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Anesthetics Target Interfacial Transmembrane Sites in Nicotinic Acetylcholine Receptors

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Abstract

General anesthetics are a heterogeneous group of small amphiphilic ligands that interact weakly at multiple allosteric sites on many pentameric ligand gated ion channels (pLGICs), resulting in either inhibition, potentiation of channel activity, or both. Allosteric principles imply that modulator sites must change configuration and ligand affinity during receptor state transitions. Thus, general anesthetics and related compounds are useful both as state-dependent probes of receptor structure and as potentially selective modulators of pLGIC functions. This review focuses on general anesthetic sites in nicotinic acetylcholine receptors, which were among the first anesthetic-sensitive pLGIC experimental models studied, with particular focus on sites formed by transmembrane domain elements. Structural models place many of these sites at interfaces between two or more pLGIC transmembrane helices both within subunits and between adjacent subunits, and between transmembrane helices and either lipids (the lipid-protein interface) or water (i.e. the ion channel). A single general anesthetic may bind at multiple allosteric sites in pLGICs, producing a net effect of either inhibition (e.g. blocking the ion channel) or enhanced channel gating (e.g. inter-subunit sites). Other general anesthetic sites identified by photolabeling or crystallography are tentatively linked to functional effects, including intra-subunit helix bundle sites and the lipid-protein interface.

Keywords

Alcohol; propofol; barbiturates; allosterism; photolabel; mutagenesis

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1. Introduction

General anesthetics are a heterogeneous group of essential clinical drugs that reversibly modulate central nervous system functions involved in memory formation, perceptive awareness, and reactivity to noxious stimuli. Since the widespread adoption of general anesthesia into clinical use in the late 19th century, researchers have sought to identify the molecular target sites where general anesthetics act and to understand how they modulate neural functions. Nicotinic ACh receptors (nAChRs), particularly the naturally abundant *Torpedo* nAChR, were among the first ligand-gated ion channel models used to investigate general anesthetic mechanisms. The ability to purify large quantities of *Torpedo* nAChR (Sobel et al., 1977) in native or reconstituted membranes enabled biochemical, tracer-ion flux, photolabeling, and biophysical studies that to this day remain extremely challenging in most related ion channels. With the widespread adoption of voltage-clamp electrophysiology and techniques for heterologous expression of cloned receptor subunits, nAChRs also were among the first receptors where “reverse pharmacology” was applied to explore which protein regions were functionally linked to anesthetic actions. This proved fortuitous, as related pentameric ligand-gated ion channels (pLGICs) in neurons, chiefly γ-aminobutyric acid type A ($GABA_A$) receptors, are key target molecules for many general anesthetics. While nAChRs may not mediate the major behavioral effects of anesthesia, anesthetic inhibition at the neuromuscular junction contributes to immobility, and effects on neuronal nAChRs may also affect neural functions such as memory. Moreover, mechanistic studies of nAChRs have provided important insight into the locations and nature of anesthetic binding sites in other targets. Of particular importance, and the theme of this review, is evidence that general anesthetics modulate nAChRs and related pLGICs through sites formed at various interfaces among the major components of the ion channel molecular system: water, lipids, and transmembrane protein helices. Allosteric principles imply that functional modulation by general anesthetics is driven by binding to sites that change conformation and drug affinity as the protein transitions from one state to another. Here, we review data focusing on the localization of anesthetic binding sites as well as how anesthetic occupation of these sites is allosterically linked to functionally important channel state transitions, particularly in peripheral nAChR subtypes that have been most informative.

2. Structure and function of nicotinic ACh receptors

The context for interpretation of experimental data derives from both structural models of peripheral nAChRs (Figure 1), as well as mechanistic models that account for major functional states and the rates at which transitions between these states occur. Significant advances in structural models have emerged from x-ray micrographic analysis (Unwin, 2005), and crystallography of related channels and proteins (Brejc et al., 2001; Corringer et al., 2012), and NMR studies (Cui et al., 2012). Nicotinic AChRs, like related pLGICs (5-HT3 receptors, $GABA_A$ receptors, glycine (Gly) receptors, and zinc-activated receptors) are formed by five homologous subunits surrounding a central ion channel, in this case cation selective. Each pLGIC subunit contains a large (~200 amino acid) extracellular domain (ECD), and a transmembrane domain (TMD) with four transmembrane helices (M1 to M4), as well as a variable-size intracellular domain (ICD) between M3 and M4. Two or more orthosteric agonist sites per pLGIC (two in muscle nAChRs) are located in interfaces

between ECDs on adjacent subunits. The transmembrane ion channel is surrounded by M2 domains in a pseudo-symmetric arrangement. A second ring of transmembrane helices is formed by M1 and M3 helices, and M4 helices are located farthest from the ion channel. Anti-channel oriented faces of M1, M3 and M4 form the lipid-protein interface. Structural data (Unwin, 2005) and substituted cysteine accessibility (Akabas and Karlin, 1995; Zhang and Karlin, 1997) indicate that significant amounts of water intercalate deeply between the transmembrane helices, not just in the ion channel.

Functionally, four major nAChR states are distinguishable: a low-affinity inactive resting (R) state, an intermediate-affinity active open-channel (O) state, and two high-affinity non-conducting desensitized states (D_{fast} and D_{slow}). Upon ACh binding, resting receptors activate in under a millisecond resulting in transmembrane ion conductance. With rapid agonist removal, most O-state receptors return to the resting state. Persistent exposure to agonist results in transitions first to D_{fast}, then to D_{slow} states. The agonist-dependent distribution of states and their transitions are consistent with Monod-Wyman-Changeux (MWC) allosteric principles (Edelstein et al., 1996). Single-channel analyses of unliganded receptors and mutants further support the relevance of MWC models to nAChR function (Auerbach, 2012). Molecular dynamics has suggested associations between structural and functional transitions at high temporal resolution (Calimet et al., 2013; Liu et al., 2008).

