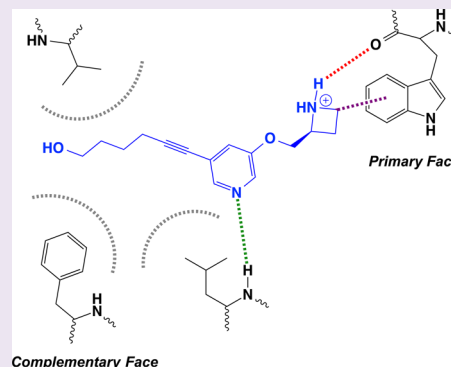


Selective Ligand Behaviors Provide New Insights into Agonist Activation of Nicotinic Acetylcholine Receptors

Christopher B. Marotta, Iva Rreza, Henry A. Lester, and Dennis A. Dougherty*

S Supporting Information

ABSTRACT: Nicotinic acetylcholine receptors are a diverse set of ion channels that are essential to everyday brain function. Contemporary research studies selective activation of individual subtypes of receptors, with the hope of increasing our understanding of behavioral responses and neurodegenerative diseases. Here, we aim to expand current binding models to help explain the specificity seen among three activators of $\alpha 4\beta 2$ receptors: sazetidine-A, cytisine, and NS9283. Through mutational analysis, we can interchange the activation profiles of the stoichiometry-selective compounds sazetidine-A and cytisine. In addition, mutations render NS9283—currently identified as a positive allosteric modulator—into an agonist. These results lead to two conclusions: (1) occupation at each primary face of an α subunit is needed to activate the channel and (2) the complementary face of the adjacent subunit dictates the binding ability of the agonist.



Nicotinic acetylcholine receptors (nAChRs) are a diverse family of the larger Cys-loop superfamily of ligand gated ion channels (LIGCs). These channels are found throughout the brain and CNS and play vital roles in the chemical and electrical communication between neurons, contributing to memory, learning, and other neural functions.^{1–3} Because of their diverse properties and widespread distribution throughout the brain, these LIGCs are prominent targets in neurological disorders, such as drug addiction, Alzheimer's disease, and Parkinson's disease.^{4,5}

Neuronal nAChRs have been extensively studied and much is known about their assembly and structure.^{2,6–8} There are two classes of subunits, identified as $\alpha 2$ – $\alpha 9$ and $\beta 2$ – $\beta 4$, which assemble into homomeric (α only) or heteromeric (α and β) channels consisting of five subunits in total.⁹ For each subunit, there is a main agonist binding pocket, denoted as the primary (+) face, which includes four of the five residues that make up the canonical "aromatic box".¹⁰ The fifth aromatic box residue and other key binding residues, including backbone contacts, are found on the complementary (–) face of the adjacent subunit.¹⁰ The five subunits then arrange in an alternating + and – fashion to form a functional receptor (Figure 1A). For decades it has been assumed that the (+) face of the agonist binding site is provided by α subunits and the (–) face by β subunits. However, recent evidence establishes the ability of α subunits to also contribute the (–) face.

The core pharmacophore of a cationic nitrogen and a hydrogen bond acceptor for agonists of the nAChR was first introduced in 1970.¹¹ Since then, the binding model has evolved to consist of three specific interactions that include a cation– π interaction on the primary subunit, along with hydrogen bonding interactions on both the primary and complementary subunits.^{12,13} Based on this model, a number of studies have aimed

to explain the efficacies and selectivities of agonists to different nAChRs of varying subunits and stoichiometries.^{12–14}

Here, we studied the activation profiles of $\alpha 4\beta 2$ receptors and their responses to mutations for the following compounds: sazetidine-A, cytisine, and NS9283 (Figure 1C). Our conclusions lead us to propose an expansion of the published structural models.^{10,12,15,16} We establish that (1) the selectivity of drug binding at subunit interfaces is largely controlled by a pocket on the complementary subunit that is hydrophobic in some subunits and hydrophilic in others and (2) an agonist must be bound at all α subunits in a given receptor to favor the activated channel. This expansion aids in our understanding of subunit- and stoichiometry-selective agents and can provide valuable insight for further development and application toward therapeutic strategies.

RESULTS AND DISCUSSION

Hydrogen Bonding: Unnatural Amino Acid Analysis.

Sazetidine-A has a unique activation profile, in that it selectively activates the $(\alpha 4)_2(\beta 2)_3$ stoichiometry over the $(\alpha 4)_3(\beta 2)_2$; these stoichiometries will be abbreviated A2B3 and A3B2, respectively.¹⁷ Unnatural amino acids are useful tools used to parse out specific chemical interactions between ligand and receptor. Previous structure–function studies of cytisine, an agonist that has the opposite activation profile for $\alpha 4\beta 2$ receptors, showed that the active drug–receptor combination (A3B2) favored the hydrogen bond to the TrpB backbone CO ("donor"), while the inactive form favored the hydrogen bond to the backbone NH on the complementary face ("acceptor") (Table 1 and Figure 2A).¹² We proposed that this difference

