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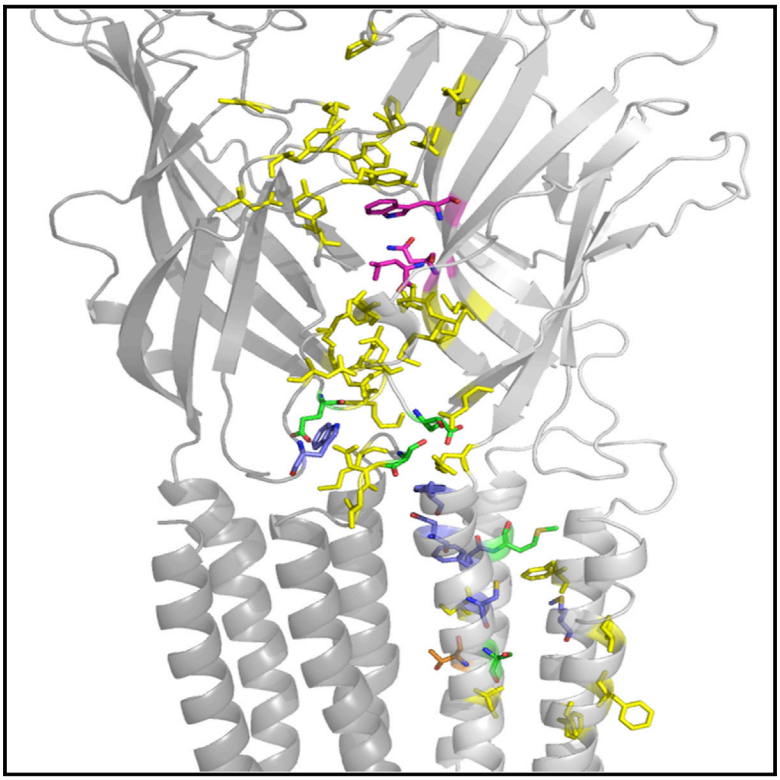
Graphical Abstract



Authors

Christopher B. Marotta, Henry A. Lester, Dennis A. Dougherty

Correspondence

dadougherty@caltech.edu

In Brief

Marotta et al. utilized non-canonical amino acid mutagenesis to establish an unaltered agonist binding site in the presence of a positive allosteric modulator, PNU-120596. Mutational analysis also discovered a network of residues vital to functional allosteric communication.

Highlights

- PNU-120596 does not alter acetylcholine binding interactions
- Functional residues in allosteric communication identified
- Global network stabilization rather than adjusted agonist binding domain

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An Unaltered Orthosteric Site and a Network of Long-Range Allosteric Interactions for PNU-120596 in α7 Nicotinic Acetylcholine Receptors

Christopher B. Marotta,[1] Henry A. Lester,[2] and Dennis A. Dougherty[1,*]
[1]Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA
[2]Division of Biology and Biological Engineering, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA
*Correspondence: dadougherty@caltech.edu
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SUMMARY

Nicotinic acetylcholine receptors (nAChRs) are vital to neuronal signaling, are implicated in important processes such as learning and memory, and are therapeutic targets for neural diseases. The α7 nAChR has been implicated in Alzheimer's disease and schizophrenia, and allosteric modulators have become one focus of drug development efforts. We investigate the mode of action of the α7-selective positive allosteric modulator, PNU-120596, and show that the higher potency of acetylcholine in the presence of PNU-120596 is not due to an altered agonist binding site. In addition, we propose several residues in the gating interface and transmembrane region that are functionally important to transduction of allosteric properties, and link PNU-120596, the acetylcholine binding region, and the receptor gate. These results suggest global protein stabilization from a communication network through several key residues that alter the gating equilibrium of the receptor while leaving the agonist binding properties unperturbed.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are pentameric ion channels that are part of the Cys-loop superfamily of ligand-gated ion channels, which includes receptors gated by other neurotransmitters such as glycine, serotonin, and γ-aminobutyric acid. The α7 nAChR displays a large and dispersed presence throughout the CNS (Millar and Gotti, 2009). It comprises five identical subunits, an uncommon arrangement for nAChRs, and each subunit contains an extracellular domain, a transmembrane domain, and a gating interface (Figure 1A) (Dougherty, 2008; Lemoine et al., 2012; Unwin, 2005).

The concepts of allostery, including cooperative transitions between two states of multi-subunit proteins (Monod et al., 1965), have been applied to nAChRs in two ways. First, the nAChR itself has been identified as a protein containing two distinct domains, a binding site for agonists and a conducting pathway (Edelstein and Changeux, 1998; Karlin, 1967).

Second, and more relevant to the present study, compounds have been identified that do not produce activation on their own, yet modulate activation and desensitization, and bind at sites distinct from both the agonist site (the "orthosteric" site) and the channel pore. These are allosteric ligands. At α7 nAChRs, positive allosteric modulators (PAMs) are especially well studied, and two classes can be distinguished. Type I PAMs increase agonist-induced activation. Type II PAMs, such as PNU-120596 (Figure 1B), increase agonist-induced activation and also vastly prolong the waveform of agonist-induced currents; in the usual interpretation, PAMs favor the active states at the expense of the desensitized states (Figure 1C) (Bertrand and Gopalakrishnan, 2007; Faghih et al., 2008; Gronlien et al., 2007; Hurst et al., 2005; Szabo et al., 2014; Williams et al., 2011a, 2011b). The existence of one or more additional, desensitized states was recognized early on (Heidmann and Changeux, 1986; Katz and Thesleff, 1957).

Inherent to models of allostery is the notion of action at a distance, and it is of interest to ask whether the orthosteric binding site, and/or the channel pore, is affected by the presence of an allosteric modulator. Unfortunately no atomic-scale structural information is available for full α7 nAChRs in any state, let alone all three states in the presence of either an agonist or allosteric modulator. However, the high functional resolution of electrophysiological data allows other approaches to this question. For example, the structurally unrelated allosteric modulator 4PB-TQS has been shown to change the kinetics of gating as well as single-channel conductance of α7 nAChRs (Pałczyńska et al., 2012), indicating that an allosteric modulator can change the structure of the conducting pore.

This study begins by determining to what extent, if any, an allosteric modulator changes the orthosteric site (Figure 2). Previous data suggested that an allosteric modulator can affect residues within the extracellular domain, but outside the orthosteric site itself (Barron et al., 2009). Non-canonical amino acid mutagenesis provides high-resolution data that complement those from X-ray crystallography. The key binding interactions at the agonist binding site of nAChRs—a cation-π interaction and two hydrogen bonds—can be probed in ways that would be sensitive to ligand displacements of <1 Å (Dougherty and Van Arnam, 2014; Tavares et al., 2012; Van Arnam et al., 2013). We have therefore applied non-canonical amino acid mutagenesis to ask whether the presence of a PAM in any way modulates these binding interactions at the orthosteric site.

