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Constraining the Expression of Nicotinic Acetylcholine Receptors by Using Pentameric Constructs

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ABSTRACT

Much of our understanding of ligand-gated ion channels comes from heterologous expression studies. However, this technique cannot produce receptors with a predetermined subunit composition for channels formed by several different subunits and cannot insert a single mutation copy if the subunit of interest is present in several copies in the channel. Here, we describe a novel approach that overcomes these problems by expressing pentameric constructs, in which the code of the five subunits is linked (i.e., $\beta4\_\beta4\_\alpha3\_\beta4\_\alpha3$). This is the first time that a concatemer of the complete pentameric receptor has been expressed for channels in the cysteine-loop superfamily. The presence of the linker did not change the agonist or antagonist sensitivity of $\alpha3\beta4$ nicotinic receptors. We show evidence that the expressed receptors were made up of $\alpha3$ and $\beta4$ subunits in one pentameric fusion protein as designed in the construct. This approach can be applied to any nicotinic superfamily receptor to produce channels with a defined subunit arrangement and to introduce specific mutations at any desired location of the pentameric fusion protein.

It is hard to exaggerate the importance of heterologous expression in the study of ion channels. *Xenopus laevis* oocytes or mammalian cells transfected with the appropriate constructs express the receptor of interest almost in isolation and in conditions ideal for electrophysiological recording. Much of our understanding of channel subunit composition and of structure-function relation comes from such work.

If the subunit combination is right, the functional properties of many synaptic channels are reproduced surprisingly faithfully by expression in non-neuronal cells. Only now are the limitations of this technique in its simplest form beginning to emerge and to constrain our experiments. One frustrating problem is that of reproducing channels with complex or variable subunit composition, such as neuronal nicotinic acetylcholine receptors (nAChRs). Functional heteromeric neuronal nAChRs can be formed by expression of an α and a β subunit (Colquhoun et al., 2003), but native receptors can contain as many as four different subunits (Conroy and Berg, 1995; Forsayeth and Kobrin, 1997; Moretti et al., 2004). Furthermore, the α-to-β ratio in the pentamer may depend on the expression system and the transfection ratio (Zwart and Vijverberg, 1998; Nelson et al., 2003).

Another problem is that channels often contain more than one copy of the same subunit. For instance, the muscle nAChR has two copies of the main binding subunit, α. This means that we cannot easily produce muscle receptors bearing just one mutant α subunit, and therefore, we cannot easily study the role of each binding site.

There have been several attempts to solve these problems by expressing concatameric constructs, namely by linking subunit cDNAs to obtain fusion proteins, which, in principle, can be incorporated in the receptor only in a restricted range of combinations (Im et al., 1995; Baumann et al., 2001, 2002, 2003; Nelson et al., 2003; Minier and Sigel, 2004). A number of problems have come to light with this approach, such as proteolysis (Nicke et al., 2003), dipentamer formation (Zhou et al., 2003), and incomplete incorporation of fusion proteins, a problem which can be hard to detect (Groot-Kormelink et al., 2004). In the nicotinic superfamily, concatemeric approaches have so far focused on lower-order concatemers (subunit dimers and trimers). Here, we report the success of an alternative approach that for the first time links all of the subunits into a pentameric construct.

Materials and Methods

Construction of Pentameric Subunit cDNAs. The human $\alpha3$ and $\beta4$ nicotinic subunits (GenBank accession numbers Y08418 and Y08416), containing only coding sequences and an added Kozak consensus sequence (*GCCACC*) immediately upstream of the start

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; PCR, polymerase chain reaction; bp, base pair(s); ACh, acetylcholine.

codon, were subcloned into the pcDNA3.1 (Invitrogen, Carlsbad, CA) and the pSP64GL vectors. Mutants were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and full-length sequenced.

