

Integrated approaches to the action of general anesthetics and alcohol

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Abstract

The use of inhalational anesthetics was first publicly demonstrated in 1846. Alcohol has been consumed for centuries and is now our most costly drug abuse problem. Despite widespread use, the molecular mechanism of action of these drugs has remained an enigma. The prevailing theory suggests that alcohol and anesthetics interact directly with neuronal membrane proteins to cause their effects. Our laboratories are using a variety of cutting edge approaches to gain insight into the mechanism of action of these drugs. Biophysical approaches such as high-resolution nuclear magnetic resonance (NMR) spectroscopy and genetic engineering approaches such as creation of designer mice are currently being used by our laboratories. These approaches are providing exciting insight into how these drugs exert their effects. This research could ultimately result in safer and better anesthetics, may lead to treatments for alcoholism, and may provide insight into basic biologic processes such as consciousness.

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1. Introduction

The practice of anesthesia by acupuncture can be traced back to the Jin dynasty in ancient China (265–420 AD). It was not until 1846, however, that the use of an exogenous compound to conquer surgical pain was first publicly demonstrated to the Western world. Despite limited side effects, toxicity, and possible long-term cognitive problems in aged or very ill patients, general anesthesia has evolved into a safe and mature medical procedure. A subclass of general anesthetics are the alcohols. Beverage alcohol has been consumed for centuries and is now our number one drug abuse problem. Many of the behavioral effects of alcohol and anesthetics are thought to be mediated through similar and/or overlapping pathways. Despite intense investigation, our understanding of the molecular mechanisms of general anesthesia and alcohol intoxication remains primitive and inadequate. For more than a century, the search for mechanisms has been directed to one of two putative targets: lipids and proteins [1]. The lipid theory focuses on the generalized perturbation of neuronal membranes by drugs

through *nonspecific* interactions. The protein theory contends that alcohol and anesthetics bind *specifically* to neuronal membrane proteins to produce effects. Compelling data support the notion that a superfamily of ligand-gated ion channel proteins is most relevant [1]. The superfamily includes nicotinic acetylcholine (nAChR), glycine (GlyR), serotonin 5-HT₃, and γ -aminobutyric acid type A (GABA_A-R) receptors. The role of these receptors in general anesthesia and intoxication has been under intense investigation in recent years. We have taken at least three different approaches to tackle the problem: (1) a spectroscopic approach to effects on protein structure and function; (2) a computational approach to effects on protein dynamics; and (3) a genetic engineering approach to the characterization of relevant mutations *in vivo*. The integration of these interdisciplinary approaches has generated useful information highly relevant to the molecular mechanisms of general anesthesia and alcohol action.

2. Structure–function relationships

The molecular nature of general anesthetic interaction with model peptides and transmembrane (TM) channel proteins has been characterized using various biophysical

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approaches, notably state-of-the-art high-resolution nuclear magnetic resonance (NMR) spectroscopy. This approach aims to identify the structure–function and dynamics–function relationships with direct binding and dynamics analysis at submolecular and atomic resolutions.

Special structural motifs are required for anesthetic binding in TM channels. The structural requirements for anesthetic binding in TM channels were first evaluated with gramicidin A (gA) [2,3]. High-resolution [¹H]-NMR and direct photoaffinity labeling have been used to characterize the volatile anesthetic binding sites in gA channels. Addition of two volatile anesthetics, halothane and 1-chloro-1,2,2-trifluorocyclobutane (F3), to gA channels caused a concentration-dependent change in resonant frequencies of the indole amide protons of specific tryptophan residues, indicating strong interaction of anesthetics with these interfacial residues. Direct [¹⁴C]-halothane photolabeling in gA yielded similar conclusions. Inspection of the 3-D structure of gA suggests that the special arrangements of the tryptophan residues of gA create a favorable anesthetic binding motif. The amphiphilicity near the interface and the cation-type of interaction may be important for anesthetic binding.

