Co-regulation of $GABA_A$ receptors by neurosteroids and protein kinases

Joanna Mary Adams

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Department of Neuroscience,
Physiology and Pharmacology
University College London
Gower Street
London
WC1E 6BT

Declaration

I, Joanna Adams, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Joanna Adams

Abstract

γ-aminobutyric acid type-A ($GABA_A$) receptors mediate inhibitory synaptic transmission in the CNS where neurosteroids and protein kinases are their most potent endogenous modulators. Acting individually, these can either enhance or depress receptor function depending on the type of neurosteroid or kinase present, and the subunit combination of the receptor. However, *in vivo*, these agents probably act in concert to modulate $GABA_A$ receptors and precisely 'fine-tune' inhibitory neurotransmission; although how this is achieved remains unclear. Therefore, the relationship between these two modulators, at α1β3γ2L and α4β3δ $GABA_A$ receptors, expressed in HEK293 cells, was investigated using whole-cell patch clamp electrophysiology.

At α1β3γ2L receptors, the potentiation of GABA responses by tetrahydrodeoxycorticosterone (THDOC) was reduced by PKC inhibition and enhanced by PKC activation, implying a role for this kinase in regulating neurosteroid potentiation. By comparison, neurosteroid potentiation was reduced at $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors, and was unaltered by PKC inhibitors or activators indicating that phosphorylation of the β3 subunit, by PKC, is important for regulating neurosteroid activity. To determine whether 'extrasynaptic-type' $GABA_A$ receptors are modulated similarly, experiments were also undertaken with α4β3δ and $\alpha4\beta3^{S408A,S409A}\delta$ receptors. Neurosteroid potentiation was significantly reduced at both receptor subtypes after treatment with the protein kinase inhibitor, staurosporine. Staurosporine was notably less effective at $\alpha4\beta3^{S408A,S409A}\delta$ receptors, suggesting that, although β3 subunit phosphorylation may play a role in the regulation of neurosteroid potentiation at α4β3δ receptors, it does not fully account for this modulation. Biochemical experiments on α4 subunits identified a new Ser/Thr phosphorylation site (S443). THDOC-mediated potentiation at $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ receptors was unaffected by protein kinase inhibition, strongly suggesting that phosphorylation of both the α4 and β3 subunits is required for the regulation of neurosteroid activity at α4β3δ receptors. Furthermore, Western blot analysis for wild-type α1β3γ2L receptors, revealed that THDOC increased phosphorylation of $\beta3^{S408,S409}$ implying a 'reverse' pathway exists for neurosteroids to modulate the phosphorylation state of the $GABA_A$ receptor.

Overall, these findings provide an important insight into the regulation of $GABA_A$ receptors *in vivo*, and into the mechanisms by which fine-tuning of GABAergic inhibitory transmission may be achieved by two endogenous neuromodulators.

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List of Abbreviations

3α-HSD	3α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase/isomerase
5-HT_3	5-Hydroxytryptamine 3 receptor
ACN	(3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile (synthetic neurosteroid)
AKAP	A-kinase anchoring protein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B285	(3α,5β,17β)-3-hydroxy-18-norandrostane-17-carbonitrile (synthetic neurosteroid)
Bis I	Bisindolylmaleimide I (PKC inhibitor)
CaMKII	Calcium-calmodulin dependent protein kinase-II
cAMP	Cyclic adenosine monophosphate
CGC	Cerebellar granule cell
CNS	Central nervous system
DAG	Diacylglycerol
DGGC	Dentate gyrus granule cell
DHEA	Dehydroepiandrosterone
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle medium
EC_{20}	Concentration eliciting 20% of the maximal response
eGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GABARAP	$GABA_A$ receptor associated protein
GC-MF	Gas chromatography-Mass fragmentography
GHB	γ-hydroxybutyrate
Glvi	*Gloeobacter violaceus*
GST	Glutathione Sepharose Transferase
HAP1	Huntingtin-associated protein 1
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney 293

