

## RESEARCH PAPER

# The fifth subunit of the $(\alpha 4\beta 2)_2\beta 2$ nicotinic ACh receptor modulates maximal ACh responses

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## BACKGROUND AND PURPOSE

The fifth subunit in the  $(\alpha 4\beta 2)_2\alpha 4$  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  $(\alpha 4\beta 2)_2\beta 2$  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  $(\alpha 4\beta 2)_2\beta 2$  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- $\beta$ -erythroidine had no effect. Alanine substitution of conserved residues that line the core of the agonist sites on  $\alpha 4(+)/\beta 2(-)$  interfaces did not impair receptor function. However, impairment of agonist binding to  $\alpha 4(+)/\beta 2(-)$  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  $(\alpha 4\beta 2)_2\beta 2$  receptor isoform modulates maximal ACh responses. This effect appears to be driven by a modulatory, and asymmetric, association with the  $\alpha 4(+)/\beta 2(-)$  agonist sites.

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## Abbreviations

ABS, agonist-binding site; Dh $\beta$ E, dihydro- $\beta$ -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

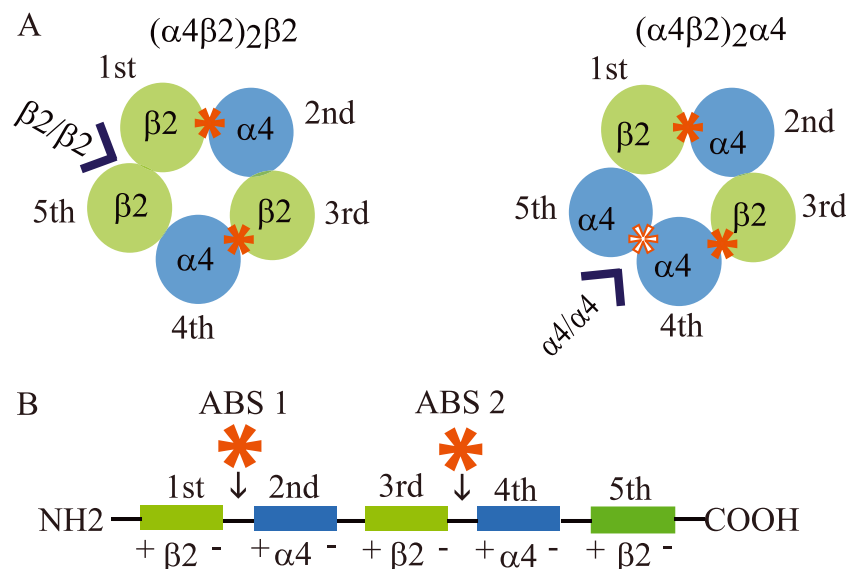
## Introduction

The  $\alpha 4\beta 2$  nicotinic 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<sub>A</sub>**, **glycine** and **5-HT<sub>3</sub> receptors**. Work on the muscle nAChR has shown that agonist binding in these proteins triggers rigid body motions, which are transduced into transient movements of the pore lining M2  $\alpha$  helices of the transmembrane domain (TMD) by a primary coupling pathway that runs along the long axis of the protein involving a series of loops of the subunit contributing the principal side of the agonist site ( $\beta 1$ – $\beta 2$  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 **ACh** and other agonists

(Nelson *et al.*, 2003; Moroni *et al.*, 2006; Harpsøe *et al.*, 2011; Mazzaferro *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; Alcaïno *et al.*, 2017; Jin *et al.*, 2017) and single-channel properties (Mazzaferro *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 (an  $\alpha 4$ ) and an adjacent  $\alpha 4$  subunit (Harpsøe *et al.*, 2011; Mazzaferro *et al.*, 2011). A triad of non-conserved E loop residues on the complementary side of the agonist site on the  $\alpha 4(+)/\alpha 4(-)$  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:  $\alpha 4H142$ ,  $\alpha 4Q150$  and  $\alpha 4T152$  (Harpsøe *et al.*, 2011; Lucero *et al.*, 2016). The fifth subunit in the  $(\alpha 4\beta 2)_2\beta 2$  isoform (a  $\beta 2$ ) forms the receptor's signature  $\beta 2(+)/\beta 2(-)$  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 ACh sensitivity (Lucero *et al.*, 2016).

Previously, we found that the agonist sites on the  $\alpha 4(+)/\beta 2(-)$  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 (Mazzaferro *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  $\alpha 4(+)/\beta 2(-)$  agonist sites of the  $(\alpha 4\beta 2)_2\alpha 4$  receptor, it found that their



## 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 ( $\beta 2(+)/\beta 2(-)$  and  $\alpha 4(+)/\alpha 4(-)$ ) are indicated by arrows. ABSs at  $\alpha 4(+)/\beta 2(-)$  interfaces are indicated by filled asterisks, whereas the ABS at the  $\alpha 4(+)/\alpha 4(-)$  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  $(\alpha 4\beta 2)_2\beta 2$  responded differently to the presence of E loop mutant  $\beta 2$  subunits (Lucero *et al.*, 2016). The most affected agonist site was the one whose complementary subunit forms the  $\beta 2(+)/\beta 2(-)$  interface with the fifth subunit. The most straightforward explanation for this finding is that the fifth subunit, likely through the  $\beta 2(+)/\beta 2(-)$  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, through an agonist site or a modulatory site on the  $\beta 2(+)/\beta 2(-)$  interface.

In the current study, the contribution of the fifth subunit to the function of  $(\alpha 4\beta 2)_2\beta 2$  receptors was examined by proving the accessibility of  $\beta 2L146C$  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- $\beta$  erythroidine** (DH $\beta$ E), a potent competitive inhibitor of nAChRs. These studies suggest that the  $\beta 2(+)/\beta 2(-)$  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  $\beta 2(+)/\beta 2(-)$  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  $\beta 2(+)/\beta 2(-)$  interface. To determine if the effect of ACh on L146C accessibility is dependent on occupancy of the  $\alpha 4(+)/\beta 2(-)$  agonist sites, we impaired the  $\alpha 4(+)/\beta 2(-)$  agonist sites by alanine substitution of a key agonist-binding residue ( $\alpha 4W182$ ), 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  $\alpha 4(+)/\beta 2(-)$  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  $\beta 2(+)/\beta 2(-)$  interface may communicate with the agonist site adjacent to the  $\beta 2(+)/\beta 2(-)$  interface to modulate the maximal responses to ACh and that this link drives the functional asymmetry of the  $\alpha 4(+)/\beta 2(-)$  agonist sites in the  $(\alpha 4\beta 2)_2\beta 2$  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 not responsive to toe pinch. Toads were then decapitated and

ovarian lobes were harvested and defolliculated by incubation in 2 mg mL<sup>-1</sup> collagenase (Type 1 C-0130, Sigma-Aldrich, UK). Defolliculated stage V-VI oocytes were sorted and injected with 100 ng of wild-type or mutant concatemeric  $\alpha 4\beta 2$  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 (NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, supplemented with 0.1 mg mL<sup>-1</sup> streptomycin, 1000 U mL<sup>-1</sup> penicillin and 50  $\mu$ g mL<sup>-1</sup> neomycin or amikacin (100  $\mu$ g mL<sup>-1</sup>) (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  $\alpha 4\beta 2$  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 ( $\alpha$   $\beta 2$  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  $\alpha 4\beta 2$  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 (L9'T 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  $\alpha 4$  and 25 for  $\beta 2$ .

