Neuropharmacology 105 (2016) 207–214

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Functional sites involved in modulation of the $GABA_A$ receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital

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ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form
15 December 2015
Accepted 3 January 2016
Available online 6 January 2016

Keywords:
$GABA_A$ receptors
Chloride channels
Electrophysiology
Xenopus oocyte
Anesthetics
Barbiturates

ABSTRACT

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the brain and are the target for many clinically important drugs. Among the many modulatory compounds are also the intravenous anesthetics propofol and etomidate, and barbiturates. The mechanism of receptor modulation by these compounds is of mayor relevance. The site of action of these compounds has been located to subunit interfaces in the intra-membrane region of the receptor. In $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors there are five such interfaces, two $\beta+/\alpha-$ and one each of $\alpha+/\beta-$, $\alpha+/\gamma-$ and $\gamma+/\beta-$ subunit interfaces. We have used reporter mutations located in the second trans-membrane region in different subunits to probe the effects of changes at these subunit interfaces on modulation by propofol, etomidate and pentobarbital. We provide evidence for the fact that each of these compounds either modulates through a different set of subunit interfaces or through the same set of subunit interfaces to a different degree. As a $GABA_A$ receptor pentamer harbors two $\beta+/\alpha-$ subunit interfaces, we used concatenated receptors to dissect the contribution of individual interfaces and show that only one of these interfaces is important for receptor modulation by etomidate.

1. Introduction

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian central nervous system. Numerous subunits have been cloned (for review see: Barnard et al., 1998; Macdonald and Olsen, 1994; Olsen and Sieghart, 2008; Rabow et al., 1995; Sigel and Steinmann, 2012), indicating that numerous receptor isoforms exist (Olsen and Sieghart, 2008). The subunits show homology to subunits of other Cys-loop receptors (Betz, 1990; Miller and Smart, 2010). Many $GABA_A$ receptors are heteromeric protein complexes consisting of five subunits, which are arranged around a central Cl^--selective channel (Macdonald and Olsen, 1994). The major receptor isoform of the $GABA_A$ receptor in the brain consists of α_1, β_2 and γ_2 subunits (Barnard et al., 1998; Macdonald and Olsen, 1994; Olsen and Sieghart, 2008; Rabow et al., 1995; Sigel and Steinmann, 2012). Different approaches have indicated a $2\alpha:2\beta:1\gamma$ subunit stoichiometry for this receptor (Baumann et al., 2001; Chang et al., 1996; Farrar et al., 1999; Tretter et al., 1997) with a subunit arrangement $\gamma\beta\alpha\beta\alpha$ anti-clockwise as seen from the synaptic cleft (Baumann et al., 2001, 2002; Baur et al., 2006). The pharmacological properties of a receptor depend on subunit composition (Sigel et al., 1990) as well as their arrangement (Minier and Sigel, 2004). $GABA_A$ receptors do not exclusively locate to synapses. Some receptor subtypes, among them the ones containing the δ subunit, have been found in extra-synaptic regions where they mediate tonic inhibition (Farrant and Nusser, 2005).

The $GABA_A$ receptor is the target of many clinically used and experimental drugs (for review see Sieghart, 1995; Sieghart and Ernst, 2005; Middendorp et al., 2014), including benzodiazepines, barbiturates, volatile anesthetics, and the intravenous anesthetics propofol and etomidate. There is good evidence that the intravenous anesthetics act near the extracellular end of the membrane-spanning domain (M) of various subunits. Amino acid residues located in the non-channel lining face of the βM2, and in αM1, βM1, βM3 and γM3 have been proposed to form part of the binding site(s) (Bali and Akabas, 2004; Chiara et al., 2013; Jayakar et al., 2014; Richardson et al., 2007; Yip et al., 2013).

Co-crystallization of homomeric *Caenorhabditis elegans* GluCl receptors with the allosteric modulator ivermectin revealed that the latter binds at each of the five interfaces in the transmembrane

Abbreviations: GABA, gamma-aminobutyric acid; $GABA_A$ receptor, gamma-aminobutyric acid type A receptor.

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http://dx.doi.org/10.1016/j.neuropharm.2016.01.003
0028-3908/

domain of the receptor. S260 (M2-15') located within M2 forms a hydrogen bond with ivermectin (Hibbs and Gouaux, 2011). The homologous position in β subunits (β_2N265) of $GABA_A$ receptors is also important for modulation by anesthetics (Belelli et al., 1997; Jurd et al., 2003; Stewart et al., 2014). A docking analysis performed with etomidate in the $GABA_A$ receptor placed this compound in a binding pocket homologous to the ivermectin-pocket in the GluCl receptor (Chiara et al., 2012). In $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors there exist in principle 5 different interfaces that harbor the corresponding pocket.

β_2N265 (βM2-15') was first identified as a determinant of the modulatory action of loreclezole (Wingrove et al., 1994). Later work has shown that this residue, located in the β+/α− interface is crucial for modulation by etomidate and propofol. Many point mutations of this residue abolished or reduced sensitivity to etomidate, among them β_3N265M, β_2N265S, β_2N265C, β_2N265M (Belelli et al., 1997; Fernandez et al., 2012; Siegwart et al., 2002; Stewart et al., 2014; Wingrove et al., 1994). In β_3N265M mutant mice its anesthetic action is impaired (Jurd et al., 2003). However, direct photo-labeling of this residue has never been observed. Moreover, propofol and etomidate failed to protect β_2N265C from covalent modification from sulfhydryl-reactive reagents (Bali and Akabas, 2004; Li et al., 2009; Stewart et al., 2014). Thus, it is not clear if the β_2N265 residue has a direct role in binding, or in allosteric effects (Stewart et al., 2014). Modulation by the barbiturate pentobarbital is not affected by the β_2N265C mutation (McCracken et al., 2010), although in a β_3N265M knock-in mouse model some of the anesthetic responses towards this agent were reduced (Zeller et al., 2007). The influence of this residue in barbiturate action remains uncertain (McCracken et al., 2010).

