An Accessory Agonist Binding Site Promotes Activation of α4β2* Nicotinic Acetylcholine Receptors*

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Background: In (α4β2)2α4 nicotinic acetylcholine receptors, there is an agonist binding site at the α4/α4 subunit interface. ns9283 at interfaces with α2, α3, and α6 accessory subunits can form an agonist site with α4. These promote activation upon agonist binding at α4/β2 agonist sites.

Results: α2, α3, and α6 accessory subunits cannot. To permit selective blockage of the accessory subunits, we therefore suggest categorizing agonists according to their site selectivity. ns9283 binds to the accessory site selectively. ns9283 binds to the accessory site.

Conclusion: Accessory subunit agonist sites greatly influence receptor function.

Significance: These sites are promising drug targets.

Neuronal nicotinic acetylcholine receptors containing α4, β2, and sometimes other subunits (α4β2* nAChRs) regulate addictive and other behavioral effects of nicotine. These nAChRs exist in several stoichiometries, typically with two high affinity acetylcholine (ACh) binding sites at the interface of α4 and β2 subunits and a fifth accessory subunit. A third low affinity ACh binding site is formed when this accessory subunit is β4 but not if it is β2. Agonists selective for the accessory ACh site, such as 3-[3-(3-pyridyl)-1,2,4-oxadiazol-5-yl]benzonitrile (NS9283), cannot alone activate a nAChR but can facilitate more efficient activation in combination with agonists at the canonical α4β2 sites. We therefore suggest categorizing agonists according to their site selectivity. NS9283 binds to the accessory ACh binding site; thus it is termed an accessory site-selective agonist. We expressed (α4β2)2 concatamers in Xenopus oocytes with free accessory subunits to obtain defined nAChR stoichiometries and α4/α4 accessory subunit interfaces. We show that α2, α3, α4, and α6 accessory subunits can form binding sites for ACh and NS9283 at interfaces with α4 subunits, but β2 and β4 accessory subunits cannot. To permit selective blockage of the accessory site, α4 threonine 126 located on the minus side of α4 that contributes to the accessory site, but not the α4β2 sites, was mutated to cysteine. Alkylation of this cysteine with a thio-reactive reagent blocked activity of ACh and NS9283 at the accessory site. Accessory agonist binding sites are promising drug targets.

nAChRs contain five homologous subunits organized to form a central cation channel whose opening is gated by the binding of ACh. Homeric α7 nAChRs have five α7 subunits and five ACh binding sites at their extracellular interfaces (1). Heteromeric α4β2 nAChRs assemble into (α4β2)2α4 or (α4β2)2β2 stoichiometries with three or two ACh binding sites (2, 3). Binding of ACh to one site is able to activate, inefficiently, both homeric and heteromeric nAChRs (4, 5). Binding to two sites in an α7 nAChR is more efficient, and binding to three is most efficient for activation. By contrast, binding to four or five sites in α7 nAChRs promotes desensitization more rapidly than activation (6). In α4β2 nAChRs, ACh can bind at the interface between α4 and β2 subunits (abbreviated as α4/β2) where the α4 subunit on the left forms the plus face of an agonist site and the β2 subunit on the right forms the minus face. In the (α4β2)2α4 stoichiometry, a third low affinity ACh site is present at the α4/α4 interface (2, 3). This results in 4-fold larger responses evoked by ACh of (α4β2)2α4 or (α4β2)2β2 stoichiometry. Because the high affinity α4/β2 agonist sites contribute less than 25% of the total response of (α4β2)2α4 nAChRs, these nAChRs appear to be low affinity if the high affinity component is not clearly resolved (7, 8). Actually, (α4β2)2α4 nAChRs can be activated by low concentrations of agonists from their intrinsic α4/β2 sites to the same extent as the (α4β2)2β2 nAChRs (2).

NS9283 is representative of a new class of selective agents targeting α4β2 nAChRs that have proven useful in aiding nAChR agonists in reducing neuropathic pain and improving cognition (9–15). It has been termed a positive allosteric modulator (PAM) because it cannot activate nAChRs by itself but enhances α4β2 nAChR activity in response to agonist stimulation (9, 10, 12). However, it was recently established that NS9283 is neither allosteric nor a modulator. It is not allosteric because NS9283 acts as a selective agonist at the ACh binding site formed at the α4/α4 interface (16, 17). NS9283 cannot activate through its action on the α4/α4 site alone. In combination with agonists at α4/β2 sites, it produces the high probability of channel opening resulting from increased binding site occupancy (12, 17). Because NS9283 achieves its effect by occupying a third ACh site, just as any other full agonist would at that site, it is not a modulator. NS9283 does not exceed the maximum...
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The activation efficiency of \((\alpha 4\beta 2)_2\alpha 4\) nAChRs by ACh (18). As expected, NS9283 is without effect on \((\alpha 4\beta 2)_2\beta 2\) nAChRs for lack of the \(\alpha 4/\alpha 4\) binding site (17, 19).

In addition to NS9283, other well known \(\alpha 4\beta 2^+\) nAChR ligands also show differential actions on \(\alpha 4/\beta 2\) and \(\alpha 4/\alpha 4\) agonist sites. The drug sazetidine is a full agonist at \(\alpha 4/\beta 2\) primary agonist sites but does not bind to the \(\alpha 4/\alpha 4\) accessory ACh binding site (20–22). Cytisine acts at both types of ACh binding sites as a partial agonist (16, 20, 22). Dihydro-\(\beta\)-erythroidine is an antagonist at both \(\alpha 4/\beta 2\) and \(\alpha 4/\alpha 4\) ACh binding sites (3).

These orthologic ligands are distinguished from PAMs that act at non-orthosteric ACh binding sites, such as the C terminus of \(\alpha 4\) (23), or transmembrane sites on \(\alpha 7\) nAChRs (24–26).

To avoid misleading nomenclature and provide nomenclature that reflects the mechanism of action, we suggest designating sazetidine and NS9283 as ACh binding site site-selective agonists (SSAgs). Here we confirm and extend what is known about accessory site activation of SSAgs, such as sazetidine. Here we investigate the ability of \((\alpha 4\beta 2)_2^+\) nAChRs to form ACh and NS9283 binding sites with the accessory subunits \(\alpha 2\), \(\alpha 3\), \(\alpha 4\), \(\alpha 6\), \(\beta 2\), and \(\beta 4\). Functional impairment by blocking accessory sites suggests that accessory agonist sites exist and that they promote channel activation from the interface between several \(\alpha\) subunits and \(\alpha 4\).

