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Subunit Interfaces Contribute Differently to Activation and Allosteric Modulation of Neuronal Nicotinic Acetylcholine Receptors

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed in the nervous system and are implicated in many normal and pathological processes. The structural determinants of allostery in nAChRs are not well understood. One class of nAChR allosteric modulators, including the small molecule morantel (Mor), acts from a site that is structurally homologous to the canonical agonist site but exists in the $\beta(+)/\alpha(-)$ subunit interface. We hypothesized that all nAChR subunits move with respect to each other during channel activation and allosteric modulation. We therefore studied five pairs of residues predicted to span the interfaces of $\alpha\beta 2$ receptors, one at the agonist interface and four at the modulator interface. Substituting cysteines in these positions, we used disulfide trapping to perturb receptor function. The pair $\alpha 3Y168-\beta 2D190$, involving the C loop region of the $\beta 2$ subunit, mediates modulation and agonist activation, because evoked currents were reduced up to 50% following oxidation (H_2O_2) treatment. The pair $\alpha 3S125-\beta 2Q39$, below the canonical site, is also involved in channel activation, in accord with previous studies of the muscle-type receptor; however, the pair is differentially sensitive to ACh activation and Mor modulation (currents decreased 60% and 80%, respectively). The pairs $\alpha 3Q37-\beta 2A127$ and $\alpha 3E173-\beta 2R46$, both in the non-canonical interface, showed increased currents following oxidation, suggesting that subunit movements are not symmetrical. Together, our results from disulfide trapping and further mutation analysis indicate that subunit interface movement is important for allosteric modulation of nAChRs, but that the two types of interfaces contribute unequally to receptor activation.

Keywords

nicotinic acetylcholine receptors; allosteric regulation; mutagenesis site specific; cysteine-mediated cross-linking; interface; oocyte; Cys-loop receptors

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1. INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are a diverse family of synaptic proteins belonging to the Cys-loop superfamily of receptors, which also includes GABA_A, serotonin, and glycine receptors (1). Numerous subtypes of nAChR are expressed throughout the central and peripheral nervous systems and are implicated in a range of neurological disorders, as well as in synaptic plasticity, learning and memory (2). Several known nAChR ligands are currently in clinical use, although their mechanisms of action are not completely understood (e.g., 3): galanthamine and rivastigmine are used to treat Alzheimer's and Parkinson's induced dementia (4, 5) and varenicline is used to treat nicotine addiction (e.g., 6, 7). Interest in nAChR allosteric modulators as therapeutic targets has grown recently, particularly because they may offer greater specificity than agonists and competitive antagonists (8, 9).

nAChRs and other Cys-loop receptors are membrane-bound, ligand-gated ion channels in which five homologous subunits form a central pore (1, 10). The general structure of these receptors, conserved across the superfamily, has been established from a variety of crystal structures of receptors and model-system proteins (e.g., 11–13). Five homologous subunits are arranged around a central pore pseudo-symmetrically. The extracellular domain of each subunit possesses a sidedness such that the (+) and (−) edges contribute to two separate, radial interfaces with neighboring subunits (11). Residues on the α(+) face, including the “C loop,” and some in the (−) face of the neighboring subunit comprise the canonical (orthosteric) agonist binding site. The diversity of nAChR subunits provides a multitude of unique interfaces which are potential ligand binding sites (e.g., 14–16).

Allostery - the interconversion of functional states - is a defining feature of ligand-gated ion channels. Allostery theory applied to nAChRs (e.g., 17) and functional characterization of the phenomenon (e.g., 18) have been quite straightforward compared to identifying the structures that give rise to allostery, let alone the movements that interconvert states. However, results such as the high-resolution structures of bacterial homologues considered to be in open and closed conformations (13, 19) are beginning to elucidate allostery at a structural level. While at present we must rely on indirect methods such as mutagenesis and disulfide trapping to deduce the nature of movement in nicotinic receptors, such techniques are now being used successfully to address questions of movement and conformation changes (e.g., 20, 21).

The pathway by which agonist binding information is transmitted to the channel gate in nAChRs has been elucidated in detail (22, 23); however, considerably less is known about the pathways that must connect allosteric modulator sites to the gate. Some structural determinants of these allosteric pathways have been identified (e.g., 24, 25), but in many cases the binding sites for the modulators are not known (26). Perhaps the most developed case to date is a site (or family of sites) for modulators of α7 receptors in the transmembrane domain just a few Ångströms from the channel gate; some mutations in this region change both agonist and modulator activity (27, 28). We note that the distance between subunit interfaces (~30 Å) in the extracellular domain is comparable to that between the canonical agonist binding site and the channel gate (~50 Å); thus, determining how interfaces

coordinate during conformational changes poses as significant a problem as understanding agonist-to-gate communication.

Our primary goal in this work was to determine the role of interface movement in allosteric modulation of neuronal nAChRs. We hypothesized that the interfaces move during channel gating, and that we could distinguish contributions to agonist activation and modulation processes. Using mutation analysis and disulfide trapping, we found evidence of movement in both the canonical and non-canonical interfaces in both types of activation, and that the interfaces contributed unequally (or asymmetrically) to these processes. Additionally, we uncovered a major role for the $\beta 2$ C loop that may parallel the function of the α subunit C loop in the canonical binding site.

2. EXPERIMENTAL PROCEDURES

2.1 Reagents

All chemicals, unless otherwise noted, were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO). Morantel is 1,4,5,6-tetrahydro-1-methyl-2-(2-[3-methyl-2-thienyl]ethenyl)pyrimidine, tartrate salt. Oxantel is 1-methyl-2-(3-hydroxyphenylethenyl)-1,4,5,6-tetrahydropyrimidine with 4,4'-methylenebis(3-hydroxy-2-naphthoic acid).

2.2 Nicotinic receptor clones and mutagenesis

Wild type rat $\alpha 3$ and $\beta 2$ subunits in the plasmid vector pGEMHE were a gift from Dr. Charles Luetje (Miami University, Miami, FL). The clones were originally isolated in the lab of Dr. Jim Patrick (Baylor College of Medicine; 29). We created mutant subunit genes in-house using the QuikChange temperature cycling method (Stratagene, La Jolla, CA) with complementary primers harboring the desired mutation (e.g., 14) or using custom mutagenesis from GenScript (Piscataway, NJ). In either case, mutants were verified by complete sequencing of the entire extracellular domain region using capillary electrophoresis of dye-detected, dideoxy-generated fragments. Unless otherwise noted, all $\alpha 3$ and $\beta 2$ residue numbering follows that in the structure a3b2.pdb (<http://www.ebi.ac.uk/compneur-srv/LGICdb/HTML/a3b2rr.html>) (30). These are smaller by two compared with numbering elsewhere in the literature; this discrepancy exists because of length differences with the ACh-binding protein, on which the a3b2.pdb homology model is based. We linearized cDNAs with unique restriction enzymes, and then made them RNase-free by phenol-chloroform extraction. RNAs were synthesized from these cDNAs using the T7 kit from Ambion (Life Technologies; Carlsbad, CA), and were diluted with RNase-free water to 0.5 $\mu\text{g}/\mu\text{L}$ and stored at -20°C .

2.3 Oocyte preparation and injection

We obtained *Xenopus laevis* oocytes either from Ecocyte Biosciences (Austin, TX) or harvested them from oocyte-positive female frogs (Nasco; Ft. Atkinson, WI) using procedures approved by the Grinnell College Institutional Animal Care and Use Committee, in accord with the National Institutes of Health guidelines. Briefly, stage V and VI oocytes were prepared by collagenase treatment and manual selection. Oocytes were maintained in

Barth's medium (88 mM NaCl, 1.0 mM KCl, 2.5 mM NaHCO₃, 0.30 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES, and 2.25 mM sodium pyruvate, pH 7.6, supplemented with 100 U/mL penicillin/streptomycin and 50 µg/mL gentamicin). We injected oocytes with 46 nL of a 1:1 (v/v) combination of the desired α and β subunits, prepared from 0.5 µg/µL stock solutions, using a Nanoject microinjector (Drummond, Broomall, PA). Injected oocytes were incubated in Barth's medium at 16°C for 2–4 days to allow for receptor expression, at which time currents could be recorded for up to 7 subsequent days. On a daily basis, Barth's medium was changed and dead cells were removed. Expression for most mutants was comparable with that for the wild type $\alpha 3\beta 2$ receptors as judged by currents in the range of 0.2–4 µA evoked with a saturating ACh concentration; some exceptions of poorly expressing mutants are discussed in Results.

