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CHARACTERIZATION OF EXTRASYNAPTIC $\alpha4\beta3\delta$ AND SYNAPTIC $\alpha4\beta3\gamma2$ GABAA RECEPTORS

by

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To my wife, Qianli (Cherry) Ma

To my parents, Hedi Yang and Xiaolong You

ABSTRACT

In the mammalian central nervous system, the γ-aminobutyric acid type A ($GABA_A$) receptor α4β3δ subtype is a putative extrasynaptic receptor that mediate tonic inhibition, while the α4β3γ2 subtype is likely to mediate phasic inhibition at the synapse. This thesis describes the use of multidisciplinary approaches to characterize the distinct pharmacological and biophysical characteristics of these two $GABA_A$ receptor subtypes.

The $GABA_A$ receptor α4β3δ subtype has higher sensitivity to GABAergic agonists than the α4β3γ2 subtype. The structural determinants underlying these differences have been investigated by generating chimeric δ/γ2 subunits that were co-expressed with the α4 and β3 subunits in *Xenopus* oocytes. A stretch of amino acids in the δ subunit, S238-V264, was shown to play an important role in determining agonist potency. Further studies suggested that the differences in agonist sensitivities are likely to arise from changes in the transduction mechanism that links agonist binding to channel activation.

The $GABA_A$ receptor α4β3δ subtype desensitizes more slowly and less completely than the α4β3γ2 receptor during the application of GABA. Structural determinants in the δ and γ2 subunits that underlie these distinct desensitization properties were investigated using a mutagenesis approach. Residues in the transmembrane domains of both subunits were identified as determinants of their desensitization profiles.

α4-Containing receptors are generally thought to be diazepam-insensitive. In this thesis, we showed that nanomolar concentrations of diazepam and flunitrazepam can significantly potentiate GABA-evoked currents mediated by the α4β3γ2 receptor

expressed in *Xenopus* oocytes, suggesting the presence of a high affinity binding site(s) for benzodiazepines. However, the $\alpha4\beta3\gamma2$ receptor expressed in mammalian cells did not show high affinity binding of benzodiazepines. The discrepancy in these results may be due to different posttranslational modifications or receptor assembly processes between the two expression systems.

A defined $GABA_A$ receptor subunit arrangement can facilitate drug development since most recognition sites for GABAergic agonists, antagonists and allosteric modulators are located at subunit-subunit interfaces. The subunit arrangement of the $GABA_A$ receptor $\alpha4\beta3\delta$ subtype, expressed in HEK cells, was studied using atomic force microscopy. Our results show a predominant subunit arrangement of α-β-α-δ-β (counter-clockwise, when viewed from the extracellular side).

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TABLE OF CONTENTS

Chapter 1 Introduction 1

GABA and $GABA_A$ Receptor Overview 2

Heterogeneous Distribution of $GABA_A$ Receptors........ 3

Stoichiometry and Arrangement of $GABA_A$ Receptor Subunits........ 5

Structure of *Lymnaea stagnalis* Acetylcholine Binding Protein (AChBP) and *Torpedo* nAChR........ 7

The Overall Structure of $GABA_AR$ and the GABA Binding Site........ 12

Benzodiazepine Binding Site on the $GABA_AR$........ 15

Receptor Desensitization........ 18

$GABA_AR$ Pharmacology........ 22

- Agonists........ 22
- Antagonists........ 24
- Allosteric Modulators: Benzodiazepines........ 25
- Other Allosteric Modulators: Barbiturates and Neurosteroids........ 26

Phasic and Tonic GABAergic Inhibition........ 28

A Role for δ-containing $GABA_ARs$ in Epilepsy........ 33

Aims of the Present Studies........ 35

Bibliography........ 57

Chapter 2 Identification of a Domain in the δ Subunit (S238-V264) of the α4β3δ $GABA_A$ Receptor that Confers High Agonist Sensitivity 79

Introduction........ 80

Materials and Methods........ 82

- Clones and Construction of δ/γ2 Chimeras........ 82
- Expression of $GABA_A$ Receptors in *Xenopus* Oocytes........ 83
- Two-electrode Voltage Clamp Recordings........ 84
- Data Analysis........ 84
- Chemicals........ 85

Results........ 85

- Expression of $GABA_A$ Receptor Subtypes........ 85

Effects of Varying cRNA Ratios on Receptor Expression.................. 86
Effects of GABA and Muscimol on Functional Responses of Wildtype and Chimeric Receptors.. 87
Effects of THIP and I4AA on Functional Responses of Wildtype and Chimeric Receptors.. 88
Effects of Competitive Antagonists and Picrotoxin on Functional Responses of Wildtype and Chimeric Receptors............................ 90
Discussion.. 90
Bibliography... 110

Chapter 3 Identification of Structural Determinants in the γ2L and δ Subunits of the $GABA_A$ Receptor that Influence Receptor Desensitization 114
Introduction.. 115
Materials and Methods.. 119
Construction of Mutated δ, γ2L Subunits and δ/γ2L Chimeras............ 119
Xenopus Oocyte Preparation and Expression of $GABA_A$ Receptors...... 119
Two-electrode Voltage Clamp Measurements.................................. 121
Data Analysis.. 121
Results... 122
Comparison of the Desensitization Properties of Wildtype α4β3δ and α4β3γ2L Subtypes... 122
Desensitization of Chimeric δ/γ2L Subunit-Containing Receptors........ 123
Effects of Substitution Mutations in the Extracellular Domain of TM1 (δV233/γ2Y235 -δQ237/γ2Q239).. 125
Effects of Substitution Mutations in the Intracellular Domain of TM1 (δA247/γ2V249 – δI255/γ2I257).. 127
Effects of Substitution Mutations in the Extracellular Domain of TM2 (δM278/γ2S280 - δI291/γ2V293).. 128
Discussion.. 131
Several Regions Containing Structural Determinants of Desensitization Revealed by δ/γ2L Chimeras.............................. 132

Desensitization Affected by Residues in the Extracellular End of TM1 Domain.......... 133
Desensitization Affected by Residues in the Extracellular End of TM2 Domain.......... 135
Impaired Desensitization by Introducing γ2L Residues into the δ Subunit.......... 137
Conclusion.......... 138
Bibliography.......... 160

Chapter 4 Benzodiazepine Modulation of the Rat GABAA Receptor α4β3γ2L Subtype Expressed in *Xenopus* Oocytes 164
Introduction.......... 165
Materials and Methods.......... 167
Chemicals.......... 167
Clones.......... 167
Expression in *Xenopus* Oocytes and Two-Electrode Voltage Clamp Recordings.......... 168
Transient Transfection and Cell Membrane Preparation.......... 169
Radioligand Binding Assays.......... 169
Data and Statistical Analysis.......... 170
Results.......... 172
Benzodiazepine Modulation of the Rat α4β3γ2L $GABA_A$ Receptor Expressed in *Xenopus* Oocytes.......... 172
Inhibition of Diazepam and Flunitrazepam Potentiation by Ro15-1788 and ZK93426.......... 173
Radioligand Binding Studies of Receptors Expressed in HEK 293 Cells 173
Discussion.......... 174
Bibliography.......... 191

Chapter 5 Atomic Force Microscopy Reveals the Stoichiometry and Subunit Arrangement of the α4β3δ $GABA_A$ Receptor 194
Introduction.......... 195

Materials and Methods.. 197
Construction of Tagged $GABA_A$ Receptor Subunits........................ 197
Expression and Functional Studies of Untagged-α4β3(M256)δ and Tagged-α4β3(M256)δ Receptors in *Xenopus* Oocytes...................... 198
Transient Transfection in HEK 293 Cells and Patch-clamp Electrophysiology.. 199
Immunofluorescence of Tagged $GABA_A$ α4β3(M256)δ Receptor......... 200
Immunoblot Analysis.. 200
AFM Imaging of Receptors and Receptor-Antibody Complexes.......... 201
Results.. 202
Functional Expression of Untagged-α4β3(M256)δ and Tagged-α4β3(M256)δ Subtypes in *Xenopus* Oocytes............................ 202
Functional Expression of Tagged-α4β3(M256)δ Subtype in HEK 293 Cells.. 203
Immunofluorescence Staining and Immunoblotting of the Tagged-α4β3(M256)δ Receptor Expressed in HEK 293 Cells....................... 203
AFM imaging.. 204
Discussion.. 207
Bibliography... 225

Chapter 6 General Discussion 229
General Discussion... 230
Future Directions.. 237
Bibliography... 244

LIST OF TABLES

Table 2-1	Activation of the wild-type and chimeric $GABA_A$ receptor subtypes by GABA and muscimol	107
Table 2-2	Activation of the wild-type and chimeric $GABA_A$ receptor subtypes by THIP and I4AA	108
Table 2-3	Inhibition of GABA-gated currents by the competitive antagonists (SR95531 and bicuculline) and the channel blocker (picrotoxin)	109
Table 3-1	Summary of desensitization kinetics of wildtype α4β3δ and α4β3γ2L receptors	154
Table 3-2	Summary of desensitization kinetics of chimeric receptors	155
Table 3-3	Summary of desensitization kinetics of mutants in the extracellular part of TM1 (δV233/γ2Y235-δQ237/γ2Q239) domain of δ and γ2L subunits	156
Table 3-4	Summary of desensitization kinetics of mutants in domain δS238/γ2T240 – δI255/γ2I257 of δ subunit	157
Table 3-5	Summary of desensitization kinetics of mutants in the extracellular part of TM2 (δM278/γ2S280-δI291/γ2V293) of δ and γ2L subunits	158
Table 3-6	Summary of the effects of δ and γ2L residues on the rate of desensitization and non-desensitizing current	159
Table 4-1	Effects of different compounds on potentiation of GABA-evoked currents in the α4β3γ2L receptor	189

Table 4-2	Ro15-1788 and ZK93426 inhibitory effects on benzodiazepine potentiation of GABA-evoked currents in the $\alpha4\beta3\gamma2L$ receptor	190
Table 5-1	Activation of untagged-$\alpha4\beta3(M256)\delta$ and tagged-$\alpha4\beta3(M256)\delta$ $GABA_A$ receptor subtypes by GABA	224

LIST OF FIGURES

Figure 1-1	Model of $GABA_AR$	39
Figure 1-2	Crystal structure of the homopentameric AChBP at 2.7 Å resolution	41
Figure 1-3	Structure of *Torpedo* nAChR determined by electron microscopy at 4 Å resolution	43
Figure 1-4	$GABA_AR$ structure	45
Figure 1-5	The GABA binding site on the $GABA_AR$	47
Figure 1-6	The benzodiazepine binding site on the $GABA_AR$	49
Figure 1-7	Residues in the TM2 domain affect receptor desensitization	51
Figure 1-8	Chemical structure of $GABA_AR$ agonists, antagonists and channel blockers	53
Figure 1-9	Chemical structures of benzodiazepines and benzodiazepine site ligands	55
Figure 2-1	Schematic representation of chimeric subunit construction	97
Figure 2-2	The effects of GABA and muscimol on activation of wildtype and chimeric receptors expressed in *Xenopus* oocytes	99
Figure 2-3	Representative currents for activation of different $GABA_A$ receptors by varying concentrations of THIP and I4AA as indicated	101

Figure 2-4	The effects of THIP and I4AA on activation of α4β3δ, α4β3γ2L and the chimeric α4β3χ277, α4β3χ255 and α4β3χ237 receptors	103
Figure 2-5	The effects of SR95531, bicuculline and picrotoxin on GABA-evoked currents in the α4β3δ, α4β3γ2L and chimeric α4β3χ277 and α4β3χ237 receptors	105
Figure 3-1	Sequence alignment of the δ and γ2L subunits containing the TM1 and TM2	139
Figure 3-2	Desensitization profiles of the wildtype α4β3δ and α4β3γ2 receptors	141
Figure 3-3	Desensitization properties of χ232-, χ237-, χ241-, χ244-, χ246-, χ255-, χ277-containing receptors	143
Figure 3-4	Effects of substitution mutations in the extracellular domain of TM1 of the δ and γ2L subunits	145
Figure 3-5	Effects of substitution mutations in the intracellular end of the TM1 domain of δ and γ2L subunits	148
Figure 3-6	Effects of substitution mutations in the extracellular end of TM2 of the δ and γ2L subunits	150
Figure 4-1	Effects of benzodiazepines on GABA-evoked currents mediated by the α4βxγ2L receptors	178
Figure 4-2	Effects of Ro15-4513, β-CCE and ZK93423 on GABA-evoked currents on the α4β3γ2L receptor	181
Figure 4-3	Effects of Ro15-1788 and ZK93426 on GABA-mediated currents on the α4β3γ2L receptor	183

Figure 4-4	Effects of Ro15-1788 and ZK93426 on GABA mediated currents	185
Figure 4-5	Displacement of [^{3}H]Ro15-4513 binding by diazepam and flunitrazepam	187
Figure 5-1	Functional expression of tagged- and untagged-α4β3(M256)δ receptor in *Xenopus* oocytes and HEK 293 cells	210
Figure 5-2	Immunofluorescent staining of tagged $GABA_A$ α4β3(M256)δ receptor expressed in HEK 293 cells	212
Figure 5-3	Immunoblotting of tagged $GABA_A$ α4β3(M256)δ receptor	214
Figure 5-4	Frequency distribution of molecular volumes of undecorated $GABA_A$ receptors	216
Figure 5-5	AFM imaging of complexes between $GABA_A$ receptors and anti-subunit antibodies	218
Figure 5-6	Determination of the absolute subunit arrangement	220
Figure 5-7	Determination of the orientation of receptor on the mica surface	222

LIST OF ABBREVIATIONS

ACh	acetylcholine
AChBP	acetylcholine binding protein
AFM	atomic force microscopy
ANOVA	analysis of variance
BDZ	benzodiazepine
BES	*N*, *N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid
β-CCE	β-carboline-3-carboxylic acid ethyl ester
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
cRNA	complementary ribonucleic acid
DMCM	methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DMSO	dimethyl sulfoxide
EC_{50}	half-maximal concentration for channel activation
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
$GABA_BR$	γ-aminobutyric acid type B receptor
GEF+	generalized epilepsy with febrile seizures plus
GFP	green fluorescence protein
HEK293	human embryonic kidney 293
HEPES	4-(hydroxyethyl)-1-piperazineethanesulfonic acid
5-HT	5-hydroxytryptamine
$5\text{-}HT_3R$	5-hydroxytryptamine type 3 receptor
I4AA	imidazole-4-acetic acid
IC_{50}	half maximal inhibitory concentration
I_{max}	maximal current
JME	juvenile myoclonic epilepsy
K_D	equilibrium dissociation constant
K_I	equilibrium inhibitor dissociation constant
LGIC	ligand-gated ion channel
MTLE	mesial temporal lobe epilepsy

P4S	piperidine-4-sulphonic acid
PBS	phosphate buffered saline
4-PIOL	5-(4-piperidyl)-3-isoxazolol
PTX	picrotoxin
SCAM	substituted cysteine accessibility modification
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
thio-4-PIOL	5-(4-piperidyl)-3-isothiazolol
THIP	4,5,6,7-tetrahydroisoxazolo[5,4]pyridin-3-ol
THDOC	allotetrahydrodeoxycorticosterone
TM	transmembrane domain
ZAPA	Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid

CHAPTER 1

Introduction

GABA AND $GABA_A$ RECEPTOR OVERVIEW

The function of the mammalian brain relies on a finely-tuned balance between neuronal excitation, which transmits and amplifies signals, and inhibition, which dampens and refines these signals. The γ-aminobutyric acid (GABA) system has been known to be a major inhibitory mechanism since the discovery of GABA as a neurotransmitter in the 1960s (Kravitz *et al.*, 1963, Krnjević and Schwartz, 1966). GABA is synthesized by the decarboxylation of L-glutamate (an excitatory neurotransmitter), by glutamic acid decarboxylase and it is accumulated in presynaptic vesicles (see Roberts and Sherman, 1992). Upon depolarization, GABA is released into the synaptic space where it elicits its effects through activation of the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. Upon dissociation of GABA from its receptor sites, it is taken up by the GABA transporters in neurons or astrocytes and subsequently metabolized to succinate by GABA aminotransferase.

$GABA_A$ receptors ($GABA_ARs$) are members of the Cys-loop ligand gated ion channel (LGIC) superfamily, which also includes the nicotinic acetylcholine receptors (nAChRs), glycine receptors and 5-hydroxytryptamine type 3 receptors (5-HT_3Rs) (Barnard *et al.*, 1998). Like other members of the LGIC superfamily, $GABA_ARs$ are pentameric protein complexes (Nayeem *et al.*, 1994) of homologous subunits arranged in a rosette conformation to form a central ion channel (Figure 1-1, A). Molecular heterogeneity of $GABA_AR$ subunits has been revealed following the first cloning and sequencing of bovine α and β subunits in 1987 (Schofield *et al.*, 1987). To date, nineteen $GABA_AR$ subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3) have been identified in the

mammalian central nervous system (CNS) (Sieghart and Sperk, 2002), with further heterogeneity arising from alternative splicing (eg. γ2S *vs.* γ2L, Whiting *et al.*, 1990).

HETEROGENEOUS DISTRIBUTION OF $GABA_A$ RECEPTORS

Mapping of the distribution of $GABA_A$ receptor subtypes in the mammalian brain is important for our understanding of the physiological actions of GABA and the pharmacological actions of drugs that target $GABA_A$ receptors. Using immunolabelling and autoradiography, the α1, β1-3 and γ2 subunits have been demonstrated to be the most abundant $GABA_AR$ subunits throughout the central nervous system (Pirker *et al.*, 2000). The γ3 subunit is also distributed in all parts of the brain but it is of lower abundance. The α2 and α3 subunits are expressed mainly in the olfactory bulb, cerebral cortex and the amygdala area (Pirker *et al.*, 2000) while the α4 and δ subunits share a similar distribution pattern in the thalamus, dentate gyrus, striatum and hippocampus (Sur *et al.*, 1999a). The α5 subunit is preferentially localized to the hippocampus but is also present at considerably lower levels in the cerebral cortex (Caraiscos *et al.*, 2004; Sur *et al*; 1999b; Fritschy and Mohler, 1995). This restricted distribution suggests that the α5 subunit may be involved in learning and memory (Collinson *et al*, 2002). The α6 subunit has the most restricted expression of all the α subunits. It has been detected only in cerebellar granule cells (Hadingham *et al.*, 1996), where the δ subunit is also expressed (Jones *et al.*, 1997). Less abundant subunits e.g. ε, θ, π and ρ, also have a restricted localization. The expression of the ε subunit is largely restricted to gonadotrophin-releasing hormone neurons in the hypothalamus and dentate gyrus (Whiting *et al.*, 1997; Jones *et al.*, 2006). Rho subunits have been detected mainly in the retina (Koulen *et al.*,

1998) and brainstem neurons (Milligan *et al.*, 2004), while the θ subunit is robustly expressed in the substantia nigra and other structures enriched with monoaminergic neurons (Bonnert *et al.*, 1999). In contrast, the π subunit can be detected only in certain peripheral tissues, such as uterus (Hedblom and Kirkness, 1997).

Despite the plethora of receptor subunits, studies of native $GABA_A$ receptors originally suggested that only 10-15 combinations exist *in vivo* (McKernan and Whiting 1996). More recent studies, however, suggest that various receptor subtypes with minor populations exist far more than previously assumed (reviewed in Sieghart and Spark, 2002). The most commonly expressed $GABA_AR$ subtype is α1β2γ2 (Barnard *et al.*, 1998). The α2β3γ2 or α3β3γ2 subtypes are less abundant but are highly expressed in areas where the α1β2γ2 receptor is present at lower levels (McKernan and Whiting 1996), such as in hippocampal pyramidal neurons (α2β3γ2) and basal forebrain cholinergic neurons (α3β3γ2). The α4 subunit is frequently coexpressed with the δ or γ2 subunits along with different subtypes of the β subunit in the hippocampus and thalamus (Brooks-Kayal *et al.*, 1999; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sur *et al.*, 1999a), whereas subtypes incorporating the α6βxδ and α6βxγ2 subunits appear to exist only in the cerebellum (Jechlinger *et al.*, 1998; Poltl *et al.*, 2003). The α5 subunit is thought to coassemble with the β3 and γ2 subunits in the hippocampus (Caraiscos *et al.*, 2004). It has also been suggested the π and θ subunits coassemble with α, β, γ subunits to form αβγπ or αβγθ receptor subtypes (McKernan and Whiting, 1996). The possibility that individual pentamers might contain two different α or two different β subunits cannot be ruled out (Pollard *et al.*, 1993; Duggan *et al.*, 1991; Luddens *et al.*, 1991).

Recently, studies have focused on the subcellular localization of $GABA_A$Rs. It has been suggested that distinct $GABA_A$Rs are differentially localized to synaptic or to extrasynaptic/perisynaptic regions (Farrant and Nusser 2005). The majority, but not all, of γ2 subunits in association with α1/2/3/4/6 and β subunits, have been found to be highly enriched in synapses (Somogyi *et al.*, 1996), whereas δ-containing receptors (α4β1/2/3δ and α6β2/3δ) are present exclusively in the extrasynaptic and perisynaptic locations (Somogyi *et al* 1989; Nusser *et al.*, 1995; Nusser *et al.*, 1998; Wei *et al.*, 2003), lying perhaps hundreds of nanometers away from the postsynaptic density. The γ2 or δ subunits are suggested to play a central role in the clustering of synaptic and extrasynaptic $GABA_A$Rs, respectively. The γ2 subunit, which is linked to the synaptic anchoring protein gephyrin (Essrich *et al.*, 1998), appears to be critical for the trafficking of γ2-containing receptors to the synapse (Schweizer *et al.*, 2003), whereas the mechanism of localization of δ-containing receptor remains to be established.

STOICHIOMETRY AND ARRANGEMENT OF $GABA_A$ RECEPTOR SUBUNITS

The most common $GABA_A$R subtype contains the α, β and γ subunits and its stoichiometry is suggested to be 2α:2β:1γ (Farrar *et al.*, 1999). A variety of methods has been used to study receptor stoichiometry. The effects of mutagenesis on the functional properties of different subunit combinations originally suggested a subunit stoichiometry of either 2α:1β:2γ or 2α:2β:1γ (Backus *et al.*, 1993). Im *et al.* (1995b) investigated the expression of the α6β2γ2 receptor using tandem constructs of α6-β2 subunits. The authors showed that monomeric γ2 is required to be co-expressed with the tandem construct to form a functional chloride channel, suggesting that a pentamaric $GABA_A$

receptor has a stoichiometry of $2\alpha:2\beta:1\gamma$. Chang *et al.* (1996) examined the relative presence of each subunit class in a $GABA_A$ $\alpha1\beta2\gamma2$ receptor using a mutagenesis approach. The authors suggested that the relative change in GABA sensitivity due to a specific mutation in each individual subunit class reflects the contribution of each. They concluded that the $GABA_A$ $\alpha1\beta2\gamma2$ receptor is composed of two α, two β and one γ subunits. Additional studies using techniques such as Western blotting (Tretter *et al.*, 1997) and density sediment centrifugation (Knight *et al.*, 2000) suggested the same stoichiometry for $GABA_A$Rs.

The arrangement of individual subunits in the heteropentomer remains a major question. Tretter *et al.* (1997) proposed two possible subunit arrangements, α-β-α-γ-β or α-β-α-β-γ (counter-clockwise, when viewed from the extracellular face). These possible arrangements were supported by structural evidence based on the localization of GABA and benzodiazepine binding pockets (see below). Using a fluorescence resonance energy transfer technique combined with a radioligand binding assay, Farrar *et al.* (1999) reported a $2\alpha:2\beta:1\gamma$ subunit stoichiometry for the $GABA_A$R $\alpha1\beta2\gamma2$ subtype and provided further evidence for its likely subunit arrangement i.e., α-β-α-γ-β (counter-clockwise, when viewed from the extracellular face).

In the last few years, additional subunit concatenation studies have provided some supporting evidence for subunit arrangement (Sigel *et al.*, 2006; Minier and Sigel 2004). By co-expressing various combinations of dimer-linked subunits, such as $\beta\rightarrow\alpha$ (with a short amino acid linker connecting the C-terminus of the β subunit to the N-terminus of the α subunit) or $\alpha\rightarrow\beta$ and trimer linked subunits such as, $\gamma\rightarrow\beta\rightarrow\alpha$ or $\beta\rightarrow\alpha\rightarrow\gamma$, it has been suggested that 1) tetramers and hexamers do not form functional receptors; and 2)

subunits in the $GABA_AR$ $\alpha1\beta2\gamma2$ subtype are arranged in the following order: α-β-α-γ-β counter-clockwise, as seen from the synaptic cleft (Figure 1-1, B). In the case of the binary $\alpha1\beta2$ subtype, a stoichiometry of 2α:3β has been proposed to form a functional receptor (Baumann *et al.*, 2001; Baumann *et al.*, 2002).

Recently, atomic force microscopy (AFM) has been applied to the study of the subunit arrangement of ligand-gated ion channels (Neish *et al.*, 2003; Barrera *et al.*, 2005a, b; Barrera *et al.*, 2008). AFM imaging of the $GABA_A$ $\alpha1\beta2\gamma2$ receptor tagged with subunit-specific antibodies (Neish *et al.*, 2003) suggested that the two α subunits are separated by a non-α subunit. However, the authors also did not preclude the possibility that two α subunits might be adjacent in a small proportion of receptors.

STRUCTURE OF *Lymnaea stagnalis* ACETYLCHOLINE BINDING PROTEIN (ACHBP) AND *Torpedo* nACHR

Members of the Cys-loop LGIC superfamily share many structural similarities. Each receptor subunit has a large N-terminal extracellular domain followed by four transmembrane (TM) domains with a small extracellular C-terminus (see Changeux and Edelstein, 1998). The N-terminal region contains two signature cysteine residues which are separated by 13 amino acids. These are linked by a disulfide bond to form the so-called Cys-loop, a hallmark feature of the LGIC superfamily. Functionally, the N-terminal extracellular domains are involved in forming ligand binding sites; the TM2 domain lines the lumen of the ion channel and forms the channel gate, whereas the intracellular domain contributed by the TM3-4 loop is predicted to form portals to allow

ions to exit the channel (Miyazawa *et al.*, 1999). In some subunits, this domain also carries sites for potential phosphorylation (Yee and Huganir, 1987; Wagner *et al.*, 1991).

Our understanding of the structural and functional characteristics of the Cys-loop LGIC family has been significantly increased in the last few years due to the resolution of an X-ray crystallographic structure of a homologous acetylcholine binding protein (AChBP) (Brejc *et al.* 2001) and also 4-Å resolution electron microscopy images of the *Torpedo* nAChR (Miyazawa *et al.*, 2003).

The *Lymnaea* AChBP is a homopentameric protein. The crystal structure of AChBP is 62 Å in length and 80 Å in diameter, which is in good agreement with the size of the extracellular domain of *Torpedo* nAChR estimated from electron microscopy (see below) (Figure 1-2, A and B). This protein is released from the glial cells into the synaptic area where it binds acetylcholine (Smit *et al.*, 2001). Although it shares only 20-30% sequence identity to the extracellular ligand binding domain of the Cys-loop LGIC family, the AChBP carries many residues that have been implicated in ligand binding to the nAChR (Smit *et al.*, 2001). However, the AChBP does not have transmembrane domains. Starting with a short N-terminal α-helix, much of the AChBP structure folds as a "sandwich", which contains ten β-strands. The luminal "inner" sheet and the abluminal "outer" sheet are connected by the signature disulfide bridge (Brejc *et al.* 2001).

The most striking feature of the AChBP is that it confirms much of the biochemical data previously obtained for the nicotinic acetylcholine receptor. Prior to crystallography of the AChBP, the acetylcholine binding pocket of the nAChR was predicted to be located at subunit interfaces where it was formed by "loops" of amino acids. The principal component of the binding pocket is formed by residues from the α

subunit (in *Torpedo* nAChR), which contributes loops A, B, and C. The complementary part of the binding pocket is formed by residues from an adjacent subunit (γ or δ subunit in *Torpedo* nAChR), which contributes loops D, E, and F (see Corringer *et al.*, 2000, Figure 1-2, C). Within the AChBP, many of the residues in the binding site previously identified by photoaffinity labeling and mutagenesis studies in the nAChR are conserved (Brejc *et al.* 2001). These key residues in AChBP include Tyr89 in loop A, Trp143 in loop B, Tyr185, the vicinal Cys187-188 residues and Tyr192 in loop C. Binding site residues on the complementary side include Trp53 and Gln55 in loop D, Arg104, Val106, Leu112, Met 114 in loop E and Try164 in loop F. When viewed from the side (Figure 1-2 B), the ligand-binding site is approximately 30 Å away from the bottom of the structure as predicted for the LGIC family (Unwin, 1993; Valenzuela *et al.*, 1994). Aromatic residues Try89 (Galzi *et al.*, 1990; Cohen *et al.*, 1991), and Try185 (Dennis *et al.*, 1988) from the principal side and residues Try164 and Trp53 (O'Leary *et al.*, 1994) from the complementary side form the bottom of the ligand-binding cavity. The top of the binding site is formed by hydrophobic residues Arg104, Val106 and Leu112 (Sine and Claudio, 1991; Sine, 1993; Wang *et al.*, 2000). Residues Try143, 145, Try192 (Dennis *et al.*, 1998) and Cys187 / 188 (Kao and Karlin, 1986) from the principal side and Gln55, Met114 (Tomizawa *et al.*, 2007) from the complementary side contribute to the wall of the ligand-binding cavity.

The crystal structure of AChBP provided the first detailed three-dimensional information about the likely folding pattern and arrangement of the ligand-binding sites of the LGIC family. However, the overall structure of an intact receptor was not apparent

until Unwin and his colleagues solved the structure of the *Torpedo* nAChR to 4 Å resolution using electron microscopy (Miyazawa 2003, Unwin 2005).

The nAChR, found in the electric organs of the *Torpedo* ray, is the prototypical receptor of the Cys-loop LGIC family. It contains five homologous subunits with a stoichiometry of 2α:1β:1γ:1δ (Raftery *et al.*, 1980). Early electron microscopy studies of the *Torpedo* nAChR revealed the overall shape and dimensions of the receptor (Mitra *et al.*, 1989; Toyoshima and Unwin 1988, Unwin 1993, Unwin, 1995). At 9 Å resolution, the *Torpedo* nAChR was revealed to be 125 Å in length and 80 Å in diameter. The extracellular part of the *Torpedo* nAChR is approximately 65 Å in length and the outer vestibule is approximately 20 Å in diameter. The acetylcholine binding cavity, which is located in the extracellular domain, is approximately 30 Å above the membrane (Unwin, 1993; Valenzuela *et al.*, 1994). The intracellular part is about 30 Å in length and forms as an inverted cone underneath the membrane spanning domain (Miyazawa *et al.*, 1999). Recently, higher resolution images of the *Torpedo* nAChR have provided a better idea of the secondary structure of the individual subunits, the channel pore and cytoplasmic domains (Miyazawa *et al.*, 1999; Miyazawa *et al.*, 2003; Unwin 2005) (Figure 1-3, A).

The nAChR ion channel pore is formed by membrane-spanning domains from each subunit. Each subunit consists of four transmembrane helices TM1-TM4 and the lumen of the pore is shaped by TM2, which tilts inwards toward the central axis in the middle of the membrane (Miyazawa *et al.*, 2003). In contrast, TM1, 3 and 4 coil around each other but tilt tangentially towards the central axis. The TM2s make no extensive contact with the other helices and are separated from the TM1, 3 and 4 by water-filled spaces. The study using electron microscopy with 4 Å resolution revealed many

structural features in the ion conduction pathway and the gate. Miyazawa *et al.* (2003) suggested that the gate of the channel is located in the middle of the membrane (Leu251 and Val255 in the α subunit) (Figure 1-3, B), The side-chains of these residues and their equivalent residues from the neighboring helices project to form a tight girdle around the pore. In the closed channel, the minimum radial distance of the gate is ~3 Å, which is too constricting for a hydrated sodium or potassium ion to pass through. Therefore it provides an energetic barrier to ion permeation (Miyazawa *et al.*, 2003). When the receptor is activated by an agonist, there is a ~ 15° clockwise rotation of the extracellular domain of both the α subunits towards its neighboring γ and β subunits. This rotational movement is further conveyed to the TM2 domain resulting in destabilizing the gate. As a result, the TM2 helices move towards the surrounding outer helices leading to the opening of the gate (Miyazawa *et al.*, 2003).

The cytoplasmic part of the *Torpedo* nAChR is composed of the TM1-2 loop and the TM3-4 loop, which includes an intracellular membrane-associated (MA) helix preceding TM4 (Finer-Moore and Stroud 1984; Miyazawa *et al.* 1999; Unwin, 2005). Although the structure of this region is poorly resolved due to the limitations of electron microscopy, the existing data suggest that the intracellular MA helices contributed by each subunit together form an inverted cone with five intervening open spaces, which provide portals for diffusing ions (Miyazawa *et al.*, 1999) (Figure 1-3, C). The intracellular vestibule is approximately 30 Å long and 20 Å wide in which the passing ions can interact electrostatically with the side-chain of wall-lining residues but without direct contact to slow their movement.

THE OVERALL STRUCTURE OF $GABA_AR$ AND THE GABA BINDING SITE

Due to the sequence similarities between the $GABA_AR$ and nAChR subunits, it was predicted, following the cDNA sequencing of the first two subunits of the $GABA_AR$ family, that the $GABA_A$ receptor subunits also have a large extracellular N terminal region, followed by four transmembrane domains (TM1-4) and a small extracellular C-terminal domain (Schofield, 1987). The TM2 domain is the most conserved region across the family and has been proposed to be the channel pore-lining segment. Between TM3 and TM4, there is a large intracellular loop containing the least amount of sequence similarity across the family but with a consensus sequence for phosphorylation by different protein kinases (Schofield *et al.*, 1987; Moss and Smart, 1996) (Figure 1-4).

Identification of the binding sites of GABA and other clinical important drugs has been one of the major $GABA_AR$ research areas for more than three decades. The binding of GABA is the initial step in receptor activation. This binding leads to conformational changes in the receptor structure that transduce the signal from the binding site to the transmembrane domain and this eventually leads to channel opening.

Although certain α and β subunit homomeric receptors are functional and can be activated by GABA (Blair *et al.*, 1988; Sanna *et al.*, 1995), it is generally predicted that the GABA binding pocket involves 6 discrete loops from both the β and α subunits (see Smith and Olsen, 1995). Loops A, B and C, which form the principal component of the binding pocket and face the neighboring α subunit, are from the β subunit (+ side), whereas loops D, E and F, which form the complementary part of the binding pocket, are contributed by the α subunit (– side). Using site-directed mutagensis, photoaffinity labeling and substituted cysteine accessibility modification (SCAM), all of which are

methods to examine the local environment of the amino acids of interest, many residues contributing to the GABA binding site have been identified. On the rat β2 subunit, Tyr97 and Leu99 (Boileau *et al.*, 2002) are located in Loop A; Tyr157; Thr160 (Amin and Weiss, 1993) are in Loop B; Thr202 (Amin and Weiss, 1993), Ser204 (Wagner and Czajkowski, 2001), Tyr205 (Amin and Weiss, 1993; Wagner and Czajkowski, 2001), Arg207 and Ser209 (Wagner and Czajkowski, 2001) are in Loop C. Similarly, on the α1 subunit, the "– side" of the binding pocket, Phe64 (Sigel *et al.*, 1992), Arg66 (Boileau *et al.*, 1999; Hartvig *et al.*, 2000) and Ser68 (Boileau *et al.*, 1999) are in Loop D. Loop E includes Arg119 (Westh-Hansen *et al.*, 1999; Hartvig *et al.*, 2000) and Ile120 (Westh-Hansen *et al.*, 1997). Val178, Val180 and Asp183 have been identified in Loop F (Newell and Czajkowski, 2003) (Figure 1-5).

Within these loops, four amino acids have been identified as primary determinants for agonist recognition: Phe64 (Sigel *et al.*, 1992) and Arg66 (Boileau *et al.*, 1999; Hartvig *et al.*, 2000) from the α1 subunit and Tyr157 and Tyr205 from the β2 subunit (Amin and Weiss, 1993). Sigel *et al.* (1992) showed that substitution of Phe64 by a leucine produced a 200-fold right shift in the concentration-dependence of GABA activation. Its equivalent position in the bovine α1 subunit can be photolabeled (with low efficiency) with [^{3}H]muscimol (Smith and Olsen, 1994). Hartvig *et al.* (2000) showed that mutation of Arg70 to lysine in the α5 subunit (homologous to Arg66 in α1) increased the EC_{50} value for GABA activation by more than 100-fold. Using SCAM, Boileau *et al.* (1999) suggested that Phe64 and Arg66 are likely to line part of the GABA binding site. Similarly, mutations of Tyr157 and Tyr205 on the β2 subunit were shown to result in at

least a 50-fold rightward shift of the concentration-response curve for GABA without altering the maximum amplitude of the current (Amin and Weiss, 1993).

Since the most common $GABA_AR$ has a stoichiometry of 2α:2β:1γ, the $GABA_AR$ is predicted to have two agonist binding sites, i.e., one lying at each of the β(+)/α(–) interfaces (Smith and Olsen, 1995). Using subunit concatenation and mutagenesis, Baumann *et al.* (2003) suggested that these two agonist binding sites (i.e., two β(+)/α(–) interfaces) have different affinities for GABA. Based on the presumed subunit arrangement of α-β-α-γ-β (counter-clockwise, when viewed from the extracellular face), the authors further suggested that the difference in affinities of the two binding sites may be due to subtly different conformations of the two sites as a consequence of the nature of the flanking subunits.

Early radioligand binding studies with $[^3H]$GABA or its rigid analogue, $[^3H]$muscimol, suggested that there are at least two classes of agonist binding sites with several orders of magnitude differences in their affinities (Enna and Snyder, 1975; Olsen *et al.*, 1981; Yang and Olsen, 1987; Agey and Dunn, 1989). These early studies were confounded by the now known receptor heterogeneity, but it was suggested that the observed differences in binding affinities were unlikely to be due to interconvertible states of a single binding site or negative cooperativity (Olsen *et al.*, 1981; Agey and Dunn, 1989). Using recombinant receptors expressed in transiently transfected cell lines (Newell *et al.*, 2000) and a stable cell line (Davies *et al.*, 1994), it was demonstrated that two classes of binding sites with high and low affinity exist in a single receptor subtype. The structural basis of a separate high affinity site on the $GABA_A$ receptor has been explored previously. Newell *et al.* (2000) suggested that a separate high affinity site is

located at the $\alpha(+)/\beta(-)$ interface with the involvement of Tyr 62 on β2 subunit (Figure 1-5), which is equivalent to α1 Phe64 in Loop D. Substitution of the tyrosine residue with phenylalanine decreases the affinity for both GABA and muscimol, while substitution of serine at this position leads to a loss of detectable high affinity binding site. Further studies suggested that this high affinity GABA binding site may play a role in maintaining the desensitized state after the onset of desensitization of $GABA_AR$ (Newell and Dunn, 2002).

BENZODIAZEPINE BINDING SITE ON THE $GABA_A$ RECEPTOR

Because of their clinical importance, the effect of benzodiazepines on the $GABA_AR$ has been extensively characterized and the binding site for benzodiazepines has been one of the major focuses of $GABA_AR$ research. Using photolabeling techniques, early studies suggested that $[^3H]$flunitrazepam photoincorporated primarily into the α subunit (Casalotti *et al.*, 1986; Stephenson *et al.*, 1990), while the γ subunit was photolabeled to a lesser extent (Stephenson *et al.*, 1990). These early data suggested that both α and γ subunits contribute to the benzodiazepine binding site (Stephenson *et al.*, 1990). Furthermore, using recombinant expression of $GABA_AR$, functional studies showed that benzodiazepine sensitivity was absent in the binary αβ receptor (Schofield *et al.*, 1987) but could be restored by the inclusion of the γ subunit (Pritchett *et al.*, 1989).

Similar to the GABA binding site, the residues that have been implicated in contributing to the benzodiazepine binding site cluster into discrete loop structures in the extracellular N-terminal domains of α (loop A, B and C, + side) and γ subunits (loop D, E, F, – side). Many of these important residues have been identified by photolabeling

techniques, radioligand binding assays and functional studies combined with mutagenesis (Figure 1-6, A). His101 in loop A was identified as a major determinant contributing to the benzodiazepine high affinity binding site on the rat α1 subunit (Wieland *et al.*, 1992; Duncalfe *et al.*, 1996; Davies *et al.*, 1998; Smith and Olsen, 2000). Further, in the rat α1 subunit, Tyr159 (Amin *et al.*, 1997), Thr162 (Wieland and Luddens, 1994; Renard *et al.*, 1999) in loop B, Ser 204 (Renard *et al.*, 1999), Ser205 (Derry *et al.*, 2004), Thr206 and Tyr209 (Buhr *et al.*, 1997b) in loop C have been identified as contributing to the binding site. In the γ subunit, residues Phe77 (Buhr *et al.*, 1997a; Wingrove *et al.*, 1997) and Ala79 (Kucken *et al.*, 2003) in loop D, Met130 (Wingrove *et al.*, 1997), Thr142 (Mihic *et al.*, 1994) in loop E and Met57 (Buhr and Sigel, 1997) and Tyr58 (Kucken *et al.*, 2000) have been identified as contributing to the binding site (Figure 1-6, A). Recently residues Asp192-Arg 197 in loop F of the γ2 subunit have been implicated in the benzodiazepine-mediated conformational change that leads to an increased response to GABA, but does not affect benzodiazepine binding *per se* (Padgett and Lummis, 2008).

The orientation of benzodiazepines in their binding pocket is still unclear. McKernan *et al.* (1998) previously investigated the effects of photoaffinity labeling with [^{3}H]flunitrazepam on the binding of various benzodiazepine site ligands. They suggested that the classical benzodiazepines interact with α subunit His 101 through their pendant 5-phenyl groups (the C-ring, see Figure 1-9). They further proposed that a possible mechanism for this interaction is hydrogen bonding or π-π interaction of the phenyl ring with the histidyl side chain. However, the proposed orientation of benzodiazepines was contradicted by the results of other studies, which suggested that the histidine residue is in the proximity of the seven-membered heterocycle (the B-ring) of benzodiazepines

where it may form a hydrogen bond (Zhang *et al.*, 1995; Davies *et al.*, 2000; Gardner *et al.*, 1992). Furthermore, based on screening of the affinity of structurally-related compounds on wildtype or mutated receptors, Sigel *et al.* (1998) suggested that the pendant phenyl group of classic benzodiazepines may be located close to γ2 Phe77. In a novel study, Berezhnoy *et al.* (2004) individually mutated a series of residues, which had been implicated in the benzodiazepine binding pocket, to cysteines. The authors then observed a covalent interaction of the receptor α1His101Cβ1γ2 with a cysteine-reactive benzodiazepine, in which an isothiocyanate was incorporated at the C7 position to replace chloride atom. This result led the authors to suggest that the C7 position in the 1,4-benzodiazepine backbone may lie close to α1His101. Recently, Mokrab *et al.* (2007) used comparative modeling approaches to dock 1,4-benzodiazepines to the α1β1γ2 receptor. In this three-dimensional structural model, the N1-methyl and the 7-nitro group of the flunitrazepam are close to α1 His101, while the pendant 5-phenyl is positioned between α1 Tyr159 and γ2 Tyr58 and the benzodiazepine A-ring is about 3 Å away from γ2 Phe77 (Figure 1-6, B).

It is of note that a benzodiazepine site distinct from that at the α-γ subunit interface has been described by others. Im *et al.* (1993) reported that several benzodiazepines and benzodiazepine site ligands can significantly potentiate GABA-evoked currents of the binary β2γ2 receptor expressed in a stable cell line. These results suggest that a benzodiazepine site may exist at subunit interface other than α-γ. Amin *et al.* (1997) and Walters *et al.* (2000) showed that GABA currents mediated by the α1β2γ2 receptor can be potentiated by diazepam in a biphasic manner, with distinct components in the nanomolar and micromolar concentration ranges. These authors further suggested

that a low affinity benzodiazepine binding site may be located in the TM2 domain since mutations in the TM2 domains of the α, β and γ subunit abolished the low affinity component of benzodiazepine modulation but not the high affinity component.

RECEPTOR DESENSITIZATION

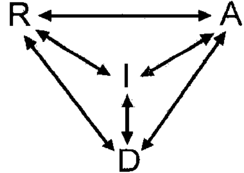
Desensitization is an intrinsic property of the LGICs. In the continued presence of agonist, the current progressively declines over the time of exposure and the channels enter a refractory or non-conducting (desensitized) state. The mechanism of desensitization remains poorly understood. Katz and Thesleff (1957) proposed a two-state model for desensitization, which originally accounted for the observation of desensitization of the nACh receptor at the neuromuscular junction.

$$\begin{array}{ccc} R + A & \overset{K_R}{\longleftrightarrow} & AR \\ L \updownarrow & & \updownarrow \\ D + A & \underset{K_D}{\longleftrightarrow} & AD \end{array}$$

This cyclic model implies that the nicotinic acetylcholine receptor pre-exists in two interconvertible conformations, the resting state (R, also known as unliganded activatable state) and the desensitized state (D). These two receptor conformations are characterized by significant differences in their affinities for agonist. For example, the apparent dissociation constant for Ach binding to the low-affinity (resting state) conformation (K_R) was determined to be > 600 nmol/L, whereas the apparent dissociation constant for binding to the high-affinity desensitized conformation (K_D) was about 2 nmol/L (Weiland *et al.*, 1976). In the absence of agonist (A), most receptors are in the resting state (R), but a small fraction of the nAChRs exists in the desensitized state (D).