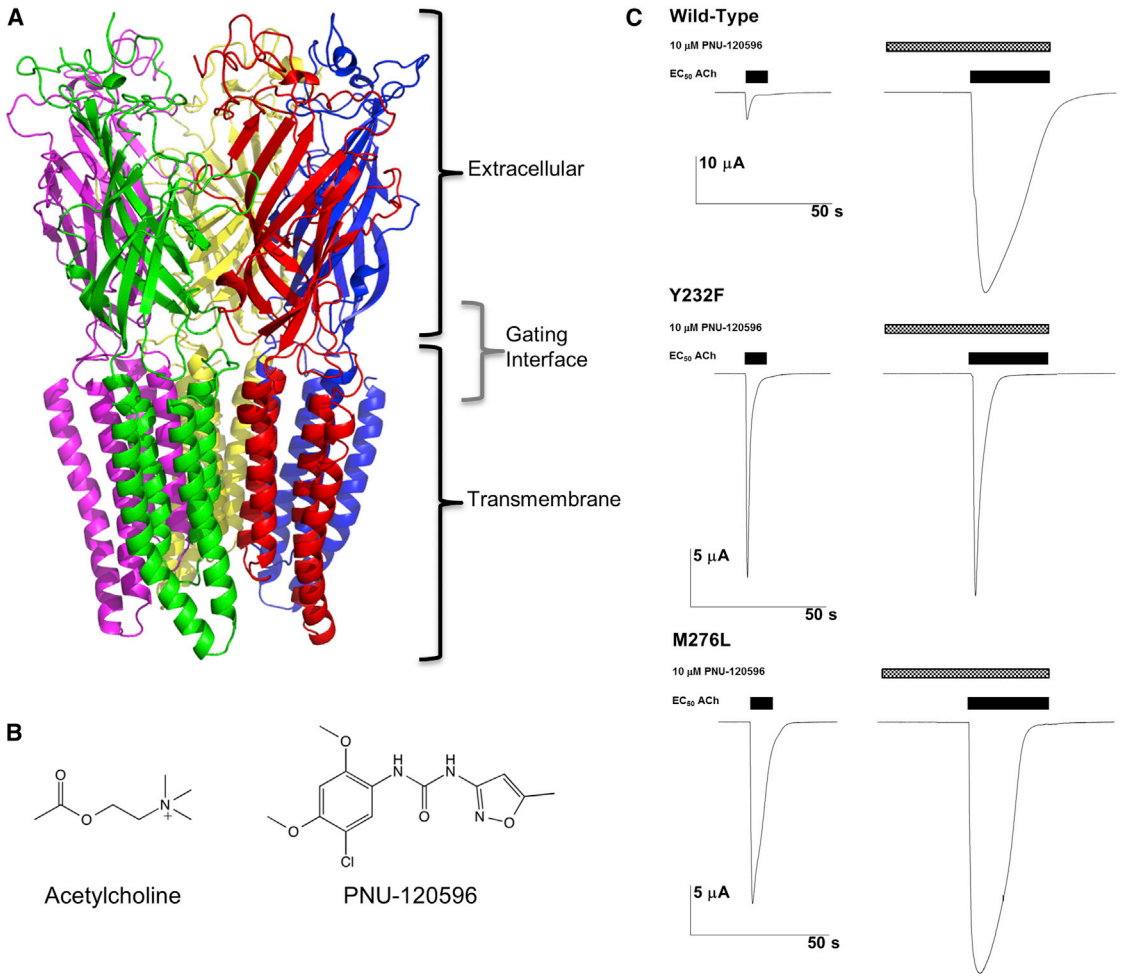

Figure 1. Rat α7 nAChR and PNU-120596 Response

(A) Homology model of the rat α7 nAChR. This ligand-gated ion channel consists of five subunits arranged in a pentameric fashion that forms a pore to transmit cations across the membrane. Each subunit consists of a large extracellular domain where the agonist binding site lies between two subunits. In addition, there is a transmembrane region consisting of four α helices and an intracellular portion used for receptor trafficking (not shown). The region where the extracellular domain and transmembrane physically interact is considered the gating interface, and is thought to be important for signal relay from the agonist binding site to the channel gate (Hanek et al., 2008; Tillman et al., 2014).

(B) Chemical structures of acetylcholine and PNU-120596.

(C) Sample traces of α7 receptor responses for wild-type and the cases designated as type A (Y232F) and type B (M276L) in Table 4. Acetylcholine is represented by a black bar and 10 μM PNU-120596 is represented by a checker-patterned bar. EC_{50} doses of acetylcholine were used for each respective mutation.

Our next goal was to map out the functional coupling pathway from the orthosteric site to the allosteric binding site of PNU-120596, which is thought to be in the transmembrane region (Bertrand et al., 2008; Young et al., 2008), and/or from the allosteric site to the channel gate. We term our strategy, which again uses functional measurements, double perturbation cycle analysis (see Figure 4 below). In this analysis, the first perturbation is mutation of the protein (with conventional or non-canonical amino acids); the second perturbation is not a mutation, but the addition of PNU-120596. Non-additivity of the two perturbations indicates that the protein mutation differentially affects receptor function depending on whether PNU-120596 is or is not present, suggesting that the residue under study plays an important role in allosteric modulation (Daeffler et al., 2012; Gleitsman et al., 2009; Miles et al., 2012). The analysis parallels that of the common double mutant cycle analysis, including the notion of an interaction energy, designated $\Delta\Delta G$. From these studies, we have identified several residues with significant $\Delta\Delta G$ values, suggesting a potential pathway of communication from the agonist binding site to the PAM binding site and then on to the receptor gate.

RESULTS

Methodology for Interpretation of Functional Coupling Comparisons

As noted in the Introduction, we sought to identify mutations of the receptor that differentially affect function when PNU-120596 is or is not present. Since we wish to evaluate a large number of sites throughout the protein, our metric is the

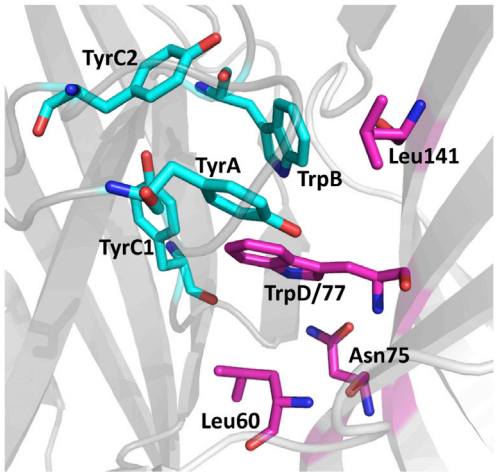

Figure 2. Agonist Binding Site for the α7 nAChR

Several tyrosine and tryptophan residues constituting the aromatic box for the agonist binding site are shown. These residues are labeled as such: TyrA (Y115), TrpB (W171), TyrC1 (Y210), and TyrC2 (Y217) lie on the principal side (cyan) of one subunit while TrpD (W77) lies on the complementary side (magenta) of the adjacent subunit. Backbone hydrogen bonding interactions have been implicated for the carbonyl of TrpB and the backbone amide of Leu141. Residues that have been shown to turn PNU-120596 into a weak agonist—Leu60, Asn75, and TrpD—are highlighted to show the proximity to the aromatic box.

half-maximal effective concentration (EC_{50}), rather than more tedious single-channel methods. We fully appreciate the composite nature of EC_{50}, and have in fact used it to our advantage in evaluating an allosteric modulator.

As noted above, for a ligand-gated ion channel one can envision two limiting modes of allosteric activation. Binding of the modulator could induce a conformational change in the protein that propagates to the orthosteric site, altering the innate affinity of that site for the natural agonist. Alternatively, the allosteric modulator could affect the gating transition of the receptor, by binding essentially at the "gate" or, again, by action through a distance.

