Pharmacological properties of GABA<sub>A</sub>- receptors containing gamma1-subunits

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Abstract

GABA<sub>A</sub> receptors composed of α<sub>1</sub>, β<sub>2</sub>, γ<sub>1</sub>-subunits are expressed in only a few areas of the brain and thus represent interesting drug targets. The pharmacological properties of this receptor subtype, however, are largely unknown. In the present study we expressed α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>-GABA<sub>A</sub> receptors in <i>Xenopus</i> oocytes and analysed their modulation by 21 ligands from 12 structural classes making use of the two-microelectrode-voltage-clamp method and a fast perfusion system. Modulation of GABA-induced chloride currents (<i>I<sub>GABA</sub></i>) was studied at GABA concentrations eliciting 5-10% of the maximal response. Triazolam, clotizepam, midazolam, CGS 20625, CGS 9896, diazepam, zolpidem, and bretazenil at 1 µM concentrations were able to significantly (>20%) enhance <i>I<sub>GABA</sub></i> in α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> receptors. 1 µM of DMCM, Cl 218,872, clobazam, flumazenil, Ru 33203, PK 9084, flurazepam, L-655,708, Ru 33356, Ru 32698 had no significant effect and flunitrazepam and PK 8165 inhibited <i>I<sub>GABA</sub></i>. The most potent compounds triazolam, clotizepam, midazolam and CGS 20625 were investigated in more detail on α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> receptors. The potency and efficiency of these compounds for modulating <i>I<sub>GABA</sub></i> was smaller for α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> than for α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> receptors and their effects on α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> could not be blocked by flumazenil. CGS 20625 displayed the highest efficiency by enhancing at 100 µM <i>I<sub>GABA</sub></i> (α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>) by 775±17% vs. 526±14% <i>I<sub>GABA</sub></i> (α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>) and 157±17% <i>I<sub>GABA</sub></i> (α<sub>1</sub>β<sub>2</sub>) (p<0.05). These data provide new insight into the pharmacological properties of GABA<sub>A</sub> receptors containing γ<sub>1</sub>-subunits and may aid design of specific ligands for this receptor subtype.
Introduction

γ-aminobutyric acid (GABA) is the principle inhibitory neurotransmitter in the mammalian brain. It mediates fast synaptic inhibition by interaction with the GABA type A (GABA_A) receptor. GABA_A receptors are ligand-gated ion channels that are modulated by a large number of clinically relevant drugs such as benzodiazepines (BZ), barbiturates, neurosteroids and anaesthetics (Sieghart, 1995). They are assembled from individual subunits forming a pentameric structure. Nineteen isoforms of mammalian GABA_A receptor subunits have been cloned: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3} and θ (Barnard et al., 1998; Simon et al., 2004). The major receptor subtype of the GABA_A receptor in adults consists of α_{1-}, β_{2-}, and γ_{2-} subunits and the most likely stoichiometry is two α-subunits, two β-subunits and one γ-subunit (Sieghart and Sperk, 2002).

The subunit composition determines the GABA-sensitivity and the pharmacological properties of the GABA_A receptor (Hevers and Lüddens, 1998; Sieghart 1995; Boileau et al., 2002). The subunit composition of the receptor also affects the time course of the GABA response (desensitization and deactivation of the chloride currents) (Boileau et al. 2003, Bianchi and MacDonald 2002, Feng et al. 2004). Mutation of amino acid residues in α and γ_{2-} subunits modulate the BZ-sensitivity of the receptor suggesting that the BZ binding pocket is located at the interface between α and γ_{2} (Sigel, 2002; Ernst et al., 2003). There is clear evidence that substitution of the γ_{2-} subunit by either γ_{1} or γ_{3} significantly alters the sensitivity for BZ (Hevers and Lüddens, 1998).

In contrast to the γ_{2-} subunit, that is ubiquitously expressed in the central nervous system, the γ_{1-} subunit is expressed in only a few areas of the brain such as the amygdala (central and medial nuclei), the pallidum, the septum, the substantia nigra and the thalamus (centrolateral and medial nuclei) (Pirker et al., 2000; Korpi et al., 2002). Compounds selectively interacting with receptors containing γ_{1-} subunits, thus, might have a substantial clinical potential.
Compared to receptors containing $\gamma_2$-subunits little is known about the pharmacological profile of GABA$_A$ channels composed of $\alpha_1$, $\beta_2$- and $\gamma_1$-subunits. Ymer et al. (1990) observed a loss in affinity for the benzodiazepine antagonist Ro 15-1788 and the inverse agonist DMCM, when the $\gamma_1$ was substituted for $\gamma_2$ in $\alpha_1\beta_1\gamma_2$- receptors. Negative modulatory effects of Ro15-4513, $\beta$-CCM and DMCM for GABA$_A$ receptors composed of $\alpha_{1/2}\beta_1\gamma_2$ subunits are changed to positive modulatory in $\alpha_{1/2}\beta_1\gamma_1$ receptors (Puia et al., 1991; Wafford et al., 1993).

Benke et al. (1996) observed a low affinity for clonazepam, zolpidem and flunitrazepam, and apparent insensitivity for flumazenil and Ro15-4513 for $\gamma_1$-containing receptors. Wafford et al. (1993) demonstrated a reduced enhancement of chloride currents through $\alpha_3\beta_2\gamma_3$ by diazepam, clonazepam and bretazenil compared to $\alpha_3\beta_2\gamma_2$ and a negative modulatory effect of zolpidem for $\alpha_2\beta_1\gamma_1$ and alpidem for $\alpha_3\beta_1\gamma_1$ receptors.

