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

Karina New, Silvia Garcia Del Villar, Simone Mazzaferro, Constanza Alcaino, Isabel Bermudez

Department of Biological and Medical Sciences, Faculty of Health and Life Sciences, Oxford Brookes University
Oxford OX3 0BP, UK.

Corresponding author:

Isabel Bermudez

Department of Biological and Medical Sciences

Faculty of Health and Life Sciences

Oxford Brookes University

Gipsy Lane, Oxford OX3 0BP

E-mail: ibermudez@brookes.ac.ul

Phone: +44 1865 483292

British Pharmacological Society

ABSTRACT

BACKGROUND AND PURPOSE:

The fifth subunit in the $(\alpha4\beta2)_2\alpha4$ nicotinic acetylcholine 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 $(\alpha4\beta2)_2\beta2$ 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 $(\alpha4\beta2)_2\beta2$ receptors.

KEY RESULTS:

Covalent modification of cysteine substituted fifth subunit with a thiol-reactive agent (MTS) caused irreversible inhibition of receptor function. ACh reduced the rate of MTS reaction but the competitive inhibitor dihydro-β-erythroidine had no effect. Alanine substitution of conserved residues that line the core of agonist sites on $\alpha4(+)/\beta2(-)$ interfaces did not impair receptor function. However, impairment of agonist binding to $\alpha4(+)/\beta2(-)$ agonist sites by mutagenesis modified the effect of ACh on the rate of MTS reaction. The extent of this effect was dependent on the position of the agonist site relative to the fifth subunit.

CONCLUSIONS AND IMPLICATIONS:

We conclude that the fifth subunit in $(\alpha4\beta2)_2\beta2$ receptor isoform modulates maximal ACh responses. This effect appears to be driven by a modulatory, and asymmetric, association with the $\alpha4(+)/\beta2(-)$ agonist sites.

Tables of Links

TARGET
Receptor
α4β2 nicotinic acetylcholine receptor

LIGANDS
Acetylcholine
Dihydro-β-erythroidine

These Tables of Links list the key protein target and ligand in this article that 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)

ABBREVIATIONS

Agonist binding site, ABS; dihydro-β-erythroidine, DHβE; extracellular domain, ECD; methanethiosulfonate or thiol-reactive reagent, MTS; methanethiolsulfonate reagent [2-(Trimethylammonium) ethyl] methanethiosulfonate, MTSET; nicotinic acetylcholine receptor, nAChR; pentameric ligand gated ion channel, pLGIC; substituted cysteine accessibility method , SCAM; transmembrane domain, TMD.

INTRODUCTION

The α4β2 nicotinic acetylcholine 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 α4β2 nicotinic acetylcholine receptor (nAChR) is a member of the pentameric ligand-gated ion channel (pLGICs) superfamily of neurotransmitter receptors that includes the muscle nAChR, GABA$_A$, glycine and serotonin type 3 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 α 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 (β1-β2 loop, the Cys loop and M2-M3 linker) at the interface between the TMD and the N-terminal extracellular domain (ECD) interface (Lee and Sine 2005; Jha et al., 2007; Lee et al., 2009). The most recent cryo-electron microscopy studies of *Torpedo* nAChRs have suggested that the fifth subunit (β1 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 α4β2 nAChR comprises two α4β2 pairs and a fifth subunit that can be β2 or α4, and this subunit difference produces two alternate receptor isoforms, the (α4β2)$_2$β2 and (α4β2)$_2$α4 nAChRs (Nelson et al., 2003; Moroni et al., 2006) (**Figure 1A).** The alternate 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; Alcaino 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 (α4β2)$_2$α4 stoichiometry housed by the interface between the fifth subunit (an α4) and an adjacent α4 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 α4(+)/α4(-) interface has been identified as critical in determining the agonist sensitivity differences between the (α4β2)$_2$β2 and (α4β2)$_2$α4 receptors: α4H142, α4Q150 and α4T152 (Harpsøe et al., 2011; Lucero et al., 2016). The fifth subunit in the (α4β2)$_2$β2 isoform (a β2) forms the receptor's signature β2(+)/β2(-) interface with an adjacent β2 subunit (**Figure 1A**). In contrast to the

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(α4β2)$_2$α4 receptors, transferring the α4 E loop to the fifth subunit in the (α4β2)$_2$β2 does not affect ACh sensitivity (Lucero et al., 2016).

Previously, we found that the agonist sites on the α4(+)/β2(-) interfaces in the (α4β2)$_2$α4 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 α4 non-conserved E loop residues to the β2 subunit, and *vice versa* (Lucero et al., 2016). Although this study did not find evidence of functional asymmetry in the α4(+)/β2(-) agonist sites of the (α4β2)$_2$α4 receptor, it found that their counterparts in the (α4β2)$_2$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, through 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)$_2$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 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

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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 is agonist binding-position dependent. Overall, our findings suggest that the fifth subunit through the β2(+)/β2(-) interface may communicates 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)₂β2 nAChR.

METHODS

Materials

The cationic methanethiolsulfonate reagent (MTS) [2-(Trimethylammonium) ethyl] methanethiolsulfonate (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.

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 anaesthetised by immersion in 0.5% tricaine until no-responsive to toe pinch. Toads were then decapitated and ovarian lobes were harvested and defolliculated by incubation in 2 mg/ml 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 α4β2 nAChR-cRNA, as previously described (Carbone et al., 2009). Injected oocytes were incubated until use 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 streptomycin, 1000 U/mL Penicillin and 50 µg/mL neomycin or amikacin (100 µg/mL) (pH 7.5, with 5 M NaOH).

Mutagenesis and Expression in Oocytes

The fully concatenated form of wild type or mutant α4β2 nAChRs was engineered as previously described (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) and

the 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, the subunit was cut from the concatemer using unique restriction enzyme sites and then its nucleotide sequence was verified by DNA sequencing (SourceBioscience, UK, Eurofins, UK). 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 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, $CaCl_2$ 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 normalised 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). ACh concentration response curve data were first fit to the one-component Hill equation, $I = I_{max}/[1 + (EC_{50}/x)^{nH}]$, where EC_{50} represents the concentration of agonist inducing 50% of the maximal response (I_{max}), x is the agonist concentration and nH the Hill coefficient. When ACh induced biphasic receptor activation, the concentration response curve data were fit to the sum of two Hill equations, as decribed previously (Moroni et al., 2006). For chimeric receptors, we measured their maximal functional expression and compare it to 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.

MTSET Modification of Substituted Cysteines

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 stabilised (<6% variance of peak current responses to ACh on four consecutive ACh applications). After stabilisation, 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 stabilised. For all mutant receptors except mutant $\beta2\_^{W182A}\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$, the concentration of ACh pulses were 30 μM (EC_{80}). For $\beta2\_^{W182A}\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$, EC_{80} was 100 μM (see Table 1). Higher concentrations of ACh were not used for the MTSET modification of substituted cysteine receptor experiments to minimise possible ion channel blockade by ACh and/or chronic receptor desensitisation. The effect of MTSET was estimated using the following equation: % Change = $[(I_{after\ MTSET}/I_{initial})$ – 1] x 100, where $I_{initial}$ is the response to ACh EC_{80} before MTSET application and I_{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 applications of sub-saturating concentrations of MTSET using a protocol previously described (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 stabilised as follows: EC_{80} 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_{80} 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 stabilised (<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 stabilisation of the ACh responses to correct for any process of desensitisation 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 40s. 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 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 EC_{80} 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_{80} 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: $y = span \times e^{-kt} + plateau$ (Graph Pad Software INC., San Diego, CA, USA), 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 (k_2) was calculated by dividing k_1 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 non-

linear 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 comparison involving more than two groups. Unpaired Student's 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 2 copies of the α4 subunit and 3 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.

