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A portable site: a binding element for 17β-estradiol can be placed on any subunit of a nicotinic α4β2 receptor

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Cellular/Molecular

A Portable Site: A Binding Element for 17β-Estradiol Can Be Placed on Any Subunit of a Nicotinic α4β2 Receptor

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Endogenous steroids can modulate the activity of transmitter-gated channels by directly interacting with the receptor. 17β-Estradiol potentiates activation of neuronal nicotinic α4β2 receptors by interacting with a 4 aa sequence at the extreme C terminus of the α4 subunit, but it is not known whether potentiation requires that the sequence be placed on a specific subunit (e.g., an α4 subunit that is involved in forming an acetylcholine-binding site). By using concatemers of subunits and chimeric subunits, we have found that the C-terminal domain can be moved from the α4 to the β2 subunit and still result in potentiation. In addition, the sequence can be placed on a subunit that contributes to an acetylcholine-binding site or on the structural subunit. The data indicate that this estradiol-binding element is a discrete sequence and suggest that the effect of 17β-estradiol is mediated by actions on single subunits and that the overall consequences for gating occur because of the summation of independent energetic contributions to overall gating of this receptor.

Introduction

Steroids are endogenous modulators of membrane channel function. Although many actions of steroids are mediated by alterations in gene expression initiated by binding to nuclear receptors, steroids can have rapid and reversible actions on both transmitter-gated and voltage-gated ion channels (Belelli and Lambert, 2005; Schlichter et al., 2006). A particularly well studied example is the GABA type A ($GABA_A$) receptor, for which neurosteroids are among the most potent and efficacious potentiators (Belelli and Lambert, 2005; Akk et al., 2007). The fact that steroids can modulate the function of synaptic receptors provides a rapid link between endocrine and nervous system functions.

The ligand-gated ion channel gene family includes the subunits for the vertebrate nicotinic, $GABA_A$, serotonin type 3, and glycine receptors, and a number of related proteins in invertebrates (Brejc et al., 2001; Akabas, 2004; Sine and Engel, 2006). These receptors form as pentamers of homologous subunits (see Fig. 1), arranged in a rosette around a central ion channel formed from membrane-spanning α-helical regions contributed from all subunits. We are studying the neuronal nicotinic receptor containing α4 and β2 subunits, to define the sites and mechanisms by which potentiating agents act on the receptor. The α4 subunit is expressed in many brain regions, and the α4β2* receptor is one of the most common receptor subtypes (Gotti et al., 2007). The major physiological role of these receptors is to modulate the release of other neurotransmitters (Dani and Bertrand, 2007). The endogenous steroid 17β-estradiol potentiates the response of the α4β2 nicotinic receptor and requires a specific amino acid sequence at the extreme C terminus of the α4 subunit (Paradiso et al., 2001; Curtis et al., 2002). We sought to better define the structural requirements for estradiol potentiation of this receptor.

The sites at which potentiators interact with receptors in the ligand-gated ion channel family have been defined for some drugs. In several cases, the recognition region for the drug is formed by residues from two subunits at an interface between subunits (Hsiao et al., 2006; Moroni et al., 2008; Seo et al., 2009). In others, binding occurs between residues in a single subunit (Jenkins et al., 2001; Hosie et al., 2006; Li et al., 2006). By using concatemeric constructs of subunits and mutated subunits, we examined the effects of placing the α4 C-terminal sequence on either the α4 or β2 subunit, or on subunits that contribute an interface to an ACh-binding site or that serve as the structural subunit (see Fig. 1). Our results show that the C-terminal sequence may be placed on either subunit and the subunit may be in either position in the assembled receptor. These observations indicate that the C-terminal domain is a discrete and transferable element underlying 17β-estradiol potentiation. Potentiation increases geometrically with the number of C-terminal domains in the receptor, which suggests that binding of 17β-estradiol has an independent effect on an individual subunit, which adds a constant amount of energy to stabilize the open-channel form of the receptor.

Materials and Methods

cDNAs and molecular biology. cDNA constructs for human nicotinic receptor α4 and β2 subunits were kindly provided by J. Lindstrom (University of Pennsylvania, Philadelphia, PA) (α4, accession number NM_000744; β2, accession number NM_000748). The constructs were transferred to pcDNA3 (Invitrogen).

Two concatemers were constructed, which linked two subunits, as previously described (Zhou et al., 2003). From the N to C termini, they are β2-EF$(AGS)_6$-α4 (abbreviated β/α) and α4-EF$(AGS)_8$-β2 (α/β).

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The signal sequence of the second subunit was deleted. The β/α concatemer is very similar to that described previously (Zhou et al., 2003) [EG(AGS)$_6$], whereas the α/β concatemer has a linker two residues longer than the α4-(AGS)$_6$-β2 concatemer described previously [QEGT(AGS)$_6$TG]. Chimeric subunits were constructed by overlap extension and smaller mutations were constructed using QuikChange (Stratagene). The locations of chimera joining points are shown in Figure 1.

All constructs were sequenced through the entire coding region.

Receptor expression and oocyte voltage clamp. cRNA was synthesized using the mMessage mMachine T7 kit (Ambion). The concentration of mRNA was estimated from the OD_{260} value. When combinations of free subunits were injected, the ratio of construct with an α4 N terminus to that with a β2 N terminus was 8:1 (mass ratio), unless otherwise specified. When concatemers were injected with free subunits, the ratio was 2:1.

Xenopus oocytes were prepared in the laboratory of Dr. C. Zorumski (Washington University, St. Louis, MO) using an approved protocol. Oocytes were injected with 12–15 ng of cRNA in a volume of 18–23 nl. Oocytes were maintained at 18°C for 2–7 d before physiological study.

Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Steinbach et al., 2000; Paradiso et al., 2001; Jin et al., 2009), using an OC-725C voltage clamp (Warner Instruments). Currents were filtered at 20 Hz, and then digitized at 50 Hz (Digidata 1200 interface; Molecular Devices) and stored using pClamp 8.0 (Molecular Devices). Transients were analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in a small chamber that 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 s. The external solution contained the following (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.

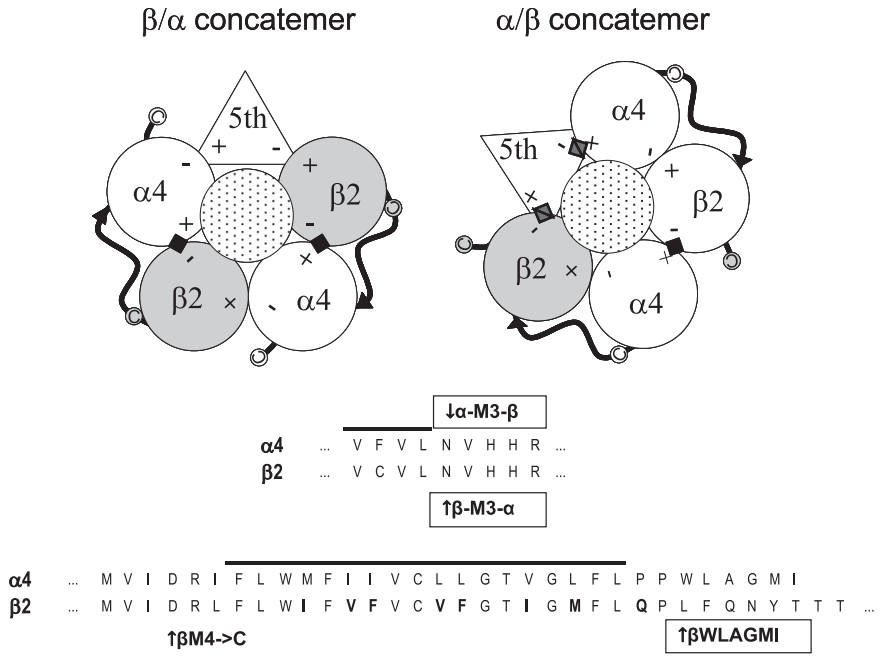

Figure 1. Schematic views of the receptor and subunits. The top panel shows the overall arrangements in pentamers containing concatemers. Subunits are arranged around the ion channel (dotted circle). ACh-binding sites are indicated by the diamonds between an α4 subunit (contributing the positive or + side of the interface) and a β2 subunit. Four subunits contribute to the agonist-binding sites, whereas the fifth occupies a "structural" position. The concatemers are indicated with linkers connecting the C terminus (denoted by a circle with C) to the N terminus, as shown by the arrowheads. The fainter diamonds on the free subunit assembled with the α/β concatemer indicate that the site is located on opposite sides, depending on the nature of the N-terminal sequence of the free subunit (note that the structural subunit in this combination is contributed from one of the concatemers). The bottom panel shows the constructs used. The top pair shows sequence for the chimeras α-M3-β and β-M3-α. The line above the sequences shows the position of the C-terminal end of the M3 region. The joining point is indicated by the arrow; the sequences were swapped for all positions including and following the indicated residue. The bottom pair shows the βM4⟶C and βWLAGMI constructs. The line shows the position of the predicted M4 region. The bold letters indicate amino acid residues in the predicted M4 region that differ between α4 and β2.

