A TRANSMEMBRANE AMINO ACID IN THE GABA<sub>A</sub> RECEPTOR β<sub>2</sub> SUBUNIT CRITICAL FOR THE ACTIONS OF ALCOHOLS AND ANESTHETICS

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ABBREVIATIONS:

GABA: $\gamma$-aminobutyric acid
TM: transmembrane
OMTS: octyl methanethiosulfonate
pCMBS$^-$: $p$-chloromercuribenzenesulfonate
PMTS: propyl methanethiosulfonate

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ABSTRACT

Alcohols and inhaled anesthetics enhance the function of GABA<sub>A</sub> receptors containing α, β, and γ subunits. Molecular analysis has focused on the role of the α subunits, however there is evidence that the β subunits may also be important. The goal of our study was to determine whether N265, which is homologous to the site implicated in the α subunit (S270), contributes to an alcohol and volatile anesthetic binding site in the GABA<sub>A</sub> receptor β<sub>2</sub> subunit. We substituted cysteine for N265 and exposed the mutant to the sulfhydryl-specific reagent octyl methanethiosulfonate (OMTS). We used two-electrode voltage clamp electrophysiology in Xenopus oocytes and found that, following OMTS application, GABA-induced currents were irreversibly potentiated in mutant α<sub>1</sub>β<sub>2</sub>(N265C)γ<sub>2S</sub> receptors (but not α<sub>1</sub>β<sub>2</sub>(I264C)γ<sub>2S</sub>), presumably due to the covalent linking of octanethiol to the thiol group in the substituted cysteine. Notably, this effect was blocked when OMTS was applied in the presence of octanol. We found that potentiation by butanol, octanol, or isoflurane in the N265C mutant was nearly abolished following the application of OMTS, suggesting that an alcohol and volatile anesthetic binding site at position 265 of the β<sub>2</sub> subunit was irreversibly occupied by octanethiol and consequently prevented butanol or isoflurane from binding and producing their effects. OMTS did not affect modulation or direct activation by pentobarbital, but there was a partial reduction of allosteric modulation by flunitrazepam and alphaxalone in mutant α<sub>1</sub>β<sub>2</sub>(N265C)γ<sub>2S</sub> receptors after OMTS was applied. Our findings provide evidence that N265 may contribute to an alcohol and anesthetic binding site.
INTRODUCTION

Recent investigations of the specific sites of action for alcohols and anesthetics have focused on the role of target proteins in the central nervous system, including the γ-aminobutyric acid type A (GABA\(_A\)) receptor. Functional GABA\(_A\) receptors are composed of five homologous subunits positioned around a central chloride channel (Hevers and Luddens, 1998), with the GABA binding site situated at the α-β interface (Newell and Czajkowski, 2003). In brain, the predominant subunit stoichiometry of GABA\(_A\) receptors is 2α, 2β, and 1γ (Tretter et al., 1997; Sieghart and Sperk, 2002), however there is a subpopulation of extrasynaptic receptors in which certain α and β subunits co-assemble with the δ subunit (Olsen & Sieghart, 2008). The structure of each subunit consists of an extracellular N-terminal domain, a transmembrane domain with four alpha helical segments (TM1, TM2, TM3, and TM4), an intracellular loop between TM3 and TM4, and an extracellular C-terminal domain (Ortells and Lunt, 1995). TM2 amino acid residues from each subunit presumably contribute to the ion pore of the chloride channel (Xu and Akabas, 1996; Miyazawa et al., 2003).

Alcohols and volatile anesthetics positively modulate GABA\(_A\) receptor function (Lovinger, 1997; Harris, 1999; Franks, 2008), and recent studies have identified alcohol-binding sites in this receptor (Mihic et al., 1997; Mascia et al., 2000; Jung et al., 2005; Perkins et al., 2009). Specifically, studies using point mutations and heterologous expression systems provide evidence that homologous TM2 positions in the GABA\(_A\) receptor α\(_1\), α\(_2\), and β\(_1\) subunits and the glycine receptor α\(_1\) subunit are important for the actions of alcohols and
anesthetics. For example, Mihic et al. (1997) found that mutant α1(S270I) β1, α2(S270I) β1, and α1β1(S265I) GABA_A receptors and α1(S267I) glycine receptors were resistant to enhancement by ethanol or enflurane. Similarly, Ueno et al. (1999) reported that the potentiating effects of ethanol were abolished in mutant α2(S270I)β1γ2L and α2β1(S265I)γ2L receptors, and Jenkins et al. (2001) found that S270 of the GABA_A receptor α subunits contributes to a common site of action for the inhalation anesthetics chloroform, halothane, and isoflurane. It has been further proposed that these residues, along with residues from the other TM domains, contribute to a water-filled, intrasubunit binding pocket (Yamakura et al., 2001; Trudell & Bertaccini, 2004; McCracken et al., 2010). In addition, residues in the γ and δ subunits may also contribute to alcohol binding sites (Perkins et al. 2009; Olsen and Sieghart, 2009), however currently, the role of these subunits is not well defined.

