Specificity Determinants of Allosteric Modulation in the Neuronal Nicotinic Acetylcholine Receptor: A Fine Line between Inhibition and Potentiation

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Switching Allosteric Inhibition and Potentiation in nAChRs

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Nonstandard abbreviations: nAChR – nicotinic acetylcholine receptor; Mor – morantel; Oxa – oxantel; Pyr – pyrantel; MTS - methanethiosulfonate
ABSTRACT

We are interested in the allosteric modulation of neuronal nicotinic acetylcholine receptors (nAChRs). We recently postulated that the anthelmintic morantel (Mor) positively modulates (potentiates) rat α3β2 receptors through a site, homologous to the canonical agonist site, located at the β(+)/α(−) interface (Seo et al., 2009, J. Neurosci. 29: 8734). On this basis, we aimed to determine the site specificity by studying differences in modulation between α3β2 and α4β2 receptors. We also compared modulation by Mor to that of the related compound oxantel (Oxa). Whereas Mor and Oxa each potentiated α3β2 receptors two-fold at saturating ACh concentrations, Mor had no effect on α4β2 receptors, and Oxa inhibited ACh-evoked responses. The inhibition was noncompetitive, but not due to open channel block. Furthermore, the nature and extent of modulation did not depend on subunit stoichiometry. We studied six positions at the α(−) interface that differ between α3 and α4. Two positions (α3I57 and α3T115) help mediate the effects of the modulators, but appear not to contribute to specificity. Mutations in two others (α3L107 and α3I117) yielded receptors with appreciable α4-character; that is, Mor potentiation was reduced compared to wild type α3β2 control and Oxa inhibition was evident. A fifth position (α3E113) was unique in that it discriminated between the two compounds, showing no change in Mor potentiation from control but substantial Oxa inhibition. Our work has implications for rational drug design for nicotinic receptors and sheds light on mechanisms of allosteric modulation in nAChRs, especially the subtle differences between potentiation and inhibition.
INTRODUCTION

The neuronal nicotinic acetylcholine receptors (nAChRs) are a diverse family of ligand-gated ion channels and are members of the Cys-loop superfamily. They are implicated in neurological disorders such as Alzheimer’s and Parkinson’s diseases (Albuquerque et al., 2009). Perhaps more importantly, nAChRs have a central role in nicotine abuse, as nicotine increases the activation of reward pathways (Dani and Bertrand, 2007). The clinical successes of drugs such as varenicline for smoking cessation (e.g., Gonzales et al., 2006) and galanthamine in reducing cognitive impairments associated with Alzheimer’s (Birks, 2006) suggest promise for pharmacological interventions targeting central cholinergic systems. While nAChRs containing \( \alpha_7 \) or \( \alpha_4/\beta_2 \) subunits have been a focus of drug development, specific roles for nAChRs in physiological processes and well-defined nAChR subtypes remain unclear (e.g., Gotti et al., 2009); thus, the search for new nAChR ligands is still broad (Jensen et al., 2005).

Substantial recent interest has focused on allosteric modulators of nAChRs (Bertrand and Gopalakrishnan, 2007). Such compounds enhance or inhibit channel activity from binding sites that are noncompetitive with the canonical (orthosteric) agonist site. Some positive modulators have the advantageous property of being at most weak partial agonists. Thus, their potentiation of responses elicited by endogenous ACh may allow for temporal and spatial coincidence activation in cholinergic pathways, thereby avoiding the tonic activation expected for drugs that are full agonists (Maelicke and Albuquerque, 2000).

Several novel compounds and their allosteric modulatory effects have been reported. Examples include PNU-120596 (Hurst et al., 2005) acting on \( \alpha_7 \) receptors, KAB-18 (Henderson et al., 2010) acting on \( \alpha_4\beta_2 \) receptors, and LY-2087101 with a less selective profile (Broad et
al., 2006). In addition, a variety of previously known compounds act as allosteric modulators, such as the anti-acetylcholinesterases galanthamine and physostigmine (e.g., Schrattenholz et al., 1996) and the anthelmintic ivermectin (Krause et al., 1998). We and others have shown that other anthelmintic compounds that are full agonists in lower species, causing spastic paralysis in the worm, also allosterically potentiate human and rat nAChRs (e.g., Bartos et al., 2006; Wu et al., 2008).

Of primary concern for a complete understanding of allosteric modulators—and crucial for any rational drug design efforts—is localizing ligand binding sites. The identification and elaboration of the canonical agonist and competitive antagonist binding site at the α(+)/non-α(−) interface of the receptor extracellular domain is a well-developed aspect of the nAChR field (Arias, 2000; Sine and Engel, 2006). Work based on X-ray crystal structures of the nAChR extracellular domain homologue acetylcholine binding protein, as well as a model of the entire muscle-type receptor and homology modeling thereof, has enriched our understanding of receptor-ligand interactions (e.g., Rucktooa et al., 2009). This information is now being employed to determine binding sites for allosteric modulators of nAChRs (e.g., Nirthanan et al., 2008; Collins and Millar, 2010). While technical limitations and subtype diversity together currently preclude an atomic-resolution picture of many neuronal nAChR systems of interest, the available models still have very strong predictive power in guiding structure-function studies of nAChRs.

On the basis of our studies of anthelmintics as allosteric modulators, we proposed as a general feature of the receptors that nAChR ligand “pseudo-sites” occur at the non-canonical subunit interfaces (e.g., β(+)/α(−) for Mor; Seo et al., 2009). From this premise, several important questions about the nature of these sites arise: Which features are common to both
canonical and non-canonical sites, thus constituting a generalized nAChR ligand site, and which features differentiate canonical (agonist/competitive antagonist) from non-canonical (modulator) sites? What are the determinants of specificity for allosteric modulators acting on different receptor subtypes? Such questions seem of increasing importance given the recent demonstration that the α(+)/α(−) interface in (α4)3(β2)2 receptors constitutes a low-sensitivity agonist site (Harpsoe et al., 2011; Mazzaferro et al., 2011). The answers to such questions are one of the keys to the development of therapeutic agents against neuropathologies involving nicotinic receptors. In this study, we identify the determinants of specificity of the β2(+)/α3(−) modulator site and discover that modulation can be inter-converted between potentiation and inhibition by point mutations.
MATERIALS AND METHODS

Reagents

All chemicals used, 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). Pyrantel is 1-methyl-2-(2-[2-thienyl]ethenyl)-1,4,5,6-tetrahydropyrimidine, with tartrate salt. Methanethiosulfonate (MTS) reagents were purchased from Toronto Research Chemicals (Toronto, Canada). Those used in this study were [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET) and imidazole-4-methyl methanethiosulfonate (MTS-His).

