Pharmacological Properties of Recombinant Human GABA$_A$ Receptors

by

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Abstract

GABA is the major inhibitory neurotransmitter in the vertebrate brain mediating its fast inhibitory action via $GABA_A$ receptors. This receptor is a hetero-oligomeric protein with the majority of receptors being composed of several distinct polypeptides (α, β, γ, δ, ε, θ, π, ρ). $GABA_A$ receptors are allosterically regulated by a large number of compounds, some of which are in clinical use as anxiolytic, sedative and antiepileptic agents. The aim of this study was to characterise the pharmacological properties of novel $GABA_A$ receptor subunits or mutated subunits and to examine, in detail, the pharmacology of specific allosteric modulators.

Receptors containing the ε subunit and the mutated $\beta2$ subunit ($\beta2L259S$) exhibit a degree of constitutive-activity while still being sensitive to activation by GABA. $\alpha1\beta1\varepsilon$ receptors were not modulated by benzodiazepines, displayed intermediate sensitivity to Zn^{2+} (cf $\alpha1\beta1$ and $\alpha1\beta1\gamma2s$), were potentiated by the general anaesthetic agents, pentobarbitone (PB), propofol and etomidate and exhibited rapid desensitization kinetics. In contrast, $\alpha1\beta2L259S\gamma2s$ receptors exhibited slow desensitization kinetics, increased sensitivity to GABA and GABA site agonists and were not modulated by various allosteric modulators.

Studying the subtype selectivity of PB revealed distinct differences in the direct activation with $\alpha6\beta2\gamma2s$ receptors being significantly more sensitive and having greater efficacy than $\alpha1-5\beta2\gamma2s$ receptors. The EC_{50} for the total modulatory effect of PB however was not influenced by the receptor subtype, unlike the efficacy which depended on the type of α subunit present within the $\alpha\beta\gamma$ receptor complex. Mutagenesis studies with the selective antagonist furosemide revealed that the $\beta2/3$ selectivity was due to Asn 265 within transmembrane domain (TM) 2, while a significant portion of the $\alpha6$ selectivity was due to Ile 228 within TM1. The allosteric modulator tracazolate displayed opposing functional effects, with $\alpha1\beta1/3\varepsilon$ receptors being inhibited while $\alpha1\beta1/3\gamma2s$ receptors were potentiated. This study has therefore provided new information on the pharmacology of a number of allosteric modulators and receptor subtypes.

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Acknowledgements

The fact that I am writing this page brings with it a sense of relief and hope that this thesis is nearly complete. From apparently nowhere, my secondary school motto pops into my head 'What I undertake I do' and seems, at this moment, rather appropriate.

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Chapter 1

General Introduction

1.1.   γ-aminobutyric acid and GABA receptors.

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian central nervous system. This amino acid was first shown to be present within brain tissue in 1950 (Awapara *et al.*, 1950; Roberts & Frankel, 1950; Udenfriend, 1950) and is synthesised from L-glutamic acid via the enzyme L-glutamic decarboxylase (GAD). GABA which is released form neurons can interact with two types of receptors: $GABA_A$ receptors that are ligand-gated anion channels and second messenger-linked $GABA_B$ receptors that are indirectly coupled to K^+ or Ca^{2+} channels via GTP-binding proteins (Bormann, 1988; Bowery, 1993). Reuptake of released GABA into neurons and glial cells occurs via a family of membrane transporters known as γ-aminobutyric acid transporter (GAT) 1-3 and BGT-1 (Soudijn & Wijingaarden, 2000) .

1.2. $GABA_A$ receptors.

$GABA_A$ receptors are members of a superfamily of ligand-gated ion channels (LGIC) that include nicotinic acetylcholine (nACh) receptors, glycine receptors, 5-hydroxy-tryptamine type 3 (5-HT$_3$) receptors and invertebrate glutamate-gated chloride channels (Cully *et al.*, 1994; Karlin & Akabas, 1995). nACh receptors have been extensively characterised and are often used as a paradigm for the whole LGIC superfamily. Evidence suggests that these receptors are comprised of five individual subunits with each subunit having a similar membrane topology (Anand *et al.*, 1991; Cooper *et al.*, 1991; Unwin, 1993, 1995, 1996; Nayeem *et al.*, 1994) (Figure 1.1 and 1.2). The subunits of the $GABA_A$ receptor are usually between 400 to 500 amino acids in length, comprising of a large extracellular N-terminal domain (approximately 200 amino acids) that contains the ligand binding site and a 15-residue cysteine loop, four hydrophobic membrane spanning domains (classified as transmembrane domain 1-4 (TM1-4)) and a small extracellular C-terminus. The second transmembrane domain of each subunit are thought to form the sides of the ion channel pore. Evidence from ACh receptors suggests that this pore is narrowest in the middle with the extracellular region and intracellular region widening out (Unwin, 1993). Based

on the permeabilities of large polyatomic anions the pore diameter of the $GABA_A$ receptor is 5.6Å (Bormann *et al.*, 1987).

Figure 1.1. Schematic diagram of a $GABA_A$ receptor subunit.
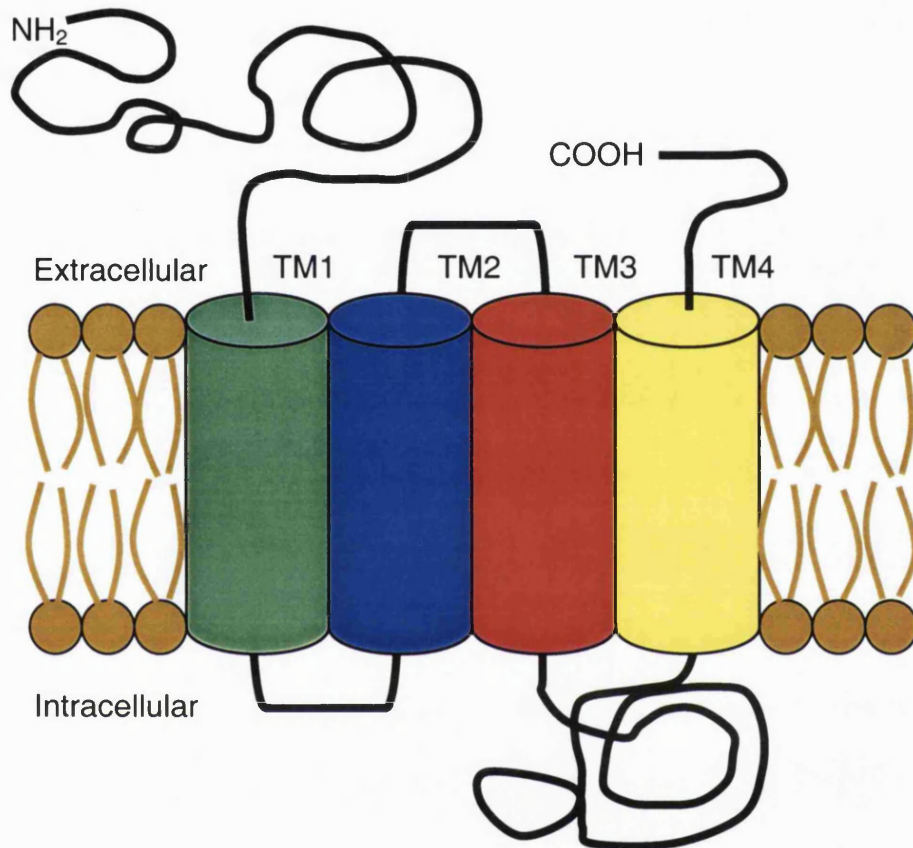
The secondary structure of the transmembrane domains for members of the ligand-gated ion channel superfamily are proposed to be alpha helical (Noda *et al.*, 1982; Xu & Akabas, 1996) however, recent evidence using structural biological techniques suggest that for TM1 this may not be the case (Blanton & Cohen, 1994; Zhang & Karlin, 1997; Corbin *et al.*, 1998).

Figure 1.2. Schematic diagram of a GABA$_A$ receptor.
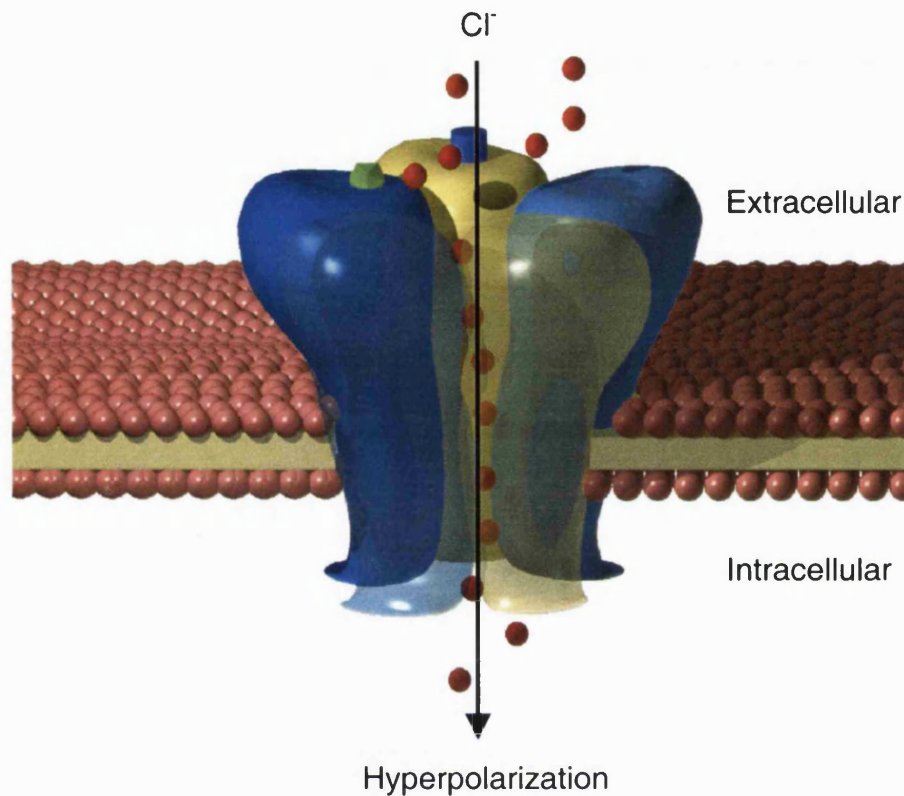
Five subunits are arranged together to form the GABA$_A$ receptor ion channel which spans the lipid membrane. The second transmembrane domain of each subunit are proposed to form the pore through which Cl$^-$ ions can flow across the cell membrane.

1.3.   GABA$_A$ receptor subunits.

The first two GABA$_A$ receptor proteins were isolated in the early 1980's (Sigel *et al.*, 1983; Sigel & Barnard, 1984) followed by the cloning and expression of these subunits in 1987 (Schofield *et al.*, 1987). Since this time many additional subunits have been isolated, sequenced, cloned and expressed, bringing the total number of human GABA$_A$ receptor subunits to 19. These subunits have been subclassified according to their degree of amino acid identity as α, β, γ, δ, ρ, θ, ϵ, and π. To date for humans there are 6 α subunits (α1-6), three β subunits (β1-3), three γ subunits (γ1-3), three ρ subunits (ρ1-3), one δ subunit, one θ subunit, one ϵ subunit and one π

subunit. The predominant location of ρ subunits within the retina and the different pharmacological properties of these receptors, has lead some authors to classify these subunits as a distinct group (GABA$_C$), however it has recently been suggested that the ρ subunits be classified as a subset of the GABA$_A$ receptors (Barnard et al., 1998).

A number of splice variants have been described, which in the human include the long and short form of the γ2 subunit (Whiting et al., 1990; Kofuji et al., 1991) and a splice variant of the β3 subunit (Kirkness & Fraser, 1993). Additional splice variants have been identified in other species namely rat α6 (Korpi et al., 1994) and chicken β2 and β4 (Bateson et al., 1991; Harvey, et al., 1994). The γ2l isoform has an insert of eight amino acids between TM3 and TM4 which provides additional phosphorylation sites and has an influence on ethanol modulation (Wafford et al., 1991).

1.4.   *In vivo* subunit combinations and their localisation.

As stated above the GABA$_A$ receptor is comprised of five subunits (pentamer) leading to a vast number of possible receptor complexes. A number of immunohistochemical and *in situ* hybridization studies have studied the distribution of GABA$_A$ receptor subunits within the brain and have shown a highly heterogenous distribution of the different subunits (Laurie et al., 1992; Wisden et al., 1992; Fritschy & Mohler, 1995; Pirker et al., 2000). From these studies, the α1, β1–3 and γ2 subunits appear to be the most predominant, being found in almost all brain regions, whereas the other subunits show a more limited distribution (See Table 1.1 for a general guide). Expression of multiple subunits in the same neurons suggests the existence of a large variety of GABA$_A$ receptor subtypes within the brain. In addition the discrete location of certain subunits suggests that different receptor subtypes may have specific functions.

Table 1.1. Distribution of GABA$_A$ receptor subunits.

Subunit	Location
α2	Olfactory bulb, dentate molecular layer, CA3 region of the hippocampus, septum, striatum, accumbens, hypothalamus, amygdala
α3	Olfactory bulb, inner layers of the cerebral cortex, endopiriform nucleus, amygdala, superior colliculus, lateral septum
α4	Hippocampus, thalamus
α5	Hippocampus, olfactory bulb
α6	Cerebellar granule cells
γ1	Central and medial nuclei of the amygdala, the pallidum, the septum, substantia nigra, hypothalamus, superior colliculus, inferior olive
γ3	Midbrain, pons, hypothalamus
δ	Cerebellar granule cells, thalamus, cortex and stratum

In vitro studies indicate that a combination of an $\alpha + \beta + \gamma$ subunit gives rise to a recombinant receptor with properties similar to that of a native receptor (Wafford *et al.*, 1995; Itier *et al.*, 1996). Current evidence supports a stoichiometry of 2 α subunits, 2 β subunits and 1 γ subunit (Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999; Knight *et al.*, 2000) with the δ, ϵ and π subunits substituting for a γ subunit and the θ subunit substituting for a β subunit (Davies *et al.*, 1997a; Whiting *et al.*, 1997; Bonnert *et al.*, 1999; Neelands & Macdonald, 1999b). Immunoprecipitation of native receptors and expression of recombinant receptors has shown that functional GABA$_A$ receptors can contain two different α subtypes (e.g. α1 and α6) (Duggan *et al.*, 1991; Lüddens *et al.*, 1991; Mertens *et al.*, 1993; Pollard *et al.*, 1993; Ebert *et al.*, 1994; Verdoorn *et al.*, 1994; Pollard *et al.*, 1995; Khan *et al.*, 1996), two different β subtypes (e.g. β1 and β3) (Fisher & MacDonald, 1997; Li & DeBlas, 1997) or two different γ subtypes (e.g. γ2 and γ3) (Khan *et al.*, 1994; Quirk *et al.*, 1994). The number of distinct GABA$_A$ receptor subtypes that may exist in the brain could therefore amount to 500 or more (Sieghart, 2000).

1.5.   Receptor assembly and anchoring.

Expression studies which have focused on $\alpha 1, \beta 1$-2 and $\gamma 2$ subunits have revealed that access to the cell surface is limited to the combinations $\alpha\beta$ and $\alpha\beta\gamma 2$ (Angelotti & MacDonald, 1993; MacDonald & Olsen, 1994; Rabow *et al.*, 1995; Connolly *et al.*, 1996). Most single subunits, with the exception of $\beta 3$ and $\beta 1$, $\alpha 1/\gamma 2$ and $\beta 2/\gamma 2$ combinations, are retained in the endoplasmic reticulum where they are degraded (Connolly *et al.*, 1996). A recent study has identified the importance of residues 58-67 within the α subunit isoforms in the assembly of receptors comprised of $\alpha\beta$ and $\alpha\beta\gamma$ subunits (Taylor *et al.*, 2000). Deletion of these residues within $\alpha 1$ or $\alpha 6$ prevented cell surface expression with the $\beta 3$ subunit implicating the importance of these residues in mediating $GABA_A$ receptor assembly.

Recent studies have identified a number of intracellular protein molecules e.g. gephyrin (Cabot *et al.*, 1995, Sassoè-Pognetto *et al.*, 1995), dystrophin (Knuesel *et al.*, 1999) and $GABA_A$-receptor-associated protein (GABARAP) (Wang *et al.*, 1999; Kneussel *et al.*, 2000), that are involved in synaptic targeting and clustering of $GABA_A$ receptors. The intracellular region between TM3 and TM4 of the $GABA_A$ subunits has the least sequence homology, leading to the possibility that different intracellular proteins can interact with different $GABA_A$ subunits. It has been suggested that interactions of intracellular proteins with specific GABA receptor subunits may have a role in mediating subcellular localizaton and synaptic targeting of $GABA_A$ receptors (Moss & Smart, 2001).

1.6.   Pharmacology of $GABA_A$ receptors.

A large number of clinically used psychoactive drugs e.g. benzodiazepines, general anaesthetic agents, anticonvulsants and ethanol, exert their effects mainly or exclusively via an interaction with $GABA_A$ receptors. In addition, a number of other substances have been shown to interact with $GABA_A$ receptors e.g. loreclezole, avermectin, furosemide, zinc, picrotoxin and lanthanum. Studies have shown that these compounds allosterically interact with the $GABA_A$ receptor via a number of binding sites. Over the last 25 years a significant effort has focused on examining

receptor subtype selectivity for a number of agents and identifying the amino acid residues that form the binding site or are involved in the transduction mechanism of these ligands to better understand their mechanism of action. The properties of a number of these compounds are outlined below:

1.6.1. GABA and GABA site ligands.

The affinity of GABA at native receptors e.g. acutely dissociated rat striatal, cerebellar and dorsal root ganglion neurons (Wafford *et al.*, 1995; Itier *et al.*, 1996) is generally similar to that reported for recombinant receptors composed of $\alpha\beta\gamma$ subunits (Ebert *et al.*, 1994). Studies with rat and human recombinant GABA$_A$ receptors have shown that the identity of the α subunit influences the apparent GABA affinity. Receptors containing the $\alpha3$ subunit are generally the least sensitive with EC$_{50}$ values ranging between 11-487μM (Sigel *et al.*, 1990; Ebert *et al.*, 1994; Smith *et al.*, 2001) whereas receptors containing the $\alpha6$ subunit are generally the most sensitive with EC$_{50}$ values ranging between 0.2-1.5μM (Korpi & Lüddens, 1993; Korpi *et al.*, 1995; Ebert *et al.*, 1997). The remaining subunits however can also influence the GABA affinity with δ and ε containing receptors being more sensitive than their γ counterparts (Whiting *et al.*, 1997; Brown *et al.*, 2001). It must be noted however, that rank orders are not always consistent between laboratories reflecting differences in experimental conditions (e.g. species differences, expression systems). Similarly the efficacy of GABA$_A$ partial agonists is influenced by receptor subunit composition with the type of α subunit being the major determinant (Ebert *et al.*, 1994).

Early studies demonstrated that co-expression of an α and β subunit produced a receptor that was sensitive to GABA (Sigel *et al.*, 1990; Angelotti & Macdonald, 1993) suggesting that the binding site for GABA could be made up from amino acid residues from both subunits. This was confirmed by mutagenesis studies which show that the GABA-binding site is formed at the interface between the α and β subunits. Mutation of Phe 64 to Leu within the $\alpha1$ subunit and co-expression with $\beta2\gamma2$ dramatically reduced the affinity for GABA and the competitive antagonists, SR95531 and bicuculline (Sigel *et al.*, 1992). In addition this same amino acid was shown to photoaffinity label [^{3}H]muscimol (Smith & Olsen, 1994) suggesting that this

amino acid forms part of the GABA binding site. Other mutagenesis experiments have identified two N-terminal regions of the β2 subunit which when conservatively mutated result in dramatic changes in GABA affinity but no change in activation by pentobarbitone (Amin & Weiss, 1993).

1.6.2. Benzodiazepine site ligands.

Benzodiazepines, whose actions include sedation, anxiolysis, anticonvulsant, hypnosis and muscle relaxation, have been in clinical practice for 40 years. The benzodiazepine binding site is reported to consist of amino acid residues of both the α subunit (Pritchett & Seeburg, 1991; Wieland et al., 1992; Duncalfe et al., 1996; Buhr et al., 1997b; Schaerer et al., 1998) and the γ subunit (Mihic et al., 1994b; Buhr et al., 1997a; Buhr & Sigel, 1997; Wingrove et al., 1997). The results from these and other studies suggest that, within the N-terminal region, there are three separate domains of the α subunit (His 101, Tyr 159-Thr 162, Gly 200-Val 211) and two domains of the γ2 subunit (Lys 41-Trp82, Arg 114-Asp 161) that are involved in benzodiazepine binding. Of these amino acid residues, photoaffinity labelling has shown that His 102 of the human α1 subunit interacts directly with the pendant phenyl group of the classical benzodiazepines diazepam, flunitrazepam and chlordiazepoxide (McKernan et al., 1998). The α4 and α6 subunits both contain an Arg residue at position 102 (human α1 subunit numbering) and receptors containing these subunits have very low affinity for classical benzodiazepines (hence the term 'diazepam-insensitive' receptors). They do however bind other benzodiazepine site ligands such as Ro15-4513 and bretazenil, although the efficacy of these compounds can be very different from α1,2,3 or 5 containing receptors (Hadingham et al., 1996; Wafford et al., 1996; Whittemore et al., 1996). Receptors containing either the δ or ε subunit do not bind benzodiazepine site ligands with high affinity (Quirk et al., 1995; Davies et al., 1997a). The influence of GABA$_A$ receptor subunits on the benzodiazepine pharmacology has been well characterised and reviewed by Whiting et al. (1995a) and Hevers & Lüddens (1998).

1.6.3. General anaesthetic agents.

A large number of structurally diverse agents such as isoflurane, enflurane, barbiturates, etomidate, propofol and steroid anaesthetics have been reported to interact with $GABA_A$ receptors (reveiwed in Franks & Lieb, 1998; Krasowski & Harrison, 1999; Thompson & Wafford, 2001; Yamakura et al., 2001). At therapeutic concentrations this interaction is primarily potentiation of the GABA response. It must be noted however that many of these agents, at therapeutic concentrations, also modulate other ligand-gated ion channels such as nACh, glycine, AMPA and kainate receptors. Over the last decade numerous studies have examined the subunit selectivity of these agents and combined with mutagenesis studies have identified residues that impair sensitivity (e.g. Harrison et al., 1993; Mihic et al, 1994a; Sanna et al., 1995b; Lambert et al., 1996; Thompson et al., 1996; see Chapter 4; Lees & Edwards, 1998). ρ receptors are the only $GABA_A$ receptors examined that are not affected by pentobarbitone, alphaxalone or propofol, (see Chapter 3 for an investigation of ε subunits and anaesthetics), they are however inhibited by alcohols and volatile anaesthetics.

Recently a photoaffinity general anaesthetic, 3-azioctanol has been synthesised (Husain et al., 1999) which potentiates $GABA_A$ receptor function and inhibits muscle nACh receptors. Photolabelling of Torpedo $\alpha 1$ nAch receptor with this compound indicated that the primary site of incorporation was E262 in the carboxy-terminal of the TM2 region (Pratt et al., 2000). Another structural biology approach has combined the labelling approach, using the anaesthetic propanethiol, with cysteine mutagenesis (Mascia et al., 2000). Residues previously shown to impair anaesthetic sensitivity were mutated to cysteine and exposed to propanethiol or propyl methanethiosulphate, which bind covalently to cysteine residues. The authors demonstrated that Ser 267 in the $\alpha 2$ subunit can be covalently modified to produce irreversible potentiation and that the receptor is subsequently insensitive to enflurane and isoflurane, suggesting a common binding pocket for these anaesthetics but not alphaxalone. Recently Jenkins et al. (2001) have proposed that the volatile anaesthetics isoflurane, halothane and chloroform occupy a small cavity, the surface of which is made up from residues from TM1 (L232) TM2 (S270) and TM3 (A291)

of the α subunit. It is hoped that within the next few years advances in structural biology techniques will confirm which amino acids are involved in the binding and mechanism of action of anaesthetic agents.

1.6.4. Loreclezole.

Loreclezole is an effective anticonvulsant agent which has been shown to potentiate the opening of GABA-gated chloride channels in cultured rat cortical neurons and *Xenopus* oocytes expressing recombinant $GABA_A$ receptors (Wafford *et al.*, 1994). Unlike benzodiazepines, whose affinity or efficacy are not influenced by the type of β subunit (Hadingham *et al.*, 1993), loreclezole displayed clear selectivity for $\beta 2/3$ containing receptors over $\beta 1$. This selectivity was subsequently identified to be due to the presence of an Asn residue at position 265 of $\beta 2/3$ of TM2 (numbering of mature polypeptide) (Wingrove *et al.*, 1994), however it has not been demonstrated if this amino acid forms part of the binding site for loreclezole. A number of additional $GABA_A$ receptor modulators such as etomidate (Belelli *et al.*, 1997), β-carbolines (Stephenson *et al.*, 1995), mefenamic acid (Halliwell *et al.*, 1999), furosemide (Thompson *et al.*, 1999a; see Chapter 5) and tracazolate (Thompson *et al.*, 2001; see Chapter 6) have been identified whose $\beta 2/3$ selectivity is similarly due to Asn 265.

1.6.5. Zinc.

The divalent cation zinc is a non-competitive antagonist of $GABA_A$ receptors whose sensitivity depends on the receptor subunit composition (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Smart *et al.*, 1994; Chang *et al.*, 1995; Fisher & Macdonald, 1998). Studies demonstrate that Zn^{2+} is significantly more potent on receptors containing $\alpha\beta$ subunits than those containing $\alpha\beta\gamma$ subunits, with $\alpha\beta\delta$ or $\alpha\beta\epsilon$ receptors having an intermediate sensitivity (Thompson *et al.*, 1997; see Chapter 4; Whiting *et al.*, 1997; see Chapter 3; Krishek *et al.*, 1998). Mutagenesis studies have identified a number of histidine residues that are important for the inhibition of $GABA_A$ receptors by Zn^{2+}. These residues include the 17' His of TM2 of the $\beta 1$ subunit (Horenstein & Akabas, 1998) and $\beta 3$ subunit (Wooltorton *et al.*, 1997a), His 273 of the $\alpha 6$ subunit located in the TM2-TM3 extracellular domain (Fisher & Macdonald, 1998) and the N-terminal

His 107 of the $\beta3$ subunit (Dunne *et al.*, 1999). Mutation of the 17' His in homomeric $\beta3$ receptors and $\alpha1\beta1$ receptors produced dramatic reductions in Zn^{2+} sensitivity (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998) leading to the suggestion that this residue is directly involved in the binding site for Zn^{2+}.

1.6.6. Picrotoxin.

Picrotoxin is a non-competitive antagonist of the $GABA_A$ receptor. The use dependent nature of this compound suggests that the binding site is located within the pore region. Mutagenesis studies have identified a number of residues, all within TM2, that when mutated affect picrotoxin inhibition. In particular Val at the 2' position (Xu *et al.*, 1995), Thr at the 6' position (Gurley *et al.*, 1995; Xu *et al.*, 1995) and Leu at the 9' position (Chang & Weiss, 1999).

1.7. Native verses recombinant $GABA_A$ receptors.

Single channel characteristics of native $GABA_A$ receptors have revealed that these channels exhibit multiple conductance levels including 12, 17-20 and 27-30 pS (Bormann *et al.*, 1987; Macdonald *et al.*, 1989; Macdonald & Olsen, 1994). The major level, accounting for approximately 95% of the current, is the 27-30 pS conductance level. Similarly the single channel characteristics of a number of recombinant receptors expressed in a variety of cells have been reported (Levitan *et al.*, 1988a; Verdoorn *et al.*, 1990; Herb *et al.*, 1992; Angelotti *et al.*, 1993; Saxena & Macdonald, 1994). In general, recombinant receptors display similar conductance levels to native receptors, however differences in the most abundant conductance level are reported.

Studies with $GABA_A$ receptor modulators have demonstrated clear differences in their mechanisms of action at the single channel level. For example the competitive antagonist bicuculline reduces the frequency and mean duration of GABA-induced single channel events (Macdonald *et al.*, 1989). Picrotoxin and benzodiazepine inverse agonists reduce the channel opening frequency (Newland & Cull-Candy, 1992; Porter *et al.*, 1992; Rogers *et al.*, 1994), whereas benzodiazepine agonists

increase the channel opening frequency (Macdonald & Twyman, 1992; Macdonald & Olsen, 1994). Unlike benzodiazepines, barbiturates increase the mean open time to exogenously applied GABA with no effect on the single-channel conductances or frequency of openings (Study & Barker, 1981; Macdonald *et al.*, 1989; Twyman *et al.*, 1989). More recent studies however have shown that in addition to increasing channel activity benzodiazepines and pentobarbitone also increase channel conductance while bicuculline has been demonstrated to reduce channel conductance (Eghbali *et al.*, 1997; Birnir *et al.*, 2000a; Eghbali *et al.*, 2000).

Recombinant receptors display very similar pharmacological characteristics to native $GABA_A$ receptors and therefore represent a powerful tool with which to characterise the pharmacological properties of $GABA_A$ receptors. Significant progress has been made over the last 25 years in understanding the pharmacological properties and the structure of recombinant $GABA_A$ receptors. The challenge for the future however is to link receptor subtype with physiological function and to the develop therapeutic molecules which target specific receptor subtypes. This goal will require the input of many disciplines including electrophysiology, development of transgenic animals, single cell PCR etc. Two recent reports by Rudolph *et al.* (1999) and McKernan *et al.* (2000) illustrate that these goals are achievable. Both groups developed genetically modified mice in which histidine 101 had been mutated to arginine within the $\alpha 1$ subunit resulting in a mouse in which the $\alpha 1$ receptor subtype was insensitive to benzodiazepines. In addition, McKernan *et al.* (2000) synthesised a benzodiazepine site ligand that selectively potentiated $\alpha 2\beta 3\gamma 2s$, $\alpha 3\beta 3\gamma 2s$ and $\alpha 5\beta 3\gamma 2s$ receptors but not $\alpha 1\beta 3\gamma 2s$ receptors. Given these tools both groups were able to conclude that the $\alpha 1$ subtype was responsible for the sedative but not the anxiolytic effects of benzodiazepines.

1.8.   The *Xenopus* oocyte expression system.

The *Xenopus laevis* oocyte was first introduced as an *in vitro* system for the transient expression of foreign mRNA for haemoglobin and mouse myeloma protein by Gurdon *et al.* (1971). In addition to mRNA, the *Xenopus* oocyte is also able to transcribe and translate cDNA which has been injected into the nucleus of the oocyte.

Advantages of using cDNA over mRNA are that it is less labile than mRNA and easier to handle. Other advantages with *Xenopus* oocytes are that they are large robust cells (up to 1.5mm in diameter) which makes isolation and penetration of the oocyte with fine injection needles and microelectrodes relatively easy. A disadvantage of the large size of the cell is that drug application times are slow. A second disadvantage of the *Xenopus* oocyte is that the cell originates from an amphibian and consequently may perform different post-translational modifications to proteins as well as containing different phosphorylase / kinase enzymes to a mammalian cell. It must also be remembered that although *Xenopus* oocytes do not contain genes encoding GABA$_A$ receptors, they do express endogenous cell surface receptors such as muscarinic acetylcholine receptors (Kusano *et al.*, 1977), voltage-gated ion channels such as the Ca^{2+}-dependent chloride channel (Miledi, 1982) and stretch-activated channels (Yang & Sachs, 1989). Overall however the *Xenopus* oocyte expression system represents a very useful robust technique which has been used throughout this study.

1.9.   Aims and Objectives.

A broad aim for this study is to enhance our pharmacological knowledge of recombinant GABA$_A$ receptors. The studies within this thesis will either examine the pharmacological properties of novel GABA$_A$ receptor subunits and mutated subunits, or characterise, in detail, the pharmacology of specific compounds that interact with GABA$_A$ receptors. This will be achieved by implementing the two-electrode voltage-clamp technique on *Xenopus* oocytes expressing wild type or mutated human recombinant GABA$_A$ receptors.

Chapter 2

Methods

2.1. Molecular biology.

The wild type and mutant cDNAs that were used in this project were all generated by members of the Molecular Biology Department, headed by Dr. P. J. Whiting, at Merck Sharp & Dohme. Standard cloning and site directed mutagenesis techniques were employed (Whiting *et al.,* 1995b; Sambrook & Russell, 2001) and the resulting cDNAs cloned into the eukaryotic expression vectors pCDM8 or pcDNA1.1Amp, which when introduced into *Xenopus* oocytes behave essentially the same, however see Chapter 3.

Figure 2.1. Pictorial representation of a GABA$_A$ receptor cDNA inserted in a eukaryotic expression vector.
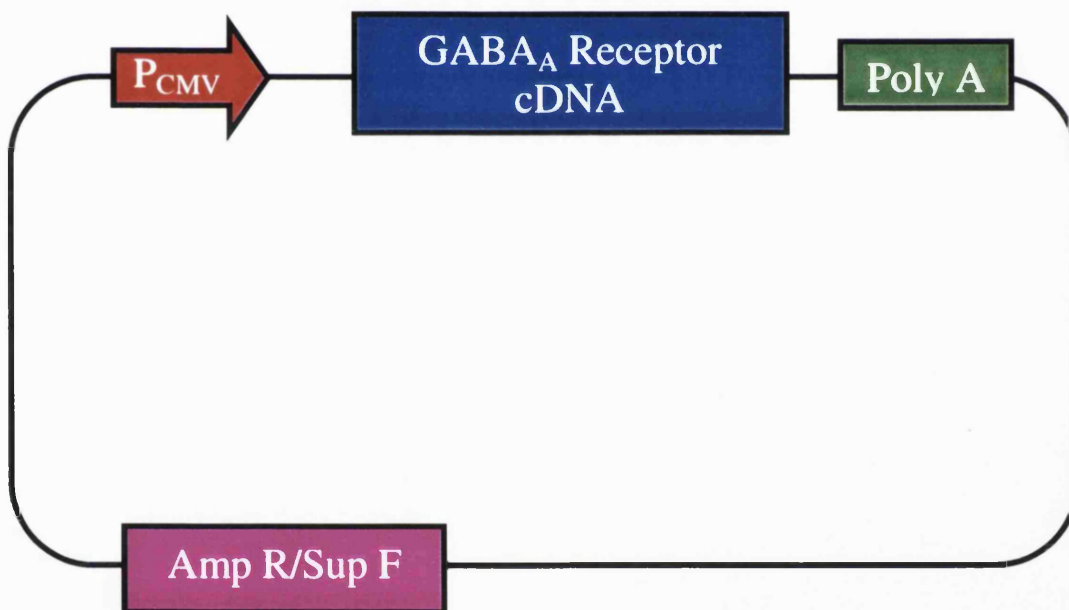
P_{CMV} = promotor, Poly A = polyadenylation/transcription termination sequences, Amp R/Sup F = ampicillin resistance gene present in pcDNA1.1Amp or Sup F sequences present in pCDM8.

2.2. *Xenopus* oocyte expression system.

The *Xenopus laevis* expression system provides a way to study some of the electrophysiological properties of recombinant receptors formed from both wild type and mutant cDNAs. Since its introduction in 1971 (Gurdon *et al.,* 1971) it has been widely used to study numerous ion channels including $GABA_A$ receptors. The techniques for the removal, isolation and injection of oocytes have been reported previously (Soreq & Seidman, 1992; Smart & Krishek, 1995; Whiting *et al.,*1995b) and are described in detail below.

2.3. Housing conditions.

Mature *Xenopus laevis* (~100g body weight) were supplied by either Blades Biological (U.K.) or African Xenopus Facility (South Africa) and housed on site for at least 3 months before use. Up to ten female *Xenopus laevis* were housed in one tank containing approximately 100 litres of water giving a depth of approximately 18cm. The environment of the *Xenopus* holding room was maintained at 20°C in a constant 12hr light-dark cycle. The toads were fed three times a week, on a diet of freeze dried Tubifex (Sinclair Ltd, U.K.) and live crickets.

During the course of this work it was demonstrated that the quality of the water, with regard to the concentration of ammonia, nitrite and nitrate, affected the quality of the oocytes harvested (determined by survival time in culture and general condition of the oocytes). For this reason the water was continuously pumped through an Eheim 2217 filter to break down ammonia to nitrite and then to the less harmful nitrate. Routine tests on the levels of ammonia, nitrite and nitrate and the pH of the water were performed. Partial water changes (up to half the total water volume) were performed three times a week or whenever the levels of ammonia, nitrite or nitrate increased above the acceptable range. The quality of the oocytes was also found to be poor if the *Xenopus laevis* was harbouring a parasitic infection. Routine post-mortem examinations identified the parasite, *Protopolystoma xenopi*, whose life cycle involved the kidney and bladder (Thurston, 1964) and another unidentified parasite of the abdominal cavity. To eliminate these parasitic infections the *Xenopus laevis* were treated on arrival and as required, depending on routine post-mortem results, with

levamisole, 12mg/litre of tank water for three days (Levacide Injection 7.5%, Norbrook Laboratories) and fenbendazole, 10mg/kg single dose p.o. (Panacur 2.5% Liquid Wormer, Hoechst Roussel Vet Ltd).

2.4.   Removal of ovary tissue.

Ovary tissue was removed from either a freshly culled *Xenopus laevis* (decapitation followed by pithing) or via a laparotomy of an anaesthetised *Xenopus laevis*. The laparotomy was performed in a Microflow fume hood, the bench of which was sterilised with 70% ethanol prior to use. Dissection equipment, consisting of 2 pairs of forceps, 1 scalpel and blade, 1 needle holder with scissors, was sterilised using 100% ethanol and flamed. An adult female *Xenopus laevis* was anaesthetised by immersion, for 30-45 min, in a 0.1% solution of 3-aminobenzoic acid ethylester (Tricaine, Sigma), the pH of which had been adjusted with 1M $NaHCO_3$ to that of the water in which the toad was housed. During this time the toads head was supported ensuring that its nostrils remained out of the anaesthetic and hence allowing it to breath. The depth of anaesthesia was tested using the righting reflex and by lightly pinching one of its back legs. Once anaesthetised the toad was placed on its back on a cork board moistened with anaesthetic solution. Throughout the surgical procedure the toads skin was kept moist with anaesthetic solution.

Using the scalpel a small incision, approximately 10mm, was made through the skin into one side of the abdomen. This incision revealed a muscle layer covered by a muscle sheath. Using a pair of forceps to hold the muscle wall away from the internal organs, an incision through the muscle was made. Lobes of ovary were then gently removed using a pair of forceps and scissors and placed in a petri dish containing isolation medium (Table 2.1). Prior to suturing, a transponder was injected into the abdominal cavity to enable future identification (only after first surgical procedure). Sterile synthetic absorbable suture (Ethicon PDS II) was used to individually stitch the muscle and the skin together. Once complete the toad was placed in a small container of tank water to recover. Its head was supported with some tissue allowing its nostrils to be above the level of the water, thus ensuring that the toad could breath whilst recovering. Once the toad was fully recovered (2-3 hours) it was returned to the home tank.

2.5.   Oocyte isolation and injection.

Ovary tissue contains oocytes at all developmental stages (stage 1 being the most immature and stage VI the most mature) however only stage V and VI were used for the expression of ion channels. Each individual oocyte is surrounded by a number of membranes and layers of cells which are gently removed using two pairs of forceps, one to hold the lobe and the other to grip the outer membrane and pull it away (Figure 2.2). Undamaged mature oocytes were transferred to fresh Modified Barth's Solution (MBS, Table 2.2). Prior to injection the isolated oocytes were placed in a collagenase solution (0.5mg/ml MBS) for 6 mins in order to remove the final layer of follicle cells which surround the vitelline membrane.

Figure 2.2. Illustration of the technique for the manual removal of oocytes from the ovary-attached follicle.
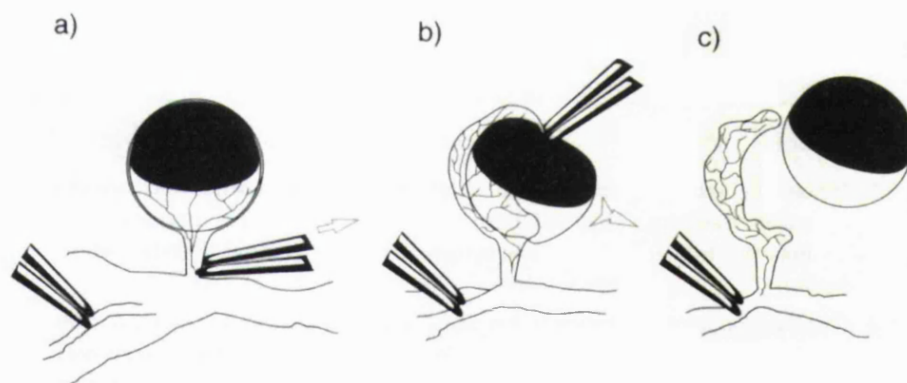
a) While holding a section of the ovary for support with one set of forceps, the second set are used to grasp and pull the outer membrane surrounding the oocyte away.

b) The oocyte protrudes from the membrane sac.

c) The oocyte becomes free from the ovary.

Picture reproduced with permission from Whiting *et al.* (1995b).

Injection needles were pulled from thin-walled borosilicate capillary glass (Laser Laboratory Systems Ltd, U.K.) using a microelectrode puller (Flaming/Brown Model P-87, Sutter Instrument Co., U.S.A.). The tip was cut using a small pair of scissors under a stereomicroscope with a graticule (magnification X50) to approximately 20-30μm diameter. The injection needle was then partially filled with mineral oil via a

needle and syringe. The injection needle was carefully loaded onto a Drummond 10-μl microinjector (Laser Laboratory Systems Ltd, U.K.) attached onto a three-dimensional course micromanipulator and secured.

Aliquots of individual stock cDNAs (at concentrations ranging from 0.3-2μg/μl) were obtained from the Molecular Biology Department of Merck Sharp & Dohme and stored at $-20°C$. Solutions containing the receptor subunit combination of interest e.g. $\alpha1\beta1\gamma2s$ were made, stored in the fridge at $4°C$ and used as required. As a general rule and when initially studying a new receptor combination, each subunit was present at the same concentration i.e. 2μg of each subunit in 300μl injection buffer (Table 2.3). For a number of receptor subtypes expression could be improved by increasing the concentration of one subunit over the remaining ($\alpha1\beta1/3\epsilon$MRK/TIGR, 1:1:3; $\alpha1\beta1\gamma1$, 1:1:10; $\alpha6\beta3\delta$, 1:1:3; $\alpha1\beta1\delta$, 1:0.5:3). All other receptor subtypes used had equimolar concentrations of the individual subunits.

Using a sterile pipette tip, 5μl of mixed cDNA solution was transferred onto a small piece of Parafilm. Using a microscope for visual assistance, the injection needle was placed above the cDNA droplet and some oil expelled from the needle before it was lowered into the cDNA. The cDNA was then carefully drawn into the needle using the Drummond microinjector. Each oocyte was carefully held between a pair of forceps and the injection needle lowered onto the middle of the animal pole until a dimple formed. Further lowering enabled the needle to penetrate the oocyte cell membrane and travel into the oocyte by approximately 100μm. Between 10-20nl of cDNA solution was injected into the oocyte using the Drummond microinjector and the needle subsequently withdrawn from the oocyte. Intranuclear injection of the cDNA was performed "blind" since the nucleus was not visible, however successful injection occurred 80-90% of the time. This procedure was repeated for the remaining oocytes. The injected oocytes were transferred to 24-well tissue culture dishes (1-2 per well) containing 1-2ml incubation medium (Table 2.4) per well and placed in an incubator set at 20°C for at least 24hrs. To improve the longevity of the oocytes, as soon as useable currents were recorded (>1000nA) they were stored at 4°C.

2.6.   Electrophysiological recordings.

An incubation period of 24hrs was normally required to enable the expression and assembly of a $GABA_A$ receptor. However, some receptor subtypes in particular δ, α4 and α6 containing receptors required an incubation period of up to five days. The oocytes were studied electrophysiologically using the two-electrode voltage-clamp technique (Figure 2.3). A healthy oocyte was placed between four metal pins mounted in a Sylgard base within a Perspex chamber of approximate volume 100μl. MBS and drug solutions were held in glass chambers and individually selected via three way taps. The oocyte was perfused at a constant flow rate of between 4-6ml/min via gravity and the solution earthed using a silver/silver chloride pellet. The tip of the electrodes were plugged with agar (1% solution of Type IX agarose in 2M KCl, Sigma) and stored at 4°C. Prior to use two electrodes were back filled with 2M KCl. The tip of the electrodes were carefully broken down by touching the metal pins until the resistance was between 1-4MΩ and then inserted into the oocyte.

Electrical recordings were made using a GeneClamp 500 amplifier (Axon instruments, U.S.A), filtered at 2kHz, and displayed on a Gould Oscilloscope 1602, a Gould Windograf chart recorder and a Compaq Deskpro IBM compatible computer containing the software package 'Oocyte' (Digitimer Ltd, U.K.). 'Oocyte' was developed by Digitimer Ltd in collaboration with Merck Sharp & Dohme as a system for acquiring and storing oocyte data. The output from the GeneClamp 500 amplifier was connected to an analogue to digital converter which was connected to the internal PC Card. After insertion of the electrodes, the resting membrane potential of the oocyte was given time to re-establish before being voltage-clamped at the required potential (usually –70mV). The resting membrane potential of uninjected oocytes was –45.3 ± 2.2mV n=8 (range –36 to –57mV) compared with –58.6 ± 4.2mV n=5 (range –44 to –60mV) from oocytes expressing α1β2γ2s receptors. Uninjected oocytes and those expressing α1β2γ2s receptors possessed similar input resistances of 0.95 ± 0.24MΩ n=8 and 1.05 ± 0.21MΩ n=5 respectively which are comparable with previous reports (Kusano *et al.*, 1977; Krishek *et al.*, 1996).

Figure 2.3. Illustration of the two-electrode voltage-clamp technique.
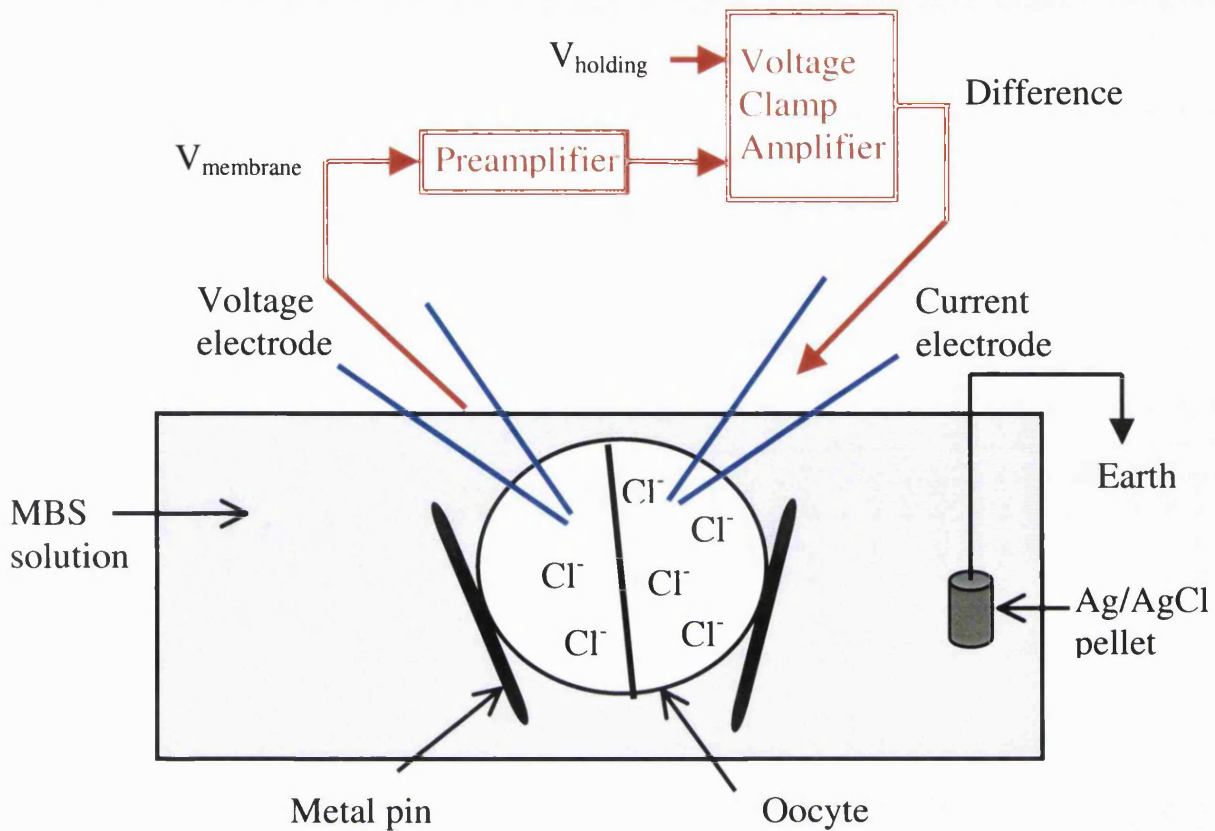
The voltage electrode records the voltage difference across the membrane while the current electrode intracellularly injects current. The electrodes are connected to a feedback circuit that compares the measured voltage across the membrane with the desired voltage. If these two values differ then current is injected to compensate for this difference. When voltage-clamped at $-70mV$ activation of $GABA_A$ receptors results in chloride ions leaving the oocyte which is compensated by injection of a negative current through the current electrode, hence the current injected is proportional to the current flowing through the activated channels.

2.7.   Experiment protocols.

In all experiments drugs were applied in the perfusate and a minimum of two *Xenopus* toads were used for each data set.

A) GABA site agonists and GABA mimetic agents.

Non-cumulative concentration-response curves to GABA site agonists (e.g. GABA, muscimol, THIP) and GABA mimetic agents (e.g. pentobarbitone) were constructed with an interval, upon recovery to baseline, of three minutes between each agonist application.

B) GABA site agonists and GABA mimetic agents in the presence of an antagonist or allosteric modulator.

Protocol as in A) except the antagonist/allosteric modulator was pre-applied 30 sec before co-application of both compounds to ensure complete equilibration.

C) Current-voltage relationships.

The holding voltage was manually adjusted on the GeneClamp 500 amplifier from − 90mV or −70mV to + 30mV in 20mV increments. The oocyte was given 2-3 minutes to equilibrate at the new holding potential before either the application of a GABA EC_{50} concentration or measurement of the holding current.

Inward or outward currents were measured by subtracting the base line immediately prior to drug application from the peak current during drug application (performed using the 'Oocyte' software).

2.8.   Data analysis.

Concentration-response curves were fitted by use of Prism version 2.01 (GraphPad Software Inc, CA, U.S.A.) using a non-linear square fitting programme to the equation:

$$Y = Min + \left[\frac{(Max - Min)}{1 + 10^{(\log EC50 - \log X)nH}} \right] \qquad (1)$$

where Y is the response (as a % of the maximum GABA response or as a % change of the control GABA EC_{20} response); Min and Max are the minimum and maximum responses; EC_{50} is the concentration of X eliciting a half maximal response; X is the concentration of drug; n_H is the Hill coefficient.

Similarly concentration-inhibition curves were fitted using the following equation:

$$Y = Min + \left[\frac{(Max - Min)}{1 + 10^{(\log IC50 - \log X)nH}} \right] \qquad (2)$$

where Y is the response (as a % change of the control GABA or picrotoxin response); Min and Max are the minimum and maximum responses; IC_{50} is the concentration of X eliciting a half maximal inhibition; X is the concentration of drug, n_H is the Hill coefficient.

In exceptional circumstances the data for the concentration-inhibition curves were better fitted using a two-site fit equation:

$$Y = Min + (Max - Min) \left[\frac{Fraction\ 1}{1 + 10^{X - \log IC50_1}} + \frac{Fraction\ 2}{1 + 10^{X - \log IC50_2}} \right] \qquad (3)$$

For competitive antagonist studies pKi values (dissociation constants) were calculated from the equation:

$$\log (K_i) = \log ([Antagonist]) - \log (DR-1) \qquad (4)$$

where DR is the ratio between EC_{50} values with or without antagonist.

For current-voltage relationships Prism version 2.01 (Graphpad software, U.S.A) was used to fit linear regression and polynomial fits to the equations below:

Linear regression $\quad\quad\quad\quad\quad\quad\quad\quad y = bx + a$ $\quad\quad\quad\quad\quad\quad$ (5)

Second order polynomial distribution $\quad\quad y = a + bx + cx^2$ $\quad\quad\quad\quad$ (6)

Third order polynomial distribution $\quad\quad y = a + bx + cx^2 + dx^3$ $\quad\quad$ (7)

EC_{50} and IC_{50} values are shown as the geometric mean (- SEM, + SEM) and all other data shown as the arithmetic mean ± SEM. The statistical significance of differences was evaluated by Student's t-test or ANOVA as appropriate and considered significant if $P<0.05$.

2.9.   Drugs and Solutions.

Outlined below is a list of the drugs used, the source, the solvent and the concentration of stock solution made. Subsequent dilutions were made in MBS.

γ-Aminobutyric acid (Sigma), THIP (gift from Prof B Ebert, Copenhagen, Denmark) and P4S (Sigma) were prepared as 1M solutions in MBS.

Oxytocin (Sigma) and muscimol (Sigma) were prepared as 10mM solutions in MBS.

$ZnCl_2$ (Sigma) was prepared as a 1M solution in 0.2M HCl.

Furosemide (Sigma) was prepared as a 1M solution in DMSO.

Bicuculline (Sigma) and picrotoxin were prepared as 100mM solutions in DMSO.

SR 95531 (Sigma), DMCM (RBI now Sigma), flunitrazepam (Sigma), CL 218,872 (Lederle), bretazenil (gift from Roche), zolpidem (ICN), abecarnil (gift from Schering AG), FG8205 (synthesised at Merck Sharp & Dohme), β-CCM (RBI now Sigma), flumazemil (synthesised at Merck Sharp & Dohme), tracazolate (RBI now Sigma), etazolate (RBI now Sigma), loreclezole (gift from Janssen), L-triiodothyronine (Sigma), thyrotropin releasing hormone (Sigma), vasopressin (Sigma) allopregnanolone (Sigma) were prepared as 10mM solutions in DMSO.

Pentobarbitone (Rhône Mérieux), etomidate (Janssen), propofol (Sigma) and growth hormone (Sigma) were provided as concentrated liquids and diluted into MBS.

With the exception of furosemide, tracazolate, bicuculline, pentobarbitone, etomidate and propofol which were made fresh when needed, all stock solutions, once made, were stored at -20°C. Control experiments with the highest concentration of the different solvents had no direct effect on the oocyte or on a GABA EC_{20} response.

The constituents of the isolation medium, incubation medium, MBS and injection buffer are outlined in Tables 2.1-2.4.