RESULTS

We examined the contribution of the fifth subunit to the agonist responses of the $(\alpha4\beta2)_2\beta2$ nAChR. The fifth subunit in the $(\alpha4\beta2)_2\beta2$ receptor is a $\beta2$ subunit, and this subunit forms the signature $\beta2(+)/\beta2(-)$ interface with a $\beta2$ subunit that contributes to an $\alpha4(+)/\beta2(-)$ agonist site (**Figure 1 A).** To circumvent ambiguities in data analysis brought about by non-targeted subunit mutagenesis, the studies described here were carried out on fully concatenated $(\alpha4\beta2)_2\beta2$ nAChRs ($\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2$ nAChRs). $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2$ nAChRs replicate the pharmacological (Carbone et al., 2009) and single channel (Mazzaferro et al., 2017) properties of $(\alpha4\beta2)_2\beta2$ nAChRs assembled from free subunits. In concatenated $(\alpha4\beta2)_2\beta2$, the first subunit in the linear sequence of the concatemer (a $\beta2$ subunit) interfaces with the fifth subunit of the linear sequence of the concatemer (a $\beta2$ subunit), establishing the $\beta2(+)/\beta2(-)$ interface **(Figure 1A, B).** The first subunit contributes the principal face of the $\beta2(+)/\beta2(-)$ interface, whilst the fifth subunit contributes the complementary side **(Figure 1A).** Agonist binding sites 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 agonist binding site 1, ABS 1) and between the third and fourth subunits (hereafter termed agonist binding site 2, ABS 2) **(Figure 1 A, B).** For clarity, mutations in the linked receptors are shown as superscript positioned in the (+) or (-) side of the mutated subunit. For example, in $\beta2^{L146C}\_\alpha4\_\beta2\_\alpha4\_\beta2$ L146C is located in the (-) side of the $\beta2$ subunit forming part of ABS 1 and, in $\beta2\_\alpha4\_\beta2\_\alpha4^{T152C}\_\beta2$, T152C is positioned in the (-) side of the $\alpha4$ subunit contributing the (+) side of ABS 2.

ACh sensitivity in $\alpha4\beta2$ nAChRs maps to the N-terminal ECD

We first examined the effect of the fifth subunit on the function of $(\alpha4\beta2)_2\beta2$ nAChRs by testing the effect of ACh on concatenated $(\alpha4\beta2)_2\beta2$ receptors containing a chimeric fifth subunit. Chimeric subunits consisted of either the amino-terminal ECD of the $\alpha4$ subunit and the remaining part (TMD and C-terminus) of the $\beta2$ subunit ($\alpha4/\beta2$), or the amino-terminal ECD of the $\beta2$ subunit and the remaining part of the $\alpha4$ subunit ($\beta2/\alpha4$) (**Figure 2A**). As shown in **Figure 2B** and **C** (see **Table 1** for estimated values of ACh potency), the ACh sensitivity of receptors containing a chimeric $\alpha4/\beta2$ subunit at the fifth position was different from wild type ($\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2$ nAChRs) but not different from that of $\beta2\_\alpha4\_\beta2\_\alpha4\_\alpha4$ nAChRs. In contrast, when the chimeric fifth subunit contained the amino-terminal ECD of the $\beta2$ subunit (i.e., $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2/\alpha4$ nAChRs), the sensitivity to ACh was comparable to wild type $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2$ nAChRs but statistically different from that of $\beta2\_\alpha4\_\beta2\_\alpha4\_\alpha4$

receptors (**Figures 2B and C; Table 1**). The amplitude of the maximal ACh responses for β2_α4_β2_α4_α4/β2 nAChRs increased by 7- and 5-times, compared to respectively, β2_α4_β2_α4_β2/α4 and β2_α4_β2_α4_β2 nAChRs (**Figure 2D**). To probe that chimeric subunit α4/β2 has the capability to form an α4(+)/α4(-) agonist site with the adjacent α4 subunit in the β2_α4_β2_α4_α4/β2 receptor, we alanine substituted the conserved agonist-binding W182 residue on the chimeric α4/β2 subunit to engineer mutant β2_α4_β2_α4_α4^{W182A}/β2 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)$_2$β2 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 α4_^{W182A}α4/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)$_2$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 β2_α4_β2_α4_ ^{W182A}α4/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, β2F144 and β2L146 to the E loop of chimeric α4/β2 subunit to engineer β2_α4_β2_α4_α4^{β2Eloop}/β2 receptors. β2V135, β2F144 and β2L146 residues are equivalent to α4 E loop residues H142, Q150 and T152 (Harpsøe et al., 2011). Previous studies have shown that transferring the β2 E loop residues to the fifth subunit in (α4β2)$_2$α4 receptors induces a left-shift in ACh sensitivity to (α4β2)$_2$β2-like levels (Harpsøe et al., 2011; Lucero et al., 2016). As shown in **Figure 2C (Table 1**), the ACh sensitivity of β2_α4_β2_α4_α4^{β2Eloop}/β2 receptors was comparable to that of β2_α4_β2_α4_β2 receptors. In accord with Lucero et al. (2016), introducing the α4 E loop residues into the β2/α4 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 (Harpsøe 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)$_2$α4 isoform plays a critical role in determining the ACh sensitivity of the (α4β2)$_2$α4 isoform (Harpsøe et al., 2011; Lucero et al., 2016) but not that of the (α4β2)$_2$β2 receptor (Lucero et al., 2011; this study), although it appears to modify functional expression.

The fifth subunit modulates ACh maximal currents in the $(\alpha4\beta2)_2\beta2$ nAChR

To further examine the effect of the fifth subunit on the amplitude of the maximal ACh current responses of the $(\alpha4\beta2)_2\beta2$ receptor, we introduced a cysteine residue in lieu of β2L146 in the fifth subunit to engineer β2_α4_β2_α4_β2 ^{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 β2^{L146C}_α4_β2_α4_β2 and β2_α4_β2^{L146C}_α4_β2 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 that does not affect the sensitivity of the $(\alpha4\beta2)_2\beta2$ receptor to activation by ACh. Application of 1mM MTSET for 1 min to oocytes expressing wild type receptors had no effect on the subsequent ACh EC$_{80}$ 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 β2_α4_β2_α4_β2 ^{L146C} receptors by 2.3-times. MTSET also modified the subsequent ACh-induced currents in β2^{L146C}_α4_β2_α4_β2 and β2_α4_β2^{L146C}_α4_β2, although in comparison to β2_α4_β2_α4_β2 ^{L146C} receptors, the effect on β2^{L146C}_α4_β2_α4_β2 or β2_α4_β2^{L146C}_α4_β2 was more pronounced (4.2- and 3.2-times, respectively) (**Figure 3C, D**). These data show that covalent modification of β2L146C by MTSET reduces subsequent ACh responses and that the extent of the reduction is β2 position-dependent, being greater when the β2 subunit forms part of an α4(+)/β2(-) agonist site. Next, we examined if the receptor could activate after MTSET modification of both α4(+)/β2(-) agonist sites. To examine this, we tested the effect of ACh on β2^{L146C}_α4_β2^{L146C}_α4_β2 receptors before and after MTSET treatment. The ACh sensitivity of β2^{L146C}_α4_β2^{L146C}_α4_β2 receptors was not different from wild type (**Table 1**) but exposure to MTSET completely abolished the responses to ACh. Thus, when both $(\alpha4\beta2)_2\beta2$ 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