Overall, in 4 different studies a total of 14 compounds from 5 different compound classes have been investigated so far for their ability to modulate GABA$_A$- receptors containing $\gamma_1$-subunits. Unfortunately, most of these studies were carried out under different experimental conditions and with receptors containing different $\alpha$ and $\beta$-subunits combined with $\gamma_1$. Thus, the relative efficacies of these compounds for $\alpha\beta\gamma_1$ are not comparable (Hevers and Lüddens, 1998).

In the present study we analysed the modulation of $\alpha_1\beta_2\gamma_1$- receptors expressed in Xenopus oocytes by 21 compounds comprising distinct chemical structures. Triazolam, clotiazepam, midazolam and CGS 20625 exhibited a significant potency and efficiency whereas the other compounds were either inactive or displayed only a low potency on $\gamma_1$- containing receptors.
Materials and Methods

Chemicals

Compounds were obtained from the following sources: flunitrazepam (7-nitro-1,3-dihydro-1-methyl-5-o-fluorophenyl-2H-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flurazepam (7-chloro-1,3-dihydro-1-ethylaminodiethyl-5-o-fluorophenyl-2H-1,4-benzodiazepin-2-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine), Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), bretazenil (r-butyl(s)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a][1,4]benzodiazepine-1-carboxylate) (Hoffmann La Roche, Basle, Switzerland); L-655,708 (ethyl-7-methoxy-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate) (Hoffmann La Roche, Basle, Switzerland); clotiazepam (5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2H-thieno[2,3-e][1,4]diazepin-2-one) (Troponwerke, Köln, Germany); clobazam (7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione) (Hoechst, Frankfurt, Germany); triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4,3-a][1,4]benzodiazepine) (Sigma, Vienna, Austria); methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) (Ferrosan, Soeborg, Denmark); CGS 9896 (2-(4-chlorophenyl)-pyrazolo[4,3-c]quinolin-3-one), CGS 20625 (2-(4-methoxyphenyl)-2,3,5,6,7,8,9,10-octahydro-cycloheptabopyrazolo[4,3-d]pyridin-3-one) (Ciba Geigy, Summit, NJ, USA); zolpidem (N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide) (Synthelabo Recherche, Bagneux, France); Cl 218,872 (3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-b]pyridazine) (American Cyanamide Comp., Wayne, NJ, USA); Ru 31719 ((7-ethyl-5-methoxyimidazo[1,2-a]quinolin-2-yl)phenyl methanone), Ru 32698 (6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)phenylmethanone, Ru 33203 (5-(6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)-3-methyl-[1,2,4]oxadiazole), Ru 33356 (2-(6-ethyl-7-
methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)-4-methyl-thiazole) (Roussel Uclaf, Romainville, France); PK 8165 (2-phenyl-4-(4-ethyl-piperidinyl)-quinoline), PK 9084 (2-phenyl-4-(3-ethyl-piperidinyl)-quinoline) (Pharmuka Laboratories, Gennevilliers, France).

For chemical structures see Ogris et al., 2004.

Expression and functional characterization of GABA\(_A\) receptors

*Xenopus laevis* oocytes were prepared and injected as previously described (Grabner et al., 1996). Female *Xenopus laevis* (NASCO, USA) were anaesthetised by exposing them for 15 minutes to a 0.2 % MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A, Sigma). Preparation of stage V-VI oocytes from *Xenopus laevis*, synthesis of capped off run-off poly(A\(^+\)) cRNA transcripts from linearized cDNA templates (pCMV vector). One day after isolation the oocytes were injected with about 10-50 nl of a solution of DEPC\(_5\) (diethyl pyrocarbonate) water containing the different cRNAs at a concentration of approximately 300 - 3000 pg/nl/subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Steinfurt, Germany). To ensure expression of the gamma-subunit in the case of \(\alpha_1\beta_2\gamma_1\) and \(\alpha_1\beta_2\gamma_2\) receptors cRNAs were mixed in a ratio of 1:1:10 and for receptors comprising only \(\alpha_1\) and \(\beta_2\) subunits in a ratio 1:1 (Boileau et al., 2002).

Oocytes were stored at 18°C in ND96 solution (Methfessel et al., 1986). Electrophysiological experiments were performed by the two-electrode voltage clamp method making use of a TURBO TEC 01C amplifier (NPI electronics) 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 a modified version of a fast perfusion system according to Hering (1998). A schematic drawing of the perfusion chamber and drug application device is shown in Fig. 1 A. As previously described the voltage-clamp experiments on *Xenopus* oocytes were performed in a small (∼15 µl) bath that was covered by a glass plate. Two angular inlet channels in the glass cover (diameter < 1 mm) enabled access of the two microelectrodes to the oocyte. A funnel for drug application surrounded both access channels for the microelectrodes compared to a funnel surrounding a single access channel in Hering (1998). This modification increased the stability of oocyte perfusion.

Drug or control solutions were applied to the funnel by means of a TECAN Miniprep 60 that was controlled by an Axon DIGIDATA 1322A (Clampex version 9.2) permitting automation of the experiments (see also supplemental materials (files methods_1.avi and methods_2.avi) for animation of the solution exchange).

In order to estimate the rate of solution exchange independent of the ligand-receptor interaction we expressed Kv1.1 channels in *Xenopus* oocytes and analysed the time course of current decay during a rapid increase of the extracellular potassium concentration from 1 to 10 mM (sodium was reduced to 80 mM respectively). Fig. 1 B illustrates a typical Kv1.1 current during a voltage clamp step from −80 mV to +20 mV. The current decrease upon fast perfusion with 10 mM potassium at a speed of 1 ml/sec is shown on the right panel in Fig. 1 B. A mean current decline time t_{10-90%} of 140.8 ± 17.5 ms (n = 7) was estimated.