Table 2.1. Constituents of Isolation Medium.

Constituent	Concentration (mM)
Sodium Chloride (NaCl)	108
Potassium Chloride (KCl)	2
EDTA	1.2
HEPES	10
Adjusted to pH 7.9 with 10M sodium hydroxide	

Table 2.2. Constituents of Modified Barth's Solution (MBS).

Constituent	Concentration (mM)
Sodium Chloride (NaCl)	88
Potassium Chloride (KCl)	1
HEPES	10
Magnesium Sulphate ($MgSO_4$)	0.82
Calcium Nitrate ($Ca(NO_3)_2$)	0.33
Calcium Chloride ($CaCl_2$)	0.91
Sodium Hydrogen Carbonate ($NaHCO_3$)	2.4
Adjusted to pH 7.5 with 10M sodium hydroxide	

Table 2.3. Constituents of Injection Buffer.

Constituent	Concentration (mM)
Sodium Chloride (NaCl)	88
Potassium Chloride (KCl)	1
HEPES	15
Adjusted to pH 7 with 10M sodium hydroxide and filtered through nitrocellulose	

Table 2.4. Constituents of Incubation Medium.

Constituent	Concentration (mM)
Sodium Chloride (NaCl)	88
Potassium Chloride (KCl)	1
HEPES	10
Magnesium Sulphate ($MgSO_4$)	0.82
Calcium Nitrate ($Ca(NO_3)_2$)	0.33
Calcium Chloride ($CaCl_2$)	0.91
Sodium hydrogen carbonate ($NaHCO_3$)	2.4
Penicillin	10units/ml
Streptomycin	0.01mg/ml
Gentamicin	0.05mg/ml
Sodium pyruvate	2
Adjusted to pH 7.5 with 10M sodium hydroxide	

Chapter 3

Pharmacological Characterisation of Human α1β1ε GABA$_A$ Receptors

3.1.   Introduction.

A cDNA encoding a novel human $GABA_A$ receptor subunit was independently cloned by four groups in 1997 (Davies *et al.,* 1997a; Garret *et al.,* 1997; Whiting *et al.,* 1997; Wilke *et al.,* 1997). Note the protein reported by Garret *et al.* (1997) was referred to as χ compared with the other three groups who each termed their protein ε. This protein has an open reading frame of 506 amino acids and contains many motifs typical of a ligand-gated ion channel subunit e.g. an 18 residue signal peptide, two cysteine residues separated by 13 amino acids, four hydrophobic regions, which are putative membrane spanning domains and putative N-glycosylation sites (Figure 3.1).

Comparison of this novel amino acid sequence with known ligand-gated ion channel sequences revealed that the highest homology was with the γ subunits of the $GABA_A$ receptor (47% with rat $\gamma 1$, 43% with human $\gamma 2$, 46% with rat $\gamma 3$ and 49% with chicken $\gamma 4$; Wilke *et al.,* 1997). In general the amino acid sequence homology between members of the same class e.g. $\alpha 1$-6, $\beta 1$-3, is greater than 70%. This homology (43-49%) with γ subunits therefore was not sufficient for it to be classified within this class and resulted in the generation of a new subunit class termed ε. (Figure 3.2).

Epsilon transcripts were detected by Northern analysis in a wide range of peripheral tissues, including heart, placenta, pancreas, spleen, testes, and in spinal cord and various brain regions (Davies *et al.,* 1997a; Garret *et al.,* 1997; Whiting *et al.,* 1997; Wilke *et al.,* 1997). Expression of the complete protein sequence however was restricted to the heart, brain and spinal cord by RNA splicing. Within the squirrel monkey brain, *in situ* hybridization demonstrated that the ε transcript was highly localised to the hypothalamus, primarily the arcuate-ventromedial area, and the hilus of the dentate gyrus in the hippocampus (Whiting *et al.,* 1997).

Figure 3.1. Comparison of the deduced amino acid sequence of the human ε subunit with sequences of other GABA_A receptor subunits.
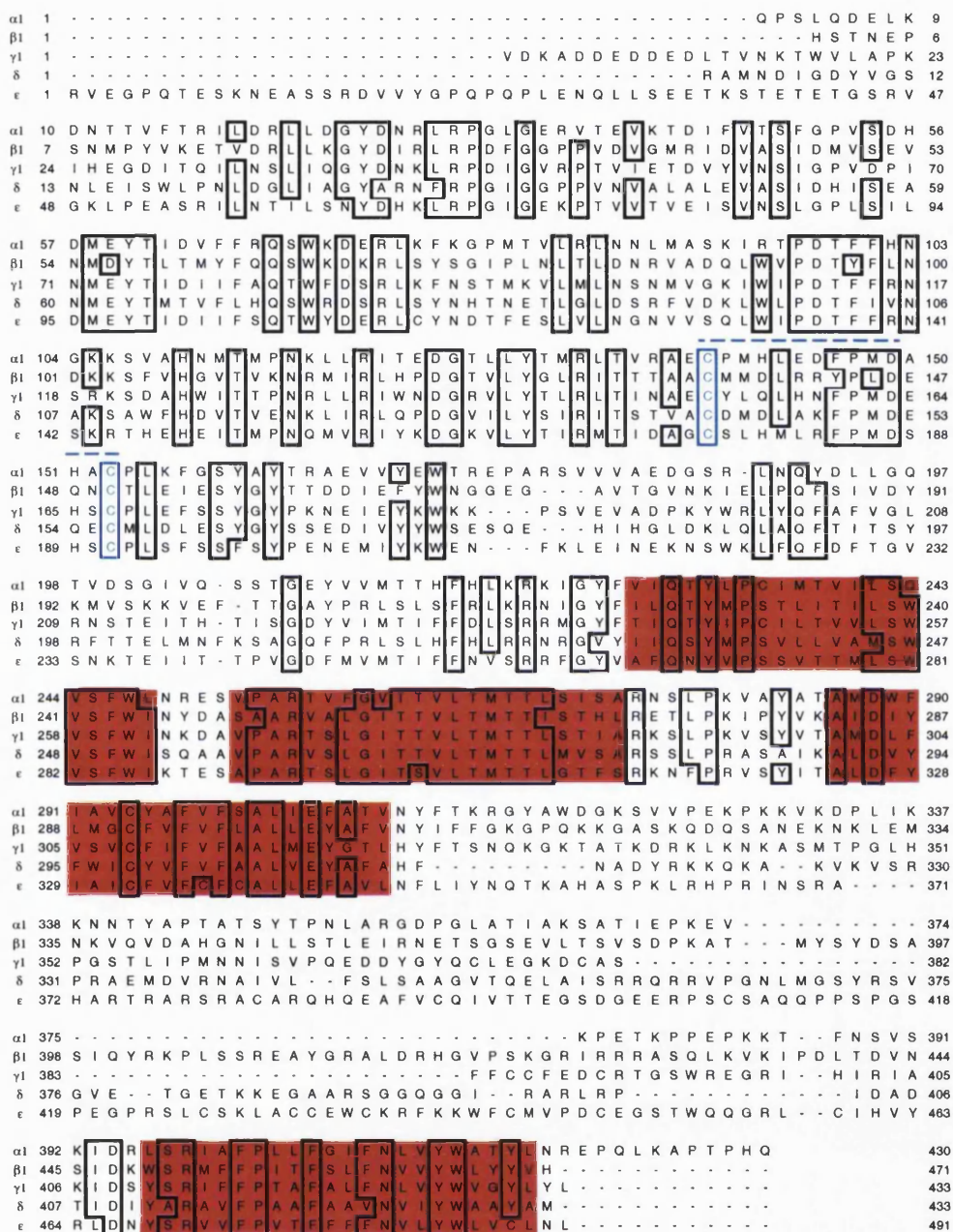
Positions in which amino acids residues are conserved in four or more sequences are boxed. Numbering corresponds to the mature polypeptide. Putative transmembrane regions TM1-TM4 are indicated by the red background and the domains contained within the two cysteine residues are indicated by the hashed blue line.

Figure 3.2. Dendrogram of the deduced amino acid sequences of the GABA$_A$ receptor family, including the ε subunit.
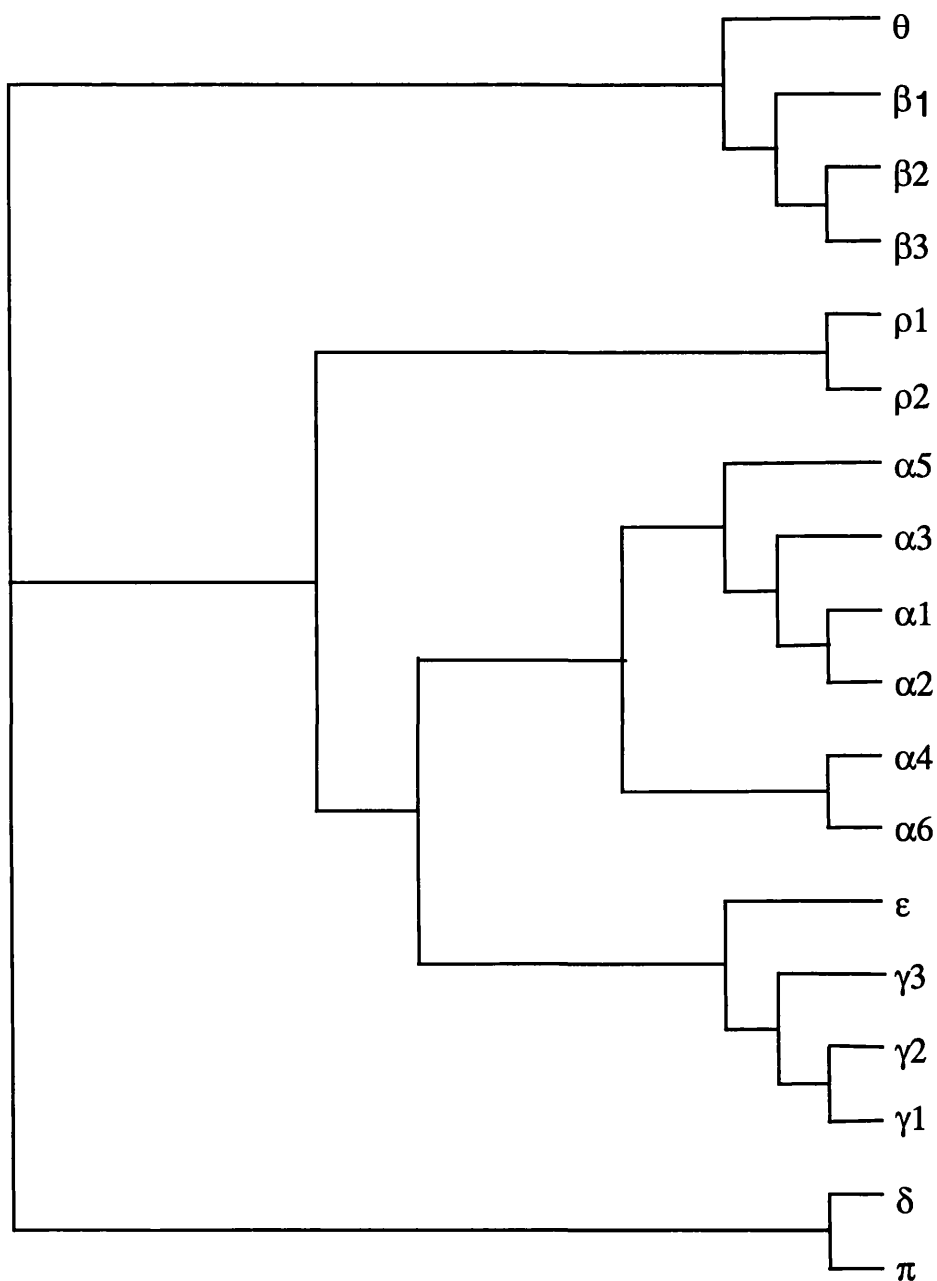
The distance along the horizontal is proportional to the differences between sequences. The analysis was performed by Dr. T. Bonnert (Merck Sharp & Dohme) using PileUp (Genetics Computer Group, University of Wisconsin) and permission for its reproduction obtained.

The aim of this chapter was to investigate the functional properties of recombinant GABA$_A$ receptors containing the ε subunit using the *Xenopus* oocyte expression system (Whiting *et al.*, 1997; Thompson *et al.*, 1998). At the time of this study it was not known if the ε subunit could form homomeric receptors or if it substituted for either an α, β or γ subunit. Initial experiments were performed to address this question.

Comparison of the results from this study (Whiting *et al.*, 1997; Thompson *et al.*, 1998) with the results of Davies *et al.* (1997a) revealed clear pharmacological differences between the two ε clones. To attempt to clarify, additional experiments were conducted and a hypothesis for the apparent differences in pharmacology between these two clones proposed.

3.2. Results.

3.2.1. ε does not form functional homomeric channels.

To see if the ε subunit could form functional homomeric channels *Xenopus* oocytes were injected with the ε subunit cDNA alone. No currents were observed to GABA (3mM) or picrotoxin (100μM)(n=7). Similarly *Xenopus* oocytes injected with α1ε (1:1) and β1ε (1:1) cDNA failed to respond to GABA or picrotoxin. Injection of α1, β1 and ε however resulted in the formation of functional GABA-gated channels. Further pharmacological analysis was required however, since co-expression of α1β1 also forms functional channels (Draguhn *et al.*, 1990; Smart *et al.*, 1991). Comparative studies on *Xenopus* oocytes injected with α1β1, α1β1γ2s and α1β1ε were therefore undertaken.

3.2.2. α1β1ε receptors are constitutively-active and are blocked by PTX.

In general, oocytes injected with α1β1ε cDNA displayed a significantly ($P<0.05$) greater leak current when voltage-clamped at -70mV (-182 ± 29nA n=5) than those injected with either α1β1 or α1β1γ2s cDNA (-61 ± 17nA n=7 and -49 ± 16nA, n=6 respectively). Furthermore, the resting membrane potential for oocytes injected with

α1β1ε (-31.6 ± 1.7mV n=5) was significantly ($P<0.05$) more depolarised than α1β1γ2s (-40.5 ± 3.1mV n=6). One possible explanation of these differences could be the presence of constitutively-active channels. Picrotoxin has previously been shown to block the constitutively-active channels present in homomeric β1 and β3 receptors (Sanna *et al.*, 1995a; Krishek *et al.*, 1996; Wooltorton *et al.*, 1997b) and α1β2L259Sγ2s receptors (Thompson *et al.*, 1999b; see Chapter 7). The effect of 100μM picrotoxin on the leak current of *Xenopus* oocytes expressing α1β1, α1β1γ2s and α1β1ε voltage-clamped at -70mV was investigated. Small outward currents (95 ± 18nA n=14) to 100μM picrotoxin were only observed with oocytes injected with α1β1ε. Picrotoxin effectively reduced the holding current of cells expressing α1β1ε to that observed in α1β1, α1β1γ2s and uninjected oocytes. Recovery from this picrotoxin effect was slow, typically taking >15 mins to return to baseline.

3.2.3. GABA concentration-response curves.

GABA concentration-response curves were constructed on oocytes injected with α1β1, α1β1γ2s and α1β1ε subunits (Figure 3.3 and Table 3.1). The EC_{50} for GABA was similar for oocytes injected with α1β1 and α1β1ε but significantly lower than α1β1γ2s. Interestingly the Hill coefficient for oocytes injected with α1β1ε was significantly lower than those injected with α1β1 and α1β1γ2s. Previous studies have shown that receptors composed of α and β subunits have a lower GABA EC_{50} than receptors containing α, β and γ subunits (Verdoorn *et al.*, 1990; Demuro *et al.*, 1999; Yamashita *et al.*, 1999). Consistent with these findings the GABA EC_{50} for α1β1 (5.3μM) was significantly lower than α1β1γ2s (16.2μM). Studying the shape of the recorded currents revealed two striking differences between oocytes injected with α1β1ε compared to α1β1 and α1β1γ2s; firstly, the responses to GABA appeared to desensitize much more rapidly and secondly, washout of GABA revealed a small transient outward current (Figure 3.4). Although not conclusive, the results for the GABA concentration-response curves did suggest that the ε subunit was co-assembling with the α1β1 subunits to form a unique receptor.

Figure 3.3. GABA concentration-response curves for α1β1, α1β1γ2s and α1β1ε receptors.
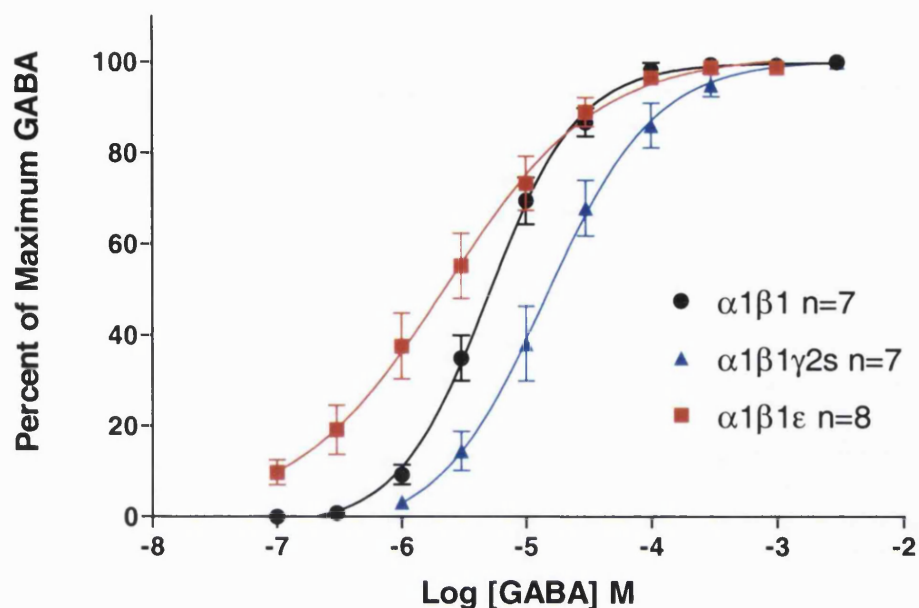
GABA concentration-response curves for α1β1, α1β1γ2s and α1β1ε receptors. Data were normalised to the maximum response obtained, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 3.1.

Table 3.1. EC_{50} and Hill coefficient values for GABA concentration-response curves on α1β1, α1β1γ2s and α1β1ε receptors.

Receptor	EC_{50} (μM)	Hill Coefficient	No. of Oocytes
α1β1	5.3 (4.4, 6.4)*	1.32 ± 0.06**	7
α1β1γ2s	16.2 (12.2, 21.4)	1.36 ± 0.18**	7
α1β1ε	2.5 (1.6, 3.8)*	0.85 ± 0.08	8

* indicates a significant difference ($P<0.01$) from α1β1γ2s, ** indicates a significant difference ($P<0.05$) from α1β1ε. Note the lower GABA EC_{50} value and lower Hill coefficient for α1β1ε receptors.

Figure 3.4. Examples of typical GABA-activated membrane currents for *Xenopus* oocytes expressing α1β, α1β1γ2s and α1β1ε receptors.

A) α1β1

B) α1β1γ2s

C) α1β1ε
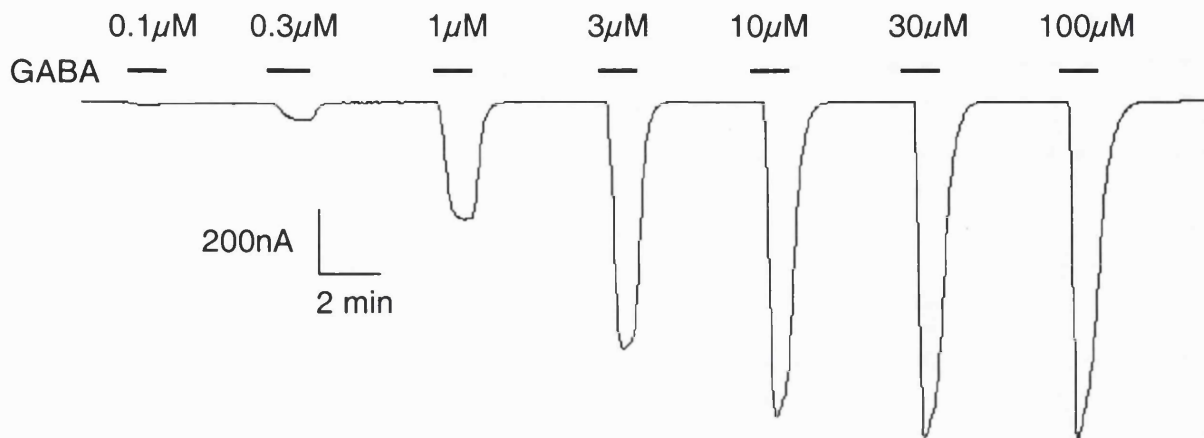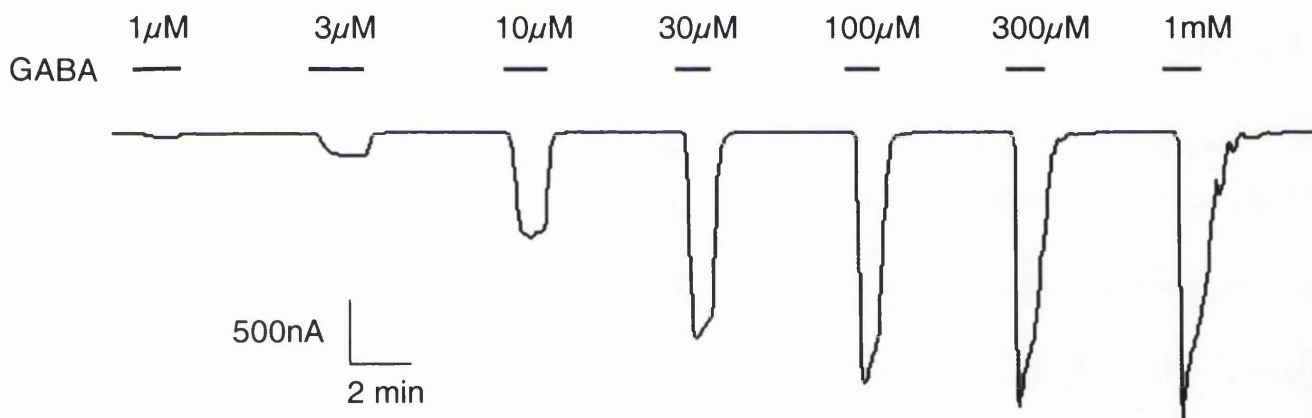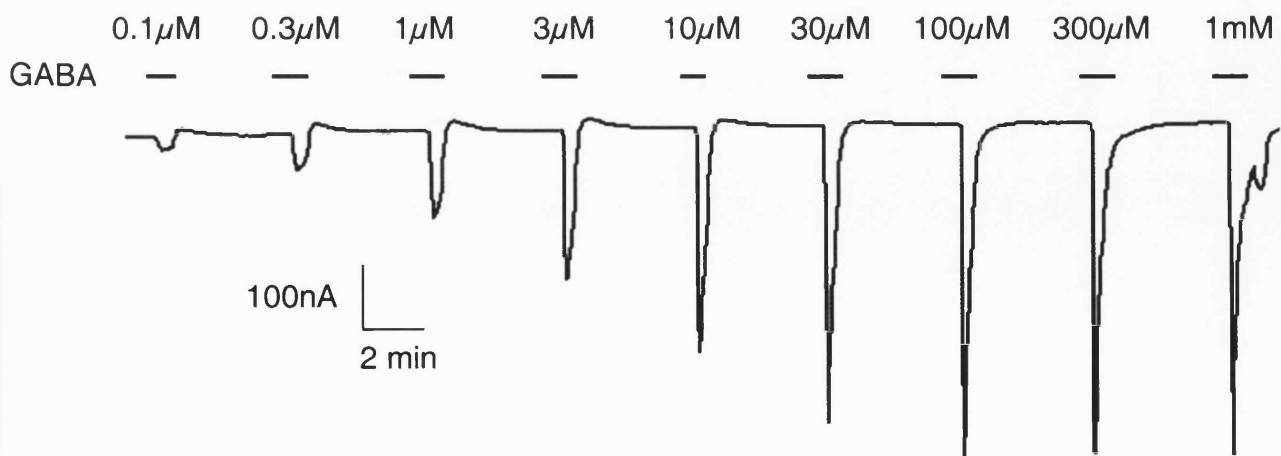
3.2.4. Current-voltage relationship of the leak current and the GABA evoked current.

Current-voltage relationships were determined for the leak current (I_{CC}) in uninjected oocytes and oocytes expressing α1β3ε receptors, and for the GABA-activated current (I_{GABA}) in oocytes expressing α1β1ε receptors, using the stepping protocol as described in Chapter 2. The leak current measured from oocytes expressing α1β3ε receptors is a summation of the leak current normally present in an uninjected oocyte and the leak from the constitutively-active channels. As can be seen in Figure 3.5, the leak current of uninjected oocytes is small and therefore represents only a minor fraction of the leak current measured for oocytes expressing α1β3ε receptors. Third order polynomial fits (equation (7) Chapter 2) revealed reversal potentials of -30.7 ± 3.5mV n=5 for I_{CC} and –37.9 ± 2.7mV n=4 for I_{GABA} (Figure 3.5). Both these reversal potentials were slightly more negative than the predicated reversal potential for Cl⁻ ions of –25.4mV in *Xenopus* oocytes with an external Cl⁻ concentration of 89.91mM (MBS used in this study) and an internal Cl⁻ concentration of 33.4mM (Barish, 1983). The presence of constitutive-activity would lead to a internal Cl⁻ concentration lower than that measured by Barish (1983) in uninjected oocytes and hence a more negative E_{Cl}. Measurement of the true internal Cl⁻ concentration in *Xenopus* oocytes expressing α1β1/3ε receptors would enable an accurate E_{Cl} to be calculated. The data however do indicate that the predominant carrier for both the I_{CC} and I_{GABA} is Cl⁻ ions.

Figure 3.5. Current-voltage relationship of the leak current and the GABA-activated current.
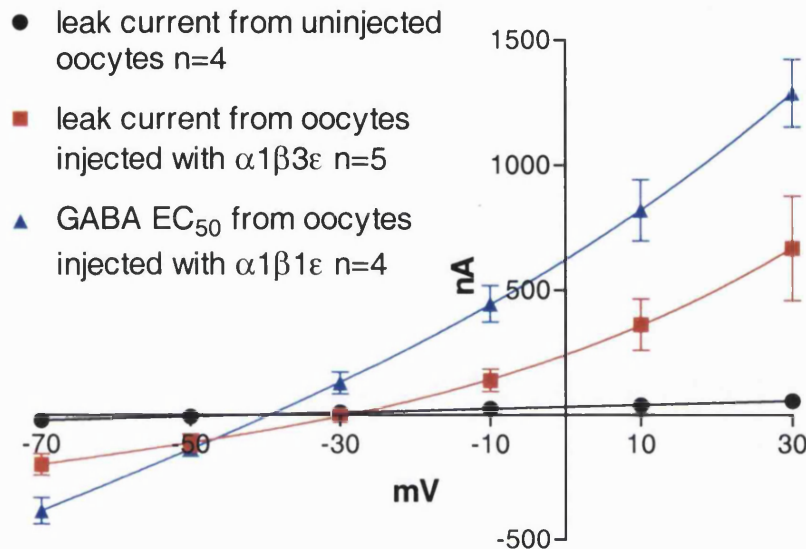
Uninjected oocytes or oocytes expressing $\alpha 1\beta 3\epsilon$ receptors were initially voltage-clamped at -70mV and the leak current, upon stabilisation, recorded. The leak current was then recorded for successive 20mV steps to more positive holding potentials (up to $+30\text{mV}$) so generating an I/V relationship. Note that oocytes expressing $\alpha 1\beta 3\epsilon$ receptors have significantly larger leak currents than uninjected oocytes at holding potentials either side of E_{Cl}. For comparison the I/V relationship for the current induced by a GABA EC_{50} concentration (not including the resting leak current) on $\alpha 1\beta 1\epsilon$ receptors is shown.

3.2.5. ϵ co-assembles with $\alpha 1\beta 1$.

Definitive evidence for co-assembly of the ϵ subunit with $\alpha 1\beta 1$ came from examining the inhibition of GABA currents by Zn^{2+} ions. It has previously been shown that Zn^{2+} behaves as a $GABA_A$ receptor antagonist and is substantially more potent on $\alpha\beta$ heteromers than on $\alpha\beta\gamma$ heteromers (Draguhn *et al.*, 1990; Smart *et al.*, 1991). Similar to previous reports a 3000-fold difference between the Zn^{2+} IC_{50} was seen with $\alpha 1\beta 1$ ($0.17\mu M$) and $\alpha 1\beta 1\gamma 2s$ ($565\mu M$). Oocytes injected with $\alpha 1\beta 1\epsilon$ subunits

had a 243-fold lower affinity for Zn^{2+} (IC_{50} = 41.4μM) compared to those injected with $\alpha 1\beta 1$. The data for Zn^{2+} on $\alpha 1\beta 1\varepsilon$ were best fitted to a single site suggesting a homogenous population of $\alpha 1\beta 1\varepsilon$ receptors rather than a mixed population of $\alpha 1\beta 1$ and $\alpha 1\beta 1\varepsilon$. The data for $\alpha 1\beta 1\gamma 2s$ however were best fitted with a two-site fit suggesting the presence of a mixed population of receptor subtypes (e.g. a proportion of $\alpha 1\beta 1$ and $\alpha 1\beta 1\gamma 2s$ receptors) or a low and high affinity binding site for Zn^{2+} on $\alpha 1\beta 1\gamma 2s$ receptors.

Figure 3.6. Concentration-inhibition curves to Zn^{2+}: definitive evidence for expression of $\alpha 1\beta 1\varepsilon$ receptors.
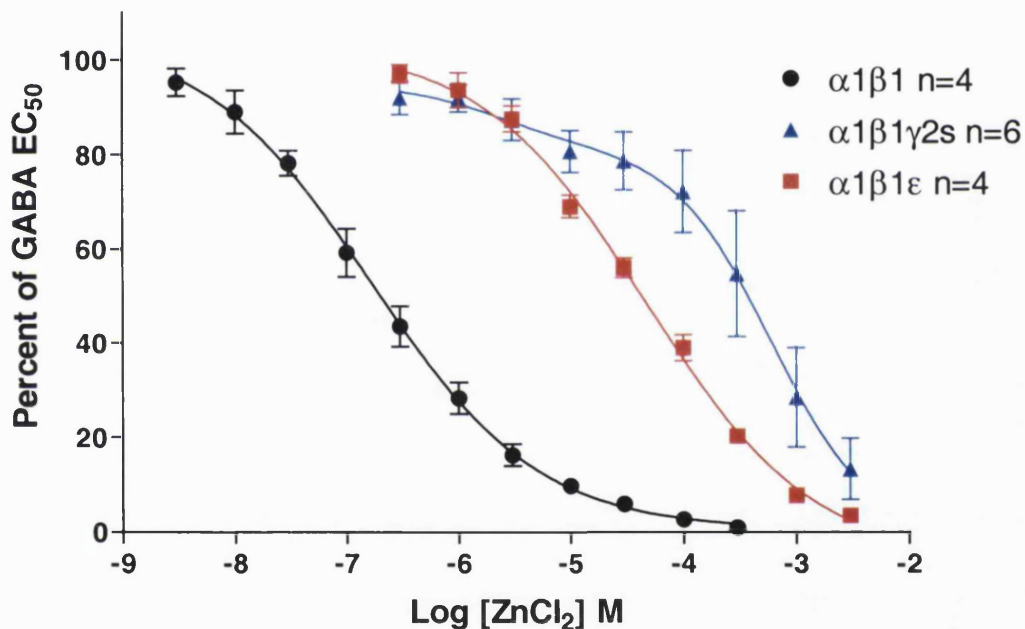
Concentration-inhibition curves to Zn^{2+} were constructed against an approximate GABA EC_{50} concentration on oocytes injected with $\alpha 1\beta 1$, $\alpha 1\beta 1\gamma 2s$ and $\alpha 1\beta 1\varepsilon$ subunits. Data were normalised with respect to the control GABA EC_{50} response, fitted using equation (2 or 3) (see Chapter 2) and the parameters shown in Table 3.2 determined.

Table 3.2. IC_{50} and Hill coefficient values for Zn^{2+} inhibition curves on α1β1, α1β1γ2s and α1β1ε receptors.

Receptor	IC_{50} (μM)	Hill Coefficient	No. of Oocytes
α1β1	0.17 (0.11, 0.27)	-0.60 ± 0.02	4
α1β1γ2s	565 (372, 857)	n.d.	6
α1β1ε	41.4 (37.8, 45.4)	-0.69 ± 0.03	4

The data obtained for oocytes injected with α1β1γ2s cDNA were best fitted using a two-site fit equation (3) (see Chapter 2). The IC_{50} quoted was that obtained for the major low affinity site. n.d. = not determined.

3.2.6. Effect of benzodiazepine site ligands on α1β1ε receptors.

The above results further reinforced the suggestion that the ε subunit co-assembled with α1β1 which prompted further pharmacological characterisation. The effect of a range of structurally diverse benzodiazepine site ligands were examined against a GABA EC_{20} concentration on α1β1ε and α1β1γ2s receptors and the results summarised in Table 3.3. Clearly, unlike α1β1γ2s receptors, α1β1ε receptors were not modulated by benzodiazepine site ligands.

Table 3.3. Modulation of α1β1γ2s and α1β1ε receptors by various benzodiazepine site ligands.

Benzodiazepine Ligand	α1β1γ2s (%)	α1β1ε (%)
Flunitrazepam	117 ± 7.3	0 ± 0
Bretazenil	29.3 ± 3.6	-1 ± 1.5
Zolpidem	159 ± 30	-5.8 ± 4.0
CL-218,872	64 ± 8.6	-0.8 ± 5.4
Abecarnil	60 ± 13.2	-5.0 ± 5.9
FG8205	47 ± 11	-1.3 ± 2.4

Table 3.3 summarises the percent modulation of the control GABA EC_{20} response by various benzodiazepine site ligands at α1β1γ2s and α1β1ε receptors. All compounds were examined at a concentration of $1\mu M$ except for CL-218,872 on α1β1ε where a concentration of $10\mu M$ was used. The data shown are the mean ± SEM of at least four oocytes. The data for flunitrazepam, zolpidem, CL-218,872 and FG8205 on α1β1γ2s receptors has been taken from Hadingham *et al.* (1993). Permission for its reproduction has been given by Dr. Keith Wafford (Merck Sharp & Dohme) who performed the experiments.

3.2.7. Effect of pentobarbitone.

Almost all general anaesthetic agents e.g. pentobarbitone, etomidate and propofol have been shown to interact with $GABA_A$ receptors. General anaesthetic agents have three distinct mechanisms of action on $GABA_A$ receptors, namely i) potentiation of the GABA response, ii) direct activation of $GABA_A$ receptors and iii) at high concentrations, a block of the GABA-activated chloride channel. For a more detailed discussion on general anaesthetics, in particular pentobarbitone, see Chapter 4 and reviews by Tanelian *et al.* (1993) and Lambert *et al.* (1995). Concentration-response curves to pentobarbitone against a GABA EC_{20} were constructed on α1β1, α1β1γ2s

and α1β1ε receptors. Two effects were measured, the total modulation of the GABA EC_{20}, which included both the potentiation and direct activation by pentobarbitone and the direct activation in the absence of GABA. The results presented in Thompson *et al.* (1996) and Chapter 4 show that potentiation of a GABA EC_{20} on α1β1γ2s receptors occurs over a lower concentration range than the direct activation. The total effect therefore includes only a small component of direct activation.

Figure 3.7. Concentration-response curves for the total modulation by pentobarbitone on α1β1, α1β1γ2s and α1β1ε GABA$_A$ receptors.
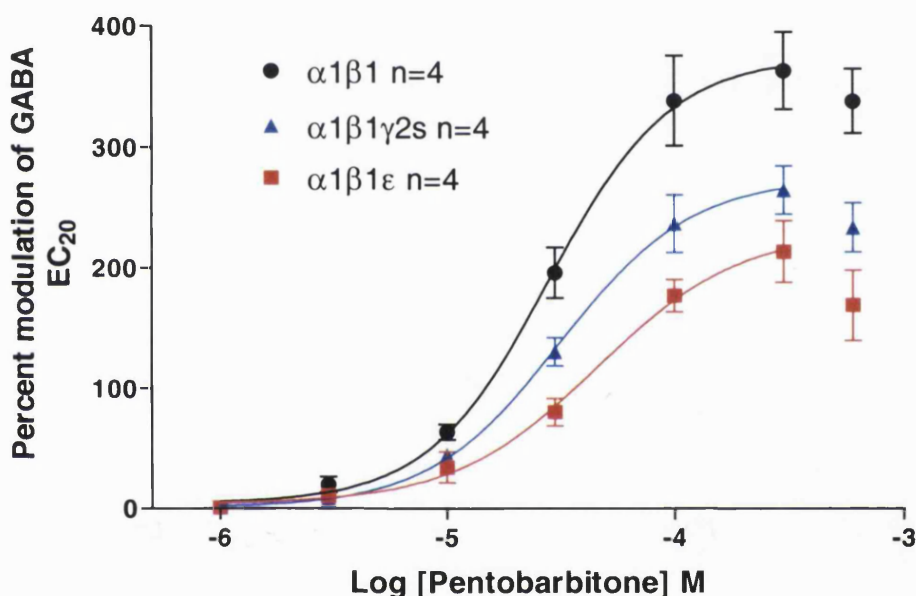
Concentration-response curves to the total effect of pentobarbitone on α1β1, α1β1γ2s and α1β1ε receptors. The data were normalised to the control GABA EC_{20} response, fitted using equation (1) (see Chapter 2) and the mean results from the individual curve fits shown in Table 3.4. Note curves were only fitted for the data obtained for 1 to 300μM with the maximum not constrained.

Table 3.4. EC$_{50}$, Hill coefficient and maximum response for total modulation by pentobarbitone on α1β1, α1β1γ2s and α1β1ε receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β1	28.3 (27.4, 29.3)	1.56 ± 0.04	384 ± 41	4
α1β1γ2s	31.9 (29.1, 35.1)	1.51 ± 0.14	275 ± 17	4
α1β1ε	43.3 (34.8, 53.9	1.50 ± 0.15	233 ± 31*	4

* indicates a significant difference ($P<0.05$) from α1β1 receptors.

All three receptor combinations were modulated by pentobarbitone (Figure 3.7). With the exception of the maximum modulation on α1β1 receptors compared to α1β1ε receptors, there were no significant differences between the EC$_{50}$ values, Hill coefficient or maximum response and receptor combination.

The GABA mimetic effect of pentobarbitone was normalised to the maximum GABA response (Figure 3.8) and the resulting concentration-response curve fitted using equation (1) (see Chapter 2) providing the parameters shown in Table 3.5.

The maximum efficacy of pentobarbitone on α1β1ε and α1β1γ2s was approximately half that observed on α1β1 receptors. The EC$_{50}$ for α1β1ε receptors was not significantly different from that obtained on α1β1 or α1β1γ2s receptors. Interestingly, similar to GABA activation, the Hill coefficient for direct activation by pentobarbitone was significantly lower on α1β1ε compared to α1β1 and α1β1γ2s receptors.

Figure 3.8. Concentration-response curves for the direct activation of α1β1, α1β1γ2s and α1β1ε receptors by pentobarbitone.
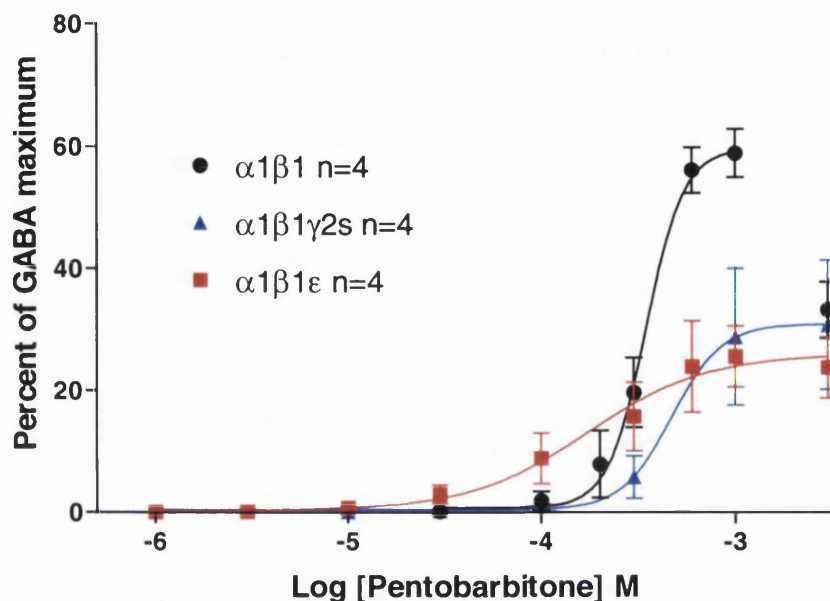
The GABA mimetic effect of pentobarbitone was normalised to the maximum GABA response. Data were fitted using equation (1) (see Chapter 2) providing the parameters shown in Table 3.5. Note for α1β1 the curve was only fitted up to 1mM.

Table 3.5. EC$_{50}$, Hill coefficient and maximum response for the GABA mimetic action of pentobarbitone on α1β1, α1β1γ2s and α1β1ε receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β1	335 (307, 365)	4.84 ± 0.91**	59.6 ± 4.2	4
α1β1γ2s	540 (476, 611)*	3.30 ± 0.32**	33.3 ± 11.88	4
α1β1ε	187 (122, 287)	1.80 ± 0.10	26.6 ± 5.4*	4

* indicates a significant difference ($P<0.05$) from α1β1, ** indicates a significant difference ($P<0.05$) from α1β1ε.

3.2.8. Effect of propofol and etomidate.

The modulatory effects of two additional anaesthetics, propofol (10μM) and etomidate (3 and 30μM), were examined on α1β1, α1β1γ2s and α1β1ε receptors (Figure 3.9A and B). The concentrations chosen have previously been shown to have little or no direct activation on α1β1γ2s receptors (propofol; Wafford *et al.*, 1996; see Chapter 4, etomidate; Hill-Venning *et al.*, 1997). Similar to pentobarbitone, both propofol and etomidate (30μM) potentiated control GABA EC_{20} responses on α1β1 receptors to a greater extent than α1β1γ2s and α1β1ε receptors. As reported previously, propofol (10μM) and etomidate (3 and 30μM) did not elicit an inward current in the absence of GABA on α1β1 and α1β1γ2s receptors, however on α1β1ε receptors both produced a direct effect in the absence of GABA. This direct effect as a percentage of the total modulatory current was 11 ± 3 % for 10μM propofol and 13 ± 1 % and 17 ± 3 % for 3μM and 30μM etomidate respectively. Etomidate potentiation has been shown to depend on the β-subunit isoform having a 9-fold lower EC_{50} on α1β2γ2L compared to α1β1γ2L receptors (Hill-Venning *et al.*, 1997). Co-expression of α1ε with the β2 isoform revealed a 10-fold increase in etomidate potency compared to α1β1ε receptors (Figure 3.9B).

Figure 3.9. Modulation of α1β1, α1β1γ2s, α1β1ε, α1β2 and α1β2ε receptors by propofol and etomidate.

A)

B)
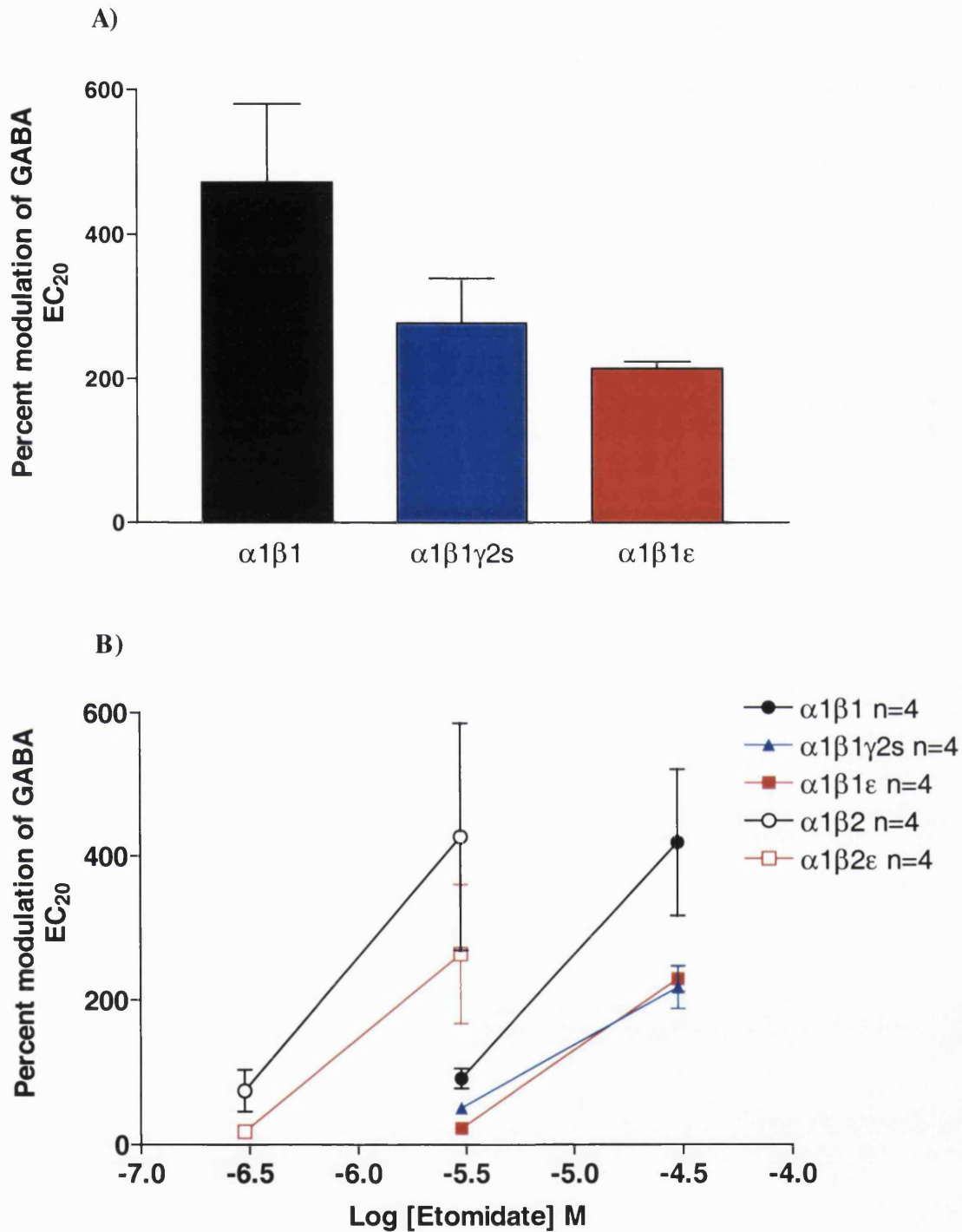
A) Potentiation of control GABA EC_{20} responses by 10μM propofol.

B) Potentiation of control GABA EC_{20} responses by etomidate.

Note the modulation by propofol and etomidate on ε containing receptors includes the small direct effect.

3.2.9. Effect of allopregnanolone.

Concentration-response curves to the neuroactive steroid, allopregnanolone (5α-pregnan-3α-ol-20-one), were obtained for the same three receptor subunit combinations (Figure 3.10). The response measured was the total effect, uncorrected for the small degree of direct activation observed at high concentrations.

Figure 3.10. Concentration-response curves for allopregnanolone on α1β1, α1β1γ2s and α1β1ε receptors.

Data were normalised with respect to the control GABA EC_{20} response, fitted using equation (2) (see Chapter 2) and the parameters shown in Table 3.6 determined.

Table 3.6. EC$_{50}$, Hill coefficient and maximum response for allopregnanolone concentration-response curves on $\alpha1\beta1$, $\alpha1\beta1\gamma2s$ and $\alpha1\beta1\epsilon$ receptors.

Receptor	EC$_{50}$ (nM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
$\alpha1\beta1$	74 (64, 85)*	1.14 ± 0.12	265 ± 38	6
$\alpha1\beta1\gamma2s$	108 (89, 131)*	1.28 ± 0.12	215 ± 35	4
$\alpha1\beta1\epsilon$	193 (181, 205)	1.16 ± 0.10	260 ± 45	4

* indicates a significant difference ($P<0.05$) from $\alpha1\beta1\epsilon$.

Unlike PB, a similar maximum potentiation of ~250% was seen for allopregnanolone on $\alpha1\beta1$, $\alpha1\beta1\gamma2s$ and $\alpha1\beta1\epsilon$, whereas a significantly lower EC$_{50}$ was observed on $\alpha1\beta1$ receptors compared to $\alpha1\beta1\epsilon$ or $\alpha1\beta1\gamma2s$.

3.2.10. Interaction of various hormones with $\alpha1\beta1\epsilon$.

Presence of ϵ mRNA in the hypothalamus may suggest a role in neuroendocrine regulation since this brain region releases a number of hormones which regulate the secretion of pituitary hormones. These hormones, in turn, negatively feedback to the hypothalamus and could modulate GABA$_A$ receptor function. The effect of a number of hormones on $\alpha1\beta1\epsilon$ receptor function was therefore examined. Oxytocin, thyrotropin releasing hormone, vasopressin and human growth hormone had little or no effect on control GABA EC$_{50}$ currents at the concentrations tested whereas L-Triiodothyronine ($30\mu M$) inhibited the GABA EC$_{50}$ by 65% (Table 3.7).

Table 3.7. Effects of various hormones on *Xenopus* oocytes expressing human α1β1ε receptors.

Compound	Percent Modulation of GABA EC$_{50}$
Oxytocin (3μM)	-2.7 ± 1.8
Thyrotropin releasing hormone (3μM)	-5.7 ± 1.5
Vasopressin (3μM)	-6.4 ± 4.4
Growth Hormone (0.04IU/ml)	-12.3 ± 4.4
L-Triiodothyronine (30μM)	-65.3 ± 6.2

Concentration-response curves (30μM being the limit of solubility) to L-triiodothyronine were constructed on α1β1 and α1β1ε receptors (Figure 3.11). In the absence of GABA, 10μM and 30μM L-Triiodothyronine elicited small outward currents (<3% of GABA maximum) on α1β1ε receptors possibly due to the inhibition of the constitutively-active current. Curves were fitted using equation (2) (see Chapter 2) assuming complete inhibition would be obtained and revealed estimated IC$_{50}$ values of 13.8 (10.5, 18.1)μM n=4 for α1β1ε compared to > 30μM for α1β1.

Figure 3.11. Concentration-response curves to L-Triiodothyronine on α1β1 and α1β1ε receptors.
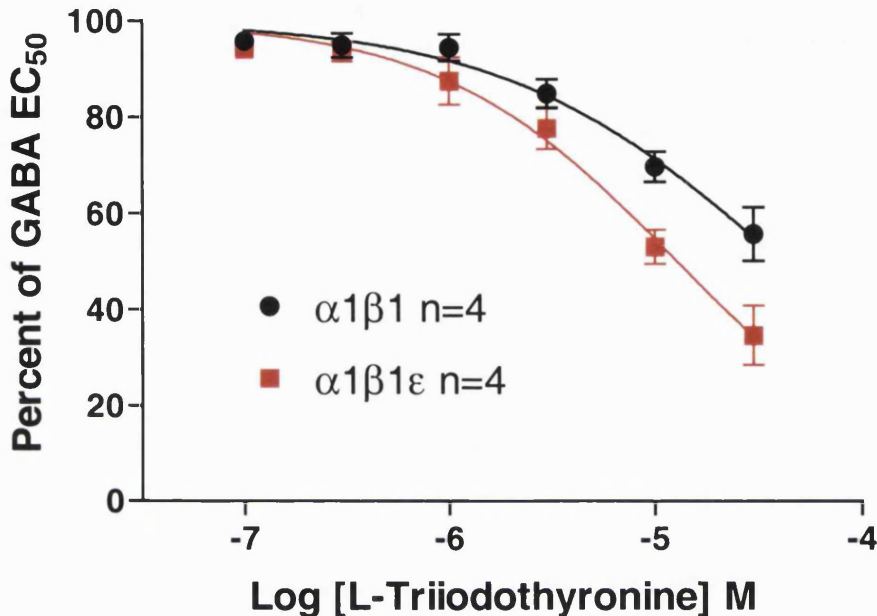
3.2.11. Comparison of the two ε clones.

The simultaneous and independent cloning of the ε subunit by colleagues at Merck Sharp & Dohme Research Laboratories and by a group at The Institute for Genomic Research should have produced clones with identical properties. However, comparison of the two papers (Davies *et al.*, 1997a; and Whiting *et al.*, 1997) revealed a striking difference in the sensitivity of ε subunit containing receptors to anaesthetic agents. The ε clone used by Davies *et al.* (1997a), (hereafter referred to as εTIGR), was insensitive to the potentiating effects of general anaesthetic agents, whereas our clone (εMRK) exhibited GABA responses clearly potentiated by general anaesthetic agents. Sensitivity to the direct activation by anaesthetic agents with εTIGR was however retained. Comparison of the amino acid sequence of the two ε clones revealed only one difference, namely a single residue switch at position 102 (serine to alanine). In addition the vector into which the ε clones were engineered and the expression system used were different between the two groups; with Davies *et al.* (1997a) preferring the vector pCDM8 and transient transfection of human embryonic

kidney cells (HEK-293) and our group using the vector pcDNA1.1Amp and transient expression in *Xenopus* oocytes. To address the clearly different pharmacological properties of the two ε clones further experiments were performed with both these clones under the same experimental conditions.

3.2.12. Allopregnanolone, etomidate and pentobarbitone do not potentiate εTIGR.

εTIGR was co-expressed with α1β1 in *Xenopus* oocytes and the effect of 1μM allopregnanolone, 30μM etomidate and 100μM pentobarbitone on the GABA EC_{20} response examined. Since the direct activation by anaesthetic agents was retained by εTIGR this effect was ignored and only the potentiation, expressed relative to the control GABA EC_{20} measured. Modulation of α1β1εTIGR by allopregnanolone, etomidate and pentobarbitone was −8.3 ± 10.5% n=4, -27 ± 13% n=4 and 13.0 ± 8.9% n=11 respectively compared to 126.9 ± 24.7% n=7, 178 ± 25 n=4 and 124.9 ± 26.6% n=8 on α1β1εMRK (Figure 3.12).

3.2.13. α1β1εMRK(S102A) is potentiated by allopregnanolone and pentobarbitone.