As determined by radiolabeled and fluorescent agonist binding studies to the nAChR in *Torpedo* membrane vesicles, the value of the allosteric constant (*L*) for the state transition between desensitized and unliganded activatable nAChR was estimated as 0.1 - 0.2 (Weiland *et al.*, 1976; Heidmann and Changeux, 1979; Neubig *et al.*, 1982), i.e., about 10% – 20% of the nAChRs are in the desensitized state in the absence of agonist. Agonist binding to the receptor in the resting state was proposed to trigger sequential allosteric conformational changes, that promote the isomerization of the complex to the active state (AR) and opening of the ion channel. Prolonged exposure to the agonist results in an increase in its affinity for the receptor and induces the conformational change(s) leading to receptor desensitization (Ochoa *et al.*, 1989).

Although the model of Katz and Thesleff has received widespread support (eg. Rang and Ritter, 1970; Feltz and Trautmann, 1982), it does not take into account the multiple agonist binding steps, and thus the original model of channel binding and gating has been extended to accommodate this now well-established property (Feltz and Trautmann, 1982). In addition, an intermediate state has been added in order to reflect the bi-exponential time course of desensitization onset and recovery (Changeux *et al.*, 1984). In this model, Changeux *et al.* (1984) proposed that the presence of high concentration of



agonists shifts the equilibrium between the resting (R) and the active (A) state more toward the active state. If the agonist is applied for a prolonged period, the intermediate

state (I) rapidly gets populated in a transient manner (within 0.1 to 1 second), and finally, the desensitized state (D) is stabilized slowly (with seconds). This two-step model of desensitization consisting of rapid and slow processes observed using electrophysiological methods (Feltz and Trautmann, 1982; Anwyl and Narahashi, 1980; Chesnut, 1983; Boyd, 1987) and by rapid kinetics measurements (Neubig and Cohen, 1980; Walker *et al.*, 1981).

The above cyclic models and their variants of desensitization generally assume that channel activation and desensitization involve binding of ACh to two ACh sites in each state of the receptor. However, based on the observations that apparent dissociation constants obtained from agonist-mediated permeability responses by the nACh receptors and those measured in direct ligand binding experiments are greatly different, Dunn and Raftery (1982a, b) proposed that the nAChR has multiple classes of binding sites with significantly different affinities for agonists. A model put forward by Raftery and his colleagues (1983) suggested that

$$2\ [\,\mathbf{R_L} + \mathbf{A} \longleftrightarrow \mathbf{AR_L} \overset{\text{fast}}{\longleftrightarrow} \text{open channel}\,]$$
$$2\ [\,\mathbf{R_H} + \mathbf{A} \longleftrightarrow \mathbf{AR_H} \overset{\text{slow}}{\longleftrightarrow} \text{desensitization}\,]$$

the interaction of high concentrations agonist (A) with intrinsically low-affinity binding sites (R_L) may induce rapid conformational changes of the receptor (AR_L) to open the channel. Over a longer time scale, the agonist binding to the two high-affinity sites (R_H) evokes considerably slower receptor conformational transitions, which induce the receptor (AR_H) to enter into the closed, desensitized state. According to this model, the

authors suggested that the receptor activation and desensitization of the nAChR are parallel, rather than sequential, processes.

The elucidation of structural determinants in receptor desensitization has been another major area of study. In $GABA_ARs$, desensitization varies between different receptor subtypes. Inclusion of the γ2 subunit in the ternary $GABA_AR$ such as the α1β3γ2 and α4β3γ2s subtype significantly increases the rate and the extent of desensitization compared to α1β3 and α4β3 subtypes, respectively (Dominguez-Perrot *et al.*, 1996; Lagrange *et al.*, 2007). However, substitution of the γ2 subunit with the δ subunit decreases the rate and the extent of desensitization (Brown *et al.*, 2002; Bianchi *et al.*, 2001). Several molecular determinants have been suggested to be involved in the desensitization of $GABA_ARs$ and many of these are located in transmembrane domain 2. In the literature, to facilitate comparisons of the residues in the TM2 segment of different subunits, an index numbering system has been used (Miller, 1989). Residues in TM2 are identified independently as 1' to 28' (Figure 1-7), from the cytoplasmic end to the extracellular end (Miyazawa *et al.*, 2003). Within TM2, Leu9' is highly conserved in the LGICs. Substituting this Leu9' to a polar residue (Ser or Thr) significantly decreases the apparent desensitization rate of these receptors, accompanied by an apparent increase in their agonist affinities (Revah *et al.*, 1991; Yakel *et al.*, 1993; Labraca *et al.*, 1995; Filatov and White, 1995; Thompson *et al.*, 1999b; Scheller and Forman, 2002). In a cell line transfected with the $GABA_AR$ α1β1 subtype, a mutation of a Leu in β1 (L9'T) does not generate functional receptors. A mutation in the α1 (L9'T) subunit, however, has a significantly decreased rate of desensitization, when compared to wildtype (Tierney *et al.*, 1996). In the $GABA_AR$ α1β3γ2 subtype, a mutation of the γ2 subunit (L9'S) results in

a slower desensitization rate, longer deactivation and increased apparent GABA affinity (Bianchi and Macdonald, 2001). These similar results suggest different roles for Leu9' on channel gating and desensitization. Other residues in TM2, such as 5' valine in the $GABA_AR$ α1 subunit, 5' isoleucine, 12' threonine in β1, and 13' threonine in α6, (Birnir *et al.*, 1997a, b; Im *et al.*, 1995) can also influence desensitization rates. Using δ/γ chimeric subunits, Bianchi *et al.* (2001) identified the N-terminal domain and first two residues in TM1 domain (V233, Y234), rather than the pore-lining TM2 domain, of the δ subunit as being important for the slow desensitization observed for the δ-containing receptor.

$GABA_AR$ PHARMACOLOGY

$GABA_A$ receptors possess a rich pharmacology. There are more than 100 drugs of many different classes that are known to act on $GABA_ARs$ (Johnston, 1996). These ligands can be broadly divided into three categories: 1) agonists, including full agonists and partial agonists, 2) antagonists, including competitive and noncompetitive antagonists, and 3) various allosteric modulators. Here I provide an overview of $GABA_AR$ pharmacology.

AGONISTS

Ligands which directly activate $GABA_ARs$ and open the chloride channel are $GABA_AR$ agonists (Figure 1-8), exemplified by GABA itself. GABA is a flexible molecule which can adopt a variety of low energy conformations to interact with different receptors, enzymes, and transporters. When GABA binds to the $GABA_AR$, the

receptor undergoes a series of conformational changes, which lead to the opening of the receptor channel and an increase in chloride ion conductance. The potencies of GABA and other GABAergic agonists vary depending on the receptor subunit composition. For example, the EC_{50} value for GABA activation of the $\alpha4\beta3\gamma2$ receptor is ~30 fold greater than for the $\alpha4\beta3\delta$ subtype (Wallner *et al.*, 2003; You and Dunn, 2007). One of the most widely used exogenous agonists is muscimol, a toxin found in *Amanita muscaria* mushrooms. Muscimol is a relatively rigid analogue of GABA and has been a prototype for the development of a range of $GABA_AR$ agonists (Krogsgaard-Larsen *et al.*, 1975) including thiomuscimol, dihydromuscimol, isoguvacine, 4,5,6,7-tetrahydroisoxazolo[5,4]pyridin-3-ol (THIP), Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA), 5-(4-piperidyl)-3-isothiazolol (thio-4-PIOL), 5-(4-piperidyl)-3-isoxazolol (4-PIOL), piperidine-4-sulphonic acid (P4S) (Krogsgaard-Larsen *et al.* 2004). In addition, several endogenous agonists have been identified, including imidazole-4-acetic acid (I4AA) (Tunnicliff, 1998), taurine and β-alanine (Quinn and Harris, 1995). Muscimol and isoguvacine have similar efficacies to GABA when acting on the most common $GABA_AR$ subtype $\alpha1\beta2\gamma2$, and these are generally recognized as full agonists. However, THIP, I4AA and P4S have been described as partial agonists due to their lower efficacies on this receptor subtype, when compared to GABA (Krogsgaard-Larsen *et al.* 2004). The efficacies of agonists and partial agonists depend on the subunit composition. Acting on the $\alpha4\beta3\delta$ subtype, for example, THIP and I4AA elicit maximum currents that are higher than those evoked by saturating concentrations of GABA (Brown *et al.*, 2002; You and Dunn, 2007).

ANTAGONISTS

Bicuculline, a plant alkaloid, was the first GABA receptor antagonist to be identified (Curtis *et al.*, 1970). Subsequent studies showed that not all GABA receptors were antagonized by bicuculline, leading to the classification of two classes of GABA receptor, i.e. $GABA_A$ and $GABA_B$ receptors (Hill and Bowery, 1981). Competitive antagonists of the $GABA_AR$ include bicuculline and SR95531 (Figure 1-8), a pyridazinyl GABA derivative. These drugs are suggested to have structural similarities with muscimol and to share at least part of the binding site with $GABA_AR$ agonists (Vestergaard *et al.*, 2007). They shift concentration-response curves to GABA to the right in a competitive manner. Recently, a number of analogues of 4-PIOL have been shown to have no direct effects on $GABA_ARs$ but to have the ability to antagonize GABA-mediated currents in a competitive way (Frølund *et al.*, 2005).

A representative non-competitive antagonist is picrotoxin (Figure 1-8), a mixture of picrotoxinin and picrotin isolated from a Southeast Asia plant, *Anamirta cocculus.* Picrotoxinin, the more potent component of the mixture, does not inhibit the binding of agonists or benzodiazepines to $GABA_ARs$ but binds to the lumen of the channel pore in $GABA_ARs$ (Chen *et al.*, 2006). Furthermore, several $GABA_AR$ non-competitive antagonists, such as lindane and fipronil used as insecticides (Bloomquist, 2003), have been suggested to occupy the same ligand binding site as picrotoxinin (Chen *et al.*, 2006). Furosemide, a noncompetitive subtype-selective $GABA_AR$ antagonist (Korpi *et al.*, 1995), has been shown to have approximately 100-fold greater sensitivity for $\alpha6$, $\beta2/3$ - containing receptors over other receptors containing different α subunits and the $\beta1$ subunit (Thompson *et al.*, 1999a).

ALLOSTERIC MODULATORS: BENZODIAZEPINES

A number of clinically important drugs exert their effects on $GABA_A$Rs through allosteric mechanisms. These compounds do not directly activate or inhibit $GABA_A$Rs but modulate the effects of agonists. The best characterized allosteric modulators of the $GABA_A$Rs are the benzodiazepines (Figure 1-9). The first such compound, chlordiazepoxide, was introduced in 1960 (Tobin and Lewis, 1960), i.e., before GABA was known to be a neurotransmitter. Many studies in the 1970s and 1980s demonstrated that the clinically relevant sedative, anxiolytic, anticonvulsant, muscle relaxant and amnesic effects of the classical benzodiazepines are due to their modulation of GABA activation of $GABA_A$Rs (Macdonald and Barker, 1978; Gavish and Snyder 1980). Single channel studies revealed that the classical 1,4-benzodiazepines, such as diazepam and flunitrazepam, enhance the frequency of GABA-gated channel opening (Twyman *et al.*, 1989). However, some benzodiazepines and benzodiazepine site ligands, such as the structurally-related imidazobenzodiazepines and β-carbolines, act as proconvulsants and stimulants. As negative allosteric modulators, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), β-carboline-3-carboxylic acid ethyl ester (β-CCE), Ro15-4513 and Ro19-4603 typically inhibit currents mediated by the $GABA_A$ $\alpha1\beta2\gamma2$ receptors (Barnard *et al.*, 1998). Furthermore, Ro15-1788 and ZK93426 are generally considered to be antagonists acting at the benzodiazepine binding site.

The heterogeneity of $GABA_A$R subtypes further complicates benzodiazepine pharmacology. Some benzodiazepine site ligands, such as the triazolopyridazine CL218872, zolpidem and certain β-carbolines, have high affinities for the $\alpha1\beta\gamma2$ subtype, but lower affinities for $\alpha2/3/5\beta\gamma2$ subtypes. Several structurally-related

imidazobenzodiazepines, such as L-655,708 and RY-80, have been identified as selective compounds for the benzodiazepine site. These drugs have 50-100 fold higher affinity for α5-containing receptors than for those containing the α1/2/3 subunits (Quirk *et al.*, 1996; Skolnick *et al.*, 1997). Acting as negative allosteric modulators, both compounds selectively inhibit GABA currents elicited by α5-containing receptors (Maubach 2003). Since α1/2/3/5βγ2 receptors all bind diazepam with high affinity, they are classified as diazepam-sensitive receptors. On the other hand, diazepam-insensitive receptors refer to the α4- and α6-containing receptors, where the affinities of classical 1,4-benzodiazepines are at least 1000-fold lower than for the diazepam-sensitive subtypes (Hadingham *et al.*, 1993). The molecular basis for this is attributed to a substitution of a histidine in position 101 (loop "A", see above) of α1/2/3/5 by an arginine residue in α4 and α6 (Wieland *et al.*, 1992). Despite their insensitivity to the classical benzodiazepines, α4/6βγ2 subtypes still retain high affinity for the imidazobenzodiazepines Ro15-4513 and Ro15-1788 and certain β-carbolines, such as β-CCE and DMCM (Derry *et al.*, 2004). However, substitution of a γ subunit by the δ subunit can totally abolish the effects of benzodiazepines and benzodiazepine site ligands (Brown *et al.*, 2002).

OTHER ALLOSTERIC MODULATORS: BARBITURATES AND NEUROSTEROIDS

$GABA_A$Rs can be modulated by a wide variety of structurally diverse general anesthetics, including barbiturates, propofol, etomidate and alphaxalone. These anesthetics can enhance $GABA_A$R function at clinically relevant concentrations. Barbiturates have been used since the early 1900s. They have three distinct effects on

$GABA_AR$ function. At low concentrations, barbiturates act as positive allosteric modulators of $GABA_AR$ to increase the response to GABA (Nicoll and Wojtowicz 1980; Schulz and Macdonald 1981). At high micromolar concentrations, they act as agonists and directly activate the receptor (Nicoll and Wojtowicz 1980; Schulz and Macdonald 1981). Single-channel analysis suggested that the barbiturates enhance GABA-mediated currents by increasing the mean open time of the channel but not affecting channel opening frequency (Twyman *et al.*, 1989). However, at millimolar concentrations, barbiturates act as channel blockers and inhibit $GABA_AR$ activity (Akaike *et al.*, 1987). These three distinct effects are possibly mediated by different interaction sites on the $GABA_AR$. Although both α and β subunits of $GABA_AR$ are thought to contribute to barbiturate effects, the modulation effects of these drugs depend largely on the particular β subunit isoform present (Thompson *et al.*, 1996; Serafini *et al.*, 2000; Chang *et al.*, 2003). A greater sensitivity to pentobarbital was observed for β2- and β3-containing receptors compared to β1-containing receptors. Several residues within the TM1 and TM2 domains of the β subunit have been identified as being responsible for these difference (Chang *et al.*, 2003; Cestari *et al.*, 2000).

Neurosteroids have anxiolytic, anticonvulsant, sedative and anesthetic effects mainly through their ability to allosterically modulate $GABA_ARs$ (Belelli and Lambert 2005). At low nanomolar concentrations, neurosteroids allosterically modulate $GABA_ARs$ by prolonging channel open times, with no effect on channel conductance. At higher concentrations (>100 nmol/L), neurosteroids directly activate $GABA_ARs$ (Herd *et al.*, 2007). Compared to benzodiazepines and barbiturates, neurosteroid effects show less $GABA_AR$ subtype specificity. Receptors containing different subtypes of α, β and γ have

a modest difference (3- to 10-fold) in neurosteroid sensitivity (for review see Herd *et al.*, 2007; Hoise *et al.*, 2007). One of only a few distinguishing features is that the neurosteroid efficacy at the δ-containing receptor is significantly increased compared to the γ2-containing receptor (Brown *et al.*, 2002; Wohlfarth *et al.*, 2002). The mechanism of neurosteroid modulation has been examined by constructing chimeric subunits containing neurosteroid-sensitive murine $GABA_AR$ α1 or β2 subunits with a neurosteroid-insensitive *Drosophila melanogaster* RDL (resistance to dieldrin) $GABA_AR$ subunit. Using this strategy, Hoise *et al.* (2006) demonstrated that neurosteroids bind to two discrete sites that play distinct roles in $GABA_AR$ activation and allosteric modulation. The binding site for direct activation is located at the outer surface of the interface between the β subunit TM3 domain and the α subunit TM1 domain. The neurosteroid allosteric modulation site is suggested to be located in a hydrophobic cavity formed by the highly conserved TM1 and TM4 domains of the α subunit (Hoise *et al.* 2006).

PHASIC AND TONIC GABAERGIC INHIBITIONS

It has long been known that GABA mediates fast synaptic inhibition in the central nervous system (see Jones and Westbrook, 1996). However, studies in the early 1990s showed the presence of a large component of tonic inhibition in hippocampal neurons (Otis *et al.*, 1991) and cerebellar granule cells (Kaneda *et al.*, 1995; Brickley *et al.*, 1996). It has now become clear that $GABA_ARs$-mediated neuronal inhibition has two distinct modes of operation: phasic inhibition, mediated by the receptors found at the synapse and

tonic inhibition, mediated by receptors found extrasynaptically. (Mody, 2001; Farrant and Nusser, 2005).

Phasic inhibition occurs when the postsynaptic $GABA_ARs$ are exposed to a transient but high concentration of GABA released from the presynatic neuron after the arrival of an action potential. This inhibition typically displays high temporal- and spatial-resolution. It has been estimated that the distance between the GABA release sites on the presynaptic neuron and its postsynaptically located receptors is about 50 nm (Fritschy and Brunig, 2003). Within this distance, it has been calculated that peak GABA concentrations may reach the hundreds of micromolar to the millimolar range (Perrais and Ropert, 1999; Maconochie *et al.*, 1994; Mozrzymas *et al.*, 1999). However, this high concentration of GABA is short-lived. A variety of experimental methods have shown that released GABA is cleared from the synapse with a time constant of 100 ~ 500 μs (Overstreet *et al.*, 2002; Mozrzymas *et al.*, 2003). A rapid onset (a few hundred microseconds) of spontaneous miniature inhibitory postsynaptic currents (mIPSCs), generated by released GABA, also reflects the proximity of the receptors to the presynaptic release site (Jones and Westbrook, 1995). In contrast, tonic GABAergic inhibition refers to the activation of a subset of $GABA_ARs$ in a manner that is temporally and spatially dissociated from phasic synaptic events. $GABA_ARs$ expressed in the extrasynaptic/perisynaptic area can be persistently or "tonically" activated by a low ambient concentration GABA that either overspills from the synaptic cleft or is released from glial cells (Farrant and Nusser, 2005).

Tonic inhibition was first demonstrated in cerebellar granule cells (Kaneda *et al.*, 1995). Application of bicuculline to these cells decreased spontaneous inhibitory

postsynaptic currents and this was accompanied by a reduction of background noise, indicating a block of stochastic ion channel openings. Subsequent studies identified similar phenomena in granule cells of the dentate gyrus (Nusser and Mody, 2002), CA1 pyramidal neurons (Bai *et al.*, 2001) and in inhibitory interneurons of hippocampus (Semyanov *et al.*, 2003). In these areas, specific $GABA_AR$ subtypes have been suggested to be responsible for tonic inhibition. In cerebellar granule cells, for example, tonic inhibition is selectively blocked by the α6-selective antagonist, furosemide, enhanced by the δ subunit sensitive neurostoid allotetrahydrodeoxycorticosterone (THDOC) (Stell *et al.*, 2003) and is insensitive to diazepam (Hamann *et al*, 2002). In α6 and δ knockout mice, the tonic conductance mediated by $GABA_ARs$ in cerebellar granule cells was abolished and there was a reduction of surface expression of the δ subunit (Brickley 2001). Immunohistochemistry has indicated the co-localization of α6 and δ in the extrasynaptic area of cerebellum granule cells (Nusser *et al.*, 1998). Moreover, the properties of slow desensitization and high sensitivity to GABA displayed by the recombinant α6βδ receptor closely mimic the biophysical and pharmacological characteristics of the tonic conductance observed in cerebellar granule cells (Saxena and Macdonald, 1996). These observations led to the suggestion that the tonic inhibition in the cerebellum is likely to be mediated by the α6βδ subtype (Brickley 2001, Stell *et al.*, 2003).

Delta-containing $GABA_ARs$ have also been implicated in mediating tonic currents in dentate granule cells, ventrobasal neurons and the dorsal lateral geniculate nucleus from the thalamus, where the δ subunit has a close partnership with the α4 subunits (Wisden *et al.*, 1992; Fritschy and Mohler, 1995 Stell *et al.*, 2003; Chandra *et al.*, 2006).

Similar to the cerebellum, the tonic conductance in the dentate gyrus granule cells is insensitive to benzodiazepines but sensitive to THDOC in a concentration range that has no effect on synaptic currents (Stell, 2003). The tonic current was significantly, but not totally, reduced in brain slices from δ knockout mice, and was accompanied by a decrease in the surface expression of the α4 subunit (Peng *et al.*, 2002). However, a residual tonic current detected in the same preparation of δ knockout mice indicated the existence of a heterogeneous population of $GABA_A$Rs that is responsible for tonic inhibition in these cells. On the other hand, α4 knockout mice have also been found to lack tonic inhibition in dentate granule cells and ventrobasal neurons of the thalamus (Chandra 2006). These studies indicated that the role of α4-, δ-containing receptors is to mediate tonic inhibition in these brain areas. A recent study using confocal microscopy imaging and electrophysiological methods suggested that the α4βδ subtype is exclusively co-assembled and predominantly co-localized at extrasynaptic sites where it contributes to tonic inhibition (Jia *et al.*, 2005). It is of note that in these areas, the α4 and β subunits are found to be co-localized with the γ2 subunit, a putative synaptic isoform. Sur *et al.* (1999a) reported that two-thirds of α4-containing $GABA_A$ receptor subtypes in the hippocampus and thalamus include the δ subunit while one-third of the α4 subunits co-associate with the γ2 subunits in the same area. Furthermore, the distribution patterns of these two receptors are complementary (Peng *et al.*, 2002) and, in many pathological situations, the changes in expression levels of one subtype are often at the expense of other subtypes (see below).

Another form of $GABA_A$R-mediated tonic currents was detected in hippocampal pyramidal neurons. The subunit composition of this $GABA_A$R was investigated using

different pharmacological approaches. Tonic current in the dentate gyrus was found to be insensitive to the imidazopyridine, zolpidem, for which the α5-containing receptor has been previously reported to have low affinity (Luddens *et al.*, 1994). This tonic current was also insensitive to the α4, α6 and ε-selective antagonist, furosemide, and was not affected by the δ-selective neurosteroid, THDOC (Caraiscos *et al.*, 2004). However, the tonic conductance was reduced by an α5-selective inverse antagonist, L-655,708, and preferentially enhanced by the β2/3-selective loreclezole and etomidate at low concentrations which left synaptic currents unchanged. The potentiation of tonic current by the benzodiazepine, midazolam, indicated the involvement of the γ2 subunit. Together, these results suggest that the tonic currents in the hippocampal pyramidal neurons are most likely mediated by $GABA_A$Rs containing the α5, β2/3 and γ2 subunits. Evidence from knockout mice has further supported these results. In the δ knockout mice, tonic current was still detected in hippocampal pyramidal neurons, whereas the tonic conductance was significantly reduced in the same area from the α5 knockout mice (Caraiscos *et al.*, 2004).

A recent study suggested that $GABA_A$Rs containing α1βxδ subunits could mediate tonic inhibition in the molecular layer interneurons of the hippocampus (Glykys *et al.*, 2007). The α1 subunit is generally co-expressed with the γ2 subunit, and is found mostly at synapses. However, in the molecular layer interneurons, colocalization of α1/δ subunits was revealed by confocal microscopic immunofluorescence studies, although no α2, α3, α4 and α5 subunits in these neurons were detected. The tonic current displayed by these neurons remained the same in both wildtype and α4 knockout mice, although a substantial decrease of δ subunit expression was observed in the α4 knockout mice.

The function of tonic inhibition is poorly understood and remains to be elucidated. The consequence of tonic activation of $GABA_A$Rs is to persistently increase the negative input conductance of the cell. In response to a given excitatory postsynaptic current, tonic inhibition can reduce the magnitude and duration of the excitatory postsynaptic potential, narrow the temporal and spatial window over which signal integration can occur and make it less likely that a subsequent action potential is generated (Farrant and Nusser, 2005).

A ROLE FOR δ-CONTAINING $GABA_A$ RECEPTORS IN EPILEPSY

Epilepsy is a common neurological disorder that is caused by the disruption of the balance between neuronal excitation and inhibition. Increased neuronal excitability caused by impaired $GABA_A$R-mediated inhibition is a pathophysiological mechanism that leads to epileptogenesis. There is considerable evidence to suggest that changes in GABAergic synaptic transmission trigger epileptic activity. Extrasynaptic $GABA_A$Rs, such as δ-containing receptors may be important in playing a role in seizure initiation, maintenance and arrest (Semyanov *et al.*, 2004; Mody, 2005).

Expression of the δ subunit is altered in several animal models of epilepsy. In a mouse model of absence seizures, the expression of the $GABA_A$R α1 and β2 subunits and the $GABA_A$R-specific synaptic anchoring protein gephyrin remained the same in the dentate granule cells. However, consistent up-regulation of the synaptic α4 and γ2 subunit expression were observed with significant reduction of δ subunit expression as illustrated by a reduction in the THDOC-sensitive $GABA_A$R-mediated tonic current (Payne *et al.*, 2006). Out of the many epilepsy syndromes, the most common form of

human epilepsy is symptomatic focal epilepsy mesial temporal lobe epilepsy (MTLE) (Brooks-Kayal *et al.*, 1998). In a cyclothiazide-induced rat model of temporal lobe epilepsy, tonic conductance was significantly reduced after epileptogenic stimulation whereas the synaptic amplitude of mIPSCs was unaffected (Qi *et al.*, 2006). In addition, the reduced expression of the δ subunit in the dentate gyrus cells was found in a pilocarpine-induced mouse model of temporal lobe epilepsy (Peng, *et al.*, 2004), accompanied by an increase of α4 and γ2 subunits in the same area. Using immunohistochemical and electrophysiological techniques, other studies (Zhang *et al.*, 2007; Schwarzer *et al.*, 1997) found that in epileptic animal models, there was significant $GABA_AR$ plasticity with decreased expression of the δ subunit and an increased expression of γ2 subunits in the dentate granule cell. Consistent with the idea that alterations in the expression of δ-containing receptors are involved in epilepsy, δ knockout mice exhibit epilepsy and other signs of hyperexcitability (Mihalek *et al.*, 1999; Spigelman *et al.*, 2002)

The plasticity of δ-containing receptors can be regulated by sex hormones and this phenomenon has been implicated in the etiology of catamenial epilepsy. Catamenial epilepsy describes a tendency for increased seizures in different stages of the menstrual cycle. Maguire (*et al.*, 2005) showed that there are dynamic alterations in $GABA_AR$ subunit composition and neuronal excitability in the hippocampus over the ovarian cycle. During dioestrus when the progesterone level is high, the expression of δ subunits is enhanced while γ2 subunit expression is decreased. This leads to increased tonic inhibition and reduced seizure susceptibility. During oestrus, when the progesterone level is low, decreased δ expression and increased γ2 expression result in reduced tonic

inhibition, and therefore, a higher seizure susceptibility has been predicted. Consistent with this finding, Lovick *et al.* (2005) reported an increased expression of α4, β1 and δ subunits in female rats in late diestrus in the dorsolateral periaqueductal gray matter, a region dominated by GABAergic interneurones.

Other evidence of δ subunit involvement in epilepsy came from a recent study by Dibbens *et al.* (2004) which described two δ subunit mutations found in human epilepsy. Glu177Ala and Arg220His, which are located in the extracellular N-terminal domain of the δ subunit, were heterozygously associated with generalized epilepsy with febrile seizures plus (GEF+) and juvenile myoclonic epilepsy (JME) in patients, respectively. When these mutants were co-expressed with the α1 and β2 subunits in human embryonic kidney (HEK) cells, there was a significant reduction of GABA-evoked currents, underlying a possible mechanism for reduced tonic inhibition (Dibbens *et al.*, 2004). When these two mutants were co-expressed with α4 and β2 subunits, a reduction of surface receptor proteins was observed with both the α4β2δ(E177A) and α4β2δ(R220H) mutations (Feng *et al.*, 2006). In addition, single-channel analysis of these two variants suggested a decreased mean channel open time compared to wildtype receptors (Feng *et al.*, 2006). Considering the function of the δ subunit in mediating tonic inhibitory current, these data suggested that reduction of δ-subunit mediated tonic inhibition would likely cause neuronal hyperexcitability and an increased susceptibility to develop epilepsy.

AIMS OF THE PRESENT STUDIES

The overall goal of my studies has been to characterize the putative extrasynaptic $GABA_AR$ subtype α4β3δ and the putative synaptic α4β3γ2 subtype. The δ subunit has attracted a great deal of attention because of its specific localization and function in the mammalian brain. It has been found in the extrasynaptic area of the thalamus and in dentate gyrus granule cells where it is thought to be associated with the α4 subunit (Sur *et al.*, 1999; Bencsits *et al.*, 1999). In addition, altered expression levels of δ- and γ2-containing receptor subtypes has been observed in different animal models related to epilepsy (Payne *et al.*, 2006; Qi *et al.*, 2006; Peng, *et al.*, 2004). The δ-containing receptor possesses a unique pharmacological and biophysical profile, which is distinct from γ2-containing receptors. The putative extrasynaptic $GABA_AR$ α4β3δ subtype can be activated by low concentrations of GABA that overspill from the synaptic cleft or is released from glial cells (Farrant and Nusser, 2005). However, the molecular mechanisms underlying its higher agonist sensitivity compared with the putative synaptic $GABA_A$ α4β3γ2 receptor are unknown. To address this question, in Chapter 2 we generated a series of δ/γ2 chimeras and co-expressed these with α4 and β3 subunits in *Xenopus* oocytes. We used different agonists (GABA, muscimol) and partial agonists (THIP, I4AA) to explore the structural determinants of the δ subunit that confer high agonist potencies. Moreover, we characterized the functional effects of two competitive antagonists, bicuculline and SR95531, and the channel blocker, picrotoxin, on these wildtype and chimeric subtypes to investigate the mechanisms underlying their differences in action.

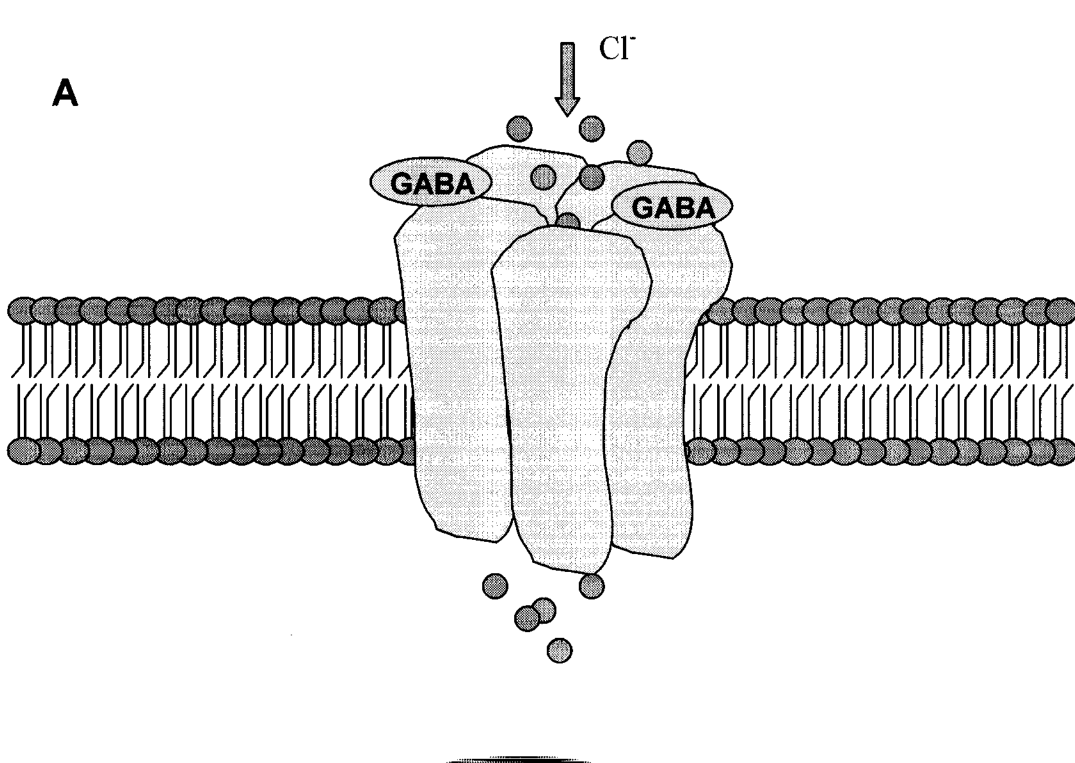
One of the basic characteristics of tonic conductance is its slow desensitization. This property is most likely conferred by inclusion of the δ subunit in the extrasynaptic $GABA_A$Rs, such as α1βδ, α4βδ and α6βδ. Compared to the γ2-containing receptors, the rate and extent of desensitization of δ-containing receptors are significantly reduced. In Chapter 3, we investigated the structural determinants in the δ and γ2L subunits that contribute to their unique desensitization properties. Using a chimeragenesis approach, we identified that the extracellular parts of both the TM1 and TM2 domains are involved in controlling receptor desensitization. Furthermore, using mutagenesis approaches, we investigated the roles of individual residues in these two areas of δ and γ2L subunits in modulating receptor desensitization.

In Chapter 4, we characterized benzodiazepine effects on the α4β3γ2 subtype. The pharmacology of benzodiazepines is largely dependent on the particular α subunit present. The α4-containing $GABA_A$ receptor is suggested to be diazepam-insensitive. This insensitivity is attributed to a single amino acid difference. The substitution of a residue, histidine 101, in the α1 subunit by an arginine residue in α4 and α6 leads to the $GABA_A$R insensitivity to classical 1,4-benzodiazepines, such as flunitrazepam and diazepam. Surprisingly, we found that diazepam and flunitrazepam can significantly potentiate GABA currents mediated by the α4β3γ2 receptor (but not α4β1/2γ2) expressed in *Xenopus* oocytes. Moreover, this potentiation can be antagonized by Ro15-1788, a classical benzodiazepine antagonist. In this chapter, we explored this phenomenon by carrying out functional studies on the α4β3γ2 receptor expressed in *Xenopus* oocytes and radioligand binding studies of the α4β3γ2 receptor expressed in HEK cells.

Although δ-containing receptors represent only a minor population of the total $GABA_A$ receptors, their possible roles in epilepsy when abnormal excitability is the underlying cause, render them attractive targets for drug development. It is evident that in $GABA_A$ receptors, recognition sites for agonists, antagonists and allosteric modulators are mainly located at subunit interfaces. Therefore, it is important to determine the stoichiometry and subunit arrangement within the δ-containing receptor. Furthermore, information about the subunit arrangement will be useful for the construction of homology models of the δ-containing receptors using the nicotinic acetylcholine receptor as a structural template, which would facilitate a rational design of new drugs that would act specifically on these particular $GABA_A$ receptor subtypes. In Chapter 5, we, in collaboration with Dr. J. M. Edwardson's laboratory (Cambridge, UK), studied the stoichiometry and subunit arrangement of the α4β3δ $GABA_A$ receptor using atomic force microscopy.

In conclusion, the purpose of the research undertaken in this study was to use multidisciplinary approaches to characterize the pharmacological and biophysical properties of the putative extrasynaptic $GABA_A$ receptor α4β3δ subtype and synaptic α4β3γ2 subtype. The overall goal was to provide a better understanding of the structure-function relationships of these two $GABA_A$ receptors.

Figure 1-1. Model of $GABA_AR$. (A) A representation of the pentameric $GABA_A$ receptor in the plasma membrane. Upon the binding of GABA, the $GABA_A$ receptor is activated and its central chloride-selective ion channel opens. (B) The putative subunit arrangement of the common $GABA_AR$ subtype, $\alpha 1\beta 2\gamma 2$, as seen from the synaptic cleft.


A
Cl-
GABA
GABA

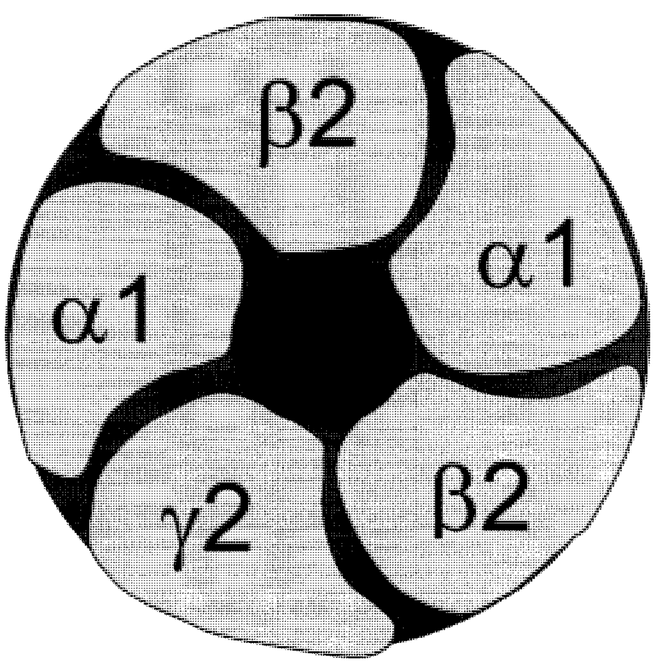
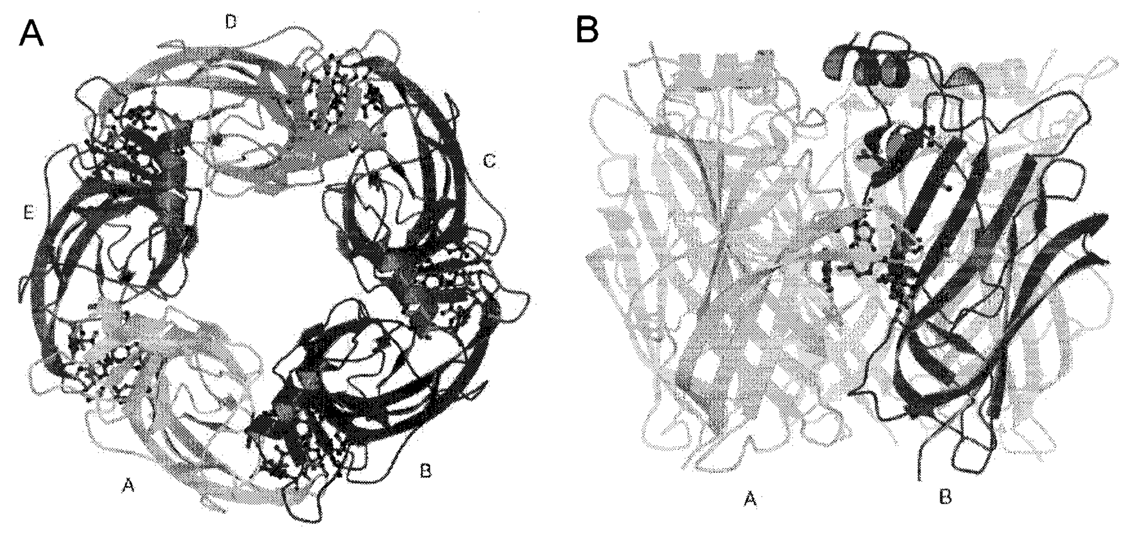

B
β2
α1
α1
γ2
β2

Figure 1-2. Crystal structure of the homopentameric AChBP at 2.7 Å resolution. (A) The pentamaric complex is viewed along the AChBP five-fold axis. Five identical subunits are illustrated in different colors and labeled with A, B, C, D and E. (B) The pentamaric complex is viewed perpendicular to the AChBP five-fold axis. The ligand binding site is depicted as lying at a subunit-subunit interface. The key residues involved in ligand binding are illustrated in a stick-representation in black (Brejc *et al.*, 2001). (C) Ribbon representation of the two adjacent AChBP subunits. The discrete loop structures which contribute the acetylcholine binding pocket located at the subunit interface is illustrated. Loops A, B, C from one subunit form the principal part of the binding pocket, whereas Loops D, E, F from another subunit contribute to the complementary part of the binding pocket. Images are modified from those of Brejc *et al.* (2001).



C

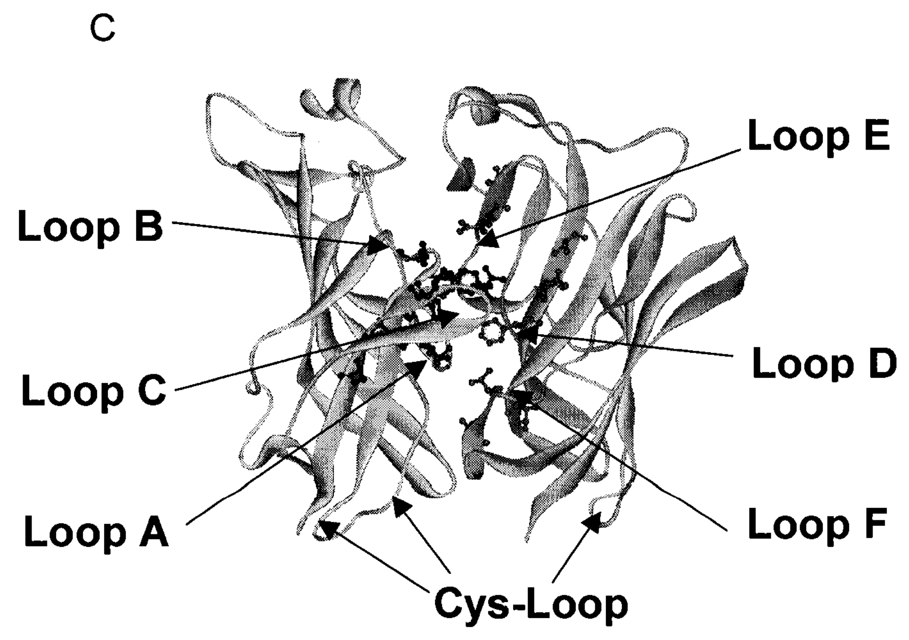
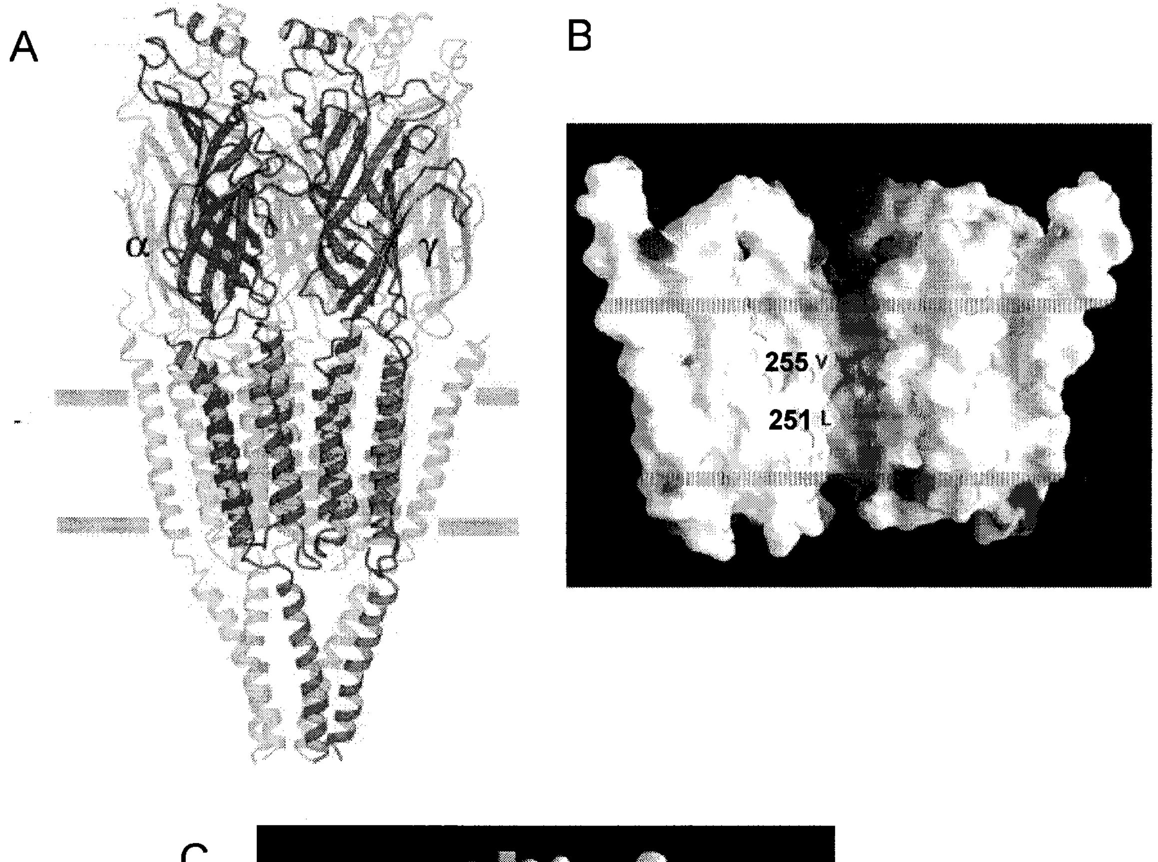


Figure 1-3. Structure of *Torpedo* nAChR determined by electron microscopy at 4 Å resolution. (A) Overall structure of nAChR as viewed parallel with the membrane plane. The nAChR is composed of 3 major parts: extracellular domain; transmembrane domain; and cytoplasmic domain. (B) The middle part of transmembrane domain 2 forms the gate of the receptor. Hydrophobic residues L251 and V255 provide cation selectivity (Miyazawa *et al.*, 2003). (C) The intracellular part of the nAChR forms the portals for ion transportation and also provides ion selectivity with its highly negative charged residues. Images are modified from those of Unwin (2005).


