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Exploring Subtype-Specific Differences in Allosteric Modulation of the γ-Aminobutyric Acid Type A Receptor

by

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Introduction

INTRODUCTION

The γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) is the major inhibitory neurotransmitter receptor in the mammalian CNS. Many aspects of its structure (e.g. ligand binding domains and the protein regions that form the ion channel structure) and function (receptor activation, desensitization and allosteric modulation) have been studied. Probing the structure and function of the receptor and its agonist and allosteric binding sites has included biochemical studies such as radioligand binding to examine ligand affinity and electrophysiological techniques to study its functional characteristics. Many diverse approaches have shed light on $GABA_AR$ properties, and the evolutionary relatedness and sequence conservation between $GABA_AR$ subunits and the better-characterized nicotinic acetylcholine receptor (nAChR; see below) have broadened this understanding. The recent identification (Smit et al., 2001) and determination of the 2.7Å atomic structure (Brejc et al., 2001) of a molluscan acetylcholine binding protein from *Lymnaea stagnalis* (AChBP) that bears sequence homology to the extracellular domains of the nicotinic acetylcholine receptor (nAChR) (and to a lesser degree the $GABA_AR$) has permitted more rational and targeted studies of the function, and the underlying structure, of these receptors. Specifically, comparative models, aligning the sequences of $GABA_AR$ subunits to that of the AChBP (e.g. Cromer et al., 2002) have begun to guide these newer approaches. A major advancement will be the determination of subtype-specific structural features that underlie differences in agonist activation and allosteric modulation of those receptors. The advent of model-based studies has begun to tease apart some of these (often subtle) subtype differences.

GABA: OUTLINE

GABA is an amino acid neurotransmitter with actions that are typically inhibitory. It is formed by the decarboxylation of another amino acid neurotransmitter, glutamate, in a reaction catalyzed by the enzyme glutamic acid decarboxylase. The metabolism of GABA shares some overlap with carbohydrate oxidation via the Krebs cycle, but enzymes of a specific pathway (the GABA shunt) exist in the CNS for GABA metabolism (see Cooper et al., 2003). GABA is unique as a neurotransmitter in that as a zwitterion, it carries no net charge at physiological pH, a feature suggested to enhance

reliability of signaling due to reduced electrostatic interaction with other charged entities at the synapse (Roberts and Sherman, 1993). Specific receptors for this neurotransmitter exist in the CNS to mediate its neurochemical effects. GABA binds to and activates a heterogeneous population of receptors that can be broadly divided into the ionotropic receptors for GABA (the $GABA_AR$ and the related $GABA_C$ receptor; $GABA_CR$) and the metabotropic $GABA_B$ receptor ($GABA_BR$).

Metabotropic $GABA_BRs$ are 7-transmembrane domain G-protein coupled receptors (GPCRs) that transduce the binding of GABA into an intracellular response, producing changes in intracellular second messenger (G-protein-mediated) signaling pathways that act on specific Ca^{2+} and K^+ ion channels (reviewed in Bowery et al., 2002). These receptors function as a dimer of two linked GPCR proteins (see Marshall et al., 1999).

The $GABA_AR$ is a ligand-gated ion channel (LGIC) receptor that incorporates a chloride-selective ion channel pore. Binding of the neurotransmitter to its recognition sites induces a conformational change in the receptor structure, thereby gating the integral channel. The resulting increase in chloride conductance generally has a hyperpolarizing effect on neurons and this membrane-stabilizing effect is the basis of the inhibitory activity. An array of $GABA_AR$ subtypes with different properties is known (see below). These diverse subtypes have been studied in cultures of neurons from different brain regions and also in recombinant systems whereby a $GABA_AR$ of defined subunit composition can be selectively expressed. Pharmacological features specific for the $GABA_AR$ include activation by the mushroom toxin, muscimol, competitive antagonism by the plant alkaloid, bicuculline, and selective blockade of ion channel function by picrotoxin (reviewed in Sieghart, 1995; Korpi et al., 2002). Other hallmark features of the $GABA_AR$ include multiple agonist binding sites (see below), concentration-dependent desensitization of the agonist response and the existence of numerous allosteric sites through which a wide range of compounds can modulate GABA-gated effects, the best characterized being the benzodiazepine (BZD) site (e.g. see Sieghart, 1995; Korpi et al., 2002).

In the mature mammalian CNS, the inhibitory actions of GABA are due to its actions at both $GABA_ARs$ and $GABA_BRs$. Although GABA acting via the $GABA_AR$

mediates the majority of inhibitory neurotransmission in the mature CNS, a reversal of chloride gradients in the embryonic stage permits excitatory activity of GABA (see Ben-Ari, 2002), the function of which may be linked to trophic or developmental signaling in embryonic or immature neuronal circuits (see Owens and Kriegstein, 2002). Furthermore, despite its widespread inhibitory actions, in select mature neuronal populations and under specific pathologic settings, GABA can act as an excitatory transmitter in the mature CNS (reviewed in Stein and Nicoll, 2003).

A role of GABA as a signaling molecule outside of the mammalian CNS is known. Although GABA effects are best characterized in the mammalian central nervous system, peripheral expression of $GABA_AR$ subtypes in mammalian enteric nervous tissue (see Galligan, 2002), the pancreas (Yang et al., 1994; von Blankenfeld et al., 1995), reproductive organs (Hedblom and Kirkness, 1997) and adrenal medulla tissue (e.g. bovine; Parramon et al., 1994) suggest a potential peripheral signaling role of the $GABA_AR$. Furthermore, its capacity as a neuronal signal is not limited to mammals, as inhibitory ionotropic GABA receptors in invertebrates have been characterized from several insect species (see Hosie et al., 1997) and the nematode *Caenorhabditis elegans* (see Schuske et al., 2004). Additionally, a specific GABA-gated cation-conducting excitatory channel, EXP-1, has been isolated from *C. elegans* (Beg and Jorgensen, 2003), adding to the diversity in ionotropic receptors for GABA. Moreover, GABA is known to act as a developmental factor in certain plant species (see Bouché and Fromm, 2004), highlighting its ubiquity as a useful chemical signal mediator.

THE $GABA_AR$: INTRODUCTION

The study of the $GABA_AR$ binding sites and receptor function began before structural information was available for the individual subunits that comprise the receptor. For example, specific BZD binding sites in the brain were shown to be associated with the $GABA_AR$ and these studies furthered the concept of it being a receptor with additional binding sites (see below). Early heterologous expression of brain mRNA in *Xenopus* oocytes was carried out to reproduce $GABA_AR$ responses for study (Houamed et al., 1984; Barnard et al., 1984). Additionally, $GABA_AR$ was isolated from native mammalian (e.g. bovine) brain membranes with a variety of detergents; use

of specific solubilization protocols permitted the retention of properties of native receptors (reviewed by Stephenson, 1988). $GABA_AR$s within these solubilized bovine brain preparations could be purified by BZD-affinity chromatography (e.g. Sigel et al., 1983; 1984) and such purification permitted an early investigation of subunit size and the protein composition of the $GABA_AR$, as well as a providing a source of receptor for examining the pharmacological properties of GABAergic ligands (e.g. Sigel et al., 1984). Purification of the $GABA_AR$ permitted the isolation and partial amino acid sequencing of two subunit proteins, the α- and the β-subunit (Schofield et al., 1987). Determination of these partial sequences allowed for the design of synthetic oligonucleotide probes for the screening of bovine brain cDNA libraries, ultimately leading to cloning of these subunits (Schofield et al., 1987).

The cloning and sequencing of the α- and β-subunits led to studies that revealed extensive $GABA_AR$ subunit diversity at the primary amino acid sequence level. Beyond separate subunit classes (e.g. α- or β-), subsequent identification of additional isoforms within one subunit class (e.g. Levitan et. al, 1988) began to reveal the assortment of subunits and the heterogeneity of $GABA_AR$ subtypes. Identification of the homologous subunit proteins in rat and human eventually followed initial studies that were mostly on bovine $GABA_AR$. Currently, 19 related $GABA_AR$ subunits, grouped into eight major classes, have been cloned from mammalian sources. These subunits are classified as α1-6, β1-3, γ1-3, δ, ε, π, θ (and ρ1-3) (reviewed in Barnard et al., 1998; Korpi et al., 2002). Specifically for the rat, the α1,2 (Seeburg et al., 1990), α3,5 (Malherbe et al., 1990), α4 (Wisden et al, 1991), α6 (Lüddens et al., 1990), β1-3 (Ymer et al., 1989), γ1 (Ymer et al., 1990), γ2 (Shivers et al., 1989), γ3 (Herb et al., 1992), δ (Shivers et al., 1989) ε (Davies et al., 1997), π (Hedblom and Kirkness, 1997) and θ (Bonnert et al., 1999) have all been cloned. Additional diversity exists due to splice variants of certain subunits (e.g. $\gamma 2_S/\gamma 2_L$; Whiting et al., 1990). Heterogeneity of receptor subtypes is underscored by a distinct brain region expression pattern for each subunit as determined by *in situ* hybridization (e.g. Wisden et al., 1992; Laurie et al., 1992a) or immunocytochemistry (Pirker et al., 2000). Developmental changes in expression are also known for individual subunit isoforms (Laurie et al., 1992b); this temporal component to subunit expression further

expands $GABA_AR$ heterogeneity by limiting which isoforms can assemble into receptors at different times during development.

$GABA_ARs$ are heteropentameric protein complexes (Figure 1-1A; reviewed in Korpi et al, 2002). Receptors are comprised of subunits from several different classes (see above) and the particular combination of the specific subunit isoforms determines the properties of the receptor that is formed. Sequence analysis of the first two identified $GABA_AR$ subunits (Schofield et al., 1987) showed homology to the subunits of the nAChR and allowed for structural predictions. In comparison to the nAChR subunits, $GABA_AR$ subunits share ~25% sequence conservation (see Barnard et al., 1998). For $GABA_AR$ subunits themselves, sequence homology between different subunit classes is ~30%, whereas a greater conservation (~70%) exists for isoforms within a class (see Korpi et al., 2002).

The three isoforms of the ρ subunit class are related in sequence to the other $GABA_AR$ subunits, but they are known to preferentially assemble with each other and to produce a receptor with unique properties. This related receptor, the $GABA_CR$, has a unique pharmacology (e.g. bicuculline-insensitivity) and is also a GABA-gated chloride channel, which has selective retinal expression (see Enz and Cutting, 1998; Bormann, 2000; Zhang et al., 2001).

The initial cloning and sequencing of subunits of the $GABA_AR$ (Schofield et al., 1987) shed light on the structure of both the individual subunit and the receptor complex. These studies indicated homology and similarity to the nicotinic acetylcholine receptor (nAChR). Subsequent characterization of the other $GABA_AR$ subunits and those from the glycine receptor (GlyR; Grenningloh et al., 1987) and the serotonin type-3 receptor ($5\text{-}HT_3R$; Maricq et al., 1991) demonstrated the homology of these LGIC receptors. The subunits of these receptors all bear a degree of sequence homology and have similar putative topologies, suggesting an evolutionary relatedness (Ortells and Lunt, 1995) with the subunits of the $GABA_AR$ belonging to a more specialized superfamily of evolutionarily related proteins for inhibitory ionotropic amino acid transmitter receptors (Xue, 1998). A recently identified novel, but distantly related, member of this receptor superfamily, a zinc-activated channel (ZAC; Davies et al., 2003) broadens the number of receptors related to the $GABA_AR$.

$GABA_AR$: SUBUNIT SEQUENCE AND TOPOLOGY

All of the $GABA_AR$ subunits are predicted to share a similar topology, with each having four putative membrane-spanning (or transmembrane; TM) domains (Schofield et al., 1987). The first TM domain (TM1) is preceded by approximately one half of the subunit sequence (~220 amino acids) (Figure 1-1B,C). This N-terminal region is the extracellular portion of the protein and it contains a disulfide link between two conserved cysteine residues (the "Cys-loop"), a hallmark feature of this LGIC receptor superfamily. The extracellular domain also has consensus sequences for *N*-linked glycosylation (e.g. Schofield et al., 1987; Pritchett et al., 1989a; Shivers et al., 1989). As the N-terminal domain is extracellular in the quaternary $GABA_AR$ structure, this soluble domain contains segments from which select amino acid residues contribute to the binding domains for GABA and other ligands for the receptor (see below). The remaining proposed topology of each subunit consists of TM1, followed by a small intracellular region, TM2, the extracellular TM2-TM3 linker region, TM3, then a large intracellular domain. This large intracellular loop is of variable length and can contain consensus sequences for phosphorylation (see below) depending on the particular subunit (Schofield et al., 1987). Lastly, the putative topology includes TM4 and a short, extracellular C-terminal domain (see Figure 1-1).

The five subunits in the $GABA_AR$ pentamer form both the extracellular neurotransmitter binding sites and the ion channel portion of the receptor. Binding of agonist at the synapse-exposed extracellular sites induces a conformational change that elicits increased chloride conductance through the channel. Early studies examining electron microscopy (EM) images of tubular crystals of the homologous native nAChR predicted that the TM2 domain adopts an α-helical conformation, whereas the secondary structure of the remaining TM segments may adopt β-strand conformations (Unwin, 1993; 1995). More recent EM imaging at higher resolution predicts that all four TM domains of the nAChR adopt an α-helical structure (e.g. Miyazawa et al., 2003), in line with a similar prediction based on the sequence of the putative TM domains of LGIC receptors (Schofield et al., 1987; Bertaccini and Trudell; 2002). The TM2 helices from each subunit are predicted to line the channel pore and contain the selectivity filter and resistance gate to impede ion flow (reviewed in Kash et al., 2004). However, recent

evidence suggests that intracellular domains, including the large TM3-TM4 segment (Kelley et al., 2003) and the TM1-TM2 region (Fillipova et al, 2004), in other members of the LGIC family contribute to the pore of the ion channel and play a role in the regulation of ionic conductance.

STOICHIOMETRY OF THE $GABA_AR$

In homology with other members of the LGIC receptor class, $GABA_ARs$ are pentameric protein complexes. The inclusion of five subunits was first demonstrated for the *Torpedo* electric organ nAChR (Raftery et al., 1980) and analysis of electron microscopy images of native porcine receptors in membrane preparations suggested the same pentameric nature for $GABA_ARs$ (Nayeem et al., 1994). With the identification of a wide diversity of receptor subunits, the potential for a seemingly incalculable number of different combinations of five subunits within a receptor pentamer would give the $GABA_AR$ extensive heterogeneity. The number of physiologically relevant subtypes, however, has been suggested to be limited to a discrete subpopulation of all of the potential subtypes, perhaps numbering as few as 10 distinct combinations (McKernan and Whiting, 1996). More recent examinations of the diversity (reviewed in Sieghart et al., 1999; Sieghart and Sperk, 2002) have, however, indicated numerous minor populations of additional subtypes that have putative brain region-specific functional roles.

The most commonly expressed $GABA_AR$ subtypes are likely comprised of α-, β-, and γ-subunits, though other subunits (e.g. δ) can substitute for the γ-subunit for co-expression in the pentamer (see Barnard et al., 1998). It is thought that subunits from these classes assemble in the receptor in a stoichiometry of 2α-, 2β- and a single γ-isoform (Figure 1-2). Evidence to support this has been gleaned from a range of studies that used different approaches to examine the relative presence of each subunit class in a receptor. For example, the relative change in potency for activation of the receptor due to a specific mutation in each individual subunit class (Chang et al., 1996) indicated the contribution of each and suggested the 2α:2β:γ stoichiometry. The same stoichiometry is suggested for receptors comprised of αβγ subunits by the interaction of subunit-specific monoclonal antibodies as detected by Western blotting (Tretter et al., 1997) or density sediment centrifugation (Knight et al., 2000). The preceding two references indicate a

stoichiometry of 2α:3β for receptors comprised of αβ subunits, implying that inclusion of the γ-subunit is accomplished by substituting for a single β-subunit. Further confirmatory evidence for this stoichiometry was achieved using fluorescence energy transfer between tagged antibodies (Farrar et al., 1999). Evidence for additional diversity of $GABA_AR$ subtypes is known, with the suggestion that the pentamer can contain different isoforms of one subunit class (e.g. α-isoforms; see Duggan et al., 1991; Pollard et al., 1993). A specific inclusion of both the α1- and the α6-isoforms in the same native receptor from cerebellar tissue has been demonstrated by co-immunopurification using isoform-selective antibodies (Pollard et al., 1995; Khan et al., 1996). These studies have capitalized on the specificity of isoform-selective antibodies to determine which subunits associate (e.g. see Jechlinger et al., 1998) and specifically for the α-subunit, have indicated the inclusion of two α-subunit proteins per $GABA_AR$ (e.g. Pollard et al., 1993, 1995). Further co-purification studies have revealed that for some receptor subtypes, as many as four or possibly five different subunits can be included (see Sieghart et al., 1999; Sieghart and Sperk, 2002).

Elucidation of the arrangement of the subunits in the pentamer is another feature of $GABA_AR$ structure that has recently been addressed. Using α-β and β-α subunit concatamers (Baumann et al., 2001) it has been shown that recombinant receptors are not expressed as tetramers or hexamers. Functional $GABA_ARs$ with these concatamers could be expressed only in combination with individual β- or γ-subunits, suggesting a pentameric assembly and supporting the proposed stoichiometries of 2α:2β:γ or 2α:3β for functional receptors (Baumann et al., 2001). Further studies with dimer and trimer linked subunits (concatamers of two and three subunits) (Baumann et al., 2002) provides evidence that in functional $GABA_ARs$ with native properties, subunits are expressed in the clockwise arrangement of γ-α-β-α-β when viewed from the synaptic cleft (see Figure 1-2, 1-4). Homology modeling of the extracellular domain of αβγ $GABA_ARs$ (Trudell, 2002), based on the atomic structure of the molluscan AChBP (Brejc et al., 2001), indicates that this same arrangement is the only pattern that agrees with all evidence to date on subunit-subunit contact points for assembly and the location of GABA agonist sites and the BZD site at subunit interfaces (see below). Additional evidence that

contributes to the understanding of subunit arrangement has come from the recent exploitation of atomic force microscopy to visualize the interaction of monoclonal antibodies with $GABA_AR$ subunits containing specific antibody tags. These studies have permitted an examination of multiple antibodies with a recombinant $\alpha1\beta2\gamma2$ $GABA_AR$ that show a specific separation of α-subunits by an angle consistent with separation by one intervening (non-α) subunit (Neish et al., 2003). Such direct visualizations are in agreement with other available data that suggest that for $\alpha1\beta2\gamma2$ $GABA_ARs$, the clockwise γ-α-β-α-β pattern accounts for the predicted stoichiometry.

$GABA_AR$ ASSEMBLY

Specific domains of certain $GABA_AR$ subunits have been identified as mediators of subunit-subunit interactions and it appears that an ordered assembly process dictates which subunits can co-assemble. Since receptors are not simply formed by a random association of any of the known subunit isoforms, only a discrete (though currently unknown) number of specific combinations can be possible.

Studies of $GABA_AR$ assembly have revealed discrete sequences of the extracellular N-terminal domain of individual subunits that mediate the protein-protein interactions between individual subunits. In particular, initial evidence of specific assembly signals was found for the β3-subunit that permits selective homo-oligomerization (see Connolly et al., 1996b) and membrane expression rather than retention in the endoplasmic reticulum (ER). Four amino acid residues in the β3 isoform (Gly^{171}, Lys^{173}, Glu^{179} and Arg^{180}) were identified as specific mediators of homo-oligomeric surface expression (Taylor et al., 1999). Using a similar approach whereby the association of truncated subunits, or proteins with specific deletions, with a second subunit was assessed, a discrete region in the α1 subunit ($\alpha1Met^{57}$-Ser^{68}), and the particular importance of $\alpha1Gln^{67}$ for the assembly of α1- with β-subunits was determined (Taylor et al., 2000). Other studies have indicated a role of the α1(80-100) extracellular segment for a specific interaction with γ2-, but not β3-subunits (Klausberger et al., 2001b); the corresponding recognition domain for α-subunits in the γ-subunit was identified as γ2(91-104) (Klausberger et al., 2000). These data complement the

observations that $\alpha 1Trp^{69}$ and $\alpha 1Trp^{94}$ are necessary for the assembly and surface expression of pentameric receptor complexes (Srinivasan et al., 1999). From these studies, it is clear that discrete domains of the extracellular portion of α-subunits underlie a selective association with both β- and γ-subunit proteins. More recent evidence highlights assembly signals for specific subunit isoform associations. Critical domains have been identified within the β3- (76-89; 85-89; Ehya et al., 2003) and γ3- (70-84; 86-95; 94-107; Sarto et al., 2003) isoforms, and subdomains are predicted to mediate interaction with different neighbouring proteins. Additionally, specific evidence from the γ2-isoform indicates an absolute requirement of $\gamma 2Ser^{171}$ for subunit-subunit interaction and receptor expression (Jin et al., 2004).

The assembly of functional $GABA_AR$s depends on both the oligomerization of subunits to form the pentameric complex and the proper neuronal membrane insertion. The association of two subunit proteins via their assembly sequences is the initial step in the process. The detection of lower-order assemblages of receptor subunits (dimers, trimers, etc.) has shed light on the likely sequence of interaction and the subcellular movement of nascent $GABA_AR$s. Initial investigations showed that whereas $\alpha 1\beta 2\gamma 2_L$ and $\alpha 1\beta 2$ receptors are functional, not all subunit associations can lead to the formation of viable receptors. Specifically, there is the retention (and presumed degradation) of $\alpha 1/\gamma 2_L$ and $\beta 2/\gamma 2_L$ dimers, as well as monomers (α-, β-, and γ-subunits) in the ER (Connolly et al., 1996a). Dimerization of these subunit pairs is dependent only on the N-terminal domain (see Klausberger et al., 2001a). Furthermore, the particular β-isoform incorporated into $GABA_AR$s was shown to mediate intracellular trafficking of the assembled receptor to different membrane regions (see Connolly et al, 1996b), indicating an additional receptor subtype-specific feature of assembly.

The body of evidence gained thus far on $GABA_AR$ assembly suggests that selective N-terminal domains mediate a defined process of receptor construction and restrict the stoichiometry and exact subunit composition of receptors (reviewed in Bollan et al., 2003b). These limited pathways rely on major assembly sequences in each class of subunit, but the formation of dimers between any two classes of α-, β- and γ-subunits suggests that multiple assembly sequences from each subunit could be involved (see

Klausberger et al., 2001b, Bollan et al., 2003a,b). Each individual subunit in the functional pentamer can be considered to have polarity; that is, it has two interfaces for association with other subunit proteins. The α1-subunit is predicted to possess at least three assembly domains, with one facing an interface for the association with a β-subunit, and two facing the other interface for interaction with either another β- or the γ-subunit. The interaction of the α- and γ- assembly domains is suggested to control receptor assembly and stoichiometry; selective use of the α-γ interaction permits the inclusion of only a single γ-subunit and sets the arrangement around the pentamer (Klausberger et al., 2001b).

Taken together, assembly of ternary complexes of subunits has a critical dependence on the α-γ subunit interaction, but functional expression is only possible for that dimer upon subsequent interaction with β-subunits (e.g. Connolly et al., 1996a; Kittler et al., 2000). An alternative interaction via additional recognition domains for a further β-subunit can replace the interaction with γ, consistent with the concatamer studies of Baumann et al. (2001) and evidence indicating functional binary receptors (αβ) expressed without a third subunit (e.g. Verdoorn et al., 1990; Bencsits et al., 1999).

The most striking aspect of the data delineating assembly domains in the individual $GABA_AR$ subunits is the concurrence of these subunit contact regions with N-terminal segments implicated in forming agonist binding sites or the BZD allosteric site at subunit-subunit interfaces (e.g. Smith and Olsen, 1995; Sigel and Buhr, 1997). The accord between subunit segments implicated in both GABA binding and BZD site binding (see below), and those predicted to interface between subunits, strengthens the placement of these binding sites at the point of apposition between subunits (see Bollan et al., 2003a,b).

Of additional note is that intracellular domains of $GABA_AR$ subunits can also mediate subunit-subunit interactions. Specifically, the segment in the γ2 subunit intracellular loop that elicits interaction with GABARAP (see below) also permits an interaction with other $GABA_AR$ complexes via the β- or γ-subunit (Nymann-Andersen et al., 2002b), indicating even greater control over the assembly of individual and clustered receptors.

$GABA_AR$ TRAFFICKING

Signaling through the $GABA_AR$ can be modulated by both the phosphorylation state of the receptor (see below), and the regulation of the relative number of receptors at a GABAergic synapse. Although the exact role of phosphorylation on all receptor subtypes is not clear and the intracellular transport pathways for the trafficking of receptors are incompletely understood, advances have been made in these areas of modulation of GABAergic signaling (reviewed in Moss and Smart, 2001; Kittler and Moss, 2003; Lüscher and Keller, 2004).

Certain proteins have been identified as $GABA_AR$-associated proteins. A specific $GABA_A$ receptor-associated protein (GABARAP; Wang et al., 1999) has been identified as a protein that can indirectly link the receptor to other intracellular proteins. GABARAP can bind the large intracellular loop of γ-subunits (Nymann-Andersen et al., 2002a) and is predicted to have a role in connecting $GABA_ARs$ to intracellular vesicular transport proteins, providing an association with pathways for receptor clustering and turnover (reviewed in Kittler and Moss, 2003). Additional proteins that associate with the $GABA_AR$ and participate in post-synaptic clustering and the dynamic cycling of receptor expression include anchoring proteins (e.g. gephyrin), proteins of the clathrin/dynamin-dependent, AP2 adapter-related endocytic machinery (Herring et al., 2003), protein kinases and related anchoring proteins (e.g. p130, NSF, RACK-1, AKAP150/79 and PKC-βII) and the ubiquitin-like Plic-1 (Bedford et al., 2001) protein that is part of the endocytic pathway (reviewed in Moss and Smart, 2001; Kittler and Moss, 2003). Other proteins that associate with receptor subunits include a β2-specific interaction of $GABA_A$ receptor interacting factor-1 (GRIF-1) (Beck et al., 2002) and a β-selective association with gC1q-R (Schaerer et al., 2001), proteins also proposed to be involved in regulating receptor trafficking and function. These proteins are proposed to play a role in dynamic receptor turnover, including endocytosis and the targeting of $GABA_ARs$ from the ER to the membrane, and also to play a role in the dynamic maintenance of the number and function of the expressed receptors, as indicated by abnormal $GABA_AR$ function with certain mutations in, or knockouts of, those proteins (reviewed in Kittler and Moss, 2003). These associated proteins themselves can have an

effect on receptor function. For example, evidence suggests that GABARAP co-expression with $GABA_AR$s alters both the kinetics of channel activation (Chen et al., 2000) as well as single channel conductance levels, bestowing native receptor conductances to recombinant receptors that otherwise do not display all native receptor conductance levels (Everitt et al., 2004). These data suggest a role for the associated proteins in the regulation of the properties of the $GABA_AR$s that they bind.

$GABA_AR$ PHOSPHORYLATION

Phosphorylation of $GABA_AR$s is a means of dynamic modulation of receptor function and stability. Select α-, β-, and γ-subunits contain consensus sequences for phosphorylation in the large intracellular loop between the TM3 and TM4 segments by a range of protein kinases, the consequence of which can have receptor subtype-specific effects (reviewed in Brandon et al., 2002; Kittler and Moss, 2003). Specifically, the β1-3 and γ2 subunits are potential targets for phosphorylation by protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), and calcium-calmodulin dependent kinase II (CaM kinase II); the tyrosine kinase *Src* is also known to phosphorylate the γ2 subunit (see Brandon et al., 2002; Kittler and Moss, 2003). CaM kinase II has also been shown to specifically phosphorylate the α1 subunit (Churn et al., 2002). Phosphorylation of these receptor subunits has been studied on purified receptor protein, recombinant receptors expressed in cells, and on neurons in culture to confirm specificity of phosphorylation, effects on the function of receptors and to detect basal levels of phosphorylation in neurons or cells.

A direct effect on the function of the $GABA_AR$ due to phosphorylation has been observed. For example, phosphorylation of different subtypes of the $GABA_AR$ by PKA (e.g. Moss et al., 1992) and PKC (e.g. Krishek et al., 1994) is known to alter current amplitude and kinetics. More recent evidence indicates that a PKA-mediated effect via β1 and β3 subunit phosphorylation has specific effects on the kinetics of $GABA_AR$ desensitization, suggested to have a role in modulating the duration of synaptic GABA currents (Hinkle and Macdonald, 2004). Studies using neuronal cultures suggest a basal, constitutive phosphorylation of the $GABA_AR$ by several kinases (e.g. *Src* tyrosine kinase;

Brandon et al., 2001), as well as the involvement of endogenous phosphatase enzymes that regulate the level of receptor phosphorylation (Minier et al., 2000).

The phosphorylation state of the $GABA_AR$ plays a role in the modulation of both receptor function and trafficking (see above). For example, levels of stable cell surface expression of recombinant receptors and receptors in cortical and hippocampal neurons, regulated through constitutive endocytosis, have been shown to be dependent on both the presence of the γ2-subunit and to be regulated by PKC-mediated phosphorylation (Connolly et al., 1999). Several kinases and kinase anchoring proteins associate with the $GABA_AR$ and phosphorylation is a mechanism for the rapid, dynamic modulation of the endocytosis and recycling pathways that regulate cell surface expression levels (reviewed in Brandon et al., 2002; Kittler and Moss, 2003).

The overall state of the neuron with respect to the activation of other metabotropic pathways that influence phosphorylation can have an effect on $GABA_AR$ function and expression. Other receptors that are linked to intracellular kinases have $GABA_ARs$ as downstream targets, and the clustering and association of kinases, kinase anchoring proteins and phosphatases with the $GABA_AR$ make these receptors signaling complexes. Specifically, modulation of $GABA_AR$ activity has been shown for signaling elicited by brain-derived neurotrophic factor (BDNF) via TrkB receptors (Jovanovic et al., 2004). The activation of metabotropic receptors for other neurotransmitters (e.g. dopamine, serotonin, acetylcholine) that alter PKA and PKC activity provides a link between these signaling pathways (see Kittler and Moss, 2003). The distinction between synaptic signaling mediated by ionotropic and metabotropic receptors is thus blurred as the common intracellular signal messengers can have regulatory roles. A further interaction is seen for the $GABA_AR$ in its association and interaction with its metabotropic counterpart, the $GABA_BR$. Recent evidence indicating that a physical association with the $GABA_AR$ alters the expression and cellular turnover of $GABA_BR$ subunits (Balasubramanian et al., 2004) suggests that there can be a direct link between the signaling mediated by both types of GABA receptors. Additionally, metabotropic G-protein signaling has been shown to affect LGIC function in a novel way; the interaction of G-protein βγ dimers can directly modulate GlyR channel function (Yevenes et al.,

2004). It remains to be seen if subtypes of the $GABA_AR$ are also susceptible to this additional form of cross talk and modulation.

Phosphorylation of the $GABA_AR$ or associated proteins can affect the response to certain modulatory compounds. Changes in the sensitivity of hippocampal neurons to neurosteroid compounds (e.g. Harney et al., 2003) or BZDs (Lilly et al., 2003) have been shown following modulation of PKA and PKC activity. Furthermore, the phosphorylation of the $\alpha 1$ subunit by CaM kinase II has been shown to subsequently enhance the binding of BZD site ligands (Churn et al., 2002). Mice with a knockout of the *Src*-related tyrosine-specific *fyn*-kinase show altered responsiveness to etomidate and a reduction in density of expressed functional $GABA_ARs$ (Boehm 2^{nd} et al., 2004). Additionally, a role of altered receptor phosphorylation as a mechanism underlying the longitudinal changes associated with the tolerance and dependence phenomena of chronic BZD administration has been proposed. Interference with PKA- and PKC- mediated phosphorylation of recombinant $GABA_ARs$ can affect the uncoupling of the GABA and BZD sites that is expected after persistent exposure to the BZD modulator (Ali and Olsen, 2001). Furthermore, diazepam (DZP) administration can cause short-term alteration in the levels of certain neuronal transcripts including those for some kinases and related signaling proteins; mice that bear a specific "knock-in" mutation that removes diazepam-sensitivity at the $\alpha 1$-containing $GABA_AR$ (see below) show differential changes in the transcript levels for certain of these intracellular signals (Huopaniemi et al., 2004). These results indicate the potential for a long-term reciprocal interaction between phosphorylation pathways that affect, and are acted upon by, $GABA_AR$-mediated activity as a mechanism of tolerance. Alterations in the function of the expressed $GABA_A$ receptors, or plasticity of GABAergic synapses due to dysregulation of receptor trafficking may be the mechanisms involved (see Kittler and Moss, 2003). Of additional note is the recent evidence from studies of neurons that contribute to the circadian rhythm in the suprachiasmatic nuclei that showed that casein kinase family proteins have a periodic association with $GABA_AR$ $\beta 2/3$ subunits and can regulate $GABA_AR$ function; this association may contribute to diurnal variation in function (Ning et al., 2004). This reinforces the view that various kinases and related proteins are

involved in modulating $GABA_AR$ function, either through direct receptor phosphorylation or the formation of signaling complexes.

SYNAPTIC VERSUS EXTRASYNAPTIC $GABA_AR$

Ionotropic $GABA_ARs$ have been localized to both synaptic densities and to extrasynaptic (or perisynaptic) regions of neurons. The activity of each type of receptor, and the subunit composition of each, are known to differ. Those $GABA_ARs$ expressed at synaptic densities are known to mediate phasic conductances in response to released GABA; extrasynaptic $GABA_ARs$ are presumed to respond to much lower background concentrations of GABA in a neuroendocrine-like fashion to elicit tonic conductances that regulate neuronal excitability (Mody, 2001). The subunits most likely to incorporate into extrasynaptic $GABA_ARs$ are generally presumed to include the α4-, α5-, α6-, and the δ- subunits (see Mody, 2001) and such tonic $GABA_AR$ inhibition has been described for cerebellar granule cells (e.g. Nusser et al., 1998) and for neuronal circuits between certain hippocampal cells (e.g. Yeung et al., 2003).

The higher potency of GABA to activate receptors that mediate the tonic conductances can be seen in recombinant $GABA_AR$ preparations that incorporate the δ-subunit (e.g. Adkins et al., 2001; Brown et al., 2002). Receptors that contain the δ-subunit also show reduced and slowed desensitization compared to γ-subunit containing receptors (Haas and Macdonald, 1999), further demonstrating unique properties of δ-containing subtypes. The δ-subunit has been identified by immunogold labeling exclusively at perisynaptic locations in cerebellar (Nusser et al., 1998) and hippocampal (Wei et al., 2003) neurons. Theoretically, inclusion of the δ- in place of a γ-subunit would be expected to have consequences for receptor synaptic clustering that is dependent on the γ-subunit (see above); whether non-synaptic localization is simply due to this substitution is not clear for all neuronal types. The exact composition of these perisynaptic receptors remains to be determined for various neuronal circuits, but recent additional evidence indicates a role of α5-containing $GABA_AR$ in tonic inhibition in the hippocampal CA1 region (Caraiscos et al., 2004). These presumed extrasynaptic $GABA_AR$ subtypes are also selectively modulated by lower (physiologically relevant)

concentrations of ethanol (e.g. Sundstrom-Poromaa et al., 2002; Wallner et al., 2003) and certain neurosteroids (Stell et al., 2003), suggesting that the perisynaptic $GABA_AR$ subtypes that regulate neuronal excitability by means of their tonic conductance may be selective targets for the action of these compounds.

The $GABA_AR$: AGONIST BINDING SITES

The association of GABA with its agonist binding domains on the $GABA_AR$ complex is the initial step in receptor activation. The step of GABA binding is suggested not to be rate limiting in channel activation (Maconochie et al., 1994). Upon agonist binding, specific conformational changes are induced that lead to channel gating and the increase in chloride conductance. Whereas there is considerable knowledge about the location of the agonist sites, the mechanism for the gating of the $GABA_AR$ is less well understood. Recent evidence has begun to suggest potential molecular mechanisms for the transduction of the binding event at the GABA agonist sites in the extracellular N-terminal domain to the ion channel gate within the transmembrane region (see Kash et al., 2003, 2004; Absalom et al., 2004). Although recent advances in molecular modeling of the $GABA_AR$ (see below) have permitted better assumptions about channel gating and associated molecular mechanisms, the most conclusive studies on the $GABA_AR$ have focused on the receptor binding sites.

