Unanticipated Structural and Functional Properties of δ-Subunit-containing GABA_A Receptors

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GABA_A receptors mediate inhibitory neurotransmission in the mammalian brain via synaptic and extrasynaptic receptors. The delta (δ)-subunit-containing receptors are expressed exclusively extra-synaptically and mediate tonic inhibition. In the present study, we were interested in determining the architecture of receptors containing the δ-subunit. To investigate this, we predefined the subunit arrangement by concatenation. We prepared five dual and three triple concatenated subunit constructs. These concatenated dual and triple constructs were used to predefine nine different GABA_A receptor pentamers. These pentamers composed of α_1-, β_3-, and δ-subunits were expressed in Xenopus oocytes and maximal currents elicited in response to 1 mM GABA were determined in the presence and absence of THDOC (3α, 21-dihydroxy-5α-pregnane-20-one). β_3-α_1-δ/α_1-β_3, and β_3-α_1-α_1/β_3-α_1 resulted in the expression of large currents in response to GABA. Interestingly, the presence of the neurosteroid THDOC uncovered α_1-β_3-α_1/β_3-δ receptors, additionally. The functional receptors were characterized in detail using the agonist GABA, THDOC, Zn^{2+}, and ethanol and their properties were compared with those of non-concatenated α_1β_3 and α_1β_3δ receptors. Each concatenated receptor isoform displayed a specific set of properties, but none of them responded to 30 mM ethanol. We conclude from the investigated receptors that δ can assume multiple positions in the receptor pentamer. The GABA dose-response properties of α_1-β_3-α_1/β_3-δ and β_3-α_1-δ/α_1-β_3 match most closely the properties of non-concatenated α_1β_3δ receptors. Furthermore, we show that the δ-subunit can contribute to the formation of an agonist site in α_1-β_3-α_1/β_3-δ receptors.

γ-Aminobutyric acid type A receptors (GABA_A receptors) mediate fast synaptic inhibitory neurotransmission in the mammalian brain. They belong to the family of ligand-gated ion channels that includes nicotinic acetylcholine, glycine, and serotonin type-3 receptors. GABA_A receptors are composed of pentameric combinations of α (1–6), β (1–4), γ (1–3), δ, ε, θ, π subunit subtypes (1–4). The five subunits are arranged pseudo-symmetrically around a central Cl^− selective channel (1). Subunit composition confers specific physiological and pharmacological properties to GABA_A receptors (5, 6). The heterogeneity of GABA_A receptors potentially provides different targets for receptor subtype-selective drugs to improve the treatment of insomnia, anxiety, and epilepsy.

Synaptic receptors mediate phasic inhibition whereas extrasynaptic receptors mediate tonic inhibition (7–9). δ-subunit-containing GABA_A receptors occur exclusively extra-synaptically and have been shown to mediate tonic inhibition in dentate gyrus granule cells (10–13), cerebellar granule cells (11), thalamic neurons (14, 15), and in pyramidal neurons (16). The δ-subunit normally shows partnership with either α_3- or α_6-subsunits (17, 18). Recently, α_δ-subunit assemblies have been shown to be present in the hippocampal interneurons (19). GABA_A receptors containing the δ-subunit are activated by persistent and usually nonsaturating ambient GABA concentrations (0.5–1.0 μM) (20).

The homology of the δ-subunit to the β_3- and γ_2-subsunits is 50 and 44%, respectively. Studies in various regions of rat brain suggest that δ- and γ_2-subsunits do not coexist in the same receptor (21–23). Therefore, δ is generally considered as a subunit of the γ_2-subunit, although one study performed with rat brain extracts suggested that δ- and γ_2-subsunits co-assemble to produce a receptor with novel pharmacology (24). αβδ forms functional receptors upon expression in heterologous expression systems (25–39). Several studies have shown that the δ-subunit is part of the GABA_A receptors that exhibit a unique pharmacology. Such receptor isoforms are Zn^{2+}-sensitive (25, 26, 29, 36) and benzodiazepine-insensitive (25). It has also been demonstrated that extrasynaptic δ-subunit-containing GABA_A receptors are particularly sensitive to modulation by neurosteroids (30–32, 37–39). There is contradictory evidence on the effect of physiological concentrations of ethanol on δ-subunit-containing GABA_A receptors (32, 34, 35, 38).

In the absence of any method able to determine membrane protein architecture in situ in the nervous system, model systems have to be used. Recently, αβδ has been proposed to be the predominant subunit arrangement around the pore when viewed from the extracellular space for α_4β_3δ GABA_A receptors expressed in tsA 201 cells (40). This work was performed at the structural level. In the present study, we have focused on the architecture of α_4β_3δ GABA_A receptors expressed in Xenopus oocytes at the functional level. To investigate active channels, we used covalently linked α_4, β_3, and δ subunits to have a defined arrangement of different subunits in a pentamer (41–46). The concatenated receptors were characterized in detail using the agonist GABA, the neurosteroid THDOC, Zn^{2+}, and ethanol, and their properties were compared with those of non-
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concatenated receptors. We provide evidence for the fact that: 
(a) the $\delta$-subunit has a promiscuous role in receptor assembly, 
(b) the $\delta$-subunit can form part of an agonist site, (c) neurosteroids uncover a receptor isoform, and (d) none of the functional receptors is modulated by ethanol.