Pentameric constructs were produced from intermediate tandem and trimer constructs. All tandem constructs (α3_β4, β4_α3, and α3_α3) were made in the same way. First, the coding region of each subunit was amplified by PCR, excluding the stop codon. The PCR primers directed to each corresponding start codon (bold) included the Kozak sequence (*GCCACC*) and the EcoRI restriction site (underlined, 5′-end PCR fragment, CTGAATTC*GCCACC***ATG**. . . .). The primer directed to the coding sequence upstream of the stop codon included the NotI restriction site (basically replacing the stop codon by the NotI site). The resulting DNA fragments were purified and subcloned into the pcDNA3.1/Myc-His version C vector (Invitrogen) using the EcoRI and NotI restriction sites. A linker DNA fragment (Im et al., 1995) was created by hybridization of two complementary oligonucleotides: 5′-GGCCGC**T**CAGCAACAGCAGCAACAGCAGCAAG-′3 and 5′-AATTCTTGCTGCTGTTGCTGCTGTTGCTG**A**GC-3′. The resulting double-strand DNA linker contains a 5′-end NotI restriction site overhang (underlined) and a 3′-end EcoRI restriction site overhang (underlined) separated by 25 nucleotides [the first nucleotide (boldface type) is inserted to bring the NotI site (8-cutter) back in the correct reading frame, whereas the next 24 nucleotides code for 8 glutamines]. The tandem constructs were created using three unique restriction sites: EcoRI (upstream of the start codon of all subunits and the 3′-end of the linker), NotI (downstream of the coding sequence of all subunits and the 5′-end of the linker), and AgeI (between the Myc and His epitope sequences in the pcDNA3.1/Myc-His version C vector). A three-way ligation resulted in the following tandem circular plasmid: * [**AgeI**. . . His-epitope. . . stop codon. . . pcDNA3.1/Myc-His C vector. . . EcoRI - *subunit A* - NotI] * [NotI - linker - *Eco*RI] * [EcoRI - *subunit B* - NotI. . . Myc-epitope. . . **AgeI**] *, where asterisks represent the ligation sites, and brackets represent purified DNA fragments digested with the restriction sites indicated in boldface type. To remove the epitope tags (Myc- and His-), all tandems were subcloned in the corresponding pcDNA3.1 vector using a unique restriction site in subunit B, for instance, cutting the tandem upstream of the start codon (of subunit A) and somewhere in subunit B and transferring this fragment in the same position of subunit B, previously cloned in the pcDNA3.1 vector. Finally, all tandem constructs were also subcloned in the pSP64GL vector. 9′-mutant tandems were created by swapping the corresponding DNA fragments (using unique restriction sites upstream and downstream of the L9′T mutation) from $\alpha3^{L279T}$ (for $\beta4\_\alpha3^{L279T)}$ and $\beta4^{L272T}$ (for $\beta4^{L272T}\_\alpha3$). The α3_β4, β4_α3, $\beta4\_\alpha3^{L279T}$, and $\beta4^{L272T}\_\alpha3$ tandem constructs cloned in the pSP64GL vector were fully sequenced.

Both trimer constructs were created by cutting and ligating different parts of the tandem and monomeric plasmids described above. The first trimer (β4_β4_α3/ pSP64GL) was created by ligating three DNA fragments: β4_α3/pSP64GL*EcoRI-XbaI (4583 bp), β4/pcDNA3.1/Myc-His*EcoRI-ApaI (1486 bp), and β4-α3/pSP64GL*XbaI/ApaI (1814 bp). The mutated [$\beta4^{L272T}$ _β4_α3/pSP64GL] trimer was created as above except for the first DNA fragment being $\beta4^{L272T}\_\alpha3$/pSP64GL*EcoRI-XbaI (4583 bp).

Pentameric constructs were created by cutting and ligating different parts of the tandem and trimer plasmid constructs described above. The first pentameric construct [β4_β4_α3_β4_α3/pSP64GL] was created by ligating three different DNA fragments: β4_β4_α3/pSP64GL*KspI-XbaI (6176 bp), α3_β4/pcDNA3.1/Myc-His*KspI-ApaI (2986 bp), and β4_α3/pSP64GL*ApaI-XbaI (1814 bp). Mutant pentameric constructs ($\beta4^{L272T}$_β4_α3_β4_α3/pSP64GL, β4_β4_α3_β4_$\alpha3^{L279T}$/pSP64GL, and $\beta4^{L272T}$_β4_α3_β4_$\alpha3^{L279T}$/pSP64GL) were created as above except for using the corresponding mutated $\beta4\_\alpha3^{L279T}$/pSP64GL and/or $\beta4^{L272T}$_β4_α3/pSP64GL DNA fragments. All trimer and pentameric constructs were checked for cloning artifacts by a minimum of five different restriction enzyme digests.

