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Low Expression in *Xenopus* Oocytes and Unusual Functional Properties of $\alpha_1\beta_2\gamma_2$ $GABA_A$ Receptors with Non-Conventional Subunit Arrangement

Roland Baur, Erwin Sigel*

Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland

* sigel@ibmm.unibe.ch

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Abstract

The major subunit isoform of $GABA_A$ receptors is $\alpha_1\beta_2\gamma_2$. The subunits are thought to surround an ion pore with the counterclockwise arrangement $\alpha_1\gamma_2\beta_2\alpha_1\beta_2$ as seen from the outside of the neuron. These receptors have two agonist sites and one high affinity drug binding site specific for benzodiazepines. Recently, this receptor was postulated to assume alternative subunit stoichiometries and arrangements resulting in only one agonist site and one or even two sites for benzodiazepines. In order to force a defined subunit arrangement we expressed a combination of triple and dual concatenated subunits. Here we report that these unconventional receptors express only small current amplitudes in *Xenopus* oocytes. We determined agonist properties and modulation by diazepam of two of these receptors that resulted in currents large enough for a characterization, that is, β_2-α_1-γ_2/α_1-γ_2 and β_2-α_1-γ_2/β_2-γ_2. The first pentamer predicted to have two benzodiazepine binding sites shows similar response to diazepam as the standard receptor. As expected for both receptors with a single predicted agonist site the concentration response curves for GABA were characterized by a Hill coefficient < 1. β_2-α_1-γ_2/β_2-γ_2 displayed a mM apparent GABA affinity for channel opening instead of the expected μM affinity. Based on their subunit and binding site stoichiometry, that contradicts all previous observations, their unusual functional properties and their very low expression levels in oocytes, we consider it unlikely that these unconventional receptors are expressed in neurons to an appreciable extent.

Introduction

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in mammalian brain [1–3]. The major isoform of this receptor is composed of α_1, β_2 and γ_2 subunits. The consensus subunit stoichiometry is $2\alpha_1$, $2\beta_2$ and $1\gamma_2$ [4,5], arranged $\alpha_1\gamma2\beta2\alpha1\beta2$ counterclockwise as seen from the synaptic cleft [6–9]. $\alpha_1\beta_2\gamma_2$ receptors have one benzodiazepine binding site and two GABA sites [10], located at the α_1/γ_2 and β_2/α_1 subunit interfaces, respectively in a homologous position [3]. The presence of more than one agonist site for GABA is also predicted from their

GABA concentration dependence that are characterized by a Hill coefficient of >1 (e.g. [8]). These $\alpha_1\beta_2\gamma_2$ receptors, unlike many other isoform containing δ or ε subunits [11–15], are thought to have a well-defined subunit arrangement. Subunit stoichiometry and arrangement are important as they govern functional properties [16]. Recently, unconventional subunit arrangements of $\alpha_1\beta_2\gamma_2$ receptors have been proposed [17]. Some of these receptors do not conform to the above stoichiometry as they contain $2\gamma_2$ subunits. Furthermore, these receptors are predicted to have one agonist site only and some of them two benzodiazepine sites. Functional properties of these novel receptors, such as EC_{50} and corresponding Hill coefficient for the agonist GABA and modulation by diazepam were not analyzed. We set out to determine these properties. While receptors conforming the conventional subunit arrangement could easily expressed in *Xenopus* oocytes, the receptors with unconventional arrangement form rather inefficiently, making their analysis difficult. Nevertheless, we describe the properties of some of them, but think that if these receptors are formed at all, they represent only a very minor part of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors.

Materials and Methods

Expression of $GABA_A$ receptors in xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80˚C. cRNA mixtures were prepared from these stock solutions and stored at −80˚C.