The two ε sequences differed by one amino acid at position 102 (numbering including signal sequence) with εMRK containing a serine and εTIGR an alanine. εMRK was modified at position 102 to see if this amino acid difference could account for the anaesthetic insensitivity. Interestingly α1β1εMRK(S102A) was still potentiated by 1μM allopregnanolone (101.5 ± 22.1% n=4) and 100μM pentobarbitone (183.3 ± 18.6% n=4) (Figure 3.13). The single amino acid difference at position 102 therefore was not responsible for the anaesthetic insensitivity of εTIGR.

Figure 3.12. Lack of potentiation of $\alpha 1\beta 1\epsilon$TIGR by general anaesthetic agents when expressed in *Xenopus* oocytes.
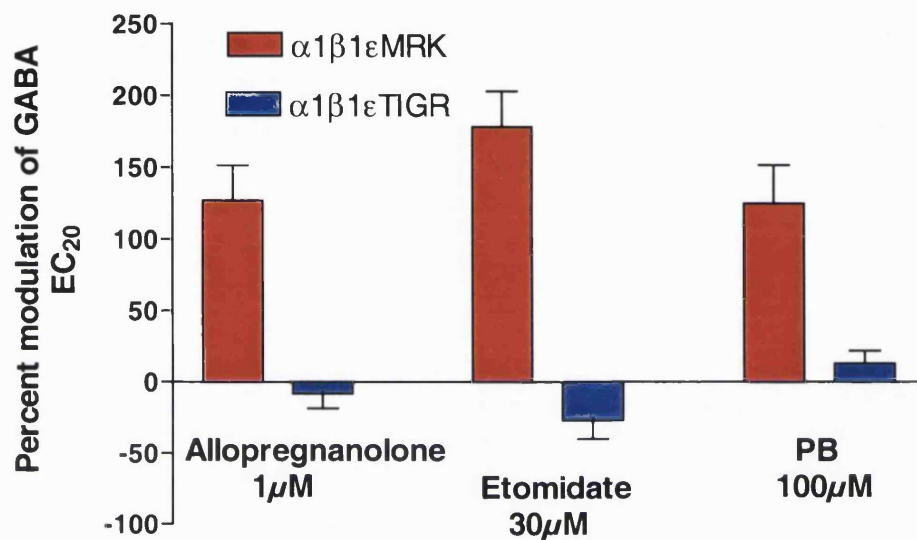
Figure 3.13. The amino acid difference at position 102 was not responsible for the lack of anaesthetic potentiation in ϵTIGR.
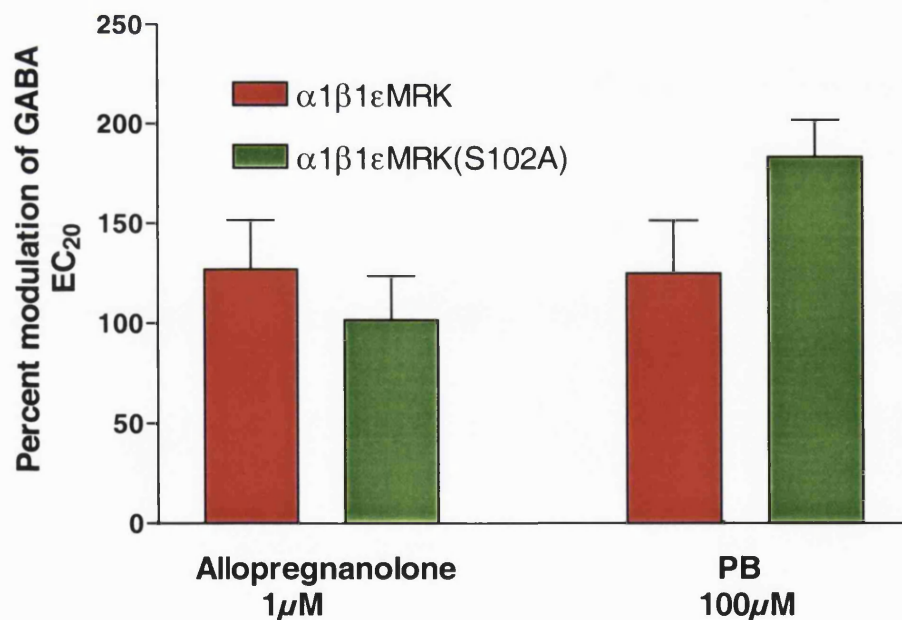
3.2.14. Expression of εTIGR in pcDNA1.1Amp restores pentobarbitone potentiation.

Since the coding region of the two constructs were identical and were now compared in the same cell type, the differences to anaesthetic agents observed between the two ε receptor constructs must be due to, either, differences in the untranslated regions or differences in the vectors in which they were engineered. To answer this question a number of different ε-constructs were made. εTIGR was transferred into pcDNA1.1Amp, with the 3' and 5' non-coding regions intact. It was also transferred into pcDNA1.1Amp with the 3' non-coding region of the εMRK clone and / or the 5' non-coding region of the εMRK clone (Figure 3.14). As essentially similar results were obtained with both the steroid and barbiturate, $100\mu M$ pentobarbitone was used to evaluate these four different constructs of the εTIGR clone. Pentobarbitone potentiated all four constructs when expressed with α1β1 (Figure 3.15) suggesting that by transferring the εTIGR clone into a different vector, sensitivity to pentobarbitone could be restored and the 3' and 5' untranslated regions did not relate to the reduced sensitivity.

Figure 3.14 Schematic diagram of the different constructs made.
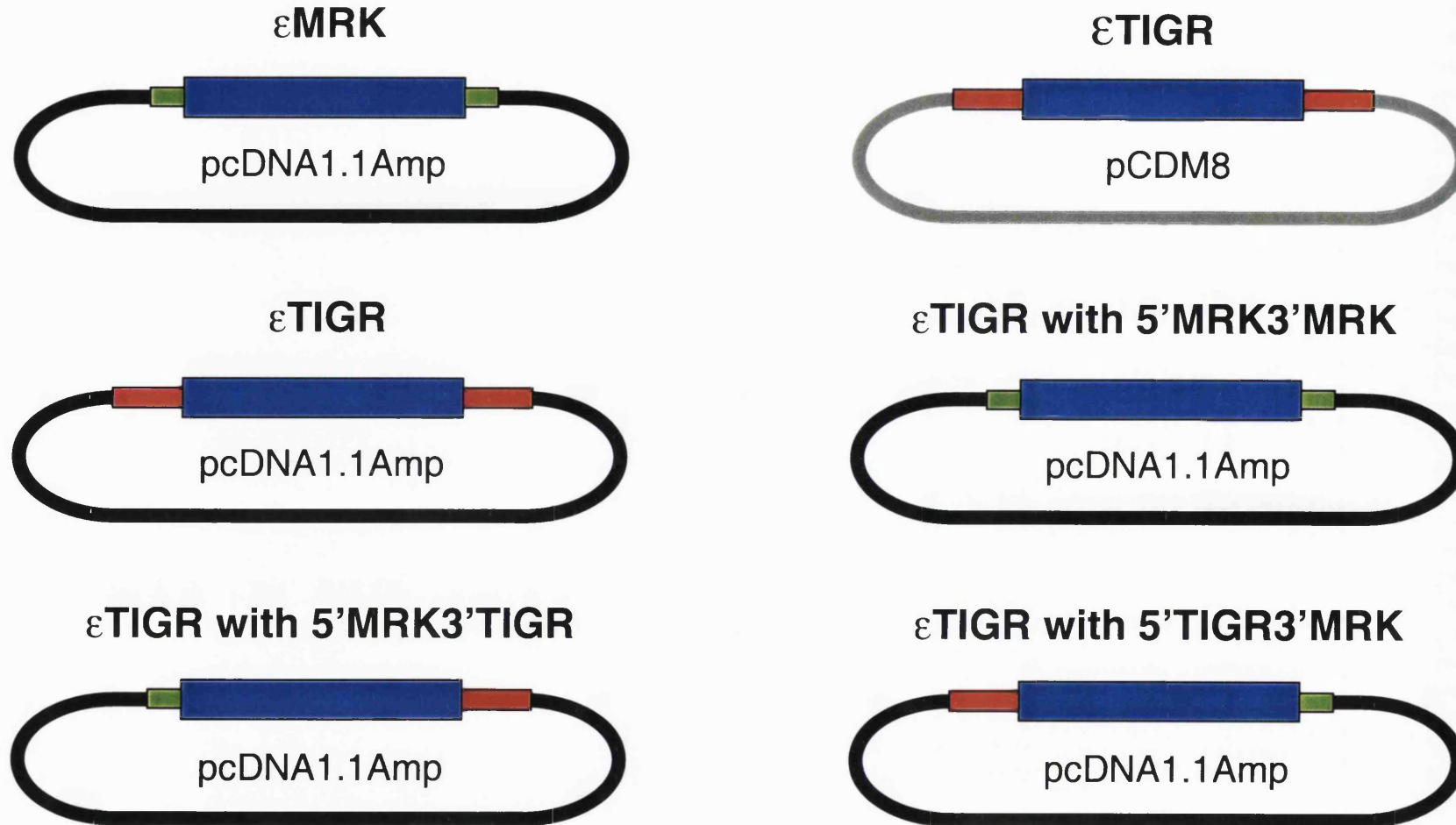
The solid blue rectangle represents the ε coding region, the green and red rectangles represent the 5' (left) and 3' (right) non-coding regions of εMRK and εTIGR and the black and grey ovals represent the vectors pcDNA1.1Amp and pCDM8.

Figure 3.15. Potentiation by pentobarbitone for is restored when εTIGR is engineered into pcDNA1.1Amp.
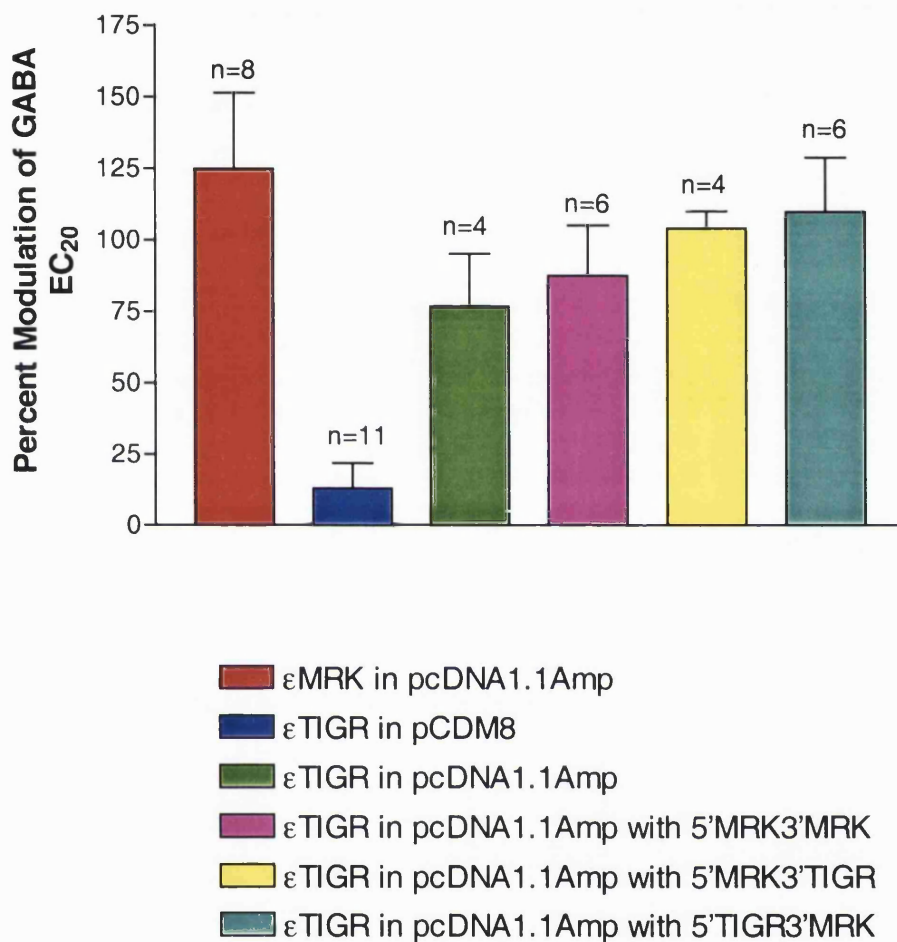
Each ε construct was co-expressed with an identical $\alpha1$ and $\beta1$ subunit in *Xenopus* oocytes. Engineering εTIGR into pcDNA1.1Amp restored potentiation to pentobarbitone.

3.2.15. α1β1εTIGR have a larger leak current and higher apparent GABA affinity.

The maximum inward current to GABA was not significantly different between the two ε clones (εTIGR 710 ± 143nA n=18, εMRK 862 ± 52nA n=52, voltage-clamped at -70mV), however the apparent GABA affinity was approximately 4-fold higher on εTIGR compared to εMRK. In the absence of full GABA concentration-response curves for εTIGR, EC_{20} and EC_{50} values were compared using only those concentrations that were between an EC_{16-29} and EC_{40-60} (Table 3.8).

Table 3.8. Comparison of the GABA EC_{20} and EC_{50} values for εTIGR and εMRK co-expressed with α1β1 in *Xenopus* oocytes.

Approx. [GABA]	α1β1εTIGR	No. of Oocytes	α1β1εMRK	No. of Oocytes
EC_{20} (μM)	0.09 (0.08, 0.10)*	15	0.30 (0.27, 0.33)	42
EC_{50} (μM)	0.35 (0.23, 0.52)*	5	1.42 (1.18, 1.70)	20

* indicates a significant difference ($P<0.01$) from α1β1εMRK.

The GABA EC_{20} and EC_{50} were significantly lower on α1β1εTIGR receptors compared to α1β1εMRK receptors. α1β1εTIGR also displayed significantly ($P<0.05$) larger outward currents to 100μM picrotoxin (233 ± 51nA n= 6 voltage clamped at – 70mV) compared to α1β1εMRK (95 ± 18 n=14 voltage clamped at –70mV) (Figure 3.16).

Figure 3.16. Typical recordings of α1β1εMRK and α1β1εTIGR receptors to 100μM GABA and 100μM PTX.
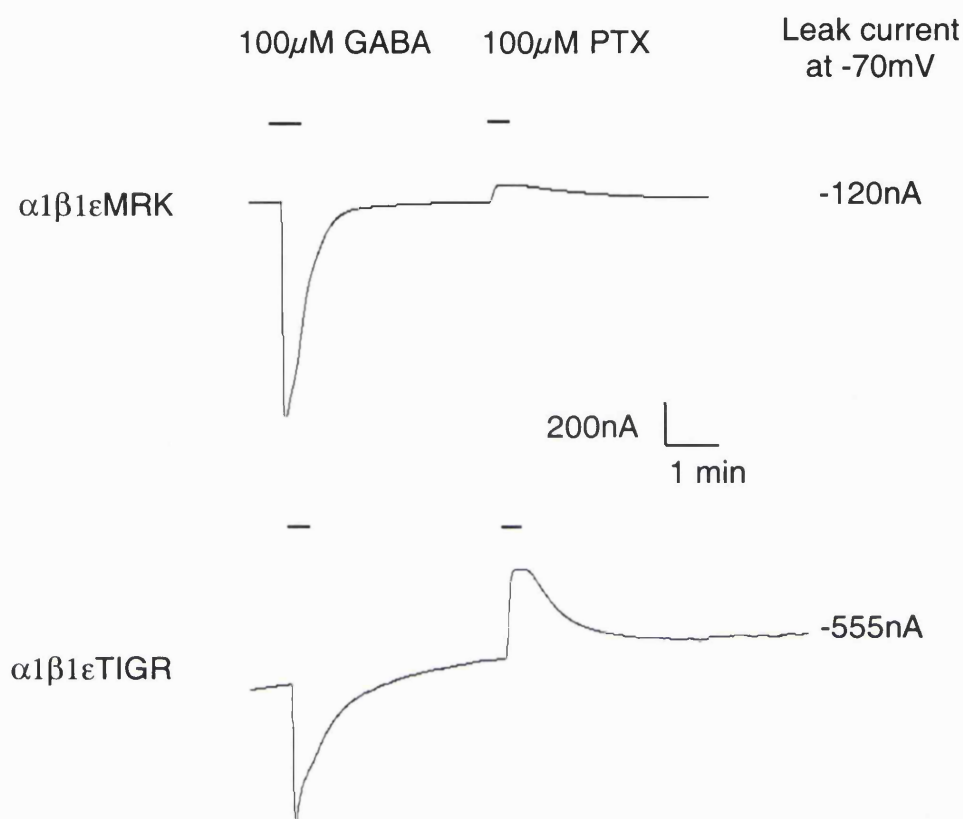
3.2.16. Insensitivity to anaesthetic agents may be due to overexpression of εTIGR.

Dr. Timothy Bonnert (Merck Sharp & Dohme) performed a Northern blotting assay which measured the RNA present in HEK-293 cells that had been transiently transfected with α1β1εTIGR and α1β1εMRK subunits in a ratio 1:1:1. Interestingly cells transfected with α1β1εTIGR had significantly greater levels of εTIGR RNA compared with α1 and β1 RNA, whereas cells transfected with α1β1εMRK showed similar levels of RNA for all 3 subunits (data not shown).

These results suggested that the ϵTIGR subunit was being overexpressed compared to the α, β and the ϵMRK subunits and this overexpression may confer anaesthetic insensitivity. To confirm this, the ratio of ϵTIGR (in its original vector) to $\alpha 1$ and $\beta 1$ was reduced to 1:1:0.1, 1:1:0.03 and 1:1:0.01 and the modulation by 100μM pentobarbitone determined. In order to show that $\alpha 1\beta 1\epsilon$ receptors were being formed and $\alpha 1\beta 1$ receptors did not predominate, 1μM Zn^{2+} was used as a diagnostic tool. All the ratios showed a low sensitivity to Zn^{2+} however the inhibition for 1:1:0.03 and 1:1:0.01 was significantly greater ($P<0.05$) than for 1:1:1 (Figure 3.17) indicating the presence of a small proportion of $\alpha 1\beta 1$ receptors. Interestingly reducing the ratio from 1:1:1 to 1:1:0.1 conferred potentiation by 100μM pentobarbitone while having no effect on the Zn^{2+} inhibition (Figure 3.17). The lower ratios (1:1:0.03 and 1:1:0.01) were also potentiated by pentobarbitone however the degree of potentiation correlated with the amount of inhibition by Zn^{2+}. This would be expected since pentobarbitone was significantly more efficacious on $\alpha 1\beta 1$ receptors than on $\alpha 1\beta 1\epsilon$ (see Figure 3.7). The anaesthetic insensitivity of $\alpha 1\beta 1\epsilon$TIGR receptors therefore appears to be due to over-expression of the ϵ subunit. This over-expression of the ϵ subunit results in receptors that have a greater incidence of spontaneous openings, a higher apparent GABA affinity and are insensitive to the potentiating actions of anaesthetic agents.

Figure 3.17. Reducing the ratio of εTIGR to α1β1 confers pentobarbitone sensitivity.
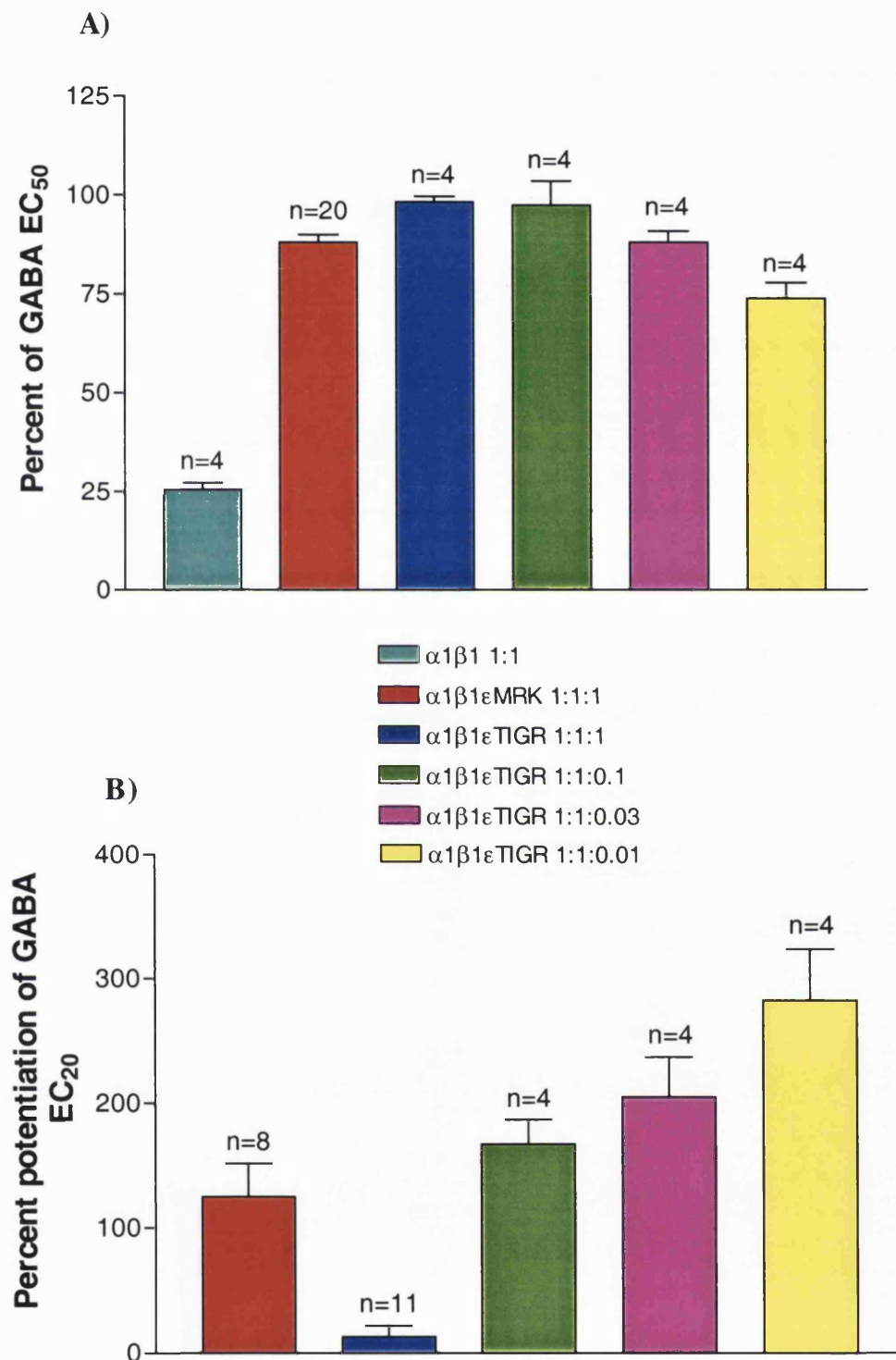
A) Modulation of a GABA EC_{50} concentration by $1\mu M$ $ZnCl_2$.

B) Potentiation of a GABA EC_{20} concentration by $100\mu M$ pentobarbitone.

3.3. Discussion.

This chapter reports on the pharmacological characterisation of a human cDNA encoding a novel GABA$_A$ receptor subunit, ε. Functional channels are formed when the ε subunit is co-expressed with α1 and β1 suggesting that the ε subunit, like the δ subunit, can substitute for a γ subunit.

3.3.1. The ε subunit can replace a γ subunit to form functional receptors.

Similar to Davies *et al.* (1997a), oocytes injected with either ε alone or α1ε and β1ε subunits did not respond to either GABA or picrotoxin. The ε subunit therefore does not behave like a typical α or β subunit and form receptors composed only of two subunits. Co-injection of ε with α1 and β1 however, did result in functional channels, which elicited inward currents to GABA and also small outward currents to PTX, the latter being indicative of constitutively-active channels.

3.3.2. GABA activation and desensitization kinetics.

Although not conclusive evidence of functional expression of the ε subunit, the concentration-response curves to GABA did reveal some novel observations. Firstly α1β1ε receptors expressed in *Xenopus* oocytes appeared to desensitize much more rapidly as compared with α1β1 or α1β1γ2s receptors. The *Xenopus* oocyte expression system is not an ideal *in vitro* system for the measurement of kinetic parameters due to the very slow drug application times. For this reason some kinetic measurements were performed on transiently transfected HEK-293 cells using whole-cell patch-clamp techniques which allow more rapid solution changes. As observed with *Xenopus* oocytes, α1β1ε receptors desensitized more rapidly than α1β1 and α1β1γ2s receptors. Single exponentials fitted to the desensitization phase of a maximum GABA concentration (30μM) revealed rate constants of 1.79 ± 0.33, 4.23 ± 0.83 and 4.55 ± 1.2 sec for α1β1ε, α1β1 and α1β1γ2s respectively. These experiments were performed by Mr. George Marshall (Merck Sharp & Dohme) and permission for reproduction of the results obtained. The rapid desensitization kinetics conferred to the GABA$_A$ receptor by the ε subunit is in contrast to the δ subunit, which appears to

decrease the rate of GABA-induced desensitization (Saxena & Macdonald, 1994; Brown *et al.*, 2001).

The EC_{50} values obtained for the GABA concentration-response curves were not significantly different between $\alpha 1 \beta 1$ and $\alpha 1 \beta 1 \epsilon$ receptors whereas the Hill coefficient for $\alpha 1 \beta 1 \epsilon$ receptors was significantly lower ($\alpha 1 \beta 1 \epsilon$ $n_H = 0.85$). A Hill coefficient of <1 is unusual for $GABA_A$ receptors. In fact native $GABA_A$ receptors (Sakmann *et al.*, 1983; Kapur & Macdonald, 1996) and most recombinant $GABA_A$ receptors (White *et al.*, 1995) show some degree of positive cooperativity (see Chapter 4 for further discussion on Hill coefficients for GABA concentration-response curves). The lower Hill coefficient observed for GABA using *Xenopus* oocytes expressing $\alpha 1 \beta 1 \epsilon$ may be as a consequence of the rapid desensitization kinetics observed with ϵ containing receptors. As previously mentioned application of GABA to the oocyte is generally regarded as being slow. Due to the large surface area of the oocyte, some receptors are exposed to GABA before others, and in fact may have entered a desensitized state while others have yet to be activated. If this occurred the maximum GABA response would be underestimated and would result in a shallow concentration-response curve. Whole-cell patch-clamp experiments on transiently transfected HEK-293 cells and a stable cell line ($\alpha 2 \beta 1 \epsilon$) in Ltk⁻ cells however also revealed significantly lower Hill coefficients for ϵ containing receptors compared to γ containing receptors (data not shown). Unlike the oocyte technique, the patch-clamp technique used employed more rapid solution changes. Whether the low Hill coefficient is as a consequence of the rapid desensitization kinetics remains to be determined.

3.3.3. Transient outward current upon washout of GABA.

A second novel finding was that washout of GABA from both *Xenopus* oocytes and HEK-293 cells expressing $\alpha 1 \beta 1 \epsilon$ receptors produced a transient outward current that was absent from $\alpha 1 \beta 1$ and $\alpha 1 \beta 1 \gamma 2s$ receptors. A similar transient outward current has been reported for a chimeric receptor given the nomenclature $\alpha 1 C2$ (Serafini *et al.*, 2000). The C2 subunit was a chimera of $\beta 3$ (223) and $\rho 1$ (265) with the join occurring in the middle of TM1. Like $\alpha 1 \beta 1 \epsilon$ receptors, cells expressing $\alpha 1 C2$ had a degree of constitutive-activity which was blocked by picrotoxin. The presence of constitutively-

active channels within these receptors may provide an explanation for the transient outward current upon washout of GABA. If it is assumed that the channels flicker between the spontaneously open and closed state and that GABA can bind to both these states, then application of high concentrations of GABA will cause a proportion of both the constitutively-active and GABA-activated channels to enter one or more desensitized / non conducting states. Subsequent washout of GABA could then reveal an apparent outward current as inactivated channels reopen to their normal constitutive level. Single channel kinetics and mathematical modelling could be used to address this theory.

3.3.4. Inhibition by Zn^{2+}: definitive evidence of expression.

Definitive evidence for co-assembly of $\alpha 1 \beta 1 \varepsilon$ receptors came from the differential effects of zinc. Previous studies have shown that receptors containing three subunits e.g. $\alpha \beta \gamma$ or $\alpha \beta \delta$ are much less sensitive to zinc than the corresponding $\alpha \beta$ receptor (Draguhn et al., 1990, Thompson et al., 1997 and Chapter 4). This pattern of reduced zinc sensitivity was also apparent when the ε subunit was introduced into the $\alpha 1 \beta 1$ heteromer. Recently two histidine residues have been described within the $\beta 3$ subunit that confer high affinity, H292 within TM2 (Wooltorton et al., 1997b) and low affinity Zn^{2+} binding, H107 within the N-terminal (Dunne et al., 1999). Interestingly, the N-terminal lower affinity histidine is present in all $GABA_A$, $GABA_C$ and glycine subunits, whereas the histidine responsible for the high affinity is only present in the $\beta 1$, 2, 3 and θ subunits. Histidine residues are thought to bind Zn^{2+} ions through an interaction with a protonatable nitrogen atom on the imidazole ring. The amino acids present at the homologous TM2 position within the γ and ε subunits are isoleucine and phenylalanine. Evidence to date suggests that the stoichiometry of $\alpha \beta \gamma$ heteromers is 2α, 2β and 1γ subunit (Chang et al., 1996, Farrar et al., 1999) and $\alpha \beta$ heteromers is 2α and 3β subunits (Tretter et al., 1997; Baumann et al., 2001). The presence of three β subunits within the $\alpha \beta$ heteromer and hence three histidine residues may explain the high Zn^{2+} sensitivity seen with $\alpha \beta$ heteromers. Differences in isoleucine and phenylalanine within the γ and ε subunit may account for the altered Zn^{2+} sensitivity observed with $\alpha 1 \beta 1 \varepsilon$ and $\alpha 1 \beta 1 \gamma 2s$ receptors.

3.3.5. Lack of modulation of α1β1ε receptors by benzodiazepines.

Unlike α1β1γ2s receptors but similar to α1β1δ (Saxena & Macdonald, 1994), α1β1ε receptors were not modulated by benzodiazepines. It has subsequently been shown that [^{3}H] flumazenil is unable to bind to α1β1ε receptors transiently transfected in HEK-293 cells (unpublished data performed by Dr. Peter Wingrove, Merck Sharp & Dohme). Previous studies have shown that the benzodiazepine binding site is made up from a number of amino acid residues within both the α and γ subunits (for a review see Sigel & Buhr, 1997). Phenylalanine at position 77 within the γ2s subunit has been shown to be critical for both the high affinity binding and efficacy of various benzodiazepine site ligands (Buhr *et al.*, 1997a; Wingrove *et al.*, 1997). Both ε and γ1 subunits contain an isoleucine residue at this homologous position. Previous reports have shown that α1β1γ1 receptors can bind benzodiazepine ligands, albeit with much lower affinities than α1β1γ2s receptors, however functionally they are not modulated (Wingrove *et al.*, 1997). Replacement of this isoleucine within the γ1 subunit with the γ2 equivalent residue phenylalanine confers modulation. It is possible therefore, that isoleucine 103 within the ε subunit is responsible for the lack of benzodiazepine modulation, however other additional amino acid residues may also be involved. It is worth noting that two additional amino acid residues within the γ2 subunit (M130 and T142) have been shown to alter benzodiazepine binding affinities and / or efficacies. Interestingly these amino acids have been conserved within the ε subunit.

3.3.6. Modulation by general anaesthetic agents.

General anaesthetic agents e.g. pentobarbitone, propofol and etomidate and the neurosteroid, allopregnanolone were able to directly activate and potentiate α1β1ε receptors. In general there was no difference between the modulatory effects of pentobarbitone, propofol, etomidate and allopregnanolone on α1β1γ2s and α1β1ε receptors. The total modulatory effect of pentobarbitone has been examined on a range of GABA$_A$ receptor combinations (Thompson *et al.*, 1996 and Chapter 4). This study showed that the EC$_{50}$ value and Hill coefficient for the total modulatory effect of pentobarbitone was relatively constant for the subunit combinations tested (see

Figure 3.7 Chapter 4). The additional data presented in this chapter, for α1β1 and α1β1ε receptors, further support this finding.

Pentobarbitone was the only general anaesthetic agent whose direct effect was fully investigated. Interestingly the threshold for the direct effects to pentobarbitone occurred at much lower concentrations on α1β1ε receptors compared to α1β1 and α1β1γ2s. Similarly concentrations of propofol, etomidate and allopregnanolone, which did not directly activate α1β1 and α1β1γ2s receptors, elicited inward currents on α1β1ε receptors in the absence of GABA. This could be due to an increased affinity of these agents for α1β1ε receptors or, more likely, due to modulation of the constitutively-active channels. Similar to GABA, the Hill coefficient for the direct effect of pentobarbitone was significantly lower on α1β1ε receptors compared to α1β1 and α1β1γ2s receptors. The reduction in Hill coefficient is much larger for pentobarbitone than GABA and is probably due to a combination of both the modulation of the constitutively-active channels at low pentobarbitone concentrations and the influence of faster desensitization kinetics.

3.3.7. No specific interaction of a number of hormones with α1β1ε receptors.

Of the five hormones examined only L-triiodothyronine had any significant effect causing 65% inhibition at 30μM. This inhibition was not specific to ε-containing receptors since Martin et al. (1996) showed that α1β2γ2 and α1β2 GABA$_A$ receptors were inhibited by L-triiodothyronine with similar IC$_{50}$'s to those in this study. The importance of the ε subunit within the hypothalamus remains to be identified.

3.3.8. Importance of the constitutively-active current.

The presence of constitutively-active channels in α1β1ε receptors perturbs the Cl⁻ distribution within the oocyte, which resulted in a more depolarised resting membrane potential. The resting membrane potential of most neurones is primarily determined by a potassium conductance, however the addition of a significant resting chloride conductance, in the form of a constitutively-active GABA$_A$ receptor, offers a mechanism to adjust the baseline excitability of neurones. This isoform (α1β1ε) is the

first example of a recombinant $GABA_A$ receptor composed of three different native $GABA_A$ receptor subunits that exhibits both constitutively-active and GABA-activated currents. It remains to be established whether neuronal preparations expressing the ϵ subunit display this constitutively-active current and what its function would be. Recent work, using brain slices and dissociated neurons, however has identified a tonic GABAergic current in the cerebellum (Brickley et al., 1996, Wall & Usowicz, 1997), cortex (Salin & Prince, 1996), thalamus (Liu et al., 1995) and hippocampus (Otis et al., 1991; Birnir et al., 2000b). At present it is not known if these tonic currents are as a result of activation of extrasynaptic receptors by low GABA concentrations present in the extracellular space or by the spontaneous opening of constitutively-active channels. The subunit composition of the receptors involved remains to be determined but in the hippocampus, for example, may include an ϵ subunit.

3.3.9. Comparison with other constitutively-active $GABA_A$ receptors.

Although $\alpha 1\beta 1\epsilon$ exhibits constitutive activity, this phenomenon has been reported for homomeric $\beta 1$ (Sigel et al., 1989; Sanna et al., 1995a; Krishek et al., 1996) and $\beta 3$ receptors (Wooltorton et al., 1997b) and for heteromers incorporating various mutations within TM2 of $GABA_A$ receptor subunits e.g. $\alpha 1\beta 2L259S\gamma 2s$ (Thompson et al., 1999b; see Chapter 7) and $\alpha 2S270W\beta 1$ (Findlay et al., 2001). General anaesthetic agents directly activated all these receptors however potentiation of a submaximal GABA response was only seen with human $\beta 1$ homomers (Sanna et al., 1995a). Whether or not potentiation is observed with general anaesthetic agents may depend on the relative proportion of constitutively-active channels to non-constitutively-active channels. This may explain why $\alpha 1\beta 1\epsilon$TIGR receptors which had a larger leak current, due to the over-expression of the ϵ subunit, than $\alpha 1\beta 1\epsilon$MRK, were not potentiated by general anaesthetic agents.

Recent research by Neelands et al. (1999a) and G. Maksay, S.A. Thompson & K.A. Wafford (unpublished data) has shown that the IC_{50} for inhibition of the constitutively-active current on $\alpha 1\beta 3\epsilon$ receptors by PTX is $1.8\mu M$. Previous studies on homomeric $\beta 1$ and $\beta 3$ receptors revealed IC_{50}'s for inhibition of the constitutively-

active current by PTX of 2.11 (Krishek *et al.*, 1996) and 0.084μM respectively (Wooltorton *et al.*, 1997b).

3.3.10. Distribution of the ε subunit.

In situ hybridization and immunohistochemical studies using squirrel monkey brain showed that the ε subunit was discreetly located within the hypothalamus (particularly within the arcuate-ventromedial area) and in the hippocampus (specifically the hilus of the dentate gyrus). Previous immunohistochemical (Fritschy & Mohler, 1995) and *in situ* hybridization (Wisden *et al.*, 1992) studies have mapped the distribution of the majority of GABA$_A$ subunits within the adult rat brain. A subsequent study by Sperk *et al.* (1997) performed a more detailed immunohistochemical study focusing solely on the hippocampus of the adult rat brain. The arcuate nucleus and ventromedial nucleus generally stained positive for the same subunits (α1, α2, α3, α5, (β1 ventromedial nucleus only), β3, γ1 and γ2) with α2, β3, γ1 and γ2 predominating (Wisden *et al.*, 1992; Fritschy & Mohler, 1995), whereas the hilus of the dentate gyrus gave positive signals for α1, β2, γ2 and δ subunits (Sperk *et al.*, 1997).

The hilus of the dentate gyrus contains GABAergic interneurons which synapse with granule cell dendrites and exert an important role in gating the impulse flow transmitted through the hippocampus to the CA3 region. The presence of an ε containing receptor with constitutive-activity within the hilus of the dentate gyrus may provide an inhibitory tone. Immunoprecipitation studies are required to provide definitive evidence of which subunits are co-expressed with ε *in vivo*. In addition, information on the location of ε containing receptors i.e. pre or postsynaptic would help to understand its role *in vivo*.

3.3.11. Overexpression of εTIGR results in insensitivity to anaesthetic agents.

The two ε clones (Davies *et al.*, 1997a and Whiting *et al.*, 1997) differed significantly with respect to potentiation of a submaximal GABA response by general anaesthetic agents. The report from Davies *et al.* (1997a) that ε containing receptors were not potentiated by general anaesthetic agents was a novel finding which differed from the

many reports using various recombinant receptor combinations and native neuronal preparations (Tanelian *et al.*, 1993; Thompson *et al.*, 1996 and Chapter 4; Belelli *et al.*, 1999). As demonstrated in this study restoration of the potentiating effects of pentobarbitone occurred upon transfer of εTIGR from its original vector (pCDM8) into pcDNA1.1Amp and upon reduction of the ratio of εTIGR (in pCDM8) to α1β1. Collectively the electrophysiological and biochemical results indicate that εTIGR is being overexpressed relative to α1β1 and this overexpression renders the receptor insensitive to anaesthetic agents. Overexpression of εTIGR may alter the assembly and hence the stoichiometry of the receptor e.g. 2 copies of α, 1 copy of β and 2 copies of εTIGR or 1 copy of α, 2 copies of β, and 2 copies of εTIGR compared with a stoichiometry of 2 copies of α, 2 copies of β and 1 copy of εMRK, which in turn leads to different pharmacological properties. Further biochemical experiments are needed to investigate the differences between εMRK and εTIGR containing receptors at the level of assembly and the stoichiometry. However the results from this study clearly demonstrate that the expression vector used in recombinant studies can significantly alter the pharmacology. It remains to be seen if endogenous ε containing GABA$_A$ receptors are modulated by general anaesthetic agents although no barbiturate insensitive native receptors have been previously reported.

Changes in pharmacology have been observed for overexpression of glycine α1 subunit in *Xenopus* oocytes. The pharmacology of the channels appeared to change at high expression levels resulting in receptors with a 5-6 fold higher affinity for glycine and resistance to block by strychnine (Taleb & Betz, 1994). These receptors also differed in their relative permeability for chloride and open channel probability, suggesting differences in channel properties (Maammar *et al.*, 1997).

3.4.   Summary.

Co-injection of human α1β1 and ε (MRK) cDNA into *Xenopus* oocytes resulted in functional receptors. These receptors displayed a leak current due to the presence of constitutively-active Cl⁻ channels. α1β1ε receptors were sensitive to GABA and modulated by various general anaesthetic agents such as propofol, pentobarbitone,

etomidate and the neurosteroid, allopregnanolone. In contrast $\alpha1\beta1\epsilon$ receptors were insensitive to the modulatory effects of benzodiazepines.

Differences in the anaesthetic pharmacology between ϵMRK and ϵTIGR were shown to be due to the vector used. It is proposed that over-expression of the ϵ subunit with respect to the $\alpha1$ and $\beta1$ subunits produced receptors which were insensitive to the potentiating effects of general anaesthetic agents, displayed a higher GABA affinity and greater leak current.

Chapter 4

Interactions of Pentobarbitone and Propofol at the Human GABA$_A$ receptor: Dependence on Receptor Subunit Combination

4.1.   Introduction.

General anaesthetic agents have been shown to interact with a number of ion channels (for recent reviews see Franks & Lieb, 1998; Krasowski & Harrison, 1999; Thompson & Wafford, 2001). A common feature of many volatile and intravenous agents however is the ability to enhance the ion channel activation of GABA$_A$ receptors (Franks & Lieb, 1994; Harrison *et al.*, 2000), with this interaction being proposed as the probable major molecular mechanism for anaesthetic action in the mammalian CNS (Olsen, 1988; Tanelian *et al.*, 1993).

The intravenous anaesthetic agents pentobarbitone and propofol have been shown to affect GABA$_A$ receptor-mediated responses in several ways. At low micromolar concentrations they potentiate GABA-evoked responses, at high micromolar concentrations they activate GABA$_A$ receptors directly and at millimolar concentrations they are inhibitory (Schulz & MacDonald, 1981; Parker *et al.*, 1986; Akaike *et al.*, 1987; Adodra & Hales, 1995). Complete separation of the three effects (potentiation, direct activation and inhibition) is difficult, since for most receptor subtypes, the concentrations over which each occurs overlap. It must be remembered, therefore, that each effect can be influenced by the other two.

This chapter focuses on the potentiation and the direct activation of pentobarbitone and propofol on recombinant GABA$_A$ receptors. The results presented represent data taken from three separate publications (Thompson *et al.*, 1996; Wafford *et al.*, 1996 and Thompson *et al.*, 1997). The initial paper was a systematic analysis of the effect of pentobarbitone on $\alpha x \beta 2 \gamma 2s$, $\alpha 1 \beta y \gamma 2s$ and $\alpha 6 \beta y \gamma 2s$ (where x = 1, 2, 3, 5 or 6 and y = 1, 2 or 3) recombinant GABA$_A$ receptors. At the time of these studies the human $\alpha 4$ subunit cDNA was unavailable. Subsequently the human $\alpha 4$ subunit was cloned, co-expressed with $\beta 1 \gamma 2s$ and functionally characterised. Co-expression with the $\beta 1$ subunit was chosen since the original paper reporting the cloning of the bovine $\alpha 4$ subunit showed that it formed functional receptors when co-expressed with $\beta 1$ (Ymer *et al.*, 1989). The final publication reports on the co-expression of the δ subunit with $\alpha 6 \beta 3$. Immunohistochemistry (Fritschy & Mohler, 1995) and *in situ* hybridization studies (Laurie *et al.*, 1992; Wisden *et al.*, 1992) have revealed strong signals for the δ

subunit within certain areas of the thalamus, and the granule cells within the dentate gyrus of the hippocampus and the cerebellum. The $\alpha6$ subunit is found exclusively within the cerebellar granule cells together with $\alpha1$, $\beta2$, $\beta3$, $\gamma2$ and δ (Laurie *et al.*, 1992; Wisden *et al.*, 1992). Co-expression of the δ subunit with $\alpha6$ and $\beta3$ subunit may therefore represent a native receptor combination.

Thus this study represents a detailed investigation of the effects of pentobarbitone and propofol on many recombinant GABA$_A$ receptor combinations but pays particular attention to the discretely located, low abundance subunits such as $\alpha4$, $\alpha6$ and δ.

4.2.   Results.

The direct action and the potentiation of a GABA-activated response (EC$_{20}$ concentration) by either pentobarbitone or propofol could be studied together in a single oocyte. Pentobarbitone or propofol were preapplied alone for 30 sec or until any direct effect had reached a plateau, followed immediately by the co-addition of the GABA EC$_{20}$. Two measurements were taken:

1) the total modulation (including both direct activation and potentiation) normalised to the control EC$_{20}$ response. At high pentobarbitone concentrations the direct response undergoes rapid desensitization or inhibition of the response. Co-addition of the GABA EC$_{20}$ during this phase elicited only a small inward current which did not exceed that of the direct response (see Figure 4.1 1mM and 3mM pentobarbitone on $\alpha6\beta2\gamma2s$). In this situation the peak of the direct response and not the peak of co-addition of the GABA EC$_{20}$ was measured.

2) the direct effect normalised to the current elicited by a maximum concentration of GABA.

4.2.1. Total modulation of the GABA EC$_{20}$ response: Effect of different α subunits.

Concentration-response curves to pentobarbitone, over the concentration range 1μM to 3mM, were constructed on *Xenopus* oocytes expressing α1β2γ2s, α2β2γ2s, α3β2γ2s, α5β2γ2s and α6β2γ2s GABA$_A$ receptors. An initial observation was that potentiation of the GABA EC$_{20}$ response by pentobarbitone occurred at approximately 10-fold lower concentrations than the direct channel activation by pentobarbitone on α1β2γ2s, α2β2γ2s, α3β2γ2s and α5β2γ2s receptors, whereas on α6β2γ2s, both effects occurred over the same concentration range (Figure 4.1). Measurement of the total modulation of pentobarbitone on α6β2γ2s receptors therefore included a significant direct activation component. Similar to other general anaesthetic agents (neurosteroids, Lambert *et al.*, 1995; isoflurane, Lees & Edwards, 1998; etomidate, Hill-Venning *et al.*, 1997; propofol, Adodra & Hales, 1995) the concentration-response curves to pentobarbitone were bell-shaped (Figure 4.1 and 4.2). Data up to the maximum response were fitted with equation (1) (see Chapter 2) and the results presented in Table 4.1. The apparent affinity (EC$_{50}$) for the total modulatory effect of pentobarbitone was similar on all subunits tested (between 20 and 35 μM), however the maximum percentage increase in GABA EC$_{20}$ varied from 236% on α1β2γ2s to 536% on α6β2γ2s (Figure 4.2 and Table 4.1). Many authors have reported on the washout phenomenon that appears upon removal of high concentration of propofol and pentobarbitone (Akaike *et al.*, 1987; Robertson, 1989; Peters, 1988; Wooltorton *et al.*, 1997b). Although this phenomenon was not studied in detail in this study, arrows have been used in Figure 4.1 to illustrate its occurrence at 1 and 3mM concentrations of pentobarbitone.

Figure 4.1. Representative traces for a concentration-response curve to pentobarbitone on α1β2γ2s and α6β2γ2 receptors.
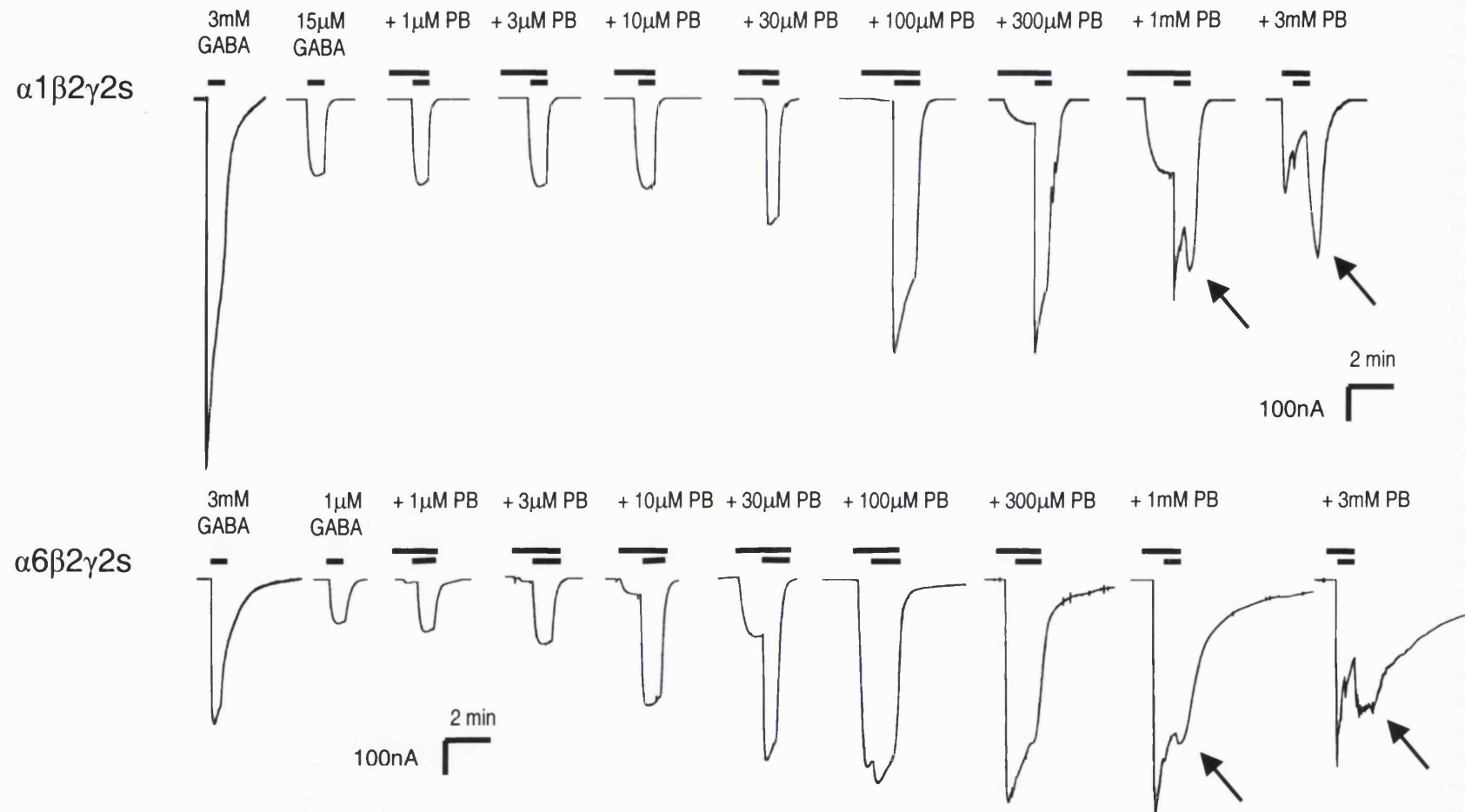
A maximum GABA response is followed by an approximate EC_{20} concentration. Subsequent responses show the effect of increasing concentrations of pentobarbitone (1μM to 3mM) on the control GABA EC_{20} response. Note the threshold for direct activation by pentobarbitone was significantly lower on α6β2γ2s than α1β2γ2s and the inhibition of the modulatory effect at mM concentrations. The black arrows illustrate the rebound phenomena which occurs upon removal of high concentrations of pentobarbitone.

Figure 4.2. Concentration-response curves for the total modulation (potentiation and direct effect) of pentobarbitone on α1β2γ2s, α2β2γ2s, α3β2γ2s, α5β2γ2s and α6β2γ2s GABA$_A$ receptors.
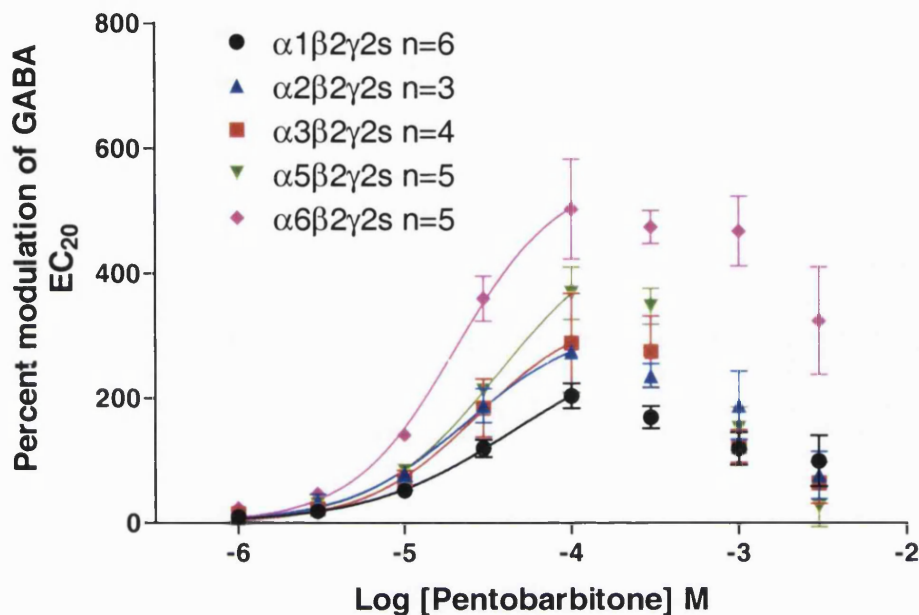
Data were normalised to the control GABA EC$_{20}$ response. Since a true maximum was not reached only the data points 1 to 100μM were fitted with equation (1) (see Chapter 2). The maximum was not constrained and the resulting parameters, as detailed in Table 4.1, therefore represent an estimate of the EC$_{50}$, Hill coefficient and maximum response.

Table 4.1. EC$_{50}$, Hill coefficient and the maximum response for the total modulation of pentobarbitone on α1β2γ2s, α2β2γ2s, α3β2γ2s, α5β2γ2s and α6β2γ2s GABA$_A$ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β2γ2s	25.6 (20.9, 31.4)	1.7 ± 0.3	236 ± 32	6
α2β2γ2s	20.2 (16.7, 24.5)	1.4 ± 0.1	299 ± 10	3
α3β2γ2s	25.4 (20.7, 31.1)	2.0 ± 0.7	313 ± 161	4
α5β2γ2s	25.7 (24.2, 27.4)	1.4 ± 0.1	403 ± 42	5
α6β2γ2s	20.7 (18.9, 22.6)	1.7 ± 0.3	536 ± 75	5

Potentiation of control GABA EC$_{20}$ responses by pentobarbitone was examined on α4β1γ2s receptors and compared to α1β1γ2s and α6β1γ2s receptors. 100μM pentobarbitone was used since the previous experiments had shown this to produce the maximum potentiation. Marked potentiation of the control GABA EC$_{20}$ response was observed with α4β1γ2s receptors, 706 ± 82% n=4 compared with 288 ± 32% n=5 on α1β1γ2s and 719 ± 52% n=6 on α6β1γ2s. Unlike α6β1γ2s but similar to α1β1γ2s, 100μM pentobarbitone did not directly activate α4β1γ2s receptors. Pentobarbitone was therefore able to potentiate α4β1γ2s receptors to an equivalent level as α6β1γ2s receptors, however on α6β1γ2s receptors this response was predominately due to direct activation by pentobarbitone rather than potentiation.

