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## Combining Mutations and Electrophysiology to Map Anesthetic Sites on Ligand-Gated Ion Channels

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Nicotinic acetylcholine receptor; GABA-A receptor; general anesthetic; cysteine; covalent modification; *Xenopus* oocyte

### Introduction:

The parallel development of methods for heterologously expressing and mutating cloned ion channel subunits, and for voltage-clamp electrophysiological assessment of ion channel function has engendered multiple structure-function approaches that contributed to discovery of general anesthetic sites in pentameric ligand-gated ion channels. Here, we review structure-function experimental strategies combining electrophysiology with ion channel subunit chimeras, single-point mutations, and covalent modification of sulfhydryls and their application in identifying general anesthetic sites on nicotinic acetylcholine receptors (nAChRs) and  $\gamma$ -aminobutyric acid type A receptors (GABA<sub>A</sub>Rs). We review detailed methodological considerations for one method that has proven useful in identifying drug-receptor contacts on GABA<sub>A</sub> receptors: substituted cysteine modification and protection (SCAMP).

### 1) Challenges for Identifying General Anesthetic Binding Sites

Many anesthetic drugs, including alcohols and volatile alkanes and ethers are low affinity and probably act through multiple molecular targets. More potent anesthetics that display stereoselectivity and greater target specificity include certain barbiturates, etomidate, and alphaxalone. These potent anesthetics present fewer challenges in defining their mechanisms of action and represent bases for developing new drugs with improved clinical utility.

Many anesthetics inhibit the activity of excitatory pentameric ligand-gated ion channels (pLGICs). A well-studied case is inhibition of the muscle-type nAChR, although nAChR activity is also positively modulated by small alcohols [1]. Mechanisms of anesthetic inhibition in nAChRs include channel blockade, desensitization, and negative allosteric modulation [2, 3]. In contrast, most general anesthetics positively modulate inhibitory pLGICs such as GABA<sub>A</sub> receptors and glycine receptors [4]. Positive modulation at a subset

of GABA<sub>A</sub> receptor isotypes is known to mediate major behavioral effects of many general anesthetics, including hypnosis, amnesia, and immobility [5]. In addition, high concentrations of some anesthetics directly agonize GABA<sub>A</sub> receptors and others also inhibit receptor activity. Thus, there are potentially many types of anesthetic binding sites mediating different anesthetic actions on each pLGIC. Identifying these sites is a first challenge, and a second is demonstrating which anesthetic actions, if any, each binding site mediates. It is also possible that one site can mediate multiple drug effects.

## 2) pLGIC Structural Models

Current structural models of heteromeric pLGICs are homology models, typically based on both cryo-electron microscopy and crystallography in channels from bacteria, nematodes, and mammals. The existence of many different subunit isotype genes indicates a very high number of potential structures, whereas a limited number of nAChRs and GABA<sub>A</sub> receptors are found in the central nervous system. The subunit arrangement of muscle nAChR is established as  $\alpha(\gamma/\epsilon)\alpha\delta\beta$ . The most common synaptic  $\alpha\beta\gamma$  GABA<sub>A</sub> receptor structure is established as  $\beta\alpha\beta\alpha\gamma$  (Fig 1). All pLGIC subunits include an N-terminal extracellular domain (ECD) of about 200 amino acids, a transmembrane domain (TMD) consisting of 4 transmembrane helices (M1 to M4), a variable-size intracellular domain (ICD) between M3 and M4, and a short extracellular C-terminus. Sequence alignments in the TMD are well-established in M2 helices, where a universally conserved leucine is the 9<sup>th</sup> residue (L9') after the positively charged residue defining the onset of the helix. However, sequence alignment of other transmembrane helices is more uncertain due to variation in the length of inter-helical linker sequences.

Structure-function studies have established that in muscle nAChRs orthosteric agonists (e.g. ACh and nicotine) bind to  $\alpha^+/\delta^-$  and  $\alpha^+/\gamma/\epsilon^-$  interfaces in the ECD, while in GABA<sub>A</sub>Rs, GABA binds to ECD  $\beta^+/\alpha^-$  interfaces. Thus, nAChR  $\alpha$ 's can be considered homologs of GABA<sub>A</sub>R  $\beta$ 's and in both pLGIC families these archetypal subunits can form functional homopentamers.

Several high-resolution structural studies have focused on anesthetic binding sites in crystallized pLGICs, all of which are homopentamers [6–10]. These confirm that small amphiphilic general anesthetics can fit into multiple types of intra-subunit or inter-subunit protein pockets formed in both ECDs and TMDs (Fig 1C).

