbital sensitivity of $GABA_A$ receptors is reduced by a point mutation of residues in TM2 (which correspond to positions critical for volatile anesthetic actions) of the β_3 subunit (37), and pentobarbital sensitivity is introduced into $GABA_C$ ρ_1 receptors by single amino acid mutations of residues in TM2 (40) and TM3 (41), which also correspond to positions critical for volatile anesthetic actions. A point mutation of residues in TM2 of the $GABA_A$ receptor β_1 subunit located three positions amino terminal of the critical site for volatile anesthetics abolishes pentobarbital potentiation of $GABA_A$ receptors (42). Conversion of a conserved glycine residue at the entrance of TM1 of $GABA_A$ receptor β_2 subunits into phenylalanine ($GABA_C$ ρ_1 residue) decreases modulatory effects of pentobarbital, alphaxalone, etomidate, and propofol (43).

By examining glutamate GluR3/GluR6 chimeric receptors and extensive point mutations, a specific amino acid in segment M4 of GluR6 kainate receptors is identified to be critical for volatile anesthetic enhancement of kainate receptors (44). Sensitivity of AMPA receptors to barbiturates is determined by an arginine residue in channel-lining segment M2 of the $\alpha 2$ (GluR2) subunit, which corresponds to amino acid residues determining Ca^{2+} permeability of AMPA receptors (45, 46). Ketamine sensitivity of NMDA receptors is determined by the conserved asparagine residue in the channel-lining segment M2 of the $\varepsilon 2$ (NR2B) and $\zeta 1$ (NR1) subunits, which constitutes the Mg^{2+} block site of NMDA receptors (46–48). This position of the asparagine residue on NMDA receptor subunits corresponds to that of arginine of the $\alpha 2$ (GluR2) subunit, which determines barbiturate sensitivity of AMPA receptors.

Point mutations of an amino acid residue in the middle of TM2 of muscle nACh receptor $\alpha 1$ and $\beta 1$ subunits enhance sensitivity to volatile anesthetics (49, 50). The sensitivity of nACh receptors to blockade by volatile anesthetics increases with increased hydrophobicity of this residue (49, 50).

Binding or Transduction?

As discussed above, site-directed mutagenesis has identified many single amino acid mutations in receptor subunits that alter anesthetic modulation. The next question of whether a key residue is involved in constituting a binding site for anesthetics or in transducing the anesthetic actions is critical to understanding molecular mechanisms of anesthetic actions. Even for effects of mutation on

agonist binding to receptors, it is not easy to differentiate between effects on binding and effects on the transductional process (51), and the same is true in anesthetic binding. Furthermore, general anesthetics have properties that limit the utility of other experimental techniques. For example, radioligand-binding assay with general anesthetics to ligand-gated ion channels has proven exceedingly difficult to demonstrate owing to low affinity, rapid kinetics, and high nonspecific binding to neuronal membranes (2, 3, 52).

Although limited progress has been made in developing anesthetic congeners useful for photoaffinity labeling or other covalent modification of receptors (3), recently a photoaffinity general anesthetic 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (3-azioctanol) was developed (53). This compound acts as an anesthetic in tadpoles, potentiates $GABA_A$ receptor function, and inhibits muscle-type nACh receptors (53). 3-Azioctanol is photoincorporated into *Torpedo* nACh receptor $\alpha 1$ subunits (53). The primary binding site in the desensitized state of nACh receptors is identified to be $\alpha 1$-E262 at the extracellular ring of TM2 (54).

Recently a new approach, using an alkanethiol anesthetic or a sulfhydryl-specific agent that forms disulfide bonds with a thiol group of a cysteine residue introduced at the specific site, was employed to show that binding at the site produces an irreversible anesthetic effect in vitro (55). The ability of an unconventional anesthetic propanethiol under conditions of oxidation by iodine or the ability of propyl methanethiosulfonate (PMTS) to form covalent bonds was examined on $GABA_A$ and glycine receptor mutants in which cysteine residues were introduced into specific amino acid positions critical for anesthetic modulation in TM2 and TM3. Propanethiol and PMTS irreversibly enhanced function of mutant receptors at the TM2 critical site, whereas they reversibly potentiate wild-type and TM3 mutant receptors, indicating that anesthetics specifically bind to the critical residues in TM2 (55). Thus, this approach provides strong evidence that anesthetic actions on $GABA_A$ and glycine receptors are due to binding at a specific site and may have general applicability for defining anesthetic binding sites on other brain receptors.

