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Expression and pharmacology of human $GABA_A$ receptors containing $\gamma 3$ subunits

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Abstract

A cDNA encoding the $\gamma 3$ subunit of the human $GABA_A$ receptor has been obtained by molecular cloning. Its deduced amino acid sequence shows a high level of sequence identity with the published mouse and rat sequences (96%). The ligand binding pharmacology of the benzodiazepine site formed by stably-expressed human $\alpha 5\beta 3\gamma 2S$ and $\alpha 5\beta 3\gamma 3$ $GABA_A$ receptor subtypes have been compared for a number of ligands. Benzodiazepine site ligands were found to be either non-selective or $\gamma 2$-selective, with the exception of CL218,872, which was found to be 10-fold selective for the $\alpha 5\beta 3\gamma 3$-containing subtype. Two benzodiazepine site ligands, Ro15-4513 and FG8205 were more efficacious at $\alpha 5\beta 3\gamma 3$ receptors than $\alpha 5\beta 3\gamma 2$ receptors expressed in *Xenopus* oocytes. CL218,872, which is a partial agonist at $\alpha 1$ containing receptors, had no intrinsic activity at either $\alpha 5\beta 3\gamma 2$ or $\alpha 5\beta 3\gamma 3$. $\alpha 1\beta 2\gamma 2S$ and $\alpha 1\beta 2\gamma 3$ human $GABA_A$ receptors were also expressed in *Xenopus* oocytes and their benzodiazepine pharmacology investigated. Both the EC_{50} and efficacy of benzodiazepine site ligands were influenced by the type of γ subunit coexpressed with $\alpha 1$ and $\beta 2$.

Keywords: $GABA_A$ receptor, $\gamma 3$ subunit; Benzodiazepine site

1. Introduction

The $GABA_A$ receptor is a Cl-gated ion channel, mediating the major inhibitory synaptic events in the vertebrate central nervous system (CNS). It is known to be the target for a wide variety of clinically-important drugs such as benzodiazepines and barbiturates which are prescribed as anxiolytics, muscle relaxants, sedatives, antiepileptic agents and hypnotics (Doble and Martin, 1992; Macdonald and Olsen, 1994). In recent years, molecular cloning has demonstrated the existence of multiple genes in the mammalian CNS ($\alpha 1$–6, $\beta 1$–3, $\gamma 1$–3, δ) encoding $GABA_A$ receptor subunits which are thought to coassemble into a family of receptor subtypes in the arrangement $\alpha x\beta x\gamma x$ or $\alpha x\beta x\delta$ (where x indicates any variant) (Macdonald and Olsen, 1994; Whiting et al., 1995).

There is good evidence that different subunit combinations expressed in recombinant systems confer distinct $GABA_A$ receptor benzodiazepine site pharmacologies, and that a minimum requirement to confer such pharmacology is $\alpha x\beta x\gamma x$ (Pritchett et al., 1989a, b; for review see Whiting et al., 1995). The type of α subunit present has been shown to have a profound effect on the affinity and efficacy of action of benzodiazepine ligands acting on the receptor (e.g. Pritchett and Seeburg, 1990; Hadingham et al., 1993a). Similarly, it has been demonstrated that the β subunit has little or no effect on benzodiazepine pharmacology (Hadingham et al., 1993b). Most studies of the effects of benzodiazepines at recombinant $GABA_A$ receptors have used $\gamma 2$ subunit containing receptors as this is the most abundant γ subunit in the CNS (Stephenson et al., 1990; Quirk et al., 1994b), and $\alpha x\beta x\gamma 2$ receptors have benzodiazepine pharmacologies comparable with that of native brain receptors (Pritchett et al., 1989a; Pritchett and Seeburg, 1990; Hadingham et al 1993a). Receptors containing $\gamma 1$ and $\gamma 3$ subunits are considerably less abundant, comprising 11% and 14% of $GABA_A$ receptors in the rat brain (Quirk et al., 1994b). There have been relatively few studies on the contribution of the $\gamma 1$ or $\gamma 3$ subunit to the benzodiazepine pharmacology of $GABA_A$ receptors. It is however clear that both $\gamma 1$ (Ymer et al., 1990; Puia et al., 1991; Wafford et al., 1993a) and $\gamma 3$ (Knoflach et al., 1991; Herb et al., 1992; Lüddens et al., 1994) containing receptors have benzodiazepine binding

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sites with pharmacologies which differ from those of receptors containing $\gamma 2$.

Although the exact subunit combinations which coassemble to form native $GABA_A$ receptors are not known, a considerable body of evidence has accumulated from studies using antibodies (e.g. Benke et al., 1991; Pollard et al., 1991; Fritschy et al., 1992; Quirk et al., 1994a), and mRNA co-localisation studies (e.g. Laurie et al., 1992a, b; Wisden et al., 1992), to give some indications as to which subunits are most likely to combine *in vivo*. There is good evidence for the coassembly in hippocampal neurons of $\alpha 5$ with α β subunit and $\gamma 2$. Both $\alpha 5$ and $\gamma 2$, and all three β subunits (primarily $\beta 1$ and $\beta 3$) are expressed in the hippocampus (Wisden et al., 1992), and the unique benzodiazepine pharmacology of $\alpha 5\beta x\gamma 2$ receptors expressed in recombinant systems (low affinity for the imidazopyridine zolpidem) (Pritchett and Seeburg, 1990; Hadingham et al., 1993a, b) is also found in radioligand binding studies using rat brain sections (Benavides et al., 1993), and can be immunoprecipitated from solubilised rat hippocampal membranes with $\alpha 5$ subunit specific antisera (McKernan et al., 1991). The $\gamma 3$ subunit is also expressed in the hippocampus (Wisden et al., 1992), and thus could coassemble with the $\alpha 5$ subunit, although there is as yet no direct evidence that this occurs. Interestingly, gene mapping studies have demonstrated the close linkage of the genes encoding $\alpha 5$, $\beta 3$ and $\gamma 3$ on both mouse chromosome 7 (Wagstaff et al., 1991a; Culiat et al., 1993; Culiat et al., 1994) and human chromosome 15q11-15q13 (Wagstaff et al., 1991b; Knoll et al., 1993; Gregor et al., 1995), which could suggest some coordinated regulation of expression *in-vivo*. We have therefore cloned the cDNA encoding the human $\gamma 3$ subunit, and generated a cell line stably expressing recombinant human $\alpha 5\beta 3\gamma 3$ $GABA_A$ receptors which we have used to characterise the pharmacology of this subtype. Additionally, we have investigated the influence of the $\gamma 3$ subunit on the pharmacology of $\alpha 1\beta 2 + \gamma 2$ or $\gamma 3$ subunit combinations expressed in *Xenopus oocytes*. We demonstrate the unique pharmacology of $\gamma 3$-containing $GABA_A$ receptors, and provide further evidence that determinants of the a and γ subunits influence the pharmacology of compounds acting at the benzodiazepine site.