4.2.2. Total modulation of the GABA EC$_{20}$ response: Effect of different β subunits.

Concentration-response curves to pentobarbitone were constructed on oocytes expressing α1βyγ2s receptors (where y = 1, 2, or 3) (Figure 4.3). Data (up to the apparent maximum) were fitted with equation (1) (see Chapter 2) and the results shown in Table 4.2. These results, at least for α1βyγ2s (where y = 1, 2, or 3) receptors, suggest that the type of β subunit present has very little influence on the total modulation by pentobarbitone.

Figure 4.3. Concentration-response curves for the total modulation (potentiation and direct activation) of pentobarbitone on α1β1γ2s, α1β2γ2s and α1β3γ2s GABA$_A$ receptors.

Data were normalised to the control GABA EC$_{20}$ response. Since a true maximum was not reached only the data points on the rising portion of the curve were fitted with equation (1) (see Chapter 2). The maximum was not constrained and the resulting parameters, as detailed in Table 4.2, therefore represent an estimate of the EC$_{50}$, Hill coefficient and maximum response.

Table 4.2. EC$_{50}$, Hill coefficient and the maximum response for the total modulation of pentobarbitone on $\alpha1\beta1\gamma2s$, $\alpha1\beta2\gamma2s$ and $\alpha1\beta3\gamma2s$ GABA$_A$ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
$\alpha1\beta1\gamma2s$	34.7 (30.7, 39.2)*	1.4 ± 0.2	308 ± 27	4
$\alpha1\beta2\gamma2s$	25.6 (20.9, 31.4)	1.7 ± 0.3	236 ± 32	6
$\alpha1\beta3\gamma2s$	19.4 (18.9, 19.9)	1.4 ± 0.3	315 ± 30	3

* indicates a significant difference ($P=0.01$) from the corresponding data on $\alpha1\beta3\gamma2s$.

4.2.3. Direct effect of pentobarbitone on receptors containing different α subunits.

The direct effect of pentobarbitone varied according to the α subunit present (Figure 4.1, 4.4 and Table 4.3). *Xenopus* oocytes expressing receptors containing $\alpha6$ subunits produced maximal currents to pentobarbitone alone which were larger than those obtainable with a maximal concentration of GABA (3mM). These currents ranged from 154% of the maximal GABA response on $\alpha6\beta1\gamma2s$ to 168% on $\alpha6\beta2\gamma2s$. The other alpha subunits ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$) only produced maximal currents to pentobarbitone which were smaller than those obtained with 3mM GABA. These maximum responses ranged from 45% of the maximal GABA response on $\alpha5\beta2\gamma2s$ to 83% on $\alpha2\beta2\gamma2s$. Interestingly pentobarbitone produced little direct activation on $\alpha4\beta1\gamma2s$ receptors (6 $\pm$ 2.4% n=4 at 1mM). The EC$_{50}$ for the direct activation by pentobarbitone on $\alpha6$ containing receptors was lower (between 53 and 77 μM) compared to those obtained on receptors containing $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ (between 140 and 540 μM) (Table 4.3). It is interesting to note that the Hill coefficients obtained for the direct action of pentobarbitone were greater than those obtained for the potentiation of GABA, suggesting a greater degree of co-operativity for direct channel activation than potentiation of GABA.

Figure 4.4. Concentration-response curves for the direct effect of pentobarbitone on α1-3,5,6β2γ2s and α1,4,6β1γ2s GABA$_A$ receptors.

A) α1–3,5,6β2γ2s

B) α1,4,6β1γ2s
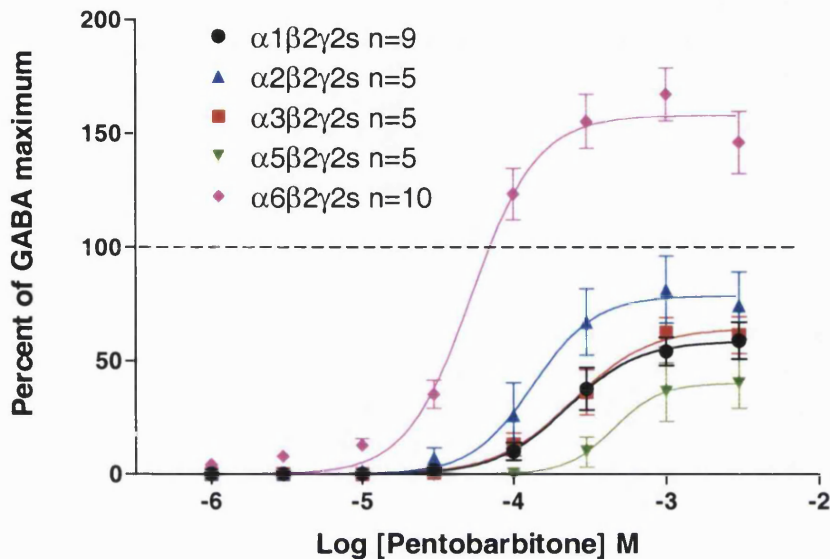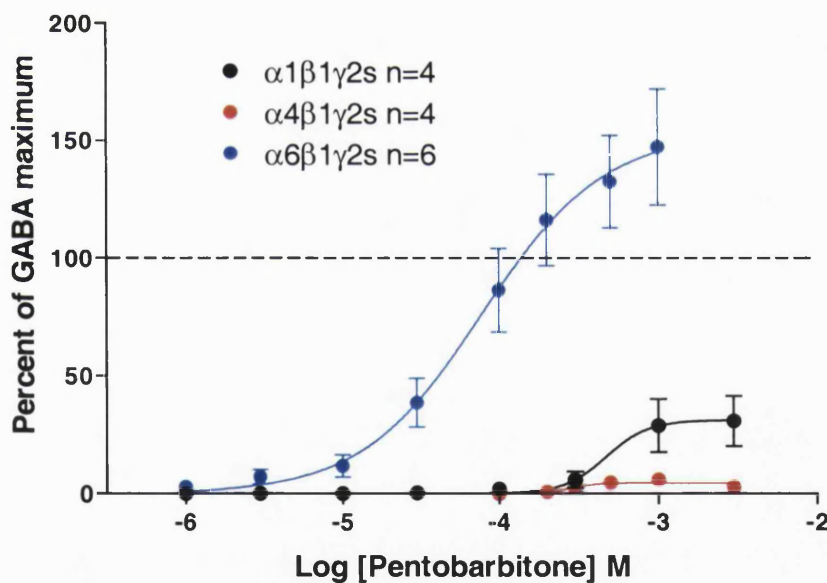
Data were normalised to the maximum GABA response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.3. Note the dashed line represents the maximum GABA response.

Table 4.3. EC$_{50}$, Hill coefficient and maximum response for the direct effect of pentobarbitone on α1-3,5,6β2γ2s and α1,4,6β1γ2s receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β2γ2s	314.1 (251.8, 391.7)	2.4 ± 0.5	65.6 ± 6.9	9
α2β2γ2s	138.7 (108.1, 177.8)	2.5 ± 0.5	82.6 ± 14.8	5
α3β2γ2s	262.4 (197.2, 349.1)	2.2 ± 0.2	67.0 ± 5.2	5
α5β2γ2s	528.4 (430.5, 648.6)	3.6 ± 0.2	45.2 ± 15.8	5
α6β2γ2s	57.8 (51.4, 65.0)	2.0 ± 0.3	168.2 ± 11.3	10
α1β1γ2s	539.5 (476.4, 610.9)	3.3 ± 0.3	33.3 ± 11.8	4
α4β1γ2s	n.d.	n.d	6 ± 2.4 @ 1mM	4
α6β1γ2s	77.3 (53.6, 111.4)	1.4 ± 0.2	154.0 ± 25.4	6

n.d. = not determined due to the small magnitude of the current induced by pentobarbitone

4.2.4. Direct effect of pentobarbitone on receptors containing different β subunits.

The contribution of the β subunit to the direct effect of pentobarbitone was examined by co-expressing different β subunits (β1, β2 or β3) with α6 and γ2s or α1 and γ2s (Figure 4.5 and Table 4.4). The type of β subunit present did not influence the direct action of pentobarbitone to the same extent as the α subunit. There were no significant differences (One-way ANOVA) between the apparent affinity (log EC$_{50}$) or efficacy (maximum response) on oocytes expressing human α6β1γ2s, α6β2γ2s and α6β3γ2s GABA$_A$ receptors. The EC$_{50}$ values of pentobarbitone on α1β1γ2s, α1β2γ2s and α1β3γ2s (540, 314 and 189μM respectively) were all significantly different (P<0.05 Student's t-test), whereas only the efficacy on α1β1γ2s (33%) was significantly different from that of α1β2γ2s (66%) and α1β3γ2s (75%). It appears that the type of β subunit present does affect affinity and efficacy obtained with

pentobarbitone, but when an α6 subunit is present it is this subunit which determines the response to pentobarbitone. These results suggest that the binding site for the direct action of pentobarbitone is influenced by, or even made up of, determinants from both the α and β subunit.

Figure 4.5. Concentration-response curves for the direct effect of pentobarbitone on α1β1-3γ2s and α6β1-3γ2s GABA$_A$ receptors.
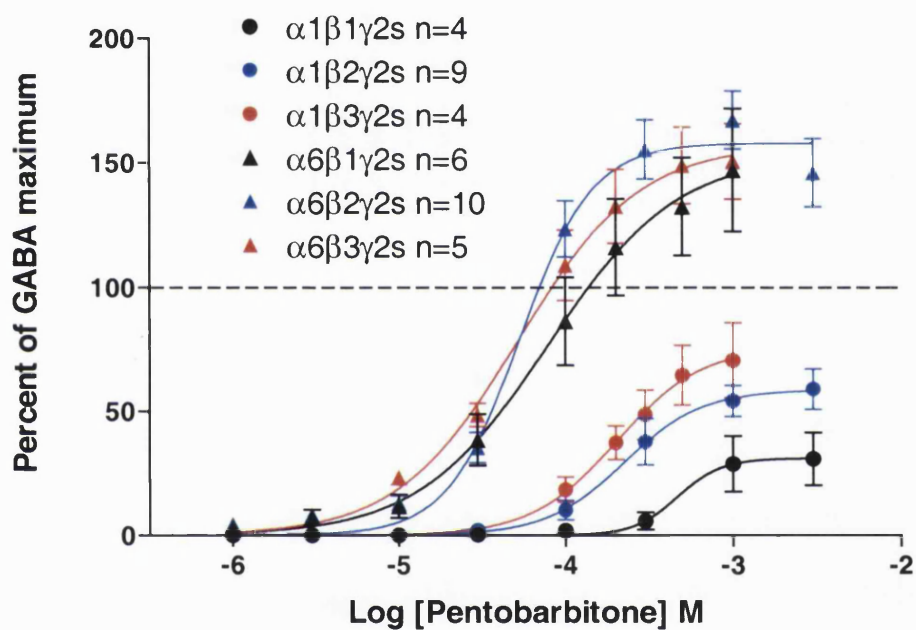
Data were normalised to the maximum GABA response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.4. Note the dashed line represents the maximum GABA response.

Table 4.4. EC$_{50}$, Hill coefficient and maximum response for the direct effect of pentobarbitone on α1β1-3γ2s and α6β1-3γ2s receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β1γ2s	539.5 (476.4, 610.9)	3.3 ± 0.3	33.3 ± 11.8	4
α1β2γ2s	314.1 (251.8, 391.7)	2.4 ± 0.5	65.6 ± 6.9	9
α1β3γ2s	189.2 (165.2, 216.8)	1.7 ± 0.1	75.3 ± 15.7	4
α6β1γ2s	77.3 (53.6, 111.4)	1.4 ± 0.2	154.0 ± 25.4	6
α6β2γ2s	57.8 (51.4, 65.0)	2.0 ± 0.3	168.2 ± 11.3	10
α6β3γ2s	52.8 (42.1, 66.4)	1.4 ± 0.1	159.0 ± 15.5	5

4.2.5. Influence of the δ subunit.

Co-expression of the δ subunit with α6 and β3 in *Xenopus* oocytes proved to be extremely difficult. The hit rate (defined by a usable inward current (>40nA) to a maximum GABA concentration, 3mM) for oocytes injected with α6β3δ was very low (<10%) compared to >80% for oocytes injected with α6β3 and α6β3γ2s. The time taken to detect any responses to GABA from oocytes injected with α6β3δ cDNAs was generally 3-5 days after injection. In addition, the current amplitude to a maximum concentration of GABA (generally 3mM) for α6β3δ was significantly lower ($P<0.001$) than for α6β3γ2s (124 ± 23nA n=23 compared with 766 ± 88nA n=22). This reduction in current amplitude to a maximum concentration of GABA has previously been reported with α1β1δ receptors when compared to α1β1γ2l receptors (Saxena & Macdonald, 1994), suggesting that substitution of a γ subunit with a δ subunit causes a reduction in receptor number or that αβδ hereomers have a lower probability of channel opening. A recent study supporting the second hypothesis has shown that the reduced current amplitude with δ containing receptors is as a consequence of lower open channel probability (Haas & Macdonald, 1999). Similarly

oocytes injected with $\alpha6\beta3$ also revealed small maximal currents to GABA (168 ± 72nA n=10) which were not significantly different ($P=0.57$) from $\alpha6\beta3\delta$.

Verification that $\alpha6\beta3\delta$ receptors were being expressed was required before the effect of pentobarbitone could be examined. GABA concentration-response curves were constructed on oocytes injected with $\alpha6\beta3$, $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ subunits however, statistical analysis (t-test) of the GABA log EC$_{50}$ values and Hill coefficients revealed no significant differences (Figure 4.6 and Table 4.5). An interesting observation and one that was noted in Chapter 3 for $\alpha1\beta1\epsilon$ receptors was that the Hill coefficients for GABA on $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ were less than 1. Most native and recombinant GABA$_A$ receptors have been reported to have Hill coefficients of between 1.2 –1.8. The GABA binding site occurs between the interface of an α and β subunit which with the generally accepted stoichiometry of 2xα subunits, 2xβ subunits and 1xγ subunit could give rise to two GABA binding sites per receptor. A Hill coefficient of less than one would be produced if there was only one binding site for GABA i.e. an altered stoichiometry to the one proposed.

Inhibition of a GABA EC$_{50}$ by Zn^{2+} ions however provided definitive evidence of expression of the δ subunit. As has previously been reported (Draguhn et al., 1990; Smart et al., 1991 and Chapter 3), Zn^{2+} ions are substantially more potent on $\alpha\beta$ heteromers than on $\alpha\beta\gamma$ subunits. Receptors composed of $\alpha6\beta3\delta$ subunits produced a Zn^{2+} sensitivity between $\alpha6\beta3$ and $\alpha6\beta3\gamma2s$ providing evidence of expression of the δ subunit (Figure 4.7 and Table 4.6). The maximum inhibition by Zn^{2+} on $\alpha6\beta3$ receptors in contrast to $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ receptors did not reach 100% suggesting an allosteric interaction. Based on the above results, all oocytes injected with $\alpha6\beta3\delta$ cDNA were tested with $1\mu M$ ZnCl$_2$ to confirm expression. Any cell whose GABA EC$_{50}$ response was inhibited by more than 25% in the presence of $1\mu M$ ZnCl$_2$ was rejected.

Figure 4.6. Concentration-response curves to GABA for α6β3, α6β3γ2s and α6β3δ receptors.
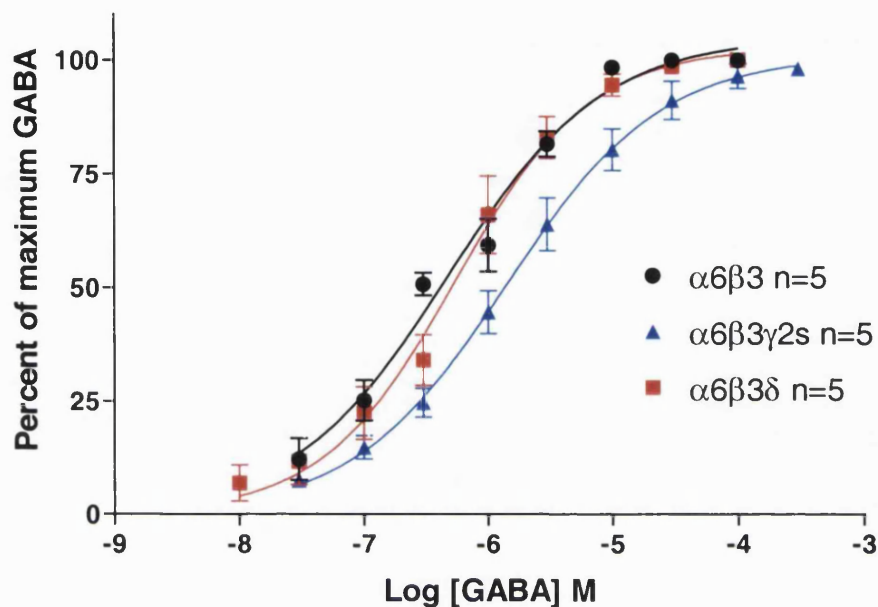
Data were normalised to the maximum GABA response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.5.

Table 4.5. EC$_{50}$ and Hill coefficients for GABA concentration-response curves on α6β3, α6β3γ2s and α6β3δ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient
α6β3	0.56 (0.43, 0.73)	1.00 ± 0.19
α6β3γ2s	1.50 (1.05, 2.13)	0.73 ± 0.06
α6β3δ	0.58 (0.42, 0.78)	0.89 ± 0.08

Figure 4.7. Concentration-response curves to Zn^{2+} for $\alpha6\beta3$, $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ receptors.
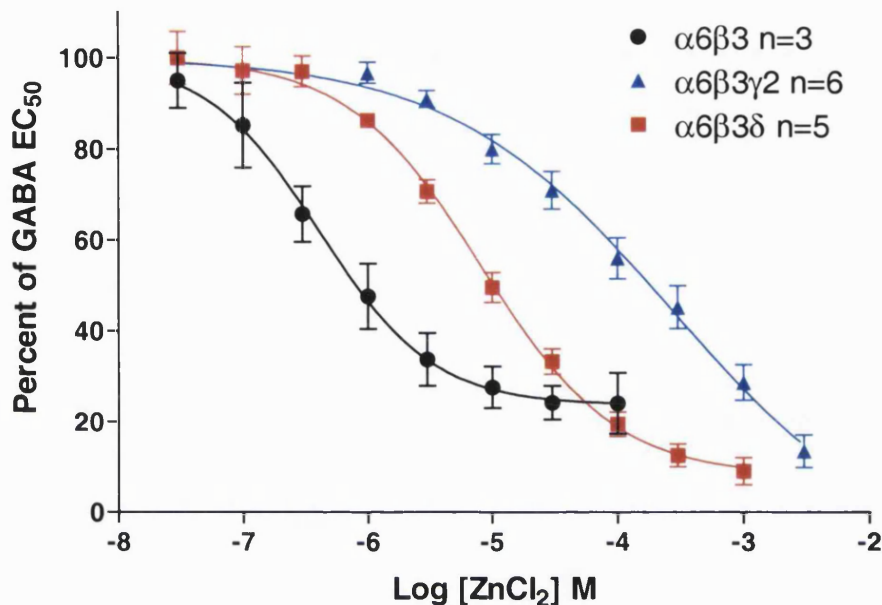
Data were normalised to the control GABA EC_{50} response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in Table 4.6.

Table 4.6. IC_{50}, Hill coefficients and maximum inhibition for concentration-response curves to Zn^{2+} for $\alpha6\beta3$, $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ receptors.

Receptor	IC_{50} (μM)	Hill Coefficient	Maximum Inhibition (%)	No. of Oocytes
$\alpha6\beta3$	0.42 (0.34, 0.52)	-1.06 ± 0.14	76 ± 4.2	3
$\alpha6\beta3\gamma2s$	143 (85, 238)	-0.61 ± 0.05	98 ± 5.7	6
$\alpha6\beta3\delta$	8.63 (7.65, 9.75)	-0.87 ± 0.05	91 ± 2.5	5

The direct effect of pentobarbitone was examined on receptors composed of $\alpha6\beta3$, $\alpha6\beta3\gamma2s$ and $\alpha6\beta3\delta$ (Figure 4.8 and Table 4.7). All three receptor subunit combinations produced a maximum pentobarbitone response greater than the maximum GABA response. Direct activation by pentobarbitone on $\alpha6\beta3\gamma2s$ and

α6β3δ receptors revealed similar EC$_{50}$ values and Hill coefficients, however the maximum response as a percentage of the maximum GABA response was significantly greater ($P<0.01$) on α6β3δ (324 ± 36 n=6) compared to α6β3γ2s receptors (159 ± 16% n=5). Interestingly the EC$_{50}$ values, maximum response and Hill coefficients for the direct effect of pentobarbitone on α6β3 heteromers were significantly greater ($P<0.05$) than those of α6β3γ2s and α6β3δ receptors. This trend for pentobarbitone to elicit a greater efficacy value on αβ receptors compared to αβγ receptors was also apparent with α1β1 and α1β1γ2s receptors (see Chapter 3 section 3.2.7). Higher Hill coefficient values were observed for the direct activation by pentobarbitone of α6β3, α6β3γ2s and α6β3δ receptors compared to those of GABA suggesting that pentobarbitone has a greater degree of cooperativity than GABA.

Figure 4.8. Concentration-response curves to the direct effect of pentobarbitone on α6β3, α6β3γ2s and α6β3δ receptors.
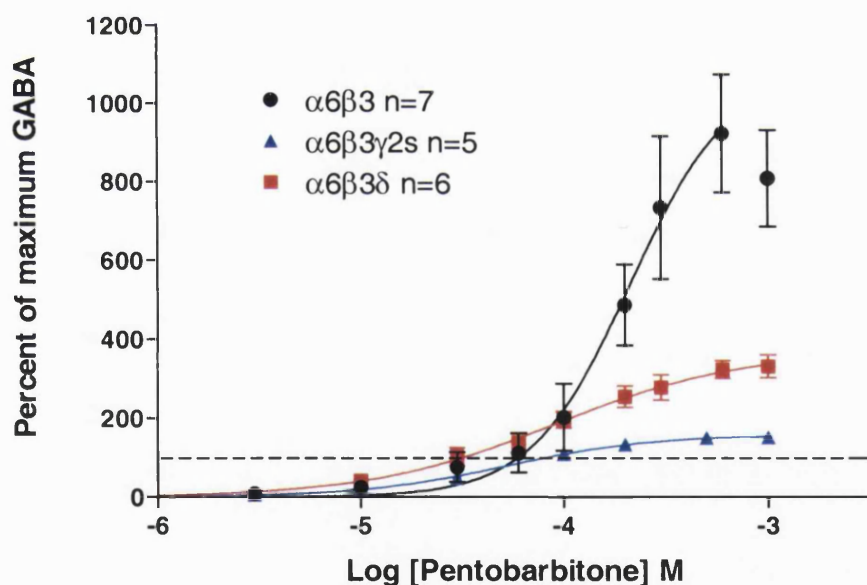
Data were normalised to the maximum GABA response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.7. Note the dashed line represents the maximum GABA response. For α6β3 receptors curves were fitted up to 300μM with the maximum not constrained.

Table 4.7. EC$_{50}$, Hill coefficient and maximum response for the direct effect of pentobarbitone on α6β3, α6β3γ2s and α6β3δ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α6β3	153 (120, 194)	2.55 ± 0.44	955 ± 146	7
α6β3γ2s	52.8 (42.1, 66.4)	1.37 ± 0.11	159 ± 16	5
α6β3δ	61.9 (47.3, 81.3)	1.36 ± 0.25	324 ± 36	6

4.2.6. Effect of picrotoxin, bicuculline and SR95531 on GABA and pentobarbitone.

As expected currents to GABA (3μM) could be completely inhibited by the competitive antagonists, bicuculline (100μM) and SR95531 (1μM), and the non-competitive antagonist, picrotoxin (100μM) in oocytes expressing α6β2γ2s receptors. Direct activation of the receptor by pentobarbitone however, was antagonised by picrotoxin (100μM) but not by bicuculline or SR95531 (Figure 4.9). Small outward currents to bicuculline, SR95531 and picrotoxin were observed which could be due to a proportion of constitutively-active channels or to the incomplete washout of GABA.

Bicuculline, at concentrations of 100μM and 1mM, produced concentration dependent parallel shifts to the right of the GABA concentration-response curve on oocytes expressing α6β3γ2s receptors (13 and 244-fold shifts respectively), suggesting this to be a classical competitive antagonist at the α6β3γ2s receptor subtype. Very small rightward shifts in the concentration-response curves to the direct action of pentobarbitone were observed with bicuculline. These shifts (1.57 and 1.43-fold with 100μM and 1mM bicuculline respectively), although statistically significant, were not concentration related (Figure 4.10 and Table 4.8).

Figure 4.9. Effects of GABA$_A$ receptor antagonists on currents elicited by GABA and pentobarbitone on human α6β2γ2s receptors.
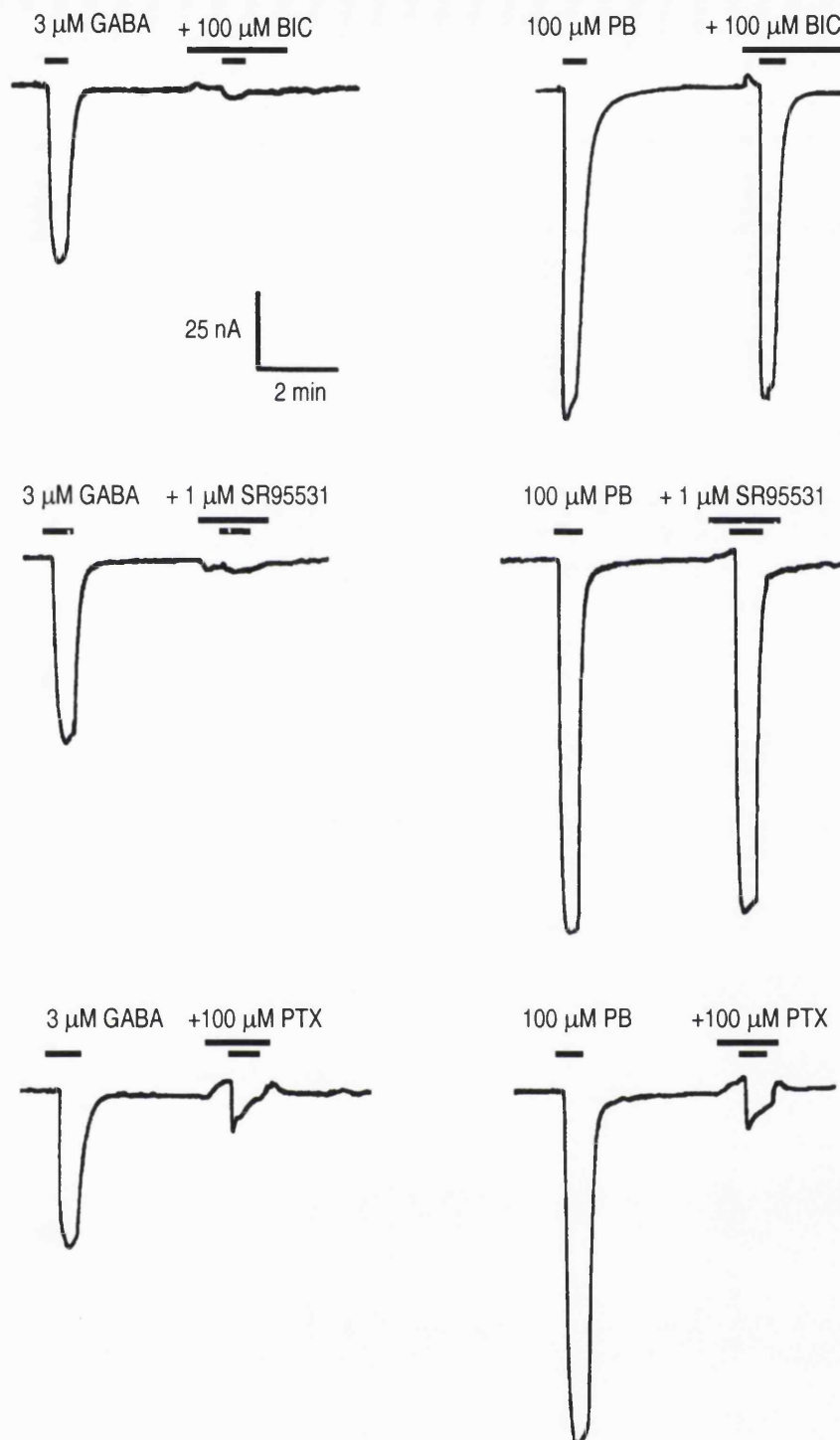
Cells were voltage-clamped at -70mV and exposed to agonists and antagonists as illustrated by the bars above each response. PB = pentobarbitone, BIC = bicuculline, PTX = picrotoxin.

Figure 4.10. Concentration-response curves for GABA and pentobarbitone in the absence and presence of 100μM and 1mM bicuculline on α6β3γ2s receptors.

A) GABA
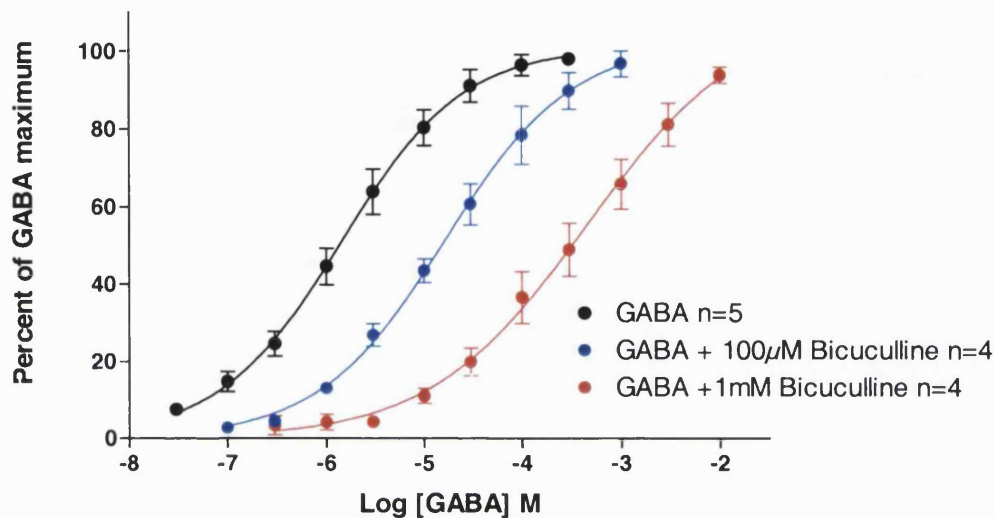
B) Pentobarbitone
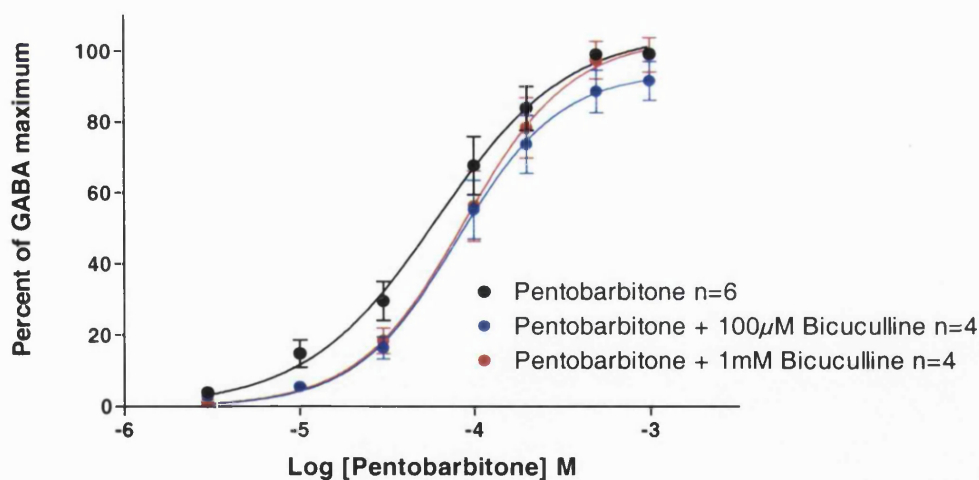
Control GABA concentration-response curves and those in the presence of bicuculline were constructed in the same oocyte, however, for some oocytes only one concentration of antagonist was tested. Data were normalised to the GABA maximum response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.8.

Table 4.8. EC$_{50}$ and Hill coefficient values for GABA and pentobarbitone concentration-response curves in the absence and presence of bicuculline on α6β3γ2s receptors.

	EC$_{50}$ (μM)	Hill Coefficient	No. of Oocytes
GABA (control)	1.5 (1.05, 2.13)	0.73 ± 0.06	5
GABA + 100μM Bicuculline	17.6 (13.3, 23.3)	0.68 ± 0.05	4
GABA + 1mM Bicuculline	361.4 (252.3, 517.6)	0.64 ± 0.07	4

	EC$_{50}$ (μM)	Hill Coefficient	No. of Oocytes
PB (control)	59.8 (47.2, 75.9)	1.42 ± 0.06	6
PB + 100μM Bicuculline	83.9 (71.9, 97.9)	1.51 ± 0.06	4
PB + 1mM Bicuculline	89.3 (70.1, 113.8)	1.56 ± 0.08	4

4.2.7. Effect of propofol on α1β1γ2s, α4β1γ2s and α6β1γ2s receptors.

For α1β1γ2s, α4β1γ2s and α6β1γ2s receptors the effect of an additional anaesthetic, propofol, was examined. 10μM propofol potentiated a GABA EC$_{20}$ response to similar levels on both α1β1γ2s and α4β1γ2s receptors (214 ± 9 % n=4 and 214 ± 32% n=8 respectively). In contrast to pentobarbitone, the total modulation by propofol on α6β1γ2s receptors (120 ± 22% n=5) was significantly smaller (P<0.05) than that observed for α1β1γ2s and α4β1γ2s. For all three receptor subtypes the total modulatory effect was predominantly due to potentiation, since 10μM propofol caused little or no direct activation.

The profile for direct activation of α1β1γ2s, α4β1γ2s and α6β1γ2s receptors by propofol was similar to that of pentobarbitone with direct activation being greater at α6β1γ2s, lower at α1β1γ2s and negligible on α4β1γ2s, up to concentrations of 1mM (Figure 4.11 and Table 4.9). Unlike pentobarbitone, the direct effect of propofol on α6β1γ2s was not greater than the maximum current to GABA.

Figure 4.11. Concentration-response curves for the direct effect of propofol on α1,4,6β1γ2s GABA$_A$ receptors.
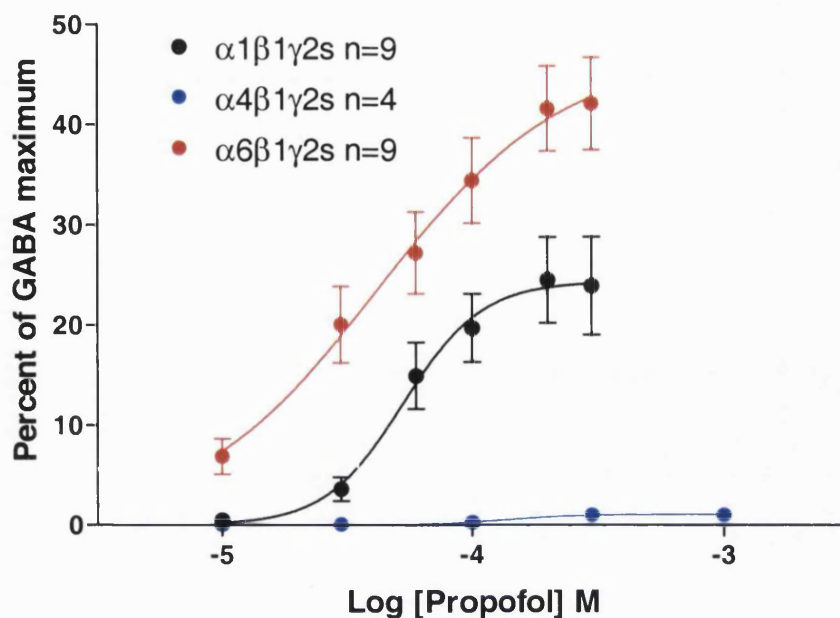
Data were normalised to the maximum GABA response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in Table 4.9.

Table 4.9. EC$_{50}$, Hill coefficient and maximum response for the direct effect of propofol on α1,4,6β1γ2s receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β1γ2s	54.6 (47.9, 62.2)	4.0 ± 0.4	24.7 ± 4.8	9
α4β1γ2s	n.d	n.d	1 ± 0.7	4
α6β1γ2s	46.5 (37.3, 57.8)	1.4 ± 0.2	49.2 ± 5.1	9

n.d. = not determined due to the small magnitude of the current induced by propofol.

4.3. Discussion.

The results in this chapter represent the first detailed study of the effects of pentobarbitone on a large range of recombinant human GABA$_A$ receptor subtypes. Similar to native GABA$_A$ receptors, pentobarbitone has three actions on recombinant GABA$_A$ receptors dependent on increasing concentration, initially a potentiation of the GABA induced current, followed by a direct activation of the receptor chloride channel, and at millimolar concentrations, a blockade of the GABA-induced current.

4.3.1. Total modulation.

The estimated EC$_{50}$ for total modulation of GABA-induced currents by pentobarbitone was found to be between 19-43μM on all the receptor combinations tested (α1–3,5,6β2γ2s and α1β1–3γ2s (this chapter) and α1β1 and α1β1ϵ (chapter 3)). Although concentration-response curves for the total modulatory effect of pentobarbitone were not constructed on α4 or δ containing receptors, a recent report by Brown *et al.* (2001) revealed EC$_{50}$ values for the total modulation of 23 and 30μM on α4β3γ2s and α4β3δ receptors respectively. These results indicate that the apparent EC$_{50}$ value for the total modulatory action of pentobarbitone is not greatly influenced by the GABA$_A$ receptor subtype. The maximum degree of total modulation, however, was dependent on the type of α subunit present within the $\alpha\beta\gamma$2s ternary complex, with α4 and α6 containing receptors eliciting the largest responses. In contrast, the type of β subunit within the α1$\beta\gamma$2s ternary complex did not influence the degree of total modulation. This is similar to a previous publication (Hadingham *et al.*, 1993) which reported no differences in the modulation of a single concentration of pentobarbitone on α1β1–3γ2s receptors. The modulatory effect of propofol was only examined at a single concentration (10μM) and only on a limited number of subunits (α1β1γ2s, α4β1γ2s and α6β1γ2s (this chapter) and α1β1 and α1β1ϵ (chapter 3)). Similar to pentobarbitone the modulation by propofol was influenced by the receptor subtype, however selectivity with this anaesthetic was for α1 and α4 containing receptors.

A subsequent study by Krasowski *et al.* (1997) has examined the effects of propofol, trichloroethanol and the barbiturate, methohexital on $\alpha 1\beta 3\gamma 2s$ and $\alpha 6\beta 3\gamma 2s$ receptors. Similar to the results for pentobarbitone, the EC$_{50}$ for GABA potentiation by propofol and trichloroethanol was not influenced by receptor subtype (data for methohexital not reported), whereas the maximum potentiation for all three anaesthetic agents was greater on $\alpha 1\beta 3\gamma 2s$ receptors than $\alpha 6\beta 3\gamma 2s$. The selectivity for the maximum potentiation by 10μM propofol being greater on $\alpha 1\beta 3\gamma 2s$ than on $\alpha 6\beta 3\gamma 2s$ agrees with the data presented in this chapter, however, the selectivity for the barbiturate, methohexital appears to be different to that shown here with pentobarbitone.

4.3.2. Direct activation.

The most marked differences between receptor subtypes were found in the direct activation of the receptors by pentobarbitone and propofol. On $\alpha 6\beta x\gamma 2$ (where x is 1,2 or 3) receptors, direct activation by pentobarbitone occurred at lower concentrations and with a much greater efficacy than on receptors containing other α subunits. In fact, the direct activation by pentobarbitone on $\alpha 6$ containing receptors exceeded that of a maximum concentration of GABA. Antagonism of receptor activation by pentobarbitone on human $\alpha 6\beta 3\gamma 2s$ GABA$_A$ receptors by picrotoxin confirmed that this action was via direct opening of GABA$_A$ receptor chloride channels. Like pentobarbitone, direct activation by propofol revealed a higher efficacy on $\alpha 6\beta 1\gamma 2s$ receptors compared to $\alpha 1\beta 1\gamma 2s$ receptors, however, unlike pentobarbitone there were no differences in potency. When expressed relative to the maximum GABA response, pentobarbitone was significantly more efficacious than propofol on $\alpha 6\beta 1\gamma 2s$ receptors. If the site for direct activation by these two compounds is identical, then propofol may be acting as a partial agonist and if co-applied with pentobarbitone on $\alpha 6\beta 1\gamma 2s$ receptors would reduce the maximum response observed. Interestingly the $\alpha 4$ subunit, which has greatest sequence homology with the $\alpha 6$ subunit, when co-expressed with $\beta 1\gamma 2s$ showed only modest direct activation to both pentobarbitone and propofol (1mM = 6% and 1% of the GABA maximum respectively). Reduced efficacy for the GABA mimetic effect of pentobarbitone and propofol on $\alpha 4\beta 1\gamma 2s$ compared to $\alpha 6\beta 1\gamma 2s$, is akin to that observed with the GABA site partial agonists, THIP and P4S (Wafford *et al.*, 1996). One hypothesis to explain these differences

may be that $\alpha 4\beta 1\gamma 2s$ receptors have a lower probability for channel opening than $\alpha 6\beta 1\gamma 2s$ receptors, however this theory needs to be tested using single channel recording.

4.3.3. Pentobarbitone does not interact at the GABA binding site.

Evidence for distinct binding sites for GABA and pentobarbitone was provided by mutagenesis studies that identified residues within the extracellular N-terminal domain of the β subunit, that when mutated had reduced GABA EC$_{50}$ values (up to 900-fold) with no change in their sensitivity for direct activation by pentobarbitone (Amin & Weiss, 1993).

In this study the direct activation by pentobarbitone was not inhibited by a single concentration of the competitive GABA recognition site antagonists, bicuculline (100μM) or SR95531 (1μM). This result is contrary to Nicoll & Wojtowicz (1980), Akaike et al. (1987), Peters et al. (1988), Robertson (1989) and Rho et al. (1996) who report blockade of the direct effect of pentobarbitone by bicuculline. These authors used mammalian or frog neuronal preparations (sensory or motor), or isolated chromaffin cells compared to the recombinant $\alpha 6\beta 3\gamma 2s$ receptors used in this study. In the present study, concentration-response curves to pentobarbitone in the absence and presence of 100μM and 1mM bicuculline were shifted to the right by 1.57 and 1.43-fold respectively. This inhibition by bicuculline was more apparent at low concentrations of pentobarbitone and, unlike GABA antagonism, was not increased with higher concentrations of bicuculline. Trace amounts of contaminating GABA, due to incomplete washout, might account for this effect, as blockade of the GABA site would produce an apparent greater block at low pentobarbitone concentrations due to its potentiation of the GABA response, and such a small effect would be unaffected by increasing bicuculline concentrations. Recently bicuculline and gabazine (SR95531) have been shown to behave as allosteric inhibitors of the GABA$_A$ receptor, in addition to behaving as competitive antagonists (Ueno et al., 1997; Thompson et al., 1999b and Chapter 7). Differences in the degree of allosteric inhibition by bicuculline on recombinant human $\alpha 6\beta 3\gamma 2s$ receptors and the neuronal preparations listed above, which do not contain $\alpha 6$ subunits, could account for the

differing degrees of inhibition of the pentobarbitone response by bicuculline. Alternatively the neuronal cultures may have been contaminated with GABA, which in the absence of bicuculline would potentiate the pentobarbitone response, but in the presence would produce apparent antagonism.

Krishek & Smart (1995) reported pA$_2$ values for the competitive antagonism of GABA by bicuculline of 5.87, 5.96 and 5.99 on murine $\alpha 1\beta 1$, $\alpha 1\beta 1\gamma 2s$ and $\alpha 1\beta 1\gamma 2l$ GABA$_A$ receptors respectively. Our experiments indicate that bicuculline is weaker on human $\alpha 6\beta 3\gamma 2s$ GABA$_A$ receptors with an estimated pA$_2$ of 5.07. A recent report (Ebert *et al.*, 1997) has demonstrated that bicuculline has a lower binding affinity for $\alpha 6\beta 3\gamma 2s$ receptors compared to $\alpha 1\beta 3\gamma 2s$, $\alpha 2\beta 3\gamma 2s$, $\alpha 3\beta 3\gamma 2s$ and $\alpha 5\beta 3\gamma 2s$ receptors which correlates with the reduced pA$_2$ value inferred from this study.

4.3.4. Comparison with previous reports.

Most reported electrophysiological studies with pentobarbitone have been performed on cell types such as mouse spinal cord neurones (Schulz & Macdonald, 1981) or hippocampal neurones (Zimmerman *et al.*, 1994), which under normal conditions, do not express the $\alpha 6$ subunit (Laurie *et al.*, 1992; Wisden *et al.*, 1992;). These studies generally report some small degree of direct activation of neurones with concentrations of pentobarbitone over $100\mu M$ (Schulz & Macdonald, 1981; Peters *et al.*, 1988; Robertson, 1989), which would be consistent with receptors containing other α-subunits. Since the $\alpha 6$ subunit is located primarily on cerebellar granule cells (Baude *et al.*, 1992; Hadingham *et al.*, 1996), it would be interesting to study the effect of pentobarbitone on these neurones.

Previous results using recombinant GABA$_A$ receptors have demonstrated both potentiation and direct activation of receptors by pentobarbitone (Sigel *et al.*, 1990). Unlike benzodiazepine modulation but similar to a number of other modulators, e.g. other anaesthetics, tracazolate, the $\gamma 2$ subunit is not required for potentiation or direct activation by pentobarbitone or propofol (Davies *et al.*, 1997b; Thompson *et al.*, 2001 and Chapter 3). A number of studies have shown that β homomeric receptors are

activated by general anaesthetic agents, indicating that the binding site or part of the binding site for these agents can be comprised from residues of the β subunit.

4.3.5. Do pentobarbitone and propofol bind at the same sites?

A number of authors have concluded that two separate sites exist for the potentiation and direct activation by pentobarbitone and propofol (Adodra & Hales, 1995; Sanna *et al.*, 1995a; Rho *et al.*, 1996; Uchida & Yang 1997; Dalziel *et al.*, 1999b). Ligand binding studies have shown that pentobarbitone and propofol produce similar effects suggesting a common mechanism of action (Davies *et al.*, 1998). The opposite functional profiles, seen with potentiation of GABA by pentobarbitone and propofol presented within this chapter, suggest that propofol and pentobarbitone interact differently, either by binding to a different pocket or effecting different processes downstream in the allosteric pathway. The similarity of the profile for direct activation may indicate a common mechanism for these compounds agonist-like behaviour.

A recent report by Fukami and colleagues (1999) has shown that mutation of tyrosine 157 to phenylalanine in the β2 subunit reduced the apparent affinity for direct activation of propofol (3-fold) but had no effect on the direct activation of pentobarbitone and etomidate or the modulatory effects of all three general anaesthetic agents. These authors suggest that these results indicate that the site for direct activation by propofol is different from pentobarbitone and etomidate. Additional mutagenesis studies have delineated a number of residues within the transmembrane domains that influence the functional responses to anaesthetic agents (Belelli *et al.*, 1997; Dalziel *et al.*, 1999b; Carlson *et al.*, 2000), however, whether these amino acids form part of the binding site(s) for these agents or whether they play a role in the transduction / gating mechanism remains to be determined. Further mutagenesis studies, structural biological techniques and the development of radiolabeled ligands are required to specifically identify the residues that form the binding sites for general anaesthetic agents.

4.3.6. Washout phenomenon.

Many groups (Akaike *et al.*, 1987; Peters, 1988; Robertson, 1989; Orser *et al.*, 1994; Adodra & Hales, 1995) have reported on the washout phenomenon observed with high concentrations of pentobarbitone and propofol. This effect, which has been termed "bounce", "surge" "rebound" or "hump", involves a marked transient increase in current during the washout period of high concentrations of pentobarbitone. We observed this same phenomena at concentrations of 1 mM and 3 mM pentobarbitone and 300μM propofol on all the subunit combinations tested. Bounce occurred in both the absence or presence of a GABA EC$_{20}$ concentration. We saw, as did Peters (1988), that this inward current was often of a greater amplitude than the initial response to pentobarbitone. A recent study, using β3 homomeric receptors expressed in HEK cells, has kinetically modelled the rebound phenomenon (Wooltorton *et al.*, 1997b). The model predicts that the homomeric β3 receptor is modulated/activated initially by pentobarbitone binding followed by further apparent silent binding which causes the complex to enter one or more inactivate states. Dissociation of pentobarbitone during washout in effect allows the receptor complex to re-enter the activated state i.e. increases the probability of channel activation, and hence gives rise to the appearance of a rebound current. Evidence suggests that for pentobarbitone and propofol, the inactive state is caused by an allosteric inhibition of channel gating (Adodra & Hales, 1995).

4.3.7. Relevance to *in vivo* anaesthetic concentration.

The estimated free aqueous concentration (EC$_{50}$) of pentobarbitone for general anaesthesia is approximately 50 μM (Franks & Lieb, 1994), whereas for propofol it ranges from 0.4-1.1μM (Franks & Lieb, 1994; Pistis *et al.*, 1997). There are, however, caveats associated with the measured plasma concentrations since we do not know how this relates to brain concentrations or what degree of receptor occupancy is required for anaesthesia. From the results in this study, we can see that the main effect of pentobarbitone at 50μM on GABA$_A$ receptors containing α1, α2, α3, α4 and α5 subunits and of propofol on all the subunits tested would be potentiation of the GABA response with little or no direct activation. On GABA$_A$ receptors containing α6

subunits, however, pentobarbitone would exert both a direct action and potentiation of the GABA response. On all receptor combinations tested, blockade of the receptor only occurred at extremely high concentrations (1 mM and above), which would be of little relevance at clinically active doses.

4.3.8. Are GABA$_A$ receptor subtypes are involved in anaesthesia?

A recent report using an α6 knockout mouse has demonstrated no difference in the sleep time of wild type animals and the knock-out animals in response to pentobarbitone, indicating that α6 containing GABA$_A$ receptors are not required to mediate the sedative/hypnotic effects of pentobarbitone (Homanics et al., 1997). Likewise a δ knockout mouse, when compared to wild type animals, revealed no differences in the sleep times induced by pentobarbitone or propofol (Mihalek et al., 1999). However, removal of the β3 subunit gene produced mice with some resistance to the immobilising actions of intravenous and volatile anaesthetics, providing good evidence for the involvement of GABA potentiation in general anaesthesia (Quinlan et al., 1998).

It is currently unknown if general anaesthesia is mediated via an interaction with a specific receptor subtype (GABA or otherwise) or a more general interaction with a number of different receptor subtypes. The lack of selectivity with regard to the EC$_{50}$ value for modulation of a GABA EC$_{20}$ response by pentobarbitone, suggests an overall potentiation of the inhibitory inputs within the brain. However, two recent positron emission tomographic studies in humans using the intravenous anaesthetic propofol (Fiset et al., 1999) and the volatile anaesthetics, halothane and isoflurane, (Alkire et al., 2000) have shown that anaesthetic agents induce behavioural changes via an interaction with specific brain regions primarily the thalamus.

A number of water-soluble analogues of propofol have recently been synthesised which retain anaesthetic activity, however, unlike the parent compound, do not inhibit [^{35}S]TBPS binding to rat whole brain membranes, suggesting that their in vivo anaesthetic activity is mediated by a non-GABAergic mechanism (Cooke et al.,

2001). The development of additional transgenic animals will help us to understand the involvement of GABA receptor subtypes in anaesthesia.

4.4.   Summary.

This study has investigated the total modulation and direct activation of GABA$_A$ receptors by the general anaesthetic agents pentobarbitone and propofol on a range of recombinant GABA$_A$ receptors. The EC$_{50}$ for total modulation by pentobarbitone of the GABA$_A$ receptor was not dependent on receptor subtype, whereas the maximum response depended on the α subunit present within the receptor, with $\alpha 6$ containing receptors having greater efficacy than $\alpha 1$ containing receptors. The efficacy and apparent affinity (EC$_{50}$) for direct activation by pentobarbitone was influenced by the nature of the α and β subunits present within the receptor, with $\alpha 6$ containing receptors having a significantly higher affinity and efficacy (greater than a maximum GABA response) than any other receptor combination.

Clear differences in the total modulation and direct activation of GABA$_A$ receptors were observed with propofol compared to pentobarbitone. Firstly, there was no difference in the EC$_{50}$ for direct activation between $\alpha 1\beta 1\gamma 2$s and $\alpha 6\beta 1\gamma 2$s and secondly, the maximum efficacy of propofol on $\alpha 6\beta 1\gamma 2$s did not exceed that of a maximum GABA response. Finally, unlike pentobarbitone the degree of total modulation by 10μM propofol on $\alpha 1\beta 1\gamma 2$s and $\alpha 4\beta 1\gamma 2$s was greater than $\alpha 6\beta 1\gamma 2$s receptors.

Chapter 5

Residues in Transmembrane Domains I and II Determine GABA$_A$ Receptor Subtype Selective Antagonism by Furosemide

5.1.   Introduction.

The effects of the diuretic compounds furosemide, amiloride and α-human atrial natriuretic polypeptide (α-hANP) were first examined on GABA-gated chloride currents in 1988 by Inomata *et al.* Using frog isolated sensory neurones perfused with Na^+, K^+ and Ca^{2+} free solutions, this group showed that both furosemide and amiloride inhibited the GABA-activated chloride current. Against an approximate GABA EC_{50} concentration, furosemide and amiloride revealed IC_{50}'s of $8 \times 10^{-4}M$ and $3 \times 10^{-4}M$ respectively while α-hANP had no effect on the GABA-activated current. This group also showed that the blockade of the GABA response with amiloride and furosemide was not voltage-dependent. In addition $3 \times 10^{-4}M$ amiloride caused a parallel shift to the right of the GABA concentration-response curve with no effect on the maximum, whereas furosemide decreased the maximum GABA current with little or no shift in the GABA concentration-response curve. The experiments described above were performed at the time when molecular cloning of GABA$_A$ subunit cDNA's was in its infancy. Currently there are no reports that characterise the subunits present in frog dorsal root ganglions (DRG). However, *in situ* hybridization and reverse transcription-polymerase chain reaction on adult rat DRG showed the presence of mRNA encoding α1, α2, β2, β3 and γ2 subunits (Persohn *et al.*, 1991; Ma *et al.*, 1993), whereas a functional study demonstrated that rat DRG neurons mimic the pharmacological profile of recombinant α3β3γ2s receptors (Wafford *et al.*, 1995).