MOLECULAR MODELING OF ANESTHETIC INTERACTIONS WITH LIGAND-GATED ION CHANNELS

Why Molecular Modeling?

Most receptor proteins of biological interest are studied after their structure is determined via X-ray crystallography. This often provides relatively exact atomic coordinates from which very accurate three-dimensional representations may be developed. This requires that an adequate amount of purified protein can be made such that reasonably sized crystals can be formed for examination. The difficulty of studying the ligand-gated ion channels is that they are transmembrane proteins. Therefore, upon removal from their membrane environment they often become

denatured such that any further purification or crystallization steps become meaningless. This difficulty renders this class of protein poorly amenable to study, with the current X-ray crystallographic methods of analysis only able to render very low-resolution structural images (56). In such a case, molecular modeling has been used to study such systems for the purposes of analyzing and visualizing protein structure as well as ligand binding (57–59).

Controversy Over the Predicted Secondary Structure of the Transmembrane Regions of Ligand-Gated Ion Channels

Our initial models assume that the common motif of the ligand-gated ion channel superfamily is a pentamer of subunits, with each subunit consisting of four antiparallel α helices (60). A series of papers by Unwin and co-workers (61–63) strongly supported the general motif of an ion channel composed of five subunits arranged around a central pore. The suggestion that the other three transmembrane segments in each subunit are also α helices is much more controversial (63–69). Here we review the evidence for each segment.

Secondary Structure of TM2 Although it seems out of order, it is appropriate to consider TM2 first because it is the pore-lining segment and is the most studied. Cryo-electron microscopy was used to demonstrate that electron density in the transmembrane region is consistent with five α helices lining the central pore of the nACh receptor (63, 70). These α helices were identified as TM2 in the nACh receptor in a series of studies in which specific amino acid residues were mutated to cysteine and labeled with water-soluble reagents (66, 67, 70).

A breakthrough for our proposed molecular modeling occurred recently when the crystal structure of a bacterial mechanosensitive ion channel was published (71). This ion channel contains five α helices arranged around a central pore with a right hand supertwist and a funnel shape that is narrowest at the intracellular face (72). This structure is entirely consistent with predictions made by Unwin and co-workers (61–63) based on electron density in the cryo-electron micrographs of nACh receptors. Although early studies predicted a "kink" at the highly conserved leucine residue lining the ion channel (61), this kink was not observed in a recent NMR study (73).

Secondary Structure of TM1 In general, transmembrane domains of proteins are either all α-helical or all β-barrel. As described above, there is strong evidence that the pore-lining TM2 segments are α helices. As a result, the other three transmembrane segments identified by hydropathy algorithms have long been assumed to be α helices as well. However, some studies suggest that this may not be the case. For example, exposure of cysteine mutants of TM1 and TM2 of the nACh receptor to a water-soluble probe revealed that TM2 was labeled in a manner consistent with an α helix (70), whereas TM1 was labeled in an irregular manner, indicating incomplete exposure to the aqueous pore region (66). In addition, this exposure

changed during channel gating, suggesting that the tertiary structure of the channel changes during gating. A similar result was obtained with the hydrophobic probe 3-trifluoromethyl-3-(m-[^{125}I]-iodophenyl)diazirine in the *Torpedo* nACh receptor (74). The probe reacted nonspecifically with residues 222, 223, 227, and 228, a pattern of incorporation inconsistent with that expected from "face" of either an α helix or a β sheet. Additional uncertainty about the secondary structure of TM1 was provided by proteolytic digestion and mass spectrometry studies of the glycine receptor (68). This study found cleavage sites within the putative TM1 and TM3 transmembrane helices. The authors interpreted these cleavage sites as evidence for a mixture of α helices and β sheets within this region (68). An alternative explanation is that the very aggressive detergents (triton X-100) used in isolation of receptors before proteolytic digestion resulted in denaturation of the receptor and exposure of inappropriate cleavage sites. There is similar concern about the FTIR spectroscopy study that found only 50% α-helical content after vigorous removal of the extracellular domain with proteinase K (69). In contrast to the conclusion of the previous study, recent studies in the nACh receptor supported an all α-helical structure in the transmembrane domain (75, 76).