We have developed a strategy to distinguish these two possibilities and, in so doing, have removed ambiguities associated with EC_{50} measurements. Over the past 20 years, we have developed methods for probing structure-function relationships at the agonist binding site of nicotinic receptors and related proteins with unprecedented precision (Dougherty and Van Arnam, 2014). Using non-canonical amino acids, we can reveal key drug-receptor contacts. We can identify cation-π interactions using fluorination, and evaluate potential hydrogen bonding interactions using backbone mutagenesis. Importantly, both approaches provide information on the magnitude of the non-covalent interaction between drug and receptor. As such, we can detect subtle changes in

Table 1. EC_{50} Values and Coupling Strengths of Aromatic Box Residues

α7 Mutants	Acetylcholine					Acetylcholine + PNU-120596 (10 μM)					
Aromatic Box	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift From WT	n	I_{max} (μA)	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift From WT	n	I_{max} (μA)	ΔΔG (kcal/mol)
Wild-type (WT)	120 ± 8	1.8 ± 0.2	–	20	1.1–23	12 ± 0.4	3.0 ± 0.2	–	21	1.7–48	0.00
TyrA: TyrOMe	130 ± 8	2.3 ± 0.23	1.1	10	2.9–48	10 ± 0.4	2.5 ± 0.2	0.8	13	4.5–51	0.15
TyrA: 3-F-TyrOMe	79 ± 5	1.7 ± 0.2	0.7	11	0.22–6.7	8.7 ± 0.5	2.9 ± 0.5	0.7	15	1.6–20	0.05
TyrA: 3,5-F_2-TyrOMe	290 ± 10	1.8 ± 0.1	2.4	18	1.0–11	20 ± 0.6	2.4 ± 0.1	1.7	23	1.0–41	0.21
TyrA: 4-F-Phe	2,400 ± 100	1.9 ± 0.1	20	17	0.11–1.4	420 ± 40	2.3 ± 0.4	35	16	0.43–24	0.32
TyrA: 4-Br-Phe	200 ± 8	1.9 ± 0.1	1.7	15	0.69–15	14 ± 1	2.8 ± 0.2	1.2	16	9.3–35	0.20
TyrA: 4-CN-Phe	1,800 ± 80	2.2 ± 0.2	15	17	0.11–5.4	240 ± 10	3.5 ± 0.8	20	17	9.5–44	0.16
TyrA: Phe	5,800 ± 200	1.7 ± 0.1	48	13	0.041–2.1	1,000 ± 80	2.7 ± 0.5	83	14	0.57–53	0.31
TyrA: 3,4,5-F_3-Phe	5,800 ± 700	2.2 ± 0.4	48	9	0.034–0.74	1,200 ± 80	4.0 (set)	100	14	0.53–25	0.41
TrpB: W	260 ± 20	1.5 ± 0.2	2.2	10	2.4–26	22 ± 0.3	3.1 ± 0.1	1.8	20	0.79–47	0.10
TrpB: 4,5,6,7-F_4W	670 ± 20	1.6 ± 0.1	5.6	14	0.050–4.8	60 ± 3	3.2 ± 0.4	5	16	0.45–68	0.06
TrpB: Wah	13 ± 1	1.2 ± 0.1	0.1 (1/10)	14	0.98–8.2	0.78 ± 0.04	2.1 ± 0.2	0.06 (1/15)	21	0.29–21	0.29
TyrC2: TyrOMe	230 ± 10	1.6 ± 0.1	2	15	0.76–36	18 ± 1	3.1 ± 0.2	1.5	15	0.19–90	0.14
TyrC2: 3-F-TyrOMe	1,200 ± 120	1.5 ± 0.2	10	15	0.32–6.1	120 ± 5	4.4 ± 0.9	10	14	0.88–20	0.00
TyrC2: 3,5-F_2-TyrOMe	1,500 ± 100	2.0 ± 0.2	12.5	13	0.091–5.1	200 ± 20	4.7 ± 1.6	17	13	0.66–44	0.16
TyrC2: 4-F-Phe	160 ± 10	1.4 ± 0.1	1.3	19	0.84–18	12 ± 0.4	4.5 ± 0.5	1	16	10–33	0.16
TyrC2: 4-Br-Phe	53 ± 2	1.7 ± 0.1	0.5 (1/2)	14	3.4–21	5.1 ± 0.1	3.0 ± 2	0.4 (1/2.5)	16	10–39	0.02
TyrC2: 4-CN-Phe	220 ± 10	1.8 ± 0.1	1.8	12	4.2–34	18 ± 1	3.1 ± 0.2	1.5	16	5.7–42	0.11
TyrC2: Phe	790 ± 40	1.9 ± 0.1	6.6	13	0.34–12	110 ± 7	5.9 ± 3	9.2	16	0.36–21	0.19
TyrC2: 3,4,5-F_3-Phe	1,400 ± 60	2.4 ± 0.2	12	16	0.1–2.4	290 ± 20	4.4 ± 1.4	24	16	5.0–23	0.41
TrpD: W	160 ± 7	1.9 ± 0.1	1.3	9	0.59–11	18 ± 0.6	3.2 ± 0.2	1.5	13	0.31–23	0.07
TrpD: 4,5,6,7-F_4W	90 ± 9	1.0 ± 0.1	0.75 (1/1.3)	20	0.15–10	4.1 ± 0.1	2.2 ± 0.1	0.3 (1/3)	17	2.8–21	0.45
TrpD: 1-Nap	450 ± 20	1.5 ± 0.1	3.7	13	0.10–4.4	40 ± 1	3.4 ± 0.3	3.3	16	3.6–17	0.07
TrpD: Wah	360 ± 20	1.3 ± 0.1	3	14	0.081–4.5	24 ± 1	4.0 (set)	2	15	0.21–24	0.23
W77A	160 ± 10	2.4 ± 0.2	1.3	15	8.2–28	15 ± 1	2.1 ± 0.2	1.2	15	9.6–51	0.04

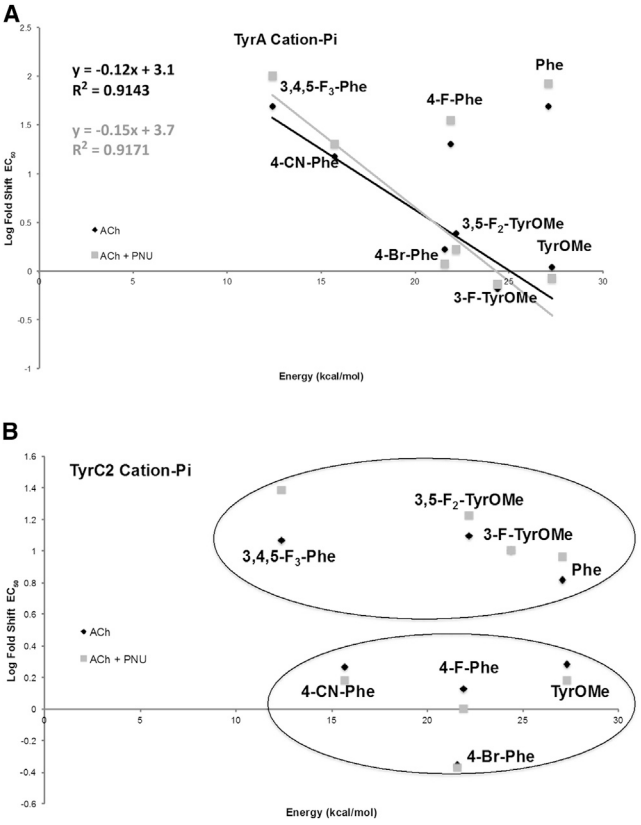

Figure 3. Cation-π Plots for TyrA and TyrC2 in the Rat α7 Receptor

ACh, acetylcholine; PNU, PNU-120596 (Puskar et al., 2011).

