The fifth subunit of the (α4β2)₂β2 nicotinic ACh receptor modulates maximal ACh responses

BACKGROUND AND PURPOSE
The fifth subunit in the (α4β2)₂α4 nicotinic ACh receptor (nAChR) plays a determining role in the pharmacology of this nAChR type. Here, we have examined the role of the fifth subunit in the ACh responses of the (α4β2)₂β2 nAChR type.

EXPERIMENTAL APPROACH
The role of the fifth subunit in receptor function was explored using two-electrode voltage clamp electrophysiology, along with subunit-targeted mutagenesis and the substituted cysteine scanning method applied to fully linked (α4β2)₂β2 receptors.

KEY RESULTS
Covalent modification of the cysteine-substituted fifth subunit with a thiol-reactive agent (MTS) caused irreversible inhibition of receptor function. ACh reduced the rate of the reaction to MTS, but the competitive inhibitor dihydro-β-erythroidine had no effect. Alanine substitution of conserved residues that line the core of the agonist sites on α4(+)/β2(−) interfaces did not impair receptor function. However, impairment of agonist binding to α4(+)/β2(−) agonist sites by mutagenesis modified the effect of ACh on the rate of the reaction to MTS. The extent of this effect was dependent on the position of the agonist site relative to the fifth subunit.

CONCLUSIONS AND IMPLICATIONS
The fifth subunit in the (α4β2)₂β2 receptor isoform modulates maximal ACh responses. This effect appears to be driven by a modulatory, and asymmetric, association with the α4(+)/β2(−) agonist sites.

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Abbreviations
ABS, agonist-binding site; DhβE, dihydro-β-erythroidine; ECD, extracellular domain; MTS, methanethiosulfonate or thiol-reactive reagent; MTSET, methanethiosulfonate reagent [2-(trimethylammonium) ethyl] methanethiosulfonate; nAChR, nicotinic ACh receptor; pLGIC, pentameric ligand-gated ion channel; SCAM, substituted cysteine accessibility method; TMD, transmembrane domain
**Introduction**

The \(\alpha 4\beta 2\) nicotinic \(\text{ACh}\) receptor (nAChR) is the most prevalent type of nAChR in the brain (Gotti et al., 2009), and this type is a key mediator of the rewarding and reinforcing effects of nicotine (Tapper et al., 2004; Maskos et al., 2005). The \(\alpha 4\beta 2\) nAChR is a member of the pentameric ligand-gated ion channel (pLGIC) superfamily of neurotransmitter receptors that includes the muscle nAChR, GABA\(\alpha\), glycine and 5-HT\(_3\) receptors. Work on the muscle nAChR has shown that agonist binding in these proteins triggers rigid body motions, which are transduced into transient changes that drive channel gating upon agonist binding (Unwin and Fujiyoshi, 2012). The most recent cryo-electron microscopy studies of the Torpedo nAChR have suggested that the fifth subunit (\(\beta 1\)-\(\beta 2\) loop, the Cys loop and M2–M3 linker) at the interface between the TMD and the N-terminal extracellular domain (ECD) (Lee and Sine, 2005; Jha et al., 2007; Lee et al., 2009). The most recent cryo-electron microscopy studies of the Torpedo nAChR have suggested that the fifth subunit (\(\beta 1\) subunit), a non-agonist-binding subunit, might play a critical role in receptor activation by being part of the pathway transmitting to the TMD the conformational changes that drive channel gating upon agonist binding (Unwin and Fujiyoshi, 2012).

The \(\alpha 4\beta 2\) nAChR comprises two \(\alpha 4\beta 2\) pairs and a fifth subunit that can be \(\beta 2\) or \(\alpha 4\), and this subunit difference produces two alternate receptor isoforms, the \((\alpha 4\beta 2)_2\beta 2\) and \((\alpha 4\beta 2)_2\alpha 4\) nAChRs (Nelson et al., 2003; Moroni et al., 2006) (Figure 1A). The alternative receptors display strikingly different sensitivities to activation by \text{ACh} and other agonists (Nelson et al., 2003; Moroni et al., 2006; Harpsøe et al., 2011; Mazzaferrro et al., 2011; Timmermann et al., 2012; Absalom et al., 2013; Lucero et al., 2016), high-affinity desensitization (Marks et al., 2010; Benallegue et al., 2013), sensitivity to allosteric modulators (Moroni et al., 2008; Olsen et al., 2013; Alcaino et al., 2017; Jin et al., 2017) and single-channel properties (Mazzaferrro et al., 2017). These differences are accounted for partly by an additional operational agonist site in the \((\alpha 4\beta 2)_2\alpha 4\) stoichiometry housed by the interface between the fifth subunit (\(\alpha 4\) and an adjacent \(\alpha 4\) subunit (Harpsøe et al., 2011; Mazzaferrro et al., 2011). A triad of non-conserved E loop residues on the complementary side of the agonist site on the \((\alpha 4(+)/\alpha 4(\alpha))\) interface has been identified as critical in determining the agonist sensitivity differences between the \((\alpha 4\beta 2)_2\beta 2\) and \((\alpha 4\beta 2)_2\alpha 4\) receptors: \(\alpha 4\)H142, \(\alpha 4\)Q150 and \(\alpha 4\)T152 (Harpsøe et al., 2011; Lucero et al., 2016). The fifth subunit in the \((\alpha 4\beta 2)_2\beta 2\) isoform (\(\beta 2\) forms the receptor’s signature \(\beta 2(+)/\beta 2(\alpha)\) interface with an adjacent \(\beta 2\) subunit (Figure 1A). In contrast to the \((\alpha 4\beta 2)_2\alpha 4\) receptors, transferring the \(\alpha 4\) E loop to the fifth subunit in the \((\alpha 4\beta 2)_2\beta 2\) does not affect \text{ACh} sensitivity (Lucero et al., 2016).

