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Toward Understanding Functional Properties and Subunit Arrangement of $\alpha_4\beta_2\delta$ γ-Aminobutyric Acid, Type A ($GABA_A$) Receptors*

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$GABA_A$ receptors are pentameric ligand-gated channels mediating inhibitory neurotransmission in the CNS. $\alpha_4\beta_x\delta$ $GABA_A$ receptors are extrasynaptic receptors important for tonic inhibition. The functional properties and subunit arrangement of these receptors are controversial. We predefined subunit arrangement by using subunit concatenation. α_4, β_2, and δ subunits were concatenated to dimeric, trimeric, and, in some cases, pentameric subunits. We constructed in total nine different receptor pentamers in at least two different ways and expressed them in *Xenopus* oocytes. The δ subunit was substituted in any of the five positions in the $\alpha_1\beta_2$ receptor. In addition, we investigated all receptors with the 2:2:1 subunit stoichiometry for α_4, β_2, and δ. Several functional receptors were obtained. Interestingly, all of these receptors had very similar EC_{50} values for GABA in the presence of the neurosteroid 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC). All functional receptors containing δ subunits were sensitive to 4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide (DS2). Moreover, none of the receptors was affected by ethanol up to 30 mM. These properties recapitulate those of non-concatenated receptors expressed from a cRNA ratio of 1:1:5 coding for α_4, β_2, and δ subunits. We conclude that the subunit arrangement of $\alpha_4\beta_2\delta$ $GABA_A$ receptors is not strongly predefined but is mostly satisfying the 2:2:1 subunit stoichiometry for α_4, β_2, and δ subunits and that several subunit arrangements result in receptors with similar functional properties tuned to physiological conditions.

$GABA_A$ receptors are the most abundant inhibitory neurotransmitter receptors in the CNS and are a therapeutic target for several drugs. They form heteropentamers made up of distinct subunit combinations selected from the following different subunit isoforms: α(1–6), β(1–4), γ(1–3), δ, ϵ, θ, π, and ρ(1–3). The five subunits form a central chloride ion-selective channel (1–3). The major isoform of the $GABA_A$ receptor is composed of α_1, β_2, and γ_2 subunits with a fixed stoichiometry of 2:2:1 (4–6), arranged as $\gamma_2\beta_2\alpha_1\beta_2\alpha_1$ anticlockwise when viewed from the extracellular space (7–9).

$GABA_A$ receptors incorporating the γ_2 subunits are considered to be localized predominantly at synaptic regions and play a crucial role in phasic inhibition, whereas those containing the δ subunit are dominantly located peri- and extrasynaptically and mediate tonic inhibition (10). δ subunits preferentially combine with either α_1 (11), α_4 (12, 13), or α_6 (14) subunits. Without experimental evidence, the δ subunit is generally assumed to substitute for the γ_2 subunit. At least in $\alpha_1\beta_3\delta$, $\alpha_6\beta_3\delta$, and $\alpha_1\alpha_6\beta_3\delta$ receptors, the δ subunit may assume different positions but predominantly the one of the β subunit between α subunits in the major isoform of $GABA_A$ receptors (15–17).

Subunit arrangement of $\alpha_4\beta_x\delta$ $GABA_A$ receptors is still incompletely understood. Atomic force microscopy of recombinant $\alpha_4\beta_3\delta$ receptors expressed in either the endoplasmic reticulum membrane or in the plasma membrane of tsA 201 cells has suggested that the δ subunit is able to substitute for the γ_2 subunit (18). Structural work on $\alpha_4\beta_2\delta$ $GABA_A$ receptors expressed in HEK cells indicated that subunit stoichiometry is highly influenced by the ratio of subunit cDNAs transfected and that more than one δ subunit can assemble into a pentamer (19). It should be noted that such structural work cannot differentiate between functional and non-functional receptors. Therefore, functional strategies are of special interest.

Several groups have observed that the functional properties of $\alpha_4\beta_x\delta$ receptors depend on the ratio of genetic information coding for different subunits used during expression (19–22), suggesting that several subunit arrangements are possible. A recent publication concluded that the subunit stoichiometry of recombinant $\alpha_4\beta_3\delta$ $GABA_A$ receptors expressed in HEK cells is 2:2:1 (23), independent of the cDNA ratio used for expression.

Receptor concatenation predefines subunit composition and arrangement. Electrophysiological analysis provides functional properties of these receptors. So far, subunit concatenation has been applied to approach the architecture of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (7–9), $\alpha_1\beta_3\delta$ (16), $\alpha_6\beta_3\delta$ (15), and $\alpha_1\alpha_6\beta_3\delta$ (17). In addition, the technique has contributed to the finding that the assembly of ϵ subunits is promiscuous (24). Initial attempts to use subunit concatenation to understand subunit arrangement in $\alpha_4\beta_2\delta$ receptors have indicated that the δ subunit simply replaces the γ_2 subunit in the classical arrangement (25). Later, the same group reported that various subunit arrangements may result in functional receptors (26).

In this work, we aimed to understand the function and architecture of $\alpha_4\beta_2\delta$ receptors by combining subunit concatenation and expression in *Xenopus* oocytes. The receptors were func-

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tionally characterized by determining their response to GABA in the presence of THDOC[3] and modulation by DS2 and ethanol. We conclude that the δ subunit, together with α_4 and β_2, does not form one defined receptor but can form, at least in the oocyte, more than one subunit arrangement and that these receptors display similar functional properties.

Results

Preliminary Considerations—We wanted to get insight into the subunit arrangement of $\alpha_4\beta_x\delta$ GABA$_A$ receptors. As a first step, we had to choose the β subunit subtype. To allow selection, we investigated whether expression of $\alpha_4\beta_1\delta$, $\alpha_4\beta_2\delta$, or $\alpha_4\beta_3\delta$ would result in the largest current amplitudes, indicating a preference for subunit assembly. Comparison of these three receptor types revealed no preference (data not shown). We restricted our analysis to functional $\alpha_4\beta_2\delta$ receptors expressed in *Xenopus* oocytes by applying electrophysiological techniques. Unless stated otherwise, 2.5 fmol cRNA/subunit was injected into an oocyte, an amount that prevents overloading artifacts. For such a study, it would be desirable to construct 30 pentameric concatenated receptors where all five subunits are covalently linked. In a realistic time period, this is impossible. We restricted ourselves to the construction of six dimeric subunit constructs, six trimeric subunit constructs, and two pentameric subunit constructs. We cannot exclude that receptors with alternative subunit arrangements are also functional.

