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*

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Background: Positive allosteric modulators (PAMs) of α4β2 nicotinic acetylcholine receptors have significant therapeutic potential.

Results: Two PAMs, NS206 and NS9283, were observed to have differential and additive pharmacological actions due to binding at distinct receptor sites.

Conclusion: Modulator binding activity is linked to the specific binding position in Cys-loop receptors.

Significance: Diverse PAM profiles increase possibilities for rational drug design and understanding of receptor function.

Positive allosteric modulators (PAMs) of α4β2 nicotinic acetylcholine receptors have the potential to improve cognitive function and alleviate pain. However, only a few selective PAMs of α4β2 receptors have been described limiting both pharmacological understanding and drug-discovery efforts. Here, we describe a novel selective PAM of α4β2 receptors, NS206, and compare with a previously reported PAM, NS9283. Using two-electrode voltage-clamp electrophysiology in Xenopus laevis oocytes, NS206 was observed to positively modulate acetylcholine (ACh)-evoked currents at both known α4β2 stoichiometries (2α:3β and 3α:2β). In the presence of NS206, peak current amplitudes surpassed those of maximal efficacious ACh stimulations (E_{max}(ACh)) with no or limited effects at potencies and current waveforms (as inspected visually). This pharmacological action contrasted with that of NS9283, which only modulated the 3α:2β receptor and acted by left shifting the ACh concentration-response relationship. Interestingly, the two modulators can act simultaneously in an additive manner at 3α:2β receptors, which results in current levels exceeding E_{max}(ACh) and a left-shifted ACh concentration-response relationship. Through use of chimeric and point-mutated receptors, the binding site of NS206 was linked to the α4-subunit transmembrane domain, whereas binding of NS9283 was shown to be associated with the αα-interface in 3α:2β receptors. Collectively, these data demonstrate the existence of two distinct modulatory sites in α4β2 receptors with unique pharmacological attributes that can act additively. Several allosteric sites have been identified within the family of Cys-loop receptors and with the present data, a detailed picture of allosteric modulatory mechanisms of these important receptors is emerging.

In the search for new drugs targeting nicotinic acetylcholine receptors (nAChRs),2 positive allosteric modulators (PAMs) have recently attracted interest due to their proposed advantages over receptor agonists, such as less tonic activation, desensitization, and up-regulation of receptors (1–3). Due to the important role of α4β2 nAChRs in normal brain function and several disorders of the central nervous system (4–7), ongoing drug discovery efforts seek to identify novel PAMs acting at these receptors. Indeed recent preclinical studies with NS9283 have shown early promise in the treatment of pain (8) and cognitive disorders (9). However, as only few α4β2 nAChR PAMs have been described in the literature to date (1), overall understanding of allosteric modulation at this receptor subtype is sparse relative to other prominent Cys-loop receptors such as the α1β2γ2 γ-aminobutyric acid type A receptor (GABA_A, R) or the hommeric α7 nAChR.

When considering Cys-loop receptors broadly, it is clear that the variously described allosteric modulators have distinct pharmacological actions. For the α1β2γ2 GABA_A, R, benzodiazepines increase GABA potency without affecting maximal GABA-evoked peak current levels (E_{max}(GABA)) (10). By contrast, barbiturates can modulate maximal efficacy to levels above E_{max}(GABA) in addition to modulating GABA potency (11). For α2 nAChRs, two broad classes of PAMs have been identified exemplified by NS1738 and PNU-120596 (12, 13). Pharmacologically, NS1738 increase ACh-evoked peak current levels above E_{max}(ACh) without otherwise affecting current...
waveforms, whereas PNU-120596 additionally delay desensitization resulting in large current waveform changes. Thus, it appears that at least three different modulatory phenotypes can be observed in the Cys-loop receptor family: (i) left-shift of the agonist CRR, (ii) increased peak current levels above \( E_{\text{max}} \) of the endogenous agonist, and (iii) delay of desensitization. Depending on the compound and receptor, these actions can be observed independently or in combination.

The binding sites for Cys-loop receptor allosteric modulators have been identified in a number of cases. In GABA\(_A\)Rs, the \( \alpha\gamma\)-subunit extracellular domain (ECD) interface contains the binding site for benzodiazepines in a position homologous to the \( \beta\alpha\)-subunit agonist binding sites for GABA (10). Similar non-orthosteric binding sites in the nAChR ECD have also been proposed for galanthamine (14) and the \( \alpha3\beta4 \) nAChR modulator morantel (15). On the other hand etomidate has been shown to bind in the transmembrane domain (TMD) of GABA\(_A\)Rs (16). In fact a cavity corresponding to the etomidate binding pocket has been suggested to be conserved across Cys-loop receptors, constituting the binding site for PU02 in 5-hydroxytryptamine type 3A receptors and ivermectin in the invertebrate GluCl (17, 18). Similarly, the \( \alpha7 \) nAChR PAMs NS1738 and PNU-120596 have been proposed to bind in an \( \alpha7 \) TMD-associated binding pocket (19, 20).