Secondary Structure of TM3 This segment is the subject of much research and controversy. There is strong evidence that the TM2-3 loop is involved in transmission of a signal from agonist binding at the agonist-binding site to the ion channel pore (77–80). If both TM2 and TM3 were α helices connected by a short loop, there would be analogy to the established mode of light activation of rhodopsin (81). In addition, it was recently shown that activation by GABA increases the water-accessibility of M3 membrane-spanning residues in GABA receptors (67). This result suggests that channel gating involves motion of the transmembrane segments relative to each other. However, initial cryo-electron microscopy by Unwin did not observe clear evidence of α helices in the region of TM3 (61). In the absence of this evidence he proposed other, more random, structures for this segment. On the other hand, a recent NMR study of a synthetic peptide corresponding to the putative TM3 from *Torpedo californica* concluded that the segment has an α-helical structure (82).

Secondary Structure of TM4 The lipid-protein interface of the *Torpedo* nACh receptor was identified with the hydrophobic probe 3-trifluoromethyl-3-(m-[^{125}I]-iodophenyl)diazirine (74). The periodicity of the resulting labeling was consistent with both TM3 and TM4 being α helices. The cholesterol-binding domain in the nACh receptor has been located near TM4 with [^{125}I] azido-cholesterol (83). Early studies demonstrated that the TM4 segment could be substituted with homologous sequences without loss of receptor function (84). However, more recent studies have shown that substitution of C418 in TM4 by tryptophan altered ion channel function (85). The latter studies suggest that although TM4 is thought to be distant from the channel pore, it is important in gating of the ion channel.

Tertiary Structure Model of the $GABA_A$ Receptor Subunit

Molecular Modeling We obtained the sequences of nine related proteins in the superfamily of ligand-gated ion channels. These were nACh receptor α_4, α_7, and torpedo α subunits; $GABA_A$ receptor α_1, α_2, β_1, and β_3 subunits; glycine receptor α_1 subunit; and GABA receptor ρ_1 subunit. We predicted the topology of the transmembrane domains of these segments with the bioinformatics techniques PHDhtm and HMMTOP. The ends of the transmembrane α helices were averaged. The sequences were simultaneously aligned with the multiple sequence alignments algorithm ClustalW. The averaged secondary structure predictions were added to the multiple sequence alignment to give a clearer picture of regions of secondary structure and helical limits that were common to all nine sequences. This analysis clearly predicted that the four transmembrane segments (TM1–4) were all α helices with reasonably well-defined helical ends.

This secondary structure information was then used in conjunction with the SeqFold algorithm to search for a modeling template based upon both sequence and fold homology/analogy. Although the SeqFold search of a modified version of the Protein Data Bank produced several well-scored alignments, there was only one template that was of mammalian origin and showed an alignment over all four transmembrane domains. This was the chain C domain 1 portion of bovine cytochrome oxidase, 1occ.pdb (Figure 2). In a qualitative search of both the SCOP (Structural Classification of Proteins) and CATH (Class, Architecture, Topology, and Homologous superfamily) fold databases, this template was again the only one found that was a tetramer of α helices, mammalian in origin and common to both databases. This template was then aligned with the sequence of $GABA_A$ receptor α_2 subunit. This alignment was initially scored based upon sequence similarity, fold similarity, and the mean force potential of Sippl (86). We used knowledge-based modeling to produce agreement with the hydrophilic and hydrophobic labeling studies (Figure 3*A*) carried out in the homologous nACh receptors (66, 67, 74) and with the proposed juxtaposition of Arg 273 and Asp 286 to form a salt bridge. We also hypothesized that the lack of a halothane effect owing to the L231F mutation (87) was due to its proximity to Ser 269 and Ala 290 (TM2 and TM3 residues critical for anesthetic actions, respectively). Conformer libraries were used to provide reasonable initial orientations of the amino acid side chains. The loops between α helices were constructed to minimize fraying of helical ends. The TM1-2 and TM2-3 loops were constructed using the loop modeling features within the Swiss Protein Data Bank Viewer (88). These are based upon loop fragment sequence similarities to loops of known three-dimensional structure. The large TM3-4 loop was removed and replaced by a series of 6 glycine residues. This substitution allowed maximum flexibility of the loop, but maintained a reasonable distance between the end of TM3 and the beginning of TM4.

