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Cloning of cDNAs Encoding the Human γ-Aminobutyric acid Type A Receptor α6 Subunit and Characterization of the Pharmacology of α6-Containing receptors

KAREN L. HADINGHAM, ELIZABETH M. GARRETT, KEITH A. WAFFORD, CORINNA BAIN, ROBERT P. HEAVENS, DALIP J. S. SIRINATHSINGHJI, and PAUL J. WHITING

Merck Sharp & Dohme Research Laboratories, Harlow, Essex, UK CM20 2QR

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SUMMARY

A cDNA encoding the human γ-aminobutyric acid$_A$ ($GABA_A$) receptor α6 subunit has been cloned and sequenced. The deduced amino acid sequence of this cDNA shows 91.4% identity with the published rat α6 subunit. *In situ* hybridization histochemistry reveals the α6 mRNA to be located within the granule cell layer of the human cerebellar cortex. Recombinant human α6β2γ2S $GABA_A$ receptors have been expressed in both stably transfected cells and *Xenopus* oocytes, and the pharmacology of the benzodiazepine binding site has been determined. The recombinant receptor has a diazepam-insensitive pharmacology, with negligible affinity for a number of classic benzodiazepines. A number of compounds that bind to the benzodiazepine site potentiated the GABA response of α6β2γ2 receptors. Most importantly, the classic benzodiazepine antagonist ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (Ro 15–1788) and the partial inverse agonist ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (Ro 15–4513) both acted as agonists at the α6 containing receptor. This observation demonstrates definitively that efficacy of benzodiazepine compounds cannot be generalized across receptor subtypes and may also help explain some of the behavioral effects that have been reported for these compounds.

The mammalian $GABA_A$ receptor is a ligand-gated ion channel; the binding of GABA to this receptor results in the opening of an intrinsic chloride ion channel, leading to hyperpolarization of the cell membrane. The receptor is the target for a number of drugs, including BZs, barbiturates, neurosteroids, and ethanol (1, 2).

Molecular cloning has demonstrated the existence of an extensive family of highly homologous $GABA_A$ receptor subunit genes, of which there are six α, three β, three γ, and one δ currently known in the mammalian brain. Combinations of these subunits are believed to assemble *in vivo*, forming a family of receptor subtypes (for a review, see Refs. 2 and 3). The use of *in vitro* expression systems has shown that an α, a β, and a γ subunit are necessary to assemble a recombinant receptor exhibiting the different functional and pharmacological properties seen in native $GABA_A$ receptors (4, 5). Pharmacologically defined BZ1/BZ2 nomenclature (6) has been shown to correlate well with α1β2γ2 (BZ1) and α2/α3/α5β2γ2 (BZ2) pharmacologies observed in recombinant systems (4, 7–9). However, the BZ site pharmacologies of rat α4- and α6-containing receptors appear markedly different (10, 11) than those seen with receptors expressing the other α subunits. The pharmacology of rat α6β2γ2 receptors corresponds to the so-called diazepam insensitive [^{3}H]Ro 15–4513 binding sites found in the rat or bovine cerebellum (12). The binding of [^{3}H]Ro 15–4513 to these sites is displaced by micromolar concentrations of some compounds, such as DMCM and Ro 15–1788, but not by other "classic BZs," such as diazepam or flunitrazepam. The α6 subunit is unique in that, at least in the rat, its expression is limited to cerebellar granule cells (10). Immunoprecipitation experiments have shown that in the rat cerebellum, the most abundant receptor subtype (36% of $GABA_A$ receptors) contains α6 in combination with γ2 (and presumably a β subunit), suggesting that it may have a physiologically important role (13).

We report the cloning and sequencing of a cDNA encoding the human α6 $GABA_A$ receptor subunit and describe the site of expression of the corresponding α6 mRNA in human brain. Also, we report the characterization of the pharmacology of human α6-containing receptors expressed in *Xenopus* oocytes and stably transfected mammalian cells.

ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid type A; BZ, benzodiazepine; DI, diazepam insensitive; DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate methyl ester; Ro 15–4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; Ro 15–1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; TM, transmembrane; PBS, phosphate-buffered saline.

Materials and Methods

Isolation and sequencing of a cDNA encoding the human α6 subunit. A rat α6 subunit cDNA was first obtained with the use of PCR. PCR was performed as described previously (14) using whole rat brain cDNA as template and oligonucleotide primers derived from the published rat α6 sequence (10), incorporating an *Eco*RI site for subcloning into pBluescript SK⁻ vector (Stratagene): 5′-GGAA-GAATTCAGGAGGGTGACCT-3′ (bp 48–72) and 5′-GAAAATAAC-GAATTCCAGTGTCCAGCTTT-3′ (bp 1376–1404). The rat α6 cDNA was labeled with ^{32}P and used to screen a human cerebellum cDNA library (Stratagene), as described elsewhere (9). Several overlapping α6 cDNA clones were isolated; DNA sequencing of the clones indicated that none contained the complete coding region. A full-length α6 cDNA was constructed from three overlapping cDNAs with the use of convenient restriction sites and sequenced completely on both strands with Sequenase II (United States Biochemicals) through a combination of restriction enzyme deletion and oligonucleotide primer walking. Management of cDNA sequences and analysis of nucleotide and translated protein sequences was performed with Intelligenetics software.

Expression vectors and transfection. Isolation of human α1, β2, β3, and γ2S $GABA_A$ subunit cDNAs have been described previously (9, 15). In *Xenopus* oocyte expression studies, each subunit cDNA was expressed from the eukaryotic vector pCDM8 (Invitrogen).