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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 $\beta 2\_\alpha 4\_\beta 2\_\alpha 4\_\beta 2^{L146C}$ before and after a 1 min exposure to 1 mM MTSET. It has been shown that derivatisation of conserved aromatic residues in the γ 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 ± 2 µM; EC_{50} after MTSET = 5.9 ± 1.1 µM; n = 5). For control purposes, we also determined the ACh concentration responses 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 derivatisation of $\beta 2^{L146C}\_\alpha 4\_\beta 2\_\alpha 4\_\beta 2$ decreased the maximal current response of ACh by 4 times without significant changes in ACh potency (EC_{50} before MTSET = 7.48 ± 3 µM; EC_{50} 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 $\beta 2(+)/\beta 2(-)$ 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 derivatisation of the substituted cysteine. If L146 is part of or nearby an ACh binding site, the presence of ACh should slow down its derivatisation by MTSET. **Figures 5A and B** show 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 of ACh (**B**). As shown in **Figure 5C** (data summarised 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 5 D, E**), although the rate of reaction in the absence or presence of ACh on these two receptors was more pronounced than on

$\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ receptors (**Table 2**). These data suggest that accessibility to L146C, in the presence or absence of ACh, is $\beta2$ position-dependent. The rank order of L146C accessibility is: ABS 1 > ABS 2 > $\beta2(+)/\beta2(-)$. For an additional inter-subunit interface control, we cysteine substituted $\alpha4T152$, the $\alpha4$ residue equivalent to $\beta2L146$, in one of the $\beta2(+)/\alpha4(-)$ interface ($\beta2\_\alpha4\_\beta2\_\alpha4^{T152C}\_\beta2$) and then measured the rate of MTSET modification in the presence and absence of ACh. The ACh EC_{50} in $\beta2\_\alpha4\_\beta2\_\alpha4^{T152C}\_\beta2$ 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 $\beta2(+)/\beta2(-)$ 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 *Lymnaean* AChBP (Shasavar et al., 2012) and functional data from mutagenesis studies of the $\alpha4\beta2$ nAChR (Iturriaga-Vásquez 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 $\alpha4(+)/\beta2(-)$ or $\alpha4(+)/\alpha4(-)$ agonist sites in the $(\alpha4\beta2)_2\alpha4$ receptor (Mazzaferro et al., 2011). Thus, if there is an ACh binding site on the $\beta2(+)/\beta2(-)$ interface formed by conserved aromatic residues, it is reasonable to expect a decrease in the rate of MTSET reaction in the presence of DHβE. **Figure 6A** shows that EC_{80} DHβE did not perturb the rate of MTSET modification of $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ receptors (k_2 - DHβE = 1876 ± 251 M^{-1}s^{-1}; n = 5); k_2 + DHβE = 1671 ± 391 M^{-1}s^{-1}; n = 5). For comparison, we also measured the rate of MTSET reaction in the presence of DHβE for $\beta2^{L146C}\_\alpha4\_\beta2\_\alpha4\_\beta2$ receptors or $\beta2\_\alpha4\_\beta2^{L146C}\_\alpha4\_\beta2$ receptors. As expected for canonical agonist sites, we found that DHβE decreased the rate of MTSET reaction with $\beta2^{L146C}\_\alpha4\_\beta2\_\alpha4\_\beta2$ (k_2 - DHβE = 5645 ± 721 M^{-1}s^{-1}; k_2 + DHβE = 787 ± 141 M^{-1}s^{-1}; n = 5) receptors (**Figure 6B**) and $\beta2\_\alpha4\_\beta2^{L146C}\_\alpha4\_\beta2$ (k_2 - DHβE = 2561 ± 800 M^{-1}s^{-1}; k_2 + DHβE = 955 ± 256 M^{-1}s^{-1}; n = 5) receptors **(Figure 6C)**.

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, Y230) are conserved in the β2 subunit (β2W176, β2Y120; β2W82 and β2Y221). We have previously shown that impairment of individual agonist sites in the $(\alpha4\beta2)_2\alpha4$ receptor isoform by alanine substitution 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 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 α4(+)/β2(-) agonist sites affect accessibility of L146C in the fifth subunit. If this is the case, impairing the α4(+)/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 $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ 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 $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ 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., $\beta2\_^{W182A}\alpha4\_\beta2\_\alpha4\_\beta2$), W182A caused a biphasic ACh sensitivity, comprising a high-affinity component (EC_{50} 1.07 ± 0.1 µM) and a low affinity component (EC_{50} 53 ± 12 µM) (**Table 1).** In contrast, incorporation of W182A into ABS 2 (i.e., $\beta2\_\alpha4\_\beta2\_^{W182A}\alpha4\_\beta2$) did not produce biphasic concentration response curves for ACh but decreased ACh potency from 8.64 ± 2.2 µM 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.

Compared to $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ receptors, the rate of MTSET reaction in $\beta2\_^{W182A}\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ receptors was 2.2 times slower, whereas in

β2_α4_β2_^{W182A}α4_β2^{L146C} receptors the rate was 1.4 times slower (**Figure 7A**; data summarised in **Table 2**). More strikingly, compared to β2_α4_β2_α4_β2^{L146C}, the accessibility of L146C in β2_^{W182A}α4_β2_α4_β2^{L146C} receptors was almost obliterated in the presence of ACh (**Figure 7A, Table 2**), whereas in β2_α4_β2_^{W182A}α4_β2^{L146C} the accessibility was reduced by 2.1 times (**Figure 7B; Table 2**). We noticed a slight potentiation of the ACh responses in to β2_^{W182A}α4_β2_α4_β2^{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.

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DISCUSSION

Here, by combining voltage-clamp electrophysiological recordings from concatenated (α4β2)$_2$β2 nAChRs, site-directed mutagenesis, along with probing with the thiol-reactive MTSET reagent, we have shown that the fifth subunit in the (α4β2)$_2$β2 nAChR, like its counterpart in the (α4β2)$_2$α4 isoform, plays an important role in the maximal ACh responses of the receptor, albeit more subtly. Our findings have also confirmed that α4(+)/β2(-) agonist sites in the (α4β2)$_2$β2 nAChR isoform function asymmetrically (Lucero et al., 2016). We found that ACh but not DHβE, 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 α4(+)/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 α4(+)/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 fifth subunit and the agonist sites might be the mechanism underlying functional asymmetry in the α4(+)/β2(-) agonist sites in the (α4β2)$_2$β2.

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 to 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)$_2$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)$_2$β2 receptor (Morales-

Hernandez et al., 2016) and these residues could potentially be part of an agonist or modulatory site on the $\beta2(+)/\beta2(+)$ interface.