The concentration–response relationship for activation by ACh was characterized by fitting the Hill equation, $Y([ACh]) = Y_{max}(1/(1 + (EC_{50}/[ACh])\hat{}n_{Hill}))$, 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 >60% of the fit value, and all parameter estimates for that fit were discarded. The relationship was analyzed for each cell, and then overall mean values were calculated for oocytes injected with that set of constructs.

Potentiation by 17β-estradiol is strongest for low concentrations of ACh (Paradiso et al., 2001; Curtis et al., 2002). 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 <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 17β-estradiol. The application was switched to bathing solution, followed by repeat of the control low concentration. The relative response in the presence of 17β-estradiol was then calculated. 17β-Estradiol was not preapplied, as the onset and offset of potentiation are rapid (Paradiso et al., 2001). ACh or ACh plus 17β-estradiol were applied for 10–20 s, and applications were separated by 3–4 min, to allow full washout.

The amplitudes of currents expressed by some combinations of constructs were much larger than for others (supplemental Table 1, available at www.jneurosci.org as supplemental material). We are exploring possible reasons for this, but do not have an explanation at present. Experimentally, to accept a value for potentiation by 17β-estradiol, the control response had to be at least 5 nA. For analysis of agonist concentration–response relationships, the maximal response had to be at least 50 nA. To avoid problems of clamp control, the maximal response also had to be <30 μA. For some constructs, the low response amplitude required us to use a higher than usual concentration of ACh, which might have reduced the estimated potentiation (supplemental Table 2, available at www.jneurosci.org as supplemental material).

Values are presented as arithmetic mean ± SE (number of observations). Statistical tests were made using Excel (Microsoft) or Systat (Systat Software). Unless otherwise indicated, statistical tests were two-tailed *t* tests with unequal variance.

Receptor extraction and Western blots. Groups of 40–50 oocytes were injected with mRNA, as described previously. Membrane proteins were extracted basically as described previously (Carbone et al., 2009). In brief, oocytes were suspended in an ice-cold homogenization buffer containing the following (in mM): 150 NaCl, 2 $CaCl_2$, 2% Triton X-100, 20 Tris-HCl, pH 7.4, supplemented with protease inhibitor mixture (Sigma-Aldrich; P8465), at a ratio of 5 μl of buffer per oocyte. Oocytes were homogenized by passing through a 20 gauge needle seven times and a 27 gauge needle three times, and then extracted for 30 min on ice. Homogenates were

Table 1. Basic characterization of receptors containing concatemers

	ACh EC_{50} (μM)			5I A85380 response			10 μM 17β-Estradiol			
Combination	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	*N*	*p*
α4&β2 1:8	6.6	1.4	14	1.44	0.06	11	2.56	0.12	7	0.000
α4&β2 8:1	131	11	25	0.19	0.03	9	2.96	0.30	12	0.000
α/β	105	23	18	0.16	0.01	16	0.85	0.06	5	0.047
β/α	121	16	48	0.44	0.04	62	3.17	0.25	6	0.000
α/β&α4	87	8	33	0.14	0.01	29	1.47	0.07	27	0.000
β/α&α4	140	14	43	0.17	0.01	46	3.77	0.20	11	0.000
α/β&β2	2.9	0.6	12	1.84	0.07	16	1.04	0.06	11	0.560
β/α&β2	4.4	0.8	29	1.59	0.07	34	3.73	0.32	7	0.000

The first column names the constructs injected. The next three columns give data on activation by ACh in terms of the EC_{50} value provided by a fit of the Hill equation. The three columns headed "5I A85380 response" give data on the response to 1 μM 5I A85380 normalized to the response to 1 mM ACh, for that cell. The columns headed "10 μM 17β-Estradiol" give data for the potentiation by 10 μM 17β-estradiol of a response to a low concentration of ACh; the column headed *p* gives the probability that the potentiation ratio differs significantly from 1 (no effect), by two-tailed *t* test.

centrifuged twice at 1000 × *g* for 5 min at 4°C to remove the yolk, and the supernatants were then recentrifuged at 10,000 × *g* for 10 min at 4°C. The cleared supernatants were collected and diluted 50:50 in 2× Laemmli sample buffer (Bio-Rad) supplemented to 100 mM DTT. Samples were placed at room temperature for 30 min, and then aliquots were loaded on precast 7.5% gels (Bio-Rad). After electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride) membranes (Millipore). Membranes were blocked for 1 h at room temperature with 100% Odyssey block solution (LI-COR Biosciences), followed by overnight incubation at 4°C in a solution of 50% Odyssey block solution/50% PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) containing 0.4% Tween 20 (Sigma-Aldrich) with primary antibody. Polyclonal rabbit antibody to α4 subunit (sc5591; Santa Cruz Biotechnology) and goat antibody to β2 (sc1449; Santa Cruz Biotechnology) were used at 1:300. Membranes were washed four times with PBS containing 0.2% Tween 20, and then incubated with secondary antibody in the dark for 45 min at room temperature. Goat anti-rabbit and donkey anti-goat labeled with IR dye 680 (LI-COR Biosciences) were used at dilutions between 1:5000 and 1:20,000. Membranes were washed five times with PBS plus 0.2% Tween 20 and scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences).

Drugs. 17β-Estradiol (CAS 50-28-2), acetylcholine chloride (ACh) (CAS 60-31-1), and 19-norpregna-1,3,5(10)-trien-20-yne-3,17-diol, (17α)-(9CI) (17α-vinylestradiol) (CAS 57-63-6) were purchased from Sigma-Aldrich. 5-Iodo-3-(2(*S*)-azetidinylmethoxy)pyridine (5I A85380) (CAS 213764-92-2) was purchased from Tocris. Steroids were 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 1 M stock solution in bath solution and stored frozen at −20°C. 5I A85380 was prepared as a 50 μM stock solution in bath solution and stored frozen at −20°C. Working solutions were prepared on the day of experiments.

Characterization of receptors containing concatemers. We wanted to control the subunit stoichiometry and subunit position in $\alpha4\beta2$ receptors. To accomplish this, we generated two concatemers, following the approach described by Zhou et al. (2003), one with the β2 subunit at the N terminus (abbreviated β/α) and the other with α4 (α/β). We expressed each concatemer in oocytes, both alone and with free α4 or β2 subunits (Table 1). We confirmed that concatemers expressed alone produce functional receptors. However, in the presence of a free subunit, the resulting receptors appear to reflect the properties of receptors composed of two concatemers plus a single free subunit (Zhou et al., 2003). Expression with a free β2 subunit results in a population of receptors with a small EC_{50} for activation by ACh, whereas free α4 subunit results in receptors with a large EC_{50} (Table 1). The values are similar to those for free subunits expressed at a low $\alpha4{:}\beta2$ ratio or a high $\alpha4{:}\beta2$ ratio, respectively (Table 1) (Zwart and Vijverberg, 1998; Moroni et al., 2006). We also examined gating by the subtype-selective agonist, 5I A85380. This agonist has different EC_{50} values for activation of the two forms: ~10 nM for $(\alpha4)_2(\beta3)_3$ versus ~18,000 nM for $(\alpha4)_3(\beta3)_2$ (Zwart et al., 2006). In addition, the maximal response is actually greater than the maximal response to ACh for the $(\alpha4)_2(\beta3)_3$ receptor (Zwart et al., 2006). We determined the response to 1 μM 5I A85380 relative to the response to 1 mM ACh. 5I A85380 activates receptors incorporating free β2 (resulting in receptors containing three copies of β2) much more strongly than those with free α4, as expected from studies with free subunits (Table 1). Overall, these results indicate that the concatemers assemble with free subunits to generate pentameric receptors with properties appropriate for the stoichiometry predicted for incorporation of the free subunit, as reported previously (Zhou et al., 2003).

