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A $GABA_A$ receptor of defined subunit composition and positioning: Concatenation of five subunits

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Abstract **We show that the five subunits of a γ-aminobutyric acid type A receptor ($GABA_A$ receptor) can be concatenated to yield a functional receptor. This concatenated receptor α_1–β_2–α_1–γ_2–β_2 has the advantage of a known subunit arrangement. Most of its functional properties are not significantly different from a receptor formed by individual subunits. Extent of expression amounted to about 40% of that of non-concatenated receptors in Xenopus oocytes, after injection of oocytes with comparable amounts of cRNA coding for concatenated and non-concatenated receptors. The ability to express receptors consisting of five subunits enables detailed studies of $GABA_A$ receptor subtype selective compounds.**

Keywords: GABA; $GABA_A$ receptor; Benzodiazepines

1. Introduction

The fast neuronal action of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) is mediated by γ-aminobutyric acid type A receptors ($GABA_A$ receptors). They are integral membrane proteins consisting of five subunits surrounding a central channel selective for chloride ions [1]. The major isoforms of the $GABA_A$ receptor are composed of two α, two β and one γ subunit(s) [2–6]. γβαβα, counter-clockwise when it viewed from the synaptic cleft, was identified as the correct subunit arrangement of the major adult $GABA_A$ receptor isoform $\alpha_1\beta_2\gamma_2$ [6,7]. Several classes of drugs, including benzodiazepines, act at $GABA_A$ receptors [8,9]. The subunit composition of a $GABA_A$ receptor determines its pharmacological properties [10].

$GABA_A$ receptors formed from concatenated subunits (for review, see [11]) have been used to study receptor architecture [6,7], to study positional effects of point mutations [12,13] and positional effects of subunit isoforms [14,15]. So far $GABA_A$ receptors made of dual and triple subunit constructs have been reported. Very recently, it has been shown that in the case of nicotinic acetylcholine receptors all five subunits may be concatenated to give a functional receptor upon expression in Xenopus oocytes [16]. However, currents could only be shown after injection of more than 100-fold the usual amount of cRNA. Here, we show for the case of $GABA_A$ receptors that such a concatenated five subunit construct may be functionally expressed in Xenopus oocytes after injection with comparable amounts of cRNA coding for non-concatenated and concatenated receptors. As currents mediated by concatenated and non-concatenated receptors have very similar properties, these concatenated receptors provide a drug assay system of $GABA_A$ receptors with perfectly defined subunit isoform composition and positioning.

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Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$ receptor, γ-aminobutyric acid type A receptor

2. Materials and methods

2.1. Construction of the pentameric subunit cDNA

For simplicity, we use in the following α for modified rat α_1, β for rat β_2 and γ for rat γ_2. The modified rat α subunit differs from the rat α by one amino acid residue, that confers the subunit specific bd24 antibody recognition [17,18] and corresponds to human α_1. This property has previously been used to exclude proteolysis of the linked constructs [6]. The antibody only reacts if the N-terminal of the α subunit is free. Tandem constructs with various linker lengths were made as described in [6,7]. In the following, numbers between two subunit symbols describe the length of the introduced synthetic linker. The following linkers have been introduced: γ–26–β: $Q_5A_3PAQ_3APA_3PA_2Q_5$, α–10–β and α–10–γ: Q_{10}, β–23–α: $Q_3(Q_2A_3PA)_2AQ_5$. Triple constructs were prepared from tandem constructs as exemplified for the β–23–α–10–γ (β–α–γ) construct: The β–23–α (β–α) tandem construct was cut by *Bam*HI in the α subunit and the vector behind the gene to yield a 7 kb fragment containing the sequence of the vector, the β subunit, the linker and the beginning of the α subunit. This vector fragment was dephosphorylated with shrimp alkaline phosphatase (USB) in 10 mM Tris–HCl, pH 8.0 and 100 mM $MgCl_2$ for 1 h at 37 °C. The α–10–γ (α–γ) tandem construct was cut by *Bam*HI in the α subunit and the vector behind the gene to yield a 2 kb fragment containing the sequence of the second half of the α subunit, the linker and the γ subunit. The two fragments were ligated and proper ligation was checked by restriction analysis. The construct α–10–β–23–α–10–γ (α–β–α–γ) was made accordingly from α–10–β and β–23–α–10–γ using *Hin*dIII to cut in β. The construct α–10–β–23–α–10–γ–26–β (α–β–α–γ–β) was made accordingly from α–10–β–23–α–10–γ and γ–26–β, using an *Age*I restriction site introduced by mutation into the γ subunit of both constructs. After ligation the restriction site was removed again.