(A) The cation-π interaction is present at TyrA and does not shift in its relative strength when PNU-120596 is added.

(B) TyrC2 shows no cation-π interaction with acetylcholine, even in the presence of PNU-120596.

the agonist binding site that would enhance (or diminish) agonist binding in ways that are not possible with conventional approaches. As described herein, we find no evidence of alteration of the agonist binding site on addition of PNU-120596.

EC_{50} describes a composite of several equilibria, some involving agonist binding, some involving channel gating. Since we can rule out alteration of binding equilibria, we can conclude that changes in EC_{50} induced by PNU-120596 reflect changes in the gating equilibria of the receptor. We have used such analyses before, removing the innate ambiguity in EC_{50} by eliminating one component of the measurement (Lummis et al., 2005; Xiu et al., 2009).

Again, our goal is to identify residues that play a role in the allosteric modulation provided by PNU-120596. To do this, we compare the impact of a mutation on wild-type function versus function when PNU-120596 is present. If the mutation does not affect PNU-120596 in any way, the impact of the mutation should be the same whether PNU-120596 is present or not. Stated differently, the effects on wild-type EC_{50} of the mutation and of PNU-120596 should simply have additive energies. Alternatively, if a mutation alters PNU-120596 function, then the effect of PNU-120596 on receptor function should be different from that of wild-type when the mutation is present. That is, the side-chain mutation and the impact of PNU-120596 should be non-additive. By analogy to conventional double mutant cycle analysis, the non-additivity can be expressed as a $\Delta\Delta G$ value (Figure 4), which if markedly different from zero signifies the presence of non-local conformational effects on a large and complex membrane bound protein.

The Orthosteric Site: Binding Interactions Are Unaffected by PNU-120596

Previous studies of the α7 nAChR show that acetylcholine makes a single cation-π interaction with TyrA (Figure 2), which is one of five aromatic residues at the orthosteric binding site (Puskar et al., 2011). One possible way in which the allosteric binding of PNU-120596 could affect receptor function is by influencing the shape of the aromatic box, such that the strength of the cation-π interaction to acetylcholine could change, or the site of the cation-π interaction could move to another aromatic residue instead of, or in cooperation with, TyrA. Other studies show that mutations outside the agonist binding site can reshape the binding site and significantly alter agonist-receptor contacts (Xiu et al., 2009). One can identify cation-π interactions using progressive fluorination of the aromatic groups that contribute the π electrons (Figure S1). We were able to probe four of the five aromatic box residues for acetylcholine cation-π interactions in the absence and presence of PNU-120596 (Table 1). TyrC1 could not be probed because of the large loss of function for any substitution made at this residue; TyrC1 has never been implicated in a cation-π interaction in the dozens of studies of Cys-loop receptors we have performed (Dougherty and Van Arnam, 2014).

The first observation is that acetylcholine continues to make a cation-π interaction with TyrA in the presence of PNU-120596 (Figure 3A). In addition, the slopes of the two fluorination plots (with and without PNU-120596) are not meaningfully different, which indicates that the strength of the cation-π interaction was also unaltered (Dougherty and Van Arnam, 2014). Since the interaction with TyrA was unchanged, TyrC2 was probed next, because previous data show that a higher sensitivity agonist, epibatidine, makes a cation-π interaction with TyrC2 in addition to TyrA in the α7 nAChR (Puskar et al., 2011). As seen in Figure 3B, a cation-π interaction still does not exist between TyrC2 and acetylcholine in the presence of PNU-120596. Two additional observations can be made regarding interactions with the TyrC2 residue. The near wild-type receptor response for bulky substituent groups (4-CN-Phe, 4-Br-Phe, and TyrOMe) and severe loss of function for small/no substituents (4-F-Phe and Phe) suggest a large substituent is needed at the 4-position in the aromatic ring to maintain proper receptor function. In addition, the receptor cannot tolerate substitutions at the 3- or 5-position in the ring system, as indicated by the large loss of function for 3-F-TyrOMe and 3,5-F_2-TyrOMe residues. These results suggest a tight steric environment for TyrC2 at the orthosteric site. Of more relevance here, however, is the fact that the pattern of responses to substitution at TyrC2 is unaltered by the presence of PNU-120596.

Since PNU-120596 did not influence the two critical Tyr residues in the α7 nAChR, the Trp residues were also studied.

