British Journal of Pharmacology (2000) 131, 843–850 

www.nature.com/bjp

# A novel positive allosteric modulator of the $GABA_A$ receptor: the action of (+)-ROD188

[1]Urs Thomet, [1]Roland Baur, [2]Rodolphe Razet, [2]Robert H. Dodd, [3]Roman Furtmüller, [3]Werner Sieghart & *,[1]Erwin Sigel

[1]Department of Pharmacology, University of Bern, CH-3010 Bern, Switzerland; [2]Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France and [3]Section of Biochemical Psychiatry, Department Psychiatry, University of Vienna, A-1090 Wien, Austria

**1** (+)-ROD188 was synthesized in the search for novel ligands of the GABA binding site. It shares some structural similarity with bicuculline. (+)-ROD188 failed to displace [$^3$H]-muscimol in binding studies and failed to induce channel opening in recombinant rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors functionally expressed in *Xenopus* oocytes.

**2** (+)-ROD188 allosterically stimulated GABA induced currents. Displacement of [$^3$H]-Ro15-1788 indicated a low affinity action at the benzodiazepine binding site. In functional studies, stimulation by (+)-ROD188 was little sensitive to the presence of 1 $\mu$M of the benzodiazepine antagonist Ro 15-1788, and (+)-ROD188 also stimulated currents mediated by $\alpha1\beta2$, indicating a major mechanism of action different from that of benzodiazepines.

**3** Allosteric stimulation by (+)-ROD188 was similar in $\alpha1\beta2$N265S as in unmutated $\alpha1\beta2$, while that by loreclezole was strongly reduced.

**4** (+)-ROD188 also strongly stimulated currents elicited by either pentobarbital or 5$\alpha$-pregnan-3$\alpha$-ol-20-one (3$\alpha$-OH-DHP), in line with a mode of action different from that of barbiturates or neurosteroids as channel agonists.

**5** Stimulation by (+)-ROD188 was largest in $\alpha6\beta2\gamma2$ ($\alpha6\beta2\gamma2 >> \alpha1\beta2\gamma2 = \alpha5\beta2\gamma2 > \alpha2\beta2\gamma2 = \alpha3\beta2\gamma2$), indicating a unique subunit isoform specificity.

**6** Miniature inhibitory postsynaptic currents (mIPSC) in cultures of rat hippocampal neurons, caused by spontaneous release of GABA showed a prolonged decay time in the presence of 30 $\mu$M (+)-ROD188, indicating an enhanced synaptic inhibitory transmission.

*British Journal of Pharmacology* (2000) **131,** 843–850

**Keywords:** GABA; $GABA_A$ receptors; *Xenopus* oocytes; HEK 293 cells; hippocampal neurons; voltage clamp; allosteric modulation

**Abbreviations:** GABA, $\gamma$-aminobutyric acid; $GABA_A$ receptors, $\gamma$-aminobutyric acid type A receptor; (+)-ROD188, (1R, 2′R)-1-(2,3,4,5-Tetrahydro-5-oxo-2-furyl)-2-N-(p-toluene-sulphonyl)-1,2,3,4-tetrahydroisoquinoline

## Introduction

GABA ($\gamma$-aminobutyric acid) is the most important inhibitory neurotransmitter, and mediates fast synaptic transmission in the central nervous system by acting at $GABA_A$ receptors. These receptors are heteropentamers formed from different subunit types (Nayeem *et al*., 1994; Chang *et al*., 1996; Tretter *et al*., 1997). So far 20 distinct isoforms have been discovered ($\alpha1-6$, $\beta1-4$, $\gamma1-3$, $\delta$, $\varepsilon$, $\pi$, $\theta$ and $\rho1-3$), with further diversity arising from alternative splicing (Burt & Kamatchi, 1991; Macdonald & Olsen, 1994; Dunn *et al*., 1994; Rabow *et al*., 1995; Davies *et al*., 1997; Hedblom & Kirkness, 1997; Barnard *et al*., 1998; Bonnert *et al*., 1999). The transmembrane subunits of the $GABA_A$ receptor belong to a superfamily of subunits of ligand gated ion channels, including those of the nicotinic acetylcholine receptor, the glycine receptor and the serotonin type 3 receptor. Biophysical and pharmacological properties of recombinant $GABA_A$ receptors depend critically on their subunit composition (Sigel *et al*., 1990; Rabow *et al*., 1995). *In situ* hybridization techniques demonstrate that different receptor subunits are expressed in distinct regions of the brain (Wisden *et al*., 1992; Persohn *et al*., 1992). Thus, it may be possible to develop novel compounds that are selective for one of the many subclasses of $GABA_A$ receptors.

The $GABA_A$ receptor bears several allosteric sites, among them those for benzodiazepines, barbiturates, neurosteroids, anaesthetics and loreclezole (Sieghart, 1995; Sigel & Buhr, 1997). In an attempt to find novel ways for activating the $GABA_A$ receptor we tested derivatives of bicuculline for action at the $GABA_A$ receptor. Bicuculline is a $GABA_A$ receptor antagonist and competitively inhibits GABA induced currents (Curtis *et al*., 1970). Additionally, it displays properties of an allosteric modulator as shown by inhibition of pentobarbital induced currents (Ueno *et al*., 1997). All derivatives surprisingly lost their ability to bind to the site for GABA and some of these interestingly potentiated currents elicited by GABA. The most potent compounds were arylsulphonyl-tetrahydro-isoquinolines, and their structure was further optimized, leading finally to a molecule that allosterically stimulated the $GABA_A$ receptor and had only a very weak affinity for the benzodiazepine binding site. One of these compounds was named (+)-ROD188 ((1R, 2′R)-1-(2,3,4,5-Tetrahydro-5-oxo-2-furyl)-2-N-(p-toluene-sulphonyl)-1,2,3,4-tetrahydroisoquinoline; Figure 1) and analysed in more detail. In the present study, we discuss the mode of action of (+)-ROD188 and compare it with that of other stimulatory ligands of the $GABA_A$ receptor.

*Author for correspondence at: Department of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland; E-mail: sigel@pki.unibe.ch

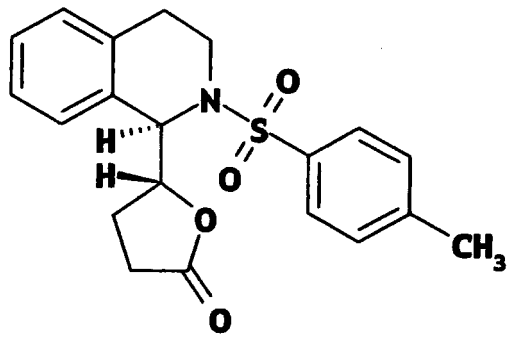

**Figure 1** Chemical structure of (+)-ROD188.

## Methods

### *Oocyte expression*

*Xenopus laevis* oocytes were removed from anaesthetized mature female frogs and manually separated from the ovary by a platinum loop under a light microscope. Prior to defolliculation by a collagenase treatment, the oocytes were injected with 50 nl of 5 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid)-KOH (pH 6.8), containing a mixture of cRNAs. All compounds were from Sigma or Fluka, unless indicated otherwise in the text. For dual subunit combinations a concentration of 100 nM was used for each transcript ($\alpha$1, $\beta$2 and the point mutant $\beta$2N265S). For triple subunit combinations 50 nM of the cRNA coding for the different $\alpha$ subunits ($\alpha$1, $\alpha$2, $\alpha$3, $\alpha$5 or $\alpha$6), 50 nM for $\beta$2 and 150 nM for $\gamma$2 or the mutant $\gamma$2F77I were injected. A detailed description of the isolation, culturing, injection and defolliculation of the oocytes is given elsewhere (Sigel, 1987; Sigel *et al*., 1990). The point mutants used were constructed as described previously (Buhr *et al*., 1997). Number of amino acid residues correspond to the mature rat subunit isoforms. After injection the oocytes were incubated at 18°C and used for electrophysiological recordings within 1–4 days.