**Experimental Procedures**

**Chemicals**—2-Aminothyl methanethiosulphonate (MTSEA) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). NS9283 was synthesized as described previously (27). A \(10 \text{mM}\) stock of NS9283 was prepared in dimethyl sulfoxide. Dilutions of NS9283 and MTSEA were prepared daily in buffer between use. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

**cDNAs and cRNAs**—Human \(\alpha 3\), \(\alpha 4\), \(\alpha 6\), \(\beta 2\), and \(\beta 4\) were cloned in this laboratory (28–31). The human \(\alpha 2\) subunit was obtained from OriGene Technologies, Inc. (Rockville, MD). The \(\alpha 2\) sequence was cut out with the restriction enzymes Sma1 and Xho1 to shorten the untranslated region and improve functional expression. The 2.0-kb DNA fragment coding for \(\alpha 2\) was subcloned into the pSP64 vector for RNA preparation or into pCDNA3.1/Zeocin (+) (Invitrogen) for human cell transfection.

Syntheses of concatamers of \(\beta 2(AGS)_{4}\alpha 4\) (abbreviated as \(\beta 2-\alpha 4\)) and \(\beta 2(AGS)_{4}\alpha 4(AGS)_{2}\beta 2(AGS)_{4}\alpha 4\) (abbreviated as \(\beta 2-\alpha 4\‌beta 2-\alpha 4\)) were described previously (32, 33). Signal peptides of \(\alpha 4\), \(\alpha 6\), and \(\beta 2\) subunits were analyzed by Signal-3L (34). The mature amino acid sequences were used to number \(\alpha 4\), \(\alpha 6\), and \(\beta 2\) subunits. Mutations in the dimeric concatamer are numbered as they are in single subunits and displayed in the upper right corner of the subunit that carries the mutation. For example, \(\beta 2-\alpha 4_{T126C}\) means that the threonine at the 126 position of the \(\alpha 4\) subunit is replaced by a cysteine. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The amino acids mutated in \(\alpha 4_{T126C}, \beta 2_{L121C}\), and \(\alpha 6_{T2508}\) are underlined: \(\alpha 4\), VQWTPPAI; \(\beta 2\), IFWLPPPAI; \(\alpha 6\), SLLSSSLTV. All mutations were confirmed by sequencing.

After linearization and purification of cDNAs, RNA transcripts were prepared in vitro using mMessage mMachine kits (Ambion, Austin, TX). Concentrations of cDNAs and cRNAs were calculated by spectrophotometry.

**Oocyte Removal and Injection**—Oocytes were removed surgically from *Xenopus laevis* as described except that a higher concentration (0.26 mg/ml) of collagenase type IA (Sigma) was used to obtain oocytes optimal for the automated voltage clamp instrument described later.

Oocyte injections were performed within 48 h after surgery. Oocytes were injected with 20 ng of \(\beta 2-\alpha 4\‌beta 2-\alpha 4\) concatamer cRNA and free single subunit at a 1:1 ratio except with \(\alpha 6\) and \(\beta 2\) subunits. To express \((\alpha 4\beta 2)_{6}\alpha 6\) and \((\alpha 4\beta 2)_{6}\beta 2\) in oocytes with high enough currents, 40 ng of total cRNA was injected in each oocyte at a 1:1 ratio of concatamer \(\beta 2-\alpha 4\‌beta 2-\alpha 4\) to \(\alpha 6\) subunit, and 30 ng of total cRNA was injected at a 2:1 ratio of concatamer \(\beta 2-\alpha 4\‌beta 2-\alpha 4\) to \(\beta 2\) subunit. Dimeric concatamer \(\beta 2-\alpha 4\) produced larger currents than tetrameric concatamers when expressed with free subunits. To obtain functional responses above 0.1 \(\mu\)A but below 20 \(\mu\)A, 1.25 ng of dimeric concatamer was co-injected with 1.25 ng of free subunit (\(\alpha 2\), \(\alpha 3\), \(\alpha 4\), and \(\beta 4\)) per oocyte. 10 ng of total cRNAs were injected for the \(\alpha 6\) or \(\beta 2\) subunit when co-expressed with \(\beta 2-\alpha 4\). Function was assayed 3–7 days after injection.

**Electrophysiology**—Currents in oocytes were measured using a manual two-electrode voltage clamp amplifier setup (oocyte clamp OC-725, Warner Instrument, Hamden, CT) or OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) (30, 35). OpusXpress is an integrated system that provides automated impalement, voltage clamp, and drug delivery for up to eight oocytes in parallel (35). Electrodes were filled with 3 M KCl and had resistances of 0.5–10 megaohms for the voltage electrode and 0.5–3 megaohms for the current electrode. Oocytes were voltage-clamped at a holding potential of \(-50\) mV. Data were collected and filtered at 50 Hz. 200 \(\mu\)l of drugs were delivered on top of oocytes for 4 s through the sidewall of the bath to minimize disturbance. Between drug applications, oocytes received a 30-s prewash and 223-s postwash with ND96 solution (96 mm NaCl, 2 mm KCl, 1.8 mm CaCl2, 1 mm MgCl2, 5 mm HEPES, pH 7.6) plus 0.5 \(\mu\)M atropine perfused through the bath at a rate of 3 ml/min unless otherwise noted.

In concentration/response experiments, each oocyte received two initial control applications of ACh (300 \(\mu\)M for concatamer expressed with free \(\alpha\) subunits and 30 \(\mu\)M for concatamer expressed with free \(\beta\) subunits) followed by application of various concentrations of ACh (from low to high). In MTSEA experiments, each oocyte received 2 mM MTSEA for 60 s at the rate of 0.9 ml/min and then was incubated with MTSEA for an additional 5 min while the ND96 wash was stopped to retain the...
reagent in the bath before being washed with buffer solution for 287 s. Oocytes were discarded after experiments involving MTSEA because they had been covalently modified. In NS9283 experiments, 10 μM NS9283 was preapplied to oocytes expressing β2-α4-β2-α4 and free subunits for 30 s at the rate of 1.8 ml/min and incubated for an additional 80 s in a static bath before its co-application with ACh for 4 s at 3 ml/min at EC_{20–40} concentrations (30 μM for α subunits, 1 μM for β2 subunit, and 3 μM for β4 subunit). Preincubation with NS9283 eliminates kinetic effects of NS9283 binding, leaving only the kinetics of ACh responses in nAChRs at equilibrium with NS9283. Control experiments were performed on the same oocytes before NS9283 applications following the same protocol in which 10 μM NS9283 was replaced with 0.1% (v/v) DMSO. Potentiation by NS9283 was calculated by increased response to ACh with 10 μM NS9283 versus response of ACh co-applied with 0.1% DMSO. In the ACh concentration/response curve experiment with NS9283, 10 μM NS9283 was co-applied with different concentrations of ACh without preapplciation to shorten the experiment duration.

