Structural and Functional Studies of the Modulator NS9283
Reveal Agonist-like Mechanism of Action at α4β2 Nicotinic Acetylcholine Receptors*

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Background: Cys loop receptors can be modulated by exogenous compounds.

Results: By combining x-ray crystallography, homology modeling, quantum mechanical calculations, and functional studies on α4β2 nAChRs, the binding mode and modulatory mechanism of the α4β2 nAChR modulator NS9283 were revealed.

Conclusion: Modulatory actions occur by mimicking agonists in the α4-α4 ACh-binding pocket.

Significance: Increased understanding of modulator actions open new possibilities for rational drug design.

Modulation of Cys loop receptor ion channels is a proven drug discovery strategy, but many underlying mechanisms of the mode of action are poorly understood. We report the x-ray structure of the acetylcholine-binding protein from Lymnaea stagnalis with NS9283, a stoichiometry selective positive modulator that targets the α4-α4 interface of α4β2 nicotinic acetylcholine receptors (nAChRs). Together with homology modeling, mutational data, quantum mechanical calculations, and pharmacological studies on α4β2 nAChRs, the structure reveals a modulator binding mode that overlaps the α4-α4 interface agonist (acetylcholine)-binding site. Analysis of contacts to residues known to govern agonist binding and function suggests that modulation occurs by an agonist-like mechanism. Selectivity for α4-α4 over α4-β2 interfaces is determined mainly by steric restrictions from Val-136 on the β2-subunit and favorable interactions between NS9283 and His-142 at the complementary side of α4. In the concentration ranges where modulation is observed, its selectivity prevents NS9283 from directly activating nAChRs because activation requires coordinated action from more than one interface. However, we demonstrate that in a mutant receptor with one natural and two engineered α4-α4 interfaces, NS9283 is an agonist. Modulation via extracellular binding sites is well known for benzodiazepines acting at γ-aminobutyric acid type A receptors. Like NS9283, benzodiazepines increase the apparent agonist potency with a minimal effect on efficacy. The shared modulatory profile along with a binding site located in an extracellular subunit interface suggest that modulation via an agonist-like mechanism may be a common mechanism of action that potentially could apply to Cys loop receptors beyond the α4β2 nAChRs.

Neuronal nicotinic acetylcholine receptors (nAChRs) and γ-aminobutyric acid type A receptors (GABA_As) are important ion channels that belong to the Cys loop family of receptors. They are expressed in the central nervous system where they contribute to excitatory and inhibitory neurotransmission, respectively (1, 2). Both receptor types are implicated as either cause or remedy in devastating diseases, including anxiety, Parkinson, and Alzheimer diseases, pain, and attention deficit hyperactivity disorder (3–5). Therefore, the development of drugs that target these receptors is a priority area for research (3, 6). Although allosteric modulators are mechanistically less well understood than agonists, they are believed to have advantages as pharmaceuticals. First and foremost, they cause less tonic activation and receptor desensitization, i.e. the normal temporal and spatial resolution of neuronal firing is maintained, which may result in better tolerated drugs (7, 8). Furthermore, modulator binding sites are often located in less structurally conserved regions than the agonist-binding sites, which suggest that it may be easier to obtain subtype-selective modulators (7–9).

It is becoming apparent that different modulator “phenotypes” exist, and at least some of these can be linked to distinct modulator binding sites (4, 10). A classic example of this is etomidate and benzodiazepine modulators at GABA_As, which work through different binding sites and have very distinct modulatory profiles (11). Etomidate binds to sites in the transmembrane region of the receptor where it modulates GABA_A receptor.

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The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; Ac, Aplysia californica; AChBP, acetylcholine-binding protein; CRR, concentration response relationship; LS, Lymnea stagnalis; 5-HT, AR, 5-hydroxytryptamine type 3A receptor; GABA_A, GABA_A receptor.

The atomic coordinates and structure factors (code 4NZB) have been deposited in the Protein Data Bank (http://wwpdb.org/).
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efficacy levels at low concentrations and directly activates at high concentrations, similarly to the barbiturate type of modulators at this receptor (12, 13). In contrast, benzodiazepines only modulate the GABA potency and are known to bind in an extracellular pocket in the receptor α-γ interface, a pocket that is structurally similar to the GABA-binding β-α-subunit pocket (6, 9).

Recently, an αβ2 nAChR modulator, NS9283 (Fig. 1A), with a benzodiazepine-like phenotype was reported. NS9283 improves cognitive function in animal models, and it enhances the actions in the ACh-binding site for NS9283 actions (10), thereby hinting that resemblance to benzodiazepines may extend beyond pharmacological actions. Given that benzodiazepine-type modulators have proven extremely useful at GABA,Rs, and modulators with similar profiles at αβ2 nAChRs now turn out to be promising drug-discovery lead compounds, further investigations of these shared modulatory mechanisms may provide important insights applicable to the whole family of Cys loop receptors.