Although recent studies have alternatively proposed the presence of a general anesthetic binding pocket at the GABA_A receptor α-β interface (Li et al., 2010), it has been shown that the 265 position of the GABA_A receptor β subunit is necessary for the actions of the injectable anesthetic etomidate (Belelli et al., 1997), and two knock-in mice, β2(N265S) and β3(N265M), have been constructed to investigate the behavioral effects of injectable anesthetics in vivo (Jurd et al., 2003; Reynolds et al., 2003; Sanchis-Segura et al., 2007). In contrast to these studies of injectable anesthetics, the role of the GABA_A receptor β2 subunit in alcohol and inhaled anesthetic action has not been as extensively studied.
Therefore, the aim of the present study was to investigate whether N265 in the GABA<sub>A</sub> receptor β<sub>2</sub> subunit, which is homologous to the implicated sites in TM2 of the α<sub>1</sub>, α<sub>2</sub>, and β<sub>1</sub> subunits, is important for alcohol and anesthetic action. Figure 1 shows the relative positions of β<sub>2</sub>(I264, 14') and β<sub>2</sub>(N265, 15') residues in TM2 of α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> GABA<sub>A</sub> receptors, based on the X-ray structure of the prokaryotic proton-gated ion channel GLIC (Bocquet et al., 2009). Specifically, we asked whether the individual mutation of this amino acid residue to cysteine affected receptor sensitivity to alcohols and if octyl methanethiosulfonate (OMTS), an alcohol/anesthetic analog, covalently reacted with the substituted cysteine and irreversibly altered GABA-induced currents. Propyl methanethiosulfonate (PMTS) covalently reacts with the substituted cysteine in mutant α<sub>2</sub>(S270C)β<sub>1</sub> receptors and irreversibly potentiates GABA-induced currents, mimicking the effects of alcohol and anesthetics (Mascia et al., 2000). We hypothesized that if the substituted cysteine in the homologous position of the β<sub>2</sub> subunit contributes to an alcohol and anesthetic binding site, then it would covalently react with OMTS, resulting in the irreversible attachment of octyl thiol to the cysteine thiol group. With the octyl thiol covalently bound at position 265, it was expected that GABA-induced current would be irreversibly potentiated and subsequently applied alcohols or anesthetics would be unable to bind and modulate receptor function.
METHODS

Materials

γ-Aminobutyric acid was obtained from Research Biomedicals, Int., and ethanol, 1-butanol, 1-octanol, alphaxalone, chloroform, flunitrazepam, and sodium pentobarbital were purchased from Sigma (St. Louis, MO). OMTS was obtained from Toronto Research Chemicals Inc. (New York, Toronto, Canada), isoflurane was purchased from Marsam Pharmaceuticals Inc. (Cherry Hill, NJ) and etomidate from Tocris (Evansville, MO).

Mutagenesis and Transcription

Mutations were achieved using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Complementary primers containing the desired mutations were designed and polymerase chain reactions (PCR) were performed. Subsequently, Dpn I was used to digest the parental DNA and TOP10/P3 competent cells were transformed with the PCR products. The plasmids of interest were obtained through minipreps and were sent for sequencing to verify the presence of the desired mutations. cRNAs were synthesized using the wild-type and mutant cDNA as a template. Specifically, pCDM8 plasmid encoding human GABA<sub>A</sub> receptor β<sub>2</sub> subunit and pGEMHE plasmids encoding rat α<sub>1</sub> or γ<sub>2S</sub> GABA<sub>A</sub> receptor subunits were employed to synthesize the cRNAs using T7 RNA polymerase (mMESSAGE mMACHINE T7 Kit, Ambion, Austin, TX).
Oocyte Isolation and Injection