Unexpectedly, DHβE slowed down the rate of modification of the $\alpha4(+)/\beta2(-)$ 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 recognising the site (Karlin and Akabas, 1998; Sullivan and Cohen, 2000). Available DHβE-bound crystal structures of *Lymnaean* 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 $\alpha4(+)/\beta2(-)$ agonist site and W82 from the (-) site (Shasavar et al., 2012). Furthermore, functional data from mutagenesis studies of the $\alpha4\beta2$ 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 $\beta2(+)/\beta2(-)$ interface do not bind agonist or antagonist, like they do in the $\alpha4(+)/\alpha4(-)$ interface of the $(\alpha4\beta2)_2\alpha4$ 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 $(\alpha4\beta2)_2\beta2$ nAChR reveals a reorganisation of conserved tyrosine residues (Y120 and Y221) in the $\beta2(+)/\beta2(-)$ 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 stabilise 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 $\beta2(+)/\beta2(-)$ 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 $\beta(+)/\alpha(-)$ interfaces that do not involve conserved aromatic residues (Moroni et al., 2008; Seo et al., 2009). More pertinently, Jain et al. (2016) reported that irreversible modification of $\alpha5/\alpha4$ and $\beta3/\alpha4$ interfaces in respectively, $(\alpha4\beta2)_2\alpha5$ and $(\alpha4\beta2)_2\beta3$ nAChRs reduces the maximal ACh responses without changes in $EC_{50.}$ These authors concluded that $\alpha5/\alpha4$ and $\beta3/\alpha4$ interfaces contain operational agonist sites of an unorthodox nature (Jain et al., 2016). $\alpha5$ and $\beta3$ nAChR subunits were thought to be incapable of forming agonist sites. However, earlier studies have shown that mutations of conserved aromatic residues in the $\alpha5$ subunit had no effect on the agonist sensitivity of

(α4β2)$_2$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)$_2$α5 and (α4β2)$_2$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 to a better understanding of the agonist sites that mediate the effects of these interfaces. In the case of the (α4β2)$_2$β2 receptor, an ACh binding site on the β2(+)/β2(-) interface seems unlikely. The high-resolution structure of the human (α4β2)$_2$β2 nAChR was obtained by co-crystallisation 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)$_2$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)$_2$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)$_2$α4 have also been proposed to account for the different patterns of single channel opening durations exhibited by the alternate α4β2 nAChR isoforms (Mazzaferro 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)$_2$β2 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 imply 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 GABA$_A$ 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 (Kloda and Czajkowski, 2007; Eaton et al., 2012). In the $GABA_A$ receptor, the binding sites are located at β/α interfaces (the β subunit is the principal subunit in the $GABA_A$ receptor) and the γ subunit. This structural and functional arrangement is equivalent to the one we propose for the $(\alpha4\beta2)_2\beta2$ receptor. Consistently with this possibility, Baumann et al. (2003) reported asymmetry in the function of the agonist sites in the $GABA_A$ 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 $(\alpha4\beta2)_2\beta2$ receptor? Since MTSET derivatisation 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 T*orpedo* 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 αγ 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 modulate the agonist responses of the $(\alpha4\beta2)_2\beta2$ nAChR receptor isoform.

In conclusion, these data suggest that the fifth subunit in the (α4β2)2β2 nAChR 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.

AUTHORS CONTRIBUTION

KN and IB designed and carried out experiments. SM designed earlier MTSET experiments. CA and SG carried out experiments. IB, KN analysed data and wrote paper.

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CONFLICT OF INTEREST

None

REFERENCES

Absalom NL, Quek G, Lewis TM, Qudah T, von Arenstorff I, Ambrus JI, *et al*. (2013). Covalent trapping of methyllycaconitine at the α4-α4 interface of the α4β2 nicotinic acetylcholine receptor: antagonist binding site and mode of receptor inhibition revealed. J Biol Chem 288: 26521-32.

Alcaino C, Musgaard M, Minguez T, Mazzaferro S, Faundez M, Iturriaga-Vasquez P, *et al*. (2017). Role of the Cys loop and transmembrane domain in the allosteric modulation of α4β2 nicotinic acetylcholine receptors. J Biol Chem 292: 551-62.

Baumann SW, Baur R., Sigel E (2003). Individual properties of the two functional agonist sites in GABA$_A$ receptors. J. Neurosci 23: 11158-66.

Benallegue N, Mazzaferro S, Alcaino C, Bermudez I (2013). The additional acetylcholine binding site at the α4(+)/α4(-) interface of the (α4β2)2α4 nicotinic acetylcholine receptor contributes to desensitisation. Br J Pharmacol 170: 304-16.

Carbone AL, Moroni M, Groot-Kormelink PJ, Bermudez I (2009). Pentameric concatenated (α4)$_2$(β2)$_3$ and (α4)$_3$(β2)$_2$ nicotinic acetylcholine receptors: subunit arrangement determines functional expression. Br J Pharmacol 156: 970-81.

Colquhoun D (1998). Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. B J Pharmacol 125:924-947.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA, *et al*. (2015) Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461-71.

Eaton MM, Lim YB, Bracamontes J, Steinbach JH, Akk G (2012). Agonist-specific conformational changes in the α1-γ2 subunit interface of the GABA$_A$ receptor. Mol Pharmacol 82: 255-63.

Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, *et al*. (2009). Structural and functional diversity of native brain neuronal nicotinic receptors. Biochem Pharmacol 78: 703-11.

Harpsøe K, Ahring PK, Christensen JK, Jensen ML, Peters D, Balle T (2011). Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. J Neurosci 31: 10759-66.

Iturriaga-Vásquez P, Carbone AL, García-Beltrán O, Livingstone PD, Biggin PC, Cassels BK, *et al*. (2010). Molecular determinants for competitive inhibition of alpha4beta2 nicotinic acetylcholine receptors. Mol Pharmacol 78: 366-75.

Jain A, Kuryatov A, Wang J, Kamenecka TM, Lindstrom J (2016) Unorthodox acetylcholine binding sites formed by α5 and β3 accessory subunits in α4β2* nicotinic acetylcholine receptors. J Biol Chem 291: 23452-63.

Jha, A, Cadugan DJ, Purohit P, Auerbach, A (2007). Acetylcholine receptor gating at extracellular transmembrane domain interface: the Cys- loop and M2-M3 linker. J Gen Physiol 130: 547-58.

Jin X, McCollum MM, Germann A, Akk G, Steinbach JH (2017). The E loop of the transmitter-binding site is a key determinant of the modulatory effects of physostigmine on neuronal nicotinic α4β2 receptors. Mol Pharmacol 91: 100-09.

Karlin A and Akabas MH (1998). Substituted cysteine accessibility method. Methods Enzymol 293: 123-45.

Kloda JH and Czajkowski C (2007). Agonist-, antagonist-, and benzodiazepine induced structural changes in the α1 Met113-Leu132 region of the GABA$_A$ receptor. Mol Pharmacol 71: 483-93.

Lee WY and Sine SM (2005). Principal pathway coupling agonist binding to channel gating in nicotinic receptors. Nature 438: 243-47.

Lee WY, Free CR, Sine SM (2009). Binding to gating transduction in nicotinic receptors: Cys-loop energetically couples to pre-M1 and M2-M3 regions. J Neurosci 29: 3189-99.

Lucero LM, Weltzin MM, Eaton JB, Cooper JF, Lindstrom JM, Lukas RJ, *et al*. (2016). Differential α4(+)/(-)β2 agonist-binding site contributions to α4β2 nicotinic acetylcholine receptor function within and between isoforms. J Biol Chem 291:2444-59.