With respect to PAMs of \( \alpha4\beta2 \) heteromeric receptors, further complicating issues relate to existence of variant stoichiometries (21–24). One stoichiometry, with a subunit ratio of \( 2\alpha:3\beta \), has relatively high sensitivity to the endogenous ligand Ach (EC\(_{50} \sim 1 \text{ \mu M} \)) whereas the other, with a subunit ratio of \( 3\alpha:2\beta \), shows a biphasic response to Ach (EC\(_{50} \sim 1 \text{ and } 70 \text{ \mu M} \)). The low sensitivity component of the biphasic response was recently shown to arise from Ach binding to a third binding site in the \( \alpha\alpha \)-interface, which is only present in \( 3\alpha:2\beta \) receptors (24). Interestingly, such receptor stoichiometry differences can greatly affect PAM actions as illustrated by findings that two \( \alpha4\beta2 \) PAMs, \( \text{Zn}^{2+} \) and NS9283, are both completely specific for \( 3\alpha:2\beta \) receptors (9, 25). No PAMs have yet been shown to modulate the \( 2\alpha:3\beta \) stoichiometry \( \alpha4\beta2 \) receptors, an unfortunate fact given their potential importance in mediating dopamine release in the striatum (26).

In the present study, we describe a novel \( \alpha4\beta2 \) nAChr PAM, NS206, which modulates Ach-evoked responses at both common \( \alpha4\beta2 \) receptor stoichiometries (i.e. \( 2\alpha:3\beta \) and \( 3\alpha:2\beta \) receptors). Pharmacological actions of NS206 and the previously described PAM, NS9283, are compared and their binding sites mapped to specific receptor regions. The two compounds had differing modulatory actions where NS9283 gave a left shift of the agonist CRR, whereas NS206 predominantly increased agonist peak current levels and, interestingly, these modulatory actions proved additive when the compounds were co-applied. Aligning pharmacological actions for a range of PAMs (literature and current study) with their proposed binding sites, it appears that binding in ECD interfaces mostly result in agonist CRR left shifts, whereas TMD binding can further result in increased agonist-evoked peak currents, delayed desensitization characteristics, and direct agonist independent receptor activation.

**Additive Allosteric Modulation of \( \alpha4\beta2 \) nAChRs**

**EXPERIMENTAL PROCEDURES**

**Materials**—3-N-Benzoyl-3-hydroxyimino-2-oxo-6,7,8,9-tetrahydro-1H-benzo[\( g \)]indole-5-sulfonamide (NS206) was synthesized at NeuroSearch A/S according to methods described in patent WO 01/55110. 3-(3-(Pyridine-3-yl)-1,2,4-oxadiazol-5-yl)benzonitrile (NS9283) was likewise synthesized at NeuroSearch A/S as described by Timmermann et al. (9). The structures of NS206 and NS9283 were confirmed using MS and NMR and both compounds are of >98% purity. ACh (A9101) and all other chemicals were of analytical grade and purchased from Sigma unless otherwise specified.

**Molecular Biology**—Human cDNA of the \( \alpha3 \), \( \alpha4 \), \( \beta2 \), and \( \beta4 \) nAChR subunits were cloned and inserted into in-house plasmid expression vectors as previously described (12). Creation of a point-mutated \( \alpha4 \)-subunit with mutations H142V, Q150F, and T152L (numbering corresponds to the uniprot canonical sequence of the nAChR \( \alpha4 \)-subunit) have been described previously (24). Chimeric subunits, where the ECD of e.g. \( \alpha4 \) was substituted with that of \( \alpha3 \) or vice versa, were described previously (27). Custom designed oligos were ordered from Eurofins MWG Operon with the following sequences in which lowercase letters indicate positions introducing nucleotide mutations: 5’-GGT GTC CAT GTC TCA GCT GGT GAA GGT cGA cGA Aaa AAA CCA GAT gAT GGA gAC CAA CCT G-3’ giving mutations V77K and I80M and further 5’-CCA TCT TTA AGA GCT CCT GTI cAA TCG ATg TGA CCT tCT CCC GTG TGG ATC acG AAA ACT GTA CCA TGA AGT tCG-3’ giving mutations K160S, Y165F, and Y170Q. Mutations were confirmed by sequencing (MWG Operon) and cRNA was produced using the Message mMachine T7 Transcription kit according to the manufacturer’s description (Ambion).