Our current understanding of the GABA binding sites proposes that there is an asymmetrical contribution of protein domains from two subunits at their interface to form a binding site. The identification of the agonist binding domains of the $GABA_AR$ has been achieved through various means, including photoaffinity labeling, examining the functional consequences of mutagenesis and probing for accessibility with substituted cysteine accessibility mutagenesis (SCAM). Initial studies with the photolabile agonist [^{3}H]muscimol indicated incorporation of the ligand into the β-subunit of the $GABA_AR$ complex (Casalotti et al., 1986; Deng et al., 1986). However, additional labeling of the α-subunit had also been reported in addition to β-subunit incorporation (e.g. Bureau and Olsen, 1988; 1990). These data indicated the accessibility of both the β- and α-subunits to agonist interaction and provided early evidence for a role of both neighbouring proteins in forming the agonist site. A specific substrate for muscimol incorporation was

eventually identified as α1Phe^{64} (Smith and Olsen, 1994). Mutation of this residue (α1F64L) had been previously shown to greatly reduce the ability of GABA to activate the $GABA_AR$; this mutation had also been identified as a rare natural mutation that altered receptor gating (Sigel et al., 1992).

The agonist binding sites on the $GABA_AR$ are predicted to lie at subunit-subunit interfaces (see Smith and Olsen, 1995), in homology to the nAChR (reviewed in Corringer et al., 2000; Karlin, 2002). For the nAChR, critical residues for agonist and antagonist interaction with the α-subunit, as determined by photolabeling studies, cluster into three discrete, non-contiguous segments of the extracellular N-terminal domain, and such knowledge permitted the suggestion of a multiple-loop model of the binding site for ligand interaction (Galzi et al., 1990; Galzi and Changeux, 1994). Other protein domains from the adjacent subunit are proposed to contain residues critical for the binding site as well. This model is extended to include at least 6 discrete regions within the N-terminal domain of every subunit that can contribute to binding pockets at subunit interfaces. Three of these domains (Loops A, B and C) are from one subunit as the principal component whereas the opposing subunit would contribute the other three domains (Loops D, E and F) as the complementary side; this is a common motif for members of the LGIC receptor family (see Corringer et al., 2000; Karlin, 2002). In this manner, each subunit is predicted to have polarity; Loops A-C face one neighbouring subunit (the + face) whereas the Loop D-F segments oppose another subunit (the – face) (see Figures 1-1C, 1-2, 1-3D, 1-4). In homology to the nAChR, identification of residues important for GABA binding are found to be distributed in the same homologous regions and some of the residues themselves are conserved across members of the LGIC superfamily (e.g. see Ernst et al., 2003), suggesting a specialized role in forming the agonist sites.

A binding site for GABA is formed at each of the two β+/α- subunit interfaces, and these sites are postulated to mediate activation of the receptor (see Smith and Olsen, 1995). Essential residues from the β-subunit (from the "+ face") include (rat β2 numbering) Loop A amino acids Tyr^{97} and Leu^{99} (Boileau et al., 2002). The second domain (Loop B) contains important residues Tyr^{157} and Thr^{160} (Amin and Weiss, 1993). Within the Loop C region, other important residues have also been determined and include Thr^{202} (Amin and Weiss, 1993), Ser^{204} (Wagner and Czajkowski, 2001), Tyr^{205}

(Amin and Weiss, 1993; Wagner and Czajkowski, 2001), Arg^{207} and Ser^{209} (Wagner and Czajkowski, 2001).

Residues from the $GABA_AR$ α-subunit implicated in forming the agonist site for channel activation can be clustered into three domains, homologous to Loops D, E and F, indicating that the "– face" of the α-subunit participates in this GABA site. In addition to (rat α1 numbering) Phe^{64} (Sigel et al., 1992), other α-subunit residues in Loop D include Arg^{66} (Boileau et al., 1999; Hartvig et al., 2000) and Ser^{68} (Boileau et al., 1999). The second segment, Loop E, includes Arg^{119} (Westh-Hansen et al., 1999; Hartvig et al., 2000) and Ile^{120} (Westh-Hansen et al., 1997). Loop F residues have been identified as Val^{178}, Val^{180} and Asp^{183} (Newell and Czajkowski, 2003) (see Table 1-1; Figure 1-5).

Although homology to the nAChR predicts the asymmetric contribution from two neighbouring subunits for the formation of the GABA agonist sites, and domains from both the β- and α-subunits are crucial for GABA binding, actual information on the structural features of the binding pocket was still lacking with only the identification of residues involved. The classification of binding domains as "loops" was not based on any structural information, and although it could be presumed that these non-contiguous segments form a binding cleft accessible to the synaptic/extracellular space, no actual structural features could be predicted solely from the concept of three critical domains from each opposing subunit. The isolation of the homopentameric molluscan AChBP (Smit et al., 2001) that is homologous to the N-terminal domains of LGIC receptor subunits, and the determination of its atomic structure (Brejc et al., 2001; see Figure 1-3, 1-4) has allowed for comparative modeling of the structure of the N-terminus of $GABA_AR$ subunits (e.g. Cromer et al., 2002; Trudell, 2002; Ernst et al., 2003; Chou, 2004). These studies have begun to guide further investigations into the actual structure of the important GABA binding site regions (see Table 1-1).

Prior to AChBP-based molecular modeling efforts, the defining of the secondary structure of LGIC subunits had been attempted. For example, the homology of sequence and proposed structure of LGIC N-terminal domains with previously identified domains of the bacterial biotin repressor protein complex permitted secondary structure models that include a substantial β-sheet component (Gready et al., 1997). Examining data from fluorescence spectroscopy and circular dichroism of the truncated α1-subunit N-terminal

protein segments overexpressed in *Escherichia coli* cells also suggested a large contribution of β-strand regions in the extracellular segment (Hang et al., 2000), a feature conserved in β-and γ-subunits (Shi et al., 2003). Although these studies suggest secondary structural features, interpretation of structural data gathered from truncated subunit proteins may not be applicable to the structure of the N-terminal domain in a complete subunit.

A comparison of the AChBP sequence and that of the N-terminal of LGIC subunits reveals about ~15-30% conservation of sequence, an amount indicative of reduced structural homology (see Ernst et al., 2003). However, certain invariant residues align between the AChBP and other LGIC members, suggesting conserved roles in structure and function; there is also the prediction of retention of the overall β-sheet-rich, immunoglobin-like shape of the subunit structure (e.g. Cromer et al., 2002; Ernst et al., 2003). The crystal structure of the AChBP (Figure 1-3) reveals that the secondary structures of each subunit is an N-terminal α-helix followed by mostly β-strands; certain loop regions as linkers between β-sheets and including the conserved Cys-loop characteristic of the receptor family are also structural features (Brejc et al., 2001; see Figure 1-3).

Recent modeling studies have used sequence alignments between $GABA_AR$ subunit N-terminal domains and the AChBP to thread the $GABA_AR$ sequence onto the known AChBP structure in an initial attempt to derive a model structure of the extracellular domain of a heteropentameric $GABA_AR$. Determination of the crystal structure of the AChBP was carried out with a HEPES buffer (Brejc et al., 2001). HEPES is serendipitously predicted to interact with the protein in a manner analogous to ACh at the nAChR (Brejc et al., 2001; Grutter and Changeux, 2001). The ligand-binding residues and surrounding structures in the AChBP are suggested to be conserved and therefore, comparative modeling of the $GABA_AR$ based on the AChBP would be predicted to resolve features of the GABA agonist sites. The recent AChBP-derived models (Cromer et al., 2002; Trudell, 2002; Ernst et al., 2003) have revealed a concordance of the model with biochemical (e.g. photoaffinity labeling) and mutational data with respect to the spatial proximity of residues that contribute to the GABA site as well as N-terminal domains that underlie subunit-subunit contact for assembly. Receptor

subtype-specific models based on the sequence of different α-subunit isoforms have also recently been put forth (Chou, 2004).

Comparative modeling supports the concept of discrete domains of each subunit forming the binding site, and the models provide an assumption of the subunit secondary structure of these segments at the β+/α– interface. An alignment of the three "loops" from the principal β-subunit (A-C), and the complementary domains (Loops D-F) from the α-subunit, with the sequence and structure of the AChBP has been performed (reviewed in Kash et al., 2004). The β2Tyr^{97}-Asp^{101} (Loop A, + face) corresponds to structural β-sheet 4 and part of structural loop 5; β2Glu^{155}-Thr^{161} (Loop B, + face) aligns to β-sheet 7 and into loop 8; β2Phe^{200}-Arg^{207} (Loop C, + face) overlaps structural loop 10 and into β-sheet 10; α1Asp^{63}-Ser^{69} (Loop D, – face) coincides with β-sheet 2; α1Ile^{118}-Thr^{130} (Loop E, – face) encompasses β-sheet 5, loop 6, and into β-sheet 6; and α1Ala^{177}-Gly^{186} (Loop F, – face) falls within loop 9 (see Kash et al., 2004; Table 1-1).

Although the secondary structure of $GABA_AR$ subunit extracellular domains has been predicted by modeling, more direct approaches have provided confirmatory evidence for the structure of individual "Loop" regions. The majority of these studies have capitalized on SCAM studies to probe for solvent accessibility of engineered cysteine mutations, and any changes in this accessibility in the presence of agonist (i.e. presumably in the open/activated state). In accordance with comparative modeling of this region, an examination of the α1 Loop D segment reveals a predicted β-strand structure from αThr^{60}-Ser^{68}, based on a pattern of alternating residues that are modified by cysteine-specific reagents (Boileau et al., 1999). The pattern of accessible residues has also allowed for an examination of the proposed secondary structure of α1 Loop E as two β-strand regions separated by an intervening loop (Holden and Czajkowski, 2001) and that of α1 Loop F as a variable coil structure in that domain (Newell and Czajkowski, 2003). Within the β2-subunit, similar SCAM studies have revealed that the β2Val^{93}-Leu^{99} segment adopts a β-strand (Boileau et al., 2002) for the Loop A domain and that the Loop C exists as a random coil (Wagner and Czajkowski, 2001), in line with homology models. Confirmatory evidence of a β-strand structure for the region homologous to Loop D has been shown for the $GABA_AR$ γ-subunit γ2Thr^{73}-Thr^{81}

(Teissére and Czajkowski, 2001). With the caveat that the engineered cysteines may alter the conformation of mutation-bearing receptors to an unnatural state, such SCAM studies have refined comparative models of the extracellular domain of $GABA_AR$ subunits by assigning secondary structure to sequences of the protein. Comparative models can incorporate this accessibility, and in this manner, the fit of the AChBP structure can be adjusted for specific $GABA_AR$ subtypes (see Table 1-1).

Another feature that has arisen from comparative modeling is a better prediction of the role of specific residues in GABA site function. In particular, SCAM studies conducted in the presence of agonist or antagonist propose that a reduction of the rate of cysteine modification is indicative of competitive interaction of the ligand with the modifying agent at a particular engineered cysteine, thereby identifying solvent-accessible residues that likely form a subdomain within the binding pocket for interaction with that ligand. These experiments have shown several accessible residues that interact differently with GABA and an antagonist, SR 95531 (e.g. Holden and Czajkowski, 2001; Holden and Czajkowski, 2002; Newell and Czajkowski, 2003), providing evidence for an antagonist-specific interaction in the GABA binding site. Antagonists can produce specific conformational changes distinct from those elicited by GABA. The SCAM studies complement additional evidence showing allosteric activity of GABA antagonists on direct $GABA_AR$ activation by anesthetics (Ueno et al., 1997) and antagonist-induced conformational changes in the receptor as assayed by the shifts in fluorescence of a tethered probe (Chang and Weiss, 2002). Overall, these data suggest that antagonists act in a more complex manner than just simply a steric hindrance of GABA interaction.

Furthermore, comparison of sulfhydryl modification both with and without compounds that can gate the receptor (e.g. pentobarbital), can reveal subsets of residues that are differently accessible in distinct receptor states (e.g. Wagner and Czajkowski, 2001). A similar approach with SCAM studies has been done to probe the changes in accessibility that occur in the TM2 ion channel region upon $GABA_AR$ gating (e.g. Horenstein et al., 2001). In this manner, an appreciation of different conformational states of the receptor can be gained; changes that occur within the GABA site upon GABA binding (see Bianchi and Macdonald, 2001) can be observed, an initial step for understanding the transduction of the binding to the gating effect within the receptor.

Modeling of the GABA site at the β(+)/α(-) interface has proved useful for further elucidation of the roles of other residues identified to contribute to GABA interaction. An assay of macroscopic GABA currents does not individually examine the underlying (and inter-related) phenomena of binding of agonist and gating of the ion channel (see Colquhoun, 1998), and therefore other kinetic studies must be carried out to separate binding from gating effects (e.g. Wagner et al., 2004). However, predictions as to the role of a residue can be made based on the modeled structure. Recently, additional α-subunit residues that underlie receptor subtype sensitivity to GABA have been identified in the α6- (α6Ser^{83}; Drafts and Fisher, 2004) and the α3- (α3Leu^{173}-Asn^{176}; Böhme et al., 2004) isoforms. These residues model towards the β-α interface, but are further towards the synaptic space (i.e. further away from the membrane-surface) than the other identified GABA site amino acid residues (see above) and lie outside of the modeled binding pocket, in line with their presumed roles of contributing to a subtype-selective architecture of the GABA site, but not a direct role in binding (see Drafts and Fisher, 2004; Böhme et al., 2004). Here, comparative modeling provides a rationale for the interpretation of the contribution of a residue to GABA activity based on presumed accessibility to the agonist.

The $GABA_AR$ is predicted to have an agonist binding site involved in channel activation (low affinity sites) at each β(+)/α(-) interface (see Smith and Olsen, 1995), consistent with models of activation that predict a requirement of two agonist molecules to gate the receptor. Based on the presumed subunit arrangement around the pentamer (clockwise from the synapse: γ-α-β-α-β), the two sites are flanked by a different subset of neighbours. Studies similar to the ones that elucidated subunit arrangement (see above) have been done whereby one concatenated dimer and one concatenated trimer are expressed; the ability to mutate individual α- or β- subunits in each of these linked subunit constructs permits an examination of the functional roles of the individual GABA sites, and has suggested non-equivalent affinities of GABA at each site (Baumann et al., 2003). Other studies have used similar approaches to replicate the heterozygous state for epilepsy-associated mutations in other domains of the α1-subunit (Gallagher et al., 2004). Additionally, SCAM studies have suggested divergence in the conformational change induced in each of the two individual α-subunits upon allosteric modulator binding

(Williams and Akabas, 2001), consistent with the notion that the two GABA sites do not have identical properties.

Additionally, binding studies have revealed a high and a low affinity component to GABA (e.g. Olsen et al., 1981) or to muscimol (e.g. Agey and Dunn, 1989) association. Binding studies on brain membrane preparations that include multiple receptor subtypes could account for these affinity differences. Also, whether the high and low affinity binding components reflect two separate sites or an interconvertible state of binding at a single class of sites on one receptor subtype is a matter not yet resolved. It is presumed that receptor activation would be mediated by low affinity sites, in agreement with higher (micromolar) concentrations required for $GABA_AR$ activation in functional studies. Evidence for a separate high affinity site has been described for a defined recombinant subtype (Newell et al., 2000) that includes a contribution from $\beta 2Tyr^{62}$, the residue from "Loop D" that is homologous to $\alpha 1Phe^{64}$ known to be critical in forming the (low affinity) GABA site. This lends support to the concept of additional GABA sites at the other subunit-subunit interfaces [e.g. $\alpha(+)/\beta(-)$], and a role of this site in stabilizing the receptor in a desensitized state has been proposed (Newell and Dunn, 2002).

THE BENZODIAZEPINE BINDING SITE

Of the multiple allosteric modulatory sites on the $GABA_AR$, the BZD binding site is the best characterized. Early evidence showed specific BZD binding sites in the brain (Braestrup and Squires, 1977; Möhler and Okada, 1977) that were suggested to influence and have overlap with the $GABA_AR$ (e.g. Macdonald and Barker, 1978; Costa et al., 1979). Co-purification of the $GABA_AR$ and BZD receptors (Gavish and Snyder, 1981) and isolation of a complex that could bind not only GABA site ligands but also BZDs and barbiturates (e.g. Sigel et al., 1984) provided early confirmation of the physical association between the BZD binding site and the $GABA_AR$.

Occupation of the BZD binding site by a ligand generates a conformational change in the ternary structure of the heteropentamer. The consequences of BZD binding are allosterically linked to the GABA binding sites, and BZD site ligands span the continuum of efficacy from negative to null to positive efficacy with respect to

modulation. Potentiation of $GABA_AR$ function by BZD occurs by increasing the frequency of GABA-gated channel openings (Study and Barker, 1981; Twyman et al., 1989), activity that is initially elicited by enhancing the affinity of GABA for at least one of the agonist binding sites (Lavoie and Twyman, 1996).

Certain BZD site ligands also act as photoaffinity labels and identification of the substrate for covalent incorporation provided some of the first data indicating the location of the site at the α(+)/γ(-) subunit-subunit interface (see Sigel and Buhr, 1997). Both the classical 1,4-BZD [^{3}H]flunitrazepam (FNZ) (Möhler et al., 1980) and the imidazobenzodiazepine (*i*-BZD) [^{3}H]Ro15-4513 (Möhler et al., 1984) are useful photolabels. Certain β-carboline ligands (or derivatives) can also photolabel the BZD site (e.g. Dellouve-Courillon et al., 1989; Zezula, 1995). FNZ was shown to incorporate into protein bands consistent with α-subunit isoforms (e.g. Sigel et al., 1984; Casalotti et al., 1986; Fuchs et al., 1988; Stephenson et al., 1990), with possibly lesser labeling of γ-subunits (Stephenson et al., 1990). In analogy to the agonist binding sites formed at subunit-subunit interfaces, these early photolabeling data support a contribution to the BZD site from both the α- and γ-subunits. Furthermore, initial recombinant expression of $GABA_ARs$ comprised of only α- and β-subunits could not accurately recreate BZD-sensitivity in functional studies (Schofield et al., 1987); sensitivity could be endowed to recombinant receptors only with the inclusion of the γ-subunit (Pritchett et al., 1989a), consistent with its contribution to the BZD binding site.

In recombinant preparations, both the specific α- (e.g. Puia et al., 1991; Wieland et al., 1992; Smith et al., 2001) and γ- (e.g. Puia et al., 1991; Lüddens et al., 1994; Davies et al., 2000) isoforms incorporated can affect BZD pharmacology, indicative of a contribution of each subunit to the BZD site. Accordingly, the BZD site itself exhibits heterogeneity, as was demonstrated very early in the classification of BZD binding in different brain regions (e.g. Sieghart and Karobath, 1980; Nielsen and Braestrup, 1980), consistent with distinctive CNS expression patterns for different receptor subtypes (e.g. Pirker et al., 2000). Based on the inclusion of a specific α-subunit isoform (α1-3,5), different classes of BZD site (BZ1 or BZ2; e.g. Pritchett et al., 1989b; Pritchett and Seeburg, 1990, 1991) with specific properties and selective ligands can be formed; these

subtypes all recognize DZP and are classified as the diazepam-sensitive (DS) subtypes. Inclusion of either the α4- or α6-isoform confers insensitivity to DZP and other classical 1,4-BZDs, leading to the designation of such subtypes as diazepam-insensitive (DI).

Several residues from both the α- and the γ-subunit have been identified as contributing to the BZD site pharmacology. Some of the initial investigations to identify the determinants of ligand interaction at the BZD site exploited differences in ligand affinity at receptor subtypes or the divergence in DS and DI BZD pharmacology to identify specific residues mediating divergent receptor properties. For example, investigations into the distinctive BZ1 and BZ2 pharmacology permitted the identification of specific α-isoform residues that affect selective BZD binding (e.g. Pritchett et al., 1989b; Pritchett and Seeburg, 1990, 1991). The creation of a series of chimeric subunits derived from the α1- and the α6-isoforms was used to probe DS pharmacology and identified (rat α1 numbering) $\alpha1His^{101}$ as a significant determinant of high affinity binding of classical BZD ligands; an arginine aligns at the homologous residue in the DI subtype α4- and α6-subunits. (Wieland et al., 1992). Further mutational analyses identified a subset of four residues that are divergent between the α1- and the α6-isoforms that at least in part mediate the differences with respect to DS and DI pharmacology (Wieland and Lüddens, 1994).

Identification of the specific domain for photoincorporation is another method that has revealed determinants of BZD binding and function. Upon photolabeling of the BZD site on $GABA_AR$s with [^{3}H]FNZ or [^{3}H]Ro15-4513, each ligand covalently incorporates within different regions of the α1-subunit, implying a differential interaction of each ligand within the BZD site. The incorporation of FNZ occurs within bovine $GABA_AR$ α1(1-103) whereas Ro15-4513 labels C-terminal to that point (Duncalfe and Dunn, 1996; Davies et al., 1996). A specific residue for the photoincorporation of [^{3}H]FNZ was identified as $\alpha1His^{101}$ (Duncalfe et al., 1996) and [^{3}H]Ro15-4513 was suggested to incorporate within the small extracellular TM2-3 domain (Davies and Dunn, 1998). More recent studies have identified the specific substrate of Ro15-4513 labeling as $\alpha1Tyr^{209}$ and the homologous tyrosine in the α2- and α3-isoforms (Sawyer et al., 2002). Isoform-specific labeling of different residues has been proposed for Ro15-4513

at bovine α1- and α6-subunits (Duncalfe and Dunn, 1996); however, this was not shown in cell lines expressing the same human $GABA_AR$ subtypes (Davies et al., 1996). Although it could be argued that the photolabeling reaction done under UV irradiation does not reflect normal pharmacological conditions, the identification of photolabeling substrates defines a direct point of contact of the ligand in its binding site, enabling further mutagenesis studies to probe for a role of the substrate residue in BZD pharmacology.

Since all the $GABA_AR$ subunits share a degree of sequence homology and a common putative topology, it can be presumed that the subunit-subunit interfaces formed with any two subunits are structurally similar. In accordance with this homology, the amino acid residues that have been implicated in contributing to the BZD site cluster into the same homologous discrete regions in the extracellular N-terminal domain that contain determinants of binding and function at the β-α GABA sites. Based on alignment of sequence, the α-subunit ("+" subunit face, Loops A, B and C) and γ-subunit ("–" subunit face, Loops D and E) contain BZD site residues at homologous positions to those from β- and α-subunits implicated in the GABA site (see Sigel, 2002; Ernst et al., 2003). Comparative modeling of the $GABA_AR$ α- and γ-subunits (e.g. Cromer et al., 2002; Trudell, 2002; Ernst et al., 2003) has allowed predictions of the secondary structure of these "loop" domains, as can be done for the β(+)-α(-) GABA binding site (see above). Confirmation of the β-strand secondary structure of the γ2-subunit $\gamma2Thr^{73}$-Thr^{81} region ("Loop D") with SCAM studies has been reported (Teissére and Czajkowski, 2001).

To date, the residues that have been implicated by photoaffinity labeling and mutagenesis studies to contribute to the BZD site from the α-subunit include (rat α1 numbering): $\alpha1Pro^{96}$ (Smith and Olsen, 2000), $\alpha1His^{101}$ (Wieland et al., 1992; Wieland and Lüddens, 1994; Duncalfe et al., 1996; Davies et al., 1998; Dunn et al., 1999; Smith and Olsen, 2000), $\alpha1Tyr^{159}$ (Amin et al., 1997), $\alpha1Thr^{162}$ (Wieland and Lüddens, 1994; Renard et al., 1999) $\alpha1Gly^{200}$ (Pritchett and Seeburg, 1991; Schaerer et al., 1998; Renard et al., 1999), $\alpha1Ser^{204}$ (Renard et al., 1999; Casula et al., 2001), $\alpha1Ser^{205}$ (Derry et al., 2004), $\alpha1Thr^{206}$ (Buhr et al., 1997a; Schaerer et al., 1998), $\alpha1Tyr^{209}$ (Buhr et al., 1997a;

Amin et al., 1997; Sawyer et al., 2002), and α1Val^{211} (Wieland and Lüddens, 1994; Strakhova et al., 2000; Casula et al., 2001).

A number of γ-subunit residues have also been implicated and include (rat γ2 numbering): γ2Met^{57} and γ2Tyr^{58} (Kucken et al., 2000), γ2Phe^{77} (Wingrove et al., 1997; Buhr et al., 1997b; Buhr and Sigel, 1997), γ2Ala^{79} and γ2Thr^{81} (Teissére et al., 2001; Kucken et al., 2003), γ2Met^{130} (Wingrove et al., 1997; Buhr and Sigel, 1997) and γ2Thr^{142} (Mihic et al., 1994).

It is of note that the presence of another, flumazenil-insensitive, site for BZD activity has been described. This low (micromolar) affinity site is distinct from the well characterized site at the α-γ interface. Mutation of certain TM2 domain residues that are important for anesthetic modulation of the $GABA_AR$ (see below) can remove this low affinity BZD component of receptor modulation, suggesting a degree of overlap of this secondary site and that for anesthetic modulation (Walters et al., 2000).

Beyond occupation of the BZD site by ligand, elements that mediate the transduction of the binding to the overall allosteric effect on receptor function have been identified as distinct from the binding site itself. Approaches using chimeric subunits derived from γ/α (Boileau et al., 1998; Boileau and Czajkowski, 1999) or γ/δ (Jones et al., 2002) subunits have identified required γ-subunit domains in the pre-TM1, TM1, TM2 and the extracellular TM2-3 loop that are critical for allosteric modulation. Additionally, specific BZD site ligands demonstrate $GABA_AR$ subtype-selective allosteric effects (e.g. between α1- and α6-containing receptors). Specifically, despite the DI pharmacology of the α6-subtype, Ro15-1788 acts as a positive modulator compared to an antagonist of the BZD site at DS subtypes (e.g. Im et al., 1997). A defined region within the extracellular N-terminal domain (α1Thr^{162}-Leu^{188}) has been determined to underlie the specific differences in Ro15-1788 modulatory effect (Im et al., 1997). This domain aligns to the α-subunit "Loop F" (structural loop 9; see Ernst et al., 2003), predicted to contribute to the GABA site at the neighbouring interface. It is proposed that this extracellular domain plays a role in the allosteric coupling of the BZD and GABA sites (Im et al., 1997; see Ernst et al., 2003).

Comparative modeling of the BZD site has been accomplished based on homology of the extracellular domains of subunits of the $GABA_AR$ to the AChBP (e.g. Cromer et al., 2002; Ernst et al., 2003). These models have helped align all of the previous data that propose the orientation of BZD site ligands in their binding pocket. In addition to mutational approaches to define the BZD pharmacophore, ligand mapping approaches have yielded three dimensional quantitative structure-activity relationships (3D-QSAR) for compounds docked in the BZD site and have revealed subtype-specific features of the BZD site formed with different α-subunits (e.g. Huang et al., 1999; He et al., 2000). Recent models have oriented DZP at the α-γ interface based on the selective modification of an engineered cysteine at α1H101C by DZP derivatized with a sulfhydryl-reactive group incorporated at the C7 point in the 1,4-BZD chemical backbone (Figure 1-7) (e.g. Berezhnoy et al., 2004). Other models orient *i*-BZD ligands in the BZD site based on the photolabeling of $\alpha 1Tyr^{209}$ by the C8 azido group of Ro15-4513 (Sawyer et al., 2002) and the presumed association of the C3′ imidazo-substituent on the opposite end of the structure (Figure 1-7) with $\gamma 2Ala^{79}$ and $\gamma 2Thr^{81}$ (Kucken et al., 2003). With a proposed orientation of a BZD ligand docked in a comparative model, relative molecular distances to other critical residues can be estimated. These docking models improve upon the predictions of orientation based upon screening the affinity of a panel of closely chemically related compounds at receptors with specific mutations (e.g. McKernan et al., 1998; Sigel et al., 1998). Overall, comparative modeling of the BZD site has permitted an alignment of some of the data from QSAR studies with molecular data from mutational work to better predict BZD ligand interaction. Of more importance will be to interpret ligand mapping data that shows $GABA_AR$ subtype differences in the BZD binding pocket with different α-subunit isoforms and to examine the consequences of mutations in the binding pocket across different receptor subtypes (see Sigel, 2002) to reveal differences in the BZD site produced with different α-subunits. Recent homology modeling has predicted subtype-specific differences in the placement and orientation of the side chain of BZD site amino acid residues within the binding pocket (Chou, 2004), implying a theoretical basis for differences in QSAR studies across receptor subtypes.

Another line of experimentation has examined the $GABA_AR$ subtype-selective function of BZD site ligands. The heterogeneity of receptor subtypes in the CNS is

partially a function of the diversity of expression of subunit isoforms, often with brain nucleus specificity. Classical BZD ligands like DZP and FNZ possess affinity for all of the DS subtypes, irrespective of the individual α-subunit isoform (α1-3,5) incorporated. Clinical use of 1,4-BZD compounds produces a spectrum of results including sedative, anxiolytic, myorelaxant and anticonvulsive effects. The use of mice with specific knock-in point mutations whereby one particular α-subunit isoform is rendered diazepam-insensitive has provided *in vivo* results that indicate which clinical aspects are mediated by which $GABA_AR$ subtypes, and has contributed to the understanding of the roles of brain regions that preferentially express these receptor subtypes (reviewed in Rudolph and Möhler, 2004). The specific substitution of α1H101R is known to underlie DI pharmacology (e.g. Wieland et al., 1992) and homologous substitutions of α2H101R, α3H126R and α5H105R abolish DZP sensitivity in recombinant preparations (Benson et al., 1998). Mice that are bred to contain individual knock-in DI mutations can be administered BZD site ligands to assess the *in vivo* behavioral changes due to lack of ligand recognition at one selective subtype.

In this manner, particular clinical outcomes of BZD administration are dissected across $GABA_AR$ subtypes. *In vivo* behavioral studies with mice bearing knock-in DI mutations have indicated that the sedative effects of BZD ligands like DZP are achieved by action at α1-containing $GABA_ARs$ (Rudolph et al., 1999). An role of the α1-subtype in mediating anterograde amnesia, and a partial anticonvulsant contribution induced by DZP has also been demonstrated (Rudolph et al., 1999). Pharmacological confirmation of the α1-subtype selective sedative effect has been achieved with a novel BZD site ligand (L-838,417) that shows no sedative effect *in vivo*; this compound is an antagonist at the α1-subtype and a positive modulator at the α2-, α3- and α5-subtypes (McKernan et al., 2000). Additionally, BZD site negative allosteric modulators that are proconvulsant and stimulatory also exert these effects via the α1-subtype (Crestani et al., 2002a). The α2-$GABA_AR$ subtype (which has selective expression in the limbic system) has been shown to mediate anxiolytic effects of DZP (Löw et al., 2000), the majority of its myorelaxant activity (with a lesser effect elicited through the α3-subtype) (Crestani et al., 2001), as well as DZP precipitation of narcosis (but not sedation) *in vivo* when

combined with alcohol (Täuber et al., 2003). In addition to the high-dose myorelaxant effects that are generated via the α3-subtype, $GABA_A$Rs with the α3-isoform are also predicted to have a selective influence on neuronal oscillations linked to absence epilepsy in thalamic nuclei and their modulation by another BZD ligand, clonazepam (Sohal et al., 2003). Roles of the α5-subtype have been predicted to include a myorelaxant influence and hippocampal effects on trace fear conditioning in associative learning (Crestani et al., 2002b). Additionally, a role of the hippocampal α5-subtype in the induction of tolerance to DZP has also been demonstrated (van Rijnsoever et al., 2004).

Examinations of the effects of knock-in mutations have also clarified the selective substrates of action of individual BZD site ligands, in particular for zolpidem (ZOL). This imidazopyridine compound has selective affinity for α1-containing $GABA_A$Rs (e.g. Pritchett and Seeburg, 1990) and similar studies with α1(H101R) knock-in mice have revealed that the sedative/hypnotic and anticonvulsant effects of ZOL are mediated by the α1-subtype *in vivo* (Crestani et al., 2000). Recent examination of a novel knock-in γ2(F77I) mouse model demonstrates the abolition of ZOL effect *in vivo*, but not that of flurazepam (another 1,4-BZD) in homozygous mutant mice (Cope et al., 2004). The γ2F77I substitution has been shown to selectively abolish ZOL binding in recombinant preparations (Wingrove et al., 1997; Buhr et al., 1997b) and the knock-in γ2F77I mouse confirms that γ2-containing $GABA_A$Rs are the subtypes through which ZOL acts. Taken together, the specificity of ZOL activity *in vivo* for the α1- (Crestani et al., 2000) and the γ2- (Cope et al., 2004) subtypes indicates that the sedative/hypnotic effect of ZOL is mediated by the BZD site formed specifically at the α1-γ2 subunit-subunit interface at $GABA_A$Rs that contain these subunits.

These studies have demonstrated subtype-selective effects of BZD site ligands. That only a subset of the clinical spectrum of BZD site ligands like DZP is mediated through one α-subunit receptor subtype illustrates the potential for therapeutic compounds that can dissect the spectrum of effects. These studies further the potential for selective sedatives/hypnotics, anxiolytics, anticonvulsants, and myorelaxant drugs with reduced side effects. Undoubtedly, there will continue to be a focus on subtype-specific BZD site ligands as novel therapeutics (see Möhler et al., 2002; Wafford et al.,

2004). A better characterization of the BZD site, and specifically the physicochemical differences in the binding site formed at all $GABA_AR$ subtypes, will be of major importance in advancing subtype-specific BZD pharmacology.

OTHER ALLOSTERIC MODULATORY SITES ON THE $GABA_AR$

A large diversity of compounds act at the $GABA_AR$ to modulate receptor activity. Many of these modulators, like BZD site ligands, help to define $GABA_AR$ pharmacology. For example, receptor modulation by barbiturate compounds, and the binding of picrotoxin and [^{35}S]*tert*-butylbicyclophosphorothionate ([^{35}S]TBPS) associated with the ion channel, are representative of $GABA_AR$ pharmacology. Whereas it has been established that the BZD binding site exists at the α-γ subunit interface, and residues important for BZD ligand interaction cluster in analogy to the GABA agonist sites (see above), the locations and properties of other modulatory sites generally have been characterized to a lesser degree.

Barbiturates

The barbiturates are another class of $GABA_AR$ modulators that have clinical use as anesthetics and anticonvulsants. The molecular effects of barbiturate modulation of the $GABA_AR$ differ from BZD modulation, as barbiturates induce a lengthening of the GABA-gated ion channel open time (Twyman et al., 1989). In addition to modulatory effects, high concentrations (e.g. >100μM) of barbiturate ligands can directly gate the $GABA_AR$ chloride channel in the absence of GABA, indicative of at least two independent sites of action through which these ligands can act (see Thompson et al., 1996). The site for direct activation is distinct from the GABA agonist site; mutations that alter the ability of GABA to activate the $GABA_AR$ preserve the ability of certain barbiturates to directly activate the receptors (e.g. Amin and Weiss, 1993) and GABA antagonists do not block this gating effect (Thompson et al., 1996).

In defining the site of interaction of barbiturates, recombinant preparations that vary the subunit composition of the expressed $GABA_ARs$ have indicated that both the particular α- and β-subunit incorporated in the receptor affects barbiturate pharmacology (Thompson et al., 1996). Different subtypes show individual sensitivities, and in

particular, receptors incorporating the α4-isoform (with β1- and γ2S) are insensitive to direct modulation by pentobarbital (Wafford et al., 1996), implying an α-subunit component to that effect. A further investigation of the site(s) of barbiturate interaction has used chimeric subunits derived from barbiturate-sensitive $GABA_AR$ subunits and insensitive GlyR subunits; these data exclude both the initial ~30 N-terminal residues and the portion of $GABA_AR$ subunits that is C-terminal to the end of TM3 (the large intracellular loop) (Koltchine et al., 1996). Mutation of a single residue in the TM2 domain in β1-subunits was shown to negate the potentiating effects of pentobarbitone (Birnir et al, 1997). Other studies have exploited the barbiturate-insensitivity of $GABA_CRs$ containing ρ-subunits to identify specific residues in the TM2 (Belelli et al., 1999) and TM3 (Amin, 1999) domains that upon substitution alter barbiturate potentiation, and both direct and modulatory effects, respectively. Another recent study has exploited chimeric β3/ρ subunits to determine an effect of the subunit segment from TM1 to the TM2-3 linking region on the sensitivity of $GABA_AR$ β3 homomers to pentobarbital potentiation; the affinity of barbiturate compounds was affected by the C-terminal portion of the TM2 domain, suggesting a binding effect (Serafini et al., 2000). Taken together, the data on the interaction of barbiturates with the $GABA_AR$ indicate a likely interaction of barbiturates within the TM domains, although the exact nature of this interaction is not well defined. Additionally, as these barbiturate-sensitive receptor segments share overlap with channel-forming domains of the receptor, it is not entirely clear if these data support a defined binding environment for barbiturates, or if these domains contribute to specific receptor architecture that permits barbiturate activity. The exact nature of the separable potentiation and modulatory effects, and how this may vary across all receptor subtypes, is currently unknown.