EXPERIMENTAL PROCEDURES

Construction of Concatenated $\delta$-Subunit-containing cDNAs—
The approach used for subunit concatenation of GABA$_A$ receptors has been described previously (41–45). The procedure is detailed here for the dual subunit construct $\alpha_1\cdot\delta$ and the triple subunit construct $\beta_3\cdot\alpha_1\cdot\delta$ and $\delta\cdot\alpha_1\cdot\delta$. The number between two subunits describes the number of amino acid residues of the introduced synthetic linker. To prepare $\delta$-subunit, the $\alpha_1$-subunit was dephosphorylated as above. The forward primer for $\alpha_1$ was complementary to the vector sequence preceding the $\alpha_1$-subunit. The reverse primer was complementary to a PinA1 site followed by the first half of the linker and to the last nucleotides of $\alpha_1$ before the stop codon. The forward primer for the $\delta$-subunit was complementary to PinA1 site followed by the first half of the linker and to the last nucleotides of $\alpha_1$ before the stop codon. The forward primer for the $\delta$-subunit was complementary to PinA1 site followed by the first half of the linker and to the last nucleotides of $\alpha_1$ before the stop codon. The forward primer for the $\delta$-subunit was complementary to PinA1 site followed by the first half of the linker and to the last nucleotides of $\alpha_1$ before the stop codon. The forward primer for the $\delta$-subunit was complementary to PinA1 site followed by the first half of the linker and to the last nucleotides of $\alpha_1$ before the stop codon.

**Expression in Xenopus Oocytes**—Capped cRNAs were synthesized (Ambion, Austin, TX) from the linearized vectors containing different non-concatenated and concatenated subunits. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (USB, Cleveland, Ohio). The concentration of the cRNA was quantified on a formaldehyde-agarose gel using Radiant Red stain (Bio-Rad) for visualization of the RNA with known concentrations of RNA ladder (GIBCO BRL) as standard on the same gel. The cRNAs were dissolved in water and stored at $-80^\circ$C. Isolation of oocytes from the frogs, culturing of the oocytes, injection of cRNA, and defolliculation were done as described earlier (47). cRNA coding for each dual and triple subunit concatenated was injected either alone or in different combinations in oocytes resulting in a total of seven different concatenated receptors. Oocytes were injected with 50 nl of RNA solution containing each dual or triple subunit construct at 50 nM and pentameric constructs at 100 nM, unless indicated otherwise in Fig. 1. Combinations of $\alpha_1\cdot\delta$, $\beta_3\cdot\delta$, and $\delta\cdot\alpha_1\cdot\delta$ were expressed at a ratio of 10:10:10 nM or 10:10:50 nM. If the $\gamma_2$-subunit is used in place of $\delta$, the latter ratio is required (48). Potentiation of currents by THDOC was similar, but current amplitudes were rather small in the former case (data not shown). Therefore, we used the second condition for detailed characterization. The injected oocytes were incubated in modified Barth’s solution (47) at 18°C for about 72 h for the determination of $I_{\text{max}}$ and for at least 24 h before the measurements for detailed characterization of the functional receptors.

**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) was applied in the absence and presence of 1 $\mu$m THDOC (Sigma) for 20 s. THDOC was prepared as a 10 mm stock solution in dimethyl sulfoxide (DMSO) and was dissolved in external solution resulting in a maximal final DMSO concentration of 0.5%. The perfusion solution (6 ml/min) was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte (5). Non-concatenated and concatenated receptors containing the $\delta$-subunit showed a pronounced decrease in response to GABA with time. This decrease amounted to about 30–70% and did not recover. The experiments were performed after the measured currents became constant. Concentration response curves for GABA were fitted with the equation $I(c) = I(\infty)/[1 + (EC_{50}/c)^n]$, where $c$ is the concentration of GABA, $EC_{50}$ the concentration of GABA eliciting half maximal current amplitude, $I_{\text{max}}$ the maximal current amplitude, $I$ the current amplitude, and $n$ the Hill coefficient.

Relative current potentiation by THDOC was determined as $(I_{\text{max}} / I(100%\text{THDOC} + 1 \text{mm GABA}/I_{\text{max GABA}} - 1)) \times 100$%. Inhibition curves for Zn$^{2+}$ were fitted with the equation $I(c) = I(0)/[1 + (IC_{50}/c)^n]$, where $I(0)$ is the control current in the absence of Zn$^{2+}$ normalized to 100%, $I$ is the relative current amplitude, $c$ is the concentration of Zn$^{2+}$, $IC_{50}$ the concentration of Zn$^{2+}$ causing 50% inhibition of the current, and $n$ the Hill coefficient. Zn$^{2+}$ was pre-applied for a minimum of 1 min prior to co-application of GABA with Zn$^{2+}$. Potentiation by ethanol was determined at $EC_{50}$ for GABA, using 30 mm ethanol. Relative current potentiation by ethanol was determined as $(I_{\text{max}} / I(100%\text{ethanol} + GABA EC_{20}/GABA IC_{20} - 1)) \times 100$%.