The peak amplitudes of experimental responses were calculated relative to the maximum ACh response or the average of the first two control ACh responses to normalize the data and compensate for variable expression levels among oocytes. Mean and S.E. were calculated from normalized responses. The numbers of oocytes tested are listed. The Hill equation was fit to the concentration/response relationship using a nonlinear least squares curve fit method (Kaleidagraph, Abelbeck/Synergy, Reading, PA): I(x) = I_{max} \left[ x^{nH}/(x^{nH} + EC_{50}^{nH}) \right] \] where I(x) is the peak current measured at the agonist concentration x, I_{max} is the maximum current peak at the saturating concentration, EC_{50} is the agonist concentration required to achieve half of the maximum response, and n_{H} is the Hill coefficient.

**Cell Culture and Transfection**—All cells were maintained as described previously (29). The human embryonic kidney tsA201 (HEK) cell lines stably expressing human α4β2 and α3β2 were described previously (8, 29, 36). To establish stable cell lines of α3β4, αβ2β, αβ4, and α4β4, equal amounts of plasmid encoding appropriate α and β subunits were transfected into HEK cells using the FuGENE 6 transfection reagent (Roche Diagnostics) at a ratio of 6 μg of DNA/18 μl of FuGENE 6/100-mm dish. A single colony expressing appropriate nAChRs was selected as described previously (36).

**FLEXstation Experiments**—For functional tests of nAChRs expressed in HEK cells, we used a FLEXstation (Molecular Devices) bench top scanning fluorometer as described by Kuryatov et al. (36). To increase the expression level of αβ2β and α3β2 nAChRs, the plates were incubated at 29 °C for 20 h before being tested. The membrane potential kit (Molecular Devices) was used according to the manufacturer’s protocols. Serial dilutions of NS9283 were manually added to the assay plate 15 min prior to recording. ACh dilutions were prepared in V-shaped 96-well plates (Fisher Scientific Co.) and added in cell culture wells at 20 μl/s during recording. Each data point was averaged from three to four responses from separate wells. The potency and efficacy of drugs were calculated from the Hill equation described above.

**Results**

NS9283 Potentiates α2* and α4* but Not α3* nAChRs—As reported by others, NS9283 augments activation of nAChRs containing three α2 or three α4 subunits per nAChR expressed in oocytes or HEK cells but not α3* nAChRs (10, 12). Here we used nAChRs expressed in HEK cells to validate the pharmacological characteristics of the NS9283 that we synthesized (27). NS9283 increased activation by ACh (at EC_{20–30} concentration) of α2* and α4* cell lines by 86–371% (Fig. 1). An α2β4 line was not affected by NS9283. Cell lines may contain mixtures of subtypes, for example (α4β2)α4 and (α2β2)β2 in the α2β2 line and (αβ4)α2 (αβ4)β4 in the αβ4 line. Subtypes containing sites at which NS9283 can bind (e.g. α4/α4 in (α4β2)α4 or α2/α2 in (α2β4)α2) exhibit increased responses with NS9283, but the (αβ4)β4 stoichiometry cannot bind NS9283 (12). We tested potentiation by NS9283 of (α2β4)α2 nAChRs expressed in oocytes (cRNA injection ratio of α2 to β4, 4:1). NS9283 increased activation by 100 μM ACh of α2β4 nAChR by 268% with an EC_{50} of 5.02 ± 1.81 μM and reached maximum potentiation at 30 μM. This suggests that our unresponsive α2β4 cell line expresses mainly the (α2β4)β4 stoichiometry, whereas the responsive α2β4, αβ4β4, and α2β2 nAChR cell lines express the (αβ)α stoichiometry and possibly the (αβ)β stoichiometry. NS9283 did not potentiate activation by ACh on α3β2 or α3β4 cell lines (Fig. 1). NS9283 did not activate any α2*, α3*, or α4* nAChR by itself as reported by others (data not shown) (10, 12).

**FIGURE 1.** Chemical structure and potentiation effect of NS9283 on activation of nAChR subtypes expressed in HEK cells. Various concentrations of NS9283 were preapplied to cell lines for 15 min before acute application of ACh at EC_{20–30} concentration (i.e. α4β2, 0.4 μM; α4β4, 1 μM; α3β2, 4 μM; α3β4, 5 μM; α2β2, 0.4 μM; α2β4, 0.8 μM). A fluorescent indicator was used to record the membrane potential changes. NS9283 potentiates α2β4, α4β4, and α2β2 nAChRs but not α2β4, α3β2, or α3β4 nAChR. Because NS9283 inhibited its own potentiation at higher concentrations, we could not fit the concentra-

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**FIGURE 2.** **NS9283 potentiates activation of α4β2 nAChRs by ACh, sazetidine, and cytisine differently.** 10 μM NS9283 was incubated for 15 min with HEK cells stably expressing α4β2 nAChRs before acute application of various concentrations of sazetidine or cytisine. A fluorescent indicator was used to record the membrane potential changes. A, concentration/response curves of ACh with and without NS9283. Note the two-component curve with ACh, reflecting a high affinity contribution from the α4β2 sites and a low affinity contribution from the α4/α4 site. The efficacy of ACh is 49.9 ± 7.1%, and the EC50 is 0.0026 ± 0.0009 μM for the α4β2 sites. The efficacy for the low sensitivity α4/α4 sites is 49.1 ± 8.5%, and the EC50 is 3.89 ± 1.35 μM. After the α4/α4 site is occupied by preapplied NS9283, sensitivity reflects acutely applied ACh activating at high affinity α4β2 sites. The efficacy of ACh with NS9283 is 102 ± 1%, and the EC50 is 0.0314 ± 0.0024 μM. B, concentration/response curves of sazetidine with and without NS9283. The efficacy of sazetidine alone is 43.5 ± 2.1%, and the EC50 is 0.00262 ± 0.00047 μM. The efficacy of sazetidine with NS9283 is 94.0 ± 2.6%, and the EC50 is 0.00107 ± 0.00012 μM. C, concentration/response curves of cytisine with and without preapplication of 10 μM NS9283. The maximum efficacy of cytisine alone (≥90 μM) is 34.3 ± 2.0%, and the concentration/response curve does not reach plateau even at 90 μM. The efficacy of cytisine with NS9283 is 57.9 ± 1.9%, and the EC50 is 0.0259 ± 0.0044 μM. Responses of 90 μM cytisine with NS9283 plunge to 41.3 ± 3.0% of the maximum responses evoked by ACh. Error bars represent the mean ± S.E.