### *Dissociated hippocampal cell culture*

Both hippocampi were dissected from the brain of one neonatal Sprague-Dawley rat (P1-3) and processed according to a protocol modified from Malgaroli & Tsien (1992) and Ryan *et al*. (1993). Briefly, the tissue was incubated in 3.4 mg $ml^{-1}$ trypsin type IX and 0.9 mg $ml^{-1}$ DNAse type IV, then mechanically dissociated in $Ca^{2+}$-free Hank's solution supplemented with 12 mM $MgSO_4$, 0.4 mg $ml^{-1}$ DNAse and 3 mg $ml^{-1}$ BSA. The cell suspension obtained was centrifuged twice ($80 \times g$) and resuspended to estimate cell density on a Coulter counter. Hippocampal cells were then plated at a density of 715 $mm^{-2}$ on poly-L-ornithine-coated coverslips within a cloning cylinder (28 $mm^2$) attached to the coverslip with high vacuum silicon grease. The cells were incubated in minimal essential medium, supplemented with 292 mg $l^{-1}$ glutamin, 5000 mg $l^{-1}$ glucose, 25 mg $l^{-1}$ insulin, 100 mg $l^{-1}$ transferrin, 5 mg $l^{-1}$ gentamycin and 10% foetal calf serum. Cultures were maintained in a $CO_2$ enriched atmosphere (6.5%) at 37°C. On the second day *in vitro*, the serum concentration in the medium was reduced to 5% and cytosine $\beta$-D-arabinofuranoside and B27 supplement were added to a final concentration of 1 $\mu$M and 2%, respectively. The medium was changed every 2–3 days.

### *Culture and transfection of HEK 293 cells*

Human embryonic kidney 293 cells (HEK 293 cells) were cultured in minimum essential medium (GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% foetal calf serum, 2 mM glutamine, 50 units $ml^{-1}$ penicillin and 50 $\mu$g $ml^{-1}$ streptomycin, using standard cell culture techniques. The cDNAs coding for the $\alpha$1, $\beta$2 and $\gamma$2 subunits of the rat $GABA_A$ receptor channel have been described elsewhere (Malherbe *et al*., 1990). Equal amounts (total of 4 $\mu$g of DNA per 35 mm dish) of subunits were transiently transfected into HEK 293 cells (American Type Culture Collection, Rockville, MD, U.S.A., CRL 1573) through the calcium phosphate precipitation method (Chen & Okayama, 1988). A plasmid encoding the green fluorescence protein (phGFP, Clontech) was cotransfected as a reporter. After overnight incubation, the cells were washed twice with serum-free medium and fed again with medium. Cells were ready for electrophysiological studies approximately 24–48 h after transfection.

### *Electrophysiological measurements in* Xenopus *oocytes*

Oocytes were placed on a nylon grid in a 0.4 ml bath and perfused by a gravity-driven system throughout the experiment at 6 ml $min^{-1}$ with a modified Barth's medium consisting of (mM): NaCl 90, KCl 1, $MgCl_2$ 1, $CaCl_2$ 1, HEPES–NaOH 5, pH 7.4. Oocytes were impaled with two 0.3–1 MΩ resistance microelectrodes containing 3 M KCl and the membrane potential was clamped at −80 mV (two-electrode voltage-clamp). Experiments were performed at room temperature (22–29°C). Modulation was quantified at a GABA concentration eliciting alone approximately 5% of the maximal current amplitude ($EC_5$) measured at 10 mM GABA ($I_{max}$). $EC_5$ was established by determining $I_{max}$ first and subsequently titrating with GABA until the desired relative response was obtained. A full GABA concentration-response curve was not determined for each of the analysed subunit combinations. But our measurements of the respective $EC_5$ of different subunit combinations are in line with previous observations that dual subunit combinations have an about 7 fold higher apparent affinity of GABA compared to triple combinations (Sigel *et al*., 1990). Drugs were bath applied 30–40 s at least until the peak of the response was observed. Between two applications, oocytes were perfused with the modified Barth's medium (see above) for at least 3 min to ensure full recovery from desensitization. GABA activated currents were recorded *via* a x-y-plotter for analysis.

### *Electrophysiological measurements in hippocampal cell cultures*

Randomly occurring miniature postsynaptic currents (mIPSCs) through $GABA_A$ receptor channels were measured using the patch-clamp technique in the whole cell configuration at a holding potential of −60 mV. Hippocampal cell cultures (days 14–21 *in vitro*) were transferred to a bath solution containing (mM): NaCl 140, KCl 5, $MgCl_2$ 4, $CaCl_2$ 1, D-(+)-glucose 10 and HEPES–NaOH 10 (pH 7.4). To block non-NMDA- and NMDA-mediated glutamatergic synaptic currents, measurements were carried out in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 $\mu$M; Tocris, Nr. 0190) and D(−)-2-amino-5-phosphonopentanoic acid (APV, 10 $\mu$M; Tocris, Nr. 0106). Action potentials were blocked by tetrodotoxin (TTX, 0.1 $\mu$M). Recording pipettes (2–5 MΩ) were filled with a solution consisting of (mM): CsCl 135, $MgCl_2$ 4, $CaCl_2$ 1, ethylene glycol-O,O-bis(2-aminoethyl)-N, N, N, N-

tetraacetic acid (EGTA) 5, $Na_2ATP$ 3 and HEPES–CsOH 10 (pH 7.4). These postsynaptic events could be blocked with 10 $\mu$M of the $GABA_A$ receptor antagonist bicuculline (not shown). Spontaneous mIPSCs either in absence or in presence of 30 $\mu$M (+)-ROD188 were recorded during periods of 1–3 min using an EPC-7 patch-clamp amplifier (List Medical Instruments, Darmstadt, Germany). Synaptic activity was recorded on digital tapes and then replayed, filtered at 2 kHz (EF5-01 Barr and Stroud low pass filter, −3 dB, 8-pole modified Butterworth) and digitized at 10 kHz (Digidata 1200 interface, Axon Instruments) for analysis. Rise and decay times (monoexponential fits) as well as peak amplitudes were measured using pCLAMP 6 software from Axon Instruments. The paired sample *t*-test was used for statistical evaluation.

### *Binding assays*

For binding studies HEK 293 cells (American Type of Culture Collection, Rockville, MD, U.S.A., CRL 1573) were maintained in Dulbecco's modified Eagle's medium (GIBCO–BRL, Grand Island, NY, U.S.A.) supplemented with 10% foetal calf serum (JHR Biosciences, Lenexa, KS, U.S.A.), 2 mM glutamine, 50 $\mu$M $\beta$-mercaptoethanol, 100 units $ml^{-1}$ penicillin G and 100 $\mu g\ ml^{-1}$ streptomycin in 75-$cm^2$ Petri dishes by using standard cell culture techniques.