More recently Korpi *et al.* (1995) showed that furosemide behaves as a subtype selective non-competitive antagonist at GABA$_A$ receptors. Using *Xenopus* oocytes expressing rat GABA$_A$ receptors, they demonstrated that furosemide was approximately 300-fold more potent on α6β2γ2 than on α1β2γ2 receptors. They also showed that [^{35}S]TBPS binding was enhanced by furosemide in the presence of $1\mu M$ GABA for HEK-293 cells expressing α6β2/3γ2 receptors, but not for α6β1γ2 receptors. This study clearly demonstrated that furosemide was selective for α6 over α1 and β2/3 over β1.

The aim of this study was to examine the effect of furosemide on the remaining α subunits and to identify the amino acids critical for the α6 and β2/3 selectivity.

5.2. Results.

Perfusion of 3mM furosemide alone for 2 minutes did not alter the current required to hold the oocyte at −70mV indicating no acute effects on the endogenous *Xenopus* oocyte Na$^+$ - K$^+$ - 2Cl$^-$ co-transporter (Shetlar *et al.*, 1990).

5.2.1. Effect of furosemide on the α-subunits.

Similar to the results of Korpi *et al.* (1995) furosemide displayed a greater sensitivity for inhibiting responses activated by an EC$_{50}$ concentration of GABA on α6β3γ2s receptors (IC$_{50}$ = 12.1μM) compared to α1β3γ2s receptors (IC$_{50}$ = 980μM). Its action on the additional α-subunits revealed that similar to α1β3γ2s receptors furosemide was a weak antagonist on α2β3γ2s, α3β3γ2s and α5β3γ2s receptors. Full inhibition curves could not be constructed due to 3mM furosemide being the limit of solubility. Interestingly α4 containing receptors, whose amino acid sequence is most similar to α6, revealed an intermediate furosemide sensitivity (IC$_{50}$ = 235μM) (Figure 5.1 and Table 5.1).

Figure 5.1. Furosemide inhibition is selective for α6β3γ2s receptors.
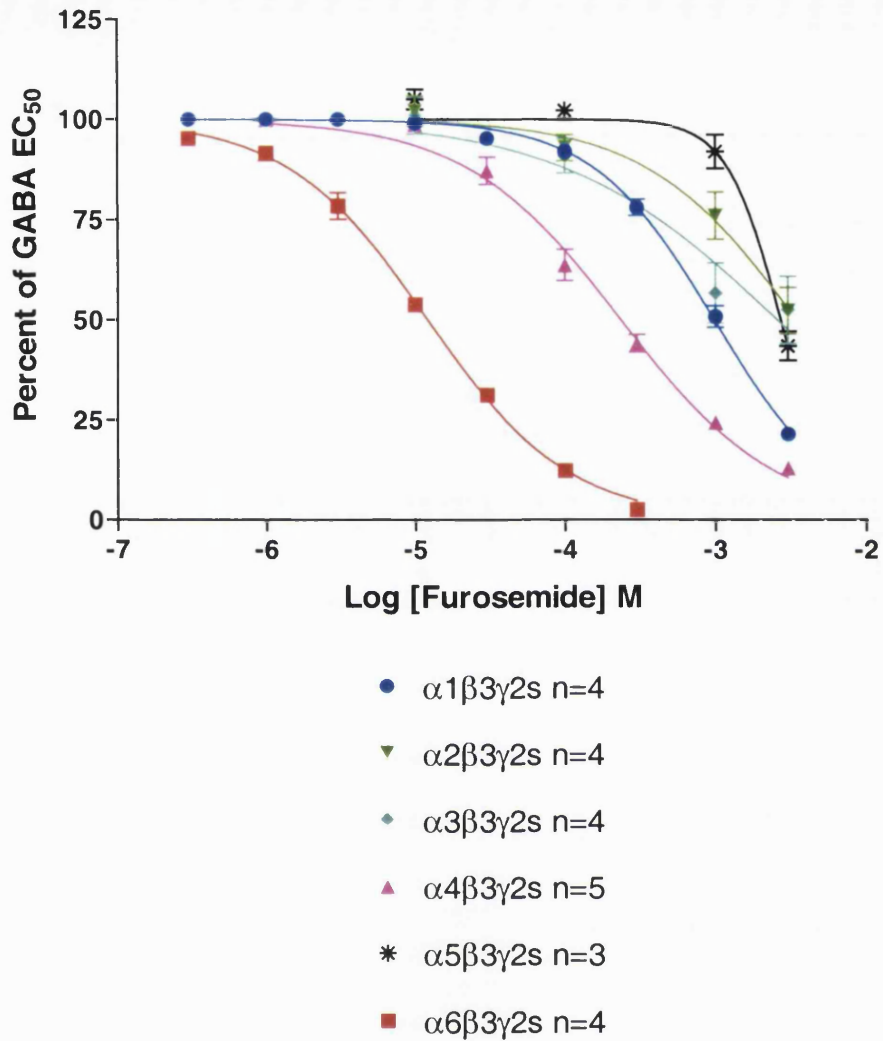
Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing α1–6β3γ2s receptors. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in Table 5.1. Note the marked selectivity of furosemide for α6β3γ2s receptors.

Table 5.1. IC$_{50}$ and Hill coefficient values of furosemide inhibition for α1-6β3γ2s GABA$_A$ receptors.

Receptor	IC$_{50}$ (μM)	Hill Coefficient	No. of Oocytes
α1β3γ2s	980 (890, 1080)	-1.09 ± 0.04	4
α2β3γ2s	~3000	n.d.	4
α3β3γ2s	~3000	n.d.	4
α4β3γ2s	235 (212, 260)	-0.85 ± 0.03	5
α5β3γ2s	~3000	n.d.	3
α6β3γ2s	12.1 (11.4, 12.9)	-0.94 ± 0.05	4

Accurate estimates of IC$_{50}$ or Hill coefficient values for α2β3γ2s, α3β3γ2s and α5β3γ2s could not be determined since there were insufficient data points on the inhibition curve. An approximation has been given for the IC$_{50}$. n.d. = not determined.

5.2.2. Identification of two regions containing residues responsible for the α6 selectivity of furosemide.

It was hypothesised that the residues that conferred selectivity of furosemide for α6β3γ2s receptors would be located either extracellularly or within the transmembrane domains. Five chimeras between α1 and α6 cDNA, encompassing different regions from the N-terminal and up to the TM2-3 loop, were generated (Figure 5.2), and co-expressed with β3 and γ2 subunits in *Xenopus* oocytes. Chimera 1 and chimera 3 both displayed α1-like furosemide sensitivity (C1 IC$_{50}$ = 1.38 (1.32, 1.45)mM and C3 IC$_{50}$ = 0.98 (0.87, 1.10)mM). The furosemide sensitivity for chimera 2 was not significantly different from α6β3γ2s receptors (17.1 (14.9, 19.7)μM, while chimera 4 and 5 displayed intermediate sensitivity (78.6 (55.8, 110.6)μM and 56.3 (37.6, 84.3)μM respectively) (Figure 5.3). These results suggested that there were at least two amino acids responsible for the high furosemide sensitivity of α6 containing receptors, the first being located within a region between amino acids 131 and 160 (region 1), and the second between 209 and 279 (region 2) (Figure 5.4).

Figure 5.2. Schematic diagram of the five α1/α6 chimeras generated for this study.
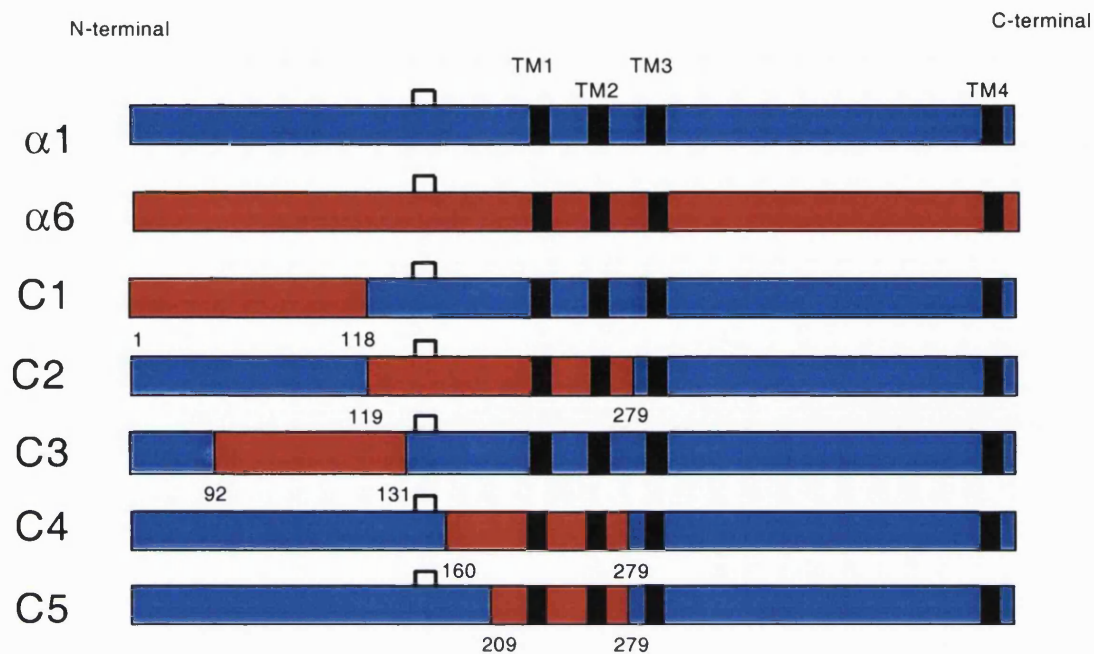
Numbering is according to mature α1 polypeptide and for each chimera the first and last amino acid of the α6 sequence are numbered. The disulfide linked cysteine residues, and transmembrane domains are also illustrated. The red area represents α6 sequence and the blue areas represents α1 sequence.

Figure 5.3. Chimeras between α1 and α6 identify two regions that are responsible for the α6 selectivity of furosemide.
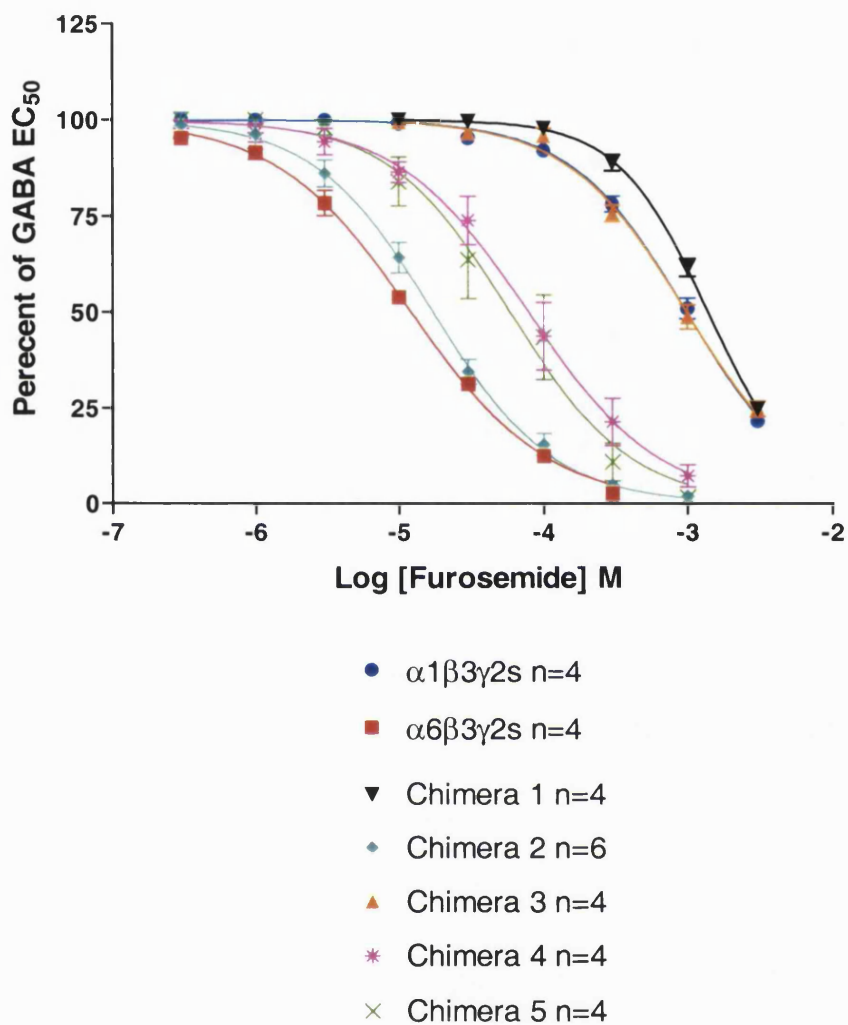
Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing chimera C1-5β3γ2s and wild type α1β3γ2s and α6β3γ2s receptors. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

Figure 5.4. Alignment of human GABA_A α1 and α6 subunits showing the two regions identified from the α1/6 chimeras that contain the amino acids responsible for the high sensitivity of furosemide at α6-containing receptors.


```
α1     1   Q P S L Q D E L K D N T T V F T R I L D R L L D G Y D N R L R P G L G E R V T E V K T D I F V T
α6     1   K . . L E V E G N F Y S E N V S R I L D N L L E G Y D N R L R P G F G G A V T E V K T D I Y V T


49    S F G P V S D H D M E Y T I D V F F R Q S W K D E R L K F K G P M T V L R L N N L M A S K I R T P D T
47    S F G P V S D V E M E Y T M D V F F R Q T W T D E R L K F G G P T E I L S L N N L M V S K I W T P D T


100   F F H N G K K S V A H N M T M P N K L L R I T E D G T L L Y T M R L T V R A E C P M H L E D F P M D A
98    F F R N G K K S I A H N M T T P N K L F R I M Q N G T I L Y T M R L T I N A D C P M R L V N F P M D G


151   H A C P L K F G S Y A Y T R A E V V Y E W T R E P A R S V V V A E D G S R L N Q Y D L L G Q T V D S G
149   H A C P L K F G S Y A Y P K S E I I Y T W K K G P L Y S V E V P E E S S S L L Q Y D L I G Q T V S S E


202   I V Q S S T G E Y V V M T T H F H L K R K I G Y F V I Q T Y L P C I M T V I L S Q V S F W I N R E S V
200   T I K S N T G E Y V I M T V Y F H L Q R K M G Y F M I Q I Y T P C I M T V I L S Q V S F W I N K E S V
                                                                                    TM1

253   P A R T V F G V T T V L T M T T L S I S A R N S L P K V A Y A T A M D W F I A V C Y A F V F S A L I E
251   P A R T V F G I T T V L T M T T L S I S A R H S L P K V S Y A T A M D W F I A V C F A F V F S A L I E
                        TM2                                                              TM3

304   F A T V N Y F T . . . K R G Y A W D G . . . . K S V V . . . . P E K P K K V K D P L I K K N N T Y . .
302   F A A V N Y F T N L Q T Q K A K R K A Q F A A P P T V T I S K A T E P L E . A E I V L H P D S K Y H L


342   A P T A T S Y T P N L A R G D P G L A T I A K S A T I E P K E V K P E T K P P E P K K T F N S V S K I
352   K K R I T S L S L P I V S S S E A N K V L T R A P I L Q S T . . . P V T P P P L P P A F G . G T S K I


393   D R L S R I A F P L L F G I F N L V Y W A T Y L N R E P Q L K A P T P H Q
399   D Q Y S R I L F P V A F A G F N L V Y W V V Y L S K D T M E V S S S V E .
                TM4
```

Numbering is according to mature α1 polypeptide. The figure shows the four putative transmembrane domains underlined in black. The two regions containing the amino acids responsible for the high sensitivity of furosemide at α6-containing receptors are shaded in yellow (131-160) (region 1) and blue (209-279) (region 2). Within both regions amino acid differences between α1 and α6 are boxed.

Within region 1 (131-160) there were 7 amino acid differences between α1 and α6 while region 2 (209-279) had 12. Fisher *et al.* (1997) described a rat α6/α1 chimera with a splice site within TM1 that conferred high furosemide sensitivity, and an α1 point mutation (α1L258T) where furosemide sensitivity was unchanged. These results eliminated 5 of the 12 amino acids identified within region 2. The seven amino acids within region 1 and the remaining seven within region 2 were mutated (in groups of 1,2 or 3) in α1 to the α6 equivalent and the furosemide IC$_{50}$ determined.

5.2.3. Determinants in region 2 that contribute to the α6 selectivity of furosemide.

α1V212I,T215V,H216Yβ3γ2s and α1K220Q,I223Mβ3γ2s receptors both displayed α1-like furosemide sensitivity (IC$_{50}$ = 2.12 (1.78, 2.51)mM and 1.15 (0.94, 1.40)mM respectively). α1V227M,T230Iβ3γ2s receptors however revealed an intermediate sensitivity (IC$_{50}$ = 51.4 (44.6, 59.2)μM) similar to that of chimeras 4 and 5. Individual point mutations produced IC$_{50}$'s of 0.7 (0.63, 0.78)mM for α1V227Mβ3γ2s and 40.9 (34.6, 48.3)μM for α1T230Iβ3γ2s (Figure 5.5 and 5.6). Thus the single point mutation α1T230Iβ3γ2s increased the sensitivity to furosemide by approximately 20-fold. The converse mutation within α6 (α6I228T), when co-expressed with β3γ2s, reduced furosemides sensitivity by 10-fold (IC$_{50}$ = 127.6 (86.3, 188.6)μM) (Figure 5.7). Hence, isoleucine at position 228 in α6 confers a significant proportion of the selective inhibition by furosemide. Interestingly, the other α-subunits have a threonine residue at this position. Mutation of this conserved threonine to isoleucine in α4 produced a 10-fold increase in furosemide sensitivity with an IC$_{50}$ of 22.3 (18.5, 26.9)μM, similar to α6β3γ2s receptors, suggesting that this residue is solely responsible for the difference in sensitivity of furosemide on α6β3γ2s and α4β3γ2s receptors (Figure 5.8).

Figure 5.5. The single point mutant α1T230I co-expressed with β3γ2s increased the sensitivity to furosemide by approximately 20-fold.

- α1β3γ2s n=4
- α6β3γ2s n=4
- α1V227Mβ3γ2s n=4
- α1T230Iβ3γ2s n=4
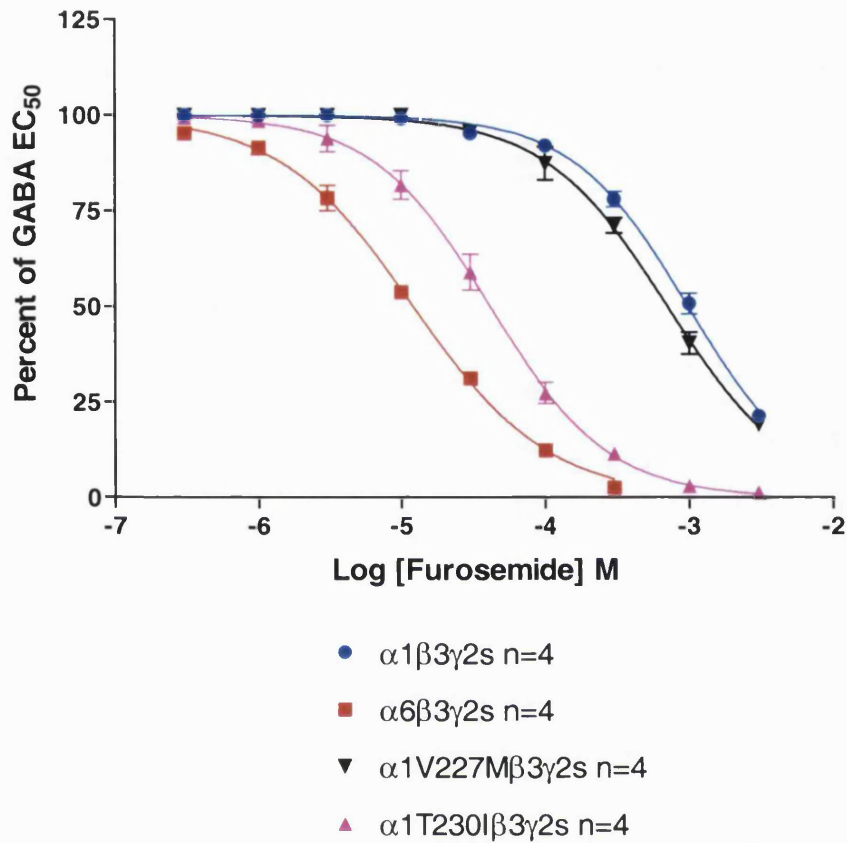
Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing α1β3γ2s, α6β3γ2s and the individual point mutants α1V227Mβ3γ2s and α1T230Iβ3γ2s. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

Figure 5.6. Increased furosemide sensitivity by the single point mutant α1T230I co-expressed with β3γ2s.
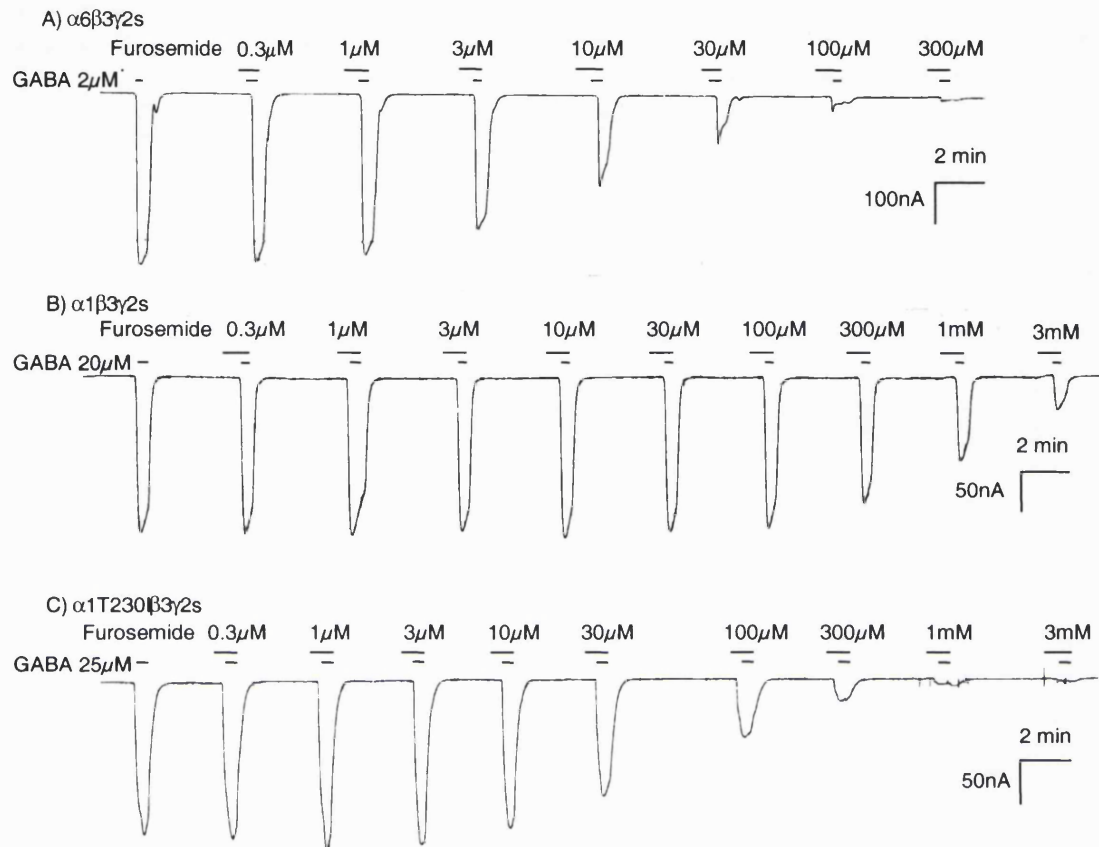
Representative current recordings illustrate the concentration-dependent inhibition by furosemide (0.3μM − 3mM) of the inward current evoked by a EC$_{50}$ concentration of GABA on *Xenopus* oocytes expressing (A) α6β3γ2s, (B) α1β3γ2s and (C) α1T230Iβ3γ2s receptors. Note inhibition of α1T230Iβ3γ2s by furosemide is intermediate of α1β3γ2s and α6β3γ2s. The horizontal bars above the current records indicate drug application.

Figure 5.7. Furosemide inhibition is reduced by replacement of α6 isoleucine 228 with threonine, the α1 counterpart.
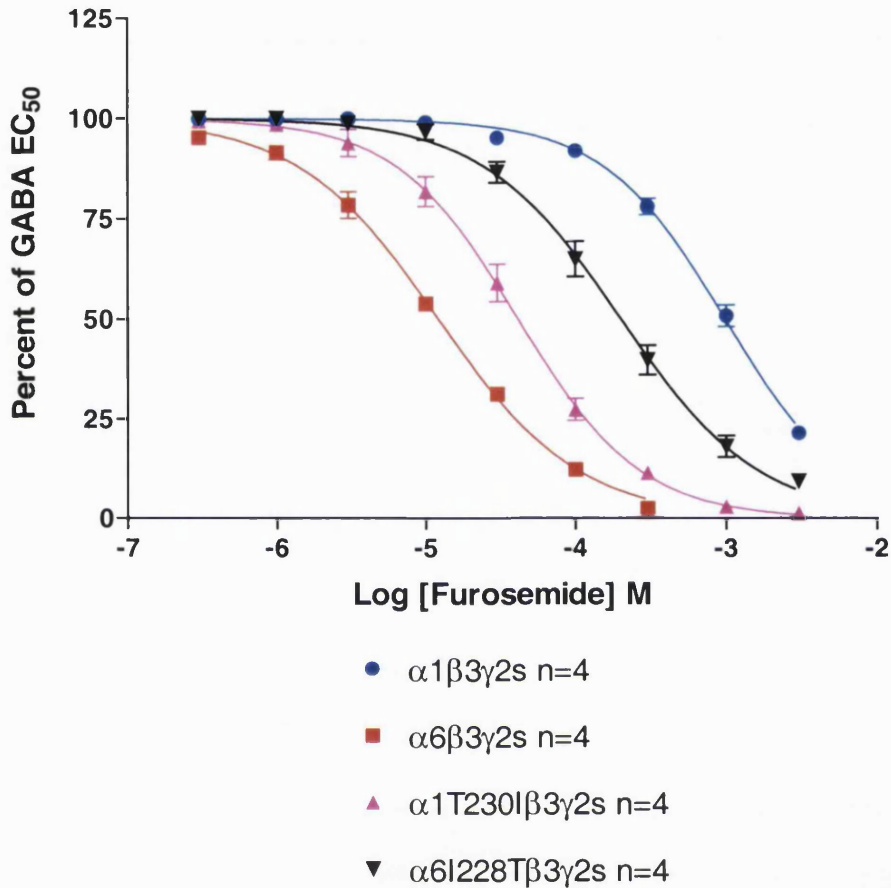
- ● α1β3γ2s n=4
- ■ α6β3γ2s n=4
- ▲ α1T230Iβ3γ2s n=4
- ▼ α6I228Tβ3γ2s n=4

Furosemide concentration-inhibition curves of a GABA EC_{50} response on *Xenopus* oocytes expressing α1β3γ2s, α6β3γ2s and the individual point mutants α1T230Iβ3γ2s and α6I228Tβ3γ2s. Replacement of theronine at position 230 within the α1 subunit with isoleucine, the α6 counterpart increased the sensitivity of furosemide whereas the converse mutation in α6 decreased the sensitivity. Data were normalised to the control GABA EC_{50} response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

Figure 5.8. Replacement of threonine within α4 at position 234 with isoleucine, the α6 counterpart, increased the sensitivity of furosemide
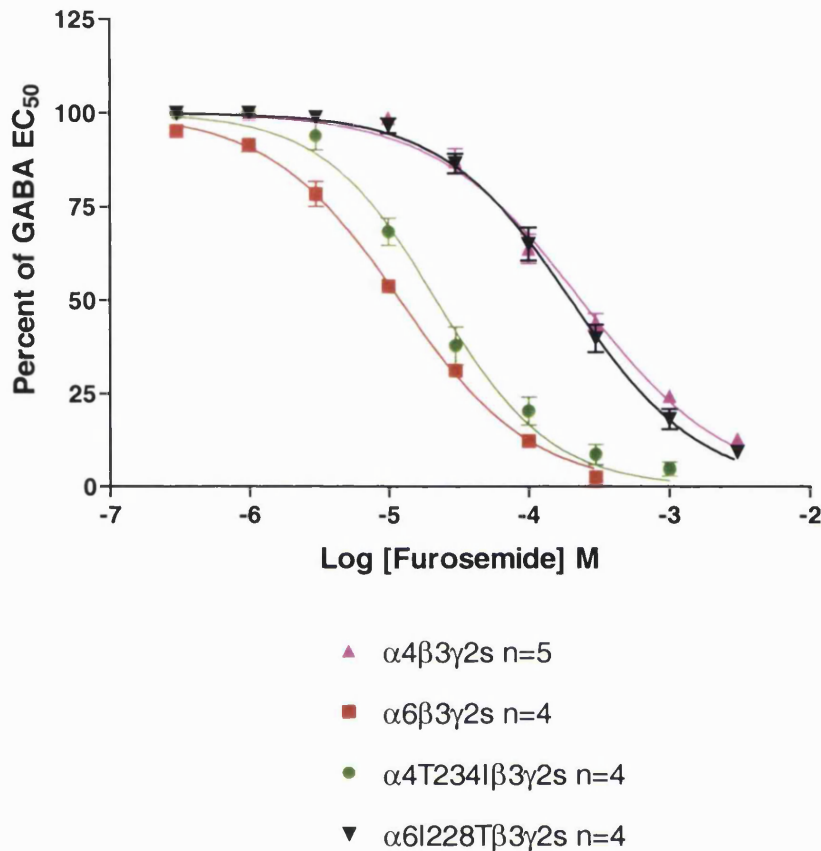
Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing α1β3γ2s, α6β3γ2s and the individual point mutants α4T234Iβ3γ2s and α6I228Tβ3γ2s. Note how the furosemide inhibition curves of these mutations almost superimpose the wild type curves suggesting that this residue is solely responsible for the difference in sensitivity of furosemide on α6β3γ2s and α4β3γ2s receptors. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

5.2.4. Determinants within region 1.

The results with the $\alpha 1/6$ chimeras identified two regions (131-160 and 209-279) within the $\alpha 6$ subunit that were responsible for the high furosemide sensitivity. Within region 2, a single amino acid which when mutated to the $\alpha 6$ equivalent, $\alpha 1T230I$, increased the sensitivity of furosemide by 20-fold. A further 5-fold increase in sensitivity would be required to bring the furosemide IC_{50} to that seen on $\alpha 6\beta 3\gamma 2s$. Based on the results with the $\alpha 1/6$ chimeras, this additional residue should be located between amino acids 131 and 160. Mutation of the non conserved amino acids in the $\alpha 1$ subunit to the $\alpha 6$ equivalent did not change the IC_{50} of furosemide (Table 5.2). In addition, replacement of the whole region (131 to 160) in $\alpha 1$ with the $\alpha 6$ sequence did not affect the furosemide IC_{50} (Table 5.2, chimera 6) and this makes trying to identify the additional determinants very difficult. The additional 5-fold increase in sensitivity may require the presence of I230, which means region 1 must be expanded to between 119 and 160 since chimera 3 is $\alpha 1$ like at position 230. This additional mutation was not constructed in this study.

Table 5.2. Furosemide IC$_{50}$ determinations and Hill coefficient values for the α1 point mutations to the α6 equivalent within the region 1 and chimera 6.

Receptor	IC$_{50}$ (μM)	Hill Coefficient	No. of Oocytes
α1β3γ2s	980 (889, 1080)	-1.09 ± 0.04	4
α6β3γ2s	12.1 (114, 12.9)	-0.94 ± 0.05	4
α1V135Iβ3γ2s	1133 (931, 1378)	-0.86 ± 0.13	3
α1R136Nβ3γ2s	883 (796, 979)	-1.04 ± 0.06	3
α1E138Dβ3γ2s	1791 (1720, 1865)	-0.84 ± 0.04	3
α1H142Rβ3γ2s	820 (762, 882)	-0.79 ± 0.04	3
α1E144Vβ3γ2s	1377 (1324,1433)	-1.09 ± 0.04	3
α1D145Nβ3γ2s	782 (703, 869)	-1.06 ± 0.05	3
α1A150Gβ3γ2s	1179 (1068, 1302)	-0.99 ± 0.08	3
Chimera 6	1520 (1350, 1700)	-0.75 ± 0.02	4

5.2.5. Identification of the determinant responsible for furosemides β2/3 selectivity.

The action of furosemide has also been shown to depend on the β subunit variant, being weaker on β1 containing receptors than on β2 and β3 containing receptors (Korpi *et al.*, 1995). Other GABA modulators which demonstrate the same selectivity are the anticonvulsant, loreclezole (Wafford *et al.*, 1994) and the anaesthetic, etomidate (Hill-Venning *et al.*, 1997). Mutagenesis studies have determined that the β2/3 selectivity for loreclezole and etomidate is dependent on the presence of asparagine 265 in the β2 or β3 subunit (Wingrove *et al.*, 1994; Belelli *et al.*, 1997). Concentration-response curves to furosemide on α6β1γ2 and α6β3γ2 receptors confirmed selectivity for α6β3γ2s over α6β1γ2s (5-fold). Co-expression of the β point mutants, β1S265N and β3N265S, with α6 and γ2s demonstrated that the β subunit selectivity was determined by the same asparagine residue as previously described for loreclezole and etomidate (Figure 5.9). Mutation of the serine within β1

to asparagine (the $\beta3$ counterpart) increased furosemide sensitivity (from an IC$_{50}$ of 66.5 (63.3, 70.0)μM to 12.3 (11.8, 12.9)μM.). Conversely, mutation of the asparagine within $\beta3$ to serine (the $\beta1$ counterpart) decreased furosemide sensitivity (from an IC$_{50}$ of 12.4 (11.4, 12.9)μM to 224 (190, 263)μM). It is interesting to note that mutation within the $\beta1$ subunit revealed an identical IC$_{50}$ as $\alpha6\beta3\gamma2s$ whereas mutation within the $\beta3$ subunit produced a significantly higher IC$_{50}$ than $\alpha6\beta1\gamma2s$.

5.2.6. Lack of involvement of the third subunit.

The γ subunit appeared not to be required for furosemide antagonism, since $\alpha6\beta3$ receptors were also highly sensitive to block by furosemide. In addition, replacement of the $\gamma2s$ subunit with the δ subunit and co-expression with $\alpha1\beta3$ and $\alpha6\beta3$ also had no effect on the inhibition by furosemide (Figure 5.10 and Table 5.3).

Figure 5.9. Selectivity of furosemide for β3 containing receptors over β1 containing receptors is conferred by asparagine 265.
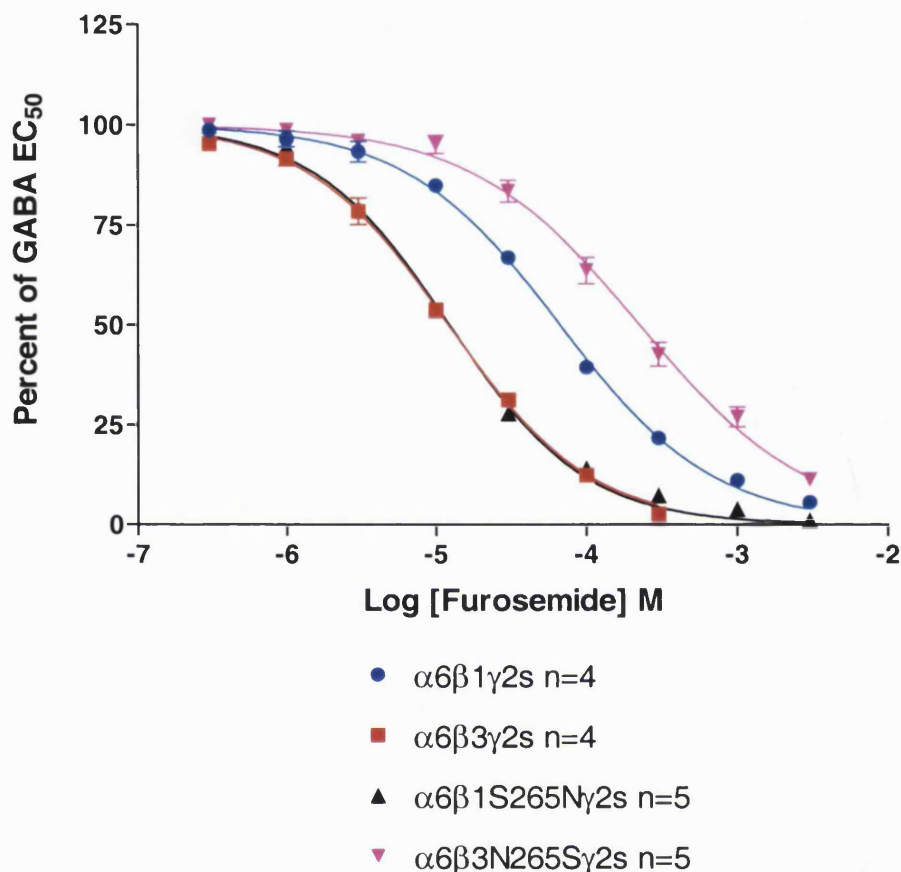
- ● α6β1γ2s n=4
- ■ α6β3γ2s n=4
- ▲ α6β1S265Nγ2s n=5
- ▼ α6β3N265Sγ2s n=5

Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing α6β1γ2s, α6β1S265Nγ2s, α6β3γ2s and α6β3N265Sγ2s receptors. Replacement of serine 265 with asparagine within β1 increased the furosemide sensitivity demonstrating that, similar to loreclezole and etomidate, selectivity for β3 containing receptors over β1 is dependent on the presence of asparagine 265. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

Figure 5.10. The sensitivity of furosemide is not influenced by the nature of the third subunit within the receptor complex.
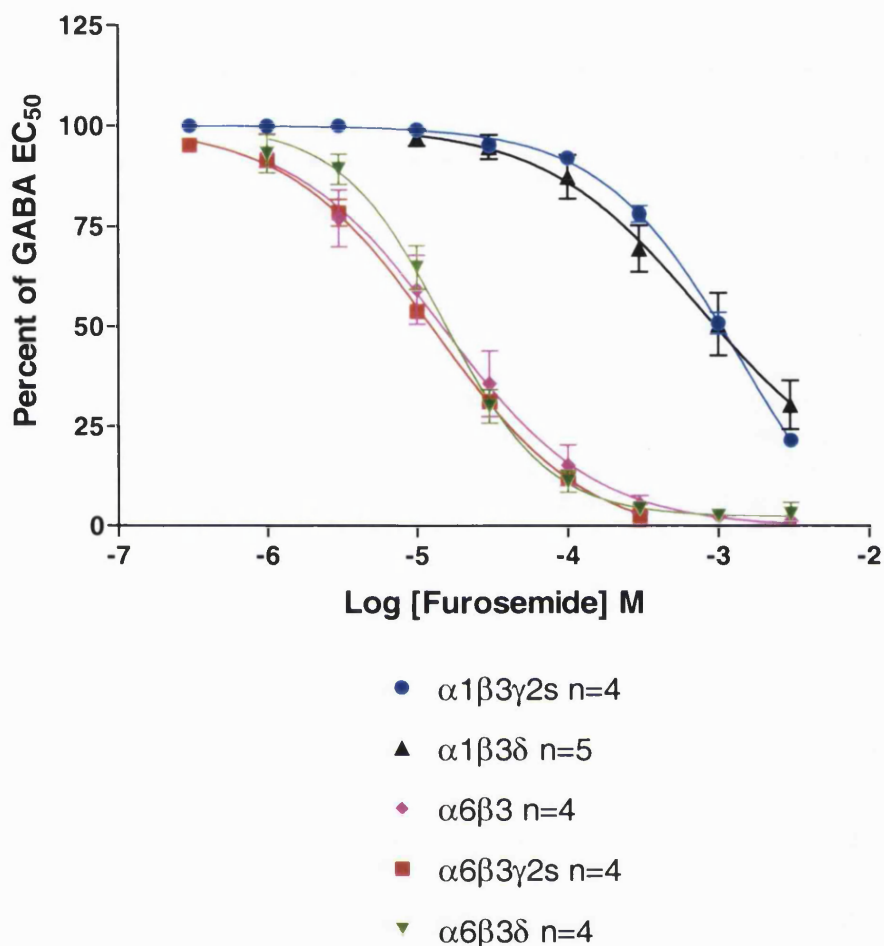
Furosemide concentration-inhibition curves of a GABA EC$_{50}$ response on *Xenopus* oocytes expressing α1β3γ2s, α1β3δ, α6β3, α6β3γ2s and α6β3δ receptors. Removal of the γ2s subunit or replacement with a δ subunit had no effect on the furosemide sensitivity. Data were normalised to the control GABA EC$_{50}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in Table 5.3.

Table 5.3. IC$_{50}$ and Hill coefficient values for furosemide inhibition of α6β3γ2s, α6β3δ, α6β3, α1β3γ2s and α1β3δ GABA$_A$ receptors.

Receptor	IC$_{50}$ (μM)	Hill Coefficient	No. of Oocytes
α6β3γ2s	12.1 (11.4, 12.9)	-0.94 ± 0.05	4
α6β3	14.4 (9.5, 21.9)	-0.94 ± 0.04	4
α6β3δ	15.3 (12.7, 18.4)	-1.32 ± 0.19	4
α1β3γ2s	980 (890, 1080)	-1.09 ± 0.04	4
α1β3δ	867 (542, 1387)	-0.95 ± 0.12	5

5.2.7. Inhibition by furosemide is not use-dependent.

With the identification of residues within transmembrane domains 1 and 2 conferring the inhibitory effects of furosemide on α6β3γ2s, experiments were performed to determine whether the inhibition by furosemide was use-dependent. As shown in Figure 5.11 inhibition of a GABA EC$_{50}$ concentration on α6β3γ2s receptors by 10μM furosemide was not use-dependent compared to that of picrotoxin (1μM) which displayed clear use-dependence on additional application of GABA.

Figure 5.11. Typical recordings showing the inhibition of a GABA EC$_{50}$ response by furosemide and picrotoxin on α6β3γ2s GABA$_A$ receptors.
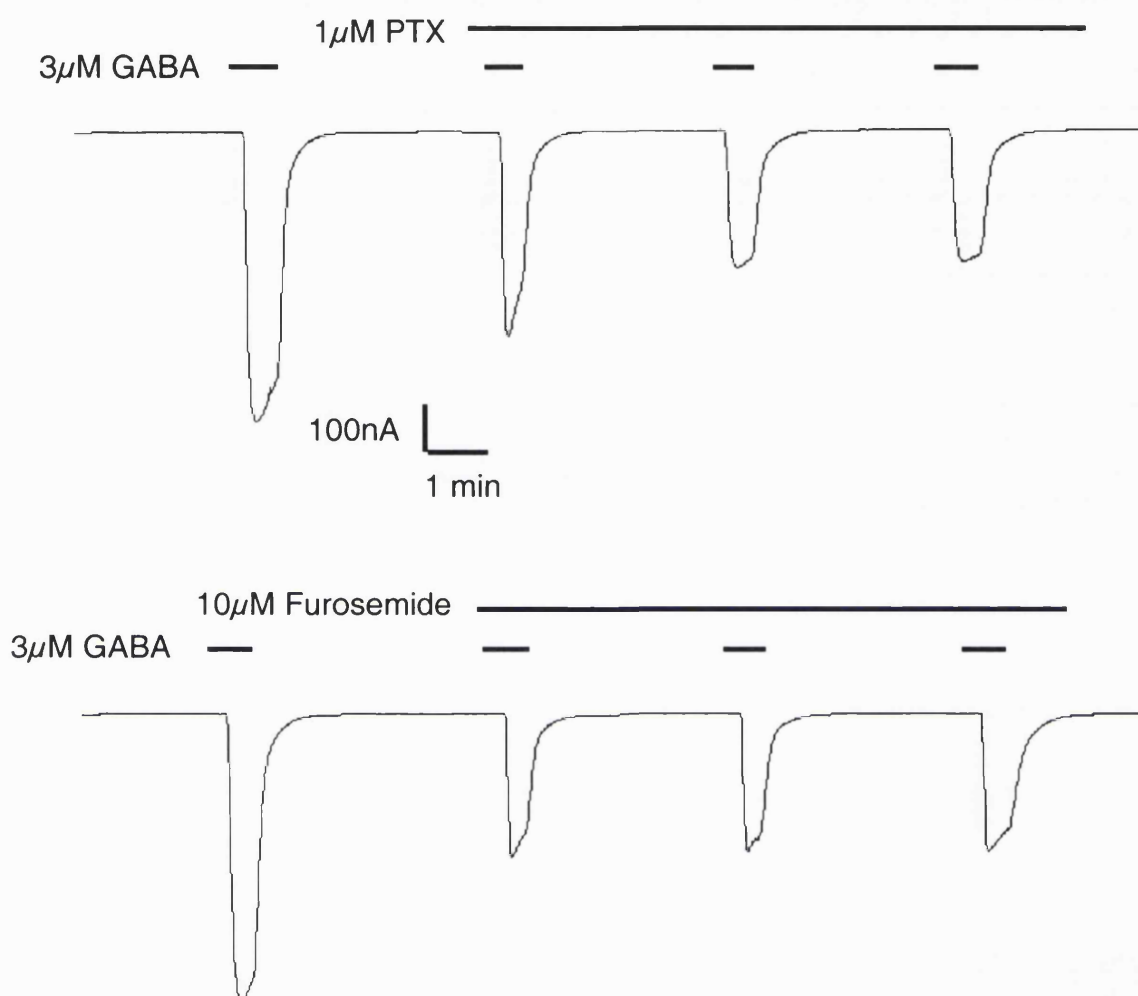
Note the greater degree of inhibition by picrotoxin (PTX) upon a second application of GABA, indicative of use-dependence, compared with the inhibition by furosemide which remained constant during the three GABA applications. The horizontal bars above the current records indicate drug application.

5.2.8. α1T230I does not alter the effects of anaesthetic agents or benzodiazepines.

Experiments were performed to investigate whether the mutation of threonine 230 to isoleucine in α1 influenced the direct activation by pentobarbitone or the potentiation of a GABA EC$_{20}$ by the benzodiazepine, flunitrazepam. Concentration-response curves to pentobarbitone revealed no significant differences in either the log EC$_{50}$ or maximum response as a percent of the maximum GABA response (189μM and 75% for α1β3γ2 compared to 191μM and 66% for α1T230Iβ3γ2s) (Figure 5.12A). Potentiation of a GABA EC$_{20}$ by the benzodiazepine flunitrazepam (1μM) was similarly unaffected by the threonine to isoleucine mutation (104 ± 13% for α1β3γ2s and 90 ± 8% for α1T230Iβ3γ2s) (Figure 5.12B). Hence, while mutation of T230 to I within the α1 subunit significantly increased the sensitivity to furosemide, it did not alter the direct activation of pentobarbitone or the potentiation elicited by flunitrazepam.

Figure 5.12. α1T230Iβ3γ2s has no effect on the direct activation by pentobarbitone or the potentiation by flunitrazepam
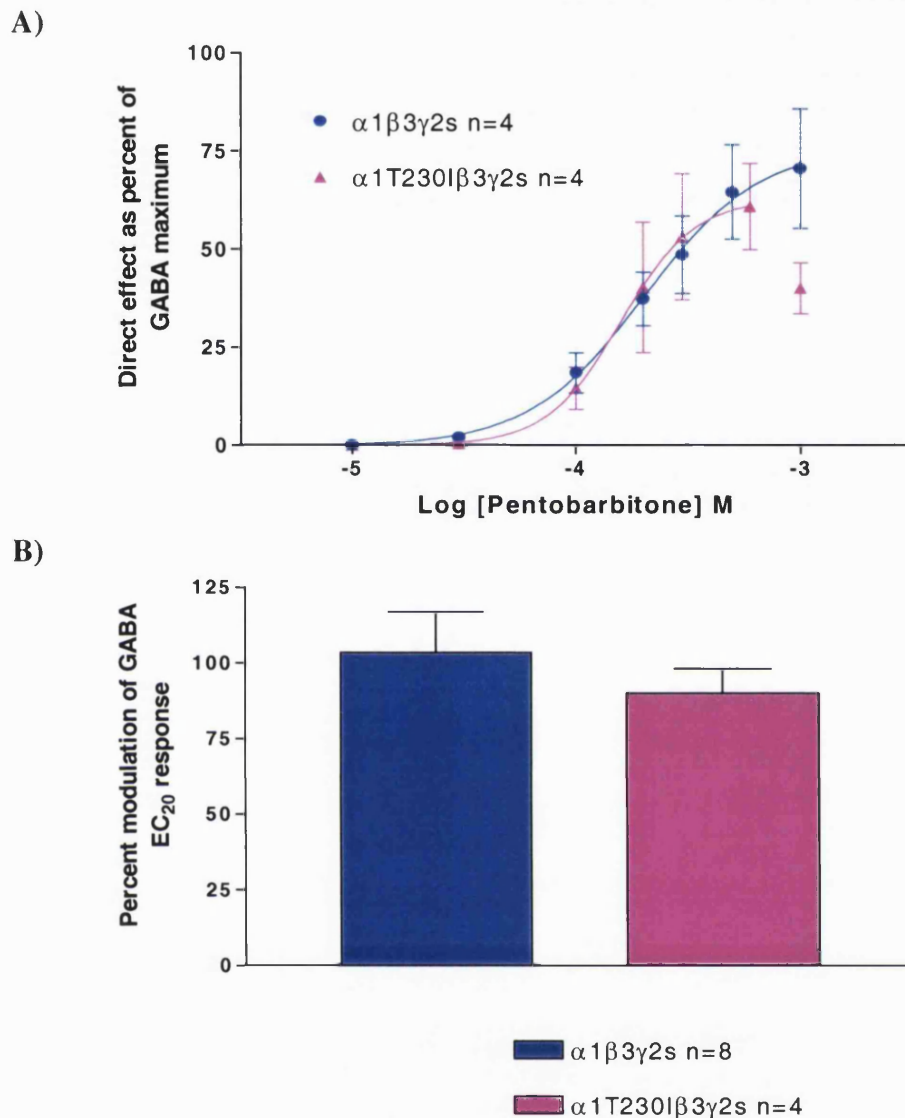
A)

B)

A) Concentration-response curves for the direct activation of pentobarbitone on α1β3γ2s and α1T230Iβ3γ2s GABA$_A$ receptors. Data were normalised to the maximum GABA response (3mM) prior to the pentobarbitone concentration-response curve, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in the text.

B) Potentiation of a GABA EC$_{20}$ response by 1μM flunitrazepam on α1β3γ2s and α1T230Iβ3γ2s GABA$_A$ receptors.

5.3.   Discussion.

This chapter reports on the subtype selectivity of furosemide for GABA$_A$ receptors and the identification of two amino acid residues within transmembrane domains 1 and 2 that confer components of this selectivity.

5.3.1.   α and β subunit selectivity.

The results demonstrate that furosemide is approximately 100 times more potent on α6β3γ2s receptors compared to α1β3γ2s, α2β3γ2s, α3β3γ2s and α5β3γ2s receptors. On α4β3γ2s receptors furosemide revealed an intermediate sensitivity being approximately 20 times less potent on α4β3γ2s than on α6β3γ2s receptors. Of all the α subunits, α6 is closest in sequence homology to the α4. Similar to the anticonvulsant, loreclezole and the anxiolytic, tracazolate (see Chapter 6) furosemide displayed 5-fold selectivity for α6β3γ2s receptors over α6β1γ2s receptors.

5.3.2.   Influence of the third subunit.

Removal of the γ2s subunit, or replacement of the γ2s subunit with a δ subunit, had no effect on the furosemide sensitivity suggesting that the residues involved in the binding of furosemide are formed from the α and β subunits. This result is in contrast to the non-competitive antagonist Zn^{2+} ions which are significantly more potent on αβ heteromers than αβδ or αβγ heteromers (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Thompson *et al.*, 1997 and Chapter 4; Whiting *et al.*, 1997 and Chapter 3).

5.3.3.   Isoleucine 228 within TM1 confers a significant portion of the furosemide selectivity for α6.

The data from the α1/α6 chimeras identified two regions that influenced sensitivity to furosemide. The first region (position 131 to 160) encompasses the cys-cys loop while the second region (position 209 to 279) includes both TM1 and TM2. The first and second regions appeared to be responsible for a 5- and 20-fold increase in furosemide sensitivity respectively. In region 2, this was confirmed since a single amino acid (α6

isoleucine 228) located within TM1 was identified, which, when mutated in α1 to the α6 counterpart (threonine to isoleucine), decreased the IC$_{50}$ for furosemide by 20-fold. However, although all possible point mutations within region 1 were constructed and evaluated in *Xenopus* oocytes, a 5-fold increase in furosemide sensitivity was not observed. The failure to identify a single residue in region 1 may be because the presence of isoleucine at the equivalent position in α1 is required, or more than one residue within this region is involved, or the chimera over this region produced an allosteric effect elsewhere in the receptor. The fact that α4 has a threonine at position 234 but has intermediate furosemide sensitivity may suggest that the amino acid or acids within the first region are conserved in both α4 and α6 but not α1.

After the generation of the chimeras used in this study, a paper appeared by Fisher *et al.* (1997) who demonstrated that the amino acid or acids required for the increased furosemide sensitivity in α6 containing receptors were located between the amino terminus to approximately half way within TM1. A recent paper by Jackel *et al.* (1998), narrowed this region down further to 34 amino acids terminating in the middle of TM1. This group generated four α1/α6 chimeras using two fragments (102bp and 258bp) which were exchanged within rat α1 and α6 to the corresponding counterpart. Equivalent fragments for the human cDNA would run from positions 194 to 279 and from 160 to 193 (numbering according to mature α1 polypeptide). The larger fragment therefore includes the whole of region 2, whereas, the smaller fragment starts at the end of region 1. The larger fragment contains the crucial isoleucine identified in this study, whereas, the smaller fragment represents an area shown to have no effect on furosemides sensitivity. Jackel *et al.* (1998) replaced the large fragment encompassing TM1 and TM2 within the α1 subunit with the corresponding α6 sequence. This chimera produced α6 like furosemide sensitivity. This result is contrary to the intermediate sensitivity expected from the results described in this chapter. The reason for the discrepancies between the data presented here and that of Jackel *et al.* (1998) is unclear at present but could be due to conformational issues with regard to the different lengths of the substitutions.