The interaction of general anesthetics with proteins has predictable effects on protein function, which is not necessarily associated with changes in protein structures. For instance, the volatile anesthetic F3 was found to alter gA channel function by enhancing Na⁺ transport [3]. We used circular dichroism and NMR spectroscopy to evaluate whether this functional change was coupled with structural alternation. The results indicated that at low millimolar concentrations of F3, changes in gA channel structure were minute. All hydrogen bonds between channel backbones were well maintained in the presence of F3, and channel structure was stable. The finding supports the notion that anesthetics can cause significant changes in protein function without necessarily producing associated changes in protein structure. The finding also suggests that in addition to structural changes, other protein properties, including dynamic characteristics of channel motions, may also be of functional significance.

Although the membrane-associated ligand-gated ion channels in their intact forms are refractory to experimental high-resolution structural determinations, an attempt has recently been made to delineate the crucial architecture of the TM pore by recapitulating the active moiety and studying the functional segments of the putative pore-lining second TM (TM2) domains in a membranous environment [4]. Among ligand-gated ion channels, GlyRs are particularly suited for this approach because the TM2 segments of the $\alpha 1$ subunit alone form homopentameric channels. Our interests on the structure determination of the TM2 domain of the GlyR $\alpha 1$ subunit were also stimulated by the finding that single-point mutation in TM2 of the GlyR (S267Y) could induce distinctly different channel sensitivity to anesthetics and alcohols [5]. Based on our NMR-derived mono-

mer TM2 structures, we further determined the plausible pore architecture by extensive energy minimization of homopentameric GlyR $\alpha 1$ TM2 channels in a model membrane using large-scale computer simulation [6]. The NMR-derived structures and computation-based homopentameric pore architecture revealed a sufficient amount of atomic details that are potentially relevant to channel gating. Information on structural and pore architecture offers a preliminary explanation as to why volatile anesthetics and short-chain alcohols can potentiate GlyR function whereas the point mutation S267Y abolishes or even reverses such potentiation. The location of S267 is unique in that its polar side-chain interfaces with the hydrophobic surface of M263. A recent study suggests that a second anesthetic sensitive mutation point in the GlyR, A288, is in the opposite TM3 domain at the same membrane level as S267 [7]. Thus, S267 and A288, along with M263, may border an amphipathic cavity that anesthetics or short-chain alcohols can preferably occupy because anesthetic molecules are amphiphilic in nature. Our model of channel gating [4] involves rotational movement that relocates the Q266 side chain within the channel pore. The corresponding movement of the next residue undoubtedly changes the shape and volume of the amphipathic cavity bordered by S267. The dynamics of TM2 movement is changed when an amphiphilic molecule occupies and stabilizes the cavity. The change leads to potentiation by stabilizing and prolonging the open channel if it is in favor of the open state. This is apparently the case for the wild-type GlyR and GABA_A-R. If, on the other hand, anesthetic occupation of the cavity favors the closed state, the results would be inhibition, as is probably the case for nAChR. Mutations at either S267 or A288 with different volumes of residue side chains can have similar effects. Bulky side chains can partially or fully fill the cavity to render open or closed (or even some intermediate) state more stable in the absence of anesthetics or alcohols and thereby abolish the channel sensitivity to these neuronal agents. This view of the molecular mechanism of anesthetic action based on channel dynamics is certainly worth further investigation.

3. Protein dynamics simulations

To reveal the molecular details of anesthetic interaction with TM ion channels, large-scale, 2.2-ns, all-atom molecular dynamics simulations were performed to study the effects of 10 halothane molecules, on a gA channel in a model membrane consisting of 182 lipid molecules and 5538 water molecules as shown in Fig. 1. At the outset, the halothane molecules were strategically placed around the channel based on our previous NMR observations [2,3,8]. The simulations revealed that while the effect of the anesthetics on channel structure is minimal, the presence of anesthetic molecules profoundly affects channel dynamics. More detailed data analysis suggests that halothane