2. Materials and methods

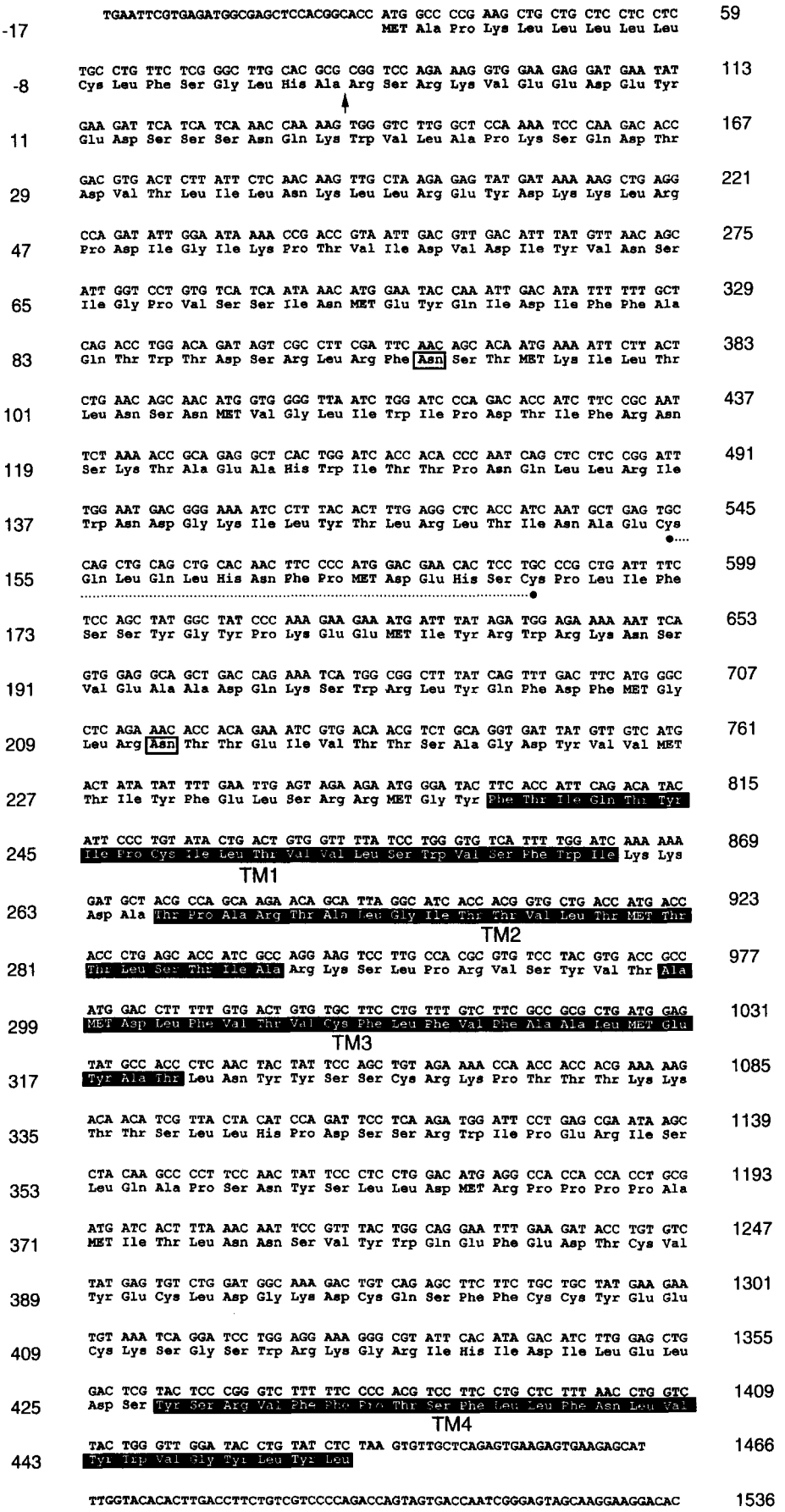
2.1. Cloning of cDNA encoding the human $\gamma 3$ subunit

A rat $\gamma 3$ cDNA probe was first generated by the polymerase chain reaction (PCR) using oligonucleotide primers derived from the rat $\gamma 3$ cDNA sequence (Knoflach et al., 1991): 5′ attcaagcttaccatggctgcaaagctgctgcttctctgcctgttctcgggg 3′ (bp 177–217, with 13 bases on the 5′ end containing a *Hin*dIII restriction site), and 5′ ggaattgtttaacgtgatcatcacgggtg 3′ (bp 1330–1358). PCR was performed as previously described (Whiting et al., 1990) using rat brain cDNA as template. A 1250 bp product was obtained which when cut with *Hin*dIII yielded 350 bp and 900 bp fragments, the latter of which was subcloned into pBluescript SK- (Stratagene). Sequencing confirmed the identity of this cDNA.

A human fetal brain cDNA library in λZAP (Stratagene) was screened as described previously (Hadingham et al., 1993a) and a single hybridising cDNA clone obtained. Sequence analysis, performed using an Applied Biosystems 373A sequencer and dye terminator chemistry, indicated that the cDNA lacked both the 5′ (approximately 200 bp) and 3′ (approximately 350 bp) ends of the coding region. These were subsequently obtained by anchored PCR. For the 3′ end, a sense oligonucleotide derived from sequence at the end of the truncated clone (5′ccagattcctcaagatgaattcctgagcgaataag 3′, incorporating an *Eco*RI site) was used in conjunction with an oligonucleotide overlapping the T7 primer sequence of pBluescript in a PCR reaction with human fetal brain cDNA library as template. A 500 bp product was obtained and subcloned into pBluescript. Sequence analysis indicated that it contained the 3′ end of the human $\gamma 3$ coding region together with 131 bp of 3′ untranslated region. The missing 5′ sequences of the $\gamma 3$ cDNA were obtained using human brain '5′ RACE ready cDNA' (Clontech) using nested oligonucleotide primers (5′ gctttttatcatatgctcttagcaac 3′ and 5′ caagacccacatatggtttgatggaga 3′). A 200 bp PCR product was subcloned into pCR-Script (Stratagene), and sequence analysis confirmed that it contained the missing 5′ coding region of the $\gamma 3$ cDNA together with 25 bp of 5′ untranslated region.