$3 \times 10^6$ HEK 293 cells were transfected with a total of 21 $\mu$g cDNA encoding for the rat $\alpha$1-, $\beta$2-, and $\gamma$2-subunits (ratio 1 : 1 : 1) subcloned individually into pCDM8 expression vectors, using the calcium phosphate precipitation method (Chen & Okayama, 1988). The medium was changed 20 h after transfection and the HEK 293 cells were harvested 48 h after transfection by scraping into phosphate buffered saline. Cells were centrifuged at $12{,}000 \times g$ for 10 min and the cell pellet was homogenized in 50 mM Tris-citrate buffer, pH 7.4 by using an Ultraturrax, followed by three centrifugation ($200{,}000 \times g$ for 20 min) resuspension cycles, and were then used for ligand binding studies or were stored at −20°C.

For binding assays, membranes from rat cerebellum and forebrain or membranes from transiently transfected HEK 293 cells were centrifuged and resuspended in 50 mM Tris-citrate buffer, pH 7.4, at a protein concentration of about 1 mg $ml^{-1}$ as measured by the BCA-protein assay kit of Pierce Chemical Co. with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 150 mM NaCl and 2 nM of [$^3$H]-Ro 15-1788 or 5 nM [$^3$H]-muscimol in the absence or presence of 10 $\mu$M diazepam or 10 $\mu$M GABA or various concentrations of (+)-ROD188 for 90 min at 4°C (Zezula *et al*., 1996). Membranes were then filtered through Whatman GF/B filters. The filters were rinsed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml Hydrofluor (National Diagnostics, NJ, U.S.A.) scintillation fluid. Non-specific binding determined in the presence of 10 $\mu$M diazepam or 10 $\mu$M GABA was subtracted from total [$^3$H]-Ro 15-1788 or [$^3$H]-muscimol binding, respectively, to result in specific binding.

### *Synthesis of (+)-ROD188*

(+)-ROD188 was prepared by reaction of 1,2,3,4-tetrahydro-N-p-toluenesulphonylisoquinolinium chloride with 2-(tert-butyldimethylsiloxy)furan (Rassu *et al*., 1997) in acetonitrile followed by reduction of the lactone ring double bond using hydrogen in the presence of palladium on carbon as catalyst. The resulting mixture of isomers was separated by high pressure liquid chromatography on a chiral-support OD column. The absolute configuration of (+)-ROD188 was deduced from the X-ray crystallographic study of its enantiomer. Details of this work will be published elsewhere.

## Results

### *Expression of $GABA_A$ receptors in* Xenopus *oocytes*

$\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta2\gamma2$, $\alpha6\beta2\gamma2$, $\alpha1\beta2\gamma2$F77I, $\alpha1\beta2$ and $\alpha1\beta2$N265S rat recombinant $GABA_A$ receptors were functionally expressed in *Xenopus laevis* oocytes. Using the two-electrode voltage-clamp method, currents in the $\mu$A range were measured for all subunit combinations in response to application of a saturating concentration of GABA (10 mM). Measurements of the respective $EC_5$ of already described subunit combinations indicated no significant change in the apparent GABA affinity relative to previous observations (Sigel *et al*., 1990; 1998; Buhr *et al*., 1997; Thomet *et al*., 1999).

### *(+)-ROD188 allosterically stimulates the $GABA_A$ receptor*

We first characterized the action of (+)-ROD188 (Figure 1) on $\alpha1\beta2\gamma2$ receptors. At the limit of solubility of 100 $\mu$M (+)-ROD188 alone induced currents amounting to less than 0.2% of the maximum current amplitude elicited by GABA. (+)-ROD188 was found to stimulate currents induced by GABA in a concentration dependent manner. Figure 2A shows original current traces and Figure 2B the average current stimulation observed in ten experiments. This stimulation must be due to a positive allosteric modulation. Maximum currents induced by 10 mM GABA were slightly inhibited by 30 $\mu$M (+)-ROD188 ($85 \pm 3$% residual current, mean ± s.e.mean of three oocytes from one batch).

HEK 293 cells were transiently transfected with rat recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors. Using the whole-cell configuration of the patch-clamp technique at a holding potential of −60 mV, currents in the nA range were measured in response of a saturating concentration of GABA (64 $\mu$M). GABA concentration response curves, either in absence or presence of (+)-ROD188 were determined. Increasing concentrations of GABA were applied in pulses of 5–10 s duration. In control experiments the GABA dose response curve was best fitted with an $EC_{50}$ value of $5.1 \pm 1.3$ $\mu$M and a Hill coefficient of $1.9 \pm 0.1$ (mean ± s.e.mean of four cells). Upon coapplication of 30 $\mu$M (+)-ROD188 a leftward shift of the GABA dose-response curve was observed. The $EC_{50}$ value and the Hill coefficient were $1.1 \pm 0.2$ $\mu$M and $1.2 \pm 0.1$, respectively. The reason for the decrease of the Hill coefficient is not clear. The dose response curves in the presence and absence of (+)-ROD188 were performed in different cells, such that an effect on the maximal current amplitude was not visible.

### *Binding studies*

In radioactive ligand binding studies (+)-ROD188 failed to displace 5 nM [$^3$H]-muscimol from membranes from the cerebellum or the forebrain (Figure 3A). This lack of inhibition of [$^3$H]-muscimol binding demonstrates that (+)-ROD188 interacts with the $GABA_A$ receptor through a binding site different from that of GABA and is in line with functional data that show that (+)-ROD188 neither has an agonistic effect nor a competitive inhibitory effect on the $GABA_A$ receptor. (+)-ROD188 displaced the total specific binding 2 nM [$^3$H]-Ro 15-