Received: December 20, 2013

Accepted: February 24, 2014

Published: February 24, 2014

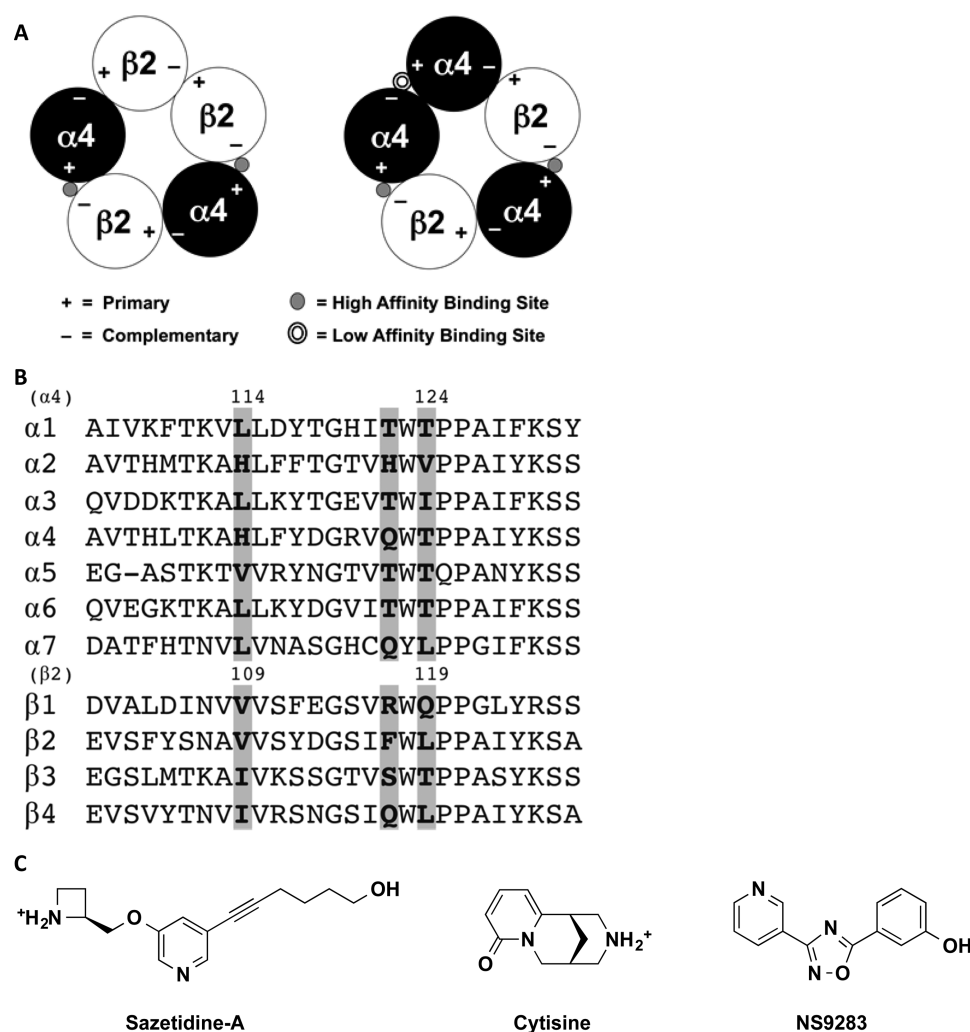


Figure 1. (A) View from the extracellular side of the high affinity (A2B3) and low affinity (A3B2) $\alpha 4\beta 2$ receptors. Agonist binding locations are indicated by smaller circles at the interfaces of $\alpha 4$ – $\beta 2$ subunits and $\alpha 4$ – $\alpha 4$ subunits. (B) Sequence alignment of the rat muscle and neuronal nAChR subunits. The three residues that greatly influence agonist affinity are highlighted in gray. (C) Structures of sazetidine-A, cytisine, and NS9283.

Table 1. Agonist Binding Model Comparison

Stoichiometry	Compound	Wild Type EC ₅₀ (μM) ^a	Relative Efficacy (%) ^b	TrpB Cation-π ^c	H-Bond Donor ^d	H-Bond Acceptor ^e
	Acetylcholine ^f	4.0	[100]	69	1.1	6.8
	Cytisine ^f	0.066	0	31	8.8	62
	Sazetidine-A	0.0046	80	22	5.7	10
	Acetylcholine ^f	87	[100]	540	1.1	8.5
	Cytisine ^f	15	7	30	27	14
	Sazetidine-A	0.14	0	58	11	5.0

^aSee Methods for wild type EC₅₀ corrections. ^bRatio of I_{\max} of compound divided by I_{\max} of acetylcholine. ^cRatio of EC₅₀ values for 4,5,6,7-tetrafluoro-Trp and Trp incorporation at $\alpha 4$ W154 (Figure 2A). ^dRatio of EC₅₀ values for Thr- α -hydroxy and Thr incorporation at $\alpha 4$ T155 (Figure 2A). ^eRatio of EC₅₀ values for Leu- α -hydroxy and Leu incorporation at $\beta 2$ L119 (Figure 2A). ^fPreviously reported values from Tavares et al.¹² [†]Measured EC₅₀ values reported in the SI Table 1.

could explain the stoichiometry selectivity of the drug. Through unnatural amino acid incorporation, we were able to characterize the cation- π binding, hydrogen bond-donating, and hydrogen-bond accepting properties of sazetidine-A and compare the results to those previously measured for cytisine. We now find,

however, that the opposite hydrogen bonding pattern is not seen for sazetidine-A and that the pattern roughly follows the one observed for cytisine: a larger affect for the hydrogen-bonding acceptor in the A2B3 stoichiometry and a larger affect for the hydrogen-bonding donor in the A3B2 stoichiometry (Table1