Previously, we found that the agonist sites on the \((\alpha 4(+)/\beta 2(\alpha)\) interfaces in the \((\alpha 4\beta 2)_2\alpha 4\) receptor responded differently to alanine substitutions of conserved aromatic residues, suggesting that this type of agonist sites may function asymmetrically, despite their structural equivalency (Mazzaferrro et al., 2011). A more recent study examined this possibility in detail in both receptor isoforms by transferring the triplet of \(\alpha 4\) non-conserved E loop residues to the \(\beta 2\) subunit, and vice versa (Lucero et al., 2016). Although this study did not find evidence of functional asymmetry in the \((\alpha 4(+)/\beta 2(\alpha)\) agonist sites of the \((\alpha 4\beta 2)_2\alpha 4\) receptor, it found that their

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**Figure 1**
Alternative forms of the \(\alpha 4\beta 2\) nACh receptor. (A) Cartoon showing the alternate \((\alpha 4\beta 2)_2\beta 2\) and \((\alpha 4\beta 2)_2\alpha 4\) forms of the \(\alpha 4\beta 2\) nAChR. Stoichiometry-specific interfaces (\(\beta 2(+)/\beta 2(\alpha)\) and \(\alpha 4(+)/\alpha 4(\alpha)\)) are indicated by arrows. ABSs at \((\alpha 4(+)/\beta 2(\alpha)\) interfaces are indicated by filled asterisks, whereas the ABS at the \((\alpha 4(+)/\alpha 4(\alpha)\) interface of the \((\alpha 4\beta 2)_2\alpha 4\) receptor is indicated by a clear asterisk. (B) Diagram showing the linear sequence and spatial orientation of \(\alpha 4\) and \(\beta 2\) subunits in concatemeric \((\alpha 4\beta 2)_2\beta 2\) nAChR. The position of canonical agonist sites (ABS 1 and ABS 2) is indicated by arrows.
counterparts in the (α4β2)β2 responded differently to the presence of E loop mutant β2 subunits (Lucero et al., 2016). The most affected agonist site was the one whose complementary subunit forms the β2(+)/β2(−) interface with the fifth subunit. The most straightforward explanation for this finding is that the fifth subunit, likely through the β2(+)/β2(−) interface, affects receptor function by asymmetrically altering the function of the agonist sites. The fifth subunit could alter the affinity for ACh or the ability of the channel to open in response to agonist occupancy, or both, in an agonist site or a modulatory site on the β2(+)/β2(−) interface.

In the current study, the contribution of the fifth subunit to the function of (α4β2)β2 receptors was examined by proving the accessibility of β2L146C in the fifth subunit using the substituted cysteine accessibility method (SCAM; Karlin and Akabas, 1998). L146 in the fifth subunit was mutated to cysteine to test the ability of a methanethiosulfonate reagent (MTS) to react with this cysteine, in the presence or absence of ACh or dihydro-β-erythroidine (DHβE), a potent competitive inhibitor of nAChRs. These studies suggest that the β2(+)/β2(−) interface may play an important role in the maximal ACh response of the receptor. We also tested for the presence of an agonist site at the β2(+)/β2(−) interface by using site-directed mutagenesis of conserved aromatic residues that line the canonical agonist sites in nAChRs, followed by two-electrode voltage clamp experiments in Xenopus oocytes. When mutated to alanine, none of the conserved residues, individually or combined, affected ACH sensitivity, suggesting that conserved aromatic residues do not form an agonist-binding site (ABS) at the β2(+)/β2(−) interface. To determine if the effect of ACh on L146C accessibility is dependent on occupancy of the α4(+)/β2(−) agonist sites, we impaired the α4(+)/β2(−) agonist sites by alanine substitution of a key agonist-binding residue (α4W182), one site at a time, and measured the rate of MTS reaction in the absence or presence of ACh. These data indicate that occupancy of α4(+)/β2(−) agonist sites decreases the accessibility of L146C in the fifth subunit and that this effect depends on the position of the agonist binding site (ABS) relative to the fifth subunit. Overall, our findings suggest that the fifth subunit through the β2(+)/β2(−) interface may communicate with the agonist site adjacent to the β2(+)/β2(−) interface to modulate the maximal responses to ACh and that this link drives the functional asymmetry of the α4(+)/β2(−) agonist sites in the (α4β2)β2 nAChR.

Methods

Animals

All animal care and experimental procedures followed the guideline from the UK Home Office at the Biomedical Services, Oxford University. Adult female Xenopus laevis were purchased from the European Xenopus Resource Center (Portsmouth, UK), Xenopus1 (MI, USA) or Nasco (WI, USA). Xenopus toads were housed in a climate-controlled, light-regulated room; 120 toads were used. Toads were anaesthetized by immersion in 0.5% tricaine until non-responsive to toe pinch. Toads were then decapitated and ovarian lobes were harvested and defolliculated by incubation in 2 mg mL−1 collagenase (Type I C-0130, Sigma-Aldrich, UK). Defolliculated stage V-VI oocytes were sorted and injected with 100 ng of wild-type or mutant concatemeric α4β2 nAChR-cRNA, as described previously (Carbone et al., 2009). Injected oocytes were incubated until used at 18 °C in Barth’s solution: 88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES, supplemented with 0.1 mg mL−1 streptomycin, 1000 U mL−1 penicillin and 50 μg mL−1 neomycin or amikacin (100 μg mL−1) (pH 7.5, with 5 M NaOH). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015).

Mutagenesis and expression in oocytes

The fully concatenated form of wild-type or mutant α4β2 nAChRs was engineered as described previously (Carbone et al., 2009; Mazzaferro et al., 2011). Briefly, the signal peptide and start codon were removed from all the subunits, but the first (a β2 subunit) subunits were bridged by AGS linkers. Only the last subunit in the construct contained a stop codon. The subunits were subcloned into a modified pCI plasmid vector (Promega, UK) using unique restriction enzyme sites flanking the N- and C-terminals of each subunit. To introduce a mutation into a specific subunit of the concatemeric α4β2 nAChR, the mutation was first introduced into the subunit subcloned into the modified pCI plasmid using the Stratagene QuikChange Site-Directed Mutagenesis Kit (Agilent, UK). The presence of the mutation and the absence of unwanted mutations were confirmed by sequencing the entire cDNA insert (SourceBioscience, UK; Eurofins, UK). The mutated subunit was then ligated into the concatemer using unique restriction enzyme sites. To confirm that the mutated subunit was incorporated into the concatemer, we cut the subunit from the concatemer using unique restriction enzyme sites and then its nucleotide sequence was verified by DNA sequencing (SourceBioscience; Eurofins). All concatemeric constructs were assayed for integrity using restriction enzyme digestion and the LT reporter mutation (L9T in M2) as previously described (Mazzaferro et al., 2011). Note that we present the numbering of the residues in terms of the full length, including the signal sequence. To obtain the position in the mature form, subtract 28 from the number for α4 and 25 for β2.

Oocyte electrophysiology

Two-electrode voltage clamp recordings on oocytes were carried out 4–10 days after injection at room temperature in Ringer’s solution (NaCl 115 mM, KCl 2.5 mM, CaCl2 1.8 mM, HEPES 10 mM, pH 7.4). Concentration–response curves for ACh were obtained as described previously (Moroni et al., 2006). The ACh responses were normalized to the maximal ACh response (1 mM) of each individual recorded oocyte. Concentration–response curves were plotted using Prism 5.0 (GraphPad, San Diego, CA, USA). ACh concentration–response curve data were first fit to the one-component Hill equation, \( I = I_{\text{max}}/[1 + (E_{\text{C50}}/x)^{n_H}] \), where \( E_{\text{C50}} \) represents the concentration of agonist inducing 50% of the maximal response (\( I_{\text{max}} \)) and \( x \) is the agonist concentration and \( n_H \) the Hill coefficient. When ACh induced biphasic receptor activation, the concentration–response
curve data were fit to the sum of two Hill equations, as described previously (Moroni et al., 2006). For chimeric receptors, we measured their maximal functional expression and compare it with that of wild-type receptors. For these experiments, wild-type and mutant maximal ACh currents were measured from oocytes of the same batch that were injected 4–5 days before the experiments with the same amount of chimeric or wild-type cRNA.