### 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, CaCl<sub>2</sub> 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,  $I = I_{\max}/[1 + (EC_{50}/x)^{n_H}]$ , where EC<sub>50</sub> represents the concentration of agonist inducing 50% of the maximal response ( $I_{\max}$ ) 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] methanethiosulfonate (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  $\beta 2_{W182A}\alpha 4_{\beta 2_{L146C}}$ , the concentration of ACh pulses was 30  $\mu$ M ( $EC_{80}$ ). For  $\beta 2_{W182A}\alpha 4_{\beta 2_{L146C}}$ ,  $EC_{80}$  was 100  $\mu$ M (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 =  $[(I_{\text{after}}/I_{\text{initial}}) - 1] \times 100$ , where  $I_{\text{initial}}$  is the response to ACh  $EC_{80}$  before MTSET application and  $I_{\text{after}}$  is the response to ACh  $EC_{80}$  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 ( $\alpha 4\beta 2$ ) $_2$  nACh receptors

Receptor	$EC_{50}$ ACh	$n_H$	$EC_{50Mut}/EC_{50WT}$	$n$
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$8.64 \pm 2.2$	$0.75 \pm 0.012$	–	11
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}/\alpha 4}}$	$8.33 \pm 1.8$	$0.67 \pm 0.09$	0.96	10
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}}}}$	$80.42 \pm 8.3^*$	$0.86 \pm 0.02$	–	10
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}/\beta 2}}$	$81.66 \pm 3^*$	$1.15 \pm 0.25$	9.5	10
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}^{W182A}/\beta 2}}$	$13.39 \pm 3^*$ $3.51 \pm 1.2$	$0.64 \pm 0.06$ $2.18 \pm 0.35$	1.54 406	8
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}^{E4loop}/\alpha 4}/\beta 2}}$	$11.51 \pm 4$	$0.87 \pm 0.09$	1.3	6
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}^{E4loop}/\beta 2}/\alpha 4}}$	$9.74 \pm 1.2$	$0.71 \pm 0.1$	1.12	6
$Y120A_{\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$10.55 \pm 2.3$	$0.94 \pm 0.24$	1.2	10
$Y221A_{\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$9.20 \pm 0.6$	$0.97 \pm 0.2$	1.06	9
$W176A_{\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$7.20 \pm 0.95$	$0.97 \pm 0.03$	0.83	9
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W82A}}}}$	$8.84 \pm 1.6$	$0.82 \pm 0.19$	1.02	9
$Y120A, W176A, Y221A_{\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W82A}}}}$	$7.26 \pm 0.5$	$0.93 \pm 0.42$	0.84	9
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}}}}$	$6.61 \pm 0.9$	$0.98 \pm 0.09$	0.76	9
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}/\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$7.24 \pm 1.9$	$0.89 \pm 0.04$	0.43	9
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}/\alpha 4_{\beta 2}}}}$	$5.96 \pm 1.2$	$0.72 \pm 0.09$	0.69	10
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}/\alpha 4_{\beta 2}^{L146C}}}}$	$4.59 \pm 2.1$	$0.86 \pm 0.09$	0.53	6
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$	$1.07 \pm 0.1^*$ $53.00 \pm 12$	$0.64 \pm 0.21$ $2 \pm 0.90$	0.12 6.1	9
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2}}}}$	$17.00 \pm 4^*$	$0.61 \pm 0.31$	1.97	7
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}}}}$	$40.26 \pm 15^{\wedge}$	$0.6 \pm 0.07$	4.7	7
$\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2}^{L146C}}}}$	$6.13 \pm 2.1$	$0.71 \pm 0.1$	0.71	7

The concentration effects of ACh on oocytes expressing heterologously wild-type or mutant concatenated ( $\alpha 4\beta 2$ ) $_2$  nAChRs were determined using two-electrode voltage clamp. The data points were used to generate concentration–response curves from which  $EC_{50}$  and Hill coefficient ( $n_H$ ) values were estimated, as described in the Methods section. Data for  $\beta 2_{W182A}\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}$  mutant receptors were best fit to a biphasic Hill equation ( $P = 0.0001$ ). The ratio between mutant  $EC_{50}$  ( $EC_{50Mut}$ ) and wild-type  $EC_{50}$  ( $EC_{50WT}$ ) is shown. Values represent the mean  $\pm$  SEM of  $n$  number of experiments. Statistic differences between  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$  and chimeric  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}/\alpha 4}}$  or  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\alpha 4}/\beta 2}}$  receptors were measured by Student's  $t$ -tests. Statistical differences between control ( $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}}}}$ ) and mutants of conserved aromatic residues were determined by one-way ANOVA with Dunnett's correction. Asterisks denote statistical difference. Statistical comparison between  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{L146C}}}$  (control) and  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2}^{L146C}}}$  and  $\beta 2_{\alpha 4_{\beta 2_{\alpha 4_{\beta 2}^{W182A}/\alpha 4_{\beta 2}^{L146C}}}$  was carried out by one-way ANOVA with Dunnett's correction; statistically different values are noted by  $\wedge$ . \*,  $\wedge$  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  $\mu\text{M}$ . The responses to ACh prior to MTSET reagent application were first stabilized as follows: EC<sub>80</sub> 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 EC<sub>80</sub> concentration to be tested later for protection (30  $\mu\text{M}$  ACh or 0.1  $\mu\text{M}$  Dh $\beta$ E) 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 to be tested for their ability to protect the introduced cysteine residues from MTSET reactions 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 Dh $\beta$ E), the following protocol was used. Peak current responses to 5 s pulses of ACh EC<sub>80</sub> 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 (EC<sub>80</sub> ACh or Dh $\beta$ E) 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:  $y = \text{span} \times e^{-kt} + \text{plateau}$  (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  $1 - \text{plateau}$ . A second-order rate constant ( $k_2$ ) was calculated by dividing  $k_1$  by the concentration of MTSET used. At least two different concentrations of

MTSET (10 and 50  $\mu\text{M}$ ) 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  $\pm$  SEM. Statistical tests with *P* < 0.05 were considered significant.

The published structure of the nicotinic receptor containing two copies of the  $\alpha 4$  subunit and three copies of  $\beta 2$  (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  $-80^\circ\text{C}$ . 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 ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChR. The fifth subunit in the ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  receptor is a  $\beta 2$  subunit, and this subunit forms the signature  $\beta 2(+)/\beta 2(-)$  interface with a  $\beta 2$  subunit that contributes to an  $\alpha 4(+)/\beta 2(-)$  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 ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChRs ( $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2$  nAChRs).  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2$  nAChRs replicate the pharmacological (Carbone *et al.*, 2009) and single-channel (Mazzaferro *et al.*, 2017) properties of ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChRs assembled from free subunits. In concatenated ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$ , the first subunit in the linear sequence of the

concatemer (a  $\beta 2$  subunit) interfaces with the fifth subunit of the linear sequence of the concatemer (a  $\beta 2$  subunit), establishing the  $\beta 2(+)/\beta 2(-)$  interface (Figure 1A, B). The first subunit contributes the principal face of the  $\beta 2(+)/\beta 2(-)$  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  $\beta 2^{L146C}\text{-}\alpha 4\text{-}\beta 2$ , L146C is located in the (-) side of the  $\beta 2$  subunit forming part of ABS 1, and in  $\beta 2\text{-}\alpha 4\text{-}\beta 2^{\alpha 4T152C}$ , T152C is positioned in the (-) side of the  $\alpha 4$  subunit contributing the (+) side of ABS 2.