In an attempt to further characterize the site of action of these compounds, photo-affinity labeling has been used. Photo-reactive etomidate analogs identified two equivalent anesthetic binding sites in the trans-membrane domain in the two β+/α− interfaces, which also contain the GABA binding sites in the extracellular domain. A second class of anesthetic binding site has been labeled using a photo-reactive derivative of barbiturates, at the α+/β− and γ+/β− subunit interfaces. In $\alpha_1\beta_3\gamma_2$ and $\alpha_1\beta_3$ receptor types, propofol produced a concentration-dependent inhibition of photolabeling by etomidate and barbiturate analogs >90%. Propofol bound with similar affinities to β+/α− sites and α+/β−/γ+/β− sites, with an IC_{50} of ~40 μM in $\alpha_1\beta_3\gamma_2$ receptors. Since this concentration is nearly 10-fold higher than the concentrations of propofol necessary to potentiate the receptor, the authors suggested that propofol may not only bind to the aforementioned sites, but additionally to other unidentified sites (Chiara et al., 2013). Barbiturates failed to inhibit with high affinity etomidate binding, and etomidate does not inhibit the labeling by the barbiturate derivative (Chiara et al., 2013; Jayakar et al., 2014). Thus, the authors propose that occupation of any of these sites is enough for anesthetic action. Given the structural diversity of intravenous anesthetics it is also unlikely that all anesthetics bind to a common class of pockets (Chiara et al., 2012).

Use of photo-affinity labeling is a direct method for the identification of amino acid residues located in or close to drug binding sites through an irreversible covalent reaction, although the lack of labeling does not provide evidence that a certain amino acid residue is not involved in drug interaction (Yip et al., 2013). Successful photo-affinity labeling alone also can not provide information on whether the corresponding site mediates weak or strong positive allosteric properties or antagonist properties. In any case functional data using low concentrations of the compounds are required.

Here we performed a detailed study of the functional consequences of the β_2N265I point mutation, and homologous mutations in other subunits (α_1S269I, γ_2S280I). All these mutations are located at subunit interfaces. Earlier work has shown that combined mutation of the three residues eliminated the low affinity potentiation by diazepam in mutated $\alpha_1\beta_2\gamma_2$ receptors (Walters et al., 2000). Our results indicate multiple sites of action all located at subunit interfaces for low concentrations of etomidate, propofol and pentobarbital. These sites mediate channel potentation to a different degree. Using subunit concatenation, we demonstrate that the two β+/α− subunit interfaces differentially affect modulation by etomidate.

2. Methods

2.1. Construction of mutated receptor subunits

The point mutations α_1S269I, β_2N265I and γ_2S280I were prepared using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

2.2. Construction of concatenated subunits

Construction of tandem and triple subunit cDNAs. The tandem construct γ_2-β_2, and triple construct α_1-β_2-α_1 has been described previously (Boulineau et al., 2005). Site-directed mutagenesis of β_2N265 to I was done in the tandem construct and the triple construct using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

2.3. Expression of $GABA_A$ receptors in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C.

Xenopus laevis oocytes were prepared, injected and defolliculated as described previously (Sigel, 1987; Sigel and Minier, 2005; Animal research permit by the Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE98/12)). They were injected with 50 nL of the cRNA solution containing wild type or mutated rat α_1, β_2 and γ_2 subunits of the $GABA_A$ receptors at a concentration of 10 nM:10 nM:50 nM (Boileau et al., 2002). For concatenated tandem and triple constructs, cRNA combinations ratios of 25: 25 nM were used. Injected oocytes were incubated in modified Barth's solution at 18 °C for at least 24 h before the measurements.

2.4. Functional characterization of the $GABA_A$ receptors

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. The holding potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4). Concentration response curves for GABA were fitted with the equation $I(c) = I_{max}/$

$[1 + (EC_{50}/c)^n]$, where c is the concentration of GABA, EC_{50} the concentration of GABA eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration–respose curves. For all receptors studied, modulation was measured at a GABA concentration eliciting 0.5–1.5% of the maximal GABA current amplitude. GABA was applied two times for 20 s alone, and 45 s in combination with the different compounds. The duration of washout periods was 4 min in between agonist or agonist/drug aplications to prevent receptor desentization. GABA application were done repeatedly when the eliceted current amplitude altered by >5%. The relative currents in presence of an modulator were expressed as potentiation calculated by the following equation: $(I_{Modulator + GABA}/I_{GABA} - 1) * 100\%$. The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination. All media contained a final concentration of 0.5% DMSO (v/v) to ensure drug solubility.

The concentrations of propofol and etomidate were chosen as 3 μM and 1 μM, respectively. These concentrations are both well below the respective EC_{50}s for potentiation of the GABA response. Potentiation by pentobarbital was small under those conditions. Therefore, a concentration of 50 μM was chosen which is slightly above this EC_{50}.

