



## Article

# Mutational Analysis of Anesthetic Binding Sites and Their Effects on GABA<sub>A</sub> Receptor Activation and Modulation by Positive Allosteric Modulators of the $\alpha 7$ Nicotinic Receptor

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**Abstract:** The positive allosteric modulators (PAMs) of the  $\alpha 7$  nicotinic receptor *N*-(5-Cl-2-hydroxyphenyl)-*N'*-[2-Cl-5-(trifluoromethyl)phenyl]-urea (NS-1738) and (*E*)-3-(furan-2-yl)-*N*-(*p*-tolyl)-acrylamide (PAM-2) potentiate the  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor through interactions with the classic anesthetic binding sites located at intersubunit interfaces in the transmembrane domain of the receptor. In the present study, we employed mutational analysis to investigate in detail the involvement and contributions made by the individual intersubunit interfaces to receptor modulation by NS-1738 and PAM-2. We show that mutations to each of the anesthetic-binding intersubunit interfaces ( $\beta + / \alpha -$ ,  $\alpha + / \beta -$ , and  $\gamma + / \beta -$ ), as well as the orphan  $\alpha + / \gamma -$  interface, modify receptor potentiation by NS-1738 and PAM-2. Furthermore, mutations to any single interface can fully abolish potentiation by the  $\alpha 7$ -PAMs. The findings are discussed in the context of energetic additivity and interactions between the individual binding sites.

**Keywords:** GABA<sub>A</sub> receptor;  $\alpha 7$  nicotinic receptor; modulation; potentiator; allostery



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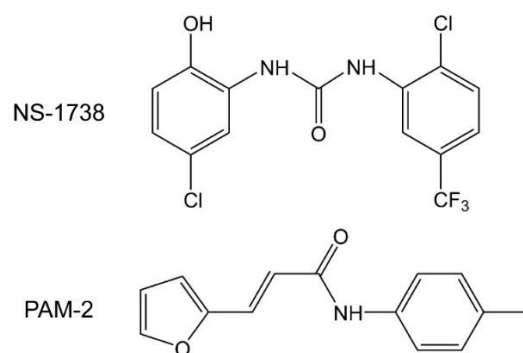
## 1. Introduction

The  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor is a Cl<sup>-</sup>-permeable transmitter-gated ion channel. Although found in select non-neural tissue, it is mostly, and widely, expressed in neurons where its activation by synaptically released or ambient GABA leads to cellular hyperpolarization or dampening of the effects of excitatory ion channels. The receptor is also activated by 2-aminoethanesulfonic acid (taurine), while various neurosteroids can potentiate (e.g., allopregnanolone) or inhibit (e.g., pregnenolone sulfate) its function. The GABA<sub>A</sub> receptor is a target for numerous anxiogenics, sedatives, and anesthetics used in clinical practice. Studies of the mechanisms of receptor activation and modulation, particularly in the simultaneous presence of multiple active compounds, are crucial to our understanding of the physiological and pathophysiological roles of the GABA<sub>A</sub> receptor.

The common  $\alpha 1\beta 2\gamma 2L$  subtype of the GABA<sub>A</sub> receptor contains two transmitter-binding (orthosteric) sites in the extracellular domain at the two  $\beta + / \alpha -$  intersubunit interfaces [1,2]. There is a homologous site at the  $\alpha + / \gamma -$  interface through which benzodiazepines act on the GABA<sub>A</sub> receptor [3]. In the transmembrane domain, the receptor contains several interfacial and intrasubunit sites for anesthetics, benzodiazepines, and neurosteroids [4–6]. Co-application of an allosteric agonist potentiates the response to subsaturating GABA or to another allosteric agonist (e.g., [7–10]). In the “co-agonist

model" [11,12], each activator is postulated to make an independent and additive energetic contribution towards the stabilization of the active state. This enables a model-based prediction of the response amplitude for agonist combinations if the properties of each agonist are known. The co-agonist model-based predictions work, within limits of experimental error, for the combinations of GABA plus propofol, etomidate, benzodiazepines, or neurosteroids [13–18]. On the other hand, discrepancies between experimental observations and predictions based on energetic additivity have been observed for several combinations of anesthetic drugs including the combinations of etomidate plus propofol and etomidate plus barbiturate [10]. Furthermore, mutations to individual anesthetic binding sites have been shown to modify the potentiating effects of the anesthetics binding at that site as well as those binding to homologous sites at other interfaces [19]. This has been interpreted as an allosteric linkage between the sites.

The anesthetic binding sites are located at intersubunit interfaces in cavities formed by the second and third transmembrane domains (TM2 and TM3) of the subunit contributing to the "+" side of the interface and the first transmembrane domain (TM1) of the subunit contributing to the "-" side of the interface. The sedative anesthetic etomidate, for example, binds to the  $\beta+$ / $\alpha-$  interface, whereas a barbiturate derivative binds with high affinity to the  $\alpha+$ / $\beta-$  and  $\gamma+$ / $\beta-$  interfaces [20,21]. We recently showed that type I and type II positive allosteric modulators (PAMs) of the  $\alpha 7$  nicotinic receptor *N*-(5-chloro-2-hydroxyphenyl)-*N'*-[2-chloro-5-(trifluoromethyl)phenyl]-urea (NS-1738) and (*E*)-3-(furan-2-yl)-*N*-(*p*-tolyl)-acrylamide (PAM-2) (Figure 1) potentiate the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor through interactions with anesthetic binding sites [22]. Here, we have employed mutational analysis to investigate in detail the involvement and contributions made by the individual intersubunit interfaces to receptor modulation by NS-1738 and PAM-2. A major observation is that mutations to any single interface can fully abolish potentiation by the  $\alpha 7$ -PAMs, which is indicative of a lack of independently acting sites. The findings are discussed in the context of energetic additivity and allosteric interactions between individual binding sites.



**Figure 1.** Structural formulas of *N*-(5-chloro-2-hydroxyphenyl)-*N'*-[2-chloro-5-(trifluoromethyl)phenyl]-urea (NS-1738) and (*E*)-3-(furan-2-yl)-*N*-(*p*-tolyl)-acrylamide (PAM-2).

## 2. Materials and Methods

### 2.1. Molecular Modeling

Modeling of the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor was done using the Prime module of the Schrödinger Suite Release 2020-3 (Schrödinger, LLC, New York, NY, USA). For the template, we used the human  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>R structure (PDB: 6X3T; [23]). The molecular structures of NS-1738 and PAM-2 were prepared and evaluated for their ionization states at pH 7.4 using 2D Sketcher and LigPrep within Schrödinger Maestro (Maestro Version 12.5.139). Each ligand was docked to the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor model using QuickVina-W [24]. The exhaustiveness parameter was set to 300. Ten docking runs, each producing twenty poses, were performed under the same conditions. The poses with more negative theoretical binding energy values, indicating higher theoretical binding affinities, were stored. The final selected docked conformers were further analyzed using molecular dynamics (MD) to determine their stability and behavior within the binding site. Membrane building

(dipalmitoylphosphatidylcholine and cholesterol), complex solvation, and ionization were carried out using CHARMM-GUI [25]. To determine the stability of the selected poses within their predicted docking sites, MD simulations of 100 ns were performed using the program NAMD, CHARMM force field [26], and CHARMM-GUI NAMD input generator with the PETE supercomputer at the High Performance Computing Center (Oklahoma State University Center for Health Sciences, Tulsa, OK, USA).

### 2.2. Receptors, Expression, and Electrophysiology

The cDNAs for rat  $\alpha 1$  (Genbank accession number NM\_183326),  $\beta 2$  (NM\_012957), and  $\gamma 2L$  (NM\_183327) subunits in the pcDNA3 vector were linearized with the XbaI (NEB Laboratories, Ipswich, MA, USA) restriction enzyme. The cRNAs were synthesized from linearized cDNA using mMessage mMachine (Life Technologies, Grand Island, NY, USA). Mutant clones were purchased from Twist Bioscience (South San Francisco, CA, USA).

Wild-type and mutant GABA<sub>A</sub>Rs were expressed in *Xenopus laevis* oocytes. The oocytes were purchased from Xenopus 1 (Dexter, MI, USA) as quarter ovaries. To remove the follicular membrane, ovaries were incubated in 2% *w/v* (mg/mL) Collagenase A (Sigma-Aldrich, St. Louis, MO, USA) solubilized in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES; pH 7.4) with supplements (2.5 mM Na pyruvate, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin and 50  $\mu$ g/mL gentamycin) at 37 °C with shaking at 250 RPM for 30 min. Following a subsequent 3–4 h incubation at 15 °C, the oocytes were injected with a total of 3.5 ng of cRNA (0.5 ng:0.5 ng:2.5 ng,  $\alpha 1:\beta 2:\gamma 2L$ ) per oocyte. The injected oocytes were incubated in ND96 with supplements for 1–2 days before commencing electrophysiological recordings.

Two-electrode voltage-clamp recordings were conducted as described in detail previously [27,28]. The oocytes were placed in a recording chamber (RC-1Z, Warner Instruments, Hamden, CT, USA) and clamped at  $-60$  mV. Borosilicate glass capillaries (G120F-4, Warner Instruments) were used as voltage and current electrodes. When filled with 3 M KCl, the electrodes had typical resistances of  $\sim 1$  M $\Omega$ . Solutions were gravity-applied from glass syringes and switched manually using 4-port bulkhead switching valves and medium-pressure 6-port bulkhead valves (IDEX Health and Science, Rohnert Park, CA, USA). The current responses were amplified with an OC-725C amplifier (Warner Instruments), digitized with a Digidata 1200 series digitizer (Molecular Devices, San Jose, CA, USA), and stored using pClamp (Molecular Devices).

### 2.3. Cysteine-Modification Experiments

Cysteine-modification experiments were done by exposing a wild-type (control) or a cysteine-mutated receptor to *p*-chloromercuribenzoic acid (pCMB). Successful modification of a cysteine residue was deduced from irreversible alteration of receptor function following the application of pCMB [29–31]. The pCMB concentration was 25  $\mu$ M and the application duration was 30 s. pCMB was applied in the presence of 1 mM GABA, and all drug applications were followed by a 4–5 min wash in ND96. In modification-protection experiments, the cells were exposed to the protecting agent (NS-1738 or PAM-2) for 60 s, followed by a 30 s application of pCMB + GABA + protective agent.

