University of Alberta

$GABA_A$ Receptors and Tonic Inhibition: Towards an Improved Understanding of Agonist Binding and *in vivo* Expression of the Extrasynaptic $\alpha_4\beta_3\delta$ Subtype

By

Benjamin Gene Nilsson

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science

Department of Pharmacology

Fall 2013
Edmonton, Alberta

ABSTRACT

The $GABA_A$ receptor is the major inhibitory neurotransmitter receptor in the central nervous system. Receptors containing the δ subunit generate tonic inhibition due to their extrasynaptic expression, high affinity for γ-aminobutyric acid (GABA), and slow densensitization kinetics. The present work had two goals: first, compare structural elements involved in agonist binding in the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, which are model synaptic and extrasynaptic receptor subtypes, respectively; second, develop an immunoassay using two-step fluorescence resonance energy transfer to detect the incorporation of three subunits into one receptor complex. The structural studies showed that the loop D region participates in agonist activity at both receptor subtypes, and that the agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) may function through a distinct subsite from that of GABA. Inadequate expression of the subunit constructs limited progress on the immunoassay, requiring more work to optimize the expression system before proceeding to proof-of-principle studies using two-step FRET.

ACKNOWLEDGEMENTS

Although she is not here to accept my thanks, I would like to extend my deepest gratitude to Dr. Susan Dunn for allowing me to work and learn in her lab. She always showed a tremendous amount of patience and a forgiving attitude when things did not go as planned.

I am also grateful for the efforts of my co-supervisors Dr. Andy Holt and Dr. Janna Kozuska, who stepped in at a difficult time to give me the opportunity to continue on and finish my degree. I would also like to thank Dr. Ian Martin for providing a tremendous amount of positive support – both in the form of wise counsel and by reminding me that research can be as enjoyable as it is stressful.

Thanks also go to my supervisory committee members, Dr. Joe Casey and Dr. Fred Tse. Thank you for your support, feedback, and guidance over the last couple of years.

I would also like to thank other members of the lab: particularly Isabelle Paulsen for providing much needed help on a day-to-day basis, as well as to Greg Plummer, Dr. Chris Carter and Sheldon Michaelson for being there to provide help when it was needed.

Finally, I would like to thank my friends and family for keeping me anchored in the real world and for reminding me why all of this was worth doing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh	acetylcholine
AChBP	acetylcholine binding protein
AFM	atomic force microscopy
CACA	cis-aminocrotonic acid
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
cRNA	complementary ribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EC_{50}	concentration of agonist eliciting a response equal to half of the maximal channel activation
ER	endoplasmic reticulum
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
$GABA_A$	GABA type A receptor
$GABA_B$	GABA type B receptor
GABARAP	GABA receptor-associated protein
5-HT	5-hydroxytryptamine
$5\text{-}HT_3$	5-HT type 3 receptor
IC_{50}	concentration of antagonist reducing agonist-induced channel activity by 50%
I_{max}	maximal current elicited by an agonist
MA helix	membrane-associated α-helix
nACh	nicotinic acetylcholine receptor
n_H	Hill coefficient
PBS	phosphate-buffered saline
4-PIOL	5-(4-piperidyl)-3-isoxazolol
SEM	standard error of the mean
TACA	trans-aminocrotonic acid
THIP	4,5,6,7-tetrahydroisoxazolo[5,4]pyridin-3-ol

CHAPTER 1

Introduction

$GABA_A$ Receptor Overview

Transmission of information in the nervous system is a complex process that occurs through rapid, precise communication mediated by electrical and chemical signals. Neuronal activity arises from electrochemical gradients produced by the selective transport of ions such as Na^+, K^+, and Cl^- across the cell membrane. If the ionic equilibrium is disturbed through an influx of cations or efflux of anions, the membrane potential will become more positive (depolarization), while an efflux of cations or an influx of anions causes the membrane potential to become more negative (hyperpolarization). Membrane depolarization past a certain threshold will regeneratively activate voltage sensitive Na^+ channels in the axon and create a wave of depolarization – called an action potential – that propagates to the axonal terminal and triggers the release of neurotransmitters. Axon terminals associate tightly with neighbouring cells through synaptic junctions, which are only ~50 nm nanometers wide, allowing neurotransmitters to rapidly activate receptors embedded in the postsynaptic membrane. Neurotransmitters that cause membrane depolarization result in increased action potential firing and are termed excitatory neurotransmitters, while those causing hyperpolarization reduce action potential firing and are thus referred to as inhibitory.

The amino acid γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system (CNS), with GABA being found at approximately 30% of all synapses (Bloom and Iversen, 1971). The

signalling role of GABA was first deduced from studies on crayfish neuromuscular synapses, where it was shown that GABA is able to inhibit stretch-activated responses by inducing a hyperpolarizing influx of chloride across the cell membrane (Kuffler and Edwards, 1958; Hagiwara et al., 1960). Later studies in the rat brain showed that GABA activates distinct populations of GABA-responsive receptors, with one acting at postsynaptic and the other at presynaptic neurons (Bowery et al., 1980). The action of GABA on mammalian postsynaptic neurons occurrs through an increase in chloride permeability across the membrane, similar to the responses seen in the crayfish, whereas the presynaptic mechanism seen in mammals is not dependent on the concentration of chloride ions and is insensitive to the classic GABA antagonist bicuculline (Bowery et al., 1980; Bowery et al., 2002). Moreover, the compound baclofen selectively targets these same bicuculline-insensitive GABA binding sites, which led to the division of GABA receptors into the bicuculline-sensitive A-type and baclofen-sensitive B-type GABA receptors (Bowery et al., 1980; Hill and Bowery, 1981). The $GABA_B$ receptor has since been shown to be a G_i/G_o protein-coupled receptor that modulates adenylate cyclase activity, suppresses Ca^{2+} influx in presynaptic neurons, and increases K^+ conductance in postsynaptic neurons. This either inhibits neuronal activity or neurotransmitter release, depending on the context of receptor activation (Bowery et al., 2002). The sole focus of this work is on the $GABA_A$ receptor, so while the unique function of $GABA_B$

receptors makes them an interesting pharmacological target, they will not be discussed further here.

$GABA_A$ receptors are ionotropic receptors that allow Cl^- ions to move across the membrane in the presence of GABA. The chloride-selective conductance produced by channel opening can either depolarize or hyperpolarize the cell membrane, depending on the relative intracellular/extracellular chloride concentration $([Cl^-]_{in})/([Cl^-]_{out})$. Generally, if the intracellular chloride concentration is high, then chloride ions will move out of the cell and depolarize the membrane, while a low $[Cl^-]_{in}$ will cause chloride ions to move into the cell and hyperpolarize the membrane (Ben-Ari, 2002; Watanabe et al., 2009). There are some examples in mature neurons where GABA mediates excitation, such as in gonadotropin releasing hormone neurons (Watanabe et al., 2009), but in most mature GABA-responsive neurons, a potassium-chloride cotransporter is expressed that acts to lower the $[Cl^-]_{in}$, thus rendering $GABA_A$ responses predominantly inhibitory (Payne et al., 1996).

Molecular Biology of $GABA_A$ Receptors

Cloning of the first $GABA_A$ receptor subunits revealed that they were part of a super family of receptors called the Cys-loop family of ligand-gated ion channels (Schofield et al., 1987), which includes nicotinic acetylcholine (nACh) receptors (Lindstrom et al., 1987; Stroud et al., 1990), serotonin type 3 ($5\text{-}HT_3$) receptors (Maricq et al., 1991 and Miyake et al., 1995), and glycine

receptors (Grenningloh et al., 1987a; Grenningloh et al., 1987b), as well as zinc-activated channels (Davies et al., 2003). The nACh and 5-HT_3 receptors are selective for cations such as Na^+ and Ca^{2+}, while the $GABA_A$ and glycine receptors are selective for the anions Cl^- and bicarbonate (Kulik et al., 2009). Each receptor family is comprised of multiple homologous subunit isoforms that assemble to form homo- or heteropentameric ion channels, with the subunits arranged in a rosette conformation containing a central ion pore (Kubalek et al., 1987; Langosch et al., 1988; Anand et al., 1991; Nayeem et al., 1994; Boess et al., 1995).

To date, a total of 19 separate $GABA_A$ receptor subunit isoforms have been described in the human genome, consisting of α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3} δ, ε, π, and θ subunits (Simon et al., 2004). Subunit families are defined by their degree of sequence similarity and typically range from 60-80% between members of the same family, 20-40% between subunits of different families, and 10-20% between subunits from different receptor families (Olsen and Tobin 1990; Simon et al., 2004). Given the large number of $GABA_A$ receptor subunits available, the theoretical number of pentameric subunit combinations if there are no constraints on receptor assembly is massive (19^5 = 2.5 million possible subunit combinations).

$GABA_A$ Receptor Assembly

Despite the large number of potential subunit combinations, there appear to be "rules" that guide receptor assembly and serve to limit the

number of complexes that express on the cell surface. For example, in the case of the α_1, β_2, and γ_2 subunits, expression of each subunit alone primarily results in the formation of monomers and homodimers that are retained in the endoplasmic reticulum (ER) and degraded (Connolly et al., 1996; Gorrie et al., 1997). Expressing binary $\alpha_1\gamma_2$ and $\beta_2\gamma_2$ subunit combinations also fails to produce GABA-responsive receptors, as they form heterodimers that are retained and degraded in the ER (Connolly et al., 1996; Tretter et al., 1997; Taylor et al., 1999). In contrast, expression of the α_1 and β_2 subunits results in robust GABA-responsive currents (Schofield et al., 1987; Connolly et al., 1996; Connor et al., 1998) – behaviour which is seen for a number of other α/β subunit combinations (Levitan et al., 1988; Ymer et al., 1989; Sigel et al., 1990; Angelotti et al., 1993) – suggesting that these two subunit families represent the minimal requirement for functional $GABA_A$ receptor formation. It is worth noting that the β_1 and β_3 subunits are both capable of forming homomeric receptors (Krishek et al., 1996; Davies et al., 1997a), but coexpression with an α subunit isoform generally produces homogeneous $\alpha\beta$ receptor populations due to slow or inefficient homomeric receptor assembly (Angelotti et al., 1993; Tretter et al., 1997; Sieghart and Sperk 2002). Combining the α and β subunits with a third subunit isoform such as the γ_2 subunit results in relatively homogeneous $\alpha\beta\gamma$ receptor populations (Angelotti et al., 1993; Tretter et al., 1997), suggesting that ternary complex formation generally occurs preferentially to that of receptors containing only two subunit isoforms. Thus, in cells where two or more subunit isoforms are

expressed, the rate at which they associate with other subunits during assembly appears to be important for limiting the diversity of receptor subtypes formed (Bollan et al., 2003).

The fact that only certain subunit combinations assemble into pentameric complexes suggests that specific structural features or subunit arrangements are required for receptor assembly. Consistent with this, specific residues or motifs have been identified that have pronounced effects on the assembly of subunits into higher order oligomers (Klausberger et al., 2000; Taylor et al., 2000; Klausberger et al., 2001; Sarto et al., 2002; Bollan et al., 2003; Sarto-Jackson et al., 2006). Some subunit-subunit interactions, for example between β-γ or α-γ subunits, are mediated through motifs on only one face of each subunit, which is consistent with their observed tendency to form heterodimers (Sarto-Jackson and Sieghart, 2008). In contrast, two separate motifs on α and β subunits mediate contacts at α-β and β-α interfaces, which explains the tendency of higher order oligomers to form between these two subunits (Bollan et al., 2003; Sarto-Jackson and Sieghart, 2008). Because these assembly motifs constrain the possible orientations in which subunits can assemble effectively, they would be expected to contribute to the formation of receptors with defined subunit arrangements. Two separate studies examining the arrangement of subunits in $GABA_A$ receptors have deduced that αβ receptor subtypes are arranged as αβαββ and αβγ receptor subtypes as αβαγβ (going counter-clockwise when viewed from the extracellular side of the membrane) (Tretter et al., 1997; Baumann

et al., 2001). Subsequent modelling of an $\alpha_1\beta_3\gamma_2$ GABAA receptor subtype has shown that this orientation places the assembly motifs appropriately based on their known roles in subunit assembly (Trudell, 2002).

The fact that the δ, ε, and π subunits require coexpression with α and β subunits suggests that they may adopt a similar subunit arrangement as $\alpha\beta\gamma$ receptors (Quirk et al., 1995; Davies et al., 1997b; Hedblom and Kirkness, 1997). Consistent with this, visualization of antibody-bound $\alpha_4\beta_3\delta$ receptors using atomic force microscopy (AFM) deduced a subunit arrangement of $\alpha\beta\alpha\delta\beta$ - analogous to that deduced for $\alpha_1\beta_2\gamma_2$ receptors (Barrera et al., 2008). However, it is possible that there are multiple subunit arrangements that occur *in vivo* that were not detected by the AFM work. Concatenated constructs containing α_1, β_3 and δ subunits or α_1, β_2, and ε subunits in different subunit arrangements have demonstrated that both the δ and ε subunits can occupy a variety of different positions and still form viable receptors (Bollan et al., 2008; Kaur et al., 2009).

The assembly patterns of the remaining subunits appears to be distinct from the γ subunit. The θ subunit can be expressed on the cell surface in $\alpha\theta$, $\alpha\beta\theta$ and $\alpha\beta\theta\gamma$ complexes, although only the $\alpha\beta\theta\gamma$ complex is functional (Bonnert et al., 1999). The $\rho_{1\text{-}3}$ subunits are unique in that they form GABA-responsive homomeric receptors (Cutting et al., 1991; Wang et al., 1994; Shingai et al., 1996) or heteromeric ρ_1/ρ_2 (Pan et al., 2006) receptors. They are not known to coassemble with α and β subunits, though there is evidence

that the ρ_1 and ρ_2 subunits can associate with the γ_2 subunit (Pan and Qian, 2005). Receptors containing ρ subunits have historically been called "$GABA_C$" receptors due to their unique subunit makeup and pharmacological behaviour, but it has recently been suggested that this practice is misleading, and that they should instead be considered a special sub-class of $GABA_A$ receptors (Olsen and Sieghart, 2008).

Expression of $GABA_A$ Receptor Subtypes

Expression of the 19 different $GABA_A$ receptor subunits is highly heterogeneous, with some subunits being abundant and widespread while others are found only in discrete regions of the brain. Data for mRNA expression of α, β, γ, and δ subunits (Wisden et al., 1992), the ρ subunits (Wang et al., 1994; Ogurusu and Shingai, 1996), ε subunit (Davies et al., 1997b) and θ subunit (Bonnert et al., 1999) are summarized in Table 1-1 and in the following paragraph. It is worth noting that although mRNA expression is described here, patterns of protein expression are generally similar, with variation mostly being found in the intensity of subunit expression in some brain regions (Fritschy and Mohler 1995; Pirker et al., 2000; Schwarzer et al., 2001).

The α_1 subunit – expressed ubiquitously throughout the brain and spinal cord – comprises the most abundant α subunit isoform. The α_2 subunit is also expressed widely in brain and spinal cord, though not to the same extent as the α_1 subunit. The expression of other α subunits is more

restricted, with the highest levels seen in the cortex and spinal cord (α_3); the cortex, thalamus, striatum, hippocampus, and dentate gyrus (α_4); the hippocampus (α_5); and the granule cell layer of the cerebellum (α_6). Expression of the β_2 subunit is robust, and generally mirrors that of the α_1 subunit. The β_1 and β_3 subunits are less widespread than the β_2 subunit, but show high levels of expression in a number of brain regions. The γ_2 subunit is by far the most abundant γ subunit, and colocalizes extensively with the α_1 and β_2 subunits. The γ_1 and γ_3 subunits show homogeneously low levels of expression throughout most of the brain, though the γ_1 subunit shows some enrichment in the pallidum, amygdala, and cerebellum. The δ subunit largely coexpresses with the α_4 and α_6 subunits, but is most abundant in the cortex, thalamus, and cerebellum. The ε and θ subunits are expressed mostly in the subthalamic nucleus and substantia nigra, respectively. The $\rho_{1\text{-}3}$ subunits appear to be expressed almost exclusively in the retina, although there is evidence there may be limited expression in some regions of the brain (Boue-Grabot et al., 1998; Lopez-Chavez et al., 2005; Rosas-Arellano et al., 2012). The π subunit is only expressed in peripheral tissues, in particular the uterus (Hedblom and Kirkness, 1997).

Knowledge of receptor subunit expression patterns, combined with biochemical and electrophysiological data, has helped to define which subunit combinations most likely exist in the CNS. A recent review by Olsen and Sieghart (2008) has suggested a list of possible receptor subtypes with

three categories: those that have been "identified", which includes $\alpha_{1\text{-}5}\beta_x\gamma_2$, $\alpha_6\beta_2\gamma_2$, $\alpha_4\beta_{2/3}\delta$, $\alpha_6\beta_3\delta$, and ρ receptors; those existing with "high probability", which are the $\alpha_1\beta_3\gamma_2$, $\alpha_1\beta_x\delta$, $\alpha_5\beta_3\gamma_2$, $\alpha_x\beta_1\gamma$, $\alpha_x\beta_1\delta$, $\alpha_x\beta_x$, $\alpha_1\alpha_6\beta_x\gamma_x$, and $\alpha_1\alpha_6\beta_x\delta$ receptor subtypes; and those that have been "tentatively" identified, such as the ρ_1, ρ_2, ρ_3, $\alpha_x\beta_x\gamma_1$, $\alpha_x\beta_x\gamma_3$, $\alpha_x\beta_x\theta$, $\alpha_x\beta_x\varepsilon$, $\alpha_x\beta_x\pi$, and $\alpha_x\alpha_y\beta_x\gamma_2$ receptor subtypes (Olsen and Sieghart 2008). Of these receptors, the $\alpha_1\beta_2\gamma_2$ receptor is by far the most abundant subtype, accounting for approximately 40% of all $GABA_A$ receptors, while $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$ receptor subtypes together comprise another 35% (McKernan and Whiting, 1996; Whiting, 2003). With respect to specific brain regions, the predominant receptor subtypes from the above list are believed to be $\alpha_1\beta_x\gamma_2$, $\alpha_1\beta_x\delta$, $\alpha_2\beta_x\gamma_2$, $\alpha_3\beta_x\gamma_2$, and $\alpha_4\beta_x\delta$ in cerebral cortex; $\alpha_1\beta_2\gamma_2$, $\alpha_4\beta_2\gamma_2$, and $\alpha_4\beta_2\delta$ in the thalamus; $\alpha_1\beta_x\gamma_2$, $\alpha_1\beta_x\delta$, $\alpha_2\beta_x\gamma_2$, $\alpha_4\beta_x\delta$, and $\alpha_5\beta_3\gamma_2$ in the hippocampus; and $\alpha_1\beta_x\gamma_2$, $\alpha_1\beta_x\delta$, $\alpha_6\beta_2\gamma_2$, $\alpha_6\beta_3\delta$, $\alpha_1\alpha_6\beta_x\gamma_x$, and $\alpha_1\alpha_6\beta_x\delta$ in the cerebellum (Olsen and Sieghart, 2008; Macdonald and Botzolakis, 2009, and references therein). There is also some evidence that $GABA_A$ receptors may be present on glial membranes in the cerebellum, hippocampus and retina, and that they consist of some combination of $\alpha_{1/3}\beta_{1/3}\gamma_1$ subunits (Yoon et al., 2012).

Functional Heterogeneity of $GABA_A$ Receptor Subtypes

The functional responses of receptor subtypes are highly dependent on their subunit composition. At the level of macroscopic currents, varying the α subunit in $\alpha_x\beta_3\gamma_2$ receptor complexes results in a 50- to 100-fold range

in the concentration of GABA that elicits a half-maximal response (EC_{50}), with the general trend in EC_{50} values being $\alpha_6 < \alpha_1/\alpha_4/\alpha_5 < \alpha_2/\alpha_3$ (Bohme et al., 2004; Mortensen et al., 2012). In contrast to the α subunit, varying the β subunit in $\alpha_1\beta_x\gamma_2$ receptors only results in a 3- to 5-fold range in EC_{50} values for responses to GABA (Hadingham et al., 1993; Mortensen et al., 2012). Experiments with the δ subunit suggest that including it in place of the γ_2 subunit in $\alpha_4\beta_x(\gamma_2/\delta)$ receptors results in higher potency GABA-responses (Brown et al., 2002; Mortensen et al., 2010), although it shows little effect when substituting in $\alpha_6\beta_3(\gamma_2/\delta)$ receptors (Feng and Macdonald., 2004; Mortensen et al., 2012).

At the level of single channel events, the conductance of γ_2- and δ-containing receptors are similar, at around 25-30 pS (Fisher and Macdonald, 1997; Mortensen et al., 2010), while those of binary $\alpha\beta$ receptor subtypes are significantly lower at 10-15 pS (Fisher and Macdonald, 1997). Receptors containing the γ_2 subunit produce longer bursts of channel activation with a higher probability of opening when compared to δ-containing receptors (Fisher and Macdonald, 1997; Bianchi and Macdonald, 2003; Keramidas and Harrison, 2008; Mortensen et al., 2010). This is consistent with the idea that GABA acts as a high affinity partial agonist at δ-containing receptors and a full agonist at γ_2-containing receptors, despite their single channel conductances being similar (Bianchi and Macdonald, 2003).

Different receptor subtypes appear to localize to distinct subcellular compartments, depending on their subunit makeup, with some forming high density clusters at the synapse and others forming extrasynaptic receptor populations. The γ_2 subunit is a major determinant of synaptic clustering, as it interacts with the synaptic scaffold protein gephyrin (Essrich et al., 1998; Schweizer et al., 2003) as well as the $GABA_A$-Receptor-Associated-Protein (GABARAP), which is associated with elements of the cytoskeleton (Wang et al., 1999a; Nymann-Andersen et al., 2002). The major receptor subtypes believed to cluster synaptically are those containing the α_1, α_2, and α_3 subunits along with $\beta_x\gamma_2$ subunits (Essrich et al., 1998; Nusser et al., 1998; Brunig et al., 2001). Major extrasynaptic receptor subtypes include the δ subunit, which is localized exclusively to peri- or extrasynaptic membrane compartments (Nusser et al., 1998; Wei et al., 2003). The δ subunit preferentially associates with the α_4 and α_6 subunits, and is believed to form significant receptor populations in the cerebral cortex, striatum, dentate gyrus, and thalamus ($\alpha_4\beta_{2/3}\delta$) (Bencsits et al., 1999; Sur et al., 1999; Pirker et al., 2000), as well as cerebellar granule cells ($\alpha_6\beta_3\delta$) (Nusser et al., 1998; Poltl et al., 2003). More recently, the δ subunit has also been shown to assemble with the α_1 subunit in hippocampal interneurons (Glykys et al., 2007). The α_5 subunit is unique in that it forms significant $\alpha_5\beta_x\gamma_2$ receptor populations, particularly in the hippocampus, yet does not exhibit significant enrichment at the synapse (Brunig et al., 2001; Crestani et al., 2002; Caraiscos et al., 2004).

Tonic and Phasic Inhibition

The subcellular localization of a given receptor subtype has a significant impact on how it contributes to neuronal signalling (Figure 1-1). Receptors that cluster at the synapse mediate phasic inhibition in response to synaptically-released GABA, which reaches peak concentrations in the millimolar range and then rapidly dissipates in a span of 100-200 μs (Clements, 1996; Mozrzymas, 2004). The time course of GABA release results in postsynaptic currents that activate in less than a millisecond and then decay over tens to hundreds of milliseconds (Maconochie et al., 1994; Haas and Macdonald, 1999). Activation of phasic currents across neuronal populations generates and synchronizes rhythmic oscillations in neuronal networks (Mann and Paulsen, 2007; Mann and Mody, 2010), which appears to be important for information processing throughout the CNS (Sejnowski and Paulsen, 2006).

Tonic inhibition is generated in response to low- to submicromolar concentrations of GABA present outside of the synapse (Brickley et al., 1996; Rossi and Hamann, 1998; Nusser and Mody, 2002). The extrasynaptic $\alpha_1\beta_x\delta$, $\alpha_4\beta_{2/3}\delta$, $\alpha_6\beta_3\delta$, and $\alpha_5\beta_3\gamma_2$ receptors respond to GABA concentrations in this range (Saxena and Macdonald, 1996; Brown et al., 2002; Caraiscos et al., 2004; Mortensen et al., 2012). Because ambient GABA is always present to some degree in the extracellular space, extrasynaptic receptors are persistently activated and therefore generate a tonic inhibitory tone (Bright et al., 2011). Tonic inhibition has been shown to modulate the threshold

required for action potential generation and appears to play a role in setting the frequency of neuronal signalling (Pavlov et al., 2009; Mann and Mody, 2010).

Breakthroughs in Determining Cys-loop Receptor Structure

Given the unique physiological responses observed for different $GABA_A$ receptor subtypes, there is a major interest in characterizing the structural features underlying differences in activity. There has historically been a lack of structural models of Cys-loop receptors due to the difficulty of growing crystals of transmembrane proteins for X-ray diffraction studies. However, two major breakthroughs in the past decade have launched the study of Cys-loop receptors into the structural age and facilitated the construction of $GABA_A$ receptor models with increasing detail and accuracy.

One breakthrough was made possible by the serendipitous discovery that some molluscan species express a soluble acetylcholine binding protein (AChBP) secreted by glial cells (Smit et al., 2001). AChBP is a 210 amino acid protein that shares approximately 30% sequence identity with the extracellular domain of the nACh receptor (Smit et al., 2001). Functional studies have shown that a number of agonists and antagonists at nACh receptors also bind to AChBP (Smit et al., 2001; Brejc et al., 2001). Moreover, a chimeric construct that replaced the $5\text{-}HT_3A$ receptor extracellular domain with the homologous AChBP residues - but maintained the residues lying at the extracellular-transmembrane domain interface - produced a fully

functional acetylcholine-responsive receptor (Bouzat et al., 2004). As a result, it appears that the agonist-induced conformational changes in AChBP are analogous to those of the extracellular domain in Cys-loop receptors, providing evidence that AChBP serves as a functionally relevant model for ligand binding in the extracellular domain. Since AChBP is a relatively small, soluble protein, it is more amenable to crystallographic techniques and has therefore provided high-resolution structural models for agonist and antagonist binding at Cys-loop receptors (Brejc et al., 2001; Celie et al., 2004; Celie et al., 2005; Hansen et al., 2005).

Another breakthrough was achieved using tissues from the electroplax organ from the *Torpedo* electric ray, where almost 50% of the membrane protein is comprised of nACh receptors (Sobel et al., 1977). Analysis by cryo-electron microscopy (cryo-EM) of tubular crystals formed using these tissues had previously provided low resolution models of the nACh receptor structure (>9 Å) (Toyoshima and Unwin, 1988; Unwin, 1993). Subsequent improvements to the technique allowed the resolution to be refined to 4 Å (Miyazawa et al., 2003; Unwin, 2005). The refined models presented in 2003 and 2005 represented the first time that detailed structural data had been obtained for the extracellular, transmembrane, and intracellular domains of Cys-loop receptors. Combining the cryo-EM data with previous structural data from AChBP and the T*orpedo* nACh receptor has allowed the binding-gating mechanism in Cys-loop ligand-gated ion channels to be examined at unprecedented levels of detail.

While structural studies of the nACh receptor have benefited directly from having abundant natural sources for study - namely the electroplax organ of the *Torpedo* electric ray and AChBP in molluscs – these structural data have also been used to inform studies of $GABA_A$ receptors. A number of comparative models have been constructed for the $GABA_A$ receptor based on its sequence conservation with the nACh receptor and AChBP (Cromer et al., 2002; Ernst et al., 2003; Ernst et al., 2005; Mokrab et al., 2007; Melis et al., 2008). This process requires alignment of both sequences to identify well-conserved structural regions, which are then used as anchor points to align less well-conserved regions (Ernst et al., 2003). At this point the model is generated using the coordinates of the known three-dimensional structure as a template (Schwede et al., 2003). Generally speaking, secondary structural elements can be modelled with greater accuracy than loop regions, as they are usually part of the core protein structure and thus have a number of constraints that influence their topology (Chothia and Lesk, 1986; Ernst et al., 2003). The initial model is then optimized using energy-minimization algorithms based on homology-derived restraints from the template structure, as well as stereochemical restraints and bond-angle preferences obtained from the molecular mechanics force field of CHARMM-22 (Fiser and Sali, 2003). This process is intended to remove steric clashes and suboptimal backbone conformations that may have been introduced during the modelling process (Ernst et al., 2003). Further steps can then be taken to optimize the structure, such as adding solvent and solute molecules and

embedding the receptor in a simulated membrane lipid environment (Law et al., 2005), and/or performing molecular dynamics simulations (Law et al., 2005; Melis et al., 2008; Sander et al., 2011).

Overview of the Cys-loop Receptor Structure

To help put the structural features of the three-dimensional model into context, the primary sequence of the $GABA_A$ α_1 subunit is shown in Figure 1-2. The dimensions of each subunit are approximately 30 Å x 40 Å x 160 Å, with the overall structure of the pentameric receptor having dimensions of roughly 70 Å x 70 Å x 160 Å. The receptor has a quaternary structure containing an extracellular ligand-binding domain, a transmembrane domain composed of four membrane spanning helices (M1-M4), and an intracellular domain that consists of a membrane-associated α-helix (MA helix) as well as an intracellular loop of >100 residues that has not yet been resolved (Figure 1-3) (Unwin, 2005). The ion channel consists of two vestibules in the intracellular and extracellular domains that have a diameter of approximately 20 Å, which are connected by an ion pore formed by the transmembrane domain that is approximately 6 Å across at its narrowest point.

The Extracellular Domain and Agonist Binding

The extracellular domain consists of 10 β-strands and an N-terminal α-helix, with loops of residues connecting adjacent β-strands (Brejc et al.,

2001). The β strands are arranged in a "β-sandwich", consisting of an inner sheet and an outer sheet connected by the Cys-loop (β6-β7 loop) (Miyazawa et al., 2003). The Cys-loop is a conserved structure in all Cys-loop receptor subunits, and consists of a 13 residue stretch of amino acids flanked on both sides by a pair of disulphide-bonded cysteine residues. Agonist molecules are believed to bind at subunit-subunit interfaces in the extracellular domain, where six non-contiguous loops (A-F) associate to form the agonist binding site (Figure 1-4). Loops A-C are formed by linkers connecting the β4-β5 (A), β7-β8 (B), and β9-β10 (C) strands and comprise the principal face of the subunit. The complementary face is formed by loops D-F, which are composed of the β2 (D) and β5-5' (E) strands and the β8-β9 loop (F). Loops D and E exist as β-sheets, but are referred to as "loops" for historical reasons (Galzi et al., 1990).

While loops A-F have all been implicated in agonist binding, movement of loop C appears to be a key element for channel gating. Structures obtained by co-crystallization of AChBP with a variety of agonists and antagonists have shown that the efficacy of a compound correlates with movements in loop C (Celie et al., 2004; Hansen et al., 2005; Hibbs and Gouaux, 2011). Agonists with high efficacy cause loop C to swing inward and cap the binding site, whereas nACh receptor antagonists fail to move loop C, and can even cause it to move outwards if they are sufficiently bulky (Figure 1-5) (Celie et al., 2004; Hansen et al., 2005). The inward movement of loop C

has been shown to decrease solvent accessibility (Shi et al., 2006) and may help to lock the agonist in the binding site (Chang and Weiss, 1999).