3. General anesthetics and their effects on nicotinic ACh receptors

General anesthetics belong to various molecular classes, including alcohols (e.g. ethanol), alkanes (e.g. halothane), ethers (e.g. isoflurane, sevoflurane, and desflurane), simple gases (e.g. N_2O and Xe), barbiturates, alkyl-phenols (propofol), phenyl esters (propanidid), imidazole esters (etomidate), and others (ketamine) (Alkire et al., 2008; Franks, 2008). Most general anesthetics are small amphiphilic molecules that can occupy numerous protein cavities (Eckenhoff, 2001). Their effects on nAChRs vary both with drug and receptor type. Most general anesthetics inhibit nAChRs, including *Torpedo*, mammalian muscle, and neuronal receptor subtypes (Forman and Miller, 2011). Potential mechanisms for inhibition include channel blockade, allosteric stabilization of closed channels, and desensitization. Evidence exists for both channel blockade and desensitization. In addition, several anesthetics, including a photolabel, TDBzl-etomidate, positively modulate nAChR responses to low concentrations of ACh (Nirthanan et al., 2008), resulting in a leftward shift of agonist concentration-response relationships similar to that observed with $GABA_A$ receptors in the presence of many general anesthetics (Rüsch et al., 2004). Ethanol and other short-chain alcohols also enhance channel gating in peripheral and certain central nAChRs (Aistrup et al., 1999; Bradley et al., 1980; Forman et al., 1989; Gage, 1965). Gating enhancement is most likely caused by stabilization of open channel (O) states *via* allosteric agonist sites.

4. Interfacial general anesthetic sites in nicotinic ACh receptors

In the early twentieth century, it was established that general anesthetic potency across a remarkably wide range is correlated with hydrophobicity based on oil/water partitioning (Meyer, 1899; Overton, 1901), leading to multiple theories postulating a common site of action in membrane lipids (Miller, 1985). However, in the late twentieth century, direct

general anesthetic actions on protein was shown to be compatible with the Meyer-Overton correlation (Franks and Lieb, 1982), at least for simple agents. Other studies established that anesthetic potency was best correlated with solubility in lecithin rather than olive oil (Taheri et al., 1991). Indeed, anesthetics were found to partition into the water-lipid interface in simple bilayer systems (North and Cafiso, 1997; Pohorille et al., 1996), establishing the concept of anesthetics as interfacially active ligands, albeit mostly low affinity and low specificity ones. Additional studies have extended this concept to anesthetic sites within membrane proteins, which are exposed to the high concentrations (~25 mM) of general anesthetics in membrane lipids (Miller, 1985). The majority of general anesthetic sites within ion channel proteins are located at the interfaces between multiple transmembrane helices or between the helices and water or lipid. Moreover, the interactions of potent general anesthetics with pLGICs, including nAChRs, demonstrate surprising selectivity among a wide variety of interfacial sites.

4.1 The ion channel (water-protein interface)

Ligand-gated ion channels regulate ion permeation across membranes. In pLGICs, the key structure controlling ion permeation is the transmembrane water-filled pore, surrounded by five M2 transmembrane helices, one from each subunit. Moreover, the pLGIC transmembrane pore is also involved in desensitization, which involves transition to a closed-channel state with high agonist affinity. Early studies of general anesthetics and nAChRs suggested that channel inhibition might be mediated by sites in the transmembrane channel. Biochemical studies of orthosteric agonist and antagonist binding to nAChRs showed that general anesthetics increase the fraction of desensitized receptors (Firestone et al., 1994; Firestone et al., 1986). Using rapid-mixing and quench-flux, early functional studies of *Torpedo* nAChR suggested that long-chain alcohols either accelerated desensitization or preferentially blocked activated nAChRs (Miller et al., 1987). Dilger and colleagues (Brett et al., 1988) were the first to use patch-clamp electrophysiology in nAChRs to demonstrate that brief single-channel closures increased in frequency with higher anesthetic concentration, and that channel closures between channel-opening bursts also increased in duration. This suggested a dual mode of inhibition involving both channel blockade and stabilization of desensitized states. Selective inhibition of open-channels was subsequently confirmed in electrophysiology studies using rapid concentration-jumps to achieve state-dependent drug application in expressed muscle nAChRs (Forman et al., 1995). Moreover, mutations that altered hydrophobicity in the pore-forming M2 domains of muscle nAChR affected sensitivity to inhibition by long-chain alcohols and volatiles, consistent with a central inhibitory pore site to which all M2 domains contributed (Forman, 1997; Forman et al., 1995). Scanning mutagenesis suggested the presence of a “hydrophobic patch” near the middle of M2 domains that interacts with many hydrophobic and amphiphilic molecules (Zhou et al., 2000). This entire region of M2 is also demonstrably water-accessible (Wilson and Karlin, 2001).