### 2.4. Functional Studies and Data Analysis

The modulatory effects of  $\alpha 7$ -PAMs were examined under two experimental protocols. In cases where the constitutive probability of being in the active state ( $P_{A, \text{constitutive}}$ ) was low ( $< 0.02$ ), modulation was established by coapplying  $\alpha 7$ -PAM during a steady-state response to a low concentration ( $P_A < 0.10$ ) of GABA. The  $P_{A, \text{low GABA}}$  was not corrected for  $P_{A, \text{constitutive}}$  when the latter was less than 0.01. Drug effects were initially quantified by calculating fold-potentiation from the ratio of the peak response to GABA +  $\alpha 7$ -PAM to the steady-state response to GABA immediately before the application of the modulator. In cases where  $P_{A, \text{constitutive}}$  was high ( $> 0.02$ ), the  $\alpha 7$ -PAMs were applied in the absence of GABA, and drug effects were calculated from the estimated probability of being in the active

state ( $P_{A,\alpha 7\text{-PAM}}$ ) as  $P_{A,\alpha 7\text{-PAM}}/P_{A,\text{constitutive}}$ .  $P_{A,\text{constitutive}}$  was estimated by comparing the effects on holding current by 200  $\mu\text{M}$  picrotoxin, which was assumed to block all receptors ( $P_A = 0$ ), and 1 mM GABA + 50  $\mu\text{M}$  propofol, which was assumed to activate all receptors ( $P_A = 1$ ) [32].

For mechanistic analysis of receptor modulation by  $\alpha 7\text{-PAMs}$ , the data were analyzed in the framework of the two-state (Resting-Active) cyclic model [11,12]. The state function of the model is as follows:

$$P_{A,\alpha 7\text{-PAM}} = \frac{1}{1 + ((1 - P_{A,\text{background}})/P_{A,\text{background}}) \left[ \frac{1 + [\alpha 7\text{-PAM}]/K_{R,\alpha 7\text{-PAM}}}{1 + [\alpha 7\text{-PAM}]/(K_{R,\alpha 7\text{-PAM}}c_{\alpha 7\text{-PAM}})} \right]^{N_{\alpha 7\text{-PAM}}}} \quad (1)$$

where  $P_{A,\alpha 7\text{-PAM}}$  and  $P_{A,\text{background}}$  are the  $P_A$  in the presence and absence, respectively, of the compound studied.  $K_{R,\alpha 7\text{-PAM}}$  is the equilibrium dissociation constant of the compound in the resting receptor,  $c_{\alpha 7\text{-PAM}}$  is the ratio of the equilibrium dissociation constant of the compound in the active receptor to  $K_{R,\alpha 7\text{-PAM}}$ ,  $[\alpha 7\text{-PAM}]$  is the concentration of the compound, and  $N_{\alpha 7\text{-PAM}}$  is the number of imposed binding sites (by convention, 2).

In all experiments, NS-1738 and PAM-2 were tested at 50  $\mu\text{M}$ , which is a near-saturating concentration in the  $\alpha 1\beta 2\gamma 2\text{L}$  receptor [22]. The parameter  $c_{\alpha 7\text{-PAM}}$  that reflects gating efficacy (ratio of equilibrium dissociation constants in the active and resting receptors) was calculated as follows:

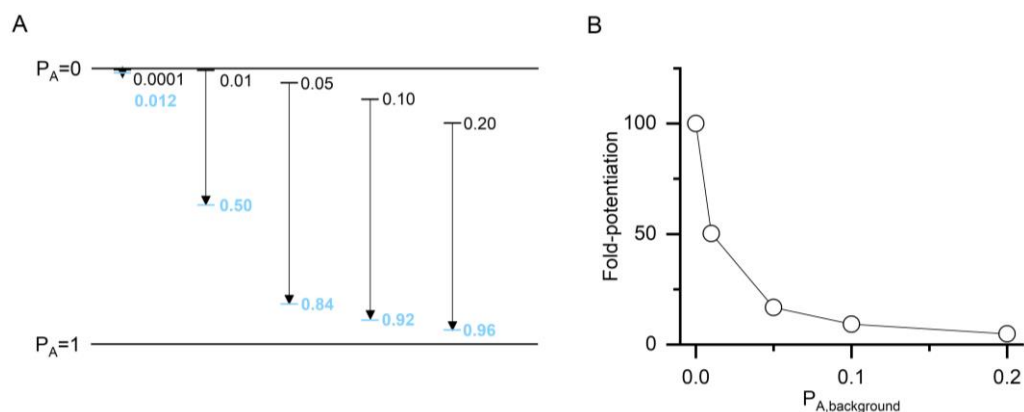
$$c_{\alpha 7\text{-PAM}} = {}^{1/N_{\alpha 7\text{-PAM}}}\sqrt{\frac{1/P_{A,\alpha 7\text{-PAM}} - 1}{(1 - P_{A,\text{background}})/P_{A,\text{background}}}} \quad (2)$$

Free energy change ( $\Delta G$ ) was calculated from  $c_{\alpha 7\text{-PAM}}$  as  $\Delta G = NRT \times \ln(c_{\alpha 7\text{-PAM}})$ . The gating efficacies of the tested anesthetic compounds were estimated by analogous calculations.

The value of  $c_{\text{compound}}$  and, consequently, that of  $\Delta G$  is a measure of the ability of a compound to increase the  $P_A$  of a response. It non-linearly expresses the difference between the  $P_A$  of the control response ( $P_{A,\text{background}}$ ) and the  $P_A$  of the response in the presence of a modulatory compound ( $P_{A,\text{compound}}$ ). The relationship between the background activity and  $c_{\text{compound}}$  is illustrated in Figure 2. The apparent potentiating effect (the calculated fold-potential) of a compound is dependent on the  $P_A$  value of the background, i.e., control response, thus introducing an error if fold-potential is compared at different  $P_{A,\text{background}}$ . The use of  $c_{\text{compound}}$  to compare effects negates this. The nominal value of  $c_{\text{compound}}$ , however, depends on the number of postulated binding sites for the compound. This precludes direct comparison of  $c$  values for compounds with differing numbers of binding sites.  $\Delta G$ , on the other hand, reflects the total stabilization energy provided by a compound, and its nominal value is independent of the number of binding sites.

## 2.5. Materials

Salts used in ND96, HEPES, GABA, propofol, and 5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one (3 $\alpha$ 5 $\beta$ P) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Etomidate was purchased from Toronto Research Chemicals (Toronto, ON, Canada). NS-1738 was obtained from Cayman Chemical (Ann Arbor, MI, USA) and Adooq Bioscience (Irvine, CA, USA). PAM-2 was synthesized as described previously [33]. A stock solution of 500 mM GABA in ND96 was stored at 4  $^{\circ}\text{C}$ . All other stock solutions were made in dimethyl sulfoxide (Sigma-Aldrich) with 200 mM propofol and 20 mM 3 $\alpha$ 5 $\beta$ P stocks stored at room temperature and 200 mM etomidate, 100 mM NS-1738, and 100 mM PAM-2 stocks stored at  $-20$   $^{\circ}\text{C}$ . Final dilutions were made on the day of the experiment.



**Figure 2.** The relationship between apparent potentiation and the value of  $c_{compound}$ . **(A)** The plot shows imposed  $P_{A,background}$  (black lines and numbers) and calculated  $P_{A,compound}$  (blue lines and numbers) for a hypothetical compound with two binding sites ( $N = 2$ ) and a  $c_{compound}$  of 0.100 ( $\Delta G = -2.72$  kcal/mol). The calculations were done at a saturating concentration ( $1000\times$  of  $K_{resting}$ ) and thus reflect maximal effects. Higher  $P_A$  is shown downward for consistency with the direction of current flow in electrophysiological recordings from GABA<sub>A</sub> receptors. **(B)** The graph shows fold-potentiation at different  $P_{A,background}$  calculated from the data in panel A. The compound has a larger apparent potentiating effect when measured at low  $P_{A,background}$ .

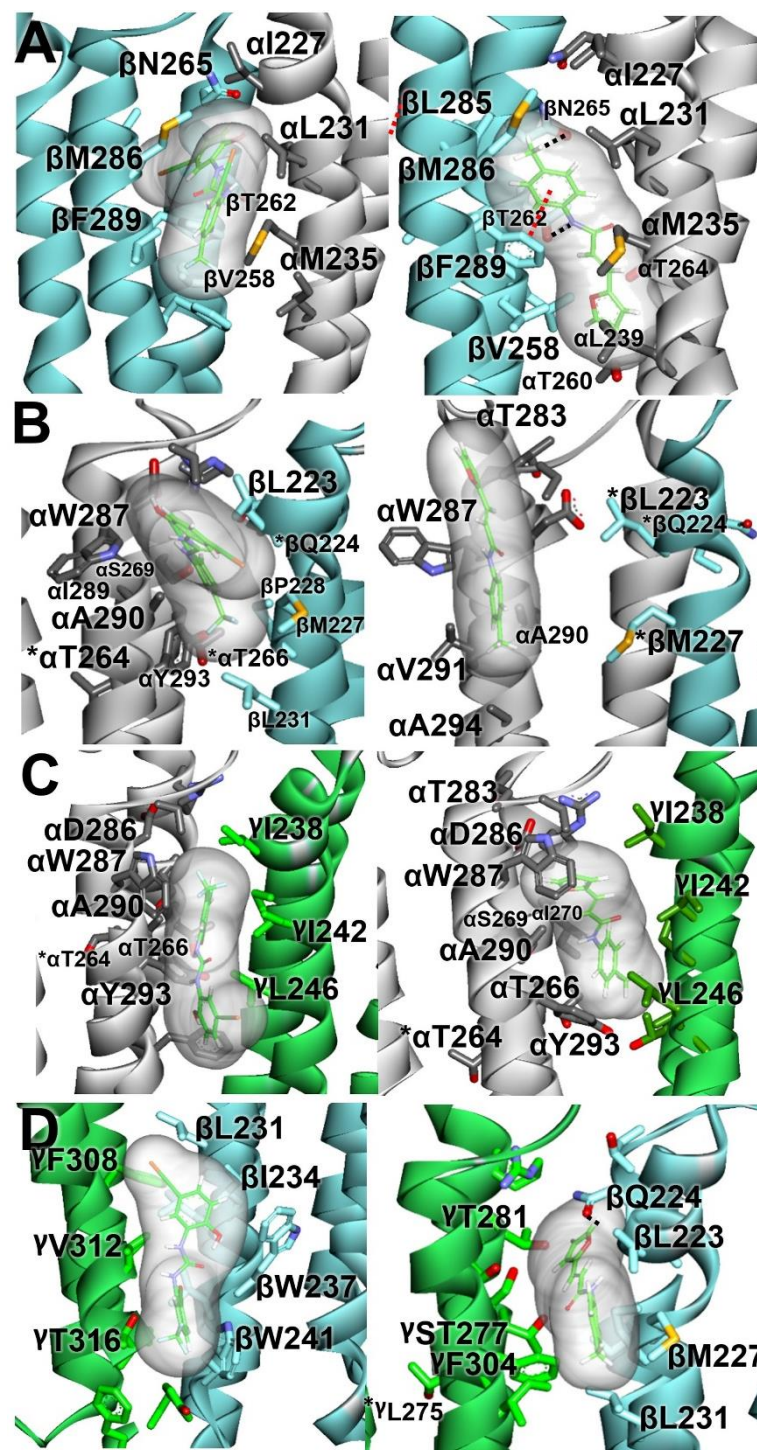
### 3. Results

#### 3.1. Molecular Docking and Molecular Dynamics Indicate Binding Sites for $\alpha 7$ -PAMs at Intersubunit Interfaces in the Transmembrane Domain

The docking experiments were based on the structure of the human  $\alpha 1\beta 2\gamma 2$  receptor (PDB: 6X3T) [23]. In the APO 6X3T structure, both NS-1738 and PAM-2 were able to dock in the anesthetic binding sites at the  $\beta +/\alpha -$ ,  $\alpha +/\beta -$ , and  $\gamma +/\beta -$  interfaces. In addition, the compounds were shown to bind at a homologous site at the  $\alpha +/\gamma -$  interface. The structure figures with docked  $\alpha 7$ -PAMs are given in Figure 3. The corresponding PDB files are provided in the Supplementary Materials. The amino acid sequences of the human and rat  $\alpha 1$  subunits differ. The human subunit has one extra residue (a leucine in position 4 in the mature subunit) in the amino-terminal region but is otherwise identical to rat  $\alpha 1$ . For convenience and consistency with electrophysiological data, we used the rat numbering when discussing specific residues in the  $\alpha 1$  subunit (hence, the true numbering in human  $\alpha 1 =$  the provided rat numbering + 1).