Nicotinic receptor clones and mutagenesis

Plasmids of the pGEMHE background bearing wild type rat α3 and β2 cDNA sequences were a gift from Dr. Charles Luetje (Miami University); the clones were originally isolated in the lab of Dr. Jim Patrick (Baylor College of Medicine; Boulter et al. 1987). Mutant genes were created either by 1) the QuikChange temperature cycling method (Stratagene; La Jolla, CA) with complementary primers harboring the mutation, or 2) custom mutagenesis from GenScript (Piscataway, NJ). Mutations were verified by complete sequencing of the entire extracellular domain region using capillary electrophoresis of dye-detected, dideoxy-generated fragments. Unless otherwise noted in the context of another receptor subtype, all α3 and β2 residue numbering follows that in the structure a3b2rr.pdb (Sallette et al., 2004); these position numbers are smaller by 2 compared to numbering used elsewhere in the literature, which discrepancy arises due to homology modeling based on a crystal structure of a protein of different sequence.
The cDNAs were linearized with a unique restriction enzyme, and then were made RNase-free by phenol-chloroform extraction. RNAs were synthesized from these cDNAs using the T7 kit from Ambion (Austin, TX). RNAs were diluted with RNase-free water to 0.5 µg/µL and stored at –20 °C.

**Oocyte preparation and injection**

Functional receptors were expressed in *Xenopus laevis* oocytes harvested from oocyte-positive female frogs or whole ovary tissue (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-VI oocytes were prepared by collagenase treatment and manual selection. Oocytes were maintained at 16 °C in Barth’s medium (in mM: 88 NaCl, 1.0 KCl, 2.5 NaHCO₃, 0.30 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 15 HEPES, 2.5 sodium pyruvate; pH 7.6, supplemented with 100 U/mL penicillin/streptomycin and 50 µg/mL gentamycin). Using a Nanoject micro-injector (Drummond; Broomall, PA), each oocyte was injected with 46 nL of a 1:1 (by volume) combination of the desired α and β subunits, prepared from 0.5 µg/µL stock solutions. In a subset of experiments, 10:1 or 1:10 combinations (by volume; total of 46 nL per oocyte) were injected following previous work (Zwart and Vijverberg, 1998; Moroni et al., 2008). Allowing 2-4 days for receptor expression, with daily changes of Barth’s medium and removal of dead cells, currents could be recorded for up to 7 subsequent days. Expression for all mutants was comparable to that for the wild type α3β2 and α4β2 receptors as judged by currents in the range 0.2 – 4 µA evoked with a saturating ACh concentration (3 mM); expression appeared to depend more on the donor/injection batch than on subtype. Likewise, current traces for all mutants, including the triple and quintuple
combinations, were very similar to wild type α3β2 (and not α4β2; See Figure 1A) at the same effective concentrations; this observation suggests that neither the mutations nor the modulators greatly affected the desensitization properties of the receptors, consistent with our previous results (Wu et al., 2008).

Macroscopic current recordings

Electrophysiology recordings were made with an Axon Instruments Gene Clamp 500B amplifier to measure evoked currents from oocytes using the two-electrode voltage clamp method, following previous work (Wu et al., 2008; Seo et al., 2009). Voltage was clamped at −60 mV, with leak currents < 200 nA, although larger leak currents were occasionally tolerated in the presence of a stable baseline. Perfusion and drug administration were controlled with Warner Instruments VC-6 solenoid valve systems. Recording electrodes were filled with 3 M KCl and selected for resistances between 0.5 and 5 MΩ. Each oocyte was perfused with drug for 5 seconds, and washed with oocyte Ringer’s medium (OR2; in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 HEPES, pH 7.3) for at least 90 seconds. Dilute drug solutions were prepared in OR2 from concentrated stocks. We determined spectrophotometrically that Oxa is fully soluble to at least 1 mM, which is consistent with the much higher calculated maximum solubility of 0.47 M under similar conditions (SciFinder; Chemical Abstracts Service: Columbus, OH, 2011; RN 58-08-2 (accessed October 20, 2011); calculated using ACD/Labs software, version 8.14; ACD/Labs 1994-2011). Both of these exceed the highest concentration used in our experiments (300 μM). In experiments using MTS reagents, we followed established procedures (Karlin and Akabas, 1998); small aliquots of the crystalline reagent were dissolved in water and kept on ice before diluting to the working concentration in OR2 immediately before use in the experiment.
Changes in current with respect to the baseline in response to the administration of drug were recorded. Current responses were recorded using Clampex 9.2 and measured with Clampfit 9.2 (Molecular Devices; Sunnyvale, CA).

Concentration-response behavior for wild type and mutant receptor subtypes, as well as potentiation for ACh plus modulator (Mor, Oxa, Pyr) was characterized across the micromolar to millimolar range. Comparisons were made by fitting the Hill equation (fractional response = $E_{\text{max}}/\left\{1+(EC_{50}/[\text{agonist}])^{n_H}\right\}$) to concentration-response data for wild type and mutant receptor subtypes; for all experiments testing modulators, currents were normalized to the response evoked by a saturating ACh concentration alone as an internal control. The ACh responses of the two wild type subunit combinations (Figure 1 and Table 1) were consistent with previous reports (Cohen et al., 1995; Hsiao et al., 2006). Unless indicated otherwise, modulation was measured via co-application of 10 μM modulator and ACh. Repeat measurements for the same oocyte were averaged, and responses for multiple oocytes were normalized as appropriate to the experiment (described in figure legends). Data are reported as means ± S.E.M.
RESULTS

We are interested in a class of anthelmintic compounds that includes morantel, pyrantel and oxantel (structures in Figure 1A) as a model of allosteric modulation of neuronal nicotinic acetylcholine receptors (nAChRs). Having previously determined that morantel (Mor) potentiation was quite specific for $\alpha_3\beta_2$ receptors (both rat and human; Wu et al., 2008), we carried out a limited structure-activity relationship study with these three anthelmintics. The two key results from this approach are demonstrated by the representative traces shown in Figure 1A. First, whereas Mor and pyrantel (Pyr) potentiate ACh-evoked currents for $\alpha_3\beta_2$ receptors, there is little to no effect of these compounds on $\alpha_4\beta_2$ receptors when co-applied with ACh. Second, while oxantel (Oxa) potentiated $\alpha_3\beta_2$ responses in the same manner as Mor and Pyr, it inhibited $\alpha_4\beta_2$ ACh-evoked currents.