5.3.4. Asparagine 265 confers furosemide β2/3 selectivity.

Furosemide displays selectivity for β2/3 receptors over β1 receptors and this selectivity is entirely determined by asparagine 265. An increasing number of structurally diverse compounds e.g. etomidate (Belelli *et al.*, 1997), β-carbolines (Stephenson *et al.*, 1995; Thomet *et al.*, 1999), loreclezole (Wafford *et al.*, 1994), mefenamic acid (Halliwell *et al.*, 1999) and tracazolate (Thompson *et al.*, 2001 and Chapter 7) display β2/3 selectivity, which has been shown to be determined by the same asparagine residue suggesting that this residue may play a role in transduction of the effects of allosteric modulators.

5.3.5. Importance of α6 isoleucine 228 and β2/3 asparagine 265.

This study has, therefore, identified two amino acids that influence the sensitivity of furosemide, namely α6 isoleucine 228, which is located near the extracellular end of TM1 and β2/3 asparagine 265, which is located near the extracellular end of TM2. These two amino acids could form part of the yet undefined furosemide binding site, or they could have a role in transduction and/or ion channel gating. The role of the putative membrane spanning TM1 has been investigated in the muscle nicotinic receptor (Akabas & Karlin, 1995) using cysteine substitution experiments. These authors suggest that the top third (N-terminal) of TM1 contributes to the lining of the ion channel, and hypothesise, that in the closed state, TM1 segments intercalate between TM2 at the extracellular end. On receptor activation, movements of TM1 and TM2 could operate a gate, possibly formed by the cytoplasmic loop between them. If the same is true in the homologous GABA$_A$ receptor, by interacting directly with TM1 and TM2, furosemide could stabilise this closed state of the ion channel gate. Inomata *et al.* (1988) showed that furosemide facilitated the inactivation phase of I$_{Cl}$. This facilitation may be attributed to open channel blockade of GABA activated Cl⁻ channel or stabilisation of the closed state of the ion channel. The results presented in this chapter suggest that open channel blockade is unlikely since inhibition by furosemide showed no use-dependence.

Cysteine substitution experiments, using mouse muscle acetylcholine receptor subunit α and β, have shown that Val 229 (homologous to Ile 228 in GABA α6 subunit) is accessible but only when the receptor is in the open state (Zhang & Karlin, 1997; Akabas & Karlin, 1995). Data from SCAM studies for the 15' residue in TM2 (homologous to Asn 265 in GABA β2/3) within the ligand-gated ion channel superfamily is conflicting, with the mouse muscle acetylcholine α subunit reported not to be accessible (Akabas *et al.*, 1994) compared to the β subunit which was reported to be accessible (Zhang & Karlin, 1998). An initial report by Xu and Akabas, (1993) using the GABA α1 subunit showed that Ser 270 was not accessible however more recent data demonstrated accessibility at a GABA EC$_{50}$ concentration but not at a maximal concentration (Williams & Akabas, 1999). At present the accessibility of homologous residues (Ile 228 in α6 and Asn 265 in β2/3) is not known. Further experiments, including cysteine substitution experiments, kinetic studies and single channel recordings are required to enhance our understanding of how Ile 228 in α6 and Asn 265 in β2/3 influences the selectivity of furosemide.

5.3.6. Relevance to clinical use.

Peripherally furosemide acts as a diuretic by inhibiting the reabsorption of sodium and chloride ions in the proximal and distal tubules and the loop of Henle in the kidney. Clinically it is prescribed for the treatment of oedema associated with congestive heart failure, cirrhosis of the liver, renal disease and hypertension. Very few centrally mediated side effects have been reported and coupled with the fact that furosemide is extensively protein bound (91-99%) suggests that furosemide is unable to cross the blood brain barrier.

5.4.   Summary.

Furosemide exhibits approximately 100-fold selectivity for α6-containing receptors over α1-3,5-containing receptors and approximately 5-fold selectivity for β2/3-containing receptors over β1-containing receptors. The generation of α1/α6 chimeras identified a single amino acid within the first transmembrane domain of α1, that when mutated from threonine to isoleucine, the α6 counterpart, increased furosemide

sensitivity by 20-fold. Similar to the anticonvulsant loreclezole and the anxiolytic tracazolate the β2/3 selectivity for furosemide was due to asparagine 265 within the second transmembrane domain. Location of these two residues within transmembrane domains leads to speculation that they may be involved in transduction or gating mechanisms.

Chapter 6

Modulation of GABA$_A$ Receptors by the Pyrazolpyridines: Functional Response is Dependent on the Receptor Subtype

6.1.   Introduction.

Since the introduction of the first benzodiazepine, chlordiazepoxide, in 1960, many laboratories have attempted to identify non-benzodiazepine anxiolytics that are devoid of the side effects of benzodiazepines, which include sedation, myorelaxation, alcohol potentiation, dependence and tolerance. One such chemical class, identified 30 years ago, is the pyrazolopyridines, which include tracazolate, etazolate and cartazolate (Figure 6.1). Encouraging results in behavioural studies led to the development of tracazolate, by ICI Pharmaceuticals, as a novel anxiolytic agent (Patel & Malick, 1982).

Biochemical studies have shown that the pyrazolopyridines enhance [^{3}H]-flunitrazepam, [^{3}H]-diazepam, [^{3}H]-GABA and [^{3}H]-muscimol binding in rat brain (Leeb-Lundberg et al., 1981; Supavilai et al., 1982; Malick et al., 1984). While electrophysiological studies, using cultured rat cortical neurones, demonstrated that etazolate (0.3 to 100μM) prolonged the duration of spontaneously occurring IPSPs (Barnes et al., 1983). Higher concentrations however, produced an increase in membrane chloride conductance, which was blocked by picrotoxin and bicuculline. In addition, changes in conductance to exogenously applied GABA were potentiated upon co-application of etazolate (Barnes et al., 1983). Collectively these results suggest that the pyrazolopyridines interact with the GABA$_A$ receptor at a site other than the benzodiazepine or GABA recognition site.

Behaviourally, tracazolate and etazolate have been shown to possess anxiolytic and anticonvulsant activity by a number of groups in several animal models (Beer et al., 1972; Patel et al., 1985; Young et al., 1987). Compared to the standard benzodiazepine chlordiazepoxide, tracazolate was 2-20 times less potent. The side effect liability of tracazolate was examined and compared with that of chlordiazepoxide. Interestingly a much larger window of separation between the anxiolytic effect and potential side effects (sedation, motor incoordination and its interaction with ethanol and barbital) was observed with tracazolate (Patel et al., 1985).

Structurally the pyrazolopyridines are similar to the classical adenosine antagonists theophylline and caffeine (Figure 6.1). It is not surprising therefore that these compounds bind to adenosine receptors with relatively high affinities (K_i at A$_1$ and A$_{2A}$ receptors, 0.46~3.4μM) and functionally behave as antagonists (Murphy & Snyder, 1981; Williams *et al.*, 1981; Psychoyos *et al.*, 1982; Shi *et al.*, 1997). In addition etazolate has been shown to inhibit brain cyclic AMP phosphodiesterases with an IC$_{50}$ of 2μM (Beer *et al.*, 1972).

Figure 6.1. Chemical structure of etazolate, tracazolate, caffeine, theophylline and chlordiazepoxide.

Etazolate

Caffeine

Tracazolate

Theophylline

Chlordiazepoxide
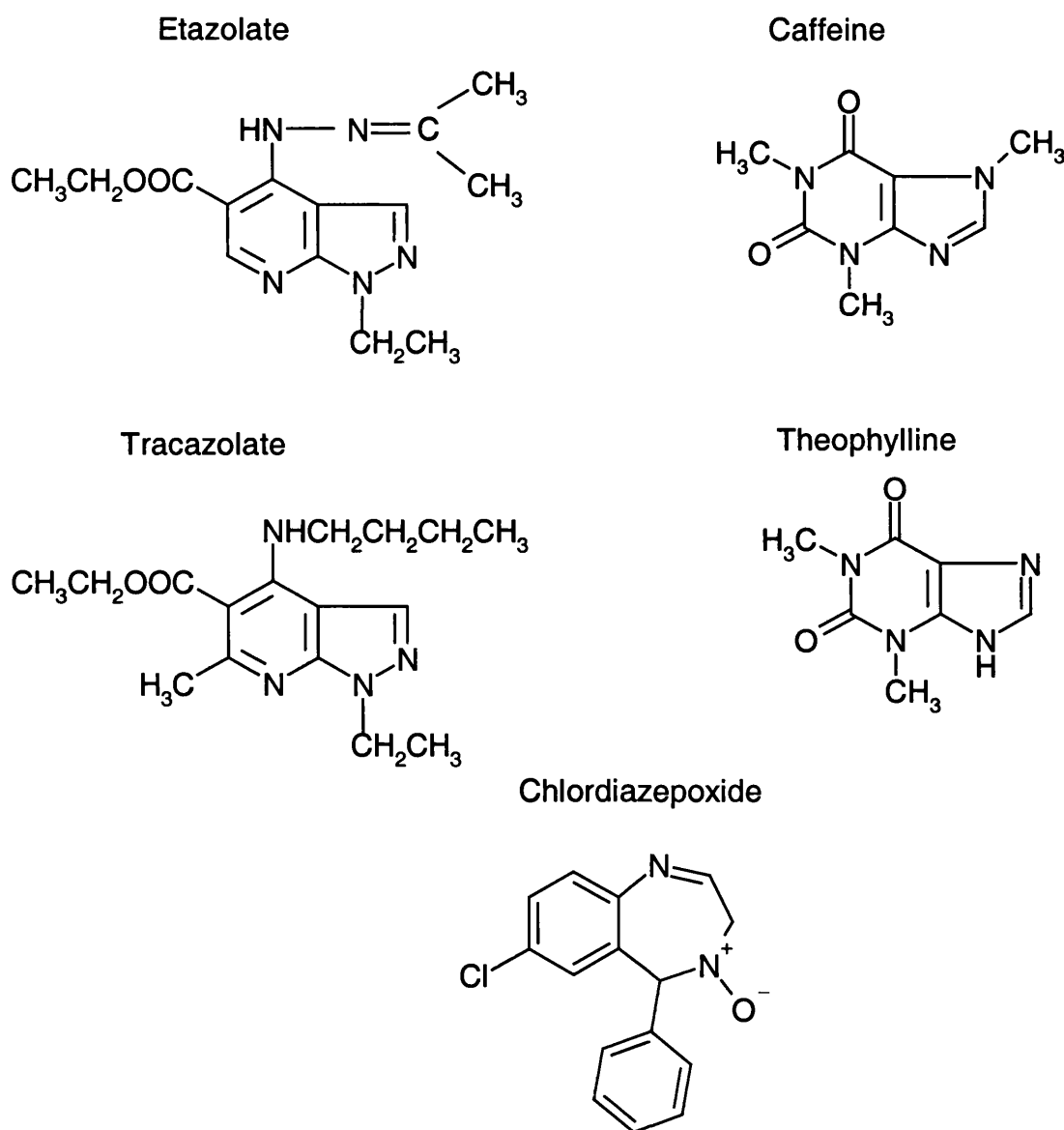
It is most likely that the anxiolytic activity of the pyrazolopyridines is due to an interaction with GABA$_A$ receptors, although both antagonism of central adenosine receptors (Williams *et al.*, 1981) and inhibition of cAMP (Beer *et al.*, 1972) have previously been implicated in this activity. In recent years anxiolytic compounds have been developed that have lower intrinsic efficacy (partial agonists) at GABA$_A$ receptor subtypes, e.g. divaplon, imidazenil, bretazenil and as a result are claimed to be devoid of many of the side effects associated with conventional anxiolytic benzodiazepines (Puia *et al.*, 1992; Auta *et al.*, 1994; Potokar & Nutt, 1994). Recent studies using transgenic animals have revealed that the sedative effect of benzodiazepines is due to an interaction with the α1 subtype (Rudolph *et al.*, 1999; McKernan *et al.*, 2000). It is possible that the reported reduced side effect liability of the pyrazolopyridines could be explained by the compounds behaving as partial agonists or showing receptor subtype selectivity. To date, a functional study examining the effect of pyrazolopyridines on recombinant GABA$_A$ receptors has not been performed. Thus, this study represents the first detailed investigation of the effects of tracazolate and etazolate on recombinant GABA$_A$ receptors.

6.2. Results.

6.2.1. Tracazolate and etazolate potentiate GABA responses on $\alpha1\beta1\gamma2s$ and $\alpha1\beta3\gamma2s$ receptors: selectivity for $\beta3$ subunit containing receptors.

GABA EC$_{20}$ responses on $\alpha1\beta1\gamma2s$ and $\alpha1\beta3\gamma2s$ receptors were potentiated by tracazolate and etazolate (Figure 6.2). The maximum potentiation obtained ranged from between 168-315% and is comparable to that seen with both full benzodiazepine agonists and many other non-benzodiazepine modulators of the GABA$_A$ receptor e.g. loreclezole (Wafford et $al.$, 1994), pentobarbitone (Thompson et $al.$, 1996 and Chapter 4) and neurosteroids (Lambert et $al.$, 1995). No significant direct activation by either tracazolate or etazolate was observed over the concentration range examined (30nM-100μM). Interestingly both compounds revealed a significant 6-8 fold selectivity for $\alpha1\beta3\gamma2s$ over $\alpha1\beta1\gamma2s$ receptors. Except for the maximum potentiation on $\alpha1\beta1\gamma2s$ there were no significant differences (Students t-test) comparing tracazolate and etazolate on either $\alpha1\beta1\gamma2s$ or $\alpha1\beta3\gamma2s$ receptors. Since structurally and functionally tracazolate and etazolate were similar further investigations were performed with tracazolate only.

Figure 6.2. Tracazolate and etazolate potentiate α1β1γ2s and α1β3γ2s receptors: selectivity for α1β3γ2s receptors.

A) Tracazolate
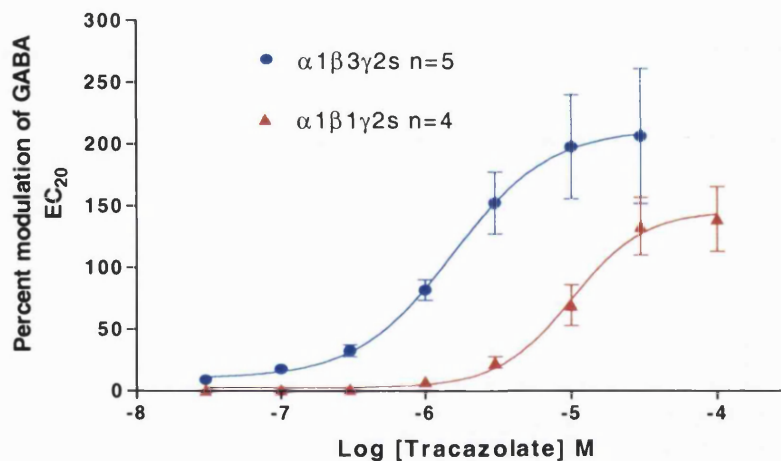
B) Etazolate
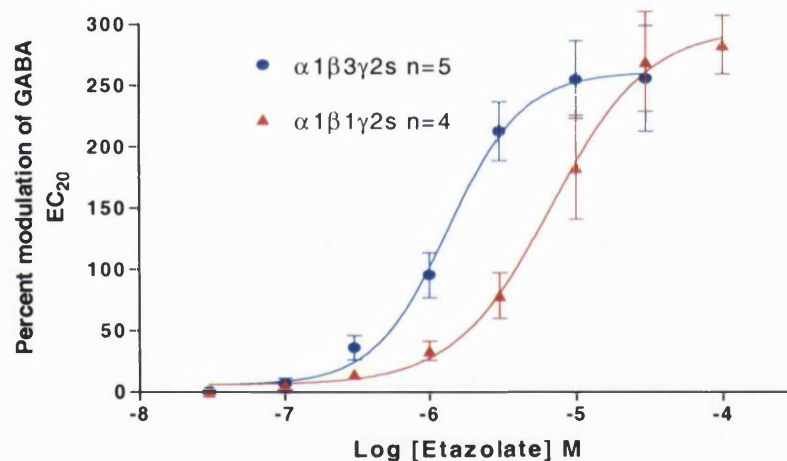
Concentration-response curves to tracazolate and etazolate on α1β1γ2s and α1β3γ2s GABA$_A$ receptors. Data were normalised to the control GABA EC$_{20}$ response, fitted using equation (1) (see Chapter 2) with the maximum not constrained and the resulting parameters detailed in the Table 6.1. Within the two groups of oocytes expressing α1β3γ2s receptors three out of the five oocytes examined produced smaller responses to 30μM tracazolate/etazolate than the previous 10μM response. These data points were ignored for the individual curve fits but included in the mean graph hence the values given for the maximum response in Table 6.1 appear higher than those in the above figure.

Table 6.1 EC$_{50}$, Hill coefficient and the maximum potentiation for tracazolate and etazolate concentration-response curves on $\alpha1\beta1\gamma2s$ and $\alpha1\beta3\gamma2s$ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
Tracazolate				
$\alpha1\beta1\gamma2s$	13.2 (10.2, 17.2)	1.54 ± 0.11	168 ± 16	4
$\alpha1\beta3\gamma2s$	1.5 (1.2, 1.8)**	1.27 ± 0.12	224 ± 46	5
Etazolate				
$\alpha1\beta1\gamma2s$	8.3 (6.8, 10.2)	1.25 ± 0.09	315 ± 32*	4
$\alpha1\beta3\gamma2s$	1.3 (1.1, 1.5)**	1.91 ± 0.47	271 ± 34	5

* indicates a significant difference ($P<0.01$) from tracazolate on $\alpha1\beta1\gamma2s$.

** indicates a significant difference ($P<0.001$) from the corresponding data on $\alpha1\beta1\gamma2s$.

6.2.2. Tracazolate's $\beta3$ selectivity is conferred by asparagine 265.

As has been discussed (see General Introduction and Chapter 5), an increasing number of compounds display selectivity for $\beta2/3$ containing GABA$_A$ receptors over $\beta1$. For loreclezole (Wingrove *et al.*, 1994) etomidate (Hill-Venning *et al.*, 1997), furosemide (Thompson *et al.*, 1999 and Chapter 5) and mefenamic acid (Halliwell *et al.*, 1999) this selectivity has been shown to be due to a critical asparagine residue at position 264 and 265 (numbering according to mature polypeptide sequence) within TM2 of the $\beta2$ and $\beta3$ subunit. It was logical, therefore, to see if this residue also determined the $\beta2/3$ selectivity of tracazolate. The two mutant β cDNA's ($\beta1S265N$ and $\beta3N265S$), as described in Chapter 5, were co-expressed with $\alpha1$ and $\gamma2s$ and concentration-response curves to tracazolate constructed (Figure 6.3 and Table 6.2). Replacement of Ser265 within the $\beta1$ subunit with Asn (the $\beta3$ counterpart) increased the sensitivity of tracazolate, whereas the opposite mutation ($\beta3Asn265$ to Ser) decreased the sensitivity to tracazolate. Hence tracazolate and, presumably, etazolate

join the list of compounds that modulate GABA$_A$ receptors with selectivity for β2/3 containing receptors and are influenced by position 265.

Figure 6.3. Tracazolate's β3 selectivity is conferred by asparagine 265.

Concentration-response curves to tracazolate on α1β1γ2s, α1β3γ2s, α1β1S265Nγ2s and α1β3N265Sγ2s GABA$_A$ receptors. Data were normalised to the control GABA EC$_{20}$ response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in the Table 6.2. The responses to increasing concentrations of tracazolate for the two mutant receptors did not plateau but infact were significantly smaller at 100μM than 30μM. Curves were fitted to the rising portion of the curve with the maximum not constrained hence the data presented in Table 6.2 represent an estimate of the EC$_{50}$, Hill coefficient and maximum response.

Table 6.2. EC$_{50}$, Hill coefficient and the maximum potentiation for tracazolate and etazolate concentration-response curves on α1β1γ2s, α1β3γ2s, α1β1S265Nγ2s, and α1β3N265Sγ2s receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β1γ2s	13.2 (10.2, 17.2)	1.54 ± 0.11	168 ± 16	4
α1β3γ2s	1.5 (1.2, 1.8)	1.27 ± 0.12	224 ± 46	5
α1β1S265Nγ2s	2.7 (2.4, 2.9)*	1.28 ± 0.12	273 ± 60	4
α1β3N265Sγ2s	6.8 (5.5, 8.5)**	1.10 ± 0.10	207 ± 24	4

* indicates a significant difference ($P<0.01$) from α1β1γ2s. ** indicates a significant difference ($P<0.01$) from α1β3γ2s

6.2.3. Interaction is not via the benzodiazepine binding site.

Concentration-response curves to tracazolate were also constructed on oocytes expressing α1β3 and α6β3γ2s GABA$_A$ receptors (Figure 6.4 and Table 6.3). Similar to α1β3γ2s receptors, control GABA EC$_{20}$ concentrations on α1β3 and α6β3γ2s receptors were potentiated by tracazolate. Statistical analysis (ANOVA) revealed no significant differences in the log EC$_{50}$, Hill coefficient or maximum potentiation for α1β3, α1β3γ2s or α6β3γ2s. Unlike compounds that interact at the benzodiazepine site, receptors lacking a γ subunit were also potentiated by tracazolate. Replacement of the α1 subunit with an α6 subunit did not alter the concentration-response curve to tracazolate. Finally Ro15-1788 (300nM), a benzodiazepine site antagonist, had no affect on the degree of potentiation of GABA EC$_{20}$ activated responses elicited by 10μM tracazolate on α1β3γ2s receptors (198 ± 42% n=5 in the absence verses 223 ± 31% n=4 in the presence of Ro15-1788).

Figure 6.4. Concentration-response curves for tracazolate on α1β3, α1β3γ2s and α6β3γ2s GABA$_A$ receptors.
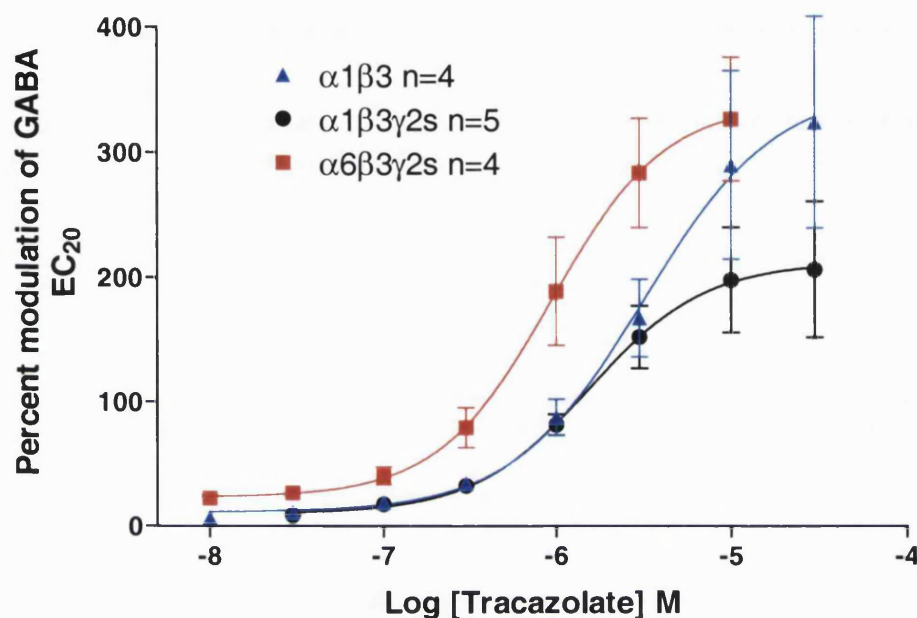
Concentration-response curves to tracazolate on α1β3, α1β3γ2s and α6β3γ2s GABA$_A$ receptors. Data were normalised to the control GABA EC$_{20}$ response, fitted using equation (1) (see Chapter 2) and the resulting parameters detailed in the Table 6.3.

Table 6.3 EC$_{50}$, Hill coefficient and maximum response for tracazolate concentration-response curves on α1β3, α1β3γ2s and α6β3γ2s GABA$_A$ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
α1β3	2.7 (2.0, 3.5)	1.24 ± 0.13	351 ± 94	4
α1β3γ2s	1.5 (1.2, 1.8)	1.27 ± 0.12	224 ± 46	5
α6β3γ2s	1.1 (0.8, 1.6)	1.42 ± 0.18	363 ± 44	4

6.2.4. Tracazolate inhibits α1β3ε GABA$_A$ receptors.

In addition to replacement of the α1 subunit with an α6 subunit and removal of the γ subunit, the effect of tracazolate on α1β3ε receptors was examined. As has been described in Chapter 3, ε containing receptors reveal some unusual properties including a proportion of constitutively-active channels and fast desensitization kinetics. Similar to the experiments described above, concentration-response curves to tracazolate were performed on oocytes expressing α1β3ε receptors using a GABA EC$_{20}$ concentration. Unlike all the receptor combinations studied above, on α1β3ε receptors tracazolate had a significant direct effect. Over the 30sec application period of tracazolate, low concentrations produced an inward current whereas higher concentrations produced an inward current followed by an outward current (Figure 6.5). To facilitate comparison with the results obtained with α1β3γ2s receptors, only the inward current to GABA in the presence of tracazolate was measured as illustrated in Figure 6.6A. Surprisingly tracazolate caused inhibition of the control GABA EC$_{20}$ response (Figure 6.6B). Data were normalised with respect to the response evoked by the control GABA EC$_{20}$ response and fitted using equation 2 (see Chapter 2). The IC$_{50}$ value for tracazolate on α1β3ε receptors (1.2 (0.9, 1.5)μM) was similar to the EC$_{50}$ obtained on α1β3γ2s receptors (1.5 (1.2, 1.8)μM). The Hill coefficient was –0.84 ± 0.07 and the maximum inhibition was 96 ± 1.6%.

Figure 6.5: Representative traces of tracazolate concentration-response curves against a GABA EC_{20} concentration on $\alpha1\beta3\varepsilon$ and $\alpha1\beta3\gamma2s$ receptors.

A) $\alpha1\beta3\varepsilon$

Tracazolate   10nM   30nM   100nM   300nM   1μM   3μM   10μM   30μM

GABA
0.3μM

50nA

2 min

B) $\alpha1\beta3\gamma2s$

Tracazolate   30nM   100nM   300nM   1μM   3μM   10μM   30μM

GABA
10μM

100nA

2 min
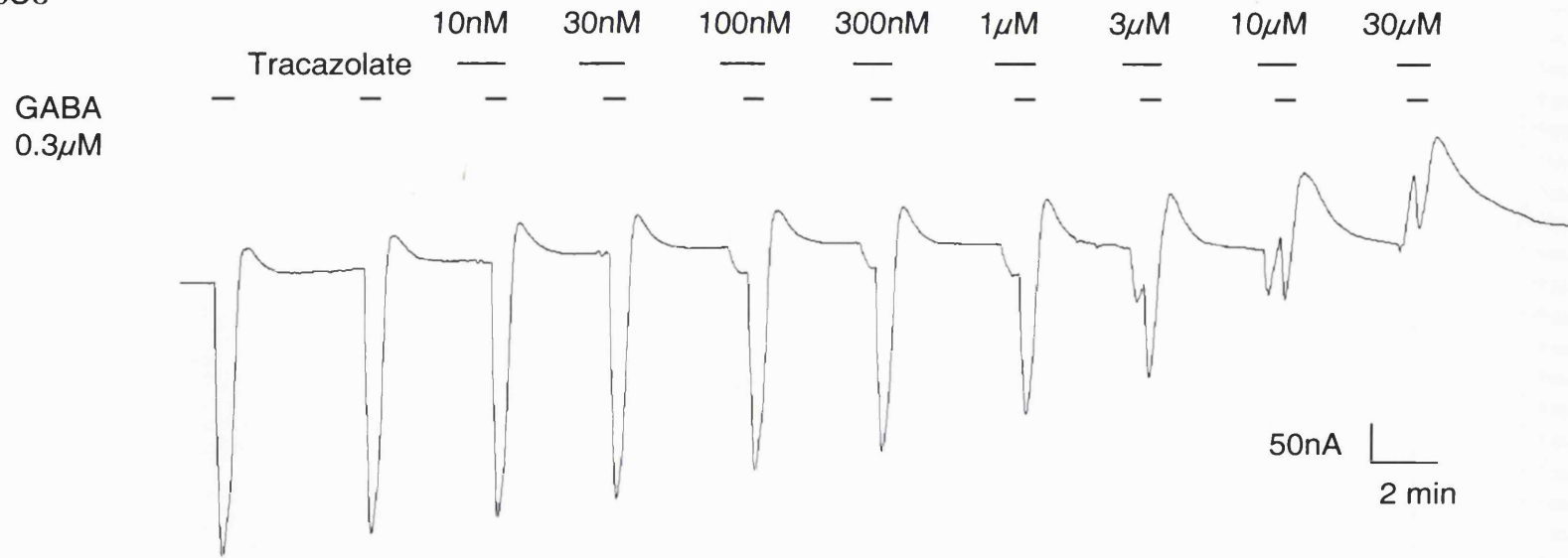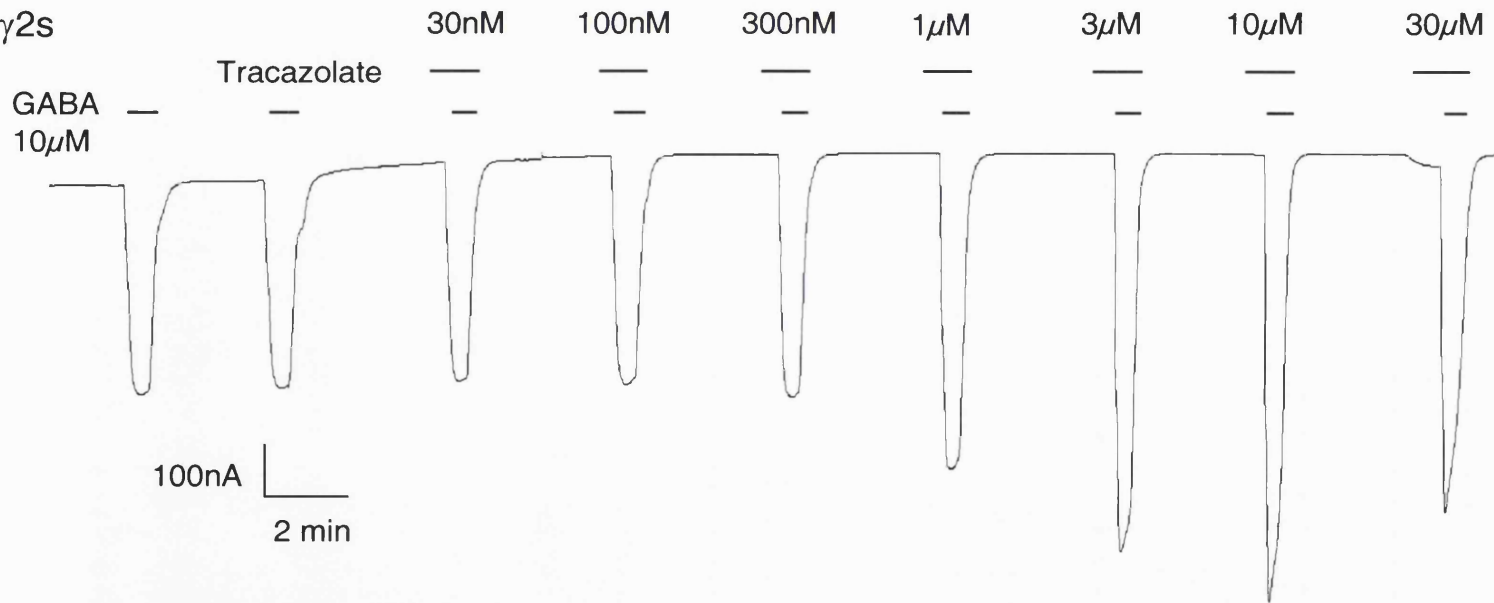
Figure 6.6. Tracazolate inhibits GABA EC$_{20}$ responses on α1β3ε receptors.

A)

B)
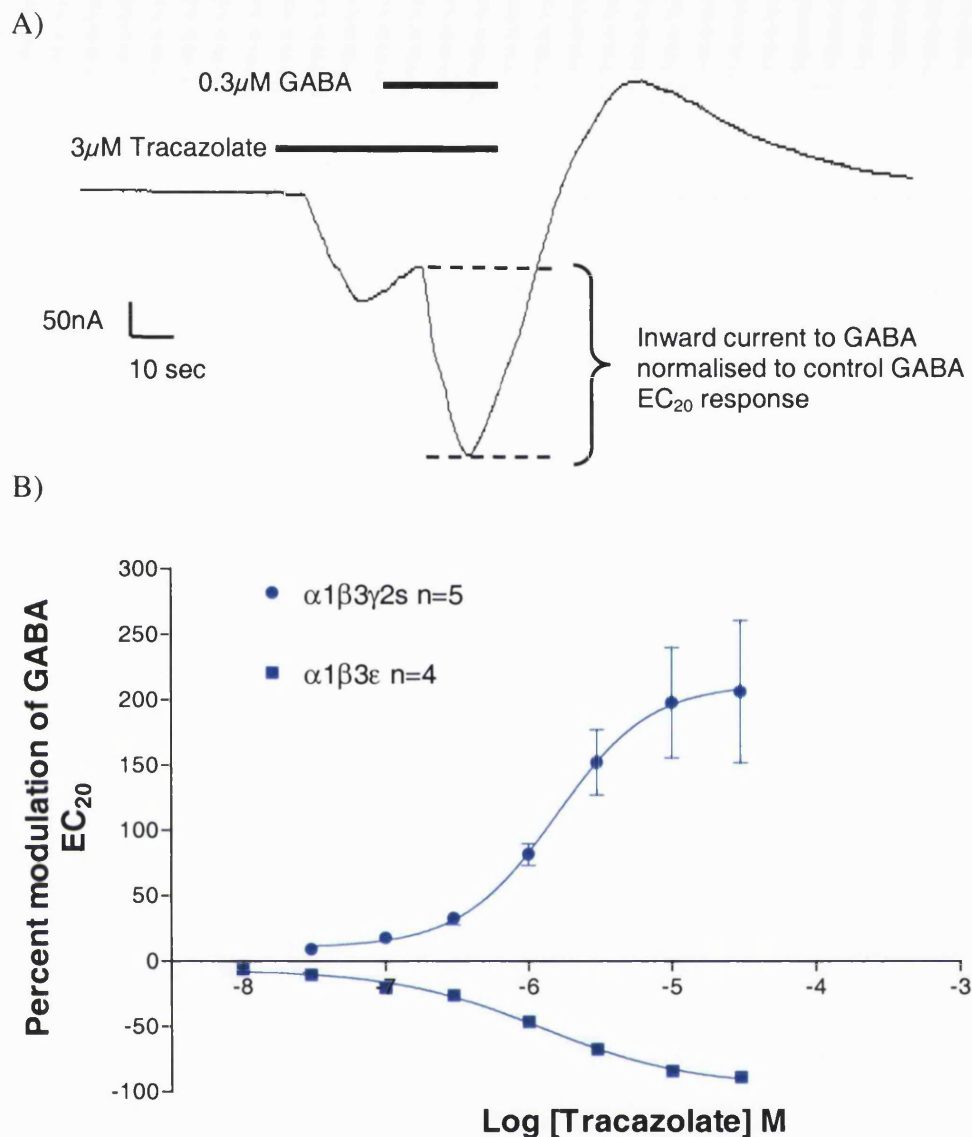
A) Representative recording of the application of 3μM tracazolate for 30sec followed by the coapplication of 3μM tracazolate and 0.3μM GABA. The inward current to GABA, as indicated by the dashed lines, was normalised to the control GABA EC$_{20}$ response.

B) Concentration-response curves for tracazolate on α1β3γ2s and α1β3ε receptors. Data were normalised to the control GABA EC$_{20}$ response, fitted using equation (1 or 2) (see Chapter 2) and the resulting parameters detailed in the text. Note the error bars for α1β3ε are concealed by the symbol.

6.2.5. β2/3 selectivity is retained with ε containing receptors.

Having determined that the ε subunit changes the functional response to tracazolate, selectivity for the β3 subunit was examined in the presence of ε. Similar to α1β1γ2s receptors, α1β1ε receptors were 4-fold less sensitive ($P<0.05$) to the modulatory effects of tracazolate (IC$_{50}$= 4.0 (2.7, 5.9)μM compared to 1.2 (0.9, 1.5)μM for α1β3ε (Figure 6.7).

Figure 6.7. Concentration-response curves for tracazolate on α1β1ε and α1β3ε receptors: β3 selectivity retained.
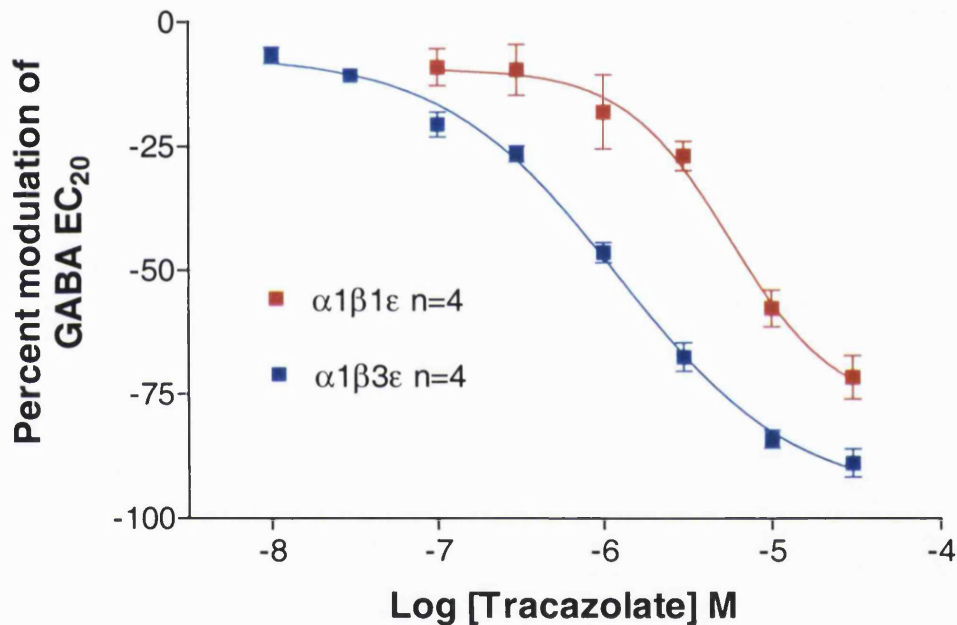
Concentration-response curves to tracazolate on α1β1ε and α1β3ε receptors. Data were normalised to the control GABA EC$_{20}$ response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

6.2.6. Outward current to tracazolate carried by Cl⁻ ions.

Concentration-response curves to tracazolate alone were constructed on α1β3ε receptors to examine the direct effect in more detail. Tracazolate was applied until the response reached a plateau and then washed out until the baseline was re-established. There were two components to the direct effect when voltage clamped at –70mV, an initial small inward current which was followed by an apparent outward current (Figure 6.8). At high concentrations the inward current was masked by the outward current and was not examined further. At high concentrations the outward current took up to 180 secs to reach a plateau followed by a washout period of up to 15 minutes to re-establish the baseline value. The outward current was measured using the peak of the inward current as the start value. 100μM picrotoxin, which it is assumed blocks all the constitutively-active channels, was applied at the end of the tracazolate concentration-response curve (see Chapter 3). Data were normalised to the response to 100μM PTX, and fitted using equation 2 (see Chapter 2). Interestingly the IC$_{50}$ value of the direct effect of tracazolate (1.4 (1.1, 1.6)μM n=4) was not significantly different from the IC$_{50}$ value for inhibition of GABA EC$_{20}$ response (1.2 (0.9, 1.5)μM n=4) (Figure 6.9). The current-voltage relationship was determined for this direct effect of tracazolate (3μM) on α1β3ε receptors using the stepping protocol as described in Chapter 2. The data were best fitted to a linear regression (see Chapter 2 equation(5)) (r^2 = 0.94 ± 0.02 n=4) and revealed a reversal potential of –25.7 ± 1.3mV n=4, which was similar to the predicted reversal potential for Cl⁻ ions of – 25.4mV in *Xenopus* oocytes (see 3.2.4), indicating that the carrier of the direct effect is Cl⁻ ions (Figure 6.10).

Figure 6.8. Representative trace examining the direct effect of tracazolate on α1β3ε receptors.
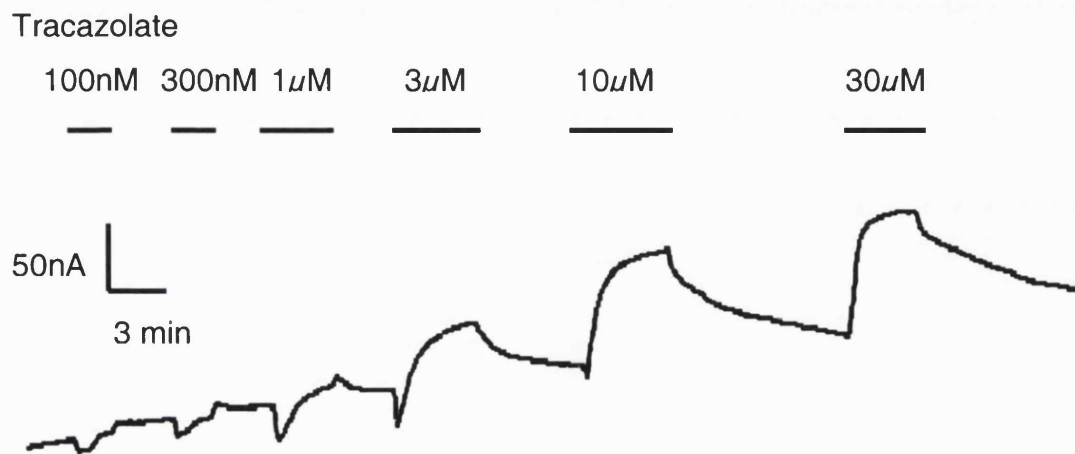
Figure 6.9. Concentration-response curves for the direct effect (2nd component) of tracazolate on α1β3ε receptors.
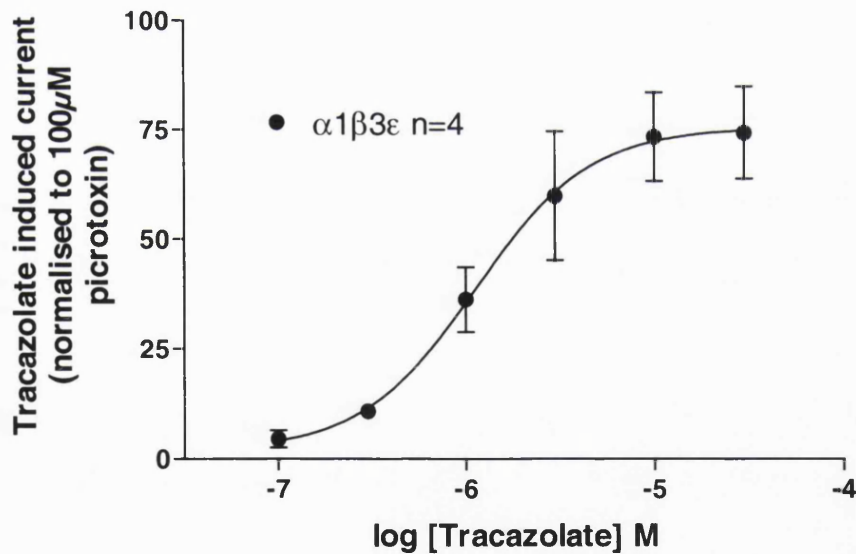
Concentration-response curves to the direct component of tracazolate on α1β3ε receptors. Data were normalised to 100μM PTX response, fitted using equation (2) (see Chapter 2) and the resulting parameters detailed in the text.

Figure 6.10. Current-voltage relationship for the direct effect of 3μM tracazolate on α1β3ϵ receptors.
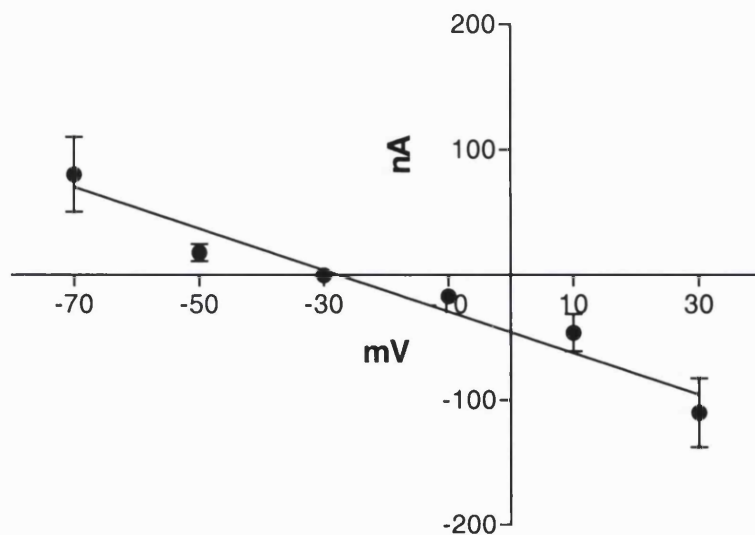
The current-voltage relationship was determined for the direct effect of tracazolate (3μM) on α1β3ϵ receptors using the stepping protocol as described in Chapter 2. Data were fitted using equation (5) (see Chapter 2) and the resulting parameters detailed in the text.

6.2.7. γ1, γ3 and δ containing receptors.

The opposing effects observed with tracazolate on γ2s and ϵ containing receptors prompted studies on γ1, γ3 and δ subunit containing receptors. These subunits were co-expressed with α1 and β1 subunits and concentration-response curves to tracazolate constructed. Co-expression with α1β1 instead of α1β3 was chosen since a previous publication had shown that functional receptors were formed when the γ1 subunit was co-expressed with α1β1 subunits and Zn^{2+} (3μM) could be used as a tool to confirm expression (Wingrove *et al.*, 1997). As can be seen in Figure 6.11, tracazolate had a completely different effect on α1β1γ1 and α1β1γ3 receptors compared to that on α1β1γ2s and α1β1ϵ. Concentrations up to 10μM produced a small degree of potentiation of the GABA EC$_{20}$ response (19% and 30% for 10μM tracazolate on α1β1γ1 and α1β1γ3 respectively) whereas higher concentrations inhibited the GABA EC$_{20}$ response. The potentiating portion of the concentration-

response curve was fitted with equation (1) as described in Chapter 2 and the resulting parameters are summarised in Table 6.3. The results must be viewed with caution because the low level of potentiation and the small number of data points made curve fitting difficult. The data obtained for $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$ receptors were not significantly different from one another ($P>0.05$ unpaired students t-test) however, comparison with $\alpha1\beta1\gamma2s$ revealed a 10-fold decrease in EC$_{50}$.

Figure 6.11. Modulation of α1β1γ1 and α1β1γ3 receptors by tracazolate.
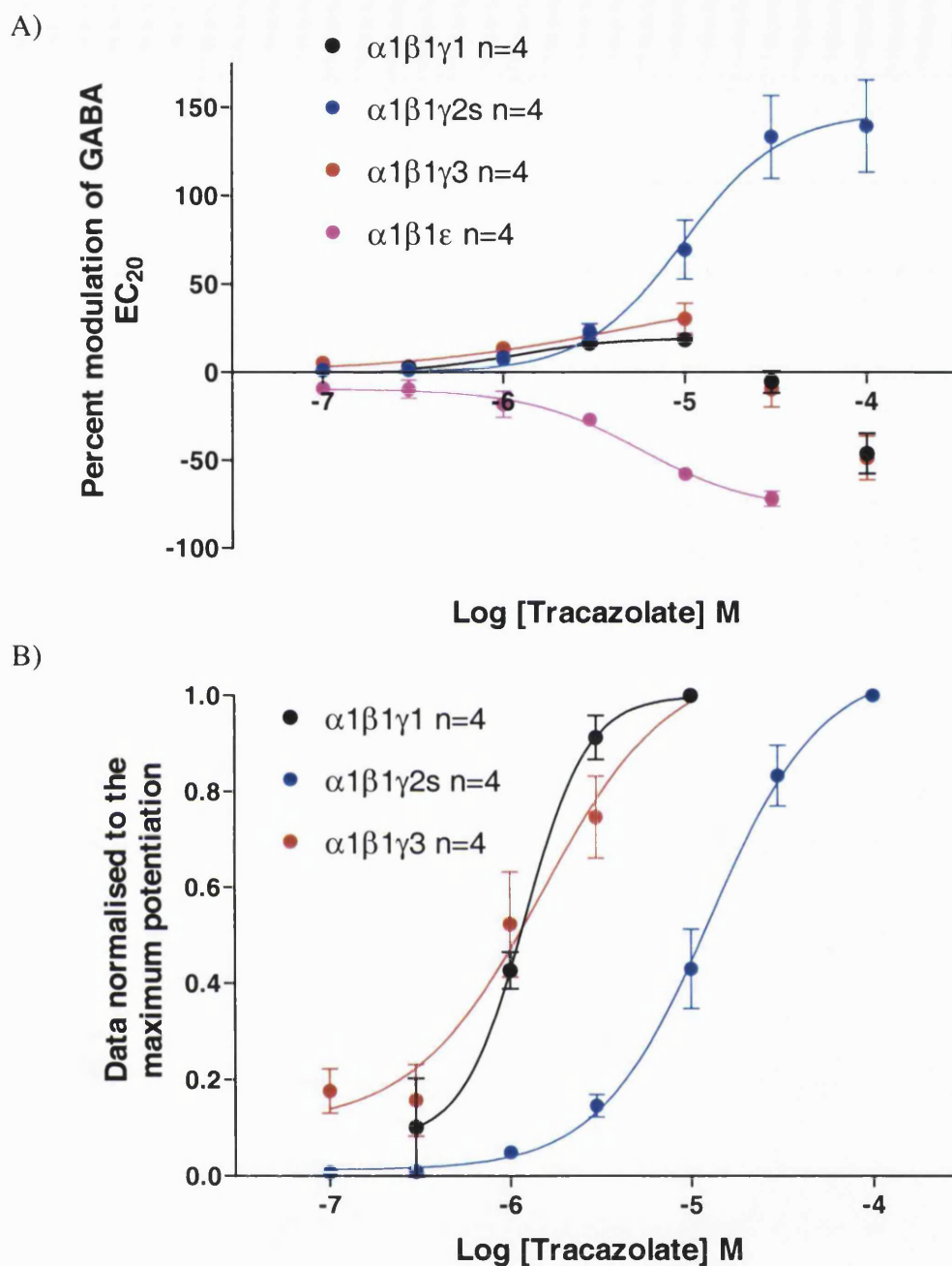
A) Concentration-response curves to tracazolate on α1β1γ1, α1β1γ2s, α1β1γ3 and α1β1ε GABA_A receptors.

B) Data for the potentiating portion of the curve were normalised to the maximum potentiation obtained on α1β1γ1, α1β1γ2s and α1β1γ3 receptors. Note the leftward shift in the concentration-response curve for α1β1γ1 and α1β1γ3 receptors.

Table 6.4. EC$_{50}$, Hill coefficient and the maximum potentiation for tracazolate concentration-response curves on $\alpha1\beta1\gamma1$ and $\alpha1\beta1\gamma3$ receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No of Oocytes
$\alpha1\beta1\gamma1$	1.3 (1.1, 1.5)	1.8 ± 0.5	21.0 ± 5.9	4
$\alpha1\beta1\gamma3$	1.4 (1.0, 2.1)	1.6 ± 0.7	34.7 ± 10.1	4

As discussed in Chapter 4, the time taken for $\alpha6\beta3\delta$ receptors to be expressed in *Xenopus* oocytes was much longer than $\alpha6\beta3\gamma2s$ receptors. Expression of $\alpha1\beta1\delta$ receptors followed this trend, taking four or more days to produce currents with amplitudes large enough for further experimentation (>100nA to 1mM GABA). Similar to $\alpha6\beta3\delta$ receptors, the maximal peak current amplitude (in response to 1mM GABA) was smaller for $\alpha1\beta1\delta$ receptors (183 ± 31nA n=19) compared to $\alpha1\beta1\gamma2s$ receptors (2598 ± 519 n=15). As discussed in Chapter 4, the presence of a δ subunit with the $\alpha\beta$ dimer could not be guaranteed. For this reason, the presence of the δ subunit was confirmed for every cell using 1μM Zn^{2+} as a diagnostic tool. Any cell whose GABA EC$_{50}$ response was inhibited by more than 25% in the presence of 1μM Zn^{2+} was rejected. Interestingly, tracazolate potentiated the GABA EC$_{20}$ response to levels substantially greater than that produced by a maximum GABA concentration (Figure 6.12). Potentiation of a GABA EC$_{20}$ concentration by tracazolate above and beyond the maximum current elicited by GABA was not observed with any other subunit combination examined. The log EC$_{50}$'s and Hill coefficients however were not significantly different between $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2s$ ($\alpha1\beta1\delta$; EC$_{50}$ = 18.1 (15.8, 20.8)μM, n$_H$ = 1.4 ± 0.08 $\alpha1\beta1\gamma2s$; EC$_{50}$= 13.2 (10.2, 17.2)μM, n$_H$ = 1.5 ± 0.11).

Figure 6.12. Modulation of α1β1δ receptors by tracazolate.
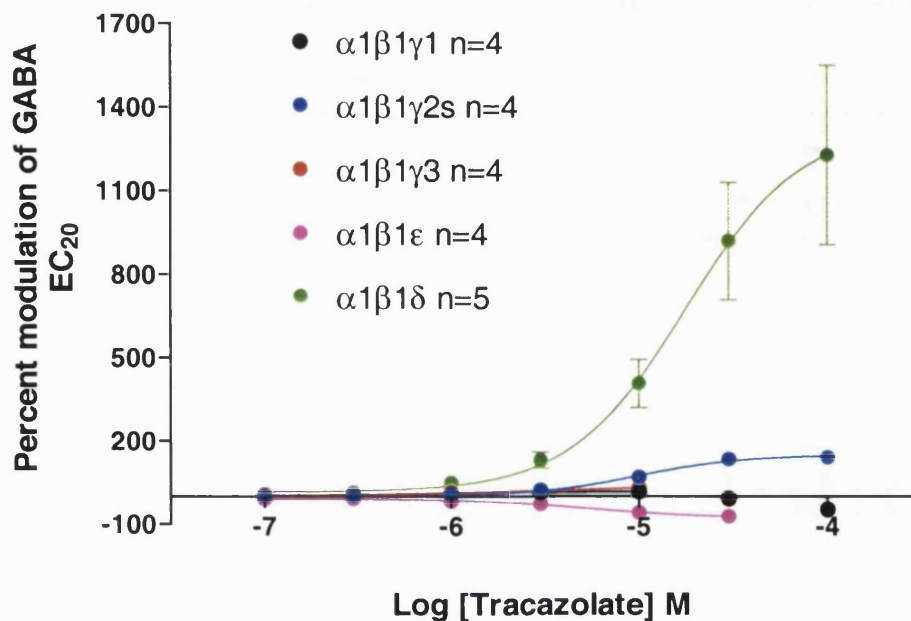
6.2.8.   Effect of tracazolate on GABA concentration-response curves.