The entire structure was subjected to sequentially restrained molecular mechanics energy optimization. Because the sequence of $GABA_A$ receptor α_2 subunit is quite different from the primary sequence of the template, we used a previously

described technique to maintain the left-handed supertwist of the template of four α helices without imposing additional restraints on the backbone atoms (89). Each α helix was divided into an upper, middle, and lower third. Then a centroid of the backbone atoms of each third was defined. Distance restraints were applied to maintain an 11 Å separation between adjacent centroids of neighboring α helices. These restraints had the appearance of three squares, one each at the upper, middle, and lower levels of the tetramer. The structure was optimized using these restraints with Discover 98 (MSI, San Diego, CA) and the CFF91 forcefield. In order to allow the side chains of the tetramer to adjust to optimum packing before the helical backbone was distorted, the initial force constant of the centroid restraints was set to 100 kcal/Å^2 with subsequent reductions to 10 and 1 kcal/Å^2 in sequential optimizations.

We then used a template-forcing algorithm to align the TM2 α helix of this tetramer onto the pore-lining α helix of the pentameric bacterial stretch receptor [1msl (mechanosensitive ion channel) in the Protein Data Bank]. This was repeated with fivefold symmetry to produce a pentamer of tetramers totaling 20 α helices. Simulated annealing with restrained molecular dynamics produced our final structure. We used two sets of restraints in these simulations. First, the distance restraints between α helices defined above were used for each subunit with a force constant of 10 kcal/Å^2. Second, the template forcing distances that were used to construct the pentamer of TM2 segments onto the 1msl template were retained and assigned a force constant of 100 kcal/Å^2. We subjected this restrained structure to molecular dynamics with sequential steps (10,000 cycles of 1 femtosecond) of heating by 100°K up to 500°K, an equilibration step of 10,000 cycles at 500°K, and then cooling steps of 100°K. Finally, we re-optimized the structure with molecular mechanics, and recorded energies of both bonded and nonbonded interactions. This procedure produced a structure in which specific amino acid residues from all four transmembrane α helices were in direct proximity to one another. These residues are Leu 231 (TM1), Ser 269 (TM2), Ala 290 (TM3), and Val 407 (TM4). Whereas residues that were homologous to Ser 269 and Ala 290 were previously studied in the glycine receptor as being fundamental to anesthetic action, it is only recently that the residue homologous to Leu 231 has been shown to be important as well (87). Lastly, this model clearly predicts that it is the Val 407 on TM4 that should prove to be important for anesthetic binding in conjunction with the other three residues (Figure 3*B*).

Size of the Anesthetic Binding Pocket We used molecular modeling to visualize the effect of site-directed mutations in the glycine and GABA receptors. A common theme was the volume of a site in the transmembrane domain. Homologous n-alcohols increase in CNS depressant potency as the length of the carbon chain increases, up to a point at which further increases in molecular size decrease (or no longer increase) alcohol potency. This is termed the cutoff. Our group found that alcohol regulation of the glycine receptor critically depends on specific amino acid residues in TM2 and TM3 of the α subunit polypeptide (29). They

demonstrated that residues in the glycine α_1 receptors and the GABA ρ_1 receptor also control alcohol cutoff. Mutation of Ser 267 to Gln (S267Q) decreased the n-alcohol cutoff in glycine α_1 receptor. Conversely, mutation of Ile 307 and/or Trp 328 to smaller residues increased the n-alcohol cutoff in the GABA ρ_1 receptor. These results are consistent with the suggestion that n-alcohol binding sites within these transmembrane proteins modulate the action of these alcohols, and that amino acid residues present at these critical positions limit the size of alcohol molecules that can interact with the binding cavity.