**MTS modification of substituted cysteines**
[2-(trimethylammonium) ethyl] methanethiosulphonate (MTSET) was used to covalently modify the introduced cysteines. Accessibility of introduced cysteines to MTSET was determined by exposing the cysteines to a maximal concentration of MTSET (1 mM). Briefly, ACh pulses (5 s) were applied every 6 min, and prior to MTSET application, the responses to ACh were stabilized (<6% variance of peak current responses to ACh on four consecutive ACh applications). After stabilization, freshly diluted 1 mM MTSET was applied for 1 min, the cell was washed for 130 s and then ACh responses were measured until the responses stabilized. For all mutant receptors except mutant β2L146C/β2W182A/α4/β2, the concentration of ACh pulses was 30 μM (EC50). For β2L146C/α4/β2L146C, EC50 was 100 μM (Table 1). Higher concentrations of ACh were not used for the MTSET modification of substituted cysteine receptor experiments to minimize possible ion channel blockade by ACh and/or chronic receptor desensitization. The effect of MTSET was estimated using the following equation: % change = [(I acidic/Ip initial) – 1] × 100, where Ip initial is the response to ACh EC50 before MTSET application and I acidic is the response to ACh EC50 after MTSET application.

**Rate of MTSET modification in the absence of ligand**
The rate of modification of substituted cysteines by MTSET was determined by measuring the effect of sequential

### Table 1
Concentration effects of ACh on wild-type and mutant concatenated (α4β2)β2 nACh receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>EC50 ACh</th>
<th>nH</th>
<th>EC50Mut/EC50WT</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>6.61 ± 0.2</td>
<td>0.75 ± 0.012</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>8.33 ± 1.8</td>
<td>0.67 ± 0.09</td>
<td>0.96</td>
<td>10</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>80.42 ± 8.3*</td>
<td>0.86 ± 0.02</td>
<td>–</td>
<td>10</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>81.66 ± 3*</td>
<td>1.15 ± 0.25</td>
<td>9.5</td>
<td>10</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>13.39 ± 3*</td>
<td>0.64 ± 0.06</td>
<td>1.54</td>
<td>8</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>3.51 ± 1.2</td>
<td>2.18 ± 0.35</td>
<td>406</td>
<td>8</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>11.51 ± 4</td>
<td>0.87 ± 0.09</td>
<td>1.3</td>
<td>6</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>9.74 ± 1.2</td>
<td>0.71 ± 0.1</td>
<td>1.12</td>
<td>6</td>
</tr>
<tr>
<td>Y120Aβ2L146C/α4β2L146C</td>
<td>10.55 ± 2.3</td>
<td>0.94 ± 0.24</td>
<td>1.2</td>
<td>10</td>
</tr>
<tr>
<td>Y221Aβ2L146C/α4β2L146C</td>
<td>9.20 ± 0.6</td>
<td>0.97 ± 0.2</td>
<td>1.06</td>
<td>9</td>
</tr>
<tr>
<td>W176Aβ2L146C/α4β2L146C</td>
<td>7.20 ± 0.95</td>
<td>0.97 ± 0.03</td>
<td>0.83</td>
<td>9</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>8.84 ± 1.6</td>
<td>0.82 ± 0.19</td>
<td>1.02</td>
<td>9</td>
</tr>
<tr>
<td>Y120A/W176A/Y221Aβ2L146C</td>
<td>7.26 ± 0.5</td>
<td>0.93 ± 0.42</td>
<td>0.84</td>
<td>9</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>6.61 ± 0.9</td>
<td>0.98 ± 0.09</td>
<td>0.76</td>
<td>9</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>7.24 ± 1.9</td>
<td>0.89 ± 0.04</td>
<td>0.43</td>
<td>9</td>
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<tr>
<td>β2L146C/α4β2L146C</td>
<td>5.96 ± 1.2</td>
<td>0.72 ± 0.09</td>
<td>0.69</td>
<td>10</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>4.59 ± 2.1</td>
<td>0.86 ± 0.09</td>
<td>0.53</td>
<td>6</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>1.07 ± 0.1*</td>
<td>0.64 ± 0.21</td>
<td>0.12</td>
<td>9</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>53.00 ± 12</td>
<td>2 ± 0.90</td>
<td>6.1</td>
<td>9</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>17.00 ± 4*</td>
<td>0.61 ± 0.31</td>
<td>1.97</td>
<td>7</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>40.26 ± 15</td>
<td>0.6 ± 0.07</td>
<td>4.7</td>
<td>7</td>
</tr>
<tr>
<td>β2L146C/α4β2L146C</td>
<td>6.13 ± 2.1</td>
<td>0.71 ± 0.1</td>
<td>0.71</td>
<td>7</td>
</tr>
</tbody>
</table>

The concentration effects of ACh on oocytes expressing heterologously wild-type or mutant concatenated (α4β2)β2 nAChRs were determined using two-electrode voltage clamp. The data points were used to generate concentration–response curves from which EC50 and Hill coefficient (nH) values were estimated, as described in the Methods section. Data for β2L146C/α4β2L146C mutant receptors were best fit to a biphasic Hill equation (P = 0.0001). The ratio between mutant EC50 (EC50Mut) and wild-type EC50 (EC50WT) is shown. Values represent the mean ± SEM of n observations in the Methods section. The statistical differences between β2L146C/α4β2L146C and mutants of conserved aromatic residues were determined by one-way ANOVA with Dunnett’s correction. Asterisks denote statistical differences. The statistical difference between control (β2L146C/α4β2L146C) and mutants of conserved aromatic residues was determined by one-way ANOVA with Dunnett’s correction; statistically different values are noted by * and ** denote P < 0.05. F-tests were carried out to determine whether concentration–response data were best fit by one-site or biphasic model; the simpler one-component model was preferred unless the extra sum-of-squares F-test had a value of P less than 0.05.
applications of sub-saturating concentrations of MTSET using a protocol described previously (Mazzaferro et al., 2014). The concentration of MTSET causing sub-saturating effects was determined separately for each mutant receptor and for all mutants tested this was 10 μM. The responses to ACh prior to MTSET reagent application were first stabilized as follows: EC80 ACh was applied for 5 s, followed by a recovery time of 95 s. Immediately after the recovery time, a pulse of a ligand at EC80 concentration to be tested later for protection (30 μM ACh or 0.1 μM DHβE) was applied for 10 s followed by a 3 min 40 s wash with Ringer solution. This cycle was repeated until the ACh responses stabilized (<6% variance of peak current responses to ACh on four consecutive applications). Ligands were tested for their ability to protect the introduced cysteine residues from MTSET reaction were applied during the stabilization of the ACh responses to correct for any process of desensitization and/or ion channel blockade that could develop during the protection assays described below. MTSET was then applied using the following sequence of reactions: at time 0, ACh was applied for 5 s, followed by a period of recovery of 95 s; MTSET was then applied for 10 s, followed by a recovery period of 20 s. Immediately after the recovery time, the protectant was applied for 10 s, after which time the cell was washed with Ringer’s solution for 3 min and 40 s. This cycle was repeated until the peak current responses to ACh no longer changed, indicating completion of the MTSET reaction. After completion of the MTSET reaction, ACh and ligand were applied as described above to demonstrate that the observed changes in ACh responses were induced by MTSET.

