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Accelerated communication

The nicotinic α5 subunit can replace either an ACh-binding or non-binding subunit in the α4β2* neuronal nicotinic receptor

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Running title: Assembly of α5 subunits in α4β2* nicotinic receptors

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5I A85380: 5-iodo-3-(2(S)-azetidinylmethoxy) pyridine

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Abstract

Heteropentameric neuronal nicotinic receptors assemble so that the canonical acetylcholine-binding sites are located at the interfaces between 2 pairs of subunits, while the 5th subunit does not participate in a canonical transmitter-binding site. Several subunits are considered to be unable to participate in forming a functional receptor when they occupy a position that would contribute to such a site, including the α5 subunit. The α5 subunit is of interest because of its apparent involvement in nicotine dependence and in the control of dopamine release. We have examined this question using α4 and β2 subunits in concatemeric constructs with the α5 subunit, expressed in *Xenopus* oocytes. Using dimeric constructs of α4 and β2 subunits expressed with free α5 and pentameric constructs incorporating a single copy of α5 we find that the α5 subunit can occupy the position of a non-binding subunit, or replace a β2 subunit participating in a canonical binding site. The resulting receptors functionally resemble pentamers assembled with 2 copies of α4 and 3 copies of β2. Functional receptors apparently cannot be formed with α5 subunits in both canonical binding sites. These observations extend the present ideas on the possible positions in the pentamer that may be occupied by the α5 subunit, and suggest that additional physiological or pharmacological subtypes of neuronal nicotinic receptors may be present in neurons.

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Introduction

Pentameric ligand-gated ion channels (pLGIC) are members of a gene family including the nicotinic, $GABA_A$, glycine and serotonin type A receptors in vertebrates and a number of related channels in invertebrates and prokaryotes. The neuronal nicotinic receptors can form as pentamers of a single subunit (the α7 subunit), but many are heteropentameric and contain both α (α2-α6) and β (β2-β4) subunits in a variety of stoichiometries (Gotti et al., 2007). Recently the α5 subunit has been of particular interest since it was found that a nonsynonymous coding variant of this subunit is significantly associated with an increased risk of developing nicotine dependence (Bierut et al., 2008). Further, the level of α5 in the medial habenula determines the aversive response to nicotine (Frahm et al., 2011), and receptors containing the α5 subunit along with α4 and β2 are critical in regulating dopamine release in the dorsal striatum (Exley et al., 2012). In heteropentameric receptors the canonical acetylcholine (ACh) binding sites are located at the interface between an α and a β subunit, in which the α subunit contributes the principal (or +) face and the β subunit the complementary (or -) face (Gotti et al., 2007; Mazzaferro et al., 2011; see Figure 1). This means that there is one subunit in the heteropentamer that does not contribute to such a canonical site (the 5th subunit). It has been proposed that the α5 subunit, in particular, cannot incorporate into a functional receptor in a position at which it contributes to a canonical binding site (Brown et al., 2007; Kuryatov et al., 2008). However it can incorporate efficiently into the 5th subunit position and thereby affect the properties of the pentameric receptor (Gerzanich et al., 1998; Groot-Kormelink et al., 2001; Kuryatov et al., 2008). It should be noted that recent studies have shown that the 5th subunit in α4β2 receptors can participate in a novel agonist-binding site; in particular adjacent α4 subunits form an ACh-binding site in which the 5th subunit contributes the principal face (Harpsoe et al., 2011; Mazzaferro et al., 2011). This expands the possible physiological importance of incorporating the α5 subunit.

We examined the ability of the α5 subunit to form functional receptors with the α4 and β2 subunits, using concatemeric constructs of subunits to define the number and position of subunits in the pentamer. The results indicate that the α5 subunit can assemble in the place of a β2 subunit forming a canonical ACh-binding site.

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Materials and Methods

Constructs and expression

We used human α4 (NM000744), β2 (NM000748) and α5 (NM000745) subunits. The generation of the dimeric constructs α4-β2 and β2-α4 is described in (Jin and Steinbach, 2011). The pentameric constructs β2-α4-β2-α4-α4 and β2-α4-β2-α4-β2 are described in (Carbone et al., 2009). The α5 subunit was inserted into a pentamer by mutational insertion of the appropriate linker sequences: the sequence for the signal peptide at the 5' end and the stop codon at the 3' end were removed and replaced by the linker. This construct was then restriction digested and inserted into the position of the middle β2 subunit in the pentamers. All constructs were fully sequenced through the inserted receptor sequence. In the pentamers, subunits were excised using the appropriate restriction enzymes and sequenced independently to verify that each copy was intact. RNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin TX). The concentration of RNA was estimated from the OD_{260} value.