A
α
γ
B
255 V
251 L

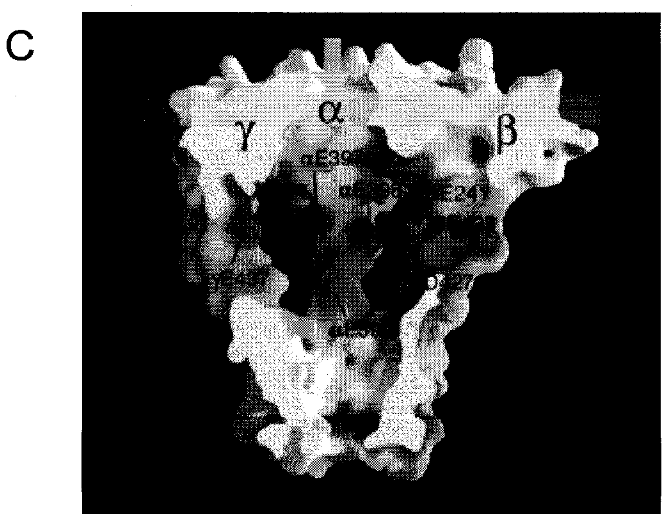

C
γ
α
β

Figure 1-4. GABA$_A$R structure. Ribbon diagram of the GABA$_A$R $\alpha1\beta2\gamma2$ subtype, constructed using comparative modeling, showing a similar structure to the nAChR. The GABA$_A$R consists of three major parts: the extracellular (EC) N-terminal domain, the transmembrane domain (TM) and the intracellular domain, which contains a substantial membrane-associated (MA) helix preceding TM4. Image is modified from Mokrab *et al.* (2007).

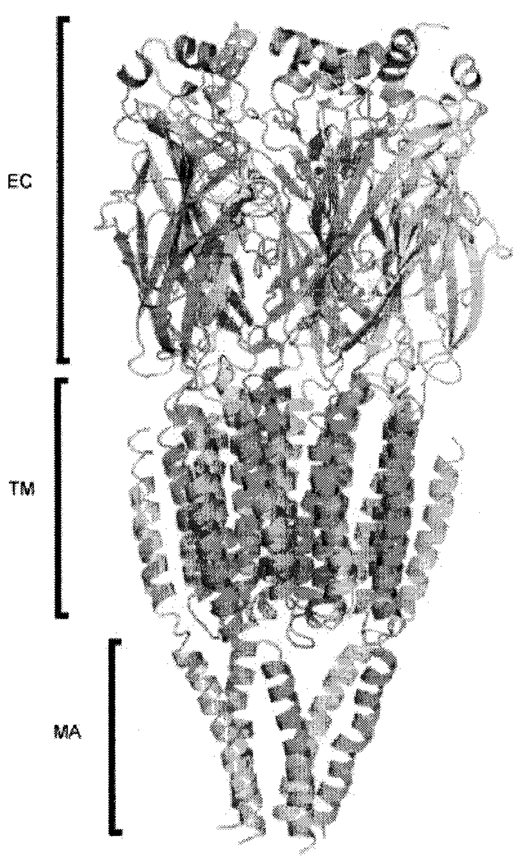

EC
TM
MA

Figure 1-5. The GABA binding site on the $GABA_AR$. The diagram shows residues involved in GABA binding. Residues (black) at the β(+)/α(-) interface are the key residues involved in low-affinity GABA binding (see text for details). Residue Y62 in β2 (red) has been suggested to contribute to high-affinity GABA binding and it is homologous to α1F64 in loop D (Newell *et al.*, 2000). The position of the benzodiazepine (BDZ) binding site is illustrated.

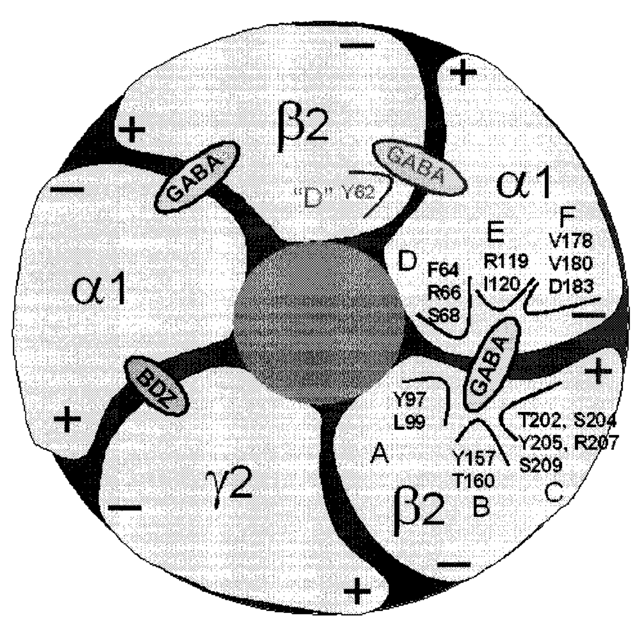
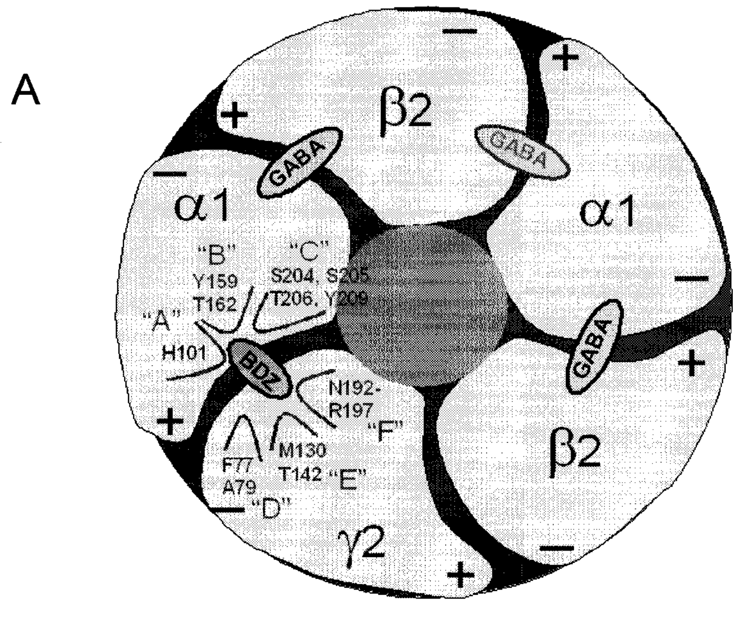

β2
GABA
GABA
"D" Y62
α1
E
F
D
F64
R66
S68
R119
I120
V178
V180
D183
α1
BDZ
GABA
Y97
L99
T202, S204
Y205, R207
S209
A
Y157
T160
B
C
γ2
β2

Figure 1-6. The benzodiazepine binding site on the $GABA_AR$. (A) This diagram shows residues involved in benzodiazepine binding. (B) The position of the benzodiazepine binding site with a docked flunitrazepam molecule (Mokrab *et al.*, 2007). The N1-methyl and 7-nitro groups of flunitrazepam are close to the α1 His101 (His129 for human α1) while the pendant 5-phenyl is positioned between α1 Tyr159 (Tyr187 for human α1) and γ2 Tyr58 (Tyr97 for human γ2). The benzodiazepine A-ring is about 3 Å away from γ2 Phe77 (Phe116 for human α1).


A
β2
GABA
GABA
α1
α1
"B"
Y159
T162
"C"
S204, S205
T206, Y209
"A"
H101
BDZ
GABA
N192-
R197
"F"
M130
T142
"E"
F77
A79
"D"
β2
γ2
+
−

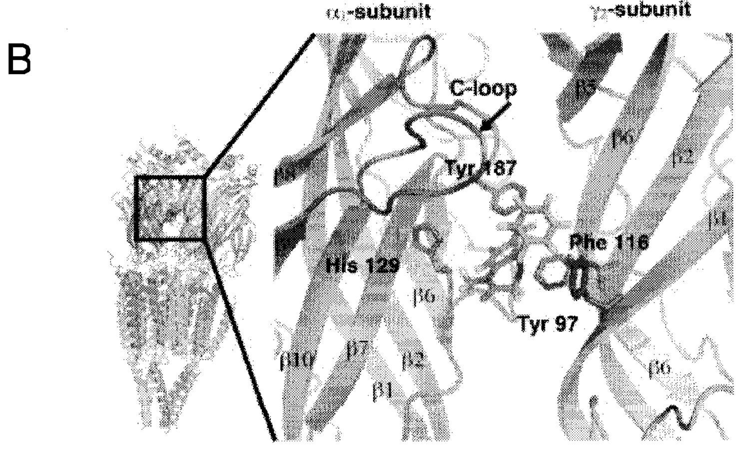

B
α1-subunit
γ2-subunit
C-loop
Tyr 187
His 129
Phe 116
Tyr 97

Figure 1-7. Residues in the TM2 domain affect receptor desensitization. Amino acid sequence alignment of the TM2 region of rat $GABA_A$ receptor α1, α6, β1, γ, δ subunits *Torpedo* nACh receptor α subunit and rat 5 HT_{3A} subunits, according to Ernst *et al.*, (2005). To facilitate comparisons of the residues in the TM2 segment of different subunits, an index numbering system has been used (Miller, 1989). Residues in the TM2 are identified independently as 1' to 28', from the cytoplasmic end to the extracellular end. Shaded residues have been suggested to affect the receptor desensitization. (Tierney *et al.*, 1996; Birnir *et al.*, 1997a, b; Labraca *et al.*, 1995; Filatov and White, 1995; Bianchi and Macdonald, 2001; Scheller and Forman, 2002)

		TM2																										
		1'	2'	3'	4'	5'	6'	7'	8'	9'	10	11'	12'	13'	14'	15'	16'	17'	18'	19'	20'	21'	22'	23'	24'	25'	26'	27'	28'
α1	256	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
α6	256	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
β1	251	V	A	L	G	I	T	T	V	L	T	M	T	T	I	S	T	H	L	R	E	T	L	P	K	I	P	Y	V
δ	262	V	S	L	G	I	T	T	V	L	T	M	T	T	L	M	V	S	A	R	S	S	L	P	R	A	S	A	I
γ2	264	T	S	L	G	I	T	T	V	L	T	M	T	T	L	S	T	I	A	R	K	S	L	P	K	V	S	Y	V
nAChR α		M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V	E	L	I	P	S	T	S	S	A
5-HT3A		V	S	F	K	I	T	L	L	L	G	Y	S	V	F	L	I	I	V	S	D	T	L	P	A	T	A	I	G

Figure 1-8. Chemical structure of $GABA_AR$ agonists, antagonists and channel blockers. $GABA_AR$ agonists: GABA, muscimol, THIP, I4AA. $GABA_AR$ antagonists: SR95531, bicuculline; $GABA_AR$ channel blocker: picrotoxin (a mixture of picrotoxinin and picrotin)

GABA

muscimol

THIP

I4AA

SR 95531

bicuculline

picrotoxinin + picrotin

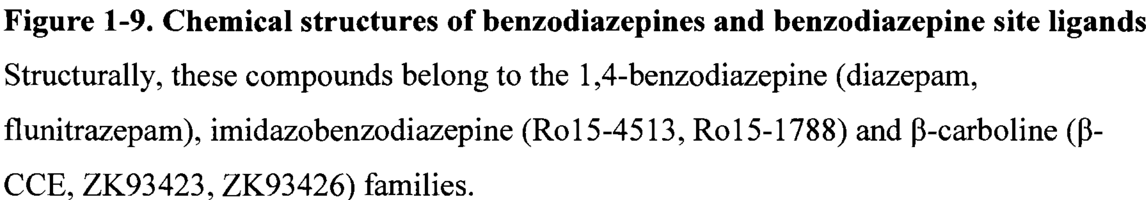


Figure 1-9. Chemical structures of benzodiazepines and benzodiazepine site ligands. Structurally, these compounds belong to the 1,4-benzodiazepine (diazepam, flunitrazepam), imidazobenzodiazepine (Ro15-4513, Ro15-1788) and β-carboline (β-CCE, ZK93423, ZK93426) families.

diazepam

flunitrazepam

Ro 15-4513

Ro 15-1788

β-CCE

ZK-93423

ZK93426

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CHAPTER 2[1]

Identification of a Domain in the δ Subunit (S238-V264) of the α4β3δ $GABA_A$ Receptor that Confers High Agonist Sensitivity

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INTRODUCTION

γ-Aminobutyric acid type A receptors ($GABA_ARs$) are the major inhibitory neurotransmitter receptors in the mammalian brain. These receptors are members of the cys-loop family of ligand-gated ion channels (LGICs) that includes the nicotinic acetylcholine (nACh) receptors, serotonin type 3 ($5HT_3$) and glycine receptors (Sieghart *et al.*, 1999). Each member of the LGIC family is likely to be a pentamer in which homologous transmembrane subunits are arranged in a rosette conformation to form a central ion channel pore (Nayeem *et al.*, 1994). Nineteen mammalian $GABA_A$ receptor subunits have now been identified i.e. α1-6, β1-3, γ1-3, ρ1-3, δ, ε, θ and π (McKernan and Whiting, 1996; Barnard *et al.*, 1998). The most common subtype of $GABA_A$ receptor in the mammalian CNS is the α1β2γ2 combination (reviewed by Sieghart *et al.*, 1999; Whiting, 2003), where the likely stoichiometry is 2α:2β:1γ (Farrar *et al.*, 1999). However, the inclusion of other subunits within the pentamer results in differences in physiological function and sensitivity to the large number of pharmacological agents that target these receptors (Sieghart, 1995).

Recently it has been suggested that there are two inhibitory neuronal signalling pathways which are mediated by $GABA_A$ receptors, namely phasic inhibition and tonic inhibition (Brickley *et al.*, 2001; Mody, 2001). Phasic inhibition is evoked by the action potential-dependent release of high concentrations of GABA (0.5 ~ 1 mmol/L) at the synapse. These high local concentrations of GABA are transient and induce rapid inhibitory responses. In contrast, tonic inhibition is mediated by the persistent but low concentrations of GABA that either overspill from the synapse or are released from glial cells. The extrasynaptic receptors that are responsible for these subtype-selective

responses are likely to include the α4, α5, α6, γ2/3 or δ subunits, with the most likely combinations in the brain being α4βxδ, α5β2/3γ2, and α6β2/3δ (see Farrant and Nusser, 2005). Sur *et al.* (1999), using immunoprecipitation techniques, suggested that approximately one-third of the α4-containing $GABA_A$ receptor subtypes in the hippocampus and thalamus include the γ2 subunit while two-thirds co-associate with the δ subunit. Another study, using a similar approach and brain membrane extracts, suggested a higher ratio of the α4γ2 containing subtypes compared to α4δ (Bencsits *et al.*, 1999). Overall, the available data suggest that a heterogeneous population of $GABA_A$ receptors containing the α4 subunit (mainly α4βxγ2 and α4βxδ subtypes) exists in the same regions of the brain.

Extrasynaptic receptor subtypes appear to have a higher sensitivity to GABA than those that mediate synaptic responses (Saxena and Macdonald, 1994). This is not unexpected since these receptors respond to low ambient concentrations of GABA. The major aim of the present study was to identify the molecular basis for the higher potency of GABA at the α4δ-containing receptors. There is considerable evidence to demonstrate that binding sites within the LGIC family lie at subunit-subunit interfaces and agonist binding sites that are involved in receptor activation have been localised to the β-α subunit interfaces (see Amin and Weiss, 1993). However, structural determinants lying outside the putative agonist binding domains have also been shown to affect agonist sensitivity. These include motifs in the α subunit that confer differential sensitivities between α6- or α1-containing receptors (Korpi and Luddens, 1993) or between α3 and other α subunits (Bohme *et al.*, 2004). The current structural and functional evidence suggests that, following formation of the receptor-agonist complex, a concerted

movement of domains within all subunits may be required to induce channel opening (see Unwin, 2005). Thus, all subunits may contribute to agonist affinity and efficacy.

In this study, we explore the role of structural determinants within the δ and/or γ2L subunit that are important for conferring differential potencies for agonists. We have generated δ/γ2L chimeric subunits and co-expressed these with α4 and β3 subunits in *Xenopus* oocytes for functional studies. The results demonstrate that a domain in the $GABA_A$ receptor δ subunit (S238-V264) confers high agonist sensitivity to the α4β3δ subtype.

MATERIALS AND METHODS

Clones and Construction of δ/γ2 Chimeras

The cDNAs encoding the rat $GABA_A$ subunits were subcloned into the pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA). The original cDNAs encoding the α4 and β3 subunits were from Dr. P. H. Seeburg's laboratory and those encoding the γ2L and δ subunits were generously provided by Drs. D. L. Weiss and R. L. Macdonald, respectively. Randomly derived δ/γ2L chimeras were created following the protocol of Moore and Blakely (1994). In brief, the δ and γ2L subunit cDNAs were engineered into multiple cloning sites in the pcDNA3.1(+) vector with the δ subunit being positioned upstream of the γ2L subunit. A *Bam*HI and an *EcoR*I restriction site were left in the polylinker between the δ and γ2L sequences. The dual plasmid DNA was digested and linearized by *Bam*HI/*EcoR*I, and was then transformed into Library Efficiency® competent DH5α *Escherichia coli* cells (Life Technologies, Gaithersburg, MD, USA). During the transformation, random crossover events occur at regions of

homology between the δ and γ2L sequences, creating a series of random chimeric subunit cDNAs. Figure 2-1 shows the 3 in-frame hybrid DNA chimeras (χ237, χ255, χ277) that were generated. In each case, the N-terminal domain was derived from the original δ subunit DNA with the remainder coming from γ2L. The chimeras were named by the point of crossover with the number representing the last residue of the δ subunit prior to the in-frame switch. Figure 2-1 also shows an alignment of the δ and γ2L sequences, the position of the crossover points and the location of the putative transmembrane domains based on the structural model of Ernst *et al.* (2005).

Expression of $GABA_A$ Receptors in *Xenopus* Oocytes

Capped cRNAs encoding rat $GABA_A$ receptor wildtype and chimeric subunits were synthesized from linearized cDNA following standard protocols and using T7 RNA polymerase (Invitrogen, San Diego, CA) for *in vitro* transcription. cRNA concentrations were calculated from their absorbance at 260 nm. Stage V-VI *Xenopus laevis* oocytes were isolated and prepared as described (Smith *et al.*, 2004). Oocytes were injected with 50 nl of 1 μg/μl of total subunit cRNAs in a 1:1:1 ratio (α4:β3:δ/γ2L mutant) or 1:1 for the α4β3. Other ratios of subunit cRNAs were also used as described in the text. Injected oocytes were incubated individually in ND96 buffer (96 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ 5 mmol/L HEPES, pH 7.4) in 96-well plates at 14°C for at least 48 hours prior to functional analysis.

Two-electrode Voltage Clamp Recordings

Oocytes were continuously bathed in frog Ringer's solution (110 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES, pH 7.4) by gravity flow (~5 ml/min) in a custom-made recording chamber. Drug-induced currents were measured by standard two electrode voltage clamp techniques using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA) at a holding potential of -60 mV. The voltage-sensing and current-passing electrodes were filled with 3 mol/L KCl and only electrodes with a resistance between 0.5 to 3.0 MΩ in frog Ringer's solution were used.

To measure the effects of GABA and other agonists (muscimol, THIP, I4AA), the agonist was applied via gravity perfusion (30 - 40 s at 5 mL/min) followed by a 10-15 min washout to ensure complete recovery from desensitization. In studies of antagonist effects, oocytes were pre-perfused with these ligands for 2 min prior to initiation of the receptor response by perfusion with GABA (at its EC_{50} concentration) and the same concentration of the antagonist as used in the pre-perfusion.

Data Analysis

Concentration-effect curves for agonist activation were analyzed by non-linear regression techniques using GraphPad Prism 4.0 software (San Diego, CA) and the following equation:

$$I = \frac{I_{\max} * [L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the amplitude of agonist-evoked current for a given concentration $[L]$, I_{max} is the maximum amplitude of current, EC_{50} is the agonist concentration that evokes half maximal receptor activation, and n is the Hill coefficient.

The inhibitory effects of antagonists were analyzed using the equation (GraphPad Prism 4.0):

$$\frac{I}{I_{\max}} = \frac{[A]^n}{[A]^n + IC_{50}}$$

where IC_{50} is the concentration of antagonist, [A], that reduces the amplitude of the GABA-evoked current by 50% and n is the Hill coefficient.

Data were analyzed by one-way ANOVA and levels of significance were determined by the Dunnett's post-test for multiple comparisons.

Chemicals

All drugs were purchased from Sigma-Aldrich (St. Louis, MO) and were made as stock solutions (1 mmol/L to 1 mol/L) in sterile water. Picrotoxin suspensions were ultrasonicated to make soluble 1 mmol/L preparations. The stock solutions for GABA, THIP, I4AA, SR95531 and picrotoxin were aliquoted and stored at –80°C until use. Muscimol and bicuculline stocks were freshly made prior to each experiment.

RESULTS

Expression of $GABA_A$ Receptor Subtypes

Many investigators have reported difficulties in co-expressing the δ subunit with other $GABA_A$ receptor subunits in recombinant systems (see Brown *et al.*, 2002). We have, therefore, looked at the expression of various combinations of subunits i.e., β3 alone, α4β3, α4δ, β3δ and α4β3δ. When injected into oocytes, only the α4β3 and α4β3δ combinations produced functional receptors as measured by detectable current responses elicited by a saturating (3 mmol/L) concentration of GABA. Using 1:1 (α4:β3) or 1:1:1

($\alpha4$:$\beta3$:δ) ratios of the encoding cRNAs, these receptor subtypes showed significant differences in both their time courses of expression and expression levels attained. Using the same batches of oocytes, the expression levels of the $\alpha4\beta3\delta$ subtype was robust 48 hours after injection but the $\alpha4\beta3$ subtype required incubation for 4-5 days before stable responses could be recorded. Six days after injection, the $\alpha4\beta3\delta$ population displayed an average maximum current that was 4.7 fold higher than that of the $\alpha4\beta3$ subtype (1419 ± 79 nA, $n = 7$ *vs.* 301 ± 76 nA, $n = 6$, respectively). The EC_{50} values for GABA activation of the two subtypes were not significantly different but, in agreement with previous results (Storustova and Ebert, 2006), the $\alpha4\beta3\delta$ subtype displayed lower sensitivity to inhibition by Zn^{2+} ($IC_{50} = 5.40 \pm 0.72$ µmol/L, $n = 3$) compared to the $\alpha4\beta3$ combination (0.18 ± 0.02 µmol/L, $n = 3$). The two receptor subtypes could also be distinguished by the effects of THIP. Although this agonist displayed similar 'super-agonism' at both receptors (see below), its EC_{50} for activation of the $\alpha4\beta3$ receptor (85.1 ± 17.7 µmol/L, $n = 3$) was ninefold higher than for the $\alpha4\beta3\delta$ subtype (see below).

Effects of Varying cRNA Ratios on Receptor Expression

It has been reported that efficient expression of trimeric $\alpha\beta\gamma$ receptors in *Xenopus* oocytes may require an increase in the relative amount of cRNA encoding the γ subunit in the injection mix (Boileau *et al.*, 2002). We have not previously observed anomalous expression of receptors containing the α, β and γ subunits when a cRNA ratio of 1:1:1 was used (unpublished results). However, to investigate the expression of the $\alpha4\beta3\delta$ subtype, different cRNA ratios have been used. When the subunit ratios ($\alpha4$:$\beta3$:δ) were changed from 1:1:1 to 1:1:5 or 1:1:10, the EC_{50} values for GABA activation were

progressively increased (1.4 ± 0.13, 3.90 ± 0.84 and 10.4 ± 0.4 μmol/L, respectively) while the Hill slope decreased significantly (0.70 ± 0.07, 0.64 ± 0.03 and 0.38 ± 0.09). In control experiments using similar higher ratios of the δ subunit cRNA, we have not been able to force the functional expression of β3δ or α4δ receptors. Possible explanations for these results are provided in the Discussion. As the properties of the α4β3δ and α4β3γ2L receptors were highly consistent when a 1:1:1 ratio was used, we have used this stoichiometry in all experiments described below.

Effects of GABA and Muscimol on Functional Responses of Wildtype and Chimeric Receptors.

Concentration-response curves (Figure 2-2, A and Table 2-1) revealed GABA to be approximately 20-fold more potent in mediating activation of the α4β3δ receptor ($EC_{50} \approx 1.4$ μmol/L) compared to the α4β3γ2L subtype ($EC_{50} \approx 27.6$ μmol/L).

Chimeric δ/γ2L receptor subunits were constructed to investigate structural domains in these subunits that contribute to the aforementioned differences in agonist potency. These chimeras (χ277, χ255, χ237; see Figure 2-1) incorporated the N-terminal sequence of the δ subunit with the remainder of the sequence corresponding to that of γ2L. When co-expressed with the α4 and β3 subunits, clear differences in GABA potency were observed. The EC_{50} value for GABA activation of the χ277-containing receptor ($EC_{50} \approx 2.3$ μmol/L), where the crossover point lies towards the middle of the TM2 domain, was not significantly different from that of the wildtype α4β3δ receptor. In contrast, the EC_{50} for GABA activation of the χ237-containing subtype ($EC_{50} \approx 21.5$ μmol/L) was not significantly different from that of the α4β3γ2L subtype. The EC_{50}

value for GABA activation of the χ255-containing subtype was intermediate between the two wildtype receptors ($EC_{50} \approx 9.05$ µmol/L), and was significantly different from both (see Table 2-1).

A similar trend in potency was seen when another "full" $GABA_A$ receptor agonist, muscimol, was investigated (Table 2-1). Incorporation of the χ277 chimeric subunit again conferred $\alpha4\beta3\delta$-like potency whereas the presence of the χ237 chimera led to characteristics similar to those of the $\alpha4\beta3\gamma2L$ receptor. The above results suggest that a domain of the δ subunit lying between residues 237 and 277 plays an important role in determining agonist sensitivity.

Effects of THIP and 14AA on Functional Responses of Wildtype and Chimeric Receptors

THIP and I4AA are often described as partial agonists of the $GABA_A$ receptor since they elicit maximum current responses at the major $GABA_A$ receptor subtype, $\alpha1\beta2\gamma2$, that are lower than those produced by "full" agonists, such as GABA and muscimol. The oocyte expression system is not ideal for determining efficacies as their large size limits the rates of agonist perfusion and thus the resolution of rapid responses. However, in agreement with previous studies that used a stably expressing cell line (Brown *et al.*, 2002), we found that THIP acts as a "super-agonist" of the $\alpha4\beta3\delta$ receptor, eliciting a maximum current that was 135.6% of that induced by 1 mmol/L GABA (Figures 2-3, A and 2-4, A). THIP, however, is only a partial agonist of the $\alpha4\beta3\gamma2L$ combination, producing a maximum current that was 47% of that mediated by GABA (Figures 2-3, C and 2-4, A). A similar pattern was seen for I4AA i.e., it is a partial

agonist of the $\alpha4\beta3\gamma2L$ subtype (Figures 2-3, D and 2-4, B) but, at the $\alpha4\beta3\delta$ combination, it elicits a maximum response that is not significantly different from GABA (Figures 2-3, B and 2-4, B). In order to investigate the possible involvement of the δ subunit in influencing agonist efficacy as well as potency, we studied the responses of the chimeric receptors to THIP and I4AA.

The effects of THIP and I4AA on wildtype and chimeric receptors are shown in Figure 2-4 and the results are summarized in Table 2-2. Inclusion of the $\delta/\gamma2L$ chimeric subunits had similar effects on the potency of these agonists to the results obtained for GABA i.e. the presence of the $\chi277$ subunit gave rise to a receptor that had similar sensitivity to the $\alpha4\beta3\delta$ subtype, while the chimeric receptor incorporating $\chi237$ displayed similar potency characteristics to the $\alpha4\beta3\gamma2L$ receptor. In these experiments, the potencies of THIP and I4AA for the $\alpha4\beta3\chi255$ receptor were not significantly different from the $\alpha4\beta3\delta$ wildtype (Table 2-2).

The effects of the chimeric subunits on agonist efficacy were more complex (see Figures 2-3 and 2-4). The efficacies reported in Table 2-2 were calculated from the percentage of the maximum current responses induced by these agonists compared to the maximum effect of GABA. In the case of THIP, inclusion of any one of the chimeric subunits resulted in maximum current amplitudes that were very similar to those of GABA. Thus all receptors displayed significantly higher efficacy when compared to the $\alpha4\beta3\gamma2L$ receptor but no receptor combination displayed a similar degree of THIP "superagonism" as the $\alpha4\beta3\delta$ wildtype. I4AA was a partial agonist at all chimeric receptors i.e., the maximum responses induced were not significantly different from those of the $\alpha4\beta3\gamma2L$ subtype.

Effects of Competitive Antagonists and Picrotoxin on Functional Responses of Wildtype and Chimeric Receptors

To investigate whether the above results obtained using the $\delta/\gamma 2L$ chimeric subunits may be explained by changes in the overall structure of the binding sites for agonists/competitive antagonists or the properties of the ion channel itself, we have characterized the functional effects of two competitive antagonists (bicuculline and SR95531) and the channel blocker, picrotoxin. In these experiments, the oocytes were pre-perfused with the antagonist for two minutes prior to challenge with a concentration of GABA equal to its EC_{50} value. The results shown in Figure 2-5 and summarized in Table 2-3 show that the apparent affinities for SR95531 and picrotoxin are not significantly different between the wildtype and chimeric receptors. Similarly, the effects of bicuculline were comparable for all subtypes, except in the case of $\alpha 4\beta 3\chi 237$ receptor where its IC_{50} value was ~3-fold higher than that of either the $\alpha 4\beta 3\delta$ or $\alpha 4\beta 3\gamma 2L$ receptor (Table 2-3).

DISCUSSION

The importance of tonic inhibitory conductances mediated by extrasynaptic $GABA_A$ receptors has become clear over the last few years (see Farrant and Nusser, 2005). At least in some cases, the extrasynaptic $GABA_A$ receptors have distinct subunit compositions from their synaptic counterparts and this confers their unique activation and pharmacological properties. Thus far, the δ subunit has been localised exclusively to extrasynaptic and perisynaptic membranes in a variety of cell types, including cerebellar granule cells (Nusser *et al.*, 1998) and the dentate gyrus of the hippocampus (Wei *et al.*,

2003). This subunit appears to be preferentially expressed with the $\alpha6$ or $\alpha4$ subunit. The $\alpha4$ and δ subunits are colocalised in restricted regions of the brain, especially the thalamus and hippocampus (Bencsits *et al.*, 1999; Sur *et al.*, 1999). Furthermore, parallel changes in the expression levels of these subunits in response to various physiological and pharmacological challenges (see e.g. Lovick *et al.*, 2005; Sundstrom-Poromaa *et al.*, 2002) suggest that the $\alpha4\beta x\delta$ subtype is likely to be a native receptor combination.

The presence of the $\gamma2$ subunit in the $GABA_A$ receptor has been implicated in clustering of receptor subtypes at the postsynaptic synapse (Essrich *et al.*, 1998) suggesting that the $\alpha4\beta3\gamma2$ receptor is located predominantly in this region. In this study, we have, therefore, used the rat $\alpha4\beta3\delta$ and $\alpha4\beta3\gamma2$ receptors as likely representatives of extrasynaptic and synaptic receptors, respectively.

The human forms of these $GABA_A$ receptors were characterized previously in stably transfected cell lines (Brown *et al.*, 2002) and it was shown that the $\alpha4\beta3\delta$ receptor was more sensitive to GABA than the $\alpha4\beta3\gamma2$ subtype. In the present study, we found a similar difference in potency (approximately 20-fold) for the rat receptor subtypes expressed in *Xenopus* oocytes. It is thus clear that inclusion of either the δ or $\gamma2$ subunit in the receptor complex has a significant effect on the potency of GABA activation. However, neither of these subunits has been implicated directly in the binding of GABA or other receptor agonists. Site-directed mutagenesis studies have been used to localise the agonist activation sites to the interfaces between the β and α subunits (Amin and Weiss, 1993). These sites appear to be formed by distinct loops of amino acids contributed by the primary (β) and secondary (α) subunits, in homologous positions to neurotransmitter binding sites in other members of the receptor family.

The aim of the present study was to identify structural determinants within the $GABA_A$ receptor δ subunit that contributes to the higher potency of GABAergic agonists on δ-containing receptors. We used a random chimeragenesis approach to form δ/γ2L subunits and expressed these with native α4 and β3 subunits in *Xenopus* oocytes. Similar approaches have been used to investigate structural requirements for the binding of benzodiazepine site ligands to other $GABA_A$ receptor subtypes (e.g. Boileau *et al.*, 1998; Derry *et al.*, 2004).

Our major finding is that a domain in the $GABA_A$ receptor δ subunit (S238-V264) confers high agonist sensitivity to the α4β3δ subtype. For all agonists studied (GABA, muscimol, THIP and I4AA) inclusion of the χ277 chimeric subunit (containing the first 277 residues of δ) imparted δ-like characteristics to the receptor activation properties. In contrast, inclusion of the χ237-subunit led to receptors whose activation characteristics were not significantly different from the wildtype α4β3γ2L receptor. These results suggest that a specific region of the δ subunit (residues 238-277) plays a role in conferring higher agonist potency. Inspection of the sequence homologies of the rat δ and γ2 subunits (Figure 2-1, C) further restricts this domain. Based on the recent receptor model and alignments of Ernst *et al.* (2005), the sequences of these subunits are identical between residues 265 and 277 (δ subunit numbering); thus the domain that confers high agonist potency can be narrowed further to residues 238 - 264. In the case of activation by either THIP or I4AA, the receptor that included the χ255 chimeric subunit displayed similar potency to the α4β3δ subtype. Again inspection of the sequence homology shows that there is conservation between residues 249 and 255 (δ subunit numbering) suggesting that the sequence S238-M248 of the δ subunit, lying in the N-terminal

segment of TM1, may be of particular importance in conferring high affinity for these agonists.

Of the 27 amino acid residues lying in the S238-V264 domain of the δ subunit, 16 are identical to those in equivalent position of γ2 (see Figure 2-1, C). Where divergences occur, homologous amino acids are found in five positions (S238T, M240I, L245I, M248L, Q257K) leaving only six semi- or non-conservative substitutions (S242C, V243T, A247V, S256N, A258D, V264T), five of which lie within the putative TM1 domain (see Figure 2-1, C). In terms of overall structure, this domain appears to be highly conserved among all subunits of the cys-loop LGIC family (see Ernst *et al*., 2005) but, despite this homology, there is increasing evidence for its influence on the binding-gating coupling properties of different receptor subtypes (see below).

The structural determinants within the δ and γ2L subunits that underlie differences in agonist efficacy are less clear. In the α4β3δ receptor, THIP has been suggested to act as a "superagonist" (see above and Brown *et al.*). No clear patterns for determining agonist efficacy have emerged from the present study. This is perhaps not surprising since many factors lying in the pathway from ligand recognition to channel opening may dictate differences in transduction efficiency.

In agreement with the earlier results of Brown *et al.* (2002) who studied the human $GABA_A$ receptor subtypes, we found that bicuculline, SR95531 and picrotoxin did not differentiate between the wildtype rat α4β3δ and α4β3γ2 receptors. Apart from a small effect (approximately 3-fold) of bicuculline on the χ237 containing receptor, none of the expressed receptors showed any significant differences in their responses to these ligands. These results suggest that the influence of the δ/γ2L subunits on agonist potency

does not involve radical changes in the architecture of the binding sites. Similarly the lack of influence of the chimeric subunit on inhibition by the channel blocker, picrotoxin, suggests that the open channel characteristics are not dramatically affected. These results are consistent with the involvement of the δ/γ2L subunits, in particular the TM1 domain, in the transduction mechanism leading from agonist binding to channel opening rather than agonist recognition *per se*.

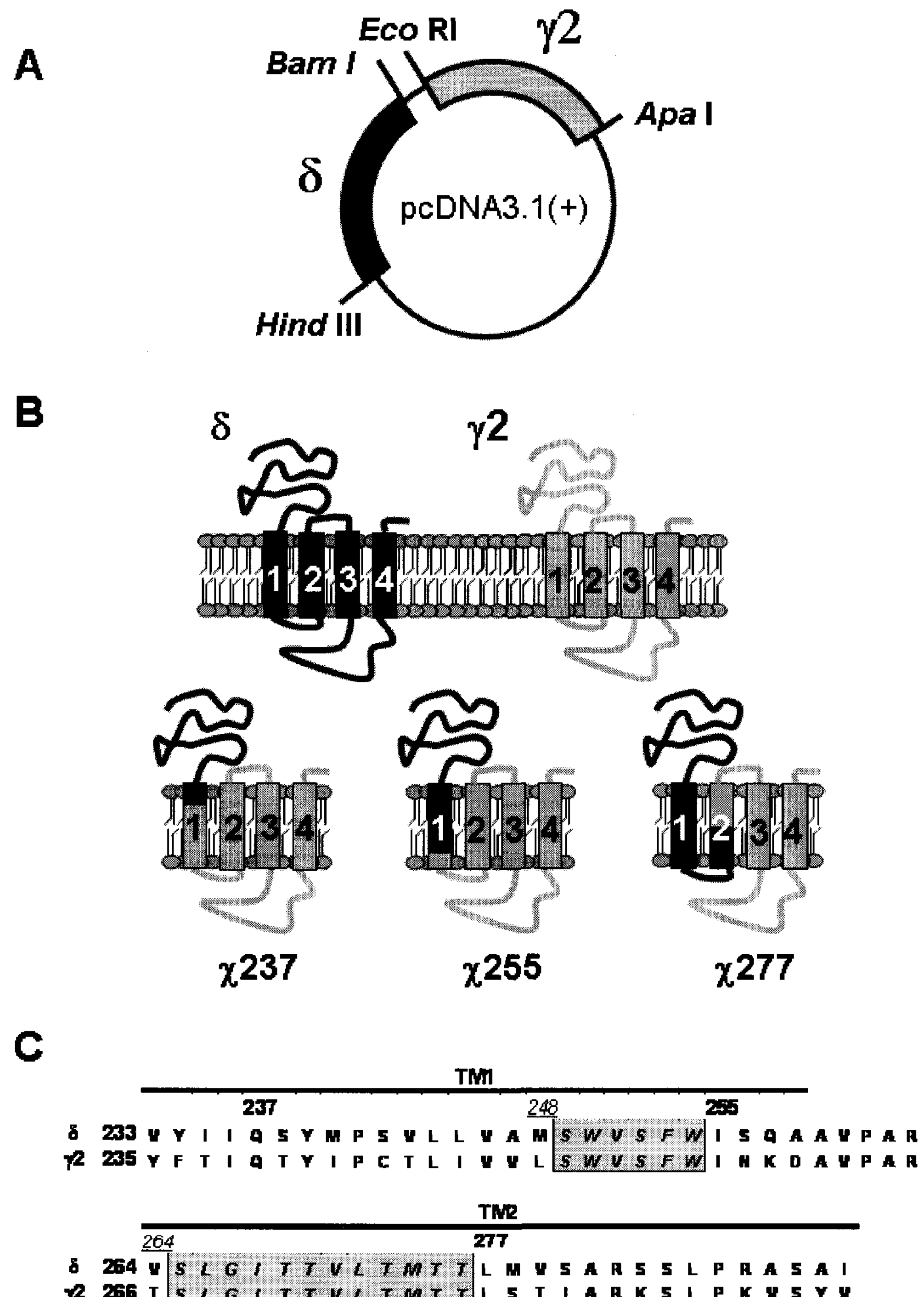
As both the α4 and δ subunits have been reported to be difficult to express in recombinant systems, the actual subunit composition of the receptors investigated in different laboratories has been questioned (see Borghese *et al.*, 2006). In the present study, we have compared the expression of different combinations of α4, β3, δ and γ2L subunits. Only the α4β3, α4β3δ and α4β3γ2L combinations resulted in the expression of functional $GABA_A$ receptors. The expressed α4β3 and α4β3δ receptors displayed distinct properties with respect to the times required for expression, block by Zn^{2+}, sensitivity to agonists and desensitization characteristics (unpublished results). However, increasing the relative amount of cRNA encoding the δ subunit affected both the EC_{50} for GABA activation and the apparent Hill coefficient of the response. Control experiments revealed that using higher than stoichiometric amounts of the δ subunit cDNA does not force the aberrant expression of other functional receptors containing only one or two subunit isoforms. These results are difficult to rationalize in terms of expression of a heterogeneous population of receptors. It seems more likely that the effects of changing cRNA ratios reflect changes in subunit stoichiometry within the pentameric α4β3δ complex. Further analysis of receptor composition under different expression conditions using biophysical approaches (see e.g. Barrera *et al.*, 2005) will be required to interpret

these observations. In the present study, although we cannot exclude the possibility that a heterogeneous population of receptors existed, the results obtained for the native receptor subtypes combinations are similar to those reported by others (see Brown *et al*, 2002; Storustova and Ebert, 2006). Furthermore, the differential responses obtained for receptors incorporating the chimeric δ/γ2L subunits suggest that these subunits were also efficiently incorporated into the receptors examined.

A major issue in studying $GABA_A$ receptor properties is how the binding of an agonist to its extracellular sites that are predicted to lie approximately 30Å above the membrane surface is communicated to the channel gate lying deep within the transmembrane domains (see Unwin, 2005). A number of previous studies have used chimeric/mutagenesis strategies to investigate the roles of the δ/γ2 subunits in signal transduction. Using a chimeragenesis approach, Jones-Davis *et al*., (2005), demonstrated that the TM1 domain of the γ2 subunit was important for benzodiazepine potentiation of the recombinant α1β2γ/δ receptors. Haas and Macdonald (1999) reported that the kinetics of desensitisation of the α1β3δ receptor were slower than those of the α1β3γ2L subtype and Bianchi *et al*. (2001) subsequently demonstrated that these results could be attributed to determinants within the extracellular N-terminal domain and two residues in the TM1 domain (V233, Y234). In studies of general anaesthetic action, it has been shown that determinants for modulation of GABA currents by propofol and pentobarbital lie within the N-terminal and TM1 domains of these subunits (Feng and Macdonald 2004; Feng *et al*., 2004). Together with our current results, the available data suggest that the TM1 domain of the δ subunit plays a significant role in conferring the unique functional and pharmacological properties of the δ containing receptors.