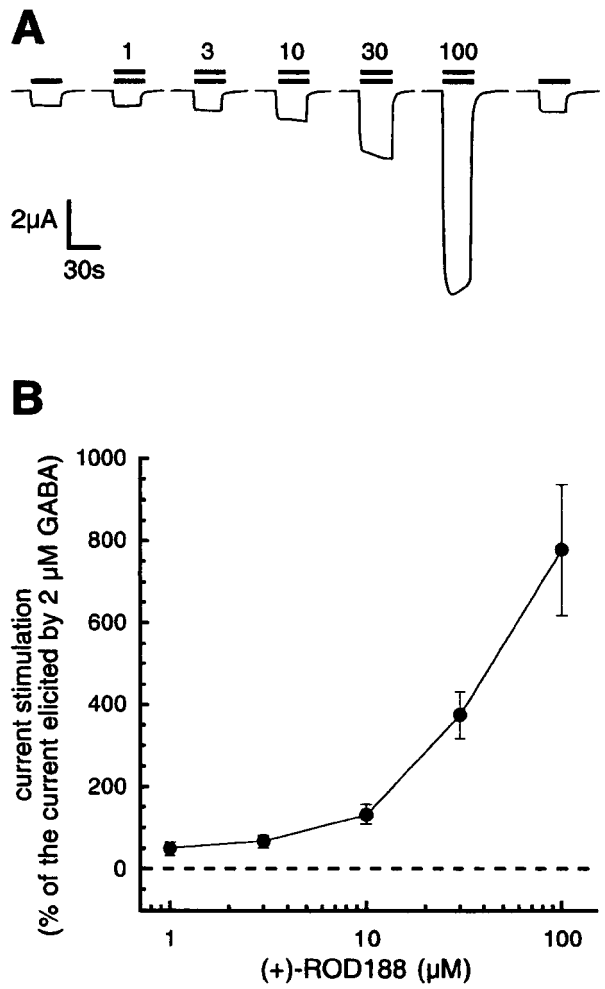


**Figure 2** (+)-ROD188 acts as a positive allosteric modulator of recombinant rat $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. (A) Potentiation of GABA responses by (+)-ROD188 at $\alpha1\beta2\gamma2$ receptors. Application of 2 $\mu$M GABA alone resulted in approximately 5% of the maximal current amplitude. Increasing concentrations of (+)-ROD188 were coapplied with GABA. The last application was again GABA alone and it demonstrated that (+)-ROD188 could be washed out almost completely. Bars above the current records indicate the periods of drug application. Concentrations of (+)-ROD188 are shown in $\mu$M. (B) Concentration response curves for (+)-ROD188 at $\alpha1\beta2\gamma2$ receptors. Values are shown as mean$\pm$s.e.mean of 10 oocytes from three batches.

1788 from membranes of the cerebellum or the forebrain with an $IC_{50}$ value of 32.9$\pm$1.2 and 52.4$\pm$2.7 $\mu$M, respectively (Figure 3B, mean$\pm$s.e.mean of three experiments, triplicates each). In HEK 293 cells transiently transfected with $\alpha1\beta2\gamma2$ receptors (+)-ROD188 inhibited the total specific binding of 2 nM [$^3$H]-Ro 15-1788 with an $IC_{50}$ value of 33.1$\pm$3.5 $\mu$M (Figure 3C, mean$\pm$s.e.mean of three experiments, triplicates each). Further experiments showed that (+)-ROD188 competes competitively with [$^3$H]-flunitrazepam for the benzodiazepine binding site (not shown). These results indicate therefore a low affinity interaction with the benzodiazepine binding site.

### *A mode of action different from that of benzodiazepines and loreclezole*

The benzodiazepine antagonist Ro15-1788 binds with nM affinity to its binding site, whereas about 30 $\mu$M (+)-ROD188 are required to displace 50% of 2 nM [$^3$H]-Ro15-1788 from recombinant $\alpha1\beta2\gamma2$ receptors. If (+)-ROD188 exerts its action exclusively through the benzodiazepine binding site, 1 $\mu$M Ro15-1788 should inhibit the stimulation by 20 $\mu$M (+)-ROD188 to 100%. We found an inhibition of only 29$\pm$5% (mean$\pm$s.e.mean of four oocytes from one batch). Representative current traces are shown in Figure 4A. We then studied the effect of the mutation $\gamma$F77I on current stimulation by (+)-ROD188. It has been shown previously that $\alpha1\beta2\gamma2$ receptors carrying the point mutation $\gamma$F77I lose affinity for Ro15-1788, methyl 6,7-dimethoxy-4-ethyl-$\beta$-carboline-3-carboxylate (DMCM) and zolpiden (Buhr *et al*., 1997). Stimulation by (+)-ROD188 was not affected by the mutation $\gamma$F77I (Figure 4B). To provide additional evidence for a mode of action of (+)-ROD188 different from that of benzodiazepines, we functionally expressed the dual subunit combination $\alpha1\beta2$, which lacks the $\gamma$ subunit required for benzodiazepine modulation (Pritchett *et al*., 1989; Sigel *et al*., 1990; Günther *et al*., 1995). The experiments were carried out using a GABA concentration eliciting about 5% of the maximal current amplitude in $\alpha1\beta2$. Current stimulation by 100 $\mu$M (+)-ROD188 in $\alpha1\beta2$ (763$\pm$89%, mean$\pm$s.e.mean of five oocytes from two batches, Figure 4C, left panel) was reduced only weakly as compared to that observed in the triple subunit combination $\alpha1\beta2\gamma2$ (859$\pm$43%, mean$\pm$s.e.mean of three oocytes from one batch). Thus, the major action of (+)-ROD188 occurs through a site of action different from the benzodiazepine site.

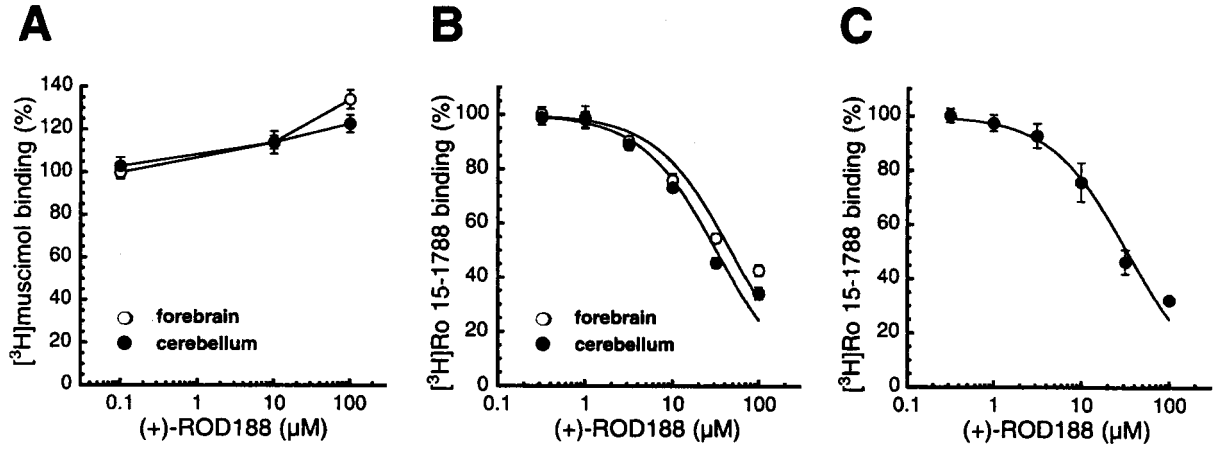


**Figure 3** Effect of (+)-ROD188 on the binding of either 5 nM [$^3$H]-muscimol (A) or 2 nM [$^3$H]-Ro 15-1788 (B, C). (A) Lack of displacement by (+)-ROD188 of binding of [$^3$H]-muscimol to membranes from rat cerebellum or forebrain. (B) (+)-ROD188 inhibited the binding of [$^3$H]-Ro 15-1788 to membranes from rat cerebellum or forebrain. (C) (+)-ROD188 also inhibited the binding of [$^3$H]-Ro 15-1788 to membranes of HEK 293 cells transiently transfected with $\alpha1\beta2\gamma2$. Values show mean$\pm$standard deviation of three determinations, triplicates each.

