Impact of subunit positioning on GABAA receptor function

E. Sigel*, R. Baur†, N. Boulineau‡ and F. Minier‡
*Department of Biochemistry and Molecular Medicine, University of Bern, CH-3012 Bern, Switzerland, †Department of Neurology, Medical University of Ohio, Toledo, OH 43614-5809, U.S.A., and ‡Équipe INSERM AVENIR 3/IFR8, Institut François Magendie, 33000 Bordeaux, France

Abstract
The major isoforms of the GABAA (γ-aminobutyric acid type A) receptor are composed of two α, two β and one γ subunit. Thus α and β subunits occur twice in the receptor pentamer. As it is well documented that different isoforms of α and β subunits can co-exist in the same pentamer, the question is raised whether the relative position of a subunit isoform affects the functional properties of the receptor. We have used subunit concatenation to engineer receptors of well-defined subunit arrangement to study this question. Although all five subunits may be concatenated, we have focused on the combination of triple and dual subunit constructs. We review here what is known so far on receptors containing simultaneously α1, α2 and γ subunits and receptors containing β1 and β2 subunits. Subunit concatenation may not only be used to study receptors containing two different subunit isoforms, but also to introduce a point mutation into a defined position in receptors containing either two α or β subunits, or to study the receptor architecture of receptors containing unconventional GABAA receptor subunits. Similar approaches may be used to characterize other members of the pentameric ligand-gated ion channel family, including nicotinic acetylcholine receptors, glycine receptors and 5-HT3 (5-hydroxytryptamine) receptors.

Introduction
During the last few years, subunit concatenation has been applied to members of the pentameric ligand-gated ion channel family. Covalently linking two or more subunits of such a channel at the level of cDNA has the advantage of predefining receptor architecture. We focus here not only on the work done on GABAA [GABA (γ-aminobutyric acid) type A] receptors, but also mention work on the nicotinic acetylcholine receptor.

GABA receptors mediate the fast neuronal action of the major inhibitory neurotransmitter GABA. They are integral membrane proteins consisting of five subunits surrounding a central channel selective for chloride ions [1]. The major isoforms of the GABAA receptor are composed of two α, two β and one γ subunit [2–7]. Several classes of drugs, notably benzodiazepines, act at GABAA receptors [8,9]. The subunit composition of a GABAA receptor determines its pharmacological properties [10]. Receptor subunits expose both C- and N-termini on the extracellular side, enabling subunit concatenation. GABAA receptors formed from concatenated subunits (for a review, see [11]) have been used to study receptor architecture [6,7,12]. As the two α- and the two β-subunits occupy non-symmetrical positions (Figure 1), receptors formed from concatenated subunits are a means to study positional effects of point mutations [13,14] and positional effects of subunit isoforms [15,16]. Precautions that have to be taken in such an approach are discussed here, followed by examples of the application of subunit concatenation.

Precautions
Artefacts are theoretically possible in receptors formed from concatenated subunits. They include (i) proteolysis in the linker and reassembly of an affected subunit, (ii) one subunit of a dimeric construct taking part in the formation of a channel, and the second subunit sticking out, (iii) a subunit interspersing a dual subunit construct, (iv) a dimeric subunit linking two pentamers, and (v) rearrangement of a dimeric construct (reviewed in [11]). Most of these artefacts may be avoided by carefully titrating and minimizing the length of the linker sequences joining the two linked subunits and avoiding inclusion of the signal sequence of all but the N-terminal subunit of a multisubunit construct. In fact most of the artefacts described have been observed in cases where two simple rules were not respected [17,18].

A second point of concern is that almost all subunit arrangements, irrespective of their physiological relevance, result in the expression of small agonist-induced currents. In many cases, this tiny amplitude may be increased by overloading the expression system Xenopus oocyte with cRNA. These currents may be due to a small amount of proteolysed constructs or, more likely, to the described low efficiency rearrangement of a tandem construct [6]. These phenomena may explain the reports that α–β constructs in combination with free γ subunits [19,20] can result in functional GABAA receptors, in spite of the fact that the subunit arrangement of the major receptor isoform has been determined to be γ2-β2-α1-β2-α1 (viewed from the synaptic cleft) by two independent

Key words: benzodiazepine, concatenated subunit, γ-aminobutyric acid (GABA), γ-aminobutyric acid type A receptor (GABA receptor), subunit positioning, subunit specificity.
Abbreviations used: GABA, γ-aminobutyric acid; GABAA, GABA type A.
*To whom correspondence should be addressed (email erwin.sigel@mci.unibe.ch).
Structure of the major adult GABA<sub>α</sub> receptor isoform