## 3) Electrophysiological Approaches to Assessing pLGIC Function

**3.1) Functional states of pLGICs.**—Electrophysiological studies have demonstrated that most pLGICs undergo a similar set of functional transitions. Binding of orthosteric agonists to resting-state closed receptors stimulates rapid transition to activated or open ion-conductive states that can be observed in voltage-clamp experiments. Prolonged agonist application results in multiple phases of channel desensitization, leading to non-conductive states with high affinity for agonists. After discontinuing agonist application, pLGICs undergo deactivation as open channels return to the resting state. Recovery of desensitized receptors to the resting state may be via “silent” pathways or return to open states, and take up to tens of seconds, depending on the degree of receptor desensitization.

### 3.2) Cellular systems for heterologous expression and electrophysiology.—

The most common cell systems used for heterologous expression and structure-function studies are *Xenopus* oocytes and cultured mammalian cell lines like HEK293. *Xenopus* oocytes are large cells (~ 1 mm diameter) that can express many receptors on the membrane surface and under ideal conditions, oocytes can provide stable currents for lengthy experiments lasting several hours. Oocytes are microinjected with messenger RNA or cDNA encoding wild-type or mutant receptor subunits. Oocytes can then be used for whole-oocyte two-electrode voltage-clamp studies or for patch-clamp studies in excised membrane patches [11]. Mammalian cells are transfected with cDNA plasmids that direct synthesis of receptor subunits that are assembled and transported to the cytoplasmic membrane and used for whole cell or patch current studies. Post-translational modification of receptors may vary between expression cell types or depending on cytoplasmic kinase activity. There are examples where either receptor assembly and/or functional properties differ when expressed in HEK293 cells vs. oocytes [12, 13].

**3.3) Electrophysiological and solution exchange equipment.—**Commercially available two-microelectrode voltage-clamp amplifiers can typically measure whole-oocyte voltage-clamped currents ranging from under 5 nA to 10  $\mu$ A. Application of various solutions containing agonists and anesthetics or other chemicals is typically controlled by manual or electronically triggered valves from multiple reservoirs feeding into a manifold with outflow directed at the oocyte in a flow-chamber and then to a waste-collection system. Special care needs to be used with volatile anesthetics to prevent evaporation and adsorption into flow-system materials. When using very hydrophobic compounds such as potent anesthetics, reservoirs and tubing should be made of impermeable materials like glass, stainless steel, or PTFE. Depending on the rate of flow and the design of the reservoir around the oocyte, solution exchange can be as fast as 10 ms, but is typically around 0.1 s or longer. This is often too slow to observe the fastest rates of desensitization and deactivation observed in nAChRs and synaptic GABA<sub>A</sub> receptors. Another important consideration in studying hydrophobic anesthetics is that the large volume of intracellular lipid and protein in oocytes acts as a sink, slowing the rate of equilibration between drug in the oocyte membrane and the surrounding solution. Pre-equilibrating oocytes with anesthetics before agonist application can reduce errors due to this process. An associated challenge is prolonged washout times to fully remove hydrophobic drugs from oocytes.

Patch-clamp amplifiers can typically measure currents in the 5 pA to 10  $\mu$ A range. When combined with “artificial synapse” techniques for rapid solution switching, solution exchange times under 1 ms can be achieved at excised membrane patches [14, 15]. Solution exchange times in whole HEK cells are typically 2 to 10 ms using rapid solution switching devices. Equilibration of hydrophobic drugs is fastest in excised patch experiments, and can be rate-limiting in measuring drug effects in voltage-clamped cells [16]. These approaches enable studies of state-dependent drug actions with analysis of desensitization and deactivation rates. Single-channel recordings can also be obtained using low-noise patch-clamp techniques.

#### 4) Chimeric Subunits

Structure-function studies based on chimeric subunits represent an unbiased approach to identifying peptide sequences within receptor subunits that influence anesthetic sensitivity. Mihic et al [17] created a set of chimeric pLGIC subunits containing complementary portions of anesthetic-sensitive  $\alpha 1$  glycine receptors and anesthetic-insensitive  $\rho$  GABA<sub>A</sub> receptors, and used electrophysiology to assess anesthetic modulation in each chimera at its EC<sub>20</sub> agonist concentration (the agonist depended on which type of receptor donated the N-terminal domain). The results identified a subunit region including M2 and M3 helices that was both necessary and sufficient to confer positive modulation by volatile anesthetics and alcohols. Subsequent point mutations exchanging the non-homologous residues in M2 and M3 helices identified two amino acids that influenced anesthetic modulation in both parent receptors: the M2-15' position, and an M3 residue 21 amino acids away (sometimes called 36'). Mutations at the homologs on GABA<sub>A</sub> receptor  $\alpha$  and  $\beta$  subunits were subsequently shown to influence sensitivity to both volatile and intravenous anesthetics.