At the  $\beta +/\alpha -$  interface, NS-1738 and PAM-2 are sandwiched, nearly in parallel with the  $\alpha$ -helical transmembrane segments, in the cavity between  $\beta$ -TM3,  $\beta$ -TM2, and  $\alpha$ -TM1. The 2-hydroxyl group of NS-1738 and the tolyl group of PAM-2 are oriented towards the extracellular side of the membrane. The  $\beta 2$ (F289) residue contributes to carbonyl–aromatic (CO- $\pi$ ) interaction with the carbonyl group of NS-1738, whereas, one  $\alpha$ -helical turn-up,  $\beta 2$ (M286) borders the chloro-hydroxyphenyl ring. At the “-” side of the interface, the  $\alpha 1$ (M235) residue abuts the trifluoromethylphenyl group of NS-1738, and  $\alpha 1$ (L231) and  $\alpha 1$ (I227) further up in  $\alpha 1$ -TM1 border the amine and phenylhydroxyl groups. The tolyl group of PAM-2 is oriented between the  $\beta 2$ (M286),  $\beta 2$ (F289), and  $\alpha 1$ (I227) residues. The carbonyl of acrylamide points towards  $\alpha 1$ (T236).

At the  $\gamma +/\beta -$  interface, NS-1738 is positioned at the protein–lipid interface between  $\gamma 2$ -TM3 and  $\beta 2$ -TM1. NS-1738 is oriented in parallel with the transmembrane helices, with the trifluoro and phenyl groups positioned between  $\gamma 2$ (T316) and  $\beta 2$ (W241), and pointed towards the cytosolic side of the membrane. The chloro-hydroxyphenyl ring is located between  $\gamma 2$ -TM3 and  $\beta 2$ -TM1, near  $\gamma 2$ (F308) and  $\beta 2$ (I234). In contrast, PAM-2 lies, nearly perpendicular to the transmembrane helices, in a cavity between  $\gamma$ -TM2,  $\gamma$ -TM3, and  $\beta$ -TM1. The furan group rests between  $\gamma 2$ (T281) and  $\beta 2$ (Q224) in  $\gamma 2$ -TM2 and  $\beta 2$ -TM1, respectively, whereas the tolyl group of PAM-2 is sandwiched between  $\gamma 2$ (F304) and  $\beta 2$ (M227).



**Figure 3.** Molecular docking of NS-1738 (left panels) and PAM-2 (right panels) to the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor model. Stable docking sites are shown at the  $\beta + / \alpha -$  (A),  $\alpha + / \beta -$  (B),  $\alpha + / \gamma -$  (C), and  $\gamma + / \beta -$  (D) interfaces. Subunits ( $\alpha$ , white;  $\beta$ , light-blue; and  $\gamma$ , green) are represented as ribbons, while ligands are represented as thin sticks surrounded by their molecular surfaces, colored by atoms, with carbon atoms in green. The interacting residues are represented by thick sticks, colored by atoms, with H atoms omitted for clarity. Black dotted lines represent hydrogen bonds. Red dotted lines represent  $\pi - \pi$  interactions. \* Residues were tested with mutational analysis but were not predicted to interact with the ligand.

At the  $\alpha+\beta-$  interface, the NS-1738 is positioned between  $\alpha$ -TM3 and  $\gamma$ -TM1. The trifluoro group points towards the cytosolic side of the membrane, surrounded by  $\alpha 1$ (Y293) from the “+” side and  $\beta 2$ (M227) and  $\beta 2$ (L231) from the “-” side of the interface. The oxygen of the chloro-hydroxyphenyl ring is within 5 Å of  $\alpha 1$ (W287). PAM-2 is oriented in parallel with the transmembrane domains with its tolyl group pointed towards the extracellular side. The molecule clinches to  $\alpha$ -TM3 with the carbonyl of acrylamide within 4 Å of  $\alpha 1$ (A290).

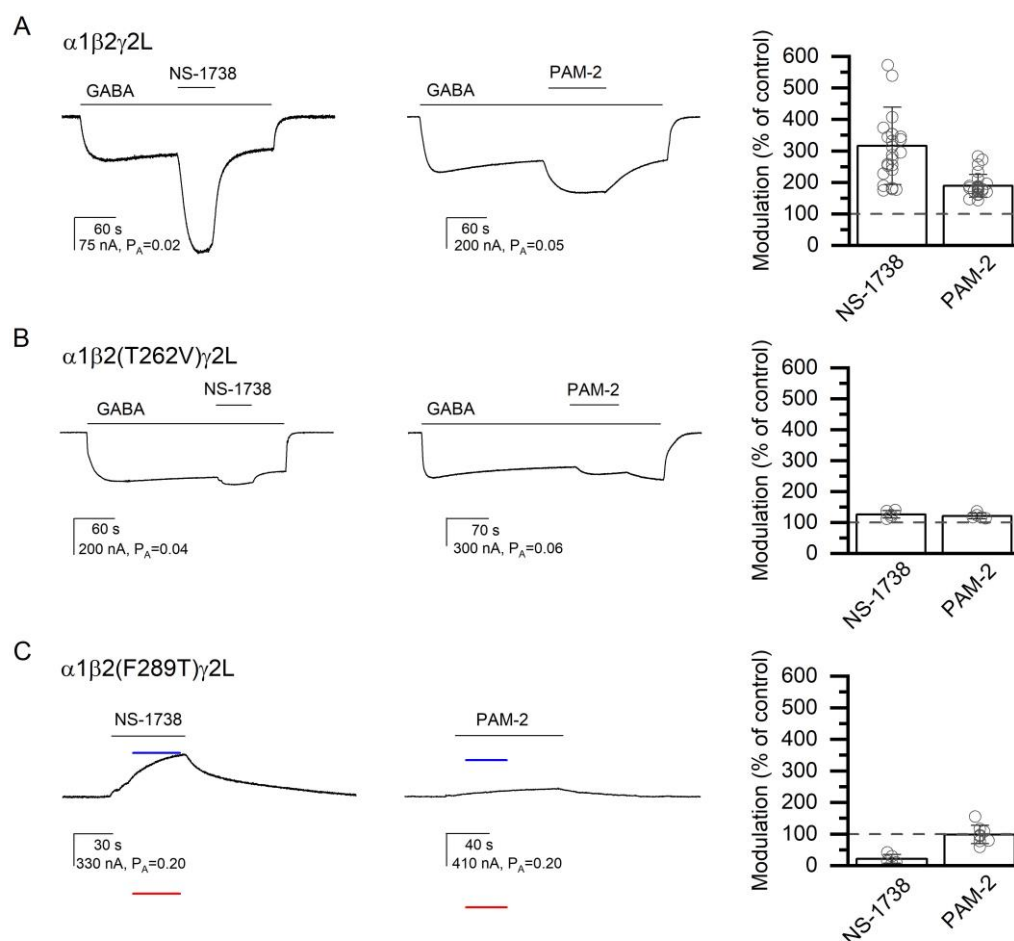
At the  $\alpha+\gamma-$  interface, NS-1738 is placed between the  $\alpha$ -TM3 and  $\gamma$ -TM1 domains. It is oriented in parallel with the  $\alpha$ -helical transmembrane segments, with the trifluoro group pointing towards the extracellular side of the membrane and located within 3.4 Å of  $\alpha 1$ (A290). The 2-hydroxyl oxygen of NS-1738 borders  $\alpha 1$ (A294), one  $\alpha$ -helical turn down towards the cytosolic side of the membrane. At the “-” side,  $\gamma 2$ (I242) and  $\gamma 2$ (L246) position near the trifluoro and carbonyl groups, respectively. Like at the  $\gamma+\beta-$  interface, PAM-2 is positioned nearly perpendicular to the transmembrane segments. The furan group oxygen is ~3 Å from  $\alpha 1$ (A290) and less than 5 Å from  $\alpha 1$ (W287). The carbonyl group of acrylamide is within 5 Å of  $\gamma 2$ (I242) and  $\gamma 2$ (L246) residues, whereas the methyl group of tolyl ring points to  $\gamma 2$ (T275) (the 10' residue in  $\gamma$ -TM2).

### 3.2. Mutations to the $\beta+\alpha-$ Interface Affect Receptor Activation and Potentiation by $\alpha 7$ -PAMs

In the wild-type  $\alpha 1\beta 2\gamma 2$ L receptor, 50  $\mu$ M NS-1738 potentiated the responses to low GABA ( $P_A = 0.05 \pm 0.03$ ,  $n = 25$ ) to  $316 \pm 123\%$  of the control. The application of 50  $\mu$ M PAM-2 potentiated the response to GABA ( $P_A = 0.06 \pm 0.03$ ,  $n = 25$ ) to  $189 \pm 36\%$  of the control. With  $N_{\alpha 7\text{-PAM}}$  constrained to two, the calculated  $c_{50 \mu\text{M NS-1738}}$  is  $0.561 \pm 0.106$  ( $\Delta G = -0.70 \pm 0.23$  kcal/mol), and the calculated  $c_{50 \mu\text{M PAM-2}}$  is  $0.721 \pm 0.061$  ( $\Delta G = -0.39 \pm 0.10$  kcal/mol). Sample current traces showing potentiation of the wild-type receptor by the  $\alpha 7$ -PAMs are given in Figure 4A, and the data are summarized in Table 1.