Rate of MTSET mediated modification in the presence of ACh
To determine whether the accessibility of the incorporated cysteines could be altered by the presence of ligands (ACh or DHβE), the following protocol was used. Peak current responses to 5 s pulses of ACh EC80 were stabilized as described above, after which time MTSET was applied using the following sequence: at time 0, ACh was applied (5 s), followed by 95 s recovery; MTSET and the protectant (EC80 ACh or DHβE) were then co-applied for 10 s, followed by a recovery period of 4 min and 10 s. This cycle was repeated nine times (90 s in total). At the end of this cycle, ACh and ligand were applied as described for the MTSET reaction rate protocol. At the end of each protection assay, the cells were exposed to maximal MTSET to ensure that the previously protected mutant cysteines were still accessible. For all rate experiments, the decrease in the peak current response to ACh was plotted versus cumulative time of MTSET exposure. The change in current was plotted versus cumulative time of MTSET exposure. Peak values at each time point were normalized to the initial peak at time 0 s, and the data points were fit with a single-exponential decay function: y = span × e^−kt + plateau (GraphPad Software Inc.), where k is the first pseudo-first-order rate constant of the reaction. Plateau is the peak ACh current at the end of the reaction and span is 1 – plateau. A second-order rate constant (k2) was calculated by dividing k1 by the concentration of MTSET used. At least two different concentrations of MTSET (10 and 50 μM) were used to determine rates of reaction, to verify that the rates were independent of the concentration of MTSET. In all cases, the second-order rate constants were independent of MTSET concentration.

Statistical analysis
The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data for wild-type or each mutant receptor studied were obtained from oocytes from at least three different donors. Statistical and nonlinear regression analyses of the data from concentration–response curves and MTSET modification were performed using Prism 5 (GraphPad, San Diego, CA). An F-test determined whether the one-site or biphasic model best fit the concentration–response data; the simpler one-component model was preferred unless the extra sum-of-squares F-test had a value of P less than 0.05. One-way ANOVA with post hoc Dunnett’s test was used for comparisons involving more than two groups. Student’s unpaired t-tests were used for comparison between two groups (control and test). Values are presented as arithmetic mean ± SEM. Statistical tests with P < 0.05 were considered significant.

The published structure of the nicotinic receptor containing two copies of the α4 subunit and three copies of β2 (5kxi.PDB; Morales-Perez et al., 2016) was viewed, and figures were made using Pymol (http://www.pymol.org).

Data and statistical analysis for all alanine and MTSET experiments were blinded.

Materials
MTSET was purchased from Toronto Chemicals (Canada); 100 mM stocks were prepared and stored at –80°C. MTSET stocks were diluted to the appropriate concentration in Ringer’s solution and used immediately.

Nomenclature of targets and ligands
Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Results
We examined the contribution of the fifth subunit to the agonist responses of the (α4β2)3β2 nAChR. The fifth subunit in the (α4β2)3β2 receptor is a β2 subunit, and this subunit forms the signature β2(+)/β2(−) interface with a β2 subunit that contributes to an α4(+)/β2(−) agonist site (Figure 1A). To circumvent ambiguities in data analysis brought about by non-targeted subunit mutagenesis, the studies described here were carried out on fully concatenated (α4β2)3β2 nAChRs (β2_α4 β2_α4 β2_α4 β2 nAChRs). β2_α4 β2_α4 β2_α4 β2 nAChRs replicate the pharmacologic (Carbone et al., 2009) and single-channel (Mazzaferro et al., 2017) properties of (α4β2)3β2 nAChRs assembled from free subunits. In concatenated (α4β2)3β2, the first subunit in the linear sequence of the
concatemer (a β2 subunit) interfaces with the fifth subunit of the linear sequence of the concatemer (a β2 subunit), establishing the β2(+)β2(−) interface (Figure 1A, B). The first subunit contributes the principal face of the β2(+)β2(−) interface, whilst the fifth subunit contributes the complementary side (Figure 1A). ABSs in the concatenated receptors form at the interface between the first subunit of the linear sequence of the concatemer and the second subunit (hereafter termed ABS 1) and between the third and fourth subunits (hereafter termed ABS 2) (Figure 1A, B). For clarity, mutations in the linked receptors are shown as superscript positioned in the (+) or (−) side of the mutated subunit. For example, in β2L146Cα4β2α4β2, L146C is located in the (−) side of the β2 subunit forming part of ABS 1, and in β2α4β2α4T152Cβ2, T152C is positioned in the (+) side of the α4 subunit contributing the (+) side of ABS 2.