Animal experiments were carried out in strict accordance to the Swiss ethical guidelines, and have been approved by the local committee of the Canton Bern Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15). Surgery of female adult *Xenopus laevis* was done under anesthesia (0.2% tricaine solution). Oocytes were prepared, injected and defolliculated as described previously [18]. 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 RNA solution containing RNA. In the case of non-concatenated $\alpha1\beta2\gamma_2$ receptors, cRNA coding for α_1, β2 and γ_2 subunits were injected at a ratio of 0.5:0.5:2.5 fMol/oocyte. In case of concatenated receptors, oocytes were injected with cRNA coding for dual and triple subunits at 1.25 fMol each. Injected oocytes were incubated in modified Barth's solution at 18˚C for at least 24 h before the measurements.

Functional characterization of the $GABA_A$ receptors

We used a two-electrode voltage clamp amplifier in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 using the computer programs Chart (ADInstruments–Europe, Oxford, England). Electrodes were filled with 3 M KCl and had resistances of 0.5–0.8 MΩ. Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. The holding membrane potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4). Concentration response curves for the compounds were fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of the compound, EC_{50} the concentration eliciting half-maximal current amplitude, I_{max}

is the maximal current amplitude, I the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration-response curves. For all receptors studied, modulation was measured at a GABA concentration eliciting 1–3% of the maximal GABA current amplitude. GABA was applied twice alone, and 30 s application in combination with the different compounds. The duration of washout periods was 3 min in between agonist or agonist/drug aplications to prevent receptor desensitization. At the beginning of the experiments, GABA applications were repeated when the elicited current amplitude altered by >5%. Potentiation was calculated by the following equation: $(I_{Modulator + GABA}/I_{GABA} - 1) * 100\%$. The perfusion solution 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. This allowed fast changes in agonist concentration around the oocyte. The rate of change was estimated 70% in less than 0.5 s. The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination.

All data are from as a minimum of three different oocytes from at least two different batches of oocytes. Data represent mean ± SEM or SD as indicated.

Results and Discussion

The subunit arrangement of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor has been derived from subunit interface studies [6] and from work with concatenated subunits [7–9]. Fig 1 shows a this arrangement characterized by the presence of two β_2/α_1 subunit interfaces each harboring agonist sites for GABA and one α_1/γ_2 subunit interface harboring a benzodiazepine binding site. This is in agreement with the observation that receptors purified from bovine brain have more than one GABA site per benzodiazepine binding site [10]. GABA concentration response curves obtained for $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors also indicate the presence of more than one agonist site (e.g. [19]), as their Hill coefficient is > 1. In addition to the consensus receptor, Fig 1 shows

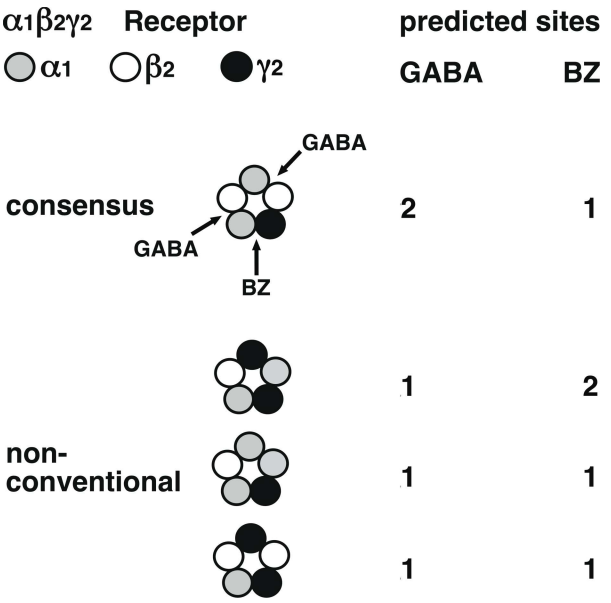

Fig 1. Consensus subunit arrangement of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors and three newly proposed receptors with unconventional subunit arrangement. Number of predicted agonist sites for GABA and modulatory sites for benzodiazepines (BZ) is shown for each receptor form. On the top left is the color code for the subunits.