2.2. Expression of concatenated subunit constructs in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX) from the linearized pCMV vectors containing the pentameric construct or the single α_1, β_2 and γ_2 subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (USB, Cleveland, OH). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Biorad) for visualization of the RNA and known concentrations of RNA ladder (Gibco-BRL) as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C. Isolation of oocytes from the frogs,

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culturing of the oocytes, injection of cRNA and defolliculation were done as described earlier [19]. Oocytes were injected with 50 nl of the cRNA solution. cRNA coding for the pentameric construct was used at 10 nM concentration. The combination of non-concatenated modified rat α_1, rat β_2 and rat γ_2 subunits was expressed at 10 nM:10 nM:50 nM. As the cRNA for concatenated receptors codes for two copies of each α_1 and β_2 subunits and one copy of γ_2 subunits, oocytes injected with this cRNA obtained twice the amount of genetic information coding for α_1 and β_2 subunits and one fifth of genetic information coding for the γ_2 subunit as compared to oocytes injected with cRNA coding for non-concatenated receptors. The injected oocytes were incubated in modified Barth's solution (10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 U/ml penicillin, 100 μg/ml streptomycin) at 18 °C.

2.3. Two-electrode voltage-clamp measurements

All measurements were done in medium containing 90 mM NaCl, 1 mM $MgCl_2$, 1 mM KCl, 1 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 at a holding potential of −80 mV. For the determination of maximal current amplitudes 1 mM GABA (Fluka, Switzerland) was applied for 20 s. GABA-evoked currents (at 8–12% of the maximal current amplitude) were inhibited by varying concentrations of bicuculline methiodide (RBI) or picrotoxin. Relative current stimulation by diazepam was determined at a GABA concentration evoking 2–5% of the maximal current amplitude in combination with varying concentrations of diazepam (DZ; Roche, Switzerland) and expressed as $((I_{(GABA+DZ)}/I_{(GABA)}) - 1) \times 100\%$. Data given in the text are expressed as mean ± S.D. Data shown in figures are given as mean ± S.E.M.

2.4. Transient transfection in HEK-293 cells

The cells were maintained in minimum essential medium (Gibco-BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin by standard cell culture techniques. Equal amounts (total of 3 μg DNA/35 mm dish) of plasmids coding for $GABA_A$ receptor subunits or 1 μg/35 mm dish of plasmids coding for a double and a triple construct, or for the pentamer, were co-transfected with 1 μg/35 mm dish of plasmid coding for the GFP into human embryonic kidney 293 cells (ATCC no. CRL 1573) by the calcium phosphate precipitation method [20]. After overnight incubation, the cells were washed twice with serum free medium and re-fed with medium.

2.5. Patch-clamp experiments

For whole-cell patch-clamp recordings, electrodes were pulled from thick-walled borosilicate glass capillaries (GC150F, Harvard apparatus, Edenbridge, UK) with DMZ-universal puller (Zeitz-Instrumente, Augsburg, Germany), fired polished and coated with Sylgard. They were filled with an internal solution containing 151 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES (pH 7.4) and had resistances of 3–5 MΩ. The extracellular solution contained 141 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM KCl, 10 mM glucose and 10 mM HEPES (pH 7.4). The chloride equilibrium potential is near 0 mV. Currents were filtered with a 2.9 kHz Bessel filter and recorded using an EPC-10 amplifier interfaced with a G4 Macintosh computer. The sampling rate was 10 kHz. Data were recorded in parallel on a *x–y* recorder. Voltage commands and current measurements were performed using Patchmaster software (HEKA, Lambrecht/Pfalz, Germany). Cells were voltage-clamped at −80 mV and the solutions containing GABA were applied using a rapid perfusion system consisting of pulled double-barrel glass flow pipes connected to a Warner Perfusion Fast-step (Warner Instrument, Hamden, USA). 10–90% rise-time to peak for currents elicited by GABA was about 20 ms. Digital data were lost and redrawn from the *x–y* recorder traces.