Because the degree of potentiation is a strong function of both agonist and modulator concentration (Wu et al., 2008), we explored the full concentration-response behavior for the three anthelmintics on both $\alpha_3\beta_2$ and $\alpha_4\beta_2$ nAChRs. As shown in Figure 1B, the three compounds potentiated ACh-evoked currents in a quantitatively indistinguishable manner; each of the three parameters characterizing the Hill equation fits are the same within error across the three drugs (See Table 1 and the Figure 1 legend). Importantly, these compounds potentiated at saturating concentrations of agonist ($I_{+\text{modulator}}/I_{\text{control}} \sim 2.3$), a macroscopic indication of increased efficacy of channel gating (Wu et al., 2008).

In contrast, Mor and Pyr failed to potentiate $\alpha_4\beta_2$ responses (Figure 1C); Hill equation fits for the modulator-added condition are indistinguishable from their matched pair ACh-alone controls (Table 1 and the Figure 1 legend). Surprisingly, 10 $\mu$M Oxa inhibited all ACh-evoked currents.
currents across this range, with an 18-fold decrease in potency for the evoked response. The increased Hill slope and decreased maximum response (at the highest ACh concentrations) are immediate indications that Oxa inhibits α4β2 receptors noncompetitively (Rang, 1981; Colquhoun, 1998). In addition to the fit to the entire data set denoting a decreased efficacy ($E_{\text{max}} = 0.85 \pm 0.13$), the ACh + Oxa responses at 1 and 3 mM ACh were significantly smaller than controls (t-test; $p < 0.05$). At this level of analysis, Mor and Pyr had identical behavior; we therefore focused only on identifying the origins of the differences between Mor and Oxa.

We next investigated in more detail the mechanism of Oxa inhibition of α4β2 responses. Figure 2A demonstrates that, while inhibition of ACh-evoked currents increased with increasing Oxa concentration, the currents could not be completely eliminated under these conditions (100 μM ACh ~EC$_{70}$), as would be expected for competitive inhibition. This incomplete inhibition is unlikely to arise from Oxa insolubility, because the highest concentrations we used are well below the maximum solubility under these conditions (See Methods). Many nAChR ligands are positively charged and inhibit by an open channel blocking mechanism at high concentration (Buisson and Bertrand, 1998; Arias et al., 2006). We therefore measured Oxa inhibition of α4β2 as a function of membrane holding potential. As can be seen in Figure 2B, Oxa inhibition was not strongly voltage-dependent, and if anything, showed a slight increase as the cell was depolarized. This behavior is opposite that expected for an open channel blocker, the inhibition by which becomes more severe at hyperpolarized potentials due to the increased driving force to cross the membrane (Buisson and Bertrand, 1998). Notice also that the degree of inhibition was greater for the lower (100 μM) ACh concentration at all potentials measured; this behavior is also opposite to that expected for competitive and open channel block mechanisms (Rang, 1981).
Numerous studies have demonstrated that the stoichiometry of α4 and β2 subunits in heterologously expressed receptors dictates their response to agonists and antagonists, as well as allosteric modulators (e.g., Zwart and Vijverberg, 1998; Moroni et al., 2008). We therefore measured Oxa inhibition of α4β2 receptors as a function of the ratio of the subunit RNAs injected, following the previous work cited above in choosing the ratios 10:1 and 1:10. Figure 2C shows that Oxa inhibition is substantial throughout the range of ACh concentrations tested, including at saturation (~1 mM). Comparison to Figure 1C reveals that Oxa inhibition of the 10:1 and 1:10 combinations is qualitatively the same as the 1:1 subunit RNA ratio. Furthermore, we obtained the same result (equivalent Oxa inhibition) when we co-expressed wild type α4 with the β2(+) mutant β2T150C in 10:1 and 1:10 ratios (data not shown). While the degree of inhibition of the 10:1 and 1:10 α4:β2 combinations may differ slightly from one another and from the 1:1 ratio, these results suggest that both α and β subunits are necessary for the effect (see also below) and that the different modes of modulation by Mor and Oxa do not arise because each compound acts preferentially on a receptor isoform of different stoichiometry.

Chemical modification studies of a series of cysteine-substituted residues provided evidence that Mor binds in the β(+)/α(−) interface, in a pocket structurally homologous to the canonical/agonist binding site (Seo et al., 2009). In particular, both tryptophans and two of the three tyrosines of the so-called aromatic box (Arias, 2000; Sine and Engel, 2006) are conserved in this interface. We therefore sought to substantiate that tryptophans 149 of β2 and 53 of α3 (See Figure 4) play a role in allosteric modulation by these anthelmintics. As shown in Figure 3, the concentration-response experiments for α3β2W149A receptors support this idea. In contrast to the very large degree of potentiation by Mor and Oxa on wild type α3β2 receptors, both
modulators gave mild inhibition of the ACh response. We found nearly identical results with the α3W53Aβ2 mutant (Table 1). Importantly, both tryptophan → alanine mutants responded to ACh just as well as wild type receptors (each has slightly larger nH; Table 1), indicating that the loss of Mor and Oxa potentiation does not arise from a general impairment of receptor function.