2.5. Statistical analysis

All data are from as a minimum of five different oocytes from at least two different batches of oocytes, except the experiments with concatenated subunits were performed with three oocytes. Data represent mean ± SEM or mean ± SD as indicated in each case. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by a Tukey *post hoc* test.

3. Results

3.1. Functional characteristics of wild-type and mutated $GABA_A$ $\alpha_1\beta_2\gamma_2$ receptors

Combined mutation in M2 of three subunits of $\alpha_1\beta_2\gamma_2$ receptors, α_1S269I, β_2N265I and γ_2S280I, has been shown to abolish low affinity effects mediated by diazepam (Walters et al., 2000). We used these mutations here and call them α_1M, β_2M and γ_2M in the following. These mutations are homologous to each other. Fig. 1 shows a scheme of the localization of the mutations at the different subunit interfaces. We studied here this triply mutated receptor and receptors containing the individual mutations. Wild-type, $\alpha_1\beta_2$, α_1M$\beta_2\gamma_2$, $\alpha_1\beta_2$Mγ_2, $\alpha_1\beta_2\gamma_2$M and α_1Mβ_2Mγ_2M were expressed in *Xenopus* oocytes, and using electrophysiological techniques we first investigated the GABA sensitivity of these receptors. In α_1M$\beta_2\gamma_2$ receptors the α+/β− and α+/γ− subunit interfaces are affected. In $\alpha_1\beta_2$Mγ_2 the two β+/α− subunit interfaces are affected. In $\alpha_1\beta_2\gamma_2$M only one subunit interface is affected, namely γ+/β−. Full concentration–response curves for GABA were determined in all receptor types (Fig. 2) and the normalized data were fitted as described in the methods section. Table 1 shows the parameters obtained from each receptor type.

Oocytes expressing α_1M$\beta_2\gamma_2$ and α_1Mβ_2Mγ_2M receptors, showed a leftward shift in the GABA concentration response curves compared to that obtained for wild-type receptors, by about one order of magnitude. Hence these mutations rendered these receptors more sensitive to opening by GABA. In contrast, the $\alpha_1\beta_2$Mγ_2 mutant receptors presented a slightly higher EC_{50} as compared to wild-type receptors. *X.* oocytes expressing the $\alpha_1\beta_2\gamma_2$M receptor, produced GABA activated currents with a GABA

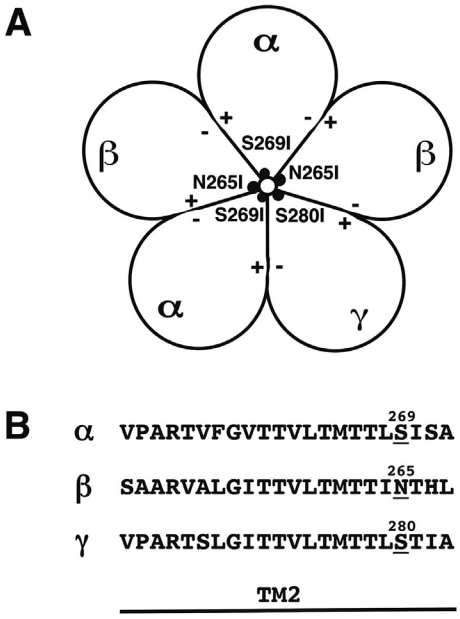

Fig. 1. Localization of mutations at different subunit interfaces. **A**, the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor is composed of two α, two β and a γ subunit(s). Two GABA binding sites are located at the two β+/α− subunit interfaces. The scheme shows the localization of the point mutations; α_1S269I, β_2N265I, and γ_2S280I, studied in this paper. + and − indicate the plus sides and the minus sides of the subunits. **B**, sequence alignment of the transmembrane domains M2 of α_1, β_2, and γ_2 subunits. The underlined residues were each mutated to Ile.

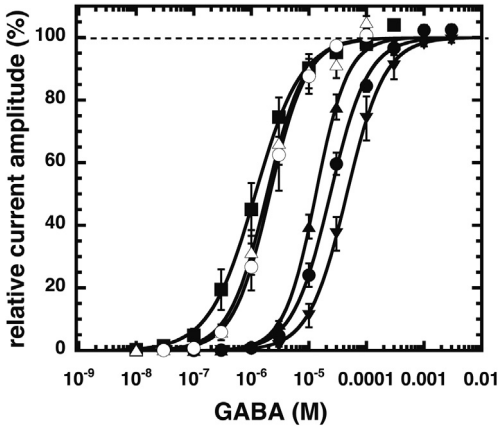

Fig. 2. GABA dose response curve of wild type and mutated receptors. Concentration-response curves in oocytes expressing wild-type $GABA_A$ $\alpha_1\beta_2\gamma_2$ (close circle) receptors, $\alpha_1\beta_2$ receptors (open triangle), and the mutated receptors α_1M$\beta_2\gamma_2$ (open circles), $\alpha_1\beta_2$Mγ_2 (inverted triangles), $\alpha_1\beta_2\gamma_2$M (triangles) and the triple mutated receptor α_1Mβ_2Mγ_2M (closed squares). GABA currents were normalized and fitted by a Hill equation. Data averaged from at least 3 dose response curves, mean ± SEM are shown.

EC_{50} comparable to that of wild-type receptors. As the α_1M mutation made the receptor more sensitive to GABA we tested it for spontaneous channel opening. The picrotoxin-sensitive current was small and amounted to only 0.8 ± 0.3% of maximal GABA elicited currents (mean ± SD, n = 3), indicating a rather low probability of spontaneous opening.