Adult female *Xenopus laevis* frogs were obtained from Xenopus Express (Plant City, FL), and portions of ovary were surgically extracted. Mature oocytes were manually isolated, treated in collagenase (type IA, 0.5 mg/ml), and subsequently injected into the cytoplasm with 30 nl of diethyl pyrocarbonated-treated water and cRNAs (3 ng/subunit/30 nl) encoding wild-type $\alpha_1$, either wild-type or mutant $\beta_2$, and wild-type $\gamma_{2S}$ subunit combinations in a 1:1:1 ratio. Injected oocytes were incubated at 13º C in sterile modified Barth’s solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 19 mM HEPES, 0.82 mM MgSO$_4$, 0.33 mM Ca(NO$_3$)$_2$, 0.91 mM CaCl$_2$, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, 220 mg/liter sodium pyruvate, pH 7.5) for 3-4 days.

Electrophysiological Recording

Two-electrode voltage clamp electrophysiology was used to measure GABA-induced currents from oocytes 3-4 days following injection with cRNAs. Each oocyte was placed in a rectangular chamber (~ 100 $\mu$l) and continuously perfused at a rate of 2 ml/min with ND96 buffer (96 mM NaCl, 1 mM CaCl$_2$, 2 mM KCl, 1 mM MgCl$_2$, 5 mM HEPES, pH 7.5) at room temperature (23ºC). Two glass electrodes containing 3 M KCl were used to achieve a -70 mV holding potential using a Warner Instrument oocyte clamp (Hamden, CT). All solutions were prepared immediately before use and applied by bath perfusion.
Concentration Response Curves. Increasing concentrations of GABA were applied to oocytes from each condition for 20-30 s, followed by a 5-15 min washout. From the resulting concentration response curves (CRCs), the concentrations eliciting the half maximal response (EC$_{50}$) were calculated for the wild-type and the mutants tested.

Effect of Alcohols and Anesthetics on GABA-induced Current. The GABA EC$_5$ GABA (the concentration that produced 5% of the maximal response) was determined for each oocyte after application of the maximal GABA concentration and served as the test GABA concentration. All test GABA applications were 30 s and were followed by a 5 min washout. The test GABA concentration was applied twice, and the modulator was then preapplied for 1 min, coapplied with GABA for 30 s, and followed by a 5 min washout. The test GABA concentration was applied again, and the percent potentiation of the GABA-induced current by the modulator was calculated for each oocyte. This procedure was used to test 200 mM ethanol, 11 mM, 22 mM and 32 mM butanol as well as 57 µM, 114 µM, and 171 µM octanol. The potentiation of EC$_5$ GABA-induced current by 1 µM etomidate and 2 mM chloroform was also measured using this protocol.

Effect of OMTS on GABA-induced Current. Two consecutive applications of the test GABA concentration were administered, with the second application representing the initial response. 50 µM OMTS was applied alone for 1 min and was followed by a 5 min washout. Subsequently, three applications of the test
GABA concentration were applied and currents were measured as percentages of the initial response. To test the effect of OMTS when the receptor was in the open/desensitized state, the same protocol was followed, with the difference that 50 μM OMTS was coapplied with maximal GABA, and the washout time immediate afterward was extended to 15 min. Additionally, we tested the ability of 114 μM octanol or 0.4 μM flunitrazepam to block the effects of OMTS on the N265C mutant using the protocol described above with the exception that octanol or flunitrazepam was pre-applied for 1 min alone and then coapplied with 50 μM OMTS.

**Effect of Allosteric Modulators Pre- and Post- OMTS Application.** Oocytes received two applications of the test GABA concentration, followed by a 30 s coapplication of the test GABA concentration and the modulator. Following a 5 min washout, the test GABA concentration was applied alone and the potentiation of GABA-induced current by the modulator was calculated. After a 1 min application of 50 μM OMTS and a 5 min washout, the maximal GABA concentration was reapplied and the test concentration was recalculated. The potentiation by the modulator was then measured again as described above. This protocol was used to test 114 μM octanol, 0.6 mM isoflurane, 0.4 μM flunitrazepam, 3 μM alphaxalone, and 50 μM pentobarbital. However, 11 mM butanol and 0.6 mM isoflurane were applied alone for 1 min prior to being coapplied with the test GABA concentration.
Direct Activation by Etomidate and Pentobarbital. The maximal GABA concentration was applied for 20 s and was followed by a 15 min washout. Etomidate (10 μM) was then applied for 1 min, and the resulting current was measured as a percentage of the maximal response. The same procedure was used to measure the direct activation after the application of 50 μM OMTS. Similarly, direct activation by 300 μM pentobarbital was also measured.