In this report, we have co-crystallized NS9283 with the ACh-binding protein from Lymnaea stagnalis (Ls-AChBP). Supported by homology modeling and mutational data on αβ2 nAChRs, the results convincingly show a binding mode overlapping the ACh binding pocket in the α4-α4-subunit interface. Furthermore, NS9283 was observed to directly activate engineered receptors with three sites capable of binding NS9283. Collectively, the data show that although NS9283 functionally appears to be a classical positive allosteric modulator, mechanistically it works the same way as an agonist, albeit constrained to a single subunit interface at the tested concentrations.

**EXPERIMENTAL PROCEDURES**

**Materials**—NS9283 (3-(3-(pyridine-3-yl)-1,2,4-oxadiazol-5-yl)benzonitrile) was synthesized at NeuroSearch A/S. ACh ER2 (A9101), and all other chemicals were of analytical grade and purchased from Sigma unless otherwise specified.

**Molecular Biology**—Mutations were introduced into plasmid expression vectors coding for human α4 and β2 nAChR subunits (19) by site-directed mutagenesis as described previously (20). Custom-designed oligonucleotides were ordered from Eurofins MWG Operon. Mutations were confirmed by sequencing (MWG Operon), and cRNA was produced using the mMessage mMachine T7 transcription kit (Ambion) according to the manufacturer’s instructions.

**Radioactive Ligand Binding**—An Ls-AChBP/5-HT4AR chimera was stably expressed in HEK293 cells (CRL-1573, American Type Culture Collection) and prepared for binding experiments as described previously (21). Briefly, cell pellets were thawed, washed in 15 ml of Tris-HCl (50 mM, pH 7.4), treated with an Ultra-Turrax homogenizer, centrifuged for 10 min at 4 °C and 25,000 relative centrifugal force, and resuspended in ice-cold Tris-HCl buffer (50 mM, pH 7.4). Affinity of NS9283 was determined by displacement of [3H]epibatidine (250 μCi, NET1102250UC, PerkinElmer Life Sciences). 800 μl of tissue suspension was mixed with 100 μl of NS9283 solution in 48% ethanol and 100 μl of an ~0.3 nm [3H]epibatidine solution in 48% ethanol. The specific concentration of the [3H]epibatidine solution was determined by linear liquid scintillation counting. Binding was terminated after incubation for 1 h by filtration over GF/C glass fiber filters (Brandel Inc.) preincubated for 30 min with 0.1% polyethyleneimine. Non-specific binding was tested by incubation with an excess of (-)-nicotine (30 μM). Filters containing protein and bound [3H]epibatidine were individually incubated for at least 4 h with 3 ml of Ultima Gold (PerkinElmer Life Sciences). Radioactivity was then measured by liquid scintillation counting on a Tri-Carb counter (PerkinElmer Life Sciences), and IC50 values were determined by nonlinear regression using GraphPad Prism, with all points in one experiment determined in triplicate. The Ki value was determined from the average IC50 values of three individual experiments by the Cheng-Prusoff equation: \[ K_i = \frac{IC_{50}}{L} \left( 1 + \frac{K_i}{L} \right) \]

where L is the concentration of [3H]epibatidine used in the assay, and Kd is the dissociation constant for [3H]epibatidine at Ls-AChBP (0.097 nm) as determined previously (22).

**Electrophysiology**—Two-electrode voltage clamp electrophysiological recordings were carried out using Xenopus laevis oocytes as described previously (23). Briefly, oocytes were injected with ~25 ng of cRNA with a ratio between α4- and β2-subunit cRNAs of 4:1 or 10:1, to give 3α:2β stoichiometry. Injected oocytes were incubated for 2–7 days at 18 °C in modified Barth’s solution (90 mM NaCl, 1.0 mM KCl, 0.66 mM NaNO3, 2.4 mM NaHCO3, 10 mM Hepes, 2.5 mM sodium pyruvate, 0.74 mM CaCl2, 0.82 mM MgCl2, 100 μg/ml gentamycin, and pH adjusted to 7.5). Oocytes were placed in a custom-designed recording chamber and voltage clamped at a holding potential ranging from −40 to −80 mV using a Geneclamp 500B amplifier (Axon). Pipette resistances were 0.6–2.0 megohms in OR2 (90 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 1.0 mM MgCl2, 5.0 mM Hepes, and pH adjusted to 7.5). Fresh solutions of NS9283 and ACh were prepared on the day of measurement in OR2 and applied to the oocytes with a flow rate of 2.0 ml/min via a glass capillary. Signals were low-pass filtered at 20 Hz and digitized at 200 Hz by a Digidata 1322A (Axon) digitizer. Traces were recorded in Clampex 10.2 and subsequently analyzed in Clampfit 10.2 (Axon). Traces were baseline-subtracted, and responses to individual applications were read as peak current amplitudes. Concentration-response relationships (CRRs) were fitted in GraphPad Prism to a monophasic Hill equation constrained with a Hill slope of 1 and starting at zero. All data sets were constructed from at least five oocytes and recorded on a minimum of 2 experimental days.

