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Does General Anesthetic-Induced Desensitization of the *Torpedo* Acetylcholine Receptor Correlate with Lipid Disordering?

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SUMMARY

We have tested the hypothesis that general anesthetics stabilize the desensitized state of the nicotinic acetylcholine receptor by disordering its surrounding lipids. Acetylcholine receptor-rich postsynaptic membranes from the electroplaques of *Torpedo* were used in this study to obtain the highest possible receptor specific activity in native membranes. We examined 18 general anesthetics, including six inhalation agents, eight 1-alcohols, the enantiomers of 2-octanol, and two intravenous general anesthetics (pentobarbital and ethylcarbamate). The degree of desensitization after preincubation with the general anesthetics was determined by brief exposure to [^{3}H]acetylcholine, making use of the facts that desensitized receptors have much higher affinity than do those in the resting state and that interconversion between the states is slow. All of the general anesthetics desensitized the receptor within minutes, exhibiting steep concentration-response curves with Hill coefficients generally within the range of 2–4. At the highest general anesthetic concentrations, almost all receptors were desensitized. The concentrations that desensitized half of the resting state receptors varied by >3000-fold. The 2-octanol enantiomers were without stereoselectivity. Membrane order was examined in parallel by using spin-labeled fatty acids doped into the native membranes. The spin label 5-doxylpalmitate reported from the most ordered part of the bilayer near the aqueous interface, whereas 12-doxylstearate reported from the less ordered region nearer the center of the bilayer. The spin label deeper in the membranes was 3 times more sensitive to a given anesthetic than was the other probe. At both depths in the membrane general anesthetics decreased lipid order linearly with increasing concentration. The range of disordering potencies (change in order parameter induced by a unit concentration of general anesthetic in the aqueous phase) was 5333 for 5-doxylpalmitate and 7143 for 12-doxylstearate, but the range of disordering compared at equally desensitizing concentrations was reduced by 875- and 1430-fold, respectively. The average degrees of disordering at concentrations that desensitized half of the resting state receptors were 1.5% and 4.4%, respectively. It is unlikely that changes in membrane order parameter *per se* cause desensitization, because the associated changes in order parameter can be reproduced by changes in cholesterol content or temperature that do not cause desensitization. We conclude that, although there is a strong association between anesthetic-induced membrane disordering and desensitization, more detailed tests of a mechanistic nature will be necessary to elucidate the mechanisms underlying the Meyer-Overton-type behavior we have observed.

General anesthetics are a remarkably diverse group of compounds, ranging from simple atoms like argon to the complex ring structures of barbiturates and sterols. In spite of this structural diversity their potency correlates well with their solubility in apolar solvents such as olive oil, octanol, or lipid bilayers. Although such correlations have remarkable predictive power, their mechanistic implications are harder to ascertain. On one hand one might simply take the correlations at face value and develop unitary theories based on lipid perturbations (reviewed in Ref. 1). On the other hand one might assume that such correlations point to hydrophobic sites on proteins (2). Both approaches have had some degree of success in accounting for the known pharmacology of anesthesia, but the relevance of the models employed, lipid bilayers and luciferases, to general anesthesia *in vivo* remains unclear. A more direct approach would be to attempt to elucidate the mechanism of general anesthetics at their primary sites of action such as ion channels. However, membranes from the central nervous system of mammals are far too heterogeneous, are available in insufficient quantities, and have too low a specific activity to permit meaningful molecular studies.

One known target for general anesthetics is a superfamily of

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ABBREVIATIONS: nAcChoR, nicotinic acetylcholine receptor; AcCho, acetylcholine; 5-PASL, 5-doxylpalmitate; 12-SASL, 12-doxylstearate.

ligand-gated ion channels that includes the γ-aminobutyric acid type A receptor, the nAcChoR, the 5-hydroxytryptamine type 3 receptor, and the *N*-methyl-D-aspartate receptor (3). Of these receptors, the one that is available both in large quantities and in a relatively homogeneous state is the nAcChoR from the electric organ of *Torpedo*. Electrophysiological studies have long demonstrated that cholinergic postsynaptic membranes are particularly sensitive to inhibition by anesthetics (4, 5), and this has led numerous workers to exploit the nAcChoR-rich membranes of *Torpedo* to elucidate the molecular mechanisms of action of anesthetics (6–8). Although considerable progress has been made in the case of local anesthetics and barbiturates, including the identification of allosteric sites, the inhalational general anesthetics have proved a more difficult problem. They exert two major actions (9). First, some, but by no means all, of them inhibit the ion channel. Second, all agents so far examined stabilize the desensitized state of the nAcChoR. At rest, the nAcChoR exists in two slowly interconverting conformations, i.e., the resting and desensitized states. Approximately 20% of the receptors are normally in the latter state, which is readily detected because of its high affinity for [^{3}H]AcCho. General anesthetics increase the fraction of nAcChoRs in the desensitized state. The concentration-response curves are steep and their midpoints correlate with general anesthetic potency (10). The effect is pressure reversible (11). Even pentobarbital, which at low concentrations binds stereoselectively to an allosteric site, exerts this desensitizing action at higher concentrations in a nonstereospecific manner (12). Desensitization, then, has a pharmacological profile similar to that of general anesthesia itself, and elucidation of its mechanism might therefore be expected to contribute to an understanding of general anesthesia *per se*.

One hypothesis is that there exist a large number of nonspecific anesthetic sites on the protein, perhaps at the lipid-protein interface (13). On the other hand, reconstitution experiments demonstrate that the resting state is stabilized only when the surrounding lipid bilayer falls within a specific "fluidity window" (14). Consistent with this notion, the ability of general anesthetics and alcohols to desensitize the nAcChoR correlates well with their ability to disorder membranes (15). In this study we have extended that work, first by using an improved method based on that of Boyd and Cohen (16) to estimate desensitization, second by measuring membrane disorder at two depths in the bilayer, and third by using a much larger and more diverse set of general anesthetics.

Materials and Methods

Reagents and chemicals. The specific activity of each batch of [^{3}H]AcCho (250–3200 Ci/mol; Amersham-Searle, Arlington Heights, IL) was determined by the isotope-dilution method (17). Radiochemical purity was determined as the fraction of total radioactivity bound to membranes after three cycles of equilibration with a 5-fold molar excess of receptor. Counts not bound after equilibration were radiochemical impurities and always amounted to <3% of total radioactivity. Diisopropylfluorophosphate was from Sigma Chemical Co. (St. Louis, MO).