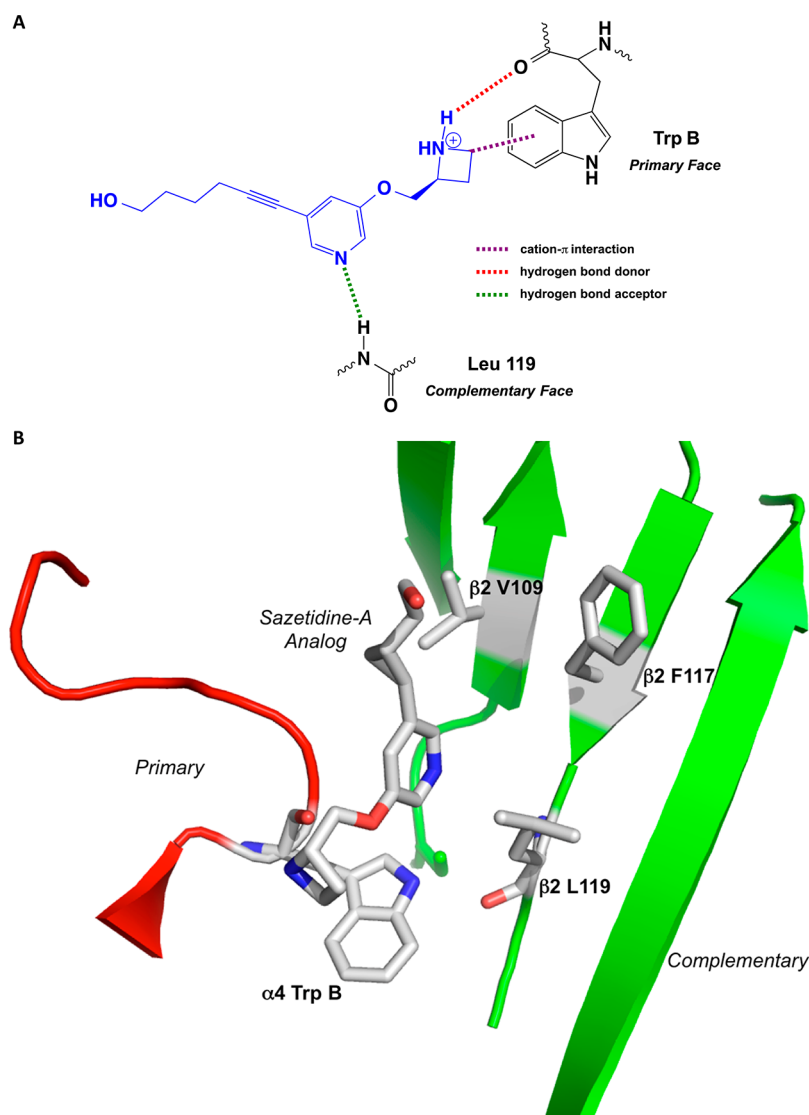


Figure 2. Binding models of sazetidine-A and analogs. (A) Binding model for sazetidine-A based on established interactions seen with nicotine.¹² The cation- π interaction is in purple, the hydrogen bond donor is in red, and the hydrogen bond acceptor is in green. (B) Crystal structure showing a sazetidine-A analog bound to Ct-AChBP (PDB: 4B5D).¹⁵ The three key residues identified for the hydrophobic pocket associated with the $\beta 2$ subunit (V109, F117, and L119) are shown as is the TrpB residue from the $\alpha 4$ subunit. These residues were mutated into the crystal structure to show general spatial locations (no residue minimizations calculated).

and Figure 2A). This pattern suggests an alternative explanation is needed to identify the properties of stoichiometry selective agonists.

Sazetidine-A and the $\beta 2$ Complementary Face. It has been shown that the unique hydrophobic appendage off of the pyridine ring of sazetidine-A gives the compound its subunit and receptor selectivity and that the alcohol group at the end of the appendage does not play a significant role.^{15,18,19} Because this aliphatic adjunct interacts mostly with the complementary side, we began by focusing on the known differences between $\alpha 4$ and $\beta 2$ subunits in this region.¹⁶ Previous investigations identified an $\alpha 4$ - $\alpha 4$ binding site and suggested the differences between the “high” affinity ($\alpha 4$ - $\beta 2$) and “low” affinity ($\alpha 4$ - $\alpha 4$) binding pockets are due to three key residues that reside on the complementary face.^{20–22} The $\beta 2$ (-) face residues (V109, F117, and L119) generate a hydrophobic pocket for the high affinity case, while the aligning $\alpha 4$ (-) face residues (H114, Q122, and T124) create a hydrophilic, low affinity pocket (Figure 1B and Figure 2B). We have evaluated the triple mutant of the $\alpha 4$ (-)

face by swapping these three residues (H114V, Q122F, and T124L) to make them resemble the $\beta 2$ (-) face. We were able to generate receptor responses and measure an EC_{50} curve for sazetidine-A in the A3B2 receptor, which was not possible with the wild type $\alpha 4$ subunit (Figure 3) (Table 2). The EC_{50} value for the triple mutant was about 5-fold larger than the wild type A2B3 response, a small difference compared to having zero response in the wild type A3B2 receptor. Single mutations at the $\alpha 4$ subunit did not give rise to sazetidine-A response at low μM doses (SI Table 2). Combinations of double mutations saw some response to low μM doses of sazetidine-A (SI Table 2). Mutations to make the β subunit more like the α subunit resulted in a large loss of function for sazetidine-A (SI Table 3).

Cytisine and the $\beta 2$ Complementary Face. Since these three residues had a large affect on receptor agonist selectivity and activation for sazetidine-A, we considered cytisine in an attempt to explain its selectivity for A3B2 over A2B3 receptors. Early chimera analysis showed that cytisine selectivity for human $\beta 4$ over $\beta 2$ subunits is strongly influenced by the extracellular

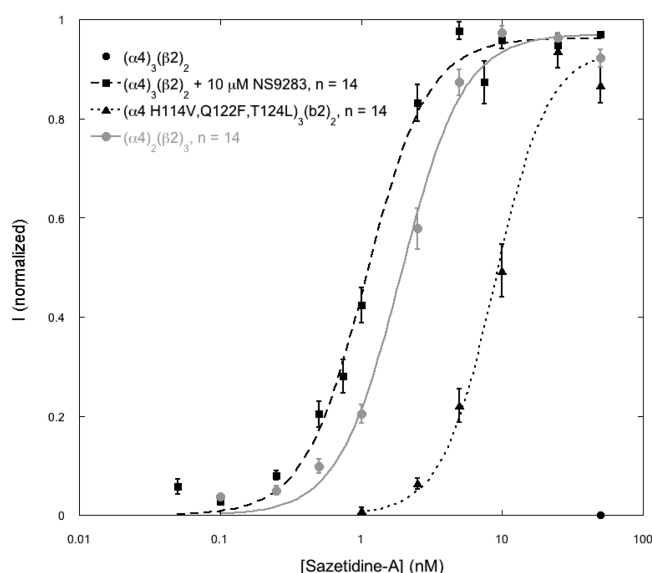


Figure 3. Sazetidine-A EC_{50} curves. $(\alpha 4)_3(\beta 2)_2$ cannot be activated by sazetidine-A. Responses can be obtained by mutating the complementary side of the $\alpha 4$ subunit to resemble the $\beta 2$ subunit as seen with $(\alpha 4 \text{ H114V, Q122F, T124L})_3(\beta 2)_2$ receptor. Also shown is wild type $(\alpha 4)_3(\beta 2)_2$ receptor exposed to a combination of sazetidine-A and NS9283.