2.4 Macroscopic current recordings

We made recordings of macroscopic evoked currents from oocytes using a Gene Clamp 500B amplifier and Digidata 1322A data acquisition system (Molecular Devices; Sunnyvale, CA) using the two-electrode voltage-clamp method, as previously described (14, 31, 32). Voltage was clamped at –60 mV, unless otherwise noted, with leak currents in the 0–200 nA range. Higher leak currents were tolerated in some cases where the baseline was reliably stable. Recording electrodes were filled with 3.0 M KCl and selected for resistances of 0.5–4.0 M Ω . Perfusion and drug administration were controlled with VC-6 solenoid valve systems (Warner Instruments; Hamden, CT). We perfused cells with oocyte Ringer's medium (OR2; 15 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.3) until the baseline stabilized prior to recording, typically, 90–120 sec. Drug applications (referred to throughout as “challenges”) were 5 sec unless otherwise noted and oocytes were washed with OR2 for approximately 100 sec between challenges or until currents had returned to a stable baseline. In some cases, additional wash time was used to avoid desensitization. All drugs were prepared in OR2 from concentrated stocks. In titration experiments (e.g., Fig. 3), 2–3 challenges chosen semirandomly in addition to the ACh concentration used for normalizing the set were repeated to ensure stability of responses throughout these 20–30 min experiments.

2.5 Disulfide cross-linking experiments

We performed experiments testing oxidation or reduction of putative disulfide bonds as previously described (31). In brief, currents were elicited with the same challenge (or set of challenges) 2–3 times prior to and following treatments. Responses to these series did not vary by more than 15% and that variation was random; thus responses were judged not to be subject to problems of desensitization or rundown in our experiments. Oxidation treatment was a 5-min perfusion with 4.4 mM H₂O₂ solution, either alone, or including 100 µM ACh, 10 µM Mor, or both drugs. To minimize degradation, this solution was usually prepared fresh for every cell, but no more than every third cell, from 30% (w/v) H₂O₂ stock kept on ice. Reduction was accomplished with a 5-min perfusion of 40 µM dithiothreitol (DTT) solution, unless otherwise noted, which was prepared fresh for every second or third cell from a concentrated stock. Following treatment with either H₂O₂ or DTT, oocytes were washed for 100 sec with OR2 before subsequent drug challenges. Specific drug challenges varied with the experiment and are reported in the figure legends and text. We refer to

responses prior to treatment as I_{control} and those after oxidation and reduction treatments as $I_{\text{H}_2\text{O}_2}$ and I_{DTT} , respectively.

2.6 Data and statistical analyses

Our standard characterization of $\alpha 3\beta 2$ mutants includes concentration-response titrations for ACh alone and in the presence of modulator (Mor or Oxa, 10 μM unless otherwise noted), typically over the μM to mM concentration range, but appropriate to cover the response fully. In many cases this consisted of five ACh concentrations with and without modulator, while in the others titrations consisted of 9–10 concentrations to extend the range or to refine the shape of the curve. For comparison of replicate measurements, peak evoked current responses were normalized to that evoked by a saturating concentration of ACh; in experiments testing other agonists, responses were also normalized to saturating ACh. We fit the Hill equation to titration data sets (14, 32):

$$\text{Response} = \frac{E_{\text{max}}}{1 + (EC_{50}/[ACh])^{n_H}}$$

where EC_{50} is the concentration evoking half-maximal response, n_H is the slope of the curve at EC_{50} and E_{max} is the response at saturating agonist. By definition, E_{max} is 1 for ACh titrations, but $E_{\text{max}} > 1$ for potentiation and $E_{\text{max}} < 1$ for inhibition in the presence of modulators; E_{max} is a measure of relative efficacy in experiments testing other agonists (e.g., Fig. 8). Given the relative errors in the measurements, we consider 2-fold differences in EC_{50} the limit of significance (31).

With the premise of studying pairs of residues interacting across interfaces, we evaluated the coupling in these pairs with thermodynamic cycles (33) by calculating

$$\Omega = \frac{EC_{50}(\text{WT}) \cdot EC_{50}(\text{mut } ij)}{EC_{50}(\text{mut } i) \cdot EC_{50}(\text{mut } j)}$$

where *mut i* and *j* represent single subunit mutations and *mut ij* represents the co-expressed double mutant. This approach is used to identify nearest-neighbor as well as long-range interactions between amino acid residues (34). In addition, depending on the substitutions used, the type and strength of the interaction can be estimated (e.g., 22). Interaction energies are the theoretical foundation of allosteric behavior at the molecular level (35). A value of $\Omega = 1$ indicates the two mutations are independent, that is, not interacting. Errors propagated in the Ω values in our experiments ranged 23–73% (ave. $46 \pm 2\%$, $n = 48$); therefore, we consider only values $\Omega < 0.2$ or $\Omega > 2$ meaningful.

By analogy to the thermodynamic cycle, we also calculated the ratio:

$$\hat{\Omega} = \frac{EC_{50}(\text{WT}, A) \cdot EC_{50}(\text{mut}, A+M)}{EC_{50}(\text{WT}, A+M) \cdot EC_{50}(\text{mut}, A)}$$

where A and A + M represent activation by ACh and ACh +10 μ M modulator, respectively, for wild type $\alpha 3(\beta 2)$ and a particular mutant (Table 4). These measures illustrate the differential effect of a mutation on modulator and agonist activation properties. This approach is based on the concept that both ligands and amino acid substitutions contribute discrete energy to channel activation, either locally, globally or both (35, 36).

For oxidation and reduction experiments with double cysteine mutants (e.g., Figs. 4, 6 and 7), we used standard statistical analyses, namely, the paired comparison t-test, and for tests of differences among the various oxidation treatments or across mutants, analysis of variance (ANOVA) followed by Tukey's post hoc test. We consider $p < 0.05$ significant.

3. RESULTS

We previously established that a class of anthelmintic compounds allosterically modulates neuronal nicotinic receptors from sites located at non-canonical interfaces (14, 32). Based on others' work demonstrating that nAChR interface interactions are important in agonist activation (e.g. 20, 23), we asked in this study whether interface movement differs for agonist activation and allosteric modulation. Fig. 1 is a schematic representation of the pentameric extracellular domain of an $(\alpha 3)_2(\beta 2)_3$ receptor, with $\alpha 3$ subunits in purple and $\beta 2$ subunits in blue. The relative locations of five pairs of residues we studied are also indicated; four occur in the $\beta 2(+)/\alpha 3(-)$ modulator interface (M) and one is in the $\alpha 3(+)/\beta 2(-)$ canonical agonist interface (A). We chose these pairs based on the reported importance in agonist activation of homologous pairs from other Cys-loop receptors. We explain these choices below as we describe the results for each location.

In the $\alpha 3\beta 2$ system, low concentrations (10 μ M) of morantel (Mor) potentiate ACh-evoked currents several fold, as shown in each of the left set of traces (in three pairs) in Fig. 2. Under the conditions of these control measurements, potentiation ($I_{ACh+Mor}/I_{ACh}$) was greater than 2.5-fold for the modulator interface M1 $\alpha 3Y168C\beta 2D190C$ mutant, canonical interface A2 $\alpha 3S125C\beta 2Q39C$ mutant and wild type receptors, respectively. Upon exposure to the oxidizing conditions of H_2O_2 (arrows), the evoked currents for the mutant receptors were greatly decreased: both currents for $\alpha 3Y168C\beta 2D190C$ decreased by ~80%, while for the $\alpha 3S125C\beta 2Q39C$ receptor, I_{ACh} decreased by 60% and $I_{ACh+Mor}$ decreased 90%. In contrast, the wild type receptor was unaffected by oxidizing treatment, as shown by the third set of traces in Fig. 2, and substantiated by replicate experiments (Supplemental Fig. S1). Together, these findings suggest that the changes in channel function following oxidation of the mutants are due to the cysteine substitutions.

3.1 Movement of the $\beta 2$ C loop is necessary for potentiation

The C loop of α subunits, which contributes several important residues to the canonical agonist/competitive antagonist binding sites, closes on these ligands during channel activation (reviewed in 10). In addition, pinning the C loop to the adjacent subunit by oxidizing two appropriately substituted cysteines significantly increases channel activity (37). We hypothesize that the homologous C loop structure of the $\beta 2$ subunit also moves during modulation of $\alpha 3\beta 2$ receptors. To test this, we substituted cysteines for residues $\alpha 3Y168$ and $\beta 2D190$ (M1; Fig. 1), predicted to be 4.6 Å apart in the homology model (30).

Fig. 3A shows the agonist concentration-response behavior of the $\alpha 3Y168C\beta 2D190C$ receptor for ACh alone, and in the presence of 10 μM Mor or 10 μM oxantel (Oxa), compared to the behavior of the wild type $\alpha 3\beta 2$ receptor. The response of the double-cysteine mutant to ACh was not statistically different from wild type ($EC_{50} = 37$ vs. 66 μM , respectively; see Table 1). While the M1 double-cysteine mutant was still potentiated by both Mor and Oxa, the extent of this potentiation was considerably lower than for wild type. For example, $E_{max} = 1.38$ for ACh + Mor activation of $\alpha 3Y168C\beta 2D190C$, compared to $E_{max} = 2.64$ for wild type. However, like wild type, Mor and Oxa modulation for this mutant were quite similar, with the same value of E_{max} , and EC_{50} values differing by only 3-fold (Table 1).