The complete nucleotide sequence of human $\gamma 3$ cDNA was determined using an Applied Biosystems 373A sequencer and dye terminator chemistry. Both strands of the cDNAs were sequenced. Sequence analysis was performed using Intelligenetics software (Palo Alto, CA). A cDNA containing the complete coding region of $\gamma 3$ was assembled from overlapping cDNAs using convenient restriction enzyme sites. The cDNA used for expression studies (see below) contains the first 30 amino acids of the rat $\gamma 3$ sequence (18 from the putative signal peptide and 12 from the putative mature polypeptide, 3 of which differ between rat and human). For generation of a stable cell line expressing the $\alpha 5\beta 3\gamma 3$ receptor, the $\gamma 3$ subunit cDNA was subcloned into pMSGneo inducible eukaryotic expression

Fig. 1. Nucleotide and deduced primary amino acid sequence of the human $GABA_A$ receptor $\gamma 3$ subunit. The arrow indicates the cleavage site of the putative signal peptide. The filled circles joined by a hatched line indicate the conserved cysteines separated by thirteen amino acids motif. Boxed residues indicate putative N-glycosylation sites. TM1–TM4 filled in sequences indicate the four hydrophobic domains. Amino acid numbering is on the left, with +1 being the first residue of the putative mature polypeptide. Nucleotide numbering is on the right.


TGAATTCGTGAGATGGCGAGCTCCACGGCACC ATG GCC CCG AAG CTG CTG CTC CTC CTC 59
-17 MET Ala Pro Lys Leu Leu Leu Leu Leu
TGC CTG TTC TCG GGC TTG CAC GCG CGG TCC AGA AAG GTG GAA GAG GAT GAA TAT 113
-8 Cys Leu Phe Ser Gly Leu His Ala Arg Ser Arg Lys Val Glu Glu Asp Glu Tyr
GAA GAT TCA TCA TCA AAC CAA AAG TGG GTC TTG GCT CCA AAA TCC CAA GAC ACC 167
11 Glu Asp Ser Ser Ser Asn Gln Lys Trp Val Leu Ala Pro Lys Ser Gln Asp Thr
GAC GTG ACT CTT ATT CTC AAC AAG TTG CTA AGA GAG TAT GAT AAA AAG CTG AGG 221
29 Asp Val Thr Leu Ile Leu Asn Lys Leu Leu Arg Glu Tyr Asp Lys Lys Leu Arg
CCA GAT ATT GGA ATA AAA CCG ACC GTA ATT GAC GTT GAC ATT TAT GTT AAC AGC 275
47 Pro Asp Ile Gly Ile Lys Pro Thr Val Ile Asp Val Asp Ile Tyr Val Asn Ser
ATT GGT CCT GTG TCA TCA ATA AAC ATG GAA TAC CAA ATT GAC ATA TTT TTT GCT 329
65 Ile Gly Pro Val Ser Ser Ile Asn MET Glu Tyr Gln Ile Asp Ile Phe Phe Ala
CAG ACC TGG ACA GAT AGT CGC CTT CGA TTC AAC AGC ACA ATG AAA ATT CTT ACT 383
83 Gln Thr Trp Thr Asp Ser Arg Leu Arg Phe Asn Ser Thr MET Lys Ile Leu Thr
CTG AAC AGC AAC ATG GTG GGG TTA ATC TGG ATC CCA GAC ACC ATC TTC CGC AAT 437
101 Leu Asn Ser Asn MET Val Gly Leu Ile Trp Ile Pro Asp Thr Ile Phe Arg Asn
TCT AAA ACC GCA GAG GCT CAC TGG ATC ACC ACA CCC AAT CAG CTC CTC CGG ATT 491
119 Ser Lys Thr Ala Glu Ala His Trp Ile Thr Thr Pro Asn Gln Leu Leu Arg Ile
TGG AAT GAC GGG AAA ATC CTT TAC ACT TTG AGG CTC ACC ATC AAT GCT GAG TGC 545
137 Trp Asn Asp Gly Lys Ile Leu Tyr Thr Leu Arg Leu Thr Ile Asn Ala Glu Cys
CAG CTG CAG CTG CAC AAC TTC CCC ATG GAC GAA CAC TCC TGC CCG CTG ATT TTC 599
155 Gln Leu Gln Leu His Asn Phe Pro MET Asp Glu His Ser Cys Pro Leu Ile Phe
TCC AGC TAT GGC TAT CCC AAA GAA GAA ATG ATT TAT AGA TGG AGA AAA AAT TCA 653
173 Ser Ser Tyr Gly Tyr Pro Lys Glu Glu MET Ile Tyr Arg Trp Arg Lys Asn Ser
GTG GAG GCA GCT GAC CAG AAA TCA TGG CGG CTT TAT CAG TTT GAC TTC ATG GGC 707
191 Val Glu Ala Ala Asp Gln Lys Ser Trp Arg Leu Tyr Gln Phe Asp Phe MET Gly
CTC AGA AAC ACC ACA GAA ATC GTG ACA ACG TCT GCA GGT GAT TAT GTT GTC ATG 761
209 Leu Arg Asn Thr Thr Glu Ile Val Thr Thr Ser Ala Gly Asp Tyr Val Val MET
ACT ATA TAT TTT GAA TTG AGT AGA AGA ATG GGA TAC TTC ACC ATT CAG ACA TAC 815
227 Thr Ile Tyr Phe Glu Leu Ser Arg Arg MET Gly Tyr Phe Thr Ile Gln Thr Tyr
ATT CCC TGT ATA CTG ACT GTG GTT TTA TCC TGG GTG TCA TTT TGG ATC AAA AAA 869
245 Ile Pro Cys Ile Leu Thr Val Val Leu Ser Trp Val Ser Phe Trp Ile Lys Lys
TM1
GAT GCT ACG CCA GCA AGA ACA GCA TTA GGC ATC ACC ACG GTG CTG ACC ATG ACC 923
263 Asp Ala Thr Pro Ala Arg Thr Ala Leu Gly Ile Thr Thr Val Leu Thr MET Thr
TM2
ACC CTG AGC ACC ATC GCC AGG AAG TCC TTG CCA CGC GTG TCC TAC GTG ACC GCC 977
281 Thr Leu Ser Thr Ile Ala Arg Lys Ser Leu Pro Arg Val Ser Tyr Val Thr Ala
ATG GAC CTT TTT GTG ACT GTG TGC TTC CTG TTT GTC TTC GCC GCG CTG ATG GAG 1031
299 MET Asp Leu Phe Val Thr Val Cys Phe Leu Phe Val Phe Ala Ala Leu MET Glu
TM3
TAT GCC ACC CTC AAC TAC TAT TCC AGC TGT AGA AAA CCA ACC ACC ACG AAA AAG 1085
317 Tyr Ala Thr Leu Asn Tyr Tyr Ser Ser Cys Arg Lys Pro Thr Thr Thr Lys Lys
ACA ACA TCG TTA CTA CAT CCA GAT TCC TCA AGA TGG ATT CCT GAG CGA ATA AGC 1139
335 Thr Thr Ser Leu Leu His Pro Asp Ser Ser Arg Trp Ile Pro Glu Arg Ile Ser
CTA CAA GCC CCT TCC AAC TAT TCC CTC CTG GAC ATG AGG CCA CCA CCA CCT GCG 1193
353 Leu Gln Ala Pro Ser Asn Tyr Ser Leu Leu Asp MET Arg Pro Pro Pro Pro Ala
ATG ATC ACT TTA AAC AAT TCC GTT TAC TGG CAG GAA TTT GAA GAT ACC TGT GTC 1247
371 MET Ile Thr Leu Asn Asn Ser Val Tyr Trp Gln Glu Phe Glu Asp Thr Cys Val
TAT GAG TGT CTG GAT GGC AAA GAC TGT CAG AGC TTC TTC TGC TGC TAT GAA GAA 1301
389 Tyr Glu Cys Leu Asp Gly Lys Asp Cys Gln Ser Phe Phe Cys Cys Tyr Glu Glu
TGT AAA TCA GGA TCC TGG AGG AAA GGG CGT ATT CAC ATA GAC ATC TTG GAG CTG 1355
409 Cys Lys Ser Gly Ser Trp Arg Lys Gly Arg Ile His Ile Asp Ile Leu Glu Leu
GAC TCG TAC TCC CGG GTC TTT TTC CCC ACG TCC TTC CTG CTC TTT AAC CTG GTC 1409
425 Asp Ser Tyr Ser Arg Val Phe Phe Pro Thr Ser Phe Leu Leu Phe Asn Leu Val
TM4
TAC TGG GTT GGA TAC CTG TAT CTC TAA GTGTTGCTCAGAGTGAAGAGTGAAGAGCAT 1466
443 Tyr Trp Val Gly Tyr Leu Tyr Leu
TTGGTACACACTTGACCTTCTGTCGTCCCCAGACCAGTAGTGACCAATCGGGAGTAGCAAGGAAGGACAC 1536