### ACh sensitivity in $\alpha 4\beta 2$ nAChRs maps to the N-terminal ECD

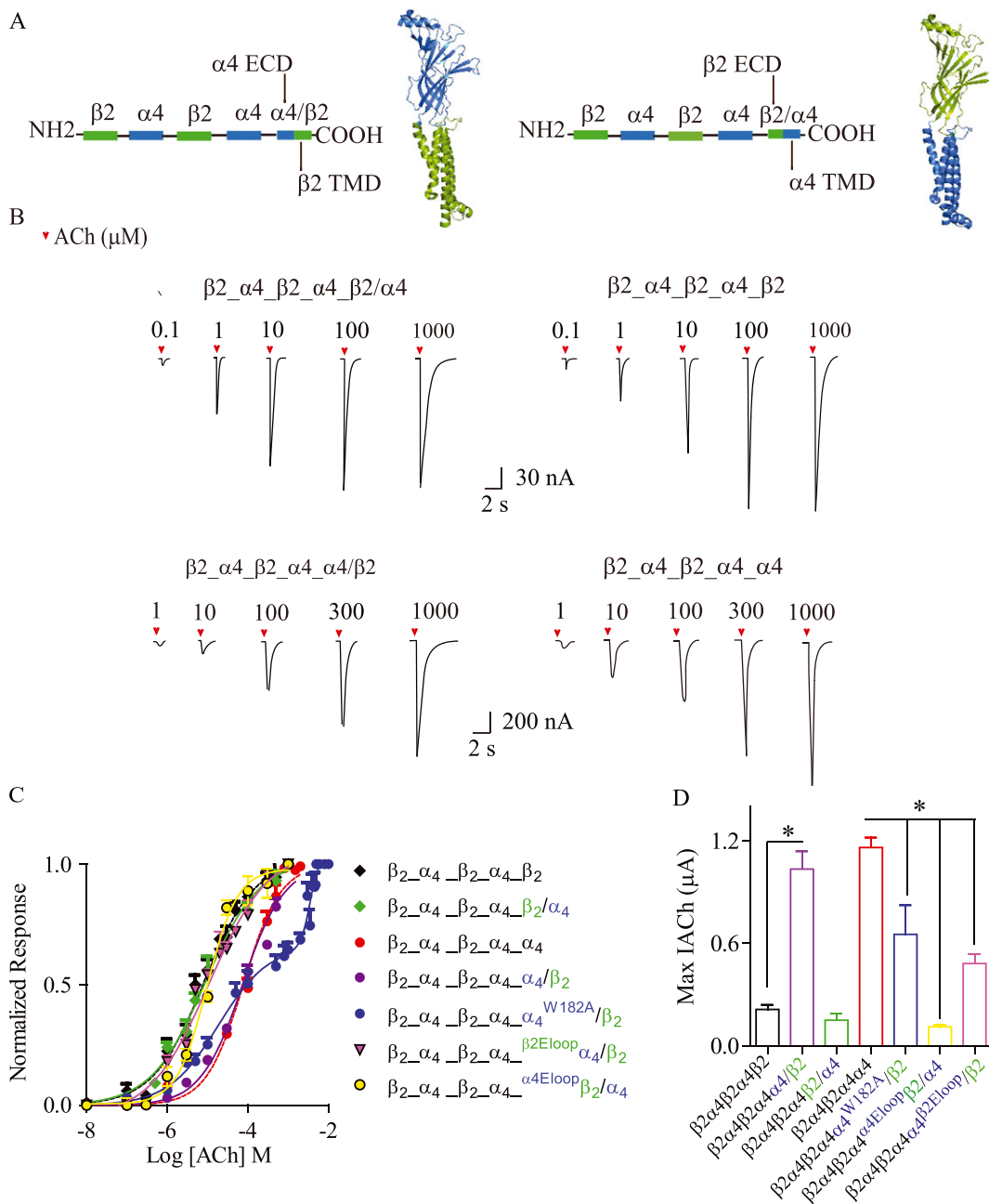
We first examined the effect of the fifth subunit on the function of  $(\alpha 4\beta 2)_2\beta 2$  nAChRs by testing the effect of ACh on concatenated  $(\alpha 4\beta 2)_2\beta 2$  receptors containing a chimeric fifth subunit. Chimeric subunits consisted of either the amino-terminal ECD of the  $\alpha 4$  subunit and the remaining part (TMD and C-terminus) of the  $\beta 2$  subunit ( $\alpha 4/\beta 2$ ) or the amino-terminal ECD of the  $\beta 2$  subunit and the remaining part of the  $\alpha 4$  subunit ( $\beta 2/\alpha 4$ ) (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  $\alpha 4/\beta 2$  subunit at the fifth position was different from wild type ( $\beta 2\text{-}\alpha 4\text{-}\beta 2$  nAChRs) but not different from that of  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4$  nAChRs. In contrast, when the chimeric fifth subunit contained the amino-terminal ECD of the  $\beta 2$  subunit (i.e.  $\beta 2\text{-}\alpha 4\text{-}\beta 2/\alpha 4$  nAChRs), the sensitivity to ACh was comparable with wild-type  $\beta 2\text{-}\alpha 4\text{-}\beta 2$  nAChRs but statistically different from that of  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4$  receptors (Figures 2B, C; Table 1). The amplitude of the maximal ACh responses for  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4/\beta 2$  nAChRs increased by seven and five times, compared with, respectively,  $\beta 2\text{-}\alpha 4\text{-}\beta 2/\alpha 4$  and  $\beta 2\text{-}\alpha 4\text{-}\beta 2$  nAChRs (Figure 2D). To probe that chimeric subunit  $\alpha 4/\beta 2$  has the capability to form an  $\alpha 4(+)/\alpha 4(-)$  agonist site with the adjacent  $\alpha 4$  subunit in the  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4/\beta 2$  receptor, we alanine-substituted the conserved agonist-binding W182 residue on the chimeric  $\alpha 4/\beta 2$  subunit to engineer mutant  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4^{\text{W182A}}/\beta 2$  receptor and then tested the functional consequences of the mutation. Unnatural amino acid mutagenesis has shown that ACh makes a cation- $\pi$  interaction with  $\alpha 4$ W182 in the  $(\alpha 4\beta 2)_2\beta 2$  nAChRs, and this interaction critically contributes to ACh-binding affinity and receptor activation (Xiu *et al.*, 2009). If an operational agonist site forms at the interface  $\alpha 4\text{-}\alpha 4^{\text{W182A}}/\beta 2$ , ACh should yield biphasic concentration-response curves. We have shown in previous studies that alanine substitution of W182 in individual agonist sites in concatenated  $(\alpha 4\beta 2)_2\alpha 4$  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  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4^{\text{W182A}}/\beta 2$

$\beta 2$  receptors was biphasic without significant changes in the amplitude of the maximal ACh current responses (Figure 2D). In addition, we also transferred  $\beta 2$  E loop residues  $\beta 2$ V135,  $\beta 2$ F144 and  $\beta 2$ L146 to the E loop of chimeric  $\alpha 4/\beta 2$  subunit to engineer  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4^{\beta 2\text{Eloop}}/\beta 2$  receptors.  $\beta 2$ V135,  $\beta 2$ F144 and  $\beta 2$ L146 residues are equivalent to  $\alpha 4$  E loop residues H142, Q150 and T152 (Harpsoe *et al.*, 2011). Previous studies have shown that transferring the  $\beta 2$  E loop residues to the fifth subunit in  $(\alpha 4\beta 2)_2\alpha 4$  receptors induces a left shift in ACh sensitivity to  $(\alpha 4\beta 2)_2\beta 2$ -like levels (Harpsoe *et al.*, 2011; Lucero *et al.*, 2016). As shown in Figure 2C (Table 1), the ACh sensitivity of  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4^{\beta 2\text{Eloop}}/\beta 2$  receptors was comparable with that of  $\beta 2\text{-}\alpha 4\text{-}\beta 2$  receptors. In accord with Lucero *et al.* (2016), introducing the  $\alpha 4$  E loop residues into the  $\beta 2/\alpha 4$  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  $\alpha 4\beta 2$  nAChRs maps to the amino-terminal ECD of the fifth subunit (Harpsoe *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  $(\alpha 4\beta 2)_2\alpha 4$  isoform plays a critical role in determining the ACh sensitivity of the  $(\alpha 4\beta 2)_2\alpha 4$  isoform (Harpsoe *et al.*, 2011; Lucero *et al.*, 2016) but not that of the  $(\alpha 4\beta 2)_2\beta 2$  receptor (Lucero *et al.*, 2016; this study), although it appears to modify functional expression.