3. Results

3.1. Functional expression in Xenopus oocytes

Robust functional expression of the construct was achieved in Xenopus oocytes. 24 h after microinjection with cRNA coding for the pentameric α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptor functional expression amounted to about 1.5 μA and 48 h after injection to about 3 μA. This latter value should be compared with about 5–9 μA obtained with the corresponding receptor composed of non-concatenated subunits. Functional properties of concatenated receptors were very similar to those of non-concatenated receptors. Cumulative concentration response curves with the agonist GABA indicated that subunit concatenation had little effect on agonist parameters with an EC_{50} of 73 ± 22 μM ($n = 4$) and a Hill coefficient of 1.4 ± 0.1 as compared with non-concatenated subunits with an EC_{50} of 58 ± 24 μM ($n = 7$) and a Hill coefficient of 1.2 ± 0.1 (Fig. 1). 1 mM pentobarbital elicited by itself currents that amounted to 27 ± 2% ($n = 3$) of the maximal current amplitudes elicited by GABA in the same oocytes. This is slightly lower than the corresponding value of 40 ± 3% ($n = 3$) in non-concatenated receptors. Concentration dependent stimulation by diazepam of currents elicited by GABA was also studied (Fig. 2). Diazepam stimulated non-concatenated and concatenated receptors with a similar EC_{50} amounting to 113 ± 18 nM ($n = 3$) and 81 ± 12 nM ($n = 3$), respectively. Maximal stimulation amounted to 200 ± 25% ($n = 3$) and 247 ± 21% ($n = 4$), respectively. This difference did not reach significance levels in this experiment, but a difference was also previously noted in receptors made of a dual and a triple subunit construct [7]. This may be due to a contamination with receptors not containing the γ_2 subunit in the case of non-concatenated receptors [21]. A detailed comparison of the stimulation by diazepam of non-concatenated and concatenated receptors justifying these conclusions has been presented elsewhere [7]. Concentration dependent inhibition of currents elicited by GABA at about EC_{10} were determined for bicuculline and picrotoxin. The IC_{50} for bicuculline was 1.5 ± 0.3 μM ($n = 4$) for concatenated and 1.2 ± 0.2 μM ($n = 4$) for non-concatenated receptors (Fig. 3). The IC_{50} for picrotoxin was 1.9 ± 0.3 μM ($n = 4$) for concatenated and 2.6 ± 0.8 μM ($n = 3$) for non-concatenated receptors (Fig. 4). The reversal potential of the current elicited by GABA amounted to

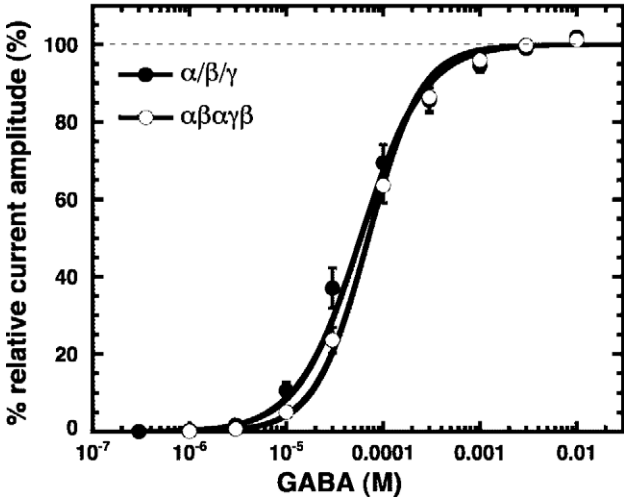

Fig. 1. Non-concatenated $\alpha_1/\beta_2/\gamma_2$ or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were expressed in Xenopus oocytes. Increasing concentrations of GABA were applied to the oocytes and the corresponding current amplitudes determined. Seven individual concentration response curves were averaged for non-concatenated receptors and four for concatenated receptors. Data are expressed as mean ± S.E.M.