vector (Whiting et al., 1991, Hadingham et al., 1992). For expression in *Xenopus* oocytes the $\gamma 3$ cDNA was subcloned into the eukaryotic expression vector pCDM8.

2.2. cDNAs

Human $\alpha 1$, $\alpha 5$, $\beta 2$, $\beta 3$ and $\gamma 2$ $GABA_A$ subunit cDNAs have been described previously (Wingrove et al., 1991; Hadingham et al., 1993a, b). The $\gamma 2S$ isoform of the human $\gamma 2$ subunit was used throughout this study.

2.3. Generation of stably transfected cell line expressing $\alpha 5\beta 3\gamma 3$ $GABA_A$ receptors

Production of a stably transfected cell line, K532 clone 5, expressing human $\alpha 5\beta 3\gamma 2$ $GABA_A$ receptors has been described previously (Hadingham et al., 1993b). Stable expression of the $\alpha 5\beta 3\gamma 3$ subtype was similarly obtained by transfection into mouse L(tk-) cells of the individual subunit cDNAs, subcloned into pMSGneo. DNA for transfection was prepared by double-banding on CsCl gradients. Cell culture and transfections were performed as described previously (Hadingham et al., 1992, 1993b). Geneticin (Gibco) -resistant cell colonies obtained from the $\alpha 5\beta 3\gamma 3$ stable transfection were isolated using cloning cylinders and individually analysed for the binding of 3nM [^{3}H]Ro15-1788 (75.3Ci/mmol; New England Nuclear), after a 5-day induction of receptor expression by the addition of 1 mM dexamethasone to culture medium lacking Geneticin. The population expressing the highest levels of [^{3}H]Ro15-1788 binding was recloned by limiting dilution. The resultant cell line, C533 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.

2.4. Radioligand binding

Cell monolayers expressing recombinant $GABA_A$ receptors were washed twice with phosphate-buffered saline (PBS) and scraped into PBS. After centrifugation ($3000 \times g$ for 20 min at 4°C), membranes were prepared as described previously (Hadingham et al., 1993b). Saturation binding curves were obtained by incubating membranes (200 μg and 300 μg of protein for K532 clone 5 and C533 clone 1, respectively) with various concentrations of [^{3}H]Ro15-1788. Non-specific binding was measured by the inclusion of 10 mM unlabelled flunitrazepam (Sigma). All binding assays were performed in triplicate in an assay volume of 0.5 ml, with an incubation time of 90 min at 4°C. Incubations were terminated by filtration through GF/B filters (Brandel, Gathersberg, MD) 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 0.5 nM [^{3}H]Ro15-1788 by various benzodiazepine binding site ligands was performed under similar conditions, and single-site dose-response curves fitted to the experimental data using the least-squares iterative fitting routine of analysis package RS/1 (BBN Research Systems, Cambridge, MA). K_i values were calculated from the results of at least three independent determinations, using the equation $K_i = IC_{50}/(1 + [[^3H]Ro15\text{-}1788]/K_D$. Other than Ro15-1788, bretazenil (both gifts from Hoffmann La Roche), CGS8216 (Ciba-Geigy, Basle, Switzerland), zolpidem (Synthelabo), abecarnil (Schering AG), CL218,872 (Lederle) and FG8205 (synthesized at Merck Sharp and Dohme), all other benzodiazepine site ligands were obtained from Research Biochemicals, Natwick, MA or Sigma.