Marks MJ, Meinerz NM, Brown RW, Collins AC (2010). $^{86}Rb^+$ efflux mediated by α4β2* nicotinic acetylcholine receptors with high and low-sensitivity to stimulation by acetylcholine display similar agonist-induced desensitisation. Biochem Pharmacol 15:1238–51.

Marotta CB, Dilworth CN, Lester HA, Dougherty DA (2014). Probing the non-canonical interface for agonist interaction with an α5 containing nicotinic acetylcholine. Neuropharmacol 77: 342-49.

Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, *et al.* (2005). Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature 436:103-107.

Mazzaferro S, Benallegue N, Carbone A, Gasparri F, Vijayan R, Biggin PC, *et al*. (2011). An additional ACh binding site at the α4(+)/α4(-) interface of the (α4β2)$_2$α4 nicotinic receptor influences agonist sensitivity. J Biol Chem 286:31043-54.

Mazzaferro S, Bermudez I, Sine SN (2017). α4β2 nicotinic acetylcholine receptors: relationships between subunit stoichiometry and function at the single channel level. J Biol Chem 292: 2729-40.

Mazzaferro S, Gasparri F, New K, Alcaino C, Faundez M, Iturriaga-Vasquez P, *et al*. (2014). Non-equivalent ligand selectivity of agonist sites in (α4β2)2α4 nicotinic acetylcholine receptors: a key determinant of agonist efficacy. J Biol Chem 289: 21795-21806.

Morales-Perez CL, Noviello CM, Hibbs RE (2016). X-ray structure of the human α4β2 nicotinic receptor. Nature 538: 411-15.

Moroni M, Vijayan R, Carbone A, Zwart R, Biggin PC, Bermudez I (2008). Non-agonist-binding subunit interfaces confer distinct functional signatures to the alternate stoichiometries of the α4β2 nicotinic receptor: an α4-α4 interface is required for Zn^{2+} potentiation. J Neurosci 28: 6884-94.

Moroni M, Zwart R, Sher E, Cassels BK, Bermudez I (2006). α4β2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. Mol Pharmacol 70: 755-768.

Nelson ME, Kuryatov A, Choi CH, Zhou Y and Lindstrom J (2003). Alternate stoichiometries of α4β2 nicotinic acetylcholine receptors. Mol Pharmacol 63**:** 332-41.

Olsen JA, Kastrup JS, Peters D, Gajhede M, Balle T, Ahring PK (2013). Two distinct allosteric binding sites at α4β2 nicotinic acetylcholine receptors revealed by NS206 and NS9283 give unique insights to binding activity-associated linkage at Cys-loop receptors. J Biol Chem 288: 35997-06.

Seo S, Henry TJ, Lewis AH, Wang N, Levandoski M (2009). The positive allosteric modulator morantel binds at noncanonical subunit interfaces of neuronal nicotinic acetylcholine receptors. J Neurosci 29: 8734-42

Shahsavar A, Kastrup JS, Nielsen E, Kristensen JL, Gajhede M, Balle T (2012). Crystal structure of *Lymnaea stagnalis* AChBP complexed with the potent nAChR antagonist DHβE suggests a unique mode of antagonism. PLoS 1 7: e40757.

Sullivan D and Cohen JB (2000). Mapping the agonist binding site of the nicotinic acetylcholine receptor: orientation requirements for activation by covalent agonist. J Biol Chem 275: 12651-60.

Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, *et al*. (2004). Nicotine activation of alpha4* receptors: sufficient for reward, tolerance, and sensitization. Science 306: 1029-32.

Timmermann DB, Sandager-Nielsen K, Dyhring T, Smith M, Jacobsen AM, Nielsen EØ, *et al*. (2012). Augmentation of cognitive function by NS9283, a stoichiometry-dependent positive allosteric modulator of α2- and α4-containing nicotinic acetylcholine receptors. Br J Pharmacol 167: 164-182.

Unwin N and Fujiyoshi Y (2012). Gating movement of acetylcholine receptor caught by plunge-freezing. J Mol Biol 422: 617-34.

Wang J, Kuryatov A, Sriram A, Jin Z, Kamenecka TM, Kenny PJ, *et al*. (2015). An accessory agonist binding site promotes activation of α4β2* nicotinic acetylcholine receptors. J Biol Chem 290: 13907-18.

Xiu X, Puskar NL, Shanata JPA, Lester HA, Dougherty DA (2009). Nicotine binding to brain receptors requires a strong cation-π interaction Nature 458: 534-37.
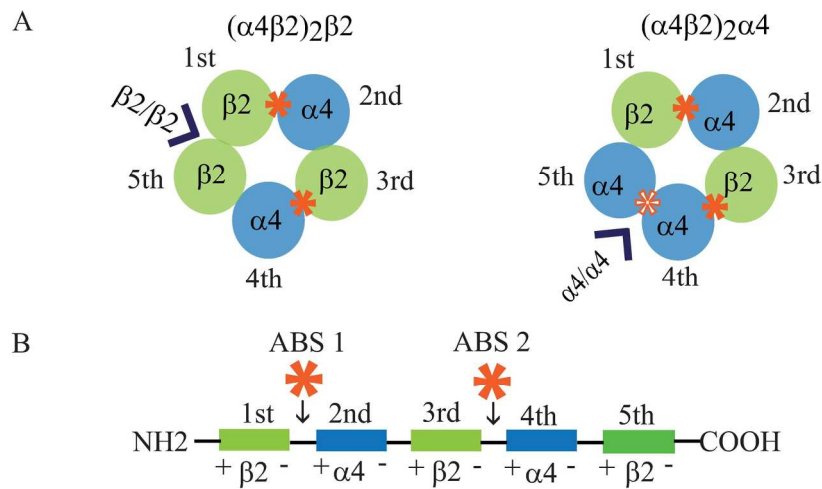
Figure 1. Alternate forms of the α4β2 nAChR. (A) Cartoon showing the alternate (α4β2)$_2$β2 and (α4β2)$_2$α4 forms of the α4β2 nAChR. Stoichiometry-specific interfaces (β2(+)/β2(-) and α4(+)/α4(-)) are indicated by arrows. Agonist binding sites at α4(+)/β2(-) interfaces are indicated by filled asterisks, whereas the agonist binding site at the α4(+)/α4(-) interface of the (α4β2)$_2$α4 receptor is indicated by a clear asterisk. **(B)** Diagram showing the linear sequence and spatial orientation of α4 and β2 subunits in concatemeric (α4β2)$_2$β2 nAChR. Arrows show the position of canonical agonist sites (agonist binding site 1, ABS 1 and agonist binding site 2, ABS 2) are indicated by arrows.
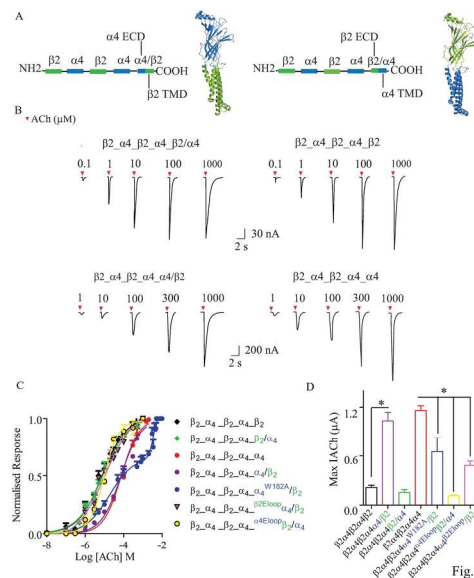
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Figure 2. Effects of the fifth subunit on the ACh responses of α4β2 nAChRs. (A) Diagram of chimeric concatenated α4β2 nAChRs. A chimeric subunit consisting of the α4 subunit extracellular domain and the remaining part of the β2 subunit (or *vice versa*) 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 extracellular domain of the fifth subunit of the alternate α4β2 nAChRs significantly affected the responses to ACh. The EC_{50} values and Hill coefficients (nHill) are summarised 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. Unpaired, two-tailed students t-tests showed significant differences (***) between wild type concatenated $(α4β2)_2β2$ and chimeric $(α4β2)_2β2/α4$ 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.%"