The fact that we produce surface receptors by combining subunit concatemers with mutated free subunits raises the possibility that some combinations assembled inappropriately (for example excluding the free subunit). We cannot rule this out, but for most combinations we determined the EC_{50} for activation by ACh and the relative gating by 1 μM 5I A85380 compared with 1 mM ACh (supplemental Table 1, available at www.jneurosci.org as supplemental material). As mentioned previously, these values provide an indication of the numbers of α4 and β2 subunits present in the functional receptors. Our unpublished data indicate that the N-terminal extracellular domain is the primary determinant for both of these parameters; that is, when three β2 extracellular domains are present in the receptor, the EC_{50} will be small (10 μM or less) and the relative response to 5I A85380 will be large (1.0 or greater), in contrast to the case when only two are present. Figure 2 shows a plot of relative response against log(EC_{50}) and shows that the data fall into two distinct groups. The groups indicate that the functional receptors contain the number of β2 extracellular domains expected if the free subunits incorporated into the pentameric receptor. As shown in Results, we also found that a single free α4 subunit confers potentiation by 17β-estradiol, indicating that it also incorporates into the assembled pentameric receptors. Accordingly, the constructs we have used behave in the expected way, and the majority of pentameric receptors include both concatemers and free subunits. We cannot rule out the possibility that there is a small fraction of receptors that did not incorporate free subunits.

We note that our data indicate that both the α/β and β/α concatemers when expressed without free subunits behave as though receptors include three α4 subunits. This differs from the results obtained by Zhou et al. (2003), who observed that the β/α receptor had a small EC_{50} for ACh.

We also confirmed by Western blot that concatemers do not significantly degrade into individual subunits in the oocytes (Fig. 3) (Zhou et al., 2003; Carbone et al., 2009).

We sought to incorporate the free subunit selectively into a defined position in the pentamer, either a position in which it contributed to an agonist-binding site or one in which it did not. Accordingly, we confirmed the arrangements proposed by Zhou et al. (2003) for subunits in receptors formed by a concatemer plus a free subunit (Fig. 1). To do this, we used a mutation in the E-loop of the agonist-binding site contributed by the β2 subunit, which we had previously shown to affect activation by 5I A85380 (β2F119Q) (Hamouda et al., 2009). We expressed β2F119Q with each concatemer and determined activation by 5I A85380 and ACh. As shown in Figure 4, when β2F119Q is expressed with the β/α concatemer, activation is indistinguishable from when wild-type β2 is expressed. In contrast, when expressed with the α/β concatemer, activation by 5I A85380 is shifted toward higher concentrations and lower efficacy. The EC_{50} for activation by ACh is not affected [5 ± 1 μM (five cells) for α/β&β2F119Q and 9 ± 3 μM (seven cells) for β/α&β2F119Q], as expected from results with free subunits (Hamouda et al., 2009). These

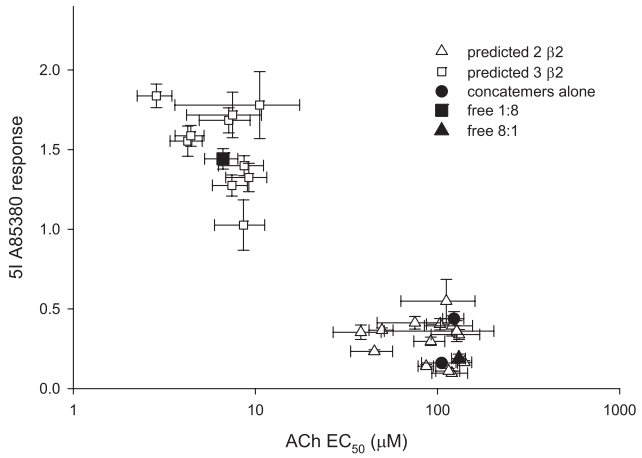

Figure 2. The free subunit participates in the assembled receptor, as indicated by responses to agonists. The relative response to 5I A85380 is plotted against the EC_{50} for ACh; when the receptor contains three $\beta2$ subunit N-terminal domains, the relative response is large, and the EC_{50} is small (free subunits with an $\alpha4$:$\beta2$ ratio of 1:8; black square). Conversely, when two $\beta2$ subunits are present, the relative response is small and the EC_{50} is large (free subunits with an $\alpha4$:$\beta2$ ratio of 8:1; black triangle). The hollow squares show data for combinations in which it is predicted that three $\beta2$ subunit N-terminal domains would be present if receptors contained two copies of a concatemer and a single copy of the free subunit, whereas hollow triangles show data for receptors with two predicted $\beta2$ N-terminal domains. The black circles show data for the two concatemers expressed without a free subunit. The means of values with two predicted $\beta2$ N termini are 5I A85380 relative response of 0.30 ± 0.04 ($N = 14$ combinations) and $EC_{50} = 97 \pm 9$ μM, whereas with three predicted $\beta2$ N termini the means are 1.47 ± 0.08 ($N = 9$ combinations) and 6.2 ± 2.7 μM. The means differ at $p < 10^{-6}$ for each parameter. Data show mean ± SE, for 27 combinations of constructs (data from 3 or more oocytes), including concatemers alone and combinations of free subunits. The full data set is shown in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

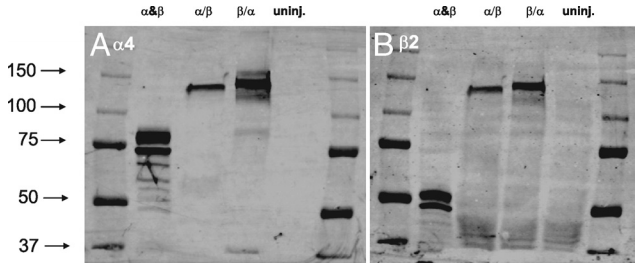

Figure 3. Concatemers do not appear to be significantly degraded. The panels show two blots of the same transfer of proteins extracted from four batches of oocytes, injected with different constructs on the same day. In ***A***, the transfer was probed with antibody to $\alpha4$ (H-133; sc5591). In ***B***, the transfer was stripped and reprobed with antibody to $\beta2$ (C-20; sc1449). Note that there appears to be some breakdown of $\alpha4$ in extracts from oocytes injected with $\alpha4$&$\beta2$ subunits. However, it does not appear that concatemers break down to a significant extent. For each preparation, 50 oocytes were used. Approximately 230 μg (~20% of preparation) of protein loaded in lanes 2, 3, and 4, and ~50 μg in lane 1 (~5% of prep). These images are representative of eight gels from three protein preparations. Images are shown in grayscale and reversed intensity scale to allow visualization of minor bands.

observations confirm the proposed arrangement (Zhou et al., 2003), that the free subunit contributes to an agonist-binding interface when expressed with the α/β concatemer but not the β/α concatemer (Fig. 1).

Results

Previous studies of the ability of 17β-estradiol to potentiate the nicotinic $\alpha4\beta2$ receptor have found that potentiation requires a specific sequence at the C-terminal of the $\alpha4$ subunit (Paradiso et al., 2001; Curtis et al., 2002). Mutagenesis of the sequence (Paradiso et al., 2001) demonstrated that not only a specific set of residues is required but that the position of the sequence with respect to the final (fourth) transmembrane helix is critical: insertion or deletion of a single residue to move the domain further from or closer to the external end of the helix abolishes potentiation. Finally, addition of even a single residue at the end of the domain also abolishes potentiation by 17β-estradiol.

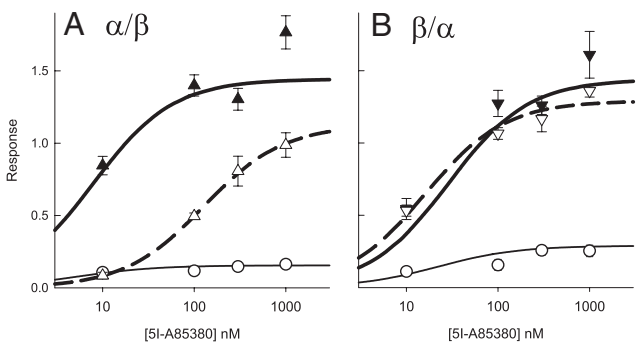

Figure 4. Activation by 5I A85380 indicates positions of subunits in receptors containing concatemers. ***A*** shows the relative gating (normalized to the response to 1 mM ACh for the tested cell) for α/β concatemers assembled with wild-type $\beta2$ (black triangles and solid black line) or with $\beta2$F119Q (hollow triangles and dashed line). The parameters for a Hill equation fit to the data are as follows: EC_{50}, 8 ± 1 nM; maximal response, 1.44 ± 0.07-fold for $\beta2$; and EC_{50}, 127 ± 14 nM; maximal response, 1.13 ± 0.11-fold for $\beta2$F119Q (the Hill coefficient was constrained to 1 for fits). The hollow circles show responses of the $\alpha4/\beta2$ concatemer expressed alone. ***B*** shows comparable data for the β/α concatemer expressed with $\beta2$ or $\beta2$ F119Q. The parameters for a Hill equation fit to the data are as follows: EC_{50}, 29 ± 12 nM; maximal response, 1.44 ± 0.10-fold for $\beta2$; and EC_{50}, 16 ± 1 nM; maximal response, 1.29 ± 0.04-fold for $\beta2$F119Q (the Hill coefficient was constrained to 1 for fits). Fit parameters and data points are mean ± 1 SE, for data from six cells.