ACH sensitivity in α4β2 nAChRs maps to the N-terminal ECD

We first examined the effect of the fifth subunit on the function of α4β2β2 nAChRs by testing the effect of ACh on concatenated (α4β2)β2 receptors containing a chimeric fifth subunit. Chimeric subunits consisted of either the amino-terminal ECD of the α4 subunit and the remaining part (TMD and C-terminus) of the β2 subunit (α4/β2) or the amino-terminal ECD of the β2 subunit and the remaining part of the α4 subunit (β2/α4) (Figure 2A). As shown in Figure 2B, C (see Table 1 for estimated values of ACh potency), the ACh sensitivity of receptors containing a chimeric α4/β2 subunit at the fifth position was different from wild type (β2α4β2α4β2 nAChRs) but not different from that of β2α4β2α4α4 nAChRs. In contrast, when the chimeric fifth subunit contained the amino-terminal ECD of the β2 subunit (i.e. β2α4β2α4β2/α4 nAChRs), the sensitivity to ACh was comparable with wild-type β2α4β2α4β2 nAChRs but statistically different from that of β2α4β2α4α4 receptors (Figures 2B, C; Table 1). The amplitude of the maximal ACh responses for β2α4β2α4α4/β2 nAChRs increased by seven and five times, compared with, respectively, β2α4β2α4β2/α4 and β2α4β2α4β2 nAChRs (Figure 2D). To probe that chimeric subunit α4/β2 has the capability to form an α(+)β(−) agonist site with the adjacent α4 subunit in the β2α4β2α4/β2 receptor, we alanine-substituted the conserved agonist-binding W182 residue on the chimeric α4/β2 subunit to engineer mutant β2α4β2α4α4W182Aβ2 receptor and then tested the functional consequences of the mutation. Unnatural amino acid mutagenesis has shown that ACh makes a cation-π interaction with α4W182 in the (α4β2)β2 nAChRs, and this interaction critically contributes to ACh-binding affinity and receptor activation (Xu et al., 2009). If an operational agonist site forms at the interface α4W182α4/β2, ACh should yield biphasic concentration–response curves. We have shown in previous studies that alanine substitution of W182 in individual agonist sites in concatenated (α4β2)α4 receptors results in biphasic ACh responses due to the co-existence of wild-type and mutated agonist sites in the mutant receptor (Mazzaferro et al., 2011). As shown in Figure 2C (concentration–response parameters shown in Table 1), the ACh concentration–response curve of β2α4β2α4α4W182Aα4/β2 receptors was biphasic without significant changes in the amplitude of the maximal ACh current responses (Figure 2D). In addition, we also transferred β2 E loop residues β2V135, β2F214 and β2L146 to the E loop of chimeric α4/β2 subunit to engineer β2α4β2α4α4E-loop/β2 receptors. β2V135, β2F214 and β2L146 are equivalent to α4 E loop residues H142, Q150 and T152 (Harspøe et al., 2011). Previous studies have shown that transferring the β2 E loop residues to the fifth subunit in (α4β2)α4 receptors induces a left shift in ACh sensitivity to (α4β2)β2-like levels (Harspøe et al., 2011; Lucero et al., 2016). As shown in Figure 2C (Table 1), the ACh sensitivity of β2α4β2α4α4E-loop/β2 receptors was comparable with that of β2α4β2α4β2 receptors. In accord with Lucero et al. (2016), introducing the α4 E loop residues into the β2α4 chimeric subunit had no significant effect on the ACh responses (Figure 2C; Table 1), although there was significant decrease in functional expression (Figure 2D). These studies confirm that the agonist sensitivity in the alternate α4β2 nAChRs maps to the amino-terminal ECD of the fifth subunit (Harspøe et al., 2011; Mazzaferro et al., 2011; Wang et al., 2015; Lucero et al., 2016). We also confirm that the E loop of the fifth subunit in the (α4β2)α4 isofrom plays a critical role in determining the ACh sensitivity of the (α4β2)α4 isofrom (Harspøe et al., 2011; Lucero et al., 2016) but not that of the (α4β2)β2 receptor (Lucero et al., 2016; this study), although it appears to modify functional expression.

The fifth subunit modulates ACh maximal currents in the (α4β2)β2 nAChR

To further examine the effect of the fifth subunit on the amplitude of the maximal ACh current responses of the (α4β2)β2 receptor, we introduced a cysteine residue in lieu of β2L146 in the fifth subunit to engineer β2α4β2α4β2L146C receptors and then tested the accessibility of the introduced cysteine to MTSET (Figure 3A). For experimental control purposes, we also introduced L146C in the complementary subunit of ABS 1 or ABS 2 to construct respectively β2L146Cα4β2α4β2 and β2α4β2L146Cα4β2 receptors. We (Mazzaferro et al., 2011, 2014) and others (Wang et al., 2015) have used the L146C substitution to study agonist-induced responses in concatenated α4β2 nAChRs. As for the α4(+)β2(−) interfaces, the side chain of L146 in the fifth subunit orientates towards the space between the fifth subunit and the opposing subunit in the β2(+)β2(−) interface (Figure 3B).

Introducing L146C into the fifth subunit or the complementary subunit of ABS 1 or ABS 2 had no effect on ACh potency (Table 1), indicating that the cysteine substitution in these sites is well tolerated and does not affect the sensitivity of the (α4β2)β2 receptor to activation by ACh. Application of 1 mM MTSET for 1 min to oocytes expressing wild-type receptors had no effect on the subsequent ACh EC50 current responses (Figure 3C, D). We concluded therefore that any changes in the function of the cysteine-substituted receptors following exposure to MTSET can be attributed to the covalent modification of the substituted cysteine. As shown in Figure 3C, D, application of 1 mM MTSET irreversibly decreased the subsequent ACh-induced currents for β2α4β2α4β2L146C receptors by 2.3 times. MTSET also modified the subsequent ACh-induced currents

Figure 2
Effects of the fifth subunit on the ACh responses of α4β2 nACh receptors. (A) Diagram of chimeric concatenated α4β2 nAChRs. A chimeric subunit consisting of the α4 subunit extracellular domain (ECD) and the remaining part of the β2 subunit (or vice versa) was introduced into the fifth subunit position of both stoichiometric forms of the α4β2 nAChRs. (B) Representative traces of the current responses of wild-type and chimeric concatenated α4β2 nAChRs to ACh. (C) Concentration–response curves for ACh current responses in concatenated wild-type, chimeric and mutated chimeric α4β2 nAChRs expressed in Xenopus oocytes. The ECD of the fifth subunit of the alternate α4β2 nAChRs significantly affected the responses to ACh. The EC50 values and Hill coefficients (nH) are summarized in Table 1. (D) Maximal ACh current responses elicited by wild-type, chimeric and mutated chimeric concatenated α4β2 nAChR. To compare maximal currents, the same amount of cRNA coding wild-type and chimeric receptors were injected on the same oocyte batch and tested for functional expression on the same day. Student’s unpaired, two-tailed t-test showed significant differences (*, P < 0.05) between wild-type concatenated (α4β2)2α4 and chimeric (α4β2)2α4/β2 receptors but not between wild-type (α4β2)2β2 and (α4β2)2β2/α4 receptors (n = 10). Mutant chimeric (E loop and W182A mutants) were compared to wild-type chimeric receptors (E loop mutants, n = 6; W182A mutants, n = 8). Bar showing the maximal current of ACh on wild-type concatemeric (α4β2)2α4 nAChR is shown for comparison.
in $\beta_2^{L146C}$, although in comparison with $\beta_2^{L146C}$, the effect on $\beta_2^{L146C}$ was more pronounced (4.2- and 3.2 times respectively) (Figure 3C, D). These data show that covalent modification of $\beta_2L146C$ by MTSET reduces subsequent ACh responses and that the extent of the reduction is $\beta_2$ position-dependent, being greater when the $\beta_2$ subunit forms part of an $\alpha 4(+)/\beta 2(-)$ agonist site. Next, we examined if the receptor could activate after MTSET modification of both $\alpha 4(+)/\beta 2(-)$ agonist sites. To examine this, we tested the effect of ACh on $\beta_2^{L146C}$ before and after MTSET treatment. The ACh sensitivity of $\beta_2^{L146C}$ was not different from wild type (Table 1) but exposure to MTSET completely abolished the responses to ACh. Thus, when both ($\alpha 4\beta 2$) agonist sites are irreversibly inactivated by MTSET, the receptors are no longer capable to activate in response to ACh (Figure 3C, D). These findings are in accord with previous studies that have suggested that activation of ($\alpha 4\beta 2$) requires occupancy of both $\alpha 4(+)/\beta 2(-)$ agonist sites (Wang et al., 2015).