2.5. Oocyte expression

Xenopus oocytes were removed from anaesthetised 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 $GABA_A$ subunit cDNAs (6 ng/ml) engineered into the expression vector pCDM8. Following incubation for 24 hr, oocytes were placed in 50 μl bath and perfused with modified Barth's medium (MBS) 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 $CaCl_2$, 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 4–6 ml/min and drugs were applied in the perfusate. GABA modulators were preapplied for 30 sec prior to the addition of GABA. GABA was applied until the peak of the response was observed, usually 30 sec or less. At least three minutes wash time was allowed between each GABA application to prevent desensitisation. Concentration-response curves were calculated using a non-linear squares fitting program to the equation $f(x) = B_{max}/(1 + (EC_{50}/x)^n)$ where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half maximal response and n is the Hill coefficient.

3. Results

3.1. Human $\gamma 3$ subunit cDNA and deduced primary amino acid sequence

The nucleotide and deduced primary amino acid sequence of human $GABA_A$ receptor $\gamma 3$ subunit is shown in Fig. 1. The polypeptide has an open reading frame of 467 amino acids, 19 and 21 of which differ from the published rat (Knoflach et al., 1991) and mouse sequences (Wilson-

Shaw et al., 1991), respectively. The human γ3 subunit has motifs found in other $GABA_A$ receptor subunits, i.e. a putative signal peptide, 2 cysteines separated by 13 residues, and four hydrophobic domains (TM1–TM4). In Fig. 2 the deduced amino acid sequences of the human γ subunits have been aligned. Overall there is significant sequence homology; the domains showing most diversity are the putative signal peptide and the putative large cytoplasmic loop between TM3 and TM4.

3.2. Comparison of the benzodiazepine pharmacology of α5β3γ2 and α5β3γ3 human $GABA_A$ receptors

A stable cell line expressing the human α5β3γ3 $GABA_A$ receptor subtype has been established in mouse L(tk-) cells, using an expression system we have described previously (Whiting et al., 1991; Hadingham et al., 1992, 1993b). Clonal cell line C533 clone 1, used in this study, expresses approximately 80fmol [^{3}H]Ro15-1788 binding sites/mg membrane protein following a 5-day induction of receptor expression. The expression of human α5, β3 and γ3 mRNA transcripts in this cell line was confirmed by isolation of mRNA, cDNA synthesis and PCR using subunit specific oligonucleotide primers (data not shown).

Scatchard analysis of the binding of [^{3}H]Ro15-1788 to α5β3γ2 (Hadingham et al., 1993b) and α5β3γ3 cell membranes gave mean K_D values of 0.45 ± 0.04 nM and 0.63 ± 0.11 nM, respectively, for this ligand at the expressed $GABA_A$ receptor benzodiazepine binding sites. Displacement of this radioligand with various other benzo-

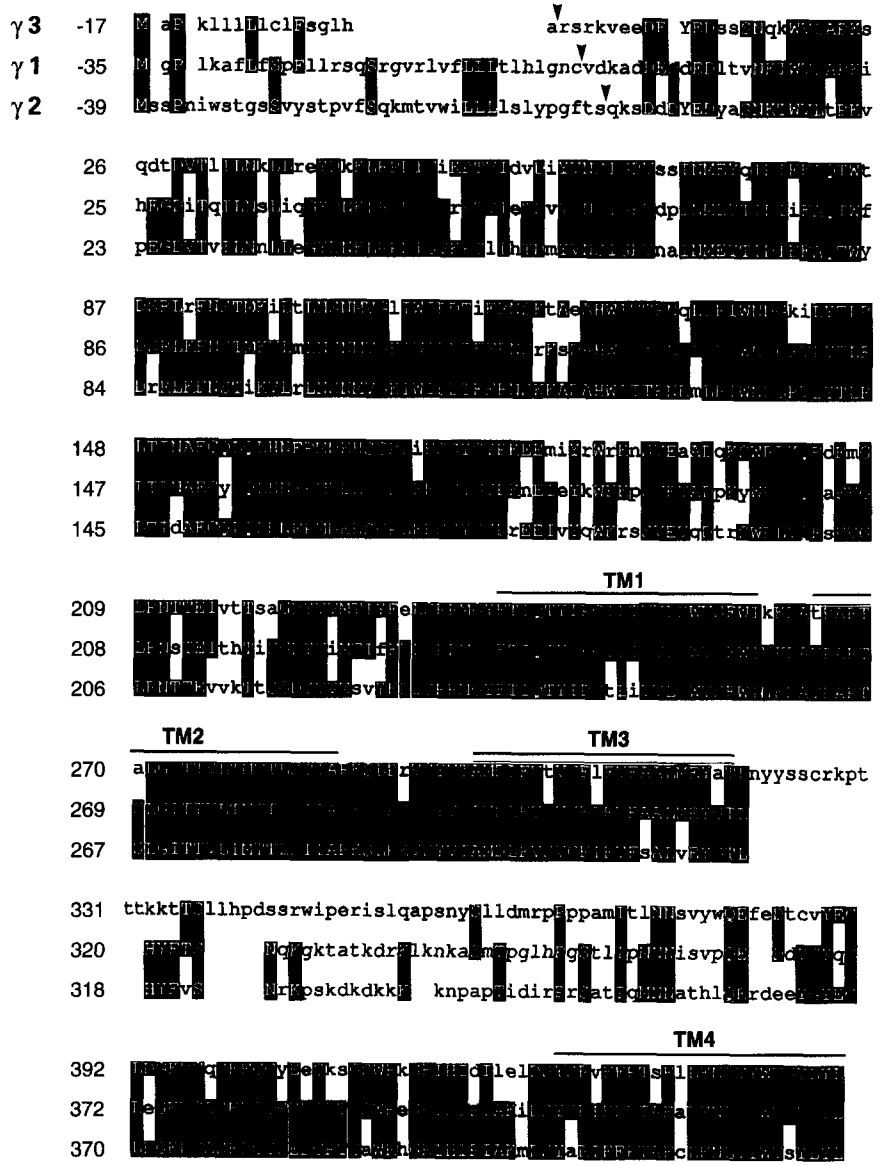


Fig. 2. Alignment of the deduced amino acid sequences of the human γ1, γ2 and γ3 subunits. Sequences were aligned using the Genalign program (Intelligenetics, Palo Alto, CA) so that the most homologous sequences are placed next to each other. The arrows indicate the site of cleavage of the putative signal peptide. The amino acid numbers are indicated on the left. TM1-TM4, hydrophobic domains.