Neurosteroids

Neurosteroids are another class of potent $GABA_AR$ allosteric modulatory compounds. These modulators can be steroid compounds from peripheral glands (e.g. ovaries, adrenal tissue) or *de novo*, neuron-synthesized, steroid molecules that can affect $GABA_ARs$ at low nanomolar (physiological) concentrations (reviewed in Lambert et al., 2003). Unlike the BZD site, a defined point of interaction for this class of compounds on

the $GABA_AR$ pentamer has not been elucidated. Early studies of pregnane steroid metabolites (Puia et al., 1990) revealed both a potentiation of submaximal GABA currents and a direct gating of the $GABA_AR$ by certain pregnane steroids, with little influence of the subunit composition. Further studies on the receptor subtype dependence of neurosteroid activity have shown that most subtypes are sensitive to the modulatory effect, with δ-subunit containing $GABA_ARs$ being the most sensitive (e.g. Wohlfarth et al., 2002). Activity of neurosteroids at δ-containing $GABA_AR$ is suggested to enhance the efficacy of gating of the receptor by partial agonists, a feature that may have consequences for the activity of endogenous partial agonists like taurine (Bianchi and Macdonald, 2003). The overall effect of neurosteroid modulation is suggested to be an increase in the duration of synaptic current (see Puia et al., 2003) but, in general, neurosteroid effects are neuron-specific (see Lambert et al., 2003). This specificity across neurons suggests that the subtypes of the $GABA_ARs$ that are selectively expressed (and the synaptic versus extrasynaptic location) lies behind the diversity.

Anesthetics and Alcohols

The modulatory effects of anesthetic compounds and alcohols on the $GABA_AR$ have also been demonstrated, and domains of the receptor have been established for their interaction. Chimeric subunits derived from ethanol- and enflurane-sensitive GlyR α1-subunits and the ethanol- and enflurane-insensitive $GABA_CR$ ρ1-subunits indicated that the TM2 and TM3 domains are necessary and sufficient for the modulatory effect of these compounds (Mihic et al., 1997). Certain residues within these domains have been implicated in ethanol (e.g. Ueno et al., 1999) and anesthetic modulatory activity (e.g. Jenkins et al., 2001). Specifically, residues Ser^{270} (TM2) and Ala^{291} (TM3) of the α-subunit and the homologous residues in the β-subunit have been shown upon substitution to alter the potentiation by ethanol more than mutation of the homologous residues in the γ-subunit (Ueno et al., 1999). Three anesthetic agents, halothane, isoflurane and chloroform, are predicted to interact with a cavity in the transmembrane region that is formed by at least Leu^{232} (TM1), Ser^{270} (TM2) and Ala^{291} (TM3) of the α-subunit (Jenkins et al., 2001). A contribution from the TM4 α-helix is also predicted for anesthetic interaction (Jenkins et al., 2002). The points of interaction of alcohols and

anesthetics appear to be physical cavities, as "cutoff" points beyond which molecules of greater molecular volume will not modulate the $GABA_AR$ are known for alcohols (e.g. Dildy-Mayfield et al., 1996; Wick et al., 1998) and certain anesthetics (e.g. Jenkins et al., 2001).

All anesthetic agents do not modulate receptor function via the same putative sites. For example, propofol is predicted to have a greater dependence for interaction with the identified TM3 residue, selectively in the β-subunit (e.g. $\beta2Met^{286}$; Krasowski et al., 2001; Bali and Akabas, 2004). Furthermore, etomidate is predicted to interact selectively with the β2- and β3- (but not β1-) isoforms, with a dependence on the $\beta3Asn^{265}$ TM2 residue in the β-subunit that is homologous to $\alpha1Ser^{270}$ (Belelli et al., 1997). In a manner similar to the dissecting of BZD subtype-specific actions *in vivo* (see above), the β3(N265S) knock-in mouse mutation selectively reduced anesthetic sensitivity to etomidate and propofol (Jurd et al., 2003). A complementary knock-in mutation of β2(N265S) illustrates that the sedative (as opposed to anesthetic) effects of etomidate are mediated by β2-containing $GABA_AR$ (Reynolds et al., 2003). Confirmatory evidence for subtype-selectivity of effects with β2-knock-out mice (O'Meara et al., 2004) demonstrates that the anesthetic effect of etomidate is still present, indicating a role of the β3-subtype in this effect. With respect to anesthetic agents and alcohols on the $GABA_AR$, it is clear that specific residues in the TM domains appear to play a role in ligand interaction and allosteric modulation. The anesthetic-specific consequences of point mutations, and *in vivo* data illustrating subtype-specific actions, both imply a complex collection of sites for anesthetic/alcohol interaction. Identification of contact points for anesthetics within the TM domains has recently been examined by SCAM studies to probe the incorporation of photolabile anesthetic compounds (Mascia et al., 2000) or the slowing of sulfhydryl modification of engineered cysteines by an anesthetic (Bali and Akabas, 2004). These more direct studies will likely reveal additional detail on the interaction of anesthetics (and alcohols) with the $GABA_AR$.

Other Modulators

The determinants of interaction of other $GABA_AR$-specific compounds have been delineated to a certain extent. For example, the anticonvulsant loreclazole has a critical

dependence on the β2/3Asn^{265} TM2 domain residue, and insensitivity of the β1-subtype is mediated by the homologous β1 serine residue (Wingrove et al., 1994). The lower affinity (micromolar) effect of certain BZD site β-carboline negative modulator compounds is also dependent on this loreclazole site (Stevenson et al., 1995). Determinants of the [^{35}S]TBPS/picrotoxin binding site have been located to the TM2 domain in the β3-subunit, including Ala^{252} and Leu^{253} (Jursky et al., 2000). The basis for divalent zinc ion interaction with the $GABA_AR$ has also been proposed and includes three distinct regions; β3His^{267} and β3Glu^{270} within the ion channel, and a subunit-interface location for β3Glu^{182} with α1Glu^{137}/α1His^{141} coordinating to bind Zn^{2+} (see Hosie et al., 2003). Additionally, the diuretic agent amiloride shows dependence on the α-subunit for its inhibition of $GABA_ARs$, with selectivity for α6-containing receptors (Fisher, 2002); critical residues within have been located in the α6-isoform to α6Ser^{83}, α6Leu^{174} and α6Tyr^{175} (Drafts and Fisher, 2004).

Many additional compounds have been identified as regulators of $GABA_AR$ function, and several of these can be described as interacting with novel sites on the $GABA_AR$. For example, β-lactam antibiotics are known to affect $GABA_AR$ function, with penicillin-G postulated to interact with a site in the ion channel near the picrotoxin site (Sugimoto et al., 2002). Other antibiotics (fluoroquinolones) can be modified to produce anxiolytic compounds that are predicted to allosterically potentiate $GABA_ARs$ via a novel site (Johnstone et al., 2004). The antiepileptic drug riluzole (He et al., 2002) and the selective serotonin reuptake inhibitor fluoxetine (FLX) (Robinson et al., 2003) have also been shown to potentiate $GABA_AR$ function in a manner independent of the BZD site. Inhibition of $GABA_AR$ receptor current by L-type calcium channel blockers of the dihydropyridine (e.g. nifedipine and nitrendipine) and phenylalkylamine (e.g. verapamil) classes has also been demonstrated via a novel binding site (Das et al., 2004). The nonsteroidal anti-inflammatory fenamate drugs mefenamic acid (Halliwell et al., 1999) and niflumic acid (Sinkkonen et al., 2003) show $GABA_AR$ subtype-dependent positive and negative modulation, with niflumic acid modulating receptor function through a γ-subunit-linked, non-BZD, novel modulatory site (Sinkkonen et al., 2003). Ligands that act through additional allosteric sites to modulate $GABA_AR$ subtype

function and/or ligand binding include a variety of compounds extracted from herbal or plant sources, such as the thyme essential oil extract thymol (Priestley et al., 2003) and ergot alkaloids (Tvrdeic and Pericic, 2003). Additionally, a range of flavonoid compounds can interact with the $GABA_AR$; binding at the BZD site has been demonstrated (e.g. Baureithel et al., 1997) and pharmacophore models of flavonoid compounds docked in the BZD site have been constructed (e.g. Marder and Paladini, 2002; Kahnberg et al., 2002). However, certain flavonoids have been shown to have a modulatory action that is flumazenil-insensitive, implying a non-BZD site of action. Examples of these flavonoids include amentoflavone (Hanrahan et al., 2003), 6-methylflavone (Hall et al., 2004) and hispidulin, a novel positive modulatory compound that has recently been confirmed to cross the blood-brain barrier and is suggested to have central modulatory effects (Kavvadias et al., 2004)

AIMS OF THE PRESENT STUDIES

The overall aim of my studies has been the investigation of the basis for subtype-specific interaction of modulatory compounds at $GABA_AR$ subtypes. Expressing $GABA_ARs$ in tsA201 cells (for use in radioligand binding studies) or in *Xenopus* oocytes (for functional assay by two-electrode voltage clamp) allows for the reproducible recombinant expression of receptors of a defined subtype. This is a key element in first reproducing subtype-specific pharmacology with respect to ligand affinity or functional effect. Furthermore, both cDNA transfection of mammalian adherent cells lines and injection of cRNA into *Xenopus* oocytes are systems amenable to the expression of wild-type and mutant receptors. Such expression systems are therefore useful for first establishing $GABA_AR$ subtype-specific differences, followed by the expression of mutant receptors to probe for the determinants of the individual subtype properties.

The BZD site is the best-characterized allosteric modulatory site on the $GABA_AR$, but its full diversity is not yet appreciated. The simple dichotomy between DS and DI pharmacology fails to represent the entire assortment of individual BZD site subtypes that vary with the inclusion of differing α- and γ-subunits. The DI BZD sites formed with either the α4- or α6-isoform are equally insensitive to classical 1,4-BZDs, but can display differences in the affinity of several classes of compounds, in particular, certain

β-carboline ligands (e.g. Yang et al., 1995; Gunnersen et al., 1996; Knoflach et al., 1996). Furthermore, other BZD site ligands that still recognize α4- and α6-containing $GABA_A$Rs (e.g. Ro15-4513) display specific differences between the DS and DI subtypes with respect to efficacy of modulation, photolabeling of the receptor proteins, and the consequences of mutation of BZD site residues on the affinity and efficacy of these ligands (see Chapter 2). Therefore, an examination of the DI BZD site is described in Chapter 2 with the goal of identifying determinants of the affinity differences for certain β-carboline ligands. An extended aim was to examine mutations in BZD site residues implicated in DI subtype affinity differences, and to compare the effects of homologous mutations in the DS α1-subtype to better characterize $GABA_A$R subtype differences in the BZD allosteric site.

Most of the data that have defined the BZD pharmacophore have been gathered by examining the DS subtype; most commonly, recombinant preparations have used the α1-isoform (see Sigel, 2002). Medicinal chemistry ligand mapping approaches have postulated that the BZD site formed with different α-isoforms will create a unique pharmacophore with a distinctive, subtype-specific volume, indicative of physicochemical differences across subtypes. The goal of Chapter 2 was to identify individual amino acid residues within the α4- and α6-isoforms that contribute to the differential affinity of β-carboline ligands as a means of finding determinants of DI BZD pharmacology for comparison to the better-understood DS BZD site.

Chapter 3 focuses on the interaction of FLX, which acts as a novel modulator of $GABA_A$R function. FLX has recently been characterized as a positive allosteric modulator of all $GABA_A$R subtypes, with the sole exception of those receptors that incorporate the α5-isoform (Robinson et al., 2003). This enhancement of submaximal GABA-mediated currents is suggested to resemble potentiation by BZD site ligands, but the ability of FLX to potentiate GABA responses in receptors lacking the γ-subunit indicates a novel site of action distinct from the BZD site. The aim of the studies detailed in Chapter 3 was to recreate the FLX-insensitivity of the α5-subtype in functional studies and to probe within this subunit isoform for the molecular determinants of the lack of modulation in comparison to the sensitive α1-isoform. By defining the requirements for

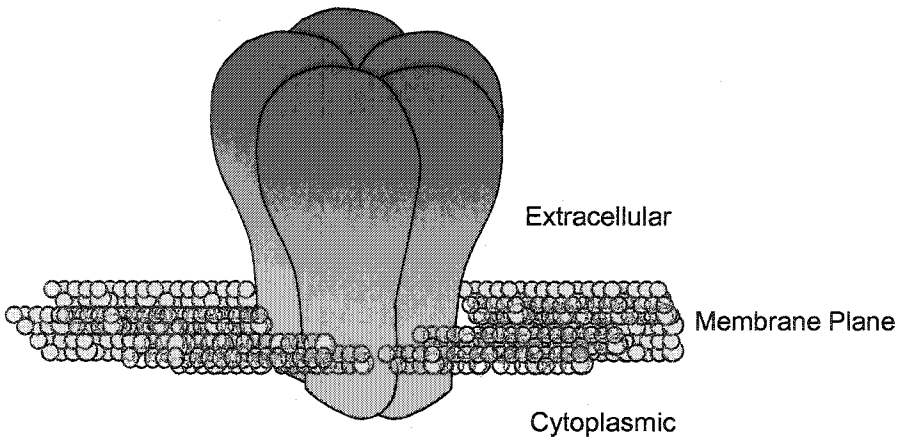
the insensitivity to FLX, the experimental approach in Chapter 3 aimed to better characterize the interaction and effect of FLX and to examine the molecular region surrounding the determinants of insensitivity to better understand $GABA_AR$ function.

In conclusion, the two main branches of the present studies have the overall aim of examining the molecular basis of subtype-selective pharmacology. The differences in the affinity of BZD site ligands and in the function of a novel modulator (acting through an as yet undefined binding site) are examined to better understand the contribution of different isoforms of the α-subunit to the pharmacology of allosteric modulators of the $GABA_AR$.

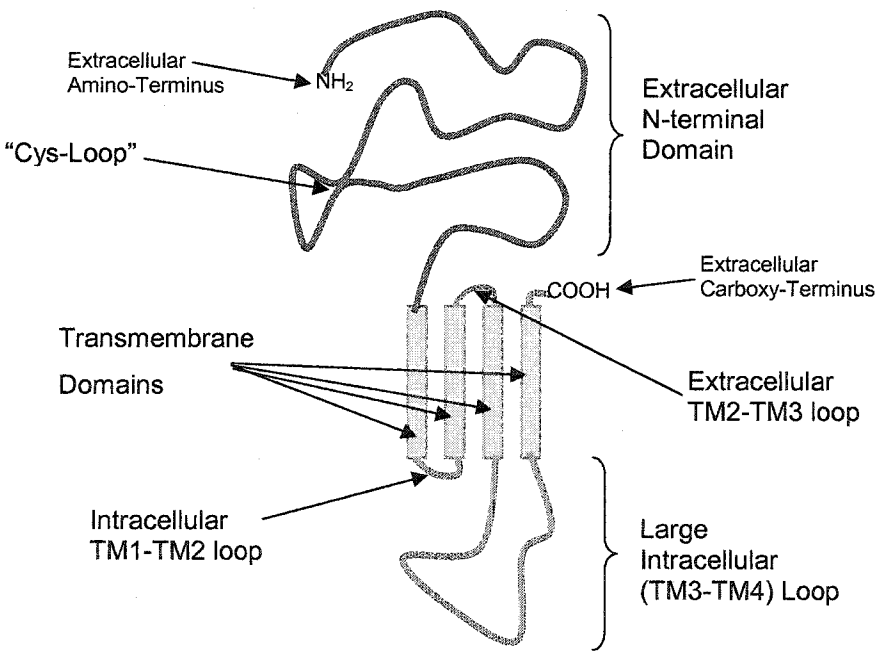
Figure 1-1

(A) A representation of a pentameric $GABA_A$ receptor complex in a neuronal membrane. Note that all of the subunits are transmembrane proteins. (B) A schematic of the topology of a single $GABA_AR$ subunit, including the large extracellular N-terminal domain (that contains the "Cys-loop" formed by a cysteine-cysteine disulfide bridge characteristic of subunits of the LGIC receptor superfamily), the four transmembrane segments (TM1-TM4), a small extracellular TM2-TM3 linker region and the intracellular (TM3-TM4) loop. The carboxy terminal tail of the subunit protein is extracellular. (C) A linear representation of a single $GABA_AR$ subunit depicting the four TM domains, the characteristic "Cys-loop" (*yellow*) and discrete regions of the extracellular N-terminal domain that have been implicated in forming ligand recognition domains in the "multiple loop model" (see Chapter 1). Three of these segments from one subunit (Loops A-C, as the principal component; *green*) and three additional regions from an adjacent subunit (Loops D-F, as the complementary domains; *light blue*) are postulated to form the binding sites for GABA and BZD site ligands at subunit-subunit interfaces (see text).

A.



B.



C.

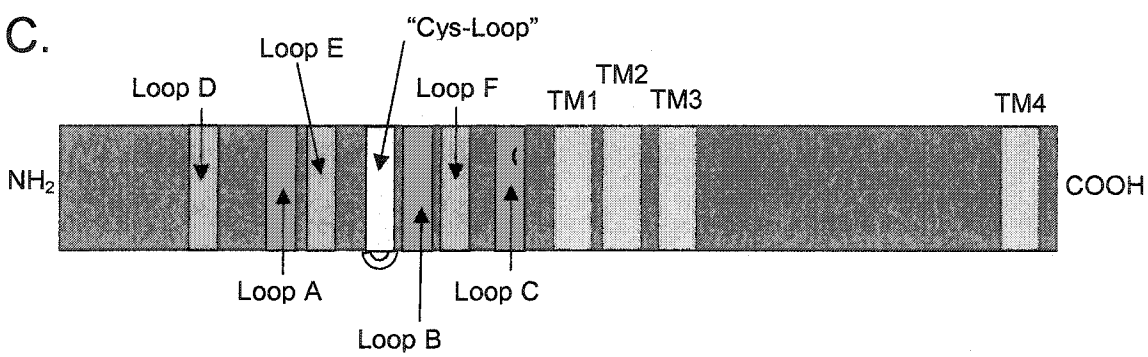


Figure 1-2

A model of the five subunits in a $GABA_A$ receptor heteropentamer. The individual proteins assemble to form a chloride-selective ion channel through the membrane. With respect to the N-terminal domain implicated in ligand association, each subunit in the receptor complex has polarity (i.e. both a "+" and a "-" face). Two binding sites for agonists (the GABA binding sites) are located at each of the β+/α- subunit interface (*yellow*). In homology to the GABA sites, the BZD binding site (*light blue*) is positioned at the α+/γ- subunit interface.

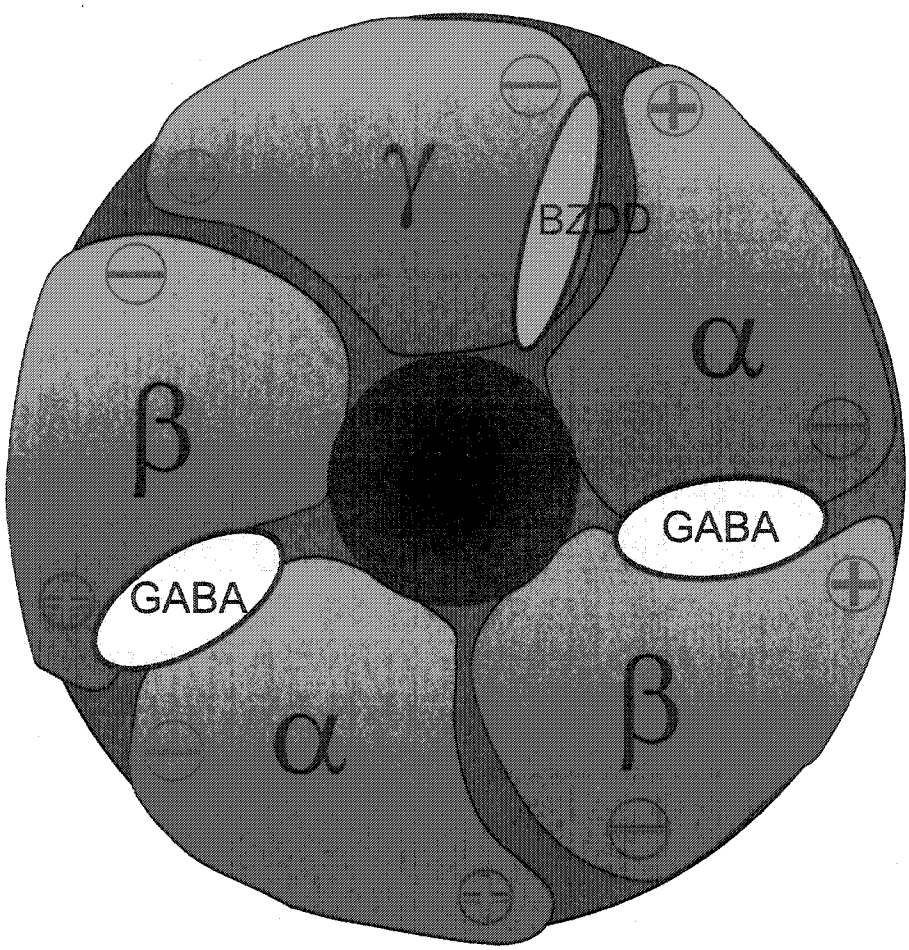


Figure 1-3

A depiction of the crystal structure of a homopentameric AChBP at 2.7Å isolated from the snail *Lymnaea stagnalis* (see Brejc et al., 2001) from the top (A) and side (B) views. The amino acid sequence of each AChBP subunit has a degree of homology to the extracellular domain of subunits of members of the LGIC receptor superfamily (C) and as such has served as a model for the structure of this region. (D) Structural models of the extracellular N-terminal domain have included putative physical correlates of the discrete "loop" regions from "+" and "-" protein faces thought to contribute to the formation of ligand (e.g. GABA and BZD) sites at subunit-subunit interfaces (see Chapter 1).

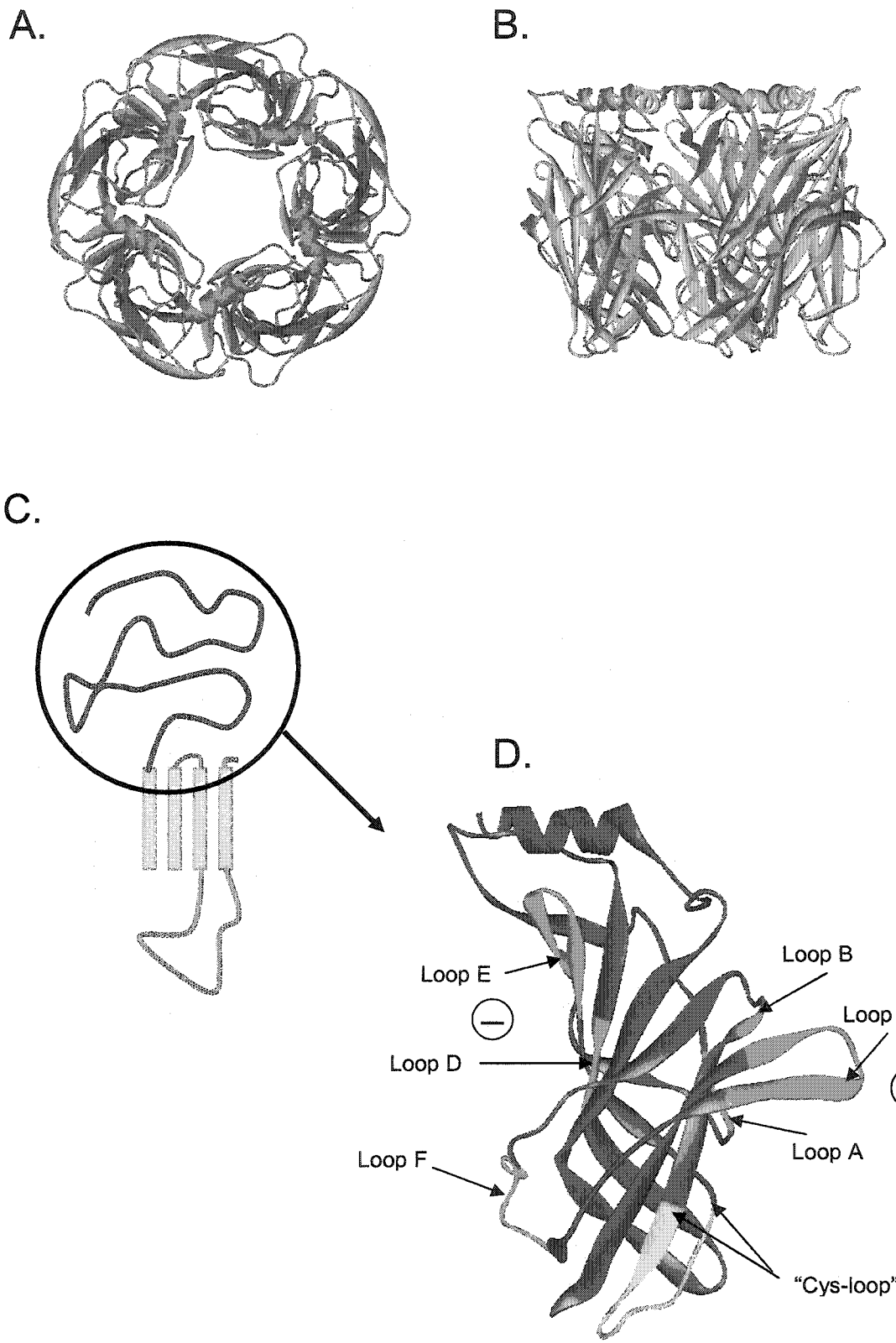
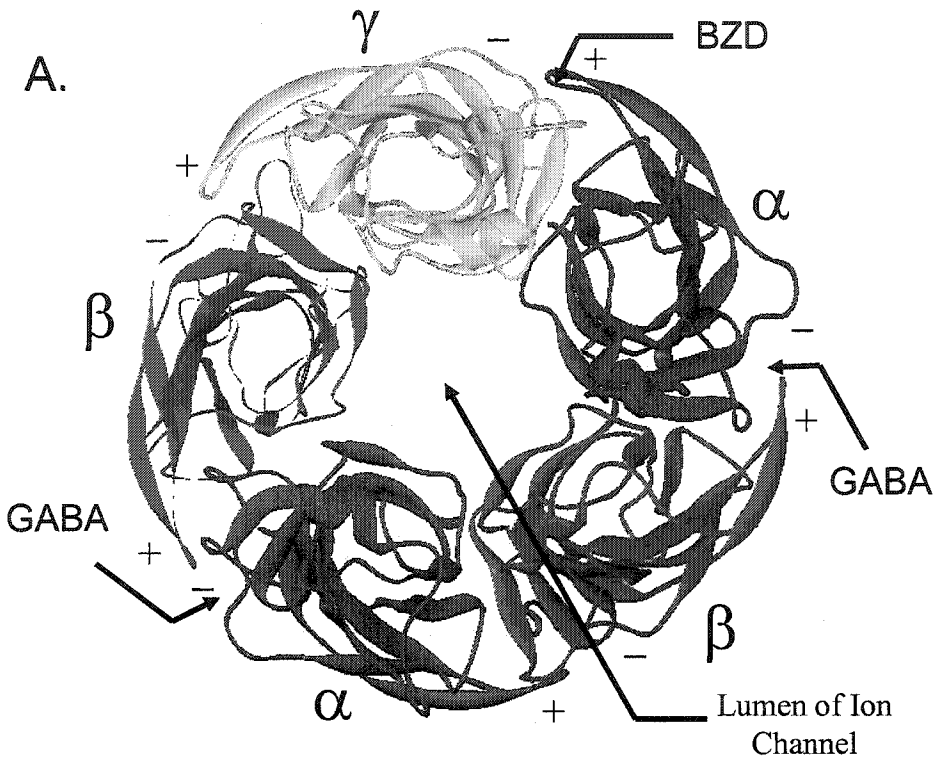


Figure 1-4

(A) A model of the extracellular portion of a $\alpha1\beta2\gamma2$ $GABA_A$ receptor derived by comparative modeling. For this model, the amino acid sequences of the $GABA_A$ receptor α- (*red*), β- (*blue*) and γ- (*yellow*) subunits are threaded onto the atomic structure of the AChBP (Brejc et al., 2001) to create an energy-minimized, best-fit comparative model of the $GABA_AR$ extracellular domain (see Ernst et al., 2003). The model depicts the polarity of each subunit in the pentameric complex and shows the GABA sites at the two $\beta+/\alpha-$ interfaces and the BZD site at the $\alpha+/\gamma-$ protein-protein interface. A side view (along the membrane plane) of the model (B) allows for the visualization of the spatial relationship of the modeled features in the extracellular domain to the membrane (below) and the extracellular/synaptic space (above).



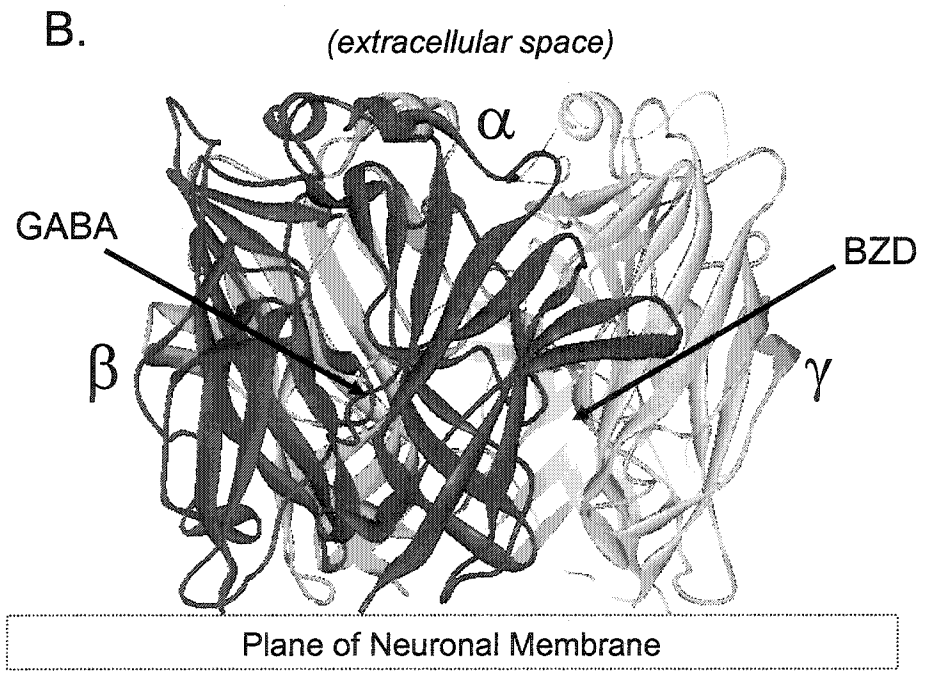
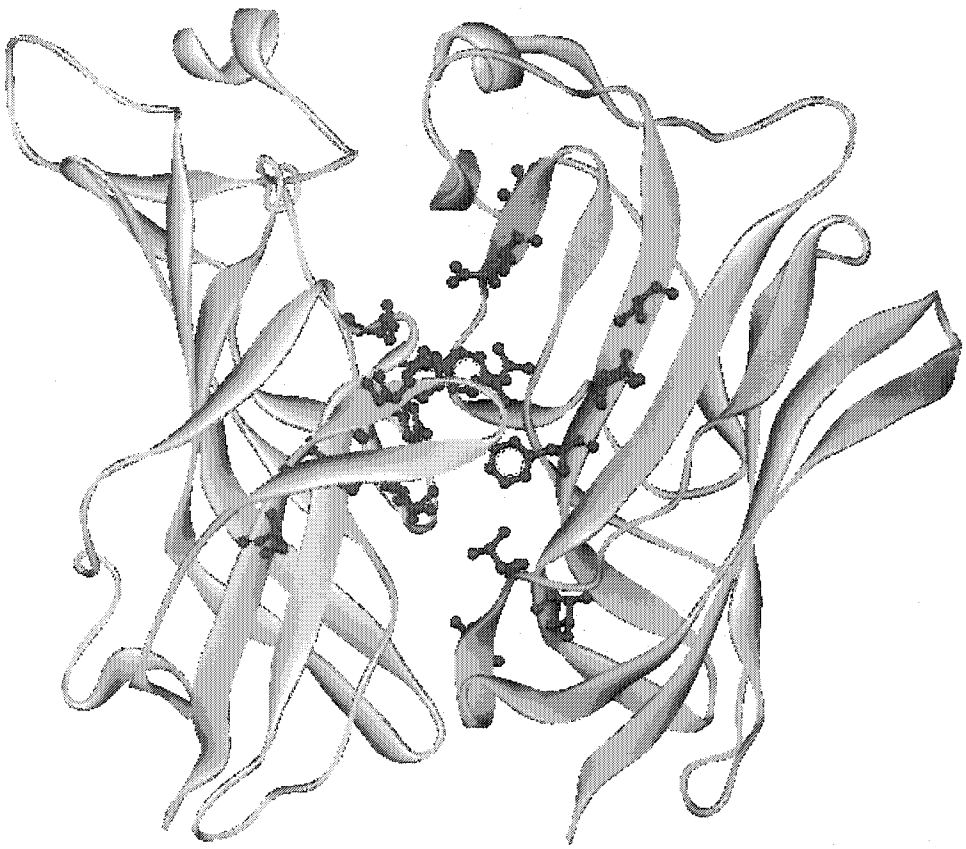


Figure 1-5

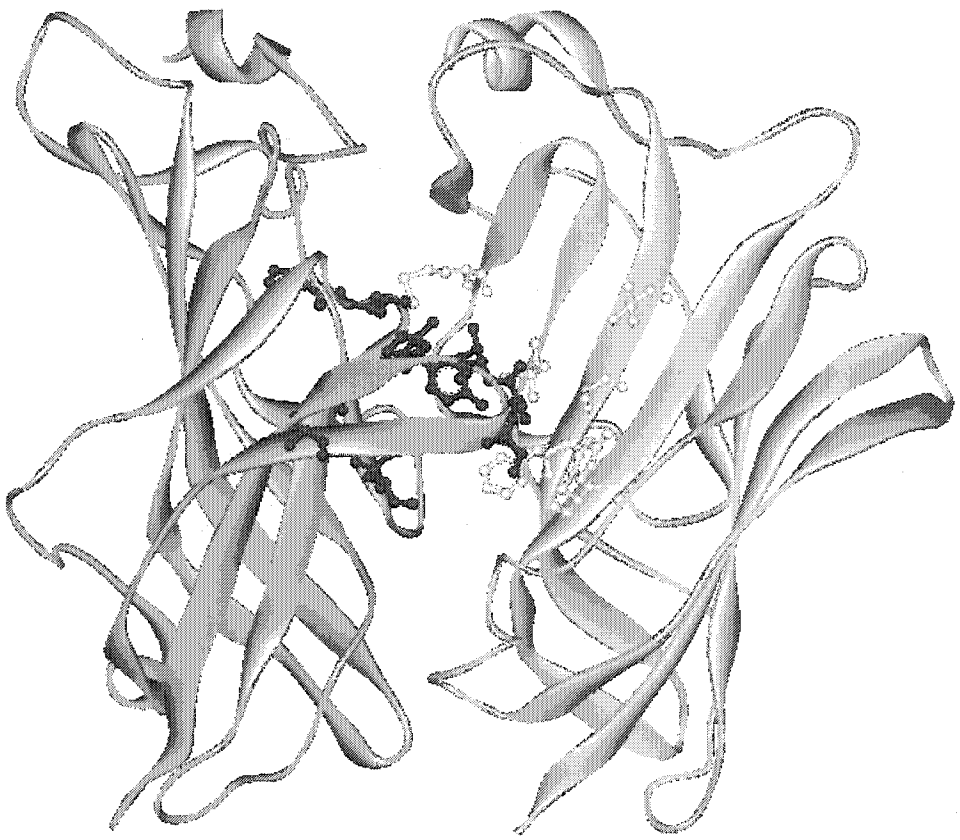
A model of the GABA binding site at the β+/α- subunit-subunit interface. The model depicts the putative structure of the β+ (*blue*) and the α- (*red*) subunits derived by comparative modeling of the $GABA_A$ receptor based on the atomic structure of the AChBP (see Ernst et al., 2003). Amino acid residues that have been implicated previously as contributing to the β+/α- GABA site (see Chapter 1) are shown in ball-and-stick format and include (rat β2 numbering) $\beta2Tyr^{97}$, $\beta2Leu^{99}$, $\beta2Tyr^{157}$, $\beta2Thr^{160}$, $\beta2Thr^{202}$, $\beta2Ser^{204}$, $\beta2Tyr^{205}$, $\beta2Arg^{207}$, $\beta2Ser^{209}$ and (rat α1 numbering) $\alpha1Phe^{64}$, $\alpha1Arg^{66}$, $\alpha1Arg^{68}$, $\alpha1Arg^{119}$, $\alpha1Ile^{120}$, $\alpha1Val^{178}$, $\alpha1Val^{180}$ and $\alpha1Asp^{183}$ (see Chapter 1).