Agonists that elicit smaller maximum responses than "full" agonists, also known as partial agonists, are generally larger than full agonists but not as bulky as most antagonists. As such, they may only partially block the capping motion of loop C, resulting in an intermediate conformation of loop C between fully open and fully closed (Hansen et al., 2005; Hibbs et al., 2009; Sander et al., 2011). Kinetic modelling of partial and full agonist activity at the glycine receptor has suggested that there may be a pre-open conformation called the "flip" state, in which the agonist-bound receptor is primed for channel gating (Burzomato et al., 2004; Lape et al., 2008). According to this model, both full and partial agonists produce similar transitions for channel opening, but differ in their ability to achieve the flipped state (Lape et al., 2008). The degree of loop C closure could be one of the major determinants to achieve the flipped state, thus providing an attractive link between the size of an agonist, its ability to elicit loop C closure, and its efficacy once bound to the receptor.

The Transmembrane Domain

The transmembrane domain consists of four stretches of hydrophobic amino acids that form membrane-spanning α-helices (M1-4). Short stretches of amino acids act as linkers between the M1-M2 and M2-M3 helices, while there is a large loop of residues between the M3-M4 helices that forms the

bulk of the intracellular domain (Jansen et al., 2008). A wealth of biochemical and structural evidence suggests that the M2 helices face towards the inside of the receptor and form the lining of the ion channel (Giraudat et al., 1986; Imoto et al., 1986; Unwin, 1993; Akabas et al., 1994; Hibbs and Gouaux, 2011). In contrast, the M1 and M3 helices are tightly associated with the M2 helix and may play a secondary role in stabilizing the ion channel structure, while the M4 helix is located on the periphery of the cluster of helices and is largely surrounded by membrane lipids.

Because they form the structure of the ion pore, the M2 helices are crucial determinants for ionic conductance and selectivity. In $GABA_A$ receptors, arginine residues on the intra- and extracellular ends of the M2 helices increase the local concentration of anions at the mouth of the ion channel (Keramidas et al., 2004). Ion selectivity is conferred by the narrow constriction of the ion pore at the intracellular portion of the M2 helices, which forces water molecules in the hydration shell to dissociate from the permeant ion, thus creating a major energetic barrier to ion translocation (Fatima-Shad and Barry, 1993). The structure and electrostatic environment of the M2 helices likely contribute to ion selectivity by forming stabilizing interactions with the ion, which compensates for energy lost due to dehydration as the permeant ion passes through the narrow ion pore (Hibbs and Gouaux, 2011). Consistent with this idea, mutations altering the geometry of the intracellular region and/or the electrostatic environment of

the M2 helix are critical for anionic versus cationic selectivity (Galzi et al., 1992; Wang et al., 1999b; Jensen et al., 2005; Hibbs and Gouaux, 2011).

In addition to forming the selectivity filter, the M2 helices also produce a channel gate that regulates the flow of ions. The M2 helices do not run exactly parallel with the axis of the channel, but rather taper from a width of 15-20 Å at the extracellular end to approximately 5.6 Å- 6.2 Å at the narrowest point of the channel (Figure 1-6) (Miyazawa et al., 2003; Sunesan et al., 2006; Hibbs et al., 2011). Some positions of the M2 helices contain hydrophobic residues, the side chains of which are believed to point into the lumen of the ion channel to form a hydrophobic girdle (Unwin, 1995; Chang and Weiss, 1998; Miyazawa et al., 2003). One model proposes that channel activation may be achieved by movements that destabilize the hydrophobic girdle and cause the hydrophobic side chains to collapse against the side of the ion pore, which would then allow ions to flow down their electrochemical gradient (Miyazawa et al., 2003).

The Intracellular Domain

The intracellular domain appears to play major roles in ion translocation and in mediating protein-protein interactions with intracellular proteins. While the structure of the full intracellular domain formed by the M3-M4 linker has not been fully elucidated, that of the MA helix has been successfully resolved. The MA helices from each subunit associate to form an intracellular vestibule containing fenestrations through which ions can

access the cytoplasm (Unwin, 2005; Mokrab et al., 2007). The fenestrations have been shown to play a role in regulating ion flow through mutations of three arginine residues on the MA helix of the 5-HT_3A subunit (Kelley et al., 2003). When these arginine residues were mutated to the homologous residues found on the 5-HT_3B subunit, the single-channel conductance increased from 0.4 pS to 25 pS in 5-HT_3A homomeric receptors (Kelley et al., 2003). Based on experimental and computational data, it has been postulated that the MA helices also influence ionic conductance in nACh, glycine and $GABA_A$ receptors (Hales et al., 2006; Mokrab et al., 2007).

The rest of the intracellular domain plays a predominantly regulatory role, influencing aspects of receptor function such as trafficking and subcellular localization. For instance, the GABA receptor-associated protein (GABARAP) has been shown to regulate cell-surface receptor expression by mediating interactions between the intracellular domain, tubulin and N-ethylmaleimide-sensitive factor (Wang et al., 1999a; Kittler et al., 2001; Leil et al., 2004). GABARAP also interacts with gephyrin during trafficking, which may have implications for synaptic clustering (Kneussel et al., 2000). Finally, proteins such as PRIP-1, CamKII, PKA, and PKC can phosphorylate the intracellular domain to modulate functional responses and receptor trafficking (Terunuma et al., 2004; Choi et al., 2008; Houston et al., 2009; Tang et al., 2010).

Activation of Cys-loop Receptors

Agonist binding initiates a wave of conformational changes that passes through the extracellular and transmembrane domains and converges on the channel gate over 30 Å away from the binding site (Grosman et al., 2000; Chakrapani et al., 2004; Purohit et al., 2007). One of the most conspicuous movements – as seen in structural models and molecular dynamics simulations of agonist-bound nACh receptors – is a rigid body rotation of approximately 15° in the extracellular domains of the principal subunits (Unwin, 1995; Unwin et al., 2002; Miyazawa et al., 2003; Law et al., 2005).

Movements in the extracellular domain are thought to be communicated to the gating region through three points of contact between the extracellular and transmembrane domains. The first is a direct connection between loop C and the M1 helix through the β10 sheet, while the second and third contacts are mediated by the Cys-loop and the β1-β2 loop on the extracellular domain, both of which contact the M2-M3 helices (Figure 1-7) (Unwin et al., 2002; Kash et al., 2003; Miyazawa et al., 2003; Kash et al., 2004). There is also evidence that the pre-M1 region, the Cys loop, and the β hairpin loop are connected by a network of salt bridges, which could allow these regions to move in a concerted manner (Chakrapani et al., 2004; Xiu et al., 2005; Purohit et al., 2007). The M2-M3 linker has been identified as a key determinant for channel gating, likely because its rigid structure allows it to transmit conformational changes directly to the M2 helix (Kash et al., 2003;

Kash et al., 2004; Lummis et al., 2005a). Movements at the extracellular/transmembrane domain interface are then transmitted to the M2 helix, causing the hydrophobic side chains constituting the gate to collapse against the lumen of the channel and increase the diameter of the ion pore (Unwin, 1995; Miyazawa et al., 2003; Law et al., 2005).

The Agonist Binding Site of $GABA_A$ Receptors

Substantial evidence suggests that agonists elicit responses by binding at β/α subunit interfaces in $GABA_A$ receptors, with the β and α subunits forming the principal and complementary faces of the binding site, respectively (Sigel et al., 1992; Amin and Weiss, 1993; Newell et al., 2000). The positively charged amino group of GABA is believed to participate in a cation-π interaction with a tyrosine residue on loop A of the β subunit (Lummis et al., 2005b; Padgett et al., 2007), which leaves the GABA molecule oriented with the carboxylate group facing towards the complementary face of the binding site. Three arginine residues, from loop C of the β subunit and loops D and E of the α subunit, are conserved in all $GABA_A$ subunit isoforms and have been identified as forming a crown of positive charges that stabilize the carboxylate end of GABA (Boileau et al., 1999; Wagner et al., 2004; Goldschenn-ohm et al., 2011). A combination of molecular modelling and electrophysiological studies in the ρ1 homomeric $GABA_A$ receptor have suggested that the loop D arginine plays a key role in forming stable hydrogen bonds with the GABA carboxylate, while the other arginine

residues may form part of the pathway for movement of GABA into or out of the binding site (Melis et al., 2008; Goldschenn-Ohm et al., 2011). In all of the $GABA_A$ α subunits, the loop D arginine residue is positioned two residues downstream of a phenylalanine residue that has also been implicated in GABA binding through electrophysiological (Sigel et al., 1992), biochemical (Boileau et al., 1999; Holden and Czajkowski, 2002), and UV photolabelling studies (Smith and Olsen, 1994).

Agonists Acting at the GABA Binding Site

GABA is a flexible molecule that can adopt a number of distinct conformations; however only some of them appear to be active at $GABA_A$ receptors. For example, cis-aminocrotonic acid (CACA) - a conformationally-restricted GABA analogue that resembles the folded conformation of GABA (Figure 1-8) - shows negligible activity on $GABA_A$ receptors in the CNS, with the exception of ρ receptors (Johnston et al., 1975; Feigenspan et al., 1993). In contrast, trans-aminocrotonic acid (TACA), which mimics the extended conformation of GABA, displays nearly identical functional responses to those of GABA (Kusama et al., 1993). It is also worth noting that muscimol, the first discovered natural analog of GABA with a high affinity for $GABA_A$ receptors, has a conformationally-restricted isoxazole ring structure that also mimics the extended conformation of GABA.

The structure of muscimol proved to be a good starting point for the synthesis of useful specific $GABA_A$ receptor agonists. These include 4,5,6,7-

tetrahydroisoxazolo[5,4]pyridin-3-ol (THIP or gaboxadol), isoguvacine, and 4-PIOL, among others (Figure 1-8) (Krogsgaard-Larsen 2004). THIP has nearly the same structure as muscimol, with the exception that the amino group is constrained in a tetrahydropyridine ring that is in turn fused to the isoxazole ring structure. THIP was thought to have potential as an effective therapeutic compound due to its analgesic and hypnotic effects, but has demonstrated limited usefulness in clinical trials due to high rates of adverse events (Kjaer and Nielson, 1983) or limited/inconsistent efficacy (Roth et al., 2010).

Despite the setbacks in the clinic, THIP has continued to be a compound of interest due to its pronounced subtype selectivity. As is the case with GABA, THIP produces more potent responses at $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ receptors compared to other receptor subtypes, particularly those containing the γ_2 subunit (Adkins et al., 2001; Brown et al., 2002; Storustovu and Ebert, 2006; You and Dunn, 2007; Mortensen et al., 2010; Meera et al., 2011). Most studies report the rank order of potencies as muscimol > GABA >> THIP, with the exception of Meera et al., who found that expression of the δ subunit with $\alpha_4\beta_3$ or $\alpha_6\beta_3$ subunits increased the potency of THIP responses down to the nanomolar range in *Xenopus* oocytes (2011). An interesting aspect of THIP is its functional selectivity for specific receptor subtypes, as it acts as a partial agonist at γ_2-containing receptors and a "superagonist" at $\alpha\beta$ receptors or δ-containing receptor subtypes (Adkins et al., 2001; Brown et al., 2002; Storustovu and Ebert, 2006). Single channel analysis of THIP activity at $\alpha_4\beta_3\delta$

receptor subtypes suggested that its superagonist activity appears to be due to the fact that it produces longer mean open times and duration of bursts of channel opening events compared to GABA (Mortensen et al., 2010), both of which are consistent with higher efficacy gating patterns (Bianchi and Macdonald, 2003).

Antagonists Acting at the GABA Binding Site

In contrast to $GABA_A$ receptor agonists, antagonists tend to be bulky hydrophobic molecules that sterically block loop C closure upon binding (Jones et al., 2001; Celie et al., 2004; Hansen et al., 2005; Shi et al., 2006; Sander et al., 2011). For example, the analog 4-PIOL is a weak partial agonist, while analogs with moderate- to large-sized hydrophobic substituents (for example 4-(3-diphenylpropyl)-4-PIOL in Figure 1-8) act as full competitive antagonists at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Mortensen et al., 2002). This shift from partial agonism to antagonism appears to be a result of increased interactions between the substituted 4-PIOL analogs and a hydrophobic cavity near the binding site (Mortensen et al., 2002; Sander et al., 2011). Kinetic modelling and mutation analysis with the structurally unrelated $GABA_A$ receptor antagonists bicuculline and gabazine also found that antagonists bind predominantly through hydrophobic effects, suggesting that this may be a common mechanism for antagonist activity at the GABA binding site (Jones et al., 2001).

Agonist Binding and Efficacy

A long-standing issue in the field of receptor pharmacology has been adequately defining the relationship between agonist binding and channel gating (Colquhoun, 1998). Agonist efficacy can be generally defined as that property of the agonist molecule that causes the receptor to change its behaviour towards the host. The major difficulties faced by experimental studies are that agonist efficacy is dependent on the total number of receptors on a cell, the number of binding sites per receptor, the coupling efficiency between receptor activation and tissue response, as well as the intrinsic efficacy of a given agonist (Kenakin, 2004). Further complicating the matter is that variations in efficacy can also manifest themselves on the apparent potency of agonist responses, since a lower concentration of the agonist is required to achieve a certain level of receptor activation (Kenakin, 2004). These difficulties are compounded when attempting to define the binding versus gating contribution of a given amino acid residue, since both the potency and efficacy of agonist responses can be affected by mutations that alter the channel gating equilibrium (Colquhoun, 1998; Bianchi and Macdonald, 2003; Mortensen et al., 2003; Purohit and Auerbach, 2009).

Despite these difficulties, there are studies using single-channel techniques that have successfully characterized the contribution of agonist binding to receptor activation. For example, studies measuring the single-channel kinetics of $GABA_A$ receptors have shown that it is possible to accurately model agonist responses, but the process requires that the life-

time and frequency of distinct open, closed, and desensitized states be measured in addition to agonist affinity and efficacy (Mortensen et al., 2010). Another approach used estimates of single-channel equilibrium constants for the closed-open transition in unliganded and diliganded receptors, which were then used to calculate dissocation constants of ligands from the closed and open receptor states, respectively (Purohit and Auerbach, 2009). The ratio of the two dissociation constants was then used to provide a measure of agonism based on a ligand's preference for the open versus closed state of the receptor, with acetylcholine showing a 16,000-fold higher affinity for binding to the open state of the receptor (Purohit and Auerbach, 2009). Both of these techniques provide detailed information about how the agonist elicits a response upon binding to the receptor, but have the drawback that they are technically complicated and require that a number of simplifying assumptions be made to generate models for agonist activity.

A more elegant solution to this problem has been proposed called the operational model, which defines an experimentally determined variable, τ, for each agonist (Black and Leff, 1983; Kenakin, 2004). The τ value incorporates aspects of the receptor concentration, the coupling efficiency between receptor activation and tissue response, as well as the intrinsic efficacy of the agonist, and is incorporated into the model in such a way that it can account for both the maximal response and apparent affinity of an agonist. The utility of this approach comes from the fact that the ratio of τ values for two agonists is tissue independent, and allows for different

agonists to be accurately compared in multiple systems. The major disadvantage of this method is that it gives limited information on the binding-gating mechanism.

Another common approach taken in studies of agonist efficacy is to compare the maximum responses elicited by saturating concentrations of two agonists. This is referred to as the relative efficacy, although it is not strictly efficacies that are being compared. This approach is simple, and is sufficient to account for variables such as receptor concentration and the efficiency of receptor-tissue coupling, and does not require a known intrinsic efficacy value to be assigned to each agonist. The drawbacks to this method are that relative efficacy measurements give limited information for each individual agonist and fail to account for the fact that changes in efficacy can cause changes in the apparent affinity of an agonist. As a result, measurements of EC_{50} values for agonist-evoked responses, including studies of wild-type and mutant receptors, need to be interpreted with caution and should not be confused with measurements of agonist affinity in these experiments.

Fluorescence and FRET

Fluorescence is a luminescent process in which a compound - called a fluorophore - absorbs light to achieve an excited state and subsequently emits a photon as it relaxes back to its ground state. Excitation of the fluorophore pushes an electron from its ground state ($_GS_0$) into a higher

energy level ($_ES_1$) (Figure 1-9). Following excitation, the electron relaxes to the lowest possible excited sublevel in $_ES_1$ through internal conversion, which releases vibrational energy that is transferred to the surrounding environment as heat. The final relaxation step from $_ES_1$ to $_GS_0$ results in the release of a photon, the wavelength of which depends on the gap in energy between the two states. The relationship between photon energy and wavelength is given by:

$$E = h \bullet c/\lambda$$

where *E* is the energy gap, in Joules, between $_ES_1$ and $_GS_0$, *h* is Planck's constant (J.s), *c* is the speed of light ($m.s^{-1}$), and λ is the wavelength (m) of the absorbed or emitted photon. Because each energy state ($_ES_1$ and $_GS_0$) has multiple sublevels, there are many possible transitions with different associated energies, resulting in a spectrum of excitation and emission wavelengths for each given fluorophore.

In addition to direct fluorescence through photon release, fluorophores can also transfer energy through a non-radiative process called fluorescence resonance energy transfer (FRET). FRET occurs when the emission spectrum of one fluorophore (the "donor" fluorophore) overlaps with the excitation spectrum of a second fluorophore (the "acceptor") (Ishikawa-Ankerhold et al., 2012) (Figure 1-10). When the donor fluorophore is excited by a photon, it can transfer that energy to an acceptor fluorophore through dipole-dipole coupling. Because dipole-dipole interactions are

dependent on distance, FRET only occurs when two fluorophores are brought into close contact, typically within 100 Å (Figure 1-11). The relationship of FRET efficiency and fluorophore distance was first modelled by Theodor Förster, and is given by the equation:

$$E_{FRET} = 1 / (1 + (r/R_0)^6)$$

where E_{FRET} is the FRET efficiency ($0 < E_{FRET} < 1$), r is the distance of separation between the two fluorophores, and R_0 is the Förster radius. The value of R_0 is a constant determined for each FRET pair, and varies with the quantum yield and the degree of spectral overlap for each fluorophore.

The sharp distance-dependence of FRET - varying inversely with the distance between fluorophores raised to sixth power – makes it particularly useful as a "molecular ruler". A variety of techniques have been developed to capitalize on the unique properties of FRET, including the use of fluorescent-labelled DNA conjugates (Clegg et al., 1993; Lam and Li, 2010), covalent labelling of proteins with fluorophores (Schuler et al., 2002), and conjugation of fluorophores with antibodies directed against proteins of interest (Maurel et al., 2004). Using the first two techniques, the number of fluorophores on each molecule can be precisely controlled, either through step-wise incorporation of labelled nucleotides during oligonucleotide synthesis or through specific labelling of cysteine residues in proteins using sulfhydryl-reactive reagents. This allows for accurate calculation of E_{FRET}, which can then be extrapolated to calculate the distance of separation between the

fluorophores. This can be extended to provide useful information on the structure or conformational rearrangements of a given protein. In contrast, FRET immunoassays are useful for qualitative detection of protein-protein contacts, but give limited information at the structural level. This limitation arises because it is difficult to control the number of fluorophores incorporated for each antibody, which results in multiple intermolecular FRET interactions between the antibody and target as well as intramolecular interactions between fluorophores on the same antibody. Additionally, the conformation in which the antibody binds the target - as well as subsequent movements following binding - increases the difficulty in isolating structural changes specific to the target protein.

FRET has traditionally been used in systems that contain only two interacting fluorophores. However, additional fluorophores can be incorporated to create a "FRET relay", where two or more FRET pairs share a common fluorophore that acts as both a donor and acceptor molecule (Watrob et al., 2003; Galperin et al., 2004). In such two-step FRET systems, three separate fluorophores are present, with a donor fluorophore (D_1), an intermediate acceptor/donor fluorophore (D_2), and a terminal acceptor fluorophore (A). In this system, excitation of D_1 results in energy transfer to D_2, which in turn excites A. One of the major complications of this system is that D_1, D_2 and A can undergo varying degrees of radiative and non-radiative decay processes, which necessitates a large number of control experiments to allow for calculation of E_{FRET} for all FRET pair combinations. To date, two-

step FRET has only been reported using oligonucleotide probes or with fluorescent protein constructs (Galperin et al., 2004; Lam and Li 2010).

Aims of the Present Studies

The overarching goals of the work presented here were to study the expression of the extrasynaptic $\alpha_4\beta_3\delta$ $GABA_A$ receptor subtype and elucidate structural elements in the binding site underlying their unique agonist pharmacology.

The work presented in Chapter 2 has a structural focus, using site-directed mutagenesis to modify amino acid residues in the binding site of $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors. Two mutations were chosen for the study – both of which are on loop D of the α subunit – and their contributions to the potency and efficacy of GABA and THIP were compared between the two receptor subtypes. Responses of wild-type and mutant receptors were measured using electrophysiological techniques and the *Xenopus* oocyte expression system. The results are discussed in the context of ligand-docking studies carried out *in silico* using homology models of both $GABA_A$ receptor subtypes, which were constructed based on the nACh receptor structure and AChBP structures discussed above (Mokrab et al., 2007).

The work presented in Chapter 3 aimed to develop an assay that would facilitate the characterization of receptor subtype expression patterns. Using FRET, it is theoretically possible to combine detection of subunit expression with detection of specific intersubunit contacts. Fluorophores can

be targeted to specific $GABA_A$ receptor subunits using antibody-fluorophore conjugates that are specific for each subunit. Since $GABA_A$ receptors exist primarily as complexes containing three distinct subunits isoforms, detection will require the use of three fluorophores and two-step FRET, a technique which has not been applied using antibody-dye conjugates. Therefore, this study aimed to optimize the technique in an *in vitro* system using mammalian cell cultures and epitope-tagged α_4, β_3, and δ subunit constructs.

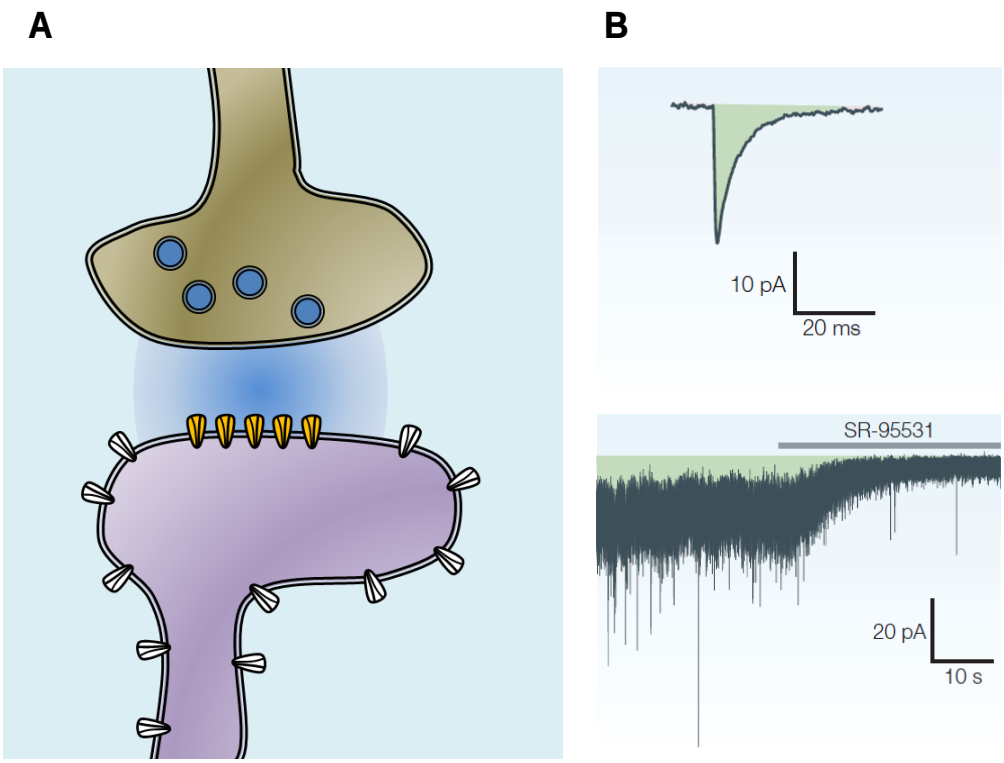


Figure 1-1. $GABA_A$ receptors can produce phasic and tonic inhibition. (A). Cartoon representation of a synapse, where the presynaptic neuron (brown) releases vesicles containing GABA to activate receptors on the postsynaptic neuron (purple). Synaptic receptors are coloured in orange, with synaptic GABA shown in dark blue. Extrasynaptic receptors are coloured white, with the low levels of extrasynaptic GABA represented by the light blue background (B) The top trace shows a typical phasic response of synaptic $GABA_A$ receptors, which activate and deactivate rapidly as synaptic GABA fluctuates. The trace on the bottom demonstrates that tonic inhibitory currents can be significant, but are only visible upon the application of the GABA antagonist SR-95531. Part B adapted by permission from Macmillan Publishers Ltd: Nature Reviews, Farrant and Nusser, 2005, copyright 2005.

MKKSRGLSDYLWAWTLILSTLSGRSYGQPSQDELKDNTTVFTRILDRLLDGYDNR
1

LRPGLGERVTEVKTDIFVTSFGPVSDHDMEYTIDVFFRQSWKDERLKFKGPMTVL
56 *Loop D*

RLNNLMASKIWTPDTFFHNGKKSVAHNMTMPNKLLRITEDGTLLYTMRLTVRAEC
111 *Loop A* *Loop E*

PMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTREPARSVVVAEDGSRLNQYDLL
166 *Cys-loop* *Loop B* *Loop F*

GQTVDSGIVQSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFWLNR
221 *Loop C* *M1 helix*

ESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEF
276 *M2 helix* *M2-M3 linker* *M3 helix*

ATVNYFTKRGYAWDGKSVVPEKPKKVKDPLIKKNNTYAPTATSYTPNLARGDPGL
331

ATIAKSATIEPKEVKPETKPPEPKKTFNSVSKIDRLSRIAFPLLFGIFNLVYWAT
386 *MA helix* *M4 helix*

YLNREPQLKAPTPHQ
441

Figure 1-2. Primary sequence of the $GABA_A$ receptor α_1 subunit.

Sequence is that from *Rattus norvegicus* (NP_034380.1). Major structural features are underlined and labelled. The numbers at the start of each line refer to the position of the first amino acid residue in the sequence.

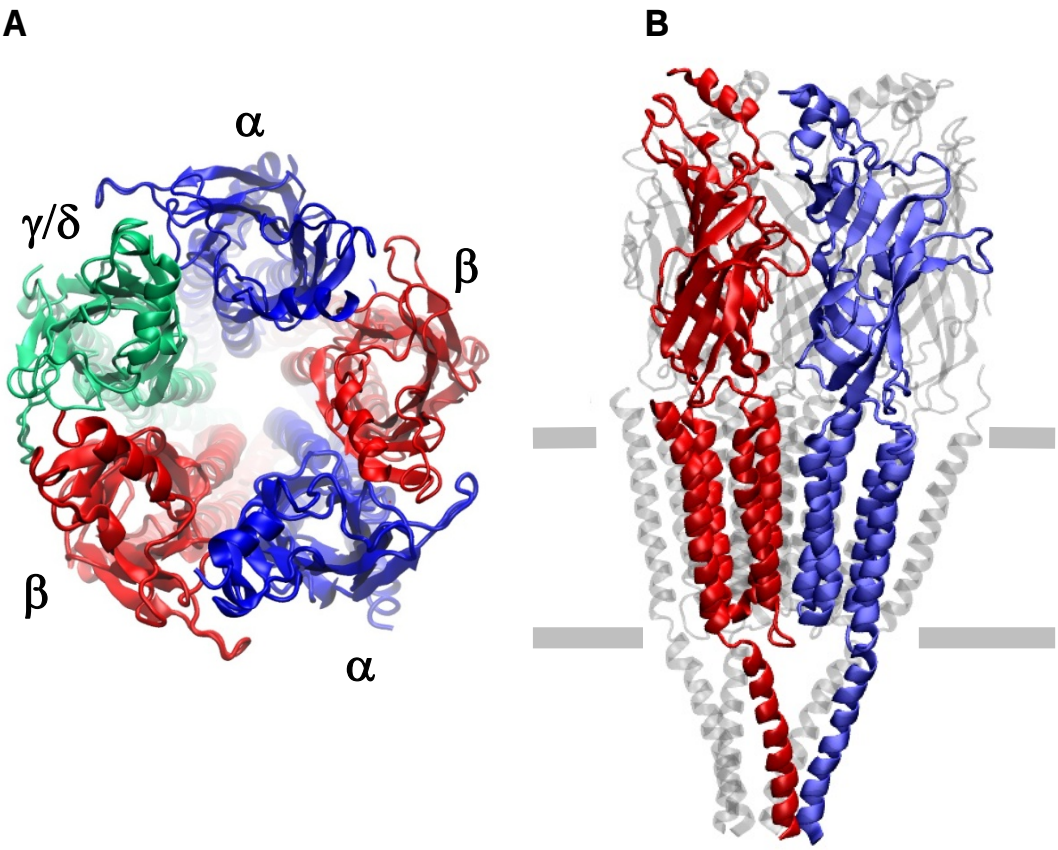


Figure 1-3. Ribbon diagram showing the general structural features of the Cys-loop receptors. The model shown is that of the nACh receptor obtained by cryo-electron microscopy of tissues from *Torpedo marmorata* (Unwin, 2005; pbd 2BG9). (A) The pentameric structure of the receptor when viewed from above the membrane looking down. The subunits have been coloured to represent the putative subunit arrangements for the $GABA_A$ receptor, with α in blue, β in red, and γ/δ in green. (B) The same receptor when viewed parallel to the membrane (shown by grey lines). The α subunit is coloured in blue and the β subunit in red.

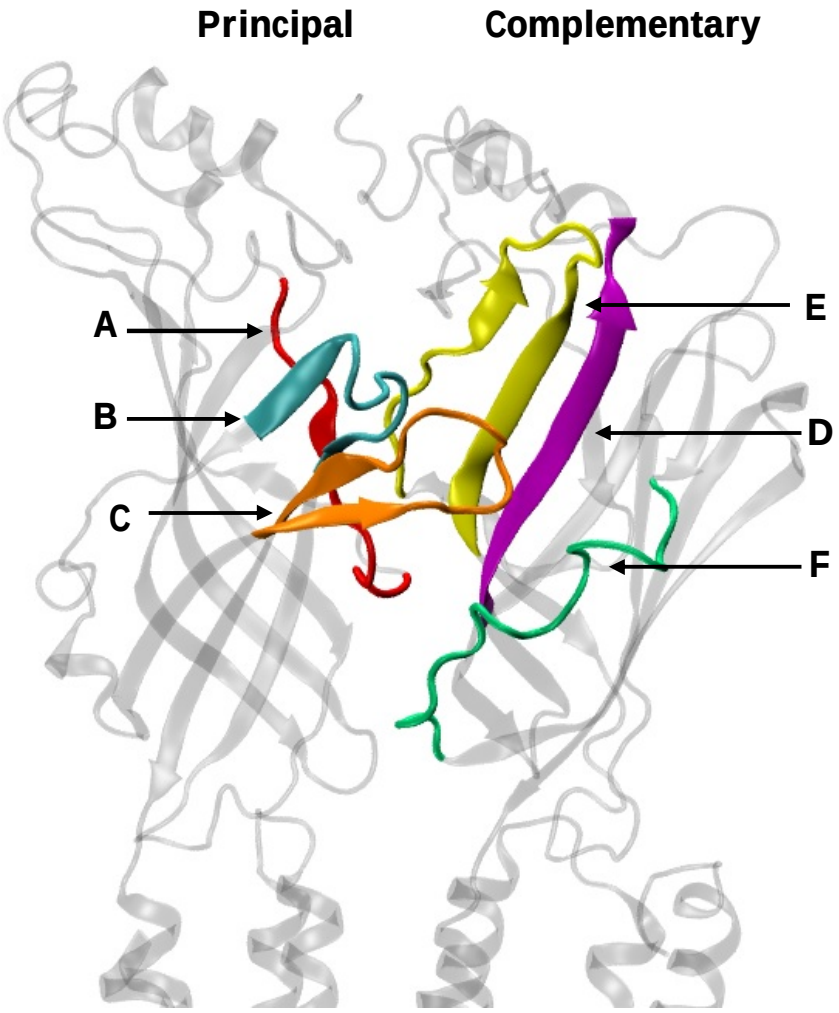


Figure 1-4. Structure of the agonist binding site in Cys-loop receptors.

