

Potentiation of a Neuronal Nicotinic Receptor via Pseudo-agonist Site

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Abbreviations

nAChR Nicotinic acetylcholine receptor
ACh Acetylcholine

Running title: Allosteric modulation of neuronal nAChRs

Abstract-

Neuronal nicotinic receptors containing $\alpha 4$ and $\beta 2$ subunits assemble in two pentameric stoichiometries, $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$, each with distinct pharmacological signatures; $(\alpha 4)_3(\beta 2)_2$ receptors are strongly potentiated by the drug NS9283, whereas $(\alpha 4)_2(\beta 2)_3$ receptors are unaffected. Despite this stoichiometry-selective pharmacology, the molecular identity of the target for NS9283 remains elusive. Here, studying $(\alpha 4)_3(\beta 2)_2$ receptors, we show that mutations at either the principal face of the $\beta 2$ subunit or the complementary face of the $\alpha 4$ subunit prevent NS9283 potentiation of ACh-elicited single channel currents, suggesting the drug targets the $\beta 2$ - $\alpha 4$ pseudo-agonist sites, the $\alpha 4$ - $\alpha 4$ agonist site, or both sites. To distinguish among these possibilities, we generated concatemeric receptors with mutations at specified subunit interfaces, and monitored the ability of NS9283 to potentiate ACh-elicited single channel currents. We find that a mutation at the principal face of the $\beta 2$ subunit at either $\beta 2$ - $\alpha 4$ pseudo-agonist site suppresses potentiation, whereas mutation at the complementary face of the $\alpha 4$ subunit at the $\alpha 4$ - $\alpha 4$ agonist site allows significant potentiation. Thus monitoring potentiation of single concatemeric receptor channels reveals that the $\beta 2$ - $\alpha 4$ pseudo-agonist sites are required for stoichiometry-selective drug action. Together with the recently determined structure of the $(\alpha 4)_3(\beta 2)_2$ receptor, the findings have implications for structure-guided drug design.

Introduction-

Nicotinic acetylcholine receptors (AChRs) are found in the central and peripheral nervous systems, and trigger moment-to-moment neuronal excitation and modulate release of neurotransmitters [1,2]. They are implicated in a variety of neurological disorders [2,3], participate in nicotine addiction [4–6], and are targets for a host of inhibitors and activators, both man-made and occurring in nature. AChRs belong to the large family of Cys-loop receptors and are pentamers formed from homologous subunits of which there are many different subtypes. At the neuromuscular junction the AChR contains four types of subunits that assemble in a fixed stoichiometry and arrangement [7], whereas in the brain, there are many different types of subunits that assemble in a variety of combinations and stoichiometry [1]. The majority of [³H] nicotine binding in the brain is associated with receptors containing $\alpha 4$ and $\beta 2$ subunits [8–11]. These subunits assemble in two pentameric stoichiometries, one with three and the other with two $\alpha 4$ subunits [12–15]. These stoichiometric variants exhibit distinct functional and pharmacological properties. In particular, receptors with three $\alpha 4$ subunits are strongly potentiated by the drug NS9283, whereas receptors with two $\alpha 4$ subunits are unaffected [14,16]. This subunit-selective pharmacology could arise through creation of a drug binding site through exchange of an $\alpha 4$ for a $\beta 2$ subunit, or through changes in inter-subunit interactions. To identify structures required for drug potentiation, studies of receptors with known stoichiometry and subunit arrangement are necessary.

Previously, we generated enriched populations of receptors with either three $\alpha 4$ and two $\beta 2$ subunits or two $\alpha 4$ and three $\beta 2$ subunits by transfecting cells with biased ratios of cDNAs encoding the subunits, and recorded ACh-elicited single channel currents

from receptors with each stoichiometry [14]. In addition, we established that pentameric concatemers recapitulated single channel biophysical properties of receptors formed from biased ratios of unlinked subunits. In particular, in the presence of ACh alone, concatemeric receptors with three nonconsecutive $\alpha 4$ subunits and two $\beta 2$ subunits activate predominantly as single channel openings flanked by long closings, whereas in the presence of ACh and NS9283, they activate in a series of many channel openings in quick succession [14]. Here, studying receptors formed from biased ratios of the subunits, we find that a mutation at either the principal face of the $\beta 2$ subunit or the complementary face of the $\alpha 4$ subunit prevents potentiation by NS9283. However, in both cases the mutation was present in multiple copies per receptor. Using concatemeric receptors formed from five subunits linked head to tail, we introduce a single copy of each mutant subunit per receptor, record ACh-elicited single currents, and identify the type, number and location of subunits required for drug potentiation.

Materials and Methods-

Mutagenesis and Expression of human $(\alpha 4)_3(\beta 2)_2$ AChRs formed from unlinked and linked subunits.

cDNAs encoding unlinked $\alpha 4$ and $\beta 2$ subunits, or linked $\beta 2$ - $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\alpha 4$ subunits, and the chaperone protein 14-3-3 were individually sub-cloned into a modified pCI mammalian expression vector (Promega), as previously described [17,18]. The 14-3-3 chaperone increases expression of $\alpha 4\beta 2$ AChRs formed by unlinked as well as linked subunits [17,19,20]. BOSC 23 cells, a cell line derived from HEK 293 cells [21], were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum, and transfected by calcium phosphate precipitation, as previously described [22–24]. For experiments with receptors formed from unlinked subunits, the stoichiometry was biased towards $(\alpha 4)_3(\beta 2)_2$ by transfecting cells with a 10:1:10 ratio of $\alpha 4$, $\beta 2$, and 14-3-3 cDNAs. Varying the ratio of $\alpha 4$ to $\beta 2$ subunit cDNAs biases the receptor population toward a single subunit stoichiometry in both mammalian cell lines [15,25] and *Xenopus laevis* oocytes [13]. The amounts of transfected $\alpha 4$ and $\beta 2$ cDNAs were respectively 3 and 0.3 μg for each 35 mm culture dish of cells. A cDNA encoding green fluorescent protein was included in all transfections. Transfections were carried out for 4 to 16 hours, followed by medium exchange. Single channel recordings were made 48-72 hours post-transfection. For experiments with receptors formed from linked $\beta 2$ - $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\alpha 4$ subunits, the total amount of cDNA was 1-10 μg per 35 mm culture dish. Furthermore, following transfection at 37°C, the cells were incubated at 30°C until use, which increases expression of receptors on the cell surface [26]. Single channel recordings from cells expressing receptors formed from linked subunits were made 72 to 96 hours post-transfection. Mutations were installed in

cDNAs encoding unlinked and linked subunits and confirmed by sequencing as described previously [13,27,28].

Drugs-

Acetylcholine (ACh) was purchased from Sigma-Aldrich (St Louis, MO, USA), and 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzotrile (NS9283) from Tocris (UK).

Patch clamp recordings-

Single-channel recordings were obtained in the cell-attached patch configuration at a membrane potential of -70 mV and a temperature of 20°C , as previously described (14, 15, 16). For all experiments, the extracellular bathing solution contained (mM): 142 KCl, 5.4 NaCl, 1.8 CaCl_2 , 1.7 MgCl_2 , and 10 HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained (mM): 80 KF, 20 KCl, 40 K-aspartate, 2 MgCl_2 , 1 EGTA, 10 HEPES, adjusted to pH 7.4 with KOH [31,32]. Concentrated stock solutions of ACh were made in pipette solution and stored at -80°C until the day of each experiment. A concentrated stock solution of NS9283 was prepared in DMSO, stored at -80°C , and added to the pipette solution the day of each experiment. Pipette solution without NS9283 contained an equivalent volume of DMSO. Patch pipettes were pulled from glass capillary tubes (No.7052, Garner Glass) and coated with Sylgard (Dow Corning).

Data Analysis-

Single-channel currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices), with a gain of 100 mV/pA and the internal Bessel filter at 10 kHz. Currents were sampled at intervals of 20 μs using a PCI-6111E acquisition card (National Instruments), and recorded to hard disk using the program Acquire (Bruxon Corporation). Channel opening and closing transitions were determined using the program TAC 4.2.0

(Bruxton Corporation), which digitally filters the data (Gaussian response, final effective bandwidth 5 kHz), interpolates the digitized points using a cubic spline function, and detects channel openings using the half-amplitude threshold criterion, as previously described [33]. Following detection, each single channel open dwell time was plotted against its time of occurrence during the recording **in order to assess** the stability of the channel opening frequency and gating kinetics.

To determine single channel current amplitudes, the variable amplitude option in TAC was used, whereas to determine open and closed dwell times, the fixed amplitude option was used. Dwell time histograms were plotted using a logarithmic abscissa and square root ordinate [34], with a uniformly imposed dead time of 40 μ s, and the sum of exponentials was fitted to the data by maximum likelihood using the program TACFit 4.2.0 [33]. Clusters of channel openings were identified as a series of closely spaced openings preceded and followed by closed intervals longer than a specified critical duration (τ_{crit}). This duration was taken as the point of intersection between consecutive components in the closed time histogram and ranged between 1 and 50 ms. A cluster duration therefore comprises the total open time of a series of openings plus that of the intervening closings briefer than τ_{crit} .