J.J. Lambert and colleagues found that GABA<sub>A</sub> receptors containing  $\beta 2$  or  $\beta 3$  subunits were sensitive to modulation and direct agonism by the intravenous drug etomidate, while those with  $\beta 1$  subunits were not [18]. Chimeras containing complementary portions of  $\beta 1$  and  $\beta 2$ , co-expressed with  $\alpha$  and  $\gamma$  subunits and studied electrophysiologically, showed that determinants of etomidate sensitivity were in the transmembrane domain [19]. Subsequent point mutation experiments showed that swapping  $\beta 2$  and  $\beta 1$  M2-15' residues determined drug sensitivity. At this position in  $\beta 2$ , a N-to-S mutation suppressed etomidate sensitivity, while the complementary  $\beta 1$  S-to-N mutation increased sensitivity to etomidate modulation.

Interpretation of data from chimera experiments is critically dependent on the experimental framework. An important illustration is a study of  $\beta 3$  sequences involved in pentobarbital agonism [20]. Chimeric GABA<sub>A</sub>  $\beta 3/\rho$  subunits were co-expressed with  $\alpha 1$  subunits and pentobarbital vs. GABA agonism was assessed electrophysiologically in the resulting receptors. In one chimeric receptor where pentobarbital efficacy was reduced relative to that of GABA, noise and single-channel analyses indicated that the structural modification had increased GABA efficacy, without altering pentobarbital efficacy. Alternative analysis based on deactivation kinetics in the various chimeric receptors suggested that pentobarbital affinity determinants were downstream (toward the C-terminus) from the M2 helix. Another study of pentobarbital actions on GABA<sub>A</sub> receptor chimeras containing complementary sequences from  $\gamma$  and  $\delta$  subunits [21] concluded that sequences upstream from M2 were critical. However, pentobarbital-induced currents were normalized to maximal GABA currents, and this study failed to account for evidence that GABA intrinsic efficacy in  $\alpha\beta\gamma$  receptors is high, but is very low in  $\alpha\beta\delta$  receptors [22].

#### 5) Hydrophobic Point Mutations in nAChRs

**5.1) Introduction.**—Single point mutations are the simplest hypothesis-based (biased) structure-function approach, because each site chosen for a mutation represents a hypothesis or set of hypotheses. Conceptually, one makes mutations and tests their effects on receptor function and sensitivity to drugs, leading to inferences about drug binding or modulation mechanisms.

**5.2) Nicotinic ACh receptors.**—Pioneering work by Henry Lester and colleagues focused on the ion conductive pore of muscle nAChRs, which was thought to be surrounded by M2 helices of the TMD [23]. They showed that M2-6′ S-to A mutations in mouse muscle nAChR alpha subunits reduced sensitivity to QX222, a charged quarternary ammonium analog of an amide local anesthetic thought to block nAChRs via open-channel block. A strength of this study was the use of single-channel kinetics, demonstrating reduced apparent QX222 residence time in its site, as evidence of reduced binding affinity. Voltage-dependent block was also demonstrated, supporting a transmembrane location of the site. Mutations at M2-10′ residues on nAChRs showed that increased hydrophobicity enhanced QX-222 affinity, suggesting that the charged end of the molecule interacted with 6′ residues, while the hydrophobic end interacted with 10′ residues.

Kinetic studies in mouse nAChRs also suggested that general anesthetics might act as open-channel blockers [24]. Following the work on QX222, Forman et al [25] tested M2-10′ mutations that enhanced hydrophobicity, and found that they dramatically increased mouse nAChR sensitivity to open state-dependent block by both alcohols and volatile anesthetics. Subsequent studies showed that mutations on all five M2-10′ residues affected sensitivity to anesthetic inhibition, suggesting a pore site [26]. Scanning mutagenesis studies defined a “hydrophobic patch” in the nAChR channel where anesthetics apparently interacted [27].

Unlike long-chain alcohols, ethanol enhances the apparent affinity of ACh for muscle and Torpedo nAChRs. Studies in nAChR mutants that impaired gating demonstrated that ethanol enhances ACh agonist efficacy rather than binding [28]. However, positive modulation by ethanol is unaffected by M2 mutations that affect alcohol inhibitory potency.

**5.3) GABA<sub>A</sub> receptors.**—In contrast to anesthetic inhibition of excitatory serotonin and nAChRs, many general anesthetics enhance activation of inhibitory glycine and GABA<sub>A</sub> receptors [4]. Moreover, high concentrations of general anesthetics directly activate (agonize) these receptors in the absence of GABA or other orthosteric agonists, which might indicate the presence of at least two classes of anesthetic sites and presented challenges in defining anesthetic potency for putative sites. A standard approach is to measure anesthetic enhancement of receptor activation by a low agonist concentration that alone generates a small fraction (5 to 20%) of maximal receptor-mediated responses.