In light of these observations, our initial hypothesis was that potentiation requires that the C-terminal domain must be placed on an $\alpha4$ subunit and, additionally, that the $\alpha4$ subunit must participate in ACh binding. To test this hypothesis, we need to control the number and position of mutated subunits in the assembled receptor. The use of concatemeric constructs of subunits (Zhou et al., 2003; Carbone et al., 2009) allows this control. We generated two concatemers, one with the $\alpha4$ subunit at the N terminus (referred to as α/β) and the other with the $\beta2$ subunit at the N terminus (β/α) using the approach developed by Zhou et al. (2003) (see Materials and Methods). Our characterization of the concatemers demonstrates that the functional receptors generated when a concatemer is expressed with a free subunit show the properties of a receptor that includes two copies of the concatemer with one copy of the free subunit (see Materials and Methods). Furthermore, the use of a point mutant in the $\beta2$ subunit confirmed the subunit positions defined by Zhou et al. (2003) (see Materials and Methods) (Fig. 1). When a free subunit is expressed with the β/α concatemer, it will occupy the structural (non-agonist-binding) position in the receptor, whereas with the α/β subunit it will occupy an agonist-binding position (Fig. 1).

The concentration of ACh used to elicit responses and to test potentiation was relatively low (0.1 to 1 μM) and, for almost all constructs, elicited <20% of the response to 1 mM ACh (supplemental Table 2, available at www.jneurosci.org as supplemental material). We used 10 μM 17β-estradiol as the standard test concentration of potentiator.

Sample traces of potentiation for the most important combinations of constructs are shown in Figure 5. The data for the potentiation ratios for all combinations studied are shown in supplemental Table 2 (available at www.jneurosci.org as supplemental material).

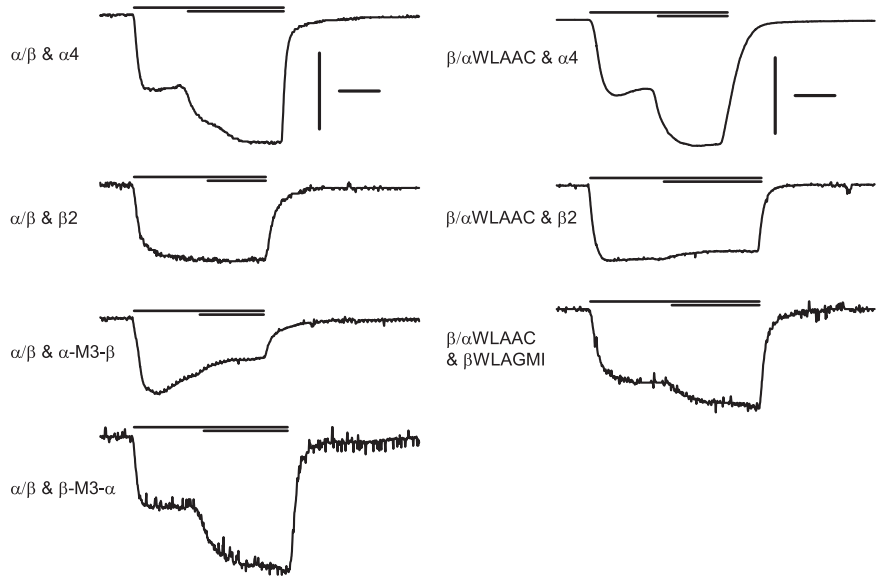

Figure 5. The responses to ACh and ACh plus 10 μM 17β-estradiol are shown for oocytes injected with selected combinations of subunits. The times of drug application are indicated by the horizontal bars above the traces: the top bar shows the application of a low concentration of ACh alone, whereas the bottom bar shows the application of ACh plus 10 μM 17β-estradiol. The left column shows responses from oocytes injected with the α/β concatemer and different free subunits; in this combination the free subunit contributes to an ACh-binding site. Note that a single copy of wild-type α4 produces a receptor that is potentiated by 17β-estradiol, whereas β2 does not, and swapping the portions of the subunit from the end of M3 to the C terminus transfers potentiation or lack thereof. Calibration (top panel): 10 s (all panels); 260 nA (α/β&α4), 34 nA (α/β&β2), 29 nA (α/β&α-M3-β), 14 nA (α/β&β-M3-α). The right column shows responses from oocytes injected with the β/αWLAAC concatemer and different free subunits; in this combination, the free subunit occupies the structural position. Note that a single copy of wild-type α4 produces a receptor that is potentiated by 17β-estradiol, whereas β2 does not, and swapping the last six residues from α4 to β2 transfers potentiation. Calibration (top panel): 10 s (all panels); 1800 nA (β/αWLAAC&α4), 68 nA (β/αWLAAC&β2), 11 nA (β/αWLAAC&βWLAGMI).

17β-Estradiol potentiation is conferred whether the α4 subunit participates in ACh binding or serves as the structural subunit

We confirmed that the α/β concatemer, expressed alone, is not potentiated by 10 μM 17β-estradiol, whereas the β/α concatemer is (Zhou et al., 2003) (Table 1). This indicates that potentiation requires one or more intact, untethered C-terminal domains on the α4 subunit. When the α/β concatemer is expressed with free β2 subunit (abbreviated as α/β&β2), potentiation is also absent, whereas coexpression of the α/β concatemer with free α4 subunit results in potentiation (Fig. 5, Table 1). This observation indicates that a single intact α4 C terminus is sufficient to allow some potentiation. Overall, comparing β/α&α4 to α4&β2 (8:1) indicates that potentiation for the β/α&α4 receptor is greater than for receptor composed of free subunits ($p = 0.04$) as well as for β/α&β2 compared with α4&β2 (1:8) receptors ($p = 0.01$).

Coexpression of the α/β concatemer with free α4 subunit results in a receptor in which the α4 subunit that has an intact C terminus also participates in forming an ACh-binding interface (Fig. 1). To determine whether this is required, we tested additional constructs.

The first manipulation tested the effects of a mutation of the α4 subunit C terminus. Potentiation by 17β-estradiol is lost when the α4 C-terminal WLAGMI is mutated to WLAAC (abbreviated αWLAAC) (Paradiso et al., 2001). Expression of αWLAAC with α/β removes potentiation (Fig. 6), as expected. We then mutated the untethered C terminus of the α4 subunit in the β/α concatemer (β/αWLAAC). When the β/αWLAAC concatemer is expressed with free α4, potentiation is present, but not when it is expressed with free β2 (Fig. 6). These observations indicate that potentiation can occur whether the α4 subunit contributes to an agonist-binding interface (when expressed with the α/β concatemer) or acts as a structural subunit (when expressed with the β/αWLAAC concatemer). They also confirm that only a single subunit need contain the WLAGMI sequence to underlie potentiation.

Incorporation of a single α4 subunit with an untethered WLAGMI domain confers estradiol potentiation on the receptor. We compared two combinations of constructs. In the case of α/β&α4 to α/β&α4WLAAC, the difference in potentiation ratio is significant at $p = 5 \times 10^{-8}$ (t test). For β/αWLAAC&α4 to β/αWLAAC&α4WLAAC, the difference is significant at $p = 8 \times 10^{-7}$. These observations support the conclusion that free α4 subunits incorporate efficiently when expressed with either concatemer.

A 17β-estradiol binding element can be placed on either α4 or β2 subunits

We then examined the question of whether potentiation required the rest of the α4 subunit or was based on the C-terminal region alone. The initial constructs were chimeric subunits between α4 and β2 subunits, with a join just after the end of the third membrane spanning region (abbreviated α-M3-β and β-M3-α) (Fig. 1). In these chimeras, the N-terminal extracellular domain and the first three transmembrane domains are from one subunit, whereas the large cytoplasmic loop, the fourth transmembrane domain and the C-terminal tail are transferred. As expected, replacing the C-terminal domain of the α4 subunit with sequence from β2 removes potentiation when expressed with the α/β concatemer (Figs. 5, 6; supplemental Table 2, available at www.jneurosci.org as supplemental material). More surprisingly, transferring the α4 sequence to β2 confers potentiation (Fig. 6). This observation indicates that the N-terminal extracellular domain and the first three transmembrane domains are not sufficient for potentiation. Potentiation is transferred whether the free subunit occupies the structural position or contributes to an agonist-binding interface (Fig. 6).