The single mutants $\alpha 3\beta 2D190C$ and $\alpha 3Y168C\beta 2$ displayed important differences in activation properties from both wild type and the double mutant $\alpha 3Y168C\beta 2D190C$ nAChRs (Fig. 3B). Like the double-cysteine receptor, ACh activation of each single mutant was similar to wild type (Table 1). ACh + Oxa modulation of the $\alpha 3Y168C\beta 2$ receptor was comparable to the double-cysteine mutant ($EC_{50} = 5.9$ vs. 9.5 μM and $E_{max} = 1.34$ vs. 1.38, respectively), as was the ACh + Mor behavior (Table 1). In contrast, Mor and Oxa potentiation were eliminated in the $\alpha 3\beta 2D190C$ mutant, and in fact showed signs of inhibition, not unlike the behavior of these compounds on wild type $\alpha 4\beta 2$ receptors (32). The EC_{50} in the presence of Mor or Oxa was shifted to a higher concentration compared to ACh alone, and for ACh + Oxa, the E_{max} (0.83 ± 0.08 ; Table 1) was significantly below one.

We also studied the $\alpha 3Y168F\beta 2$, $\alpha 3\beta 2D190Q$, and double $\alpha 3Y168F\beta 2D190Q$ mutants. Phenylalanine and glutamine occupy the homologous positions in $\alpha 4$ and $\beta 4$, respectively, and are thus of interest because Mor potentiation in $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors is minor and considerably less than that observed $\alpha 3\beta 2$ (32, 38). We predicted that loss of a possible hydrogen-bonding interaction would decrease modulation. Indeed, the Mor and Oxa modulation profiles for the $\alpha 3Y168F\beta 2$ and $\alpha 3\beta 2D190Q$ mutants paralleled the cysteine mutants, as did ACh activation in the two single mutants and the double mutant (Table 1). However, phenylalanine in position 168 discriminates between Mor and Oxa more strongly than cysteine (21- and 3.7-fold differences in EC_{50} , respectively). Interestingly, Mor potentiation for $\alpha 3Y168F\beta 2D190Q$ was very similar to wild type $\alpha 3\beta 2$, but Oxa potentiation was substantially decreased. Therefore, with the $\alpha 3Y168$ single mutants behaving more like their respective double mutants (as well as wild type $\alpha 3\beta 2$), $\alpha 3Y168$ appears to compensate for the loss of potentiation in the $\beta 2D190$ mutants, or $\alpha 3Y168$ dominates in determining the behavior of the double mutants.

Thermodynamic cycle analysis compares the effects of two single mutants to that of the double mutant, and thus measures putative residue interactions in terms of energetic coupling (33). The approach is typically used to evaluate residues predicted from structural data to be nearest neighbors, but it has been applied to long-range interactions in ion channels (34, and references therein). The EC_{50} from the Hill equation is a composite parameter that includes information about binding and gating (36), but such values have been used to evaluate the coupling parameter Ω when the more rigorous ligand binding constants and gating equilibrium constants are not available (e.g., 39, 40). Hence, in

applying this analysis, we assume that the mutations do not radically alter the mechanisms of activation, and we limit our interpretation to differences in activation by agonist alone vs. allosteric modulation.

As indicated in Table 4, with all $\Omega > 2$, the two mutant cycles in location M1 reveal an improvement in channel function; these values are driven by the EC_{50} s for the single mutants being smaller (left-shifted) than wild type, indicating improved potency. The coupling is modest ($\Omega = 2.1\text{--}4.9$) for modulation by Mor and Oxa, and surprisingly is less than for ACh activation in the case of the $\alpha 3Y168F\text{--}\beta 2D190Q$ pair. Extending this approach to the interaction of each mutation with modulator (referenced to ACh activation; two right-hand columns of Table 4) shows that the two residues of the M1 pair mediate modulation, but that the mutations have opposite effects. For wild type $\alpha 3\beta 2$ receptors, the ratio $EC_{50}(\text{ACh})/EC_{50}(\text{ACh} + \text{Mor})$ is 6.6 (Table 1); thus, the $R(\text{mut-Mor})$ values of 0.29 and 0.066 for the two $\alpha 3Y168$ single mutations signify a much greater left-shift upon adding Mor than for wild type. In contrast, the $\beta 2D190$ single mutation values ($R = 7.1\text{--}19$) reflect the effective inhibition discussed above (Fig. 3B, Table 1). Hence, whereas mutations in $\alpha 3Y168$ may “loosen” the receptor, making it more sensitive to Mor and (somewhat) Oxa, mutations in $\beta 2D190$ appear to disconnect the modulatory mechanism from activation or favor the closed state over the open (relative to wild type).

We next used a disulfide trapping approach to test whether cross-linking the $\beta 2$ C loop to the $\alpha 3(-)$ face would disrupt channel function. We tested four oxidation conditions on $\alpha 3Y168C\beta 2D190C$ (the absence and presence of agonist and modulators), as we and others have previously done (25, 31), to determine whether disulfide reactivity depended on the activation state of the receptor. Fig. 4 shows that, consistent with the example illustrated in Fig. 2, oxidation decreased currents evoked by ACh alone, ACh + Mor, and ACh + Oxa, all to the same extent (per treatment). Currents decreased 20–30% for oxidation with H_2O_2 alone, $H_2O_2 + \text{ACh}$ or $H_2O_2 + \text{Mor}$ (panels C, B and D, respectively), but currents decreased by 48% upon oxidation with $H_2O_2 + \text{ACh} + \text{Mor}$ (panel A). All but two of these decreases were significant (paired t-test). The larger effect for the $H_2O_2 + \text{ACh} + \text{Mor}$ oxidation was significantly different from the H_2O_2 alone treatment for currents evoked by ACh and ACh + Mor (ANOVA, Tukey's; $F = 4.36$, $p = 0.016$; $F = 3.19$, $p = 0.046$, respectively). Similarly, in a separate experiment testing the oxidation onset kinetics (Supplemental Fig. S2A), the final fraction of current remaining at long times was significantly different for oxidation as H_2O_2 alone, $n = 5$) vs. $H_2O_2 + \text{ACh} + \text{Mor}$ ($I_{\text{oxidation}}/I_{\text{control}} = 0.61 \pm 0.05$ and 0.43 ± 0.04 , respectively), while the time course was the same for both ($t_{1/2} = 60$). Together, these results suggest that movement in the vicinity of the M1 position is necessary for channel activation and that residues $\alpha 3Y168$ and $\beta 2D190$ are closer and/or are more reactive when the receptor is potentiated compared to the resting state.

We further explored the properties of the putative disulfide bond formed in M1 $\alpha 3Y168C\beta 2D190C$ receptors with several control experiments. We found 1) that the decrease in currents following oxidation was completely reversible (Supplemental Fig. S2B); 2) that this decrease arose from decreased efficacy of the channels (Supplemental Fig. S2C); and 3) importantly that oxidation treatments for the single mutants $\alpha 3Y168C\beta 2$ and

$\alpha 3\beta 2$ D190C expressed separately failed to changed evoked currents significantly (Supplemental Fig. S3A and S3B).

In sum, our analysis of the M1 position suggests a) a strong interaction between these residues, b) a direct role of the residues in mediating modulation effects, in that for only one of 12 cases is modulation equivalent to wild type, c) a role for these residues in discriminating between Mor and Oxa, and d) relative movement of these residues during channel activation and modulation.

We also attempted to study the $\alpha 3$ L107C $\beta 2$ T150C double-cysteine mutant (M5 in Fig. 1) because the predicted separation of 3.4 Å for the pair suggests an interaction. We previously showed that $\beta 2$ T150 and $\alpha 3$ L107 are involved in allosteric modulation of $\alpha 3\beta 2$ receptors (14, 32). T150, adjacent to W149 of the “aromatic box,” is highly conserved in (–)-interface nAChR subunits, while $\alpha 3$ L107 is not, making it one of the determinants of modulator specificity for $\alpha 3\beta 2$. All attempts to express the M5 $\alpha 3$ L107C $\beta 2$ T150C double-cysteine mutant failed to produce currents large enough for viable study. In total, we tried expressing $\alpha 3$ L107C $\beta 2$ T150C thirteen times, including several attempts to reduce putative pre-existing disulfide bonds with DTT, without success. This failure must be due to the co-expression of the cysteine pair, because the single cysteine mutants were readily expressed and fully characterized (14, 32).

3.2 $\alpha 3$ S125 in the canonical interface is a hotspot for receptor activation

Extensive study has elucidated the transformation of agonist binding energy into channel gating in the muscle-type nAChR system 35, and references therein). For example, Mukhtasimova and Sine (20) demonstrated that the $\alpha 1$ Y127- ϵ Q39/ δ Q41 pairs in canonical interfaces couple agonist binding with rapid and efficient gating. Based on the conservation of a hydroxyl-bearing residue in this $\alpha(+)$ position and the completely invariant glutamine in the (–)-face, neighboring subunit, we studied the role of this location (A2; Fig. 1) in $\alpha 3\beta 2$ modulation.