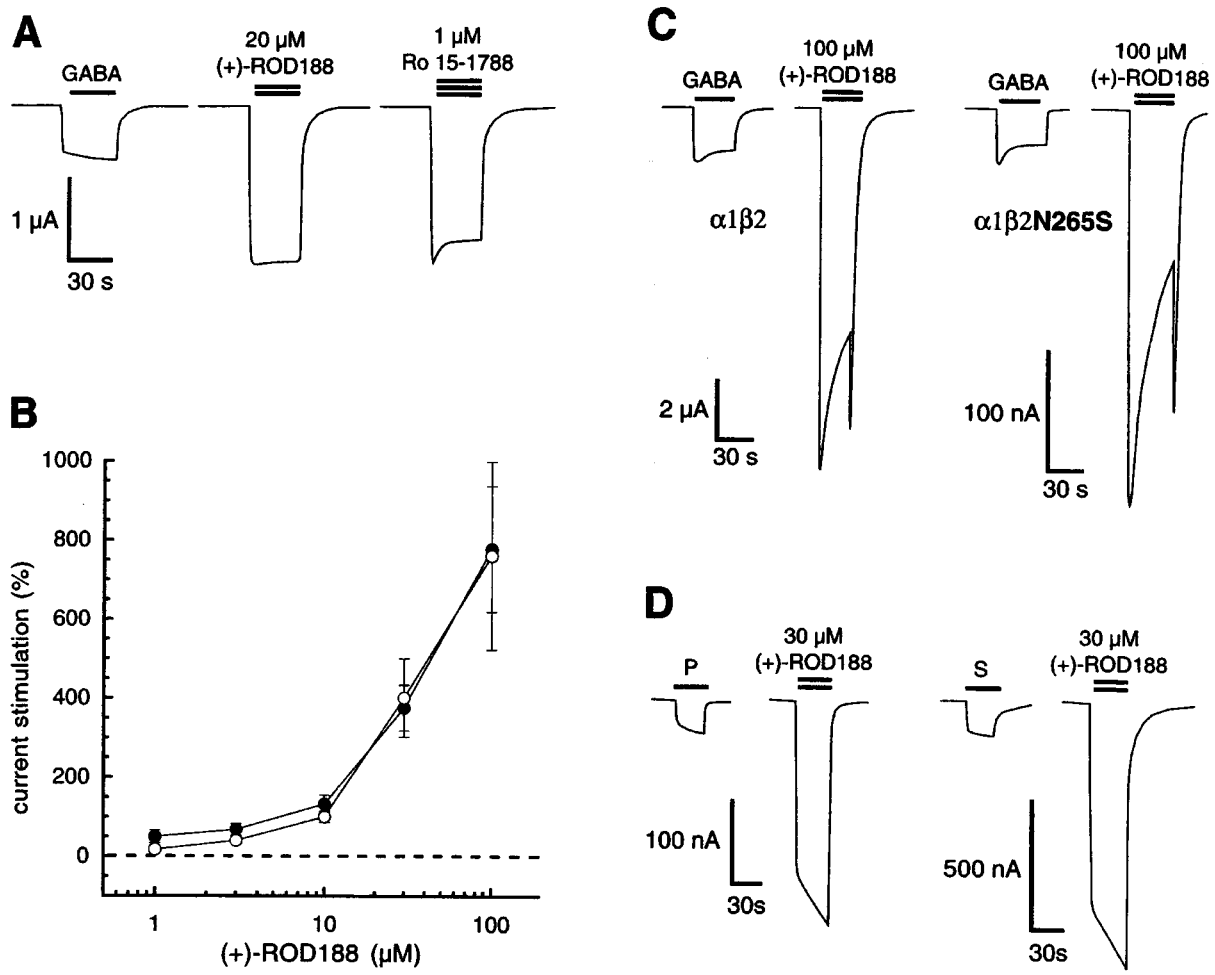


**Figure 4** The mode of action of (+)-ROD188 is different from that of benzodiazepines, loreclezole and pentobarbital and 3α-OH-DHP as agonists. (A) The concentration of GABA eliciting approximately 5% of the maximal current amplitude at recombinant rat $\alpha1\beta2\gamma2$ GABA$_A$ receptors was determined first. 20 μM (+)-ROD188 markedly stimulated these currents. When 1 μM Ro 15-1788 was coapplied with 20 μM (+)-ROD188, the stimulation was only inhibited by a small extent. Three additional experiments gave very similar results. (B) Effect of the point mutant $\alpha1\beta2\gamma2$F77I on the concentration response curve for (+)-ROD188. No difference in (+)-ROD188 potentiation between the mutated (○) and the wild type receptors (●) could be observed. Values are presented as mean±s.e.mean of at least three experiments. (C) Stimulation of currents elicited by GABA by 100 μM (+)-ROD188 at $\alpha1\beta2$ and $\alpha1\beta2$N265S receptors. 100 μM (+)-ROD188 strongly potentiated these currents in either the dual subunit combination $\alpha1\beta2$ or the mutant $\alpha1\beta2$N265S that reduces stimulation by loreclezole. In contrast to the triple subunit combinations investigated, a rebound current at the end of application was obvious. 3–4 additional experiments gave similar results. Bars above the current records indicate the periods of drug application. (D) (+)-ROD188 strongly stimulated GABA$_A$ receptor mediated currents induced by either 100 μM pentobarbital (P) or 3 μM of the steroid 3α-OH-DHP (S) in $\alpha1\beta2\gamma2$ receptors. Bars above the current records indicate the periods of drug application. Two additional experiments gave similar results in both experiments.

To see whether the observed potentiation by (+)-ROD188 displays similar properties as the current stimulation by loreclezole, we tested (+)-ROD188 for modulation of GABA induced currents at the dual subunit combination containing the point mutant $\beta$2N265S, which strongly interferes with loreclezole potentiation (Wafford *et al*., 1994; Wingrove *et al*., 1994; Thomet *et al*., 1999). Current stimulation by 100 μM (+)-ROD188 on $\alpha1\beta2$N265S receptors (600±46%, mean± s.e.mean of four oocytes from one batch) was not significantly different from that on the wild type receptor, in line with a mode of action of (+)-ROD188 different from that of loreclezole. Representative current traces are shown in Figure 4C (right panel).

### *A mode of action different from pentobarbital and neurosteroids as channel agonists*

Barbiturates and steroids modulate GABA$_A$ receptor currents and additionally activate the channel in the absence of GABA. In $\alpha1\beta2\gamma2$, 30 μM (+)-ROD188 strongly stimulated currents elicited by either 100 μM pentobarbital (588±218%, mean± s.e.mean of three oocytes from one batch) or 3 μM 5α-pregnan-3α-ol-20-one (3α-OH-DHP; 644±47%, mean±s.e.mean of three oocytes from one batch). Representative current traces are shown in Figure 4D. Our experiments suggest that the modulatory binding site for (+)-ROD188 is different from the sites for pentobarbital and neurosteroids leading to the opening of the channel.

We tested also whether the stimulation of GABA induced currents by (+)-ROD188 and pentobarbital are additive. Stimulation was determined at a GABA concentration eliciting about 0.4% of the maximal current amplitude to allow large values of stimulation to occur. Fifty μM pentobarbital stimulated currents evoked by GABA in $\alpha1\beta2\gamma2$ by 941±63% (mean±s.e.mean of three oocytes from one batch). 100 μM (+)-ROD188 displayed a potentiation of 1560±106% (mean±s.e.mean of three oocytes from one batch). No additional potentiation was observed when pentobarbital was coapplied with 100 μM (+)-ROD188 (1599±83%, mean± s.e.mean of three oocytes from one batch). Pentobarbital and

(+)-ROD188 either share the binding site through which pentobarbital stimulates currents induced by GABA, or the subsequent conformational changes of the two stimulators are in common.