Production of a stably transfected cell line (H132 clone 16) expressing the human α1β3γ2S $GABA_A$ receptor subtype has been described previously (15). Stable expression of the α6β3γ2S subtype was similarly obtained by transfection of the individual subunit cDNAs in the dexamethasone-inducible eukaryotic expression vector pMSGneo (16, 17) into mouse L(tk−) cells. DNA for transfection was prepared by double-banding on CsCl gradients. Cell culture and transfections were performed as described previously (15, 17). Geneticin-resistant (GIBCO-BRL) cell colonies obtained from the α6β3γ2S stable transfection were isolated using cloning cylinders and analyzed individually for the binding of [^{3}H]Ro 15–4513 (28.8 Ci/mmol; NEN) after a 5-day induction of receptor expression by the addition of 1 μM dexamethasone to culture medium lacking geneticin. The population expressing the highest levels of [^{3}H]Ro 15–4513 binding was recloned by limiting dilution. The resultant cell line, M632 clone 1, was initially maintained in medium containing Geneticin (2 mg/ml) but was subsequently cultured in normal growth medium and incubated only every 2–3 weeks in medium containing geneticin.

Transient expression of human α1β1γ2S, α6β1γ2S, and α1α6β1γ2S cDNAs in human embryonic kidney 293 cells was performed essentially as described previously, with the exception that $GABA_A$ receptor cDNAs (5–10 μg/10-cm dish) were supplemented with an equal amount of pAdvantage vector (Promega).

Membrane preparation and ligand binding. Cells were washed twice with PBS and scraped into PBS. After centrifugation (3000 × *g* for 20 min at 4°), membranes were prepared as described previously (15). Saturation binding curves were obtained by incubating membranes (100–200 μg of protein) with various concentrations of [^{3}H]Ro 15–1788 (α1β3γ2S stably transfected cells) or [^{3}H]Ro 15–4513 (α6β3γ2S stably transfected cells). Nonspecific binding was measured by the inclusion of 10 μM unlabeled Ro 15–1788 (α1β3γ2S stably transfected cells) or Ro 15–4513 (α6β3γ2S stably transfected cells) (both from Research Biochemicals International). All binding assays were performed in triplicate in an assay volume of 0.5 ml, with an incubation time of 90 min at 4°. Incubations were terminated by filtration through GF/B filters (Brandel) on a Tomtec cell harvester, followed by three washes in ice-cold assay buffer. After drying, filter-retained radioactivity was measured by liquid scintillation counting. Displacement of [^{3}H]Ro 15–4513 (5 nM) or [^{3}H]Ro 15–1788 (0.75 nM), as appropriate, by various BZ binding site ligands was performed under similar conditions, and single-site dose-response curves were fitted to the experimental data using the least-squares iterative fitting routine of analysis package RS/1 (BBN Research Systems). K_i values were calculated from the results of at least three independent determinations by using the following equation: $K_i = IC_{50}/(1 + [\text{radioligand}]/K_d$, where K_d is the mean value for the particular radioligand/receptor combination used, as given in Table 1. Other than Ro 15–1788 and bretazenil (both gifts from Hoffmann-La Roche), zolpidem (Synthelabo), abecarnil (Schering AG), CL 218,872 (Lederle), and FG 8205 (synthesized at Merck Sharp & Dohme), all other BZ-site ligands were obtained from Research Biochemicals or Sigma.

TABLE 1

Affinities of selected BZ binding site ligands for human α1β3γ2S and α6β3γ2S $GABA_A$ receptor subunit combinations stably expressed in mouse L(tk−) fibroblasts

Affinities are shown (K_i; K_d where indicated[a]) for 11 BZ site ligands. K_d values were obtained by Scatchard isotherm analysis of radioligand binding ([^{3}H]Ro 15–1788 for α1β3γ2S and [^{3}H]Ro 15–4513 for α6β3γ2S). The K_i values indicated were obtained by displacement of sub-K_d concentrations of the appropriate radioligand by various ligands, as described in Materials and Methods. All values given are the mean ± standard error from at least three independent determinations. n.d. = not determined.

	K_i	
	α1β3γ2S	α6β3γ2S
	nM	
[^{3}H]Ro 15–1788[a]	0.92 ± 0.04	n.d.
[^{3}H]Ro 15–4513[a]	n.d.	6.5 ± 0.6
Flunitrazepam	5.2 ± 0.2	>10,000
Methyl β-carboline-3 carboxylate	1.0 ± 0.2	2053 ± 471
Ro 15–4513	2.6 ± 1.1	n.d.
Ro 15–1788	n.d.	148 ± 3
FG 8205	0.68 ± 0.17	227 ± 40
CL 218,872	89.5 ± 4	>10,000
Zolpidem	20.7 ± 2.4	>10,000
Triazolam	0.68 ± 0.11	>10,000
Bretazenil	0.35 ± 0.07	12.7 ± 1.3
Diazepam	16.8 ± 2.0	>10,000
DMCM	11.2 ± 0.9	134 ± 21

Displacement of [^{3}H]Ro 15–4513 (6 nM) from the transiently expressed human $GABA_A$ receptors by flunitrazepam was performed as described above, with single- and multiple-site dose-response curves fitted to the data with a nonlinear squares fitting program (Grafit; Erithacus Software Ltd.).

Oocyte expression. *Xenopus* oocytes were removed from anesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment to remove follicle cells [Type IA (0.5 mg/ml) for 8 min], the oocyte nuclei were then directly injected with 10–20 nl of injection buffer [88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7.0 (nitrocellulose filtered)] containing different combinations of human $GABA_A$ receptor subunit cDNAs (20 ng/μl) engineered into the expression vector pCDM8 or pcDNAI/Amp. After incubation for 24 hr, oocytes were placed into a 50-μl bath and perfused with modified Barth's medium consisting of 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, and 2.4 mM $NaHCO_3$, pH 7.5. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltage-clamped between −40 and −70 mV. The cell was continuously perfused with saline at a rate of 4–6 ml/min, and drugs were applied in the perfusate. GABA modulators were preapplied for 30 sec before the addition of GABA. GABA was applied until the peak of the response was observed, usually within 30 sec. At least 3 min wash time was allowed between each GABA application to prevent desensitization. Concentration-response curves were calculated with a nonlinear squares fitting program to the following equation: $f(x) = B_{max}/(1 + (EC_{50}/x)^{n_H})$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n_H is the Hill coefficient.