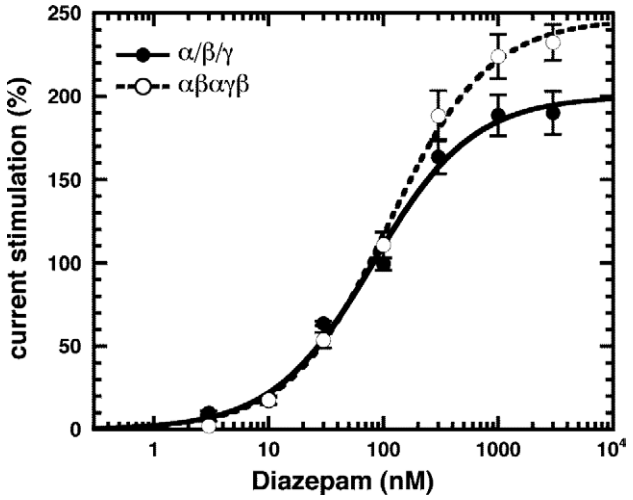

Fig. 2. Non-concatenated $\alpha_1/\beta_2/\gamma_2$ or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were expressed in Xenopus oocytes. Increasing concentrations of diazepam in combination with 5 μM GABA were applied to the oocytes and the corresponding current amplitudes determined. Three individual concentration inhibition curves were averaged for each, non-concatenated receptors and for concatenated receptors. Data are expressed as mean ± S.E.M.

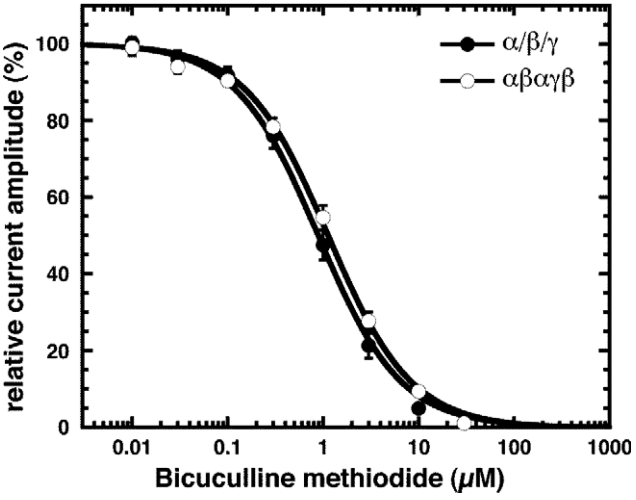

Fig. 3. Non-concatenated $\alpha_1/\beta_2/\gamma_2$ or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were expressed in Xenopus oocytes. Increasing concentrations of bicuculline in combination with 20 μM GABA were applied to the oocytes and the corresponding current amplitudes determined. Four individual concentration inhibition curves were averaged for each, non-concatenated receptors and for concatenated receptors. Data are expressed as mean ± S.E.M.

−29 ± 4 mV. This value is very similar as observed for non-concatenated receptors [22]. While so far all the investigated parameters were very similar in concatenated and non-concatenated receptors, some differences were observed in the desensitization properties. Fig. 5 illustrates the time course of the current elicited by 10 mM GABA in non-concatenated and concatenated receptors. The time course of the decay of the current elicited by 10 mM GABA in concatenated receptors could be fitted with two exponentials. A first component amounting to 42.5 ± 7.3% was fitted with $\tau = 7.0 \pm 1.0$ s and a second component amounting to 57.5 ± 7.3% was fitted with $\tau = 30.9 \pm 3.4$ s. The residual non-desensitizing current

