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PERSPECTIVE

Pretty Subunits All in a Row: Using Concatenated Subunit Constructs to Force the Expression of Receptors with Defined Subunit Stoichiometry and Spatial Arrangement

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ABSTRACT

The members of the Cys-loop ligand-gated ion channel (LGIC) gene family play a major role in fast synaptic transmission, and these receptors represent an important class of targets for therapeutic agents. Each member of this gene family is a pentameric complex containing one or more different subunits, and a large number of subunits for each member have been identified. This large number of subunits could give rise to a bewildering array of possible subunit compositions and spatial arrangements within a single complex, not all of which may occur in vivo. Heterologous expression systems have been used to create specific combinations of individual subunits to mimic naturally occurring receptors. However, this approach is not without its problems. In this issue of *Molecular Pharmacology*, Groot-Kormelink et al. (page 559) describe a method for constructing "concatameric" receptors, in which five individual subunits are arranged in a predetermined order connected by a flexible linker. Expression of this construct results in the formation of receptors with a unique, predefined subunit stoichiometry and subunit arrangement within the receptor complex. Receptors formed from this construct are fully functional and have properties essentially identical to those formed from individual subunits. The application of this very general approach to other members of the LGIC family should markedly enhance our ability to understand how subunit composition influences receptor function, as well as provide a means for the expression of receptors of predefined subunit composition and arrangement as tools for the development of novel selective pharmacological and therapeutic agents.

The Cys-loop ligand-gated ion channel (LGIC) family is a group of neurotransmitter receptors that are involved in fast synaptic transmission and includes the muscle and neuronal nicotinic acetylcholine receptors (AChR), the serotonin type 3 receptor, the $GABA_A$ receptor ($GABA_AR$), and the glycine receptor (Connolly and Wafford, 2004; Lester et al., 2004). All members of the LGIC family are believed to be pentameric in structure, with one or more different homologous subunits arranged around a central pore that forms the channel itself. Binding of the appropriate agonist to the receptor induces a conformational change in the protein, resulting in the opening of the ion channel, and initiating the sequelae of channel activation.

So far, a large number of subunits for each type of LGIC have been cloned (Table 1), and many studies have been carried out using heterologous expression systems such as *Xenopus laevis* oocytes or transfected mammalian cells to understand how subunit composition affects the functional properties of the receptor. For example, coexpression of the $GABA_AR$ α_1 and β_1 subunits in human embryonic kidney 293 cells results in receptors that display much of the pharmacology of bona fide $GABA_ARs$, including potentiation by barbiturates, but these receptors are insensitive to benzodiazepines. However, coexpression of the γ_2 subunit with the α_1 and β_1 subunits produces receptors with benzodiazepine binding sites and functional responses to benzodiazepines (Pritchett et al., 1989), demonstrating the crucial role that γ subunits play in $GABA_AR$ function.

An equally important goal of the expression studies is to duplicate various receptor subunit combinations found in vivo to use these refined systems as tools for developing receptor (sub)subtype-selective pharmacological agents.

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ABBREVIATIONS: LGIC, Cys-loop ligand gated ion channel; AChR, nicotinic acetylcholine receptor; $GABA_AR$, $GABA_A$ receptor.

These tools could then be used not only for analysis of the properties of receptors themselves but also for the development of novel therapeutic agents targeted toward a specific receptor (sub)subtype (Gotti et al., 2000; Bunnelle et al., 2004). However, for this approach to be truly fruitful, one must first determine the subunit stoichiometry of a given target and then develop methods for the expression of receptors with this exact stoichiometry and subunit arrangement. Neither of these tasks is by any means easy or straightforward.

Consider the simplest nontrivial situation—a pentameric receptor that contains two different subunits, A and B. There are six possible subunit stoichiometries (A_5, A_4B, A_3B_2, A_2B_3, AB_4, and B_5). With identical handedness governed by *l*-amino acid chirality, there are eight possible unique subunit arrangements in the complex (Fig. 1). In the absence of a detailed understanding of the "rules" for receptor assembly, it is not possible a priori to predict the exact subunit stoichiometry and/or subunit arrangement in the complex when two different types of subunits could be incorporated into the complex. When there are more than two different subunits that could potentially be incorporated into the receptor (as is more often the case; see Table 1), the situation becomes even more complex. In either situation, experimental techniques must be developed to address the issue of subunit stoichiometry and subunit arrangement.

In the case of naturally occurring receptors, subunit-specific antibodies have been used to determine subunit stoichiometry (but not arrangement) from tissue homogenates (Benke et al., 1996; Li and De Blas, 1997; Turner and Kellar, 2005). Although this approach can certainly determine which subunits are associated with each other in a complex, it may not be able to identify all complexes that occur naturally because of the sensitivity of detection methods and may not be quantitative enough to unambiguously determine subunit stoichiometry. Nonetheless, studies of this type have identified a number of potential subunit combinations and stoichiometries of naturally occurring receptors, and provide the groundwork for studies in heterologous expression systems using more defined subunit composition.