Channel-lining M2 residues are homologous among the various nAChR subunits, forming rings at 2′, 6′, 9′, 13′, 17′, and 20′ (Table 1). A variety of anesthetic photo-affinity reagents, including 3-azi- octanol, m-azi-propofol, azi-etomidate, pTFD-etomidate, TDBzl-etomidate, and mTFD-MPAB label residues in the *Torpedo* nAChR ion channel (Hamouda et al., 2014;

Hamouda et al., 2011; Jayakar et al., 2013; Nirthanan et al., 2008; Pratt et al., 2000; Ziebell et al., 2004) (Figure 2). Halothane is the exception, probably because this drug selectively photolabels tyrosines (Chiara et al., 2003). The extracellular M2-20′ pore residues (α1E262, β1D268, δQ276) are far from the mid-pore hydrophobic patch, and depending on their orientation, might interact with transmembrane helix residues on adjacent subunits. Anesthetic photolabeling of M2-20′ is frequently enhanced by desensitization and mutations at M2-20′ suggest that these residues may be linked more strongly to desensitization than to channel block (Chiara et al., 2009; Forman et al., 2007; Pratt et al., 2000).

The mid-pore hydrophobic patch was also photolabeled with the non-anesthetic hydrophobic compound 3-trifluoromethyl-3-(m-[^{125}I]-iodophenyl)diazirine ([^{125}I]TID) (White and Cohen, 1992; White et al., 1991), which was thought to selectively stabilize the closed-nAChR channel (Chiara et al., 2001). However, rapid superfusion electrophysiology reveals that TID, like general anesthetics, blocks open channels better than closed channels, but that rapid open-channel block requires pre-exposure to TID and develops slowly during co-application of TID with ACh (Forman, 1999). Further evidence of TID open-channel block comes from mutations in the hydrophobic patch that alter sensitivity to inhibition (Forman, 1999). Time-resolved photolabeling also reveals that TID has hindered access to the ion channel in the closed state (Chiara et al., 2001). The rate of channel labeling is comparable to the onset of cation flux inhibition in resting state *Torpedo* nAChR in native vesicles (Wu et al., 1994), further linking this site to inhibition. Given that TID partitions into lipids and photolabels the lipid- protein interface within milliseconds of its introduction (Chiara et al., 2001), this suggests access to its channel site is affected by TID uptake into surrounding lipids and diffusion through the protein.

4.2 Transmembrane inter-subunit (protein-protein) interfaces

While most anesthetics inhibit nAChRs, a few, including short-chain alcohols, also positively modulate (potentiate) nAChR channel activity, resulting in a leftward shift of agonist concentration-response curves (Aistrup et al., 1999; Bradley et al., 1980; Forman et al., 1989; Gage, 1965). This effect mirrors that of most anesthetics on inhibitory anion-conducting pLGICs that mediate anesthetic actions in brain ($GABA_A$ receptors) and spinal cord (Gly receptors). Potentiation by anesthetics in $GABA_A$ receptors is linked to allosteric sites that also mediate direct receptor activation (allosteric agonism) by anesthetics (Rüsch et al., 2004). Based on both pharmacological and mutational studies, nAChR sites mediating anesthetic potentiation by ethanol are distinct from sites mediating inhibition (Wood et al., 1991; Wood et al., 1995). Mutations in the mid-pore “hydrophobic patch” affect ethanol and hexanol inhibition but not ethanol potentiation of gating, and reveal that positive modulation is associated with enhanced gating activation of agonist-bound receptors (Forman and Zhou, 1999; Forman and Zhou, 2000; Zhou et al., 2000). Similar gating enhancement by ethanol is observed in neuronal nAChRs (Aistrup et al., 1999; Nagata et al., 1996). Anesthetic gating enhancement in nAChRs is most likely mediated by inter-subunit transmembrane sites, such as those first identified in $GABA_A$ receptors for azi-etomidate (Li et al., 2006) and o-propofol diazirine (Yip et al., 2013). Similar sites also likely exist in Gly receptors, which also show gating potentiation in the presence of alcohols and small general anesthetics (Murail et al., 2011).

A number of photoreactive anesthetics label homologous transmembrane *Torpedo* nAChR sites between α and γ subunits (Figure 3; Table 1), including the positive modulator TDBzl-etomidate (Husain et al., 2006; Nirthanan et al., 2008). Like inhibitory anesthetic photolabels, TDBzl-etomidate also labels within the cation pore and at the lipid-protein interface. However, its potency for displacement of channel blockers is low (millimolar range), presumably resulting in dominant positive modulation at low micromolar concentrations. None of the four other nAChR inter-subunit transmembrane sites are photolabeled by anesthetics, whereas four such sites have been photolabeled in heteropentameric $GABA_A$ receptors (Chiara et al., 2013). In addition, positive modulation is limited to n-alcohols shorter than pentanol in *Torpedo* nAChRs and propanol in neuronal nAChRs (Zuo et al., 2001). Removal of hexanol inhibition with a mid-pore mutation in muscle nAChRs does not unmask positive modulation (Zhou et al., 2000). These findings suggest restrictive size [length more than volume (Wood et al., 1993)] limitations within most of the nAChR inter-subunit pockets that mediate positive modulation, with the exception of the site accessible to TDBzl-etomidate. While general anesthetics may enter inter-subunit pockets *via* lipids where they concentrate, cysteine accessibility and modification studies indicate that these pockets are also accessible *via* aqueous routes from the extracellular space (Zhang and Karlin, 1997).

Inter-subunit anesthetic sites were also identified in high-resolution crystallographic studies of bromoform bound to *Erwinia chrysanthemi* ligand-gated ion channel (ELIC) (Spurny et al., 2013). Additional evidence linking transmembrane inter-subunit sites to positive allosteric modulation and allosteric agonism comes from crystallographic studies of ivermectin bound to *Caenorhabditis elegans* glutamate-gated chloride channel alpha (GluCl) (Hibbs and Gouaux, 2011) and *Gloeobacter violaceus* ion channel (GLIC) mutants that enlarge five homologous inter-subunit cavities between M2 helices and sensitize the receptor to positive allosteric modulation by ethanol and small general anesthetics (Sauguet et al., 2013).