To elicit GABA-induced chloride currents (I_{GABA}) the chamber was perfused with 120 µl of GABA-containing solution at the same volume rate (1 ml/sec). The rise time of I_{GABA} ranged usually between 100 and 250 ms (see Fig. 1C) which is comparable with the rate of solution exchange estimated in Fig. 1 B.

After the initial fast perfusion step for rapid agonist application the chamber was continuously perfused at a rate of 1 µl/second for a total of 18 seconds. Before rapid washout of agonist
and/or drug the funnel was emptied by a suction pulse applied to the two funnel outlets (Fig. 1 A). The time between this suction pulse and the application of new solution to the funnel was < 1 s to avoid evaporation of the bath surrounding the oocyte.

Duration of washout periods was extended from 3 to 30 minutes with increasing concentrations of applied GABA to account for slow recovery from increasing levels of apparent desensitization. Oocytes with maximal current amplitudes > 3 µA were discarded to minimise voltage clamp errors.

**Analysing concentration – response curves**

Enhancement of chloride currents by modulators of the GABA<sub>A</sub> receptor was measured at a GABA concentration eliciting between 5 and 10 % of the maximal current amplitude (EC<sub>5-10</sub>). The EC<sub>5-10</sub> (usually ranging between 3 and 5 µM) was determined at the beginning of each experiment.

Enhancement of the chloride current (I<sub>GABA</sub>) was defined as (I<sub>(GABA+Comp)/I<sub>GABA</sub>)) – 1, where I<sub>(GABA+Comp)</sub> is the current response in the presence of a given compound and I<sub>GABA</sub> is the control GABA current. To measure the sensitivity of the GABA<sub>A</sub> receptor for a given compound it was applied for an equilibration period of 1 minute before concomitant application of GABA (EC<sub>5-10</sub>) and increasing concentrations of the compound. None of the compounds investigated was able to induce chloride flux in the absence of GABA.

Concentration-response curves were generated and the data were fitted by non-linear regression analysis using Microcol ORIGIN software. Data were fitted to the equation: 1/(1+(EC<sub>50</sub>/[Comp])<sup>n_H</sup>), where EC<sub>50</sub> is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% and n<sub>H</sub> is the Hill coefficient. Data are given as mean ± SE from at least 4 oocytes and ≥ 2 oocyte batches. Statistical significance was calculated using unpaired Student t-test with a confidence interval of p < 0.05.
Results

Modulation of $\alpha_1\beta_2\gamma_1$-receptors

The aim of the present study was to investigate the pharmacological properties of compounds interacting with $\alpha_1\beta_2\gamma_1$ GABA$_A$ receptors. We have therefore analyzed the modulation of this GABA$_A$ receptor subtype by 21 compounds from 12 different structural classes comprising 1,4-benzodiazepines (flunitrazepam, diazepam, flurazepam, midazolam, triazolam), 1,4-thienodiazepines (clotiazepam), 1,5-benzodiazepines (clobazam), imidazobenzodiazepines (flumazenil, bretazenil, L-655,708), $\beta$-carbolines (DMCM), pyrazoloquinolines (CGS 9896), pyrazolopyridines (CGS 20625), imidazopyridines (zolpidem), triazolopyridazines (Cl 218,872), imidazoquinolines (Ru 31719), imidazopyrimidines (Ru 32698, Ru 33203, Ru 33356) and quinolines (PK 8165, PK 9084).

In a first step GABA$_A$ receptors were activated by GABA concentrations corresponding to EC$_{5-10}$ and drug effects were screened at a single concentration of 1 $\mu$M. As illustrated in Fig. 2 (white bars) only the benzodiazepines triazolam, midazolam and diazepam, the thienodiazepine clotiazepam, the pyrazolopyridine CGS 20625 and the pyrazoloquinoline CGS 9896 induced an enhancement of $> 20\%$ at this concentration. The other compounds induced either a very small, but statistically significant enhancement (zolpidem and bretazenil), no statistically significant effect (DMCM, Cl 218,872, clobazam, flumazenil, Ru 33203, PK 9084, flurazepam, L-655,708, Ru 31719, Ru 33356 and Ru 32698) or even an inhibition of GABA-induced chloride currents (flunitrazepam and PK 8165), details are given in Tab. 1.

In order to establish compounds displaying low potency but high efficiency all compounds were subsequently tested at higher concentrations (10 $\mu$M, 100 $\mu$M) (see Fig. 2A). Modulation of GABA-receptors at low GABA concentrations (EC$_{5-10\%}$ close to “tonic concentrations”) can substantially differ from modulation at high concentrations (i.e. millimolar “synaptic concentrations”). We have, therefore, analysed the effects of all 21
compounds at 1 mM GABA (Fig. 3 A). None of the compounds, however, substantially enhanced or inhibited \( I_{\text{GABA}} \). Representative chloride currents induced by 1 mM in the absence or presence of 1 µM triazolam or CGS 20625 are shown in Fig. 3B.

**Contribution of \( \alpha_{1}\beta_{2} \)-receptors**

Previous studies have clearly shown that the extent of the incorporation of \( \gamma \)-subunits into heterologously expressed GABA\( _{A} \) receptors may vary between oocyte batches and decline with time (Boileau et al., 2002). To clarify whether effects observed were caused by \( \alpha_{1}\beta_{2}\gamma_{1} \) or by \( \alpha_{4}\beta_{2} \)-comprising receptors, we analysed the effect of these compounds on GABA\( _{A} \) channels composed of \( \alpha_{1}\beta_{2} \) subunits. With the exception of CGS 20625 (+20±5%), CGS 9896 (+16±7%), flumazenil (+13±5%) and flurazepam (+26±4%) all compounds (1 µM) were either inefficient in enhancing \( I_{\text{GABA}} \) or even induced significant inhibition (triazolam, clotiazepam, midazolam, Cl 218,872 and PK 8165, Tab. 1).