Given this further support for a modulator binding site located near, and possibly involving, β2W149 and α3W53, we turned our attention to elucidating the specificity differences between α3- and α4-containing receptors and between Mor and Oxa. Figure 4 shows a homology structural model of the β(+)/α(−) interface (Sallette et al., 2004), based on the acetylcholine binding protein X-ray crystal structure (Brejc et al., 2001), and an alignment of the rat α3 and α4 sequences for the equivalent of loops D and E from the canonical binding site. We reasoned that residues differing between the two subunits give rise to the specificity differences; those we selected for study are color-coded with the side chains shown in the structure (Figure 4). Notice that the residues differing between α3 and α4 alternate with residues identical in the two, and that these identical amino acids are largely hydrophobic in character. This observation is consistent with the structural prediction that these stretches form β-strands with the hydrophobic residues directed toward the interior and that the alternating residues constitute diverse surfaces in this region for the two subunits (cf. Teissére and Czajkowski, 2001).

Figure 5 illustrates the effects of substituting α4 residues into the α3 subunit for three of the six positions we examined. For the mutants α3L107H, α3E113R and α3I117T (Figures 5A-C, respectively), the ACh-control concentration-response behavior was quite similar to that for wild type α3β2 (See Table 1), although the parameters trended toward the α4 behavior. Again, these results indicate that the mutations do not significantly disrupt receptor function. In contrast
to the wild type-like ACh activation for these three mutants, each had a different pattern of response for Mor and Oxa modulation.

Mor potentiation was markedly reduced in α3L107Hβ2 as compared to wild type α3β2 (Figure 5A), but not to the extent seen in wild type α4β2. Our standard test concentration of 10 μM Oxa was only slightly inhibitory for α3L107Hβ2, but this behavior was considerably closer to that of α4 considering that Oxa substantially potentiates α3 responses at this concentration. Five-fold higher Oxa concentration gave more α4-like inhibition, with a four-fold decrease in potency and a significant increase in the Hill coefficient (Table 1). We also studied the cysteine-substitution in this position, α3L107Cβ2 (Table 1). Its behavior on the whole was quite similar to wild type α3β2, in particular maintaining potentiation by both Mor and Oxa; note the left-shifted EC50 values relative to ACh alone and Emax values > 1.5. While cysteine is polar and smaller than leucine, it does not introduce an aromatic group or the expected positive charge of the α4-based histidine substitution. Following treatment with methanesthosulfonate (MTS) reagents that introduce either a fixed positive charge or an imidazole group, currents evoked by ACh + Mor or ACh + Oxa in α3L107Cβ2 were reduced relative to controls, indicating that the position is accessible to the reagent and involved in modulation (LCC and MML, unpublished observations). Together, our results on the mutant α3L107Hβ2 and α3L107Cβ2 receptors strongly support a role of this position in modulation by Mor and Oxa.

In contrast to the α3L107 position, the mutation in position 113 (glutamate → arginine) showed marked discrimination between Mor and Oxa. Mor potentiation for α3E113Rβ2 was nearly identical to wild type α3β2 (Figure 5B; Table 1), although it had a slightly lower maximum effect at saturating agonist concentrations. However, Oxa effects were much more
like wild type α4β2, with a two-fold right-shift of EC50 and increased nH relative to control. In addition, 10 μM Oxa inhibited ACh-evoked currents (side by side measurements; paired t-test, p < 0.05) at all concentrations but the highest (3 mM).

The α3I117Tβ2 receptors had yet a third profile, with modulation by Mor and by Oxa much more like wild type α4 than α3 receptors, without discriminating between the two (Figure 5C; Table 1). Not only was Mor potentiation abolished for this receptor, but Mor inhibited at non-saturating ACh concentrations. Similarly, like wild type α4, Oxa inhibited most at lower ACh concentrations, shifting the response curve to the right with a three-fold increase in EC50.

We also studied three other positions in the α3 loop D and E regions. The best-fit parameters for these mutants are given in Table 1. The α3K109F substitution removes a positive charge and introduces an aromatic moiety. Despite this substantial change in physical character, Mor and Oxa still potentiated the ACh response like wild type α3β2, if not better. The impact of another charge substitution – neutral and nonpolar to negatively-charged – in the α3I57Eβ2 receptor was similar. Both Mor and Oxa potentiated the receptor with substantial 18-fold left-shifts in EC50 for the concentration-response curve relative to ACh alone activation (cf. six-fold for wild type α3β2). However, the overall improvement in efficacy in the presence of the modulator was less than for the wild type, as indicated by Emax of 1.7 vs. 2.6 for Mor and 1.2 vs. 2.3 for Oxa. Finally, two mutations at position 115 of α3 gave rather surprising results. For substitutions of the α3 threonine with cysteine and with the α4-based glutamine, both Mor and Oxa still potentiated ACh-evoked responses; the left-shift in EC50 (relative to ACh control) ranged from 13- to 125-fold. While the relative efficacy of both modulators was about the same as wild type α3β2 receptors for α3T115Cβ2 (a fairly conservative substitution), the Emax
increased for both in the case of α3T115Qβ2. Along with our previous demonstration of substantial effects of MTS modification on Mor activity for α3T115Cβ2 (Seo et al., 2009), these results support a role of position 115 in modulation of these receptors. However, this position does not appear to discriminate between the two compounds.

Not surprisingly, no single mutation that we studied converted α3 to α4 in terms of its modulation, so we next sought to generate α4-like modulation with combination mutants. To this end, we made α3/α4[3], the triple mutant α3L107H/E113R/I117T, and α3/α4[5], the quintuple mutant that adds α3T115Q and α3I57E to the triple mutant. Again we measured the effects of 10 μM Mor or 10 μM Oxa on the ACh concentration-response for these mutant subtypes (Figure 6). There were no apparent adverse effects of these mutations in combination on receptor function (for example, on protein folding or receptor assembly), as suggested by ACh-concentration-response behavior like the two wild type receptors (Table 1) and by typical expression levels (evoked current magnitudes). In fact, the α3-based combination mutants interestingly had ACh responses very similar to wild type α4β2, indicating an impact of this interface in channel activation (cf. Seo et al., 2009). Both combination mutants also had Mor and Oxa modulation behavior more like α4 than any of the single mutants, but with some interesting differences (Table 1). The Mor-added condition gave ACh-responses indistinguishable from control for the two combination mutants; that is, Mor did not potentiate these mutants. For the α3/α4[3] construct, the Oxa-added titration resulted in an EC50 shifted to lower potency (about three-fold) and an efficacy much lower than control (Emax ~ 0.5); this efficacy was also lower than for Oxa inhibition of wild type α4 receptors. While the potency
shift for the Oxa-added condition was larger at four-fold for $\alpha_3/\alpha_4[5]$, the efficacy was the same as wild type $\alpha_4\beta_2$ receptors ($E_{\text{max}} = 0.88$), but importantly less than control response.