***In situ* hybridization histochemistry.** *In situ* hybridization histochemistry was performed on 10-μm-thick frozen human brain

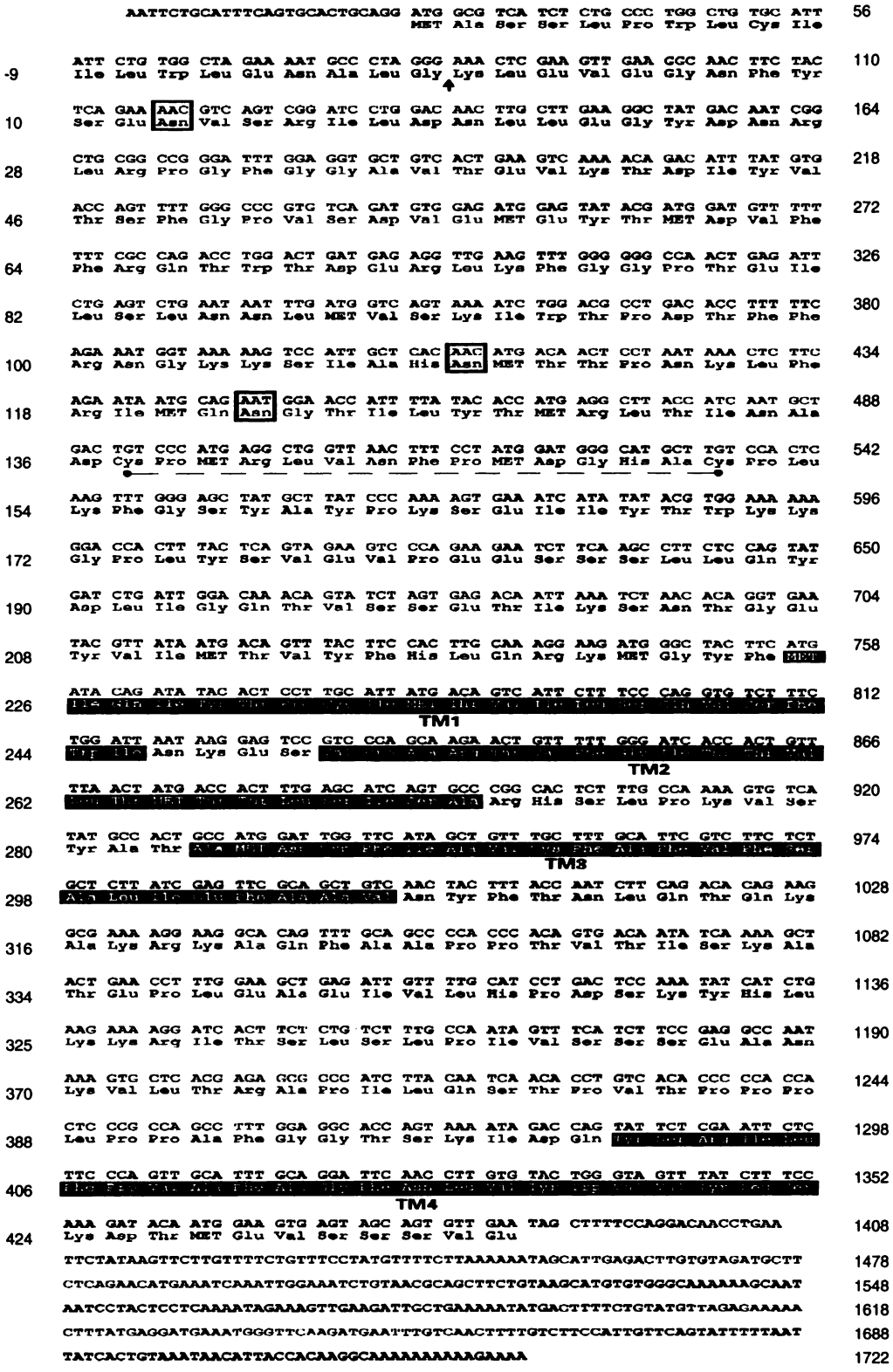

Fig. 1. Nucleotide and deduced amino acid sequences of human $GABA_A$ receptor α6 subunit. *Left*, amino acid numbers; negative numbers refer to the putative signal peptide, with the first amino acid of the putative mature polypeptide being residue +1. *Arrow*, position of the putative signal sequence cleavage site. *Dashed line*, the two cysteine residues separated by 13 amino acids. *Boxes*, putative *N*-glycosylation sites. *Black boxes*, putative transmembrane domains (TM1–TM4).

sections as described previously (18). The brain was obtained from a male heart attack victim aged 54 years. Briefly, blocks of human postmortem brain were frozen on dry ice, and 10-μm-thick sections from various brain regions were cut with a cryostat. After fixation in 40% paraformaldehyde in PBS, the sections were dehydrated in a series of alcohols and stored in 95% alcohol at 4° until used. Hybridization was performed overnight at 37° on air-dried sections with an antisense oligonucleotide corresponding to bases 1194–1241 of the human α6 sequence, which had been labeled to high specific radioactivity with ^{35}S-dATP using terminal transferase. After stringent washing (1× standard saline citrate, 55° and 60°, 30 min each), the sections were dehydrated again before air-drying. Sections were apposed to Hyperfilm max (Amersham) for 10 days. High resolution autoradiograms were achieved by dipping sections in Ilford K5 liquid emulsion and, after being developed 2 months later, counterstained with methylene blue, dehydrated, cleared with xylene, and mounted.

