Regulatory Domains of $GABA_A$ Receptors

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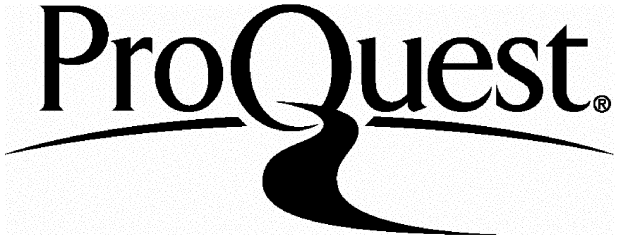

ABSTRACT

$GABA_A$ receptors are the principal mediators of rapid, synaptic inhibition within the mammalian central nervous system. These receptors are hetero-oligomeric proteins that can be assembled from α(1-6), β(1-3), γ(1-3), δ, ε, π and θ subunit classes (Mehta & Ticku, 1999; Bonnert *et al.*, 1999). Each subunit comprises a putative extracellular N-terminus, four putative transmembrane domains (TM), a large intracellular domain linking TM3 and TM4, and an extracellular C-terminus. The present study investigated these domains to identify molecular determinants affecting modulation of $GABA_A$ receptor function.

An N-terminal histidine (H107) was implicated in mediating lower affinity Zn^{2+} binding on the β3 subunit and also a possible role in homomeric receptor assembly/protein folding. Interestingly, mutation of the aligned residue on the α1 subunit (H109) strongly influenced GABA but not Zn^{2+}potency. Substitution of $\alpha1^{H141}$ located in the cysteine loop region of the N-terminal domain reduced Zn^{2+} sensitivity of heteromeric $GABA_A$ receptors. An electrophysiological approach based on the relative insensitivity to Zn^{2+} generated by a TM2 mutation (H267A) was used to predict a tetrameric stoichiometry for β3 subunit homomeric $GABA_A$ receptors. In addition, the substituted histidine accessibility method (SHAM) was developed to probe TM2 by individually substituting putative channel lining residues with histidines in homomeric $\beta3^{H267A}$ $GABA_A$ receptors. It was inferred that Zn^{2+} is able to penetrate the anion channel at least as far as residue 263. Finally, an initial investigation of residues in the large intracellular TM3-TM4 domain responsible for tyrosine phosphorylation of α1β1γ2S receptors discovered that external application of protein tyrosine kinase (PTK) inhibitors directly modulate $GABA_A$ receptors independently of tyrosine kinases.

The present study into regulatory domains of the $GABA_A$ receptor revealed important novel N-terminal residues for Zn^{2+}inhibition and a novel non-specific interaction of PTK inhibitors with the $GABA_A$ receptor. In addition, a method for probing ion channel properties using histidine was devised and the stoichiometry of a $GABA_A$ receptor complex was predicted using the binomial theorem.

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CHAPTER 1

Introduction.

1.1 INTRODUCTION.

γ-Aminobutyric acid (GABA), a nonprotein amino acid, is widely distributed throughout the central nervous system (CNS) of both vertebrates and invertebrates. GABA is synthesised in neuronal cell bodies and terminals from L-glutamate by glutamate decarboxylase (GAD). Upon nerve terminal stimulation, GABA is released by exocytosis into the synaptic cleft where it binds to postsynaptic receptors. GABA levels are closely regulated by GABA transporters located on both neurons and astrocytes, which take up GABA for subsequent metabolism via GABA-aminotransferase (GABA-T) to succinic semialdehyde and then succinate (Waagepetersen *et al.*, 1999). Furthermore, cloning has revealed two isoforms of GAD (GAD_{65} and GAD_{67}) exhibiting diverse subcellular localisation in GABAergic neurons which also appears important in regulating GABA synthesis. A small increase in neuronal GABA concentration inhibits GAD_{65}, which is mainly localised in nerve endings. Interestingly, GAD is also expressed in glutamatergic neurons, particularly after seizures, suggesting a putative inhibitory capacity for excitatory neurons (Waagepetersen *et al.*, 1999 for review). GABA is an important neurotrophin and neurodifferentiative agent in early development, playing a vital role in synaptogenesis (for review see Meier *et al.*, 1991; Belhage *et al.*, 1998). However, the major function of GABA is as a neurotransmitter at the majority of inhibitory synapses within the vertebrate CNS.

GABA mediates synaptic inhibition via interaction with either ionotropic ($GABA_A$ and $GABA_C$) or metabotropic ($GABA_B$) receptors. Historically, GABA receptors displaying sensitivity to the competitive antagonist bicuculline and insensitivity to the GABA analogue baclofen have been termed $GABA_A$ receptors. $GABA_B$ receptors were initially classified according to stimulation by baclofen and insensitivity to bicuculline (Hill & Bowery, 1981). A third class of GABA receptor, insensitive to both bicuculline and baclofen, was designated as the $GABA_C$ receptor (Feigenspan *et al.*, 1993; Qian & Dowling, 1993). The properties of $GABA_A$ receptors, the subject of the present study, will be discussed in detail following brief summary of $GABA_B$ and $GABA_C$ receptors.

1.1.1. $GABA_B$ receptors

$GABA_B$ receptors are located both pre- and post-synaptically in vertebrate and invertebrate nervous systems, in addition to a widespread distribution in peripheral tissues (Bowery, 1993). Recent cloning has revealed that $GABA_B$ receptors share certain structural similarities with family 3 G-protein coupled receptors which includes metabotropic glutamate receptors (mGluR; Kaupmann *et al.*, 1997; Bettler *et al.*, 1998). However, unusually, the functional expression of $GABA_B$ receptors appears to require the coexpression of two seven-transmembrane domain receptor subunits (GBR1 and GBR2) which interact through C-terminal coiled-coil domains to form a heterodimer (Kaupmann *et al.*, 1998; Galvez *et al.*, 1999; Marshall *et al.*, 1999). GBR1 and GBR2 are G-protein coupled. Agonist binding to $GABA_B$ receptors stimulates G-proteins which in turn regulate the activity of voltage-operated Ca^{2+} and K^+ channels; evoking a decrease in membrane Ca^{2+} conductance and an increase in K^+ conductance. This results in slow and prolonged synaptic inhibition (Bowery, 1993).

1.1.2. $GABA_C$ receptors.

$GABA_C$ receptors have been identified on several retinal cell populations illustrating a possible important role in the visual pathway (Bormann & Feigenspan, 1995; Johnston, 1996; Lukasiewicz, 1996). Although $GABA_A$ and $GABA_C$ receptors possess integral Cl^- channels, they demonstrate distinct channel and pharmacological properties. $GABA_C$ receptors are composed from either homo-oligomeric or hetero-oligomeric combinations of ρ subunit isoforms (ρ1-3) which share approximately 30-38% sequence homology with $GABA_A$ receptor subunits (Cutting *et al.*, 1991, 1992; Wang *et al.*, 1994; Enz *et al.*, 1995; Zhang *et al.*, 1995; Shingai *et al.*, 1996). Compared to $GABA_A$ receptors, GABA receptors comprised of ρ subunits are activated by lower concentrations of GABA, desensitise at a much slower rate and display a different agonist and antagonist specificity and efficacy. In addition these receptors are not affected by $GABA_A$ modulators including bicuculline, barbiturates and benzodiazepines (reviewed by Bormann & Feigenspan, 1995; Johnston, 1996; Lukasiewicz, 1996). Recently, ρ subunits have been classified as $GABA_A$ receptor subunits (Barnard *et al.*, 1998).

However, ρ subunits do not co-assemble with other $GABA_A$ receptor subunits *in vitro* (Hackam *et al.*, 1998) and are more typically classified as $GABA_C$ receptor subunits.

1.2. $GABA_A$ receptors.

$GABA_A$ receptors are the major mediators of fast inhibitory neurotransmission within the vertebrate CNS. Activation by GABA opens an integral Cl^- channel and the ensuing Cl^- influx drives the membrane potential towards the equilibrium potential for Cl^-. This frequently results in a slight membrane hyperpolarization in conjunction with diminished neuronal excitability because the increased Cl^- conductance counteracts the effects of depolarization by excitatory stimuli (Rabow *et al.*, 1995; Sieghart, 1995). However, in the developing nervous system, the Cl^- equilibrium potential is frequently depolarized relative to the resting potential of neurones (Rivera *et al.*, 1999). As such, activation of $GABA_A$ receptors elicits depolarization which may be important in the formation of GABAergic synapses (Leinekugel *et al.*, 1999).

The $GABA_A$ receptor is a heteromeric transmembrane protein, expressed in both the central and peripheral nervous systems. $GABA_A$ receptors share a common amino acid sequence homology (20 – 30%) with other transmitter-gated ion channels in the nicotinic acetylcholine (nACh) receptor superfamily, which also includes glycine and $5\text{-}HT_3$ receptors. Molecular cloning has identified several genes coding discrete but related $GABA_A$ receptor subunit classes. Mammalian $GABA_A$ receptors form a pentameric assembly constructed from seven subunit classes: α1–α6, β1-β3, γ1–γ3, δ, ε, π, and θ (Nayeem *et al.*, 1994; Mehta & Ticku, 1999; Bonnert *et al.*, 1999) creating the possibility for considerable receptor heterogeneity. Splice variants exist for α6-, γ2– and human β2/3 subunits generating short and long forms (Whiting *et al.*, 1990; Kofuji *et al.*, 1991; Kirkness & Fraser, 1993; Korpi *et al.*, 1994; McKinley *et al.*, 1995). Furthermore, additional $GABA_A$ receptor subunit isoforms (β4 and γ4) have been identified in chick brain (Bateson *et al.*, 1991; Harvey *et al.*, 1993). Typically, subtypes within a class of receptor subunit share approximately 70% amino acid homology whereas only 20-25% sequence identity exists between subunit classes. These subunit

classes and isoforms are differentially distributed throughout the brain (Wisden *et al.*, 1992; Mehta & Ticku, 1999 for review). For example, α6 and δ subunits are found exclusively in cerebellar granule cells, while the other α isoforms have more widespread, but specific, patterns of distribution. The differential distribution of the subunits within brain regions suggests that variations in subunit composition may have functional significance, for example by conferring differences in agonist affinity or the kinetics of channel opening; and this is supported by studies of recombinant receptors (Gingrich *et al.,* 1995). To date, the π subunit has only significantly been detected in peripheral tissues including lung, thymus, prostate and uterus (Hedblom & Kirkness, 1997).

The exact subunit stoichiometry of native $GABA_A$ receptors continues to prove controversial. Although β1 and β3 subunits are capable of forming functional homomeric $GABA_A$ receptors, these ion channels are spontaneously active and not gated by GABA (Sigel *et al.*, 1989; Sanna *et al.*, 1995; Slany *et al.*, 1995; Connolly *et al.*, 1996a,b; Krishek *et al.*, 1996b; Wooltorton *et al.*, 1997b). In recombinant systems, a minimum combination of αβ is required for the effective gating of the receptor by GABA whereas inclusion of a γ subunit is required for benzodiazepine modulation and channel properties similar to those of native receptors (Draguhn *et al.*, 1990, Sigel *et al.*, 1990, 1992; Verdoorn *et al.*, 1990; Amin & Weiss, 1993; Angelotti & Macdonald, 1993; Connolly *et al.*, 1996a). The expression of other binary combinations, such as αγ and βγ, appears to be impaired (Sigel *et al.*, 1989; Draguhn *et al.*, 1990, Sigel *et al.*, 1990; Verdoorn *et al.*, 1990; Angelotti & Macdonald, 1993; Mihic *et al.*, 1995; Connolly *et al.*, 1996a). Evidence suggests that the vast majority of $GABA_A$ receptors in the brain comprise a combination of α and β subunits along with one or more of the γ, δ, ε or θ subunits. The combination of α1, β2/3 and γ2 subunits appears to be particularly prevalent in many neuronal preparations (Mehta & Ticku, 1999). It is however possible that receptors composed of only α and β may form, albeit rarely. In addition, two different isoforms of the α,β or γ subunit may coassemble in the same $GABA_A$ receptor complex. Certain subunit combinations (as shown for δ and γ subunits) may not be found in individual $GABA_A$ receptor molecules *in vivo,* while other combinations may preferentially assemble (Quirk *et al.*, 1995; Araujo *et al.*, 1998).

In support of this view, the cerebellum of α6 subunit deficient mice ($\alpha6^{-/-}$) displayed a substantial reduction in the level of δ subunit protein, suggesting a specific interaction between α6 and δ subunits in these cells (Jones *et al.*, 1997). Over-expression of other $GABA_A$ receptor subunits in cerebellar granule cells (α1, β2, β3 and γ2) could not rescue the levels of δ subunit protein. Interestingly, the motor skills of the $a6^{-/-}$ mice were not obviously impaired despite a significant reduction in $GABA_A$ receptors in the cerebellum (Jones *et al.*, 1997). Furthermore, αβγθ is the only combination of θ-subunit containing receptors thought to be functionally expressed, presumably reflecting the co-localisation of α2, β2, γ1 and θ subunits in the rat striatum (Bonnert *et al.*, 1999). These data suggest that the actual $GABA_A$ receptor subunit combinations found *in vivo* may be relatively limited, and that different subunit classes may have specific roles in receptor function.

The ratio of subunit types within the presumed pentameric complex is controversial and may differ depending on the brain region and the subunit isoforms present (see Chapter 5 for discussion). Subunit stoichiometries of 2α: 1β: 2γ (Backus *et al.*, 1993) or 2α: 2β: 1γ (Im *et al.*, 1995; Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999) have been reported for the $GABA_A$ receptor complex.

1.3. Regulatory domains of the $GABA_A$ receptor.

Hydropathy profiles suggest $GABA_A$ receptor subunits possess a large, extracellular domain, four hydrophobic membrane spanning regions or transmembrane domains (TMs), a large hydrophilic intracellular domain linking TM3 and TM4 and a short extracellular C-terminal region (Rabow *et al.*, 1995; Fig. 1.1.). Based on this structure, it has been estimated that 60% of the $GABA_A$ receptor is extracellular whilst the remaining 40% resides in the transmembrane and intracellular domains (Wooltorton *et al.*, 1997a). Each of these domains contain molecular determinants vital for the specific aspects of $GABA_A$ receptor function.

1.3.1. The N-terminal domain.

The N-terminal domain constitutes the largest extracellular domain of the $GABA_A$ receptor and forms the binding sites for GABA and many of the molecules which modulate the activity of $GABA_A$ receptors. This domain contains several potential sites for N-linked glycosylation and a putative loop formed by the disulphide bonding of two highly conserved cysteine residues (Barnard *et al.*, 1987; Rabow *et al.*, 1995). Although the functional cell surface expression of $GABA_A$ receptors appears to be independent of N-linked glycosylation (Connolly *et al.*, 1996a), mutation of a conserved cysteine prevents functional subunit expression (Amin *et al.*, 1994). As with other members of this receptor superfamily (nACh and glycine), sequences within the N-terminal domain are crucial for the folding of subunit proteins and subsequent receptor assembly. The α6 subunit gene is alternatively spliced, with the short form lacking 10 amino acids found in all other α subunits. These residues appear essential for receptor expression as receptors containing the α6S subunit fail to reach the cell surface (Korpi *et al.*, 1994). Recently, four amino acids (glycine (G) 171, lysine (K) 173, glutamate (E) 179 and arginine (R) 180) in the N-terminal domain of the β3 subunit have been identified as vital for the assembly of functional homomeric β3 and heteromeric β3γ2 subunit constructs, putative intermediates in the assembly of αβγ subunit $GABA_A$ receptors (Taylor *et al.*, 1999). Substitution of these residues with the analogous residues in the β2 subunit (known to be incapable of homomer or βγ heteromer formation; Connolly *et al.*, 1996a,b; Gorrie *et al.*, 1997) prevented functional assembly. Recent evidence also suggests a role in assembly for two conserved N- terminal tryptophan residues ($\alpha1^{W69}$ and $\alpha1^{W94}$; Srinivasan, 1999). The extracellular part of the receptor, as formed by the N-terminal domains on each subunit, contains the binding sites for GABA and many allosteric modulators, including benzodiazepines, which regulate $GABA_A$ receptor function (Fig. 1.2; Macdonald & Olsen, 1994; Mehta & Ticku, 1999)

1.3.1.1. The GABA binding site.

Early photoaffinity-labelling of native $GABA_A$ receptors with the GABA agonist [^{3}H] muscimol revealed agonist binding domains on α (Asano *et al.*, 1983; Cavalla & Neff,

1985) and β subunits (Casalotti *et al.*, 1986; Deng *et al.*, 1986; Browning *et al.*, 1990). The GABA binding site is considered to lie at the interface between the α and β subunits (Galzi & Changeux, 1994; Smith & Olsen, 1995).

Experiments utilising photoaffinity-labelling and site-directed mutagenesis have implicated the contribution of several discrete regions on the N-terminal domains of α and β subunits to the formation of an agonist binding pocket (Smith & Olsen, 1995; Fig. 1.2.). Mutation of a phenylalanine (F) to leucine (L) at position 64 in the N-terminal domain of the rat α1 subunit elicited a 210-fold reduction in GABA potency when co-expressed with β2 and γ2 subunits in *Xenopus* oocytes (Sigel *et al.*, 1992). Similarly, the $\alpha1^{F64L}$ mutation conferred a substantial reduction in the potencies of competitive GABA antagonists bicuculline methiodide and SR95531 whereas homologous mutations in either the β2 or γ2 subunits produced negligible effects on the apparent affinity of either compound. The equivalent residue in the bovine α1 subunit (F65) was photoaffinity-labelled with the GABA analogue [^{3}H] muscimol (Smith & Olsen, 1994), and the analogous mutation in the β2 subunit decreased the affinity for [^{3}H] muscimol (Newell *et al.*, 1999) further supporting an important role for this residue in GABA binding. Recently, using the substituted cysteine accessibility method, F64 and neighbouring arginine (R66) and serine (S68) residues have been implicated in lining part of the GABA binding site on the α1 subunit (Boileau *et al.*, 1999). These residues were systematically mutated to cysteine (C) and the resulting mutant α1 subunits were then co-expressed with wild-type β2 subunits in either *Xenopus* oocytes or human embryonic kidney (HEK) cells. Both $\alpha1^{F64C}$ and $\alpha1^{R66C}$ mutations generated a substantial reduction in GABA potency (75- and 320-fold respectively). Pre-treatment of $\alpha1^{S68}\beta2$ $GABA_A$ receptor constructs with a sulphydryl reagent reduced [^{3}H]muscimol and [^{3}H]SR95531 binding. Conversely, the covalent modification of the substituted cysteine by sulphydryl reagents in all three mutant receptor constructs was significantly inhibited in the presence of GABA, suggesting that these residues are in or near the GABA binding site. The mutation $\alpha5^{R70K}$ (equivalent to $\alpha1^{R66}$) substantially reduced GABA potency and when combined with another arginine mutation ($\alpha5^{R123K}$) resulted in a near complete elimination of GABA sensitivity (Hartvig *et al.*, 1999). Additional evidence for the involvement of arginine residues in GABA binding is provided by the time- and

concentration-dependent elimination of [^{3}H]muscimol binding in purified $GABA_A$ receptors following treatment with 2,3-butanedione, an arginine specific reagent (Widdows *et al.*, 1987). The accessibility of sulphydryl reagents to react with alternating residues from $\alpha1^{Y59}$ to $\alpha1^{S68}$ suggests that this region forms a β strand, part of which lines the GABA binding domain (Boileau *et al.*, 1999). This is in agreement with structural predictions of this region using secondary structure modelling alogorithms (Chou & Fasman, 1978; Smith & Olsen, 1995).

In addition, two separate regions of the β2 subunit N-terminal domain (tyrosine (Y) 157 and threonine (T) 160; T202 and Y205), located between the cysteine loop and the predicted start of TM1, have been identified as important for the binding of GABA (Amin & Weiss, 1993). Mutation of these residues significantly reduced the apparent affinity for agonists and antagonists when coexpressed with α1 and γ2 in *Xenopus* oocytes. GABA potency was virtually unaffected by equivalent mutations in the α1 and γ2 subunits. Furthermore, pentobarbitone activation was unaffected by any of these mutations suggesting that the channel structure and gating remained intact and that PB and GABA activate the $GABA_A$ receptor via different binding sites. Conceivably, since the residues in either region are three residues apart, these GABA binding domains on the β2 subunit may represent two α-helices (Boileau *et al.*, 1999). Alternatively, the conserved glycine located in both of these binding domains may form a hairpin turn in order to correctly orientate the crucial threonine and tyrosine residues for agonist binding. Therefore the multiple loop model proposed for the agonist-binding domain in other members of the nicotinic acetylcholine receptor family may also apply to the GABA binding site (Galzi & Changeux, 1994), in which case other residues in the N-terminal domains of both α and β subunits may contribute to the binding site.

1.3.1.2. The benzodiazepine binding domain.

Benzodiazepines are clinically important agents, commonly prescribed as anxiolytics, sedatives, anti-convulsants and muscle relaxants (Mehta & Ticku, 1999). The therapeutic effects of these compounds result at least in part from the allosteric modulation of $GABA_A$ receptors. Benzodiazepines do not activate the channel in the

absence of GABA, instead they potentiate GABA-activated conductance by increasing the probability of Cl^- channel opening (Smith & Olsen, 1995). Functional studies using recombinant $GABA_A$ receptors revealed that the γ subunit is essential for benzodiazepine modulation (Pritchett *et al.*, 1989b, Schofield *et al.*, 1987, Sigel *et al.*, 1990). Depletion of the γ2 subunit in γ2 subunit deficient ($\gamma2^{-/-}$) mice (Gunther *et al.*, 1995) or by infusion of the antisense oligodeoxynuleotide (Karle & Nielsen, 1995; Karle *et al.*, 1995), almost completely inhibited the formation of benzodiazepine binding sites, whilst only slightly affecting the number of GABA binding sites.

In addition, the α subunit subtype greatly influences both the affinity and efficacy of benzodiazepine ligands (Mehta & Ticku, 1999). $GABA_A$ receptor constructs containing the α1 subtype are highly sensitive to modulation by classical benzodiazepine ligands (termed type I benzodiazepine sites) and while α2 and α3 subunit-containing receptors are less sensitive (type II sites), receptors containing α4 or α6 subunits are typically insensitive (Luddens *et al.*, 1990; Mehta & Ticku, 1999). Benzodiazepine affinity and efficacy can be further modified by the γ-subunit subtype present in the receptor complex (Ymer *et al.*, 1990; Wafford *et al.*, 1993; Luddens *et al.*, 1994; Hadingham *et al.*, 1995; Benke *et al.*, 1996). The contribution from both α and γ subunits to benzodiazepine modulation implies the benzodiazepine binding domain is located at the interface of these two subunits.

Structure-function studies of the $GABA_A$ receptor indicate which residues in α and γ subunits contribute to the benzodiazepine binding site (Smith & Olsen, 1995; Mehta & Ticku, 1999; Fig. 1.2). To date, three separate regions of the α subunit N-terminal domain have been identified as being important for benzodiazepine binding (H101, Y159 – T162 and G200 – V211; Mehta & Ticku, 1999). A histidine (H) located at position 101 on the α1 subunit was photoaffinity labelled by the benzodiazepine [^{3}H]flunitrazepam (Smith & Olsen, 1994; Duncalfe *et al.*, 1996). Mutation of this histidine to arginine, found in the equivalent position in both α4 and α6 subunits, abolished both benzodiazepine binding (Wieland *et al.*, 1992) and benzodiazepine potentiation of GABA-activated currents (Kleingoor *et al.*, 1993) when coexpressed with β2 and γ2 subunits in HEK cells. In addition, H101 (also present in α2, α3 and α5

subtypes) has been shown to directly interact with the pendant phenyl group of classical benzodiazepines such as diazepam, flunitrazepam and chlordiazepoxide (McKernan *et al.*, 1998; Zhang *et al.*, 1995). Recently, the $\alpha1^{H101R}$ point mutation has been introduced into the germline of mice by homologous recombination (Rudolph *et al.*, 1999). Purified $GABA_A$ receptors from the mutant mice displayed an essential lack of affinity for diazepam. The $\alpha1^{H101R}$ mice, however, did not display an overt distinct phenotype, although the sedative and amnesic effects of diazepam were abolished, and the anti-convulsant action partially reduced. Interestingly, the anxiolytic, myorelaxant and ethanol-potentiating effects of diazepam were comparable for wild-type and $\alpha1^{H101R}$ mutant mice, suggesting that these actions may be exclusively mediated by α2, α3 or α5 subunit-containing $GABA_A$ receptors, an important observation for drug design. Furthermore, a naturally occurring arginine-to-glutamine mutation at the corresponding position in the α6 subunit of alcohol non-tolerant (ANT) rats, restores benzodiazepine sensitivity, thus indicating the importance of the nature of this residue in benzodiazepine modulation. A glycine residue (G200) in the α1 subunit appears important for the high affinity of benzodiazepines for α1-subunit containing receptors (Pritchett & Seeburg, 1991). Interestingly, substituting either $\alpha1^{Y159}$, $\alpha1^{T162}$, $\alpha1^{T206}$or $\alpha1^{Y209}$ dramatically impaired benzodiazepine potentiation and binding affinity (Wieland & Luddens, 1994; Buhr *et al.*, 1996; Amin *et al.*, 1997; Buhr *et al.*, 1997b). These residues are directly equivalent to the amino acids identified as part of the GABA binding domain on the β2 subunit (Amin & Weiss, 1993, Fig. 1.2). In addition neighbouring serine, tyrosine and valine residues ($\alpha1^{Y161}$, $\alpha1^{S204}$ and $\alpha1^{V211}$) also influence benzodiazepine sensitivity (Buhr & Sigel, 1997; Renard *et al.*, 1999; Schaerer *et al.*, 1998). Aromatic residues (tyrosine and phenylalanine) thus appear to be key components of both GABA and benzodiazepine binding domains. These residues are also homologous to residues implicated in agonist binding domains on other members of the nicotinic acetylcholine receptor family (Smith & Olsen, 1995).

So far, two domains on the γ2 subunit (K41 – W (tryptophan) 82 and R114 – N (asparagine)161) have been implicated as crucial for high affinity benzodiazepine binding (Boileau *et al.*, 1998). In particular, mutation of $\gamma2^{F77}$ dramatically alters the benzodiazepine binding affinity and allosteric modulation (Buhr *et al.*, 1996; Buhr *et al.*,

1997a; Wingrove *et al.*, 1997). This residue is homologous to F64 in the α1 subunit, involved in GABA binding (Sigel *et al.*, 1992). Other residues in the first benzodiazepine binding domain on the γ2 subunit, notably M (methionine)57, Y58, N60, I (isoleucine)62, A (alanine)79 and T81, have been implicated in high affinity binding of benzodiazepine ligands (Buhr & Sigel, 1997; Kucken *et al.*, 1999). In the second putative domain, $\gamma2^{M130L}$ and $\gamma2^{T142S}$ mutations affected the sensitivity of benzodiazepines (Mihic *et al.*, 1995; Buhr & Sigel, 1997;Wingrove *et al.*, 1997). As yet, no equivalent to this residue has been identified as contributing to the GABA binding site. Given the marked similarities in the distribution of GABA and benzodiazepine site residues it has been suggested that the benzodiazepine binding site has evolved from an agonist binding site which has lost the ability to bind GABA but is still capable of allosterically modulating channel activity/GABA binding (Smith & Olsen, 1995).

1.3.2. The Transmembrane domains.

The four transmembrane domains are the most highly conserved regions of $GABA_A$ receptor subunits. These regions contribute to the lining of the ion channel, as well as the channel gate. As such they are intimately involved in determining the channels ion selectivity and in regulating the allosteric transitions associated with channel gating. In addition, certain residues in the transmembrane domains have a central role in determining the potency and efficacy of a wide range of allosteric modulators.

1.3.2.1. The ion-conducting channel.

The binding of GABA evokes a conformational change within the receptor to an open channel state that permits the selective passage of anions. In addition to Cl^-, $GABA_A$ receptor channels are permeable to other inorganic anions (the halides) and polyatomic anions as large as propionate (Bormann *et al.*, 1987; Akaike *et al.*, 1989; Fatima-Shad & Barry, 1993) implying an effective pore diameter of 5.6Δ for open $GABA_A$ receptor channels (Bormann *et al.*, 1987). The majority of binding sites and allosteric interactions between binding sites on $GABA_A$ receptors can be modulated by various anions including Cl^-, Br^-, I^-, NO_3^-, SCN^-, and ClO_4^- (Sieghart, 1995). In mouse spinal

neurons, the halide conductance sequence (Cl^-> Br^-> I^-> SCN^-> F^-) is the inverse of the halide permeability sequence, with the exception of F^- (Bormann *et al.*, 1987). This implies the existence of anion binding sites, within or near the putative channel-lining TM2, which attract permeating anions. Channel conductance is mainly determined by anion binding to the channel and their hydration energies as opposed to pore size. Increasing SCN^- concentration relative to Cl^- (i.e. a constant total anion concentration) did not uniformly alter channel conductance. This would not be expected if SCN^- and Cl^- compete for a single site and is best explained by a two-site model.

By analogy to nicotinic ACh receptors, the channel lining of GABA receptors is most likely formed by TM2 (Galzi & Changeux, 1994; Karlin & Akabas, 1995). Indeed, the TM2 domains of GABA and glycine receptors (also anion selective) are very similar. In $\alpha1\beta1\gamma2S$ $GABA_A$ receptors, mutation of a conserved arginine ($\alpha1^{R273A}$; $\beta1^{R269A}$; $\gamma2S^{R287A}$) at the extracellular end of TM2 resulted in a significant increase in the relative permeability to I^- and a decrease in the outward rectification (Wooltorton *et al.*, 1997c, 1998). The degree of reduction in outward rectification was proportional to the number of mutant subunits included in the $GABA_A$ receptor construct, suggesting that this conserved arginine may influence an anion binding site. However, arginine is not found at the equivalent position in ρ subunits or insect RDL subunits, all of which bear asparagine at the equivalent location, yet form anion-selective homomeric receptors. Thus ion-selectivity and binding must also occur elsewhere in the channel.

While $GABA_A$ receptors are anion selective, the tertiary structure of their TM2 domains appears similar to that of nACh receptors, as demonstrated by the substituted cysteine accessibility method (SCAM). Substitution of residues in TM2 of the $GABA_A$ receptor $\alpha1$ subunit to cysteine, followed by extracellular application of membrane impermeable sulphydryl reagents identified channel lining residues forming a kinked α-helix (Xu & Akabas, 1993; 1996). The location of these residues is very similar to that of accessible residues in the α subunit of nACh receptors (Akabas *et al.*, 1992, 1994a). Detailed studies of mutated nACh receptors suggest that the presence of a proline at the beginning of TM2, and a threonine towards the middle of the domain are required for anion selectivity of GABA receptors (Galzi *et al.*, 1992; Corringer *et al.*, 1999).

Although a complementary study has yet to be performed on GABA receptors Xu & Akabas' (1996) SCAM study of $GABA_A$ receptors placed the putative ion selectivity filter and channel gate location towards the cytoplasmic end of the ion channel, suggesting a conserved structure-function relationship amongst members of this receptor superfamily.

In the nACh receptor, residues located towards the extracellular end of TM1 are also exposed in the channel (Akabas & Karlin, 1995). The presence of agonist alters the exposure of residues to the channel lumen. Therefore agonist binding appears to propagate conformational changes in TM1 and TM2, and the movement of the latter domain relative to the rest of the receptor could open the channel gate.

Not surprisingly, a number of residues in TM2 are crucial in propagating the allosteric transitions that underlie channel gating (Chang *et al.*, 1996; Tierney *et al.*, 1996; Birnir *et al.*, 1997a, b; Buhr *et al.*, 1999; Hosie *et al.*, 1999; Thompson *et al.*, 1999b; Chapter 6 for discussion). The functional effects of mutagenesis have also implicated TM2 residues in determining the action of a range of modulators including picrotoxin (Gurley *et al.*, 1995; Xu *et al.*, 1995; Chapter 6 for discussion), pentobarbitone (Birnir *et al.*, 1997a), loreclezole (Wingrove *et al.*, 1994), etomidate (Belelli *et al.*, 1997), mefenamic acid (Halliwell *et al.*, 1999), tracazolate and etazolate (Thompson *et al.*, 1999c); furosemide (Thompson *et al.*, 1999a) and Zn^{2+} (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998; Fig. 1.3.). While some of these compounds may bind to residues in TM2, it unclear if all these compounds have discrete binding sites in the channel. Thus some of these residues may be crucial to the allosteric modes of action of these compounds.

1.3.2.2. A potential target for general anaesthetics.

The agonist responses of $GABA_A$ receptors are potentiated by clinically relevant concentrations of a number of structurally diverse general anaesthetic agents including barbiturates, steroid anaesthetics, etomidate and propofol (2,6-diisopropoylphenol) (Belelli *et al.*, 1999b; Mehta & Ticku, *et al.*, 1999). In addition, a range of volatile

anaesthetics including halothane, isoflurane and enflurane also modulate $GABA_A$ receptors.

At clinically relevant concentrations, these compounds greatly enhance the magnitude of GABA-activated conductances. For example, barbiturates potentiate the action of GABA by increasing the average channel open time by increasing the incidence of long duration open states but without altering the maximum current response or frequency of channel openings. In addition, the presence of barbiturates appears to increase the receptors' agonist affinity as measured by [^{3}H]GABA binding (Sieghart, 1995; Rabow *et al.*, 1995). Concentrations of anaesthetic greater than those required for potentiation directly activate the $GABA_A$ receptor, while even higher, although probably not clinically relevant, doses depress the GABA response possibly through channel blockade (Rabow *et al.*, 1995, Sieghart, 1995).

It is not known where on the receptor general and volatile anaesthetics bind. However it seems unlikely to be the GABA binding site as point mutations in the β2 subunit abolish GABA but not pentobarbitone (PB) activation of the $GABA_A$ receptor (Amin & Weiss, 1993; see also DeLorey & Olsen, 1992; Sieghart, 1995; Davies *et al.*, 1997b). Although a point mutation ($\beta2^{Y157F}$) implicated in the binding of GABA (Amin & Weiss, 1993) impaired the agonist and not the modulatory action of propofol (Fukami *et al.*, 1999), as β3 homomers are activated by propofol and not GABA, it seems likely that these agonists act though different mechanisms (Davies *et al.*, 1997b). Furthermore, PB activation of the $GABA_A$ receptor was unaffected by the competitive GABA antagonists bicuculline and SR95531 (Thompson *et al.*, 1996), although others have observed bicuculline inhibition of PB-induced currents, suggesting that bicuculline acts as an inverse agonist at the GABA site (Robertson, 1989; Ueno *et al.*, 1996; Uchida *et al.*, 1996). What is clear however, is that residues in TM1-3 are vital in mediating the actions of both general and volatile anaesthetics.

Unlike benzodiazepines, anaesthetic action is not dependent on the presence of any particular subunit (e.g. γ subunit). However, the potency and efficacy of these compounds reflects the subunit composition of the $GABA_A$ receptor. The α subunit subtype isoform influences the potency and efficacy of PB and propofol potentiation and

direct activation of $GABA_A$ receptors (Thompson *et al.*, 1996; Wafford *et al.*, 1996; Krasowski *et al.*,1998; Lam & Reynolds, 1998). In contrast to benzodiazepine modulation, α6β2γ2S receptor currents were potentiated to a greater extent by PB than α1β2γ2S receptor currents (Thompson *et al.*, 1996), while the presence of the α4 subunit abolishes the direct activation of $GABA_A$ receptors by PB and propofol (Wafford *et al.*, 1996).

While the influence of different γ subunit isoforms has not been reported in detail, constructs in which γ was replaced with ε (αβε) were found to be insensitive to the modulatory actions of propofol, barbiturates and steroid anaesthetics yet could still be directly activated by these compounds (Davies *et al.*, 1997a). However, these findings were disputed by Whiting *et al.* (1997) and Neelands *et al.* (1999) who found little difference in the actions of PB, steroids and loreclezole on αβε and αβγ constructs. The modulatory actions of steroids and barbiturates are depressed in αβδ receptors although GABA-mimetic actions appear unaffected (Zhu *et al.*, 1996; Saxena & Macdonald, 1994). Interestingly, δ subunit-deficient mice demonstrated a dramatic reduction in the sensitivity to neuroactive steroids whereas responses to other anaesthetic agents remained unaltered (Mihalek *et al.*, 1999).

1.3.2.3. Determinants of general anaesthetic action on β subunits.

As spontaneous currents transduced by homomeric β1 and β3 $GABA_A$ receptors are potentiated by PB, propofol, etomidate and steroid anaesthetics, it seems likely that β subunits form a substantial part of their binding site(s) (Sigel *et al.*, 1989; Davies *et al.*, 1997b; Sanna *et al.*, 1995; Slany *et al.*, 1995; Krishek *et al.*, 1996; Wooltorton *et al.*, 1997b). Potentiation and activation by propofol, steroids and barbiturates is relatively independent of β subunit isoform (Thompson *et al.*, 1996; Hadingham *et al.*, 1993; Hill-Venning *et al.*, 1997). By contrast, the potencies of etomidate, the anticonvulsant loreclezole, and the non-steroidal anti-inflammatory drug mefenamic acid, are markedly greater (~ 300-fold) on receptors containing β2/3 isoforms than on those containing β1 (Wingrove *et al.*, 1994; Belelli *et al.*, 1997; Halliwell *et al.*, 1999; Table 1.1.). This

Table 1.1. Effects of residues in TM2 and TM3 on allosteric modulators.

Residue	Etomidate		Loreclezole	Pentobarbitone		Propofol		Enflurane/Isoflurane		Ethanol	Furosemide
	Modulatory	Direct	Modulatory	Modulatory	Direct	Modulatory	Direct	Modulatory	Direct	Modulatory	Antagonism
TM2											
$\beta1^{S15'I}$	no effect	reduced				no effect	reduced	abolished		reduced	
$\beta1^{S15'N}$	enhanced	enhanced	enhanced								enhanced
$\beta3^{N15'S}$	reduced	reduced	reduced								reduced
$\beta3^{N15'M}$	abolished	abolished	abolished	reduced	reduced	reduced	reduced				
$RDL^{M15'S}$				enhanced	introduced	enhanced	introduced				
$RDL^{M15'N}$	introduced	no effect				enhanced	introduced				
$\alpha2^{S15'I}$	no effect	no effect				no effect	no effect	abolished		reduced	
Gly $\alpha1^{S15'I}$								little effect	-	abolished	
$\rho1^{I15'S}$				introduced	no effect	no effect	-				
TM3											
$\beta1^{M36'W}$						abolished	no effect	abolished			
$\rho1^{W36'M}$				introduced	introduced						

Modified from Belelli *et al.*, 1999b. Mutations in ionotropic GABA ($\alpha2$, $\beta1$, $\beta3$, $\rho1$ and RDL) and glycine (Gly $\alpha1$) receptor subunits influencing the modulatory and direct effects of allosteric modulators. Lack of direct activation of wild-type receptors is indicated (-). A clear box indicates the effect was not determined. See text for references.

isoform selectivity results from a non-conserved residue at the extracellular end of TM2: serine 265 in β1, and asparagine 265 in β2/3. Mutation of this residue to serine (the analogous reside in β1 subunit) substantially reduced loreclezole sensitivity (Wingrove *et al.*, 1994). This asparagine has also been implicated in the modulation of GABA-activated currents by the anaesthetic etomidate (Belelli *et al.*, 1997), the non-steroidal anti-inflammatory agent mefenamic acid (Halliwell *et al.*, 1999), the anxiolytics tracazolate and etazolate (Thompson *et al.*, 1999c) and the diuretic furosemide (Thompson *et al.*, 1999a). In accordance with the dependence upon the β subunit *in vitro*, the loss of righting reflex evoked by etomidate is attenuated in β3-subunit deficient mice (Quinlan *et al.*, 1998).

Interestingly, replacement of the methionine residue with either serine or asparagine at the equivalent location in *Drosophila* RDL subunits greatly enhanced the actions of etomidate and surprisingly propofol and PB on these receptors, which are relatively insensitive to anaesthetics (McGurk *et al.*, 1998; Pistis *et al.*, 1999). Conversely, introduction of methionine into β3 subunits, abolished the etomidate and loreclezole sensitivity of $GABA_A$ receptors, and greatly reduced that of PB. Only the potency of steroid anaesthetics was insensitive to manipulation of this residue (Wingrove *et al.*, 1994; Belelli *et al.*, 1997; Pistis *et al.*, 1999).

ρ1 receptors are insensitive to PB. However, the replacement of the isoleucine found at the same position, with serine (as in β3) conferred PB-modulation on ρ1 homo-oligomers (Belelli *et al.*, 1999a). In addition, the mutation (T262Q) in TM2 of the β1 subunit abolished PB modulation of α1β1 $GABA_A$ receptor constructs whereas the analogous mutation in the α1 subunit was ineffective (Birnir *et al.*, 1997). Mutation of a conserved glycine residue (G219) at the putative entrance to TM1 in the β2 subunit reduced the potentiation of agonist binding by four structurally diverse anaesthetics (pentobarbitone, alphaxalone, etomidate and propofol; Carlson *et al.*, 1999).

Interestingly, a hydrophobic residue in TM3 of both α1 and β2 subunits has been implicated in PB sensitivity (Amin, 1999). Substitution of a tryptophan (W) at position 328 on the ρ1 subunit with either a methionine or an alanine residue (located in the

corresponding position in α1 and β2 subunits respectively) conferred PB sensitivity to the typically insensitive ρ1 subunit. Conversely, the M328W mutation abolished the PB sensitivity of β2 homomeric receptors. Mutation of the equivalent residue in TM3 of the β1 subunit ($\beta1^{M286W}$) eliminated the potentiating effect of propofol on GABA-activated conductance but not the direct activation of the $GABA_A$ receptor (Krasowski *et al.*,1998). Thus residues in TM1, 2 and 3 of β subunits have a profound effect on the actions of general anaesthetics.

1.3.2.4. Determinants of modulation by volatile anaesthetics.

The TM2 and TM3 residues discussed above, also profoundly affect the actions of volatile anaesthetics. The mutations S270I (TM2) and A291W (TM3) in the α2 subunit, or homologous mutations in the β1 subunit (S265I, M286W) eliminated the potentiation of GABA-activated Cl^- currents by clinically relevant concentrations of enflurane and isoflurane (Mihic *et al.*, 1997; Krasowski *et al.*, 1998). Interestingly, mutations in the α subunit did not affect the potencies of propofol or etomidate (Krasowski *et al.*, 1998), distinguishing the structural determinants of general and volatile anaesthetic action. It has recently been proposed that the side chain volume of this amino acid ($\alpha2^{S270}$) is crucial for both agonist gating and isoflurane sensitivity (Koltchine *et al.*, 1999). Amino acid residues with small side chain volumes (such as S or A) enabled $GABA_A$ receptor modulation by isoflurane, whereas larger amino acid side chains (such as C or T) impaired the modulatory effect. In support of this, mutations of the equivalent residues in glycine receptors α subunits which have very similar TM domains to GABA receptors, profoundly affect the actions of ethanol, enflurane and isoflurane (Mihic *et al.*, 1997; Krasowski *et al.*, 1998; Yamakura *et al.*, 1999). It is therefore possible that these regions form the binding site for volatile anaesthetics, while TM2 of the β subunit is involved in mediating the allosteric transitions induced by general anaesthetics which may bind outside the channel. In support of this view, α subunit isoforms of $GABA_A$ receptors affect anaesthetic potency, yet the primary structure of TM2 is absolutely conserved between α subunit isoforms, suggesting that residues outside TM2 may

contribute to the anaesthetic binding site. However, the locations of these binding sites are still contentious (see Belelli *et al.,* 1999b for review).

1.3.2.5. Determinants of modulation by ethanol.

Ethanol potentiates $GABA_A$ receptor function and shares several pharmacological properties with both barbiturates and benzodiazepines, displaying anticonvulsant, anxiolytic and sedative activity (Sieghart, 1995; Mehta & Ticku, 1999). The ability of ethanol to modulate the GABA response also appears to be dependent on the subunit composition of the receptor (Sieghart, 1995). The γ2L subunit, and in particular a protein kinase C (PKC) phosphorylation site, was previously considered a crucial determinant for ethanol modulation (Wafford *et al.*, 1991; Wafford & Whiting, 1992) and a recent study of mice deficient in an isoform of PKC (PKCε) demonstrated an increased sensitivity to the acute behavioural effects of ethanol, associated with an enhanced ethanol sensitivity of $GABA_A$ receptors *in vitro* (Hodge *et al.*, 1999). However, other studies using recombinant $GABA_A$ receptors (Sigel *et al.*, 1993; Marszalec *et al.*, 1994) and generation of γ2L-subunit deficient mice (Homanics *et al.*, 1999) have contradicted this view. Although structurally different, the potentiating action of ethanol on $GABA_A$ receptors was impaired by mutating residues previously implicated in mediating enflurane sensitivity ($\alpha1^{S270}$ or $\beta1^{S265}$; Mihic *et al.*, 1997). Coexpression of the two TM2 mutant subunits generated a $GABA_A$ receptor complex that was inhibited by ethanol.

1.3.3. The Intracellular domain.

The main regions of structural diversity between the $GABA_A$ receptor subunits are located within the large intracellular loop domain between TM3 and TM4 (Macdonald & Olsen, 1994). Presumably inaccessible to extracellularly applied modulators, this domain contains numerous consensus sites for phosphorylation by endogenous protein kinases, the phosphorylation of which can regulate receptor function (Moss & Smart,

1996; Smart, 1997). Alternatively spliced forms of the γ2 subunit (Whiting *et al.*, 1990; Kofuji *et al.*, 1991) and the human and chick β2 subunits contain small insertions within the TM3-TM4 loop conferring additional kinase consensus sequences (Harvey *et al.*, 1994; McKinley *et al.*, 1995). The alternative splicing of these subunits may thus create a further level of receptor diversity by generating $GABA_A$ receptors with similar agonist sensitivities and kinetic properties which differential in their sensitivity to intracellular regulators.

The intracellular domains of β and γ subunits are phosphorylated *in vitro* by a number of serine/threonine kinases including PKA (cAMP-dependent protein kinase), PKC, PKG (cGMP protein-dependent kinase) and CaM-kinase II (calcium/calmodulin dependent protein kinase II) and the tyrosine kinase *src* (Whiting *et al.*, 1990; Moss *et al.*, 1992; Machu *et al.*, 1993; McDonald & Moss, 1994; Moss *et al.*, 1995; Valenzuela *et al.*, 1995). Protein kinases may interact with the intracellular domains of $GABA_A$ receptor subunits via kinase anchoring proteins such as RACK-1 (receptor for activated C kinase) and AKAPs (A kinase –anchoring proteins (Pawson & Scott, 1997; Mochly-Rosen & Gordon, 1998). However, it has recently been demonstrated that PKC-βII can directly bind to β1 and β3 subunits independently of RACK-1 (Brandon *et al.*, 1999). These anchor proteins may therefore serve to locate regulatory enzymes close to their target molecule.

Despite possessing common consensus sites, $GABA_A$ receptors are differentially regulated depending on the protein kinase activated and the receptor subunit composition (Moss & Smart, 1996; Smart, 1997). Activation of PKC by phorbol esters inhibits both recombinant and native neuronal $GABA_A$ receptor function. However, PKA-mediated phosphorylation results in either up or down regulation of $GABA_A$ receptor function depending on the neuronal preparation. Recently, the differential regulation of $GABA_A$ receptor function by PKA was shown to be dependent upon the β subunit isoform (McDonald *et al.*, 1998). PKA-mediated phosphorylation of α1β3γ2S $GABA_A$ receptors resulted in a potentiation of GABA-activated currents whereas α1β1γ2S receptor currents were inhibited. The intracellular loop of the β3 subunit contains two adjacent serine residues (S408, S409) whereas the β1 subunit possesses a

sole serine (S409). Mutation of $\beta3^{S408}$ to alanine, the homologous residue in β1 subunits, evoked an inhibitory effect of PKA-mediated phosphorylation comparable to β1 subunit-containing receptors. Interestingly, the β2 subunit has identical residues at these positions to β1 subunits but is not phosphorylated by PKA in recombinant systems. Therefore the presence of phosphorylation consensus sequences does not necessarily imply that the subunit can be phosphorylated. Recently, GTAP34 ($GABA_A$ receptor-tubulin complex-associated protein of molecular mass 34kDa), a novel $GABA_A$ receptor-associated serine kinase, has been identified as specifically phosphorylating S409 of $GABA_A$ receptor β3 subunits and reducing receptor function, while not phosphorylating the adjacent S408 (Kannenberg *et al.*, 1999).

In contrast, the γ subunit appears to be vital for the enhancement in GABA-activated conductance mediated by tyrosine kinases (Moss *et al.*, 1995; Valenzuela *et al.*, 1995; Moss & Smart, 1996; Wan *et al.*, 1997; Huang *et al.*, 1999; see Chapter 7 for discussion). Although the β subunit is phosphorylated by tyrosine kinases *in vitro,* this does not appear to result in any functional effect (Moss *et al.*, 1995).

The protein kinases are themselves regulated by the activation of a variety of other receptors, for example receptors coupled to G-proteins (Smart, 1997). Therefore protein kinases permit the regulation of ionotropic receptor function by metabotropic receptors. In addition to modulation of $GABA_A$ receptor function, phosphorylation has been implicated in other aspects of regulation. PKA may be involved in the assembly of the receptor and protein kinases may also be important for effective trafficking of receptor subunits to the cell membrane and subsequent turnover of cell surface receptors (Moss & Smart, 1996; Smart, 1997). It remains to be determined what proteins are phosphorylated in these processes.

Recent experiments suggest that the intracellular domain of $GABA_A$ receptors can directly bind to cytoskeletal proteins, which may be vital for synaptic localisation of $GABA_A$ receptors in addition to inserting and/or stabilising receptors at the cell surface (Bedford *et al.*, 1999; Kneussel *et al.*, 1999; Wang *et al.*, 1999). The regulation of synaptic localisation of $GABA_A$ receptors is vital for correct neuronal function.

Gephyrin, a tubulin binding protein which clusters glycine receptors at synapses, has been found to bind to the large intracellular TM3-TM4 domain of β1 and γ2S $GABA_A$ receptor subunits, although it is not clear whether this is a direct interaction or one mediated by an intermediate protein (Bedford *et al.*, 1999). Interestingly, the intracellular domains of these subunits show no primary sequence identity to the gephyrin binding site located on glycine receptor β subunits (Meyer *et al.*, 1995; Kneussel *et al.*, 1999). Nonetheless, gephyrin appears to play a crucial role in $GABA_A$ receptor clustering (Betz, 1998). Removal of gephyrin either by antisense treatment or gene targeting (gephyrin$^{-/-}$ mice) abolished synaptic clustering of α2 and γ2 subunit-containing $GABA_A$ receptors (Essrich *et al.*, 1998; Kneussel *et al.*, 1999). Conversely, gephyrin clusters were substantially attenuated in γ2 subunit deficient mice ($\gamma2^{-/-}$; Essrich *et al.*, 1998). The γ3 subunit can partially restore the synaptic localization of gephyrin and $GABA_A$ receptors in $\gamma2^{-/-}$ mice, implying the existence of a conserved sequence motif in the large TM3-TM4 intracellular domains of both γ2 and γ3 subunits required for gephyrin-mediated clustering (Baer *et al.*, 1999). GABARAP ($GABA_A$ receptor-associated protein) interacts with the $GABA_A$ receptor γ2 subunit both *in vitro* and *in vivo* (Wang *et al.*, 1999) and could conceivably mediate interaction between γ2 subunits and gephyrin. GABARAP binds to a region of the intracellular TM3-TM4 domain (R394 – aspartate (D) 411) of the γ2 subunit that is highly conserved amongst the γ subunits, indicating that GABARAP might also bind to γ3 subunit containing receptors (Wang *et al.*, 1999). In addition, rapsyn, a cellular protein involved in postsynaptic ACh receptor clustering at the neuromuscular junction has been found to cluster $GABA_A$ receptors *in vitro* by unknown mechanisms (Yang *et al.*, 1997). The identification of direct links between the cytoskeleton and receptor subunits represents a new route for investigating the regulation of GABA signalling - for example by regulating the stability and size of clusters of synaptic $GABA_A$ receptors.

1.4. Ionic Regulation of the $GABA_A$ receptor.

$GABA_A$ receptors are targets of numerous pharmacological agents and clinically relevant compounds, however, their activity is also regulated by physiological

concentrations of cations binding to sites on the extracellular part of the protein (Fig. 1.4.). These ions can have a profound effect on GABA receptor function, however, the molecular determinants of ion modulation have been less well characterised than the modulators discussed above. The identification of residues involved in ionic modulation is one of the aims of the present study.

1.4.1. Effects of divalent cations.

1.4.1.1. Zinc.

Zinc (Zn^{2+}) is an important dietary constituent and is essential for the correct development of the nervous system (Hurley & Shrader, 1972; Dvergsten *et al.*, 1984). Zn^{2+} deficiency can result in behavioural disorders including lethargy and depression. In addition, Zn^{2+} has been linked with several neurological disease states including epilepsy and Pick's dementia (Harrison & Gibbons, 1994). Since Zn^{2+} is concentrated within neurons in certain regions of the CNS and is released during synaptic activity, Zn^{2+} may have an important physiological role in regulating neuronal excitability (Frederickson, 1989; Harrison & Gibbons, 1994; Smart *et al.*, 1994).

The Zn^{2+} sensitivity of native $GABA_A$ receptors depends on the type and developmental stage of the neuron tested (Table 1.2; Westbrook & Mayer, 1987; Smart & Constanti, 1990; Celentano *et al.*, 1991; Legendre & Westbrook, 1991; Smart, 1992). This diversity may be partly explained by the dependence of Zn^{2+} sensitivity on $GABA_A$ receptor subunit composition (Table 1.3; See Chapters 3.1 and 4.1 for discussion). The Zn^{2+} sensitivity of $\alpha\beta x$ $GABA_A$ receptor constructs (where x is γ,δ or ε) is markedly less than that of $\alpha\beta$ constructs (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Whiting *et al.*, 1997; Thompson *et al.*, 1997; Krishek *et al.*, 1998; Gingrich & Burkat, 1998). Furthermore, the α subunit isoform influences Zn^{2+} sensitivity (White & Gurley, 1995; Burgard *et al.*, 1996; Knoflach *et al.*, 1996; Fisher *et al.*, 1997). $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents were approximately 6-fold less sensitive to Zn^{2+} than $\alpha6\beta3\gamma2L$ receptors (Fisher *et al.*, 1997; Fisher & Macdonald, 1998). To investigate the structural basis for this difference, $\alpha1$ and $\alpha6$ subtype chimeras were constructed with a splice site

Table 1.2. Effect of Zn^{2+} on native $GABA_A$ receptors.

SPECIES/CELL TYPE	EFFECT	CONCENTRATION	IC_{50}	COMMENTS	REFERENCES
Lobster muscle	$\downarrow \Delta G_{GABA}$	10μM – 1mM	~25μM	Non-competitive inhibition of GABA-induced conductance ($\downarrow \Delta G_{GABA}$), reduced at low external pH.	Smart & Constanti, 1982.
Bullfrog dorsal root ganglia	$\downarrow I_{GABA}$	100 – 300μM	~ 100μM	Competitive, voltage-independent inhibition.	Akaike *et al.*, 1987; Yakushiji *et al.*, 1987.
Chick spinal neurons: embryonic	$\downarrow I_{GABA}$	0.1μM – 3mM	50μM	Non-competitive inhibition, reduced by the presence of Cd^{2+}, Ni^{2+} or Mn^{2+}. Intracellular application ineffective.	Celentano *et al.*, 1991.
Mouse hippocampus: embryonic	$\downarrow I_{GABA}$	2 – 300μM	11μM	Voltage-independent inhibition of GABA-activated current (I_{GABA}), saturating at 80-90% inhibition.	Westbrook & Mayer, 1987; Mayer & Vylicky, 1989.
Rat hippocampus: postnatal	$\downarrow I_{GABA}$ $\downarrow P_O$	30μM	N.D.	Non-competitive inhibition of GABA currents recorded from dissociated cultures, decreased open probability ($\downarrow P_O$) for GABA channels.	Legendre & Westbrook, 1991.
Rat sympathetic ganglia: Adult Postnatal/embryonic	N.E. (no effect) $\downarrow I_{GABA}$	100 – 500μM 100 – 300μM	N.D. (not determined) N.D.	Intact superior cervical ganglion. Non-competitive, voltage-independent inhibition, dissociated cultures and intact ganglion; embryonic GABA responses more susceptible to inhibition than adult responses.	Smart & Constanti, 1982. Smart & Constanti, 1990; Smart, 1992.
Rat cortex	N.E.	0.2M	N. D.	*In vivo* recording from adult.	Wright, 1984.
Rat pyriform cortex	N.E.	200μM	N.D.	Adult brain slices.	Hori *et al.*, 1987.
Rat cerebellar neurons	$\downarrow I_{GABA}$ $\downarrow P_O$	10 – 30μM	N.D.	Non-competitive, voltage–independent inhibition.	Smart, 1992; Kilic *et al.*, 1993.
Rat dorsal root ganglion neurons	$\downarrow I_{GABA}$	≤1mM	19μM	Non-competitive, voltage–independent inhibition, attenuated in the presence of increasing concentrations of Cu^{2+}.	Ma & Narahashi, 1993a.

Modified from Smart *et al.*, 1994.

Table 1.3. Modulation of recombinant $GABA_A$ receptors by Zn^{2+}.

RECOMBINANT RECEPTORS:		COMMENTS	REFERENCES
Effect of Zn^{2+}		Typically $\downarrow I_{GABA}$, voltage-independent, non-competitive inhibition.	See below.
Dependence on number of different subunits present		Characteristically, αβ receptor constructs are inhibited with high affinity whereas inclusion of a third subunit type lowers the potency for Zn^{2+}, in particular the addition of a γ subunit which leads to relative insensitivity to Zn^{2+}. Typical IC_{50} values (μM): α1β1, 0.2 ± 0.1; α1β1ε, 42 ± 4; α1β1γ2S, 712 ± 296 (Whiting *et al.*, 1997); α1β1, 1.2 ± 0.4; α1β1δ, 16 ± 4; α1β1γ2S, 640 ± 35; α1β1γ2Sδ, 615 ± 84 (Krishek *et al.*, 1998); α2β1γ1, 11 ± 3; α2β1θγ1, 24±12 (Bonnert *et al.*, 1999).	Draguhn *et al.*, 1990; Smart *et al.*, 1991; Saxena & Macdonald, 1994; Thompson *et al.*, 1997; Whiting *et al.*, 1997; Gingrich & Burkat, 1998; Krishek *et al.*, 1998; Bonnert *et al.*, 1999.
Dependence on α subtype		Receptors containing α1-subunits appear the least sensitive to Zn^{2+}. Typical maximal inhibition: α1β3γ2L, 19 ± 2%; α2β3γ2L, 51 ± 1%; α3β3γ2L, 53 ± 2% (White & Gurley, 1995). Typical IC_{50} values (μM): α1β3γ2L, 150 ± 34; α6β3γ2L, 26 ± 4 (Fisher & Macdonald, 1998).	White & Gurley, 1995; Burgard *et al.*, 1996; Knoflach *et al.*, 1996; Fisher *et al.*, 1997; Fisher & Macdonald, 1998.
Dependence on amino acid residues	α subunit	$\alpha6^{H274}$ responsible for increased Zn^{2+} sensitivity of α6 subunit-containing receptors. IC_{50} values (μM): α6β3γ2L, 26 ± 4; $\alpha6^{H274}$β3γ2L, 114 ± 12.	Fisher & Macdonald, 1998.
	β subunit	$\beta1^{H267}$ and $\beta3^{H267}$ important for high affinity Zn^{2+} inhibition. Typical IC_{50} values (μM): β3, 0.31 ± 0.02, $\beta3^{H267A}$, 307 ± 33; α1β3, 0.11 ± 0.01; $\alpha1\beta3^{H267A}$, 23 ± 4 (Wooltorton *et al.*, 1997a)	Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998.

within TM1, and co-expressed with wild-type β3 and γ2L subunits in L929 fibroblasts (Fisher *et al.*, 1997). The Zn^{2+} sensitivity of receptors containing the α1/α6 chimera, that includes the N-terminal domain of α1, was equivalent to that of wild-type α6-containing receptors. Similarly, the α6/α1-containing construct displayed comparable Zn^{2+} sensitivity to wild-type α1 subunit-containing receptors, implying that regions C-terminal to TM1 are important for the increased sensitivity to Zn^{2+} conferred by the α6 subtype (Fisher *et al.*, 1997). Histidine residues are known to contribute to Zn^{2+} binding sites and the substitution a histidine residue (H273) in the TM2-TM3 loop with an asparagine found in the analogous position in the α1 subunit (N274) reduced Zn^{2+} sensitivity to almost that of wild-type α1β3γ2L levels. Likewise, $\alpha1^{N274H}\beta3\gamma2L$ receptor currents displayed a similar Zn^{2+} sensitivity to currents gated by wild-type α6β3γ2L $GABA_A$ receptors (Fisher & Macdonald, 1998). The importance of histidine residues were first demonstrated in studies of β1 and β3 subunits, showing that a histidine residue located in TM2 of all β subunits is a vital determinant of Zn^{2+} potency (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998).

1.4.1.2. Copper.

Copper (Cu^{2+}) also represents a vital trace element in our dietary intake (1-2mg/day) and may also play an important physiological role in the regulation of neurotransmission. $Cu^{2+}$is concentrated in specific regions of the brain, in particular the hypothalamus (Donaldson *et al.*, 1973; Chan *et al.*, 1983). The endogenous release of Cu^{2+} from nerve terminals following depolarization has been reported, reaching concentrations of 100-250μM in the synaptic cleft (Hartter & Barnea, 1988; Kardos *et al.*, 1989). In addition, Cu^{2+} has been shown to accumulate abnormally in the brain in neurodegenerative disorders (Bouras *et al.*, 1991).

Cu^{2+} was first shown to inhibit $GABA_A$ receptor function on lobster muscle (Smart & Constanti, 1982; Table 1.4.). Cu^{2+} also non-competitively inhibited GABA-activated Cl^- currents from rat dorsal root ganglion (DRG) neurons independently of membrane potential (Ma & Narahashi, 1993a). The inhibition was rapid in onset and readily reversible and the sensitivity of the $GABA_A$ receptors to Cu^{2+} was comparable to that of

Table 1.4. Modulation of $GABA_A$ receptors by divalent cations.

SPECIES/CELL TYPE	EFFECT	CONCENTRATION	IC_{50} (μM)	COMMENTS	REFERENCES
Cu^{2+}					
Lobster muscle	$\downarrow \Delta G_{GABA}$	≤500μM	N. D.	Non-competitive inhibition.	Smart & Constanti, 1982.
Rat dorsal root ganglion (DRG) neurons	$\downarrow I_{GABA}$	3μM – 1mM . Maximum inhibition, 96% (1mM Cu^{2+}).	16 ± 2	Non-competitive, voltage-independent inhibition. Cu^{2+} (300μM) completely eliminates the inhibitory effect of Zn^{2+} (10μM).	Ma & Narahashi, 1993a.
Rat septal cholinergic neurons	$\downarrow I_{GABA}$	100μM; 50% inhibition.	N.D.	Inhibition of I_{GABA} comparable in the absence and presence of Zn^{2+} (100μM).	Kumamoto & Murata, 1995.
Recombinant $GABA_A$ receptors	$\downarrow I_{GABA}$	α1 containing receptors more sensitive than α6 containing constructs; α1β3γ2L, 9 ± 3μM (52%)*, 2 ± 3mM (48%)*; α6β3γ2L, 13 ± 2μM (16%)*, 1.7 ± 0.3mM (84%)*. Construction of chimeras indicate high Cu^{2+} sensitivity is mediated via N-terminal region of the α1 subunit.			Fisher & Macdonald, 1998.
Ni^{2+}					
Rat DRG neurons	$\downarrow I_{GABA}$	1mM; 80% inhibition.	N.D.	Reversible.	Ma & Narahashi, 1993a.
Chick spinal neurons: embryonic	$\downarrow I_{GABA}$	10mM; 80% inhibition.	N.D.	Ni^{2+} (10mM) partially relieves the inhibition of the response to 20μM GABA by Zn^{2+}(3mM).	Celentano *et al.*, 1991.
Recombinant $GABA_A$ receptors	$\downarrow I_{GABA}$	α6β3γ2L, 108± 9μM; α1β3γ2L, 1.3 ± 0.3mM. Construction of chimeras indicate high Ni^{2+} sensitivity is mediated via extracellular regions C-terminal to TM1 on the α6 subunit; $\alpha6^{H273}$ partially responsible, IC_{50}: $\alpha6^{H274N}$ β3γ2L, 212 ± 16μM.			Fisher & Macdonald, 1998.
Cd^{2+}					
Rat DRG neurons	$\downarrow I_{GABA}$	1mM; 82% inhibition.	N.D.	Reversible.	Ma & Narahashi, 1993a.
Chick spinal neurons: embryonic	$\downarrow I_{GABA}$	5mM; 71% inhibition.	N.D.	Cd^{2+} partially relieves the inhibition of the response to 3 - 5μM GABA by 3mM Zn^{2+}. Cd^{2+} inhibition is eliminated by application of Ni^{2+}(3mM).	Celentano *et al.*, 1991.
Rat septal cholinergic neurons	$\downarrow I_{GABA}$	100μM; 20% inhibition.	N.D.	Cd^{2+} inhibition of 20μM GABA response eliminated in the presence of 100μM Zn^{2+}	Kumamoto & Murata, 1995.
Recombinant $GABA_A$ receptors	$\downarrow I_{GABA}$	α1β3γ2L, 103 ± 34μM; α6β3γ2L, 134 ± 19μM. Construction of chimeras indicate high Cd^{2+} sensitivity is mediated via extracellular regions C-terminal to TM1 on the α6 subunit ($\alpha6^{H273}$ is important, IC_{50}: $\alpha6^{H274N}$ β3γ2L, 432 ± 56μM ± 16μM) and the N-terminal domain of the α1 subunit.			Fisher & Macdonald, 1998.

(* denotes relative contributions of IC_{50} values obtained from a two-population logistic equation).

Zn^{2+} (IC_{50}s: 16μM and 19μM respectively). Increasing the Cu^{2+} concentration reduced the antagonism of GABA-activated Cl^- currents by Zn^{2+}(10μM) until the inhibition by Zn^{2+} was completely eliminated (300μM Cu^{2+}), suggesting the existence of a common site of action (Ma & Narahashi, 1993a). However, the inhibitory action of Cu^{2+} (100μM) on GABA-activated Cl^- currents in rat septal cholinergic neurons persisted in the presence of Zn^{2+} (100μM; Kumamoto & Murata, 1995), possibly reflecting a variation in the $GABA_A$ receptor subunit composition in these neurons.

In contrast to the α subtype dependence of Zn^{2+} sensitivity, α1β3γ2L $GABA_A$ receptors were more sensitive to inhibition by Cu^{2+} than α6β3γ2L $GABA_A$ receptors (Fisher & Macdonald, 1998). GABA concentration-response curves constructed for both receptor constructs were best fitted with a two-site inhibition equation and indicated that the increased Cu^{2+} sensitivity of α1β3γ2L $GABA_A$ receptors was mainly due to a larger contribution of the higher affinity site than observed for α6-containing receptors. To investigate the location of a putative Cu^{2+} binding site, the α1 and α6 subtype chimeras (see 1.4.1.1.) were co-expressed with wild-type β3 and γ2L subunits in L929 fibroblasts (Fisher & Macdonald, 1998). Cu^{2+} inhibited the GABA response of receptors containing α1/α6 chimeras to an equivalent degree as α1- containing receptors. Similarly, the degree of inhibition by Cu^{2+} of α6/α1- and α6-containing receptors was analogous, indicating that the N-terminal domain of the α1 subunit is responsible for the high Cu^{2+} sensitivity. In agreement, the TM2-TM3 loop mutations $\alpha6^{H273N}$ and $\alpha1^{N274H}$ did not alter Cu^{2+} sensitivity. This differs from the region identified as important in the higher Zn^{2+} sensitivity on the α6 subunit (Fisher *et al.*, 1997; Fisher & Macdonald, 1998). The contribution of the high affinity Zn^{2+} binding site on the β subunit (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998) to Cu^{2+} sensitivity has not been investigated to date. Therefore, whilst some Zn^{2+} and Cu^{2+} sites may coincide, it appears there is a divergence of the sites on the α subunits.