Table 2. EC_{50} Values and Coupling Strengths of Extracellular Residues

α7 Mutants	Acetylcholine					Acetylcholine + PNU-120596 (10 μM)					
Extracellular	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift from WT	n	I_{max} (μA)	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift from WT	n	I_{max} (μA)	ΔΔ*G* (kcal/mol)
WT	120 ± 8	1.8 ± 0.2	–	20	1.1–23	12 ± 0.4	3.0 ± 0.2	–	21	1.7–48	0.00
L60A	27 ± 2	1.3 ± 0.1	0.2 (1/4)	15	1.2–44	2.0 ± 0.2	2.3 ± 0.3	0.2 (1/6)	15	3.9–50	0.17
Q61A	44 ± 4	1.4 ± 0.2	0.3 (1/3)	16	4.1–34	4.0 ± 0.1	3.2 ± 0.2	0.3 (1/3)	18	1.3–89	0.05
M63A	32 ± 1	1.9 ± 0.1	0.27 (1/4)	13	0.42–19	2.5 ± 0.03	3.4 ± 0.2	0.2 (1/5)	15	2.6–39	0.14
E67A	830 ± 30	1.8 ± 0.1	7	15	0.13–1.1	190 ± 30	3.6 ± 1.6	16	24	0.24–11	0.47
E67N	720 ± 100	1.5 ± 0.3	6	10	0.027–0.40	240 ± 20	2.4 ± 0.4	20	14	0.085–1.6	0.69
K68A	300 ± 20	1.9 ± 0.2	2.5	18	0.24–39	59 ± 1	3.7 ± 0.2	5	16	11–44	0.39
N69A	400 ± 30	1.8 ± 0.2	3.3	16	1.3–39	52 ± 3	2.7 ± 0.3	4.3	16	4.4–38	0.15
Q70A	450 ± 30	1.3 ± 0.1	3.8	20	0.10–6.8	29 ± 1	5.4 ± 1	2.4	15	0.56–18	0.25
T73A	210 ± 10	1.4 ± 0.1	1.8	13	0.19–8.6	22 ± 0.8	3.5 ± 0.4	1.8	16	0.98–23	0.03
N75A	8 ± 1	1.0 ± 0.1	0.067 (1/15)	15	1.6–27	0.29 ± 0.02	1.7 ± 0.2	0.025 (1/40)	16	4.6–25	0.58
P103G	180 ± 10	1.9 ± 0.2	1.5	15	2.3–26	21 ± 1	2.4 ± 0.3	1.8	16	5.7–45	0.09
H127A	48 ± 3	1.9 ± 0.2	0.4 (1/2.5)	14	2.4–35	5.8 ± 0.2	3.0 ± 0.3	0.5 (1/2)	16	3.8–42	0.11
Q139F	220 ± 10	1.8 ± 0.1	1.8	14	1.3–35	25 ± 1	2.5 ± 0.3	2.1	16	0.96–37	0.07
L141:Leu	120 ± 7	1.9 ± 0.2	1	15	0.48–17	15 ± 0.6	2.8 ± 0.2	1.2	16	1.3–41	0.13
L141:Lah[a]	180 ± 10	1.9 ± 0.2	1.5	14	0.23–31	27 ± 1	5.0 ± 1.3	1.8	15	1.5–26	0.10
Y151A	120 ± 20	1.2 ± 0.1	1	15	3.2–43	8.3 ± 0.3	2.3 ± 0.2	0.7	12	11–37	0.21
D153A	170 ± 10	1.8 ± 0.2	1.4	15	0.27–9.6	25 ± 1	4.0 (set)	2.1	14	1.6–17	0.22
W156A	240 ± 30	1.9 ± 0.4	2	12	0.16–8.8	65 ± 3	1.9 ± 0.2	5.4	15	0.18–23	0.57
S172:Ser	480 ± 20	1.3 ± 0.1	4	15	0.21–4.8	23 ± 0.5	3.2 ± 0.2	1.9	16	0.30 - 7.8	0.42
S172:Sah[a]	130 ± 10	1.2 ± 0.1	0.3 (1/3)[a]	15	0.088–3.1	8.4 ± 0.6	3.5 ± 0.8	0.36 (1/3)[a]	15	0.54–18	0.17
S172:Thr	120 ± 10	1.2 ± 0.1	1	15	0.28–35	8.0 ± 0.5	2.8 ± 0.5	0.67 (1/1.5)	16	5.3–31	0.23
S172:Tah[a]	48 ± 10	0.93 ± 0.2	0.4 (1/2.5)[a]	13	0.18–7.9	1.6 ± 0.05	3.5 ± 0.2	0.2 (1/5)[a]	16	0.33–26	0.39
G175K	7.5 ± 0.7	1.6 ± 0.2	0.06 (1/17)	17	0.42–30	1.0 ± 0.2	2.5 ± 0.9	0.08 (1/12)	19	2.1–42	0.16
S189A	210 ± 20	1.4 ± 0.1	1.8	15	0.20–48	16 ± 0.7	2.9 ± 0.2	1.3	16	4.6–49	0.15
N193A	210 ± 5	1.7 ± 0.1	1.8	14	0.22–12	18 ± 1	2.4 ± 0.2	1.5	21	0.18–18	0.09
E195A	620 ± 60	1.7 ± 0.3	5.2	13	0.050–0.17	170 ± 8	3.6 ± 0.4	14	15	0.54–13	0.57
E195N	530 ± 20	1.4 ± 0.1	4.4	22	0.17–14	40 ± 2	2.7 ± 0.2	3.3	15	0.56–25	0.16
D197A	130 ± 10	1.6 ± 0.2	1.1	14	0.54–19	16 ± 0.4	3.0 ± 0.1	1.3	16	2.6–40	0.12
D219A	860 ± 20	2.1 ± 0.1	7.2	13	0.37–31	130 ± 5	5.1 ± 0.7	11	16	3.6–23	0.24

[a]Fold shift from canonical amino acid incorporation (Ser and Thr) using the non-canonical amino acid method.

Note that for most combinations of agonist and nAChR, the cation-π interaction is to TrpB; α7 is unusual in employing TyrA. For both TrpB and TrpD in the presence and absence of PNU-120596, there was no meaningful shift in function when we expressed the highly perturbing residue 4,5,6,7-F_4-Trp (Table 1) (Figure S1). This indicates that acetylcholine is not making a cation-π interaction with either of these residues. Previous studies showed that a much more dramatic mutation of TrpD—to Ala—converted PNU-120596 into a partial agonist, while maintaining its function as a positive allosteric modulator (Barron et al., 2009; Papke et al., 2014). We find that this mutation shows a ΔΔ*G* of essentially zero (Table 1).

We also applied a previously described strategy for evaluating the two key hydrogen bonding interactions at the agonist binding site (Tavares et al., 2012; Van Arnam et al., 2013). In brief, α-hydroxy analogs of α-amino acids are incorporated in ways that are known to strongly modulate the hydrogen bonding ability of the protein backbone (Figure S1). We found that perturbation of the hydrogen bond acceptor (L141) or the hydrogen bond donor (S172) had little impact on PNU-120596 modulation (Figure 2) (Table 2).

Overall, the high-precision methodology of non-canonical amino acid mutagenesis allows us to conclude that the presence PNU-120596 does not measurably alter the key binding interactions to acetylcholine (ACh) at the orthosteric site.

A Double Perturbation Cycle Analysis to Identify Residues Critical to PNU-120596 Function

We assessed numerous residues throughout the α7 subunit to determine whether they play a role in the allosteric modulation by PNU-120596. Previous studies have emphasized how mutations affect the potentiation produced by PNU-120596, an approach that has produced useful insights such as

$$\Delta\Delta G = Abs(-RT\ln\Omega)$$

$$\Omega = \frac{EC_{50}WT * EC_{50}Mut1,2}{EC_{50}Mut1 * EC_{50}Mut2}$$

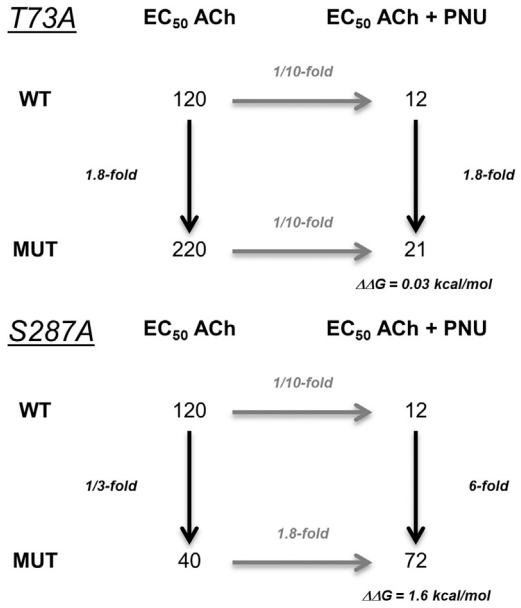

Figure 4. Example of the Double Perturbation Cycle Analysis Based on the Mutant Cycle Analysis

ACh, acetylcholine; PNU, PNU-120596. Two examples are given, showing both negligible and significant ΔΔ*G* values. Room temperature (25°C) was used for the value *T*.

identification of the PNU-120596 binding site in the transmembrane region. We extended previous data by adopting the double perturbation cycle analysis, which is appropriate for identifying long-range communication between two sites of interest (described in Figure 4). Results are tabulated for all residues studied in Tables 1, 2, and 3. If the absolute value of the calculated ΔΔ*G* is ≥0.5 kcal/mol, we consider the protein residue to be functionally important to the allosteric modulatory activity of PNU-120596. This approach may minimize possible complications arising from changes to the receptor that do not influence the allosteric communication pathway (Gleitsman et al., 2009).

Previous experiments probing agonist binding interactions in α7 nAChRs were aided by the inclusion of a pore mutation (T6′S) that produces a modest gain of function and slows α7 desensitization, allowing more precise waveform analysis (Puskar et al., 2011; Van Arnam et al., 2013). However, this mutation is coupled to PNU-120596, with a ΔΔ*G* of 0.68 kcal/mol. As such, this mutation was not employed except in two cases where the introduced mutations generated a non-functional receptor that was recoverable through the introduction of a gain-of-function pore mutation (Zhang et al., 2011).

