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Estimating the efficiency of benzodiazepines on $GABA_A$ receptors comprising γ1 or γ2 subunits

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Background and purpose: Heterologous expression of α1, β2 and γ2S(γ1) subunits produces a mixed population of $GABA_A$ receptors containing α1β2 or α1β2γ2S(γ1) subunits. GABA sensitivity (lower in receptors containing γ1 or γ2S subunits) and the potentiation of GABA-activated chloride currents (I_{GABA}) by benzodiazepines (BZDs) are dependent on γ2S(γ1) incorporation. A variable γ subunit incorporation may affect the estimation of I_{GABA} potentiation by BZDs. We propose an approach for estimation of BZD efficiency that accounts for mixed population of α1β2 and α1β2γ2S(γ1) receptors.
Experimental approach: We investigated the relation between GABA sensitivity (EC_{50}) and BZD modulation by analysing triazolam-, clotiazepam- and midazolam-induced potentiation of I_{GABA} in *Xenopus* oocytes under two-microelectrode voltage clamp.
Key results: Plotting EC_{50} versus BZD-induced shifts of GABA concentration-response curves (ΔEC_{50}(BZD)) of oocytes injected with different amounts of α1, β2 and γ2S(γ1) cRNA (1:1:1–1:1:10) revealed a linear regression between γ2S(γ1)-mediated reduction of GABA sensitivity (EC_{50}) and ΔEC_{50}(BZD). The slope factors of the regression were always higher for oocytes expressing α1β2γ1 subunit receptors (1.8 ± 0.1 (triazolam), 1.6 ± 0.1 (clotiazepam), 2.3 ± 0.2 (midazolam)) than for oocytes expressing α1β2γ2S receptors (1.4 ± 0.1 (triazolam), 1.4 ± 0.1 (clotiazepam), 1.3 ± 0.1 (midazolam)). Mutant $GABA_A$ receptors (α1β2-R207Cγ2S) with lower GABA sensitivity showed higher drug efficiencies (slope factors = 1.1 ± 0.1 (triazolam), 1.1 ± 0.1 (clotiazepam), 1.2 ± 0.1 (midazolam)).
Conclusions and implications: Regression analysis enabled the estimation of BZD efficiency when variable mixtures of α1β2 and α1β2γ2S(γ1) receptors are expressed and provided new insights into the γ2S(γ1) dependency of BZD action.
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Keywords: $GABA_A$ receptor; benzodiazepine modulation; concentration–response curve; *Xenopus* oocytes; two-microelectrode voltage clamp

Abbreviations: BZD, benzodiazepine; DEPC, diethylpyrocarbonate; I_{GABA}, GABA-induced chloride current

Introduction

GABA (γ-aminobutyric acid) mediates fast inhibitory transmission by interacting with GABA type A ($GABA_A$) receptors in the central nervous system. These ligand-gated ion channels are assembled from individual subunits forming a pentameric structure. A total of 19 isoforms of mammalian $GABA_A$ receptor subunits have been cloned: α1–6, β1–3, γ1–3, δ, ε, π, ρ1–3 and θ (Barnard *et al*., 1998; Simon *et al*., 2004). The subunit composition determines GABA sensitivity, sensitivity for benzodiazepines (BZDs), barbiturates, neurosteroids and anaesthetics (Sieghart, 1995; Hevers and Luddens, 1998; Sigel, 2002; Ernst *et al*., 2003, 2005) and also the gating properties of $GABA_A$ receptors (Feng *et al*., 2004; Boileau *et al*., 2003, 2005). The N-terminal parts of α- and β-subunits participate in the formation of the two agonist sites (Sigel *et al*., 1992; Amin and Weiss, 1993; Boileau and Czajkowski, 1999; Wagner and Czajkowski, 2001; Newell and Czajkowski, 2003). GABA binding to these sites leads to pore opening.

BZDs interact with amino-acid residues located at the interface between α- and γ-subunits (Macdonald and Barker, 1978; Sigel and Buhr, 1997). These drugs allosterically modulate activation of $GABA_A$ receptors either by increasing apparent affinity of at least one agonist-binding site (Gallager and Tallman, 1983; Serfozo and Cash, 1992; Lavoie and Twyman, 1996) or affecting the pore opening (Baur and Sigel, 2005). $GABA_A$ receptors carry two GABA-binding sites at the respective αβ interfaces (see Twyman *et al*., 1990). By selective disruption of the one or the other of these sites in concatenated $GABA_A$ receptors, it recently has been demonstrated (Baur and Sigel, 2005) that chloride currents were potentiated by diazepam in both cases.

Heterologous expression systems are the basis for drug development and pharmacological characterization of

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different $GABA_A$ receptor subtypes. Transfection of mammalian cells or cRNA injection into *Xenopus* oocytes with α1, β2 and γ2 subunits may, however, result in different mixtures of α1β2 and α1β2γ2 receptors (Baumann *et al*., 2001; Boileau *et al*., 2002, 2003), which complicates the unequivocal estimation of potencies and efficiencies of BZDs in heterologous expression systems. This problem can be partially overcome by injecting larger amounts of γ2 subunit cRNA (Boileau *et al*., 2002) or, alternatively, by making use of concatenated $GABA_A$ receptor subunits (Baumann *et al*., 2002; Minier and Sigel, 2004; Boileau *et al*., 2005).

We have previously reported that different efficiencies of BZDs to enhance chloride currents through $GABA_A$ receptors comprising γ1 or γ2S subunits are related to their ability to shift the GABA concentration–response curves towards higher GABA sensitivities (Khom *et al*., 2006). Here we analyse the relation between γ-induced inhibition of GABA sensitivity and GABA-induced chloride current (I_{GABA}) potentiation by three BZDs (triazolam, clotiazepam and midazolam) in oocytes expressing different populations of α1β2 and α1β2γ2S(γ1) subunit receptors. Correlation analysis yielded regression lines with slope factors reflecting higher drug efficiency in γ2S than in γ1 subunit-comprising receptors. GABA sensitivity and I_{GABA} potentiation of concatenated subunits (γ2-β2-α1 and β2-α1) fitted the regression lines of non-concatenated receptors supporting the hypothesis that their lower GABA sensitivity is due to complete γ2S incorporation (rather than forced subunit arrangement). A simulation supports the hypothesis that even under conditions where a higher ratio of γ2S cRNA relative to α1 and β2 cRNA is injected oocytes contain a significant population of α1β2 receptors.

Smaller slope factors of the regression lines were estimated for oocytes expressing receptors with lower GABA sensitivity (mutant β2-R207C; Wagner *et al*., 2004).