We constructed two additional chimeric subunits. These involved transferring the fourth transmembrane segment plus the WLAGMI sequence (βM4→C) from the α4 subunit to the β2 subunit, or only the WLAGMI sequence (βWLAGMI). The 6 aa sequence was transferred, as previous work has indicated that the length of the C-terminal sequence is critical for potentiation (Paradiso et al., 2001). Both of these chimeras allow potentiation when expressed with the β/αWLAAC or the α/β concatemer (Fig. 6). These data indicate that the terminal residues are critical for potentiation. However, the amount of potentiation is significantly greater for the βM4→C construct than for α4 or βWLAGMI when expressed with either the α/β or β/αWLAAC concatemers, and for β-M3-α expressed with α/β (one-way ANOVA for each concatemer separately, with Bonferroni's cor-

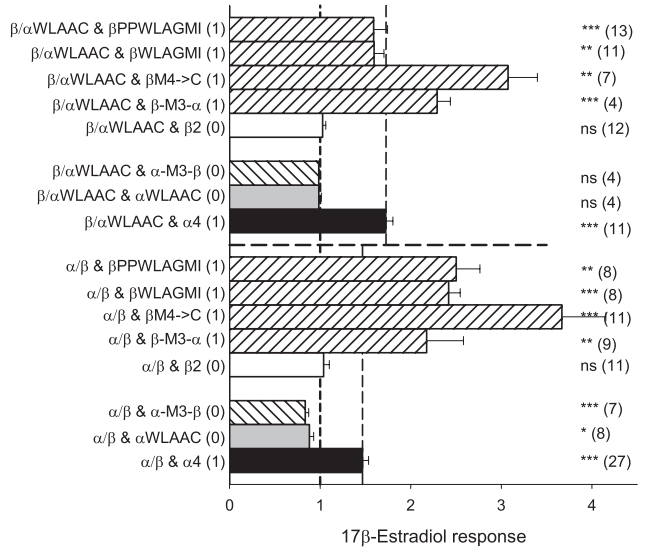

Figure 6. Transferring the β2 C terminus to α4 removes potentiation by 17β-estradiol, and transferring the α4 C-terminal domain to β2 confers potentiation. Combinations of constructs were chosen in which there are no free WLAGMI domains except in the added free subunit. When expressed with the α/β concatemer, the free subunit occupies a position in which it contributes to an ACh-binding site, whereas when expressed with the β/αWLAAC concatemer it occupies the structural position (Fig. 1). The figure shows the mean response ratio in the presence of 10 μM 17β-estradiol (+1 SE). The labels for the bar show the combination of constructs expressed and the number in parentheses shows the number of free C-terminal domains in the postulated pentamer. The significance levels are shown on the right for the probability that the ratio differs from 1 (no effect, shown by the heavy dashed line): ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The numbers of oocytes tested is shown in parentheses after the significance level. The two dashed lines indicate a response ratio of 1 (no effect; heavy line) or the mean level for free wild-type α4 expressed with that concatemer (thin line). Full data are shown in supplemental Table 2 (available at www.jneurosci.org as supplemental material).

rection). We note, however, that mutation of WLAGMI to WLAAC in the intact α4 subunit removes potentiation.

We noted that transferring the WLAGMI sequence to β2 resulted in the C-terminal sequence QPWLAGMI. Previous work (Paradiso et al., 2001) showed that mutation of both prolines in α4 (PPWLAGMI to AAWLAGMI) abolishes potentiation, so we mutated the glutamine to proline in β2WLAGMI to produce β2PPWLAGMI. Expression of the resulting construct did not increase potentiation to the same level as seen with the βM4→C mutation (Fig. 6).

Overall, these data indicate two points. First, transfer of the TM4 region plus the tail produces more potentiation than the simple transfer of the C-terminal tail. Second, the cytoplasmic loop of the α4 subunit (from the end of TM3 to the start of TM4) appears to diminish potentiation compared with the amount produced when only the TM4 to the C terminus is transferred.

The observation that the WLAGMI domain can be moved from one subunit to another is quite surprising. Our data suggest that potentiation does not differ greatly depending on whether the tail is placed on the α4 or β2 subunit. Pooling the data for receptors containing single copies of WLAGMI, the mean values for potentiation are, for β2 N-terminal constructs, 2.3 ± 0.3-fold (mean ± SE; $N = 8$ combinations of constructs), and, for α4 N-terminal constructs, 1.6 ± 0.1-fold ($N = 2$) ($p = 0.03$). Similarly, it does not appear to matter whether the domain is placed on the structural subunit (mean potentiation, 2.4 ± 0.5-fold; four combinations) or on a subunit contributing to an ACh-binding interface (2.0 ± 0.2-fold; six combinations) ($p = 0.8$). These conclusions are tentative, given the relatively small number of cases examined and the possibility for confounding factors.

Our observations indicate that potentiation can occur when only a single copy of a free WLAGMI domain is incorporated in the assembled receptor. Furthermore, the WLAGMI sequence can be placed on the α4 or β2 subunit, and the subunit can participate in forming an agonist-binding interface or serve as the fifth, structural, subunit. There are trends suggesting that potentiation may be larger when the receptor includes concatemers, and may be larger when the domain is present on a β2 subunit rather than an α4 subunit. There is also an indication that some portions of the α4 cytoplasmic loop (between the third and fourth transmembrane regions) might reduce potentiation, whereas some portions of the fourth transmembrane region may enhance potentiation. These observations suggest the existence of additional factors that influence the extent of potentiation, which will have to be examined in additional experiments.

Relationship between copy number and potentiation

There is a significant increase in potentiation as the total number of WLAGMI C-terminal domains increases in a pentameric receptor. The average value for potentiation by 10 μM 17β-estradiol increases from 0.97 ± 0.02 (0 domains, 10 combinations tested) to 2.2 ± 0.2 (1 domain, 10), 2.4 ± 0.4 (2 domains, 5), 3.3 ± 0.2 (3 domains, 4), to 3.7 ± 0.3 (5 domains, 3). Regression of potentiation on the number of untethered domains gives a slope of 0.6 ($p < 10^{-7}$ that the slope is zero).

Our data were obtained using a constant concentration of 17β-estradiol, and it is possible that changes in both potency and efficacy occurred for some of the constructs. Accordingly, we determined the concentration–effect relationship for 17β-estradiol for combinations of subunits that have one to five untethered WLAGMI sequences. Two combinations of constructs were tested for each number of untethered WLAGMI. The combinations were chosen to keep the number of β2 N termini constant at 2 in the assembled pentamers, but some combinations were formed without the use of concatemers and the domains were placed on agonist-binding or structural subunits. The concentration–potentiation data are shown in Figure 7, and the fit parameters are given in Table 2.

The fit maximal potentiation is plotted against the number of untethered WLAGMI domains in Figure 8. There is a clear increase in maximal potentiation with increasing untethered WLAGMI. The increase is greater than linear, with each added untethered WLAGMI increasing the potentiation by ~1.6-fold. The geometric fit shown in Figure 8 is a better description than the linear fit, although there is an indication that the increase from three to five domains is not as great as might be expected. This could result from several possible factors. Some are technical, for example as a result of particular constructs providing a somewhat greater or lesser amount of potentiation than others. However, it is also possible that there is a "ceiling" on the amount of potentiation. This could arise because there is a maximal potentiation possible by the mechanism used by estradiol (so that five domains occupied by 17β-estradiol would not be 1.6^2 as efficacious as three), or it could be that there is some interaction among bound estradiols so that there is a maximal possible number that can bind. Additional experiments will be necessary to distinguish among these possibilities.