The muscle-type receptor has near-perfect efficacy (e.g., 18) compared to $\alpha 3\beta 2$ (38). We therefore studied the $\alpha 3$ S125Y $\beta 2$ receptor, predicting increased efficacy for the tyrosine substitution because this amino acid is invariant in $\alpha 1$ sequences. Remarkably, as shown in Fig. 5A, Mor alone was as good an agonist as ACh, with EC_{50} for activation of 2.3 μ M (vs. 7.6 μ M for ACh) and E_{max} of 1.7 relative to saturating ACh (Table 2). In contrast, Mor alone activates wild type $\alpha 3\beta 2$ in the same micromolar range, but with E_{max} only 0.2 relative to ACh (Table 2; 38). For $\alpha 3$ S125Y $\beta 2$, we omitted from the fit data collected in the range 30–300 μ M Mor (not shown) because these responses were considerably lower than E_{max} and showed evidence of open channel block (cf. 38). Relative potency for ACh was also increased 8-fold for $\alpha 3$ S125Y $\beta 2$, consistent with improved efficacy (36). $\alpha 3$ S125Y $\beta 2$ demonstrated Mor potentiation of ACh-evoked currents, indicating an interaction of the two compounds over a very broad range (EC_{50} = 9 nM, Table 2); the ACh + Mor curve was shifted to higher potency than for ACh and Mor alone, but the ACh + Mor and Mor alone E_{max} values were similar. In other words, cooperativity between ACh and Mor persists, because the response was not constant at the level evoked by 10 μ M Mor alone, and it

increased monotonically with increasing ACh concentration. Exploring the unusual effect of the tyrosine mutation on ACh + Mor modulation further is beyond the scope of this work.

A possible explanation of the strong agonist behavior for Mor is that the tyrosine substitution unmasks Mor activation from the $\alpha(+)/\beta(-)$ canonical agonist site, despite the evidence that Mor binds at the $\beta2(+)/\beta3(-)$ interface (14, 32). To test this idea, we co-expressed $\alpha3S125Y$ with $\beta2W149A$, a mutation in the non-canonical interface that greatly reduces Mor activity (32). These results are shown in Fig. 5B and Table 2. Although ACh activation did not differ from the $\alpha3S125Y\beta2$ receptor (EC_{50} left-shifted less than 2-fold), Mor activation and Mor potentiation were drastically reduced for $\alpha3S125Y\beta2W149A$ compared to the single mutant; these profiles are in fact very similar to wild type $\alpha3\beta2$ (Table 2). In other words, the $\beta2W149A$ substitution selectively decreases Mor activities. Coupling analysis (Table 4) indicates that $\alpha3S125\beta2W149$ do not interact in ACh activation, but do so in Mor modulation, an effect clearly driven by a strong correlation between Mor and the $\alpha3S125$ position. Thus, we conclude that Mor still acts through the $\beta2(+)/\alpha3(-)$ site in the $\alpha3S125Y\beta2$ receptor, suggesting that Mor-binding information is fed into the “principal gating pathway” (22) at or near the A2 pair.

With this evidence of the importance of the A2 pair (3.6 Å separation), we substituted $\alpha3S125$ and $\beta2Q39$ with cysteines and tested oxidation and reduction. Fig. 6 (left) shows that treatment with H_2O_2 alone decreased currents evoked by ACh, ACh + Mor and Mor alone, by 62–89% (also as in Fig. 2). These decreases were all highly significant as measured by paired comparison t-test (p values ranged 0.00022–0.0033), and the effect on I_{ACh} was different from $I_{ACh+Mor}$ and I_{Mor} (ANOVA; $F = 29.6$, $p = 2.5 \times 10^{-8}$ and Tukey's post hoc test). The decreases in activation for disulfide trapping of $\alpha3S125C\beta2Q39C$ (Fig. 6) were also greater than for $\alpha3Y168C\beta2D190C$ (Fig. 4; all conditions) (ANOVA; $F = 62.3$, $p = 9.8 \times 10^{-15}$), indicating that the A2 pair is more solvent-accessible, or that cross-linking in this location decreases efficacy more substantially. We again attribute the changes in currents for the double mutant to formation of a disulfide bond, because H_2O_2 oxidation treatment led to no changes in evoked currents for the single mutant $\alpha3S125C\beta2$ (Supplemental Fig. S3C).

The effective resistance of the $\alpha3S125C$ - $\beta2Q39C$ disulfide bond to reduction by DTT (Fig. 6, right), especially compared to the ready reversibility of the M1 bond, is partial evidence against the A2 pair being easily accessible. In a subset of experiments having first oxidized $\alpha3S125C\beta2Q39C$, we were able to test reduction (40–100 μM DTT). Although differences (I_{DTT} vs. $I_{H_2O_2}$) registered as significant by paired comparison for currents evoked by ACh ($p = 0.034$) and ACh + Mor ($p = 0.028$), but not for Mor alone ($p = 0.089$; $n = 7$ all three measures), these changes are clearly very far from returning the receptor to original control levels of activation. Though the cysteine substitutions for this A2 location did not exhibit strong coupling (Table 4), the very significant decreases in receptor function upon oxidation suggest an important interaction. Together, the effects of the substitutions in the A2 location implicate these residues not only in agonist activation, but also in allosteric modulation.

3.3 Interface movement is not symmetrical

In order to examine whether the subunit interfaces contribute symmetrically to channel activation, we studied mutations in the β 2A127 and α 3Q37 positions (M3), which are homologous to α 3S125 and β 2Q39C, respectively, and predicted to be 4.4 Å apart. We hypothesized that perhaps this pair functions to move Mor-binding information “down” to the channel gate directly, in analogy to agonist-binding information moving through the principal gating pathway of the α 1(+) interface (22).

Oxidation of the M3 α 3Q37C β 2A127C double-cysteine mutant had dramatic effects on receptor activation properties. Currents evoked by ACh alone, ACh + Mor and Mor alone, shown as representative traces in Fig. 7A, were all increased by oxidation, whether as H₂O₂ alone (data collated in Fig. 7C) or in the presence of ACh and Mor (data collated in Fig. 7B). These increases were all significant in paired comparison ($p = 2.5 \times 10^{-5}$ –0.035), except for Mor alone currents in oxidation with H₂O₂ only ($p = 0.132$), but the six ratios were not different from one another (ANOVA; $F = 2.94$, $p = 0.130$). Given that the currents increased following oxidation for the α 3Q37C β 2A127C receptor, these effects were clearly different from those in the α 3Y168C β 2D190C and α 3S125C β 2Q39C receptors (ANOVA; $F = 23.6$, $p = 1.2 \times 10^{-12}$). However, like the homologous α 3S125C- β 2Q39C (A2) pair, the α 3Q37C- β 2A127C disulfide bond was essentially resistant to reduction by DTT ($p = 0.096$ –0.457 for paired t-test). The effects of the cysteine substitutions in these M3 residues on ACh activation and Mor modulation were modest, with slightly decreased Mor potentiation (Table 3 and Table 4). Nonetheless, given the increased currents upon oxidation of the α 3Q37C β 2A127C receptor, we conclude that these M3 residues participate in channel gating, but that their role is likely different from the canonical A2 position.

To further characterize the contributions the interfaces make to gating, we compared activation and modulation for several agonists. Nicotinic receptor subtypes display characteristic pharmacological profiles, including differences in efficacy for some agonists (38, 41, 42). We aimed to exploit such efficacy differences to determine whether Mor modulation is parallel for all agonists or in some way discriminates among them. Fig. 8 shows traces of currents evoked by ACh $\pm$ Mor, nicotine $\pm$ Mor, epibatidine $\pm$ Mor and Mor alone for α 3S125Y β 2, α 3 β 2A127Y, and the double mutant α 3S125Y β 2A127Y. These are representative of repeated experiments of this drug profiling, the collated results of which are reported numerically in the figure (means $\pm$ SEM; $n = 3$ –8). The concentrations of the three traditional nAChR agonists were saturating, as determined in independent experiments (Table 2 and Table 3, and not shown). For all three subtypes, 10 μ M Mor modestly potentiated ACh responses, but inhibited responses evoked by nicotine and epibatidine. This inhibition might make sense in the case of α 3S125Y β 2 where the greater efficacy for nicotine and epibatidine (than ACh) might be approaching a ceiling (cf. 32); however, this would not explain the inhibition for the α 3 β 2A127Y and α 3S125Y β 2A127Y subtypes. For all three mutants, the responses to Mor alone were very different, ranging from full agonist for α 3S125Y β 2 as noted above (Fig. 5A) to the nearly negligible current for α 3 β 2A127Y, which was considerably smaller than for α 3 β 2 (Table 2). Although analyzing them quantitatively is beyond the scope of this study, the kinetics of inactivation were also clearly different for the three subtypes. Interestingly, α 3S125Y and β 2A127Y appear to couple in

ACh activation and for Mor alone activation ($\Omega = 3.4$), and $\beta 2A127Y$ mediates Mor modulation (Table 2, Table 3 and Table 4). In sum, the degree of Mor potentiation or inhibition is effectively independent of the agonist for these three subtypes, despite the agonist activities being different. Accordingly, these substitutions (which are in homologous positions) contribute equally to Mor modulation, or their role is predominantly in agonist activation. However, for Mor acting as an agonist, the interfaces may contribute equally in this location, because the response of the $\alpha 3S125Y\beta 2A127Y$ is intermediate between that of the two single mutants, not dominated by one or the other.