GABA acts only as a partial agonist for many δ subunit-containing receptors (15, 16, 27, 28). We made similar observations in non-concatenated $\alpha_4\beta_2\delta$ receptors expressed at a subunit ratio of 1:1:5. Fig. 1*A* shows current traces from an application of 1 mM GABA followed by application of 1 μM THDOC and, subsequently, by the combination of the two. 1 mM GABA elicited 17% ± 3% (mean ± S.D., $n = 5$) of the current elicited in the combined presence of 1 mM GABA/1 μM THDOC. Fig. 1*B* shows a similar experiment with the concatenated receptor β_2-α_4-δ/α_4-β_2 (R1). Here the relative current was 32% ± 13% (mean ± S.D., $n = 5$). For P1 with the same subunit arrangement but all five subunits covalently linked, this value amounted to 19% ± 3% (mean ± S.D., $n = 5$). THDOC has been reported to potentiate current mediated by δ subunit-containing receptors with no or maximally a 2.6-fold decrease of the EC$_{50}$ for GABA (16, 29, 30). Additionally, it has been reported that, in the presence of THDOC, receptors are uncovered that are silent in its absence (16, 30). The concentration of endogenous neurosteroids near the GABA$_A$ receptors in the brain is not known, but it may be assumed that at least part of the receptors are activated by these compounds. For all experiments shown below, except when DS2 was present, we included 1 μM THDOC in the solutions containing GABA. In the following, we first report our observations on the expression of non-concatenated single subunits and combinations of two or three subunits, followed by observations on concatenated receptors.

Functional expression of the individual subunits α_4, β_2, or δ did not result in appreciable currents (Fig. 2). The same was observed for the expression of the δ subunit in combination with α_4 or β_2 subunits (Fig. 2). The combination of α_4 with β_2 subunits resulted in robust currents, characterized by an EC$_{50}$ for GABA of 0.25 ± 0.03 μM ($n = 4$). It is interesting to note that expression of 5-fold lower amounts of cRNA produced only very tiny currents, but inclusion of small amounts of cRNA coding for the δ subunit (0.25 fmol) to $\alpha_4\beta_2$ rescued functional expression (Fig. 2).

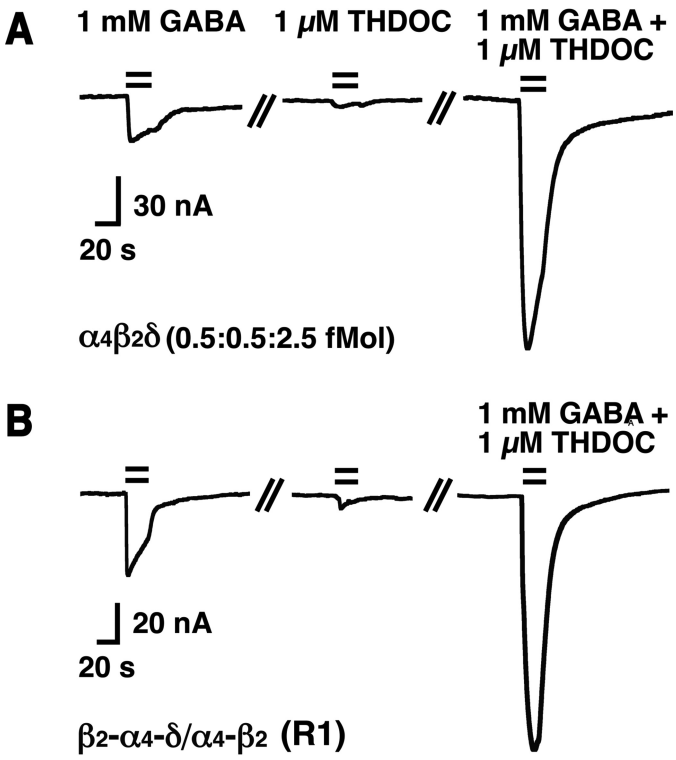

FIGURE 1. **Current traces recorded from oocytes expressing either non-concatenated $\alpha_4\beta_2\delta$ receptors or β_2-α_4-δ/α_4-β_2 (R1) receptors.** *A*, current traces recorded upon application of 1 mM GABA, followed by 1 μM THDOC and co-application of 1 mM GABA/1 μM THDOC to non-concatenated $\alpha_4\beta_2\delta$ receptors. *B*, analogous experiment with R1 receptors. The bars indicate the time period of drug perfusion. Both experiments were repeated four times with similar results.

We investigated the triple subunit combination $\alpha_4\beta_2\delta$ injected with genetic information coding for the individual subunits at two different stoichiometries that are often used in work with these receptors (21, 22, 26). Both stoichiometries, 0.5:0.5:2.5 fmol/oocyte and 2.5:0.5:2.5 fmol/oocyte, resulted in robust current expression. Although the first receptor was characterized by an EC$_{50}$ for GABA of 0.41 ± 0.12 μM ($n = 3$), the second receptor displayed a biphasic behavior with nanomolar affinities (Fig. 2). As discussed below, we do not think that such a receptor is expressed *in vivo*. Detailed averaged concentration-response curves are shown in Fig. 3.

Expression of Concatenated Receptors—First we tested all dual- and triple-concatenated subunit constructs individually for current expression. In case of a subunit hanging out, pentameric receptors could be built. The dual subunit constructs α_4-β_2, α_4-δ, β_2-δ, δ-α_4, and δ-β_2 and the triple subunit constructs α_4-β_2-α_4, α_4-δ-α_4, β_2-δ-β_2, and δ-β_2-α_4 did not result in current expression (Fig. 2). β_2-α_4-δ produced very small currents. Surprisingly, 2.5 fmol cRNA/oocyte coding for β_2-α_4 and 2.5 fmol cRNA/oocyte coding for β_2-α_4-β_2 both resulted in

[3] The abbreviations used are: THDOC, 3α, 21-dihydroxy-5α-pregnan-20-one; DS2, 4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide.

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Receptor	GABA+THDOC I (nA)	(n)	EC_{50} (μM)	Hill coefficient	(n)
α4	6 ± 1	10	-	-	-
β2	11 ± 6	7	-	-	-
δ	2 ± 1	10	-	-	-
α4β2 (2.5:2.5 fMol)	307 ± 162	10	0.25 ± 0.03	0.95 ± 0.09	4
α4β2 (0.5:0.5 fMol)	11 ± 6	5	-	-	-
α4δ	4 ± 5	5	-	-	-
β2δ	5 ± 3	5	-	-	-
α4β2δ (0.5:0.5:2.5 fMol)	490 ± 358	10	0.41 ± 0.12	0.76 ± 0.04	3
α4β2δ (0.5:0.5:0.25 fMol)	473 ± 150	10	-	-	-
α4β2δ (2.5:0.5:2.5 fMol)	2620 ± 1960	6	0.057 ± 0.034[a]	0.58 ± 0.11	3
			0.002 ± 0.001[b]	-	
			0.17 ± 0.14[b]	-	
α4-β2	3 ± 1	10	-	-	-
α4-δ	4 ± 5	5	-	-	-
β2-α4	312 ± 46	15	1.28 ± 0.46	0.75 ± 0.09	4
β2-δ	1 ± 1	9	-	-	-
δ-α4	0 ± 0	9	-	-	-
δ-β2	6 ± 2	10	-	-	-
α4-β2-α4	2 ± 2	7	-	-	-
α4-δ-α4	2 ± 1	10	-	-	-
β2-α4-β2	194 ± 103	10	0.83 ± 0.32	0.88 ± 0.06	5
β2-α4-δ	21 ± 6	9	-	-	-
β2-δ-β2	0 ± 0	5	-	-	-
δ-β2-α4	3 ± 1	10	-	-	-
α4-β2/δ	0 ± 1	10	-	-	-
α4-δ/δ	0 ± 1	10	-	-	-
β2-δ/δ	0 ± 1	10	-	-	-
δ-α4/δ	0 ± 0	10	-	-	-
δ-β2/δ	1 ± 1	10	-	-	-