The volatile anesthetics used were as follows: halothane (clinical grade; Ayerst, New York, NY), chloroform (reagent grade; American Scientific Products, McGraw Park, IL), methoxyflurane (clinical grade; Abbott, North Chicago, IL), and diethyl ether (reagent grade; Mallinckrodt, Paris, KY). Thiomethoxyflurane and fluroxene were gifts of Dr. R. C. Terrell, Research and Development, Anaquest, Inc. (Murray Hill, NJ). Urethane (ethylcarbamate) was from Fisher Scientific (Fairlawn, NJ), sodium pentobarbital was from Sigma, and all primary alcohols were spectroscopic grade from Aldrich (Milwaukee, WI). Optical isomers of secondary octanol (Norse Laboratories, Newbury Park, CA) had chemical purity of >98%, which was confirmed in our laboratory by gas chromatography. Optical purity, which was determined by optical rotatory dispersion by the manufacturer for each numbered batch, was >96%. Chemical purity of all volatile agents was reconfirmed in our laboratory by gas chromatography [Beckman GC-72 with a 6-foot × 0.25-inch column packed with Poropak P (Waters Co., Milford, MA); flame-ionization detector temperature, 250°; oven temperature, 110–215°]. The spin label 12-SASL [12-(2,2-dimethyl-*N*-oxyloxazolidine)stearate] was obtained from Molecular Probes (Eugene, OR). The spin label 5-PASL [5-(2,2-dimethyl-*N*-oxyloxazolidine)palmitate] was synthesized in our laboratory. The purity of these probes was confirmed by thin layer chromatography with a solvent of hexane/diethyl ether/methanol/acetic acid (60:20:2:3, by volume).

Preparation of nAcChoR-rich membranes. Electroplaque tissue from freshly dissected *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) was homogenized, and nAcChoR-rich membranes were obtained by differential and sucrose density gradient centrifugation as described in detail elsewhere (11). Typical preparations contained 0.5–2.0 μmol of AcCho sites/g of protein and were stored at −80° until used. Purified membranes were resuspended in *Torpedo* Ringer solution (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na_2PO_4, pH 7.0), containing 0.02% NaN_3.

Determination of specific activity and receptor concentration in suspension. The concentration of nAcChoR sites (R_{Total}) present in membrane suspensions was determined by incubating an aliquot of membranes, which had been preincubated for 30 min with 10^{-4} M diisopropylfluorophosphate, with a saturating concentration of [^{3}H]-AcCho until equilibrium was reached (30 min at 20°) (10). Protein and lipid phosphorus concentrations were measured by colorimetric methods as described previously (18).

Determination of the fraction of desensitized nAcChoRs. The nAcChoR exists in an equilibrium between the desensitized state, with high affinity (R_{Hi}) for AcCho, and the resting state, with low affinity (R_{Lo}) (13). At rest, 80–85% of receptors are in the resting state. At room temperature, AcCho-induced conversion to the desensitized state takes several minutes and brief exposure to low concentrations of AcCho leads to rapid binding to pre-existing desensitized nAcChoRs without causing any immediate conformational interconversion. Therefore, suspensions of nAcChoRs (final concentration, 25 nM in AcCho sites) were preincubated, with or without general anesthetics, at 20° for 30 min in gas-tight vials. The vials were opened to allow rapid addition of 50 μl of [^{3}H]AcCho solution (final concentration, 45 nM), capped, and vortexed and, after a 5-sec incubation, the suspension was vacuum filtered through dry glass fiber filter discs (Whatman GF/F; effective retention, 0.7 μm). Samples of the filtrate were counted in Liquiscint (National Diagnostics, Somerville, NJ) with a Beckman LS 8100 liquid scintillation counter; counting efficiency was determined by the channel ratio method, using tritiated water as a standard. The high rate of dissociation of AcCho from its receptor (16) and the lability of volatile anesthetics in membranes precluded the use of any procedure involving washing and counting the filters. The concentration of bound [^{3}H]AcCho was determined as the difference between the total and free concentrations. Corrections for nonspecific binding to the filter and membrane, which amounted to 4% of the bound [^{3}H]AcCho concentration, were determined using controls in which membranes were preincubated with α-bungarotoxin, as described previously (10). Counting efficiency was found to be unaffected by the presence of anesthetics in the counting samples, even at the highest concentrations used during this study. Volatile and alcohol anesthetic concentrations were monitored by gas chromatography and were found to remain within 10% of their initial concentrations during the time course of all assays.

The increase in the fraction of high affinity sites with general anesthetic concentration was established for each agent from duplicate determinations with a minimum of eight different concentrations of

anesthetic. Entire studies were repeated and the data were pooled after correction for minor variations in the control levels of desensitized receptors. The general anesthetic concentrations that caused half-maximal conversion of nAcChoRs initially in the low affinity resting state to the higher affinity desensitized state (R_{Hi}^{50}), the slopes (Hill coefficients), and estimates of their respective standard deviations were obtained by fitting the data to a logistic equation using the method of nonlinear least-squares fitting,

$$R_{Hi}^{50} = \left(R_{Hi}^{Max} - R_{Hi}^{Min}\right) \cdot \left(\frac{[GA]^{n_H}}{[GA]^{n_H} + (R_{Hi}^{50})^{n_H}}\right) + R_{Hi}^{Min} \quad (1)$$

where R_{Hi}^{Max} and R_{Hi}^{Min} are the maximum and minimum fractions of high affinity receptors attained, [GA] is the free anesthetic concentration in the buffer, and n_H is the Hill coefficient.