We further explored the possibility of direct channel activation from the $\beta 2(+)/\alpha 3(-)$ interface by studying the pair $\alpha 3E173\beta 2R46$ (M4; Fig. 1). $\alpha 3E173$ and $\beta 2R46$ might be intimate enough to share an electrostatic attraction (4.8 Å), and the homologous positions are implicated in channel activation processes in other systems (24, 25, 43, 44). In only one of five attempts to express the $\alpha 3E173C\beta 2R46C$ mutant did we observe currents large enough for study. This allowed us to characterize ACh activation and Mor modulation properties (Table 3) and perform one experiment testing oxidation and reduction. The apparent poor expression of the double-cysteine M4 mutant ($I_{\text{average}} = 60 \pm 10$ nA evoked by 2 mM ACh, $n = 8$, for the one successful expression attempt) was likely due to the cysteines themselves, because we observed robust expression of the M4 double mutant $\alpha 3E173A\beta 2R46V$ ($I_{\text{average}} = 990 \pm 130$ nA evoked by 800 μ M ACh, $n = 8$) and of the single-cysteine mutants.

Like the M3 pair $\alpha 3Q37C\beta 2A127C$, just 8–10 Å “above,” toward the Mor site, oxidation of $\alpha 3E173C\beta 2R46C$ with H_2O_2 alone significantly increased currents (Supplemental Fig. S4). Unlike the M3 pair, reduction of this double mutant with DTT following oxidation did decrease currents, but not completely to the control level (Supplemental Fig. S4). Table 4 indicates that $\alpha 3E173C$ and $\beta 2R46C$ are coupled modestly in ACh activation and more substantially in Mor modulation; the pair is dominated by $\alpha 3E173C$, as both mutants have effectively lost Mor potentiation. In contrast, the $\alpha 3E173A\beta 2R46V$ pair, which substitutions we based on the literature (25, 43), do not interact in Mor modulation, but are strongly coupled in ACh activation (Table 4). Interestingly, the double mutant has also lost Mor potentiation, but in this case is dominated by $\beta 2R46V$ (Table 3). Our findings about the M4 position parallel both those of the M1 location, in that the mutations overall seem to “loosen” the receptor for ACh activation and “disconnect” Mor potentiation, and those of the M3 location, in that oxidation of engineered cysteines increases currents. These results suggest the M4 position, and perhaps this region of the receptor more broadly, deserves further study, for example, with charge reversal substitution.

4. DISCUSSION

In this study, we aimed to demonstrate that allosteric modulation of neuronal nicotinic receptors is mediated by movement between subunits. Using mutation analysis and disulfide trapping of cysteine-substituted receptors, we found support for the potentiator Mor enhancing the transmission of agonist binding information, rather than contributing to gating more directly. In addition, interaction of the C loop region of $\beta 2$ with the α subunit is also

integral to modulation. Each type of interface contributes to both agonist activation and allosteric modulation, but the contributions are not equal.

4.1 Allosteric modulation requires movement of all five subunits

Our primary observation supporting the conclusion that interface movement underlies general channel activation is that oxidation of pairs of cysteine residues engineered across these interfaces perturbs evoked currents. For two mutants $\alpha 3Y168C\beta 2D190C$ (M1) and $\alpha 3S125C\beta 2Q39C$ (A2), oxidation treatments decreased currents evoked by ACh alone and by the potentiating combination of ACh + Mor/Oxa (Fig. 4 and Fig. 6). For two others, $\alpha 3Q37C\beta 2A127C$ (M3) and $\alpha 3E173C\beta 2R46C$ (M4), oxidation increased currents (Fig. 7 and Supplemental Fig. S4).

Our previous work in the $\alpha 3\beta 2$ system (31) and numerous other studies of Cys-loop family receptors (e.g., 20, 45, 46) demonstrate that changes in current caused by oxidation are due to disulfide bond formation between the substituted cysteines. Accordingly in this study, decreases in $\alpha 3Y168C\beta 2D190C$ currents were completely reversed by subsequent treatment with the reducing agent DTT (Supplemental Fig. S2B), and several single cysteine mutants were unaffected by oxidation (Supplemental Fig. S3). Disulfide bond formation alters channel efficacy, because maximum responses (at saturating agonist concentrations) change while relative potencies do not. Hence, we conclude that movement of the subunits with respect to each other is necessary for optimal channel activation. This interpretation is straightforward for the M1 and A2 pairs for which disulfide bonds impair function. The improved function upon disulfide formation for the M3 and M4 pairs suggests that these bonds favor the open state. This in itself does not mean these pairs must move; however, that the unlinked residues interact comparatively less does suggest a change in orientation or distance at some point during gating. With movement implicated for the non-canonical $\beta 2(+)/\alpha 3(-)$ [M1, M3, M4] and canonical $\alpha 3(+)/\beta 2(-)$ [A2] interfaces, we infer that all subunits contribute to channel activation, including allosterically modulated states. We did not probe directly the role of homomeric α/α or β/β interfaces that should exist in our system; however, all five subunits contribute to the four interfaces we did examine, and such homomeric interfaces have recently been shown to impact or harbor agonist activities (15, 16).

Although we have no chemical or structural demonstration of disulfide bond formation, we observe large and varied effects of oxidation for cysteine pairs, but no such changes for the wild type or single mutant receptors. Desensitization cannot explain our results because responses were stable before and after treatments, decreases in currents upon oxidation were reversible, and for two pairs oxidation increased currents. Furthermore, we have made no assumptions about channel activation mechanism in regard to the relative occupancy of agonist and modulator sites. Thus, only in the unlikely case that a double mutant greatly perturbed the subunit stoichiometry would comparison of the effects of mutants be open to misinterpretation.

4.2 Subunit interfaces do not contribute symmetrically to channel activation

Previous work has demonstrated that movement in the agonist binding site interface underlies channel activation of Cys-loop receptors (e.g., 20, 47). The Auerbach and Sine groups, through exhaustive mutagenesis and rigorous quantitative modeling, have elucidated the route that agonist binding information follows from the canonical site to the gate, which includes residues in this interface, primarily in the α subunit (22, 23, and references therein). This work has also allowed dissection of the activation process (binding $\rightarrow$ gating) into discrete components (21, 37, 48). Drawing from these ideas, we postulated that Mor and Oxa, acting from the $\beta 2(+)/\alpha 3(-)$ interface, whether as a modulator or partial agonist, could communicate with the gate in a parallel fashion (i.e., “directly down”). The bulk of our evidence in this study, however, weighs against this hypothesis.

Mutations in locations A2 and M1–4 have different effects (from one another) on activation properties, and in some cases discriminate between agonist activation and modulation. Changes in EC_{50} for ACh activation were generally modest, with the largest increase (2-fold for $\alpha 3\beta 2Q39C$; Table 2) and largest decrease (60-fold for $\alpha 3S125C\beta 2Q39C$; Table 2) occurring for A2 mutants. Most changes in Mor modulation (EC_{50} comparisons) were in a similar range and, as might be expected, occurred in the $\beta 2(+)/\alpha 3(-)$ interface: 4-fold increase ($\alpha 3\beta 2W149A$; Table 2) and 70-fold decrease ($\alpha 3Y168F\beta 2$; Table 1). However, the largest change in Mor modulation (1000-fold decrease in EC_{50}) was for $\alpha 3S125Y\beta 2$ (A2) receptors, and improvements in ACh potency (2- to 10-fold) were realized for the six mutants studied in the M4 location (Table 3). Furthermore, the tyrosine substitution of $\alpha 3S125$ (perfectly conserved as tyrosine in all $\alpha 1$ sequences at this position) improved activation by Mor alone, to the point that it was a full agonist, while replacement in $\beta 2A127$ decreased Mor efficacy relative to wild type (Figs. 5 and Fig. 8; Table 3). Thus, the structural differences between canonical and non-canonical (heteromeric) interfaces in $\alpha 3\beta 2$ dictate unequal contributions to channel activation.