Heterologous expression systems, such as *X. laevis* oocytes or transfected mammalian cells, have been used to determine subunit stoichiometry and to create receptors of defined subunit composition by introducing RNA or DNA for one or more subunits. Subunit stoichiometry can then be determined by analyzing the properties of the expressed receptors using a variety of techniques, including immunoprecipitation with subunit-selective antisera (Anand et al., 1991; Kellenberger et al., 1996; Knight et al., 2000), analysis of the effects of mutations in the pore-forming domain that alter receptor properties (Cooper et al., 1991; Backus et al., 1993; Chang et al., 1996; Boorman et al., 2000; Burzomato et al., 2003), and, in one case, atomic force microscopy (Barrera et al., 2005). Studies such as these provide most of what we know about subunit stoichiometry of receptors containing various subunits.

However, although one assumes/hopes that the receptors expressed in these systems will be faithful surrogates for naturally occurring receptors, there is some evidence that in heterologous expression systems, the ratio of subunits in a given receptor complex depends on the ratios of exogenous subunit RNA or DNA introduced into the expression system, resulting in variable subunit stoichiometry (Hedblom and Kirkness, 1997; Zwart and Vijverberg, 1998; Nelson et al., 2003). In addition, it is clear that these systems can produce receptors that probably do not exist in vivo—either homomeric receptors (Beato et al., 2002) or subunit-deficient complexes (Jackson et al., 1990; Charnet et al., 1992). Furthermore, the presence of endogenous subunits in the expression system may further complicate the analysis of the effects of altering subunit composition on receptor function (Buller and White, 1990). Therefore, these systems may not always produce the type of receptors expected, and conclusions obtained from such studies may not be as firm as one would like.

One method to obtain the expression of receptors of known/predefined subunit stoichiometry and even subunit orientation is the use of concatameric subunit constructs. This forces subunits to be in a particular stoichiometry and spatial arrangement by expressing a polyprotein containing more than one subunit sequences in the mature protein. This is essentially what is seen naturally in some voltage-gated channels. In the case of K^+ channels, the channel is a true tetramer formed from four individual subunits (MacKinnon, 1991; Doyle et al., 1998), whereas in Na^+ and Ca^{2+} channels, the "tetramer" is formed by four homologous repeat domains within a single large polypeptide chain encoding the main subunit (Catterall, 1995).

Originally applied to voltage-gated K^+ channels (Liman et al., 1992), this approach has been used for several members of the LGIC family (Im et al., 1995; Baumann et al., 2001; Zhou et al., 2003; Grudzinska et al., 2005). In most cases, tandem dimeric subunits were created with a polyglutamine linker containing 10 to 25 glutamines between the two subunits. At this length, polyglutamine is water-soluble and assumes a random coil conformation (Altschuler et al., 1997), making it an ideal linker for this purpose. Expression of the dimeric construct with one or more individual subunits can then be used to determine not only the subunit stoichiometry

TABLE 1

Human LGIC subunit genes/gene products

Various human genes/gene products for each receptor cloned to date and listed in the current version of the ligand-gated ion channel database [http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php (Le Novere and Changeux, 2001)]. Please note that not all of the gene products have been unequivocally demonstrated to be incorporated into LGICs in vivo.

Serotonin type 3 receptor	HTR_3A, HTR_3B, HTR_3C, HTR_3D, HTR_3E
$GABA_AR$	α_1, α_2, α_3, α_4, α_5, α_6, β_1, β_2, β_3, γ_1, γ_2, γ_3, δ, ϵ, π, ρ_1, ρ_2, ρ_3, θ
Glycine receptor	α_1, α_2, α_4, β
Muscle AChR	α_1,b_1, γ, δ, ϵ
Neuronal AChR	α_2, α_3, α_4, α_5, α_6, α_7, α_9, α_{10}, β_2, β_3, β_4

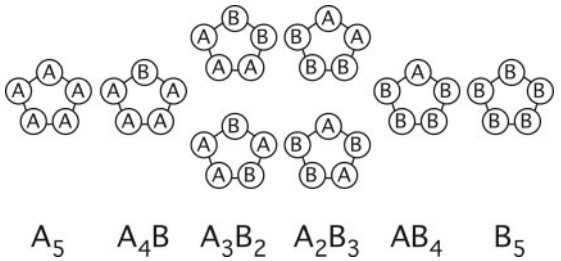

Fig. 1. Possible subunit stoichiometries and spatial arrangement of a receptor containing five subunits, each of which can be either an "A" subunit or a "B" subunit. Note that even for this simple system, there are six possible stoichiometries and eight potential arrangements of the subunits in the pentamer.

but also the spatial arrangement of the subunits in the complex. For example, if expression of A→B tandem subunits (where the carboxyl terminus of the A subunit is joined to the amino terminus of the B subunit via the linker) alone does not produce functional receptors, then one can rule out dimers, tetramers, or hexamers as forming the entire receptor complex (which would not be surprising given the fact that numerous studies analyzing receptors from both tissues and heterologous systems have demonstrated that these receptors are pentamers). If coexpression of the A→B dimer with the B subunit formed functional receptors but coexpression of A subunit with the dimer did not, then (assuming a pentameric complex) one would conclude that the stoichiometry of the receptor was A_2B_3 and the subunit arrangement in the complex was A→B→A→B→B (and then back to the initial "A" in the sequence to complete the loop).