**Protein Expression and Crystallization**—Ls-AChBP was expressed in Sf-9 cells using the Bac-to-Bac baculovirus expression system and purified by ion-exchange chromatography as described previously (24). A solution of ~0.2 mg/ml protein and 0.4 mM NS9283 in 0.1 M Tris, pH 8.0, 0.2 M NaCl, and 5% DMSO was equilibrated at 4 °C for 20 h, with some precipitate...
form. The solution was subsequently concentrated to an estimated protein concentration of 5 mg/ml and used immediately for crystallization experiments. Crystals were obtained by sitting drop vapor diffusion at 20 °C by mixing 1 μl of protein/ligand solution with 1 μl of crystallization solution containing 0.1 m Tris, pH 8.0, 1.9 μl (NH₄)₂SO₄, and 4% PEG 400. Crystals were supplemented with glycerol containing crystallization solution to a final glycerol concentration of ~25% before being mounted on cryo-loops and cooled in liquid nitrogen. Data were collected at MAX-lab in Lund, Sweden, on beamline I911-3 at 101 K using a wavelength of 1.0 Å. Images were collected as 0.3° φ slices on a mosaico 225 detector.

Data Processing and Refinement—XDS (54) was used for initial data processing and SCALa in CCP4 (25) for scaling and creation of an Rfree set from 5% of the total data. The structure of Ls-AChBP in complex with NS9283 was determined by molecular replacement using the PHENIX program suite (26) based on a template from Protein Data Bank code 3ZDG (chains A to E) (27) with loop C removed. The subsequent model was refined in iterative steps of manual model adjustment in COOT (28) and refinement in PHENIX. B factors were refined as individual isotropic values. NS9283 was built in Maestro, and geometry restraints and cif-file were generated with eLBOW in PHENIX. Noncrystallographic symmetry (NCS) restraints were used in refinement steps; however, residues within 5 Å of the modulator-binding pocket were excluded as well as residues 22–25 (loop A) and 155–163 (loop F). For statistics on data collection and refinement, see Table 1.

Molecular Modeling—Models of the extracellular domain of a dimer between two α4 nAChR subunits were created and evaluated based on the procedure described previously (20), but with the NS9283 co-crystal structure replacing the 1UW6 template and NS9283 replacing nicotine. In this way, the refined structure between NS9283 and Ls-AChBP was used to define the binding site region in the model. One hundred models of the dimer with NS9283 bound in the interface were prepared in MODELLER Version 9.12 (29), and the highest ranked model according to Discrete Optimized Protein Energy (DOPE) score (30) was selected. Subsequently, nonconserved residues within 5 Å of NS9283 were sampled in PRIME (31) to predict sidechain conformations while taking electrostatics of the ligand into consideration. Quantum mechanical calculations were performed on the NS9283 molecule extracted from the chain C-D interface of the Ls-AChBP co-crystal structure. After preparation of the modulator, the geometry was optimized using the B3LYP/6–31G* method keeping ring-connecting dihedrals frozen. Subsequently, the electrostatic potential was calculated using the B3LYP/CC-PVTZ + + functional and basis set implemented in Jaguar (version 7.9, Schrödinger, LLC). The SCF convergence criteria was set to “ultrafine.”

RESULTS

The ACh-binding protein (AChBP) has been used frequently as a model for α4β2 nAChRs (22, 32) to investigate interactions of agonists and antagonists with extracellular binding sites and, less frequently, modulators (33). An extracellular binding site for the α4β2-positive allosteric modulator NS9283 is suggested from several datasets. Experiments with chimeric receptors link efficacy to the extracellular domain of α4β2 receptors (10). Furthermore, mutation of three residues, His-142, Gln-150, and Thr-152, in the complementary α4-β-subunit interface, abolishes all NS9283 actions, which indicate binding in this region (10). Interestingly, these same point mutations were previously shown to affect potency of ACh for the α4-α4 site, which could indicate partly or fully overlapping binding sites for NS9283 and ACh (20).

Previously, NS9283 was shown to not displace or otherwise affect [3H]cytosine binding at rat cortical membranes (15). To determine whether NS9283 displaces [3H]epibatidine from Ls-AChBP, initial experiments were performed on membranes from HEK293 cells expressing an Ls-AChBP/5-HT₃AR chimera (22). Surprisingly, NS9283 did in fact clearly displace epibatidine with a calculated Kᵢ of 67 μM (Fig. 1B), but due to precipitation at high concentrations (>300 μM), full displacement could not be obtained under the experimental conditions.

Ls-AChBP was next successfully co-crystallized in presence of an ~400 μM suspension of NS9283. The resulting crystals in the space group C2 diffracted to 2.7 Å resolution (Table 1). In the crystal structure, subunits were in the expected pentameric arrangement with three pentamers in the asymmetric unit of the crystal, each subunit consisting of a core β-sandwich structure and several important loop regions (Fig. 1, C and D) (32). NS9283 Binds to Ls-AChBP in the ACh Binding Pocket—Following structure determination by molecular replacement and initial refinement, additional electron density was visible at all interfaces in the electron-rich aromatic box around Trp-143. This region is homologous in sequence to the ACh binding pocket in nAChRs and is also the binding site for other α4β2 ligands, including nicotine, carbamoylcholine, and dihydro-β-erythroidine (10, 29, 34). NS9283 was unambiguously built into six interfaces out of the total of 15 that compose the three pentamers of the asymmetric crystallographic unit (Fig. 1, D–F). As the electron density in the remaining sites did not allow unambiguous positioning of NS9283, they were left unmodeled. For AChBP complexes with low affinity ligands, it is common that only some sites have ligands modeled, e.g. in some binding sites with neonicotinoids, cocaine, galanthamine, and carbamoylcholine (24, 33, 35). In the sites where NS9283 is modeled, it binds in a single well defined conformation, but the exact location varies slightly between sites (root mean square deviation = 0.4 Å) (Fig. 1G) as does capping of the binding site by the tip of loop C (Fig. 1G).