Spin labeling of nAcChoR-rich membranes. Spin label (either 5-PASL or 12-SASL) was deposited in a thin film from methanolic stock solution by drying for 6 hr under vacuum. An aliquot of nAcChoR-rich membranes was then added so that the final probe concentration was 1% of the membrane phospholipid, and the mixture was gently shaken for 6 hr at 4°. To minimize signal from unbound spin label, aliquots of spin-labeled membranes (100 μl, 8 mg of protein/ml) were then washed by 10 cycles of pelleting (6000 × *g* for 3 min at 4°) and resuspended in ice-cold *Torpedo* Ringer solution (at a concentration of 15–20 mg of protein/ml) and were finally added to buffer with or without an anesthetic. Membrane-anesthestic mixtures were quickly flame-sealed in 1-mm (i.d.) glass capillary tubes (2-mm o.d.; Corning) while being kept in ice. For each anesthetic studied, quadruplicate capillary samples were prepared at each of four different anesthetic concentrations, and entire studies were repeated up to four times. All samples were pelleted in the sealed capillary tubes (250 × *g* for 30 min at 4°), and the pellets were centered in the microwave cavity (to maximize signal strength) and equilibrated at 20° for 10 min. After spectroscopy, the samples, still in the sealed capillary tubes, were centrifuged (250 × *g* for 6 hr) and duplicate 10-μl aliquots of the supernatant were then injected into a Beckman GC-72 gas chromatograph [column packing, Poropak P (Waters); flame-ionization detector temperature, 250°; oven temperature, 100–215°]. The anesthetic concentrations so determined correspond to free aqueous concentrations, because protein or lipid phosphorus was undetectable in the clarified supernatants of the capillary tubes.

ESR spectroscopy. ESR spectroscopy was performed at 20° using a Varian E109 Century series spectrometer operating at 9.3 GHz, with a magnetic field strength of 3260 G. Microwave power was set at 10 mW, and the filter time constant, modulation frequency, and amplitude were set at 0.32 sec, 100 kHz, and 3.2 G, respectively. Instrument settings were then kept constant for all subsequent assays. Spectra were scanned over a 100-G range in 8 min and their first derivatives were recorded. Cavity temperature was maintained at 20 ± 0.1° by passing a thermostatted stream of dry nitrogen through a Dewar insert, which supported the sample. A lipid order parameter (S) was derived for each of the samples, as described (19),

$$S = \frac{(T_\parallel - T_\perp)}{[T_{xx} - 0.5(T_{xx} + T_{yy})]} \quad (2)$$

where T_{xx}, T_{yy}, and T_{zz} are single-crystal values for the principal elements of the T tensor and $T_\parallel$ and $T_\perp$ are, to a first approximation, half the separation of the outer and inner extrema, respectively (see Fig. 2). To correct for the difference between the polarity of nAcChoR-rich membranes and that corresponding to the single-crystal measurement, S was multiplied by:

$$\frac{(T_{xx} + T_{yy} + T_{zz})}{(T_\parallel + 2T_\perp)} \quad (3)$$

Results

General anesthetic desensitization of membrane-bound nAcChoRs. Our control data confirmed the findings (reviewed in Ref. 13) that in the absence of anesthetic approximately 20% of nAcChoRs pre-exist in the high affinity desensitized conformation. Preincubation of nAcChoR-containing membranes with the anesthetics increased the percentage of desensitized nAcChoRs. When a half-effective concentration of butanol (45 mM) was preincubated for various times, the percentage of desensitized receptors, which was initially 20%, increased to 30% at 10 sec, 47% at 3 min, and 53% at 30 min. The rate of butanol-induced desensitization varied with the butanol concentration and a standard preincubation time of 30 min was chosen based on these experiments.

General anesthetics produced a steep, sigmoidal, concentration-dependent enhancement of [^{3}H]AcCho specifically bound to membranes, indicating an increase in the percentage of the high affinity, desensitized nAcChoR (R_{Hi}) up to nearly 100%. For example, in a single experiment diethyl ether caused little desensitization between 15 and 32 mM, but the percentage of desensitized receptors rose from 16 to 28% at 50 mM, to 50% at 63 mM, and finally to a value of 90% at 100–120 mM. Typical data from single experiments are shown in Fig. 1. Two or three separate experiments were performed for each agent. The reproducibility of the runs may be illustrated by the three experiments performed with thiomethoxyflurane, where the R_{Hi}^{50} varied from 580 to 670 μM, the R_{Hi}^{Max} from 98% to 100%, and the Hill coefficient from 3.5 to 5.4 (the Hill coefficient was the most variable parameter because the steepness of the curves meant that it was often defined by few points). The results of fitting the combined data simultaneously were 650 ± 18 μM, 98 ± 2.8%, and 4.5 ± 0.66, respectively. Results of the analyses for all agents are summarized in Table 1.

The six volatile agents (two haloalkanes, three haloethers, and diethyl ether) behaved as a group. All increased the R_{Hi}^{Max} from control values around 20% to close to 100%, and their Hill coefficients were never significantly less than 4. Thiomethoxyflurane was the most potent agent in this group, being twice as potent as both its oxy analog and halothane. Halogenation increased potency and the haloethers were all at least 1 order of magnitude more potent than diethyl ether, although they varied among themselves by only 4-fold.

The potency of the 1-alkanols increased regularly with chain

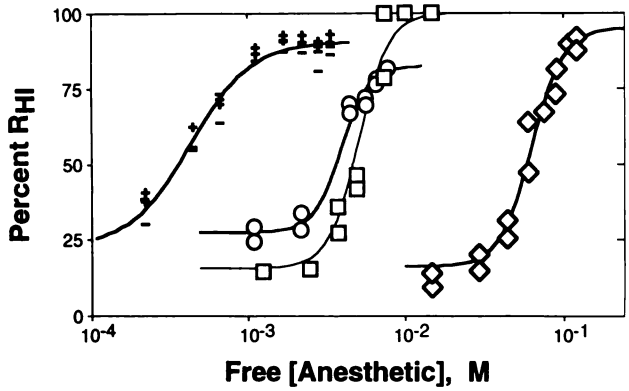

Fig. 1. General anesthetic-induced desensitization of the nAcChoR. Data are presented as the percentage of total receptors that are in the high affinity conformation [(R_{Hi}/R_{Total}) × 100%] as a function of anesthetic concentration in the buffer. The results for each anesthetic are from a representative experiment, and each *point* represents a single determination. +, (+)-2-Butanol; −, (−)-2-butanol; ○, pentobarbital; □, fluroxene; ◇, diethyl ether. The *solid line* was drawn according to eq. 1. The control values for R_{Hi} are not shown but the minimum values of the curves indicate their magnitude.

length by >1000-fold, from 1.3 M for methanol to 490 μM for octanol; the change in free energy with each additional methylene group was −637 ± 12 cal. The shorter alcohols were much less potent than the volatile clinical anesthetics, and 1-butanol was comparable in potency to its formula isomer diethyl ether. Compared with the volatile anesthetics, there appeared to be more variability in the R_{Hi}^{Max} among the alcohols, with ethanol achieving a value of only 81%. However, no regular trends were observed with chain length. The Hill coefficients were generally in the range of 2–3. The enantiomers of 2-octanol were equivalent in their actions on desensitization (Fig. 1; Table 1) and their potency was close to that of 1-octanol, which suggests that the number of carbon atoms rather than the chain length is related to potency.

