# GABA- and Drug-induced Conformational Changes Detected in the $GABA_A$ Receptor Channel-Lining Segments

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## Introduction

$GABA_A$ receptors appear to be a major target for the actions of many general anesthetics. In order to understand the mechanism of action of these anesthetics at a molecular level we need to identify the anesthetic binding sites within the receptor and elucidate the conformational changes that anesthetic binding induces. The $GABA_A$ receptors are members of the neurotransmitter-gated ion channel gene superfamily that includes receptors for glycine, acetylcholine and $5HT_3$ (serotonin)[1-3]. They are the major inhibitory neurotransmitter receptors in the central nervous system. Binding of GABA to the $GABA_A$ receptors stimulates the opening and subsequent desensitization of an anion-selective channel that is an integral part of the receptor protein. At low concentrations many general anesthetics including volatile anesthetics, such as isoflurane and halothane, and intravenous anesthetics, such as propofol, barbiturates, etomidate and neurosteroids potentiate the currents induced by submaximal GABA concentrations. At higher concentrations these same anesthetics directly activate $GABA_A$ receptors. The goals of our research are to elucidate: 1) how the structure of the $GABA_A$ receptor determines its functional properties, 2) the conformational changes the channel undergoes during gating, and 3) the mechanisms by which drugs, such as general anesthetics and benzodiazepines, modulate the activity of the receptor.

The $GABA_A$ receptors are formed by the assembly of five homologous subunits. Within the $GABA_A$ receptor gene family the cloned subunits have been divided into multiple sub-families based on the extent of sequence identity[1,3]. All of the subunits have a similar transmembrane topology with an ~200 amino acid extracellular N-terminal domain

that forms the agonist binding sites and a similar sized C-terminal domain with four membrane-spanning segments (M1, M2, M3, M4) that form the transmembrane ion channel[1,2]. The high resolution x-ray crystal structure of an acetylcholine binding protein homologous to the extracellular domain has been determined[4]. This provides a molecular basis for homology modeling of the structure of the GABA binding sites to guide future experiments. A lower resolution (9 Å) structure of the membrane-spanning domain of the homologous *Torpedo* nicotinic acetylcholine receptor has also been determined by cryo-electron microscopy[5]. It suggests that one membrane-spanning segment contributed by each of the five subunits forms the major portion of the channel lining. Considerable evidence from the homologous nicotinic acetylcholine receptor indicated that the M2 membrane-spanning segment was the primary channel-lining segment[2].

## Channel-lining Residues

The amino acids lining the ion channel are likely to be major determinants of the functional properties of the channel. We developed the substituted-cysteine-accessibility method (SCAM) to provide a systematic approach to identifying putative channel-lining residues[6,7]. Applying SCAM to the 26 residues in and flanking the $GABA_A$ receptor $\alpha_1$ M2 segment we showed that ten of the residues are on the water-accessible surface of the protein (Fig. 1)[8,9]. An $\alpha$ helical wheel plot of the M2 segment residues shows that most of the water-accessible residues lie on one side of the helix (Fig. 1A)[9]. We conclude that the M2 segment secondary structure is largely $\alpha$ helical. (We will discuss the implications of the accessibility of $\alpha_1$S270C below.) We infer that most of the M2 segment water-accessible residues line the ion channel because the reaction rate of the negatively charged sulfhydryl reagent p-chloromercuribenzenesulfonate ($pCMBS^-$) with a cysteine substituted for the most cytoplasmic M2 segment water-accessible residue, $\alpha_1$Val257 (Fig. 1A) was voltage dependent (Horenstein and Akabas, unpublished results). This implies that the access pathway to $\alpha_1$V257C is via the ion channel.

Using SCAM we have also identified the water-accessible residues in the M2 segments of the homologous muscle acetylcholine receptor $\alpha$ subunit and the $5\text{-}HT_{3A}$ receptor subunit[6,10]. In general, the aligned residues are water accessible but there are some differences (Fig. 1C)[10].