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three unconventional receptors [17] with one predicted agonist site each, two of them with one, one of them with two predicted benzodiazepine binding sites. All these unconventional receptors do not conform to the well-established subunit stoichiometry of 2:2:1 for α_1, β_2 and γ_2 subunits [4,5]. We attempted to characterize the functional properties of these unconventional receptors.

Non-concatenated $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *Xenopus* oocytes. Current amplitudes elicited by 1 mM GABA were determined as about 15 μA. This current amplitude was compared with those resulting from three concatenated dual subunit constructs and three triple subunit constructs used later as part of pentameric receptors (Fig 2). While α_1-γ_2, β_2-γ_2, γ_2-β_2, α_1-β_2-α_1 and β_2-α_1-β_2 all resulted in the relative expression of less than 0.5% of the above current amplitude, β_2-α_1 and β_2-α_1-γ_2 resulted in 4.6% and 0.8%, respectively. This extent of current expression from single concatenated subunits was not observed in previous work [7]. The reason for the discrepancy is not clear.

Non-concatenated receptors were also compared with three concatenated receptors with the consensus subunit arrangement and with four concatenated receptors with unusual subunit arrangement (Fig 2). Data on β_2-α_1-γ_2/β_2-α_1 should be taken with care as the individual concatenated subunits result in current expression. All receptors with unusual subunit arrangement formed very inefficiently with expression levels well below 1%, with the exception of β_2-α_1-γ_2/β_2-γ_2 with 1.95% (Fig 2). For two of these receptors, namely β_2-α_1-γ_2/α_1 and α_1-γ_2/β_2, currents were too small for a further analysis. The observation that β_2-α_1-γ_2/α_1 fails to express has been made before [7]. In control experiments, β_2-α_1-γ_2/α_1 was expressed at a RNA ratio of 1:2. The current amplitude elicited by 1 mM GABA amounted to 108 ± 13 nA (mean ± SEM, n = 5). This is not significantly different from the same receptors expressed at an 1:1 ratio (Fig 2).

Agonist concentration-response curves were created for the non-conventional β_2-α_1-γ_2/α_1-γ_2 and β_2-α_1-γ_2/β_2-γ_2 receptors and for the triple construct β_2-α_1-γ_2. Both pentameric receptors are predicted to have only one agonist site (Fig 1). Fig 3A shows original current traces for β_2-α_1-γ_2/β_2-γ_2. After wash-out of GABA the current decreased in a bi-phasic manner. This unusual kinetic behavior was not observed with β_2-α_1-γ_2 and β_2-α_1-γ_2/α_1-γ_2. Fig 3B shows averaged concentration-response curves for β_2-α_1-γ_2, β_2-α_1-γ_2/α_1-γ_2 and β_2-α_1-γ_2/β_2-γ_2 receptors. EC_{50s} and Hill coefficients characterizing the corresponding curves are shown in Table 1. Parameters describing the concentration response curve of β_2-α_1-γ_2/α_1-γ_2 indicate efficient isomerization to the open state of the singly ligated receptor. The concentration dependence of β_2-α_1-γ_2/β_2-γ_2 receptors differs strongly from receptors built from non-concatenated α_1, β_2 and γ_2 subunits with an EC_{50} of about 40 μM and a Hill coefficient of about 1.4 [8]. This indicates that little if any β_2-α_1-γ_2/β_2-γ_2 receptors are built from non-concatenated α_1, β_2 and γ_2 subunits.

The fact that amplitude and agonist dependent properties of currents resulting from β_2-α_1-γ_2/α_1-γ_2 are similar to the ones resulting from β_2-α_1-γ_2 could suggest that two copies of β_2-α_1-γ_2 form a pentameric receptor with one β_2 hanging out, such that the pentamer would be β_2-α_1-γ_2/α_1-γ_2. However, the Hill coefficient characterizing the concentration response curve for GABA at β_2-α_1-γ_2 indicates that at least in some receptors the γ_2 subunit must hang out, to allow formation of a second agonist site.