1.4.1.3. Other divalent cations.

Although only Zn^{2+} and Cu^{2+} appear to be endogenously released into synapses (Assaf & Chung, 1984; Howell *et al.*, 1984; Hartter & Barnea, 1988; Kardos *et al.*, 1989; Xie

& Smart, 1991) other divalent cations may have a role in certain physiological or pathological states (Carpenter, 1994). Investigating their effects on synaptic activity may prove useful in understanding their neurotoxicity. It is not known whether divalent cations inhibit GABA-activated Cl^- currents by acting at the same site on the $GABA_A$ receptor or whether multiple allosteric modulatory sites are present.

Other divalent cations including cadmium (Cd^{2+}), nickel (Ni^{2+}), cobalt (Co^{2+}) and lead (Pb^{2+}) inhibit GABA-activated Cl^- currents with varying sensitivities depending on the developmental stage and type of neurons considered (Ma and Narahashi, 1993a; Narahashi *et al.*, 1994; Kumamoto and Murata, 1995; Table 1.4.). GABA-activated Cl^- currents recorded in rat DRG neurons were rapidly and reversibly inhibited by extracellular application of 1mM Cd^{2+}, Ni^{2+}, $Co^{2+}$and Pb^{2+} to 18, 20, 29 and 35% of control respectively (Ma & Narahashi, 1993a). However, manganese (Mn^{2+}; 1mM) did not significantly alter the GABA response. Similar results were obtained in embryonic chick spinal cord neurons (Celentano *et al.*, 1991). Ni^{2+} and Cd^{2+} substantially inhibited the GABA response (84% and 72% maximal inhibition respectively) whereas GABA-activated currents were suppressed by less than 40% by high millimolar concentrations of Mn^{2+}. The inhibitory action of high (millimolar) concentrations of Zn^{2+} and Cd^{2+} on the GABA response was not additive (Celentano *et al.*, 1991). Indeed the inhibitory effect of Zn^{2+} (3mM) on the response to 20μM GABA was partially alleviated by co-application with either Cd^{2+}(5mM), Ni^{2+}(10mM) and Mn^{2+}(10mM) suggesting that the sites of action of these divalent cations may interact or overlap. This is supported by the observation that Cd^{2+} (100μM) sensitivity is abolished in the presence of Zn^{2+} (100μM) in rat septal cholinergic neurons (Kumamoto & Murata, 1995).

The effects of altering the α subunit subtype on Ni^{2+} and Cd^{2+} sensitivity has also been investigated by constructing α1/α6 chimeras in which the N-terminal domains have been swapped (Fisher & Macdonald, 1998). Comparable to the α subtype dependence of Zn^{2+} sensitivity, α6β3γ2L $GABA_A$ receptor currents were 12-fold more sensitive to Ni^{2+} than α1β3γ2L GABA responses. The degree of inhibition of wild-type α6- and α1/α6 chimeric-containing $GABA_A$ receptors by Ni^{2+} was equivalent. Similarly α1- and α6/α1 chimeric-containing $GABA_A$ receptors demonstrated comparable sensitivity to Ni^{2+},

indicating that extracellular domains located C-terminal to the splice site in TM1 already known to regulate Zn^{2+} sensitivity also regulate the high Ni^{2+} sensitivity of α6 subunit (Fisher *et al.*, 1997; Fisher & Macdonald, 1998). However, the $\alpha6^{H273N}$ mutation only slightly reduces the sensitivity of the receptor to Ni^{2+}, and $\alpha1^{N274H}$subunit-containing receptors display an intermediate sensitivity to Ni^{2+}, suggesting that although a histidine in this position contributes to the higher nickel sensitivity of the α6 subtype, other residues are likely to be involved (Fisher & Macdonald, 1998).

Analysis of α1 and α6 subunit-containing $GABA_A$ receptors has indicated the structural dependence of cadmium sensitivity differs from that of Zn^{2+}(Fisher & Macdonald, 1998). Both α6β3γ2L and α1β3γ2L receptors display an equivalent sensitivity to cadmium (IC_{50}s: 134 ± 19.3μM and 103 ± 34μM respectively). Although the Cd^{2+} sensitivity of α1/α6 chimeric containing receptors was comparable to both wild-type α1- and α6-containing receptors, the Cd^{2+} sensitivity of α6/α1 chimeric-containing receptors was dramatically reduced (IC_{50}: 696 ± 203μM) implicating residues in the α1 N-terminus and C-terminal to TM1 in the α6 subunit as crucial for coordinating Cd^{2+}. Interestingly, the $\alpha6^{H273N}$ mutation reduced Cd^{2+} sensitivity although not to the extent observed with the α6/α1 chimera implicating a possible contribution from other residues. The Cd^{2+} sensitivity of $GABA_A$ receptors containing the $\alpha1^{N274H}$ mutation was equivalent to wild-type α1β3γ2L constructs. Since the α1/α6 chimera appears to contain regulatory domains for Cd^{2+} sensitivity of both α subtypes, the observation that the α1/α6 chimera does not enhance Cd^{2+} sensitivity above wild-type values may indicate that these sites are not additive or that only one of these sites was functional (Fisher & Macdonald, 1998).

The ability to inhibit the $GABA_A$ receptor via extracellular application is not a general property of divalent cations. Barium (Ba^{2+}; 5mM) did not affect the GABA response of rat DRG neurons although partially alleviated the inhibition produced by either Zn^{2+}(100μM) or Cd^{2+}(500μM; Celentano *et al.*, 1991). Removal of both calcium (Ca^{2+}) and magnesium (Mg^{2+}) from the extracellular recording solution did not affect the Zn^{2+} or Cd^{2+} sensitivity of these $GABA_A$ receptors. Similarly extracellular Ca^{2+}, Mg^{2+} and Ba^{2+} did not affect the GABA response in either lobster muscle (Smart & Constanti,

1982) or frog DRG neurons (Yakushiji *et al.*, 1987; Akaike *et al.*, 1989). In addition, GABA-activated currents recorded from rat septal cholinergic neurons were not altered by the extracellular application of strontium (Sr^{2+}; 100μM; Kumamoto & Murata, 1995). Whereas intracellular application of Zn^{2+} is ineffective (Celentano *et al.*, 1991), intracellular application of Ca^{2+} reduces the affinity of the $GABA_A$ receptor (Inoue *et al.*, 1986; Kaila, 1994 for review) indicating a further diversity in the sites of action of divalent cations. Furthermore, Hg^{2+} (0.1-100μM) evoked a large enhancement of GABA-activated Cl^- currents in rat DRG neurons which proved irreversible (Arakawa *et al.*, 1991) and may play a crucial role in Hg^{2+} toxicity.

1.4.2. Effect of trivalent cations.

1.4.2.1. The lanthanides.

Lanthanides are trivalent cations commonly referred to as rare earth metals. There are fifteen members ranging from lanthanum (La^{3+}, atomic number 57) to lutetium (Lu^{3+}, atomic number 71). Clinically, the therapeutic potential of lanthanides has been widespread including use in dentistry and in treating tuberculosis and cancer, as anti nausea, anti-inflammatory, and anti-viral agents and as anti-coagulants (Reviewed by Ma & Narahashi, 1993b). In addition, there has been an increase in the industrial usage of lanthanides, generating investigation into the pharmacological and cytotoxic actions of these compounds (Das *et al.*, 1988). Cytotoxic effects have been observed using micromolar concentrations of lanthanum in both cell culture and animal systems (Haley, 1965). In addition, lanthanides have been shown to interact with amino acids, nucleoproteins, phospholipids, enzymes and intermediary metabolites (Das *et al.*, 1988) providing a variety of potential targets for modification of cellular function. Therefore, it is important to understand the possible effects of these compounds on the excitability of the nervous system and to determine their mechanism of action.

Functional studies using recombinant $GABA_A$ receptors have been used to investigate a possible dependence on subunit composition for lanthanide modulation of GABA-activated currents. La^{3+} reversibly potentiated GABA-activated currents in $\alpha1\beta2\gamma2$

receptors expressed in HEK cells with an EC_{50} of 21μM and a maximum potentiation of 240% of control (Im *et al.*, 1992). A weak voltage-sensitivity was observed as the degree of potentiation was reduced at depolarized membrane potentials, which is consistent with an extracellular site of action. However, α1β2 receptors were substantially less sensitive to La^{3+}. Concentrations of La^{3+} greater than 100μM were required to elicit potentiation, and a maximum potentiation of only 70% was observed, implying that the γ subunit is important for high affinity La^{3+} binding (Im *et al.*, 1992). This is in direct contrast to the Zn^{2+} sensitivity of $GABA_A$ receptors which is drastically reduced by the inclusion of a γ subunit in the receptor complex (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Gingrich & Burkat, 1998).

La^{3+} was shown to potentiate GABA-gated Cl^- currents in rat DRG neurons in a dose-dependent manner with an EC_{50} of 231μM and a maximal enhancement to approximately 300% of control (Ma & Narahashi, 1993a). The potentiating effect was readily reversible on the removal of La^{3+} from the bath. La^{3+} (100μM) did not alter the reversal potential and the potentiation induced by La^{3+} was only weakly voltage-dependent, with a slight reduction in potentiation at depolarized membrane potentials, which is consistent with observations from recombinant $GABA_A$ receptors (Im *et al.*, 1992). Furthermore, La^{3+} did not affect benzodiazepine, barbiturate, picrotoxin, Zn^{2+} or Cu^{2+} modulation of the GABA-induced response indicating a novel site of action on the $GABA_A$ receptor (Ma & Narahashi, 1993a). In addition to potentiating the GABA response, application of millimolar concentrations of lanthanides directly activated Cl^- currents that are potentiated by benzodiazepines and barbiturates and inhibited by PTX, Zn^{2+}, penicillin and bicuculline (Ma & Narahashi, 1993b). The rank order of efficacies of the seven lanthanides tested as both modulators and activators of $GABA_A$ receptors was: Lutetium (Lu^{3+}) > erbium (Er^{3+}) > terbium (Tb^{3+}) > europium (Eu^{3+}) > neodymium (Nd^{3+}) > cerium (Ce^{3+})> lanthanum (La^{3+}; Ma & Narahashi, 1993b). As the effective ionic radii of these ions gradually decrease with increasing atomic mass (Shannon, 1976) the efficacy lanthanide ions correlates inversely with their ionic radii. The rapid onset/offset of both potentiation and direct activation of the $GABA_A$ receptor suggests an extracellular site of action, supported by the relative impermeability of the cell membrane to lanthanides (Lesseps, 1967; Langer & Frank, 1972). Further support for an extracellular site of action comes from single channel responses to Tb^{3+} recorded from

outside/out patches from rat DRG neurons. These revealed an increase in the relative proportions of the longest open and burst duration time constants in the presence of Tb^{3+}, in conjunction with a decrease in the relative proportion of the longest closed time constant (Ma *et al.*, 1994). Thus lanthanides increase the apparent mean open time of $GABA_A$ receptors. These observations suggested that lanthanides induced allosteric changes which increased the affinity of GABA for its binding site and/or increased the likelihood of the receptor assuming open conformations (Ma *et al.*, 1994).

The sensitivity of $GABA_A$ receptors to La^{3+} is also dependent on the α subunit subtype present. La^{3+} strongly potentiated GABA-activated currents in α1β3γ2L receptors expressed in L929 fibroblasts (Saxena *et al.*, 1997) with an EC_{50} of 210μM. Maximum potentiation was achieved with 1mM La^{3+} (164% of control GABA response), although greater concentrations of La^{3+} evoked less potentiation and 10mM La^{3+} was virtually ineffective. Substituting the α1 subunit with α6 reversed the modulatory effect of La^{3+} such that La^{3+} weakly inhibited α6β3γ2L GABA-activated currents, with an IC_{50} of 117μM and a maximum inhibition of 32% achieved at 3mM La^{3+}(Saxena *et al.*, 1997). Interestingly, replacing the γ2L subunit with a δ subunit increased the level of maximal inhibition. La^{3+} (600μM) produced a maximal inhibition of 80% of α6β3δ $GABA_A$ receptor currents (IC_{50}: 29μM). This substitution generates a similar effect on Zn^{2+} sensitivity (see Chapter 4 for discussion). Interestingly, α1β1δ receptors were insensitive to La^{3+} (Saxena & Macdonald, 1994) suggesting a possible counteraction of potentiating and inhibitory effects of the α1 and δ subunits respectively (Saxena *et al.*, 1997). Thus far, the influence of β subunit subtypes on La^{3+} sensitivity has not been investigated.

The La^{3+} binding site(s) is likely to consist of extracellular amino acid residues from at least the α and γ/δ subunits. Lanthanide binding sites have previously been determined on proteins to be near aromatic amino acid residues, particularly in proximity to a motif of tryptophan, tyrosine and phenylalanine residues (Martin & Richardson, 1979). Therefore, these residues may play an important role in the La^{3+} sensitivity of the $GABA_A$ receptor. As with Zn^{2+} and Cu^{2+}, the role of the N-terminal domain in La^{3+} sensitivity has been investigated by constructing α1 and α6 subtype chimeras by

exchanging their N-terminal domains via a splice site within the putative TM1 (Fisher *et al.*, 1997). The chimeric constructs were co-expressed with β3 and γ2L subunits in L929 fibroblasts. The α1/α6 chimeric construct conferred an equivalent degree of potentiation and sensitivity to the wild-type α1 subunit (EC_{50}s: 190μM and 233μM respectively) indicating that the positive modulatory site is located on the N-terminal domain of the α1 subunit. Although La^{3+} inhibited α6β3γ2L-gated Cl^- currents, La^{3+} did not inhibit receptors containing either of the chimeric constructs. In fact the α6/α1β3γ2L receptor displayed an intermediate sensitivity to La^{3+} (very slight potentiation) suggesting that structural domains from both N- and C-terminal regions of the α6 subunit are required to form a functional inhibitory site of action for La^{3+} (Fisher *et al.*, 1997). Further construction of α1/α6 chimeras identified the region between the cysteine loop and the beginning of TM1 in the α1 subunit as important for positive modulation by La^{3+} (Kim & Macdonald, 1999).

1.4.2.2. Aluminium.

Aluminium (Al^{3+}) alloys and compounds are widely used both at home and in industry. The abnormal accumulation of Al^{3+} in the brain has been detected in neurodegenerative disorders (Bouras *et al.*, 1991). Preliminary investigations have demonstrated that Al^{3+} (1mM) suppressed the GABA-activated Cl^- current from rat dorsal root ganglion neurons to approximately 78% of control response to 30μM GABA (Ma & Narahashi, 1993a), suggesting that Al^{3+} could regulate inhibitory synapses in certain pathological states.

1.4.3. Effect of H^+.

Protons are ubiquitously distributed throughout the CNS. Certain pathological conditions such as ischaemia, epileptic seizures and anoxia (Chesler, 1990; Chesler & Kaila, 1992) produce a reduction in external pH. Transient alterations in extracellular pH feature in normal synaptic transmission (Chen & Chesler, 1992) which could affect $GABA_A$ receptor mediated inhibition and the control of neuronal excitability. Furthermore, external pH may be altered following bicarbonate efflux upon activation

of $GABA_A$ receptors (Kaila & Voipio, 1987; Kaila, 1994) and could modulate GABA-activated conductance.

Differential effects of altering pH have been reported on GABA-activated Cl^- currents in neuronal $GABA_A$ receptors. Increased extracellular H^+ concentration enhanced GABA responses recorded in cat dorsal root ganglia and rat cerebellar granule neurons (Gallagher *et al.*, 1983; Robello *et al.*, 1994) and inhibited GABA responses recorded in rat sympathetic ganglia, spinal and reticulospinal neurons (Groul *et al.*, 1980; Smart, 1992; Krishek *et al.*, 1996a). These differences in H^+ sensitivity may partly reflect variation in subunit composition of the native $GABA_A$ receptors present in different neurons. Interestingly, increased extracellular H^+ concentration inhibited GABA-activated currents on rat hippocampal neurons at low GABA concentrations, yet enhanced the GABA response at saturating GABA concentrations, suggesting the coexistence of two discrete populations of $GABA_A$ receptors (Pasternack *et al.*, 1996).

Functional studies using recombinant $GABA_A$ receptors indicate that sensitivity to external H^+ concentration is in fact highly dependent on the subunit composition of the receptor construct (Krishek *et al.*, 1996a). Lowering external pH to 5.4 decreased the apparent affinity for GABA and increased the maximum response to saturating concentrations of GABA in $\alpha1\beta1$ receptors expressed in *Xenopus* oocytes. The potentiating effect of increasing H^+ concentration was voltage-independent and the reversal potential of the GABA response remained unaltered. Conversely, raising external pH to 9.4, non-competitively inhibited the GABA concentration-response curve. The pH titration relationship predicted that H^+ could interact with two discrete sites with apparent pK_a values of 6.6 and 7.5 (presumably histidine residues). Interestingly, $\alpha1\beta1\gamma2S$ $GABA_A$ receptors were virtually insensitive to alterations in external pH (4.4-9.4), though altering the β subunit subtype restored H^+ sensitivity (Krishek *et al.*, 1996a). Either increasing or decreasing extracellular H^+ concentration diminished the GABA-activated conductance in $\alpha1\beta2\gamma2S$ receptor constructs, with predicted pK_a values of 5.2 and 9.4, potentially corresponding with aspartate/glutamate and tyrosine residues. Exchanging the $\gamma2S$ for the δ subunit also restored H^+ sensitivity although decreasing the external pH increased the maximum GABA response in $\alpha1\beta1\delta$ receptors without affecting the GABA EC_{50}. Raising external pH non-

competitively inhibited the GABA response and pK_a values of 6.6 and 9.9 were obtained. Finally, either increasing or decreasing external pH diminished the GABA response in oocytes expressing $\alpha1\beta1\gamma2S\delta$ receptor constructs. H^+ appeared to be acting at sites with presumed pK_a values of 5.5 and 9.4 (Krishek *et al.*, 1996a). The differential modulation of $GABA_A$ receptors presumably reflects multiple sites of action for H^+. The pK_a values estimated from pH titration plots provide an indication of the amino acids involved in mediating H^+ sensitivity, although in a mature protein pK_a values may be distorted by the local microenvironment (Antosiewicz *et al.*, 1994). Cysteine residues have recently been implicated in mediating H^+ sensitivity, since the effect of reducing pH on $GABA_A$ receptor function is impaired in the presence of the alkylating reagent N-ethylmaleimide (NEM; Dillon *et al.*, 1999).

Protons are most likely to mediate the GABA response via a direct interaction with the extracellular domains of $GABA_A$ receptors. Firstly, it is doubtful that the sensitivity of the GABA response is due to the charge distribution of the GABA molecule itself, since in the pH 5-9.5, the zwitterion constitutes the greatest proportion of GABA molecules present (92-100%). In addition, it appears that external H^+ concentration does not affect $GABA_A$ receptor function by a subsequent change in intracellular H^+ concentration. Changes in the external pH resulted in negligible alterations to internal pH (Krishek *et al.*, 1996a) and GABA-activated Cl^- currents in crayfish muscle fibres were insensitive to changes in internal pH (Pasternack *et al.*, 1992). Finally, the reduction in Zn^{2+} sensitivity of $GABA_A$ receptors generated by increasing extracellular H^+, raises the possibility of overlapping sites of action on the $GABA_A$ receptor (Smart & Constanti, 1982; Krishek *et al.*, 1998). Interestingly, $\alpha1\beta1\gamma2S$ $GABA_A$ receptors demonstrate a marked insensitivity to both Zn^{2+} (Draguhn *et al.*, 1990; Smart *et al.*, 1991) and H^+ (Krishek *et al.*, 1996a). Therefore perturbations in extracellular H^+ concentration may play an important role in regulating neuronal excitability in regions where Zn^{2+}-containing nerve terminals impinge on GABAergic synapses.

1.5. Aims and objectives.

The aim of the present study is to identify molecular determinants affecting the modulation of $GABA_A$ receptor function by both exogenous and endogenous agents. This will predominantly be achieved by utilising site-directed mutagenesis and subsequent expression of murine $GABA_A$ receptor subunits (wild-type and mutant) in *Xenopus* oocytes for analysis using the two-electrode voltage clamp technique. To assess the efficiency of the *Xenopus* oocyte expression system, recombinant $GABA_A$ receptors will also, in certain cases, be expressed in human embryonic kidney cells and assessed using whole-cell patch clamp recording.

The initial objective focuses on the divalent cation Zn^{2+}. Zn^{2+} is an endogenous inhibitor of $GABA_A$ receptor function and may have an important physiological role in modulating neuronal excitability (Smart *et al.*, 1994). Since external application of Zn^{2+} is required for the inhibition of $GABA_A$ receptor function (Celentano *et al.*, 1991), the extracellular domain must contain amino acid residues crucial for co-ordinating Zn^{2+} binding. This study will examine the contribution of extracellular residues on the β3 $GABA_A$ receptor subunit to the inhibitory effect of Zn^{2+}. Similarly, the possible involvement of amino acids on the $GABA_A$ receptor α1 subunit in Zn^{2+} inhibition, which has not been investigated to date, will also be addressed.

A histidine residue (H267) in TM2, located at the extracellular end of the putative ion channel, has previously been identified as crucial for the Zn^{2+} inhibition of β3 and α1β (1 or 3) $GABA_A$ receptors (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). This discovery will be exploited in an attempt to probe the structure of the $GABA_A$ receptor anion channel, by developing the novel substituted histidine accessibility method (SHAM). Selected putative channel lining residues in the $GABA_A$ $\beta3^{H267A}$ mutant subunit receptor will be individually mutated to histidine and the accessibility of these substituted histidines to Zn^{2+} will be assessed using electrophysiological methods. The unique characteristics of the mutant channel, in particular the relative insensitivity of

$\beta3^{H267A}$ subunit receptors to Zn^{2+}, will also be used as the basis for an electrophysiological approach, based on the binomial theorem, to determine receptor subunit stoichiometry for a β3 subunit homomer.

Finally, the intracellular domain represents a region of structural diversity between the $GABA_A$ receptor subunits. The large intracellular loop between TM3 and TM4 of the γ2S subunit contains consensus sequences for tyrosine phosphorylation (Moss & Smart, 1996). To assess whether the γ2S subunit is modulated by tyrosine phosphorylation, the effects of membrane permeable protein tyrosine kinase inhibitors on α1β1γ2S $GABA_A$ receptors (wild-type and mutant) will be determined electrophysiologically.

Figure 1.1. $GABA_A$ receptor subunit structure. Schematic diagram of a $GABA_A$ receptor subunit. Each subunit is composed of an extracellular hydrophilic N-terminal domain, four hydrophobic transmembrane domains (TM), a large intracellular hydrophilic domain linking TM3 and TM4, and an extracellular C-terminal domain. The two highly conserved cysteine residues forming the putative disulphide bridge are also indicated (yellow).

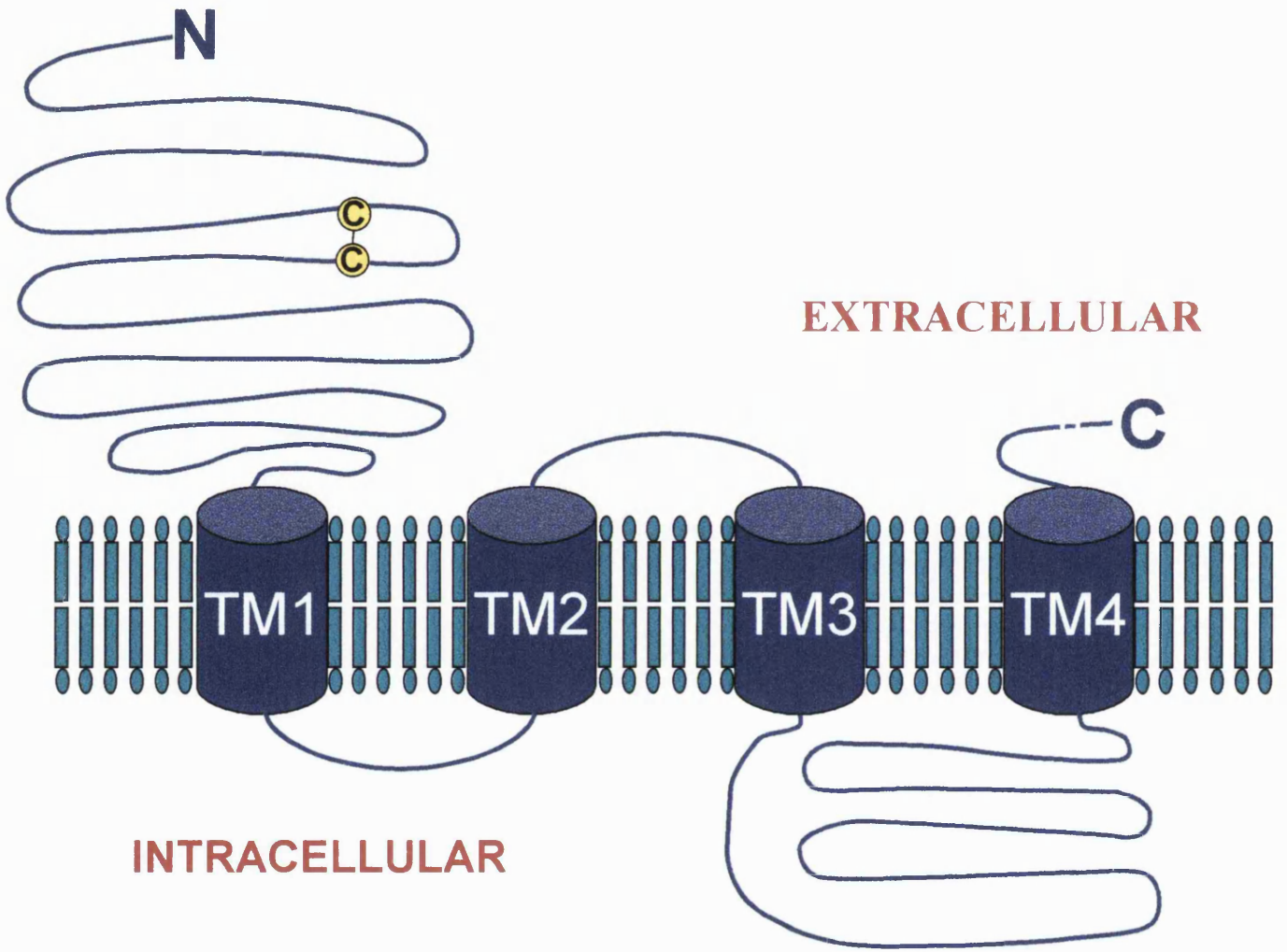
N
C
C
EXTRACELLULAR
C
TM1
TM2
TM3
TM4
INTRACELLULAR

Figure 1.2. Functional domains of the $GABA_A$ receptor N-terminus. (A) Comparison of N-terminal amino acid sequences for rat α1, β2 and γ2 subunits. Residues conserved between subunit classes are highlighted in yellow. Underlined residues represent the proposed start of TM1. The two highly conserved cysteine residues, which by analogy to nicotinic ACh receptors, are believed to form a disulphide bridge are indicated (blue). Putative components of the GABA binding domain are highlighted in green (Mehta & Ticku, 1999 and references therein; Newell *et al.*, 1999; Boileau *et al.*, 1999; Hartvig *et al.*, 1999). The boxed residue was identified in mediating GABA potency on α5-subunit containing receptors (Hartvig *et al.*, 1999). Amino acids influencing benzodiazepine sensitivity are shaded in blue (Schaerer *et al.*, 1998; Mehta & Ticku, 1999 and references therein; Kucken *et al.*, 1999; Renard *et al.*, 1999). Important determinants of $GABA_A$ receptor assembly are shown in pink (Srinivasan *et al.*, 1999; Taylor *et al.*, 1999). (B) Schematic diagram illustrating the presumed location of GABA and benzodiazepine (BZ) binding sites at subunit interfaces. The subunit stoichiometry of the $GABA_A$ receptor is controversial. Possible arrangements of 2α: 2β: 1γ (Chang *et al.*, 1996; Tretter *et al.*, 1997) and 2α: 1β: 2γ (Backus *et al.*, 1993) would presumably alter the number of GABA and BZ binding sites. Various residues implicated in ligand binding are pictured in a putative multiple loop model as proposed for the agonist-binding domain in other members of the nicotinic acetylcholine receptor family (Galzi & Changeux, 1994).

A

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α1  35  ERVTEVKTDI FVTSFGPVSD HDMEYTIDVF FRQSWKDERL KFKGPMTVLR  84
β2  33  GPPVAVGMNI DIASIDMVSE VNMDYTLTMY FQQAWRDKRL SYNVIPLNLT  82
γ2  48  VKPTLIHTDM YVNSIGPVNA INMEYTIDIF FAQTWYDRRL KFNSTIKVLR  97

α1  95  LNNLMASKIW TPDTFFHNGK KSVAHNMTMP NKLLRITEDG TLLYTMRLTV 134
β2  93  LDNRVADQLW VPDTYFLNDK KSFVHGVTVK NRMIRLHPDG TVLYGLRITT 132
γ2 108  LNSNMVGKIW IPDTFFRNSK KADAHWITTP NRMLRIWNDG RVLYTLRLTI 147

α1 135  RAECPMHLED FPMDAHACPL KFGSYAYTRA EVVYEWTREP ARSVVVAEDG 184
β2 133  TAACMMDLRR YPLDEQNCTL EIESYGYTTD DIEFYW-RGD DNAVTGVTKI 180
γ2 148  DAECQLQLHN FPMDEHSCPL EFSSYGYPRE EIVYQWKRSS VE--VGDTRS 195