Our test concentration of 17β-estradiol (10 μM) is not a saturating concentration for any of these constructs. Accordingly, the potentiation ratios we calculate for the screening data may reflect both efficacy and potency. However, the qualitative con-

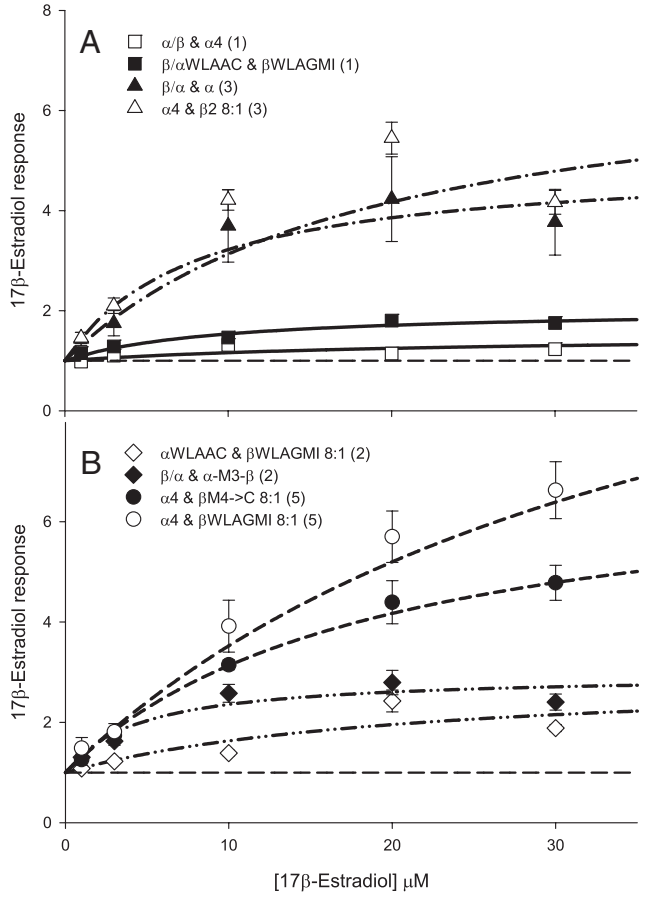

Figure 7. An increased number of free WLAGMI domains results in increased maximal potentiation by 17β-estradiol. Potentiation is plotted against the concentration of 17β-estradiol for eight combinations of constructs. ***A*** shows data for receptors containing one (β/αLAAC&βWLAGMI and α/β&α4; solid lines show fits) or three (α4&β2 8:1 and β/α&βM4⟶C; dash-dot lines) free WLAGMI domains. ***B*** shows data for two (αWLAAC&βWLAGMI 8:1 and β/α&β-M3-α; dash-dot-dot lines) or five (α4&β2WLAGMI 8:1 and α4&βM4⟶C 8:1; dashed lines) domains. The number of free WLAGMI domains in each combination is shown in parentheses. The lines show fits of the equation $r = 1 + R_{max}([\beta Est]/(EC_{50} + [\beta Est])$, where R is the response ratio, R_{max} is the maximal ratio, $[\beta Est]$ is the concentration of 17β-estradiol, and EC_{50} is the concentration producing a half-maximal effect. (The Hill coefficient was constrained to 1.) The fit values are shown in Table 2. The points show the mean ± SE, and the dashed line at a ratio of 1 shows no effect.

clusions about placement of the untethered WLAGMI domain are clear.

Potentiation by 17α-vinylestradiol

The steroid analog 17α-vinylestradiol also potentiates the α4β2 receptor. Previous work (Paradiso et al., 2001) has found that potentiation by this analog does not require the final four residues of the α4 C terminus, although potentiation is larger when the AGMI sequence is present. To remove potentiation, it is necessary to also mutate a tryptophan residue just preceding the terminal sequence (i.e., to convert WLAGMI to LLAAC). Mutation only of the tryptophan (to LLAGMI) does not reduce potentiation by either 17α-vinylestradiol or 17β-estradiol, emphasizing the overall greater importance of the AGMI sequence for potentiation (Paradiso et al., 2001). Assuming that the transduction mechanism is the same for potentiation by 17α-vinylestradiol or 17β-estradiol, these observations indicate that neither the AGMI sequence nor the critical tryptophan is required for the transduction of molecular recognition into functional potentiation. Accordingly, this steroid analog provides a control to indicate that the transduction mechanism for potentiation is preserved when potentiation by 17β-estradiol is removed.

Table 2. Concentration dependence for potentiation by 17β-estradiol

Combination		$R_{max} + 1$		EC_{50}		
Constructs	No. free WLAGMI	Mean	SE	Mean	SE	*N*
α/β&α4	1	1.54	0.1	23.3	5.7	6
β/αWLAAC&α4	1	2.04	0.1	9.4	3.1	4
β/α&α-M3-β	2	2.96	0.2	4.5	0.6	9
αWLAAC&βWLAGMI 8:1	2	2.97	0.2	21.1	3.5	7
α4&β2 8:1	3	5.00	1.0	8.0	1.1	4
β/α&α4	3	5.81	0.3	6.3	1.0	7
α4&βWLAGMI 8:1	5	7.19	0.6	19.0	3.4	4
α4&βM4⟶C 8:1	5	13.44	1.0	39.2	13.6	4

The first column names the constructs injected. The next column lists the number of free C-terminal WLAGMI sequences predicted to be in the assembled receptor. The next columns give the parameters obtained from fitting the concentration–effect relationships (Fig. 7) with the equation $R = 1 + R_{max}([\beta Est]/(EC_{50} + [\beta Est])$, where R is the response ratio, R_{max} is the maximal ratio, $[\beta Est]$ is the concentration of 17β-estradiol, and EC_{50} is the concentration producing a half-maximal effect. The mean values shown are the means of fits to data from *N* oocytes injected with that combination. An ANOVA with Bonferroni's correction indicates that the value for EC_{50} is larger for α4&βM4⟶C than for all other combinations except for α/β&α, αWLAAC&βWLAGMI, and α4&βWLAGMI. EC_{50} for α/β&α4 is larger than for β/α&α-M3-β, but no other differences are significant. For values of R_{max}, the value for α4&βM4⟶C is larger than that for all other combinations. Otherwise, values for combinations with one or two free WLAGMI do not differ significantly from each other, nor do values for three or five free WLAGMI. All values for combinations with one or two free WLAGMI were significantly less than all combinations with three or five, except for αWLAAC&βWLAGMI 8:1 whose value for R_{max} does not differ significantly from that for α4&β2 8:1.

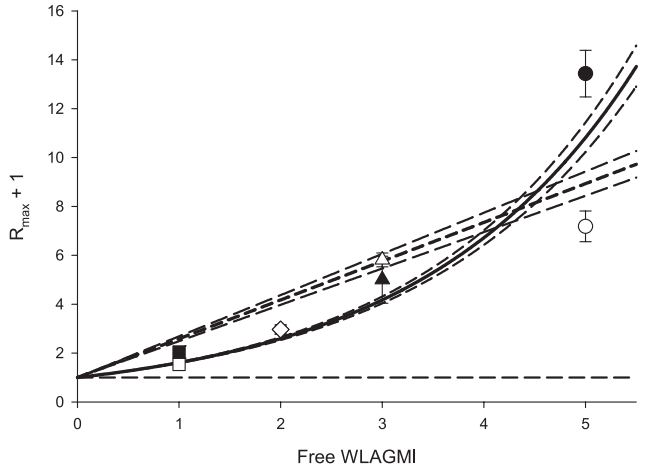

Figure 8. An increased number of free WLAGMI domains results in increased maximal potentiation by 17β-estradiol. The fit $R_{max} + 1$ is plotted against the number of free WLAGMI domains predicted to be in the assembled receptor. The data were fit with two simple equations. The first is a linear increase ($R_{max} + 1$) = 1 + sM, where R_{max} is the fit maximal potentiation, s is the constant of proportionality, and M is the number of free WLAGMI domains. The second is a geometric increase ($R_{max} + 1$) = r^M, where r is the relative increase in R_{max} conferred by adding one WLAGMI domain. The lines show the predicted dependence (dashed linear, solid geometric) and the dashed lines show ±1 SE of the fits. The fit values are $s = 1.6 \pm 0.1$ (best fit ± 1 SE of parameter estimate) and $r = 1.62 \pm 0.02$. The geometric fit was better than the linear fit ($p = 0.02$, F test). Points show mean ± SE for data shown in Table 2. Note that the symbol is sometimes larger than the error bar, and that the two data points at two free WLAGMI overlap. The symbols match those in Figure 7.

We tested a number of the combinations described above (a total of 17) (supplemental Table 3, available at www.jneurosci.org as supplemental material) and found that 17α-vinylestradiol potentiates all of them. For the five cases in which there are no untethered AGMI and two or three critical tryptophans, the mean potentiation by 17α-vinylestradiol is 2.3 ± 0.6, whereas 17β-estradiol on average has no effect (0.94 ± 0.04). Overall, potentiation increases with increasing numbers of critical tryptophans (associated with increasing numbers of untethered AGMIs) from 3.1 ± 1.5 ($N = 2$) for two tryptophans, to 6.1 ± 1.7

($N = 11$) for three tryptophans, and 7.7 ± 2.7 ($N = 4$) for five tryptophans, although the regression coefficient (1.2 ± 1.3; fit value ± estimated SE of parameter) is not significantly different from 0 ($p = 0.39$).