### *Selectivity for $GABA_A$ receptors containing the α6-subunit*

(+)-ROD188 also potentiated currents induced by GABA in oocytes expressing $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors (Figure 5). Current potentiation was measured at a GABA concentration eliciting 2–5% of the maximal current amplitude in the respective subunit combination. Interestingly, at $\alpha6\beta2\gamma2$ receptors, current potentiation by (+)-ROD188 was much larger than at the other subunit combinations. $\alpha2\beta2\gamma2$ and $\alpha3\beta2\gamma2$ showed a smaller stimulation than $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$. Potentiation of currents elicited by GABA reached a maximum at approximately 100 μM (+)-ROD188 at the subunit combinations $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$ and $\alpha5\beta2\gamma2$ whereas concentration response curves of $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors were not saturated at the limit of solubility of (+)-ROD188.

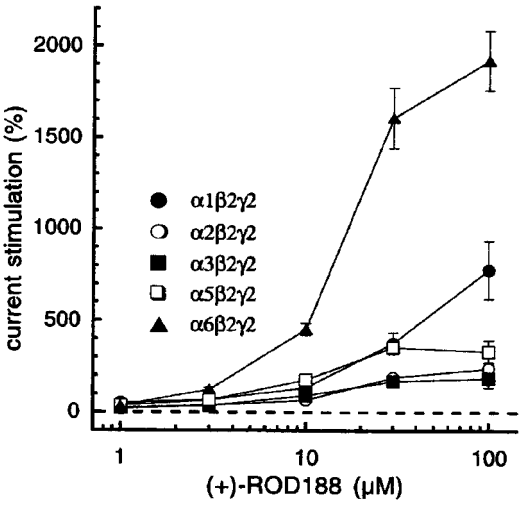


**Figure 5** α-isoform-selective modulation of $GABA_A$ receptor currents by (+)-ROD188. The concentration response curves for (+)-ROD188 at recombinant rat $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors are shown. Experiments were carried out as described under Figure 2A. Values are presented as mean ± s.e.mean of at least three oocytes from 1–3 batches.

### *Enhanced duration of mIPSCs in hippocampal neurones*

To evaluate the action of (+)-ROD188 on synaptic transmission, spontaneous miniature inhibitory postsynaptic currents (mIPSCs) through $GABA_A$ receptors were recorded in rat hippocampal neurones at −60 mV. Five μM CNQX, 10 μM APV and 0.1 μM TTX were present to inhibit glutamatergic currents and action potentials. Synaptic activity was measured before (control) and during the application of 30 μM (+)-ROD188. The effect of (+)-ROD188 on the peak amplitude, rise time and decay time ($\tau$) of the mIPSCs were determined. The latter two could be fitted monoexponentially. As judged by eye, fitting with a double exponential function was far less satisfactory. Thirty μM (+)-ROD188 significantly increased the decay time of events (191 ± 7% of control, mean ± s.e.mean of four cells, $P<0.001$). Representative current traces are shown in Figure 6A. Histograms of $\tau$ of all mIPSCs analysed are presented in Figure 6B. Both the amplitude of 45.8 ± 2.0 pA and rise time of 0.96 ± 0.07 ms were not changed significantly upon application of 30 μM (+)-ROD188 (109 ± 18% ($P>1.0$) and 109 ± 11% ($P>1.0$) of control, respectively; mean ± s.e.mean of four cells). The mIPSC frequency was also not visibly altered.

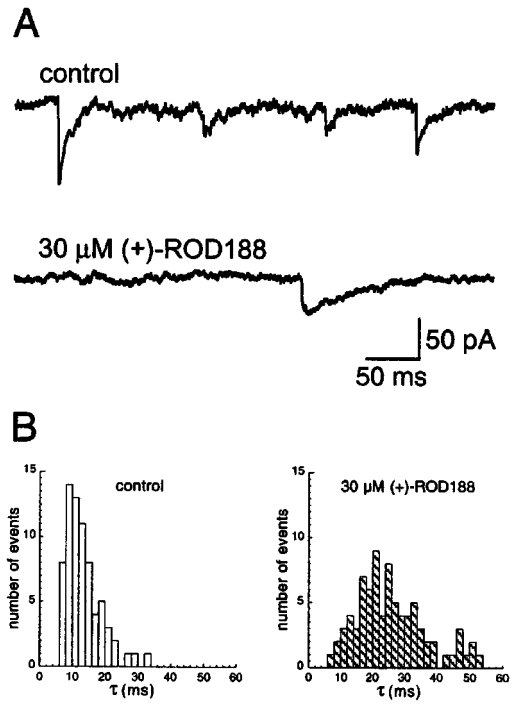


**Figure 6** Effect of 30 μM (+)-ROD188 on spontaneous mIPSCs recorded in rat hippocampal neurones at −60 mV in the whole-cell configuration of the patch-clamp technique. (A) Representative current traces. (B) Histograms of the decay times ($\tau$) of miniature IPSCs recorded in four cells.

## Discussion

In attempts to develop novel ligands for the GABA binding site on the $GABA_A$ receptor, we used the competitive antagonist bicuculline as inspiration for the synthesis of novel compounds. All the studied derivatives themselves induced negligible currents. Moreover, some bicuculline analogues display a potentiation rather than an inhibition of GABA induced currents. By optimizing the function of these derivatives we finally discovered (+)-ROD188. An action of (+)-ROD188 *via* the GABA binding site could be excluded in displacement studies, where we found a lack of inhibition of [$^3$H]-muscimol binding by (+)-ROD188. (+)-ROD188 stimulated currents elicited by GABA in a concentration dependent fashion without inducing sizeable currents by itself. These findings indicate that the observed stimulation by (+)-ROD188 is due to a positive allosteric modulation. In the following we discuss the site of action of (+)-ROD188 and its subunit selectivity. Electrophysiological studies and binding experiments were combined to characterize this unique mode of action.

In displacement studies (+)-ROD188 was found to inhibit the binding of 2 nM [$^3$H]-Ro 15-1788 either in membranes of the forebrain and the cerebellum or from membranes of HEK 293 cells transiently transfected with $\alpha1\beta2\gamma2$, the $IC_{50}$ values ranging from 33 to 52 μM. This indicated a very weak interaction of (+)-ROD188 with the benzodiazepine site.

Stimulation by benzodiazepines can be counteracted on a functional level by coapplication of 1 $\mu$M of the benzodiazepine antagonist Ro 15-1788. When such experiments were carried out with (+)-ROD188, current potentiation by (+)-ROD188 was largely uninhibited, suggesting that the major part of stimulation occurs through a separate binding site and that Ro 15-1788 cannot allosterically prevent stimulation. Also at the mutated $\alpha1\beta2\gamma2$F77I receptor, which loses affinity for Ro 15-1788, zolpidem or DMCM (Buhr *et al*., 1997), current potentiation by higher concentrations of (+)-ROD188 was not affected. Stimulation by (+)-ROD188 in dual subunit combinations lacking a $\gamma$ subunit was similar to that measured in the triple subunit combination $\alpha1\beta2\gamma2$. In contrast, benzodiazepine modulation depends entirely on the presence of a $\gamma$ subunit (Pritchett *et al*., 1989; Sigel *et al*., 1990; Günther *et al*., 1995).