We modeled the double site-directed mutation of Ile to Ser at position 307 (I307S) in TM2, and Trp to Ala at position 328 (W328A) in TM3 in the human GABA receptor ρ_1 subunit. The change in van der Waals volume resulting from the double site-directed mutation, assuming that the inter-helical packing does not allow movement of the backbone atoms of the helices, is $162 - 109 = 53$ Å^3 plus $224 - 99 = 125$ Å^3, a total volume change of 178 Å^3. This volume corresponds to that of $C_7H_{15}OH$. In the series of alcohols studied, each additional CH_2 adds approximately 20 A^3 of van der Waals volume. The volume of 178 Å^3 is equivalent to 8.9 additional methylenes (178 Å^3/20 Å^3). On this basis, we predicted that the double site-directed mutation could increase the alcohol cutoff by 8 to 9 methylene groups. We observed that the double site-directed mutation increased the alcohol cutoff by at least 7 methylenes (29) (alcohols greater than C-12 were too insoluble to test).

Interactions with Anesthetics Residues in our current model of $GABA_A$ receptor α_2 subunit were replaced at several positions in an effort to elucidate the effect of side chain volume characteristics on the anesthetic binding regions noted above. These mutations were S269A, S269W, and S269W + A290W (A Jenkins, EP Greenblatt, HJ Faulkner, E Bertaccini, A Light, A Andreasen, A Viner, JR Trudell, NL Harrison, unpublished data). The resulting three new models were re-optimized as above. We defined a site midway between the Ca carbons of residues 269 and 290. At this site we manually inserted halothane, isoflurane, and either one or two chloroform molecules to produce a set of 16 ligand-receptor complexes. All protein backbone atoms in these models were constrained to their initial positions. Only movement of the ligand and those amino acids residues within 5 Å of this ligand was allowed. Each of these complexes was subjected to simulated annealing with restrained molecular dynamics. The many restraints in these calculations cause the resulting energies of these ligand-receptor complexes to be of only qualitative value. However, they suggest that all three ligands bind well to sites near S269 or A290. The single tryptophan mutant (S269W) binds only one chloroform well, suggesting that the native binding pocket, with a smaller amino acid residue, could contain two chloroform molecules. The double tryptophan mutant (S269W + A290W) produces an unstable complex in the forced presence of all ligands.

Therefore, based upon our knowledge of transmembrane mutations and their effects on anesthetic activity, the way in which transmembrane amino acids can be chemically labeled, the physicochemical characteristics among series of

anesthetics that are favorable for activity, and the ways in which anesthetics bind to generic proteins, one can look for sites in ligand-gated ion channels that meet these criteria. With this, we can show that the region that is both necessary and sufficient for anesthetic activity is the region within the tetrameric subunit of ligand-gated ion channels that is bounded by four specific amino acids: Leu 231 (TM1), Ser 269 (TM2), Ala 290 (TM3), and Val 407 (TM4).

CORRELATION WITH IN VIVO ANESTHETIC ACTION

Stereoselectivity

General anesthetic stereoselectivity poses the greatest challenge to traditional lipid theories of anesthetic actions because optical isomers of general anesthetics behave identically with respect to their ability to disorder lipid bilayers, despite significant differences in their in vivo potency (90, 91). Furthermore, stereoselectivity represents a powerful test for the relevance of a putative anesthetic target, i.e. if a given receptor is one of the important anesthetic targets, the rank order and ratio of potency of the optical isomers should be similar for producing anesthetic actions and for modulating the receptor function.

R(+) etomidate is ~17 times as potent as S(−) etomidate to induce a loss of righting reflex in tadpoles, and the R(+) isomer is 6–10 times more effective than the S(−) isomer at potentiating GABA-induced currents (91).

Steroid anesthetics such as 3α-hydroxy-5α-pregnan-20-one are enantioselective both for inducing a loss of righting reflex in tadpoles and mice and for potentiating GABA-induced currents, with (+) isomer exhibiting greater potency than (−) isomer (92, 93).

S isomers of barbiturates are generally more potent than R isomers for their anesthetic activities (94). $GABA_A$ receptor responses are potentiated by S isomers of hexobarbital, pentobarbital, and thiopental more potently than by their R isomers by a factor of 2–4 (95, 96), whereas neuronal $\alpha_4\beta_2$ nACh receptor is inhibited by R(+) thiopental more effectively than by S(−) thiopental, and inhibition of neuronal α_7 and muscle $\alpha_1\beta_1\gamma\delta$ nACh receptors by thiopental isomers shows no stereoselectivity (97). Furthermore, $GABA_A$ receptor function is enhanced by an anesthetic barbiturate R(−) 1-methyl-5-phenyl-5-propyl barbiturate (MPPB), but is inhibited by its convulsant stereoisomer S(+) MPPB (98, 99), whereas both R(−) and S(+) MPPB inhibited AMAP (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor current responses, with R(−) MPPB being only slightly potent compared with S(+) MPPB (99).