RNA Gel Electrophoresis. Capped cRNA was prepared as described previously (Boorman et al., 2000). For RNA electrophoresis (1.5% agarose gel, 1× Gel Prep/Running buffer, NorthernMax-Gly; Ambion, Austin, TX), RNA samples (including the 0.24–9.5-kb RNA ladder; Invitrogen) were diluted 1:1 with Glyoxal sample loading dye (Ambion) and incubated at 50°C for 30 min before loading. Samples were separated at 5 V/cm for 3 h, and RNAs were visualized by UV transillumination.

Electrophysiological Recording and Analysis. *X. laevis* oocyte harvesting, preparation, and two-electrode voltage-clamp recording were performed as described previously (Boorman et al., 2000). The quantity of cRNA injected was from 0.05 to 0.9 ng for α3, $\alpha3^{L279T}$, β4, $\beta4^{L272T}$, and $\beta4\_\alpha3^{L279T}$ constructs and up to 100 ng cRNA for the pentameric constructs. Recording was done at −70 mV in nominally 0-calcium Ringer solution (150 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 0.5 μM atropine sulfate, pH 7.2; Sigma Chemical , St. Louis, MO). Trimetaphan was bath-applied for 25 min before responses to ACh were obtained (in the continued presence of antagonist). ACh concentration-response curves were obtained in a descending dose protocol, and the resulting data were fitted with a Hill equation with the program CVFIT (Colquhoun and Vais, http://www.ucl.ac.uk/pharmacology/dc.html). Values reported are means (±S.D. of the mean) of the results of fitting each dose-response curve separately (Boorman et al., 2000).

Results

Receptors Expressed from Pentameric Constructs Are Similar to Those Expressed from Monomer Constructs. Figure 1, a and b, shows ACh current responses from oocytes expressing human α3β4 neuronal nAChRs from monomer α3 + β4 constructs (top) or from the pentamer construct β4_β4_α3_β4_α3. Agonist responses were very similar for the two receptors both in time course and agonist sensitivity. Analysis of the concentration-response curves (Fig. 1c) showed a small difference in EC_{50} values (137.8 ± 13.7 versus 95.0 ± 10.7 μM, $n = 4$ and 6, for the monomer and pentamer constructs, respectively; $P = 0.038$, two-tailed Student's t test) but not in Hill slope (1.79 ± 0.14 and 1.71 ± 0.13, respectively).

Measuring agonist EC_{50} values is a fairly unsatisfactory way of comparing receptors. A much more robust technique for determining the integrity of the binding site is to measure the shift produced by a competitive antagonist (such as trimetaphan) in the concentration dependence of agonist responses (i.e., dose ratios). Dose ratios to 0.2 μM trimetaphan are very similar in monomer- and pentamer-expressed receptors with values of 4.35 ± 0.38 and 5.01 ± 0.37, respectively (monomer construct values from Boorman et al., 2003). Because the magnitude of this shift is a direct expression of antagonist affinity, this indicates that the (overlapping) agonist/antagonist binding site is very similar in the two types of recombinant receptors.

Functional Receptors Expressed from Pentameric Constructs Are Not Formed by Breakdown Products or by Incomplete Incorporation of the Fusion Protein. We recently showed (Groot-Kormelink et al., 2004) that tandem subunit incorporation in nAChRs can be incomplete and that this problem is hard to detect. Hence, we extensively tested whether this problem occurs for pentameric construct receptors and whether these receptors contain the correct complement of subunits, as dictated by the construct design.

First, we checked whether the in vitro synthesized cRNA was of the correct length and that no RNA breakdown had

happened. Conceivably, if there is sufficient contamination by breakdown products, the functional receptors we observed may not be produced by the linked, five-subunit construct as we wish but by the contaminant. cRNA gel electrophoresis (Fig. 2) indicated that construct impurity or breakdown was not a problem, because all constructs used showed one clean band of the appropriate molecular weight.

Of course, degradation may occur after injection into the oocyte or at protein level and could still make pointless our effort to constrain receptor topology and composition. A straightforward way of testing this would be to check the size of either the individual proteins (by Western blotting) or the assembled complex (by sucrose-gradient separation). The other possibility was to devise a functional test for the presence of breakdown products and for incomplete pentamer incorporation. We chose to do that in the hope that a functional test would be more sensitive in detecting what may be relatively low levels of breakdown products. Furthermore, such a test would detect only those channels that are on the surface of the oocyte and are functional. The approach we chose relies on a reporter mutation: incorporation of the mutation we selected (L9′T in the second transmembrane domain) into $\alpha3\beta4$ nAChRs increases agonist sensitivity (Boorman et al., 2000).