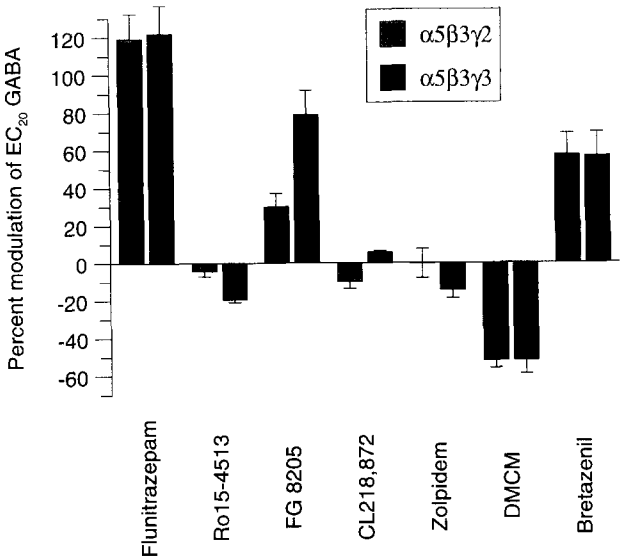


Fig. 3. Efficacy of benzodiazepine compounds at $\alpha5\beta3\gamma2$ and $\alpha5\beta3\gamma3$ receptors expressed in *Xenopus* oocytes A GABA concentration of approximately 20% of maximum was used on each oocyte. Each column is the mean ± standard error of at least four oocytes. Concentrations of each benzodiazepine site compound which should give a maximal effect, as determined from the affinity values in Table 1, were applied: flunitrazepam, 1 μM; Ro15-4513, 0.1 μM; FG8205, 1 μM; CL218,872, 3 μM; zolpidem, 10 μM; methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), 0.1 μM; bretazenil, 0.1 μM.

diazepine binding site ligands was performed for each cell line, and their respective K_i values calculated. The mean K_i values obtained for each ligand at the two receptor subtypes are listed in Table 1. It can be seen that whilst a few ligands (e.g. Ro15-1788, Ro15-4513) show no selectivity between the two receptor subtypes, a majority have higher affinity for receptors containing the γ2 subunit, whilst only one compound, CL218,872, exhibited a higher affinity for $\alpha5\beta3\gamma3$.

Table 1
Affinities of selected benzodiazepine site ligands for human $\alpha5\beta3\gamma2$ and $\alpha5\beta3\gamma3$ $GABA_A$ receptor subunit combinations stably expressed in mouse L(tk-) cells

Ligand	Affinity (nM)	
	$\alpha5\beta3\gamma2$	$\alpha5\beta3\gamma3$
[^{3}H]Ro15-1788 [a]	0.45 ± 0.04	0.63 ± 0.11
Ro15-4513	0.24 ± 0.05	0.40 ± 0.10
Flunitrazepam	2.11 ± 0.23	11.0 ± 2.2
Triazolam	0.48 ± 0.04	8.77 ± 1.44
FG8205	1.54 ± 0.31	2.73 ± 0.25
Bretazenil	0.55 ± 0.09	1.56 ± 0.19
CL218,872	561 ± 111	52.2 ± 5.6
Zolpidem	> 10000 [d]	> 10000 [d]
β-CCM [b]	52.6 ± 6.3	599 ± 111
DMCM [c]	1.0 ± 0.2	26.8 ± 6.2
CGS8216	0.48 ± 0.08	5.79 ± 0.61

Affinities (K_i; K_d or IC_{50} where indicated) for eleven benzodiazepine site ligands are shown. The K_d value shown was obtained by Scatchard isotherm analysis of radioligand binding. The K_i and IC_{50} values indicated were obtained by displacement of 0.5 nM [^{3}H]Ro15-1788 by various ligands. All values are the mean standard error from at least 3 independent determinations. [a] K_D value; [b] β-CCM, methyl-carboline-3-carboxylate; [c] methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; [d] IC_{50} values.

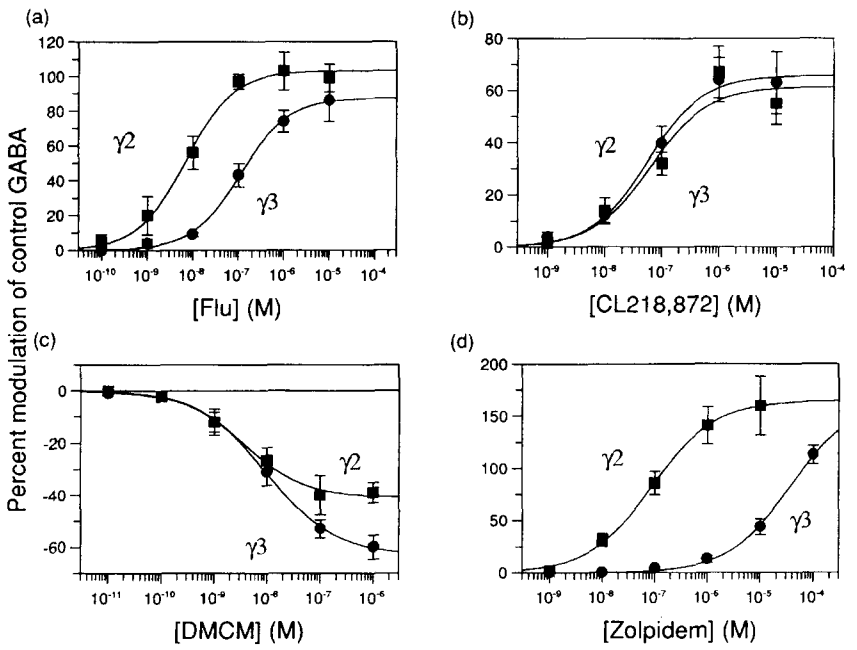


Fig. 4. Concentration-response curves for compounds acting at the benzodiazepine binding site of $\alpha1\beta2\gamma2$ (filled squares) on $\alpha1\beta2\gamma3$ (filled circles) recombinant human $GABA_A$ receptors expressed in *Xenopus* oocytes. A GABA concentration approximately 20% of maximum was used on each individual oocyte. Each point represents the mean ± standard error of at least four oocytes and represents the modulation of a control GABA current for (a) flunitrazepam, (b) CL218 872, (c) zolpidem, and (d) methyl-6,7-dimethoxy-4-ethyl-b-carboline-3-carboxylate (DMCM). Curves were fitted as described in Materials and methods.