We tested a subset of additional constructs in which the critical tryptophan was mutated. These were αLLAAC and β/αLLAAC. These constructs allowed us to test the effect of increasing numbers of critical tryptophans in the absence of an untethered AGMI sequence, again holding the number of subunits with β2 N termini constant at 2. The mean potentiation increased steadily with increasing numbers of tryptophans: β/αLLAAC&αLLAAC (zero tryptophans: 1.16 ± 0.03, five oocytes); β/αLLAAC&αWLAAC (one: 1.20 ± 0.09, four); β/αWLAAC&αLLAAC (two: 1.56 ± 0.15, four); β/αWLAAC&αWLAAC (three: 1.76 ± 0.05, four). Linear regression of potentiation on the number of critical tryptophans for this subset gives a regression coefficient of 0.22 ± 0.04, which differs from zero ($p = 0.04$).

Overall, the most significant observation is that removal of potentiation by 17β-estradiol does not remove potentiation by 17α-vinylestradiol, indicating that the transduction mechanism is retained.

Discussion

The goal of this study was to identify the critical portions of the nicotinic α4 and β2 subunits required for potentiation by 17β-estradiol. We extended previous studies that had determined that the C-terminal tail of the α4 subunit is necessary (Paradiso et al., 2001; Curtis et al., 2002). Our starting hypothesis was that potentiation would be subunit specific and likely would require that the specific sequence be present on a subunit in a particular position (e.g., on an α4 subunit involved in forming an agonist-binding site). To our surprise, this hypothesis is incorrect in both respects. The results indicate that the WLAGMI domain can be placed at the C terminus of either the α4 or β2 subunit to subserve potentiation. In addition, the subunit can participate in forming an agonist-binding site or serve as the fifth, structural, subunit in the receptor. As a corollary to these observations, the subunit with the domain can be placed between β2 and α4 subunits or α4 and β2 subunits in the assembled receptor, so potentiation does not appear to require a particular neighbor subunit.

Several observations support the idea that 17β-estradiol interacts with the receptor at the AGMI sequence. Because 17β-estradiol is a hydrophobic molecule, it can interact with the lipid membrane. However, the enantiomer of 17β-estradiol does not potentiate (Paradiso et al., 2001). This enantioselectivity indicates that potentiation requires interaction of steroid with an optically active site, perhaps on the receptor. Potentiation is also extremely sensitive to the structure of the WLAGMI domain—mutations, insertions, or deletions can greatly reduce potentiation. In addition, potentiation by 17α-vinylestradiol is reduced but not removed by mutation of AGMI (Paradiso et al., 2001; this study). To remove potentiation by 17α-vinylestradiol, it is necessary to mutate both the AGMI sequence and the neighboring tryptophan. This observation indicates that the AGMI sequence is not required for transduction, in that 17α-vinylestradiol still is capable of potentiation. Furthermore, the finding that complementary changes in steroid and receptor structures affect potentiation supports the idea that the D ring of the steroid associates with the C-terminal WLAGMI tail to underlie potentiation. Finally, the present observations indicate that moving this defined domain from one subunit to another can transfer potentiation. Overall, these data support the conclusion that the C-terminal domain is involved in molecular recognition (binding) rather than the conversion of binding at a different site into functional potentiation (transduction). However, previous work from our laboratory (Paradiso et al., 2001) has shown that the molecular structure of the A ring (the "other end" of the steroid molecule) also is important for potentiation. The binding domain for the A ring has not been localized, although the present data suggest that it must be either to a sequence that is found in both the α4 and β2 subunits, or possibly lies in the membrane. Accordingly, it seems most appropriate to call the C-terminal sequence a binding element or binding domain.

It is surprising that potentiation can be transferred between subunits simply by moving the WLAGMI sequence. Previous studies of potentiating drugs show more specificity in sites. Several potentiators interact with receptors in this gene family at subunit interfaces in the extracellular region. The classic example is benzodiazepine potentiation of $GABA_A$ receptor function (Sigel and Buhr, 1997), but potentiation of nicotinic α3β4* receptors by morantel (Seo et al., 2009) or $\alpha4_3\beta2_2$ receptors by Zn^{2+} ions (Moroni et al., 2008) also requires specific residues in the two subunits forming an interface. For more hydrophobic compounds, etomidate appears to interact with transmembrane regions of two subunits in the $GABA_A$ receptor to potentiate (Li et al., 2006), whereas potentiating neurosteroids are proposed to interact with two transmembrane domains of a single $GABA_A$ subunit (Hosie et al., 2006). This estradiol-binding element appears to be remarkably discrete and effective at transferring potentiation.

There are additional parts of the subunit(s) that appear to influence the amount of potentiation (Curtis et al., 2002). The cytoplasmic loop of the α4 subunit may reduce the amount of potentiation transferred. This effect might result from an action of the loop on transduction of potentiation, as there have been reports in receptors in this family that the cytoplasmic loop can affect channel function (Bouzat et al., 1994; Wang et al., 2000; Kuo et al., 2005; Hales et al., 2006) or modulation (Swope et al., 1999; Yevenes et al., 2008). In contrast, the TM4 helix of the α4 subunit appears to increase potentiation. As will be discussed below, the action of 17β-estradiol to potentiate responses may result from an effect on the transmembrane helices to stabilize the open-channel state of the receptor. Accordingly, the structure of the TM4 region may influence the transduction mechanism. Alternatively, it might be that the A ring of the steroid interacts with some of the residues in the TM4 region. Additional experiments will be required to elucidate the bases for these effects.

The mechanism by which potentiation is produced is not known. Single-channel studies have shown that there is no increase in the single-channel conductance (Curtis et al., 2002). However, the probability of being open is increased, perhaps because of an increase in the duration of openings [Curtis et al. (2002), their Fig. 4]. The increased probability of being open is reminiscent of the effects of mutations of the conserved leucine at the ninth residue in the second membrane-spanning region. Numerous studies have reported that mutation of TM2 L9′ to more hydrophilic residues increases the open probability of nicotinic and related receptors (Revah et al., 1991; Labarca et al., 1995; Chang et al., 1996). The increase is produced by a mutation in any of the five subunits in the receptor, and the overall effect increases with number of mutated subunits (Labarca et al., 1995; Chang et al., 1996; Moroni et al., 2006). These observations indicate that all five subunits contribute to a conformational change before the channel becomes permeable for ions and that the L9′ mutation in any subunit therefore can shift the overall gating equilibrium. Perhaps the interaction with estradiol produces a conformational

change in the transmembrane regions of that subunit that results in a similar stabilization of the open state. The observation that the extent of potentiation (efficacy) increases with increased numbers of untethered WLAGMI regions is consistent with this suggestion. The data suggest that each untethered WLAGMI increases the maximal potentiation by ~1.6-fold. The idea of an independent action of 17β-estradiol on any subunit that carries an available WLAGMI sequence accounts well for the essential features of our observations.

Drugs that enhance the response of synaptic receptors, without directly interacting with the agonist-binding site, are of increasing interest as therapeutic agents that do not produce a response directly but enhance endogenous signaling. A number of drugs that enhance $GABA_A$ receptor activity are in clinical use as tranquilizers, sedatives, or hypnotics, first represented by the benzodiazepines (Rudolph and Möhler, 2004). More recently, potentiators of the nicotinic α7 receptor have received attention as possible agents to enhance cognition and memory (Lightfoot et al., 2008). The nicotinic α4 subunit is quite prevalent in the mammalian brain; the receptor comprising α4 and β2 subunits is the most common heteromeric receptor and the α4 subunit also participates in forming a variety of receptors of more complex stoichiometry (Gotti et al., 2007). These receptors have their major physiological effects by modulating the release of other neurotransmitters, rather than directly mediating postsynaptic responses in the brain (Dani and Bertrand, 2007). In particular, a role for α4-containing receptors in control of dopamine release has been proposed (Exley and Cragg, 2008), providing a possible link to the reward pathway. The actions of endogenous compounds, particularly steroids, are more difficult to define compared with the effects of exogenously added drugs. There are two principal difficulties in demonstrating a physiological role for estradiol potentiation specifically of α4-containing nicotinic receptors. The first is that the concentration of 17β-estradiol required for potentiation is high (>1 μM), much higher than the levels in the brain (~1 nM) (Mukai et al., 2006), although local synthesis clearly occurs (Mukai et al., 2006; Cornil and Charlier, 2010) and could result in higher local levels. The second is that 17β-estradiol has a multiplicity of effects in the nervous system, both in sculpting development (cf. Gillies and McArthur, 2010) and in more rapid changes in function (cf. Mukai et al., 2006; Cornil and Charlier, 2010; Gillies and McArthur, 2010).