Thus, we added an excess of a single mutant monomer cRNA, either $\alpha3^{LT}$ or $\beta4^{LT}$, to the pentamer construct. The addition of mutant monomers would reveal the presence of a significant level of (functional) degradation products (such as lower-order concatamers or single subunits) by incorporating them into receptors. Such receptors would carry mutations and would be distinguishable from the wild-type receptors produced by the pentamer construct alone. Figure 3 shows that no such mutant receptors could be detected when either $\alpha3^{LT}$ or $\beta4^{LT}$ subunits were expressed together with the pentamer. Thus EC_{50} values were not changed by expression with mutant subunits and were 99.6 ± 10.7 and 84.6 ± 7.6 μM for pentamer constructs coinjected with $\alpha3^{LT}$ or $\beta4^{LT}$, respectively (n = 4 for both).

To maximize the sensitivity of this test, we chose to inject an excess of monomer mutant subunit constructs. Because efficiency of expression and incorporation are much greater for the monomer construct, considering simply the ratio between the quantity of cRNA injected for monomer and pentamer grossly underestimates the "functional" excess in monomer. A better quantification is given by the level of functional expression achieved by the different constructs. Thus, we determined empirically the amount of cRNA to be injected (Fig. 3, top) to have a functional expression of monomers [estimated by measuring maximum ACh responses (i.e., to 1 mM ACh)] approximately an order of magnitude larger than that of monomer plus pentamer. Thus, 0.25 ng of $\alpha3^{LT}$ or $\beta4^{LT}$ cRNA produced several microamperes of current if injected together with 0.25 ng of the appropriate wild-type monomer (5570 ± 890 nA, n = 10, and 2342 ± 393 nA, n = 6, respectively) but only approximately 0.5 μA if injected with 100 ng of pentamer cRNA (570 ± 151 and 621 ± 97 nA, respectively). As detailed above, this approximately 10-fold excess of mutant monomer failed to affect the receptor dose-response curve.

Another concern was that degradation of the construct or of the fusion protein could lead to the formation of a fragment, such as $\beta4\_\beta4\_\alpha3$, that would require both an α and a β subunit for functional assembly and detection. This was

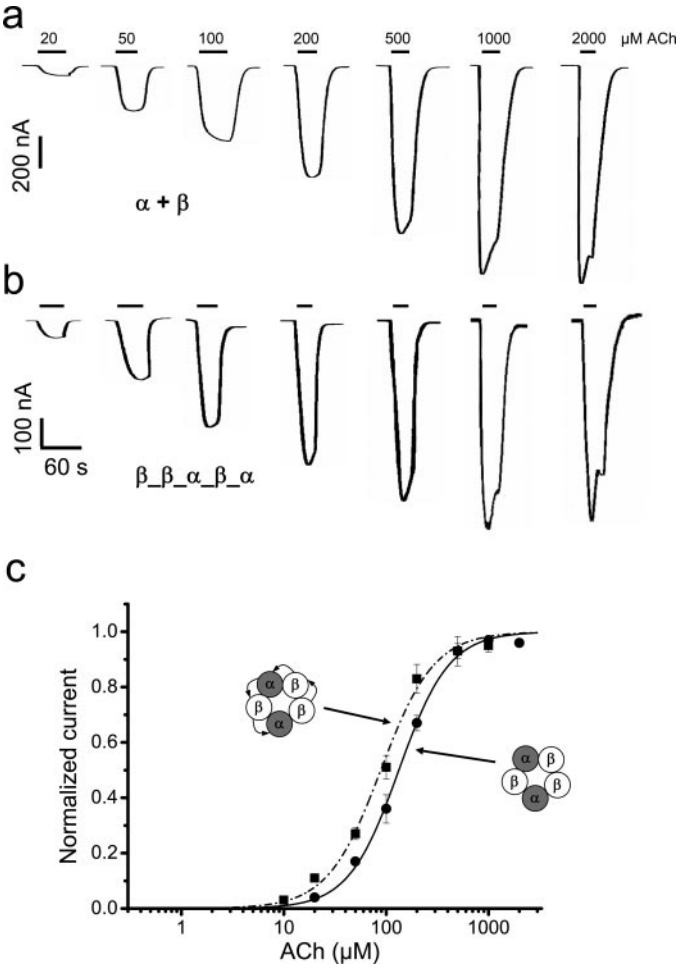

Fig. 1. $\alpha3\beta4$ neuronal nicotinic receptors expressed from the pentameric construct have ACh sensitivity similar to that of receptors expressed from monomeric constructs. a and b, examples of current responses elicited by increasing ACh concentrations on the two types of receptors. c, concentration-response curves for $\alpha3$ + $\beta4$ (monomeric) receptors and for the $\alpha3$ + $\beta4$ (pentameric) receptors (n = 4 and 6, respectively). Lines are fits of the data with the Hill equation. All responses were recorded from *X. laevis* oocytes voltage-clamped at −70 mV in nominally 0-calcium solution.