**Electrophysiology**—Experiments were performed as described previously (28). In brief, two-electrode voltage-clamp (TEVC) electrophysiology recordings were carried out using *Xenopus laevis* oocytes injected with ~25 ng of cRNA. Following injection, oocytes were incubated for 2–7 days at 18 °C in modified Barth’s solution containing: 90 mm NaCl, 1.0 mm KCl, 0.66 mm NaNO\(_3\), 2.4 mm NaHCO\(_3\), 10 mm HEPES, 2.5 mm sodium pyruvate, 0.74 mm CaCl\(_2\), 0.82 mm MgCl\(_2\), 100 μg/ml of gentamycin, and pH adjusted to 7.5. For measurements, an oocyte was placed in a custom designed recording chamber where compound solutions could be added directly to the oocyte via a glass capillary. Pipette resistances were 0.6–2.0 MΩ when submerged in OR2 containing: 90 mm NaCl, 2.5 mm KCl, 2.5 mm CaCl\(_2\), 1.0 mm MgCl\(_2\), 5.0 mm HEPES, and pH adjusted to 7.5. Following impalement the oocyte membrane potential was voltage clamped at a holding potential ranging from ~40 to ~80 mV using a Geneclamp 500B amplifier (Axon) and oocytes
Additive Allosteric Modulation of α4β2 nAChRs

NS206 Is a Novel PAM of Both α4β2nAChR Stoichiometries—To evaluate the ability of NS206 to act as a modulator at α4β2 nAChRs, the compound was tested on receptors expressed in oocytes injected with cRNA in ratios previously shown appropriate to give uniform populations of either a 2α:3β or a 3α:2β receptor stoichiometry (24). From representative traces at 2α:3β receptors, NS206 were seen to modulate ACh-control currents in a concentration-dependent manner (Fig. 2A). In fact, at the highest tested concentration of NS206 (10 μM), the modulated response surpassed that of the $E_{\text{max}}$(ACh) response. Experiments carried out with the 3α:2β receptor revealed qualitatively similar observations (Fig. 2B). Although oocytes are not well suited for studying details of receptor desensitization kinetics, it was noted that current waveforms were not visually observed to differ in these experiments.

The concentration dependence of NS206 modulation at the two receptor stoichiometries was well approximated by the empirical Hill equation (Fig. 2C), suggesting the presence of a specific and saturable NS206 binding site. From the fitted curves, however, it is clear that full saturation is not observed at the highest tested concentration but precipitation within the NS206 solutions prohibited tests with higher concentrations. Whereas it could appear that NS206 gives higher modulatory efficacy at the 3α:2β receptor, this cannot be firmly concluded based on these data as percentage modulation, in experiments such as these, is influenced by the specific ACh-control concentrations chosen. From the fitted graphs very similar $E_{\text{50}}$ values of ~2–4 μM are observed at the two receptor stoichiometries (Table 1), suggesting that NS206 binding is largely unaffected by receptor stoichiometry. NS206 was unable to evoke any responses when applied by itself, i.e. without co-application of ACh at both receptor stoichiometries (Fig. 2, D and E) and engendered no displacement of [3H]cytisine nor [3H]epibatidine binding in native mouse and rat brain tissue (data not shown). Together, these data provide strong evidence of an allosteric mechanism of action and NS206 is therefore considered a PAM.

NS206 Is Selective for α4 Containing Receptors—To investigate the selectivity of NS206, the compound was tested in oocytes on additional common nAChRs.

At the homomeric α7 receptor, NS206 displayed no modulation of the ACh-control response (Table 1). When tested at various heteromeric receptors, these were expressed by inject-
Additive Allosteric Modulation of $\alpha_4\beta_2$ nAChRs

![Diagram](image-url)

**Figure 2.** Allosteric modulation of $\alpha_4\beta_2$ nAChR currents by NS206 in *X. laevis* oocyte TEVC electrophysiology. A and B, representative traces of ACh-, Ach + NS206-, and NS206-evoked $\alpha_4\beta_2$ currents. Oocytes were injected with cRNA for $\alpha_4$- and $\beta_2$-subunits in 1:4 (A) or 10:1 (B) ratios to yield ($\alpha_4$)$_2$(/$\beta_2$)$_2$ and ($\alpha_4$)$_2$(/$\beta_2$)$_2$ receptors respectively, as indicated by insets. Modulatory efficacy of NS206 was evaluated by co-applications with a submaximal concentration of ACh typically giving rise to EC$_{10}$-EC$_{30}$ current levels (ACh-control, Table 1). Applications of ACh or ACh + NS206 (co-application) are indicated by bars above each trace. C, NS206 modulates both $\alpha_4\beta_2$ stoichiometries with comparable potencies. Background subtracted peak current amplitudes from experiments described above were normalized to the respective ACh-control current ($\Delta$I$_{ACh-control}$) and depicted as a function of increasing NS206 concentrations of n = 8 or 4–10 experiments, respectively. Plotted data points were fitted to the empirical Hill equation using non-linear regression. D and E, NS206 does not activate ($\alpha_4$)$_2$(/$\beta_2$)$_2$ or ($\alpha_4$)$_2$(/$\beta_2$)$_2$ receptors in the absence of ACh. Oocytes were injected as described above and stimulated with ACh (left) or NS206 (right). */ denotes that applications are separated by washing steps or intermediate applications.