HPLC	High performance liquid chromatography
IC_{50}	Concentration eliciting 50% of the total inhibition
KIF5	Kinesin family motor protein 5
nAch	Nicotinic acetylcholine receptor
NSF	N-ethylmaleimide-sensitive factor
P5	Postnatal day 5
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKA	Protein Kinase A (cAMP-dependent protein kinase)
PKAI	Myristoylated PKA inhibitor peptide 14-22 amide
PKC	Protein Kinase C
PLC	Phospholipase C
PKG	Protein Kinase G (cGMP-dependent protein kinase)
PMA	Phorbol-12-myristate-13-acetate (Phorbol ester)
PMDD	Premenstrual dysphoric disorder
P_{open}	Probability of channel opening
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PP2B	Protein Phosphatase 2B (also known as calcineurin)
PRIP	Phospholipase C-related, but catalytically inactive protein
PYK	Protein Tyrosine Kinase
RACK	Receptor for activated C-kinase
SCAM	Substituted cysteine accessibility method
SCG	Superior cervical ganglion
Ser	Serine
StAR	Steroidogenic acute regulatory protein
TBST	Tris buffered saline + 0.1% Tween
THDOC	Tetrahydrodeoxycorticosterone
THIP	4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol
Thr	Threonine
TM	Transmembrane
TSPO	Transporter protein (also known as the peripheral benzodiazepine receptor)
Tyr	Tyrosine

Chapter 1

Introduction

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the mammalian CNS, with the activity of almost all mature neurons being inhibited by applied GABA (Burt and Kamatchi, 1991). These inhibitory effects of GABA are mediated through one of two subclasses of GABA receptor: the ionotropic $GABA_A$ or metabotropic $GABA_B$ receptors, of which, the $GABA_A$ receptor is the most extensively studied.

1.1 The $GABA_A$ receptor

$GABA_A$ receptors mediate the majority of fast synaptic inhibition in the brain and therefore the actions of GABA at these receptors are critically important for controlling CNS excitability. $GABA_A$ receptors are pentameric assemblies (Nayeem *et al*., 1994; Tretter *et al*., 1997), consisting of five subunits arranged to form a chloride-selective ion channel (see Fig. 1.1). As members of the Cys-loop family of ligand-gated ion channels, $GABA_A$ receptors possess a similar structural architecture to nicotinic acetylcholine receptors (nACh), serotonin ($5\text{-}HT_3$) receptors and glycine receptors (Schofield *et al*., 1987; Nayeem *et al*., 1994; Rothlin *et al*., 1999; Korpi *et al*., 2002; Connolly and Wafford, 2004).

1.1.1 Receptor structure

A wide range of $GABA_A$ receptor subunits have been identified to date which are divided into classes based on their sequence homology. These include: α1-α6, β1-β3, γ1-γ3, δ, ε, π and θ, and also ρ1-3 (Barnard *et al*., 1998; Sieghart *et al*., 1999; Korpi *et al*., 2002). There is typically 70-80% identity within subunit families and 30-40% between classes (Burt and Kamatchi, 1991; Korpi *et al*., 2002). Additional diversity is also created through the alternative splicing of certain receptor subunits, with the most well characterised being the γ2 subunit, which has been shown to exist in two forms, γ2 short (γ2S) and γ2 long (γ2L). The γ2L subunit differs from the γ2S by the presence of an 8 amino acid insertion within the major TM3-4 intracellular domain (Whiting *et al*., 1990; Kofuji *et al*., 1991) and contains an additional consensus site for PKC (Moss *et al*., 1992; Machu *et al*., 1993), which generates the potential for additional functional modulation by protein kinases.