The model displayed here is a homology model of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor based on the nACh receptor and AChBP structures (Mokrab et al., 2007). The principal subunit is on the left, and contributes loops A (red), B (cyan) and C (orange). The complementary subunit is on the right, which contributes loops D (purple), E (yellow), and F (green).

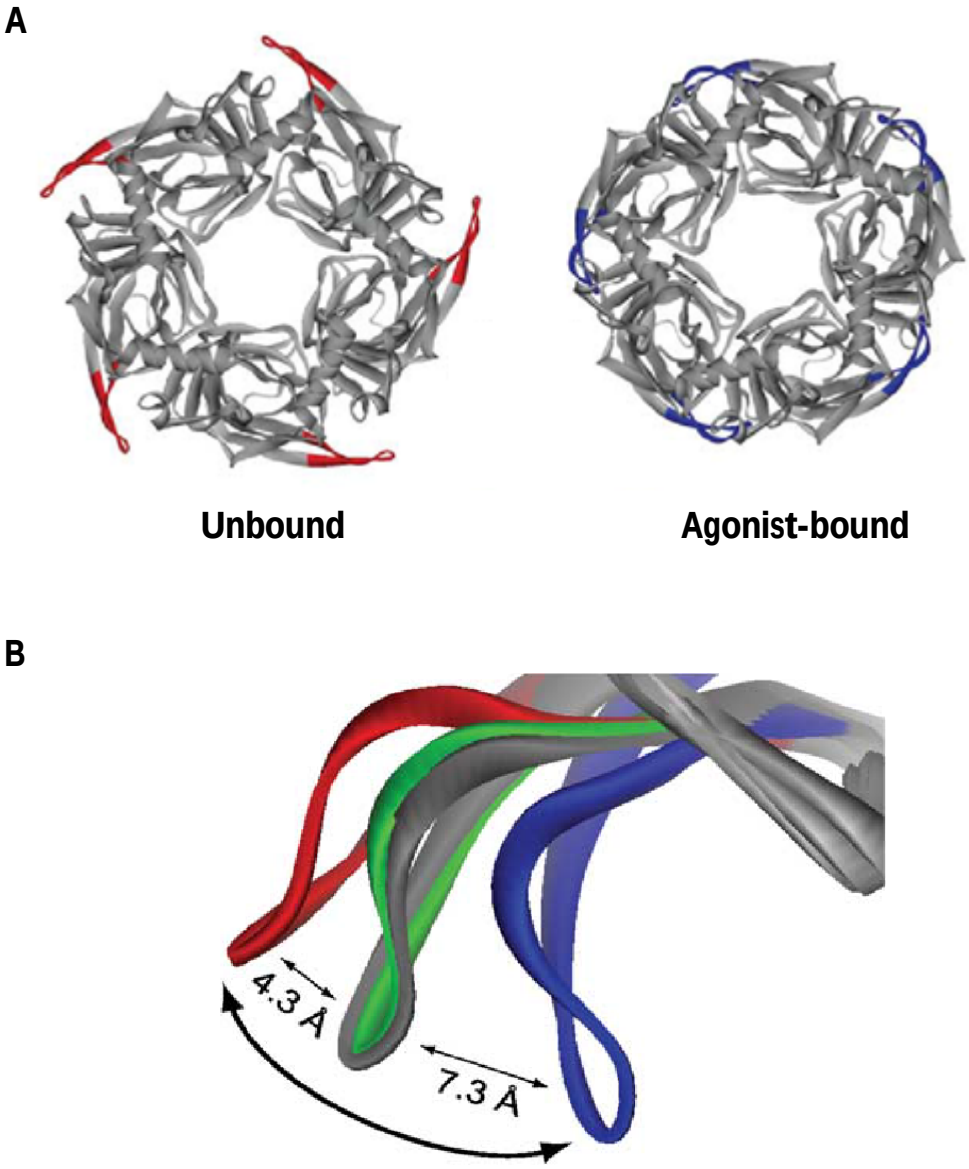


Figure 1-5. Movement of loop C in AChBP correlates with the efficacy of the bound compound. (A) Structure of the AChBP pentamer in the unbound and agonist-bound states. The loop C region has been colored to highlight its capping motion. (B) An overlay of the loop C conformations when bound by a large peptide antagonist (red), a small molecule antagonist (green), and an agonist (blue). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews, Hansen et al., 2005, copyright 2005.

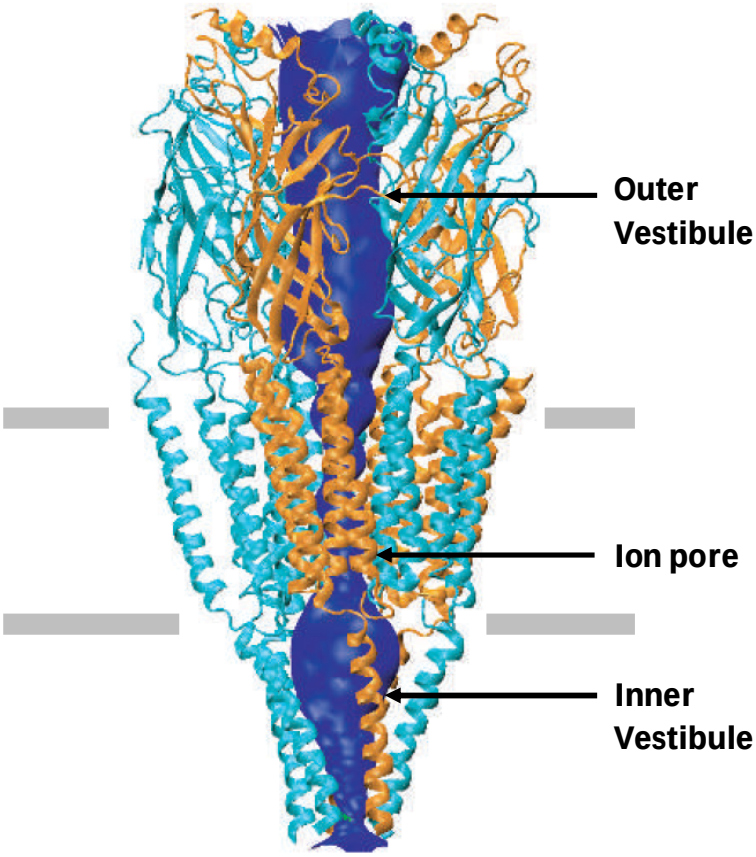


Figure 1-6. Structural model of the *Torpedo* nACh receptor model showing the ion translocation pore. The α subunits are coloured in orange, non-α subunits in pale blue and the outer surface of the ion channel is shown in dark blue. The approximate location of the membrane is shown with grey lines. The ion channel is widest at the inner and outer vestibules in the intracellular and extracellular domains, while the M2 helices cause the channel to constrict to a narrow pore. The ion pore is narrowest near the cytoplasmic end of the M2 helices. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews, Sine and Engel, 2006, copyright 2006.

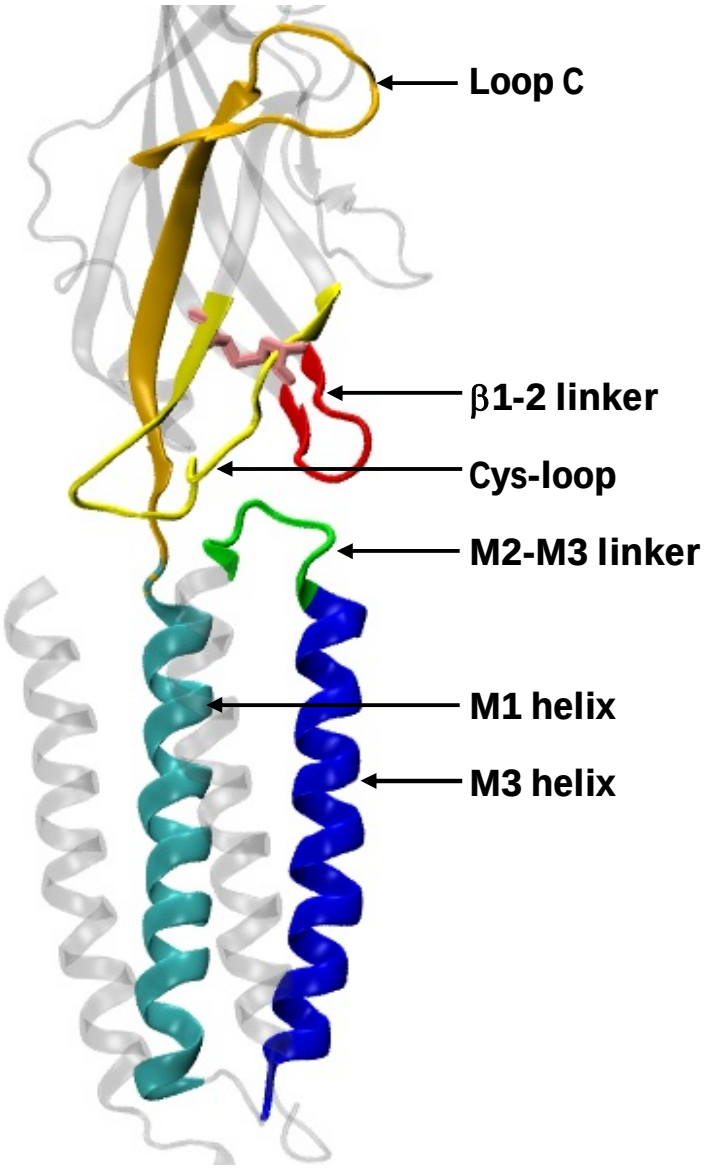
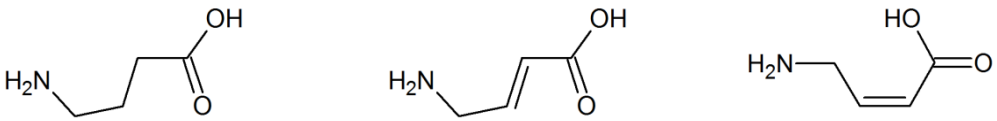
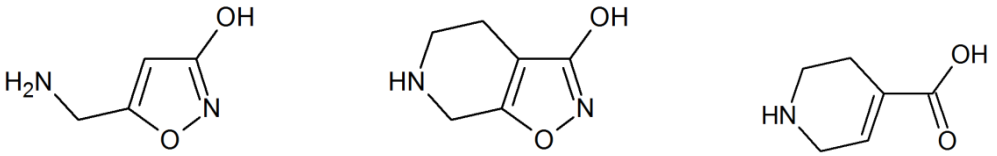


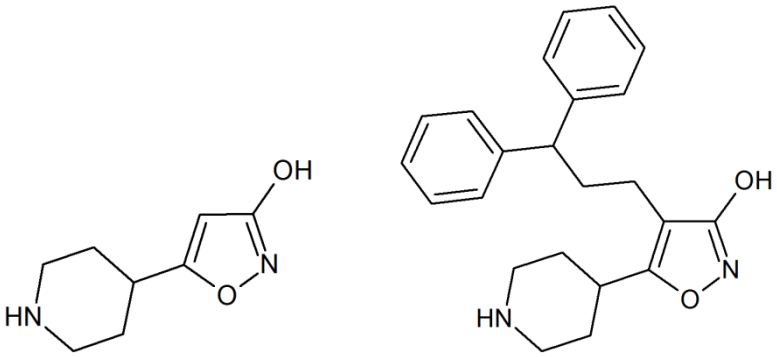
Figure 1-7. The extracellular/transmembrane domain interface in a model of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor. Loop C (orange) is connected directly to the M1 helix (cyan) through the β10 linker, while the Cys-loop (yellow) and β1-β2 loop (red) contact the M2-M3 linker (green) situated at the top of the M2 helix (blue). The disulfide bond in the Cys-loop is shown using a stick representation (pink). Model provided courtesy of Mokrab et al. (2007).



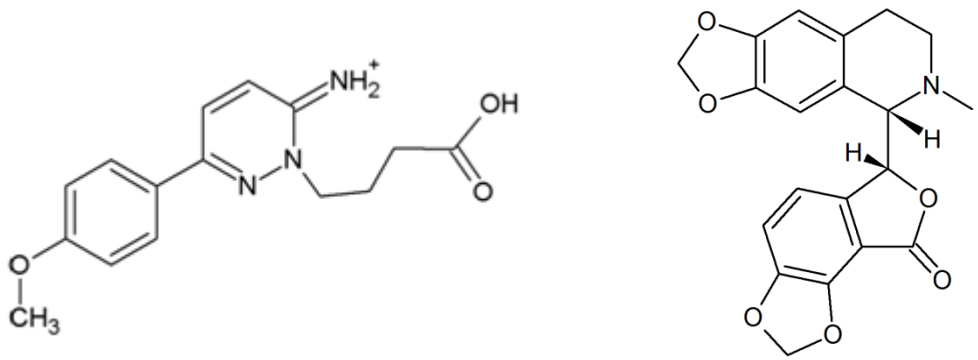
GABA **TACA** **CACA**



Muscimol **THIP** **Isoguvacine**



4-PIOL **4-(3-diphenylpropyl)-4-PIOL**



Gabazine (SR-95531) **Bicuculline**

Figure 1-8. Structures of GABA, muscimol, 4-PIOL and their analogues, as well as the classic antagonists gabazine and bicuculline.

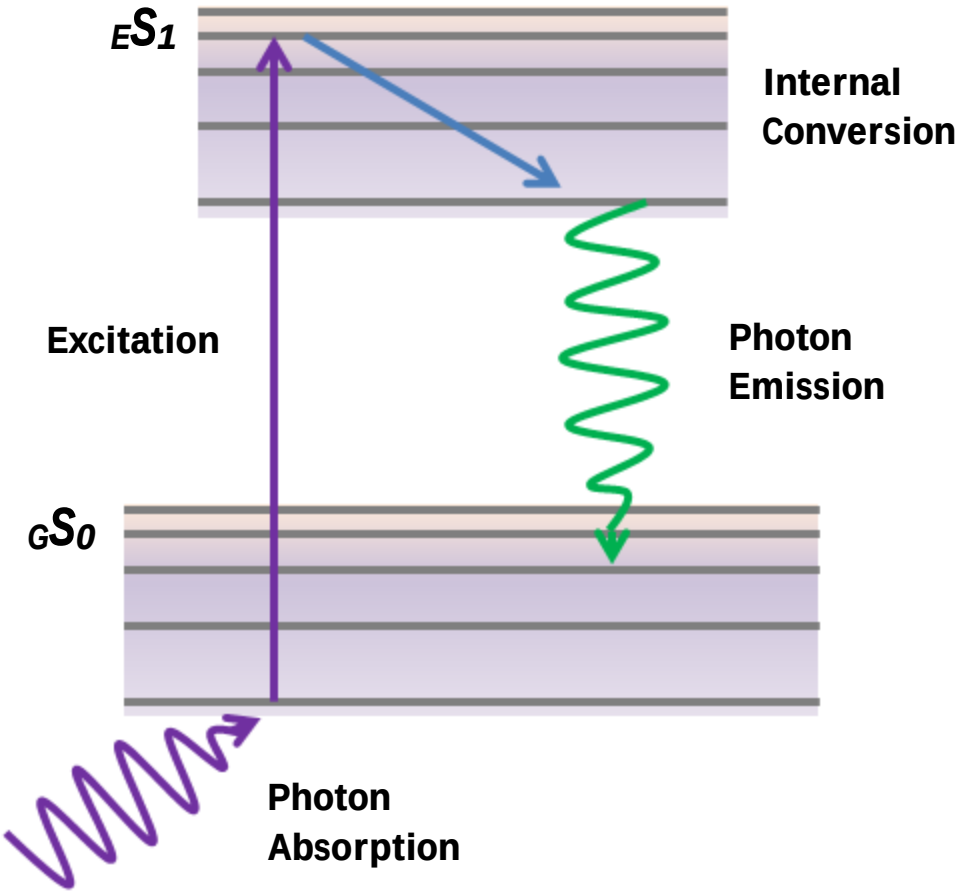


Figure 1-9. Jablonski diagram depicting the electron transitions involved in fluorescence. Absorption of a photon promotes a valence electron from the ground state ($_GS_0$) to an excited state ($_ES_1$). The electron relaxes to the lowest sub-level of energy in $_ES_1$, and then returns to $_GS_0$, resulting in the release of a photon with energy equal to the gap between $_ES_1$ and $_GS_0$. Note that Jablonski diagrams typically also include transitions involved in phosphorescence, which are excluded here for the purpose of clarity.

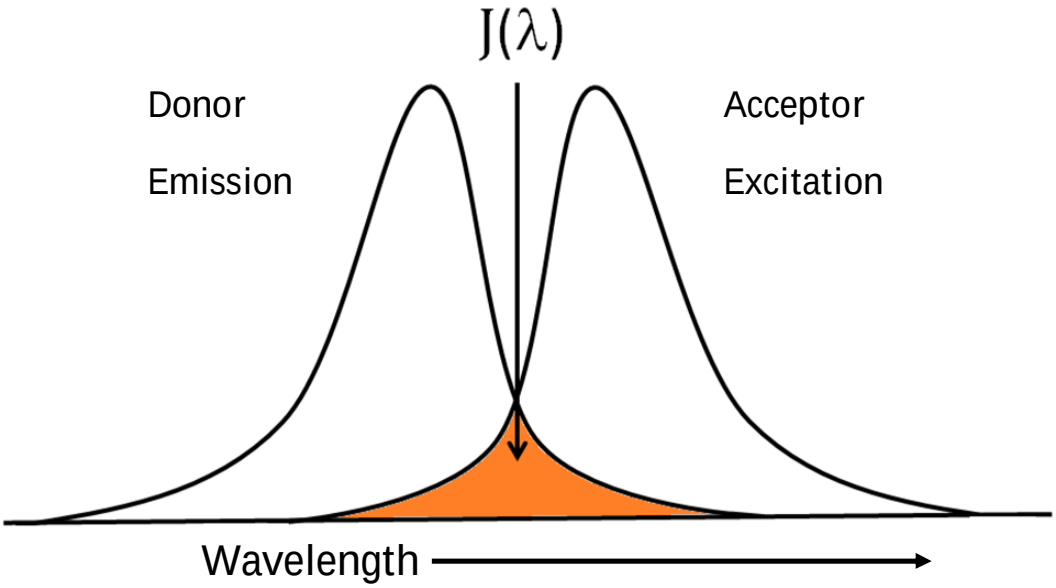


Figure 1-10. Emission and excitation curves of theoretical donor and acceptor fluorophores. The region of overlap between the donor emission and acceptor excitation wavelengths, denoted by J(λ), is a major determinant for the strength of the FRET interaction.

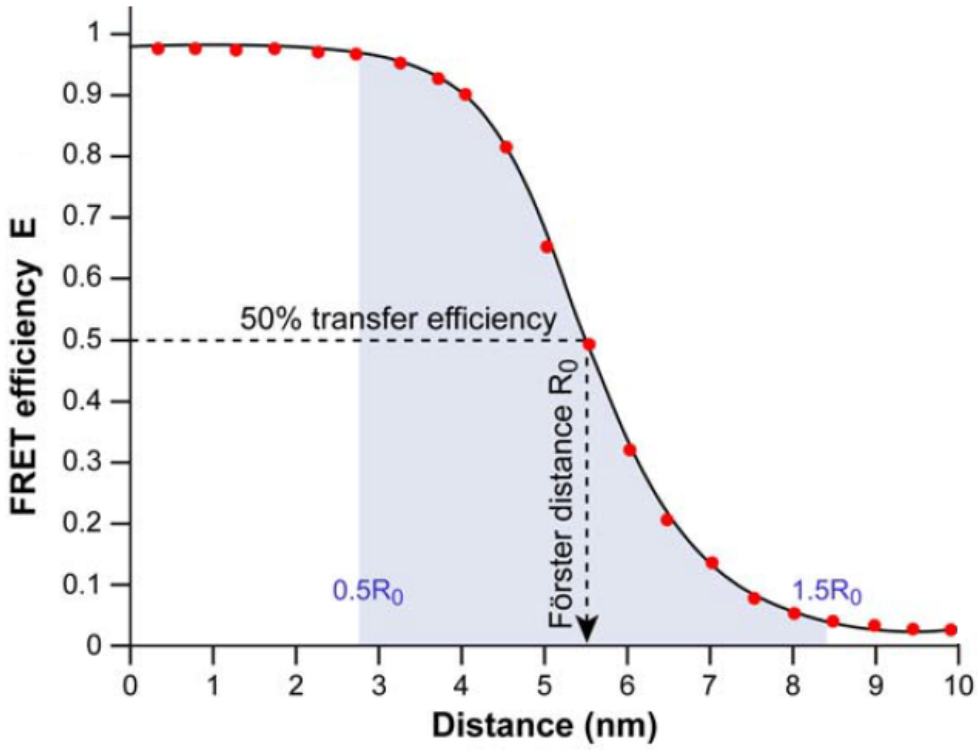


Figure 1-11. Graph of the distance-dependence relationship of FRET efficiency for two theoretical fluorophores. The theoretical FRET efficiency (E) is closest to 1 when there is minimal separation between the two fluorophores and drops off sharply as the distance is increased, approaching 0 around 10 nm (100 Å) distance. The dashed lines indicate the Förster radius, R_0, where the FRET efficiency is equal to 0.5. The values of $0.5R_0$ and $1.5R_0$ denote the range in which FRET can typically be measured experimentally. Adapted from Ishikawa-Ankerhold et al. (2012).

Table 1-1. Summary of subunit mRNA expression in the CNS. Data summarized from Wisden et al. (1992).

Subunit isoform	High subunit expression	Low subunit expression
Olfactory system	α_1 α_2 α_4 α_5 $\beta_{1\text{-}3}$ γ_2	α_3 γ_3 δ
Cortex	$\alpha_{1\text{-}4}$ $\beta_{2/3}$ γ_2 δ	α_5 γ_3 δ
Striatum	α_2 α_4 β_3	$\gamma_{2/3}$ δ
Pallidum	α_1 β_2 $\gamma_{1/2}$	α_2
Substantia nigra	α_1 β_2 θ	α_3 α_4 β_3 $\gamma_{1/2}$
Hippocampus	α_1 α_2 α_4 α_5 $\beta_{1\text{-}3}$ γ_2	γ_1 δ θ
Dentate gyrus	α_1 α_2 α_4 $\beta_{1\text{-}3}$ γ_2	α_3 α_5 $\gamma 1$ δ
Amygdala	α_1 α_2 $\beta_{2/3}$ $\gamma_{1/2}$	α_3 α_4 β_1 ε θ
Thalamus	α_1 α_4 β_2 δ	α_2 α_3 $\gamma_{2/3}$ ε
Subthalamic nucleus	α_1 β_2 γ_2 δ ε	
Hypothalamus	α_2 β_3 $\gamma_{1/2}$	α_1 α_3 α_5 β_1 δ θ
Cerebellum	α_1 α_6 $\beta_{2/3}$ $\gamma_{1/2}$ δ	
Retina	ρ_1 ρ_2 ρ_3	
Spinal cord	α_1 α_2 α_3 $\beta_{2\text{-}3}$ γ_2	

REFERENCES

Adkins CE, Pillai GV, Kerby J, Bonnert TP, Haldon C, McKernan RM, Gonzalez JE, Oades K, Whiting PJ and Simpson PB. (2001). $\alpha 4\beta 3\delta$ $GABA_A$ receptors characterized by fluorescence resonance energy transfer-derived measurements of membrane potential. *J. Biol. Chem.* **276**, 38934-38939.

Akabas MH, Kaufmann C, Archdeacon P and Karlin A. (1994). Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the α subunit. *Neuron* **13**, 919-927.

Amin J and Weiss DS. (1993). $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature* **366**, 565-569.

Angelotti TP, Uhler MD and Macdonald RL. (1993). Assembly of $GABA_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescence substrate/marker gene technique. *J. Neurosci.* **13**, 1418-1428.

Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SMJ and Edwardson JM. (2008). Atomic force microscopy reveals the stoichiometry and subunit arrangement of the $\alpha_4\beta_3\delta$ $GABA_A$ receptor. *Mol. Pharmacol.* **73,** 960-967.

Baumann SW, Baur R and Sigel E. (2001). Subunit arrangement of γ-aminobutyric acid type A receptors. *J. Biol. Chem.* **276**, 36275-36280.

Ben-Ari Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nature Rev. Neurosci.* **3**, 728-739.

Bencsits E, Ebert V, Tretter V and Sieghart W. (1999). A significant part of native γ-aminobutyric $acid_A$ receptors containing $\alpha 4$ subunits do not contain γ or δ subunits. *J. Biol. Chem.* **274**, 19613-19616.

Bera AK, Chatav M and Akabas MH. (2002). $GABA_A$ receptor M2-M3 loop secondary structure and changes in accessibility during channel gating. *J. Biol. Chem.* **45**, 43002-43010.

Bianchi MT and Macdonald RL. (2003). Neurosteroids shift partial agonist activation of $GABA_A$ receptor channels from low- to high-efficacy gating patterns. *J. Neurosci.* **23**, 10934-10943.

Black JW and Leff P. (1983). Operational models of pharmacological agonism. *Proc. Roy. Soc. Lond. B* **220**, 141-162.

Bloom FE and Iversen LL. (1971). Localizing ^{3}H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. *Nature* **229**, 628-630.

Boess FG, Beroukhim R and Martin IL. (1995). Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. *J. Neurochem.* **64**, 1401-1405.

Bohme I, Rabe H and Luddens H. (2004). Four amino acids in the α subunits determine the γ-aminobutyric acid sensitivities of $GABA_A$ receptor subunits. *J. Biol. Chem.***279**, 35193-35200.

Boileau AJ, Evers AR, Davis AF and Czajkowski C. (1999). Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. *J. Neurosci.* **19**, 4847-4854.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ and Connolly CN. (2003). $GABA_A$ receptor composition is determined by distinct assembly signals with α and β subunits. *J. Biol. Chem.* **278**, 4747-4755.

Bollan K, Baur R, Hales TG, Sigel E and Connolly CN. (2008). The promiscuous role of the epsilon subunit in $GABA_A$ receptor biogenesis. *Mol. Cell. Neurosci.* **37**, 610-621.

Bonnert TP, McKernan RM, Farrar S, le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJS, Brown N, Wafford KA and Whiting PJ. (1999). θ, a novel γ-aminobutyric acid type A receptor subunit. *PNAS* **96**, 9891-9896.

Boue-Grabot E, Roudbaraki M, Bascles L, Tramu G, Bloch B and Garret M. (1998). Expression of $GABA_A$ receptor ρ subunits in rat brain. *J. Neurochem.* **70**, 899-907.

Bouzat C, Gumilar F, Spitzmaul G, Wang H-L, Rayes D, Hansen SB, Taylor P and Sine SM. (2004). Coupling of agonist binding to channel gating in an ACh-binding protein linked to an ion channel. *Nature* **430**, 896-900.

Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J and Turnbull M. (1980). (-) Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. *Nature* **283**, 92-94.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI and Enna SJ. (2002). International union of pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. *Pharmacol. Rev.* **54**, 247-264.

Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van der Oost J, Smit AB and Sixma TK. (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411**, 269-276.

Brickley SG, Cull-candy SG and Farrant M. (1996). Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. *J. Physiol.* **497.3**, 753-759.

Bright DP, Renzi M, Bartram J, McGee TP, MacKenzie G, Hosie AM, Farrant M and Brickley SG. (2011). Profound desensitization by ambient GABA limits activation of δ-containing GABAA receptors during spillover. *J. Neurosci.* **31**, 753-763.

Brown N, Kerby J, Bonnert TP, Whiting PJ and Wafford KA. (2002). Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br. J. Pharmacol.* **136**, 965-974.

Brunig I, Scotti E, Sidler C and Fritschy J-M. (2001). Intact sorting, targeting, and clustering of γ-aminobutyric acid receptor subtypes in hippocampal neurons in vitro. *J. Comp. Neurol.* **443**, 43-55.

Burzomato V, Beato M, Groot-Kormelink PJ, Colquhoun D and Sivilotti LG. (2004). Single-channel behavior of heteromeric $\alpha 1\beta$ glycine receptors: an attempt to detect a conformational change before the channel opens. *J. Neurosci.* **24**, 10924-10940.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF and Orser BA. (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *PNAS* **101**, 3662-3667.

Celie PHN, van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB and Sixma TK. (2004). Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP structures. *Neuron* **41**, 907-914.

Celie PHN, Kasheverov IE, Mordvinstev DY, Hogg RC, van Nierop P, van Elk R, van Rossum-Fikkert SE, Zhmak MN, Bertrand D, Tsetlin V, Sixma TK and Smit AB. (2005). Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an α-conotoxin PnIA variant. *Nature Struct.Mol. Biol.* **7**, 582-588.

Chakrapani S, Bailey TD and Auerbach A. (2004). Gating dynamics of the acetylcholine receptor extracellular domain. *J. Gen. Physiol.* **123**, 341-356.

Chang Y and Weiss DS. (1998). Substitutions of the highly conserved M2 leucine create spontaneously opening ρ1 γ-aminobutyric acid receptors. *Mol. Pharmacol.* **53**, 511-523.

Chang Y and Weiss DS. (1999). Channel opening locks agonist onto the $GABA_C$ receptor. *Nature Neurosci.* **2**, 219-225.

Choi D-S, Wei W, Deitchman JK, Kharazia VN, Lesscher HMB, McMahon T, Wang D, Qi Z-H, Sieghert W, Zhang C, Shokat KM, Mody I and Messing RO. (2008). Protein kinase Cδ regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. *J. Neurosci.* **28,** 11890-11899.

Chothia C and Lesk AM. (1986). The relation between the divergence of sequence and structure in proteins. *EMBO J* **5**, 823-826.

Clegg RM, Murchie AIH, Zechel A and Lilley DMJ. (1993). Observing the helical geometry of double-stranded DNA in solution by fluorescence resonance energy transfer. *PNAS* **90**, 2994-2998.

Clements JD. (1996). Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *TiNS* **19**, 163-171.

Colquhoun D. (1998). Binding, gating, affinity, and efficacy: the interpretation of structure-activity relationships for agonists and the effects of mutating receptors. *Br. J. Pharmacol.* **125**, 923-947.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG and Moss SJ. (1996). Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. *J. Biol. Chem.* **271**, 89-96.

Connor JX, Boileau AJ and Czajkowski C. (1998). A $GABA_A$ receptor α_1 subunit tagged with green fluorescent protein requires a β subunit for functional surface expression. *J. Biol. Chem.* **273**, 28906-28911.

Crestani F, Keist R, Fritschy J-M, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H and Rudolph U. (2002). Trace fear conditioning involves hippocampal α5 $GABA_A$ receptors. *PNAS* **99**, 8980-8985.

Cromer BA, Morton CJ and Parker MW. (2002). Anxiety over $GABA_A$ receptor structure relieved by AChBP. *TiBS* **27**, 280-287.

