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., \( \beta_4 \beta_4 \alpha_3 \beta_4 \alpha_3 \)). This is the first time that a concatamer 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 \( \alpha_3 \beta_4 \) nicotinic receptors. We show evidence that the expressed receptors were made up of \( \alpha_3 \) and \( \beta_4 \) 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 \( \alpha \) and a \( \beta \) 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 \( \alpha \)-to-\( \beta \) 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, \( \alpha \). This means that we cannot easily produce muscle receptors bearing just one mutant \( \alpha \) 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 superfamilly, concatemeric approaches have so far focused on lower-order concatamers (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 \( \alpha_3 \) and \( \beta_4 \) nicotinic subunits (GenBank accession numbers Y08418 and Y08416), containing only coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon.
Pentameric constructs were produced from intermediate tandem and trimer constructs. All tandem constructs (α3, β4, α3, 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 EcoRI restriction site (upstream of the L9* and downstream of the L9* and downstream of the L9*), and the pSP64GL vectors. Mutants were created using the corresponding mutated EcoRI and NotI restriction sites indicated in boldface type. To remove the epitope tags of subunit B, previously cloned in the pSP64GL vector using a unique restriction site in subunit B, for (Myc- and His-), all tandems were subcloned into the corresponding pcDNA3.1/Myc-His version C vector. A three-way ligation resulted in the following tandem circular plasmid: α3 (Myc- His-epitope...) & stop codon... pcDNA3.1/Myc-His C vector... EcorI - subunit A - NotI] * [NotI - linker - EcoRI] * [EcoRI - subunit B - NotI] * Myc-epitope... α3 (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 tandem constructs were created by swapping the corresponding DNA fragments (using unique restriction sites upstream and downstream of the L9* mutation) from α3L279T (for α3, α3, α3, and α3) and α3L279T (for α3, α3, and α3). The α3, β4, α3, and α3, β4, α3 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 [(α3, β4, α3) pSP64GL] was created by ligating three DNA fragments: (α3, α3, pSP64GL)EcorI-XbaI (4583 bp), (β4,pSP64GL)1My-His-EcorI-Apal (1486 bp), and (p4,3pSP64GL)XbaI/Apal (1814 bp). The mutated (β4L272T, β4, pSP64GL)EcorI-Apal-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 [(α3, β4, α3, β4, pSP64GL)] was created by ligating three different DNA fragments: (α3, β4, α3, pSP64GL)KspI- XbaI (6176 bp), (α3, β4,pSP64GL)1My-His-KspI-Apal (2896 bp), and (p4,3pSP64GL)Apal-XbaI (1814 bp). Mutant pentameric constructs (β4L272T, β4, α3, β4, α3, pSP64GL), (β4, β4, pβ4, α3, α3), and (β4L272T, β4, pβ4, α3, β4, α3, pSP64GL) were created as above except for using the corresponding mutated (β4, α3, α3, pSP64GL) and/or (β4L272T, β4, pβ4, α3, β4, α3, pSP64GL) DNA fragments. All trimer and pentameric constructs were checked for cloning artifacts by a minimum of five different restriction enzyme digests.

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RNA Gel Electrophoresis. Capped cRNA was prepared as described previously (Boorman et al., 2000). For RNA electrophoresis (1.5% agarose gel, 1x 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, β4, β4L272T, and β4, α3L279T 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 MgCl2, 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 Vains, 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, β4, α3, 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 EC50 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 EC50 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 α3β4 nAChRs increases agonist sensitivity (Boorman et al., 2000).

Thus, we added an excess of a single mutant monomer cRNA, either α3LT or β4LT, 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 α3LT or β4LT subunits were expressed together with the pentamer. Thus EC50 values were not changed by expression with mutant subunits and were 99.6 ± 10.7 and 84.6 ± 7.6 μM for pentamer constructs co-injected with α3LT or β4LT, 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 α3LT or β4LT 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 β4_α3, that would require both an α and a β subunit for functional assembly and detection. This was
tested by injecting the pentamer construct together with an excess of $\beta_4\alpha_3$ construct. No shift in the resulting dose-response curves was observed, indicating that $\beta_4\alpha_3$ does not incorporate and therefore a $\beta_4\beta_4\alpha_3$ 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 pentamer construct that we tested ($\alpha_3\beta_4\alpha_3\beta_4\alpha_3$) failed to produce functional receptors.

These experiments not only suggest that breakdown does not occur or is very limited but also confirm that pentamer 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).

**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 subunits 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 L9T mutations in neuronal nAChRs. In unlinked $\alpha_3\beta_4$ receptors (Boorman et al., 2000), interpolation of the effect of mutating all $\alpha$ or $\beta$ subunits (with the assumption of equivalence within subunit subtype) gave similar unitary shifts for $\alpha$ and $\beta$ of 5.6- and 6.2-fold, respectively. We have now tested the effect of introducing a single L9T mutation in the $\alpha_3\beta_4$ receptor.

Concentration-response curves (Fig. 4) show that the effect of a single mutation is much greater if it is carried by the $\alpha$ subunit ($\beta_4\alpha_3\beta_4\alpha_3$ construct) than if it is carried by the $\beta$ subunit ($\beta_4\alpha_3\beta_4\beta_4\alpha_3$ construct). Receptors with a mutation in $\alpha$ have an ACh sensitivity 16-fold greater than wild type (EC$_{50}$ value = $5.9 \pm 0.2$ $\mu$M, $n = 4$), whereas for receptors with one $\beta$ mutation, sensitivity increases only roughly 3-fold (EC$_{50}$ value = $28.5 \pm 1.8$ $\mu$M, $n = 5$). Note that for both $\alpha$ and $\beta$, the effect of a single mutation is different from the 6-fold shift predicted by the results of mutating all $\alpha$s or all $\beta$s (shown by the broken lines in the graphs in Fig. 4). These data strongly suggest that both the two $\alpha$ and the three $\beta$ 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 $\alpha$ and one $\beta$ subunit multiply (i.e., add logarithmically). Thus the EC$_{50}$ value of $\beta_4\alpha_3\beta_4\beta_4\alpha_3$ receptors was $2.0 \pm 0.6$ $\mu$M, $n = 4$, 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 pentamer construct, in which all of the five subunits that form the receptor have been artificially linked. Although expression of such a complete receptor concatamer 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 concatamers, such as tandems (expressed with monomers) or trimers (expressed with tandems; Minier and Sigel, 2004). We recently found that expressing β4_α3 tandem together with β4 does produce functional α3β4-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 concatamers 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.

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 α3LT or β4LT or tandem β4_α3LT. 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 EC50 values and were identical in their response to the competitive antagonist triphen, 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; Grudzińska 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 concatamers at producing defined mutant receptors when dealing with homomeric receptors, such as some glycine receptors and α7 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.

References


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