Table 2
EC_{50} values and maximum potentiation of a GABA EC_{20} response produced by selected benzodiazepine site ligands at human $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma3$ $GABA_A$ receptors expressed in *Xenopus* oocytes

	$\alpha1\beta2\gamma2$		$\alpha1\beta2\gamma3$	
	Maximum potentiation of GABA EC_{20}	EC_{50} (nM)	Maximum potentiation of GABA EC_{20}	EC_{50} (nM)
Flunitrazepam	103 ± 3.2	6.9 ± 1.4	88 ± 2.3	110.7 ± 14.3
CL218 872	61 ± 10.7	57.4 ± 6.2	66 ± 3.0	56.7 ± 13.4
Zolpidem	165 ± 5.1	86.6 ± 14.1	165 *	36 500 ± 1928
[a] DMCM	−40.9 ± 1.7	3.7 ± 0.9	−63 ± 1.3	9.8 ± 1.1
Abecarnil	74 ± 20	–	96 ± 28	–
FG8205	54 ± 9.0	–	47 ± 8.3	–

Values are calculated from data in Fig. 3 using the Hill equation as described in the materials and methods and represent the mean ± standard errors of at least 4 individual oocytes. * As a true maximum could not be reached for zolpidem on $\alpha1\beta2\gamma3$, the EC_{50} was estimated by fitting a curve and constraining the maximum (B_{MAX}) to the value obtained for $\alpha1\beta2\gamma2$. [a] Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate.

The efficacy of a number of benzodiazepine site ligands was compared at $\alpha5\beta3\gamma2$ and $\alpha5\beta3\gamma3$ receptors expressed in *Xenopus* oocytes (Fig. 3). Flunitrazepam, zolpidem, CL218,872, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and bretazenil did not distinguish between the two receptor subunit combinations. Ro15-4513 was a more efficacious inverse agonist, and FG8205 a more efficacious agonist, at $\alpha5\beta3\gamma3$. Interestingly CL218,872, which is a partial agonist at $\alpha1$ containing receptors (Wafford et al., 1993b) (Fig. 4), had no intrinsic efficacy at either $\alpha5\beta3\gamma2$ or $\alpha5\beta3\gamma3$.

3.3. Characterization of the functional pharmacology of $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma3$ human $GABA_A$ receptors expressed in Xenopus oocytes

Human $GABA_A$ receptor subunit combinations $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma3$ were expressed in *Xenopus* oocytes and their pharmacology compared. Although not discussed here the $\gamma3$ variant confers a slightly higher GABA affinity compared to receptors containing a $\gamma2$, as has been previously reported (Ebert et al., 1994). EC_{20} GABA responses were determined for each oocyte and benzodiazepine ligands were co-applied to study the relative affinity and efficacy of these compounds for the two different receptor subtypes. Six different benzodiazepine site ligands were examined and concentration response curves determined for four of these compounds (Fig. 4). Maximum potentiation and EC_{50} values determined from these experiments are shown in Table 2. Flunitrazepam had a 16-fold lower affinity for $\alpha1\beta2\gamma3$ over $\alpha1\beta2\gamma2$, with a small reduction in efficacy. Interestingly CL218,872 had an identical affinity and efficacy at both receptor subtypes, suggesting that the $\alpha1$ subunit contributes the primary determinants for the binding of this compound. The contribution made by the γ-subunit appears to depend on the α-variant present, as CL218,872 affinity is different between receptors containing $\gamma2$ or $\gamma3$ when co-expressed with the $\alpha5$-subunit (Table 1). Zolpidem had a considerably lower affinity at $\alpha1\beta2\gamma3$ compared to $\alpha1\beta2\gamma2$ receptors with a maximum potentiation of 165% on $\alpha1\beta2\gamma2$, greater than that with flunitrazepam. Because the lack of solubility of zolpidem at concentrations higher than 100 μM precluded a maximum level of potentiation being reached for $\alpha1\beta2\gamma3$, the curve fit was extrapolated to the same maximum as $\alpha1\beta2\gamma2$ to obtain an estimate of affinity (Table 2). Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) inhibited the GABA response to a greater maximum level on $\alpha1\beta2\gamma3$ (−63%) than $\alpha1\beta2\gamma2$ (−40.9%), with a small decrease in affinity. The effects of some other benzodiazepine site ligands were also investigated. The β-carboline derivative abecarnil elicited approximately the same degree of potentiation on both subunit combinations at a concentration of 1 μM, $\alpha1\beta2\gamma2$ (74 ± 20%) and $\alpha1\beta2\gamma3$ (96 ± 28%). The partial agonist FG8205 (Tricklebank et al., 1990) (1 μM) also potentiated both subunit combinations to the same extent, $\alpha1\beta2\gamma2$ (54 ± 9%) and $\alpha1\beta2\gamma3$ (57 ± 8.3%). This compound also had similar affinities at $\alpha5\beta3\gamma2$ and $\alpha5\beta3\gamma3$ (Table 1). The high affinity ligand Ro15-1788, which is an antagonist at $\alpha1\beta2\gamma2$ receptors (Wafford et al., 1993a), was tested for antagonism on $\alpha1\beta2\gamma3$. 10 μM Ro15-1788 gave a small degree of potentiation alone but reduced the potentiation induced by 3 μM flunitrazepam from 86 ± 9.6% ($n = 4$) to 19 ± 6.3% ($n = 4$), consistent with this compound behaving as an antagonist on $\gamma3$ containing receptors.