For the most potent stimulators of \( \alpha_{1}\beta_{2}\gamma_{1} \)-receptors triazolam, clotiazepam and midazolam we also analysed the inhibition of \( I_{\text{GABA}} \) in oocytes expressing only \( \alpha_{4}\beta_{2} \) subunits at higher (10 µM) concentrations. Triazolam inhibited the GABA-induced chloride flux in \( \alpha_{4}\beta_{2} \) receptors by -33±4%, (n=12), clotiazepam by -30±8% (n=7) and midazolam by -31±9% (n=5) respectively (experiments not shown).

**Comparing the effects of benzodiazepine site ligands on \( \alpha_{1}\beta_{2}\gamma_{1} \)- and \( \alpha_{1}\beta_{2}\gamma_{2} \)-receptors**

Triazolam, clotiazepam, midazolam and CGS 20625 were subsequently analysed in more detail by comparing their effects on \( \alpha_{4}\beta_{2}\gamma_{1} \) and \( \alpha_{4}\beta_{2}\gamma_{2} \) receptors. Fig. 4 illustrates the concentration dependency of the enhancement of the currents (EC\(_{50} \)) by triazolam, clotiazepam and midazolam. The half maximum enhancement (EC\(_{50} \)) was determined by fitting the concentration effect data to the Hill equation. Triazolam enhanced the maximum
chloride current of $\alpha_1\beta_2\gamma_1$ receptors by 85% while displaying the highest potency ($EC_{50} \approx 90$ nM) of all tested benzodiazepines. Clotiazepam elicited an enhancement of the GABA response of about 170%, but had a 19-fold lower potency ($EC_{50} \approx 1.7 \mu M$) than triazolam. Midazolam was more potent than clotiazepam ($EC_{50} = 1.2 \mu M$), but 13 times less potent than triazolam with a maximum enhancement (92%) comparable to triazolam.

A comparison with the concentration response data obtained on GABA$_A$ channels containing $\gamma_2S$-subunits reveals a 3-fold higher efficiency of triazolam, an 1.5-fold higher efficiency of clotiazepam and a 3.7-fold higher efficiency of midazolam on $\alpha_1\beta_2\gamma_2S$-receptors. The ratio of the $EC_{50}$ values for triazolam, clotiazepam and midazolam ($EC_{50}^{\alpha_1\beta_2\gamma_1}/EC_{50}^{\alpha_1\beta_2\gamma_2S}$) reflect 4-, 9- and 8-fold lower potencies of these compounds for $\alpha_1\beta_2\gamma_1$ receptors, respectively (see Table 2). The apparent $EC_{50}$ values, maximum enhancement, and the corresponding ratios for the benzodiazepines tested are given in Table 2.

**Modulation of $I_{GABA}$ by triazolam, clotiazepam and midazolam at different GABA concentrations**

In order to gain insight into the mechanism of $I_{GABA}$ enhancement we studied the GABA dose effect curves in the absence and presence of the modulators. The results are shown on Fig. 5 A-C. In control the mean $EC_{50}$ for GABA was $39 \pm 3 \mu M$ in $\alpha_1\beta_2\gamma_1$ receptors and $50 \pm 3 \mu M$ in $\alpha_1\beta_2\gamma_2S$ receptors. The three benzodiazepine receptor ligands shifted the dose effect curves to the left without affecting the maximal response (see also Fig. 3 A). Interestingly, the drug-induced shift was more pronounced for $\alpha_1\beta_2\gamma_2S$ than for $\alpha_1\beta_2\gamma_1$ receptors reflecting the higher efficiency of these ligands on $\alpha_1\beta_2\gamma_2S$.

**Effect of the pyrazolopyridine CGS 20625 on $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_1$- and $\alpha_1\beta_2\gamma_2S$-receptors**
CGS 20625 elicited maximum enhancement of chloride currents through $\alpha_1\beta_2\gamma_1$-receptors of approximately 645% with a half maximal enhancement occurring at about 20 µM (Fig. 6B). CGS 20625 thus, represents a low potency, but highly efficient positive modulator of $\alpha_1\beta_2\gamma_1$-receptors. This compound enhanced the GABA response of $\alpha_1\beta_2\gamma_{2S}$-receptors with comparable efficiency ($I_{\text{max/}2}/I_{\text{max/}1} \approx 1.12$) and was less efficient on $\alpha_1\beta_2$-receptors (Fig. 6B). At higher CGS 20625 concentrations ($\geq 300$ µM) we observed weaker enhancement of the GABA-induced chloride flux than at 100 µM for all subunit compositions (Fig. 6B).

**Effect of flumazenil on $\alpha_1\beta_2\gamma_1$-receptors**

Flumazenil is a ligand of the BZ-binding site of $\alpha_1\beta_2\gamma_{2-}$ GABA$_A$- receptors and competitively inhibits the enhancement of GABA-induced chloride currents by benzodiazepine agonists (Wafford et al., 1993). It was thus interesting whether this compound would also inhibit the effects of triazolam and clotiazepam on $\alpha_1\beta_2\gamma_1$-receptors.

Fig. 7 A, B (left panels) illustrates the inhibition by flumazenil of triazolam- or clotiazepam-induced $I_{\text{GABA}}$ enhancement in $\alpha_1\beta_2\gamma_{2S}$-receptors. As shown on the right panels, the effects of triazolam or clotiazepam on $\alpha_1\beta_2\gamma_1$-receptors, however, were not inhibited by 1 µM flumazenil. Moreover, we were unable to study possible antagonistic effects at higher concentrations because flumazenil induced significant enhancement of $I_{\text{GABA}}$ in oocytes expressing $\alpha_1\beta_2\gamma_1$-subunits at 10 µM (22±2%, n=4) and 100 µM (64±8%, n=4, see also Fig. 7C).