4.3 Transmembrane intra-subunit four-helix bundle sites

An intriguing implication that emerges from studies of inter-subunit allosteric potentiator sites is that channel gating is affected by high-order subunit-subunit interfacial movements. However, there is also evidence that intra-subunit anesthetic pockets exist and may be allosterically linked to receptor function. The most complete study of small ligand binding sites in different conformation states is based on rapid mixing combined with freeze-quench to achieve time-resolved TID photolabeling of *Torpedo* nAChR in native plasma membranes (Addona et al., 1999). This approach has revealed a unique intra-subunit site on the δ subunit of *Torpedo* nAChR (Arevalo et al., 2005). The labeled residues include δF232 on M1, δT274 and δL278 on M2 contralateral to the ion channel, and δI288 predicted to be located just before M3 or in M3, depending on the homology model template. These residues define a hydrophobic pocket at the extracellular end of the δ transmembrane four-helix bundle (Figure 4). Brief (1 to 10 ms) exposure to carbamylcholine increases TID photo-incorporation in this site, whereas longer agonist exposures (1 sec to over 1 hour) reduce TID photoincorporation relative to resting state control levels. Thus, interactions between TID and the δ helix bundle are apparently enhanced in the open state, and reduced

in desensitized states, indicating conformational changes. The relative affinities of TID for various nAChR functional states (O > R > D) suggest that TID binding might allosterically stabilize the open-state. However, there is no evidence of such an effect, while TID binding in the open channel inhibits ion translocation.

The δ four-helix bundle site is only 10 to 15 Å from the α-carbon of αE262 where azi-octanol and azi-etomidate interact with the *Torpedo* nAChR cation channel (Figure 4; Table 1). TID does not photolabel any transmembrane four-helix bundle sites on other nAChR subunits. However, several anesthetic photolabels, including halothane, azi-etomidate, TFD-etomidate, and m-azi-propofol also modify residues in the δ intra-subunit helix bundle. Propofol also competitively inhibits azi-propofol photolabeling at this site (Jayakar et al., 2013). Halothane also photolabels one residue, αY213 on M1, predicted to project into a homologous pocket on α subunits. Homologous intra-subunit sites for modulators of α7 neuronal nAChRs were also suggested by mutational analysis (daCosta et al., 2011; Taly et al., 2009).

Crystallographic studies in GLIC (Nury et al., 2011) and ELIC (Spurny et al., 2013) reveal that propofol and inhaled anesthetics occupy intra-subunit helical bundle sites. The location of the propofol binding site in GLIC was confirmed with m-azi-propofol photolabeling (Chiara et al., 2014). Propofol produces apparent conformational changes in both intra-subunit and inter-subunit transmembrane pockets in GLIC functional studies (Ghosh et al., 2013). Wild-type GLIC channels are inhibited by anesthetics and mutations in intra-subunit anesthetic sites alter the inhibitory potency of anesthetics. However, anesthetic binding in crystallized GLIC is not associated with detectable change in the protein structure, including in the transmembrane pore (Nury et al., 2011). Moreover, the crystallized GLIC pore is occupied by detergent, potentially masking other inhibitory anesthetic binding sites.

4.4 The extracellular domain-transmembrane domain interface

Coupling between agonist-induced conformational changes in pLGIC ECDs and those in the TMD pore is thought to involve steric and electrostatic interactions between extracellular loops 2 and 7 (the cys-loop) and the M2-M3 linker (Absalom et al., 2003; Grutter et al., 2003; Kash et al., 2003). While general anesthetics do not appear to interact with these extracellular loops to any significant degree, their sites at the extracellular ends of M1, M3 and M4 are in close proximity to the proposed extracellular-transmembrane coupling mechanism, and TFD-etomidate photolabeling is found in one M2-M3 loop (δI288). This residue, also labeled by TID, is thought to form part of an intra-subunit transmembrane helix bundle site, and therefore represents a possible nexus point that couples anesthetic binding to altered channel gating without requiring relative movement of the intra-subunit transmembrane helices. Parallel evidence of ECD-TMD coupling is found in GLIC mutants with an engineered disulfide cross-link between extracellular loop 2 and the M2-M3 linker, leading to a “locally closed” configuration (Prevost et al., 2012). Gating defective mutant Gly receptors with “locally closed” disulfide cross-links retain gating potentiation by propofol, suggesting that the ECD-TMD interface does not mediate this anesthetic effect (Prevost et al., 2013). Instead, based on the evidence presented above (section 4. 2), Gly gating potentiation by propofol is likely mediated by inter-subunit transmembrane sites.