The major adult isoform of the GABA<sub>α</sub> receptor is composed of two α<sub>1</sub>, two β<sub>1</sub>, and one γ<sub>2</sub> subunits. The subunit arrangement is shown as seen from the synaptic cleft. In space filling, the homologous amino acid residues contributing to the formation of the agonist site for GABA on the γ<sub>2</sub> subunit, Phe<sup><b>64</b></sup>, to the formation of the modulatory site for benzodiazepines on the α<sub>1</sub> subunit, Phe<sup><b>32</b></sup>, and the homologous residue in the β<sub>2</sub> subunit, Tyr<sup><b>62</b></sup>, are shown (numbering of the rat GABA<sub>α</sub> receptor). In general, if a subunit occurs twice in a pentameric receptor and the receptor is not symmetrical, the positions of these two subunits are not equivalent and may confer different functional properties. The figure is based on the homology model discussed in [24].

In contrast, the relative positioning of β<sub>1</sub> and β<sub>2</sub> subunits was found to be irrelevant for the current stimulation by two β<sub>2</sub>-selective agents, the anaesthetic etomidate and loxapine [16]. This positional insensitivity of the effect by etomidate is illustrated in Figure 3. The fact that the number of β<sub>2</sub> subunits in a receptor determines the size of the stimulatory effect argues for an additive effect of etomidate.

The expression of such receptor isoforms will also open the way for the screening of the effects of potentially useful substances on defined GABA<sub>α</sub> receptor subtypes. This principle may be applied to other members of the family of pentameric ligand-gated ion channels.

Application of subunit concatenation

Receptor architecture

Im et al. [19] first prepared a tandem construct, where the α<sub>6</sub> subunit of the GABA<sub>α</sub> receptor was linked to the β<sub>3</sub> subunit and expressed it along with individual subunits in HEK-293 cells (human embryonic kidney cells). The connection between the two subunits included the signal sequence of the β<sub>3</sub> subunit of 24 amino acid residues in length. As discussed above, the consequences of such a signal sequence in the middle of a protein are difficult to predict. Interpretation of the observations was complicated by the fact that the extent of functional expression of receptors was very low [19]. With improved linkers and the use of triple subunits, we attempted to define the architecture of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>α</sub> receptors [6,7]. A number of dual and triple subunits were combined and expressed in Xenopus oocytes using cRNA injection. Exclusively, combinations of triple and dual constructs, which resulted in the arrangement γ<sub>2</sub>β<sub>1</sub>α<sub>1</sub>β<sub>3</sub>α<sub>1</sub>, were functionally expressed with properties very similar to receptors made from non-concatenated subunits. Combinations of subunits and/or concatenated subunit constructs that would result in a different stoichiometry or in a different subunit arrangement were not functional [6,7]. Expression of different constructs was always compared using similar quantities of cRNA and was standardized to the expression of voltage-gated Na<sup>+</sup> channels to avoid any bias by volume and quality of cRNA solution injected and by individual oocytes. Final proof of the subunit arrangement γ<sub>2</sub>β<sub>1</sub>α<sub>1</sub>β<sub>3</sub>α<sub>1</sub> was provided by the expression of a pentameric construct in which all five subunits were covalently linked at the DNA level [12]. Subunit concatenation is currently being used to study positioning of non-conventional GABA<sub>α</sub> receptor subunits, such as the δ subunit (Figure 2), which in principle may replace any of the subunits. As the δ subunit has never been shown to co-exist with a γ subunit, it is generally assumed to replace it.

Figure 1 | Structure of the major adult GABA<sub>α</sub> receptor isoform

In both reports on α–β constructs [19,20], only relatively small current amplitudes were observed.

Similar reservations apply to a recent report suggesting that in the case of nicotinic acetylcholine receptors all five subunits may be concatenated to give a functional receptor upon expression in Xenopus oocytes [21]. However, even small currents could only be shown after injection of more than 100-fold the usual amount of cRNA. It is mandatory that any multisubunit constructs result in expression levels comparable with receptors expressed from non-concatenated subunits after injection of Xenopus oocytes with comparable amounts of cRNA. In the case of GABA<sub>α</sub> receptors, such concatenated five-subunit constructs may be functionally expressed in Xenopus oocytes fulfilling these criteria [12].