Discussion

In the present study we made use of a fast and automated perfusion technique (Fig. 1) to test a selection of 21 modulators on GABA\textsubscript{A} receptors composed of \(\alpha_1\beta_2\gamma_1\)-subunits. Solution exchange occurred between 100 and 250 ms (see Methods section, Fig. 1 C) which reduced effects of desensitization on peak current detection compared to conventional bath perfusion.

\(I_{GABA}\) of \(\alpha_1\beta_2\gamma_1\)-subunit receptors is enhanced by some benzodiazepines and the pyrazolopyridine CGS 20625

The 21 compounds tested comprised benzodiazepines and representatives of other structural classes of ligands of the BZ binding site of GABA\textsubscript{A} receptors. In order to determine the most potent modulators of \(\alpha_1\beta_2\gamma_1\)-subunit receptors we first tested the compounds at a concentration of 1 µM. 6 out of the 21 compounds induced an enhancement of the GABA-response (EC\textsubscript{5-10}) by more than 20% with the following order of potency: triazolam > clotiazepam > midazolam > CGS 20625 > CGS 9896 > diazepam. Zolpidem and bretazenil induced a small enhancement (\(\approx 13\%\) and \(\approx 10\%\) respectively). The effects of DMCM, CI 218,872, clobazam, flumazenil, Ru 33203, PK 9084, flurazepam, L-655,708, Ru 31719, Ru 33356, Ru 32698 were not significantly different from control. In contrast, flunitrazepam and PK 8165 significantly inhibited the GABA- induced chloride flux. Application of higher concentrations (10 and 100 µM) revealed a moderate enhancement at high concentrations (low potency modulation) by bretazenil, DMCM, flumazenil, Ru 33203, Ru 32698 and flunitrazepam. All compounds were tested in the absence of GABA (data not shown). None of them induced measurable currents even at high concentrations (up to 100 µM).

Thus, the 1,4- benzodiazepines triazolam and midazolam, the 1,4-thienodiazepine clotiazepam and the pyrazolopyridine CGS 20625 appeared as the most promising candidates for further detailed analysis.
Contribution of the $\gamma_1$-subunit to the enhancement of I_GABA

On injection of *Xenopus* oocytes with $\alpha$, $\beta$, and $\gamma$ subunits not only receptors containing all three subunits are formed but possibly also receptors composed of $\alpha$ and $\beta$ subunits, only (Boileau et al., 2002). To investigate whether the observed drug effects were due to effects on $\alpha_1\beta_2\gamma_1$ receptors or could also be explained by effects on $\alpha_1\beta_2$ receptors, the effects of drugs on the latter receptors were also investigated. A comparison of drug effects on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors - revealed that the $\gamma_1$-subunit was essential for enhancement of I_GABA by triazolam, clotiazepam and midazolam (Table 1, Fig. 2) because these compounds at 1 $\mu$M concentration induced a significant inhibition by $-21\pm 4\%$, ($n=12$, $p<0.05$), $(-14\pm 2\%$, $n=6$), and $(-20\pm 5\%$, $n=6$), respectively, of I_GABA through $\alpha_1\beta_2$-subunit receptors. In addition, the effects of several other drugs were clearly different in $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors. Thus, CGS 20625, CGS 9896, diazepam, zolpidem and bretazenil exhibited an enhancement of I_GABA that was higher in $\alpha_1\beta_2\gamma_1$ than in $\alpha_1\beta_2$ receptors, supporting the importance of the $\gamma_1$ subunit for the effects observed. Finally, CI 218,872 and flurazepam exhibited effects in opposite direction in $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors, again supporting the conclusion that the drug effects observed on injection of oocytes with $\alpha_1$, $\beta_2$, and $\gamma_1$ subunits were predominantly due to $\alpha_1\beta_2\gamma_1$ receptors (Tab.1).

Our finding that triazolam, clotiazepam and midazolam significantly inhibit I_GABA through $\alpha_1\beta_2$-receptors suggests that we might have underestimated the enhancement of I_GABA in $\alpha_1\beta_2\gamma_1$- receptors by these drugs due to simultaneously occurring inhibition of $\alpha_1\beta_2$-subunit receptors. The differential effects of various BZ binding site ligands on receptors composed of $\alpha_1\beta_2$-subunits is highly interesting by itself and significantly extends previous evidence for the existence of a low affinity benzodiazepine binding site at $\alpha\beta$ receptors (Walters et al., 2000; Wafford et al., 1993; Thomet et al., 1999).
In order to determine the potency and efficiency (maximum ability to enhance the GABA (EC$_{5-10}$ response) of the 3 most potent BZ (Table 2) we studied their concentration-effect for receptors composed of $\alpha_1\beta_2\gamma_1$-subunits (Fig. 3). The 1,4- benzodiazepine triazolam displayed the highest potency (EC$_{50}$ $\approx 90$ nM), followed by midazolam (EC$_{50}$=1.2 µM) and the 1,4-thienodiazepine clotiazepam (EC$_{50}$=1.7 µM, Table 2). A comparison of the concentration-effect curves for these benzodiazepine type ligands with receptors composed of $\alpha_1\beta_2\gamma_2S$-receptors revealed a significantly lower efficiency and potency for $\gamma_1$-containing receptors (Fig. 3, Tab. 2).