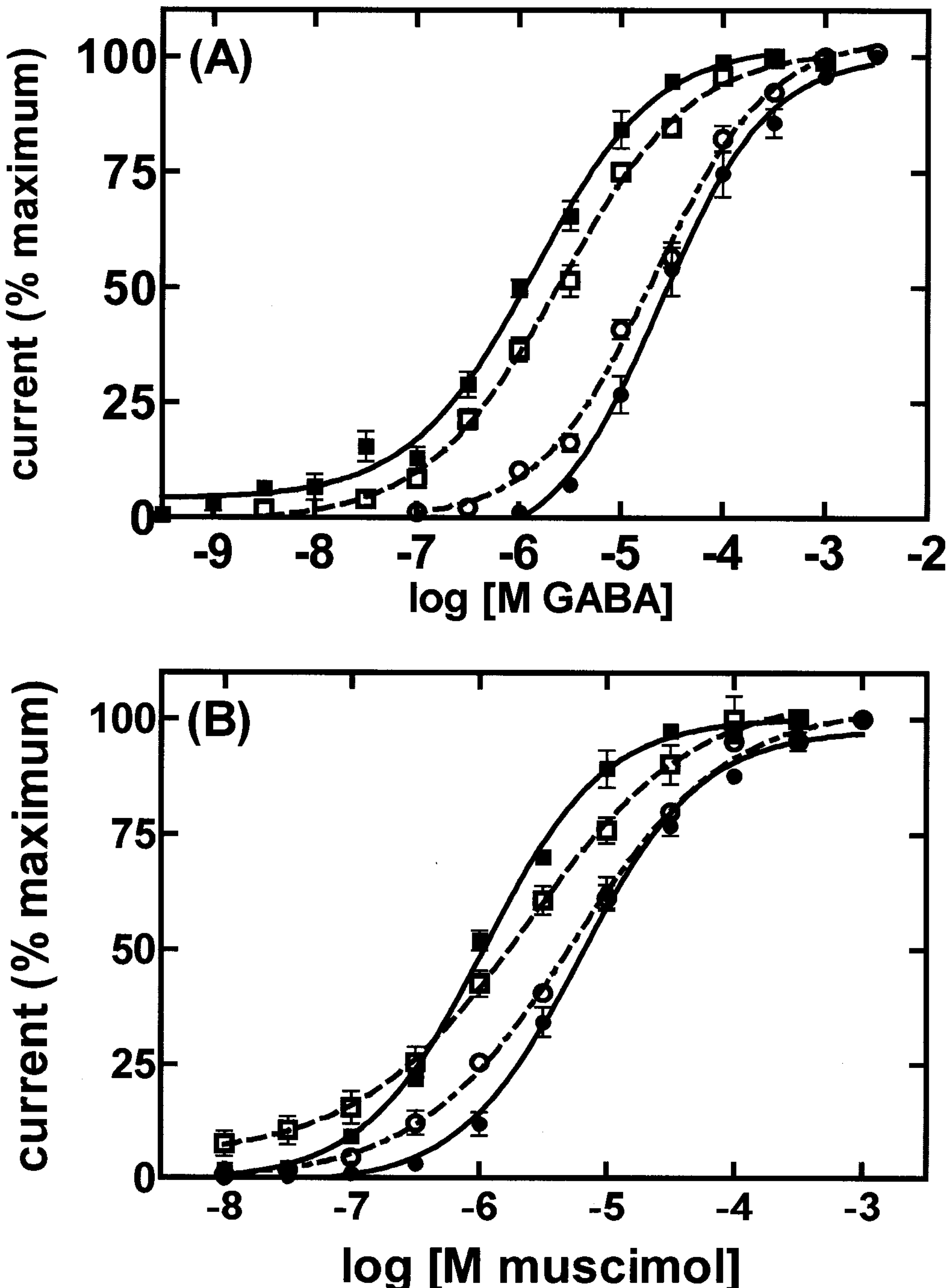
In conclusion, we have identified a structural domain within the $GABA_A$ receptor δ subunit (S238-V264) that confers high agonist sensitivity to the α4β3δ subtype. Further, we have shown that these effects are agonist-dependent and are likely to involve changes in the transduction mechanism that links agonist binding to channel activation.

Figure 2-1. Schematic representation of chimeric subunit construction. (A) The wildtype δ subunit cDNA (*black*) was subcloned upstream of the wildtype γ2L subunit cDNA (*grey*) into pcDNA3.1(+). This dual plasmid was doubly digested using *Bam*HI/*Eco*RI and the linearized plasmid was subsequently transformed into DH5α *Escherichia coli* cells. During the transformation, the linearized plasmid was recircularized by recombination events in homologous regions between the δ and γ2L sequences. (B) Three chimeric subunits (χ277, χ255 and χ237) were isolated and named according to the last residue of δ sequence before the crossover point. The wildtype δ subunit is shown in black and the γ2L subunit is shown in grey. The diagrams show the approximate positions of the crossover points within the predicted transmembrane domains of the subunit. (C) Partial amino acid sequence alignments of the δ and γ2L subunits including the transmembrane domains, TM1 and TM2, according to Ernst *et al.* (2005). The positions of the chimeric crossover points (χ277, χ255 and χ237) are shown. The shaded areas illustrate stretches of amino acids that are conserved between the δ and γ2L subunits showing that in the χ255 and χ277, the switch to the unique γ2L sequence occurs after M248 and V264 respectively.



A
Eco RI
γ2
Bam I
Apa I
δ
pcDNA3.1(+)
Hind III
B
δ
γ2
1 2 3 4
1 2 3 4
χ237
χ255
χ277
C
TM1
237
248
255
δ 233 V Y I I Q S Y M P S V L L V A M S W V S F W I S Q A A V P A R
γ2 235 Y F T I Q T Y I P C T L I V V L S W V S F W I N K D A V P A R
TM2
264
277
δ 264 V S L G I T T V L T M T T L M V S A R S S L P R A S A I
γ2 266 T S L G I T T V L T M T T L S T I A R K S L P K V S Y V

Figure 2-2. The effects of (A) GABA and (B) muscimol on activation of wildtype and chimeric receptors expressed in *Xenopus* oocytes. Concentration effect curves for α4β3δ (■), α4β3γ2L (●) and the chimeric receptors α4β3χ277 (□),α4β3χ237 (○) are shown. The effects of the agonists on the α4β3χ255 subtype are omitted for clarity. Data represent the mean ± SEM from at least 3 independent experiments and the parameters obtained from curve-fitting are summarized in Table 2-1.



(A)
current (% maximum)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
log [M GABA]
(B)
current (% maximum)
100
75
50
25
0
-8
-7
-6
-5
-4
-3
log [M muscimol]

Figure 2-3. Representative currents for activation of different $GABA_A$ receptors by varying concentrations of THIP and I4AA as indicated. Control responses of the same oocytes elicited by 1 mmol/L or 3mmol/L GABA are shown for comparison.

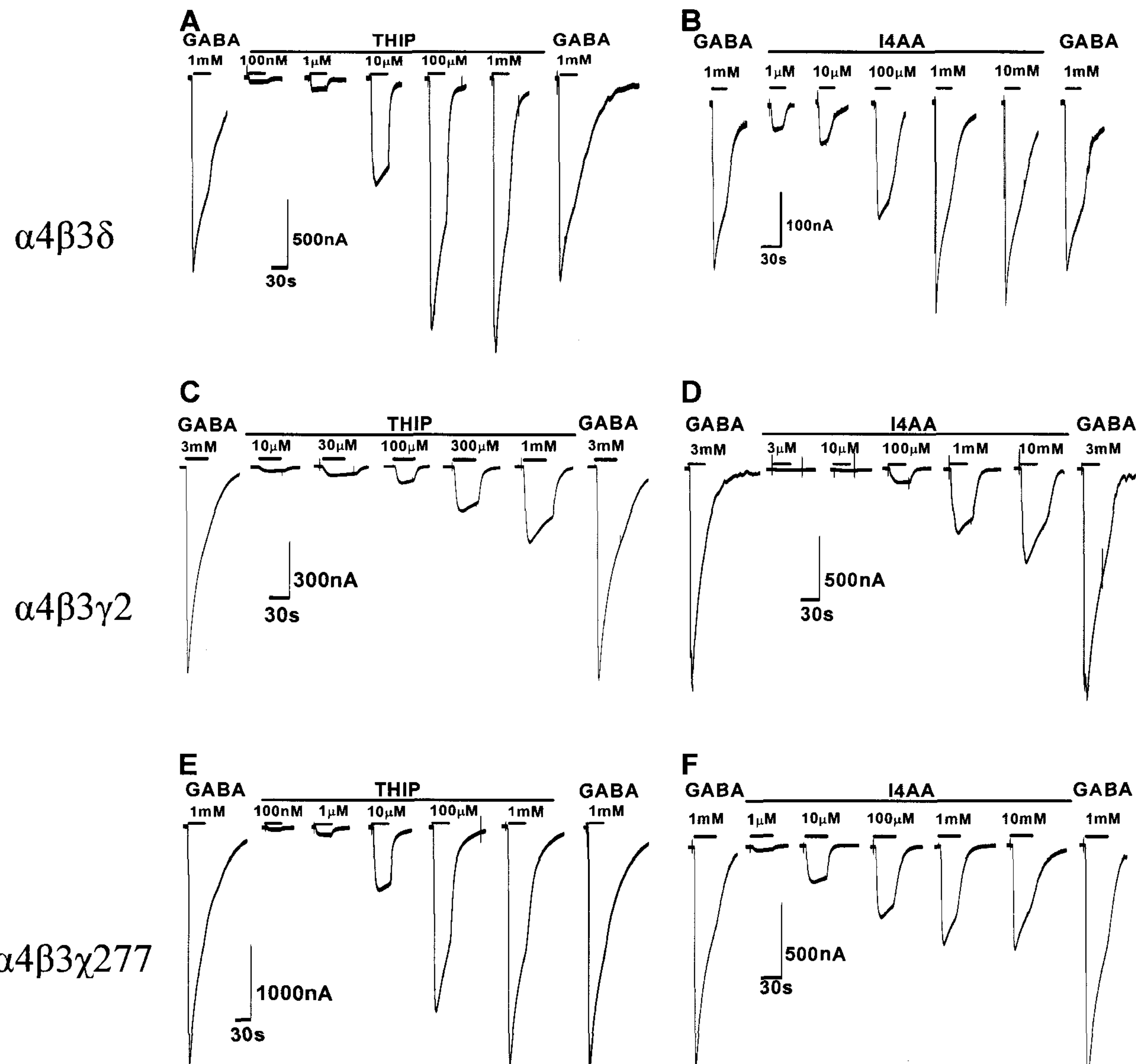

A
GABA
THIP
GABA
1mM
100nM
1μM
10μM
100μM
1mM
1mM
500nA
30s
B
GABA
I4AA
GABA
1mM
1μM
10μM
100μM
1mM
10mM
1mM
100nA
30s
α4β3δ
C
GABA
THIP
GABA
3mM
10μM
30μM
100μM
300μM
1mM
3mM
300nA
30s
D
GABA
I4AA
GABA
3mM
3μM
10μM
100μM
1mM
10mM
3mM
500nA
30s
α4β3γ2
E
GABA
THIP
GABA
1mM
100nM
1μM
10μM
100μM
1mM
1mM
1000nA
30s
F
GABA
I4AA
GABA
1mM
1μM
10μM
100μM
1mM
10mM
1mM
500nA
30s
α4β3χ277

Figure 2-4. The effects of (A) THIP and (B) I4AA on activation of α4β3δ (■), α4β3γ2L (•) and the chimeric receptors α4β3χ277 (□), α4β3χ255 (▲), α4β3χ237 (○). The data represent the mean ± SEM from at least 3 independent experiments and the results from data fitting are summarized in Table 2-2. The amplitudes are normalised to the magnitude of the response induced by a maximal concentration of GABA (100%, dashed line in figure).

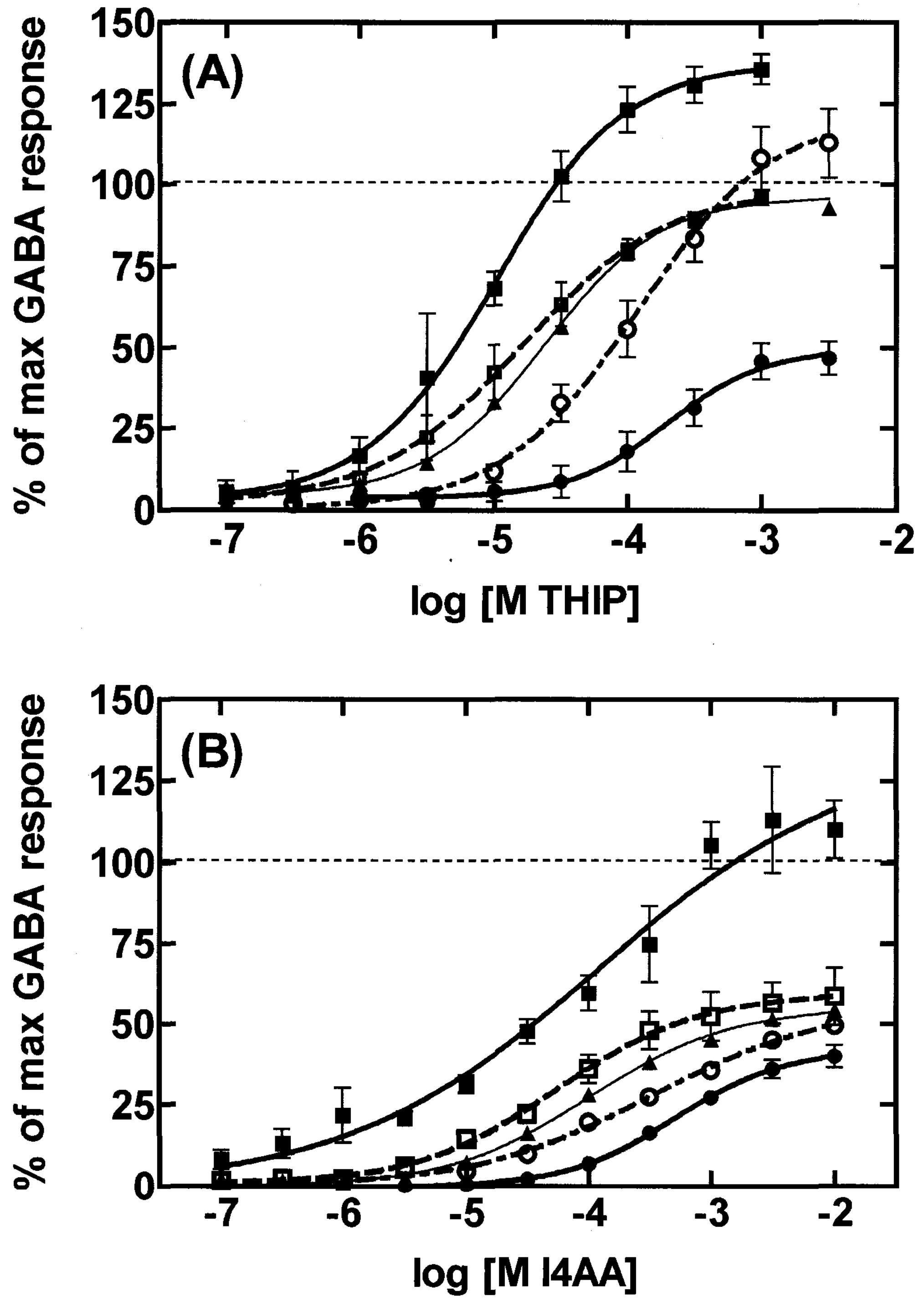
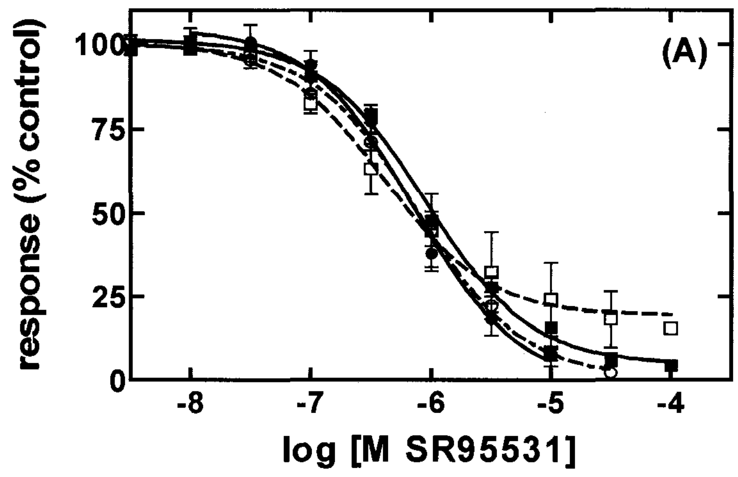
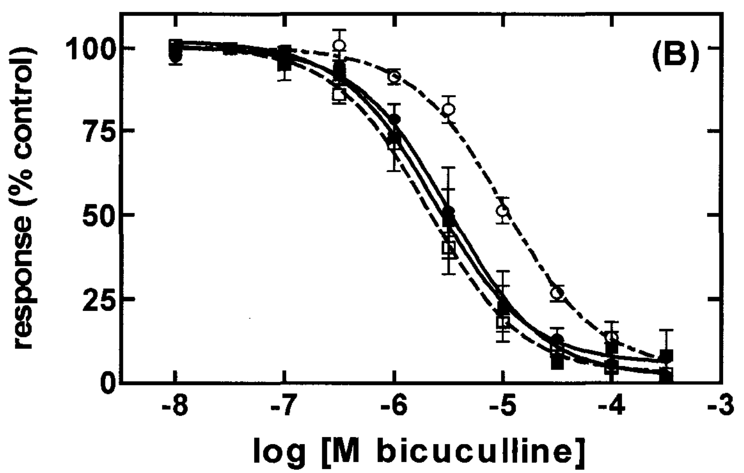

(A)
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
% of max GABA response
log [M THIP]
(B)
150
125
100
75
50
25
0
-7
-6
-5
-4
-3
-2
% of max GABA response
log [M I4AA]

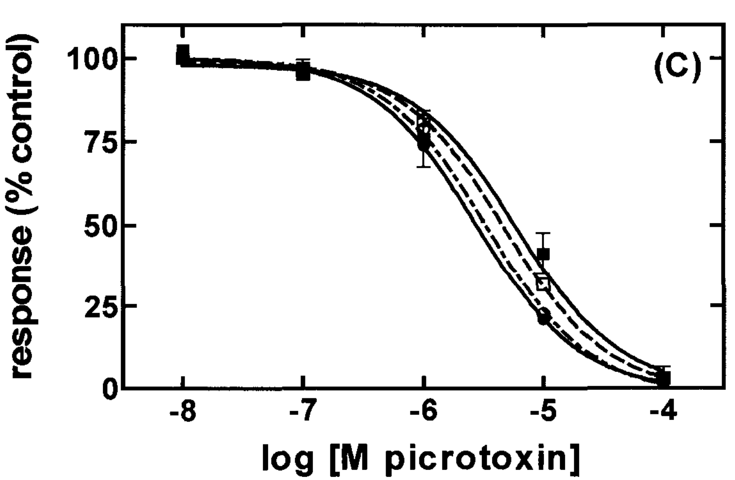
Figure 2-5. The effects of (A) SR95531, (B) bicuculline and (C) picrotoxin on GABA-evoked currents in the α4β3δ (■), α4β3γ2$_L$ (●) and chimeric α4β3χ277 (□) and α4β3χ237 (○) receptors. In each experiment, the oocyte was pre-perfused with the antagonist for 2 minutes before challenged with GABA and the same concentration of antagonist as used in the pre-perfusate. The GABA concentration used was equivalent to its EC_{50} value for each receptor subtype and the data were normalized to the GABA response in the absence of antagonist. Data represent the mean ± SEM from at least 3 independent experiments. The data obtained from curve-fitting are summarized in Table 2-3.



response (% control)
100
75
50
25
0
(A)
-8
-7
-6
-5
-4
log [M SR95531]



response (% control)
100
75
50
25
0
(B)
-8
-7
-6
-5
-4
-3
log [M bicuculline]



response (% control)
100
75
50
25
0
(C)
-8
-7
-6
-5
-4
log [M picrotoxin]

Table 2-1. Activation of the wild-type and chimeric $GABA_A$ receptor subtypes by GABA and muscimol. Log EC_{50} values were obtained from pooled data from at least three independent concentration-effect curves using different batches of oocytes and representthe mean ± SEM. Hill coefficients (n_H) from individual curves were averaged to give the final estimates indicated. * $p < 0.01$ compared to wildtype α4β3γ2 receptor. † $p < 0.01$ compared to wildtype α4β3δ receptor. †† $p < 0.05$ compared to wildtype α4β3δ receptor.

	GABA			Muscimol		
	log EC_{50}	EC_{50} (μmol/L)	n_H	log EC_{50}	EC_{50} (μmol/L)	n_H
α4β3δ	$-5.85 \pm 0.05^{*}$	1.41	0.70 ± 0.07	$-5.97 \pm 0.02^{*}$	1.08	0.93 ± 0.05
α4β3χ277	$-5.64 \pm 0.10^{*}$	2.29	0.69 ± 0.05	$-5.67 \pm 0.06^{*\dagger}$	2.15	0.71 ± 0.07
α4β3χ255	$-5.04 \pm 0.02^{*\dagger}$	9.05	0.71 ± 0.04	$-5.74 \pm 0.07^{*\dagger\dagger}$	1.82	0.72 ± 0.10
α4β3χ237	$-4.67 \pm 0.07^{\dagger}$	21.5	0.77 ± 0.07	$-5.27 \pm 0.04^{\dagger}$	5.35	0.72 ± 0.02
α4β3γ2	$-4.56 \pm 0.11^{\dagger}$	27.6	0.91 ± 0.09	$-5.21 \pm 0.06^{\dagger}$	6.17	0.93 ± 0.09

Table 2-2. Activation of the wild-type and chimeric $GABA_A$ receptor subtypes by THIP and I4AA. Log EC_{50} values were obtained from pooled data from at least three independent concentration-effect curves using different batches of oocytes and represent the mean ± SEM. Hill coefficients (n_H) from individual curves were averaged to give the final estimates indicated. The efficacy values reported are the amplitudes of the maximum current induced by the agonist relative to the maximum current induced by GABA (100%) for that receptor subtype. * $p < 0.01$ compared to wildtype α4β3γ2 receptor; † $p < 0.01$ compared to wildtype α4β3δ receptor; †† $p < 0.05$ compared to wildtype α4β3δ receptor.

	THIP				I4AA			
	log EC_{50}	EC_{50} (μmol/L)	n_H	Efficacy	log EC_{50}	EC_{50} (μmol/L)	n_H	Efficacy
α4β3δ	-5.02 ± 0.08*	9.62	0.92 ± 0.08	135.6 ± 4.7*	-4.00 ± 0.05*	100.2	0.66 ± 0.06	110.3 ± 8.7*
α4β3χ277	-4.83 ± 0.17*	14.7	0.86 ± 0.05	96.5 ± 2.0*†	-4.22 ± 0.03*	60.3	0.78 ± 0.08	58.7 ± 5.2†
α4β3χ255	-4.62 ± 0.07*	24.2	0.95 ± 0.07	96.0 ± 1.5*†	-3.96 ± 0.11*	110.2	0.74 ± 0.05	54.3 ± 4.0†
α4β3χ237	-4.02 ± 0.11†	94.6	1.00 ± 0.10	110.5 ± 11.0*††	-3.46 ± 0.05†	345.9	0.57 ± 0.01	49.6 ± 2.9†
α4β3γ2	-3.70 ± 0.10†	199.1	1.30 ± 0.19	46.9 ± 5.1†	-3.28 ± 0.06†	520.0	0.97 ± 0.11	40.0 ± 3.5†

TABLE 2-3. Inhibition of GABA-gated currents by the competitive antagonists (SR95531 and bicuculline) and the channel blocker (picrotoxin). Log IC_{50} values were determined from at least three independent experiments and represent mean ± SEM. The concentration of GABA used was its EC50 value for each receptor subtype (see Methods) ** $p < 0.05$ compared to wildtype α4β3γ2 receptor; † $p < 0.01$ compared to wildtype α4β3δ receptor.

	SR95531		Bicuculline		Picrotoxin	
	IC_{50} (μM)	logIC_{50} ± SEM	IC_{50} (μM)	logIC_{50} ± SEM	IC_{50} (μM)	logIC_{50} ± SEM
α4β3δ	0.90	-6.05 ± 0.08	2.44	-5.61 ± 0.19	4.85	-5.31 ± 0.25
α4β3χ277	0.51	-6.29 ± 0.18	2.10	-5.68 ± 0.02	4.83	-5.32 ± 0.02
α4β3χ255	0.59	-6.23 ± 0.04	3.28	-5.48 ± 0.02	2.82	-5.55 ± 0.02
α4β3χ237	0.79	-6.10 ± 0.06	10.5**†	-4.98 ± 0.07	3.41	-5.47 ± 0.04
α4β3γ2	0.74	-6.13 ± 0.06	3.24	-5.49 ± 0.12	2.83	-5.55 ± 0.01

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CHAPTER 3[1]

Identification of Structural Determinants in the γ2L and δ Subunits of the $GABA_A$ Receptor that Influence Receptor Desensitization

[1]Ms. Jacqueline Montpetit contributed to the studies of $GABA_A$ receptor α4β3δ, α4β3γ2L, α4β3χ237, α4β3χ255 and α4β3χ277 subtypes.

INTRODUCTION

Neuronal inhibition in the mammalian central nervous system is mediated largely by the γ-aminobutyric acid type A ($GABA_A$) receptor. $GABA_A$ receptors are members of the ligand-gated ion channel superfamily to which the nicotinic acetylcholine receptors, glycine receptors, and the serotonin type 3 receptors belong (Barnard *et al.*, 1998). To date, 19 different mammalian $GABA_A$ receptor subunits have been identified, including six α, three β, three γ, three ρ and one each of the δ, ε, π and θ subunits (McKernan and Whiting, 1996). Recently, $GABA_A$ receptor mediated inhibition has been further categorized into two groups, namely phasic and tonic inhibition. Phasic inhibition has been well characterized in many different cell types in the CNS and results from the action-potential induced release of GABA from the presynaptic cell. The postsynaptic receptors that respond to these high concentrations of GABA are of relatively low sensitivity (EC_{50}s of 20-30 μmol/L, Akaike *et al.*, 1985; Maconochie *et al.*, 1994) and usually show rapid desensitization. Many $GABA_A$ receptors at the synapse are thought to contain the $\gamma 2$ subunit in association with two α and two β subunits, with the $\alpha 1\beta 2\gamma 2$ composition being the most abundant subtype (Barnard *et al.*, 1998). Recently, the importance of tonic inhibition mediated by extrasynaptic receptors has become clear. These receptors are activated by the relatively low concentrations of GABA that either overspill from the synapse or are released from glial cells (Mody, 2001). Tonic inhibition plays an important role in shaping the postsynaptic response to GABA and has been studied most extensively in cerebellar granule cells (Brickley *et al.*, 1996) and hippocampal dentate granule cells (Nusser and Mody 2002; Mtchedlishvili and Kapur 2006). The $\alpha 4\beta x\delta$, $\alpha 6\beta 2/3\delta$ and $\alpha 5\beta 3\gamma 2/3$ combinations are likely to be the major

subtypes involved (see Farrant and Nusser, 2005). These receptors display higher GABA sensitivity and desensitize more slowly than their synaptic counterparts.

In recombinant expression systems, several studies have demonstrated that inclusion of the δ subunit in the $GABA_A$ receptor confers characteristics that mimic the pharmacological and biophysical properties of tonic inhibition observed *in vivo* (Saxena and Macdonald, 1996). When co-expressed with the α4 and β3 subunits in *Xenopus* oocytes or a stable cell line, the putative extrasynaptic receptor, α4β3δ, showed 20-40 fold higher sensitivity to GABA than the α4β3γ2 subtype (Brown *et al.*, 2002, Wallner *et al.*, 2003, You and Dunn 2007). Moreover, both the rate and extent of desensitization of the δ-containing receptor were significantly reduced compared to the γ2-containing receptor (Haas and Macdonald, 1999; Bianchi *et al.*, 2001).

The structural determinants of the δ subunit that play a role in its slower desensitization properties have been studied previously. Using a rapid application system and brief (400 ms) pulses of a saturating concentration of GABA, Bianchi *et al.*, (2001) suggested that the N-terminal domain and the extracellular region of transmembrane (TM) domain 1 are responsible for the differences in the fast component of desensitization displayed by δ and γ2-containing receptors. In a subsequent study using a longer (28 s) application of GABA, the TM2 domain was suggested to be important for the less extensive desensitization seen in the δ-containing receptor (Bianchi and Macdonald 2002).

Although there have been several attempts to understand the different desensitization properties of δ- and γ2-containing receptor, their structural basis remains unclear (Bianchi *et al*, 2001; Bianchi and Macdonald, 2001, 2002). The role of individual

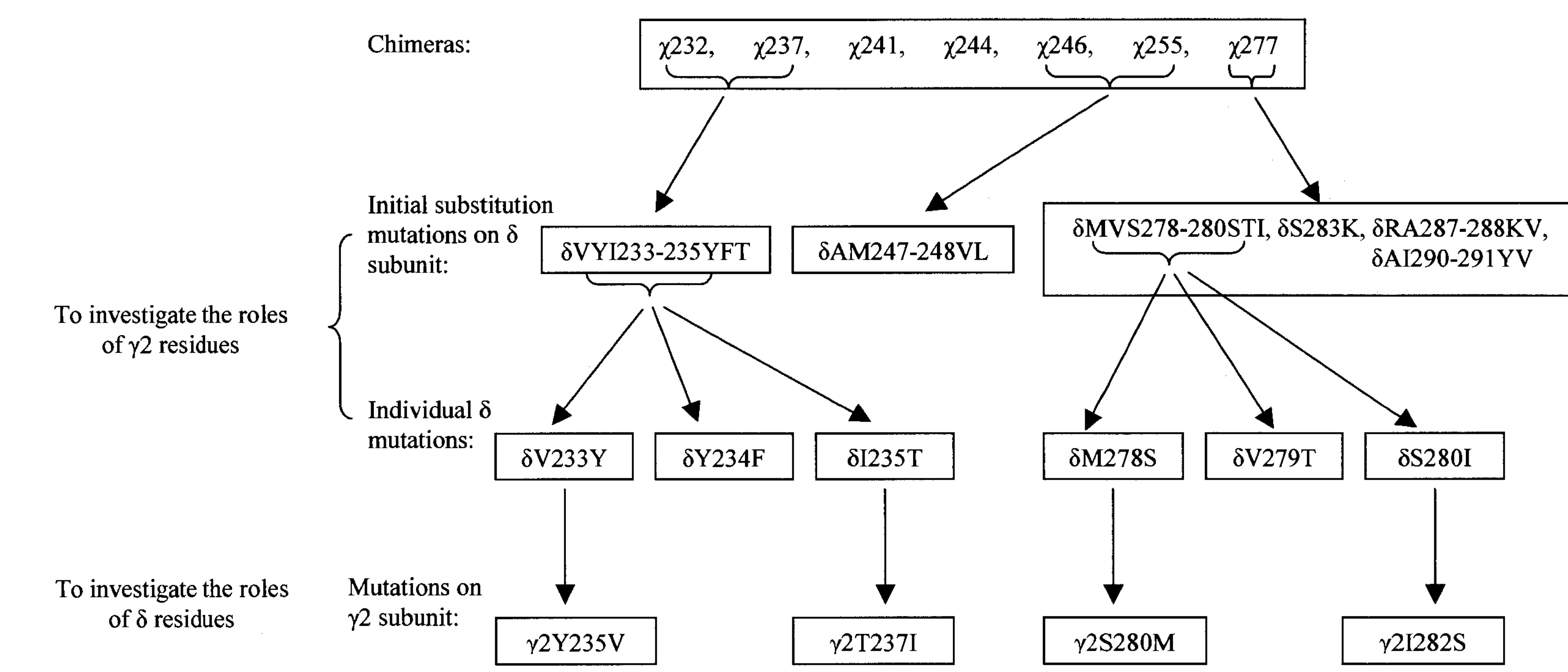
residues within TM1 and TM2 in modulating receptor desensitization have been investigated previously but not fully elucidated (Bianchi and Macdonald 2002).

In the present study, we have examined structural determinants in the TM1 and TM2 domains of the δ and γ2L subunits that contribute to their unique desensitization properties. In order to achieve our goal, we

- have generated a panel of chimeric δ/γ2 subunits and co-expressed these chimeric subunits with wildtype α4 and β3 subunits in *Xenopus* oocytes. The desensitization properties of chimeric receptors were investigated.
- have constructed a series of substitution mutations in the TM1 and TM2 domains of the δ and γ2 subunits based on the results of chimera studies, and have further determined their desensitization characteristics (see the schematic illustration of this study in the next page).

Our results demonstrate that structural determinants within the extracellular part of both the TM1 and TM2 domains play important roles in receptor desensitization.

Schematic Illustration of the Investigation of Structural Determinants in the γ2 and δ Subunits of the $GABA_A$ Receptor that Influence Receptor Desensitization



MATERIALS AND METHODS

Construction of Mutated δ, γ2L Subunits and δ/γ2L Chimeras

The cDNAs encoding the α4, β3, δ and γ2L subunits of the rat $GABA_A$ receptor have been described elsewhere (You and Dunn, 2007, Chapter 2) and all were subcloned into the pcDNA3.1 vector. Single or multiple mutations were introduced into the δ and γ2L subunits using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Three in-frame hybrid DNA chimeras (χ237, χ255, χ277) were generated using the protocol of Moore and Blakely (1994, see details in You and Dunn, 2007; Chapter 2). Chimeras χ232, χ241 and χ244 were engineered by site-directed mutagenesis of chimera χ237, while χ246 was constructed by mutations of chimera χ255. In each chimera, the sequence from the N-terminus to the crossover point is derived from the original δ subunit cDNA, whereas the remainder of the sequence comes from γ2L. The numbering of the chimeras corresponds to the last residue on the δ subunit prior to the in-frame switch. Figure 3-1 shows an alignment of the δ and γ2L sequences, the position of the crossover points and the location of the putative transmembrane domains based on the structural model of Ernst *et al.* (2005). All chimeras and mutations were verified by restriction digestion and DNA sequencing (Molecular Biology Facility, Department of Biological Sciences, University of Alberta).

Xenopus Oocyte Preparation and Expression of $GABA_A$ Receptors

Mature oocytes (stage V–VI) from *Xenopus laevis* were prepared by Dr. J. D. Young's laboratory (Department of Physiology, University of Alberta) and the methods for preparation were described previously (Smith *et al.*, 2004). Briefly, female *Xenopus*

laevis (Biological Sciences Vivarium, University of Alberta) was anaesthetized by immersion in 0.2% tricaine methanesulphonate (pH 7.4; Sigma, Oakville, ON, Canada) and humanely euthanized in compliance with guidelines approved by the Canadian Council on Animal Care. A portion of the ovary lobe was removed from the toad through a lateral incision. Lobes were broken up with forceps and isolated oocytes were transferred immediately to a petri dish containing Ca^{2+}-free ND96 buffer (96 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES, pH 7.4). To remove the follicle cell layer (defolliculation), oocytes were transferred to fresh Ca^{2+}-free ND96 buffer containing 0.25 mg/ml collagenase P and 0.25 mg/ml trypsin inhibitor and shaken gently for 2 hours at room temperature. Defolliculated oocytes were then washed extensively with fresh Ca^{2+}-free ND96 buffer to remove the follicle cells completely. The defolliculated oocytes were stored in ND96 buffer at 14°C before injection.

Capped cRNAs encoding rat $GABA_A$ wildtype, chimeric or mutated receptor subunits were synthesized from linearized cDNA by *in vitro* transcription using T7 RNA polymerase (Invitrogen, San Diego, CA). The concentrations of cRNAs were quantified from their absorbance at 260 nm. Oocytes were injected with 50 nl of 1 μg/μl total subunit cRNA in a 1:1:1 ratio (α4:β3:δ, α4:β3:γ2L, chimeric or mutated receptors). Injected oocytes were incubated in ND96 buffer supplemented with 100 μg/ml gentamicin in 96-well plates at 14°C for at least 48 hours prior to functional analysis.

Two-Electrode Voltage Clamp Measurements

All experiments were carried out using frog Ringer's solution (110 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES, pH 7.4). GABA-evoked currents were recorded by standard two-electrode voltage clamp techniques using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA) at a holding potential of –60 mV. The electrodes were filled with 3 mol/L KCl and only electrodes with a resistance between 0.5 to 3.0 MΩ in frog Ringer's solution were used.

In desensitization experiments, a saturating concentration (3 mmol/L) of GABA was applied via gravity perfusion for 5 minutes. During this time, most receptors desensitized to a steady state level. GABA application was followed by at least a 2-minute washout period until the current returned to baseline. The desensitization profile for each oocyte was recorded 1-3 times. In the case of multiple GABA applications, the oocyte was perfused with frog Ringer's solution for at least 20 minutes between applications to ensure complete recovery from desensitization.

Data Analysis

Currents evoked by saturating concentrations of GABA were analyzed using pClamp 9.0 (Axon Instruments). In all experiments reported here, the desensitizing phase of the current was adequately described by a two exponential model:

$$A = A_f e^{(-t/\tau_f)} + A_s e^{(-t/\tau_s)} + C$$

where A is the peak current, τ_f and τ_s are the fast and slow time constants of the two phases, A_f and A_S are their corresponding amplitudes and t is time. C is the residual (non-desensitizing current) current measured at the end of the two observed phases of

desensitization. This model provided satisfactory agreement between the theoretical curves and experimental traces (correlation > 0.99 in most cases). The weighted time constant, τ_w, is defined as:

$$\tau_W = \frac{\tau_f A_f}{A_f + A_s} + \frac{\tau_S A_s}{A_f + A_s}$$

where the parameters are as described above. (Haas and Macdonald, 1999; Wagner *et al.*, 2005). The relative amplitude contributions of the fast and slow phase desensitization are defined as $\%A_f$ and $\%A_s$ and are equal to $A_f / (A_f + A_s)$ and $A_s / (A_f + A_s)$, respectively. The fraction of non-desensitizing current is defined as %C, which is calculated as C / peak current A.

Data from multiple oocytes (n = 3 - 11) expressing the same receptor subtype were pooled and values are reported as the mean ± SEM. Data were analyzed by one-way ANOVA and levels of significance were determined by Dunnett's post-test for multiple comparisons (GraphPad Prism 4.0).

RESULTS

Comparison of the Desensitization Properties of Wildtype α4β3δ and α4β3γ2L Subtypes

Typical desensitization profiles of the α4β3δ and α4β3γ2L subtypes are shown in Figure 3-2 A. As noted in the Methods, a two exponential model gave the best fit to the desensitization curves recorded for each receptor subtype. When comparing the weighted time constants for the desensitization process (see Table 3-1, Figure 3-2 B), it is clear that the α4β3δ receptor subtype desensitizes more slowly ($\tau_w = 53.0 \pm 2.5$ s) than the α4β3γ2L subtype ($\tau_w = 29.8 \pm 0.8$ s, $P<0.01$, Student t-test). Detailed analysis of the kinetics of

desensitization (see Table 3-1) reveals that the time constants of both the fast (τ_f) and slow (τ_s) phases of desensitization of the wildtype α4β3γ2L are significantly faster than those of the α4β3δ subtype. However, the proportions of the fast and slow components of desensitization of the α4β3γ2L receptor were not significantly different from those of the α4β3δ subtype. Thus, the overall rapid desensitization of the α4β3γ2L receptor can be attributed to its faster rates (τ_f and τ_s) in both phases of desensitization.

Another important feature of the desensitization characteristics displayed by different $GABA_A$ receptor subtypes is the magnitude of the current that persists in the presence of continued GABA application. Currents mediated by the α4β3γ2L desensitized almost completely (95-96%) after 5 minutes of exposure to 3 mmol/L GABA. However, in the α4β3δ combination, a significant non-desensitizing current (21.2% of peak current) persisted under these conditions (Table 3-1, Figure 3-2 C).

Desensitization of Chimeric δ/γ2L Subunit-Containing Receptors

Several chimeric δ/γ2 subunits with crossover points within the TM1 and TM2 domains (χ232, χ237, χ241, χ244, χ246, χ255 and χ277) were constructed (Figure 3-1) to investigate differences in the desensitization properties that are conferred by the δ and γ2L subunits. The current traces shown in Figure 3-3 A (see also Table 3-2) demonstrate significant differences in the desensitization characteristics of these chimeric receptors. Chimera χ232 which incorporates the extracellular domain of the δ subunit with the remaining sequence coming from γ2L displays desensitization characteristics of the γ2L-containing receptor i.e., almost complete desensitization during current application and a relatively fast rate of desensitization (τ_w of 29.1 ± 1.5 s). In contrast, as the amount of

δ sequence was extended towards the middle of TM1 (χ237, χ241, χ244, χ246), the desensitization kinetics became more similar to those of δ-containing receptors i.e. slower rates (τ_ws in the range of 70 - 120 s which are slower than those observed for the α4β3δ receptor) and a significant (17-37%) non-desensitizing current (Table 3-2). Given the changes in desensitization kinetics observed with the chimera χ232- and χ237-containing receptors, we speculated that there may be specific amino acids within this 5-residue sequence lying in the extracellular portion of TM1 that may confer differences in the δ/γ2 desensitization kinetics (see below).

Surprisingly, as the N-terminal δ subunit sequence was extended further to include most of the TM1 domain (χ255) and also towards the middle of TM2 (χ277), the desensitization kinetics became indistinguishable from those of the γ2-containing receptor (see Figures 3-1, 3-3 and Table 3-2). This suggests that residues within this region (see Figure 3-1) may also contribute to difference in the observed desensitization kinetics.

Taken together, the desensitization results of the δ/γ2 chimera-containing receptors suggest that structural determinants controlling receptor desensitization may reside in two separate domains: (1) the first 5 residues in TM1, as indicated by the different desensitization kinetics of the χ232- and χ237-containing receptors and (2) the intracellular end of the of TM1 domain (between δ247 and 255), as indicated by differences in the desensitization characteristics of the χ246 and χ255/χ277-containing receptors (Figure 3-1).

Effects of Substitution Mutations in the Extracellular Domain of TM1 (δV233/γ2Y235 -δQ237/γ2Q239)

Within this 5-residue sequence there are only 3 divergent residues between the δ and γ2L subunits (δVYI/γYFT; see Figure 3-1). A triple mutation in the δ subunit was therefore constructed (δVYI233-235YFT) and this mutated δ subunit was co-expressed with wildtype α4 and β3 subunits in *Xenopus* oocytes. Our results showed that the desensitization properties of the δVYI233-235YFT-containing receptor were dramatically changed (Table 3-3, Figure 3-4 A). This δVYI233-235YFT-containing receptor has a fast desensitization rate (τ_w of 18.5 ± 1.1 s), which is approximately 3-fold faster than that of the α4β3δ subtype.

To further probe the roles of individual residues in modulating desensitization, three single mutations (δV233Y, δY234F and δI235T) were generated. When co-expressed with the α4 and β3 subunits, the δV233Y and δI235T-containing receptors had fast desensitization characteristics similar to the δVYI233-235YFT-containing receptor (Figure 3-4 B). The rates of desensitization (τ_w) of the δV233Y- and δI235T-containing receptor were significantly faster than the rate of desensitization of the α4β3δ subtype (see Table 3-3, Figure 3-4 D). Furthermore, the fraction of non-desensitizing current (%C) of δV233Y- and δI235T-containing receptors (14.1 ± 0.9 % and 16.7 ± 1.4 %, respectively) was significantly decreased compared to the α4β3δ subtype but still 3-fold larger than that of the α4β3γ2L subtype (Figure 3-4 E). In contrast to the δV233Y- and δI235T-containing receptor, mutation δY234F does not have any significant effect on the desensitization properties of wildtype α4β3δ subtype.

In summary, these results suggest that two γ2L residues, Y235 and T237 (as indicated by mutations δV233Y and δI235T, respectively), located at the extracellular end of the TM1 play major roles in determining fast rate of desensitization. However, failure to confer a γ2L-like small non-desensitizing currents (4.6% of peak current, see Table 3-1) suggests these two γ2L residues may play only minor roles in determining the non-desensitizing currents and that other structural determinants may be involved.

With the identification of γ2 residues Y235 and T237 as determinants of fast desensitization, it was interesting to determine whether their equivalents in the δ subunit, the δ residues V233 and I235, were also the structural determinants involved in controlling slow desensitization. By introducing the equivalent δ residue V233 into a γ2L subunit, mutation γ2Y235V clearly resulted in a slower rate of desensitization ($\tau_w = 49.7 \pm 1.5$ s), which had no difference from that of the wildtype α4β3δ receptor (Table 3-3, Figure 3-4 C, D). The fraction of non-desensitizing currents of γ2Y235V (13.2 ± 1.1 %) was significantly increased (Table 3-3, Figure 3-4 E). In contrast, the desensitization of γ2T237I-containing receptors was indistinguishable from that of wildtype α4β3γ2L receptor desensitization (Figure 3-4 C). These results indicate that of these two δ residues (V233 and I235), the valine residue is a major determinant for slow desensitization and plays a minor role in determining non-desensitizing current of δ-containing receptors.

Taken together, the results from δ and γ2L subunit mutations suggest that the extracellular part of the TM1 plays an important role in determining the desensitization profiles of the α4β3δ and α4β3γ2L receptors. Introduction of the γ2L residues (Y235, T237) into the δ subunit resulted in faster rate of desensitization and smaller non-

desensitizing currents whereas introduction of δ residue V233 into γ2L subunit led to a slower rate of desensitization and larger non-desensitizing current.

Effects of Substitution Mutations in the Intracellular domain of TM1 (δA247/γ2V249 – δI255/γ2I257)

As mentioned in the chimera studies, differences in the desensitization characteristics of the χ246- and χ255-containing receptors suggest that structural determinants controlling receptor desensitization may also reside in the intracellular end of the of TM1 domain (between δ247 and 255, see Figure 3-1). Of the 9 amino acid residues lying in the A247- I255 domain of the δ subunit, A247 and M248 are divergent from those in equivalent position of γ2L (V249 and L250). We, therefore, constructed a double mutation (δAM247-248VL) to substitute these two δ residues with their equivalent γ2L residues. Interestingly, when co-expressed with the α4 and β3 subunits, mutation δAM247-248VL resulted in a slow rate of desensitization ($\tau_w = 69.2 \pm 4.2$ s), which is much slower than that observed for both of the α4β3δ and α4β3γ2L receptors (Figure 3-5 A, B). This greater weighted time constant can be mainly attributed to a significantly increased fraction of slow phase desensitization ($\%A_s$). In addition, the fraction of non-desensitizing current (41.8 ± 3.1 %) of this receptor is significantly increased and it is approximately 2-fold greater than that of wildtype α4β3δ receptor (Figure 3-5 C, Table 3-4). The study of δAM247-248VL mutant did not give a clear explanation for why there is a difference in the desensitization properties of the χ246- and χ255-containing receptors. However, these results may imply that introducing γ2

residues V249-L250 into the δ subunit impairs δ-containing receptor desensitization and leads to a much slower desensitization process (see Discussion).