Modeling
The relative positions of β2(I264, 14') and β2(N265, 15') residues in α1β2γ2S GABA receptors were modeled using the best available template for the ion pore of the Cys-loop superfamily of receptors; the prokaryotic proton-gated ion channel GLIC (PDB ID 3EAM) (Bocquet et al., 2009). In that the structure of GLIC now provides the best information about the likely orientation and position of residues in TM2 (Nury and Corringer, 2010), the model of GABA β2 residues was made by simply substituting the two corresponding residues in GLIC with I264 and N265. The positions of the side chains were then optimized automatically with the side chain refinement module of Discovery Studio 2.5.5 using the CHARMM force field (Accelrys Inc, San Diego, CA).
RESULTS

The goal of our study was to determine whether N265 (15’) contributes to an alcohol and anesthetic binding site in the GABA_\textsubscript{A} receptor β\textsubscript{2} subunit. We substituted cysteine for the candidate amino acid and exposed the cysteine mutant to sulphydryl-specific reagents. Additionally, we constructed a second mutant in which cysteine was substituted at the neighboring residue, I264. Two tryptophan mutants, I264W and T266W, were previously constructed by Nishikawa et al. (2002), and the effects of volatile anesthetics were tested. Although submaximal GABA currents were significantly potentiated by both halothane and isoflurane in the mutant tryptophan receptors, the effects were small in the I264W compared to the wild-type. Therefore, we included the I264C mutant (14’) in our study to rule out the possibility that I264 also participates in alcohol and volatile anesthetic binding.

The relative positions of β\textsubscript{2}(I264, 14’) and β\textsubscript{2}(N265, 15’) residues in α\textsubscript{1}β\textsubscript{2}γ\textsubscript{2S} GABA\textsubscript{A} receptors based are shown in a model based on the prokaryotic proton-gated ion channel GLIC (Bocquet et al., 2009) (Figure 1). Although less is known about the structure of GABA\textsubscript{A}R subunits, compared to nAChR, there is a consensus that residues in TM2 at positions 13’ and 17’ face into the ion pore. Since the C-alpha atoms of an alpha helix rotate approximately 100 degrees clockwise with respect to the long axis of the helix; it is reasonable that I264 (14’) should face towards TM1 of the same subunit and N265 (15’) should face into the center of the subunit, but slightly toward TM3. We should note that Figure 1 depicts the alpha helices as a ribbon that traces the helical backbone carbons.
This representation provides a clear depiction of I264 and N265, but is somewhat incomplete because, when all the side chains are rendered as space-filling surfaces, the entire TM domain is essentially filled with atoms, except for spaces in the center of each four-helical bundle and spaces at the interfaces between subunits.

The GABA concentration-response curves show that the β2(I264C) and β2(N265C) mutations both produced an increase in apparent affinity for GABA (Figure 2). This increase was larger for the I264C mutation than N265C; the GABA EC₅₀ values were 1.5 μM for α₁β₂(I264C)γ₂S and 17 μM for α₁β₂(N265C)γ₂S vs. 36 μM for the wild-type. In each of the cysteine mutants tested, there was also a slight decrease in the maximal current elicited by saturating concentrations of GABA (5337 nA for α₁β₂(I264C)γ₂S and 6114 nA for α₁β₂(N265C)γ₂S vs. 7361 for the wild-type).

We next examined whether the β2(I264C) or β2(N265C) point mutations had an effect on alcohol modulation of GABA-induced currents. The β2(N265C) mutation eliminated the enhancement of GABA-induced current by 200 mM ethanol and instead, inhibition was produced (-14 ± 3% vs. 51 ± 5% in wild-type) (data not shown). Additionally, the potentiation of GABA-induced current by butanol was significantly reduced in mutant α₁β₂(N265C)γ₂S receptors compared to the wild-type at all concentrations tested (Figure 3A and B). Similarly, potentiation by octanol was markedly reduced in mutant α₁β₂(N265C)γ₂S receptors (Figure 3C). In contrast to the β2(N265C) mutation, the β2(I264C)
mutation did not significantly alter receptor modulation by any of the alcohols tested (Figure 3B and C).