The pyrazolopyridine CGS 20625 displays the highest efficiency in enhancing GABA-induced chloride flux of $\alpha_1\beta_2\gamma_1$-subunit receptors

CGS 20625 was identified as the most efficient compound in terms of maximum enhancement of the GABA induced chloride currents through $\alpha_1\beta_2\gamma_1$-subunit receptors (Fig. 6). This compound induced a maximum enhancement of 645±55 % above control which is about 3.75-fold the enhancement achieved with clotiazepam (172±24 % above control), more than 7 times the enhancement induced by midazolam (92±8 % above control) or by triazolam (85±7 % above control), respectively (Fig. 3). CGS 20625 had, however, an about 200-times lower potency for $\alpha_1\beta_2\gamma_1$- receptors (EC$_{50}$ $\approx 20$ µM) than triazolam and a 10-20 times lower potency than the other benzodiazepines (Tab. 2).

A closer inspection of the subunit composition specificity of CGS 20625 action revealed that this drug potentiates $\alpha_1\beta_2\gamma_1$- and $\alpha_1\beta_2\gamma_2S$-subunit receptors to an almost similar extent (Fig. 6B). A significantly lower efficiency on $\alpha_1\beta_2$-receptors revealed, however, an essential role of a $\gamma$-subunit.

CGS 20625 at 300 µM caused less enhancement than at 100 µM in $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_1$- and $\alpha_1\beta_2\gamma_2S$-subunit receptors (Fig. 6B) suggesting that this compound might inhibit chloride
currents at high concentrations. Similar behaviour was previously shown for the action of another pyrazolopyridine (tracazolate) on GABA<sub>A</sub> channels (Thompson et al., 2002).

CGS 20625 thus represents a low potency (EC<sub>50</sub> ≈ 20 µM) but high efficiency modulator of α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>- and α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub>-subunit containing receptors (Fig. 6B). This compound was almost not selective for either γ<sub>1</sub>- or γ<sub>2</sub>-subunits. α<sub>1</sub>β<sub>2</sub>-subunit receptors were, however, stimulated to a significantly lesser extent (∼160%) as compared with α<sub>1</sub>β<sub>2</sub>γ<sub>1/2S</sub>-subunit receptors (640-730%) (Fig. 6B).

The competitive antagonist flumazenil (1 µM) inhibited I<sub>GABA</sub> enhancement of α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub>-receptors but failed to affect the enhancement of I<sub>GABA</sub> through α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>-receptors by triazolam and clotiazepam (Fig. 7, right panels). These data suggest that flumazenil exhibits either no or a very low affinity for the BZ binding site of α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>-receptors or that flumazenil interacts with a binding site different from that for triazolam and clotiazepam at these receptors. The first conclusion is consistent with the observation that the affinity of flumazenil for its binding site was reduced about 1000 fold in GABA<sub>A</sub> receptors in which phenylalanine 77 of the γ<sub>2</sub> subunit was mutated to the corresponding residue (isoleucine) of the γ<sub>1</sub> subunit (γ<sub>2</sub>F77I, Wingrove et al., 1997; Buhr et al., 1997; Ogris et al., 2004). At higher concentrations flumazenil displayed properties of a low affinity agonist on α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>-receptors (10 µM potentiated I<sub>GABA</sub> by 22±2 % and 100 µM by 64±9%).

In addition to flumazenil, the affinity of bretazenil, L-655,708, DMCM, zolpidem, Cl 218,872, and PK 8165 was drastically reduced in receptors containing the γ<sub>2</sub>F77I point mutation (Ogris et al., 2004) as measured by [<sup>3</sup>H]flunitrazepam binding studies. A low affinity of these compounds for α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub>-receptors could have contributed to their small effects on these receptors observed in the present study. In contrast, replacement of phenylalanine (γ<sub>2</sub>F77) by the corresponding isoleucine of the γ<sub>1</sub>-subunit only weakly (2-7 fold) reduced the affinity of the classical 1,4-benzodiazepines, of the 1,4-thienodiazepine clotiazepam, the 1,5-
benzodiazepine clobazam, or the pyrazoloquinoline CGS 9896 (Ogris et al., 2004). The small (4-9 fold) reduction in potency of triazolam, midazolam and clotiazepam for enhancing $\alpha_1\beta_2\gamma_1$- as compared to $\alpha_1\beta_2\gamma_2$-receptors could be explained by a reduced apparent affinity of these compounds for $\alpha_1\beta_2\gamma_1$-receptors underlining the importance of F77 for high affinity BZ binding (Buhr et al., 1997). For other compounds such as flunitrazepam (1 µM) we observed significant inhibition of $I_{GABA}$ in $\alpha_1\beta_2\gamma_1$-receptors indicating the importance of additional amino acids for drug binding and gating.

The different efficiency of triazolam, midazolam, clotiazepam and CGS 20625 are explained by the different amounts of shifts of the GABA concentration effect curves (Fig. 5 A-C). Larger shifts induced on $\alpha_1\beta_2\gamma_{2S}$- receptors reflect the higher apparent efficiency.