Picrotoxin is a $GABA_A$ receptor inhibitor. Picrotoxin protected the cysteine substituted for $\alpha_1$Val257 from reaction with $pCMBS^-$ applied extracellularly. It did not protect cysteines substituted at more extracellular channel-lining positions[11]. We inferred that picrotoxin binds in the channel near the cytoplasmic end at the level of $\alpha_1$Val257. Because picrotoxin is a rigid, roughly spherical molecule ~9 Å in diameter the channel lumen must be ~9 Å in diameter down to the level of $\alpha_1$Val257 in the open state. The narrowest point in the channel may be at a more cytoplasmic position.

$Zn^{2+}$ inhibits $GABA_A$ receptors formed by coexpression of the $\alpha_1$ and $\beta_1$ subunits with an $IC_{50}$ of ~0.5 μM[12,13]. We showed that mutation of $\beta_1$His267, a water-exposed M2 segment residue, eliminated the high affinity $Zn^{2+}$ binding site[14]. Based on these and

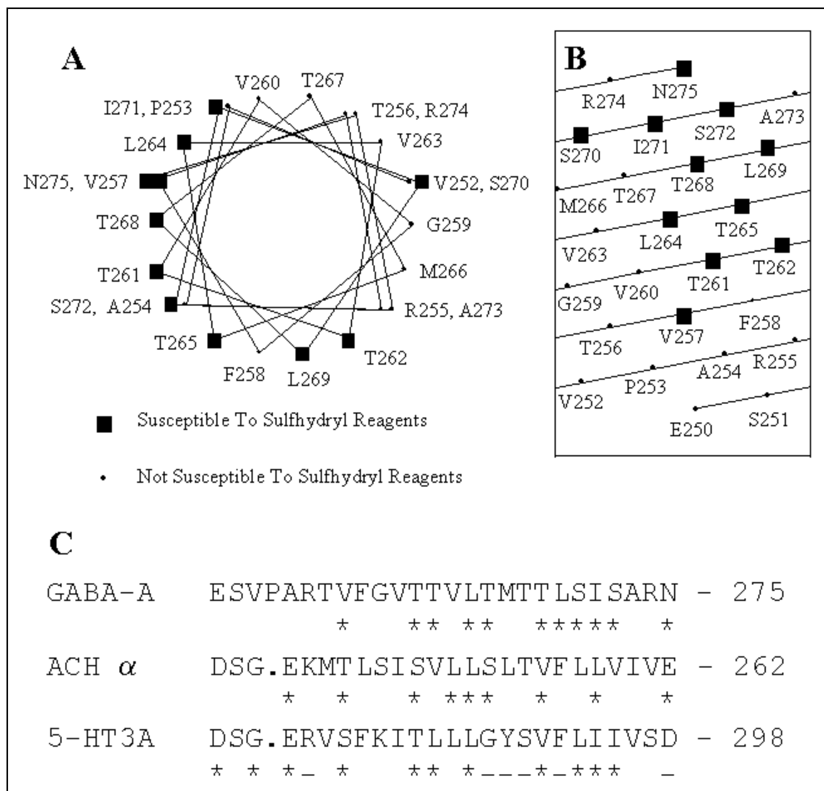



*Fig. 1: Results of Substituted-Cysteine-Accessibility studies on the $GABA_A$ receptor M2 segment. (**A**) α helical wheel representation of the residues in the $GABA_A$ receptor $\alpha_1$ M2 segment. Black squares indicate residues at which charged, sulfhydryl reagents reacted with cysteine-substitution mutants. (**B**) α helical net representation of the $\alpha_1$ M2 segment residues. Positions that align vertically are on the same face of the helix. Black squares indicate residues at which charged, sulfhydryl reagents reacted with cysteine-substitution mutants. Extracellular is at the top. (**C**) Sequence alignment of the M2 segments of the $GABA_A$ receptor $\alpha_1$ subunit, mouse muscle nicotinic acetylcholine receptor α subunit and $5\text{-}HT_{3A}$ receptor subunits. Sulfhydryl-reactive positions indicated by * underneath sequence. Positions at which cysteine-substitution mutants did not express indicated by dash (-) underneath residue. Amino acid number of last residue indicated to the right of the sequences. (Modified from Xu and Akabas, 1996 with permission)*