3.2. Asymmetry in channel modulation

In order to determine the effect of these point mutations on the potentiation of $GABA_A$ receptor by the anesthetics propofol and

Table 1
GABA concentration–response curves of wild-type $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2$ and mutated receptors.

GABA receptor	EC_{50} (μM)	Hill	I_{max} (μA)
$\alpha_1\beta_2\gamma_2$ (WT)	25.6 ± 7.4	1.2 ± 0.2	7.5 ± 2.2
α_1(S270I)β_2(N265I)γ_2(S280I)	1.4 ± 0.9	1.2 ± 0.9	10.3 ± 1.3
α_1(S270I)$\beta_2\gamma_2$	2.2 ± 0.5	1.2 ± 0.2	3.8 ± 0.4
$\alpha_1\beta_2$(N265I)γ_2	50 ± 19	1.4 ± 0.2	9.1 ± 1.8
$\alpha_1\beta_2\gamma_2$(S280I)	14.5 ± 7.0	1.7 ± 0.2	6.9 ± 0.5
$\alpha_1\beta_2$	2.2 ± 0.6	1.4 ± 0.1	7.5 ± 1.5

EC_{50}, Hill coefficient, and I max are given as mean ± SEM (n = 4–6). Data from two injections each.

etomidate, and by the barbiturate pentobarbital, wild-type and mutated receptors were expressed in *X. oocytes* and the effect of GABA responses was examined, using a concentration of GABA eliciting 0.5–1.5% of the maximal current amplitude. Fig. 3 shows original traces and Fig. 4 summarizes the results.

As shown in Fig. 4, the mutation in the α_1 subunit did not significantly affect the degree of potentiation by 3 μM propofol observed in wild-type receptors. Modulation by propofol was strongly impaired in the $\alpha_1\beta_2M\gamma_2$ receptor. Potentiation of the GABA response by propofol in wild-type receptors amounted to 1292 ± 200% (mean ± SEM, n = 5) and was reduced to 32 ± 15% (mean ± SEM, n = 5) in this latter receptor subtype. Moreover, potentiation in $\alpha_1\beta_2\gamma_2M$ receptors was strongly decreased, indicating that propofol may act at the γ+/β− subunit interface. In $\alpha_1\beta_2$ receptors potentiation at GABA $EC_{0.5\text{-}1.5}$ amounted to 1443 ± 364% (mean ± SD, n = 3), which is similar as in $\alpha_1\beta_2\gamma_2$ receptors.

In $\alpha_1M\beta_2\gamma_2$ receptors potentiation by 1 μM etomidate amounted to 974 ± 112% and did not significantly differ from the potentiation observed in wild-type receptors, which amounted to 1011 ± 47% (mean ± SEM, n = 5). Potentiation of the GABA response by etomidate in $\alpha_1\beta_2M\gamma_2$ and in $\alpha_1M\beta_2M\gamma_2M$ receptors was totally abolished. In the $\alpha_1\beta_2\gamma_2M$ receptor type, potentiation was significantly enhanced as compared to wild-type receptors, amounting to 1746 ± 93% (mean ± SEM, n = 5). Thus, etomidate may also act at the γ+/β− subunit interface. Potentiation in $\alpha_1\beta_2$ receptors was