Effects of Substitution Mutations in the Extracellular domain of TM2 (δM278/γ2S280 - δI291/γ2V293)

In additional experiments, we set out to investigate the role of residues in the extracellular domain of TM2 in determining receptor desensitization. As mentioned in the chimera study, χ277, which incorporates the longest N-terminal sequence from the δ subunit, conferred a γ2-like fast weighted time constant with a small fraction of non-desensitizing current. Results from χ277 infer that amino acids lying between the middle of TM2 (γ2S280) to the C-terminus of γ2L subunit, which comprises the extracellular part of TM2 and the whole TM3 and TM4 segments, may contain additional structural determinants involved in modulating receptor desensitization. The extracellular part of TM2 was previously implicated in controlling non-desensitizing currents. However, the roles of individual residues in this region are not clear (Bianchi and Macdonald, 2002). Therefore, we chose to focus our studies on the extracellular part of TM2. There are eight residues that are not conserved between the δ and γ2L subunits (Figure 3-1) in this area. We initially made four single or multiple amino acid substitutions in this region of the δ subunit (δMVS278-280STI, δS283K, δRA287-288KV and δAI290-291YV), replacing δ residues with the amino acids at the equivalent position of the γ2L subunits. The intention was to identify γ2L residues, which would confer a γ2L-like fast rate of desensitization and small non-desensitizing current.

Of all four mutations, δMVS278-280STI is the only one that affects both the weighted time constant and non-desensitizing current of receptor desensitization.

Currents evoked on δMVS278-280STI-containing receptors displayed a fast rate of desensitization ($\tau_w = 13.7 \pm 0.9$ s, see Table 3-5, Figure 3-6 A, D), which was 4-fold faster than that of the wildtype α4β3δ receptor. In parallel, the fraction of non-desensitizing current of δMVS278-280STI (8.0 ± 0.3 %) was significantly decreased and was indistinguishable from that of wildtype γ2L-containing receptor (Figure 3-6 A, E). However, other mutations (δS283K, δRA287-288KV and δAI290-291YV) in the extracellular part of TM2 domain of δ subunit only affect part of the desensitization process (i.e., either weighted time constant or non-desensitizing current, see Figure 3-6 A, D, E and Table 3-5), suggesting the presence of differential roles of these γ2L residues in modulating receptor desensitization.

Next we chose to focus on the segment δM278/γ2S280 - δS280/γ2I282 to investigate the roles of individual residues in controlling receptor desensitization. Three δ subunit mutations (δM278S, δV279T and δS280I) were produced by replacing individual residues with those found in the equivalent position of the γ2L subunit. The weighted time constants of the desensitizing currents of δM278S- and δS280I-containing receptors (21.2 ± 3.0 s and 13.0 ± 1.5 s, respectively) were about 2- and 4-fold faster than those measured for the α4β3δ receptor (Table 3-5, Figure 3-6 B, D). Upon closer inspection, the desensitization kinetics showed that these two mutations result in a significant increase in the relative amplitudes of the fast phase desensitization ($\%A_f$) when compared to the α4β3δ receptor. Although both of these mutations led to significant reductions in non-desensitizing currents (see Table 3-5, Figure 3-6 E), neither of them resembled the small fraction of non-desensitizing current of the wildtype α4β3γ2L receptor.

Interestingly, the presence of the δV279T mutation exerted an unexpected desensitization profile. The desensitization of δV279T-containing receptors had a significantly slower weighted time constant (τ_w of 102.9 ± 11.7 s) and an increased fraction of non-desensitizing current (%C of 32.3 ± 2.0 %) compared to the α4β3δ receptor (Table 3-5, Figure 3-6 B, D, E). This may suggest that the introduction of the γ2L threonine residue into the δ subunit may impair the desensitization process of the α4β3δ receptor (see Discussion).

In summary, the results of these three δ mutations indicate that (1) two individual γ2L residues S280 and I282 (as indicated by mutations δM278S and δS280I) located in the middle of TM2 play major roles in determining faster desensitization process; and (2) three consecutive residues γ2L STI280-282 (as indicated by mutation δMVS278-280STI) are required to confer a γ2L-like small non-desensitizing current.

We further probed the roles of δ residues M278 and S280 to investigate whether they are necessary in controlling the slow desensitization process. These residues were introduced into equivalent positions in the γ2L subunit as mutations γ2S280M and γ2I282S (Figure 3-1). Our data show that neither of these two mutations affected the rate of desensitization (τ_w) when compared to wildtype α4β3γ2L (Table 3-5, Figure 3-6 C, D). However, the fraction of non-desensitizing current of γ2I280S-containing receptors (23.2 ± 1.1 %) was significantly increased compared to the wildtype α4β3γ2L receptor and was similar to that of the α4β3δ receptor (Table 3-5, Figure 3-6 C, E). Thus, these results indicate that δ residues in this segment do not have significant influence on the rate of desensitization compared to their counterparts in the γ2L subunit, but clearly δ residue S280 is a structural determinant for the large non-desensitizing currents.

In conclusion, the roles of δ and γ2L residues in the TM1 and TM2 domain in determining receptor desensitization are summarized in Table 3-6.

DISCUSSION

The work in this chapter has identified structural determinants in the TM1 and TM2 domains of γ2L and δ subunits that influence receptor desensitization. Desensitization is an intrinsic property of the ligand gated ion channels. For $GABA_A$ receptors, it has been suggested that desensitization may play an important role in determining the magnitude and the shape of GABAergic inhibitory postsynaptic currents, both by truncating activation and prolonging deactivation (Jones and Westbrook 1995, 1996; Overstreet *et al.*, 2000). Recently, there has been growing evidence that certain subtypes of $GABA_A$ receptors are located in the extrasynaptic area and mediate tonic inhibitory currents in response to low ambient concentrations of GABA present around the postsynaptic neurons. In hippocampal dentate granule cells, this tonic inhibition is likely mediated by the extrasynaptic α4-, δ-containing receptors, whereas the synaptic inhibition, or the phasic inhibition, is likely mediated by the α4-, γ2-containing receptors (Sur *et al.*, 1999). The desensitization properties of the extrasynaptic receptors are distinct from those of the synaptic receptors. Notably, these extrasynaptic receptors desensitize at a slower rate and to a lesser extent (Brown *et al.*, 2002). Although this difference is still under investigation, this slow desensitization process may allow a persistent influx of chloride ions into postsynaptic neurons and may, therefore, play an important role in maintaining low excitability in those postsynaptic neurons (Overstreet *et al.*, 2000).

Several Regions Containing Structural Determinants of Desensitization Revealed by δ/γ2L Chimeras

In the present study, we investigated the structural basis for the difference in desensitization profiles between δ- and γ2L-containing receptors. At the beginning of this research, several domains of interest were indicated by the chimera studies. First, inclusion of the χ237 chimeric subunit (containing the N-terminal extracellular domain and first five residues from TM1 domain of δ subunit with the remainder coming from γ2L sequence) imparted δ-like characteristics to the receptor in terms of desensitization properties. In contrast, inclusion of the χ232-subunit led to a receptor whose desensitization characteristics were not significantly different from the wildtype α4β3γ2L. These results point to a specific region of δ or γ2L subunit (δV233/γ2Y235 - δQ237/γ2Q239), which plays a role in receptor desensitization. Secondly, a clear switch of desensitization properties between χ246- (δ-like) to χ255- (γ2-like) containing receptor suggests that the intracellular end of the TM1 domain (between δ247 and 255) may also contains structural determinants controlling receptor desensitization. Therefore, we initially set out to investigate structural determinants of receptor desensitization in two functional domains involved in receptor desensitization:

(1) the extracellular domain of TM1 and;

(2) the intracellular domain of TM1

In additional experiments, we explored the structural determinants in the extracellular domain of TM2 since both of our chimera studies (χ277) and previous reports from another group (Bianchi and Macdonald, 2002) inferred the importance of this region in controlling receptor desensitization.

With further mutagenesis studies, our results demonstrated that structural determinants for receptor desensitization are located in the extracellular domain of TM1 and TM2. However, the roles of residues in the intracellular domain of TM1 in receptor desensitization are still not clear (see below).

Desensitization Affected by Residues in the Extracellular End of TM1 Domain

We have identified one δ residue (δV233) and two γ2L residues (γ2Y235 and γ2T237) in the extracellular part of TM1 domain, which are involved in modulating desensitization of δ- and γ2L-containing receptors, respectively (Table 3-6). δV233 and γ2Y235 are in a homologous position at the beginning of TM1. Sequence alignments of α, β, γ, π, ρ and ε $GABA_AR$ subunits within this region show that the tyrosine residue in the γ2L subunit is conserved throughout the family with the exception that the δ subunit has a valine and ρ1 subunit has a phenylalanine in this position. Interestingly, the ρ1 subunit also forms slow-desensitizing homomeric receptors (Kusama *et al.*, 1993). The sequence comparison between subunits suggests that the δV233/γ2Y235 may play an important role in determining receptor desensitization. Indeed, substitution mutations of these two residues result in significant changes in the weighted time constants and non-desensitizing currents, suggesting that both of them are important to control the respective desensitization characteristics of their subunits. Furthermore, we demonstrated that the γ2L residue T237 has similar effects on desensitization of the γ2L-containing receptor. These results are inconsistent with a previous study (Bianchi *et al.*, 2001), in which the authors did not observe any significant changes in desensitization caused by introducing δ residues alone into γ2L subunit ($\gamma 2L_{(Y\Delta V)}$ termed by these authors; the same

as our γ2Y235V mutation). They suggested that inclusion of the entire extracellular N-terminal domain and the first two residues of TM2 (δV233 and δY234) of δ sequence are required to abolish fast desensitization in γ2L, which is not the case in the present study.

How residues in the extracellular end of TM1 regulate receptor desensitization is still unclear. However, recent studies on the pre-TM1 domain of LGIC receptor subunits may shed some light on this issue. The pre-TM1 domain, which is the link between the N terminus and TM1 domain, has been implicated in transducing binding signal to channel gating in a number of LGIC, including the nAChR (Lee and Sine *et al.*, 2005), $GABA_A$ (Kash *et al.*, 2004; Keramidas *et al.*, 2006; Mercado and Czajkowski 2006), $GABA_C$ receptor (Wang *et al.*, 2006; Price *et al.*, 2007), and the 5-HT_3 receptor (Hu *et al.*, 2003; Price *et al.*, 2007). Several charged residues in the pre-TM1 domain have been shown to form electrostatic interactions with other residues located in the loop structures (loop 2 and 7) of the extracellular domain. These pair-wise interactions are suggested to be critical for receptor activation and controlling the gating process (see the references above). Interestingly, mutating residues in this area often results in altered desensitization process. Engblom *et al.* (2002) reported that a conserved glycine residue of the β2 subunit, which is in an equivalent position of δG232 (see Figure 3-1), is involved in controlling desensitization. Altered desensitization properties were observed when they mutated this glycine residue to a phenylalanine. In 5-HT_{3A} receptors, it was shown that Arg222, which lies two residues away from the extracellular end of TM1, is involved in the transduction process and but also plays a role in desensitization (Hu *et al.*, 2003). In case of δ- and γ2L-containing receptors, given the proximity of δV233, γ2Y235 and γ2T237 to the pre-TM1 domain, it is likely that they may also be involved in the coupling between ligand

binding and channel gating (also see below). Changes of the side-chain size or hydrophobicity of the residues (eg. valine *vs.* tyrosine) in this domain may cause structural perturbation, and consequently may alter the energy barrier for the receptor entering the conformational state of desensitization.

Desensitization Affected by Residues in the Extracellular End of the TM2 Domain

Previous studies have identified many residues in the TM2 domain that influence macroscopic desensitization rate of recombinant GABA receptor current (Tierney *et al.*, 1996; Birnir *et al.*, 1997a, b; Bianchi and Macdonald, 2001; Scheller and Forman, 2002, see also Chapter 1). However, these identified residues are conserved between δ and γ2L subunit. In the present study, we have clearly demonstrated that residues in the extracellular part of the TM2, where the δ and γ2L sequences are less conserved, play pivotal roles in controlling the rate of desensitization as well as the completion of desensitization. Of those divergent residues between δ and γ2L subunit in this area, a triple set of amino acids of γ2L subunit (STI280-282) has been shown to have the strongest influence in controlling fast desensitization and small non-desensitizing current (Table 3-6). Substitution of this triplet in the δ subunit (as mutation δMVS278-280STI) can completely convert the desensitization properties of α4β3δ receptor to that of α4β3γ2L. The roles of the individual residues within this triplet were further probed. We showed that the introduction of γ2 S280 and I282 residues into the δ subunit (as mutations δM278S and δS280I, respectively) resulted in fast rate desensitization, which is similar to the triplet mutation. However, their effects on the non-desensitizing currents are not as strong as the triplet mutation (Figure 3-6 E), indicating that the completion of

desensitization of wildtype α4β3γ2 receptor is more likely relies on an orchestrated action of three residues (γ2L STI280-282) rather than single residues. Interestingly, we observed "asymmetric" effects of δ residues in this area. None of the δ residues in this domain has an effect on the rate of desensitization. However, δ residue S280 has been identified as a structural determinant in controlling large non-desensitizing current (Table 3-6). These observations simply reflect differential roles of the equivalent δ and γ2 residues played in receptor desensitization.

The extracellular domain of TM2 has been suggested to be loosely packed and highly mobile (Horenstein *et al.*, 2001; Goren *et al.*, 2004) and it may undergo translational motion across the channel lumen in the absence and the presence of GABA (Horenstein *et al.*, 2005). Based on electron microscopy of the nACh receptor, which shares structural homology with $GABA_A$ receptors, it is suggested that agonist binding induces a rotation of extracellular domain, which in turn transduces a rotation of the extracellular part of TM2 domain, leading to the opening of channel gate (Miyazawa *et al.*, 2003). Although there is no clear structural model available for the receptor desensitization, it is likely that rotations of the extracellular end of TM2 domains of all subunits may also be required during the conformational changes of receptor desensitization. Therefore, it is not unexpected that those divergent residues in δ and γ2L subunits in this region play roles in controlling desensitization.

Our results from the TM1 and TM2 domain support the suggestion that separate domains modulate the desensitization process (Bianchi and Macdonald, 2001). How these two separate domains coordinate to affect desensitization remains to be investigated. Previously, using cysteine-scanning mutagenesis, it was shown that TM1 and TM2 of

nACh receptor may be interleaved at their extracellular ends (Akabas *et al.*, 1994; Akabas and Karlin, 1995). Based on a $GABA_A$ receptor model, Trudell and Bertaccini, (2004) indicated that the extracellular end of TM1 domain (before the conserved δP241/γ2P243, see Figure 3-1) intercalate with TM2 to form a tight channel and therefore that the ion pore is lined by residues contributed from both TM1 and TM2. It was suggested by Akabas and Karlin (1995) that the extracellular end of TM1 domain works in tandem with the TM2 domain to elicit the conformational changes of the gating process including activation, desensitization and deactivation. Thus, the residues we identified in the extracellular end TM1 and TM2 may contribute in a concerted fashion to the receptor desensitization.

Impaired Desensitization by Introducing γ2L Residues into the δ Subunit

A curious finding in our experiments is that introducing certain γ2L residues into the δ subunit resulted in slower weighted time constants and larger fractions of non-desensitizing current compared to those of the wildtype α4β3δ. Interestingly, when inspecting the positions of these residues, it was found that they are located in the proximity of the center of TM1 (δ AM247-248) and TM2 (δ V279) domains. It has been suggested that there are potential interactions of residues in the middle of TM2 domain with residues of TM1 domain and that these interactions are likely to have effects on relative movements of these two transmembrane domains during the gating (Miyazawa *et al.*, 2003). Based on these suggestions, one possible explanation for our results is that introduction of these γ2L residues may break the original interactions in the middle part of TM1 and TM2 in the wildtype δ subunit and the relative movements of TM1 and TM2

in this region are likely disrupted, which in turn lead to an impaired "super-slow" desensitization. In the future, introducing δ residues into γ2L subunit and additional mutations on both subunits with amino acids of different size, polarity and hydrophobicity will clarify the roles of these residues in the regulation of desensitization.

Conclusion

In this chapter, we set out to investigate the structural determinants in the δ and γ2L subunits of the $GABA_A$ receptor that influence receptor desensitization. To this end we have clearly demonstrated that:

(1) δ residues V233 in TM1, S280 in TM2 and;

(2) γ2L residues Y235, T237 in TM1 and a triplet STI280-282, K285, KV289-290, YV292-293 in TM2

play differential roles in controlling the rate of desensitization and the non-desensitizing current.

Figure 3-1. Sequence alignment of the δ and γ2L subunits containing the TM1 and TM2, according to Ernst *et al*., (2005). The positions of the chimeric crossover points (χ232, χ237, χ241, χ244, χ246, χ255 and χ277) are shown. Identical residues shared by these subunits are shaded. The underlined residues indicate the position where the substitution mutations in the δ and γ2L subunits were constructed. Brackets indicate the domains implicated in controlling receptor desensitization (see Results for details).

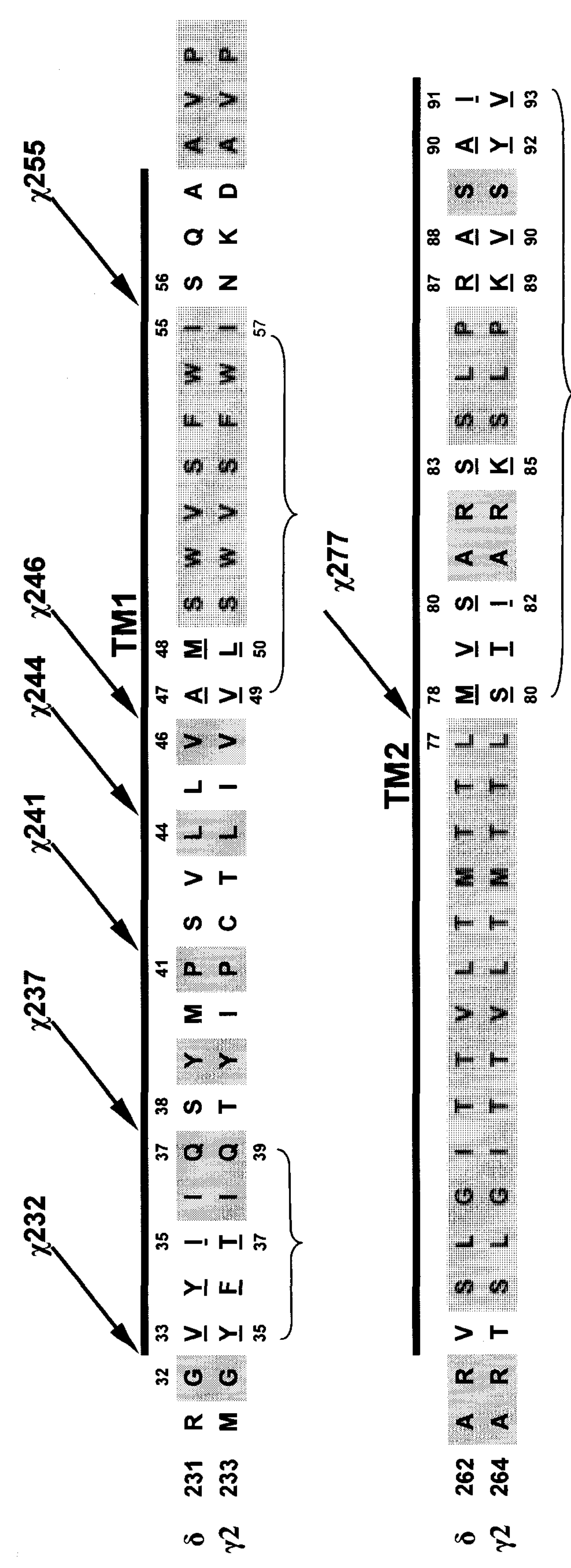
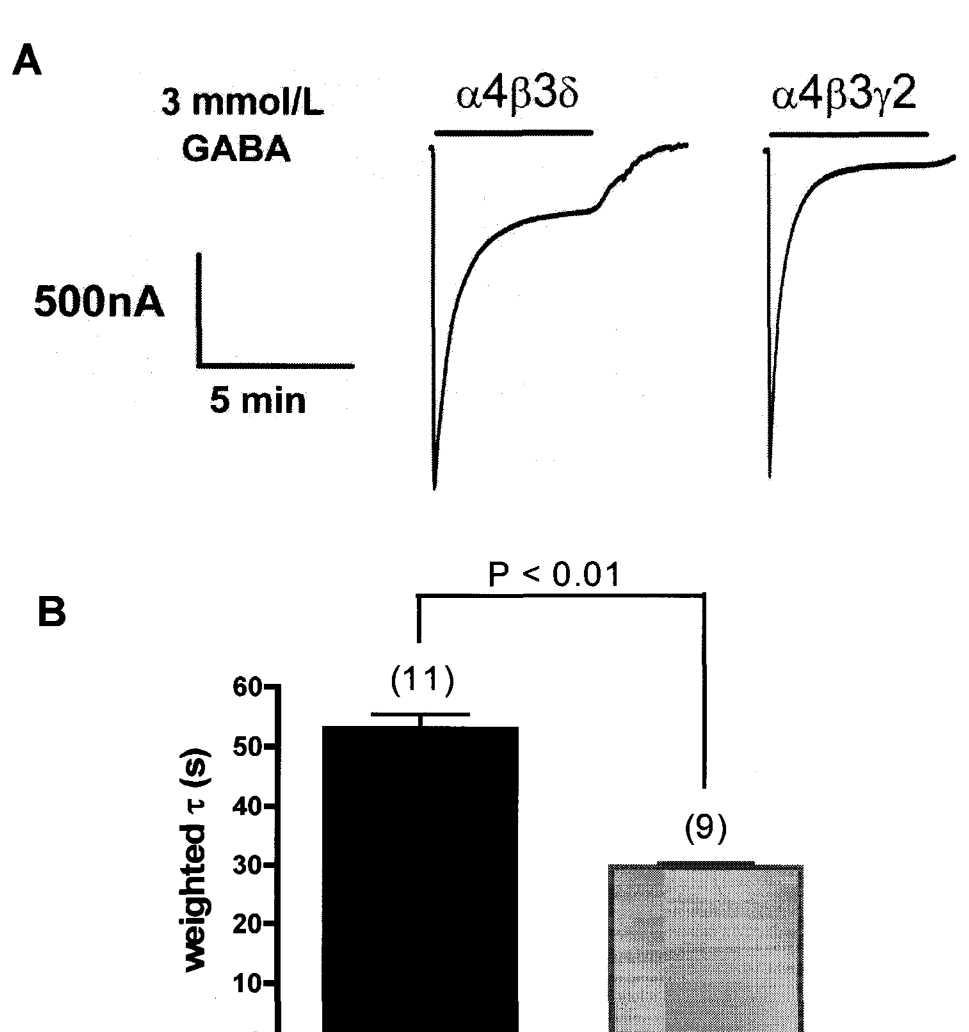

χ232
χ237
χ241
χ244
χ246
χ255
χ277
TM1
TM2
δ
γ2

Figure 3-2. Desensitization profiles of the wildtype α4β3δ and α4β3γ2 receptors. (A) Representative traces of GABA-evoked currents on different subtypes of $GABA_A$ receptors. In the experiments, a saturating concentration (3 mmol/L) of GABA was applied via gravity perfusion for 5 minutes. (B) A comparison of weighted time constants of these subtypes. (C) A comparison of the fractions of non-desensitizing currents of these subtypes. Data represent the mean ± SEM. The values in parentheses are the number of oocytes used for each receptor subtype.


A
3 mmol/L
GABA
α4β3δ
α4β3γ2
500nA
5 min
B
P < 0.01
(11)
(9)
weighted τ (s)
60
50
40
30
20
10
0
α4β3δ
α4β3γ2

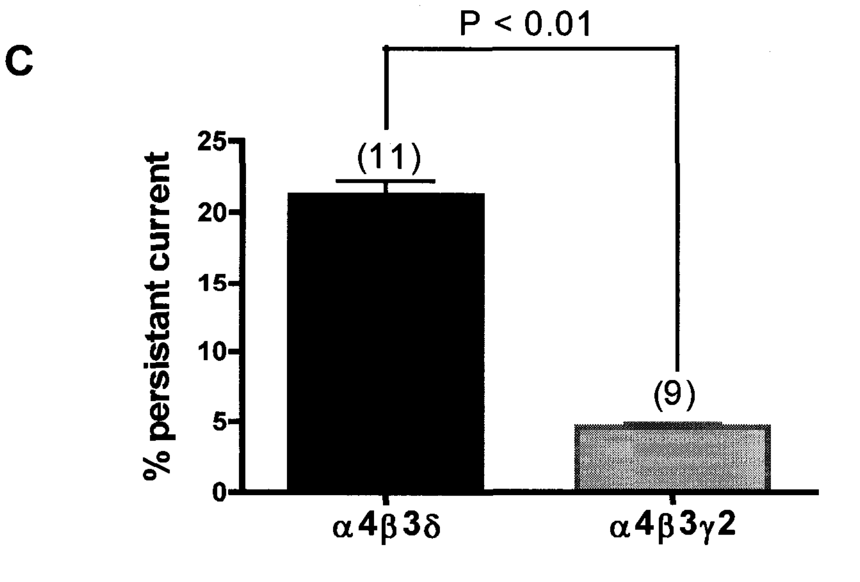
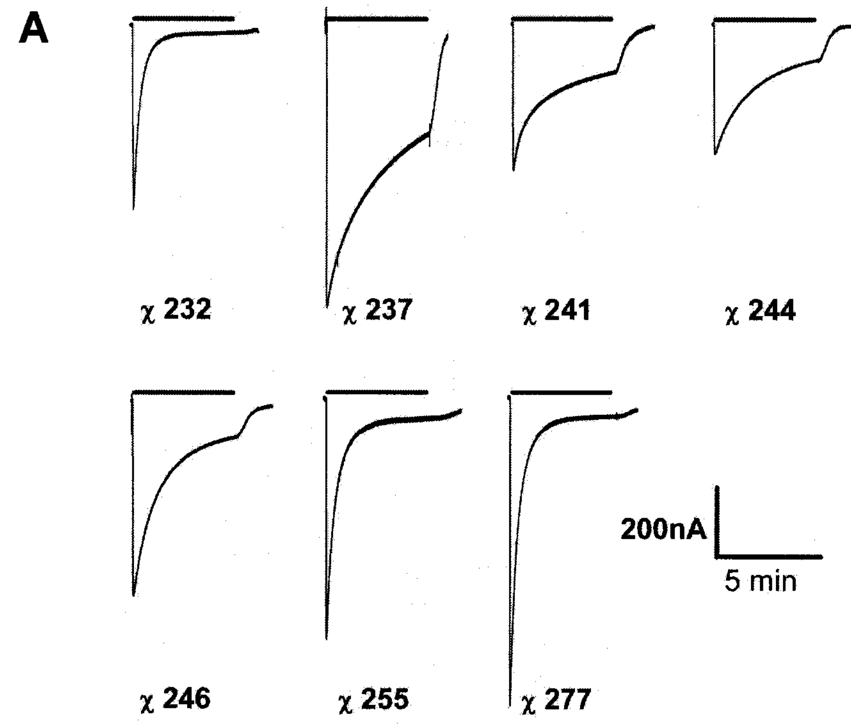

C
P < 0.01
(11)
(9)
% persistant current
25
20
15
10
5
0
α4β3δ
α4β3γ2

Figure 3-3. Desensitization properties of χ232-, χ237-, χ241-, χ244-, χ246-, χ255-, χ277-containing receptors. (A) Representative traces of GABA-evoked currents on different chimeric $GABA_A$ receptors. (B) A comparison of weighted time constants of chimeric subtypes with those of wildtype α4β3δ and α4β3γ2 receptors. (C) A comparison of the fractions of non-desensitizing currents of chimeric subtypes with those of wildtype α4β3δ and α4β3γ2 receptors. Data represent the mean ± SEM. The values in parentheses are the number of oocytes used for each receptor subtype. ## $P<0.01$ compared to wildtype α4β3γ2 receptor. ** $P<0.01$ compared to wildtype α4β3δ receptor. * $P< 0.05$ compared to wildtype α4β3δ receptor


A
χ 232
χ 237
χ 241
χ 244
χ 246
χ 255
χ 277
200nA
5 min

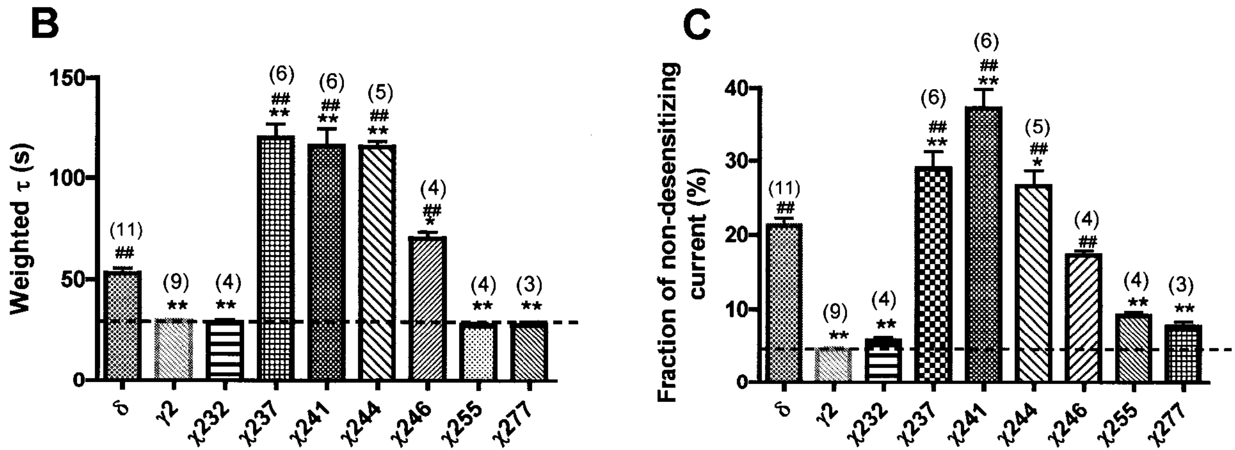
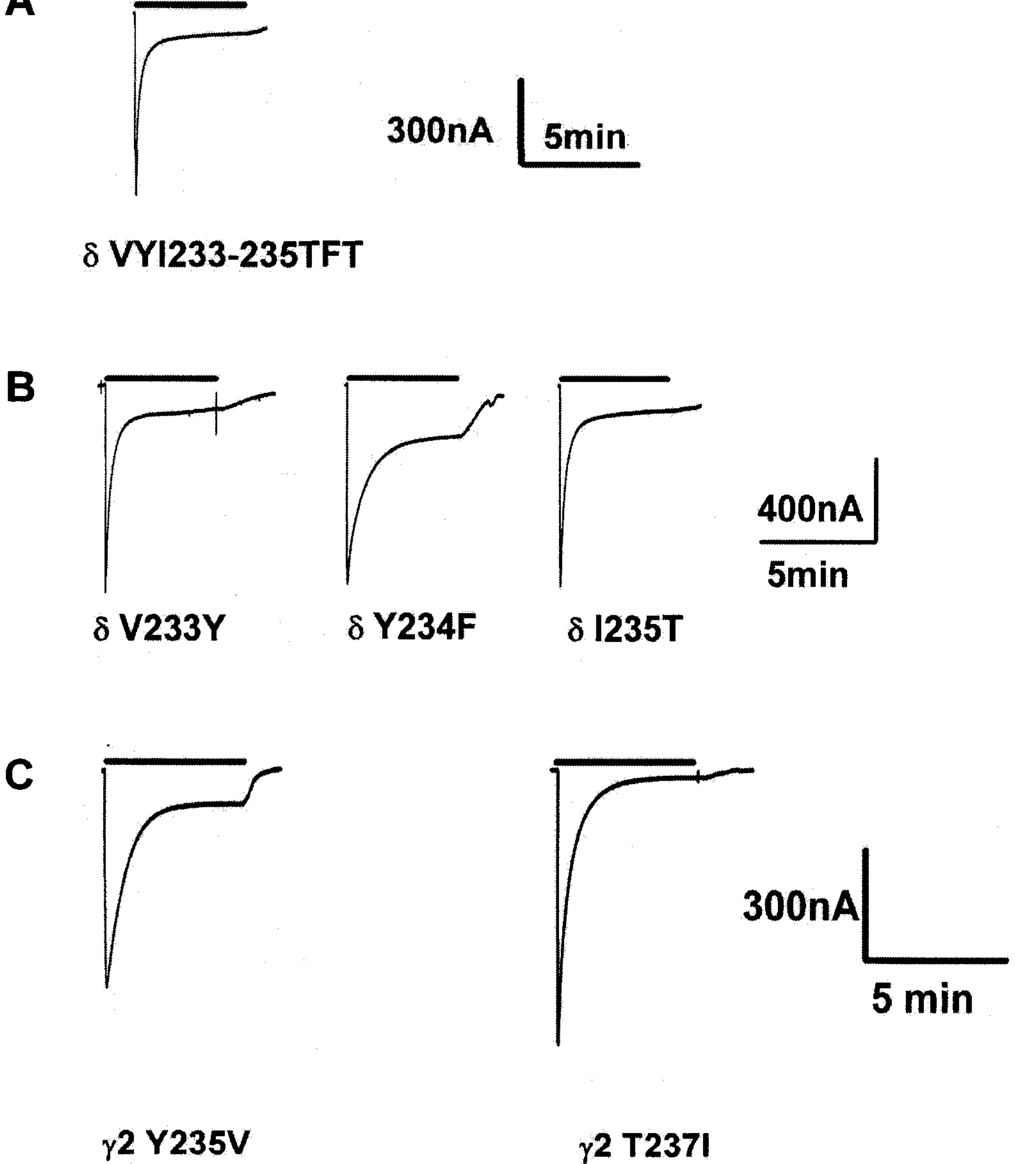
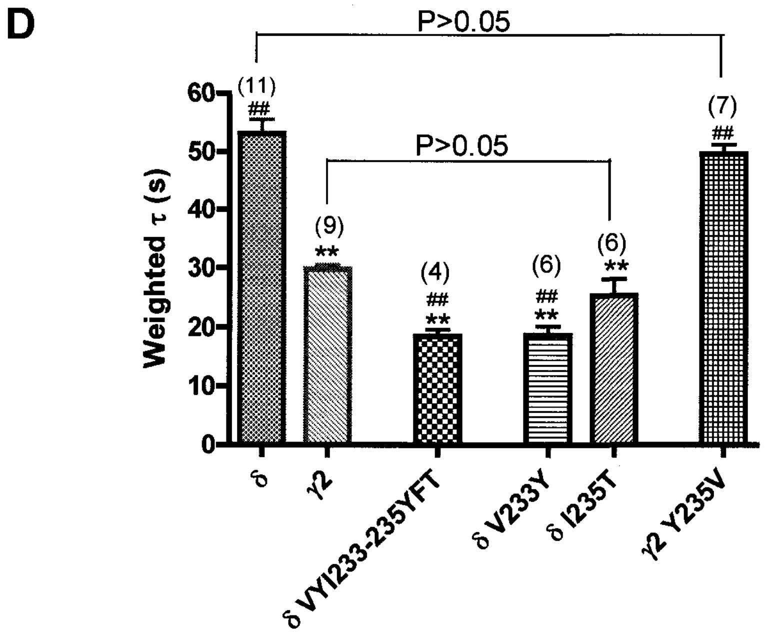

B
Weighted τ (s)
150
100
50
0
(11)
##
(9)
**
(4)
**
(6)
##
**
(6)
##
**
(5)
##
**
(4)
##
*
(4)
**
(3)
**
δ
γ2
χ232
χ237
χ241
χ244
χ246
χ255
χ277
C
Fraction of non-desensitizing current (%)
40
30
20
10
0
(11)
##
(9)
**
(4)
**
(6)
##
**
(6)
##
**
(5)
##
*
(4)
##
(4)
**
(3)
**
δ
γ2
χ232
χ237
χ241
χ244
χ246
χ255
χ277

Figure 3-4. Effects of substitution mutations in the extracellular domain of TM1 of the δ and γ2L subunits. (A) Representative trace of δVYI233-235YFT-containing receptor, showing a fast desensitization profile. Subsequently, single mutations δV233Y, δY234F and δI235T (B) were constructed and co-expressed with α4 and β3 subunits. Mutations δV233Y and δI235T resulted in dramatic changes in desensitization properties. Finally the effects of their equivalent mutations on the γ2L subunit were investigated by constructing mutations γ2Y235V and γ2T237I, respectively (C). (D) A comparison of weighted time constants of these mutations with those of wildtype α4β3δ and α4β3γ2L receptors. (E) A comparison of the fractions of non-desensitizing currents of these mutations with those of wildtype α4β3δ and α4β3γ2 receptors. Data represent the mean ± SEM. The values in parentheses are the number of oocytes used for each receptor subtype. * $P<0.05$ compared to wildtype α4β3δ receptor. ** $P<0.01$ compared to wildtype α4β3δ receptor. $^{\#\#}$ $P<0.01$ compared to wildtype α4β3γ2 receptor. Note: δY234F- and γ2T237I-containing receptors do not have significant changes of desensitization properties from their respective wildtype receptor. Therefore, data of δY234F and γ2T237I are omitted in (D) and (E) for clarity.


A
300nA
5min
δ VYI233-235TFT
B
400nA
5min
δ V233Y
δ Y234F
δ I235T
C
300nA
5 min
γ2 Y235V
γ2 T237I


D
P>0.05
P>0.05
Weighted τ (s)
60
50
40
30
20
10
0
(11)
##
(9)
**
(4)
##
**
(6)
##
**
(6)
**
(7)
##
δ
γ2
δ VYI233-235YFT
δ V233Y
δ I235T
γ2 Y235V

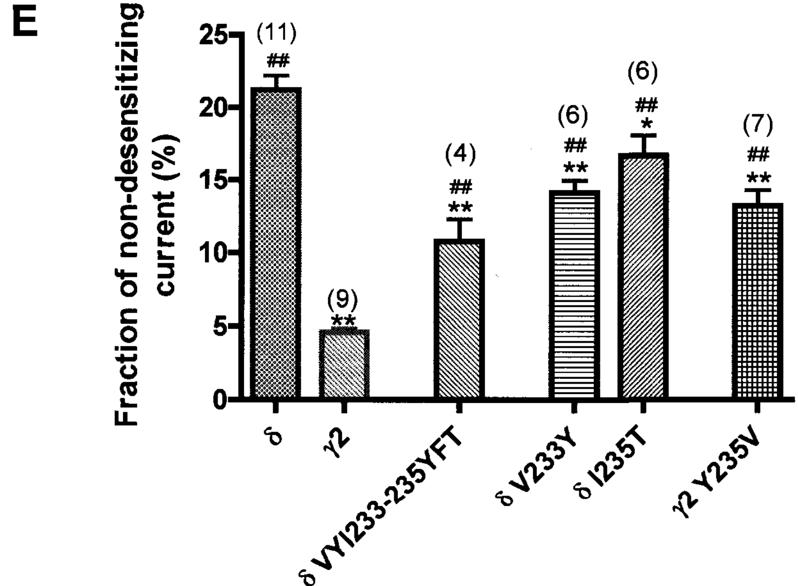

E
Fraction of non-desensitizing current (%)
25
20
15
10
5
0
(11)
##
(9)
**
(4)
##
**
(6)
##
**
(6)
##
*
(7)
##
**
δ
γ2
δ VYI233-235YFT
δ V233Y
δ I235T
γ2 Y235V

Figure 3-5. Effects of substitution mutations in the intracellular end of the TM1 domain of δ and γ2L subunits. (A) Representative trace of GABA-evoked current on δAM247-248VL-containing receptor. (B) A comparison of weighted time constant of δAM247-248VL-containing receptor with those of wildtype α4β3δ and α4β3γ2 receptors. (C) A comparison of the fraction of non-desensitizing current of δAM247-248VL-containing receptor with those of wildtype α4β3δ and α4β3γ2 receptors. Data represent the mean ± SEM. The values in parentheses are the number of oocytes used for each receptor subtype. ## $P<0.01$ compared to wildtype α4β3γ2 receptor. ** $P<0.01$ compared to wildtype α4β3δ receptor.