Xenopus oocytes were prepared in Dr. C. Zorumski's laboratory (Washington University, St. Louis MO) using an approved protocol. Oocytes were injected with 12 to 20 ng of cRNA in a volume of 18 to 23 nL. Oocytes were maintained at 18 C for 2 to 7 days before physiological study.

Electrophysiology

Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Jin and Steinbach, 2011), using an OC-725C voltage clamp (Warner Instruments, Hamden CT). Oocytes were clamped at -50 mV, and all recordings were made at room temperature (23 - 25 ^{o}C). Currents were filtered at 20 Hz, then digitized at 50 Hz (Digidata 1200 interface; Molecular Devices, Sunnyvale, CA) and stored using pClamp 8.0 (Molecular Devices). Transients were analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in a small chamber which was continuously perfused with saline. Drug applications were made using a manually controlled perfusion system. The system was made with glass, stainless steel or Teflon components, to reduce steroid adsorption. The applications were relatively slow, with bath exchange times of ~1 sec. The external solution contained (in mM): 96 NaCl, 2 KCl, 1.8 $BaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH 7.3. External Ca^{2+} was replaced with Ba^{2+}, to avoid activation of Ca^{2+} activated channels. We did not use atropine to block muscarinic receptors, as it potentiates α4β2 receptors (Zwart and Vijverberg, 1997). Occasional oocytes showed delayed responses to ACh; these oocytes were discarded.

The concentration-response relationship for activation by ACh was characterized for data from each cell using non-linear regression in SigmaPlot (Systat Software, Chicago IL) by fitting the Hill equation

$(Y([ACh]) = Y_{max} (1 / (1 + (EC_{50}/[ACh])^n_{Hill}))$,

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where Y is the response to a concentration of ACh, Y_{max} is the maximal response, EC_{50} is the concentration producing half-maximal activation, and n_{Hill} is the Hill coefficient. Concentration-response data were collected for an individual cell, and data were normalized to the response to 1 mM ACh. The fit was rejected if the estimated error in any fit parameter was greater than 60% of the fit value, and all parameter estimates for that fit were discarded. The relationship was analyzed for each cell, then overall mean values were calculated for oocytes injected with that set of constructs.

Potentiation by 17β-estradiol or physostigmine is strongest for low concentrations of ACh (Curtis et al., 2002; Paradiso et al., 2001; Smulders et al., 2005). Since the EC_{50} for activation by ACh depends on the subunit combinations expressed (see Results), each oocyte was tested with 1 mM ACh, to estimate the maximal response. A low concentration of ACh, chosen to be able to evoke less than 20% of the maximal current, was then applied. After the response to ACh had reached a stable level, the application was switched to ACh plus 10 μM drug. The application was switched to bathing solution, followed by repeat of the control low concentration. The relative response in the presence of drug to that in the absence of drug was then calculated. Drug was not preapplied. ACh or ACh plus drug were applied for 10 to 20 seconds, and applications were separated by 3 to 4 minutes, to allow full washout.

Values are presented as arithmetic mean ± SE (number of observations).

Drugs.

17β-estradiol (CAS 50-28-2) and acetylcholine chloride (ACh; CAS 60-31-1) were purchased from Sigma-Aldrich (St. Louis, MO). 5-iodo-3-(2(S)-azetidinylmethoxy) pyridine (5I A85380; CAS 213764-92-2) and physostigmine hemisulfate (physostigmine; CAS 64-47-1) were purchased from Tocris (Ellisville, MO). 17β-estradiol was prepared as a 20 mM stock solution in DMSO and diluted into external solution on the day of an experiment. ACh was prepared as a 1M stock solution in bath solution and stored frozen at -20 ^{o}C. 5I A85380 was prepared as a 50 μM stock solution in bath solution and stored frozen at -20 ^{o}C. Physostigmine was prepared as a 10 mM stock in deionized water and stored frozen at -20 ^{o}C. Working solutions were prepared on the day of experiments.

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Results and Discussion

We used concatemeric constructs to express receptors containing α5 subunits in a defined stoichiometry and position in nicotinic α4β2* receptors. Receptors were expressed in *Xenopus* oocytes and responses were determined using 2-electrode voltage clamp.