β+ α-

Figure 1-6

A model of the BZD binding site at the α+/γ- subunit-subunit interface. The model depicts the putative structure of the α+ (*red*) and the γ- (*yellow*) subunits derived by comparative modeling of the $GABA_A$ receptor based on the atomic structure of the AChBP (see Ernst et al., 2003). Amino acid residues that have been implicated previously as contributing to the α+/γ- BZD site (see Chapter 1) are shown in ball-and-stick format and include (rat α1 numbering) $\alpha1Pro^{96}$, $\alpha1His^{101}$, $\alpha1Tyr^{159}$, $\alpha1Thr^{162}$, $\alpha1Gly^{200}$, $\alpha1Ser^{204}$, $\alpha1Ser^{205}$, $\alpha1Thr^{206}$, $\alpha1Tyr^{209}$, $\alpha1Val^{211}$ and (rat γ2 numbering) $\gamma2Met^{57}$, $\gamma2Tyr^{58}$, $\gamma2Phe^{77}$, $\gamma2Ala^{79}$, $\gamma2Thr^{81}$, $\gamma2Met^{130}$ and $\gamma2Thr^{142}$.

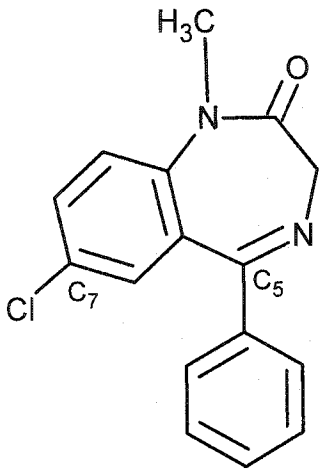


α+ γ-

Figure 1-7

A depiction of the chemical structures of (A) diazepam (DZP) and of (B) Ro15-4513. Numbering of positions on the chemical backbone at selected points is shown to indicate the position of derivative groups for compounds that have assisted in orienting BZD ligands in their binding pocket (see Chapter 1).

A.



B.

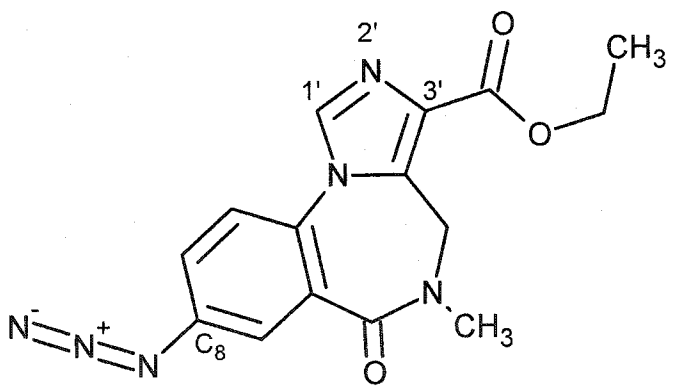


Table 1-1. A correlation of protein domains in the extracellular domain of $GABA_AR$ subunits with the secondary structure of the molluscan AChBP from *Lymnaea stagnalis* (Brejc et al., 2001). A multiple loop model of the proposed ligand binding sites at subunit-subunit interfaces on the $GABA_AR$ (see Figure 1-1C; Chapter 1) can be aligned by sequence homology with the secondary structural features of the AChBP crystal structure (Figure 1-3). Loops A-C are predicted to contribute the principal component of binding whereas the complementary component of the binding site is mediated by Loop D-F of the opposing subunit. See Chapter 1 text for details and references on the specific residues that have been implicated in forming the β(+)/α(-) low affinity GABA sites, and by homology, the α(+)/γ(-) BZD site (see Figures 1-2, 1-4, 1-5, 1-6). No residue at the γ(-) Loop F domain has been implicated in contributing to the BZD site to date. Numbering shown reflects rat α1, β2 and γ2 subunits. Table 1-1 is modified from Kash et al., 2004.

Loop Model	Protein Domain	GABA Residues	Protein Domain	BZD Residues	AChBP Structural Correlate
A	β2Tyr^{97}-Asp^{101}	(β2) Tyr^{97} Leu^{99}	α1Phe^{99}-Gly^{103}	(α1) His^{101}	β-sheet 4, loop 5
B	β2Glu^{155}-Thr^{161}	(β2) Tyr^{157} Thr^{160}	α1Tyr^{157}-Thr^{162}	(α1) Tyr^{159} Thr^{162}	β-sheet 7, loop 8
C	β2Phe^{200}-Arg^{207}	(β2) Thr^{202} Ser^{204} Tyr^{205} Arg^{207}	α1Val^{202}-Tyr^{209}	(α1) Ser^{204} Ser^{205} Thr^{206} Tyr^{209}	loop 10, β-sheet 10
D	α1Asp^{62}-Ser^{68}	(α1) Phe^{64} Arg^{66} Ser^{68}	γ2Asp^{75}-Thr^{81}	(γ2) Phe^{77} Ala^{79} Thr^{81}	β-sheet 2
E	α1Leu^{117}-Thr^{129}	(α1) Arg^{119} Ile^{120}	γ2Met^{130}-Thr^{142}	(γ2) Met^{130} Thr^{142}	β-sheet 5, loop 6, β-sheet 6
F	α1Ala^{175}-Gly^{184}	(α1) Val^{178} Val^{180} Asp^{183}	γ2Val^{188}-Ser^{195}		loop 9

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CHAPTER 2[†]

Identification of a Residue in the γ-Aminobutyric Acid Type A Receptor α Subunit that Differentially Affects Diazepam-Sensitive and -Insensitive Benzodiazepine Site Binding

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INTRODUCTION

γ-Aminobutyric acid type A ($GABA_A$) receptors are the major inhibitory receptors in the mammalian CNS. These receptors are members of the ligand-gated ion channel (LGIC) family that includes the nicotinic acetylcholine, glycine, and serotonin type-3 ($5\text{-}HT_3$) receptors (Ortells and Lunt, 1995). Each $GABA_A$ receptor is a heteropentamer of homologous subunits that assemble to form a GABA-gated, chloride-selective ion pore. Several different classes of receptor subunits have been cloned (α1-6, β1-3, γ1-3, δ, ε, π and θ) (for reviews, see Barnard et al., 1998; Korpi et al., 2002) and different combinations of these subunits in the oligomer determine the pharmacology of the receptor subtype. Although the number of subtypes remains unknown, most receptors appear to be formed by α-, β- and γ- subunits in a 2:2:1 stoichiometry (Tretter et al., 1997; Farrar et al., 1999). Allosteric modulation is a hallmark feature of the $GABA_A$ receptor and this is best exemplified by compounds that act at the benzodiazepine (BZD) binding site. Ligands of many chemical classes recognize this site and these compounds display a spectrum of efficacy from positive to negative allosteric modulation of GABA-gated Cl^- currents (see Barnard et al., 1998).

All receptors in the LGIC family share common structural features including a similar subunit topology and overall receptor conformation. In all members of the family, agonist binding sites are thought to lie at subunit-subunit interfaces and to be formed by six distinct stretches or 'loops' of amino acids; three of these domains (A-C) are contributed by one subunit, while the other three (D-F) are provided by the adjacent subunit in the pentamer (reviewed in Corringer et al., 2000). In the case of the $GABA_A$ receptor, GABA binding sites are predicted to lie at the interfaces between the β–α (see Smith and Olsen, 1995) and α–β (Newell et al., 2000) subunits. The BZD binding site is predicted to be formed by homologous loops of amino acids that lie at the interface between α- and γ- subunits (Sigel and Buhr, 1997; Cromer et al, 2002; Ernst et al., 2003). BZD pharmacology is determined by the specific α- and γ-subunit isoforms that are present in the receptor oligomer. The incorporation of the α4- or α6-subunit in the receptor, for example, bestows insensitivity to diazepam and other classical 1,4-BZDs, a property that has been ascribed to the substitution of a histidine (H101, rat α1 subunit

numbering) by an arginine (Wieland et al, 1992). However, other ligands from diverse classes retain affinity for these diazepam-insensitive (DI) receptors and some display differential affinity for those containing the α4- or α6-isoform. Certain β-carboline and pyrazoloquinolinone ligands, for example, have higher affinity for DI receptors in the cortex compared to the cerebellum (Ito et al., 1995). Studies with recombinant receptors suggest that this may be due to the higher levels of expression of the α4-subunit in the cortex compared to the cerebellum where the α6-subunit is preferentially expressed (see Yang et al., 1995; Gunnersen et al., 1996; Knoflach et al., 1996).

This study initially focused on the identification of specific residues in the α4- and α6-subunits that confer the differential affinity for certain β-carbolines. In order to identify regions of interest, we used a chimeric approach similar to that used previously in the analysis of binding requirements of BZD ligands (e.g. Boileau et al, 1998). Randomly derived chimeras from the α4- and α6-subunits were created and, based on their observed binding characteristics, individual residues were targeted for site-directed mutagenesis. This led to the identification of a specific residue (α6N204/α4I203) that bestows the differential affinity for β-carbolines. By mutating the homologous residue in the α1-subunit to that found in α6 (S205N), we show that although this residue has little influence on binding of the classical agonist, flunitrazepam, it has significant effects on the binding of both β-carbolines and the imidazobenzodiazepines (*i*-BZD), Ro15-1788 and Ro15-4513.

MATERIALS AND METHODS

Materials

Ro15-4513 and Ro15-1788 were gifts from Hoffmann-La Roche and Co. (Basel, Switzerland). β-CCE was a generous gift from Dr. Brian Jones (GlaxoSmithKline, Harlow, UK). DMCM and GABA were obtained from RBI/Sigma (Natick, MA). [^{3}H]Ro15-4513 (23.06 Ci/mmol) and [^{3}H]flunitrazepam (84.5 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA). See Figure 2-1 for structures of the BZD ligands used.

Production of Alpha-Subunit Chimeras

Several randomly derived α-subunit chimeras (χ) were created following the protocol of Moore and Blakely (1994). Sequences of both the rat α6- and α4-subunit cDNA were subcloned into the same pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA) (Figure 2-2). The complete α6 cDNA was inserted (as a *Hind*III fragment) upstream of a partial α4 cDNA sequence (an *Eco*RI – *Xba*I fragment containing an N-terminally truncated α4 sequence). A *Bam*HI restriction site was left in the polylinker between the inserts. Restriction digestion was used to excise the intervening polylinker sequence as well as a portion of the α6 insert, thereby limiting the amount of α6 insert that was homologous to the α4 cDNA. Digestion with *Bam*HI and *Hpa*I or with *Eco*RI and *Bst*XI limited the region of homology with the α4 insert to a sequence in the α6 cDNA that represents the extracellular N-terminus of the mature α6 subunit. In this manner, random crossover events were targeted to occur in regions of the cDNA that represent the extracellular, ligand-binding domain of the α-subunits, a technique that has been used previously to produce targeted random chimeras of $GABA_A$ receptor subunits (Boileau *et al.*, 1998). Linearized plasmid DNA was transformed into Library Efficiency® competent DH5α *Escherichia coli* cells (Life Technologies, Gaithersburg, MD) where random crossover events occurred at regions of homology in the α-subunit cDNA sequences, producing in-frame hybrid cDNA. Individual colonies were isolated and the extracted plasmid DNA was assessed for size to indicate a potential recombinant crossover event. For those plasmids showing a chimeric crossover, the switch point was

assessed by restriction digestion analysis and confirmed by DNA sequencing. Chimeras are named based on the point of crossover; the nomenclature represents the last residue of N-terminal α6-subunit contribution (rat subunit numbering) prior to the in-frame switch. Individual chimeras were used for transfection with rat β2 and $\gamma 2_L$ plasmid constructs (Figure 2-3, and see below).

Mutagenesis

Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene, La Jolla, CA). Wild-type rat α6 or α1 cDNA inserts were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and were used as the templates in separate PCR-mediated mutagenesis protocols. Complementary 27-mer mutagenic oligonucleotide primer pairs were synthesized (Department of Biochemistry, DNA Core Laboratory, University of Alberta) for each substitution reaction. PCR amplification with *Pfu* polymerase (Stratagene, La Jolla, CA) was carried out under the recommended conditions. Plasmid DNA was digested with *Dpn*I endonuclease to degrade methylated template DNA and was subsequently transformed into competent *E. coli* cells for propagation of the plasmid. Individual colonies were isolated and plasmid DNA extracted. Successful substitutions within the cDNA insert of the plasmid were confirmed by DNA sequencing.

Transient Transfection

Recombinant $GABA_A$ receptor subtypes were expressed in tsA201 cells. The tsA201 cell line (European Collection of Cell Cultures, ECACC 96121229) is a derivative of the human embryonic kidney 293 (HEK 293) cell line that has been transformed with the SV40 T antigen. Cells were maintained in low-glucose Dulbecco's modified Eagle's medium (GibcoBRL, Grand Island, NY) supplemented with 10% (vol/vol) Fetal Clone III bovine serum (HyClone, Logan, UT) and were provided with fresh medium prior to transient transfection (Davies et al, 2000). Briefly, subunit cDNAs were subcloned into the pcDNA3.1 expression vector (Invitrogen, San Diego, CA). Plates of cells were transiently co-transfected with 10μg each of wild-type, mutant, or chimeric α-subunit plasmid together with wild-type β2 and $\gamma 2_L$ vector constructs in a

1:1:1 ratio. The DNA was added to an appropriate volume of 250mM $CaCl_2$, to which was added an equal volume of *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (pH 7.02). Solutions were mixed and added dropwise to plates of cells. Cells were incubated at 37°C in a 3% CO_2 incubator for 48 h. After incubation, cells were scraped into ice-cold harvesting buffer (50mM Tris, 250mM KCl, pH 7.4) containing protease inhibitors (1mM benzamidine, 0.1mg/ml bacitracin, 0.01mg/ml chicken egg white trypsin inhibitor and 0.5mM phenylmethylsulfonyl fluoride). Harvested cells were homogenized with two pulses (10-s, 13,500 rpm) of an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany). Crude homogenates were recovered by centrifugation (Sorvall SS34 rotor, 18,000 rpm, 39100 RCF, 30 minutes) and were resuspended in ice-cold harvesting buffer and transferred to microfuge tubes. The crude homogenate was vortexed (30 seconds) and subjected to a further centrifugation (benchtop centrifuge, 13,000 rpm, 30 seconds) to pellet cellular debris. The supernatant (enriched in membrane fragments) was isolated and stored at –80°C until the day of experiments.

Radioligand Binding

Radioligand binding was performed as described previously (Davies et al., 2000). In brief, for equilibrium binding assays, aliquots of cell homogenates were incubated in duplicate with increasing concentrations (0.5nM – 80nM) of [^{3}H]Ro15-4513 or [^{3}H]flunitrazepam in binding assay buffer (50mM Tris, 250mM KCl, 0.02% NaN_3, pH 7.4) at 4°C for 60 min. Non-specific binding was determined in the presence of excess unlabeled Ro15-4513 or flunitrazepam as appropriate. For competition binding assays, aliquots of cell homogenates were incubated with a constant concentration of radioligand (at a concentration equal to its K_d for the receptor subtype) and increasing concentrations of displacing ligand. Parallel samples were filtered through GF/B filters (Whatman, Maidstone, England) using a cell harvester (Brandel, Gaithersburg, MD). The filters were immediately washed three times with 3 ml of ice-cold assay buffer prior to scintillation counting.

Expression of $GABA_A$ Receptors in *Xenopus* oocytes

Mature (stage V-VI) *Xenopus laevis* oocytes were isolated after collagenase treatment of a freshly isolated ovary lobe, as described previously (Goldin, 1992). Oocytes were maintained at 14°C in ND96 buffer (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ and 5mM HEPES, pH 7.4) supplemented with 100μg/ml gentamicin (GibcoBRL, Grand Island, NY). Capped (m7G(5')ppp(5')G) (GibcoBRL) cRNA transcripts were prepared for each $GABA_A$ receptor subunit. Plasmids containing the individual $GABA_A$ subunit rat cDNAs were linearized and used as templates for *in vitro* transcription with T7 RNA polymerase (Invitrogen, San Diego, CA) under the recommended conditions. Wild type or mutant α-subunit transcripts were mixed in a 5:1:1 (see Discussion) ratio with wild-type rat β3 and $\gamma 2_L$ transcripts (final RNA concentration of 1μg/μL in DEPC-treated water); 20-50nL (20-50ng) of total RNA was microinjected per oocyte. Oocytes were incubated in ND96 in 96-well plates at 14°C.

Two-Electrode Voltage Clamp

Electrophysiological recordings were made 2-14 days following injection. Oocytes were placed in a recording chamber (~0.5mL) and were continuously perfused with ND96 buffer (pH 7.4) at a rate of ~5mL/min. Using a GeneClamp500B amplifier (Axon Instruments Inc., Foster City, CA), oocyte membrane potentials were held at a membrane potential of -60mV with a standard two-electrode voltage clamp arrangement. The voltage-sensing and current-passing microelectrodes were filled with 3M KCl (final resistance between 0.5-2.5 MΩ in ND96). Solutions of GABA were prepared in ND96 buffer and were administered to oocytes by gravity perfusion. Oocytes were used for experiments when current responses were stable (±5%) between successive agonist applications. Additionally, after agonist challenge, oocytes were perfused with buffer for >12 minutes to permit sufficient time for recovery from desensitization. GABA-gated currents were recorded using Axoscope 9.0 data acquisition software (Axon Instruments, Inc.). Concentration-effect experiments were conducted for GABA on oocytes expressing $GABA_A$ receptor combinations whereby the currents elicited by several GABA concentrations (ranging at least four orders of magnitude) were measured.

Data and Statistical Analysis

Saturation and competition ligand binding data were analyzed using the least-squares nonlinear regression curve fitting programs of GraphPad Prism 3.0 (GraphPad, San Diego, CA). Binding parameters (K_d or K_i) were determined as the mean ± S.E.M. of at least three independent experiments. Data were analyzed by one-way ANOVA and levels of significance were determined by the Dunnett post-test for multiple comparisons or by a two-tailed *t*-test between two groups.

To determine the potency of GABA for receptor activation at each $GABA_AR$ subtype expressed, concentration-effect data were fitted to the following the four-parameter logistic equation using GraphPad Prism 3.0:

$$I = (I_{max} * [L]^n) / (EC_{50}{}^n + [L]^n)$$

where I is the measured GABA-evoked current, $[L]$ is the GABA concentration, EC_{50} is the GABA concentration that evokes half the maximal current (I_{max}) and n is the Hill slope coefficient. Data were analyzed by one-way ANOVA and levels of significance were determined by the Dunnett post-test for multiple comparisons.

RESULTS

Binding to Wild-type α4β2γ2 and α6β2γ2 $GABA_A$ Receptors

The α4- and α6- $GABA_A$ receptor subunits were each recombinantly expressed with the β2 and $\gamma2_L$ subunits in tsA201 cells for radioligand binding assays. Each receptor subtype recognized [^{3}H]Ro15-4513 with nanomolar affinity (Figure 2-5; Table 2-1). This ligand was, therefore, used in competition studies to determine β-carboline affinities for these receptors. Both β-CCE and DMCM displayed a greater affinity (approximately 6- and 9-fold higher, respectively) for the α4-containing subtype (Table 2-1, see also Figures 2-5C, 2-8).

Chimeric α6/α4 Subunits

Chimeric α-subunits were created from random recombination events at the cDNA level. Three chimeras were initially analyzed and, of these, two were identical. The two chimeric subunits with different crossover points (χ141 and χ207) were isolated for transient transfection with β2 and $\gamma2_L$ subunits (Figure 2-3). Expression of receptors containing the chimeric α-subunits was verified by the retention of high affinity binding for [^{3}H]Ro15-4513 (Figure 2-6; Table 2-1) although, in the case of the χ207-containing receptor, the affinity for this ligand was reduced by ~4-fold compared to the wild-type $\alpha6\beta2\gamma2_L$ receptor ($P < 0.001$). Competition assays were used to screen for the effect of the chimeric α-subunit on β-CCE affinity. The isolation of the initial two chimeras was fortunate, as inclusion of the χ141- or χ207- subunit produced receptor subtypes with an affinity for β-CCE that was α4-like or α6-like, respectively (Table 2-1, see also Figure 2-6C). Thus the stretch of amino acids between the two crossover points, α6(L141-E207), was implicated as containing residues that may confer the differential affinities of the α4- and α6-containing receptor subtypes.

Effect of Substitution Mutations in the α6-Subunit

The α6(L141-E207) region has a high degree of sequence homology with the same domain in the wild-type α4-subunit, with only eight divergent residues. Of these eight amino acids, one (α6N143) lies within the characteristic cys-loop region and another (α6N204) lies within the loop C segment previously implicated in BZD binding (Figure 2-4). To investigate their potential roles in β-carboline recognition, these residues in the α6-isoform were individually substituted by their homologues in the α4-subunit, i.e., α6N143D and α6N204I. Expression of receptors bearing these α-subunit mutations was again verified by the retention of high affinity binding of [^{3}H]Ro15-4513 (Figure 2-7; Table 2-1). Although the α6N143D substitution had no significant effect on the affinity for β-CCE, the α6N204I substitution resulted in a greater than 10-fold increase in its apparent affinity (Table 2-1; Figure 2-7C) i.e., this mutation bestowed α4-like higher affinity to the wild-type α6-subtype. This substitution also conferred higher affinity α4-like binding of another β-carboline, DMCM (Table 2-1; Figure 2-8). Thus a single residue in Loop C can account for the differences in β-CCE and DMCM affinities for the α4- and α6-receptor subtypes.

In the other α-subunits (α1-3, 5) the residue in the equivalent position to α6N204 is a serine (Figure 2-4). A further substitution was, therefore, the α6N204S mutation. Receptors carrying this mutation again retained high affinity binding of [^{3}H]Ro15-4513 (Figure 2-9; Table 2-1). The affinity of this mutant receptor for both β-CCE and DMCM was increased approximately 5-fold compared to their respective affinities for wild-type α6-containing receptors again implicating this residue in β-carboline recognition (see Figure 2-9C; Table 2-1).

Effect of Substitution Mutations in the α1-Subunit

In additional experiments, we characterized the binding of β-CCE and DMCM to the wild-type $\alpha1\beta2\gamma2_L$ receptor (Table 2-2). Within the α1-subunit, a substitution with the α6-subunit asparagine for the homologous serine residue (α1S205N), produced a mutant receptor with reduced affinity for β-CCE and DMCM (~6.5- and ~8-fold, respectively), compared to the wild-type α1-containing receptors. Although this

α1S205N substitution led to a slight increase (~3-fold) in the affinity for [^{3}H]flunitrazepam, there were considerable decreases in the affinities for both of the *i*-BZD compounds, Ro15-1788 (~19-fold) and Ro15-4513 (~38-fold) (Figure 2-10; Table 2-2).

Expression of α4- and α6-containing $GABA_AR$ in *Xenopus* Oocytes

Supplementary experiments were carried out to examine the functional properties of α4- and α6-containing $GABA_AR$ expressed in *Xenopus* oocytes. Robust expression was observed for the α6β2γ2 and the α6(N204I)β2γ2 subunit combinations, but not for the α4β2γ2 $GABA_AR$ (data not shown). Substitution of the β2-isoform with the β3-permitted expression of all three of these subtypes. GABA was ~10-fold more potent for the activation of the α6β3γ2 subtype compared to the α4β3γ2; inclusion of the mutant α6(N204I) subunit reduced GABA potency by ~8-fold (Figure 2-11; Table 2-3).

Modulation of GABA-gated currents at the α4- and α6-containing receptors by DMCM and β-CCE via the BZD site was attempted, but no discernable modulatory effect was observed at concentrations of these ligands up to 1.0μM (data not shown). As higher concentrations of β-carboline ligands are known to have non-BZD site effects in the micromolar range (see Stevenson et al., 1995), we were unable to observe subtype differences in BZD site modulatory activity.

DISCUSSION

Beyond the simple dichotomy of DS and DI $GABA_A$ receptor subtypes, ligand binding to the BZD site of the DI subtypes can be pharmacologically differentiated by the inclusion of either the α4- or the α6-subunit. In agreement with previous reports (Yang et al., 1995; Gunnersen et al., 1996; Knoflach et al., 1996), we found that β-carbolines (β-CCE and DMCM) display higher affinity for receptors containing the α4-subunit. We then used two subunit chimeras randomly derived from both the α4- and α6-isoforms and site-specific mutagenesis to probe the basis of this differential affinity. Substitution of a single residue within the Loop C segment (α6N204I) was sufficient to bestow higher α4-like affinity to the lower affinity α6-containing receptor. Several residues from the α-subunit Loop C segment have been shown previously to play a role in BZD binding (see Sigel, 2002). The present results now implicate an additional residue in this domain of the α4- and α6-subunits as a determinant of β-carboline recognition.

The results from additional mutagenesis studies show that the amino acid in the equivalent position in the α1-subunit also influences β-carboline binding. Introduction of a serine (conserved in α1-3,5) into the α6-isoform (α6N204S) resulted in an approximately 5-fold increase in the affinity for both β-CCE and DMCM. The reciprocal substitution at the homologous position in the α1-subunit (α1S205N) reduced the affinity (~7-8-fold) of these ligands compared to wild-type α1-containing receptors. Thus structural determinants for the binding of these ligands occur in similar domains of both DI and DS receptors. These results are consistent with previous reports of altered β-carboline pharmacology upon mutation of other residues within the loop C domain of the α1 subunit. Substitutions at the neighbouring α1T206 (T206A, Buhr et al., 1997; T206V, Schaerer et al., 1998) increase DMCM affinity by 5- and 40- fold, respectively.

The present study also demonstrates the importance of Ser205 in the α1-subunit in the binding of different classes of BZD site ligands. In addition to the effects on β-carboline affinity, the α1S205N substitution led to a modest increase (~3-fold) in the affinity for [^{3}H]flunitrazepam, consistent with a previous report where the α1S205A mutation did not compromise the ability of classical 1,4-BZDs to potentiate GABA-gated chloride currents (Buhr et al., 1996). In contrast, the α1S205N substitution significantly

decreased the affinity for the *i*-BZDs, Ro15-1788 and Ro15-4513, by ~19- and ~38-fold, respectively. The large reduction in the affinity for Ro15-4513 was unexpected since homologous mutations in the α6-subunit (α6N204I, α6N204S) had no appreciable effect on the binding of this ligand, permitting its use as a reporter ligand in displacement studies of β-carboline binding. In previous studies we have found that the binding of Ro15-4513 is much more resistant to other α1-subunit mutations than the binding of flunitrazepam and Ro15-1788 (e.g. Davies et al., 1998). Additionally, several mutations within the α1-subunit Loop C domain have smaller effects on the binding of Ro15-1788 and Ro15-4513; substitutions at the adjacent α1S204 and α1T206 residues (Buhr et al., 1997; Schaerer et al., 1998; Casula et al., 2001) alter the affinity of Ro15-1788 and Ro15-4513 by only ~2-4-fold. The considerable effect of the α1S205N substitution on *i*-BZD affinity suggests a specific interaction of the α1Ser205 residue with these ligands. As mutation at the homologous residue in the α6-isoform did not change the affinity of Ro15-4513, there is an apparent differential contribution of this residue to *i*-BZD binding at the DS and DI subtypes.

The results of the reciprocal α6/α1 mutations emphasize the divergent requirements for ligand recognition at the DI compared to the DS subtypes, in particular for Ro15-4513. Several lines of evidence, including opposite efficacies at DI and DS receptors (see Hadingham et al., 1996; Dunn et al, 1999), suggest that Ro15-4513 interacts differently with each receptor subtype. Distinct domains of α-subunits are photolabeled by [^{3}H]Ro15-4513 in native bovine cortical DS receptors compared to cerebellar DI receptors (Duncalfe and Dunn, 1996); however, this difference was not reproduced in recombinant receptors containing the α1- or α6-subunits (Davies et al., 1996). The substrate of [^{3}H]Ro15-4513 labeling in cortical receptors has been mapped to the extracellular loop between transmembrane domains 2 and 3 (Davies and Dunn, 1998), but more recent investigations have identified α1Y209 (and the homologous tyrosine in the α2- and α3-subunits) as a major site of Ro15-4513 labeling (Sawyer et al., 2002). The proximity of this residue to α1S205 supports a role of this domain in the recognition of Ro15-4513 at α1-containing receptors. Determination of the site for covalent incorporation of Ro15-4513 into the α6- (and α4-) subunits remains to be determined.

Recent homology models of the BZD site based on the molluscan acetylcholine binding protein (AChBP) (Brejc et al., 2001) propose a specific orientation of Ro15-4513 within the binding cleft at the α1/γ2-subunit interface (Sawyer et al., 2002; Kucken et al., 2003) and predict the proximity of the photo-reactive 8′-azide for interaction with α1Y209. Ligand mapping modeling of the BZD pharmacophore predicts structural differences of the DI BZD site, including a smaller binding pocket created with the α6-isoform (e.g. Huang et al., 2000; He et al., 2000). Further predictions suggest that the 8′-substituent of *i*-BZD ligands underlies DS/DI subtype selectivity and that the 8′-azide moiety of Ro15-4513 must adopt different diastereomeric conformations for lowest energy docking within the DS and DI binding sites (Wong et al., 1993; Huang et al., 2000). Although the present mutational results cannot be directly compared to the pharmacophore models, disparate effects of the α1S205N/α6N204S substitutions on Ro15-4513 binding are consistent with a differential interaction of the 8′-azide with the Loop C domain of the α-subunit at DS and DI subtypes.

Comparative modeling of the BZD site (Cromer et al., 2002; Ernst et al., 2003) suggests that although there exists conservation of the overall hairpin structure of Loop C, low sequence homology with the AChBP (Brejc et al., 2001) in this region leads to high model variability. In particular, the low homology prevents the exact placement of the preceding β-strand segment (β9) and hampers the determination of the length of the bend (L10; centered on Loop C) between the β9 and β10 structural β-strands of the proposed α-subunit structure (see Figure 2-4; Ernst et al., 2003). As a consequence, a unified model across all α-isoforms is not possible. The variable L10 region contains the identified α1S205/α6N204 divergence; structural differences in this region at DI and DS BZD sites may underlie the disparate effects of substitution on Ro15-4513 recognition. Additional residues from Loop C that have been implicated in BZD ligand selectivity (see Sigel, 2002) align to the β-sheet structures that flank the L10 hairpin, indicating a more complex region that influences the structure and pharmacology of the binding site. Interestingly, inclusion of the χ207-subunit (with the crossover point in Loop C) reduced the affinity of Ro15-4513 compared to either wild-type DI receptor, implying that the exact structure of this region influences Ro15-4513 recognition. The reduced affinity of

Ro15-4513 at χ207-containing receptors does not negate the usefulness of that chimeric subunit in identifying α6N204 as a mediator of β-carboline affinity at the DI subtypes, and emphasizes the complex contributions to ligand recognition from multiple residues at the α-γ subunit interface. Further investigation of the differences in the binding sites produced with the different α-isoforms (see Sigel, 2002) is warranted to better understand ligand recognition, especially at the DI BZD binding site.

With respect to the expression of α4- and α6-containing $GABA_AR$ in *Xenopus* oocytes for functional studies of β-carboline interaction, the low affinity of these compounds for the BZD site (especially for β-CCE) hampered the analysis of modulatory effects. Beyond the selectivity for the α4- over the α6-subtype that is explored in this chapter, the affinities of β-CCE and DMCM for the BZD site on DI $GABA_AR$ are considerably lower than for the site on DS subtypes (e.g. Table 2-1; see also Yang et al., 1995; Gunnersen et al., 1996; Knoflach et al., 1996). In particular, the affinity with which β-CCE recognizes the BZD site on the α6-subtype is in the low micromolar range (Table 2-1), a concentration sufficient to produce non-BZD site effects (Stevenson et al., 1995). The activity of negative modulator β-carboline ligands to potentiate receptor function through a separate lower-affinity site (Stevenson et al., 1995) that overlaps the loreclazole modulatory site (Wingrove et al., 1994) complicates analysis of a complete range of β-CCE and DMCM concentrations. Other studies with BZD site ligands have indicated a general concordance of ligand EC_{50} values for modulation with affinity constants as determined by radioligand assay (e.g. Davies et al., 2000). Considering this, demonstrable modulation of the DI $GABA_AR$ subtypes through the BZD site by β-CCE and DMCM would most likely occur in the high nanomolar to low micromolar concentration range. In this manner, isolating the functional effects of DMCM and β-CCE that are mediated through the BZD site on the DI $GABA_AR$ subtypes is not possible, nor is the determination of the functional consequences of the inclusion of the mutations or the chimeric α-subunits described in this chapter.

Robust expression of both DI subtypes was only observed for receptors that incorporated the β3-isoform. Although heterologous expression of the α4β2γ2 subtype could be achieved in tsA201 cells for radioligand binding experiments, no expression of

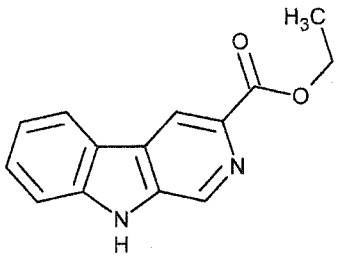
α4-containing $GABA_AR$ was observed when the β2-isoform was used for co-injection into oocytes. Here we observe a cell system-dependent selectivity for the assembly or expression of the α4β2γ2 $GABA_AR$ subtype. Additionally, higher levels of expression (as assayed by maximal GABA-gated current) were only seen for transcript injections with a proportionally higher amount of the α4-subunit (e.g. 5:1:1 ratio of α:β:γ), in accordance with earlier studies that demonstrated better expression of the α4-subtype with the injection of more α4-isoform transcript (Whittemore et al., 1996).

The α6(N204I) mutation was shown to reduce the potency of GABA for receptor activation by ~8-fold. This was not predicted, as this α6-subunit Loop C residue is postulated to contribute to the α-subunit component of the BZD site, and not to the α-subunit segments that orient towards the GABA binding pocket. Recent studies on the α6-subtype have revealed a specific residue in the α6-isoform ($\alpha6Ser^{83}$), from a domain that has not been previously implicated in affecting the agonist response, that contributes to the higher potency of GABA for activation of α6-containing $GABA_AR$ (Drafts and Fisher, 2004). The effect of mutation at $\alpha6Asn^{204}$ on the GABA response warrants further investigation into the role of this residue in the pharmacology of α6-containing $GABA_AR$.

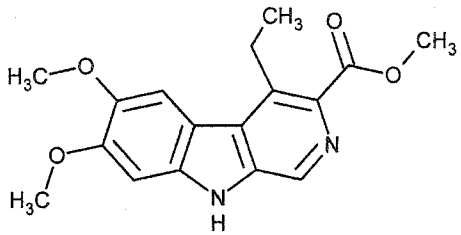
Figure 2-1

Chemical structures of the BZD site ligands used in these studies. Both β-CCE (A.) and DMCM (B.) were chosen as ligands from the β-carboline class. Two imidazobenzodiazepine (*i*-BZD) ligands, Ro15-4513 (C.) and Ro15-1788 (flumazenil, D.) and the 1,4-BZD flunitrazepam (FNZ, E.) were also used for radioligand binding experiments.

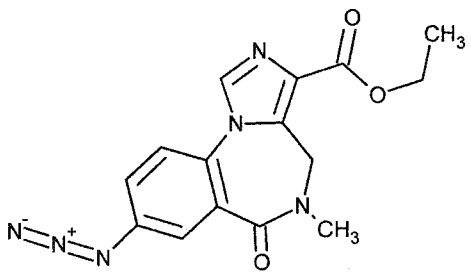
A.