other results we inferred that the stoichiometry of $\alpha_1\beta_1$ receptors is 2 α and 3 β subunits and that $\beta_1$His267 from adjacent β subunits form a bidentate $Zn^{2+}$ binding site. In order to form a bidentate $Zn^{2+}$ binding site the Cα carbons of two histidine residues must be less than 13 Å apart. Thus, this provides a distance constraint on the maximum separa-

tion of channel-lining residues in adjacent M2 segments at the level of $\beta_1$His267. Experiments by others are consistent with $\beta_1$His267 forming the high affinity $Zn^{2+}$ binding site[15].

## Mobility and Proximity of the M2 Channel-lining Segments in Different Subunits

In order to investigate the quaternary structure and thermal protein mobility of the M2 channel-lining segments in different subunits and ligand-induced conformational changes in the channel lining we used disulfide trapping experiments[16]. In disulfide trapping experiments the ability to form a disulfide bond between pairs of engineered cysteine residues is used to assay the relative proximity, mobility and flexibility of different protein regions. Disulfide trapping has been used to study protein mobility and structure in several proteins[17-20]. In order to form a disulfide bond the Cα carbons of the two cysteines must approach to within ~5.6 Å; this measures their closest approach not necessarily their average separation[17,20].

We investigated the ability to form disulfide bonds between cysteines substituted at aligned channel-lining positions in the $\alpha_1$ and $\beta_1$ M2 segments. Using electrophysiological assays we demonstrated that disulfide bonds formed at three levels in the M2 segment, two near the extracellular end, at the 17′ and 20′ positions, and one below the middle of the segment (6′ position) (Fig. 2A)[16]. Western blot analysis showed the formation of disulfide-linked dimers at the same positions and under similar oxidizing conditions to the electrophysiological assays[16]. Near the extracellular end of the channel, disulfide bonds formed at similar rates both in the presence and in the absence of GABA. The ability to form disulfide bonds and the similarity of the rates with and without GABA suggests that thermal protein motion at the extracellular ends of the M2 segments moves the engineered cysteines into close proximity to allow disulfide bond formation. The presence of GABA does not seem to significantly alter this motion.

In contrast, at the 6′ position disulfide bond formation only occurred in an oxidizing environment in the presence of GABA. Furthermore, disulfide bond formation only required the presence of an engineered cysteine in the $\beta_1$ subunit. Because the channels were locked in an open state we infer that the disulfide bond formed between cysteines in adjacent subunits (Fig. 2B); disulfide bond formation between cysteines in non-adjacent subunits would obliterate the channel lumen. This implies that there are adjacent β subunits in the functional receptors. This is consistent with a subunit stoichiometry of 2 α and 3 β subunits in $\alpha_1\beta_1$ receptors as was inferred from the $Zn^{2+}$ experiments (Fig. 2B)[14]. Thus, the γ subunit in αβγ receptors is replaced by a β subunit in αβ receptors as was also inferred by other investigators[21].

Our data supports the hypothesis that gating from the closed to the open state involves a rotation of the M2 segments. Disulfide bond formation at the 6′ position locked the channels in the open state. We assume that the disulfide bond is trapping the channel in a conformation similar to the open state and that in the closed state the subunits are arranged relatively symmetrically around the central channel axis (Fig. 2C, left panel).