Initially we used dimeric concatemers containing a single α4 and a single β2 subunit, in either α4-β2 or β2-α4 orientation. Previous work (Jin and Steinbach, 2011; Zhou et al., 2003) has shown that these concatemers assemble in the pentamer in a counter-clockwise fashion (Figure 1), so that a free additional subunit either takes the position of the 5th (non-binding) subunit when expressed with the β2-α4 concatemer or of a subunit contributing to the canonical ACh-binding site interface when expressed with the α4-β2 concatemer. To assess the number and position of subunits in the receptor pentamer, we used pharmacological tests. We determined the concentration of ACh that produced a half-maximal response (ACh EC_{50}), the relative current elicited by a 1 μM concentration of 5-iodo-A85380 (compared to 1 mM ACh) and the ability of 17β-estradiol to potentiate responses to a low concentration of ACh. Previous work has shown that receptors containing 3 copies of α4 have an ACh EC_{50} near 100 μM while those with 2 have an ACh EC_{50} less than 10 μM (Jin and Steinbach, 2011; Moroni et al., 2006; Nelson et al., 2003). 5-iodo-A85380 (5I-A85380) is a more efficacious agonist on receptors containing 3 copies of β2, with the response to 1 μM 5I-A85380 larger than that to high ACh (~1.4x), while for receptors with 3 copies of α4 the response is less (~0.3x) (Jin and Steinbach, 2011; Zwart et al., 2006). Finally, for 17β-estradiol to potentiate responses there must be a free (untethered) carboxy terminus for an α4 subunit (Jin and Steinbach, 2011; Paradiso et al., 2001; Zhou et al., 2003). In addition, we tested the ability of physostigmine to potentiate responses to a low concentration of ACh. As shown in Table 1 and Figure 2, potentiation by physostigmine is seen when there are two adjacent α4 subunits. We also determined the response to a high concentration of ACh (1 mM) as an estimate of the level of expression of functional receptors. The results are presented in Figure 2 and Table 1.

We will use abbreviations for the constructs studied. Concatemers are named with subunits in order from the amino terminus, so α4-β2 indicates that the dimer begins with α4 subunit sequence. When more than one construct was used, the constructs are separated by &, so α4&β2 1:8 indicates that both α4 and β2 subunit cRNA were injected, at a 1:8 mass ratio.

The inclusion of 3 copies of α4 (α4&β2 8:1, α4-β2 & α4, β2-α4 & α4 or β2-α4-β2-α4-α4) resulted in receptors that had a large EC_{50} for ACh (~100 μM), a small relative response to 5I A85380 (< 0.3) and that showed potentiation by physostigmine (ratio > 1.2). In addition, the response to 17β-estradiol reflected whether there was a free (untethered) carboxy terminus for at least one α4 subunit (e.g. comparing α4-β2&β2 to β2-α4&β2). In contrast, inclusion of 3 copies of β2 (α4&β2 1:8, α4-β2 & β2, β2-α4 & β2 or β2-α4-

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β2-α4-β2) resulted in receptors with a small EC_{50} for ACh (<10 μM), a large relative response to 5I A85380 (> 1.0) and that showed block by physostigmine (< 0.7). We also tested responses to the dimeric concatemers injected alone. Overall, these properties were more similar to receptors containing 3 copies of α4 (Figure 2, Table 1), but clearly distinct from either dimer expressed with free β2 subunits.

We then expressed dimeric constructs with free α5 subunits. We tested both wild-type α5 and α5(D398N), as the α5(D398N) variant is associated with an increased risk of developing nicotine dependence (Bierut et al., 2008). However, we saw no difference between these constructs in any of the parameters measured, so the results have been pooled. Injected oocytes expressed functional receptors, although the response to saturating concentrations of ACh was reduced compared to when α4 was used as the free subunit (Table 1). In this case the pharmacological properties of the receptors were very similar to those of receptors containing 3 copies of β2 (Figure 2, Table 1). The properties of the receptors containing free α5 subunits are clearly distinct from those of receptors when the dimers are expressed in the absence of a free subunit, as shown in Figure 2 and Table 1. Accordingly, even though the dimeric constructs can assemble to produce functional surface receptors, in the presence of free α5 subunits the majority of the functional receptors contain the free subunit in addition to the dimer. These results suggest that the α5 subunit can replace either the subunit that does not contribute to a canonical binding site (when expressed with the β2-α4 dimer) or a subunit contributing to a binding site (when expressed with the α4-β2 dimer). However, it is also possible that the presence of the α5 subunit caused the dimers to assemble in different orientations, so that in association with the β2-α4 dimer the α5 subunit actually occupied the position that does not contribute to a canonical binding site.