196x125mm (300 x 300 DPI)

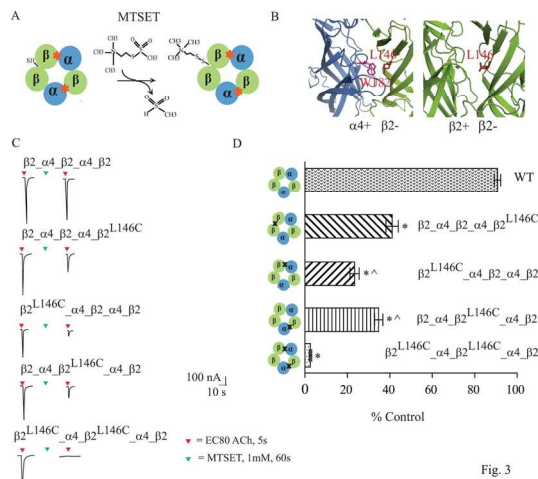**Figure 3. Effects of MTSET on ACh EC_{80} responses in wild type and cysteine substituted concatenated $(\alpha4\beta2)_2\beta2$ nAChRs. (A)** Cartoon depicting covalent labelling of cysteine substituted $(\alpha4\beta2)_2\beta2$ nAChRs by MTSET. **(B)** Structure of the $\alpha4(+)/\beta2(-)$ (right panel) and $\beta2(+)/\beta2(-)$ (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 $\alpha4(+)/\beta2(-)$ interface. The $\alpha4$ subunit is shown in blue and $\beta2$ 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 $(\alpha4\beta2)_2\beta2$ nAChRs. **(D)** 1 mM MTSET decreased significantly the responses to ACh EC_{80} in all mutant receptors. The amplitude of the currents remaining after MTSET were calculated using the equation [(Iafter MTSET/Iinitial - 1) x 100], as described in Methods. Significant differences between the cysteine substituted receptors and control ($\beta2$ _$\alpha4$_$\beta2$ _$\alpha4$_$\beta2$) are shown by asterisk and were determined with one-way ANOVA with Dunnett's post-test. The sign + indicates that unpaired Student's *t* tests showed that the maximal inhibition of $\beta2^{L146C}$_$\alpha4$_$\beta2$_$\alpha4$_$\beta2$ and $\beta2$ _$\alpha4$_$\beta2^{L146C}$ _$\alpha4$_$\beta2$ receptors by MTSET are significantly different. The data shown represent n = 8 for each type of receptor tested. ‖ +

157x75mm (300 x 300 DPI)