Overall, these observations indicate that the interaction between 17β-estradiol and the nicotinic α4β2 receptor is mostly determined by the discrete, C-terminal tail of a subunit. The ability to transfer potentiation between subunits and the relationship between numbers of WLAGMI domains and efficacy of potentiation suggest that the effect of 17β-estradiol is mediated by actions on single subunits and that the overall consequences for gating occur because of the summation of independent energetic contributions to overall gating of this receptor.

References

Akabas MH (2004) $GABA_A$ receptor structure-function studies: a reexamination in light of new acetylcholine receptor structures. Int Rev Neurobiol 62:1–43.

Akk G, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S (2007) Mechanisms of neurosteroid interactions with $GABA_A$ receptors. Pharmacol Ther 116:35–57.

Belelli D, Lambert JJ (2005) Neurosteroids: endogenous regulators of the $GABA_A$ receptor. Nat Rev Neurosci 6:565–575.

Bouzat C, Bren N, Sine SM (1994) Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. Neuron 13:1395–1402.

Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411:269–276.

Carbone AL, Moroni M, Groot-Kormelink PJ, Bermudez I (2009) Pentameric concatenated $\alpha4_2\beta2_3$ and $\alpha4_3\beta2_2$ nicotinic acetylcholine receptors: subunit arrangement determines functional expression. Br J Pharmacol 156:970–981.

Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant $GABA_A$ receptor. J Neurosci 16:5415–5424.

Cornil CA, Charlier TD (2010) Rapid behavioural effects of oestrogens and fast regulation of their local synthesis by brain aromatase. J Neuroendocrinol 22:664–673.

Curtis L, Buisson B, Bertrand S, Bertrand D (2002) Potentiation of human α4β2 neuronal nicotinic acetylcholine receptor by estradiol. Mol Pharmacol 61:127–135.

Dani JA, Bertrand D (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol 47:699–729.

Exley R, Cragg SJ (2008) Presynaptic nicotinic receptors: a dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. Br J Pharmacol 153 [Suppl 1]:S283–S297.

Gillies GE, McArthur S (2010) Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Pharmacol Rev 62:155–198.

Gotti C, Moretti M, Gaimarri A, Zanardi A, Clementi F, Zoli M (2007) Heterogeneity and complexity of native brain nicotinic receptors. Biochem Pharmacol 74:1102–1111.

Hales TG, Dunlop JI, Deeb TZ, Carland JE, Kelley SP, Lambert JJ, Peters JA (2006) Common determinants of single channel conductance within the large cytoplasmic loop of 5-hydroxytryptamine type 3 and α4β2 nicotinic acetylcholine receptors. J Biol Chem 281:8062–8071.

Hamouda AK, Jin X, Sanghvi M, Srivastava S, Pandhare A, Duddempudi PK, Steinbach JH, Blanton MP (2009) Photoaffinity labeling the agonist binding domain of α4β4 and α4β2 neuronal nicotinic acetylcholine receptors with [^{125}I]epibatidine and 5[^{125}I]A-85380. Biochim Biophys Acta 1788:1987–1995.

Hosie AM, Wilkins ME, da Silva HM, Smart TG (2006) Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. Nature 444:486–489.

Hsiao B, Mihalak KB, Repicky SE, Everhart D, Mederos AH, Malhotra A, Luetje CW (2006) Determinants of zinc potentiation on the α4 subunit of neuronal nicotinic receptors. Mol Pharmacol 69:27–36.

Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, Andreasen A, Viner A, Trudell JR, Harrison NL (2001) Evidence for a common binding cavity for three general anesthetics within the $GABA_A$ receptor. J Neurosci 21:RC136(1–4).

Jin X, Covey DF, Steinbach JH (2009) Kinetic analysis of voltage-dependent potentiation and block of the glycine α3 receptor by a neuroactive steroid analogue. J Physiol 587:981–997.

Kuo YP, Xu L, Eaton JB, Zhao L, Wu J, Lukas RJ (2005) Roles for nicotinic acetylcholine receptor subunit large cytoplasmic loop sequences in receptor expression and function. J Pharmacol Exp Ther 314:455–466.

Labarca C, Nowak MW, Zhang H, Tang L, Deshpande P, Lester HA (1995) Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. Nature 376:514–516.

Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB (2006) Identification of a $GABA_A$ receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. J Neurosci 26:11599–11605.

Lightfoot AP, Kew JN, Skidmore J (2008) α7 nicotinic acetylcholine receptor agonists and positive allosteric modulators. Prog Med Chem 46:131–171.

Moroni M, Zwart R, Sher E, Cassels BK, Bermudez I (2006) α4β2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. Mol Pharmacol 70:755–768.

Moroni M, Vijayan R, Carbone A, Zwart R, Biggin PC, Bermudez I (2008) Non-agonist-binding subunit interfaces confer distinct functional signatures to the alternate stoichiometries of the α4β2 nicotinic receptor: an α4-α4 interface is required for Zn^{2+} potentiation. J Neurosci 28:6884–6894.

Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S (2006) Local neurosteroid production in the hippocampus:

influence on synaptic plasticity of memory. Neuroendocrinology 84:255–263.

Paradiso K, Zhang J, Steinbach JH (2001) The C terminus of the human nicotinic $\alpha4\beta2$ receptor forms a binding site required for potentiation by an estrogenic steroid. J Neurosci 21:6561–6568.

Revah F, Bertrand D, Galzi JL, Devillers-Thiéry A, Mulle C, Hussy N, Bertrand S, Ballivet M, Changeux JP (1991) Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. Nature 353:846–849.

Rudolph U, Möhler H (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol 44:475–498.

Schlichter R, Keller AF, De Roo M, Breton JD, Inquimbert P, Poisbeau P (2006) Fast nongenomic effects of steroids on synaptic transmission and role of endogenous neurosteroids in spinal pain pathways. J Mol Neurosci 28:33–51.

Seo S, Henry JT, Lewis AH, Wang N, Levandoski MM (2009) The positive allosteric modulator morantel binds at noncanonical subunit interfaces of neuronal nicotinic acetylcholine receptors. J Neurosci 29:8734–8742.

Sigel E, Buhr A (1997) The benzodiazepine binding site of $GABA_A$ receptors. Trends Pharmacol Sci 18:425–429.

Sine SM, Engel AG (2006) Recent advances in Cys-loop receptor structure and function. Nature 440:448–455.

Steinbach JH, Bracamontes J, Yu L, Zhang P, Covey DF (2000) Subunit-specific action of an anticonvulsant thiobutyrolactone on recombinant glycine receptors involves a residue in the M2 membrane-spanning region. Mol Pharmacol 58:11–17.

Swope SL, Moss SJ, Raymond LA, Huganir RL (1999) Regulation of ligand-gated ion channels by protein phosphorylation. Adv Second Messenger Phosphoprotein Res 33:49–78.

Wang HL, Ohno K, Milone M, Brengman JM, Evoli A, Batocchi AP, Middleton LT, Christodoulou K, Engel AG, Sine SM (2000) Fundamental gating mechanism of nicotinic receptor channel revealed by mutation causing a congenital myasthenic syndrome. J Gen Physiol 116:449–462.

Yevenes GE, Moraga-Cid G, Peoples RW, Schmalzing G, Aguayo LG (2008) A selective G $\beta\gamma$-linked intracellular mechanism for modulation of a ligand-gated ion channel by ethanol. Proc Natl Acad Sci U S A 105:20523–20528.

Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, Lindstrom J (2003) Human $\alpha4\beta2$ acetylcholine receptors formed from linked subunits. J Neurosci 23:9004–9015.

Zwart R, Vijverberg HP (1997) Potentiation and inhibition of neuronal nicotinic receptors by atropine: competitive and noncompetitive effects. Mol Pharmacol 52:886–895.

Zwart R, Vijverberg HP (1998) Four pharmacologically distinct subtypes of $\alpha4\beta2$ nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. Mol Pharmacol 54:1124–1131.

Zwart R, Broad LM, Xi Q, Lee M, Moroni M, Bermudez I, Sher E (2006) 5-I A-85380 and TC-2559 differentially activate heterologously expressed $\alpha4\beta2$ nicotinic receptors. Eur J Pharmacol 539:10–17.