The intravenous agents pentobarbital and ethylcarbamate (urethane) both enhanced desensitization. Analysis (Table 1) showed that pentobarbital behaved like the other general anesthetics, causing a steep enhancement of the fraction of desensitized receptors (Fig. 1). The potency of urethane was comparable to that of diethyl ether.

Effects of anesthetics on order parameter. The mean control lipid order parameters reported for 5-PASL and 12-SASL at 20.0 ± 0.1° in >200 individual experiments with *Torpedo* nAcChoR-rich membranes were 0.668 ± 0.0030 (mean ± standard deviation) and 0.469 ± 0.0026, respectively. For a given experiment the four individual determinations yielded an order parameter with a typical standard deviation of about 5%, so that over several experiments a change in order parameter of 0.01 could be reliably detected.

The order parameter varied linearly with temperature over the range of 4–28° and showed no discontinuities. The order parameters for 5-PASL and 12-SASL changed by 0.006/degree and 0.012/degree, respectively. Therefore, errors introduced by temperature variations in the cavity of ±0.1° were not detectable.

Fig. 2 shows typical ESR spectra of native *Torpedo* membranes spin labeled with 5-PASL and exposed to increasing concentrations of diethyl ether. The outer extrema moved systematically towards the center of the spectrum, so that $2T_\parallel$ decreased as the concentration of general anesthetic increased. This decrease in $T_\parallel$ was matched by an increase in $T_\perp$, with the result that the order parameter decreased. The other anesthetics behaved similarly.

Disordering effects were found to be reversible with representative agents from each category, even after exposure to high anesthetic concentrations (Table 2). For example, in three separate experiments using membranes labeled with 5-PASL, 6.5 mM halothane produced a change in lipid order parameter (ΔS) of −0.026 ± 0.0010 (mean ± standard deviation), but the control value for S was restored after complete evaporation of the halothane (confirmed by gas chromatography). In analogous experiments, also with 5-PASL, control S values (coefficient of variation of 6%) were recovered from membrane samples after washing out of 500 mM butanol, a concentration that produced a ΔS value of 0.141 ± 0.0044.

General anesthetic-induced disordering was a linear function of increasing aqueous general anesthetic concentration, as illustrated for typical data in Fig. 3, and regression correlation coefficients were always −0.90 or better. This remained true even when concentrations exceeding 10 times the R_{Hi}^{50} were used in some studies designed to test whether disordering effects reached a plateau (for example, chloroform). In membrane suspensions, the total and free aqueous concentrations of some anesthetics were not the same due to high partition coefficients and membrane concentrations; consequently, free concentrations of agents in those categories were always measured by gas chromatography and used in the analysis. With the most volatile agents (i.e., diethyl ether, halothane, and fluroxene), entire studies were repeated up to four times.

The change in order parameter, ΔS, was calculated for each

TABLE 1

Effects of anesthetics on desensitization of the AcChoR

All data are mean ± standard deviation.

Agent	Concentration range	R_{Hi}^{50}[a]	R_{Hi}^{Max}[b]	n_H[c]
	mM	*mM*	%	
Chloroform	0.5–17	2.6 ± 0.11	99 ± 2.1	4.2 ± 0.80
Halothane	0.3–9	1.20 ± 0.070	93 ± 3.7	4.3 ± 1.0
Diethyl ether	15–105	63 ± 3.5	94 ± 5.8	4.3 ± 0.82
Fluroxene	1–15	6.8 ± 0.45	100 ± 6.1	3.6 ± 0.58
Methoxyflurane	0.3–4	1.29 ± 0.040	96 ± 2.7	5.3 ± 2.7
Thiomethoxyflurane	0.01–0.3	0.65 ± 0.018	98 ± 2.8	4.5 ± 0.66
Methanol	500–4000	1300 ± 100	93 ± 4.7	2.6 ± 0.50
Ethanol	120–2400	570 ± 13	81 ± 1.0	3.2 ± 0.22
Propanol	40–400	135 ± 23	107 ± 9.5	1.5 ± 0.24
Butanol	24–300	47 ± 1.4	87 ± 1.7	4.2 ± 0.48
Pentanol	6–60	10.6 ± 0.49	93 ± 1.7	2.5 ± 0.33
Hexanol	2–28	4.0 ± 0.15	96 ± 1.4	2.9 ± 0.29
Heptanol	0.8–6	1.30 ± 0.050	96 ± 1.8	2.7 ± 0.32
Octanol	0.1–3	0.49 ± 0.016	97 ± 2.5	3.0 ± 0.41
(−)-2-Octanol	0.1–3	0.43 ± 0.020	89 ± 1.7	2.3 ± 0.30
(+)-2-Octanol	0.1–3	0.38 ± 0.020	92 ± 1.1	2.1 ± 0.18
Pentobarbital	1–8	3.95 ± 0.84	80 ± 3.8	4.6 ± 1.5
Urethane	31–220	75 ± 21	80 ± 13	1.9 ± 0.71

[a] R_{Hi}^{50}, concentration of anesthetic that produces a half-maximal increase in R_{Hi} beyond the control level of 15–20%. Note that this is different from the anesthetic concentration needed to produce 50% R_{Hi} (see Fig. 2).

[b] R_{Hi}^{Max}, plateau value of R_{Hi} at highest anesthetic concentration.

[c] n_H, Hill coefficient.