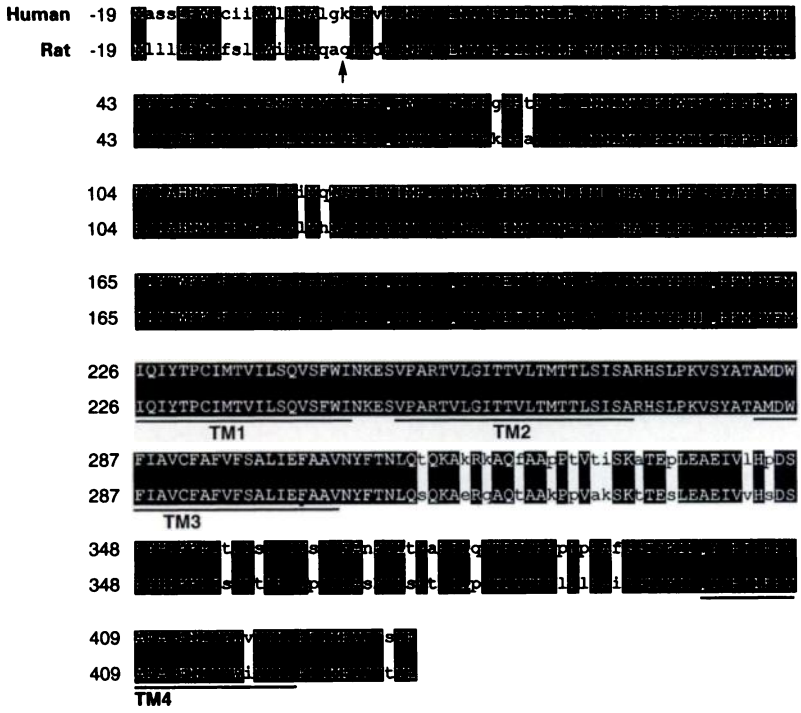

Fig. 2. Alignment of the deduced amino acid sequences of the rat (10) and human α6 $GABA_A$ receptor subunits. Sequences were aligned using the Intelligenetics Genalign program so that the most homologous sequences were placed next to each another. *Arrow*, position of the putative signal sequence cleavage site. *Black boxes*, amino acid identity between the sequences. *Left*, amino acid numbers, with the putative mature polypeptide beginning at position +1. *Underline*, putative transmembrane domains (TM1–TM4).

Results and Discussion

Nucleotide and deduced amino acid sequence of human α6 subunit. The nucleotide and deduced primary amino acid sequence of human α6 cDNA is shown in Fig. 1. The amino acid sequence (454 residues) contains all of the motifs seen in members of the ligand-gated ion channel family: a putative signal peptide (19 residues), a large putative extracellular domain containing two cysteines separated by 13 residues, and four hydrophobic putative TM domains. There are three putative *N*-glycosylation sites (Asn^{12}, Asn^{109}, and Asn^{122}), one putative cAMP-dependent protein kinase phosphorylation site (Thr^{356}), and two putative protein kinase C phosphorylation sites (Thr^{313} and Thr^{395}). Fig. 2 shows the alignment of the deduced amino acid sequences of the rat and human α6 subunits. There are 39 amino acid differences (91.4% identity), most of which are in the putative signal peptide and putative intracellular loop region between TM3 and TM4. It is interesting to note that this degree of species divergence is somewhat greater than that found for other human/rat α subunits, which show 99%, 97%, 98%, and 94% deduced amino acid sequence identity (α1, α2, α3, and α5 subunits, respectively) (9, 19–21). It is of importance that Arg^{100}, which determines the diazepam insensitivity of α6-containing rat $GABA_A$ receptors (22), is conserved in human α6.

Expression of the α6 subunit mRNA in human brain. *In situ* hybridization histochemistry has been performed on 10-μm-thick human cerebellar coronal sections with the use of an antisense oligonucleotide probe corresponding to bases 1194–1241 of the human α6 sequence. As shown in Fig. 3, dense hybridization signals for human α6 mRNA were found in the human cerebellum, with the signal being restricted to the granule cell layer of the cerebellar cortex. This is identical to the expression observed in rat brain (23, 24). High resolution autoradiograms (Fig. 3b) showed no expression of the mRNA in the Purkinje cells.

Pharmacology of human α6-containing receptors. To allow determination of the BZ pharmacology of human α6-containing receptors, a stable cell line expressing the α6β3γ2S $GABA_A$ receptor subtype has been established in mouse L(tk−) cells with an approach we described previously (15–17). The clonal cell line used in these studies, M632 clone 1, expresses 0.5–0.7 pmol [^{3}H]Ro 15–4513 binding sites/mg protein after a 7-day induction in medium containing dexamethasone, as determined from binding isotherms such as that shown in Fig. 4.

Scatchard analysis of the binding of [^{3}H]Ro 15–4513 to α6β3γ2S human $GABA_A$ receptors gave a K_d value of 6.5 ± 0.6 nM. Displacement of this radioligand was performed with a number of BZ binding site ligands, and their K_i values were calculated. The mean K_i values obtained are listed in Table 1, together with those obtained for the displacement of [^{3}H]Ro 15–1788 from similarly expressed α1β3γ2S $GABA_A$ receptors. It can be seen that the α6-containing receptor, although binding Ro 15–4513 with high affinity, shows negligible affinity for classic BZs, as typified by diazepam. This DI pharmacology, which has also reported for rat α4-containing receptors (11), is very different from that seen for α1-containing receptors, which exhibit a BZ1 pharmacology, binding BZs and the β-carbolines methyl-β-carboline-3-carboxylate and DMCM with high affinity. The DI pharmacology of the human α6-containing receptor is not unexpected given the conservation of the Arg^{100} residue among species, as this residue is known to be a major contributor to the DI pharmacology of rat α6-containing receptors (22). The 1,4-diazepinone carboxylate bretazenil (Ro 16–6028), like Ro 15–4513, exhibits a high affinity for human α6β3γ2S-containing receptors. This compound has been reported to have a high affinity for DI $GABA_A$ receptors in rat cerebellar membranes (25). The high affinity of Ro 15–4513 and bretazenil and the measurable affinities of Ro 15–1788 and FG 8205 may reflect their structural similarity; they are all imidazobenzodiazepines.