**Table 1**

| Subunits | cRNA ratio | Modulator | ACh-control | EC$_{50}$ | $E_{max}$ | n |
|----------|------------|-----------|-------------|----------|-----------|---|
| $\alpha_4 + \beta_2$ | 1:4 | NS206 | 1 | 4.2 (2.7–6.6) | 420 (340–500) | 8 |
| $\alpha_4 + \beta_2$ | 10:1 | NS206 | 10 | 2.2 (1.3–3.6) | 600 (490–700) | 4–10 |
| $\alpha_4 + \beta_4$ | 4:1 | NS206 | 100 | 2.0 (0.77–5.0) | 370 (200–540) | 8 |
| $\alpha_4 + \beta_4$ | 4:1 | NS206 | 30 | NA$^*$ | NA | 10 |
| $\alpha_3 + \beta_4$ | 4:1 | NS206 | 100 | 1.3 (1.0–1.8) | 330 (290–360) | 9 |
| $\alpha_3 + \beta_4$ | 4:1 | NS206 | 10 | 1.5 (0.63–3.7) | 80 (59–100) | 7 |
| $\alpha_3 + \beta_4$ | 4:1 | NS206 | 10 | NA | NA | 8 |
| $\alpha_3 + \beta_4$ | 4:1 | NS206 | 100 | 1.5 (1.0–2.2) | 680 (580–780) | 4–11 |
| $\alpha_4 + \beta_2$ | 4:1 | NS206 | 10 | 3.4 (1.5–7.9) | 680 (534–834) | 6 |
| $\alpha_4 + \beta_2$ | 4:1 | NS206 | 10 | NA | NA | 5 |
| $\alpha_4 + \beta_2$ | 4:1 | NS206 | 100 | 5.4 (2.2–13) | 440 (340–540) | 8 |

$^*$ NA, no modulatory activity could be noticed. $\alpha_4$35M has the following mutations: H142V, Q150F, and T152L, and $\alpha_4$43M has the following mutations: H142V, Q150F, and T152L.

ing cRNA in ratios to give the 3$\alpha$2$\beta$ stoichiometry. NS206 showed no modulation of the ganglionic $\alpha_3$$\beta_4$ nAChRs, however, the $\alpha_4$$\beta_4$ receptor was positively modulated with a potency of $\sim 2 \mu M$ (Table 1). This is similar to the potency observed at $\alpha_4$$\beta_2$ receptors and the maximal efficacy likewise appeared to be within the same range. Collectively, these data indicate that the presence of the $\alpha_4$-subunit is mandatory for PAM actions of NS206, whereas there is no dependence on the $\beta$-subunit.

**Pharmacologically NS206 Increases Agonist Efficacy**—Recently, the mechanism of NS9283 PAM action was described as a left-shift of the ACh CRR such that ACh appeared more potent in the presence of NS9283 at 3$\alpha$2$\beta$ receptors. Importantly, no noticeable differences were observed for $E_{max}(ACh)$ in µM and $E_{max}$ in % with 95% confidence intervals from the indicated number of experiments.
current levels in the presence or absence of NS9283 (9). To investigate the modulatory actions of NS206, similar experiments were conducted. Initially, two ACh CRRs were obtained from the same oocytes, the first without concomitant NS206 application and the second in the continued presence of NS206 from the same oocytes, the first without concomitant NS206 application and the second in the continued presence of NS206 from the same oocytes. Normalized data points are depicted as the ratio between the maximum fitted response in the presence of modulator and the maximum fitted ACh CRRs (see legend for Fig. 3). Data were fitted to either mono- or biphasic versions of the empirical Hill equation using non-linear regression with a fixed bottom of 0 and (a) Hill slope(s) of 1. Data are presented as EC50 in μM and E_max as the ratio between the maximum fitted response in the presence of modulator and the maximum fitted ACh CRRs (see footnote a) or EC100 ACh reference traces with 95% confidence intervals from the indicated number of experiments. With biphasic fits, the fraction corresponding to the highest affinity component is indicated.