We next examined the mechanism underlying the effect of MTSET by determining the ACh concentration–response curve for $\beta_2^{L146C}$ before and after a 1 min exposure to 1 mM MTSET. It has been shown that derivatization of conserved aromatic residues in the $\gamma$ subunit of the muscle nAChR by MTS reagents reduces the maximum agonist response without changes in sensitivity (Sullivan and...
Cohen, 2000). Figure 4A shows that exposure to MTSET decreased the maximal ACh response in β2_α4_β2_α4_β2L146C by 2.5 times (n = 5; P < 0.05) without significant changes in the ACh EC50 (EC50 before MTSET = 5.41 ± 2 μM; EC50 after MTSET = 5.9 ± 1.1 μM; n = 5). For control purposes, we also determined the ACh concentration–response curve before and after MTSET treatment of β2_α4_β2_α4_β2 receptors. As shown in Figure 4B, MTSET derivatization of β2L146C_α4_β2_α4_β2 decreased the maximal current response of ACh by four times without significant changes in ACh potency (EC50 before MTSET = 7.48 ± 3 μM; EC50 after MTSET = 7.27 ± 2 μM; n = 5). These findings are consistent with irreversible inhibition of receptor function through removal of cysteine-substituted ACh sites by MTSET modification.

**ACh decreases accessibility of L146C in agonist sites and the β2(+)/β2(–) interface**

A role in receptor activation could account for the effects of the fifth subunit on the amplitude of the maximal ACh currents of the (αβ2)β2 receptor, and this effect could be driven by an operational agonist or a modulatory site on the β2(+)/β2(–) interface. To examine this possibility, we measured the accessibility of the introduced cysteine in the presence or absence of ACh to establish whether the presence of ACh impeded the derivatization of the substituted cysteine. If L146 is part of or nearby an ACh-binding site, the presence of ACh should slow down its derivatization by MTSET. Figure 5A, B shows current traces from a representative rate of MTSET reaction measurement using the cysteine-substituted β2_α4_β2_α4_β2L146C receptor in the absence (A) or presence (B) of ACh. As shown in Figure 5C (data summarized in Table 2), the rate of MTSET reaction decreased in the presence of ACh. For comparison, we determined the rate of MTSET reaction in the absence and presence of ACh for β2L146C_α4_β2_α4_β2 and β2_α4_β2L146C_α4_β2 receptors. As for β2_α4_β2_α4_β2L146C, ACh decreased the modification of β2L146C_α4_β2_α4_β2 and β2_α4_β2L146C_α4_β2 receptors (Figure 5D, E), although the rate of reaction in the absence or presence of ACh on these two receptors was more pronounced than that on β2_α4_β2_α4_β2L146C receptors (Table 2). These data suggest that accessibility to L146C, in the presence or absence of ACh, is β2 position-dependent. The rank order of L146C accessibility is ABS 1 > ABS 2 > β2(+)β2(–). For an additional inter-subunit interface control, we cysteine-substituted α4T152, the α4 residue equivalent to β2L146, in one of the β2(+)α4(–) interface (β2_α4_β2_α4_β2L146C_α4T152C β2) and then measured the rate of MTSET modification in the presence and absence of ACh. The ACh EC50 in β2_α4_β2_α4_β2L146C_α4T152C β2 was no different from wild type (Table 1). As shown in Figure 5F, the rate of MTSET reaction (2829 ± 610 M–1 s–1; n = 5) was not significantly different from the rate measured in the presence of ACh (2171 ± 715 M–1 s–1; n = 5) (Table 2).

Demonstrating that competitive antagonists decrease the rate of MTSET modification of L146C in the fifth subunit would support the presence of an ACh-binding site on the β2(+)β2(–) interface. If ACh and antagonists occupy the same site in the fifth subunit, the presence of either should alter the rate of MTSET modification of the cysteine-substituted fifth subunit in a similar manner. We therefore measured the rate of MTSET modification in the presence or absence of the nAChR inhibitor DHβE. Available DHβE-bound crystal structures of Lymnaea AChBP (Shahsavari et al., 2012) and functional data from mutagenesis studies of the α4β2 nAChR (Iturriaga-Vasquez et al., 2010) have shown that DHβE and agonists interact with the same conserved aromatic residues in canonical agonist sites. Furthermore, we have found in a previous study that DHβE slows down the rate of MTSET reaction in cysteine-substituted α4(+)/β2(–) or α4(+)/α4(–) agonist sites in the (α4β2)2α4 receptor (Mazzaferrro et al., 2011). Thus, if there is an ACh-binding site on the β2(+)β2(–) interface
Conserved aromatic residues in the β2(+)/β2(−) interface do not affect ACh sensitivity

Conserved aromatic residues in the β2(+)/β2(−) interface do not affect ACh sensitivity. Consistent with the presence of an agonist site on the β2(+)/β2(−) interface, key aromatic α4 subunit agonist-binding residues (W182, Y120, W88 and Y230) are conserved in the β2 subunit (β2W176, β2Y120; β2W82 and β2Y221). We have previously shown that impairment of individual agonist sites in the (α4β2)2 receptor isoform by alanine substitutions of conserved aromatic residues yields biphasic ACh concentration–response curves (Mazzaferro et al., 2011, 2014). Thus, if the β2(+)/β2(−) interface houses an ACh-binding site formed by conserved aromatic residues, alanine mutations of these residues should yield biphasic ACh concentration–response curves. Table 1 shows that individual or simultaneous alanine substitutions of conserved aromatic residues in the β2(+)/β2(−) interface do not affect ACh sensitivity.
aromatic residues in the β2(+)β2(−) interface had no effect on potency of ACh. This finding indicates that conserved aromatic residues do not engage ACh in the β2(+)/β2(−) interface.