The effect of NS9283 on the probability a channel will re-open was quantified by plotting the fraction of channel opening episodes with greater than N openings against the number of openings per episode. A channel opening episode was defined as a series of one or more openings separated by closings shorter than τ_{crit} . The re-opening distributions were fitted with exponential decay functions, and an F-test was used to determine whether a single or a bi-exponential decay best described the re-opening data; a single-exponential

decay was preferred unless the sum-of-squares F-test had a p-value less than 0.01. Both the exponential fitting and F-test were carried out using the Prism software package (GraphPad Software). In addition, the mean number of openings per episode was calculated as the reciprocal of the decay constant, and an F-test was used to determine whether the fitted decay constants and fractional areas differed significantly between pairs of recordings under different experimental conditions. Parameters were considered significantly different if the p-value was less than 0.01.

Results-

Overview-

The present work identifies sites required for NS9283 potentiation through a combination of single channel recording, mutations of candidate drug sites and concatemeric receptors. First we establish experimental conditions to demonstrate, and analysis methods to quantify, potentiation of $(\alpha 4)_3(\beta 2)_2$ receptors by NS9283 at the level of single channel currents. Second we define concentrations of ACh and NS9283 that maximize potentiation. Third we determine the impact on potentiation of mutations at either the principal face of the $\beta 2$ or the complementary face of the $\alpha 4$ subunit in receptors formed from unlinked subunits. Finally we determine the impact on potentiation of a single copy of each mutation in concatemeric receptors composed of five subunits linked head to tail.

NS9283 potentiation at the single channel level-

To generate an enriched population of AChRs with three $\alpha 4$ and two $\beta 2$ subunits, we transfected BOSC 23 cells, a variant of the 293 HEK cell line, with a 10:1 ratio of cDNAs encoding the $\alpha 4$ and $\beta 2$ subunits, as described previously [14]. Using a patch pipette filled with extracellular solution containing a known concentration of ACh, we established a gigaohm seal to a cell and recorded single channel currents in the cell-attached patch configuration. The recordings reveal a single conductance class of channel openings with 4.1 ± 0.3 pA unitary current amplitude (Fig. 1A), an electrical signature indicating receptors with three $\alpha 4$ and two $\beta 2$ subunits [14]. In the presence of ACh alone, the majority of channel openings appears as solitary current pulses flanked by long periods of baseline current (Fig. 1B), while a minority appears as several pulses in quick succession, which we

call bursts (Fig. 1D). By contrast, in the presence of ACh and NS9283, the majority of channel openings appears as a series of current pulses in quick succession (Fig. 1C), which we call clusters (Fig. 1D), while a minority appears as solitary current pulses flanked by long periods of baseline current. Thus, qualitatively, NS9283 potentiates $(\alpha 4)_3(\beta 2)_2$ receptors by enhancing the ability of a receptor channel that just closed to reopen [14].

Dwell time analysis of un-potentiated channel openings-

To quantify potentiation, we developed methods to distinguish un-potentiated from potentiated channel openings. The first step was to analyze histograms of closed dwell times from recordings obtained in the presence of ACh alone. A representative histogram, obtained from a recording in the presence of 10 μ M ACh, is well fitted by the sum of exponentials: two major components with brief and long mean durations, and a third minor component with intermediate duration. The components with brief and intermediate mean durations represent closings within bursts of openings by the same receptor channel, whereas the component with longest mean duration represents closings between both solitary and bursts of openings primarily from different receptor channels (Fig. 1B). To distinguish solitary from bursts of channel openings, we established a discriminating closed time, τ_{crit} , from the point of intersection between the components with longest and intermediate mean durations. For the recording illustrated in Fig. 1B, the discriminating closed time was 0.7 ms. However, owing to different numbers of receptors in each patch, the component with long mean duration varied, and in patches with low opening frequency, or in which the opening frequency declined as the recording progressed, the discriminating closed time extended to 1 ms. Thus for uniformity, we

applied a discriminating closed time of 1 ms to distinguish solitary from bursts of channel openings. After assigning each channel opening to one of the two classes, we find that some 67 % of openings are solitary channel openings, while 33 % are bursts of channel openings. In addition, for both classes of channel openings, the open time histogram contains three exponential components with mean durations and relative areas that are similar between the two classes (Fig. 1B). Thus although the two classes of openings are readily distinguished by their flanking closed dwell times, their open time distributions are very similar. This analysis of channel openings in the presence of ACh alone provides a frame of reference to distinguish potentiated channel openings in the presence of ACh and NS9283.

Dwell time analysis of potentiated channel openings-

In the presence of submaximal concentrations of ACh and NS9283, a mixture of potentiated and un-potentiated channel openings is expected. Un-potentiated channel openings comprise either solitary or bursts of openings, as just described, whereas potentiated openings comprise clusters of many successive channel openings. Thus we devised a three-step procedure to distinguish potentiated from un-potentiated channel openings. In the first step, openings separated by closings briefer than 1 ms were joined to yield composite events composed of N openings and N-1 closings; a portion of these composite events corresponds to un-potentiated openings, whereas the remaining portion corresponds to potentiated openings (Fig. 1C; [Table 1](#)). In the second step, to distinguish composite events that are potentiated from those that are un-potentiated, the closed duration histogram was again fitted by the sum of exponentials, and a discriminating closed time of 30 ms was determined from the point of intersection between successive exponential components, by

analogy to the analysis described for recordings in the presence of ACh alone; composite events flanked by closings briefer than 30 ms were classified as potentiated, whereas composite events flanked by closings longer than 30 ms were classified as un-potentiated (Fig. 1D). In the third step, the two classes of composite events were separated, closings briefer than 1 ms that were masked initially were re-introduced, and a histogram of open durations was generated for each class of channel openings (Fig. 1C, shaded and un-shaded histograms). The number of channel openings in the potentiated and un-potentiated classes was then determined from the total number of openings in the histogram for each class. The analysis reveals that in the presence of 10 μ M ACh and 10 μ M NS9283, some 58 % of channel openings are potentiated (Fig. 1C, shaded portion of the bar; [Table 1](#)). In addition, openings in both the potentiated and un-potentiated classes contain three exponential components with mean durations that are similar between the two classes. Thus NS9283 does not affect open to closed transitions, but instead increases both the probability and rate of closed to open transitions.

Potentiation by NS9283 as a function of ACh concentration-

To determine the functional consequences of mutations of candidate targets for NS9283, we first established experimental conditions that maximize potentiation. Thus we recorded single channel currents in the presence of a maximally effective concentration of NS9283, 30 μ M, but with a range of ACh concentrations. We then quantified the percentage of potentiated and un-potentiated channel openings, as just described. In the presence of 1 μ M ACh alone, a minimum concentration to elicit channel opening, the majority of openings are solitary events flanked by long closings (Fig. 2A), while a minority are bursts of several

openings in quick succession. Because both classes of channel openings are un-potentiated, for clarity they are combined into a single class here and in subsequent sections (Fig. 2A, un-shaded bar). In the presence of 1 μM ACh and NS9283, the majority of channel openings appear as clusters of many openings flanked by brief closings, and a minority appear as solitary or bursts of openings flanked by long closings (Fig. 2B). After applying discriminating closed times to distinguish potentiated from un-potentiated channel openings, we find that potentiated openings comprise some 61 % of all openings (Fig. 2B, shaded portion of the bar; [Table 1](#)). Furthermore, open duration histograms for both potentiated and un-potentiated openings contain three exponential components, which again mirror those observed in the presence of ACh alone (Fig. 2A). Thus in the presence of a minimal concentration of ACh and a maximal concentration of NS9283, a substantial percentage of the channel openings is potentiated.

Increasing the ACh concentration, while maintaining a maximal concentration of NS9283, yields channel openings and closings that qualitatively mirror those in the presence of lower concentrations of ACh (Fig. 2C-I). In the presence of 10 and 50 μM ACh, the percentage of potentiated channel openings increases to 78 and 74 %, respectively (Fig. 2D,F; [Table 1](#)). However, in the presence of 100 μM ACh, the percentage of potentiated channel openings declines to 55 % (Fig. 2I; [Table 1](#)); this decline may arise from enhanced desensitization of potentiated relative to un-potentiated channel openings owing to the increased ACh concentration. These results establish concentrations of ACh and NS9283 that maximize potentiation.