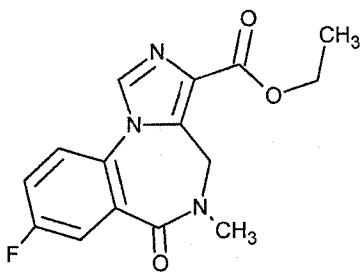
B.



C.



D.



E.

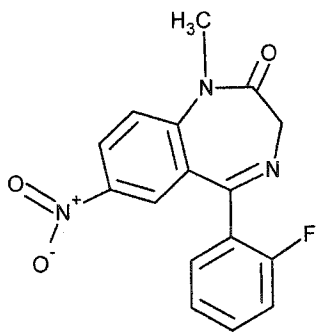


Figure 2-2

Schematic of the vector construct that was used to create targeted random chimeras from rat $GABA_A$ α6- and α4-subunit cDNA, based on the protocol of Moore and Blakely (1994). The entire α6 subunit cDNA was subcloned into pcDNA3.1(+) upstream of a partial α4 sequence (that excludes the signal sequence and the first seven amino acids). This dual plasmid was digested with restriction enzymes pairs (*Bam*HI and *Hpa*I; *Eco*RI and *Bst*XI) to limit the amount of homologous cDNA that was capable of participating in a recombination event between the subunit sequences. *Escherichia coli* were transformed with linearized cDNA wherein the recombination events occurred (see Materials and Methods).

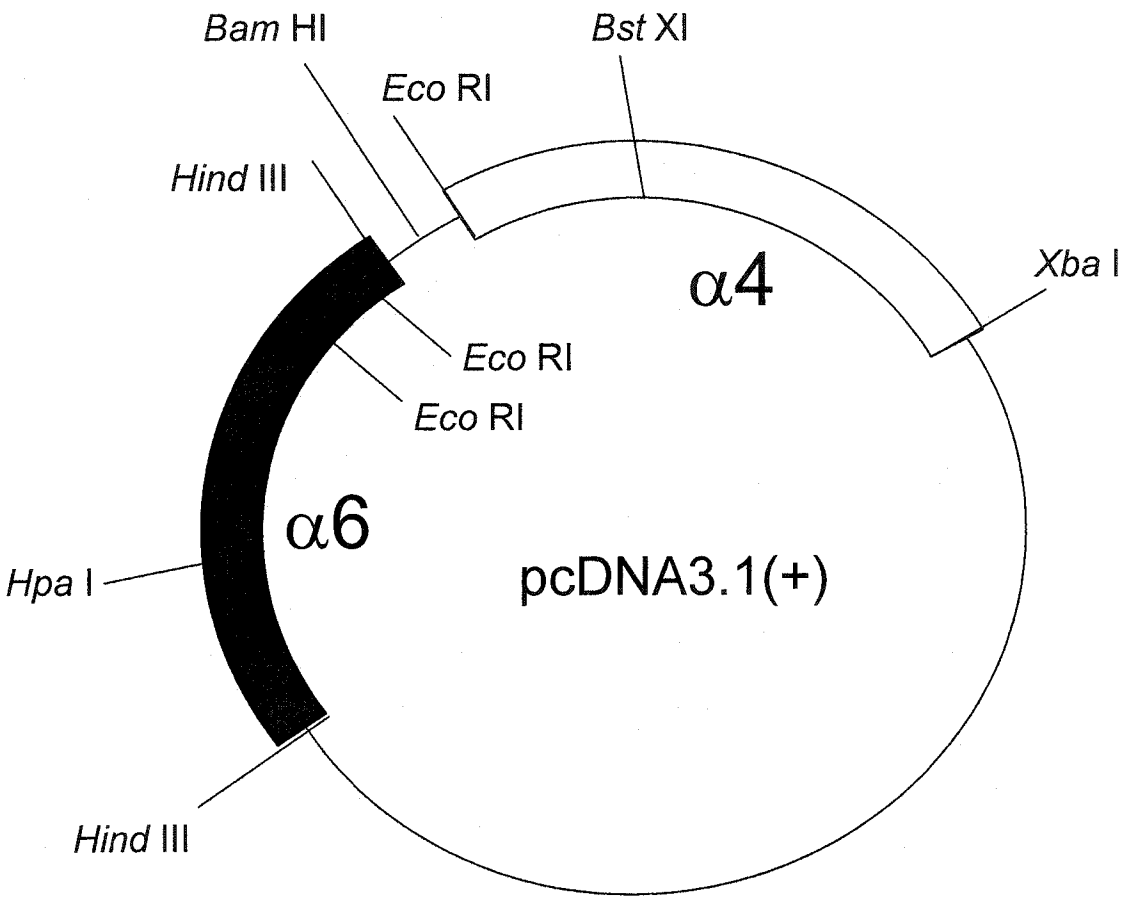


Figure 2-3

Two chimeric alpha subunits (χ) were isolated and named according to the last residue of α6 sequence prior to the switch point. A linear depiction of the wild-type α4-, α6- and the χ141- and χ207- subunits is presented. *White*, α4 sequence. *Black*, α6 sequence. *Gray*, putative transmembrane domains.

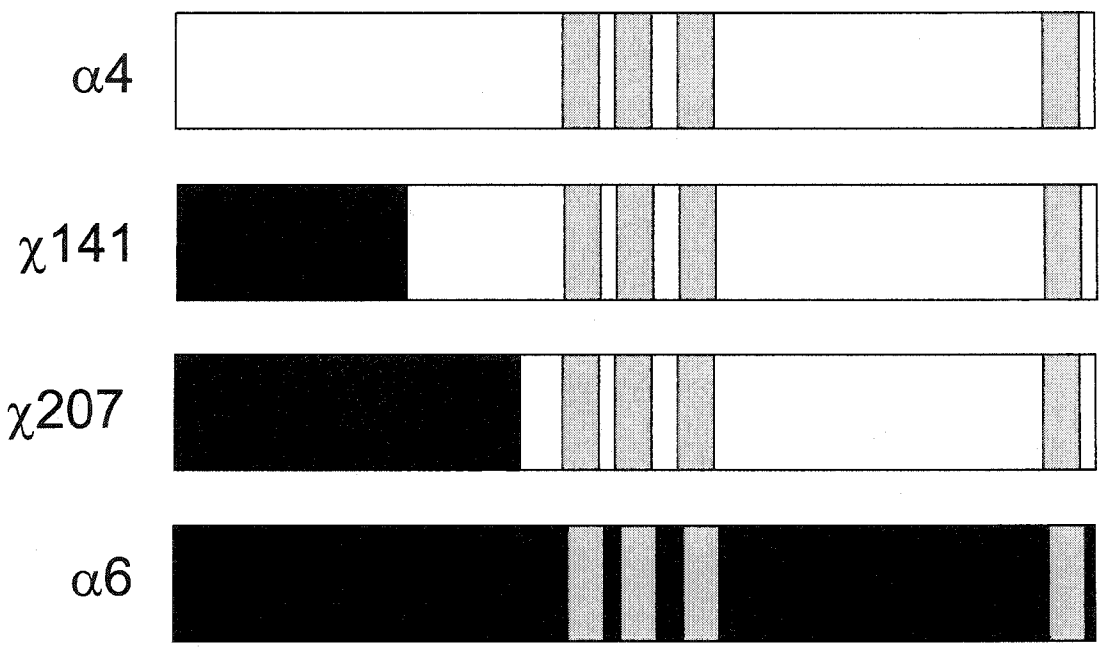


Figure 2-4

Sequence alignment of the α1-, α4- and α6-subunit Loop C region of the extracellular N-terminus. Numbering above the figure represents the rat α1 sequence whereas the number below the figure depicts the rat α6 subunit numbering. Letters in boldface type indicate residues that have been implicated previously in BZD binding (see Sigel, 2002). The boxed letters indicate the α-subunit residues that are the focus of this current study. The boxes above the sequence represent the structural features proposed by comparative modeling of the $GABA_A$ receptor (Ernst et al, 2003) based on the structure of the AChBP (Brejc et al., 2001). *White*, putative β-strands, β9 and β10. *Black*, structural loop 10 (L10). *Gray*, sequence corresponding to the Loop C domain.

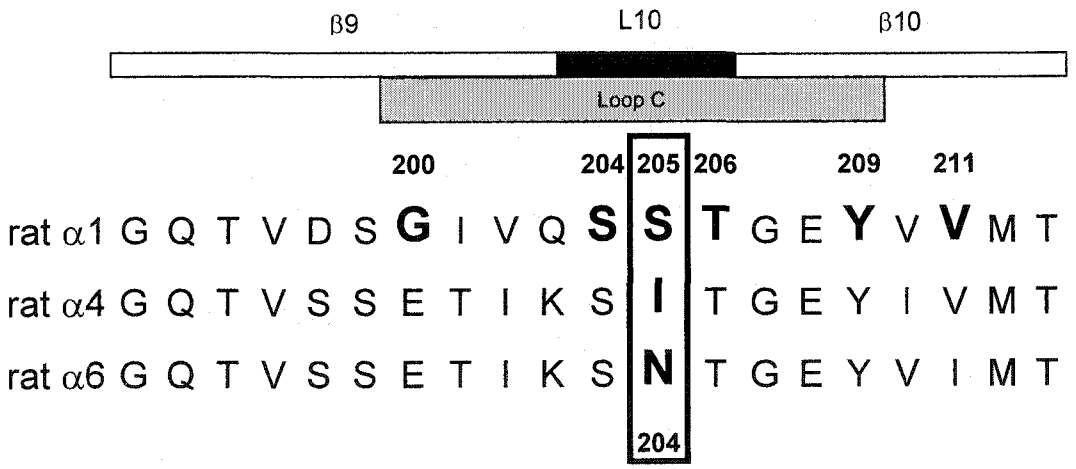
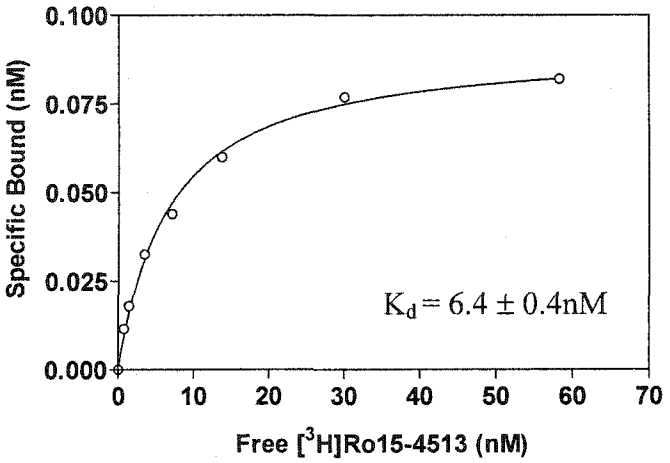


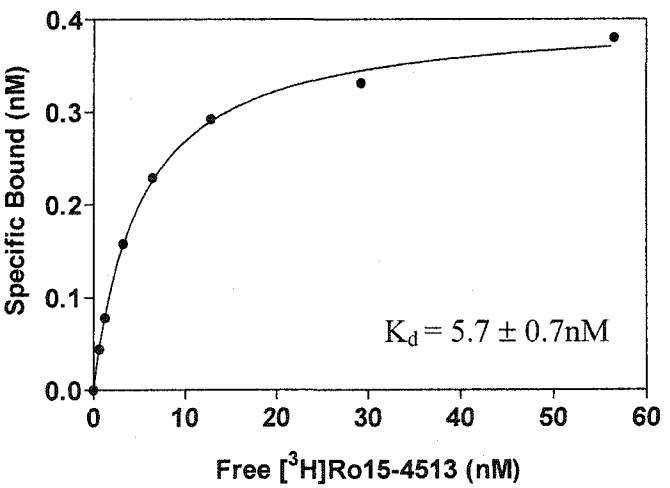
Figure 2-5

The affinity of [^{3}H]Ro15-4513 at the (A.) α4β2γ2 and (B.) α6β2γ2 $GABA_AR$ subtypes as determined by saturation binding. A representative curve for each subtype is shown; affinity (K_d) represents the mean ± S.E.M. of at least three independent experiments. (C.) Competition binding curves of the displacement of [^{3}H]Ro15-4513 by β-CCE. The β-carboline was used to displace the radioligand from membrane-enriched fractions of cell homogenates prepared from cells expressing either wild-type α4β2γ2 (○) or α6β2γ2 (●) $GABA_A$ receptors. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for each receptor subtype. Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate. These ligand affinity data are summarized in Table 2-1.

A.



B.



C.

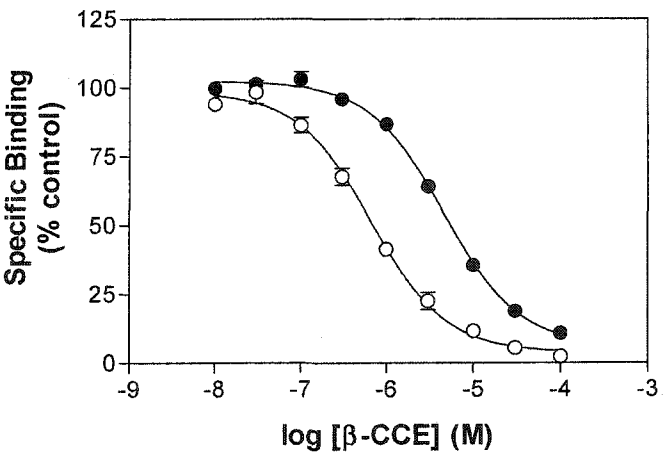
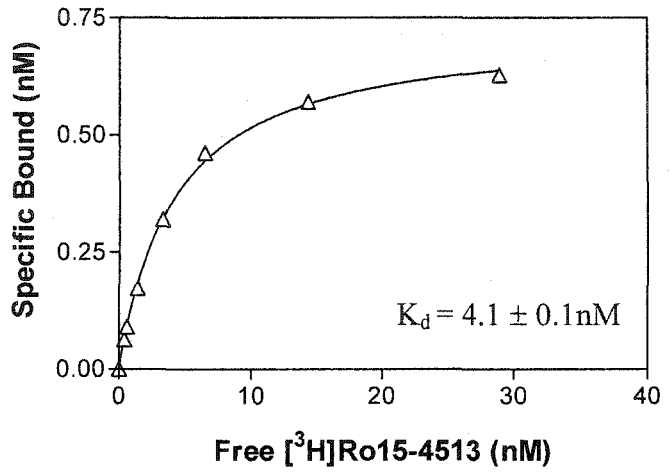


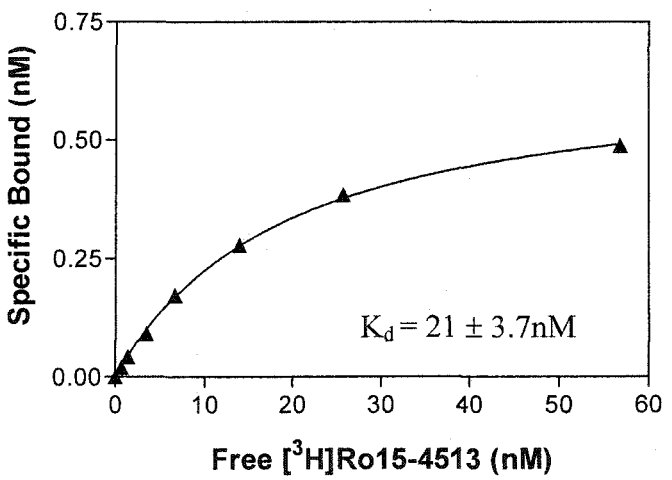
Figure 2-6

The affinity of [^{3}H]Ro15-4513 at the (A.) χ141β2γ2 and (B.) χ207β2γ2 $GABA_AR$ subtypes as determined by saturation binding. A representative curve for each subtype is shown; affinity (K_d) represents the mean ± S.E.M. of at least three independent experiments. (C.) Competition binding curves of the displacement of [^{3}H]Ro15-4513 by β-CCE. The β-carboline was used to displace the radioligand from membrane-enriched fractions of cell homogenates prepared from cells expressing $GABA_A$ receptors incorporating the α-subunit chimeras χ141 (△) or χ207 (▲). [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for each receptor subtype. Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate. These ligand affinity data are summarized in Table 2-1.

A.



B.



C.

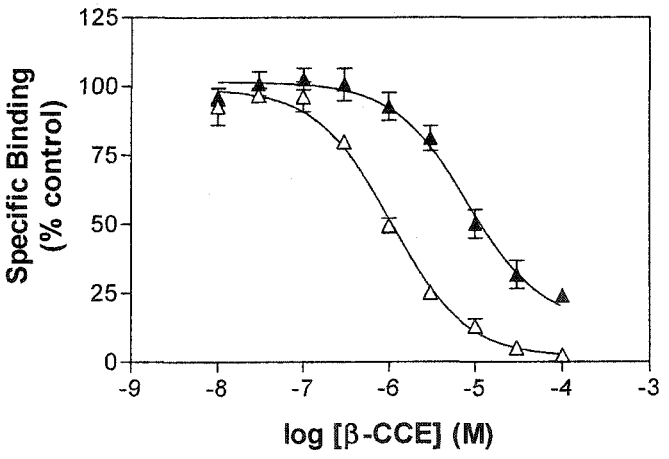
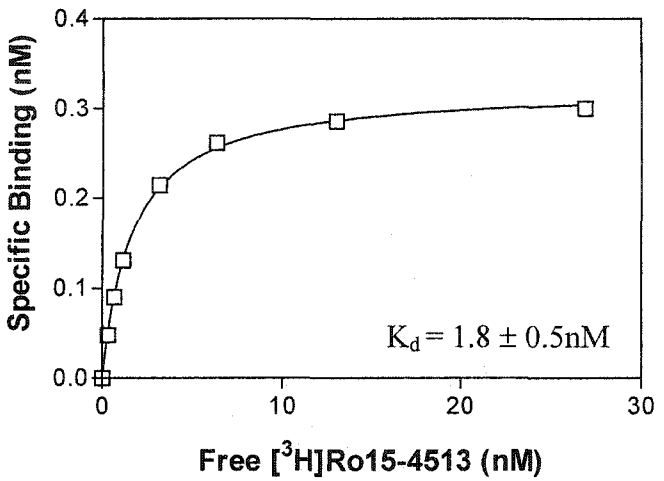


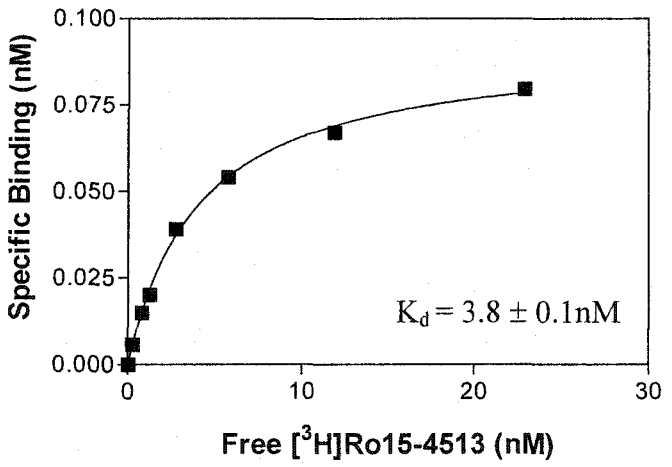
Figure 2-7

The affinity of [^{3}H]Ro15-4513 at the (A.) α6(N204I)β2γ2 and (B.) α6(N143D)β2γ2 $GABA_AR$ subtypes as determined by saturation binding. A representative curve for each subtype is shown; affinity (K_d) represents the mean ± S.E.M. of at least three independent experiments. (C.) Competition binding curves of the displacement of [^{3}H]Ro15-4513 by β-CCE. The β-carboline was used to displace the radioligand from membrane-enriched fractions of cell homogenates prepared from cells expressing $GABA_A$ receptors incorporating the α6(N204I)β2γ2 (□) or α6(N143D)β2γ2 (■) mutant α-subunits. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for each receptor subtype. The curve for β-CCE affinity at wild-type α6β2γ2 receptors (from Figure 2-5) is included for comparison (dashed line). Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate. These ligand affinity data are summarized in Table 2-1.

A.



B.



C.

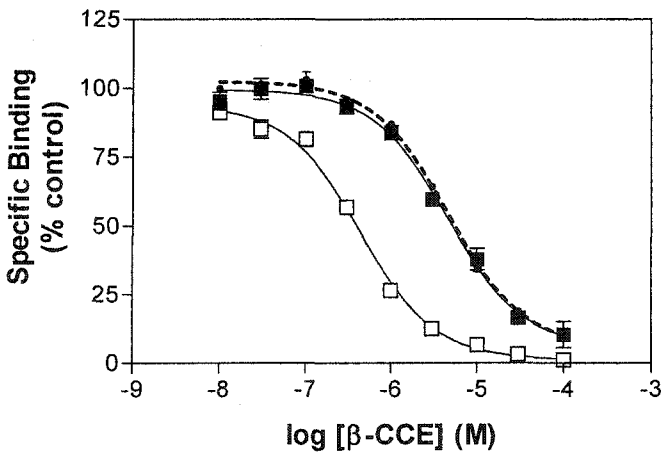


Figure 2-8

Competition binding curves of the displacement of [^{3}H]Ro15-4513 by DMCM. The competition curves show DMCM displacement of the radioligand at wild-type α4β2γ2 (○), α6β2γ2 (●) and α6(N204I)β2γ2 (□) $GABA_A$ receptors. [^{3}H]Ro15-4513 was present at a concentration equivalent to its K_d value for each receptor subtype. High affinity binding of the radioligand to the mutant receptors was retained as shown by saturation binding (see Table 2-1). Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate.

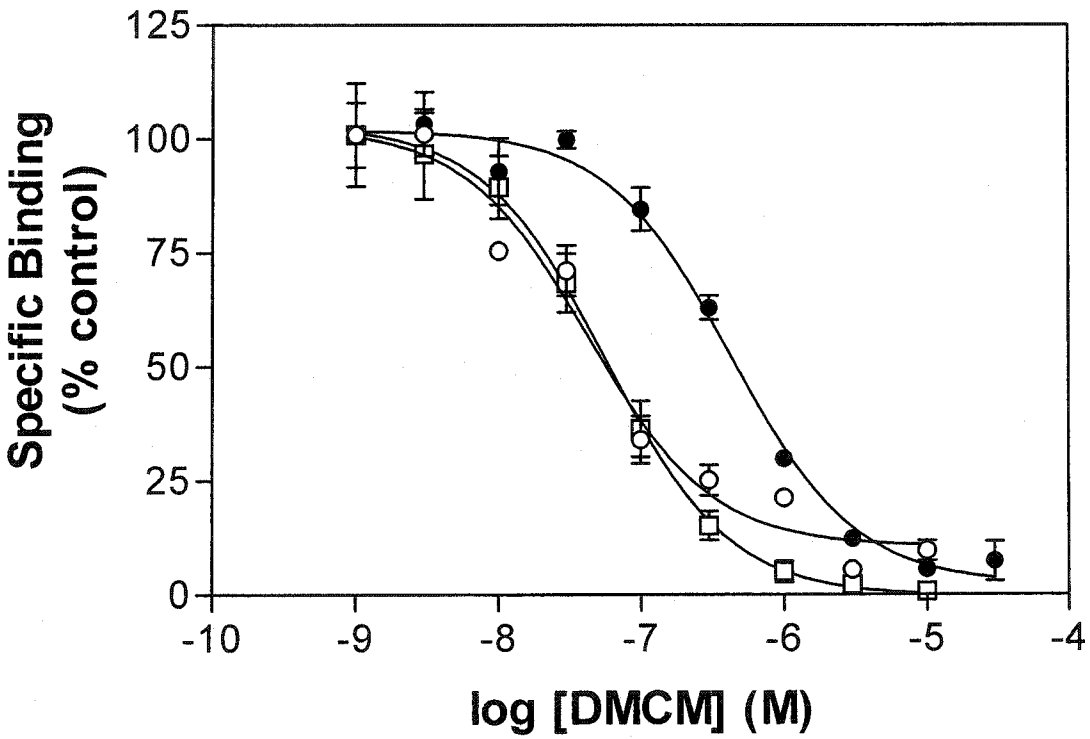
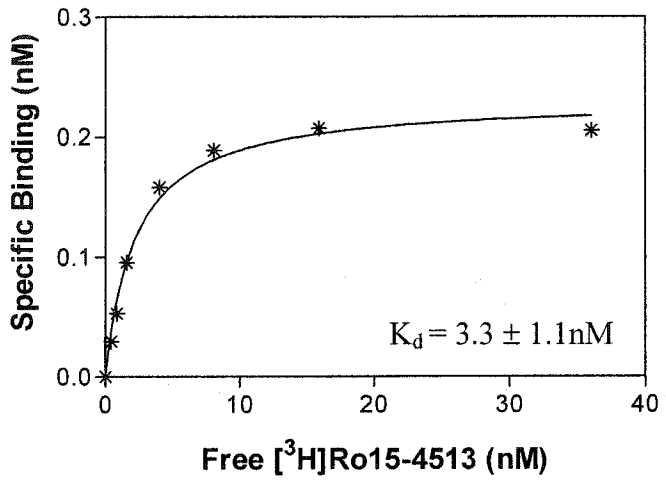


Figure 2-9

The affinity of [^{3}H]Ro15-4513 at the (A.) α6(N204S)β2γ2 $GABA_AR$ subtype as determined by saturation binding. A representative curve is shown; affinity (K_d) represents the mean ± S.E.M. of at least three independent experiments. (B.) Competition binding curves of the displacement of [^{3}H]Ro15-4513 by β-CCE (◇) and DMCM (◆) from membranes prepared from cells expressing mutant α6(N204S)β2γ2 $GABA_A$ receptors. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value for the mutant receptor subtype. Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate. These ligand affinity data are summarized in Table 2-1.

A.



B.

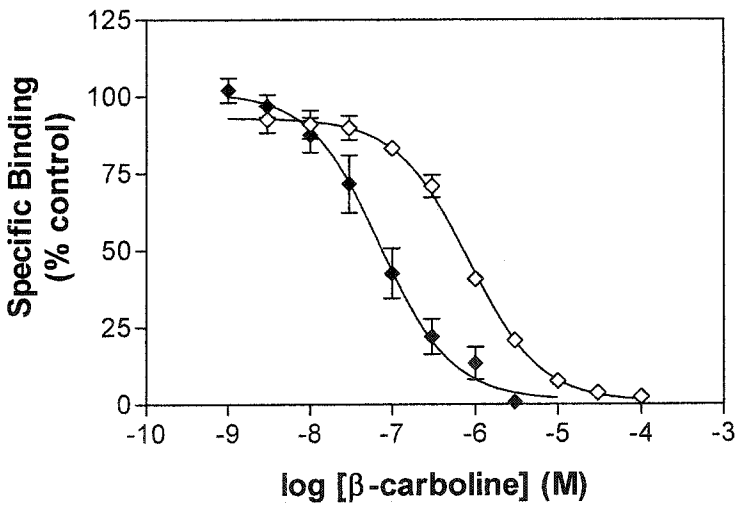


Figure 2-10

Displacement of [^{3}H]flunitrazepam binding by Ro15-1788 and Ro15-4513. Competition binding curves show the displacement of [^{3}H]flunitrazepam from wild-type $\alpha1\beta2\gamma2_L$ receptors (open symbols) and from mutant $\alpha1$(S205N)$\beta2\gamma2_L$ receptors (filled symbols) by Ro15-1788 (○,●) and Ro15-4513 (□,■). [^{3}H]Flunitrazepam was present for competition binding experiments at a concentration equal to its K_d at the receptor subtypes (Table 2-2). Data shown represent the mean ± S.E.M. of three independent experiments performed in duplicate. *Inset*, representative curve of [^{3}H]flunitrazepam saturation binding at the $\alpha1$(S205N)$\beta2\gamma2$ subtype (K_d = 2.0 ± 0.5 nM).

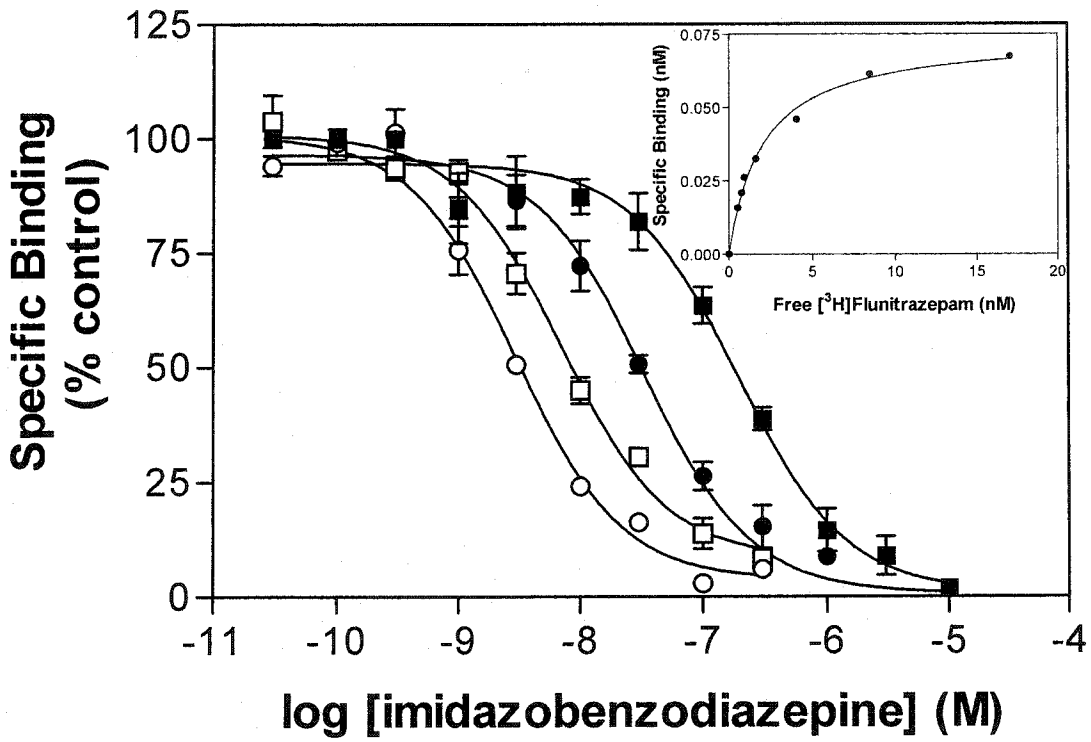


Figure 2-11

Concentration-effect relationships for receptor activation by GABA on receptor subtypes that include the wild-type α4 (○), wild-type α6 (●) or the mutant α6(N204I) subunit (■) in combination with wild-type β3 and $\gamma 2_L$ subunits. Receptor subtypes were expressed in *Xenopus* oocytes and the current evoked by a range of concentrations of GABA of at least four orders of magnitude was tested to determine the concentration for half-maximal activation (EC_{50} concentration). Data represent the mean ± S.E.M. of at least three independent curves from individual oocytes and are summarized in Table 2-3.

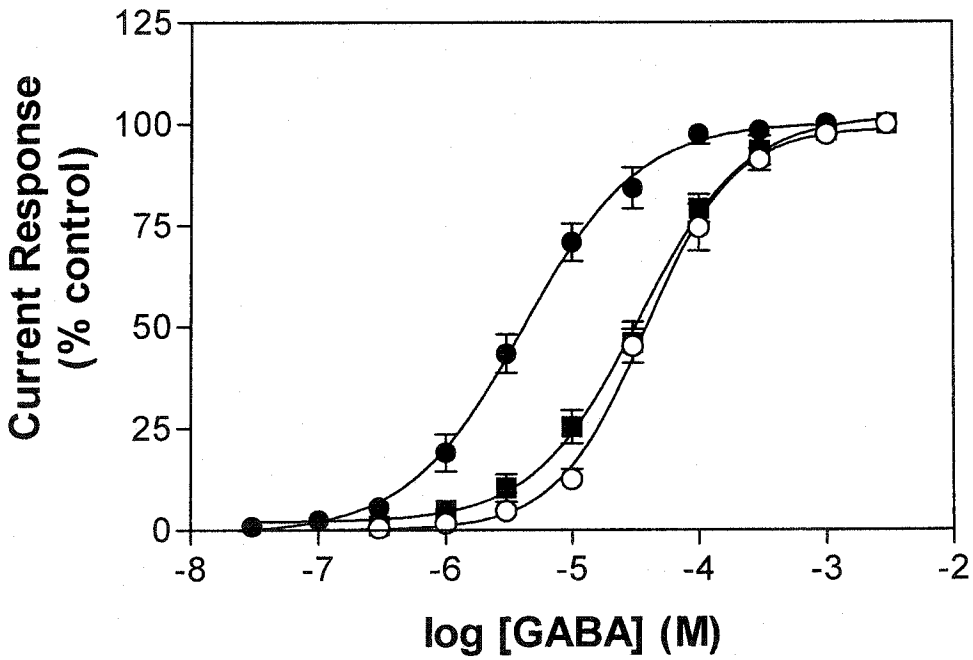


Table 2-1. Binding affinities of BZD site ligands at $GABA_A$ receptor subtypes that incorporate wild-type, mutant and chimeric α-subunits with β2 and $\gamma2_L$ subunits. Data shown are mean ± S.E.M. from at least three independent experiments performed in duplicate. [a]Affinity values for β-CCE and DMCM are obtained from studies of the displacement of [^{3}H]Ro15-4513 and are represented as K_i. [b]Affinity of Ro15-4513 from saturation analysis is given as K_d. N.D. = not determined. Data were analyzed by one-way ANOVA followed by the Dunnett post-test for levels of significance. $^*P < 0.05$, $^{***}P < 0.001$ compared to wild-type α6β2γ2; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to wild-type α4β2γ2.

	Affinity (nM)		
α Subunit	β-CCE[a]	DMCM[a]	Ro15-4513[b]
α4	$347 \pm 28^{*}$	$24 \pm 5^{***}$	6.4 ± 0.4
α6	$2300 \pm 200^{\#}$	$220 \pm 27^{\#\#\#}$	5.7 ± 0.7
χ141	$540 \pm 59^{*}$	N.D.	4.1 ± 0.1
χ207	$5400 \pm 1000^{***, \#\#\#}$	N.D.	$21 \pm 3.7^{***}$
α6(N143D)	$2200 \pm 200^{\#}$	N.D.	3.8 ± 0.1
α6(N204I)	$210 \pm 17^{*}$	$30 \pm 7^{***}$	1.8 ± 0.5
α6(N204S)	$442 \pm 27^{*}$	$41 \pm 11^{***}$	3.3 ± 1.1

Table 2-2. Binding affinities of BZD site ligands at $GABA_A$ receptor subtypes that incorporate wild-type and mutant α1-subunits with β2 and $\gamma2_L$ subunits. Data shown are mean ± S.E.M. from at least three independent experiments performed in duplicate. [a]Affinity of flunitrazepam (FNZ) was determined by saturation binding analysis and is given as the K_d value. [b]Affinity values of Ro15-4513, Ro15-1788, β-CCE and DMCM are given as K_i values determined from the displacement of [^{3}H]flunitrazepam or [^{3}H]Ro15-4513. [c]Data from Davies et al. (1998). Data were analyzed by a two-tailed *t*-test between wild-type control and mutant for each ligand. $*P < 0.05$, $**P < 0.01$ compared to wild-type α1β2γ2.

	Affinity (nM)				
α Subunit	FNZ[a]	Ro15-4513[b]	Ro15-1788[b]	β-CCE[b]	DMCM[b]
α1	6.1 ± 0.4[c]	3.2 ± 0.3	1.3 ± 0.1	0.43 ± 0.02	11 ± 1
α1(S205N)	2.0 ± 0.5**	121 ± 15**	25 ± 5.0*	2.8 ± 0.2**	88 ± 13**

Table 2-3. Concentration-effect relationships for $GABA_AR$ activation by GABA on receptor subtypes expressed in *Xenopus* oocytes. The cRNA transcripts for different α-subunits were co-injected into oocytes with transcripts for wild-type β3 and $\gamma 2_L$ subunits. The current evoked by a range of concentrations of GABA of at least four orders of magnitude was tested to determine the GABA concentration for half-maximal channel activation (EC_{50}) and the Hill slope (n) of the concentration-effect curve [see Materials and Methods; $I = (I_{max}*[L]^n) / (EC_{50}^n + [L]^n)$]. Data represent the mean ± S.E.M. of at least three independent curves from individual oocytes. All data were analyzed by one-way ANOVA followed by the Dunnett post-test for levels of significance. $^{***}P < 0.001$ compared to wild-type α6-containing receptors.