Table 2. Sazetidine-A EC_{50} (nM) Values

receptor	EC_{50} (nM)	Hill	<i>n</i>	I_{max} (μ A)
$(\alpha 4)_2(\beta 2)_3$	1.9 ± 0.1	2.0 ± 0.2	14	$0.059\text{--}0.26$
$(\alpha 4)_3(\beta 2)_2$	NR			
$(\alpha 4)_2(\beta 2)_3 + 10 \mu\text{M}$ NS9283	2.0 ± 0.2	1.7 ± 0.2	13	$0.067\text{--}1.6$
$(\alpha 4)_3(\beta 2)_2 + 10 \mu\text{M}$ NS9283	1.1 ± 0.1	2.0 ± 0.2	14	$0.4\text{--}7.2$
$(\alpha 4 \text{ H114V, Q122F, T124L})_2(\beta 2)_3$	4.8 ± 0.3	1.8 ± 0.2	13	$0.18\text{--}1.3$
$(\alpha 4 \text{ H114V, Q122F, T124L})_3(\beta 2)_2$	9 ± 1	2.3 ± 0.6	14	$0.71\text{--}6.2$

Agonist = Sazetidine-A. NR = No Response.

region,²³ and more recent analyses provide further details.²⁴ Sequence alignment shows that of the three residues considered here, the only difference lies in the 117 position – $\beta 2$ F117 and $\beta 4$ L117 for the human subunits. For the rodent subunits considered here, $\beta 4$ is Q117, which is identical to the $\alpha 4$ residue at this same position (Figure 1). In the rodent wild type $\alpha 4\beta 2$ receptors, cytosine is a partial agonist with a biphasic response for the A3B2 receptor. No response is observed for the A2B3 receptor (Table 3), although signal can be obtained in receptors with hypersensitive mutations (see Methods). However, with the single mutation of F117Q in the $\beta 2$ subunit, cytosine generated a sizable response for the A2B3 receptor. The mutation also raised

the efficacy of the A3B2 receptor compared to the wild type response (Table 3).

NS9283 and the $\alpha 4$ Complementary Face. We next considered NS9283, which has a binding preference for the $\alpha 4\text{--}\alpha 4$ interface.²⁵ This compound has been previously characterized as a benzodiazepine-like positive allosteric modulator (PAM) for only the A3B2 stoichiometry of receptors containing either $\alpha 2$ or $\alpha 4$ subunits.^{25,26} In addition, its effects are lost when the $\alpha 4(-)$ face is mutated to resemble the $\beta 2(-)$ face in the region of the classical agonist binding site.²⁷ Since we have molecules that selectively associate with $\alpha 4\text{--}\alpha 4$ (NS9283) and $\alpha 4\text{--}\beta 2$ (sazetidine-A) interfaces, coapplication should generate an A3B2 receptor response. As shown in Figures 3 and 4 and Table 2, we find that individual applications of NS9283 and sazetidine-A show essentially no activation of wild type A3B2 receptors. However, coapplication generates full activation of the receptor, compared to acetylcholine. The A3B2 $\alpha 4$ triple mutant (H114V, Q122F, T124L) was then exposed to similar conditions. The sazetidine-A response for the mutant was preserved, but the effect of NS9283 was completely lost (Figure 4) (Table 2). The mutations eliminate the ability of NS9283 to bind at the $\alpha 4\text{--}\alpha 4$ interface and allow sazetidine-A to replace it in binding. These data suggest that occupation of an agonist at each α subunit is necessary for a receptor response. We generated the A3B2 $\beta 2$ triple mutant (V109H, F117Q, L119T) to test if NS9283 could alone activate the channel. A response was seen in a dose dependent manner, suggesting the drug is a partial agonist, albeit, not potent or highly efficacious (Figure 4). Because NS9283 is sparingly soluble, a full EC_{50} curve could not be obtained. Nevertheless, we were able to transform a compound once designated as a PAM into an agonist, suggesting it could be binding to the canonical aromatic binding site. Also, due to its low potency and lower receptor expression, the corresponding A2B3 $\beta 2$ triple mutant (V109H, F117Q, L119T) was inconclusive with regard to activation via NS9283.

CONCLUSIONS

The present work confirms and expands upon recent studies on the $(-)$, complementary face of the agonist binding site of nAChRs. In particular, the long-held belief that agonist binding sites are formed only at $\alpha(+)/\beta(-)$ interfaces has been challenged by increasing evidence for a viable agonist binding site at $\alpha(+)/\alpha(-)$ interfaces. Here we use several drugs that show some subtype specificity to probe this issue. The novel agonist sazetidine-A can only activate $\alpha 4\beta 2$ receptors with an A2B3 stoichiometry. In the alternative A3B2 stoichiometry, an $\alpha(+)/\alpha(-)$ interface exists. The $(-)$ face of such an interface is relatively polar, and evidently it is incompatible with the hydrophobic side chain of sazetidine-A that is expected to project into this region (recall that the OH group of sazetidine-A is not necessary for function). By mutating three residues in this region to be more hydrophobic and thus more like a $\beta 2(-)$ face,

Table 3. Cytisine-A EC_{50} (μ M) Values

receptor	EC_{50} (μ M) [1]	Hill [1]	EC_{50} (μ M) [2]	Hill [2]	% [1]	<i>n</i>	Cyt I_{max} (μ A)	ACh I_{max} (μ A)	efficacy
$(\alpha 4)_2(\beta 2)_3$	NR							$0.15\text{--}2.4$	
$(\alpha 4)_3(\beta 2)_2$	0.047 ± 0.005	1.8 ± 0.3	6.0 ± 0.3	1.3 ± 0.1	22	16	$0.052\text{--}2.9$	$0.94\text{--}33$	$7 \pm 0.1\%$
$(\alpha 4)_2(\beta 2 \text{ F117Q})_3$	0.019 ± 0.001	1.6 ± 0.1				11	$0.026\text{--}0.10$	$0.084\text{--}2.5$	$22 \pm 0.1\%$
$(\alpha 4)_3(\beta 2 \text{ F117Q})_2$	0.03 ± 0.01	1.0 ± 0.3	3.3 ± 0.7	1.1 ± 0.2	33	17	$0.055\text{--}4.0$	$0.51\text{--}20$	$16 \pm 0.3\%$

Agonist = Cytisine. NR = No Response.