Mutations in $\alpha 4$ and $\beta 2$ subunits block potentiation-

The recent cryo-EM structure of the $(\alpha 4)_3(\beta 2)_2$ receptor is shown in Fig. 3A,B. There are two $\alpha 4$ - $\beta 2$ subunit interfaces that form orthosteric agonist binding sites, one $\alpha 4$ - $\alpha 4$ subunit interface that forms a third orthosteric site, and two $\beta 2$ - $\alpha 4$ subunit interfaces that form pseudo-agonist sites. The agonist nicotine, included during preparation of the receptor protein for cryo-EM, is present at the $\alpha 4$ - $\beta 2$ and $\alpha 4$ - $\alpha 4$ interfaces, but not at the $\beta 2$ - $\alpha 4$ interfaces. When each subunit interface is viewed from the side, the subunit on the left forms the principal face, while the subunit on the right forms the complementary face. The principal face contains structural motifs known as loops A, B and C, from which conserved aromatic residues extend into the binding pocket. The complementary face contains loops D, E, F and G, from which aromatic, hydrophobic, polar and anionic residues extend into the pocket. Furthermore, each pair of $\alpha 4$ - $\beta 2$ and $\beta 2$ - $\alpha 4$ subunits is asymmetric owing to differences in the flanking subunits; one $\alpha 4$ - $\beta 2$ pair is flanked by $\beta 2$ and $\alpha 4$ subunits, and the other pair is flanked $\alpha 4$ and $\alpha 4$ subunits; one $\beta 2$ - $\alpha 4$ pair is flanked by $\alpha 4$ and $\alpha 4$ subunits, and the other pair is flanked by $\alpha 4$ and $\beta 2$ subunits (Fig. 3A, right panel).

To identify subunit interfaces required for NS9283 potentiation, we generated mutations at either the complementary face of the $\alpha 4$ subunit or the principal face of the $\beta 2$ subunit. Previous work showed that the mutation $\alpha 4$ H142V, either alone or combined with mutations of two nearby residues, prevented NS9283 potentiation of ACh-elicited macroscopic currents [35–37]. Our choice of the mutation $\beta 2$ W176A was based on the structures of the $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$ receptors in which $\beta 2$ W176 establishes close contact with $\alpha 4$ H142 at each $\beta 2$ - $\alpha 4$ subunit interface (Fig. 3B, right panel). Each mutant

subunit was co-transfected with the complementary wild type subunit using biased ratios of subunit cDNAs to promote expression of $(\alpha 4)_3(\beta 2)_2$ receptors. In the presence of 50 μM ACh and 30 μM NS9283, receptors comprised of wild type subunits open in clusters of many channel openings flanked by brief closings, with some 74 % of channel openings classified as potentiated (Fig. 3C, shaded bar and histogram; [Table 1](#)), as in Fig. 2F. In addition, to provide a second measure of potentiation, a histogram of cluster durations was generated; clusters were defined as a series of channel openings separated by closings shorter than 30 ms. The histogram of cluster durations is well described as the sum of four major exponential components, with the component with longest mean duration representing maximally potentiated channel openings (Fig. 3C).

For receptors containing the mutant $\alpha 4\text{H142V}$, the two measures of NS9283 potentiation are markedly reduced (Fig. 3D). The percentage of channel openings classified as potentiated falls below our limits of detection, and the exponential component of clusters with longest mean duration is eliminated. Because the mutation is present at the $\alpha 4$ - $\alpha 4$ subunit interface, as well as the two $\beta 2$ - $\alpha 4$ interfaces, one or both types of interfaces are required for NS9283 potentiation.

For receptors containing the mutant $\beta 2\text{W176A}$, the two measures of potentiation are also markedly reduced (Fig. 3E; [Table 1](#)). The percentage of channel openings classified as potentiated falls from 74 % to 10 %, and the exponential component of clusters with longest mean duration appears as a small tail rather than a distinct component. Because the mutant $\beta 2$ subunit is present at only $\beta 2$ - $\alpha 4$ subunit interfaces, one or both of these interfaces is required for NS9283 potentiation.

Mutations of individual $\alpha 4$ and $\beta 2$ subunits in pentameric concatemers-

Previously, we showed that NS9283 strongly potentiated ACh-elicited single channel currents from concatemeric receptors composed of three non-consecutive $\alpha 4$ and two $\beta 2$ subunits [14]. Here, in the presence of optimal concentrations of ACh and NS9283, the same concatemeric receptors activate as clusters of many channel openings separated by brief closings (Fig. 4A, B), which mirror potentiated channel openings by $(\alpha 4)_3(\beta 2)_2$ receptors formed from unlinked subunits. The analysis to quantify the percentage of potentiated and un-potentiated channel openings reveals that for concatemeric receptors in the presence of optimal concentrations of ACh and NS9283, some 81 % of channel openings are potentiated (Fig. 4B; **Table 1**). In addition, the histogram of cluster durations contains a fourth, prolonged component representing maximally potentiated channel openings. Thus receptors formed from concatemeric subunits recapitulate key measures of potentiation observed for receptors formed from unlinked subunits.

We then installed the $\beta 2W176A$ mutation at either of the two $\beta 2$ - $\alpha 4$ subunit interfaces of the concatemer. Recordings in the presence of optimal concentrations of ACh and NS9283 show predominantly solitary channel openings flanked by long closings, and the percentage of potentiated channel openings markedly decreases; when the $\beta 2W176A$ mutation is present at one of the $\beta 2$ - $\alpha 4$ interfaces, the percentage of potentiated channel openings decreases to 16 %, whereas when the mutation is present at the other $\beta 2$ - $\alpha 4$ interface, the percentage of potentiated channel openings decreases to 29 % (Figs. 4C, D; **Table 1**). In addition, for each of the receptors containing a single $\beta 2W176A$ mutation, the fourth, prolonged component of clusters is eliminated. In a third construct containing the $\beta 2W176A$ mutation at both $\beta 2$ - $\alpha 4$ subunit interfaces, the percentage of potentiated

channel openings decreases to 14 %, and the fourth, prolonged component of clusters is eliminated (Fig. 4E; Table 1).

When a mutation is generated in only one subunit of a concatemer, the particular subunit interface to which the mutation localizes depends on whether the subunits assemble in a clockwise or anticlockwise direction. However, in constructs containing a mutant $\beta 2$ subunit, an $\alpha 4$ subunit is present on both sides, so that regardless of the direction in which the subunits assemble, the mutation localizes to a $\beta 2$ - $\alpha 4$ interface. Thus mutating the principal face of the $\beta 2$ subunit markedly suppresses potentiation by NS9283, showing that both $\beta 2$ - $\alpha 4$ subunit interfaces are required for potentiation.

We next installed the $\alpha 4$ H142V mutation into the concatemer at the presumed $\alpha 4$ - $\alpha 4$ subunit interface, and in the presence of optimal concentrations of ACh and NS9283, observed plentiful clusters of channel openings (Fig. 4F). The percentage of channel openings classified as potentiated decreases compared to that for the concatemer comprised of wild subunits, but some 55 % of channel openings remain drug potentiated (Fig. 4F, shaded bar and histogram; Table 1). Also, a prolonged component of cluster durations is present with a mean duration similar to that for the concatemer composed of wild type subunits. If the subunits assembled in a clockwise direction, the $\alpha 4$ H142V mutation would be located at the $\alpha 4$ - $\alpha 4$ interface, whereas if they assembled in an anti-clockwise direction the mutation would be located at the $\beta 2$ - $\alpha 4$ interface. Nevertheless, receptors containing the $\alpha 4$ H142V mutation still potentiate, suggesting one of two scenarios. If the mutation is at the $\alpha 4$ - $\alpha 4$ interface this interface is not required for

potentiation, or if the mutation is at the $\beta 2$ - $\alpha 4$ interface, the mutated residue is not required.

Channel re-opening as a measure of drug potentiation: receptors formed from unlinked subunits-

To illustrate channel re-opening as a measure of drug potentiation, Fig. 5A shows recording **segments** from the wild type $(\alpha 4)_3(\beta 2)_2$ receptor formed from unlinked subunits in the presence of either ACh alone or ACh plus NS9283. In the presence of ACh alone, one channel opening episode comprises 3 successive openings and another just one opening, whereas in the presence of ACh and NS9283, one channel opening episode comprises 7 openings and another 17 openings. Thus to further quantify potentiation, we plotted the fraction of channel opening episodes with greater than N openings against the number of openings per episode, in either the presence of ACh alone or in the presence of ACh and NS9283. For a recording in the presence of ACh alone, the re-opening plot decays bi-exponentially; the mean of the major component is 0.7 re-openings per episode, while the mean of the minor component is 2.8 re-openings per episode (Fig. 5B; Table 2). However, in the presence of ACh and NS9283, the mean of the major component is 10 re-openings per episode, while the mean of the minor component is 2 re-openings per episode. In addition, NS9283 alters the fractional weights of the two components; the weight of the component with fewest openings per episode declines from 71 % to 59 %, while that of the component with greatest openings per episode increases from 29 % to 41 % (Table 2). Application of the F-test reveals that the means and fractional weights of the two components are significantly different without **versus** with NS9283 (Materials and Methods; Table 2). Because the component with a mean of 10 re-openings per episode is

observed only in the presence of ACh and NS9283, we conclude it corresponds to potentiated channel openings. These determinations of channel re-opening in Fig. 5B mirror those reported previously for wild type $(\alpha 4)_3(\beta 2)_2$ receptors formed from unlinked subunits [14].