At 2αβ3 receptors, ACh on its own was found to have an EC50 value of ~1 μM, whereas at 3α2β2 receptors the response was, as expected, biphasic with EC50 values of ~1 and ~100 μM, respectively (Fig. 3, A and B, and Table 2). The biphasic response in a uniform population of 3α2β2 receptors reflects the existence of two high sensitivity sites at the αβ-interfaces and a low sensitivity site at the αα-interface and the observed potencies are, with the resolution taken into account, comparable with previous studies (21, 23, 24). Although not clearly evident in the graph (Fig. 3B), previous experiments demonstrate that 1 mM ACh is sufficient to give E_max (ACh) responses (24). Interestingly, with NS206 present, the ACh CRRs were affected at both receptor stoichiometries. For the 2α3β stoichiometry, the peak current amplitudes were potentiated ~3–4-fold at the highest ACh concentration accompanied with only a minor change in EC50 value (Fig. 3A and Table 2). Qualitatively similar potentiating effects of ~1.5 times were noted at the highest ACh concentrations for the 3α2β stoichiometry. However, the ACh CRR for this combination still appeared biphasic, albeit with an altered fraction between the two phases, and a 2–3-fold potency increase was noted for both EC50 values (Fig. 3B and Table 2).

Upon visual inspection of representative traces from the maximal ACh concentrations (1 mM) in the above experiments, it is seen that NS206 efficacy is not due to large changes in current waveform characteristics (Fig. 3, A and B, insets). The total application time for the ACh pulse is ~30 s and during this time period only limited current decay is observed for 2α3β receptors, whereas some decay is observed for 3α2β receptors. In the presence of NS206, the increases in maximal currents are readily evident; however, the overall current waveforms appear similar. Based on this it appears that NS206 is unlikely to have
The modulatory actions of NS9283 match previously published results, i.e. a monophasic left-shifted CRR with no significant increase in maximum peak currents at the highest ACh concentrations (Fig. 5). However, simultaneous application of both NS206 and NS9283 gives a unique mixture of modulatory effects not observed for either compound alone (Fig. 5 and Table 2). (i) The ACh CRR is monophasic, whereas the CRR with NS206 alone is biphasic (Fig. 4B), (ii) at high ACh concentrations, the modulated response show efficacy levels that significantly surpasses that of NS9283 alone by a factor of \(~1.7\) \((F_{1,160} = 633, p < 0.0001;\) maximal efficacy NS9283 + NS206 versus NS9283 alone). Compared with the NS9283 alone addition, NS206 further left-shifted the CRR of ACh albeit only by a factor of \(~2\). Interestingly, in these experiments preincubation of the two modulators simultaneously gave rise to small currents of \(~10\%\) of \(E_{\text{max}}(\text{ACh})\), i.e. the Hill fit does not approach zero. Results from all Hill fits are seen in Tables 1 and 2.

Additive Allosteric Modulation of \(\alpha_4\beta_2\) nAChRs

**FIGURE 4.** Detailed analysis of NS206 pharmacological actions in \(X.\ laevis\) oocyte TEVC electrophysiology. Oocytes were injected as described in the legend to Fig. 2. A, a detailed CRR of ACh + NS206 at \((\alpha_4)_2(\beta_2)_3\) receptor displays monophasic fit. Peak current amplitudes of ACh-evoked currents in the presence of preincubated \(3.16 \mu M\) NS206 were normalized to an \(E_{\text{max}}(\text{ACh})\) reference ACh trace \((n = 5–12, \text{SE})\). Biphasic fitting to the data points was not justified over a monophasic fitting \((F_{2,12} = 2.2, p = 0.12)\). A, a detailed CRR of ACh + NS206 at \((\alpha_4)_2(\beta_2)_3\) receptor displays a biphasic fit. Experimental conditions from \(n = 5\) experiments were similar to (A) except for the ACh concentration used for the \(E_{100}\) reference ACh trace \((n = 5–6)\). Data points were best fitted to a biphasic Hill equation \((F_{2,10} = 56, p < 0.0001)\).

**FIGURE 5.** NS206 and NS9283 additively modulate \((\alpha_4)_2(\beta_2)_3\) receptors in \(X.\ laevis\) oocyte TEVC electrophysiology. Oocytes were injected as described in the legend to Fig. 2. Peak current amplitudes of ACh-evoked currents in the presence of preincubated \(3.16 \mu M\) NS9283 or \(3.16 \mu M\) NS206 were normalized to an \(E_{100}\) control ACh trace \((n = 5–12, \text{SE})\). A detailed CRR of ACh + NS206 at \((\alpha_4)_2(\beta_2)_3\) receptor displays a monophasic fit. Peak current amplitudes of ACh-evoked currents in the presence of preincubated \(3.16 \mu M\) NS9283 or \(3.16 \mu M\) NS206 were normalized to an \(E_{100}\) control ACh trace \((n = 5–12, \text{SE})\). Data points were best fitted by a monophasic Hill equation \((F_{2,10} = 56, p < 0.0001)\).
Additive Allosteric Modulation of α4β2 nAChRs

FIGURE 7. Allosteric modulation of αα-interface mutants by NS206 and NS9283 in X. laevis oocyte TEVC electrophysiology. Oocytes were injected with either α4- or α43604-subunits in a 1:1 RNA ratio with the wild-type β2-subunit. 3M indicates the three mutations: H142V, Q150F, and T152L. At the αα-interface. Modulatory efficacy of NS9283 modulation at (α4)(β2) and (α43604)(β2) receptors was recorded as described in the legend to Fig. 2, depicted ± S.E. as a function of increasing concentrations of NS9283 of n = 9 or 5 experiments, respectively, and fitted to the Hill equation. B, NS206 modulation of α4β2 receptor currents is not affected by mutations in the αα-site. Experimental conditions and data treatment of n = 9–10 experiments mirror the description in A.