Our disulfide trapping results also indicate differences among the contributions of the probe locations to channel gating, particularly the homologous A2 and M3 sites. Oxidation decreased currents in the M1 and A2 pairs to different degrees (Fig. 4 and Fig. 6). While this may reflect different solvent accessibility of the cysteine pairs, our control experiments indicate that reactions were complete. Thus, the differences are more likely due to different efficacies in the cross-linked receptors, meaning that these locations do not mediate activation the same way. Furthermore, for the $\alpha 3S125\text{-}\beta 2Q39$ (A2) pair, the current decreases were greater for $I_{ACh + Mor}$ and I_{Mor} than for I_{ACh} (Fig. 6). More importantly, oxidation has opposite effects at the homologous $\alpha 3S125\text{-}\beta 2Q39$ (A2) and $\alpha 3Q37\text{-}\beta 2A127$ (M3) locations, possibly suggesting opposite directions of relative movements at these interfaces (cf. 49).

With five unique interfaces, the muscle-type nAChR is inherently asymmetric, and even its two ACh binding sites can be distinguished with various ligands (e.g., 18, 50). Similarly, benzodiazepine-sensitive GABA_A receptors have a high degree of asymmetry; interestingly, the benzodiazepine site has a unique position with respect to the two GABA sites (46) vis-a-vis the relationships of modulator and agonist sites in the $\alpha 3\beta 2$ system. In this light, it is perhaps not surprising that our results suggest the various $\alpha 3\beta 2$ interfaces contribute

unequally to agonist activation and modulation, but it begs the questions of the number and relative arrangement of agonist and modulator sites necessary for optimal channel activity. In ongoing work we are addressing these questions with subunit concatemers to control the number and relative order of sites.

4.3 The $\alpha 3$ subunit transduces modulator information

If modulator binding information does not travel “down” the $\beta 2(+)/\alpha 3(-)$ interface to the channel gate, by what route does it flow in order to enhance receptor activity? The finding that the $\alpha 3S125Y$ mutation dramatically enhanced the ability of Mor to activate the receptor on its own was unexpected (Fig. 5A), but it suggests that the modulation pathway includes this location. We have recently demonstrated that residues on the outer face of $\alpha 3$, at the junction of the two sheets constituting the β sandwich structure of the extracellular domain, mediate modulation and channel activation (31). Furthermore, residues in the $(-)$ -face of $\alpha 3$ govern modulator specificity (32), probably in most cases by direct contact, but mutations of several of these residues also substantially alter agonist activation (14, 32). Combining these observations, we propose that modulation consists of Mor binding information routed across or through the $\alpha 3$ subunit and added to the “principal gating pathway” (22) at or near $\alpha 3S125$, which itself is immediately adjacent to one of the cysteines of the Cys-loop.

4.4 The $\beta 2$ C loop governs modulation

Although we conclude that the $\alpha 3$ subunit contains the primary determinants of the modulation pathway for Mor and Oxa, the $\beta 2$ C loop also makes an essential contribution. Our data indicate that relative movement in the $\beta 2$ C loop impacts modulation and that its interaction with the $\alpha 3$ subunit may be involved in discriminating between Mor and Oxa. These findings lead us to an emerging hypothesis about the differences between potentiation and inhibition (positive and negative allosteric modulation) in this system.

Cross-linking the $\alpha 3$ and $\beta 2$ subunits at location M1 decreased channel function (Fig. 4); hence, movement in this area is necessary for optimal gating, in accord with our central hypothesis, and this disulfide bond favors the closed state. Although the differences are not large, the results suggest that oxidation is more effective in the presence of both ACh and Mor than in the resting state (Fig. 4A), which may indicate that $\alpha 3Y168$ and $\beta 2D190$ are closer or more reactive in a fully activated state. These observations alone do not enable us to determine a direction for the movement of the $\beta 2$ C loop relative to the $\alpha 3(-)$ face. Restriction of movement of the benzodiazepine interface also decreases potentiation by those modulators of GABA_A receptors (46).

The cysteine and phenylalanine substitutions in position 168 of $\alpha 3$ discriminated between Mor and Oxa modulation; when these were co-expressed with their respective $\beta 2D190$ mutant partners, discrimination was lost for the double-cysteine mutant but maintained for $\alpha 3Y168\beta 2D190Q$ (Fig. 3 and Table 1). The $(-)$ -face single mutations $\alpha 3E113R$, $\alpha 3Q30R$ and $\alpha 3E32G$, based on the $\alpha 4$ sequence, also differ in selectivity for Mor and Oxa (32, and unpublished observations). Thus, with further delineation of modulator specificity, we may find mutants that differentiate these modulators because specific modulator-receptor

interactions have been perturbed directly, or because in binding, the modulator promotes or disrupts discrete interactions between $\beta 2$ C loop and $\alpha 3(-)$ face residues.

In a forthcoming study we have extended the disulfide trapping approach to several cysteine pairs between the $\beta 2$ C loop and $\alpha 3(-)$ face. Cross-linking of at least one pair ($\alpha 3T115-\beta 2S192$) increases currents and including Mor during oxidation alters the changes for another pair ($\alpha 3Y168-\beta 2S192$; unpublished observations). Thus, with judicious choices of pairs, we think it possible to determine the direction of movement of the $\beta 2$ C loop with respect to $\alpha 3$, as well as pinpoint which residues discriminate between modulators. In turn, such studies may enable us to dissect contributions to gating mechanisms, in particular elucidating the determinants of positive and negative modulation (32), which will facilitate future drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

nAChR	nicotinic acetylcholine receptor
Mor	morantel
Oxa	oxantel
OR2	oocyte Ringer's
DTT	dithiothreitol
ANOVA	analysis of variance

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HIGHLIGHTS

Some nAChR allosteric modulators bind to subunit interfaces

Disulfide trapping and mutations affect receptor activation and modulation

Receptor movement during allosteric modulation is distinct from agonist activation

Novel finding of non-canonical interface motion mediating nAChR allosteric modulation

Figure 1.

Locations of residue pairs studied. The schematic represents the modulator site (M), at the $\beta 2(+)/\alpha(-)$ interfaces, and the canonical agonist site (A) at the $\alpha(+)/\beta 2(-)$ interfaces, and indicates the relative locations of the pairs of residues examined in this study. The extracellular domain of a pentameric $(\alpha 3)_2(\beta 2)_3$ receptor is shown, where the $\alpha 3$ subunits are purple and the $\beta 2$ subunits are blue. The choices of pairs are described in the text. We coded the pair locations as M1: $\alpha 3Y168/\beta 2D190$, A2: $\alpha 3S125/\beta 2Q39$, M3: $\alpha 3Q37/\beta 2A127$, M4: $\alpha 3E173/\beta 2R46$ and M5: $\alpha 3L107/\beta 2T150$ to facilitate referencing these locations and because we studied multiple mutants for some pairs. Numbers next to the pair codes indicate the distance ($\text{\AA}$) between nearest heavy atoms for the pairs in the rat homology model a3b2rr.pdb (30).

Figure 2.

Oxidation of double-cysteine receptors decreases activation. Representative current traces are shown for two double-cysteine receptors and the wild type $\alpha 3\beta 2$ receptor (independent oocytes), voltage-clamped at -60 mV. For each set, overlaid responses to ACh alone (black) and ACh + $10 \mu M$ Mor (red) prior to an oxidizing treatment (arrow) are on the *left* and responses following the treatment are on the *right*. Currents were evoked with 1 mM ACh for $\alpha 3Y168C\beta 2D190C$ and $300 \mu M$ for $\beta 2Q39C$ and wild type. The oxidation treatments were 5 min constant perfusion with 4.4 mM $H_2O_2/100 \mu M$ ACh/ $10 \mu M$ Mor for $\alpha 3Y168C\beta 2D190C$ and 4.4 mM H_2O_2 alone for $\alpha 3S125C\beta 2Q39C$ and wild type $\alpha 3\beta 2$. Scale bars are 50 sec (horizontal) for all traces; 50 nA (vertical) for $\alpha 3S125C\beta 2Q39C$ and 200 nA (vertical) for $\alpha 3Y168C\beta 2D190C$ and wild type $\alpha 3\beta 2$. Collated data for these three experiments are shown in Figs. 4, 6 and Supplemental S1 for $\alpha 3Y168C\beta 2D190C$, $\alpha 3S125C\beta 2Q39C$ and wild type, respectively.

Figure 3.

An interaction involving the C loop β 2 is needed for potentiation. Current responses evoked by ACh alone (squares; solid curves), ACh + 10 μ M Mor (circles; dashed curves) and ACh + 10 μ M Oxa (triangles; dotted curves) for three mutant receptor subtypes are plotted as a function of agonist concentration. **A.** Activation profiles for the double-cysteine receptor α 3Y168C β 2D190C are shown with black symbols and black fitted curves. Curves shown in red depict the best-fit parameters of the Hill equation for wild type α 3 β 2 (symbols omitted; data from 32). **B.** Activation profiles for the single mutant α 3Y168C β 2 and α 3 β 2D190C

receptors are shown in black and red, respectively; the ACh concentrations for $\alpha 3\gamma 168C\beta 2$ are slightly offset for clarity. Symbols represent means ($\pm$ SEM). Parameters for the Hill equation fits and replicates (n) are given in Table 1.