For receptors containing either the $\alpha 4H142V$ or $\beta 2W176A$ mutations in the presence of ACh alone, channel re-opening is similar to that observed for the wild type receptor (Fig. 5 B). However, for the $\alpha 4H142V$ mutant receptor in the presence of ACh and NS9283, channel re-opening is similar to that in the presence of ACh alone, and statistical analyses show that the fitted parameters do not differ without or with NS9283 (Table 2). Similarly, for the $\beta 2W176A$ mutant receptor in the presence of ACh and NS9283, channel re-opening does not increase relative to that in the presence ACh alone; in fact channel re-opening is modestly reduced in the presence of NS9283 (Table 2). These results, obtained for receptors formed from unlinked subunits, confirm that a mutation in either the $\alpha 4$ or the $\beta 2$ subunit suppresses potentiation. However, because the receptors are formed from unlinked subunits, each mutation is present at multiple subunit interfaces per receptor.

Channel re-opening as a measure of drug potentiation: receptors formed from linked subunits-

In the presence of ACh alone, the pentameric $(\alpha 4)_3(\beta 2)_2$ concatemer composed of wild type subunits exhibits two exponential components of channel re-opening with means of 0.5 and 2.7 re-openings per episode (Fig. 5C; Table 2), similar to that observed for wild type receptors formed from unlinked subunits (Fig. 5B). In the presence of ACh and NS9283, the two components show means of 1.7 and 7.7 re-openings per opening episode, which again

are similar to those observed for wild type receptors formed from unlinked subunits (Table 2). Statistical comparison of the re-opening plots, without **versus** with NS9283, reveals that the decay rates and relative weights of the two components differ significantly with $p < 0.01$ (Table 2), as observed for receptors formed from unlinked subunits. For the concatemer with the $\beta 2W176A$ mutation at either of the $\beta 2-\alpha 4$ subunit interfaces, channel re-opening in the presence of NS9283 approaches that in the presence of ACh alone, but this depends on the subunit interface that contains the mutation. When the $\beta 2W176A$ mutation is present at one of the $\beta 2-\alpha 4$ interfaces, the major and minor components show means of 0.6 and 2.9, which do not differ significantly from those in the presence of ACh alone (Table 2), indicating essentially complete suppression of potentiation. On the other hand, when the mutation is at the other $\beta 2-\alpha 4$ interface, the means are 1.3 and 3.9 re-openings per episode (Table 2), which differ significantly from that in the presence of ACh alone, so that although potentiation is reduced it remains significant. Furthermore, when the $\beta 2W176A$ is present at both $\beta 2-\alpha 4$ subunit interfaces, re-opening is reduced further, with means of 0.6 and 2.3 re-openings per episode for the major and minor components; however statistical comparison of the fitted parameters is precluded because in the presence of ACh alone re-opening is described by a single exponential decay, whereas in the presence of ACh and NS9283 re-opening is described as a double exponential decay (Table 2). By contrast, for the concatemer containing the $\alpha 4H142V$ mutation at the presumed $\alpha 4-\alpha 4$ subunit interface, NS9283 still increases channel re-opening, although the extent of the increase is reduced compared to that of the wild type concatemer; the major and minor components show means of 1.2 and 5.4 re-openings per episode, which differ significantly from that in the presence of ACh alone (Table 2). Thus as measured by channel re-opening, the

β 2W176A mutation suppresses potentiation to a greater extent than the α 4H142V mutation.

Discussion-

The diversity of nicotinic AChR subunits, together with their ability to assemble in different combinations, enables a wide variety of functional and pharmacological signatures. A major challenge in the field is to understand how the combination and stoichiometry of subunits endow a receptor with its signature function and pharmacology. This understanding is required not only to understand receptor-mediated neuronal signaling, but also to design therapeutic drugs to target a particular signaling pathway. It is this overall context that frames the present study.

Previous studies showed that the drug NS9283 selectively potentiates heteromeric nicotinic AChRs containing either $\alpha 2$ or $\alpha 4$ subunits, but not $\alpha 3$ or $\alpha 7$ subunits [53]. Studies in laboratory animals showed that NS9283 enhances cognitive function, and the enhancement was blocked by the cholinergic antagonist mecamylamine [53]. NS9283 also attenuated nicotine self-administration and reinstatement, and **it had no** effect when sucrose was substituted for nicotine [38]. Cholinergic agonists such as nicotine and epibatidine are potent analgesics, but they suffer from side effects. However, administration of the synthetic nicotinic agonist ABT 595 together with NS9283 enhanced the potency of this agonist against nociception [39], providing **a** rationale for combination therapy. Owing to their selective ability to target AChRs with particular subunits, potentiators such as NS9283 are promising drugs to treat cognitive impairment, nicotine dependence and nociception. Thus defining molecular targets of AChR potentiators are essential steps toward developing more potent and target-selective drugs.

Seminal studies showed that when $\alpha 4$ and $\beta 2$ subunits were co-expressed, the agonist dose-response relationship, based on recordings of voltage clamped macroscopic

current, exhibited high and low sensitivity components [12,13,15,40]. The high sensitivity component was shown to arise from receptors with two $\alpha 4$ and three $\beta 2$ subunits, whereas the low sensitivity component was shown to arise from receptors with three $\alpha 4$ and two $\beta 2$ subunits [13,17]. In addition, receptors with low sensitivity exhibited robust potentiation by the drug NS9283, whereas receptors with high agonist sensitivity were unaffected by the drug [14,16,35,41]. Thus for a receptor with composition $(\alpha 4)_3(\beta 2)_2$, a third $\alpha 4$ subunit in place of a $\beta 2$ subunit endowed drug sensitivity. Insight into this stoichiometry-specific drug sensitivity emerged from mutagenesis studies showing that mutations at the complementary face of the $\alpha 4$ subunit abolished potentiation [35,36,42]. An appealing interpretation was that NS9283 bound to the $\alpha 4$ - $\alpha 4$ subunit interface, and the mutation prevented drug binding and thus blocked potentiation. Thus a co-agonist mechanism emerged in which the agonist binds to the two $\alpha 4$ - $\beta 2$ interfaces, and potentiation resulted from binding of NS9283 to the $\alpha 4$ - $\alpha 4$ interface [36]. However, an equally plausible interpretation is that the agonist binds to the $\alpha 4$ - $\beta 2$ and $\alpha 4$ - $\alpha 4$ interfaces, while NS9283 binds to the $\beta 2$ - $\alpha 4$ interfaces. The present work distinguishes between these two interpretations, showing that although a third $\alpha 4$ subunit is necessary for NS9283 potentiation, a mutation at the presumed $\alpha 4$ - $\alpha 4$ interface still permits potentiation. By contrast, a mutation at either of the two $\beta 2$ - $\alpha 4$ interfaces markedly curtails potentiation. Because the agonist does not bind to the $\beta 2$ - $\alpha 4$ interface, as shown by recent crystal and cryo-EM structures of the $\alpha 4\beta 2$ receptor with bound nicotine [43,44], NS9283 could bind to the $\beta 2$ - $\alpha 4$ interfaces and potentiate through a mechanism analogous to benzodiazepine potentiation of GABA_A receptors [45–48].

The $\beta 2$ - $\alpha 4$ subunit interface harbors four of the five conserved aromatic residues present at the $\alpha 4$ - $\beta 2$ subunit interface that forms the orthosteric ligand binding site. However, the $\beta 2$ - $\alpha 4$ interface also harbors an Arg residue, analogous to a bound agonist, and thus may be considered a pseudo-agonist site [43,49]. For receptors formed from unlinked subunits, when the mutant $\alpha 4$ H142V subunit is co-expressed with the $\beta 2$ subunit, the mutation is present at the $\alpha 4$ - $\alpha 4$ as well as the two $\beta 2$ - $\alpha 4$ interfaces. By contrast, when the mutant $\beta 2$ W176A subunit is co-expressed with the $\alpha 4$ subunit, the mutation is present only at the $\beta 2$ - $\alpha 4$ interfaces. Because both the $\beta 2$ W176A and $\alpha 4$ H142V mutations markedly curtail potentiation in receptors formed from unlinked subunits, a possible interpretation is that the subunit interface common to both mutations, $\beta 2$ - $\alpha 4$, is required for NS9283 potentiation. To test this possibility, we studied receptors formed from concatemeric subunits.