Cutting GR, Lu L, O'hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR and Kazazian HH. (1991). Cloning of the γ-aminobutyric acid (GABA) ρ1 cDNA: a GABA receptor subunit highly expressed in the retina. *PNAS* **88**, 2673-2677.

Davies PA, Kirkness EF and Hales TG. (1997a). Modulation by general anaesthetics of rat $GABA_A$ receptors comprised of α1β3 and β3 subunits expressed in human embryonic kidney 293 cells. *Br. J. Pharmacol.* **120**, 899-909.

Davies PA, Hanna MC, Hales TG and Kirkness EF. (1997b). Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. *Nature* **385**, 820-823.

Davies PA, Wang W, Hales TG and Kirkness EF. (2003). A novel class of ligand-gated ion channel is activated by Zn^{2+}. *J. Biol. Chem.* **278**, 712-717.

Ernst M, Brauchart D, Boresch S and Sieghart W. (2003). Comparative modeling of $GABA_A$ receptors: limits, insights, future developments. *Neuroscience* **119**, 933-943.

Ernst M, Bruckner S, Boresch S and Sieghart W. (2005). Comparative models of $GABA_A$ receptor extracellular and transmembrane domains: important insights in pharmacology and function. *Mol. Pharmacol.* **68**, 1291-1300.

Essrich C, Lorez M, Benson JA, Fritschy J-M and Luscher B. (1998). Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. *Nature Neurosci.* **1**, 563-571.

Farrant M and Nusser Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nature Rev.* **6**, 215-229.

Fatima-Shad K and Barry PH. (1993). Anion permeation in GABA- and glycine-gated channels of mammalian cultured hippocampal neurons. *Proc. Biol. Sci.* **253**, 69-75.

Feigenspan A, Wassle H and Bormann J. (1993). Pharmacology of GABA receptor Cl^- channels in rat retinal bipolar cells. *Nature* **361**, 159-162.

Feng H-J and Macdonald RL. (2004). Multiple actions of propofol an $\alpha\beta\gamma$ and $\alpha\beta\delta$ $GABA_A$ receptors. *Mol. Pharmacol.* **66**, 1517-1524.

Fiser A and Sali A. (2003). MODELLER: Generation and refinement of homology-based protein structure models. *Macromol. Crystallog. Pt. D* **374**, 461-491.

Fisher JL and Macdonald RL. (1997). Single channel properties of recombinant $GABA_A$ receptors containing γ2 or δ subtypes expressed with α1 and β3 subtypes in mouse L929 cells. *J. Phys.* **505**, 283-297.

Fritschy J-M and Mohler H. (1995). $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.* **359**, 154-194.

Galperin E, Verkhusha VV and Sorkin A. (2004). Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nature Methods* **1**, 209-217.

Galzi J-L, Revah F, Black D, Goeldner M, Hirth C and Changeux J-P. (1990). Identification of a novel amino acid alpha-tyrosine 93 within the cholinergic ligands-binding sites of the acetylcholine receptor by photoaffinity labeling. Additional evidence for a three-loop model of the cholinergic ligands-binding site. *J. Biol. Chem.* **25,** 10430-10437.

Galzi J-L, Devillers-Thiery A, Hussy N, Bertrand S, Changeux J-P and Bertrand D. (1992). Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. *Nature* **359**, 500-505.

Giraudat J, Dennis M, Heidmann T, Chang J-Y and Changeux J-P. (1986). Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: serine-262 of the δ subunit is labeled by [^{3}H]chlorpromazine. *PNAS* **83**, 2719-2723.

Glykys J, Peng Z, Changda D, Homanics GE, Houser CR and Mody I. (2007). A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nature Neurosci.* **10**, 40-48.

Goldschen-Ohm MP, Wagner DA and Jones MV. (2011). Three arginines in the $GABA_A$ receptor binding pocket have distinct roles in the formation and stability of agonist- versus antagonist-bound complexes. *Mol. Pharmacol.* **80**, 647-656.

Gorrie GH, Vallis Y, Stephenson A, Whitfield J, Browning B, Smart TG and Moss SJ. (1997). Assembly of $GABA_A$ receptors composed of α1 and β2 subunits in both cultured neurons and fibroblasts. *J. Neurosci.* **17**, 6587-6596.

Grenningloh G, Rienitz A, Schmitt B, Methfessel C, Zensen M, Beyreuther K, Gundelfinger ED and Betz H. (1987a). The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. *Nature* **328**, 215-220.

Grenningloh G, Gundelfinger E, Schmitt B, Betz H, Darlison MG, Barnard EA, Schofield PR and Seeburg PH. (1987b). Glycine vs $GABA_A$ receptors. *Nature* **330**, 25-26.

Grosman C, Zhou M and Auerbach A. (2000). Mapping the conformational wave of acetylcholine receptor channel gating. *Nature* **403**, 773-776.

Haas KF and Macdonald RL. (1999). $GABA_A$ receptor subunit $\gamma 2$ and δ subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. *J. Physiol.* **514.1**, 27-45.

Hadingham KL, Wingrove PB, Wafford KA, Bain C, Kemp JA, Palmer KJ, Wilson AW, Wilcox AS, Sikela JM, Ragan CI and Whiting PJ. (1993). Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44**, 1211-1218.

Hagiwara S, Kusano K and Saito S. (1960). Membrane changes in crayfish stretch receptor neuron during synaptic inhibition and under action of gamma-aminobutyric acid. *J. Neurophysiol.* **23**, 505-515.

Hales TG, Dunlop JI, Deep TZ, Carland JE, Kelley SP, Lambert JJ and Peters JA. (2006). Common determinants of single channel conductance within the large cytoplasmic loop of 5-hydroxytryptamine type 3 and $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J. Biol. Chem.* **281**, 8062-8071.

Hansen SB, Sulzenbacher G, Huxford T, Marchot P, Taylor P and Bourne Y. (2005). Structures of *Aplysia* AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* **24**, 3635-3646.

Hedblom E and Kirkness EF. (1997). A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. *J. Biol. Chem.* **272**, 15346-15350.

Hibbs RE, Sulzenbacher G, Shi J, Talley TT, Conrod S, Kem WR, Taylor P, Marchot P and Bourne Y. (2009). Structural determinants for interaction of partial agonists with acetylcholine binding protein and neuronal $\alpha 7$ nicotinic acetylcholine receptor. *EMBO J* **28**, 3040-3051.

Hibbs RE and Gouaux E. (2011). Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* **474**, 54-62.

Hill DR and Bowery NG. (1981). ^{3}H-baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. *Nature* **290**, 149-152.

Holden JH and Czajkowski C. (2002). Different residues in the $GABA_A$ receptor $\alpha 1$T60-$\alpha 1$K70 region mediate GABA and SR-95531 actions. *J. Biol. Chem.* **277**, 18785-18792.

Houston CM, He Q and Smart TG. (2009). CaMKII phosphorylation of the $GABA_A$ receptor: receptor subtype- and synapse-specific modulation. *J. Physiol.* **587.10**, 2115-2125.

Imoto K, Methfessel C, Sakmann B, Mishina M, Mori Y, Konno T, Fukuda K, Kurasaki M, Bujo H, Fujita Y and Numa S. (1986). Location of a δ-subunit region determining ion transport through the acetylcholine receptor channel. *Nature* **324**, 670-674.

Imoto K, Busch C, Sakmann B, Mishina M, Konno T, Nakai J, Bujo H, Mori Y, Fukuda K and Numa S. (1988). Rings of negatively charged amino acids determine the acetylcholine channel conductance. *Nature* **335**, 645-648.

Ishikawa-Ankerhold HC, Ankerhold R and Drummen GPC. (2012). Advanced fluorescence microscopy techniques – FRAP, FLIP, FLAP, FRET and FLIM. *Molecules* **17**, 4047-4132.

Jansen M, Bali M and Akabas MH. (2008). Modular design of Cys-loop ligand-gated ion channels: functional 5-HT_3 and GABA ρ1 receptors lacking the large cytoplasmic M3M4 loop. *J. Gen. Physiol.* **131**, 137-146.

Jensen ML, Pedersen LN, Timmermann DB, Schousboe A and Ahring PK. (2005). Mutational studies using a cation-conducting $GABA_A$ receptor reveal the selectivity determinants of the Cys-loop family of ligand-gated ion channels. *J. Neurochem.* **92**, 962-972.

Johnston GAR, Curtis DR, Beart PM, Game CJA, McCullouch RM and Twitchin B. (1975). Cis- and trans-4-aminocrotonic acid as GABA analogs of restricted conformation. *J. Neurochem.* **24**, 157-160.

Jones MV, Jonas P, Sahara Y and Westbrook GL. (2001). Microscopic kinetics and energetics distinguish $GABA_A$ receptor agonists from antagonists. *Biophysical J.* **81**, 2660-2670.

Kash TL, Jenkins A, Kelley JC, Trudell JR and Harrison NL. (2003). Coupling of agonist binding to channel gating in the $GABA_A$ receptor. *Nature* **421**, 272-275.

Kash TL, Dizon M-J.F, Trudell JR and Harrison NL. (2004). Charged residues in the β2 subunit involved in $GABA_A$ receptor activation. *J. Biol. Chem.* **279**, 4887-4893.

Kaur KH, Baur R and Sigel E. (2009). Unanticipated structural and functional properties of δ-subunit-containing $GABA_A$ receptors. *J. Biol. Chem.* **284**, 7889-7896.

Kelley SP, Dunlop JI, Kirkness EF, Lambert JJ and Peters JA. (2003). A cytoplasmic region determines single-channel conductance in $5\text{-}HT_3$ receptors. *Nature* **424**, 321-324.

Kenakin T. (2004). Principles: receptor theory in pharmacology. *TiPS* **25**, 186-192.

Keramidas A, Moorhouse AJ, Schofield PR and Barry PH. (2004). Ligand-gated ion channels: mechanisms underlying ion selectivity. *Prog. Biophys. Mol. Biol.* **86**, 161-204.

Keramidas A and Harrison NL. (2008). Agonist-dependent single channel current and gating in $\alpha_4\beta_2\delta$ and $\alpha_1\beta_{2\gamma 2S}$ $GABA_A$ receptors. *J. Gen. Physiol.* **131**, 163-181.

Kittler JF, Rostaing P, Schiavo G, Fritschy J-M, Olsen R, Triller A and Moss SJ. (2001). The subcellular distribution of GABARAP and its ability to interact with NSF suggest a role for this protein in the intracellular transport of $GABA_A$ receptors. *Mol. Cell. Neurosci.* **18**, 13-25.

Kjaer M and Nielsen H. (1983). The analgesic effect of the GABA-agonist THIP in patients with chronic pain of malignant origin. A phase I-II study. *Br. J. Clin. Pharmacol.* **16**, 477-485.

Klausberger T, Fuchs K, Mayer B, Ehya N and Sieghart W. (2000). $GABA_A$ receptor assembly. *J. Biol. Chem.* **275**, 8921-8928.

Klausberger T, Sarto I, Ehya N, Fuchs K, Furtmuller R, Mayer B, Huck S and Sieghart W. (2001). Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. *J. Neurosci.* **21**, 9124-9133.

Kneussel M, Haverkamp S, Fuhrmann JC, Wang H, Wassle H, Olsen RW and Betz H. (2000). The γ-aminobutyric acid type A receptor ($GABA_AR$)-associated protein GABARAP interacts with gephyrin but is not involved in receptor anchoring at the synapse. *PNAS* **97**, 8594-8599.

Krishek BJ, Moss SJ and Smart TG. (1996). Homomeric β1 γ-aminobutyric acid$_A$ receptor-ion channels: evaluation of pharmacological and physiological properties. *Mol. Pharmacol.* **49**, 494-504.

Krogsgaard-Larsen P, Frolund B, Liljefors T and Ebert B. (2004). GABA$_A$ agonists and partial agonists: THIP (Gaboxadol) as a non-opioid analgesic and a novel type of hypnotic. *Biochem. Pharmacol.* **68**, 1573-1580.

Kubalek E, Ralston S, Lindstrom J and Unwin N. (1987). Location of subunits within the acetylcholine receptor by electron image analysis of tubular crystals from *Torpedo marmorata*. *J. Cell. Biol.* **105**, 9-18.

Kuffler SW and Edwards C. (1958). Mechanism of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. *J. Neurophysiol.* **21**, 589-610.

Kulik A, Nishimaru H and Ballanyi K. (2009). Role of bicarbonate and chloride in GABA- and glycine-induced depolarization and $[Ca^{2+}]_i$ rise in fetal rat motoneurons *in situ*. *J. Neurosci.* **20**, 7905-7913.

Kusama T, Spivak CE, Whiting P, Dawson VL, Schaeffer JC, Uhl GR. (1993). Pharmacology of GABA Rho-1 and GABA alpha/beta receptors expressed in *Xenopus* oocytes and Cos cells. *Br. J. Pharmacol.* **109**, 200-206.

Lam JCF and Li Y. (2010). Influence of a cleavage site on global folding of an RNA-cleaving DNAzyme. *Chembiochem* **11**, 1710-1719.

Langosch D, Thomas L and Betz H. (1988). Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer. *PNAS* **85**, 7394-7398.

Lape R, Colquhoun D and Sivilotti LG. (2008). On the nature of partial agonism in the nicotinic receptor superfamily. *Nature* **454**, 722-728.

Law RJ, Henchman RH and McCammon JA. (2005). A gating mechanism proposed from a simulation of a human α7 nicotinic acetylcholine receptor. *PNAS* **102**, 6813-6818.

Leil TA, Chen Z-W, Chang C-S.S and Olsen RW. (2004). $GABA_A$ receptor-associated protein traffics $GABA_A$ receptors to the plasma membrane in neurons. *J. Neurosci.* **24**, 11429-11438.

Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Kohler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG, Barnard EA and Seeburg PH. (1988). Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature* **335**, 76-79.

Lindstrom J, Schoepfer R and Whiting P. (1987). Molecular studies of the neuronal nicotinic acetylcholine receptor family. *Mol. Neurobiol.* **1**, 281-337.

Lopez-Chavez A, Miledi R and Martinez-Torres A. (2005). Cloning and functional expression of the bovine $GABA_C$ $\rho 2$ subunit. Molecular evidence of a widespread distribution in the CNS. *Neurosci. Res.* **53**, 421-427.

Lummis SCR, Beene DL, Lee LW, Lester HA, Broadhurst RW and Dougherty DA. (2005a). Cys-trans isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. *Nature* **438**, 248-252.

Lummis SCR, Beene DL, Harrison NJ, Lester HA and Doughert DA. (2005). A cation-π binding interaction with a tyrosine in the binding site of the $GABA_C$ receptor. *Chem. Biol.* **12**, 993-997.

Macdonald RL and Botzolakis EJ. (2009). $GABA_A$ receptor channels. In *Physiology and pathology of chloride transporters and channels in the nervous system First edition*. Alvarez-Leefmans, F., and Delpire, E. (Eds.). Academic Press, London, UK. 257-282.

Maconochie DJ, Zempel JM and Steinbach JH. (1994). How quickly can $GABA_A$ receptors open? *Neuron* **12**, 61-71.

Mann EO and Paulsen O. (2007). Role of GABAergic inhibition in hippocampal network oscillations. *TiNS* **30**, 343-349.

Mann EO and Mody I. (2010). Control of hippocampal gamma oscillation frequency by tonic inhibition and excitation of interneurons. *Nature Neurosci.* **13**, 205-213.

Maurel D, Kniazeff J, Mathis G, Trinquet E, Pin J-P and Ansanay H. (2004). Cell surface detection of membrane protein interaction with homogeneous time-resolved fluorescence resonance energy transfer technology. *Anal. Biochem*. **329**, 253-262.

Maricq AV, Peterson AS, Brake AJ, Myers RM and Julius D. (1991). Primary structure and functional expression of the $5HT_3$ receptor, a serotonin-gated ion channel. *Science* **254**, 432-437.

McKernan RM and Whiting PJ. (1996). Which $GABA_A$-receptor subtypes really occur in the brain? *TiNS* **19**, 139-143.

Meera P, Wallner M and Otis TS. (2011). Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic $GABA_A$ receptors. *J. Neurophysiol.* **106**, 2057-2064.

Melis C, Lummis SCR and Molteni C. (2008). Molecular dynamics simulations of GABA binding to the $GABA_C$ receptor: the role of Arg104. *Biophysical J.* **95**, 4115-4123.

Miller C. (1989). Genetic manipulation of ion channels: a new approach to structure and mechanism. *Neuron* **2**, 1195-1205.

Miyake A, Mochizuki S, Takemoto Y and Akuzawa S. (1995). Molecular cloning of 5-hydroxytryptamine3 receptor: heteregeneity in distribution and function among species. *Mol. Pharmacol.* **48**, 407-416.

Miyazawa A, Fujiyoshi Y and Unwin N. (2003). Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423**, 949-955.

Mokrab Y, Bavro VN, Mizuguchi K, Todorov NP, Martin IL, Dunn SMJ, Chan SL and Chau P-L. (2007). Exploring ligand recognition and ion flow in comparative models of the human GABA type A receptor. *J. Mol. Graphics and Modelling* **26**, 760-774.

Mortensen M, Frolund B, Jorgensen AT, Liljefors T, Krogsgaard-Larsen P and Ebert B. (2002). Activity of novel 4-PIOL analogues at human $\alpha1\beta2\gamma2S$ $GABA_A$ receptors – correlation with hydrophobicity. *Eur. J. Pharmacol*. **451**, 125-132.

Mortensen M, Wafford KA, Wingrove P and Ebert B. (2003). Pharmacology of $GABA_A$ receptors exhibiting different levels of spontaneous activity. *Eur. J. Pharmacol.* **476**, 17-24.

Mortensen M, Ebert B, Wafford K and Smart TG. (2010). Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. *J. Physiol.* **588**, 1251-1268.

Mortensen M, Patel B and Smart TG. (2012). GABA potency at $GABA_A$ receptors found in synaptic and extrasynaptic zones. *Front. Cell. Neurosci.* **6**, 1-10.

Mozrzymas JW. (2004). Dynamism of $GABA_A$ receptor activation shapes the "personality" of inhibitory synapses. *Neuropharmacology* **47**, 945-960.

Nayeem N, Green TP, Martin IL and Barnard EA. (1994). Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. *J. Neurochem.* **62**, 815-818.

Newell JG, Davies M, Bateson AN and Dunn SMJ. (2000). Tyrosine 62 of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. *J. Biol. Chem.* **275**, 14198-14204.

Nusser Z, Sieghart W and Somogyi P. (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J. Neurosci*. **18**, 1693-1703.

Nusser Z and Mody I. (2002). Selective modulation of tonic and phasic inhibition in dentate gyrus granule cells. *J. Neurophysiol*. **87**, 2624-2628.

Nymann-Andersen J, Wang H, Chen L, Kittler JT, Moss SJ and Olsen RW. (2002). Subunit specificity and interaction domain between $GABA_A$ receptor-associated protein (GABARAP) and $GABA_A$ receptors. *J. Neurochem.* **80**, 815-823.

Ogurusu T and Shingai R. (1996). Cloning of a putative γ-aminobutyric acid (GABA) receptor subunit ρ3 cDNA. *Biochimica et Biophysica Acta* **1305**, 15-18.

Olsen RW and Sieghart W. (2008). International union of pharmacology. LXX. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev.* **60**, 243-260.

Olsen RW and Tobin AJ. (1990). Molecular biology of GABA$_A$ receptors. *FASEB J* **4**, 1469-1480.

Padgett CL, Hanek AP, Lester HA, Dougherty DA and Lummis SCR. (2007). Unnatural amino acid mutagenesis of the GABA$_A$ receptor binding site residues reveals a novel cation-π interaction between GABA and β2Tyr97. *J. Neurosci.* **27**, 886-892.

Pan Y and Qian H. (2005). Interactions between ρ and γ$_2$ subunits of the GABA receptor. *J. Neurochem.* **94**, 482-490.

Pan Y, Ripps H and Qian H. (2006). Random assembly of GABA ρ1 and ρ2 subunits in the formation of heteromeric GABA$_C$ receptors. *Cell. Mol. Neurobiol.* **26**, 289-305.

Pavlov I, Savtchenko LP, Kullmann DM, Semyanov A and Walker MC. (2009). Outwardly rectifying tonically active GABA$_A$ receptors in pyramidal cells modulate neuronal offset, not gain. *J. Neurosci.* **29**, 15341-15350.

Payne JA, Stevenson TJ and Donaldson LF. (1996). Molecular characterization of a putative K-Cl cotransporter in rat brain. *J. Biol. Chem.* **271**, 16245-16252.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W and Sperk G. (2000). GABA$_A$ receptors: immunocytochemical distribution of 13 subunits in the adult brain. *Neuroscience* **101**, 815-850.

Poltl A, Hauer B, Fuchs K, Tretter V and Sieghert W. (2003). Subunit composition and quantitative importance of GABA$_A$ receptor subtypes in the cerebellum of mouse and rat. *J. Neurochem.* **87**, 1444-1455.

Purohit P, Mitra A and Auerbach A. (2007). A stepwise mechanism for acetylcholine receptor channel gating. *Nature* **446**, 930-933.

Purohit P and Auerbach A. (2009). Unliganded gating of acetylcholine receptor channels. *PNAS* **106**,

Quirk K, Whiting PJ, Ragan CI and McKernan RM. (1995). Characterisation of δ-subunit containing $GABA_A$ receptors from rat brain. *Eur. J. Pharmacol.* **290**, 175-181.

Rosas-Arellano A, Machuca-Parra AI, Reyes-Haro D, Miledi R and Martinez-Torres A. (2012). Expression of $GABA_\rho$ receptors in the neostriatum: localization in aspiny, medium spiny neurons and GFAP-positive cells. *J. Neurochem.* **122**, 900-910.

Rossi DJ and Hamann M. (1998). Spillover-mediated transmission at inhibitory synapses promoted by high affinity α6 subunit $GABA_A$ receptors and glomerular geometry. *Neuron* **20**, 783-795.

Roth T, Lines C, Vandormael K, Ceesay P, Anderson D and Snavely D. (2010). Effect of gaboxadol on patient-reported measures of sleep and waking function in patients with primary insomnia: results from two randomized, controlled, 3-month studies. *J. Clin. Sleep Med.* **6**, 30-39.

Sander T, Frolund B, Bruun AT, Ivanov I, McCammon JA and Balle T. (2011). New insights into the $GABA_A$ receptor structure and orthosteric ligand binding: receptor modeling guided by experimental data. *Proteins* **79**, 1458-1477.

Sarto I, Wabnegger L, Dogl E and Sieghart W. (2002). Homologous sites of $GABA_A$ receptor α_1, β_3 and γ_2 subunits are important for assembly. *Neuropharmacology* **43**, 482-491.

Sarto-Jackson I, Ramerstorfer J, Ernst M and Sieghart W. (2006). Identification of amino acid residues important for assembly of GABAA α_1 and γ_2 subunits. *J. Neurochem.* **96**, 983-995.

Sarto-Jackson I and Sieghart W. (2008). Assembly of $GABA_A$ receptors (review). *Mol. Mem. Biol.* **25**, 302-310.

Saxena NC and Macdonald RL. (1996). Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. *Mol. Pharmacol.* **49**, 567-579.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Real V, Glencorse TA, Seeburg PH and Barnard EA. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* **328**, 221-227.

Schuler B, Lipman EA and Eaton WA. (2002). Probing the free-energy surface for protein folding with single-molecule fluorescenc spectroscopy. *Nature* **419**, 743-749.

Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W and Sperk G. (2001). Distribution of the major γ-aminobutyric acid$_A$ receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. *J. Comp. Neurol.* 433, 526-549.

Schwede T, Kopp J, Guex N and Peitsch MC. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* **31**, 3381-3385.

Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy J-M, Mohler H and Luscher B. (2003). The γ2 subunit of GABA$_A$ receptors is required for maintenance of receptors at mature synapses. *Mol. Cell. Neurosci.* **24**, 442-450.

Sejnowski TJ and Paulsen O. (2006). Network oscillations: emerging computational principles. *J. Neurosci.* **26**, 1673-1676.

Shi J, Koeppe JR, Komives EA and Taylor P. (2006). Ligand-induced conformational changes in the acetylcholine-binding protein analyzed by hydrogen-deuterium exchange mass spectrometry. *J. Biol. Chem.* **281**, 12170-12177.

Shingai R, Yanagi K, Fukushima T, Sakata K and Ogurusu T. (1996). Functional expression of GABA ρ3 receptors in *Xenopus* oocytes. *Neurosci. Res.* **26**, 387-390.

Sieghart W and Sperk G. (2002). Subunit composition, distrubution and function of GABA$_A$ receptor subtypes. *Curr. Top. Med. Chem.* **2**, 795-816.

Sigel E, Baur R, Trube G, Mohler H and Malherbe P. (1990). The effect of subunit composition of rat brain GABA$_A$ receptors on channel function. *Neuron* **5**, 703-711.

Sigel E, Baur R, Kellenberger S and Malherbe P. (1992). Point mutations affecting antagonist affinity and agonist dependent gating of GABA$_A$ receptor channels. *EMBO J* **11**, 2017-2023.

Simon J, Wakimoto H, Fujita N, Lalande M and Barnard EA. (2004). Analysis of the set of $GABA_A$ receptor genes in the human genome. *J. Biol. Chem.* **279**, 41422-41435.

Sine SM and Engel AG. (2006). Recent advances in Cys-loop receptor structure and function. *Nature* **440**, 448-455.

Smit AB, Syed NI, Schaap D, van Minnen J, Klumperman J, Kits KS, Lodder H, van der Schors RC, van Elk R, Sorgedrager B, Brejc K, Sixma TK and Garaerts WPM. (2001). A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature* **411**, 261-268.

Smith GB and Olsen RW. (1994). Identification of a [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric $acid_A$ receptor α subunit. *J. Biol. Chem.* **269**, 20380-20387.

Sobel A, Weber M and Changeux J-P. (1977). Large-scale purification of the acetylcholine-receptor protein in its membrane-bound and detergent-extracted forms from *Torpedo marmorata* electric organ. *Eur. J. Biochem.* **80**, 215-224.

Storustovu S and Ebert B. (2006). Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ2. *JPET* **316**, 1351-1359.

Stroud RM, McCarthy MP and Shuster M. (1990). Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry* **29**, 11009-11023.

Sunesan M, de Carvalho LP, Dufresne V, Grailhe R, Savatier-Duclert N, Gibor G, Peretz A, Attali B, Changeux J-P and Paas Y. (2006). Mechanism of Cl^- selection by a glutamate-gated chloride (GluCl) receptor revealed through mutations in the selectivity filter. *J. Biol. Chem.* **281**, 14875-14881.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR and McKernan RM. (1999). Preferential coassembly of α4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. *Mol. Pharmacol.* **56**, 110-115.

Tang X, Hernandez CC and Macdonald RL. (2010). Modulation of spontaneous and GABA-evoked tonic $\alpha4\beta3\delta$ and $\alpha4\beta3\gamma2L$ GABAA receptor currents by protein kinase A. *J. Neurophysiol.* **103**, 1007-1019.

Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG and Moss SJ. (1999). Identification of amino acid residues within $GABA_A$ receptor β subunits that mediate both homomeric and heteromeric receptor expression. *J. Neurosci.* **19**, 6360-6371.

Taylor PM, Connolly CN, Kittler JT, Gorrie GH, Hosie A, Smart TG and Moss SJ. (2000). Identification of residues within $GABA_A$ receptor α subunits that mediate specific assembly with receptor β subunits. *J. Neurosci.* **20**, 1297-1306.

Terunuma M, Jang I-S, Ha SH, Kittler JF, Kanematsu T, Jovanovic JN, Nakayama KI, Akaike N, Ryu SH, Moss SJ and Hirata M. (2004). $GABA_A$ receptor phospho-dependent modulation is regulated by phospholipase C-related inactive protein type 1, a novel protein phosphatase 1 anchoring protein. *J. Neurosci.* **24**, 7074-7084.

Toyoshima C and Unwin N. (1988). Ion channel of acetylcholine receptor reconstructed from images of postsynaptic membranes. *Nature* **336**, 247-250.

Tretter V, Ehya N, Fuchs K and Sieghart W. (1997). Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. *J. Neurosci.* **17**, 2728-2737.

Trudell JR. (2002). Unique assignment of inter-subunit association in $GABA_A$ $\alpha1\beta3\gamma2$ receptor determined by molecular modeling. *Biochimica et Biophysica Acta* **1565**, 91-96.

Unwin N. (1993). Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* **229**, 1101-1124.

Unwin N. (1995). Acetylcholine receptor channel imaged in the open state. *Nature* **373**, 37-43.

Unwin N, Miyazawa A, Li J and Fujiyoshi Y. (2002). Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the α subunits. *J. Mol. Biol.* **319**, 1165-1176.

Unwin N. (2005). Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. *J. Mol. Biol.* **346**, 967-989.

Wagner DA, Czajkowski C and Jones MV. (2004). An arginine involved in GABA binding and unbinding but not gating of the $GABA_A$ receptor. *J. Neurosci.* **24**, 2733-2741.

Wang T-L, Guggino WB and Cutting GR. (1994). A novel γ-aminobutyric acid receptor subunit (ρ2) cloned from human retina forms bicuculline-insensitive homooligomeric receptors in *Xenopus* oocytes. *J. Neurosci.* **14**, 6524-6531.

Wang H, Bedford FK, Brandon NJ, Moss SJ and Olsen RW. (1999a). $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. *Nature* **397**, 69-72.

Wang C-T, Zhang H-G, Rocheleau T, Ffrench-Constant RH and Jackson MB. (1999). Cation permeability and cation-anion interactions in a mutant GABA-gated chloride channel from *Drosophila*. *Biophysics J.* **77**, 691-700.

Watanabe M, Sakuma Y and Kato M. (2009). $GABA_A$ receptors mediate excitation in adult rat GnRH neurons. Biol. Reprod. **81**, 327-332.

Watrob HM, Pan C-P and Barkley MD. (2003). Two-step FRET as a structural tool. *J. Am. Chem. Soc.* **125**, 7336-7343.

Wei W, Zhang N, Peng Z, Houser CR and Mody I. (2003). Perisynaptic localization of δ subunit-containing $GABA_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. *J. Neurosci.* **23**, 10650-10661.

Whiting PJ. (2003). GABA-A receptor subtypes in the brain: a paradigm for drug discovery? *Drug Discovery Today* **8**, 445-450.

Wisden W, Laurie DJ, Monyer H and Seeburg PH. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesenchephalon. *J. Neurosci.* **12**, 1040-1062.

Xiu X, Hanek AP, Wang J, Lester HA and Dougherty DA. (2005). A unified view of the role of electrostatic interactions in modulating the gating of Cys-loop receptors. *J. Biol. Chem.* **280**, 41655-41666.

Ymer S, Draguhn A, Kohler M, Schofield PR and Seeburg PH. (1989a). Sequence and expression of a novel $GABA_A$ receptor α subunit. *FEBS Lett.* **258**, 119-122.

Yoon B-E, Woo J and Lee CJ. (2012). Astrocytes as GABA-ergic and GABA-ceptive cells. *Neurochem. Res.* **37**, 2474-2479.

You H and Dunn SMJ. (2007). Identification of a domain in the δ subunit (S238-V264) of the $\alpha4\beta3\delta$ $GABA_A$ receptor that confers high agonist sensitivity. *J. Neurochem.* **103**, 1092-1101.