In order to confirm that Oxa inhibits the $\alpha_3$ mutant receptors by the same mechanism as it does $\alpha_4\beta_2$ receptors, we also studied the voltage-dependence of Oxa inhibition. These results are shown in Figure 7A, which is a plot of the degree of inhibition as a function of holding potential. For the three point mutants showing the greatest $\alpha_4$-character (Figure 5), we studied Oxa inhibition at two ACh concentrations. As was the case for wild type $\alpha_4\beta_2$, these data sets have effectively zero slopes. The only exception was the condition of 100 $\mu$M ACh for $\alpha_3I117T\beta_2$ (open triangles), which showed a shallow negative slope. We found similar results for the two combination mutants (data not shown in Figure 7A for clarity). As was the case with Oxa inhibition of wild type $\alpha_4\beta_2$ receptors, these results are contrary to an open channel block mechanism.

Similarly, we confirmed that changing the subunit stoichiometry of $\alpha_3$-based receptors does not substantially alter Oxa inhibition. Figure 7B shows that Oxa inhibited indistinguishably receptors expressed by co-injecting the triple mutant $\alpha_3/\alpha_4[3]$ and wild type $\beta_2$ RNAs in ratios of 10:1 and 1:10. As was the case with wild type $\alpha_4\beta_2$ receptors, different subunit stoichiometries do not appear to give rise to different modes of modulation by Mor and Oxa.
DISCUSSION

We studied the effects of mutations at the non-canonical \( \beta(+) / \alpha(-) \) interface on the allosteric modulation of neuronal nicotinic receptors by anthelmintic compounds. Our results support our previous hypothesis that this interface constitutes the modulator binding site, and we have identified several residues in the rat \( \alpha_3 \) subunit that not only give rise to the subunit specificity for these compounds but also discriminate between morantel (Mor) and oxantel (Oxa). We have also unexpectedly discovered that modulation in this system can shift between the extremes of potentiation and inhibition with ostensibly minor structural changes in the receptor.

*The Modulator Binding Site*

Our data and previous reports in the literature support the conclusion that the \( \beta(+) / \alpha(-) \) interface contains an allosteric modulator binding site. As shown in Figure 3 and Table 1, mutation of the two highly conserved tryptophans to alanine (\( \alpha_3W53 \) and \( \beta_2W149 \)) abolished the potentiation by Mor and Oxa seen in wild type \( \alpha_3\beta_2 \). While it is tempting to interpret these results as substantially reducing modulator affinity (thereby supporting a direct role in binding), the effects are no doubt more complicated: For example, the 2.5-fold increase in \( EC_{50} \) for ACh activation and \( E_{max} = 0.90 \) in the presence of Oxa indicate inhibition.

We previously demonstrated that residues in this interface, particularly \( \beta_2T150 \) and \( \alpha_3T115 \), contribute to allosteric potentiation (Seo et al., 2009). The gross structural homology of this interface pocket to the canonical agonist site and that four of the five residues of the
“aromatic box” are conserved further substantiate this as a ligand binding site. Additionally, the Luetje and Bermudez groups found that residues in this same vicinity of non-canonical interfaces in neuronal nAChRs are responsible for modulation by divalent cations (Hsiao et al., 2006; Moroni et al., 2008).

Furthermore, the modulation of nAChRs by anthelmintic compounds parallels that of GABA<sub>A</sub> receptors by benzodiazepines, the non-canonical interface binding sites of which are well-established (e.g., Miller and Smart, 2010). For example, radioligand binding assays, evoked-current measurements and chemical modification experiments for receptors with mutations in these positions indicate that γ2T81, γ2R132 and γ2T142 play roles in benzodiazepine/hypnotic binding or modulation (Kucken et al., 2000; Teissére and Czajkowski, 2001; Hanson et al., 2008). These residues are homologous to the α3I57, α3L107 and α3I117 nAChR residues, respectively, which we found govern Mor and Oxa activities (Figures 5 and 6; Table 1). While it is possible that Mor and Oxa bind elsewhere to the nicotinic receptor, all our results are consistent with a binding site at the β(+)/α(−) interface. This demonstration suggests that five (potential) interface ligand binding sites are a general feature of the Cys-loop receptor superfamily.

**Specificity of Modulation**

The major goal of this study was to understand the specificity of modulator–binding site interactions. Our limited structure-activity relationship design, exploring both the compounds and subunits, yielded specificity information about both. Five of the six positions differing between α3 and α4 that we studied (Figure 4) had essentially parallel effects on Mor and Oxa
activity, suggesting little ability to discriminate between the two. In contrast, α3E113Rβ2 showed unambiguous differences for the two compounds.

The positions α3I57 and α3T115, though involved in modulation, do not appear to impact modulation specificity. α3I57Eβ2 receptors were clearly still potentiated by Mor and Oxa, given the left-shifted EC₅₀s, but the E_max values were lower than wild type α3β2 (Table 1). The conservative cysteine and the α4-based glutamine substitutions for α3T115 gave receptors that were potentiated substantially (E_max > 1 and left-shifted EC₅₀) by both Mor and Oxa (Table 1; see also Seo et al., 2009). However, α3T115Qβ2 was potentiated better than wild type α3β2, judging by the elevation in E_max and the degree of the EC₅₀ shift. In contrast, the α3K109Fβ2 receptor displayed Mor and Oxa potentiation very similar to wild type α3β2 (Table 1) and may play no role in modulation by these compounds. Both α3I57 and α3T115 are likely closer to the two tryptophans than α3K109 (Figure 4).