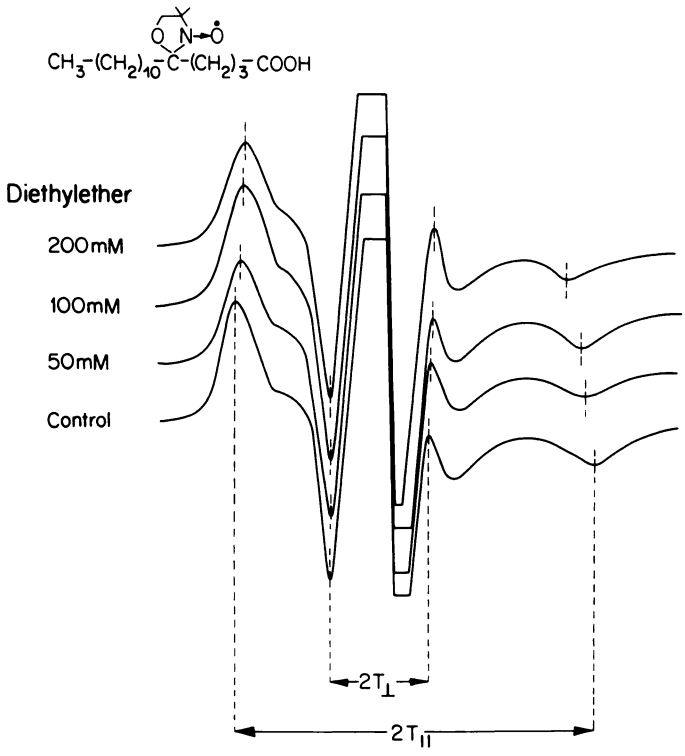

Fig. 2. EPR spectra for 5-PASL incorporated in *Torpedo* nAcChoR-rich membranes. The *horizontal scale* is given by the spectra, each of which was scanned over 100 G. The inner and outer hyperfine splittings, $2T_{\perp}$ and $T_{\parallel}$, respectively, are indicated with *vertical dashed lines*. The effects of increasing concentrations of diethyl ether are shown. The central peaks of the spectra are truncated for convenience of scaling.

TABLE 2

General anesthetic-induced membrane lipid disordering

Order parameters were measured in the presence of increasing concentrations (determined in the buffer) of general anesthetics over the ranges shown. The decrease in order parameter was fitted by linear least-squares fitting constrained to pass through the control values, and the slopes and standard deviations of these lines are reported for the spin labels 5-PASL and 12-SASL.

Agent	Concentration range	$-\Delta S$	
		5-PASL	12-SASL
	mM	M^{-1}	
Chloroform	4–41	2.5 ± 0.45	7 ± 1.0
Halothane	1–15	4 ± 1.1	16 ± 4
Diethyl ether	25–475	0.16 ± 0.034	0.7 ± 0.12
Fluroxene	2–21	2.0 ± 0.40	4 ± 1.1
Methoxyflurane	1–6	5 ± 1.7	16 ± 5
Thiomethoxyflurane	0.4–4	21 ± 6	17 ± 6
Methanol	1000–4000	0.006 ± 0.001	0.007 ± 0.0025
Ethanol	100–1000	0.016 ± 0.0056	0.05 ± 0.015
Propanol	100–400	0.049 ± 0.008	0.09 ± 0.031
Butanol	30–600	0.28 ± 0.038	0.37 ± 0.087
Pentanol	10–80	0.87 ± 0.054	1.8 ± 0.23
Hexanol	2–45	2.4 ± 0.20	5 ± 1.1
Heptanol	1–8	9 ± 1.5	16 ± 2.3
Octanol	0.3–2	32 ± 4.8	50 ± 9.1
(±)-2-Octanol	1–4	26 ± 4.3	42 ± 4.1
Pentobarbital	0.7–6	5 ± 1.5	7 ± 1.8
Urethane	125–1000	0.043 ± 0.0096	0.18 ± 0.03

experiment. Pooled ΔS data for each agent were plotted against its aqueous, or free, concentrations and were fit to straight lines through the origin by the method of least-squares fitting. The slopes are reported in Table 2. The values for these slopes remained relatively constant between preparations; for example, for butanol the mean ± standard deviation of the slopes obtained by individually fitting each of four experiments, using four different membrane preparations, was 0.28 ± 0.035 M^{-1} when 5-PASL was used.

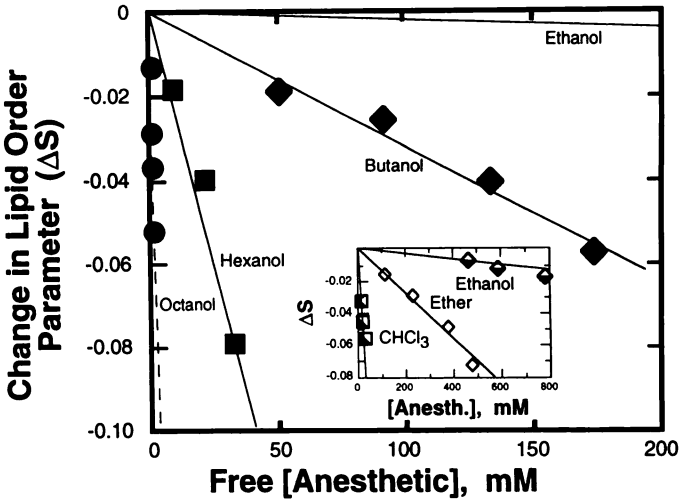

Fig. 3. General anesthetic-induced disordering of *Torpedo* nAcChoR-rich membranes. The changes in the order parameter of 5-PASL with increasing free aqueous concentrations of some representative anesthetics are shown. The data are from single experiments, and the *lines* are least-squares fits through the origin. The insert contains similar data plotted over an extended concentration range.

The slopes for the six volatile agents varied 130-fold and 24-fold for 5-PASL and 12-SASL, respectively. These slopes varied systematically with chain length in the 1-alkanol series. Octanol was the most potent and methanol the least potent of all of the agents we studied; octanol was 5300- and 7100-fold more potent than methanol for 5-PASL and 12-SASL, respectively. 2-Octanol had essentially the same potency as 1-octanol. The enantiomers were examined with 5-PASL in paired experiments. No stereoselectivity was found, with the slopes of the order parameter being -25 ± 4.8 M^{-1} for the (+)-enantiomer and -27 ± 3.7 M^{-1} for the (−)-enantiomer; the mean of these values is reported in Table 2.

In general, the changes recorded for each agent tended to be greater for the spin label deeper in the membrane, but this effect never exceeded 4-fold and was usually closer to 2-fold. The average ratio was 2.3 ± 1.1 (mean ± standard deviation), which differed from 1 ($p < 0.001$). The alcohols tended to have lower ratios (1.9 ± 0.60) than the inhalation agents (2.9 ± 1.3), but this effect did not achieve significance ($p > 0.05$).