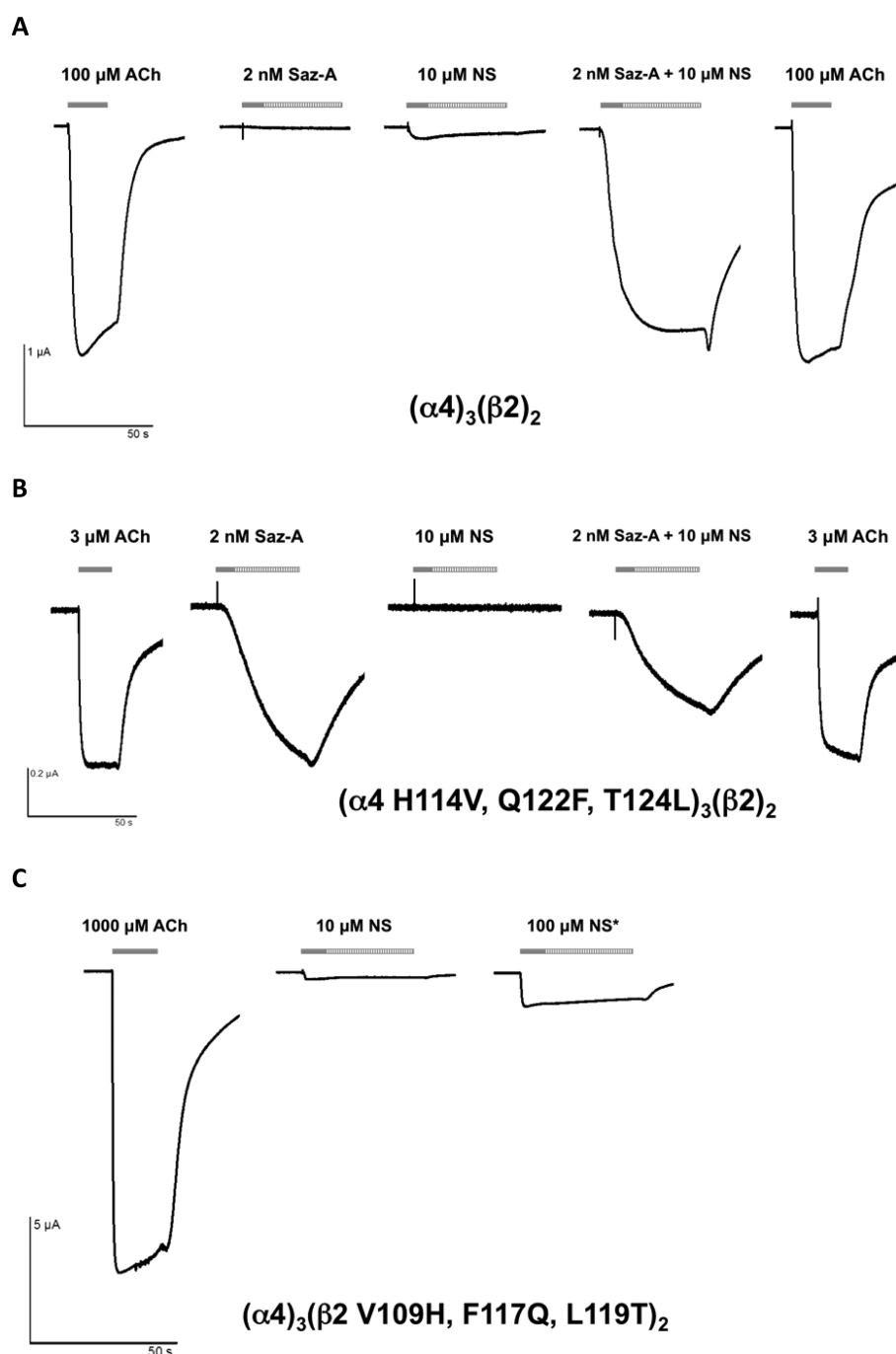


Figure 4. Sample traces of responses to acetylcholine (ACh), sazetidine-A (Saz-A), and NS9283 (NS) to A3B2 receptors. Solid gray bars indicate drug application and dashed bars indicate a pause where drug remains present but the buffer wash has not started. Gaps between traces indicate buffer washes (see Methods for duration of drug application and buffer washes). (A) Activation of wild type receptor by ACh at its EC₅₀ value and Saz-A and NS at the concentrations shown. (B) Activation of (α4 H114V, Q122F, T124L)₃(β2)₂. (C) Application of I_{max} concentrations of acetylcholine and two concentrations of NS to (α4)₃(β2 V109H, F117Q, L119T)₂. The * indicates a 1% by volume DMSO drug solution.

we can prepare A3B2 receptors that are quite responsive to sazetidine-A (Table 2).

In a complementary series of experiments, we considered the drug NS9283, which binds only to α(+)/α(−) interfaces. It is unable to activate the receptor on its own, and it is thus an allosteric modulator. We reasoned that a combination of NS9283 and sazetidine-A would activate A3B2 receptors, with the former binding to the α(+)/α(−) interface and the latter to the α(+)/β(−) interfaces. Indeed, a mixture of NS9283 and sazetidine-A is quite potent at A3B2 receptors, while neither compound alone

can activate the receptor. Taking this one step further, by mutating all interfaces so they resemble α(+)/α(−) interfaces, NS9283 becomes an agonist, rather than the allosteric modulator it is for the wild type receptor.