α-Subunit	EC_{50} (μM) (replicates)	Hill Slope (n_H)
α4	41 ± 6.8 (4)***	1.3 ± 0.1
α6	4.3 ± 0.7 (3)	1.0 ± 0.1
α6(N204I)	35 ± 4.7 (4)***	1.1 ± 0.1

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CHAPTER 3*

Identification of a Domain in the α5-Subunit Of the $GABA_A$ Receptor That Underlies Insensitivity to Modulation by Fluoxetine

* *A version of this chapter is in preparation for submission to* The Journal of Neurochemistry. DERRY, J.M.C., DAVIES, M., AND DUNN, S.M.J.

INTRODUCTION

In addition to the binding sites for GABA, there exist several distinct sites on the $GABA_A$ receptor through which a broad variety of substances can act allosterically to modulate agonist-evoked receptor function. Examples of $GABA_A$ receptor modulators include benzodiazepine (BZD) site ligands, barbiturates, neurosteroids, certain ions (e.g. Zn^{2+}), volatile anaesthetics, and some alcohols (e.g. Barnard et al., 1998; Korpi et al., 2002). Whereas the BZD site is well defined at the $\alpha(+)/\gamma(-)$ subunit interface (in homology to the low affinity GABA binding site at the $\beta(+)/\alpha(-)$ interface; see Sigel and Buhr, 1997; Ernst et al, 2003) and specific receptor domains involved in the interaction of other compounds have been identified (e.g. Zn^{2+}; see Hosie et al., 2003), the structural basis for interaction of many other modulators is either poorly characterized or not yet defined.

The characterization of a specific allosteric site on the $GABA_A$ receptor is complicated by the heterogeneity of receptor subtypes. The $GABA_A$ receptor is a heteropentamer of homologous subunits that together form a GABA-gated, chloride-selective ion pore. Subunits from several different classes have been identified (α1-6, β1-3, γ1-3, δ, ε, π and θ) (see Korpi et al, 2002) and the specific combination that assembles defines the pharmacology of the receptor subtype. Most $GABA_A$ receptors are predicted to include α-, β-, and γ- subunits in a 2:2:1 stoichiometry (e.g. Tretter et al., 1997; Farrar et al., 1999), but other subunits can substitute, often for the γ-subunit in the pentamer (see Korpi et al., 2002). However, this multiplicity of subtypes can be advantageous for the characterization of sites through which modulators act. Recombinant expression of defined subtypes permits an investigation of the absolute subunit requirements for allosteric modulation. Recently, this approach has been used in an initial effort to identify specific subunit requirements for potentiation by fluoxetine (FLX), acting as a novel $GABA_A$ modulator (Robinson et al., 2003).

Beyond its activity as a selective-serotonin re-uptake inhibitor (SSRI) antidepressant, FLX has additional interactions with several ligand-gated ion channels (LGIC). Non-competitive functional antagonism of the nicotinic acetylcholine (nAChR; e.g. Fryer and Lucas, 1999) and serotonin type-3 ($5\text{-}HT_3R$; e.g. Eisenamer et al., 2003) receptors are known in addition to the ability of FLX to allosterically potentiate $GABA_A$

receptor responses (Robinson et al., 2003). FLX potentiation of currents elicited by submaximal GABA concentrations has been shown to be independent of the β-, γ, and δ-subunits; all recombinant $GABA_A$ receptor subtypes examined, with the sole exception of α5-containing receptors, are sensitive to modulation by FLX (Robinson et al., 2003). This FLX-sensitivity is independent of the presence of a γ-subunit, suggesting that FLX is not acting through the allosteric BZD binding pocket formed at the α(+)/γ(-) subunit-subunit interface. These recombinant expression studies have concluded that FLX acts through a novel allosteric site associated, at least in part, with the α-subunit isoform in the receptor complex (Robinson et al., 2003).

The original aim of this investigation was to determine the region of the α5-isoform that underlies FLX insensitivity in order to better define the α-subunit requirements for FLX modulation. We have created several chimeric subunits derived from the wild-type α1- and α5-isoforms to probe for the structural basis of the lack of potentiation by FLX at α5-containing receptors. We have found that a chimeric subunit that incorporates a portion of the extracellular N-terminus of the α1-isoform is sufficient to confer FLX-sensitivity to the α5-isoform. Expression of $GABA_A$ receptors that incorporate additional chimeric α-subunits with separate crossover points in the extracellular domain yields receptors that differ in their ability to be modulated by FLX. We have used these differential responses to delineate a discrete region in the N-terminal domain of the α-subunit that appears to have a role in mediating the allosteric potentiation by FLX.

The differential responsiveness of chimeric α-subunits with nearby crossover points identifies a section of the α-subunit polypeptide that contains molecular determinants of the FLX-insensitivity. To further probe for the basis of this response, comparative modeling of the $GABA_A$ receptor α-subunit (see Ernst et al., 2003) was carried out to identify additional segments of the α-isoform that are predicted to be in close spatial approximation with our identified protein region. Site-specific mutagenesis, whereby the homologous residue from the α1-isoform was substituted into the α5-isoform, was done in an attempt to create gain-of-function mutations that bestow FLX-sensitivity. Substitution mutation of an additional residue from a non-contiguous protein

domain (modeled in close molecular proximity to our segment) was also prepared to examine the role of this adjacent domain.

We created several mutant α5-subunits within the delimited extracellular domain to probe for the molecular basis of insensitivity to FLX. Only one individual substitution mutation (α5T179A) was sufficient to confer sensitivity, identifying a single amino acid residue ($\alpha5Thr^{179}$) as a determinant of the lack of response to FLX in the α5-subtype. The reciprocal mutation (α1A175T) was without effect on the ability of FLX to potentiate $GABA_AR$ currents. Mutation of a residue in the α1-isoform that is modeled as closest from a non-contiguous segment (α1F45A) had no appreciable effect on FLX modulation, but reduced GABA potency by ~20-fold. Comparative modeling studies propose that this region of the α-subunit may contribute to the accessible surface of the GABA binding site (Kucken et al., 2000; Ernst et al., 2003). The effect on GABA potency lends support to the notion that this region affects GABA interaction and identifies the most N-terminal α-subunit residue implicated in affecting the agonist binding site. The panel of randomly derived chimeric α1/5-subunits permitted the identification of a domain that contributes to allosteric modulation by FLX; comparative modeling of the α-subunit enabled a prediction of an adjacent domain in the α-subunit and the proposed contribution of a residue within to the agonist binding site. Therefore, within this three-dimensional portion of the α-subunit, different residues mediate an effect on the GABA- and the FLX-response.

MATERIALS AND METHODS

Materials

GABA and FLX were obtained from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON). FLX was also received as a generous gift from Eli Lilly and Co. (Indianapolis, IN, USA). See Figure 3-1 for the chemical structures of these compounds.

Production of Alpha-Subunit Chimeras

Chimeric subunits (χ) derived from the rat α1- and α5- subunit isoforms were produced as described previously (Derry et al., 2004). In brief, the protocol of Moore and Blakely (1994) was followed to allow for the creation of a series of random chimeric subunit cDNAs. A partial cDNA sequence of the rat α1-isoform (as a *Hind*III – *Bam*HI fragment) was subcloned into the pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA) upstream of the complete cDNA sequence encoding the rat α5-subunit (inserted into the same vector as a *Xho*I fragment) (see Figure 3-3). The partial α1-subunit sequence was a C-terminally truncated fragment that excludes the subunit sequence downstream of the predicted third transmembrane domain. In this manner, any random crossover events at regions of sequence homology between the two α-subunit sequences would be targeted to occur in the sequence proximal to that encoding the putative intracellular TM3-TM4 loop. Restriction digestion with *Bam*HI and *Eco*RV (cutting between the subunit inserts) was followed by transformation of the linearized plasmid DNA into Library Efficiency® competent DH5α *Escherichia coli* cells (Life Technologies, Gaithersburg, MD) wherein the recombination events occurred to produce in-frame hybrid cDNA. Separate colonies were chosen and their plasmid DNA was extracted and assessed for size. For those plasmids of a size indicative of a recombinant crossover event, the extracted DNA was examined with restriction digestion analysis and the exact point of crossover was confirmed by DNA sequencing. For the series of chimeric α-subunits, the numbering of individual chimeric constructs represents the last residue of α1-sequence (rat subunit numbering) prior to the in-frame crossover.

Mutagenesis

Site-directed mutagenesis was performed as described previously (Derry et al., 2004). Briefly, the QuikChange protocol (Stratagene, La Jolla, CA) was used to introduce specific residue substitutions into the wild-type rat α1- or α5- cDNA sequences. The individual subunit cDNA sequences were subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA) and these plasmids were used as the templates in separate PCR-mediated mutagenesis protocols. Successful substitutions within the cDNA insert of the plasmid were confirmed by DNA sequencing.

Expression of $GABA_A$ Receptors in *Xenopus* oocytes

Mature (stage V-VI) *Xenopus laevis* oocytes were isolated after collagenase treatment of a freshly isolated ovary lobe, as described previously (Goldin, 1992). Oocytes were maintained at 14°C in ND96 buffer (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ and 5mM HEPES, pH 7.4) supplemented with 100μg/ml gentamicin (GibcoBRL, Grand Island, NY). Capped (m7G(5')ppp(5')G) (GibcoBRL) cRNA transcripts were prepared for each $GABA_A$ receptor subunit. Plasmids containing the individual $GABA_A$ subunit rat cDNAs were linearized and used as templates for *in vitro* transcription with T7 RNA polymerase (Invitrogen, San Diego, CA) under the recommended conditions. Wild type, chimeric or mutant α-subunit transcripts were mixed in a 1:1:1 ratio with wild-type rat β2 and $\gamma 2_L$ transcripts (final RNA concentration of 1μg/μL in DEPC-treated water); 20-50nL (20-50ng) of total RNA was microinjected per oocyte. Oocytes were incubated in 96-well plates at 14°C prior to recording.

Two-Electrode Voltage Clamp

Electrophysiological recordings were made 2-14 days following injection. Oocytes were placed in a recording chamber (~0.5mL) and were continuously perfused with ND96 buffer (pH 7.4) at a rate of ~5mL/min. Using a GeneClamp500B amplifier (Axon Instruments Inc., Foster City, CA), oocyte membrane potentials were held at a membrane potential of -60mV with a standard two-electrode voltage clamp arrangement. The voltage-sensing and current-passing microelectrodes were filled with 3M KCl (final

resistance between 0.5-2.5 MΩ in ND96). Both FLX (<5mg/mL; 5mM stock) and GABA were dissolved in ND96 and all dilutions were made in the same buffer. Drug solutions were administered to oocytes by switching to the drug solution from buffer by gravity perfusion. Oocytes were used for experiments when current responses were stable (±5%) between successive agonist applications. After agonist challenge, oocytes were perfused with buffer for >12 minutes to permit sufficient time for recovery from desensitization. Washout times were increased to >15 minutes after exposure to FLX. GABA-gated currents were recorded using Axoscope 9.0 data acquisition software (Axon Instruments, Inc.) Concentration-effect experiments were conducted for GABA on oocytes expressing $GABA_A$ receptor combinations whereby the current elicited by several GABA concentrations (ranging at least four orders of magnitude) was measured. For FLX modulation experiments, oocytes were pre-exposed to 10mL of FLX solution (for ~2 min) immediately followed by the same concentration of FLX combined with a submaximal concentration of GABA (3μM or 10μM GABA, dependent on each receptor combination, EC_{5-15}). Exposure of oocytes to concentrations of FLX >300μM caused a consistent loss of stable voltage clamp of membrane potential. Therefore, the highest concentration of FLX that oocytes were exposed to was limited to 300μM.

Structural Modeling

All predictions about the structure of the $GABA_A$ receptor α-subunit were made based on a previously published, best-fit, comparative model of the α1β2γ2 $GABA_A$ receptor subtype (Ernst et al., 2003). This model, ultimately derived from comparison of sequence and predicted structure of the subunits of the $GABA_AR$ with the molluscan acetylcholine binding protein (AChBP; Brejc et al., 2001), has been made publicly available (http://www.univie.ac.at/brainresearch) and was used as the template for the modeling studies in this chapter. The model was viewed in WebLab ViewerLite 4.0 (Accelrys, Inc., San Diego, CA). Use of the modeling software permitted the identification and visualization of amino acid residues within a defined atomic radius from a focus. Since the model (Ernst et al., 2003) is of a $GABA_AR$ that incorporates the α1-isoform, all modeled predictions are for this isoform.

Data and Statistical Analysis

To determine the potency of GABA for receptor activation at each $GABA_AR$ subtype expressed, concentration-effect data were fitted by the following four-parameter logistic equation using GraphPad Prism 3.0:

$$I = (I_{max} * [L]^n) / (EC_{50}^n + [L]^n)$$

where I is the measured GABA-evoked current, $[L]$ is the GABA concentration, EC_{50} is the GABA concentration that evokes half the maximal current (I_{max}) and n is the Hill slope coefficient. Data were analyzed by a two-tailed t-test between two receptor subtypes.

The GABA current elicited subsequent to FLX exposure ($I_{GABA+FLX}$) was measured and compared to the current evoked by the application of the control concentration of GABA alone (I_{GABA}). Extent of modulation by FLX is expressed as a percentage of the control current and is defined as:

$$\text{Potentiation} = (I_{GABA+FLX} / I_{GABA}) * 100$$

for each concentration of FLX. Data were analyzed as the mean ± S.E.M. of at least three independent responses (on individual, stable oocytes) per FLX concentration, and are plotted as a histogram. A comparison was made for the modulatory response to the highest concentration (300μM) of FLX tested; data were analyzed by a one-way ANOVA and levels of significance were determined by the Dunnett *post hoc* test for multiple comparisons.

RESULTS

FLX Activity at Wild-type $\alpha1\beta2\gamma2_L$ and $\alpha5\beta2\gamma2_L$ $GABA_A$ Receptors

Wild type $\alpha1\beta2\gamma2_L$ and $\alpha5\beta2\gamma2_L$ $GABA_A$Rs were recombinantly expressed in *Xenopus* oocytes. Concentration-effect curves yielded similar EC_{50} values for GABA-evoked currents of 32.8 ± 2.5μM and 31.4 ± 0.8μM, respectively (Figure 3-6; Table 3-1). Since FLX potentiation of currents has been reported to be GABA concentration-dependent and to occur at only submaximal GABA concentrations (Robinson et al., 2003), a submaximal test concentration of 10μM GABA (within ~EC_{5-15}) was used for testing FLX effects on GABA-gated currents at these receptor subtypes. Exposure of the oocytes to concentrations of FLX >300μM was consistently deleterious to the cells, causing disintegration of the oocyte or loss of a stable voltage clamp and is the system-dependent maximum concentration that was tested. A series of FLX concentrations up to 300μM was tested on both wild-type $GABA_A$R subtypes and a dichotomy in the response of each to the modulator was seen (e.g. Figures 3-2, 3-7). Whereas the GABA response at the $\alpha1\beta2\gamma2_L$ subtype was dose-dependently enhanced by FLX (maximal enhancement with 300μM FLX to 144 ± 10% current compared to GABA current alone), the $\alpha5\beta2\gamma2_L$ subtype showed an insensitivity to potentiation. A slight decrement in the GABA-evoked current (85 ± 14 % of the GABA-alone response) was observed with exposure to 300μM FLX (Figure 3-2, 3-7, Table 3-1).

Recombinant $GABA_A$ Receptors with Chimeric α1/α5 Subunits

A panel of five chimeric α1/α5 subunits was produced and isolated for expression with wild-type β2 and $\gamma2_L$ subunits (Figure 3-4). Separate receptor subtypes were expressed by the inclusion of individual α-subunit chimeras (χ60, χ103, χ164, χ183 and χ281) and were exposed to 100-300μM FLX to assess sensitivity. Upon initial screening, only inclusion of the χ281- and χ183-subunits produced recombinant $GABA_A$ receptors that were sensitive to potentiation by >100μM FLX (Figure 3-4). The lack of a robust effect of FLX on receptors containing the χ164-subunit was taken as insensitivity of that subtype (depicted as "–"; Figure 3-4). Recombinant receptors incorporating the χ164-

and χ183-subunits had negligible effects on the EC_{50} values for GABA-gated currents (50.5 ± 6.9μM and 28.8 ± 2.8μM, respectively; see Figure 3-6; Table 3-1). This permitted the use of 10μM GABA as a submaximal (~EC_{5-15}) test concentration. The receptor subtype incorporating the χ183-subunit exhibited dose-dependent potentiation by FLX (maximal enhancement with 300μM FLX of 168 ± 16% of control current of 10μM GABA alone; Figure 3-7). Inclusion of the χ164-subunit was insufficient to impart full FLX potentiation (300μM FLX altered test current to 118 ± 5% of 10μM GABA alone; Figure 3-7). The nearby crossover point of two differently sensitive α-subunit chimeras delimits a region influencing FLX-insensitivity to α1Ala^{164}-Asp^{183}, equivalent to α5Ser^{168}-Asp^{187}.

Effect of Substitution Mutations in the α5-Subunit

Within the domain between the chimeric crossover points, there are six residues that are not conserved between the α1- and α5-isoforms (Figure 3-5). We made site-specific mutations whereby the homologous residue from the FLX-sensitive α1-subtype was substituted for the residue in the α5-isoform. Our initial approach was to target the segment of five consecutive non-conserved residues from α5Asp^{176}-Lys^{180}. Five individual mutations (α5N176R, α5G177E, α5S178P, α5T179A and α5K180R) were produced. GABA potency on each subtype was determined (data not shown), and for each mutant receptor a concentration of 3μM GABA was found to be in the submaximal (~EC_{5-15}) range. This test concentration of 3μM GABA was used to assess the effect of 100-300μM FLX on GABA-elicited currents in these mutant α5-containing receptors. Only the α5T179A mutation was sufficient to bestow sensitivity to potentiation by FLX to the α5-subtype (Figures 3-7, 3-8); the other individual α5-isoform mutants did not confer FLX sensitivity (data not shown).

Effect of Substitution Mutations in the α1-Subunit

With the identification of a residue that underlies FLX-insensitivity of the α5-subtype, we made the reciprocal substitution mutation to determine if a single residue mediates sensitivity of the α1-subtype. The α1A175T mutation did not remove the

ability of FLX to enhance currents evoked by a submaximal concentration (within EC_{5-15}) of GABA (data not shown).

Using a comparative structural modeling approach, we identified all residues within a defined atomic radius of $\alpha 1Ala^{175}$. As mutation of the adjacent residues to this point had no effect on FLX sensitivity, we made measurements within the model of Ernst et al., (2003) to determine the nearest, non-contiguous domain. An additional α-subunit domain to the segment homologous to $\alpha 1Arg^{172}$-$\alpha 1Arg^{176}$ (see Figure 3-5) lies within 6Å of $\alpha 1Ala^{175}$. The residues that are predicted to lie within this 6Å radius of $\alpha 1Ala^{175}$ are the adjacent $\alpha 1Arg^{172}$, $\alpha 1Glu^{173}$, $\alpha 1Pro^{174}$, $\alpha 1Arg^{176}$, $\alpha 1Ser^{177}$ amino acids, as well as $\alpha 1Phe^{45}$ and one residue from the neighbouring β2 subunit, $\beta 2Ser^{201}$ (see Figure 3-9). Mutation of the $\alpha 1Phe^{45}$ residue (α1F45A) produced a mutant receptor subtype with markedly reduced potency for activation by GABA (EC_{50}=600μM; $P < 0.001$ compared to wild type $\alpha 1\beta 2\gamma 2_L$) (Figure 3-10). Currents elicited by submaximal concentrations of GABA at the α1F45A mutant subtype were still sensitive to potentiation by 300μM FLX (data not shown).

DISCUSSION

The recent characterization of FLX as a positive modulator of the $GABA_A$ receptor has indicated activity through a novel α-subunit-linked modulatory site (Robinson et al., 2003). Submaximal GABA currents were enhanced to a similar level by FLX at all receptor subtypes examined, with the singular exception of receptors that incorporate the α5-isoform (Robinson et al., 2003). Our present studies demonstrate the unique insensitivity of the α5-subtype of the $GABA_A$ receptor and we have exploited this to probe further the molecular determinants for the lack of FLX effect. Although we could not achieve the same extent of potentiation on receptors in oocytes as was observed in Robinson et al. (2003), and high FLX concentrations (>300μM) were not tolerated by the oocytes, we have reproduced the dichotomy in sensitivity between the wild-type α1- and α5-isoforms. Full potentiation was observed with a chimeric α1/5 subunit that includes the initial 183 residues of α1-sequence (χ183-) suggesting that mediators of the allosteric response exist in this portion of the α-subunit. Inclusion of the χ164- subunit produces receptors with an apparent intermediate FLX-sensitivity; the differential responses of χ164- and χ183-containing $GABA_A$Rs to FLX were the basis of our further probing within the intervening α1Ala^{164}-Asp^{183} segment (homologous to α5Ser^{168}-Asp^{187}) for the basis of FLX-insensitivity. This segment is proximal (N-terminal) to the α5His^{195} and α5Ile^{215} residues that were examined and excluded as α5-specific residues possibly involved in the selective α5-isoform FLX-insensitivity (Robinson et al., 2003).

Within this domain in the α5-isoform, we have identified a single residue that when substituted by the homologous residue of the FLX-sensitive α1-isoform (α5T179A) was sufficient to remove insensitivity to potentiation by FLX. This effect was limited to this single threonine residue, as single point substitutions at adjacent residues did not confer sensitivity. Within the defined α5Ser^{168}-Asp^{187}, an additional non-conserved residue between the α1- and α5-isoforms, α1Glu^{169}/α5Val^{173} was not examined, as the α5(T179A) mutation alone was sufficient to impart α1-like FLX sensitivity (see Figure 3-5). Whereas the substitution of a single α5-isoform residue was adequate to reverse FLX-insensitivity, the reciprocal mutant (α1A175T) was insufficient to eliminate FLX-sensitivity.

Comparative modeling of the extracellular domain of the $GABA_AR$ has been undertaken based on the defined atomic structure of a related molluscan acetylcholine binding protein (AChBP; Brejc et al., 2001). Such models have postulated putative features of secondary structure for regions of the N-terminus of $GABA_AR$ subunits based on homology to the AChBP (e.g. Cromer et al., 2002; Ernst et al., 2003; see also Chapter 1). These modeling efforts have given a putative secondary structure to the non-adjacent segments of the extracellular domain that contribute to the binding sites formed at subunit-subunit interfaces (see Chapter 1).

The identified segment that contains a mediator of FLX-insensitivity partially overlaps the α-subunit "Loop F" domain. The $\alpha1Ala^{164}$-Asp^{183} segment partly covers the α1 Loop F region from residue 175 to 184 (see Table 1-1) that by sequence alignment to the AChBP (Brejc et al., 2001) corresponds to structural Loop 9 (see Ernst et al., 2003). This loop region is predicted to contribute to the complementary component of the low affinity GABA binding pocket at the interface of the α(-) with the adjacent principal contribution from residues in the β(+) subunit (see Figures 1-5 and 3-8).

Comparative modeling has suggested a putative secondary structure for the α-subunit at the Loop F domain. In accordance with the comparative models, the segment from $\alpha1Pro^{174}$-$\alpha1Asp^{191}$ is predicted to exist as a random-coil structure that may exhibit flexion during gating of the receptor (Newell and Czajkowski, 2003). Within this region, aqueous accessibility of $\alpha1Pro^{174}$ and $\alpha1Arg^{176}$ (but not $\alpha1Ala^{175}$) has been specifically shown and several residues from Loop 9 were identified ($\alpha1Val^{178}$, $\alpha1Val^{180}$, $\alpha1Asp^{183}$) as contributing to the agonist binding site (Newell and Czajkowski, 2003). A specific analysis of the secondary structure of this domain in α5-containing receptors awaits further subtype-specific experiments. Recent homology models of the extracellular domain of the $GABA_A$ receptor with different α-isoforms have proposed subtype-specific differences in the orientation of side chains of certain identified GABA binding site residues (Chou 2004). These models however, did not examine the recently identified Loop 9 ("Loop F") residues to suggest any differences in this region, thereby preventing specific predictions of the structure of the α5-isoform in this domain.

Of note is the lack of sequence conservation in this protein segment between subunits of all LGIC superfamily receptors and in particular for $GABA_A$ receptor α-subunits, with no residues common amongst any of the α-isoforms at the point homologous to $\alpha1Ala^{175}/\alpha5Thr^{179}$ (Ernst et al., 2003). Comparative modeling of the $GABA_A$ receptor based on the AChBP is complicated by this lack of sequence conservation in structural loop 9. Recent comparative models (Cromer et al., 2002; Ernst et al., 2003) identify this region between proposed structural β-strands β8 and β9 as the most poorly defined of the proposed $GABA_A$ receptor α-subunit model structure. We hypothesize that a specific configuration of this Loop 9 segment of the α5-isoform, with a dependence on the nature of $\alpha5Thr^{179}$, underlies the lack of FLX response.

Earlier investigation into FLX interaction with the $GABA_AR$ had indicated potential interaction of FLX with the BZD site. High concentrations (>100μM) of FLX were shown to displace binding at the BZD site (Tunnicliff et al., 1999). The wide range of receptor subtypes that were examined by Robinson et al. (2003) indicated that FLX potentiation of the $GABA_AR$ was independent of the γ-subunit, arguing against a specific interaction at the α(+)/γ(-) BZD allosteric site. Furthermore, our identification of a domain that contains mediators of FLX sensitivity partially overlaps the Loop F / structural loop 9 segment; this region is postulated to orient towards the opposite subunit [i.e. α(-)] interface (see Figures 1-4, 1-5, 3-8).

Thus, the $\alpha1Ala^{164}$-Asp^{183} ($\alpha5Ser^{168}$-Asp^{187}) segment most likely faces the neighbouring β-subunit, placing the domain that affects FLX-sensitivity at a site of subunit apposition. Support for this suggestion arises from the sequence homology of $\alpha1Ala^{175}$ with $\beta3Lys^{173}$, a residue shown to be a critical assembly signal for selective homo-oligomerization of β3-$GABA_AR$ (Taylor et al., 1999).

Additional evidence points to a role of the region around $\alpha1Ala^{175}/\alpha5Thr^{179}$ in the activity of other allosteric modulators. The homologous $\alpha6Leu^{174}$ and the adjacent $\alpha6Tyr^{175}$ were recently identified as mediators of selective effects of amiloride on the α6-containing subtype (Drafts and Fisher, 2004). An earlier study with chimeric α1/6 subunits indicated a role of the segment homologous to the $\alpha1Thr^{162}$-Asn^{188} domain in mediating subtype-specific modulatory effects of Ro15-1788 acting via the BZD site (Im

et al., 1997). Comparative models place this domain (encompassing Loop 9/Loop F) between the α-subunit "Loop B" and "Loop C" segments thought to contribute to the BZD site at the adjacent α(+) subunit-subunit interface. A role of this linking segment in allosteric cross talk between the BZD and GABA sites (via Loop F) has been suggested (see Ernst et al., 2003). As FLX acted in an analogous manner to BZD site positive modulators, potentiating only submaximal GABA currents (Robinson et al., 2003), it is possible that although the requirements for ligand interaction differ with BZD ligands, FLX shares common mechanisms of allosteric transduction.

Our present results suggest an indirect effect of the Loop 9 segment on modulation by FLX. Within the α1-isoform, the prediction of no aqueous accessibility of α1Ala^{175} (Newell and Czajkowski, 2003) and the continued FLX sensitivity of the α1(A175T) do not support a direct interaction of FLX with this single residue in this domain. The lack of conservation of residues within all other α-isoforms at the point homologous to α1Ala^{175}/α5Thr^{179} argues against a specific conserved binding site at that point. The identified region containing the determinants of FLX-insensitivity exists adjacent to identified GABA binding site residues from Loop F, but FLX alone was insufficient to gate recombinant $GABA_A$ receptor subtypes (Robinson et al., 2003). However, by analogy to α6-specific effects of Ro15-1788 (Im et al., 1997) we propose that the exact configuration of the Loop 9 domain in the α5-isoform, in part due to the physicochemical properties of α5Thr^{179} with its local environment, prevents or dampens allosteric transitions required for positive allosteric modulation of GABA-gated currents by FLX.

To probe beyond the Loop 9 domain, the nearest residue from outside of that structural loop, α1Phe^{45}, models within 6Å and is positioned in the proposed β1 β-sheet segment (Ernst et al., 2003). Although FLX-sensitivity was retained with the α1F45A mutation, the substantial ~20-fold reduction in GABA potency and the altered Hill slope coefficient of the concentration-effect relationship (Figure 3-9) suggest a specific effect on the agonist interaction with the mutant receptor. A reduction in the Hill coefficient may reflect mutational changes in GABA affinity, co-operativity of binding between the two agonist sites, or may indicate an effect on the receptor gating mechanisms. Although

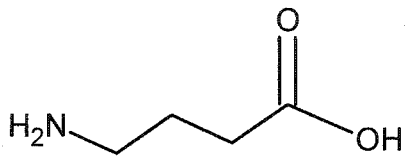
it can be difficult to assess whether mutations affect ligand association, the subsequent receptor gating process or a combination of both (see Colquhoun, 1998), the α1F45A mutation evidently has consequences for the interaction of GABA. Other substitution mutations, or the inclusion of chimeric α1/5-subunits, only altered GABA EC_{50} by ~2-3-fold, whereas the effect of the α1F45A mutation was markedly greater. This residue is homologous to the $GABA_A$ receptor γ2Tyr[58], a residue implicated (along with γ2Met[57]) in high affinity FNZ binding at the BZD site (Kucken et al., 2000). As residues implicated in BZD site binding from Loops A, B and C from the α-subunit and Loops D and E from the γ-subunit are generally at homologous positions to residues from these Loops predicted to contribute to the GABA binding site at the neighbouring β(+)/α(-) interface (Sigel and Buhr, 1997; Sigel 2002; Cromer et al., 2002; Ernst et al., 2003), it could be predicted that γ-subunit residues implicated in BZD binding will have a functionally important counterpart at the structurally homologous agonist binding site. The γ2Tyr[58] residue lies in a segment of the subunit that is predicted by homology modeling to fall within the β1 sheet and to contain certain residues with side chains accessible within the BZD binding site; by convention to older nomenclature, this small segment has been dubbed "Loop G" (Ernst et al., 2003). By homology, this Loop G region in the α-subunit would also be predicted to contribute to the solvent-accessible complement of residues (Ernst et al., 2003). The substantial effect of mutation on the GABA agonist response to the α1Phe[45] is in line with a role of this residue in agonist interaction. Although further probing within the proposed Loop G is warranted for the α-subunit, the predictive power of comparative modeling (see Ernst et al., 2003) with respect to other domains of the $GABA_A$ receptor lends support to a role of this α-subunit Loop G segment in GABA interaction. It is of note that the aromatic nature of the residue at the position homologous to α1Phe[45]/γ2Tyr[58] is conserved as a tyrosine in all α-, β-, γ- and the δ-subunit isoforms of the $GABA_A$ receptor. Several of the residues that are implicated in GABA site binding are aromatic in nature (α1Phe[64], Sigel et al., 1992; β2Tyr[97], Boileau et al., 2002; β2Tyr[157], Amin and Weiss, 1993; and β2Tyr[205], Wagner and Czajkowski, 2001).

Although the present results cannot unequivocally determine the nature of the interaction of GABA with α1Phe^{45}, we have identified the most N-terminal α-subunit residue implicated in affecting the GABA response. Within a radius of 6Å of α1Ala^{175} it is apparent that different residues in this spatial domain have selective effects on either GABA activation or the FLX modulatory response.

Figure 3-1

Chemical structures of the two compounds used in these studies. Activation of the receptors by (A.) GABA and modulation of receptor activity by the SSRI antidepressant (B.) fluoxetine (FLX) was studied.

A.



B.

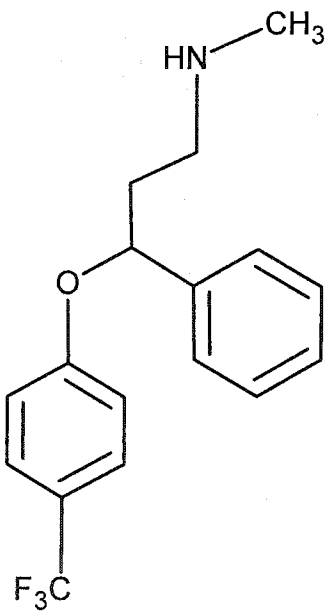
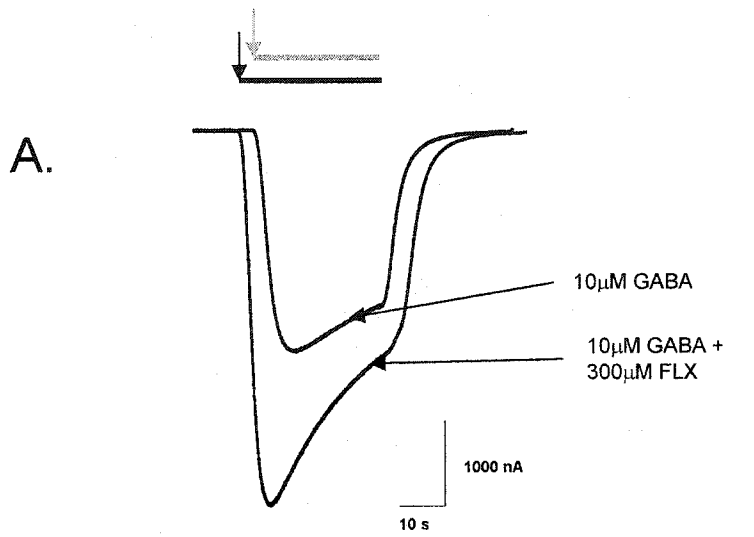


Figure 3-2

Representative traces of GABA-evoked current (10μM GABA) for both before and after exposure to 300μM FLX (see Materials and Methods for protocol) for the (A.) $\alpha1\beta2\gamma2_L$ and (B.) $\alpha5\beta2\gamma2_L$ $GABA_AR$ subtypes. For FLX modulation, the oocyte was exposed to FLX in buffer for 2 minutes prior to the application of the same concentration of FLX in solution with the GABA test concentration (30 second exposure, bar above traces). The arrow represents the time of initial exposure to GABA (± FLX). *Gray* bar, GABA alone; *black* bar, GABA + FLX. Washout with buffer occurred at the time point represented by the end of the bar above the traces.



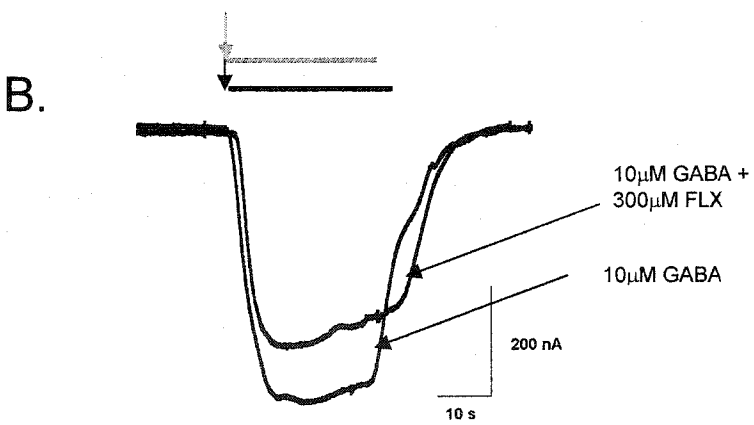


Figure 3-3

Diagram of the plasmid from which targeted random chimeras from rat $GABA_A$ α1- and α5-subunit cDNA were derived. The complete α5-subunit cDNA was subcloned into pcDNA3.1(+) and a partial α1-sequence (that excludes sequence distal to the third putative transmembrane domain) was inserted upstream. This dual plasmid was digested with restriction enzymes between the individual cDNA sequences (see Materials and Methods). Recombination events occurred within DH5α *Escherichia coli* cells upon transformation of these cells with the linearized plasmid. *White*, α1 sequence. *Black*, α5 sequence. *Gray*, T7 promoter region in pcDNA3.1(+) that was used for *in vitro* preparation of subunit transcripts.