Measuring the Coupling at the Proposed PNU-120596 Binding Site

Several studies of the impact of mutations on PNU-120596 potentiation of acetylcholine response suggested that PNU-120596 binds in the transmembrane region, across α helices M1 (S245 and A248), M2 (M276), and M4 (F478 and C482) of a single subunit (Figure 5) (Collins et al., 2011; Young et al., 2008). Since the goal of the present work was to map out the functional coupling pathway between the binding site for PNU-120596 and the agonist binding site, we mutated a large number of residues throughout the receptor (Tables 1, 2, and 3). For the purposes of discussion, we will begin at the "bottom" and work our way up to the agonist binding site.

Of the five residues thought to contribute to the PNU-120596 binding site, only two show a meaningful functional coupling. However, several nearby residues did show meaningful coupling (Table 3). Interestingly, these residues generally lie "above" the three residues that were previously implicated in binding but show no coupling (A248, F478, C482). Further exploration of the area around this region yielded several other residues—C241 (M1), F275 (M2), and M301 (M3)—that resulted in a large coupling, as reflected in the ΔΔ*G* values. As seen in Figure 5, these residues lie outside the previously proposed PNU-120596 binding pocket (Williams et al., 2011a, 2011b).

Some of these mutations produced altered response waveforms. F275A showed responses that, regardless of the addition of PNU-120596, resembled the examples of wild-type α7 acetylcholine waveforms in Figure 1 (referred to as Type A in Table 4). In contrast, M276L (example in Figure 1) and M301A markedly lengthened the waveform for application of acetylcholine alone (Type B).

Important Residues in the Gating Interface and the Extracellular Domain

Previous cysteine-labeling experiments identified several extracellular residues positioned at the interface of two adjacent subunits that underwent conformational changes in the α7 nAChR when exposed to either PNU-120596 or acetylcholine (Barron et al., 2009). These residues (L60, M63, E67, N75, N193, and E195) were evaluated along with several others in the surrounding region (Table 2). Figure 5 shows the residues in the gating interface with $|\Delta\Delta G| \geq 0.5$ kcal/mol (Hanek et al., 2008; Tillman et al., 2014). Tillman et al. (2014) showed through chimera analysis that specific loops and linkers were necessary for PNU-120596 potentiation of α7 receptors. Here, we were able to isolate specific residues on some of these identified regions: in loop 2, E67; in loop 9, E195; and in the M2-M3 Linker, S287. Again, we observed distinct response waveforms. Results for E67A/N, E195A, Y232F, N236A, and S287A all resembled α7 wild-type acetylcholine waveforms, even in the presence of PNU-120596 (Figure 1; Table 4).

All the mutations that had $|\Delta\Delta G| \geq 0.5$ kcal/mol, or that rendered PNU-120596 an agonist, define a network of residues necessary for propagation of the PNU-120596 effects throughout the full receptor (Figure 5). These results suggest a conformational wave of movement upon activation, and give insight to the molecular motions that potentially take place between the closed and open forms of the receptor.

DISCUSSION

The present work aims to evaluate the influences of the positive allosteric modulator PNU-120596 on α7 nAChRs. Through the

Table 3. EC_{50} Values and Coupling Strengths of Transmembrane Residues

α7 Mutants	Acetylcholine					Acetylcholine + PNU-120596 (10 μM)					
Transmembrane	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift (WT)	n	I_{max} (μA)	EC_{50} (μM ± SEM)	Hill (± SEM)	Fold Shift (WT)	n	I_{max} (μA)	ΔΔ*G* (kcal/mol)
WT	120 ± 8	1.8 ± 0.2	–	20	1.1–23	12 ± 0.4	3.0 ± 0.2	–	21	1.7–48	0.00
T6′S	64 ± 1	2.4 ± 0.1	0.5 (1/2)	15	4.8–67	21 ± 2	3.2 ± 0.7	1.8	16	0.48–23	0.68
R229A	78 ± 8	1.3 ± 0.1	0.6	12	0.42–42	8.1 ± 0.3	2.6 ± 0.2	0.7	14	0.12–8.1	0.02
Y232F	52 ± 2	1.9 ± 0.2	0.4 (1/2.5)	15	2.4–20	73 ± 2	2.1 ± 0.1	6	17	0.85–20	1.50
N236A	480 ± 40	1.2 ± 0.1	4	17	0.32–6.8	430 ± 40	1.1 ± 0.1	36	16	0.087–0.77	1.25
C241A	180 ± 10	3.1 ± 0.4	1.5	16	0.19–31	73 ± 3	4.1 ± 0.5	6	15	0.61–15	0.80
S245A	160 ± 10	1.3 ± 0.1	1.3	15	1.2–15	3.2 ± 0.2	2.5 ± 0.4	0.27 (1/4)	15	13–57	0.92
A248D	92 ± 5	1.3 ± 0.1	0.8	12	0.16–7.7	10 ± 0.4	2.8 ± 0.3	0.8	16	0.089–10	0.05
S271A	110 ± 7	2.2 ± 0.2	0.9	14	9.1–32	14 ± 0.3	2.6 ± 0.1	1.2	15	1.2–58	0.14
F275A	280 ± 20	1.8 ± 0.2	2.3	15	0.35–23	510 ± 30	1.4 ± 0.1	43	18	0.16–18	1.65
M276L	46 ± 1	3.0 ± 0.1	0.4 (1/2.5)	15	9.9–39	32 ± 2	2.0 ± 0.2	2.7	16	7.3–43	1.10
M283A T6′S[a]	2,100 ± 70	2.3 ± 0.1	33[a]	14	0.52–18	440 ± 30	4.4 ± 1.2	21[a]	16	3.6–37	0.26
S287A	40 ± 2	2.0 ± 0.2	0.3 (1/3)	16	8.7–36	72 ± 7	1.3 ± 0.1	6	15	3.1–31	1.65
D288A T6′S[a]	750 ± 30	2.3 ± 0.2	12[a]	13	4.8–46	230 ± 10	5.7 ± 3	11[a]	16	1.6–84	0.04
S289A	52 ± 2	1.7 ± 0.1	0.4 (1/2.5)	16	3.3–51	6.6 ± 0.5	2.1 ± 0.3	0.5 (1/2)	15	6.4–42	0.14
F297A	150 ± 10	1.8 ± 0.2	1.3	15	0.93–52	16 ± 1	2.6 ± 0.2	1.3	16	6.9–45	0.04
M301A	19 ± 1	2.1 ± 0.1	0.2 (1/5)	10	11–53	23 ± 1	1.7 ± 0.1	1.9	15	8.2–60	1.42
F475A	78 ± 3	1.8 ± 0.1	0.6	16	1.3–22	5.8 ± 0.7	2.0 ± 0.4	0.5 (1/2)	16	13–30	0.17
F478A	74 ± 1	2.3 ± 0.1	0.6	16	1.3–30	12 ± 0.7	3.4 ± 0.6	1.0	15	8.1–48	0.28
C482Y	210 ± 20	2.0 ± 0.3	1.8	19	0.84–27	44 ± 1	3.0 ± 0.2	3.7	16	2.4–37	0.42

[a]Fold shift from the T6′S value, since it is a background mutation for the point in question.

use of non-canonical amino acids, the binding region of acetylcholine was probed for potential changes in interactions with acetylcholine when PNU-120596 was introduced. These studies produced three key results: (1) the cation-π interaction with the TyrA residue is not perturbed by the introduction of PNU-120596; (2) no other cation-π interactions are gained when PNU-120596 is present; and (3) the overall shape of the agonist binding site seems unperturbed. The aromatic fluorination series and introduction of α-hydroxy acid residues give compelling evidence that no significant rearrangements propagate to the orthosteric binding site from the allosteric binding site of PNU-120596.