Based on the findings of chimera studies [17], mutations on various GABA<sub>A</sub> receptor subunit M2-15′ and 36′ residues have been tested for effects on anesthetic modulation. These studies showed that βM286W mutations weaken modulation by propofol and related alkyl-phenols [29, 30] and that the size of the α1 M2-15′ (S270) sidechain determines volatile anesthetic sensitivity [31]. Tryptophan mutant sensitivity has been applied as a scanning method to discover anesthetic contacts [32, 33]. Similar approaches have also been used to discern selectivity for multiple GABA<sub>A</sub> receptor binding sites by various anesthetics [34].

**5.4) MWC co-agonism as an interpretive framework for anesthetic actions on GABA<sub>A</sub> receptors.**—The interpretive framework for mutant function studies is also critically important, because it is difficult, if not impossible, to rule out allosteric effects

based on binding studies and functional analysis alone. This is particularly difficult when studying agonist sites [35]. Reframing anesthetic actions at GABA<sub>A</sub> receptors as allosteric co-agonism was based on pharmacological analysis using a formal Monod-Wyman-Changeux (MWC) two-state model [36–38]. These models provide a mechanistic framework for structure-function experiments that accounts for both direct anesthetic agonism and modulation of GABA-evoked responses based on drug interaction with a single class of allosteric agonist sites, providing simplified analysis in many cases. This approach has also proven insightful in explaining results that might otherwise lead to the conclusion that direct activation and modulation are mediated by independent sites. For example, based on etomidate photolabeling results [39], we examined the effects of tryptophan mutations at both  $\alpha 1M236$  and  $\beta 2M286$  in  $\alpha 1\beta 2\gamma 2L$  receptors [40]. Oocyte electrophysiology showed that  $\alpha 1M236W\beta 2\gamma 2L$  receptors were spontaneously active and that etomidate potently and efficaciously activated these receptors, whereas etomidate modulation of GABA responses was much weaker than that in wild-type receptors. MWC models predict that spontaneously active mutant channels should display increased sensitivity to agonists (both orthosteric and allosteric), and model-based analysis showed that all of the etomidate-dependent results were consistent with reduced drug efficacy. More importantly, this example illustrates how mutations that alter receptor gating or orthosteric agonist efficacy may produce the appearance of altered anesthetic sensitivity, even when underlying anesthetic site interactions are unaltered.

However, even though MWC mechanisms distinguish binding affinity to resting receptors from agonist efficacy as separate model parameters, we have consistently found that mutations alter apparent efficacy far more than binding affinity, even at residues thought to be important binding contact points. This is likely because agonist efficacy in MWC is defined as the ratio of binding dissociation constants in open vs. closed receptors, and molecules sufficiently efficacious to activate receptors bind much more tightly to open state receptors. At the same time, the very high anesthetic concentrations needed to occupy the majority of low-affinity resting state sites usually produce mixed functional effects that include positive modulation and inhibition. Some mutations that produce total insensitivity to anesthetics provide no data for differentiating binding vs. efficacy changes [41]. Thus, even using an analytical approach that differentiates binding and efficacy, identifying binding determinants is a challenge.

**5.5) Comparing mutant sensitivity and anesthetic photolabeling.**—Given the possibility that mutations allosterically alter apparent anesthetic sensitivity, an important question is how well hydrophobic mutagenesis and functional analysis performs in identifying drug-receptor contacts. We recently compared results of studies based on GABA<sub>A</sub> receptor photolabeling with derivatives of etomidate, propofol, mephobarbital, and alphaxalone to tryptophan mutagenesis experiments at two photolabeled amino acids [42]. With eight possible interactions (4 positive and 4 negative photolabeling results), Trp mutant studies agreed with photolabeling in 6 of 8 cases, with one false positive and one false negative. This correlation was too weak to support mutant function analysis as a reliable approach to discovering anesthetic contact residues in GABA<sub>A</sub> receptors.

## 6) Substituted Cysteine Accessibility Method: SCAM

**6.1) Introduction.**—Cysteine mutation-based approaches based on covalent sulfhydryl modification are potentially more sensitive to local steric rather than allosteric effects, because implicit in this method is a bi-molecular interaction between a chemical probe and the sulfhydryl group. These approaches include the Substituted Cysteine Accessibility Method (SCAM), disulfide mapping, and Substituted Cysteine Modification Protection (SCAMP). Below, we focus on how these methods have been applied to pLGIC receptor structure and mapping of anesthetic sites.

**6.2) Nicotinic ACh receptors.**—Akabas and Karlin were first to combine real-time agonist-dependent cysteine modification of pLGICs with electrophysiological analysis in nAChRs [43]. Their focus was cysteine accessibility, using a positively charged methane thiosulfonate-ethylammonium probe (MTS-EA), which they presumed was accessible only to the water-filled cation-conductive pore. Water is usually required for disulfide bond formation, which can also be influenced by the acidity of the targeted –SH group. At the time of these pioneering experiments, it was assumed that water was generally excluded from access to the M1, M3, and M4 helices that surrounded the M2s. Akabas and Karlin assumed that MTSEA modification in the nAChR pore would inhibit cation conduction. Thus, they compared maximal ACh-activated voltage-clamp currents mediated by cysteine-substituted receptors before and after MTSEA exposure. When inhibition was found, they inferred sidechain access from the water-filled ion pore. Other water-soluble reagents have also been used to probe water-accessible portions of pLGIC receptors. Using various modifying reagents at accessible substituted cysteines also enables studies of the effect of size, hydrophobicity, and charge on function.