4.5 The lipid-protein interface

The ability to purify large quantities of receptor protein made *Torpedo* nAChR the focus of much research into how membrane lipids influence receptor structure and function. The experimental approach generally involved solubilization of receptor protein in detergent to remove native lipids, followed by reconstitution into liposomes or vesicles of known lipid composition (Heidmann et al., 1980). Reconstitution studies demonstrate that allosteric transitions between low and high agonist affinity states requires the presence of both neutral sterols such as cholesterol, and negatively charged phospholipids (Criado et al., 1984; Epstein and Racker, 1978; Heidmann et al., 1980). The hydrophobic photolabel ^{125}I-TID was used to map the lipid-protein interface of *Torpedo* nAChR (Blanton and Cohen, 1992, 1994), revealing that parts of M1, M3 and M4 are lipid-exposed and likely adopting α-helical structures. Mutations in M4 also influence nAChR gating (Ortiz-Miranda et al., 1997), implying allosteric coupling. Structural models of *Torpedo* nAChR based on electron micrography (Unwin, 2005) reveal spaces between transmembrane helices M1, M3, and M4 that could accommodate lipids, and molecular dynamics simulations suggest that cholesterol may prevent collapse of these cavities that results in transition to agonist-unresponsive states (Brannigan et al., 2008). One such state is the high-affinity desensitized state. Another, recently recognized has been named the “uncoupled” state, which is characterized by low agonist affinity, similar to that of resting-state receptors (Baenziger and Corringer, 2011; daCosta and Baenziger, 2009). Uncoupled receptors do not activate upon agonist binding. Biophysical studies indicate they have increased solvent exposure, leading to the hypothesis that the C-terminus beyond M4 participates in coupling of agonist binding between the ECD and opening of the ion channel (daCosta and Baenziger, 2009). Further evidence that the lipid-protein interface regulates pLGIC function comes from crystal structures of GLIC. These reveal several native phospholipids that co-purify with protein, presumably bound to high-affinity sites at several transmembrane domain interfaces near both the extracellular and intracellular membrane leaflets (Bocquet et al., 2009). GLIC is now emerging as a new pLGIC model for studies of lipid-protein interaction. Labriola et al (2013) demonstrated that GLIC is readily reconstituted into defined lipid environments, but, unlike *Torpedo* nAChR, does not adopt an uncoupled configuration. Thus, lipid-protein interactions or sites in this interface may variably affect different pLGICs.

All anesthetic photolabels covalently modify *Torpedo* nAChR at the lipid-protein interface (Table 1). The specific residues show overlap with those identified using TID. Moreover, some steroids inhibit nAChR function (Bouzat and Barrantes, 1996) and cholesterol analogs photolabel the nAChR lipid-protein interface (Hamouda et al., 2006). Given the linkages between the lipid-protein interface and two non-conducting states (desensitized and uncoupled), such anesthetic sites most likely mediate receptor inhibition in nAChRs. One possibility is that anesthetics displace lipids from sites at the nAChR surface, but spin-label studies suggest that displacement of lipids (or at least the spin-labeled probe portions deep in the bilayer) is weak at clinically relevant anesthetic concentrations (Abadji et al., 1993; Fraser et al., 1990). Nonetheless, anesthetics accumulate at the lipid-water interface (Pohorille et al., 1996), and could interpose between lipid head-groups and nAChR, affecting protein dynamics as well as local electrostatic fields (Xiu et al., 2005).

5. Asymmetry and allosteric mechanisms in hetero-oligomers

Allosteric principles and the four established functional states of nAChRs were introduced above (sections 2 and 3). All subunits are usually envisioned to undergo state transitions in concert in the simplest (two-state) allosteric models. However, the muscle nAChR pentamer is composed of four different types of subunits arranged αγαδβ counter clockwise when viewed from the synaptic space (Figure 1). The asymmetric aspects of nAChR structure thus enable asymmetric and non-concerted state transitions at the subunit level, which may also involve anesthetic sites.

5.1 State-dependence of anesthetic sites in different locations

Mechanistic insights deriving from differential linkages between anesthetic sites and receptor states are best illustrated by the interactions of photolabels such as TID, which interact with various sites on *Torpedo* nAChR, and which have been investigated in all four major functional states. As noted above, TID binds at the lipid-protein interface, within the nAChR transmembrane channel (M2 9′, 13′ and 16′ residues), and within the δ-subunit four-helix bundle near the extracellular ends of M1, M2, and M3 helices. The latter two sites are separated by a single transmembrane δ-M2 helix. Each TID/anesthetic site is defined both by structural proximity and the parallel state–dependent photolabeling of its multiple residues.

In the resting state, TID equilibrates with membrane lipids in the dead time of the rapid-mixing freeze-quench system (~1 ms) and with the lipid-protein interface within a few milliseconds (Chiara et al., 2001). The pore site is also labeled in the resting state, but the lipid-protein interface and pore can be resolved kinetically, as channel labeling proceeds slowly (~15 s^{-1}) in resting receptors and is only 10% complete 2 ms after adding ACh (Addona et al., 1999; Chiara et al., 2001). In contrast, the δ-subunit helix bundle site does not bind TID in the absence of agonist, but it is fully occupied 1.5 ms after addition of ACh, whether membranes are pre-equilibrated with TID or TID is added simultaneously with the ACh. Thus, the δ-helix bundle site is occupied by TID before the channel is inhibited. During fast desensitization, TID photoincorporation in the channel lumen drops ~3–fold whereas that in the δ-subunit helix bundle remains unchanged. During slow desensitization, TID photoincorporation decreases dramatically in the δ-subunit helix bundle, but little or none in the channel lumen.

The TID sites in both the nAChR pore and δ-helix bundle are also accessed by general anesthetics (Table 1). Azi-etomidate labels δC236 in the helix bundle strongly in the open state, but weakly in the resting and fast desensitized states (Chiara et al., 2009; Ziebell et al., 2004), behavior that is consistent with that of TID. In addition, azioctanol and azietomidate photolabel residues at the outer end of M2 (20′ loci) much more in the open state than in the resting state. However, unlike labeling in the 9′ to 16′ region of the pore, no decrease in 20′ photolabeling is observed upon slow desensitization.

5.2 Asymmetric and non-concerted state transitions and anesthetic sites

A two-gate, four-state model of channel activation and desensitization has been proposed for nAChRs (Auerbach and Akk, 1998; Purohit and Grosman, 2006). In the resting state, the desensitization gate is open and the activation gate is closed. In the open state both gates are open. During fast desensitization, the desensitization gate closes while the activation gate remains open, and it too finally closes during slow desensitization. This model is consistent with TID photoincorporation in the middle of the M2 domain (9′ to 16′), which differs in the resting, open, and fast-desensitized states. Cysteine substitution and accessibility of M2 helix residues also reveals differential structures in these three states (Wilson and Karlin, 2001).