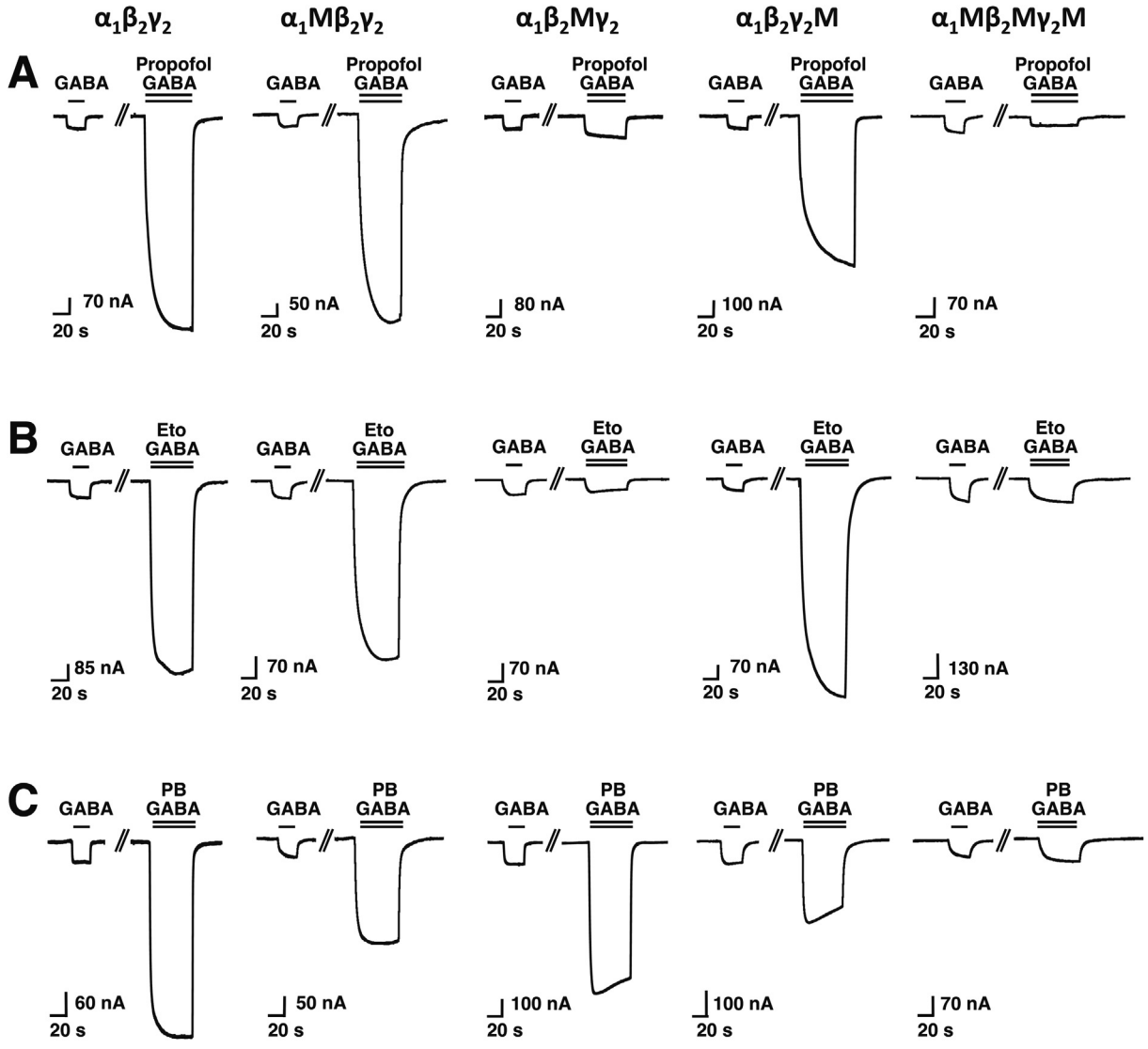

Fig. 3. Potentiation of the GABA response by propofol, etomidate and pentobarbital. Original current traces showing potentiation of GABA current responses by **A** 3 μM propofol, **B** 1 μM etomidate, and **C** 50 μM pentobarbital, in wild-type receptors and the mutated receptors $\alpha_1M\beta_2\gamma_2$, $\alpha_1\beta_2M\gamma_2$, $\alpha_1\beta_2\gamma_2M$, $\alpha_1M\beta_2M\gamma_2M$. Currents were activated several times with a concentration of GABA eliciting 0.5–1.5% of the maximal current amplitude in the corresponding receptor until a constant response was observed, the last application is shown. Subsequently drug was applied in combination with GABA. Horizontal bars represent 20 s of GABA application and 45 s of combined GABA plus drug application.

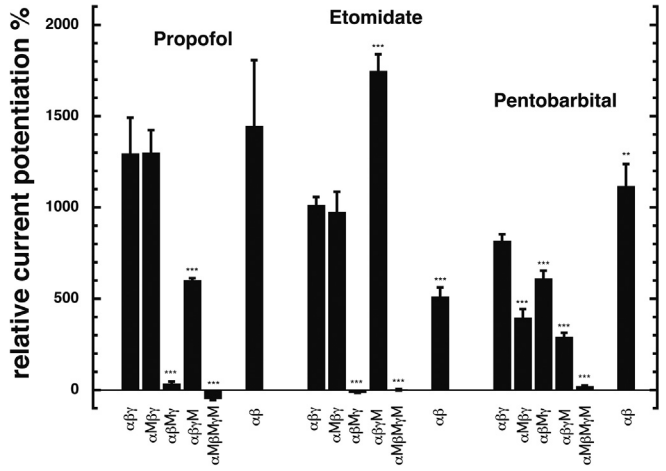

Fig. 4. Potentiation of the GABA response by propofol, etomidate and pentobarbital. Modulation by propofol, etomidate, and pentobarbital in wild type $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2$, single mutant and triple mutant receptors. The bars indicate mean ± SEM, n = 4–5. ***, $p < 0.0001$; **, $p < 0.001$.

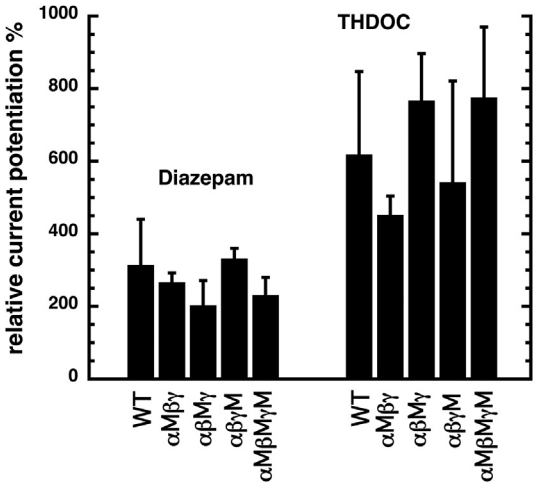

Fig. 5. Effect of the mutations on the potentiation by diazepam and THDOC. After a reproducible response to GABA was obtained, currents were potentiated by 1 μM diazepam and 1 μM THDOC. The bars indicate mean ± SD, n = 3. Modulation by both diazepam and THDOC did not differ significantly between wild type and any mutated receptor.

510 ± 52% (mean ± SD, n = 3), significantly lower than in $\alpha_1\beta_2\gamma_2$ receptors.

Modulation of both propofol and etomidate is abolished by the mutation in the β_2 subunit, while the mutation in the α_1 subunit does not modify the response. Additionally the γ_2 mutation had different consequences for each; potentiation by propofol was reduced, whereas that by etomidate was increased. Thus, the site at the γ+/β− subunit interface has different effects on potentiation by these two compounds.