Data are given as mean $\pm$ S.E. for the $I_{\text{max}}$ values for GABA with and without THDOC and as mean $\pm$ S.D. for analysis of properties of receptors using GABA, Zn$^{2+}$, and ethanol. The perfusion system was cleaned between two experiments by
washing with 100% dimethyl sulfoxide (DMSO) after application of THDOC and with 10 mM HCl for Zn2+ experiments to avoid contamination.

RESULTS

Preparation of Concatenated δ-Subunit-containing GABA\textsubscript{A} Receptors—We used the subunit concatenation approach to determine the architecture of δ-subunit-containing GABA\textsubscript{A} receptors. We assumed that the δ-subunit would either occupy the position of the γ\textsubscript{2} subunit, one of the two α subunits, or one of the two β subunits in the major isoform of GABA\textsubscript{A} receptors that is arranged γ\textsubscript{2}β\textsubscript{1}α\textsubscript{1}β\textsubscript{1}α\textsubscript{1} counter-clockwise when viewed from the synaptic cleft (41, 42). We also analyzed receptors containing two δ subunits in the same receptor with δ at γ position and one of the β positions. Five dual and three triple concatenated constructs were prepared to force the δ-subunit into defined positions to form nine different GABA\textsubscript{A} receptor pentamers (Fig. 1). For the design of the links, we applied the rule that the sum of the predicted C-terminal protrusion of a preceding subunit and the artificial linker has to be minimally 23 residues in length. Shorter linkers do not result in receptor expression (41, 46).

Functional Expression of δ-Subunit-containing GABA\textsubscript{A} Receptors—Concatenated receptors R1-R9 (Fig. 1) were expressed in Xenopus oocytes. The non-concatenated subunit combinations α\textsubscript{1}δ, β\textsubscript{1}δ, α\textsubscript{1}β\textsubscript{2}δ, α\textsubscript{1}β\textsubscript{3}δ and concatenated dual and triple subunit constructs were used as a control. GABA has been shown to be a partial agonist for δ-subunit-containing receptors (31, 32), and the maximal current evoked by GABA could be enhanced by a neurosteroid. Here we estimated receptor expression in the presence of the neurosteroid THDOC. Currents were determined at saturating concentration of GABA (1 mM) in the absence and presence of 1 μM THDOC (Fig. 1). The non-concatenated α\textsubscript{1}δ and β\textsubscript{1}δ receptors resulted in currents < 10 nA in either case. Both, α\textsubscript{1}δ- and β\textsubscript{1}δ-subunits were required to obtain robust expression of δ-subunit-containing receptors (Fig. 1). Further, non-concatenated α\textsubscript{1}β\textsubscript{3} receptors were expressed to compare their properties with those of α\textsubscript{1}β\textsubscript{3}δ to ensure that δ-subunit was being expressed in the latter receptors. α\textsubscript{1}β\textsubscript{3} and α\textsubscript{1}β\textsubscript{3}δ displayed a different sensitivity toward THDOC. Current potentiation was 6- and 17-fold, respectively. This difference, together with the different sensitivity to Zn\textsuperscript{2+} (see below), confirms that δ-subunit was indeed being incorporated into α\textsubscript{1}β\textsubscript{3}δ receptors, although we cannot completely rule out that a subpopulation of α\textsubscript{1}β\textsubscript{2}δ receptors is expressed along with α\textsubscript{1}β\textsubscript{3}δ. To our surprise and for reasons we can only speculate (see “Discussion”) the concatenated β\textsubscript{3}α\textsubscript{1} construct in the absence and presence of THDOC and the β\textsubscript{3}α\textsubscript{1}δ construct in the presence of THDOC, themselves, resulted in substantial current expression, unlike all the other concatenated subunits (Fig. 1). For the β\textsubscript{3}α\textsubscript{1} construct this functional expression was analyzed further. When 50 nM construct were injected, the current was about 500 nA. Expression of 25 nM of β\textsubscript{3}α\textsubscript{1} resulted in about 30% of this current, and 10 nM gave currents less than 7% of the currents observed using 50 nM of cRNA (Fig. 1). So clearly this artifactual signal was only prominent at higher cRNA concentrations used. Unfortunately, high concentrations of cRNA were required for the δ-subunit-containing receptors to achieve significant expression. To exclude ambiguities in the interpretation of the properties observed for the receptors β\textsubscript{3}α\textsubscript{1}δ/α\textsubscript{1}β\textsubscript{3} (R1) and β\textsubscript{2}α\textsubscript{1}δ/β\textsubscript{3}α\textsubscript{1} (R5), we prepared the β\textsubscript{3}α\textsubscript{1}δ/α\textsubscript{1}β\textsubscript{3} (P1) and β\textsubscript{2}α\textsubscript{1}δ/β\textsubscript{3}α\textsubscript{1} (P5) pentamers. Notably, receptors containing the δ-subunit in different positions resulted in the current expression. The concatenated receptors with the subunit arrangement β\textsubscript{2}α\textsubscript{1}δ/α\textsubscript{1}β\textsubscript{3} (R1), β\textsubscript{2}α\textsubscript{1}δ/α\textsubscript{1}β\textsubscript{3} (P1), α\textsubscript{1}β\textsubscript{3}δ/β\textsubscript{3}α\textsubscript{1} (R2), β\textsubscript{2}α\textsubscript{1}δ/δ/β\textsubscript{3}α\textsubscript{1} (R5), pentamer β\textsubscript{2}α\textsubscript{1}δ/β\textsubscript{3}α\textsubscript{1} (P5), and β\textsubscript{2}α\textsubscript{1}δ/β\textsubscript{3}α\textsubscript{1} (R7) resulted in currents > 240 nA, whereas α\textsubscript{1}β\textsubscript{3}δ/α\textsubscript{1}δ/β\textsubscript{3} (R3), α\textsubscript{1}β\textsubscript{3}δ/α\textsubscript{1}δ/β\textsubscript{3} (R4), β\textsubscript{2}α\textsubscript{1}δ/β\textsubscript{3}α\textsubscript{1} (R6), β\textsubscript{2}α\textsubscript{1}δ/α\textsubscript{1}δ/β\textsubscript{3} (R8) and β\textsubscript{2}α\textsubscript{1}δ/α\textsubscript{1}δ/β\textsubscript{3} (R9) resulted in currents < 45 nA on co-application of GABA and THDOC. None of the functional receptors was directly activated by 1 μM THDOC alone (data not shown). With the exception of β\textsubscript{3}α\textsubscript{1}-