α1 185  SRLNQYDLLG QTVDSGIVQS STGEYVVMTT HFHLKRKIGY FVIQTYLPCI 234
β2 181  -ELPQFSIVD YKLITKKVVF STGSYPRLSL SFKLKRNIGY FILQTYMPSI 230
γ2 196  WRLYQFSFVG LRNTTEVVKT TSGDYVVMSV TFDLSRRMGY FTIQTYIPCT 245
```

B

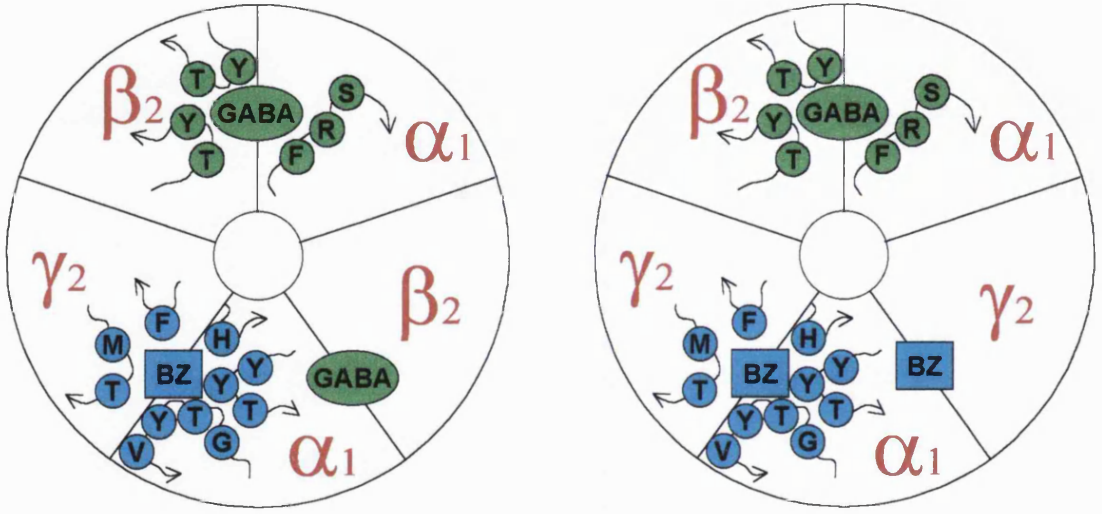


Figure 1.3. Importance of residues in the transmembrane domains of $GABA_A$ receptor α and β subunits for the modulation of receptor function. Alignment of amino acid sequences for the rat $GABA_A$ receptor α(1-6) and β(1-3) subunits. Underlined residues constitute transmembrane domains (TM1-TM3). X denotes a difference in amino acid residue between subunit isoforms. Residues conferring sensitivity to anaesthetic agents or ethanol are highlighted in green (Birnir *et al.*, 1997; Mihic *et al.*, 1997; Krasowski *et al.*, 1998 Amin, 1999; Carlson *et al.*, 1999; Koltchine *et al.*, 1999). An asparagine residue (N265) underlying the β2/β3 subunit selectivity of a range of agents is highlighted in pink (Wingrove *et al.*, 1994; Belelli *et al.*, 1997; Halliwell *et al.*, 1999; Thompson *et al.*, 1999a, c). Putative ion channel lining residues (in bold) were identified using the substituted cysteine accessibility method (Xu & Akabas, 1993, 1996). Amino acids altering picrotoxin sensitivity are denoted in red (Gurley *et al.*, 1995, Xu *et al.*, 1995). Determinants of Zn^{2+} sensitivity are highlighted in blue (Wooltorton *et al.*, 1997a; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998). A highly conserved arginine residue implicated in anion permeability is italicised (Wooltorton *et al.*, 1997c, 1998).

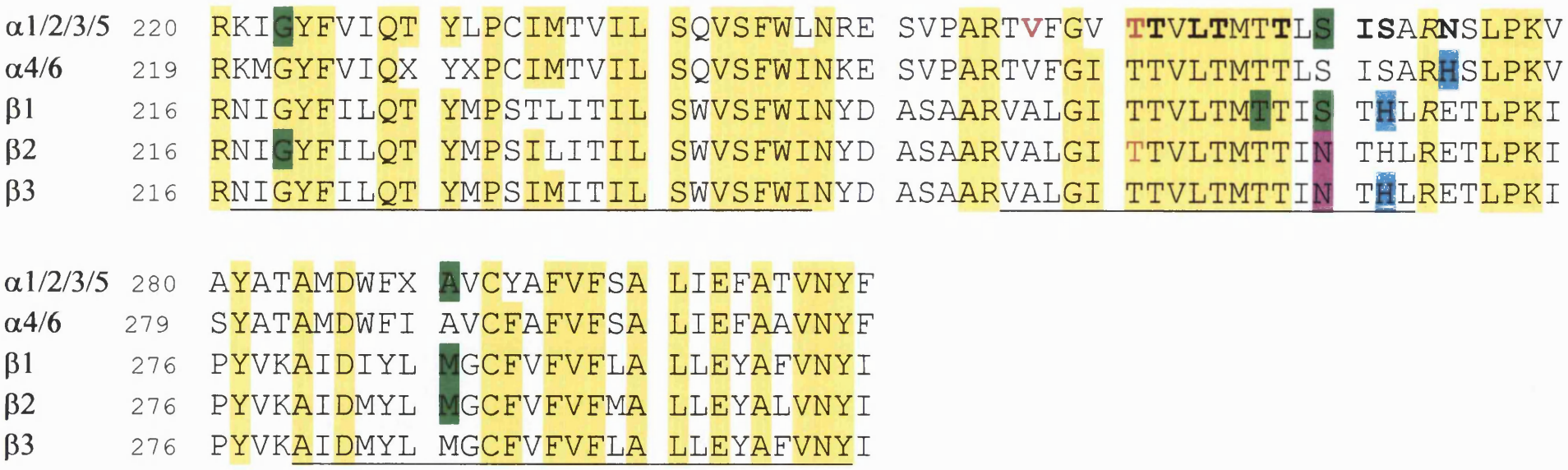
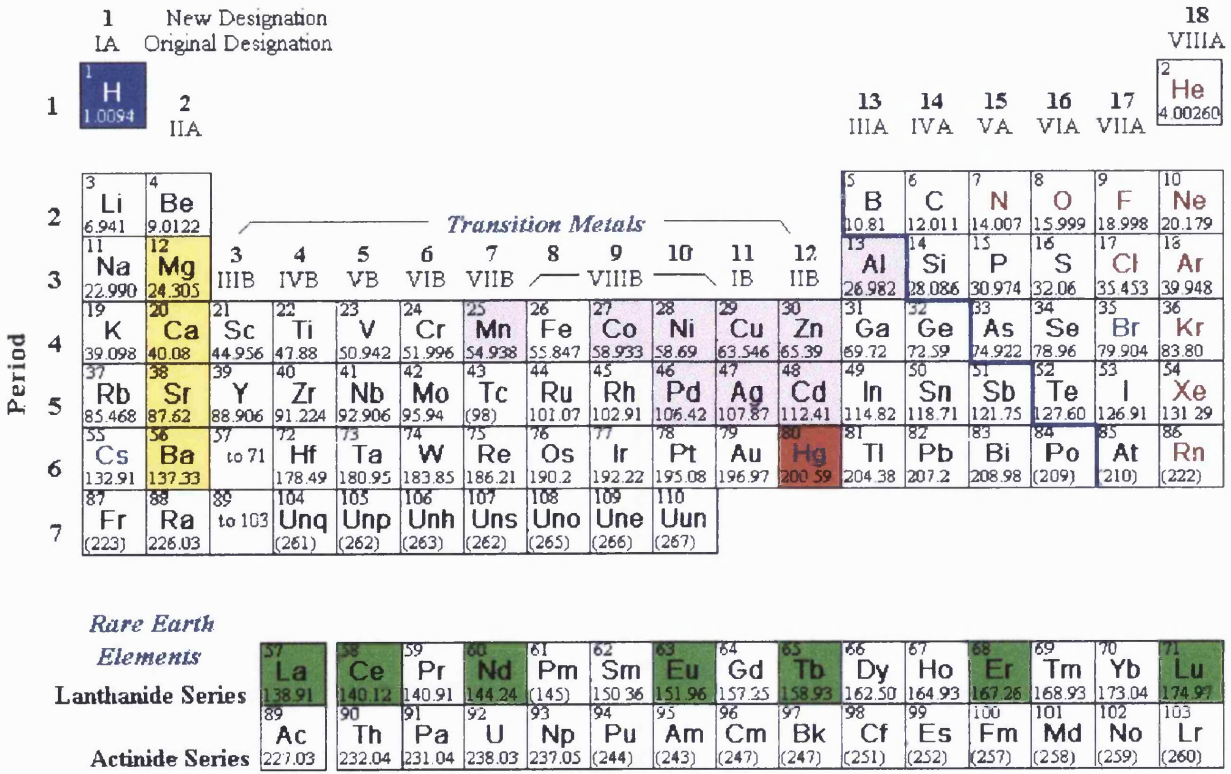

α1/2/3/5 220 RKIGYFVIQT YLPCIMTVIL SQVSFWLNRE SVPARTVFGV TTVLTMTTLS ISARNSLPKV
α4/6 219 RKMGYFVIQX YXPCIMTVIL SQVSFWINKE SVPARTVFGI TTVLTMTTLS ISARHSLPKV
β1 216 RNIGYFILQT YMPSTLITIL SWVSFWINYD ASAARVALGI TTVLTMTTIS THLRETLPKI
β2 216 RNIGYFILQT YMPSILITIL SWVSFWINYD ASAARVALGI TTVLTMTTIN THLRETLPKI
β3 216 RNIGYFILQT YMPSIMITIL SWVSFWINYD ASAARVALGI TTVLTMTTIN THLRETLPKI
α1/2/3/5 280 AYATAMDWFX AVCYAFVFSA LIEFATVNYF
α4/6 279 SYATAMDWFI AVCFAFVFSA LIEFAAVNYF
β1 276 PYVKAIDIYL MGCFVFVFLA LLEYAFVNYI
β2 276 PYVKAIDMYL MGCFVFVFMA LLEYALVNYI
β3 276 PYVKAIDMYL MGCFVFVFLA LLEYAFVNYI

Figure 1.4. A summary of the ionic regulation of $GABA_A$ receptors. The effects of the extracellular application of ions on $GABA_A$ receptor function are summarised on the periodic table. Cations producing a reversible inhibitory effect on GABA-activated currents are illustrated in pink (Ma & Narahashi, 1993a; for further references, see tables 1.2, 1.3 and 1.4), whereas mercury (Hg^{2+}), which produces an irreversible block is depicted in red (Arakawa *et al.*, 1991) and cations which are ineffective when extracellularly-applied are shown in yellow (Celentano *et al.*, 1991; Kaila, 1994 and references therein; Kumamoto & Murata, 1995). Lanthanides, shown to directly activate and modulate, either potentiating or inhibiting $GABA_A$ receptor function are shown in green (Im *et al.*, 1992; Ma & Narahashi, 1993a,b; Saxena *et al.*, 1997). H^+, which modulates certain $GABA_A$ receptor complexes is denoted in blue (Kaila, 1994 and references therein; Krishek *et al.*, 1996a).


1 IA
New Designation
Original Designation
18 VIIIA
1 H 1.0094
2 IIA
13 IIIA
14 IVA
15 VA
16 VIA
17 VIIA
2 He 4.00260
Transition Metals
3 IIIB
4 IVB
5 VB
6 VIB
7 VIIB
8
9 VIIIB
10
11 IB
12 IIB
Period
1
2
3
4
5
6
7
3 Li 6.941
4 Be 9.0122
5 B 10.81
6 C 12.011
7 N 14.007
8 O 15.999
9 F 18.998
10 Ne 20.179
11 Na 22.990
12 Mg 24.305
13 Al 26.982
14 Si 28.086
15 P 30.974
16 S 32.06
17 Cl 35.453
18 Ar 39.948
19 K 39.098
20 Ca 40.08
21 Sc 44.956
22 Ti 47.88
23 V 50.942
24 Cr 51.996
25 Mn 54.938
26 Fe 55.847
27 Co 58.933
28 Ni 58.69
29 Cu 63.546
30 Zn 65.39
31 Ga 69.72
32 Ge 72.59
33 As 74.922
34 Se 78.96
35 Br 79.904
36 Kr 83.80
37 Rb 85.468
38 Sr 87.62
39 Y 88.906
40 Zr 91.224
41 Nb 92.906
42 Mo 95.94
43 Tc (98)
44 Ru 101.07
45 Rh 102.91
46 Pd 106.42
47 Ag 107.87
48 Cd 112.41
49 In 114.82
50 Sn 118.71
51 Sb 121.75
52 Te 127.60
53 I 126.91
54 Xe 131.29
55 Cs 132.91
56 Ba 137.33
57 to 71
72 Hf 178.49
73 Ta 180.95
74 W 183.85
75 Re 186.21
76 Os 190.2
77 Ir 192.22
78 Pt 195.08
79 Au 196.97
80 Hg 200.59
81 Tl 204.38
82 Pb 207.2
83 Bi 208.98
84 Po (209)
85 At (210)
86 Rn (222)
87 Fr (223)
88 Ra 226.03
89 to 103
104 Unq (261)
105 Unp (262)
106 Unh (263)
107 Uns (262)
108 Uno (265)
109 Une (266)
110 Uun (267)
Rare Earth Elements
Lanthanide Series
Actinide Series
57 La 138.91
58 Ce 140.12
59 Pr 140.91
60 Nd 144.24
61 Pm (145)
62 Sm 150.36
63 Eu 151.96
64 Gd 157.25
65 Tb 158.93
66 Dy 162.50
67 Ho 164.93
68 Er 167.26
69 Tm 168.93
70 Yb 173.04
71 Lu 174.97
89 Ac 227.03
90 Th 232.04
91 Pa 231.04
92 U 238.03
93 Np 237.05
94 Pu (244)
95 Am (243)
96 Cm (247)
97 Bk (247)
98 Cf (251)
99 Es (252)
100 Fm (257)
101 Md (258)
102 No (259)
103 Lr (260)

CHAPTER 2

Materials and Methods.

2.1. cDNA construction.

Murine α1, β1, β3 and γ2S $GABA_A$ receptor subunit cDNAs were cloned into the mammalian cytomegalovirus-based expression vector pGW1 as described previously (Moss *et al.*, 1990; Kamatchi *et al.*, 1995).

2.1.1. Site-directed mutagenesis.

Mutations were created by the Quickchange method (Strategene Corp., La Jolla, CA; as described by Harvey *et al.*, 1999). Briefly, pairs of oligonucleotides (30mer) complementary to the opposite strands of the subunit cDNA and bearing the desired mutation were used as primers in the polymerase chain reaction (PCR). High-fidelity *Pfu* DNA polymerase was used to extend the oligonucleotides yielding copies of both strands of the parental plasmid. The reaction mixture used for this procedure was: 5μl 10x reaction buffer, 50ng template plasmid (pRK5β3, pRK5α1), 125ng each oligonucleotide primer (approximately 15pmol), 1μl deoxynucleotide triphosphate (dNTP) mix (each at 2.5mM), 2.5U cloned *Pfu* DNA polymerase, water to a final volume of 50μl. Thermocycling parameters: 95°C, 5 min; followed by 16 cycles of 95°C, 45 s; 55°C, 45 s; 72°C, 14min. The PCR generates nicked replicates of the parental plasmid bearing the desired mutation. Following thermocycling, the mixture was chilled on ice for two minutes. The PCR products were treated with the restriction endonuclease *Dpn* I (10U), which only restricts methylated or hemi-methylated DNA. Reaction mixtures were mixed and incubated at 37 °C for at least 1 hr. The parental plasmids, which were isolated from a methylating strain of *E. coli* (TOP10, Invitrogen), were susceptible to *Dpn* I digestion whereas the PCR products were unmethylated and therefore unaffected by the endonuclease. Digestion thus eliminated the parental DNA, and the nicked vector DNA was transformed into *E. coli.*

Briefly, 1μl of the digested PCR product was gently mixed with 100μl of *E. coli* (TOP10, Invitrogen) and incubated on ice for 30 min. The transformation reaction was then placed in a heating block at 42 °C for 45s and returned to the ice for a further 2 min. SOB medium (250μl) was added to the reactions, which were subsequently incubated at 37 °C for 1 hr, shaking at 225 rpm. The transformed cells (100-200μl) were

plated on LB-ampicillin agar. Plasmids bearing the mutated subunit cDNAs were purified from *E. coli* using a standard alkaline lysis technique (Sambrook *et al.*, 1989). Site directed mutagenesis was performed in collaboration with S. J. Moss and R. J. Harvey.

2.2. Cell preparation.

2.2.1. *Xenopus laevis* oocytes.

Female *Xenopus laevis* (Blades Biological, Edenbridge, Kent, UK) were anaesthetised by immersion in 0.5% (w/v) tricaine (ethyl-m-aminobenzoate, Sigma) and oocytes were aseptically removed as reported previously (Smart & Krishek, 1995) and stored in modified Barth's medium (MBM) containing (in mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $McSO_4$ 0.82, Gentamycin 50mg/l; pH 7.6. Stage IV-V folliculated oocytes were manually separated and centrifuged (700-1100*g* for 7-8min at 10° C) to reveal the nuclei of individual oocytes. Undamaged oocytes were nuclear microinjected with 10nl of 1mg/ml cDNA solution encoding for the appropriate combinations of wild-type/mutant $GABA_A$ receptor subunits and then incubated at 18°C for 24h. Oocytes were subsequently stored at 10°C and replenished with fresh MBM every 2-3 days.

2.2.2. Human embryonic kidney (HEK) cells.

HEK cells (American Tissue Culture Collection CRL 1573) were grown in Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% (v/v) fetal calf serum, 0.12% (w/v) $NaHCO_3$, 100U/ml penicillin G and 100U/ml streptomycin at 37°C in a humidified 95% air/5% CO_2 atmosphere (Smart *et al.*, 1991). Exponentially growing cells were electroporated (Gene Pulser II: BioRad, Hemel Hempstead, UK; optimal parameters: 400mV, 125μF, infinite resistance) with a total of 15μl cDNA (0.5-1mg/ml). For electrophysiological analysis, the reporter plasmid for the S65T mutant jellyfish green fluorescent protein (GFP; Heim *et al.*, 1995) was co-electroporated into the cells with the $GABA_A$ receptor subunit

cDNAs to facilitate the identification of transfected cells. Transfected cells were maintained in culture for 18-72h before use. The fluorescent signal generated by GFP(S65T) was visualised on a Nikon Diaphot microscope using an epifluorescence unit and filter block B-2A (Nikon, Kingston-upon-Thames, UK).

2.3. Immunocytochemistry.

HEK 293 cells were transfected with either β3 or $\beta3^{H107A}$ by electroporation and plated on poly-l-lysine coated coverslips as described above. After 24hr they were washed twice with phosphate buffered saline (PBS, Sigma) and fixed by a 15min incubation in 4% paraformaldehyde in PBS at room temperature. The fixative was quenched by a 10 min incubation in 50mM NH_4Cl (in PBS) and the cells were then washed twice with PBS. When necessary, cells were permeablised by a 20min incubation with 0.2% v/v Tween-20 in PBS containing foetal calf serum (FCS, 10% v/v) and bovine serum albumin (0.5% w/v). Cells were then washed twice with PBS and once with 10% FCS in PBS.

The primary antibody, BD17 (Boehringer Mannheim) was used at a concentration of 5 μg/ml (in 10% FCS-PBS). Each coverslip was inverted onto 50μl of antibody solution that had been pipetted onto parafilm, and incubated at room temperature for 30min. Coverslips were then washed three times with 10% FCS-PBS. An identical procedure was used to apply the secondary antibody, anti-mouse IgG conjugated to TRITC (Sigma), which was diluted 1/50 in 10% FCS-PBS. Incubation was performed for 30 min in the dark to avoided photobleaching of the conjugated fluorophore. The coverslips were washed three times in 10% FCS-PBS, once in PBS, drained on tissue paper and mounted on slides with *n*-propylgalate.

Immunofluorescence was detected using a MRC1024 confocal system equipped with an argon-krypton laser (Bio-Rad, Hercules, CA) and a Nikon Optiphot microscope. Digitised images were first loaded into Photoshop (Adobe Software,) and then into Powerpoint (Microsoft Corporation). Other than the removal of areas where no cells were present, no alterations were made to the images. I am grateful to Ian Duguid,

Alastair Hosie and Josef Kittler for help with antibody staining and confocal microscopy.

2.4. Electrophysiology.

2.4.1. Intracellular recording.

Membrane currents and conductances were recorded from *Xenopus laevis* oocytes under two-electrode voltage clamp in conjunction with an Axoclamp 2B amplifier and a Gould 2400S ink-jet pen recorder. Oocytes were placed in a Perspex chamber, secured between insect pins fixed to a Sylgard base and continuously superfused (using constant gravity perfusion) with an amphibian Ringer solution comprising (in mM) 110 NaCl, 2 KCl, 5 HEPES, 1.8 $CaCl_2$ (pH 7.4) at 8-10 ml/min (bath volume, 0.5ml). The voltage and current microelectrodes were filled with 0.6M K_2SO_4 (1-2 MΩ).

2.4.2. Whole-cell recording.

HEK cells were continuously superfused with a Krebs' solution consisting of (in mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 11 glucose (pH7.4). Experiments were performed using a List EPC7 patch amplifier in the whole-cell recording mode at a holding potential of –40mV using patch electrodes (1-5 MΩ) filled with the following electrolyte (mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 EGTA and 2 adenosine triphosphate (pH 7.1). Series resistance compensation of 70-80% was achieved and currents were filtered at 3-5 kHz (-3dB, six-pole Bessel filter, 36 dB/octave) and recorded on a Gould 2200 pen recorder. Whole-cell recording was performed in collaboration with T. G. Smart.

2.5. Analysis of ligand-induced membrane currents and conductances.

Membrane currents recorded from HEK cells represent peak current measurements. For *Xenopus* oocytes, membrane input conductances were determined by briefly applying

hyperpolarizing voltage command steps (1s duration, -10mV amplitude, 0.2Hz frequency) superimposed on the holding potential (–40mV) in the absence and presence of ligand. Curve fitting was performed using either Origin 4.1 (Microcal Software) or Fig P version 6 (Biosoft) on a Viglen PC. Theoretical curves used in the binomial stoichiometry model analogues were performed using Mathcad Version 6 (Adept Scientific).

2.5.1. Agonist concentration-response relationships.

To construct equilibrium concentration-response relationships for GABA and pentobarbitone (PB), the change in membrane conductance (ΔG) as a result of ligand application was calculated by subtracting the resting membrane conductance from the maximum conductance measured in the presence of each concentration of ligand. All conductances were subsequently normalized (ΔG_N) to the maximum change in conductance ($\Delta G_{N,\,max}$) induced by a saturating concentration of ligand, unless where stated, and fitted with the equation:

$$\Delta G_N/\Delta G_{N,\,max} = [1/1 + (EC_{50}/[A])^{n_H}],$$

where EC_{50} denotes the concentration of ligand ([A]) generating 50% of the maximal conductance induced by a saturating concentration of ligand and n_H represents the Hill coefficient.

2.5.2. Antagonist concentration-inhibition relationships.

The reduction in membrane conductance by Zn^{2+} was used to construct concentration-inhibition relationships. For β3 wild-type and mutant subunit receptors, the antagonist-sensitive conductance (ΔG_N; equivalent to the β3 subunit receptor-gated spontaneous membrane conductance) was determined by subtracting the residual membrane conductance following the addition of a saturating concentration of Zn^{2+} from the resting membrane conductance in the absence of antagonist. The inhibition of this

conductance by intermediate concentrations of Zn^{2+} were fitted according to the following equation:

$$\Delta G_N'/\Delta G_N = [1-([B]^{n_H}/([B]^{n_H} + IC_{50}^{n_H}))],$$

where $\Delta G_N'$ and ΔG_N represent the normalized GABA-induced (at a particular concentration of GABA) or β3 subunit receptor-gated conductance in the presence and absence of Zn^{2+} respectively. IC_{50} denotes the concentration of Zn^{2+} ([B]) eliciting 50% inhibition of either the GABA-induced or β3 subunit receptor-gated conductance. Statistical significance ($p<0.05$) was assessed using an unpaired *t*-test.

2.5.3. Current-voltage relationships.

Current-voltage (I-V) relationships for the response to GABA were established under voltage clamp. The membrane potential was increased in 10mV steps from –100mV to 60mV. I-V plots were obtained in control Ringer solution and after reaching a steady state conductance in the presence of 10μM GABA, then subsequently fitted with fifth-order polynomials. The GABA I-V relationship was determined by subtracting the control I-V plot from the I-V plot constructed in the presence of GABA. To analyse the action of protein tyrosine kinase (PTK) inhibitors, I-V plots (∀GABA) were obtained in the presence of PTK inhibitors after achieving a steady-state reduction in the response to 10μM GABA.

2.6. Drugs and solutions.

Drugs and solutions were bath-applied in experiments using *Xenopus* oocytes. Drugs were rapidly applied to HEK cells using a modified U-tube which ensured rapid recovery after exposure to a drug (Wooltorton *et al.*, 1997b). Stock solutions of genistein, daidzein, tyrphostin A1 and tyrphostin A25 (Calbiochem-Novabiochem; La Jolla, CA) were dissolved in dimethylsulphoxide (DMSO). The maximal concentration of DMSO applied to the oocytes was 1% v/v and did not affect resting membrane conductance. A stock solution of PB (100mM; Sigma) was prepared by dissolution in 50% ethanol. The concentration of ethanol in the Ringer solution (maximum 2% v/v) had no effect on the resting membrane conductance. All drugs were dissolved in either

Ringer or Krebs' solutions before application to either oocytes or HEK cells. GABA, diazepam, picrotoxin and DEPC were obtained from Sigma. Zn Cl_2 was obtained from BDH, Poole, Dorset, UK. For experiments involving wild-type and mutant $\alpha1\beta3$ and $\alpha1\beta1\gamma2S$ $GABA_A$ receptor constructs, modulators (i.e. PB, diazepam and Zn^{2+}) were pre-applied for 2min before subsequent application in conjunction with a GABA dose. Diethylpyrocarbonate (DEPC) was pre-applied for 2min. Genistein, daidzein, tyrphostin A1 and tyrphostin A25 were constantly superfused. For experiments assessing the effects of Ringer pH, the Ringer was returned to pH7.4 in between each pH experiment and ligand sensitivity re-assessed before altering to a different Ringer pH.

To investigate the effect of Zn^{2+} on $\beta3$ subunit homomers in *Xenopus* oocytes, a cumulative application protocol was followed. A threshold concentration of Zn^{2+} was applied and once the maximum inhibitory effect was observed, a higher Zn^{2+} concentration was applied. This protocol was continued until the application of a saturating concentration of Zn^{2+} was applied. The total Zn^{2+} sensitive conductance was calculated by subtracting the residual membrane conductance observed after application of the saturating concentration of Zn^{2+} from the resting membrane conductance. The inhibitory effect of each individual Zn^{2+} concentration was expressed as a percentage of the total Zn^{2+} sensitive conductance. Previously this procedure was validated using saturating concentrations of picrotoxin which resulted in a block of the membrane conductance of similar magnitude to that observed using saturating concentrations of Zn^{2+} (Wooltorton *et al.*, 1997b).

CHAPTER 3

Regulation of Zn^{2+} inhibition on the $GABA_A$ receptor: involvement of the N-terminal domain of the β3 subunit?

3.1. INTRODUCTION

Zinc (Zn^{2+}) is differentially distributed in the mammalian central nervous system, being most abundant in the mossy fibre terminals of the hippocampus (Frederickson, 1989). Zn^{2+} is concentrated in synaptic vesicles and is released in a Ca^{2+}-dependent manner into the synaptic cleft following nerve terminal stimulation, potentially reaching concentrations of 100-300μM in the extracellular space (Assaf & Chung, 1984; Howell *et al.*, 1984; Xie & Smart, 1991) implying a significant physiological role. Here Zn^{2+} may act as an endogenous neuromodulator, affecting neuronal excitability via interaction with a large array of voltage- and ligand-gated ion channels including the $GABA_A$ receptor (reviewed by Smart *et al.*, 1994; Harrison & Gibbons, 1994).

The sensitivity of native $GABA_A$ receptors to inhibition by Zn^{2+} varies according to neuronal preparation, with Zn^{2+} having little or no effect on the GABA-activated Cl^- currents recorded from adult rat sympathetic ganglia, cortex and pyriform cortex (reviewed by Smart *et al*, 1994). Furthermore, Zn^{2+} sensitivity differs according to neuronal age, with younger neurones displaying a greater sensitivity to Zn^{2+} (Smart & Constanti, 1990; Smart, 1992). This diversity may be explained by studies utilising recombinant $GABA_A$ receptors, which indicate the potency of Zn^{2+} inhibition is dependent on receptor subunit composition. In particular the presence of a γ subunit dramatically reduces Zn^{2+} sensitivity compared with the corresponding αβ subunit-containing receptor (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Gingrich & Burkat, 1998). Inclusion of the ε subunit in αβ subunit-containing receptors causes a similar reduction in the potency of Zn^{2+} (Whiting *et al.*, 1997). The relative insensitivity of γ subunit containing receptors can be diminished by co-expression with the δ subunit (Saxena & Macdonald, 1994, 1996) and by alteration of the α subunit isoform (White & Gurley, 1995; Knoflach *et al.*, 1996; Saxena & Macdonald, 1996; see Chapter 4 for discussion). The mechanism of antagonism is also variable. Zn^{2+} may act as a non-competitive inhibitor of $GABA_A$ receptors on hippocampal and postnatal sympathetic ganglion neurons (Westbrook & Mayer, 1987; Mayer & Vyklicky, 1989; Smart & Constanti, 1990; Legendre & Westbrook, 1991), whereas apparent competitive inhibition of

$GABA_A$ receptors has been reported for bullfrog dorsal root ganglion neurons (Akaike *et al.*, 1987; Yakushiji *et al.*, 1987). In addition Zn^{2+} can act as a competitive inhibitor (Calvo *et al.*, 1994) or as a mixed inhibitor (Chang *et al.*, 1995; Dong & Werblin, 1995) of homomeric ρ1 subunit-containing receptors and native $GABA_C$ receptors on catfish retinal cells.

The search for Zn^{2+} binding sites on $GABA_A$ receptors has been hindered by several problems including the heterogeneity of native $GABA_A$ receptors and their differential sensitivity to Zn^{2+}, which can also vary, during neuronal development/maturation. Hydropathy profiles of the $GABA_A$ receptor indicate that approximately 60% of the receptor is extracellular (Rabow *et al.*, 1995). A single $GABA_A$ receptor subunit comprises approximately 500 amino acids and therefore a pentameric receptor would comprise approximately 2500 amino acid residues, of which 1500 would reside externally. Many of these residues will possess charged side chains presenting numerous opportunities for binding or allosterically contributing to the actions of Zn^{2+}. Recently, putative binding sites for Zn^{2+} have been determined on $GABA_A$ receptor β3 (Wooltorton *et al.*, 1997a), β1 (Horenstein & Akabas, 1998) and α6 subunits (Fisher & Macdonald, 1998) and $GABA_C$ receptor ρ1 subunits (Wang *et al.*, 1995b). Both β1 and β3 subunits can form functional homomeric ion channels which exhibit a spontaneously-gated Cl^- current and can also be activated or positively modulated by pentobarbitone and inhibited by picrotoxin or Zn^{2+} (Sigel *et al.*, 1989; Sanna *et al.*, 1995; Krishek *et al.*, 1996b; Wooltorton *et al.*, 1997b). The IC_{50}s determined for Zn^{2+} are in the nanomolar range, indicating the likelihood of a major role for the β subunit in determining the high affinity Zn^{2+} binding site in αβ receptors. Homomeric β3 receptors have been used to obviate the many uncertainties regarding receptor heterogeneity evident from using native $GABA_A$ receptors or indeed multi-subunit recombinant $GABA_A$ receptors in expression systems, in the search for a Zn^{2+} binding site and revealed a single histidine (H) residue (H267) located in the second transmembrane domain as a major determinant for Zn^{2+} inhibition of both spontaneous and pentobarbitone-gated currents (Wooltorton *et al.*, 1997a). Although mutation of this residue produced a 1000-fold reduction in the potency of Zn^{2+} inhibition (IC_{50}s: $\beta3^{wild\text{-}type}$, 0.31μM; $\beta3^{H267A}$, 307μM), application of higher doses of Zn^{2+} (≥1mM) still caused

complete inhibition of the current gated by β3 homomeric receptors, suggesting that an additional, possibly lower affinity, binding site was still present. Similar experiments were conducted on α1β*i* heteromeric receptors (where *i* = 1 or 3). The mutant receptor $\alpha 1\beta i^{H267A}$demonstrated a marked reduction (~ 200-fold) in Zn^{2+} potency although increasing the Zn^{2+} concentration still diminished the GABA-gated current (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). These results indicated the presence of additional amino acid residues forming binding sites for Zn^{2+} on these homomeric and heteromeric $GABA_A$ receptors.

Studies on Zn^{2+}-containing metalloenzymes have revealed a biochemical variability in Zn^{2+} binding sites (Vallee & Falchuk, 1993). Zn^{2+} can form a complex with 2 – 8 ligands although Zn^{2+} binding sites are most commonly formed by 4 – 6 ligands, involving predominantly cysteine, histidine, glutamate or aspartate residues (Vallee & Falchuk, 1993; Berg & Shi, 1996). Only external residues were considered as candidates for the additional Zn^{2+} binding site on the β3 subunit as internal application of Zn^{2+} has been shown to be ineffective on GABA-gated currents (Celentano *et al.*, 1991). It is thought that the external cysteine residues are unlikely to be available to interact with Zn^{2+} as extracellular application of charged, water-soluble, sulphydryl reactive reagents had no effect on $GABA_A$ receptors (Xu & Akabas, 1993, 1996) and two highly conserved N-terminal cysteines are believed to form a disulphide bridge (Barnard *et al.*, 1987; Pan *et al.*, 1995) and are thereby unavailable to bind to Zn^{2+}. In addition, it has been demonstrated that the inhibitory action of Zn^{2+} on $GABA_A$ receptors is sensitive to pH, suggesting a common binding site for H^+ and Zn^{2+}. A favoured candidate for the interaction between Zn^{2+} and amino acids is the protonable nitrogen atom in the imidazole ring of a histidine residue (Smart & Constanti, 1982; Krishek *et al.*, 1998). To date, the Zn^{2+} binding sites elucidated on $GABA_A$ and $GABA_C$ receptor subunits have all been histidine residues (Wang *et al.*, 1995b; Wooltorton *et al.*, 1997a; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998).

In order to identify the additional Zn^{2+} binding site on the β3 subunit, the role of histidine residues in Zn^{2+} sensitivity was examined using site-directed mutagenesis and

expression of recombinant murine β3 subunit $GABA_A$ receptors in combination with electrophysiological techniques (Dunne *et al.*, 1999a, b).

3.2. RESULTS

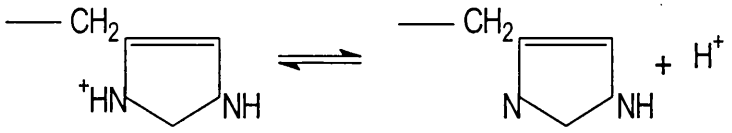
3.2.1. Existence of an additional Zn^{2+} binding site on the $GABA_A$ receptor β3 subunit.

A histidine residue (H267) was previously identified as crucial for Zn^{2+} inhibition of both spontaneous and pentobarbitone (PB)-gated currents of β3 homomeric receptors (Wooltorton *et al.*, 1997a). Mutation of this residue to alanine (A) greatly reduced the sensitivity of the receptor to Zn^{2+}. Zn^{2+} inhibition curves for the spontaneous β3 conductance were established using a cumulative dose application protocol (see Chapter 2). Briefly, increasing concentrations of Zn^{2+} were applied until further inhibition of the conductance could not be achieved. This saturating concentration of Zn^{2+} (β3 wild-type: 10μM; β3 mutants: 2mM) was assumed to have achieved maximal block. The residual leak conductance was then subtracted from each response and the resulting data points plotted and fitted with the four parameter logistic equation for inhibition shown in chapter 2. Zn^{2+} concentration-inhibition curves were constructed for the spontaneously-gated conductance (Fig. 3.1.). As reported previously (Wooltorton *et al.*, 1997a), the β 3^{H267A} mutation caused a large rightward shift in the Zn^{2+} concentration-inhibition curve and an approximate 1200-fold increase in the Zn^{2+} IC_{50} (β3 $^{wild\text{-}type}$, 0.16 ± 0.03μM; β, 194.58 ± 16.17μM; n = 3 – 7 oocytes). An increase in the Hill coefficient (n_H) was also observed (β3 $^{wild\text{-}type}$, 0.51± 0.06; $β3^{H267A}$, 2.39 ± 0.46; n = 3 – 7 oocytes). In order to control for a possible non-specific action of Zn^{2+}, high Zn^{2+} concentrations were applied to uninjected oocytes from the same donor *Xenopus*. Application of 1mM Zn^{2+} had no effect on uninjected oocytes (102 ± 3% control, n = 5 oocytes) which is in direct contrast to the inhibition observed for oocytes injected with $β3^{H267A}$ cDNA (6 ± 4% of the control conductance, n = 3 oocytes).

As previously described (Wooltorton *et al.*, 1997a), introduction of this mutation generated a small but significant reduction in the PB-induced modulation of the ion channel ($\beta3^{wild\text{-}type}$, EC_{50}: 80.76 ± 12.7μM, n_H: 1.44 ± 0.32; $\beta3^{H267A}$, EC_{50}: 53.79 ± 7.92μM, n_H: 1.62 ± 0.35; n = 3 – 7 oocytes; Fig. 3.6A, C). In addition to inhibiting the spontaneous conductance, the PB modulation of $\beta3^{H267A}$ receptors was also susceptible to Zn^{2+} inhibition. Application of 200μM Zn^{2+} produced a mixed/non-competitive block of the PB concentration-response curve, depressing the maximum response (56 ± 8% inhibition of control response to 500μM PB) and shifting the PB EC_{50} (control, 53.79 ± 7.92μM; + Zn^{2+}, 188.11 ± 44.21μM; n = 3 oocytes). Clearly mutation did not abolish Zn^{2+} inhibition indicating the likely presence of an additional lower affinity binding site, or conceivably the incomplete disruption of the Zn^{2+} binding pocket in the environment of H267.

3.2.2. Inhibitory effect of Zn^{2+} on $\beta3^{H267A}$ receptors: sensitivity to H^+.

Lowering external pH reduces Zn^{2+} antagonism of GABA-activated currents recorded from recombinant α1β1 receptors (Krishek *et al.*, 1998). The Zn^{2+} sensitivity of the β 3^{H267A} receptor was examined in the presence of low pH Ringer to determine the importance of histidine residues for the additional low affinity Zn^{2+} binding site as H^+ will compete with Zn^{2+} for binding to the imidazole groups on histidine residues (Vallee & Falchuk, 1993).



At physiological pH 7.4, pentobarbitone (1μM – 2mM) increased the membrane conductance for $\beta3^{H267A}$ subunit expressing oocytes. Zn^{2+} (300μM) acts as a non-competitive/mixed antagonist: the PB equilibrium concentration–response curve is laterally displaced, increasing the EC_{50} from 46 ± 9.8μM to 95.6 ± 9.7 μM, and the maximum response is reduced (Fig. 3.2A; n = 3 oocytes). Increasing the H^+ concentration by reducing Ringer pH to 5.4 resulted in a slight increase in the potency of PB for modulating the Cl^- conductance for $\beta3^{H267A}$ homomeric receptors; however,

more importantly, low pH entirely abolished the residual Zn^{2+} inhibition of the PB-modulated conductance. Accordingly, the PB concentration-response curves in the absence and presence of Zn^{2+} (300μM) were coincident (EC_{50}, PB control, 21.1 ± 4.1μM; + Zn^{2+}, 19.4 ± 3.5μM; n = 3 oocytes; Fig. 3.2B.).

Likewise, the Zn^{2+} sensitivity (5μM – 2mM) of the spontaneous conductance for β 3^{H267A} subunit homomers was also eliminated by reducing the external pH from 7.4 to 5.4 (Fig. 3.3., n = 3 – 4 oocytes) intimating that H^+ and Zn^{2+} are likely to be competing for similar amino acid residues. Since increasing H^+ concentration 100-fold completely abolished inhibition by Zn^{2+}, this pH change presumably traversed the pK_a for the amino acid(s) involved in the Zn^{2+} binding site. As histidine is the only amino acid with a pK_a predicted to be affected by this pH change (pK_a 6.5) then it is probable that a histidine residue(s) is responsible for the low affinity binding site and/or allosteric effect of Zn^{2+} on the $GABA_A$ β3 subunit.

3.2.3. Diethylpyrocarbonate abolishes the Zn^{2+} sensitivity of the $\beta3^{H267A}$ receptor.

Diethylpyrocarbonate (DEPC) is a useful experimental tool in studies attempting to deduce whether histidine residues have a functional role in biological mechanisms. At pH 7.4 DEPC is reasonably specific for reacting with histidine residues by covalently, and irreversibly converting their imidazole groups to N-carboxyhistidyl derivatives. (Miles, 1977; Lundblad & Noyes, 1984). As *Xenopus* oocytes were intolerant to DEPC (1mM) the effects of this compound were assayed on HEK cells transiently transfected with $\beta3^{H267A}$ under whole-cell patch clamp. Inward currents induced by 1mM PB were inhibited by Zn^{2+} (100μM; 59 ± 12% inhibition; Fig. 3.4A, B). Application of DEPC (1mM; 2 min) alone did not significantly alter the amplitude of PB-induced currents, but attenuated the inhibition by Zn^{2+} (29 ± 17% inhibition; n = 4 cells; Fig. 3.4A, B). This provides further evidence for the involvement of histidine residues in the inhibitory action of Zn^{2+}.

3.2.4. External histidine residues in the $\beta3^{H267A}$ receptor: candidates for co-ordinating Zn^{2+} inhibition.

Three external histidine residues, H107, H119 and H191 are found in the N-terminal domain of the β3 subunit in addition to H267 located in TM2, the ion channel pore forming region (Fig. 3.5.). Comparison of amino acid sequences revealed that whilst H107 is highly conserved throughout $GABA_A$, $GABA_C$, glycine and nicotinic acetylcholine receptors, H119 is conserved only within $GABA_A$ receptor β subunits (and the $GABA_C$ ρ3 subunit), and H191 is unique to the $GABA_A$ receptor β3 subunit. Since the β1 subunit possesses a similar lower affinity Zn^{2+} binding site but lacks a histidine at residue 191 (Horenstein & Akabas, 1998), H107 and H119 were considered as prime candidates for this additional site. Each of the external histidines was subjected to systematic mutation to alanines leaving the background mutation H267A intact to preclude the high affinity binding of Zn^{2+}.

Nuclear injection of DNA encoding $\beta3^{H119A,\ H267A}$ in *Xenopus* oocytes resulted in functional receptors exhibiting both spontaneous and PB-modulated currents. Equilibrium concentration-response curves for PB were constructed and the EC_{50} for PB was slightly increased by the H119A mutation (Fig. 3.6A; $\beta3^{H119A,\ H267A}$, 97.13 ± 5.03μM; $\beta3^{H267A}$, 53.79 ± 7.92μM; β3, 80.76 ± 12.7μM; n = 5 – 8 oocytes). However, the H119A mutation did not generate any further reduction in the sensitivity of the β $3^{H119A,\ H267A}$ subunit to Zn^{2+}. Construction of Zn^{2+} inhibition curves for the spontaneous β3 current yielded similar IC_{50}s of 194.11 ± 0.04μM for $\beta3^{H119A,\ H267A}$ receptors and 194.58 ± 16.17μM for $\beta3^{H267A}$ receptors (n = 5 – 8 oocytes; Fig. 3.6B.). The Zn^{2+} inhibition curves also displayed similar Hill coefficients ($\beta3^{H119A,\ H267A}$, 2.84 ± 0.001; β 3^{H267A}, 2.39 ± 0.46; n = 5 – 8 oocytes).

Xenopus oocytes injected with $\beta3^{H191A,\ H267A}$ cDNA also resulted in functional receptor expression. As for the H119A mutation, the PB equilibrium concentration response curves displayed considerable overlap and the PB EC_{50} remained unchanged ($\beta3^{H191A,\ H267A}$, 66.14 ± 7.06μM; $\beta3^{H267A}$, 53.79 ± 7.92μM; β3, 80.76 ± 12.7μM; n = 3 – 7 oocytes; Fig. 3.6C.). The H191A mutation also had little effect on the potency of Zn^{2+}

and the Zn^{2+} inhibition curves of the spontaneous β3 current for $\beta3^{H191A,\ H267A}$ and $\beta3^{H267A}$ constructs were coincident. The Zn^{2+} IC_{50}s were: $\beta3^{H191A,\ H267A}$, 154 ± 15.25μM and $\beta3^{H267A}$, 194.58 ± 16.17μM (n = 3 – 5 oocytes; Fig. 3.6D.). The Hill coefficient remained unchanged ($\beta3^{H191A,\ H267A}$, 2.35 ± 0.51; $\beta3^{H267A}$, 2.39 ± 0.46; n = 3 – 5 oocytes).

These results implied that the remaining histidine residue, the highly conserved H107 is the most likely candidate for co-ordinating Zn^{2+} binding. Injection of $\beta3^{H107A,\ H267A}$ cDNA into oocytes did not result in the formation of functional β3 subunit receptors (n = 17 oocytes). In addition, single mutant $\beta3^{H107A}$ subunits also failed to form functional receptors (n = 15 oocytes). In order to examine whether the expression system utilized determined the ability of this mutant to form functional receptors, HEK cells were transfected with either $\beta3^{H107A}$ or $\beta3^{H107A,\ H267A}$ cDNAs. On each occasion however, these cells failed to express functional receptors (n = 6 cells). As a positive control for DNA transcription, these cells were co-transfected with the reporter DNA encoding *Aquorea* green fluorescent protein (GFP; n = 6). These cells exhibited the characteristic epifluorescence image, indicating successful transfection. In addition, parallel transfections in the same batch of HEK cells with αβ subunit cDNAs resulted in the expression of functional $GABA_A$ receptors (n = 5 cells).

The highly conserved nature of H107 throughout members of the ligand-gated ion channel superfamily implied this amino acid is critical for the expression and/or function of homomeric $GABA_A$ receptors and may play an important role in subunit assembly, protein folding and/or cell surface transport. Confocal immunofluorescence suggested that $\beta3^{H107A}$ receptors did not exit the endoplasmic reticulum, and were thus unable to gain access to the cell surface (Fig. 3.7.). In order to increase the chance of functional ion channel formation, two $\beta3^{H107X}$ mutants were constructed where X preserved and increased the positively-charged nature of the imidazole group in the histidine residue by incorporating either an arginine, $\beta3^{H107R}$, or lysine residue, $\beta3^{H107K}$. Despite possessing positively charged side chains, arginine and lysine are different to histidine, being strongly basic with pK_as of approximately 12 and 10.2 respectively. At a physiological pH 7.4, it is expected that 100% of arginine and 99.8% of lysine side

chains will be charged compared to only 4.77% for histidine side chains. However transfection of either $\beta3^{H107R}$ or $\beta3^{H107K}$ mutant subunit cDNAs together with GFP cDNA into HEK cells (n = 6 cells) did not result in the expression of functional receptors even with the preservation (even possible over-compensation) of positive charge.

3.2.5. H107: a prime candidate for mediating Zn^{2+} inhibition.

To provide more evidence for the contribution of H107 to the lower affinity Zn^{2+} inhibition, a triple mutant $\beta3^{H119A,\ H191A,\ H267A}$ subunit was expressed in *Xenopus* oocytes. The rationale for this construct was to investigate whether H119 and H191 could compensate for Zn^{2+} binding when either one is mutated to alanine. This mutant formed a functional receptor that was modulated by PB (Fig. 3.8B,D.) although the spontaneous conductance was markedly reduced. These combined mutations produced a rightward shift in the PB equilibrium concentration response curve, increasing PB EC_{50} ($\beta3^{H119A,\ H191A,\ H267A}$: EC_{50}, 130.9 ± 6.7μM; $\beta3^{H267A}$: EC_{50}, 54.9 ± 12.5μM; n = 3 – 7 oocytes). Despite this alteration in PB potency, Zn^{2+} sensitivity is unaffected by this triple mutation. Application of 100μM Zn^{2+} resulted in a depression of the PB concentration-response curve for the triple mutant $\beta3^{H119A,\ H191A,\ H267A}$ receptor (Fig. 3.8D; 59 ± 7% inhibition of control response to 500μM PB) comparable to that observed with the $\beta3^{H267A}$ construct (Fig. 3.8C; 56 ± 8% inhibition of control response to 500μM PB) with little change in the PB EC_{50} (control, 123.9 ± 6.62μM; + Zn^{2+} 145.42 ± 11.75μM; n = 3 oocytes), consistent with non-competitive antagonism. A non-competitive mode of antagonism is further demonstrated by the upper graphs in Fig 3.8C and D, which show that the degree of inhibition exerted by Zn^{2+} was almost entirely independent of PB concentration. Therefore Zn^{2+} was still exerting a considerable inhibitory effect on the mutant β3 subunit despite removing two of the three external and available histidine residues.

In order to investigate the nature of the residual Zn^{2+} inhibition of the $\beta3^{H119A,\ H191A,\ H267A}$ mutant receptor, the effect of H^+ on Zn^{2+} inhibition was examined. This was studied for the $\beta3^{H119A,\ H191A,\ H267A}$ construct using an IC_{50} concentration of Zn^{2+} (200μM) in combination with an EC_{50} concentration (100μM) of PB over a Ringer pH

range 5.4 – 8.4. The Zn^{2+} inhibitory effect was then calculated as a percentage of the control response to 100μM PB (= 100%) at each external pH. The resulting pH titration curve (Fig. 3.9A.) produced a pK_a of 6.79 ± 0.003 (n = 3 oocytes). This pK_a value is entirely consistent with the hypothesis that a histidine residue is involved in the additional low potency inhibitory action of Zn^{2+}. Since H107 is the only external histidine present in these β3 mutants, it is a prime candidate for mediating Zn^{2+} inhibition.

3.3. DISCUSSION

This study strongly suggests that H107 is involved in mediating low affinity Zn2+ inhibition of β3 homomers. H107 also appears to be critical for the surface expression of homomeric β3 receptors. $GABA_A$ receptors are differentially sensitive to Zn^{2+} and the subunit composition has dramatic effects on receptor affinity for Zn^{2+} and/or the ability of Zn^{2+} to interfere with channel gating. Furthermore, evidence suggests that than both α and β subunits may contribute to the Zn^{2+} binding site(s). Indeed such sites have been identified on α6 (Fisher & Macdonald, 1998), β1 (Horenstein & Akabas, 1998) and β3 (Wooltorton *et al.*, 1997a) $GABA_A$ receptor subunits. However, the apparent disruption of these high affinity binding sites did not abolish Zn^{2+} antagonism raising the possibility that each subunit may have more than one Zn^{2+} binding site, or that inhibition from a single site was not entirely disrupted. Other ligand-gated ion channels are presumed to possess two discrete Zn^{2+} binding sites. Native NMDA receptors in cortical neurones (Christine & Choi, 1990) and recombinant NR1/NR2A subunit NMDA receptors (Williams, 1996; Chen *et al.*, 1997; Paoletti *et al.*, 1997) were demonstrated to possess one voltage-insensitive higher affinity site, presumably located outside the membrane field and operating by decreasing ion channel opening frequency, possibly by inhibiting glycine binding (Yeh *et al.*, 1990). In addition, these studies also defined a voltage-dependent lower affinity site, thought to reside within the channel and interfere directly with ion flux. Two distinct Zn^{2+} binding sites have also been ascribed to glycine receptors (Bloomenthal *et al.*, 1994; Laube *et al.*, 1995) and P_2X purinoceptors (Cloues *et al.*, 1993). Zn^{2+} modulates both of these receptors in a biphasic

manner, causing current potentiation at low doses and inhibition at high doses. To date, Zn^{2+} does not appear to cause any potentiation of currents gated by $GABA_A$ receptors. The present study utilised homomeric β3 receptors to simplify the search for further determinants of Zn^{2+} inhibition by precluding the contribution of the residues from the α subunit. In addition β homomeric receptors are presumed to form ion channels with a symmetrical distribution of amino acid residues, therefore selected mutations, particularly in the ion channel pore should have clearly observable effects on receptor function.

3.3.1. Determining the nature of the residues influencing the residual Zn^{2+} inhibition.

Although the $\beta3^{H267A}$ mutation caused a clear increase in the Zn^{2+} IC_{50}, it was apparent from constructing Zn^{2+} inhibition curves that complete inhibition of the conductance gated by $\beta3^{H267A}$ subunit receptors was still achieved at high Zn^{2+} concentrations. Zn^{2+} chelation of the agonist cannot play a role in the inhibition of β3 homomers since Zn^{2+} reduces a spontaneous conductance not induced by ligands. Furthermore, application of high doses of Zn^{2+} (≤ 2mM) had no effect on the resting membrane conductances of control uninjected oocytes acquired from the same donor *Xenopus*, ruling out a non-specific action of Zn^{2+}. Therefore the residual Zn^{2+} inhibition must be due an additional amino acid residue(s), acting either as a Zn^{2+} binding site or contributing to the inhibition via an allosteric mechanism.

The potential for H^+ and Zn^{2+} to interact at $GABA_A$ receptors was realised from experiments conducted on invertebrate muscle $GABA_A$ receptors (Smart & Constanti, 1982). Increasing external pH markedly reduced the antagonistic effects of Zn^{2+}, implying that H^+ and Zn^{2+} modulate $GABA_A$ receptor function via the same binding site, with a pK_a for sensitivity to H^+ of 6.1. This implicated histidine residues (pK_a 6.5) in the binding of Zn^{2+} to the $GABA_A$ receptor. External H^+ concentration also affects the Zn^{2+} antagonism of vertebrate $GABA_A$ receptors in cultured cerebellar granule neurones and recombinant α1β1 and α1β1δ subunit-containing receptors (Krishek *et al.*, 1998)

underlining the importance of external histidine residues in mediating the Zn^{2+} inhibition. Furthermore histidine residues have been demonstrated to be likely Zn^{2+} binding sites in α6 (H273; Fisher & McDonald, 1998), β1 and β3 (H267; Horenstein & Akabas, 1998; Wooltorton *et al.*, 1997a) $GABA_A$ receptor subunits, and in ρ1 $GABA_C$ receptor subunits (H156; Wang *et al.*, 1995b). Mutation of H267 in the β3 subunit revealed the likely existence of additional residues involved in the Zn^{2+} modulation of this subunit. External histidine residues were targeted as prime candidates in order to locate the complete spectrum of residues involved in Zn^{2+} binding on the murine β3 $GABA_A$ receptor subunit.

The search for these additional residues therefore centred on histidines located in the extracellular domain since lowering the Ringer pH abolished all inhibition by Zn^{2+} at concentrations up to 2mM. The pH reduction from 7.4 to 5.4 encompasses the pK_a for histidine (6.5) but not those of other amino acids capable of forming Zn^{2+} binding sites have pK_as outside of this range (cysteine, 8.5; aspartate and glutamate, 4.4). It was therefore assumed that H^+ and Zn^{2+} are competing for similar binding sites involving histidine residues. The present study implicated the histidine residue, H107 as a determinant of Zn^{2+} on the $GABA_A$ receptor β3 subunit. As mutation of this residue prevented functional expression, its role in Zn^{2+} was deduced indirectly, the evidence being that the separate and concurrent mutation of residues H119, H191 along with H267 all failed to have any effect on the residual Zn^{2+} inhibition. In addition the Zn^{2+} sensitivity of the mutant receptor subunit $\beta3^{H119A,\ H191A,\ H267A}$ was reduced by raising the H^+ concentration. Constructing a pH titration curve produced a pK_a of approximately 6.79, implicating the remaining histidine, H107.

However, it is implicitly assumed that pK_as of amino acid residues are maintained within the microenvironment of the receptor protein. There is evidence that pK_as can be perturbed by juxtaposed charged groups in proteins and pockets of hydrophobicity/hydrophilicity (Antosiewicz *et al.*, 1994; Nakamura, 1996; Mehler & Guarnieri, 1999). A pK_a of 8.12 was calculated for a histidine residue in close proximity to negative charges in Ribonuclease St (Miyamoto *et al.*, 1981). Furthermore, substantial shifts in pKa values for a histidine (pKa 4.0) and an aspartate residue (pKa

6.4) in triclinic lysozyme (Bashford & Karplus, 1990). Thus although the pH experiment suggests the involvement of histidines, other residues cannot be completely discounted. Yet the use of the histidine-modifying reagent, DEPC, provided additional evidence for the involvement of histidine residues. Treatment of $\beta3^{H267A}$ receptors with DEPC resulted in the abolition of Zn^{2+} inhibition of PB-gated currents. The involvement of each of the external histidine residues was examined by systematic mutation using the background mutation of H267A to remove the potent inhibitory effect of Zn^{2+}.

3.3.2. A role for H107 in receptor assembly?

Mutation of H107 prevented functional expression of β3 subunit ion channels suggesting that H107 may have a prime role in receptor assembly, protein folding or transport of the receptor to the cell surface. Confocal immunofluorescence demonstrated that these receptors were unable to gain access to the cell surface, being retained in the endoplasmic reticulum of HEK cells. This failure to produce a functional receptor was not unexpected since this histidine is highly conserved across $GABA_A$, $GABA_C$ and glycine receptor subunits and is also found in the comparable position in members of the nicotinic acetylcholine receptor family. Even nominally conservative substitutions of H107 to lysine ($\beta3^{H107K}$) or arginine ($\beta3^{H107R}$), residues with positively charged side chains, failed to result in functional cell surface expression of these mutant β3 receptors. These results agree with the observation that substitution of the aligned residue in $GABA_C$ ρ1 homomers, H163, prevented the formation of functional GABA-gated channels (Wang *et al.*, 1995b). This suggests a critical role for this specific histidine residue in the formation and/or function of ionotropic GABA receptors that cannot be performed by an alternative amino acid residue, even allowing for a charged side chain.

A role in receptor assembly for H107 is thought to be unlikely. It appears that H^+, DEPC and presumably Zn^{2+} are able to access this residue in the β3 subunit homomer and modulate receptor function. This level of accessibility appears inconsistent with H107 lying at the interface of two juxtaposed subunits, for example, forming part of an inter-subunit contact or assembly box. H107 has not been identified as an important assembly box amino acid in studies of homomeric and heteromeric $GABA_A$ receptor expression

(Taylor *et al.,* 1999). However, it should be noted that this study was so designed as to identify residues which prevented β2 forming homomers, rather than those residues conserved between β2 and β3 that are necessary for β homomer formation. Thus a role in protein folding for this highly conserved histidine may be more likely. The correct folding of the $GABA_A$ receptor protein to stabilise a particular conformation, is presumably conditional upon the arrangement of polar and nonpolar amino acid side chains. Therefore $\beta3^{H107}$, a highly polar residue, might be essential for the structural conformation of the receptor, due to the formation of hydrogen bonds, between histidine side chains and either peptide bonds or other polar side chains. Highlighting the importance of certain basic amino acids for correct receptor biogenesis, an arginine residues (conserved throughout $GABA_A$ receptor subunits) is important for the correct structure and function of glycine receptor α1 subunit homomers (Langosch *et al.*, 1993). Mutation of $\alpha1^{R219}$ (0'), located at the cytoplasmic end of TM2, resulted in the retention of the mutant subunits in the endoplasmic reticulum. It has been suggested that the 0' arginine may be important in terminating the passage of TM2 through the membrane as a charged lysine residue is found at the equivalent position in cation selective receptors (Langosch et al., 1993). Clearly H107 cannot have such a role in subunit folding, however it may perform a crucial role in subunit formation whilst also retaining accessibility to modulatory agents. Unfortunately this presumed role in functional homomeric receptor expression precludes investigation using site-directed mutagenesis. Despite this complication, the use of different approaches known to affect histidine residues, namely H^+, DEPC and Zn^{2+} provided reasonably compelling evidence for the involvement of a histidine residue in Zn^{2+} inhibition for which H107 is the only remaining candidate.

3.3.3. Possible mechanisms for H107 in regulating Zn^{2+} sensitivity.

The mechanism by which H107 is involved in the sensitivity of the β3 subunit to Zn^{2+} is unclear. Given that Zn^{2+} antagonism of PB on $\beta3^{H267A}$ was non-competitive, and the apparent lack of a suitable binding site in the channel, its actions are probably allosteric as they are on αβ receptors (Smart, 1990). H107 may constitute a second discrete

binding site for Zn^{2+}, with each juxtaposed subunit supplying H107 to co-ordinate the binding pocket. The Hill coefficients for the Zn^{2+} inhibition plots were affected by the introduction of the H267A mutation. The Hill coefficient for the Zn^{2+}inhibition of wild-type β3 receptors is 0.59 ± 0.03 suggesting that only a single Zn^{2+} ion may be capable of antagonizing the receptor or that Zn^{2+} ions bind to the receptor with negative co-operativity. However, the deduced Hill coefficient of 2.55 ± 0.47 from the Zn^{2+}inhibition curve for $\beta3^{H267A}$ subunit receptors is consistent with co-operative binding of three (or more) Zn^{2+} ions, indicating that inhibition by the higher and lower affinity sites may be mediated by different mechanisms. However, this deduction from the Hill slopes may be too simplistic. The Hill slope can be affected by conformational changes associated with channel gating and also by changes solely to the Zn^{2+} binding site.

Alternatively, since the tertiary structure of the β3 homomeric receptor is unknown, the H107 region of the N-terminus may lie in close proximity to the entrance of the ion channel. The imidazole groups of H107 and H267 could then co-ordinate with Zn^{2+} and form a *single* binding site. Thus the low potency and high cooperativity of Zn^{2+} on $\beta3^{H267}$ could reflect its binding to the remnants of this site, rather than an second low affinity binding site elsewhere on the receptor. However for Zn^{2+} binding sites identified on other proteins, the distance between amino acid ligands is remarkably regular. Zn^{2+} is usually co-ordinated by four (or occasionally six) ligands. There is nearly always one, two, or three residues between the first and second amino acids in the binding site. This common consensus sequence for Zn^{2+} binding sites, $HX_{1-3}H$, is termed the short spacer (where H is a binding histidine residue and X_{1-3} denotes the spacer of up to three other amino acids). The short spacer is able to form a bidentate Zn^{2+} complex which stabilizes both local and overall protein structure, similar to disulphide bond formation (Vallee & Falchuk, 1993). The third ligand of a Zn^{2+} binding site is separated from the second ligand by several amino acids (18 – 123) termed the long spacer, which adds further structural stability and helps align the residues that bind Zn^{2+}. Therefore, the typical consensus sequence for Zn^{2+} binding sites is $H_{1-3}HX_{18-123}H$. The fourth ligand is usually an activated water molecule. It is therefore unlikely, from previous metalloenzyme studies (Vallee & Falchuk, 1993), that H107 and H267 participate in the

formation of a single Zn^{2+} binding site given the number of intervening amino acid residues.

Two neighbouring histidine residues (H42 and H44) in the N-terminus of the NR2A subunit have recently been identified as critical for high affinity, voltage-independent Zn^{2+} inhibition of NMDA receptors (Choi & Lipton, 1999). Mutation of either or both residues resulted in a similar shift in the Zn^{2+} IC_{50} (by ~200-fold). These histidines were thought likely to form a short spacer since mutation of one histidine would be enough to disrupt a bidentate Zn^{2+} complex. A similar Zn^{2+} binding motif has been identified in the glycine receptor α1 subunit. Zn^{2+} has a dual regulatory role causing potentiation of glycine-gated currents at low Zn^{2+} concentrations (<10μM) and then inhibition at higher Zn^{2+} concentrations (>10μM) (Bloomenthal *et al.*, 1994; Laube *et al.*, 1995). Experiments using α1 homomeric glycine receptors have noted that two histidines, H107 and H109, located in the N-terminal domain are involved in the inhibitory effects of Zn^{2+} (Harvey *et al.*, 1999). Mutation of either or both residues to alanine is sufficient to abolish Zn^{2+} inhibition, suggesting that H107 and H109 are forming a bidentate complex with Zn^{2+}. The potentiating effect of Zn^{2+} is more complex. Potentiation of glycine-gated currents was abolished by mutating H109 but unaffected by mutating H107. In addition, several other point mutations can also remove Zn^{2+} induced potentiation (Lynch *et al.*, 1998) suggesting that the H109A mutation may affect this potentiation via allosteric mechanisms (Harvey *et al.*, 1999). Therefore a single histidine residue can be capable of regulating the Zn^{2+} modulation of a receptor via different mechanisms. Interestingly, this important histidine, H109, is the highly conserved histidine found throughout $GABA_A$, $GABA_C$ and glycine receptor subunits and equivalent to $\beta3^{H107}$. Whilst mutation of this residue produces a loss of function in homomeric $GABA_A$ β3 (Dunne *et al.*, 1999a, b) and $GABA_C$ ρ1 (Wang *et al.*, 1995b) receptors, substitution of this histidine is tolerated in glycine receptor α1 subunits allowing the formation of functional homomeric ion channels (Harvey *et al.*, 1999).

A final possibility is that H107 does not bind zinc directly buts contribute to either the transduction mechanism or the structure of an as yet unknown site. Thus H107 may be crucial in stabilising the receptor in closed conformations following Zn^{2+} binding with other distinct amino acids, such as glutamate and aspartate. The loss of function induced

by mutating this residue supports such a structural role. As discussed previously, the aligned residue in the glycine receptor α1 subunit has been implicated in the allosteric modulation of Zn^{2+} regulation (Harvey *et al.,* 1999). Other residues that have been implicated in Zn^{2+} binding on ion channels include aspartate and glutamate (α1 glycine receptor subunit; Laube *et al.*, 1995). Indeed, as Zn^{2+} inhibition of GABA-activated currents evoked from sympathetic neurones is insensitive to external pH (Smart, 1992), different amino acids may be involved on other subunits.

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3.3.4. Summary.

This study has expanded the concept of a second site involved in the regulation of Zn^{2+} sensitivity of the $GABA_A$ receptor β3 subunit, additional to the previously identified histidine residue (H267) positioned within the external entrance of the ion channel. This site is sensitive to external pH. Lowering the pH to 5.4 abolished the Zn^{2+} inhibition of both the PB-gated and spontaneous currents on $\beta3^{H267A}$ receptors. Alteration of the Ringer pH over a range from 5.4 to 8.4 enabled the titration of the site involved in the additional Zn^{2+} sensitivity. The pK_a was calculated at approximately 6.79 intimating the involvement of histidine residues. Mutating H119 and/or H191 did not further reduce the Zn^{2+} sensitivity of the $\beta3^{H267A}$ receptor. The only remaining external histidine, H107 in the N-terminal domain, is therefore a prime candidate for mediating Zn^{2+} inhibition in the β3 subunit. H107 is likely to regulate Zn^{2+} sensitivity either by forming a discrete binding site or via an allosteric mechanism rather than co-ordinating with H267 in a single binding site. Mutation of this residue (H107A, H107K, H107R) disrupted the functional expression of β3 subunit ion channels proposing a further, crucial role for this residue in assembly and/ or protein folding of the receptor. Therefore this study has highlighted the importance of an N-terminal histidine, H107 in the mediation of Zn^{2+} inhibition and receptor function in the β3 $GABA_A$ receptor. It may be that $\beta3^{H107}$ or its equivalent plays a role in the Zn^{2+} sensitivity of other $GABA_A$ receptor subunits; this possibility is investigated in the next chapter.

Figure 3.1. Inhibition of spontaneous Cl^- currents through β3 subunit $GABA_A$ receptors by Zn^{2+}. Representative whole-cell membrane currents recorded from *Xenopus* oocytes expressing either $\beta3^{wild\text{-}type}$ (A) or $\beta3^{H267A}$ (B) subunit receptors in the absence and presence of 10μM Zn^{2+}. The transient downward deflections represent current pulses induced by brief (1s) hyperpolarizing commands (-10mV, 0.2Hz) applied to the holding potential of –40mV. (C) Zinc concentration-inhibition curves for blocking the spontaneous Cl^- current mediated by $\beta3^{wild\text{-}type}$ (■) and $\beta3^{H267A}$ mutant (●) $GABA_A$ receptors. The IC_{50}s were 0.16 ± 0.03μM (β3) and 194.58 ± 16.17μM ($\beta3^{H267A}$) and the Hill coefficients were 0.51± 0.06 (β3) and 2.39 ± 0.46 ($\beta3^{H267A}$). Data points represent the mean ± s.e.m. from 3 – 7 oocytes. The curves describe logistic equation fits to the experimental data (see Chapter 2).

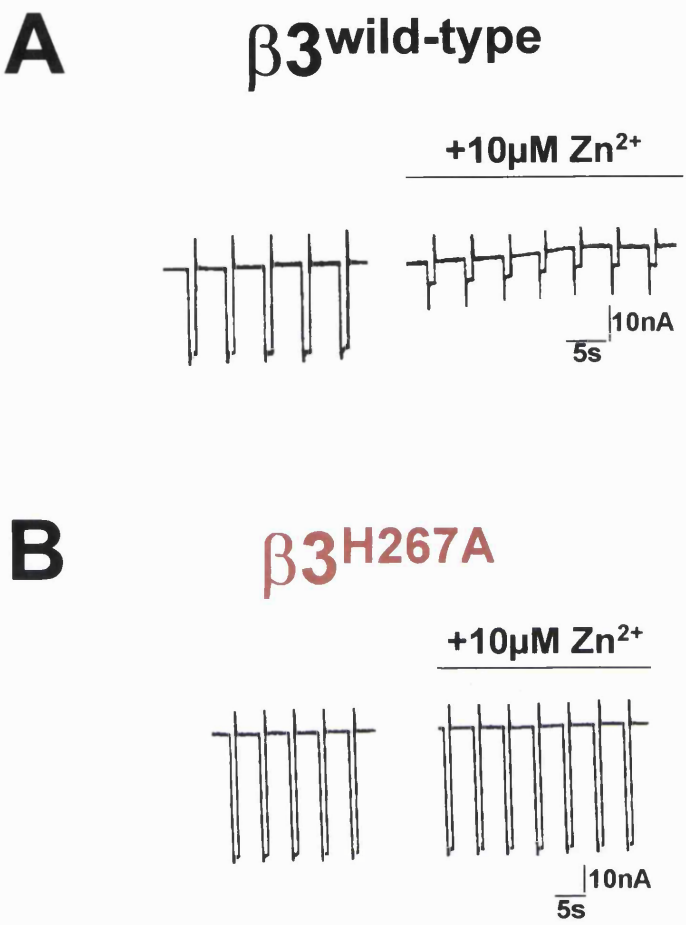
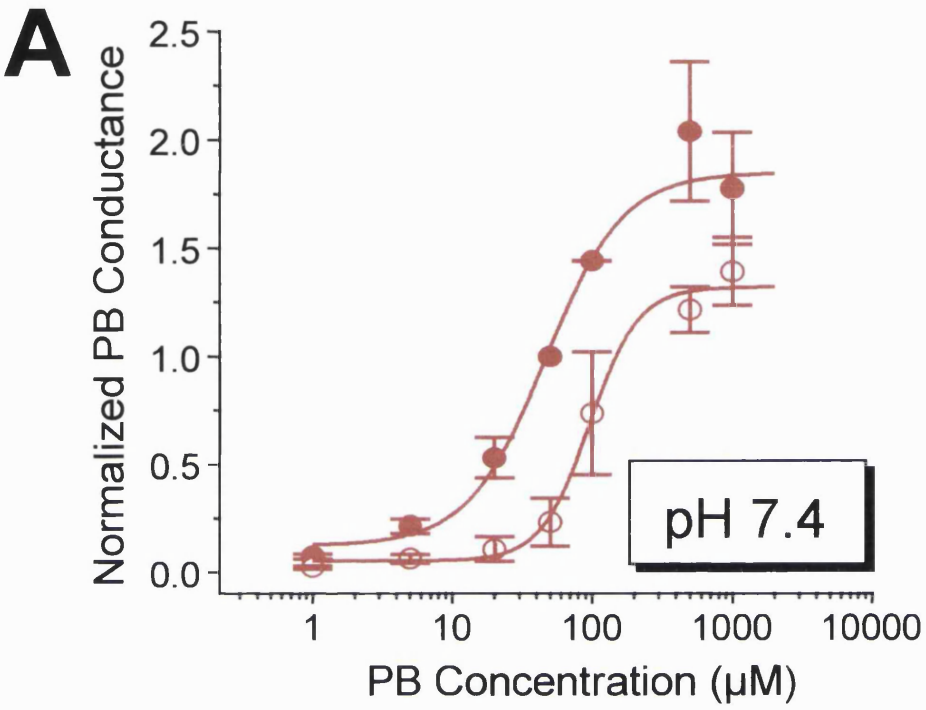

A
β3wild-type
+10μM Zn2+
10nA
5s
B
β3H267A
+10μM Zn2+
10nA
5s
C
% Zinc-sensitive Conductance
100
80
60
40
20
0
β3wild-type
β3H267A
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

Figure 3.2. Low external pH eliminates the inhibitory action of Zn^{2+} on the pentobarbitone modulation of the $\beta3^{H267A}$ $GABA_A$ receptor. Concentration response curves were constructed for pentobarbitone (PB) modulated currents in the absence (●) and presence (○) of 300μM Zn^{2+} recorded from oocytes expressing $\beta3^{H267A}$ homomers in Ringer at pH 7.4 (A) and 5.4 (B). Curves were fit to the data as described in the methods, no allowance was made for the small inhibitory effect of PB often observed at high concentrations (500μM – 1mM) resulting in a bell-shaped maximum to the concentration response curve (see Halliwell *et al.,* 1999). This effect of PB was present even after the addition of Zn^{2+}. Data points represent the mean ± s.e.m. from 3 oocytes and are normalized to the conductance induced by 50μM PB in the absence of Zn^{2+} at either pH7.4 or pH5.4.


A
Normalized PB Conductance
2.5
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
10000
PB Concentration (μM)
pH 7.4

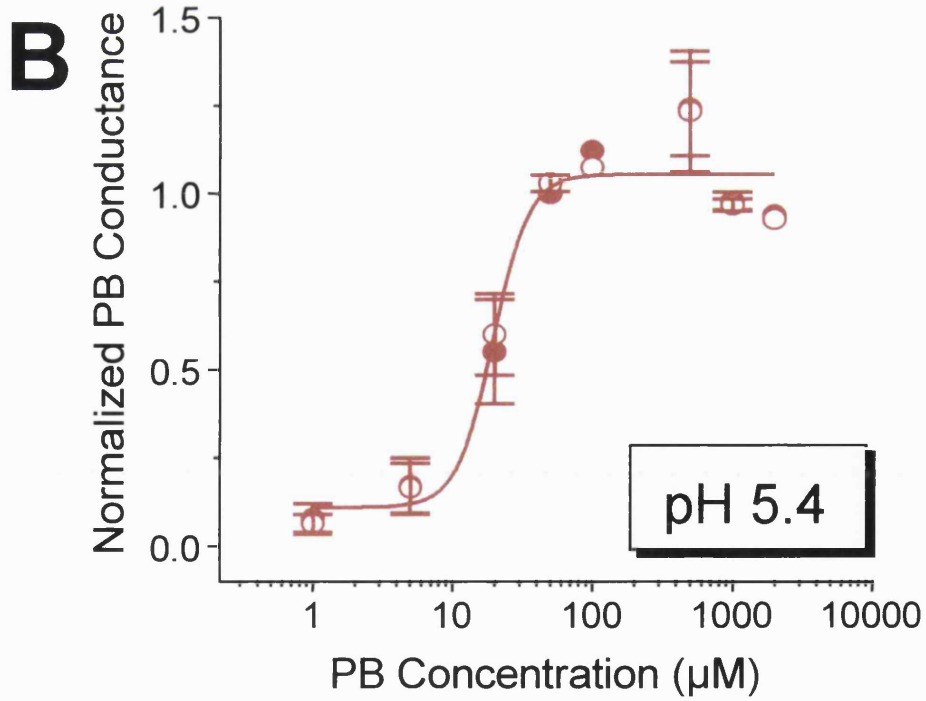

B
Normalized PB Conductance
1.5
1.0
0.5
0.0
1
10
100
1000
10000
PB Concentration (μM)
pH 5.4

Figure 3.3. Zn^{2+} inhibition of spontaneous currents mediated by $\beta3^{H267A}$ $GABA_A$ receptors is affected by H^+. Zinc concentration-inhibition relationships for spontaneous currents mediated by $\beta3^{H267A}$ homomeric receptors exposed to Ringer at pH 7.4 (●) and 5.4 (○). Zn^{2+} (5μM – 2mM) had no effect on the receptor at pH 5.4 indicating that amino acid residues involved in the residual Zn^{2+} sensitivity of $\beta3^{H267A}$ receptors are pH sensitive. Data (mean ± s.e.m.) were obtained from n = 3 – 4 oocytes. Data acquired at pH7.4 were fitted using the equation described in the methods (Chapter 2) and for the pH5.4 data, a linear regression line was applied.

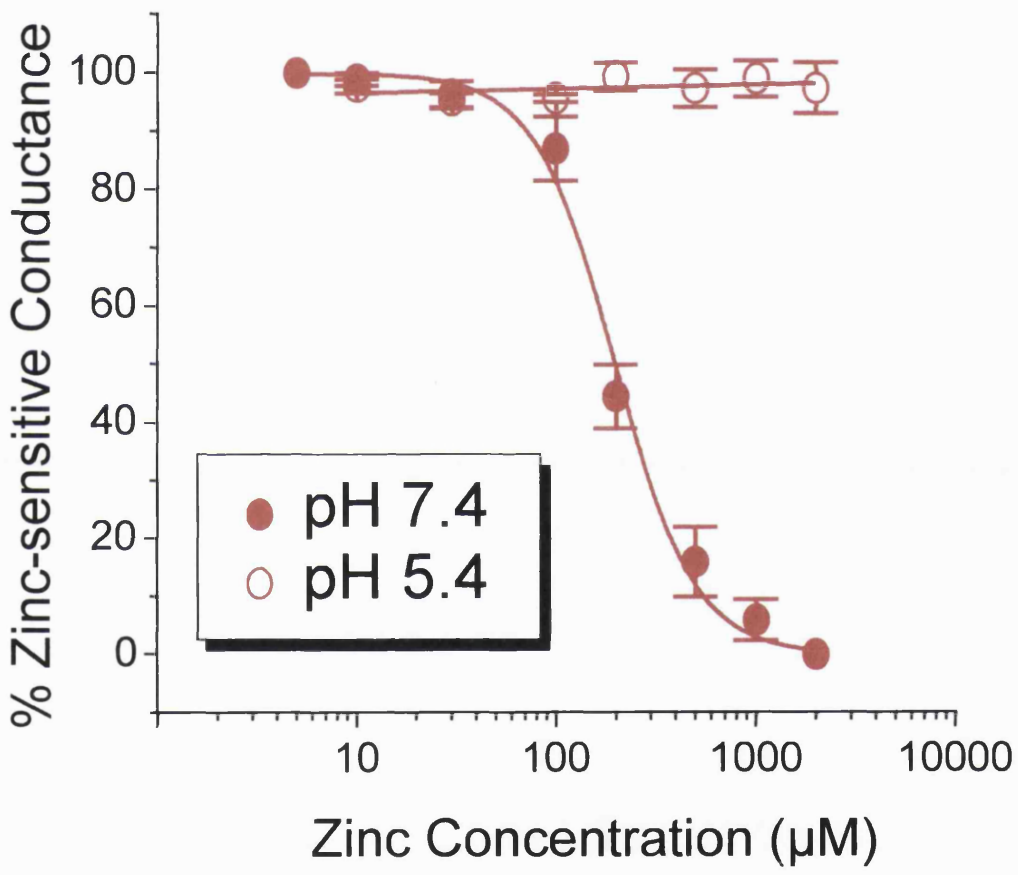

% Zinc-sensitive Conductance
100
80
60
40
20
0
pH 7.4
pH 5.4
10
100
1000
10000
Zinc Concentration (μM)

Figure 3.4. Diethylpyrocarbonate attenuates the Zn^{2+} sensitivity of $\beta3^{H267A}$ homomers. (A) Typical whole-cell membrane currents recorded from $\beta3^{H267A}$ subunit homomers expressed in HEK cells under whole-cell patch clamp at a holding potential of –40mV. Application of 1mM PB (black bar) induces an inward current which is reversibly inhibited by 100μM Zn^{2+} (green bar). Full recovery of the PB response was observed within 3 min (middle trace). DEPC (1mM, dashed red line) was applied for 2 min prior to co-application with 1mM PB and 100μM Zn^{2+}. DEPC attenuated the Zn^{2+} inhibition of the PB-induced currents. A rebound current was observed on the removal of PB (Wooltorton *et al.*, 1997b). (B) A histogram summarising the effects of DEPC. Data are normalized to the peak PB- induced current in the absence of Zn^{2+} or DEPC, and represent mean ± s.e.m from n=4 cells..

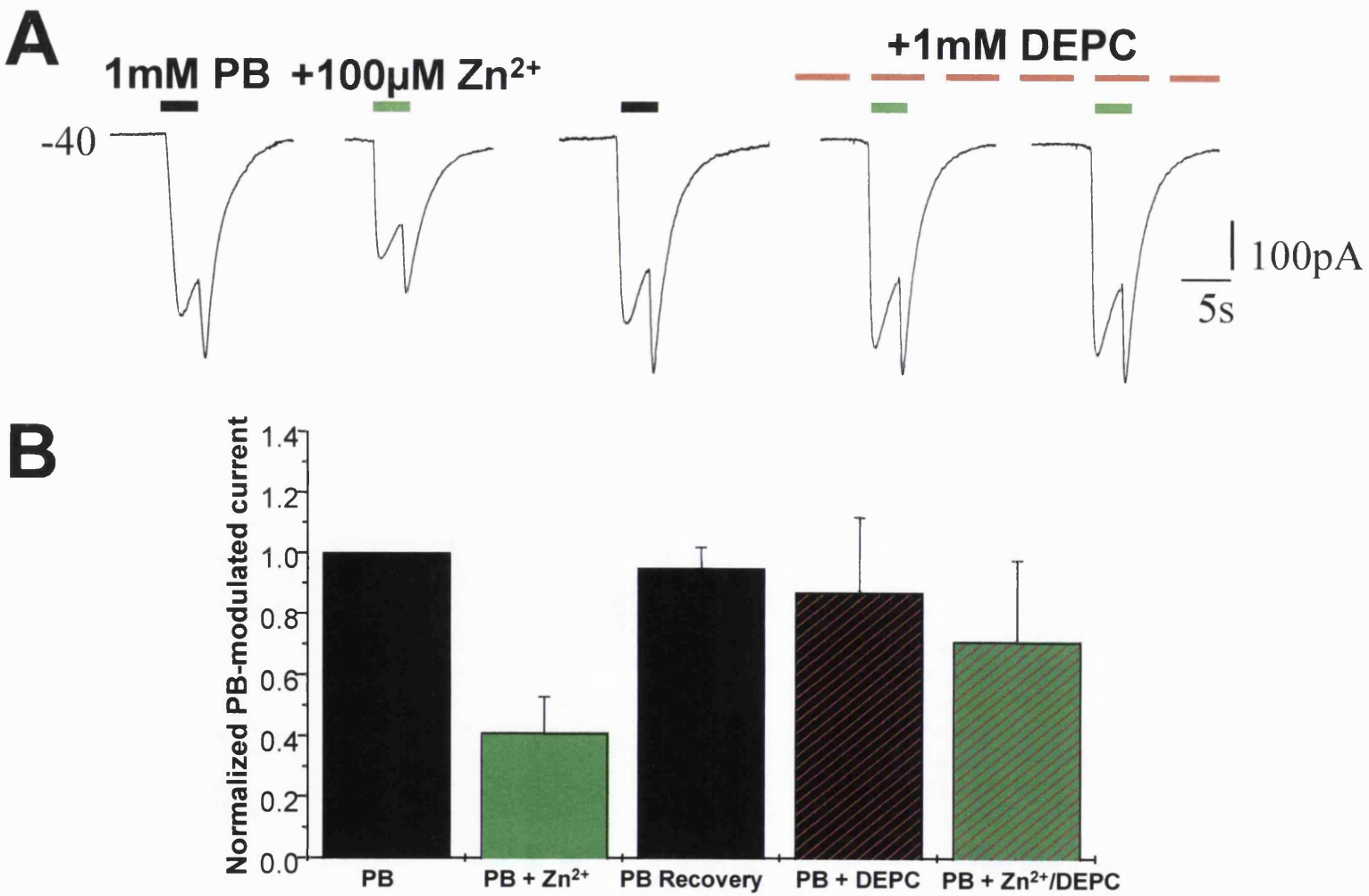
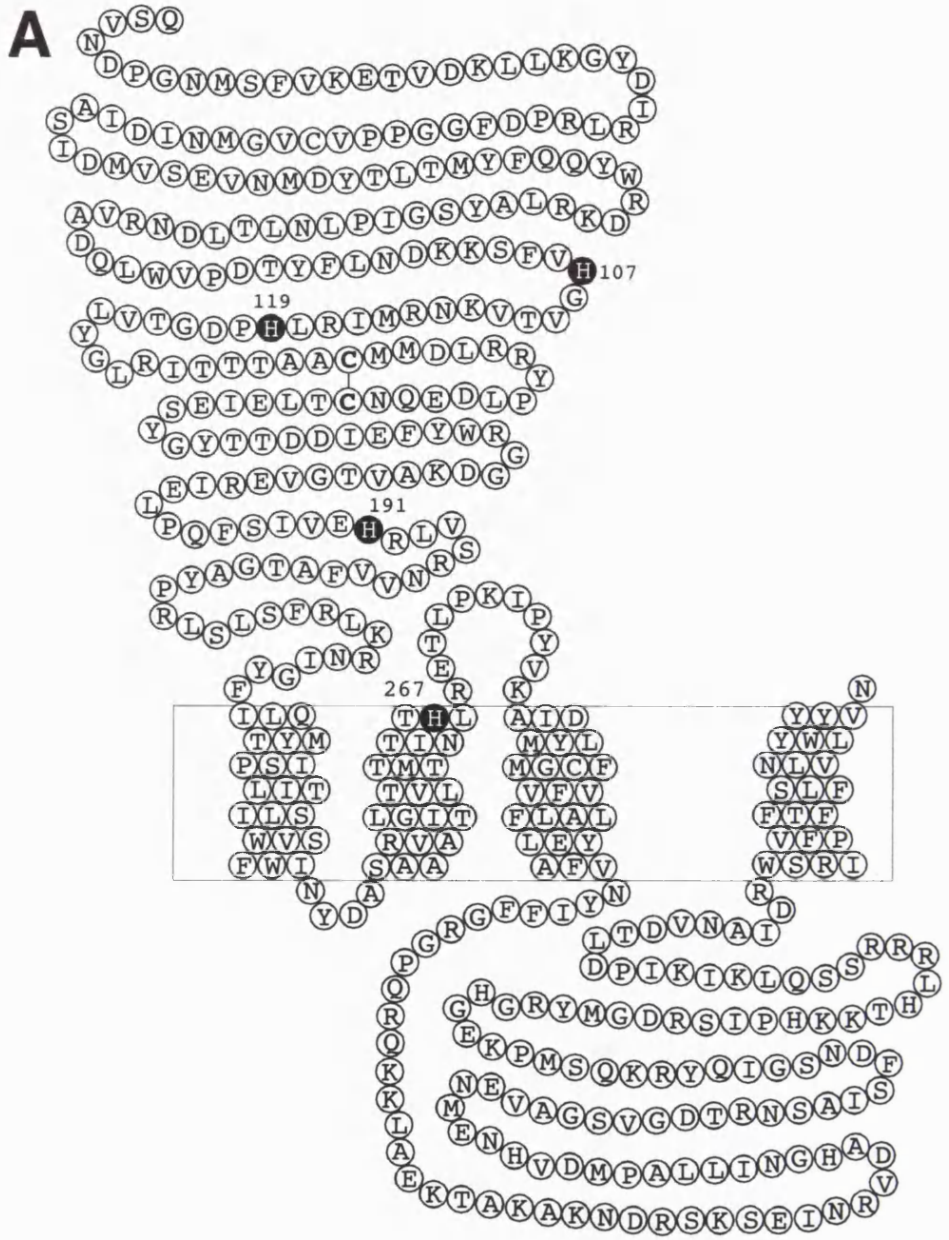

A
1mM PB
+100μM Zn2+
+1mM DEPC
-40
100pA
5s
B
Normalized PB-modulated current
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PB
PB + Zn2+
PB Recovery
PB + DEPC
PB + Zn2+/DEPC

Figure 3.5. Location of external histidine residues in the β3 subunit and comparison of amino acid sequences with other GABA ionotropic receptor subunits. (A) Schematic diagram of the $GABA_A$ receptor β3 subunit illustrating the transmembrane domains (TM) and external N- and C-termini and large intracellular loop between TM3 and TM4. The external histidine residues and the single histidine (H267) in TM2 are highlighted. (B) Comparison of amino acid sequences of the N-terminal and first three TM domains of $GABA_A$ and $GABA_C$ receptor subunits that influence receptor sensitivity to Zn^{2+} indicates the relative positions of these external histidines (Wang *et al.*, 1995b; Wooltorton *et al.*, 1997a; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998). Histidines are depicted as bold characters and those previously demonstrated to affect Zn^{2+} modulation are also italicised. TMs 1-3 are underlined.



B

```
α1   WTPDTFFHNGKKSVAHNMTMPNKLLRITEDGTLLYTMRLT  133
α6   WTPDTFFRNGKKSIAHNMTTPNKLFRLMHNGTILYTMRLT  132
β1   WVPDTYFLNDKKSFVHGVTVKNRMIRLHPDGTVLYGLRIT  131
β3   WVPDTYFLNDKKSFVHGVTVKNRMIRLHPDGTVLYGLRIT  131
ρ1   WVPDMFFVHSKRSFIHDTTTDNVMLRVQPDGKVLYSLRVT  172

α1   VRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTRE  173
α6   INADCPMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKKG  172
β1   TTAACMMDLRRYPLDEQNCTLEIESYGYTTDDIEFYWN--  169
β3   TTAACMMDLRRYPLDEQNCTLEIESYGYTTDDIEFYWR--  169
ρ1   VTAMCNMDFSRFPLDTQTCSLEIESYAYTEDDLMLYWK--  210

α1   PARSVVVAEDGSRLNQYDLLGQTVDSGIVQSS-TGEYVVM  212
α6   PLYSVEVPEESSSLLQYDLIGQTVSSETIKSN-TGEYVIM  211
β1   GGEGAVTGVNKIELPQFSIVDYKMVSKKVEFT-TGAYPRL  208
β3   GGDKAVTGVERIELPQFSIVEHRLVSRNVVFA-TGAYPRL  208
ρ1   KGNDSLKTDERISLSQFLIQEFHTTTKLAFYSSTGWYNRL  250

α1   TTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFWLNRESVP  252
α6   TVYFHLQRKMGYFMIQIYTPCIMTVILSQVSFWINKESVP  251
β1   SLSFRLKRNIGYFILQTYMPSTLITILSWVSFWINYDASA  248
β3   SLSFRLKRNIGYFILQTYMPSILITILSWVSFWINYDASA  248
ρ1   YINFTLRRHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVP  290