Potentiation by 50 μM of the barbiturate pentobarbital was considerably reduced in all mutated receptors subtypes (Fig. 4), thus displaying a different pattern from etomidate and propofol. Potentiation by pentobarbital in wild-type receptors amounted to 816 ± 37% (mean ± SEM, n = 5) and was significantly reduced to 17 ± 8% (mean ± SEM, n = 5) in the $\alpha_1M\beta_2M\gamma_2M$ receptor. For the individual mutated receptors stimulated with pentobarbital potentiation amounted to 395 ± 49% in $\alpha_1M\beta_2\gamma_2$, 608 ± 46% in $\alpha_1\beta_2M\gamma_2$ and to 289 ± 24% in $\alpha_1\beta_2\gamma_2M$ receptor subtypes (mean ± SEM, n = 5), all being statistically different from the value of potentiation obtained in wild-type receptors. In $\alpha_1\beta_2$ receptors potentiation was increased compared to wild type receptors and amounted to 1114 ± 124% (mean ± SEM, n = 4).

The above data seem to suggest the presence of modulatory sites for barbiturates at least at one of the β+/α− subunit interfaces, at one of the α+/β− or α+/γ− subunit interfaces and at the γ+/β− subunit interface, respectively.

To establish that these mutations do not interfere with potentiation by all possible compounds, we tested whether 1 μM THDOC and 1 μM diazepam were sensitive towards them. Fig. 5 shows that, single as well as combined mutations did not affect significantly potentiation by these compounds at the concentrations tested.

3.3. The two β+/α− subunit interfaces have different properties

Each $GABA_A$ receptor contains two β_2 subunits in different positions (Fig. 6), both at β+/α− subunit interfaces. As shown above, combined mutation in these subunits has been shown to greatly reduce the modulatory effects of propofol and etomidate. We wanted to study at the functional level the properties of individual mutations, in order to uncover the effects of both subunit interfaces. We called the mutation in the β_2 subunit next to the γ_2 subunit interface 1 M, and the mutation in the β_2 subunit located

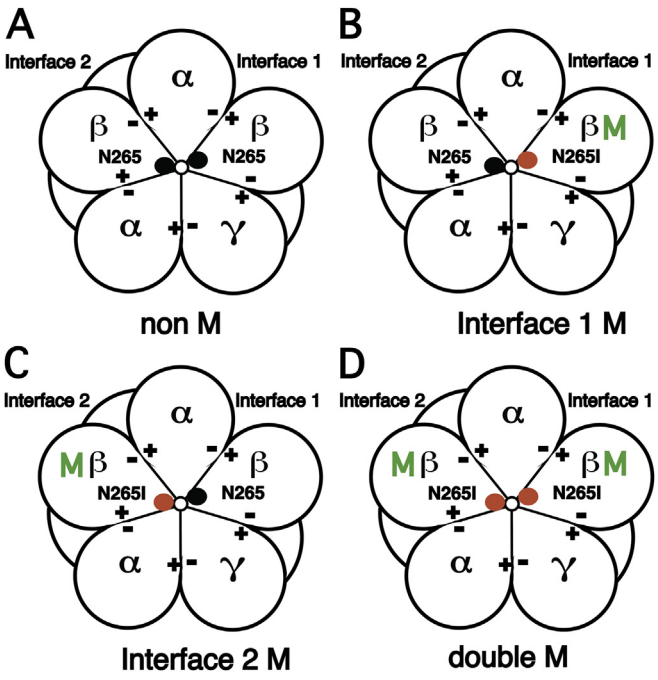

Fig. 6. Positional effect of the mutation in the β_2 subunit. With the help of subunit concatenation, receptors were prepared containing either no mutation (**A**), a mutation at interface 1 (**B**), a mutation at interface 2 (**C**), or in both sites (**D**). Interface 2 has previously been shown to display a binding site for GABA with higher apparent affinity for channel gating than that located at the interface 1 (Baumann et al., 2003).

between the α_1 subunits interface 2 M. Using subunit concatenation, we introduced the β_2M mutation selectively in either Site 1 or Site 2. Double and triple concatenated subunits were built and expressed in *X. oocytes* to form the receptors (Fig. 6). The α_1-β_2M-α_1 or γ_2-β_2M constructs were co-expressed with corresponding non mutated dual or triple subunit constructs (α_1-β_2M-α_1/γ_2-β_2 and α_1-β_2-α_1/γ_2-β_2M), or were expressed together to form the double mutant (α_1-β_2M-α_1/γ_2-β_2M). Wild-type concatenated receptors α_1-β_2-α_1/γ_2-β_2 were also expressed. This construct has been previously

characterized by an EC_{50} value for GABA of approximately 120 μM (Baumann et al., 2003). Thus, we were able to study the influence of the position of the β_2M mutation in the receptor pentamer on modulation by etomidate, propofol and pentobarbital. In non-mutated concatenated $GABA_A$ receptors, propofol, etomidate, and pentobarbital potentiated currents induced by GABA to a similar extent as in non-concatenated wild-type receptors (Fig. 7).