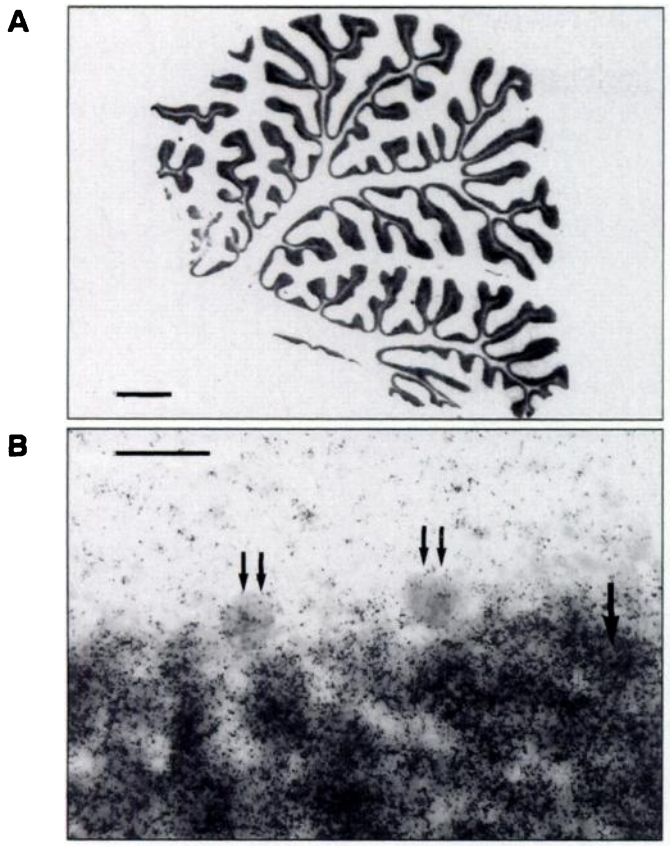

Fig. 3. Low (A) and high (B) resolution autoradiograms showing expression of α6 $GABA_A$ receptor mRNA in the human cerebellum. *In situ* hybridization of an antisense probe to the human α6 sequence to frozen human brain sections was performed as described in Materials and Methods. Scale bars: A, 1 mm; B, 50 μm. *Single arrow*, granule cell intensely labeled for α6 mRNA. *Double arrows*, Purkinje cells not labeled for α6 mRNA.

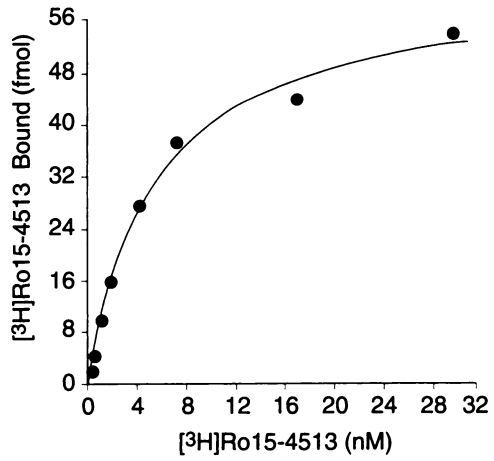

Fig. 4. Saturation isotherm of [^{3}H]Ro 15–4513 binding to membranes of α6β3γ2S permanently transfected cell membranes. In this experiment, 100 μg cell membrane protein bound a maximum of 61.3 fmol [^{3}H]Ro 15–4513 with a K_d of 5.3 nM. From four independent experiments, a mean K_d value of 6.5 ± 0.6 nM was obtained.

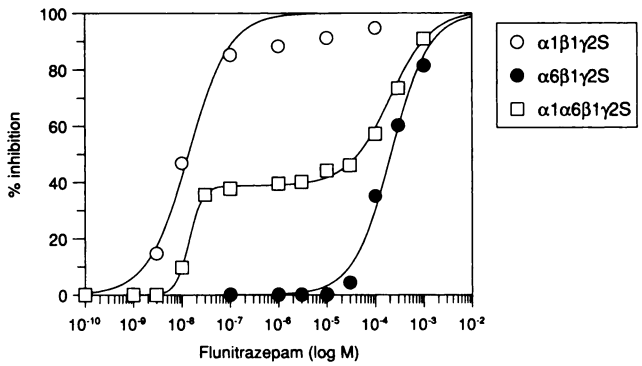

Fig. 5. Displacement by flunitrazepam of [^{3}H]Ro 15–4513 binding from membranes of human embryonic kidney 293 cell transiently transfected with α1β1γ2S (○), α6β1γ2S (●), or α1α6β1γ2S (□) cDNAs.

Cerebellar granule cells express both α1 and α6 subunits (24). It has been suggested that some $GABA_A$ receptor macromolecules in the cerebellum do contain both α1 and α6 subunits (26), although Quirk *et al.* (13) presented data indicating that the vast majority of receptors in the cerebellum contain either α1 or α6 subunits but not both. There have also been reports that native $GABA_A$ receptors can contain both α1 and α3 subunits or both α2 and α3 subunits (8, 27). To further investigate whether receptors can contain both an α1 and an α6 subunit, we transiently transfected 293 cells with α1β1γ2S, α6β1γ2S, and α1α6β1γ2S cDNAs and determined the affinities of these subunit combinations for flunitrazepam, which has markedly different affinities for α1- and α6-containing receptors (Table 1). Fig. 5 shows the result of a typical experiment. Membranes from α1α6β1γ2S-transfected cells show a biphasic curve with high affinity (IC_{50} = 14 nM) and low affinity (IC_{50} = 380 μM) sites for flunitrazepam. Membranes from cells transfected with α1β1γ2S and α6β1γ2S had affinities for flunitrazepam (IC_{50} = 14 nM and 210 μM, respectively) that were essentially identical to the affinities at the high and low affinity sites of α1α6β1γ2S-transfected cells. In Fig. 5, 40% of the [^{3}H]Ro 15–4513 binding sites in the α1α6β1γ2S-transfected cells have high affinity for flunitrazepam, whereas 60% have low affinity. The ratio of low to high affinity sites could be varied by altering the ratio of α1 to α6 cDNAs in the transfection (data not shown). These data clearly suggest that in 293 cells transfected with α1, α6, β1, and γ2S cDNAs, two populations of receptors are formed: α1β1γ2S and α6β1γ2S. The other possible interpretation is that an α1α6β1γ2S receptor is assembled, the BZ pharmacology of which is identical to that of α1β1γ2S or α6β1γ2S receptors.