We also applied this interface concept to cytosine, which has the reverse activation profile of sazetidine-A, in that it cannot activate the A2B3 receptor. By mutating one residue of the β2 subunit to that of the α4 subunit, we find that cytosine can activate the A2B3 receptor. In addition, this same mutation increased

efficacy of cytosine for the A3B2 receptor from 7% to 16% (Table 3).

In sum, this work shows the relevance of the $\alpha(+)/\alpha(-)$ interface of nAChRs to achieving full receptor activation. This knowledge could be of great value to efforts to develop selective agonists for specific nAChR subtypes.

METHODS

Molecular Biology. Rat nAChR $\alpha 4$ and $\beta 2$ subunits were in pAMV (unnatural mutagenesis) and pGEMhe (natural mutagenesis) vectors. Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). Circular DNA of $\alpha 4$ and $\beta 2$ in pAMV was linearized with the NotI restriction enzyme and the plasmids in pGEMhe were linearized with the SbfI restriction enzyme. After purification (Qiagen), the T7 mMessage Machine kit (Ambion) was used to *in vitro* transcribe mRNA from linearized DNA templates. QIAGEN's RNeasy RNA purification kit was used to isolate the transcribed mRNA product.

For unnatural amino acid incorporation, the amber (UAG) stop codon was used for all $\alpha 4$ subunit incorporation and the opal (UGA) stop codon was used for the $\beta 2$ subunit incorporation. 74-nucleotide THG73 tRNA (for UAG) and 74-nucleotide TQOpS' tRNA (for UGA) were *in vitro* transcribed using the MEGAscript T7 (Ambion) kit and isolated using Chroma Spin DEPC-H₂O columns (Clontech). Synthesized unnatural amino acids coupled to the dinucleotide dCA were enzymatically ligated to the appropriate 74-nucleotide tRNA as previously described.^{13,28}

Oocyte Preparation and Injection. *Xenopus laevis* stage V and VI oocytes were harvested via standard protocols.²⁸ For unnatural amino acid incorporation to the α -subunit, the $\alpha 4$ and $\beta 2$ mRNAs were mixed in a 3:1 ratio by mass to obtain the A2B3 receptor, and in a 100:1 ratio to obtain the A3B2 receptor. Unnatural amino acid incorporation to the β -subunit used $\alpha 4$ and $\beta 2$ mRNA ratios of 1:20 and 10:1 to obtain the A2B3 and A3B2 receptors, respectively. mRNA mixtures and deprotected (photolysis) tRNA were mixed in a 1:1 volume ratio, and 50 nL were injected into each oocyte. After injection, the oocytes were incubated at 18 °C in ND96+ medium for 24 h. For the unnatural amino acids with reduced cation- π binding ability, a second round of injections following the same procedure was performed followed by incubation for an additional 24 h. The reliability of the unnatural amino acid incorporation was confirmed through read-through/reaminoacylation tests as previously performed.¹²

For the natural mutagenesis experiments, the $\alpha 4$ and $\beta 2$ mRNAs were mixed in 1:2 or 10:1 ratios by mass to obtain the A2B3 and A3B2 receptors, respectively.²⁹ A total of 50 nL were injected to each oocyte, delivering a mRNA mass total of 25 ng. Oocytes were incubated at 18 °C in ND96+ medium for 24–72 h.

Chemical Preparation. Acetylcholine chloride was purchased from Sigma-Aldrich and dissolved to 1 M stock solutions in ND96 Ca²⁺ free buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES at pH 7.5). Sazetidine-A dihydrochloride and (–)-cytosine were purchased from Tocris Bioscience and dissolved to 10 mM stock solutions in ND96 Ca²⁺ free buffer.

NS9283 was synthesized following a patented protocol.³⁰ 3-Pyridylamidoxime and 3-cyanobenzoyl chloride were purchased from Sigma-Aldrich. 3-Pyridylamidoxime (0.5 g) was dissolved in 5.4 mL of pyridine. Then, 0.6 g of 3-cyanobenzoyl chloride was added while stirring. The mixture was heated at reflux for 90 min and then cooled to RT. 200 mL of water was added and the white powder was filtered with two subsequent washes with water. The resulting powder was lyophilized overnight to remove the excess water. The reaction resulted in 60% yield, and the product was pure by LC-MS and NMR. ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.26 (dd, *J* = 2.2, 0.9 Hz, 1H), 8.82 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.64 (td, *J* = 1.7, 0.7 Hz, 1H), 8.50 (ddd, *J* = 8.0, 1.9, 1.1 Hz, 1H), 8.47 (dt, *J* = 6.0, 2.1 Hz, 1H), 8.22 (dt, *J* = 7.9, 1.4 Hz, 1H), 7.88 (td, *J* = 7.9, 0.7 Hz, 1H), 7.65 (ddd, *J* = 7.9, 4.8, 0.9 Hz, 1H); MS (+ESI-AP) *m/z* 249 (M+H)⁺.

The purified compound, NS9283, was then dissolved to a 10 mM stock solution in DMSO. All drug solutions containing NS9283 were

0.1% DMSO (v/v) with the exception of the 100 μ M dose, which had 1% DMSO (v/v). Appropriate controls of 1% DMSO (v/v) in ND96 Ca²⁺ free buffer only were applied to expressing cells to show no receptor response to the higher DMSO concentration.