Mutations in positions α3L107 and α3I117 were similar in giving more α4-like modulation than the aforementioned mutants. Both α3L107Hβ2 and α3I117Tβ2 receptors lost Mor potentiation and exhibited partial inhibition by Oxa (Figure 5; Table 1), but neither distinguished between the two compounds to any large degree. Interestingly, the α3L107Cβ2 mutant was modulated like wild type α3β2, implicating positive charge and/or aromaticity at this location for inhibition.

Mutation of α3E113 also led to inhibition, but only by Oxa (Figure 5; Table 1). The left-shifted EC₅₀ and E_max ~2 in the presence of Mor demonstrate clear potentiation, whereas the right-shifted EC₅₀ and E_max ~1 with Oxa signify inhibition. These results indicate either that Oxa
contacts α.3E113 whereas Mor does not, or that Oxa and Mor interact differentially with this residue. Compared to positions α.3I57, α.3K109 and α.3T115, the residues α.3L107, α.3E113 and α.3I117 appear to be dominant in dictating inhibition as the mode of modulation, because Oxa inhibition was more pronounced for the triple mutant than for the quintuple mutant or wild type α.4β2 receptors (Figure 6).

The Bouzat group has studied the differences in pharmacological activity of these same anthelmintics on mammalian muscle-type and α7 receptors (Bartos et al., 2006, 2009). They found that α.7Q57 was important for specificity of morantel, but not oxantel (Bartos et al., 2009); this position is homologous to α.3K55, which is identical in α4 (Figure 4). Nonetheless, their results are consistent with our finding of the importance of the complementary face, D loop residues for the activity of these anthelmintics. Because these compounds are full or partial agonists, acting at canonical sites, of α7 and muscle-type nAChRs (Bartos et al., 2009), an interesting question arises regarding the specificity differences between canonical and non-canonical sites. While there are other differences between α.3 and α.4 in these loop D and E equivalents and we may not have completely delimited the anthelmintic modulator binding site, the residues we identified account for the majority of the α4-like responses to Mor and Oxa, as indicated by the combination mutants (Figure 6).

Switching the Modulation Mechanism

How can we explain our findings in a comprehensive way, especially in regard to a mechanistic switch between potentiation and inhibition? Non-canonical interfaces govern
modulation of α4-containing receptors by divalent cations, but whether the modulation is inhibition or potentiation appears to depend on the receptor stoichiometry (Hsiao et al., 2006; Moroni et al., 2008). In our studies, altering the ratio of injected subunit RNAs did not change Oxa inhibition substantially for either wild type or mutant receptors (Figures 2 and 7). This suggests that differential subunit stoichiometry selectivity cannot explain the differences in Mor and Oxa modulation, which is consistent with our general conclusion that Mor/Oxa modulation requires both α and β subunits.

We previously demonstrated that Mor potentiates α3β2 by enhancing gating efficacy, likely by increasing the rate of channel opening (Wu et al., 2008), similar to the finding that zinc enhances α4 channel open probability through increased burst duration (Hsiao et al., 2008). In other words, a Mor-bound receptor favors the open state of the channel preferentially over the closed state, relative to the control of activation by ACh alone. We are intrigued by an observation for all the mutants in the current study that the shift in EC\textsubscript{50} and E\textsubscript{max} in the presence of Mor or Oxa were each correlated with the shift in EC\textsubscript{50} for ACh activation relative to wild type α3β2. For example, the mutations α3I57E and α3T115Q reduced ACh potency, and potentiation was greater than for the wild type α3β2 receptor, whereas α3L107H, α3I117T and the triple and quintuple combinations increased ACh potency, and potentiation was greatly reduced or inhibition obtained (Table 1). If the modulator, when bound, establishes approximately the same closed ↔ open equilibrium independent of other factors, then inhibition (which we found to be noncompetitive; Figures 2 and 7) is merely a decrease in efficacy relative to control. Further work is required to substantiate this mechanism, for example, by finding
mutations in this region of α3 that would yield definitive Mor inhibition, as well as recording single-channel activity to measure efficacy directly.

Our work indicates that adjacent residues can favor preferentially either the closed or open state of the receptor, and that such residues are located in the complementary (–) side of binding site interfaces. In light of previous studies, neither of these ideas is surprising. Collins and Millar (2010) demonstrated that mutations clustered in the transmembrane domain of α7 receptors could convert potentiation by ivermectin into inhibition. Similarly, many studies support a role of the complementary face of the canonical binding site in channel activation (e.g., Mukhtasimova and Sine, 2007; Young et al., 2007). That such apparently important contributions to gating arise in the non-canonical interfaces (our work) raises questions about the symmetry requirements of activation for nAChRs (cf. Rayes et al., 2009). Similarly, mapping the spatial distribution of residues promoting channel opening vs. closing in this region may help illuminate how subunits move in converting between these conformations (Corringer et al., 2010). In addition to these issues fundamental to ion channel function, our work suggests a very specific region of α3 and α4 nAChRs to target for drug design.
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Conducted experiments:

Cesa, Higgins, Sando, Kuo, Levandoski

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Cesa, Higgins, Sando, Kuo, Levandoski

Performed data analysis:

Cesa, Higgins, Sando, Kuo, Levandoski

Wrote or contributed to the writing of the manuscript:

Cesa, Higgins, Sando, Kuo, Levandoski
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Hanson SM, Morlock EV, Satyshur KA, Czajkowski C (2008) Structural requirements for eszopiclone and zolpidem binding to the γ-aminobutyric acid type-A (GABA_A) receptor are different. *J Med Chem* 51: 7243-52.