**NS9283 Increases Activation by Agonists—** Both sazetidine and cytisine are partial agonists for α4β2 expressed in HEK cells (20). Our α4β2 cell line expresses a mixture of (α4β2)2α4 and (α4β2)β2 stoichiometries as illustrated by the biphasic activation curve by ACh alone in Fig. 2A (8). Partial agonism of sazetidine arises from its exclusive and high affinity action at the α4β2 agonist sites; i.e. sazetidine is a primary SSAg (21, 22). Cytisine is an intrinsic partial agonist that non-selectively binds to both the α4β2 and α4/α4 agonist sites (20). ACh is defined as a full agonist for both the α4/α4 site and α4β2 site. We investigated whether NS9283, which is an accessory SSAg, assists activation by ACh, sazetidine, and cytisine. At low concentrations of ACh, NS9283 from the α4/α4 site increased activation of α4β2 nAChRs, but at high concentrations of ACh, NS9283 did not exceed the efficacy of ACh (18). This is because NS9283 cannot compete with ACh to bind to the α4/α4 sites, or if it does, it is functionally indistinguishable from ACh bound to this site.

NS9283 increases activation of nAChRs by partial agonists, but its potentiation profiles differ between the α4β2 primary SSAg sazetidine and the non-selective partial agonist cytisine. NS9283 increased the efficacy of both sazetidine and cytisine (Fig. 2, B and C). Interestingly, NS9283 made sazetidine a full agonist at α4β2 nAChRs without changing its potency. This is most likely because NS9283 activates the α4/α4 site (the critical aspect of potentiation), a site that is not bound by sazetidine. NS9283 (10 μM) also potentiated activation of α4β2 nAChRs by cytisine up to 10 μM but not at higher concentrations of cytisine (Fig. 2C). Because NS9283 is an agonist at the α4/α4 site and cytisine is a partial agonist at both α4β2 and α4/α4 sites, NS9283 likely augments activation by cytisine at α4β2 sites, whereas increasing concentrations of cytisine compete with NS9283 for binding to the α4/α4 site, thereby preventing the full agonist effect of NS9283 at this site. Channel block by cytisine could also contribute to reduced responses at high concentrations of cytisine.
In summary, NS9283, which binds only to the α4/α4 site, increases efficacies of SSAGs that bind only to the two α4/β2 sites and the potencies of agonists that bind to all three sites. Its effect is lost if it is displaced from the α4/α4 site by the partial agonist cytisine but not by the full agonist ACh; i.e. NS9283 behaves like a full agonist for the α4/α4 site.

**Activation and Potentiation of (α4β2)\textsubscript{2} nAChRs with Different Accessory Subunits**—Residues on the minus side of the α4 subunit determine agonist sensitivity and selectivity of the α4/α4 agonist site (2, 3, 19). To investigate the pharmacological requirement for the plus side of this interface, we expressed (α4β2)\textsubscript{2} nAChRs with various accessory subunits by injecting oocytes with mRNAs of β2-α4-β2-α4 concatamers and a free accessory subunit (Fig. 3A). The concatamer alone gives minimal 300 μM ACh-evoked current, less than 40 nA on average (n = 17) (Fig. 3B). Concatamer expressed with a free subunit produces maximum ACh-evoked responses ranging from 673 nA to 3.17 μA (Fig. 3B). Higher total current and responses dominated by the low affinity agonist site at the α4/α4 interface characterize (α4β2)\textsubscript{2}α4 nAChRs (2). Like α4, other α structural subunits except α6 also show higher absolute currents than β subunits when assembling into the accessory position (Fig. 3B). Because of expression variation between individual oocytes, the standard errors of absolute currents are large. Because of assembly, trafficking, and functional issues, it is difficult to express α6 in heterologous systems (37). The gain-of-function mutant α6\textsubscript{L250S} can, however, be more readily expressed with the concatamer β2-α4-β2-α4. This produced an average maximum ACh-evoked current of 3.00 ± 0.50 μA, which is as high as the other α subunits tested. These data suggest that an additional ACh site can form at interfaces of an accessory α with the α4 subunits to increase activation. This additional accessory α6/α4 site in (α4β2)\textsubscript{2}α6\textsubscript{L250S} resulted in 64.8 ± 6.4% inhibition of ACh (300 μM) responses by the α6-selective antagonist conotoxin MII (100 nM) (38).

Absolute current differences could be due to different protein expression levels, assembly or maturation efficiencies, and/or channel electrophysiological properties. However, besides absolute current, we observed another characteristic of the agonist site at the accessory α/α4 sites, i.e. low sensitivity to ACh activation (Fig. 3C and Table 1). β2 and β4 subunits produced nAChRs highly sensitive to ACh when expressed with β2-α4-β2-α4 with EC\textsubscript{50} values of 1.02 ± 0.10 μM for (α4β2)\textsubscript{2}β2 and 4.96 ± 1.53 μM for (α4β2)\textsubscript{2}β4 nAChRs. α subunits (α2–4 and α6 wild type and mutant) as accessory subunits display low sensitivities to ACh with EC\textsubscript{50} values higher than 100 μM. Note that (α4β2)\textsubscript{2}α nAChRs have two high affinity agonist sites at α4/β2 interfaces. This usually results in ACh concentration/
response curves fitting better to a biphasic curve with a high and a low EC_{50} value (2, 3). Unfortunately, our data set could not resolve the two EC_{50} values very well for all of the nAChR subtypes; thus all dose/response curves were fit to a monophasic curve for comparison. The Hill coefficient values of (α4β2)_{2}α2, (α4β2)_{2}α4, and (α4β2)_{2}α6 calculated in this way are less than 1 (0.625–0.762), consistent with the presence of agonist sites with different affinities.