The best defined electron density for NS9283 (Fig. 1, E and F) was observed in the interface between chains B and C and is consequently used in the following analysis (deviations observed in other, largely similar, interfaces are indicated in Fig. 1G). The ligand mainly interacts with conserved residues on the principal side of the interface (Fig. 1E) as follows: (i) the pyridine ring of NS9283 stacks with Tyr-89 at a centroid-centroid distance of 4.0 Å; (ii) the oxadiazole moiety interacts with the backbone carbonyl oxygen of Trp-143 at a distance of 3.1 Å, measured to C5 of the oxadiazole; (iii) the pyridine nitrogen is within hydrogen bonding distance (2.7 Å) of Tyr-185; and (iv) the nitrogen of the nitrile is within hydrogen bonding distance (3.1 Å) of Tyr-192. On the complementary side of the interface, the N2 of the oxadiazole is located 4.0 Å away from the centroid.
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FIGURE 1. NS9283, chemical structure, binding, and co-crystal structure with Ls-AChBP. A, chemical structure of NS9283. B, [3H]epibatidine displacement by NS9283 from an Ls-AChBP/S-HT4R chimera expressed in HEK293 cells. NS9283 was not soluble at concentrations exceeding 300 μM under the experimental conditions. Data were fitted to a one-site competitive binding equation giving an IC50 value of 67 μM (pKa = 4.17 ± 0.43 (S.D.), n = 6). C, top view of the crystal structure of Ls-AChBP with NS9283 determined at 2.7 Å resolution. D, side view of the C and D interface with NS9283 bound. NS9283 is shown as a space-filling model. E, binding mode of NS9283 (gray sticks) in the crystal structure. Residues conserved with respect to the α4-α4 interface in nAChRs are shown as sticks and nonconserved residues within 5 Å of NS9283 as lines. Green residues are from the principal (+) subunit, and blue residues are from the complementary (−) subunit. Interactions are highlighted as dashed black lines. The binding mode is representative of three interfaces where loop C is the most closed. The water molecule seen interacting with the conserved Trp-53, and modulator binding is further supported by van der Waals interactions to Arg-104, Leu-112, and Met-114. Among the observed interactions, the contact between the oxadiazole and the Trp-143 carbonyl oxygen appeared unusual and was therefore further investigated by quantum mechanical calculations to map the molecular surface properties of NS9283. This revealed that the area around C5 of the oxadiazole ring and C3 of the benzonitrile is electron-deficient as evident from the positive electrostatic potential (Fig. 2), suggesting that favorable interaction to the carbonyl oxygen is contributing to binding.

NS9283 Forms an Inter-subunit Bridge in α4-α4 Interfaces—The principal side residues forming direct contacts to NS9283 are fully conserved between Ls-AChBP and the α4 nAChR subunit. However, when comparing contact residues on the complementary subunit interface of Ls-AChBP with those in the classical agonist binding pockets of α4-β2 or α4-α4 interfaces, only Trp-53 corresponding to Trp-88 and Trp-82 in α4 and β2,

| TABLE 1 | Crystallography statistics |
|--------------------------------|----------------------------|
| Data collection                | C2                         |
| Space group                    | 231.73, 140.38, 119.55      |
| a, b, c (Å)                    | 90.00, 89.98, 90.00         |
| Reflections, overall           | 378,581                    |
| Reflections, unique            | 102,572                    |
| Resolution range (Å)           | 28.37–2.68 (2.82–2.68°)    |
| I/σr                          | 8.9 (2.0)                  |
| Completeness (%)               | 98.1 (87.6)                |
| Multiplicity                   | 3.6 (2.5)                  |
| Rmerge (%)                     | 7.2 (37.9)                 |
| Mosaicity (%)                  | 0.19                       |
| Pentamers/AU                   | 3                         |

Refinement

| No. of residues | Protein | 2963 |
|-----------------|---------|------|
| Water           | 6       |      |
| R(work) (%)     | 21.0    |      |
| R(free) (%)     | 24.6    |      |
| r.m.s.d. bonds (Å) | 0.014 |      |
| Ramachandran outliers (%) | 0.1 |      |
| Ramachandran favored (%) | 97.3 |      |
| Average B (Å²)  | Protein | 50   |
| Water           | 68      |      |
| Water           | 40      |      |