Unlike in the triple subunit combination, currents stimulated by (+)-ROD188 in the dual subunit combination showed a rebound current phenomenon after removal of the ligands. The presence of the $\gamma2$ subunit seems to repress these rebound currents and we did not further investigate this phenomenon. Rebound currents have been described by Wooltorton *et al*. (1997) upon removal of pentobarbital from murine homomeric $\beta3$ $GABA_A$ receptors gated by pentobarbital. The authors interpreted the rebound current in this case as channels returning from a desensitized state.

Currents elicited by pentobarbital in the absence of GABA were also strongly stimulated by (+)-ROD188. This excludes that (+)-ROD188 exerts its action through the same site as pentobarbital elicits channel opening. We were interested to see whether currents elicited by GABA are stimulated by (+)-ROD188 and by pentobarbital in an additive manner. In these experiments we used a concentratioan of pentobarbital that produced modulatory effects and itself only activated the receptor to a very small extent. In case of coapplication of both compounds, we measured a potentiation of the same size as that caused by (+)-ROD188 alone. The two pentobarbital sites that elicit current themselves on one hand and the site that confers stimulation of currents elicited by GABA on the other hand may be identical, as Amin (1999) has found that a single point mutation W328M confers both properties to the otherwise quite pentobarbital insensitive $\rho1$ receptor. Pentobarbital also stimulates the maximum response elicited by GABA (Malherbe *et al*., 1990; Horne *et al*., 1992). In contrast (+)-ROD188 slightly inhibited this maximum current. This might be an indication of the fact that both drugs modulate the receptor through different mechanisms.

Neurosteroids are another class of positive modulators of the $GABA_A$ receptor which also activate the receptor in the absence of GABA (Sieghart, 1995). When currents were induced by the steroid 3$\alpha$-OH-DHP we observed a strong stimulation upon coapplication of the steroid with (+)-ROD188. Thus, (+)-ROD188 does not act through the steroid site effecting channel opening.

Loreclezole is known to interact *via* a unique site of the $GABA_A$ receptor (Wafford *et al*., 1994). In several studies it could be demonstrated that loreclezole effects are strongly reduced in receptors carrying the point mutation $\beta$2N265S (Wingrove *et al*., 1994; Stevenson *et al*., 1995; Belelli *et al*., 1997). We used this mutant to see whether modulation by (+)-ROD188 displays similar properties as loreclezole. Potentiation by (+)-ROD188 in $\alpha1\beta$2N265S was not significantly different from that measured in the wild type receptor, in line with a mode of action of (+)-ROD188 different from that of loreclezole. It has also been demonstrated that $\gamma$-butyrolactones and $\gamma$-thiobutyrolactones interact with the $GABA_A$ receptor (Holland *et al*., 1990; 1995; Williams *et al*., 1997). The mechanism of action of this class of compounds is presently not clearly understood. Additional studies will be required to address whether modulation by these substances shares similarity with that by (+)-ROD188.

(+)-ROD188 was to a certain extent selective for the $\alpha6$ isoform in $\alpha x\beta2\gamma2$ receptors, where x = 1, 2, 3, 5, 6. This selectivity for $\alpha6$ containing receptors is in contrast to the properties of benzodiazepines that are inactive in receptors containing the $\alpha6$ subunit (Stevenson *et al*., 1995; Sieghart, 1995). This selectivity is also not seen in displacement studies of [$^3$H]-Ro 15-1788 from membranes of rat forebrain where no $\alpha6$ is expressed and cerebellum where about 50% of the receptors contain $\alpha6$, as the affinity for (+)-ROD188 was similar in both cases.

As a model for synaptic transmission we studied the effect of (+)-ROD188 on spontaneous postsynaptic currents through $GABA_A$ receptors in cultured rat hippocampal neurones. (+)-ROD188 increased the decay time constant without affecting either the amplitude or rise time of these events. This provides evidence that (+)-ROD188 increases GABAergic neuronal transmission. For comparison, the benzodiazepine zolpidem enhanced both, the decay time constant as well as the amplitude of $GABA_A$ receptor mediated mIPSCs in rat V pyramidal neurons of the visual cortex (Perrais *et al*., 1999).

In conclusion, we show that (+)-ROD188 acts as a positive allosteric modulator of $GABA_A$ receptor currents through a locus of action different from that of GABA, channel opening by pentobarbital or by the steroid 3$\alpha$-OH-DHP, from that of loreclezole or that of the benzodiazepines and displays a certain selectivity for $\alpha6$ containing receptors. Further studies are required to establish the therapeutic potential of this drug.

We thank Ms Charlotte Becker for her unfailing preparation of the hippocampal cultures and Dr Frantisek Jursky for performing some of the initial binding experiments. This study was supported by the European Union grant BIO4-CT96-0585 (BBW 96.0010) and grant 3100-053599.98/1 from the Swiss National Science Foundation.

## References

AMIN, J. (1999). A single hydrophobic residue confers barbiturate sensitivity to $\gamma$-aminobutyric acid type C receptor. *Mol. Pharmacol.*, **55,** 411 – 423.

BARNARD, E.A., SKOLNICK, P., OLSEN, R.W., MÖHLER, H., SIEGHART, W., BIGGIO, G., BRAESTRUP, C., BATESON, A.N. & LANGER, S.Z. (1998). International Union of Pharmacology. XV. Subtypes of $\gamma$-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.*, **50,** 291 – 313.

BELLELI, D., LAMBERT, J.J., PETERS, J.A., WAFFORD, K. & WHITING, P.J. (1997). The interaction of the general anaesthetic etomidate with the $\gamma$-aminobutyric acid type A receptor is influenced by a single amino acid. *Proc. Natl. Acad. Sci. U.S.A.*, **94,** 11031 – 11036.

BONNERT, T.P., MCKERNAN, R.M., FARRAR, S., LE BOURDELLÈS, B., HEAVENS, R.P., SMITH, D.W., HEWSON, L., RIGBY, M.R., SIRINATHSINGHJI, D.J.S., BROWN, N., WAFFORD, K.A. & WHITING, P.J. (1999). $\theta$, a novel $\gamma$-aminobutyric acid type A receptor subunit. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 9891–9896.

BUHR, A., BAUR, R. & SIGEL, E. (1997). Subtle changes in residue 77 of the $\gamma$ subunit of $\alpha1\beta2\gamma2$ $GABA_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site and suggest the presence of two sites. *J. Biol. Chem.,* **272,** 11799–11804.

BURT, D.R. & KAMATCHI, G.L. (1991). $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.,* **5,** 2916–2923.

CHANG, Y., WANG, R., BAROT, S. & WEISS, D.S. (1996). Stoichiometry of a recombinant $GABA_A$ receptor. *J. Neurosci.,* **16,** 5415–5424.

CHEN, C.A. & OKAYAMA, H. (1988). Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques,* **7,** 632–638.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A.R. (1970). GABA, bicuculline and central inhibition. *Nature,* **226,** 1222–1224.

DAVIES, P.A., HANNA, M.C., HALES, T.G. & KIRKNESS, E.F. (1997). Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. *Nature,* **385,** 820–823.

DUNN, S.M.J., BATESON, A.N. & MARTIN, I.L. (1994). Molecular neurobiology of the $GABA_A$ receptor. *Int. Rev. Neurobiol.,* **36,** 51–96.