Although the large number of subunits present could potentially enable vast receptor heterogeneity, numerous studies of both native and recombinant receptors have indicated

that the majority of synaptic $GABA_A$ receptors are composed of α, β and γ subunits (Sieghart *et al*., 1999; Whiting, 1999; Olsen and Sieghart, 2008). *In vitro* studies have shown that co-expression of recombinant α, β and γ subunits is required to produce receptors with pharmacological properties akin to many native $GABA_A$ receptors (Pritchett *et al*., 1989; Sieghart *et al*., 1995) and furthermore, immunocytochemical and electron microscopic analysis of subunit expression *in vivo* has indicated a significant co-localisation of α, β and γ subunits in numerous regions of the brain (Benke *et al*., 1991; Fritschy *et al*., 1992; Somogyi *et al*., 1996). However, although most receptors are thought to be heteromers of α, β and γ subunits, due to the number of different isoforms within each subunit class, this still leaves the potential for numerous different receptor subtypes to be expressed *in vivo*. In fact, studies have shown that there is a broad spatial and temporal heterogeneity of subunit expression, with different neuronal populations expressing different complements of $GABA_A$ receptor subunits during different stages of development (Laurie *et al*., 1992a, 1992b; Wisden *et al*., 1992; Sperk *et al*., 1997; Pirker *et al*., 2000).

Each receptor subunit consists of four transmembrane domains (TM1-4), with TM2 thought to line the ion channel (Xu and Akabas, 1996; Goren *et al*., 2004). The extracellular N-terminal domain contains two cysteine residues purported to form the 'Cys-loop' important for GABA binding (Connolly and Wafford, 2004), along with two to four N-linked glycosylation sites. A large, poorly conserved intracellular domain is present between TM3-4 and has been shown to contain a number of consensus sites for phosphorylation (Moss and Smart, 1996; see Fig. 1.1).

Although the exact subunit combination of any $GABA_A$ receptor expressed *in vivo* is yet to be determined, a number of studies using recombinant receptors have indicated that a subunit stoichiometry of 2α:2β:1γ (Chang *et al*., 1996; Tretter *et al*., 1997; Farrar *et al*., 1999) is most likely. However, some studies have shown that co-expression of α and β subunits alone can form functional receptors, although these possess different pharmacological and biophysical properties to those incorporating the γ subunit. αβ receptors exhibit reduced single channel conductance (13-15pS) compared to αβγ receptors (27-29pS; Moss *et al*., 1991; Angelotti and MacDonald, 1993; Fisher and MacDonald, 1997), as well as displaying altered kinetic properties (Haas and MacDonald, 1999; Boileau *et al*., 2003). Furthermore, receptors consisting of αβ subunits are insensitive to benzodiazepines, a modulation which requires the presence of a γ subunit within the receptor complex (Pritchett *et al*., 1989; Sigel *et al*., 1990; Sigel and Buhr, 1997). In addition, the incorporation of the γ subunit also results in the enhancement of the maximal GABA-activated current recorded (Angelotti *et al*., 1993). As the

presence of the γ subunit has been shown to be important for the clustering of $GABA_A$ receptors at post-synaptic sites (Essrich *et al*., 1998), αβ receptors are likely to be excluded from the synapse and instead located extrasynaptically, where they contribute to the generation of a slower 'tonic' form of GABAergic inhibition (Mortensen and Smart, 2006; see Chapter 5).