To address this concern question we utilized pentameric concatemers. We first confirmed that the β2-α4-β2-α4-β2 and β2-α4-β2-α4-α4 constructs behaved as would be predicted from the expected structure (Carbone et al., 2009; Mazzaferro et al., 2011). With the β2-α4-β2-α4-α4 the properties were consistent with there being 3 copies of α4 present: the ACh EC_{50} was high, the response to 1 μM 5I A85380 was low. Furthermore, 17β-estradiol potentiated the response, indicating that the carboxy-terminal α4 sequence was present, and physostigmine potentiation indicated that there were adjacent α4 subunits. In contrast, with β2-α4-β2-α4-β2 the properties of the receptor were consistent with only 2 copies of α4 being present (Figure 2 and Table 1), without either a free α4 carboxy terminus (indicated by the absence of potentiation by 17β-estradiol) or adjacent α4 subunits (indicated by the absence of potentiation by physostigmine). To examine the properties of receptors containing α5 we replaced the central β2 subunit in each pentamer so that α5 was flanked on both sides by an α4 subunit (Figure 1). In these constructs α5 would occupy the position of β2 irrespective of whether the concatemer assembled in a clockwise direction (as found by Mazzaferro et al, 2011) or counterclockwise direction in the pentameric receptor. As shown in Figures 2 and 3, and Table 1, the inclusion of the α5 subunit resulted in receptors whose properties resemble those of the receptors

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formed by the original, β2-containing, pentameric concatemers. That is, the pharmacological properties of the receptors containing α5 in place of β2 support the idea that each receptor contains the subunits in a single pentameric construct. They are not the result of receptors assembled with contributions from multiple concatemers or concatemers with subunits derived from proteolysis of concatemers. These results strongly support the idea that the α5 subunit can assemble to produce functional receptors even when it occupies the position of a β2 subunit at a canonical ACh-binding site.

A concern in using subunit concatemers is that the linker sequences or the physical constraints imposed in linking subunits will alter receptor properties. In the case of these constructs this does not seem to be the case. Examination of the data in Table 1 indicates that for receptors containing α4 and β2 subunits, the properties of receptors containing 3 copies of α4 are indistinguishable for receptors derived from free subunits, dimers and pentamers except for the expected effects on potentiation by 17β-estradiol. Similarly, for receptors containing 2 copies of α4 the properties are indistinguishable (again, except for 17β-estradiol). Accordingly, there is no indication that the linkers *per se* affect properties for the present studies.

We also generated pentameric β2-α5-β2-α4-α4 concatemers in which the second position (occupied by α4) was replaced by α5. In this case the α5 subunit would be constrained to replace an α4 subunit that contributes to a canonical binding site. These constructs expressed so poorly that reliable data could not be obtained (data not shown). Previous work has shown that the α5 subunit does not assemble to produce functional receptors when expressed with the β2, β3 or β4 subunits (Boulter et al., 1990), so it seems unlikely that the α5 subunit can replace an α4 subunit at a position producing a canonical ACh-binding site. We found that no functional receptors were produced when we injected free α4 and free α5 subunits (data not shown), that indicates that there must be at least one α4/β2 interface in an α4β2α5 receptor.

The efficiency of expression of functional surface receptors containing the α5 subunit appears to be low. A previous study (Kuryatov et al., 2008) found that surface expression of α4β2* was reduced by α5, although total (surface plus intracellular) expression was actually increased. They proposed that the α5 subunit might enhance formation of unproductive oligomers that could not associate to form pentamers and successfully traffic to the cell surface (Kuryatov et al., 2008).

In sum, the α5 subunit is not restricted to occupying only the position in a receptor of the subunit that does not contribute to a canonical binding site. In the pentameric constructs we used, the α5 subunit contributes to both α4/α5 and α5/α4 interfaces, in the first case replacing a β2 subunit at an interface containing a canonical site and in the second case possibly contributing to a non-canonical (α4/α4) site (Harpsoe et al., 2011; Mazzaferro et al., 2011). When expressed with the α4-β2 dimeric construct, the α5 subunit would contribute to interfaces containing a canonical site, most likely replacing β2 to generate an α4/α5 site. However, it is not clear that the α4/α5 interface actually forms a functional ACh-binding site. Previous work has shown that it is possible to mutate residues in a canonical binding site to render that site

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ineffective, and still form functional (albeit impaired) receptors (Mazzaferro et al., 2011). Still, functional receptors can form with α5 replacing a subunit contributing to a canonical site. The physiological consequences of an α4β2* receptor containing the α5 subunit in place of a β2 subunit at a canonical binding site are not yet known, since in the assays that we have used the α4β2* receptor containing the α5 subunit in place of a β2 subunit at a canonical binding site is indistinguishable from one with β2 or α5 in the 5th subunit position. Additional experiments will be required to define the consequences, including a possible effect on the ability of nicotine to upregulate the surface expression of the receptor (Kishi and Steinbach, 2006; Kuryatov et al., 2008; Mao et al., 2008).

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Authorship Contributions.