* Numbers in parentheses correspond to the outer resolution bin.
* A measure of agreement among multiple measurements of the same reflections.
Rmerge is calculated as follows: I(hkl) is the intensity of an individual measurement of the reflection with Miller indices hkl, and I(hkl) is the intensity from multiple observations Rmerge = ΣI(hkl) − 1/hkl I(hkl) − I(hkl). AU means asymmetric unit of the crystal.
Rmerge is calculated as follows: I(hkl) is the intensity of an individual measurement of the reflection with Miller indices hkl, and I(hkl) is the intensity from multiple observations Rmerge = ΣI(hkl) − 1/hkl I(hkl) − I(hkl).
* r.m.s.d. means root mean square deviation.
* The Ramachandran plot was calculated using PHENIX. The outliers are at the borderline of allowed regions.
respectively, are conserved. The remaining contact residues, Arg-104 (which only exposes its hydrophobic carbon chain to the binding site), Leu-112, and Met-114 make the complementary binding interface hydrophobic in nature, and thus it best resembles the $\beta_2(-)$ interface with Val-136, Phe-144, and Leu-146 in corresponding positions (22, 32). When considering the $\alpha_4-\alpha_4$ interface, where the corresponding residues His-142, Gln-150, and Thr-152 are hydrophilic in nature (20), the use of $L_s$-AChBP as a model system to predict important ligand-receptor interactions is complex.

Therefore, as NS9283 binds in the $\alpha_4-\alpha_4$ interface of $\alpha_4\beta_2$ nAChRs, a homology model of this interface was constructed using the NS9283 x-ray structure as one of the templates. Particular focus was placed on defining the binding site region, and a procedure similar to that previously reported by Harpsøe et al. (20) was used. This model (Fig. 3) is highly comparable with the $L_s$-AChBP on the principal side with interactions similar to those in the B-C interface of the x-ray structure; however, it also suggests potential interactions of NS9283 with nonconserved residues on the complementary side of the $\alpha_4$-subunit. In particular, hydrogen bonding is predicted from His-142 to the nitrile group of NS9283. This interaction appears to involve peripheral residues Arg-112 and Phe-144, stacking with the histidine and shielding the polar histidine and arginine from the surrounding solvent. In summary, the model supports an $\alpha_4-\alpha_4$ interface (Trp-88 in $\alpha_4$ versus Ls-AChBP) are shown as sticks, and conserved residues are shown as lines. Residues on the principal $\alpha_4(\text{+})$ and complementary $\alpha_4(-)$ side are colored green and blue, respectively. 8, alignment of human nAChR subunits $\alpha_4$ and $\beta_2$ and $L_s$-AChBP based on a multiple alignment from Ref. 32.

Mutations Validate NS9283 Contacts—To investigate the observed binding mode of NS9283, we performed a mutational scan of residues on the complementary side of the $\alpha_4-\alpha_4$ interface that were predicted, based on the x-ray structure, to be in proximity of the compound. By only introducing mutations on the $\alpha_4$ complementary interface, the $\alpha_4-\beta_2$ interfaces are left intact for ACh binding. The mutant constructs were expressed in oocytes in 3Xenopus oocytes in 3

To investigate whether the detrimental effect of H142V was due to lack of interaction with the histidine or rather a disruptive steric effect of the more bulky valine, an alanine was next tested in this position. At $\alpha_4-\alpha_4$ interface, the $\alpha_4(\text{+})$ and complementary $\alpha_4(-)$ side are colored green and blue, respectively. 8, alignment of human nAChR subunits $\alpha_4$ and $\beta_2$ and $L_s$-AChBP based on a multiple alignment from Ref. 32.

As mentioned, an $\alpha_4$ complementary side mutant, where three residues were changed to the corresponding residues in $\beta_2$, H142V, Q150F, and T152L, obliterates all NS9283 activity (10). When mutated individually, a similar effect was seen with the H142V mutant, whereas the Q150F and T152L mutations resulted in decreased functional potency by a factor of 2 and 3, respectively (Table 2 and Fig. 5A). To investigate whether the detrimental effect of H142V was due to lack of interaction with the histidine or rather a disruptive steric effect of the more bulky valine, an alanine was next tested in this position. At $\alpha_4$-mutated receptors, NS9283 showed modulatory efficacy albeit with an ~10-fold decreased EC_{50} value (Fig. 5, B and C), which indicates that a combination of both above-mentioned explanations causes the obliteration of efficacy at the H142V construct.