Modulation of the currents by 1 μM diazepam was determined. As reported before [8] concatenated receptors with consensus subunit arrangement showed a larger stimulation than non-concatenated receptors (Fig 2). Presumably, injection of α_1, β_2 and γ_2 subunits even at a ratio of 1:1:5 results in diazepam responsive $\alpha_1\beta_2\gamma_2$ receptors and diazepam non-responsive $\alpha_1\beta_2$ receptors. As expected, receptors composed of concatenated β_2-α_1 and β_2-α_1-γ_2 showed reduced extent of modulation, as the current may partly be produced by the individual concatenated constructs, specifically by β_2-α_1.

$\alpha_1\beta_2\gamma_2$ Receptor α_1 β_2 γ_2		GABA I (nA)	n	%	DZ stim. (%)	n
α_1-γ_2		1 ± 1	15	0.01 %	n.d.	
α_1-β_2-α_1		65 ± 16	11	0.42 %	n.d.	
β_2-α_1		715 ± 112	11	4.6 %	n.d.	
β_2-γ_2		12 ± 2	10	0.08 %	n.d.	
β_2-α_1-β_2		3 ± 1	10	0.02 %	n.d.	
β_2-α_1-γ_2		125 ± 19	13	0.80 %	see below	
γ_2-β_2		42 ± 14	11	0.27 %	n.d.	
non-concatenated subunits						
α_1 / β_2 / γ_2		15580 ± 2050	6	100 %	223 ± 12	6
consensus receptors						
β_2-α_1-γ_2 / β_2-α_1		3240 ± 371	11	20.8 %	124 ± 58	7
β_2-α_1-β_2 / α_1-γ_2		14340 ± 1124	8	92.0 %	347 ± 46	9
α_1-β_2-α_1 / γ_2-β_2		14500 ± 790	12	93.0 %	371 ± 27	6
non-conventional receptors						
β_2-α_1-γ_2 / α_1-γ_2		102 ± 14	21	0.65 %	295 ± 48	7
α_1-γ_2 / β_2		30 ± 4	15	0.19 %	n.d.	
β_2-α_1-γ_2 / α_1		74 ± 9	15	0.47 %	n.d.	
β_2-α_1-γ_2 / β_2-γ_2		304 ± 71	15	1.95 %	222 ± 23	6

Fig 2. Dual and triple concatenated constructs used for the construction of pentameric receptors were expressed in *Xenopus* oocytes. Currents were standardized to the amplitude elicited in oocytes expressing non-concatenated α_1, β_2 and γ_2 subunits (non-concatenated receptors). The same parameters are shown for receptors with consensus subunit arrangement (consensus receptors) that

were built in three different ways and for three receptors with non-consensus subunit arrangement (non-consensus receptors), one of them built in two different ways. For some receptors, stimulation by 1 μM diazepam is also shown.

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Effects of diazepam at the non-conventional β_2-α_1-γ_2/α_1-γ_2, α_1-γ_2/β_2 and β_2-α_1-γ_2/β_2-γ_2 receptors were also determined. The first two receptors are predicted to have two sites for benzodiazepines. 1 μM diazepam by itself did not elicit any currents in β_2-α_1-γ_2/α_1-γ_2 receptors (n = 5) and α_1-γ_2/β_2 receptors (n = 3). Modulation of GABA current by the same concentration of diazepam in β_2-α_1-γ_2/α_1-γ_2 receptors was comparable to that in concatenated consensus receptors, in spite of the predicted presence of two benzodiazepine binding sites in a receptor. In β_2-α_1-γ_2/β_2-γ_2 receptors extent of modulation was slightly lower (Fig 2). Fig 4 shows averaged diazepam concentration-response curves from oocytes expressing β_2-α_1-γ_2, β_2-α_1-γ_2/α_1-γ_2, β_2-α_1-γ_2/β_2-γ_2 or α_1/β_2/γ_2 receptors. The curves were characterized by EC_{50}s of 65 ± 9 nM (mean ± SD, n = 3), 77 ± 20 nM (mean ± SD, n = 3), 88 ± 18 nM (mean ± SD,