α1   ARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVC  292
α6   ARTVFGITTVLTMTTLSISARHSLPKVSYATAMDWFIAVC  291
β1   ARVALGITTVLTMTTISTHLRETLPKIPYVKAIDIYLMGC  288
β3   ARVALGITTVLTMTTINTHLRETLPKIPYVKAIDMYLMGC  288
ρ1   ARVPLGITTVLTMSTIITGVNASMPRVSYIKAVDIYLWVS  330
```

Figure 3.6. Effects of mutating histidine residues H119 and H191 in $GABA_A$ receptor $\beta3^{H267A}$ homomers. Concentration-response curves for pentobarbitone (PB) were determined for β3, $\beta3^{H267A}$ and either $\beta3^{H119A, H267A}$ (A) or $\beta3^{H191A, H267A}$ (C) $GABA_A$ receptor constructs. Data for each construct were normalised to the maximum PB-evoked response recorded in each oocyte and represent mean ± s.e.m. from n = 3 – 11 oocytes. Concentration-inhibition relationships for Zn^{2+} antagonising the spontaneous Cl^- current of β3, $\beta3^{H267A}$ and either $\beta3^{H119A, H267A}$ (B) or $\beta3^{H191A, H267A}$ (D) constructs. Data are mean ± s.e.m. from n = 3 – 8 oocytes. Curves were fitted to the data according to the methods (Chapter 2).

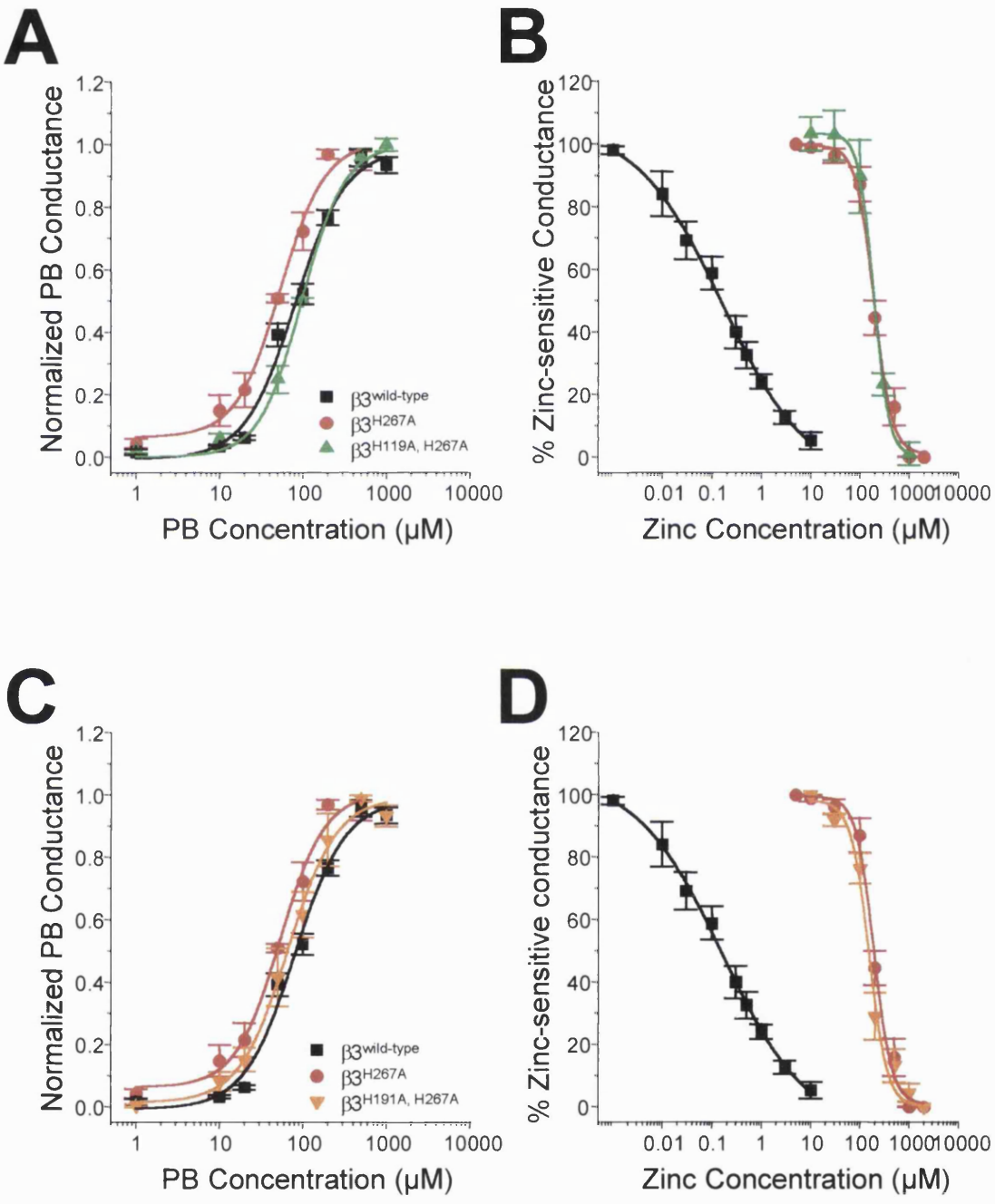
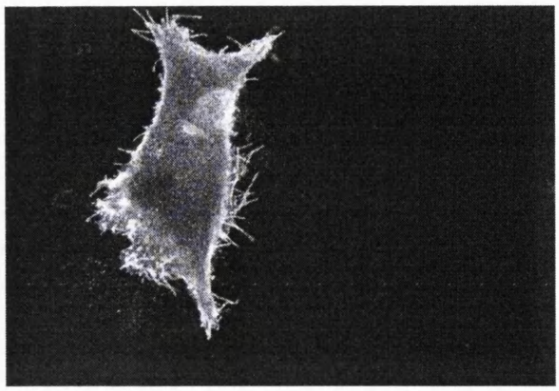

A
Normalized PB Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
10000
PB Concentration (μM)
β3wild-type
β3H267A
β3H119A, H267A
B
% Zinc-sensitive Conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)
C
Normalized PB Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
10000
PB Concentration (μM)
β3wild-type
β3H267A
β3H191A, H267A
D
% Zinc-sensitive conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

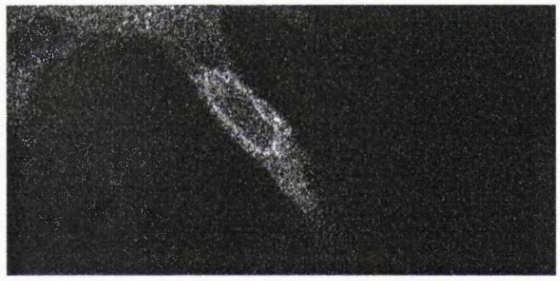
Figure 3.7. Mutation of H107 prevents cell surface expression of β3 homomers. Confocal images of HEK cells transfected with wild type β3, or $\beta3^{H107A}$ subunit cDNAs. HEK cells were incubated with the primary anti-β subunit antibody BD17 and then with a TRITC-conjugated secondary antibody. Surface staining was clearly seen in cells transfected with wild type β3 (A). By contrast, the surface immunofluorescence of cells transfected with $\beta3^{H107A}$ did not exceed the background levels of fluorescence (B). However, a bright, ring-like structure, could be seen in permeablised cells transfected with the mutant subunit (C, D). This pattern of immunofluorescence corresponds to the characteristic shape of the endoplasmic reticulum of HEK cells and suggests that $\beta3^{H107A}$ mutants are retained within this organelle.

A



B

C



D

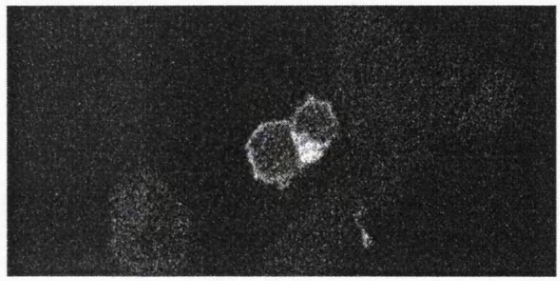


Figure 3.8. The $\beta3^{H119A, H191A, H267A}$ $GABA_A$ receptor retains Zn^{2+} sensitivity. Whole-cell membrane currents evoked by 100μM PB in the absence and presence of pre-applied 100μM Zn^{2+} in oocytes expressing $\beta3^{H267A}$ (A) or $\beta3^{H119A, H191A, H267A}$ (B) constructs. The transient downward deflections represent current pulses induced by brief hyperpolarizing voltage commands (-10mV, 1s, 0.2Hz) applied to the holding potential of –40mV. Note the different time calibrations on recovery from PB due to the slowing of the chart recorder speed (1: 5s; 2: 12.5s; 3: 50s). Concentration-response curves for PB were established in the absence (closed symbol) or presence (open symbol) of 100μM Zn^{2+} for $\beta3^{H267A}$ (C) or $\beta3^{H119A, H191A, H267A}$ subunit receptors (n = 3 oocytes). Data are normalized to the maximum PB-evoked response recorded in control Ringer for each oocyte. Data points are mean ± s.e.m. The curves describe logistic equation fits to the experimental data (Chapter 2). A non-competitive mode of antagonism is indicated by the upper graphs in Fig 3.8C and D, which show that the degree of inhibition exerted by Zn^{2+} is virtually independent of PB concentration.

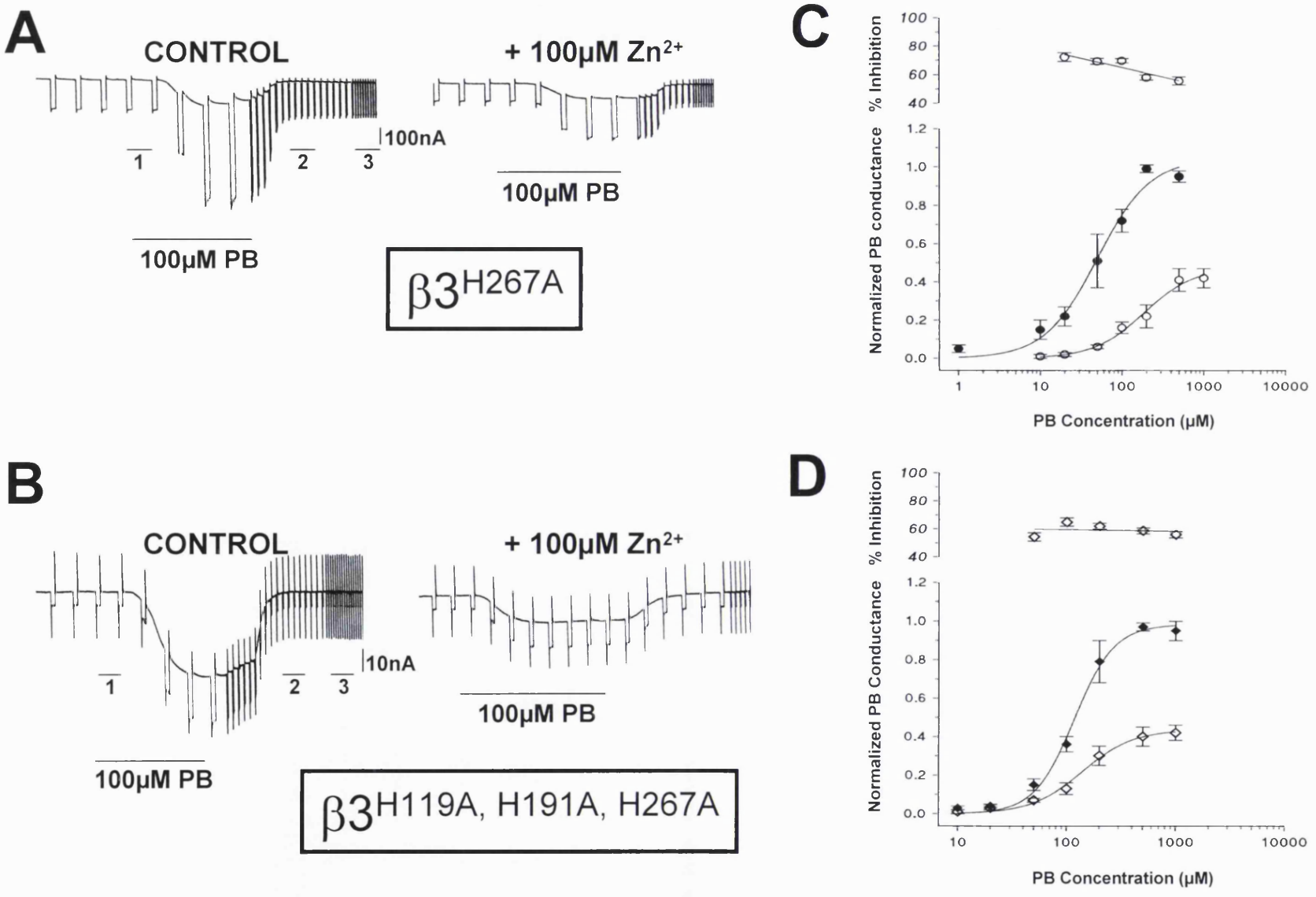
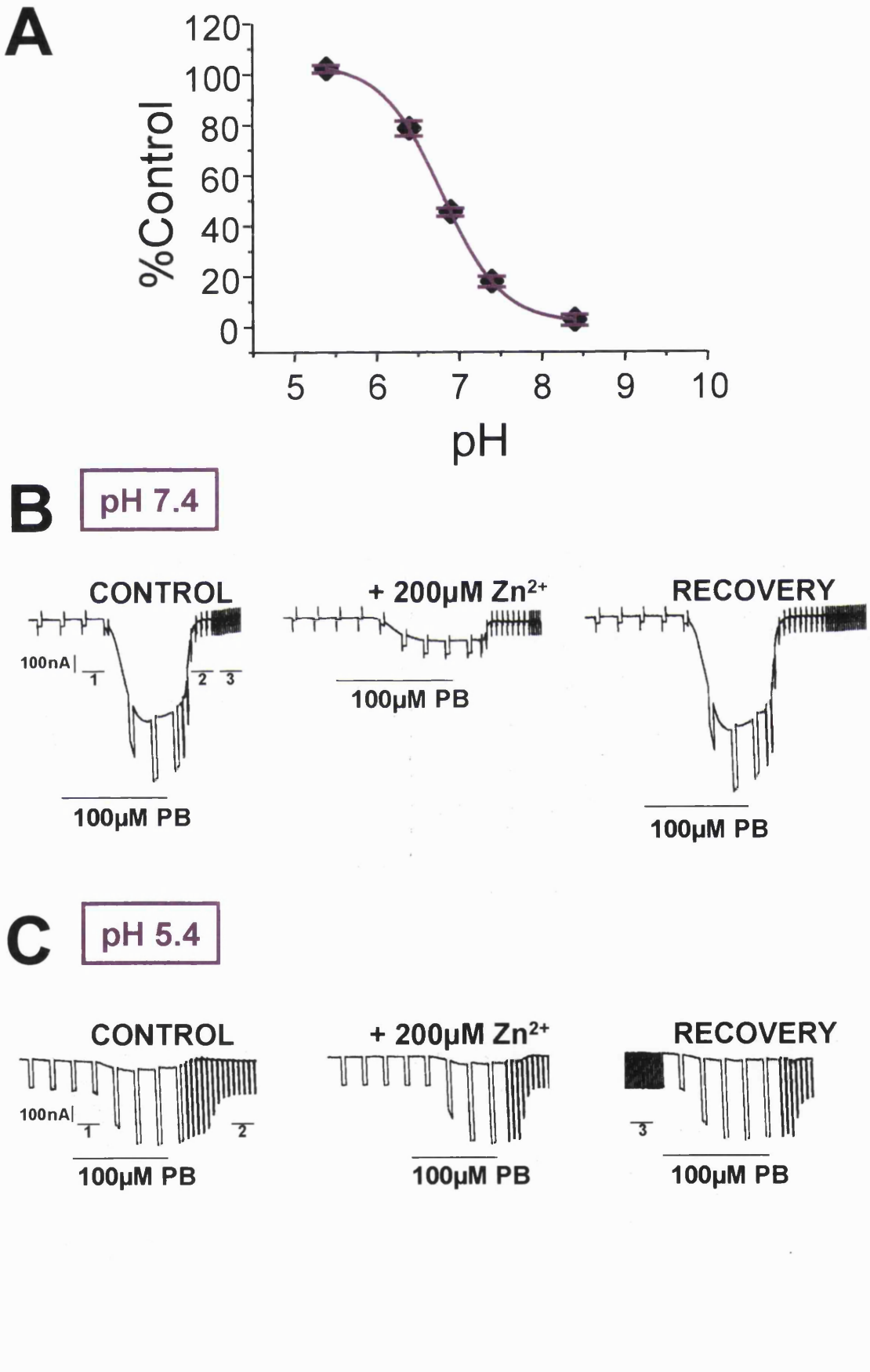

A
CONTROL
+ 100μM Zn2+
100nA
1
2
3
100μM PB
100μM PB
β3H267A
B
CONTROL
+ 100μM Zn2+
10nA
1
2
3
100μM PB
100μM PB
β3H119A, H191A, H267A
C
% Inhibition
Normalized PB conductance
PB Concentration (μM)
D
% Inhibition
Normalized PB Conductance
PB Concentration (μM)

Figure 3.9. The Zn^{2+} inhibition of the $\beta3^{H119A,\ H191A,\ H267A}$ $GABA_A$ receptor is sensitive to external pH. Control responses to 100μM PB were recorded in the absence and then in the presence of 200μM Zn^{2+} over the external Ringer pH range 5.4 to 8.4. The data are presented in the form of a pH titration (A). The reduced inhibitory effect of Zn^{2+} as the external pH decreased was determined as a percentage of the control PB response measured at each pH in the absence of Zn^{2+}. The curve fit was performed according to the inhibition model described in the methods (Chapter 2). The pK_a determined from the curve fit was 6.79 ± 0.003. Data are mean ± s.e.m. from n = 3 oocytes. Whole-cell membrane currents evoked by 100μM PB in the presence and absence of pre-applied 200μM Zn^{2+} in oocytes expressing $\beta3^{H119A,\ H191A,\ H267A}$ constructs at Ringer pH 7.4 (B) or 5.4 (C). The transient downward deflections represent current pulses induced by brief hyperpolarizing voltage commands (-10mV, 1s, 0.2Hz) applied to the holding potential of –40mV. Zn^{2+} was pre-applied for 2 minutes in addition to application with the PB dose. Traces showing recovery of the PB response were recorded 5 minutes after the removal of Zn^{2+}. Note the different time calibrations on recovery from PB (1: 5s; 2: 12.5s; 3: 50s).


A
120
100
80
60
40
20
0
%Control
5
6
7
8
9
10
pH
B
pH 7.4
CONTROL
+ 200µM Zn2+
RECOVERY
100nA
1
2
3
100µM PB
100µM PB
100µM PB
C
pH 5.4
CONTROL
+ 200µM Zn2+
RECOVERY
100nA
1
2
3
100µM PB
100µM PB
100µM PB

CHAPTER 4

Identification of an α1 subunit histidine important for regulating Zn^{2+} inhibition of heteromeric $GABA_A$ receptors.

4.1. INTRODUCTION

Zn^{2+} is a negative allosteric modulator of the $GABA_A$ receptor, believed to stabilise the closed state of the channel (Smart *et al.*, 1994) and reduce the frequency of channel opening (Legendre & Westbrook, 1991; Smart, 1992; Kilic *et al.*, 1993). Whilst experiments using β homomeric $GABA_A$ receptors enable detailed investigation of the Zn^{2+} modulation of the subunit in relative isolation, free from the problems of receptor heterogeneity and the specific functional roles of different subunits, it is unlikely that β homomeric channels exist, in abundance, *in vivo*, as functional receptors require coexpression of α and β subunits (and a γ subunit for benzodiazepine potentiation) (Schofield *et al.*, 1987; Pritchett *et al.*, 1989; Gorrie *et al.*, 1997). The inhibition of recombinant $GABA_A$ receptors by Zn^{2+} was first observed on α1β1 (Smart *et al.*, 1991) and α1β2 (Draguhn *et al.*, 1990) constructs. These receptors are inhibited with high affinity (IC_{50}s: α1β1, 1.5μM; α1β2, 0.56μM). Inclusion of a third subunit type lowers the potency for Zn^{2+}, in particular the addition of a γ subunit which leads to relative insensitivity to Zn^{2+} (Draguhn *et al.*, 1990; Smart *et al.*, 1991; Gingrich & Burkat, 1998; Table 1.3.). Coexpression of the ε subunit with α1 and β1 subunits results in a receptor exhibiting 175-fold reduction in Zn^{2+} potency as compared to an α1β1 receptor combination (Whiting *et al.*, 1997). Inclusion of the δ subunit with either α1β1 (Krishek *et al.*, 1998) or α6β3 combinations (Thompson *et al.*, 1997) produces a 14-20-fold reduction in Zn^{2+} potency. Interestingly, coexpression of the δ subunit alleviates the relative insensitivity to Zn^{2+} of the αβγ construct, although α1β1γ2Lδ receptors are still less sensitive to Zn^{2+} than α1β1δ receptors (Saxena & Macdonald, 1994). In contrast, inclusion of a θ subunit has no effect on the Zn^{2+} antagonism of α2β2γ1 $GABA_A$ receptors (Bonnert *et al.*, 1999).

The α subunit subtype influences the sensitivity of $GABA_A$ receptors to inhibition by Zn^{2+} (Table 1.3.). The maximal Zn^{2+} block of the GABA-gated current is smaller in α1β3γ2L receptors (19%) than in α2- or α3-subunit containing receptors (~50%; White & Gurley, 1995). Similarly the α4 and α6 subtypes confer a greater sensitivity to Zn^{2+} (~ 6-fold reduction in IC_{50}) than the α1 subtype (Knoflach *et al.*, 1996; Fisher *et al.*, 1997).

The α5 subtype also confers relatively high sensitivity to Zn^{2+} (Burgard *et al.*, 1996). Construction of α1/α6 subunit chimeras implicated the extracellular TM2 - TM3 loop in the differential Zn^{2+} sensitivity of these $GABA_A$ subunits (Fisher *et al.*, 1997). A histidine residue in the α6 subunit, H273, (which is replaced by an asparagine in the α1 subunit; Fig. 1.3.) was identified as crucial for the enhanced Zn^{2+} sensitivity (Fisher & Macdonald, 1998). However receptors containing mutant α6 subunits are still sensitive to Zn^{2+} blockade. As α6-containing receptors will necessarily also contain a β subunit, this suggests the existence of a second lower affinity site on either the α or β subunit. From the data presented in Chapter 3, the N-terminal histidine, H107, may underlie residual Zn^{2+} inhibition of $\beta3^{H267}$ homomers and may therefore be involved in residual inhibition of αβ heteromers.

In the present chapter investigates the importance of $\beta3^{H107}$ and a possible role for the α1 subunit in the additional Zn^{2+} binding site on α1β3 receptors. Amino acids with ionizable groups form complexes with metal ions and the greater the basicity of the functional group, the more stable the resultant complex (cysteine > histidine > aspartate/glutamate). However the rare occurrence of a free sulphydryl group on cysteine residues in proteins makes histidine the principal amino acid residue involved in metal ion binding sites (Higaki *et al.*, 1992; Regan, 1993). Therefore histidine residues in the α1 subunit may also contribute to Zn^{2+}binding sites. Zn^{2+} inhibition of GABA-activated currents was analysed in α1β3 heteromeric receptors using the two electrode voltage-clamp technique following the injection of murine α1 (wild-type or mutant) cDNAs in a 1:1 ratio with murine β3 (wild-type or mutant) cDNAs into *Xenopus* oocytes.

4.2. RESULTS

4.2.1. The role of $\beta3^{H107}$ in Zn^{2+} inhibition of $\alpha1\beta3^{H267A}$ heteromers.

As determined previously, H267 in the TM2 of the β subunit is important for high affinity Zn^{2+} inhibition of both α1β1 and α1β3 $GABA_A$ receptors (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). Coexpression of $\alpha1\beta3^{H267A}$ subunits produced a functional receptor with a slightly altered sensitivity to GABA compared with the wild-type α1β3 receptor (α1β3, EC_{50}: 3.14 ± 0.23μM, n_H: 1.04 ± 0.08; $\alpha1\beta3^{H267A}$, EC_{50}: 4.48 ± 0.5μM, n_H: 1.02 ± 0.12; n = 6 - 8 oocytes; Fig. 4.1A). However the potency of Zn^{2+} on the $\alpha1\beta3^{H267A}$ was dramatically reduced compared to the wild-type receptor (α1β3, IC_{50}: 0.14 ± 0.02μM, n_H: 1.14 ± 0.21; $\alpha1\beta3^{H267A}$, IC_{50}: 20.05 ± 3.48μM, n_H: 0.54 ± 0.05; n = 5 oocytes; Fig. 4.1B). The observed 150–fold reduction in Zn^{2+} sensitivity is in accordance with previous studies (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). Histidine 107, located in the N-terminus of the β3 subunit, has been implicated in mediating low affinity Zn^{2+} inhibition in the $GABA_A$ β3 homomeric receptor (Dunne *et al.*, 1999a, b; Chapter 3). To determine whether this histidine is involved in regulating Zn^{2+} sensitivity in a heteromeric $GABA_A$ receptor complex, *Xenopus* oocytes were injected with $\alpha1\beta3^{H107A,\ H267A}$ cDNAs resulting in functional receptor expression. Equilibrium concentration-response curves for GABA were constructed and the GABA EC_{50} was moderately increased by the H107 mutation ($\alpha1\beta3^{H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM, n_H: 1.1 ± 0.12; n = 4 oocytes; Fig. 4.1A). Interestingly, the H107 mutation did not further reduce the potency of Zn^{2+} on $\alpha1\beta3^{H267A}$ receptors with the inhibition curve for Zn^{2+} antagonism of GABA-activated currents providing an IC_{50} of 17.12 ± 6μM (n_H: 0.58 ± 0.09; n = 4 oocytes; Fig. 4.1B). Therefore either $\beta3^{H107}$ is not involved in regulating Zn^{2+} inhibition in α1β3 receptors, a different amino acid on the β3 subunit is responsible, or a higher affinity Zn^{2+} binding site on the α1 subunit is masking the contribution of $\beta3^{H107}$.

4.2.2. The additional Zn^{2+} inhibition involves histidine residues.

The Zn^{2+} sensitivity of the $\alpha1\beta3^{H107A, H267A}$ receptor was further assessed over a range of external pH in order to ascertain the involvement of histidine residues, as H^+ will present as a competitor ion binding to imidazole groups on histidines. The IC_{50} concentration of Zn^{2+} (20μM; Fig. 4.1B) was applied in combination with an approximate EC_{50} concentration of GABA (5μM; Fig. 4.1A) and the inhibitory effect titrated at Ringer pH 5.4, 6.4, 6.9, 7.4 and 8.4 (Fig. 4.2.). The Zn^{2+} inhibitory effect was then calculated as a percentage of the control response to 5μM GABA (=100%) at each Ringer pH. This enabled the construction of a pH titration curve which provided a pK_a of 6.76 ± 0.03 (n = 4 oocytes; Fig. 4.3.). Therefore Zn^{2+} is interacting with an amino acid residue that, if it has a similar pK_a in the receptor protein as in solution, is most likely a histidine residue (pK_a 6.5). As Zn^{2+} externally modulates the $GABA_A$ receptor (Celentano *et al.*, 1991), histidine residues in the extracellular domains of the α1 and β3 subunits were considered the primary candidates for binding Zn^{2+}. Interestingly, the pH titration curve revealed that Zn^{2+} inhibition (at this IC_{50} concentration) reached a saturated point at approximately 60% inhibition despite increasing the pH beyond 7.4.

The above data demonstrate that H107 in the β3 subunit does not mediate Zn^{2+} inhibition when α1 and β3 are co-expressed. It is possible the tertiary structure of β subunits differs in β homomers and αβ receptors, thus an additional, compensatory histidine residue in the β3 subunit may be available for mediating Zn^{2+} inhibition in an αβ complex. Since α1β1 receptors also possess low affinity Zn^{2+} inhibition, the most likely candidate is $\beta3^{H119}$ as this is the only other external histidine also present in β1 subunits. Expression of $\alpha1\beta3^{H107A, H119A, H267A}$ subunits resulted in functional receptors, with EC_{50} values (6.75 ± 0.61μM; n_H: 1.16 ± 0.11; n = 4 oocytes) equivalent to $\alpha1\beta3^{H107A, H267A}$ receptors (8.08 ± 0.74μM). However the H119 substitution did not further reduce Zn^{2+} potency; construction of Zn^{2+} inhibition curves for antagonism of GABA-gated currents yielded an IC_{50} of 15.34 ± 4.88μM (n_H: 0.64 ± 0.14; n = 3 oocytes). These results imply that histidine residues in the α1 subunit are probably

important in mediating Zn^{2+} inhibition in the $\alpha1\beta3$ $GABA_A$ receptor, rather than histidine residues in the $\beta3$ subunit.

4.2.3. Contribution of histidines in the N- and C-terminal domains of the $GABA_A$ receptor $\alpha1$ subunit to Zn^{2+} potency.

Analysis of the amino acid sequence for the $GABA_A$ receptor $\alpha1$ subunit revealed eight external histidine residues. Seven of these are located in the N-terminal domain (H55 – H217) and one histidine resides at the C-terminus (H427; Fig. 4.4.). Comparison of amino acid sequences with other $GABA_A$, $GABA_C$ and glycine receptor subunits demonstrated that whilst H55 and H427 are unique to the $\alpha1$ subunit, the other histidines show varying degrees of conservation amongst the subunits (Fig. 4.5.). H101 is also present in $GABA_A$ receptor $\alpha2$, $\alpha3$, and $\alpha5$ subunits; H141 is conserved in $GABA_A$ $\alpha2$, $\alpha3$, and ε subunits; H150 is conserved within all $GABA_A$ receptor α subunits, $\gamma1$ and ε subunits; H215 is also found in $\alpha2$, $\alpha3$, $\alpha5$, and δ $GABA_A$ subunits and H217 is present in all α and the δ $GABA_A$ subunits and the α glycine receptor subunits. Most interesting is H109, which is entirely conserved throughout the $GABA_A$, $GABA_C$, and glycine receptor subunits and corresponds to H107 in the $\beta3$ subunit. This was considered to be the major candidate for affecting Zn^{2+} potency. Each of the $GABA_A$ receptor $\alpha1$ subunit external histidines was subjected to systematic mutation to alanine. In case a higher affinity Zn^{2+} binding site on the $\alpha1$ subunit is masking the contribution of $\beta3^{H107}$, each $\alpha1$ mutant subunit was co-expressed with the $\beta3^{H107A,\ H267A}$ mutant subunit. All of the mutant subunit combinations produced functional $GABA_A$ receptors when injected into *Xenopus* oocytes.

$\alpha1^{H109A}\beta3^{H107A,\ H267A}$ receptors were dramatically less sensitive to GABA than either $\alpha1\beta3^{H107A,\ H267A}$ mutant or $\alpha1\beta3^{wild\text{-}type}$ receptors (Fig 4.6A). Construction of GABA concentration- response curves indicated that the $\alpha1^{H109A}$ mutation laterally shifted the curve, increasing the GABA EC_{50} approximately 50-fold ($\alpha1^{H109A}\beta3^{\ H107A,\ H267A}$, EC_{50}: 389.77 ± 27.53μM, n_H: 0.89 ± 0.04; $\alpha1\beta3^{H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM, n_H: 1.1 ± 0.12; $\alpha1\beta3$, EC_{50}: 3.14 ± 0.23μM, n_H: 1.04 ± 0.08; n = 4 - 8 oocytes). Despite this reduction in GABA potency, the $\alpha1^{H109A}$ mutation did not alter Zn^{2+} sensitivity, and the

Zn^{2+} inhibition curves of the GABA–gated conductance (activated by an EC_{50} GABA concentration) overlapped ($\alpha1^{H109A}\beta3^{H107A,\ H267A}$, IC_{50}: 25.06 ± 3.65μM, n_H: 0.48 ± 0.04; $\alpha1\beta3^{H107A,\ H267A}$, IC_{50}: 17.12 ± 6μM, n_H: 0.58 ± 0.09; $\alpha1\beta3^{H267A}$, IC_{50}: 20.05 ± 3.48μM, n_H: 0.54 ± 0.05; n = 3 - 5 oocytes; Fig. 4.6B). Other potential candidates for mediating the Zn^{2+} inhibitory effect included H215 and H217, since these residues could form a recognized Zn^{2+} binding site, H-X-H, termed the short spacer (Vallee & Falchuk, 1993). If these residues formed such a site then mutation of either would be sufficient to disrupt Zn^{2+} binding. However, the $\alpha1^{H215A}$ mutation did not substantially affect the sensitivity of the receptor to GABA ($\alpha1^{H215A}\beta3^{H107A,\ H267A}$, EC_{50}: 5.23 ± 0.36μM; n_H: 1.3 ± 0.13; $\alpha1\beta3^{H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM, n_H: 1.1 ± 0.12; n = 4 oocytes; Fig. 4.7A). Similarly the $\alpha1^{H215A}$ mutation had little effect on the potency of Zn^{2+} ($\alpha1^{H215A}\beta3^{H107A,\ H267A}$, IC_{50}: 14.28 ± 5.52μM; n_H: 0.61 ± 0.1; $\alpha1\beta3^{H107A,\ H267A}$, IC_{50}: 17.12 ± 6μM, n_H: 0.58 ± 0.09; n = 4 oocytes; Fig. 4.7B).

Table 4.1. EC_{50} and n_H values for GABA concentration-response curves determined for wild-type and mutant α1 subunit-containing receptors.

Construct	EC_{50} (μM)	n_H	No. of oocytes
$\alpha1\beta3^{wild\text{-}type}$	3.14 ± 0.23	1.04 ± 0.08	8
$\alpha1\beta3^{H267A}$	4.48 ± 0.50*	1.02 ± 0.12	6
$\alpha1\beta3^{H107A,\ H267A}$	8.08 ± 0.74**	1.10 ± 0.12	4
$\alpha1^{H55A}\beta3^{H107A,\ H267A}$	3.20 ± 0.13	1.45 ± 0.07	4
$\alpha1^{H101A}\beta3^{H107A,\ H267A}$	4.92 ± 0.20	1.20 ± 0.06	3
$\alpha1^{H109A}\beta3^{H107A,\ H267A}$	389.8±27.5***	0.89 ± 0.04	4
$\alpha1^{H141A}\beta3^{H107A,\ H267A}$	2.43± 0.26	1.34 ± 0.15	5
$\alpha1^{H150A}\beta3^{H107A,\ H267A}$	6.30 ± 0.33	1.16 ± 0.08	3
$\alpha1^{H215A}\beta3^{H107A,\ H267A}$	5.23 ± 0.36	1.30 ± 0.13	4
$\alpha1^{H217A}\beta3^{H107A,\ H267A}$	1.94 ± 0.14**	1.15 ± 0.09	3
$\alpha1^{H427A}\beta3^{H107A,\ H267A}$	4.64 ± 0.39	1.40 ± 0.15	5

N.B. Statistical significance was assessed using an unpaired t-test: *P<0.03, **P<0.004, ***P<0.0001. '$\alpha1\beta3^{H267A}$ was compared to $\alpha1\beta3^{wild\text{-}type}$; $\alpha1\beta3^{H107A,\ H267A}$ was compared to $\alpha1\beta3^{H267A}$; $\alpha1^{H109A}\beta3^{H107A,\ H267A}$ and $\alpha1^{H217A}\beta3^{H107A,\ H267A}$ were compared to $\alpha1\beta3^{H107A,\ H267A}$.

GABA concentration-response curves were established for the remaining α1 histidine mutant constructs and the EC_{50}s determined (Table 4.1). Of the remaining α1 subunit

mutations, only $\alpha1^{H217A}$ generated a small, yet significant, change in the apparent affinity for GABA. In order to examine the contribution of these external α1 subunit histidines to Zn^{2+} potency, the inhibitory effect of two concentrations of Zn^{2+} (a near IC_{50} concentration: 10μM, and a near saturating concentration: 300μM) on the control EC_{50} GABA response was observed for each mutant receptor. The Zn^{2+} inhibitory effect was then calculated as a percentage of the control EC_{50} GABA response (=100%; Fig. 4.8A). The only α-subtype mutation to significantly affect the sensitivity of Zn^{2+} was $\alpha1^{H141A}$. The $\alpha1^{H141A}\beta3^{H107A, H267A}$ receptor was insensitive to 10μM Zn^{2+} (104 ± 2.65% of the control response; $\alpha1\beta3^{H107A, H267A}$, 52.66 ± 3.93% of the control response; n = 3 – 5 oocytes) and the inhibition evoked by 300μM Zn^{2+} was dramatically reduced (64.33 ± 10.09% of the control response; $\alpha1\beta3^{H107A, H267A}$, 12.33 ± 3.93% of the control response; n = 3 - 5 oocytes; Fig. 4.8). Therefore H141 in the N-terminus of the α1 subunit is clearly important in mediating Zn^{2+} inhibition of the Cl^- currents gated by the $\alpha1\beta3$ $GABA_A$ receptor.

4.2.4. Effect of the $\alpha1^{H141A}$ mutation on Zn^{2+} potency.

To further investigate the effect of the $\alpha1^{H141A}$ mutation on Zn^{2+} inhibition, the Zn^{2+}dose-inhibition relationship of $\alpha1^{H141A}\beta3^{H107A, H267A}$ receptors was determined at EC_{50} GABA . The H141A mutation caused a near 25-fold lateral displacement of the curve increasing the Zn^{2+} IC_{50} to 477.02 ± 41.4μM, (n_H: 1.16 ± 0.08;cf. $\alpha1\beta3^{H107A, H267A}$, IC_{50}: 17.12 ± 6μM, n_H: 0.58 ± 0.09; n = 3 - 5 oocytes; Fig. 4.9.). To determine the contribution of $\beta3^{H107}$ in mediating Zn^{2+} inhibition, $\alpha1^{H141A}\beta3^{H267A}$ subunits were coexpressed in *Xenopus* oocytes. The Zn^{2+} concentration-inhibition curves for $\alpha1^{H141A}\beta3^{H267A}$ and $\alpha1^{H141A}\beta3^{H107A, H267A}$ were coincident ($\alpha1^{H141A}\beta3^{H267A}$, IC_{50}: 439.98 ± 34.11μM, n_H: 1.16 ± 0.08; $\alpha1^{H141A}\beta3^{H107A, H267A}$, IC_{50}: 477.02 ± 41.4μM, n_H: 1.16 ± 0.08; n = 3 – 4 oocytes; Fig. 4.9.) suggesting that $\beta3^{H107}$ is not involved in regulating the Zn^{2+} modulation of the $\alpha1\beta3$ $GABA_A$ receptor.

4.2.5. Further Zn^{2+} binding sites?

Despite the large reduction in Zn^{2+} potency observed for the $\alpha1^{H141A}\beta3^{H267A}$ and $\alpha1^{H141A}\beta3^{H107A, H267A}$ receptors, application of higher concentrations of Zn^{2+} (300μM – 5mM) were still able to substantially inhibit the GABA-gated conductance (Fig. 4.9.). This implicates yet a further site of regulation. The identity of the residue(s) involved in this additional Zn^{2+} binding was investigated using external pH as a probe. GABA concentration-response curves for the $\alpha1^{H141A}\beta3^{H267A}$ receptor were determined in the absence and presence of 1mM Zn^{2+} at pH 7.4 and at pH 5.4. Application of Zn^{2+} (1mM) at pH 7.4 produced a mixed/non-competitive displacement of the GABA concentration-response curve. The GABA EC_{50} was increased (control: 2.33 ± 0.33μM, n_H: 1.24 ± 0.19; $+Zn^{2+}$: 5.38 ± 0.44μM, n_H: 1.4 ± 0.16; n = 3 - 5 oocytes;) and the maximum response decreased (+ Zn^{2+}: 35 ± 6% inhibition of the control response to 100μM GABA; n = 3 - 5 oocytes; Fig. 4.10A). However increasing H^+ concentration (Ringer pH 5.4) abolishes Zn^{2+} antagonism of the GABA-gated conductance, and both the GABA EC_{50} and maximum response are unaffected by application of 1mM Zn^{2+} (control: EC_{50}: 8.25 ± 0.78μM, n_H: 1.12 ± 0.15; $+Zn^{2+}$: EC_{50}: 10.39 ± 0.68μM, n_H: 1.29 ± 0.12, 9 ± 8% inhibition of the control response to 100μM GABA; n = 3 oocytes; Fig. 4.10B).

In order to further examine the pH dependence of the Zn^{2+} antagonism of $\alpha1^{H141A}\beta3^{H267A}$ receptors, Zn^{2+} (1mM) was co-applied with an approximate EC_{50} concentration of GABA (3μM) over a Ringer pH range of 5.4 - 8.4 (Fig. 4.11B, C). The Zn^{2+} inhibitory effect was then calculated as a percentage of the control response to 3μM GABA (=100%; n = 3 oocytes) at each Ringer pH. A pH titration curve was constructed for the data and provided a pK_a of 6.19 ± 0.12 (Fig. 4.11A). This pK_a is again in accordance with Zn^{2+} binding to a histidine residue (pK_a 6.5). It therefore appears that other histidine residues may be involved in mediating the inhibitory action of Zn^{2+}.

4.3. DISCUSSION

This study has identified a residue important for Zn^{2+} inhibition on the murine $GABA_A$ receptor α1 subunit, revealing the existence and potential identity of a lower affinity

site. There is considerable diversity in the location of regulatory sites for Zn^{2+} inhibition on ligand-gated ion channels with the N-terminal domain being the most common site of action. Zn^{2+} binds to N-terminal histidine residues in $GABA_c$ receptor ρ1 (Wang *et al.*, 1995b), glycine receptor α1 (Harvey *et al.*, 1999) and NMDA receptor NR2A (Choi & Lipton, 1999) subunits. Despite similar locations in the N-terminal domain, the binding sites determined for Zn^{2+} on the $GABA_C$ receptor ρ1 and glycine receptor α1 subunits are distinct. TM2 is also an important regulatory domain for Zn^{2+} inhibition. A histidine within this pore lining region (H267), previously thought only to be accessible to anions, has been identified as a high affinity binding site for Zn^{2+} on the $GABA_A$ receptor β subunit (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). Finally, the putative extracellular loop between TM2 and TM3 is important for the Zn^{2+} sensitivity of the $GABA_A$ receptor α6 subunit. Substitution of H273 in the α6 subunit with the asparagine found in the homologous location in the α1 subtype (N274) reduced the Zn^{2+} sensitivity whereas replacing N274 with a histidine in the α1 subunit produced α6-like sensitivity to Zn^{2+} (Fisher & Macdonald, 1998). Overall, histidine appears to be the favoured amino acid involved in the inhibitory action of Zn^{2+}. Furthermore, unlike zinc metalloenzymes (Vallee & Falchuk, 1993), an invariant binding motif does not appear to be important for coordinating the Zn^{2+} inhibition on ligand-gated ion channels since beyond the presence of a histidine residue, there is no similarity in the primary sequence around the determinants of Zn^{2+} regulation in the N-terminal domain, within TM2, and on the TM2 - TM3 extracellular loop. Thus, the structural requirements for Zn^{2+} binding to these proteins is unknown.

It is quite possible that residues from different subunits may combine to form Zn^{2+} binding pockets. Indeed, it has already been shown that the α1 subunit is able to interact with the β1 subunit to form a high affinity Zn^{2+} binding site. Serine 272 in TM2 of the α1 subunit was mutated to the histidine found in the equivalent position in the β1 subunit (H267) and previously identified as crucial for high affinity Zn^{2+} binding (Horenstein & Akabas, 1998). Coexpression of $\alpha1^{S272H}\beta1$ subunits resulted in a receptor with a significantly higher apparent affinity for Zn^{2+} than wild-type α1β1 receptors, suggesting that the aligned residues in the different subunits are in close proximity and able to coordinate Zn^{2+} inhibition. The contribution of putative Zn^{2+} binding residues in

the N-terminal domains of both α1 and β3 subunits was determined in order to investigate the regulation of Zn^{2+} inhibition in heteromeric α1β3 $GABA_A$ receptors.

4.3.1. H107 on the β3 subunit is not critical for Zn^{2+} sensitivity of α1β3 receptors.

The role of H107, located in the N-terminus of the β3 subunit, in mediating the residual Zn^{2+} inhibition observed for $\alpha1\beta3^{H267A}$ receptors was examined. This residue has been implicated in the low affinity Zn^{2+} inhibition of β3 homomeric $GABA_A$ receptors (Dunne *et al.*, 1999a, b; Chapter 3) and the aligned histidine in glycine α1 subunit receptors has been identified as a key residue for the Zn^{2+} inhibitory binding site of glycine α1 receptors (Harvey *et al.*, 1999). In addition, the low Zn^{2+} IC_{50} observed for β subunit homomers (Krishek *et al.*, 1996b; Wooltorton *et al.*, 1997b) implicates the β subunit as responsible for the high potency of Zn^{2+} on αβ receptors. Following mutagenesis studies, $\beta3^{H107}$ was also considered a prime candidate for regulating the Zn^{2+} inhibition of α1β3 $GABA_A$ receptor constructs. Surprisingly, mutation of this residue to alanine did not further reduce Zn^{2+} sensitivity of $\alpha1\beta3^{H267A}$ constructs and may reflect the fact that H107 is inaccessible for Zn^{2+} binding when α and β subunits coassemble, although this residue has not been identified so far as contributing to an 'assembly box' (Srnivasan *et al.*, 1999; Taylor *et al.*, 1999).

Metal ion binding does require the correct orientation of histidine side chains, achieved via the formation of hydrogen bonds with other side chains or backbone carbonyl and amide groups (Higaki *et al.*, 1992; Regan, 1993). Typically, the interacting atoms involved in Zn^{2+} binding are held firmly in the plane of the imidazole ring of the histidine residue (Christianson & Alexander, 1989). Assuming H107 mediates low affiinty zinc inhibition of β3 homomers, it is possible that in an αβ complex, hydrogen bond formation may be disrupted in the region of $\beta3^{H107}$ thus preventing the correct positioning of this residue and subsequent binding of Zn^{2+}. Alternatively, the distance between these histidine residues in an αβ $GABA_A$ receptor complex may be too great for Zn^{2+} binding. Studies of metalloproteins have revealed the maximum separation of the α-carbons of two histidine residues bound to Zn^{2+} must be less than 13Å (Higaki *et*

al., 1992). Diversity in the tertiary structures of the N-terminal domains of β3 and α1β3 $GABA_A$ receptor complexes may result in different distances of separation between histidines 107 and whatever residues other residues form the binding site in β3 homomers. Mutation of H107, alone or in conjunction with other histidine mutations, is not tolerated in β3 homomers suggesting a putative structural role in protein folding (Chapter 3). Since substitution of $\beta3^{H107}$ with alanine resulted in functional receptors when coexpressed with α1 subunits, it appears that there are differences in the tertiary structure of β3 homomeric and αβ heteromeric $GABA_A$ receptors. Therefore when α and β subunits coassemble, $\beta3^{H107}$ may be unable or unnecessary to stabilise the αβ subunit receptor conformation and could be incapable of forming part of a Zn^{2+} binding site. Alternatively, the contribution of H107 to the Zn^{2+} sensitivity may be masked by a higher affinity Zn^{2+} binding site on the α1 subunit.

4.3.2. Importance of H141 on the α1 subunit in mediating Zn^{2+} inhibition.

Construction of a pH titration curve for the Zn^{2+} inhibition of $\alpha1\beta3^{H107A,\ H267A}$ receptors produced a pK_a of 6.76 ± 0.03, implicating a histidine residue(s) as a Zn^{2+} binding ligand. On the β3 subunit, H119 is a possible candidate since this residue is also present on β1 subunits, and α1β1 receptors demonstrate similar low affinity inhibition by Zn^{2+} (Horenstein & Akabas, 1998). However, mutation of $\beta3^{H119}$ did not reduce Zn^{2+} potency in α1β3 constructs, which is in accordance with data obtained from β3 homomers (Chapter 3). Therefore, a histidine in the external domain of the α1 subunit was concluded to be responsible for the inhibitory action of Zn^{2+}. The separate mutations of the eight external α1 subunit histidines and subsequent coexpression with $\beta3^{H107A,\ H267A}$ subunits, resulted in functional receptor expression. However only one mutation, $\alpha1^{H141A}$, altered the Zn^{2+} sensitivity and greatly increased the Zn^{2+} IC_{50} (~ 25-fold).

H141 may reduce Zn^{2+} sensitivity through various mechanisms. This histidine may conceivably form: part of a Zn^{2+} binding site; allosterically modify the properties of a remote binding site by altering receptor conformation; or regulate the transduction pathway via which Zn^{2+} binding inhibits GABA-gated conductance. The hypothesis that $\alpha1^{H141}$ coordinates with Zn^{2+} appears the most likely. Mutating $\alpha1^{H141}$ did not influence

the gating effects of GABA (Table 4.1). In addition, increasing external H^+ concentration abolished the inhibition by Zn^{2+}, implying competition at the same binding site. This site is not masking a lower affinity site consisting of $\beta3^{H107}$ since Zn^{2+} inhibition curves constructed for the antagonism of the GABA-gated conductances of both $\alpha1^{H141A}\beta3^{H107A,\ H267A}$ receptors and $\alpha1^{H141A}\beta3^{H267A}$ receptors were coincident.

It is possible that $\alpha1^{H141}$ forms a single Zn^{2+} binding site with $\beta3^{H267}$. $\alpha1^{H141}$ may contribute to stabilising the binding of Zn^{2+} to $\beta3^{H267}$, therefore explaining the smaller shift in Zn^{2+} potency observed in $\alpha1\beta3$ receptors compared to $\beta3$ homomeric receptors (Wooltorton *et al.*, 1997a). There is however no tertiary structural evidence to support this possibility and although $\alpha1^{H141}$ and $\beta3^{H267}$ lie on different subunits, the relative number of residues between them does not correspond to any previously determined consensus for Zn^{2+} binding sites (Vallee & Falchuk, 1993). It is suggested that at least two histidine residues are required for high affinity Zn^{2+} binding in the $\alpha1\beta1$ receptor (Horenstein & Akabas). However despite controversy over receptor stoichiometry and subunit number, at least two β subunits are present in an $\alpha\beta$ receptor (Im *et al.*, 1995; Kellenberger *et al.*, 1996; Gorrie *et al.*, 1997; Tretter *et al.*, 1997), which would be sufficient to form an independent high affinity Zn^{2+} binding site. Since Zn^{2+} binding sites are formed by a minimum of two ligands (Vallee & Falchuk, 1993), the presence of at least two $\alpha1$ subunits in an $\alpha1\beta3$ receptor would allow the formation of a second discrete Zn^{2+} binding site. In addition, an aspartate in the equivalent position to $\alpha1^{H141}$ in the β subunits could complete a Zn^{2+} binding pocket for $\alpha\beta$ receptors.

H141 is located within the cysteine loop, a highly conserved region of the N-terminal domain. The cysteine loop consists of two disulphide-bonded cysteine residues separated by 13 amino acids. The significant amino acid homology between $GABA_A$, $GABA_C$, glycine and nicotinic acetylcholine receptor subunits has implicated this region as important for channel function. Early modelling studies suggested that this structural motif could form the agonist binding site of GABA-, glycine- and nicotinic acetylcholine-gated ion channels (Smart *et al.*, 1984; Luyten, 1986; Cockcroft *et al.*, 1990). However this is not supported by a wealth of more recent data. Mutation of amino acids within the cysteine loop, or alteration of the redox state of the receptor had

little effect on GABA sensitivity (Amin *et al.*, 1994; Amato *et al.*, 1999). Indeed, preventing the cysteine loop forming appeared to preclude receptor subunit expression in both $GABA_A$ and nACh receptors (Amin *et al.*, 1994). A similar study using site-directed mutagenesis concluded that the cysteine loop did not form the agonist binding site on the glycine α1 receptor (Vandenberg *et al.*, 1993), instead implying a role in receptor assembly and in antagonist binding. Furthermore, evidence now suggests that this region does not constitute the agonist binding site in nicotinic acetylcholine receptors (Criado *et al.*, 1986). Mutating H141 in the $GABA_A$ receptor α1 subunit did not significantly affect GABA sensitivity; consistent with the present view that this region does not form the agonist binding site in ligand-gated channels.

4.3.3. Further candidates for mediating Zn^{2+} inhibition

Despite mutating the two identified putative Zn^{2+} binding sites, application of Zn^{2+} (300μM–5mM) still substantially blocked the GABA-gated conductance of $\alpha1^{H141A}$ $\beta3^{H267A}$ receptors.. This residual inhibition may be due to other lower affinity Zn^{2+} binding sites or incomplete disruption of Zn^{2+} binding to one (or both) of the identified binding sites. Lowering the Ringer pH prevented Zn^{2+} antagonism of the GABA-gated conductance of the $\alpha1^{H141A}\beta3^{H267A}$ receptor, and construction of a pH titration curve for this inhibition produced a pK_a of 6.19 ± 0.12, implying a further contribution of histidine residues in mediating Zn^{2+} inhibition.

Introduction of the $\beta3^{H267A}$ and $\alpha1^{H141A}$ mutations affects the Hill coefficents of the Zn^{2+} concentration-inhibition curves. Wild-type α1β3 receptors have a Hill coefficient of 1.14 ± 0.21 whilst $\alpha1\beta3^{H267A}$ receptors generate a reduced Hill coefficient of 0.54 ± 0.05. Zn^{2+} antagonism of $\alpha1^{H141A}\beta3^{H267A}$ receptors resulted in a Hill coefficient of 1.16 ± 0.08, similar to the wild-type value, indicating that probably only one Zn^{2+} ion is required to inhibit the receptor. This difference in Hill coefficients may reflect that the different binding sites may have different requirements for Zn^{2+} binding in order to effect receptor inhibition; however altered Hill coefficients can also be related to

changes in channel gating rather than effects on binding sites (see Chapter 3 for discussion).

Substitution of the other seven external histidines in the α1 subunit did not unduly effect Zn^{2+} potency. However it is conceivable that the contribution of one (or more) of these residues to a lower affinity Zn^{2+} binding site is not discernible when a higher affinity Zn^{2+} binding site, H141 is present. Alternatively, since the affinity of a Zn^{2+} binding site depends on the number of complexing residues and their relative positions (Higaki *et al.*, 1992; Regan, 1993; Berg & Shi, 1996) one (or more) of these histidines may stabilise the binding of Zn^{2+} at H141. The H-X-H arrangement of H215 and H217 is a common Zn^{2+} binding motif termed the short spacer (Vallee & Falchuk, 1993). This motif is able to form a bidentate Zn^{2+} complex, and has recently been identified as a Zn^{2+} binding site in the N-terminus of both glycine α1 receptors (H107 and H109; Harvey *et al.*, 1999) and NMDA NR2A receptors (H42 and H44; Choi & Lipton, 1999). It is possible that H215 and H217 form a previously undisclosed lower affinity Zn^{2+} binding site, although the mutation of either histidine, had no effect on Zn^{2+} potency and a histidine aligned to H217 had no effect on Zn^{2+} inhibition of glycine α1 receptors (Harvey *et al.*, 1999). H101 is in the adjacent position to H156 in $GABA_C$ ρ1 subunits, previously determined to be a high affinity Zn^{2+} binding site (Wang *et al.*, 1995b). Histidine 101 in the $GABA_A$ receptor α1 subunit has been previously identified as a major determinant for high affinity binding of benzodiazepine (BZ) agonists (Wieland *et al.*, 1992; Kleingoor *et al.*, 1993). In addition, substitution of this histidine did not influence the GABA sensitivity of the α1β2γ2 receptor complex (Kleingoor *et al.*, 1993), confirmed by the observation that the $\alpha1^{H101A}$ mutation did not alter the GABA potency on α1β3 receptors in this study. Since Zn^{2+} and BZ ligands are considered to act at discrete sites (Legendre & Westbrook, 1991; Celentano *et al.*, 1991; Smart, 1992) it is unlikely that H101 plays any significant role in Zn^{2+} antagonism. Histidine 150, located only nine residues C-terminal to H141, may stabilise the conformation of the Zn^{2+} binding site via the formation of hydrogen bonds with H141, thus increasing the affinity of the site (Vallee & Falchuk, 1993). Furthermore, this residue is present in all $GABA_A$ receptor α subunits and so may represent a common Zn^{2+} binding site.

H109 is another possible candidate for this residual Zn^{2+} sensitivity. The aligned histidine in glycine α1 receptors regulates both inhibition and potentiation by Zn^{2+}(Harvey *et al.*, 1999). Interestingly, the H109A substitution produced a 50-fold shift in GABA potency ($\alpha1^{H109A}\beta3^{H107A,\ H267A}$, EC_{50}: 389.77 ± 27.53μM; n_H: 0.89 ± 0.04; α1 $\beta3^{H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM; n_H: 1.1 ± 0.12; n = 3 - 5 oocytes). The GABA binding site has been proposed to lie at the interface between the α and β subunits (Galzi & Changeux, 1994; Smith & Olsen, 1995). The mutation $\alpha1^{F64L}$ produces a 200-fold increase in GABA EC_{50} (Sigel *et al.*, 1992). Phenylalanine 64, arginine 66 and serine 68 are suggested to form a β strand and line part of the GABA binding site on the α1 subunit (Boileau *et al.*, 1999). Two separate domains (Y157 and T160; T202 and Y205) have been identified as important for GABA binding on the β subunit (Amin & Weiss, 1993). Although conservative substitutions of these residues resulted in a 50-fold reduction in GABA potency, non-conservative mutations abolished the GABA response (#200mM GABA). The entirely conserved H109 may therefore form part of a novel binding domain for GABA on the α1 subunit. Alternatively, H109 may allosterically stabilise the previously determined binding site or affect the transduction pathway between GABA binding to extracellular N-terminal sites and the channel gate, located close to the cytoplasmic end of the channel (Xu & Akabas, 1996). Interestingly, in both $GABA_A$ receptor β3 (Dunne *et al.*, 1999a, b; Chapter 3) and $GABA_C$ receptor ρ1 (Wang *et al.*, 1995b) subunit homomers, mutation of this conserved histidine resulted in a complete loss of function.

4.3.4 Possible involvement of H141 in the Zn^{2+} antagonism of other $GABA_A$ receptor subunits

Since α2 and α3 subunits share H141, it is possible that this residue is in part responsible for the Zn^{2+} sensitivity of receptors containing these subunits. However, the maximal Zn^{2+} block of the GABA-gated current is larger in both α2- and α3- containing receptors than in α1 subunit-containing receptors (maximal inhibition: 51, 53 and 19% respectively) although the IC_{50} value for α1 subunit-containing receptors was the smallest (~6-fold; White & Gurley, 1995). This may either be due to an additional Zn^{2+} binding site that is not present on the α1 subunit, an increased affinity for the binding

site or an improved allosteric inhibitory effect. The number and position of chelating residues and the local electrostatic environment can affect the affinity of a Zn^{2+} binding site (Higaki *et al.*, 1992; Regan, 1993; Berg & Shi, 1996). Although the amino acid sequence surrounding H141 is virtually identical in these subunits, there is an additional histidine located six residues N-terminal to H141 in the α3 subunit which may contribute to the increased inhibitory effect of Zn^{2+}. However, this additional histidine is not present in α2 subunits that demonstrate a similar sensitivity to Zn^{2+}, further highlighting the diversity in the sites of action for Zn^{2+} on different receptor subunits.

As discussed previously, H273 in the TM2-TM3 loop has been identified as responsible for the increased sensitivity of α6 subunit-containing receptors compared with α1 subunit-containing receptors which have an asparagine in the homologous position (Fisher & Macdonald, 1998). They observed that $\alpha6^{H273N}\beta3\gamma2L$ receptor currents had the reduced sensitivity of α1β3γ2L receptor currents and hypothesised that the homologous histidine in α4 was also responsible for the increased Zn^{2+} sensitivity of receptors containing α4 subunits. However, since H141 is not present in α6 or α4 subunits, the residual inhibition by Zn^{2+} is due to the contribution from a different site than in α1 subunits. In addition, α5 subunit-containing receptors possess an increased sensitivity to Zn^{2+} (Burgard *et al.*, 1996), although like α1subunit-containing receptors, they contain an asparagine at position 273. Furthermore, α5 subunits contain a glutamine residue at position 141, implying a novel Zn^{2+} binding site for this receptor subunit.

4.3.5. Zn^{2+} sensitivity of $GABA_A$ receptors consisting of three (or more) different subunits.

Coexpression of a third subunit with αβ subunits reduces the potency of Zn^{2+}. It is possible that only one β subunit exists in a tertiary complex (Backus *et al.*, 1993), which may preclude the formation of a high affinity Zn^{2+} binding site. However, more recent studies have inferred a 2α:2β:1γ stoichiometry (Im *et al.*, 1995; Chang *et al.*, 1996; Tretter *et al.*, 1997, Farrar *et al.*, 1999). In addition, the nature of the third subunit

affects the degree of reduction in Zn^{2+} sensitivity. Inclusion of a δ subunit produced a small reduction (14 – 20-fold; Thompson *et al.*, 1997; Krishek *et al.*, 1998), the ε subunit produced a moderate reduction (175-fold; Whiting *et al.*, 1997) and inclusion of a γ subunit generated a dramatic reduction in the potency of Zn^{2+}(> 600 –1000-fold; Whiting *et al.*, 1997; Krishek *et al.*, 1998).

A variation in amino acid sequence at the extracellular end of TM2 may be responsible for the reduction in Zn^{2+} potency. In β subunits there is a glutamate (E270), only three residues extracellular to H267, which could contribute to the high affinity Zn^{2+} inhibition. This glutamate is in the aligned position to H273, responsible for Zn^{2+} inhibition on the α6 subunit (Fisher & Macdonald, 1998). The γ and ε subunits contain a positively charged lysine (K) in this position, which would be expected to repel cation binding. Furthermore, the δ subunit has a serine residue in this position which would not be predicted to affect Zn^{2+} binding. However, the $\gamma2^{K285A}$ mutation did not alter the relative insensitivity to Zn^{2+} of α1β1γ2 receptors (S. J. Moss & T. G. Smart, unpublished observations reported in Smart *et al.*, 1994). Interestingly, co-expression of the δ subunit with α1β2γ2L subunits increased the susceptibility of these receptors to block by Zn^{2+} (Saxena & Macdonald, 1994) although the Zn^{2+} sensitivities of α1β1γ2S and α1β1γ2Sδ receptors were comparable (Krishek *et al.*, 1998). Inclusion of a third subunit type may sterically hinder the other subunits from adopting the conformation that binds Zn^{2+}. Alternatively Zn^{2+} may still bind to the receptor complex but the presence of an additional subunit could restrict the conformational change induced by Zn^{2+} in an αβ receptor and the degree of restriction would depend on the nature of the third subunit.