CHAPTER 2

Identification of Structural Determinants that Influence Agonist Activity at $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ Receptor Subtypes

INTRODUCTION

$GABA_A$ receptors have been shown to produce one of two distinct modes of neurotransmission, termed phasic and tonic inhibition, depending on their subunit composition (Mody, 2001; Farrant and Nusser, 2005). Phasic inhibition is mediated by receptors containing the γ_2 subunit, which allows receptors to cluster at the synapse and respond to the rapidly fluctuating GABA concentration elicited by synaptic GABA release and re-uptake (Clements, 1996; Essrich et al., 1998; Schweizer et al., 2003). The most common γ_2-containing subtype is the $\alpha_1\beta_2\gamma_2$ receptor, which is estimated to constitute approximately 40% of all $GABA_A$ receptors in the CNS (Mckernan and Whiting, 1996; Whiting, 2003). In contrast, tonic inhibition is associated with the δ subunit, which is localized exclusively to peri- or extrasynaptic compartments of the membrane, where GABA concentrations are in the low micromolar range (Nusser et al., 1998; Wei et al., 2003; Cope et al., 2005; Jia et al., 2005; Bright et al., 2011). The δ subunit is believed to assemble with the α_1, α_4, and α_6 subunits to form $\alpha_1\beta_x\delta$, $\alpha_4\beta_x\delta$ and $\alpha_6\beta_3\delta$ receptors (Sur et al., 1999; Tretter et al., 2001; Glykys et al., 2007). Receptors containing the δ subunit generally show high agonist affinity (Brown et al., 2002; Storustovu and Ebert 2006; Mortensen et al., 2010), slow desensitization (Saxena and Macdonald, 1994; Haas and Macdonald, 1999), and low efficacy gating patterns (Bianchi and Macdonald, 2003), which is consistent with a role in producing persistent inhibitory currents.

Since δ-containing receptors have a unique functional role in neuronal signalling, they may be useful for the development of novel therapeutics through the use of subtype-selective drugs. Extrasynaptic receptors have been identified as key targets for anaesthetics, hypnotics, neurosteroids, and alcohol, and may underlie conditions such as mood disorders, schizophrenia, epilepsy, and Parkinson's disease (Brickley and Mody, 2012). Adding to their appeal as a drug target is the fact that δ-containing receptors exhibit distinct pharmacological properties, particularly with respect to γ_2-containing receptors (Brown et al., 2002; Storustovu and Ebert, 2006; Wafford et al., 2009; Mortensen et al., 2010). One agonist of particular interest is THIP, due to its increased potency and efficacy at δ-containing receptors; acting as a partial agonist at γ_2-containing receptors and a "super" agonist at δ-containing receptors (Storustovu and Ebert, 2006; Mortensen et al., 2010).

At present, it is not clear whether THIP binds to the same site as GABA, but the structural similarity of the two suggests that this might be the case. THIP consists of a 3-hydroxyl-substituted isoxazole ring fused with a tetrahydropyridine ring. The functional groups of THIP mimic those found on GABA - namely an acidic oxygen and basic amine (pKa 4.4 and 8.5, respectively, in THIP versus 4 and 10.5 for GABA) separated by four carbon atoms - but fixes the geometry of these groups due to limited conformational flexibility of the fused ring structure (Krishek et al., 1996; Brehm et al., 1997). Evidence that the binding sites for GABA and THIP at least partially overlap has been provided by the fact that thio-THIP, which substitutes the isoxazole

oxygen with sulphur, acts as a competitive antagonist of GABA at spinal $GABA_A$ receptors (Brehm et al., 1997). Therefore it appears reasonable to suggest that both agonists act through a conserved mechanism at the β/α interface.

The aim of the present study was to compare the roles for binding site residues in GABA and THIP activity at both synaptic $\alpha_1\beta_2\gamma_2$ and extrasynaptic $\alpha_4\beta_3\delta$ receptors. The first residues chosen for study were α_1(F92) and α_4(F98) (Figure 2-1 and 2-2), as previous work has demonstrated a functional role for the α_1(F92) residue in agonist binding at the $\alpha_1\beta_2\gamma_2$ receptor (Sigel et al., 1992; Boileau et al., 1999). Another residue of interest was identified through studies on the 5-HT_3 receptor conducted by others in Dr. Dunn's lab, where it was found that substituting a loop D glutamate residue in the 5-HT_3AB receptor with glutamine - as found in the 5-HT_3A homomer - increased the relative efficacy of the agonist mCPBG compared to 5-HT (Michaelson and Dunn, 2010). This residue is conserved as Q95 in the α_1 subunit and Q101 in the α_4 subunit (Figure 2-1 and 2-2). Measurement of functional responses at receptors containing mutations at these two positions demonstrated that the general mechanism of agonist activity is conserved at $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, although specific interactions may differ. These results are discussed in the context of docking studies carried out using homology models constructed for the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, which suggest that THIP may function through a subsite that is unique from that of GABA.

MATERIALS AND METHODS

Materials

All drugs were purchased through Sigma-Aldrich (St Louis, MO, USA) and stock solutions were prepared in water. GABA stocks were prepared as 1M solutions and stored as aliquots at -20°C while THIP was prepared fresh as a 10 mM stock for each experiment. In experiments using a concentration of THIP greater than 3 mM, stock solutions were adjusted to pH 7.4 with 5M NaOH. Restriction enzymes were purchased from New England Biolabs (Pickering, ON, Canada) and Invitrogen (Burlington, ON, Canada), and materials for cRNA transcription were purchased from Invitrogen, Ambion (Streetsville, ON, Canada) and Promega (Madison, WI, USA). Pfu turbo polymerase used for site-directed mutagenesis was purchased from Stratagene (La Jolla, CA, USA), and custom primers were ordered from IDT (Coralville, IA, USA).

Site-Directed Mutagenesis

The cDNAs encoding the rat $GABA_A$ receptor subunits were previously subcloned into the pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA, USA), except for the β_2 subunit, which was inserted into the pcDNA3.1(-) vector. The F92 and F98 mutations of the α_1 and α_4 subunits, respectively, were prepared using the QuickChange protocol developed by Stratagene. Mutant plasmids were screened by restriction digestion and confirmed by

DNA sequencing (Molecular Biology Service Unit, University of Alberta, AB, Canada).

Expression of $GABA_A$ Receptors in *Xenopus* Oocytes

Capped RNA was synthesized using T7 RNA polymerase (Invitrogen) following manufacturer's protocols. The $GABA_A$ receptor subunit cDNAs were linearized by restriction digestion prior to transcription. Stage V-VI *Xenopus laevis* oocytes were isolated and prepared as previously described (Smith et al., 2004). For expression of $\alpha_4\beta_3\delta$ receptors, oocytes were injected with 50 ng total cRNA at a 1:1:1 subunit ratio, while expression of $\alpha_1\beta_2\gamma_2$ receptors was carried out using 5 ng total cRNA. The lower total cRNA was used for the $\alpha_1\beta_2\gamma_2$ receptor to avoid amplifier saturation resulting from receptor overexpression. Following injection, oocytes were individually placed in 96-well plates containing ND96 buffer (in mM: 96 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4) with 50 μg/mL gentamicin (GIBCO, Grand Island, NY, USA) at 12°C and allowed to express for at least 48 hours prior to functional analysis.

Electrophysiological Recordings

Oocytes were bathed in frog Ringer's solution (in mM: 110 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 HEPES, pH 7.4) using a gravity flow perfusion chamber running at approximately 10 mL/min. Currents evoked by agonist were measured using a two electrode voltage clamp with a GeneClamp 500B amplifier (Axon Instruments Inc., Foster City, CA, USA) and a holding potential of -60 mV.

Both electrodes were filled with 3 M KCl and only electrodes with a resistance between 0.5 and 1.5 MΩ in frog Ringer's solution were used. Agonists were applied by continuous perfusion until currents reached their peak values, followed by an 8 minute washout period to allow recovery from desensitization. Before starting a concentration-response curve, oocytes were exposed to a concentration of GABA eliciting a maximal response. This response was measured in succession a minimum of three times to determine whether the response of the oocyte was stable and reproducible within 10% error. Maximum GABA currents were measured between each test response during data collection and used to normalize concentration-response curves.

Data Analysis

Concentration-response curves for agonist activation were fitted by non-linear regression analysis, using GraphPad Prism 5.0 software (San Diego, CA, USA) and the Hill equation:

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

where I_L is the amplitude of agonist-evoked current for a given concentration [L], I_{max} is the maximum amplitude of current, EC_{50} is the agonist concentration that evokes half maximal receptor activation, and n_H is the Hill coefficient. Values from each individual curve were used in calculating the

mean and standard error for the log EC_{50} and Hill coefficient. Efficacy values for THIP were determined by generating a concentration-response curve with THIP, and comparing the fit value for the I_{max} of THIP to that obtained with a concentration of GABA previously determined to elicit a maximal response. In the case of the α_4(F98A)$\beta_3\delta$ receptor, GABA curves did not reach a plateau and therefore a theoretical maximum response had to be approximated by fitting a GABA concentration-response curve and comparing the GABA I_{max} to that of THIP. Statistical significance was determined by comparing the mean values at the mutant receptors to the respective wild-type control using one-way analysis of variance with Dunnett's post-test for multiple comparisons.

Docking Simulations

Homology models for the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors were constructed as described (Mokrab et al., 2007) and used courtesy of Dr. Younes Mokrab. Briefly, the models are based on the general structure of the nACh receptor obtained by cryo-electron microscopy of the electroplaque organ of *Torpedo marmorata*. The structure of the extracellular domain was further refined by alignment with structures obtained with the soluble Acetylcholine Binding Protein (AChBP), which contains significant homology to the extracellular domain of the nACh receptor. The extracellular domain, transmembrane domain and MA helix of the intracellular domain were successfully modelled for the $\alpha_1\beta_2\gamma_2$ receptor, while only the extracellular

domain and transmembrane domain of the $\alpha_4\beta_3\delta$ receptor contained sufficient homology with the nACh receptor to be modelled.

Docking was carried out by Dr. N.P. Todorov for GABA and THIP in both models using the genetic algorithm GOLD (Jones et al., 1997) and easyDock (Todorov et al., 2003) as described by Mokrab et al. (2007). Multiple docking simulations were carried out with each agonist-receptor complex to produce a number of binding modes. The energy associated with each binding mode was calculated in terms of a binding score that approximates steric and hydrogen-bonding interactions between the ligand and protein molecules (Gehlhaar et al., 1995; Mokrab et al., 2007). These energy scores do not have any physical meaning (such as for calculating the affinity of a compound), but are useful to identify which agonist conformations represent the most stable binding modes, with lower or more negative scores corresponding to more favourable interactions (Todorov et al., 2003). For each round of docking, the coordinates of the lowest scoring conformation were compared to all of the previous lower-scoring conformations. If the root-mean square deviation was greater than 3.0 Å, then the conformation was defined as a distinct binding mode. Clusters were then generated by Dr. P-L. Chau, by assigning each conformation to a binding-mode with the closest root mean square deviation. Separate clusters are represented by the docked configuration that generated the lowest calculated binding score.

RESULTS

GABA Pharmacology

A summary of the concentration-response data for the wild-type and mutant $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors is shown in Tables 2-1 and 2-2, and Figures 2-3 and 2-4. The mean EC_{50} of 35 μM observed for responses to GABA at $\alpha_1\beta_2\gamma_2$ receptors is close to values previously reported (Newell et al., 2000). The mean EC_{50} value of 8.1 μM observed for GABA activation of $\alpha_4\beta_3\delta$ receptors is higher than what was expected based on the literature (~2 μM) (Storustovu and Ebert, 2006), but is still consistent with the δ subunit forming receptors with higher affinity for GABA than those containing the γ_2 subunit. The two α_1 mutations, α_1(F92L) and α_1(Q95E) have mean EC_{50} values of 166 μM and 62 μM for GABA activation, an increase of 4.7 and 1.8 times, respectively, compared to the wild-type EC_{50} value (35 μM). These results contrast with a previous study on the α_1(F92L)$\beta_2\gamma_2$ receptor, which reported a 200-fold increase in the EC_{50} for responses to GABA (Sigel et al., 1992). Both mutations also affected the Hill coefficient for GABA activation, showing a decrease from 1.12 to 0.71 as a result of the α1(F92L) mutation, and an increase to 1.28 as a result of the α_1(Q95E) mutation. In contrast to the α_1(F92L) mutation, the α_4(F98L) mutation failed to produce any significant change in the EC_{50} or Hill coefficient observed for GABA activation of $\alpha_4\beta_3\delta$ receptors. Given the relatively conserved side chain volume between the phenylalanine and leucine side chains, an α_4(F98A) point mutation was introduced to address whether further reducing the side chain volume would

affect agonist activity. The α_4(F98A) mutation produced a marked change in the EC_{50} for GABA activation, increasing approximately 400-fold over the wild-type value to 3 mM, and also produced a significant decrease in the Hill coefficient to 0.52 from 0.88. The EC_{50} for GABA activation of the α_4(Q101E) mutant was significantly decreased to 4.4 μM, an effect which is opposite to that observed for the α_1(Q95E) mutation.

THIP Pharmacology

During the initial characterization of THIP activity at $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors, it was noticed that current responses started to decrease when concentrations over 3 mM were applied (Figure 2-5). Measuring the pH of the THIP dilutions used for constructing curves showed that high concentrations of THIP significantly alter the pH of the solution (Figure 2-6), similar to what was seen by Mortensen et al. (2010). To limit artefacts resulting from buffer acidification, the pH of THIP was adjusted to 7.4 with 5 M NaOH in experiments using THIP concentrations greater than 3 mM.

The results for the concentration-response curves of THIP with $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors are summarized in Tables 2-3 and 2-4 and Figures 2-7 and 2-8. The mean EC_{50} for THIP activation of wild-type $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors were 582 μM and 63.5 μM, respectively. The EC_{50} value at $\alpha_4\beta_3\delta$ receptors is similar to previously published values (Storustovu and Ebert, 2006), while the EC_{50} of THIP at $\alpha_1\beta_2\gamma_2$ receptors is approximately 3-4 times higher than what others have observed (Ebert et al., 1997). The EC_{50} and Hill

coefficient for responses to THIP were not changed for $\alpha_1\beta_2\gamma_2$ receptors containing either the α_1(F92L) or α_1(Q95E) mutations, in contrast to the differences observed for responses to GABA. There was a significant change in the relative efficacy of THIP compared to the maximum GABA response at α_1(F92L)$\beta_2\gamma_2$ receptors, increasing from 58% in the wild-type to 73% in the mutant. Responses to THIP were unchanged by either the α_4(F98L) or α_4(Q101E) mutations in $\alpha_4\beta_3\delta$ receptors, while the α_4(F98A) mutation significantly right-shifted the EC_{50}, causing a 22-fold increase to 1.4 mM, but did not alter the Hill coefficient. The impact on the relative efficacy of THIP was pronounced, decreasing from 156% in the wild-type receptor to 38% in the α_4(F98A) mutant.

Docking and Homology Modelling

The docking pose of GABA at the $\alpha_1\beta_2\gamma_2$ receptor model shown in Figure 2-9 is that described by Mokrab et al. (2007), and shows the GABA carboxylate group facing the α_1(F92)/α_1(R94) residues. For THIP, 524 docked poses were produced, with 132 poses forming 15 clusters, with cluster 1 and cluster 6 account for 32% and 24% of all docked configurations, respectively. These clusters show THIP docking closer to β_2(Y183), as opposed to β_2(Y181) or β_2(Y229) on loop B and C, respectively, as observed for GABA. This upper site does not come into close contact with either α_1(F92) or α_1(R94), although rotation of α_1(R94) may bring it close enough to interact with THIP. In the $\alpha_4\beta_3\delta$ receptor, 518 configurations were

produced for GABA, of which 173 formed clusters. In this case, three clusters were produced that accounted for the majority of docked poses, with cluster 1, cluster 2 and cluster 14 accounting for 23%, 18%, and 16% of the docked poses, respectively. One of these clusters docks closely to β_3(Y182) and β_3(Y230) on loops B and C, respectively, and orients the GABA carboxylate towards the α_4(F98) side chain. A second cluster docks to a site similar to that seen for THIP in the $\alpha_1\beta_2\gamma_2$ model, only in this case the α_4(R100) side chain is oriented to form potential hydrogen bonding interactions with GABA. For THIP, 490 docked configurations were produced in the $\alpha_4\beta_3\delta$ receptor, with 128 grouping into 13 clusters. The major cluster for THIP in the $\alpha_4\beta_3\delta$ model appears to dock in the upper site, similar to what is seen in the $\alpha_1\beta_2\gamma_2$ model.

DISCUSSION

The Loop D Phenylalanine Residue Plays a Structural Role in the Binding Site of $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ Receptors

The data presented here support a role for the α_4(F98) residue in agonist activity, as both the relative efficacy and EC_{50} values for GABA and THIP responses are altered considerably as a result of the α_4(F98A) mutation. In light of these results, the failure of the α_4(F98L) mutation to impact either GABA- or THIP-evoked responses suggests that the key contribution of this residue is through the aliphatic structure of the side chain, and not the aromatic π electrons of the phenyl group. This is similar to the role of the α_1(F92) residue as proposed by Padgett et al. (2007), who observed that adding electron-withdrawing groups to the α_1(F92) aromatic ring structure through unnatural amino acid mutagenesis did not significantly alter the EC_{50} for GABA activation. Thus, the aliphatic structure of the α_1(F92) and α_4(F98) residues are important for binding site function while the aromatic component does not play a significant role.

The fact that agonist activity is sensitive to the volume of the α_1(F92)/α_4(F98) side chains suggests that they primarily carry out a structural role in the binding site. Both phenylalanine residues are predicted to lie just below a loop D arginine residue that forms key hydrogen bonding interactions with the GABA carboxylate group (Boileau et al., 1999; Hartvig et al., 2000; Holden and Czajkowski, 2002; Melis et al., 2008). Following agonist binding, the cavity formed in the β/α interface is believed to constrict, while

loop C swings in to lock the agonist in the binding site (Chang and Weiss, 1999; Muroi et al., 2006; Kloda and Czajkowski, 2007). One outcome of this conformational change is a general decrease in solvent accessibility for residues in the binding site (Holden and Czajkowski, 2002; Shi et al., 2006; Kloda and Czajkowski, 2007). This could be important for agonist binding, as solvent water molecules may change the polarity of the binding site environment (Gao et al., 2009) or compete directly for bonding partners in the binding site (Voet and Voet, 2004), both of which could alter key electrostatic or hydrogen bonding interactions. Given that the phenylalanine side chain is likely positioned directly underneath the agonist in the binding site, it may function primarily by capping the bottom of the binding site following loop C closure. This would imply that the α_1(F92L) and α_4(F98A) mutations decrease agonist potency by reducing the barrier for agonist dissociation or increasing the accessibility of solvent molecules in the binding site.

The Docking Data Suggest Subsites for Agonist Binding

The docking data presented here suggest that the complete picture for agonist binding could be more complicated than that described above (Figure 2-9). The major implication of these results is that GABA and THIP may bind to different subsites at the β/α interface. A consistent feature with both models is that THIP appears to preferentially dock in a pocket that is distant from the α_1(F92)/α_4(F98) residues, but still close enough to maintain

hydrogen bonding interactions with the loop D arginine residue – in the $\alpha_4\beta_3\delta$ model it contacts α_4(R100) directly, while in the $\alpha_1\beta_2\gamma_2$ model it would be close enough to contact α_1(R94) if the sidechain were rotated. In contrast, GABA is seen to form a cluster in both models with its amino group sandwiched between two tyrosine residues on loop B and C and the carboxyl group oriented towards the loop D arginine/phenylalanine pair of residues. GABA appears to dock closer to the phenylalanine residue than THIP in both models, which may explain why its potency was affected more strongly by both the α_1(F92L) and α_4(F98A) mutations compared to that of THIP. Therefore, these docking results suggest that the exact location of agonist binding may be an additional variable underlying the different sensitivities of GABA and THIP activity in response to the α_1(F92L) and α_4(F98A) mutations.

The presence of distinct subsites mediating the activity of different agonists is not an entirely new concept, as work on the nACh receptor previously showed that there are two allosterically linked subsites (Dunn and Raftery, 1997). Moreover, bisquaternary ligands, which consist of two acetylcholine groups separated by an aliphatic linker of varying length, are able to cross-link these two separate subsites when the linker length is 15-20 Å (Dunn and Raftery, 1997; Carter et al., 2007). The length of the linker region in bisquaternary ligands also contributes to their efficacy, with analogues containing less than 4 or more than 7 methyl groups in their linker region displaying much weaker partial agonist activity (Carter et al., 2007). Characterization of a tryptophan to phenylalanine mutation at the putative

subsite failed to significantly alter acetycholine-evoked responses but caused a 200-fold increase in the EC_{50} observed for responses to suberyldicholine, suggesting that this is a functionally relevant binding site for bisquaternary compounds (Kapur et al., 2006).

The Role of the Phenylalanine Residue In Agonist Efficacy

An interesting aspect of the α_1(F92L) and α_4(F98A) mutations is that both produced significant effects on the relative efficacy of THIP and GABA. The changes observed for the α_4(F98A)$\beta_3\delta$ receptor were especially striking, with the relative efficacy of THIP decreasing from approximately 155% of that for GABA to less than 40%. In contrast to the α_4(F98A)$\beta_3\delta$ receptor, the relative efficacy of THIP actually increased slightly at α_1(F92L)$\beta_2\gamma_2$ receptors. These results suggest that the loop D region is involved in some component of channel gating following agonist binding.

One of the limitations of the current experiments is that the efficacy of THIP and GABA is defined in relative terms. As a result, it is not possible to determine whether the efficacy of THIP, GABA, or both THIP and GABA was affected. Techniques such as single channel analysis could be used to determine which aspects of the channel gating kinetics for each agonist have been altered, such as channel open/close times, burst duration, and channel opening probability (Mortensen and Smart, 2007). While these parameters do not give a direct measure of agonist efficacy, such information would be useful to determine how each agonist was affected by each mutation and

provide some clues as to how the mutations may have altered the ability of GABA and/or THIP to elicit channel activation. However, it should be noted that it is not trivial to isolate the contributions of individual residues to agonist affinity versus agonist efficacy in ligand-gated ion channels, as the energetics of these two parameters are intrinsically linked (Colquhoun, 1998).

The fact that any changes were observed on agonist efficacy stands in contrast to an earlier study that concluded that the α_1(F92) residue does not contribute to receptor gating (Sigel et al., 1992). This earlier conclusion had been reached through the observation that the EC_{50} of GABA changed by a similar magnitude as the IC_{50} for the antagonists SR-95531 and bicuculline methiodide at α_1(F92L)$\beta_2\gamma_2$ receptors. The assumption made was that antagonists bind through the same mechanism as agonists, but do not elicit the conformational rearrangements that lead to channel gating and thus represent a binding-only phenomenon (Sigel et al., 1992). However, kinetic modelling, *in silico* docking, and biochemical studies have suggested that agonist binding is driven by the formation of specific bonding interactions with binding site residues, whereas antagonist binding is driven through hydrophobic interactions at a cavity just below the agonist binding site (Jones et al., 2001; Holden and Czajkowski, 2002; Sander et al., 2011). In light of these observations, it appears that agonists and antagonists do not bind through equivalent mechanisms. Therefore, it may not be appropriate to rule

out a role for the α_1(F92)/α_4(F98) residues in determining agonist efficacy based on a comparison of GABA EC_{50} with SR-95531 IC_{50} values.

The Role of α_1(Q95)/α_4(Q101) in Agonist Activity

The results for the α_1(Q95E)$\beta_2\gamma_2$ and α_4(Q101E)$\beta_3\delta$ $GABA_A$ receptors did not show a comparable pattern of changes to those observed in the 5-HT_3AB(E86Q) receptor by my colleagues (Michaelson and Dunn, 2010). In the 5-HT_3AB(E86Q) receptor, both the EC_{50} and Hill coefficient for 5-HT activation increased, while the EC_{50} for mCPBG activation decreased and its efficacy relative to 5-HT increased (Michaelson and Dunn, 2010). In contrast, the α_1(Q95E) mutation produced a less than 2-fold decrease in the EC_{50} value and a small but significant increase in the Hill coefficient for responses to GABA, while the α_4(Q101E) mutation produced a 2-fold increase in the EC_{50} of GABA activation with no effect on the Hill coefficient. Neither the EC_{50}, Hill coefficient nor the relative efficacy of THIP responses changed significantly at either α_1(Q95E)$\beta_2\gamma_2$ or α_4(Q101E)$\beta_3\delta$ receptors, suggesting that the residue at this position plays a lesser role in THIP activity. While the overall magnitude of the changes in potency were small in both cases, the qualitatively different responses seen for GABA at the two receptor subtypes suggests that the function of this residue differs, depending on the context of the agonist and subunit composition of the receptor.

The loop D region of the alpha subunit (α_1(Q95E) and α_4(Q101E)) is well conserved (Figure 2-2), suggesting that the distinct effects on GABA

potency observed for the α_1(Q95E)$\beta_2\gamma_2$ and α_4(Q101E)$\beta_3\delta$ receptors are likely due to structural differences outside of loop D on the α subunit. The homology models constructed for both the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors show that the α_1(Q95) and α_4(Q101) residues face towards the opposite side of the α subunit, and could be situated to anchor the upper portion of the loop A region (Figure 2-10). It is not clear based on the model whether the glutamine side chain predominantly forms hydrogen bonds to backbone atoms or with the side chains of other residues, but in either case alterations of these interactions through the α_1(Q95E) and α_4(Q101E) mutations could underlie the observed changes in GABA potency. The role of loop A in the complementary subunit is not clear, as the α/β, α/γ, and α/δ interfaces have not been implicated as major sites for agonist-evoked receptor activation. However, one study on the linker region between loop A and loop E has shown that it is a key determinant for GABA activity, suggesting that changes to the conformation of this region have a significant effect on agonist activity (Hanek et al., 2010).

The loop D glutamine residue could also play a structural role to help stabilize the β-sheet structure of the extracellular domain. In each subunit, loops D, E and F contribute to a sheet of five β-strands, while loops A, B and C form a separate sheet of four β-strands. Molecular dynamics simulations of a homomeric α_7 nACh receptor model have shown that the backbone structure of these β-sheets is rigid, and allows for concerted movements throughout

the extracellular domain during conformational rearrangements (Law et al., 2005). Contacts between these two sets of β-sheets, such as those potentially mediated by α_1(Q95) and α_4(Q101) between loop D and loop A, could be important for contributing to such rigid body motions. Introducing the α_1(Q95E)/α_4(Q101E) mutations may therefore interfere with the concerted movements in the extracellular domain mediated by interactions between the two sets of β-sheets.

Taken together, these data show that the loop D region is important for aspects of agonist binding as well as channel gating. The results with the α_1(F92)/α_4(F98A) mutations showed that these residues play a structural role in the binding site, possibly by capping the bottom of loop C following agonist binding. This could be an important step to prevent agonist dissociation from the binding site and limit solvent accessibility. The *in silico* docking results, combined with the electrophysiological data, suggests that GABA and THIP may bind to unique subsites at β/α interfaces. The results obtained with the α_1(Q95E)/α_4(Q101E) mutations suggest that contacts between the two β-sheets making up the extracellular domain may be important for agonist binding. The α_1(Q95E)/α_4(Q101E) mutations also had qualitatively different effects on the potency of GABA activation, suggesting that the functional role of these residues is dependent on the identity of the neighbouring subunits.

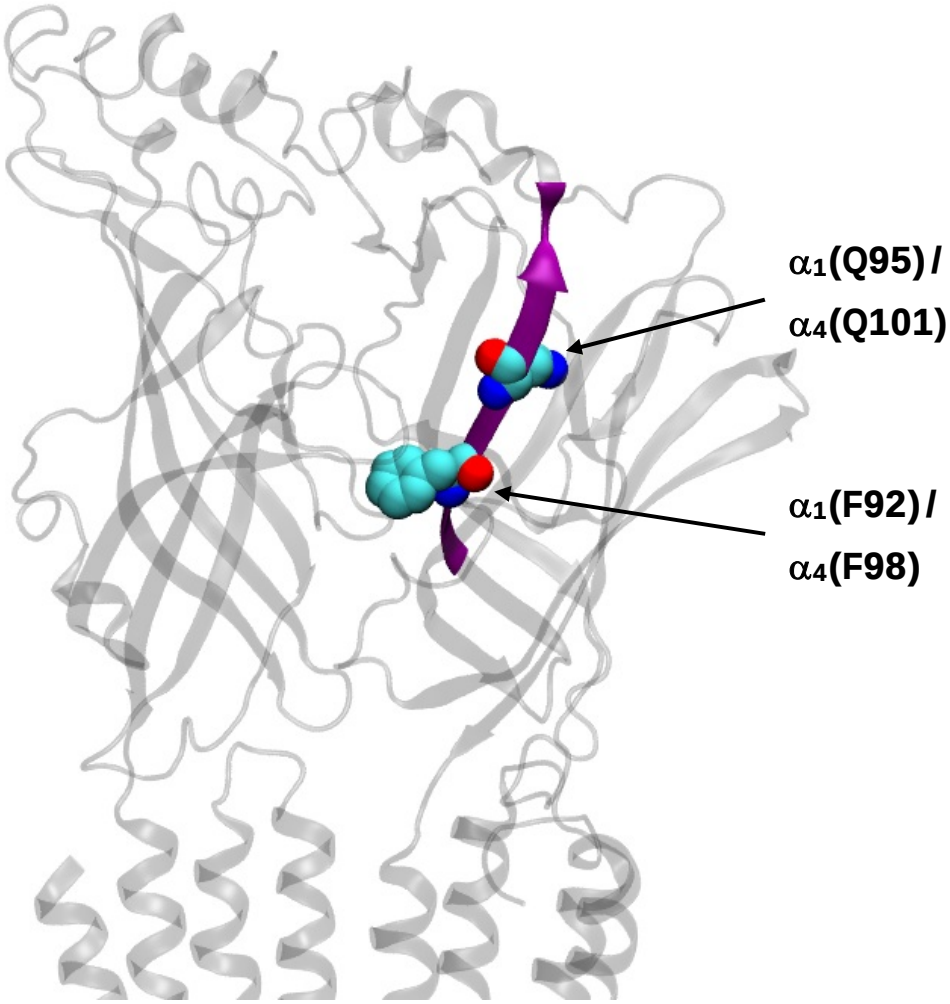


Figure 2-1. View of the loop D residues targeted for mutation in the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors. The loop D region is highlighted in magenta. The sidechains of the α_1(F92)/α_4(F98) residue can be seen pointing into the binding site pocket, while those of the α_1(Q95)/α_4(Q101) residues face towards the opposite side of the α subunit. The pdb coordinates for the receptor structure were obtained courtesy of Dr. Younes Mokrab.

```
alpha-1         MEYTIDVFFRQSWKDER
alpha-2         MEYTIDVFFRQKWKDER
alpha-3         MEYTIDVFFRQTWHDER
alpha-4         MEYTMDVFFRQTWIDKR
alpha-5         MEYTIDVFFRQSWKDER
alpha-6         MEYTMDVFFRQTWTDER
                ****:******.* *:*
```

Figure 2-2. Alignment of the loop D region of rat $GABA_A$ receptor α subunits. An asterisk (*) denotes a single strictly conserved residue, a colon (:) is conservation of amino acids with strongly similar properties, and a full stop (.) is conservation of amino acids with weakly similar properties. Loop D residues are underlined, starting at T88 and extending to K98 in the α_1 subunit and T94 to I104 in the α_4 subunit. The phenylalanine and glutamine residues investigated in this study are highlighted in grey. The alignment was performed using ClustalW2 (Larkin et al., 2007)

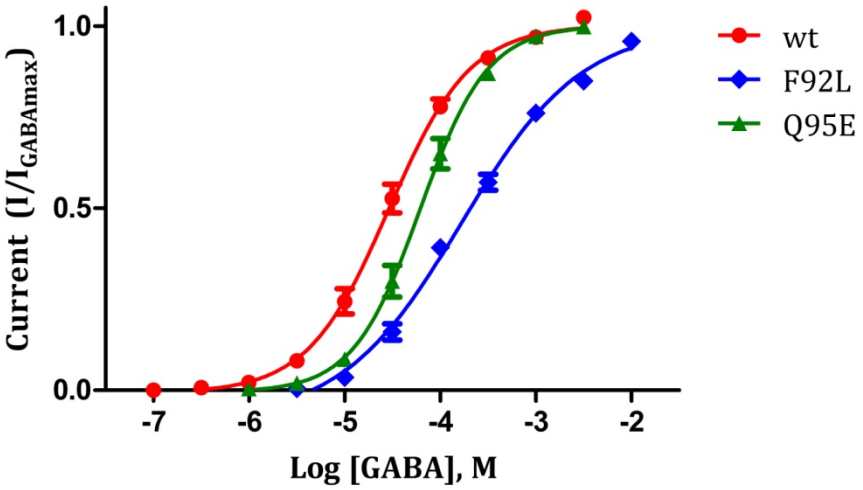


Figure 2-3. Normalized concentration-response curves of GABA at mutant and wild-type $\alpha_1\beta_2\gamma_2$ receptors. The error bars represent the standard error of the mean (SEM) for the normalized response measured at each agonist concentration, where n= 10 (wt), 5 (F92L), and 5 (Q95E).