Materials and methods

Expression of $GABA_A$ receptors

Stage V–VI oocytes from *Xenopus laevis* were prepared and cRNA was injected as previously described by Khom *et al*. (2006). Female *X. laevis* (NASCO, WI, USA) were anaesthetized by exposing them for 15 min to a 0.2% MS-222 (methanesulphonate salt of 3-aminobenzoic acid ethyl ester; Sandoz, Germany) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymically digested with 2 mg mL^{-1} collagenase (Type 1A; Sigma, Vienna, Germany). Synthesis of capped run-off poly(A^+) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). At 1 day after enzymatic isolation, the oocytes were injected with 50 nL of water treated with diethylpyrocarbonate (Sigma) containing the different rat cRNAs at a concentration of approximately 300–3000 pg nL^{-1} per subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-Biotech, Steinfurt, Germany). To ensure expression of the γ-subunit with different incorporation in the case of α1β2γ1 and α1β2γ2S receptors cRNAs were mixed in a ratios: 1:1:1, 1:1:3 and 1:1:10. The double β2-23-α1 (β2-α1) and triple γ2-26-β2-23-α1 (γ2-β2-α1) concatemers (kindly provided by E Sigel) and the β2-R207C mutant (kindly provided by C Czajkowski) have been described previously (Baumann *et al*., 2001, 2002; Wagner *et al*., 2004). Oocytes were stored at 18 °C in ND96 solution (Methfessel *et al*., 1986). Voltage clamp measurements were performed between days 1 and 5 after cRNA injection.

Two-microelectrode voltage clamp studies

Electrophysiological experiments were performed by the two-microelectrode voltage clamp method making use of a TURBO TEC 01C amplifier (npi electronic GmbH, Tamm, Germany) at a holding potential of −70 mV. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7.4).

Perfusion system

GABA was applied by means of an automated fast perfusion system according to Baburin *et al*. (2006). To elicit I_{GABA} the chamber was perfused with 120 μL of GABA-containing solution at volume rates of 300 μL s^{-1}. Duration of washout periods was extended from 3 to up to 20 min with increasing concentrations of applied GABA to account for slow recovery from increasing levels of desensitization (see Khom *et al*., 2007 for details).

Analysing concentration–response curves

Concentration–response curves were generated and the data were fitted by non-linear regression analysis using Origin software (OriginLab Corporation, Northampton, MA, USA). Data were fitted to the equation: $1/(1+(EC_{50}/\mathrm{GABA})^{n_H})$, where EC_{50} is the concentration of the GABA that induces half-maximal GABA-evoked current and n_H is the Hill coefficient.

Concentration–response data for each oocyte were normalized to the maximum control GABA current for that oocyte. Statistical significance was calculated using unpaired Student's *t*-test with a confidence interval of $P<0.05$.

Calculation of EC_{50} and ΔEC_{50} of mixed population

The EC_{50} values and BZD-induced shifts in GABA sensitivity in oocytes expressing mixed receptor populations (different fractions of high GABA sensitive α1β2 versus low-sensitive α1β2γ2S receptors) are described by the concentration–response curves for each receptor population by:

$$R_{\alpha\beta} = \frac{i_{\alpha\beta} N_{\alpha\beta}}{1 + (EC_{50,\alpha\beta}/[\mathrm{GABA}])^{H_{\alpha\beta}}} \quad (1)$$

and

$$R_{\alpha\beta\gamma} = \frac{i_{\alpha\beta\gamma} N_{\alpha\beta\gamma}}{1 + (EC_{50,\alpha\beta\gamma}/[\mathrm{GABA}])^{H_{\alpha\beta\gamma}}} \quad (2)$$

where $R_{\alpha\beta}$ and $R_{\alpha\beta\gamma}$ are peak I_{GABA} currents (number of open channels in each population) at a given GABA concentration,

$i_{\alpha\beta}$ and $i_{\alpha\beta\gamma}$, amplitudes of single channel currents, $N_{\alpha\beta}$ and $N_{\alpha\beta\gamma}$ are numbers of channels characterized by $EC_{50,\alpha\beta}$ and $EC_{50,\alpha\beta\gamma}$, midpoints of concentration–response curves, $H_{\alpha\beta}$ and $H_{\alpha\beta\gamma}$—Hill coefficients.

The total normalized I_{GABA} is a weighted sum of partial current responses:

$$R_{total} = FR_{\alpha\beta\gamma} + (1 - F)R_{\alpha\beta} \quad (3)$$

where F is the fraction ($0<F<1$) of current through γ-containing $GABA_A$ receptors at saturating GABA concentrations: $F = (i_{\alpha\beta\gamma}N_{\alpha\beta\gamma})/(i_{\alpha\beta}N_{\alpha\beta} + i_{\alpha\beta\gamma}N_{\alpha\beta\gamma})$.

Under-application of a saturating concentration of BZD the receptor population consists of two subpopulations:

(1) $\alpha\beta$ receptors with unchanged $EC_{50,\alpha\beta}$ and $H_{\alpha\beta}$

and

(2) modulated $\alpha\beta\gamma$ receptors with enhanced GABA sensitivity (midpoint, $EC_{50,mod}$ and Hill coefficient, H_{mod}):

$$R_{mod} = \frac{i_{\alpha\beta\gamma}N_{\alpha\beta\gamma}}{1 + (EC_{50,mod}/[GABA])^{H_{mod}}} \quad (4)$$

The total current response is described now by:

$$R_{total} = FR_{mod} + (1 - F)R_{\alpha\beta} \quad (5)$$

The apparent midpoints of the GABA concentration–response curves (EC_{50}) for oocytes expressing a given fractions (F) of $\alpha1\beta2\gamma2S$ receptors and the shift of this curve (ΔEC_{50}(BZD)) at saturating concentrations of triazolam, clotiazepam and midazolam can be obtained by varying the fractions (F) from 0 to 1 by maximum likelihood.

Chemicals

Compounds were obtained from the following sources: triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4,3-a][1,4]benzodiazepine; Sigma), clotiazepam (5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2H-thieno[2,3-e][1,4]diazepin-2-one; Troponwerke, Köln, Germany), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine; Hoffmann La Roche, Basel, Switzerland).

Results

Modulation by BZDs of chloride currents through $GABA_A$ receptors expressed in *Xenopus* oocytes is dependent on the incorporation of a γ-subunit (Boileau *et al*., 2002). On the one hand injection of increasing amounts of γ-encoding cRNA (that is, $\alpha1$:$\beta2$:$\gamma2S$ ratios of 1:1:3 or 1:1:10) gradually reduces GABA sensitivity and, on the other hand, the increased fraction of $\alpha1\beta2\gamma2S$ receptors results in an increased I_{GABA} potentiation by BZDs. To quantify the relation between γ-subunit-mediated inhibition of GABA sensitivity and BZD action we have analysed modulation of GABA-induced chloride currents (I_{GABA}) in a large population of oocytes injected with different mixtures (1:1:1, 1:1:3, 1:1:10) of wild-type or mutated $\alpha1$, $\beta2$ and $\gamma2S$ or $\gamma1$ $GABA_A$ receptor subunits, or $\gamma2$-$\beta2$-$\alpha1$/$\beta2$-$\alpha1$ concatenated constructs. Triazolam, clotiazepam and midazolam were chosen because these drugs modulate $GABA_A$ receptors incorporating $\gamma2S$ and $\gamma1$ subunits that would allow us to compare their action at different efficiencies.