The subunit combinations α6β2γ2S and α1β2γ2S were expressed in *Xenopus* oocytes, and their functional properties were characterized with regard to GABA and a number of different modulators of the $GABA_A$ receptor at the BZ site, as well as the steroid and barbiturate sites. GABA concentration-response curves revealed EC_{50} for α1β2γ2S to be 19.8 μM ± 4.6 (seven oocytes), with n_H = 1.36 ± 0.18, whereas EC_{50} for α6β2γ2S was 10.1 ± 6.0 μM (eight oocytes) with n_H = 1.06 ± 0.13. A number of different compounds that act via the BZ site were compared for these two subunit combinations at a concentration of 1 μM, which would be expected from the binding studies shown in Table 1 to give a maximum effect on α1β2γ2S (Fig. 6). A classic BZ such as flunitrazepam, which potentiated α1β2γ2S receptors, had no effect on α6β2γ2S, which is consistent with its extremely low affinity at this subunit combination (Table 1). The β-carboline DMCM had moderate affinity for α6-containing receptors and potentiated the GABA response at 1 μM. This is consistent with

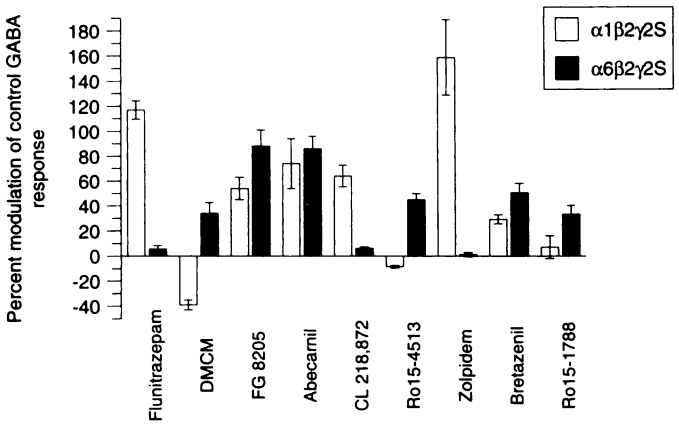

Fig. 6. Modulation of control GABA currents by BZ ligands on $\alpha1\beta2\gamma2S$ and $\alpha6\beta2\gamma2S$ $GABA_A$ receptor subunit combinations in a comparison of the degree of potentiation or inhibition with each ligand. Each value is the mean ± standard error of at least four oocytes. Control GABA responses were obtained by selecting a GABA concentration ~20% of maximum for each individual oocyte, usually 1–10 μM. All drugs were applied at a concentration of 1 μM.

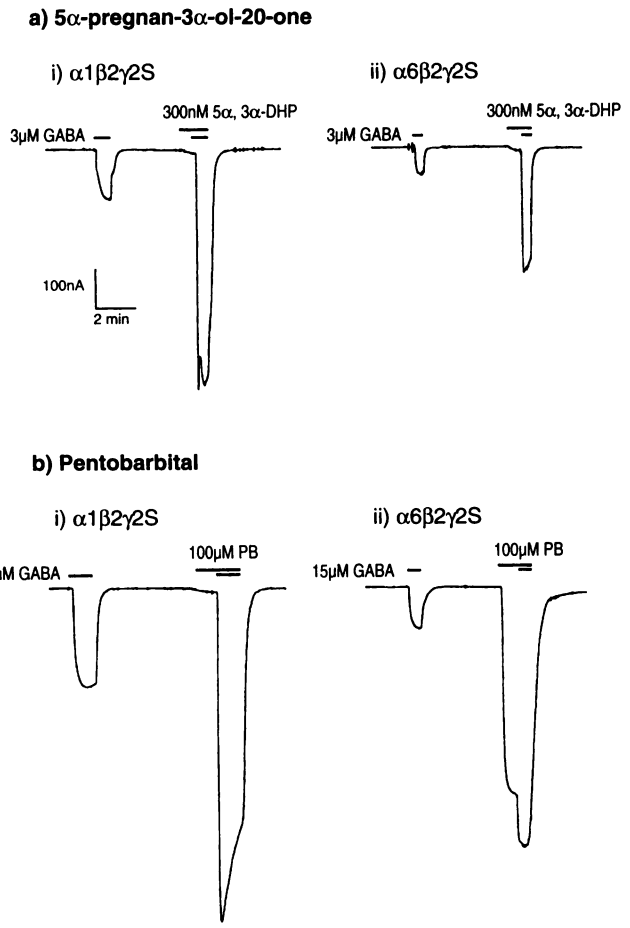

Fig. 7. a, Effects of the steroid 5α-pregnan-3α-ol-20-one (300 nM) on control GABA currents (EC_{20} response) in oocytes expressing the $GABA_A$ receptor combinations (i) $\alpha1\beta2\gamma2S$ and (ii) $\alpha6\beta2\gamma2S$. b, Effects of the barbiturate pentobarbital (100 μM) on oocytes expressing (i) $\alpha1\beta2\gamma2S$ and (ii) $\alpha6\beta2\gamma2S$. Shown is a large direct response on $\alpha6\beta2\gamma2S$ $GABA_A$ receptors. *Bars*, modulators were applied for 30 sec before the coapplication of GABA (EC_{20} predetermined for each oocyte).