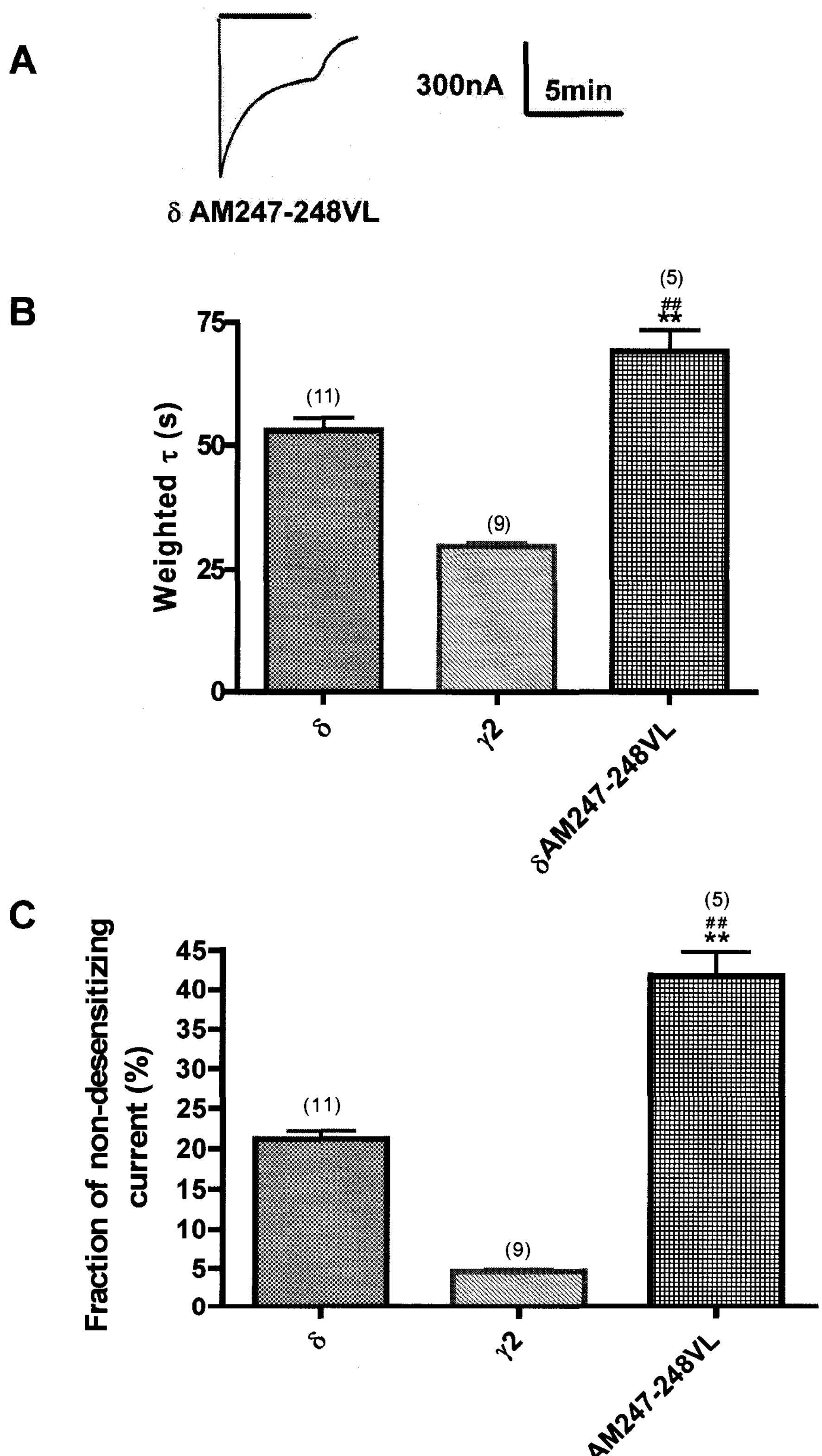
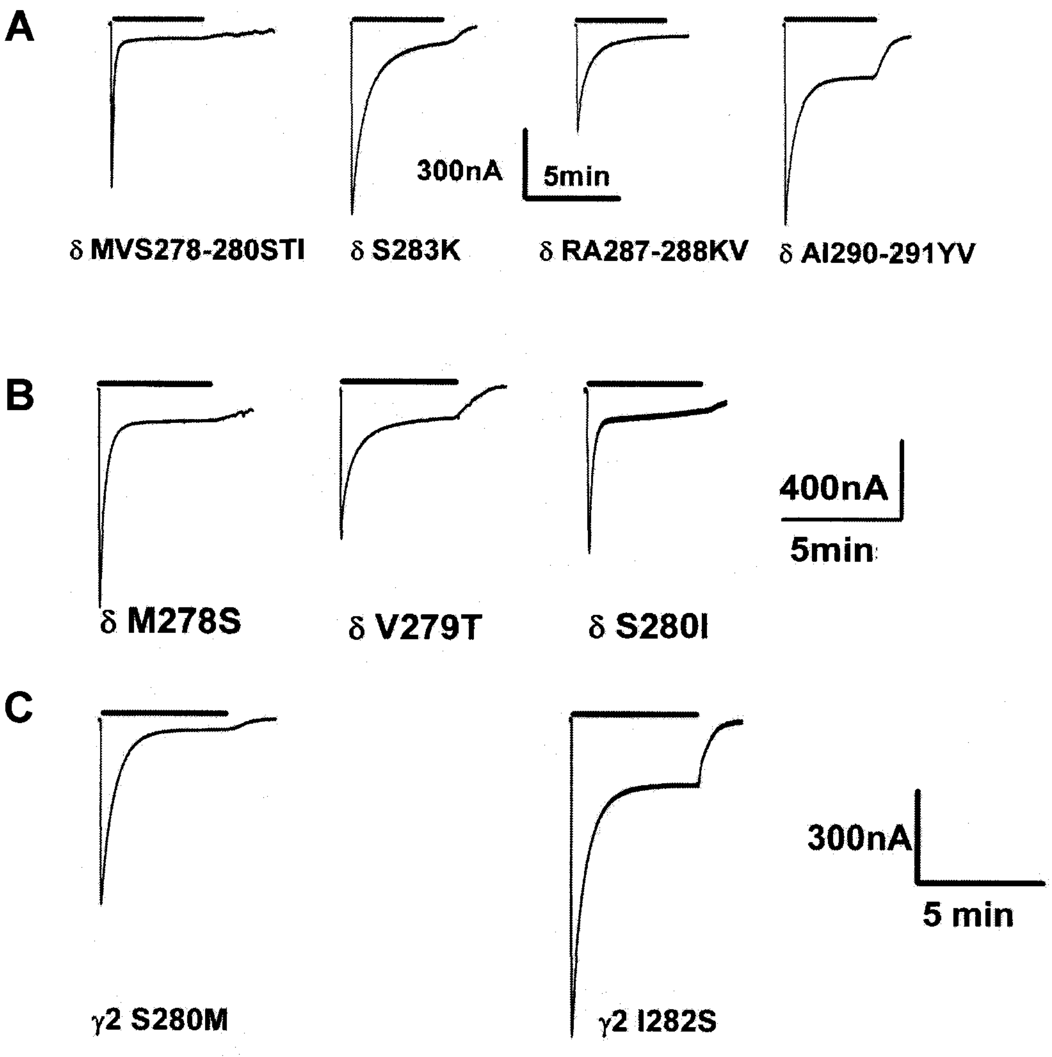
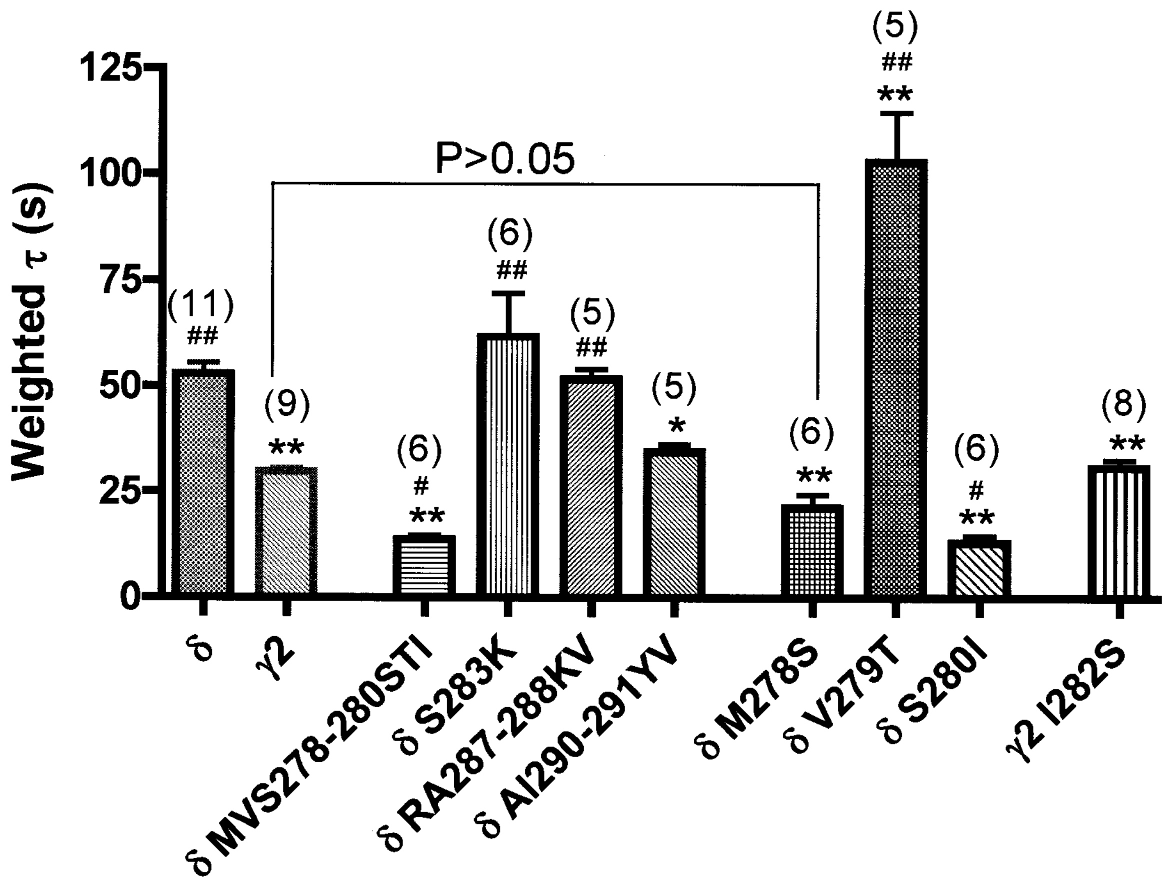

A
300nA
5min
δ AM247-248VL
B
Weighted τ (s)
75
50
25
0
(11)
(9)
(5)
##
**
δ
γ2
δAM247-248VL
C
Fraction of non-desensitizing current (%)
45
40
35
30
25
20
15
10
5
0
(11)
(9)
(5)
##
**
δ
γ2
δ AM247-248VL

Figure 3-6. Effects of substitution mutations in the extracellular end of TM2 of the δ and γ2L subunits. (A) Representative trace of δMVS278-280STI, δS283K, δRA287-288KV and δAI290-291YV-containing receptors. In further studies, single mutations δM278S, δV279T and δS280I (B) were constructed and co-expressed with α4 and β3 subunits. δM278S- and δS280I-containing receptors have fast desensitization properties, whereas δV279T-containing receptors has significant slower desensitization compared to the wildtype α4β3δ. (C) Finally, the effects of the equivalent residues of δ subunit on controlling receptor desensitization were investigated by constructing mutations γ2S280M and γ2I282S. (D) A comparison of weighted time constants of these mutations with those of wildtype α4β3δ and α4β3γ2L receptors. (E) A comparison of the fractions of non-desensitizing currents of these mutations with those of wildtype α4β3δ and α4β3γ2 receptors. Data represent the mean ± SEM. The values in parentheses are the number of oocytes used for each receptor subtype. ** $P<0.01$ compared to wildtype α4β3δ receptor. $^{\#\#}$ $P<0.01$ compared to wildtype α4β3γ2 receptor. Note: Mutation of γ2S280M does not result in significant changes of desensitization properties from wildtype γ2L-containing receptor. Therefore, data of γ2S280M is omitted in (D) and (E) for clarity.


A
300nA
5min
δ MVS278-280STI
δ S283K
δ RA287-288KV
δ AI290-291YV
B
400nA
5min
δ M278S
δ V279T
δ S280I
C
300nA
5 min
γ2 S280M
γ2 I282S


D
Weighted τ (s)
125
100
75
50
25
0
P>0.05
(11)
##
(9)
**
(6)
#
**
(6)
##
(5)
##
(5)
*
(6)
**
(5)
##
**
(6)
#
**
(8)
**
δ
γ2
δ MVS278-280STI
δ S283K
δ RA287-288KV
δ AI290-291YV
δ M278S
δ V279T
δ S280I
γ2 I282S

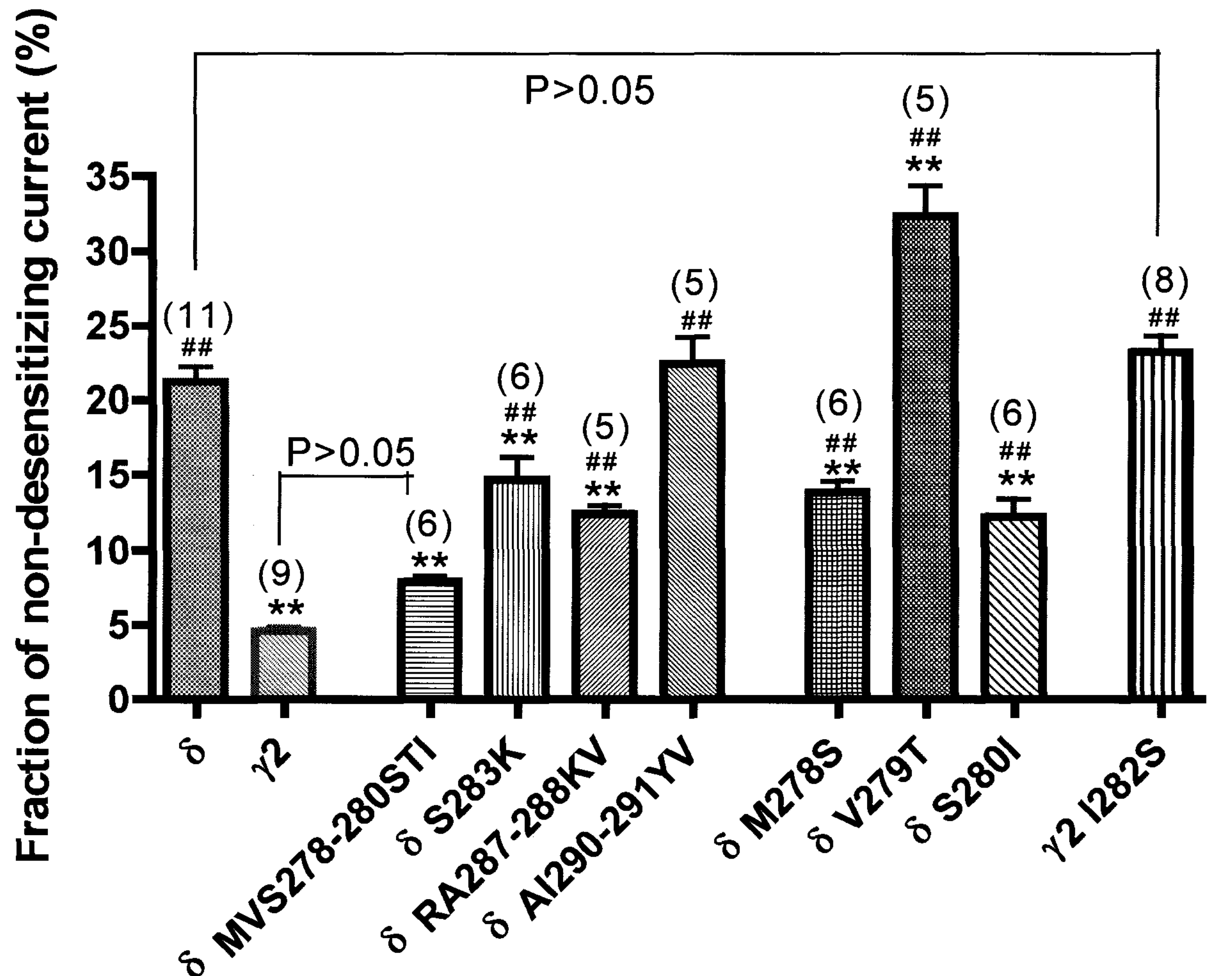

E
Fraction of non-desensitizing current (%)
P>0.05
P>0.05
35
30
25
20
15
10
5
0
(11)
##
(9)
**
(6)
**
(6)
##
**
(5)
##
**
(5)
##
(6)
##
**
(5)
##
**
(6)
##
**
(8)
##
δ
γ2
δ MVS278-280STI
δ S283K
δ RA287-288KV
δ AI290-291YV
δ M278S
δ V279T
δ S280I
γ2 I282S

Table 3-1. Summary of desensitization kinetics of wildtype α4β3δ and α4β3γ2L receptors. τ_f and τ_s are the fast and slow time constants. $\%A_f$ and $\%A_s$ are the relative amplitude contributions of fast and slow desensitization. τ_w is the weighted time constant. %C is the fraction of non-desensitizing current (see Materials and Methods). Data are presented as mean ± SEM. *n* indicates the number of oocytes used for each receptor subtype. ** indicate significant differences from α4β3δ, $P< 0.01$

Receptor subtype	τ_f (s)	% A_f	τ_s (s)	% A_s	τ_w (s)	%C	*n*
α4β3δ	16.7±0.9	32.8±2.2	72.9±3.7	67.2±2.2	53.0±2.5	21.2±1.0	11
α4β3γ2L	10.4±0.6	38.8±4.3	36.7±0.9	61.2±4.3	29.8±0.8**	4.6±0.3**	9

Table 3-2. Summary of desensitization kinetics of chimeric receptors. Data are presented as mean ± SEM. *n* indicates the number of oocytes used for each receptor subtype. ** indicates significant differences from α4β3δ, $P < 0.01$; * indicates significant differences from α4β3δ, $P < 0.05$; ## indicates significant differences from α4β3γ2L, $P<0.01$; # indicates significant differences from α4β3γ2L, $P<0.05$.

Receptor subtype	τ_f (s)	% A_f	τ_s (s)	% A_s	τ_w (s)	%C	*n*
χ232	12.9±0.5	40.3±2.8	38.4±1.6	59.7±2.8	29.1±1.5**	5.8±0.4**	4
χ237	24.0±1.6	29.6±2.7	159.4±7.5	70.4±2.7	120.1±7.2$^{**##}$	29.0±2.2$^{**##}$	6
χ241	21.6±0.7	28.9±2.9	157.2±7.9	71.1±2.9	116.3±8.6$^{**##}$	37.3±2.6$^{**##}$	6
χ244	33.9±1.9	21.4±1.9	141.6±3.9	78.6±1.9	115.8±2.8$^{**##}$	26.6±2.1$^{*##}$	5
χ246	27.6±1.6	35.0±1.2	93.4±3.8	65.0±1.2	70.4±3.3$^{*##}$	17.3±1.0$^{##}$	4
χ255	11.6±0.6	32.7±3.1	35.9±1.4	67.3±3.1	27.9±10.5**	8.9±3.8**	4
χ277	9.4±0.9	38.1±4.4	39.0±1.3	61.9±4.4	28.2±1.6**	7.8±1.0**	3

Table 3-3. Summary of desensitization kinetics of mutants in the extracellular part of TM1 (δV233/γ2Y235 -δQ237/γ2Q239) domain of δ and γ2L subunits. Data are presented as mean ± SEM. *n* indicates the number of oocytes used for each receptor subtype. ** indicates significant differences from α4β3δ, P< 0.01; * indicates significant differences from α4β3δ, P< 0.05; ## indicates significant differences from α4β3γ2L, P<0.01; # indicates significant differences from α4β3γ, P<0.05. [a] denoted the δ mutations with no difference from wildtype α4β3γ2L receptor, P>0.05. [b] denoted the γ2L mutations with no difference form wildtype α4β3δ receptor, P>0.05.

Receptor subtype	τ_f(s)	% A_f	τ_s (s)	% A_s	τ_w (s)	%C	*n*
δ VYI233-235YFT	8.0±0.5	79.1±1.9	47.6±4.8	20.9±1.9	18.5±1.1**##	10.8±1.5**	4
δ V233Y	9.1±1.2	66.2±7.2	38.0±10.6	33.7±7.3	18.7±1.5**##	14.1±0.9**	6
δ Y234F	13.9±2.3	24.9±4.9	62.6±4.5	75.1±4.9	49.3±1.4	25.0±1.5	6
δ I235T	12.9±2.0	53.5±11.3	63.2±1.4	46.5±11.3	25.4±2.8**[a]	16.7±1.4*	6
γ2 Y235V	16.6±1.8	24.0±3.1	50.6±2.5	76.0±3.1	49.7±1.5## [b]	13.2±1.1##	7
γ2 T237I	15.2±1.0	56.2±6.4	51.9±9.6	43.8±6.4	29.1±2.5	5.1±0.7	4

Table 3-4. Summary of desensitization kinetics of mutants in domain δS238/γ2T240 – δI255/γ2I257 of δ subunit. Data are presented as mean ± SEM. *n* indicates the number of oocytes used for each receptor subtype. ** indicates significant differences from α4β3δ, $P < 0.01$.

Receptor subtype	τ_f(s)	% A_f	τ_s (s)	% A_s	τ_w (s)	%C	*n*
δ AM247-248VL	9.7±1.7	12.3±0.1	77.5±4.6	87.7±0.1	69.2±4.2**	41.8±3.1**	5

Table 3-5. Summary of desensitization kinetics of mutants in the extracellular part of TM2 (δM278/γ2S280 - δI291/γ2V293) of δ and γ2L subunits. Data are presented as mean ± SEM. *n* indicates the number of oocytes used for each receptor subtype. ** indicates significant differences from α4β3δ, P< 0.01; ## indicates significant differences from α4β3γ2L, P<0.01. [a] denoted the δ mutations with no difference from wildtype α4β3γ2L receptor, P>0.05. [b] denoted the γ2L mutations with no difference form wildtype α4β3δ receptor, P>0.05.

Receptor subtype	τ_f(s)	% A_f	τ_s(s)	% A_s	τ_w(s)	%C	*n*
δ MVS278-280STI	9.1±0.4	86.7±1.3	45.1±4.3	13.3±1.3	13.7±0.9**	8.0±0.3**[a]	6
δ S283K	20.0±1.2	39.1±2.7	71.9±6.5	60.9±2.7	61.8±10.3	14.7±1.5**	6
δ RA287-288KV	19.2±0.8	50.7±2.3	85.8±4.6	49.3±2.3	51.8±2.4	12.4±0.6**	5
δ AI290-291YV	12.0±1.8	29.3±4.6	43.7±3.4	70.7±4.6	34.1±1.8*	22.5±1.8	5
δ M278S	10.6±0.5	83.4±1.3	54.2±10.8	16.6±1.8	21.2±3.0**[a]	13.9±0.8**	6
δ V279T	17.4±1.1	31.5±1.7	118.5±8.0	68.5±1.7	102.9±11.7**	32.3±2.0**	5
δ S280I	7.8±0.3	85.7±3.9	52.2±8.0	14.3±3.9	13.0±1.5**	12.2±1.2**	6
γ2 S280M	11.2±3.3	41.8±1.7	41.3±5.4	58.2±1.7	29.0±1.6	7.6±0.9	4
γ2 I282S	18.1±1.8	61.3±8.0	59.3±8.9	38.7±8.0	30.9±1.9	23.2±1.1## [b]	8

Table 3-6. Summary of the effects of δ and γ2L residues on the rate of desensitization and non-desensitizing current. + denotes that residues are the structural determinants for a fast desensitization. – denotes that residues are the structural determinants for a slow desensitization. ↑↑ and ↑ denote that residues are the structural determinants for large fraction of non-desensitizing current. ↓↓ and ↓ denote that residues are the structural determinants for small fraction of non-desensitizing current. N.E denotes that residues do not have significant effects on the rate of desensitization and/or the fraction of non-desensitizing current.

	δ residues			γ2L residues	
	rate of desensitization	non-desen. current		rate of desensitization	non-desen. current
TM1 domain					
δ V233	–	↑	γ2 Y235	+	↓
			γ2 T237	+	↓
TM2 domain					
			γ2 STI280-282	+	↓↓
			γ2 S280	+	↓
δ S280	**N. E**	↑↑	γ2 I282	+	↓
			γ2 K285	**N. E**	↓
			γ2 KV289-290	**N. E**	↓
			γ2 YV292-293	+	**N. E**

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Birnir B, Tierney ML, Lim M, Cox GB and Gage PW. (1997b). Nature of the 5' residue in the M2 domain affects function of the human alpha 1 beta 1 $GABA_A$ receptor. *Synapse.* **26,** 324-327.

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CHAPTER 4[1]

Benzodiazepine Modulation of the Rat $GABA_A$ Receptor $\alpha 4\beta 3\gamma 2L$ Subtype Expressed in *Xenopus* Oocytes

[1]Ms. Isabelle M. Paulsen contributed to the electrophysiological study of diazepam modulation of $GABA_AR$ $\alpha 4\beta 3\gamma 2L$ subtype.

INTRODUCTION

Since their introduction in the 1960s, the benzodiazepines have been prescribed widely as effective anxiolytics, hypnotics and muscle relaxants (see Korpi *et al.*, 1997 for review). Benzodiazepines act as allosteric modulators of the γ-aminobutyric acid type A ($GABA_A$) receptor, the major inhibitory neurotransmitter receptor in the mammalian brain. The $GABA_A$ receptor belongs to the Cys-loop family of ligand-gated ion channel that includes the nicotinic, serotonin type 3, and glycine receptors (Sieghart *et al.*, 1999). Each receptor in the family is a pentamer of homologous subunits that assemble to form a central ion channel. The anion-selective $GABA_A$ receptors are heterogeneous, and nineteen mammalian $GABA_A$ receptor subunits have been identified to date i.e., α1-6, β1-3, γ1-3, ρ1-3, π, δ, ε and θ (McKernan and Whiting 1996; Barnard *et al.*, 1998). The major receptor isoform in the mammalian brain is the α1β2γ2 combination in a likely stoichiometry of 2:2:1 (Baumann *et al.*, 2001; Baumann *et al.*, 2002, Farrar *et al.*, 1999) with an anti-clockwise arrangement of α–β–α–γ–β when viewed from the synaptic cleft (Baumann *et al.*, 2002).

The pharmacology of benzodiazepines is determined mainly by the particular α subunit isoform that is present in the pentamer. $GABA_A$ receptors containing α1, α2, α3 or α5, along with a β and γ subunit, are sensitive to classical benzodiazepines, such as diazepam and flunitrazepam. In contrast, receptors containing the α4 or α6 subunits are generally thought to be "diazepam-insensitive" although they retain high affinity for the imidazobenzodiazepines, Ro15-4513 and Ro15-1788 (Derry, *et al.*, 2004, Knoflach, *et al.*, 1996). The results of many biochemical and mutagenesis studies (Wieland *et al.*, 1992; Duncalfe *et al.*, 1996) have attributed this diazepam sensitivity primarily to a single

amino acid substitution in the diazepam-sensitive α subunits i.e., a histidine at position H101 (rat α1 subunit numbering) to an arginine in the equivalent position of the α4 or α6 subunits.

There is abundant evidence to suggest that the high affinity benzodiazepine site lies at the α–γ subunit interface (see Sigel and Buhr, 1997) and, not surprisingly, the γ subunit has been shown to play a major role in benzodiazepine binding. Replacement of the γ2 subunit in α4β3γ2 subtype by the δ subunit was shown to abolish benzodiazepine binding to recombinant receptors expressed in mammalian cells (Brown *et al.*, 2002). Receptors containing the α4-subunit represent a small percentage of the total $GABA_A$ receptor population in the mammalian brain. The α4 subunit is colocalized with γ2 subunits in the hippocampus and thalamus (Sur *et al.*, 1999) and appears to play an important role in epileptogenesis. Animal models of epilepsy suggest that there is a consistent up-regulation of the α4βγ2 subtype in dentate granule cells and that this parallels a decreased expression of the α4βδ subtype (Payne *et al.*, 2006; Peng *et al.*, 2004).

In the present study, we have re-examined the interactions of benzodiazepines with the rat α4β3γ2L subtype expressed in both *Xenopus* oocytes and HEK 293 cells. We show that, at nanomolar concentrations, classical benzodiazepines can significantly potentiate GABA currents mediated by the α4β3γ2L receptor expressed in oocytes and that this potentiation is inhibited by Ro15-1788 and ZK93426. However, consistent with previous reports (Ebert *et al.*, 1996; Scholze *et al.*, 1996), we did not detect high affinity binding of diazepam or flunitrazepam to this subtype expressed in HEK 293 cells. These

results suggest that benzodiazepine modulation of the recombinant α4β3γ2L subtype may be an oocyte-specific phenomenon.

MATERIALS AND METHODS

Chemicals

[^{3}H]Ro15-4513 was purchased from NEN Life Science Products (Boston, MA). GABA and flunitrazepam were obtained from Sigma-Aldrich (St. Louis, MO). Diazepam was a gift from Dr. Glen Baker (Psychiatry, University of Alberta) and β-CCE from Dr Brian Jones (GlaxoSmithKline, Harlow, UK). Ro15-4513 and Ro 15-1788 were provided by Hoffman-La Roche and Co. (Basel, Switzerland), and ZK93423 and ZK93426 were gifts fom Schering. GABA was dissolved in frog Ringer's buffer (110 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES, pH 7.4) and the other modulators were dissolved in DMSO to make concentrated stock solutions. The final concentration of DMSO in all experiments was constant at 0.01% (v/v) and this concentration was shown to have no effect on GABA responses.

Clones

The original cDNAs encoding the rat α4, β1 and β3 subunits were from Dr P. H. Seeburg's laboratory, and those encoding the γ2L and β2 subunits were provided by Dr D. L. Weiss. All cDNAs were subcloned into the pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA) except for the β2 subunit cDNA which was subcloned into pcDNA3.1(-). To improve the expression levels of α4β3γ2L receptors in HEK293 cells,

the signal peptide sequence of β3 was substituted by that of β2. The coding regions of all subunit clones used in these studies were verified by sequencing.

Expression in *Xenopus* Oocytes and Two-Electrode Voltage Clamp Recordings

Capped cRNAs encoding rat $GABA_A$ receptor subunits were prepared as described (You and Dunn, 2007). Stage V-VI Xenopus laevis oocytes were prepared as described by Smith *et al.* (2004). Oocytes were injected with 50 nL of 1 μg/μL total subunit cRNA in a 1:1:1 ratio (α4:βx:γ2L). The injected oocytes were maintained in ND96 buffer (96 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES, pH 7.4) supplemented with gentamicin (1%, v/v) in 96-well plates at 14°C for at least 48 h prior to functional analysis.

Oocyte recordings were carried out using standard two-electrode voltage clamp techniques at a holding potential of −60 mV. Oocytes were continuously perfused with frog Ringer's solution (110 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES, pH 7.4). Currents were recorded using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA). The microelectrodes were filled with 3 mol/L KCl, and had a resistance between 0.5 - 3.0 MΩ. In studies of modulator effects, oocytes were pre-perfused with 10 ml of these ligands for ~2 min prior to activation of the receptor by perfusion with GABA at its EC_{20} concentration (or EC_{50} for β-CCE modulation of the α4β3γ2L subtype) and the same concentration of the modulator as used in the pre-perfusion. After each current measurment, oocytes were washed with buffer for > 15 min to ensure complete recovery from desensitization.

Transient Transfection and Cell Membrane Preparation

Human embryonic kidney (HEK-293) cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% bovine growth serum (Hyclone) at 37 °C in a 5% CO_2 incubator. Transient transfection was carried out using the calcium phosphate method described previously (Derry *et al.*, 2004, Hansen *et al.*, 2005). cDNAs encoding α4, β3, γ2L subunits were added in a 1:1:1 mass ratio to an appropriate volume of 250 mmol/L $CaCl_2$, followed by the addition of an equal volume of *N*, *N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) buffer (pH 7.04). The solutions were mixed well and added dropwise to the cells. 44 to 48 hours after transfection, cells were harvested by scraping into ice-cold harvesting buffer (50 mmol/L Tris, 250 mmol/L KCl, pH7.4) supplemented with benzamidine (1 mmol/L), bacitracin (0.1 mg/ml), trypsin inhibitor (0.01 mg/ml) and phenylmethylsulfonyl fluoride (0.5 mmol/L). The cells were then homogenized, and following centrifugation (Sorvall SS34 rotor, 18,000 rpm, 30 min), the pellets were resuspended in harvesting buffer and stored at -80°C.

Radioligand Binding Assays

Radioligand binding experiments were performed as described previously (Newell and Dunn 2002, Derry *et al.*, 2004). For saturation assays, cell homogenates were incubated in duplicate with increasing concentrations (1 - 80 nmol/L) of [^{3}H]Ro15-4513 in Tris-HCl buffer (50 mmol/L Tris, 250 mmol/L KCL, 0.02% NaN_3, pH 7.4) in a final volume of 0.5 ml at 4°C for 1 hour. Non-specific binding was determined in the presence of a high concentration (250 μmol/L) of unlabeled Ro15-4513. For competition

binding assays, membranes were incubated with a constant concentration of $[^3H]$Ro15-4513 at a concentration equal to its K_D value and increasing concentrations of unlabeled flunitrazepam or diazepam. Following incubation, the membrane preparations were filtered through GF/B filters (Whatman, Maidstone, UK) using a cell harvester (Brandel, Gaithersburg, MD) and washed twice with 3 ml aliquots of ice-cold Tris-HCl buffer. Filters were transferred to scintillation vials and subjected to scintillation counting (LS6500 Scintillation System, Beckman Instruments Inc., Fullerton, CA) after addition of 5 mL scintillation fluid.

Data and Statistical Analysis

All electrophysiological and radioligand binding data were analyzed by non-linear regression techniques using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

The potentiation of GABA-induced currents by modulators was compared to the current evoked by the application of a control concentration of GABA (at its EC_{20} or EC_{50} value as described above). The extent of modulation is expressed as a percentage of the control current using the following equation:

$$\text{Potentiation (\%)} = [(I_{GABA+modulator}/I_{GABA}) \times 100] - 100$$

Normalized data were then used to construct concentration-response curves using the equation:

$$P = (P_{max} + [L]^n)/(EC_{50}^n + [L]^n)$$

where P is the normalized the potentiation elicited by modulator; [L] is the modulator concentration, EC_{50} is the concentration of the modulator producing 50% of the maximal response (P_{max}), and n is the Hill coefficient.

In experiments estimating antagonist affinities, IC_{50} values were calculated from the equation:

$$I/I_{max} = [A]^n / ([A]^n + IC_{50}^n)$$

where IC_{50} is the concentration of antagonist, [A], that reduces the amplitude of benzodiazepine potentiation of GABA-evoked current by 50% and n is the Hill coefficient.

$[^3H]$Ro15-4513 binding data were fit by the equation:

$$B = B_{max}[R]/(K_D + [R])$$

where B is the amount of $[^3H]$Ro15-4513 specifically bound at concentration [R], B_{max} is the maximal binding at saturating concentration of $[^3H]$Ro15-4513 and K_D is the equilibrium dissociation constant.

K_I values of the unlabelled ligands from competition experiments (one site competition) with $[^3H]$Ro15-4513 were calculated using the Cheng-Prusoff (Cheng and Prusoff, 1973) equation:

$$K_I = IC_{50}/(1 + [R]/K_D)$$

LogEC_{50}, K_D and K_I values were calculated from at least three independent experiments and expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA and levels of significance were determined by multiple comparisons.

RESULTS

Benzodiazepine Modulation of the Rat α4β3γ2L $GABA_A$ Receptor Expressed in *Xenopus* Oocytes

We have previously reported that GABA activation of the recombinant α4β3γ2L $GABA_A$ receptor expressed in oocytes is characterized by an $EC_{50} \approx 27.6$ μmol/L and a Hill slope of about 0.91 (You and Dunn, 2007, Chapter 2). We have now investigated the functional effects of two classical benzodiazepine agonists, diazepam and flunitrazepam, on this receptor subtype. Following perfusion with these agents (see Methods), the response to a challenge concentration of GABA was potentiated in a concentration-dependent manner (Figures 4-1, A, C, D and Table 4-1). The estimated EC_{50} values for this potentiation were 12.3 nmol/L for diazepam and 2.25 nmol/L for flunitrazepam. Using an EC_{20} concentration of GABA, the maximal potentiation induced by diazepam (101.5%) was higher than the flunitrazepam potentiation (70.3%).

We also investigated diazepam modulation of the α4β1γ2L and α4β2γ2L receptor subtypes (Figure 4-1, B). No significant effect of diazepam on these receptors was observed, suggesting that the presence of the β3 subunit is an important determinant of benzodiazepine effects on the α4βxγ2L receptor.

To further characterize benzodiazepine modulation of the α4β3γ2L receptor, we investigated other benzodiazepine site ligands (Figure 4-2, see Chapter 1). These included Ro15-4513 and β-CCE (negative modulators of the most abundant α1β2γ2 subtype), in addition to ZK93423 (a partial positive modulator of the α1β2γ2 subtype). These compounds all displayed some positive modulation of GABA-evoked currents on the α4β3γ2L receptor. The data presented in Table 4-1 show that the EC_{50}s for Ro15-

4513, β-CCE and ZK93423 were 62.7, 891.3 and 5.87 nmol/L respectively. The maximal levels of potentiation by Ro15-4513 (26.1%), β-CCE (19.3%) and ZK93423 (18.3%) were similar.

Inhibition of Diazepam and Flunitrazepam Potentiation by Ro15-1788 and ZK93426

To investigate the pharmacological specificity of the potentiation of GABA-evoked currents described above, we also examined the effects of two drugs (Ro15-1788 and ZK93426) that are generally considered to be antagonists acting at the benzodiazepine binding site. Consistent with previous reports (Kelly *et al.*, 2002), the direct effects of Ro15-1788 on the α4β3γ2L receptor were not significant (Figure 4-3, A). However, in competition experiments (Figure 4-4, A and Table 4-2), inclusion of Ro15-1788 in the benzodiazepine pre-perfusion inhibited the potentiating effects of 100 nM diazepam and flunitrazepam with estimated IC_{50} values of 7.9 nmol/L and 8.7 nmol/L, respectively. The effects of ZK93426 acting on the α4β3γ2L subtype were more complicated. At high concentrations (> 30 nmol/L), ZK93426 caused a potentiation of GABA-evoked currents (Figure 4-3, B). The effects of ZK93426 on diazepam- and flunitrazepam-induced potentiation were biphasic (Figure 4-4, B). Low concentrations (< 100 nmol/L) inhibited the effects of diazepam and flunitrazepam but potentiation by the latter ligands was restored at higher concentrations of ZK93426.

Radioligand Binding Studies of Receptors Expressed in HEK 293 Cells

The *Xenopus* oocyte expression system has limited usefulness for direct characterisation of the binding properties of recombinant receptors using radiolabelled

ligand binding techniques. The α4β3γ2L receptor subtype was, therefore, also expressed in HEK 293 cells to facilitate such analysis. Initial problems of poor expression of this receptor subtype were overcome by substituting the signal peptide sequence of the rat $GABA_A$ receptor β3 subunit with that of β2 (Derry, *et al.*, 2004). In equilibrium binding assays, [^{3}H]Ro15-4513 was shown to bind with high affinity (K_D of 4.3 ± 0.4 nmol/L, Figure 4-5, inset) which is consistent with previous results (Derry *et al*, 2004). Therefore, we used [^{3}H]Ro15-4513 as a reporter ligand in competition studies to estimate the affinities for diazepam and flunitrazepam for this receptor subtype. These experiments (Figure 4-5) show that displacement of [^{3}H]Ro15-4513 occurs only at high micromolar concentrations of diazepam (K_I = 671 ± 330 μmol/L) and flunitrazepam (K_I = 60.3 ± 23.7 μmol/L). In addition, [^{3}H]flunitrazepam saturation binding studies did not show any significant specific binding (data not shown). Thus, when expressed in mammalian cells, no high affinity binding of these classical benzodiazepine agonists was detected, although robust expression of the α4β3γ2L receptor subtype was demonstrated by the high affinity binding of [^{3}H]Ro15-4513 to cell membrane preparations.

DISCUSSION

The interaction of benzodiazepines with the $GABA_A$ receptor has been extensively studied because of the therapeutic importance of many of these ligands and their broad spectrum of activities in allosterically modulating receptor function. Since the cloning of multiple $GABA_A$ receptor subunits, it has become clear that multiple receptor subtypes exist and that the pharmacological profile of each subtype depends on its precise subunit complement (McKernana and Whiting, 1995). Early studies suggested a

subdivision between two groups of receptors; those that included the α1-, α2-, α3- or α5-subunits were sensitive to classical benzodiazepines such as diazepam whereas receptors carrying the α4- or α6-subunits were diazepam-insensitive (Malminiemi and Korpi, 1989).

In the present study, we show that both diazepam and flunitrazepam potentiate GABA-induced currents mediated by the rat α4β3γ2L receptor expressed in *Xenopus* oocytes. We also show that the observed effects appear to be dependent on the isoform of the β subunit that is present. Unlike the receptor containing the β3 subunit, the α4β1γ2L and α4β2γ2L subtypes did not display such modulation. Although the β subunits have not been implicated previously in the direct binding of benzodiazepines, the overall receptor structure is likely to be shaped by all subunits present in the pentamer since these confer distinct properties to the binding site interfaces.

It is surprising that the ability of diazepam and flunitrazepam to modulate α4β3γ2L receptor function in the present study occurred over a similar concentration range to the reported EC_{50}s for the well-characterized "diazepam-sensitive" α1β2γ2L subtype (Dunn *et al.*, 1999). Furthermore, IC_{50} values for inhibition of agonist responses by Ro15-1788 and ZK93426 were also similar to those observed for the α1β2γ2L receptor (Dunn *et al.*, 1999; Davies *et al.*, 1998).

Some of our results on the effects of other benzodiazepine site ligands are consistent with previous reports. Harvey *et al.* (2002) reported that a high concentration of ZK93426 potentiated the GABA-mediated currents of the rat α4β3γ2 subtype expressed in *Xenopus* oocytes. Although Ro15-4513 has been shown to be a poor inverse agonist of GABA responses in both human (Hadingham *et al.*, 1996) and rat (Dunn *et al.*,

1999) recombinant $\alpha1\beta2\gamma2$ receptors, Ro15-4513 is a positive allosteric modulator of $\alpha4$- and $\alpha6$-containing receptors (Kelly, *et al.*, 2002; Knoflach *et al.*, 1998). β-CCE is also an inverse agonist at the classical benzodiazepine site, but has been reported to be a positive modulator of the human $\alpha4\beta3\gamma2$ subtype (Adkins *et al.*, 2001). However, as found here, this modulation occurred only at micromolar concentrations, and Adkins *et al.* (2001) suggested that the β-CCE effect may have been due to its binding to a low affinity, non-benzodiazepine site as previously reported (Stevenson *et al.*, 1995).

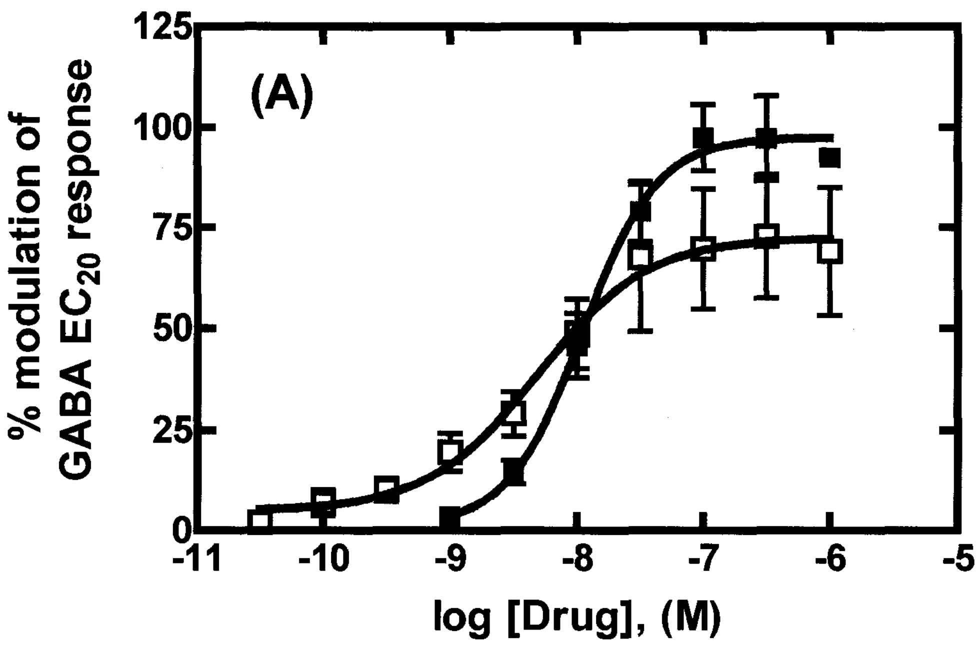
The most controversial finding of the present study is the ability of diazepam and flunitrazepam to modulate the $\alpha4\beta3\gamma2L$ receptor. Not only did the effects of these ligands occur in a similar concentration range to that reported for the $\alpha1\beta2\gamma2L$ subtype, but also inhibition by Ro15-1788 and ZK93426 was similar between the two receptor subtypes. To our knowledge, there is only one previous report of benzodiazepine potentiation of the human $\alpha4\beta3\gamma2$ receptor expressed in the *Xenopus* oocytes (Kelly *et al.*, 2002). Although not discussed in detail, these authors reported a 40-50% potentiation of the GABA current (at its EC_{20} concentration) by 300 nmol/L flunitrazepam and a 5-10% potentiation by diazepam (1 μmol/L).

Our results showing a lack of high affinity of diazepam and flunitrazepam binding to the $\alpha4\beta3\gamma2L$ receptor expressed in mammalian cells are consistent with many previous reports (Ebert *et al.*, 1996; Scholze *et al.*, 1996; Kelly *et al.*, 2002; Sur *et al.*, 1999). Functional studies have also found no evidence for benzodiazepine modulation of this receptor subtype expressed in cells (Adkins *et al.*, 2001; Brown *et al.*, 2002). Our binding studies show that high micromolar concentrations of flunitrazepam and diazepam can displace bound [^{3}H]Ro15-4513. Although this may suggest that the binding sites for

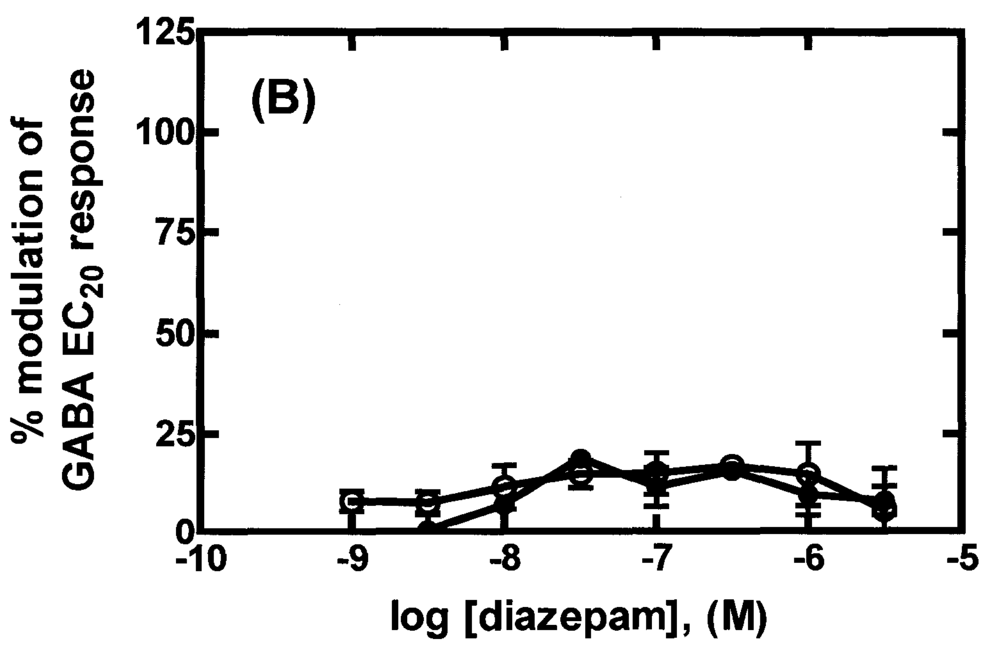
classical benzodiazepines are retained in the cell-expressed receptor, it is more likely that these effects could be explained by their action at a distinct low affinity site(s) (see e.g. Dunn *et al.* 1999) that is distinct from, but allosterically coupled to, the high affinity site for Ro15-4513.

In conclusion, in contrast to many previous reports (Brown *et al*, 2002; Adkins *et al.*, 2001; Ebert *et al.*, 1996; Scholze *et al.*, 1996), we show that the rat $\alpha4\beta3\gamma2L$ $GABA_A$ receptor (expressed in oocytes) can be modulated by nanomolar concentrations of diazepam and flunitrazepam. The reasons for disparate results obtained in different laboratories are unclear. To exclude possible experimental error, all of the cDNAs used in the present study were verified by sequencing. Another curious finding is that the observed potentiation is specific for receptors expressed in *Xenopus* oocytes. Although results obtained with the different recombinant systems are usually comparable, significant discrepancies have been described (see Lovinger and Homanics, 2007), and the reasons underlying the present results are under investigation.