Modulation of I_{GABA} by BZDs in oocytes expressing variable ratios of $\alpha1\beta2$ versus $\alpha1\beta2\gamma1$ or $\alpha1\beta2\gamma2S$ receptors

Figure 1a illustrates the effect of triazolam on the concentration–response curves of I_{GABA} through $GABA_A$ receptors in oocytes injected with different ratios of cRNAs encoding for $\alpha1$, $\beta2$ and $\gamma2S$ subunits. A saturating concentration (1 μM) of triazolam shifted the GABA concentration–response curves leftwards without significant effects on the maximal response. Higher $\gamma2S$ expression and thus, presumably higher $\gamma2S$ incorporation resulted in reduced GABA sensitivity and larger triazolam-induced shifts of the GABA EC_{50} values (ΔEC_{50}(triazolam): 16 μM (cRNA ratio $\alpha1$:$\beta2$:$\gamma2S$ 1:1:1), 24 μM (1:1:3), 50 μM (1:1:10; Figure 1a, see also legend to Figure 1 for individual EC_{50} values).

The typical pattern of I_{GABA} modulation of an oocyte ($\alpha1$:$\beta2$:$\gamma2S$ cRNA ratio 1:1:3) is illustrated in Figure 1b. Triazolam enhanced I_{GABA} predominantly at low GABA concentrations corresponding to EC_{5-10} (concentrations of GABA that induce 5–10% of maximal GABA-evoked current) and had almost no effects at a saturating GABA concentration. Similar observations were made for $\alpha1\beta2\gamma1$ receptors (Figure 1c).

Different efficiencies of I_{GABA} potentiation in oocytes expressing $\alpha1\beta2\gamma1$ or $\alpha1\beta2\gamma2S$ receptors

Figure 1 illustrates that GABA potency (EC_{50}) and I_{GABA} modulation by triazolam were both dependent on the amount of $\gamma2S$ (or $\gamma1$) incorporation (see also Boileau *et al*., 2002). These experiments confirmed that apparent higher γ-expression and correspondingly lower GABA sensitivity were always associated with larger BZD-induced shifts of the curves. In line with Boileau *et al*. (2002, 2003) we observed a higher GABA sensitivity at cRNA ratios 1:1:1 and 1:1:3 compared to 1:1:10. The range of GABA EC_{50}s for a given cRNA ratio reflected differences in γ-incorporations.

EC_{50}s of individual oocytes were plotted versus the BZD-induced shifts of the GABA concentration–response curves (ΔEC_{50}(BZD)s; Figure 2). For all three compounds applied at saturating concentrations, we obtained a clear correlation between EC_{50}s and ΔEC_{50}(BZD)s. The slope factors for oocytes expressing $\alpha1\beta2\gamma1$ subunit receptors (1.8 ± 0.1 (triazolam; Figure 2a), 1.6 ± 0.1 (clotiazepam; Figure 2b) and 2.3 ± 0.2 (midazolam; Figure 2c)) were always higher than for oocytes expressing $\alpha1\beta2\gamma2S$ receptors (1.4 ± 0.1 (triazolam; Figure 2d), 1.4 ± 0.1 (clotiazepam; Figure 2e) and 1.3 ± 0.1 (midazolam; Figure 2f)). The regression line approached the ordinate in the range of the EC_{50} of $\alpha1\beta2$ receptors. The data suggest that the slope factors may reflect the efficiency of I_{GABA} potentiation for these compounds (see Table 1). For clotiazepam, we estimated similar slopes for $\gamma1$- and $\gamma2S$-incorporating receptors (difference statistically not significant; $P>0.05$) whereas for triazolam and