For a receptor comprised of five covalently linked subunits, there is the issue of whether the subunits assemble in a clockwise or counter-clockwise direction. A recent study based on the ability of NS9283 to potentiate concatemeric receptors with mutations in individual subunits suggested the subunits assembled in a counter-clockwise direction [50]. On the other hand, studies of the effects of single residue mutations [18,51–53], and the ability of agonists to protect against covalent reaction **of a methanethiosulfonate reagent with** a substituted cysteine [54], suggested the subunits assembled in a clockwise direction. Thus to interpret our findings, we consider both scenarios of subunit assembly. In a $\beta 2$ - $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\alpha 4$ concatemer, if the subunits assemble clockwise, a mutation at the complementary face of the penultimate $\alpha 4$ subunit will be located at the $\alpha 4$ - $\alpha 4$ interface, whereas if they assemble counter-clockwise, the mutation will be located at a $\beta 2$ - $\alpha 4$

interface. The two possible locations of the mutation arise because the mutant $\alpha 4$ subunit is flanked by a $\beta 2$ subunit on one side and an $\alpha 4$ subunit on the other side. Conversely, a mutation at the principal face of a $\beta 2$ subunit will always be located at a $\beta 2$ - $\alpha 4$ interface because each $\beta 2$ subunit is flanked by two $\alpha 4$ subunits. Thus our conclusion that the $\beta 2$ - $\alpha 4$ interface is required for potentiation is independent of the direction of subunit assembly.

Our studies of concatemeric receptors show that each of the $\beta 2$ - $\alpha 4$ subunit interfaces is required for potentiation, and that the contribution of each interface to potentiation is not equivalent. This functional asymmetry may originate from differences in the subunits that flank the $\beta 2$ - $\alpha 4$ interfaces, as suggested in previous work in which mutations at nominally equivalent $\alpha 4$ - $\beta 2$ subunit interfaces had different functional consequences [25,52]. For the $\beta 2$ - $\alpha 4$ interface flanked by $\alpha 4$ and $\beta 2$ subunits, the $\beta 2$ W176A mutation reduces the percentage of channel openings that are potentiated from 81 to 29. By contrast, for the $\beta 2$ - $\alpha 4$ interface flanked by two $\alpha 4$ subunits, the $\beta 2$ W176A mutation reduces the percentage of openings that are potentiated from 81 to 16. When the $\beta 2$ W176A mutation is present at both $\beta 2$ - $\alpha 4$ interfaces, the percentage of potentiated channel openings is reduced to 14, close to the reduction to 10 percent observed when the $\beta 2$ W176A mutation is incorporated into receptors formed from unlinked subunits. In these three mutant receptors, the residual potentiation may arise from an incomplete effect of mutating only one residue at the $\beta 2$ - $\alpha 4$ interface.

We also find that the mutation $\alpha 4$ H142V prevents potentiation in receptors formed from unlinked subunits; in these receptors the mutation is present at each of the $\beta 2$ - $\alpha 4$ interfaces, as well as the $\alpha 4$ - $\alpha 4$ interface. However, when the $\alpha 4$ H142V mutation is

incorporated into the presumed $\alpha 4$ - $\alpha 4$ interface of a concatemeric receptor, the percentage of channel openings that is potentiated is still 55 percent. In addition, two other measures of NS9283 potentiation are preserved; the exponential component of clusters with prolonged mean duration remains, and channel re-opening is increased relative to that in the presence of ACh alone. Further studies are required to determine whether the $\alpha 4$ H142V mutation is located at the $\alpha 4$ - $\alpha 4$ versus the $\beta 2$ - $\alpha 4$ interface. By contrast, our results show that potentiation is essentially eliminated when the $\beta 2$ W176A mutation is present at the $\beta 2$ - $\alpha 4$ interface.

Although our results demonstrate NS9283 potentiation requires the $\beta 2$ - $\alpha 4$ interface, each receptor stoichiometry, $(\alpha 4)_3(\beta 2)_2$ and $(\alpha 4)_2(\beta 2)_3$, contains two copies of this interface. The major difference between the two stoichiometries is an $\alpha 4$ subunit in place of a $\beta 2$ subunit. This exchange of a single subunit creates two subunit interfaces that differ between each stoichiometry: $\alpha 4$ - $\alpha 4$ and $\alpha 4$ - $\beta 2$ interfaces in the $(\alpha 4)_3(\beta 2)_2$ stoichiometry, versus $\alpha 4$ - $\beta 2$ and $\beta 2$ - $\beta 2$ interfaces in the $(\alpha 4)_2(\beta 2)_3$ stoichiometry. Thus the contributions of the $\beta 2$ - $\alpha 4$ interfaces to NS9283 potentiation may depend on subunit interfaces novel to the $(\alpha 4)_3(\beta 2)_2$ stoichiometry.

Studies of potentiation at the single channel level provide novel insights into the underlying mechanism. We find that the open channel lifetime contains three exponential components, indicating three stable open states, in both the presence and absence of NS9283. Thus potentiation does not affect transitions from open to closed states. On the other hand, potentiation increases the probability a channel that just closed will reopen, as well as the speed with which it reopens. Moreover, by distinguishing potentiated from un-

potentiated channel openings, our single channel measurements provide a quantitative measure of potentiation. In addition, potentiation is assessed from an increase in the mean duration of clusters of channel openings, as well as an increase in the number of channel re-openings per **opening** episode. Thus, by contrast to measurements of macroscopic currents, measurements of single channel currents not only distinguish between unpotentiated and potentiated receptor channel openings, but **they** also provide several quantitative measures of potentiation.

On the other hand, whether NS9283 affects slower processes such as desensitization or deactivation is best assessed through measurements of macroscopic currents. In fact, measurements of the time courses of desensitization onset and recovery show that NS9283 does not affect onset, and promotes only a modest slowing of recovery [16]. However, measurements of deactivation following a brief pulse of agonist reveal that NS9283 slows deactivation [16]. In accord with a slowing of deactivation, our results show that in the presence of ACh alone the receptor channel opens primarily as single isolated openings, whereas in the presence of NS9283 it opens in clusters of many successive openings. Thus, the overall findings from macroscopic and single channel current measurements suggest NS9283 stabilizes activatable receptor states.

Our results suggest NS9283 potentiates $(\alpha 4)_3(\beta 2)_2$ receptors through binding to the $\beta 2$ - $\alpha 4$ subunit interfaces. In support, W176 from the principal face of the $\beta 2$ subunit contacts H142 from the complementary face the $\alpha 4$ subunit [49], and together the two residues could contribute to an interfacial drug binding site. In addition, NS9283 did not compete against [^3H]-cytisine binding to rat cortical tissue [55], a region rich in $(\alpha 4)_3(\beta 2)_2$ receptors [40], suggesting it does not bind to the orthosteric sites. Nevertheless, the $\beta 2$ - $\alpha 4$

subunit interfaces might not be the binding sites for NS9283, but may instead serve as transduction elements for potentiation. Among the family of nicotinic receptors, and the larger family of pentameric ligand gated channels, sites for drug modulation have been identified in a variety of locations: the extracellular domain [35,45,47,56–58], transmembrane domain [59–61], and the junction between the two domains [35]. Atomic scale structures of drug-receptor complexes will be required to distinguish whether the focal determinants we have identified mediate drug binding or transduction of drug binding to potentiation.

Footnotes-

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Table 1- Potentiated versus un-potentiated channel openings for various types of AChRs in the presence of ACh and NS9283. Averaged proportions of potentiated versus un-potentiated openings are given for the indicated number of patches (n) along with \pm the Standard Error (S.E.). For each experimental condition, the total number of openings from n patches is indicated. The table summarizes results displayed in Figs. 1-4.

Receptor-type (number of patches)	[ACh]	[NS9283]	Total Openings (n)	Proportion of openings		S.E.
	μ M	μ M		Un-potentiated	Potentiated	
(α 4) ₃ (β 2) ₂ (n=3)	10	10	4895	0.42	0.58	\pm 0.01
(α 4) ₃ (β 2) ₂ (n=6)	1	30	2214	0.39	0.61	\pm 0.04
(α 4) ₃ (β 2) ₂ (n=4)	10	30	3462	0.22	0.78	\pm 0.06
(α 4) ₃ (β 2) ₂ (n=6)	50	30	27025	0.26	0.74	\pm 0.03
(α 4) ₃ (β 2) ₂ (n=4)	100	30	1885	0.45	0.55	\pm 0.02
(α 4) ₃ (β 2 ^{W176A}) ₂ (n=6)	50	30	1752	0.90	0.10	\pm 0.01
β 2 α 4 β 2 α 4 α 4 (n=6)	50	30	2776	0.19	0.81	\pm 0.04
β 2 ^{W176A} α 4 β 2 α 4 α 4 (n=6)	50	30	1403	0.84	0.16	\pm 0.01
β 2 α 4 β 2 ^{W176A} α 4 α 4 (n=8)	50	30	810	0.71	0.29	\pm 0.04
β 2 ^{W176A} α 4 β 2 ^{W176A} α 4 α 4 (n=10)	50	30	473	0.86	0.14	\pm 0.05
β 2 α 4 β 2 α 4 ^{H142V} α 4 (n=8)	50	30	2080	0.45	0.55	\pm 0.09

Table 2- NS9283 promotes ACh-elicited channel re-opening. Mean number of re-openings and the relative percentage of the corresponding phase are given along with the 95% confidence interval (95% IC). An F-test was used to determine whether a mono- or bi-phasic model best described the data, and to determine whether the fitted decay rates and fractional areas differed significantly without versus with NS9283. The table summarizes results displayed in Fig. 5.