**6.3) GABA<sub>A</sub> receptors.**—Akabas and colleagues extended the SCAM approach to M2 helices of GABA<sub>A</sub> receptors, and using EC<sub>50</sub> GABA instead of high GABA when testing mutant receptors, found that modification both enhanced receptor activation and inhibited it at various positions [44]. The finding of enhanced GABA responses after modification meant that inferences regarding accessibility via the central ion pore were uncertain. Accessibility to water remained the sole inferential outcome of these studies. Importantly however, results in other TM helices showed that water could reach close to the presumed inner ends of TM helices, implying that these helices are flexible and that water intercalates between them, and not just in the ion-conductive pore [45, 46]. In short, SCAM revealed water-accessible pockets formed among the various transmembrane helices.

## 7) Disulfide Bond Formation Used to Probe Helix-Helix Interactions in GABA<sub>A</sub> Receptors.

Janson & Akabas [47] extended the use of cysteine substitutions in order to gain more information about the orientation of residues on adjacent transmembrane helices in the GABA  $\alpha 1$  subunits. In this study, they inserted cysteines at different positions in both  $\alpha 1$ -M2 and  $\alpha 1$ -M3 helices, and used oocyte electrophysiology and chemical manipulations to either promote or reverse disulfide bond formation between the two helices. Their results helped constrain structural models and also suggested possible relative movements of M2 and M3 during channel gating.

Bali et al [48] utilized similar methods, but focused on creating disulfide linkages between two transmembrane helical faces across the  $\beta^+/\alpha^-$  inter-subunit cleft. Their results study further constrained structural models and were consistent with azi-etomidate photolabeling data suggesting  $\beta^+/\alpha^-$  inter-subunit etomidate binding sites based on photolabeling of residues on both  $\beta$ -M3 and  $\alpha$ -M1 helices [39].

## 8) Substituted Cysteine Modification and Protection: SCAMP

**8.1) Introduction.**—Because covalent modification of a substituted cysteine requires bimolecular interactions between the probe reagent and the engineered sulfhydryl, this reaction can be hindered by non-covalent ligands that bind nearby. This inhibition of cysteine modification or “protection” has been developed as an approach to both complement and supplement other techniques, particularly photolabeling, in identifying anesthetic contacts on GABA<sub>A</sub> receptors. The first such study was that of Bali and Akabas [49], testing whether propofol contacted  $\beta$ N265 (M2-15') or  $\beta$ M286 (36') residues that had been identified in chimera and point mutation studies. To do this, they used oocyte electrophysiology to estimate the rates of modification at  $\beta$ N265C or  $\beta$ M286C by the sulfhydryl-reactive probe p-chloromercuribenzenesulfonate (pCMBS). They then tested whether addition of propofol slowed the rate of modification, and found protection at  $\beta$ M286C, but not  $\beta$ N265C.

**8.2) SCAMP experimental design considerations.**—For the SCAMP technique to enable strong inferences of steric interactions, a number of experimental conditions must be met [50]:

- 8.2.1** The background receptor must be insensitive to probe exposure. This may require removing native cysteines, usually by mutation to alanines or serines.
- 8.2.2** The cysteine-substituted mutant receptor should retain sensitivity to the anesthetic drug (usually measured as EC<sub>50</sub> enhancement by a standard drug concentration), ideally similar to wild-type sensitivity. Loss of drug sensitivity might be caused by the cysteine mutation dramatically reducing drug binding, which could lead to false negative results. For example, we studied  $\beta$ N265C protection by etomidate, but also found that the mutation totally eliminated etomidate sensitivity [51]. Thus, the observed absence of etomidate protection could have been due to absence of drug binding, rather than binding at a site distant from  $\beta$ N265. It is possible that the lack of propofol protection at  $\beta$ N265C [49] was also due to low occupancy of these sites. In cases where drug sensitivity is moderately reduced, protection may still be tested, but using higher drug concentrations.
- 8.2.3** Modification of the mutant receptor must reliably produce quantifiable functional changes. Multiple probes may be tested to determine which produce the largest functional effects. However, very large probes may increase sensitivity to inhibition by ligands that bind farther from the study residue. Electrophysiological signals indicating GABA<sub>A</sub> receptor modification can include: a) spontaneous receptor activity, determined with picrotoxin inhibition; b) maximal receptor current; or c) EC<sub>50</sub> GABA response, usually normalized to



maximal response. In our experience, carefully designed SCAMP studies can lead to valid inferences in cases where receptor modification produces functional changes with magnitudes as small as two-fold.