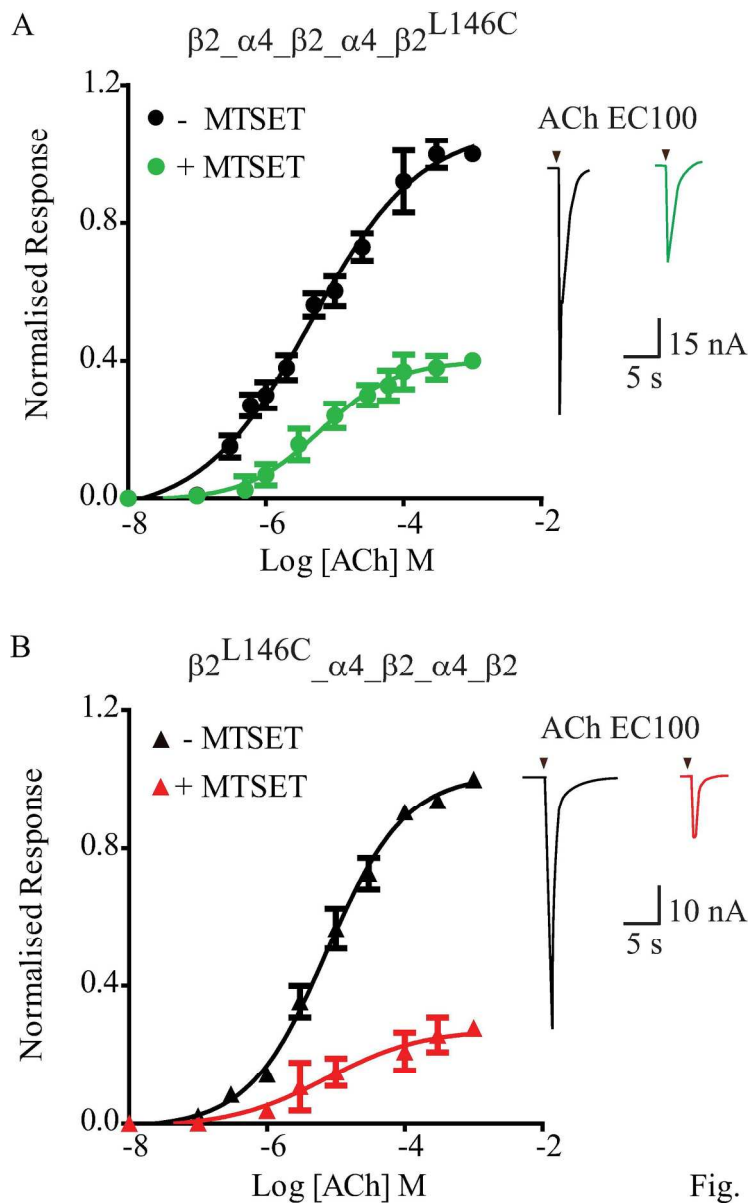**Figure 4. MTSET modification decreases the maximal ACh responses of concatenated $(\alpha4\beta2)_2\beta2$ receptors.** Derivatisation of $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ or $\beta2^{L146C}\_\alpha4\_\beta2\_\alpha4\_\beta2$ receptors by 1 mM of MTSET reduced the maximal ACh responses without changes in the potency of ACh. The ACh concentration response curve for $\beta2\_\alpha4\_\beta2\_\alpha4\_\beta2^{L146C}$ **(A)** or $\beta2^{L146C}\_\alpha4\_\beta2\_\alpha4\_\beta2$ **(B)** receptors was obtained before and after 1 min exposure to 1 mM MTSET. Data points represent the means ± SEM of 5 experiments. Data were fit by nonlinear regression, as described under Methods.
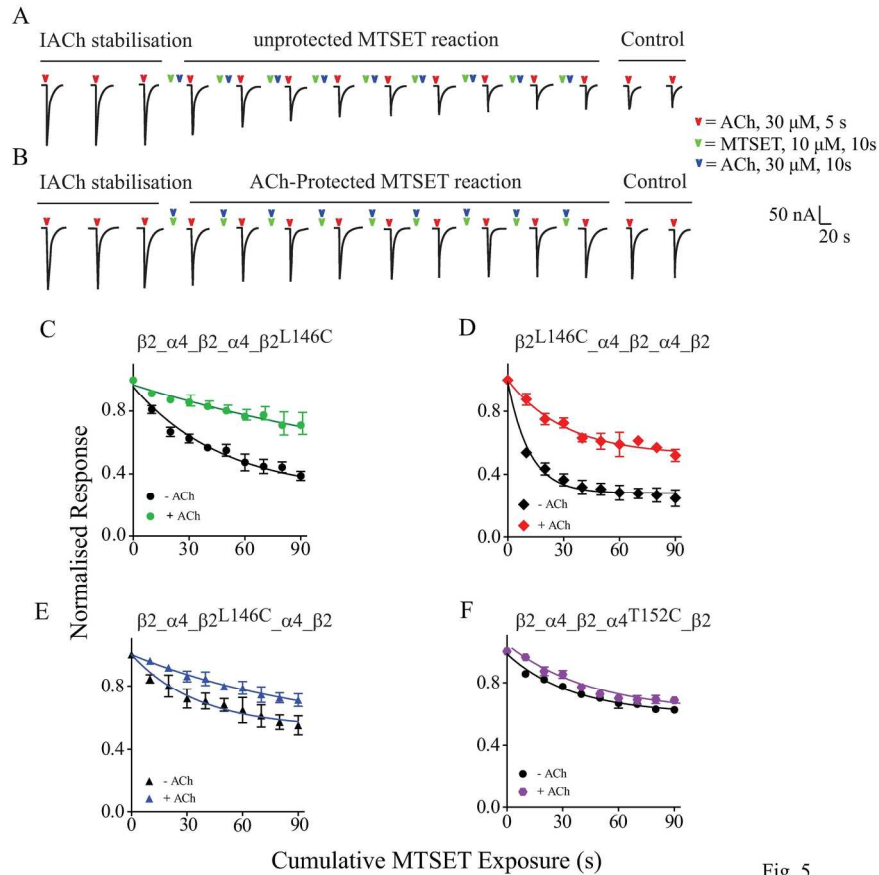
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Figure 5. Effect of ACh on the rate of MTSET modification of substituted concatenated (α4β2)₂β2 nAChRs. Representative traces of responses to ACh EC$_{80}$ before and after cumulative MTSET application in the absence **(A)** or presence of ACh EC$_{80}$ **(B)**. The traces shown were obtained from β2_α4_β2_α4_β2^{L146C} receptors. Rates of MTSET modification of β2_α4_β2_α4_β2^{L146C} **(C)**, β2^{L146C}_α4_β2_α4_β2 **(D)**, β2_α4_β2^{L146C} _α4_β2 **(E)** or β2_α4_β2_α4^{T152C}_β2 **(F)** receptors in the absence (black curves) or presence of ACh (green, red or blue and purple curves, respectively). For C, D and E n = 8. For F, n = 5. Data were normalised and fit to a single phase exponential decay, as described in Methods. Second order rate constants for MTSET modification of L146C are summarised in Table 2.
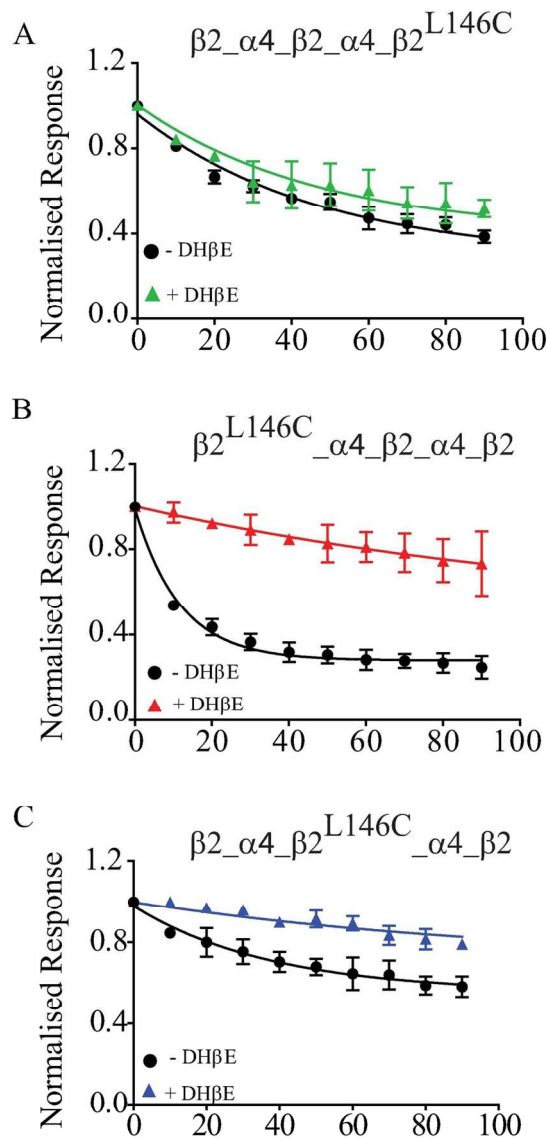
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Fig. 6   Cumulative MTSET Exposure (s)

Figure 6. Effect of the competitive antagonist DHβE on the rate of MTSET modification of cysteines substituted β2(+)/β2(-) or α4(+)/β2(-) interfaces in concatenated (α4β2)$_2$β2 nAChRs. **(A)** The rate of MTSET derivatisation of cysteine substituted β2_α4_β2_α4_β2^{L146C} receptors was not affected by the presence of IC$_{20}$ 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 (unpaired Student's *t*-tests). Data were normalised and fit to a single phase exponential decay, as described in Methods.
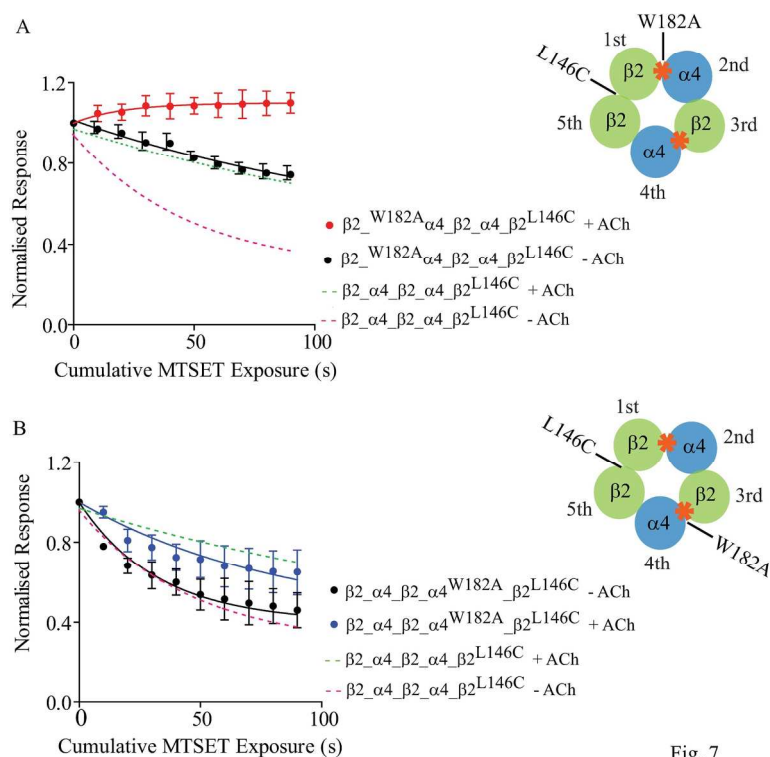
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Fig. 7