Participated in research design: Jin, Steinbach

Conducted experiments: Jin

Contributed new reagents or analytic tools: Bermudez, Jin

Performed data analysis: Jin, Steinbach

Wrote or contributed to the writing of the manuscript: Bermudez, Jin, Steinbach

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Structure of α4β2 receptor pentamers. Panel A shows receptors composed of free α4 and β2 subunits (left) and receptors expressed when a dimeric concatemer is expressed with a free α5 subunit (middle and right). The receptors are diagrammed as viewed from the extracellular space. The subunits are arranged in a rosette around the ion channel. Canonical ACh-binding sites (indicated by stars) are located at the interface between the α4 (contributing the principal or + face) and the β2 subunit. The 5th subunit (indicated by the square) does not participate in an interface contributing to a canonical site. The middle panel shows the pentamer formed with β2-α4 dimers are expressed with a free α5 subunit (indicated by a triangle). The free subunit occupies the position of the 5th subunit. The right panel shows the pentamer formed with α4-β2 dimers are expressed with a free subunit. The free subunit occupies a position participating in a canonical binding interface, indicated by stars with question marks. The dimers are shown with the linking sequence as a curved line with the arrowhead indicating the amino- to carboxy terminal direction. Panel B shows the β2-α4-α5-α4-(α4 or β2) pentameric concatemer containing the α5 subunit in the middle position. In this construct one α4/β2 interface is formed (indicated by the stars with dashed outline), and an α4/α5 interface. The subunit order is as proposed in Mazzaferro et al. (2011), but as the α5 has an α4 subunit on either side the same α4/α5 interface would be formed irrespective of a reversal of order.

FIGURE 2. Graphical depiction of the pharmacological profiles. Panels A and B show responses to agonists (A: ACh EC_{50} (note logarithmic abscissa), B: response to 1 μM 5I A85380 relative to 1 mM ACh), while panels C and D show the effects of two modulating drugs (C: effect of 10 μM 17β estradiol, D: 10 μM physostigmine). The panels are formatted in the same way: at the bottom are free subunits at two different cRNA ratios, then a group of 4 receptors containing the α4-β2 concatemer, 4 receptors containing the β2-α4 concatemer and 4 pentamers. The position of the α5 subunit is emphasized in bold and underlined. The bars are filled to indicate predicted structural features. In each panel the bars corresponding to dimers expressed without a free subunit are filled with gray. In panels A and B the solid fill indicates receptor predicted to have 2 copies of α4 and diagonal hatching indicates 3 copies of α4. In panel C solid fill indicates no free (untethered) α4 carboxy termini, diagonal hatching 1 free C-terminus and cross-hatching 2 or 3 free C-termini. In panel D solid fill indicates no adjacent α4 subunits, while diagonal hatching indicates a pair of adjacent α4 subunits. The bars in the panels show mean values ± SE. Data values are presented in Table 1.

FIGURE 3. Responses of pentamers containing α5. The left column shows responses of receptors formed

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from β2-α4-α5-α4-β2 pentamers, and the right column from β2-α4-α5-α4-α4. Panel A shows responses to agonists; note that in the left column 5I-A85380 produces a larger response than 1 mM ACh, and that 10 μM ACh is clearly a more than half-maximal concentration. In contrast, in the right column 5!-A85380 produces a smaller response than 1 mM ACh, and 10 μM ACh is clearly a less than half-maximal concentration. Panels B and C show responses 0.3 μM ACh, then the application is switched to 0.3 μM ACh plus 15 μM physostigmine (PHY; Panel B) or 10 μM 17β-estradiol (βEST; Panel C). Both drugs inhibit responses of β2-α4-α5-α4-β2 pentamers but potentiate responses of β2-α4-α5-α4-α4. Each frame in Panel A shows responses from one oocyte, while each frame in Panels B and C show responses from separate oocytes.