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LEGENDS FOR FIGURES

Figure 1. Differential Modulation by Three Anthelmintic Compounds Depends on the \( \alpha \) Subunit.
A. Representative traces for responses evoked by ACh and ACh + modulators are shown for oocytes expressing wild type \( \alpha 3 \beta 2 \) (top row) or wild type \( \alpha 4 \beta 2 \) receptors (bottom row). In each case, the control response to 100 \( \mu M \) ACh is shown as a solid trace and the response to 100 \( \mu M \) ACh + 10 \( \mu M \) modulator is overlaid as a dashed trace; the red traces for the Oxa-added condition highlight the differences in potentiation (\( \alpha 3 \beta 2 \)) vs. inhibition (\( \alpha 4 \beta 2 \)). The horizontal scale bar is 20 sec and applies to all experiments. The vertical scale bar is 1.0 \( \mu A \), except or \( \alpha 3 \beta 2 / \text{pyrantel} \) (0.4 \( \mu A \)), \( \alpha 4 \beta 2 / \text{morantel} \) (0.5 \( \mu A \)) and \( \alpha 4 \beta 2 / \text{pyrantel} \) (0.2 \( \mu A \)). B and C. The effects of the three anthelmintic compounds as a function of ACh concentration are displayed for wild type \( \alpha 3 \beta 2 \) (panel B) and wild type \( \alpha 4 \beta 2 \) receptors (panel C). Responses evoked by ACh alone are shown as solid squares, whereas responses to ACh + 10 \( \mu M \) modulator are given as open squares (Mor), open triangles (Pyr) or red open circles (Oxa). All currents were normalized to the response to 3 \( mM \) ACh; values plotted are means ± SEM. Solid curves represent best fits of the Hill equation to the data sets. For clarity, only fits for ACh alone (black) and Oxa-added (red) are shown. Control ACh responses were collected separately for each experiment, but these were then analyzed in aggregate. Replicates were \( n = 3-12 \). Fits for the Pyr-added experiments, not reported elsewhere, were \( EC_{50} = 5.5 \pm 1.7 \ \mu M \), \( n_H = 1.24 \pm 0.42 \), \( E_{max} = 2.8 \pm 0.1 \) (\( \alpha 3 \beta 2 \)) and \( EC_{50} = 40 \pm 10 \ \mu M \), \( n_H = 0.78 \pm 0.17 \), \( E_{max} = 1.1 \pm 0.1 \) (\( \alpha 4 \beta 2 \)). See Table 1 for the parameters of other fits.
Figure 2. Oxantel Inhibition is Noncompetitive and Independent of Subunit Stoichiometry.
Panel A plots the fractional degree of inhibition by Oxa \([I_{ACH} - I_{ACH+Oxa}] / I_{ACH}\] as a function of Oxa concentration. The control, uninhibited response was elicited with 100 μM ACh with the holding potential at –60 mV. Data for a separate experiment to determine the voltage-dependence of Oxa inhibition are shown in panel B. Currents were evoked with two ACh concentrations (100 μM and 1 mM) and inhibition by co-application was measured. Symbols represent means ± SEM for n = 4 oocytes for both experiments. Panel C shows concentration-response data for ACh alone (filled diamonds) and in the presence of 10 μM Oxa (open diamonds) for oocytes injected with RNA ratios of 10:1 α4:β2, and for ACh + 10 μM Oxa for oocytes injected with 1:10 α4:β2 (open circles). Symbols represent mean currents normalized to the response evoked by 3 mM ACh alone (± SEM). Curves represent best fits of the Hill equation to the data. The parameters, not reported elsewhere, were: EC50 = 64 ± 7 μM, nH = 1.32 ± 0.17, Emax = 0.75 ± 0.02 (dashed curve, Oxa inhibition for 10:1 ratio) and EC50 = 105 ± 12 μM, nH = 1.17 ± 0.13, Emax = 0.57 ± 0.02 (dashed curve, Oxa inhibition for 1:10 ratio); EC50 = 9.2 ± 0.8 μM, nH = 0.75 ± 0.05 (ACh control for 10:1 ratio, omitted for clarity) and EC50 = 3.4 ± 0.4 μM, nH = 0.86 ± 0.07 (solid curve, ACh control for 1:10 ratio). The replicates were n = 3 (ACh alone) and n = 4 and 5 (Oxa inhibition).

Figure 3. A Role for Tryptophans in Modulation. The effects of Mor and Oxa on the concentration-response behavior of mutant α3β2W149A receptors are shown. The two experiments (as 5-point ACh ± modulator titrations) were collected separately, each with the series of control ACh responses, but the controls were then analyzed in aggregate. Symbols
represent mean currents normalized to 3 mM ACh alone (± SEM), and fits of the Hill equation are reported in Table 1. The replicates were n = 5 (+ Mor, open squares and dashed curve), n = 6 (+ Oxa, open circles and dotted curve), and n = 11 (ACh controls, filled squares and solid curve).

Figure 4. The Non-canonical Interfaces of α3β2 and α4β2 Diverge. A homology model of the extracellular domain β2(+)/α3(−) interface is shown in ribbon format; β2 (left) is green and α3 (right) is gold. The tryptophan side chains (magenta, W149 of β2 and W53 of α3) conserved in the canonical/agonist binding site are shown in stick format. Below the structure is an alignment of the rat α3 and α4 sequences in the regions of the D and E loops. The side chains (stick format) chosen for specificity studies are color-coded in the α3 sequence; numbering is for the mature α3 protein. This depiction is derived from a3b2rr.pdb (Sallette et al., 2004).

Figure 5. α3 Single Mutants Reveal Morantel and Oxantel Specificity. The three panels show concentration-response data for ACh alone controls, in the presence of 10 μM Mor, and in the presence of 10 μM Oxa for the single point mutants α3L107Hβ2 (A), α3E113Rβ2 (B) and α3I117Tβ2 (C). As described for the data in Figure 4, the experiments with the two modulators were conducted separately but the ACh control sets were analyzed in aggregate (per mutant). Symbols represent mean currents normalized to 3 mM ACh alone (± SEM), and fits of the Hill equation and numbers of replicates are reported in Table 1. The experiments were: filled squares and solid curves for ACh controls, open squares and dashed curves for + Mor, and open circles and dotted curves for + Oxa (panels B and C) or filled triangles (10 μM Oxa) and open triangles.
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(50 μM Oxa) in panel A. For α3L107Hβ2, the dotted curve is the fit to the ± 50 μM Oxa data set. The fit parameters for α3L107Hβ2 with 10 μM Oxa, not reported elsewhere, were EC50 = 63 ± 9 μM, nH = 1.32 ± 0.20, Emax = 1.22 ± 0.04 (n = 6).