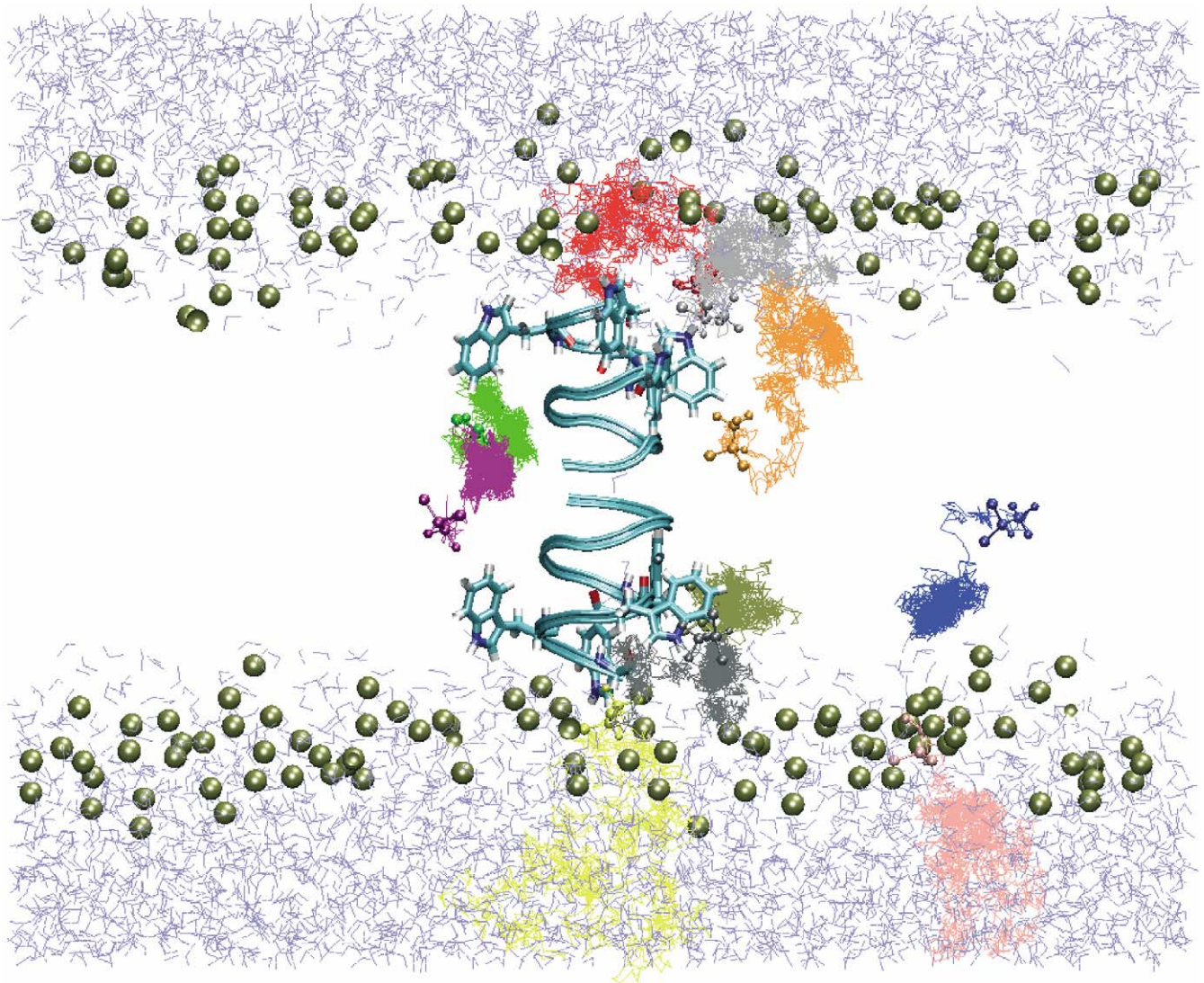


Fig. 1. Halothane motion trajectories over the simulations in a channel–membrane system, which includes a gA channel (cyan ribbon showing only the backbone and tryptophan side chains), 182 lipid molecules (showing only the phosphorus atoms as gold spheres to mark the interface of the membrane), and more than 5000 water molecules. The initial positions of halothane molecules are marked in licorice. The preference of halothane molecules to the membrane interface is noticeable.

has no effects on the subpicosecond librational motion of the channel, prolongs the backbone autocorrelation time in the 10- to 100-ps timescale, and significantly decreases the asymptotic values of generalized order parameter and the correlation time of the nanosecond motions for the inner but not the outer residues of the channel.