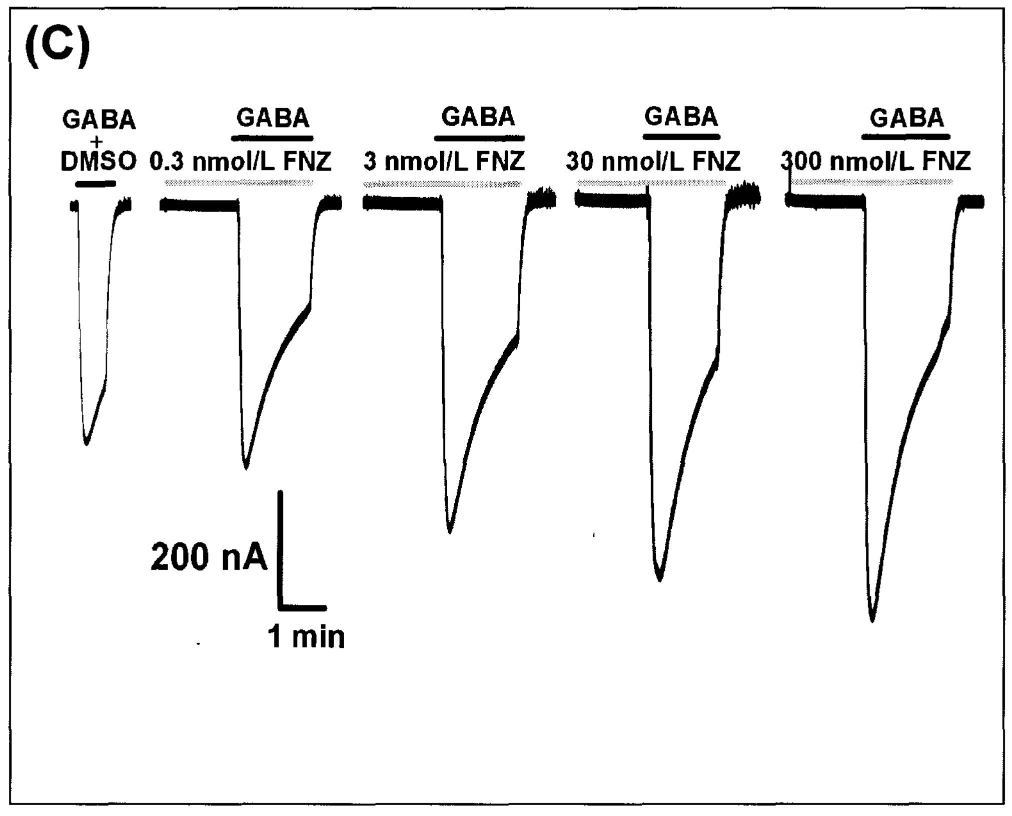
Figure 4-1. Effects of benzodiazepines on GABA-evoked currents mediated by the α4βxγ2L receptors. (A) The concentration dependence of diazepam (■) and flunitrazepam (□) on potentiation of responses to an EC_{20} concentration of GABA on α4β3γ2L receptor. (B) Diazepam does not potentiate GABA responses of the α4β1γ2L (○) or α4β2γ2L (●) receptors. Data represent the mean ± SEM from at least 3 independent experiments and the parameters obtained from curve-fitting are summarized in Table 4-1. (C) and (D), representative traces of flunitrazepam and diazepam effects, respectively, on GABA-evoked currents on the α4β3γ2L receptor. The final concentration of DMSO in these experiments was constant at 0.01% (v/v) and this concentration was shown to have no effect on GABA responses.



(A)
% modulation of
GABA EC_{20} response
125
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log [Drug], (M)



(B)
% modulation of
GABA EC_{20} response
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log [diazepam], (M)


(C)
GABA
+
DMSO
GABA
0.3 nmol/L FNZ
GABA
3 nmol/L FNZ
GABA
30 nmol/L FNZ
GABA
300 nmol/L FNZ
200 nA
1 min

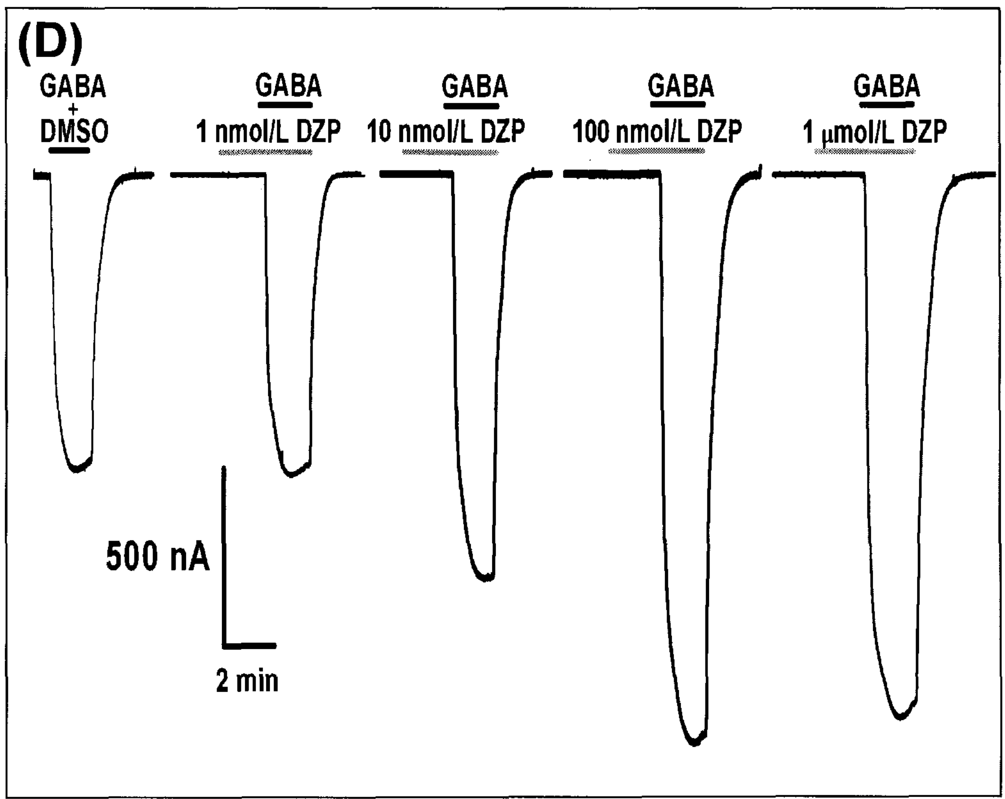

(D)
GABA
+
DMSO
GABA
1 nmol/L DZP
GABA
10 nmol/L DZP
GABA
100 nmol/L DZP
GABA
1 μmol/L DZP
500 nA
2 min

Figure 4-2. Effects of Ro15-4513 (□), β-CCE (○) and ZK93423 (■) on GABA-evoked currents on the α4β3γ2L receptor. Concentration dependence of Ro15-4513 (□) and ZK93423 (■) effects induced by a concentration of GABA approximately equal to its EC_{20} value. Concentration dependence of β-CCE (○) effects induced by a concentration of GABA approximately equal to its EC_{50} value. Data represent the mean ± SEM from at least 3 independent experiments and the parameters obtained from curve-fitting are summarized in Table 4-1.

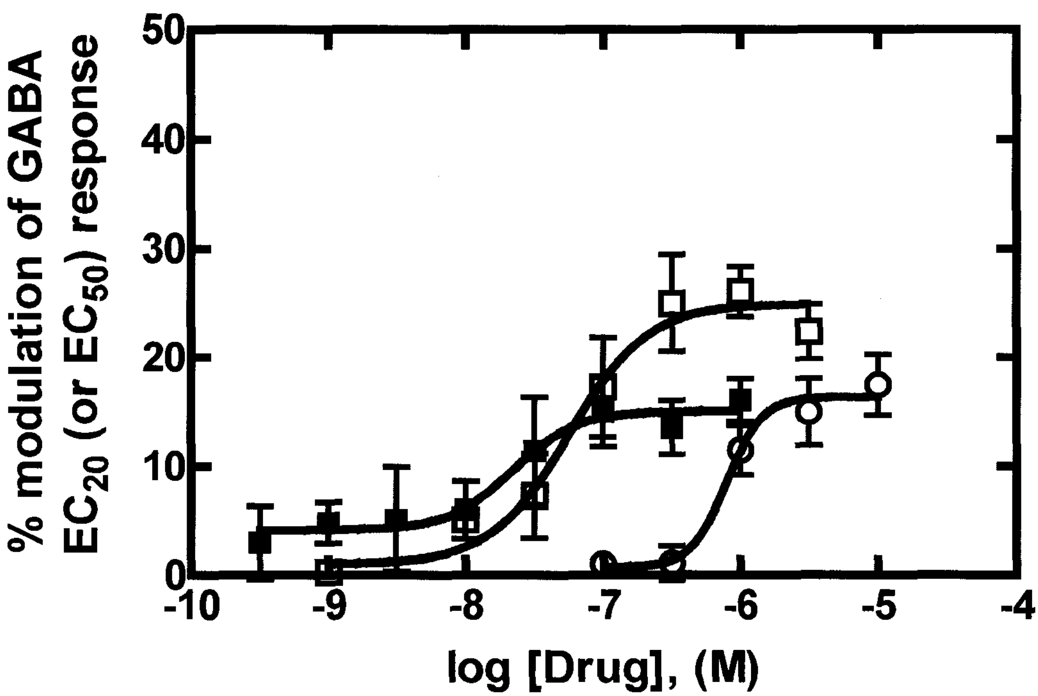

% modulation of GABA
EC_{20} (or EC_{50}) response
50
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
log [Drug], (M)

Figure 4-3. Effects of Ro15-1788 and ZK93426 on GABA-mediated currents on the α4β3γ2L receptor. (A) At 1 μmol/L concentration, Ro15-1788 does not have an effect on the GABA current while ZK93426 significantly potentiated the GABA-evoked current. The numbers in brackets indicate the number of oocytes used in the experiments. (B) Concentration dependence of ZK93426 effects induced by a concentration of GABA approximately equal to its EC_{20} value. Data represent the mean ± SEM from at least 3 independent experiments.

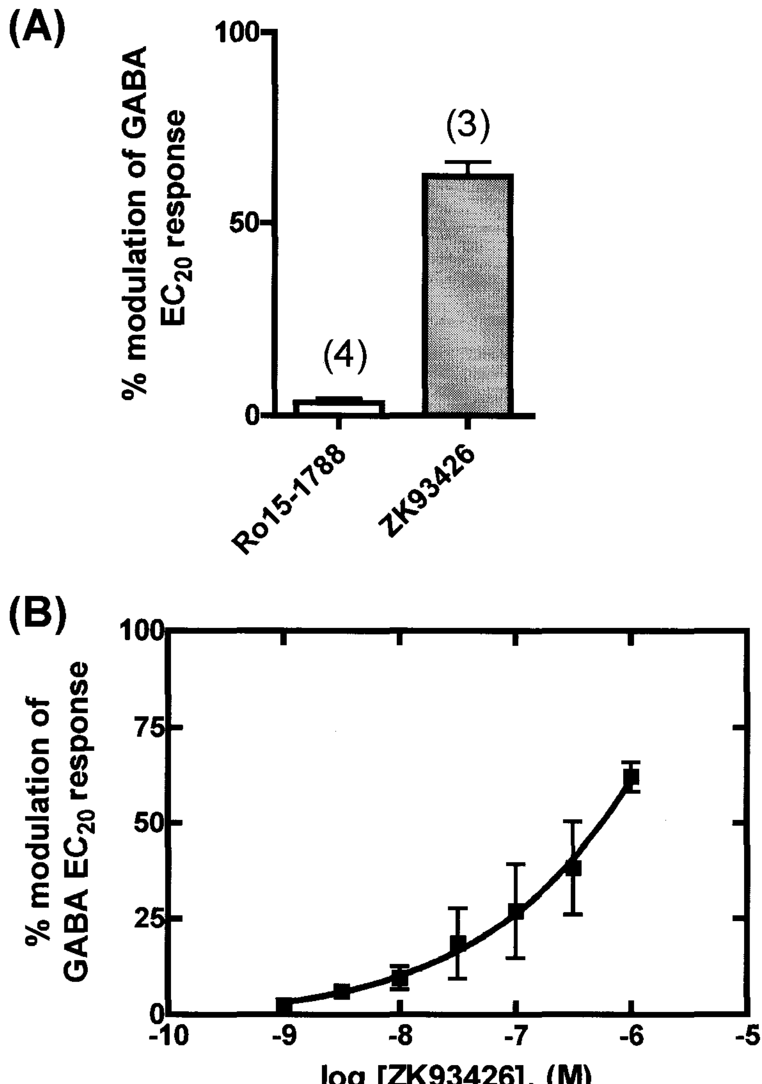

(A)
100
50
0
% modulation of GABA EC_{20} response
(4)
(3)
Ro15-1788
ZK93426
(B)
100
75
50
25
0
% modulation of GABA EC_{20} response
-10
-9
-8
-7
-6
-5
log [ZK93426], (M)

Figure 4-4. Effects of Ro15-1788 and ZK93426 on GABA mediated currents . (A) Inhibition of diazepam- (■) and flunitrazepam- (□) evoked potentiation by Ro15-1788 on the α4β3γ2L receptor. (B) Inhibition of diazepam- (■) and flunitrazepam- (□) evoked potentiation by ZK934326 on α4β3γ2L receptor. The data were obtained using a GABA concentration equal to its EC_{20} values and a concentration of 100 nmol/L for both diazepam and flunitrazepam. Data represent the mean ± SEM from at least 3 independent experiments and the parameters obtained from curve-fitting are summarized in Table 4-2.

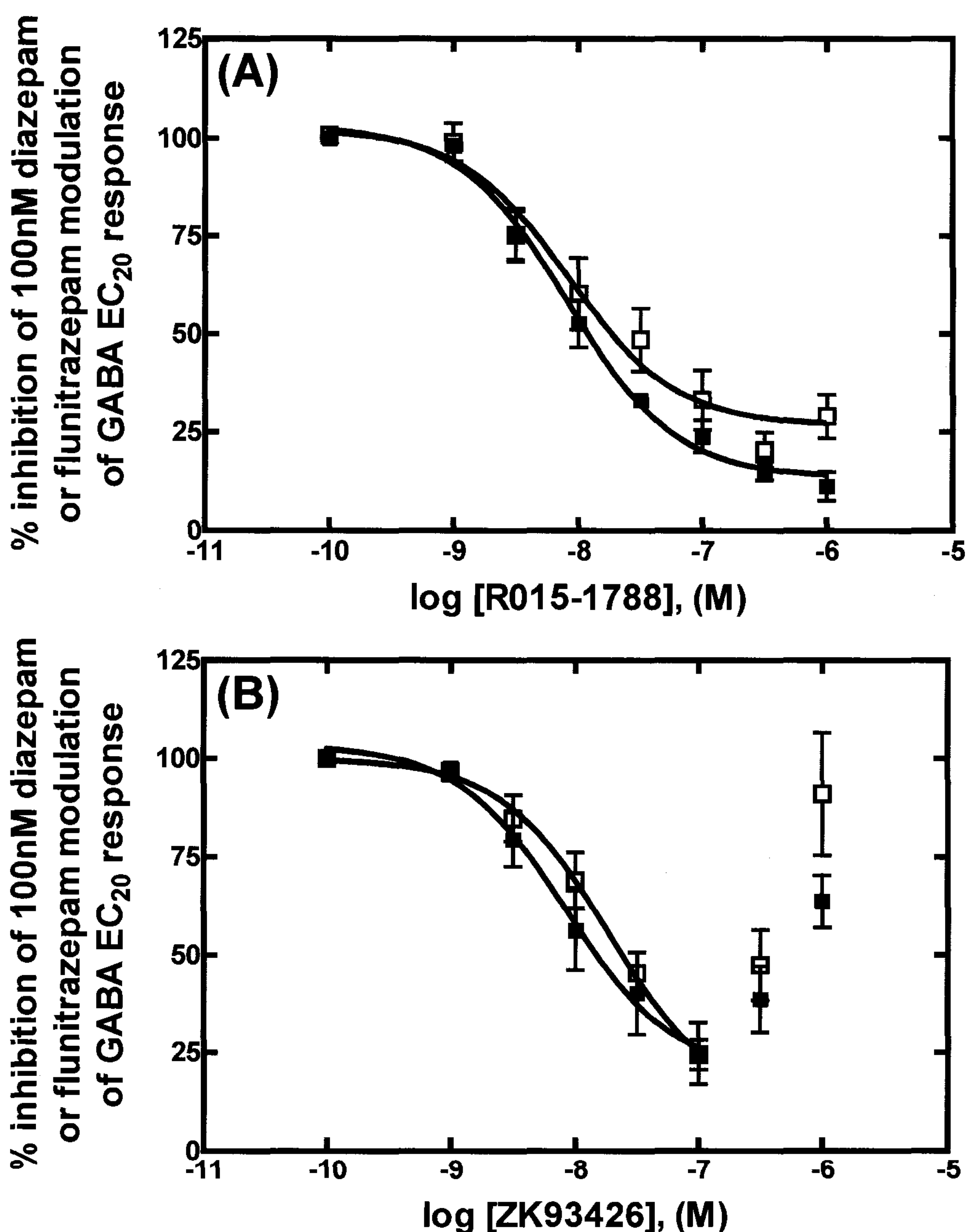

(A)
% inhibition of 100nM diazepam or flunitrazepam modulation of GABA EC20 response
125
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log [R015-1788], (M)
(B)
% inhibition of 100nM diazepam or flunitrazepam modulation of GABA EC20 response
125
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log [ZK93426], (M)

Figure 4-5. Displacement of [^{3}H]Ro15-4513 binding by diazepam and flunitrazepam. Competition binding curves show the displacement of [^{3}H]Ro15-4513 binding from the α4β3γ2L receptor by diazepam (■) and flunitrazepam (□). [^{3}H]Ro15-4513 was present in the competition binding experiments at a concentration equal to its K_D value for binding to the α4β3γ2L receptor (K_D = 4.3 ± 0.4 nmol/L). Data shown represent the mean ± SEM of three independent experiments performed in duplicate. Inset, representative curve of [^{3}H]Ro15-4513 saturation binding at the α4β3γ2L receptor.

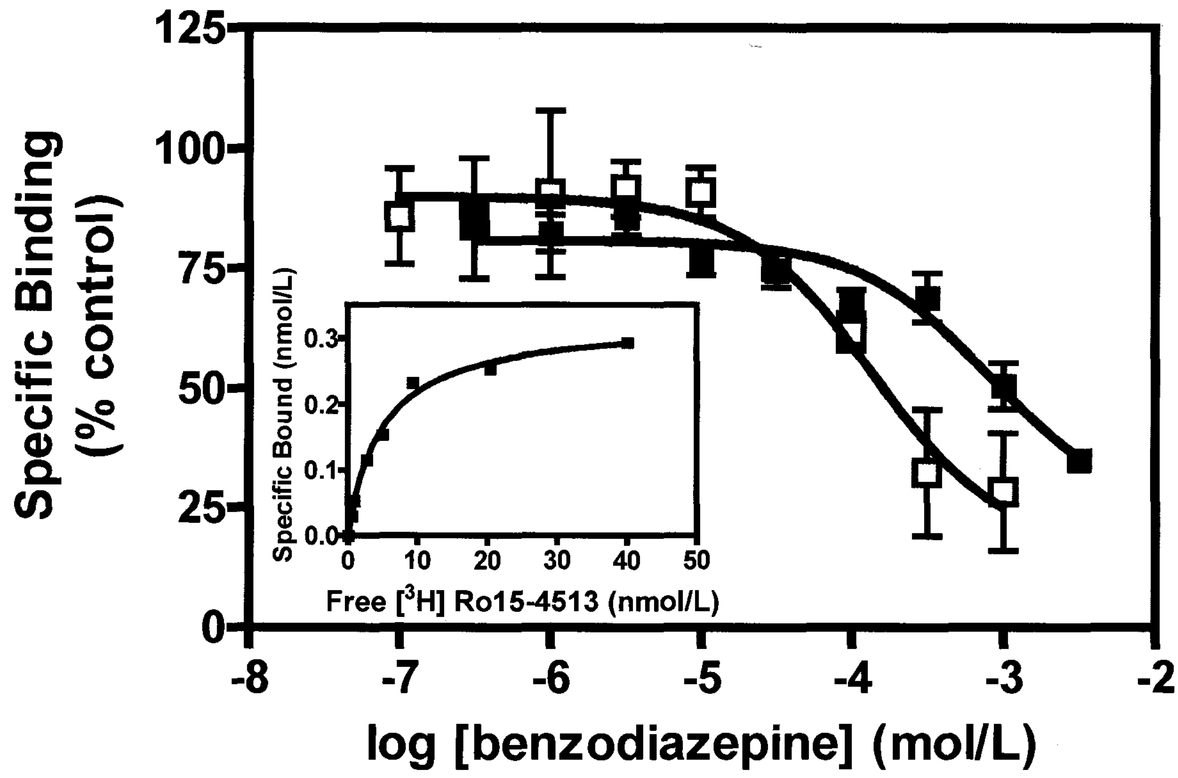

Specific Binding
(% control)
125
100
75
50
25
0
-8
-7
-6
-5
-4
-3
-2
log [benzodiazepine] (mol/L)
Specific Bound (nmol/L)
0.0
0.1
0.2
0.3
0
10
20
30
40
50
Free [3H] Ro15-4513 (nmol/L)

Table 4-1. Effects of different compounds on potentiation of GABA-evoked currents in the α4β3γ2L receptor.

	log EC_{50}	EC_{50} (nmol/L)	Efficacy	n
diazepam	−7.91 ± 0.11	12.3	101.5 ± 6.5%	4
flunitrazepam	−8.65 ± 0.22	2.25	70.3 ± 15.2%	6
Ro15-4513	−7.20 ± 0.18	62.7	26.1 ± 2.3 %	3
ZK93423	−8.23 ± 0.69	5.87	18.3 ± 0.9%	3
β-CCE	−6.05 ± 0.02	891.3	19.3 ± 3.3%	3

Table 4-2. Ro15-1788 and ZK93426 inhibitory effects on benzodiazepine potentiation of GABA-evoked currents in the α4β3γ2L receptor.

	log IC_{50}	IC_{50} (nmol/L)	n
diazepam (100nM)			
Ro15-1788	−8.10 ± 0.13	7.9	3
ZK93426	−7.99 ± 0.20	10.2	3
flunitrazepam (100nM)			
Ro15-1788	−8.06 ± 0.20	8.7	3
ZK93426	−7.73 ± 0.15	18.6	3

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CHAPTER 5[1] [2]

Atomic Force Microscopy Reveals the Stoichiometry and Subunit Arrangement of the α4β3δ $GABA_A$ Receptor

[1] Part of this chapter has been published: Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SMJ and Edwardson JM. (2008). *Mol. Pharmacol.* **73**, 960-967.
[2] Dr. J. L. Kozuska contributed to the patch-clamp recording of GABA-evoked current on tagged α4β3δ receptor.

INTRODUCTION

The $GABA_A$ receptor, which mediates fast neuronal inhibition in the CNS, is a member of the Cys-loop ligand-gated channel superfamily that also includes the nicotinic acetycholine, glycine, and $5HT_3$ receptors (Barnard *et al.*, 1998). Each member of the family is a pentamer of homologous subunits assembled to form a central ion pore. To date, nineteen mammalian $GABA_AR$ subunit isoforms have been identified (Sieghart and Sperk, 2002). The major isoform of the $GABA_AR$ is composed of two α1, two β2 and one γ2 subunits (Farrar *et al.*, 1999). Using concatenated subunits expressed in *Xenopus* oocytes, Sigel and his colleagues suggested a subunit arrangement of α-β-α-γ-β of the $GABA_AR$ α1β2γ2, reading counter-clockwise around the pore when viewed from the extracellular face of the membrane (Baumann *et al.*, 2001; Baumann *et al.*, 2002). With this arrangement, the $GABA_AR$ provides two agonist (GABA) binding pockets lying at the interface of β-α subunits and a benzodiazepine binding pocket at the interface of the α-γ subunits.

Although a minor component of the total $GABA_ARs$ in the CNS, δ-containing receptors play a major role in mediating tonic inhibition, characterized by high sensitivity to GABA and a slow rate of desensitization (Farrant and Nusser, 2005). δ-containing receptors are predominantly extrasynaptic and are preferentially co-expressed with the α4 and β subunits in dentate gyrus and thalamus granule cells and with the α6 and β subunits in cerebellar granule cells (Farrant and Nusser, 2005). The expression levels of the $\alpha4\beta_x\delta$ receptor are highly plastic. They have been found to change in various animal models of epilepsy (Brooks-Kayal *et al.*, 1998; Payne *et al.*, 2006; Qi *et al.*, 2006) and to show different expression during phases of the ovarian cycle in rat (Lovick *et al.*, 2005; Maguire *et al.*, 2005). Thus, there are potential roles for these receptors in catamenial

epilepsy and in pre-menstrual disturbances in women. Therefore, these δ-containing receptors may be a novel target for the development of anti-epilepsy drugs.

To gain insight into the unique pharmacological and biophysical characteristics of α4-, δ-containing receptor, it is important to understand the stoichiometry and subunit arrangement of the α4βδ receptor. Using the α4β3δ receptor subtype as a model system, we have collaborated with Dr. J. Michael Edwardson (Cambridge), who has developed a method, based on atomic force microscopy (AFM) imaging, to determine the arrangement of subunits within multimeric proteins. AFM is a technique that yields sub-nanometer resolution images and provides structural information at the single molecule level (Edwardson and Henderson, 2004). It has been applied previously to the study of the subunit arrangement of other ligand-gated ion channels, such as the $GABA_AR$ α1β2γ2 receptor (Neish *et al.*, 2003), the $5\text{-}HT_3$ receptor (Barrera *et al.*, 2005a) and the P2X receptor (Barrera *et al.*, 2005b). In AFM studies of ionotropic receptors, specific epitope tags are engineered onto each receptor subunit. Receptors isolated from transfected cells by affinity chromatography are then incubated with antibodies to the tags. The size of the AFM-imaged receptor-antibody complex provides an estimate of the subunit stoichiometry, whereas the geometry of the complexes reveals more detailed information about the receptor architecture. An early AFM study was used to determine the subunit arrangement of the $GABA_A$ α1β2γ2 receptor and it was concluded that the two α1 subunits are separated by a non-α subunit (Neish *et al.*, 2003). However, some uncertainty remained. From this work it was not clear about the position of β and γ subunits within the pentamer. Resolution of the true subunit arrangement of a ternary $GABA_A$ receptor requires the ability to distinguish three types of subunits

simultaneously. To accomplish this task, we initially constructed epitopes tagged α4, β3 and δ subunits and verified their functional expression in both *Xenopus* oocytes and HEK cells. We also performed Western blot analysis combined with immunohistochemical detection of the tagged receptor expressed in HEK cells. Subsequently, AFM imaging and analysis was conducted in Dr. Edwardson's laboratory. In this chapter, I provide detailed information of the related work that I have done and a summary of the AFM imaging results from Dr Edwardson's laboratory.

MATERIALS AND METHODS

Construction of Tagged $GABA_A$ Receptor Subunits

The original cDNAs encoding the $GABA_A$ receptor α4 and β3 subunits were from Dr. P. H. Seeburg's laboratory. The cDNA encoding the δ subunit was generously provided by Dr. R. L. Macdonald. To generate epitope-tagged constructs, cDNA encoding the β3 subunit was subcloned into the vector pcDNA3.1/V5-His A (Invitrogen, San Diego, CA) using *Kpn*I and *Xba*I. The α4 subunit sequence was first engineered to include a C-terminal FLAG epitope tag and then subcloned into the pcDNA3.1/V5-His A vector using *Hind*III and *Age*I, in order to delete the V5 epitope tag. Similarly, the δ subunit with a C-terminal HA epitope tag was subcloned into the same vector using *Kpn*I and *Age*I, so as to delete the V5 epitope tag.

Two different sequences of the rat β3 subunit appear on the UniProtKB/Swiss-Prot and NCBI databases. In the UniProtKB/Swiss-Prot database, the rat β3 subunit has a leucine 256 (sequence number: P63079, Lolait et al., 1989), whereas it has a methionine 256 in the NCBI database (sequence number: β_3 NM-017065.1, Ymer et al., 1989). In our

previous studies (Chapters 2, 3 and 4), all the β3 cDNAs used contained leucine 256. However, in the present chapter, the β3 subunit carrying a methionine in position 256 (denoted as β3(M256)) was used.

Expression and Functional Studies of Untagged-α4β3(M256)δ and Tagged-α4β3(M256)δ Receptors in *Xenopus* Oocytes

Capped cRNAs of the α4, β3 and δ subunits were synthesized from their linearized cDNAs following standard protocols using T7 RNA polymerase (Invitrogen) for *in vitro* transcription. Preparations of *Xenopus laevis* oocytes were as described earlier (You and Dunn, 2007). Oocytes were injected with 50 nL of 1 μg/μl total subunit cRNAs in a 1:1:1 ratio and incubated individually in ND96 buffer (96 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5 mmol/L HEPES, pH 7.4) in 96-well plates at 14°C for at least 48 h before functional analysis.

For two-electrode voltage-clamp analysis of the concentration dependence of GABA activation, injected oocytes were clamped at a holding potential of –60mV using a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA) and continuously bathed in frog Ringer's buffer (110 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 5 mmol/L HEPES, pH 7.4). GABA-evoked currents were recorded using Axoscope 9.0 data acquisition software (Axon Instruments Inc.). Concentration-effect curves were analyzed by non-linear regression techniques using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA) as described earlier (You and Dunn 2007). The log EC_{50} values, Hill coefficients and maximal current amplitudes mediated by GABA of the untagged-α4β3(M256)δ and tagged-α4β3(M256)δ subtypes were compared. Levels of

significance were determined by the Student two-tailed *t*-test using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

Transient Transfection in HEK 293 Cells and Patch-clamp Electrophysiology

The cells were grown on 35 mm plates and maintained in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% bovine growth serum (Hyclone) at 37°C in 5% CO_2. HEK293 cells were transiently transfected with 0.38 μg/plate of each of the tagged α4, β3 and δ subunit cDNAs, using a modified calcium phosphate precipitation method (Chen and Okayama, 1988). 1 μg/plate of green fluorescence protein (GFP) cDNA was co-transfected. Transfected HEK 293 cells were incubated for 24-72 hours before analysis.

Recording of whole cell GABA-activated membrane currents was conducted by Dr. J. L. Kozuska using an Axopatch 200B amplifier. Electrodes were pulled from borosilicated glass capillaries (A-M Systems. Everett, WA) with a Flaming Brown P-87 micropipette puller (Sutter Instrument Company, Novato, CA) to a resistance of 3-5 MΩ. They were filled with an internal solution containing 140 mmol/L KCl, 2 mmol/L $MgCl_2$, 5 mmol/L EGTA, 10 mmol/L HEPES and 3 mmol/L Mg-ATP (pH 7.4). The extracellular solution contained 140 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 11 mmol/L glucose and 10 mmol/L HEPES (pH 7.4). Cells were voltage-clamped at a holding potential of −50 mV and GABA was applied by gravity flow from a micropipette approximately 500 μm away from the clamped cells. Data were analyzed using Strathclyde electrophysiology software (John Dempster, WinWCP2.4.9).

Immunofluorescence of Tagged $GABA_A$ α4β3(M256)δ Receptor

Cell staining was used to examine receptor expression on the cell surface. Transfected HEK cells were cultured on coverslips in 35 mm dishes with media. After 36 hours incubation, cells were washed twice with phosphate buffered saline (PBS) at room temperature and then fixed by incubation in 4% (w/v) paraformaldehyde in the same buffer for 1 hour and this was followed by another two washes with PBS. For staining of permealized cells, primary antibodies were incubated with the fixed cells for 1 hour in PBS solution containing 1% normal goat serum and 1% saponin. Primary antibodies were detected using a fluorescent secondary antibody, Alexa555 (red, Invitrogen), which was incubated with the cells for 1 hour. The cells were then washed twice with PBS and visualized by fluorescence microscopy. For staining of non-permealized cells, the cells were incubated in PBS buffer (without saponin) with both primary and secondary antibodies.

Immunoblot Analysis

Cells were grown in 150 mm dishes. The culturing, transfection and harvesting of the cells followed the same protocols as described in Chapter 4. Cell membranes were collected and washed twice with cold PBS and then mixed with Laemmli sample buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 mol/L Tris-HCl, pH = 6.8) at 65°C for 20 minutes and the membranes were then centrifuged at 14,000 g at room temperature. 20 μL samples were separated using 7.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred electrophoretically to a nitrocellulose membrane. The

membrane was incubated in blocking buffer containing 5% nonfat milk and 0.1% Tween 20 for 1 hour at room temperature. The membrane was then incubated with mouse anti-V5 (Invitrogen), anti-HA (Covance) and anti-Flag (Sigma) monoclonal antibody IgGs in blocking buffer for either 1 h or overnight at 4°C. After washing, the nitrocellulose membrane was incubated with horseradish peroxidase conjugated secondary antibody. Immunoreactivity was detected using a chemiluminescence detection system (Pierce, CA).

AFM Imaging of Receptors and Receptor-antibody Complexes

AFM imaging was performed in Dr. J. M. Edwardson's laboratory (Cambridge, UK). After cell lysis, a crude membrane fraction was solubilized in 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and the $GABA_AR$ was purified by Ni^{2+}-affinity chromatography (Invitrogen, UK). The receptors were eluted with increasing concentrations of imidazole. Purified receptors were bound to a mica substrate and imaged either alone or with various monoclonal antibodies. Receptors were also incubated with Fab fragments of the anti-FLAG antibody, generated by papain digestion (ImmunoPure Kit; Pierce). Imaging was performed with a Multimode atomic force microscope (Digital Instruments).

The molecular volumes of a large number of receptor protein particles were determined from particle dimensions based on AFM images. After adsorption of the receptors onto the mica support, the particles adopted the shape of a spherical cap. The heights and half-height diameters were measured from the particle and the molecular volume was calculated by using the equation:

$$V_m = (\pi h/6)(3r^2+h^2) \quad \text{(Equation 1)}$$

where h is the particle height and r is the radius. A predicted molecular volume for an individual subunit can be calculated based on its molecular mass using the equation

$$V_c = (M_0/N_0)(V_1+dV_2) \quad \text{(Equation 2)}$$

(Schneider *et al*., 1998), where M_0 is the molecular mass, N_0 is Avogadro's number, V_1 and V_2 are the partial specific volumes of the particle (0.74 cm^3/g, Durchschlag and Zipper, 1997) and water (1 cm^3/g), respectively, and d is the extent of protein hydration, of which the value is 0.4 g H_2O/g protein reported for a typical globular protein (human serum albumin) (Grant, 1957)

A frequency distribution of the molecular volumes (V_m) imaged from AFM was generated and the peak volumes were compared with their predicted V_c to give an estimate of the likely stoichiometry of the receptor.

When the receptor-antibody complexes were examined, receptors decorated by two antibodies were identified in the AFM images, and a frequency distribution of the angle between the two bound antibodies was generated. The peak of the frequency distribution curve reflected the size of the angle between those two subunits in the receptor.

RESULTS

Functional Expression of Untagged-α4β3(M256)δ and Tagged-α4β3(M256)δ Subtype in *Xenopus* Oocytes

To investigate the expression of untagged-α4β3(M256)δ and tagged-α4β3(M256)δ receptors, cRNAs encoding the respective subunits were injected into

Xenopus oocytes. GABA-evoked currents from these oocytes were recorded by standard two-electrode voltage clamp techniques. Compared to the untagged-α4β3(M256)δ receptor, the concentration-response curves of the tagged-α4β3(M256)δ were shifted to the right (Figure 5-1, A). The EC_{50} for GABA activation of the tagged-α4β3(M256)δ is 9.8 μmol/L (log $EC_{50} = -5.01 \pm 0.03$, n = 3), which is approximately 2-fold greater than that of the untagged-α4β3(M256)δ subtype (EC_{50} = 4.3 μmol/L, log $EC_{50} = -5.37 \pm 0.03$, n = 2). However, Hill coefficients and the maximal currents elicited by a saturating concentration of GABA (1 mmol/L) were not significantly different between these two subtypes (see Table 5-1).

Functional Expression of Tagged-α4β3(M256)δ Subtype in HEK 293 Cells

The whole cell current of HEK 293 cells expressing tagged-α4β3(M256)δ receptor is illustrated in Figure 5-1, B. The average maximum current evoked by 1 mmol/L GABA was 532.5 ± 152.4 pA (n = 5). The current trace shows the slow desensitization and deactivation that are characteristics of this receptor subtype.

Immunofluorescence Staining and Immunoblotting of the Tagged-α4β3(M256)δ Receptor Expressed in HEK 293 Cells

In cells transfected with cDNAs encoding the tagged α4, β3 and δ subunits (at a 1:1:1 ratio by weight), anti-FLAG, anti-V5 and anti-HA antibodies all gave positive immunofluorescence signals in both permeabilized and non-permeabilized cell preparations (Figure 5-2). In permeabilized cells, the immunofluorescence signals were significantly stronger than those from non-permeabilized cells. These results suggest that

only a small population of receptors are expressed on the cell surface. It is likely that many of the tagged subunits may be retained in the endoplasmic reticulum.

The expression of tagged α4, β3 and δ subunits was confirmed by immunoblotting using the same antibodies. As illustrated in Figure 5-3, crude membrane fraction separated by SDS-PAGE gel gave positive signals on immunoblots with anti-FLAG, anti-V5 and anti-HA antibodies. The major bands in all three lanes had apparent molecular weights of 50-75 kDa (in a size order of $\alpha 4 > \beta 3 \approx \delta$), which is consistent with the expected sizes of the three subunits.

AFM imaging

A detailed description of the AFM results is available in Barrera *et al.* (2008) and only a brief summary is provided below.

The isolated $GABA_A$ receptors in AFM imaging appeared as a spread of particles. The frequency distribution of the calculated molecular volumes (Equation 1) had two discernable peaks, at 120 ± 4 nm^3, ($n = 431$) and 593 ± 31 nm^3 ($n = 110$) (Figure 5-4). The smaller peak is likely to correspond to single receptor subunits since the molecular volume is close to 105 nm^3 (Equation 2) that is predicted for a $GABA_A$ receptor subunit, based on its molecular mass of 55 kDa. The larger peak corresponds to the expected pentameric receptor.

$GABA_A$ receptors were incubated with anti-FLAG mouse monoclonal antibodies directed against FLAG-tagged α4 subunit or anti-V5 antibodies against the V5-tagged β3 subunit or anti-HA antibodies against the HA-tagged δ subunit. AFM imaging of these receptor-antibody complexes suggested that 7-8% of receptor protein particles were

doubly decorated by either anti-FLAG or anti-V5 antibody. Further analysis of these doubly decorated subunits showed that the peak values of angles between bound antibodies were $137 \pm 4°$ (n = 54) and $142 \pm 4°$ (n = 54) for anti-FLAG and anti-V5 antibodies, respectively, indicating that there are two α and two β subunits separated by another subunit (expected angle 144°) (Figure 5-5). In contrast, a majority of receptor proteins showed only a single subunit that was decorated by anti-HA antibody. These results suggest that the subunit stoichiometry of the receptor is 2α:2β:1δ.

To determine the absolute subunit arrangement, receptors were incubated with Fab fragments of the anti-FLAG antibody, and normal anti-HA antibodies, to decorate both α and δ subunits simultaneously. In imaging of the receptor-antibody complexes, the subunits decorated with Fab fragments can be clearly distinguished from the whole antibody decorated subunits on the basis of their smaller size (Figure 5-6, A). AFM results showed that the angles from whole antibody to Fab and from Fab to Fab were 83° and 122°, respectively (Figure 5-6, B). These results, together with AFM results of the position of two β subunits, suggest a predominant subunit arrangement of α-β-α-β-δ (counter-clockwise) on the mica surface.

However, because α-β-α-β-δ and α-β-α-δ-β are two mirror images, it is important to know the orientation of the receptor on the grid i.e., whether the extracellular or the intracellular domain has bound to mica surface. To answer this question, mica surfaces were coated with either poly-L-lysine (as usual) or poly-L-glutamate, to give either a positively- or a negatively-charged surface. The extracellular domain of the receptor contains negatively charged oligosaccharides (Ymer *et al.*, 1989; Wisden *et al.,* 1991; Shivers *et al.*, 1989), which may bind to a positively charged surface, such as the poly-L-

lysine coated mica; in contrast, the intracellular domain of the receptor contains many positively charged amino acids (54 positively charged residues within the intracellular domain of the α4β3δ receptor for the stoichiometry described above) (Ymer *et al.*, 1989; Wisden *et al.,* 1991; Shivers *et al.*, 1989), which may bind to a negatively charged surface, such as the poly-L-glutamate coated mica surface. To determine the orientation of the receptors, the receptors were bound to these two surfaces and incubated with either concanavalin A (Im *et al.*, 1989) or monoclonal antibody bd17 (Richards *et al.*, 1987) (Figure 5-7). Concanavalin A binds to oligosaccharides present in the extracellular domain of the receptor. Monoclonal antibody bd17 recognizes an epitope at the N-terminus of the β-subunit. If the extracellular face of the imaged receptors binds face down to mica surface, the binding sites for these two reagents will be occluded. The AFM results showed that for the receptors which were incubated with either concanavalin A or antibody bd17 there was about twice as much binding when the receptor was bound to poly-L-glutamate than to poly-L-lysine. This result led to the conclusion that the receptors normally bind predominantly with their negatively charged extracellular face down to poly-L-lysine-coated mica surface. Therefore, with this receptor orientation, the previous results with anti-FLAG Fabs (for α subunits) and anti-HA whole antibody (for δ subunit) indicated a predominant subunit arrangement of α-β-α-δ-β, counter-clockwise, when seen from the extracellular space; however, some 21% of the population exhibited a distinct subunit arrangement.

DISCUSSION

As part of this project to elucidate the stoichiometry and subunit arrangement of the $GABA_A$ α4β3δ receptor, we constructed epitope-tagged α4, β3 and δ subunits and examined their assembly on the membrane surface. Our data from tagged-α4β3(M256)δ subtype show that addition of epitope tags to the C-terminus of α4, β3 and δ subunits results in receptor that is less sensitive to GABA than its untagged counterpart. In other studies, epitope-tagged receptors have similar binding and electrophysiological properties to their untagged receptors (Connolly *et al.*, 1996; Neish *et al.*, 2003; Boyd *et al.*, 2002; Barrera *et al*, 2005), suggesting that the addition of small epitope tags to the receptor subunits usually appears to be functionally silent. The reason why addition of epitope tags on the receptor subunits results in a decreased GABA sensitive in the present study is unknown. However, the maximal current amplitudes elicited by GABA of the untagged and tagged receptors are not different, indicating that these subtypes may have similar levels of membrane surface expression in *Xenopus* oocytes. When expressed in HEK cells, the maximal currents of the tagged receptor were similar to those reported elsewhere for untagged α4β3δ receptors. In addition, the tagged receptor well retained the slow desensitization and deactivation properties that are characteristics of this extrasynaptic receptor and similar to the results of wildtype α4β3δ receptor reported by others (Brown *et al.*, 2002 and Feng *et al.*, 2006). Thus, we are confident that the tagged-α4β3(M256)δ receptor examined in AFM study is functional.

The α4βxδ receptor has proved to be difficult to express in transient recombinant systems, especially in mammalian cell lines (Brown *et al.*, 2002 and Feng *et al.*, 2006). Consistent with previous studies, the maximal whole cell currents of the tagged-

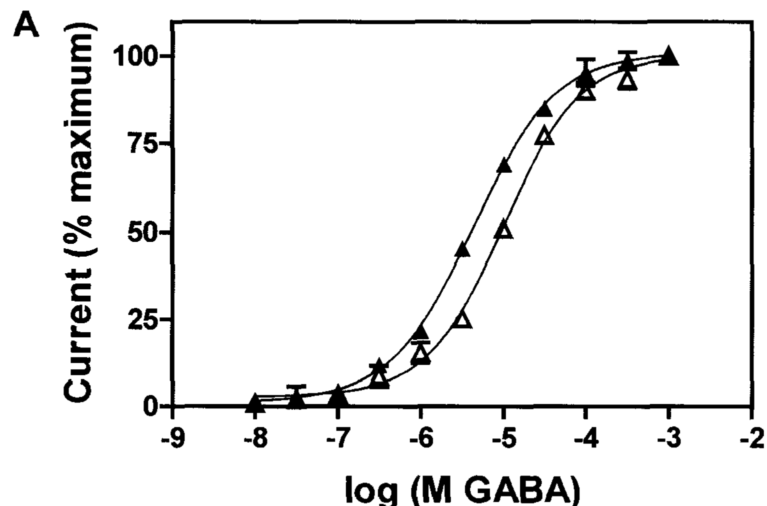
$\alpha4\beta3(M256)\delta$ receptors expressed in HEK cells were typically 10-fold smaller than for the recombinant $\alpha1\beta2\gamma2$ subtype using the same transfection protocol (Kozuska *et al.*, unpublished data). Assembly of Cys-loop receptors, such as the peripheral nAChR (Green and Claudio 1993) and the $GABA_AR$ (Connolly *et al.*, 1996), occurs in the endoplasmic reticulum before the mature receptor is translocated to the cell surface. Our immunofluorescence results using non-permeabilized cells show that only a small fraction of tagged subunits are detected on the cell surface, suggesting that a large proportion of subunits remained in the endoplasmic reticulum. Results from Dr. J M Edwardson's laboratory using AFM imaging also suggest that 44% of the total subunits do not assemble into pentameric receptors. Such a lack of receptor assembly was not seen in previous AFM studies of the $GABA_A$ $\alpha1\beta2\gamma2$ receptor (Neish *et al.*, 2003).