Next, we tested the $\beta_2(I264C)$ and $\beta_2(N265C)$ mutants in order to determine whether the substituted cysteines reacted with an MTS reagent consequently altered receptor function. We found that following a 1 min application of 50 $\mu$M OMTS, the GABA-induced currents of mutant $\alpha_1\beta_2(N265C)\gamma_2$ receptors were irreversibly enhanced (Figure 4A and B), suggesting that OMTS reacted with the substituted cysteine and resulted in the covalent attachment of octanethiol to the cysteine thiol group. The GABA responses of the $\beta_2(I264C)$ mutant, like the wild-type, were not altered following OMTS application (Figure 4B). Moreover, there was no effect of OMTS on the $\beta_2(N265C)$ mutant when it was applied in the presence of 114 $\mu$M octanol (Figure 4C), suggesting a competitive interaction between octanol and OMTS at the 265 site. However, 0.4 $\mu$M flunitrazepam, which presumably acts at a distinct site from 265, did not block the reaction of OMTS with the substituted cysteine (Figure 4D).

In order to address the possibility that I264 is accessible only when the receptor is in the activated or desensitized state, we measured $E_{C5}$ GABA responses before and after applying 50 $\mu$M OMTS in the presence of maximal GABA. These results were similar to our findings when OMTS was applied while the receptor was in the resting state, and again we observed an irreversible enhancement of the GABA responses of the $\beta_2(N265C)$ mutant, but no change in the GABA responses of the $\beta_2(I264C)$ mutant or the wild-type (data not shown).
PMTS, a smaller sulfhydryl-specific reagent, was also tested on the I264C and N265C mutants in the resting and activated/desensitized states. The potentiating effects on the N265C mutant were similar to those observed with OMTS, though not as robust, and there was no effect on the I264C mutant or the wild-type (data not shown). Because the β2(I264C) mutation did not affect receptor modulation by alcohols, and MTS reagents had no apparent effect on the function of these mutant receptors, we focused on the α1β2(N265C)γ2S mutant in the remainder of our experiments.

Subsequently, we examined whether the N265C mutation or the application of OMTS altered allosteric modulation. Notably, all of the compounds tested (11 mM butanol, 0.4 μM flunitrazepam, 0.6 mM isoflurane, 50 μM pentobarbital, and 3 μM alphaxalone) produced less potentiation of GABA-induced current in α1β2(N265C)γ2S receptors compared to the wild-type prior to the application of OMTS. Nevertheless, the potentiation was still measurable in all cases. In wild-type receptors, OMTS did not alter the potentiation of GABA responses by any of the modulators tested (Figure 5A). In mutant α1β2(N265C)γ2S receptors, the application of OMTS abolished the effects of octanol and isoflurane, and the potentiation by flunitrazepam and alphaxalone was reduced (Figure 5B). However, OMTS did not significantly affect mutant receptor modulation by pentobarbital (Figure 5B).

The effects of the cysteine mutation on direct channel activation by 10 μM etomidate and 300 μM pentobarbital were also measured (Table 1). Activation by etomidate was significantly less in mutant α1β2(N265C)γ2S receptors
compared to the wild-type. However, activation by pentobarbital did not differ significantly from the wild-type, and this effect was not altered following the application of OMTS.
DISCUSSION