- 8.2.4** Modification conditions must enable estimation of modification rates. Typically, sequential cycles of receptor modification are separated by repeated electrophysiological assessments of receptor function, with each cycle resulting in approximately 10% of the full modification effect (Fig 2). With many such cycles, full modification can be achieved and the electrophysiological results fitted to an exponential function to determine a rate parameter. Alternatively, three or four partial modification cycles can be used to establish an initial “apparent” modification rate based on a linear fit to the electrophysiological results. Normalization to each individual cell’s maximal current is essential when using “apparent” modification rate analysis.
- 8.2.5** The distribution of receptor states should be similar in both control modification experiments and in drug protection experiments. This aspect of study design can be challenging when using anesthetics that act as agonists at GABA<sub>A</sub> receptors. In our experience, the rate of pCMBS modification at cysteines in transmembrane helices of GABA<sub>A</sub> receptors is usually faster when pCMBS is co-applied with GABA than when applied alone [42, 52, 53]. If adding anesthetics activates receptors, then using a “closed state” modification control is inappropriate, because a fraction of receptors will be activated during protection experiments. To avoid this problem, we use control modification conditions with pCMBS + GABA. GABA-bound receptors also bind more avidly to anesthetics (allosteric agonists), resulting in higher anesthetic binding site occupancy. However, GABA may become a partial agonist at with introduction of some cysteine mutations, introducing the possibility that the fractions of closed vs. open vs. desensitized receptors differ in control experiments and anesthetic protection experiments. In such cases, addition of another positive modulator that binds to distinct sites may help establish similar distributions of receptor states in control modification and protection experiments [52]. For example, pharmacological and photolabeling evidence indicates that alphaxalone and some barbiturate sites are distinct from those for propofol or etomidate.
- 8.2.6** Drug site occupancy should correlate with the degree of observed protection. Demonstrating concentration-dependent protection, particularly when anesthetic functional effects parallels protection, supports the presumed local steric hindrance mechanism [52]. By extension, SCAMP will be more sensitive when high anesthetic concentrations are used, leading to higher binding site occupancy. As noted above, this is particularly important when studying mutations that are relatively insensitive to modulation by one or more study drugs. Including GABA usually enhances mutant receptor affinity for anesthetics, and increases drug site occupancy (see above).

- 8.2.7** In protection experiments, potent anesthetics should be pre-applied before adding modifiers, to assure anesthetic site occupancy *before* modifiers reach their target. This approach is particularly important when studying very hydrophobic anesthetics that slowly equilibrate in oocyte membranes due to intracellular binding to protein and lipid. Higher sulfhydryl probe concentrations will more rapidly reach the target sulfhydryl, so selecting conditions where lower probe concentrations are used (i.e. apply GABA with the modifier) will also reduce this potential artifact.
- 8.2.8** Even with all these conditions met, it is important to perform additional studies to help rule out allosteric drug inhibition of modification at each substituted cysteine. Allosteric inhibition of modification at a particular cysteine should correlate with receptor functional state, rather than anesthetic site occupancy. Thus, all anesthetics that modulate the mutant receptor should inhibit modification of that cysteine. Our approach has been to compare multiple anesthetics that all produce similar functional effects in GABA<sub>A</sub> receptors, but that are also known to bind to distinct sets of sites, based on pharmacological and photolabeling evidence. Sets of such drugs perform as negative controls for each other, and lend support to a steric hindrance mechanism in most cases where SCAMP experiments show inhibition.

**8.3) SCAMP Limitations.**—SCAMP doesn't enable every hypothesized residue to be tested against every drug of interest. Some cysteine mutations prevent functional expression of receptors (e.g.  $\beta$ 3Y284C) and some (e.g.  $\alpha$ 1I239C and  $\alpha$ 1Q242C) show no functional effects when exposed to sulfhydryl modifiers [53, 54]. Several cases where cysteine mutants are totally insensitive to drugs of interest have also been reported. In the case of  $\beta$ 2N265, we used an indirect SCAMP approach, based on protection of  $\alpha$ 1M236C by etomidate, to show that  $\beta$ 2N265M, which also eliminates etomidate sensitivity, dramatically reduces apparent etomidate affinity for its site in both closed and GABA-activated states. Indirect SCAMP experiments may also be used to distinguish between allosteric and steric effects of other mutations. Despite negative control experiments, we have also encountered cases where anesthetics only partially inhibit sulfhydryl modification. These may represent cases where substituted cysteines are located near the periphery of anesthetic sites, so that steric hindrance is incomplete. This possibility may be tested with different size modifiers that should show size-dependent interactions with bound anesthetic.