Several lines of evidence have lead to proposals that subunit level rearrangements may not be symmetrical or concerted during heteromeric nAChR state transitions. The only rapid time-resolved structural imaging data available, from Unwin and coworkers (Unwin and Fujiyoshi, 2012), suggests that the *Torpedo* nAChR β subunit moves more than other subunits during channel activation, introducing asymmetry into this process. Nicotinic AChR agonists display different affinities for αδ and αγ sub-sites, and some evidence suggests that these two agonist-binding subunit pairs may switch between functional states independently (Prince and Sine, 1999). This concept was extended to suggest that each agonist-binding subunit pair also has only one desensitized structure, and that fast and slow desensitization differ merely in the number of desensitized pairs (Prince and Sine, 1999). Rapid kinetics studies with a fluorescent agonist provide supporting evidence that the αδ agonist site desensitizes more slowly than the αγ site (Andreeva et al., 2006; Song et al., 2003). Thus, αγ subunit pair rearrangement should accompany fast desensitization, while αδ rearrangement accompanies slow desensitization. This is remarkably consistent with anesthetic and TID photolabeling of the δ-subunit helix bundle residues, which changes during slow desensitization but not fast desensitization. Moreover, TID does not affect the nAChR fast desensitization rate, presumably because its binding site on the d-subunit is unchanged during fast desensitization. Together, evidence from subunit-selective agonist binding to ECD and subunit-selective photolabeling of TMD suggests that these domains within a single subunit (or even agonist binding subunit pairs) undergo linked or concerted transitions. Thus, certain anesthetics that selectively interact with the αγ TMD interface (Table 1) could be sensitive to fast desensitization, but not slow desensitization. A broader array of hydrophobic photolabels with selectivity for different heteromeric pLGIC subunits or interfaces would provide tools to further investigate these complex mechanisms.

6. Summary

Studies of general anesthetic effects in nAChRs and related pLGICs have revealed the presence of a variety of anesthetic interaction sites, typically located at interfaces between the transmembrane helices, lipid, and water. The nAChR ion channel (protein-water interface) forms inhibitory anesthetic sites, while gating enhancement is mediated by inter-subunit transmembrane sites. Transmembrane intra-subunit sites and the lipid-protein interface also form anesthetic sites which may also contribute to functional inhibition. A deeper understanding of the structural and functional interactions between general

anesthetics and pLGICs is leading researchers in a number of important directions. First, we are discovering that different anesthetics display selectivity for different allosteric sites, providing a mechanistic basis for understanding subtle differences in pharmacology while developing more selective drugs. Anesthetic sites within or adjacent to different subunits of heteromeric pLGICs also contribute to subunit-selective receptor pharmacology. Second, allosteric principles state that modulatory drug sites must undergo conformational changes associated with different nAChR states. Thus, investigating how these interfacial sites change in size and shape is an important step toward a deeper understanding of how drugs modulate pLGICs. High resolution crystal structures of pLGICs in different functional states, biophysical studies of receptor state-transitions, and other structural probe techniques are being applied to this question. Third is the humbling recognition that our understanding of lipid-protein interactions and the related steroid modulation of pLGICs remains a largely unexplored frontier. Nicotinic ACh receptors remain one of the most accessible and best understood pLGICs, in which discoveries have often preceded similar findings in related channels. Thus, together with new experimental pLGIC models, nAChRs will continue as a focus of important future research.

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Abbreviations

nAChR	nicotinic acetylcholine receptor
ECD	extracellular domain
TMD	transmembrane domain
pLGIC	pentameric ligand-gated ion channel
$GABA_A$	γ-amino-acid type A
Gly	glycine
MWC	Monod-Wyman-Changeux
ELIC	*Erwinia chrysanthemi* ligand-gated ion channel
GLIC	*Gloeobacter violaceus* ion channel
GluCl	*Caenorhabditis elegans* glutamate-gated chloride channel

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Highlights

- General anesthetics bind at interfacial sites abutting nAChR transmembrane helices.
- Functionally important anesthetic sites show state-dependent drug interactions.
- Anesthetics bind in the nAChR pore, inhibiting receptor function.
- Inter-subunit transmembrane anesthetic sites mediate nAChR gating enhancement.
- Intra-subunit and lipid-protein interface sites are most likely inhibitory.

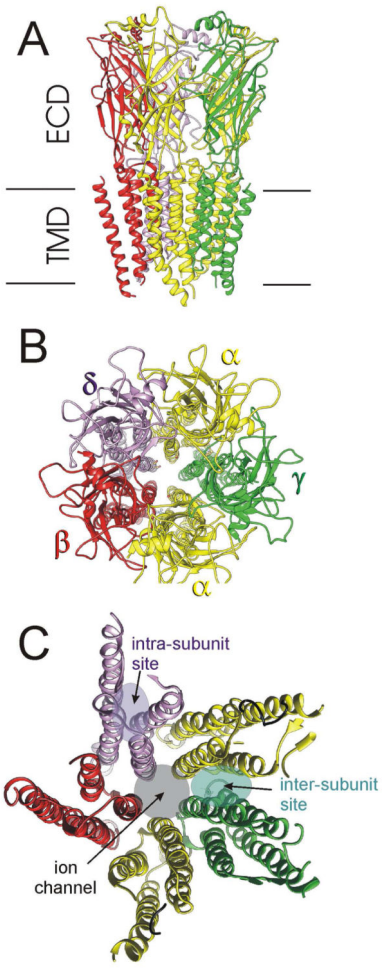

Figure 1. Transmembrane anesthetic sites on nAChRs

A structural model of *Torpedo* nAChR (Hamouda et al., 2014) showing peptide backbone as ribbons. Molecular graphics and analyses were performed with the UCSF Chimera package (Pettersen et al., 2004). **A)** A side view depicts the transmembrane domain (TMD) and extra-cellular domain (ECD). **B)** A view from the extracellular space. Subunits are colored as follows: α, yellow; β, red; γ, green; δ, purple. **C)** The TMD viewed from above, depicting three anesthetic sites: the ion channel, an intra-subunit helix bundle, and an inter-subunit site.