At the  $\beta+\alpha-$  interface, we tested the effects of mutations to the  $\beta 2$ (V258),  $\beta 2$ (T262),  $\beta 2$ (F289),  $\alpha 1$ (I227), and  $\alpha 1$ (M235) residues. In the  $\beta 2$ (V258M)-containing receptor, NS-1738 potentiated the response to GABA ( $P_A = 0.09 \pm 0.02$ ,  $n = 5$ ) to 720% of the control (S.D. and numbers of oocytes are given in Table 1). Application of PAM-2 enhanced the response to GABA ( $P_A = 0.10 \pm 0.04$ ,  $n = 5$ ) to 301% of the control. Replacement of the polar threonine with hydrophobic valine at position 262 in the  $\beta 2$  subunit ( $\beta 2$ (T262V)) reduced the potentiating actions of both  $\alpha 7$ -PAMs. Co-application of 50  $\mu$ M NS-1738 increased the response to 3–4  $\mu$ M GABA ( $P_A = 0.08 \pm 0.03$ ;  $n = 5$ ) to 126% of the control ( $P_{A,50 \mu\text{M NS-1738}} = 0.10 \pm 0.03$ ). Exposure to 50  $\mu$ M PAM-2 increased the response to GABA ( $P_A = 0.05 \pm 0.03$ ;  $n = 5$ ) to 121% of the control ( $P_{A,50 \mu\text{M PAM-2}} = 0.06 \pm 0.04$ ). Both values are statistically significantly lower than the magnitude of potentiation observed in the wild-type  $\alpha 1\beta 2\gamma 2$ L receptor. The summary of the effects of mutations on GABA<sub>A</sub> receptor potentiation or direct activation by  $\alpha 7$  PAMs on mutant receptors is provided in Table 1. Sample current traces for the  $\alpha 1\beta 2$ (T262V) $\gamma 2$ L receptor are given in Figure 4B.

The  $\beta 2$ (F289A) mutant receptor exhibited a considerable level of constitutive activity ( $P_{A,\text{const}} = 0.025 \pm 0.019$ ,  $n = 25$ ). The direct-activating and potentiating actions of NS-1738 and PAM-2 were abolished in the mutant receptor. In the absence of GABA, exposure to NS-1738 or PAM-2 reduced  $P_{A,\text{const}}$  from  $0.035 \pm 0.030$  ( $n = 5$ ) to  $0.016 \pm 0.009$  (74% of the control) or from  $0.035 \pm 0.021$  ( $n = 5$ ) to  $0.029 \pm 0.013$  (87% of the control), respectively. In the presence of 0.1  $\mu$ M GABA, the application of NS-1738 reduced the  $P_A$  from  $0.06 \pm 0.02$  ( $n = 5$ ) to  $0.02 \pm 0.01$  (29% of the control), and the application of PAM-2 decreased  $P_A$  from  $0.06 \pm 0.04$  ( $n = 5$ ) to  $0.04 \pm 0.03$  (81% of the control).



**Figure 4.** The modulatory effects of NS-1738 and PAM-2. The current traces show the potentiating effects of 50  $\mu\text{M}$  NS-1738 or PAM-2 in the  $\alpha 1\beta 2\gamma 2L$  receptor activated by 2  $\mu\text{M}$  GABA (A), or in the  $\alpha 1\beta 2(T262V)\gamma 2L$  receptor activated by 4  $\mu\text{M}$  GABA (B). Panel (C) shows direct activation by NS-1738 or PAM-2 of the constitutively active  $\alpha 1\beta 2(F289T)\gamma 2L$  receptor. The blue and red lines show the current levels in the same cell in the presence of 200  $\mu\text{M}$  picrotoxin (assumed  $P_A = 0$ ) or 1 mM GABA + 50  $\mu\text{M}$  propofol (assumed  $P_A = 1$ ), respectively. The modulatory effects are summarized in the column graphs. In (A,B), modulation is calculated as the amplitude of the peak response to GABA +  $\alpha 7$ -PAM divided by the amplitude of the response to GABA alone at the time of peak response to GABA +  $\alpha 7$ -PAM. In (C), modulation is calculated as the ratio of the estimated  $P_A$  in the presence and absence of  $\alpha 7$ -PAM. Drug application durations are given with horizontal lines.

A threonine substitution at  $\beta 2(F289)$  ( $P_{A,\text{const}} = 0.25 \pm 0.06$ ,  $n = 13$ ) similarly reduced the activating actions of  $\alpha 7$ -PAMs. The application of NS-1738 reduced the  $P_A$  from  $0.28 \pm 0.05$  ( $n = 5$ ) to  $0.04 \pm 0.05$  (17% of the control). Exposure to PAM-2 had a minimal effect; the  $P_A$  was  $0.23 \pm 0.06$  ( $n = 8$ ) in the absence and  $0.22 \pm 0.05$  (98% of the control) in the presence of the compound. Sample current traces are given in Figure 4C. Thus, in relative terms, amino acid substitutions at  $\beta 2(F289)$  had greater effects on the actions of NS-1738 for which the mutations are predicted to lead to the loss of the carbonyl–aromatic (CO- $\pi$ ) interaction, compared to the actions of PAM-2 that makes  $\pi$ - $\pi$  interactions with  $\beta 2(F289)$ .

The introduction of the  $\beta 2(V289M)$  mutation increased the potentiating effects of  $\alpha 7$ -PAMs (Table 1). The mechanism of this is unclear but is likely to be indirect; the residue is  $>9\text{\AA}$  from the trifluoro group of NS-1738 although only  $\sim 3\text{\AA}$  from the furan oxygen in PAM-2.



**Table 1.** Summary of effects of mutations on GABA<sub>A</sub> receptor potentiation or direct activation by the  $\alpha 7$ -PAMs NS-1738 and PAM-2.

Receptor	Modulation, 50 $\mu$ M NS-1738, % of Control ( <i>n</i> )	<i>c</i> <sub>50 <math>\mu</math>M NS-1738</sub>	$\Delta G$ <sub>50 <math>\mu</math>M NS-1738</sub> , kcal/mol	Modulation, 50 $\mu$ M PAM-2, % of Control ( <i>n</i> )	<i>c</i> <sub>50 <math>\mu</math>M PAM-2</sub>	$\Delta G$ <sub>50 <math>\mu</math>M PAM-2</sub> , kcal/mol
$\alpha 1\beta 2\gamma 2L$	316 $\pm$ 123 (25)	0.561 $\pm$ 0.106	−0.70 $\pm$ 0.23	189 $\pm$ 36 (25)	0.721 $\pm$ 0.061	−0.39 $\pm$ 0.10
<u><math>\beta + / \alpha -</math> interface</u>						
$\beta 2(V258M)$	720 $\pm$ 140 (5)	0.307 $\pm$ 0.044	−1.40 $\pm$ 0.16 *	301 $\pm$ 87 (5)	0.559 $\pm$ 0.110	−0.70 $\pm$ 0.22 *
$\beta 2(T262V)$	126 $\pm$ 12 (5)	0.883 $\pm$ 0.045	−0.15 $\pm$ 0.06 *	121 $\pm$ 9 (5)	0.906 $\pm$ 0.032	−0.12 $\pm$ 0.04 *
$\beta 2(F289T)$ #	21 $\pm$ 14 (5)	2.980 $\pm$ 1.097	1.22 $\pm$ 0.47 *	98 $\pm$ 29 (8)	1.058 $\pm$ 0.206	0.05 $\pm$ 0.23 *
$\beta 2(F289A)$ #	74 $\pm$ 55 (5)	1.525 $\pm$ 0.845	0.37 $\pm$ 0.60 *	87 $\pm$ 20 (5)	1.094 $\pm$ 0.123	0.10 $\pm$ 0.13 *
$\alpha 1(I227W)$	207 $\pm$ 56 (5)	0.769 $\pm$ 0.163	−0.33 $\pm$ 0.24	135 $\pm$ 18 (5)	0.856 $\pm$ 0.059	−0.19 $\pm$ 0.08 *
$\alpha 1(L231C)$	349 $\pm$ 133 (5)	0.532 $\pm$ 0.105	−0.76 $\pm$ 0.23	197 $\pm$ 19 (5)	0.691 $\pm$ 0.037	−0.44 $\pm$ 0.06
$\alpha 1(M235W)$ #	196 $\pm$ 46 (5)	0.713 $\pm$ 0.108	−0.41 $\pm$ 0.17	174 $\pm$ 20 (5)	0.749 $\pm$ 0.041	−0.34 $\pm$ 0.06
<u><math>\alpha + / \beta -</math> and <math>\gamma + / \beta -</math> interfaces</u>						
$\beta 2(L223W)$	215 $\pm$ 26 (5)	0.635 $\pm$ 0.054	−0.54 $\pm$ 0.10	48 $\pm$ 10 (5)	1.540 $\pm$ 0.157	0.50 $\pm$ 0.13 *
$\beta 2(Q224A)$	389 $\pm$ 182 (6)	0.492 $\pm$ 0.118	−0.87 $\pm$ 0.28	169 $\pm$ 26 (5)	0.752 $\pm$ 0.058	−0.34 $\pm$ 0.09
$\beta 2(M227C)$	335 $\pm$ 62 (5)	0.533 $\pm$ 0.055	−0.75 $\pm$ 0.12	143 $\pm$ 18 (5)	0.833 $\pm$ 0.051	−0.22 $\pm$ 0.07
<u><math>\gamma + / \beta -</math> interface</u>						
$\gamma 2L(L275C)$	283 $\pm$ 66 (5)	0.569 $\pm$ 0.082	−0.67 $\pm$ 0.16	251 $\pm$ 21 (5)	0.611 $\pm$ 0.029	−0.58 $\pm$ 0.06 *
$\gamma 2L(T277I)$ #	511 $\pm$ 178 (5)	0.375 $\pm$ 0.082	−1.18 $\pm$ 0.26 *	117 $\pm$ 6 (5)	0.919 $\pm$ 0.025	−0.10 $\pm$ 0.03 *
$\gamma 2L(T281I)$	262 $\pm$ 61 (6)	0.592 $\pm$ 0.078	−0.63 $\pm$ 0.16	255 $\pm$ 40 (5)	0.687 $\pm$ 0.051	−0.45 $\pm$ 0.09
$\gamma 2L(F304C)$ #	34 $\pm$ 17 (5)	1.917 $\pm$ 0.449	0.74 $\pm$ 0.29 *	40 $\pm$ 17 (5)	1.791 $\pm$ 0.428	0.66 $\pm$ 0.28 *
<u><math>\alpha + / \gamma -</math> and <math>\alpha + / \beta -</math> interfaces</u>						
$\alpha 1(T264V)$	295 $\pm$ 63 (5)	0.530 $\pm$ 0.079	−0.76 $\pm$ 0.17	240 $\pm$ 51 (5)	0.620 $\pm$ 0.075	−0.57 $\pm$ 0.14
$\alpha 1(T266M)$	151 $\pm$ 39 (5)	0.189 $\pm$ 0.107	−0.24 $\pm$ 0.16 *	147 $\pm$ 66 (6)	0.855 $\pm$ 0.133	−0.20 $\pm$ 0.21 *
$\alpha 1(W287A)$	292 $\pm$ 81 (5)	0.584 $\pm$ 0.091	−0.65 $\pm$ 0.18	182 $\pm$ 57 (6)	0.747 $\pm$ 0.092	−0.35 $\pm$ 0.16
$\alpha 1(A290C)$	262 $\pm$ 41 (5)	0.596 $\pm$ 0.042	−0.61 $\pm$ 0.08	215 $\pm$ 45 (5)	0.659 $\pm$ 0.082	−0.50 $\pm$ 0.14
$\alpha 1(Y293F)$	311 $\pm$ 140 (5)	0.567 $\pm$ 0.133	−0.70 $\pm$ 0.29	184 $\pm$ 56 (5)	0.735 $\pm$ 0.095	−0.37 $\pm$ 0.16
$\alpha 1(Y293C)$	62 $\pm$ 14 (5)	1.310 $\pm$ 0.164	0.31 $\pm$ 0.14 *	122 $\pm$ 7 (6)	0.899 $\pm$ 0.025	−0.13 $\pm$ 0.03 *
<u><math>\alpha + / \gamma -</math> interface</u>						
$\gamma 2L(I242W)$	314 $\pm$ 44 (5)	0.541 $\pm$ 0.045	−0.73 $\pm$ 0.10	166 $\pm$ 12 (5)	0.766 $\pm$ 0.030	−0.31 $\pm$ 0.05
$\gamma 2L(I242S)$	376 $\pm$ 106 (6)	0.493 $\pm$ 0.099	−0.85 $\pm$ 0.21	174 $\pm$ 12 (5)	0.744 $\pm$ 0.031	−0.35 $\pm$ 0.05
$\gamma 2L(L246W)$ #	150 $\pm$ 23 (5)	0.800 $\pm$ 0.081	−0.27 $\pm$ 0.12 *	115 $\pm$ 5 (5)	0.928 $\pm$ 0.022	−0.09 $\pm$ 0.03 *
$\gamma 2L(L246N)$ #	40 $\pm$ 25 (5)	2.161 $\pm$ 1.150	0.80 $\pm$ 0.55 *	75 $\pm$ 5 (5)	1.205 $\pm$ 0.047	0.22 $\pm$ 0.05 *