Receptor-type (number of patches)	[ACh] μM	[NS9283] μM	Best model (p<0.01)	Mean re-opening (95% IC) [% phase (95% IC)]		Significance (p<0.01)		
				1 st phase	2 nd phase	1 st phase	2 nd phase	% 1 st phase
(α4) ₃ (β2) ₂ (n=6)	50	30	Biphasic	2 (1.9-2.2) [59 (56-62)]	10 (9.6-10.6) [41 (38-44)]	Yes	Yes	Yes
(α4) ₃ (β2) ₂ (n=4)	50		Biphasic	0.7 (0.69-0.73) [71 (69-72)]	2.8 (2.7-2.9) [29 (28-31)]			
(α4) ₃ (β2 ^{W176A}) ₂ (n=6)	50	30	Biphasic	0.57 (0.5-0.6) [60 (56-64)]	2.2 (2.0-2.3) [40 (36-44)]	Yes	n.d.	n.d.
(α4) ₃ (β2 ^{W176A}) ₂ (n=4)	50		One phase	0.63 (0.57- 0.71) [1]				
(α4 ^{H142V}) ₃ (β2) ₂ (n=17)	50	30	Biphasic	0.58 (0.5-0.6) [75 (66-81)]	2.6 (2.0-3.6) [25 (19-34)]	No	No	No
(α4 ^{H142V}) ₃ (β2) ₂ (n=4)	50		Biphasic	0.58 (0.56-0.6) [75 (72-78)]	1.6 (1.5-1.7) [25 (22-28)]			
β2α4β2α4α4 (n=6)	50	30	Biphasic	1.7 (1.5-1.9) [37 (34-40)]	7.7 (7.4-8.0) [63 (60-66)]	Yes	Yes	Yes
β2α4β2α4α4 (n=5)	50		Biphasic	0.5 (0.4-0.6) [47 (43-52)]	2.7 (2.5-2.8) [53 (48-57)]			
β2 ^{W176A} α4β2α4α4 (n=6)	50	30	Biphasic	1.3 (1.2-1.4) [70 (64-75)]	3.9 (3.5-4.4) [30 (25-36)]	Yes	Yes	No
β2 ^{W176A} α4β2α4α4 (n=4)	50		Biphasic	0.4 (0.2-0.5) [67 (46-82)]	1.6 (1.2-2.4) [33 (18-54)]			
β2α4β2 ^{W176A} α4α4 (n=8)	50	30	Biphasic	0.58 (0.52- 0.64) [63 (58-68)]	2.9 (2.5-3.2) [37 (32-42)]	No	No	Yes
β2α4β2 ^{W176A} α4α4 (n=6)	50		Biphasic	0.5 (0.45-0.57) [77 (72-82)]	3 (2.5-3.7) [23 (18-28)]			
β2 ^{W176A} α4β2 ^{W176A} α4α4 (n=10)	50	30	Biphasic	0.55 (0.5-0.6) [65 (60-70)]	2.3 (2.1-2.5) [35 (30-40)]	No	n.d.	n.d.
β2 ^{W176A} α4β2 ^{W176A} α4α4 (n=7)	50		One phase	0.76 (0.4-1.2) [1]				
β2α4β2α4 ^{H142V} α4 (n=8)	50	30	Biphasic	1.2 (1.0-1.3) [63 (57-69)]	5.4 (4.7-6.2) [37 (43-31)]	Yes	Yes	No
β2α4β2α4 ^{H142V} α4 (n=9)	50		Biphasic	0.5 (0.4-0.6) [62 (56-68)]	2.2 (1.9-2.4) [38 (32-44)]			

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Figure legends-

Figure 1- NS9283 potentiates single channel currents through $(\alpha 4)_3(\beta 2)_2$ AChRs.

(A) BOSC 23 cells transfected with a tenfold excess of $\alpha 4$ over $\beta 2$ subunit cDNAs predominantly express $(\alpha 4)_3(\beta 2)_2$ AChRs. Amplitude histogram from a cell-attached patch recording in the presence of 10 μ M ACh reveals a single conductance class of channel openings with 4.1 pA unitary current amplitude (holding potential -70 mV). (B) Single channel currents were recorded with 10 μ M ACh in the recording pipette (holding potential -70 mV, Gaussian filter 4 kHz). *Left* panel shows a closed duration histogram fitted by the sum of exponentials. The point of intersection of adjacent components (arrow) yields a critical closed time τ_{crit} of 0.7 ms. *Center* panel shows a histogram of channel openings separated by closings longer than τ_{crit} fitted by the sum of three exponential components (dashed curves); an example channel opening is shown. *Right* panel shows a histogram of channel openings separated by closings briefer than τ_{crit} ; an example channel opening episode is shown. (C) Single channel currents were recorded in the presence of 10 μ M ACh and 10 μ M NS9283 as in panel B. *Left* panel shows a closed duration histogram fitted by the sum of exponentials. The point of intersection of adjacent components (arrow) yields a critical closed time τ_{crit} of 30 ms that was used to distinguish episodes of un-potentiated from potentiated channel openings. Closed duration components that selectively flank potentiated channel openings are shaded. *Middle* panel shows a histogram of channel openings separated by closings longer than τ_{crit} ; example channel openings are shown. *Right* panel shows a histogram of channel openings separated by closings briefer than τ_{crit} ; example channel opening episodes are shown. Exponential components corresponding to potentiated openings are shaded. Percentage of channel openings that are potentiated is

indicated by the shaded portion of the bar. (D) Panel summarise the classes of **channel** opening episodes recorded in the presence of ACh without (*left* traces) or with NS9283 (*right* traces).

Figure 2- Dependence of NS9283 Potentiation on ACh concentration.

(A-I) Single channel currents from $(\alpha 4)_3(\beta 2)_2$ AChRs were recorded in the presence of the indicated concentrations of ACh, without or with 30 μ M NS9283 (holding potential -70 mV, Gaussian filter 4 kHz). To the right of each trace are histograms of un-potentiated and potentiated channel openings, respectively, determined as described in the text and Fig. 1, fitted by the sum of three exponentials. Exponential components of potentiated channel openings are shaded. Percentage of **channel openings that are potentiated** is indicated by the shaded portion of the bar.

Figure 3. For AChRs formed from unlinked subunits, mutations in both $\beta 2$ and $\alpha 4$ subunits block NS9283 potentiation.

(A, B) Panels show the recent cryo-EM structure of the $(\alpha 4)_3(\beta 2)_2$ AChR (PDB code 6CNK). (A) Side (*left*) and top (*right*) views of the $(\alpha 4)_3(\beta 2)_2$ AChR complex. (B) *Left* panel shows the structure of an $\beta 2$ - $\alpha 4$ subunit interface within the $(\alpha 4)_3(\beta 2)_2$ AChR. *Right* panel shows a close up view of the $\beta 2$ - $\alpha 4$ subunit interface with residues subjected to mutation, $\beta 2$ W176 and $\alpha 4$ H142, highlighted as spheres. (C-E) Single channel currents from wild type or mutant $(\alpha 4)_3(\beta 2)_2$ AChRs were recorded in the presence of 50 μ M ACh and 30 μ M NS9283 (holding potential -70 mV, Gaussian filter 4 kHz). Red asterisks indicate locations of mutations. To the right of each trace is a histogram of cluster durations, corresponding to

successive channel openings and intervening closings, fitted by the sum of exponentials (dashed curves); the component with longest mean duration, present for the wild type AChR, is reduced or absent for the mutant AChRs. To the right of the cluster duration histograms are histograms of channel openings classified as either un-potentiated (un-shaded) or potentiated (shaded) fitted by the sum of three exponentials. **Above each histogram, the shaded portion of the bar indicates the percentage of channel openings that are potentiated.**

Figure 4. Potentiation of $(\alpha 4)_3(\beta 2)_2$ AChRs formed from linked subunits is blocked by mutation in the $\beta 2$ but not the $\alpha 4$ subunit.

(A) Schematic diagram of the plasmid encoding the five linked subunits that form the $(\alpha 4)_3(\beta 2)_2$ AChR; subunits are labeled 1-5 indicating the order of subunit linkage. (B-F) Single channel currents from the indicated wild type or mutant $(\alpha 4)_3(\beta 2)_2$ AChRs were recorded in the presence of 50 μM ACh and 30 μM NS9283 (holding potential -70 mV, Gaussian filter 4 kHz). Red asterisks indicate locations of mutations. To the right of each trace is a histogram of cluster durations, corresponding to successive channel openings and intervening closings, fitted by the sum of exponentials; the component with longest mean duration, present for the wild type (B) and $\alpha 4\text{H142V}$ mutant AChRs (F), is absent for the $\beta 2\text{W176A}$ mutant AChR (C-E). To the right of the cluster duration histograms are histograms of channel openings classified as either un-potentiated (un-shaded) or potentiated (shaded) fitted by the sum of three exponentials. The shaded portion of the bar above each histogram indicates the percentage of channel openings **that are potentiated.**

Figure 5- Enhanced channel re-opening by NS9283 is blocked by mutation in the $\beta 2$ but not the $\alpha 4$ subunit.