![FIGURE 1. Structure and functional expression in Xenopus oocytes of the GABA\textsubscript{A} receptors investigated. The code for the subunits is given on the top line of the table (read subunit sequence of concatenated receptors anti-clockwise). The figure shows subunit composition and current amplitude (nA) evoked by 1 mM GABA in the absence and presence of 1 μM THDOC of non-concatenated and concatenated receptors 3 days after injection with RNA. Mean values with S.E. for each subunit combination are shown. n, number of oocytes.](https://example.com/fig1.png)
\( \delta/\alpha_1/\beta_3 \) (R1), THDOC significantly potentiated the maximal current amplitudes elicited by GABA. \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) (R2) is especially remarkable in this respect as its expression was not strongly evident with GABA alone and was only uncovered in the presence of THDOC. Potentiation of maximal currents by THDOC amounted to about 22-fold in this case.

It was interesting to see if it was possible to place \( \delta \) in one of the \( \alpha \) positions. While \( \beta_3/\delta/\alpha_1/\beta_3 \) (R6) did not result in current expression, \( \beta_3/\delta/\beta_3/\alpha_1 \) (R7) resulted in currents of slightly larger amplitude as \( \beta_3/\alpha_1 \). The current showed likewise relatively little stimulation by THDOC. The EC50 was determined as 40 ± 18 mM and the Hill coefficient as 0.8 ± 0.1 (n = 5). Again these parameters are reminiscent of the current mediated by \( \beta_3/\alpha_1 \). Therefore, we assume that R7 is probably not formed, but its existence cannot be fully excluded.

**Pharmacological Properties of \( \delta \)-Subunit-containing GABA \( \alpha \) Receptors**—First, the functional receptors were characterized for their response to the natural agonist GABA. Current traces obtained with increasing concentrations of GABA in oocytes expressing \( \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \) (R1) are shown in Fig. 2A. Averaged GABA concentration-response curves for the concatenated \( \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \) (R1), \( \beta_3/\alpha_1/\delta/\beta_3/\alpha_1 \) (R5), pentamer \( \beta_3/\alpha_1/\delta/\beta_3/\alpha_3 \) (P5), and non-concatenated \( \alpha_1/\beta_3/\delta \) and \( \alpha_1/\beta_3 \) receptors are illustrated in Fig. 2B. The comparison between concatenated and non-concatenated receptors reveals that the corresponding EC50 for concatenated \( \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \) (R1) receptors is similar to non-concatenated \( \alpha_1/\beta_3/\delta \) receptors, while there was a 13-fold shift to the right for the concatenated pentamer \( \beta_3/\alpha_1/\delta/\beta_3/\alpha_1 \) (P5), with \( \delta \) at the \( \gamma \) position. A similar shift was observed for \( \beta_3/\alpha_1/\delta/\beta_3/\alpha_1 \) (R5). The GABA dose-response properties of all studied receptors are summarized in Fig. 3.