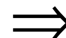
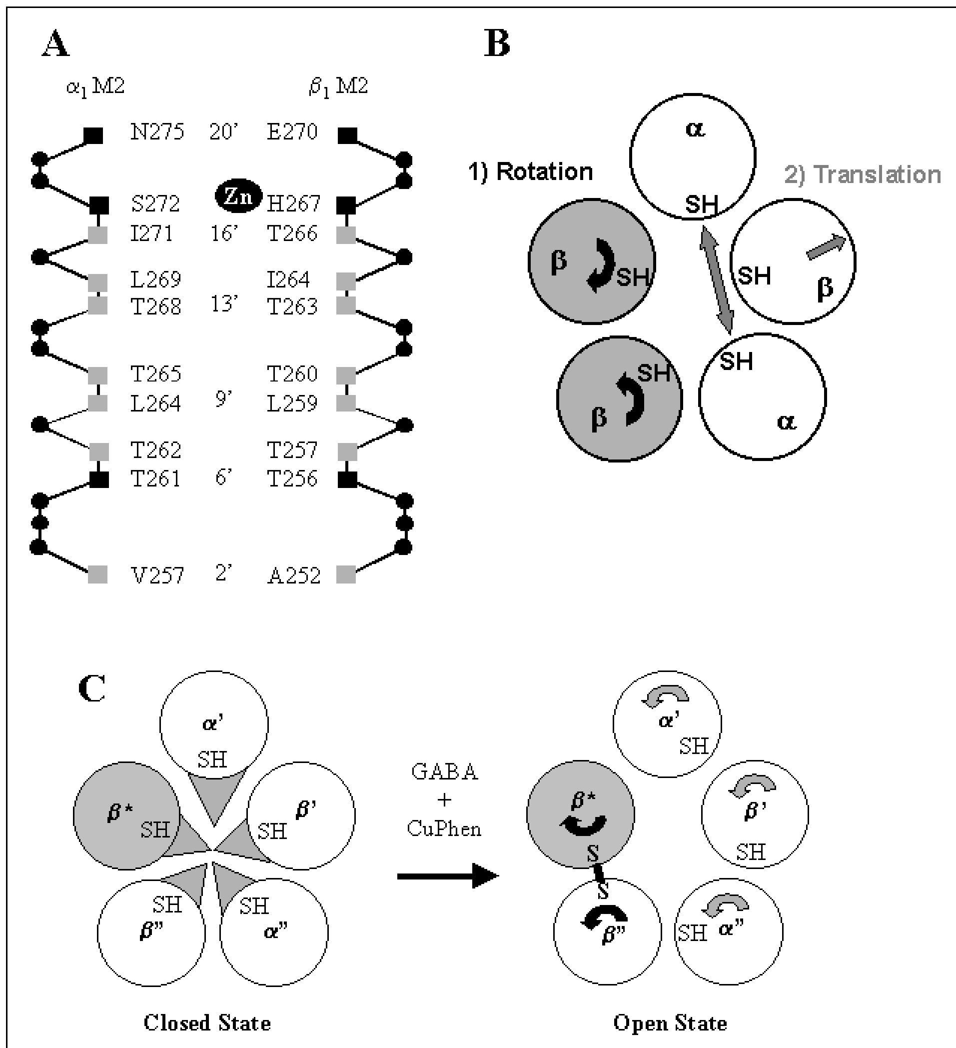