FIGURE 2. **Functional expression of single subunits, concatenated subunits, and non-concatenated $\alpha_4\beta_2\delta$ receptors in *Xenopus* oocytes.** Unless indicated otherwise, oocytes were injected with 2.5 fmol cRNA coding for a non-concatenated subunit or a concatenated construct. The figure shows subunit composition and current amplitudes (in nanoamperes) elicited by 1 mM GABA in the presence of 1 μM THDOC (mean ± S.E.) and, in some cases, EC_{50} for GABA/THDOC (mean ± S.D.). *n*, number of oocytes; −, not analyzed. *a* and *b*, the $\alpha_4\beta_2\delta$ receptor expressed at a subunit ratio of 2.5:0.5:2.5 fmol was fitted either assuming a single phasic (*a*) or a two-phasic (*b*) concentration-response curve, the two components amounting to about 31% and 69%, respectively.

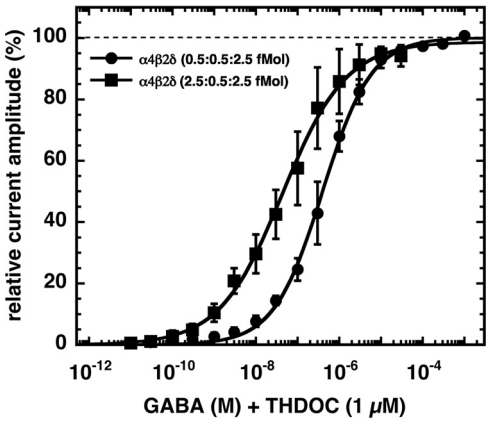

FIGURE 3. **GABA concentration dependence of non-concatenated $\alpha_4\beta_2\delta$ receptors expressed at different subunit ratios.** Oocytes were injected with x fmol of cRNA coding for α_4, 0.5 fmol of β_2, and 2.5 fmol of δ, where x was either 0.5 or 2.5. GABA concentration-response curves of these receptors were recorded in the presence of 1 μM THDOC. Individual curves were first normalized to the fitted maximal current amplitude and subsequently averaged. Concentration-response curves obtained from both receptors were fitted with a single phase. Data are expressed as mean ± S.D. (n = 3).

expression of robust currents, possibly by squeezing out one subunit of a hexameric receptor. Therefore, all pentameric receptors where one of these two constructs forms part of the pentamer should be judged with care. It is worth mentioning that, in previous work using the dual subunit construct β_2-α_4, the conclusion has been reached that several subunit arrangements can be formed from α_4, β_2 and δ (25, 26). The authors of this study report that >40 fmol cRNA coding for β_2-α_4 alone did not form functional channels. The reason for the discrepancy is not clear.

None of the combinations of the δ subunit with either α_4-β_2, α_4-δ, β_2-δ, δ-α_4, or δ-β_2 resulted in current expression (Fig. 2). Please note that α_4-β_2/δ may assemble into α_4-β_2/α_4-β_2/δ (called R2 below). This fact is discussed below.

For the investigation of the putative pentameric arrangement of $\alpha_4\beta_2\delta$ receptors, we followed two rationales. First, we assumed that the δ subunit would occupy any of the five positions in the classical subunit arrangement of $\alpha_1\beta_2$ GABA$_A$ receptors (Fig. 4, *top*) (7–9). Second, we assumed a 2:2:1 stoichiometry of α_4:β_2:δ subunits. As discussed above, this stoichiometry has been found for $\alpha_4\beta_3\delta$ receptors expressed in HEK cells and claimed to be independent of the ratio of genetic information introduced into a cell (23). These two approaches are discussed in the following.

For the first approach, we constructed each receptor, R1 to R5 (Fig. 4), combining free subunits and dual or triple subunit constructs in two to three different ways. For R1, where the δ subunit replaces the β subunit between the two α subunits, both combinations, β_2-α_4-δ/α_4-β_2 and α_4-δ-α_4/β_2, could be functionally expressed. The former receptor was characterized by an EC_{50} for GABA of 0.55 ± 0.11 μM (n = 3). We confirmed these results by constructing the concatenated pentamer P1 and found an EC_{50} for GABA of 1.45 ± 0.39 μM (n = 3). An about 2-fold increased EC_{50} for GABA compared with the receptor composed of dual and triple subunit constructs has been observed previously in similar cases (9).

We conclude that R1 may be one of the formed receptor configurations. It should be noted that this also seems to be a preferred configuration of $\alpha_1\beta_3\delta$, $\alpha_6\beta_3\delta$, and $\alpha_1\alpha_6\beta_3\delta$ receptors (15–17).

An example of a concentration-response curve is given for β_2-α_4-δ/α_4-β_2 (R1) as crude current traces (Fig. 5*A*). Averaged concentration-response curves for β_2-α_4-δ/α_4-β_2 (R1), δ-β_2-α_4/β_2-α_4 (R5), β_2-α_4-δ-α_4-β_2 (P1), and δ-β_2-α_4-β_2-α_4 (P5) are shown in Fig. 5*B*.

For R2, where the δ subunit replaces the second β subunit of the two subsequent β subunits, two of the combinations, α_4-β_2-α_4/β_2-δ and α_4-β_2/δ, were silent, whereas the other combination, β_2-α_4-β_2/δ-α_4, was functionally expressed and was characterized with an EC_{50} for GABA of 1.11 ± 0.26 μM (n = 4). This combination contains β_2-α_4-β_2 producing current by itself with similar functional properties as found here. As the EC_{50} for GABA of the combination was similar to that of the single concatenated subunit, we think that R2 may not be formed.