NS9283 can activate nAChRs into which three α4/α4 sites have been engineered (16, 17). However, NS9283 did not activate wild type nAChRs when delivered alone (12) (Fig. 4). We confirm that 10 μM NS9283 selectively potentiates wild type nAChRs through the α4/α4 agonist site. 1) It increases activation of (α4β2)_{2}α4 nAChRs (i.e. β2-α4-β2-α4 + α4) by 30 μM ACh (225 ± 43%; Fig. 4, A and B). 2) It fails to potentiate activation by ACh of (α4β2)_{2}β2 nAChRs (i.e. β2-α4-β2-α4 + β2; Fig. 4, A and C). Such potentiation selectivity applies to other α and β subunits. NS9283 increased activation by ACh (30 μM) of (α4β2)_{2}α2, (α4β2)_{2}α3, and (α4β2)_{2}α6 nAChRs by 208–477% but not activation of (α4β2)_{2}β4 nAChRs (Fig. 4A). These data suggest that accessory α/α interfaces form an agonist site for ACh and NS9283, whereas accessory β/α interfaces do not (Fig. 3A).

**Selecting Blockage of Subunit Interfaces—**To enable pharmacological study of accessory sites, we mutated threonine 126 on the minus face of α4 to cysteine to allow selective blockage of the agonist binding site at this interface by the thio-reactive agent MTSEA (39) (Fig. 5A). We also mutated the corresponding amino acid in β2 subunit, leucine 121, to cysteine to check the efficiency of blocking ACh activation at other subunit interfaces using this mutation and MTSEA. We used ACh at con-

### TABLE 1

**Summary of potencies of ACh activation of (α4β2)_{2}* nAChRs** (α4β2)_{2} nAChRs were expressed from β2-α4-β2-α4 concatamer and free subunits in oocytes. n, number of oocytes tested; N.D., not detected.

| Subtypes | EC_{50} (μM) | n | n_{H} | n |
|----------|--------------|---|-------|---|
| β2-α4-β2-α4 | N.D. | N.D. | 17 |
| β2-α4-β2-α4 + α2 | 120 ± 48 | 0.710 ± 0.098 | 4 |
| β2-α4-β2-α4 + α3 | 101 ± 20 | 0.977 ± 0.141 | 6 |
| β2-α4-β2-α4 + α4 | 106 ± 45 | 0.762 ± 0.126 | 10 |
| β2-α4-β2-α4 + α6 | 109 ± 80 | 0.625 ± 0.166 | 5 |
| β2-α4-β2-α4 + α6^{T-256} | 92.7 ± 24.4 | 0.528 ± 0.032 | 2 |
| β2-α4-β2-α4 + β2 | 1.02 ± 0.10 | 0.959 ± 0.114 | 7 |
| β2-α4-β2-α4 + β4 | 4.96 ± 1.53 | 0.801 ± 0.146 | 5 |

**FIGURE 4: Potentiation of activation of (α4β2)_{2}* nAChRs by NS9283.** nAChRs were expressed from β2-α4-β2-α4 concatamers and free accessory subunits in oocytes. 10 μM NS9283 was preapplied to oocytes for 2 min before its co-application with EC_{30–40} concentrations of ACh (i.e. 30 μM for all (α4β2)_{2} nAChRs, 1 μM for (α4β2)_{2}β2 subtype, and 3 μM for (α4β2)_{2}β4 subtype). A, potentiation of NS9283 was evaluated as increased response relative to ACh applied with vehicle 0.1% DMSO (v/v). NS9283 potentiates activation of (α4β2)_{2}α nAChRs, but not (α4β2)_{2}β subtypes, by ACh. B and C, representative response kinetics for (α4β2)_{2}α4 and (α4β2)_{2}β2 nAChRs. Error bars represent the mean ± S.E.
centrations producing maximal activation to evaluate blockage by MTSEA in the following experiments. Because repeated subunit sequences in the tetrameric concatamer would complicate mutagenesis, we expressed mutated dimeric β2-α4 concatamers with free accessory subunits to allow blockage of different subunit interfaces (Fig. 5A). When we engineered such a cysteine mutant at all of the agonist sites such as in (α4β2L121C)2β2 nAChRs, 2 mM MTSEA abolished activation of these nAChRs by ACh (Fig. 5B). Note that, although (α4β2L121C)4 nAChRs retained an intact agonist site at the accessory α4/α4 agonist site after MTSEA treatment, they failed to respond to ACh significantly after MTSEA treatment (Fig. 5B). This suggests that ACh, like NS9283, cannot activate nAChRs solely from the accessory α4/α4 agonist site. This is consistent with the notion that potentiation results from the presence of three rather than two agonist sites (12, 17, 19). We next expressed (α4T126Cβ2)2α4 nAChRs. These nAChRs have intact agonist sites at the α4/β2 interfaces, a blockable site at the α4/α4 interface, and a blockable site at one of the two β2/α4 interfaces (Fig. 5A). MTSEA decreases the activation by 300 μM ACh of these nAChRs by 50 ± 9%. A cysteine mutant at interfaces that do not form an agonist site at the accessory sites after MTSEA treatment, they failed to respond to ACh signals. No MTSEA blockage was observed in wild type nAChRs without MTSEA-reactive sites such as (α4β2)2α4 and (α4β2)2β2 nAChRs (data not shown).

**MTSEA Blocks Activation from Many Accessory α/α4 Sites**—If an agonist site is present at an accessory site, MTSEA should selectively block this interface of (α4T126Cβ2)2α4 nAChRs, leaving only the α4/β2 sites (Fig. 5A), i.e. decreasing the efficacy and leaving only the high affinity responses. To test this idea, we expressed the mutant concatamer β2-α4T126C with free subunits in oocytes and investigated the effect of MTSEA on activation by ACh of these nAChRs. Fig. 6 and Table 2 summarize the effects of MTSEA on the potency and efficacy of ACh of these nAChRs.