As shown in Fig. 7, in interface 1 and interface 2 mutated receptors, the modulation by propofol was reduced significantly compared to wild-type receptors (806 ± 41%), to a similar degree (247 ± 102% and 294 ± 85%, mean ± SD n = 3, respectively, and was highly reduced in the double mutant, amounting to 24 ± 4% (mean ± SD, n = 3). Hence both binding sites seem to participate similarly in the modulation by propofol. In contrast, modulation by etomidate was sensitive to the mutation of both sites to a different degree. In the interface 2 mutant receptor the potentiation was reduced from 761 ± 41% in wild type receptors to 498 ± 62% (mean ± SD, n = 3), while the potentiation in the interface 1 mutated receptor was more strongly affected with 129 ± 19% residual potentiation (mean ± SD, n = 3). In the double mutant potentiation was completely abolished. In this case, although both subunits are necessary for the full modulatory effect of etomidate, the contribution of interface 1 is more important than the other.

We also tested pentobarbital modulation in all concatenated receptors. In non-mutated receptors, pentobarbital potentiated currents induced by GABA to a comparable degree as non-concatenated wild-type receptors, 831 ± 180% (mean ± SD, n = 3). In the double mutant, potentiation was reduced to 440 ± 30% (mean ± SD, n = 3). Potentiation in interface 1 mutated receptor was 604 ± 118% (mean ± SD, n = 3) and 449 ± 141% (mean ± SD, n = 3) in interface 2 mutated receptors. Thus, only mutation at interface 2 reduces potentiation.

4. Discussion

In this study we investigated how modification of subunit interfaces affects potentiation of $GABA_A$ receptor by intravenous anesthetics. A detailed study of the functional properties of receptors carrying the β_2N265I point mutation, and homologous mutations in other subunits (α_1S269I, γ_2S280I) was performed. All these mutations are located at subunit interfaces. The individual contribution of the two different β+/α− subunit interfaces was also dissected.

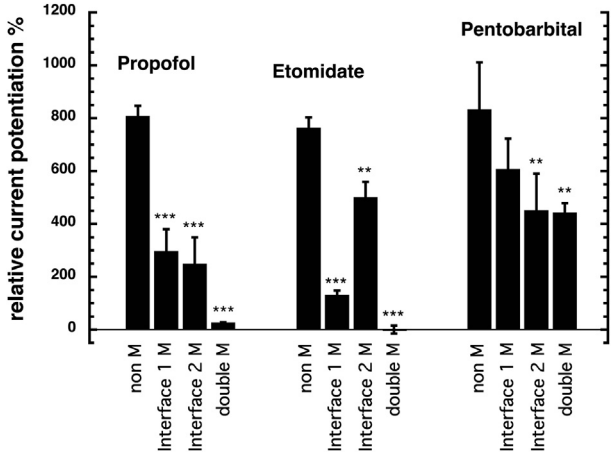

Fig. 7. Individual roles of the two β_2M residues in channel modulation. Potentiation by 3 μM propofol, 1 μM etomidate, or 50 μM pentobarbital, using a concentration of GABA eliciting 0.5–1.5% of the maximal current amplitude in the corresponding concatenated receptor subtype. The bars indicate mean ± SD, n = 3. ***p < 0.0001; **, p < 0.001. Mutation at interface 1 is nearly enough to abolish modulation by etomidate, while mutation at interface 2 has almost no consequences. In contrast, modulation by propofol is similarly sensitive to both mutations.

If point mutation of a residue decreases modulation by a compound it is not clear if the mutated residue is required for binding of the compounds or is involved in the allosteric transduction required for modulation. Inhibition of photo-affinity labeling is similarly ambiguous. In any case, we assume here that if a mutation located at a subunit interface affects the functional response, this indicates an action at that interface. If the mutation fails to affect the functional response, this residue could still be in contact with the compound or act at the same subunit interface, but could act as antagonist.

Earlier work on the location of anesthetic sites has included photo-affinity labeling experiments. This has the advantage of identification of single amino acid residues involved in the interaction, but the disadvantage of largely ignoring functional aspects and making use of modified anesthetic compounds that may orient in a different way from unmodified compounds. Competition with unmodified compounds has been interpreted as an overlap with the reacted compound, but may also reflect an allosteric interaction.

Amino acid residues located at the following subunit interfaces have been identified by direct labeling with a reactive compound. Alternatively, a low affinity site of action of an anesthetic has putatively been identified by competition with labeling. High affinity sites for propofol and etomidate (Li et al., 2006; Yip et al., 2013) and low affinity sites for barbiturates have been found at the β+/α− subunit interfaces (Chiara et al., 2013; Jayakar et al., 2014). It should be pointed out here that there are two β+/α− subunit interfaces which have not been differentiated in the above work. High affinity sites for photo-reactive barbiturate derivatives have been found at the α+/β− and γ+/β− subunit interfaces (Chiara et al., 2013; Jayakar et al., 2014). A site for propofol has been located to α+/β− in $\alpha_1\beta_3$ receptors (Jayakar et al., 2014). Displacement of labeling by a barbiturate derivative by etomidate at the γ+/β− interface has been attributed to an allosteric effect (Jayakar et al., 2015). No site has been reported for the α+/γ− subunit interface.

Here, we first determined how these mutations affect gating by GABA. For this, we expressed the mutant receptors in *X. oocytes*. As previously demonstrated, the triply mutated receptor showed a decrease in the EC_{50} for GABA as compared to wild type receptors (Walters et al., 2000), which has later been attributed to the mutation in the α_1 subunit (Nishikawa et al., 2002; Middendorp et al., 2015; this work). Thus, introduction of the point mutation α_1S269I into $\alpha_1\beta_2\gamma_2$ receptors led to a similar decrease. Earlier mutation studies of this residue have similarly found such a decrease in receptors carrying mutations to histidine, tryptophane, or isoleucine (Scheller and Forman, 2001; Nishikawa et al., 2002). A negative correlation between the EC_{50} value for GABA and the molecular size of the amino acids in this position was observed (Nishikawa et al., 2002). Similar to other studies using the point mutations β_2N265S (Fernandez et al., 2012), β_2N265I (Nishikawa et al., 2002), and β_2N265M (Siegwart et al., 2003), we found that mutation of β_2N265I led to a marginally higher EC_{50} as compared wild-type receptors. The γ_2S280I mutation that has never been studied before fails to significantly affect gating by GABA.