FIGURE 8. Allosteric modulation of nAChR chimeras by NS206 and NS9283 in X. laevis oocyte TEVC electrophysiology. Oocytes were injected with (α3)(α3) or (α4)(α4) or (α43603)(α3) in a 1:1 RNA ratio with the wild-type β2-subunit. 3M indicates the five mutations: V77K, I80M, K160S, Y165F, and Y170Q. At the αα-interface. Modulatory modulation of NS9283 modulation shows linkage to the extracellular domain of α4. Data from (α4)(α3)(β2) and (α3)(α4)(β2) receptors were obtained as described in the legend to Fig. 2, depicted ± S.E. of n = 12–13 or 10 experiments, respectively, and fitted to the Hill equation. B, NS206 modulation of nAChR depends on amino acids in the interface between the ECD and TMD. Experimental conditions and treatment of (α4)(α3)(β2), (α3)(α4)(β2), and (α3)(α4)(β2) receptor data from n = 5–8, 10, and 9–10 experiments, respectively, mirrors the description in A.

At wild-type 3α2β receptors, NS9283 is a PAM with ~700% efficacy (relative to the EC10–30 ACh-control) and an EC50 value of ~3 μM (Fig. 7A and Table 1), which is similar to previously reported data of Emax = 638% and EC50 = 3.3 μM (9). However, NS9283 loses all its modulatory actions at the 3α3β2 receptor (Fig. 7A and Table 1). Modulatory actions by NS206, on the other hand, were unaffected by the mutations (Fig. 7B and Table 1), as both efficacy and EC50 values at the mutated receptor are similar to the values obtained at wild-type receptors. Hence, consistent with the lack of subunit stoichiometry selectivity, NS206 binds to a site entirely distinct from the NS9283 binding site.

Determinants of NS206 Modulatory Action—In another attempt to identify the receptor region(s) important for NS206 modulatory actions, the compound was tested on chimeric receptors where the ECD of α4 was substituted with that of α3 or vice versa (Fig. 6B). These chimeras have previously been shown to assemble into fully functional nAChRs with characteristics dominated by the ECDs (27). Of particular importance, the ACh sensitivities are very similar to the respective wild-type receptors enabling regular selection of ACh-control concentrations (Table 1). For the experiments described below, chimeric receptors were expressed in a 3α2β stoichiometry.

At the α4/α3 + β2 chimera, i.e. an α4β2 ECD coupled with an α3β2 TMD, efficacy and potency of NS9283 were observed similar to that at wild-type receptors (Fig. 8A and Table 1). No activity was seen with the reverse α3/α4 + β2 chimera in agreement with the described lack of efficacy at α3-containing receptors (data not shown) (9). Hence, modulatory actions of NS9283 appear entirely dependent on the ECD of α4 fully consistent with the above mapping of its binding site to the αα-interface of α4β2 receptors. Furthermore, the normal modulatory behavior of NS9283 at the α4/α3 + β2 chimera underscores the usefulness of chimeras in this context.

NS206 positively modulated the α3/α4 + β2 chimera with an EC50 of ~2 μM albeit with very low efficacy (Fig. 8B and Table 1). In contrast, no modulation was observed with the reverse chimeric receptor α4/α3 + β2 (Fig. 8B and Table 1). Because
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the potency at the α3/α4 + β2 chimera was similar to that at
wild-type receptors it appears reasonable to assume that NS206
bind in the TMD region. However, for this argument to hold,
the lack of efficacy must be explained by suboptimal propa-
gation of structural changes upon compound binding. Given that
the chimera contains entire domains from two different sub-
units, this hints to determinants in the interface between the
two domains. ECD regions believed to be important for this
interface include loop61–62, the Cys-loop (loop46–47), and
loop68–69 (Fig. 6A, bottom inset) (29). An alignment of α3 and
α4 with the α4-subunit of the Torpedo marmorata AChR iden-
tifies a number of amino acid differences. Because a 4-Å reso-