Since no distinct change to the agonist binding motif was observed, we suggest that PNU-120596 does not alter the binding step of the agonist to the receptor, and instead exerts its effects only on the gating equilibrium. As explained above, this result effectively removes the ambiguity in comparing EC_{50} values for various mutations. Since the agonist binding site is unperturbed, it is reasonable to assume that PNU-120596 primarily, if not solely, influences receptor function by perturbing gating equilibria. The binding of PNU-120596 apparently affects important residues required for signal transduction from the agonist binding site to the channel gate. In evaluating long-range interactions between residues, functional coupling comparisons based on a double perturbation cycle analysis provide an appropriate and rigorous method (Daeffler et al., 2012; Gleitsman et al., 2009; Miles et al., 2012). The functional coupling comparisons were used to probe for mutations that coupled acetylcholine and PNU-120596 together, thus allowing for identification of residues necessary for proper PAM function and influence. Table 4 summarizes the major results of this study. From these data, several observations and conclusions can be drawn to elicit new information on allosteric modulation of α7 nAChRs by PNU-120596.

An interesting observation concerned the mutations M276L and M301A, which changed the decay current rate of acetylcholine-only waveforms (Table 4). This effect is quite similar to that seen with the T6′S mutation. Coupling was seen in the double perturbation cycle analysis for all three residues, because all of these mutations apparently alter the gating equilibrium and thus diminish the total effect that can be exerted on the system by PNU-120596. M301A (adjacent to the putative PNU-120596 binding site) and M276L (on the M2 pore-lining helix) most likely contribute to structural rearrangements in the transmembrane region. In addition, the mutation L60 shows a large gain of function for acetylcholine alone, which suggests a possible restructuring coupled to the agonist binding site. Thus, the allosteric propagation between acetylcholine and PNU-120596 is disrupted, and PNU-120596 becomes a weak agonist.

Another surprising result seen here is that several residues previously implicated in PNU-120596 binding do not show functional coupling with respect to transmitting the effects of PNU-120596. Previously, the binding pocket had been proposed to lie in the transmembrane region and to interact with residues on the M1, M2, and M4 α helices (Collins et al., 2011; Young et al., 2008). Even though these residues were implicated in

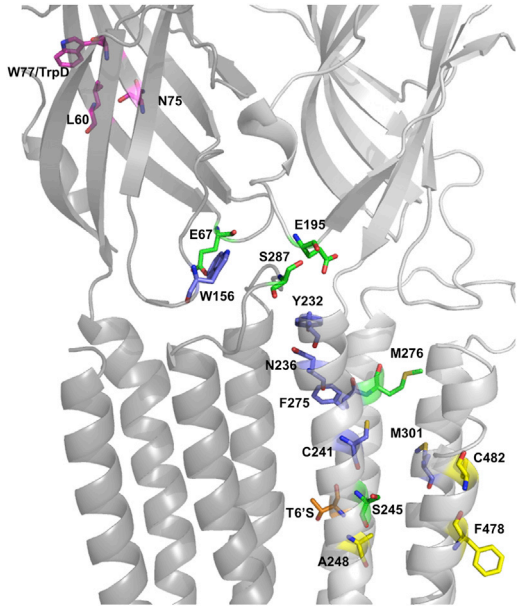

Figure 5. Summary of Residues that Generated a $\Delta\Delta G$ Greater than 0.5 kcal/mol in the Double Perturbation Cycle Analysis

Only two subunits are shown for clarity. The color scheme is as follows. Magenta: PNU-120596 becomes a partial agonist; orange: T6′S on the M2 helix; blue: new residues discovered to have a $|\Delta\Delta G| \geq 0.5$ kcal/mol; green: residues previously studied that also show a $|\Delta\Delta G| \geq 0.5$ kcal/mol; yellow: residues previously implicated in the PNU-120596 binding site that do not show a $|\Delta\Delta G| \geq 0.5$ kcal/mol.

comprising the PNU-120596 binding, they may not contribute to allosteric propagation in the protein, which is analogous to the residues critical for acetylcholine binding shown above. Residues adjacent to the proposed binding pocket were implicated in the communication pathway: N75 lies on the β2 strand connected to loop 2; C241 and S245 lie on the M1 helix, and F275 on the M2 helix. Remarkably, both of these edges of the binding sites seem to be oriented for interaction with residues at the gating interface. The residues E195 (loop 9), Y232 (M1), and N236 (M1) of one subunit are located in the vicinity of E67 (loop 2), W156 (loop 7), and S287 (M2-3 linker) of the adjacent subunit. This suggests the presence of a collection of residues in the gating interface region that communicates the allosteric potentiation between the extracellular and transmembrane regions (Figure 5). This interpretation is reinforced by the fact that five of these six residues (excluding W156) show nearly α7 wild-type response waveforms even when PNU-120596 is present (Table 4). Even though the residue N193 is not included in this complex despite its proximity, a possible explanation for the effects previously seen by Barron et al. is that its physical location can be reorienting because the aforementioned complex movements change the solvent exposure (Barron et al., 2009).

PNU-120596 thus exerts positive allosteric modulation by binding in the transmembrane region, which stabilizes the gating interface and changes the gating equilibrium of the receptor, allowing for a lower concentration of the agonist to open the channel. Also, stabilizing this interaction prolongs channel activation, presumably by decelerating desensitization of the α7 receptor, which is the most rapidly desensitizing nAChR known (Zhang et al., 2011). Naturally this suggests a global interaction involving a complex change in the stability of the wild-type receptor after agonist binding. Here, we have provided a quantitative analysis for identification of residues necessary for proper propagation of allosteric effects.

SIGNIFICANCE

nAChRs are critical contributors to neuronal communication, which also implicates them in vital normal brain processes and neural diseases. The α7 nAChR in particular has been implicated in Alzheimer's disease and schizophrenia; thus, the molecular understanding of how compounds affect this receptor has attracted much interest (Narla et al., 2013; Pandya and Yakel, 2013; Parri et al., 2011; Tong et al., 2011; Young and Geyer, 2013). Attempts to design small molecules that are specific to α7 have yielded numerous agonists, with some therapeutic success (Horenstein et al., 2008). As an alternative to selective agonist design, ongoing research has targeted the development and understanding of allosteric modulators, which have the potential to be more target specific and thus produce fewer side effects (Christopoulos, 2002; Williams et al., 2011a, 2011b). In this study, the use of non-canonical amino acids allowed individual chemical interactions of the agonist binding to the protein to be probed in the presence of the α7-specific positive allosteric modulator PNU-120596. The conclusion from this analysis is that PNU-120596 does not alter the agonist binding pocket. To further probe the molecular basis of the properties of PNU-120596, conventional mutagenesis throughout the receptor was performed. Several gating interface residues as well as transmembrane residues were identified as vital for propagating PNU-120596 properties throughout the receptor. This network of residues links the agonist binding site to the PNU-120596 binding site and to the channel gate in the pore of the receptor, influencing the global stabilization of the gating equilibria.