In addition to altered sensitivity, the mechanism of Zn^{2+} antagonism is also influenced by the γ subunit . Zn^{2+} non-competitively inhibits αβ receptors (Draguhn *et al.*, 1990; Smart *et al.*, 1991) whereas αβγ receptors exhibit apparent competition (Chang *et al.*, 1995) and a state-dependent mechanism for Zn^{2+} antagonism has recently been suggested (Gingrich & Burkat, 1998). Zn^{2+} appeared to selectively inhibit resting α1β2 receptors yet selectively inhibit ligand-exposed α1β2γ2 receptors. To account for the variety in the mechanism of Zn^{2+} antagonism, a general model of inhibition was

proposed in which Zn^{2+} binds to a single extracellular site that allosterically induces two non-conducting states. Thus the Zn^{2+} binding site affinity was suggested to be state dependent and this state dependence is determined by the γ subunit (Gingrich & Burkat, 1998).

4.3.6. Effects of $\alpha1^{H109}$ on GABA potency

A potentially significant finding of the present study was that mutation of $\alpha1^{H109}$ reduced GABA potency 50-fold. To date, this region of the α subunit has not been implicated in contributing to the agonist binding site, however residue 109 lies between two important determinants of $GABA_A$ receptor pharmacology, $\alpha1^{H101}$ and $\alpha1^{R119}$. Mutation of H101 has a profound influence on benzodiazepine pharmacology (Wieland *et al.*, 1992; Kleingoor *et al.*, 1993; Zhang *et al.*, 1995; McKernan *et al.*, 1998) while mutation of R119 (or R120 in bovine α1 subunits) has recently been shown to greatly reduce GABA potency (180-fold) and abolish the binding of the GABA analogue [^{3}H]muscimol and the competitive antagonist [^{3}H]SR 95531 (Westh-Hansen *et al.*, 1999).

The tertiary structure of the N-terminal domain of GABA, Glycine and nACh receptors appear to be well conserved. It is clear that each subunit contributes more than one region of its N-terminal domain to the ligand binding site and the known determinants of agonist pharmacology align very closely even between pharmacologically distinct receptors (Smith & Olsen , 1995; Galzi & Changeux, 1995). In nACh receptors, 3 such regions have been found in the α subunit (termed loop A, B and C) and 3 in the δ,γ and ε subunits (loops D, E and the pre-disulphide region) (Galzi & Changeux, 1995; Prince & Sine, 1996). In $GABA_A$ receptors, loops B and C are found on the β subunit (Y157, T160 and T202, Y205 respectively; Amin & Weiss, 1993) while F64 corresponds to loop D on the α subunit (Sigel *et al.*, 1992; Smith & Olsen, 1994). Interestingly the benzodiazepine binding site appears to have evolved from a GABA binding site as loops A, B and C lie on the α subunit, indeed α1H101 corresponds exactly with loop A in nACh α subunits. Similarly F77 (Buhr *et al.*, 1996) on the γ2 subunit corresponds to loop D, while $\gamma2^{M130}$ (Buhr & Sigel, 1997; Wingrove *et al.*, 1997) and $\gamma2^{T142}$ (Mihic *et*

al., 1994) aligns closely to $\alpha1^{R119}$ and determinants of agonist and antaognist binding in the pre-disulphide region of δ,γ and ε subunits of nACh receptors (Fig. 4.6; FIG 4.12). Given these similar distributions it is possible that residues around $\alpha1^{R119}$ may contribute to the GABA binding site. Furthermore, the equivalent of H109 lies between two residues that greatly affect the interactions of glycine receptors with competitive antagonists (Schmieden *et al.*, 1999). What role $\alpha1^{H109}$ has in GABA, and possibly benzodiazepine, binding remains to be determined but it could serve to stabilise the structure of the receptor around this crucial region perhaps through hydrogen bonding.

4.3.7. Summary

Injection of $\alpha1\beta3^{H267A}$ subunit cDNAs into *Xenopus* oocytes, resulted in functional GABA-gated receptors which demonstrated a 150-fold reduction in Zn^{2+} potency, although these receptors remained susceptible to Zn^{2+} antagonism. A similar reduction in Zn^{2+} sensitivity was observed for $\alpha1\beta3^{H107A,\ H267A}$ mutant receptors. Lowering the Ringer pH to 5.4 entirely abolished this additional Zn^{2+} sensitivity and construction of a pH titration curve for the Zn^{2+} antagonism of the GABA-gated conductance produced a pK_a of 6.76 ± 0.03, implicating the involvement of histidine residues. The α1 subunit is responsible for mediating the majority of this lower affinity Zn^{2+} inhibition. The subsequent introduction of the $\alpha1^{H141A}$ mutant produced a further 25-fold reduction in Zn^{2+} potency. This histidine may directly bind Zn^{2+} and this site could be common to other $GABA_A$ receptor subunits. This study therefore represents the first description of a residue on the α1 subunit, or the N-terminal domain of any α subunit, as important for regulating Zn^{2+} antagonism. This study also identified the existence and possible nature of the lower affinity Zn^{2+} inhibition. Mutation of H141 revealed a residual Zn^{2+} sensitivity, also influenced by H^+ and with a pK_a of 6.19 ± 0.12. Therefore further histidine residues are likely to be involved either as discrete Zn^{2+} binding sites or by allosterically enhancing Zn^{2+} binding to either $\alpha1^{H141}$ or $\beta3^{H267}$. In addition this study also revealed a residue in the α1 subunit, H109, that strongly influences GABA potency and could conceivably influence the structure of a previously unknown component of the GABA binding site.

Figure 4.1. Mutating $\beta3^{H107}$ has a minimal effect on GABA and Zn^{2+} sensitivity of $\alpha1\beta3^{H267A}$ receptors. (A) GABA concentration-response curves were constructed for $\alpha1\beta3$, $\alpha1\beta3^{H267A}$ and $\alpha1\beta3^{H107A,\ H267A}$ $GABA_A$ receptor constructs. Data were normalized to the maximum GABA conductance for each cell. Data shown are mean ± s.e.m. (n = 6 - 8 oocytes) and fitted using the equation described in the methods (Chapter 2). (B) Concentration – inhibition curves were constructed by expressing the inhibition by Zn^{2+} as a percentage of the control response to a near EC_{50} concentration of GABA (2μM or 5μM) obtained in the absence of Zn^{2+} for each cell. Data points represent mean ± s.e.m. from 5 oocytes. The curves describe inhibition equation fits to the experimental data (see Chapter 2).

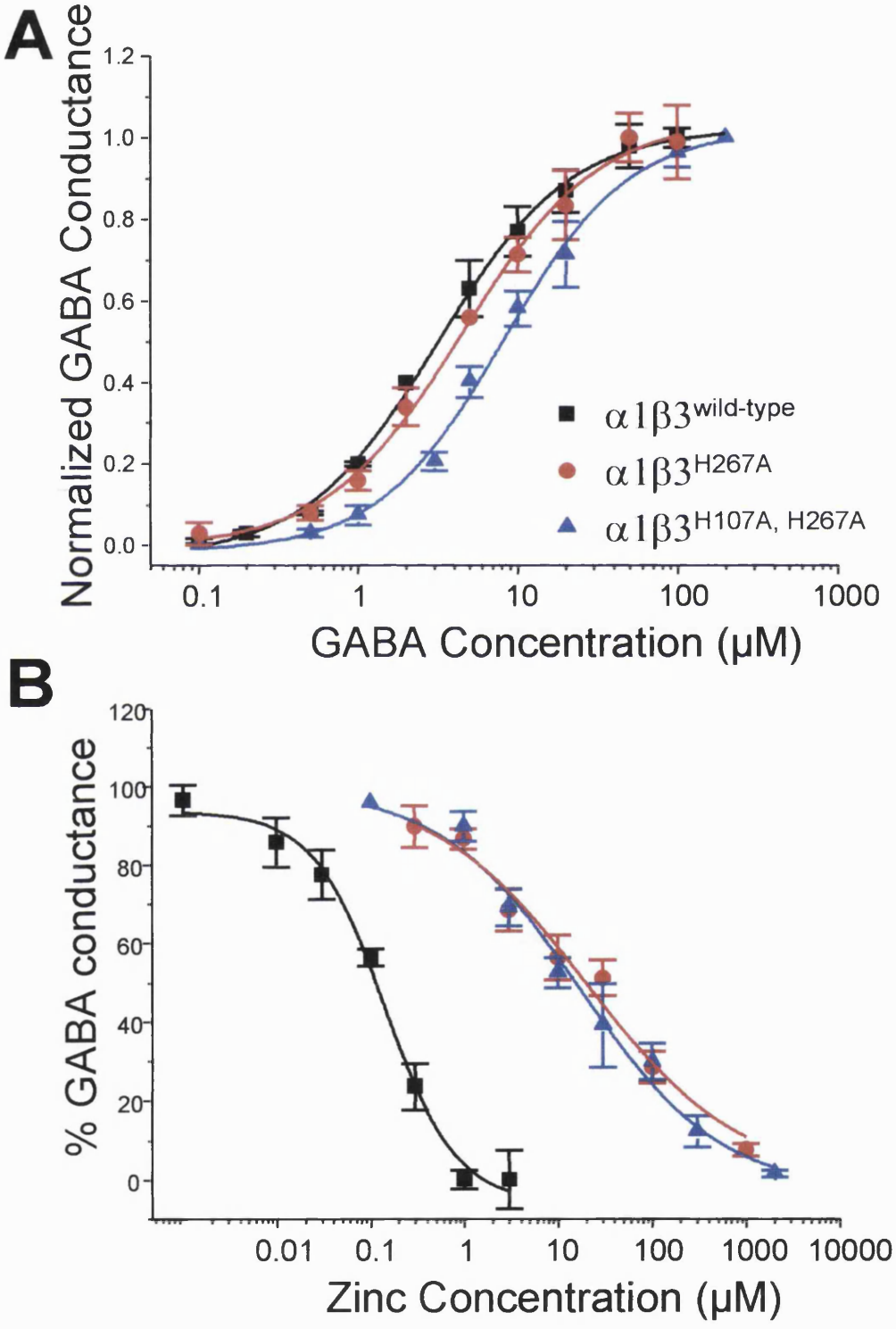
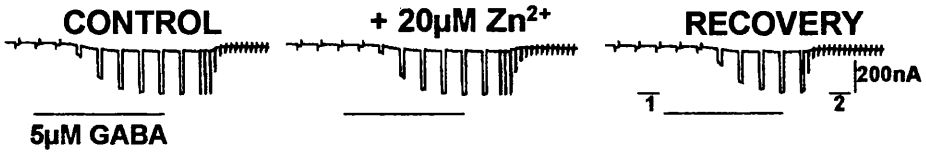
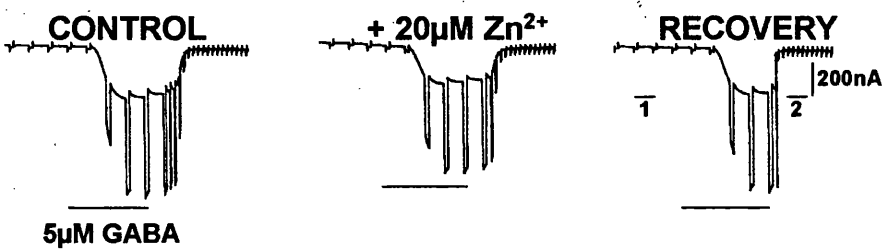

A
Normalized GABA Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
GABA Concentration (μM)
α1β3wild-type
α1β3H267A
α1β3H107A, H267A
B
% GABA conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

Figure 4.2. Zn^{2+} antagonism of $\alpha1\beta3^{H107A, H267A}$ $GABA_A$ receptors is sensitive to H^+. Representative whole-cell recordings evoked by 5μM GABA in the absence and presence of 20μM Zn^{2+} from oocytes expressing $\alpha1\beta3^{H107A, H267A}$ receptor constructs over a Ringer pH range from 5.4 to 8.4. The transient downward deflections represent current pulses induced by brief hyperpolarizing voltage commands (-10mv, 1s, 0.2 Hz) applied to the holding potential of –40mV. Zn^{2+} was pre-applied for 2 minutes then co-applied with GABA. Recovery of the GABA response was observed after 5 minutes. Note the different time calibrations on recovery from GABA (1: 5s; 2: 12.5s; 3: 50s). Reducing the H^+ concentration dramatically increased the Zn^{2+} sensitivity (pH 5.4: + Zn^{2+}, 101.5 ± 1.5% of control GABA response; pH 8.4: + Zn^{2+}, 36.7± 2.33% of control GABA response, n = 4 oocytes) implying a common site of action for these ions.

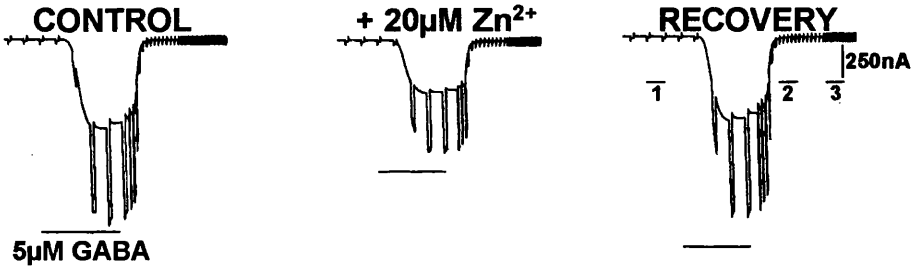
pH 5.4



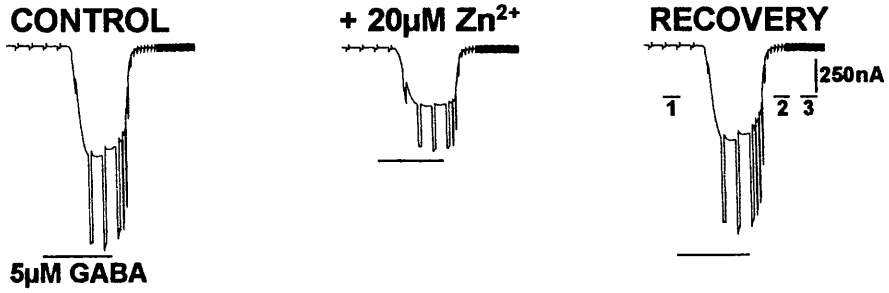
pH 6.4



pH 6.9



pH 7.4



pH 8.4

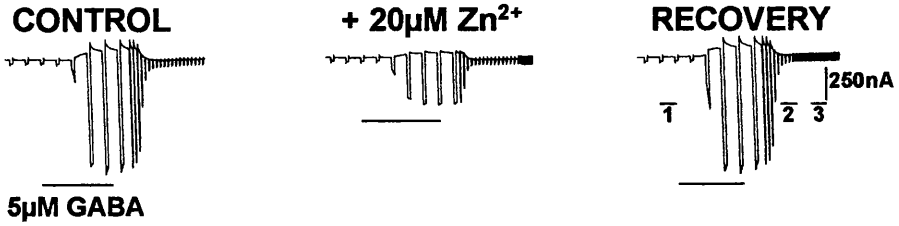


Figure 4.3. Evidence for the involvement of histidine residues mediating Zn^{2+} sensitivity. Whole-cell membrane conductances evoked by 5μM GABA were determined in the absence and presence of 20μM Zn^{2+} over the external Ringer pH range 5.4 to 8.4. As described in the previous figure, the inhibitory effect of Zn^{2+} was calculated as a percentage of the control GABA response in the absence of Zn^{2+} at each pH. The data are presented in the form of a pH titration relationship and represent mean ± s.e.m. from n = 4 oocytes. Curve fitting was performed as described in the methods (Chapter 2), and generated a pK_a of 6.76 ± 0.03 implicating the involvement of histidine(s).

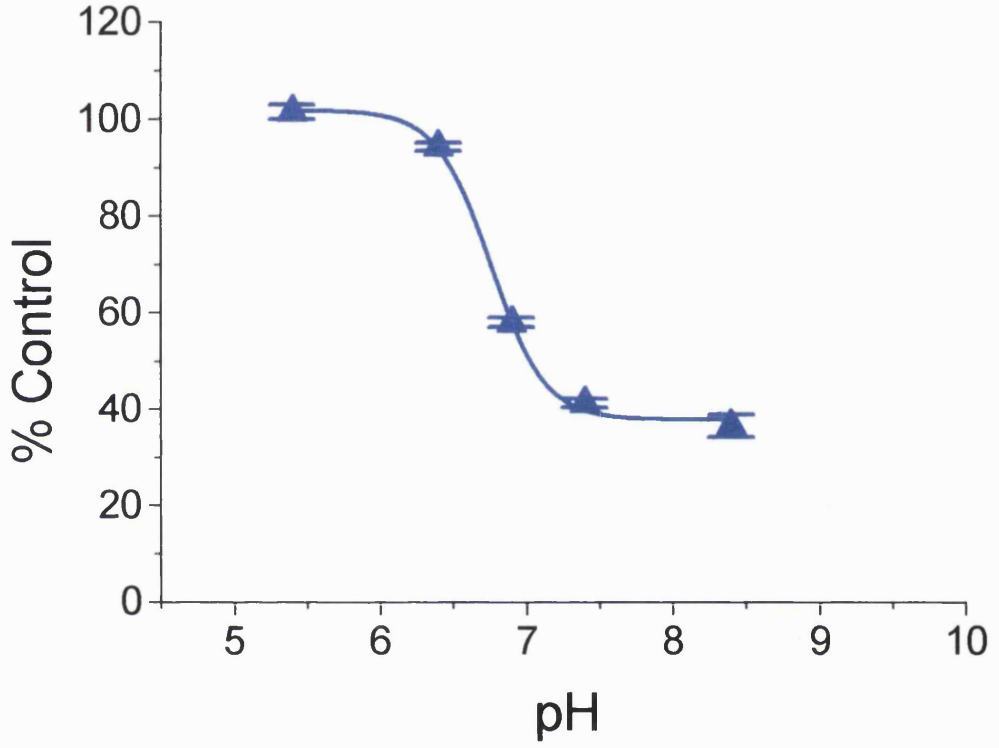

120
100
80
60
40
20
0
% Control
5
6
7
8
9
10
pH

Figure 4.4. Location of external histidine residues in the α1 subunit in relation to histidines implicated in Zn^{2+} binding domains on other GABA ionotropic receptor subunits. Schematic diagram of the $GABA_A$ receptor α1 subunit portraying the four transmembrane (TM) domains in addition to the intracellular and extracellular loop domains. The eight extracellular histidine residues present in the α1 subunit are highlighted (purple). Histidine residues previously demonstrated to be important in Zn^{2+} antagonism of $GABA_A$ and $GABA_C$ receptors are illustrated (red) in addition to the subunit involved and the sequence number of the identified residue. The two highly conserved cysteine residues forming the putative disulphide bridge are also indicated (yellow). Data obtained from Rabow *et al.*, 1995; Wang *et al.*, 1995b; Wooltorton *et al.*, 1997a; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998.

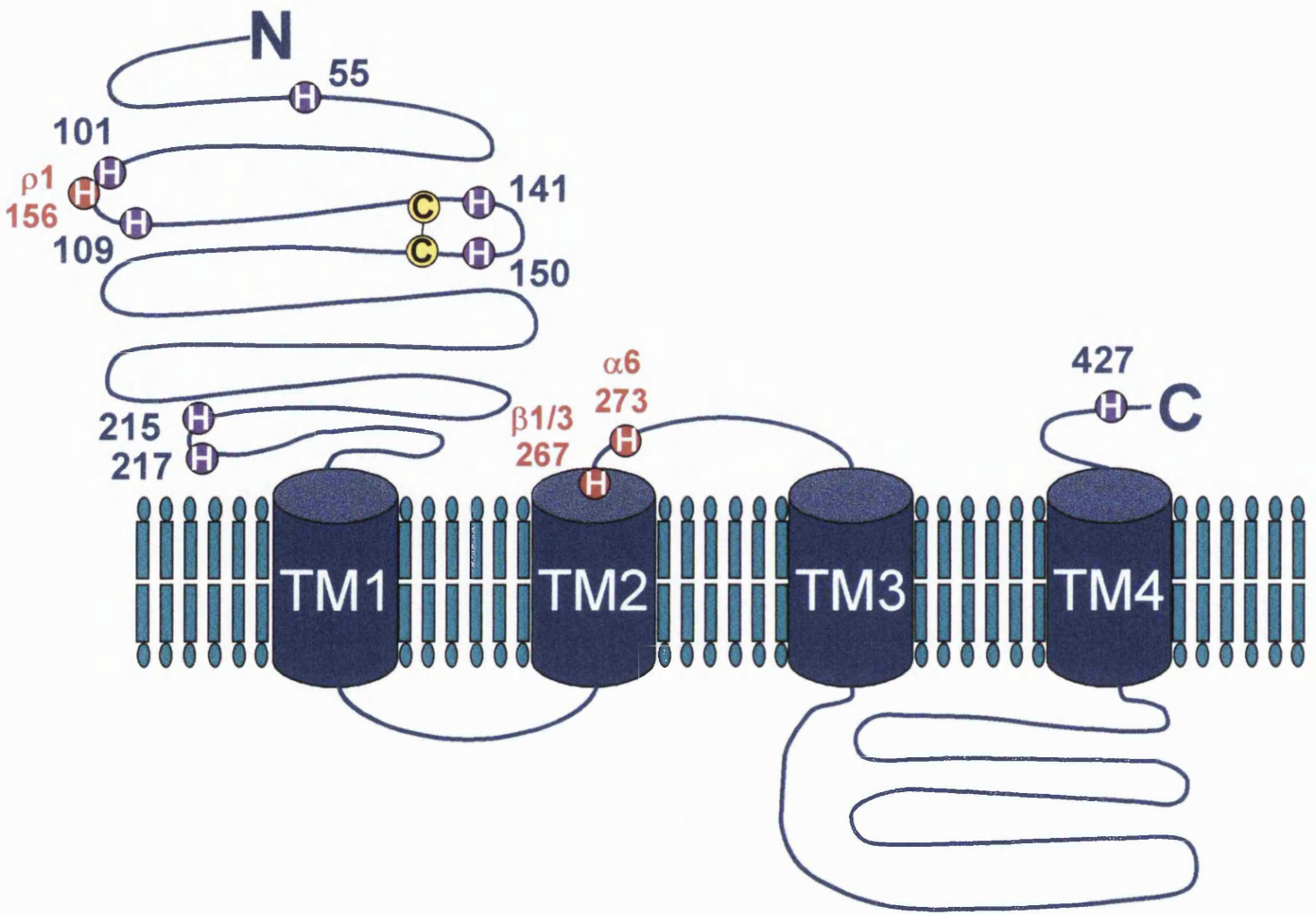

N
55
101
ρ1
156
109
141
150
215
217
α6
273
β1/3
267
427
C
TM1
TM2
TM3
TM4

Figure 4.5. Comparison of N-terminal amino acid residues on the $GABA_A$ receptor α1 subunit with other GABA and glycine ionotropic receptor subunits. Alignment of N-terminal amino acid sequences of rat $GABA_A$, $GABA_C$ and glycine (Gly) receptor subunits. The first 14 residues of the mature ρ1 subunit are not shown. Histidine residues are marked blue; those mutated in the α1 subunit are marked red and numbered. The putative cysteine loop and start of TM1 are indicated. Known determinants of receptor pharmacology and assembly are shown as follows: Agonist/antagonist X, Benzodiazepine X, Zinc X, Assembly X. References: Agonists; Sigel *et al.*, 1990; Amin & Weiss, 1993; Amin *et al.*, 1994; Boileau *et al.*, 1999; Rajendra *et al.,* 1995; Schmieden *et al.*, 1992, 1993, 1999; Vafa *et al.,* 1999; Vandenberg *et al.,* 1992a, b; Westh-Hansen *et al.,* 1999; Benzodiazepines: Buhr *et al.*, 1996, 1997a, 1997b; Buhr & Sigel, 1997; Wieland *et al.*, 1992; Pritchett & Seeburg, 1991; Wingrove *et al.*, 1997; Amin *et al.*, 1997; Mihic *et al.*, 1995; McKernan *et al.*, 1998; Zinc: Wang *et al.*, 1995b; Harvey *et al.*, 1999; Assembly: Srnivasan *et al.*, 1999; Taylor *et al.*, 1999.

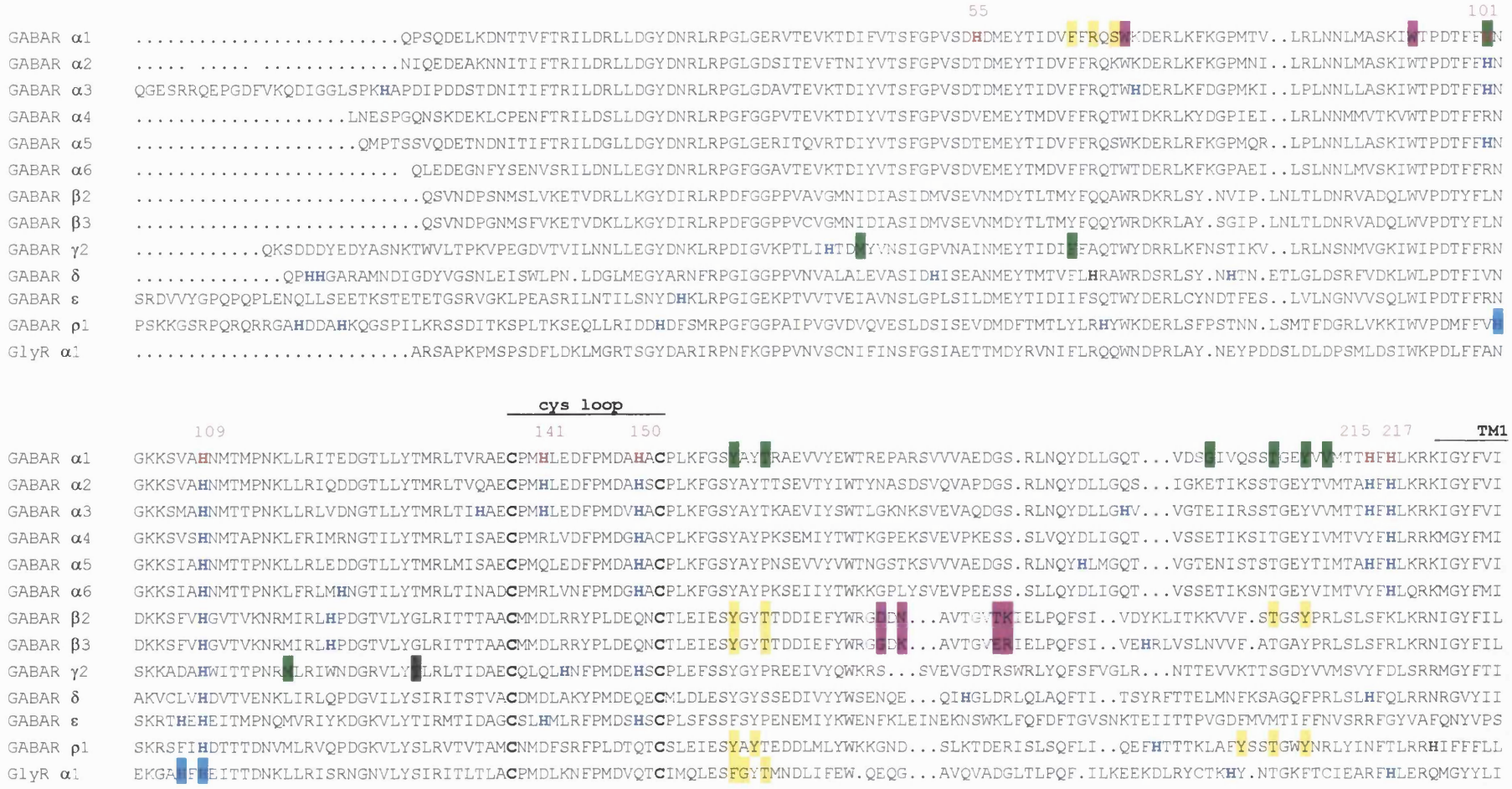
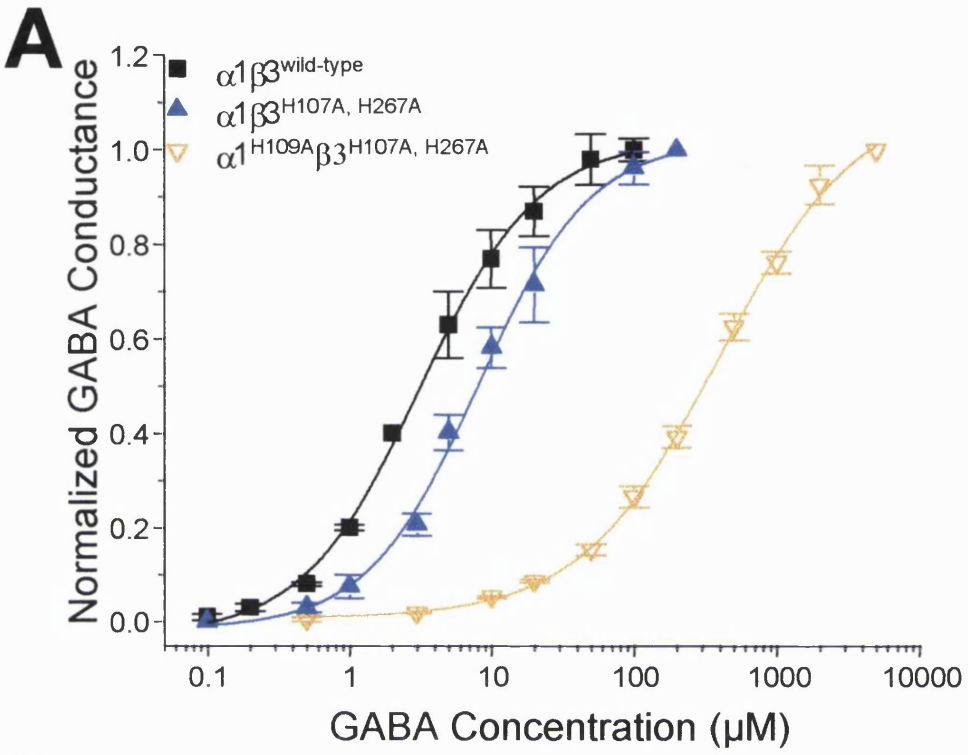

55
101
GABAR α1
GABAR α2
GABAR α3
GABAR α4
GABAR α5
GABAR α6
GABAR β2
GABAR β3
GABAR γ2
GABAR δ
GABAR ε
GABAR ρ1
GlyR α1
cys loop
109
141
150
215 217
TM1

Figure 4.6. Mutation of H109 on the $GABA_A$ receptor α1 subunit dramatically reduces GABA potency but does not affect Zn^{2+} sensitivity. (A) GABA concentration-response curves were constructed for α1β3, $\alpha1\beta3^{H107,267A}$ and $\alpha1^{H109A}\beta3^{H107A,\ H267A}$ $GABA_A$ receptor constructs. Data were normalized to the maximum GABA conductance for each cell. Data shown are mean ± s.e.m. (n = 6 - 8 oocytes) and fitted using the equation described in the methods (Chapter 2). The $\alpha1^{H109A}$ mutation produces a 50-fold reduction in GABA potency ($\alpha1^{H109A}\beta3^{\ H107A,\ H267A}$, EC_{50}: 389.77 ± 27.53μM, n_H: 0.89 ± 0.04; $\alpha1\beta3^{\ H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM, n_H: 1.1 ± 0.12; α1β3, EC_{50}: 3.14 ± 0.23μM, n_H: 1.04 ± 0.08; n = 3 - 8 oocytes). (B) Concentration – inhibition curves were constructed by expressing the inhibition by Zn^{2+} as a percentage of of the control response to a near EC_{50} dose of GABA (2μM, 5μM or 400μM) in the absence of Zn^{2+} for each cell. Data points represent mean ± s.e.m. from n = 3 - 5 oocytes. The curves describe inhibition equation fits to the experimental data. The inhibition curves for $\alpha1\beta3^{H107A,\ H267A}$ and $\alpha1^{H109A}\beta3^{H107A,\ H267A}$ $GABA_A$ receptor constructs are coincident.


A
Normalized GABA Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
α1β3wild-type
α1β3H107A, H267A
α1H109Aβ3H107A, H267A
0.1
1
10
100
1000
10000
GABA Concentration (μM)

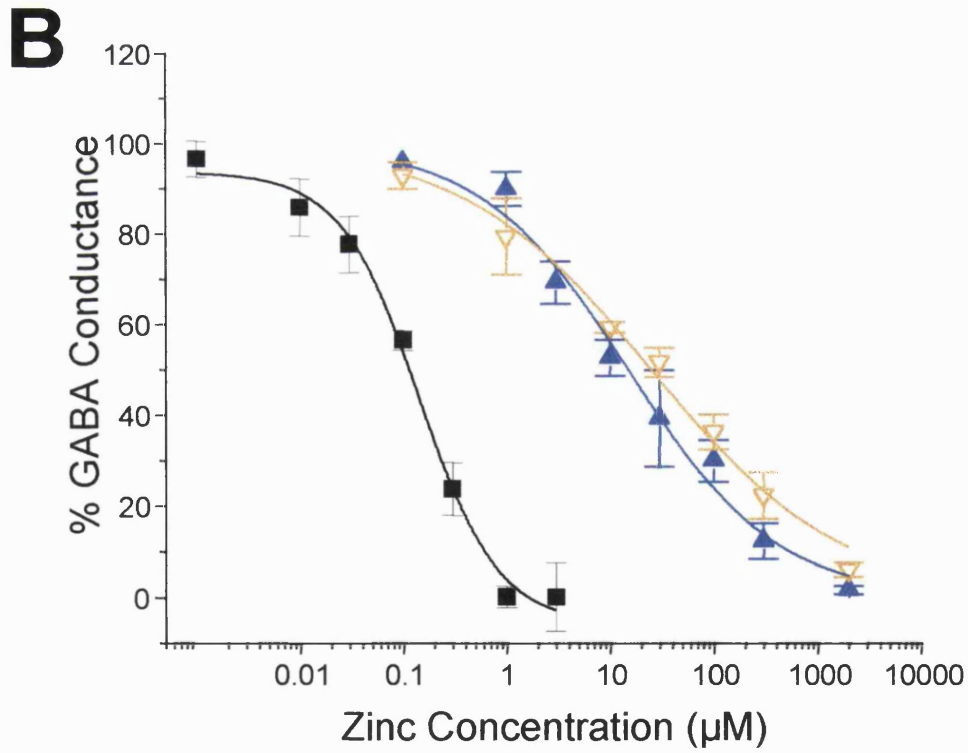

B
% GABA Conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

Figure 4.7. Mutating $\alpha1^{H215}$, part of a recognised Zn^{2+} binding motif, does not affect Zn^{2+} sensitivity of $\alpha1\beta3^{H107A,\ H267A}$ receptors. (A) GABA concentration-response curves were determined for $\alpha1\beta3$, $\alpha1\beta3^{H107A,\ H267A}$ and $\alpha1^{H215A}\beta3^{H107A,\ H267A}$ $GABA_A$ receptor constructs. Data were normalized to the maximum GABA conductance for each cell. Data represent mean ± s.e.m. (n = 4 oocytes) and fitted using the equation described in the methods (Chapter 2). (B) Concentration – inhibition curves were constructed by expressing the inhibition by Zn^{2+} as a percentage of the control response to a near EC_{50} concentration of GABA (2μM or 5μM) in the absence of Zn^{2+} for each cell. Data points shown are mean ± s.e.m. from 5 oocytes. The curves describe inhibition equation fits to the experimental data.

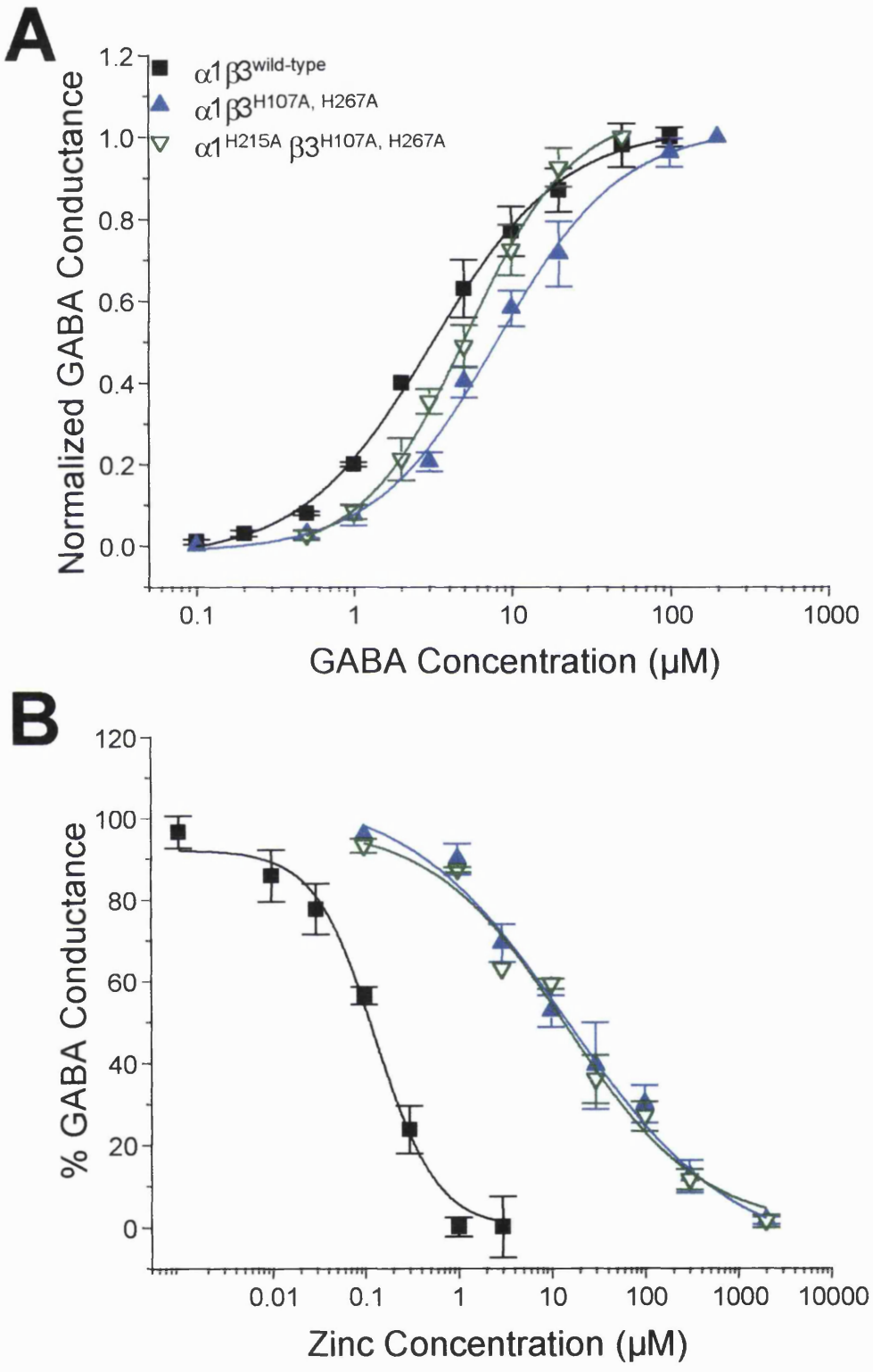
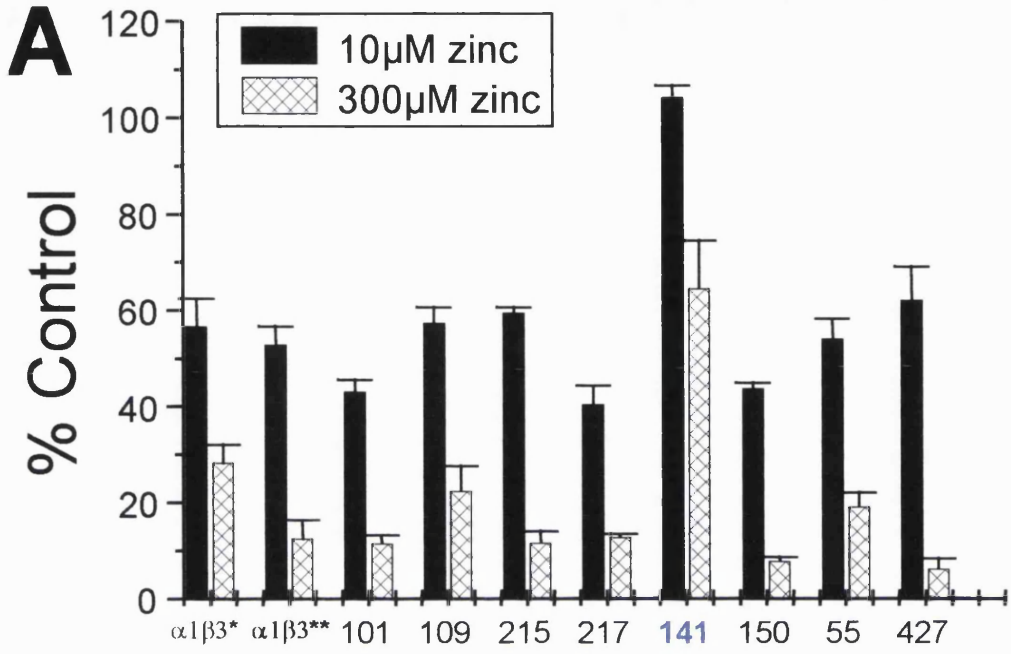
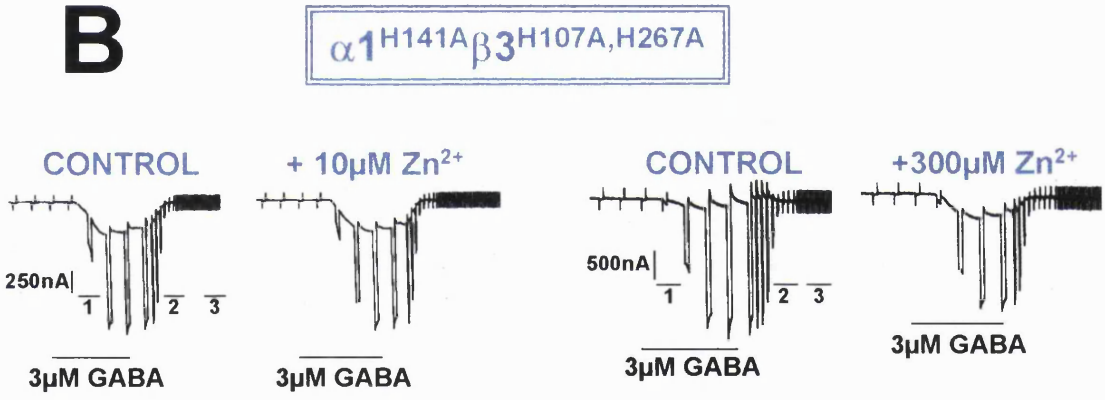

A
Normalized GABA Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
α1β3wild-type
α1β3H107A, H267A
α1H215A β3H107A, H267A
0.1
1
10
100
1000
GABA Concentration (μM)
B
% GABA Conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

Figure 4.8. H141 in the $GABA_A$ receptor α1 subunit affects the Zn^{2+} sensitivity of $\alpha1\beta3^{H107A,\ H267A}$ receptors. Each extracellular histidine in the α1 subunit was systematically mutated to alanine (A) and coexpressed with $\beta3^{H107A,\ H267A}$ mutant subunits. Zn^{2+} sensitivity was assessed by measuring responses evoked by an EC_{50} GABA concentration in the absence and presence of Zn^{2+} (10μM or 300μM). **A**: The Zn^{2+} inhibitory effect was then calculated as a percentage of the control EC_{50} GABA response (=100%). $\alpha1\beta3^{*}$ represents the $\alpha1\beta3^{H267A}$ construct, $\alpha1\beta3^{**}$ represents the $\alpha1\beta3^{H107A,\ H267A}$ construct. The remaining numbers denote the sequence numbers of the external histidines in the α1 subunit. The only α-subunit mutation to significantly affect the sensitivity of Zn^{2+} was $\alpha1^{H141A}$. The $\alpha1^{H141A}$ mutation abolished the sensitivity to 10μM Zn^{2+} (104 ± 2.65% of the control response; $\alpha1\beta3^{H107A,\ H267A}$, 52.66 ± 3.93% of the control response; n = 3 – 5 oocytes) and dramatically reduced the inhibition produced by 300μM Zn^{2+} (64.33 ± 10.09% of the control response; $\alpha1\beta3^{H107A,\ H267A}$, 12.33 ± 3.93% of the control response; n = 3 - 5 oocytes). **B, C:** Representative whole-cell membrane current responses to an EC_{50} GABA concentration (3μM or 5μM) in the absence and presence of Zn^{2+} (10μM or 300μM) for oocytes expressing $\alpha1^{H141A}\beta3^{H107A,\ H267A}$ (**B**) and $\alpha1\beta3^{H107A,\ H267A}$ (**C**) $GABA_A$ receptors. The time calibrations on recovery from GABA represent, 1: 5s; 2: 12.5s; 3: 50s.


A
% Control
120
100
80
60
40
20
0
10μM zinc
300μM zinc
α1β3*
α1β3**
101
109
215
217
141
150
55
427


B
α1H141Aβ3H107A,H267A
CONTROL
+ 10μM Zn2+
CONTROL
+300μM Zn2+
250nA
500nA
1
2
3
3μM GABA
3μM GABA
3μM GABA
3μM GABA

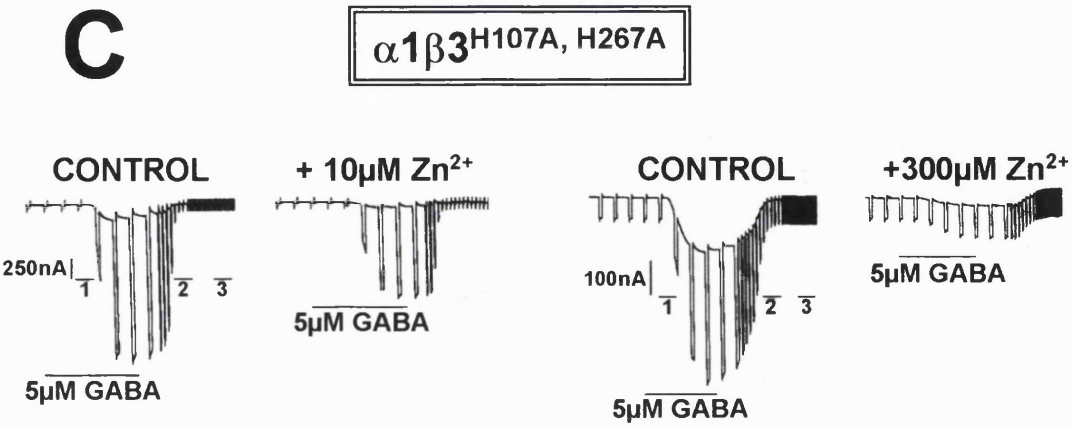
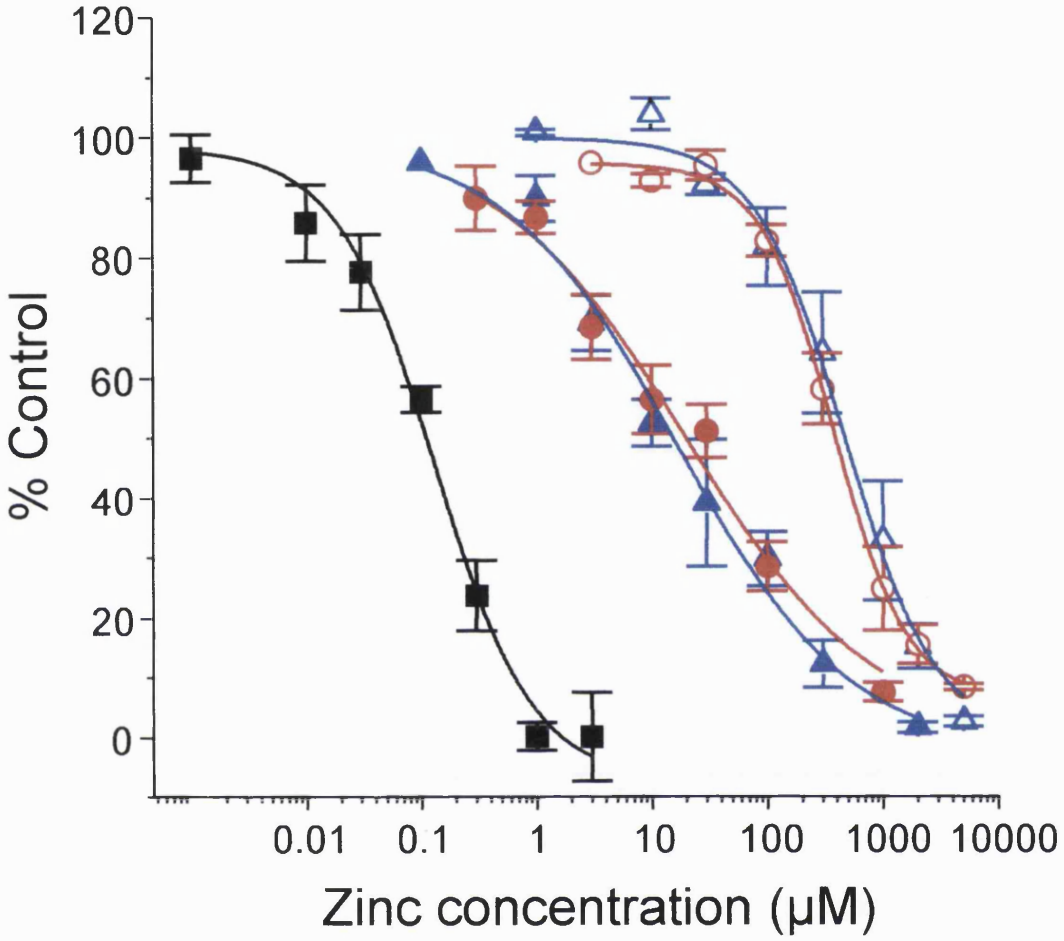

C
α1β3H107A, H267A
CONTROL
+ 10μM Zn2+
CONTROL
+300μM Zn2+
250nA
100nA
1
2
3
5μM GABA
5μM GABA
5μM GABA
5μM GABA

Figure 4.9. Mutation of $\alpha1^{H141}$ results in a 25-fold reduction in Zn^{2+} potency. Concentration – inhibition curves were established by expressing the inhibition by Zn^{2+} as a percentage of the control response to a near EC_{50} concentration of GABA (2μM, 5μM or 400μM) in the absence of Zn^{2+} for each cell. Data points shown are mean ± s.e.m. from 5 oocytes. The curves describe equation fits to the experimental data. The Zn^{2+} concentration-inhibition curves for $\alpha1^{H141A}\beta3^{H267A}$ and $\alpha1^{H141A}\beta3^{H107A,\ H267A}$ overlap ($\alpha1^{H141A}\beta3^{H267A}$, IC_{50}: 439.98 ± 34.11μM, n_H: 1.16 ± 0.08; $\alpha1^{H141A}\beta3^{H107A,H267A}$, IC_{50}: 477.02 ± 41.4μM, n_H: 1.16 ± 0.08; n = 3 – 4 oocytes) further implying that $\beta3^{H107}$ is not involved in regulating Zn^{2+} sensitivity for $\alpha1\beta3$ heteromers.


120
100
80
60
40
20
0
% Control
0.01
0.1
1
10
100
1000
10000
Zinc concentration (μM)

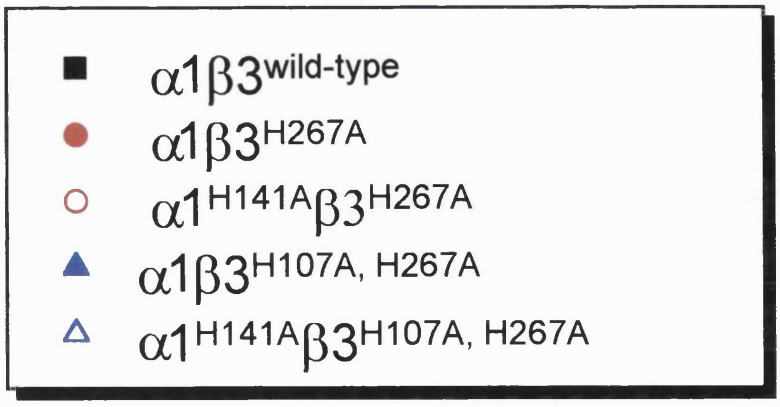

α1β3wild-type
α1β3H267A
α1H141Aβ3H267A
α1β3H107A, H267A
α1H141Aβ3H107A, H267A

Figure 4.10. Low external pH abolishes the Zn^{2+} antagonism of the GABA conductance for $\alpha1^{H141A}\beta3^{H267A}$ receptors. GABA concentration-response curves were constructed for $\alpha1^{H141A}\beta3^{H267A}$ receptors in the absence (○) and presence (▽) of 1mM Zn^{2+} in Ringer pH 7.4 (A) and pH 5.4 (B). At physiological pH 7.4, application of 1mM Zn^{2+} produces a rightward shift and depression of the GABA concentration response curve. Lowering the external Ringer pH to 5.4 reduces GABA potency (pH 7.4, - Zn^{2+}: GABA EC_{50}: 2.33 ± 0.33μM, n_H: 1.24 ± 0.19; pH 5.4, - Zn^{2+}: GABA EC_{50}: 8.25 ± 0.78μM, n_H: 1.12 ± 0.15). However at pH 5.4, Zn^{2+} inhibition of $\alpha1^{H141A}\beta3^{H267A}$ receptors is prevented, and application of 1mM Zn^{2+} does not alter either the GABA EC_{50} or maximum response. Data shown are mean ± s.e.m. from 3 – 5 oocytes and curves were fit to the data as described in the methods (Chapter 2).

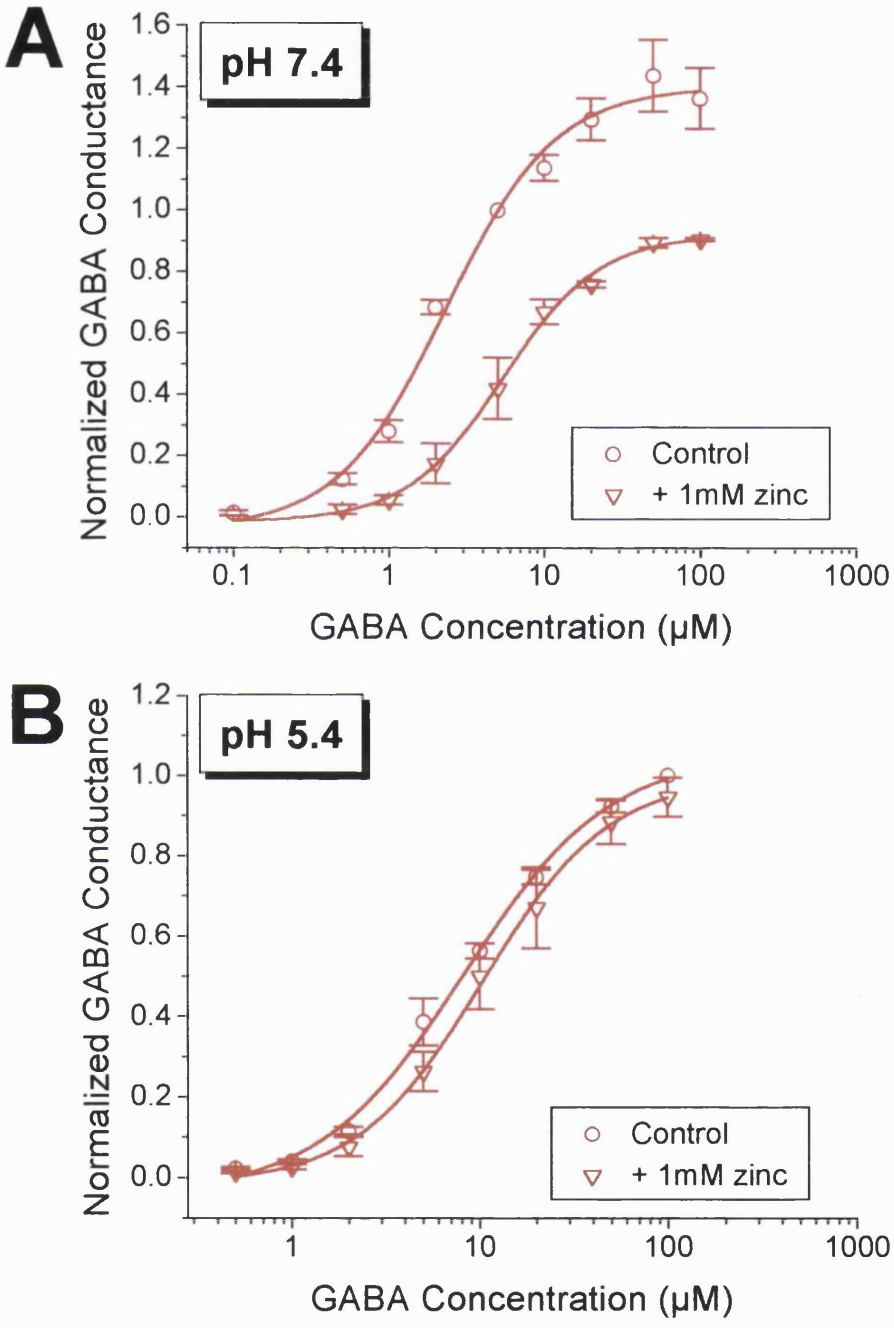
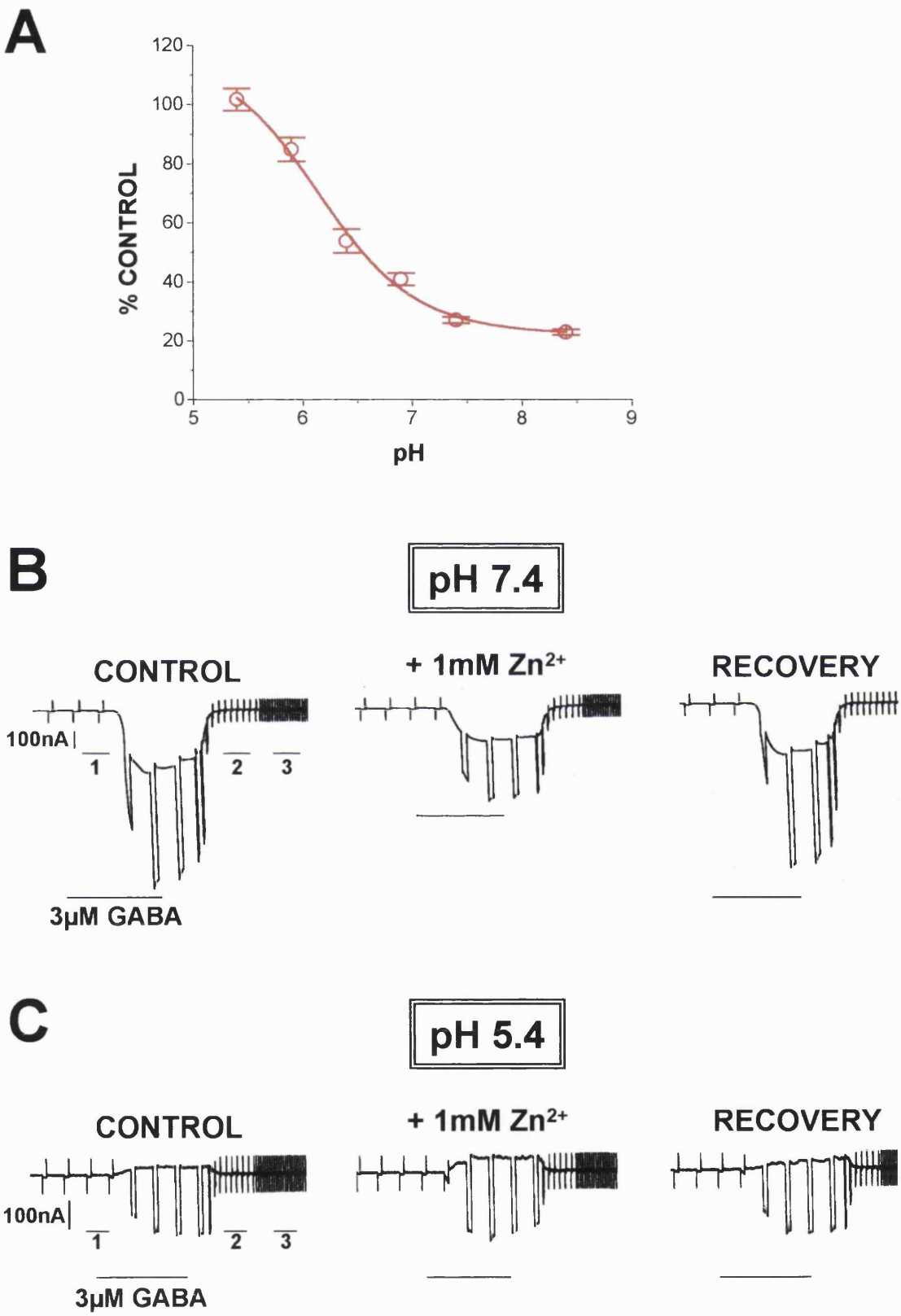

A
pH 7.4
Normalized GABA Conductance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
GABA Concentration (μM)
Control
+ 1mM zinc
B
pH 5.4
Normalized GABA Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
GABA Concentration (μM)
Control
+ 1mM zinc

Figure 4.11. The relevance of histidine residues in the Zn^{2+} sensitivity of $\alpha1^{H141A}\beta3^{H267A}$ receptors. (A)Whole-cell membrane conductances evoked by 3μM GABA were determined in the absence and presence of 1mM Zn^{2+} over an external Ringer pH range 5.4 to 8.4. The inhibitory effect of Zn^{2+} was calculated as a percentage of the control GABA response in the absence of Zn^{2+} at each pH, enabling the construction of a pH titration curve. Data represent mean ± s.e.m. from n = 3 oocytes. Curve fitting (as described in Chapter 2) produced a pK_a of 6.19 ± 0.12 pertaining to a further involvement of histidine residues in mediating Zn^{2+} inhibition. (B, C) Typical whole-cell membrane current responses to 3μM GABA in the absence and presence of Zn^{2+} (1mM) for oocytes expressing $\alpha1^{H141A}\beta3^{H107A, H267A}$ $GABA_A$ receptors at pH 7.4 (B) and pH 5.4 (C). Downward deflections represent membrane conductances evoked by hyperpolarizing voltage commands (1s duration, -10mV amplitude, 0.2Hz frequency) superimposed on the holding potential (–40mV). The time calibrations on recovery from GABA represent, 1: 5s; 2: 12.5s; 3: 50s.


A
120
100
80
60
40
20
0
% CONTROL
5
6
7
8
9
pH
B
pH 7.4
CONTROL
+ 1mM Zn2+
RECOVERY
100nA
1
2
3
3µM GABA
C
pH 5.4
CONTROL
+ 1mM Zn2+
RECOVERY
100nA
1
2
3
3µM GABA

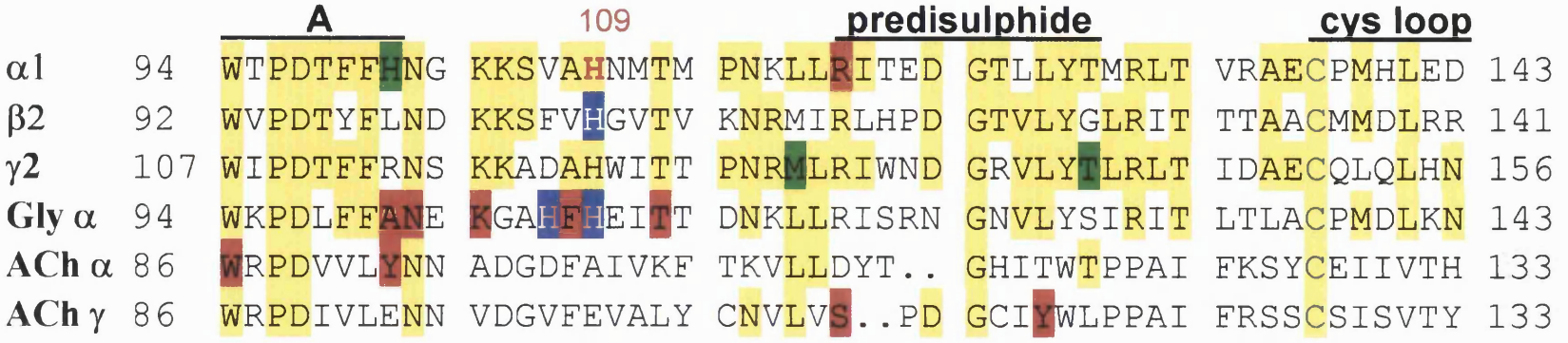


Figure 4.12. $\alpha1^{H109}$ lies between two major determinants of $GABA_A$ receptor pharmacology. Alignment of the N-terminal regions between loop A and the cysteine loop of mouse $GABA_A$ receptor α1, β2 and γ2, glycine α1 and nACh receptor α and γ subunits. Residues conserved in at least two classes of $GABA_A$ receptor subunit are highlighted in yellow and when also conserved in glycine or nACh receptors. For comparison to nACh receptors, the positions of N-terminal loops that may contribute to the agonist binding site are indicated above the alignment and labelled 'A' and 'pre-disulphide' (*cf.* Galzi & Changeux, 1995; Prince & Sine, 1996). The beginning of the disulphide loop is also shown ('cys loop'). Determinants of agonist or competitive antagonist potency are marked X, and those of benzodiazepine and zinc action X and X respectively. Note how closely the determinants of agonist pharmacology align even between pharmacologically distinct receptors. $\alpha1^{H109}$ lies between putative components of two binding sites, namely H101 and R199. References as for Fig 4.5.

CHAPTER 5

Estimating the stoichiometry of the $GABA_A$ receptor β3 subunit homomer using the $\beta3^{H267A}$ mutation.

5.1 INTRODUCTION

$GABA_A$ receptors are presumed to be pentamers, typically comprising a combination of α, β, and γ subunits. Initially, this arrangement was simply deduced from membership of the ligand-gated ion channel superfamily and following the precedent set by the nicotinic acetylcholine receptor (Arias, 1997). However, in the absence of electron microscopic data (*cf.* Nayeem *et al.*, 1994) the subunit stoichiometry of the $GABA_A$ receptor is controversial. The stoichiometry of recombinant $GABA_A$ receptors has been probed by a variety of methods including immunoprecipitation (Kellenberger *et al.*, 1996; Tretter *et al.*, 1997), electrophysiology (Backus *et al.*, 1993; Im *et al.*, 1995; Chang *et al.*, 1996) and fluorescence (Farrar *et al.*, 1999).

A common approach involves site-directed mutagenesis to generate mutant subunit isoforms with a distinct physiological and/or pharmacological property that can be analysed using electrophysiological techniques. For example, a highly conserved leucine residue was replaced with serine in TM2 of the $GABA_A$ receptor α1, β2 and γ2 subunits (Chang *et al.*, 1996). Mutation of this leucine in any of these subunits substantially increased the sensitivity to GABA. Co-injection experiments in which wild-type and mutant subunits of each subtype (e.g. α1 + $\alpha1^{L263S}$) were expressed with wild-type subunits of the remaining two subtypes (e.g. β2 + γ2) in *Xenopus* oocytes, generated mixed populations of $GABA_A$ receptors with unique, diagnostic GABA EC_{50} values. Analysis of the compound GABA concentration-response curves for the different cRNA injection mixtures revealed the number of each subunit incorporated into the $GABA_A$ receptor complex by ascertaining the number of distinct components of the concentration response curve. By mixing a mutant α subunit with wild-type α, β, and γ subunits (equimolar ratios), the concentration-response curve possessed three components indicating the likelihood of two α subunits in the receptor complex. Similar experiments with mutant β and γ subunits suggested a pentameric stoichiometry of 2α: 2β: 1γ. This approach makes several assumptions. Normal receptor stoichiometry is thought to be unaffected by the mutation since the authors considered a single point mutation of a putative channel lining residue to be unlikely to dramatically affect

subunit-subunit assembly. Furthermore, it was assumed that subpopulations of $GABA_A$ receptors (e.g. $\alpha1\beta2$) were not responsible for the observed changes in GABA potency. Using non-equivalent injection ratios of cRNAs to increase the relative availability of the different subunits did not alter the number of each subtype included into the $GABA_A$ receptor complex, implying that the pentameric $2\alpha:2\beta:1\gamma$ stoichiometry is invariant and therefore a preferred assembly product.

An electrophysiological approach was also used to assess the subunit stoichiometry of recombinant $\alpha3\beta2\gamma2$ subunit $GABA_A$ receptors expressed in HEK cells (Backus *et al.*, 1993). Residues at two analogous positions flanking TM2 in the three subunits were non-conservatively replaced with negatively charged glutamate. Wild-type and mutant subunits were then coexpressed and the degree of outward rectification of the GABA-evoked current, caused by the reduced positive charge, was compared with the total change in charge generated by the mutation(s) for each potential subunit stoichiometry. By assuming that the $\alpha3:\beta3:\gamma2$ $GABA_A$ receptor is a pentamer, this method inferred that $2\alpha:2\beta:1\gamma$, $2\alpha:1\beta:2\gamma$ and $1\alpha:2\beta:2\gamma$ were feasible subunit arrangements although the most likely $GABA_A$ receptor stoichiometry was $2\alpha:1\beta:2\gamma$. The possibility of tetrameric combinations of subunits was not considered. This model also assumed that the alteration of charge in any of the three subunits has the same effect on the outward rectification. This may not be the case as the amino acids replaced with glutamate in the TM1-TM2 loop are different in each of the subunits ($\alpha3$, arginine; $\beta2$, tyrosine; $\gamma2$, lysine). Furthermore, the model assumes that there is a simple relationship between the addition of a negative charge near TM2 and the rectification of the GABA I-V relationship.

This present study employs a different, functional approach to determine the subunit stoichiometry of $\beta3$ homomeric $GABA_A$ receptors. $\beta3$ homomeric $GABA_A$ receptors are highly sensitive to inhibition by Zn^{2+}. A single point mutation in the putative TM2 generates a 1200-fold reduction in Zn^{2+} potency ($\beta3^{H267A}$; Wooltorton *et al.*, 1997a; Chapter 3). The substantial difference in the Zn^{2+} sensitivity of the wild-type and mutant $\beta3$ receptors provides the basis of the assay to ascertain the subunit stoichiometry. *Xenopus* oocytes were co-injected with different cDNA ratios of $\beta3$ wild-type and $\beta3^{H267A}$ subunits. The Zn^{2+} sensitivities of the resulting receptors were determined and

used to infer the subunit stoichiometry following the formalism proposed by MacKinnon (1991).

5.2. RESULTS

5.2.1. Analysis of β3 subunit receptor stoichiometry: Zn^{2+} concentration-inhibition curves.

Co-injection of different β3:$β3^{H267A}$ cDNA ratios into *Xenopus* oocytes resulted in the expression of functional heteromeric receptors with intermediate sensitivities to Zn^{2+} (Fig. 5.1.). Zn^{2+}concentration-inhibition curves were constructed for the spontaneously-gated conductance transduced by wild-type β3, $β3^{H267A}$ and β3:$β3^{H267A}$ mixed receptors (Fig. 5.2). The H267A mutation resulted in a dramatic lateral shift in the Zn^{2+} curve and an increase in the Zn^{2+} IC_{50} ($β3^{wild\text{-}type}$, 0.16 ± 0.03μM; $β3^{H267A}$, 194.58 ± 16.17μM; n = 3 – 7 oocytes; Chapter 3). Increasing the fraction of $β3^{H267A}$subunit cDNA progressively reduced Zn^{2+} potency (Fig. 5.2; Table 5.1).

The Zn^{2+} inhibition curves were fitted with an inhibition equation (see Chapter 2) modified to allow the presence of a number of receptor species resulting from mixtures of wild-type and mutant β subunits. Two cases were specifically analysed where either four (Fig. 5.2A) or five (Fig. 5.2B) subunits were considered to assemble. Two sets of dissociation constants were estimated providing curve fits to the experimental data (Fig. 5.2A, B). It was predicted that any further increase in the number of components to the inhibition equation would simply increase the degrees of freedom of the fit and not necessarily reflect the underlying physical stoichiometry. The objective was to determine the minimum number of dissociation constants required to account for all the experimental data-sets. Both tetrameric and pentameric models fitted the data well and were subsequently used in the theoretical binomial analysis described below.

5.2.2. Theoretical binomial analysis of β3 subunit receptor stoichiometry.

In this stoichiometric assay, the $\beta3^{H267A}$ mutant is referred to as the 'insensitive subunit A', and β3 wild-type as the 'sensitive subunit B'. The fractions of $\beta3^{H267A}$ and $\beta3^{wild\text{-}type}$ subunits available for assembly are assumed to correspond to the ratio of cDNA injected and are denoted f_A and f_B respectively, where $f_A + f_B = 1$. Assuming insensitive and sensitive subunits have equal probabilities of co-assembling into one complex with n subunits, co-injection of insensitive and sensitive subunit cDNAs should result in three populations of receptors; homomeric receptors containing either n A subunits or n B subunits, or heteromeric receptors containing both A and B subunits, the fraction of each dependent on f_A and f_B.

Table 5.1. Comparison of IC_{50}s obtained for Zn^{2+} antagonism on receptors containing $\beta3^{H267A}$ and $\beta3^{wild\text{-}type}$ subunits.

f_A	IC_{50} (μM)	Number of oocytes
0	0.15 ± 0.01	7
0.3	1.33 ± 0.21	5
0.5	7.91 ±1.24	3
0.8	100.11 ± 16.77	3
1	198.89 ± 11.34	3

The unblocked fraction of the current of a mixture of receptors (U_{mix}) containing various combinations of insensitive and sensitive subunits in the presence of inhibitor is:

$$Umix = \sum_{i=0}^{n} Fi\left\{\frac{Ki}{Ki + B}\right\} \qquad \text{.....(1)}$$

where F_i is the fraction of receptors of i type, K_i is the inhibition constant for the ith species, B is the Zn^{2+} concentration and n denotes subunit stoichiometry. Assuming

wild-type and mutant subunits express to the same extent and are able to mix and assemble randomly, coexpression should follow a binomial distribution. Therefore,

$$F_i = \left[\frac{n!}{i!(n-i)!}\right] f_A{}^i (1-f_A)^{n-i}$$

which represents the order-independent fraction of receptors that contain i mutant subunits (f_A) and n- i wild-type subunits (1 - f_A). Substituting this into equation 1 yields,

$$U_{mix} = \sum_{i=0}^{n} \left\{ \left[\frac{n!}{i!(n-i)!}\right] f_A{}^i (1-f_A)^{n-i} \right\} \left\{\frac{K_i}{K_i + B}\right\}$$

.....(2)

Equation 2 can then be expanded according to the binomial theorem:

$$U_{mix} = (1-f_A)^n \left\{\frac{K_0}{K_0+B}\right\} + \left\{\frac{n!}{1(n-1)!}\right\} f_A (1-f_A)^{n-1} \left\{\frac{K_1}{K_1+B}\right\} + \left\{\frac{n!}{2(n-2)!}\right\} f_A{}^2 (1-f_A)^{n-2} \left\{\frac{K_2}{K_2+B}\right\} + \ldots\ldots f_A{}^n \left\{\frac{K_n}{K_n+B}\right\}$$

.....(3)

where K_0, K_1, K_2 and K_n are dissociation constants for Zn^{2+} binding to β3 homomeric receptors containing 0, 1, 2 or n mutant subunits. Simplifying equation 3 by summing all terms except the last one in the binomial expansion:

$$U_{mix} = \sum_{i=0}^{n-1} \left[\left\{\frac{n!}{i!(n-i)!}\right\} f_A{}^i (1-f_A)^{n-i} \right] \left\{\frac{K_i}{K_i+B}\right\} + f_A{}^n \left\{\frac{K_n}{K_n+B}\right\}$$

.....(4)

To facilitate the identification of n, the two terms in equation 4 can be separately defined. Let

$$R = \sum_{i=0}^{n-1} \left[\left\{\frac{n!}{i!(n-i)!}\right\} f_A{}^i (1-f_A)^{n-i} \right] \left\{\frac{Ki}{K_i+B}\right\}$$

which represents the unblocked response in the presence of inhibitor B of all receptors containing one or more wild-type subunits, and define

$$U_{mut} = \left\{ \frac{Kn}{Kn + B} \right\}$$

which represents the unblocked fraction of current in the presence of inhibitor B of all receptors composed entirely of n mutant subunits.

Therefore equation 4 becomes:

$$U_{mix} = R + f_A{}^n U_{mut} \qquad \text{.....(5)}$$

Rearranging equation 5, stepwise:

$$\frac{U_{mix} - R}{U_{mix}} = f_A{}^n \left(\frac{U_{mut}}{U_{mix}} \right)$$

$$\ln\left(\frac{U_{mix} - R}{U_{mix}} \right) = n \ln f_A + \ln\left(\frac{U_{mut}}{U_{mix}} \right)$$

$$-\ln\left(\frac{U_{mut}}{U_{mix}} \right) = n \ln f_A - \ln\left(\frac{U_{mix} - R}{U_{mix}} \right)$$

$$\ln f_A{}^{-1} \ln\left(\frac{U_{mix}}{U_{mut}} \right) = n - \frac{\ln\left(\frac{U_{mix} - R}{U_{mix}} \right)}{\ln f_A}$$

$$\ln f_A{}^{-1} \ln\left(\frac{U_{mix}}{U_{mut}} \right) = n - \frac{\ln\left(1 - \frac{R}{U_{mix}} \right)}{\ln f_A} \qquad \text{.....(6)}$$

The left-hand side of equation 6 can easily be determined. U_{mix} can be directly resolved from the y ordinate of the Zn^{2+} concentration-inhibition curve (Fig. 5.2.) for each given concentration of Zn^{2+}. Likewise, the value of U_{mut} can be determined at the same Zn^{2+}

concentration for cells injected only with $\beta3^{H267A}$ cDNA. The fraction of the receptor composed of insensitive subunits, f_A, can be deduced from the injected cDNA ratio, allowing $\ln f_A^{-1}\ln(U_{mix}/U_{mut})$ to be calculated and plotted against $[B]$ (Zn^{2+} concentration; Fig. 5.3.). The plot is predicted to asymptote towards n, the subunit stoichiometry. The data points representing $\ln f_A^{-1}\ln(U_{mix}/U_{mut})$ for β3 homomers were plotted against Zn^{2+} concentration and theoretical curves were generated for either a tetramer or pentamer model using the dissociation constants obtained from figure 5.2A and B. It is clear from figure 5.3 that only the tetramer model provides a good fit to the experimental data suggesting four β3 subunits can co-assemble to form functional ion channels.

5.2.3. Analysis of the asymptote: How accurate is the estimation of *n*?

The asymptote will differ from n by a term dependent on the value of R (the unblocked response of all receptors containing one or more wild-type subunits).

$$asymptote = n - \frac{\ln\left[1 - \frac{R}{Umix}\right]}{\ln f_A} \qquad \text{.....(7)}$$

The assumption made in the original treatise by MacKinnon considered that if the proportion of mutant subunits is high, i.e. $f_A \rightarrow 1$, and consequently R becomes very small, then the asymptote in equation 7 will approach n. However, close inspection of this assumption and actual derivation of the asymptote demonstrates that n is likely to be always underestimated. Rearranging Equation 7,

$$asymptote = n - \frac{\ln\left[\frac{Umix - R}{Umix}\right]}{\ln f_A} \qquad \text{.....(8)}$$

Expanding the terms U_{mix} and R,

$$asymptote = n - \frac{\ln\left[\dfrac{\sum_{i=0}^{n-1}\left\{\left(\dfrac{n!}{i!(n-i)!}\right)f_A{}^i(1-f_A)^{n-i}\right\}\left(\dfrac{K_i}{K_i+B}\right)+f_A{}^i\left(\dfrac{K_n}{K_n+B}\right)-\sum_{i=0}^{n-1}\left\{\left(\dfrac{n!}{i!(n-i)!}\right)f_A{}^i(1-f_A)^{n-i}\right\}\left(\dfrac{K_i}{K_i+B}\right)}{\sum_{i=0}^{n-1}\left\{\left(\dfrac{n!}{i!(n-i)!}\right)f_A{}^i(1-f_A)^{n-i}\right\}\left(\dfrac{K_i}{K_i+B}\right)+f_A{}^i\left(\dfrac{K_n}{K_n+B}\right)}\right]}{\ln f_A}$$

.....(9)

At increasing concentrations of Zn^{2+}, i.e. B→∞

$$\frac{K_n}{K_n+B} \rightarrow \frac{K_n}{B}$$

Therefore at high concentrations of Zn^{2+}, equation 9 becomes

$$asymptote = n - \frac{\ln\left[\dfrac{f_A{}^n K_n}{\sum_{i=0}^{n-1}\left\{\dfrac{n!}{i!(n-i)!}f_A{}^i(1-f_A)^{n-i}\right\}K_i+f_A{}^n K_n}\right]}{\ln f_A}$$

.....(10)

Therefore the asymptote is always less than n. When the fraction of mutant subunits (f_A) is high, R (unblocked response of all receptors containing wild-type subunits) will be small and the asymptote will approach (but underestimate) n.

5.2.4. Conditions enabling accurate estimation of *n*.

Inspection of equation 10 reveals that the accurate estimation of the asymptote will be determined by the values of f_A, K_n and K_i. It was therefore instructive to investigate exactly how the asymptote depends on these constraints.

5.2.4.1. Effect of f_A on the asymptote.

The asymptote was measured assuming that receptor stoichiometry, n, was 4 and the dissociation constants for Zn^{2+} binding to the various wild-type and mutant forms of the β3 subunit receptor were as previously determined (Fig. 5.2.), namely (in μM): K_0 0.1; K_1 0.3; K_2 1.5; K_3 15 and K_n 212. With these constraints the asymptote was assessed by varying the fraction of the mutant subunits (f_A) from 0.05-0.95. It is clear that for the asymptote to approach n, f_A should be not less than 0.5 and preferably closer to 0.9 otherwise n is seriously underestimated. (Fig. 5.4.).

5.2.4.2. Effect of the dissociation constants, *K*, on the asymptote.

To examine the influence of K on the asymptote n, a simplifying case was analysed. It was assumed (supported by experimental evidence, Chapter 3) that the dissociation constant for Zn^{2+} binding to the full mutant β3 subunit receptor, K_n, was much greater than dissociation constants for Zn^{2+} binding to wild-type and part-mutant β3 receptors, i.e. $K_n >> K_3, K_2, K_1$, and K_0. Thus let

$$r = \frac{K_n}{K_i}$$

where $i = 1, 2.....n\text{-}1$. Then equation 10 can be defined accordingly:

$$asymptote = n - \frac{\ln\left[\dfrac{f_A{}^n r}{\sum_{i=0}^{n-1}\left[\left(\dfrac{n!}{i!(n-i)!}\right) f_A{}^i (1-f_A)^{n-i}\right] + f_A{}^n r}\right]}{\ln f_A} \qquad \text{.....(11)}$$

r simply relates how much larger K_n is compared to K_i and the effect this has (from r = 2-100) on the asymptote was assessed using equation 11 at $n = 4$, and at three different values of f_A: 0.5, 0.75 and 0.9 (Fig. 5.5.). The graphical examination clearly shows that the larger K_n is compared to K_i, the closer the asymptote comes to n. Accuracy is further increased if a large r ratio is combined with a high mutant:wild-type ratio, f_A.

For the experimental analysis of β3 subunit stoichiometry, both of the above criteria were fulfilled, i.e. K_n for Zn^{2+} binding to $\beta3^{H267A}$ mutant receptors is far greater than most of the K_i values, at best approaching 1200-fold higher. Moreover, the analysis also employed a high f_A of 0.8. Thus the estimate of $n = 4$ would appear to be close to the actual subunit stoichiometry of the β3 receptor.

5.3. DISCUSSION

5.3.1. Using a functional assay to determine subunit stoichiometry: assumptions and limitations

In this study, the subunit stoichiometry of homomeric β3 $GABA_A$ receptors was predicted using a functional assay based on the dramatic difference in sensitivities to Zn^{2+} of $\beta3^{wild\text{-}type}$ and $\beta3^{H267A}$ mutant subunit-containing receptors. This assay was developed to determine the subunit stoichiometry of voltage-dependent *Shaker* K^+ channels (MacKinnon, 1991). A single point mutation in the *Shaker* K^+ channel subunit results in a 250-fold reduction in charybdotoxin (CTX) sensitivity. Co-injection of a mixture of wild-type and mutant subunits resulted in hybrid receptors with distinct toxin sensitivities. Plotting $\ln f_A^{-1}$ ln (*Umix/Umut*) against CTX concentration, produced a curve that asymptotes towards 4, consistent with a tetrameric structure for this K^+ channel. MacKinnon observed that the asymptote underestimated *n*, but approached *n* as *R* approached zero and as U_{mix} and U_{mut} are determined at high concentrations of CTX. However, the asymptote was not further defined past equation 6. The present study observed that for the asymptote to converge towards *n*, K_n must also be significantly greater than K_i since *R* will not actually ever achieve zero.

The model makes several assumptions and so there are potential sources of error. The model depends on how accurately f_A can be determined. It is assumed that the fractions of $\beta3^{H267A}$ subunits (f_A) and $\beta3^{wild\text{-}type}$ (1- f_A) subunits are directly proportional to the ratio of injected subunit cDNAs. The model is also based on subunit assembly following a binomial distribution, thereby assuming that both types of subunits are able

to mix and coassemble randomly into a single receptor complex. These assumptions appear to be true since both subunits displayed similar membrane conductances and only the Zn^{2+} sensitivity of the β3 receptor was dramatically altered by the point mutation, H267A. The H267A mutation only minimally affected both the PB modulation ($\beta3^{wild\text{-}type}$, EC_{50}: 80.76 ± 12.7μM, n_H: 1.44 ± 0.32; $\beta3^{H267A}$, EC_{50}: 53.79 ± 7.92μM, n_H: 1.62 ± 0.35; n = 3 – 7 oocytes; Chapter 3) and the PTX antagonism of the channel (3-4 fold difference; Wooltorton *et al.*, 1997a). Furthermore, similar control I-V relationships displaying outward rectification were observed for both the wild-type and mutant receptors (Wooltorton *et al.*, 1997a). Therefore the point mutation H267A is unlikely to affect the assembly and expression of subunits. Furthermore, the location of H267 within the putative channel pore is unlikely to be involved in subunit assembly and has not been identified to date as an important 'assembly box' amino acid in $GABA_A$ receptors (Srinivasan *et al.*, 1999; Taylor *et al.*, 1999).

By defining the asymptote (equations 6-11), certain conditions can be met to ensure that the asymptote is close to *n*. The present study meets several of these conditions. Firstly, K_n must be greater than K_i, and the asymptote will converge towards *n* when there is a substantial separation between the two inhibition dissociation constants. In this case there is a large (~1200-fold) separation between the inhibition dissociation constants; a much greater degree of separation than used in other studies of subunit stoichiometry employing this assay (MacKinnon, 1991; Ferrer-Montiel & Montal, 1996; Mano & Teichberg, 1998). In addition, since the asymptote differs from *n* by a term dependent on *R* (the unblocked response of receptors containing one or more wild-type subunits), the asymptote will approach *n* as f_A (the fraction of mutant subunits in the receptor) increases. Therefore f_A must be high. A substantially different result is derived when equal fractions of wild-type and mutant subunits are considered (f_A=0.5) suggesting a much lower asymptote (*cf.* Fig. 5.4; 5.5.). From the data obtained, the most probable stoichiometry of the homomeric β3 $GABA_A$ receptor is a tetramer. This is in agreement with biochemical studies that observed the formation of tetrameric but not pentameric β3 homomers when HEK cells were transfected with α1 and β3 $GABA_A$ receptor subunit cDNAs (Tretter *et al.*, 1997).

This analytical model has also been applied to determine the subunit stoichiometry of homomeric AMPA receptors composed of GluR1 subunits, although two independent studies using this approach interestingly reached different conclusions. One study coexpressed different ratios of two mutant GluR1 subunits which displayed a 300-fold difference in the sensitivity to the open channel blockers phencyclidine (PCP) and dizolcipine (MK-801; Ferrer-Montiel & Montal, 1996). Application of MacKinnon's formalism (MacKinnon, 1991) implied a pentameric stoichiometry. In contrast, application of the analysis to mixed receptors consisting of wild-type GluR1 subunits and mutant subunits that display a 100-fold reduction in quisqualate sensitivity indicated that a tetrameric stoichiometry is approximately 900-fold more likely than a pentameric complex (Mano & Teichberg, 1998). The present study on β3 $GABA_A$ receptors has demonstrated the importance of a good separation between K_n and K_i (equation 11; Fig. 5.5.) in order for the asymptote to converge towards n. The study assaying subunit stoichiometry using open channel blockers achieves a greater separation of inhibition constants and might therefore provide a more accurate determination of n. Alternatively, the model makes several assumptions and this may account for the discrepancies between these two studies. It is assumed that the subunits are equivalently expressed. However this may not be the case in the study by Ferrer-Montiel & Montal, since the current amplitudes of the open channel blocker mutant receptors varied by up to 10-fold. In addition the mutants differed in their permeability to divalent ions. Therefore the fractions of the two mutant subunits in a mixed receptor may not be entirely equivalent to the ratio of cRNAs injected and so f_A and the subsequent determination of subunit stoichiometry would be inaccurate.

5.3.2. Other methods to determine the subunit stoichiometry of $GABA_A$ receptors.

The stoichiometry of recombinant α6β2γ2 and α6β2 $GABA_A$ receptors was established by generating a tandem construct of the α6 and β2 subunit cDNAs, with the C-terminus of the α6 cDNA linked, via a synthetic oligonucleotide containing 10 glutamine residues, to the N-terminus of the β2 subunit cDNA (Im *et al.*, 1995). Transfection of HEK cells with the tandem construct, alone or co-transfected with β2 subunit cDNAs,

failed to result in functional $GABA_A$ receptor expression. However, co-transfection of the tandem construct in conjunction with either α6 or γ2 subunit cDNAs generated functional $GABA_A$ receptors, supporting pentameric stoichiometries of either 3α:2β or 2α:2β:1γ, forming an α–β–α–β–γ arrangement.

Biochemical methods have also been used to determine the subunit composition of $GABA_A$ receptors. Recombinant α1β3γ2 $GABA_A$ receptors were expressed in HEK cells and then affinity-purified to permit only the analysis of completely assembled receptors (Tretter *et al.*, 1997). Subsequent SDS PAGE and Western blot analysis using specific polyclonal antibodies (of known relative reactivity) inferred a pentameric stoichiometry of 2α: 2β: 1γ unaffected by varying the cDNA transfection ratio from 1:1:1 to 1:1:4. A subunit arrangement of four α and β subunits separated by a single γ subunit was suggested. Equivalent analysis of α1β3 receptors derived a subunit stoichiometry of 2α: 3β. Analysis of the assembly of α1β3γ2 $GABA_A$ receptors by density gradient centrifugation, revealed that the α1β3γ2 subunit receptor protein sedimented at a single peak comparable with sedimentation coefficients observed for native adult $GABA_A$ receptors and the pentameric nicotinic acetylcholine receptor. Interestingly density gradient centrifugation of α1β3 receptors revealed the presence of homomeric β3 tetramers in addition to α1β3 tetramers and pentamers (Tretter *et al.*, 1997). The subunit stoichiometry of α1β3 receptors was investigated by the metabolic labelling of these receptors expressed in *Xenopus* oocytes with [^{35}S] methionine (Kellenberger *et al.*, 1996). The $GABA_A$ receptors were isolated from the cell surface using direct immunoprecipitation and the subunits were then separated by SDS PAGE and Western blot analysis. The subunit stoichiometry was then resolved by quantitating the amount of [^{35}S] methionine incorporated into each subtype of $GABA_A$ receptor subunits. However, the data obtained were unable to distinguish between a tetrameric (2α:2β) or pentameric (3α:2β) stoichiometry, indeed, even a subunit stoichiometry of 2α:3β was also possible.