Subsequently, we examined the effect of these point mutations located at subunit interfaces on the potentiation by etomidate, propofol, and pentobarbital. Combined mutation at all interfaces nearly abolished potentiation by all compounds studied here and also by SJM-3 described earlier (Middendorp et al., 2015). Similarly, it has been reported that the low affinity site for benzodiazepines is abolished in receptors carrying these combined mutations (Walters et al., 2000). However, this triple mutant did not affect the

potentiation by low concentrations of diazepam (1 μM) and by 1 μM of the neurosteroid THDOC (Fig. 5). At low concentration, diazepam interacts with a very specific modulatory site that is located extracellularly at the α+/γ− subunit interface (Sigel and Buhr, 1997; Sigel and Lüscher, 2011). Published observations are in agreement with our findings. First, THDOC failed to inhibit labeling of an etomidate photoanalog in $GABA_A$ receptors (Li et al., 2009, 2010) and second, the β_2N265 residue is not implicated in the modulation of the receptor by neurosteroids (Belelli and Lambert, 2005).

We also investigated the effect on potentiation by anesthetics in receptors carrying the single mutations. We separately discuss in the following the findings made in α_1S269I$\beta_2\gamma_2$, $\alpha_1\beta_2$N265Iγ_2 and $\alpha_1\beta_2\gamma_2$S280I receptors.

α_1S269I$\beta_2\gamma_2$ receptors showed a reduced potentiation by pentobarbital, while that by propofol and etomidate remained unaffected. In contrast, potentiation by volatile anesthetics was highly reduced in receptors carrying different point mutations of α_1S269 (Nishikawa et al., 2002). Each $GABA_A$ receptor pentamer contains two α+ subunit interfaces that could contribute to the modulatory effect (Fig. 1). Our results do not differentiate between α+/β− or α+/γ− interfaces in barbiturate action. As stated above, photo-labeling with barbiturate derivatives also indicated involvement of the α+/β− interface.

In receptors containing the β_2N265I mutations potentiation by etomidate and propofol was nearly abolished and that by pentobarbital reduced. As stated above, photo-labeling data indicated sites for etomidate and propofol and low affinity sites for pentobarbital at β+/α− interfaces (Li et al., 2010; Jayakar et al., 2014; Li et al., 2006; Yip et al., 2013). We used subunit concatenation to alter selectively one of the two interfaces. Concatenated receptors without mutation, or containing a single β_2N265I subunit in both positions (Fig. 6), or doubly mutated receptors were assembled. As expected, in doubly mutated receptors, potentiation by etomidate and propofol was nearly abolished. Pentobarbital modulation in the double mutant was reduced to about half of the potentiation observed in non-mutated receptors. Potentiation by propofol was reduced to the similar extent in both mutated receptors, indicating that both subunit interfaces participate to a similar degree in the effect of this anesthetic. Potentiation by etomidate, was affected by alteration at both subunit interfaces to a different extent. Mutation at interface 1 nearly abolished potentiation while mutation at interface 2 only led to a small reduction. Thus, contribution of interface 1 to the modulatory effect was more dominant than that by interface 2.

Our observation is surprising as earlier work with our concatenated receptors carrying the α_1M236W point mutation has led to the conclusion that the two sites for etomidate at β+/α− interfaces contribute equally to modulation and that no cooperative interaction occurs between the two sites (Guitchounts et al., 2012). Obviously, a mutation located in α_1M1 leads to different consequences than a mutation in β_2M2. The reason for this is not known.

Introduction of the γ_2S280I mutation located at the γ+/β− subunit interface altered potentiation by all studied compounds. While the mutation negatively affected the potentiation by propofol and pentobarbital, potentiation by etomidate was surprisingly enhanced. These observations indicate that all the investigated substances interact with the γ+/β− subunit interface. Interestingly, as mentioned above, no photo-labeling at this subunit interface using a propofol or an etomidate analog has been observed, but etomidate inhibited labeling by a barbiturate derivative of a residue located at the γ+ side (Jayakar et al., 2015).

The $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor has five subunit interfaces, γβ+/α−β (interface 1), αβ+/α−γ (interface 2), α+/β−, α+/γ− and γ+/β−. We conclude here that etomidate acts predominantly at γβ+/α−β (interface 1) and γ+/β− subunit interfaces, propofol acts predominantly at γβ+/α−β (interface 1), αβ+/α−γ (interface 2), γ+/β− and pentobarbital acts predominantly at interface 2 and additionally at α+/β− and/or α+/γ− subunit interfaces. Thus, at least four of the five subunit interfaces harbor sites for these compounds which are important for functional modulation, but each compound uses a different set of subunit interfaces. Thus, we observe an asymmetry in anesthetic potentiation of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor.

Acknowledgments

We thank Dr. V. Niggli for carefully reading the manuscript. This work was supported by the Swiss National Science Foundation grant 315230_156929/1. M.C.M. is a recipient of a fellowship (Beca Chile Postdoctorado from CONICYT, Ministerio de Educacion, Chile).

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