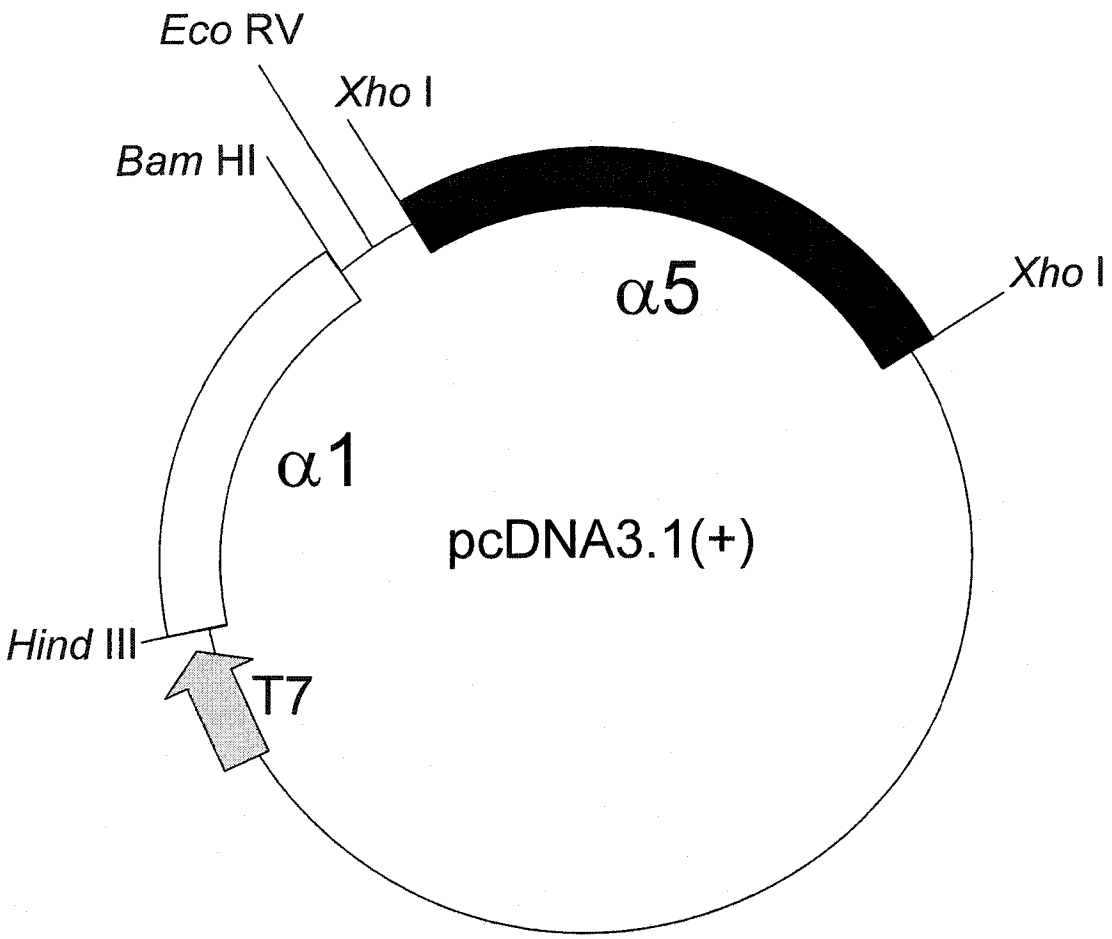


Figure 3-4

A linear depiction of the α1- and α5- wild type subunit isoforms as well as the five separate chimeric α1/5 subunits (χ) isolated. Individual chimeric subunit proteins are named according to the last residue of α1-sequence (rat α1 numbering) prior to the switch point. Sensitivity of receptors expressing these chimeric α-subunits to modulation by 100-300μM FLX was assessed; the (+) symbol represents functional modulation whereas the (-) character signifies insensitivity to modulation or an incomplete response (see Discussion). *White*, α1 sequence. *Black*, α5 sequence. *Gray*, putative transmembrane domains.

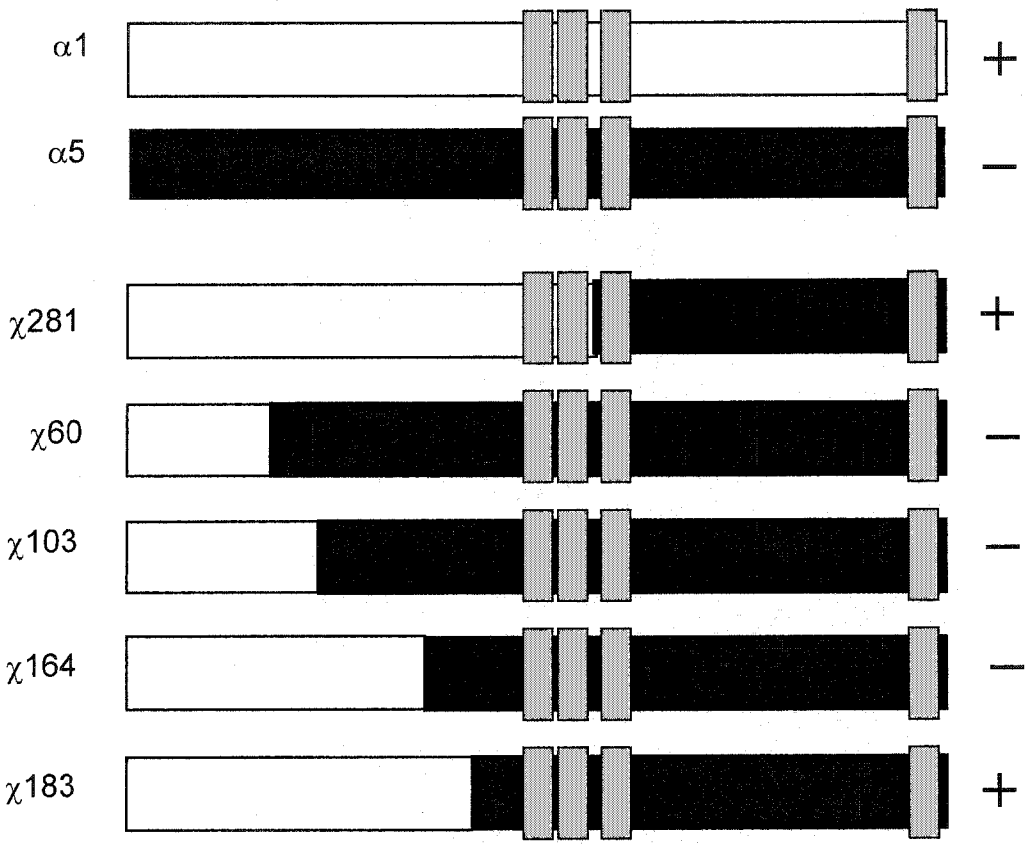


Figure 3-5

A partial sequence alignment of the α1- and α5-subunit extracellular N-terminal domain. Numbering above the figure represents the rat α1 sequence whereas the number below the figure depicts the rat α5-subunit numbering. Vertical lines represent the chimera crossover points and are named for the last residue of α1 sequence. Letters in boldface type indicate Loop F residues that have been implicated previously as contributing to the agonist site (see Newell and Czajkowski, 2003). The boxed letters indicate the α-subunit residues that are the focus of this current study. The boxes above the sequence represent the structural features of the α-subunit proposed by comparative modeling of the $GABA_A$ receptor (Ernst et al, 2003) based on the structure of the AChBP (Brejc et al., 2001). *White*, putative β-strand, β8. *Black*, structural loops 8 (L8) and 9 (L9). *Gray*, sequence corresponding to the Loop B and Loop F domains.

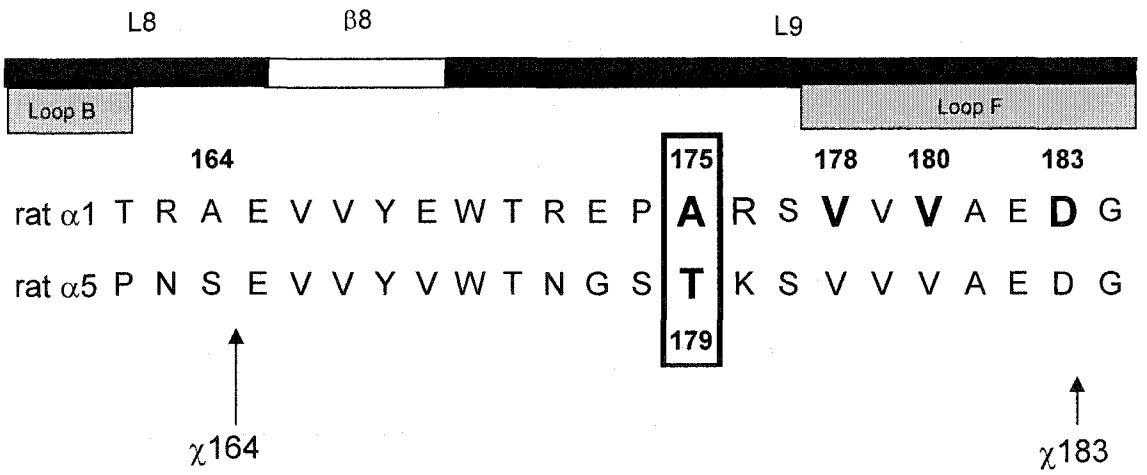


Figure 3-6

Concentration-effect relationships for receptor activation by GABA on receptor subtypes that include the wild-type α1- (●), wild-type α5- (○) or the chimeric χ183- (■) or χ164- (□) subunits in combination with wild-type β2 and $\gamma 2_L$ subunits. Receptor subtypes were expressed in *Xenopus* oocytes and the current evoked by a range of concentrations of GABA of at least four orders of magnitude was tested to determine the concentration for half-maximal activation (EC_{50} concentration). Data represent the mean ± S.E.M. of at least three independent curves from individual oocytes and are summarized in Table 3-1.

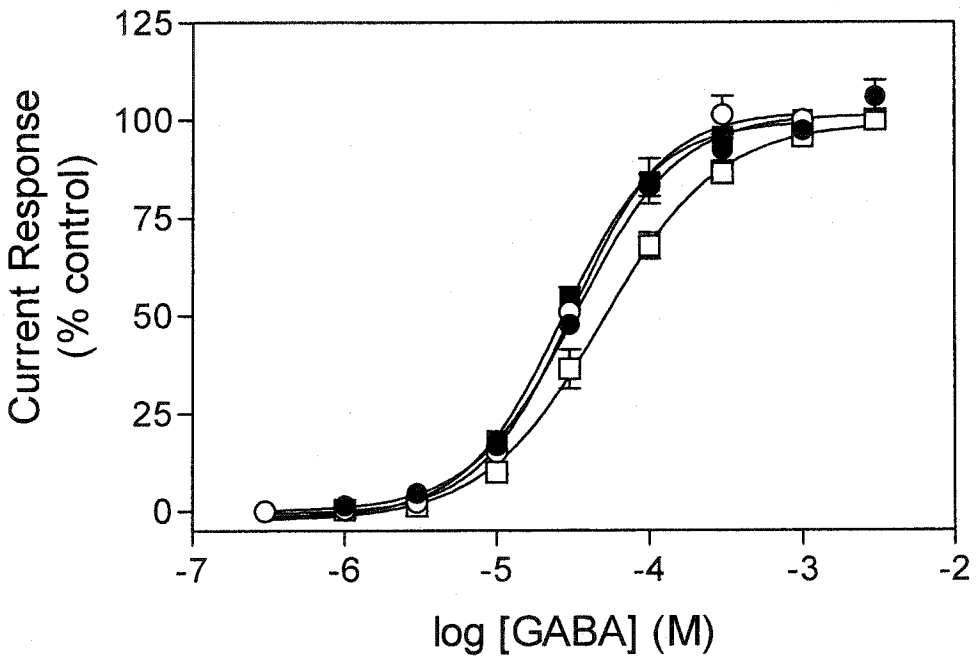


Figure 3-7

Dose-dependent potentiation by FLX of submaximal (~EC_{5-15}) GABA-elicited currents. Recombinant $GABA_A$ receptor subtypes, comprised of rat β2 and $\gamma2_L$ subunits in combination with a single wild-type, chimeric or mutant α-subunit, were expressed in *Xenopus* oocytes. The effect of a range of FLX concentrations on GABA currents was assessed. Individual bars represent the GABA current evoked following exposure to FLX compared to control GABA current (dashed line). Each bar represents the effect of a separate concentration of FLX (10, 30, 100, 178 or 300μM, left to right) on the expressed receptor subtypes that include α1- (■), α5- (□), χ183- (▨), χ164- (▤) or α5(T179A) (▩) subunits. Data were analyzed for the maximum FLX concentration (300μM) only by a one-way ANOVA followed by the Dunnett *post hoc* test for multiple comparisons to determine levels of significance. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to wild type $\alpha5\beta2\gamma2_L$; $^{@}P < 0.05$ compared to χ183; $^{\#}P < 0.05$ compared to α5(T179A); $^{\%}P < 0.001$ compared to $\alpha1\beta2\gamma2_L$.

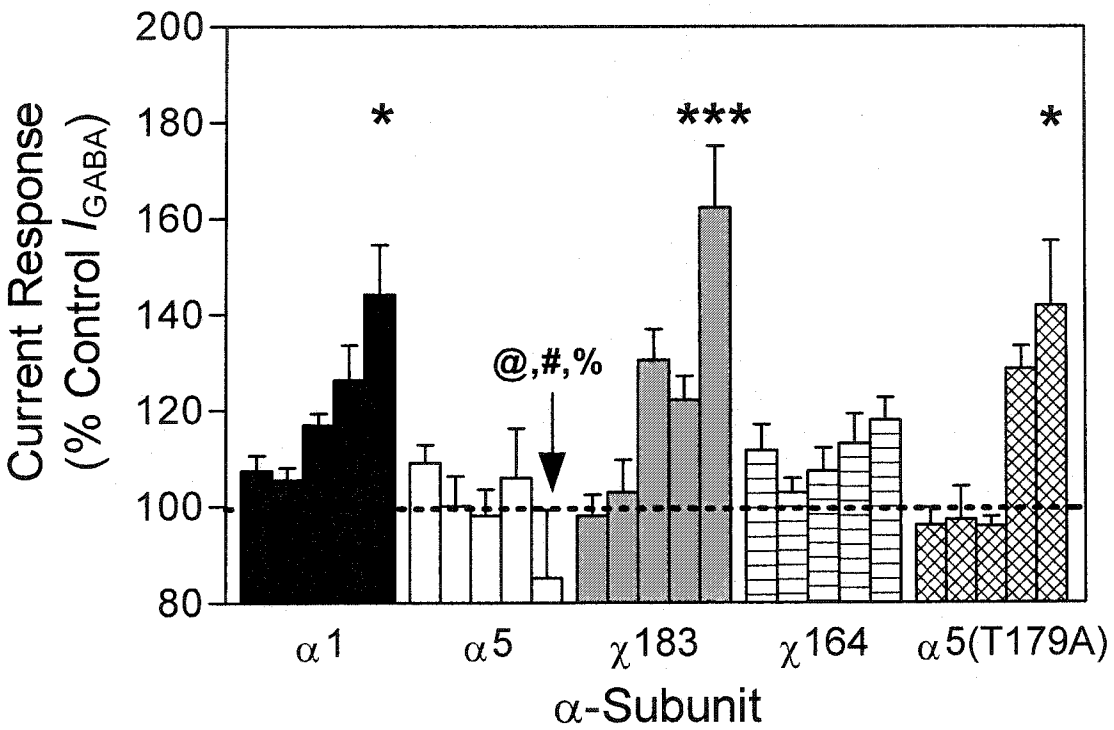
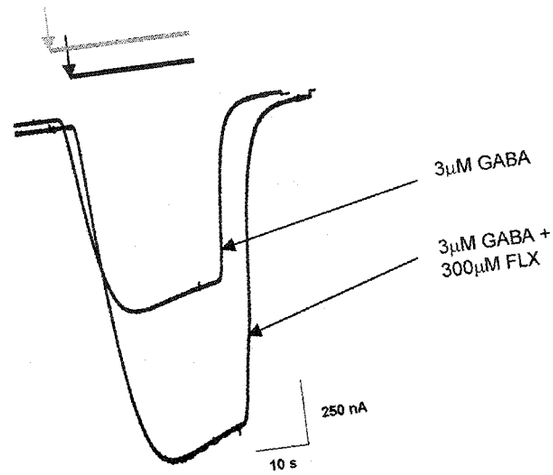


Figure 3-8

Representative traces of GABA-evoked current (3μM GABA) for both before and after exposure to 300μM FLX (see Materials and Methods for protocol) for the (A.) $\alpha5(T179A)\beta2\gamma2_L$ subtype. For FLX modulation, the oocyte was exposed to FLX in buffer for 2 minutes prior to the application of the same concentration of FLX in solution with the GABA test concentration (30 second exposure, bar above traces). The arrow represents the time of initial exposure to GABA (± FLX). *Gray* bar, GABA alone; *black* bar, GABA + FLX. Washout with buffer occurred at the time point represented by the end of the bar above the traces. The $\alpha5(T179A)$-containing $GABA_AR$ showed sensitivity to modulation by 300μM FLX, whereas the wild type α5-subtype was not potentiated. Representative traces for the wild type $\alpha5\beta2\gamma2_L$ subtype (B.) from Figure 3-2 are reproduced here for comparison.

A.



B.

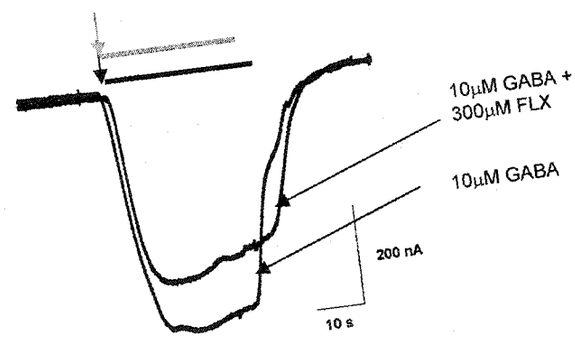


Figure 3-9

A model of the adjacent β- and α- subunits at the point of their interface illustrating the predicted locations of α1Ala^{175} and α1Phe^{45}. The upper figure depicts the low affinity GABA binding pocket formed at the interface of the β(+) (*blue*) and α(-) (*red*) subunits. Ball-and-stick representations of individual residues from each subunit that have been implicated in forming the GABA site are shown (see Chapter 1; Figure 1-5). The lower portion of the figure represents an exploded view of the local domain surrounding α1Ala^{175}, and shows the residues that are modeled to be within an atomic radius of 6Å of this point. These residues include α1Arg^{172}, α1Glu^{173}, α1Pro^{174}, α1Arg^{176}, α1Ser^{177}, α1Phe^{45} and β2Ser^{201}. The putative location (upper figure) and orientation (lower figure) of α1Ala^{175} and α1Phe^{45} (*lower figure*; *light blue*) are indicated.

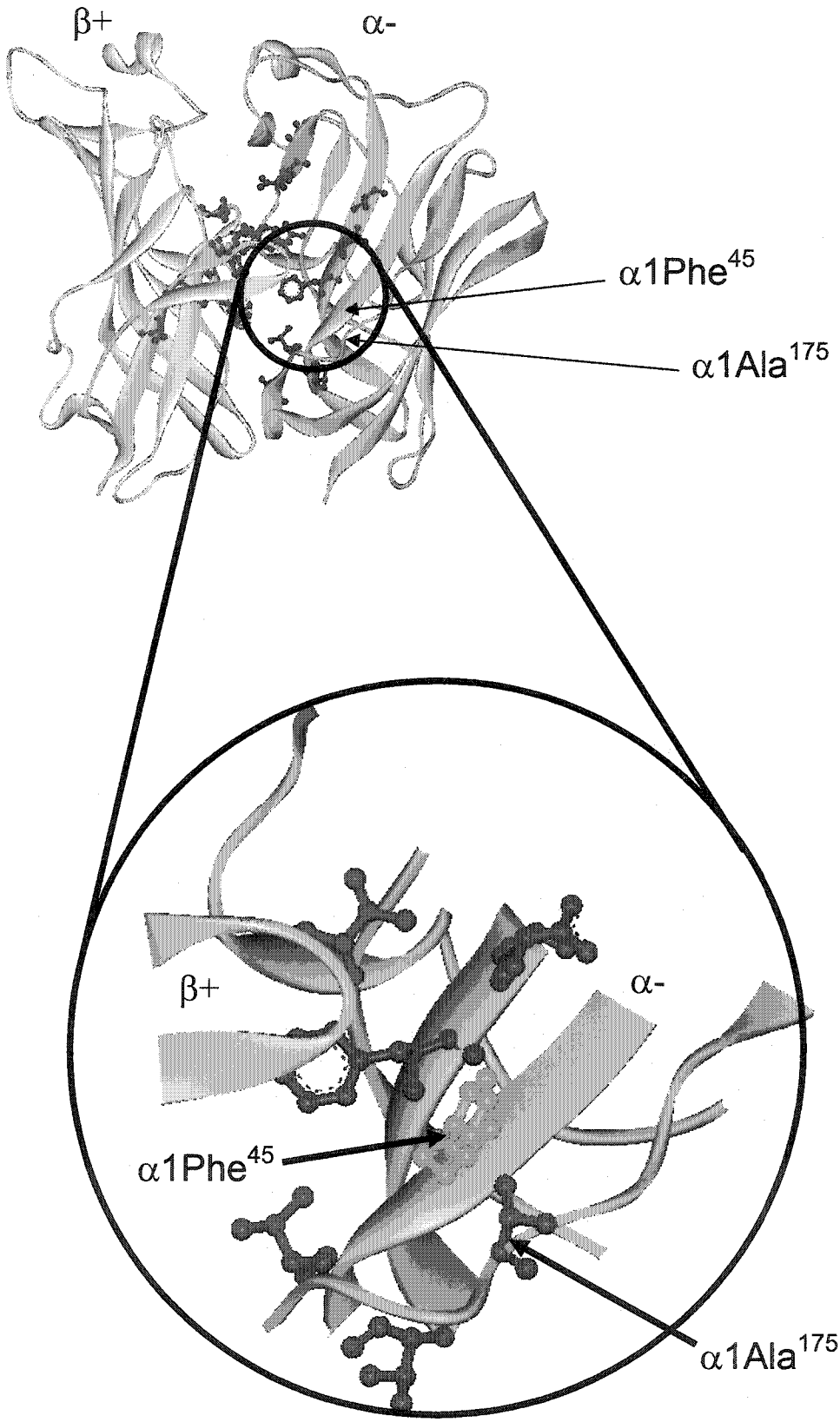


Figure 3-10

GABA current-response relationships at receptors incorporating wild-type and mutant α1-subunits. Mutation in the putative "Loop G' domain (see Kucken et al., 2000; Ernst et al., 2003; also Chapter 3 Discussion) in the α1-isoform (α1F45A, ○) caused a substantial rightward shift in the concentration-effect curve for GABA and reduced the Hill slope factor compared to wild-type α1-containing receptors (●) (see Table 3-1). Data shown for these two receptor subtypes represent the averaged curve fit for at least three independent responses on separate oocytes for each GABA concentration tested. A curve of a single experiment with the α1(A175T) mutation (■) (EC_{50}=19.4μM) is included for comparison.

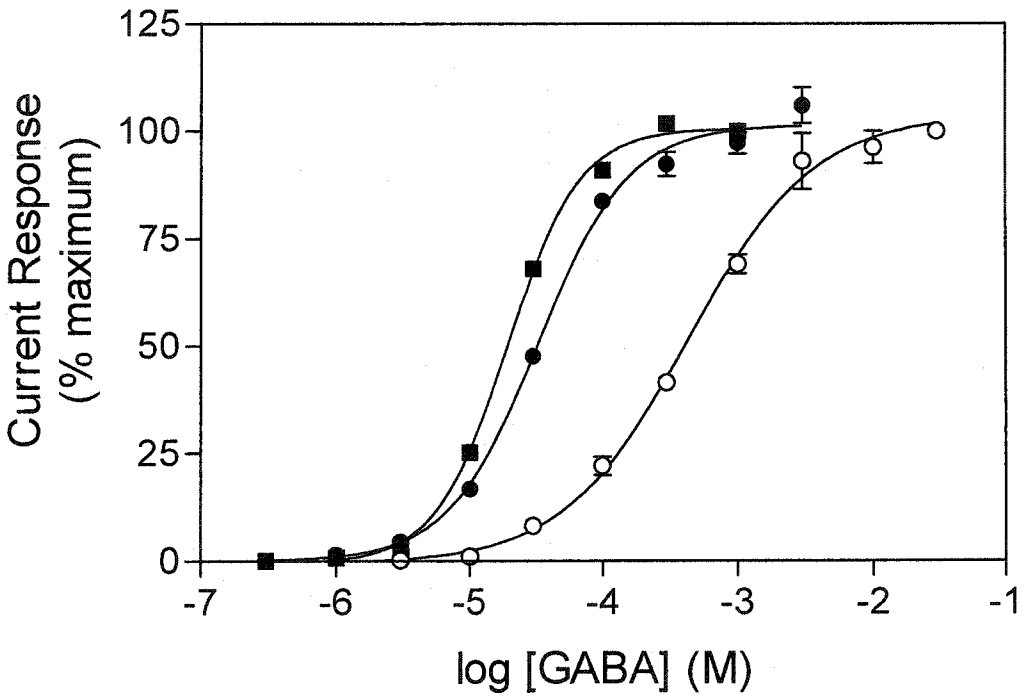


Table 3-1. Functional responses of $GABA_A$ receptor subtypes incorporating wild-type, chimeric and mutant α-subunits in combination with β2 and $\gamma2_L$ subunits.

α Subunit	Potency (GABA EC_{50}, μM)	Hill Slope	[b]Maximum FLX Response % (GABA + 300μM FLX)
α1	33 ± 2.5[a]	1.3 ± 0.1	144 ± 10[*]
α5	31 ± 0.8	1.4 ± 0.1	85 ± 14[@,#,%]
χ164	51 ± 6.9	1.1 ± 0.1	118 ± 5
χ183	28 ± 2.8	1.4 ± 0.2	162 ± 13[***]
α5(T179A)	11.6 [18.1, 5.2]	1.55 [1.78, 1.32]	142 ± 13[*]
α1(F45A)	600 ± 99[†††]	0.80 ± 0.04[†]	§§§

$GABA_A$ receptor subtypes were expressed in *Xenopus* oocytes and exposed to a series of concentrations of GABA to define potency (EC_{50}) and Hill slope (n) data for each [see Materials and Methods; $I = (I_{max}*[L]^n) / (EC_{50}^n + [L]^n)$]. Data shown are mean ± S.E.M. from at least three independent experiments for the α1-, α5-, χ183-, and χ164-subunits. Concentration-response GABA curves were done in duplicate for the α5(T179A) mutation and the value reported is the average. [a]Data from Newell et al., 2000. [b]The response after pre-exposure to 300μM FLX was measured and currents were expressed as a percentage of the control GABA-elicited current; responses to 300μM were determined on at least three different oocytes for the α1-, α5-, χ183-, χ164- and α5(T179A) subunits and data are expressed as mean ± S.E.M. Data were analyzed for the maximum FLX concentration (300μM) only by a one-way ANOVA followed by the Dunnett *post hoc* test for multiple comparisons to determine levels of significance. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to wild type $\alpha5\beta2\gamma2_L$; $^{@}P < 0.05$ compared to χ183; $^{\#}P < 0.05$ compared to α5(T179A); $^{\%}P < 0.001$ compared to $\alpha1\beta2\gamma2_L$. §§§ Due to the large loss in GABA potency, it was difficult to accurately define the submaximal (~$EC_{5\text{-}15}$) range for testing that would yield stable GABA currents; observed potentiation was noted to be ~145% of control GABA current. $^{\dagger}P < 0.05$; $^{\dagger\dagger\dagger}P < 0.001$ compared to wild type α1β2γ2L, as determined by a two-tailed *t*-test.

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CHAPTER 4

General Discussion

GENERAL DISCUSSION

Heterogeneity is a defining aspect of the $GABA_AR$, and the plethora of cloned receptor subunits is only one indicator of the molecular diversity of the receptor. Specific spatial and temporal expression patterns for subunits, post-translational modifications, and precise targeting and clustering of receptors at particular neuronal membrane locations are all factors that contribute to the variations in observed $GABA_AR$ pharmacology across receptor subtypes. Studies such as those described in the preceding chapters capitalize on the advantages of recombinant expression to examine subtype diversity at the subunit protein level, i.e., differences in ligand selectivity with respect to affinity or functional allosteric modulation can be recreated in a system that permits the heterologous expression of a defined receptor subtype. In this manner, the intrinsic properties of the subunit isoform proteins that underlie the pharmacological differences, and not the differences due to expression patterns, can be addressed.

The recombinant expression systems used permit not only the expression of a defined $GABA_AR$ subtype, but they are also amenable to the expression of modified subunit proteins. Probing for the molecular determinants of isoform-specific differences made use of a chimeric-subunit approach (Chapters 2 and 3) followed by expression of $GABA_ARs$ that incorporate residue-specific substitution mutations. All of the mutated receptors were viable in their respective expression systems (see Chapters 2 and 3).

The method chosen to create a group of chimeric α-subunit proteins was an approach that relies on random crossover events between the cDNA of the parental subunit coding sequences (Moore and Blakely, 1994). The strength of this technique is that the experimenter does not define the crossover points for individual chimeric proteins and does not need to directly create the hybrid coding sequences; isolation of a panel of hybrid subunits with distinctive crossover points reduces the identification of the molecular determinants of isoform-specific pharmacology to a more simple screening process. This approach has been used successfully for creating chimeras from other subunits of the LGIC receptor family, including other $GABA_AR$ subunits (e.g. Boileau et al., 1998) and the 5-HT_3R A and B subunits[1]. For the study on β-carboline interaction at the BZD site on the DI $GABA_AR$ subtypes (Chapter 2), the initial aim was to identify

[1] Dr. Martin Davies, Department of Pharmacology, University of Alberta; personal communication.

regions of the extracellular N-terminus that contribute to the BZD binding site. To accomplish this, selective excision by endonuclease restriction digestion of cDNA regions that encode for protein regions downstream of TM1 of one subunit cDNA sequence (α6) was carried out, thereby targeting any random crossover events to regions of homology in the remaining sequence (representing the extracellular N-terminus). Such targeted random chimera production (TRCP) has been successfully demonstrated for $GABA_AR$ subunit chimeras (Boileau et al., 1998). With respect to the studies that examined FLX modulation of $GABA_AR$ subtypes, no *a priori* assumption of FLX interaction with a defined point on the receptor was made. The studies of Robinson et al. (2003) had limited the differential FLX modulation strictly to the α-subunit (with the further exclusion of a few individual residues in the N-terminal domain); therefore, a preparation wherein a broader range of random crossovers could occur was created (this technique excluded only the distal end of the TM3-TM4 loop up to the C-terminus). With both the stricter targeting (Chapter 2) and the availability of more subunit sequence (Chapter 3) for the random production of subunit chimeras, a useful series of hybrid proteins permitted the narrowing of protein regions (and residues within) that have an effect on isoform-specific pharmacology.

Although the expression systems chosen to examine the α-isoform selective actions of β-carbolines, *i*-BZD ligands and FLX were useful to recreate $GABA_AR$ subtype-specific differences, key drawbacks for these systems were also noted. In particular, heterologous expression of both of the DI $GABA_AR$ subtypes was achieved in tsA201 cells; however, expression of α4-containing receptors was variable, and generally lower than for α6-containing receptors (Chapter 2; data not shown). The extent of expression was still sufficient for quantitative affinity measurements as assayed in radioligand binding experiments using β-carbolines and the *i*-BZD ligands Ro15-1788 and Ro15-4513, but subtype differences in expression levels highlight an inherent complexity of using this expression system. For competition binding studies to determine inhibitory binding constant (K_i) values (Cheng and Prussof, 1973), the observed levels of expression (e.g. nmol binding per mg protein) in the experiment can be normalized to an internal control of total specific binding for samples of one harvested batch of cellular membranes, thereby negating inter-batch variation. However, poorer

expression of one combination of subunits could reflect a natural incompatibility of those proteins to assemble into one $GABA_AR$ subtype, or may more simply reflect an unfavourable association of these proteins within that particular cell system. In the studies of Chapter 2, the attempted analysis of currents of the $\alpha4\beta2\gamma2_L$ $GABA_AR$ combination in *Xenopus* oocytes was prevented by the lack of functional receptors in oocytes, whereas the use of tsA201 cells allowed reproducible expression of that subtype for radioligand binding analysis of ligand affinity (e.g. Table 2-1). Early examination of the expression of ternary $\alpha\beta\gamma$ $GABA_AR$ containing the $\alpha4$-isoform in HEK293 cells had shown that a heterogeneous population of receptors is produced (Ebert et al., 1996). Additionally, reconstitution of a DI BZD binding site, and the pharmacology of ligands such as Ro15-4513, could also be observed for the two-subunit combination of $\alpha4\text{-}\gamma2_S$ (Scholze et al., 1996). Other studies have observed BZD site ligand potentiation of GABA currents in an $\alpha\text{-}\gamma$ dimer combination in HEK293 cells (e.g. Im et al., 1993). However, with respect to subunit assembly and receptor expression, it is known that assemblages of $\alpha\text{-}\gamma$ dimers are retained in the ER and are not expected to reach the cell surface (e.g. Connolly et al., 1996a). Therefore, it is possible that with the methodology to harvest cell membranes from transfected tsA201 cells that involves the homogenization of these cells, the collected fractions may include $\alpha4\text{-}\gamma2_L$ dimers that originated in the ER, and not receptors on the cell surface membrane. This would be equally applicable to the expression of the other DI receptor subtype, the $\alpha6\beta2\gamma2_L$ combination, even though the expression of this $GABA_AR$ subtype was generally more consistent. However, as the association of an $\alpha\text{-}\gamma$ dimer still permits the formation of a BZD binding site with properties similar to the site found in ternary $\alpha\beta\gamma$ receptors (e.g. Scholze et al., 1996), differences in binding could be suggested to still be linked to the particular inclusion of one α-isoform in the transfection of the mammalian cells.

The use of *Xenopus* oocytes as an expression system presented additional challenges for the studies of FLX modulation of $GABA_AR$ subtypes in Chapter 3. As described, the use of this heterologous expression system had the drawback of a system-dependent maximum concentration of FLX that could be used on the oocytes. Exposure of intact oocytes to concentrations of FLX in excess of 300μM was consistently

deleterious to the oocyte preparation, resulting in degradation of the cell or loss of the stable voltage clamp. The initial studies that examined the concentration dependence of FLX modulation revealed a much larger maximum potentiation of submaximal GABA currents (>350%) by concentrations of FLX up to 1mM (Robinson et al., 2003), whereas in the studies of Chapter 3, the FLX-sensitive $\alpha1\beta2\gamma2_L$ combination had an observable maximum current of ~144% of control upon exposure to 300μM FLX. Although the extent of potentiation that was observed for recombinant receptors expressed in HEK293 or mouse L929 cells was greater (Robinson et al., 2003), the use of oocytes reconstituted the observed dichotomy between α1- and α5-containing subtypes with respect to sensitivity to modulation by FLX. This was the basis for further experimentation with chimeric and mutant α5-subunits to further examine this insensitivity.

One additional caveat to the expression of $GABA_AR$ in *Xenopus* oocytes is the potential of a heterogeneous population of receptor subtypes. As noted for α4-containing receptors (Ebert et al., 1996), a heterogeneous population can be expressed following injection of the cRNA transcripts for three subunits. Variations in the ratio of injected α-subunits can alter expression (e.g. Whittemore et al., 1996), and the relative over-injection of the amount of the γ-subunit transcript has also been shown to elevate the comparative levels of ternary αβγ compared to binary αβ containing $GABA_AR$ (e.g. Boileau et al., 2002). For the studies in Chapter 3, a unitary ratio of wild type, chimeric or mutant α- to β- to γ-subunit was chosen as the basis of receptor expression in an initial attempt to express the different subtypes to screen for mediators of FLX responsiveness. As the studies of Robinson et al. (2003) had indicated that differential FLX-sensitivity was an α-subunit-linked feature of $GABA_AR$, and binary αβ receptors were equally FLX-sensitive, even if expression levels of ternary αβγ receptors were to be sub-optimal, the various α-subunits should still affect the FLX-sensitivity. Use of the chimeric α1/5 subunits was effective at narrowing down a particular region of interest, even though receptor expression may not have been optimal and may have reflected a heterogeneous population across the range of chimera- and mutant-containing $GABA_AR$ subtypes expressed.