Figure 4.

Oxidation of the $\alpha 3\text{Y168C}\beta 2\text{D190C}$ receptor decreases activation. Currents evoked by 1 mM ACh alone (open bars) or in combination with 10 μM Mor (gray bars) or 10 μM Oxa (black bars) were measured before and after four oxidation treatments for the double-cysteine mutant $\alpha 3\text{Y168C}\beta 2\text{D190C}$; the fractional current remaining ($I_{\text{post}}/I_{\text{pre}}$) is plotted. In each case, the H_2O_2 concentration was 4.4 mM and the oxidation treatment lasted 5 min; each condition was tested in a separate experiment. The treatments were **A.** H_2O_2 co-applied with 100 mM ACh and 10 μM Mor ($n = 6$); **B.** H_2O_2 with 100 mM ACh ($n = 5$); **C.** H_2O_2

alone ($n = 8$) and **D**. H_2O_2 with $10\ \mu M$ Mor ($n = 5$). All changes upon treatment were significant at $p < 0.05$ (range 0.0045–0.047) and are denoted with an asterisk, except **C**. ACh alone ($p = 0.115$) and **B**. ACh + Oxa ($p = 0.069$). Representative traces for the $4.4\ mM$ $H_2O_2/100\ \mu M$ ACh/ $10\ \mu M$ Mor oxidation condition are shown in Fig. 2.

Figure 5.

A putative trigger for activation involves α 3S125 and β 2Q39. Receptor activation profiles for the two mutant receptors α 3S125Y β 2 and α 3S125Y β 2W149A are shown in panels **A** and **B**, respectively. Currents evoked by ACh alone (squares), ACh with 10 mM Mor (circles) or Mor alone (diamonds), each collected in separate experiments, were normalized to the response evoked by 1 mM ACh (α 3S125Y β 2) or 300 mM ACh (α 3S125Y β 2W149A). The best fits of the Hill equation to the data are indicated by solid (ACh alone), dashed

(ACh + Mor) and dotted curves (Mor alone), respectively; parameters for these fits and replicates (n) are given in Table 2.

Figure 6.

Oxidation of the $\alpha 3\text{S125C}\beta 2\text{Q39C}$ receptor decreases activation. The effects of sequential 4.4 mM H_2O_2 (alone) and 40–100 μM DTT treatments on $\alpha 3\text{S125C}\beta 2\text{Q39C}$ receptors are plotted as currents after treatment normalized to the original (naïve) controls, as evoked by 300 μM ACh alone (open bars), 300 μM ACh + 10 μM Mor (gray bars) or 10 μM Mor alone (hashed bars). Bars represent means (+ SEM). All changes upon oxidation treatment (H_2O_2 ; $n = 13$, all three challenges) were significant at $p < 0.05$ (range 2.2×10^{-4} – 3.3×10^{-3}), and are denoted with an asterisk. The changes following reduction treatment (DTT; $n = 7$, all three challenges) were significant (denoted with a dagger) for I_{ACh} ($p = 0.034$) and $I_{\text{ACh+Mor}}$ ($p = 0.028$), but not for I_{Mor} (0.089). Representative traces for the 4.4 mM H_2O_2 oxidation condition are shown in Fig. 2.

Figure 7.

Oxidation of the α 3Q37C β 2A127C receptor increases activation. The effects of sequential oxidation and reduction treatments on α 3S125C β 2Q39C receptors are shown as representative sample traces (A) and as collated data for currents after treatment normalized to the original (naïve) controls using two oxidation treatments of (B) 4.4 mM H₂O₂ + 100 μ M ACh + 10 μ M Mor and (C) 4.4 mM H₂O₂ alone. In panel A, currents were evoked by 1 mM ACh alone (black), 1 mM ACh + 10 μ M Mor (red) or 10 μ M Mor alone (blue) with the oxidation treatment (first arrow) as 5 min 4.4 mM H₂O₂ and reduction (second arrow) with

5 min of 40 μ M DTT. For panels B and C, collated data of the type shown in panel A for currents evoked by ACh alone (open bars), ACh + Mor (gray bars) and Mor alone (hashed bars), at the concentrations given above, are plotted as bar graphs; reduction treatment was with 40 μ M DTT in both cases. Bars represent means (+ SEM). For experiments of $n = 7$ (H_2O_2 /ACh/Mor; all three challenges in **B**), $n = 5$ (DTT in **B**) and $n = 4$ (both treatments; all three challenges in **C**), all changes upon oxidation were significant at $p < 0.05$ (range 2.5×10^{-5} –0.035), except **C**. Mor alone ($p = 0.132$), and are denoted with an asterisk. DTT did not produce significant changes in any of the three currents for either oxidation treatment ($p = 0.096$ –0.457).

Figure 8.

The agonist interface dominates receptor activation. Representative current traces are shown for the two single mutant $\alpha 3S125Y\beta 2$ and $\alpha 3\beta 2A127Y$ receptors and the co-expressed double mutant $\alpha 3S125Y\beta 2A127Y$ (independent oocytes), voltage-clamped at -60 mV. For each set, responses to agonist alone (black) and agonist + $10 \mu\text{M}$ Mor (red) are overlaid for ACh, nicotine (Nic) and epibatidine (Epi); currents evoked by $10 \mu\text{M}$ Mor alone are shown in blue. Overlaid traces are offset for clarity. All responses are normalized to that evoked by ACh alone, the magnitudes of which were 2.1 , 1.1 and $1.1 \mu\text{A}$ for $\alpha 3S125Y\beta 2$, $\alpha 3\beta 2A127Y$

and $\alpha 3S125Y\beta 2A127Y$, respectively. Saturating agonist concentrations were 300 μM ACh, 300 mM nicotine and 10 μM epibatidine. In all cases responses were evoked by a 5-sec application of drugs; traces are 60 sec in duration, except for the $\alpha 3S125Y\beta 2$ samples which are 45 sec. The numbers adjacent to the traces are means ($\pm$ SEM; $n = 3-8$), color coded to the traces, for replicate measurements in these experiments. For the agonists Nic, Epi and Mor, the values are relative to ACh, whereas for the +Mor condition for ACh, Nic and Epi, the values are relative to agonist alone.

Table 1

Agonist and modulator activation involve the $\beta 2$ C loop

Subtype	ACh Activation		ACh + Mor Modulation		ACh + Oxa Modulation	
	EC ₅₀ (μ M)	n _H	EC ₅₀ (μ M)	n _H	EC ₅₀ (μ M)	n _H
$\alpha 3\beta 2^a$	66 $\pm$ 9	0.75 $\pm$ 0.07	10 $\pm$ 2	1.37 $\pm$ 0.80	2.64 $\pm$ 0.29	1.54 $\pm$ 0.25
$\alpha 3Y168C\beta 2D190C$	37 $\pm$ 6	0.68 $\pm$ 0.08 [10]	2.9 $\pm$ 0.6	1.22 $\pm$ 0.33 [4-5]	1.37 $\pm$ 0.06	1.13 $\pm$ 0.45 [5]
$\alpha 3Y168C\beta 2$	36 $\pm$ 8	0.70 $\pm$ 0.11 [11]	1.6 $\pm$ 0.6	1.17 $\pm$ 0.47 [6]	1.46 $\pm$ 0.08	1.01 $\pm$ 0.27 [6]
$\alpha 3\beta 2D190C$	12 $\pm$ 2	1.05 $\pm$ 0.14 [9]	19 $\pm$ 8	1.08 $\pm$ 0.42 [4]	0.95 $\pm$ 0.08	1.13 $\pm$ 0.44 [5]
$\alpha 3Y168F\beta 2$	14 $\pm$ 2	0.61 $\pm$ 0.04 [12]	0.14 $\pm$ 0.03	0.50 $\pm$ 0.08 [6]	1.15 $\pm$ 0.03	1.73 $\pm$ 0.66 [6]
$\alpha 3\beta 2D190Q$	12 $\pm$ 2	1.00 $\pm$ 0.15 [8]	13 $\pm$ 6	0.73 $\pm$ 0.25 [4]	1.20 $\pm$ 0.11	1.19 $\pm$ 0.21 [4]
$\alpha 3Y168F\beta 2D190Q$	46 $\pm$ 7	0.83 $\pm$ 0.09 [11]	4.0 $\pm$ 1.2	1.17 $\pm$ 0.33 [4]	2.57 $\pm$ 0.14	1.10 $\pm$ 0.09 [4]
					10 $\pm$ 1	1.06 $\pm$ 0.02

Fits to the Hill equation for experiments with ACh alone, ACh + 10 μ M Mor or ACh + 10 μ M Oxa. In all experiments for the $\alpha 3Y168C\beta 2D190C$ double mutant, recordings were made on naive oocytes, having had no previous oxidation or reduction treatment [n] indicates the number of replicate oocytes for each measurement.

^aData for wild type $\alpha 3\beta 2$ from 32.