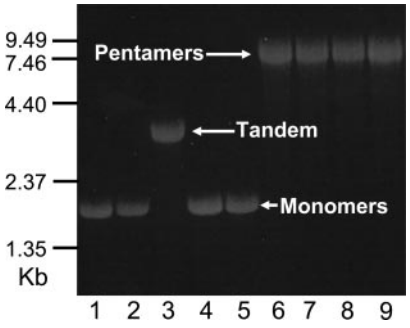

Fig. 2. RNA gel-electrophoresis of capped cRNA constructs used for *X. laevis* oocyte injections. Approximately 1 μg of $\alpha3$ (1), $\beta4$ (2), $\beta4\_\alpha3^{LT}$ tandem (3), $\alpha3^{LT}$ (4), $\beta4^{LT}$ (5), $\beta4\_\beta4\_\alpha3\_\beta4\_\alpha3$ penta (6), $\beta4^{LT}\_\beta4\_\alpha3\_\beta4\_\alpha3$ mutant penta (7), $\beta4\_\beta4\_\alpha3\_\beta4\_\alpha3^{LT}$ mutant penta (8), and $\beta4^{LT}\_\beta4\_\alpha3\_\beta4\_\alpha3^{LT}$ mutant penta (9) were separated on a 1.5% agarose gel. The 0.24- to 9.5-kb RNA ladder is indicated on the left. The predicted cRNA sizes are 1.8 kb for $\alpha3$ and $\beta4$ monomers, 3.4 kb for $\beta4\_\alpha3$ tandem, and 8 kb for the pentameric constructs.

tested by injecting the pentamer construct together with an excess of $\beta4\_\alpha3^{LT}$ construct. No shift in the resulting dose-response curves was observed, indicating that $\beta4\_\alpha3^{LT}$ does not incorporate and therefore a $\beta4\_\beta4\_\alpha3$ fragment is not present (data not shown). More indirect evidence that functional expression is not caused by the assembly of lower-order breakdown products comes from the fact that another pentameric construct that we tested ($\alpha3\_\beta4\_\alpha3\_\beta4\_\alpha3$) failed to produce functional receptors.

These experiments not only suggest that breakdown does not occur or is very limited but also confirm that pentameric construct receptors contain the pentameric fusion protein in its entirety, as intended. If this was not the case and more than one pentamer participated to the formation of a single receptor, for instance, contributing one or two subunits, we would expect to see preferential incorporation of (mutant) monomer constructs into such receptors and therefore shifts in the dose-response curves. This is because monomer subunits incorporate with high efficiency when competing with tandem constructs, even when they are not in excess (Groot-Kormelink et al., 2004).