The currents elicited by 1 mM GABA in \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) (R2) receptors were not large enough to determine the EC50 of this receptor. THDOC potentiates the maximal GABA \( \alpha \) receptor currents without changing the GABA EC50 significantly (30). Thus, for \( \alpha_1/\beta_3/\delta \) an EC50 of 5.8 mM has been reported in the absence of THDOC and 6.6 mM in its presence (30). Therefore, we investigated \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) (R2) receptors in the presence of 1 mM THDOC with increasing concentrations of GABA. Current traces are shown in Fig. 4A, and averaged results are illustrated in Fig. 4B. The EC50 obtained for \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) receptors was 8.3 ± 2.5 mM comparable to the EC50 of non-concatenated \( \alpha_1/\beta_3/\delta \) receptors, and the Hill coefficient was 1.30 ± 0.13 (n = 4). Fig. 4C shows that already submicromolar concentrations of THDOC strongly stimulate \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) (R2) receptors in the presence of 1 mM GABA.

The Hill coefficient >1 of \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) (R2) receptors hinted at the presence of more than one binding site for the agonist GABA in this receptor. As the agonist site is usually located at the \( \beta/\alpha \) interface, this suggests that the \( \delta \)-subunit contributes part of an agonist site. A point mutation Y205S in the homologous \( \beta_3 \)-subunit has been shown to strongly enhance GABA agonist properties of the affected site (43, 49). We introduced the point mutation Y205S into one of the two \( \beta_3 \)-subunits individually i.e. \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \) and \( \alpha_1/\beta_3/\alpha_1/\beta_3/\delta \).

\[ \text{FIGURE 2. GABA concentration dependence.} \]

\[ A, \text{current traces from a GABA concentration response curve obtained from a Xenopus oocyte expressing } \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \text{ receptors. Bars indicate the time period of GABA perfusion. GABA concentrations are indicated above the bars.} \]

\[ B, \text{averaged GABA concentration response curves of } \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \text{ (R1), } \beta_3/\alpha_1/\delta/\beta_3/\alpha_1 \text{ (R5), pentamer } \beta_3/\alpha_1/\delta/\beta_3/\alpha_1 \text{ (P5), } \alpha_1/\beta_3/\delta \text{ and } \alpha_1/\beta_3 \text{ receptors. Because of overlap, the curve for } \beta_3/\alpha_1/\delta/\alpha_1/\beta_3 \text{ (P1) is omitted. Individual curves were first normalized to the observed maximal current amplitude and subsequently averaged. Mean } \pm \text{ S.D. of experiments carried out with } 3-4 \text{ oocytes from two batches for each subunit combination are shown.} \]

\[ \text{FIGURE 3. Summary of the data from GABA concentration response curves and } Zn^{2+} \text{ concentration inhibition curves. } n, \text{ number of oocytes (from two different batches). A receptor was analyzed in the presence of } 1 \mu \text{M THDOC.} \]

3 E. Sigel, unpublished observations.
were normalized to wild-type receptor current expression. The curves for wild-type and mutated receptors were fitted as has been described previously (43). In each case, currents observed at lower GABA concentrations were drastically reduced as expected for receptors that only gate efficiently upon occupation of both agonist sites (43).

Most of the receptors had a Hill coefficient ≤ 1. This cannot be taken as proof for the absence of a second agonist site in these receptors, because depending on the gating mechanism, the Hill coefficient may underestimate this number.

The concatenated \( \beta_3\alpha_1\delta/\alpha_1\beta_3\delta \) (R1), \( \beta_1\beta_3\alpha_1\beta_3\delta \) (R2), \( \beta_3\alpha_2\delta/\beta_2\alpha_3\delta \) (R5), \( \beta_2\alpha_3\delta/\beta_3\alpha_2\delta \) (P5), \( \alpha_1\beta_3\delta \) and \( \alpha_1\beta_3 \) receptors. Experiments with R2 were carried out in the presence of 1 \( \mu M \) THDOC. Data obtained for inhibition by Zn\(^{2+} \) were standardized to the current amplitude elicited by GABA alone in the same oocyte and subsequently averaged from different experiments. Inhibition by Zn\(^{2+} \) was determined at EC\(_{50} \) GABA. Mean ± S.D. of 3–4 oocytes from two batches for each subunit combination are shown.

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Diazepam is a positive allosteric modulator that acts on receptors containing the \( \gamma_2 \)-subunit. As expected \( \beta_3\alpha_1\delta/\alpha_1\beta_3 \) (R1) and \( \beta_2\alpha_3\delta/\beta_2\alpha_3\delta \) (R5) receptors lacking \( \gamma_2 \)-subunit were insensitive to diazepam (data not shown).