The columns give receptor specifics, the modulatory effect (number of oocytes), and the calculated values of *c* and  $\Delta G$ . All values are given as mean  $\pm$  S.D. Modulation is expressed in % of the control response (100% = no effect) to low GABA. For receptors with  $P_{A, \text{constitutive}} > 0.02$  (marked with #), the effects of  $\alpha 7$ -PAMs were measured in the absence of GABA as an effect on holding current, and modulation is calculated as  $P_{A, \alpha 7\text{-PAM}} / P_{A, \text{constitutive}}$ . *c*, the ratio of the equilibrium dissociation constants in active and resting receptors, is a measure of gating efficacy. A value less than one indicates that the compound is an activator.  $\Delta G$  (in kcal/mol) expresses the free energy change contributed by the compound. A negative value indicates that the compound stabilizes the active state. Statistical significance between the effects of  $\alpha 7$ -PAMs in wild-type and mutant receptors was determined by one-way ANOVA (NS-1738:  $F(24,124) = 32.11, p < 0.001$ ; PAM-2:  $F(24,126) = 33.77, p < 0.001$ ), and followed by Dunnett's post-hoc multiple comparisons test (\*,  $p < 0.05$ ).

At the “−” side of the  $\beta + / \alpha -$  interface, the  $\alpha 1(I227W)$  and  $\alpha 1(M235W)$  mutations minimally reduced potentiation or direct activation by  $\alpha 7$ -PAMs. In the  $\alpha 1(I227W)$  mutant, exposure to NS-1738 or PAM-2 increased the response to 0.05–0.08  $\mu$ M GABA ( $P_A = 0.07 \pm 0.03$ ) to 207% of the control (indistinguishable from  $\alpha 1\beta 2\gamma 2L$ ) or 135% ( $p < 0.05$  vs.  $\alpha 1\beta 2\gamma 2L$ ), respectively. The  $\alpha 1(M235W)$  mutant was constitutively active ( $P_{A, \text{const}} = 0.048 \pm 0.025$ , total  $n = 18$ ). Application of NS-1738 increased the  $P_A$  from  $0.053 \pm 0.023$  ( $n = 5$ ) to  $0.096 \pm 0.027$  (196% of the control), and application of PAM-2 increased the  $P_A$  from  $0.042 \pm 0.026$  ( $n = 5$ ) to  $0.071 \pm 0.040$  (174% of the control). Neither differed from the potentiation observed in  $\alpha 1\beta 2\gamma 2L$ . We also tested the effect of the  $\alpha 1(L231C)$  mutation on the  $\beta + / \alpha -$  interface. Potentiation by  $\alpha 7$ -PAMs was not affected by the mutation. These results are described in detail in the next section. In sum, we infer from the mutational-functional experiments that the  $\beta + / \alpha -$  interface participates in the potentiation of the ternary  $\alpha 1\beta 2\gamma 2L$  receptor by  $\alpha 7$ -PAMs.

### 3.3. Cysteine-Modification Experiments Indicate the Binding of $\alpha 7$ -PAMs at the $\beta + / \alpha -$ Interface

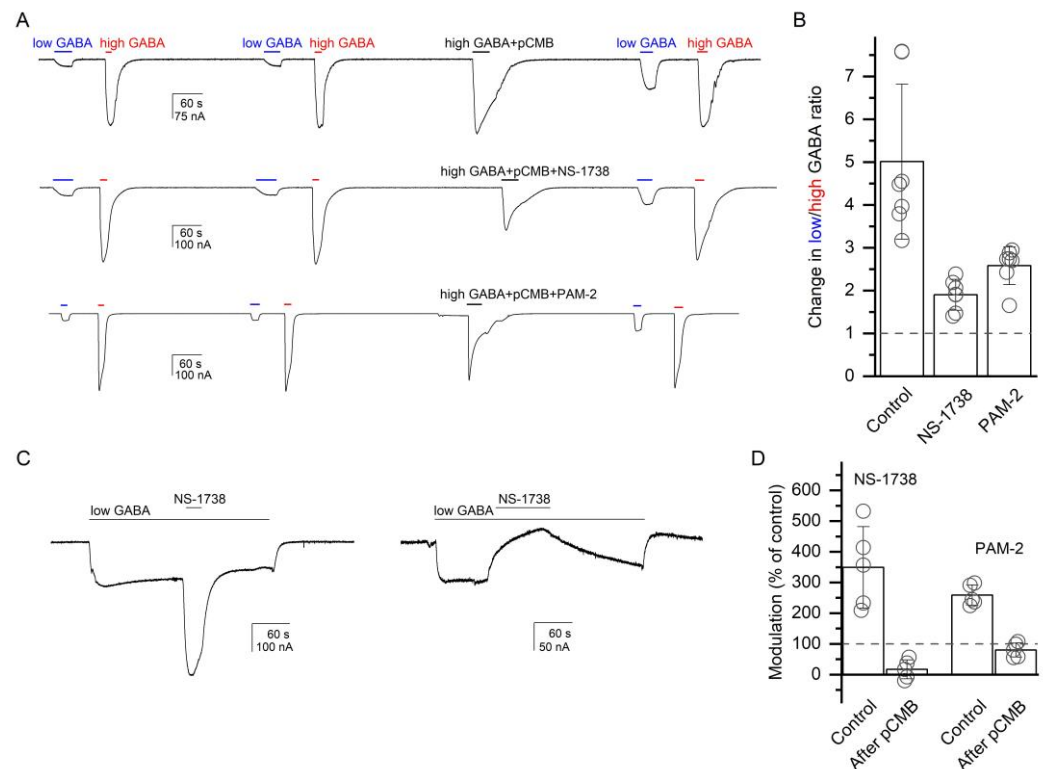
To verify the binding of  $\alpha 7$ -PAMs in the anesthetic binding site at the  $\beta + / \alpha -$  interface, we used substituted cysteine modification-protection (SCAMP). In this approach, a receptor containing a cysteine substitution in the region of interest is exposed to a cysteine-modifying agent whose effect on receptor function is compared in the absence and presence of a protective drug [29,30]. Here, we examined the protective effects of NS-1738 and PAM-2 on the functional effect of *p*-chloromercuribenzoic acid (pCMB) on receptor function. In all experiments, pCMB was co-applied with 1 mM GABA.

The experiments were done on the  $\alpha 1(L231C)\beta 2\gamma 2L$  receptor. We employed two related experimental protocols. In the first, we compared the ratio of low (3  $\mu$ M) and high (1 mM) GABA, before and after exposure to pCMB, which was applied alone (control) or in the presence of NS-1738 or PAM-2. In seven cells, the ratio of low-to-high GABA was  $0.12 \pm 0.04$  before and  $0.57 \pm 0.15$  (a  $5.0 \pm 1.8$ -fold increase) after a 30 s exposure to 25  $\mu$ M pCMB. The increase in the GABA response ratio was due to an increase in the amplitude of the response to low GABA. We interpret this observation as labeling of the  $\alpha 1(L231C)$  residue with pCMB modifying receptor function.

In the presence of 50  $\mu$ M NS-1738, the application of pCMB increased the low-to-high GABA ratio from  $0.12 \pm 0.04$  ( $n = 7$ ) to only  $0.23 \pm 0.09$  (a  $1.9 \pm 0.4$ -fold increase). Additionally, in the presence of 50  $\mu$ M PAM-2, exposure to pCMB enhanced the low-to-high GABA ratio from  $0.12 \pm 0.06$  ( $n = 7$ ) to  $0.31 \pm 0.16$  (a  $2.6 \pm 0.4$ -fold increase). Sample current traces are given in Figure 5A. There was a statistically significant difference between pCMB applied in the absence or presence of  $\alpha 7$ -PAMs as determined by one-way ANOVA ( $F(2,18) = 24.25$ ,  $p < 0.001$ ). A Bonferroni post-hoc test revealed that the effect of pCMB in the absence of  $\alpha 7$ -PAMs was statistically significantly different compared to that in the presence of NS-1738 or PAM-2 ( $p < 0.001$  for each comparison). We infer that NS-1738 and PAM-2 can protect against pCMB-induced labeling of the  $\alpha 1(L231C)$  residue.

In the second experiment, we compared the potentiation of the  $\alpha 1(L231C)\beta 2\gamma 2L$  receptor by  $\alpha 7$ -PAMs before and after exposure to pCMB. We reasoned that if NS-1738 and PAM-2 potentiate the GABA<sub>A</sub> receptor through interactions with the  $\beta + / \alpha -$  interface then labeling of  $\alpha 1(L231C)$  with pCMB may occlude the site and reduce  $\alpha 7$ -PAM-induced potentiation. Application of 50  $\mu$ M NS-1738 potentiated the response to low (3–4  $\mu$ M) GABA ( $P_A = 0.06 \pm 0.02$ ,  $n = 5$ ) to  $349 \pm 133\%$  of the control. Following a 30 s exposure to 50  $\mu$ M pCMB, NS-1738 inhibited the response to low GABA to  $17 \pm 31\%$  ( $n = 5$ ) of the control. As noted above and illustrated in Figure 5A, exposure to pCMB leads to an increase in the relative response to low GABA. Accordingly, GABA concentration in this experiment was lowered to 0.05  $\mu$ M, which generated a response with  $P_A$  of  $0.13 \pm 0.09$  following exposure to pCMB. PAM-2 potentiated the response to 2  $\mu$ M GABA ( $P_A = 0.10 \pm 0.02$ ,  $n = 5$ ) to  $197 \pm 19\%$  of the control. Following exposure to pCMB, exposure to PAM-2 resulted in a reduction ( $80 \pm 23\%$  of the control,  $n = 5$ ) in the current response to 0.05  $\mu$ M GABA ( $P_A = 0.13 \pm 0.11$ ). We conclude that the  $\beta + / \alpha -$  interface mediates receptor potentiation by NS-1738 and PAM-2, and that occlusion of the site at this interface by pCMB abolishes potentiation.