For R3, where the δ subunit replaces the α subunit between two subsequent β subunits and the single β subunit, one of the combinations, β_2-δ-β_2/α_4-β_2, was silent, whereas the other

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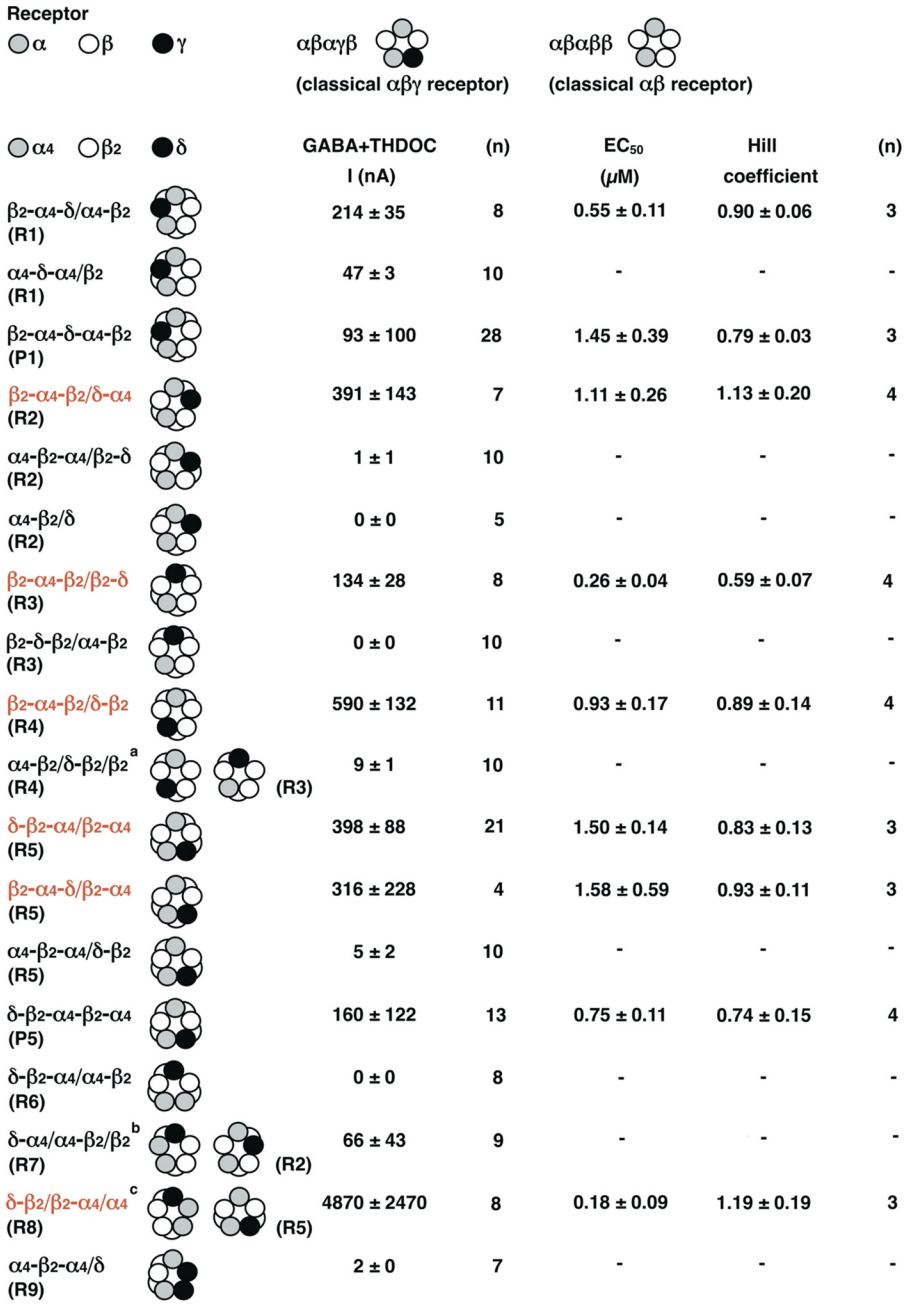

FIGURE 4. **Functional expression of concatenated $\alpha_4\beta_2\delta$ receptors in *Xenopus* oocytes.** For comparison, the *top line* shows the classical subunit arrangements of $\alpha\beta\gamma$ and $\alpha\beta$ receptors. Nine pentamers with different subunit arrangements were built, as indicated under "Results." Pentamers were either composed of concatenated subunits, or these were combined loose subunits (R1-R9). Also, two pentameric constructs (P1 and P5) were built. The figure shows subunit composition and current amplitudes (in nanoamperes) elicited by 1 mM GABA in the presence of 1 μM THDOC (mean ± S.E.) and, in some cases, EC$_{50}$ for GABA/THDOC (mean ± S.D.). *n*, number of oocytes; −, not analyzed. *a*, this subunit configuration might also assemble into R3. *b*, this subunit configuration might also assemble into R2. *c*, this subunit configuration might also assemble into R5. The receptor configurations shown in *red* contain one concatenated subunit resulting in current expression by itself.

combination, β_2-α_4-β_2/β_2-δ, was functionally expressed. This combination contains β_2-α_4-β_2 producing current by itself and is characterized by an EC$_{50}$ for GABA of 0.26 ± 0.04 μM ($n = 4$). This value is significantly different (Student's t test, $p < 0.02$) from that characterizing the β_2-α_4-β_2 construct. Therefore, it is not unlikely that the configuration R3 is formed. The fact that β_2-δ-β_2/α_4-β_2 is not functionally expressed may indicate that the β/α interface is assembled inefficiently. In line with this hypothesis is the observation that α_4-δ-α_4/β_2 (R1) expresses less efficiently than β_2-α_4-δ/α_4-β_2 (R1) and α_4-β_2-α_4/δ-β_2 (R5) not at all.

For R4, where the δ subunit replaces the α subunit preceding the two subsequent β subunits, one of the combinations, which can also form R3, α_4-β_2/δ-β_2/β_2, was silent, whereas the other combination, β_2-α_4-β_2/δ-β_2, was functionally expressed. This combination contains β_2-α_4-β_2 producing current by itself and is characterized by an EC$_{50}$ for GABA of 0.93 ± 0.17 μM ($n = 4$), similar to the EC$_{50}$ of β_2-α_4-β_2 expressed alone. We think that

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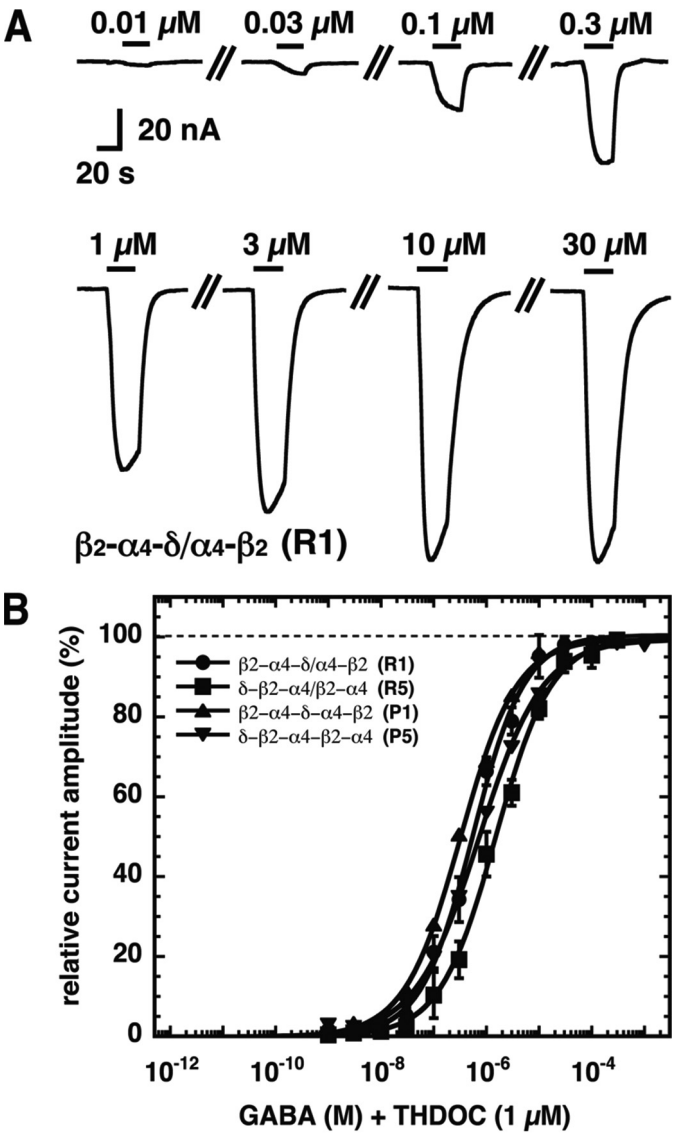