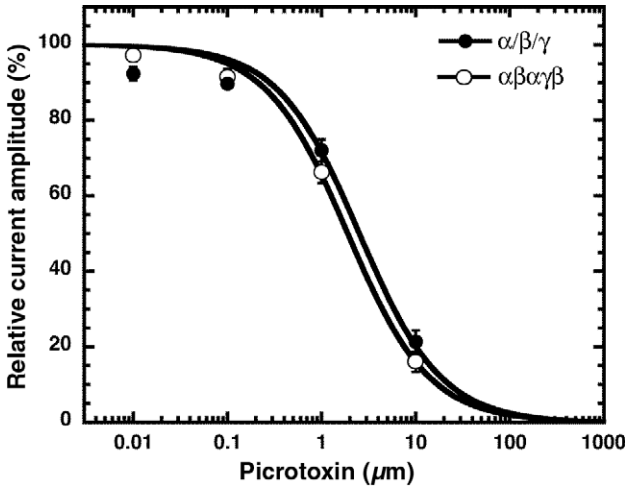

Fig. 4. Non-concatenated $\alpha_1/\beta_2/\gamma_2$ or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were expressed in Xenopus oocytes. Increasing concentrations of picrotoxin in combination with 10 μM GABA were applied to the oocytes and the corresponding current amplitudes determined. Four individual concentration inhibition curves were averaged for each, non-concatenated receptors and for concatenated receptors. Data are expressed as mean ± S.E.M.

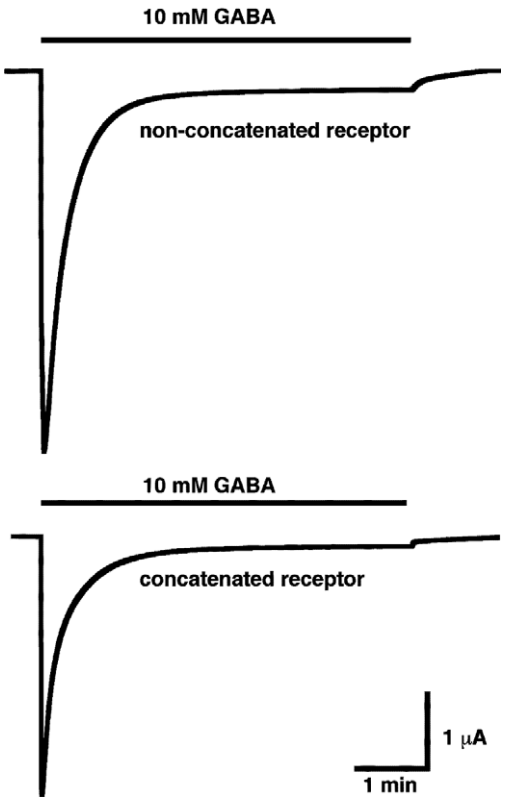

Fig. 5. Non-concatenated $\alpha_1/\beta_2/\gamma_2$ or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were expressed in Xenopus oocytes. The time course of current desensitization was determined by applying 10 mM GABA during a time period of 5 min.

amounted to 4.1 ± 0.7% of I_{max}. This value was similar for non-concatenated receptors with 5.0 ± 2.0% of I_{max}. However, in these receptors decay of the current followed a mono-exponential time course characterized by $\tau = 22.1 \pm 4.1$ s.

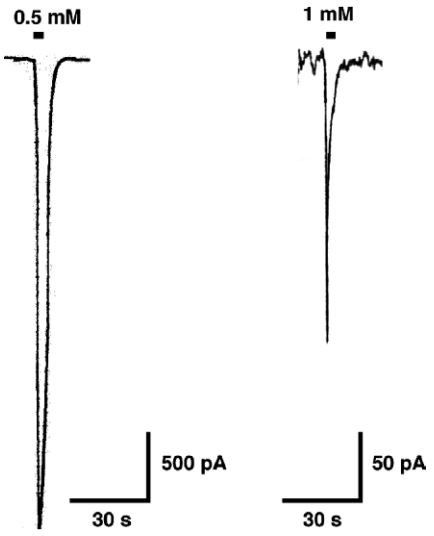

Fig. 6. Concatenated β_2–α_1–γ_2/β_2–α_1 or concatenated α_1–β_2–α_1–γ_2–β_2 $GABA_A$ receptors were transiently expressed in HEK-293 cells. Brief pulses of saturating concentrations of GABA were applied to the cells. Currents were measured with the whole-cell patch-clamp technique as described under Section 2.