Recently, the stoichiometry of cell surface $GABA_A$ receptor subunits has been probed using fluorescence resonance energy transfer (Farrar *et al.*, 1999). Recombinant $GABA_A$ receptors were tagged with a c-myc epitope on either the α1, β2 or γ2 subunit and expressed in HEK cells. These cells were then incubated with c-myc monoclonal

antibodies, which were labelled with a fluorescence donor (europium cryptate, EuK). Receptors containing the c-myc epitope on either the α1 or β2 subunit produced a maximum fluorescence that was twice that observed from receptors that were tagged on the γ2 subunit. Additional incubation of these receptors with an excess of c-myc monoclonal antibodies labelled with a fluorescence acceptor (XL665) generated a signal in response to laser excitation, only for receptors containing either c-myc tagged α1 or β2 subunits. Since the energy transfer requires the two c-myc epitopes to be very closely associated, i.e. in the same receptor complex, a subunit stoichiometry of 2α:2β:1γ was predicted (Farrar *et al.*, 1999).

5.3.3. Determination of the subunit stoichiometry of other ligand and voltage-gated channels.

In addition to GABA-gated Cl^- channels, the stoichiometry of other Cl^- channels has been investigated. The pore stoichiometry of a voltage-gated Cl^- channel, human ClC-1 has been determined by substituting amino acid residues located within the putative ion selectivity filter (Fahlke *et al.*, 1998). Application of methanethiosulphonate (MTS) reagents covalently modifies the thiol groups of the substituted cysteines (the SCAM technique, see Chapter 6) that project into the channel pore causing channel block. In order to ascertain whether the channel is comprised of one pore or two pores as formed by *Torpedo* ClC isoforms (Jentsch, 1994), heterodimers in which only one subunit contained the substituted cysteine were expressed. Since the degree of block observed in the presence of MTS reagents for the heterodimeric receptor was equivalent to the degree of block of homodimers in which both subunits contained the mutation, it was inferred that both subunits face a single pore. The observation that substituted cysteines in different subunits can co-operatively bind one Cd^{2+} ion provides further evidence for a single pore stoichiometry (Fahlke *et al.*, 1998).

Covalent cross-linking of glycine receptor subunits indicated a putative pentameric stoichiometry of 3α: 2β (Langosch *et al.*, 1988). The subunit stoichiometry of the glycine receptor has further been investigated using an electrophysiological approach (Kuhse *et al.*, 1993). Co-injection of wild-type α1 subunits with α2 subunits (containing a point mutation that confers a 100-fold reduction in apparent affinity for glycine) in

Xenopus oocytes resulted in receptors with intermediate sensitivities to activation by glycine. Altering the cRNA ratio injected greatly altered the glycine EC_{50} of the resulting receptor implying that the stoichiometry of α1/ α2 receptors is variable and dependent on subunit availability. However co-injection of β subunits with either α1 wild-type or α2 mutant subunits resulted in the expression of receptors with glycine sensitivities that were not altered by increasing the fraction of β subunit cRNA injected, inferring that αβ heteromeric glycine receptors have an invariant stoichiometry (3α:2β). Divergent amino acid sequences in the N-terminal domains of the α and β subunits were found to be responsible for the variation in assembly.

An electrophysiological approach was also used to examine the subunit stoichiometry of the neuronal nicotinic acetylcholine receptor (Cooper *et al.*, 1991). An analogous residue flanking TM2 in either the α4 or the nα1 (non-α, i.e. β) subunit was mutated to a residue with an opposite charge in order to alter the single channel conductance. To assess the α4 stoichiometry, α4 wild-type, α4-mutant and nα1-mutant subunits were co-injected into *Xenopus* oocytes. The number of α4 subunits in the receptor complex was deduced from the number of hybrid single channel conductances observed. Three different conductances were recorded; one corresponding to α4/ nα1-mutant receptors, another corresponding to α4-mutant/ nα1-mutant receptors and a third, intermediate conductance inferred to correspond to a receptor containing α4/α4-mutant/ nα1-mutant subunits. A comparable approach was used to deduce the number of nα1 subunits, and concluded that neuronal nicotinic acetylcholine receptors are pentamers with a 2α:3nα subunit stoichiometry (Cooper *et al.*, 1991).

A comparable method has been used to determine the subunit stoichiometry of an NMDA receptor composed of NR1 and NR2B subunits (Laube *et al.*, 1998). Point mutations were made in each subunit that substantially reduced (NR1, ~10,000 fold; NR2, ~250fold) the apparent agonist affinity. Co-injection of wild-type and mutant NR1 with wild-type NR2B subunits resulted in receptors displaying three independent components of glycine sensitivity, which were unaltered by varying the fraction of mutant cRNA in the injection mixture. The relative fractions of the three species of channel generated by injecting varying cRNA ratios was calculated using the binomial theorem and was consistent with the existence of two NR1 subunits in the NMDA

receptor. Using an identical protocol, analysing the glutamate sensitivity of hybrid receptors formed from co-injecting wild-type NR1 and wild-type and mutant NR2B subunits, indicated that the receptor contains two invariant NR2B subunits. Equivalent results were also obtained using NR1 and NR2B subunits containing a negative dominant mutation. It is therefore inferred that NMDA receptors are tetrameric with a 2NR1:2NR2 stoichiometry (Laube *et al.*,1998).

A different electrophysiological approach applied to AMPA receptors inferred a tetrameric stoichiometry (Rosenmund *et al.*, 1998), which is consistent with the study using a functional assay by Mano and Teichberg (1998). This approach is based on the changes in the mean single channel current evoked by the number of receptor binding sites having bound agonist. It is assumed that the number of subunits is analogous to the number of binding sites, and that the binding sites are equivalent and to ensure all the receptors were closed, a competitive antagonist was applied first prior to agonist exposure. The receptor was observed to pass through three electrophysiologically distinct states as the binding sites are successively occupied, and so following these assumptions the AMPA receptor would presumably have a trimeric structure. However, since the transition into the first state has two time constants whereas only a single time constant is observed for the other two states, the receptor was deduced to be a tetramer (Rosenmund *et al.*, 1998).

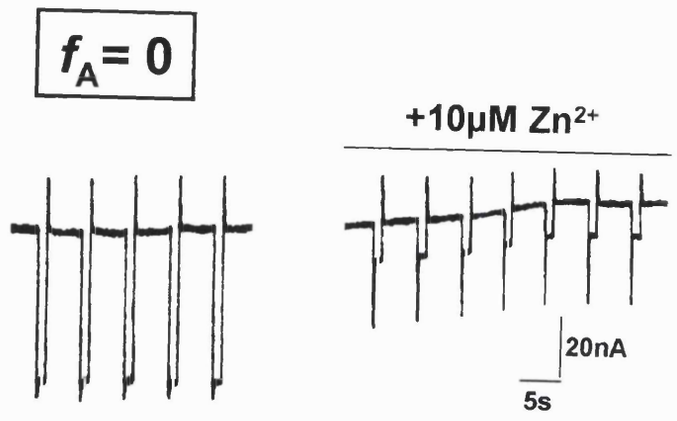
The proposal by MacKinnon of a tetrameric subunit stoichiometry for a voltage-gated K^+ channel (MacKinnon, 1991) has been supported by a variety of approaches including low resolution electron microscopy imaging (Li *et al.*, 1994), cross-linking studies (Schulteis *et al.*, 1996) and yeast two-hybrid analysis (Xu *et al.*, 1998). In addition, subunit stoichiometry of the delayed rectifier K^+ channel (Kv1.1) has been predicted by linking subunit cDNAs (Liman *et al.*, 1992). Wild-type Kv1.1subunit cDNAs were linked via the addition of 17 amino acids between the C-terminus of one subunit and the N-terminus of a further subunit, to form constructs containing 2-5 subunits. A point mutation conferring a decreased sensitivity to tetraethylammonium (TEA) was used to tag the expression of individual subunits. Expression of tetrameric cDNAs containing 3 wild-type subunits and 1 mutant subunit in *Xenopus* oocytes resulted in a channel with reduced TEA sensitivity, fitted by a single inhibition constant. The position of the mutant subunit in the construct was irrelevant indicating a random mixing of subunits.

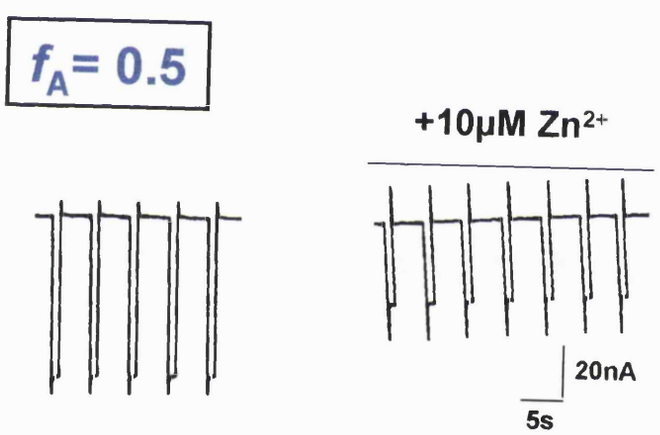
In addition, co-injection of a wild-type tetramer with a mutant monomer (unable to form functional homomers) generated receptors with equivalent TEA sensitivity to receptors resulting from the injection of wild-type tetramers alone (Liman *et al.*, 1992). Conversely, wild-type trimers coexpressed with monomeric mutant subunits resulted in a significant shift in TEA sensitivity compared to the wild-type trimer, providing further evidence that the K^+ channel is a tetramer. Furthermore, analysis of the crystal structure of a highly conserved region of the *Shaker* K^+ N-terminal domain, referred to as the tetramerization domain revealed that four identical subunits are symmetrically arranged around a central aqueous pore, constituting the ion channel (Kreusch *et al.*, 1998). Polar interactions between conserved amino acids at the interface of the tetramerization domains were able to determine subfamily-specific assembly. Therefore, by independent validation, the functional assay developed by MacKinnon (1991) can provide an accurate prediction of subunit stoichiometry.

5.3.4. Summary.

The present study describes an electrophysiological approach to determine the subunit stoichiometry of homomeric β3 subunit $GABA_A$ receptors. A point mutation, $\beta3^{H267A}$, conferred a relative insensitivity to Zn^{2+} (~1200-fold reduction in Zn^{2+} IC_{50}). Co-injection of wild-type and mutant β3 subunits into *Xenopus* oocytes resulted in receptors with intermediate sensitivities to Zn^{2+} that were susceptible to varying the cDNA ratio injected. The fraction of subunit cDNA injected was assumed to be directly proportional to the fraction of that subunit in the expressed receptor. Assuming wild-type and mutant subunits are able to assemble randomly into a single receptor complex and that both subunits express to the same extent, the data obtained from the co-injection experiments were applied to a model based on the binomial theorem developed by MacKinnon (1991) which was re-assessed. Ensuring certain conditions of the model are met, i.e. the fraction of mutant subunit cDNA in the injection mixture is high, the model generated a saturation curve that asymptotes towards *n,* the subunit stoichiometry. From the data obtained, the homomeric β3 subunit $GABA_A$ receptor is most probably a tetramer.

Figure 5.1. Differential inhibitory effects of Zn^{2+} on $GABA_A$ receptors generated by expressing different ratios of $\beta3^{wild\text{-}type}$ and $\beta3^{H267A}$ subunit cDNA ratios. Typical whole-cell membrane currents recorded from *Xenopus* oocytes injected with either $\beta3^{wild\text{-}type}$ (f_A=0), $\beta3^{H267A}$ (f_A=1) or a 1:1 ratio of $\beta3^{wild\text{-}type}$: $\beta3^{H267A}$ (f_A=0.5) subunit cDNAs in the absence and presence of 10μM Zn^{2+}. The transient downward deflections represent current pulses induced by brief hyperpolarizing commands (-10mV, 1s, 0.2Hz) applied to the holding potential of –40mV.


$f_A = 0$
+10μM Zn^{2+}
20nA
5s


$f_A = 0.5$
+10μM Zn^{2+}
20nA
5s

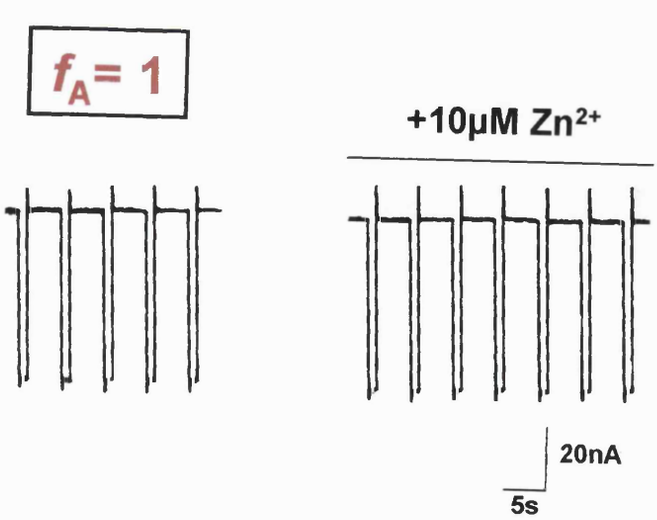
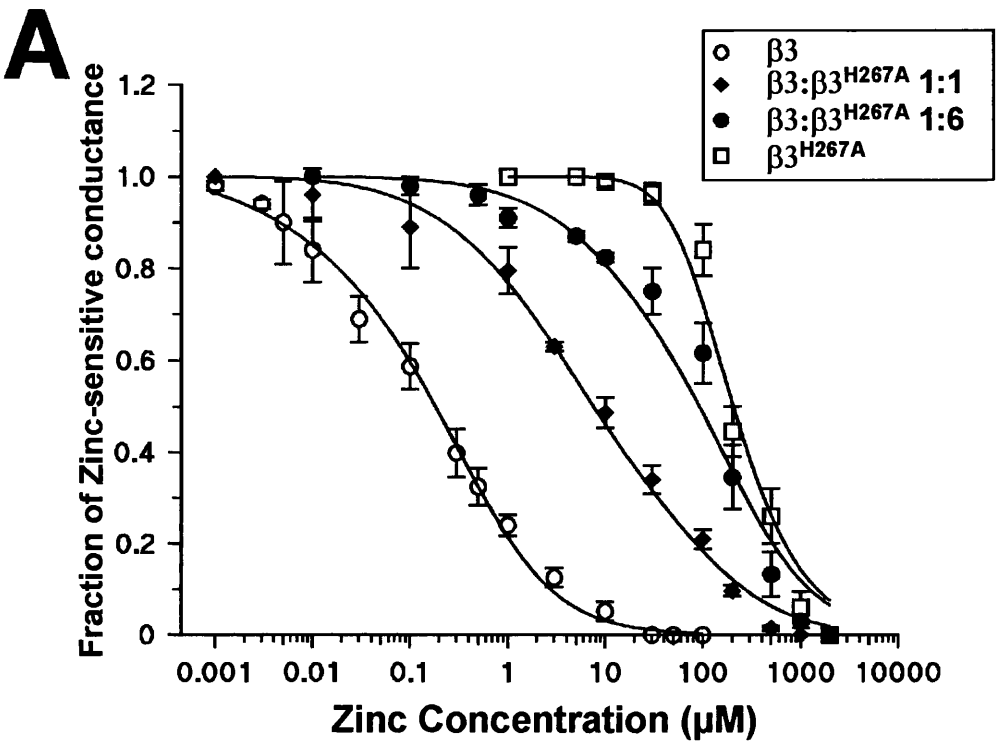

$f_A = 1$
+10μM Zn^{2+}
20nA
5s

Figure 5.2. Zn^{2+} concentration-inhibition relationships for wild-type β3, $β3^{H267A}$ and mixed β3:$β3^{H267A}$ subunit homomers. Zinc concentration-inhibition curves for blocking the spontaneous Cl^- current transduced by wild-type β3, $β3^{H267A}$ mutant and mixed β3:$β3^{H267A}$ $GABA_A$ receptors (generated by co-injection of wild-type and mutant cDNAs in either a 1:1 or 1:6 ratio). Data points represent the mean ± s.e.m. from 3 – 7 oocytes and are fitted with equation 3 (see Section 5.2.) assuming either tetrameric, n = 4 (A) or pentameric, n = 5 (B) assembly. Dissociation constants were obtained; where f_A = 0.8 and n = 4: K_0, 0.1; K_1, 0.3; K_2, 15; K_3, 20; K_4, 212μM and where f_A = 0.8 and n = 5: K_0, 0.1; K_1, 0.3; K_2, 1.5; K_3, 1.5; K_4, 22; K_5, 238μM. The constants used in these curve fits were determined by initially estimating them empirically from the data points plotted in figures 5.2A and B. These preliminary estimates were then used in equation 1 (Section 5.2.2) and more confident estimates of dissociation constants were obtained by iteration using non-linear least squares analyses.


A
β3
β3:β3H267A 1:1
β3:β3H267A 1:6
β3H267A
Fraction of Zinc-sensitive conductance
1.2
1.0
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

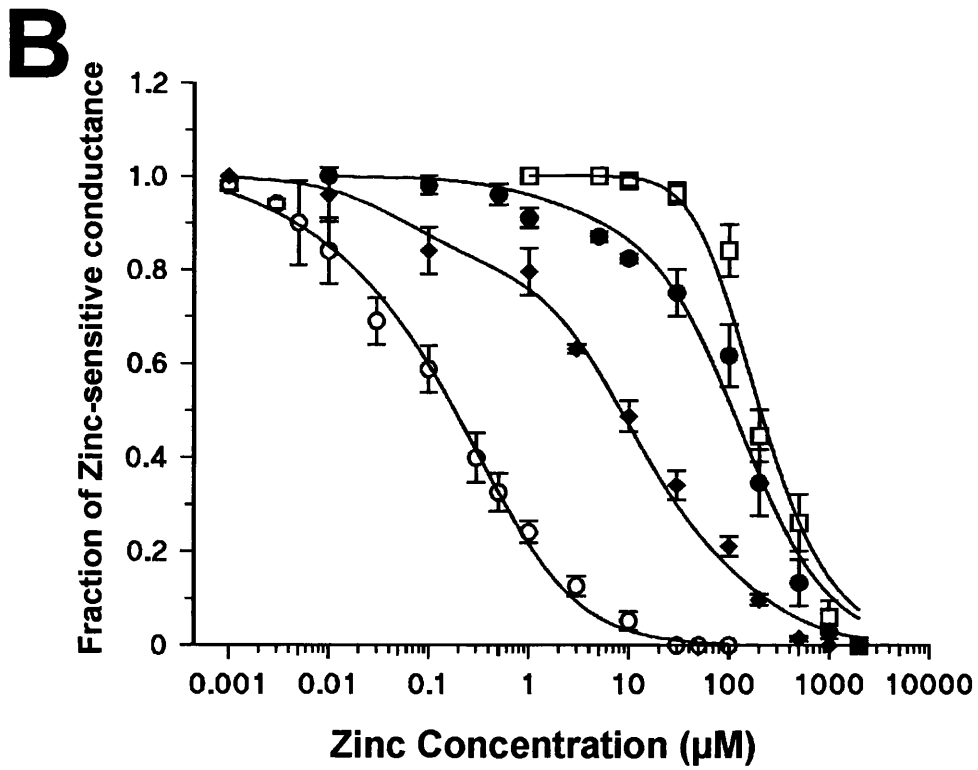

B
Fraction of Zinc-sensitive conductance
1.2
1.0
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)

Figure 5.3. Predicting the subunit stoichiometry of β3 subunit homomers. The fraction of $\beta3^{H267A}$ subunits (f_A) in a mixed $\beta3{:}\beta3^{H267A}$ receptor is assumed to correspond to the ratio of cDNA injected. U_{mix}, the unblocked fraction of the current of receptors comprising a mixture of wild-type and mutant subunits, and U_{mut}, the unblocked fraction of current of all receptors composed entirely of n mutant subunits, can be determined (Fig. 5.2.) for each given concentration of Zn^{2+}. According to equations 6 – 11 (see Section 5.2.), a plot of $\ln f_A^{-1}$ $\ln(U_{mix}/U_{mut})$ against Zn^{2+} concentration asymptotes towards n, the subunit stoichiometry. Data obtained, where f_A = 0.8, are best fitted with a model assuming n = 4, rather than n = 5.

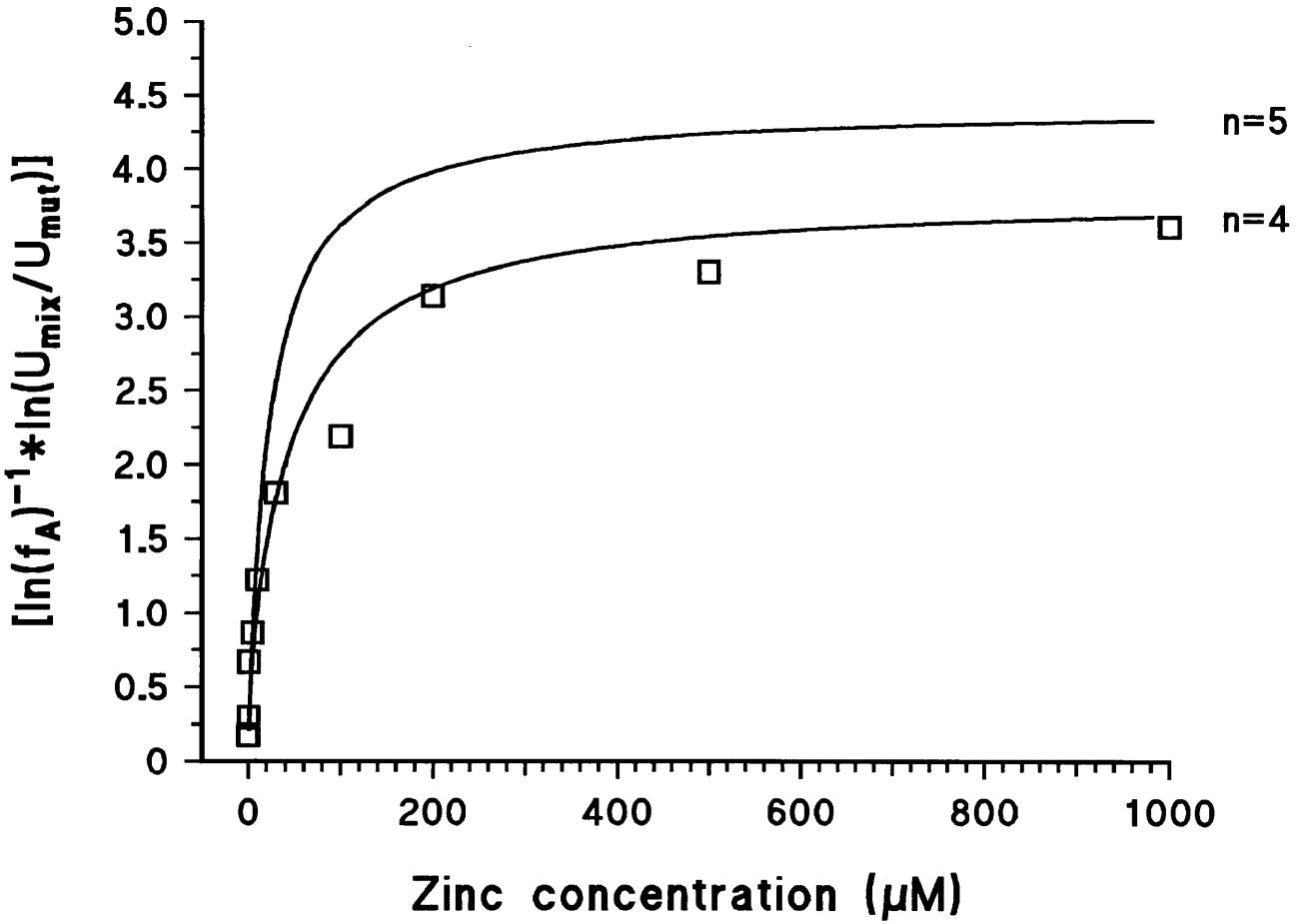

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
200
400
600
800
1000
[ln(f_A)^-1 * ln(U_mix/U_mut)]
Zinc concentration (μM)
n=5
n=4

Figure 5.4. Effect of varying f_A on predicting subunit stoichiometry. $\ln f_A^{-1}$ $\ln(U_{mix}/U_{mut})$ approaches n, the subunit stoichiometry (equations 6 – 11; Section 5.2.). The effect of altering the fraction of the mutant subunits (f_A) was determined over the range 0.05-0.95, assuming a saturating concentration of Zn^{2+} (3500μM) and using inhibitory dissociation constants obtained from Fig.5.2A. In order to avoid seriously underestimating n, f_A should be greater than 0.5, and preferably closer to 0.9. This theoretical prediction was performed using Mathcad version 6.

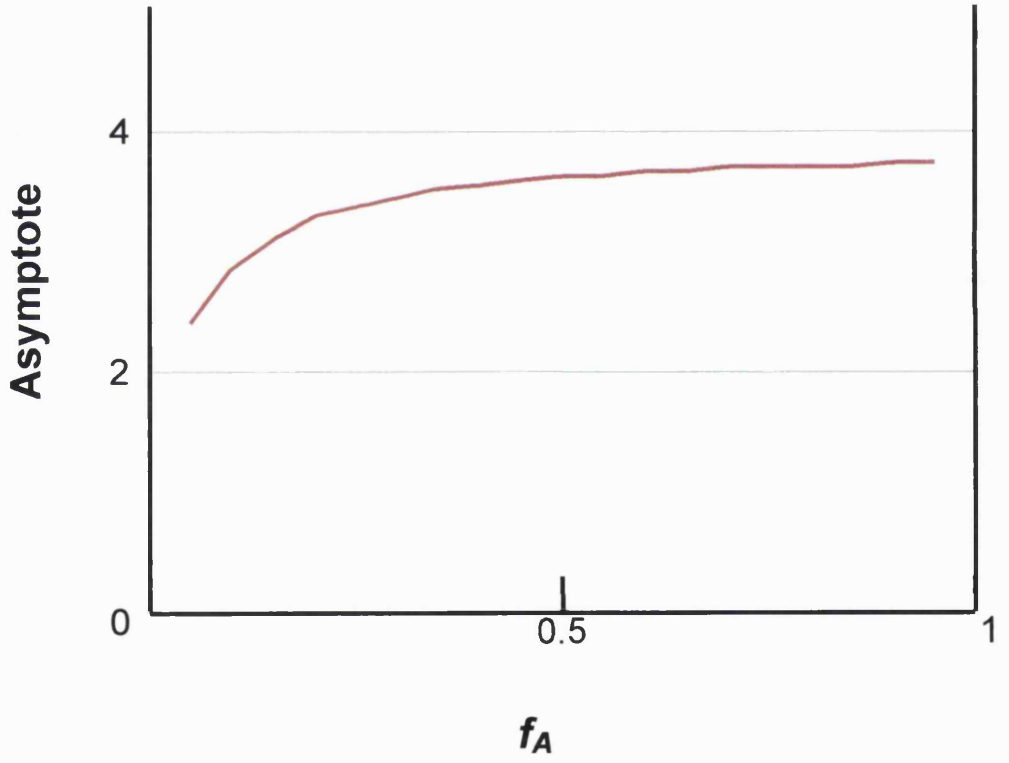
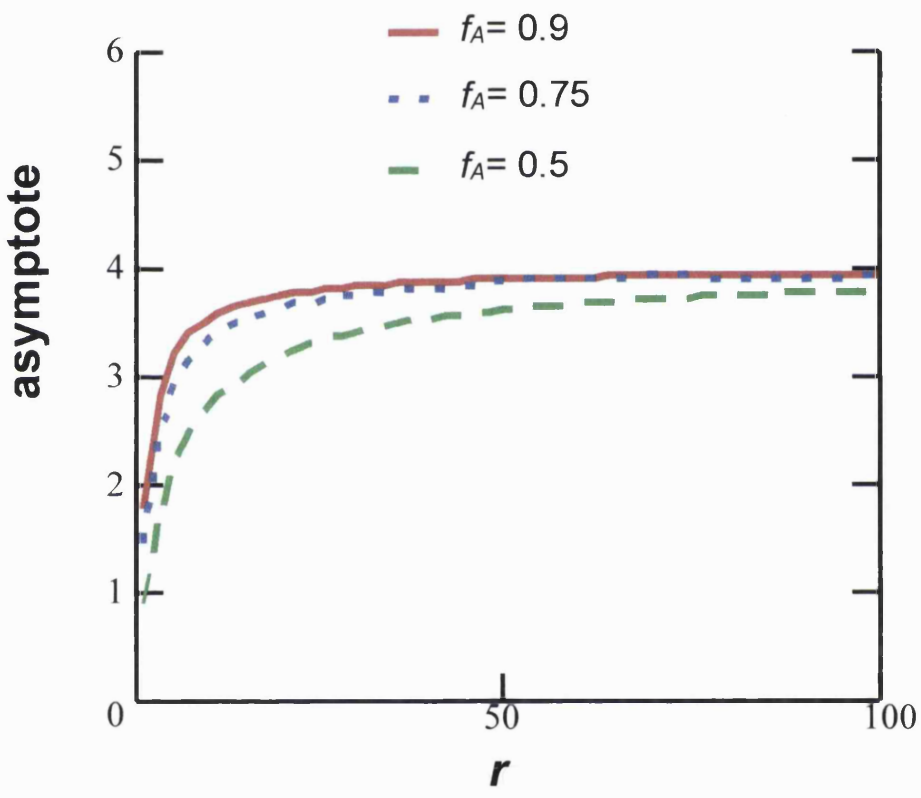

Asymptote
4
2
0
0.5
1
f_A

Figure 5.5. Effect of varying *K* on predicting subunit stoichiometry. The importance of the degree of separation between dissociation constants for Zn^{2+} binding to the mutant $\beta3^{H267A}$ receptor (K_n), and to wild-type and mixed β3 receptors (K_i) was determined by plotting $\ln f_A^{-1}$ $\ln(U_{mix}/U_{mut})$ against r, where r is K_n/K_i and $i = 1,2..n\text{-}1$ at three separate values of f_A (refer to equations 6 – 11; Chapter 5.2.). Increasing r, i.e. the larger the separation between K_n and K_i , the closer the asymptote is to n, the subunit stoichiometry. Theoretical predictions were performed using Mathcad version 6.0.


f_A= 0.9
f_A= 0.75
f_A= 0.5
asymptote
6
5
4
3
2
1
0
50
100
r

CHAPTER 6

Substituted Histidine Accessibility Method: an investigation into Zn^{2+} binding in TM2 of the $GABA_A$ receptor β3 subunit.

6.1 INTRODUCTION

The anion selective channel of the $GABA_A$ receptor is formed by residues of the membrane spanning segments. Previously, residues have been implicated in the structure and function of ligand-gated ion channels by three main approaches: examining the functional effects of mutating putative ion channel residues (Galzi & Changeux, 1994 for review; Chang *et al.*, 1996; Tierney *et al.*, 1996; Birnir *et al.*, 1997a, b; Wooltorton *et al.*, 1997c, 1998; Buhr *et al.*, 1999; Hosie *et al.*, 1999; Thompson *et al.*, 1999b); covalent labelling of residues by photoactivated channel blockers (Galzi & Changeux, 1994; Karlin & Akabas, 1995 for review); and more recently application of the substituted-cysteine accessibility method (SCAM; Xu & Akabas, 1993, 1996; Xu *et al.*, 1995; Karlin & Akabas, 1995 for review). Although each method makes certain assumptions, amino acids in the second transmembrane domain (TM2) have consistently been identified as major contributors to the formation of the ion channel.

SCAM has been used to investigate how the structure of the $GABA_A$ receptor governs functional characteristics including anion conductance, selectivity and gating by systematic identification of residues that face or line the channel lumen (Xu & Akabas, 1993; 1996; Akabas & Karlin, 1998). Consecutive residues in TM2 of the rat $\alpha 1$ $GABA_A$ receptor subunit were mutated individually to cysteine (C) and expressed with wild-type $\beta 1$ and $\gamma 2$ subunits in *Xenopus* oocytes. If the functional responses of the mutant receptors were comparable to wild-type $\alpha 1\beta 1\gamma 2$ responses, then the ability of small charged, hydrophilic sulphydryl-specific reagents to react covalently with the introduced cysteine was determined. The sulphydryl reagents used in the study were water soluble derivatives of methanethiosulphonate (MTS); methanethiosulphonate ethylammonium (MTSEA) and methanethiosulphonate ethylsulphonate (MTSES) and each molecule is less than 0.6nm in diameter (Stauffer & Karlin, 1994), allowing access to the open channel. Application of SCAM revealed that nine residues in TM2 of the rat $\alpha 1$ subunit were exposed in the channel (Fig. 6.1; Xu & Akabas, 1993; 1996).

The successful use of SCAM makes several assumptions. Firstly, if mutant receptor function is similar to wild-type then the structure of the mutant receptor is considered to

be equivalent to the wild-type. Additionally, it is assumed that residues facing the channel lumen represent a section of the water accessible surface of the protein and that in the transmembrane domains, only these luminal facing residues are accessible to charged sulphydryl reagents. Finally, it is inferred that if these reagents react with a luminal facing residue then the agonist-evoked ion conductance will be irreversibly altered (Karlin & Akabas, 1995).

SCAM has been extensively applied to the nicotinic acetylcholine (nACh) receptor to determine a structural basis for receptor function. Channel lining residues have been identified in TM1 (Akabas & Karlin, 1995), the TM1-TM2 loop (Wilson & Karlin, 1998) and TM2 of the mouse muscle nACh α receptor subunit (Akabas *et al.*, 1992; 1994a). In addition, SCAM has been used to investigate a number of other ion channels including a cyclic nucleotide-gated channel (Sun *et al.*, 1996), $P2X_2$ purinergic receptor (Egan *et al.*, 1998), the cystic fibrosis transmembrane regulator (CFTR; Akabas *et al.*, 1994b), voltage-gated potassium channels (Kurz *et al.*, 1995; Lu & Miller, 1995; Pascual *et al.*, 1995) and a voltage-gated sodium channel (Yang & Horn, 1995).

As discussed previously (Chapter 3,4, and 5) a single histidine (H) residue (H267) located in the TM2 of either β1 (Horenstein & Akabas, 1998) or β3 (Wooltorton *et al.*, 1997a) $GABA_A$ receptor subunits is a major determinant for Zn^{2+} inhibition. Mutation of this residue to alanine in β3 homomeric receptors resulted in a near 1200-fold reduction in Zn^{2+} potency (Chapter 3; Wooltorton *et al.*, 1997a). The location of the putative high affinity Zn^{2+} binding site within the TM2 domain implies that Zn^{2+} is able to access a region of the channel previously considered only to be accessible to anions indicating that ion selection is most likely executed nearer to the cytoplasmic entrance of the ion channel. This view is supported by applying SCAM to $\alpha1\beta1\gamma2S$ $GABA_A$ receptors. Positively charged sulphydryl reagents applied externally were able to penetrate the ion channel as far as $\alpha1^{T261}$ (Xu & Akabas, 1996; Fig.6.1.).

The present study developed the concept of SHAM, the substituted histidine accessibility method in order to probe TM2 of the β3 subunit. Based on SCAM, individual residues are systematically mutated to histidine (instead of cysteine) leaving the background mutation H267A intact in order to remove the high affinity binding of

Zn^{2+}. β3 homomeric $GABA_A$ receptors exhibit a spontaneously-gated Cl^- current and can also be activated / modulated by pentobarbitone (PB) and antagonised by both picrotoxin (PTX) and Zn^{2+} (Wooltorton *et al.*, 1997b). The functional properties of the mutant homomeric channels are investigated and then Zn^{2+} is applied externally. If Zn^{2+} is able to reach the new histidine residue in the ion channel, an increase in Zn^{2+} inhibitory potency is expected since the residue is assumed to be facing the channel lumen and therefore available for binding with the cation. In this preliminary study, four residues in the β3 subunit were chosen for investigation, T263, L259, T256 and A252 selected because they align with luminal facing residues in the α1 subunit deduced from SCAM (Fig. 6.1; Xu & Akabas, 1996). These residues were substituted for histidine one at a time by site-directed mutagenesis and expressed as homomeric $GABA_A$ receptors in *Xenopus* oocytes or HEK cells and analysed using two-electrode voltage clamp or patch clamp recording. β3 subunit homomers were used since the effects of substituting histidine in an ion channel with a symmetrical distribution of amino acids should be clearly observable.

6.2. RESULTS

6.2.1. Effect of the $\beta3^{T263H}$ mutation on ion channel properties.

Expression of the mutant $\beta3^{T263H,\ H267A}$ subunits resulted in the formation of functional ion channels. Concentration-response curves for the PB enhancement of the spontaneous conductance demonstrated that the T263H mutation produced a small rightwards shift in the curve, increasing the EC_{50} for PB ($\beta3^{wild\text{-}type}$: EC_{50}, 81.56 ± 10.66μM, n_H, 1.46 ± 0.23; $\beta3^{H267A}$: EC_{50}, 53.14 ± 5.55μM, n_H, 1.66 ± 0.23; $\beta3^{T263H,\ H267A}$: EC_{50}: 131.40 ± 8.06μM, n_H, 1.8 ± 0.19; n = 3-7 oocytes; Fig. 6.2A). Since the PB modulation of the mutant receptor subjected to SHAM was not substantially altered compared to the modulation of the wild-type receptor, the structure of the mutant receptor was assumed to be similar to the wild-type receptor structure.

Zn^{2+} inhibition curves for the spontaneous β3 conductance were constructed using a cumulative dose application protocol (see Chapter 2). Increasing concentrations of Zn^{2+}

were applied until further inhibition of the conductance could not be achieved. This concentration ($\beta3^{wild\text{-}type}$: 10μM; $\beta3^{mutants}$: 2mM) was assumed to have achieved maximal block. The residual leak conductance was then subtracted from each response and the resulting data points plotted and fitted with an inhibition equation. Comparison of Zn^{2+} concentration-inhibition curves revealed that the introduction of a histidine residue at position 263 in TM2 resulted in a near 100-fold increase in Zn^{2+} potency compared to the $\beta3^{H267A}$ mutant receptor, implying that Zn^{2+} is able to penetrate the anion channel at least as far as this residue. However, the Zn^{2+} sensitivity of the SHAM mutant is still 10-fold lower than wild-type β3 receptors ($\beta3^{wild\text{-}type}$: IC_{50}, 0.17 ± 0.03μM, n_H, 0.58 ± 0.04; $\beta3^{H267A}$: IC_{50}, 193.94 ± 13.96μM, n_H, 2.36 ± 0.38; $\beta3^{T263H, H267A}$: IC_{50}: 2.16 ± 0.47μM, n_H, 0.44 ± 0.04; n = 3-7 oocytes; Fig. 6.2A, C). Interestingly, introduction of the T263H mutation reduced the Hill coefficient for the Zn^{2+}antagonism to the value obtained for wild-type β3 receptors.

6.2.2. $\beta3^{L259H, H267A}$ subunits do not form functional receptors.

Injection of $\beta3^{L259H, H267A}$ cDNA into *Xenopus* oocytes failed to result in the formation of functional β3 homomeric receptors (n = 20 oocytes). No response to PB (#1mM) was observed. Furthermore, the resting conductance was not blocked by either Zn^{2+} (#1mM) or PTX (#100μM; IC_{50}s: $\beta3^{wild\text{-}type}$, 84nM, $\beta3^{H267A}$, 24nM, Wooltorton *et al.*, 1997a). In order to investigate whether the expression system utilized affected functional receptor formation, HEK cells were co-transfected with the SHAM mutant cDNA and the reporter DNA encoding for green fluorescent protein (GFP; Heim *et al.*, 1995), a positive control for DNA transcription. Although a proportion of these cells exhibited the characteristic green fluorescence image, they failed to express functional receptors (n = 8 cells).

6.2.3. Mutating $\beta3^{T256}$ abolished the pentobarbitone modulation of the homomeric β3 subunit receptor.

Expression of $\beta3^{T256H,\ H267A}$ subunits in *Xenopus* oocytes resulted in receptors which were not modulated by PB (#1mM; n = 12 oocytes; Fig. 6.3C). However, the spontaneous Cl^- conductance of these receptors was blocked by both PTX (100μM) and Zn^{2+} (10μM – 2mM). The potency of Zn^{2+} on $\beta3^{T256H,\ H267A}$ receptors was comparable to that for $\beta3^{H267A}$ homomeric receptors, with concentration-inhibition curves for the Zn^{2+} antagonism of the spontaneous conductance producing an IC_{50} of 192.54 ± 26.22μM (n_H: 1.16 ± 0.16; n = 5 oocytes; $\beta3^{H267A}$, IC_{50}: 193.94 ± 13.96μM, n_H, 2.36 ± 0.38; n = 3 oocytes; Fig. 6.3A, B). In order to examine whether the reduction in membrane conductance was due to a nonspecific action of Zn^{2+}, uninjected oocytes from the same donor *Xenopus* were treated with high Zn^{2+} concentrations (200μM – 2mM). The membrane conductance of uninjected oocytes was unaffected by the presence of 1mM Zn^{2+} (101 ± 3% control, n = 6 oocytes) whereas the conductance of oocytes injected with $\beta3^{T256H,\ H267A}$ cDNA was almost abolished (9 ± 1% control conductance, n = 5 oocytes).

6.2.4. Consequence of the A252H mutation on $GABA_A$ receptor β3 subunit function.

Injection of mutant $\beta3^{A252H,\ H267A}$ cDNA into *Xenopus* oocytes resulted in functional receptors exhibiting both spontaneous and PB-modulated currents. PB concentration-response curves were established and the PB EC_{50} for $\beta3^{A252H,\ H267A}$ receptors was equivalent to $\beta3^{H267A}$ homomeric $GABA_A$ receptors ($\beta3^{H267A}$: EC_{50}, 53.14 ± 5.55μM, n_H, 1.66 ± 0.23; $\beta3^{A252H,\ H267A}$: EC_{50}: 46.62 ± 1.82μM, n_H, 1.27 ± 0.06; n = 3-5 oocytes; Fig. 6.4A).

In addition, the spontaneous conductance transduced by $\beta3^{A252H,\ H267A}$ mutant receptors was sensitive to Zn^{2+}. Construction of Zn^{2+} concentration-inhibition curves yielded an IC_{50} of 164.53 ± 6.47μM (n_H: 1.85 ± 0.13; n = 5 oocytes, Fig. 6.4B), which is not significantly lower than the IC_{50} for the Zn^{2+} antagonism of $\beta3^{H267A}$ receptors (193.94 ±

13.96μM, n_H, 2.36 ± 0.38; n = 3 oocytes; P>0.05, unpaired t-test). Comparable to $\beta3^{H267A}$ receptors, $\beta3^{A252H,\ H267A}$ mutant receptors were insensitive to 10μM Zn^{2+}, a concentration that maximally inhibits wild-type β3 receptors (Fig. 6.4C). Interestingly, $\beta3^{A252H,\ H267A}$ receptors were not susceptible to antagonism by PTX (#100μM).

6.3. DISCUSSION

In this study, the substituted histidine accessibility method (SHAM) has been developed and applied to four amino acids that are presumed to be located on adjacent turns of the postulated α-helical structure of TM2 and are believed to line the channel lumen (Xu & Akabas,1993, 1996; Fig. 6.1). The effect of substituting histidines for TM2 residues was examined using $\beta3^{H267A}$ homomeric $GABA_A$ receptors since these mutant receptors display a 1000-fold reduction in Zn^{2+} sensitivity compared to wild-type β3 receptors (Wooltorton *et al.*, 1997a; Chapter 3). Therefore an increase in Zn^{2+} sensitivity due to a substituted histidine should be easily discernible. Similar to SCAM, several assumptions are made in the application of SHAM. It is assumed that Zn^{2+} is only able to access the residues which are exposed in the channel. In addition, it is inferred that if the Zn^{2+} sensitivity of the receptor is increased by the introduction of a histidine, then Zn^{2+} is able to access the anion channel and react with that residue. However, probing the accessibility of substituted cysteines in ion channels with sulphydryl reagents is irreversible, whereas the inhibitory effect of Zn^{2+} is readily removed.

In addition to investigating the accessibility of the channel to Zn^{2+}, the effects of introducing a highly polar residue into regions of the putative ion channel pore are also observed. This is in contrast to SCAM, which substitutes a non-polar cysteine for ion channel residues. In addition, histidine has a much larger side-chain volume (70Å) compared to cysteine (38Å; Zuhlke *et al.*, 1994). Recent investigations of TM2 indicate that the side-chain volume (rather than side-chain polarity, hydrophilicity or hydropathicity) can affect certain channel properties including agonist gating and isoflurane potentiation (Koltchine *et al.*, 1999). By applying SHAM to heteromeric αβ receptors in which the high affinity Zn^{2+} binding site has been removed, the effect of substituting histidines on both Zn^{2+} permeability and GABA gating could be

investigated. Furthermore, as other membrane spanning domains have been implicated in forming the ion channel pore (Akabas & Karlin, 1995; Zhang & Karlin, 1997) SHAM could also be applied to residues in these regions, providing these residues are not located intracellular to the putative charge selectivity filter.

In order to simplify comparisons between TM2 residues of different ligand-gated ion channels, the terminology in which the arginine at the proposed N-terminus (i.e. intracellular end) of the TM2 region is assigned the position 0' is used (Miller, 1989; Charnet *et al.*, 1990; Fig. 6.5.). Thus the effects of substituting histidines for 13' (T263H), 9' (L259H), 6' (T256H) and 2' (A252H) residues was investigated (Fig. 6.6.).

6.3.1. 13' mutations.

Introduction of a histidine residue at the 13' position in TM2 resulted in functional β3 homomeric $GABA_A$ receptors that were modulated by PB. Although the T13'H mutation resulted in an approximate 2-fold reduction in the potency of PB, this mutation was assumed not to have substantially altered receptor structure. However, the mutant $\beta3^{T263H,\ H267A}$ receptor demonstrated a 100-fold *increase* in sensitivity to Zn^{2+}compared to $\beta3^{H267A}$ receptors, implying that a new Zn^{2+} binding site has been introduced into the ion channel and that Zn^{2+} is able to permeate this deep into the anion channel. Interestingly, the Zn^{2+} sensitivity of this SHAM mutant is still 10-fold lower than the wild-type β3 receptor in which the histidine is present at position 267 (17') although the Hill coefficients for the Zn^{2+} antagonism of both receptors were equivalent. A possible explanation for the difference in Zn^{2+} potency observed when a histidine is present either at the 13' or 17' position may be due to the requirements of a Zn^{2+} binding site. The affinity of a Zn^{2+} binding site is dependent on the number and relative position of chelating residues and also the local electrostatic environment (Higaki *et al.*, 1992; Regan, 1993; Berg & Shi, 1996). Metal ion binding sites often have electrostatic surfaces and are in a region of high negative potential, which enhances the affinity for metal ions (Regan, 1993). Furthermore, histidine residues acting as Zn^{2+} binding sites commonly form a hydrogen bond with the carboxylate side chain of a nearby aspartate/glutamate residue, which serves to increase the basicity of histidine and thus

increase the affinity of the site (Christianson & Alexander, 1989). In the wild-type β3 receptor, a glutamate residue is located three residues extracellular to the 17' histidine and may therefore influence the interaction between the histidines and Zn^{2+} and increase the affinity of the binding site. Metal binding histidine residues are also able to form hydrogen bonds with carbonyl side chains, usually from glutamine or asparagine residues, although this interaction is only half as effective in increasing the basicity of the histidine residue (Christianson & Alexander, 1989). In the SHAM mutant receptor, the 13' histidine is flanked by an asparagine residue (15'; $\beta3^{N265}$) which could contribute towards Zn^{2+} binding, although not mediate the high affinity Zn^{2+} binding observed in wild-type β3 subunits. (Fig. 6.5.).

Alternatively, Zn^{2+} may not penetrate the anion channel. Instead the T13'H mutation may produce a conformational alteration that allosterically enhances Zn^{2+} binding elsewhere on the receptor (i.e. the low affinity Zn^{2+} site). However, this appears unlikely for several reasons. The PB modulation of the channel is not dramatically altered, suggesting that the T13'H mutation does not evoke a substantial change in receptor conformation *per se*. Furthermore, the Hill coefficient for the Zn^{2+} antagonism of the spontaneous conductance transduced by $\beta3^{H267A}$ receptors (2.36 ± 0.38) is substantially reduced by the introduction of a histidine at the 13' position (0.44 ± 0.04), to a level comparable with wild-type β3 receptors (0.58 ± 0.04). This reduced Hill coefficient may be due either to a lower cooperativity of Zn^{2+} binding at the introduced site or to a change in channel gating.

In addition, SCAM has demonstrated that this residue in the $GABA_A$ receptor α1 subunit (T268) is accessible to the positively charged sulphydryl reagent MTSEA in both the absence and presence of GABA, indicating that cations are able to penetrate the channel this far (Xu & Akabas, 1993; 1996). Similarly, SCAM has been applied to the muscle nACh receptor α subunit, and a negatively charged sulphydryl reagent was able to react with the 13' valine in the presence, but not in the absence, of acetylcholine (Akabas *et al.*, 1994a). Therefore the charge selectivity filter in both $GABA_A$ and nACh receptors appears to be more cytoplasmic than the 13' residue. This is in agreement with the view that Zn^{2+} is able to reach and bind to a substituted binding site in the anion channel.

Other studies have found the nature of the 13' residue to be important for receptor function. Amino acid residues believed to line the ion channels of glycine and $GABA_A$ receptor α1 subunits were substituted for the aligned residues in the α7 neuronal nACh subunit and expressed as homomers in *Xenopus* oocytes (Galzi *et al.*, 1992). Mutation of the 13' valine (V) to threonine resulted in a near 200-fold increase in apparent affinity for ACh, altered response desensitization and current rectification and converted dihydro-β-erythroidine (DHβE), a competitive antagonist of wild-type α7 nACh receptors, into an agonist. Receptors containing the V13'T mutation were still selective for cations, but subsequent insertion of a proline (P) between positions –1' and –2' and substitution of a neutral alanine at position 2' converts the ion selectivity from cationic to anionic. The nature of the 13' residue is important since mutating the 2' residue and inserting a proline residue between positions –1' and –2', whilst leaving the valine intact does not result in functional receptor expression (Galzi *et al.*, 1992). Equivalent mutations of the $5\text{-}HT_3$ receptor also increased the agonist sensitivity and eliminated most of the desensitization in response to 5-HT (Gunthorpe *et al.*, 1996). Wild-type $5\text{-}HT_3$ receptors are principally cation selective channels; reducing the extracellular concentration of NaCl altered the reversal potential to a more negative potential. However, when exposed to low extracellular NaCl, the reversal potential of the $5\text{-}HT_3$ mutant receptor was altered to a more positive potential suggesting that the mutant channel was substantially permeable to Cl^-. Therefore, the 13' threonine, conserved throughout all $GABA_A$ and $GABA_C$ receptor subunits and glycine α receptor subunits appears to be important, although not solely responsible, for the anion selectivity of these receptors.

6.3.2. 9' mutations.

Introduction of the L9'H mutation into $\beta3^{H267A}$ receptors did not result in functional receptor expression and no detectable responses to either modulators or blockers of $GABA_A$ receptors were obtained, indicating that the nature of the residue at this position is critical for ion channel function. It is not clear from these experiments whether $\beta3^{L259H,\ H267A}$ receptors are expressed at the cell surface and exhibit impaired gating or whether the mutation effects receptor assembly /transport to the cell surface. However,

the 9' leucine is highly conserved throughout nACh, glycine, $GABA_A$ and $5HT_3$ receptors, and appears to have a key role in the gating of these channels.

Substitution of this leucine with cysteine, resulted in functional nACh (Akabas *et al.*, 1994a) and $GABA_A$ receptors (Xu & Akabas, 1996), which were susceptible to modification by extracellular application of sulphydryl reagents implying a channel luminal facing position. Therefore, the hydrophobicity of the residue at the 9' position appears to be important for receptor function as cysteine is also nonpolar but with a smaller side chain than leucine. There have been many investigations on the effect of mutating the 9' leucine on the functional properties of several receptors. In accordance with the present study, introduction of a polar residue (threonine) at this position in heteromeric $\alpha1\beta1$ $GABA_A$ receptors dramatically alters receptor function (Tierney *et al.*, 1996). Cells in which one or both subunits were mutated (L9'T) expressed receptors that form constitutively open Cl^- channels in the absence of ligand. This spontaneous Cl^- conductance was blocked by 10mM penicillin, but bicuculline (100μM), PTX (100μM) and PB (100μM) were ineffective. This would agree with the present study assuming $\beta3^{L9'H,\ H17'A}$ receptors are expressed at the cell surface. The L9'T mutation also effects the GABA-activated response in a subunit-specific manner. When the mutation was present in the β1 subunit, no response to GABA was detected, although these receptors bound [^{3}H]-muscimol, indicating the formation of heteromeric receptors at the cell surface. This implies that the β subunit is a key determinant in coupling GABA binding to the receptor with opening of the ion channel (see Amin & Weiss, 1993) and that the L9'T mutation disrupts that transduction linkage. Heteromeric receptors which only contained the mutation in the α1 subunit were sensitive to GABA but the rise time and decay of GABA-activated currents were slower. The GABA-evoked conductance was sensitive to bicuculline, PTX, and penicillin but interestingly was not potentiated by PB. This 9' leucine residue has also been replaced with serine (a polar residue) in either α1, β2 or γ2 $GABA_A$ receptor subunits. In contrast to the study by Tierney and colleagues, coexpression of a mutant subunit with the two other wild-type receptors resulted in functional ion channels with an increased sensitivity to GABA and partial agonists, even when the mutation is in the β subunit, and spontaneous openings which were blocked by PTX and bicuculline (Chang *et al.*, 1996; Thompson *et al.*, 1999b). The difference observed in GABA sensitivity in mutating $\beta^{L9'}$ in either αβ or αβγ receptors

may reflect possible differences in receptor stoichiometry. The $\beta2^{L9'S}$ mutation also increased the sensitivity of the $\alpha1\beta2\gamma2$ receptor to direct activation by PB indicating that observed increases in agonist sensitivity is not limited to compounds acting at the GABA binding site (Thompson *et al.*, 1999b). Interestingly, $\beta2^{L9'S}$ subunits failed to form functional homomeric receptors (e.g. displaying spontaneously openings) which is in agreement with the comparable β3 subunit mutation in the present study. However, evidence suggests that wild-type β2 subunits themselves do not form functional homomeric receptors (Connolly *et al.*, 1996b *cf.* Cestari *et al.*, 1996).

Recently, the replacement of the 9' leucine with either a small (alanine, valine, glycine) or a polar residue (serine, threonine, or tyrosine) resulted in ρ1 $GABA_C$ receptors which were spontaneously open in the absence of GABA (Pan *et al.*, 1997; Chang & Weiss, 1998). The PTX sensitivity of the receptor was reduced by these mutations to varying degrees and GABA antagonised several of the mutant receptors although an apparent correlation between either PTX or GABA sensitivity and the nature of the residue at the 9' position was not established. Substitution of L9' with other hydrophobic residues (isoleucine or phenylalanine) did not increase the resting Cl^- conductance (Chang & Weiss, 1998). This study supports the view that this highly conserved leucine plays a crucial role in channel gating.

Introduction of a polar residue at the 9' position of cation-selective ligand-gated ion channels also resulted in a significant alteration of receptor function, increasing agonist sensitivity and reducing the rate of desensitization in homomeric α7 nACh receptors (Revah *et al.*, 1991; Bertrand *et al.*, 1997) and homomeric 5-HT_3 receptors (Yakel *et al.*, 1993). Each additional L9'T (or S) mutation in the pentameric muscle nACh receptor increased the EC_{50} for ACh by approximately 10-fold, so that a receptor containing four mutant subunits was nearly 10,000-fold more sensitive to ACh than the wild-type nACh receptor (Filatov & White, 1995; Labarca *et al.*, 1995). Introduction of a polar residue at the 9' position is thought to stabilise the open state of the channel by reducing channel closing rate (Filatov & White, 1995). Likewise, increasing the hydrophobicity of the side chain at the 9' position (achieved either by the addition of a methyl group or replacing an oxygen with a methyl group) reduced ACh sensitivity (Kearney *et al.*, 1996). Side chain stereochemistry further alters the effects of the polarity of the side

chain at the 9' position (Kearney *et al.*, 1996). Substituting L9' for threonine in homomeric α7 nACh receptors transformed 5-HT from a non-competitive antagonist to an agonist (Palma *et al.*, 1996) whereas the antagonist DHβE is converted to a full agonist by this mutation (Bertrand *et al.*, 1992). Consistent with studies on other receptors, a significant fraction of the L9'T mutant α7 channels spontaneously open in the absence of agonist (Bertrand *et al.*, 1997). Interestingly, construction of a double mutant α7(L9'T, V13'T) receptor resulted in ion channels with a substantially greater level of spontaneous opening suggesting that the identity of nearby residues is also important in channel gating processes.

Structural data for the nACh receptor implies that TM2 is an α helix and that the conserved L9' residues occur at a kink in each of the M2 helices and point into the closed channel forming the channel gate (Unwin, 1993, 1995). Agonist binding causes these leucines to rotate, opening the channel. Photoincorporation experiments using the noncompetitive antagonist 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine further suggest the conserved leucines from each subunit interact with each other to form a physico-chemical barrier that restricts ion flow in the resting state of the receptor (White & Cohen, 1992). Introducing a polar residue at this position would destabilise the leucine ring, causing a reduction in the channel closure rate. The receptor would then favour the open conformation, explaining the observed decrease in desensitization and presence of constitutively open channels. However, homomeric nACh, 5-HT_3 and $GABA_C$ ρ1 receptors containing a polar residue in the 9' position still display some gating activity (Revah *et al.*, 1991; Yakel *et al.*, 1993; Bertrand *et al.*, 1997; Pan *et al.*, 1997; Chang & Weiss, 1998), indicating that the gate may be elsewhere. Indeed, application of SCAM to nACh and $GABA_A$ receptors implies that the gate is much more cytoplasmic than the 9' position (Akabas *et al.*, 1994a; Xu & Akabas, 1996; Wilson & Karlin, 1998). Either the nature of the amino acid at the 9' channel gate is not entirely essential for the gating mechanism or other regions of the channel pore also determine channel gating. Therefore the present study supports an important role for the 9' leucine in channel gating whether or not this residue is the actual gate.

6.3.3. 6' mutations.

Expression of $\beta3^{T256H,\ H267A}$ receptors resulted in ion channels that are also no longer modulated by PB, but in contrast to the L9'H mutation, these receptors are still antagonised by PTX and Zn^{2+}. However, the Zn^{2+} sensitivity is equivalent to the sensitivity of $\beta3^{H267A}$ receptors suggesting that: the 6' residue is not luminal facing; Zn^{2+} is unable to penetrate this far into the anion channel; or Zn^{2+} penetrates, binds, but does not affect the current through the channel (i.e. silent binding). It is likely that the 6' residue in the β3 subunit is luminal facing since application of SCAM to both the α subunit of the nACh receptor and the $GABA_A$ receptor α subunit identified this residue as lining the ion channel pore (Akabas *et al.*, 1992; 1994a; Xu & Akabas, 1996). Sulphydryl reagents applied in the either the absence or presence of ligand were able to modify this residue implying the gating element in these receptors are closer to the cytoplasmic side of the channel. Furthermore, extracellular application of the positively charged sulphydryl reagent MTSEA modified the 6' threonine in the $GABA_A$ receptor α1 subunit implying the charge selectivity filter is at least as cytoplasmic as the 6' residue in the channel (Xu & Akabas, 1996). Therefore on the basis of charge, Zn^{2+} should be able to access the residue in the 6' position. Correct orientation of histidine side chains is required for Zn^{2+} binding (Higaki *et al.*, 1992). When a histidine residue is introduced at the 6' position, the imidazole group may be prevented from adopting the preferred orientation and therefore be ineligible as a binding site for Zn^{2+}. However, since other pharmacological properties of the GABA channel are dramatically altered (i.e. the PB modulation of the channel is abolished) the T6'H mutation may result in a global change of the receptor conformation. Introduction of a charged, highly polar residue at this position may distort the channel structure and this residue may no longer be accessible from the channel lumen. Alternatively Zn^{2+} may bind to the substituted histidine but be subsequently unable to produce inhibition. Interestingly although the IC_{50} is unaltered, the Hill coefficient for the Zn^{2+} antagonism of the spontaneous conductance is reduced by the 6' substitution (n_H: $\beta3^{T256H,\ H267A}$, 1.16 ± 0.16; $\beta3^{H267A}$, 2.36 ± 0.38).