FIGURE 5. **GABA concentration dependence of concatenated $\alpha_4\beta_2\delta$ receptors.** *A*, current traces from a GABA concentration-response curve in the presence of 1 μM THDOC obtained from a *Xenopus* oocyte expressing the β_2-α_4-δ/α_4-β_2 (R1) receptor. The *bars* indicate the time period of GABA/1 μM THDOC perfusion. Increasing concentrations of GABA were applied to the oocytes, and the corresponding current amplitudes were determined. GABA concentrations are indicated above the *bars*. *B*, averaged concentration-response curves of β_2-α_4-δ/α_4-β_2 (R1), δ-β_2-α_4/β_2-α_4 (R5), β_2-α_4-δ-α_4-β_2 (P1), and δ-β_2-α_4-β_2-α_4 (P5). Individual curves were first normalized to the fitted maximal current amplitude and subsequently averaged. Data are expressed as mean ± S.D. (n = 3–4).

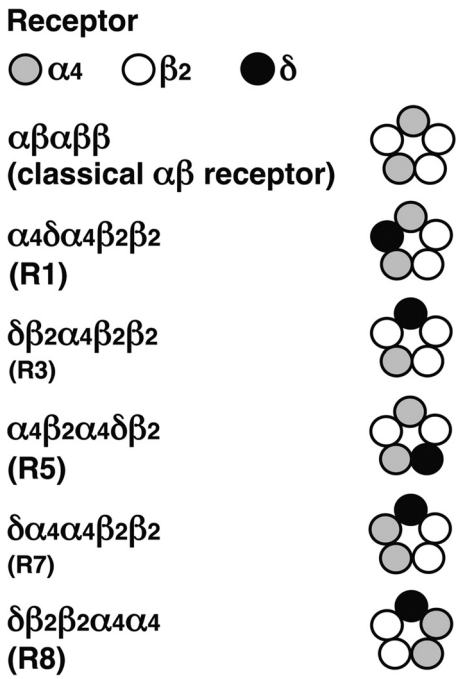

FIGURE 6. **Summary of the receptor configurations resulting in functional expression.** R1, R5, and R8 are candidates, and R3 and R7 (*smaller font*) are likely candidates for the subunit arrangement of $\alpha_4\beta_2\delta$ receptors.

R4 may either not be formed or is indistinguishable from β_2-α_4-β_2.

For R5, where the δ subunit replaces the first of the two subsequent β subunits in the classical $\alpha\beta$ receptor or the γ subunit in the classical $\alpha\beta\gamma$ receptor, α_4-β_2-α_4/δ-β_2 did not result in active channel formation, but, as discussed above, this combination requires formation of a β/α subunit interface. δ-β_2-α_4/β_2-α_4 and β_2-α_4-δ/β_2-α_4 did functionally express, but each contains β_2-α_4, a construct that results in current formation by itself. The two receptors were characterized by an EC$_{50}$ for GABA of 1.50 ± 0.14 μM (n = 3) and 1.58 ± 0.59 μM (n = 3), respectively. Both values are similar to the EC$_{50}$ of β_2-α_4. As this receptor has previously been claimed to represent the subunit arrangement of $\alpha_4\beta_2\delta$ receptors (25), we decided to construct the pentameric concatenated receptor δ-β_2-α_4-β_2-α_4 (P5). To our surprise, we found functional expression of the pentameric construct with an EC$_{50}$ for GABA of 0.75 ± 0.11 μM (n = 4). Successful expression of a pentameric concatenated construct suggested that R5 may be a possible receptor configuration.

Additionally, we studied $\alpha_4\beta_2\delta$ GABA$_A$ receptors with a 2:2:1 subunit stoichiometry of α_4, β_2, and δ subunits. In theory, six possible subunit arrangements exist with this stoichiometry. It should be noted that the subunit arrangements R1, R2, and R5 are identical to three of these six receptors. We did not observe functional expression of δ-β_2-α_4/α_4-β_2 (R6). A small functional expression was detected for δ-α_4/α_4-β_2/β_2 (R7). Please note that this combination may also be assembled into R2. A large current expression was observed for δ-β_2/β_2-α_4/α_4 (R8). This construct contains β_2-α_4, which results in current expression by itself and may also assemble to R5. The large current expression exceeds by far the one expected for β_2-α_4. Control experiments with β_2-α_4/α_4 resulted in a 6-fold lower current expression and a reduced sensitivity to DS2, ensuring formation of R8 upon injection of δ-β_2/β_2-α_4/α_4. R8 was characterized by an EC$_{50}$ for GABA of 0.18 ± 0.09 μM (n = 3). This EC$_{50}$ differs from that of R5 and the constituent β_2-α_4. R8 and possibly R7 are candidates for the subunit arrangement of $\alpha_4\beta_2\delta$ GABA$_A$ receptors. Theoretically, the components used to construct R8 could also assemble to R5. However, the formation of a β/α subunit interface would be required. An additional receptor not conforming to the above stoichiometry with two δ subunits did not result in current expression. Fig. 6 summarizes our findings on the possible subunit arrangement of concatenated $\alpha_4\beta_2\delta$ receptors containing one or several δ subunits.