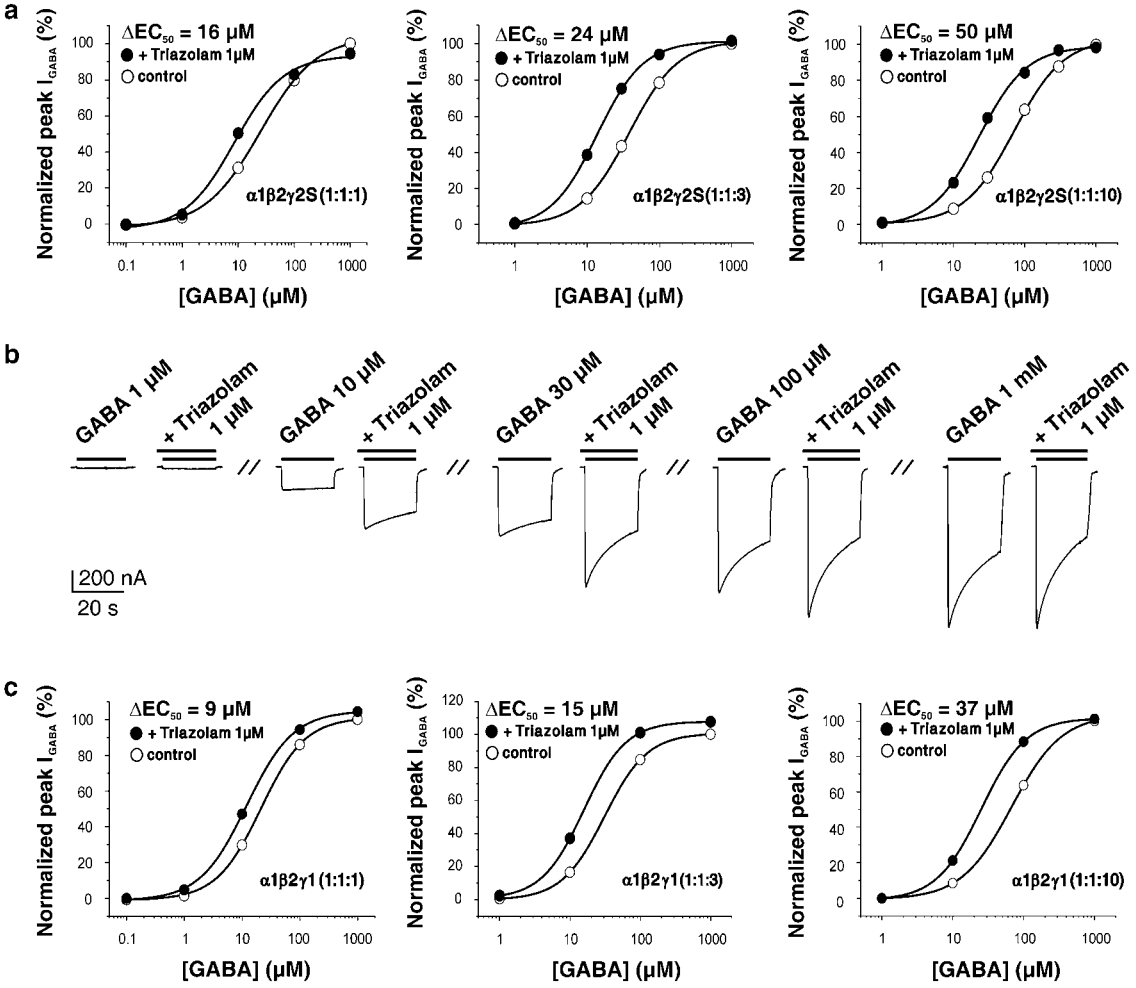

Figure 1 (**a**) Typical GABA concentration–response curves of oocytes injected with cRNAs of α1, β2 and γ2S subunits (cRNA stoichiometry: 1:1:1, left panel; 1:1:3, middle panel and 1:1:10, right panel) in the absence (control) and presence of 1 μM triazolam. The corresponding EC_{50} values for 1:1:1 were 24 μM (control), 8 μM (triazolam); for 1:1:3: 38 μM (control), 14 μM (triazolam) and for 1:1:10: 71 μM (control), 21 μM (triazolam). (**b**) Corresponding I_{GABA} through α1β2γ2S (1:1:3) channels modulated by 1 μM triazolam at indicated GABA concentrations. (**c**) GABA concentration–response curves of oocytes injected with cRNAs of α1, β2 and γ1 subunits (1:1:1, left panel; 1:1:3, middle panel and 1:1:10, right panel) in the absence (control) and presence of 1 μM triazolam. The corresponding EC_{50} values for 1:1:1 were: 21 μM (control), 12 μM (triazolam); for 1:1:3: 31 μM (control), 16 μM (triazolam); for 1:1:10: 66 μM (control), 29 μM (triazolam). Each graph in (**a**) and (**c**) represents one experiment on one oocyte.

midazolam the slopes of the regression lines for the two receptor subtypes were significantly different ($P<0.05$).

Pharmacological properties of concatenated subunits fit the extrapolated regression lines

If the inhibition of GABA sensitivity caused by γ-incorporation correlates with I_{GABA} potentiation, then complete γ-incorporation in concatenated subunits is expected to result in larger EC_{50}s. To test this hypothesis we first injected higher amounts of γ1 and γ2S subunit cRNA (1:1:20; see Figure 3). We obtained, however, only a non-significant ($P<0.05$) further reduction in GABA sensitivity (α1β2γ1: $EC_{50}=61\pm3$ μM, $n=4$ and α1β2γ2S: $EC_{50}=62\pm4$ μM, $n=4$, see dashed lines in Figure 3). Next we expressed a mixture of concatenated subunit constructs (γ2-β2-α1 and β2-α1, 1:1 ratio; Baumann *et al.*, 2002) and analysed the GABA concentration–response curves. Figure 3 compares the mean GABA concentration–response curves obtained for oocytes injected with cRNAs for α1β2 (1:1, $n=6$), α1β2γ1 (1:1:10, $n=24$), α1β2γ1 (1:1:20, $n=4$), α1β2γ2S (1:1:10, $n=27$), α1β2γ2S (1:1:20, $n=4$) or concatenated γ2-β2-α1/β2-α1 receptors (1:1, $n=18$). In line with previous studies (Baumann *et al.*, 2002) oocytes expressing concatenated subunits in *Xenopus* oocytes displayed a lower GABA sensitivity. The EC_{50} of concatenated subunits was substantially shifted to the right ($EC_{50}=186\pm13$ μM, $n=18$; Figures 3 and 4). The EC_{50}s and corresponding shifts of the concentration–response curve (ΔEC_{50}(BZD)s) for all three compounds are shown in Figures 4a–c. When EC_{50}s and corresponding ΔEC_{50}(BZD)s were added to the graph shown in Figure 2 (Figures 4d–f) they fitted the extended linear correlation obtained for non-concatenated subunits.

Estimation of α1β2 and α1β2γ2S receptor fractions

In an independent approach we theoretically calculated the behaviour of a mixed population of $GABA_A$ receptors with a