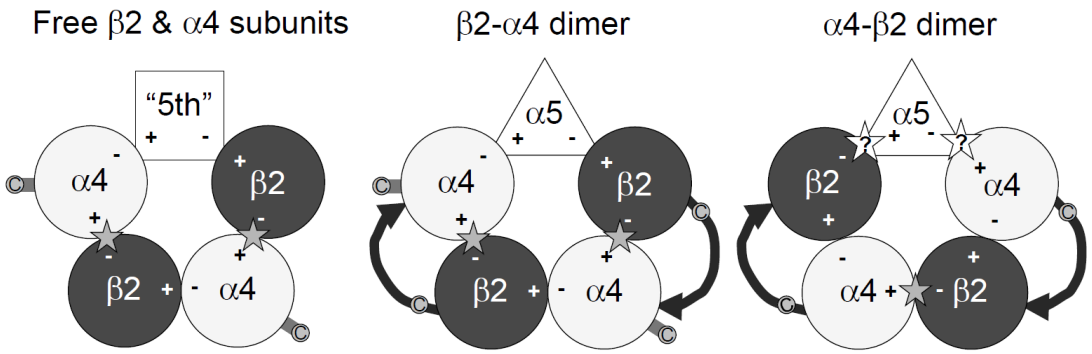
TABLE 1

Subunits	# α4	ACh EC_{50} (μM)	1000 5IA	Adjacent α4?	Physostigmine effect	# Free α4 C term	17 β-Estradiol effect	Maximal response (-nA)
α4&β2 8:1	3	135 ± 11 (32)	0.17 ± 0.02 (17)	yes	1.52 ± 0.09 (18)	3	2.54 ± 0.18 (23)	13451 ± 1459 (45)
α4-β2&α4	3	87 ± 7 (46)	0.16 ± 0.01 (41)	yes	1.57 ± 0.18 (21)	1	1.38 ± 0.05 (35)	7238 ± 1179 (68)
β2-α4&α4	3	135 ± 12 (52)	0.17 ± 0.01 (56)	yes	1.33 ± 0.04 (34)	3	3.77 ± 0.20 (11)	14454 ± 1301 (77)
β2-α4-β2-α4-α4	3	153 ± 12 (9)	0.15 ± 0.01 (12)	yes	1.43 ± 0.23 (8)	1	1.13 ± 0.02 (20)	2020 ± 265 (25)
α4&β2 1:8	2	6 ± 1 (18)	1.49 ± 0.13 (18)	no	0.67 ± 0.04 (11)	2	2.70 ± 0.11 (4)	1103 ± 208 (43)
α4-β2&β2	2	3 ± 1 (14)	1.83 ± 0.07 (19)	no	0.71 ± 0.06 (6)	0	1.04 ± 0.03 (5)	341 ± 98 (32)
β2-α4&β2	2	5 ± 1 (40)	1.72 ± 0.05 (60)	no	0.56 ± 0.03 (15)	2	3.73 ± 0.32 (7)	819 ± 114 (73)
β2-α4-β2-α4-β2	2	3 ± 1 (16)	1.79 ± 0.11 (7)	no	0.64 ± 0.03 (14)	0	0.88 ± 0.02 (8)	110 ± 23 (20)
β2-α4-α5-α4-α4	3	34 ± 9 (2)	0.47 ± 0.03 (8)	yes	1.87 ± 0.20 (6)	1	1.43 ± 0.12 (12)	24 ± 5 (30)
β2-α4&α5	2	4 ± 1 (20)	1.07 ± 0.06 (31)	no	0.64 ± 0.04 (20)	2	1.68 ± 0.06 (7)	399 ± 67 (45)
α4-β2&α5	2	4 ± 1 (9)	1.15 ± 0.08 (15)	no	0.56 ± 0.02 (19)	0	0.69 ± 0.03 (5)	436 ± 124 (20)
β2-α4-α5-α4-β2	2	9 ± 1 (13)	1.65 ± 0.06 (17)	no	0.78 ± 0.11 (9)	0	0.86 ± 0.03 (10)	27 ± 4 (36)
β2-α4	?	127 ± 15 (52)	0.41 ± 0.04 (72)	no	1.09 ± 0.10 (13)	>=2	3.17 ± 0.25 (6)	5155 ± 781 (82)
α4-β2	?	111 ± 18 (24)	0.19 ± 0.01 (24)	no	0.98 ± 0.09 (9)	0	0.87 ± 0.05 (8)	859 ± 160 (37)

TABLE 1. Summary of results. The first column gives the subunits injected. The first set of 4 rows gives results for receptors predicted to contain 3 copies of α4 and 2 copies of β2, while the second set gives results for 2 copies of α4 and 3 of β2. The third set of rows shows data for receptors containing the α5 subunit, again ordered based on the predicted number of copies of α4. The final set of 2 rows shows data for dimeric concatemers expressed in the absence of a free subunit; the pattern of properties is distinct from a dimer expressed with a free subunit (although closest to receptors formed with a free α4 subunit). The second column gives the number of α4 subunits predicted to be in the receptor, while the 3rd and 4th columns give the ACh EC_{50} and the response to 1 μM 5I A85380 relative to the response of the same oocyte to 1 mM ACh. The 5th and 6th columns give whether the predicted receptor has adjacent α4 subunits and the observed effect of physostigmine. The 7th and 8th columns give the predicted number of untethered α4 carboxy termini in the receptor and the observed effect of 17β estradiol. The final column gives the response to 1 mM ACh. All data are mean ± SE (number of observations).

Figure 1

A


Free β2 & α4 subunits
β2-α4 dimer
α4-β2 dimer
"5th"
α4
β2
α5
?