other reports that at micromolar concentrations DMCM is able to potentiate the effect of GABA via an additional site on the $GABA_A$ receptor (28) that has been identified as the loreclezole modulatory site (29). On $\alpha1\beta2\gamma2S$, however, DMCM behaved as a typical full inverse agonist, inhibiting the response to GABA to a maximum of −40% with an affinity of 3.6 nM (data not shown). The partial inverse agonist (at $\alpha1\beta2\gamma2S$) Ro 15–4513 has high affinity at α6-containing receptors, but rather than inhibiting GABA responses, this compound behaved as a partial agonist, potentiating by 50% the response of $\alpha6\beta2\gamma2S$ to an EC_{20} concentration of GABA. Similarly, the structurally related BZ Ro 15–1788 is an antagonist at $\alpha1\beta2\gamma2S$ but behaved as a partial agonist (i.e., the potentiation at maximal concentrations of Ro 15–4513 is less than that of other compounds such as FG 8205 and abecarnil) on $\alpha6\beta2\gamma2S$. Atypical non-BZ compounds that bind to the BZ site also differed in their effects on $\alpha6\beta2\gamma2S$. The α1-selective compounds zolpidem and CL 218,872 were, not surprisingly, without effect at α6-containing receptors, reflecting their affinity at this receptor (Table 1). However, bretazenil, which is a partial agonist on $\alpha1\beta2\gamma2S$, maintained a reasonable affinity for α6-containing receptors (Table 1) and potentiated $\alpha6\beta2\gamma2S$ receptors to a greater extent than $\alpha1\beta2\gamma2S$. Abecarnil potentiated the two receptor combinations to the same extent at 1 μM, and the imidazobenzodiazepine FG 8205, which is a partial agonist on $\alpha1\beta2\gamma2S$, also potentiated $\alpha6\beta2\gamma2S$ at 1 μM but to a greater degree than $\alpha1\beta2\gamma2S$.

Steroids and barbiturates modulate the $GABA_A$ receptor, and these compounds were also found to potentiate the $\alpha6\beta2\gamma2S$ receptor combination. No difference was observed in the extent of potentiation by the steroid 5α-pregnan-3α-ol-20-one (300 nM) when compared for $\alpha1\beta2\gamma2S$ receptors (298 ± 60% potentiation; four oocytes) and $\alpha6\beta2\gamma2S$ receptors (312 ± 79% potentiation; three oocytes) (Fig. 7a). When 100 μM pentobarbital was applied to $\alpha1\beta2\gamma2S$, a very small direct effect was observed, followed by 335 ± 41% (four oocytes) potentiation of the GABA response. At $\alpha6\beta2\gamma2S$, 100 μM pentobarbital elicited a large response in the absence of GABA, ~500% of the GABA EC_{20}. Coapplication of GABA in the presence of pentobarbital resulted in a saturating response larger than that of a maximum GABA concentration (Fig. 7b). Further studies have revealed two separate sites for pentobarbital on the $GABA_A$ receptor: an agonist site and a modulatory site, the former of which is dependent on the α subunit variant (30).

In the present study, we investigated for the first time the efficacy of BZ-site compounds at the α6-containing receptor. This also differs significantly from α1- (Fig. 6) and α2-, α3-, and α5-containing receptors (data not shown) in that the inverse agonist Ro 15–4513 and the antagonist Ro 15–1788 act as BZ agonists at α6-containing receptors. It is known that Arg^{100} of α6 determines its low affinity for diazepam. This residue or, more likely, other amino acid residues in α6 that are not conserved in other α subunits must be responsible for the agonism of Ro 15–1788 and Ro 15–4513 at this subtype. This observation has implications for the interpretation of *in vivo* experiments that use these compounds. The agonism of Ro 15–1788 at α6-containing receptors presumably explains the activity of this compound in the discriminative stimulus behavioral paradigm, which is believed to be acting through the DI (i.e., α6 containing) receptor (31). Ro

15–1788 has also been observed to have behavioral effects when administered to normal human subjects (32); these include impairment of motor activity, which could be a result of the BZ agonist activity of Ro 15–1788 at α6-containing receptors, leading to enhancement of the cerebellar GABAergic inhibition.

In conclusion, we describe the structure of human $GABA_A$ receptor α6 subunit and its exclusive expression in the cerebellum of human brain. We demonstrate that, like rat α6-containing $GABA_A$ receptors (10), human α6-containing receptors have a unique BZ pharmacology compared with receptors containing α1, α2, α3, and α5 (coexpressed with a β and γ2S subunit) (7, 9) in having low affinity for several of the classic BZ compounds such as diazepam and flunitrazepam. Furthermore, we demonstrate that Ro 15–4513 and Ro 15–1788 are BZ agonists at α6-containing receptors, an observation that may elucidate the molecular mechanism behind some of the behavioral effects that have been reported for these compounds.