Electrophysiology. The OpusXpress 6000A (Axon Instruments) in two-electrode voltage clamp mode was used for all electrophysiological recordings. The holding potential was set to –60 mV, and the running buffer used was ND96 Ca²⁺ free solution for all experiments. All acetylcholine drug applications used 1 mL of drug solution applied over 15 s followed by a 2.5 min buffer wash at a rate of 3 mL min^{–1}. All sazetidine-A, cytosine, NS9283, and coapplications used 1 mL of drug solution applied over 8 s with a 30 s pause before a 5 min buffer wash at a rate of 3 mL min^{–1}. Dose–response measurements utilized a series of ~3-fold concentration steps, spanning several orders of magnitude, for a total of 8 to 18 doses. Data were sampled at 50 Hz and then low-passed filtered at 5 Hz. Experiments testing activity of compounds involved two to three acetylcholine doses of either EC₅₀ or *I*_{max} values, followed by the test doses of compounds being probed, followed by one to two doses of the previous acetylcholine concentrations.

Averaged and normalized data were fit to one or two Hill terms to generate EC₅₀ and Hill coefficient (nH) values. All currents for the activity testing were normalized to the highest acetylcholine dose applied precompound testing. The efficacy of compounds was measured as the ratio of the *I*_{max} of the compound divided by the *I*_{max} of acetylcholine. All acetylcholine EC₅₀ values for the conventional mutations made in pGEMhe are reported in (SI Table 4). Error bars represent standard error of the mean (SEM) values.

Hypersensitive Mutation (L9'A). In the case of unnatural amino acid incorporation and mutagenesis scanning, EC₅₀ values were obtained using a hypersensitive mutation in the $\alpha 4$ subunit (L9'A). This mutation serves two purposes in the experimental setup: (1) the gain of function mutation gives a larger concentration window to probe effects of introduced mutations and (2) the pore mutation causes differences in rectification between the two stoichiometries, which can be probed via voltage jump experiments to confirm which stoichiometry is being observed.^{13,31} Since the EC₅₀ is shifted from true wild type, a correction factor was applied according to the procedure of Moroni et al. to obtain the wild type EC₅₀ value.³²

ASSOCIATED CONTENT

Supporting Information

Additional tables as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Tel.: 626-395-6089. Fax: 626-564-9297. E-mail: dadougherty@caltech.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the National Institutes of Health (NIH) (NS034407, DA017279, DA280382), NIH/NRSA (GM07616), and the California Tobacco-Related Disease Research Program from the University of California (19XT-0102) for support of this work.

REFERENCES

- (1) Miwa, J. M., Freedman, R., and Lester, H. A. (2011) Neural systems governed by nicotinic acetylcholine receptors: Emerging hypotheses. *Neuron* 70, 20–33.
- (2) Albuquerque, E. X., Pereira, E. F., Alkondon, M., and Rogers, S. W. (2009) Mammalian nicotinic acetylcholine receptors: From structure to function. *Physiol. Rev.* 89, 73–120.
- (3) Dani, J. A., and Bertrand, D. (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* 47, 699–729.