### The fifth subunit modulates ACh maximal currents in the $(\alpha 4\beta 2)_2\beta 2$ nAChR

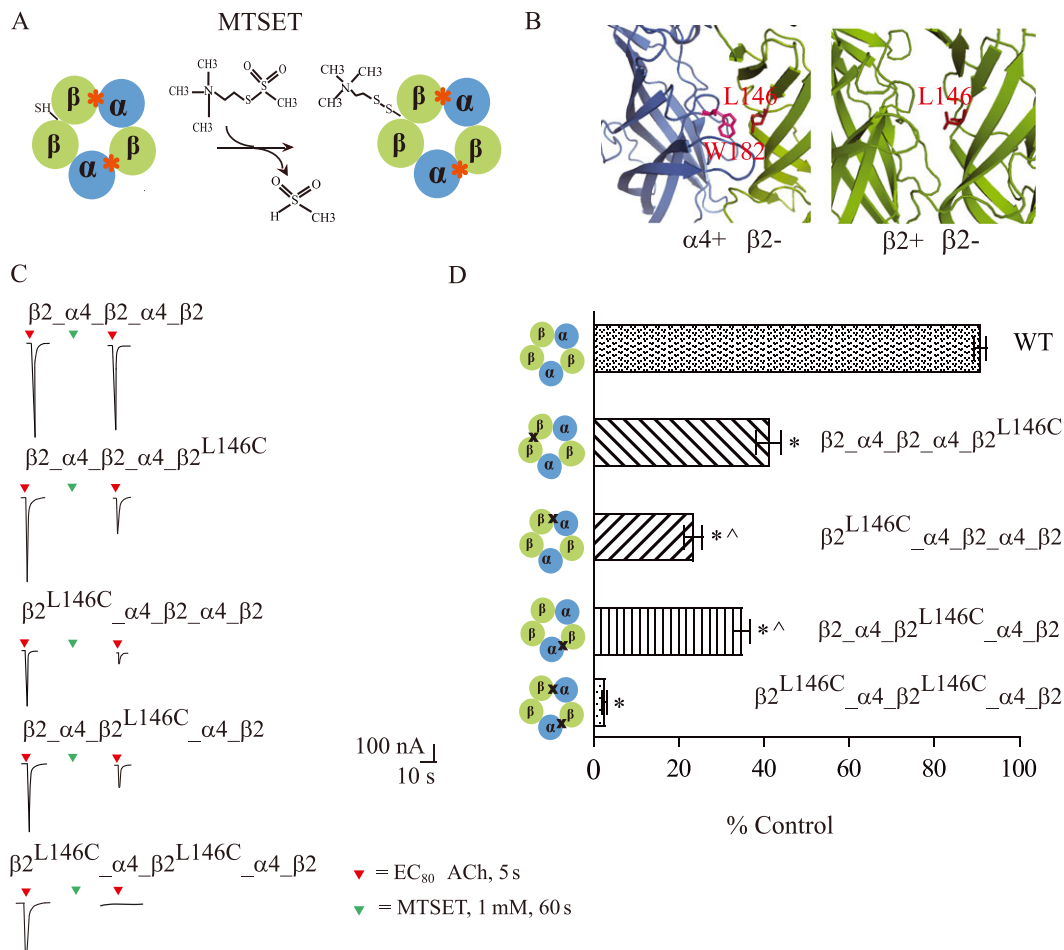
To further examine the effect of the fifth subunit on the amplitude of the maximal ACh current responses of the  $(\alpha 4\beta 2)_2\beta 2$  receptor, we introduced a cysteine residue in lieu of  $\beta 2$ L146 in the fifth subunit to engineer  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4\text{-}\beta 2^{\text{L146C}}$  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  $\beta 2^{\text{L146C}}\text{-}\alpha 4\text{-}\beta 2$  and  $\beta 2\text{-}\alpha 4\text{-}\beta 2^{\text{L146C}}\text{-}\alpha 4$  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  $\alpha 4\beta 2$  nAChRs. As for the  $\alpha 4(+)/\beta 2(-)$  interfaces, the side chain of L146 in the fifth subunit orientates towards the space between the fifth subunit and the opposing subunit in the  $\beta 2(+)/\beta 2(-)$  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  $(\alpha 4\beta 2)_2\beta 2$  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 EC<sub>80</sub> 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 cysteines. As shown in Figure 3C, D, application of 1 mM MTSET irreversibly decreased the subsequent ACh-induced currents for  $\beta 2\text{-}\alpha 4\text{-}\beta 2\text{-}\alpha 4\text{-}\beta 2^{\text{L146C}}$  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  $\alpha 4\beta 2$  nAChRs. (A) Diagram of chimeric concatenated  $\alpha 4\beta 2$  nAChRs. A chimeric subunit consisting of the  $\alpha 4$  subunit extracellular domain (ECD) and the remaining part of the  $\beta 2$  subunit (or *vice versa*) was introduced into the fifth subunit position of both stoichiometric forms of the  $\alpha 4\beta 2$  nAChRs. (B) Representative traces of the current responses of wild-type and chimeric concatenated  $\alpha 4\beta 2$  nAChRs to ACh. (C) Concentration–response curves for ACh current responses in concatenated wild-type, chimeric and mutated chimeric  $\alpha 4\beta 2$  nAChRs expressed in *Xenopus* oocytes. The ECD of the fifth subunit of the alternate  $\alpha 4\beta 2$  nAChRs significantly affected the responses to ACh. The  $EC_{50}$  values and Hill coefficients ( $n_H$ ) are summarized in Table 1. (D) Maximal ACh current responses elicited by wild-type, chimeric and mutated chimeric concatenated  $\alpha 4\beta 2$  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 ( $\alpha 4\beta 2$ ) $_2\beta 2$  and chimeric ( $\alpha 4\beta 2$ ) $_2\beta 2/\alpha 4$  receptors but not between wild-type ( $\alpha 4\beta 2$ ) $_2\beta 2$  and ( $\alpha 4\beta 2$ ) $_2\beta 2/\alpha 4$  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 concatenated ( $\alpha 4\beta 2$ ) $_2\alpha 4$  nAChR is shown for comparison.



### Figure 3

Effects of MTSET on ACh  $EC_{80}$  responses in wild-type (WT) and cysteine-substituted concatenated ( $\alpha_4\beta_2$ ) $_2\beta_2$  nAChRs. (A) Cartoon depicting covalent labelling of cysteine-substituted ( $\alpha_4\beta_2$ ) $_2\beta_2$  nAChRs by MTSET. (B) Structure of the  $\alpha_4(+)/\beta_2(-)$  (right panel) and  $\beta_2(+)/\beta_2(-)$  (left panel) showing the position of L146. L146 is shown as stick. For reference, W182, a key agonist-binding residue, is also shown in the  $\alpha_4(+)/\beta_2(-)$  interface. The  $\alpha_4$  subunit is shown in blue and  $\beta_2$  in green. (C) Representative traces showing the effects of 1 mM MTSET on ACh  $EC_{80}$  current responses in wild-type or cysteine-substituted concatenated ( $\alpha_4\beta_2$ ) $_2\beta_2$  nAChRs. (D) MTSET 1 mM decreased significantly the responses to ACh  $EC_{80}$  in all mutant receptors. The amplitude of the currents remaining after MTSET (% Control) were calculated using the equation [(latter MTSET/initial - 1)  $\times$  100], as described in the Methods section. Significant differences between the cysteine-substituted receptors and control ( $\beta_2\_α_4\_β_2\_α_4\_β_2$ ) are shown by an asterisk and were determined with one-way ANOVA with Dunnett's post test. ^Indicates that Student's unpaired *t*-test showed that the maximal inhibition of  $\beta_2^{L146C}\_α_4\_β_2\_α_4\_β_2$  and  $\beta_2\_α_4\_β_2^{L146C}\_α_4\_β_2$  receptors by MTSET are significantly different. The data shown represent  $n = 8$  for each type of receptor tested. \* and ^ indicate  $P < 0.05$  level of statistical significance.

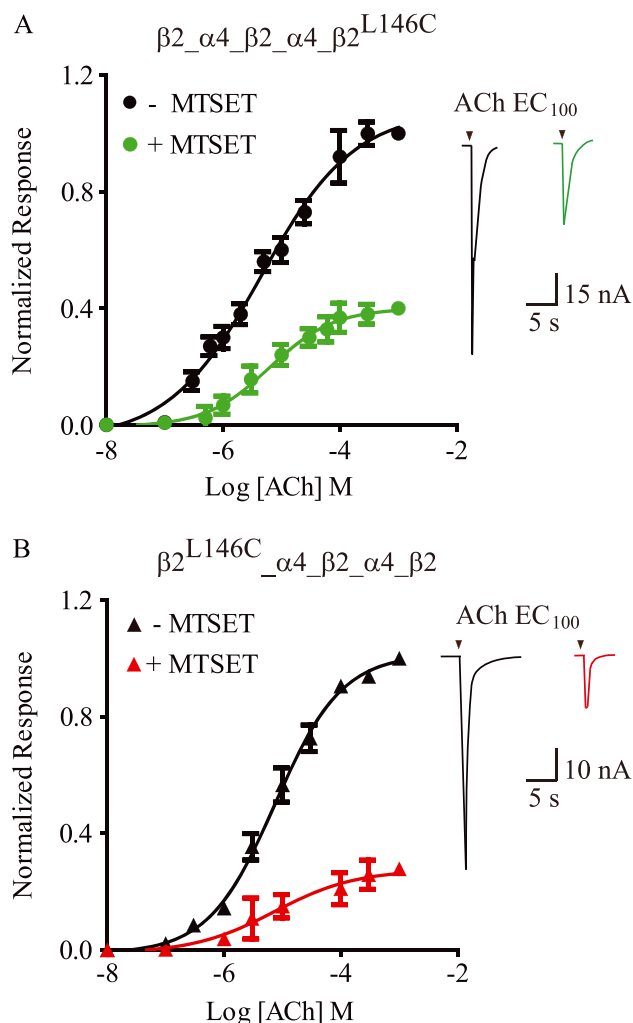
in  $\beta_2^{L146C}\_α_4\_β_2\_α_4\_β_2$  and  $\beta_2\_α_4\_β_2^{L146C}\_α_4\_β_2$ , although in comparison with  $\beta_2\_α_4\_β_2\_α_4\_β_2^{L146C}$  receptors, the effect on  $\beta_2^{L146C}\_α_4\_β_2\_α_4\_β_2$  or  $\beta_2\_α_4\_β_2^{L146C}\_α_4\_β_2$  was more pronounced (4.2- and 3.2 times respectively) (Figure 3C, D). These data show that covalent modification of  $\beta_2^{L146C}$  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  $\beta_2^{L146C}\_α_4\_β_2^{L146C}\_α_4\_β_2$  receptors before and after MTSET treatment. The ACh sensitivity of  $\beta_2^{L146C}\_α_4\_β_2^{L146C}\_α_4\_β_2$  receptors was not different from wild type (Table 1) but