**Agonist sites affect MTSET modification of the fifth subunit asymmetrically**

So far, the findings suggest that agonist-bound a4(+)/β2(−) agonist sites affect accessibility of L146C in the fifth subunit. If this is the case, impairing the a4(+)/β2(−) agonist site by mutagenesis should alter the rate of MTSET modification of the cysteine-substituted fifth subunit. We tested this possibility by introducing W182A in ABS 1 or ABS 2 of the β2_α4_β2_β2L146C receptor and then measuring the rate of modification of L146C by MTSET in the presence or absence of ACh.

Introducing W182A impacted the sensitivity of β2_α4_β2_α4_β2L146C to activation by ACh. The extent of the effect depended on which agonist site (ABS 1 or ABS 2) carried the W182A mutation. When W182A was introduced into ABS 1 (i.e. β2_α4_β2_α4_β2_W182A), W182A caused a biphasic ACh sensitivity, comprising a high-affinity component (EC50 1.07 ± 0.1 μM) and a low-affinity component (EC50 53 ± 12 μM) (Table 1). In contrast, incorporation of W182A into ABS 2 (i.e. β2_α4_β2_α4_β2_W182A) did not produce biphasic concentration–response curves for ACh but decreased ACh potency from 8.64 ± 2.2 to 17.00 ± 4 μM (Table 1). In accord with our findings, Lucero et al. (2016) found that β2 E loop mutations impair more drastically the function of ABS 1 than that of ABS 2.

2.1 times (Figure 7B; Table 2). We noticed a slight potentiation of the ACh responses in to β2_α4_β2_α4_β2L146C receptors after MTSET reaction, but this effect was not significant. These findings show that agonist-bound canonical sites alter the accessibility of the substituted cysteine in the fifth subunit, suggesting that agonist sites, particularly ABS 1, link to the fifth subunit.

### Discussion

Here, by combining voltage clamp electrophysiological recordings from concatenated (α4β2)2β2 nAChRs, site-directed mutagenesis, along with probing with the thiol-reactive MTSET reagent, we have shown that the fifth subunit in the (α4β2)2β2 nAChR, like its counterpart in the (α4β2)4 isoform, plays an important role in the maximal ACh responses of the receptor, albeit more subtly. Our findings have also confirmed that a4(+)/β2(−) agonist sites in the (α4β2)2β2 nAChR isoform function asymmetrically (Lucero et al., 2016). We found that ACh but not DHPE, a selective competitive inhibitor of the α4β2 nAChR that contacts the same conserved aromatic residues as agonists in nAChR canonical agonist sites, had no effect on the MTSET modification of the fifth subunit. In addition, alanine substitutions of conserved aromatic residues in the β2(+)/β2(−) interface had no effect on ACh responses. Together, these findings suggest that the effect of ACh on the chemical modification of the fifth subunit is not mediated by binding to a site contributed by conserved aromatic residues in the β2(+)/β2(−) interface. By impairing the function of the a4(+)/β2(−) agonist sites by mutagenesis, we found that in the presence or absence of ACh, chemical modification of the fifth subunit slowed down. The extent of this effect depended on which agonist site carried the mutation. Thus, together, our data suggest that the fifth subunit links with the a4(+)/β2(−) agonist sites to modulate the maximal ACh responses of the receptor. Since the effect of the agonist sites on the rate of modification of the fifth subunit is unequal, the relationship between the
ACh maximal responses in α4β2 nicotinic receptors

Figure 6
Effect of the competitive antagonist DHβE on the rate of MTSET modification of cysteine-substituted β2(+)/β2(−) or α4(+)/β2(−) interfaces in concatenated (α4β2)β2 nACh receptors. (A) The rate of MTSET derivatization of cysteine-substituted β2_α4_β2_α4_β2 L146C receptors was not affected by the presence of an IC20 concentration of DHβE (n = 5). In contrast, DHβE slowed down the rate of MTSET modification of β2 L146C_α4_β2_α4_β2 (n = 5) (B) or β2_α4_β2 L146C_α4_β2 (n = 5) (C) receptors (Student’s unpaired t-test). Data were normalized and fit to a single-phase exponential decay, as described in the Methods section.

Irreversible modification of L146C in the fifth subunit by MTSET reduced the amplitude of the subsequent maximal ACh responses without changes in the ACh potency. Although less pronounced, these effects were comparable with those observed for MTSET-treated ABS 1. Together, these findings highlight the fifth subunit as an important component of the mechanisms determining the maximal ACh responses of (α4β2)β2 receptors. Significantly, ACh reduced the rate of MTSET reaction with L146C in the fifth subunit. ACh also slowed down the rate of MTSET reaction with cysteine-substituted α4(+)/β2(−) agonist sites. The fifth subunit through the β2(+)/β2(−) interface could contribute to the ACh responses of the receptors by forming part of an additional agonist site or a site capable of modulating the mechanisms that affects the ability of the ion channel to open in response to agonist occupancy of the α4(+)/β2(−) agonist sites. The β2 subunit conserves key α4 aromatic residues that line the core of α4(+)/β2 agonist sites in the (α4β2)β2 receptor (Morales-Perez et al., 2016), and these residues could potentially be part of an agonist or modulatory site on the β2(+)/β2(−) interface.

Unexpectedly, DHβE slowed down the rate of modification of the α4(+)/β2(−) agonist sites but not the rate of reaction with the cysteine-substituted fifth subunit. A key assumption of SCAM is that MTS modification of cysteine-substituted residues located within or close to agonist sites alters in the presence of agonists or antagonists recognizing the site (Karlin and Akabas, 1998; Sullivan and Cohen, 2000). Available DHβE-bound crystal structures of Lymnaea AChBP have shown that DHβE and agonists contact the same conserved residues in the agonist site: Y126, W182, Y223 and Y230 from the (+) side of the α4(+)β2(−) agonist site and W82 from the (−) side (Shahsavari et al., 2012). Furthermore, functional data from mutagenesis studies of the α4β2 nAChR have shown that alanine substitution of these residues reduce the inhibitory potency of DHβE (Iturriaga-Vásquez et al., 2010). Thus, the most straightforward explanation for our findings is that the conserved aromatic residues in the β2(+)/β2(−) interface do not bind agonist or antagonist, like they do in the α4(+)/α4(−) interface of the (α4β2)α4 receptor (Harpsøe et al., 2011; Mazzaferrro et al., 2011; Wang et al., 2015; Jain et al., 2016). Significantly, the recently resolved X-ray structure of the human (α4β2)β2 nAChR reveals a reorganization of conserved tyrosine residues (Y120 and Y221) in the β2(+)/β2(−) and the sandwiching of the positively charge guanidinium group of a non-conserved arginine residue between the aromatic rings of the tyrosine residues. This arrangement would stabilize the electron-rich π environment of the region, thus preventing agonist binding (Morales-Perez et al., 2016).