In the $L_s$-AChBP structure, the Trp-53 residue was seen as the only conserved interaction from NS9283 to the complementary interface (Trp-88 in $\alpha_4$). To investigate the impor-
tance of Trp-88 for NS9283 activity, three point mutants W88A, W88F, and W88R were tested. W88R resulted in receptors that were unresponsive to NS9283, whereas W88A and W88F resulted in greatly reduced modulator potency (Fig. 5, B and C). It is noteworthy that these three mutations, in contrast to the other mutations tested here, also clearly affect the ability of ACh to activate the receptor through the \( \alpha_4\beta_4 \)-binding site (Fig. 4 and Table 3). Full ACh CRRs with the W88R and W88F mutants were similar to previous data observed for the W88A mutant (36), but the mutational effects appear more pronounced. For both mutants, ACh activation via the \( \alpha_4\beta_4 \)-site is uncompromised as evidenced by the EC\( _{50} \) values close to 1 \( \mu M \) (Table 3). In the W88F mutant, ACh activation through the low affinity \( \alpha_4\alpha_4 \) site is clearly visible although with an \( \sim 30 \)-fold decreased EC\( _{50} \) value; however, in the W88R mutant \( \alpha_4\alpha_4 \) site activation is severely compromised. For the Trp-88 mutants, the high affinity component of the biphasic Hill curve fit accounts for a larger proportion compared with the WT receptor (Table 3), which is to be expected because the mutations partly or completely disrupt ACh-evoked receptor activation through the \( \alpha_4\alpha_4 \) site. Thus, Trp-88 appears to be central for ACh actions in the \( \alpha_4\alpha_4 \) site, and the relative effects of these mutations W88R > W88F > W88A match the order observed for NS9283. This is expected given the

**FIGURE 4.** Representative traces from two-electrode voltage clamp experiments on \( \alpha_4\beta_2 \) nAChRs and mutants thereof expressed in *X. laevis* oocytes. ACh-evoked responses in WT and point-mutated \( \alpha_4\beta_2 \) receptors were recorded in *X. laevis* oocytes as described in Table 2 legend. For all mutants, representative traces are shown from applications of 10 \( \mu M \) and 1 mM ACh to the same oocyte. All mutants respond to ACh in a concentration-dependent manner and with current waveforms resembling WT receptors (upper left corner) as inspected visually. The ratio between ACh-evoked currents at 1 mM and 10 \( \mu M \) concentrations, \( \frac{I_{1\text{mM ACh}}}{I_{10\mu M ACh}} \), can be seen as an indicator of whether a particular mutation results in a functional \( \alpha_4\alpha_4 \) site with respect to ACh activation. \( \frac{I_{1\text{mM ACh}}}{I_{10\mu M ACh}} \) ratios are seen in Table 2. Note that the W88A, W88F, and W88R mutants give significantly lowered ratios compared with WT indicating a compromised site. These mutants are discussed in the text.
binding mode of NS9283 and suggests that NS9283 has
direct interaction with Trp-88, as was clearly indicated by the
AChBP binding mode.

### TABLE 2

| Mutation | WT (1 mM ACh) | µEC<sub>50</sub> | % | WT (10 µM ACh) | µEC<sub>50</sub> | % | WT (100 µM ACh) | µEC<sub>50</sub> | % |
|----------|---------------|----------------|---|---------------|----------------|---|---------------|----------------|---|
| None (WT) | 4.6 ± 1.1 | 3.4 (1.5–7.9) | 680 (530–830) | 6 | | | | | |
| W88A     | 1.6* ± 0.2 | 110 (6.1–210) | 560 (10.0–1800) | 6 | | | | | |
| W88F     | 1.9* ± 0.3 | 51 (12–220) | 410 (6.4–820) | 7 | | | | | |
| W88R     | 1.8* ± 0.5 | NA | NA | 9 | | | | | |
| T109A    | 5.1 ± 1.2 | 27 (1.8–4.1) | 580 (520–650) | 6 | | | | | |
| S110A    | 9.2* ± 1.5 | 39 (1.9–8.0) | 1700 (1300–2100) | 4 | | | | | |
| R112A    | 5.6 ± 1.0 | 47 (10–90) | 240 (110–370) | 7 | | | | | |
| T139A    | 4.2 ± 0.9 | 51 (2.6–10) | 400 (310–490) | 8 | | | | | |
| H142A    | 8.1* ± 1.5 | 36 (16–78) | 780 (420–1100) | 6 | | | | | |
| H142V    | 6.8 ± 2.2 | NA | NA | 14 | | | | | |
| F144A    | 7.5* ± 1.5 | 22 (10–50) | 900 (550–1300) | 8 | | | | | |
| H145A    | 5.9 ± 2.4 | 1.6 (0.89–2.7) | 430 (380–480) | 4 | | | | | |
| Q150A    | 4.2* ± 1.6 | 57 (2.1–16) | 469 (310–630) | 5 | | | | | |
| Q150F    | 3.5 ± 1.4 | 6.9 (3.5–14) | 570 (430–700) | 12 | | | | | |
| T152A    | 5.0 ± 1.3 | 40 (16–99) | 210 (130–290) | 6 | | | | | |
| T152L    | 4.3 ± 0.9 | 11 (6.1–22) | 360 (270–440) | 9 | | | | | |

* Data are from Ref. 10.

### FIGURE 5

Mutations on the complementary side of the α4-α4 interface affecting NS9283 modulatory potency. A, CRRs of NS9283 modulation of WT and point-mutated α4β2 receptors were recorded in X. laevis oocytes as described in Table 2 legend. B, pEC<sub>50</sub> values of NS9283 modulation at WT (black) and point-mutated α4β2 nAChRs (Table 2). For ease of reading, the dotted black line denotes the WT potency against which all bars are compared. Data bars for amino acids marked with * and colored purple were significantly different from WT (one-way analysis of variance, α = 0.01), and data bars marked in gray were not. C, residues for which the mutation resulted in statistically significant loss of potency are shown as sticks and the remaining as lines in the α4-α4 homology model.