The studies above have only examined the effect various concentrations of tracazolate have on a single low concentration of GABA (EC_{20}). To further understand the mechanism of action of tracazolate, its effect on a range of GABA concentrations was examined. GABA concentration-response curves were constructed in the absence and then the presence of a single concentration of tracazolate on oocytes expressing α1β3ε, α1β3γ2s and α1β1δ receptors. The maximum current obtained for the initial GABA concentration-response curve was taken as the control value (100%) and data normalised to this value. Significant differences were assessed using a paired Students *t*-test.

On α1β3γ2s receptors, 1μM tracazolate (which approximates to its EC_{50} using a GABA EC_{20} concentration) produced a significant ($P<0.01$) 2.5 ± 0.3 fold shift to the left of the GABA EC_{50} with no significant effect on the maximum or Hill coefficient (Figure 6.13 and Table 6.4). Higher concentrations of tracazolate (10μM and 30μM)

further increased this leftward shift of the GABA concentration-response curve (21.6 ± 5.5 and 39.3 ± 15.8 fold respectively). In addition, the maximum response of GABA in the presence of 10μM and 30μM tracazolate was significantly reduced compared to the maximum obtained for the control GABA concentration-response curve (67.6 ± 4 and 40.0 ± 2.9% respectively). 30μM tracazolate also significantly reduced the Hill coefficient of the GABA concentration-response curve compared with the control (1.54 ± 0.03 vs 0.98 ± 0.14 $P<0.05$).

As above (6.2.4), for $\alpha1\beta3\epsilon$ receptors the inward current alone to GABA and tracazolate was measured ignoring any direct effect (Figure 6.14). The log EC$_{50}$ values and Hill coefficients for the control GABA concentration-response curves compared to those in the presence of 1 and 3μM tracazolate were not significantly different. The maximum response obtainable to GABA in the presence of 1 and 3μM tracazolate however were significantly lower ($P<0.0001$) (Figure 6.14 and Table 6.5).

Similar to $\alpha1\beta3\gamma2s$ receptors, concentration-response curves to GABA on $\alpha1\beta1\delta$ receptors were shifted to the left by 10μM and 30μM tracazolate. However, unlike $\alpha1\beta1\gamma2s$, the maximum response to GABA in the presence of 10 and 30μM tracazolate was significantly larger (Figure 6.15 and Table 6.6).

Note in Figures 6.13 to 6.15 the data for the control GABA concentration-response curves have been combined.

Figure 6.13. Concentration-response curves for GABA in the absence and presence of 1,10 and 30μM tracazolate on α1β3γ2s receptors.
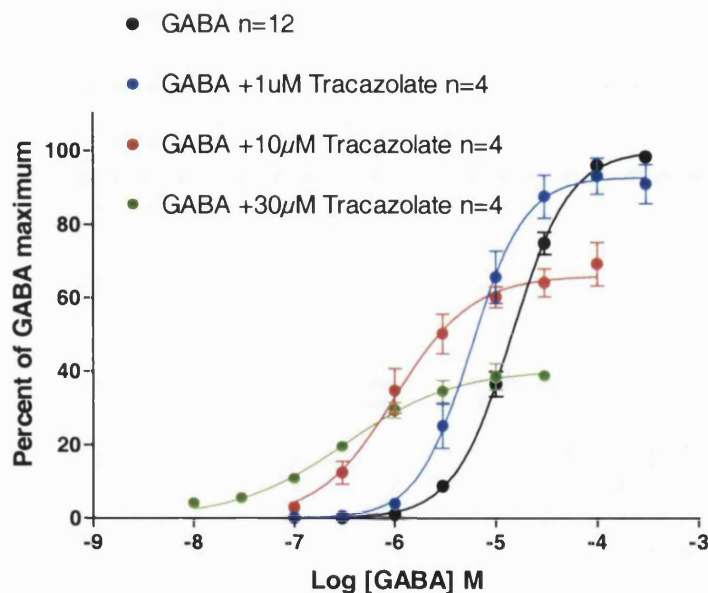
Table 6.5. EC_{50} values, Hill coefficients and maximum responses for GABA concentration-response curves in the absence and presence of 1, 10 and 30μM tracazolate on α1β3γ2s receptors.

α1β3γ2s	EC_{50} (μM)	Hill Coefficient	Maximum Response (%)
GABA	14.2 (11.5, 17.5)	1.57 ± 0.03	101.8 ± 0.9
GABA + Tracazolate 1μM	5.9 (5.1, 6.9)	1.68 ± 0.12	94.1 ± 5.2
GABA	19.5 (17.8, 21.5)	1.51 ± 0.04	101.6 ± 0.7
GABA + Tracazolate 10μM	1.01 (0.75, 1.54)	1.25 ± 0.13	67.1 ± 3.9
GABA	12.0 (10.6, 13.6)	1.56 ± 0.03	101.5 ± 1.3
GABA + Tracazolate 30μM	0.35 (0.26, 0.47)	0.84 ± 0.14	41.3 ± 3.4

Each concentration of tracazolate was examined on four individual oocytes.

Figure 6.14. Concentration-response curves for GABA in the absence and presence of 1 and 3μM tracazolate on α1β3ε receptors.
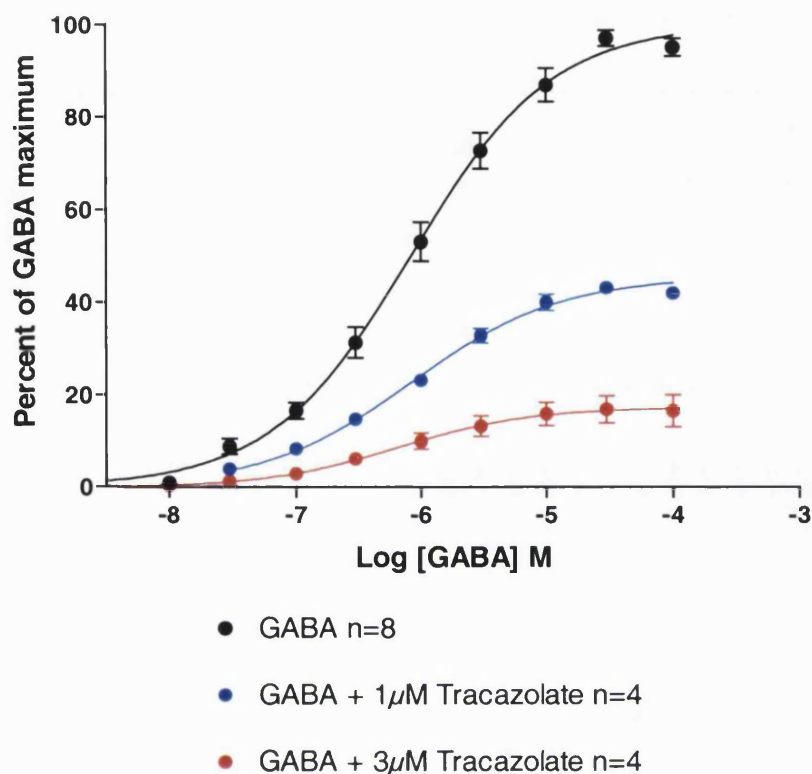
Table 6.6. EC$_{50}$ values, Hill coefficients and maximum responses for GABA concentration-response curves in the absence and presence of 1 and 3μM tracazolate on α1β3ε receptors.

α1β3ε	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)
GABA	0.72 (0.47, 1.12)	0.77 ± 0.09	104.0 ± 2.1
GABA + Tracazolate 1μM	0.90 (0.77, 1.04)	0.73 ± 0.07	46.5 ± 1.03
GABA	1.40 (1.19, 1.65)	0.72 ± 0.02	108.2 ± 1.2
GABA + Tracazolate 3μM	0.71 (0.55, 0.91)	0.84 ± 0.11	17.5 ± 2.65

Each concentration of tracazolate was examined on four individual oocytes.

Figure 6.15. Concentration-response curves for GABA in the absence and presence of 10 and 30μM tracazolate on α1β1δ receptors.
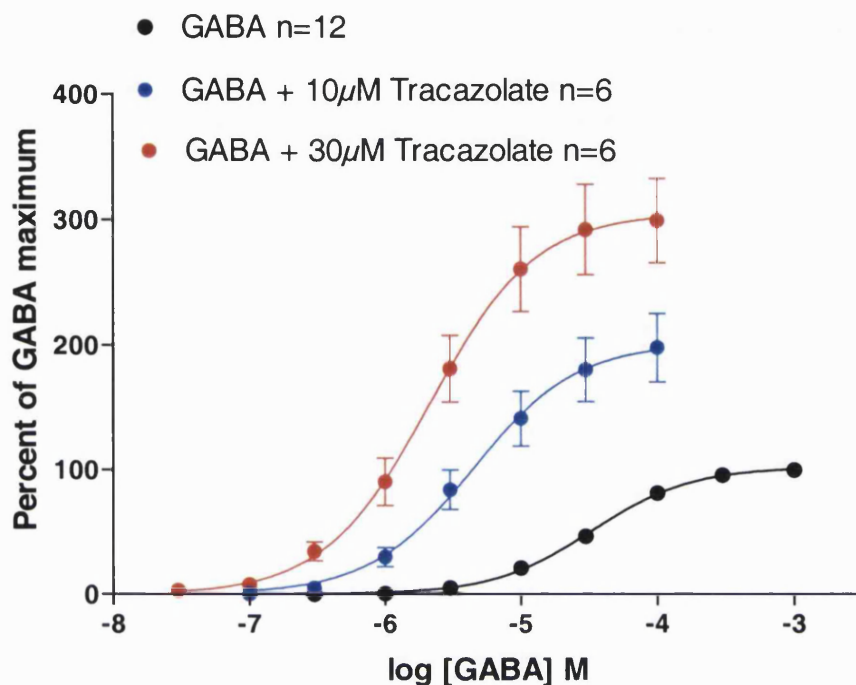
Table 6.7. EC$_{50}$ values, Hill coefficients and maximum responses for GABA concentration-response curves in the absence and presence of 10 and 30μM tracazolate on α1β1δ receptors.

α1β1δ	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)
GABA	31.8 (28.2, 35.8)	1.28 ± 0.06	101.5 ± 0.4
GABA + Tracazolate 10μM	4.7 (3.9, 5.6)	1.19 ± 0.07	202.9 ± 27.7
GABA	33.0 (28.3, 38.5)	1.24 ± 0.05	101 ± 0.5
GABA + Tracazolate 30μM	2.2 (1.8, 2.6)	1.15 ± 0.04	305.1 ± 34.2

Each concentration of tracazolate was examined on six individual oocytes.

6.3. Discussion

Although the pyrazolopyridines were first synthesised 30 years ago, this is the first detailed electrophysiological study of their effects on recombinant GABA$_A$ receptors. The data obtained support the idea that the anxiolytic effect of these compounds could be mediated via an interaction with GABA$_A$ receptors, however it must be remembered that the compounds also behave as adenosine antagonists and inhibitors of phosphodiesterase with similar IC$_{50}$/EC$_{50}$'s. Inhibition of phosphodiesterase however, has only been demonstrated in subcellular preparations (e.g. rat brain Horovietz et al., 1972; rabbit brain Chasin et al., 1972; rat liver Ingebretsen et al., 1974). In the in vivo situation elevated cAMP levels were only detected in rat plasma and urine and not in the brain leading to the conclusion that inhibition of phosphodiesterase was not responsible for the anxiolytic action (Weinryb et al., 1975).

6.3.1. Tracazolate does not bind to the benzodiazepine binding site.

Tracazolate produced concentration related potentiation of control GABA EC$_{20}$ responses on oocytes expressing the dimer, $\alpha 1\beta 3$. This result is in contrast to benzodiazepine compounds which do not modulate $\alpha\beta$ dimers (Levitan et al., 1988a and b; Pritchett et al., 1988). In addition, tracazolate did not displace [^{3}H]-Ro15-1788 from $\alpha 3\beta 3\gamma 2s$ GABA$_A$ receptors stably expressed in Ltk$^-$ cells (data not shown) nor was the functional response on $\alpha 1\beta 3\gamma 2s$ receptors inhibited by Ro15-1788.

The type of β subunit present within the receptor complex has previously been shown not to effect the modulation obtained with benzodiazepine site ligands (Hadingham et al., 1993). Tracazolate and etazolate however were significantly more potent on $\alpha 1\beta 3\gamma 2s$ receptors compared to $\alpha 1\beta 1\gamma 2s$ receptors. Over the last seven years an increasing number of structurally unrelated compounds have been identified that also show this selectivity e.g. loreclezole, etomidate, furosemide, mefenamic acid. For each compound this selectivity has been shown to be due to the presence of an asparagine residue at the homologous position 264 and 265 within TM2 of the $\beta 2$ and $\beta 3$ subunit. Experiments with the single point mutants $\beta 3$Asn265Ser and

β1Ser265Asn co-expressed with α1γ2s subunits confirmed that this same residue was responsible for the β2/3 selectivity seen with tracazolate. Collectively these results demonstrate that tracazolate does not interact with the benzodiazepine site and are in agreement with the previous biochemical and electrophysiological data obtained for the pyrazolopyridines (Wiliams & Risley, 1979; Ticku & Davis, 1982; Barnes *et al.,* 1983).

It is currently not known whether Asn265 forms part of a common binding site for the many compounds whose functional response it influences (e.g. tracazolate, loreclezole, etomidate, furosemide, mefenamic acid) or alternatively if these compounds share a common transduction mechanism which is hindered in β1 when Asn is replaced with Ser. As discussed in the previous chapter the orientation of the 15' residue within TM2 of the GABA β subunit is currently unknown.

6.3.2. Improved anxiolytic / sedation window is not due to reduced efficacy at α1 containing receptors.

Both etazolate and tracazolate potentiated control GABA EC$_{20}$ responses on α1β3γ2s receptors with an EC$_{50}$ of ~1.5μM and a maximum potentiation of ~250%. The evidence to date using α1 knock-out mice and an α2/3 selective benzodiazepine site ligands suggests that the sedative effect observed with classical benzodiazepines is mediated via the α1 subtype (Rudolph *et al.,* 1999; McKernan *et al.,* 2000). The results presented in this chapter suggest that the improved window between the anxiolytic and sedation effect of tracazolate is not due to a reduction in efficacy at the α1 subtype.

A number of alternative hypotheses that could account for this improved window exist:

1) Tracazolate may have higher affinity and therefore a lower EC$_{50}$ for other GABA$_A$ receptor subtypes such as α2–5β1–3γ2. Although the effect of tracazolate and etazolate on the complete range of α subunits was not investigated, similar EC$_{50}$ and maximum potentiation values were observed for tracazolate on α1β3γ2s and α6β3γ2s receptors.

2) The apparent GABA EC$_{50}$ for recombinant $\alpha 3$ containing receptors is an order of magnitude higher than $\alpha 1$ containing receptors, suggesting a difference in GABA binding affinity (Smith *et al.*, 2001). In addition to differences in GABA affinity, measurements of the peak GABA concentrations in the synaptic cleft of different brain areas range from 0.3 to 3mM (Maconochie *et al.*, 1994; Jones & Westbrook, 1995; Mozrzymas *et al.*, 1999; Perrais & Ropert, 1999). Both these factors will determine the level of response from the respective GABA concentration-response curve. The synaptic GABA response for $\alpha 3$ containing receptors to tracazolate may be potentiation whereas, for other α subunits it may be no change or inhibition. Potentiation of $\alpha 3$ containing receptor subtypes may elicit the anxiolytic effect.

3) It has been shown that the deactivation rate for GABA on $\alpha 3 \beta 3 \gamma 2s$ receptors is three times slower ($0.68 \pm 0.1sec$) than on $\alpha 1 \beta 3 \gamma 2s$ receptors ($0.22 \pm 0.1sec$) (Gingrich *et al.*, 1995). Meadows *et al.*, (1997), using cultured rat cerebellar granule cells, have demonstrated that tracazolate and the compound SB-205384 both prolonged the half-life for decay of the GABA response whereas diazepam, pentobarbitone, THDOC had no effect. This group further showed (Meadows *et al.*, 1998) that this increase in the decay rate of the GABA response for SB-205384 was subunit specific with selectivity for $\alpha 3$ containing receptors compared to $\alpha 1$ and $\alpha 2$ containing receptors. A third explanation for the improved anxiolytic/sedative window with tracazolate is specific prolongation of the deactivation time constants for receptors containing α subunits other than $\alpha 1$. This effect would result in an overall greater chloride current for these receptor subtypes as compared to $\alpha 1 \beta 3 \gamma 2s$ receptors. Whether the prolongation of deactivation by tracazolate (if it occurs) shows any subunit selectivity has not been demonstrated.

6.3.3. Functional response determined by the nature of the third subunit.

This study has examined the effect of tracazolate on a range of receptor subunit combinations including $\alpha 1 \beta 1 \gamma 1$, $\alpha 1 \beta 1 \gamma 2s$, $\alpha 1 \beta 1 \gamma 3$, $\alpha 1 \beta 1 \varepsilon$ and $\alpha 1 \beta 1 \delta$. The nature of the third subunit within the receptor complex was critical in determining the functional response to tracazolate. For $\alpha 1 \beta 1 / 3 \gamma 2s$, receptors tracazolate produced concentration related potentiation of control GABA EC$_{20}$ responses however, for

$\alpha 1\beta 1/3\epsilon$ receptors GABA EC$_{20}$ responses were inhibited by tracazolate. Receptors containing a $\gamma 1$ or a $\gamma 3$ subunit produced an intermediate profile with low concentrations of tracazolate potentiating, to a small degree, the GABA EC$_{20}$, while higher concentrations caused inhibition. Interestingly, the apparent affinity of tracazolate for the potentiating component was approximately 10-fold higher for $\gamma 1$ and $\gamma 3$ compared to $\gamma 2$. This accuracy of this apparent EC$_{50}$ must be viewed with some caution since the low level of potentiation made curve fitting difficult. The largest degree of potentiation was observed with $\alpha 1\beta 1\delta$ receptors. On this receptor subtype tracazolate potentiated the GABA EC$_{20}$ response by 1368 $\pm$ 377%. This current was approximately three times that elicited by a maximum concentration of GABA. Potentiation of a GABA EC$_{10-25}$ response beyond that of the maximum GABA response has previously been demonstrated for isoflurane on $\alpha 1\beta 1\delta$ receptors (Lees & Edwards, 1998). One explanation of these results is that on $\alpha 1\beta 1\delta$ receptors GABA behaves as a partial agonist with a low probability of opening. This probability of opening is increased in the presence of tracazolate and isoflurane giving rise to a supermaximal response. Further evidence for GABA behaving as a partial agonist has been demonstrated using an Ltk$^-$ cell line stably expressing $\alpha 4\beta 3\delta$ receptors. Concentration-response curves to THIP elicit significantly larger responses than concentration-response curves to GABA (Adkins et al., 2001; Brown et al., 2001). Generation of chimeras between either the γ and ϵ or δ and ϵ subunits may identify amino acid residues responsible for the opposing functional effects of tracazolate.

6.3.4. Differing effects on GABA concentration-response curves.

Concentration-response curves to GABA in the absence and presence of tracazolate on $\alpha 1\beta 3\gamma 2s$, $\alpha 1\beta 3\epsilon$ and $\alpha 1\beta 1\delta$ receptors revealed further insights to the mechanism of action of tracazolate. GABA concentration-response curves on both $\alpha 1\beta 3\gamma 2s$ and $\alpha 1\beta 1\delta$ receptors were shifted to the left with significantly lower EC$_{50}$ values. The maximum response to GABA however, in the presence of increasing concentrations of tracazolate, were shifted in opposing directions; on $\alpha 1\beta 3\gamma 2s$ tracazolate reduced the maximum response to GABA while on $\alpha 1\beta 1\delta$ this was increased. The leftward shift in the GABA concentration response curve with a reduction in the maximum response for $\alpha 1\beta 3\gamma 2s$ receptors is similar to that reported for loreclezole (Wafford *et*

al., 1994) and SB-205384 (Meadows *et al.*, 1997) and may indicate a common mechanism of action of these compounds. The similarities of these three compounds also extend to the selectivity for β2/3 containing receptors over β1 containing receptors. Benzodiazepine site ligands also produce a leftward shift in the GABA concentration-response curve however, they cause no reduction in the maximum response (Sigel & Baur, 1988; Maksay *et al.*, 2000).

On α1β3ε receptors tracazolate behaved as a non-competitive antagonist reducing the maximum response to GABA with no change in the log EC$_{50}$ value or Hill coefficients. The biochemical and electrophysiological data suggests that tracazolate is behaving as an allosteric modulator of the GABA$_A$ receptor at a site other than the benzodiazepine binding site. Reports have identified a number of allosteric modulators that can produce opposing effects depending on the receptor subtype e.g. Ro15-4513 which potentiates α6β2γ2s receptors and inhibits α1β2γ2s receptors (Hadingham *et al.*, 1996; Hauser *et al.*, 1997) and mefenamic acid which potentiates α1β2γ2s receptors but inhibits α1β1 receptors (Halliwell *et al.*, 1999).

The results presented in this chapter have shown that the functional response observed to a concentration of tracazolate is dependent on both the receptor subtype and concentration of GABA used.

6.3.5. Possible effects of tracazolate *in vivo* on ε and δ containing receptors.

As discussed in chapter 3 the ε subunit is highly localised to the hypothalamus and hippocampus. Inhibition of GABAergic systems within these areas will lead to a net excitability which could effect hormonal feedback systems and / or the function of the hippocampus i.e. learning and memory. Like the ε subunit, the δ subunit also displays restricted distribution being found in the cerebellar granule cell layer, the thalamus and the dentate gyrus of the hippocampus (Benke *et al.*, 1991). Evidence to date suggests that δ containing receptors are located extrasynaptically (Nusser *et al.*, 1998) and are exposed to low concentrations of GABA. Tracazolate will cause a significant degree of potentiation of δ containing receptors which will result in an increase in the overall inhibitory tone.

6.3.6. Putative kinetic model of the mechanism of action of tracazolate.

At the macroscopic level, the effect of benzodiazepine agonists, barbiturates, such as pentobarbitone, and neurosteroids is to increase the peak amplitude of submaximal concentrations of GABA. Studies at the single channel level however, have identified different molecular mechanisms of action. Barbiturates increase the mean open time of the channel (Twyman *et al.*, 1989), benzodiazepine agonists increase the frequency of channel opening (Study & Barker, 1981) while neurosteroids do both (Puia *et al.*, 1990). Similar kinetic and single channel studies for tracazolate will help in the understanding of its molecular mechanism of action.

The data obtained in this study combined with our current knowledge of GABA$_A$ receptors has enabled a putative kinetic model for the mechanism of action of tracazolate to be proposed. Previous studies using transiently transfected HEK-293 cells or stably expressing Ltk⁻ cells have shown that $\alpha\beta\epsilon$, $\alpha\beta\gamma2$ and $\alpha\beta\delta$ receptors have significantly different desensitization rates, with $\alpha\beta\epsilon$ receptors desensitizing significantly faster than $\alpha\beta\gamma2$ which in turn desensitize faster than $\alpha\beta\delta$ receptors (Saxena & Macdonald, 1994; Whiting *et al.*, 1997; Brown *et al.*, 2001). Figure 6.15 shows the various states a receptor such as $\alpha1\beta3\gamma2s$ can enter upon application of GABA. In the absence of GABA the receptor is in an unbound closed conformation depicted R. Binding of two GABA molecules produces a GABA bound conformation (AR) in which the channel is still closed. This GABA bound closed conformation can then isomerise to GABA bound open conformation as depicted AR* which, in turn, isomerises to a GABA bound desensitized conformation in which the channel is closed (ARD). For $\alpha1\beta3\epsilon$ receptors, which have a level of constitutive-activity, two additional steps (R* and RD) are included.

It is hypothesised that tracazolate facilitates the isomerisation from the closed to open state (i.e. enhances GABA gating) and from the open to desensitized state with a greater affinity for the second isomerisation step. In situations where the receptor rapidly enters the desensitized state (e.g. $\alpha1\beta1/3\epsilon$ or high GABA concentrations on $\alpha1\beta3\gamma2s$) the functional response to tracazolate is inhibition, whereas situations in which very few channels are entering the desensitized state (e.g. $\alpha1\beta1\delta$ or low GABA

concentrations on α1β3γ2s) the functional response is potentiation. This hypothesis however has not considered any effects tracazolate may be having on the association or deactivation rate of the GABA response which has been discussed above. Higher affinity for the desensitized state over the agonist-bound state has been reported for ifenprodil on NMDA receptors (Kew *et al.*, 1996).

Figure 6.15. Putative kinetic model of the mechanism of action of tracazolate on GABA$_A$ receptors.

A) α1β1/3γ2s

B) α1β1/3ε
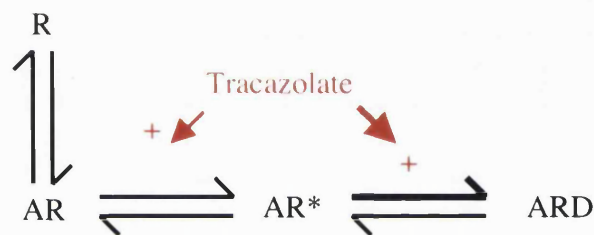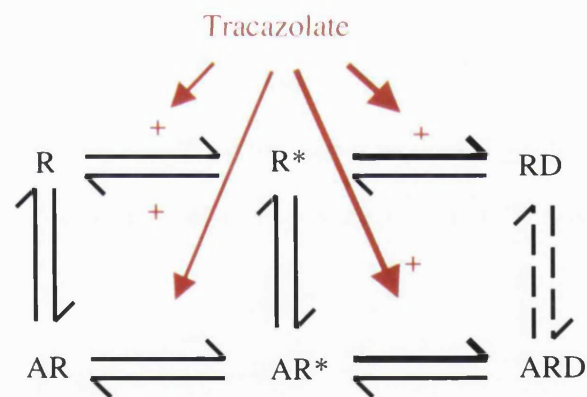
Note the bias for isomerisation from the open to desensitized state as depicted by thicker black arrows and the bias for tracazolate for this step as depicted by the thicker red arrows.

6.4.   Summary.

This is the first detailed study examining the effects of tracazolate on recombinant human GABA$_A$ receptors. Tracazolate does not bind to either the benzodiazepine or

GABA binding site but to another as yet unidentified site. Its potency (EC_{50}) is influenced by the nature of the β subunit but more importantly its intrinsic efficacy i.e. whether it potentiates or inhibits GABA, is determined by the nature of the third subunit within the $\alpha\beta$ complex. The mechanism of action at the $GABA_A$ receptor may be due to stabilisation of the open and desensitized state. It may prove to be a useful tool and aid identification of receptor subtypes within neuronal preparations.

Chapter 7

Mutation at the putative GABA$_A$ ion channel gate reveals changes in allosteric modulation

7.1. Introduction.

The high affinity binding of radiolabelled agonists for ligand-gated ion channels usually contrasts with the relatively low functional affinity they have at receptors (Edgar *et al.*, 1992) and this has recently been compared on recombinant $GABA_A$ receptors (Ebert *et al.*, 1997). The binding affinity (pKi) for GABA agonists such as muscimol, GABA, and 4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) is generally two to three orders of magnitude higher than the pEC_{50}'s measured electrophysiologically (Ebert *et al.*, 1997). A functional measure is more complex than a binding measure since the functional measure reflects binding, transduction, channel opening and desensitization. Receptors in a binding assay are thought to enter a long-term, high affinity, desensitized state which is rarely present in functional studies and hence could account for the observed differences.

Mutation of the conserved leucine at the 9' position of the putative second membrane-spanning domain of several ligand-gated ion channels has produced receptors with up to 1000-fold increased agonist sensitivity (Labarca *et al*, 1995; Chang *et al.*, 1996). These mutations bring the functional EC_{50} close to the affinity measured by ligand binding. The report by Chang *et al.* (1996) studied the stoichiometry of the $GABA_A$ receptor, however it did not address the effects the mutation had on allosteric modulators or antagonists of the $GABA_A$ receptor.

The aim of this study was therefore to investigate the effects mutation of the conserved leucine to serine, within the second transmembrane domain of the $\beta 2$ subunit of the $\alpha 1\beta 2\gamma 2s$ receptor, had on the actions of a number of $GABA_A$ agonists, antagonists and allosteric modulators (Thompson *et al.*, 1999b). The mutation was generated within the $\beta 2$ subunit and not $\alpha 1$ or $\gamma 2s$, since in the study of Chang *et al.* (1996) this caused the greatest leftward shift in the GABA concentration-response curve.

7.2.   Results.

7.2.1.   α1β2L159Sγ2s receptors have decreased agonist EC$_{50}$ values and slower desensitization kinetics.

As previously reported (Chang *et al.*, 1996), this mutation decreased the GABA EC$_{50}$ (147-fold) resulting in mean EC$_{50}$ values of 136nM (n=6) on α1β2L259Sγ2s compared to 20μM on α1β2γ2s (n=7) (Figure 7.1A and Table 1). In addition significantly smaller currents to a maximum concentration of GABA were observed in α1β2L259Sγ2s receptors (92.5 ± 6.3 nA at 30μM, n=62) compared to α1β2γ2s (2164 ± 304 nA at 3mM, n=17). Similarly the full agonist muscimol and the partial agonists, THIP and P4S, also exhibited a leftward shift in the EC$_{50}$ values for the mutant receptor compared to the wild type (Figure 7.1B and C and Table 1). Interestingly the maximum efficacy for P4S was significantly higher (*P*<0.001) on the mutant receptor compared to the wild type, whereas the efficacy for THIP was unchanged.

There were also marked differences in the rate of desensitization of GABA$_A$ receptors measured in oocytes containing the β2L259S mutant (Figure 7.2). The desensitization following maximum GABA concentrations was fitted best in both wild type and mutant by a single exponential curve. The mutation slowed the rate of desensitization (tau) from 49.7 ± 10.2 sec (n=4) in wild type to 143 ± 17.5 sec in the mutant receptor (n=4).

Figure 7.1. Concentration-response curves to GABA, THIP and P4S on α1β2L259Sγ2s and α1β2γ2s receptors.
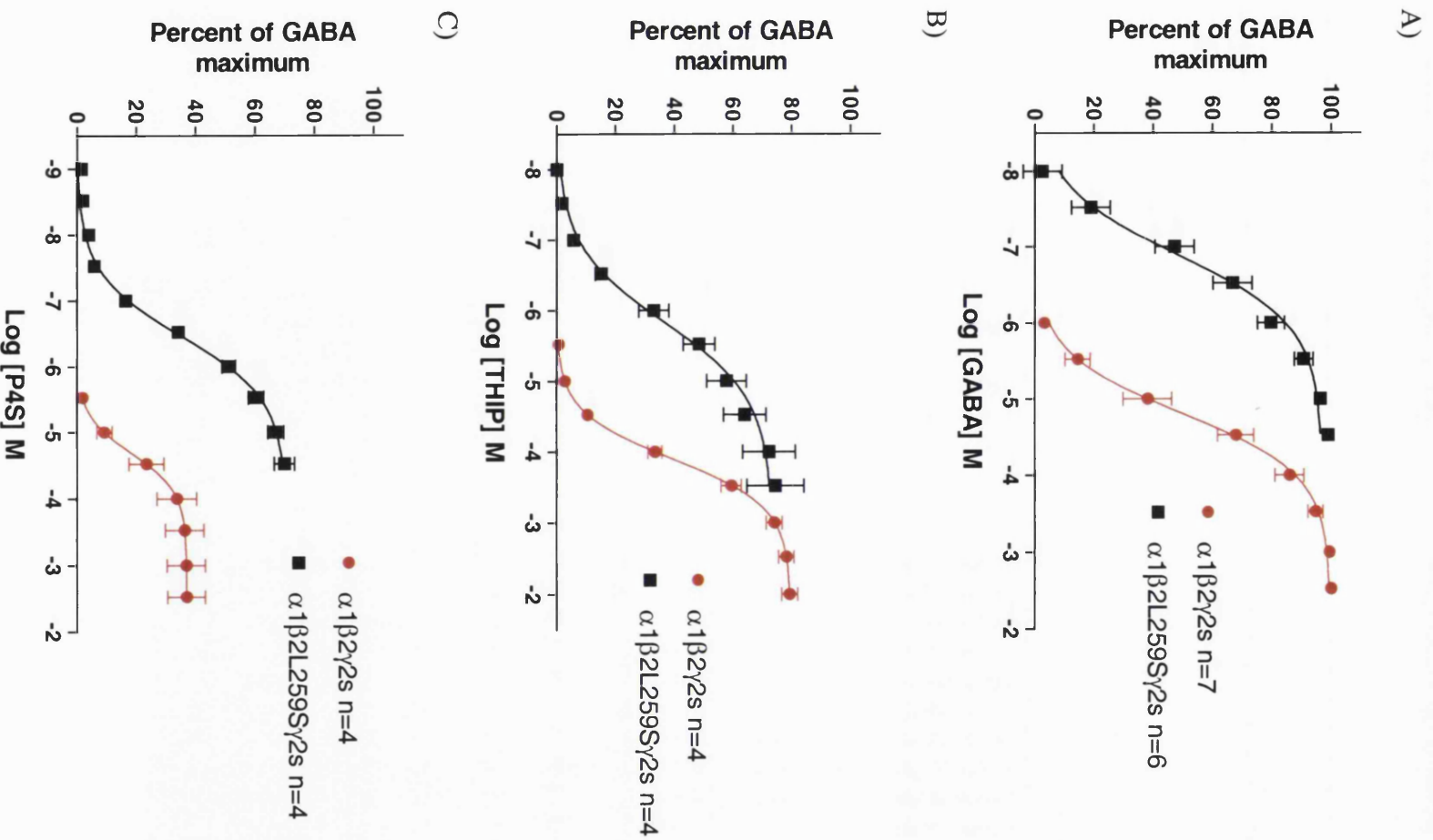
Table 7.1. EC$_{50}$, Hill coefficient and maximum response for concentration-response curves to GABA, muscimol, THIP and P4S on α1β2γ2s and α1β2L259Sγ2s receptors.

Receptor	EC$_{50}$ (μM)	Hill Coefficient	Maximum Response (%)	No. of Oocytes
GABA				
α1β2γ2s	20 ± 3	NR	100	7
α1β2L259Sγ2s	0.136 (0.097, 0.191)	0.84 ± 0.05	100	6
Muscimol				
α1β2γ2s	3.6 (2.9, 4.4)	1.52 ± 0.15	99 ± 3.1	4
α1β2L259Sγ2s	0.101 (0.081, 0.126)	0.68 ± 0.09	107 ± 3.0	4
THIP				
α1β2γ2s	143 ± 15	NR	76 ± 4	4
α1β2L259Sγ2s	1.5 (1.3, 1.8)	0.84 ± 0.08	74 ± 10	4
P4S				
α1β2γ2s	25 ± 4	NR	38 ± 5	4
α1β2L259Sγ2s	0.347 (0.310, 390)	0.90 ± 0.05	71 ± 3	4

NB The data for GABA, THIP and P4S on α1β2γ2s were generated by Prof. Bjarke Ebert (Ebert *et al.*, 1994) and permission for reproduction obtained. NR = not reported.

Figure 7.2. Response to a maximum concentration of GABA on α1β2L259Sγ2s and α1β2γ2s receptors.
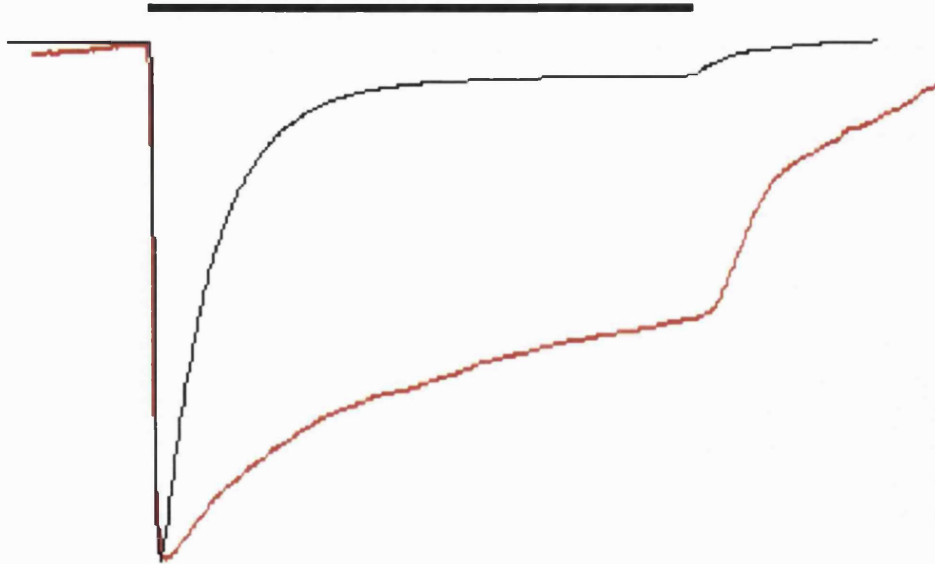
The black trace represents the inward current to 1mM GABA on α1β2γ2s receptors while the red trace represents the inward current to 30μM GABA on α1β2L259Sγ2s receptors. The solid black horizontal line represents the agonist application time of 5 mins. The current for α1β2L259Sγ2s has been scaled to the wild type amplitude to illustrate the differences in time courses.

7.2.2. α1β2L159Sγ2s receptors display a high level of constitutive-activity.

The mutant receptors also displayed a greater leak current (between 200-400nA) compared to the wild type (between 30-100nA) when voltage-clamped at –70mV. In the absence of GABA, the non-competitive GABA$_A$ antagonist picrotoxin (100μM) produced apparent outward currents on α1β2L259Sγ2s receptors (181 ± 17nA n=41) while having no effect on oocytes expressing α1β2γ2s receptors or uninjected oocytes. These observations suggest that the mutant receptor has a proportion of constitutively-active channels whereas wildtype and uninjected oocytes do not. Interestingly the outward current to picrotoxin was larger than the inward current to a maximum concentration of GABA. Current-voltage relationships were determined for

the leak current and GABA EC_{50} activated current on α1β2L259Sγ2s and α1β2γ2s receptors using the stepping protocol as described in Chapter 2 (Figure 7.3). As discussed in Chapter 3, the leak current of an oocyte injected with $GABA_A$ receptor subunits represents the summation of any constitutive-activity which may be present and the leak current normally present in an uninjected oocyte. Second order polynomial fits (see Chapter 2 equation (6)) to the leak current revealed reversal potentials of -28.5 ± 3.9mV (n=4) for α1β2L259Sγ2s and –36.2 ± 7.8 (n=4) for α1β2γ2s compared with reversal potentials for the GABA EC_{50} activated current of -26.3 ± 3.9mV (n=4) for α1β2L259Sγ2s and -21.3 ± 4.2 mV (n=5) for α1β2γ2s. These reversal potentials were not significantly different from one another (ANOVA *P*=0.26).

Figure 7.3. Current-voltage relationships for the leak current and GABA EC$_{50}$ activated current on α1β2L259Sγ2s and α1β2γ2s receptors.
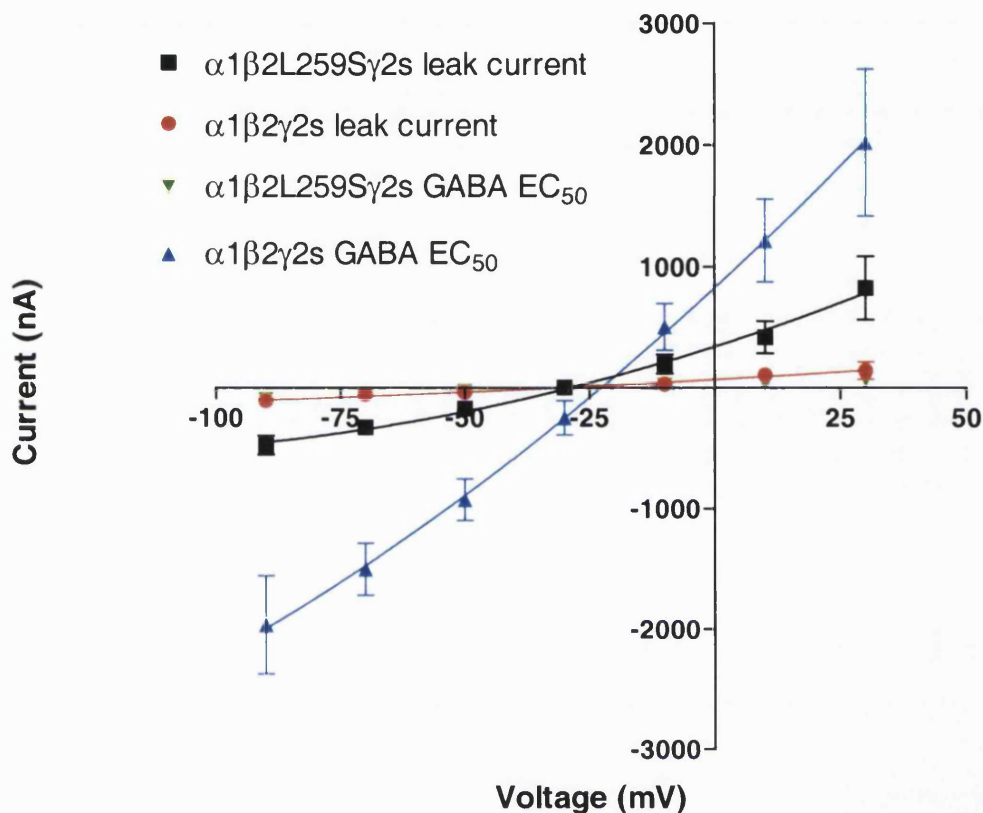
The leak current represents the current required to maintain the oocyte at the holding voltage in the absence of GABA, whereas the GABA EC$_{50}$ represents the inward or outward current in response to the application of the GABA EC$_{50}$ concentration i.e. the leak current has been subtracted. Note that the mutant receptor has a larger leak current and smaller GABA-activated currents compared to wild type receptor.

7.2.3. Effects of competitive antagonists.

Similar to picrotoxin, the competitive antagonists bicuculline and SR95531, also produced apparent outward currents on $\alpha 1\beta 2L259S\gamma 2s$ receptors. The degree of block of constitutive-activity to increasing concentrations of competitive antagonists bicuculline and SR95531 were normalized to the outward current produced by $100\mu M$ picrotoxin. Bicuculline produced a maximum inhibition of 85 ± 4% with a pIC_{50} of 5.5 ± 0.09 (n=4), while SR95531 only produced a maximal inhibition of 13 ± 1 % with a pIC_{50} of 6.8 ± 0.05 (n=4) (Figure 7.4A). The block of constitutive-activity by bicuculline could be reversed by co-application of SR95531 (Fig. 7.4B), demonstrating the competitive nature of these compounds. Concentration-response curves to GABA in the absence and presence of bicuculline ($10\mu M$ and $100\mu M$) and SR95531 ($1\mu M$ and $10\mu M$) were performed on both the mutant and wild type receptor (Figure 7.5). As expected these two compounds produced parallel shifts to the right of the GABA concentration-response curves on both wild type and mutant receptors. The values obtained for the shift in the GABA EC_{50} were used to derive the pKi of the antagonist (see Chapter 2 equation (4)) (Table 7.2).

Table 7.2. pKi values for bicuculline and SR95531 on $\alpha 1\beta 2\gamma 2s$ and $\alpha 1\beta 2L259S\gamma 2s$ receptors.

Receptor	Bicuculline	SR95531
$\alpha 1\beta 2\gamma 2s$	5.6 ± 0.05	6.5 ± 0.02
$\alpha 1\beta 2L259S\gamma 2s$	5.0 ± 0.09	6.9 ± 0.06

The data represents the mean ± SEM from four oocytes.

Figure 7.4. Inhibitory effects of bicuculline and SR95531 on α1β2L259Sγ2s receptors.

A) Concentration-response curves for the outward current of bicuculline and SR95531 normalised to the outward current generated by 100μM picrotoxin on α1β2L259sγ2s receptors.
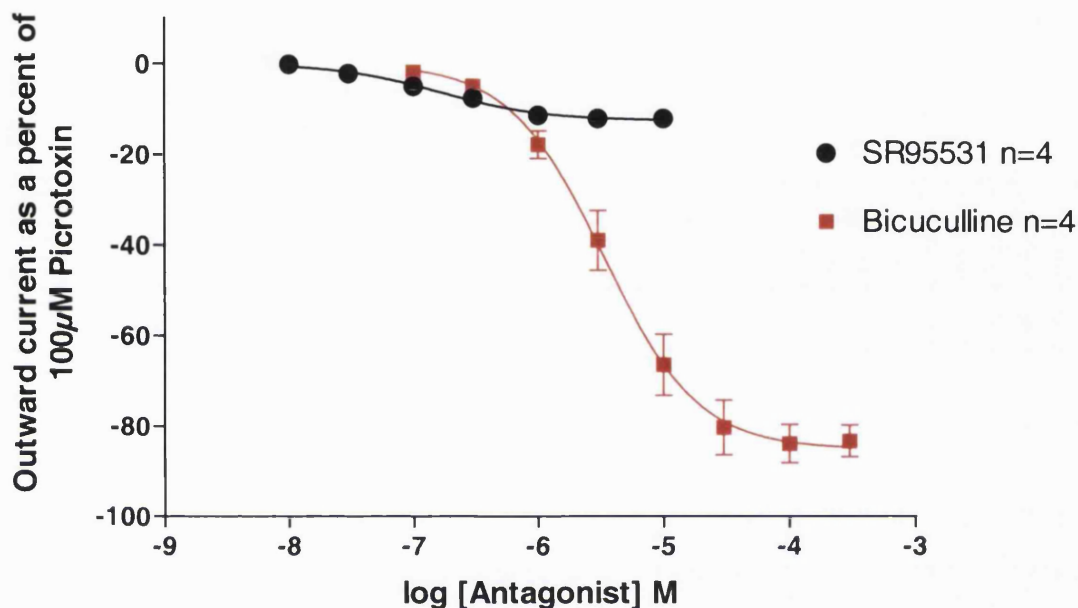
B) Outward currents to 30μM bicuculline were inhibited by co-application of 10μM SR95531.
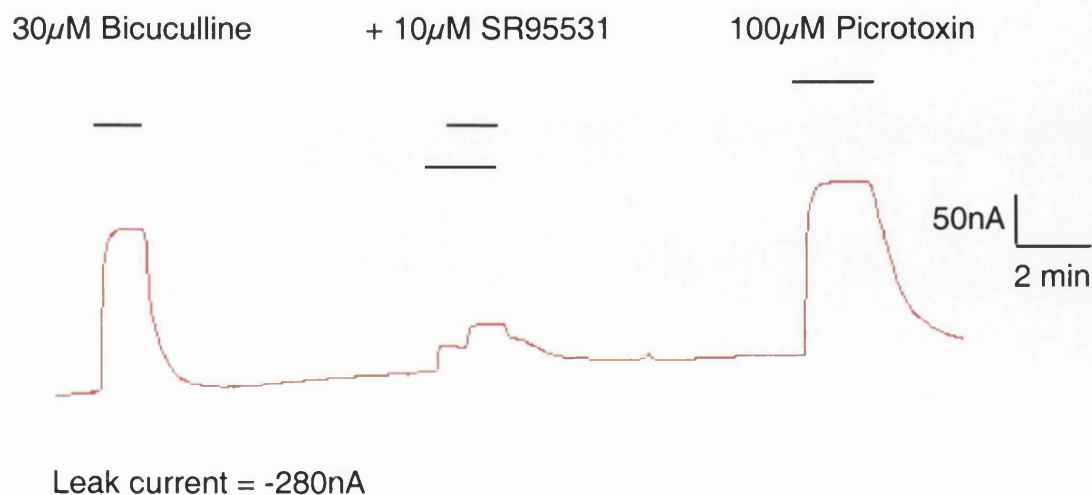
Figure 7.5. Concentration related shifts of the GABA concentration-response curves by SR95531.

A) α1β2γ2s
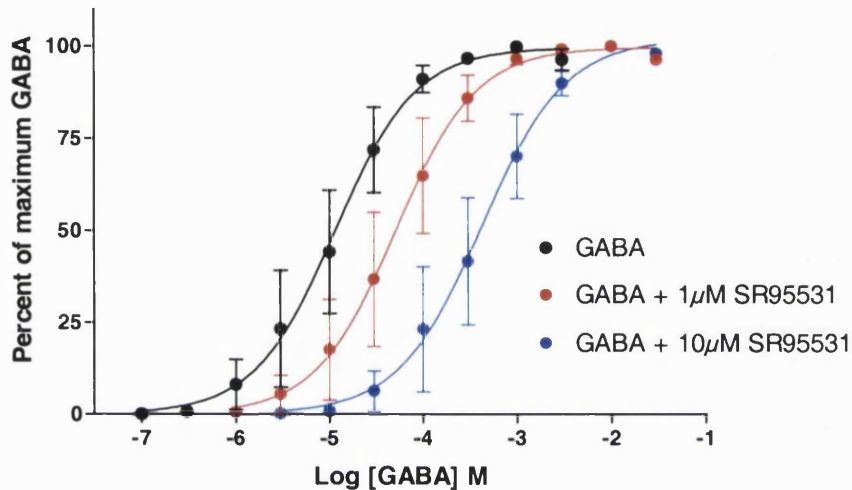
B) α1β2L259Sγ2s
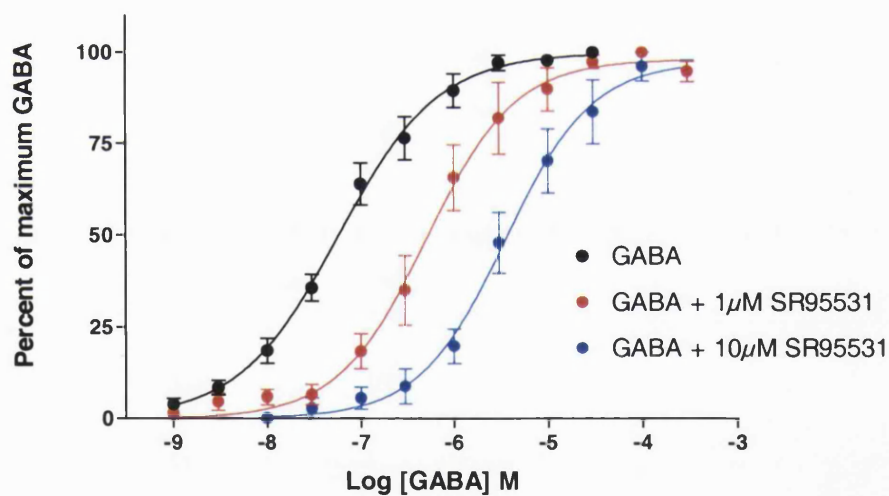
Concentration-response curves to GABA in the absence and presence of SR95532 (1μM and 10μM) on *Xenopus* oocytes expressing A) α1β2γ2s and B) α1β2L259Sγ2s receptors. All three concentration-response curves (control GABA, + 1μM SR95531, + 10μM S95531) were performed in an individual oocyte. Data were normalised to the maximum GABA response obtained for the control GABA concentration-response curve and fitted using equation (1) (see Chapter 2).

7.2.4. Allosteric modulation by benzodiazepines.

Five benzodiazepine site ligands were selected for study on the mutant receptor, reflecting structural diversity, as well as differing levels of intrinsic efficacy, ranging from full agonists such as flunitrazepam, through partial agonists such as CL218,872 to the inverse agonist, DMCM. The modulation of submaximal GABA concentrations (EC_{20}) by maximally effective concentrations of each compound were examined. While exhibiting no direct effects, but marked modulation of GABA currents on wild type α1β2γ2s receptors, the EC_{20} response of α1β2L259Sγ2s receptors was not potentiated by any of the positive allosteric modulators, however they all produced apparent direct effects in the absence of GABA (Figure 7.6). Ignoring the direct effect, the GABA EC_{20} response in the presence of the positive allosteric modulators was in fact inhibited when compared to the GABA EC_{20} control (Figure 7.6 and Figure 7.8). In comparison, the benzodiazepine inverse agonists DMCM and β-CCM, produced outward currents in the absence of GABA, but again did not modulate the GABA EC_{20}. These direct effects of benzodiazepines were blocked by the benzodiazepine antagonist Ro15-1788 (1μM) and by 30μM bicuculline (Figure 7.7), suggesting that these currents resulted from modulation of the constitutively-active channels. 30μM bicuculline was chosen since this concentration produced a near maximal block of the constitutive current (as Figure 7.4 illustrates this equates to 80 ± 6% of the outward current induced by 100μM picrotoxin). Block of the direct effect of the benzodiazepines by 30μM bicuculline did not however restore the potentiation of GABA currents by benzodiazepine agonists (Figure 7.8). Interestingly, it did restore the inverse modulation of the GABA EC_{20} by DMCM and β-CCM (Figure 7.8).