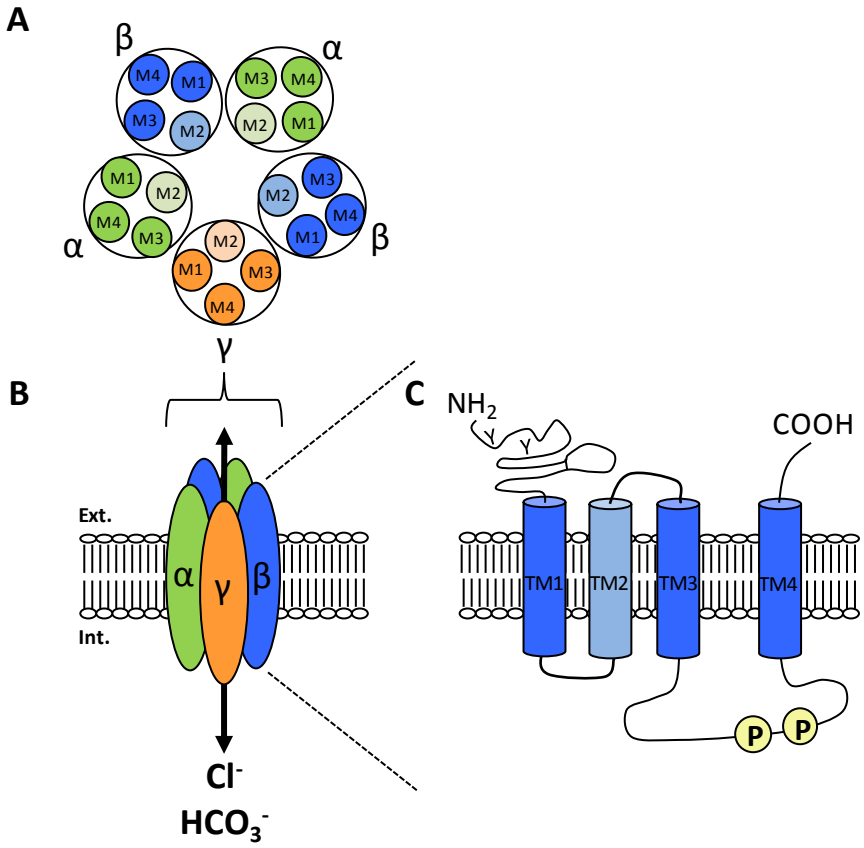

Figure 1.1 Proposed structure of the $GABA_A$ receptor
(A/B) Schematic showing the proposed 2α:2β:1γ stoichiometry of $GABA_A$ receptor heteropentamers (as viewed from the top; A) and their proposed membrane topology (B). The subunits are arranged pseudosymmetricaly to form an ion channel which, when activated by GABA, is permeable to Cl^- and HCO_3^- ions. (C) Schematic showing the proposed structure and membrane topology of a single $GABA_A$ receptor subunit. Each subunit consists of an extracellular N-terminal domain, which contains the Cys-loop and two to four putative N-linked glycosylation sites (Y), 4 transmembrane domains (TM1-4), with TM2 thought to form the channel lining (as shown in A) and a large cytoplasmic domain which houses a number of consensus sites for phosphorylation.

1.1.2 Receptor localization and trafficking

Following assembly, a process which has been shown to occur in the endoplasmic reticulum (ER; Connolly *et al*., 1996), $GABA_A$ receptors must be transported to the cell surface and clustered at synaptic sites where they will be exposed to rapid, transient releases of GABA from nearby pre-synaptic terminals. $GABA_A$ receptors exhibit a high degree of spatial diversity,

with different neuronal populations expressing different complements of receptor subunits (Laurie *et al*., 1992a; Wisden *et al*., 1992; Sperk *et al*., 1997; Pirker *et al*., 2000). In addition, different subunits have also been shown to be targeted to specific subsets of synapses within the same neuron. For example, in hippocampal pyramidal neurons, α1 and γ2 subunits exhibit a uniform distribution across all pyramidal neuron synapses, whereas α2 is preferentially localised to the axo-axonic synapses at the axon initial segment and α5 is largely found around the soma and proximal dendrites (Nusser *et al*., 1996; Fritschy *et al*., 1998). Therefore, the processes controlling membrane targeting must be highly regulated in order to ensure that these specific subtypes of $GABA_A$ receptor reach their correct locations. However, due to the vast heterogeneity of $GABA_A$ receptors *in vivo*, it has proved difficult to identify proteins that are involved in transporting receptors to the cell surface. One protein which has been implicated in this process is the $GABA_A$ receptor-associated protein, GABARAP. GABARAP has been shown to interact directly with γ2 subunits as well as with microtubules *in vitro* (Wang *et al*., 1999), which, along with further evidence indicating an interaction with the proposed $GABA_A$ receptor scaffold protein, gephyrin (Kneussel *et al*., 2000), led to the initial conclusion that GABARAP was involved in the clustering of $GABA_A$ receptors at post-synaptic sites. However, in neurons, GABARAP has been shown to be predominately located within intracellular structures, particularly the ER, Golgi apparatus and other vesicular bodies (Kneussel *et al*., 2000; Kittler *et al*., 2001). Furthermore, in the hippocampus, negligible levels of co-localisation between GABARAP and γ2 subunits were observed at the cell surface, whereas, significant co-localisation was seen intracellularly, around the peri-nuclear region (Kittler *et al*., 2001; Leil *et al*., 2004). Therefore, it has been proposed that GABARAP is more likely to be involved in the trafficking of $GABA_A$ receptors to the cell surface (see Fig. 1.2). This is supported by further studies which have shown that GABARAP can interact with N-ethylmaleimide-sensitive factor (NSF), a protein which plays a critical role in membrane fusion (Kittler *et al*., 2001).