Figure 7. Effect of ACh-bound mutated agonist sites on MTSET modification of the fifth subunit of (α4β2)₂β2 nAChRs. The rate of MTSET modification of β2_α4_β2_α4_β2^{L146C} in the absence or presence of ACh was altered when the W182A mutation was introduced in the α4(+)/β2(-) agonist sites to form β2_α4_β2_α4^{W182A}_β2^{L146C} **(A)** or β2_^{W182A}α4_β2_α4_β2^{L146C} **(B)**. Data were normalised and fit to a single phase exponential decay, as described in Methods. For A and B, n = 8. Rate constants are summarised in Table 2. The cartoon adjacent to the exponential decay plots shows the position of incorporation of W182A in the cysteine substituted β2_α4_β2_α4_β2^{L146C} receptors. For comparison we show the curves for the rate of MTSET reaction in the absence (dark pink dashed lines lines) or presence (green dashed lines) for β2_α4_β2_α4_β2^{L146C} receptors.

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Receptor	EC_{50} ACh	nHill	EC_{50Mut}/EC_{50WT}	N
β2_α4_β2_α4_β2	8.64 ± 2.2	0.75 ± 0.012		11
β2_α4_β2_α4_β2/α4	8.33 ± 1.8	0.67 ± 0.09	0.96	10
β2_α4_β2_α4_α4	$80.42 \pm 8.3*$	0.86 ± 0.02		10
β2_α4_β2_α4_α4/β2	$81.66 \pm 3*$	1.15 ± 0.25	9.5	10
β2_α4_β2_α4_α4^{W182A}/β2	$13.39 \pm 3*$ 3.51 ± 1.2	0.64 ± 0.06 2.18 ± 0.35	1.54 406	8
β2_α4_β2_α4_^{β2Eloop}α4/β2	11.51 ± 4	0.87 ± 0.09	1.3	6
β2_α4_β2_α4-^{α4Eloop}β2/α4	9.74 ± 1.2	0.71 ± 0.1	1.12	6
^{Y120A}β2_α4_β2_α4_β2	$10.55 \pm .2.3$	0.94 ± 0.24	1.2	10
^{Y221A}β2_α4_β2_α4_β2	9.20 ± 0.6	0.97 ± 0.2	1.06	9
^{W176A}β2_α4_β2_α4_β2	7.20 ± 0.95	0.97 ± 0.03	0.83	9
β2_α4_β2_α4_β2^{W82A}	8.84 ± 1.6	0.82 ± 0.19	1.02	9
$^{Y120A, W176A, Y221A}$β2_α4_β2_α4_β2^{W82A}	7.26 ± 0.5	0.93 ± 0.42	0.84	9
β2_α4_β2_α4_β2^{L146C}	6.61 ± 0.9	0.98 ± 0.09	0.76	9
β2^{L146C}_α4_β2_α4_β2	7.24 ± 1.9	0.89 ± 0.04	0.43	9
β2_α4_β2^{L146C}_α4_β2	5.96 ± 1.2	0.72 ± 0.09	0.69	10
β2^{L146C}_α4_β2^{L146C}_α4_β2	4.59 ± 2.1	0.86 ± 0.09	0.53	6
β2_^{W182A}α4_β2_α4_β2	$1.07 \pm 0.1*$ 53.00 ± 12	0.64 ± 0.21 2 ± 0.90	0.12 6.1	9
β2_α4_β2_^{W182A}α4_β2	$17.00 \pm 4*$	0.61 ± 0.31	1.97	7
β2_^{W182A}α4_β2_α4_β2^{L146C}	$40.26 \pm 15\text{\textasciicircum}$	0.6 ± 0.07	4.7	7
β2_α4_β2_^{W182A}α4_β2^{L146C}	6.13 ± 2.1	0.71 ± 0.1	0.71	7

Table 1. Concentration effects of ACh on wild type and mutant concatenated $(\alpha4\beta2)_2\beta2$ nAChRs. The concentration effects of ACh on oocytes expressing heterologously wild type or mutant concatenated $(\alpha4\beta2)_2\beta2$ 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 (nHill) were estimated, as described in Methods. Data for β2_^{W182A}α4_β2_α4_β2 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 β2_α4_β2_α4_β2 and chimeric β2_α4_β2_α4_β2/α4 or β2_α4_β2_α4_α4/β2 receptors were measured by Student′s t-tests. Statistical differences between control (β2_α4_β2_α4_/β2) and mutants of conserved aromatic residues were determined by one-way ANOVA with Dunnett′s correction. Asterisks denote statistical difference. Statistical comparison between β2_α4_β2_α4_β2 ^{L146C} (control) and β2_α4_β2_^{W182A}α4_β2^{L146C} and β2_^{W182A}α4_ β2_α4_ β2^{L146C} was carried out by one way ANOVA with Dunnett's correction; statistically different values are noted by ^. *,^ 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.

Receptor	Control k_2 $(M^{-1}s^{-1})$	n	+ ACh EC_{80} k_2 $(M^{-1}s^{-1})$	n	k_2c/k_2+ACh
β2_α4_β2_α4_β2 ^{L146C}	2089 ± 310$^{\wedge+}$	8	551 ± 141*$^{\wedge+}$	8	3.8
β2^{L146C}_α4_β2_α4_β2	5543 ± 541$^{\wedge}$	8	1009 ± 125*$^{\wedge+}$	8	5.5
β2_α4_β2^{L146C}_α4_β2	2751± 510$^{\wedge+}$	8	992 ± 101*$^{\wedge+}$	8	4
β2_^{W182A}α4_β2_α4_β2^{L146C}	861 ± 1115^{+}	8	901 ± 180^{+}	8	0.96
β2_α4_β2_^{W182A}α4_β2^{L146C}	3010±593^{+}	8	1164± 168*$^{+}$	8	2.1
β2_α4_β2_^{T152C}α4_β2	2829±610	5	2171±715	5	1.3

Table 2. Rates of covalent modification of cysteine substituted $(\alpha4\beta2)_2\beta2$ nAChRs by MTSET. Rates of MTSET reaction with introduced cysteine were measured, and second-order rate constant (k_2; $M^{-1}s^{-1}$) were calculated as described in Methods. Second order rate constants represent the mean ± SEM of n number of experiments. $k_{2c}/k_{2 + ACh}$ represents the ratio of second-order rates of MTSET reactions obtained in the control rate (k_{2c}) and in the presence of ACh ($k_{2 + ACh\ EC80}$). 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 unpaired Student's t-tests (differences are noted by *). Statistical differences between the rate of reaction (in the absence or presence of ACh) of β2_α4_β2_α4_β2^{L146C}, β2^{L146C}_α4_β2_α4_β2 and β2_α4_β2 ^{L146C}_α4_β2 was measured using one way ANOVA tests and differences are noted by $^{\wedge}$. Comparison between the rates of β2_α4_β2_α4_β2 ^{L146C} , β2_^{W182A}α4_β2_α4_β2^{L146C} and β2_α4_β2_^{W182A}α4_β2^{L146C} receptors was measured using one way ANOVA tests and differences are noted by +. *, $^{\wedge}$ and + denote $p < 0.05$.