Our results from cysteine mutagenesis and the sulfhydryl-specific reagent OMTS support the role of N265 in the binding of alcohols and volatile anesthetics. Although the residue at position 265 (15') of the β2 subunit is presumably facing away from the channel, the charged sulfhydryl-specific reagent p-chloromercuribenzencesulfonate (pCMBS−) has previously been shown to covalently react when cysteine is substituted at this site, indicating that the residue at position 265 of the β2 subunit is water accessible (Bali et al., 2004). Accordingly, we observed an irreversible enhancement of submaximal GABA-induced current in mutant α1β2(N265C)γ2S receptors following OMTS application in both the open and closed states. This suggests that the substituted cysteine was water accessible and covalently reacted with OMTS, resulting in the irreversible attachment of octanethiol to the cysteine thiol group. Our finding is also consistent with previous work showing that the position homologous to GABA A β2(N265) in the α1 subunit of the glycine receptor (S267) is accessible to OMTS (Lobo et al., 2004). Additional studies on glycine receptors and GABA A receptors have also shown that MTS reagents, such as propyl MTS or hexyl MTS, produce similar effects on receptor function when cysteine is substituted at important sites for alcohol and volatile anesthetic action (Mascia et al., 2000; Jung et al., 2005; Crawford et al., 2007). Notably in our study, the ability of octanol, but not flunitrazepam, to block the effects of OMTS in the β2(N265C) mutant is consistent with a competitive interaction and provides compelling evidence that N265 participates in an alcohol and volatile anesthetic binding
pocket. Knock-in mice containing a $\beta_2$(N265S) mutation have been constructed and are resistant to the sedative effects of etomidate, but the effects of alcohols or volatile anesthetics have not been tested in these animals (Reynolds et al., 2003). In addition, $\beta_3$(N265M) mice have also been constructed, however this subunit does not appear to have a major role in the effects of ethanol or isoflurane (Liao et al., 2005; Sanchis-Segura et al., 2007).

The effects of the I264C and N265C mutations on alcohol and anesthetic modulation further suggest that N265 is a critical site for the effects of these drugs. Previous studies have shown that the ability of alcohols to modulate receptor function is dependent upon specific amino acids located in TM2 of the glycine receptor and GABA$_A$ receptor subunits (Mihic et al., 1997; Wick et al., 1998), and amino acid volume has been hypothesized to be an important factor governing alcohol and anesthetic enhancement of GABA$_A$ and glycine receptors (Ye et al., 1998; Jenkins et al., 2001; Nishikawa et al., 2002). Although cysteine and asparagine differ in volume by only about 5 Å$^3$, we found that the single point mutation in $\alpha_1\beta_2$(N265C)$\gamma_2S$ receptors dramatically reduced alcohol and anesthetic potentiation of GABA-induced currents, despite causing only a modest change in receptor sensitivity to GABA. The N265C mutation did, however, produce a change in polarity such that a nonpolar amino acid was substituted for an uncharged polar amino acid. In contrast, isoleucine and cysteine are both nonpolar amino acids but differ in volume by about 35 Å$^3$, and the substitution of cysteine for isoleucine at position 264 in our study had a greater effect on receptor sensitivity to GABA than the N265C mutation. However, despite the
previous finding that volatile anesthetic potentiation is reduced in mutant 
$\alpha_1\beta_2(I264W)\gamma_{2S}$ receptors expressed in HEK 293 cells (Nishikawa et al. 2002), the 
I264C mutation in our study did not significantly affect alcohol or volatile 
anesthetic modulation of mutant $\alpha_1\beta_2(I264C)\gamma_{2S}$ receptors. Because alcohol or 
anesthetic potentiation of GABA-induced current was not affected by the I264C 
mutation, and there were not apparent functional effects of OMTS on mutant 
$\alpha_1\beta_2(I264C)\gamma_{2S}$ receptors, we propose that I264 is not critical for alcohol and 
anesthetic action on the GABA$_A$ receptor. As depicted in Figure 1, it is possible 
that I264 is buried between adjacent TM2 alpha helices and TM1 of its own 
subunit. As a result, it may not be available for interaction with alcohols or 
reaction with MTS reagents. Therefore, we focused exclusively on N265 in the 
remainder of our experiments.

Additional evidence that N265 is a critical site of action for alcohols and 
volatile anesthetics is provided by our experiments examining the effects of 
OMTS on allosteric modulation. Notably, we found that butanol, octanol, and 
isoflurane potentiation were reduced by the cysteine mutation alone and were 
nearly abolished following the application of OMTS. We propose that an alcohol 
and volatile anesthetic binding site at position 265 of the $\beta_2$ subunit was 
irreversibly occupied by the covalent attachment of octanethiol to N265C, and 
consequently prevented butanol, octanol, or isoflurane from binding and further 
enhancing receptor function. Our finding is in accordance with previous studies 
on other GABA$_A$ receptor subunits, glycine receptors, and nicotinic acetylcholine 
receptors suggesting that the addition of an alkylthiol group to a substituted
cysteine at an alcohol and volatile anesthetic binding site causes the site to no longer be accessible to alcohols and anesthetics (Mascia et al., 2000; Borghese et al. 2003; Crawford et al., 2007). Direct activation of the N265C mutant by pentobarbital did not differ from the wild-type and was not altered after OMTS was applied. These findings are supported by previous work proposing that the pre-TM1 region of the β2 subunit contributes to a pentobarbital-mediated activation pathway (Mercado & Czajkowski, 2008).