Figure 7.6. Representative traces showing the effects of flunitrazepam, CL-218,872, zolpidem and DMCM on α1β2γ2s and α1β2L259Sγ2s receptors.
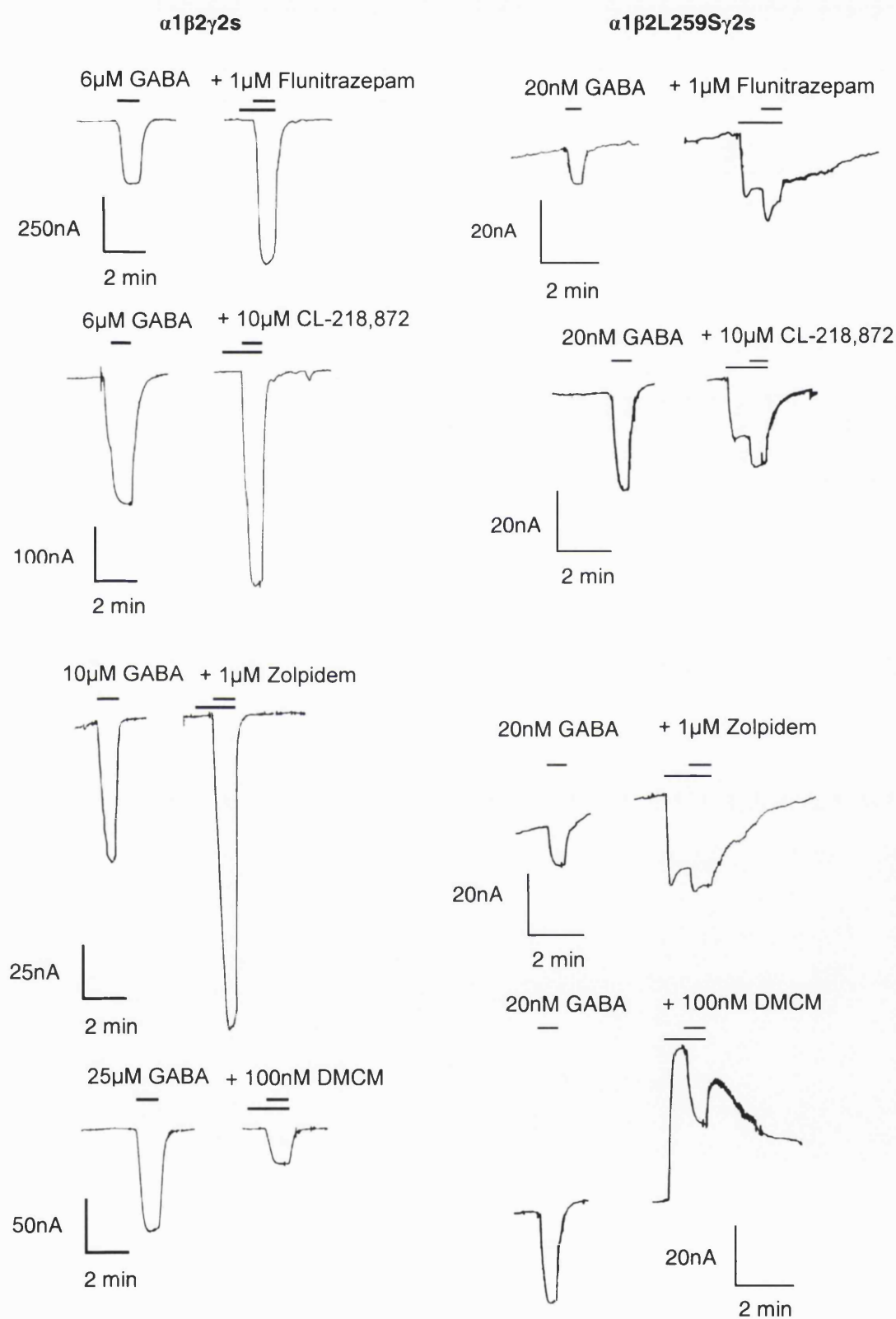
Figure 7.7. Direct currents to the benzodiazepine site ligands on α1β2L259Sγ2s receptors are inhibited by co-application of Ro15-1788 and bicuculline.

A)
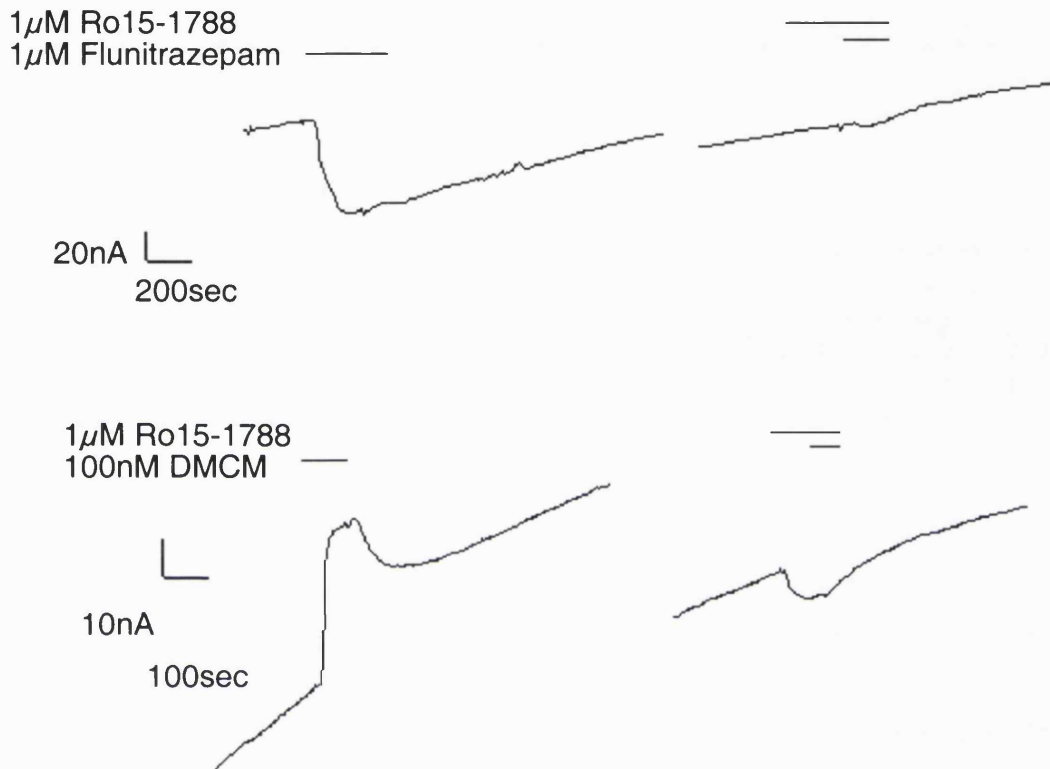
A) Inward currents to flunitrazepam and outward currents to DMCM are completely inhibited by Ro15-1788. Note the apparent inward current to Ro15-1788 following DMCM is most likely due to the very slow dissociation of previously applied DMCM, as no currents were observed when Ro15-1788 was applied alone.

B)
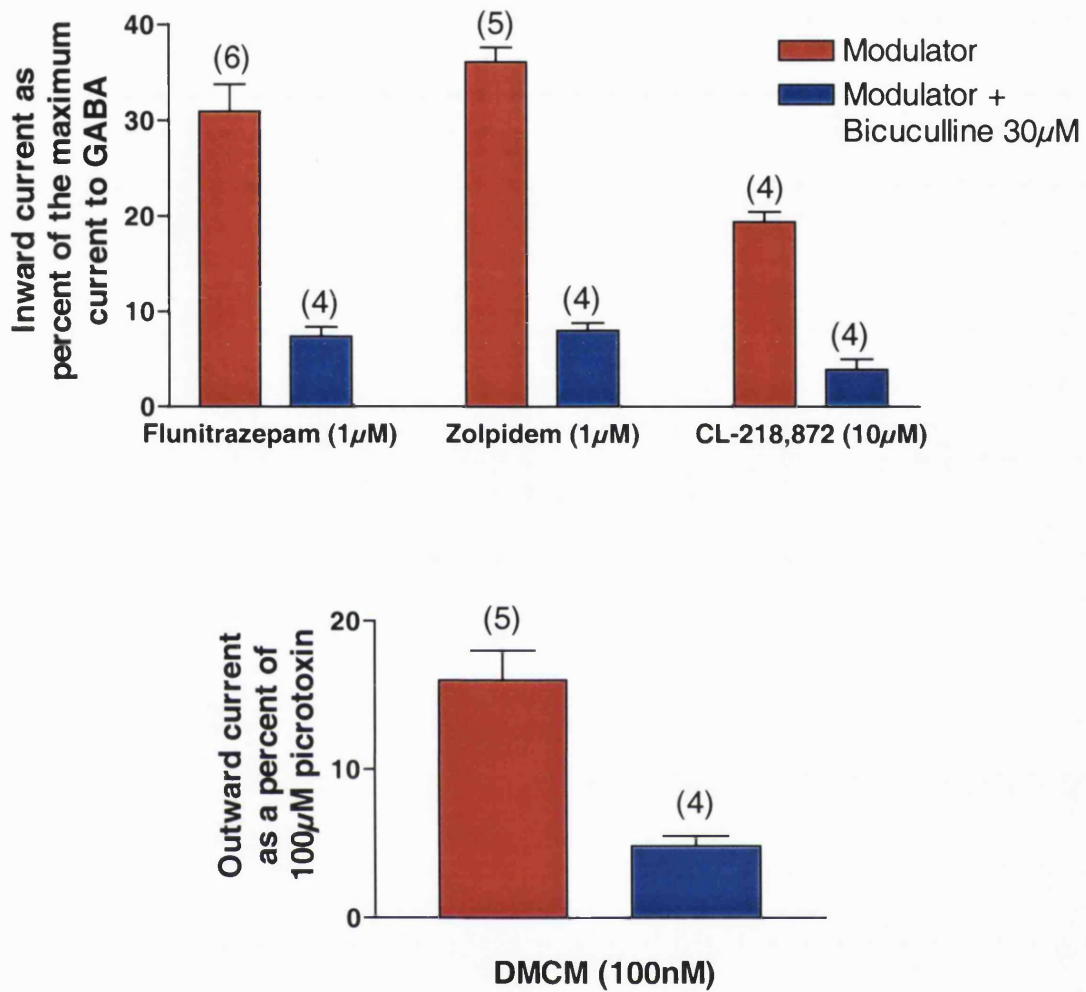
B) Inward currents to flunitrazepam, zolpidem and CL-218,872, normalised to the maximum GABA current (100%), and outward currents to DMCM, normalised to the outward current with 100μM picrotoxin (100%), were inhibited by co-application of bicuculline. Number in parenthesis above each bar represent the number of oocytes.

Figure 7.8. Modulation of the GABA EC_{20} response by the benzodiazepine ligands on $\alpha1\beta2\gamma2s$ and $\alpha1\beta2L259S\gamma2s$ receptors and $\alpha1\beta2L259s\gamma2s$ in the presence of bicuculline.
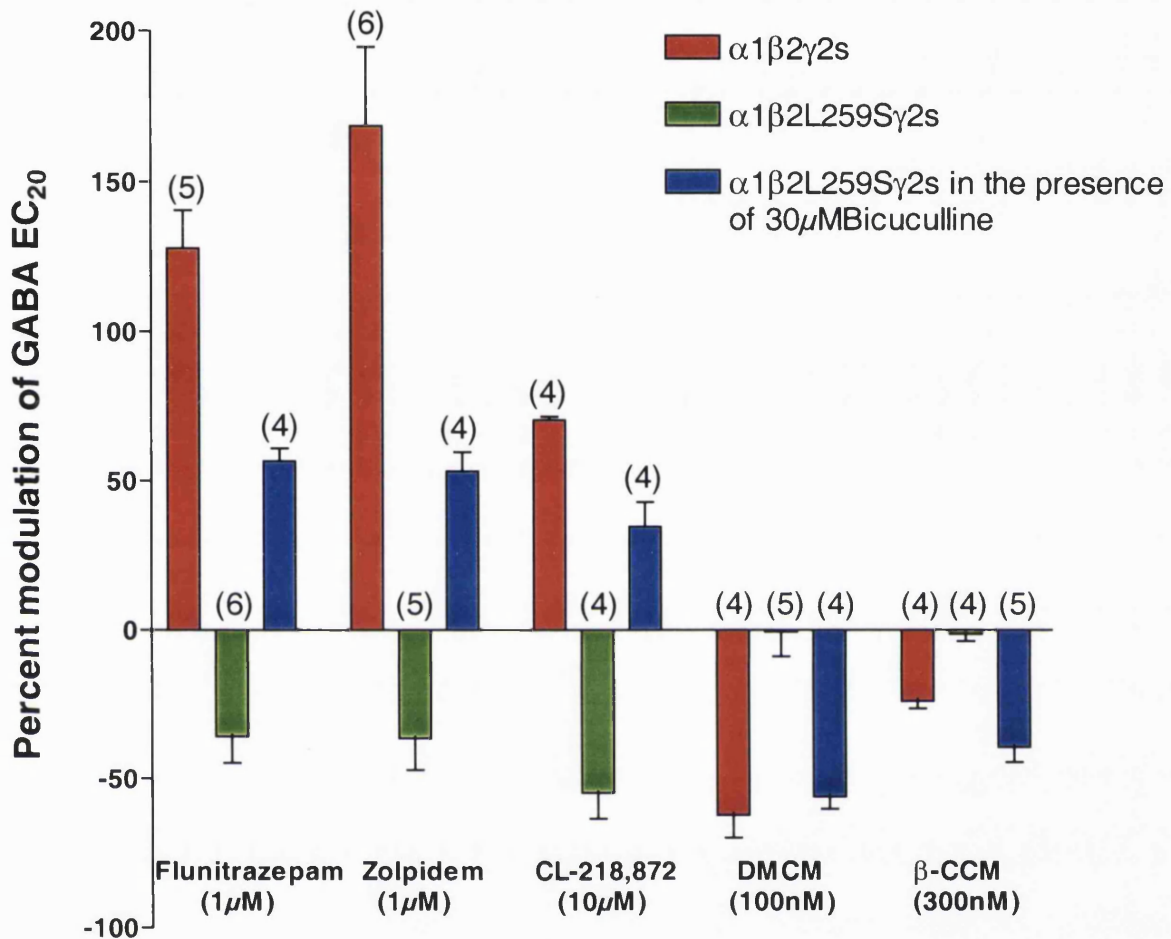
The percent modulation was calculated ignoring any direct effect i.e. the direct effect of the benzodiazepine site ligand became the baseline from which the inward or outward response to the GABA EC_{20} plus benzodiazepine site ligand was measured. Note the direct effect was greatest for $\alpha1\beta2L259S\gamma2s$ in the absence of bicuculline, followed by $\alpha1\beta2L259S\gamma2s$ in the presence of bicuculline and absent from $\alpha1\beta2\gamma2s$ receptors.

7.2.5. Effects of other allosteric modulators.

As demonstrated in Chapter 4, pentobarbitone potentiates α1β2γ2s receptors at concentrations between 10μM and 100μM, and at higher concentrations directly activates the receptor. On α1β2L259Sγ2s receptors, concentration-response curves for the direct activation by pentobarbitone showed a significant, ($P<0.001$) 10-fold decrease in EC_{50} (36.7 (32.6, 41.3)μM) compared to wild type (314 (252, 392) μM) (Figure 7.9). As figure 7.9 illustrates the direct activation on α1β2L259Sγ2s receptors was compromised by the marked inhibition seen at concentrations over 100μM. Hence, on the mutant receptor, both the direct activation and inhibition induced by pentobarbitone were laterally displaced to lower pentobarbitone concentrations. The rebound phenomenon (see Chapter 4) that occurs upon washout of pentobarbitone however, was not shifted being observed at 1mM pentobarbitone on both the wild type and mutant receptor. The potentiation of the GABA EC_{20} response observed in wild type receptors with pentobarbitone was absent in the mutant, and as can be seen from Figure 7.10, 3μM pentobarbitone was equally ineffective at potentiating a GABA EC_{20} suggesting that there is not a corresponding shift in EC_{50} for potentiation on α1β2L259Sγ2s. Similar to benzodiazepine modulation, potentiation by pentobarbitone was not restored when constitutive-activity was inhibited using 30μM bicuculline (Fig 7.10).

The neuroactive steroid allopregnanolone potentiated α1β2γ2s $GABA_A$ receptors at 1μM with no marked direct activation. In contrast large direct currents (36 ± 4 % of maximum GABA, n=4) were observed on α1β2L259Sγ2s receptors (Figure 7.11), and no potentiation of the GABA EC_{20}. Similar results were obtained with the β2/3-subunit selective compound loreclezole (10μM) and the anaesthetic compound propofol (10μM) i.e. potentiation of α1β2γ2s receptors with no direct activation compared with marked direct activation of α1β2L259Sγ2s receptors (loreclezole = 12 ± 1% of maximum GABA, n=4; propofol = 49.5 ± 3.9% of maximum GABA, n=4) and no potentiation (Figure 7.11). Unlike any of the other $GABA_A$ receptor modulators, co-application of GABA after the direct effect to propofol had reached a plateau produced no further receptor activation. This result is in contrast to 100μM

pentobarbitone which elicited a larger direct response than propofol but still produced a small degree in receptor activation on co-application of GABA.

Figure 7.9. Concentration-response curve for the direct activation of α1β1γ2s and α1β2L259Sγ2s receptors by pentobarbitone.
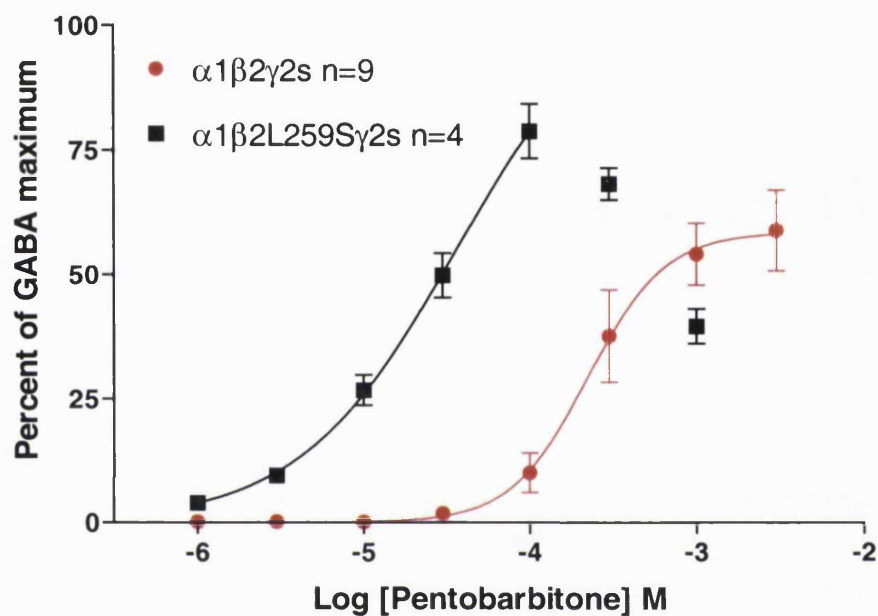
Note the curve for α1β2L259Sγ2s was only fitted up to 100μM with the maximum not constrained and therefore the EC_{50} quoted in the text represents an approximation.

Figure 7.10. Effects of 3 and 100μM pentobarbitone on a GABA EC$_{20}$ response on α1β2γ2s and α1β2L259Sγ2s receptors.
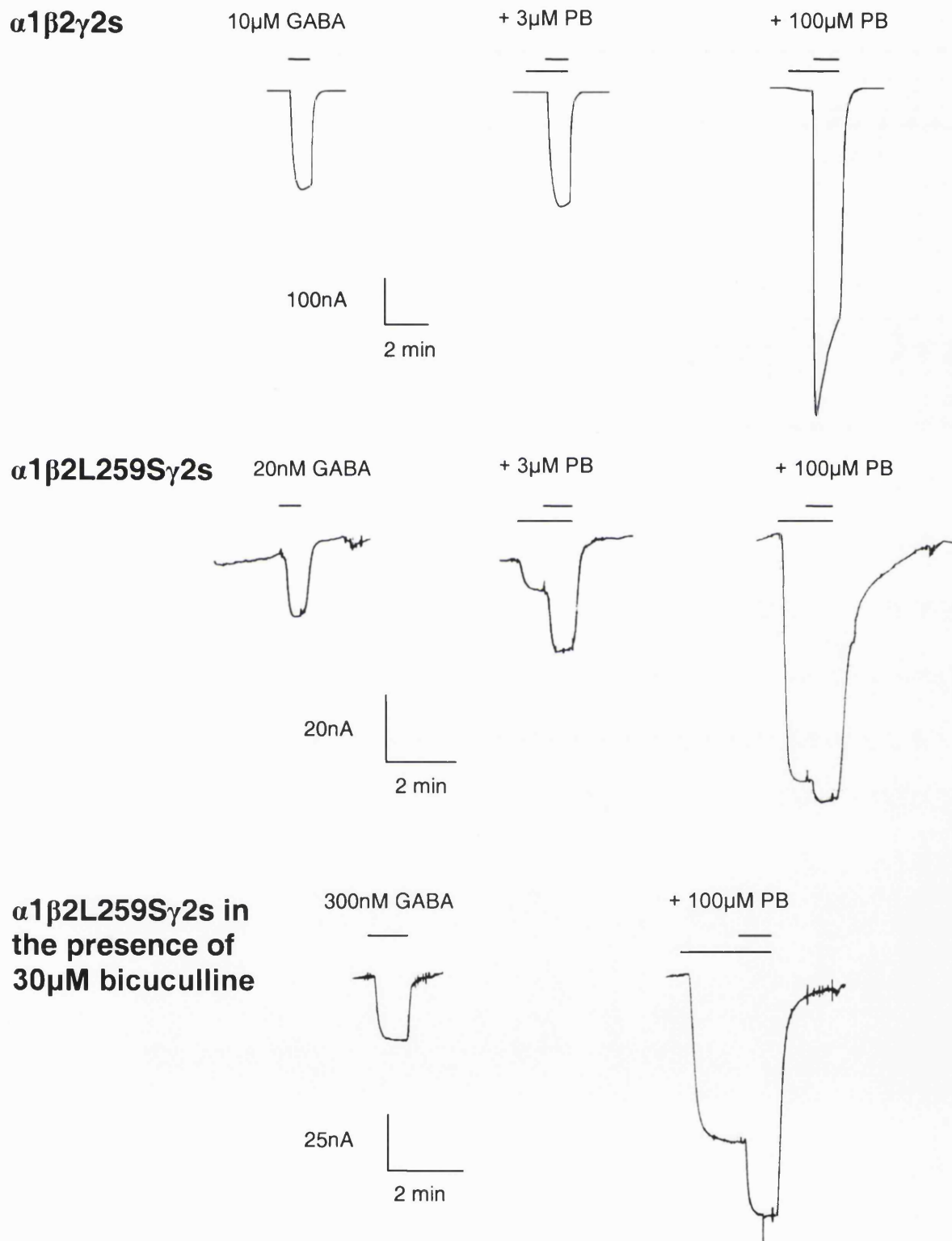
Figure 7.11. Effects of allopregnanolone, loreclezole and propofol on α1β2γ2s and α1β2L259Sγ2s receptors.
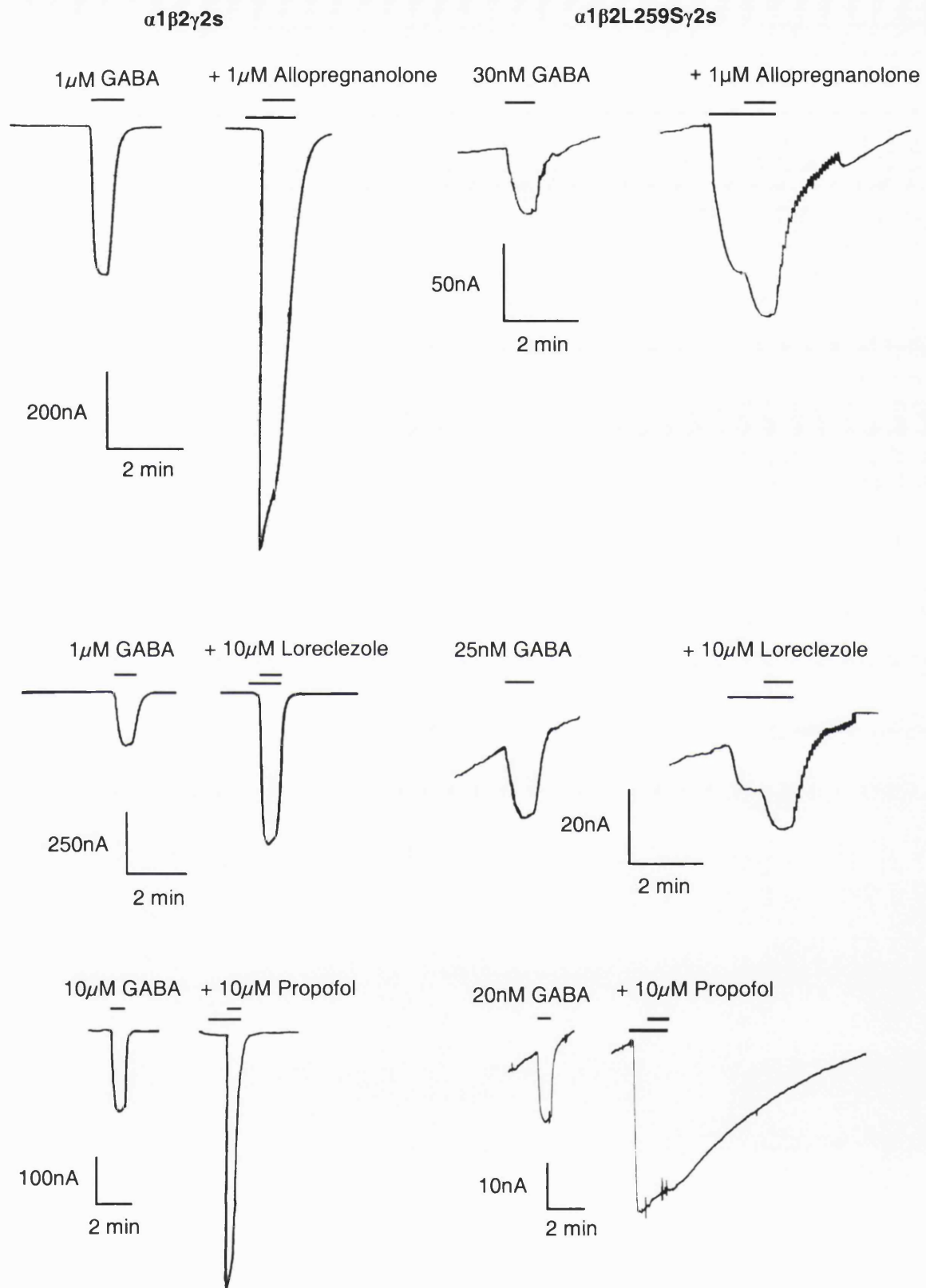
7.3.   Discussion.

The structure of the putative transmembrane domain 2 (TM2) of the GABA$_A$ receptor is thought to be close to that of other members of the ligand-gated ion channel family. Studies using the nicotinic acetylcholine receptor have demonstrated that the 9' leucine appears to be an important residue forming the ion channel gate (Unwin, 1995). Mutation of this residue to phenylalanine, valine, threonine or serine increases apparent acetylcholine sensitivity and reduces desensitization (Revah *et al.*, 1991). Mutation of the 9' leucine to serine within the β2 subunit of the GABA$_A$ receptor confers similar results (Chang *et al.*, 1996) and has been utilised to determine receptor stoichiometry. This chapter reports on the actions of a number of GABA$_A$ receptor agonists, antagonists and modulators on a human α1β2γ2s receptor in which the conserved 9' leucine within the β2 subunit was mutated to a serine.

7.3.1. Increased   affinity   of   GABA   agonists   and   pentobarbitone   on α1β2L259Sγ2s receptors.

Similar to the findings of Chang *et al.* (1996) expression of α1β2L259Sγ2s receptors resulted an increased sensitivity to GABA compared with wild type (147-fold). Corresponding increases in sensitivity were obtained with muscimol (36-fold), THIP (95-fold) and P4S (72-fold). Increased agonist sensitivity has also been reported for mutation of this conserved leucine in other members of the ligand-gated ion channel superfamily (α7 neuronal nAch, Revah *et al.*, 1991; 5-HT$_3$, Yakel *et al.*, 1993 and heteromeric muscle nAch, Akabas *et al.*, 1992; Filatov & White, 1995; Labarca *et al.*, 1995). In addition to a reduction in the EC$_{50}$ value, P4S was significantly more efficacious on α1β2L259Sγ2s than α1β2γ2s receptors. To explain partial agonism, del Castillo & Katz (1957) proposed that the binding step was separate from the conformational change:

$$A + R \;\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}\; AR \;\underset{\alpha}{\overset{\beta}{\rightleftharpoons}}\; AR^*$$

The agonist, A, binds to the receptor, R, to form a complex AR (the channel is closed). AR can then change conformation to the open state, AR*. K_1 is the equilibrium dissociation constant for the binding step while β represents the equilibrium constant for shut-open isomerisation. Assuming that the mutation of leucine to serine in $\beta 2$ has not altered the binding affinity, and this is unlikely as the mutation is not located near residues proposed to form the GABA binding site, one possible explanation for the increase in efficacy for P4S is that the mutation has increased β.

As illustrated in Chapter 3 pentobarbitone is able to directly activate the GABA$_A$ receptor via a site other than the GABA binding site. Interestingly $\alpha 1 \beta 2 L259S \gamma 2s$ receptors were more sensitive to the direct effect of pentobarbitone than wild type however, the leftward shift (10-fold) was not as marked as that observed with the GABA. This result supports the hypothesis that the agonist binding-site itself is not affected by the mutation.

In a recent study, Chang & Weiss, (1999) have mutated the conserved leucine in $\alpha 1$(L263), $\beta 2$(L259) and $\gamma 2$(L274) to serine and co-expressed all possible combinations with their wild type and/or mutant counterparts. The GABA EC_{50} value for $\alpha 1 \beta 2 L259S \gamma 2s$ when compared to wild type was shifted to the left by 881-fold, a value greater than the shift observed in this study (147-fold). Of all the possible mutant receptor combinations only $\alpha 1 L263S \beta 2 L259S \gamma 2s$ revealed a larger leftward shift (1205-fold) than $\alpha 1 \beta 2 L259S \gamma 2s$ (Chang & Weiss, 1999). These results demonstrated that there was not a clear stepwise relationship between the number of mutant subunits and the GABA EC_{50} i.e. there is a subunit asymmetry in either the role these leucines play in activation or in the degree of perturbation imparted by the position of the mutation. This is in contrast to the nACh receptor where mutation of each additional subunit imparted a ~10-fold increase in ACh sensitivity (Filatov & White, 1995; Labarca et al., 1995).

7.3.2. Mutation of the conserved leucine to serine decreased receptor desensitization.

Comparison of the desensitization rates to a maximum concentration of GABA on wild type and mutant receptors revealed that the mutant receptors had a significantly slower desensitization rate. This observation is also common to studies on other ligand-gated ion channels mutated at this position. The nicotinic ACh α7 receptor (Revah *et al.*, 1991, Bertrand *et al.*, 1992), muscle nicotinic ACh receptors (Labarca *et al.*, 1995), and 5HT₃ receptors (Yakel *et al.*, 1993) all show reduced desensitization compared to their respective wild type receptors when the corresponding leucine mutations are made. This observation would support the hypothesis that a normally desensitized state of the receptor becomes conducting when leucine is mutated to a serine.

7.3.3. Constitutive-activity of α1β2L259Sγ2s receptors.

In addition to increased sensitivity to agonists, the mutant channels were constitutively-active, reflected in large leak currents. This leak current reversed at the same potential as GABA indicating that the carrier was Cl⁻ and was blocked by the non-competitive inhibitor picrotoxin. Unwin (1993) has proposed that for the nACh receptor the leucine side-chains project into the pore forming a tight hydrophobic ring which closes the channel by making a barrier that hydrated ions cannot cross. Replacement of the non polar leucine residue (side chain volume 76 Å³) with the smaller polar serine residue (side chain volume 25 Å³) may reduce the effectiveness of this barrier allowing ions to flow when the receptor is in the inactive state. Outward currents to picrotoxin were also observed with α1β1ε receptors (Chapter 3) however, these were significantly smaller (*P*=0.001) than those measured for the mutant receptor. Unlike α1β1ε receptors (Chapter 3) the outward current to 100μM picrotoxin on α1β2L259Sγ2s receptors was larger than the maximum GABA elicited current. These results suggest that α1β2L259Sγ2s receptors have a greater degree of constitutive-activity than α1β1ε. Since the 9' leucine residue is conserved within all the subunits of the ligand-gated ion channels, including ε, additional, as yet

unidentified residues, must confer the constitutive-activity observed with α1β1ε receptors.

Interestingly, the constitutive-activity showed a very slow drift to less negative holding currents as can be seen in Figures 7.5, 7.6 and 7.7, the reason for this is unknown. One possible explanation could be the slow redistribution of chloride through the open channels when voltage-clamped at −70mV.

Constitutive channel activity, as indicated by outward currents to picrotoxin, is also observed on expression of GABA$_A$ β1 homomeric receptors (Sigel *et al.,* 1989; Krishek *et al.,* 1996), β3 homomeric receptors (Wooltorton *et al.,* 1997b) and for 9' mutations in the GABA ρ1 receptor (Pan *et al,* 1997, Chang & Weiss, 1998). On injection of the GABA$_A$ β2L259S alone, no currents were observed to GABA, and no leak conductances were present (data not shown), suggesting that unlike β1, β3 and ρ1 this construct did not form homomeric channels. This result is not surprising since homomeric murine β2 subunits are retained in the endoplasmic reticulum (Connolly *et al.,* 1996). Unlike ρ1, the α1β2L259Sγ2s receptor did not exhibit inhibition of constitutive-activity by low concentrations of GABA.

Mutation of the β1Leu259 to Thr has been reported to cause constitutive channel opening when expressed with α1 in Sf9 cells, but in this case GABA did not activate the receptor, and unlike the results here, were not blocked by picrotoxin or bicuculline (Tierney *et al.,* 1996). The equivalent mutation in the α1 subunit did result in GABA currents when expressed with β1, but in contrast to Chang *et al.,* (1998) no decrease in EC$_{50}$ was reported.

7.3.4. Mutation causes a reduction in the level of receptor expression.

Maximum currents to GABA were significantly reduced with receptors containing the mutant β2-subunit. In addition, assuming picrotoxin blocks all the constitutive current, maximum inward currents to GABA plus the outward current to 100μM picrotoxin were also significantly smaller than the maximum GABA response for wild-type receptors. On transfection into HEK-293 cells, no measurable

[³H]muscimol or [³H]flunitrazepam binding could be detected (data not shown), suggesting that the mutation may be affecting the level of receptor expression. This was consistent with the findings of Tierney *et al.* (1996) and Chang *et al.* (1998) who also described a decrease in expressed receptor following L9T in either the α1 or β1 subunit. It is, however, unlike that found with equivalent mutants in the nicotinic receptor where no reduction in current size was observed (Bertrand *et al.*, 1992).

7.3.5. The affinity of competitive antagonists was not affected by mutation of β2L259S.

The competitive antagonists, bicuculline and SR95531, produced rightward shifts of the GABA concentration-response curves on α1β2γ2s and α1β2L259Sγ2s receptors, with similar pKi values indicating that the antagonist affinity was not altered by the mutation. Interestingly Chang & Weiss (1999) have recently shown that the IC_{50} for picrotoxin mediated antagonism is increased with receptors containing the leucine 9' to serine mutation and that a correlation between the picrotoxin IC_{50} of block of the constitutive current and the number of mutated subunits within the pentamer exists, suggesting that the 9' leucine residue may play a role in the picrotoxin mediated antagonism.

In contrast to wild type receptors, bicuculline and SR95531 produced outward currents on the mutant receptors. These pIC_{50} values correlate well with the pKi's for competitive antagonism of wild type and mutant GABA receptors, suggesting this action to be via the same site. SR95531 showed only partial inhibition of the constitutive channel activity however, and antagonised the effect of bicuculline, indicating that this compound has less inverse activity than bicuculline and competes at the same site. These results suggest that bicuculline is acting as an allosteric inhibitor or inverse agonist at the GABA site, confirming the hypothesis previously postulated by Ueno *et al.* (1997) who showed allosteric inhibition of pentobarbitone currents by bicuculline on rat α1β2γ2 GABA_A receptors. The levels of inverse activity for bicuculline and SR95531 on receptors containing β2L259S correlate well with that shown on wild type pentobarbitone-gated currents, providing further evidence that bicuculline can allosterically inhibit channel activity.

7.3.6. Allosteric modulators fail to modulate submaximal GABA responses on α1β2L259Sγ2s receptors.

The action of several benzodiazepine site ligands with different intrinsic efficacy were tested on the mutant receptor and compared to the wildtype. Interestingly modulation of the GABA EC_{20} response was lost for both agonist and inverse agonists following mutation of β2L259. It was clear however, that these compounds maintained activity at the benzodiazepine binding-site, as all compounds showed apparent direct activation of the receptor, with efficacy correlating with that at the benzodiazepine site, including outward currents in response to the inverse agonist DMCM and β-CCM. Direct activation by the benzodiazepine ligands was completely inhibited by the benzodiazepine antagonist flumazenil, suggesting that the BZ site remained unaffected by the mutation. This is in agreement with the hypothesis that the BZ binding site is located at the α/γ interface (Sigel & Buhr, 1997, Wingrove *et al.*, 1997). One possible explanation for these BZ mediated currents is that the constitutive channel activity is being modulated by benzodiazepines, producing apparent BZ activated currents. This appears to be the case, as blocking the spontaneous activity with bicuculline and applying benzodiazepines significantly reduces the benzodiazepine-mediated currents. It does not, however, restore the potentiation of GABA-mediated currents, suggesting that the abolition of allosteric modulation is not due to the presence of constitutively open channels. Interestingly, the negative modulation by two inverse agonists, DMCM and β-CCM, is observable in the presence of bicuculline, which indicates that it is only the positive allosteric modulation that is affected.

In addition to the benzodiazepine site ligands the effects of pentobarbitone, propofol, the anaesthetic steroid allopregnanolone, and the β2/3-subunit selective modulator loreclezole were examined on mutant receptors. Similar to benzodiazepine modulation, the mutation also abrogates potentiation by any of these modulators, but again, the presence of constitutively-active channels reveals apparent direct effects with these compounds, most prominent with pentobarbitone, where the EC_{50} is decreased by 10-fold. As a consequence, 100μM pentobarbitone alone produces almost a saturating response overwhelming any additional GABA mediated response,

resulting in an apparent decrease in GABA modulation. Reducing the concentration of pentobarbitone to one that did not directly activate the receptor produced no potentiation, demonstrating that the EC_{50} for potentiation was not shifted in parallel with that for direct activation. In contrast, loreclezole produced only a small direct current (12% of maximum GABA) but did not potentiate GABA. Similar experiments performed in the presence of bicuculline to abolish constitutive-activity did not restore anaesthetic potentiation.

7.3.7. Comparison of the constitutive-activity, GABA EC_{50} and allosteric modulation.

A recent study in which the 15' serine has been mutated to tryptophan in the α2 and β1 subunit revealed some similarities to the results reported for the 9' leucine to serine mutation (Findlay *et al.*, 2001). α2S270Wβ1, α2β1S265W and α2S270Wβ1γ2s receptors were more sensitive to GABA, had constitutive channel activity and were not potentiated by pentobarbitone, allopregnanolone or flunitrazepam. Interestingly when compared to wild type receptors, the maximal GABA response and desensitization rates of these mutant receptors was unchanged. Similarly α1β1εTIGR receptors have an increased sensitivity to GABA, constitutive-activity and are not modulated by anaesthetic agents (see Chapter 3). The commonality between these receptors is therefore the presence of constitutive-activity, increased GABA sensitivity and the lack of modulation by allosteric agents.

The GABA EC_{50} values for each of these receptors are similar; α1β2L259Sγ2s $0.13\mu M$, α2S270Wβ1 $0.4\mu M$ α2β1S265W $1.6\mu M$ (Findlay *et al.*, 2001) and α1β1εTIGR $0.35\mu M$. As the GABA EC_{50} is shifted to the left, increasing the likelihood of channel opening, it approaches that measured by radioligand binding or the highest achievable affinity. If the mechanism of allosteric potentiation is to increase channel activity, either by increasing channel opening frequency or mean open time, there must be a limit beyond which no further leftward shift is possible. This window could be defined as that between the functional EC_{50} and that measured by radioligand binding. By shifting the GABA EC_{50} closer to the intrinsic binding affinity, it is likely that no further shift is possible and positive allosteric potentiation

is reduced. If this is the case, then positive allosteric modulators would be inactive but negative allosteric modulation would still be possible. This does appear to be the case with DMCM and β-CCM, which, in the presence of bicuculline, produces an effect identical to the wild type receptor, although it does not explain the lack of effect of DMCM and β-CCM in the absence of bicuculline. It must be noted however that a number of allosteric modulators have been shown to cause enhancement, albeit around 2-fold, of GABA receptor binding suggesting that further leftward shift is possible (Meiners & Salama, 1982; Davies *et al.*, 1998).

7.3.8. Role of the conserved 9' leucine in ligand-gated ion channels.

There has been much speculation on the role played by the conserved leucine and the effects of mutations of this residue. One possible explanation is that a normally desensitized receptor state becomes conducting in the mutant, additional conductance states have been identified in the α7 nicotinic acetylcholine receptor containing this mutant (Revah, *et al.*, 1991, Bertrand *et al.*, 1992). This explanation however, does not fit with recent observations of agonist induced closure of constitutively open channels (Pan *et al.*, 1997, Chang & Weiss, 1998). The evidence here and from others does suggest that it dramatically affects the activation and desensitization of the receptor through any binding-site which can open the channel. It also shifts the receptor into a state that can no longer be positively modulated by allosteric agonists, while these compounds are clearly still binding to their respective sites.

It has been suggested that the 9' leucine of ligand-gated ion channels lies at the most constricted position in the channel, where the transmembrane-lining helices are kinked and hence occludes the channel (Unwin, 1995). However this finding was not supported in a recent NMR study (Opella *et al.*, 1999). As the channel is likely to be pentameric, the equivalent residues from each subunit line up to form a cluster that can be translocated on agonist binding to the open channel conformation, and back again on channel closure. The introduction of a polar side chain in serine or threonine is hypothesised to destabilize the cluster, reducing the channel closure rate and favours the open conformation. This hypothesis explains the reduced desensitization and constitutively-active channels. Other evidence from cysteine substitution

experiments in nicotinic (Akabas *et al.*, 1994) and GABA$_A$ receptors (Xu & Akabas, 1996) however suggests that the gate may be more cytoplasmic than the 9' residue.

It must be remembered that mutation of the conserved 9' leucine within TM2 is not the only transmembrane residue known to alter pharmacological and biophysical properties of GABA$_A$ receptors. Other studies have identified additional amino acids within TM1, TM2 and TM3 that can also influence the pharmacological and biophysical properties of GABA$_A$ receptors (Im *et al.*, 1995; Tierney *et al.*, 1998; Dalziel *et al.*, 1999a; Harrison *et al.*, 2000; Krasowski & Harrison, 2000). Further studies into the kinetics and single-channel properties of these mutant receptors will reveal more about the relationship between receptor binding and transduction to open channels.

7.4. Summary.

Mutation of the conserved leucine to serine within TM2 of the β2 subunit and co-expression with α1γ2s resulted in smaller maximum inward currents to GABA compared to α1β2γ2s receptors, and large leak currents resulting from constitutively-active channels. As reported, this mutation significantly decreased the GABA EC$_{50}$ (147-fold) and reduced desensitization. Muscimol and the partial agonists, THIP and P4S also displayed a decrease in EC$_{50}$. In addition to competitively shifting GABA concentration-response curves, the antagonists bicuculline and SR95531 both inhibited the constitutive channel activity on α1β2L259Sγ2s receptors, with different degrees of maximum inhibition. The effects of a range of allosteric modulators, including benzodiazepines and anaesthetics, were examined on a submaximal GABA concentration (EC$_{20}$). Compared to wild type, none of these modulators potentiated the EC$_{20}$ response of α1β2L259Sγ2s receptors, however they all directly activated the receptor in the absence of GABA. Block of the constitutive-activity by bicuculline did not restore modulation by positive allosteric modulators, however, did restore that of negative allosteric modulators.

Chapter 8

General Discussion

8.1.   Overview of current study.

This study has characterised the effects of three structurally distinct GABA$_A$ receptor allosteric modulators; pentobarbitone, furosemide and tracazolate, and examined in detail the pharmacological characteristics of a novel GABA$_A$ receptor subtype, $\alpha1\beta1\varepsilon$, and a mutant receptor, $\alpha1\beta2L259S\gamma2s$, both of which are constitutively-active. Over the last decade numerous different GABA$_A$ receptor subtypes have been expressed and pharmacologically characterised. Interestingly almost all GABA agonists and allosteric modulators display some degree of selectivity (with regards to binding affinity, functional affinity or efficacy) for different GABA$_A$ receptor subtypes. One major aim for the future is to identify which receptor subtypes actually exist in the brain and to define their functional roles. Compounds that display subtype selectivity, such as furosemide and tracazolate, and / or the development of transgenic animals e.g. knock-out or knock-in will help answer these questions. Data from recombinant studies, such as those within this thesis, will assist in answering these questions. Several of the key issues which were identified from this study are outlined below:

8.2.   GABA$_A$ receptor subunits with restricted distribution.

As molecular biological techniques have improved researchers have been able to isolate, clone and express novel less ubiquitous GABA$_A$ receptor subunits such as ε and θ. The data presented within this thesis characterised the pharmacological properties of recombinant GABA$_A$ receptors containing the ε subunit. Interestingly the ε subunit appears to substitute for a γ subunit and for the first time produces recombinant receptors which show constitutive-activity. The resulting receptors ($\alpha1\beta1\varepsilon$) were similar to δ containing receptors in being insensitive to benzodiazepines, and more sensitive to GABA than $\alpha\beta\gamma$ receptors and had an intermediate sensitivity to Zn^{2+} ($\alpha\beta > \alpha\beta\delta/\varepsilon > \alpha\beta\gamma$). However these receptors differed from δ containing receptors in that they displayed rapid desensitization kinetics in response to GABA, compared to δ containing receptors which exhibit slow desensitization kinetics (Saxena & Macdonald, 1994; Brown et al., 2001). They were also inhibited by tracazolate compared to $\alpha1\beta1\delta$ receptors which were potentiated. In

situ hybridization studies demonstrated that the ε transcript was highly localised to the hypothalamus and the hippocampus. These studies with recombinant receptors provide us with important pharmacological data and useful tools, however, there are still a number of questions to be answered for example, what is the physiological role of ε containing receptors? Based on their location are they involved in memory formation or neuroendocrine regulation? Is the constitutive-activity also present *in vivo* and if so, what is the physiological importance of this? Hopefully answers to these questions and others will be forthcoming in the next few years.

To date 19 subunits of the GABA$_A$ receptor have been isolated giving rise to a large number of possible *in vivo* receptor subtypes. Whether this is the total repertoire of GABA$_A$ receptor subunits or whether there are any additional, as yet unidentified, subunits within the human brain also remains to be answered. The human genome project, which is nearing completion, may answer this question.

8.3.   The challenge of correct expression of recombinant receptors.

The experiments which examined the pharmacology of the ε construct from Merck Sharp & Dohme (MRK) and that from The Institute for Genomic Research (TIGR), demonstrated that the differences in anaesthetic pharmacology were accounted for by overexpression of ε in the vector pCDM8 relative to α1 and β1. It is generally assumed that, within an expression system, the vectors pCDM8 and pcDNA1.1Amp behave essentially the same and in fact have been used interchangeably at Merck Sharp & Dohme for a number of years. It is unclear, at present, why the ε cDNA is transcribed more efficiently in pCDM8 than in pcDNA1.1Amp. Whether or not native ε containing receptors are potentiated by anaesthetic agents will depend on how these receptors are expressed *in vivo* and remains an unanswered question. The data presented illustrates the importance of noting the vector used, when comparing data between different groups, and highlights that this can have profound effects on the pharmacology of ligand-gated receptors.

8.4.   General anaesthetic agents: Importance of GABA$_A$ receptors.

General anaesthetic agents have been in clinical practice for over 100 years. Behavioral hallmarks of general anaesthesia include immobility, amnesia, unconsciousness, hypnosis, analgesia, muscle relaxation and depression of autonomic reflexes. In recent years the general consensus of the mechanism of action has changed from an interaction with membrane lipids to an interaction with membrane proteins. Studies over the last 10-15 years have implicated a number of ligand-gated ion channels and more recently the two pore K$^+$ channels as potential sites of action (Franks & Lieb, 1998; Krasowski & Harrison, 1999; Thompson & Wafford, 2001; Yamakura *et al.*, 2001). A number of questions however need to be answered before conclusions can be drawn about the role of these channels in anaesthesia.

1. Sensitivity: Which ligand-gated ion channels are sufficiently sensitive to clinically relevant concentrations of general anaesthetics? Do these anaesthetic agents display receptor subtype selectivity and stereoselectivity?

2. Mechanism: Where do anaesthetic agents bind? What is the molecular mechanism by which general anaesthetics affect the function of ion channels?

3. *In vivo* importance: Which ion channels determine the specific behavioral actions of general anaesthetics? Are specific brain regions involved in the behavioral effects? How do we link potentiation (or inhibition) of receptors to unconsciousness, immobility, amnesia etc?

As demonstrated in this thesis all of the GABA$_A$ receptor subtypes examined were modulated by pentobarbitone at clinically relevant concentrations (Thompson *et al.*, 1996). Similarly the volatile anaesthetic agents, isoflurane and enflurane, and propofol have been shown to modulate a variety of GABA$_A$ receptor subtypes at clinically relevant concentrations (Harrison *et al.*, 1993; Mihic *et al.*, 1994a; Sanna *et al.*, 1995b; Wafford *et al.*, 1996; Lees & Edwards, 1998). Of all the ligand-gated ion channels examined only GABA$_A$ receptors are sensitive at clinically relevant concentrations to most volatile and intravenous anaesthetic (exceptions being ketamine, xenon, cyclopropane and nitrous oxide) suggesting an involvement of the

GABAergic system in anaesthesia. Due to the low affinity and high non specific binding of general anaesthetic agents, a radioligand-binding assay is currently not available, hindering the identification of a binding site or sites.

Mutagenesis studies, however, have identified a number of amino acid residues located within the transmembrane domains that influence anaesthetic modulation. One must exhibit caution in the interpretation of the results from these studies and remember that the identified residues could form part of an anaesthetic binding site or alternatively have a role in the transduction mechanism. Data presented in this thesis ($\alpha1\beta2L259S\gamma2s$ (9') and $\alpha1\beta1\epsilon_{TIGR}$) and that of Findlay *et al.* 2001 ($\alpha2S270W\beta1$, $\alpha2\beta1S265W$ and $\alpha2S270W\beta1\gamma2s$ (15')) produced receptors were directly activated but not modulated by general anaesthetic agents. The commonality between these receptors was an increase in GABA sensitivity and a proportion of constitutive activity. As P_{open} increases the functional EC_{50} decreases and spontaneous openings increase. Eventually a point is reached where allosteric modulators cannot increase P_{open} any further and hence no potentiation is observed as was seen with $\alpha1\beta2L259S\gamma2s$ and $\alpha1\beta1\epsilon_{TIGR}$. These results support the hypothesis that general anaesthetic agents allosterically interact with GABA$_A$ receptors and can also gate the channel independently of GABA.

Structural biological approaches have identified Glu 262 (20') within the carboxyl-terminal of TM2 of the $\alpha1$ subunit of the nACh receptor (Pratt *et al.*, 2000) and Ser 267 (15') within TM2 of the GABA $\alpha2$ subunit (Mascia *et al.*, 2000) as contributing to the binding site of certain anaesthetic agents. Whereas recent imaging studies on human volunteers have identified regions of the thalamus and midbrain reticular formation that are specifically suppressed during anaesthesia (Fiset *et al.*, 1999; Alkire *et al.*, 2000). Hopefully future studies will enhance our knowledge further and provide answers to the questions above.

8.5. Pharmacological characterisation of pentobarbitone, furosemide and tracazolate: potential pharmacological tools?

Although furosemide is a clinically used diuretic agent inhibiting the $Na^+/2Cl^-/K^+$ co-transporter within the loop of Henle, it also behaves as a non-competitive antagonist of $GABA_A$ receptors suggesting a similar binding domain on these two membrane proteins. While selectivity for $\alpha 6$ over $\alpha 1$ and $\beta 2/3$ over $\beta 1$ was demonstrated by Korpi *et al.* (1995) characterisation on a wide range of subtypes was lacking. Equally the anxiolytic compound tracazolate and the anaesthetic pentobarbitone had never been extensively characterised on recombinant $GABA_A$ receptor subtypes. All three compounds displayed important $GABA_A$ receptor subtype selectivity, indicating a potential use for characterisation of native receptors.

8.6. Importance of residues with the transmembrane domains.

Both furosemide and tracazolate displayed selectivity for $\beta 2/3$ containing receptors over $\beta 1$ containing receptors. As with many other $GABA_A$ receptor modulators (loreclezole, etomidate, mefenamic acid) this selectivity was identified to be due to asparagine 265 within TM2. It is currently not known whether Asn265 forms part of a common binding site for these structurally diverse group of compounds whose functional response it influences, or alternatively if these compounds share a common transduction mechanism which is hindered in $\beta 1$ when Asn is replaced with Ser. Studies using the substituted cysteine accessibility method (SCAM) for the 15' residue in TM2 (homologous to Asn 265 in GABA $\beta 2/3$) within the ligand-gated ion channel superfamily is conflicting, with the mouse muscle acetylcholine α subunit reported not to be accessible (Akabas *et al.*, 1994) compared to the β subunit which was reported to be accessible (Zhang & Karlin, 1998). An initial report by Xu and Akabas, (1993) using the GABA $\alpha 1$ subunit showed that Ser 270 was not accessible, however more recent data demonstrated accessibility at a GABA EC_{50} concentration but not at a maximal concentration (Williams & Akabas, 1999). Future experiments therefore include SCAM studies on the $\beta 2/3$ subunit.

The mutagenesis studies with furosemide identified isoleucine 228 within TM1 and a second N-terminal domain 131-160 of the α6 subunit which conferred selectivity for furosemide. It is currently unknown if isoleucine 228 within the α6 subunit is accessible to furosemide and hence forms part of a binding site or whether it plays a role in transduction and or ion channel gating. Experimental evidence with mouse muscle acetylcholine receptor subunits α and β however indicate that Val 229 (homologous to Ile 228 in GABA α6 subunit) is accessible but only when the receptor is in the open state (Zhang & Karlin, 1997; Akabas & Karlin, 1995). Future studies are planned to examine the accessibility of isoleucine 228 within TM1 of the α6 GABA$_A$ receptor subunit.

Over the last fifteen years mutagenesis studies have identified many residues within the transmembrane domains (particularly TM2) which influence the potency and / or functional response of a number of GABA$_A$ receptor modulators. Mutation of the conserved leucine at the 9' position of TM2 to serine within β2 and co-expression with α1γ2s produced receptors with constitutive-activity. This constitutive-activity may result from the smaller amino acid serine altering the presumed channel gate so that it can never completely close. Why and what causes α1β1ε receptors to be constitutively-active is currently unknown although differences in residues within TM2 or the GABA binding site could be responsible.

8.7.   Concluding Remarks.

This study has focused on recombinant human GABA$_A$ receptors expressed in *Xenopus* oocytes as a system to enhance our understanding of the pharmacological properties of GABA$_A$ receptors. The detailed characterisation of pentobarbitone, furosemide and tracazolate and their subunit selectivity suggests a use for these compounds in the characterisation of native receptors. Most of the pharmacological characteristics of recombinant receptors are applicable to native receptors highlighting the importance of studies with recombinant receptors. The future direction of GABA$_A$ receptor research is to identify the function of the different receptor subtypes *in vivo* and hence the development of clinically useful compounds. To achieve these goals studies using recombinant expression systems, such as the *Xenopus* oocyte, will be

required. It has been fifty years since GABA was first identified in the brain and, as this thesis has described, the number of $GABA_A$ receptor subunits and allosteric modulators of the receptor continues to increase.

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