Ethanol has been reported to stimulate currents elicited by \( \alpha_1\beta_3\delta \) receptors (34). We tested the effect of 30 mM ethanol on non-concatenated \( \alpha_1\beta_3\delta \) receptors and concatenated \( \beta_3\alpha_2\delta/\beta_2\alpha_3\delta \) (R1), \( \beta_2\alpha_3\delta/\beta_2\alpha_3\delta \) (R2), \( \beta_2\alpha_3\delta/\beta_2\alpha_3\delta \) (R5), and \( \beta_3\alpha_2\delta/\beta_2\alpha_3\delta \) (P5) receptors. Results are summarized in Fig. 6. We
**DISCUSSION**

We investigated the architectural role of the δ-subunit in GABA<sub>δ</sub> receptor pentamers. Preliminary experiments showed that both α<sub>1</sub>- and β<sub>3</sub>-subunits were required to form a functional channel together with the δ-subunit. We focused on the triple subunit combination α<sub>1</sub>β<sub>3</sub>δ. Functional expression of non-concatenated α<sub>1</sub>β<sub>3</sub>δ might theoretically result in multiple subunit arrangements. In this case, subunit concatenation (41–46) is a powerful approach to predefine receptor structure. Two or more subunits can be linked at the DNA level to control subunit composition and arrangement. To construct all possible subunit arrangements and of all studied receptors the pentameric concatenate clearly exceeded our work capacity. As discussed earlier the δ-subunit is thought to be a γ-subunit substitute, although it displays the highest degree of homology to the β-subunit. Nevertheless, we investigated all variants of the major GABA<sub>δ</sub> receptor isoform γβαβα, where the γ-subunit, one of the α- or one of the β-subunits was replaced by the δ-subunit.

*The δ-Subunit May Assume Several Positions in a Receptor Pentamer*—First, we investigated the receptors composed of non-concatenated subunit combinations, concatenated dual and triple subunit constructs alone, or in different combinations for their functional expression. As it is well documented that neurosteroids may profoundly affect δ-subunit-containing receptors, we used THDOC in combination with GABA. In analogy with α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors (41, 42) we expected only one single receptor combination to form a functional receptor. To our surprise four different receptor combinations, i.e. β<sub>3</sub>-α<sub>1</sub>-δ/α<sub>1</sub>-β<sub>3</sub> (R1), α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2), β<sub>3</sub>-α<sub>1</sub>-δ/β<sub>3</sub>-α<sub>1</sub>-δ (R5), and β<sub>3</sub>-δ/β<sub>3</sub>-α<sub>1</sub>-δ (R7) resulted in expression of sizeable currents. The observations with R7 should be taken with care (see below).

*Formation of a δ-Subunit-containing Receptor Silent in the Absence of Neurosteroids*—The application of 1 μM THDOC alone did not activate the δ-subunit-containing receptors (not shown). However, upon co-application with GABA, 1 μM THDOC produced a dramatic increase in the current amplitude in most of the non-concatenated and concatenated α<sub>1</sub>-, β<sub>3</sub>-, and δ-subunit-containing receptors. Potentiation by THDOC for non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors was about 17-fold. This is higher than that reported by Zheleznova *et al.* (39) who reported an about 3-fold potentiation of maximal GABA currents in α<sub>1</sub>β<sub>3</sub>δ receptors. Potentiation for β<sub>3</sub>-α<sub>1</sub>-δ/α<sub>1</sub>-β<sub>3</sub> (R1), β<sub>3</sub>-α<sub>1</sub>-δ-α<sub>1</sub>-β<sub>3</sub> (P1), α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2), β<sub>3</sub>-α<sub>1</sub>-δ/β<sub>3</sub>-α<sub>1</sub>-δ (R5), and pentamer β<sub>3</sub>-α<sub>1</sub>-δ-β<sub>3</sub>-α<sub>1</sub>-δ (P5) was about 1.3-fold, 5-fold, 22-fold, 5-fold, and 7-fold, respectively. We cannot explain the unusually low potentiation of R1 receptors. Especially interesting was α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2) that showed very small currents in response to GABA alone, but was uncovered upon co-application of 1 μM THDOC. This shows that in the absence of neurosteroid some of the αβδ GABA receptors may remain almost silent and therefore contribute little to inhibition. However, in the presence of THDOC these silent receptors get activated and thereby could exert a profound inhibitory influence on the neuronal activity. The *in vivo* concentration of neurosteroids in brain has been estimated 3–100 nM (37). At these concentrations we already observed strong potentiation of currents in α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2) receptors (Fig. 4C).

*Properties of δ-Subunit-containing Receptors in Response to GABA, Zn<sup>2+</sup>, and Ethanol*—The functional properties of the concatenated receptors were compared with those of non-concatenated receptors (Fig. 3). First, the EC<sub>50</sub> for channel opening by natural agonist GABA of concatenated and non-concatenated receptors were compared. Results show that concatenated β<sub>3</sub>-α<sub>1</sub>-δ/α<sub>1</sub>-β<sub>3</sub> (R1) and α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2) receptors had a similar sensitivity to GABA as non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors, whereas β<sub>3</sub>-α<sub>1</sub>-δ-β<sub>3</sub>-α<sub>1</sub>-δ (P5) showed a 13-fold lower EC<sub>50</sub> than non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors. Our results obtained for the EC<sub>50</sub> of non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors expressed in *Xenopus* oocytes differ from a study by Hanchar *et al.* (34) that reported an EC<sub>50</sub> of 0.56 μM. In two other studies, an EC<sub>50</sub> for α<sub>1</sub>β<sub>3</sub>δ receptors expressed in L929 cells (28) and HEK293T cells (33) was determined to be 3.5 μM and 4.6 μM, respectively.