Positioning of α or β subunit isoforms

Two α subunit isoforms or two β subunit isoforms can co-exist in the same GABA<sub>α</sub> receptor (for a review, see [10]). It is difficult to study functional properties of such receptors unless forced assembly is being used. Thus concatenated receptors carrying two α subunit isoforms, α<sub>1</sub> and α<sub>6</sub>, in defined positions were prepared, γ<sub>2</sub>β<sub>1</sub>α<sub>1</sub>β<sub>2</sub>α<sub>6</sub>, γ<sub>2</sub>β<sub>1</sub>α<sub>6</sub>β<sub>2</sub>α<sub>1</sub>, and γ<sub>2</sub>β<sub>1</sub>α<sub>6</sub>β<sub>3</sub>α<sub>1</sub>. These receptors were expressed in Xenopus oocytes and functionally characterized. It turns out that each receptor type has distinct functional properties [15], especially with respect to benzodiazepines. If an α<sub>1</sub> subunit, but not an α<sub>6</sub> subunit, neighbours a γ<sub>2</sub> subunit, allosteric modulation by benzodiazepines is observed. This set of properties that differ for the different receptors can be used as a diagnostic tool for currents detected in cerebellar granule cells that express both the α<sub>1</sub> and α<sub>6</sub> subunits.
**Figure 2 | GABA<sub>a</sub> structural variations**

The δ subunit can either replace the γ subunit (top row), as favoured in the literature, or one of the α subunits (not shown), or one of the β subunits. Note that the δ subunit has never been detected in the same pentamer as the γ subunit.

**Figure 3 | Effect of the relative positioning of the β<sub>1</sub> and β<sub>2</sub> isoforms on the impact of etomidate on the concentration-dependence of GABA**

Open symbols: in the absence of 10 µM etomidate; closed symbols: in the presence of 10 µM etomidate. As the identity of β subunits was without significant effect on the concentration-dependence of GABA in the absence of etomidate, only one of the curves is shown. While receptors containing two β<sub>2</sub> subunits (○) showed a large shift to the left, this shift was relatively small in receptors containing two β<sub>1</sub> subunits (■). Receptors containing one copy of each β<sub>1</sub> and β<sub>2</sub> (▲ and ◇) showed an intermediate shift, irrespective of the relative positioning of the subunits.

**Defined point mutations**

Subunit concatenation allows targeted introduction of a mutation in only one defined subunit if this occurs in several copies in a receptor. This is illustrated in the following example of the two agonist sites in GABA<sub>a</sub> receptors. The sites are both located at β subunit/α subunit interfaces, suggesting similar properties. One pair of subunits is flanked by γ and β and the other by α and γ, the different environment possibly affecting the binding sites. The sites were individually mutated and functional consequences of these mutations were studied. It turned out that the two sites have subtly different properties [13]. Similarly, it was shown that occupation of both agonist sites affects channel opening in the presence of benzodiazepines [14].

Similar to the approach outlined above, disease mutations in the GABA<sub>a</sub> receptor causing autosomal dominant juvenile myoclonic epilepsy have been studied for their positional effect [22]. Interestingly, in the heteromeric situation, the point mutation A322D in the α<sub>1</sub> subunit leads to differential effects on retention in the endoplasmic reticulum depending on the subunit position.

**Incorporation of subunits**

It has earlier been observed that stimulation by diazepam of currents elicited by GABA in oocytes expressing α/β/γ = 1:1:1 is smaller and more variable than in oocytes expressing α/β/γ = 1:1:5 [23], presumably due to expression of receptors consisting only of α and β subunits. In concatenated receptors where the γ<sub>2</sub> subunit is forced to be present in the receptor pentamer, the stimulation is expected to be larger and more constant. In fact we found that while stimulation by diazepam of currents elicited by GABA in oocytes expressing α/β/γ = 1:1:5 centred at approx. 170% and was quite variable in different oocytes, a value of approx. 270% with little variation was observed, provided the γ<sub>2</sub> subunit was covalently linked to other subunits to give γ<sub>2</sub>-β<sub>2</sub>-α<sub>1</sub>/β<sub>2</sub>-α<sub>1</sub> receptors [7].

**Concluding remarks**

Subunit concatenation, if applied with sufficient care, can contribute to our knowledge of pentameric ligand-gated ion channels. Specifically, its application to receptor architecture and to the study of the specific position-related functional role of those subunits occurring in multiple copies in a receptor is difficult to replace by alternative techniques.
References

Received 16 June 2006