*Fig. 2: Probing the mobility, flexibility and proximity of $GABA_A$ receptor M2 segment channel-lining residues in different subunits. (**A**) Aligned sequences of the rat $\alpha_1$ and $\beta_1$ M2 segments. Squares indicate channel-lining positions based on cysteine accessibility studies on the $\alpha_1$ subunit and the aligned positions in the $\beta_1$ subunit. Black squares indicate positions where disulfide bonds formed between engineered cysteines. Circles indicate non-channel-lining positions. Numbers in the center are an indexing number system to allow comparison of M2 segment positions across different receptor subunits[39]. The 0′ position is assigned to a highly conserved cationic residue at the cytoplasmic end of the M2 segment. Numbers increase towards the extracellular end of the M2 segment. $Zn^{2+}$ indicates the location of the $Zn^{2+}$ binding site at $\beta_1$His267. (**B**) Illustration of the two possible motions that could bring aligned channel-lining residues on different subunits into close proximity. The view is a cross-section through the channel. The circles represent the M2 segments, SH indicates the position of the cysteine sulfhydryl. Note that there are 2 $\alpha$ subunits and 3 $\beta$ subunits, the stoichiometry indicated by our results and others for $\alpha\beta$ receptors[21,14,40,16]. The left two adjacent M2 segments (gray circles) are shown undergoing an asymmetrical rotation that brings the cysteines into close proximity. The right three M2 segments (white circles) are undergoing a translational motion that allows disulfide bond formation between cysteines in non-adjacent subunits. We believe that because disulfide bond formation at the 6′ position locks the channels in an open state it is likely to be a rotation forming the bond between cysteines on adjacent subunits[16]. (**C**) Cartoon illustrating the asymmetric rotation of adjacent $\beta$ subunits upon channel opening. Left panel shows a top view cross section of the channel in the closed state. Circles represent the five M2 segments, SH indicates the position of the 6′ engineered Cys residues, gray triangles represent the closed channel gate (This should not be taken to imply that the gate is at the 6′ level). The white circles represent the M2 segments from the subunits involved in forming the two GABA binding sites. The gray circle ($\beta$*) indicates the third $\beta$ subunit that is not involved in forming the GABA binding sites. Right panel shows the open state following treatment with Cu:phen in the presence of GABA. The four subunits forming the GABA binding sites have rotated in a counterclockwise direction whereas the shaded subunit has rotated in a clockwise direction. Arrows indicate the direction of rotation relative to the initial starting position. (The handedness of rotation, clockwise vs counterclockwise, was chosen arbitrarily, our data do not provide information on the direction of rotation.) Such a movement, at least of the two adjacent $\beta$ subunits would be necessary to bring sulfhydryls at aligned positions on adjacent subunits into close proximity to allow disulfide bond formation. As discussed in the text, the two adjacent $\beta$ subunits may not rotate to a similar extent. Note the disulfide bond indicated between the sulfhydryls on the adjacent $\beta$ subunits after Cu:phen. (Panel C reproduced from Horenstein et al., 2001 with permission).*




Thus, in order to move aligned cysteines on different subunits into close contact in the open state the M2 segments in adjacent subunits must rotate asymmetrically towards each other as illustrated in Fig. 2C, right panel. At present we cannot distinguish whether both subunits rotate towards each other or whether one rotates and the other does not. Either way, the motion must be asymmetric. We hypothesize that it is the motion of the adjacent β subunits that is different. The two GABA binding sites are located at the αβ subunit interfaces. Thus, two α and two β subunits participate in forming the GABA binding sites. The fifth subunit, the third β subunit in our case, is the subunit not involved in forming a GABA binding site. We hypothesize that its motion