As mentioned earlier non-concatenated receptors lacking the γ-subunit have a high sensitivity to inhibition by Zn<sup>2+</sup> (25, 26, 29, 36). Indeed, all the investigated receptors displayed a high sensitivity. α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2), β<sub>3</sub>-α<sub>1</sub>-δ/β<sub>3</sub>-α<sub>1</sub>-δ (R5), and pentamer β<sub>3</sub>-α<sub>1</sub>-δ-β<sub>3</sub>-α<sub>1</sub>-δ (P5) showed a similar sensitivity to inhibition by Zn<sup>2+</sup> as non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors. For unknown reasons β<sub>3</sub>-α<sub>1</sub>-δ/α<sub>1</sub>-β<sub>3</sub> (R1) receptors showed about 7-fold lower IC<sub>50</sub> for inhibition by Zn<sup>2+</sup>.

We investigated the effect of a relatively high, but still physiological concentration of ethanol on non-concatenated and concatenated receptors. 30 mM (corresponding to 1.38‰ (w/v) or 1.75‰ (v/v)) ethanol failed to affect currents mediated non-concatenated α<sub>1</sub>β<sub>3</sub>δ receptors and concatenated β<sub>3</sub>-α<sub>1</sub>-δ/α<sub>1</sub>-β<sub>3</sub> (R1), α<sub>1</sub>-β<sub>3</sub>-α<sub>1</sub>-β<sub>3</sub>-δ (R2), β<sub>3</sub>-α<sub>1</sub>-δ/β<sub>3</sub>-α<sub>1</sub>-δ (R5), and β<sub>3</sub>-α<sub>1</sub>-δ-β<sub>3</sub>-α<sub>1</sub>-δ (P5) receptors. This is in contrast to the observation by Hanchar *et al.* (34) who reported a potentiation by the same concentration of ethanol of α<sub>1</sub>β<sub>3</sub>δ receptors expressed in *Xenopus* oocytes amounting to 88%. In principle different receptor pentamers could be formed upon injection of genetic information coding for α<sub>1</sub>-, β<sub>3</sub>- and δ-subunits, some of them ethanol-sensitive. With subunit concatenation, these receptors may be expressed individually. However, we observed that none of the three candidate receptors was potentiated by ethanol. The reason for these divergent observations is not known. A stimula-
tion of about 160% by 30 mM ethanol of extra-synaptic currents in hippocampal interneurons was also observed (19). This current was attributed to GABA_\text{A} receptors containing \(\alpha_1\) and \(\delta\)-subunits in combination with unknown \(\beta\)-subunits. In the latter case, a receptor-associated protein could be responsible for the observed stimulation.

**Assembly of Receptors Following Injection of Individual Dual and Triple Subunit Constructs**—Most of the dual and triple subunit constructs when injected alone did not result in current expression, with the exception of concatenated subunits \(\beta_3\alpha_1\) and \(\beta_3\alpha_1\delta\) (Fig. 1). It is not clear whether these constructs are able to form tetramers or hexamers, or whether one of the subunits is hanging out, not being incorporated in the pentamer (46). Expression of \(\beta_3\alpha_1\) alone resulted in a current that was potentiated less than 2-fold by THDOC. The current mediated by \(\beta_3\alpha_1\delta/\beta_3\alpha_1\) (R5), which contains this dual subunit construct was potentiated about 7-fold. Also \(\beta_3\alpha_1\delta\) differed from \(\beta_3\alpha_1\alpha_1\beta_3\) (R1) in this respect. These observations indicate that in the presence of suitable assembly partners, the mis-formation does not take place. To prove this, we constructed the pentamers \(\beta_3\alpha_1\delta/\alpha_3\beta_1\) (P1) and \(\beta_3\alpha_1\delta/\beta_3\alpha_1\) (P5). The functional properties of pentameric receptors were found to be similar as for the respective receptors composed of dual and triple subunit constructs with respect to sensitivity for GABA. In summary, we conclude that the assembly pathway is influenced by the co-expressed subunits.

The situation in the case of \(\beta_3\delta\beta_3\beta_3\alpha_1\) (R7) is less clear. This receptor resulted in currents of slightly larger amplitude as \(\beta_3\alpha_1\). The current showed likewise relatively little stimulation by THDOC. The EC_{50} was determined as 40 ± 18 \(\mu\text{M}\), and the Hill coefficient as 0.8 ± 0.1. Again these parameters are reminiscent of the current mediated by \(\beta_3\alpha_1\). Therefore, we assume that R7 is probably not formed, but its formation with similar properties as \(\beta_3\alpha_1\) cannot be fully excluded.