(A) Traces of single channel currents and detected channel openings in the presence of 50 μM ACh (upper) or 50 μM ACh and 30 μM NS9283 (lower). Red vertical lines indicate detected channel openings. (B) Plots of the fraction of channel opening episodes with greater than N openings against the number of openings per episode. Data shown are for the indicated wild type or mutant AChRs formed from unlinked subunits, in the presence of ACh (open symbols) or ACh and NS9283 (filled symbols), fitted by the sum of two exponentials. Red asterisks indicate locations of mutations. Note that for the wild type AChR, NS9283 markedly enhances channel re-opening over that in the presence of ACh alone, whereas for the mutant AChRs channel re-opening is similar in the presence of ACh alone and ACh plus NS9283. (C) Plots of channel re-opening as in (B) for the indicated wild type or mutant AChRs formed from linked subunits in the presence of ACh (open symbols) or ACh plus NS9283 (filled symbols) fitted by the sum of two exponentials. For the wild type AChR formed from linked subunits, NS9283 markedly enhances channel re-opening over that in the presence of ACh alone, whereas for AChRs containing the $\beta 2\text{W176A}$ mutation, at either position 3 or positions 1 and 3, channel re-opening is similar in the presence of ACh alone and ACh plus NS9283. However for AChRs containing one $\alpha 4\text{H142V}$ mutant subunit, channel re-opening, though reduced, is still significant.