A threonine is found at the 6' position throughout all $GABA_A$ and $GABA_C$ receptor subunits and glycine receptor α subunits. As reported for 9' mutations, substitution of T6' results in constitutive channel opening and the reversal of agonist action (i.e.

becomes an antagonist) on channel gating (Pan *et al.*,1997). Replacement of T6' with alanine in rat ρ1 receptors expressed in *Xenopus* oocytes resulted in a spontaneous inward current which was partially blocked by low concentrations of GABA. Higher concentrations of GABA evoked inward currents. However substitution of T6' with valine or leucine (also hydrophobic residues but possess larger side chains than alanine) or glycine (G; neutral with the smallest side chain volume, e.g. -H) does not result in spontaneous channel opening suggesting that both the hydrophobicity and the sidechain volume of the residue introduced at the 6' position is important for this phenomenon. Interestingly the magnitude of the spontaneous current generated by $\rho1^{T6'A}$ receptors was equivalent to the spontaneous current observed for $\rho1^{L9'A}$receptors. Construction of the double mutant $\rho1^{T6'A,\ L9'A}$ receptor resulted in substantially increased spontaneous currents which were almost completely inhibited by GABA, due to channel closure rather than receptor desensitization, and were not re-opened by high GABA concentrations (mM) (Pan *et al.*,1997).The 6' position is located only one turn of the α helix away from the 9' leucine previously thought to constitute the channel gate in nACh receptors (Unwin 1993; 1995). The effects of mutating T6' are consistent with changes in channel gating. However, the double mutant $\rho1^{T6'A,\ L9'A}$ receptor still displays some gating activity. This further suggests that the side chains of the 6' and 9' residues are not entirely crucial for the gating mechanism or still further residues are also involved in the gating of the channel (Pan *et al.*,1997).

Mutation of T6' to phenylalanine in either subunit of rat α1β2γ2 $GABA_A$ receptors conferred PTX insensitivity (≤100μM; Gurley *et al.*, 1995). Similarly, in rat ρ1 receptors mutating this threonine to methionine, found at the corresponding site of the rat ρ2 subunit produced a dramatic (~ 600-fold) reduction in PTX sensitivity (Zhang *et al.*, 1995), suggesting that the polarity of the 6' residue is important for PTX binding. This would support the observation that $\beta3^{T256H,\ H267A}$ mutant channels were blocked by PTX. However, $GABA_A$ receptors containing α1T6'C mutant subunits were inhibited by PTX (Xu *et al.*, 1995) indicating that a hydrophobic residue in this position does not necessarily confer PTX insensitivity.

The 6' residue has also been implicated in aiding certain permeant organic cations to pass through this narrow region of the channel in mouse nACh receptors expressed in

Xenopus oocytes (Cohen *et al.*, 1992). Mutation of the 6' residue (either serine or threonine) to alanine increased the relative permeability of some organic cations which contain both hydrophobic and hydrophilic moieties. By increasing the hydrophobicity of the 6' residue, this residue is able to act more strongly with the hydrophobic moiety of the organic cation and the 2' threonine is able to interact with the hydrophilic moiety thus aligning the cations axially in the channel lumen.

6.3.4. 2' mutations.

Injection of $\beta3^{A252H,\ H267A}$ mutant subunits into *Xenopus* oocytes resulted in the expression of functional receptors, which had comparable PB sensitivity to $\beta3^{H267A}$ receptors. Furthermore, the Zn^{2+} sensitivity of the SHAM mutant was not substantially higher than $\beta3^{H267A}$ receptors, implying that if this residue is accessible from the channel then Zn^{2+} is either unable to reach or bind to the introduced histidine or it binds but does not affect channel function. Since the PB modulation is unaltered by the A2'H mutation, it is unlikely that the unchanged Zn^{2+} sensitivity is due to a global distortion of receptor structure. Indeed the Zn^{2+} concentration-inhibition plots for the antagonism of both $\beta3^{A252H,\ H267A}$ and $\beta3^{H267A}$ are coincident, suggesting that lower affinity Zn^{2+} binding site(s) has not been affected. It appears most likely that Zn^{2+} is unable to permeate the anion channel as far as the 2' histidine. Extracellular application of the positively charged sulphydryl reagent MTSEA either in the presence or absence of GABA did not modify the V2'C mutant in the $GABA_A$ receptor $\alpha1$ subunit (Xu & Akabas, 1996). This implies that the charge selectivity filter is located extracellular to the 2' position and so Zn^{2+} would be prevented from penetrating this far into the channel lumen. In comparison, negatively charged sulphydryl reagents were able to modify V2'C in the closed state of the channel, indicating that the channel gate is probably located intracellular to this position (Xu & Akabas, 1996).

The 2' position is a structural determinant of the sensitivity of the $GABA_A$ receptor to PTX. In support of this, $\beta3^{A252H,\ H267A}$ receptors were insensitive to PTX ($\leq$100μM, representing at least a 4000-fold reduction in sensitivity compared to $\beta3^{H267A}$ receptors). A naturally occurring mutation (A2'S) found in the *Drosophila* homomeric $GABA_A$ receptor RDL dramatically reduces the sensitivity (~100-fold) of the receptor to PTX

and cyclodiene insecticides (Ffrench-Constant *et al.*, 1993). Introducing a polar serine residue at the 2' position in the RDL receptor may sterically or electrostatically impede the interaction of PTX with its binding site in the channel lumen (Ffrench-Constant *et al.*, 1993). Introduction of a histidine residue at the 2' position would change the net charge in addition to increasing polarity and so might be expected to prevent PTX binding in a similar manner. However, replacing alanine with serine differs from the A2'H mutation in that it does not entirely eliminate PTX sensitivity. The 2' residue is occupied by a serine in wild-type $GABA_A$ receptor $\gamma1, \gamma2, \delta$ subunits and $GABA_C$ receptor $\rho2$ and $\rho3$ subunits. The presence of $\gamma2^{S2'}$ does not prevent PTX antagonizing $\alpha1\beta1\gamma2$ receptors expressed in *Xenopus* oocytes (Xu *et al.*, 1995), although it is possible that $\gamma2^{S2'}$ does not structurally correspond to the 2' residue in either α or β residues, i.e. it may not line the channel lumen. Interestingly, $\rho2$ and $\rho3$ receptors are sensitive to PTX (Wang *et al.*, 1994; Shingai *et al.*, 1996) although less sensitive than homo- or heteromeric $GABA_A$ receptors. Human homomeric $\rho1$ receptors, which contain a proline at the 2' position, are 10-fold less sensitive to PTX than $\rho2$ receptors (Wang *et al.*, 1995a). The $\rho1^{P2'S}$ mutation increases the PTX sensitivity comparable to that for $\rho2$ receptors. Replacement of the proline with glycine or alanine, amino acids found at the 2' position in some $GABA_A$ and glycine receptor subunits, produced a dramatic increase in PTX sensitivity and almost eliminated the agonist-dependent components of PTX inhibition observed with wild-type ρ receptors (Wang *et al.*, 1995a). Therefore the nature of the residue at the 2' position is crucial for both elements of PTX antagonism. In RDL GABA receptors expressed in *Xenopus* oocytes, isoflurane potentiates the GABA response but at high concentrations produces a depression of the conductance (Edwards & Lees, 1997). However, in mutant A2'S RDL receptors GABA–induced currents are enhanced at high isoflurane concentrations and show no tendency towards saturation. It is thought that the inhibitory effect of isoflurane is due to low affinity channel block via the PTX site of action (Edwards & Lees, 1997).

SCAM has been applied to a region of TM2 in the $GABA_A$ receptor $\alpha1$ subunit in order to examine the mechanism of PTX inhibition (Xu & Akabas, 1995). Coexpression of mutant $\alpha1$ subunits ($\alpha1^{V2'C}$ or $\alpha1^{T6'C}$) with wild-type $\beta1$ and $\gamma2$ subunits in *Xenopus* oocytes resulted in functional receptors. Coapplication of 100μM PTX with GABA resulted in a complete inhibition of the GABA-gated current of both mutant receptors.

A sulphydryl reagent was applied in addition to GABA and PTX following a 'residue protection' protocol. After washing, the GABA-gated currents of receptors containing the $\alpha1^{V2'C}$ mutant subunit were equivalent in magnitude to initial control levels, implying that PTX protected this residue from covalent modification by the sulphydryl reagent. However the GABA-gated currents recorded from $\alpha1^{T6'C}$ mutant subunit-containing receptors were irreversibly altered (Xu & Akabas, 1995). This is in agreement with the PTX binding site being located in the ion channel at the level of the 2' position.

Residues at the 2' position are considered to be at the narrowest part of the nACh receptor pore (reviewed by Lester, 1992). Substituting amino acids at this position in nACh receptors, alters single channel conductance (Imoto *et al.*, 1991; Villarroel *et al.*, 1991; Cohen *et al.*, 1992b) ion selectivity (Cohen *et al.*, 1992b) and the permeability ratios of organic and alkali metal cations (Cohen *et al.*, 1992a). Introduction of an acidic residue (T2'D) in the equivalent position in α7 neuronal nACh receptors, resulted in a higher permeability to divalent cations over monovalent cations (Ferrer-Montiel *et al.*, 1994). The mutant channels were permeable to Mg^{2+}, a blocker of wild-type α7 channels. In addition, the T2'D mutation increased the sensitivity to ACh and decreased the rate of desensitization, a result also seen in mutating 13' and 9' residues in these receptors (see 6.3.1 and 6.3.2.) suggesting a tight coupling between agonist binding sites and the channel pore. In the nACh receptor, introducing residues with larger side chains reduced conductance (Imoto *et al.*,1991; Villaroel *et al.*, 1992). However, an equivalent introduction of a methyl side chain at this position in α1 subunit glycine receptors (G2'A) did not decrease conductance (Borman *et al.*, 1993). Instead the mutant channel adopted an additional higher main state conductance typical of α2 and α3 subunits in which alanine is found at the 2' position. The authors suggested that the side chain exerted a conformational rather than a steric effect.

6.3.5. Summary.

Since a Zn^{2+} binding site has been identified within the anion channel of the $GABA_A$ receptor (Wooltorton *et al.*,1997a; Horenstein & Akabas, 1998), the substituted histidine accessibility method (SHAM) was developed as a concept in order to

investigate whether Zn^{2+} was able to further access this region. This preliminary study investigated the effects of individually substituting four putative channel lining residues with histidines in homomeric $\beta3^{H267A}$ $GABA_A$ receptor constructs in which the high affinity binding site for Zn^{2+} has been removed. A 100-fold increase in Zn^{2+} potency was observed when a histidine was substituted at the 13' position (T268H) inferring that Zn^{2+} is able to penetrate into the anion channel at least as far as this residue. A loss of receptor function resulting from substituting histidine at the 9' position (L259) although consistent with a key role of this conserved residue in channel gating, precludes investigating the ability of Zn^{2+} to access this site. Introducing a histidine at the 6' position (T256H) did not increase the Zn^{2+} sensitivity of the mutant receptor. However, inferring that Zn^{2+} is unable to access this residue may be over simplistic since PB sensitivity is abolished suggesting that receptor conformation is substantially altered. Assuming the 6' residue is luminal facing, increasing the polarity of this 6' site combined with the introduction of a residue containing a positively charged side chain may distort the channel conformation. Thus this residue may no longer be accessible for binding Zn^{2+}, or alternatively Zn^{2+} binding to the substituted histidine may be unable to produce inhibition. Substituting histidine at the 2' position (A252H), the most cytoplasmic residue tested, also failed to increase Zn^{2+} potency. Since PB and PTX pharmacology were largely unaffected, it was assumed that Zn^{2+} is unable to access this residue, a view consistent with other approaches. In conclusion, SHAM represents a novel approach for examining the accessibility of the $GABA_A$ receptor ion channel to Zn^{2+}. Although the introduction of a highly polar, charged histidine is not tolerated at some positions, further application of SHAM to the TM2 domain provides a useful insight into charge selectivity and the location of the ion channel gate.

Figure 6.1. Putative channel lining residues in TM2 of the $GABA_A$ receptor. Schematic diagram illustrating the TM2 regions of the $GABA_A$ receptor α1 and β3 subunits. The α1 subunit residues accessible to methanethiosulfonate (MTS) derivatives (■) using the substituted cysteine accessibility method are assumed to face the channel pore (Xu & Akabas, 1993, 1996). The accessibility of the ion channel to the negatively charged methanethiosulphonate ethylsulphonate (MTSES) and the positively charged methanethiosulphonate ethylammonium (MTSEA) is shown (limit of yellow arrows; Xu & Akabas, 1993, 1996). Aligned residues in the β3 subunit (□) were therefore presumed to also line the channel and are numbered according to the terminology in which the arginine residue at the proposed N-terminus of the TM2 region is assigned the position 0' (Miller, 1989; Charnet *et al.*, 1990). Mutation of a putative channel lining residue (17';□) in the β3 subunit ^{H267A} dramatically reduces Zn^{2+} potency (Wooltorton *et al.*, 1997a; Chapter 3). In order to investigate the accessibility of the channel pore to Zn^{2+}, four putative channel lining residues (■) were systematically mutated to histidine, leaving the background H267A mutation intact. The mutant subunits were then expressed as homomeric receptors and probed with Zn^{2+}.

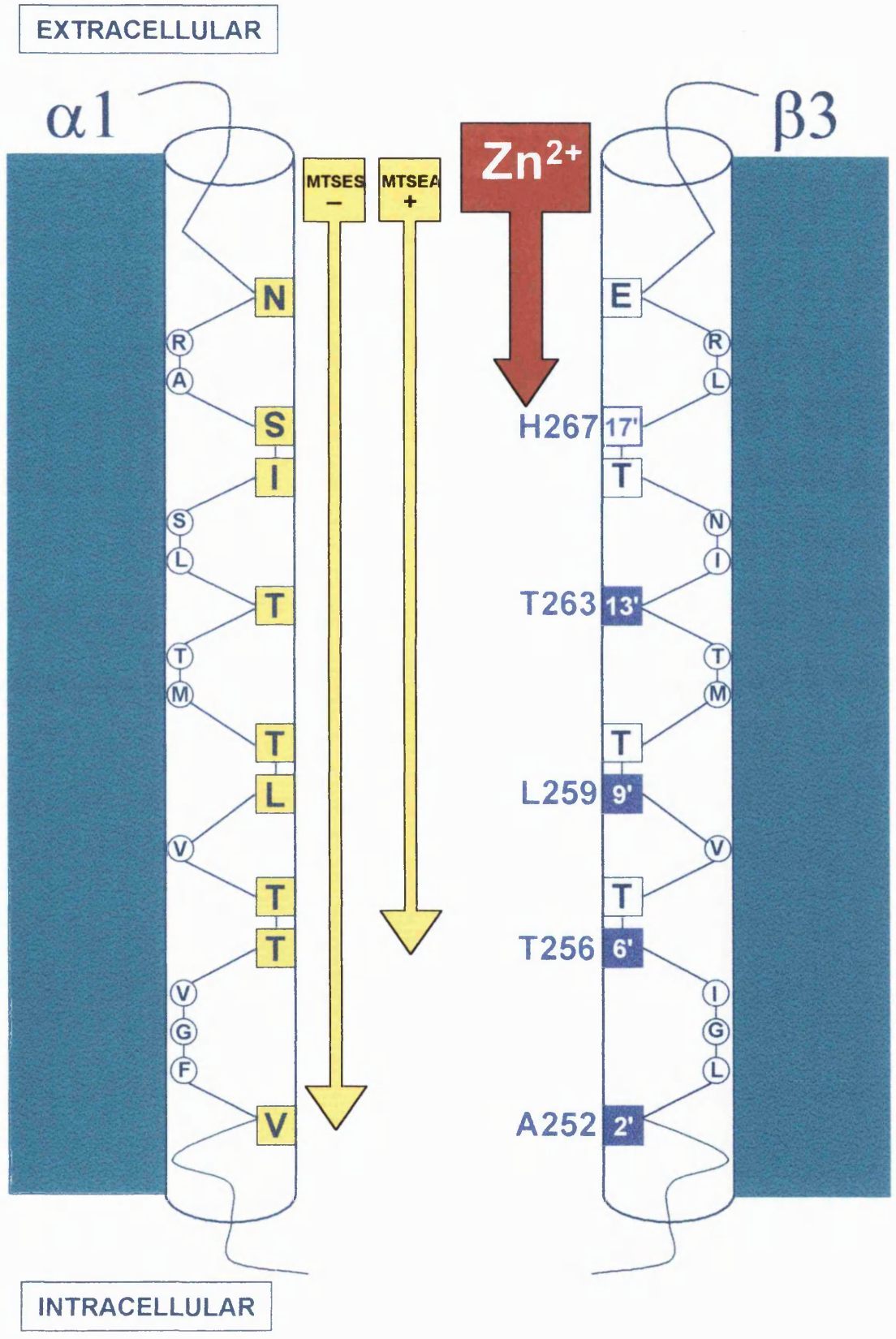

EXTRACELLULAR
α1
β3
MTSES
−
MTSEA
+
Zn2+
N
R
A
S
I
S
L
T
T
M
T
L
V
T
T
V
G
F
V
E
R
L
H267
17'
T
N
I
T263
13'
T
M
T
L259
9'
V
T
T256
6'
I
G
L
A252
2'
INTRACELLULAR

Figure 6.2. $\beta3^{T263H,\ H267A}$ receptors possess an intermediate sensitivity to Zn^{2+}. (A) Concentration-response curves for PB were constructed for $\beta3^{wild\text{-}type}$, $\beta3^{H267A}$ and $\beta3^{T263H,\ H267A}$ subunit receptors. Data were normalised to the maximum PB-evoked response from individual cells for each construct and represent mean ± s.e.m. from n = 3 – 7 oocytes. (B) Concentration-inhibition curves were determined for the inhibition of the spontaneous Cl^- current by Zn^{2+} (1nM – 2mM) for $\beta3^{wild\text{-}type}$, $\beta3^{H267A}$ and $\beta3^{T263H,\ H267A}$ subunit receptors. The Zn^{2+} sensitivity of $\beta3^{T263H,\ H267A}$ receptors is approximately 100-fold greater than $\beta3^{H267A}$ receptors. Data are fitted using the equation described in the Methods (Chapter 2) and represent mean ± s.e.m. from n = 3 – 7 oocytes. (C) Representative whole-cell membrane currents recorded from *Xenopus* oocytes expressing either $\beta3^{wild\text{-}type}$, $\beta3^{H267A}$ or $\beta3^{T263H,\ H267A}$ subunit receptors in the absence and presence of 10μM Zn^{2+}. The transient downward deflections represent current pulses induced by brief hyperpolarizing commands (-10mV, 1s, 0.2Hz) applied to the holding potential of –40mV.

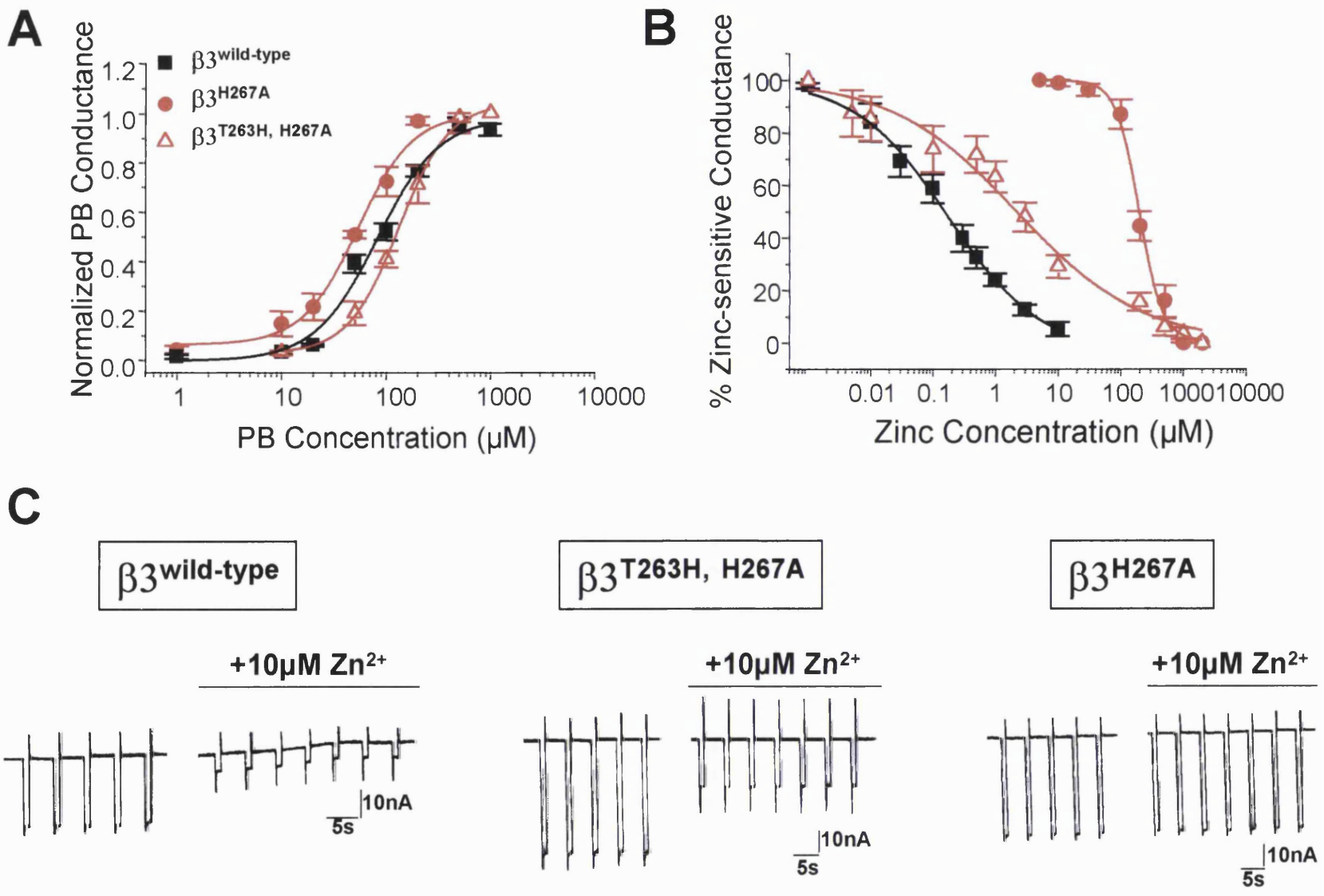
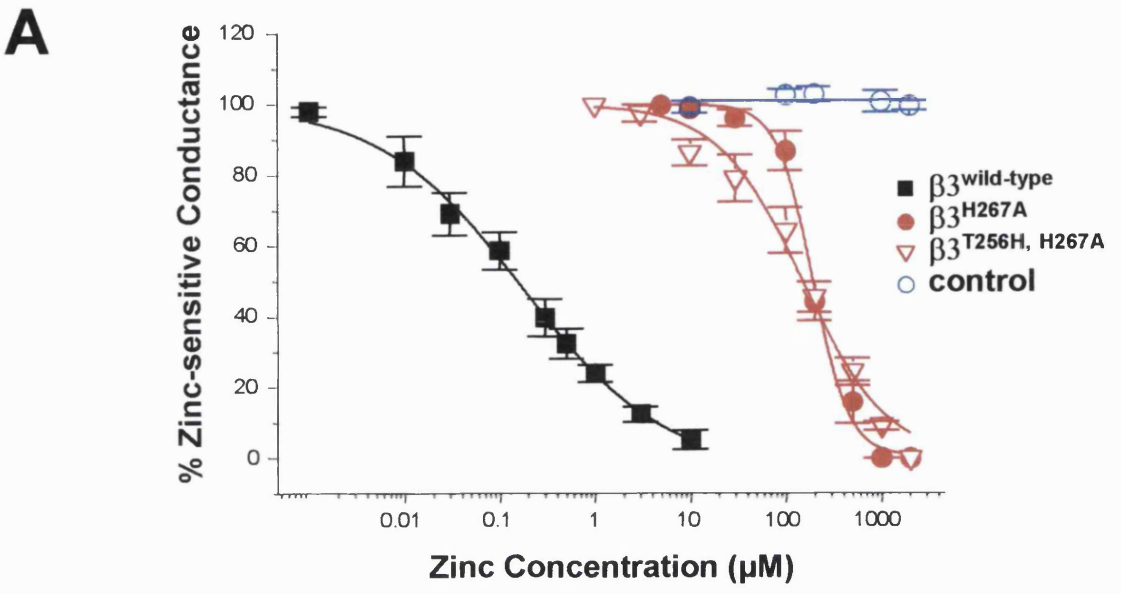
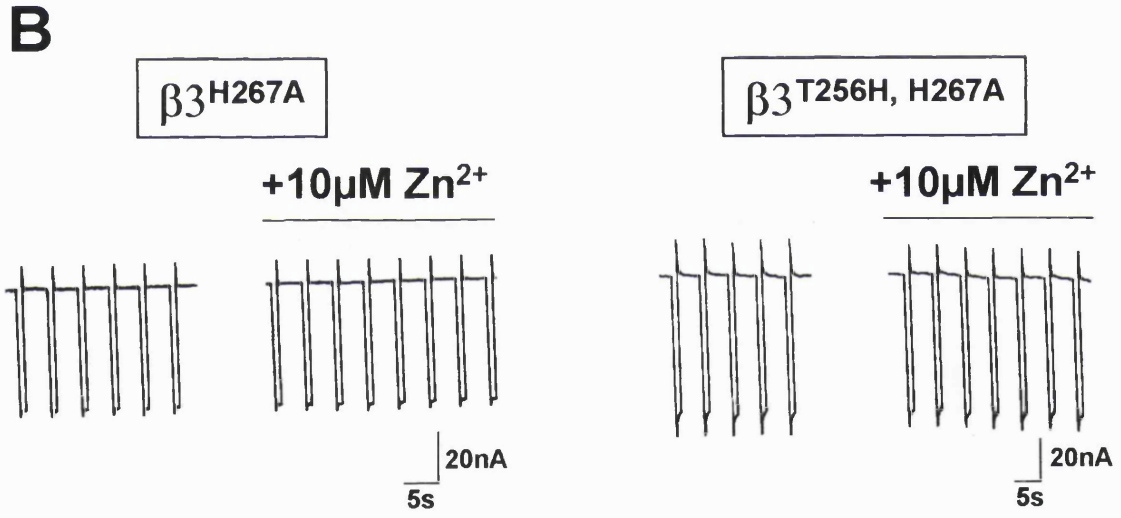

A
Normalized PB Conductance
β3wild-type
β3H267A
β3T263H, H267A
PB Concentration (μM)
B
% Zinc-sensitive Conductance
Zinc Concentration (μM)
C
β3wild-type
β3T263H, H267A
β3H267A
+10μM Zn2+
10nA
5s

Figure 6.3. The $\beta3^{T256H}$ mutation does not increase Zn^{2+} sensitivity. (A) Concentration-inhibition curves were constructed for the antagonism of the spontaneous Cl^- current by Zn^{2+} (1nM – 2mM) for β3 wild-type, $\beta3^{H267A}$ and $\beta3^{T256H, H267A}$ subunit receptors. The Zn^{2+} sensitivity of $\beta3^{H267A}$ and $\beta3^{T256H, H267A}$ subunit receptors is equivalent (IC_{50}s: $\beta3^{T256H, H267A}$, 192.54 ± 26.22μM; $\beta3^{H267A}$, 193.94 ± 13.96μM). Curves were fitted to the data using the equation described in the Methods. Data points represent mean ± s.e.m. from n = 3 – 5 oocytes. The lack of effect of Zn^{2+} (10μM – 2mM) on the resting membrane conductance of uninjected oocytes from the same donor *Xenopus* is illustrated (control). (B, C) Typical whole-cell membrane currents recorded from $\beta3^{H267A}$ or $\beta3^{T256H, H267A}$ subunit receptors expressed in *Xenopus* oocytes in the absence and presence of (B) 10μM Zn^{2+} and (C) 100μM PB. The transient downward deflections represent current pulses induced by brief hyperpolarizing commands (-10mV, 1s, 0.2Hz) applied to a holding potential of -40mV. Note the different time calibrations on application and recovery from PB (1: 5s; 2: 12.5s; 3: 50s) due to the alteration of chart recorder speed. $\beta3^{T256H, H267A}$ receptors retained a relative insensitivity to 10μM Zn^{2+} and to 100μM PB.


A
% Zinc-sensitive Conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Zinc Concentration (μM)
β3wild-type
β3H267A
β3T256H, H267A
control


B
β3H267A
+10μM Zn2+
20nA
5s
β3T256H, H267A
+10μM Zn2+
20nA
5s

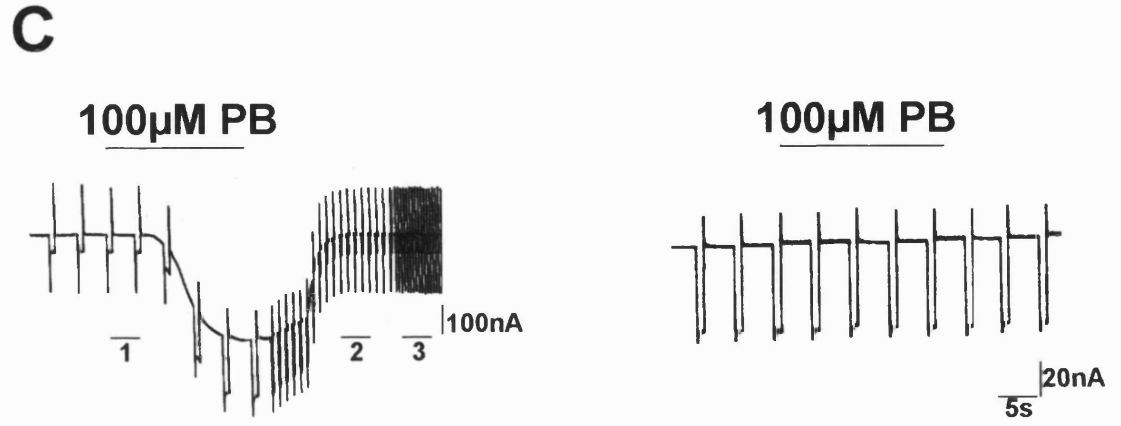

C
100μM PB
100nA
1
2
3
100μM PB
20nA
5s

Figure 6.4. Effects of the $\beta3^{A252H}$ mutation on Zn^{2+} inhibition. (A) Concentration-response curves for PB (1μM – 1mM) were established for $\beta3^{wild\text{-}type}$, $\beta3^{H267A}$ and $\beta3^{A252H,\ H267A}$ subunit receptors. Data were normalised to the maximum PB-evoked response from individual cells for each construct. Data shown are mean ± s.e.m. from n = 3 – 7 oocytes and are fitted with a logistic equation (see Chapter 2). (B) Concentration-inhibition curves were constructed for the Zn^{2+} inhibition of spontaneous Cl^- currents transduced by $\beta3^{wild\text{-}type}$, $\beta3^{H267A}$ and $\beta3^{A252H,\ H267A}$ subunit receptors. The Zn^{2+} sensitivity of $\beta3^{A252H,\ H267A}$ receptors does not significantly differ from that of $\beta3^{H267A}$ receptors (IC_{50}s: $\beta3^{A252H,\ H267A}$, 164.53 ± 6.47μM; $\beta3^{H267A}$, 193.94 ± 13.96μM; $P>0.05$, unpaired t-test). Data represent mean ± s.e.m. from n = 3 – 7 oocytes and are fitted using the equation described in the Methods. (C) Whole-cell membrane currents recorded from $\beta3^{H267A}$ or $\beta3^{A252H,\ H267A}$ subunit receptors expressed in *Xenopus* oocytes in the absence and presence of 10μM Zn^{2+}. The transient downward deflections depict current pulses evoked by brief hyperpolarizing commands (-10mV, 1s, 0.2Hz) applied to the holding potential of -40mV. $\beta3^{T256H,\ H267A}$ receptors remain insensitive to 10μM Zn^{2+}, a concentration that maximally inhibits β3 wild-type receptors.

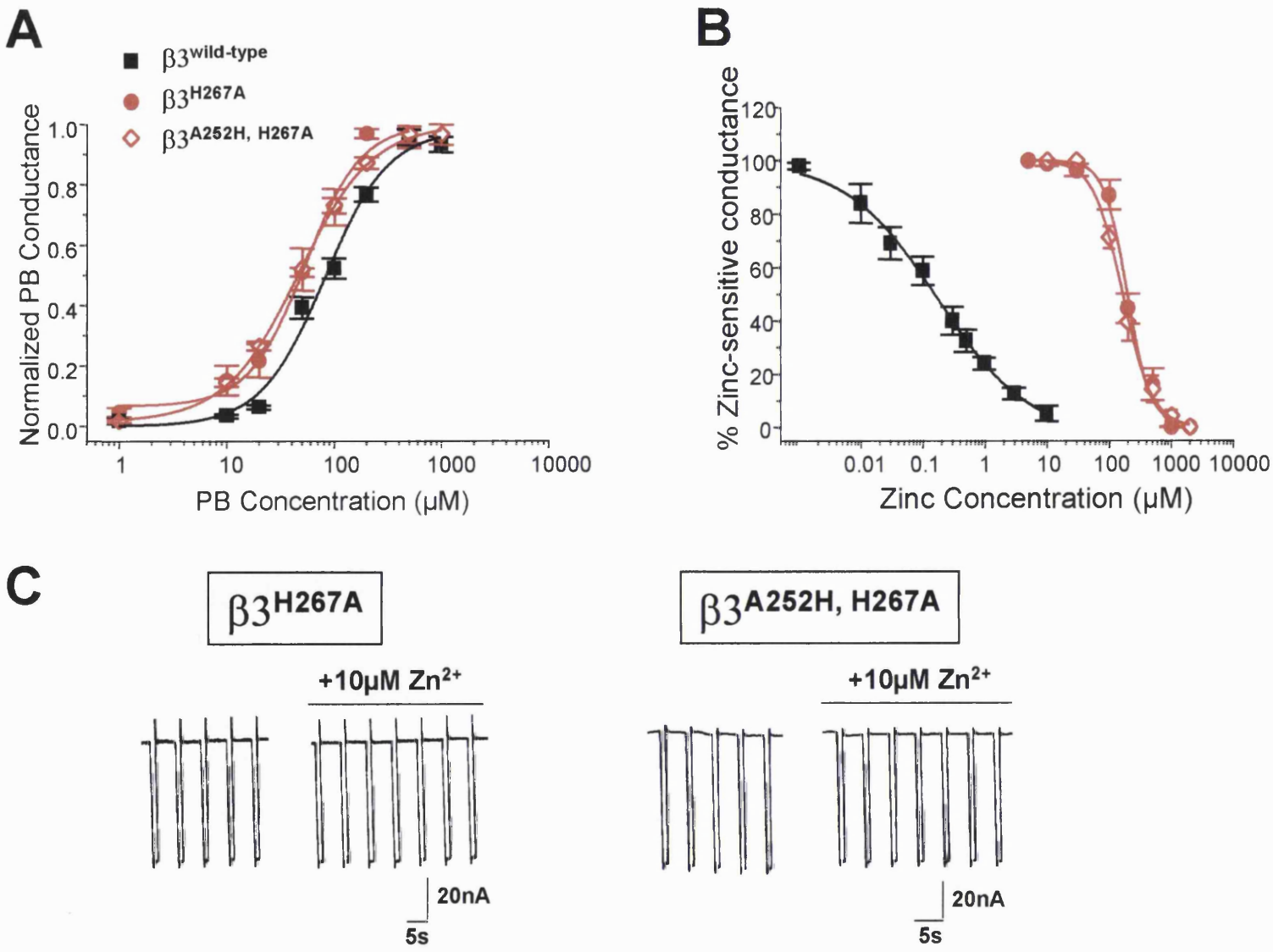

A
β3wild-type
β3H267A
β3A252H, H267A
Normalized PB Conductance
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
10000
PB Concentration (μM)
B
% Zinc-sensitive conductance
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
Zinc Concentration (μM)
C
β3H267A
+10μM Zn2+
20nA
5s
β3A252H, H267A
+10μM Zn2+
20nA
5s

Figure 6.5. Comparison of amino acid sequences in the highly conserved second transmembrane domain for GABA, glycine and ACh ionotropic receptors. Alignment of amino acid sequences for $GABA_A$, $GABA_C$, glycine (gly) and nicotinic acetylcholine (nACh) receptor subunits (*Mm, Musculus musculus*; *Rn, Rattus norvegicus*; *Tc, Torpedo californica*). Residues identified to be channel facing by the substituted cysteine accessibility method are in bold (Akabas *et al.*, 1992, 1994a; Xu & Akabas, 1993, 1996). For the $GABA_A$ receptor, these residues were all accessible in both the presence and absence of GABA (Xu & Akabas, 1993, 1996). However, the exposure of certain nACh receptor residues was altered in the presence/absence of ACh. Residues only accessible to sulphydryl reagents in the presence of ACh are italicised, whereas those residues only accessible in the absence of ACh are boxed (Karlin & Akabas, 1995). The putative ACh receptor channel gate, as determined by SCAM and the application of intracellular and extracellular sulphydryl reagents, is highlighted (pink; Wilson & Karlin, 1998). The four β3 subunit residues probed by SHAM are highlighted in green. The histidine residues implicated in the high affinity binding of Zn^{2+}on the $GABA_A$ receptor are shown in blue (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). Residues conserved in at least three classes of $GABA_A$ receptor subunits are highlighted in yellow. TM2 is underlined and residues are numbered according to the terminology in which the arginine residue at the proposed N-terminus of the TM2 region is assigned the position 0' (Miller, 1989; Charnet *et al.*, 1990).

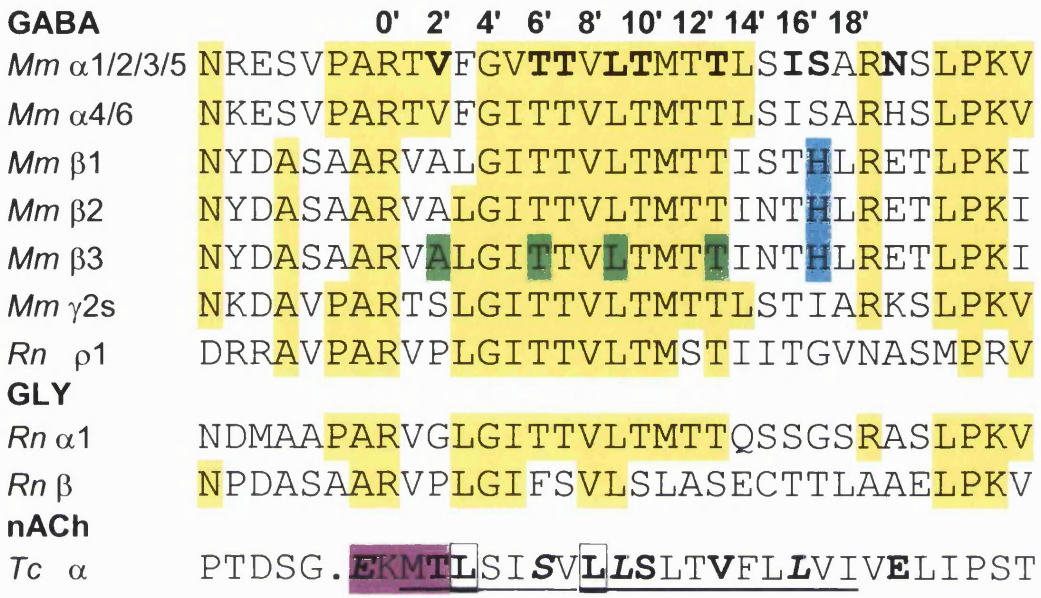
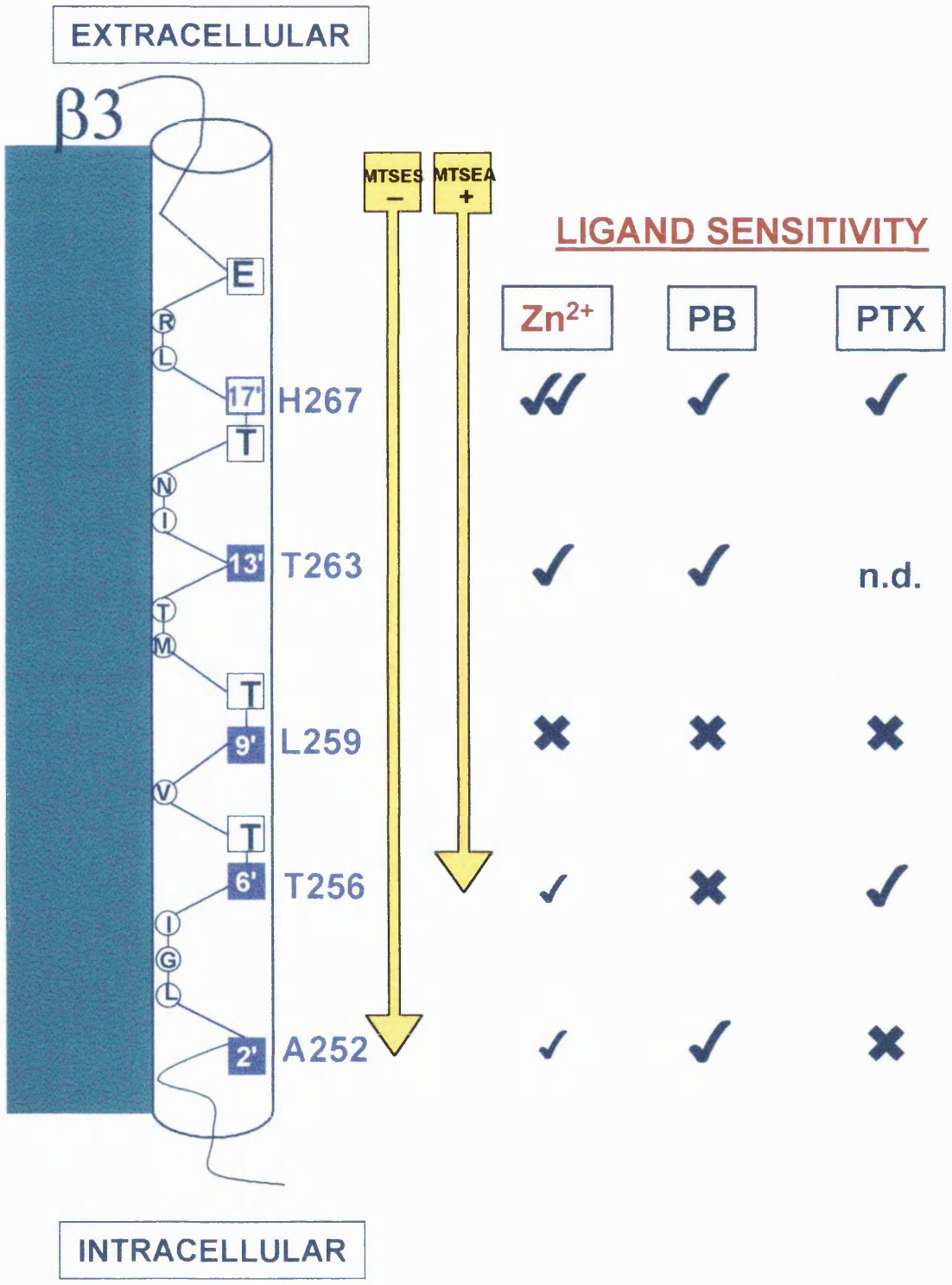

GABA
0' 2' 4' 6' 8' 10' 12' 14' 16' 18'
Mm α1/2/3/5 NRESVPARTVFGVTTVLTMTTLSISARNSLPKV
Mm α4/6 NKESVPARTVFGITTVLTMTTLSISARHSLPKV
Mm β1 NYDASAARVALGITTVLTMTTISTHLRETLPKI
Mm β2 NYDASAARVALGITTVLTMTTINTHLRETLPKI
Mm β3 NYDASAARVALGITTVLTMTTINTHLRETLPKI
Mm γ2s NKDAVPARTSLGITTVLTMTTLSTIARKSLPKV
Rn ρ1 DRRAVPARVPLGITTVLTMSTIITGVNASMPRV
GLY
Rn α1 NDMAAPARVGLGITTVLTMTTQSSGSRASLPKV
Rn β NPDASAARVPLGIFSVLSLASECTTLAAELPKV
nACh
Tc α PTDSG.EKMTLSISVLLSLTVFLLVIVELIPST

Figure 6.6. The effects of substituting TM2 residues with histidine on the ligand sensitivity of $GABA_A$ receptor β3 subunit homomers. A schematic diagram depicting TM2 of the $GABA_A$ receptor β3 subunit and a summary of ligand sensitivities. Residues in the β3 subunit (□), aligned with channel lining residues in the $GABA_A$ receptor α1 subunit as identified by SCAM (Xu & Akabas, 1993, 1996), were also presumed to line the channel. Mutation of a putative channel lining residue, (□)$\beta3^{H267A}$, significantly reduces Zn^{2+} potency (Wooltorton *et al.*, 1997a; Chapter 3). Four putative channel lining residues (■) were systematically mutated to histidine, leaving the background H267A mutation intact. The mutant subunits were then expressed as homomeric receptors in order to investigate the accessibility of the channel pore to Zn^{2+}. The effect of a histidine at each position on the sensitivity to Zn^{2+}, pentobarbitone (PB) and picrotoxin (PTX) is summarised. The relative sensitivities to Zn^{2+} are denoted; the sensitivity of $\beta3^{T256H,\ H267A}$ and $\beta3^{A252H,\ H267A}$ subunit homomers (✓) was not significantly different to $\beta3^{H267A}$ constructs, suggesting that Zn^{2+} cannot access these histidines; H267A is a putative high affinity Zn^{2+} binding site (✓✓); $\beta3^{T263H,\ H267A}$ displays an intermediate sensitivity to Zn^{2+} (✓). For PB and PTX, an observable response to a modulator is denoted (✓) as is a lack of effect (✖). The effect of PTX on $\beta3^{T263H,\ H267A}$ subunit homomers was not determined (n.d.). The accessibility of the negatively charged methanethiosulphonate ethylsulphonate (MTSES) and the positively charged methanethiosulphonate ethylammonium (MTSEA) to corresponding α1 subunit residues are shown for comparison (Xu & Akabas, 1993, 1996). Residues are numbered according to the terminology in which the arginine residue at the proposed N-terminus of the TM2 region is assigned the position 0' (Miller, 1989; Charnet *et al.*, 1990).


EXTRACELLULAR
β3
MTSES
–
MTSEA
+
LIGAND SENSITIVITY
Zn2+
PB
PTX
E
R
L
17'
H267
T
N
I
13'
T263
n.d.
T
M
T
9'
L259
V
T
6'
T256
I
G
L
2'
A252
INTRACELLULAR

CHAPTER 7

Protein tyrosine kinase inhibitors directly modulate $GABA_A$ receptors.

7.1. INTRODUCTION

Protein tyrosine phosphorylation plays an important role in the regulation of synaptic transmission within the central nervous system, modulating both voltage- and ligand-gated ion channel function. Activation of intracellular transduction pathways can lead to the covalent modification of receptor structure by phosphorylation of tyrosine residues. This process is catalysed by protein tyrosine kinases (PTKs) and balanced by protein tyrosine phosphatases (PTPs) which catalyse the cleavage of the phosphate group from the phosphotyrosine (Moss & Smart, 1996; Smart, 1997). Both PTKs and PTPs are highly expressed in the central nervous system (Wagner *et al.*, 1991), consistent with an important role for tyrosine phosphorylation in modulating neuronal function. In addition, protein tyrosine phosphorylation has been implicated in a variety of processes including synaptogenesis (Catarsi & Drapeau, 1993), long-term potentiation (LTP: O'Dell *et al.*, 1991; Grant *et al.*, 1992; Rosenblum *et al.*, 1996; Rostas *et al.*, 1996; Lu *et al.*, 1998), ischaemia (Kindy, 1993; Yokota *et al.*, 1994) and epilepsy (Stratton *et al.*, 1991).

Conserved consensus sites for tyrosine phosphorylation have been identified in the large intracellular domain between transmembrane (TM) domains three and four of the $GABA_A$ receptor $\gamma1$ and $\gamma2$ subunits (Moss & Smart, 1996; Smart, 1997). Consensus sites represent the minimum complement of amino acids required for substrate recognition, although a functional involvement in protein phosphorylation should be confirmed experimentally. However, investigating phosphorylation of the $\gamma2$ subunit has been hindered by the susceptibility of the subunit to proteolysis and problems in raising high affinity antisera to the subunit. Recently, the inclusion of reporter epitopes within the N-terminus of the $\gamma2L$ subunit has allowed tyrosine phosphorylation of the subunit to be investigated when expressed in HEK cells (Moss *et al.*, 1995). These cells have very low levels of phosphotyrosine implying either low intrinsic PTK or high PTP activity enabling $GABA_A$ receptor tyrosine phosphorylation to be studied. Coexpression of the PTK *v-src* with $\alpha1\beta1\gamma2L$ receptors resulted in the phosphorylation of tyrosine (Y) residues 365 and 367 on the $\gamma2L$ subunit and enhanced GABA-activated current (Moss *et al.*, 1995). Intracellular application of genistein, a PTK inhibitor, reduced the amplitude of the GABA response and decreased the level of phosphorylation. Mutation

of these tyrosines to phenylalanine (F) completely eliminated phosphorylation of the γ2L subunit and removed the effect of genistein. Intracellular application of genistein to cultured superior cervical ganglion (SCG) neurons diminished the amplitude of the GABA response, implying that neuronal $GABA_A$ receptors are dynamically regulated by tyrosine phosphorylation and this appears to enhance receptor function (Moss *et al.*, 1995). Similarly, PTK inhibitors reduced GABA-gated currents in *Xenopus* oocytes expressing α1β1 and α1β1γ2L $GABA_A$ receptors and diminished GABA-mediated Cl^- flux from mouse cortical brain microsacs (Valenzuela *et al.*, 1995).

The role of tyrosine phosphorylation on the $GABA_A$ receptor β subunit is more controversial. The large cytoplasmic loop of the β1 subunit is phosphorylated *in vitro* by the PTK $pp60^{v\text{-}src}$ (Valenzuela *et al.*, 1995). Immunoprecipitation experiments reveal that tyrosine residues in β2/β3 subunits are phosphorylated in neuronal cultures by endogenous PTKs and that pretreatment with externally-applied genistein reduces the level of tyrosine phosphorylation (Wan *et al.*, 1997). Although the tyrosine residues in the large cytoplasmic loop of the β1 subunit (Y370 and Y372) are also phosphorylated (albeit to a much lower stoichiometry than the γ2L subunit), no functional effects were observed (Moss *et al.*, 1995).

To determine whether the alternatively spliced short form of the γ2 subunit (γ2S) is also modulated by tyrosine phosphorylation (Fig. 7.1.), murine recombinant α1β1γ2S $GABA_A$ receptors (wild-type and mutant) were expressed in *Xenopus* oocytes or HEK cells and the effects of membrane permeable PTK inhibitors were assessed using two-electrode voltage clamp or patch clamp recording (Dunne *et al.*, 1998a,b).

7.2. RESULTS

7.2.1. Genistein inhibits GABA-gated conductance in α1β1γ2S receptors.

Genistein, a membrane permeable PTK inhibitor, acts at the adenosine triphosphate (ATP) binding site on PTKs (Akiyama *et al.*, 1987, 1991). External application of genistein (100μM) inhibited the GABA response of oocytes expressing wild-type

$\alpha1\beta1\gamma2S$ $GABA_A$ receptors after 10 minutes incubation. However, to ensure maximal inhibition of the kinase, oocytes were pretreated for up to 20 – 30 minutes with 100μM bath-applied genistein. Although genistein did not produce an observable effect on the resting membrane conductance, the response evoked by 40μM GABA was reduced (Fig. 7.2A). GABA concentration-response curves were constructed in control Ringer and in 100μM genistein (Fig. 7.2C). Genistein depressed the GABA concentration-response curve with no evidence of any lateral displacement. Both the maximum normalized response to a saturating dose of GABA (control, 1.83 ± 0.04; + 100μM genistein, 0.71 ± 0.04) and the GABA EC_{50} were reduced by 100μM genistein (EC_{50}s: control, 35.7 ± 2.1μM; + 100μM genistein, 15.1± 3.9μM; n = 3 – 21 oocytes). This suggested that $\alpha1\beta1\gamma2S$ receptors are modulated by tyrosine phosphorylation and the observed antagonism was quite likely due to the inhibition of PTKs by genistein.

To assess the selectivity of genistein, the tyrosine mutant $GABA_A$ receptor $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ was expressed in *Xenopus* oocytes. Remarkably, treatment with 100μM genistein inhibited the response to 40μM GABA (Fig. 7.2B) and produced a greater depression of the GABA concentration response curve (maximum normalized response: control, 1.81 ± 0.03; + 100μM genistein, 0.26 ± 0.01; GABA EC_{50}: control, 33.1 ± 2.3μM; + 100μM genistein, 5.8 ± 2.2μM; n = 3 – 10 oocytes; Fig. 7.2D). This implied that the inhibition induced by genistein may be non-specific. In support of this apparent non-specific action, the inhibition of both wild-type $\alpha1\beta1\gamma2S$ and mutant $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ $GABA_A$ receptors was readily reversible after washing in control Ringer for only 10 minutes. The degree of inhibition exerted by genistein was almost entirely independent of GABA concentration (upper graphs, Fig 7.2C, D) indicating a non-competitive mode of antagonism .

To further investigate the possible non-specificity of genistein, 100μM daidzein (the inactive analogue of genistein) was bath-applied to both wild-type $\alpha1\beta1\gamma2S$ and mutant receptors. Although one batch of daidzein inhibited the GABA-gated conductance with variable potency (Dunne *et al.*, 1998a), experiments with three different batches of daidzein noncompetitively depressed the GABA concentration-response curve without greatly affecting the GABA EC_{50} in both the wild-type (maximum normalized response to GABA: control, 1.83 ± 0.04; +100μM daidzein 0.87 ± 0.01; GABA EC_{50}: control,

35.65 ± 2.09μM; + 100μM daidzein, 27.79 ± 1μM; Fig. 7.3A) and mutant receptors (maximum normalized response to GABA: control, 1.81 ± 0.03 ; +100μM daidzein 1.17 ± 0.03; GABA EC_{50}: control, 33.73 ± 2.54μM; + 100μM daidzein, 30.87 ± 2.18μM; n = 3 – 10 oocytes; Fig. 7.3B). These data suggested that genistein and daidzein are interacting non-specifically with the $GABA_A$ receptor.

7.2.2. Modulation of α1β1γ2S recombinant $GABA_A$ receptors by tyrphostins.

In order to investigate tyrosine phosphorylation of α1β1γ2S $GABA_A$ receptors and to obviate the problems of the apparent non-specificity of genistein, comparative experiments were performed using alternative PTK inhibitors that target a different binding site. Tyrphostins are competitive inhibitors of substrate binding on PTKs (Gazit *et al.*, 1989). External application of Tyrphostin A25 (a membrane permeable inhibitor; 200μM) did not alter the resting membrane conductance of wild-type α1β1γ2S $GABA_A$ receptors but the conductance evoked by 40μM GABA was greatly reduced (Fig. 7.4A). GABA equilibrium concentration-response curves were constructed in control Ringer and in 200μM tyrphostin A25. Tyrphostin A25 (200μM) depressed the maximum normalized response to GABA (control, 1.95 ± 0.05; + 200μM tyrphostin A25, 0.48 ± 0.04) and reduced the GABA EC_{50}s (control, 45.6 ± 3.9μM; +200μM tyrphostin A25, 23.9 ± 6.3μM; n = 3 – 19 oocytes; Fig. 7.4C). GABA concentration-response curves were also constructed in the presence of 200μM tyrphostin A1, a negative control for tyrphostins. Surprisingly, application of this inactive tyrphostin also depressed the GABA concentration-response curve (73% inhibition of maximum normalized response to GABA; n = 3 oocytes; Fig. 7.4C).

Similarly, treatment of *Xenopus* oocytes expressing the tyrosine mutant receptor α1β1γ2S$^{Y365F,\ Y367F}$ with 200μM tyrphostin A25 substantially inhibited the response evoked by 40μM GABA (Fig. 7.4B) and noncompetitively depressed the GABA concentration response curve (maximum normalized response to GABA: control, 1.82 ± 0.04; + 200μM tyrphostin A25, 0.33 ± 0.01; EC_{50}: control, 37.2 ± 3.8μM; + 200μM tyrphostin A25, 31.7 ± 4.1μM; n = 3 – 19 oocytes; Fig 7.4D). Likewise, application of

the inactive tyrphostin A1 (200μM) resulted in a large depression of the GABA concentration-response curve (64% inhibition of the maximum normalized response to GABA; n = 3 oocytes; Fig. 7.4D). The degree of inhibition effected by tyrphostin A25 appeared to be independent of GABA concentration (upper graphs, Fig 7.4C, D) suggesting a non-competitive mode of antagonism .

7.2.3. Voltage dependence of the inhibition of the GABA-gated conductance by PTK inhibitors.

Membrane current-voltage (I-V) relationships for the responses evoked by 10μM GABA on α1β1γ2S $GABA_A$ receptors were established in control Ringer and exhibited a degree of outward rectification. Rectification ratios (*R*) were calculated from a ratio of currents (I_{20}/I_{-60}) measured at membrane potentials of 20 and –60mV. Values of *R* greater than 1 signified outward rectification, values less than 1 indicated inward rectification and an *R* value equivalent to 1 was expected for zero rectification. An *R* value of 1.39 was obtained for I-V relationships to10μM GABA in the presence of 100μM genistein compared to *R* = 1.33 in control Ringer. The reversal potential of the wild-type α1β1γ2S receptor also remained unchanged in the presence of 100μM genistein (control, -22.6 ± 5.1mV; +100μM genistein, -21.8 ± 4.6mV; n = 3 oocytes; Fig. 7.5A). The relative block induced by 100μM genistein at each membrane potential displayed weak voltage sensitivity to the inhibition. At hyperpolarized membrane potentials, genistein had a reduced inhibitory effect (40% inhibition; -70mV to -100mV) compared to the inhibition of the 10μM GABA response observed at depolarised membrane potentials (72%; 30mV to 50mV; n = 3 oocytes; Fig. 7.5B).

Similarly, membrane current-voltage (I-V) relationships for the responses evoked by 10μM GABA were obtained in the absence and presence of 200μM tyrphostin A25, demonstrating a degree of outward rectification (control, *R* = 1.53; +200μM tyrphostin A25, *R* = 1.34; n = 3 oocytes). Application of tyrphostin A25 did not affect the reversal potential of the wild-type α1β1γ2S $GABA_A$ receptor (control, -23.1 ± 5.4mV; + tyrphostin A25, -22.9 ± 5mV; n = 3 oocytes; Fig.7.5C). Analysis of the relative block of the 10μM GABA response evoked by tyrphostin A25 at each membrane potential

demonstrated no apparent voltage sensitivity (-70mV to –90mV, 39% inhibition; 20mV to 40mV, 36% inhibition; n = 3 oocytes; Fig. 7.5D).

7.2.4. Effects of PTK inhibitors on the allosteric modulation of α1β1γ2S $GABA_A$ receptors.

To determine whether the two groups of PTK inhibitors were interfering with some of the known binding sites of allosteric modulators of $GABA_A$ receptors, the modulatory effects of the barbiturate pentobarbitone (PB) and the benzodiazepine diazepam were examined in the absence and presence of the PTK inhibitors (Fig. 7.6A, B). Steady-state responses to an EC_{20} dose of GABA were obtained for oocytes expressing wild-type α1β1γ2S receptors. PB (50μM) was preapplied for 3 minutes and then the enhancement of the EC_{20} GABA response was subsequently measured. After recovery of the control EC_{20} GABA response, the oocyte was incubated with 100μM genistein (bath-applied) for up to 30 minutes, until a steady-state inhibition of the EC_{20} GABA response was observed. In the presence of genistein, 50μM PB was reapplied and the enhancement of the EC_{20} GABA response was determined. All PB-induced enhancements were normalized to the enhancement induced by 50μM PB in control Ringer. In addition, the GABA concentration was increased to 100μM to obtain a similarly matched response amplitude in the presence of genistein compared to the control, 20μM GABA-activated response, and the potentiation by 50μM PB determined. Genistein did not affect the PB modulation of the GABA-gated conductance of wild-type α1β1γ2S receptors (normalized enhancement, 1.02 ± 0.01; n = 3 oocytes; Fig. 7.6C). In addition, 200μM tyrphostin A25 did not alter PB modulation (normalized enhancement, 1.28 ± 0.21; n = 3 oocytes; Fig. 7.6C). An identical protocol was used to ascertain the effects of PTK inhibitors on the benzodiazepine binding site on the wild-type α1β1γ2S $GABA_A$ receptor. Application of either genistein (100μM) or tyrphostin A25 (200μM) failed to alter the enhancement of an EC_{20} GABA-activated response by 0.5μM diazepam (normalized enhancement: + 100μM genistein, 0.95 ± 0.02; + 200μM tyrphostin A25, 0.95 ± 0.05; n = 3 oocytes; Fig. 7.6C).

7.2.5. Involvement of the tyrosine residues on the β1 subunit in mediating inhibition by genistein.

The large intracellular loop contains consensus sequences and putative substrates for tyrosine phosphorylation (Moss & Smart, 1996; Smart, 1997). Although evidence suggests that they are not functional (Moss *et al.*, 1995), it is possible that tyrosine residues on the β1 subunit are responsible for the inhibitory action of genistein. Alternatively, the observed inhibition of the GABA-gated conductance may unusually be due to the *Xenopus* oocyte expression system affecting post-translational processing of the expressed receptor. To address these issues, the phosphorylated tyrosines in the β1 subunit (Y370, Y372) were mutated to phenylalanines and coexpressed with α1 and $\gamma2S^{Y365F,\ Y367F}$ subunits in HEK cells. Expression of wild-type α1β1γ2S receptors in HEK cells resulted in functional responses to GABA (0.1 – 100μM). Coapplication of genistein (100μM) resulted in a rapid onset of inhibiton of the GABA-gated current that was readily reversible, indicating a likely external site of action. GABA equilibrium concentration-response curves for the quadruple tyrosine mutant receptor $\alpha1\beta1^{Y370F,\ Y372F}\gamma2S^{Y365F,\ Y367F}$ were established in the absence and presence of externally applied 100μM genistein (control: EC_{50}, 5.8 ± 0.12μM; n_H, 1.1 ± 0.02; + 100μM genistein: EC_{50}, 4.4 ± 0.5μM; n_H, 1.46 ± 0.21; n = 4 cells; Fig. 7.7.). Coapplication of 100μM genistein resulted in a large depression of the GABA concentration response curve. The degree of inhibition was independent of GABA concentration (approximately 57% inhibition of the control responses to GABA). This indicates that the tyrosine residues on the β1 subunit are not involved in mediating the observed inhibitory action of genistein and that this antagonism is not a feature of the *Xenopus* oocyte expression system.

7.3. DISCUSSION

Tyrosine phosphorylation is important in regulating neuronal function (Moss & Smart, 1996; Smart, 1997) and specific PTK inhibitors are commonly used to implicate the involvement of this process. However, this study has revealed that α1β1γ2S $GABA_A$ receptors can be modulated by PTK inhibitors via a PTK independent mechanism and

highlights the importance of using mutant receptor subunits, devoid of phosphorylation substrates, as a rigorous negative control.

7.3.1. Interaction of genistein with $GABA_A$ receptors.

PTK inhibitors are routinely used to implicate the involvement of tyrosine phosphorylation in modulating $GABA_A$ receptor function. There are two classes of PTK inhibitors, one class targets the ATP binding site (Akiyama *et al.*, 1987, 1991) and the other binds to the substrate binding site on the enzyme (Gazit *et al.*, 1989). The most frequently used PTK inhibitor competing with ATP binding is genistein (4',5,7-trihydroxyflavone) together with daidzein (4',7-dihydroxyflavone) as an inactive control (Fig. 7.8A,B). Since genistein is structurally dissimilar to ATP, the PTK inhibition may not be due to true competition for the ATP binding site and may reflect genistein binding in various places in the reaction pathway (Akiyama *et al*, 1987, 1991). Genistein is a broad range PTK inhibitor, reducing epidermal growth factor receptor (EGFR), $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ tyrosine kinase activity.

External application of genistein produced a substantial reduction in the GABA-activated response of both wild-type $\alpha1\beta1\gamma2S$ and tyrosine mutant $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ $GABA_A$ receptors. The inactive analogue, daidzein, also inhibited the GABA-activated response when applied via the bath solution. Applying genistein internally, via the patch pipette electrolyte, appeared to avoid a nonselective action as mutation of both PTK substrates (Y365, Y367) abolished the inhibitory action of genistein on $\alpha1\beta1\gamma2L$ $GABA_A$ receptors (Moss *et al.*, 1995). However, genistein is most commonly applied externally. Extracellular application of genistein to rat spinal dorsal horn neurons (and cultured dorsal medulla neurons), produced a rapid and reversible inhibition of GABA-gated currents, without affecting the I-V relationship or the reversal potential (Wan *et al.*, 1997). Application of genistein also inhibited $\alpha1\beta2$ and $\alpha1\beta2\gamma2S$ $GABA_A$ receptors expressed in HEK cells. Consistent with the present study, this inhibition was rapid in both onset and offset (Wan *et al.*, 1997), however daidzein had little effect on the GABA response. Similarly in the study by Valenzuela and colleagues (1995), GABA–gated currents recorded from oocytes expressing $\alpha1\beta1$ and $\alpha1\beta1\gamma2L$ $GABA_A$ receptors were inhibited after bath-application of genistein (10 minutes). Curiously, negative

controls were not used. In the same study genistein inhibited muscimol-stimulated $^{36}Cl^-$ uptake in cortical brain microsacs, although daidzein did not significantly affect the uptake. More recently, muscimol activation of $GABA_A$ receptor currents from basal forebrain neurons was attenuated by inclusion of 100μM genistein in the bath solution, and unaffected by the presence of daidzein (Jassar *et al.*, 1997).

Tyrosine phosphorylation has been implicated in the "run-down" of the GABA-activated current observed with whole-cell patch clamp recording from cultured neurons (Amico *et al.*, 1998). Inclusion of the PTP inhibitor sodium vanadate in the patch pipette electrolyte prevented the run-down of the GABA response. However, genistein was applied externally and produced a substantial inhibition of the GABA response after 4 minutes. The inactive control daidzein was not used. Although the effect of internally applied sodium vanadate supports a role for tyrosine phosphorylation in GABA-gated current run-down, the rapid action of externally applied genistein may be substantially due to a direct modulation of the $GABA_A$ receptor via a PTK-independent mechanism.

Subsequent to the present study, a direct modulation of recombinant $GABA_A$ receptors was revealed by Huang and colleagues (1999). Extracellular application of either genistein (100μM) or daidzein (100μM) produced a rapid and reversible inhibition of GABA-activated Cl^- currents recorded from either α1β2γ2S or α1β2 $GABA_A$ receptors expressed in HEK cells. The inhibitory action of externally applied genistein was observable even when genistein was pre-equilibrated in the intracellular solution. A slight voltage sensitivity, with no effect on the reversal potential, was also observed for the direct inhibition of $GABA_A$ receptor function by genistein. The effects of tyrphostins were not examined, although intracellular (and not extracellular) application of lavendustin A (a mixed inhibitor antagonizing both ATP and substrate binding; Onoda *et al.*, 1989; Hsu *et al.*, 1991) progressively inhibited the GABA response of α1β2γ2S (and not α1β2) $GABA_A$ receptor constructs implying a specific action for this compound (Huang *et al.*, 1999).

7.3.2. Modulation of the $GABA_A$ receptor by tyrphostins.

In an attempt to avoid nonspecific actions of genistein and daidzein, the effects of a different class of PTK inhibitors on $GABA_A$ receptors were examined. Tyrphostins selectively inhibit PTKs by binding to the substrate binding site (Gazit *et al.*, 1989; 1991). These compounds structurally resemble tyrosine and have hydrophobic characteristics that allow permeation of the cell membrane. Tyrphostin A25 (α-cyano-(3,4,5-trihydroxy)cinnamonitrile) was selected as a commonly used PTK inhibitor in conjunction with tyrphostin A1 (α-cyano-(4-methoxy)cinnamonitrile) as a negative control (Fig. 7.8C,D). However both the active and inactive tyrphostins acted as antagonists of the α1β1γ2S receptor, producing a substantial depression of the GABA-concentration response curves. Since tyrphostins bear little structural similarity to genistein, either structure is not important for the nonspecific inhibition or else multiple binding sites exist for these agents on the $GABA_A$ receptor complex. In comparative studies, external application of the active inhibitor tyrphostin B44 reduced the muscimol-activated response recorded from basal forebrain neurons (Jassar *et al.*, 1997). Micro-injected tyrphostin B44 inhibited the GABA response of *Xenopus* oocytes expressing α1β1 and α1β1γ2L receptors, although the effects of an inactive tyrphostin were not tested (Valenzuela *et al.*, 1995). Similarly, tyrphostin B44 inhibited muscimol-stimulated $^{36}Cl^-$ uptake in cortical brain microsacs to a greater degree than in lysed microsacs that were resealed under conditions unfavourable for phosphorylation (Valenzuela *et al.*, 1995). However, tyrphostin B44 still produced a sizeable inhibition (27 ± 4%) of the Cl^- flux under conditions unfavourable for phosphorylation, leading the authors to suggest the possibility of a direct inhibitory action on the $GABA_A$ receptor that does not involve phosphorylation.