Like (α4β2)2α nAChRs (Fig. 3B), mutant (α4T126Cβ2)2α nAChRs produced larger total ACh-evoked currents than (α4T126Cβ2)2β nAChRs presumably because of the low affinity agonist site at the accessory α/α4 interfaces (Fig. 6 and Table 2). EC<sub>50</sub> values of ACh activating (α4T126Cβ2)2α nAChRs range from 21.0 to 37.3 μM, which are lower than the EC<sub>50</sub> values of wild type (α4β2)2α nAChRs obtained from β2-α4-β4-α4 concatamer (Table 1) but much higher than the EC<sub>50</sub> values of...
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FIGURE 6. Effects of MTSEA on sensitivity and efficacy of ACh activating \((\alpha_4\beta_2)_2\alpha\) and \((\alpha_4\beta_2)_2\beta\) nAChRs. Mutant concatamer \(\beta_2\alpha_4^{T126C}\) was expressed with free subunit in oocytes to investigate the effect of MTSEA on ACh activating from different accessory interfaces. In all cases, the cysteine is on the minus side of the \(\alpha_4\) subunit interacting with the plus side of the accessory subunit. \(A\), \((\alpha_4^{T126C}\beta_2)_2\alpha_2\); \(B\), \((\alpha_4^{T126C}\beta_2)_2\alpha_3\); \(C\), \((\alpha_4^{T126C}\beta_2)_2\alpha_4\); \(D\), \((\alpha_4^{T126C}\beta_2)_2\alpha_6\); \(E\), \((\alpha_4^{T126C}\beta_2)_2\beta_2\); \(F\), \((\alpha_4^{T126C}\beta_2)_2\beta_4\). All responses are normalized to the average of the two initial ACh controls (300 \(\mu M\) for \((\alpha_4\beta_2)_2\alpha\) nAChRs and 30 \(\mu M\) for \((\alpha_4\beta_2)_2\beta\) nAChRs) without 2 mM MTSEA treatment. Error bars represent the mean ± S.E.

TABLE 2
Summary of potencies and efficacies of ACh activating \((\alpha_4\beta_2)_2^*\) nAChRs with and without NS9283 before and after MTSEA blockage \((\alpha_4\beta_2)_2^*\) nAChRs were expressed from mutant \(\beta_2\alpha_4^{T126C}\) concatamer and free subunit in oocytes. They have an accessory interface vulnerable to blockage by MTSEA. \(I_{\text{max}}\), maximum current normalized to the average of the two initial ACh controls; current, maximum absolute current evoked by ACh; \(n\), number of oocytes tested.

| Subtype | \(E_{\text{C50}} (\mu M)\) | \(n_{\text{Hill}}\) | \(I_{\text{max}}\) | \(I_{\text{max}}\) (\(\mu A\)) | \(n\) |
|---------|-----------------|--------|-----------------|-----------------|-----|
| \((\alpha_4^{T126C}\beta_2)_2\alpha_2\) | \(2.03±0.10\) | 0.92±0.03 | 0.97±0.04 | 4.2±1.7 | 6 |
| with NS9283 | 0.82±0.15 | 0.91±0.03 | 1.03±0.03 | 3.0±1.3 | 7 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_3\) | 3.34±0.11 | 0.84±0.16 | 1.05±0.09 | 4.5±1.1 | 5 |
| with NS9283 | 3.18±0.18 | 1.00±0.04 | 1.02±0.04 | 3.0±1.3 | 8 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_4\) | 3.72±0.67 | 1.02±0.11 | 0.96±0.02 | 3.0±1.3 | 7 |
| with NS9283 | 0.17±0.03 | 0.98±0.16 | 1.17±0.04 | 5.0±1.6 | 5 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_6\) | 2.67±0.39 | 0.76±0.07 | 1.26±0.04 | 3.8±1.6 | 6 |
| with NS9283 | 2.10±0.38 | 0.81±0.09 | 0.97±0.03 | 7.0±1.3 | 7 |
| \((\alpha_4^{T126C}\beta_2)_2\beta_2\) | 2.59±0.45 | 1.60±0.38 | 0.74±0.05 | 5.1±1.6 | 7 |
| \((\alpha_4^{T126C}\beta_2)_2\beta_4\) | 2.68±0.52 | 0.87±0.05 | 1.14±0.02 | 1.9±0.35 | 8 |

| After MTSEA | \(E_{\text{C50}} (\mu M)\) | \(n_{\text{Hill}}\) | \(I_{\text{max}}\) | \(I_{\text{max}}\) (\(\mu A\)) | \(n\) |
|-------------|-----------------|--------|-----------------|-----------------|-----|
| \((\alpha_4^{T126C}\beta_2)_2\alpha_2\) | \(4.76±1.18\) | 0.65±0.09 | 0.33±0.03 | 1.0±0.13 | 10 |
| with NS9283 | 0.92±0.09 | 1.01±0.09 | 0.40±0.07 | 7.0±1.3 | 7 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_3\) | 12.3±2.1 | 0.62±0.04 | 0.63±0.01 | 3.0±1.3 | 7 |
| with NS9283 | 17.7±7.3 | 0.55±0.09 | 0.85±0.06 | 6.0±1.6 | 6 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_4\) | 2.58±0.25 | 0.96±0.08 | 0.46±0.07 | 6.0±1.6 | 6 |
| with NS9283 | 1.78±0.45 | 0.69±0.04 | 0.34±0.01 | 4.0±1.3 | 6 |
| \((\alpha_4^{T126C}\beta_2)_2\alpha_6\) | 4.38±1.54 | 0.66±0.13 | 0.36±0.02 | 4.0±1.3 | 4 |
| with NS9283 | 6.90±1.12 | 0.59±0.07 | 0.30±0.01 | 6.0±1.6 | 6 |
| \((\alpha_4^{T126C}\beta_2)_2\beta_2\) | 3.50±0.86 | 0.97±0.17 | 0.84±0.05 | 8.0±1.6 | 8 |
| \((\alpha_4^{T126C}\beta_2)_2\beta_4\) | 1.71±0.23 | 1.06±1.25 | 1.14±0.04 | 4.0±1.3 | 4 |

\((\alpha_4^{T126C}\beta_2)_2\beta_2\) and \((\alpha_4^{T126C}\beta_2)_2\beta_4\) nAChRs at 2.59 ± 0.45 and 2.68 ± 0.25 \(\mu M\), respectively. Therefore, although this T126C mutation affects activation by ACh of \((\alpha_4\beta_2)_2\alpha\), it has retained the additional agonist site at the accessory \(\alpha/\alpha_4\) sites.