- (4) Jensen, A. A., Frolund, B., Lijefors, T., and Krogsaard-Larsen, P. (2005) Neuronal nicotinic acetylcholine receptors: Structural revelations, target identifications, and therapeutic inspirations. *J. Med. Chem.* 48, 4705–4745.
- (5) Taly, A., Corringer, P. J., Guedin, D., Lestage, P., and Changeux, J. P. (2009) Nicotinic receptors: Allosteric transitions and therapeutic targets in the nervous system. *Nat. Rev. Drug Discov.* 8, 733–750.
- (6) Dougherty, D. A. (2008) Cys-Loop neuroreceptors: Structure to the rescue? *Chem. Rev.* 108, 12.
- (7) Dougherty, D. A. (2008) Physical organic chemistry on the brain. *J. Org. Chem.* 73, 7.
- (8) Unwin, N. (2005) Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. *J. Mol. Biol.* 346, 967–989.
- (9) Gotti, C., Zoli, M., and Clementi, F. (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci.* 27, 482–491.
- (10) Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van der Oost, J., Smit, A. B., and Sixma, T. K. (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* 411, 269–276.
- (11) Beers, W. H., and Reich, E. (1970) Structure and activity of acetylcholine. *Nature* 228, 917.
- (12) Tavares Xda, S., Blum, A. P., Nakamura, D. T., Puskar, N. L., Shanata, J. A., Lester, H. A., and Dougherty, D. A. (2012) Variations in binding among several agonists at two stoichiometries of the neuronal, $\alpha 4 \beta 2$ nicotinic receptor. *J. Am. Chem. Soc.* 134, 11474–11480.
- (13) Xiu, X., Puskar, N. L., Shanata, J. A., Lester, H. A., and Dougherty, D. A. (2009) Nicotine binding to brain receptors requires a strong cation– π interaction. *Nature* 458, 534–537.
- (14) Puskar, N. L., Xiu, X., Lester, H. A., and Dougherty, D. A. (2011) Two neuronal nicotinic acetylcholine receptors, $\alpha 4 \beta 4$ and $\alpha 7$, show differential agonist binding modes. *J. Biol. Chem.* 286, 14618–14627.
- (15) Zhang, H. K., Eaton, J. B., Yu, L. F., Nys, M., Mazzolari, A., van Elk, R., Smit, A. B., Alexandrov, V., Hanania, T., Sabath, E., Fedolak, A., Brunner, D., Lukas, R. J., Vistoli, G., Ulens, C., and Kozikowski, A. P. (2012) Insights into the structural determinants required for high-affinity binding of chiral cyclopropane-containing ligands to $\alpha 4 \beta 2$ -nicotinic acetylcholine receptors: An integrated approach to behaviorally active nicotinic ligands. *J. Med. Chem.* 55, 8028–8037.
- (16) Billen, B., Spurny, R., Brams, M., van Elk, R., Valera-Kummer, S., Yakel, J. L., Voets, T., Bertrand, D., Smit, A. B., and Ulens, C. (2012) Molecular actions of smoking cessation drugs at $\alpha 4 \beta 2$ nicotinic receptors defined in crystal structures of a homologous binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9173–9178.
- (17) Zwart, R., Carbone, A. L., Moroni, M., Bermudez, I., Mogg, A. J., Folly, E. A., Broad, L. M., Williams, A. C., Zhang, D., Ding, C., Heinz, B. A., and Sher, E. (2008) Sazetidine-A is a potent and selective agonist at native and recombinant $\alpha 4 \beta 2$ nicotinic acetylcholine receptors. *Mol. Pharmacol.* 73, 1838–1843.
- (18) Liu, Y., Richardson, J., Tran, T., Al-Muhtasib, N., Xie, T., Yenugonda, V. M., Sexton, H. G., Rezvani, A. H., Levin, E. D., Sahibzada, N., Kellar, K. J., Brown, M. L., Xiao, Y., and Paige, M. (2013) Chemistry and pharmacological studies of 3-alkoxy-2,5-disubstituted-pyridinyl compounds as novel selective $\alpha 4 \beta 2$ nicotinic acetylcholine receptor ligands that reduce alcohol intake in rats. *J. Med. Chem.* 56, 3000–3011.
- (19) Liu, J., Yu, L. F., Eaton, J. B., Caldarone, B., Cavino, K., Ruiz, C., Terry, M., Fedolak, A., Wang, D., Ghavami, A., Lowe, D. A., Brunner, D., Lukas, R. J., and Kozikowski, A. P. (2011) Discovery of isoxazole analogues of sazetidine-A as selective $\alpha 4 \beta 2$ -nicotinic acetylcholine receptor partial agonists for the treatment of depression. *J. Med. Chem.* 54, 7280–7288.
- (20) Eaton, J. B., Lucero, L. M., Stratton, H., Chang, Y., Cooper, J. F., Lindstrom, J. M., Lukas, R. J., and Whiteaker, P. (2013) The unique $\alpha 4(+)/(-)\alpha 4$ agonist binding site in ($\alpha 4$)3(β)2 subtype nicotinic acetylcholine receptors permits differential agonist desensitization pharmacology vs the ($\alpha 4$)2(β)3 subtype. *J. Pharmacol. Exp. Ther.*
- (21) Mazzaferro, S., Benallegue, N., Carbone, A., Gasparri, F., Vijayan, R., Biggin, P. C., Moroni, M., and Bermudez, I. (2011) Additional acetylcholine (ACh) binding site at $\alpha 4/\alpha 4$ interface of ($\alpha 4 \beta 2$) $\alpha 4$ nicotinic receptor influences agonist sensitivity. *J. Biol. Chem.* 286, 31043–31054.
- (22) Harpsøe, K., Ahring, P. K., Christensen, J. K., Jensen, M. L., Peters, D., and Balle, T. (2011) Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. *J. Neurosci.* 31, 10759–10766.
- (23) Figl, A., Cohen, B. N., Quick, M. W., Davidson, N., and Lester, H. A. (1992) Regions of $\beta 4 \beta 2$ subunit chimeras that contribute to the agonist selectivity of neuronal nicotinic receptors. *FEBS Lett.* 308, 245–248.
- (24) Harpsøe, K., Hald, H., Timmermann, D. B., Jensen, M. L., Dyhring, T., Nielsen, E. O., Peters, D., Balle, T., Gajhede, M., Kastrup, J. S., and Ahring, P. K. (2013) Molecular determinants of subtype-selective efficacies of cytosine and the novel compound NS3861 at heteromeric nicotinic acetylcholine receptors. *J. Biol. Chem.* 288, 2559–2570.
- (25) Timmermann, D. B., Sandager-Nielsen, K., Dyhring, T., Smith, M., Jacobsen, A. M., Nielsen, E. O., Grunnet, M., Christensen, J. K., Peters, D., Kohlhaas, K., Olsen, G. M., and Ahring, P. K. (2012) Augmentation of cognitive function by NS9283, a stoichiometry-dependent positive allosteric modulator of $\alpha 2$ - and $\alpha 4$ -containing nicotinic acetylcholine receptors. *Br. J. Pharmacol.* 167, 164–182.
- (26) Grupe, M., Jensen, A. A., Ahring, P. K., Christensen, J. K., and Grunnet, M. (2013) Unravelling the mechanism of action of NS9283, a positive allosteric modulator of ($\alpha 4$)3(β)2 nicotinic ACh receptors. *Br. J. Pharmacol.* 168, 2000–2010.
- (27) Olsen, J. A., Kastrup, J. S., Peters, D., Gajhede, M., Balle, T., and Ahring, P. K. (2013) Two distinct allosteric binding sites at $\alpha 4 \beta 2$ nicotinic acetylcholine receptors revealed by NS206 and NS9283 give unique insights to binding-activity associated linkage at Cys-loop receptors. *J. Biol. Chem.*, 35997.
- (28) Nowak, M., Gallivan, J. P., Silverman, S., Labarca, C. G., Dougherty, D. A., and Lester, H. A. (1998) In vivo incorporation of unnatural amino acids into ion channels in xenopus oocyte expression system. *Methods Enzymol.* 293, 26.
- (29) Nelson, M., Kuryatov, A., Choi, C., Zhou, Y., and Lindstrom, J. (2003) Alternate stoichiometries of the $\alpha 4 \beta 2$ nicotinic acetylcholine receptors. *Mol. Pharmacol.* 63, 10.
- (30) Ji, J., Lee, C.-H., Sipky, K. B., Li, T., and Gopalakrishnan, M. (July 16, 2013) Novel [1,2,4]oxadiazole compounds as $\alpha 4 \beta 2$ positive allosteric modulators and their preparation and use in the treatment of diseases. U.S. Patent No. 8486979.
- (31) Marotta, C. B., Dilworth, C. N., Lester, H. A., and Dougherty, D. A. (2014) Probing the non-canonical interface for agonist interaction with an $\alpha 5$ containing nicotinic acetylcholine receptor. *Neuropharmacology* 77, 342–349.
- (32) Moroni, M., Zwart, R., Sher, E., Cassels, B. K., and Bermudez, I. (2006) $\alpha 4 \beta 2$ nicotinic receptors with high and low acetylcholine sensitivity: Pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol. Pharmacol.* 70, 755–768.