Results from the AFM study indicate that the predominant subunit arrangement of the $GABA_A$ $\alpha4\beta3\delta$ receptor is as α-β-α-δ-β, counter-clockwise, when viewed from the extracellular surface. This arrangement is analogous to the $\alpha1\beta2\gamma2$ receptor predicted by a series of concatamer studies (i.e., α-β-α-γ-β, counter-clockwise. Baumann *et al.*, 2001; Baumann *et al.*, 2002). Since the GABA binding sites are predicted to lie at the $\beta(+)/\alpha(-)$ interface (see Chapter 1), the predominant subunit arrangement of the $\alpha4\beta3\delta$ receptor would suggest the presence of two GABA activating sites. It should be noted, however, that the present AFM study also suggested that one fifth of the total receptor population expressed in the HEK cells might adopt a subunit arrangement of α-β-α-β-δ (counter-clockwise, viewed from the extracellular surface), which would provide only one $\beta(+)/\alpha(-)$ interface for the binding of GABA molecule. It is interesting that the Hill coefficients for GABA activation of the $\alpha4\beta3\delta$ receptor seem to be dependent on the

recombinant expression system. The Hill coefficient of GABA activation of the α4β3δ receptor expressed in a mammalian cell line has been reported to lie between 1.3 and 1.5 (Brown *et al.*, 2002; Adkins *et al.*, 2001), suggesting that there must be more than one binding site on this receptor. However, the Hill coefficients obtained from several independent studies (including the present study) in which the α4β3δ receptor was expressed in *Xenopus* oocytes, are less than one (0.7 ~ 0.9, Storustova and Ebert, 2006; You and Dunn, 2007). It is, therefore, an attractive possibility that the discrepancy in Hill coefficients might be explained by different subunit arrangements of the α4β3δ receptor expressed in the mammalian cell lines and *Xenopus* oocytes and hence the differences in the number of agonist binding sites.

In conclusion, the AFM study shows a predominant α-β-α-δ-β (counter-clockwise, when viewed from the extracellular side) subunit arrangement in the $GABA_AR$ α4β3δ subtype expressed in HEK cells. The elucidation of the subunit arrangement in the α4β3δ subtype provides an opportunity to develop structural models for this putative extrasynaptic receptor in order to investigate its unique pharmacological and biophysical properties, thus facilitating the design of ligands that will allow the differential modulation of tonic and phasic inhibitory neurotransmission.

Figure 5-1. Functional expression of tagged- and untagged-α4β3(M256)δ receptor in *Xenopus* oocytes and HEK 293 cells. (A) Concentration effect curves for untagged-α4β3(M256)δ receptor (▲) and tagged-α4β3(M256)δ receptor (Δ) expressed in *Xenopus* oocytes. Data represent the mean ± SEM from 2 - 3 independent experiments. (B) Representative whole cell current for activation of tagged α4β3δ receptors expressed in HEK cells by 1 mmol/L GABA.


A
Current (% maximum)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
log (M GABA)

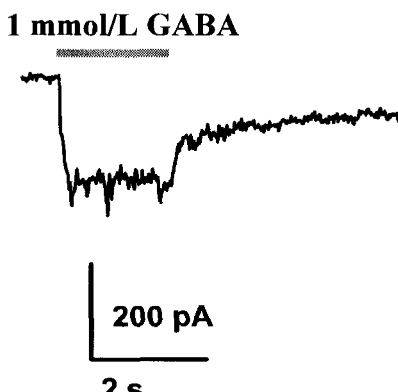

B
1 mmol/L GABA
200 pA
2 s

Figure 5-2. Immunofluorescent staining of tagged $GABA_A$ α4β3(M256)δ receptor expressed in HEK 293 cells. Permeabilized or non-permeabilized cells were fixed and incubated with primary monoclonal antibodies, followed by Alexa555 secondary antibody. In control experiments, the primary antibody was omitted. Cells were imaged by fluorescence microscopy.

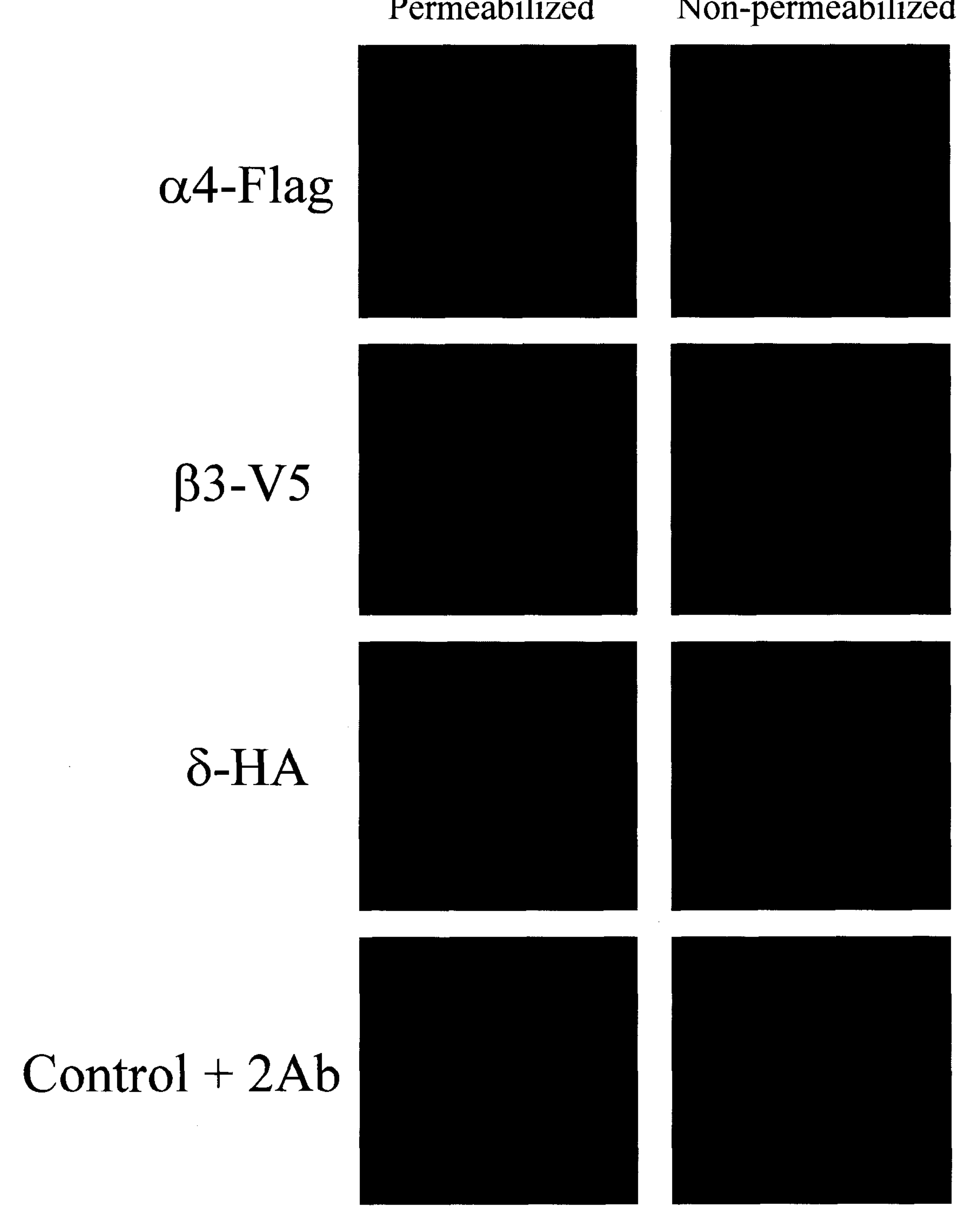

Permeabilized
Non-permeabilized
α4-Flag
β3-V5
δ-HA
Control + 2Ab

Figure 5-3. Immunoblotting of tagged $GABA_A$ α4β3(M256)δ receptor. Cell membrane fractions were separated through a 7% SDS/PAGE and immunoblotting, using monoclonal anti-FLAG, -V5, -HA primary antibodies followed by a horseradish peroxidase conjugated secondary antibody. Immunoreactive bands were detected by a chemiluminescence detection system.

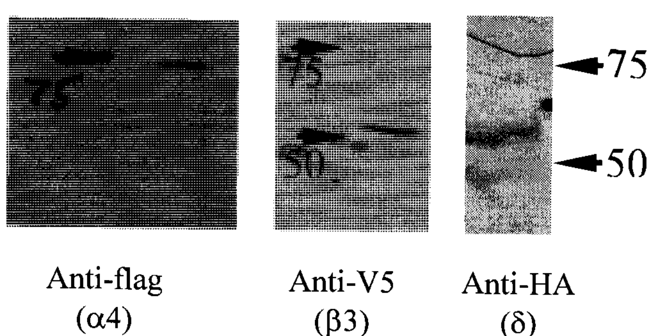

75
75
50
75
50
Anti-flag
(α4)
Anti-V5
(β3)
Anti-HA
(δ)

Figure 5-4. Frequency distribution of molecular volumes of undecorated $GABA_A$ receptors. The curve indicates the fitted double-Gaussian function. Two peaks, one at 120 ± 4 nm^3 and another at 593 ± 31 nm^3, were determined from the curve.

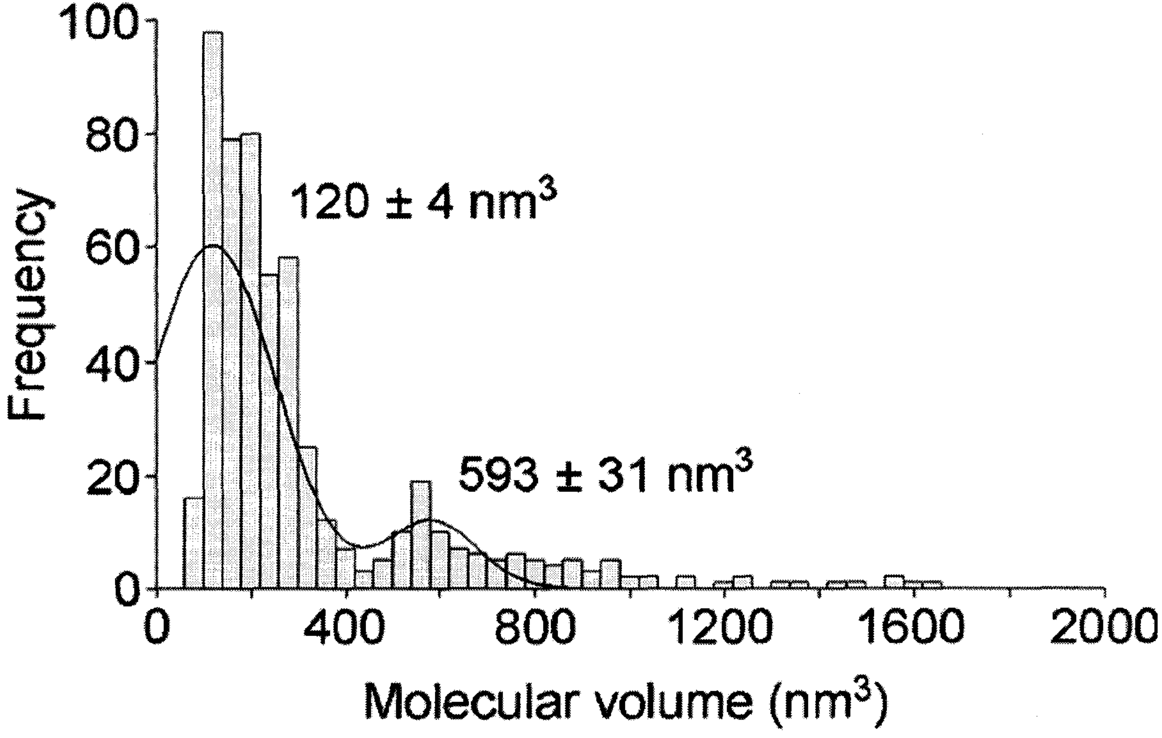

Frequency
100
80
60
40
20
0
0
400
800
1200
1600
2000
Molecular volume (nm3)
120 ± 4 nm3
593 ± 31 nm3

Figure 5-5. AFM imaging of complexes between $GABA_A$ receptors and anti-subunit antibodies. (A) and (B) Zoomed images of receptors that are uncomplexed (*top*), or bound by one (*middle*) or two (*bottom*) anti-FLAG (A) and anti-V5 (B) antibodies. A shade-height scale is shown at the right. (C) and (D) Frequency distributions of angles between anti-FLAG (C) and anti-V5 (D). The curves indicate the fitted single Gaussian functions with peak angles at $137 \pm 4°$ and $142 \pm 4°$ for anti-FLAG (C) and anti-V5 (D) antibodies, respectively.

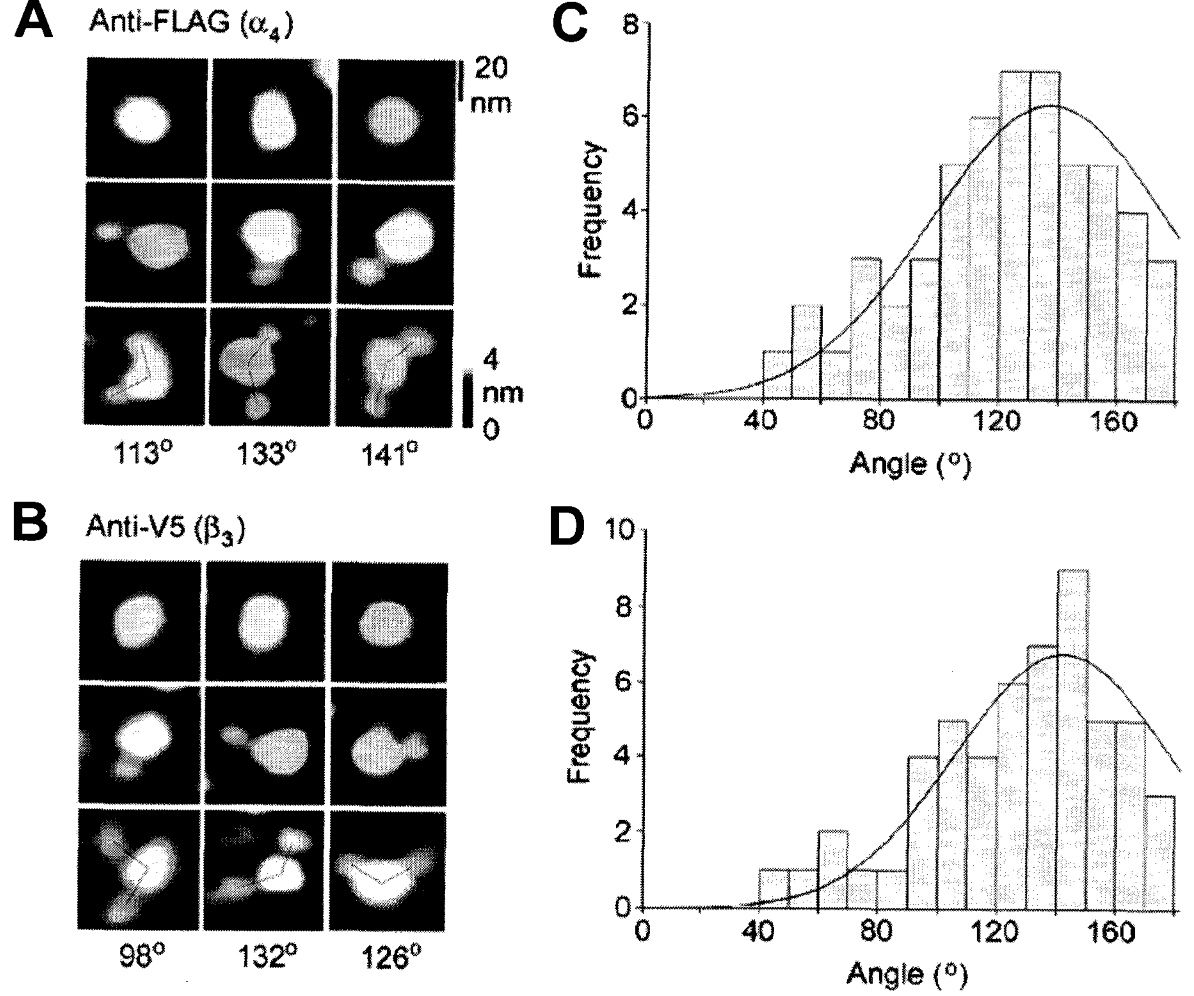

A
Anti-FLAG (α_4)
20 nm
4 nm 0
113°
133°
141°
B
Anti-V5 (β_3)
98°
132°
126°
C
Frequency
Angle (°)
D
Frequency
Angle (°)

Figure 5-6. Determination of the absolute subunit arrangement. (A) representative images of receptors decorated with two anti-FLAG Fab fragments and one anti-HA antibody. The *left-hand* image indicates a subunit arrangement of α-β-α-δ-β, counter-clockwise, and the *right-hand* image indicates an arrangement of α-β-α-β-δ, counter-clockwise. The two subunit arrangements are illustrated above the corresponding AFM images. (B) Progression of angles around the receptor for fourteen receptors decorated with two Fabs and one antibody. The angles are read counter-clockwise around the receptor, beginning at the δ-subunit, which is decorated by the anti-HA antibody. The mean angles, 83° from antibody to Fab and 122° from Fab to Fab, indicate a predominant subunit arrangement of δ-β-α-β-α, clockwise.

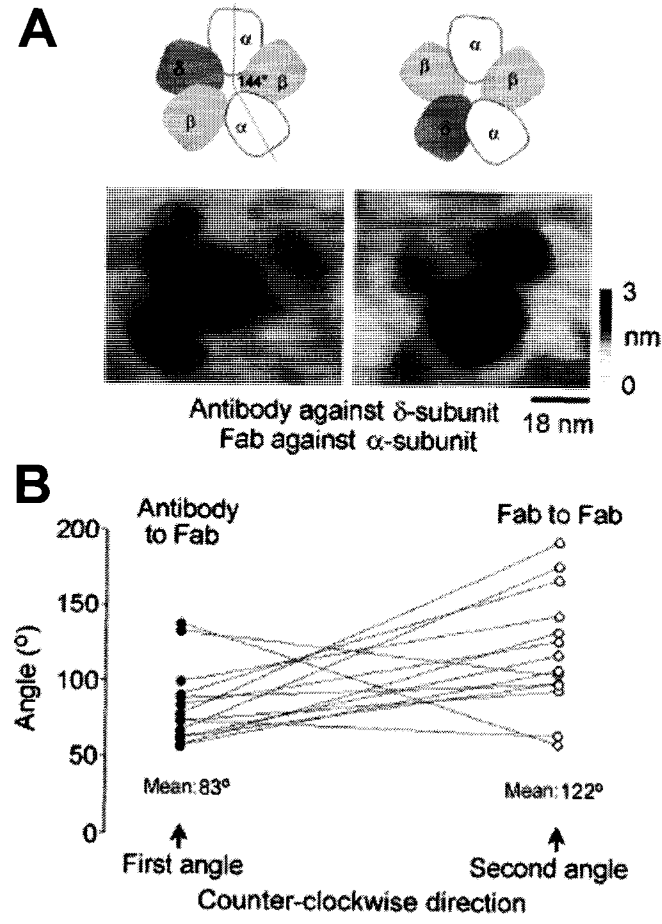
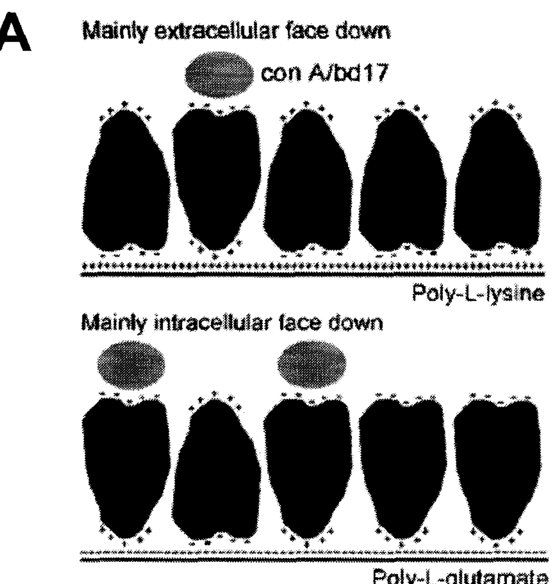

A
α
δ
144°
β
β
α
α
β
β
δ
α
3
nm
0
Antibody against δ-subunit
Fab against α-subunit
18 nm
B
Antibody to Fab
Fab to Fab
200
150
100
50
0
Angle (°)
Mean: 83°
Mean: 122°
First angle
Second angle
Counter-clockwise direction

Figure 5-7. Determination of the orientation of receptor on the mica surface. (A) Schematic illustration of the experiment to determine the orientation of the receptor on the mica support (Barrera *et al.*, 2007). Without the binding of concanavalin A or bd17 to negative-charged extracellular surface, the receptors will face down with their extracellular surface to the positive poly-L-lysine coated mica support. However, positive residue-enriched intercellular part of the receptor will preferentially face down to the negative poly-L-glutamate coated mica support. (B) Representative images of receptors that had been bound to mica coated with either poly-L-lysine (*top*) or poly-L-glutamate (*bottom*) and then decorated with either concanavalin A (*left*) or antibody bd17 (*right*).


A
Mainly extracellular face down
con A/bd17
Poly-L-lysine
Mainly intracellular face down
Poly-L-glutamate

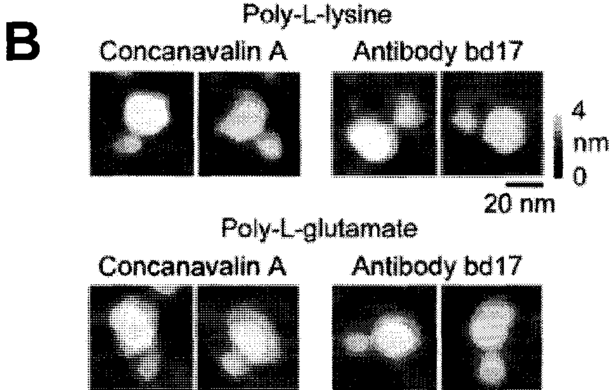

B
Poly-L-lysine
Concanavalin A
Antibody bd17
4
nm
0
20 nm
Poly-L-glutamate
Concanavalin A
Antibody bd17

Table 5-1. Activation of untagged-α4β3(M256)δ and tagged-α4β3(M256)δ $GABA_A$ receptor subtypes by GABA. Data represent the mean ± SEM. Values for log EC_{50} and Hill coefficient were determined from concentration-effect curves. Log EC_{50} and Hill coefficient from individual curves were averaged to generate final mean estimates. The values in parentheses are the number of oocytes used for each receptor subtype. Statistical analysis was performed by comparing the log EC_{50}, Hill coefficient and maximal current amplitude of the untagged-α4β3(M256)δ to those of the tagged-α4β3(M256)δ using the Student *t*-test to determine the level of significance. ** $p<0.01$ compared to tagged-α4β3(M256)δ.

	log EC_{50}	EC_{50} (μmol/L)	Hill coefficient	Maximal current (nA)
untagged-α4β3(M256)δ	−5.37 ± 0.03** (2)	4.3	0.86 ± 0.01	1050.0 ± 209.8 (5)
tagged-α4β3(M256)δ	−5.01 ± 0.03 (3)	9.8	0.90 ± 0.03	871.1 ± 220.0 (12)

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CHAPTER 6

General Discussion

GENERAL DISCUSSION

It has become clear over the last few years that $GABA_ARs$ are located not only at the synapse where they mediate phasic inhibitory currents, but also extrasynaptically, where they mediate tonic inhibition (Farrant and Nusser, 2005). Phasic inhibition results from the intermittent release of high concentrations of GABA from the presynaptic terminals. It serves important functions *in vivo,* allowing neurons to integrate synaptic inputs in a spatially and temporally-restricted manor (Jones and Westbrook, 1996). Tonic inhibition results from the activation of extrasynaptic receptors by low ambient concentrations of GABA. Consequently, tonic inhibition mediated by the $GABA_AR$ leads to a persistent influx of chloride ions into postsynaptic neurons. The total current can be several times larger than that arising from phasic inhibition, and thus plays an important role in maintaining the low excitability of the postsynaptic neurons. A large population of extrasynaptic $GABA_ARs$ contains the δ subunit, which is suggested to co-assemble with $\alpha4$ and β subunits, especially in dentate gyrus granule cells and thalamic neurons. Notably, in these areas, $GABA_ARs$ containing the $\alpha4$ and β subunits are also found to be coexpressed with the $\gamma2$ subunit, which is a putative synaptic isoform. *In vivo*, the expression levels of the putative $\alpha4\beta\delta$ and $\alpha4\beta\gamma2$ receptors in dentate gyrus and thalamus are thought to maintain a delicate balance in the modulation of neuronal function. It has been suggested that one-third of $\alpha4$-containing $GABA_A$ receptor subtypes in the hippocampus and thalamus include the $\gamma2$ subunit while two-thirds co-associate with the δ subunit (Sur *et al.*, 1999). Since their distribution patterns are complementary to each other (Peng *et al.*, 2002), it has been suggested that $\gamma2$- and δ-containing receptors play different roles in neuronal regulation.

In δ knockout animals, the expression of the γ2 subunit is significantly increased in brain areas in which the δ subunit is normally present (Peng *et al.*, 2002). Also, research on animal models of cyclothiazide- or pilocarpine-induced epilepsy demonstrated that the δ subunit in the molecular layer of the dentate was decreased and its expression level remained low throughout the period of chronic seizures (Peng *et al.*, 2004; Qi *et al.*, 2006). In contrast, the expression of α4 and γ2 subunits was increased during the same period (Peng *et al.*, 2004). The α4βδ and α4βγ2 receptors possess significantly different pharmacological and biophysical properties. In recombinant expression systems, the GABA α4β3δ receptor has been shown to have higher agonist sensitivity and to exhibit slower desensitization compared to the α4β3γ2 subtype (Brown *et al.*, 2002). These findings and other similar reports (Wallner *et al.*, 2003; Bianchi *et al.*, 2001) led to my initial studies to identify the structural determinants responsible for these properties in the δ subunit.

Studies described in Chapters 2 and 3 illustrate the use of chimeras to investigate structure-function relationships of the α4β3δ receptor. The original chimeras were constructed using the procedures of Moore and Blakely (1994). In this method, the two parental subunit cDNAs are placed in a head-to-tail fashion in the vector and are separated by a linker sequence containing two unique restriction sites. After cutting both sites, the resulting linear construct is transformed in *E. coli* with a recombinase genotype (e.g. the DH 5α strain). The bacteria are unable to recircularize the complete vector due to mismatching ends. Instead, the two similar sequences from the individual parental subunits are overlaid and a circular construct is produced by alignment of their sequences and swapping of parts to give a circular vector that contains a single chimeric subunit

cDNA. The advantage of this technique, compared to the PCR overlay method to engineer chimeric subunits, is that it does not require the labor-intensive design of PCR primers to introduce compatible restriction sites to generate individual chimeras. In a modified version of this technique, selective excision of one of the parental subunit cDNAs can lead to more targeted random crossover events occurring in the remaining sequence (such as the extracellular N-terminal domain). This approach has been successfully used to identify several structural determinants for benzodiazepine (Boileau *et al*., 1998) or β-carboline (Derry *et al*., 2004) interactions with $GABA_A$ receptors. The limitation of this chimera construction method is that random crossover occurs only in areas with high sequence identity between the two parental cDNAs. Moore and Blakely initially suggested that the crossover happens in a region that has at least 10-15 identical flanking amino acids. This presented a challenge in our construction of δ-γ2 chimeras, because there is a low degree of amino acid homology (~30%) between these two subunits. Thus, it is not surprising that the crossover points of the δ-γ2 chimeras generated are all located within the TM1-2 area, where the two subunits have the highest degree of amino acid identity.

Beyond the advantages and disadvantages of different molecular techniques to construct chimeras and mutations, the interpretation of the results from these studies should be approached with caution. Given the importance of tertiary and quaternary protein folding, it is quite possible that even a single mutation will cause global structural changes in the receptor rather than a localized change. Furthermore, it is often difficult to tell whether any mutation has affected binding, signal transduction, or some other allosteric change perhaps distant from the binding site. As described by Colquhoun

(1998), a single point mutation that results in a 100-fold increase in agonist sensitivity could result from changes in the binding site or channel gating efficacy or both of these effects. In Chapter 2, functional studies of the δ-γ2 chimeric receptors reveal a ~20-fold difference in agonist potency. Our suggestion is that these results are less likely to be due to a global structural change in the binding site, since neither of δ and γ2 subunits has been implicated directly in the binding of GABA or other receptor agonists. This idea is supported by functional characterization of the effects of two competitive antagonists (bicuculline and SR95531), which showed that the apparent affinities for these antagonists in the wildtype and chimeric receptors were not significantly different. Another major issue regarding the mutagenesis studies is how to explain the lack of effect of mutations. However, single amino acids do not act alone and the process of activation inevitably involves many interactions among different structural domains of the protein. This issue is exemplified in the study of Bohme *et al.* (2004). Using α1/α3 subunit chimeras, a segment in the extracellular N-terminal region of α subunits was shown to determine differences in their GABA sensitivity. However, subsequent point mutation analyses suggested that no single amino acid could be implicated in this effect. Instead, the authors identified that a sequential 4-amino-acid substitution within this segment was required to confer the GABA sensitivity switch.

Studies in this thesis used two well-established heterologous expression systems, i.e., the transient expression of cRNAs in *Xenopus* oocytes and the transient expression of cDNA in mammalian cells. In studies of the LGIC family, both of these expression systems have been important in aiding our understanding of the roles of different subunits in the actions of drugs on the receptor complex. Most of the studies described here have

involved the *Xenopus* oocyte expression system. *Xenopus* oocytes are easily maintained and receptors can be expressed by microinjection of cRNAs. Oocytes remain viable for ~10 days after injection and can be voltage-clamped for more than 8 hours with continuous perfusion of buffer. Oocytes express a low number of endogenous membrane transporters and ion channels because they are virtually independent of exogeous nutrients. Furthermore, their large size facilitates electrophysiological analysis of heterologously expressed channels, receptors and transporters. However, *Xenopus* oocyte expression has many limitations. Because of its large size and problems arising from slow perfusion, the *Xenopus* oocyte is not ideal for the study of fast desensitization of LGICs, and peak currents elicited by agonist application cannot be accurately determined. Expression in mammalian cells is the preferred system for detailed kinetic analyses of receptor-ligand interactions. As an example, using excised patches from transfected HEK cells, together with a fast perfusion system, Bianchi and Macdoanld (2001) were able to identify four different stages in $GABA_AR$ desensitization, which we cannot detect using oocyte expression.

A large body of evidence indicates that studies using *Xenopus* oocytes and mammalian cell expression provide comparable results. Using a microtransplantation technique, Palma *et al.* (2003) demonstrated that the rat glutamate receptor 1 (GluR1) and the human neuronal $\alpha4\beta2$ nicotinic AChR expressed in HEK293 cells could be transplanted into *Xenopus* oocytes and they retained the properties of those originally demonstrated in HEK cells. In $GABA_AR$ studies, mutations which affect benzodiazepine affinity have been characterized by electrophysiological studies using both *Xenopus* oocytes and mammalian cells and the results generally agree with radiolabelled ligand

binding studies using cell expression systems (Wieland *et al.*, 1992; Benson *et al.*, 1998; Davies *et al.*, 1998; Dunn *et al.*, 1999; Buhr *et al.*, 1996; Buhr *et al.*, 1997). Relevant to our current studies is the fact that the increase of agonist sensitivity as a result of inclusion of the δ subunit has been well documented in both expression systems (Brown *et al.*, 2002; Wallner *et al.*, 2003; You and Dunn 2007). However, discrepancies in the results obtained using these two systems have also been reported (Khanna et al., 2001; Delisle *et al.*, 2004; Lovinger and Homanics, 2007, see below). One possibility is that mammalian cells provide a more physiological environment for a mammalian protein. Signalling pathways involving second messengers that affect channel modulation also differ between these two expression systems. Other differences include changes in glycosylation that may affect the function and trafficking of ion channels (Thornhill and Levinson 1992). It has been reported that *N*-linked glycosylation is not required for the cell surface expression of functional *Shaker* potassium channels in *Xenopus* oocytes (Santacruz-Toloza *et al.*, 1994), whereas, in HEK cells, glycosylation is essential for maintaining their stability and cell surface expression (Khanna *et al.*, 2001). In addition, the culturing temperatures are different between the two systems (14~19 °C for *Xenopus* oocytes and 37 °C for mammalian cells), and this may affect the expression of temperature-sensitive proteins (Delisle *et al.*, 2004). One example in the $GABA_AR$ research field that illustrates differences between the two expression systems is the controversy over the ability of ethanol to potentiate GABA-induced currents. On the γ2L (but not the γ2S) -containing receptor expressed in *Xenopus* oocytes, GABA-evoked currents were reported to be potentiated by ethanol in the high millimolar range (50-200 mmol/L), concentrations that cause severe intoxication and acute alcohol toxicity in

humans (Wafford *et al.*, 1991; Wafford and Whiting, 1992; Mihic *et al.*, 1997; Ueno *et al.*, 2001; Harris *et al.*, 1997). The $\gamma 2L$ subunit differs from the $\gamma 2S$ with an extra eight amino acids in the region between TM3-TM4 intracellular loop due to alternative splicing. These additional residues in the $\gamma 2L$ subunit contain a consensus phosphorylation site for protein kinase C (Whiting *et al.*, 1990). Wafford and Whiting (1992) suggested that phosphorylation of this site in the $\gamma 2L$ subunit is required for the observed ethanol potentiation on the $\gamma 2L$-containing receptor. The authors further showed that disrupting this phosphorylation site by site-directed mutagenesis results in an ethanol-insensitive receptor when co-expressed with $\alpha 1$ and $\beta 2$ subunits. However, when examining the wildtype $\gamma 2L$-containing $GABA_AR$ expressed in the mammalian cells, very few electrophysiological studies have reproduced the same results. The mechanism underlying this discrepancy is still unknown. Harris *et al.* (1995) showed that alterations in protein kinase C activity can affect the modulation of the $GABA_A$ receptor by ethanol. The authors suggested that the difference of posttranslational modification, such as phosphorylation, of the $GABA_A$ receptor in the two expression systems may be responsible. In addition, intracellular microtubule organization, which is species-dependent, was found to be important for ethanol effects on $GABA_A$ receptor (Whatley *et al.*, 1996). Microtubule depolymerization can significantly decrease ethanol enhancement of muscimol-evoked current on the $GABA_A$ $\alpha 1\beta 1\gamma 2L$ receptors (Whatley *et al.*, 1996). Therefore, the observed discrepancy in ethanol effects on the $GABA_AR$ between two expression systems may also result from their different intracellular microtubule organizations.

In Chapter 4 of my thesis, I report differences in benzodiazepine effects when they act on the $GABA_AR$ $\alpha4\beta3\gamma2$ receptor expressed in either *Xenopus* oocytes or HEK cells. In oocytes, functional studies showed that GABA-mediated currents displayed by the $\alpha4\beta3\gamma2$ receptor were significantly potentiated by diazepam and flunitrazepam. However, binding studies using these two compounds failed to detect high affinity binding of these ligands to the $\alpha4\beta3\gamma2L$ receptor expressed in mammalian cells. The reasons for these disparate results remain unknown. It is possible that there is a difference in receptor assembly in the two expression systems, and that this may explain the differences in pharmacology observed. In addition, the functional properties of the recombinant $GABA_AR$ may be affected by different properties of the membrane, the intracellular ionic composition and differences in intracellular signaling pathways.

FUTURE DIRECTIONS

My thesis research provides insights into our understanding of the differences between the putative extrasynaptic $GABA_AR$ $\alpha4\beta3\delta$ and synaptic $\alpha4\beta3\gamma2$ subtypes. Many more questions have been raised than answered by this work.

With regard to Chapter 2, further studies are required to narrow down the domain responsible for the agonist sensitivity switch determined by the $\delta/\gamma2L$ subunits. There are 11 amino acid residues lying in the S238-V264 domain of the δ subunit that are different from the $\gamma2L$ subunit (see Figure 2-1, C). Six of these residues are semi- or non-conservative substitutions. It will be of interest to examine this region in detail to identify key residue(s) that confer the higher agonist sensitivity resulting from the presence of the δ subunit. However, as discussed in Chapter 2, a concerted movement of the whole S238-

V264 segment is possibly required for the change in agonist sensitivity. In this case, substitution of this whole segment of the δ subunit with the corresponding sequence of the γ2 subunit may be required. Differences in agonist efficacy of the α4β3δ subtype have also not been fully explored. Recently several studies have demonstrated that on the α4β3 subtype, THIP acts as "superagonist" (Storustovu and Ebert, 2006; You and Dunn, 2007). These results suggest that the δ subunit is not the only determinant for agonist efficacy and may explain why no clear pattern for the changes in agonist efficacy emerged from our studies of δ/γ2 chimeric subunits (You and Dunn, 2007, Chapter 2). In addition, it would be of interest in the future to investigate the structural determinants in the binary α4β3 receptor which may influence agonist efficacy.

With respect to our desensitization analysis (Chapter 3), our mutagenesis studies provided valuable information for further research. As previously discussed, the *Xenopus* oocyte expression system is not ideal for the study of fast desensitization because of the large size of the cell and slow drug perfusion that leads to a lack of temporal resolution. The expression and analysis of constructs expressed in HEK cells when used together with a fast perfusion system may be useful to verify our existing results. Furthermore, to achieve comparable data to our macroscopic analysis, single channel analysis would be useful to provide a more quantitative interpretation of receptor desensitization (Quick and Lester, 1994). Detailed analysis of the single channel kinetics of mutant receptors will reveal several important parameters of 'gating efficacy', including open time, distribution of open states and burst duration. This will undoubtedly enhance the understanding of the structure–function relationships of the receptor that are involved in the process of desensitization.

With respect to the benzodiazepine effects on the $\alpha4\beta3\gamma2$ receptor illustrated in Chapter 4, the roles of β subunits have not been established. As noted in this chapter, the $GABA_A$ $\alpha4\beta3\gamma2$ receptor, but not the $\alpha4\beta2\gamma2$ and $\alpha4\beta1\gamma2$ subtypes, can be allosterically modulated by diazepam and flunitrazepam. It is generally considered that the benzodiazepine binding site is located at the interface of the α-γ subunits. In the $\alpha4\beta_x\gamma2$ receptors, the $\alpha4$-$\gamma2$ subunit interface fails to provide a binding site for classical benzodiazepines due to a substitution of a histidine residue (H101) in $\alpha1$ subunit by an arginine residue in $\alpha4$ (Wieland *et al.*, 1992; Duncalfe *et al.*, 1996). However, several lines of evidence suggested that a benzodiazepine site distinct from that at the α-γ subunit interface may exist (Sigel *et al.*, 1990; Im *et al.*, 1993; Amin *et al.*, 1997; Dunn *et al.*, 1999, Walters *et al.*, 2000). Walters *et al.* (2000) showed that a low affinity benzodiazepine binding site may be located in the TM2 domains of the α, β and γ subunits. In addition, an alternative benzodiazepine binding site was suggested to reside on the β-γ subunit interface based on the observations depicting the benzodiazepine allosteric modulation on the binary $GABA_A$Rs, which contain only β and γ subunits (Sigel *et al.*, 1990; Im *et al.*, 1993). Furthermore, our present studies also indicate that the $\beta3$ subunit may play a role in determining benzodiazepine activity on the $\alpha4\beta3\gamma2L$ subtype. The role of the $\beta3$ subunit could be investigated by constructing chimeric subunits containing $\beta3$ and $\beta2$ (or $\beta1$) co-expressed with wildtype $\alpha4$ and $\gamma2L$ subunits. Functional studies of these chimeric β subunit-containing receptors could possibly reveal some structural determinants in the $\beta3$ subunit which contributes to the benzodiazepine effect. It will also be interesting to further investigate whether these structural

determinants are involved in the binding of benzodiazepines, signal tranduction or channel gating mechanisms.

Beyond the scope of this thesis study, the *in vivo* characteristics of δ-containing receptors remain to be fully explored. One of the major questions is why the δ subunits are expressed exclusively in the extrasynaptic area. Several lines of evidence suggest that $GABA_AR$-associated proteins might be critical in guiding the receptors to their synaptic or extrasynaptic destinations. One protein that has received considerable attention is gephyrin, a scaffolding protein, which is suggested to mediate the clustering of α2 and γ2-containing receptors to the synaptic area (Essrish *et al.*, 1998). Gephyrin has recently been illustrated to interact directly with the α2 subunit through a segment located in the TM3-4 intercellular loop (Tretter *et al.*, 2008). Inhibition of gephyrin expression using antisense oligonucleotides against gephyrin leads to a dramatic decrease in the density of clusters of α2 and γ2 subunits of $GABA_A$ receptors in the postsynaptic area. In gephyrin knockout mice, the α2 and γ2 subunit were also completely absent in hippocampal cultures. In addition, Jacob *et al.* (2005) suggested that gephyrin may play a role in retaining α2-containing receptors to the synaptic area and thus prevent their diffusion to the extrasynaptic site. In contrast, cluster formation of α5-containing $GABA_ARs$ in the extrasynaptic area requires radixin, an ERM (ezrin, radixin, moesin) family protein that links receptors to the actin cytoskeleton (Loebrich *et al.*, 2006). The α5-containing receptor is predominantly localized extrasynaptically in hippocampus, where it is thought to be important in mediating tonic inhibition (Caraiscos *et al.*, 2004). It has been shown that radixin can locate to, cluster and co-localize with the α5 subunit at extrasynaptic positions. Results from radixin knockout mice and reduction of the expression of radixin

with antisense oligonucleotides showed dramatic decreases of the α5 subunit cluster but not the α1 subunit (Loebrich *et al.*, 2006).

To date, there is no evidence suggesting that radixin can interact with the δ subunit, and whether the extrasynaptic localization of δ subunits is mediated by some $GABA_AR$-associated protein remains to be established. A method to probe δ subunit associated protein would be to use a yeast two-hybrid system. Since most, if not all, $GABA_AR$-associated proteins have been found to interact with the intracellular TM3-4 loops of the $GABA_AR$ subunits, it would be required to fuse the sequence of the δ subunit intracellular TM3-4 loop with a DNA-binding domain and transfect a yeast host cell bearing a reporter gene which is controlled by the DNA-binding domain. However, this fusion protein cannot activate transcription of a reporter gene on its own. Therefore it can be used as "bait" to screen cDNA clones of an adult rat brain library that are fused to an activation domain. If any encoded proteins (the potential δ associated protein) from the cDNA library can interact with the δ subunit intracellular TM3-4 loop, their fused activation domains can then activate the reporter gene in the yeast host cell (Criekinge and Beyaert, 1999). If any associated proteins are identified, then to substantiate the interaction data biochemically, a co-immunoprecipitation experiment using rat brain extracts and a pull-down assay would be required. Further experiments, such as immunogold labeling of δ-associated protein with electron microscopy, would be critical to demonstrate an extrasynaptic localization of this protein. Using antisense oligonucleotide or siRNA to inhibit the expression of the associated protein and then examining the electrophysiological characteristics of the neurons with different pharmacological agents would reveal the function of the associated protein.

Another interesting area that remains to be investigated is the plasticity of δ subunit expression under the influence of neurosteroids. It has become evident that physiological and pathophysiological neurosteroid alterations during the menstrual/estrous cycle, pregnancy and puberty can induce alterations of δ subunit expression (Maguire *et al.*, 2005 Lovick *et al.*, 2005, Griffiths and Lovick, 2005, Shen *et al.*, 2007). Changes of the expression of δ subunits have also been seen during the treatment with neurosteroids and after the withdrawal of neurosteroids (Smith *et al.*, 1998a, b; Gulinello and Smith 2003). These observations may be particularly relevant to menstrual cycle-related disorders, such as premenstrual dysphoric disorder and catemenial epilepsy. Recently it has been shown that changes in the expression of δ subunits can occur rapidly in acute stress situations and these changes have been suggested to be mediated directly by GABA-active neurosteroid metabolites and not *via* an interaction with hormone receptors (Shen *et al.*, 2005; Maguire and Mody, 2007). In future, further experiments are required to elucidate how neurosteroids and their metabolites regulate δ subunit expression. There is some evidence that neurosteroids may have direct effects on the activation of various protein kinases and subsequently can modulate the phosphorylation of their substrates (Rebas *et al.*, 2005). Phosphorylation is often required to activate $GABA_AR$-associated proteins such as radixin for the α5 subunit (Loebrich *et al.*, 2006). If the unknown δ subunit-associated protein (as discussed above) has similar characteristics, then neurosteroid modulation of this protein through phosphorylation process should be investigated.

In conclusion, the data in this thesis research show an effort to use multidisciplinary approaches to study the putative extrasynaptic α4β3δ receptor and the

putative synaptic $\alpha4\beta3\gamma2$ receptor. Present studies and ongoing research focusing on these subtypes will undoubtedly provide an enhanced appreciation with respect to receptor pharmacology, biophysics, structure-function relationships and receptor expression which could have tremendous therapeutic potential in the treatment of numerous neurological and psychiatric disorders and will be beneficial to subtype-specific drug design.

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