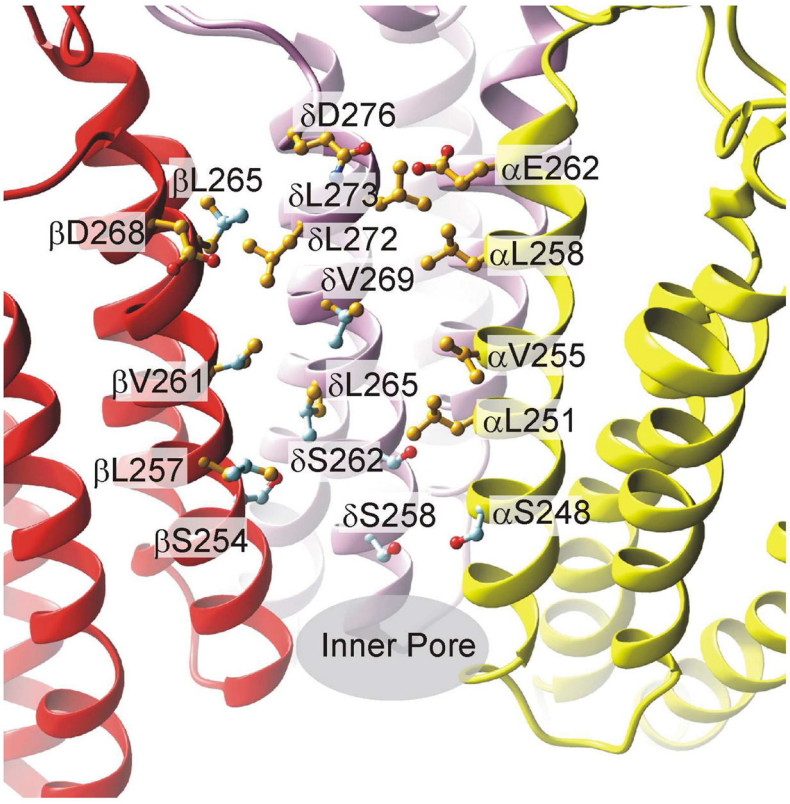

Figure 2. Anesthetic photolabeling in the nAChR ion channel

The *Torpedo* nAChR TMD structure is depicted as backbone ribbons, viewed from the membrane with the γ and one α subunit removed. Anesthetic photolabeled residues are shown as ball-and-stick structures, colored according to which anesthetics modified them: azi-etomidate, TDBzl-etomidate, or TFD-etomidate = gold; propofol or mTFD-MPAB = light blue.

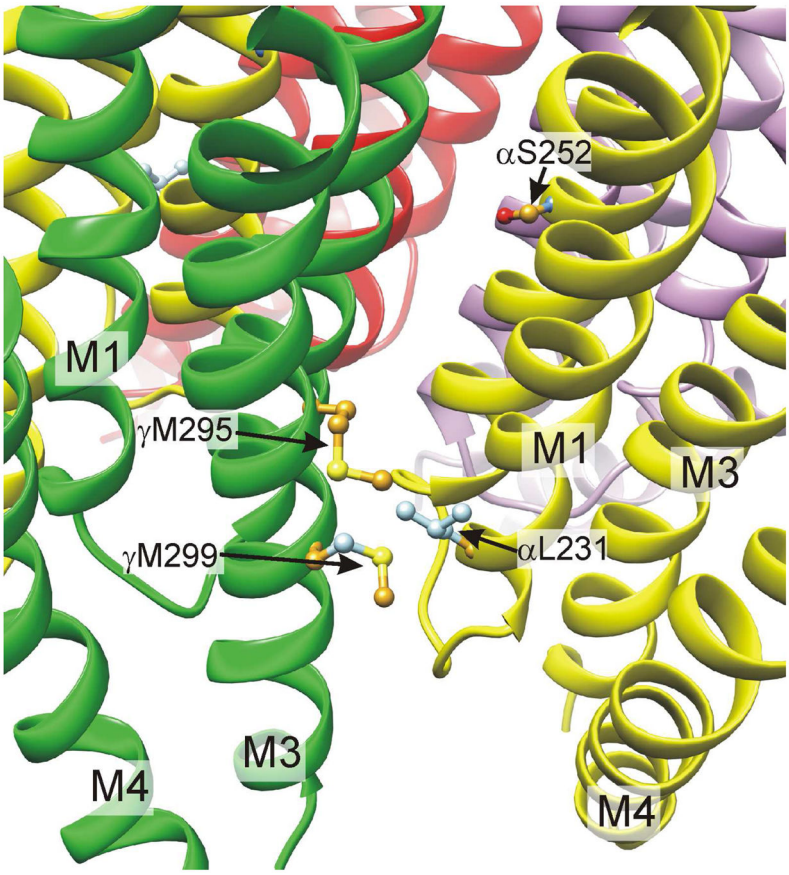

Figure 3. The γ+/α-inter-subunit site

The *Torpedo* nAChR TMD structure is viewed from the membrane. Anesthetic photolabeled residues are shown as ball-and-stick structures, colored according to which anesthetics modified them: azi-etomidate, TDBzl-etomidate, or TFD-etomidate = gold; mTFD-MPAB = light blue.