In control experiments on the wild-type  $\alpha 1\beta 2\gamma 2L$  receptor, a 30 s exposure to 100  $\mu$ M pCMB was without effect on the  $P_A$  of the response to low GABA and the magnitude of potentiation in the presence of NS-1738 or PAM-2. In five cells, 50  $\mu$ M NS-1738 potentiated the response to low GABA to  $253 \pm 72\%$  of the control before exposure to pCMB and to  $249 \pm 57\%$  of the control after exposure to pCMB. The  $P_A$  of the response to low (2–3  $\mu$ M) GABA was  $0.11 \pm 0.08$  before and  $0.09 \pm 0.02$  after exposure to pCMB. In a different set of five cells, 50  $\mu$ M PAM-2 potentiated the response to GABA to  $154 \pm 15\%$  of the control before exposure to pCMB and to  $157 \pm 18\%$  of the control after exposure to pCMB. The  $P_A$  of the responses to low (1–3  $\mu$ M) GABA were  $0.16 \pm 0.10$  and  $0.14 \pm 0.08$  before and after pCMB, respectively. We infer that pCMB does not functionally modify native cysteines in the  $\alpha 1\beta 2\gamma 2L$  receptor.



**Figure 5.** Chemical modification of the  $\alpha 1(L231C)$  residue at the  $\beta + / \alpha -$  interface modifies receptor activation by GABA and modulation by  $\alpha 7$ -PAMs. (A) Exposure to pCMB (30 s, 25  $\mu M$ ) increases the ratio of responses to low (3  $\mu M$ ) and high (1 mM) GABA in the  $\alpha 1(L231C)\beta 2\gamma 2L$  receptor. The pCMB-induced increase in the low-to-high GABA ratio is reduced when pCMB is co-applied with 50  $\mu M$  NS-1738 (middle trace) or PAM-2 (bottom trace). The data are summarized in the column graph (B). We infer that chemical modification of the  $\alpha 1(L231C)$  residue modifies responses to the transmitter and that co-application of an  $\alpha 7$ -PAM protects against labeling with pCMB. (C) Exposure to 50  $\mu M$  NS-1738 potentiates the response to 4  $\mu M$  GABA ( $P_A = 0.03$ ) in the  $\alpha 1(L231C)\beta 2\gamma 2L$  receptor (left trace). NS-1738 inhibits the response to 0.05  $\mu M$  GABA ( $P_A = 0.01$ ) following a 30 s exposure to 25  $\mu M$  pCMB. The traces are from different oocytes. Drug application durations are given with horizontal lines. The data for NS-1738 and PAM-2 are summarized in the column graph (D). We infer that pCMB-labeling of the  $\alpha 1(L231C)$  residue abolishes receptor potentiation by  $\alpha 7$ -PAMs.

### 3.4. Mutations to the Combinations of $\alpha + / \beta -$ , $\gamma + / \beta -$ , and $\alpha + / \gamma -$ Interfaces Affect Receptor Activation and Potentiation by $\alpha 7$ -PAMs

Mutations unique to the  $\alpha + / \beta -$  and  $\gamma + / \beta -$  interfaces can be made by mutating the “-” side (TM1 domain) of the  $\beta 2$  subunit. Specifically, we tested the effects of the L223W, Q224A, and M227W mutations on the  $\beta 2$  subunit. In addition, we mutated the L275, T277, T281, and F304 residues at the “+” side of the  $\gamma 2L$  subunit. These residues are unique to the  $\gamma + / \beta -$  interface. At the “+” side of the  $\alpha 1$  subunit, we mutated the T264, T266, W287, A290, and Y293 residues, which introduced mutations to the  $\alpha + / \beta -$  interface and, additionally, to the  $\alpha + / \gamma -$  interface.

At the “-” side of the  $\beta 2$  subunit, potentiation by NS-1738 was not affected by any of the tested mutations. PAM-2 inhibited responses to GABA in  $\beta 2(L223W)$ , reducing the response to low GABA ( $P_A = 0.26 \pm 0.13$ ,  $n = 5$ ) to 48% of the control. The constitutively active ( $P_{A, \text{constitutive}} = 0.15 \pm 0.07$ ,  $n = 10$ )  $\gamma 2L(F304C)$  mutation to the  $\gamma + / \beta -$  interface converted NS-1738 (34% of the control) and PAM-2 (40% of the control) into inhibitory compounds, while  $\gamma 2L(T277I)$  ( $P_{A, \text{constitutive}} = 0.08 \pm 0.05$ ,  $n = 10$ ) increased potentiation by NS-1738 (511% of the control) but reduced potentiation by PAM-2 (117% of the control) (Table 1).

At the “+” side of the  $\alpha 1$  subunit (mutations to  $\alpha +/\beta -$  and  $\alpha +/\gamma -$  interfaces), the  $\alpha 1(Y293C)$  mutation turned NS-1738 into an inhibitory compound (62% of the control) and reduced the potentiating effect of PAM-2 (122% of the control). The  $\alpha 1(Y293F)$  mutation did not affect the actions of either drug (Table 1), which is suggestive of continued aromatic interactions between the  $\alpha 7$ -PAMs and the  $\alpha 1(Y/F293)$  residue. We conclude that the  $\alpha +/\beta -$ ,  $\gamma +/\beta -$ , and/or  $\alpha +/\gamma -$  interfaces contribute to GABA<sub>A</sub> receptor potentiation by NS-1738 and PAM-2.

### 3.5. Mutations to the $\alpha +/\gamma -$ Interface Affect Receptor Activation and Potentiation by $\alpha 7$ -PAMs

Mutations to the “+” side of the  $\alpha 1$  subunit described above do not allow distinction between the  $\alpha +/\beta -$  and  $\alpha +/\gamma -$  interfaces. To mutate solely the  $\alpha +/\gamma -$  interface, we tested the effects of mutations on the “-” side of the  $\gamma 2L$  subunit. The  $\gamma 2L(I242)$  residue was mutated to a serine and a tryptophan, and the  $\gamma 2L(L246)$  residue was mutated to an asparagine and a tryptophan. The  $\gamma 2L(I242W)$  and  $\gamma 2L(I242S)$  mutations were without effect on the potentiating actions of NS-1738 and PAM-2 (Table 1). The  $\gamma 2(L246N)$  and  $\gamma 2L(L246W)$  mutations, which showed considerable constitutive activity ( $P_{A, \text{const}} = 0.25 \pm 0.05$  and  $0.07 \pm 0.04$ , respectively), were tested for direct activation by NS-1738 and PAM-2. Constitutive activity in the  $\gamma 2(L246N)$ -containing receptor was inhibited by NS-1738 to 40% of the control and by PAM-2 to 75% of the control. In the  $\gamma 2(L246W)$ -containing receptor, constitutive activity was enhanced to 150% of the control in the presence of NS-1738 and to 115% of the control in the presence of PAM-2. The calculated  $c_{\alpha 7\text{-PAM}}$  and  $\Delta G$  values are given in Table 1. Overall, we conclude that the  $\alpha +/\gamma -$  interface contributes to the actions of  $\alpha 7$ -PAMs.

### 3.6. Effects of Mutations to Intersubunit Interfaces on Receptor Activation and Potentiation by Selected Anesthetics

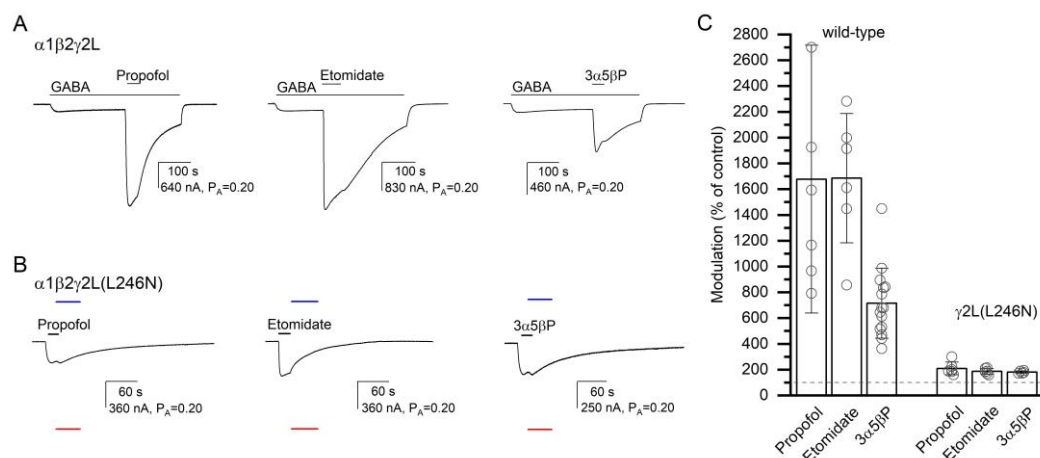
The high-affinity binding sites for the anesthetics propofol and etomidate are located in the transmembrane domain at the  $\beta +/\alpha -$  intersubunit interfaces. Additionally, propofol binds to homologous pockets at the  $\alpha +/\beta -$  and  $\gamma +/\beta -$  interfaces [23,34,35]. Potentiating neuroactive steroids make a major contribution through a site near the cytosolic end of the transmembrane helices at the  $\beta +/\alpha -$  interface [36,37].