4. Discussion

Here we report the cloning and sequence of the human $GABA_A$ receptor $\gamma3$ subunit. We also report characterisation of the benzodiazepine pharmacology of recombinant human $\gamma3$ containing receptors, expressed in both permanently transfected mammalian cells and in *Xenopus* oocytes.

Previous studies using recombinant $GABA_A$ receptors have demonstrated that the type of α subunit present determines the benzodiazepine pharmacology, both in terms

of affinity (Pritchett et al., 1989a; Pritchett and Seeburg, 1990; Hadingham et al., 1993a) and efficacy (Wafford et al., 1993b). In contrast, the type of β subunit present does not seem to significantly influence the affinity or efficacy of benzodiazepine site compounds at $GABA_A$ receptor subtypes (Hadingham et al., 1993b). Having a γ subunit present in the $GABA_A$ receptor complex is an absolute requirement for benzodiazepine binding (Pritchett et al., 1989b). Indeed, like the α subunit, the type of γ subunit present in the $GABA_A$ receptor affects both affinity (Wafford et al., 1993a; Lüddens et al., 1994) and efficacy (Puia et al., 1991; Knoflach et al., 1991; Wafford et al., 1993a) of benzodiazepine compounds. In this study we have confirmed the observations of previous studies, which used rat rather than human $GABA_A$ receptor subunit cDNAs, that $\gamma 3$ containing receptors have a benzodiazepine binding site (Knoflach et al., 1991; Herb et al., 1992; Lüddens et al., 1994).

It is clear from the data in Tables 1 and 2 that whether the $GABA_A$ receptor has a $\gamma 2$ or $\gamma 3$ subunit affects the affinity of some but not all benzodiazepine site ligands. For instance, Ro15-1788, Ro15-4513, FG8205 and bretazenil have very similar affinities at $\alpha 5\beta 3\gamma 2$ and $\alpha 5\beta 3\gamma 3$. It is interesting to note that these compounds also tend to be the least selective in their affinities for receptors which vary in their α subunit (i.e. $\alpha 1$–$\alpha 6$ coexpressed with a β subunit and $\gamma 2$) (Hadingham et al., 1993a; Hadingham et al., unpublished observations). Most compounds tested had a higher affinity for $\gamma 2$ over $\gamma 3$ containing receptors. A similar trend was also observed in a previous study comparing $\alpha 2\beta 1\gamma 1$ with $\alpha 2\beta 1\gamma 2$ (Wafford et al., 1993a). This '$\gamma 2$ selectivity' may not be surprising since all benzodiazepine site compounds will have been originally selected for their affinity at native brain $GABA_A$ receptors, the majority of which contain the $\gamma 2$ subunit. An interesting observation, as previously observed by Lüddens et al. (1994), is the higher affinity of CL218,872 for $\alpha 5\beta 3\gamma 3$ receptors over $\alpha 5\beta 3\gamma 2$. This compound is the prototypic benzodiazepine Type 1 selective compound and is atypical in being a triazolopyridazine structure (see Doble and Martin, 1992). However when the affinity of CL218,872 is examined on receptors containing an $\alpha 1$ subunit (Fig. 4, Table 2) the selectivity for $\gamma 3$ containing receptors is lost, demonstrating the complex nature of the benzodiazepine binding site, and confirming that determinants from both the α and γ subunit contribute intimately to its structure. Similar conclusions can be reached from examination of the affinities of the benzodiazepine Type 1 selective imidazopyridine zolpidem. This compound has high affinity at $\alpha 1\beta x\gamma 2$ receptors, lower affinity at $\alpha 2\beta x\gamma 2$ or $\alpha 3\beta x\gamma 2$ receptors, and very low affinity at $\alpha 5\beta x\gamma 2$ receptors (Pritchett et al., 1989b; Hadingham et al., 1993a, b). Substitution of a $\gamma 3$ for $\gamma 2$ subunit, when expressed with $\alpha 1$ (Fig. 4 and Table 2) results in a greater than 1000-fold loss in affinity. Interestingly, substitution of $\gamma 2$ for $\gamma 1$ ($\alpha 2\beta 2\gamma 1$ versus $\alpha 2\beta 1\gamma 2$) results in a 5-fold increase in affinity for zolpidem (Wafford et al., 1993a).

Whether there is a $\gamma 2$ or $\gamma 3$ subunit present in the receptor can also affect the efficacy of benzodiazepine compounds. In this study we observed that the degree of efficacy of the inverse agonist Ro15-4513 and the agonist FG8205 was influenced by whether $\gamma 2$ or $\gamma 3$ was coexpressed with $\alpha 5$ and $\beta 3$ (Fig. 3), and that the β-carboline inverse agonist DMCM was more efficacious at $\alpha 1\beta 2\gamma 3$ compared to $\alpha 1\beta 2\gamma 2$ (Fig. 4). Perhaps even more surprisingly CL218,872, which is an agonist at $\alpha 1\beta 1\gamma 2$ (Wafford et al., 1993b), $\alpha 1\beta 2\gamma 2$ (Fig. 4), $\alpha 1\beta 2\gamma 3$ (Fig. 4), $\alpha 2\beta 1\gamma 1$ (Wafford et al., 1993a) and $\alpha 2\beta 1\gamma 2$ (Wafford et al., 1993a), had no intrinsic efficacy at $\alpha 5\beta 3\gamma 2$ or $\alpha 5\beta 3\gamma 3$ (Fig. 3). Previous studies have demonstrated that the presence of a $\gamma 1$ subunit in the receptor also appears to have a profound effect on efficacy; benzodiazepine site agonists are in general less efficacious at $\alpha 2\beta 1\gamma 1$ compared to $\alpha 2\beta 1\gamma 2$, and antagonists and inverse agonists at the latter subunit combination act as agonists at the former (Wafford et al., 1993a). Thus it is clear that the type of α subunit and the type of γ subunit present in a $GABA_A$ receptor influences both the affinity and efficacy of benzodiazepine site compounds.

An additional complication is the recent observation that a small population of $GABA_A$ receptors in the rat brain contain both a $\gamma 2$ and a $\gamma 3$ subunit (Quirk et al., 1994b). The benzodiazepine pharmacology of receptors containing a $\gamma 2$ and a $\gamma 3$ subunit is unknown, but this finding potentially adds a further dimension of complexity to the benzodiazepine binding site, and merits further investigation.

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