More recent studies have identified two further proteins thought to be involved in the transport of $GABA_A$ receptors to the cell surface, PRIP (Phospholipase C-related, but catalytically inactive protein), which is thought to form a complex with the $GABA_A$ receptor and GABARAP to facilitate membrane trafficking (Mizokami *et al*., 2007) and KIF5 (Kinesin family motor protein 5), which is linked to $GABA_A$ receptors via mutual interactions with huntintin-associated protein 1 (HAP1) and acts to transport receptors to the cell membrane independently of GABARAP (Twelvetrees *et al*., 2010). In addition, the ubiquitin-like protein, Plic-1 is thought to be important for membrane insertion of receptors at the cell surface, with

studies showing the abolition of the interaction between $GABA_A$ receptors and Plic-1 results in a reduction in the number of receptors at the cell surface (Bedford *et al*., 2001).

The post-synaptic clustering of $GABA_A$ receptors is considered to be crucial for mediating efficient synaptic transmission. However, once again, the identification of proteins involved in the clustering of $GABA_A$ receptors has been problematic. A potential candidate is gephyrin, which, although initially identified as a scaffold protein involved in the clustering of glycine receptors (Pfeiffer *et al*., 1982; Moss and Smart, 2001), has also been shown to co-localise with $GABA_A$ receptors in synapses devoid of glycinergic input (Essrich *et al*., 1998; Sassoè-Pognetto *et al*., 2000; Lüscher and Keller, 2004). Furthermore, gephyrin has been shown to bind tubulin (Kirsch *et al*., 1991) and studies of knock-out mice have indicated that deletion of the γ2 subunit results in the disruption of receptor clustering as well as a concurrent loss of postsynaptic gephyrin clusters (Essrich *et al*., 1998; Schweizer *et al*., 2003), suggesting that the close association between γ2 subunits and gephyrin may be important for the clustering of $GABA_A$ receptors at post-synaptic sites (see Fig. 1.2). However, although the abolition of γ2 subunit expression causes defects in the clustering of $GABA_A$ receptors at all synapses, ablation of gephyrin expression results in a selective loss of clustering at synapses containing α2 or α3 subunits only (Essrich *et al*., 1998; Kneussel *et al*., 1999, 2001), implying the existence of gephyrin dependent and independent mechanisms for regulating the clustering of $GABA_A$ receptors. However, it is now possible that $GABA_A$ receptors and gephyrin may interact directly since α2 subunits have been shown to bind gephyrin (Tretter *et al*., 2008).