B

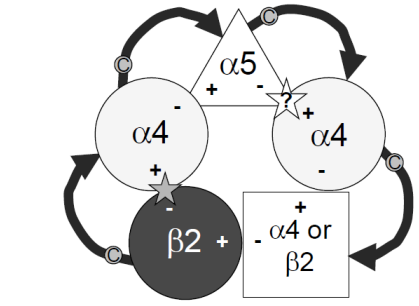

β2-α4-α5-α4-(α4 or β2) pentamer
α5
α4
α4
β2
α4 or
β2

Figure 2

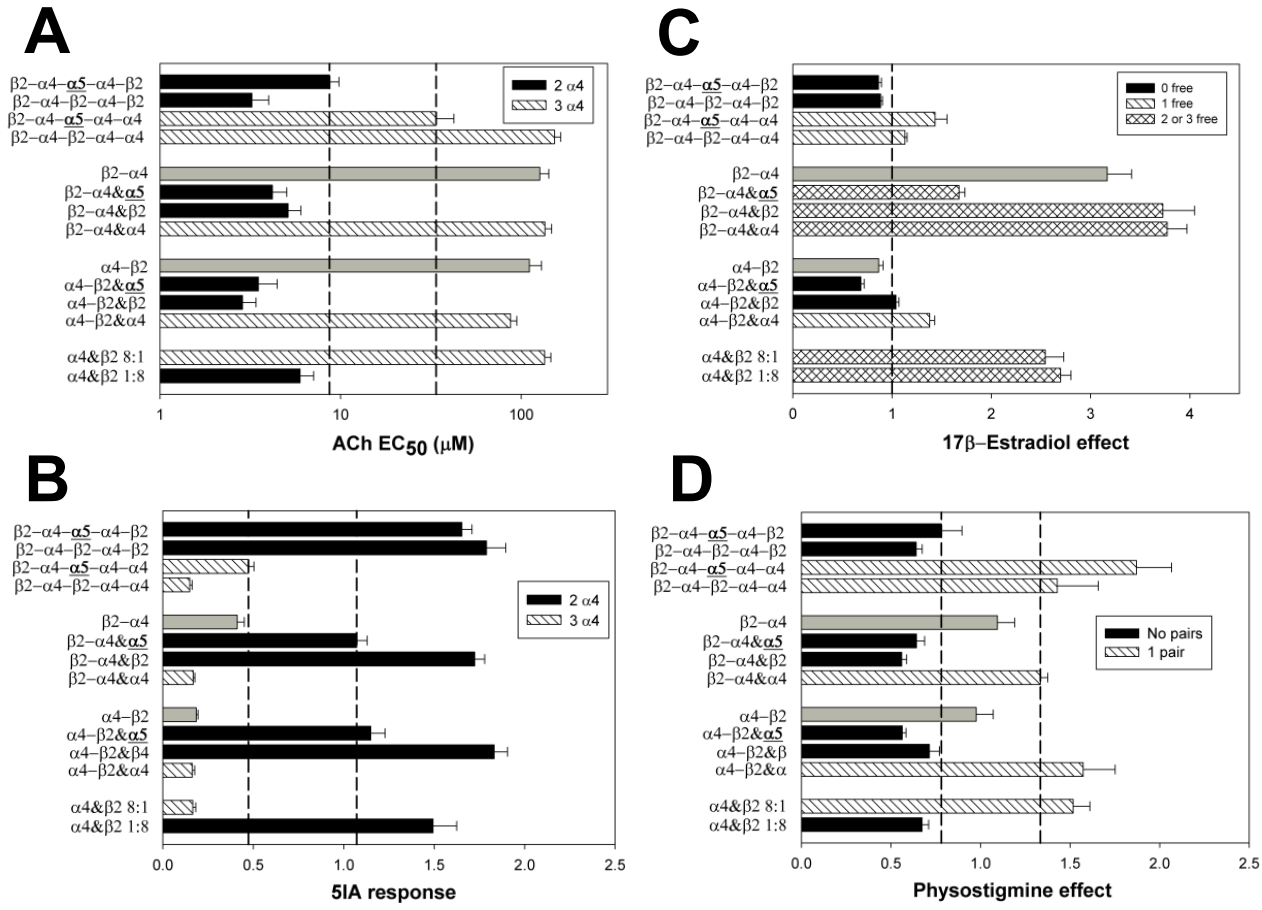
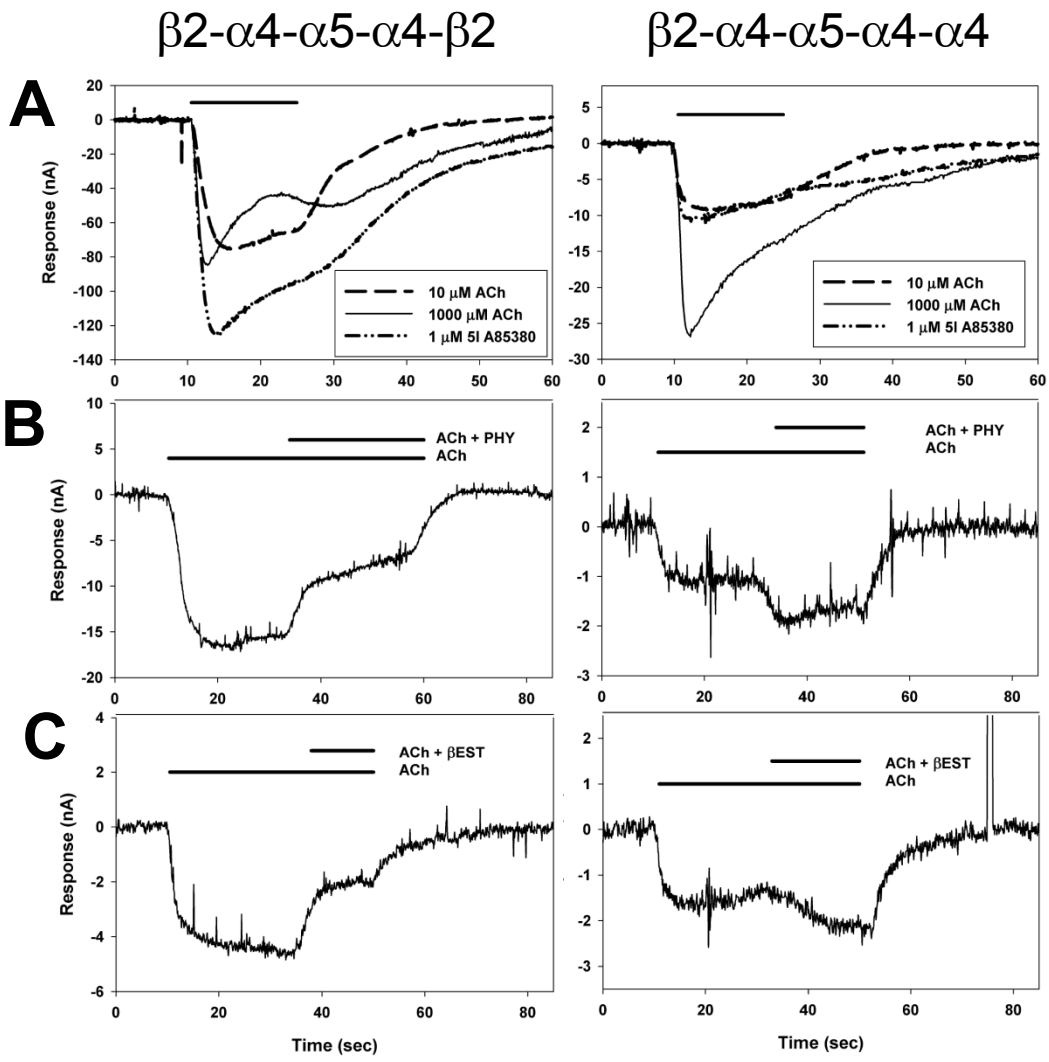