exposure to MTSET completely abolished the responses to ACh. Thus, when both ( $\alpha_4\beta_2$ ) $_2\beta_2$  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 ( $\alpha_4\beta_2$ ) $_2\beta_2$  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  $\beta_2\_α_4\_β_2\_α_4\_β_2^{L146C}$  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  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$  by 2.5 times ( $n = 5$ ;  $P < 0.05$ ) without significant changes in the ACh  $EC_{50}$  ( $EC_{50}$  before MTSET =  $5.41 \pm 2 \mu\text{M}$ ;  $EC_{50}$  after MTSET =  $5.9 \pm 1.1 \mu\text{M}$ ;  $n = 5$ ). For control purposes, we also determined the ACh concentration–response curve before and after MTSET treatment of  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  receptors. As shown in Figure 4B, MTSET derivatization of  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  decreased the maximal current response of ACh by four times without significant changes in ACh potency ( $EC_{50}$  before MTSET =  $7.48 \pm 3 \mu\text{M}$ ;  $EC_{50}$  after MTSET =  $7.27 \pm 2 \mu\text{M}$ ;  $n = 5$ ). These findings are



**Figure 4**

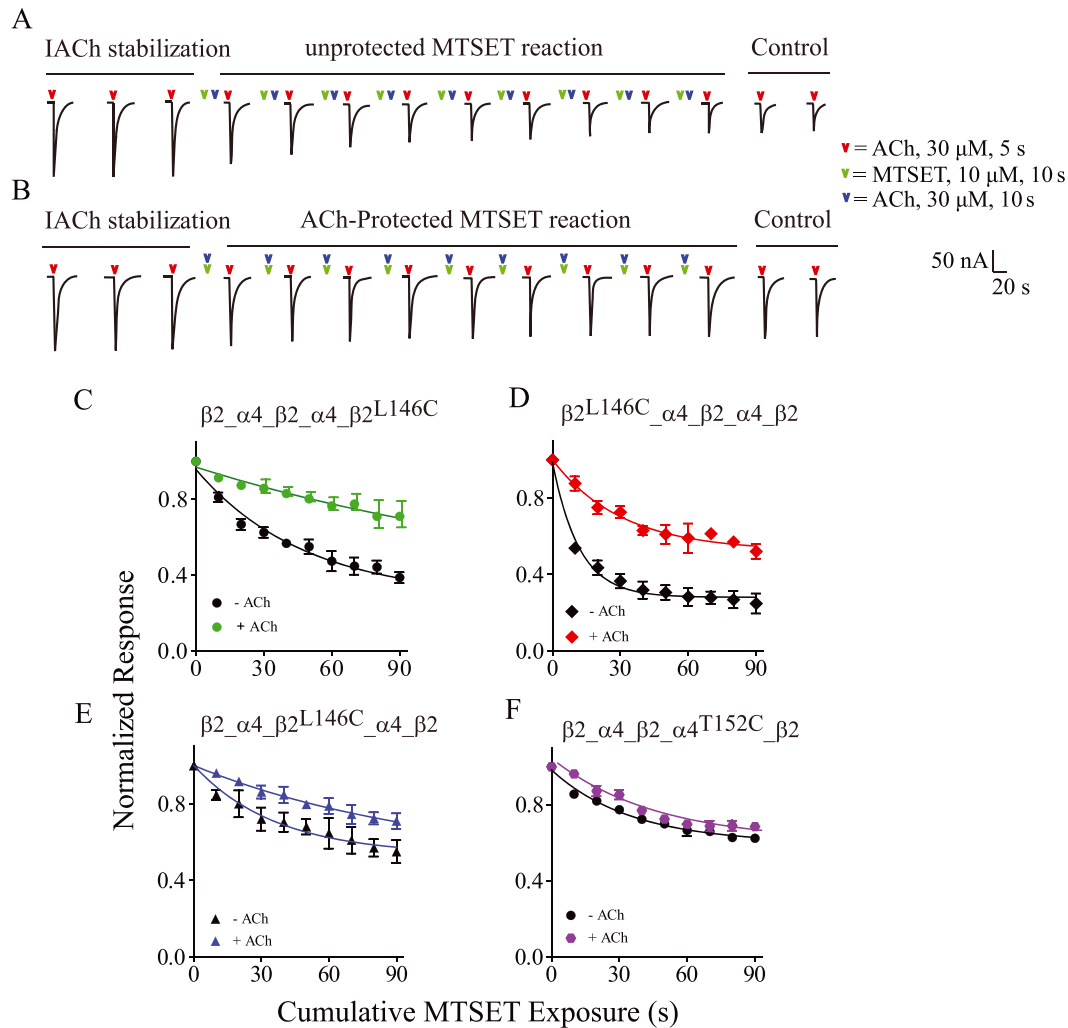
MTSET mediated modification decreases the maximal ACh responses of concatenated  $(\alpha_4\beta_2)_2\beta_2$  receptors. Derivatization of  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$  or  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  receptors by 1 mM MTSET reduced the maximal ACh responses without changes in the potency of ACh. The ACh concentration–response curve for  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$  (A) or  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  (B) receptors was obtained before and after 1 min exposure to 1 mM MTSET. Data points represent the means  $\pm$  SEM of five experiments. Data were fit by nonlinear regression, as described in the Methods section.

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 $\beta_2(+)/\beta_2(-)$ 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  $(\alpha_4\beta_2)_2\beta_2$  receptor, and this effect could be driven by an operational agonist or a modulatory site on the  $\beta_2(+)/\beta_2(-)$  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  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$  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  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  and  $\beta_2_{\alpha 4}\beta_2^{L146C}\alpha_4\beta_2$  receptors. As for  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$ , ACh decreased the modification of  $\beta_2^{L146C}\alpha_4\beta_2_{\alpha 4}\beta_2$  and  $\beta_2_{\alpha 4}\beta_2^{L146C}\alpha_4\beta_2$  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  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}\beta_2^{L146C}$  receptors (Table 2). These data suggest that accessibility to L146C, in the presence or absence of ACh, is  $\beta_2$  position-dependent. The rank order of L146C accessibility is ABS 1 > ABS 2 >  $\beta_2(+)/\beta_2(-)$ . For an additional inter-subunit interface control, we cysteine-substituted  $\alpha_4T152$ , the  $\alpha_4$  residue equivalent to  $\beta_2L146$ , in one of the  $\beta_2(+)/\alpha_4(-)$  interface ( $\beta_2_{\alpha 4}\beta_2_{\alpha 4}^{T152C}\beta_2$ ) and then measured the rate of MTSET modification in the presence and absence of ACh. The ACh  $EC_{50}$  in  $\beta_2_{\alpha 4}\beta_2_{\alpha 4}^{T152C}\beta_2$  was no different from wild type (Table 1). As shown in Figure 5F, the rate of MTSET reaction ( $2829 \pm 610 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ) was not significantly different from the rate measured in the presence of ACh ( $2171 \pm 715 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ) (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  $\beta_2(+)/\beta_2(-)$  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 DH $\beta$ E. Available DH $\beta$ E-bound crystal structures of *Lymnaean* AChBP (Shahsavari *et al.*, 2012) and functional data from mutagenesis studies of the  $\alpha_4\beta_2$  nAChR (Iturriaga-Vásquez *et al.*, 2010) have shown that DH $\beta$ E and agonists interact with the same conserved aromatic residues in canonical agonist sites. Furthermore, we have found in a previous study that DH $\beta$ E slows down the rate of MTSET reaction in cysteine-substituted  $\alpha_4(+)/\beta_2(-)$  or  $\alpha_4(+)/\alpha_4(-)$  agonist sites in the  $(\alpha_4\beta_2)_2\alpha_4$  receptor (Mazzaferro *et al.*, 2011). Thus, if there is an ACh-binding site on the  $\beta_2(+)/\beta_2(-)$  interface