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An alanine scan of further residues clustered around the binding pocket of NS9283 revealed that Arg-112 and Phe-144 also contribute to NS9283 potency in good agreement with the predicted roles of these amino acids in the homology model (Fig. 5, B and C). In contrast, alanine mutations in Thr-109, Ser-110, Thr-139, and His-145 did not result in EC<sub>50</sub> values of NS9283 significantly different from those at WT receptors.

NS9283 Activates Receptors with More Than One Compatible Binding Site—Because the results show a binding mode of NS9283 at the α4-α4 interface overlapping that of ACh and with interactions to residues known to interact with agonists, we hypothesized that NS9283 might be able to directly activate receptors containing more than one NS9283-binding site. To investigate this, a previously described point mutated β2-subunit was expressed in combination with α4 (20). Essentially, this β2 mutant has the reverse mutations of the above described α4 triple mutant (V136H, F144Q, and L146T), thereby giving a receptor in 3α:2β stoichiometry with two pseudo α4-α4 sites besides the native α4-α4 binding site. Although the pseudo α4-α4 sites are obviously not native α4-α4 sites, e.g. Phe-144 is missing, the mutations replace the valine found to be incompatible with NS9283 function and introduce the important histidine in its place.

When comparing the effect of NS9283 applications in absence of ACh on WT and α4β2 (V136H, F144Q, and L146T) receptors, a clear difference is noted (Fig. 6, A and B). At WT receptors, no response was seen, whereas at the mutant receptor, NS9283 acted as an agonist with a concentration-depen-
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Table 3

| Receptor | EC50,1 µM | EC50,2 µM | Fraction | n |
|----------|----------|----------|----------|---|
| α4β2    | 0.95     | 0.083    | 0.2      |   |
| α4Phe-88/β2 | 1.87     | 0.150    | 0.7      |   |
| α4Trp-88/β2 | 1.5 (1.0–2.3) | 1.9 (1.0–3.7) | 0.6 (0.5–0.6) | 9–10 |
| α4W88A/β2 | 0.96 (0.65–1.4) | 11 (6.0–22) | 0.6 (0.5–0.6) | 3–8 |

Figure 6. Agonist activity of NS9283 at mutant α4β2(3M) nAChR receptors. Representative traces of ACh- and NS9283-evoked currents in WT (A) and α4β2(3M) (B) receptors. 3M signifies the mutations V136H, F144Q, and H142Q. The short distance to Trp-143, which acts as a hydrogen bond acceptor, e.g., the 2-oxadiazole ring of nicotine, is important in light of NS9283 not being able to form a cation-π interaction to Trp-143 like nicotine (37).

Discussion

The aim of this study was to investigate the binding site and mode of action of the positive allosteric modulator NS9283 to further our understanding of how modulators interact with their target proteins. In a previous study (10), it was shown that the action of NS9283 is obliterated after mutation of three amino acids located in the ACh binding pocket of the α4–α4 nAChR interface. To explore the possibility that NS9283 could bind in close conjunction or even partly or fully inside the ACh binding pocket itself, we initially investigated displacement of [3H]epibatidine binding from Ls-AChBP. Ls-AChBP was chosen because, contrary to other AChBPs, it contains all the five aromatic binding pocket residues that are fully conserved in nAChRs (36). Importantly, NS9283 was found to displace epibatidine, albeit with a low inhibition constant (Ki of ~67 µM) compared with its functional potency at α4β2 receptors (EC50 = 3.4 µM). With this in mind, crystallization experiments with purified Ls-AChBP were set up with the highest concentrations of NS9283 possible, and this resulted in a successful crystallized protein with the modulator bound.

In the crystal structure of Ls-AChBP with NS9283, we observe a binding mode where NS9283 interacts with residues known to be important for agonist action. Key interactions are observed with residues conserved between Ls-AChBP and an α4–α4 interface. The pyridine ring stacks with Tyr-89, which also forms close contacts, e.g., to nicotine and carbamoylcholine in their respective structures. The oxadiazole ring of NS9283 interacts with the carbonyl group of Trp-143 at a distance of 2.9 Å. The short distance to Trp-143, which acts as a hydrogen bond acceptor, e.g., to the 2-nitropiperidinol part of nicotine (24), can be explained by electrostatic interactions, as the electron withdrawing properties of the nitrile effectively de-shields C5 of the oxadiazole and C3 of the benzonitrile, leaving a partial positive charge to interact with the lone pairs of the carbonyl oxygen. This is likely important in light of NS9283 not being able to form a cation-π interaction to Trp-143 like nicotine (37).

On the complementary side of the interface, the crystal structure reveals close contact (4 Å) from N2 of the oxadiazole ring of NS9283 to the centroid of Trp-53, which may appear counter-intuitive. However, interactions between lone pairs and centroids of aromatic rings have been confirmed to be favorable by quantum mechanical calculations and have been observed in several x-ray structures (38, 39).