Table 2

Allosteric modulation involves the canonical agonist interface

Subtype	ACh Activation		ACh + Mor Modulation		Mor Activation			
	EC ₅₀ (μM)	n _H	EC ₅₀ (μM)	n _H	E _{ax}	EC ₅₀ (μM)	n _H	E _{max}
α3β2 ^a	66 ± 9	0.75 ± 0.07 [5–11]	10 ± 2	1.37 ± 0.80	2.64 ± 0.29 [5]	20 ± 2 ^b	3.1 ± 1.6	0.20 ± 0.04
α3S125Yβ2	7.6 ± 2.1	0.84 ± 0.19 [3–6]	8.8 ± 4.9nM	0.31 ± 0.05	1.41 ± 0.11 [3–6]	2.3 ± 0.7	1.58 ± 0.42 [3–4]	1.69 ± 0.27
α3S125Y·β2W149A	4.5 ± 1.4	0.61 ± 0.10 [6]	4.9 ± 2.0	0.72 ± 0.16 [5]	1.33 ± 0.10	25 ± 9	1.01 ± 0.29 [5–6]	0.21 ± 0.02
α3β2W149A ^a	40 ± 6	0.99 ± 0.12 [5]	44 ± 6	1.38 ± 0.19	0.86 ± 0.03 [5]	N.F.	N.F.	0.015 ± 0.004 [3]
α3S125C·β2Q39C	1.1 ± 0.2	0.66 ± 0.09 [3–7]	0.75 ± 0.40	0.62 ± 0.18 [4]	2.57 ± 0.17	0.53 ± 0.21	0.92 ± 0.30 [4–8]	1.00 ± 0.14
α3S125Cβ2	3.5 ± 1.3	0.64 ± 0.13 [5–7]	0.71 ± 0.20	0.85 ± 0.17	1.48 ± 0.07 [5]	92 ± 33	0.54 ± 0.11 [4]	0.028 ± 0.006
α3β2Q39C	130 ± 30	0.78 ± 0.12 [4]	2.0 ± 0.4	0.91 ± 0.15	2.54 ± 0.09 [6]	N.D.		

Fits to the Hill equation for experiments with ACh alone, ACh + 10 μM Mor or Mor alone. In all experiments for the α3S125Cβ2Q39C double mutant, recordings were made on naive oocytes, having had no previous oxidation or reduction treatment [n] indicates the number of replicate oocytes for each measurement. N.F. indicates no fit was possible for data collected in experiment; N.D. indicates no data were collected for experiment.

^aData of ACh and ACh + Mor titrations for wild type α3β2, and all data α3β2W149A from 32.

^bData of Mor titration for wild type α3β2 from 38.

Table 3

Agonist and modulator interface contributions to activation are not symmetrical

Subtype	ACh Activation		ACh + Mor Modulation		Mor Activation		
	EC ₅₀ (μM)	n _H	EC ₅₀ (μM)	n _H	EC ₅₀ (μM)	n _H	E _{max}
α3Q37Cβ2	45 ± 11	1.67 ± 0.44 [4]	36 ± 11	0.84 ± 0.15	1.40 ± 0.11 [6]	N.D.	
α3β2A127C	71 ± 4	2.09 ± 0.34 [4]	35 ± 14	0.80 ± 0.17	2.83 ± 0.28 [5]	N.D.	
α3Q37C-β2A127C	31 ± 5	0.97 ± 0.13 [6]	8.1 ± 3.4	0.99 ± 0.38	1.73 ± 0.15 [6]	N.D.	
α3β2A127Y	38 ± 4	1.46 ± 0.20 [5]	54 ± 12	1.50 ± 0.47	1.50 ± 0.09 [3]	4.8 ± 2.4	1.08 ± 0.40 0.05 ± 0.01 [5]
α3S125Yβ2 ^a	7.6 ± 2.1	0.84 ± 0.19	8.8 ± 4.9 μM	0.31 ± 0.05	1.41 ± 0.11	2.3 ± 0.7	1.58 ± 0.42 1.69 ± 0.27
α3S125Y-β2A127Y	14 ± 3	1.06 ± 0.19 [3]	2.6 ± 0.8	0.87 ± 0.19	1.13 ± 0.06 [3-4]	1.9 ± 0.2	3.18 ± 0.63 0.16 ± 0.01 [3]
α3E173Cβ2	13 ± 1	1.16 ± 0.12 [7]	6.4 ± 1.7	0.99 ± 0.22	1.02 ± 0.05 [6]	N.D.	
α3β2R46C	30 ± 6	0.88 ± 0.13 [5]	3.7 ± 1.0	1.22 ± 0.28	4.33 ± 0.22 [5]	N.D.	
α3E173C-β2R46C	14 ± 2	0.85 ± 0.10 [5-6]	19 ± 8	0.55 ± 0.09	1.05 ± 0.08 [5-6]	N.D.	
α3E173Aβ2	6.6 ± 0.7	1.77 ± 0.26 [3-6]	1.4 ± 0.5	1.15 ± 0.33	4.14 ± 0.27 [3]	N.F.	0.16 ± 0.01 [4]
α3β2R46V	5.0 ± 0.7	0.92 ± 0.14 [5-7]	3.2 ± 0.8	0.85 ± 0.21	1.06 ± 0.06 [5-7]	N.F.	0.03 ± 0.01 [7]
α3E173A-β2R46V	7.3 ± 0.8	0.82 ± 0.06 [7-8]	9.9 ± 2.1	1.07 ± 0.23	0.95 ± 0.05 [6-7]	N.D.	

Fits to the Hill equation for experiments with ACh alone, ACh + 10 μM Mor or Mor alone. In all experiments for the α3Q37Cβ2A127C double mutant, recordings were made on naive oocytes, having had no previous oxidation or reduction treatment [n] indicates the number of replicate oocytes for each measurement. N.F. indicates no fit was possible for data collected in experiment; N.D. indicates no data were collected for experiment.

^a Data for α3S125Yβ2 repeated from Table 2 for comparison.

Table 4

Thermodynamic cycles parse the contributions of interfaces to activation

Location	Subtype	$\Omega(\text{ACh})^a$	$\Omega(\text{ACh+Mor})^a$	Rmut-Mor ^b	Rmut-Oxa ^b
M1	$\alpha 3\text{Y168}\beta 2\text{D190C}$	5.7	2.2	0.52	1.9
	$\alpha 3\text{Y168C}\alpha\beta 2$			0.29	1.2
	$\alpha 3\beta 2\text{D190C}$			10	19
M1	$\alpha 3\text{Y168}\beta 2\text{D190Q}$	18	4.9	0.57	1.6
	$\alpha 3\text{Y168F}\alpha\beta 2$			0.066	1.5
	$\alpha 3\beta 2\text{D190Q}$			7.1	19
A2	$\alpha 3\text{S125Y}\beta 2\text{W149A}$	0.98	2.9	7.2	
	$\alpha 3\text{S125Y}\beta 2$			0.0076	
	$\alpha 3\beta 2\text{W149A}$			7.3	
A2	$\alpha 3\text{S125C}\beta 2\text{Q39C}$	0.16	1.8	4.5	
	$\alpha 3\text{S125C}\beta 2$			1.3	
	$\alpha 3\beta 2\text{Q39C}$			0.10	
M3	$\alpha 3\text{Q37C}\beta 2\text{A127C}$	0.64	1.15	1.7	
	$\alpha 3\text{Q37C}\beta 2$			5.3	
	$\alpha 3\beta 2\text{A127C}$			3.3	
M4	$\alpha 3\text{E173C}\beta 2\text{R46C}$	2.4	8.0	4.7	
	$\alpha 3\text{E173C}\beta 2$			8.7	
	$\alpha 3\beta 2\text{R46C}$			1.7	
M4	$\alpha 3\text{E173A}\beta 2\text{R46V}$	15	0.57	9.0	
	$\alpha 3\text{E173A}\beta 2$			1.4	
	$\alpha 3\beta 2\text{R46V}$			4.2	
A2/M3	$\alpha 3\text{S125Y}\beta 2\text{A127Y}$	3.2	1.4	1.2	
	$\alpha 3\text{S125Y}\beta 2$			0.0076	
	$\alpha 3\beta 2\text{A127Y}$			9.4	

^aUsing values given in Tables 1–3, thermodynamic cycle Ω calculated as $\Omega = \frac{EC50(WT) \cdot EC50(mut\ ij)}{EC50(mut\ i) \cdot EC50(mut\ j)}$ for activation by ACh alone, ACh + 10 μM Mor or ACh + 10 μM Oxa; all with reference to wild type $\alpha 3(32)$ receptors.

^bUsing values given in Tables 1–3, mutation-modulator R calculated as $\hat{\alpha}\hat{s}\hat{d} = \frac{EC50(WT, A) \cdot EC50(mut, A + M)}{EC50(WT, A + M) \cdot EC50(mut, A)}$ for activation by Mor or Oxa; all with reference to wild type $\alpha 3(32)$ receptor.