Changes in channel dynamics are possible through the following three mechanisms: (1) rapid binding and unbinding of anesthetic molecules alter the association of anchoring residues with lipids and water; (2) channels experience more rapid motion of anesthetics at the channel–lipid interface instead of the relatively slower motion of the ordered interfacial lipids; and (3) anesthetic molecules stabilize structured water clusters at membrane depths where water resident time would be normally brief.

The simulation results [9] may discount the viewpoint of the structure–function paradigm that overrates the importance of structural fitting between a diverse range of general anesthetics and yet unidentified hydrophobic pockets in proteins. Instead, the simulation results underscore the global, as opposed to local, effects of anesthetics on protein dynamics as the underlying mechanisms for the action of general anesthetics, alcohol, and possibly other low-affinity drugs.

The lessons learned from the simplified model about anesthetic effects on channel backbones and side-chain dynamics, about the role of interfacial lipids in anesthetic effects, and about the organization of structured water clusters deep within the membrane in the presence of anesthetics may be able to be generalized for the under-

standing on much more complicated ligand gated ion channels.

4. Designer mice

The third approach we are using to understand alcohol and anesthetic action involves the creation and analysis of so-called designer mice [10,11]. Recently developed techniques that enable one to create whole animals (mice) with precise genetic modifications in individual genes of interest allow one to investigate the involvement of putative drug targets in the context of whole animal behavioral responses. Since hypotheses concerning putative targets must ultimately explain behavioral responses (e.g., incoordination, amnesia, immobility, etc.), such whole-animal experiments represent the most rigorous test of relevance. Only when molecular, cellular, and pharmacologic investigations are combined with whole animal behavioral studies will a clear understanding of alcohol and anesthetic mechanisms be elucidated.

These genetically engineered animals are created using gene targeting and embryonic stem cell technologies. Gene targeting is the process by which a predetermined genetic change is introduced into a specific location in an endogenous gene (Fig. 2A). Embryonic stem cells are cell lines derived from preimplantation embryos (Fig. 2B). These cell lines can be grown in culture in an undifferentiated state, genetically manipulated via gene targeting, and ultimately used to produce mice that can transmit the genetic modification to the next generation. Thus, designer mice can be created that harbor virtually any genetic change imaginable in any known gene of interest (Fig. 2C).

The majority of our work to date has focused on mice with genetic alterations in GABA_A-Rs. GABA is the main inhibitory neurotransmitter in the central nervous system and is critical for the bulk of fast inhibitory neurotransmission. GABA_A-Rs are very sensitive to clinically relevant concentrations of alcohol and anesthetics [12]. These drugs potentiate the effects of GABA at the receptor and lead to increased neuronal inhibition. However, the importance of effects at the GABA_A-R to whole animal behavioral responses to these drugs is largely unknown. To gain insight into their involvement into alcohol and anesthetic action, we create genetically engineered mice in which individual GABA_A-R subunit genes have been modified. These GABA_A-R designer mice are subsequently analyzed at the molecular, cellular, and behavioral levels for changes in drug responsiveness.

GABA_A-Rs are composed of multiple subunits. To date, at least 18 subunits have been identified. We have made numerous lines of designer mice that individually lack many of the subunits including the $\alpha 6$, $\beta 3$, $\gamma 2L$, δ , and $\alpha 1$ subunits of the GABA_A-R (for review, see Ref. [13]). These knockout mice have provided novel insights into their involvement in alcohol and anesthetic action. For example, knockout of the $\beta 3$ subunit has revealed divergent responses