**Figure 5**

Effect of ACh on the rate of MTSET modification of substituted concatenated ( $\alpha_4\beta_2$ ) $_2\beta_2$  nACh receptors. Representative traces of responses to ACh EC<sub>80</sub> before and after cumulative MTSET application in the absence (A) or presence (B) of ACh EC<sub>80</sub>. The traces shown were obtained from  $\beta_2_{\alpha_4}\beta_2_{\alpha_4}\beta_2^{L146C}$  receptors. Rates of MTSET modification of  $\beta_2_{\alpha_4}\beta_2_{\alpha_4}\beta_2^{L146C}$  (C),  $\beta_2^{L146C}_{\alpha_4}\beta_2_{\alpha_4}\beta_2$  (D),  $\beta_2_{\alpha_4}\beta_2^{L146C}_{\alpha_4}\beta_2$  (E) or  $\beta_2_{\alpha_4}\beta_2_{\alpha_4}^{T152C}\beta_2$  (F) receptors in the absence or presence of ACh. For (C–E),  $n = 8$ . For (F),  $n = 5$ . Data were normalized and fit to a single-phase exponential decay, as described in the Methods section. Second-order rate constants for MTSET modification of L146C are summarized in Table 2.

formed by conserved aromatic residues, it is reasonable to expect a decrease in the rate of MTSET reaction in the presence of DH $\beta$ E. Figure 6A shows that EC<sub>80</sub> DH $\beta$ E did not perturb the rate of MTSET modification of  $\beta_2_{\alpha_4}\beta_2_{\alpha_4}\beta_2^{L146C}$  receptors ( $k_2 - \text{DH}\beta\text{E} = 1876 \pm 251 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ;  $k_2 + \text{DH}\beta\text{E} = 1671 \pm 391 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ). For comparison, we also measured the rate of MTSET reaction in the presence of DH $\beta$ E for  $\beta_2^{L146C}_{\alpha_4}\beta_2_{\alpha_4}\beta_2$  receptors or  $\beta_2_{\alpha_4}\beta_2^{L146C}_{\alpha_4}\beta_2$  receptors. As expected for canonical agonist sites, we found that DH $\beta$ E decreased the rate of MTSET reaction with  $\beta_2^{L146C}_{\alpha_4}\beta_2_{\alpha_4}\beta_2$  ( $k_2 - \text{DH}\beta\text{E} = 5645 \pm 721 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $k_2 + \text{DH}\beta\text{E} = 787 \pm 141 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ) receptors (Figure 6B) and  $\beta_2_{\alpha_4}\beta_2^{L146C}_{\alpha_4}\beta_2$  ( $k_2 - \text{DH}\beta\text{E} = 2561 \pm 800 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $k_2 + \text{DH}\beta\text{E} = 955 \pm 256 \text{ M}^{-1}\cdot\text{s}^{-1}$ ;  $n = 5$ ) receptors (Figure 6C).

### Conserved aromatic residues in the $\beta_2(+)/\beta_2(-)$ interface do not affect ACh sensitivity

Consistent with the presence of an agonist site on the  $\beta_2(+)/\beta_2(-)$  interface, key aromatic  $\alpha_4$  subunit agonist-binding residues (W182, Y120, W88 and Y230) are conserved in the  $\beta_2$  subunit ( $\beta_2$ W176,  $\beta_2$ Y120;  $\beta_2$ W82 and  $\beta_2$ Y221). We have previously shown that impairment of individual agonist sites in the ( $\alpha_4\beta_2$ ) $_2\alpha_4$  receptor isoform by alanine substitution of conserved aromatic residues yields biphasic ACh concentration–response curves (Mazzaferro *et al.*, 2011, 2014). Thus, if the  $\beta_2(+)/\beta_2(-)$  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

**Table 2**Rates of covalent modification of cysteine-substituted ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nACh receptors by MTSET

Receptor	Control $k_2$ ( $M^{-1}\cdot s^{-1}$ )	$n$	+ ACh EC <sub>80</sub> $k_2$ ( $M^{-1}\cdot s^{-1}$ )	$n$	$k_{2c}/k_2 + \text{ACh}$
$\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$	2089 ± 310 <sup>^+</sup>	8	551 ± 141 <sup>*^+^+</sup>	8	3.8
$\beta 2^{L146C}_{\alpha 4}\beta 2_{\alpha 4}\beta 2$	5543 ± 541 <sup>^</sup>	8	1009 ± 125 <sup>*^+^+</sup>	8	5.5
$\beta 2_{\alpha 4}\beta 2^{L146C}_{\alpha 4}\beta 2$	2751 ± 510 <sup>^+</sup>	8	992 ± 101 <sup>*^+^+</sup>	8	4
$\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2^{L146C}$	861 ± 1115 <sup>+</sup>	8	901 ± 180 <sup>+</sup>	8	0.96
$\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2^{L146C}$	3010 ± 593 <sup>+</sup>	8	1164 ± 168 <sup>*^+</sup>	8	2.1
$\beta 2_{\alpha 4}\beta 2^{T152C}_{\alpha 4}\beta 2$	2829 ± 610	5	2171 ± 715	5	1.3

Rates of the reaction to MTSET with introduced cysteine were measured, and second-order rate constant ( $k_2$ ;  $M^{-1}\cdot s^{-1}$ ) were calculated as described in the Methods section. Second-order rate constants represent the mean ± SEM of  $n$  number of experiments.  $k_{2c}/k_2 + \text{ACh}$  represents the ratio of second-order rates of MTSET reactions obtained in the control rate ( $k_{2c}$ ) and in the presence of ACh ( $k_2 + \text{ACh EC}_{80}$ ). Statistical differences between rate constants in the absence of ACh (control rate) and the rate in the presence of ACh were estimated for all receptors by Student's unpaired  $t$ -test (differences are noted by \*). Statistical differences between the rates of reaction (in the absence or presence of ACh) of  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$ ,  $\beta 2^{L146C}_{\alpha 4}\beta 2_{\alpha 4}\beta 2$  and  $\beta 2_{\alpha 4}\beta 2^{L146C}_{\alpha 4}\beta 2$  were measured using one-way ANOVA tests and differences are noted by ^. Comparison between the rates of  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$ ,  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  and  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2^{L146C}$  receptors was measured using one-way ANOVA and differences are noted by +. \*, ^ and + denote  $P < 0.05$ .

aromatic residues in the  $\beta 2(+)/\beta 2(-)$  interface had no effect on potency of ACh. This finding indicates that conserved aromatic residues do not engage ACh in the  $\beta 2(+)/\beta 2(-)$  interface.

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

So far, the findings suggest that agonist-bound  $\alpha 4(+)/\beta 2(-)$  agonist sites affect accessibility of L146C in the fifth subunit. If this is the case, impairing the  $\alpha 4(+)/\beta 2(-)$  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  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptor and then measuring the rate of modification of L146C by MTSET in the presence or absence of ACh.