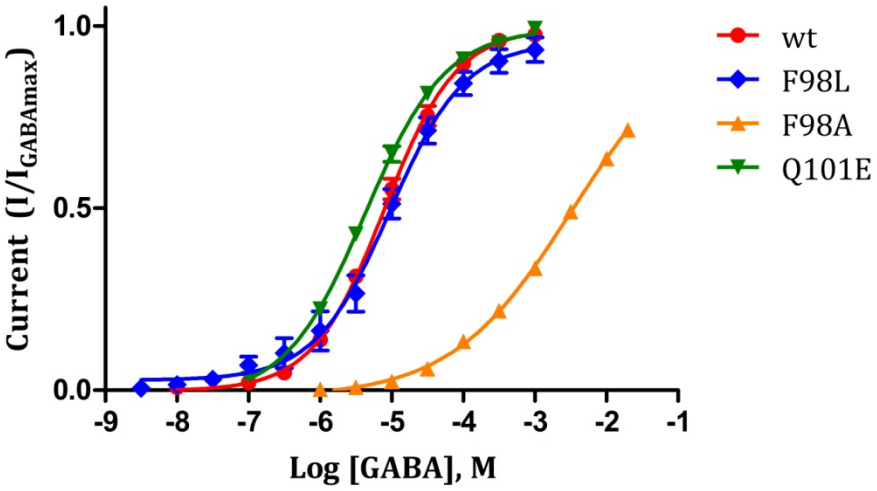


Figure 2-4. Normalized concentration-response curves of GABA at mutant and wild-type $\alpha_4\beta_3\delta$ receptors. The error bars represent the SEM for the normalized response measured at each agonist concentration, where n= 5 (wt), 5 (F98L), 4 (F98A) and 6 (Q101E).

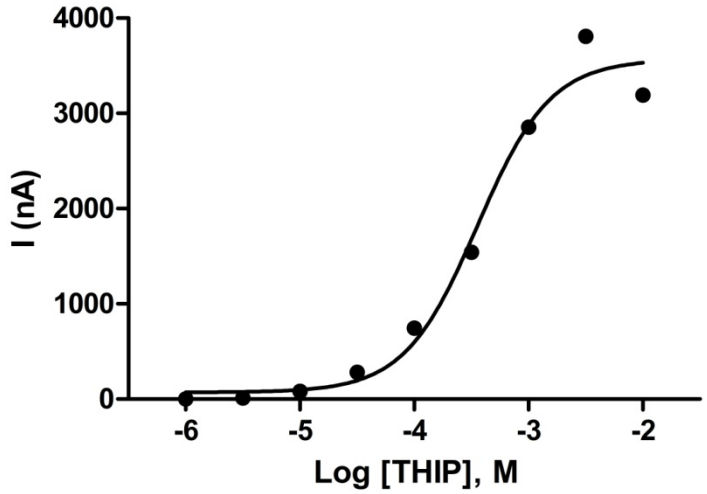


Figure 2-5. Concentration-response curve of THIP at wild-type $\alpha_1\beta_2\gamma_2$ receptors without pH adjustment. The curve presented is a single experiment demonstrating a commonly observed decrease in responses with 10 mM THIP.

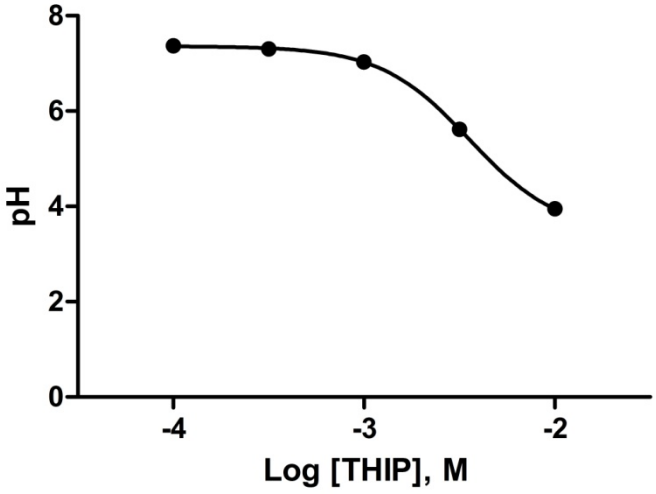


Figure 2-6. Measurement of pH in drug solutions produced by serial dilution of THIP. The curve shown is a single experiment performed by diluting THIP into Ringer's buffer (pH 7.4). The drug solutions used to measure responses to 3 mM and 10 mM THIP concentrations have a significantly decreased pH.

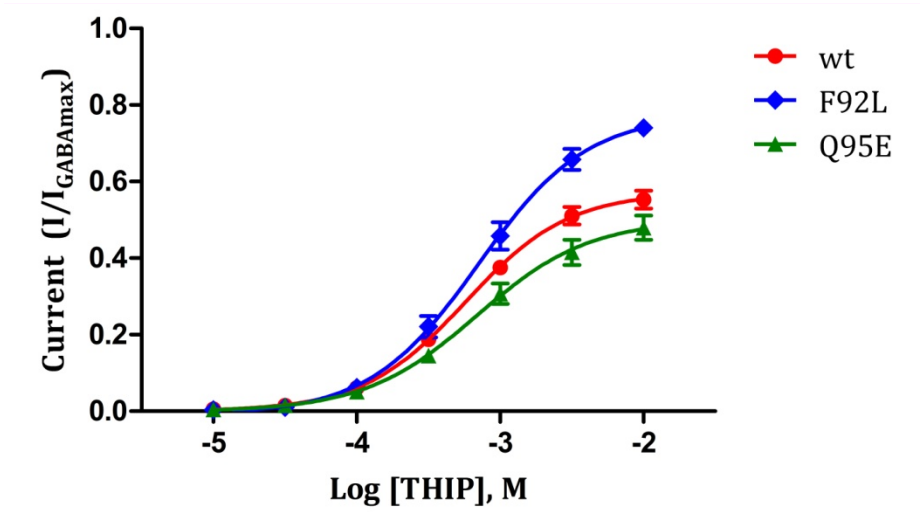


Figure 2-7. Normalized concentration-response curves of THIP at mutant and wild-type $\alpha_1\beta_2\gamma_2$ receptors. The error bars represent the SEM for the normalized response measured at each agonist concentration, where n= 5 (wt), 5 (F92L), and 5 (Q95E).

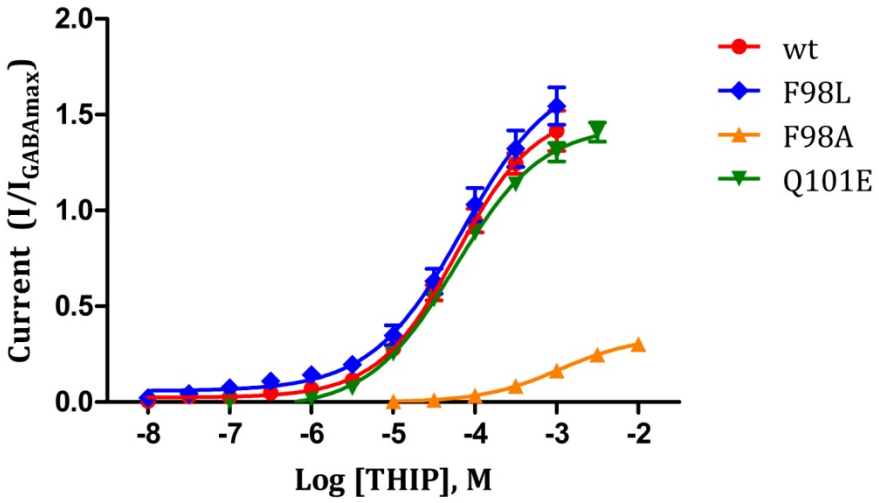


Figure 2-8. Normalized concentration-response curves of THIP at mutant and wild-type $\alpha_4\beta_3\delta$ receptors. The error bars represent the SEM for the normalized response measured at each agonist concentration, where n= 5 (wt), 5 (F98L), 3 (F98A) and 5 (Q101E).

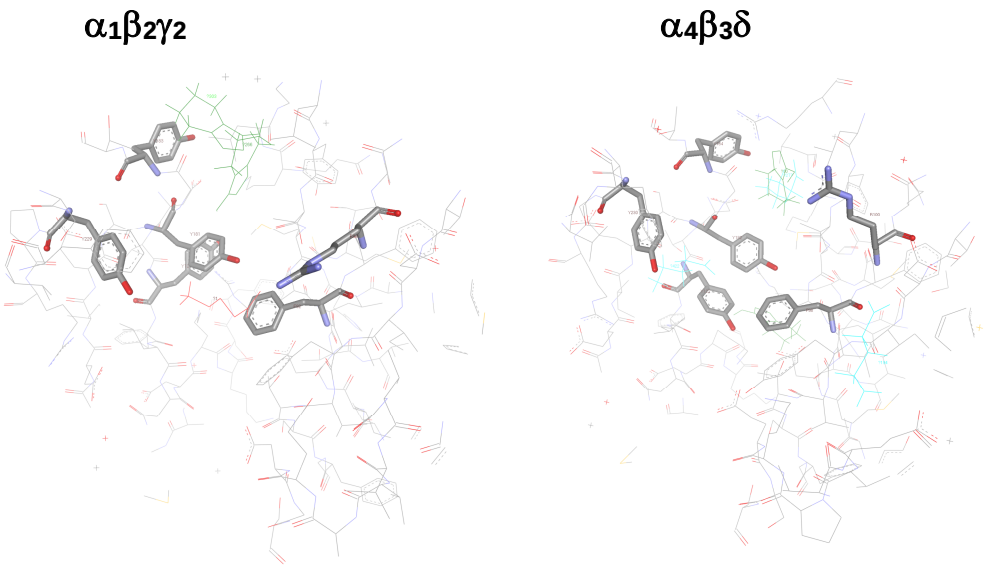


Figure 2-9. GABA and THIP docking in homology models of the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors. Residues from the β subunit are shown on the left and the α subunit on the right for each receptor subtype. The highest scoring docking poses are shown for GABA (red in α_1, cyan in α_4) and THIP (green). Residues that are implicated in agonist binding are labelled with their structures highlighted. This figure was obtained courtesy of Dr. Younes Mokrab, Dr. Nikolai P. Todorov and Dr. P-L. Chau.

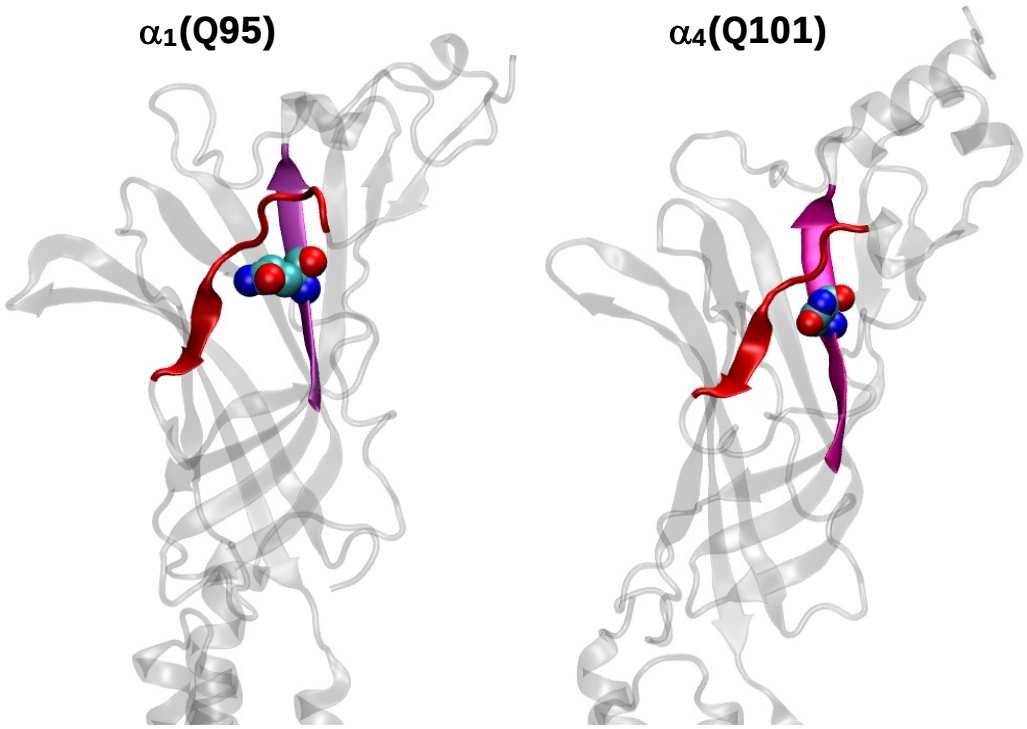


Figure 2-10. View of the α_1(Q95) and α_4(Q101) residues in the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ models, respectively. The loop D region is highlighted in magenta, and loop A in red. The pdb coordinates for the receptor structure were obtained courtesy of Dr. Younes Mokrab.

Table 2-1. Summary of concentration-response curve values for GABA at wild-type and mutant $\alpha_1\beta_2\gamma_2$ receptors. * ($p<0.05$), and *** ($p<0.001$) compared to the wild-type $\alpha_1\beta_2\gamma_2$ receptor.

Receptor	n	LogEC_{50} (SEM)	EC_{50} (μM)	n_H (SEM)
$\alpha_1\beta_2\gamma_2$ wt	10	-4.46 (0.06)	34.7	1.12 (0.04)
$\alpha_1\beta_2\gamma_2$ F92L	5	-3.78 (0.05)***	166	0.71 (0.05)***
$\alpha_1\beta_2\gamma_2$ Q95E	5	-4.21 (0.06)*	61.7	1.28 (0.03)*

Table 2-2. Summary of concentration-response curve values for GABA at wild-type and mutant $\alpha_4\beta_3\delta$ receptors. * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$) compared to the wild-type $\alpha_4\beta_3\delta$ receptor.

Receptor	n	LogEC_{50} (SEM)	EC_{50} (μM)	n_H (SEM)
$\alpha_4\beta_3\delta$ wt	5	-5.09 (0.05)	8.13	0.85 (0.03)
$\alpha_4\beta_3\delta$ F98L	5	-5.04 (0.11)	9.12	0.77 (0.10)
$\alpha_4\beta_3\delta$ F98A	4	-2.48 (0.09)***	3311	0.52 (0.04)**
$\alpha_4\beta_3\delta$ Q101E	6	-5.36 (0.04)*	4.37	0.77 (0.03)

Table 2-3 – Summary of concentration-response curve values for THIP at wild-type and mutant $\alpha_1\beta_2\gamma_2$ receptors. ** ($p<0.01$) compared to the wild-type $\alpha_1\beta_2\gamma_2$ receptor. Relative efficacy here is defined as $I_{THIPmax}/I_{GABAmax}$.

Receptor	n	$LogEC_{50}$ (SEM)	EC_{50} (μM)	n_H (SEM)	Relative Efficacy (SEM)
$\alpha_1\beta_2\gamma_2$ wt	5	-3.24 (0.03)	575	1.23 (0.09)	0.58 (0.03)
$\alpha_1\beta_2\gamma_2$ F92L	5	-3.15 (0.06)	708	1.18 (0.07)	0.73 (0.03)**
$\alpha_1\beta_2\gamma_2$ Q95E	5	-3.16 (0.03)	692	1.13 (0.03)	0.50 (0.03)

Table 2-4 – Summary of concentration-response curve values for THIP at wild-type and mutant $\alpha_4\beta_3\delta$ receptors. *** ($p<0.001$) compared to the wild-type $\alpha_4\beta_3\delta$ receptor.

Receptor	n	$LogEC_{50}$ (SEM)	EC50 (μM)	n_H (SEM)	Relative Efficacy (SEM)
$\alpha_4\beta_3\delta$ wt	5	-4.20 (0.08)	63.1	0.88 (0.03)	1.56 (0.09)
$\alpha_4\beta_3\delta$ F98L	5	-4.12 (0.06)	75.9	0.77 (0.05)	1.79 (0.20)
$\alpha_4\beta_3\delta$ F98A	3	-2.85 (0.16)***	1413	0.86 (0.11)	0.38 (0.03)***
$\alpha_4\beta_3\delta$ Q101E	5	-4.27 (0.08)	53.7	0.86 (0.04)	1.41 (0.07)

REFERENCES

Bianchi MT and Macdonald RL. (2003). Neurosteroids shift partial agonist activation of $GABA_A$ receptor channels from low- to high-efficacy gating patterns. *J. Neurosci.* **23**, 10934-10943.

Boileau AJ, Evers AR, Davis AF and Czajkowski C. (1999). Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. *J. Neurosci.* **19**, 4847-4854.

Brehm L, Ebert B, Kristiansen U, Wafford KA, Kemp JA and Krogsgaard-Larsen P. (1997). Structure and pharmacology of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (thio-THIP), an agonist/antagonist at $GABA_A$ receptors. *Eur. J. Med. Chem.* **32**, 357-363.

Brickley SG and Mody I. (2012). Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* **73**, 23-34.

Bright DP, Renzi M, Bartram J, McGee TP, MacKenzie G, Hosie AM, Farrant M and Brickley SG. (2011). Profound desensitization by ambient GABA limits activation of δ-containing GABAA receptors during spillover. *J. Neurosci.* **31**, 753-763.

Brown N, Kerby J, Bonnert TP, Whiting PJ and Wafford KA. (2002). Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br. J. Pharmacol.* **136**, 965-974.

Carter CRJ, Cao L, Kawai H, Smith PA, Dryden WF, Raftery MA and Dunn SMJ. (2007). Chain length dependence of the interactions of bisquaternary ligands with the *Torpedo* nicotinic acetylcholine receptor. *Biochem. Pharmacol.* **73**, 417-426.

Chang Y and Weiss DS. (1999). Channel opening locks agonist onto the $GABA_C$ receptor. *Nature Neurosci.* **2**, 219-225.

Chou PY and Fasman GD. (1978). Empirical predictions of protein conformation. *Ann. Rev. Biochem.* **47**, 251-276.

Clements JD. (1996). Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *TiNS* **19**, 163-171.

Colquhoun D. (1998). Binding, gating, affinity, and efficacy: the interpretation of structure-activity relationships for agonists and the effects of mutating receptors. *Br. J. Pharmacol.* **125**, 923-947.

Cope DW, Hughes SW and Crunelli V. (2005). $GABA_A$ receptor-mediated tonic inhibition in thalamic neurons. *J. Neurosci.* **25**, 11553-11563.

Dunn SMJ and Raftery MA. (1997). Agonist binding to the *Torpedo* nicotinic acetylcholine receptor. I. Complexities revealed by dissociation kinetics. *Biochemistry* **36**, 3846-3853.

Ebert B, Thompson SA, Saounatsou K, McKernan R, Krogsgaard-Larsen P and Wafford KA. (1997). Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **52**, 1150-1156.

Ebert B, Mortensen M, Thompson SA, Kehler J, Wafford KA and Krogsgaard-Larsen P. (2001). Bioisosteric determinants for subtype selectivity of ligands for heteromeric $GABA_A$ receptors. *Bioorganic Med. Chem. Lett.* **11**, 1573-1577.

Essrich C, Lorez M, Benson JA, Fritschy J-M and Luscher B. (1998). Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. *Nature Neurosci.* **1**, 563-571.

Farrant M and Nusser Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nature Rev.* **6**, 215-229.

Gao J, Bosco DA, Powers ET and Kelly JW. (2009). Localized thermodynamic coupling between hydrogen bonding and microenvironment polarity substantially stabilizes proteins. *Nature Struct. Mol. Biol.* **16**, 684-691.

Gehlhaar DK, Verkhivker GM, Rejto PA, Sherman CJ, Fogel DB, Fogel LJ and Freer ST. (1995). Molecular recognition of the inhibitor AG-1343 by HIV-1 protease: conformationally flexible docking by evolutionary programming. *Chem. Biol.* **2**, 317-324.

Glykys J, Peng Z, Changda D, Homanics GE, Houser CR and Mody I. (2007). A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nature Neurosci.* **10**, 40-48.

Haas KF and Macdonald RL. (1999). $GABA_A$ receptor subunit $\gamma 2$ and δ subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. *J. Physiol.* **514.1**, 27-45.

Hanek AP, Lester HA and Dougherty DA. (2010). Photochemical proteolysis of an unstructured linker of the $GABA_AR$ extracellular domain prevents GABA but not pentobarbital activation. *Mol. Pharmacol.* **78**, 29-35.

Hartvig L, Lukensmejer B, Liljefors T and Dekermendjian K. (2000). Two conserved arginines in the extracellular N-terminal domain of the $GABA_A$ receptor α_5 subunit are crucial for receptor function. *J. Neurochem.* **75**, 1746-1753.

Holden JH and Czajkowski C. (2002). Different residues in the $GABA_A$ receptor α1T60-α1K70 region mediate GABA and SR-95531 actions. *J. Biol. Chem.* **277**, 18785-18792.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL and Goldstein PA. (2005). An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. *J. Neurophysiol.* **94**, 4491-4501.

Jones G, Willett P, Glen RC, Leach AR and Taylor R. (1997). Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **267**, 727-748.

Jones MV, Jonas P, Sahara Y and Westbrook GL. (2001). Microscopic kinetics and energetics distinguish $GABA_A$ receptor agonists from antagonists. *Biophysical J.* **81**, 2660-2670.

Kapur A, Davies M, Dryden WF and Dunn SMJ. (2006). Tryptophan 86 of the α subunit in the *Torpedo* nicotinic acetylcholine receptor is important for channel activation by the bisquaternary ligand suberyldicholine. *Biochemistry* **45**, 10337-10343.

Kloda JH and Czajkowski C. (2007). Agonist-, antagonist-, and benzodiazepine-induced structural changes in the α_1Met^{113}-Leu^{132} region of the $GABA_A$ receptor. *Mol. Pharmacol.* **71**, 483-493.

Krishek BJ, Amato A, Connolly CN, Moss SJ and Smart TG. (1996). Proton sensitivity of the $GABA_A$ receptor is associated with the receptor subunit composition. *J. Physiol.* **492**, 431-443.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

Law RJ, Henchman RH and McCammon JA. (2005). A gating mechanism proposed from a simulation of a human α7 nicotinic acetylcholine receptor. *PNAS* **102**, 6813-6818.

Lovick TA, Griffiths JL, Dunn SMJ and Martin IL. (2005). Changes in $GABA_A$ receptor subunit expression in the midbrain during the oestrus cycle in wistar rats. *Neuroscience* **131**, 397-405.

McKernan RM and Whiting PJ. (1996). Which $GABA_A$-receptor subtypes really occur in the brain? *TiNS* **19**, 139-143.

Melis C, Lummis SCR and Molteni C. (2008). Molecular dynamics simulations of GABA binding to the $GABA_C$ receptor: the role of Arg104. *Biophysical J.* **95**, 4115-4123.

Michaelson SD and Dunn SMJ. Activation of the human 5-HT_3A and 5-HT_3AB receptors by meta-chlorophenylbiguanide. Program No. 40.25. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience 2010. Online Abstract.

Mody I. (2001). Distinguishing between $GABA_A$ receptorsresponsible for tonic and phasic conductances. *Neurochem. Res.* **26**, 907-913.

Mokrab Y, Bavro VN, Mizuguchi K, Todorov NP, Martin IL, Dunn SMJ, Chan SL and Chau P-L. (2007). Exploring ligand recognition and ion flow in comparative models of the human GABA type A receptor. *J. Mol. Graphics and Modelling* **26**, 760-774.

Mortensen M and Smart TG. (2007). Single-channel recording of ligand-gated ion channels. *Nature Protocols* **2**, 2826-2841.

Mortensen M, Ebert B, Wafford K and Smart TG. (2010). Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. *J. Physiol.* **588**, 1251-1268.

Muroi Y, Czajkowski C and Jackson MB. (2006). Local and global ligand-induced changes in the structure of the $GABA_A$ receptor. *Biochemistry* **45**, 7013-7022.

Nusser Z, Sieghart W and Somogyi P. (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J. Neurosci*. **18**, 1693-1703.

Padgett CL, Hanek AP, Lester HA, Dougherty DA and Lummis SCR. (2007). Unnatural amino acid mutagenesis of the $GABA_A$ receptor binding site residues reveals a novel cation-π interaction between GABA and β2Tyr97. *J. Neurosci.* **27**, 886-892.

Rossi DJ and Hamann M. (1998). Spillover-mediated transmission at inhibitory synapses promoted by high affinity α_6 subunit $GABA_A$ receptors and glomerular geometry. *Neuron* **20**, 783-795.

Sander T, Frolund B, Bruun AT, Ivanov I, McCammon JA and Balle T. (2011). New insights into the $GABA_A$ receptor structure and orthosteric ligand binding: receptor modeling guided by experimental data. *Proteins* **79**, 1458-1477.

Saxena NC and Macdonald RL. (1996). Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. *Mol. Pharmacol.* **49**, 567-579.

Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy J-M, Mohler H and Luscher B. (2003). The γ2 subunit of $GABA_A$ receptors is required for maintenance of receptors at mature synapses. *Mol. Cell. Neurosci.* **24**, 442-450.

Shi J, Koeppe JR, Komives EA and Taylor P. (2006). Ligand-induced conformational changes in the acetylcholine-binding protein analyzed by hydrogen-deuterium exchange mass spectrometry. *J. Biol. Chem.* **281**, 12170-12177.

Sigel E, Baur R, Kellenberger S and Malherbe P. (1992). Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J* **11**, 2017-2023.

Smith KM, Ng AML, Yao SYM, Labedz KA, Knaus EE, Wiebe LI, Cass CE, Baldwin SA, Chen X-Z, Karpinski E and Young JD. (2004). Electrophysiological characterization of a recombinant human Na^+-coupled nucleoside transporter (hCN1) produced in *Xenopus* oocytes. *J. Physiol.* **558.3**, 807-823.

Storustovu S and Ebert B. (2006). Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ2. *JPET* **316**, 1351-1359.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR and McKernan RM. (1999). Preferential coassembly of α4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. *Mol. Pharmacol.* **56**, 110-115.

Todorov NP, Mancera RL, Monthoux PH. (2003). A new quantum stochastic tunnelling optimisation method for protein-ligand docking. *Chem. Physics Lett.* **369**, 257-263.

Tretter V, Hauer B, Nusser Z, Mihalek RM, Hoger H, Homanics GE, Somogyi P and Sieghart W. (2001). Targeted disruption of the $GABA_A$ receptor δ subunit gene leads to an up-regulation of γ2-containing receptors in cerebellar granule cells. *J. Biol. Chem.* **276**, 10532-10538.

Voet D and Voet JG. (2004). *Biochemistry, Third Edition*, 260-261. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Wafford KA and Ebert B. (2006). Gaboxadol – a new awakening in sleep. *Curr. Opin. Pharmacol.* **6**, 30-36.

Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D and Lambert JJ. (2009). Novel compounds selectively enhance δ subunit containing $GABA_A$ receptors and increase tonic currents in the thalamus. *Neuropharmacology* **56**, 182-189.

Wei W, Zhang N, Peng Z, Houser CR and Mody I. (2003). Perisynaptic localization of δ subunit-containing $GABA_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. *J. Neurosci.* **23**, 10650-10661.

Whiting PJ. (2003). GABA-A receptor subtypes in the brain: a paradigm for drug discovery? *Drug Discovery Today* **8**, 445-450.

Yeung JYT, Canning KJ, Zhu G, Pennefather P, Macdonald JF and Orser BA. (2003). Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol. Pharmacol.* **63**, 2-8.

CHAPTER 3

Developing a FRET Immunoassay to Detect Extrasynaptic $GABA_A$ Receptor Expression

INTRODUCTION

One of the major challenges in studying the physiology of $GABA_A$ receptors is that these receptors form heterogeneous populations of receptor subtypes with diverse functional characteristics (Olsen and Sieghart, 2008). On the positive side, the diversity of $GABA_A$ receptors increases the number of potential drug targets available for the development of pharmaceuticals that are either more effective or have a reduced side-effect profile compared to existing compounds. The class of receptors containing the δ subunit, which is comprised by the $\alpha_1\beta_x\delta$, $\alpha_4\beta_2\delta$, $\alpha_4\beta_3\delta$, and $\alpha_6\beta_3\delta$ subtypes (Farrant and Nusser, 2005; Glykys et al., 2007), have drawn increasing interest for their therapeutic potential in conditions ranging from insomnia to epilepsy, mood disorders, schizophrenia and Parkinson's disease (Winsky-Sommerer, 2009; Brickley and Mody, 2012). A major reason for their pharmacological potential is that receptors containing the δ subunit localize to extra- or perisynaptic compartments, where they mediate tonic inhibition in a number of neuronal populations (Nusser et al., 1998; Wei et al., 2003; Cope et al., 2005; Jia et al., 2005; Bright et al., 2011). Compounds that selectively enhance or inhibit tonic inhibition through δ-containing receptors can therefore modulate neuronal activity without directly targeting synaptic neurotransmission (Pavlov et al., 2009).

To date, a great deal of effort has been directed towards studying the pharmacology of $\alpha_4\beta_3\delta$ receptors, including studies in *Xenopus* oocytes (Storustovu and Ebert, 2006; You and Dunn, 2007) and mammalian cells

(Adkins et al., 2001; Brown et al., 2002; Wafford et al., 2009; Mortensen et al., 2010). However, there is still no direct evidence that $\alpha_4\beta_3\delta$ receptors exist *in vivo*. A technique that could identify which subunits associate with each other in a given receptor complex would be valuable for its ability to clarify which receptor subtypes are the most physiologically relevant for functional studies as well as for future drug development.

The work described in this chapter aimed to develop an assay using two-step FRET to detect the coassembly of up to three specific $GABA_A$ receptor subunits in a single complex. The short interaction distance of FRET makes it a reliable indicator for the incorporation of the fluorophores into the same protein complex. Incorporation of the fluorophores can be achieved in multiple ways, but the use of covalently modified antibody-dye conjugates is well-suited for the assay proposed here, as they can be introduced exogenously and bind to individual $GABA_A$ receptor subunits with high specificity. FRET-based immunoassays using two fluorophores have been used successfully to detect protein interactions in a variety of complexes, including the G-protein coupled $GABA_B$ receptor (Maurel et al., 2004). However, the study here focused on whether three specific subunits assemble into one complex (α_4, β_3, and δ), thus requiring the use of three separate fluorophores (Figure 3-1).