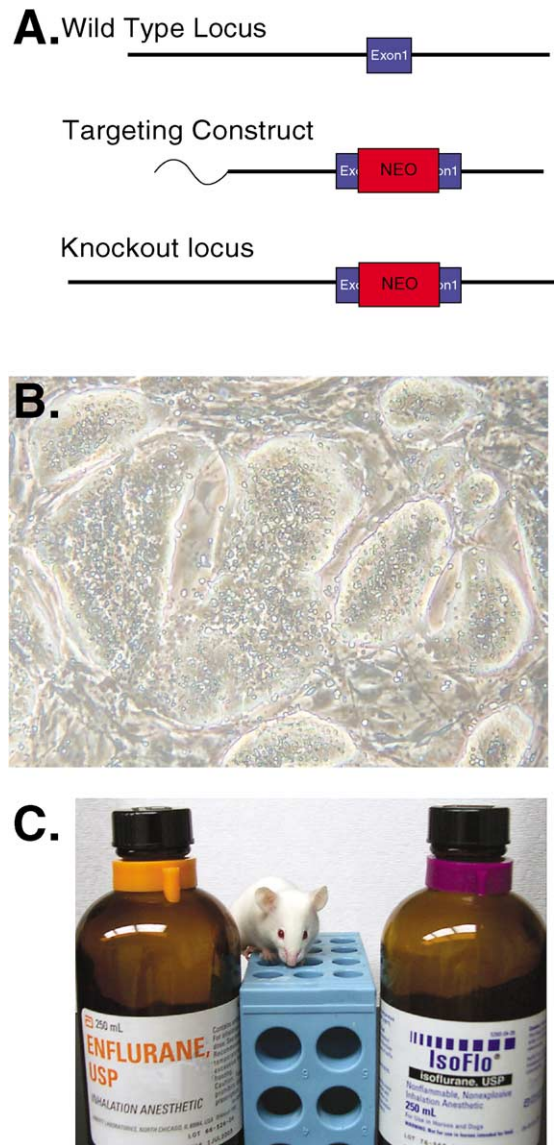


Fig. 2. (A) Generic gene-targeting strategy for creating a gene knockout. (B) Photomicrograph of mouse embryonic stem cells in culture. (C) Genetically engineered mouse derived from embryonic stem cells.

on various behavioral endpoints to various anesthetics [14]. Knockout of the δ subunit led to the surprising observation that this subunit is critically important for mediating/modulating specifically the effects of neurosteroid anesthetics [15]. However, a limitation of knockout technology is that targeted inactivation of one gene throughout the life of the animal often leads to compensatory changes in expression of other genes [16,17]. This can make interpretation of observed phenotypes difficult.

An alternative strategy for investigating drug action at these receptors involves more subtle changes in receptor function. Instead of making an animal that completely lacks a gene product, we also use gene-targeting technology to make mice that express a mutant receptor that differs from the wild-type receptor by a single amino acid, for example.

These designer mice are referred to as gene knockin animals because a genetic change has been introduced into a gene and that gene continues to be expressed. This helps to minimize the possibility of compensation.

A number of key amino acids in the GABA_A-R have been identified that appear to be critical for drug action at the receptor. For example, on benzodiazepine-sensitive α subunits (i.e., α 1, α 2, α 3, and α 5), amino acid 101 is histidine. In contrast, on benzodiazepine-insensitive α subunits (i.e., α 4, α 6), this amino acid is arginine. To understand the role of each of the sensitive subunits to behavioral responses to benzodiazepines, knockin mice have been created by other investigators that change amino acid 101 to arginine [18]. These studies have revealed fascinating insight into the role of each subunit for specific behavioral responses to these drugs. For example, the α 1 subunit is critical to the sedative and amnestic effects of these drugs, but is unimportant to the anxiolytic and myorelaxant effects.

In a series of analogous *in vitro* studies, a few other amino acids (e.g., S270, A291) have been identified that appear to be key regulators of volatile anesthetic interaction with the GABA_A-R [5]. It is currently unknown, however, whether these sites are important for clinically relevant behaviors. To investigate the importance of such sites, we are currently creating gene knockin mice that harbor many of these same mutations in GABA_A-R subunits. It is expected that mice that harbor GABA_A-Rs that respond normally to GABA but fail to be potentiated by anesthetics will reveal the contribution of those subunits to the mechanism of action of these drugs.

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