To gain insight into the specificity of the effect of the  $\gamma 2L(L246N)$  ( $\alpha +/\gamma -$  interface) mutation on receptor activation by  $\alpha 7$ -PAMs, we recorded activation of the  $\alpha 1\beta 2\gamma 2L(L246N)$  receptor by propofol, etomidate, or  $3\alpha 5\beta P$ . Previous work has suggested that the anesthetics or a neuroactive steroid do not interact with the  $\alpha +/\gamma -$  interface [30]. In six cells exposed to propofol and etomidate, and for normalization purposes to 1 mM GABA + 50  $\mu M$  propofol and 200  $\mu M$  picrotoxin, 50  $\mu M$  propofol or etomidate increased channel activity from a  $P_{A, \text{constitutive}}$  of  $0.33 \pm 0.07$  to a  $P_A$  of  $0.69 \pm 0.16$  (209% of the control; S.D. and numbers of oocytes are given in Table 2) or  $0.63 \pm 0.17$  (187% of the control), respectively. With the postulated numbers of binding sites of four for propofol and two for etomidate, the calculated  $c_{50 \mu M \text{ drug}}$  are 0.677 ( $\Delta G = -0.96$  kcal/mol) and 0.525 ( $\Delta G = -0.87$  kcal/mol) for propofol and etomidate, respectively. For comparison, in the  $\alpha 1\beta 2\gamma 2L$  receptor, 50  $\mu M$  propofol increased the response to 1–2  $\mu M$  GABA ( $P_A = 0.07 \pm 0.04$ ;  $n = 6$ ) to 1677% of the control ( $P_{A, 50 \mu M \text{ propofol}} = 0.86 \pm 0.08$ ,  $c_{50 \mu M \text{ propofol}} = 0.302$ ,  $\Delta G = -2.84$  kcal/mol), and co-application of 50  $\mu M$  etomidate increased the response to low GABA ( $P_A = 0.05 \pm 0.02$ ,  $n = 6$ ) to 1685% of the control ( $P_{A, 50 \mu M \text{ etomidate}} = 0.75 \pm 0.06$ ,  $c_{50 \mu M \text{ etomidate}} = 0.129$ ,  $\Delta G = -2.45$  kcal/mol). The  $\Delta G$  values for both propofol and etomidate are statistically significantly different ( $t$ -test,  $p < 0.001$  for each comparison) in  $\alpha 1\beta 2\gamma 2L$  and the  $\gamma 2(L246N)$  mutant. Sample current traces and data summary are given in Figure 6 and Table 2.

**Table 2.** Summary of effects of the  $\gamma 2(L246N)$  mutation to the  $\alpha + / \gamma -$  interface on GABA<sub>A</sub> receptor potentiation or direct activation by propofol, etomidate, and 3 $\alpha 5\beta$ P.

Receptor	Modulation, 50 $\mu$ M Propofol, % of Control ( <i>n</i> )	$c_{50 \mu\text{M propofol}}$	$\Delta G_{50 \mu\text{M propofol}}$ , kcal/mol	Modulation, 50 $\mu$ M Etomidate, % of Control ( <i>n</i> )	$c_{50 \mu\text{M etomidate}}$	$\Delta G_{50 \mu\text{M etomidate}}$ , kcal/mol	Modulation, 10 $\mu$ M 3 $\alpha 5\beta$ P, % of Control ( <i>n</i> )	$c_{10 \mu\text{M } 3\alpha 5\beta \text{P}}$	$\Delta G_{10 \mu\text{M } 3\alpha 5\beta \text{P}}$ , kcal/mol
$\alpha 1\beta 2\gamma 2\text{L}$	1677 $\pm$ 1039 (6)	0.302 $\pm$ 0.039	-2.84 $\pm$ 0.33	1685 $\pm$ 501 (6)	0.129 $\pm$ 0.030	-2.45 $\pm$ 0.27	715 $\pm$ 271 (15)	0.303 $\pm$ 0.069	-1.45 $\pm$ 0.34
$\gamma 2\text{L}(L246\text{N})^\#$	209 $\pm$ 49 (6)	0.677 $\pm$ 0.122	-0.96 $\pm$ 0.45 *	187 $\pm$ 22 (6)	0.525 $\pm$ 0.184	-0.87 $\pm$ 0.63	181 $\pm$ 9 (5)	0.604 $\pm$ 0.050	-0.60 $\pm$ 0.10

The columns give receptor specifics, the modulatory effect (number of oocytes), and the calculated values of *c* and  $\Delta G$ . All values are given as mean  $\pm$  S.D. Modulation is expressed in % of the control response (100% = no effect) to low GABA in  $\alpha 1\beta 2\gamma 2\text{L}$ . For the constitutively active ( $\#$ )  $\gamma 2(L246\text{N})$ -containing receptor, the effects were measured in the absence of GABA as an effect on holding current, and modulation is calculated as  $P_{A,\text{compound}}/P_{A,\text{constitutive}}$ , the ratio of the equilibrium dissociation constants in active and resting receptors, is a measure of gating efficacy. A value less than one indicates that the compound is an activator. The number of imposed binding sites was four for propofol and two for etomidate and 3 $\alpha 5\beta$ P.  $\Delta G$  expresses the free energy change contributed by the compound. A negative value indicates that the compound stabilizes the active state. Statistical significance between the effects of the compounds in wild-type and mutant receptors was determined by *t*-test (\*,  $p < 0.001$ ).



**Figure 6.** The modulatory effects of propofol, etomidate, and 3 $\alpha 5\beta$ P. The current traces show the potentiating effects of 50  $\mu$ M propofol, 50  $\mu$ M etomidate, or 10  $\mu$ M 3 $\alpha 5\beta$ P in the  $\alpha 1\beta 2\gamma 2\text{L}$  receptor activated by 2–4  $\mu$ M GABA (A) or the direct-activating effects of the compounds in the  $\alpha 1\beta 2\gamma 2\text{L}(L246\text{N})$  receptor (B). The blue and red lines show the current levels in the same cell in the presence of 200  $\mu$ M picrotoxin (assumed  $P_A = 0$ ) or 1 mM GABA + 50  $\mu$ M propofol (assumed  $P_A = 1$ ), respectively. The modulatory effects are summarized in the column graph (C). The effects of the compounds are significantly reduced in the mutant receptor. In (A), modulation is calculated as the amplitude of the peak response to GABA +  $\alpha 7$ -PAM divided by the amplitude of the response to GABA alone at the time of peak response to GABA +  $\alpha 7$ -PAM. In (B), modulation is calculated as the ratio of the estimated  $P_A$  in the presence and absence of  $\alpha 7$ -PAM. Drug application durations are given with horizontal lines.

We also recorded receptor activation and potentiation by the steroid 3 $\alpha 5\beta$ P. In five cells expressing  $\alpha 1\beta 2\gamma 2\text{L}(L246\text{N})$  receptors, the application of 10  $\mu$ M 3 $\alpha 5\beta$ P increased the  $P_A$  from  $0.29 \pm 0.06$  to  $0.53 \pm 0.11$  (181% of the control). Sample current traces are given in Figure 6. The calculated  $c_{10 \mu\text{M } 3\alpha 5\beta \text{P}}$  is 0.604, and the  $\Delta G$  is  $-0.60$  kcal/mol. For comparison, in the  $\alpha 1\beta 2\gamma 2\text{L}$  wild-type receptor, co-application of 10  $\mu$ M 3 $\alpha 5\beta$ P increased the response to low GABA ( $P_A = 0.07 \pm 0.04$ ,  $n = 15$ ) to 715% of the control ( $c_{10 \mu\text{M } 3\alpha 5\beta \text{P}} = 0.303$  and  $\Delta G = -1.45$  kcal/mol). The effects are statistically significantly different (*t*-test,  $p < 0.001$ ). In sum, we infer that the  $\gamma 2\text{L}(L246\text{N})$  mutation at the  $\alpha + / \gamma -$  interface disrupts the potentiating/activating effects of propofol, etomidate, and 3 $\alpha 5\beta$ P.

To determine if other intersubunit interfaces similarly influence the actions of 3 $\alpha 5\beta$ P, we recorded steroid-mediated potentiation or direct activation in receptors containing mutations to the anesthetic-binding interfaces. The  $\beta 2(F289\text{A})$  mutation at the  $\beta + / \alpha -$

interface, and the  $\gamma 2L(T277I)$  and  $\gamma 2L(F304C)$  mutations at the  $\gamma + / \beta -$  interface, significantly reduced receptor activation by  $3\alpha 5\beta P$ . The magnitudes of effects, however, were relatively small (Table 3), which is suggestive of a less prominent interplay between the anesthetic-binding interfaces and the neurosteroid binding sites.

**Table 3.** Summary of effects of mutations on GABA<sub>A</sub> receptor potentiation or direct activation by  $3\alpha 5\beta P$ .

Receptor	Modulation, 10 $\mu M$ $3\alpha 5\beta P$ , % of Control (n)	$c_{10 \mu M 3\alpha 5\beta P}$	$\Delta G_{10 \mu M 3\alpha 5\beta P}$ , kcal/mol
$\alpha 1\beta 2\gamma 2L$	733 $\pm$ 183 (15)	0.330 $\pm$ 0.038	−1.31 $\pm$ 0.14
	<u><math>\beta + / \alpha -</math> interface</u>		
$\beta 2(F289A)$ #	463 $\pm$ 118 (5)	0.453 $\pm$ 0.043	−0.94 $\pm$ 0.12 *
$\alpha 1(M235W)$ #	614 $\pm$ 116 (6)	0.304 $\pm$ 0.046	−1.42 $\pm$ 0.19
	<u><math>\gamma + / \beta -</math> interface</u>		
$\gamma 2L(T277I)$ #	471 $\pm$ 262 (5)	0.473 $\pm$ 0.110	−0.91 $\pm$ 0.29 *
$\gamma 2L(F304C)$ #	255 $\pm$ 31 (6)	0.493 $\pm$ 0.091	−0.86 $\pm$ 0.26 *
	<u><math>\alpha + / \beta -</math> and <math>\gamma + / \beta -</math> interfaces</u>		
$\beta 2(L223W)$ #	653 $\pm$ 186 (5)	0.298 $\pm$ 0.057	−1.45 $\pm$ 0.22
	<u><math>\alpha + / \gamma -</math> and <math>\alpha + / \beta -</math> interfaces</u>		
$\alpha 1(Y293C)$	666 $\pm$ 209 (5)	0.266 $\pm$ 0.055	−1.58 $\pm$ 0.23
	<u><math>\alpha + / \gamma -</math> interface</u>		
$\gamma 2L(L246N)$ #	181 $\pm$ 9 (5)	0.604 $\pm$ 0.050	−0.60 $\pm$ 0.10 *

The columns give receptor specifics, the modulatory effect (number of oocytes), and the calculated values of  $c$  and  $\Delta G$ . All values are given as mean  $\pm$  S.D. Modulation is expressed in % of the control response (100% = no effect) to low GABA. For receptors with  $P_{A, \text{constitutive}} > 0.02$  (marked with #), the effect of  $3\alpha 5\beta P$  was measured in the absence of GABA as an effect on holding current, and modulation is calculated as  $P_{A, \alpha 7\text{-PAM}} / P_{A, \text{constitutive}}$ .  $c$ , the ratio of the equilibrium dissociation constants in active and resting receptors, is a measure of gating efficacy. A value less than one indicates that the compound is an activator. The number of imposed steroid binding sites was two.  $\Delta G$  (in kcal/mol) expresses the free energy change contributed by the compound. A negative value indicates that the compound stabilizes the active state. Statistical significance between the effects of  $\alpha 7\text{-PAMs}$  in wild-type and mutant receptors was determined by one-way ANOVA ( $F(7,44) = 11.43, p < 0.001$ ), and followed by Dunnett's post-hoc multiple comparisons test (\*,  $p < 0.01$ ). Data on  $\gamma 2L(L246N)$  is replicated from Table 2.