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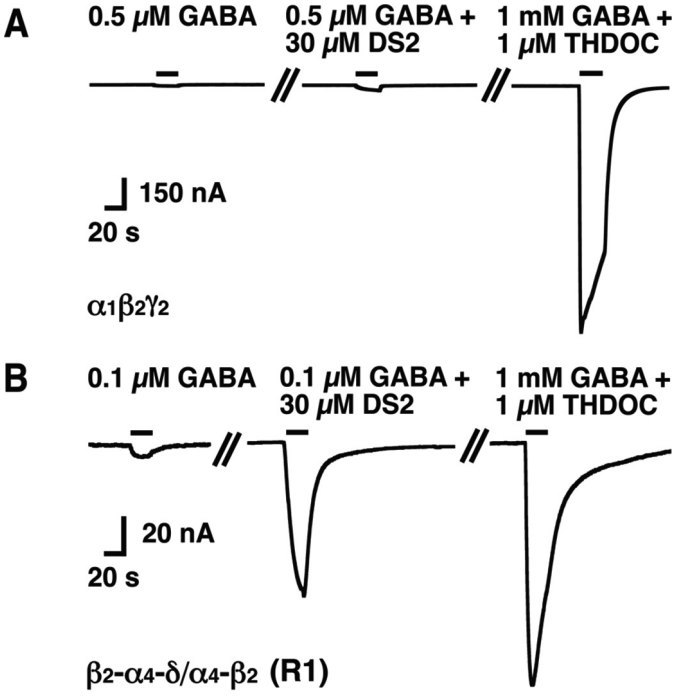

FIGURE 7. **DS2 sensitivity of non-concatenated $\alpha_1\beta_2\gamma_2$ and β_2-α_4-δ/α_4-β_2 (R1) receptors.** *A* and *B*, original current traces recorded upon application of 0.5 or 0.1 μM GABA alone, followed by application of 30 μM DS2 together with the same concentrations of GABA and subsequent co-application of 1 mM GABA/1 μM THDOC from a representative non δ subunit-containing GABA$_A$ receptor, $\alpha_1\beta_2\gamma_2$ (*A*) and a representative δ subunit-containing GABA$_A$ receptor, β_2-α_4-δ/α_4-β_2 (R1, *B*), respectively. The *bars* indicate the duration of drug application. These experiments were repeated three to four times with similar results.

Receptor (α4 β2 δ)	pot. DS2 (%)	rel. DS2 (%)	(n)
α4β2	234 ± 112	9 ± 2	5
α1β2γ2 (0.5:0.5:2.5 fMol)	549 ± 250	2 ± 2	4
α4β2δ[a] (0.5:0.5:2.5 fMol)	3340 ± 4710	53 ± 15	3
β2-α4	420 ± 290	9 ± 5	4
β2-α4-β2	515 ± 200	4 ± 1	4
β2-α4-δ/α4-β2[a] (R1)	2550 ± 1580	49 ± 16	5
β2-α4-δ-α4-β2[a] (P1)	2400 ± 680	28 ± 6	5
β2-α4-β2/δ-α4[a] (R2)	2570 ± 1060	25 ± 13	7
β2-α4-β2/β2-δ (R3)	1572 ± 410	46 ± 14	5
β2-α4-β2/δ-β2 (R4)	900 ± 570	33 ± 4	5
δ-β2-α4/β2-α4 (R5)	3100 ± 1730	25 ± 11	4
δ-β2-α4-β2-α4[a] (P5)	3100 ± 2400	31 ± 8	4
δ-α4/α4-β2/β2[b] (R7) (R2)	1615 ± 578	58 ± 13	5
δ-β2/β2-α4/α4[c] (R8) (R5)	1700 ± 670	77 ± 6	4

FIGURE 8. **Positive allosteric modulation by DS2.** The functional receptors were tested for sensitivity to DS2 by measuring the potentiation by 30 μM DS2 of current evoked by GABA$_{EC10}$ (*pot. DS2*) and the current elicited by GABA$_{EC10}$ + 30 μM DS2 relative to the maximal current elicited by 1 mM GABA in the presence of 1 μM THDOC (*rel. DS2*). *a*, the current amplitude elicited by GABA$_{EC10}$ in $\alpha_4\beta_2\delta$ (0.5:0.5:2.5 fmol), β_2-α_4-δ/α_4-β_2 (R1), β_2-α_4-δ-α_4-β_2 (P1), β_2-α_4-β_2/δ-α_4 (R2), and δ-β_2-α_4-β_2-α_4 (P5) receptors was less than 4 nA in each case. We assumed that these amplitudes amounted to 4 nA to be able to calculate potentiation by DS2. Therefore, the corresponding values represent underestimates. *b*, this subunit configuration might also assemble into R2. *c*, this subunit configuration might also assemble into R5. Data are expressed as mean ± S.D. *n*, number of oocytes.

Sensitivity to DS2—DS2 has been described to be specific for GABA$_A$ receptors containing a δ subunit (31, 32). We made similar observations. This is illustrated for $\alpha_1\beta_2\gamma_2$ and β_2-α_4-δ/α_4-β_2 (R1) (Fig. 7). The non-concatenated receptors $\alpha_4\beta_2$, $\alpha_1\beta_2\gamma_2$, and $\alpha_4\beta_2\delta$, concatenated subunit constructs that result themselves in current expression, β_2-α_4 and β_2-α_4-β_2, and the concatenated receptors β_2-α_4-δ/α_4-β_2 (R1), β_2-α_4-δ-α_4-β_2 (P1), β_2-α_4-β_2/δ-α_4 (R2), β_2-α_4-β_2/β_2-δ (R3), β_2-α_4-β_2/δ-β_2 (R4), δ-β_2-α_4/β_2-α_4 (R5), δ-β_2-α_4-β_2-α_4 (P5), δ-α_4/α_4-β_2/β_2 (R7), and δ-β_2/β_2-α_4/α_4 (R8) were tested for sensitivity to DS2 (Fig. 8). Sensitivity to DS2 was determined as potentiation by 30 μM DS2 of currents elicited by GABA$_{EC10}$ and as relative current GABA$_{EC10}$ + 30 μM DS2 divided by the maximal current elicited by GABA in the presence of 1 μM THDOC. Whenever the δ subunit was present, sensitivity to DS2 was high (relative current, 25–77%), and whenever the δ subunit was absent, sensitivity to DS2 was low (relative current, <2–9%).

Effect of Ethanol—$\alpha_4\beta_2\delta$ GABA$_A$ receptors have been claimed to be a site of action of low concentrations of ethanol (28, 33). Therefore, we were interested to investigate this with non-concatenated receptors and some of our receptors with defined subunit arrangement. A concentration dependence of ethanol was investigated in non-concatenated $\alpha_4\beta_2\delta$ GABA$_A$ receptors expressed at two different subunit stoichiometries, 0.5:0.5:2.5 fmol and 2.5:0.5:2.5 fmol, and the two pentameric concatenated receptors P1 and P5. In the concentration range of 0.1–30 mM ethanol, no significant effect on the current amplitude by GABA$_{EC10}$ was found (Fig. 9).

Discussion

In this work, we attempted to get insight into the function and subunit arrangement of $\alpha_4\beta_2\delta$ GABA$_A$ receptors. They are located extrasynaptically and are responsible for tonic inhibition (10, 34). As a first step, we expressed mRNA coding for α_4, β_2, and δ at different stoichiometries. It is well known from the literature that different stoichiometries result in different functional properties, in some cases with a nanomolar EC$_{50}$ value for GABA. The ambient concentration of GABA in the extracellular space is not known precisely (for a discussion, see Ref. 35). Nevertheless, the concentration is estimated to be in the range of 0.1–0.4 μM (36–38). A receptor with an EC$_{50}$ for GABA in the lower nanomolar range would be always fully activated under these conditions. Thus, we think that the *in vivo* existence such of a receptor is unlikely (see also below). It should be noted that the receptor resulting from the expression of 2.5:0.5:2.5 fmol mRNA/oocyte, coding for α_4, β_2, and δ subunits, results in such properties.