Discussion

Evidence that general anesthetic-induced desensitization is associated with lipid disordering. In this work we aimed to test the hypothesis that general anesthetic action is related to disordering of the surrounding lipid bilayer. Such a model has previously been tested in studies that compare disordering in lipid bilayers with anesthetic potency in animals (reviewed in Ref. 1). Here we have carried out a much more specific test, examining in parallel anesthetic actions on lipid order in purified synaptic plasma membranes and on desensitization of the nAcChoRs in the same membranes. In the native membrane fraction used here, there are only about 650 lipid molecules (phospholipids and cholesterol)/nAcChoR molecule (18), of which approximately 50 at any moment are in contact

with each receptor (20, 21). Consequently, on average each nAcChoR is separated from its neighbors by about a dozen lipid molecules. Therefore, our work constitutes a relatively direct test of the lipid hypothesis.

The conventional way of presenting a test of this hypothesis is a Meyer-Overton plot, in which the logarithm of the anesthetic potency is compared with the logarithm of the lipid solubility or lipid-perturbing potency. In the case considered here, the appropriate plot compares the R^{50}_{Hi} with the disordering potency ($-\Delta S$, in M^{-1}). The data taken from Tables 1 and 2 are presented in this way in Fig. 4. The split plot facilitates direct comparison of the correlations for the two spin labels. The data for both spectroscopic probes are highly correlated and the slopes of the lines fitted to the data do not differ from the expected value of −1 (see the legend to Fig. 4).

Considering the range of general anesthetic structures included in Fig. 4, the high degree of correlation is impressive and demands a more detailed analysis. Fig. 4 is equivalent to the statement that the product of $-\Delta S$ and R^{50}_{Hi} is constant and independent of the anesthetic agent. The range of $-\Delta S$ values for 5-PASL and 12-SASL is 5333 and 7143, respectively, and the range of R^{50}_{Hi} values is 3163, yet when these two parameters are multiplied together the range of the product is reduced by about 1000-fold, to 6.1 and 5.0 for 5-PASL and 12-SASL, respectively. This is quite remarkable and, indeed, if the standard deviations of the individual ΔS and R^{50}_{Hi} values are used to propagate the errors of their products, then all values lie within 2 SD of the mean order parameter change at R^{50}_{Hi}.

Thus, we can conclude that overall the association between disordering and desensitization is very strong for this group of agents. This confirms and extends our preliminary work in which the ability of four normal alcohols to produce desensitization was compared with their ability to disorder 12-SASL

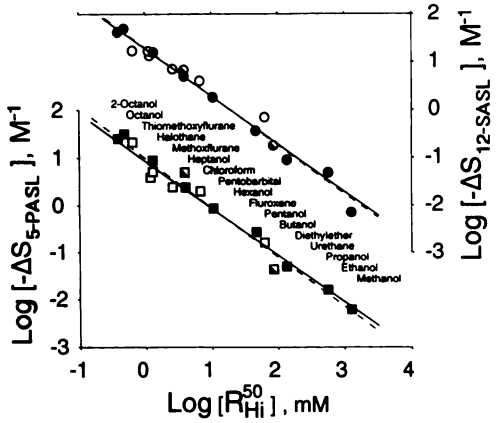

Fig. 4. Correlation between desensitizing potency (R^{50}_{Hi}) and membrane-disordering potency ($-\Delta S$) for the agents listed in Tables 1 and 2. *Upper*, correlation for the spin label 12-SASL (*circles*); *lower*, correlation for 5-PASL (*squares*). *White symbols*, 1-alkanols; *gray symbols*, 2-octanol; *black symbols*, inhalation anesthetics; *half-filled symbols*, intravenous anesthetics. The names of the agents are listed from *top* to *bottom* in the order of increasing R^{50}_{Hi}. The *lines* are linear least-squares fits to the data; the *dashed line* was fitted without constraint and the *solid line* was fitted with the slope confined to the theoretical value of −1. For 5-PASL, the slope and intercept (mean ± standard deviation) were −1.05 ± 0.043 and 1.01 ± 0.061 (r = −0.988), respectively, and for the fit with the slope constrained to −1 the intercept was 1.0 ± 0.18. For 12-SASL, the corresponding values were −1.02 ± 0.039, 1.30 ± 0.0656 (r = −0.989), and 1.3 ± 0.16. The probability that the free-fit slopes are different from 1 is negligible ($p > 0.2$).

(15). In another study we examined five general anesthetics using a much cruder assay of desensitization and found a fair correlation for both 5-PASL and 12-SASL (9). Other workers, using various assays for estimating the degree of general anesthetic-induced desensitization, have noted correlations with lipid solubility (8, 22). Our present study is distinguished by the use of a less perturbing assay (the agonist, which itself can induce desensitization, was present only for a short time and at low concentration), the direct measurement of lipid perturbation in the same nAcChoR-rich membrane fractions that were used for the desensitization studies, and the inclusion of a larger number of agents. Furthermore, we have been careful here to measure all parameters, including membrane disordering, relative to the free concentration in the buffer for each general anesthetic. This enables us to accurately compare these parameters without the complications encountered when total concentrations are used and without needing to know the membrane/buffer partition coefficient.

Possible molecular mechanisms of general anesthetic-induced desensitization. Our study was not designed to directly elucidate the underlying mechanism by which general anesthetics induce desensitization. However, had we failed to find an association between lipid disordering and desensitization we would have been able to rule out a number of possibilities. Clearly we cannot do this. Therefore, we now consider our work in relation to what is known about the nAcChoR and desensitization, to better define the steps that need to be taken to come to a deeper understanding of the underlying mechanisms.

Treatments that perturb the membrane lipid, like detergent solubilization or reaction with a phospholipase, irreversibly impair the ability of the nAcChoR to undergo agonist-induced desensitization (reviewed in Ref. 20). In contrast, this capability is stable to removal of nAcChoR-associated peripheral proteins by alkaline extraction (23). The importance of lipid-receptor interactions in modulating the relative stability of the different receptor conformations is elegantly confirmed by reconstitution experiments. In one study in which nAcChoRs were reconstituted into lipid bilayers of many different compositions, the resting to desensitized state transition was maintained, with few exceptions, only in bilayers that had order parameters (reported using 5-SASL) in a narrow range of ±0.025 (14). This compares with a change in order parameter at R^{50}_{Hi} in our work of −0.010 ± 0.0042 for 5-PASL and −0.021 ± 0.0083 for 12-SASL. Although the two studies differed in their assessments of desensitization, the magnitudes of the effects observed are not inconsistent with the idea of an optimum "fluidity" being important (14). However, we feel it is unlikely that changes in membrane order parameter *per se* may cause desensitization, for two reasons. First, depletion of the cholesterol content of native membranes by about one third causes an order parameter change of 0.032 (reported using 5-PASL) without perceptible change in desensitization (18). Second, a very small temperature difference changes the order parameter by the same amount as does the $R^{50}{}_{Hi}$ concentration of a general anesthetic. The temperature effect resembles anesthetic effects, in that temperature too is more effective at changing the order parameter deeper (12-SASL versus 5-PASL; see Results) in the bilayer. Thus, the temperature causing a change in order parameter equivalent to that associated with R^{50}_{Hi} is the same for

both spin labels and is 1.7°, a change that by itself exerts little action on desensitization (15).