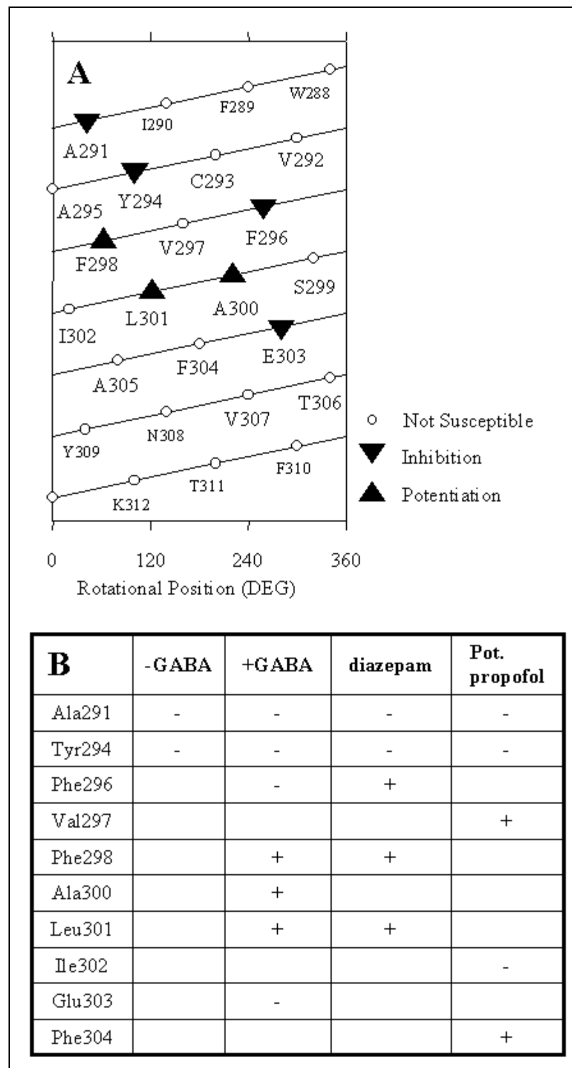
during channel opening is different than the motion of the subunits involved in forming GABA binding sites. Thus, we hypothesize that $GABA_A$ receptor gating involves asymmetric rotation of the M2 channel-lining segments in different subunits. Unwin had previously suggested that channel gating in the acetylcholine receptor involved a symmetrical rotation of the M2 segments[22]. Of note, in voltage-gated channels evidence suggests that the S4 voltage sensor undergoes rotation during gating[23].

## Conformational Dependent Accessibility of M3 Segment Substituted Cysteine Residues

The reactivity of engineered cysteine residues in the M3 membrane-spanning segment with $pCMBS^-$ applied extracellularly is state dependent[24,25]. We have used the state-dependent accessibility of the M3 engineered cysteine residues as a reporter in order to probe the effects of drug binding on receptor conformation. We mutated each residue, one at a time, in the $\alpha_1$ M3 segment (Ala291 to Val307) to cysteine. The mutant $\alpha_1$ subunits were co-expressed with wild type $\beta_1$ and $\gamma_{2S}$ subunits in *Xenopus* oocytes. The reactivity of the engineered cysteine residues was assayed by the effect of a 1-min application of 0.5 mM $pCMBS^-$ in the perfusion bath on the subsequent GABA-induced currents. Because $pCMBS^-$ reacts with the ionized thiolate form of cysteine ($S^-$) 1000 times faster than with the unionized thiol (SH)[26] and because only cysteines on the water-accessible surface will ionize to any significant extent we infer that reactive cysteines are, at least transiently, on the water-accessible protein surface. (For a more extensive discussion of the assumptions of cysteine accessibility studies see[7].)

When applied in the resting state of the receptor, in the absence of GABA, $pCMBS^-$ only altered the subsequent GABA-induced currents of two mutants near the extracellular end of M3, $\alpha_1$A291C and $\alpha_1$Y294C (Fig. 3A)[24]. In contrast, when $pCMBS^-$ was applied in the presence of GABA five more cysteine-substitution mutants became reactive, $\alpha_1$F296C, $\alpha_1$F298C, $\alpha_1$A300C, $\alpha_1$L301C and $\alpha_1$E303C (Fig. 3A and B). Thus, we infer that as the membrane-spanning segments undergo GABA-induced conformational changes water-filled crevices extend from the extracellular surface into the interior of the protein. Although it was traditionally thought that water is excluded from the interior of proteins considerable evidence now indicates that cavities in the interior of proteins are water-filled and that water molecules in cavities exchange with bulk water on a nanosecond time scale[27]. In addition, there is evidence of water movement into the bacteriorhodopsin membrane-spanning domain as it undergoes light driven conformational changes during proton pumping[28,29]. Thus, the movement of water into crevices and cavities that form in the membrane-spanning domain during $GABA_A$ receptor gating may facilitate the conformational changes by preventing the formation of “vacuums” that nature abhors. This may also explain how anesthetics, by preferentially filling these crevices/cavities, may stabilize receptor conformations other than the resting state thereby increasing channel opening.