While previous studies have indicated the feasibility of this approach, two-step FRET has yet to be extended to the use of immunoassays (Watrob et al., 2003; Galperin et al., 2004; Lopez-Gimenez, 2007). Due to the complexity

of the technique, a proof-of-principle study was designed using tsA201 cells to express C-terminally epitope-tagged α_4(FLAG), β_3(V5) and δ(HA) subunits (Figure 3-2). These specific constructs were chosen as they form functional receptors *in vitro*, and produce subunit-specific binding in the presence of antibodies directed against each epitope (Barrera *et al*., 2008). The defined position of the epitope tags and relative ease of receptor expression using cell culture techniques were intended to provide a simpler system for optimizing the immunofluorescence and two-step FRET techniques. However, the results presented here show that the α_4(FLAG) and possibly the δ(HA) constructs appear to interfere with receptor expression, suggesting that a different system may be required to develop the two-step FRET protocol.

MATERIALS AND METHODS

Cell Culture and Transient Transfection

The cDNAs encoding C-terminal epitope-tagged α_4(FLAG), β_3(V5) and δ(HA) subunits had been prepared previously (Barrera et al., 2008). Cultures of tsA201 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% v/v fetal bovine serum (FBS) (Sigma-Aldrich) and passaged every 2-3 days. Prior to transfection, the medium was aspirated and replaced with DMEM containing 2% v/v FBS. For transfections, a total of 50 μg of cDNA for wild-type and/or mutant α_4, β_3 and δ subunits were mixed at varying ratios in 5 mL of DMEM without FBS. The cDNA solution was incubated with 75 μg of sterile-filtered polyethylenimine for 10 minutes and then added to the tsA cells. At 24 hours post-transfection cells were trypsinized and 1 x 10^5 cells were added to 35 mm dishes containing glass coverslips, while the remaining cells were replated for radioligand binding studies. Transfected cells were maintained in DMEM with 2% v/v FBS for another 16-24 hours at which point they were harvested for radioligand binding or fixed for confocal microscopy.

Radioligand Binding

Cells for radioligand binding were suspended in a harvest buffer of Tris-HCl (250 mM KCl, 50 mM Tris, 0.02% NaN_3, pH 7.4) with protease inhibitors (1 mM benzamidine, 0.1 mg/mL bacitracin, 0.01 mg/mL chicken

egg white trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride). Harvested cells were homogenized using an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany). Homogenates were recovered by centrifugation and resuspension in harvest buffer. Total binding was measured by incubating 200 μg/mL membrane protein with 20 nM [^{3}H]muscimol (PerkinElmer, Waltham, MA, USA) for 60 minutes on ice. Non-specific binding was measured by including 100 μM unlabelled GABA in a parallel series of incubations. Both incubation protocols were carried out in quadruplicates for each sample. Following incubation, unbound ligand was removed by vacuum filtration through Whatman GF/B filter paper using a cell harvester, and [^{3}H]muscimol activity was measured by liquid scintillation counting. Positive expression was defined as a significant increase ($p < 0.05$) in total [^{3}H]muscimol binding compared to non-specific binding using a Student's t-test in GraphPad Prism 5.0 (San Diego, CA, USA). The output of the Student's t-test also gave a 95% confidence interval for the specific binding value. This value was used to determine whether the amount of specific binding observed between experiments was significantly different; if the 95% confidence intervals overlapped then the difference in specific binding was not deemed significant.

Immunofluorescence

The anti-FLAG::Cy3 antibody-dye conjugate was purchased from Sigma-Aldrich, and the anti-HA::Alexa Fluor 488 and anti-V5::Alexa Fluor 647

antibodies were purchased from Invitrogen (Burlington, ON, Canada). Cells on coverslips were fixed using 10% formalin and blocked with 0.2% gelatin in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, adjusted to pH 7.4 with NaOH). Cells were incubated in a 1:600 dilution of the appropriate antibody by placing the coverslip face down on 100 μL antibody solution on parafilm for 90 minutes. The coverslips were then washed with blocking solution and finally PBS alone before mounting slides using ProLong Gold Antifade reagent (Invitrogen). Cells expressing tagged and untagged $\alpha_4\beta_3\delta$ receptors were imaged using spinning disc confocal microscopy and Volocity 6.0 software (PerkinElmer, Woodbridge, ON, Canada). Cells expressing wild-type $\alpha_4\beta_3\delta$ receptors were stained with the antibody-dye conjugates and imaged first to adjust the acquisition parameters to minimize signals originating from cellular autofluorescence and non-specific antibody staining. In some experiments, it was not clear whether a lack of cell surface expression was due to a lack of any expressed protein in the cell, or if significant intracellular receptor populations were being formed (ie. in the ER). To assess this possibility, cell membranes were permeabilized by incubating the coverslips in 0.1% Triton X-100 for 1 minute to allow for detection of intracellular receptor populations.

RESULTS

Radioligand binding results for cells transfected with 1:1:1 cDNA ratios are shown for single- and triple-tagged $\alpha_4\beta_3\delta$ receptor combinations in Figures 3-3 and 3-4. Significant specific [^{3}H]muscimol binding was seen with wild-type $\alpha_4\beta_3\delta$ receptors as well as the single-tagged $\alpha_4\beta_3$(V5)δ and $\alpha_4\beta_3\delta$(HA) receptors (all values significant at $p < 0.001$). Transfection of the α_4(FLAG) construct with wild-type β_3 and δ subunits failed to produce significant specific [^{3}H]muscimol binding. Similar results were seen in cells transfected with all three α_4(FLAG), β_3(V5), and δ(HA) subunit constructs. Cells were also transfected with 1:1 ratios of the α_4 and β_3 subunits without the δ subunit to attempt to improve expression of the α_4(FLAG) construct. Significant [^{3}H]muscimol binding was observed for wild-type and β_3(V5) tagged receptors, while inclusion of the α_4(FLAG) tag once again failed to produce specific binding (Figure 3-5).

Since $\alpha_4\beta_3$ receptors have been shown to assemble, both here (Figure 3-5) and elsewhere (Storustovu and Ebert, 2006), it is possible that significant binding may be observed in cells transfected with α_4, β_3, and δ subunit cDNAs without the δ subunit being incorporated into functional receptors. To account for this possibility, the ratio of the wild-type and HA-tagged δ subunit cDNA was increased to 1:1:4 to promote ternary $\alpha_4\beta_3\delta$ complex formation. Increasing the δ subunit ratio to 1:1:4 resulted in significant binding in cells expressing both $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\delta$(HA) receptors (p

< 0.001 for both) (Figure 3-6). The 95% confidence intervals for specific [^{3}H]muscimol binding did not overlap between the two experiments (375-858 dpm for δ wild-type and 230-313 dpm for δ(HA)), indicating that the specific binding observed for cells expressing $\alpha_4\beta_3\delta$(HA) receptors was significantly lower than that observed for the wild-type $\alpha_4\beta_3\delta$ receptor.

Incubating the anti-V5::Alexa Fluor 647 antibody with cells expressing $\alpha_4\beta_3$(V5)δ receptors produced a distinct fluorescence signal in 11 out of 48 cells imaged (Figure 3-7). The anti-FLAG::Cy3 antibody was tested against cells transfected with α_4(FLAG)$\beta_3\delta$ at a 1:1:1 cDNA ratio but failed to produce an observable signal with or without membrane permeabilization. Incubation with the anti-HA::Alexa Fluor 488 antibody with permeabilized cells transfected with $\alpha_4\beta_3\delta$(HA) receptors produced occasional bright staining when transfected at a 1:1:1 or 1:1:4 cDNA ratio (Figure 3-8), although the frequency was too low to determine accurately. Most cells showed staining both on the cell membrane and inside the cell, suggesting that the δ(HA) subunit may be retained in the ER.

Since the α_4(FLAG) subunit was never detected by immunofluorescence, the ability of the FLAG::Cy3 conjugate to bind to its epitope was evaluated independently by Mr. Greg Plummer. A positive control consisting of a FLAG-conjugated UBC9 protein demonstrated that this antibody recognizes the FLAG-tag and produces a fluorescence signal under the same conditions as these experiments (data not shown). Therefore the

lack of α_4(FLAG) staining is most likely due to low expression or improper folding of the protein, rather than a result of impaired antibody activity.

DISCUSSION

Expression of the Tagged Constructs

The radioligand binding experiments show that the α_4(FLAG) construct has a strong negative impact on receptor expression. Cells expressing wild-type $\alpha_4\beta_3$ or $\alpha_4\beta_3\delta$ receptors consistently displayed significant [^{3}H]muscimol binding, while inclusion of the α_4(FLAG) construct resulted in a lack of significant binding. One explanation is that the FLAG epitope tag interferes with the [^{3}H]muscimol binding site, which is proposed to lie at the interface between the β and α subunits (Sigel et al., 1992; Amin and Weiss, 1993; Smith and Olsen, 1994). However, the failure of both permeabilized and non-permeabilized cells expressing α_4(FLAG)$\beta_3\delta$ receptors to produce an observable signal with the fluorescent anti-FLAG::Cy3 conjugate suggests that the α_4(FLAG) subunit is not physically present in these cells to start with. This could arise through retention and degradation of the α_4(FLAG) protein in the ER, or the inability of the cells to take up and/or express the α_4(FLAG)-encoding cDNA. However, the latter possibility seems more likely, since no significant intracellular staining was observed in any of the cells.

In contrast to the α_4(FLAG) construct, transfecting cells with the δ(HA) construct at a 1:1:1 cDNA ratio does not significantly impair [^{3}H]muscimol binding. However, the data presented in Figure 3-5 shows that transfecting cells with the α_4 and β_3 subunits alone is sufficient for the formation of receptors that bind [^{3}H]muscimol. It is therefore possible that

the δ(HA) subunit is not incorporated into receptors and the [^{3}H]muscimol binding detected in these cells was due to the formation of $\alpha_4\beta_3$ receptors. To assess this possibility, the HA-tagged and wild-type δ cDNA ratio was increased to 1:1:4. As a result, the specific [^{3}H]muscimol binding with the δ(HA) subunit was found to decrease compared to the wild-type δ subunit. Since the relative amounts of the cDNAs were equivalent in both experiments, the most likely reason for the difference in specific [^{3}H]muscimol binding is incorporation of the HA-tagged versus the wild-type δ subunit into receptor complexes. This implies that in cells expressing $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\delta$(HA) receptors transfected at a 1:1:1 ratio, which showed similar levels of specific [^{3}H]muscimol binding, the δ(HA) subunit may not have been incorporated as efficiently into $\alpha_4\beta_3\delta$(HA) receptors.

Since increasing the δ(HA) subunit cDNA ratio caused a significant impact on [^{3}H]muscimol binding without abolishing it completely, it was possible that this was evidence for the formation of $\alpha_4\beta_3\delta$(HA) receptors with reduced [^{3}H]muscimol affinity. However, cells expressing $\alpha_4\beta_3\delta$(HA) receptors transfected at both 1:1:1 and 1:1:4 cDNA ratios failed to consistently produce staining following incubation with the anti-HA::Alexa Fluor 488 conjugate, suggesting that the δ(HA) protein was physically absent in most cells under both conditions. As with the α_4(FLAG) subunit, this could be due either to retention in the ER or a lack of cDNA uptake and expression. However, unlike the α_4(FLAG) construct, the δ(HA) construct did produce

visible staining in a small population of cells, providing evidence that the δ(HA) subunit can be expressed, albeit with low efficiency. The immunofluorescence staining pattern showed that a substantial portion of the δ(HA) protein is found intracellularly, which is consistent with retention in the ER. This provides one mechanism by which the δ(HA) subunit may decrease observed [^{3}H]muscimol binding by forming mis-folded complexes with the α_4 and/or β_3 subunits that are subsequently retained in the ER and degraded. The reduction of observed [^{3}H]muscimol binding could therefore be a result of degradation of the α_4 and/or β_3 subunits along with the δ(HA) subunit, which reduces the number of functional $\alpha_4\beta_3$ receptors available to bind to [^{3}H]muscimol. These results suggest that cDNA uptake and expression, as well as receptor assembly and cell surface trafficking, could be impaired with the δ(HA) subunit construct.

The only tagged construct that expresses consistently is that of the β_3(V5) subunit. The high levels of specific [^{3}H]muscimol binding in cells expressing the α_4, β_3(V5), and δ subunits indicates that at least $\alpha_4\beta_3$(V5) receptors are present, as the β_3 subunit requires co-expression with the α subunit to form muscimol binding sites (Davies et al., 1997). The presence of the β_3(V5) subunit was also confirmed by immunofluorescence staining, where a significant fraction of cells produced an obvious fluorescence signal following incubation with the anti-V5::Alexa Fluor 647 antibody. It should be noted that β_3 subunits form homo-oligomers, which could contribute to the

immunofluorescence observed in cells expressing $\alpha_4\beta_3$(V5)δ receptors (Taylor et al., 1999). However, β_3 homooligomers do not produce high affinity muscimol binding sites (Davies et al., 1997), while GABA fails to produce functional responses at β_3 receptors or displace [^{35}S]-TBPS binding as observed in other GABA-responsive receptors (Davies et al., 1997; Taylor et al., 1999). Thus, the β_3 homooligomers might have been detected in the immunofluorescence experiments, but they are not likely to have contributed to the observed specific [^{3}H]muscimol binding.

Potential Reasons for Low Expression of α_4(FLAG) and δ(HA) Constructs

It is not clear why the V5 tag is tolerated on the β_3 subunit, while the FLAG and HA tags interfere with α_4 and δ subunit expression. Folding and assembly of $GABA_A$ receptor subunits is a complicated process requiring a number of chaperone proteins as well as compatible receptor assembly signals between subunits (Barnes, 2000; Sarto-Jackson et al., 2008). Previous studies have found that homologous sequences encompassing loop D on the (-) face of α and β subunits as well as the (+) face of the α subunit are involved in receptor assembly, and that they are at least partially responsible for defining the specific subunit arrangement of the receptor (Sarto et al., 2002; Bollan et al., 2003). The C-terminal insertion of the epitope tags may place them in the general location of one or more of these assembly signals, which could interfere with the subunit-subunit contacts important for receptor formation. If this were the case, then placing the epitope tags at the

N-terminus could improve expression, though care would have to be taken to ensure that the N-terminal signal sequences are not also disrupted by insertion of the epitope tags.

Another possibility is that the specific HA and FLAG peptide structures interfere with subunit folding and/or receptor assembly to a greater extent than the V5 tag. The V5 tag notably consists of a high proportion of hydrophobic residues (GKPIPNPLLGLDST), while the HA (YPYDVPDYA) and FLAG (DYKDDDDK) sequences contain a high proportion of polar or charged residues. While it is not possible to say precisely how each epitope tag interacts with the surrounding receptor structure, it may be that hydrophobic sequences are tolerated better in the C-terminal region of the receptor. This would be another reason for trying N-terminal fusion tags, as the N-terminal region is exposed to solvent and may be more amenable to insertions containing polar and ionizable residues.

Another factor that could be reducing expression is the fact that, in addition to the subunit-specific epitope tags, a C-terminal hexa-his tag was included in all epitope-tagged constructs. The tags were included for the purposes of a previous experiment conducted using these constructs, which required purification of receptor complexes during sample preparation for atomic force microscopy (Barrera et al., 2008). It is possible that these tags also interfere with, or impair, receptor expression. A study using a variety of tags with 32 different human proteins has shown that the hexa-his tag has a propensity to promote the formation of aggregates (Hammorstrom et al.,

2002). In general, it does not appear that the hexa-his tag in particular is any worse than other affinity tags, but including it in addition to the subunit-specific epitope tags is likely to increase the probability that mis-folding or aggregation of the tagged protein will occur (Graslund et al., 2008). Given that the hexa-his tag is not required for these experiments, it may be beneficial to remove it from the subunit constructs before attempting further expression studies.

The fact that the α_4(FLAG) and δ(HA) subunit constructs do not express well is surprising, given that past work has shown that they express in mammalian cells (Barrera et al., 2008). One of the biggest differences between the current and past experiments appears to be the method of transfection. The current experiment used a polyethylenimine-mediated transfection protocol, as it has been shown to produce consistently high levels of expression from one batch of cells to the next (Aricescu et al, 2006). In contrast, the calcium-phosphate transfection protocol used by Barrera et al. requires careful optimization of the proton, calcium, and phosphate concentrations, at which point it can still give mixed results from one batch of cells to the next (Jordan et al., 1996). The molecular mechanisms underlying the transfection of mammalian cells are still not completely understood, and despite the drawbacks of the calcium-phosphate method, it is possible that the means by which exogenous cDNA is introduced could impact subsequent protein expression. It may be worth investigating the different methods of

transfection more closely to determine whether they impact the expression of the tagged subunits.

The data presented here indicate that the epitope-tagged constructs were not expressed efficiently in tsA201 cells, and that some aspect of the system may need to be altered before proceeding to optimization of the FRET protocol. Future work could focus on using other transfection methods, such as the calcium-phosphate transfection protocol, or modifying the receptor subunit constructs. Modifications could include removing the hexa-his tag and/or moving the α_4(FLAG) and δ(HA) tags to the N-terminus. A final possibility would be to eschew the advantages of using the epitope tags and proceed straight to immunofluorescence with native $GABA_A$ receptor subunits, which were seen in these experiments to produce robust expression that can be reliably detected using [^{3}H]muscimol binding.

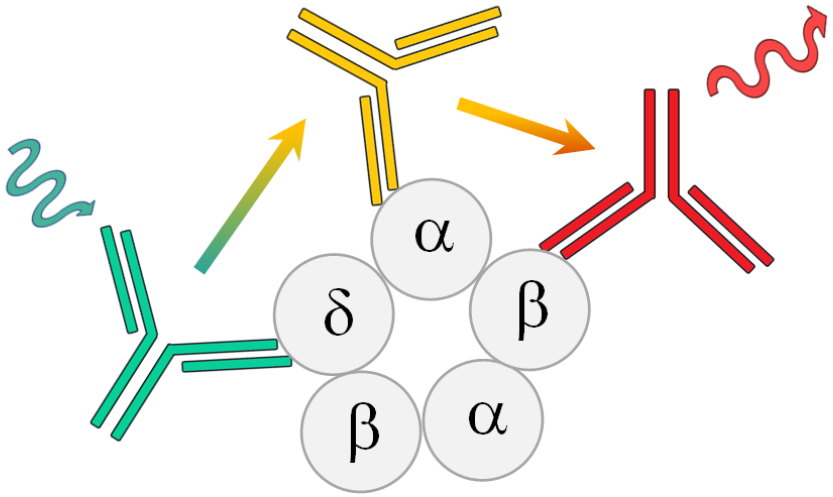


Figure 3-1. Schematic representation of the proposed three-way immuno-FRET assay. Antibody-dye conjugates would be used to detect the expression of epitope-tagged α_4(FLAG)β_3(V5)δ(HA) receptors. The antibody::fluorophore conjugates are as follows: anti-HA::Alexa Fluor 488, anti-FLAG::Cy3 and anti-V5::Alexa Fluor 647. Incident and emitted photons are depicted by oscillating arrows, while the direction of energy transfer is depicted by straight arrows.

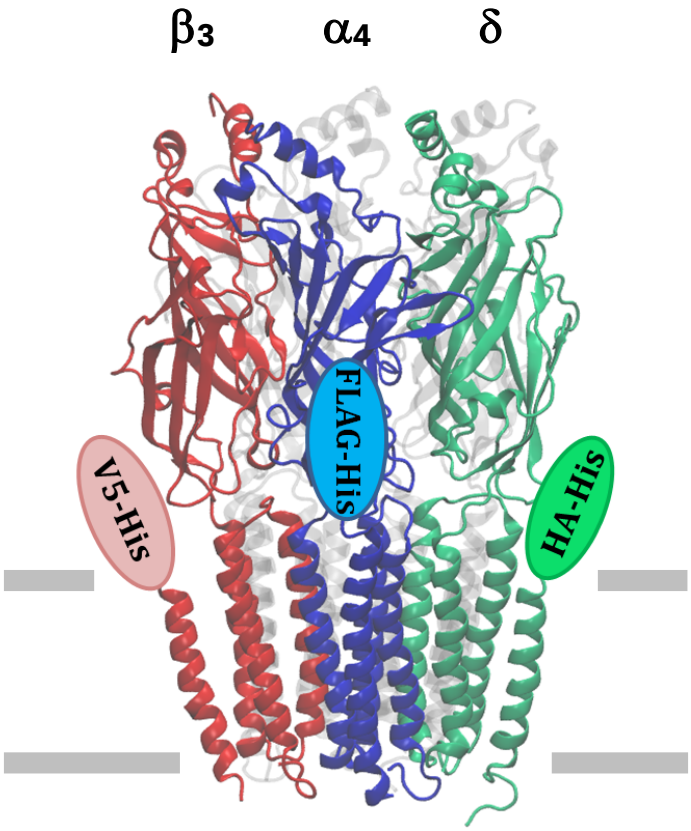


Figure 3-2. Approximate location of epitope tags in α_4(FLAG), β_3(V5), and δ(HA) constructs. The ribbon diagram shows the structure of the $\alpha_4\beta_3\delta$ receptor homology model. The α_4 subunit is coloured blue, β_3 is red, and δ is green. The coloured ovals represent the approximate location of the C-terminal epitope tags on each subunit. The grey lines show the approximate location of the cell membrane. The pdb coordinates for the receptor structure were obtained courtesy of Dr. Younes Mokrab.

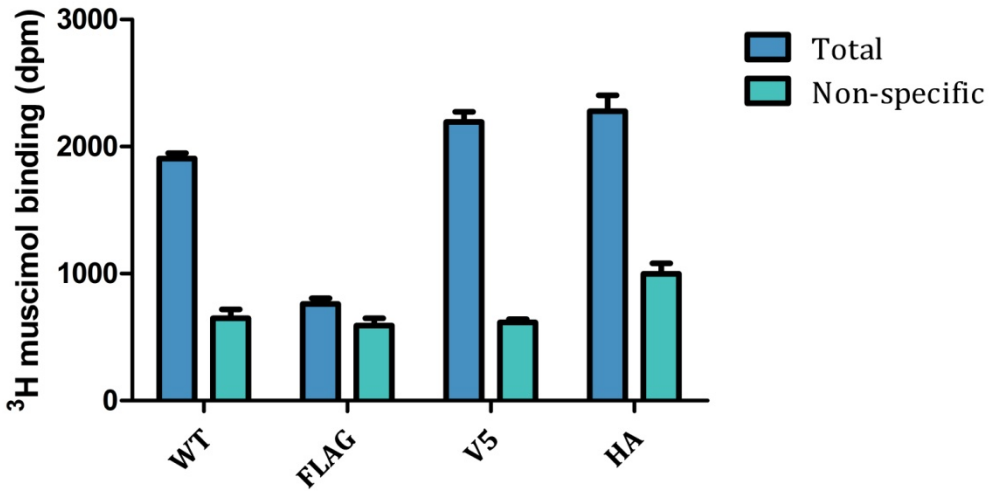


Figure 3-3. [^{3}H]muscimol binding to cell membranes expressing single-tagged α_4, β_3 and δ subunits. WT stands for all wild-type subunits, while FLAG, V5, and HA refer to whether the α_4(FLAG), β_3(V5) or δ(HA) subunit constructs were substituted for the corresponding native subunit. The error bars represent the SEM for ^{3}H muscimol binding, measured in decays per minute, for 4 replicate samples.

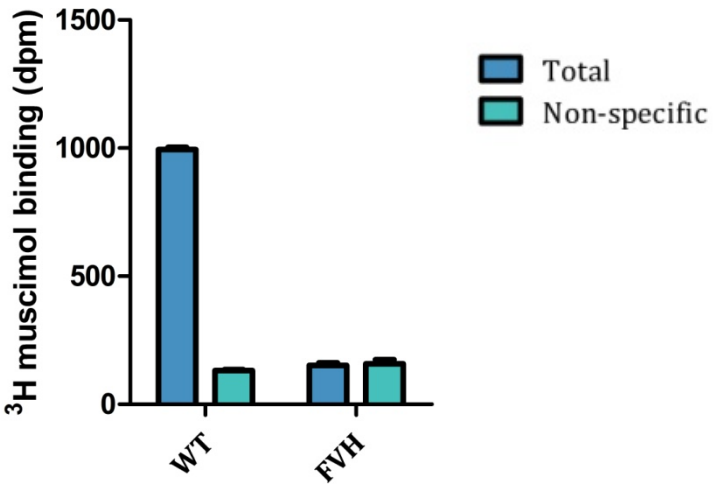


Figure 3-4. [^{3}H]muscimol binding to cell membranes expressing triple-tagged α_4, β_3 and δ subunits. WT stands for all wild-type subunits, while FVH refers to the incorporation of all three of the α_4(FLAG), β_3(V5) and δ(HA) epitope-tagged subunits. The error bars represent the SEM for ^{3}H muscimol binding, measured in decays per minute, for 4 replicate samples.

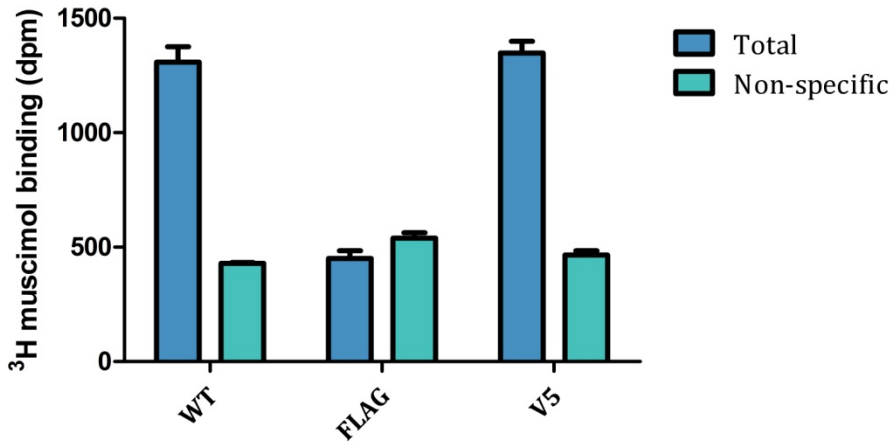


Figure 3-5. [^{3}H]muscimol binding to cell membranes expressing equal ratios of α_4 and β_3 subunit cDNAs. WT stands for all wild-type subunits, while FLAG, and V5 refer to whether the α_4(FLAG) or β_3(V5) subunit constructs were substituted for the corresponding native subunit. The error bars represent the SEM for ^{3}H muscimol binding, measured in decays per minute, for 4 replicate samples.

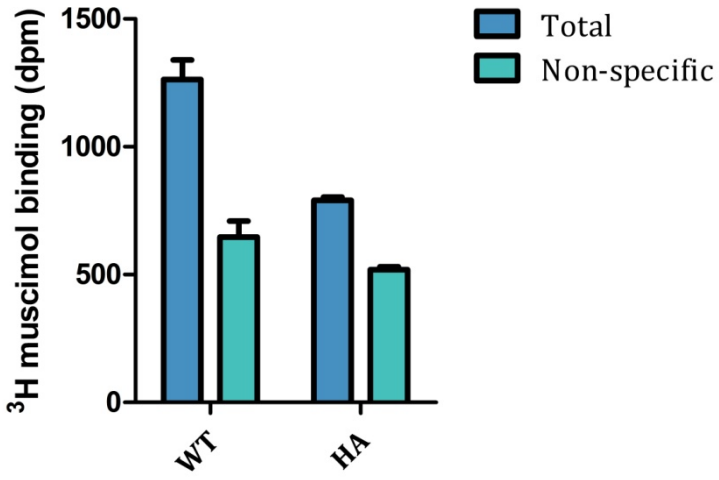


Figure 3-6. [^{3}H]muscimol binding to cell membranes expressing α_4 and β_3 subunit cDNAs with either the wild-type (WT) or HA-tagged (HA) δ subunit. Cells were transfected using 1:1:4 cDNA ratios in both cases. The error bars represent the SEM for ^{3}H muscimol binding, measured in decays per minute, for 4 replicate samples.

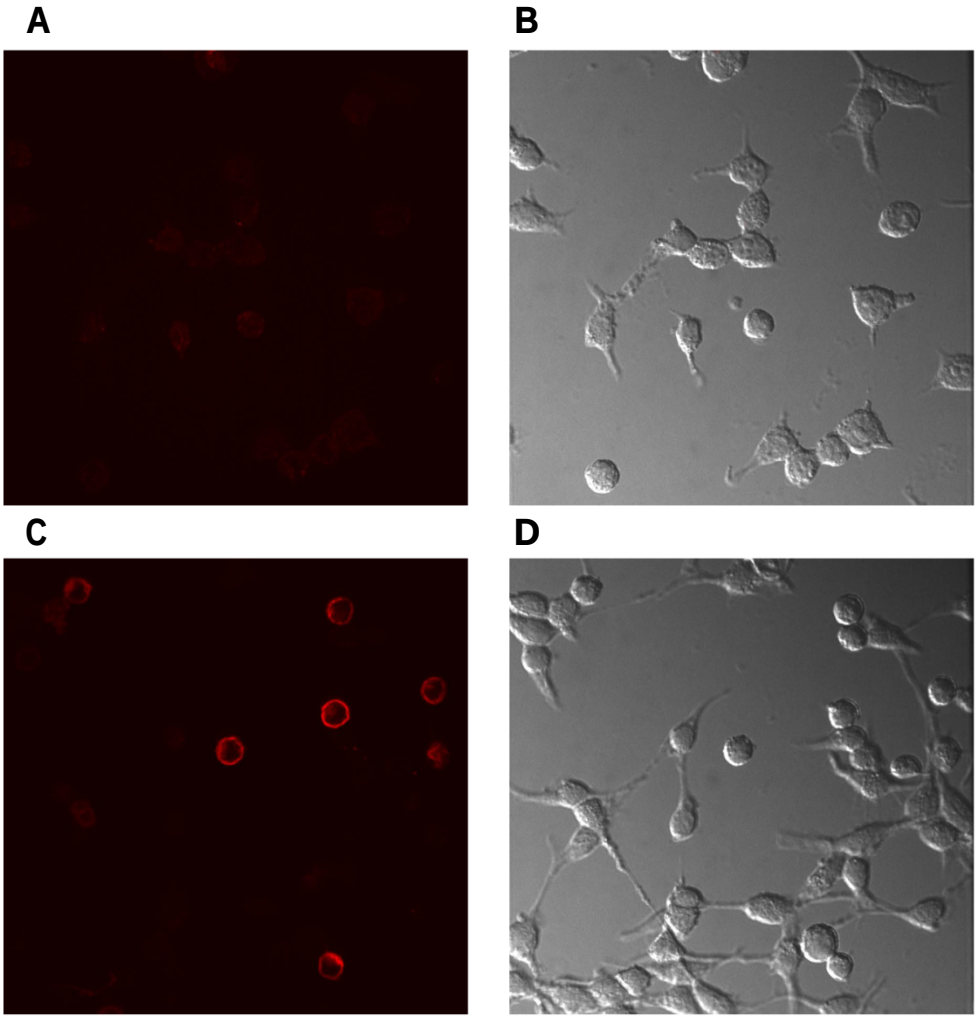


Figure 3-7. Immunofluorescence imaging of $\alpha_4\beta_3$(V5)δ receptors. (A and C) Confocal images of cells expressing wild-type $\alpha_4\beta_3\delta$ (A) or $\alpha_4\beta_3$(V5)δ receptors (C), following incubation with the anti-V5::Alexa Fluor 647 conjugate. (B and D) Differential interference contrast images of the same cells in (A) and (C), respectively.