The reason for the existence of the fluidity window in reconstituted nAcChoRs is not understood. Order parameters are experimentally accessible and give molecular level information, but many other properties of the bilayer are linearly related to them and might be more directly involved in nAcChoR dynamics. For example, headgroup charge, acyl chain length, steroid content, and packing properties of the lipid have all been implicated (reviewed in Ref. 20).

Recent advances have made it feasible to probe the lipid domain immediately adjacent to the nAcChoR protein. These "nearest neighbor" lipid molecules are in slow exchange with the bulk lipid pool and could directly modulate the receptor protein. The effects of general anesthetics on this domain of membrane lipid are only beginning to be studied. In native nAcChoR membranes, where the experimental difficulties are greatest, a small and selective displacement of some lipids at desensitizing concentrations has been observed (24, 25). In nAcChoRs reconstituted into a single lipid no effects on motions on the EPR time scale have been observed, although there are some indications that motions on a slower time scale may be important (21, 26). Overall, there is still no strong evidence for the idea that direct perturbation of lipid-protein interactions is implicated in the desensitizing action of general anesthetics on the nAcChoR; however, for the Ca^{2+}-ATPase from sarcoplasmic reticulum, halothane-induced fluidity changes may modulate the degree of aggregation, and hence activity, of the protein (27). Others have developed the concept that bilayer surface curvature might modulate lipid-protein interactions (28).

However, general anesthetics may perturb more selective lipid-protein interactions. Recently, cholesterol-nAcChoR interactions have been implicated in drug action (reviewed in Ref. 29), because cholesterol has a role in maintaining the resting to desensitized state transition (30). This is of particular interest because there is evidence that cholesterol occupies approximately five to 10 interstitial sites on the nAcChoR (31), a number that is not inconsistent with the Hill coefficients we observe (Table 1), suggesting a possible role for general anesthetics at these sites.

Kinetic limitations of our work. A limitation of our work is that we have measured only the amplitude of desensitization induced by general anesthetics. Although this is adequate for the analysis presented above, more detailed conclusions cannot be drawn without identification of the microscopic rate constants involved. Our methods are based on the detailed study by Boyd and Cohen (16), which showed that alcohols and agonists each stabilize the same desensitized state of the nAcChoR. However, agonist-induced desensitization takes place in two phases, one occuring over a few hundred milliseconds and the other over minutes (reviewed in Ref. 13). Whether general anesthetics cause desensitization in one or two phases is unknown, and the question is difficult to answer because of the need to use an agonist to probe the affinity state of the nAcChoR. Thus, ethanol enhances agonist-induced fast desensitization without causing it itself (32). Studies of rates of desensitization may reveal actions dependent on lipid viscosity (33), as they have done for the sodium channel (34). However, there are indications that the outcome of more detailed studies may be more complex than the simple relationships we have observed suggest. For example, for the sodium channels of squid axon the half-blocking concentrations of a comparable group of general anesthetics exhibited a Meyer-Overton relationship with the lipid bilayer/buffer partition coefficients of the anesthetics. However, the mode by which these anesthetics produced inhibition varied among the classes of agent and consequently the Meyer-Overton relationship could not be taken as evidence for a unitary hypothesis of general anesthetic action (34).

Conclusions. We have demonstrated a very close pharmacological association between general anesthetic-induced desensitization and membrane disordering in nAcChoR-rich membranes from *Torpedo*. We are suggesting not that general anesthesia results from desensitization of nAcChoRs but that the pharmacologies of the two phenomena are clearly related, even including pressure reversal of general anesthetic-induced desensitization (11), and consequently the underlying mechanisms may be similar. Thus, if the mechanisms responsible for the desensitization studied in our work can be defined, it is probable that they will illuminate the larger problem of general anesthesia. Elucidation of these molecular mechanisms will require a more detailed study of general anesthetic-induced desensitization, carried out on a faster time scale, and the development of a deeper understanding of the relationship between integral membrane protein conformational states and their associated lipid bilayers. In this respect the high specific activity of the nAcChoR in *Torpedo* membranes and the ability to reconstitute the nAcChoR into bilayers of known composition should prove to be great advantages.

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References

1. Janoff, A. S., and K. W. Miller. A critical assessment of the lipid theories of general anaesthetic action, in *Biological Membranes* (D. Chapman, ed.). Academic Press, London, 417–469 (1982).
2. Franks, N. P., and W. R. Lieb. Molecular mechanisms of general anaesthesia. *Nature (Lond.)* **300**:487–493 (1982).
3. Stroud, R. M., M. P. McCarthy, and M. Shuster. Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry* **29**:11009–11023 (1990).
4. Gage, P. W., and O. P. Hamill. Effects of several inhalation anaesthetics on the kinetics of postsynaptic conductance changes in mouse diaphragm. *Br. J. Pharmacol.* **57**:263–272 (1976).
5. Dilger, J. P., R. S. Brett, and L. A. Lesko. Effects of isoflurane on acetylcholine receptor channels. 1. Single-channel currents. *Mol. Pharmacol.* **41**:127–133 (1992).
6. Cohen, J. B., N. D. Boyd, and N. S. Shera. Interactions of anesthetics with nicotinic post-synaptic membranes isolated from *Torpedo* electric tissue, in *Molecular Mechanisms of Anesthesia* (B. R. Fink, ed.). Raven Press, New York, 165–173 (1980).
7. Sauter, J. F., L. M. Braswell, and K. W. Miller. Action of anesthetics and high pressure on cholinergic membranes, in *Molecular Mechanisms of Anesthesia* (B. R. Fink, ed.). Raven Press, New York, 199–208 (1980).
8. Young, A. P., and D. S. Sigman. Allosteric facilitation of *in vitro* desensitization of the acetylcholine receptor by volatile anesthetics, in *Molecular Mechanisms of Anesthesia* (B. R. Fink, ed.). Raven Press, New York, 209–227 (1980).
9. Miller, K. W., L. M. Braswell, L. L. Firestone, B. A. Dodson, and S. A. Forman. General anesthetics act both specifically and nonspecifically on acetylcholine receptors, in *Molecular and Cellular Mechanisms of Anesthetics* (S. H. Roth and K. W. Miller, eds.). Plenum, New York, 125–138 (1986).
10. Firestone, L. L., J. F. Sauter, L. M. Braswell, and K. W. Miller. Actions of general anesthetics on acetylcholine receptor-rich membranes from *Torpedo californica*. *Anesthesiology* **64**:694–702 (1986).
11. Braswell, L. M., K. W. Miller, and J. F. Sauter. Pressure reversal of the action of octanol on postsynaptic membranes from *Torpedo*. *Br. J. Pharmacol.* **83**:305–311 (1984).
12. Roth, S. H., S. A. Forman, L. M. Braswell, and K. W. Miller. Actions of