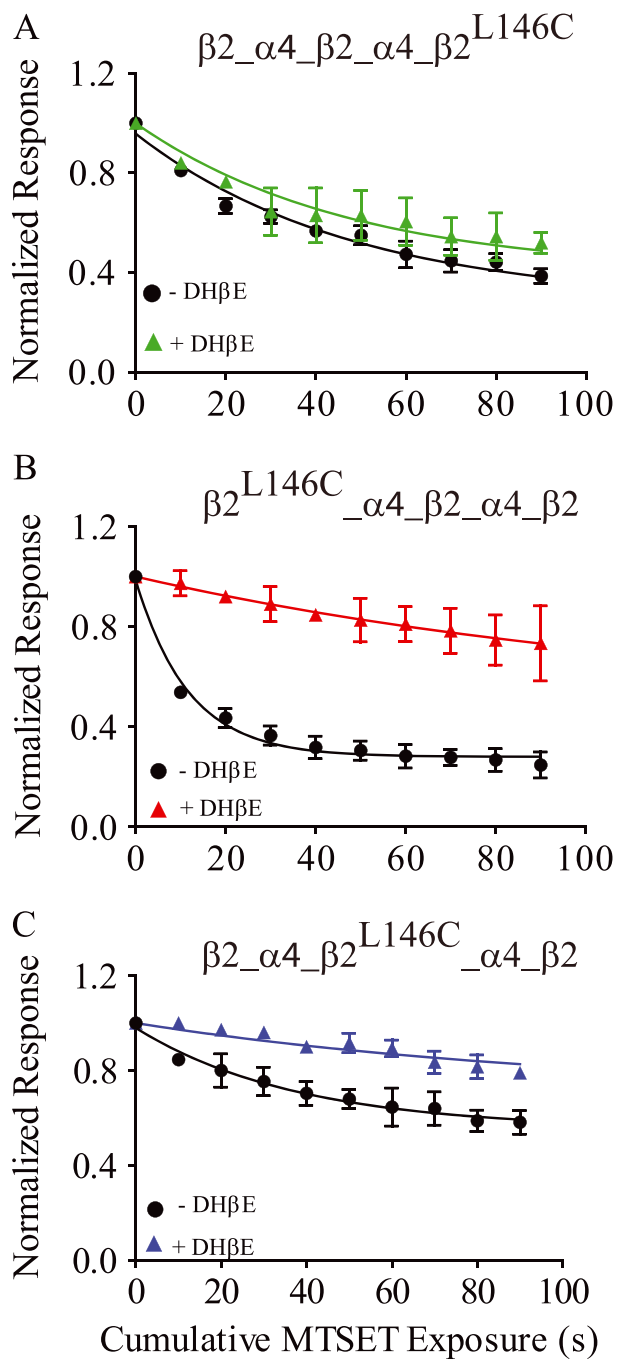
Introducing W182A impacted the sensitivity of  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  to activation by ACh. The extent of the effect depended on which agonist site (ABS 1 or ABS 2) carried the W182A mutation. When W182A was introduced into ABS 1 (i.e.  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2_{\alpha 4}\beta 2$ ), W182A caused a biphasic ACh sensitivity, comprising a high-affinity component ( $EC_{50}$  1.07 ± 0.1  $\mu$ M) and a low-affinity component ( $EC_{50}$  53 ± 12  $\mu$ M) (Table 1). In contrast, incorporation of W182A into ABS 2 (i.e.  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2$ ) did not produce biphasic concentration–response curves for ACh but decreased ACh potency from 8.64 ± 2.2 to 17.00 ± 4  $\mu$ M (Table 1). In accord with our findings, Lucero *et al.* (2016) found that  $\beta 2$  E loop mutations impair more drastically the function of ABS 1 than that of ABS 2.

Compared with  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptors, the rate of MTSET reaction in  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptors was 2.2 times slower, whereas in  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2^{L146C}$  receptors, the rate was 1.4 times slower (Figure 7A; data summarized in Table 2). More strikingly, compared with  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$ , the accessibility of L146C in  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptors was almost obliterated in the presence of ACh (Figure 7A, Table 2), whereas in  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2^{L146C}$ , the accessibility was reduced by

2.1 times (Figure 7B; Table 2). We noticed a slight potentiation of the ACh responses in to  $\beta 2_{\alpha 4}\beta 2^{W182A}_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  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 ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChRs, site-directed mutagenesis, along with probing with the thiol-reactive MTSET reagent, we have shown that the fifth subunit in the ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChR, like its counterpart in the ( $\alpha 4\beta 2$ )<sub>2</sub> $\alpha 4$  isoform, plays an important role in the maximal ACh responses of the receptor, albeit more subtly. Our findings have also confirmed that  $\alpha 4(+)/\beta 2(-)$  agonist sites in the ( $\alpha 4\beta 2$ )<sub>2</sub> $\beta 2$  nAChR isoform function asymmetrically (Lucero *et al.*, 2016). We found that ACh but not DH $\beta$ E, a selective competitive inhibitor of the  $\alpha 4\beta 2$  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  $\beta 2(+)/\beta 2(-)$  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  $\beta 2(+)/\beta 2(-)$  interface. By impairing the function of the  $\alpha 4(+)/\beta 2(-)$  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  $\alpha 4(+)/\beta 2(-)$  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



**Figure 6**

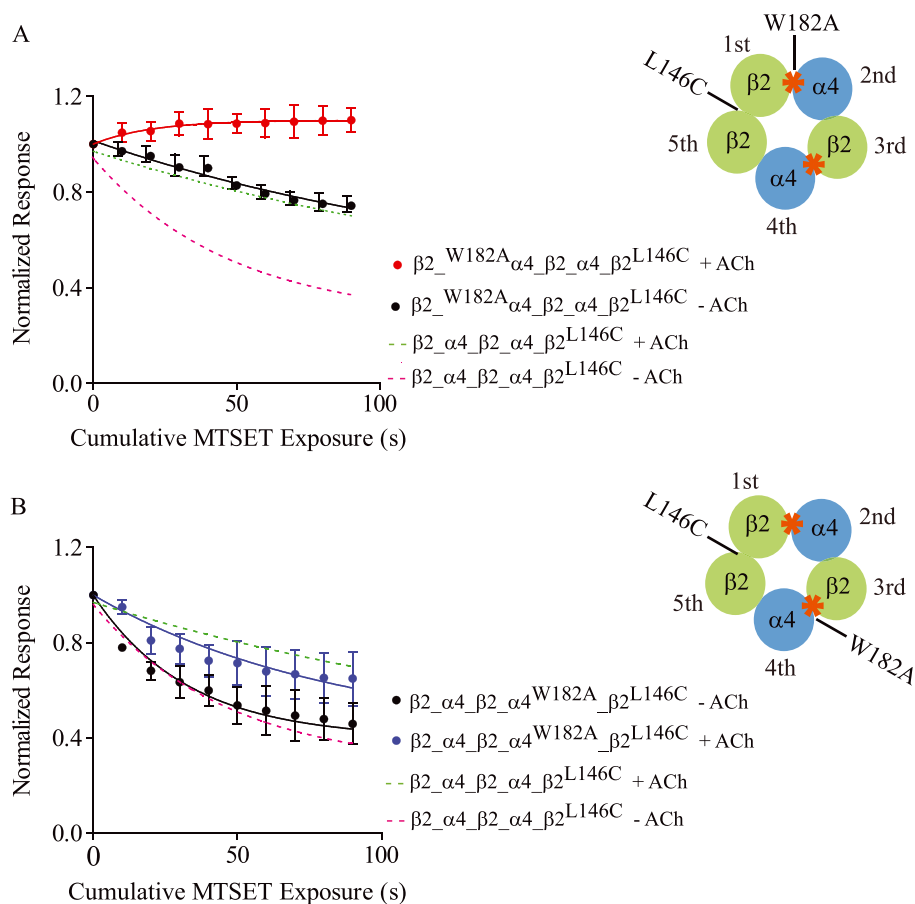
Effect of the competitive antagonist DH $\beta$ E on the rate of MTSET modification of cysteine-substituted  $\beta 2(+)/\beta 2(-)$  or  $\alpha 4(+)/\beta 2(-)$  interfaces in concatenated  $(\alpha 4\beta 2)_2\beta 2$  nACh receptors. (A) The rate of MTSET derivatization of cysteine-substituted  $\beta 2_{\alpha 4}_{\beta 2}_{\alpha 4}_{\beta 2}^{L146C}$  receptors was not affected by the presence of an IC<sub>20</sub> concentration of DH $\beta$ E ( $n = 5$ ). In contrast, DH $\beta$ E slowed down the rate of MTSET modification of  $\beta 2^{L146C}_{\alpha 4}_{\beta 2}_{\alpha 4}_{\beta 2}$  ( $n = 5$ ) (B) or  $\beta 2_{\alpha 4}_{\beta 2}^{L146C}_{\alpha 4}_{\beta 2}$  ( $n = 5$ ) (C) receptors (Student's unpaired  $t$ -test). Data were normalized and fit to a single-phase exponential decay, as described in the Methods section.

fifth subunit and the agonist sites might be the mechanism underlying functional asymmetry in the  $\alpha 4(+)/\beta 2(-)$  agonist sites in the  $(\alpha 4\beta 2)_2\beta 2$ .