**8.4) Comparing SCAMP and anesthetic photolabeling.**—We have also formally compared SCAMP results against photolabeling at two positions with four different drugs. The two approaches show 100% agreement for the eight residue-drug pairs we examined, a correlation that is statistically significant and strengthened when other photolabeled residues studied with SCAMP are added. However, in one case,  $\beta$ 3H267, SCAMP disagrees with photolabeling, reminding us that both approaches are based on structural variants of either the receptor or the ligand, and therefore potentially inaccurate [55]. The preponderance of evidence supports SCAMP as a technique that is useful both for confirming photolabeling results and for testing potential drug contacts that have not been photolabeled. Indeed,

SCAMP experiments have substantially expanded the list of anesthetic contact residues in GABA<sub>A</sub> receptors beyond those based on photolabeling alone.

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## Abbreviations:

<b>SCAM</b>	Substituted Cysteine Accessibility Method.
<b>SCAMP</b>	Substituted Cysteine Modification-Protection.
<b>GABA<sub>A</sub></b>	gamma-aminobutyric acid type A.

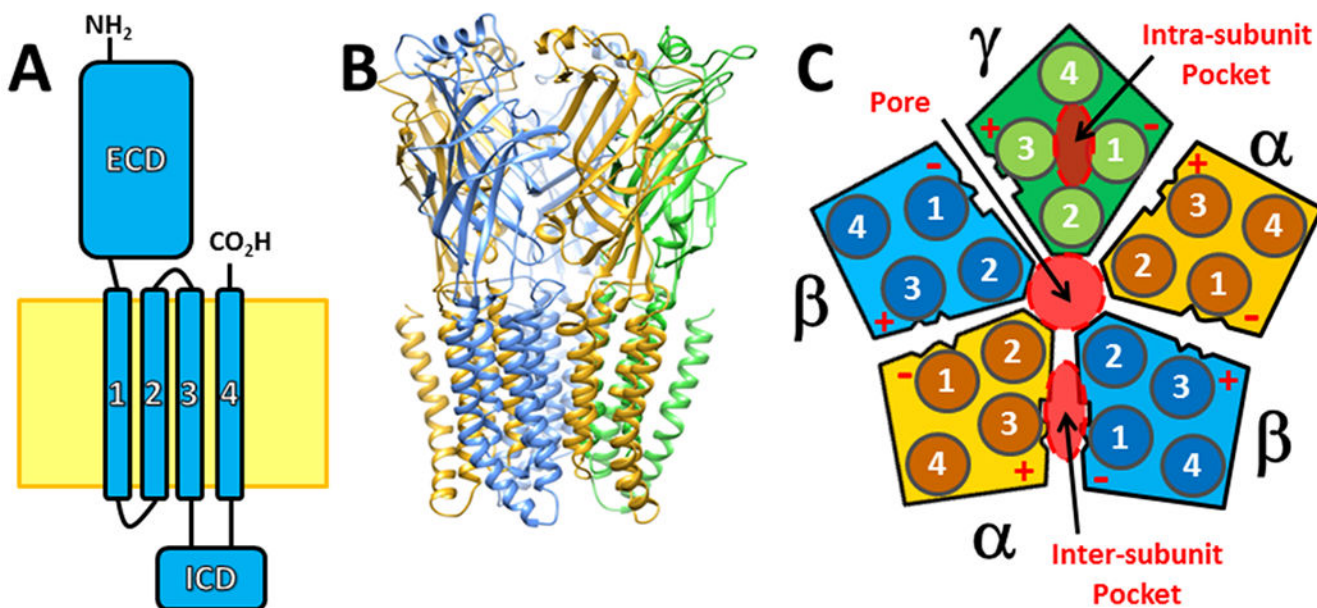
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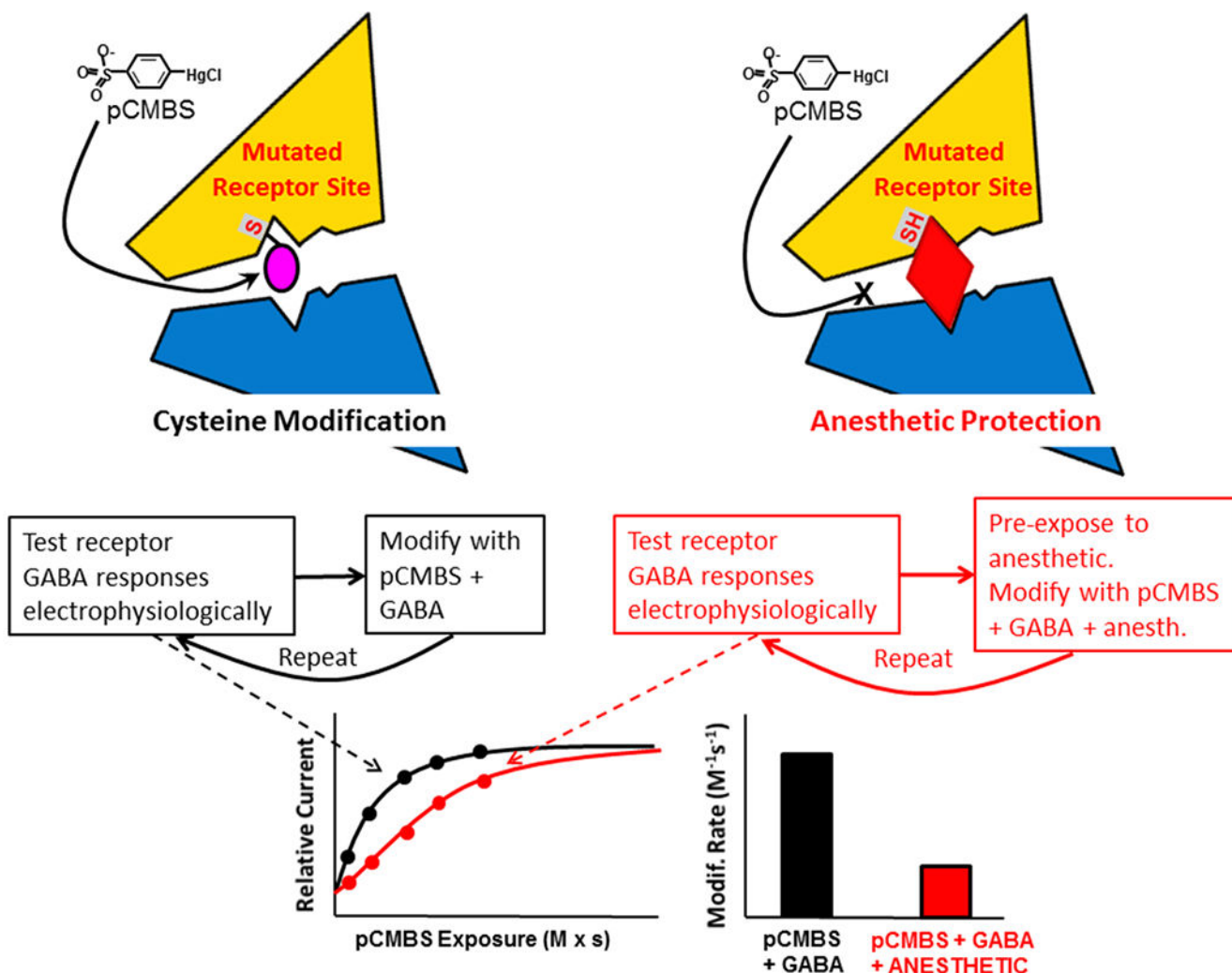
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**Figure 1: Pentameric Ligand-Gated Ion Channel Structure and Types of Anesthetic Binding Sites.**