In summary we systematically investigated 21 ligands of the BZ-binding site from chemically distinct classes in order to obtain insight in the pharmacological profile of GABA_A- receptors comprising a $\gamma_1$- subunit. Triazolam was identified as a high-potency and CGS 20625 as a high- efficiency modulator of this receptor- subtype. Different potencies of triazolam, midazolam, clotiazepam and CGS 20625 can be explained by different shifts of the GABA dose effect curve reflecting different apparent affinities of these compounds.
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Footnotes

a.)
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Legends for figures

Figure 1
(A) Cross-section view of the oocyte perfusion chamber. Two microelectrodes (M1 and M2) are inserted into the angular access inlets in a glass plate (the inlets serve simultaneously as perfusion holes) covering the small (≈15 µl) oocyte chamber. Drug was applied by a TECAN Miniprep 60 application tube (AT) to a quartz funnel surrounding the microelectrode access holes (MAH). Residual solution was removed from the funnel before drug application via the funnel outlets. (see Hering, 1998 and two video files in supplementary materials for details).
(B) Estimation of the rate of solution exchange on an oocyte expressing Kv 1.1 the extracellular potassium concentration was rapidly increased from 1 to 10 mM during a potassium outward current (voltage step from -80 mV to +20 mV). The time of current decrease (right panel) from 10 to 90% (t10-90%) was taken as a measure of the volume rate of oocyte perfusion.
(C) Typical time courses of I_{GABA} (α_1β_2γ_2s-receptors) activated by different GABA concentrations. Upon application of 300 µM GABA I_{GABA} increased from 10 to 90% within 100 ms. At 100, 30 and 10 µM GABA t_{10-90%} was 140 ms, 180 ms and 230 ms respectively.

Figure 2
(A) Modulation of chloride currents through GABA_A receptors composed of α_1β_2γ_1- subunits by 1 µM (white bars), 10 µM (grey bars) and 100 µM (black bars) of the indicated compounds. Each value represents the mean ± S.E. from at least 4 oocytes and ≥ 2 oocyte batches. (*) indicates significantly different from zero (p < 0.05, t-test by ANOVA)
(B) Typical traces for enhancement of chloride currents through α_1β_2γ_1- channels by triazolam, clotiazepam and midazolam at EC_{50-10}. Control currents (GABA, single bar) and
corresponding currents elicited by co-application of GABA and the indicated compound (double bar) are shown.

**Figure 3**

(A) Modulation of chloride currents through GABA_A receptors composed of α_1β_2γ_1 subunits at 1 mM GABA by 1 µM of the indicated compounds. (*) indicates statistically significant differences from zero (p < 0.05, t-test by ANOVA)

(B) Typical enhancement of I_{GABA} through α_iβ_2γ_1- channels induced by 1 mM GABA in the absence (control, left traces) and presence of 1 µM triazolam and 1 µM CGS 20625.

**Figure 4**

Concentration-effect curves for triazolam, clotiazepam and midazolam on α_1β_2γ_1- (●) and α_1β_2γ_2s-receptors (■) using an EC_{50} GABA concentration (EC_{50} values are given in Table 2). Data points represent means ± S.E. from at least 4 oocytes from ≥ 2 batches.

**Figure 5**

Modulation of the GABA concentration response curve of α_1β_2γ_1- (left panels) and α_1β_2γ_2s-receptors (right panels) by triazolam 1 µM (A), clotiazepam 10 µM (B) and midazolam 10 µM (C). The corresponding mean EC_{50} values were (A) 37 ± 2 µM (control), 26 ± 4 µM (triazolam) for α_1β_2γ_1 and 52±7 µM (control), 17±3 µM (triazolam) for α_1β_2γ_2s, (B) 40 ± 8 µM (control), 25 ± 4 µM (clotiazepam) for α_1β_2γ_1 and 50± 5 µM (control), 19± 3 µM (clotiazepam) for α_1β_2γ_2s and (C) 41 ± 6 µM (control), 22 ± 2µM (midazolam) for α_1β_2γ_1 and 47±11 µM (control), 13±6 µM (midazolam) for α_1β_2γ_2s.
**Figure 6**

Modulation of $I_{\text{GABA}}$ by CGS 20625

(A) Typical $I_{\text{GABA}}$ recordings illustrating concentration-dependent modulation of GABA elicited chloride currents through $\alpha_1\beta_2\gamma_1$-containing receptors.

(B) Concentration-effect curves for CGS 20625 on (●) $\alpha_1\beta_2\gamma_{2S}$-, (▲) $\alpha_1\beta_2\gamma_1$- and (■) $\alpha_i\beta_2$- receptors. EC$_{50}$ and corresponding Hill coefficient were (●): 11.2 ± 0.7 µM, n$_H$=1.8 ± 0.1, (▲): 23.7 ± 6.8 µM; n$_H$=0.9 ± 0.1, (■): 4.3 ± 1.2 µM and n$_H$=1.4 ± 0.2 respectively. Each data point represents mean ± S.E. from at least 4 oocytes and ≥ 2 batches. $I_{\text{GABA}}$ at 300 µM (open symbols) were excluded from the fit.

(C) Modulation of the GABA concentration response of $\alpha_1\beta_2\gamma_1$- (left panel) and $\alpha_i\beta_2\gamma_2$- receptors (right panel) by 100 µM CGS 20625. The corresponding EC$_{50}$ values were in $\alpha_1\beta_2\gamma_1$-receptors 39 ± 16 µM (control) and 7 ± 2 µM (CGS 20625) and in $\alpha_i\beta_2\gamma_{2S}$-receptors 56 ± 14 µM (control) and 15 ± 3 µM (CGS 20625).

**Figure 7**

(A, B) Effect of flumazenil on the enhancement of $I_{\text{GABA}}$ by triazolam and clotiazepam.

The left graphs illustrates the enhancement of $I_{\text{GABA}}$ through $\alpha_1\beta_2\gamma_{2S}$-receptors by triazolam and clotiazepam in the absence and presence of flumazenil. The right graphs illustrate the lack of inhibition of $I_{\text{GABA}}$ through $\alpha_1\beta_2\gamma_1$-receptors.

(C) Typical $I_{\text{GABA}}$ through $\alpha_i\beta_2\gamma_1$-receptors in the absence (GABA) and presence of the indicated concentrations of flumazenil.
Table 1

Modulation of the GABA induced chloride currents by 1 µM of the indicated compounds for receptors composed of α₁β₂γ₁-subunits (second column) and α₁β₂-subunits (third column).