In addition to the use of suitable systems for the expression of recombinant $GABA_AR$ subtypes, comparative modeling of the extracellular domains of receptor subunits based on the atomic structure of a homologous AChBP has furthered the study of $GABA_AR$ subtype pharmacology. The crystal structure of the AChBP isolated from *Lymnaea stagnalis* (Brejc et al., 2001) has been useful in providing a structural reference for the extracellular domain of all LGIC subunits (reviewed in Sixma and Smit, 2003). Furthermore, studies using recombinantly expressed AChBP to examine the association of nAChR ligands (as assayed by quenching of intrinsic tryptophan fluorescence) suggest that, at the AChBP, the properties of ligand association are similar to the nAChR (Hansen et al., 2002). Although sequence conservation between the AChBP subunit monomer and the extracellular domain of $GABA_AR$ subunits is low (~18%; see Cromer et al., 2002), modeling efforts have suggested that ~60-75% of amino acid residues in the extracellular N-terminal domain of $GABA_AR$ subunits have structural equivalents in the AChBP, thereby providing a basis for any comparative model of subunit structure (see Ernst et al., 2003). In addition to homology models of the $GABA_AR$ (e.g. Cromer et al., 2002; Trudell, 2002; Ernst et al., 2003; Chou, 2004), the AChBP has provided a framework for modeling efforts of other LGIC receptors including neuronal nicotinic acetylcholine receptor subtypes (e.g. Costa et al., 2003) and the $5\text{-}HT_3R$ (e.g. Reeves et al., 2003). The recent isolation of a distinct but related pentameric ACh-binding protein from *Aplysia californica* (Hansen et al., 2004) with selective differences in ligand binding characteristics as compared to the orthologous protein from *L. stagnalis* indicates another protein whose structure, once identified, may yield useful information regarding other LGIC receptor subunit structure.

Modeling of the LGIC receptor subunit extracellular domains based on the crystal structure of the AChBP cannot accurately reproduce all aspects of receptor structure. The 2.7Å atomic structure of the AChBP (Brejc et al., 2001) represents a conformational state of a homopentameric protein complex and cannot accurately reflect all LGIC receptor extracellular domains in all receptor states. Recent studies have examined the crystal structures of the AChBP when bound by nicotine and carbamylcholine (Celie et al., 2004) to begin to elucidate definitive structures for a ligand-bound state. With respect to the $GABA_AR$, how its quaternary structure may vary across all receptor subtypes (with

different subunit combinations) is unknown. Additionally, different receptor conformations reflecting the resting (unliganded), activated (ligand-bound) and desensitized states all cannot be accurately revealed by the same unique AChBP crystal structure. Allosteric modulation by various classes of ligands acting through discrete binding sites further increases the diversity of potentially distinct conformational states that a $GABA_AR$ can adopt. All of these factors are caveats for the strict interpretation of the structure of the $GABA_AR$ based on homology models. However, although one cannot accurately define the structure of the extracellular domain of one particular receptor subunit isoform in any given state based on the crystal structure of the AChBP, its greatest utility in probing $GABA_AR$ pharmacology is as a rough approximation of structure that can guide further studies needed to directly address the function of a particular subunit domain and its underlying structure. It is in this manner that such homology modeling is viewed in the preceding chapters.

The unique distribution of the α6-subunit in cerebellar granule cells, and the restricted patterns of expression of the α4-isoform suggest that these $GABA_AR$ have specific functional roles (see Introduction). Additionally, these α-subunit isoforms are predicted to be expressed in extrasynaptic receptors that contribute to the tonic (rather than the phasic) GABA-mediated inhibitory responses (see Introduction) further highlighting the potentially unique properties of α6- and α4-containing $GABA_AR$. Extrasynaptic $GABA_AR$ are additionally presumed to express the δ-subunit (e.g. Mody, 2001; Wallner et al., 2003), inclusion of which, when substituted for a γ-subunit, would render the receptor BZD-insensitive. Also, there exists a pairing of the α6- and the δ-subunits with respect to expression of each; a recombinant mouse α6-knock-out model has shown that lack of α6-isoform expression selectively reduces expression of the δ-subunit protein in the cerebellum (Jones et al., 1997). Therefore, not all α6-containing $GABA_AR$ will exhibit DI BZD pharmacology. However, given that the α6-isoform can be expressed in receptors of differing subunit composition (i.e. with a γ- or with a δ-subunit), with individual subtypes targeted to distinct regions of cerebellar granule cells (e.g. Nusser et al., 1998), the studies in Chapter 2 that focus on DI BZD ligand interaction are of particular importance for examining pharmacological features of a

certain subset (i.e. those that include the γ-subunit) of the α6-containing $GABA_AR$ subtypes.

CONCLUSIONS

The production of a series of α-subunit chimeras was successfully achieved from both the α6/4- and the α1/5-isoform pairs. Use of the protocol of Moore and Blakely (1994) to create randomly-derived chimeric α-subunits allowed for the identification of discrete segments of the extracellular, N-terminal domain of the α-subunit that contribute to BZD ligand selectivity at DI $GABA_AR$ subtypes and to differential sensitivity to modulation by FLX acting through a novel modulatory site.

Probing within the DI BZD site for the molecular basis of higher affinity for negative modulator β-carboline ligands revealed that selective affinity of β-CCE and DMCM at α4-containing receptors is attributable to the $\alpha4Ile^{203}/\alpha6Asn^{204}$ divergence in the α(+) interface Loop C (homology modeled structural loop 10) domain. The nature of the residue at this position in the α1-, α4- and α6-isoforms has an effect on β-carboline ligand affinity. The α6N204S mutation, wherein the asparagine is substituted with the serine from the homologous position in the α1-isoform, increased the affinity of both β-CCE and DMCM. This residue alone could not bestow the much higher affinity of the α1-subtype, consistent with previous studies indicating that a complement of several residues together underlies DI and DS pharmacology (Wieland and Lüddens, 1994). Substitution with either a serine or an isoleucine residue at the α6204 position was effective at increasing the affinity of β-CCE and DMCM by approximately the same extent (see Table 2-1), suggesting that the physicochemical nature of the asparagine side chain is somehow not permissive for β-carboline recognition. The reciprocal mutation (α1S205N) reduced affinity for these ligands (the change was approximately the same ~8-fold factor) further implying a contribution of the side chain at this position to β-carboline recognition at both the DS and DI $GABA_AR$ subtypes.

The identification of the $\alpha4Ile^{203}/\alpha6Asn^{204}$ residue pair specifically determines a critical point for BZD site ligand interaction at the DI subtypes, providing specific evidence for the role of this region in the α4- and α6-containing receptors. This broadens

the understanding of the BZD site at lesser-studied receptor subtypes (see Sigel, 2002). Additionally, differences in the DI subtypes compared to the better-characterized DS subtypes are described for the interaction of the *i*-BZD ligands, in particular Ro15-4513. That mutation at a homologous residue in different α-isoforms can be deleterious to Ro15-4513 affinity in the α1-containing DS subtype but without effect in the DI subtypes suggests a differential interaction of the ligand with this subunit domain. Collected evidence (see Chapter 2) suggests that the 8′-azide moiety on the *i*-BZD backbone may be interacting with different conformations of the Loop C (structural loop 10) domain in the DS versus the DI subtypes. A structural divergence in the domains that form the BZD site may be the foundation for differences in ligand affinity, efficacy, and photo-incorporation (see Chapter 2). Further study is required to determine whether such a difference can be correlated to proposed diversity in the BZD pharmacophore as described by a ligand mapping approach (e.g. Huang et al., 1999; He et al., 2000) that posits a different volume of the BZD site across $GABA_AR$ subtypes. These pharmacophore models indicate a smaller binding site with the α6-isoform, and in particular, a smaller size of one lipophilic domain (L_2) within the binding pocket; the azido-moiety of Ro15-4513 is modeled to interact with this L_2 domain (see He et al., 2000). A proposed differential interaction of the azido group with the identified $\alpha6Asn^{204}/\alpha1Ser^{205}$ suggests that this residue shares overlap with the L_2 domain and may be one of the residues that contributes to the physicochemical properties of the L_2 subsection of the pharmacophore. In this manner, it can be predicted that the Loop C / structural loop 10 segment forms part of the L_2 domain. This allows for a degree of harmony between ligand mapping models and models of protein structure at the BZD site to better predict ligand orientation in the binding pocket.

The unique insensitivity of α5-containing $GABA_AR$ to potentiation by FLX was recreated for receptors expressed in *Xenopus* oocytes (Chapter 3) and a specific domain of the α-subunit was suggested to underlie this selective pharmacology. Inclusion of the initial 183 residues of α1-isoform sequence in a hybrid α1/5-protein was sufficient to confer sensitivity to FLX potentiation to the α5-subtype, whereas an α1-contribution of only 103 residues did not impart sensitivity. A chimeric α-subunit with the first 164 residues of α1-sequence in the hybrid protein bestows reduced FLX-sensitivity, but not to

the extent seen in the α1-subtype. Examination of divergent residues in the domain homologous to α1Ala^{164}-Asp^{183} reveals that only 6 residues are not conserved between the α1- and α5-isoforms. Mutagenesis whereby the homologous residue from α1 was introduced into the α5-isoform was conducted and the mutant α5-subtypes were assessed for a gain of sensitivity to FLX. The α5T179A mutation was the only single point mutant tested that was sufficient to endow the mutant α5-subtype with sensitivity to FLX modulation. The reciprocal α1A175T mutation did not remove the ability of FLX to potentiate the receptor. These results argue against a dependence upon a single residue in this region for FLX interaction; insensitivity to modulation of the α5-subtype is likely not due to the selective lack of FLX binding within this domain. The exact structure of this Loop F (structural loop 9) segment, with a particular importance on the physicochemical properties of α5Thr^{179}, prevents FLX potentiation. A homologous domain in the α1- and α6- isoforms has been previously implicated in subtype differences in the efficacy of Ro15-1788 as it is postulated to physically link the GABA and BZD sites at opposite subunit faces of the α-subunit (Im et al., 1997). It could be suggested that the same domain mediates the allosteric transitions induced by FLX association with its novel modulatory site to cause potentiation of the $GABA_AR$. These studies, however, cannot conclusively define the site of FLX binding (or the lack thereof on the α5-isoform).

Using homology modeling, a broader examination of the α-subunit protein regions that surround the α1Ala^{175} residue indicated an adjacent domain likely involved in agonist interaction. The closest non-contiguous residue from another part of the protein (modeled structural β-sheet β1) was modeled within 6Å at α1Phe^{45}. Mutation of this residue (α1F45A), a point homologous to a γ-subunit residue implicated in contributing a unique segment to the BZD site (see Chapter 3) had a large effect (~20-fold rightward shift) in the potency of receptor activation by GABA. This β1-sheet residue is predicted to form part of the accessible surface of the α-subunit for interaction with ligands in the extracellular space (see Ernst et al., 2003) and by naming convention has been dubbed "Loop G" from the complementary (-) subunit face. These studies indicate that within a defined atomic radius of 6Å from a residue that has a critical effect on FLX modulatory activity, another protein domain has a separable effect on agonist

interaction. These data suggest that the Loop G segment in the α-subunit may contribute to the GABA site at the β(+)/α(-) interface, thereby identifying the most N-terminal element contributing to this agonist binding pocket.

FUTURE DIRECTIONS

A major challenge of $GABA_AR$ pharmacology still remains to fully explore the heterogeneity of all physiologically relevant receptor subtypes. For these diverse subunit combinations, it will be of great importance to determine which subtype-specific features can be attributed to the subunit proteins that form the receptor, and which to the adjunct regulatory mechanisms (e.g. receptor phosphorylation and trafficking) that act on $GABA_ARs$. The recent use of knock-in mutations to dissect specific clinical features of BZD site ligands mediated by select $GABA_AR$ subtypes highlights the necessity to better understand the molecular foundation of subtype-specific pharmacology, both at receptor agonist and allosteric modulatory binding sites.

The complexity of the $GABA_AR$ with respect to its heterogeneous subunit composition and the multiple conformational states that the receptor complex can adopt (i.e. resting, activated, desensitized, bound or unbound by modulator, etc.) confounds the use of a narrow approach in determining receptor structure. In the absence of a specific crystal structure of a $GABA_AR$ subtype, definitive structural features still must be indirectly inferred from the biochemical, pharmacological, and molecular biology studies on native and recombinant receptors that have been useful in probing receptor function and its underlying molecular structure. Attaining a crystal structure of the $GABA_AR$ is complicated by the hydrophobicity of the transmembrane subunit proteins and the heterogeneity of receptor subtypes in native tissue sources, which causes subsequent difficulty in the isolation of a defined receptor subtype. Furthermore, unlike the related nAChR that can be isolated in large quantities from the *Torpedo* electric organ to permit structural studies (e.g. by electron microscopy of tubular crystals of receptor protein) that approach atomic resolution (e.g. Unwin, 1995; Miyazawa et al., 2003), no readily available source enriched with sufficient $GABA_AR$ for similar studies is known.

The isolation (Smit et al., 2001) and determination of the atomic structure (Brejc et al., 2001) of the AChBP from *L. stagnalis* has provided a novel foundation from which

to launch investigations into the structure of the ligand-binding domains of $GABA_AR$ subunits. Although homology models are not definitive, and cannot accurately reproduce subunit-isoform structural differences, they have become a way of predicting the structure of protein segments and a method to guide the types of biochemical and mutagenesis studies from which the majority of information on ligand association with the receptor has been gleaned. Studies that aim to confirm the predicted secondary structure of extracellular, N-terminal domains of $GABA_AR$ subunits using SCAM experiments to define patterns of accessibility of subunit residues (e.g. Boileau et al., 1999; Newell and Czajkowski, 2003) have given credibility to predictions of structure. A rigorous expansion of these types of accessibility studies to examine patterns of aqueous-exposed residues in all $GABA_AR$ subunits, and their individual isoforms, would reveal if isoform-specific secondary features exist for the subunit segments that contribute to ligand binding. Types of SCAM experiments whereby the rate of modification of engineered cysteine residues is assessed in the absence and presence of ligand can indicate residues that likely have a direct interaction with ligands in the binding site. Also, the comparison of the accessibility of introduced cysteines in the presence and absence of agonist or modulator can be useful in inferring dynamic movements of protein domains upon receptor gating or modulation. A broader study across all subunit isoforms would expand upon the data largely obtained for the α1- (e.g. Boileau et al., 1999; Newell and Czajkowski, 2003) and β2- (e.g. Wagner and Czajkowski, 2001) isoforms. In addition to SCAM studies, other experiments that use lysine scanning mutagenesis to assess the consequences of individual lysine substitution mutations on ligand interaction have been conducted for the nAChR (e.g. Sine et al., 2002) and can also shed light on underlying secondary structural features.

With particular emphasis on the methodology used in Chapters 2 and 3, additional studies could be done to further elucidate isoform-specific features at the DI BZD site and for novel allosteric modulators. Other differences in α4- compared to α6-containing $GABA_AR$ have been observed and the examination of the molecular basis of these differences could also be appropriately addressed with the same chimeric α6/4-subunits (or others randomly derived from the preparation detailed in Chapter 2). In particular, experiments with the human α4-subunit in recombinant preparations have shown a

comparable potentiation of α4- and α6-containing receptors by pentobarbital and by propofol, but a lack of direct receptor gating by higher concentrations of these compounds only for the α4-subtype (Wafford et al., 1996). Chimeric α6/4 proteins would be useful in determining the residues or protein segments in the α4-isoform that prevent this direct $GABA_AR$ gating (which presumably represents activity at a site separate from the site that mediates receptor potentiation). Similar studies have indicated a reduced potency and efficacy of $GABA_AR$ partial agonists THIP and P4S at α4-containing receptors compared to the α6-subtype (Wafford et al. 1996), the foundation of which could be explored with hybrid α6/4 subunits. Additionally, ligands from the pyrazoloquinolone class (e.g. CGS 9895, CGS 9896; see Yang et al., 1995; Gunnersen et al., 1996) also show selectivity for α4-compared to α6-containing receptors; it would be a worthwhile aim to ascertain whether the selective affinity for a different class of BZD site ligands shares the dependence with β-carboline ligands on the Loop C $\alpha4Ile^{203}/\alpha6Asn^{204}$ divergence, and if there are any functional subtype differences that can be attributed to the nature of that residue. Furthermore, the results in Chapter 2 indicate greater levels of expression of $GABA_ARs$ that incorporate chimeric α6/4-subunits, as compared to wild-type α4β2γ2 receptors (see Figures 2-5; 2-6). This could likely be due to higher efficiency of receptor assembly and expression with increased α6-contribution to the mutant α-subunit (resembling the higher levels of expression for wild-type α6-containing receptors; see Figure 2-5). The molecular basis for α-subunit-linked differences in receptor expression could be examined using these chimeric α-subunit proteins to delineate protein regions that mediate this effect.

The suggestion from the results of Chapter 2 that certain BZD site ligands like Ro15-4513 may have a differential interaction with the Loop C / structural loop 10 domain in DS and DI BZD sites (Derry et al., 2004) warrants further study to probe these specific differences. Photolabeling with Ro15-4513 has been shown to covalently label the $\alpha1Tyr^{209}$ residue (Sawyer et al., 2002), but the substrate for incorporation of Ro15-4513 into the α6-subunit from native cerebellar preparations has been suggested to be within the initial 101 N-terminal residues (Duncalfe and Dunn, 1996) (although this was not reproduced for photolabeling of recombinant α6β3γ2 receptors; Davies et al., 1996).

Identification of the substrate of photoincorporation of Ro15-4513 in the α6- (and the α4-) subunit may reveal isoform-specific differences in ligand interaction. It is of note that certain additional β-carboline ligands are also BZD site photolabels (Dellouve-Courillon et al., 1989; Zezula et al., 1996). Also, the negative modulator β-CCE can itself be chemically modified to an azido-deriviatve that can undergo a photolabeling reaction.[2] Deduction of the α-subunit residues (or secondary structure elements) that are labeled across α-isoforms may shed light on further subtype-specific BZD site differences that occur with inclusion of a particular α-subunit protein. Advances in analytical protein chemistry may provide easier methods for the deduction of the substrates of photoincorporation. For example, peptide purification and microsequencing by mass spectrometry techniques can detect amino acid residues that are covalently linked to incorporated ligands with extremely small samples of labeled protein (e.g. in the picomolar range) (see Schriemer et al., 1998). These systems would be useful for the direct determination of potential additional sites of photoincorporation of BZD ligands into the α- (see Duncalfe et al., 1996) and possibly the γ- (Stephenson et al., 1990) subunits.

Additional experimentation would be useful to advance the concepts addressed in Chapter 3. A further examination of the proposed secondary structure in the α5-isoform would be of most benefit for comparison to the predicted random coil of the α1 Loop F domain (Newell and Czajkowski, 2003). A similar approach whereby the accessibility of the engineered cysteines in the homologous region in the α5-sequence is determined may indicate any putative differences in this domain of the α-subunit, and may provide a rationale for the lack of FLX potentiation, and the removal of this insensitivity upon mutation of α5Thr^{179}. The creation of a series of substitution mutations at the α1Ala^{175}/α5Thr^{179} pair may reveal a reliance on the nature of the side chain of that residue (or of those clustered nearby) for FLX activity, thereby further stressing the role of this amino acid residue in the FLX response. The postulate that this Loop F domain is involved in allosteric transition of distal binding events to the GABA activation sites (as

[2] Dr. Robert H. Dodd, Centre National de la Recherche Scientifique, Gif-sur-Yvette Cedex, France; personal communication.

has been suggested for this domain in the α1-/α6- isoforms; see Im et al., 1997; Ernst et al., 2003) could be better addressed with chimeric α-subunits wherein the Loop F domain is exchanged between the α1- and α5-isoforms. Such studies may implicate a broader region that underlies the molecular transitions involved in allosteric potentiation by FLX. Another potentially useful approach would be the examination of the $GABA_AR$ modulatory effects of norfluoxetine, the primary metabolite of FLX, as well as the characterization of receptor modulation elicited by the individual *R*- and *S*- enantiomers of each. These studies, coupled with the use of derivatives based on the FLX chemical structure, would be an attempt to define chemical features of the ligand that are critical for allosteric activity. Such experiments may shed light on the requirements for the binding of FLX as a first step for interaction with the $GABA_AR$. The use of chemically related compounds may shed additional light on the deleterious interaction of FLX with *Xenopus* oocytes. The consistent toxic effect of >300μM FLX to the physical integrity of the oocyte membrane, or to the ability of the oocyte to be maintained under stable whole cell voltage clamp (even with control, un-injected oocytes), indicates a potential deleterious interaction of FLX with the amphibian cell membrane itself, or interactions with endogenous proteins (e.g. ion channels) that have negative effects. Given that the initial characterization of FLX modulation of $GABA_AR$ responses (Robinson et al., 2003) was for receptors expressed in mammalian cell lines (HEK 293; mouse L929), it may be that species differences with respect to the composition of the cellular membrane (or complement of membrane proteins) prevents the tolerance of *Xenopus* oocytes to high concentration of FLX (and perhaps chemically related compounds).

Another method to probe FLX interaction at $GABA_AR$ subtypes would be to screen for any allosteric effects of FLX on the interaction of other well-characterized radiolabeled $GABA_AR$ compounds. The $GABA_AR$ exhibits allosteric co-operativity between distinct classes of binding sites (e.g. between GABA and BZD site ligand binding); the interaction of FLX with a receptor subtype therefore may affect binding at another site. An initial attempt to probe for disparity in the saturation binding response of [^{3}H]FNZ in both the absence and the presence of 100μM FLX revealed an interesting suggestion of a dichotomy between the wild-type α1β2γ2 and α5β2γ2 subtypes (data not shown). In very preliminary studies (these experiments would require careful

replication), an apparent increase in the total amount of specific binding of [^{3}H]FNZ was induced upon incubation of the binding preparation for the $\alpha1\beta2\gamma2$ subtype in a solution of 100μM FLX. An opposite effect of reduced saturation binding levels was seen for the $\alpha5\beta2\gamma2$ subtype. For both receptor combinations, no obvious shift in [^{3}H]FNZ affinity was seen. These very early data indicate the potential for examining the interaction of FLX with a receptor subtype through its effect on the binding characteristics of ligands at other receptor binding sites. Repeating these saturation binding experiments on subtypes containing chimeric $\alpha1/5$ subunits may better reveal requirements for FLX association with the $GABA_AR$.

Further studies that examine the potential for interaction of GABA with the "Loop G" segment at the $\beta(+)/\alpha(-)$ subunit interface are also required to better understand the results presented in Chapter 3. To examine both the suggestion that the $\alpha1Phe^{45}$ residue is in a β-sheet (modeled at β1 structural sheet), and that residues in this local domain are accessible to the aqueous environment, SCAM studies in parallel to those that have examined other Loop domains (e.g. Boileau et al., 1999) would provide useful results. This would not only provide evidence for the structural features of the N-terminal domain that flanks $\alpha1Phe^{45}$, but would allow for a better comparison to the homologous residues from the $\gamma(-)$ subunit that are predicted to contribute to the BZD site (Kucken et al., 2000). Ultimately this would allow for better assumptions of the structural homology of the binding clefts formed at all subunit-subunit interfaces. Additionally, creation of a series of mutations at $\alpha1Phe^{45}$ with a panel of diverse side chain substitutions may indicate a reliance on the nature of the side chain in this domain for interaction with GABA and other agonists (and antagonists). An examination of any effect on the binding of an antagonist (like bicuculline or SR 95331) that recognizes this site with high affinity may suggest a role of this residue as well. Other studies that use rapid administration of agonist to the $GABA_AR$ to examine the kinetics of receptor activation can predict whether mutations have a greater effect on ligand binding compared to channel gating (see Wagner et al., 2004); these studies would provide more evidence for the potential role of this domain in the GABA site at the $\beta(+)/\alpha(-)$ interface.

In conclusion, the use of chimeric subunit proteins is an effective method of examining pharmacological differences between $GABA_AR$ subtypes. Recent advances in comparative modeling, in predicting ligand orientation within the BZD site, and the dissecting of $GABA_AR$ subtype-selective clinical effects of BZD site ligands have advanced the understanding of receptor function as well as increased the potential for the development of novel therapeutics based on selective affinity for discrete $GABA_AR$ subtypes. The use of transgenic mice has advanced the understanding of $GABA_AR$ subtypes and allosteric modulation of receptor activity. For example, in addition to DI knock-in mutations, specific knockout α6 mice have been bred and these animals display altered tonic GABA conductances in the cerebellum (reviewed in Rudolph and Möhler, 2004). The development of transgenic mice bearing an altered α4-isoform (or of knockout α4- mice) would be of great benefit for a comparison of the *in vivo* roles of the α4- and α6-containing DI $GABA_AR$ subtypes. The promise of more selective BZD ligands raises the possibility of advances in the treatment of anxiety and convulsive disorders, and the development of better clinical sedative-hypnotics with reduced side effect profiles. An enhanced appreciation of the structural nature of the BZD pocket formed with different combinations of α- and γ-subunits will permit a deeper understanding of not only the interaction of clinically useful positive modulators, but as well it should enhance the appreciation of the binding and efficacy of negative modulators and compounds that antagonize the BZD site (see Sigel, 2002). With this a better appreciation of the nature of allosteric modulation of $GABA_AR$ subtypes will undoubtedly follow. Studies that probe for the structural basis of subtype-selective pharmacology of other modulatory ligands will also surely broaden not only the knowledge of subunit isoform-specific structure, but the comprehension of novel allosteric modulatory sites on the $GABA_AR$ as well.

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APPENDIX 1

Supplemental Methods and Protocols

Subcloning of the α6-subunit

The α6-subunit was subcloned into the pcDNA3.1(-) expression vector (Invitrogen, San Diego, CA) for use in expression studies of Chapter 2. The coding sequence of the α6-isoform was isolated by digestion from its original plasmid (unknown to our laboratory) using restriction enzymes that cut upstream (*Bam*HI) and downstream (*Hind*III) of the sequence. Following purification of the isolated insert and parallel digestion of the expression vector with the same restriction enzymes, ligation with T7 DNA ligase (Invitrogen) completed the insertion of the α6-sequence into the expression vector in the correct orientation

For the preparation of the dual α6- and α4-plasmid used in the random creation of chimeras (Chapter 2), amplification of the α6-sequence was achieved by PCR to produce a fragment that incorporated the upstream (5′) *Hind*III restriction site and introduced an additional *Hind*III site downstream (3′) of the coding sequence; the following primers were used:

Sense: 5′ GGC ATT TCA GTG **AAG CTT** AGG ATG CTG TTG 3′ (*Hind*III)

Antisense: 5′ CCA ATT ACA GTG TC**A AGC TT**T TCC TGG CTG 3′ (*Hind*III)

The intervening fragment was amplified with an optimized PCR protocol. For this protocol, the initial step was one time interval of 95°C for 45 seconds, a second step, with 25 cycles, with three components to a cycle: 95°C for 45 seconds, primer annealing at 54°C for 45 seconds, and DNA extension at 72°C for 3 minutes. Following the above steps, an additional duration of 72°C for 10 minutes was carried out.

Following amplification of the α6-sequence, this product was purified and digested with *Hind*III; the pcDNA3.1(+) [that already contained the α4 sequence between the *Eco*RI and the *Xba*I sites (see Chapter 2)] was digested with *Hind*III and CIAP (calf intestinal alkaline phosphatase (Invitrogen), an enzyme that catalyzes the dephosphorylation of the 5′ terminus of the linearized vector). This treatment reduces the ability of the self-complementary ends of the vector to associate, and thereby increases the efficiency of incorporation of the insert with a vector cut at a single restriction site. Ligation with T7 DNA ligase (Invitrogen) completed the subcloning into the expression

vector. DNA sequencing provided confirmation that the α6-sequence was inserted in the correct (5′-3′) orientation.

Subcloning of the α5-subunit

The α5-subunit isoform was subcloned into the pcDNA3.1(+) expression vector (Invitrogen) for use in the studies of Chapter 3. The coding sequence was amplified by PCR from the pCEP4 plasmid; oligonucleotide primers for both upstream (5′) and downstream (3′) were mutagenic and included introduced the restriction enzyme recognition sequence for *Xho*I.

Sense: 5′ GAA AGC TTC AAG **CTC GAG** TTG GAG GAA AGG 3′ (*Xho*I)
Antisense: 5′ CTT TGA CCT CTC **CTC GAG** TTC ATT TCT GTG 3′ (*Xho*I)

The intervening fragment was amplified with an optimized PCR protocol. For this amplification, the initial step was one time interval of 95°C (45 seconds), a second step, with 25 cycles, with three components to a cycle: 95°C (45 seconds), primer annealing at 54°C (45 seconds) and DNA extension at 72°C (3 minutes). Following the above steps, an additional duration of 72°C for 10 minutes was carried out.

After amplification of the α5-sequence, this product was purified and digested with *Eco*RI (which cuts within the α5-coding sequence) and *Xho*I. This was carried out to isolate a C-terminal portion of the coding sequence to be used in the initial step of a 2-step subcloning process. The expression vector was also digested with *Eco*RI and *Xho*I and purified. Ligation with T7 DNA ligase (Invitrogen) completed the first portion of the subcloning into the expression vector. The N-terminal fragment of the α5-sequence was isolated from the source α5pCEP4 vector with *Hind*III and *Eco*RI and a secondary digestion of the vector and a ligation reaction was carried out incorporate the remaining sequence. Confirmation of incorporation of the entire α5-coding sequence was accomplished by restriction enzyme analysis and with DNA sequencing.

For the preparation of the double α1- and α5-plasmid, amplification of the PCR product with the primers above provided a source of the α5-sequence. A separate

subcloning reaction was conducted whereby an N-terminal fragment of the α1-sequence (see Chapter 3) was subcloned into the pcDNA3.1(+) vector between the *Hind*III and *Bam*HI restriction sites. Amplification of the α5-sequence between two *Xho*I sites with the above primers was followed by digestion with *Xho*I and purification of this insert. A separate parallel digestion of the α1[*Hind*III/*Bam*HI]pcDNA3.1(+) vector with both *Xho*I and CIAP was used to prepare the vector; ligation with T7 DNA ligase (Invitrogen) completed the subcloning into the expression vector, with DNA sequencing confirming the correct (5′-3′) orientation of the α5-sequence.

Subcloning of the β3-subunit

The β3-subunit isoform was subcloned into the pcDNA3.1(+) expression vector (Invitrogen) for use in the laboratory. The coding sequence was amplified by PCR from the pCEP4 plasmid; oligonucleotide primers for both upstream (5′) and downstream (3′) were mutagenic and included introduced the restriction enzyme recognition sequence for *Xba*I.

Sense: 5′ TGC CGG GGC GCG **TCT AGA** GGA TGT GGG GCT 3′ (*Xba*I)
Antisense: 5′ ACT GGT ATA AAA **TCT AGA** CAG GCA GGG TAA 3′ (*Xba*I)

The corresponding fragment was amplified with the following optimized PCR protocol. For this amplified product, the initial step was one time interval of 95°C (for 45 seconds), a second step (with 25 cycles), with three components to a cycle: 95°C (45 seconds), primer annealing at 57°C (45 seconds), and DNA extension at 72°C (for 3 minutes). Following the above steps, an additional duration of 72°C for 10 minutes was carried out.

After amplification of the α5-sequence, this product was purified and digested with *Xba*I; the pcDNA3.1(+)vector was also digested with *Xba*I and CIAP (Invitrogen), to increase efficiency of incorporation of insert with the vector linearized at a single restriction site. Ligation with T7 DNA ligase (Invitrogen) completed the subcloning into the expression vector, with DNA sequencing confirming that the β3-sequence is in the correct (5′-3′) orientation.

Screening of Potential Chimeric α-Subunits

After transformation of DH5α *Escherichia coli* cells with the linearized dual plasmid and the subsequent random crossover event within the bacteria (see Chapters 2 and 3), the cells were plated on ampicillin-selective media. To facilitate the process of identifying those resulting colonies that contained plasmids with a chimeric α-subunit, a screening process was carried out on the size of the plasmids contained within the bacteria. Each colony to be tested was propagated by dipping a sterile toothpick into the colony and subsequently transferring each to a fresh ampicillin-selective media plate. The remaining bacteria from each colony (on individual toothpicks) were transferred into separate, sterile microfuge tubes and subjected to the following colony-cracking protocol to assess the size of the plasmids:

1. (to each tube containing bacteria on a toothpick) add 50μL of 10mM EDTA (pH 8.0)
2. vortex to resuspend bacteria
3. add 50μL of cracking buffer
   (cracking buffer: 40μL 5N NaOH + 50μL 10%(w/vol) SDS + 0.2g sucrose in 800μl sterile distilled water)
4. vortex to mix
5. place tubes in a 70°C water bath (for 5 minutes)
6. let tubes cool to room temperature
7. to each tube, add 1.5μL of 4M KCl and 1.0μL of 0.4% bromophenol blue solution (or agarose gel loading buffer)
8. vortex to mix
9. place samples on ice (10 minutes)
10. centrifuge samples (benchtop): 13000rpm (for 5minutes)
11. load supernatant (as much volume as possible without taking particulate matter from the pellet) onto an 0.5% agarose gel
12. use supercoiled (uncut) plasmids (~250ng total) as reference standards for size
    (use dual plasmid as the standard for no crossover event and a plasmid with a single insert for the target size of a successful crossover)

Although the amount of DNA plasmid contained within a single colony is very small, it is sufficient to visualize under UV light after staining the agarose gel with ethidium bromide. Electrophoresis on an 0.5% agarose gel is also sufficient to separate supercoiled plasmids of differing sizes (for example, ~5.4kb for the pcDNA3.1(+) vector, ~7.0kb for the α6[*Bam*HI/*Hind*III]pcDNA3.1(-), and ~8.4kb for the dual α6/α4 plasmid (Chapter 2)). Plasmids that contain potential chimeric crossovers can be propagated from

the bacterial colony replicated on the reference media plate. DNA sequencing was used to confirm points of crossover in the chimeric α-subunit DNA sequence.

Site-directed Mutagenesis

All site-specific substitution mutations were accomplished using the QuikChange protocol (Stratagene, La Jolla, CA) (see Materials and Methods, Chapters 2 and 3). For each substitution mutation, a pair of complementary mutagenic primers was synthesized to incorporate the residue change. The following PCR protocol was used in each reaction: a single denaturing step at 95°C (30 seconds), followed by cycling pattern of temperatures (16 cycles), each of 95°C (30 seconds), the optimized primer annealing temperature per mutant (see Appendix 1, Table A1-1 for each mutant) (1 minute), and the DNA extension temperature at 68°C (15 minutes).

Table A1-1. A summary of the mutagenic primer sequences and annealing temperatures that were used for the PCR-mediated mutagenesis reactions that produced the substitution mutations in Chapters 2 and 3.

Substitution Mutation	Primer (5′to 3′)	Optimized Annealing Temperature (°C)
α6(N143D)	GAG ACT GGT TGA CTT CCC TAT GG	51
α6(N204I)	CTA TTA AAT CGA TCA CAG GTG AAT ATG	50
α6(N204S)	ACT ATT AAA TCG AGC ACA GGT GAA TAT	50
α1(S205N)	ATT GTT CAG TCC AAT ACT GGA GAA TAT	50
α1(F45A)	AAG ACG GAC ATC GCG GTC ACC AGT TTC	53
α1(A175T)	ACA AGG GAG CCA ACC CGC TCA GTG GTT	52
α5(N176R)	TAT GTT TGG ACC CGG GGT TCC ACC AAG	52
α5(G177E)	GTT TGG ACC AAT GAA TCC ACC AAG TCT	52
α5(S178P)	TGG ACC AAT GGT CCC ACC AAG TCT GTG	52
α5(T179A)	ACC AAT GGT TCC GCG AAG TCT GTG GTG	52
α5(K180R)*	TAT GTT TGG ACC AGG GAA CCC GCC AGG TCT GTG GTG GTG	52

For each mutant engineered, only one of the complementary strands is shown (in 5′-3′ orientation). The annealing temperature that was used reflects a reaction-specific optimized temperature for each PCR-mediated mutagenesis reaction. *Note that the primer used to engineer the α5(K180R) mutation was initially designed to introduce the multiple substitutions α5(NGSTK→REPAR), but upon DNA sequencing of the resulting PCR product, only the α5(K180R) substitution had occurred. Although the multiple substitutions were not created in a single mutant, the resulting α5(K180R) mutant was retained and used as the single substitution.