S(+) isoflurane are slightly (~20–50%) more potent than R(−) isoflurane for measurements of sleep time and MAC (100–102). S(+) isoflurane is ~2 times more potent than R(−) isoflurane to potentiate $GABA_A$ receptor function (103–105), and ~1.5 times more potent to inhibit neuronal nACh receptor currents (90). In contrast, two optical isomers of isoflurane are equally effective in potentiating glycine receptor currents (106).

S(+) ketamine is 3–4 times more potent than R(−) ketamine as an analgesic agent (107–108) and 1.5–4 times more potent in terms of hypnotic activity (107–109). S(+) ketamine is 2–4 times more potent than R(−) ketamine in inhibiting NMDA receptor currents (110–112), whereas S(+) ketamine is 1.5 times as potent as R(−) ketamine in reducing neuronal excitation by acetylcholine (113).

Thus, in general, the consistent correlation of in vivo anesthetic potencies of optical isomers of several anesthetics with their potencies for modulating receptor function is observed only for potentiation of $GABA_A$ receptor activity (except for ketamine and NMDA receptors), supporting the plausibility of $GABA_A$ receptors as anesthetic targets.

Nonimmobilizers

Certain highly lipid-soluble halogenated cyclobutanes and alkanes are unable to induce immobility at concentrations predicted by the Meyer-Overton rule to be in the anesthetic range (114). The nonimmobilizers provide clues to the relevance of a putative anesthetic target, i.e. if a given receptor is one of the anesthetic targets, anesthetics should affect the receptor, but nonimmobilizers should not modulate the receptor function. The nonimmobilizer 1,2-dichlorohexafluorocyclobutane (F6) exhibits no modulatory effects on $GABA_A$ (115), glycine (13), $GABA_C$ ρ (10), 5-HT_3 (116), neuronal nACh (117), AMPA (18), kainate (18), or NMDA (T Yamakura & RA Harris, unpublished data) receptors. Muscle nACh receptors are affected by F6 through mechanisms distinct from anesthetics (118–119). On the other hand, F6 inhibits responses of G protein–coupled receptors such as metabotropic glutamate (120), muscarinic ACh (121), or 5-HT_{2A} (122) receptors the way volatile anesthetics do. These results would seem to exclude these G protein–coupled receptors as viable targets for producing immobility. These receptors may certainly play a role in other actions, such as amnesia, because nonimmobilizers may depress learning and memory (123).

Gene Targeting

Gene targeting in mice is very powerful for elucidating the in vivo roles of certain ligand-gated ion channel subunits in mediating diverse behavioral actions of general anesthetics. Several knockout mice lacking specific subunits for ligand-gated ion channels have already been created, and anesthetic sensitivities have been determined in some of these mice (124).

Mice lacking the β_3 subunits of $GABA_A$ receptors exhibit some resistance to the immobilizing actions of halothane and enflurane, but sensitivity to obtunding (loss of righting reflex) effects of volatile anesthetics is not altered (125). These mice are also more resistant to obtunding effects of etomidate and midazolam, but not to those of pentobarbital and ethanol, compared with wild-type mice (125).

Mice lacking the α_6 subunits of $GABA_A$ receptors exhibit sensitivities to the immobilizing actions of enflurane and obtunding effects of halothane, enflurane,

pentobarbital, and ethanol, which are not significantly different from those of wild-type mice (126).

Mice deficient in the δ subunits of $GABA_A$ receptors exhibit a selective attenuation of sensitivities to obtunding effects of neuroactive steroids such as alphaxalone and pregnanolone (127). This is surprising because the presence of the δ subunits reduces modulation of recombinant $GABA_A$ receptors by neurosteroids (128). Sensitivities to halothane, propofol, midazolam, etomidate, and ketamine are unaffected by the δ subunit knockouts (127).

Mice deficient in GluR2 subunits of AMPA receptors show increased sensitivity to behavioral effects of pentobarbital (loss of righting reflex and loss of corneal, pineal, and toe-pinch withdrawal reflexes) despite reduced pentobarbital inhibition of AMPA receptors in their hippocampal neurons (129).