7.3.3. Modulation of the $GABA_A$ receptor by PTK inhibitors does not involve tyrosine phosphorylation of the β1 subunit.

Although many studies investigating tyrosine phosphorylation use PTK inhibitors, the use of inactive analogues is not a guaranteed negative control. This study compared the activities of the PTK inhibitors on receptors devoid of tyrosine kinase consensus sequences following site-directed mutagenesis. Unexpectedly, $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$

receptors expressed in *Xenopus* oocytes were still susceptible to inhibition by PTK inhibitors. A potential explanation is that the PTK inhibitors are preventing tyrosine phosphorylation of the β1 subunit. Although the β1 subunit tyrosine residues are phosphorylated (Moss *et al.*, 1995; Valenzuela *et al.*, 1995), these sites were shown to be relatively unimportant in the modulation of $\alpha1\beta1\gamma2L$ $GABA_A$ receptor function (Moss *et al.*, 1995). However, application of PTK inhibitors to HEK cells expressing $\alpha1\beta1^{Y370F,\ Y372F}\gamma2S^{Y365F,\ Y367F}$ receptors resulted in substantial inhibition of the GABA response. This confirms a nonspecific, tyrosine kinase independent mechanism of inhibition.

7.3.4. Direct modulation of enzymes and other ion channels by genistein.

Genistein is a commonly used PTK inhibitor which can cause non-specific inhibition of the serine/threonine kinases, although the IC_{50} for the inhibition of the tyrosine kinase $pp60^{c\text{-}src}$ (26μM) is over 10-fold lower than for the inhibition of PKC (IC_{50}: >370μM). PKA activity was only slightly inhibited by 370μM genistein (Akiyama *et al.*, 1987, 1991). However, a possible inhibition of serine/threonine kinases would not account for the inhibition of GABA-gated currents by the inactive analogue daidzein or by the selective PTK inhibitors, the tyrphostins. Genistein also inhibits other ATP-hydrolysable enzymes. Genistein noncompetitively inhibits the ATP binding site in histidine kinases which is in contrast to the apparent competitive inhibition of ATP binding on PTKs (Huang *et al.*, 1992). In addition, genistein inhibits mammalian DNA topoisomerase II possibly via a consensus sequence for ATP binding (single amino acid code: GXGXXG....K; where X signifies any amino acid; Markovits *et al.*, 1989). Similarly both genistein and daidzein inhibit the ATP-dependent step of phospholipase C activation (Higashi & Ogawara, 1992).

Genistein has also been used to examine tyrosine phosphorylation of excitatory ligand-gated ion channels. Bath-application of genistein to rat spinal dorsal horn neurons produced a rapid inhibition of NMDA currents (2 minutes), which quickly recovered on removal of genistein (Wang & Salter, 1994). Whilst not discounting an effect on PTKs, this rapid onset/offset of inhibition is consistent with genistein directly affecting receptor function via a PTK-independent mechanism. *Xenopus* oocytes contain

endogenous receptors for insulin, which possess inherent tyrosine kinase activity (Chuang *et al.*, 1993). Bath-application of genistein to oocytes expressing murine ξ1/ε4 NMDA receptors (equivalent to NR1/NR2D subunits) also reversed the potentiation of NMDA currents by insulin, although the time course was much greater (30 - 60 minutes; Chen & Leonard, 1996). However, experiments using anti-phosphotyrosine antibodies have shown that NR1 subunits (the rat homologue of ξ1) are not directly phosphorylated (Lau & Huganir, 1995), which may suggest an indirect action of PTK inhibitors on NMDA receptors. In agreement with this view, experiments using the PTKs *src* and *fyn* revealed that the tyrosine phosphorylation site is located in the C-terminal domain of the NR2A NMDA receptor subunit (equivalent to the murine ε1 subunit) and these kinases did not alter NR1-NR2D (ξ1/ε4 equivalent) NMDA receptor function (Kohr & Seeburg, 1996).

In addition to influencing $GABA_A$ receptor function, genistein can modulate the Cl^- flux of the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR protein consists of two membrane spanning nucleotide binding domains (NBDs) linked by a highly charged cytoplasmic R domain, containing several consensus sequences for PKA and PKC (Riordan *et al.*, 1989; Riordan, 1993). PKA phosphorylation of the R domain activates the CFTR Cl^- channel and subsequent ATP binding and hydrolysis at the NBDs are coupled to channel opening and closing. Several studies of CFTR channel activity have indicated a direct interaction of genistein with the CFTR protein. Genistein enhanced the activation of Cl^- currents of CFTR channels expressed in NIH/3T3 fibroblasts and inclusion of either vanadate (a PTP inhibitor) or PKA inhibitor peptide failed to impair the effect of genistein (Wang *et al.*, 1998). Replacement of ATP with GTP, a poor substrate for PTKs, also did not alter the action of genistein (French *et al.*, 1997). Similarly, genistein (and not the active tyrphostin B47) produced a vanadate-insensitive enhancement of activated Cl^- currents in CFTR channels expressed in *Xenopus* oocytes (Weinrich *et al.*, 1997). Comparable studies on the cardiac isoform of CFTR in guinea pig ventricular myocytes have demonstrated that the enhancement of the activation of CFTR channels by genistein is similarly unaffected by inclusion of vanadate in the patch pipette (Obayashi *et al.*, 1999). Interestingly, daidzein, the inactive analogue of genistein, produces an equivalent enhancement of current. Furthermore, treatment with other PTK inhibitors, lavendustin A, tyrphostins B42 and

B51 does not effect the activation of CFTR Cl^- channels (Chiang *et al.*, 1997; Obayashi *et al.*, 1999) implying that a direct modulation of the protein may be specific to genistein. Deletion of a single amino acid (F508) in the first NBD of the CFTR protein occurs in cystic fibrosis patients (Riordan *et al.*, 1989). Mutant (ΔF508) CFTR channels expressed in NIH/3T3 cells have a lower open channel probability (P_O) when stimulated by cAMP than wild-type CFTR channels (Hwang *et al.*, 1997). Application of genistein increased the P_O of mutant CFTR channels to a level identical to wild-type CFTR channels and a putative direct modulatory role of genistein, whereby binding of genistein to the CFTR (presumably via the NBDs) facilitates PKA-dependent phosphorylation of the R domain and affects ATP hydrolysis at the NBDs. The authors suggested a putative therapeutic role for genistein, however given the interaction of genistein with the $GABA_A$ receptor and other ion channels it is unlikely to prove clinically useful. If genistein were able to gain access to the CNS, then it would be expected to have latent convulsive effects due to a reduction in the level of synaptic inhibition mediated by $GABA_A$ receptors.

The modulatory action of genistein is not limited to Cl^- channels. Inclusion of genistein in the bath solution inhibits voltage-gated K^+ currents in both rat and rabbit pulmonary artery cells whereas application of daidzein had no effect on current amplitude (Smirnov & Aaronson, 1995). However, the inhibitory action of genistein was independent of internal ATP and was unaffected by incubation (either intra- or extracellular) with a PTP inhibitor, orthovanadate. Interestingly, external application of the inactive tyrphostin A1 produced a slight inhibition of the K^+ current. This implies that genistein is directly modulating the K^+ channel independently of tyrosine kinases and provides further evidence that inactive analogues are not reliable controls. A similar study on ATP-sensitive K^+ channels in rabbit portal vein smooth muscle observed a reduction in current amplitude when genistein (but not daidzein) was bath-applied (Ogata *et al.*, 1997). However, application of other potent PTK inhibitors, herbimycin A (irreversibly binds to reactive thiol groups of PTKs; Uehara *et al.*, 1989; Fukazawa *et al.*, 1991), lavendustin A, and tyrphostin A23 did not affect K^+ currents, indicating a possible direct inhibitory action of genistein on these channels.

Voltage-gated calcium channel currents in rabbit vascular smooth muscle were rapidly reduced by application of genistein, although unaffected in the presence of daidzein (Wijetunge *et al.*, 1992). However, application of both the active tyrphostin A23 and the inactive analogue tyrphostin A1 resulted in a fast, substantial reduction in calcium current amplitude, suggesting a putative direct blocking action. Furthermore, L-type calcium currents recorded from guinea pig ventricular myocytes were rapidly depressed by application of both genistein and daidzein, indicating a direct action of these compounds on the calcium channel (Chiang *et al.*, 1996).

Recently, a direct interaction of genistein with voltage-sensitive Na^+ channels was demonstrated (Paillart *et al.*, 1997). Addition of 100μM genistein to the extracellular solution produced a rapid inhibition of Na^+ currents recorded from cultured cerebellar granule neurons and prevented neurotoxin induced $^{22}Na^+$ uptake through voltage-sensitive Na^+ channels in cultured foetal rat brain neurons. Daidzein also inhibited $^{22}Na^+$ influx whereas other PTK inhibitors, lavendustin A and tyrphostin A47, were ineffective.

7.3.5. PTK inhibitors: possible mechanism of action on the $GABA_A$ receptor.

The mechanism of antagonism of the GABA-gated conductance appeared to be similar for both classes of PTK inhibitors despite structural differences. GABA concentration-curves were non-competitively depressed, suggesting that competition between GABA and the PTK inhibitors at the GABA binding site was unlikely. It is possible that the PTK inhibitors are opportunistically binding to the $GABA_A$ receptor potentially involving the ion channel. Genistein is essentially a planar molecule with some charge delocalization expected. Daidzein only differs by the deletion of a hydroxyl group on the A ring (Fig. 7.8.). The substrate binding inhibitors typified by the tyrphostins used in this study are structurally different to genistein and are based on a hydroxycinnamonitrile nucleus (Fig. 7.8.). Both tyrphostins A1 and A25 are also mainly planar molecules with a greater charge delocalization compared to genistein and daidzein. The overall negative charge delocalization may account for the noncompetitive nature of these molecules if they are able to target the Cl^- selective ion channel of the $GABA_A$ receptor. I-V relationships were established to determine

whether the inhibition was due to a change in the Cl^- driving force or to a change in the GABA-activated conductance. Genistein did not alter the reversal potential of the GABA-activated response but reduced the slope of the I-V curve suggesting that the reduction of the current by genistein is due to a change in the GABA-activated conductance rather than an alteration in driving force. This is in accordance with other studies on both $GABA_A$ and NMDA receptors on dorsal horn neurons (Wan *et al.*, 1997; Wang & Salter, 1994). Similarly, tyrphostin A25 did not alter the reversal potential of the GABA I-V curve and the inhibition did not display any voltage sensitivity.

Phosphorylation by PKC may alter the coupling between allosteric modulatory sites on $GABA_A$ receptors (Leidenheimer *et al.*, 1993). The diazepam potentiation of the GABA response of $\alpha1\beta1\gamma2L$ receptors expressed in *Xenopus* oocytes was increased following treatment with phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC (control potentiation, ~160%; potentiation in the presence of PMA, ~333%). A similar increase in PB potentiation was observed following PMA treatment. Comparable results were obtained for the $\alpha1\beta1\gamma2S$ $GABA_A$ receptor. Although this implies that phosphorylation may regulate the allosteric modulation of the $GABA_A$ receptor, the experimental design does not allow for a possible shift in the GABA concentration response curve in the presence of PMA. Since barbiturate and benzodiazepine potentiation is maximal at lower GABA concentrations, an increase in GABA potency during treatment with PMA would also account for enhanced potentiation, although a previous study by the same authors indicated that PMA did not affect GABA potency, only efficacy (Leidenheimer *et al.*, 1993).

The present study investigated whether tyrosine kinase inhibitors could target barbiturate or benzodiazepine binding sites. In order to allow for a possible shift in GABA potency, an EC_{20} response to GABA was determined in the presence of the PTK inhibitors and the PB/diazepam potentiation recorded. This potentiation was then normalised to the potentiation of an EC_{20} GABA response in control Ringer. However genistein and tyrphostin A25 failed to affect the allosteric modulation of the $\alpha1\beta1\gamma2S$ $GABA_A$ receptor.

Non-specific effects have been reported for membrane permeable kinase activators, including the adenylate cyclase activator forskolin, which can directly inhibit $GABA_A$ receptor function independently of phosphorylation (Leidenheimer *et al.*, 1991). Phorbol esters may also demonstrate non-specificity, although this can be relatively controlled for by the inactive α–phorbols (Moss & Smart, 1996). Furthermore, intracellular dialysis of purified, constitutively active kinases (e.g. PKM) or second messengers (e.g. cAMP) could activate other cell signalling pathways, producing differential effects on $GABA_A$ receptor function (Moss & Smart, 1996; Smart, 1997). Therefore, in addition to previously identified non-specific actions of kinase modulators, the present study now adds genistein, daidzein and at least two tyrphostins.

7.3.6. Summary.

The effect of PTK inhibitors on α1β1γ2S and $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ receptors expressed in *Xenopus* oocytes was examined in order to investigate tyrosine phosphorylation of the γ2S subunit. External application of genistein, a PTK inhibitor acting at the ATP binding site, resulted in a substantial reduction of the GABA-activated response. However daidzein, the inactive structural analogue of genistein, also depressed the GABA concentration-response curve. Both compounds were also effective in reducing GABA-gated currents of the tyrosine mutant receptor $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ in which both "functionally active" substrate tyrosine residues are mutated. Tyrphostin A25 (and the inactive analogue A1), PTK inhibitors targeting the substrate binding site, also inhibited the wild-type and tyrosine mutant receptors. The antagonism of GABA-activated responses was not significantly voltage sensitive. Furthermore, genistein or tyrphostin A25 did not alter the enhancement of GABA-activated responses by either diazepam or PB. The "functionally silent" substrate tyrosine residues in the β1 subunit, Y370 and Y372 were mutated. However, externally applied genistein rapidly and reversibly inhibited $\alpha1\beta1^{Y370F,\ Y372F}\gamma2S^{Y365F,\ Y367F}$ receptors expressed in HEK cells. In conclusion, this study has demonstrated that PTK inhibitors and their inactive analogues can directly interact with $GABA_A$ receptors via a tyrosine-independent mechanism(s).

Figure 7.1. The $GABA_A$ receptor γ2S subunit contains a conserved consensus sequence for tyrosine phosphorylation. Schematic diagram of the $GABA_A$ receptor illustrating the four transmembrane domains, the external N- and C-terminal domains and the large intracellular domain. Consensus sequences surrounds the site(s) of tyrosine phosphorylation and represents the minimum amino acids required for substrate recognition (E/DEEIYG/EEF; Moss & Smart, 1996). The large intracellular loop between TM3 and TM4 of the γ2S subunit (*cf.* β1 subunit) contains a consensus sequence for tyrosine phosphorylation, conserved within murine, rat, bovine and human $GABA_A$ receptors. (illustrated in black). The putative phosphorylated tyrosines, Y365 and Y367 for γ2S-, Y370 and Y372 for β1-subunits, are highlighted in white.

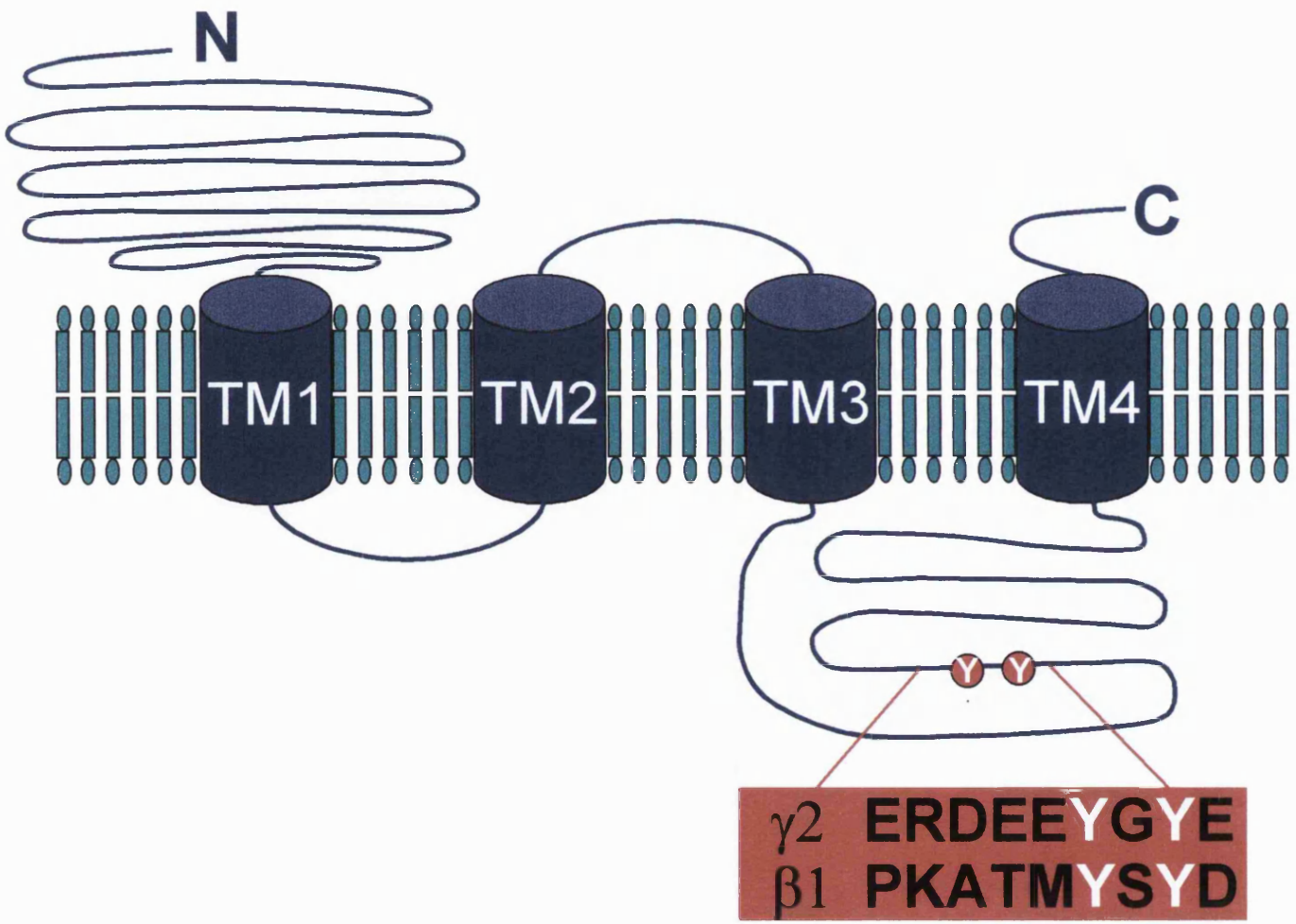

N
C
TM1
TM2
TM3
TM4
Y
Y
γ2 ERDEEYGYE
β1 PKATMYSYD

Figure 7.2. Modulation of recombinant $GABA_A$ receptors by genistein. (A,B) Representative whole-cell membrane currents evoked by 40μM GABA in control Ringer and in 100μM genistein in *Xenopus* oocytes expressing wild-type α1β1γ2S (A) and mutant $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ (B) $GABA_A$ receptors. Although inhibition of the GABA response was observed after 10 minutes, oocytes were incubated with genistein for 30 minutes to ensure maximal inhibition. The transient downward deflections represent current pulses induced by brief hyperpolarizing voltage commands (-10mV, 1s, 0.2Hz) applied to the holding potential of - 40mV. Note the different time calibrations on application and recovery from GABA due to slowing of the chart recorder speed (1: 5s; 2: 12.5s; 3: 50s). Recovery of the GABA response was observed after 10 minutes. (C,D) GABA concentration-response curves were constructed in the absence (■) and presence (●) of 100μM genistein for α1β1γ2S (C) and $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ (D) receptor constructs (n = 3 – 21 oocytes). Genistein produces a substantial inhibition of the GABA-activated response (inhibition of the response evoked by 1mM GABA: wild-type, 61%; mutant, 86%). Data are normalized to the response evoked by 40μM GABA in control Ringer and represent mean ± s.e.m. Curves were fitted according to the logistic equation in the methods (Chapter 2). A non-competitive mode of antagonism is indicated by the upper graphs in C and D, which show that the degree of inhibition exerted by genistein is, in essence, independent of GABA concentration.

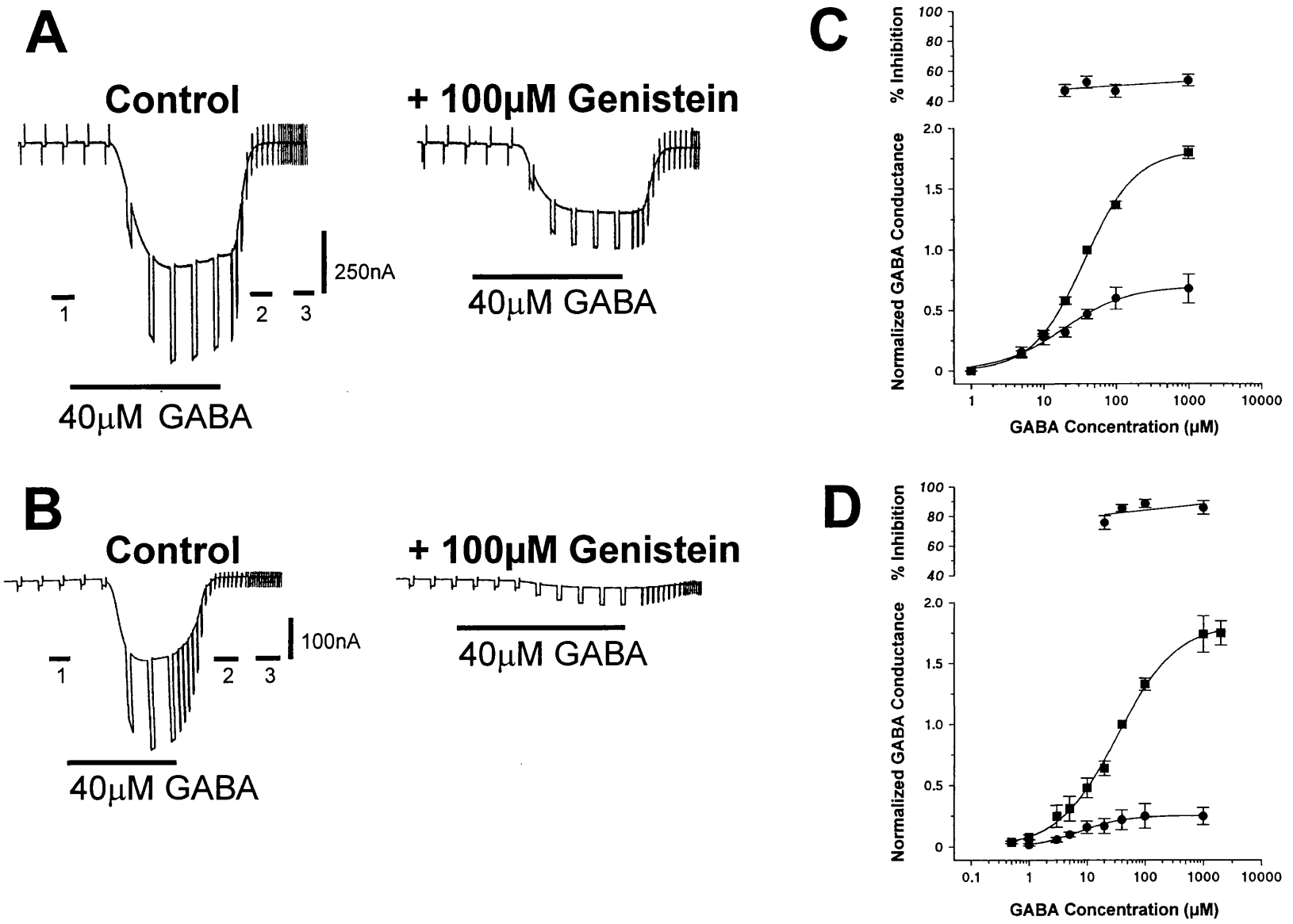
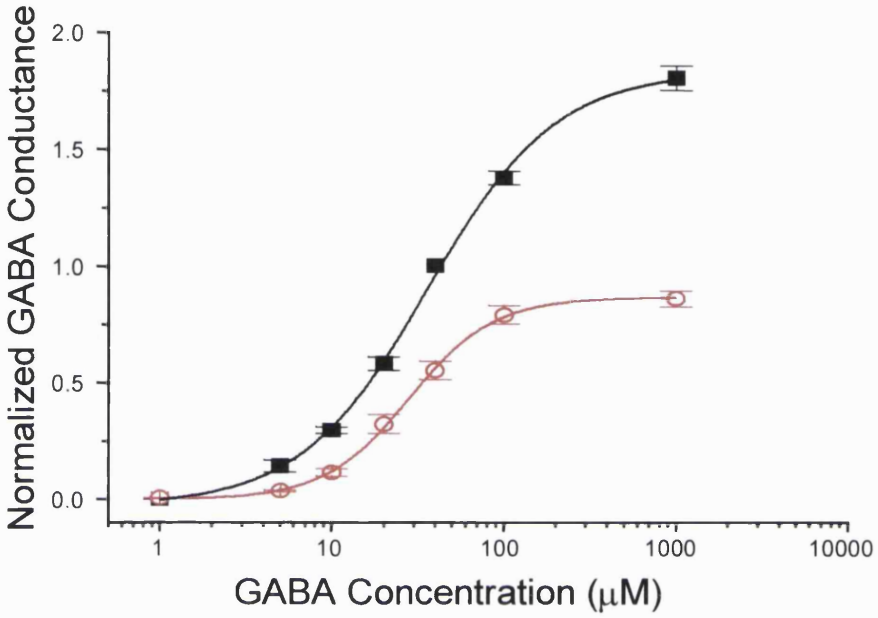

A
Control
+ 100μM Genistein
250nA
1
2
3
40μM GABA
40μM GABA
B
Control
+ 100μM Genistein
100nA
1
2
3
40μM GABA
40μM GABA
C
% Inhibition
Normalized GABA Conductance
GABA Concentration (μM)
D
% Inhibition
Normalized GABA Conductance
GABA Concentration (μM)

Figure 7.3. Daidzein inhibits recombinant $GABA_A$ receptor function. GABA concentration-response curves were established in the absence (■) and presence of 100μM daidzein (○) for wild-type α1β1γ2S (A) and mutant $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ (B) $GABA_A$ receptors expressed in *Xenopus* oocytes. External application of daidzein, an inactive PTK inhibitor, produces a noncompetitive depression of the GABA concentration-response curves (inhibition of response evoked by 1mM GABA: wild-type, 52%; mutant, 35%). Data points shown represent mean ± s.e.m. Data are normalized to the response to 40μM GABA in control Ringer and curves fitted to the data points as described in the methods (Chapter 2).

A


Normalized GABA Conductance
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
10000
GABA Concentration (μM)

B

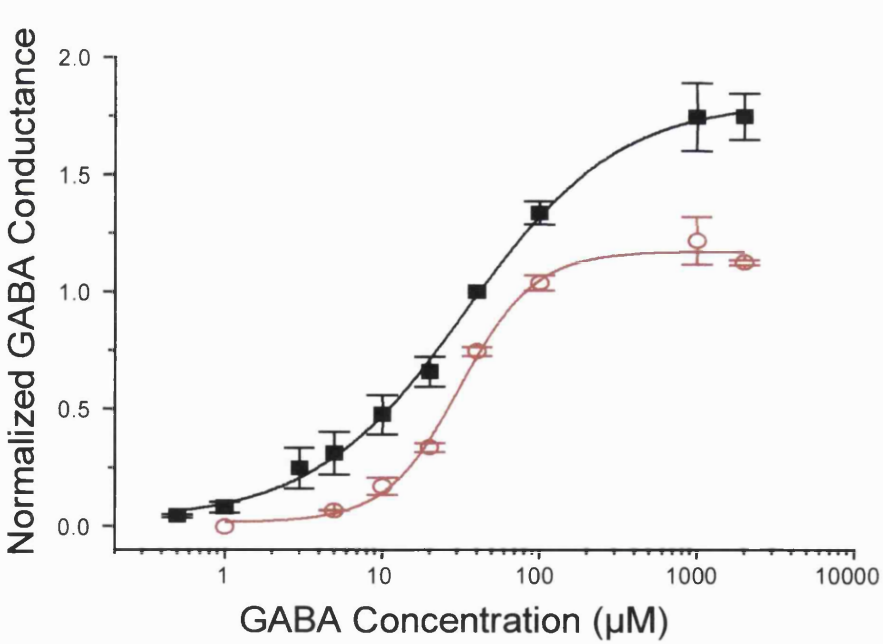

Normalized GABA Conductance
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
10000
GABA Concentration (μM)

Figure 7.4. Tyrphostins A1 and A25 reduce GABA-aetivated currents. (A, B) Typical whole-cell membrane currents induced by 40μM GABA in control Ringer and in 200μM tyrphostin A25 recorded from *Xenopus* oocytes expressing wild-type α1β1γ2S (A) and mutant $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ (B) $GABA_A$ receptors. The transient downward deflections represent current pulses induced by brief hyperpolarizing voltage commands (-10mV, 1s, 0.2Hz) applied to the holding potential of - 40mV. The different time calibrations on application and recovery from GABA refer to, 1: 5s; 2: 12.5s; 3: 50s. Oocytes were pre-treated with tyrphostin A25 in the bath solution for 30 minutes to ensure maximum inhibition of the $GABA_A$ receptor. Recovery of the response to 40μM GABA was observed after 10 minutes from removal of genistein. (C, D) GABA concentration-response curves were determined in the absence (■) and presence of either 200μM tyrphostin A1 (Δ) or tyrphostin A25 (▲) for α1β1γ2S (C) and $\alpha1\beta1\gamma2S^{Y365F,\ Y367F}$ (D) receptor constructs (n = 3 – 21 oocytes). Both tyrphostin A25 and the inactive analogue tyrphostin A1 generated a significant depression of the GABA concentration-response curve. Data are normalized to the response evoked by 40μM GABA in control Ringer and represent mean ± s.e.m. Curves were fitted according to the logistic equation in the methods. A non-competitive mode of antagonism is demonstrated by the upper graphs in C and D, which show that the degree of inhibition exerted by tyrphostin A25 is virtually independent of GABA concentration.

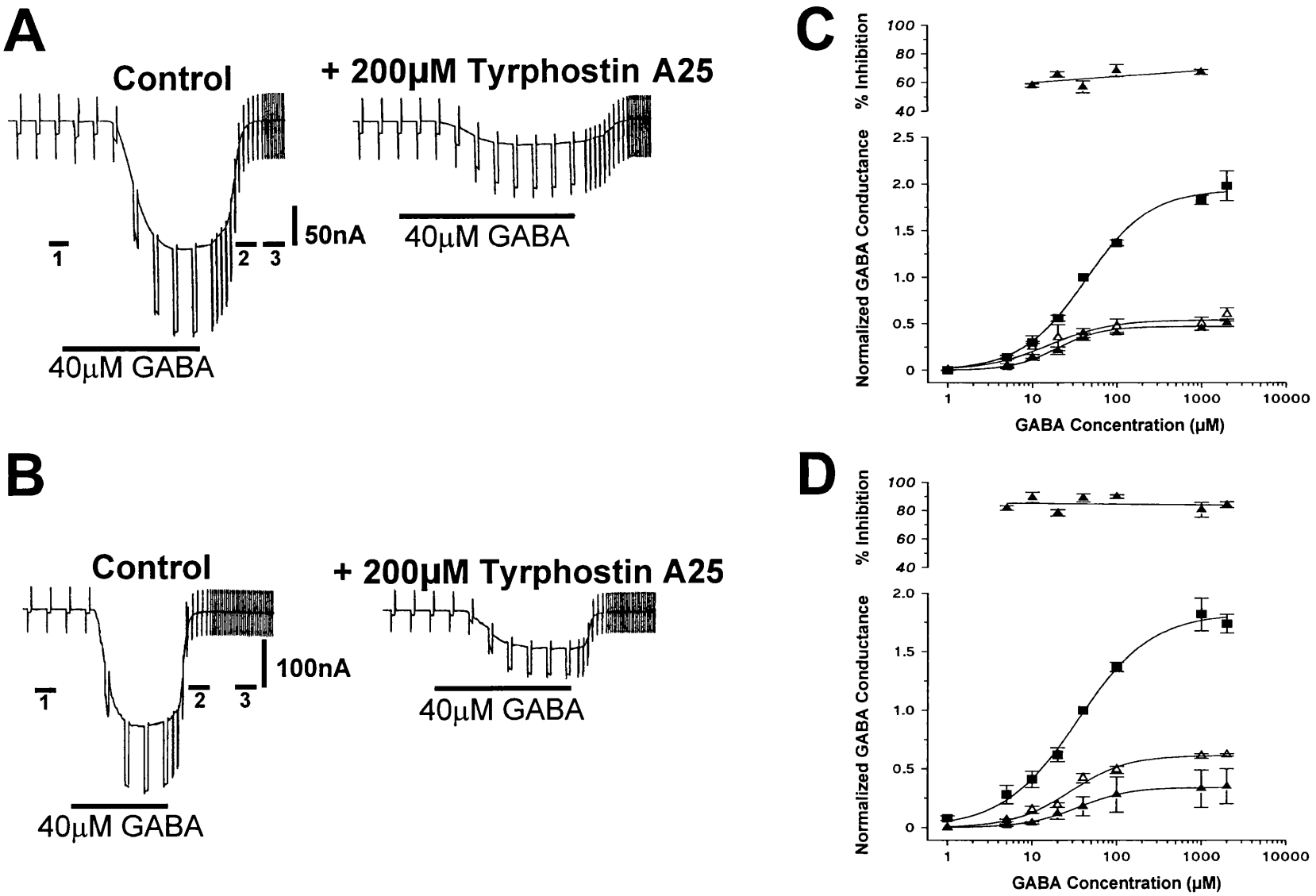
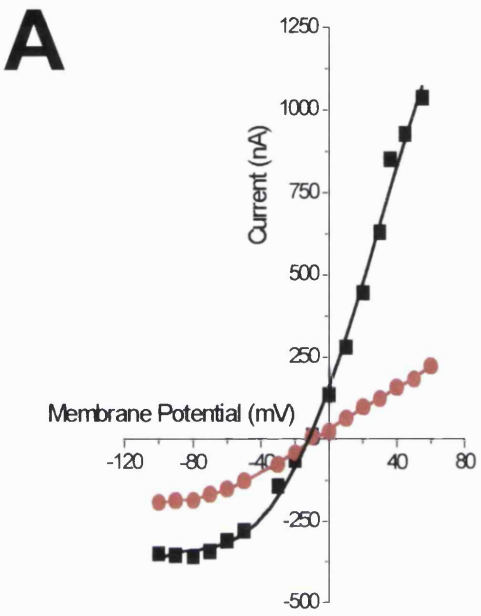
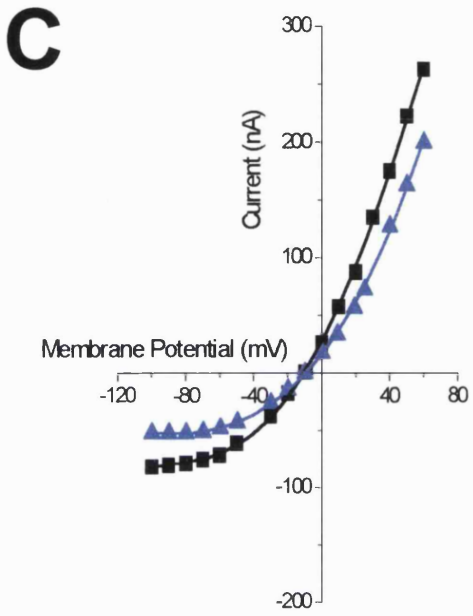
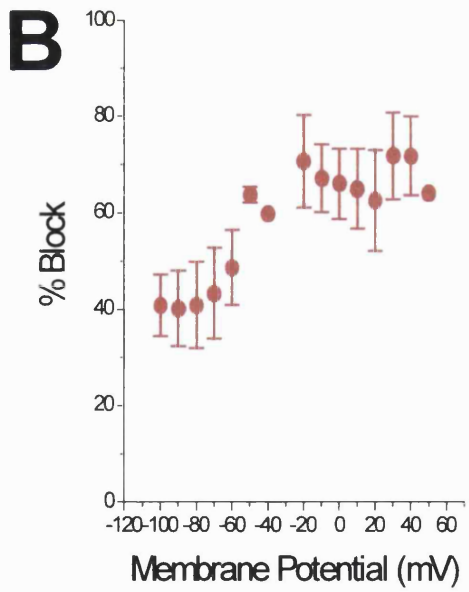

A
Control
+ 200µM Tyrphostin A25
50nA
1
2
3
40µM GABA
40µM GABA
B
Control
+ 200µM Tyrphostin A25
100nA
1
2
3
40µM GABA
40µM GABA
C
% Inhibition
Normalized GABA Conductance
GABA Concentration (µM)
D
% Inhibition
Normalized GABA Conductance
GABA Concentration (µM)

Figure 7.5. Voltage sensitivity of the antagonism of $GABA_A$ receptors by PTK inhibitors. (A, C) Representative current-voltage (I-V) relationships for the response evoked by 10μM GABA prior to (■) and during exposure to 100μM genistein (A, ●) or 200μM tyrphostin A25 (C, ▲) for wild-type α1β1γ2S $GABA_A$ receptors expressed in *Xenopus* oocytes. The reversal potential of the wild-type receptor remained unchanged in the presence of either genistein (A: control, -22.6 ± 5.1mV; + 100μM genistein, -21.8 ± 4.6mV; n = 3 oocytes) or tyrphostin A25 (B: control, -23.1 ± 5.4mV; +200μM tyrphostin A25, -22.9 ± 5mV; n = 3 oocytes). Both I-V relationships are leak-subtracted and the curves are generated by fifth order polynomials. The relative degree of inhibition evoked by 100μM genistein (B) and 200μM tyrphostin A25 (D) at each membrane potential was obtained by subtraction between the individual I-V plots (control – inhibitor treated), and the difference expressed as a percentage of the control GABA I-V relationship. Data points close to and at the reversal potential have been omitted from plots B and D. Data points shown are mean ± s.e.m. from n = 3 oocytes.


A
Current (nA)
1250
1000
750
500
250
-250
-500
Membrane Potential (mV)
-120
-80
-40
40
80


C
Current (nA)
300
200
100
-100
-200
Membrane Potential (mV)
-120
-80
-40
40
80


B
% Block
100
80
60
40
20
0
-120 -100 -80 -60 -40 -20 0 20 40 60
Membrane Potential (mV)

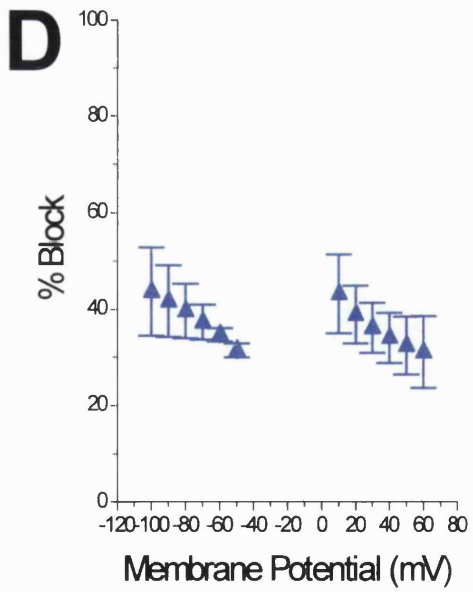
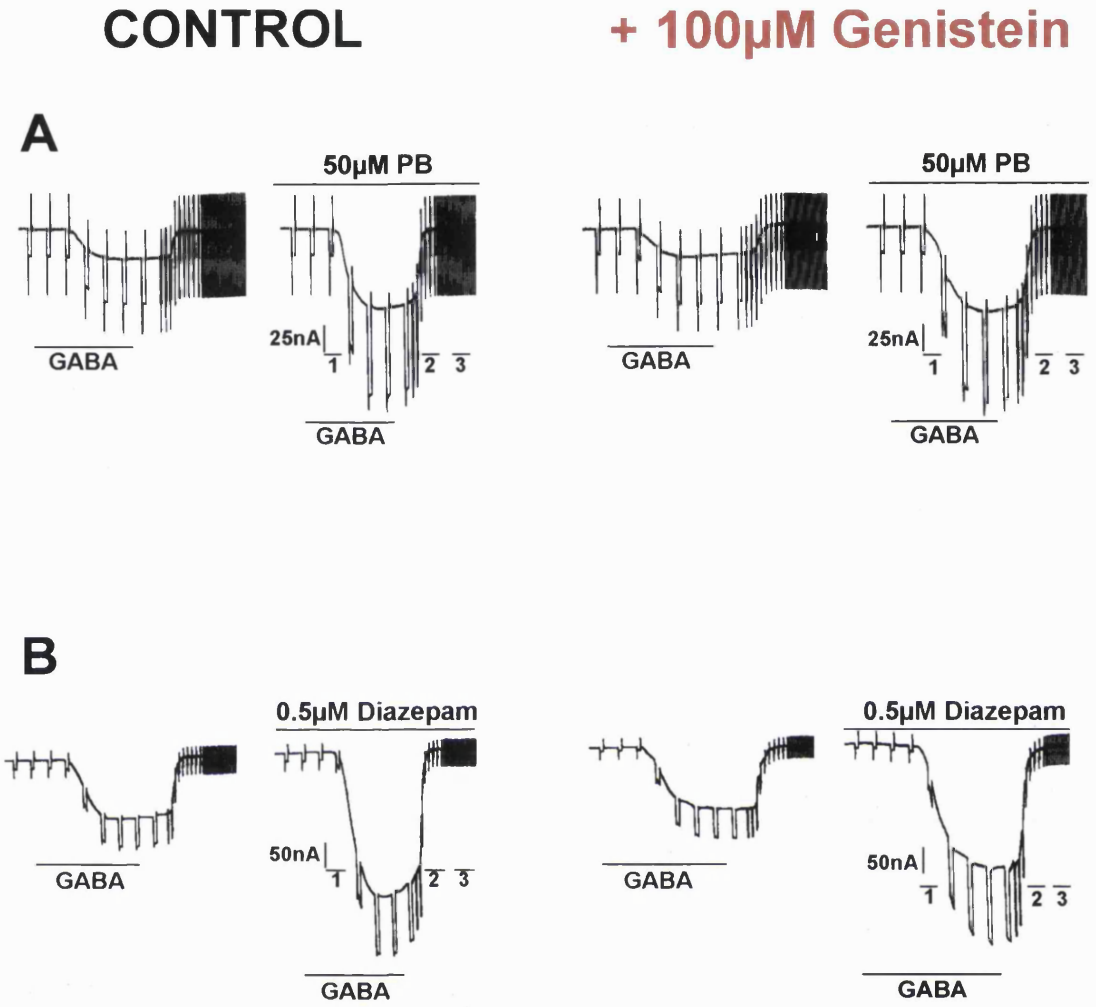

D
% Block
100
80
60
40
20
0
-120 -100 -80 -60 -40 -20 0 20 40 60 80
Membrane Potential (mV)

Figure 7.6. Potentiation of wild-type α1β1γ2S receptor function by PB and diazepam is unaffected by PTK inhibitors. Characteristic whole-cell membrane currents induced by GABA prior to and during exposure to either 50μM PB (A) or 0.5μM diazepam (B) in the presence and absence of 100μM genistein. In order to easily compare the potentiating effect, the responses shown are evoked by either 20μM GABA (control) or 100μM GABA (+ genistein). Genistein does not affect the enhancement of the GABA-activated response induced by PB and diazepam. The transient downward deflections represent leak current pulses induced by brief hyperpolarizing voltage commands (-10mV, 1s, 0.2Hz) applied to the holding potential of - 40mV. The different time calibrations on application and recovery from GABA represent, 1: 5s; 2: 12.5s; 3: 50s. (C) The relative potentiation of the EC_{20} GABA response by 50μM PB (closed bar) and 0.5μM diazepam (open bar) in the presence of either 100μM genistein or 200μM tyrphostin A25 (n = 3 oocytes). The potentiation is normalized to the control potentiation of an EC_{20} GABA response by PB or diazepam in control Ringer (defined as 1).


CONTROL
+ 100μM Genistein
A
50μM PB
GABA
25nA
1
2
3
50μM PB
GABA
25nA
1
2
3
GABA
B
0.5μM Diazepam
GABA
50nA
1
2
3
GABA
0.5μM Diazepam
GABA
50nA
1
2
3
GABA

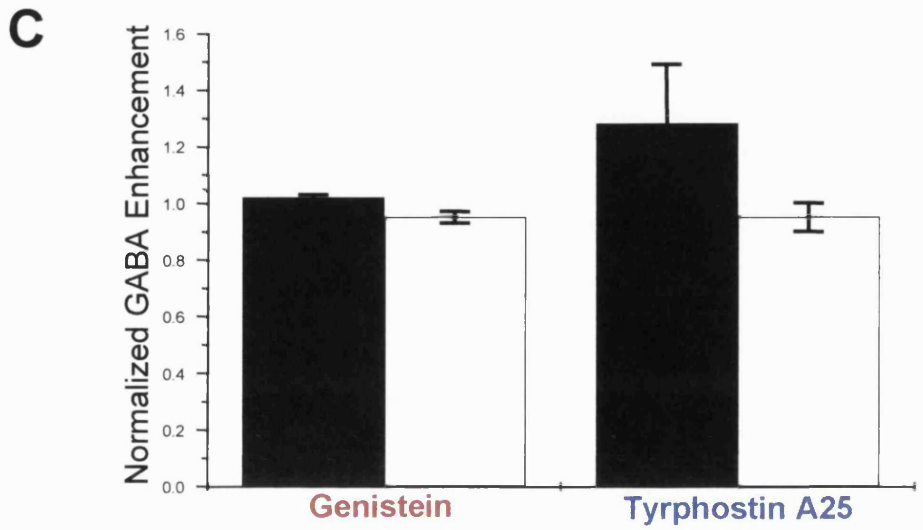

C
Normalized GABA Enhancement
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Genistein
Tyrphostin A25

Figure 7.7. The substrate tyrosine residues on the β1 subunit are not involved in the antagonism of $GABA_A$ receptors by externally applied genistein. GABA concentration-response curves were obtained in the absence (■) and presence (●) of 100μM genistein (applied via the bath solution) for HEK cells expressing $\alpha 1\beta 1^{Y370F, Y372F}\gamma 2S^{Y365F, Y367F}$ $GABA_A$ receptors. Despite mutating all the substrate tyrosine residues known to be phosphorylated by protein tyrosine kinases, genistein significantly inhibited the GABA-activated current (approximately 57% inhibition at each GABA concentration). All points represent mean ± s.e.m. from n = 4 cells. Curves are fitted according to the logistic equation in the methods (Chapter 2).

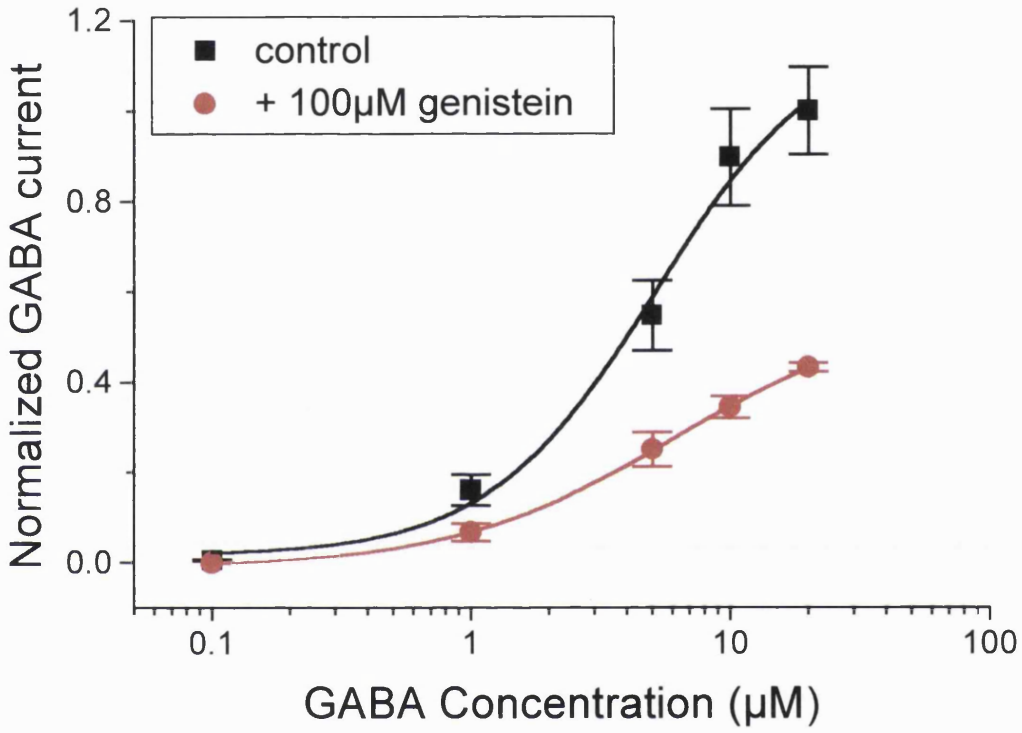
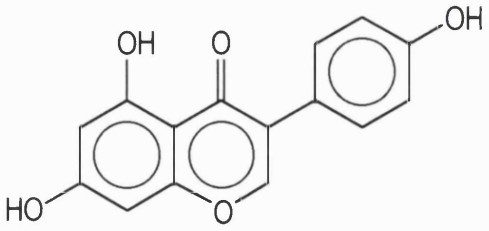
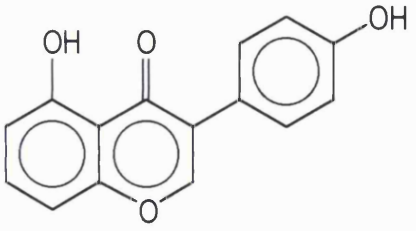

control
+ 100μM genistein
Normalized GABA current
1.2
0.8
0.4
0.0
0.1
1
10
100
GABA Concentration (μM)

Figure 7.8. Structure of typical PTK inhibitors. Genistein (4',5,7-trihydroxyflavone) is a commonly used inhibitor, acting at the ATP binding site on PTKs. Daidzein (4',7-dihydroxyflavone) is used as an inactive control for genistein, differing only by the deletion of a hydroxyl group on the A ring. The tyrphostins are substrate binding inhibitors and are structurally different to genistein, based on a hydroxycinnamonitrile nucleus. Tyrphostin A25 (α-cyano-(3,4,5-trihydroxy) cinnamonitrile) was chosen as a frequently used PTK inhibitor in conjunction with tyrphostin A1 (α-cyano-(4-methoxy)cinnamonitrile) as an inactive control.

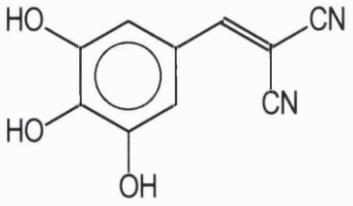
Genistein



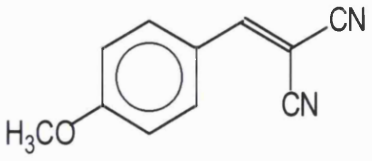
Daidzein



TYROSINE KINASE INHIBITORS



Tyrphostin A25



Tyrphostin A1

CHAPTER 8

General Discussion.

8.1. Overview of the current study.

The present study primarily addressed the interactions of zinc with the $GABA_A$ receptor. This led to the identification of residues that have a marked effect on the potency of zinc and GABA, raising the possibility that they contribute to, or at least influence, the binding sites for these compounds or directly affect the ion-channel transduction mechanisms. In addition, zinc antagonism was used as the basis of a novel method to probe the structure of the channel and a zinc-resistant receptor mutant was used to address the stoichiometry of β subunit homo-oligomers. Furthermore, the study demonstrated that certain widely-used tyrosine kinase inhibitors antagonise the $GABA_A$ receptor independently of PTK, suggesting caution in the use of these compounds.

8.2. Non-specific actions of tyrosine kinase inhibitors.

This investigation revealed a novel and direct interaction of genistein, daidzein and some tyrphostins with the $GABA_A$ receptor. These membrane permeable PTK inhibitors, when extracellularly applied, are able to inhibit $GABA_A$ receptor function via a PTK independent mechanism. Genistein (and daidzein, an inactive analogue) are ATP binding inhibitors and structurally different to the tyrphostins which inhibit substrate binding. Although, the precise site of the non-specific interaction is unresolved, the non-competitive nature of the inhibition by these compounds discounts a direct interaction with the GABA binding domain. In addition, potentiation of $GABA_A$ receptor function by pentobarbitone and diazepam is unaltered by the external application of PTK inhibitors indicating a novel site of action, which could conceivably be the ion channel. This investigation highlights the importance of disrupting phosphorylation consensus sites by site-directed mutagenesis as a stringent negative control for apparent kinase regulation since $\alpha 1\beta 1^{Y370F,\ Y372F}\gamma 2S^{Y365F,\ Y367F}$ $GABA_A$ receptors, completely devoid of substrates for tyrosine phosphorylation, were still susceptible to inhibition. Inhibition of GABA-activated conductance resulting from the extracellular application of these PTK inhibitors has routinely been used to deduce the regulation of $GABA_A$ receptor function by endogenous protein kinases (Valenzuela *et al.*, 1995; Jassar *et al.*, 1997; Wan *et al.*, 1997). However, this study provides the first indication that such a deduction may be inaccurate. Future investigations into the

tyrosine phosphorylation of the large intracellular TM3-TM4 domain of the $GABA_A$ receptor should therefore be conducted via intracellular application of these inhibitors.

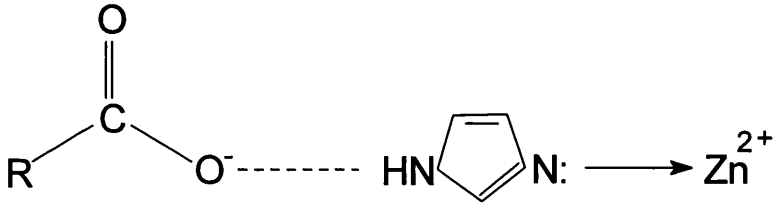
8.3. The N-terminal domain.

8.3.1. Determinants of Zn^{2+} antagonism on the N-terminal domain.

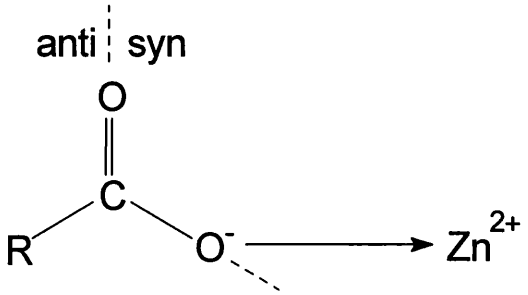
The role of the N-terminal domain in the responsiveness of the $GABA_A$ receptor to Zn^{2+} inhibition was investigated. Previously a TM2 histidine residue on the β3 subunit has been identified as important for Zn^{2+} inhibition of $GABA_A$ receptor function (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998). This study represents the first demonstration that N-terminal domain residues mediate Zn^{2+} inhibition of $GABA_A$ receptors. Using $GABA_A$ receptor β3 subunit homomers, a highly conserved N-terminal histidine ($\beta3^{H107}$) was implicated in mediating Zn^{2+} inhibition. Although this study ruled out the involvement of other histidine residues in the β subunit, the contribution of residue H107 was deduced indirectly since even conservative mutations of this residue resulted in a loss of receptor function. However, substituting $\beta3^{H107}$ did not reduce the Zn^{2+} sensitivity of $\alpha1\beta3^{H267A}$ $GABA_A$ receptors, implying that in the αβ hetero-oligomer this residue may be inaccessible to Zn^{2+}, or that its effects are masked by residues in the α1 subunit. This may highlight a difference in the surface structure of β homomeric receptors and αβ heteromeric receptors. However, an N-terminal histidine (H141) on the α1 subunit was shown to influence the inhibitory effect of Zn^{2+}, reducing the Zn^{2+} sensitivity of $\alpha1\beta3^{H267A}$ receptor constructs approximately 25-fold. The present study is the first to identify a putative Zn^{2+} binding site on the α1 subunit.

Interestingly, complete inhibition of the GABA-activated conductance from $\alpha1^{H141A}$ $\beta3^{H267A}$ constructs was still achieved by high concentrations of Zn^{2+}. Future work to elucidate the residues involved in this residual inhibition should clearly target both α1 and β3 subunits. Although the residual Zn^{2+} inhibition on both $\beta3^{H267A}$ and $\alpha1^{H141A}$ $\beta3^{H267A}$ receptors was eliminated by low external pH implicating the further involvement of histidines, other non-histidine residues could conceivably be important

as pK_a values may not be preserved within the microenvironment of the $GABA_A$ receptor protein. For example, the pK_a of histidine can be altered by the formation of hydrogen bonds with neighbouring amino acids and the degree of this shift is dependent upon the group with which the histidine bonds and whether the residue is partly buried within the folded receptor protein (Christianson & Alexander, 1989). Potential candidates for mediating low affinity Zn^{2+} inhibition include $\beta3^{E270}$, located in close proximity to H267 in the putative ion channel pore. This glutamate is in the aligned position to $\alpha6^{H273}$; a residue previously identified as contributing to the inhibitory action of Zn^{2+} (Fisher & Macdonald, 1998). In metalloproteins, histidine residues commonly bridge both Zn^{2+} and the carboxylate side chain of a nearby aspartate/glutamate residue (Christianson & Alexander, 1989).



Therefore $\beta3^{E270}$ may simply be involved in mediating the inhibitory effects of Zn^{2+} by increasing the basicity of $\beta3^{H267A}$ towards Zn^{2+}. Alternatively, though less common in metalloproteins, Zn^{2+} may directly interact with the carboxylate group of $\beta3^{E270}$ provided the carboxylate group adopts the *syn* orientation (Christianson & Alexander, 1989).



In addition, it is possible that the involvement of other extracellular histidine residues in lower affinity Zn^{2+} binding is not observable when $\alpha1^{H141}$ is present. Therefore, further work involving site-directed mutagenesis is needed to identify the full complement of amino acids involved in mediating the Zn^{2+}inhibition of $GABA_A$ receptor function.

Interestingly, $\alpha1^{H141}$ may play a role in the inhibitory effect of Cu^{2+} on α1 subunit-containing $GABA_A$ receptors. Cu^{2+}, like Zn^{2+} is a putative endogenous modulator of $GABA_A$ receptor function and therefore may be involved in physiologically regulating synaptic inhibition. Studies using $GABA_A$ receptor chimeras identified the N-terminal region of α1 subunits as responsible for Cu^{2+} sensitivity (Fisher & Macdonald, 1998). Potentially, this observation may be due to $\alpha1^{H141}$. Conflicting reports on the existence of a common site of action on the $GABA_A$ receptor for Zn^{2+} and Cu^{2+} appear to reflect the fact that different neuronal preparations have been tested (Ma & Narahashi, 1993a *cf.* Kumamoto & Murata, 1995). Therefore, the α1 subunit isoform may be the predominant isoform in $GABA_A$ receptors in which the inhibitory action of Zn^{2+} is competitively antagonised by Cu^{2+}.

Furthermore, the N-terminal domain of the α1 subunit has been implicated in coordinating the inhibitory effect of Cd^{2+} (Fisher & Macdonald, 1998). Although the endogenous release of Cd^{2+} is unlikely to be demonstrated, investigating the molecular determinants of Cd^{2+} modulation of $GABA_A$ receptor function may be useful in understanding the neurotoxic effects of this divalent cation. As a single residue located on the extracellular TM2-TM3 loop of the α6 subunit (H273) confers both Zn^{2+} and Cd^{2+} sensitivity to α6 subunit-containing $GABA_A$ receptors (Fisher & Macdonald, 1998), it is conceivable that a single residue on the α1 subunit (i.e. H141) mediates the inhibitory effects of both these divalent cations. Therefore, it appears that sites for divalent cations are located on different regulatory domains depending on the α subunit isoform present in the $GABA_A$ receptor construct. Future work will determine the precise involvement of $\alpha1^{H141}$ in regulating the actions of various divalent cations.

8.3.2. Identification of an N-terminal residue involved in β homo-oligomer formation

The present study identified a highly conserved, polar N-terminal residue, $\beta3^{H107}$ critical for correct homomeric $GABA_A$ receptor function. The mutation $\beta3^{H107A}$ resulted in a loss of channel function and retention of mutant subunits in the ER, implicating a putative role in protein folding. The retention of $\beta3^{H107A}$ was probably via an association with chaperone proteins such as BiP, which interacts with exposed

hydrophobic domains in misfolded proteins (Pelham, 1989; Connolly *et al.*, 1996a). Furthermore, conservative substitutions of $\beta3^{H107}$ with highly polar lysine ($\beta3^{H107K}$) or arginine ($\beta3^{H107R}$) residues failed to result in functional β3 subunit receptors indicating that this structurally important histidine residue cannot be replaced by an alternative amino acid with a polar side chain.

While protein-protein interactions are frequently mediated by hydrophobic residues, there is evidence that polar residues may have a role in the folding and assembly of neurotransmitter receptor subunits. The formation of $GABA_A$ receptor β3 homomers is dependent on N-terminal lysine, glutamate and arginine residues (Taylor *et al.*, 1999). Although these residues are situated some distance from H107, it is likely that more than one region of the receptor will be involved. Furthermore, an arginine residue, located at the putative start of TM2 and conserved in all GABA and glycine receptor subunits, is important for the correct biogenesis of glycine receptor α1 subunit homomers (Langosch *et al.*, 1993). Substitution of this residue, R219E/Q, resulted in the intracellular retention of the mutant subunits suggesting incorrect biogenesis of the subunit. However, it has been suggested that this residue may halt the transfer of TM2 through the lipid bilayer during polypeptide synthesis, and as such its role is distinct from that proposed for $\beta3^{H107}$. Nevertheless, the result of the present study points a potential N-terminal subdomain that may be important in the folding and/or oligomerisation of $GABA_A$ receptor subunits. Confocal microscopy demonstrated that $\beta3^{H107}$ was not inserted in the plasma membrane of HEK 293 cells appearing to be retained in the endoplasmic reticulum, suggesting the same would probably be true in *Xenopus* oocytes. Thus it appears that H107 is possibly involved in the biogenesis or assembly of $GABA_A$ receptors. This is consistent with the failure of PB to induce inward currents, and the inability of Zn^{2+} or picrotoxin to induce outward currents in oocytes injected with this subunit.

8.3.3. A novel determinant of GABA potency.

GABA binding regions have been identified on the N-terminal domains of α and β subunits, presumably located at the interface of these two subunits (Mehta & Ticku, 1999). The present study identified an N-terminal residue, $\alpha1^{H109}$, which strongly

influences GABA potency. The mutation $\alpha1^{H109A}$ produced a 50-fold reduction in GABA potency ($\alpha1^{H109A}\beta3^{H107A,\ H267A}$, EC_{50}: 389.77 ± 27.53μM; n_H: 0.89 ± 0.04; $\alpha1\beta3^{H107A,\ H267A}$, EC_{50}: 8.08 ± 0.74μM; n_H: 1.1 ± 0.12; n = 3 - 5 oocytes), which is comparable to the reduction generated by substituting other residues implicated in GABA binding. $\alpha1^{H109A}$ is not located in the known GABA binding domain of the α1 subunit ($\alpha1^{F64}$, $\alpha1^{R66}$, $\alpha1^{S68}$), however it is quite conceivable that more than one region of α subunits may contribute to the GABA binding site. This is the case for the γ, δ and ε and α7 subunits of nACh receptors, and it is notable that one of the three regions of these subunits which determine agonist potency aligns with F64 in $GABA_A$ receptor α (Cohen *et al.*, 1990; O'Leary *et al.*, 1994; Corringer *et al.*, 1995; Prince & Sine, 1997). Furthermore, a recent study of glycine receptors demonstrated that residues spanning the equivalent of $\alpha1^{H109}$ were found to disrupt the binding of competitive antagonists (Schmieden *et al.*, 1999). One of these residues glycine $\alpha1^{F108}$, aligns adjacent to GABA $\alpha1^{H109}$.

Although the key components of ligand binding domains frequently appear to be aromatic (Y, F) rather than polar (H) amino acid side chains, conservative substitution of a nearby arginine residue ($\alpha1^{R120K}$) has recently been shown to abolish [^{3}H]muscimol binding to α1β2γ2s. (Westh-Hansen *et al.*, 1999). Mutation of the equivalent residue into the α5 subunit (R123K), resulted in an approximate 100-fold reduction in the apparent affinity for GABA (Hartvig *et al.*, 1999). It is possible that the histidine and arginine residues disrupt the GABA bind site/ transduction mechanism allosterically. However, these highly polar residues help co-ordinate the correct folding of a previously unknown part of the GABA binding site on the α subunit. Further investigation of these possibilities should involve radioligand binding studies, the co-expression of $\alpha1^{H109A}$ and wild-type β3 and γ2 subunits and possibly mutation of other residues in the region.

8.4. The ion channel.

The second transmembrane domain,TM2, contributed by each subunit, is considered to constitute the majority of the $GABA_A$ receptor anion channel. The present study

investigated ion channel properties of $GABA_A$ receptor β3 subunit homomers. A putative Zn^{2+} binding site has been identified at the extracellular end of the ion channel (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998), therefore the present study developed a novel substituted histidine accessibility method (SHAM) in order to identify the further accessibility of the $GABA_A$ receptor ion channel to Zn^{2+}. Four putative channel lining residues, selected to be on discrete turns of a α-helix were individually substituted with histidine in $\beta3^{H267A}$ $GABA_A$ receptor homomers. The only substitution to increase Zn^{2+} potency was at the 13' position, suggesting Zn^{2+} is able to penetrate into the anion channel at least as far as this residue (see Chapter 6 for discussion). This observation also provides information on the structure of the channel in this region. In order for Zn^{2+} to interact with histidines (presumably up to four residues, given a tetrameric stoichiometry) the α carbons of the histidines must be less than 13Å apart (Higaki *et al.*, 1992).

The substituted residues on the β3 subunit were chosen because they are analogous to putative channel lining residues on the α1 subunit identified by applying SCAM to α1β3 $GABA_A$ receptors (Xu & Akabas, 1996). Some of these residues may not in fact line the channel in β3 subunit homomers. Given the likely difference in the subunit stoichiometry of β3 subunit homomers (probably tetrameric) and α1β3 receptor constructs (probably pentameric, Kellenberger *et al.*, 1996; Tretter *et al.*, 1997), it is conceivable that there may be some variation in ion channel structure. It could prove useful to first identify channel lining residues on the β3 subunit using SCAM (see Karlin & Akabas, 1998, for review), to fully ascertain whether a lack of Zn^{2+} sensitivity was due to a lack of penetration or whether the residues faced the interior of the receptor. Alternatively, Zn^{2+}'s lack of effect may reflect the importance of where in the channel it binds, rather than how deep into the pore the ion penetrates. A residue just N-terminal to $\beta3^{H267}$, $\beta3^{N265}$, profoundly affects the potency of a variety of allosteric modulators, and to a lesser extent that of GABA (Belelli *et al.*, 1999b for review; McGurk *et al.*, 1998). Thus, it is likely that this region of the receptor is important in regulating the conformational changes associated with gating. It is therefore possible that the binding of Zn^{2+} at position 267 is optimal for inhibiting channel opening, while addition of charge at residue 263 (in $\beta3^{T263H}$ mutants) has a lesser effect on channel gating.

The present study also confirmed previous reports on the effect of mutating certain TM2 residues on $GABA_A$ receptor function, including impaired channel gating (Chang et al., 1996; Tierney *et al.*, 1996; Thompson *et al.*, 1999b) and sensitivity to PTX (Ffrench-Constant *et al.,* 1993; Gurley *et al.*, 1995; Xu *et al.*, 1995). Future applications of SHAM could include investigating ion channel function in heteromeric $GABA_A$ receptors, using $\alpha1^{H141A}\beta3^{H267A}$ receptor constructs, which demonstrate more than 3100-fold reduction in Zn^{2+} sensitivity compared to wild-type $\alpha1\beta3$ $GABA_A$ receptors. In addition, mutation of T256 abolished PB gating of β3 homomers. This is the first report of the 6' residue affecting the actions of PB and it will be of interest to determine how this mutation affects PB modulation and direct activation of heteromeric $GABA_A$ receptors.

8.5. Subunit stoichiometry.

Identifying the precise subunit stoichiometry and arrangement is important for investigating $GABA_A$ receptor structure and function. The precise stoichiometry of heteromeric $GABA_A$ receptors is still unresolved. Homo-oligomers of $GABA_A$ receptor β(1or3) subunits are commonly used to avoid problems regarding receptor heterogeneity experienced when different subunits are coexpressed. Since homomeric β subunit ion channels are presumably symmetrical, identifying molecular determinants affecting the modulation of $GABA_A$ receptor function by site-directed mutagenesis should be easily observable. β subunits contain residues influencing receptor assembly (Taylor *et al.*, 1999) and GABA binding (Amin & Weiss, 1993), as well as sites of action for several modulators of $GABA_A$ receptor function (including Zn^{2+} (Wooltorton *et al.*, 1997a; Horenstein & Akabas, 1998), general anaesthetics (Belelli *et al.*, 1999b for review) and serine/threonine kinases (Moss & Smart, 1996; Smart, 1997 for review). Although β3 homomeric channels are formed with low efficiency (Slany *et al.*, 1995; Connolly *et al.*, 1996b) and therefore probably form only a minor population of $GABA_A$ receptors *in vivo*, β3 subunit homomers might become relatively enriched in cells following co-transfection of α1 and β3 subunits (Tretter *et al.*, 1997). A model based on the binomial theorem and developed originally by MacKinnon (1991) was critically re-assessed in the present study and used to predict a tetrameric stoichiometry for β3

subunit homomers. Therefore the present study, representing the first electrophysiological analysis of the subunit stoichiometry of $GABA_A$ receptor β3 subunit homomers, has important implications for examining the structure and function of these receptors.

8.6. Summary.

The present study investigating regulatory domains identified several novel determinants affecting the structure and function of the $GABA_A$ receptor. Novel N-terminal residues regulating the inhibitory effect of Zn^{2+} were identified, which may prove to influence the actions of other divalent cations. In addition, novel determinants of receptor folding and GABA potency were revealed to be present also on this domain. A functional assay based on the binomial theorem designed by MacKinnon (1991) was re-developed and predicted a tetrameric stoichiometry for $GABA_A$ receptor β3 subunit homomers. A new method was developed to probe ion channel properties and assess the accessibility of this region to Zn^{2+}. Finally, a novel non-specific interaction with the $GABA_A$ receptor, conceivably via the channel pore, was demonstrated for PTK inhibitors. Therefore, the present study has described novel aspects of the molecular structure and function of select regulatory domains of the $GABA_A$ receptor important for function, expression and allosteric modulation.

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