MTSEA attenuated activation by ACh of \((\alpha_4^{T126C}\beta_2)_2\alpha\) nAChRs (where \(\alpha = \alpha_2, \alpha_3, \) or \(\alpha_6\)) similarly to the attenuated response in \((\alpha_4^{T126C}\beta_2)_2\alpha_4\) nAChRs. MTSEA reduced maximum ACh-evoked responses of these nAChRs by 42–89% (Figs. 6 and Table 2). After MTSEA blockage of this low sensitivity \(\alpha/\alpha_4\) ACh site, only the responses of the high sensitivity sites were observed (Fig. 6, \(A–D\)). The sensitivities to ACh of \((\alpha_4^{T126C}\beta_2)_2\alpha\) nAChRs after MTSEA blockage are similar to those of \((\alpha_4^{T126C}\beta_2)_2\beta\) nAChRs (Table 2), suggesting complete blockage of the accessory agonist site in these nAChRs. The \(EC_{50}\) value of ACh activating \((\alpha_4^{T126C}\beta_2)_2\alpha_3\) nAChRs after MTSEA blockage is 12.3 ± 2.1 \(\mu M\), which is 2.7-fold smaller than that before MTSEA blockage but larger than those of \((\alpha_4^{T126C}\beta_2)_2\beta\) nAChRs. The increase in sensitivity and decrease in efficacy by MTSEA blockage imply that an additional agonist site is present at the accessory \(\alpha_3/\alpha_4\) site (Fig. 6B). ACh binding and/or activation of \((\alpha_4^{T126C}\beta_2)_2\alpha_3\) nAChRs is slightly different from that of the other \((\alpha_4\beta_2)_2\alpha\) nAChRs. Perhaps MTSEA cannot fully block the activation by ACh at this \(\alpha_3/\alpha_4\) interface, or residual low sensitivity activation might partially obscure the responses from high sensitivity sites.

In contrast to the above effects, MTSEA did not affect ACh-induced activation of \((\alpha_4^{T126C}\beta_2)_2\beta\) nAChRs where \(\beta = \beta_2\) or \(\beta_4\). MTSEA did not change the maximum ACh-evoked responses of \((\alpha_4^{T126C}\beta_2)_2\beta\) nAChRs (Fig. 5). The \(EC_{50}\) values
before and after MTSEA treatment are similar for (α4T126Cβ2)2β2 and (α4T126Cβ2)2β4 nAChRs (Fig. 6 and Table 2). These data suggest that there are no ACh sites present at accessory β/α4 interfaces. NS9283 selectively binds to the accessory α/α4 agonist site, acting as a third agonist to increase the probability of channel opening (16, 17) (Fig. 4). We investigated whether MTSEA blockage at α4/α4 and other accessory α/α4 agonist sites affects potentiation of (α4β2)2α nAChRs by NS9283. 10 μM NS9283 increases sensitivity to ACh of (α4T126Cβ2)2α4 nAChRs and shifts the EC50 of ACh from 37.2 ± 4.67 to 1.78 ± 0.45 μM (Fig. 7A). After MTSEA blockage, EC50 values of ACh are similar with or without NS9283 at 1.78 ± 0.45 and 2.58 ± 0.26 μM, respectively (Fig. 7B). Similarly, MTSEA blockaded potentiation by NS9283 on other (α4T126Cβ2)2α nAChRs (Fig. 8 and Table 2). MTSEA completely abolished the increase of ACh sensitivity on (α4T126Cβ2)2α3 and (α4T126Cβ2)2α6 nAChRs by NS9283 and reduced the increase of ACh potency by NS9283 on (α4T126Cβ2)2α2 nAChRs from 26- to 5.1-fold. These data suggest that NS9283 augments nAChR activation through the accessory α/α4 agonist sites as illustrated in Fig. 5A; i.e. it is an accessory SSAg for many (α4β2)2α nAChRs.

**Discussion**

There is interest in developing PAM drugs for nAChRs because agonists both activate and desensitize nAChRs (24, 40). PAMs bind away from agonist binding sites to enhance function when agonists are bound. Benzodiazepines are PAMs for γ-aminobutyric acid (GABA) receptors and have proven clinically useful in modulating their function (41). There is optimism that the same principle can be applied to nAChRs. PAMs offer the potential to modulate endogenous patterns of signaling rather than constantly activating or desensitizing as results from sustained exposure to agonist drugs. Here we have defined another group of drugs, accessory SSAgs, such as NS9283, that functionally behave like a PAM but adopt an agonist-like mechanism to potentiate nAChR activation.

NS9283 was understandably designated as a PAM initially because it appeared not to have intrinsic activity at nAChRs or to compete with agonist for binding to nAChRs (9, 10, 12). We confirm that NS9283 does not act as a PAM but instead potentiates activation of nAChRs by binding to a low affinity ACh binding site at the α4/α4 subunit interface (17, 19) (Figs. 4, 5A, and 7). The low affinity of this site and the site selectivity of NS9283 explain why this compound demonstrated PAM-like activity during its initial characterization (12, 17) (Figs. 1 and 3). Demonstration of agonist activation by NS9283 (16, 17) and identification of selectivity for a particular ACh site (17, 19) (Figs. 3 and 7) disqualify NS9283 as a PAM because it is neither a modulator nor allosteric.