Figure 6. Combination Mutants Have α4 Character. Concentration-response behavior for the two combination mutants are shown; α3/α4[3] (panel A) is the triple mutant α3L107H/E113R/I117T and α3/α4[5] (panel B) is the quintuple mutant α3I57E/L107H/E113R/T115Q/I117T. Data were analyzed as described in Figures 3 and 5 with the parameters for the fits reported in Table 1. The replicates were: n = 4 (+ Mor, open squares and dashed curve), n = 5 (+ Oxa, open circles and dotted curve), and n = 9 (ACh controls, filled squares and solid curve) for α3/α4[3] (panel A); and n = 4 (+ Mor, open squares and dashed curve), n = 6 (+ Oxa, open circles and dotted curve), and n = 10 (ACh controls, filled squares and solid curve) for α3/α4[5] (panel B).

Figure 7. Oxantel Inhibition of α3 Mutants is Noncompetitive and Independent of Subunit Stoichiometry. A. The fractional degree of Oxa inhibition [(I_ACh - I_{ACh+Oxa})/ I_{ACh}] is plotted vs. holding potential for three point mutants (α3L107Hβ2, squares; α3E113Rβ2, circles; α3I117Tβ2, triangles). In all cases, the filled symbols represent the lower ACh concentration (20 μM) and the open symbols the higher (100 μM). Control and Oxa co-applied responses at each ACh concentration were recorded on the same set of oocytes; the Oxa concentration was 10 μM for α3E113Rβ2 and α3I117Tβ2, and 50 μM for α3L107Hβ2. Symbols represent means (±
SEM) for n = 3 or 4; in some cases error bars are smaller than the symbol. B. The effect of subunit stoichiometry on Oxa inhibition of the triple mutant α3/α4β2 is shown. Responses evoked by ACh alone are shown as solid diamonds (10:1 RNA injection ratio) and solid circles (1:10), whereas responses to ACh + 10 μM Oxa are given as open diamonds (10:1) and open circles (1:10). All currents were normalized to the response to 1.5 mM ACh alone; values plotted are means ± SEM. For clarity, fits of the Hill equation to only the 10:1 ratio data are shown, with the solid curve for the ACh alone responses and the dashed curve for the Oxa inhibition case. The parameters of the Oxa inhibition, not reported elsewhere, were EC$_{50}$ = 42 ± 13 μM, n$_H$ = 1.08 ± 0.29, E$_{max}$ = 0.84 ± 0.07 (10:1 ratio) and EC$_{50}$ = 40 ± 5 μM, n$_H$ = 1.09 ± 0.11, E$_{max}$ = 0.90 ± 0.03 (1:10 ratio). The parameters for the control ACh responses were EC$_{50}$ = 14 ± 1 μM, n$_H$ = 1.01 ± 0.09 (10:1 ratio) and EC$_{50}$ = 15 ± 2 μM, n$_H$ = 1.06 ± 0.17 (1:10 ratio). The replicates were n = 5 for each experiment.
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#### Table 1. Agonist and Modulator Evoked Response Characteristics

<table>
<thead>
<tr>
<th>Subtype</th>
<th>ACh Response</th>
<th>Mor Modulation Response</th>
<th>Oxa Modulation Response</th>
</tr>
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<tr>
<td></td>
<td>EC50 (µM)</td>
<td>nH</td>
<td>Emax</td>
</tr>
<tr>
<td>α3β2</td>
<td>66 ± 9</td>
<td>0.75 ± 0.07 [5-11]</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>α4β2</td>
<td>21 ± 4</td>
<td>0.59 ± 0.06 [6-12]</td>
<td>41 ± 9</td>
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<td>1.08 ± 0.06 [6-10]</td>
<td>20 ± 3</td>
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<tr>
<td>α3β2W149A</td>
<td>40 ± 6</td>
<td>0.99 ± 0.12 [5]</td>
<td>44 ± 6</td>
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<tr>
<td>α3K109Fβ2</td>
<td>86 ± 14</td>
<td>0.66 ± 0.07 [12]</td>
<td>7 ± 2</td>
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<tr>
<td>α3L107Cβ2</td>
<td>62 ± 7</td>
<td>0.71 ± 0.06 [6-9]</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>α3L107Hβ2</td>
<td>27 ± 4</td>
<td>1.02 ± 0.13 [17]</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>α3E113Rβ2</td>
<td>40 ± 5</td>
<td>1.01 ± 0.12 [10]</td>
<td>10 ± 6</td>
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<tr>
<td>α3T115Cβ2</td>
<td>120 ± 20</td>
<td>0.71 ± 0.07 [9]</td>
<td>5 ± 2</td>
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<td>α3T115Qβ2</td>
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<td>2.2 ± 0.6</td>
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<td>α3I117Tβ2</td>
<td>16 ± 2</td>
<td>0.93 ± 0.08 [15]</td>
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<td>α3I57Eβ2</td>
<td>110 ± 10</td>
<td>0.83 ± 0.05 [5-13]</td>
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<tr>
<td>α3/α4[3]β2</td>
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<td>0.93 ± 0.13 [9]</td>
<td>26 ± 8</td>
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<tr>
<td>α3/α4[5]β2</td>
<td>20 ± 3</td>
<td>1.15 ± 0.19 [10]</td>
<td>18 ± 5</td>
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</tbody>
</table>

[n] indicates the number of replicate oocytes for each measurement.

Fits to the Hill equation for experiments with ACh + 10 µM Mor or ACh + 10 µM Oxa in all cases, except for α3L107Hβ2, which was ACh + 50 µM Oxa.
Figure 2

A) 

Fractional Inhibition

10^{-7} 10^{-6} 10^{-5} 10^{-4} [Oxantel] (M)

B) 

100 μM ACh
1 mM ACh

-80 -60 -40 -20 Holding Potential (mV)

C) 

Normalized Response

10^{-7} 10^{-6} 10^{-5} 10^{-4} [ACh] (M)

α4β2

- 10:1 ACh alone
- 10:1 ACh + 10 μM Oxa
- 1:10 ACh + 10 μM Oxa
Figure 3
Figure 5

Graphs showing the normalized response of different receptors (α3L1071β2, α3E113Rβ2, α3I1171β2) to various concentrations of ACh (M), with and without the addition of Mor (10 μM) and Oxa (10 and 50 μM).
Figure 6
Figure 7