Mutation of the M3 segment residue $\alpha_1$Ala291 and the M2 segment residue $\alpha_1$Ser270 to larger amino acids reduces the efficacy of volatile general anesthetics and alcohols to




| B | -GABA | +GABA | diazepam | Pot. propofol |
|---|---|---|---|---|
| Ala291 | - | - | - | - |
| Tyr294 | - | - | - | - |
| Phe296 |  | - | + |  |
| Val297 |  |  |  | + |
| Phe298 |  | + | + |  |
| Ala300 |  | + |  |  |
| Leu301 |  | + | + |  |
| Ile302 |  |  |  | - |
| Glu303 |  | - |  |  |
| Phe304 |  |  |  | + |

*Fig. 3: Accessibility of $\alpha_1$ M3 segment cysteine substitution mutants serves as a reporter for receptor conformation. (**A**) $\alpha$ helical net plot of the residues in and flanking the M3 segment. Black triangles indicate positions where pCMBS$^-$ reacted with substituted cysteines when applied in the presence of GABA. When applied in the absence of GABA, pCMBS$^-$ only reacted with the two most extracellular positions. (Modified from Williams and Akabas, 1999 with permission) (**B**) Table summarizes positions at which pCMBS$^-$ reacted with the corresponding substituted cysteine mutant when applied in the presence of the reagent at the top of the column. Blank spaces indicate no effect of pCMBS$^-$ application; (-) indicates inhibition of the subsequent GABA-induced currents and (+) indicates potentiation of the subsequent GABA-induced currents.*

potentiate GABA-induced currents[30,31]. $\alpha_1$Ser270 is predicted to lie on the non-channel-lining side of the M2 segment $\alpha$ helix (Fig. 1A) and would therefore be expected to face into the interior of the protein. It, however, like $\alpha_1$Ala291, is on the water-accessible surface in the absence and in the presence of GABA[24] (Bali and Akabas, unpublished results). Thus, $\alpha_1$Ser270 is likely to face into the water-filled crevice that we infer to exist between the membrane-spanning segments. Covalent modification of $\alpha_2$S270C$\beta_1$ receptors by addition of a thiopropane moiety was reported to potentiate the subsequent GABA responses[32]. There are several possible mechanisms by which mutation of these residues might alter general anesthetic efficacy. One, they might be part of the anesthetic binding site either as contact residues or in maintaining the structure of the binding site. Two, the anesthetic binding site may be deeper in the membrane-spanning domain and these residues may form part of the access pathway to the anesthetic binding site. Substituting larger amino acids may effectively close the door into the binding site or obstruct binding. Three, these residues may be part of the transduction machinery between a distant anesthetic binding site and the channel gating mechanism. Further experiments will help to distinguish between these possibilities.

## Diazepam and Propofol Stabilize Different Conformational States of the Receptor

Both diazepam and propofol potentiate currents induced by submaximal GABA concentrations[1,3,33]. We have used the state-dependent accessibility of M3 substituted cysteine residues as a reporter for the conformational changes induced by potentiating concentrations of diazepam and propofol. When applied in the presence of diazepam, $pCMBS^-$ reacted with $\alpha_1$A291C, $\alpha_1$Y294C, $\alpha_1$F296C, $\alpha_1$F298C, and $\alpha_1$L301C (Fig. 3B)[25]. This suggests that although diazepam binds in the extracellular domain at the $\alpha$-$\gamma$ subunit interface it induces a conformational change in the membrane-spanning domain that has similarities to the conformational change induced by GABA but it does not appear to be as extensive. Furthermore, at position 296 modification of the substituted cysteine in the presence of GABA caused inhibition of the subsequent currents whereas modification in the presence of diazepam caused potentiation of the subsequent currents[25,34]. Experiments sequentially applying $pCMBS^-$ in the presence of diazepam and GABA indicate that in the presence of diazepam $pCMBS^-$ only reacts with the engineered cysteine on one of the two $\alpha_1$ subunits[34]. This suggests that diazepam binding may only alter the conformation of one of the two $\alpha_1$ subunits in each receptor, presumably the $\alpha$ subunit that forms part of the diazepam binding site. Based on single channel kinetic experiments and models, others have concluded that diazepam binding only increases the on-rate of the first GABA molecule suggesting that it may only influence the structure of one of the two GABA binding sites[35,36]. Thus, our experiments may provide structural evidence consistent with the kinetic results.