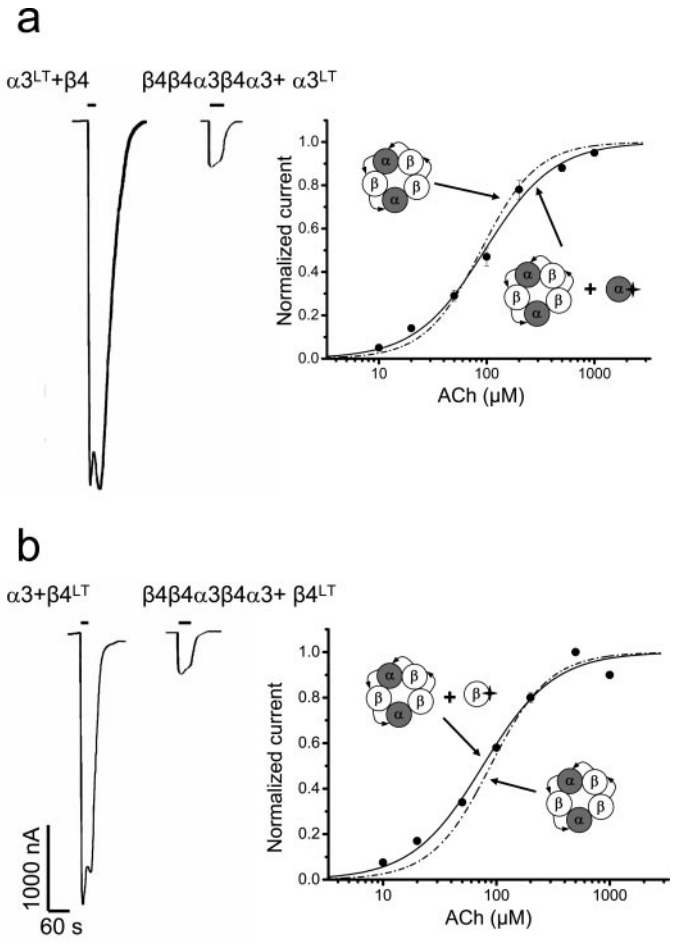

Fig. 3. Expression of an excess of $\alpha3^{LT}$ or $\beta4^{LT}$ together with the pentamer construct does not result in incorporation of the mutant monomers into functional receptors. Example traces (a and b, left-hand side) give an estimate of the extent of the functional excess of monomer subunit cRNA injected by showing that the same quantity of monomer cRNA produced very large functional response if coexpressed with the appropriate monomer. The concentration-response curves obtained from receptors expressed after coinjection of mutant monomer with wild-type pentamer constructs (filled circles and continuous lines, $n = 4$ for both) are identical with those obtained after expression of wild-type pentamer alone (broken curves).

Exploiting the Potential of Pentamer Constructs: Subunit Nonequivalence. Many ligand-gated channels of the nicotinic superfamily contain multiple copies of one subunit, and with conventional expression techniques, it is impossible to mutate only one of these subunit at a time and choose the location of the mutant subunit. This difficulty is overcome by using pentamer constructs, which allow us to determine the number and position of the mutations inserted.

We met with this limitation in our previous characterization of L9′T mutations in neuronal nAChRs. In unlinked $\alpha3\beta4$ receptors (Boorman et al., 2000), interpolation of the effect of mutating all α or β subunits (with the assumption of equivalence within subunit subtype) gave similar unitary shifts for α and β of 5.6- and 6.2-fold, respectively. We have now tested the effect of introducing a single L9′T mutation in the $\alpha3\beta4$ receptor.

Concentration-response curves (Fig. 4) show that the effect of a single mutation is much greater if it the mutation is carried by the α subunit ($\beta\_\beta\_\alpha\_\beta\_\alpha^{LT}$ construct) than if it is carried by the β subunit ($\beta^{LT}\_\beta\_\alpha\_\beta\_\alpha$ construct). Receptors with a mutation in α have an ACh sensitivity 16-fold greater than wild type (EC_{50} value = 5.9 ± 0.2 μM, $n = 4$), whereas for receptors with one β mutation, sensitivity increases only roughly 3-fold (EC_{50} value = 28.5 ± 1.8 μM, $n = 5$). Note that for both α and β, the effect of a single mutation is different from the 6-fold shift predicted by the results of mutating all αs or all βs (shown by the broken lines in the graphs in Fig. 4). These data strongly suggest that both the two α and the three β subunits are nonequivalent in their pore-lining domain. Previously, asymmetry in the contribution of each subunit's transmembrane 2 residues to gating has been studied only in muscle nicotinic receptors (Labarca et al., 1995; Grosman and Auerbach, 2000).

Figure 4 (bottom graph) shows that the shifts in the position of the dose-response curve produced by mutating one α and one β subunit multiply (i.e., add logarithmically). Thus the EC_{50} value of $\beta^{LT}\_\beta\_\alpha\_\beta\_\alpha^{LT}$ receptors was 2.0 ± 0.6 μM, $n = 4$, corresponding to a 46-fold increase in agonist sensitivity.

All dose-response curves obtained for mutant pentameric constructs were well-fitted by one component fits. This suggests that they are likely to be produced by a single population of mutant receptors containing the number of copies of the mutation intended when the construct was designed. This further confirms that these constructs express a single fusion protein that contains five subunits (i.e., there is no significant breakdown) and that these subunits are all assembled into one receptor that is functionally expressed (i.e., the incomplete incorporation seen with our tandem approach does not occur; Groot-Kormelink et al., 2004).