We define NS9283 and other agonists selective for this accessory ACh binding site as accessory SSAgs. NS9283 binds to the extracellular domain of nAChRs (17, 19) differently from PAMs...
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and allosteric agonists like PNU-120596 and 4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide, which bind to transmembrane sites near the channel gate whose opening they influence (24–26). Both NS9283 and benzodiazepines bind to the extracellular domain of accessory subunits. However, the accessory SSaG NS9283 binds to the other side of the accessory subunit relative to the benzodiazepine PAM site in GABA<sub>A</sub> receptors, and the benzodiazepine PAM site is not a GABA binding site (42). Through binding to the α4/α4 agonist site, NS9283 cannot activate wild type nAChRs by itself because single agonist site occupancy is not sufficient to efficiently activate nAChRs (6). Neither can ACh activate from binding only to the accessory α4/α4 agonist site (Fig. 5C). Hence, the accessory agonist site appears to serve as a novel potentiation site that binds agonists and potentiates activity in response to ACh binding at the other agonist sites. Binding only to the accessory site is not sufficient to induce desensitization nor can it prevent or reverse desensitization resulting from agonist binding to α4β2 sites (18). However, NS9283 increases sensitivity of ACh desensitization because it occupies the α4/α4 site as an agonist (18). Binding only to the accessory site cannot up-regulate nAChRs. Agonists and antagonists at α4/β2 sites are thought to increase the amount of nAChRs by promoting a conformation of (α4β2)<sub>2</sub> that assembles more efficiently with accessory subunits to form mature nAChRs (36). A binding site for NS9283 does not exist in the (α4β2)<sub>2</sub> assembly intermediate. Therefore, NS9283 did not up-regulate α4β2 nAChRs expressed in HEK cells (data not shown). The potentiation mechanism of accessory SSaGs like NS9283 provides a target mechanism for development of novel therapeutics.

Developing a new Food and Drug Administration-approved drug can take more than 7 years on average to pass through clinical trials, not to mention their high cost, low success rate, and high preclinical expenses (43). Drugs like NS9283 could be cost-effective because they offer unique properties when combined with already approved agonists and partial agonists. Indeed, by enhancing agonist activation and desensitization affinities, such drugs have the potential to reduce side effects of high doses of nAChR agonists such as nausea and motor or cardiovascular impairment, which result from nonspecific interaction with other receptors (9–11). This property could also enhance older drugs to treat new diseases or syndromes by enhancing their activity without the need to increase their dose (10). Our in vitro characterization of NS9283 has provided insights in guiding design of combined therapy for SSaGs and other known nAChR agonists. NS9283 increases agonist site occupancy by binding to accessory agonist sites (17) (Figs. 7 and 8), which results in gain of function of both full and partial agonists (Fig. 2). Because of different binding site selectivity, NS9283 binds to the accessory α4/α4 site independently from binding of sazetidine to α4/β2 sites, thus turning this primary SSaG into a full agonist without altering its potency. Conversely, ACh and the partial agonist cytisine bind to both the α4/β2 and α4/α4 agonist sites (20). NS9283 primes the α4/α4 agonist sites for these non-selective agonists binding to the α4/β2 sites but competes with them binding to the α4/α4 sites, thus increasing their potencies but retaining partial agonism of cytisine and maximum efficacy of ACh.

α4β2<sup>n</sup> nAChRs are the most abundant nAChR subtypes in the mammalian brain, accounting for 90% of high affinity nAChRs (44). They assemble with additional subunits <i>in vivo</i> and form more complex α4β2<sup>n</sup> subtypes, including but not limited to α4β2α2<sup>a</sup>, α4β2α3<sup>a</sup>, α4β2α6<sup>a</sup>, and α4β2β4<sup>a</sup> (37, 45, 46). These nAChRs could form one accessory α4/α4 agonist site and two identical or different α/β agonist sites, e.g. (α4β2)(α2β2)α4 and (α4β2)(α4β2)α2. Like α4, other accessory α subunits (α2, α3, and α6) can form binding sites for ACh and NS9283 (Figs. 6, 7, and 8). These α/α sites have low affinity for ACh (Fig. 3 and Table 1) but contribute greatly to nAChR activation efficacy (Fig. 6 and Table 2). Accessory agonist sites that promote much greater activation of nAChRs could be good drug targets. NS9283, which targets α/α4 accessory sites, has proven beneficial in improving cognition and reducing pain in rodents (9–15). Both α2<sup>a</sup> and α6<sup>a</sup> are more prevalent in primates than rodents (37, 47); thus drugs specifically targeting accessory α2/α4 and α6/α4 agonist sites could be even more beneficial in humans.

Like ACh, NS9283 binds to the interface between the minus face of α4 subunit and plus face of α2, α3, α4, and α6 (Fig. 4). These α subunits share a C-loop and several aromatic residues that are critical for agonist binding. In the crystal structure of NS9283 and ACh-binding protein (17), these residues from the plus sides interact with the pyridine ring of NS9283 that is partially protonated under physiological pH and resembles the charged nitrogen of an agonist. Therefore, when MTSEA prevents agonist binding at accessory α/α4 interfaces via electric repulsion (39), it can also disrupt potentiation of NS9283 binding to these agonist sites (Figs. 7 and 8).

Here we showed the relationship between activation of the two kinds of ACh binding sites. 1) The α4/β2 sites are necessary for activation by accessory α/α4 sites. 2) The accessory α/α4 sites promote activation of nAChRs from α4/β2 agonist sites. ACh cannot activate (α4<sup>121C</sup>β2)α4 nAChRs after MTSEA blocks their α4/β2 agonist sites (Fig. 5). This is consistent with the observation that occupying one agonist site does not efficiently activate nAChRs (6). It is possible that MTSEA induces an inactive conformation of nAChRs after reacting with (α4<sup>121C</sup>β2)<sup>b</sup>, preventing further activation of nAChRs. However, other ligands selective for the α4/α4 sites, such as NS9283, cannot activate wild type nAChRs (Fig. 4), which also supports the low occupancy theory. Therefore, accessory SSaGs like NS9283 that bind only to one site are unlikely to activate nAChRs by themselves and will behave pharmacologically like a PAM <i>in vivo</i>. Maximum activation by ACh is about equal to NS9283 with other agonists and much better than cytisine with or without NS9283 because cytisine is a very low efficacy partial agonist at both α4/α4 and α4/β2 agonist sites (Fig. 2). Perhaps that is because the bridged bicyclic ring of cytisine precludes the C-loop from closing properly to fully activate nAChRs (48). A partial agonist structure like cytisine should be avoided in designing accessory SSaGs with high efficacies.

In conclusion, we confirm and extend the mechanism proposed by others (2, 3, 16, 17) that there is a binding site for ACh and NS9283 at many α/α4 subunit interfaces that promotes...
activation by providing a third agonist site in addition to ACh binding sites at the α4/β2 subunit interfaces in (α4β2)α4 nAChRs. Low affinity accessory ACh binding sites may not encounter sufficient ACh concentrations to activate them during volume transmission in the brain. Postsynaptic brain nAChRs like those in muscle may transiently be exposed to encounter sufficient ACh concentrations to activate them during postsynaptic brain nAChRs like those in muscle may transiently be exposed to

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