A distinct subset of the $\alpha_1$ M3 segment cysteine-substitution mutants react with $pCMBS^-$ applied with a potentiating concentration of propofol (Williams and Akabas, manuscript in preparation). The reactive mutants include $\alpha_1$A291C, $\alpha_1$Y294C, $\alpha_1$V297C, $\alpha_1$I302C

and $\alpha_1$F304C (Fig. 3B). 2,6 di-tert-butyl phenol is a non-anesthetic compound of similar structure and hydrophobicity to propofol[37]. $\alpha_1$V297C, $\alpha_1$I302C and $\alpha_1$F304C did not react with pCMBS$^-$ in the presence of this compound. This implies that the effect of propofol on the accessibility of M3 segment cysteine mutants may be related to the anesthetic effects of propofol not simply to its hydrophobicity. Our cysteine accessibility studies provides structural evidence that the propofol-bound state is structurally distinct from the resting, GABA-activated and diazepam-bound states. Consistent with this hypothesis, experiments on the effects of propofol on $GABA_A$ receptor kinetics concluded that propofol stabilized a doubly liganded pre-open state[38]. Thus, the distinct subsets of M3 cysteine residues that pCMBS$^-$ reacts with in the resting state and in the presence of GABA, diazepam and potentiating concentrations of propofol implies that these three compounds stabilize different states of the receptor or different ensembles of states. Further understanding of the structural differences between these states will provide new insights into the processes of signal transduction by $GABA_A$ receptors and its modulation by anesthetics.

## Conclusions

Our studies of the structure and conformational changes occurring in the $GABA_A$ receptor membrane-spanning domain have demonstrated that the transmembrane channel is mainly lined by residues from the M2 segment. The M2 segment secondary structure is largely $\alpha$ helical[8,9]. We have identified the face of the M2 segment that lines the channel and the location of the picrotoxin and $Zn^{2+}$ inhibitory binding sites near the cytoplasmic and extracellular end of the M2 segment respectively[11,14]. Identification of these sites has provided distance constraints on the channel diameter (~9 Å) and the maximum separation of the $\alpha$ carbons on adjacent M2 segments (13 Å). Disulfide crosslinking experiments have led us to hypothesize that channel gating involves an asymmetric rotation of the M2 segment of the subunit that does not form the GABA binding sites relative to its neighboring subunits[16]. In the M3 segment, cysteine accessibility studies have demonstrated that a water-filled crevice extends from the extracellular surface into the interior of the membrane-spanning domain[24]. GABA binding increases the water-accessibility of the M3 segment and we infer that the water-filled crevice extends deeper into the protein interior in the presence of GABA. We suggest that general anesthetics may preferentially fill these GABA-induced crevices/cavities thereby stabilizing activated states of the receptor. Finally, the reaction of pCMBS$^-$ with distinct subsets of M3 segment substituted cysteine residues in the resting state and in the GABA-, diazepam- and potentiating propofol-bound states implies that these ligands stabilize distinct receptor conformations or ensembles of conformations[25,34]. These studies provide a foundation upon which to investigate and understand the structural bases of the functional properties and pharmacological modulation of the $GABA_A$ receptors and related neurotransmitter-gated ion channels.

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