**Abundance of the Different \(\delta\)-Subunit-containing Receptors**—Our functional study on \(\alpha_3\beta_3\delta\) GABA\text{A} receptors should be compared with a structural study on \(\alpha_4\beta_3\delta\) GABA\text{A} receptors. Using atomic force microscopy Barrera et al. (40) determined stoichiometry and subunit arrangement of these receptors expressed in tsA 201 cells. They showed that \(\alpha_4\beta_3\beta_3\delta\) counter-clockwise is the predominant subunit arrangement around the pore when viewed from the extracellular space with 21% of the population exhibiting a distinct subunit arrangement of \(\alpha\beta\alpha\beta\delta\). Only a very small number of receptor entities were analyzed, and these numbers should therefore be taken with care. The above study was done at a structural level, whereas we focused on the function of \(\delta\)-subunit-containing receptors. If it is assumed that \(\alpha_3\) is similar to \(\alpha_4\), \(\alpha\beta\alpha\beta\delta\) receptors correspond to \(\beta_3\alpha_1\delta/\beta_3\alpha_1\) (R5/P5) and \(\alpha\beta\alpha\beta\delta\) to \(\alpha_1\beta_3\alpha_1/\beta_3\delta\) (R2) in our study. From the present experiments, it is difficult to conclude the relative abundance of the three expressing receptors. Subunit concatenation may affect expression levels. Although the \(\beta_3\alpha_1\delta/\beta_3\alpha_1\) (P5) receptor with the \(\delta\)-subunit in the \(\gamma\)-subunit position produces the largest current amplitudes, this receptor has an EC_{50} for GABA about 12-fold higher than that of non-concatenated \(\alpha_1\beta_3\delta\) receptors. Nevertheless, active, non-concatenated \(\alpha_1\beta_3\delta\) receptors probably constitute a mixture of \(\beta_3\alpha_1\delta/\alpha_3\beta_3\) (R1), \(\alpha_1\beta_3\alpha_1/\beta_3\delta\) (R2), and \(\beta_3\alpha_1\delta/\beta_3\delta\alpha_1\) (R5), where R2 is only active in the presence of neurosteroids. It should be noted that we cannot fully exclude that in addition \(\beta_3\delta\beta_3\alpha_1/\beta_3\alpha_1\) (R7) or other subunit arrangements that were not analyzed could also be formed. Evidence for the expression of multiple receptors has been obtained for another \(\delta\)-subunit-containing receptor, namely \(\alpha_2\beta_3\delta\) (50). Taken together, our findings reveal a unique assembly profile for the \(\delta\)-subunit that resembles that of the \(\epsilon\)-subunit (51) with respect to the fact that both subunits can assume multiple positions in a receptor.

**Ability of \(\delta\)-Subunit to Contribute to the Formation of an Agonist Site**—\(\alpha_3\beta_3\alpha_1/\beta_3\delta\) had a Hill coefficient greater than 1, hinting at the presence of more than one agonist site. The major isoform of GABA\text{A} receptors has two different agonist binding sites located both at the interface of the \(\beta\)- and \(\alpha\)-subunits (43). Assuming that the binding site is formed at the \(\beta_3\delta\) and \(\beta_3\alpha_1\) interfaces in \(\alpha_1\beta_3\alpha_1/\beta_3\delta\), we introduced a homologous point mutation \(\beta_3Y205S\) (49) into either of the \(\beta_3\)-subunits to disrupt both agonist binding sites selectively. Our results indicate the existence of two agonist sites involving the \(\beta_3\delta\) and \(\beta_3\alpha_1\) interfaces in the \(\alpha_1\beta_3\alpha_1/\beta_3\delta\) (R2) receptor. Channel opening also occurs when the receptor is occupied with a single agonist molecule, but is promoted more than 30-fold if occupied by two agonists. Thus, the minus side of the \(\delta\)-subunit may contribute to an agonist site, but we cannot exclude that the effect of the mutation in the \(\beta\)-subunit is allosterically propagated to the plus side of the \(\delta\)-subunit (52). Whether or not the \(\delta\)-subunits assume the role of the \(\alpha\)-subunit in the agonist site is not clear from these data. It is however intriguing that the residue \(\alpha_1F64\) crucial in the agonist site of \(\alpha_2\beta_3\gamma\) receptors (52) is conserved in the homologous position in \(\delta\)-subunits. Mutation of this residue will clarify the question.

**Summary**—In summary, we have shown that GABA\text{A} receptors containing \(\alpha_3\beta_3\delta\) and \(\delta\)-subunit have a stoichiometry of \(2\alpha_1\beta_3\beta_3\delta\) and that the \(\delta\)-subunit exhibits the ability to promiscuously assemble into different ethanol-insensitive subunit arrangements at least in the Xenopus oocytes. Further, we show that at least one of these \(\delta\)-subunit-containing receptors remains silent in the absence of neurosteroid. We have also found that the \(\delta\)-subunit can contribute to the formation of an agonist site. In the future, it would be interesting to determine how the \(\delta\)-subunit assembles in the brain. It is possible that the arrangement of \(\delta\)-subunit-containing receptors in brain is controlled in a region-specific manner.

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