It would be desirable to establish function and subunit arrangement in neuronal cells, but unfortunately we had to

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Receptor ◯α4 ◯β2 ●δ	ethanol 0.1 mM (%)	(n)	0.3 mM (%)	(n)	1 mM (%)	(n)	3 mM (%)	(n)	10 mM (%)	(n)	30 mM (%)	(n)
α4β2δ (0.5:0.5:2.5 fMol)	2.5 ± 2.5	4	-1.0 ± 3.6	4	0 ± 0	4	0 ± 0	4	0 ± 0	4	-1.3 ± 1.3	4
α4β2δ (2.5:0.5:2.5 fMol)	1.6 ± 1.1	5	3.7 ± 3.2	5	0 ± 0	5	-1.4 ± 1.5	4	0 ± 0	4	0 ± 0	4
β2-α4-δ-α4-β2 (P1)	1.9 ± 1.9	4	7.5 ± 4.8	4	6.9 ± 4.7	4	1.8 ± 4.8	4	5.3 ± 8.5	4	2.5 ± 4.9	4
δ-β2-α4-β2-α4 (P5)	-3.3 ± 3.7	4	-4.9 ± 3.4	4	-7.9 ± 4.3	4	-2.8 ± 0.9	4	1.1 ± 2.9	4	6.4 ± 4.0	3

FIGURE 9. **Lack of an effect by physiological concentrations of ethanol.** Non-concatenated $\alpha_4\beta_2\delta$ (0.5:0.5:2.5 and 2.5:0.5:2.5), concatenated β_2-α_4-δ-α_4-β_2 (P1), and δ-β_2-α_4-β_2-α_4 (P5) receptors were expressed in *Xenopus* oocytes. The receptors were activated by a concentration of GABA eliciting EC$_{10}$ in the presence of 1 μM THDOC, followed by application of the same concentration of GABA/THDOC subsequently in combination with 0.1, 0.3, 1, 3, 10, and 30 mM ethanol. The relative current amplitude of the responses in the presence of ethanol compared with GABA/THDOC alone is given. Neither currents mediated by the non-concatenated $\alpha_4\beta_2\delta$ or concatenated receptors were significantly modulated by ethanol in the concentration range of 0.1–30 mM. Data are expressed as mean ± S.E. *n*, number of oocytes.

resort to expression systems lacking endogenous GABA$_A$ receptors and hope that the properties observed here reflect the situation in neurons. Results on function and subunit arrangement could in principle be obscured by endogenous expression of GABA$_A$ receptor subunits in the expression system used. In HEK cells, such an expression is well documented (39). To our knowledge, there are no such reports for *Xenopus* oocytes, which we used as an expression system.

To establish function and subunit arrangement, we used subunit concatenation. This is an elegant technique to prepare receptors with predefined subunit arrangement. The functional properties of such receptors can then be compared with the properties of non-concatenated receptors. For a detailed discussion of possible pitfalls, see Refs. 40, 41. Maybe the biggest threat to this method is proteolysis. It should be noted that, despite an intensive search, such a proteolysis of concatenated subunits has never been found. In case proteolysis should take place with a subunit, the construct is likely to be functionally silent. Therefore, we only consider proteolysis in the linker. We would like to discuss this possibility for the present case. The linkers contain no protease sites, and, in concatenated constructs, presequences were removed from all subunits except from the first one. Expression of the individual non-concatenated subunits α_4, β_2, and δ or α_4 or β_2 in combination with δ did not result in functional receptors (Fig. 2). Therefore, liberation of α_4, β_2, and δ each alone or δ in combination with α_4 or β_2 would not result in current. Exclusively, large amounts of α_4 and β_2 would do so. However, this current would be insensitive to DS2, unlike the current by receptor candidates listed in Fig. 7. The dual subunit constructs α_4-β_2, α_4-δ, β_2-δ, δ-α_4, and δ-β_2 and the triple subunit constructs α_4-β_2-α_4, α_4-δ-α_4, β_2-δ-β_2, and δ-β_2-α_4 did not result in current expression (Fig. 2). Furthermore, none of the combinations of the δ subunit with either α_4-β_2, α_4-δ, β_2-δ, δ-α_4, or δ-β_2 and α_4-β_2-α_4/β_2-δ (R2), β_2-δ-β_2/α_4-β_2 (R3), α_4-β_2/δ-β_2/β_2 (R4), α_4-β_2-α_4/δ-β_2 (R5), δ-β_2-α_4/α_4-β_2 (R6), and α_4-β_2-α_4/δ (R9) resulted in current expression. α_4, β_2, and δ must all be liberated simultaneously to produce a current sensitive to DS2. We conclude that the possibility that proteolysis affects our observations is highly unlikely.

Rearrangement of a dual construct has been documented (7). Rearrangement was quite inefficient (26%), and the resulting receptor was reported to respond rather weakly to positive allosteric modulators. Also, if rearrangement was efficient, then all configurations containing α_4-β_2 would be expected to result in β_2-α_4 and, thus, in current expression. This is not the case. Therefore, we think that subunit rearrangement is not a likely explanation for our observations.

Interpretation of our results is made more difficult by two facts. First, current expression levels are small in most cases. Second, some of the used concatenated constructs result in current expression themselves. Nevertheless, we can draw a number of conclusions. R1, R5, R8, and possibly R3 and R7, are candidates for the subunit arrangement of $\alpha_4\beta_2\delta$ GABA$_A$ receptors. The assembly properties of $\alpha_4\beta_2\delta$ GABA$_A$ receptors are less defined than in the case of $\alpha_1\beta_3\delta$, $\alpha_6\beta_3\delta$, and $\alpha_1\alpha_6\beta_3\delta$ receptors, but the subunit stoichiometry of α_4, β_2, and δ subunits mostly satisfies 2:2:1. Remarkably, all the receptors resulting in functional expression are characterized by a similar EC$_{50}$ for GABA/THDOC in the range of 0.2–1.6 μM, tuned to local physiological concentrations of GABA.

Maybe one of the most important observations is that, in the many subunit arrangements described that result in functional expression, we never encountered receptors with an EC$_{50}$ for GABA in the presence of THDOC of <180 nM. As discussed above, a receptor with an EC$_{50}$ for GABA of <100 nM would not make physiological sense. Observations of such receptors in recombinant systems most probably are the unnatural result of the use of large proportions of genetic information coding for the α subunit.