Interestingly, allosteric modulation of GABA<sub>A</sub> receptor function was less in mutant α1β2(N265C)γ2S receptors compared to the wild-type for all of the modulators tested prior to the application of OMTS. Additionally, the modulation produced by both flunitrazepam and alphaxalone was reduced in the N265C mutant following the application of OMTS. Because there is considerable evidence that modulators such as benzodiazepines and neurosteroids bind to sites distinct from N265 on the GABA<sub>A</sub> receptor (Teissére et al., 2001; Hosie et al., 2006), we have considered the alternative hypothesis that our results are entirely a consequence of altered channel gating due to the allosteric effects of a cysteine mutation at position 265. Although this possibility cannot be completely eliminated based on the current study, our finding that octanol, but not flunitrazepam, blocks the effects of OMTS in the β2(N265C) mutant provides strong evidence for a competitive interaction at position 265. Therefore, we favor the hypothesis that N265 is a critical site for alcohol and volatile anesthetic binding. Based on previous work implicating the α subunit as a site of alcohol and volatile anesthetic action and by our finding that N265 of the β2 subunit also
appears to contribute to an alcohol/anesthetic binding site, we speculate that water-filled cavities need to be present in both the $\alpha$ and $\beta$ subunits in order for alcohols or volatile anesthetics to bind and produce their effects.

In summary, we conclude that N265 of the GABA$_A$ receptor $\beta_2$ subunit contributes to an intrasubunit alcohol and volatile anesthetic binding site.
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**LEGENDS FOR FIGURES**

**Figure 1.** The five subunits that make up the TM domain of an $\alpha_1\beta_2\gamma_2S$ GABA<sub>A</sub> receptor are rendered as ribbons structures that trace the alpha helical backbone. For clarity, the ligand-binding and intracellular domains are not shown. The view is from the extracellular surface, directly down the chloride ion pore. In one subunit, residues I264 and N265 are rendered with space-filling surfaces. The model was built by replacing the corresponding residues in the prokaryotic proton-gated ion channel GLIC, as described in Methods.

**Figure 2.** GABA concentration response curves for wild-type $\alpha_1\beta_2\gamma_2S$ and mutant GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. All values are presented as mean ± SEM from 4-5 oocytes.

**Figure 3.** The $\beta_2$(N265C) point mutation reduced alcohol modulation of GABA<sub>A</sub> receptor function. A) Representative tracings showing the potentiation of EC<sub>50</sub> GABA-induced current by 11 mM butanol in wild-type $\alpha_1\beta_2\gamma_2S$ and mutant $\alpha_1\beta_2$(N265C)$\gamma_2S$ GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. B) Wild-type and mutant $\alpha_1\beta_2$(I264C)$\gamma_2S$ receptors were potentiated by butanol (11 mM, 22 mM, and 32 mM) in a concentration-dependent manner, unlike mutant $\alpha_1\beta_2$(N265C)$\gamma_2S$ receptors which were significantly less sensitive to the potentiating effects of butanol compared to the wild-type at all concentrations tested. C) The enhancement of GABA<sub>A</sub> receptor function by octanol was also less in the $\alpha_1\beta_2$(N265C)$\gamma_2S$ mutant than the wild-type, and this effect was
statistically significant at 114 μM and 171 μM octanol concentrations. Octanol modulation of mutant α₁β₂(I264C)γ₂S receptors did not differ significantly from the wild-type. All values are presented as mean ± SEM from 4-5 oocytes. *p < 0.05, **p < 0.01, ***p < 0.001 denotes values significantly different from the wild-type as tested by two-way ANOVA with Bonferroni’s post-test.