The above analysis assumes that both parts of the dimeric subunit are incorporated into the same receptor complex. However, two recent studies suggest that this may not always be the case, complicating the analysis of the results. Through the use of α_4→β_2 and β_2→α_4 nicotinic AChR receptor subunit dimer constructs, Zhou et al. (2003) showed that in some cases, the subunits in the dimer may be incorporated into two separate complexes, resulting in a dimer of pentameric complexes held together by the linker. Groot-Kormelink et al. (2004) used a wide variety of dimers containing various α-subunits of neuronal AChRs combined with various β-subunits in both orientations (i.e., α→β and β→α) and analyzed the properties of the expressed receptors. Surprisingly, they found that some dimer constructs expressed alone in *X. laevis* oocytes gave rise to functional receptors, which is at odds with the known pentameric structure of the receptor. Further analysis using mutations in the β subunit led the authors to conclude that in some cases, only one subunit in the dimer was incorporated into the complex, with the remaining subunit "floating in the wind" as an appendage outside of the complex.

These two studies demonstrate that the use of tandem dimeric subunits is not as clean as originally anticipated, and set the groundwork for the study by Groot-Kormelink et al. (2006) in the current issue. In this work, the authors expressed not tandem dimers, but concatameric *pentamers* containing α_3 and β_4 nicotinic AChR subunits in the order β_4→β_4→α_3→β_4→α_3 and compared the functional properties of these receptors with those expressed from the monomeric α_3 and β_4 subunits together. The functional properties of the receptors expressed from the pentameric construct were essentially identical to those expressed from the monomers. Through the coexpression of α_3 or β_4 subunits with a mutation in the pore domain that alters channel gating (L9′T; Revah et al., 1991; Filatov and White, 1995; Labarca et al., 1995) as a probe for incorporation of monomeric subunits, the authors were able to rule out proteolytic breakdown of the pentameric construct and subsequent reincorporation of monomeric subunits into the complex. Thus, the pentameric construct produces a fully functional receptor.

In a demonstration of the usefulness of this technique, the authors constructed a receptor in which only one of the two α_3 subunits in the complex contained the L9′T mutation. Previous work suggested that when multiple subunits contained this change that the mutation in each subunit made more or less equal contributions to the overall change in receptor gating (Filatov and White, 1995; Labarca et al., 1995). However, because it was not possible in the original studies to ensure that only a single mutant α subunit was incorporated into the complex, this notion could not be tested in an unambiguous fashion. In the present study, the authors were able to ensure incorporation of a single mutant α_3 subunit into the receptor, leaving the other α_3 subunit as a wild type, and they found that mutations in each subunit did not produce equivalent effects. This type of experiment could never be carried out without the use of the pentameric constructs.

Although this study provides interesting AChR-specific findings as well as a general approach that should be applicable to other LGICs, two types of additional experiments should be carried out before this method is elevated to panacea status. First, do other linear sequences that should produce the same arrangement of subunits in the complex (e.g., α_3→β_4→β_4→α_3→β_4) give the same results? One might expect that they would, but it would be nice to demonstrate this. If the alternate sequences do not result in the formation of functional receptors, this might provide an opening to a more detailed analysis of the contributions of particular regions of each subunit that contribute to the subunit-subunit interactions that hold the complex together. Second, do other subunit arrangements (e.g., β_4→β_4→β_4→α_3→α_3) *not* produce functional receptors? If the receptor has a unique stoichiometry and subunit arrangement, concatamers such as this should not work. If other constructs do work, it would demonstrate that there is not a unique arrangement of a given group of subunits and lays the groundwork for future studies of how subunit arrangement affects receptor function.

The construction of pentameric subunit concatamers now allows several types of studies that could not be carried out before. First, as demonstrated in this study, one can incorporate a mutation into just one subunit of the receptor to examine the role of individual subunits—even those that may be present in multiple copies in the complex. Although one might consider this a situation that would occur only in experiments designed to probe receptor structure-function relationships, this also might be observed in the case of polymorphisms in heterozygotes for a particular mutation. Application of this approach to the ligand-binding site regions [which are believed to be located at subunit-subunit interfaces (Karlin, 2004)] should allow the delineation of the role of residues on either half of a specific ligand-binding site in the receptor in the actions of agonists and antagonists. Second, and perhaps of much wider interest, one can now create receptors of predefined subunit stoichiometry and arrangement and use these not only to test which stoichiometries/arrangements produce functional receptors but also to create well defined targets for the development of novel experimental probes and therapeutic agents with receptor (sub)subtype selectivity. It is now time for the fun stuff to begin!

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