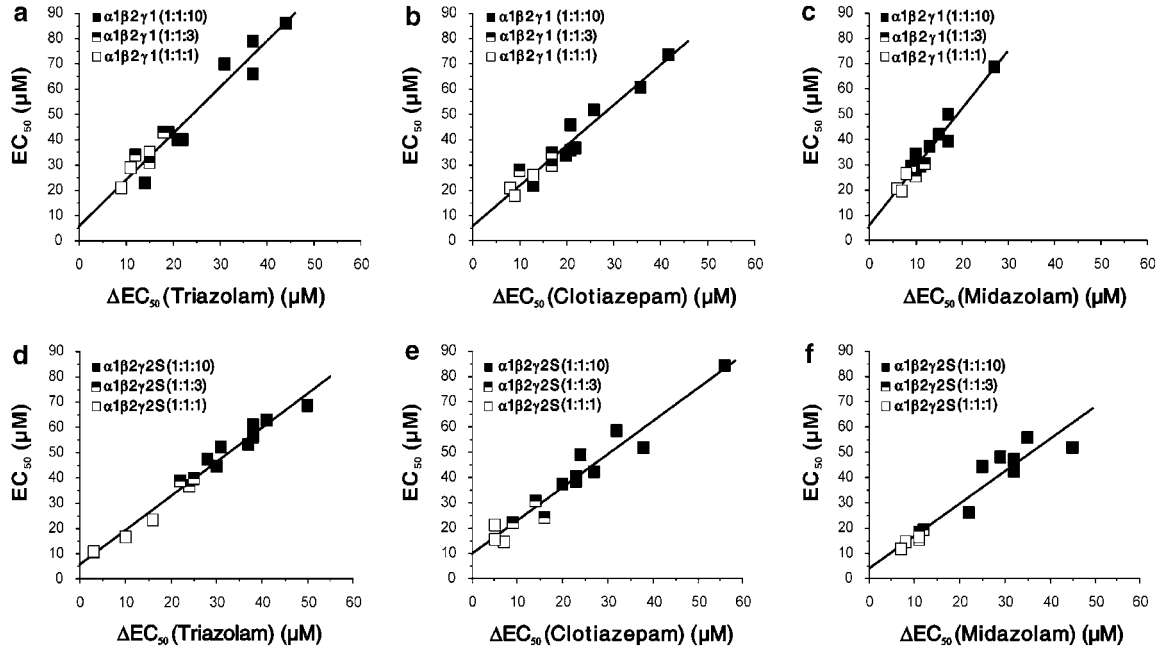

Figure 2 Correlation between EC_{50} values of the GABA concentration–response curves (EC_{50}s) of oocytes expressing $\alpha1\beta2\gamma1$ receptors (**a**–**c**), $\alpha1\beta2\gamma2S$ receptors (**d**–**f**) and shifts of these EC_{50} values (ΔEC_{50}(BZD)s) by modulation of the GABA concentration–response curve by 1 μM triazolam (**a**, **d**), 10 μM clotiazepam (**b**, **e**) and 10 μM midazolam (**c**, **f**); α-, β- and γ-subunit cRNA stoichiometries are 1:1:1, 1:1:3 and 1:1:10. Correlation coefficients were 0.94 (triazolam), 0.94 (clotiazepam), 0.94 (midazolam) ($P<0.0001$ in all cases, $\alpha1\beta2\gamma1$) and 0.97 (triazolam), 0.94 (clotiazepam), 0.90 (midazolam) ($P<0.0001$ in all cases, $\alpha1\beta2\gamma2S$). Each data point represents one experiment on one oocyte.

Table 1 Comparison of slope factors and maximal potentiation by BZDs

BZD	*α1β2γ1*		*α1β2γ2S*	
	Slope	*Maximum I_{GABA} potentiation (%)*[a]	*Slope*	*Maximum I_{GABA} potentiation (%)*[a]
Triazolam	1.8 ± 0.1	85 ± 7	1.4 ± 0.1	253 ± 12
Clotiazepam	1.6 ± 0.1	172 ± 24	1.4 ± 0.1	260 ± 27
Midazolam	2.3 ± 0.2	92 ± 8	1.3 ± 0.1	342 ± 64

Abbreviations: BZD, benzodiazepine; I_{GABA}, GABA-activated chloride currents.
[a]Data from Khom *et al*. (2006).

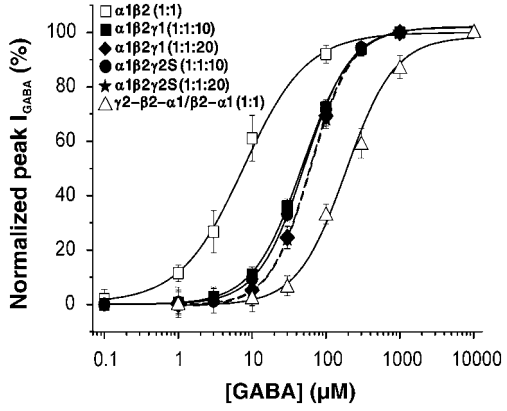

Figure 3 GABA concentration–response curves for oocytes expressing $\alpha1\beta2$ (cRNA injection 1:1), $\alpha1\beta2\gamma1$ (1:1:10), $\alpha1\beta2\gamma1$ (1:1:20), $\alpha1\beta2\gamma2S$ (1:1:10), $\alpha1\beta2\gamma2S$ (1:1:20) and γ2-β2-α1/β2-α1 (1:1) receptors. The corresponding mean EC_{50} values and Hill coefficients were $\alpha1\beta2$ (1:1): 8 ± 2 μM, $n_H = 1.0 \pm 0.2$ ($n=6$); $\alpha1\beta2\gamma1$ (1:1:10): 48 ± 3 μM, $n_H = 1.3 \pm 0.1$ ($n=24$); $\alpha1\beta2\gamma1$ (1:1:20): 61 ± 3 μM, $n_H = 1.5 \pm 0.1$ ($n=4$); $\alpha1\beta2\gamma2S$ (1:1:10): 51 ± 3 μM, $n_H = 1.4 \pm 0.1$ ($n=27$); $\alpha1\beta2\gamma2S$ (1:1:20): 62 ± 4 μM, $n_H = 1.5 \pm 0.1$ ($n=4$); γ2-β2-α1/β2-α1 (1:1): 186 ± 13 μM, $n_H = 1.3 \pm 0.1$ ($n=18$).

high ($\alpha1\beta2$) and a low ($\alpha1\beta2\gamma2S$) GABA sensitivity. The total normalized I_{GABA} is the weighted sum of partial current responses of the individual receptors and only $\alpha1\beta2\gamma2S$ are sensitive to BZDs (see Materials and methods). The simulated curves are shown in Figure 4 (solid lines). To calculate the putative fractions of $\alpha1\beta2$ and $\alpha1\beta2\gamma2S$ receptors for oocytes displaying different GABA sensitivities we made use of the concentration of half-maximal activation of the simulated curve of αβ receptors ($EC_{50,\alpha1\beta2} = 8 \pm 2$ μM, $n=6$; see also Sigel and Baur, 2000; Baumann *et al*., 2001) and $EC_{50,\alpha1\beta2\gamma2S}$ (186 μM) of concatenated receptors and corresponding Hill coefficients ($H_{\alpha1\beta2} = 1.0$, $H_{\alpha1\beta2\gamma2S} = 1.3$) from our experiments (see Figure 3).

The maximum likelihood fit of the EC_{50} versus ΔEC_{50} relation predicts 100% γ2S incorporation for the concatenated receptors (Figures 4d–f). Our calculations suggest, however, that even at a 1:1:10 cRNA ratio the fraction (F) of current through $\alpha1\beta2\gamma2S$ receptors accounts for only between 50 and 70% of the total current. If we assume that the single channel currents through γ incorporating receptors is n times larger than through $\alpha1\beta2$ receptors,