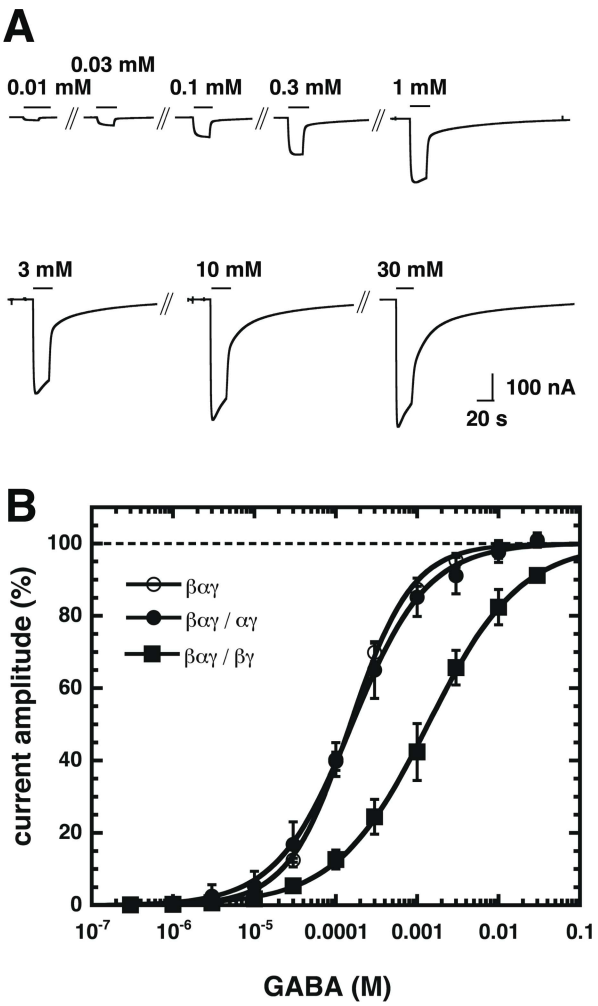

Fig 3. Concentration-response curves for β_2-α_1-γ_2, β_2-α_1-γ_2/α_1-γ_2 and β_2-α_1-γ_2/β_2-γ_2 receptors. Receptors were exposed to subsequently higher concentrations of GABA and the elicited current amplitude was determined. Individual curves were first normalized to the fitted maximal current amplitude and subsequently averaged. Data are expressed as mean ± S.D., n = 3–5 from two batches of oocytes. A) Original current traces recorded in an oocyte expressing β_2-α_1-γ_2/β_2-γ_2. B) Averaged Concentration-response curves for β_2-α_1-γ_2, β_2-α_1-γ_2/α_1-γ_2 and β_2-α_1-γ_2/β_2-γ_2.

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Table 1. Properties of GABA concentration response curves.

receptor	EC_{50} (μM)	Hill coeff.	n
β_2-α_1-γ_2	142 ± 18	1.14 ± 0.03	3
β_2-α_1-γ_2/α_1-γ_2	163 ± 35	0.97 ± 0.23	5
β_2-α_1-γ_2/β_2-γ_2	1420 ± 530	0.78 ± 0.02	3

EC_{50s} are given as mean ± SD.

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n = 3) and 59 ± 13 nM (mean ± SD, n = 3), respectively. Maximal current stimulation was similar for all constructs varying between 209 ± 31% for β_2-α_1-γ_2/ β_2-γ_2 and 285 ± 29% for α_1/β_2/γ_2. The fact that the curve for β_2-α_1-γ_2/α_1-γ_2 looks similar to that for α_1/β_2/γ_2 [8] indicates that the two predicted sites show no cooperativity or additivity of modulatory effects.