pentobarbital enantiomers on nicotinic cholinergic receptors. *Mol. Pharmacol.* **36**:874–880 (1989).
13. Changeux, J. P., T. A. Devillers, and P. Chemouilli. Acetylcholine receptor: an allosteric protein. *Science (Washington D. C.)* **225**:1335–1345 (1984).
14. Fong, T. M., and M. G. McNamee. Correlation between acetylcholine receptor function and structural properties of membranes. *Biochemistry* **25**:830–840 (1986).
15. Miller, K. W., L. L. Firestone, and S. A. Forman. General anesthetic and specific effects of ethanol on acetylcholine receptors. *Ann. N. Y. Acad. Sci.* **492**:71–87 (1987).
16. Boyd, N. D., and J. B. Cohen. Desensitization of membrane-bound *Torpedo* acetylcholine receptor by amine noncompetitive antagonists and aliphatic alcohols: studies of [^{3}H]acetylcholine binding and $^{22}Na^+$ ion fluxes. *Biochemistry* **23**:4023–4033 (1984).
17. Neubig, R. R., and J. B. Cohen. Equilibrium binding of [^{3}H]tubocurarine and [^{3}H]acetylcholine by *Torpedo* postsynaptic membranes: stoichiometry and ligand interactions. *Biochemistry* **18**:5464–5475 (1979).
18. Leibel, W. S., L. L. Firestone, D. C. Legler, L. M. Braswell, and K. W. Miller. Two pools of cholesterol in acetylcholine receptor-rich membranes from *Torpedo*. *Biochim. Biophys. Acta* **897**:249–260 (1987).
19. Hubbell, W. L., and H. M. McConnell. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93**:314–326 (1971).
20. Barrantes, F. J. The lipid environment of the nicotinic acetylcholine receptor in native and reconstituted membranes. *Crit. Rev. Biochem. Mol. Biol.* **24**:437–478 (1989).
21. Abadji, V. C., D. E. Raines, A. Watts, and K. W. Miller. The effect of general anesthetics on the dynamics of phosphatidylcholine-acetylcholine receptor interactions in reconstituted vesicles. *Biochim. Biophys. Acta* **1147**:143–153 (1993).
22. El Fakahany, E. F., A. T. Eldefrawi, and M. E. Eldefrawi. Nicotinic acetylcholine receptor desensitization studied by [^{3}H]perhydrohistrionicotoxin binding. *J. Pharmacol. Exp. Ther.* **221**:694–700 (1982).
23. Neubig, R. R., E. K. Krodel, N. D. Boyd, and J. B. Cohen. Acetylcholine and local anesthetic binding to *Torpedo* nicotinic postsynaptic membranes after removal of nonreceptor peptides. *Proc. Natl. Acad. Sci. USA* **76**:690–694 (1979).
24. Fraser, D. M., S. R. Louro, L. I. Horvath, K. W. Miller, and A. Watts. A study of the effect of general anesthetics on lipid-protein interactions in acetylcholine receptor-enriched membranes from *Torpedo nobiliana* using nitroxide spin-labels. *Biochemistry* **29**:2664–2669 (1990).
25. Firestone, L. L., and C. Furguson. Actions of ethanol and general anesthetics at boundary lipid. *Anesthesiology* **73**:A709 (1990).
26. Dalton, L. A., D. E. Raines, and K. W. Miller. Ethanol disrupts slow motions of the protein-associated lipid near the surface of acetylcholine receptors. *Biophys. J.* **66**:A56 (1994).
27. Karon, B. S., and D. D. Thomas. Molecular mechanism of Ca-ATPase activation by halothane in sarcoplasmic reticulum. *Biochemistry* **32**:7503–7511 (1993).
28. Keller, S. L., S. M. Bezrukov, S. M. Gruner, M. W. Tate, I. Vodyanoy, and V. A. Parsegian. Probability of alamethicin conductance states varies with nonlamellar tendency of bilayer phospholipids. *Biophys. J.* **65**:23–27 (1993).
29. Barrantes, F. J. Structural-functional correlates of the nicotinic acteylcholine receptor and its lipid microenvironment. *FASEB J.* **7**:1460–1467 (1993).
30. Sunshine, C., and M. G. McNamee. Lipid modulation of nicotinic acetylcholine receptor function: the role of neutral and negatively charged lipids. *Biochim. Biophys. Acta* **1108**:240–246 (1992).
31. Jones, O. T., and M. G. McNamee. Annular and nonannular binding sites for cholesterol associated with the nicotinic acetylcholine receptor. *Biochemistry* **27**:2364–2374 (1988).
32. Forman, S. A., D. L. Righi, and K. W. Miller. Ethanol increases agonist affinity for nicotinic receptors from *Torpedo*. *Biochim. Biophys. Acta* **987**:95–103 (1989).
33. Miller, K. W. Multiple actions of ethanol on acetylcholine receptors, in *Alcohol, Cell Membranes, and Signal Transduction in Brain* (C. Alling, I. Diamond, S. Leslie, G. Sun, and W. G. Wood, eds.). Plenum Publishing Corp., New York, 83–96 (1993).
34. Urban, B. W. Modifications of excitable membranes by volatile and gaseous anesthetics, in *Effects of Anesthesia* (B. G. Covino, H. A. Fozzard, K. Rehder, and G. R. Strichartz, eds.). American Physiological Society, Bethesda, MD, 13–28 (1985).

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