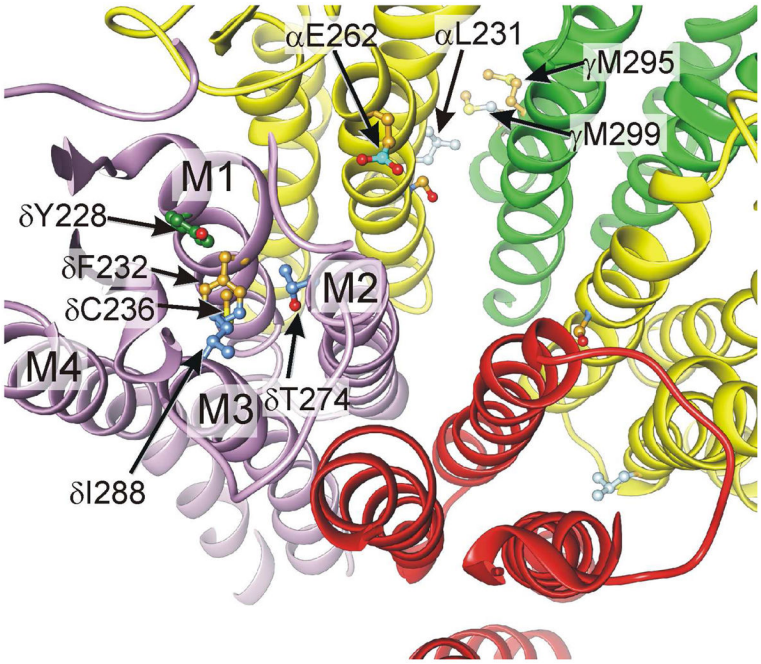

Figure 4. The δ-subunit four-helix bundle site

The nAChR TMD structure is depicted as backbone ribbons, viewed from above. The δ subunit four-helix bundle is shown along with another perspective on the γ+/α-interfacial site. Anesthetic photolabeled residues are shown as ball-and-stick structures, colored according to which anesthetics modified them: halothane = green; azi-etomidate = gold; TID or aziPm = dark blue; azi-octanol = cyan.

Table 1

General Anesthetic Photolabel Incorporation into Allosteric Sites of Torpedo nAChR[a]

Photolabel	Pore Residues	Helix Bundle Residues	Subunit Interface Residues	Lipid-Protein Interface Residues	Notes[b]
Halothane		αY213 (M1) δY228 (M1)		αC412 (M4)	γY111 in ACh site δY212 in ECD δY228 labeling inhibited by isoflurane. (Chiara et al., 2003)
Azi-Octanol	αE262 (M2-20′)			αH408 (M4) αC412 (M4)	αE262 labeling increased in D-state ECD sites αY190, αY198 (Pratt et al., 2000)
Azi-Etomidate	αE262 (M2-20′) βD268 (M2-20′) δS258 (M2-2′) δQ276 (M2-20′)	δC236 (M1)		αE390 (MA) αH408 (M4) αC412 (M4)	Open-state selective blockade. ECD sites αY190, αY198 (Chiara et al., 2009) (Ziebell et al., 2004)
TDBzl- Etomidate	αL251 (M2-9′) αV255 (M2-13′) δL265 (M2-9′) δV269 (M2-13′) δL272 (M2-16′) δL273 (M2-17′) δQ276 (M2-20′)		αS252 (M2-10′) γM299 (M3)	αC412 (M4) αM415 (M4)	Positive modulation at ≈ 10 μM, inhibition at ≈1 mM. Some channel sites labeled only in D-state. (Nirthanan et al., 2008)
TFD-Etomidate	αL251 (M2-9′) αV255 (M2-13′) αL258 (M2-16′) βL257 (M2-9′) βV261 (M2-13′) δL265 (M2-9′) δV269 (M2-13′)	δF232 (M1) δC236 (M1) δI288 (M3)	αS252 (M2-10′) γM295 (M3) γM299 (M3)		IC_{50} = 4 μM, but no displacement of channel blockers. (Hamouda et al., 2011)
m-Azi-Propofol (AziPm)	αS248 (M2-6′) βS254 (M2-6′) βV261 (M2-13′) δV269 (M2-13′)	δF232 (M1) δC236 (M1) δT274 (M2 18′)	αS252 (M2-10′)	αC412 (M4) αC418 (M4) γQ300 (M3) δQ305 (M3)	(Jayakar et al., 2013)
R-mTFD-MPAB	αS248 (M2-6′) βS254 (M2-6′) βL257 (M2-9′) βV261 (M2-13′) βL265 (M2-17′) γS257 (M2-6′) γL260 (M2-9′) δS258 (M2-2′) δS262 (M2-6′) δL265 (M2-9′) δV269 (M2-13′)		αL231 (M1) γM299 (M3)	αC412 (M4)	(Hamouda et al., 2014)
Azi-Isoflurane				V31 in M4 (near ECD) is photolabeled by aziPm* and azi- Isoflurane	NMR study of αTMD model peptide (Cui et al., 2012)

[a] Photolabel incorporation sites are listed (along with their subunit and transmembrane helix) on a grid organized by photo-label anesthetic drug and site classification (transmembrane pore, helix bundle, subunit-subunit interface, and lipid-protein interface. AziPm is 2-isopropyl-5-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenol.

[b] Notes include relevant references, state-dependent photolabeling and drug displacement details, and identify drugs that also label the extracellular domain (ECD).