Discussion

We report the successful functional expression of neuronal nAChRs from a pentameric construct, in which all of the five subunits that form the receptor have been artificially linked. Although expression of such a complete receptor concatemer has been obtained for other channel types, notably potassium channels (Liman et al., 1992), TRP channels (Hoenderop et

al., 2003), and P2X channels (Stoop et al., 1999), to our knowledge, this is the first time that this approach has been used for channels in the nicotinic superfamily.

Other work with linked constructs has focused on lower-order concatemers, such as tandems (expressed with monomers) or trimers (expressed with tandems; Minier and Sigel, 2004). We recently found that expressing $\beta4\_\alpha3$ tandem together with $\beta4$ does produce functional $\alpha3\beta4$-type nAChRs, but that these are not a homogeneous population, because a proportion suffers from incomplete incorporation of the tandem protein (Groot-Kormelink et al., 2004). The fact that these pentamers did not contain one copy of monomer and two of tandem, as planned, was revealed only by an extensive series of reporter mutation tests. Incomplete incorporation was probably caused by differences in the efficiency with which monomers and concatemers incorporate in the receptor. Therefore, although it is somewhat surprising that the more ambitious approach of a single full-length construct did produce functional channels, this approach should be less vulnerable to the problem of competition between monomers and different-order concatamers.

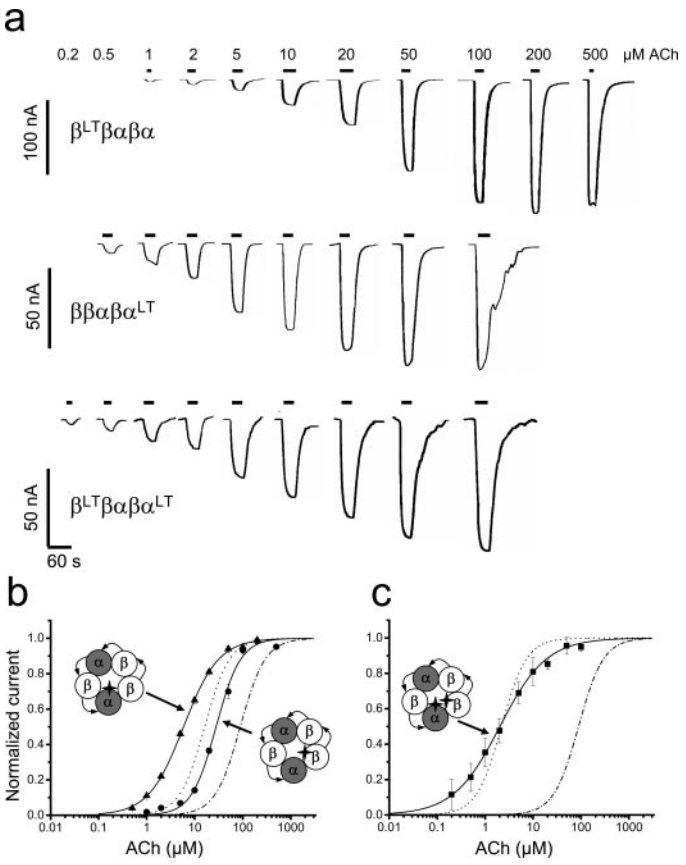

Fig. 4. Nonequivalent effects of L9′T mutations inserted in the second transmembrane domain of a single α or a single β subunit. a, example of current responses elicited by increasing concentrations of ACh. b and c, comparison of the concentration-response relation for pentamer-expressed receptors bearing an L9′T mutation on a single α subunit ($\beta4\_\beta4\_\alpha3\_\beta4\_\alpha3^{LT}$), a single β subunit ($\beta4^{LT}\_\beta4\_\alpha3\_\beta4\_\alpha3$), or both ($\beta4^{LT}\_\beta4\_\alpha3\_\beta4\_\alpha3^{LT}$). Note that the increase in agonist sensitivity produced by a single mutation copy is greater if the mutation is in α (▲, n = 4). This single-mutation shift is greater than the approximately 6-fold shift expected from previous experiments in which all α or all β were mutated (shown for reference by the broken line; Boorman et al., 2000). Conversely, the increase in ACh sensitivity is less than expected when the mutation is inserted in β (●, n = 5). When the two mutations are combined, their effects sum (on the logarithmic scale, that is, the shifts multiply), as shown by the plot in c (■, continuous line, n = 4), in which the broken line is the 36-fold shift expected on the basis of the previous data (Boorman et al., 2000).