EXPERIMENTAL PROCEDURES

Residue Numbering and Protein Modeling

Residue numbering was based on the full-length protein containing the signaling sequence as found on the Ligand Gated Ion Channel Database (http://lenoverelab.org/LGICdb/LGICdb.php). The figures were generated using PyMOL and a homology model (generated via MODELLER) of the rat α7 receptor based on the GluCl crystal structure (PDB: 3RHW) (Hibbs and Gouaux, 2011).

Molecular Biology

The QuikChange protocol (Stratagene) was used for site-directed mutagenesis of the rat nAChR α7 subunit (pAMV vector). *NotI* was used to linearize the circular plasmid. The DNA was purified (Qiagen), and in vitro transcription of mRNA from the linearized DNA templates was performed using the T7 mMessage Machine kit (Ambion). The resulting mRNA was purified and isolated using the RNeasy RNA purification kit (Qiagen). The same linearization and mRNA synthesis protocols were used for the human Ric3 (pAMV) accessory protein.

Table 4. Summary of Residues that Show Coupling of PNU-120596 and Acetylcholine

Coupled Mutations (Location)	$\|\Delta\Delta G\|$ (kcal/mol)	Previously Probed: Reference	Electrophysiology	Uncoupled Mutations (Location)	$\|\Delta\Delta G\|$ (kcal/mol)	Previously Probed: Reference	Electrophysiology
F275A (TM)	1.65	–	type A	L60A (EC)	0.17	[a]	PNU partial agonist
S287A (GI)	1.65	[b]	type A	M63A (EC)	0.14	[a]	
Y232F (GI)	1.50	–	type A	W77A (EC)	0.04	[c]	PNU partial agonist
M301A (TM)	1.42	–	type B	N193A (GI)	0.09	[a]	
N236A (GI)	1.25	–	type A	A248D (TM)	0.05	[d,e]	
M276L (TM)	1.10	[d,e]	type B	F478A (TM)	0.28	[d,e]	
S245A (TM)	0.92	[d,e]		C482Y (TM)	0.42	[d,e]	
C241A (TM)	0.80	–					
E67N (GI)	0.69	[a,b]	type A				
T6′S (TM)	0.68	–					
N75A (EC)	0.58	[a]	PNU partial agonist				
E195A (GI)	0.57	[a,b]	type A				
W156A (GI)	0.57	–					
E67A (GI)	0.47	[a,b]	type A				

The first four columns list, in descending order, mutations found to have a $|\Delta\Delta G| \geq 0.5$ kcal/mol, and thus are considered here to be important for allosteric modulation. They also contain the abbreviation for the relative location on the protein (Figure 5). The second four columns contain the residues (listed in residue numerical order) previously probed and implicated in PNU-120596 function but did not show a $|\Delta\Delta G| \geq 0.5$ kcal/mol. Both the left and right parts of the table also contain references associated with each mutation and the effect on the electrophysiology observations that were seen when comparing traces from the EC_{50}s measured (see Figure 1C for examples). Mutations without a reference are newly discovered locations that showed importance to allosteric function.

TM, transmembrane; EC, extracellular; GI, gating interface.

Type A: Addition of PNU-120596 does not alter waveform compared with acetylcholine alone.

Type B: Mutation alters waveform for application of acetylcholine alone.

[a]Barron et al. (2009).

[b]Tillman et al. (2014).

[c]Papke et al. (2014).

[d]Collins et al. (2011).

[e]Young et al. (2008).

For non-canonical amino acid incorporation, the amber (UAG) stop codon was used for all α7 subunit incorporation. The 74-nt THG73 tRNA and 76-nt THG73 tRNA were in vitro transcribed using the MEGAshortscript T7 (Ambion) kit and isolated using Chroma Spin DEPC-H_2O columns (Clontech). Synthesized non-canonical amino acids coupled to the dinucleotide dCA were enzymatically ligated to the 74-nt tRNA as previously described (Nowak et al., 1998; Xiu et al., 2009).

ND96 medium was used for all experimental running/wash buffers (96 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES [pH 7.5]). ND96+ medium was used for oocyte storage media (2.5 mM sodium pyruvate and 6.7 mM theophylline). No gentamicin was added to the ND96+ storage medium to avoid distorting acetylcholine-induced responses (Amici et al., 2005).

Oocyte Preparation and Injection

Xenopus laevis stage V and VI oocytes were harvested via standard protocols (Nowak et al., 1998) and in compliance with policies approved by the Caltech Institutional Animal Care and Use Committee (IACUC). For conventional mutagenesis, mRNA mixtures of α7 and Ric3 (Ben-Ami et al., 2005; Castillo et al., 2005; Cheng et al., 2005; Halevi et al., 2002; Williams et al., 2005) were mixed a ratio of 1:1 by weight to a final concentration of 0.8 ng/nl. Each oocyte received a 50-nl injection for a 40-ng total mRNA mass delivery. Oocytes were incubated at 18°C for 24–48 hr. For non-canonical amino acid incorporation, mRNA mixtures of α7 and Ric3 were made in a 1:1 ratio to a final concentration of 1.6 ng/nl. These mRNA mixtures were then mixed in a 1:1 volume ratio with deprotected (photolysis) tRNA, and 50 nl was injected into each oocyte. Oocytes were incubated at 18°C for 24 hr. For non-canonical amino acids that showed no response after 24 hr, the oocyte was subjected to a second injection and incubation following the aforementioned procedure. Readthrough/re-aminoacylation tests (76-nt THG73 tRNA) were performed to confirm non-canonical amino acid incorporation (Van Arnam et al., 2013).

Chemical Preparation

Acetylcholine chloride (Sigma-Aldrich) was dissolved to 1 M stock solutions in ND96 buffer. PNU-120596 (Selleckchem) was dissolved in DMSO to 150 mM stock solutions. Further dilution was performed to make a solution of 10 μM and 0.1% v/v DMSO for experimentation.

Electrophysiology

The two-electrode voltage clamp mode of an OpusXpress 6000A (Axon Instruments) was used. A holding potential of −60 mV and ND96 medium for running buffer were used.

For acetylcholine EC_{50} measurements, 2-fold and 2.5-fold acetylcholine concentration steps were applied over several orders of magnitude for dose-response measurements. Drug applications consisted of applying 1 ml of solution over 8 s. Then the oocytes were washed with buffer for 3 min at a rate of 3 ml min^{-1} before the next application of drug. For the acetylcholine and PNU-120596 EC_{50} measurements, a similar protocol was used. PNU-120596 at 10 μM was pumped in at a rate of 3 ml min^{-1} for 30 s (1.5 ml total volume per oocyte) prior to the co-application of acetylcholine and 10 μM PNU-120596. The co-application dose was 1 ml of solution over 15 s followed by an additional 15-s pause to allow each response to reach its maximum value. Then the oocytes were washed with buffer for 5 min at a rate of 3 ml min^{-1} before the next co-application of drug. Again,

2-fold and 2.5-fold acetylcholine concentration steps were used over several orders of magnitude.

Data were sampled at 50 Hz and then low-passed filtered at 5 Hz. Data were normalized on a per-cell basis, response was averaged on a per-concentration basis, and then fit to a single Hill term to generate EC_{50} and Hill coefficient (nH) values. Error bars represent the SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.06.018.

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