3.2. Expression in HEK-293 cells

Whole-cell patch-clamp experiments were performed 48 h after transfection. For the non-concatenated subunits α_1, β_2 and γ_2, the maximal current amplitude elicited by GABA was typically 5–10 nA. For receptors consisting of dual and triple concatenated subunits γ_2–β_2–α_1/β_2–α_1 reduced peak current amplitudes were observed, amounting to 0.26 ± 0.17 nA ($n = 6$) at 10 μM GABA and 2.2 ± 0.9 nA ($n = 6$) at 500 μM GABA. Similar expression levels and a detailed kinetic characterization of these receptors expressed in HEK-293 cells have been described by Gallagher et al. [23]. An example of a current trace recorded after application of 500 μM GABA is shown in Fig. 6. Pentameric α_1–β_2–α_1–γ_2–$\beta_2$$GABA_A$ receptors resulted in a further reduction of currents. Functional expression amounted to 0.12 ± 0.05 nA ($n = 6$) as determined at 1 mM GABA (Fig. 6).

4. Discussion

We have previously shown [6,7,14,15] that $GABA_A$ receptors consisting of dual and triple concatenated subunits result in receptors with similar properties as receptors composed of non-concatenated subunits. Here, we extend this approach to concatenated receptors consisting of a single entity composed of five subunits. Most of the studied properties, GABA concentration response curve, sensitivity to inhibition by bicuculline and picrotoxin and reversal potential, were similar to those of wild type receptors. One exception is the sensitivity to stimulation by diazepam, that is higher, though not significantly, in concatenated receptors. A likely reason has been discussed in the result section. A second exception is the desensitization properties that show small but significant alteration. It might be the case that one or several of the linkers affects the rate of channel opening or closing.

A major difference concerns the relative current amplitude in comparison to non-concatenated receptors. In Xenopus oocytes, concatenated receptors produce about 40% of the current amplitude detected upon expression of non-concatenated receptors, after injection with comparable amounts of cRNA coding for concatenated receptors as used for non-concatenated α and β subunits (0.5 fmol/oocyte). In principle this reduced amplitude could be due to a decreased single channel amplitude, to an altered rate of channel opening or closing or due to an altered efficiency of expression. We consider it unlikely that the single channel amplitude is affected. Altered kinetic properties are also unlikely to be the cause as the weighted desensitization properties are very similar. Most probably either the efficiency of translation, trafficking or surface insertion is reduced in concatenated receptors. This slight reduction of expression should be compared to the 4–10-fold reduction after injection with the 100-fold higher amount of cRNA (about 40 fmol/oocyte) coding for concatenated receptors compared to non-concatenated α and β subunits (about 0.4 fmol/oocyte) in the case of pentameric concatenated nicotinic acetylcholine receptors as recently reported [16]. In HEK-293 cells current amplitudes upon transfection of the concatenated $GABA_A$ receptor are reduced to larger extent. For discussion we assume here that the same considerations as in the case of oocytes apply and that this reduction is due to reduced efficiency of expression. Expression level of a concatenated receptor consisting of a dual and a triple subunit construct amounts to about 30% of the receptor composed of non-concatenated subunits. Concatenation to a pentameric construct further reduces this number to about 2%. The reason for this reduction that seems to be cell specific is not clear.

Evidence has been provided that upon expression of three subunits a mixture of receptors consisting of three or two subunits are formed [21]. Also, positional effects of subunit placement in the receptor pentamer have been observed [14]. Using concatenated receptors composed of dual and triple subunit constructs can deal with these complications, but a small residual uncertainty due to theoretically possible rearrangement of dual subunit constructs [6] remains. Whether or not this small uncertainty justifies the extra work linking dual and triple subunit constructs should be left open. It is in any case interesting that a pentameric construct is functional. The fact that all five subunits of a $GABA_A$ receptor may be concatenated will allow detailed studies of $GABA_A$ receptor subtype selective compounds.

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