GÜNTHER, U., BENSON, J., BENKE, D., FRITSCHY, J.M., REYES, G., KNOFLACH, F., CRESTANI, F., AGUZZI, A., ARIGONI, M., LANG, Y., BLUETHMANN, H., MÖHLER, H. & LÜSCHER, B. (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7749–7753.

HEDBLOM, E. & KIRKNESS, E.F. (1997). A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. *J. Biol. Chem.,* **272,** 15346–15350.

HOLLAND, K.D., FERRENDELLI, J.A., COVEY, D.F. & ROTHMAN, S.M. (1990). Physiological regulation of the picrotoxin receptor by $\gamma$-butyrolactones and $\gamma$-thiobutyrolactones in cultured hippocampal neurons. *J. Neurosci.,* **10,** 1719–1727.

HOLLAND, K.D., MATHEWS, G.C., BOLOS-SY, A.M., TUCKER, J.B., REDDY, P.A., COVEY, D.F., FERRENDELLI, J.A. & ROTHMAN, S.M. (1995). Dual modulation of the $\gamma$-aminobutyric acid type A receptor/ionophore by alkyl-substituted $\gamma$-butyrolactones. *Mol. Pharmacol.,* **47,** 1217–1223.

HORNE, A.L., HADINGHAM, K.L., WHITING, P.J. & KEMP, J.A. (1992). The pharmacology of recombinant $GABA_A$ receptors containing $\alpha1$, $\beta1$, $\gamma2$L subunits stably transfected into mouse L-cells. *Br. J. Pharmacol.,* **108,** 711–716.

MACDONALD, R.L. & OLSEN, R.W. (1994). $GABA_A$ receptor channels. *Annu. Rev. Neurosci.,* **17,** 569–602.

MALGAROLI, A. & TSIEN, R.W. (1992). Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. *Nature,* **357,** 134–139.

MALHERBE, P., SIGEL, E., BAUR, R., PERSOHN, E., RICHARDS, J.G. & MÖHLER, H. (1990). Functional characteristics and sites of gene expression of the $\alpha1\beta2\gamma2$ isoform of the rat $GABA_A$ receptor. *J. Neurosci.,* **10,** 2330–2337.

NAYEEM, N., GREEN, T.P., MARTIN, I.L. & BARNARD, E.A. (1994). Quarternary structure of the native $GABA_A$ receptor determined by electron microscope image analysis. *J. Neurochem.,* **62,** 815–818.

PERRAIS, D. & ROPERT, N. (1999). Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. *J. Neurosci.,* **19,** 578–588.

PERSOHN, E., MALHERBE, P. & RICHARDS, J.G. (1992). Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *J. Comp. Neurol.,* **326,** 193–216.

PRITCHETT, D.B., SONTHEIMER, H., SHIVERS, B.D., YMER, S., KETTENMANN, H., SCHOFIELD, P.R. & SEEBURG, P.H. (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature,* **338,** 582–585.

RABOW, L.E., RUSSEK, S.J. & FARB, D.H. (1995). From ion currents to genomic analyses: recent advances in $GABA_A$ receptor research. *Synapse,* **21,** 189–274.

RASSU, G., ZANARDI, F., BATTISTINI, L., GAETANI, E. & CASIRAGHI, G. (1997). Expeditious syntheses of sugar-modified nucleosides and collections thereof exploiting furan-, pyrrolo, and thiophene-based siloxy dienes. *J. Med. Chem.,* **40,** 168–180.

RYAN, T.A., REUTER, H., WENDLAND, B., SCHWEIZER, F.E., TSIEN, R.W. & SMITH, S.J. (1993). The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron,* **11,** 713–724.

SIEGHART, W. (1995). Structure and pharmacology of $\gamma$-aminobutyric $acid_A$ receptor subtypes. *Pharmacol. Rev.,* **47,** 181–233.

SIGEL, E. (1987). Properties of single sodium channels translated by Xenopus oocytes after injection with messenger ribonucleic acid. *J. Physiol.,* **386,** 73–90.

SIGEL, E. & BUHR, A. (1997). The benzodiazepine binding site on $GABA_A$ receptors. *Trends Pharmacol. Sci.,* **18,** 425–429.

SIGEL, E., BAUR, R., NETZER, R. & RUNDFELDT, C. (1998). The antiepileptic drug AWD 131-138 stimulates different recombinant isoforms of the rat $GABA_A$ receptor through the benzodiazepine binding site. *Neurosci. Lett.,* **245,** 85–88.

SIGEL, E., BAUR, R., TRUBE, G., MÖHLER, H. & MALHERBE, P. (1990). The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron,* **5,** 703–711.

STEVENSON, A., WINGROVE, P.B., WHITING, P.J. & WAFFORD, K.A. (1995). $\beta$-carboline $\gamma$-aminobutyric $acid_A$ receptor inverse agonists modulate $\gamma$-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Mol. Pharmacol.,* **48,** 965–969.

THOMET, U., BAUR, R., SCHOLZE, P., SIEGHART, W. & SIGEL, E. (1999). Dual mode of stimulation by the $\beta$-carboline ZK 91085 of recombinant $GABA_A$ receptor currents: molecular determinants affecting its action. *Br. J. Pharmacol.,* **127,** 1231–1239.

TRETTER, V., EHYA, N., FUCHS, K. & SIEGHART, W. (1997). Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. *J. Neurosci.,* **17,** 2728–2737.

UENO, S., BRACAMONTES, J., ZORUMSKI, C., WEISS, D.S. & STEINBACH, J.H.(1997). Bicuculline and Gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J. Neurosci.,* **17,** 625–634.

WAFFORD, K.A., BAIN, C.J., QUIRK, K., MCKERNAN, R.M., WINGROVE, P.B., WHITING, P.J. & KEMP, J.A. (1994). A novel allosteric modulatory site on the $GABA_A$ receptor $\beta$ subunit. *Neuron,* **12,** 775–782.

WILLIAMS, K.L., TUCKER, J.B., WHITE, G., WEISS, D.S., FERRENDELLI, J.A., COVEY, D.F., KRAUSE, J.E. & ROTHMAN, S.M. (1997). Lactone modulation of the $\gamma$-aminobutyric acid A receptor: Evidence for a positive modulatory site. *Mol. Pharmacol.,* **52,** 114–119.

WINGROVE, P.B., WAFFORD, K.A., BAIN, C.J. & WHITING, P.J. (1994). The modulatory action of loreclezole at the $\gamma$-aminobutyric acid type A receptor is determined by a single amino acid in the $\beta2$ and $\beta3$ subunit. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 4569–4573.

WISDEN, W., LAURIE, D.J., MONYER, H. & SEEBURG, P.H. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon and mesencephalon. *J. Neurosci.,* **12,** 1040–1062.

WOOLTORTON, J.R.A., MOSS, S.J. & SMART, T.G. (1997). Pharmacological and physiological characterizatioan of murine homomeric $\beta3$ $GABA_A$ receptors. *Eur. J. Neurosci.,* **9,** 2225–2235.

ZEZULA, J., SLANY, A. & SIEGHART, W. (1996). Interaction of allosteric ligands with $GABA_A$ receptors containing one, two or three different subunits. *Eur. J. Pharmacol.,* **301,** 207–214.

*(Received May 12, 2000*
*Revised June 15, 2000*
*Accepted June 26, 2000)*