**Figure 4.** Effect of OMTS on the EC₅ GABA responses of wild-type and mutant GABAₐ receptors expressed in *Xenopus* oocytes. **A)** Representative tracings showing EC₅ GABA-induced current from wild-type α₁β₂γ₂S and mutant α₁β₂(N265C)γ₂S GABAₐ receptors before and after 1min application of 50 μM OMTS. **B)** OMTS irreversibly enhanced the GABA responses of mutant α₁β₂(N265C)γ₂S GABAₐ receptors. The GABA responses of wild-type and mutant α₁β₂(I264C)γ₂S GABAₐ receptors were not affected by OMTS. **C)** The effect of 50 μM OMTS on the α₁β₂(N265C)γ₂S mutant was blocked in the presence of 114 μM octanol. **D)** The GABA responses of α₁β₂(N265C)γ₂S receptors were irreversibly enhanced in the presence of 0.4 μM flunitrazepam. All values are presented as mean ± SEM from 3-6 oocytes. * p < 0.05 denotes values significantly different from the pre-OMTS condition as tested by one-way ANOVA with Bonferroni’s post-test.

**Figure 5.** Allosteric modulation of wild-type α₁β₂γ₂S and mutant α₁β₂(N265C)γ₂S GABAₐ receptors expressed in *Xenopus* oocytes before and after the application of 50 μM OMTS. **A)** In wild-type α₁β₂γ₂S receptors, there was no change in the
potentiation of EC$_5$ GABA responses by 114 $\mu$M octanol, 0.4 $\mu$M flunitrazepam, 0.6 mM isoflurane, 50 $\mu$M pentobarbital, or 3 $\mu$M alphaxalone following a 1 min application of OMTS. B) In mutant $\alpha_1\beta_2(\text{N265C})\gamma_2s$ receptors, OMTS abolished the effects of 11 mM butanol, 114 $\mu$M octanol, and 0.6 mM isoflurane. The potentiating effects of 0.4 $\mu$M flunitrazepam and 3 $\mu$M alphaxalone were also reduced after OMTS was applied. However, OMTS did not effect the enhancement of EC$_5$ GABA responses by 50 $\mu$M pentobarbital. All values are presented as mean $\pm$ SEM from 4-7 oocytes. * p < 0.05, ** p < 0.01 denotes values significantly different from the pre-OMTS condition as tested by Student’s t-test.
### TABLES

**Table 1**

Direct activation of wild-type $\alpha_1\beta_2\gamma_2S$ and mutant $\alpha_1\beta_2(N265C)\gamma_2S$ GABA$_A$ receptors expressed in *Xenopus* oocytes. All values represent percentages of the maximal GABA response and are given as the mean ± SEM from 4-5 oocytes.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_1\beta_2\gamma_2S$</th>
<th>$\alpha_1\beta_2(N265C)\gamma_2S$ Post-OMTS</th>
<th>$\alpha_1\beta_2\gamma_2S$ Post-OMTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µM etomidate</td>
<td>10 ± 2</td>
<td>9 ± 1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>300 µM pentobarbital</td>
<td>18 ± 1</td>
<td>16 ± 2</td>
<td>19 ± 4</td>
</tr>
</tbody>
</table>

*Significant effect of the $\beta_2(N265C)$ mutation, p < 0.001 comparing $\alpha_1\beta_2(N265C)\gamma_2S$ to the wild-type before OMTS application.*
Figure 3

A

\[ \alpha_1\beta_2\gamma_{2S} \]

GABA (EC\textsubscript{5})
11 mM Butanol

\[ 100 \text{nA} \]
30 s

\[ \alpha_1\beta_2(N265C)\gamma_{2S} \]

GABA (EC\textsubscript{5})
11 mM Butanol

\[ 100 \text{nA} \]
30 s

B

\[ \alpha_1\beta_2(1264C)\gamma_{2S} \]

\[ \alpha_1\beta_2(N265C)\gamma_{2S} \]

\[ \alpha_1\beta_2\gamma_{2S} \]

\% Potentiation

\begin{align*}
\text{Butanol (mM)} & : 10, 20, 30 \\
\text{% Potentiation} & : 100, 200, 300
\end{align*}

C

\[ \alpha_1\beta_2(1264C)\gamma_{2S} \]

\[ \alpha_1\beta_2(N265C)\gamma_{2S} \]

\[ \alpha_1\beta_2\gamma_{2S} \]

\% Potentiation

\begin{align*}
\text{Octanol (\text{\mu M})} & : 50, 100, 150 \\
\text{% Potentiation} & : 100, 200, 300
\end{align*}