References

1. Doble, A., and I. L. Martin. Multiple benzodiazepine receptors: no reason for anxiety. *Trends Pharmacol. Sci.* **13:**76–81 (1992).
2. Whiting, P. J., R. M. McKernan, and K. A. Wafford. Structure and pharmacology of vertebrate $GABA_A$ receptor subtypes. *Int. Rev. Neurobiol.* **38:**95–138 (1995).
3. Macdonald R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17:**569–602 (1994).
4. Pritchett, D. B., H. Lüddens, and P. H. Seeburg. Type I and type II $GABA_A$ benzodiazepine receptors produced by transfected cells. *Science (Washington D. C.)* **245:**1389–1392 (1989)
5. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338:**582–585 (1989).
6. Squires, R. F., D. I. Benson, C. Braestrup, J. Coupet, V. Myers, and B. Beer. Some properties of brain specific benzodiazepine receptor: new evidence for multiple receptors. *Pharmacol. Biochem. Behav.* **10:**825–830 (1979).
7. Pritchett, D. B., and P. H. Seeburg. γ-Aminobutyric $acid_A$ receptor α5 subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54:**1802–1804 (1990).
8. McKernan, R. M., K. Quirk, R. Prince, P. A. Cox, N. P. Gillard, C. I. Ragan, and P. Whiting. $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7:**667–676 (1991).
9. Hadingham, K. L., P. Wingrove, B. Le Bourdelles, K. J. Palmer, C. I. Ragan, and P. J. Whiting. Cloning of cDNA sequences encoding human α2- and α3-aminobutyric $acid_A$ receptor subunits and characterization of the benzodiazepine pharmacology of recombinant α1-, α2-, α3-, and α5-containing human γ-aminobutyric $acid_A$ receptors. *Mol. Pharmacol.* **43:** 970–975 (1993).
10. Lüddens, H., D. B. Pritchett, M. Kohler, I. Killisch, K. Keinanen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature (Lond.)* **346:**648–651 (1990).
11. Wisden, W., A. Herb, H. Wieland, K. Keinanen, H. Lüddens and P. H. Seeburg. Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α4 subunit. *FEBS Lett.* **289:**227–230 (1991)
12. Turner, D. M., D. W. Sapp, and R. W. Olsen. The benzodiazepine/alcohol antagonist Ro 15–4513: binding to a $GABA_A$ receptor subtype that is insensitive to diazepam. *Mol. Pharmacol.* **257:**1236–1992 (1991).
13. Quirk, K., N. P. Gillard, C. I. Ragan, P. J. Whiting, and R. M. McKernan. Model of subunit composition of γ-aminobutyric acid A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. *J. Biol. Chem.* **269:**16020–16028 (1994).
14. Whiting, P., R. M. McKernan, and L. L. Iversen. Another mechanism for creating diversity in the γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of the γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. USA* **87:** 9966–9970 (1990)
15. Hadingham, K. L., P. B. Wingrove, K. A. Wafford, C. Bain, J. A. Kemp, K. J. Palmer, A. W. Wilson, A. S. Wilcox, J. M. Sikela, C. I. Ragan, and P.J. Whiting. The role of the β subunit in determining the pharmacology of human $GABA_A$ receptors. *Mol. Pharmacol.* **44:**1211–1218 (1993).
16. Whiting, P., R. Schoepfer, J. Lindstrom, and T. Priestley. Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol. Pharmacol.* **40:**463–472 (1991).
17. Hadingham, K. L., P. C. Harkness, R. M. McKernan, K. Quirk, B. Le Bourdelles, A. L. Horne, J. A. Kemp, E. A. Barnard, C. I. Ragan, and P. J. Whiting. Stable expression of mammalian type A-aminobutyric acid receptors in mouse cells: demonstration of functional assembly of benzodiazepine-responsive sites. *Proc. Natl. Acad. Sci. USA* **89:**6378–6382 (1992).
18. Sirinathsinghji, D. J. S., and S. B. Dunnett. Imaging gene expression in neural grafts, in *Molecular Imaging in Neuroscience* (N. A. Sharif, ed.). IRL Press, New York (1993).
19. Seeburg, P. H, W. Wisden, T. A. Verdoorn, D. B. Pritchett, P. Werner, A. Herb, H. Lüddens, R. Sprengel, and B. Sakmann. The $GABA_A$ receptor family: molecular and functional diversity. *Cold Spring Harb. Symp. Quant. Biol.* **55:**29–40 (1990) .
20. Schofield, P. R., D. B. Pritchett, H. Sontheimer, H. Kettenmann, and P. H. Seeburg. Sequence and expression of human $GABA_A$ receptor α1 and β1 subunits. *FEBS Lett.* **244:**361–364 (1989).
21. Wingrove, P., K. Hadingham, K. Wafford, J. A. Kemp, C. I. Ragan, and P. Whiting. Cloning and expression of a cDNA encoding the human $GABA_A$ receptor α5 subunit. *Biochem. Soc. Trans.* **20:**17S (1991).
22. Wieland, H. A., H. Luddens, and P. H. Seeburg. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.* **267:**1426–1429 (1992).
23. Wisden, W., D. J. Laurie, H. Monyer, and P. H. Seeburg. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12:**1040–1062 (1992).
24. Laurie, D. J., P. H. Seeburg, and W. Wisden. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.* **12:**1063–1076 (1992).
25. Wong, G., and P. Skolnick. High affinity ligands for 'diazepam-insensitive' benzodiazepine receptors. *Eur. J. Pharmacol.* **225:**63–68 (1992).
26. Pollard, S., M. J. Duggan, and F. A. Stephenson. Further evidence for the existence of α subunit heterogeneity within discrete γ-aminobutyric $acid_A$ receptor subpopulations. *J. Biol. Chem.* **268:**3753–3757 (1993).
27. Duggan, M. J., S. Pollard, and F. A. Stephenson. Immunoaffinity purification of $GABA_A$ receptor α-subunit iso-oligomers. *J. Biol. Chem.* **266:** 24778–24784 (1991).
28. Im, H. K., W. B. Im, D. B. Carter, and D. D. McKinley. Interaction of β-carboline inverse agonists for the benzodiazepine site with another site on $GABA_A$ receptors. *Br. J. Pharmacol.* **114:**1040–1044 (1995)
29. Stevenson, A., P. B. Wingrove, P. J. Whiting, and K. A. Wafford. β-carboline $GABA_A$ receptor inverse agonists modulate GABA via the loreclezole binding site as well as the benzodiazepine site. *Mol. Pharmacol.* **48:**965–969 (1995)
30. Thompson, S. A., P. J. Whiting, and K. A. Wafford. Barbiturate interactions at the human $GABA_A$ receptor are dependent on receptor subunit combination. *Br. J. Pharmacol.*, in press.
31. Wong, G., P. Skolnick, J. L. Katz, and J. M. Witkin. Transduction of a discriminative stimulus through a diazepam insensitive γ-aminobutyric $acid_A$ receptor. *J. Pharmacol. Exp. Ther.* **266:**570–576 (1993).
32. Higgitt, A., M. Lader, and P. Fonagy. The effects of the benzodiazepine antagonist Ro15–1788 on psychophysiological performance and subjective measures in normal subjects. *Psychopharmacology* **89:**395–403 (1986).

Send reprint requests to: Dr. Paul J. Whiting, Merck Sharp & Dohme Research Laboratories, P.O. Box 1675, Harlow, Essex, UK CM20 2QR.