Although knockout mice may provide initial clues to the nature of anesthetic targets, results can be difficult to interpret because compensation for the absent subunits by alterations in other subunits may occur. Furthermore, aberrations in neural development, grossly abnormal motor behavior, and lethality may preclude analyzing the anesthetic sensitivity. These shortcomings of global gene knockouts may be circumvented by conditional gene knockouts, which enable gene knockouts only in limited brain regions and/or specified developmental stages (130). Another strategy overcoming the limitations of global gene knockouts is the gene knock-in technique. This technique introduces, for example, a mutation into receptor subunits that eliminates anesthetic modulation but does not alter other physiological function. This approach has recently applied to benzodiazepines, and revealed that the α_1 subunit of $GABA_A$ receptors mediates sedative, hypnotic, and partly anticonvulsant actions of diazepam, but not anxiolytic, myorelaxant, motor-impairing, and ethanol-potentiating effects of diazepam (131).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

As reviewed above, the most plausible target of general anesthetics among ligand-gated ion channels is the $GABA_A$ receptor. However, there are also some anesthetics that do not affect the $GABA_A$ receptors at clinical concentrations, i.e. gaseous anesthetics nitrous oxide and xenon, dissociative anesthetic ketamine, and fluorinated alcohols that have in vivo anesthetic effects (20–22, 24, 25, 132–134). Thus, it is unlikely that $GABA_A$ receptors are universal anesthetic targets involved in producing general anesthesia. General anesthesia, however, is a behavioral state that requires varying degrees of immobility, amnesia, hypnosis/unconsciousness, analgesia, and so on. Considering that different classes of anesthetics have distinct behavioral effects, it may not be surprising that a wide variety of anesthetics has a diverse pattern of actions not only on ligand-gated ion channels, but also on other brain receptors/proteins. These issues are related to the key question of whether the neuronal function mediating certain behavioral actions of anesthesia is common to every general anesthetic or not (the unitary theory). In vivo studies of specific

anesthetic behaviors are required to address this question, and the most convincing investigation to directly correlate the anesthetic behaviors and neuronal function will be the analysis of gene knock-in mice with a mutation in target proteins that eliminates anesthetic actions but does not otherwise change the physiological function. This experiment may be complicated if multiple subunit isoforms are involved in producing an anesthetic behavior, but recent knock-in studies showing a specific role of the α_1 subunit of $GABA_A$ receptors in certain types of behavioral actions of benzodiazepines (131) are encouraging for future anesthetic research.

Through the elucidation of molecular mechanisms of general anesthesia in conjunction with structural biology and rational drug design, it may become possible to develop specific behavior- or target site–oriented anesthetic strategies without interfering with other physiological functions.

ACKNOWLEDGMENTS

The authors thank Drs. Edmond I Eger, II (University of California, San Francisco) and Neil L Harrison (Weill Medical College, Cornell University) for helpful discussions. Funding was generously provided by National Institutes of Health grants AA06399 and GM47818.

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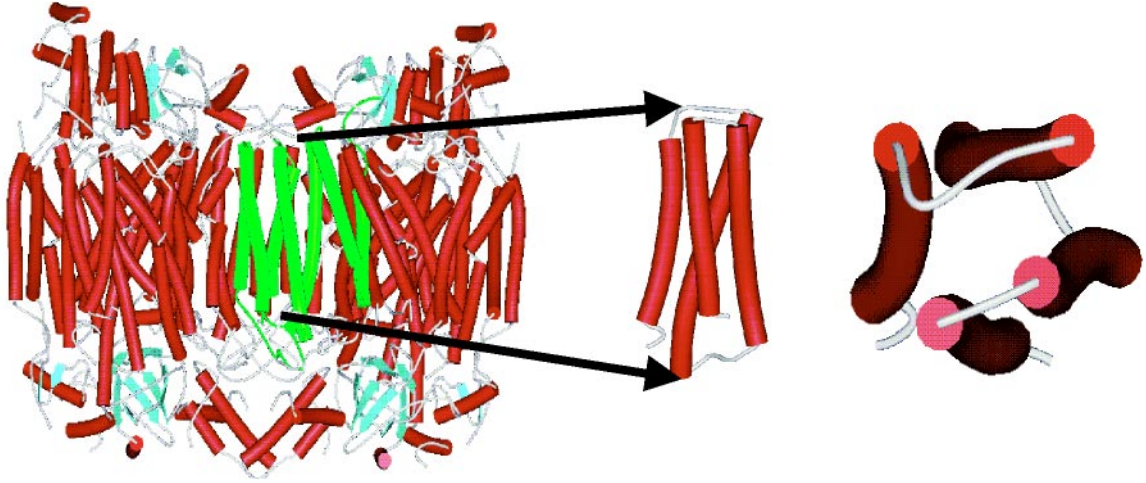