Figure 1

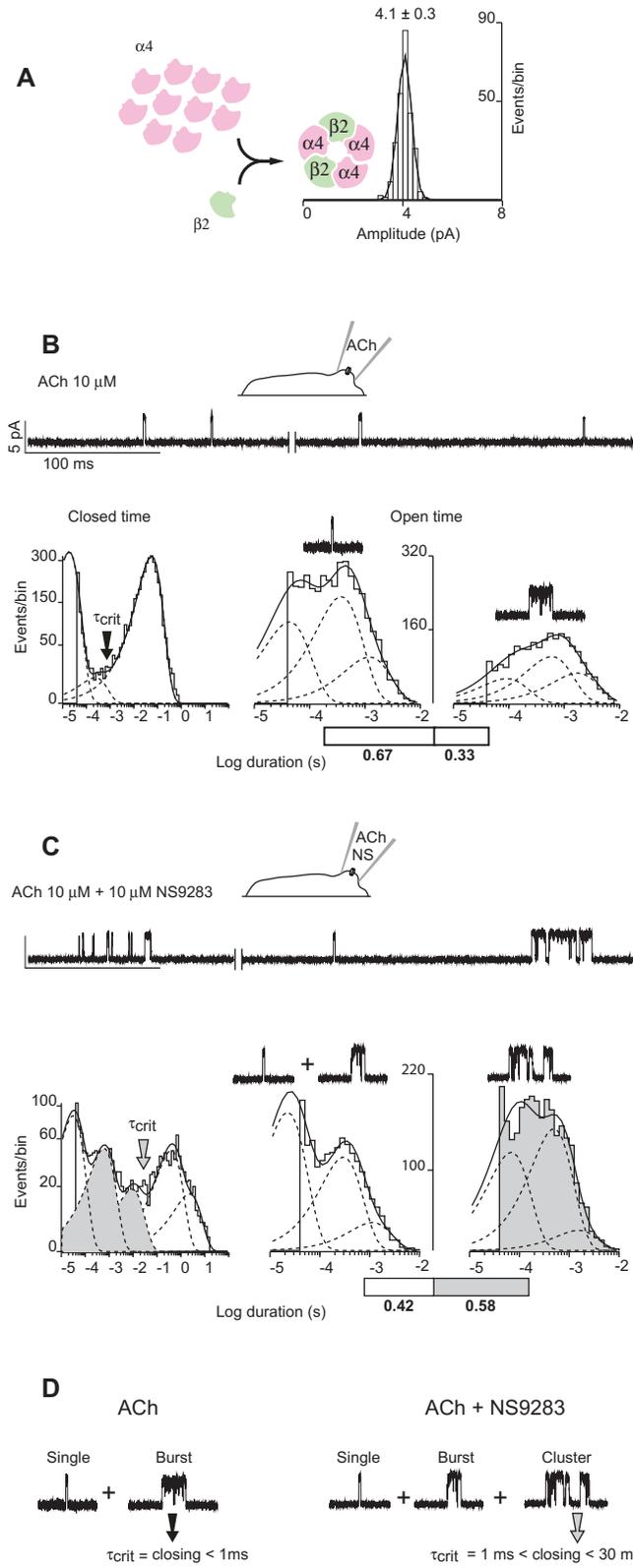


Figure 2

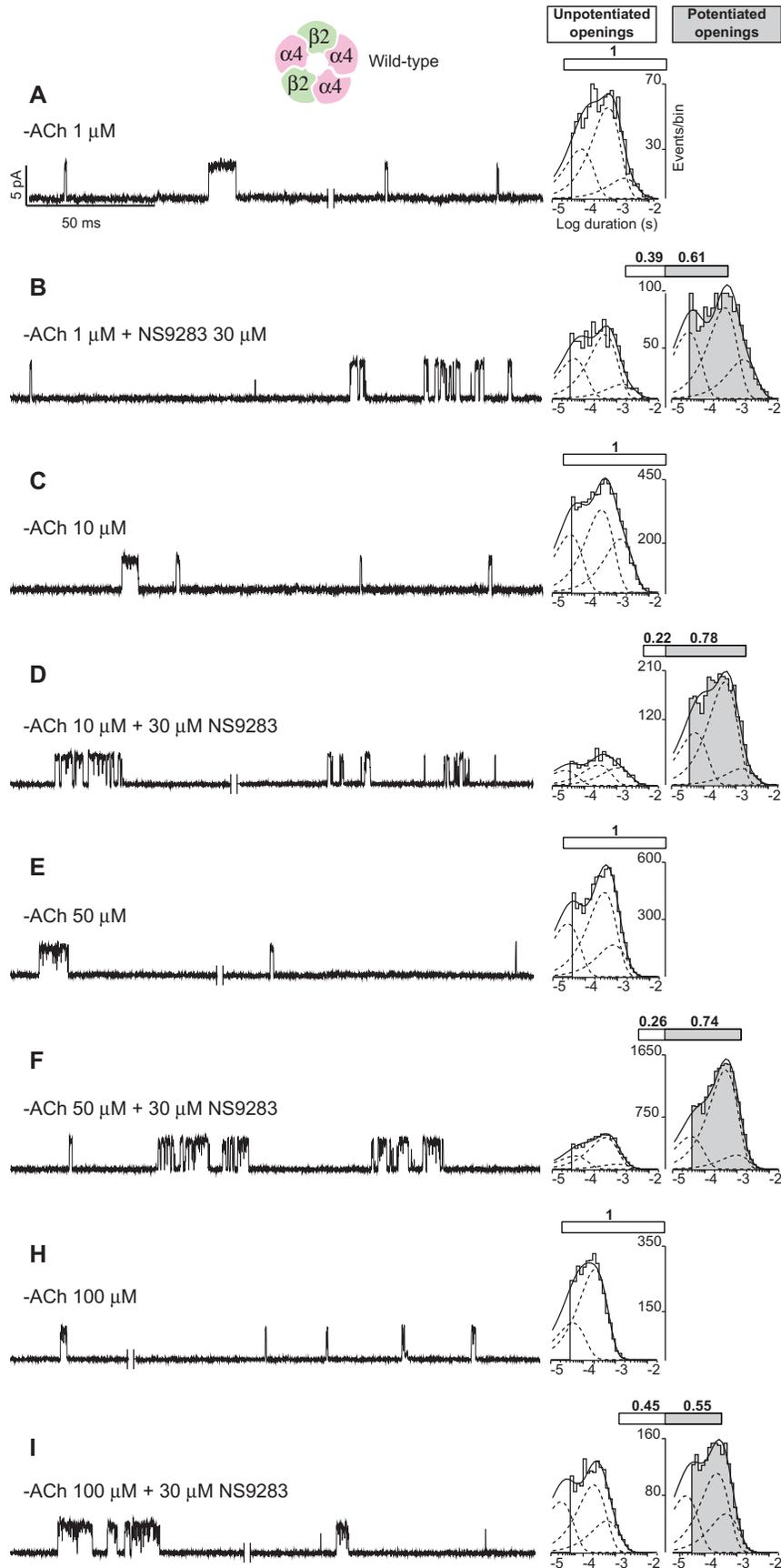


Figure 3

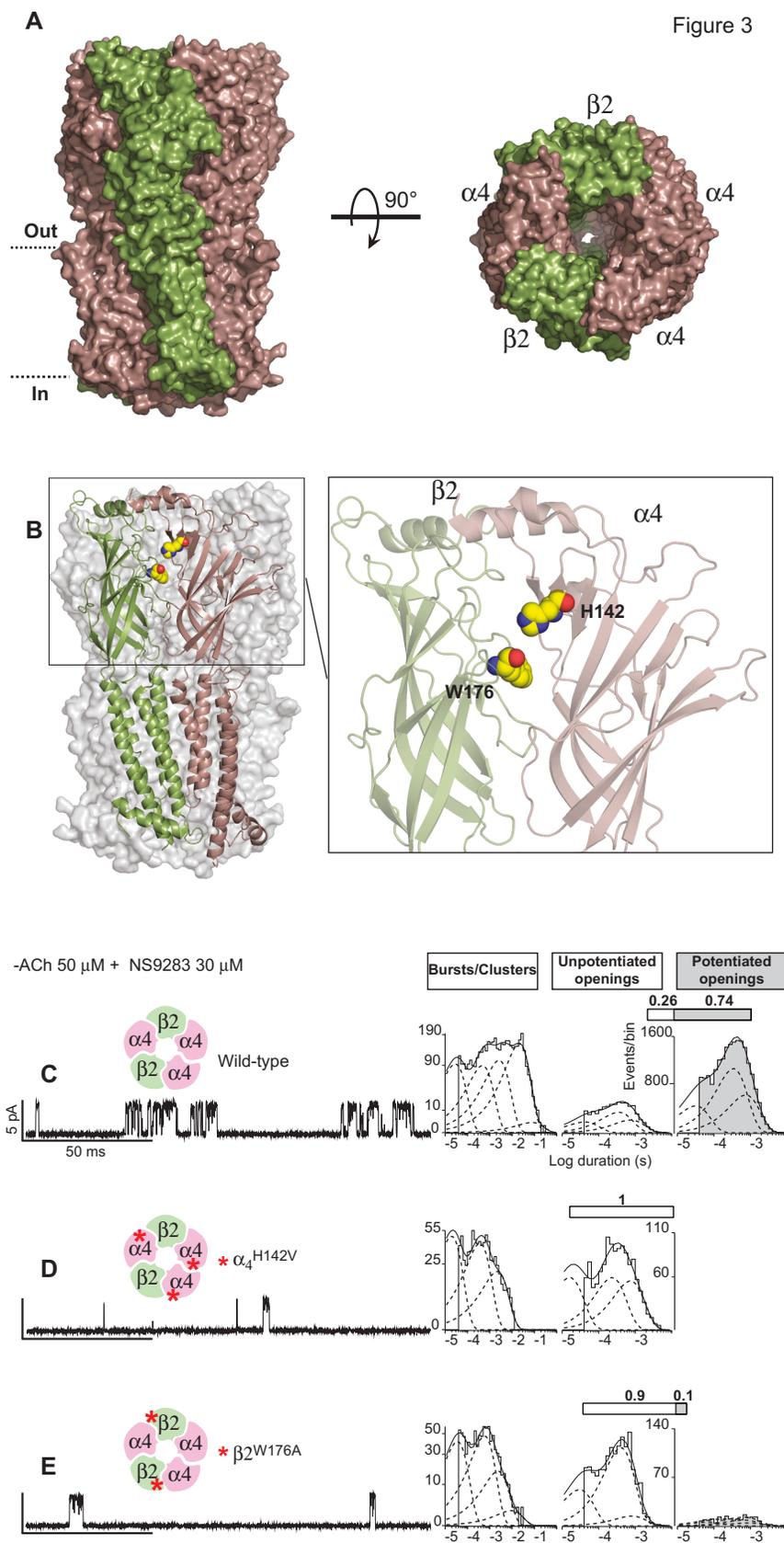
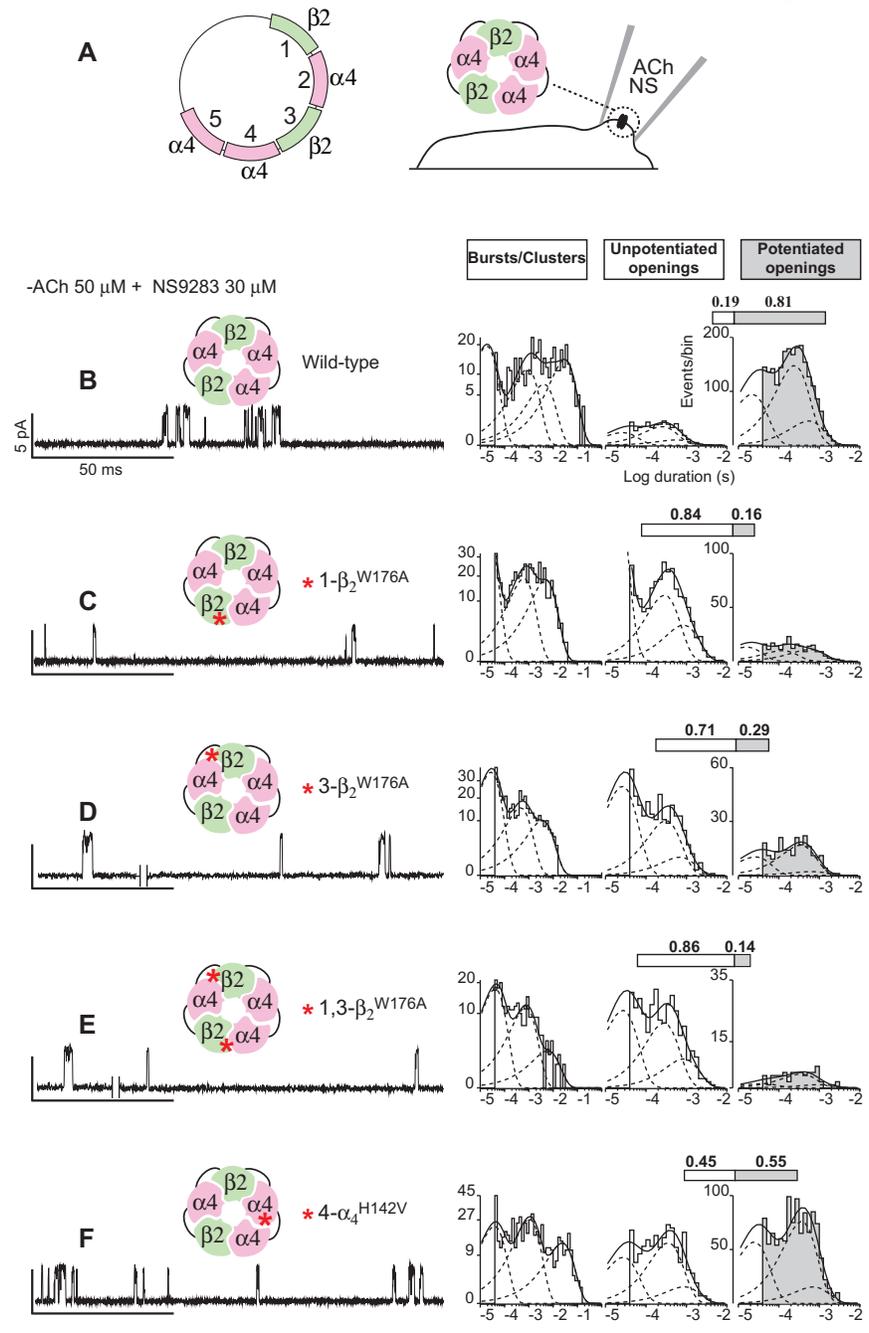
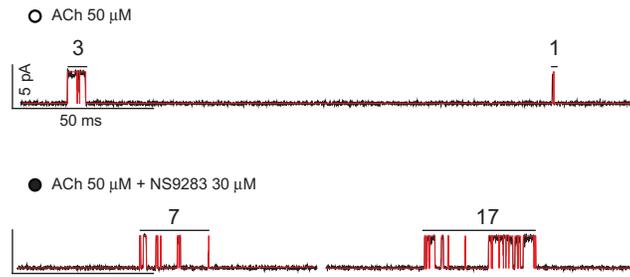
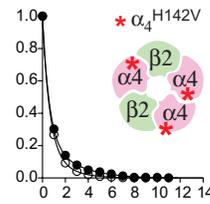
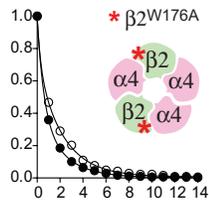
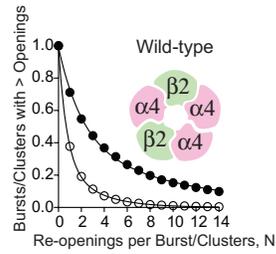
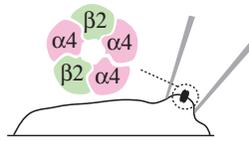


Figure 4



A

Figure 5

**B** $-(\alpha 4)_3(\beta 2)_2$ (unlinked subunits)**C** $-(\alpha 4)_3(\beta 2)_2$ (Concatamers)