Each value represents the mean ± S.E. of at least 4 oocytes from ≥ 2 oocyte batches.

*difference from “0” was calculated by ANOVA.

<p>| Compound  | ( \alpha_1\beta_2\gamma_1 ) &amp; ( \alpha_1\beta_2 ) |
|-----------|-----------------|-----------------|
|           | ( I_{GABA(EC_{50})} ) potentiation (%) &amp; ( I_{GABA(EC_{50})} ) potentiation (%) |
|           | (by 1 µM)       &amp; (by 1 µM)       |
| Triazolam | 82 ± 10 *       &amp; -21 ± 4 *       |
| Clotiazepam| 53 ± 8 *       &amp; -14 ± 2 *       |
| Midazolam | 43 ± 4 *       &amp; -20 ± 5 *       |
| CGS 20625 | 42 ± 4 *       &amp; 20 ± 5 *        |
| CGS 9896  | 24 ± 3 *       &amp; 16 ± 7          |
| Diazepam  | 20 ± 3 *       &amp; 2 ± 2           |
| Zolpidem  | 13 ± 3 *       &amp; -5 ± 3          |
| Bretazenil| 10 ± 1 *       &amp; -1 ± 5          |
| DMCM      | 8 ± 4          &amp; 4 ± 2           |
| Cl 218872 | 6 ± 5          &amp; -10 ± 1 *       |
| Clobazam  | 5 ± 2          &amp; 7 ± 3           |
| Flumazenil| 4 ± 3          &amp; 13 ± 5          |
| Ru 33203  | 2 ± 2          &amp; -5 ± 5          |
| PK 9084   | -3 ± 3         &amp; -6 ± 4          |
| Flurazepam| -5 ± 3         &amp; 26 ± 4 *        |
| L-655708  | -7 ± 2         &amp; -8 ± 5          |</p>
<table>
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<th>Compound</th>
<th>Value 1 ± Standard Error</th>
<th>Value 2 ± Standard Error</th>
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</thead>
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<tr>
<td>Ru 31719</td>
<td>-7 ± 3</td>
<td>-10 ± 5</td>
</tr>
<tr>
<td>Ru 33356</td>
<td>-7 ± 3</td>
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<td>Ru 32698</td>
<td>-8 ± 2</td>
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<tr>
<td>Flunitrazepam</td>
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<td>-9 ± 5</td>
</tr>
<tr>
<td>PK 8165</td>
<td>-16 ± 2 *</td>
<td>-14 ± 2 *</td>
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Table 2

Potency and efficiency of triazolam, midazolam, clotiazepam and CGS 20625 for GABA_A receptors composed of α_1β_2γ_1- or α_1β_2γ_2s-subunits. The maximum enhancement (in percent of control) and EC_{50} values were calculated by fitting the data points to a standard concentration response curve. Right column represents ratios between EC_{50} values of α_1β_2γ_1- and α_1β_2γ_2s-receptors.

<table>
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<th>Compound</th>
<th>α_1β_2γ_1</th>
<th></th>
<th>α_1β_2γ_2s</th>
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<th>EC_{50}(γ_1)/EC_{50}(γ_2s)</th>
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<tr>
<td></td>
<td>EC_{50}, nM</td>
<td>Maximum potentiation, %</td>
<td>EC_{50}, nM</td>
<td>Maximum potentiation, %</td>
<td></td>
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<tr>
<td>Triazolam</td>
<td>92 ± 17</td>
<td>85 ± 7</td>
<td>22 ± 3</td>
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<tr>
<td>Midazolam</td>
<td>1150 ± 259</td>
<td>92 ± 8</td>
<td>143 ± 88</td>
<td>342 ± 64</td>
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<tr>
<td>Clotiazepam</td>
<td>1681 ± 432</td>
<td>172 ± 24</td>
<td>184 ± 88</td>
<td>260 ± 27</td>
<td>9.14</td>
</tr>
<tr>
<td>CGS 20625</td>
<td>23712 ± 6835</td>
<td>645 ± 55</td>
<td>11220 ± 722</td>
<td>724 ± 25</td>
<td>2.11</td>
</tr>
</tbody>
</table>
Figure 4

A

B

C

[Percent potentiation of I_{GABA}] vs [Triazolam] (nM)

[Percent potentiation of I_{GABA}] vs [Clotiazepam] (nM)

[Percent potentiation of I_{GABA}] vs [Midazolam] (nM)
Figure 5

A

\[ \alpha_1\beta_2\gamma_1 \]

Normalized peak \( I_{\text{GABA}} \) (%)

\[ \alpha_1\beta_2\gamma_{2s} \]

Normalized peak \( I_{\text{GABA}} \) (%)

[GABA] (μM)

B

Normalized peak \( I_{\text{GABA}} \) (%)

[GABA] (μM)

C

Normalized peak \( I_{\text{GABA}} \) (%)

[GABA] (μM)
Figure 7

A

\[ \alpha_1\beta_2\gamma_{2S} \]

\[ \alpha_1\beta_2\gamma_1 \]

Percent potentiation of \( I_{\text{GABA}} \)

- **Triazolam 20 nM**
- ++ Flumazenil 1 \( \mu \)M

B

Percent potentiation of \( I_{\text{GABA}} \)

- **Clotiazepam 300 nM**
- ++ Flumazenil 1 \( \mu \)M

C

- GABA
- GABA + 1 \( \mu \)M Flumazenil
- GABA
- GABA + 10 \( \mu \)M Flumazenil
- GABA
- GABA + 100 \( \mu \)M Flumazenil

\[ 20 \text{ s} \]