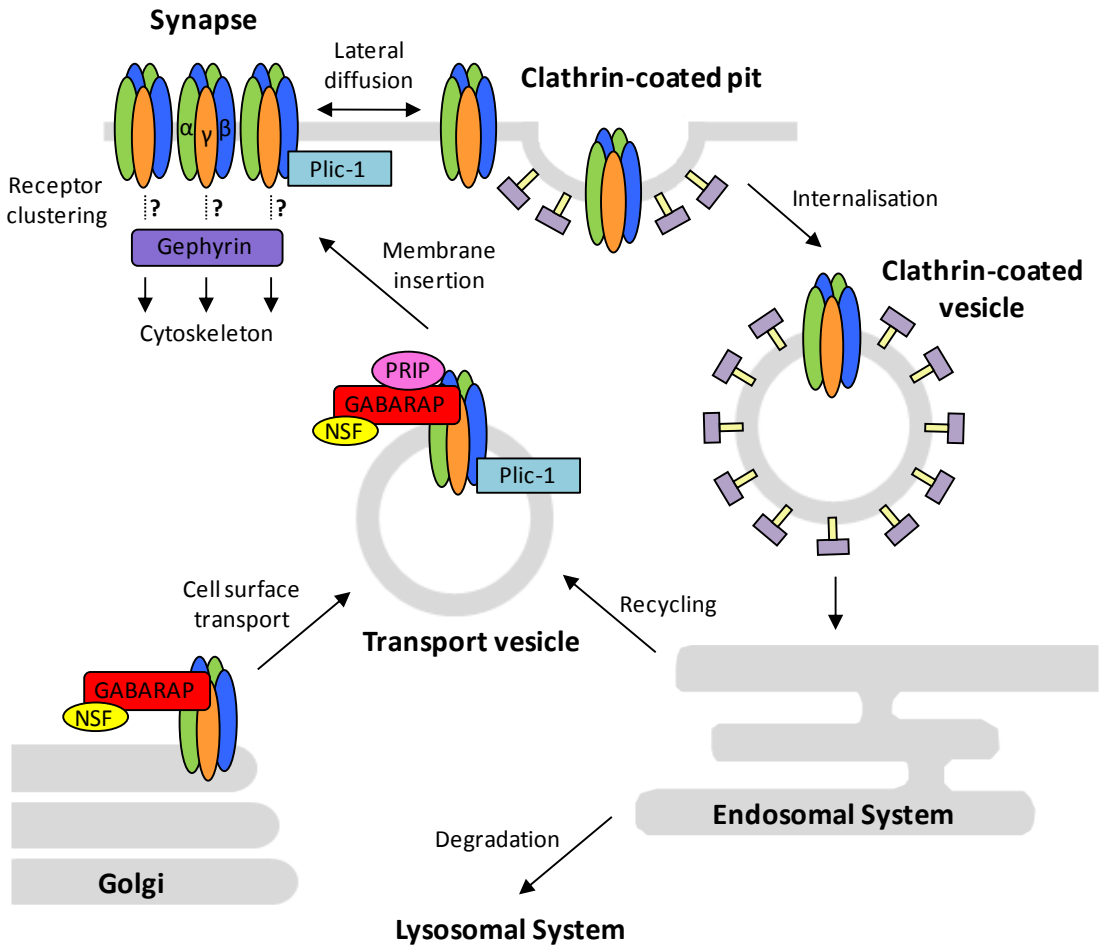

Figure 1.2 $GABA_A$ receptor trafficking - the current hypothesis
$GABA_A$ receptors are assembled in the endoplasmic reticulum and matured as they pass through the Golgi. From here they are packaged into vesicles and transported to the cell membrane. Proteins including GABARAP, NSF, PRIP, Plic-1 and the HAP1/KIF5 complex (not shown) are reported to be involved in this process. Receptors are then inserted into the membrane and clustered at synaptic sites. The γ2 subunit along with the scaffold protein, gephyrin are thought to play at least some role here. To alter synaptic efficacy, receptor number is dynamically regulated through both constitutive endocytosis and the lateral diffusion of receptors between synaptic and extrasynaptic sites. Once internalised, receptors are transported to the endosomal system where they will either be recycled back to the cell surface or targeted for degradation via the lysosomal pathway.