volution structure of the Torpedo AChR is available (Protein Data
Bank 2BG9), this alignment further allows qualified guesses as
to the proximity of these differing amino acids to the TMD (Fig.
6A, bottom inset). Within 12-Å distance of the TMD, α3 and α4
differ in only five amino acid positions, three in the Cys-loop
and two in loop68–69 (Fig. 6C). To investigate whether the iden-
tity of these five amino acids affect modulatory actions of
NS206, an α35M/α4 chimera was created, where the five resi-
dues in the ECD of α3 were point mutated to the corresponding
α4 residues (V77K, 180M, K160S, Y165F, and Y170Q). Injecting
cRNA for α35M/α4 + β2 in oocytes gave rise to receptors that
behaved like the regular non-mutated α3 + β2 receptor with
respect to ACh sensitivity (EC50 of 380 (320–450) μM versus
EC50 of 290 (240–340) μM, respectively; for simplicity these
values are from monophasic Hill fits, although both receptors
clearly show biphasic behavior). Furthermore, NS9283 showed
no modulatory efficacy consistent with the lack of an α4α4-
interface (Fig. 8A). Nevertheless, a large difference was noted
when testing modulatory actions of NS206 at this chimera, as
the efficacy level at this mutant receptor was restored to values
comparable with that at wild-type α4β2 (Fig. 8B, Table 1). These
data indicate that NS206 indeed does bind in the TMD in a
manner dependent on the α4-subunit. Furthermore, whereas
NS9283 is fully capable of propagating its modulatory actions
across a chimeric ECD/TMD interface, NS206 needs an inter-
face that closely resembles that of the α4-subunit for full
efficacy.

DISCUSSION

Drug discovery efforts aimed at identifying novel PAMs of
α4β2 nAChRs are progressing due to the important role of this
receptor class in normal brain function. However, to date very
few new chemical entities modulating these receptors have
everged in the literature limiting both the current understand-
ing but also new investigative opportunities. To this end, the
present study identifies the novel compound NS206 as a potent
and efficacious PAM of α4β2 nAChRs. Structurally, this com-
pound does not resemble the previously described NS9283 and
comparison of these two compounds indeed reveals important
pharmacological differences.

NS206 displays all the hallmark features of a PAM at α4β2
receptors. When applied alone to α4β2 expressing oocytes in
electrophysiological experiments, it evokes no detectable cur-
rent; however, when co-applied with ACh, clear potentiation of
current levels is observed with no apparent change in current
waveforms. The level of potentiation is concentration-depen-
dent and well approximated by the empiric Hill equation, indi-
cating a saturable binding site for NS206. Moreover, no radio-
ligand displacement was noted in binding studies. With respect
to selectivity profile, NS206 was highly selective for α4* over
α3β4 and α7 nAChRs. No apparent differences in PAM activity
were noted between α4β2 and α4β4, indicating that NS206
activity is linked to the presence of the α4-subunit. Hetero-
meric nAChRs such as α4β2 are known to express in two stoi-
chiometries with ratios between the subunits being either
2α:3β or 3α:2β. Whereas NS9283 only modulates 3α:2β recep-
tors, NS206 displayed allosteric modulation at both receptor
stoichiometries.

To study the pharmacological actions of NS206 further, ACh
CRRs were run in the absence and presence of the compound.
At 2α:3β stoichiometry for α4β2 receptors, NS206 increased
ACh-evoked peak current levels and fitted curvatures of the
two CRRs remained identical with no change in ACh potency.
Thus, modulatory actions of NS206 caused an ACh concen-
tration-independent fixed increase in evoked currents by a factor
of 3–4. For 3α:2β receptors, deciphering ACh CRR data are
more complicated due to the existence of two αβ-sites and one
αα-site. This causes an ACh CRR to be biphasic with observed
potencies at the two components of ~1 and ~70 μM (αβ-site
activation and αβ-site plus αα-site activation, respectively)
(24). Interestingly, in the presence of NS206 the allosterically
modulated ACh CRR remains biphasic albeit with an altered
ratio between the components and small increases in potency.
Current levels at EC100 of ACh were increased by a factor of
~1.6, which is less than seen for the 2α:3β stoichiometry but,
nevertheless, similar type of effect.

Collectively these data show that the main effect of NS206 is
modulation of ACh-evoked peak current levels, i.e. increased
“gain.” Incidentally, the 2α:3β receptor, where NS206 induces a
proportionally larger increase in maximal peak current ampi-
tudes, is also the stoichiometry with the lowest unitary con-
ductance (21). This can be reconciled with a notion that
ACh-evoked channel activation via two agonist sites (2α:3β
receptor) gives lower gating efficiency compared with activa-
tion via three sites (3α:2β receptor). Thus, the lower gating
efficiency at 2α:3β receptors allows for a greater potential for
modulation by e.g. NS206. This again explains the altered ratio
between the two ACh CRR components at 3α:2β receptors. In
support of this, recent nAChR α7 data show that activation via
two binding sites results in intermediate mean channel open
times, whereas activation via three binding sites arranged non-
consecutively (similar to the arrangement for 3α:2β receptors)
results in maximal mean channel open times (30).