Our first concern was to make sure that the receptors were formed as designed in the construct (i.e., that each pentamer contained all of the five subunits linked in the fusion protein and only those subunits). Other "unwanted" receptor assemblies could form through the incorporation of subunits produced by breakdown of the linked cRNA or protein (a problem reported for P2X concatamers; Nicke et al., 2003), through inefficient incorporation of the linked proteins (Groot-Kormelink et al., 2004), and through the sharing of linked proteins across different receptors (Zhou et al., 2003).

Because we wanted to focus on functional receptors, we tested for the formation of such unwanted pentamers by expressing with the pentamer construct a large excess of mutant monomer, either α^{LT} or β^{LT} or tandem $\beta4\_\alpha3^{LT}$. Our failure to detect incorporation of the mutation in pentamer-expressed receptors shows that the monomer subunit cannot insert itself into the final functionally expressed pentamer. This also implies that incorporation of the "tail end" of the pentamer construct is preferred to the incorporation of the monomer subunit in completing the receptor. In addition, breakdown does not produce enough single subunits to give a detectable level of functional channels, even when this is facilitated by an excess of added monomer cRNA. Because of our choice of experimental design, we cannot exclude that such "unwanted" pentamers do form but are not functional. Nevertheless, this is not important for our purpose, which is to do functional receptor work.

The properties of linked subunit nAChRs seem similar to those of nAChRs expressed from traditional monomer constructs, suggesting that linking does not significantly distort receptor structure: linked and unlinked receptors differed by only approximately 30% in their EC_{50} values and were identical in their response to the competitive antagonist trimetaphan, indicating that their binding site is very similar. These results compare well with those obtained by Sigel and coworkers in their expression of $GABA_A$ receptors by combining trimer and dimer constructs (Baumann et al., 2002).

Multimeric constructs for nicotinic superfamily receptors will allow us to produce receptors with a specified composition and subunit topology. This is particularly important for neuronal nAChRs and for $GABA_A$ receptors, because these channels can exist in a number of possible combinations of subunit isoforms (Colquhoun et al., 2003; Whiting, 2003) and, in the case of neuronal nAChRs, also in different α:β stoichiometries (Nelson et al., 2003). Being able to obtain and characterize pure receptor populations with predefined subunit stoichiometry or composition will aid in dissecting them out in vivo. Pentameric constructs are likely to be useful also in clarifying the open question of the subunit stoichiometry of heteromeric glycine receptors (Kuhse et al., 1993; Burzomato et al., 2003; Grudzinska et al., 2005).

Another important experimental route opened by concatamer techniques is the possibility of introducing a defined number of mutations in subunits present in multiple copies in a receptor and to choose the location of such mutations. Being able to introduce single mutation copies in such receptors is important in understanding how channelopathies with autosomal dominant inheritance produce their phenotype in heterozygotes. In addition, one of the reasons for

testing the pentamer-construct approach was that it could be better than lower-order concatemers at producing defined mutant receptors when dealing with homomeric receptors, such as some glycine receptors and $\alpha 7$ neuronal nAChRs. This is an important point for future work.

As an example of the power of this approach, we tested the effect of introducing single copies of the 9′ leucine-to-threonine mutation in the second transmembrane domain of the α or the β subunit. Although the effects of each copy of the mutation seem to add (on log scale, as was assumed in our work with monomer constructs; Boorman et al., 2000), being able to mutate one subunit at a time shows that the effects of the mutation are much bigger when it is carried by an α subunit.

In conclusion, we demonstrated the feasibility of expressing pentameric constructs for neuronal nAChRs. The pharmacology of these linked receptors is similar to that of receptors expressed by the conventional monomer technique. These full-length linked receptors do not seem to suffer from the problems of lower-order concatamers such as breakdown in the linker or faulty, incomplete incorporation of the linked subunits (Nicke et al., 2003; Groot-Kormelink et al., 2004). This powerful technique can, in principle, be used for any receptor in the nicotinic superfamily and could be extended to any of the channels that have been studied with lower-order concatamers (Minier and Sigel, 2004), and this will allow for the first time the expression of defined subunit complexes, formed by a planned subunit combination and topology and containing the desired number of mutations in the desired location.

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