Panel A depicts the canonical subunit structure shared by all pLGICs, including a large N-terminal extracellular domain (ECD), a transmembrane domain consisting of four alpha-helices, and an intracellular domain (ICD) between M3 and M4. The cytoplasmic membrane is depicted as a yellow zone. Panel B shows a structural homology model of a synaptic GABA<sub>A</sub> receptor consisting of two α subunits (gold), two β subunits (blue), and one γ subunit (green). Peptide backbones are depicted as ribbons to highlight secondary structural folding. Subunit ICDs have been removed because their structure is not well-defined. Panel C is a diagram showing a cross-section of an αβγ GABA<sub>A</sub> receptor through the TMD. Each subunit is color-coded as in panel B, the arrangement of subunits is details, as are the positions of numbered alpha-helices in each subunit. Three types of anesthetic binding pockets in the TMD are highlighted with red overlays: the central transmembrane pore, intra-subunit pockets formed by subunit four-helix bundles, and inter-subunit pockets. Inter-subunit anesthetic binding pockets have also been imaged in the ECD. The faces of each subunit are also labeled “+” or “-” depending on whether they include M3 or M1 helical elements.



**Figure 2: Substituted Cysteine Modification Protection (SCAMP) Experimental Approach.** The top and middle left panels depict a mutant receptor with a cysteine-substitution in an inter-subunit anesthetic binding pocket as it is covalently modified by para-chloromercuribenzenesulfonate (pCMBS). Repeated cycles of partial modification produce functional changes that are measured electrophysiologically. The top and middle right panels depict similar experiments on anesthetic-bound receptors, with inhibition of the bimolecular reaction between pCMBS and the engineered sulfhydryl group on the receptor. The bottom left panel shows how electrophysiological measurements for both control modification (black dots) and protection (red dots) conditions are plotted against cumulative pCMBS exposure and fitted with exponential functions (black and red lines) to determine modification rates. The bottom right panel shows a summary of the analyzed rates indicating that bound anesthetic “protects” this cysteine from modification.