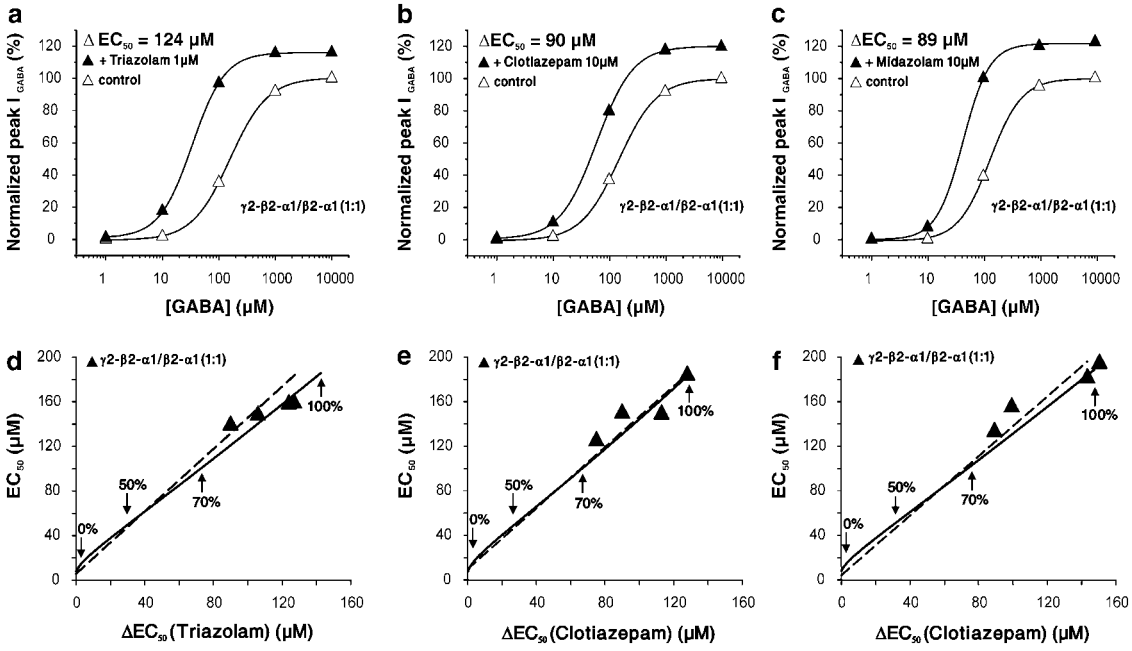

Figure 4 GABA concentration–response curves of oocytes injected with concatenated subunits (γ2-β2-α1/β2-α1) in the absence (control) and presence of 1 μM triazolam (**a**), 10 μM clotiazepam (**b**) and 10 μM midazolam (**c**). The corresponding EC_{50} values were: 158 μM (control), 34 μM (triazolam); 150 μM (control), 60 μM (clotiazepam); 132 μM (control), 43 μM (midazolam). EC_{50} values of the GABA concentration–response curves of oocytes expressing concatenated subunit constructs (γ2-β2-α1 and β2-α1 in 1:1 ratio) and ΔEC_{50}(BZD)s induced by (**d**) 1 μM triazolam, (**e**) 10 μM clotiazepam and (**f**) 10 μM midazolam were added to the regression lines from Figures 2d–f. Corresponding regression lines (dashed) were taken from Figures 2d–f and extended towards the values of the concatenated subunits. Solid lines (**d–f**) represent the non-linear fit of Equations 1–5 (see Materials and methods) by the maximal likelihood. Calculated current fractions of γ-incorporating receptors (0, 50, 70 and 100%) are indicated. Each graph (**a**, **b** and **c**) and each data point (**d**, **e** and **f**) represent one experiment on one oocyte.

then the fraction (f) of γ incorporating receptors is given by $f = F/(n - F(1-n))$.

This formula accounts for differences in single channel conductance. The frequency of openings and mean open time also influences mean conductance of both subpopulations. The equation describing concentration–response curve used in our calculation (see Materials and methods) estimates the fraction of open channels that in turn reflects the mean open time of single channels at different GABA concentrations and thus accounts for differences in open time. Assuming a twofold greater single channel conductance for α1β2γ2S receptors (that is, $n = 2$, see Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997) and $F \approx 70\%$ would predict $f \approx 54\%$. In other words, our analysis suggests that even under conditions where α1:β2:γ2S were injected at a ratio of 1:1:10 only about 50% of the expressed receptors contain a γ2S subunit. This may explain the large difference in EC_{50}s of concatenated and non-concatenated receptors (even at a ratio of 1:1:20; Figure 3).

Relation between EC_{50} and ΔEC_{50}(BZD) on a $GABA_A$ receptor mutant with reduced GABA sensitivity

Larger slope factors of γ1- versus lower slope factors of γ2S-containing receptors indicated that this parameter provides a measure of drug efficiency. To further validate the sensitivity of this parameter, we made use of a mutation (β2-R207C; located in the GABA-binding site), known to induce a reduction in GABA sensitivity (see also Wagner and Czajkowski, 2001; Wagner *et al.*, 2004). $GABA_A$ receptors composed of α1 and β2-R207C subunits displayed a reduced GABA sensitivity (Figure 5a; EC_{50} for α1β2-R207C = 486 ± 81 μM, $n = 6$; see also Wagner *et al.*, 2004). Co-expression of α1, β2-R207C with the γ2S subunit induced a further reduction of GABA sensitivity (Figure 5a; EC_{50} for α1β2-R207Cγ2S = 3217 ± 378 μM, $n = 18$). A saturating concentration of clotiazepam (10 μM) significantly increased chloride currents at GABA EC_{5-10} (Figure 5b). The corresponding correlation between individual EC_{50} values of the GABA concentration–response curves (EC_{50}s) of oocytes

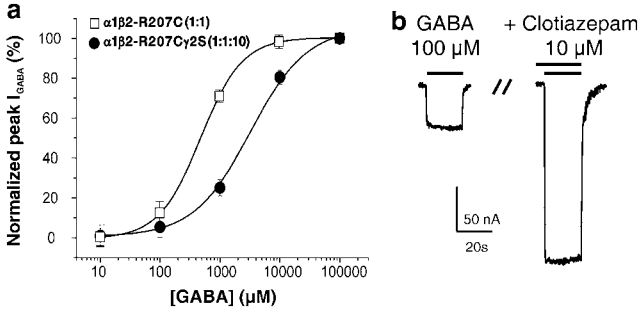

Figure 5 (**a**) GABA concentration–response curves of oocytes expressing α1β2-R207C (1:1, $EC_{50} = 486 \pm 81$ μM, $n_H = 1.2 \pm 0.2$, $n = 6$) and α1β2-R207Cγ2S (1:1:10, $EC_{50} = 3217 \pm 378$ μM, $n_H = 0.9 \pm 0.1$, $n = 18$) receptors. (**b**) Representative traces for enhancement of I_{GABA} through α1β2-R207Cγ2S. Control currents (GABA) in the absence of clotiazepam and corresponding currents elicited by co-application of GABA and clotiazepam are shown.