Concerning receptor subunit stoichiometry, the subset of $GABA_A$ receptors containing binding site for benzodiazepines, mainly $\alpha_1\beta_x\gamma_2$, $\alpha_2\beta_x\gamma_2$, $\alpha_3\beta_x\gamma_2$ and $\alpha_5\beta_x\gamma_2$, purified from bovine brain using a benzodiazepine affinity column [10] show a stoichiometry of agonist sites to benzodiazepine binding sites of 1.2–2.4. The stoichiometry of non-conventional $GABA_A$ receptors $\beta_2\alpha_1\gamma_2\alpha_1\alpha_1$, $\beta_2\alpha_1\gamma_2\alpha_1\gamma_2$ and $\beta_2\alpha_1\gamma_2\beta_2\gamma_2$ would be predicted to be 1:1, 0.5:1 and 1:1, respectively [17] and that of conventional $GABA_A$ receptors 2:1. The subunit stoichiometries of non-conventional $GABA_A$ receptors clearly do not agree to the reported stoichiometry.

In summary, the non-conventional $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors are not in agreement with literature data on subunit and binding site stoichiometry and functional properties. They show low expression levels in *Xenopus* oocytes. Functional properties, e.g very low GABA affinity for β_2-α_1-γ_2/β_2-γ_2 channel gating and low Hill coefficient for β_2-α_1-γ_2/α_1-γ_2 differ from those of non-concatenated $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in various expression systems. This together makes it very unlikely that non-conventional $GABA_A$ receptors of the subunit arrangement $\beta_2\alpha_1\gamma_2\alpha_1\alpha_1$, $\beta_2\alpha_1\gamma_2\alpha_1\gamma_2$ and $\beta_2\alpha_1\gamma_2\beta_2\gamma_2$ are being formed to any appreciable extent. Non-concatenated α_1, β_2 and γ_2 subunits as previously concluded most likely assemble to $\beta_2\alpha_1\gamma_2\beta_2\alpha_1$ $GABA_A$ receptors.

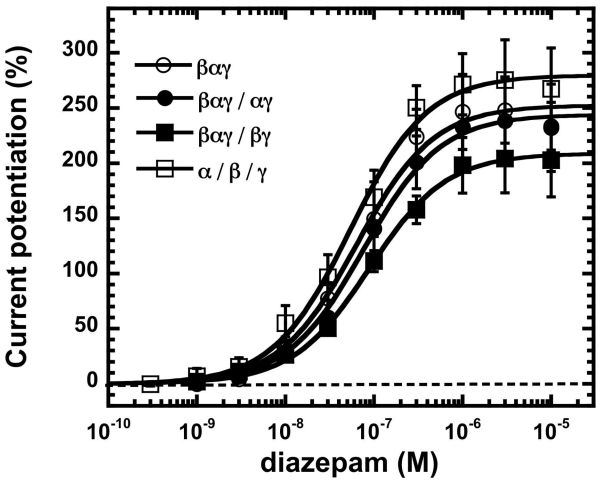

Fig 4. Stimulation of β_2-α_1-γ_2, β_2-α_1-γ_2/α_1-γ_2, β_2-α_1-γ_2/β_2-γ_2 and α_1/β_2/γ_2 receptors by diazepam. Receptors were exposed to subsequently higher concentrations of diazepam in combination with an EC_2 GABA concentration and the elicited current amplitude was determined. Individual curves were first normalized to the fitted maximal current amplitude and subsequently averaged. Data are expressed as mean ± S.D., n = 3 from two batches of oocytes.

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Author Contributions

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Data curation: ES.

Formal analysis: ES RB.

Funding acquisition: ES.

Investigation: RB.

Methodology: ES RB.

Project administration: ES.

Resources: ES.

Supervision: ES.

Validation: ES.

Visualization: ES RB.

Writing – original draft: ES.

Writing – review & editing: ES.

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