By comparing modulatory actions of NS206 to those of
NS9283 (9) it is clear that the compounds behave very differ-
ently. NS9283 only works on the 3α:2β receptor stoichiometry
and its modulatory actions left-shift the ACh CRR and give
monophasic properties. The exact left-shift degree appears to
be somewhat methodology dependent, because a ~600-fold
shift is seen in oocytes but only a ~60-fold shift in patch clamp
(31). The reasons for this discrepancy are not clear at the
moment, but irrespective of which testing regime was used,
peak current levels in the presence of NS9283 did not surpass
that of EC100 of ACh. As discussed above, this is in contrast to
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the pharmacological actions of NS206 and, interestingly, these two mechanisms can work additively. An ACh CRR at 3α:2β receptors in the presence of both NS9283 and NS206 displays the core characteristics of both compounds, i.e. large left-shift in potency and monophasic fitted curvature (NS9283 actions) combined with current levels above that of EC100 of ACh (NS206 actions). The differences in modulatory actions and additive effects make it likely that NS9283 and NS206 bind in distinct sites at the receptor. Previously, etomidate has been shown to modulate [3H]flunitrazepam binding in the GABAAR benzodiazepine site, indicating that binding to separate sites in Cys-loop receptors can indeed have additive or even synergistic effects (32).

Given that NS9283 is dependent on the αα-interface it was speculated that this interface presents the binding site (9). Indeed, three mutations associated with the binding of ACh in the αα-site turned out to be of vital importance for NS9283 modulatory actions. This indicates a shared mechanism of action with other modulators of Cys-loop receptors that bind in non-canonical ECD interfaces. The most prominent example is benzodiazepine modulation at GABAARs (10), but interface binding sites have also been proposed for galanthamine and morantel (14, 15). Whereas the case of NS9283 appears similar to that of benzodiazepines there is one important difference: the αα-interface binds ACh and is hence canonical. This suggests two possibilities: either NS9283 directly substitutes for ACh in the orthosteric binding pocket or NS9283 binds in addition to ACh and through local interactions acts to increase ACh binding potency. Regardless of which of these possibilities is correct both would lead to a full contribution of the αα-interface in gating and are hence mechanistically consistent with an ACh CRR left-shift as the only pharmacological observation.

Modulatory actions of NS206 on the other hand were unaffected by mutations in the αα-interface, however, in studies with chimeric α-subunits (α4/α3 and α3/α4), modulation was only seen with the α3/α4 chimera. Efficacy levels were severely impaired but the potency remained similar to that at wild-type α4β2 receptors. This indicates retention of key binding determinants in the α4 TMD and hints that efficacy determinants reside on both the ECD and TMD. To address this, an α3/α4 chimera was constructed in which five α3 ECD amino acids in close proximity of the TMD were mutated to the respective amino acids in α4. These mutations turned out to give “gain of function,” i.e. full modulatory efficacy of NS206 was restored in the α3SM/α4 + β2 receptor. Given that only efficacies change in these experiments it appears reasonable to assume that binding is dominated by the α4 TMD and that amino acids at the interface between the ECD and TMD are involved in propagating modulatory actions to other parts of the receptor. This would be consistent with the proposed binding sites observed for etomidate at GABAARs, PU02 at 5-hydroxytryptamine type 3 A receptors, ivermectin at an invertebrate GluCl channel, and NS1738 and PNU-120596 at a7 (16–20). Future studies will show whether NS206 in fact bind to a binding site that is the α4β2 nAChR homologue of e.g. the PU02 site.

Summing up, it is clear that α4β2 nAChRs can be allosterically modulated by two distinct mechanisms. Of the three phenotypes of allosteric modulation mentioned under the Intro-

duction, the two modulators tested here each give rise to only one of the phenotypes, neither of which dramatically changed desensitization characteristics. Allosteric modulators that exhibit several modulatory phenotypes in one molecule have previously been described (see Introduction) but the fact that the actions of two “single phenotype” modulators are additive indicates limited cross-talk between their binding sites, i.e. the actions of one is fully independent on the other. Aligning the data obtained here with that of modulators at other Cys-loop receptors, a picture begins to emerge. Notably, the pharmacological action of a modulator that binds in an ECD interface is likely one of agonist potency left shifting (benzodiazepines, galanthamine, and NS9283), whereas a modulator that binds in the ECD/TMD interface likely gives increased “gain” (etomidate, NS1738, PNU-120596, and NS206). This knowledge adds another layer of refinement in drug discovery that again can have therapeutic implications. For instance, galantamine is used for treatment of mild Alzheimers and a compound with actions similar to NS206 would based on the current data be expected to act additively, whereas a compound similar to NS9283 would instead compete for binding.

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