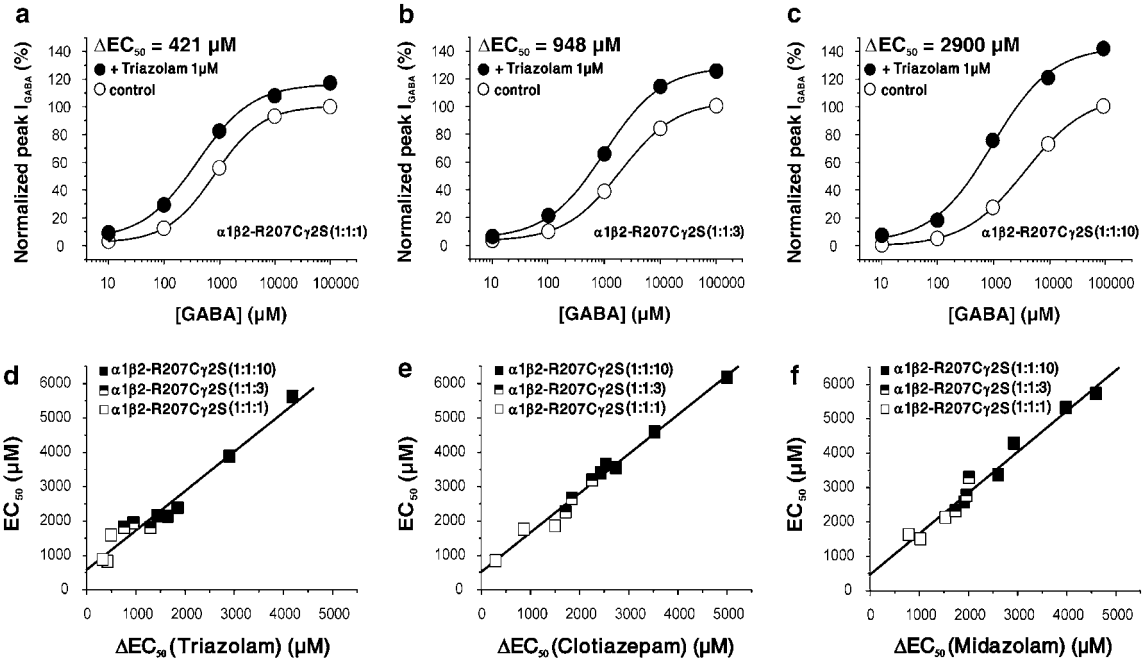

Figure 6 GABA concentration–response curves of oocytes injected with cRNA ratios of α1, β2-R207C and γ2S subunits of 1:1:1 (**a**), 1:1:3 (**b**) and 1:1:10 (**c**) in the absence (control) and presence of 1 μM triazolam. The corresponding EC_{50} values for 1:1:1 were: 827 μM (control), 406 μM (triazolam); for 1:1:3: 1945 μM (control), 997 μM (triazolam); for 1:1:10: 3891 μM (control), 991 μM (triazolam). (**d–f**) Correlation between individual EC_{50} values of the GABA concentration–response curves (EC_{50}s) of oocytes expressing α1β2-R207Cγ2S receptors and shifts of these EC_{50} values (ΔEC_{50}(BZD)s) caused by modulation of the GABA concentration–response curve by 1 μM triazolam (**d**), 10 μM clotiazepam (**e**) and 10 μM midazolam (**f**). The slopes of regression lines were 1.1 ± 0.1 (triazolam), 1.1 ± 0.1 (clotiazepam) and 1.2 ± 0.1 (midazolam). Correlation coefficients were 0.96 (triazolam), 0.98 (clotiazepam), 0.97 (midazolam) ($P<0.0001$ in all cases). Each graph (**a**, **b** and **c**) and each data point (**d**, **e** and **f**) represent one experiment on one oocyte.

expressing α1β2-R207Cγ2S receptors and shifts of these EC_{50} values (ΔEC_{50}(BZD)s) caused by three BZDs is shown in Figures 6d–f. The regression lines approach the EC_{50} axis in a range (460–587 μM; Figures 6d–f) close to the EC_{50} of α1β2-R207C receptors (Figure 5a). Figures 6a–c illustrate typical shifts of the dose–response curves by a saturating concentration of triazolam (1 μM) for different α1:β2-R207C:γ2S cRNA ratios (1:1:1—ΔEC_{50} = 421 μM, (a); 1:1:3—ΔEC_{50} = 948 μM, (b) and 1:1:10—ΔEC_{50} = 2900 μM, (c); see also legend to Figure 6 for individual EC_{50} values). The slopes of regression lines were 1.1 ± 0.1 (triazolam), 1.1 ± 0.1 (clotiazepam) and 1.2 ± 0.1 (midazolam). Interestingly, the three BZDs enhanced I_{GABA} at saturating GABA concentrations (10–100 mM) where peak currents in wild-type receptors were hardly affected (compare triazolam action in Figures 1 and 6).

Discussion

This study revealed a linear correlation between the γ-subunit-mediated suppression and BZD-induced increase of GABA sensitivity in α1β2γ2S and α1β2γ1 receptors. We made use of a statistical approach because direct evaluation of this relationship is complicated by variable γ-incorporation even under conditions where high ratios of γ-subunit cRNA relative to α1 and β2 are injected. Injection of higher cRNA ratio of α1:β2:γ2S than 1:1:10 induced only a slight further reduction in GABA sensitivity suggesting a non-linear relationship between cRNA ratios and γ-subunit incorporation (see 1:1:20 ratio in Figure 3 and also Boileau *et al*., 2002). We have therefore induced different γ-expression by injecting different cRNA ratios and analysed the relation between γ-mediated inhibition and BZD-induced enhancement of GABA sensitivity.

Slopes of regression lines are inversely related to BZD efficiency

An initially observed trend that larger BZD induced shifts of the concentration–response curves (ΔEC_{50}(BZD)s) in oocytes expressing larger fractions of γ-subunit-containing receptors (induced by injection of increasing amounts of cRNA encoding for γ-subunits, Figure 1) was confirmed by correlation analysis (Figure 2). The linear relationship between EC_{50} values and ΔEC_{50}(BZD) suggests that BZDs reduce a γ-subunit-mediated inhibition of GABA sensitivity. A slope of 1.0 would correspond to complete elimination of a γ-subunit-induced inhibition of GABA sensitivity and BZDs would correspondingly shift the concentration–response curve of α1β2γ1(2S) receptors to the position of α1β2 receptors. The observation that the tested BZDs never increased GABA sensitivity to that or above that of α1β2 receptors indicates that these drugs only partially can compensate for the γ-subunit-induced inhibition.

The estimated slope factors ranged from 1.1 ± 0.1 in α1β2-R207Cγ2S (triazolam, clotiazepam) to 2.3 ± 0.2 (midazolam) in α1β2γ1 subunit receptors. To evaluate the significance of the slopes we compared the slopes on oocytes expressing

$\alpha1\beta2\gamma1$ and $\alpha1\beta2\gamma2S$ subunit receptors. The present results support previously estimated maximal I_{GABA} potentiation by midazolam ($342 \pm 64\%$) > clotiazepam ($260 \pm 27\%$) ≈ triazolam ($253 \pm 12\%$) for oocytes expressing $\alpha1\beta2\gamma2S$ receptors (Khom *et al*., 2006) and indicate a reversed order of regression slopes (midazolam: 1.3 ± 0.1 < clotiazepam: 1.4 ± 0.1 = triazolam: 1.4 ± 0.1). Furthermore, steeper slopes of the regression lines for $\alpha1\beta2\gamma1$ compared to $\alpha1\beta2\gamma2S$ receptors specify that midazolam and to a lesser extent triazolam and clotiazepam increase GABA sensitivity less efficiently in $\alpha1\beta2\gamma1$ than in $\alpha1\beta2\gamma2S$ receptors (Figure 2), which confirms our previous studies (Khom *et al*., 2006). Taken together our data suggest that the slopes of the regression lines between ΔEC_{50}(BZD)s and EC_{50}s are inversely related to drug efficiency.