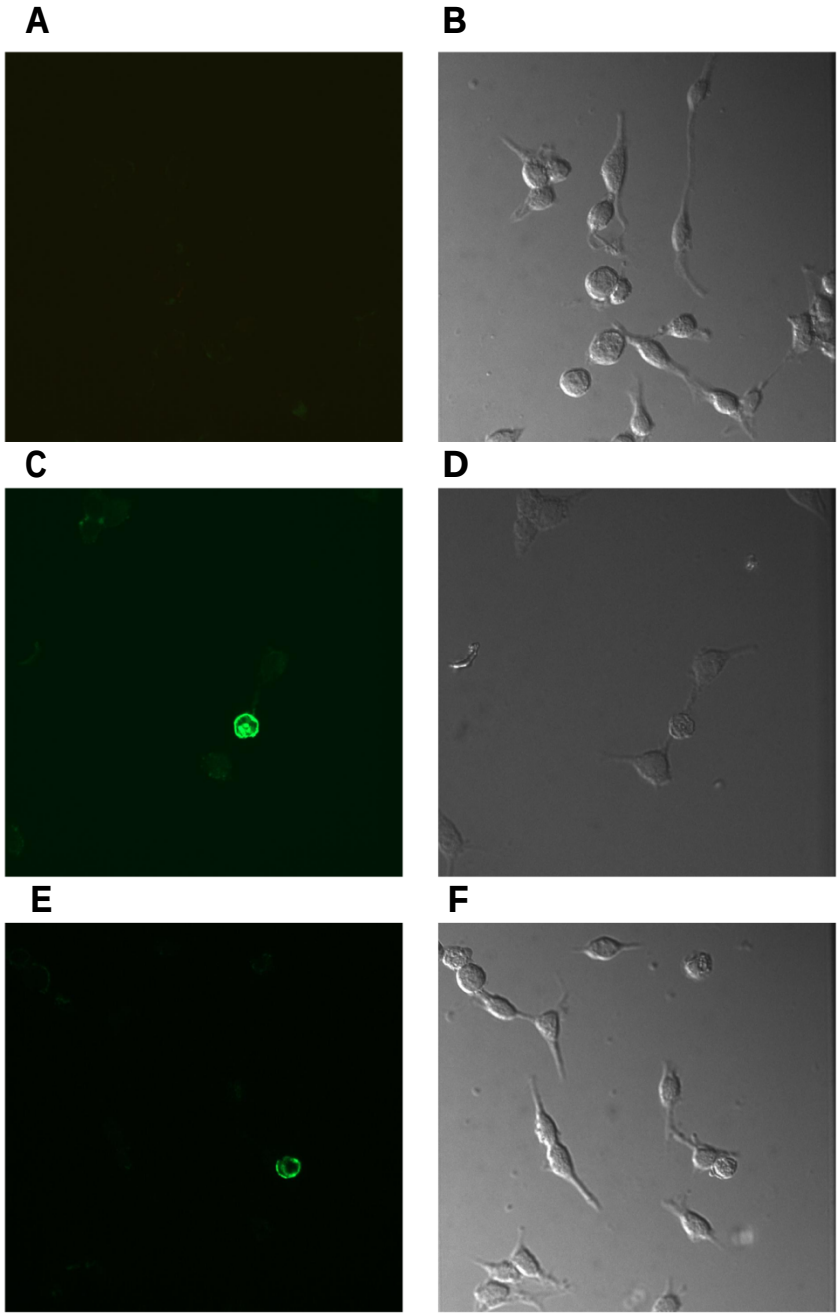


Figure 3-8. Immunofluorescence imaging of $\alpha_4\beta_3\delta$(HA) receptors. (A, C and E) Cells expressing $\alpha_4\beta_3\delta$ cDNAs at a 1:1:1 ratio (A) and $\alpha_4\beta_3\delta$(HA) cDNAS at 1:1:1 (C) and 1:1:4 (E) cDNA ratios. (B, D, and F) Differential interference contrast images of the same cells in (A), (C), and (E), respectively.

REFERENCES

Adkins CE, Pillai GV, Kerby J, Bonnert TP, Haldon C, McKernan RM, Gonzalez JE, Oades K, Whiting PJ and Simpson PB. (2001). $\alpha4\beta3\delta$ $GABA_A$ receptors characterized by fluorescence resonance energy transfer-derived measurements of membrane potential. *J. Biol. Chem.* **276**, 38934-38939.

Amin J and Weiss DS. (1993). $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature* **366**, 565-569.

Aricescu AR, Lu W and Jones EY. (2006). A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallographica Section D* **62**, 1243-1250.

Barnes EM. (2000). Intracellular trafficking of $GABA_A$ receptors. *Life Sci.* **66**, 1063-1070.

Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SMJ and Edwardson JM. (2008). Atomic force microscopy reveals the stoichiometry and subunit arrangement of the $\alpha_4\beta_3\delta$ $GABA_A$ receptor. *Mol. Pharmacol.* **73**, 960-967.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ and Connolly CN. (2003). $GABA_A$ receptor composition is determined by distinct assembly signals with α and β subunits. *J. Biol. Chem.* **278**, 4747-4755.

Brickley SG and Mody I. (2012). Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* **73**, 23-34.

Bright DP, Renzi M, Bartram J, McGee TP, MacKenzie G, Hosie AM, Farrant M and Brickley SG. (2011). Profound desensitization by ambient GABA limits activation of δ-containing GABAA receptors during spillover. *J. Neurosci.* **31**, 753-763.

Brown N, Kerby J, Bonnert TP, Whiting PJ and Wafford KA. (2002). Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br. J. Pharmacol.* **136**, 965-974.

Cope DW, Hughes SW and Crunelli V. (2005). $GABA_A$ receptor-mediated tonic inhibition in thalamic neurons. *J. Neurosci.* **25**, 11553-11563.

Davies PA, Kirkness EF and Hales TG. (1997). Modulation by general anaesthetics of rat $GABA_A$ receptors comprised of $\alpha 1\beta 3$ and $\beta 3$ subunits expressed in human embryonic kidney 293 cells. *Br. J. Pharmacol.* **120**, 899-909.

Farrant M and Nusser Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nature Rev.* **6**, 215-229.

Galperin E, Verkhusha VV and Sorkin A. (2004). Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nature Methods* **1**, 209-217.

Glykys J, Peng Z, Changda D, Homanics GE, Houser CR and Mody I. (2007). A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nature Neurosci.* **10**, 40-48.

Graslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, et al. (2008). Protein production and purification. *Nature Methods* **5**, 135-146.

Hammorstrom M, Hellgren N, van den Berg S, Berglund H and Hard T. (2002). Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*. *Protein Sci.* **11**, 313-321.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL and Goldstein PA. (2005). An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. *J. Neurophysiol.* **94**, 4491-4501.

Jordan M, Schallhorn A and Wurm FM. (1996). Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucl. Acids Res.* **24**, 596-601.

Lopez-Gimenez JF, Canals M, Pediana JD and Milligan G. (2007). The α1b-adrenoceptor exists as a higher-order oligomer: effective oligomerization is required for receptor maturation, surface delivery, and function. *Mol. Pharmacol.* **71**, 1015-1029.

Maurel D, Kniazeff J, Mathis G, Trinquet E, Pin J-P and Ansanay H. (2004). Cell surface detection of membrane protein interaction with homogeneous time-resolved fluorescence resonance energy transfer technology. *Anal. Biochem*. **329**, 253-262.

Mortensen M, Ebert B, Wafford K and Smart TG. (2010). Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. *J. Physiol.* **588**, 1251-1268.

Nusser Z, Sieghart W and Somogyi P. (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J. Neurosci*. **18**, 1693-1703.

Olsen RW and Sieghart W. (2008). International union of pharmacology. LXX. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev.* **60**, 243-260.

Pavlov I, Savtchenko LP, Kullmann DM, Semyanov A and Walker MC. (2009). Outwardly rectifying tonically active $GABA_A$ receptors in pyramidal cells modulate neuronal offset, not gain. *J. Neurosci.* **29**, 15341-15350.

Sarto I, Wabnegger L, Dogl E and Sieghart W. (2002). Homologous sites of $GABA_A$ receptor α_1, β_3 and γ_2 subunits are important for assembly. *Neuropharmacology* **43**, 482-491.

Sarto-Jackson I and Sieghart W. (2008). Assembly of $GABA_A$ receptors (review). *Mol. Mem. Biol.* **25**, 302-310.

Sigel E, Baur R, Kellenberger S and Malherbe P. (1992). Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J* **11**, 2017-2023.

Smith GB and Olsen RW. (1994). Identification of a [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric $acid_A$ receptor α subunit. *J. Biol. Chem.* **269**, 20380-20387.

Storustovu S and Ebert B. (2006). Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ2. *JPET* **316**, 1351-1359.

Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG and Moss SJ. (1999). Identification of amino acid residues within $GABA_A$ receptor β subunits that mediate both homomeric and heteromeric receptor expression. *J. Neurosci.* **19**, 6360-6371.

Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D and Lambert JJ. (2009). Novel compounds selectively enhance δ subunit containing $GABA_A$ receptors and increase tonic currents in the thalamus. *Neuropharmacology* **56**, 182-189.

Watrob HM, Pan C-P and Barkley MD. (2003). Two-step FRET as a structural tool. *J. Am. Chem. Soc.* **125**, 7336-7343.

Wei W, Zhang N, Peng Z, Houser CR and Mody I. (2003). Perisynaptic localization of δ subunit-containing $GABA_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. *J. Neurosci.* **23**, 10650-10661.

Winsky-Sommerer R. (2009). Role of $GABA_A$ receptors in the physiology and pharmacology of sleep. *Eur. J. Neurosci.* **29**, 1779-1794.

You H and Dunn SMJ. (2007). Identification of a domain in the δ subunit (S238-V264) of the α4β3δ $GABA_A$ receptor that confers high agonist sensitivity. *J. Neurochem.* **103**, 1092-1101.

CHAPTER 4

General Discussion

GENERAL DISCUSSION

The major theme that has emerged in the $GABA_A$ receptor field over the last 25 years is one of molecular diversity. The isolation and cloning of the first α and β subunits were followed by a period of rapid discovery as 17 additional subunits were identified in just over a decade. The full complement of subunits can assemble to produce a considerable number of different receptor subtypes, with estimates suggesting that the number of subtypes could be as high as 500-800 (Barnard et al., 1998; Sieghart and Sperk, 2002). The receptor subtypes underlying tonic inhibition, particularly δ-containing receptors, have emerged as candidates for drug development due to their unique pharmacological properties and potential for treating conditions such as hormone-related mood disorders, insomnia, and epilepsy (Brickley and Mody, 2012).

Identification of the major δ-containing receptor subtypes is an ongoing process that still faces many challenges. Perhaps the strongest associations have been found between the α_6 and δ subunits, which are thought to form approximately 20-30% of the receptors in cerebellar granule cells (Jechlinger et al., 1998; Poltl et al., 2003). Evidence for the formation of receptors containing α_4 and δ subunits is relatively strong, as these two subunits are extensively colocalized in a number of brain regions (Wisden et al., 1992; Pirker et al., 2000) and have been shown to interact through immunoprecipitation of receptor complexes from the cortex, thalamus, and dentate gyrus (Bencsits et al., 1999; Sur et al., 1999; Jia et al., 2005). Recently,

it has been found that hippocampal interneurons express the δ subunit in the absence of the α_4 subunit, suggesting that an α_1/δ receptor subtype mediates tonic inhibition there. However, identification of the β subunit in these complexes has been more elusive and has generally relied on functional studies using subunit-specific genetic deletions (Huntsman et al., 1999; Herd et al., 2008; Peden et al., 2008). Immunoprecipitation of receptor complexes has also shown that multiple α and β subunits may be incorporated in some complexes, adding further complexity to the identification of specific receptor populations (Benke et al., 1997; Jechlinger et al., 1998; Bencsits et al., 1999; Poltl et al., 2003).

While falling short of detecting all five subunits in a receptor complex, the approach outlined in Chapter 3 fills a long-standing need to probe the subunit architecture of $GABA_A$ receptor subunits *in vivo*. For instance, by assaying for two-step FRET using fluorescent antibody conjugates targeting the α_4, δ, and either β_1, β_2 or β_3 subunits, it would be possible to test directly the hypothesis that the β_2 subunit is preferentially incorporated into $\alpha_4\beta_x\delta$ receptors in the thalamus and dentate gyrus (Herd et al., 2008; Peden et al., 2008). An additional advantage is the ability to assess the subunit complement of receptors *in situ*, without requiring genetic knockouts or purification of receptor complexes from tissue preparations. This experimental protocol could be extended to other regions such as the striatum and basal ganglia, where $\alpha_4\beta_x\delta$ receptors are likely expressed, but

data on the direct association of these subunits *in vivo* is limited (Schwarzer et al., 2001; Goetz et al., 2007).

A more general application of the proposed assay would be to assess the expression of receptor subtypes that are less common, but may be functionally important in discrete neuronal populations. While the association of the α_4/δ or α_6/δ subunits appears to be preferred for receptor assembly, the finding that δ-containing receptor subtypes form with other α subunits suggests that this is not a strict requirement (Glykys et al., 2007). This is consistent with the suggestion that subunits may assemble with each other through a number of distinct pathways, and that less favourable receptor complexes may only express when preferred subunit partnerships are not present (Bollan et al., 2003). This may be relevant for regions of the brain such as the subthalamus, hypothalamus, midbrain, and pons, where δ subunit immunoreactivity has been detected in the absence of α_4 and α_6 subunits (Pirker et al., 2000; Schwarzer et al., 2001).

Another issue that has presented a significant challenge for the study of δ-containing receptors has been in determining their subunit stoichiometry and arrangement once assembled. The most direct assessment on the arrangement of $\alpha_4\beta_3\delta$ receptors to date is a study using AFM to visualize the binding of antibodies directed against individual subunits (Barrera et al., 2008). This study deduced an arrangement of $\alpha\beta\alpha\delta\beta$, which is analogous to that proposed for the $\alpha_1\beta_2\gamma_2$ receptor. A concatameric construct for the expression of $\alpha_4\beta_2\delta$ receptors has also demonstrated that the $\alpha\beta\alpha\delta\beta$

arrangement produces receptors with characteristics similar to those expected for α_4/δ receptors, suggesting that this subunit arrangement is functionally relevant (Shu et al., 2012). However, other studies found that δ-containing receptors can form with multiple different subunit arrangements, and that in some of these complexes the δ subunit may even contribute to the formation of a functional binding site (Baur et al., 2010; Kaur et al., 2009; Karim et al., 2012).

This problem has been further complicated by the significant variations observed between labs when characterizing $\alpha_4\beta_x\delta$ receptors; including large differences in GABA potency (Mortensen et al., 2012; Karim et al., 2012), THIP potency (Storustovu and Ebert, 2006; You and Dunn, 2007; Meera et al., 2011), and ethanol sensitivity (Wallner et al., 2003; Hanchar et al., 2006; Borghese et al., 2007). Recently, a group has published observations with the $\alpha_4\beta_3\delta$ receptor that differ markedly from those made in Chapter 2, despite using a similar experimental protocol using *Xenopus* oocytes with a two-electrode voltage clamp and bath-applied agonist to characterize functional responses (Karim et al., 2012). In their study, Karim et al. characterized the activity of GABA at $\alpha_1\beta_3$, $\alpha_1\beta_3\delta$, $\alpha_4\beta_{1\text{-}3}$, $\alpha_6\beta_{1\text{-}3}$, $\alpha_4\beta_{1\text{-}3}\delta$, and $\alpha_6\beta_{1\text{-}3}\delta$ receptors. For the $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ receptors, two distinct receptor populations were observed with GABA EC_{50} values of approximately 1 μM and 10 nM; the latter value being about 100-fold lower than what has been seen in any previous studies. Their observation that $\alpha_4\beta_3\delta$ receptors form distinct sub-populations is consistent with a previous study that also

observed two distinct receptor sub-populations, one of which produced EC_{50} values for THIP responses in the nanomolar range (Meera et al., 2011).

The biphasic concentration-response curves for GABA and THIP that result from these distinct receptor populations were not observed in the experiments reported in Chapter 2, or by a number of other groups that have done rigorous controls to demonstrate δ subunit incorporation in a variety of expression systems, including *Xenopus* oocytes (Storustovu et al., 2006; Borghese et al., 2007). This suggests there may be some factor(s) other than δ-subunit expression influencing the production of such high affinity $\alpha_4\beta_3\delta$ receptors, including phosphorylation of the intracellular domain by PKA or PKC or altered subunit arrangement and/or stoichiometry of the assembled receptors. Underlying these discrepancies may be that many groups use increased δ cRNA/cDNA ratios to force δ subunit incorporation (Wallner et al., 2003; Borghese et al., 2006; Storustovu and Ebert, 2006; Meera et al., 2011; Karim et al., 2012). A study in HEK293 cells found that transfections using a 2:1:0.25 subunit cDNA ratio give rise to receptors with a stoichiometry of $2\alpha_4$:$2\beta_2$:1δ, while transfections using a 2:1:5 subunit cDNA ratio resulted in receptors with a stoichiometry of $1\alpha_4$:$1\beta_2$:3δ (Wagoner and Czajkowski, 2010). Therefore, while it is often assumed that increasing the ratio of δ subunit cRNA yields homogeneous $\alpha_4\beta_3\delta$ receptor populations, this may result in unintended receptor complexes that are not normally found *in vivo*.

The study by Karim et al. (2012) also tested the impact of α_4, β_3, and δ subunit mutations on GABA activity at $\alpha_4\beta_3\delta$ receptors, including the α_4(F98L) mutation (note that the numbering system in their paper differs from the one used here). Despite the large discrepancy in EC_{50} values observed for GABA at the wild-type receptor, their study also found that the α_4(F98L) mutation does not have a large impact on GABA potency. On the β subunit, they found that β_3(Y205A) and β_3(R207A) mutations produced over 10,000-fold increases in GABA EC_{50} values, while the β_3(Y97A) mutation had little impact. This led to the conclusion that the (+) face of the β_3 subunit is important for agonist activity at $\alpha_4\beta_3\delta$ receptors, as it is in other receptor subtypes, but that GABA may act through a unique binding mode compared to other receptors (Karim et al., 2012). The work here suggests that this conclusion may need to be modified, as it supports the involvement of the α_4(F98) residue in agonist activity and reinforces the general mechanism for GABA activity at the β/α interface. However, their observation that the δ(R218A) mutation resulted in a 670-fold shift in GABA EC_{50} suggests that there may be an additional functional GABA site on the (+) face of the δ subunit (Karim et al., 2012). Given the possibility that the δ subunit may assume multiple positions in $\alpha_4\beta_3\delta$ receptors, it cannot be discounted that the α_4 subunit also participates in binding at δ/α_4 interfaces in some receptors.

CONCLUSIONS AND FUTURE DIRECTIONS

The work described in Chapter 2 was an attempt to assess whether residues known to be important for GABA activity at the $\alpha_1\beta_2\gamma_2$ receptor are also important in extrasynaptic $\alpha_4\beta_3\delta$ receptors, and whether these residues play a similar role for the activity of the subtype-selective agonist THIP. The functional data obtained with the α_1(F92L), α_4(F98L), and α_4(F98A) mutations are consistent with the idea that the α_1(F92) and α_4(F98) residues both play a structural role in the agonist binding site. In contrast to previous work on the α_1(F92) residue, these residues also appeared to contribute to channel gating, as the efficacy of THIP compared to that of GABA was altered by both the α_1(F92L) and α_4(F98A) mutations (Sigel et al., 1992). Single channel analysis may be useful to identify which steps of agonist binding or receptor activation are affected by these mutations (Mortensen and Smart, 2007; Goldschen-Ohm, 2011). Such analysis may reveal information about the mode of binding for each agonist and whether they act at each receptor subtype through equivalent mechanisms. The data obtained for the α_1(Q95E) and α_4(Q101E) mutations showed that they had small but qualitatively opposite effects on the potency for GABA activation. This could suggest that the context of the β_2/γ_2 versus the β_3/δ subunits influences the functional role of these residues.

Interpretation of the data produced by this study was facilitated by the recent advances in the structural determination of the nACh receptor and AChBP, which allowed the $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ $GABA_A$ receptors to be modelled

based on their homology to these two proteins (Mokrab et al., 2007). The docking data produced using these models complemented the functional studies by showing that THIP activity may be mediated by a second subsite located 5-10 Å away from that of GABA at the β/α interface. Candidate residues that may be involved in this unique binding site are the β_2(Y183) and β_3(Y184) residues, which were seen to interact with THIP in the docked poses. Mutation of these residues to phenylalanine and/or leucine would provide some insight into the role of this putative subsite in agonist activity at $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_3\delta$ receptors.

As outlined in the above discussion, there are a number of valid questions that can be addressed by the technique outlined in Chapter 3. However, since poor expression limited the progress that could be made on the proposed FRET system using epitope-tagged α_4(FLAG), β_3(V5), and δ(HA) subunits, it was not possible to carry out definitive proof-of-principle studies. Perhaps the biggest question that remains moving forward is whether the size of the molecules involved are within the limits at which FRET can be reliably measured. The maximum distance at which energy transfer can usually be detected is approximately 100-120 Å, while the radius of the $GABA_A$ receptor extracellular domain is approximately 80 Å and the antibodies themselves can span up to 160 Å (Harris et al., 1997). This problem is compounded by the requirement that three labeled antibodies bind to a receptor complex for a signal to be observed. Barrera et al. (2008) found that in $\alpha_4\beta_3\delta$ receptors containing a hexa-his tag on each subunit, only

2.5 % of the receptors were bound by three or more anti-his antibodies. The resulting combination of low 2-step FRET efficiency and low proportion of receptor complexes bound by three antibodies might strain the limits for reliable detection. However, the impact of this problem cannot be assessed until a reliable expression system is in place.

It is apparent that $GABA_A$ receptor subtypes are still poorly understood, particularly the δ-containing receptors. It is somewhat surprising that, despite the fact that the $\alpha_4\beta_3\delta$ is already being used as a model for the development of subtype-selective compounds (Wafford et al., 2009; Hoestgaard-Jensen et al., 2010), the existence of this receptor *in vivo* has yet to be proven unequivocally. In fact, as studies have accumulated using genetic knockout animals, it appears possible that the $\alpha_4\beta_3\delta$ receptor may not normally contribute to tonic inhibition in the brain (Herd et al., 2008; Peden et al., 2008). The literature currently contains conflicting lines of experimental data for the activity of GABA (Karim et al., 2012), THIP (Meera et al., 2011), and ethanol (Borghese et al., 2007) at recombinant $\alpha_4\beta_3\delta$ receptors, highlighting problems with obtaining proper expression with *in vitro* systems as well. Moving forward, it is imperative that answers are found for basic questions such as which receptors are expressed *in vivo* and in what form they are expressed. This will facilitate future studies aimed at determining how given $GABA_A$ receptor subtypes influences neuronal signaling in the brain.

As molecular studies have advanced over the last 25 years, it has become apparent that a number of important drugs act at $GABA_A$ receptors through a variety of mechanisms and distinct binding sites. The work described here aimed to elucidate some of the details of drug binding at a model synaptic and extrasynaptic receptor, and to develop an assay to study the expression of distinct receptor subtypes at a greater level of detail. Further progress on both of these fronts is expected to contribute to the rational development of pharmacological agents that improve on the selectivity, efficacy, and safety of those currently used for a wide range of clinical interventions.

REFERENCES

Adkins CE, Pillai GV, Kerby J, Bonnert TP, Haldon C, McKernan RM, Gonzalez JE, Oades K, Whiting PJ and Simpson PB. (2001). $\alpha4\beta3\delta$ $GABA_A$ receptors characterized by fluorescence resonance energy transfer-derived measurements of membrane potential. *J. Biol. Chem.* **276**, 38934-38939.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN and Langer SZ. (1998). International union of pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharm. Rev.* **50**, 291-313.

Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SMJ and Edwardson JM. (2008). Atomic force microscopy reveals the stoichiometry and subunit arrangement of the $\alpha_4\beta_3\delta$ $GABA_A$ receptor. *Mol. Pharmacol.* **73**, 960-967.

Baur R, Kaur KH and Sigel E. (2010). Diversity of structure and function af $\alpha1\alpha6\beta3\delta$ $GABA_A$ receptors. *J. Biol. Chem.* **285**, 17398-17405.

Bencsits E, Ebert V, Tretter V and Sieghart W. (1999). A significant part of native γ-aminobutyric $acid_A$ receptors containing $\alpha4$ subunits do not contain γ or δ subunits. *J. Biol. Chem.* **274**, 19613-19616.

Benke D, Michel C and Mohle H. (1997). $GABA_A$ receptors containing the $\alpha4$-subunit: prevalence, distribution, pharmacology, and subunit architecture in situ. *J. Neurochem.* **69**, 806-814.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ and Connolly CN. (2003). $GABA_A$ receptor composition is determined by distinct assembly signals with α and β subunits. *J. Biol. Chem.* **278**, 4747-4755.

Borghese CM, Storustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA and Harris RA. (2006). The δ subunit of γ-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. *JPET* **316**, 1360-1368.

Borghese CM and Harris RA. (2007). Studies of ethanol actions on recombinant δ-containing γ-aminobutyric acid type A receptors yield contradictory results. *Alcohol* **41**, 155-162.

Brickley SG and Mody I. (2012). Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* **73**, 23-34.

Glykys J, Peng Z, Changda D, Homanics GE, Houser CR and Mody I. (2007). A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nature Neurosci.* **10**, 40-48.

Goetz T, Arslan A, Wisden W and Wulff P. (2007). $GABA_A$ receptors: structure and function in the basal ganglia. *Prog. Brain Res.* **160**, 21-41.

Goldschen-Ohm MP, Wagner DA and Jones MV. (2011). Three arginines in the $GABA_A$ receptor binding pocket have distinct roles in the formation and stability of agonist- versus antagonist-bound complexes. *Mol. Pharmacol.* **80**, 647-656.

Hanchar HJ, Chutsrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M and Olsen R. (2006). Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to $\alpha_{4/6}\beta_3\delta$ $GABA_A$ receptors. *PNAS* **103**, 8546-8551.

Harris LJ, Larson SB, Hasel KW and McPherson A. (1997). Refined structure of an intact IgG2a monoclonal antibody. *Biochemistry* **36**, 1581-1597.

Herd MB, Haythornthwaite AR, Rosahl TW, Wafford KA, Homanics GE, Lambert JJ and Belelli D. (2008). The expression of $GABA_A$ receptor β subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. *J. Physiol.* **586.4**, 989-1004.

Hoestgaard-Jensen K, Dalby NO, Wolinsky TD, Murphey C, Jones KA, Rottlander M, Frederiksen K, Watson WP, Kensen K and Ebert B. (2010). Pharmacological characterization of a novel positive modulator at $\alpha4\beta3\delta$-containing extrasynaptic $GABA_A$ receptors. *Neuropharmacology* **58**, 702-711.

Huntsman MM, Porcello DM, Homanics GE, DeLorey TM and Huguenard JR. (1999). Reciprocal inhibitory connections and network synchrony in the mammalian thalamus. *Science* **283**, 541-543.

Jechlinger M, Pelz R, Tretter V, Klausberger T and Sieghart W. (1998). Subunit composition and quantitative importance of hetero-oligomeric receptors: $GABA_A$ receptors containing $\alpha6$ subunits. *J. Neurosci*. **18**, 2449-2457.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL and Goldstein PA. (2005). An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. *J. Neurophysiol.* 94, 4491-4501.

Karim N, Wellendorph P, Absalom N, Bang LH, Jensen ML, Hansen MM, Lee HJ, Johnston GAR, Hanrahan JR and Chebib M. (2012). Low nanomolar GABA effects at extrasynaptic $\alpha4\beta1/3\delta$ $GABA_A$ receptor subtypes indicate a different binding mode for GABA at these receptors. *Biochemical Pharmacology* 84, 549-557.

Kaur KH, Baur R and Sigel E. (2009). Unanticipated structural and functional properties of δ-subunit-containing $GABA_A$ receptors. *J. Biol. Chem.* 284, 7889-7896.

Meera P, Wallner M and Otis TS. (2011). Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic $GABA_A$ receptors. *J. Neurophysiol.* 106, 2057-2064.

Mokrab Y, Bavro VN, Mizuguchi K, Todorov NP, Martin IL, Dunn SMJ, Chan SL and Chau P-L. (2007). Exploring ligand recognition and ion flow in comparative models of the human GABA type A receptor. *J. Mol. Graphics and Modelling* 26, 760-774.

Mortensen M and Smart TG. (2007). Single-channel recording of ligand-gated ion channels. *Nature Protocols* 2, 2826-2841.

Mortensen M, Patel B and Smart TG. (2012). GABA potency at $GABA_A$ receptors found in synaptic and extrasynaptic zones. *Front. Cell. Neurosci.* 6, 1-10.

Peden DR, Petitjean CM, Herd MB, Durakoglugil MS, Rosahl TW, Wafford K, Homanics GE, Belelli D, Fritschy J-M and Lambert JJ. (2008). Developmental maturation of synaptic and extrasynaptic $GABA_A$ receptors in mouse thalamic ventrobasal neurones. *J. Physiol.* 586.4, 965-987.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W and Sperk G. (2000). $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult brain. *Neuroscience* 101, 815-850.

Poltl A, Hauer B, Fuchs K, Tretter V and Sieghert W. (2003). Subunit composition and quantitative importance of $GABA_A$ receptor subtypes in the cerebellum of mouse and rat. *J. Neurochem.* 87, 1444-1455.

Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W and Sperk G. (2001). Distribution of the major γ-aminobutyric $acid_A$ receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. *J. Comparative Neurology* 433, 526-549.

Shu H-J, Bracamontes J, Taylor A, Wu K, Eaton MM, Akk G, Manion B, Evers AS, Krishnan K, Covey DF, Zorumski CF, Steinbach JH and Mennerick S. (2012). Characteristics of concatameric $GABA_A$ receptors containing α4/δ subunits expressed in *Xenopus* oocytes. *Br. J. Pharmacol.* 165, 2228-2243.

Sieghart W and Sperk G. (2002). Subunit composition, distrubution and function of $GABA_A$ receptor subtypes. *Curr. top. med. chem.* 2, 795-816.

Storustovu S and Ebert B. (2006). Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ2. *JPET* 316, 1351-1359.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR and McKernan RM. (1999). Preferential coassembly of α4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. *Mol. Pharmacol.* 56, 110-115.

Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D and Lambert JJ. (2009). Novel compounds selectively enhance δ subunit containing $GABA_A$ receptors and increase tonic currents in the thalamus. *Neuropharmacology* 56, 182-189.

Wagoner KR and Czajkowski C. (2010). Stoichiometry of expressed alpha(4)beta(2)delta gamma-aminobutyric acid type A receptors depends on the ratio of subunit cDNA transfected. *J. Biol. Chem.* 285, 14187-14194.

Wallner M, Hanchar HJ and Olsen RW. (2003). Ethanol enhances α4β3δ and α6β3δ γ-aminobutyric acid type A receptors at low concentrations known to affect humans. *PNAS* 100, 15218-15223.

Wisden W, Laurie DJ, Monyer H and Seeburg PH. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesenchephalon. *J. Neurosci.* 12, 1040-1062.

You H and Dunn SMJ. (2007). Identification of a domain in the δ subunit (S238-V264) of the $\alpha4\beta3\delta$ $GABA_A$ receptor that confers high agonist sensitivity. *J. Neurochem.* 103, 1092-1101.