Figure 2 Template for a four-helix subunit. A four-helix bundle was identiÆedin cytochrome oxidase (left image) and excised for use as a template (center image, a view from the plane of the membrane; right image, a view from the extracellular surface).

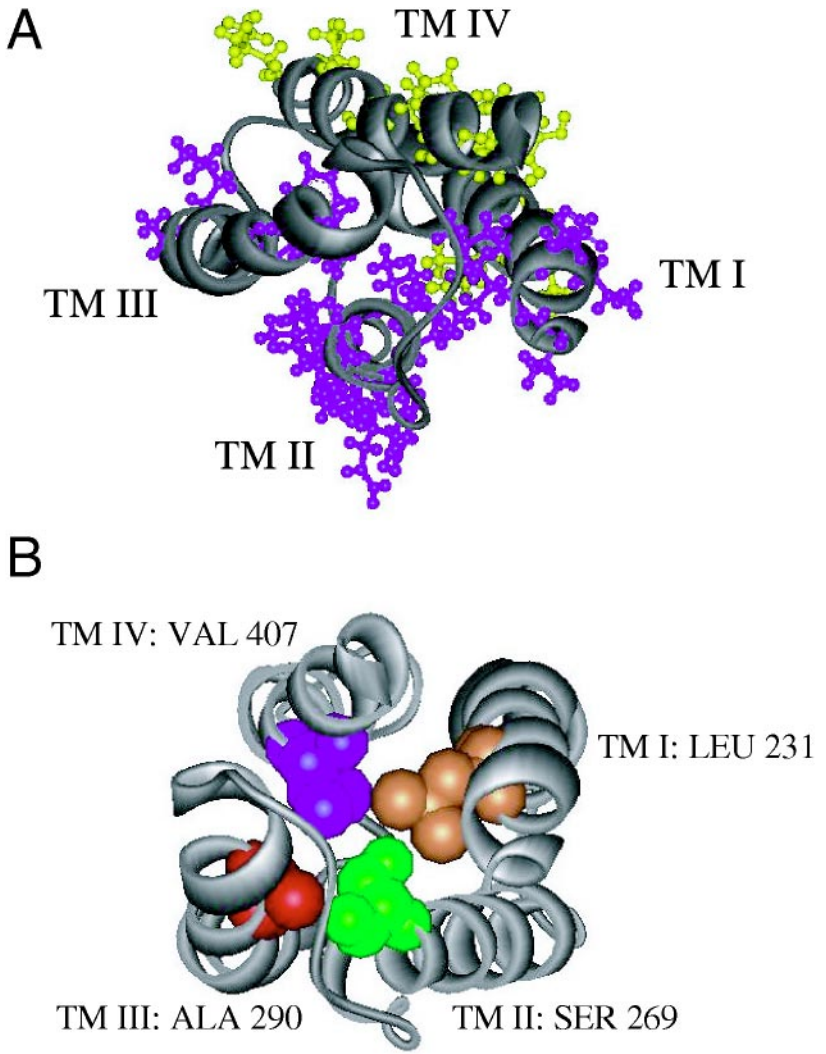

Figure 3 (*A*) Satisfying restraints based on labeling studies. View of subunit from the extracellular side. The pore-lining TM2 α helix is at the bottom of image. The alignment for lipid-facing and pore-facing labels: blue = hydrophilic label, yellow = hydrophobic label. (*B*) Proximity of residues in which mutation affects anesthetic potency. In this model of a $GABA_A$ receptor α_2 subunit, viewed from the extracellular side, three known residues are shown (S269, A290, L231). A fourth residue is predicted to form part of the boundary of the putative binding cavity (V407).