The described approach is time consuming as it requires the injection of different cRNA ratios and the measurements of a large number of concentration–response curves. Our experiments under different conditions (including different BZDs, γ-subunits and a mutation in the β2 subunit; Figures 2, 4 and 6) revealed, however, that the regression lines intercept the *y*-axis close to the EC_{50} of $\alpha1\beta2$ receptors. This finding prompted us to evaluate the possibility to use the *y*-intercept and three randomly selected data points (cRNA ratio 1:1:10; Figures 2d–f) for correlation analysis. The simplified procedure yielded regression lines with slopes that were statistically not significantly different from larger data sets (data not shown).

The relevance of the slope factor was further evaluated making use of the mutation β2-R207C that is known to induce a more than 60-fold reduction in GABA sensitivity (Wagner *et al*., 2004). Figure 5a illustrates that co-expression of γ2S decreased the GABA sensitivity analogously to wild-type receptors (Figure 3). The estimated slope factors (1.1 ± 0.1 (triazolam), 1.1 ± 0.1 (clotiazepam) and 1.2 ± 0.1 (midazolam); Figure 6) were always smaller than in wild type (though only in the case of triazolam and clotiazepam that difference was significant, $P < 0.05$), suggesting even higher BZD efficiencies compared to wild type.

Concatenated receptor subunits fit the regression lines

Forced γ2 subunit incorporation in oocytes was previously shown to be associated with a significant loss in GABA sensitivity compared to oocytes injected with 1:1:10 cRNA ratios (Baumann *et al*., 2002; see also Figure 3; but see Baur *et al*., 2006). Here we show that the higher EC_{50}s and corresponding ΔEC_{50}(BZD)s of concatenated subunits fit the predictions of the regression lines of non-concatenated subunits (Figures 4d–f, closed triangles). The mean EC_{50} of the concatenated γ2-β2-α1/β2-α1 subunits (Figure 3) corresponds to the data of Baumann *et al*. (2002). Lower GABA sensitivity and higher BZD efficiencies of concatenated subunits expressed in *Xenopus* oocytes may accordingly result from more complete γ-incorporation (rather than from forced subunit arrangement or interactions). Our data suggest that the studied $GABA_A$ receptor composed of two concatenated constructs may represent a model of completely assembled receptor with intact GABA- and BZD-binding sites. This hypothesis (based on the extrapolation of the regression line; Figure 4) requires, however, further studies.

These experimental data could be reproduced by a mathematical model describing BZD modulation in oocytes expressing different current fractions of high GABA sensitive $\alpha1\beta2$ versus low-sensitive $\alpha1\beta2\gamma2S$ receptors. Maximum likelihood analysis predicted (in line with the experimental observations) a linear relationship that enables the calculation of a putative fraction of $\alpha1\beta2\gamma2S$ receptors at a given EC_{50} and ΔEC_{50}(BZD). This calculation had, however, to account for the different single channel conductance of the two receptor subtypes (Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997). Interestingly these independent calculations reproduced not only the linear correlation but predicted the expected 100% γ2S incorporation for the concatemers and thus would support the experimental findings of the concatenated receptors and our conclusions.

Percentage of $\alpha1\beta2\gamma2$ receptors in oocytes expressing these subunits

Boileau *et al*. (2002) have clearly demonstrated that injecting higher ratios of γ2 subunit cRNA results in a purer population of $\alpha1\beta2\gamma2$ subunit receptors. In addition, they observed a remarkable run-down of BZD modulation, suggesting a decay of γ2 subunit-incorporating receptors over time after injection into oocytes that makes it difficult to compare data from different labs. Furthermore, small changes in the applied effective GABA concentration (usually between EC_3 and EC_{10}) can substantially affect the apparent BZD efficiency, thus further confounding a calculation of the percentage of expressed $\alpha1\beta2\gamma2S$ receptors. The method suggested above for determining the slope of the regression line, however, also allows the determination of the percentage of $\alpha1\beta2\gamma2S$ receptors. After correction for single channel conductances (assuming a twofold difference in the single channel conductance of $\alpha1\beta2$ and $\alpha1\beta2\gamma2S$ receptors, Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997), a 70% current ratio would correspond to about 50% of γ2S subunit incorporation.

The regression slopes are independent from variations in expression of $\alpha1\beta2$ versus $\alpha1\beta2\gamma2S(\gamma1)$ fractions or variations in experimental conditions (GABA concentration). The statistical analysis described here is based on the different shifts of the concentration–response curves and, therefore, independent of these possible experimental errors. The low percentage of $\alpha1\beta2\gamma2S$ receptors formed under the conditions used indicate that at least in the *Xenopus* oocyte system (but not necessarily in other heterologous expression systems or under native conditions) the efficiency of assembly of $\alpha1\beta2$ receptors might be comparable to or even higher than that of $\alpha1\beta2\gamma2S$ receptors, resulting in comparable amounts of $\alpha1\beta2$ or $\alpha1\beta2\gamma2S$ receptors even when high concentrations of γ-subunits are available.

Implications for the mechanism of BZD action

The different shifts in the GABA concentration–response curves for $GABA_A$ receptors with different subunit compositions or mutants can be interpreted in terms of a mechanism

where BZDs increase the microscopic affinity of the GABA-binding site or, alternatively, by a mechanism where BZDs facilitate channel gating (for example, Jones-Davis *et al*., 2005). The second scenario is supported by previous findings suggesting that BZD-like modulators enhance the amplitude of the GABA response by stabilizing the open channel conformation (Downing *et al*., 2005; Rusch and Forman, 2005; Campo-Soria *et al*., 2006).

We are tempted to speculate that the BZD-induced I_{GABA} increase reflects an increase in GABA efficacy (as defined by Colquhoun, 1998) by a direct transduction of the BZD effect to the channel region (Akabas, 2004; Ernst *et al*., 2005). An increase of efficacy from apparent low level (for example, in mutant β2-R207C or concatemers) could explain an increase of the maximal GABA response ('over-potentiation' of the BZDs on the α1β2-R207Cγ2S isoform or concatenated receptors) and a simultaneous shift of the concentration–response curve (Figures 4a–c and 6a–c; Colquhoun, 1998; Downing *et al*., 2005; Rusch and Forman, 2005; Campo-Soria *et al*., 2006). These theoretical considerations need, however, more experimental validation including the investigation of further mutations (for example, in the putative GABA-binding site and/or pore region) and the use of BZDs with a broad range of efficiencies.

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Conflict of interest

The authors state no conflict of interest.

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