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  $(\alpha 4\beta 2)_2\beta 2$  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  $\alpha 4(+)/\beta 2(-)$  agonist sites. The fifth subunit through the  $\beta 2(+)/\beta 2(-)$  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  $\alpha 4(+)/\beta 2(-)$  agonist sites. The  $\beta 2$  subunit conserves key  $\alpha 4$  aromatic residues that line the core of  $\alpha 4(+)/\beta 2$  agonist sites in the  $(\alpha 4\beta 2)_2\beta 2$  receptor (Morales-Perez *et al.*, 2016), and these residues could potentially be part of an agonist or modulatory site on the  $\beta 2(+)/\beta 2(+)$  interface.

Unexpectedly, DH $\beta$ E slowed down the rate of modification of the  $\alpha 4(+)/\beta 2(-)$  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 (Karlín and Akabas, 1998; Sullivan and Cohen, 2000). Available DH $\beta$ E-bound crystal structures of *Lymnaean* AChBP have shown that DH $\beta$ E and agonists contact the same conserved residues in the agonist site: Y126, W182, Y223 and Y230 from the (+) side of the  $\alpha 4(+)/\beta 2(-)$  agonist site and W82 from the (-) site (Shahsavari *et al.*, 2012). Furthermore, functional data from mutagenesis studies of the  $\alpha 4\beta 2$  nAChR have shown that alanine substitution of these residues reduce the inhibitory potency of DH $\beta$ E (Iturriaga-Vásquez *et al.*, 2010). Thus, the most straightforward explanation for our findings is that the conserved aromatic residues in the  $\beta 2(+)/\beta 2(-)$  interface do not bind agonist or antagonist, like they do in the  $\alpha 4(+)/\alpha 4(-)$  interface of the  $(\alpha 4\beta 2)_2\alpha 4$  receptor (Harpsoe *et al.*, 2011; Mazzaferro *et al.*, 2011; Wang *et al.*, 2015; Jain *et al.*, 2016). Significantly, the recently resolved X-ray structure of the human  $(\alpha 4\beta 2)_2\beta 2$  nAChR reveals a reorganization of conserved tyrosine residues (Y120 and Y221) in the  $\beta 2(+)/\beta 2(-)$  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  $\pi$  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  $\beta 2(+)/\beta 2(-)$  interface that does not include the conserved aromatic residues and that excludes DH $\beta$ E. Recent studies have indicated that the pharmacology of nAChRs is influenced by sites located at  $\beta(+)/\alpha(-)$  interfaces that do not involve conserved aromatic residues (Moroni *et al.*, 2008; Seo *et al.*, 2009). More pertinently, Jain *et al.* (2016) reported that



### Figure 7

Effect of ACh-bound mutated agonist sites on MTSET modification of the fifth subunit of  $(\alpha 4\beta 2)_2\beta 2$  nACh receptors. The rate of MTSET modification of  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  in the absence or presence of ACh was altered when the W182A mutation was introduced in the  $\alpha 4(+)/\beta 2(-)$  agonist sites to form  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}^{W182A}\beta 2^{L146C}$  (A) or  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}^{W182A}\beta 2^{L146C}$  (B). Data were normalized and fit to a single-phase exponential decay, as described in the Methods section. For A and B,  $n = 8$ . Rate constants are summarized in Table 2. The cartoon adjacent to the exponential decay plots shows the position of incorporation of W182A in the cysteine-substituted  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptors. For comparison, we show the curves for the rate of MTSET reaction in the absence or presence of ACh for  $\beta 2_{\alpha 4}\beta 2_{\alpha 4}\beta 2^{L146C}$  receptors.

irreversible modification of  $\alpha 5/\alpha 4$  and  $\beta 3/\alpha 4$  interfaces in respectively  $(\alpha 4\beta 2)_2\alpha 5$  and  $(\alpha 4\beta 2)_2\beta 3$  nAChRs reduces the maximal ACh responses without changes in  $EC_{50}$ . These authors concluded that  $\alpha 5/\alpha 4$  and  $\beta 3/\alpha 4$  interfaces contain operational agonist sites of an unorthodox nature (Jain *et al.*, 2016).  $\alpha 5$  and  $\beta 3$  nAChR subunits were thought to be incapable of forming agonist sites. However, earlier studies have shown that mutations of conserved aromatic residues in the  $\alpha 5$  subunit had no effect on the agonist sensitivity of  $(\alpha 4\beta 2)_2\alpha 5$  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  $\alpha 5/\alpha 4$  and  $\beta 3/\alpha 4$  interfaces affect the agonist responses of  $(\alpha 4\beta 2)_2\alpha 5$  and  $(\alpha 4\beta 2)_2\beta 3$  nAChRs. For example, probing the ability of agonists and antagonists to reduce accessibility of cysteines incorporated into putative agonist-binding residues in the  $\alpha 5/\alpha 4$  and  $\beta 3/\alpha 4$  interfaces should help in better understanding the agonist sites that mediate the effects of these interfaces. In the case of the  $(\alpha 4\beta 2)_2\beta 2$  receptor, an ACh-binding site on the  $\beta 2(+)/\beta 2(-)$  interface seems unlikely. The high-resolution structure of the human  $(\alpha 4\beta 2)_2\beta 2$  nAChR was

obtained by co-crystallization with nicotine, and this agonist was found bound only to  $\alpha 4(+)/\beta 2(-)$  agonist sites (Morales-Perez *et al.*, 2016).

Impairment of  $\alpha 4(+)/\beta 2(+)$  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  $\beta 2(+)/\beta 2(-)$  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  $(\alpha 4\beta 2)_2\beta 2$  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  $(\alpha 4\beta 2)_2\beta 2$  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  $\alpha 4(+)/\alpha 4(-)$  and  $\alpha 4(+)/\beta 2(-)$  interfaces in the



isoform ( $\alpha 4\beta 2$ ) $\alpha 4$  have also been proposed to account for the different patterns of single-channel opening durations exhibited by the alternate  $\alpha 4\beta 2$  nAChR isoforms (Mazzaferro *et al.*, 2017). Here, on the basis of our findings, we propose that ABS 1 and the fifth subunit, through the  $\beta 2(+)/\beta 2(-)$ -interface, constitute a functional unit and that this arrangement modulates the maximal ACh responses in the ( $\alpha 4\beta 2$ ) $\beta 2$  nAChR and confers functional asymmetry to the  $\alpha 4(+)/\beta 2(-)$  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 structural changes initiated at the  $\alpha 4(+)/\beta 2(-)$  can extend over considerable distance. Allosteric signals can propagate over long distances in pLGIC. In the GABA<sub>A</sub> receptor, an inhibitory pLGIC, the GABA-binding site positioned anti-clockwise to the  $\gamma$  subunit undergoes structural rearrangement upon binding of the positive benzodiazepine modulator flurazepam (Kloda and Czajkowski, 2007; Eaton *et al.*, 2012). In the GABA<sub>A</sub> receptor, the binding sites are located at  $\beta/\alpha$  interfaces (the  $\beta$  subunit is the principal subunit in the GABA<sub>A</sub> receptor) and the  $\gamma$  subunit. This structural and functional arrangement is equivalent to the one we propose for the ( $\alpha 4\beta 2$ ) $\beta 2$  receptor. Consistently with this possibility, Baumann *et al.* (2003) reported asymmetry in the function of the agonist sites in the GABA<sub>A</sub> 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- $\beta 2(+)/\beta 2(-)$  unit modulate the maximal responses of ( $\alpha 4\beta 2$ ) $\beta 2$  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- $\beta 2(+)/\beta 2(-)$  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 DH $\beta$ E, 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 ( $\beta 1$  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  $\alpha 4(+)/\beta 2(-)$  agonist sites, Unwin and Fujiyoshi (2012) reported that although both agonist sites contribute to the movement of  $\beta 1$ , the agonist site at the  $\alpha 4$  subunit interface appears to be the most prominent driving

force behind the displacement of  $\beta 1$  (Unwin and Fujiyoshi, 2012). This asymmetry is consistent with our findings and supports our view that ABS 1 and the  $\beta 2(+)/\beta 2(-)$  interface form a functional unit that modulates the agonist responses of the ( $\alpha 4\beta 2$ ) $\beta 2$  nACh receptor isoform.

In conclusion, these data suggest that the fifth subunit in the ( $\alpha 4\beta 2$ ) $\beta 2$  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  $\alpha 4(+)/\beta 2(-)$  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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