A
β2–α4–α5–α4–β2
β2–α4–β2–α4–β2
β2–α4–α5–α4–α4
β2–α4–β2–α4–α4
β2–α4
β2–α4&α5
β2–α4&β2
β2–α4&α4
α4–β2
α4–β2&α5
α4–β2&β2
α4–β2&α4
α4&β2 8:1
α4&β2 1:8
2 α4
3 α4
1
10
100
ACh EC50 (μM)
C
0 free
1 free
2 or 3 free
0
1
2
3
4
17β–Estradiol effect
B
β2–α4–α5–α4–β2
β2–α4–β2–α4–β2
β2–α4–α5–α4–α4
β2–α4–β2–α4–α4
β2–α4
β2–α4&α5
β2–α4&β2
β2–α4&α4
α4–β2
α4–β2&α5
α4–β2&β4
α4–β2&α4
α4&β2 8:1
α4&β2 1:8
2 α4
3 α4
0.0
0.5
1.0
1.5
2.0
2.5
5IA response
D
β2–α4–α5–α4–β2
β2–α4–β2–α4–β2
β2–α4–α5–α4–α4
β2–α4–β2–α4–α4
β2–α4
β2–α4&α5
β2–α4&β2
β2–α4&α4
α4–β2
α4–β2&α5
α4–β2&β
α4–β2&α
α4&β2 8:1
α4&β2 1:8
No pairs
1 pair
0.0
0.5
1.0
1.5
2.0
2.5
Physostigmine effect

Figure 3


β2-α4-α5-α4-β2
β2-α4-α5-α4-α4
A
B
C
Response (nA)
10 μM ACh
1000 μM ACh
1 μM 5I A85380
ACh + PHY
ACh
ACh + βEST
ACh
Time (sec)