An alternative explanation for our findings is that ACh binds a site close or including L146 within the β2(+)/β2(−) interface that does not include the conserved aromatic residues and that excludes DHβE. Recent studies have indicated that the pharmacology of nAChRs is influenced by sites located at β(+)/α(−) interfaces that do not involve conserved aromatic residues (Moroni et al., 2008; Seo et al., 2009). More pertinently, Jain et al. (2016) reported that
irreversible modification of α5/α4 and β3/α4 interfaces in respectively (α4β2)2α5 and (α4β2)β3 nAChRs reduces the maximal ACh responses without changes in EC₅₀. These authors concluded that α5/α4 and β3/α4 interfaces contain operational agonist sites of an unorthodox nature (Jain et al., 2016). α5 and β3 nAChR subunits were thought to be incapable of forming agonist sites. However, earlier studies have shown that mutations of conserved aromatic residues in the α5 subunit had no effect on the agonist sensitivity of (α4β2)α5 receptors, although a reduction in maximal agonist responses was observed (Marotta et al., 2014). Further studies are necessary to get a better understanding on how the α5/α4 and β3/α4 interfaces affect the agonist responses of (α4β2)α5 and (α4β2)β3 nAChRs. For example, probing the ability of agonists and antagonists to reduce accessibility of cysteines incorporated into putative agonist-binding residues in the α5/α4 and β3/α4 interfaces should help in better understanding the agonist sites that mediate the effects of these interfaces. In the case of the (α4β2)β2 receptor, an ACh-binding site on the β2(+)/β2(−) interface seems unlikely. The high-resolution structure of the human (α4β2)β2 nAChR was obtained by co-crystallization with nicotine, and this agonist was found bound only to α4(+)/β2(−) agonist sites (Morales-Perez et al., 2016).

Impairment of α4(+)β2(+) agonist sites reduced the accessibility of L146C in the fifth subunit, in the presence or absence of ACh. A plausible explanation for this observation is that the fifth subunit through the β2(+)/β2(−) interface communicates with the agonist sites. This link might be necessary and sufficient for the effect of the fifth subunit on ACh maximal responses of the (α4β2)β2 nAChR. Importantly, the accessibility of L146C was obliterated by impairment of ABS 1 but not by impairment of ABS 2. This implies that the fifth subunit links asymmetrically with the agonist sites and that the link is stronger with ABS 1. Lucero et al. (2016), based on the unequal effects of E loop substitutions in the (α4β2)β2 receptor, proposed that there may be a strong interaction between adjacent subunits so that the structure at one interface (influenced by the structure of the E loop) can alter activation mediated by binding of ACh to neighbouring subunits. Allosteric effects between the agonist sites on the α4(+)/α4(−) and α4(+)/β2(−) interfaces in the
isoform (α4β2)2α4 have also been proposed to account for the different patterns of single-channel opening durations exhibited by the alternate α4β2 nAChR isoforms (Mazzaferrro et al., 2017). Here, on the basis of our findings, we propose that ABS 1 and the fifth subunit, through the β2(+)/β2(−)-interface, constitute a functional unit and that this arrangement modulates the maximal ACh responses in the (α4β2)2β2 nAChR and confers functional asymmetry to the α4(+)/β2(−) agonist sites.

Changes in the accessibility of the cysteine-substituted fifth subunit suggest that L146 and/or residues in close proximity undergo conformational rearrangement in the presence of ACh. This implies that that structural changes initiated at the α4(+)/β2(−) can extend over considerable distance. Allosteric signals can propagate over long distances in pLGIC. In the GABAΑ receptor, an inhibitory pLGIC, the GABA-binding site positioned anti-clockwise to the γ subunit undergoes structural rearrangement upon binding of the positive benzodiazepine modulator flurazepan (Klodza and Czajkowski, 2007; Eaton et al., 2012). In the GABAΑ receptor, the binding sites are located at β/α interfaces (the β subunit is the principal subunit in the GABAΑ receptor) and the γ subunit. This structural and functional arrangement is equivalent to the one we propose for the (α4β2)β2 receptor. Consistently with this possibility, Baumann et al. (2003) reported asymmetry in the function of the agonist sites in the GABAΑ receptor, which these authors proposed could arise from differences in the subunits flanking the agonist sites. Asymmetry in the function of structural equivalent agonist sites and agonist site-fifth subunit modulatory links to regulate agonist-binding function might be a common feature of heteromeric pLGICs.

How might the ABS 1-β2(+)β2(−) unit modulate the maximal responses of (α4β2)2β2 receptor? Since MTSET derivatization of the fifth subunit decreased the maximal ACh responses without effects on ACh potency, a possible scenario is that the ABS 1-β2(+)β2(−) functional unit modulates the propagation of the conformational transitions induced by agonist occupancy to the TMD without affecting agonist-binding affinity. Although the interpretation of our findings is inevitably confounded by the problem of separating effects on agonist binding (affinity) and gating (Colquhoun, 1998), the observation that DHβE, an antagonist that inhibits receptor function, had no effects on the rate of MTSET reaction with cysteine-substituted fifth subunit supports this possibility. It appears that for the fifth subunit to exert its effects on receptor function, the canonical agonist sites of the receptor must be agonist-bound. In this respect, it is interesting that cryo-images of Torpedo nAChRs suggest that as a consequence of agonist occupation, the fifth subunit (β1 subunit) undergoes structural changes, which might be critically important for transmitting to the TMD the conformational changes driving channel gating (Unwin and Fujiyoshi, 2012). This scenario could explain why the fifth subunit modulates the maximal ACh responses without noticeable changes in ACh sensitivity, despite being functionally linked to ABS 1. Of relevance to the functional asymmetry of the α4(+)/β2(−) agonist sites, Unwin and Fujiyoshi (2012) reported that although both agonist sites contribute to the movement of β1, the agonist site at the α7 subunit interface appears to be the most prominent driving force behind the displacement of β1 (Unwin and Fujiyoshi, 2012). This asymmetry is consistent with our findings and supports our view that ABS 1 and the β2(+)β2(−) interface form a functional unit that modulates the agonist responses of the (α4β2)β2 nACh receptor isoform.

In conclusion, these data suggest that the fifth subunit in the (α4β2)β2 nACh receptor isoform plays an important role in both modulating the maximal ACh response of the receptor and conferring functional asymmetry to the agonist sites on the α4(+)/β2(−) interfaces.

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Author contributions

K.N. and I.B. designed and carried out experiments. S.M. designed earlier MTSET experiments. C.A. and S.G. carried out experiments. I.B. and K.N. analysed data and wrote paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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