### 3.7. The Effects of Mutations Indicate Lack of Additivity and Independence between Intersubunit Anesthetic Binding Sites

Results from mutated receptors indicate that mutations to each of the intersubunit interfaces affect potentiation by NS-1738 and PAM-2. To estimate the energetic additivity of the effects of mutations, we employed mutant cycle analysis in which the free energy change ( $\Delta G_{\alpha 7\text{-PAM, WT}}$ ) in the wild-type receptor was compared with the sum of changes in free energy changes ( $\Delta \Delta G_{\alpha 7\text{-PAM, MT}}$ ) in receptors containing mutations to individual interfaces. In the case of energetic additivity,  $\Delta G = -\sum \Delta \Delta G_i$ .

We selected the  $\beta 2(F289T)$  mutation to the two  $\beta + / \alpha -$  interfaces, the  $\alpha 1(Y293C)$  mutation to the  $\alpha + / \beta -$  and  $\alpha + / \gamma -$  interfaces, and the  $\gamma 2L(F304C)$  mutation to the  $\gamma + / \beta -$  interface. For NS-1738, the  $\Delta \Delta G$ s with 95% confidence intervals are 1.92 [1.50 to 2.34] kcal/mol, 1.01 [0.86 to 1.16] kcal/mol, and 1.44 [1.17 to 1.71] kcal/mol for mutations in  $\beta + / \alpha -$ ,  $\alpha + / \beta -$  and  $\alpha + / \gamma -$ , and  $\gamma + / \beta -$ , respectively. The sum of  $\Delta \Delta G$ s is 4.37 [3.85 to 4.89] kcal/mol. The 95% confidence interval for  $-\sum \Delta \Delta G_i$  does not include the inverse of  $\Delta G_{\text{NS-1738, WT}}$  (0.70 kcal/mol, Table 1), indicating that the effects of mutations are not additive or independent. Intuitively, this is evident from the observation that rather than the individual mutations incrementally reducing the potentiating effect of NS-1738 and the sum of effects of mutations predicting a complete loss of potentiation, each of the individual mutations actually leads to functional inhibition in the presence of NS-1738.

For PAM-2, the  $\Delta \Delta G$ s with 95% confidence intervals are 0.44 [0.28 to 0.60] kcal/mol, 0.26 [0.21 to 0.31] kcal/mol, and 1.05 [0.80 to 1.30] kcal/mol for  $\beta 2(F289T)$ ,  $\alpha 1(Y293C)$ , and  $\gamma 2L(F304C)$ , respectively. The sum of  $\Delta \Delta G$ s is 1.75 [1.45 to 2.05] kcal/mol. The 95% confidence interval for  $-\sum \Delta \Delta G_i$  does not include the inverse of  $\Delta G_{\text{PAM-2, WT}}$  (0.39, Table 1), indicating that the effects of mutations are not additive or independent.

#### 4. Discussion

Our molecular docking and molecular dynamics studies indicate that the  $\alpha 7$ -PAMs NS-1738 and PAM-2 bind to the intersubunit interfaces in the transmembrane domain of the GABA<sub>A</sub> receptor. This is supported by previous functional data demonstrating a reduction or loss of potentiation by  $\alpha 7$ -PAMs of receptors activated by drugs binding to the anesthetic binding sites in the transmembrane domain [22]. In the present study, we have also shown that NS-1738 and PAM-2 protect against pCMB-induced chemical modification of the  $\alpha 1$ (L231C) residue at the  $\beta + / \alpha -$  interface.

The major finding from our mutational analysis is that mutations to the putative binding sites at individual intersubunit interfaces can strongly alter the modulatory effect of  $\alpha 7$ -PAMs, with effects ranging from a significant increase in potentiation to inhibition of receptor function. This is surprising and unexpected if we assume that the mutations only act locally without modifying drug interactions at other interfaces or global receptor functions. With a few exceptions, the mutations similarly affected receptor potentiation by NS-1738 and PAM-2. The exceptions were  $\beta 2$ (L223W) at the  $\alpha + / \beta -$  and  $\gamma + / \beta -$  interfaces, which allowed for potentiation by NS-1738 but converted PAM-2 into an inhibitory compound, and  $\alpha 1$ (Y293C), which caused NS-1738 to inhibit receptor function but merely reduced the ability of PAM-2 to potentiate. We considered the possibility that  $\alpha 7$ -PAMs act as potentiators of the  $\alpha 1\beta 2\gamma 2L$  GABA<sub>A</sub> receptor through one or more interfaces and as inhibitors through other interfaces. For example, the  $\beta 2$ (F289T) mutant or the chemical modification of the  $\alpha 1$ (L231C) residue (both at the  $\beta + / \alpha -$  interface) result in NS-1738 inhibiting receptor function, potentially suggesting that the  $\beta + / \alpha -$  interface mediates potentiation whereas the remaining, intact interfaces mediate receptor inhibition. This, however, is contradicted by the observation that mutations to other interfaces ( $\gamma 2L$ (F304C) to  $\gamma + / \beta -$ ,  $\gamma 2L$ (L246N) to  $\alpha + / \gamma -$ , and  $\alpha 1$ (Y293C) to  $\alpha + / \beta -$  and  $\alpha + / \gamma -$  interfaces) could also reverse the polarity of effect.

The data summarized in Table 1 indicate that the effects of mutations to individual interfaces are not local and independent, or, alternatively, that the binding sites for  $\alpha 7$ -PAMs are allosterically linked. In the case of local and independent action, individual mutations may be expected to have an incremental effect on overall potentiation, while complete loss of potentiation may be observed when there are no intact sites remaining. Such a scenario has been observed previously with mutations affecting GABA<sub>A</sub> receptor activation by the transmitter or potentiation of receptor function by propofol, etomidate, or allopregnanolone [38–41]. Instead, we observed that a single mutation to any individual interface could fully abolish potentiation by either  $\alpha 7$ -PAM. Possible explanations for the findings are long-range effects of mutations and effects on the channel gating process shifting the equilibrium between resting and active states. The latter idea is supported by the finding that the  $\gamma 2L$ (L246N) mutation additionally reduces the gating efficacy of the anesthetics propofol and etomidate and the neurosteroid  $3\alpha 5\beta P$ , neither of which is expected to bind at the  $\alpha + / \gamma -$  interface (Table 2). Furthermore, the tryptophan substitution of this residue reduces the gating efficacy of the etomidate and the transmitter GABA [30].

In previous work, mutations to anesthetic sites at the  $\beta + / \alpha -$ ,  $\gamma + / \beta -$ , and  $\alpha + / \beta -$  interfaces modified receptor potentiation by the anesthetic drugs propofol, etomidate, and a barbiturate as well as activation by the transmitter GABA [19,30]. Similar to what we have observed with NS-1738 and PAM-2, the sum of losses of potentiating effects in individual mutants was consistently greater than the potentiating effect observed in the wild-type receptor, which is potentially indicative of allosteric crosstalk between the interfaces. GABA<sub>A</sub> receptor potentiation by neuroactive steroids is mediated by two classes of membraneous binding sites, one at the  $\beta + / \alpha -$  intersubunit interface and the other within the  $\alpha$  subunit [37]. Mutations to the two classes of binding sites have been shown to demonstrate independence and additivity in the  $\alpha 1\beta 2\gamma 2L$  receptor and lack of it in the  $\alpha 1\beta 3$  receptor [41,42]. Mutation of either one of the two transmitter binding sites reduces gating efficacy for GABA approximately, equally, and independently [38].

The conclusions of this study ultimately arise from the comparison of the magnitude of potentiation at a single concentration of a compound. The concentrations of the  $\alpha 7$ -PAMs, anesthetics, and  $3\alpha 5\beta P$  used are saturating in the wild-type receptor [14,15,18,22,43], and thus reflect maximal potentiation and a true value of  $c_{\text{compound}}$ . There is, however, no definite evidence that the same concentrations of the compounds are saturating in the mutant receptors. Thus, the estimated  $c_{\text{compound}}$  in mutant receptors should be treated as “effective”  $c_{\text{compound}}$ , while the changes in  $c_{\text{compound}}$  and  $\Delta G$  presented in Tables 1–3 may be due to altered affinity, efficacy, or both.

PAMs of the  $\alpha 7$  nicotinic receptor may be clinically useful in the treatment of cognitive symptoms in schizophrenia. In rodents, administration of  $\alpha 7$ -PAMs such as PNU-120596 or PAM-2 improves the auditory gating deficit caused by amphetamine and recognition memory and cognitive flexibility in the MK-801 model of schizophrenia [44–46]. The present, as well as prior [22], data indicate that  $\alpha 7$ -PAMs can potentiate several common subtypes of the GABA<sub>A</sub> receptor through interactions with the classic anesthetic binding sites. However, given their relatively low efficacies,  $\alpha 7$ -PAMs are expected to competitively inhibit GABA<sub>A</sub> receptor potentiation by propofol or etomidate and any resulting behavior.

## 5. Conclusions

The PAMs of the  $\alpha 7$  nicotinic receptor, NS-1738 and PAM-2, potentiate the GABA<sub>A</sub> receptor through interactions with the classic anesthetic binding sites. Mutations to each of the four classes of intersubunit interfaces in the transmembrane domain of the  $\alpha 1\beta 2\gamma 2L$  receptor can modify potentiation. Overall, there was good agreement between predictions made based on molecular docking studies, SCAMP, and mutational-functional analysis. Comparison of the magnitudes of effects of mutations to the individual intersubunit interfaces suggests that the sites mediating the actions of  $\alpha 7$ -PAMs are allosterically linked.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biom13040698/s1>, pdb structures: PDB files showing docked NS-1738 and PAM-2 at each of the four interfaces ( $\beta + / \alpha -$ ,  $\alpha + / \beta -$ ,  $\alpha + / \gamma -$ , and  $\gamma + / \beta -$ ) in the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor.

**Author Contributions:** Conceptualization, H.R.A. and G.A.; formal analysis, S.R.P., A.L.G., S.Q.X., S.L.M. and M.O.O.; investigation, S.R.P., A.L.G., S.Q.X., S.L.M. and M.O.O.; writing—original draft, G.A.; writing—review and editing, S.R.P., A.L.G., S.Q.X., M.O.O. and H.R.A.; project administration, M.O.O. and G.A.; funding acquisition, H.R.A. and G.A. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

$3\alpha 5\beta P$	5 $\beta$ -pregnan-3 $\alpha$ -ol-20-one
$c$	ratio of the equilibrium dissociation constant in the active state to that in the resting state (a measure of efficacy)
$\Delta G$	free energy change
GABA	$\gamma$ -aminobutyric acid



GABA <sub>A</sub>	γ-aminobutyric acid type A
K <sub>R</sub>	equilibrium dissociation constant of a compound in the resting receptor
NS-1738	N-(5-Cl-2-hydroxyphenyl)-N'-[2-Cl-5-(trifluoromethyl)phenyl]-urea
P <sub>A</sub>	probability of being in the active state;
PAM	positive allosteric modulator
PAM-2	(E)-3-(furan-2-yl)-N-(p-tolyl)-acrylamide.

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