Besides the key interactions, numerous van der Waals interactions are observed to residues on the complementary side that are not conserved residues, and their importance is therefore less obvious. To evaluate interactions between NS9283 and the complementary side of an α4–α4 interface, two approaches were pursued as follows: 1) a homology model based on the crystal structure obtained here was built, and 2) potentially important amino acids identified by the model were investigated by functional studies on point mutated α4 constructs. Based on the model and Ls-AChBP structure, the α4 residues Trp-88, Arg-112, His-142, Phe-144, Glu-150, and Thr-152 were all shown to interact with NS9283, and in fact, point mutations of all these amino acids affected the NS9283 modulatory potency. Other α4 residues, Thr-109, Ser-110, Thr-139, and His-145, located in or around the binding pocket and are not expected to interact with the modulator, were confirmed to be functionally silent with respect to NS9283 potency.

Of the residues on the complementary side identified to be important in the mutational studies, only Trp-88 is conserved in Ls-AChBP (Trp-53), where it is located ~4 Å from the oxadiazole ring of NS9283. Mutating Trp-88 to an alanine or phenylalanine impairs NS9283 modulatory potency by an order of magnitude, indicating that tryptophan-specific interactions are important. Substituting Trp-88 with the longer arginine abolished modulation, as expected for steric reasons based on the crystal structure with Ls-AChBP. It is noteworthy here that ACh activation through the α4–α4 interface is similarly affected (Fig. 4 and Table 3) by these mutations. This give emphasis to the observation that the binding mode of NS9283 mimic those of agonists.

Of the important residues not conserved in Ls-AChBP, His-142 was especially interesting as NS9283 potency was reduced upon mutation to alanine and abolished by mutation to valine. Because a valine is present in the corresponding β2-subunit position, this single amino acid is therefore sufficient for and likely the primary determinant of the α4–α4 versus α4–β2 interface selectivity of NS9283. In fact, the corresponding residue in α2 is likewise a histidine, but is a leucine in α3, consistent with...
the notion that this residue position is a critical determinant of modulator selectivity given that NS9283 works on α2- and α4- but not α3-containing receptors (15). That mutation of His-142 to a valine versus alanine should be so unfavorable may seem surprising but can be rationalized based on the binding mode of NS9283 in Ls-AChBP and the homology model. A branched amino acid such as valine is, for steric reasons, not compatible with binding of NS9283. Next, the homology model suggests that the histidine side chain itself interacts with NS9283 and is in close proximity to the functionally important charged Arg-112 and Phe-144. Based on the homology model and supported by mutations, Arg-112, His-142, and Phe-144 therefore contribute significantly to anchoring the ligand to the complementary interface, which may explain the functional effect of mutation to alanine of these residues.

Although NS9283 does not structurally resemble known agonists, it does, as mentioned, show α4-α4 interface contacts resembling contacts formed by agonists. This apparent agonist-like binding mode of NS9283 leads to the question of why it has no agonist activity on its own. The most logical explanation is linked to the selectivity of the compound. As discussed above, the presence of a histidine versus a valine residue at the complementary interface creates a high selectivity for the presence of a histidine linked to the selectivity of the compound. As discussed above, the most logical explanation is that of an agonist-like mechanism to help distinguish compounds such as NS9283 from true allosteric modulators that act through distinct sites. Unfortunately, the nomenclature recommendations from the “International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification” in their current form do not appear to contain a term that fully covers such a molecule (40).

On nAChRs, the described type of modulation may adhere to other modulators, including galantamine and morantel, which all enhance potency through what is thought to be extracellular binding sites (16, 33, 41). Similarly, benzodiazepines acting on GABA<sub>A</sub>Rs also produce potentiation of GABA-evoked currents without an increase in maximum efficacy. Their binding site also resides in the extracellular domain, and they form contacts across the binding interface to residues corresponding to those responsible for GABA binding in the GABA-binding site (42). The similarities in functional profiles and ligand-receptor contacts suggest that the concept may in fact also apply to benzodiazepines.

CONCLUSION

From a therapeutic perspective, positive modulators of nAChRs are of significant pharmaceutical interest. To date, only a few modulators of α4β2 receptors have been reported, and NS9283 is arguably the most promising as a drug discovery lead molecule. This study reports an x-ray structure with NS9283 bound to Ls-AChBP in excellent agreement with a comprehensive set of mutational data. Collectively, the data show that NS9283 binds in the α4-α4 interface in (α4)2(β2)2 receptors where it occupies the binding site for ACh and forms contacts to residues known to be important determinants for agonist binding and function. This suggests that the mode of action of NS9283 is that of an agonist selective for a single subunit interface that is supported by data showing that NS9283 can directly activate receptors engineered to contain three compatible binding sites.

As mentioned in the Introduction, different phenotypes of allosteric modulators exist for Cys loop receptors, and their precise mode of action appears linked to the binding site they occupy. This study greatly aids in understanding the molecular actions of compounds that bind and behave like NS9283. Interestingly, an implication of this is that insights from structure-activity studies of agonists can be used in further drug discovery.
efforts for new positive modulators. Hence, this can provide a basis for rational development of new drugs for nAChRs as well as other members of the Cys loop receptor family.

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