Our results should be compared with those obtained earlier using concatenated subunits (25, 26). The comparison is made difficult by the fact that, in our hands, the β_2-α_4 construct resulted in current expression by itself. In our work, oocytes were injected with 2.5 fmol cRNA coding for β_2-α_4 and, in the cited work, with >40 fmol (25) or 3–15 fmol (26). Despite the large quantities injected, Shu *et al.* (25) did not observe current expression in this case. The reason for the discrepancy is far from clear. Most of the receptors reported in the above references contain this construct. Nevertheless, the authors conclude that R2, R4, (26) and R5 (25, 26) (our nomenclature) are candidate subunit arrangements. The EC$_{50}$ values for GABA in the absence of THDOC for these receptors were reported to be about 55, 1.2, and 3.5 μM, respectively. In contrast to the above conclusions, we think that R2 and R4 may not represent receptor configurations expressed from limited amounts of cRNA.

In conclusion, the processes governing assembly of α_4, β_2, and δ subunits to form pentameric $\alpha_4\beta_2\delta$ GABA$_A$ receptors

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seem much less defined than assembly of α_1, β_2, and γ_2 subunits. The δ subunit can assume different positions, and the resulting receptors with different subunit arrangement have remarkably similar functional properties reflecting the properties of non-concatenated receptors expressed from a ratio of genetic information of 1:1:5 coding for α_4, β_2, and δ subunits. While all δ subunit-containing receptors were sensitive to the positive allosteric modulator DS2, we did not find any evidence for sensitivity to low concentrations of ethanol for the tested non-concatenated and concatenated $\alpha_4\beta_2\delta$ GABA$_A$ receptors.

Experimental Procedures

Construction of cDNAs—The cDNA coding for the rat δ subunit was generously provided by Dr. Hartmut Lüddens (Department of Psychiatry, University of Mainz, Mainz, Germany). The approach used for subunit concatenation of GABA$_A$ receptors has been described in detail previously (7–9, 40, 42). We prepared the dual constructs α_4-12-β_2, α_4-12-δ, β_2-23-α_4, δ-23-α_4, β_2-26-δ, and δ-26-β_2 and the triple subunit constructs α_4-12-β_2-23-α_4, α_4-12-δ-23-α_4, β_2-23-α_4-12-β_2, β_2-23-α_4-12-δ, β_2-26-δ-26-β_2, and δ-26-β_2-23-α_4. In addition, two pentameric constructs, β_2-23-α_4-12-δ-23-α_4-12-β_2 and δ-26-β_2-23-α_4-12-β_2-23-α_4, were built. The number between two subunits describes the number of amino acid residues of the introduced synthetic linker. Our strategy to design the linkers was to apply 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. Constructs containing shorter linkers did not result in receptor expression (7, 8). The linkers were Q^6TGQ^4 for α_4-β_2 and α_4-δ, $Q^5A^3PTGQA^3PA^2Q^5$ for β_2-α_4 and δ-α_4, and $Q^5A^3PTGQ^2AQA^3PA^2Q^5$ for β_2-δ and δ-β_2.

Expression in Xenopus Oocytes—The cDNA was subcloned into a eukaryotic expression pcDNA3.1 vector (Invitrogen). Capped cRNAs were synthesized (Ambion) 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). The concentration of the cRNA was quantified on a formaldehyde-agarose gel using Radiant Red stain (Bio-Rad) for visualization of the cRNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. The cRNAs were dissolved in water and stored at −80 °C. Frog oocytes obtained from *Xenopus laevis* (stages V-VI) were isolated, injected, and defolliculated as described earlier (43, 44). All animal experiments have been reviewed and approved by the Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15). cRNA coding for each dual and triple subunit concatemer was injected either alone or in different combinations in oocytes. Oocytes were injected with 50 nl of solution containing RNA. In the case of non-concatenated $\alpha_4\beta_2\delta$ receptors, cRNAs coding for α_4, β_2, and δ subunits were injected at a ratio of 0.5:0.5:2.5 fmol/oocyte or 2.5:0.5:2.5 fmol/oocyte as indicated, and, in the case of $\alpha_1\beta_2\gamma_2$ receptors, cRNA coding for α_1, β_2, and γ_2 subunits a ratio of 0.5:0.5:2.5 fmol/oocyte. In the case of concatenated receptors, oocytes were injected with cRNA coding for dual and triple subunits at 2.5 fmol each or pentameric constructs at 2.5 fmol. The injected oocytes were incubated in modified Barth's solution (43) at 18 °C for 1–2 days in case of $\alpha_1\beta_2\gamma_2$ receptors and 5–7 days for other receptors before recording.

Two-electrode Voltage Clamp Measurements—Electrophysiological studies were performed using a two-electrode voltage clamp amplifier (Oocyte Clamp OC-725C, Warner Instruments) in combination with a XY recorder (90% response time, 0.1 s) or digitized at 100 Hz using a Powerlab 2/20 (AD- Instrument GmbH, Spechbach, Germany), and data were recorded with the computer program Chart (AD Instruments GmbH). All measurements were performed 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. 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 (45). In initial experiments, 1 mM GABA (Sigma-Aldrich, Switzerland) was applied alone, followed by 1 μM THDOC (Sigma-Aldrich), and then the combination of the two. Relative current potentiation by THDOC was determined as $(I_{1\ \mu M\ THDOC\ +\ 1\ mM\ GABA}/I_{1\ \mu M\ GABA} - 1) \times 100\%$. For the determination of maximal current amplitudes, 1 mM GABA was applied in the presence of 1 μM THDOC for 20 s. THDOC was prepared as a 10 mM stock solution in DMSO and dissolved in external solution, resulting in a final DMSO concentration of 0.01%. Individual concentration-response curves for GABA in the presence of 1 μM THDOC were fitted with the equation $I(c) = I_{max} / (1 + (EC_{50} / c)^n)$, where c is the concentration of GABA, EC_{50} the concentration of GABA (in the presence of 1 μM THDOC) eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I the current amplitude, and n the Hill coefficient. The individual curves were fitted and standardized to I_{max} and subsequently averaged.

Sensitivity to DS2 was measured as potentiation by 30 μM DS2 of current evoked by $GABA_{EC10}$ and as $GABA_{EC10}$ + 30 μM DS2 divided by the current elicited by 1 mM GABA in the presence of 1 μM THDOC. DS2 was prepared as a 10 mM stock solution in DMSO and dissolved in external solution, resulting in a final DMSO concentration of 0.3%. Potentiation by ethanol was determined at EC_{10} for GABA using 0.1, 0.3, 1, 3, 10, and 30 mM ethanol.

Data are given as mean ± S.E. for the I_{max} values for GABA in the presence of 1 μM THDOC and for analysis of properties of receptors using ethanol and as mean ± S.D. for analysis of properties of receptors using DS2. To avoid contamination, the perfusion system was cleaned between drug applications by washing with 100% DMSO.

Author Contributions—N. W. conducted most of the electrophysiological experiments, analyzed the data, prepared the figures, and wrote the manuscript. R. B. produced all cRNA constructs and performed the electrophysiological experiments. E. S. conceived the idea for this project, designed the experiments, supervised the experiments, prepared the figures, and wrote the manuscript.

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