The number of $GABA_A$ receptors present at the synapse has been shown to be a critical determinant of synaptic efficacy (Nusser *et al*., 1997, 1998; Kittler *et al*., 2000; Smith *et al*., 2008; Arancibia-Cárcamo *et al*., 2009; Bannai *et al*., 2009) and therefore, the processes which regulate insertion and removal of receptors from the cell surface are of particular interest. A number of studies have indicated that $GABA_A$ receptors can undergo constitutive endocytosis, shuttling between the cell surface and intracellular compartments as a mechanism of rapidly altering inhibitory synaptic strength (Lüscher and Keller, 2004; see Fig. 1.2). Studies conducted in heterologous expression systems and neuronal cultures have indicated that endocytosis is

mediated through a clathrin-dependent mechanism (Tehrani and Barnes, 1993; Kittler *et al*., 2000), with a direct interaction demonstrated between $GABA_A$ receptor β and γ subunits and the clathrin adaptor protein, AP2 (Kittler *et al*., 2000; Smith *et al*., 2008). Further studies have indicated that PRIP may also be involved in the constitutive internalisation of $GABA_A$ receptors, with disruption of the binding of PRIP to β subunits resulting in the inhibition of receptor endocytosis (Kanematsu *et al*., 2007). Furthermore, co-immunoprecipitation studies have shown that $GABA_A$ receptors form a complex with PRIP and clathrin/AP2, indicating that PRIP is likely to play a role in clathrin-dependent receptor endocytosis (Kanematsu *et al*., 2007). Once internalised, receptors enter the endosomal system where they can either be recycled back to the cell surface via a direct interaction with HAP1 (Kittler *et al*., 2004) or targeted for degradation, a process thought to be mediated by ubiquitination at the γ2 subunit (Arancibia-Cárcamo *et al*., 2009). The balance between receptor internalisation and their delivery back to the cell surface is the key determinant of receptor number and processes which can interfere with this balance can have significant implications for inhibitory synaptic strength and overall neuronal network activity. For example, the actions of protein kinases have been reported to interfere with the recycling of receptors from endosomal compartments back to the cell surface, resulting in a reduction in the cell surface expression of $GABA_A$ receptors (Connolly *et al*., 1999; Brandon *et al*., 2002). In addition to receptor internalisation, rapid control of synaptic efficacy can also be achieved via the lateral diffusion of $GABA_A$ receptors between synaptic and peri- and extra-synaptic sites (Thomas *et al*., 2005).

1.1.3 Physiological and pathophysiological roles of $GABA_A$ receptors

Upon binding of GABA, the intrinsic $GABA_A$ receptor ion channel is opened, causing a rapid influx of chloride ions into the cell. The resulting hyperpolarisation leads to a concurrent enhancement of inhibitory tone, thus $GABA_A$ receptors are important for regulating neuronal excitability. GABAergic neurotransmission is also important for the generation and maintenance of rhythmic network activities in a number of brain regions including cortex, hippocampus, thalamus and olfactory bulb (Farrant and Nusser, 2005). For example, studies of the hippocampus have shown that the generation of both theta and gamma oscillatory activity is dependent upon the spatially and temporally precise firing of GABAergic interneurons (Jonas *et al*., 2004; Farrant and Nusser, 2005). These rhythmic activities occur during a number of behaviours including movement, exploratory behaviour, memory tasks and REM sleep and are thought to be important for working memory as well as the storage and retrieval of long term memories, providing a temporal coding system for information storage (Gonzalez-Burgos and Lewis, 2008).

In contrast to its inhibitory effects in the adult nervous system, GABA acts as an excitatory neurotransmitter during embryonic and early postnatal development (Ben-Ari *et al*., 1989; Cherubini *et al*., 1990; Luhmann and Prince, 1991; Ben-Ari, 2002). During this time, neurons express the NKCC1 transporter (Plotkin *et al*., 1997; Li *et al*., 2002), which leads to an accumulation of chloride within the neuron, thus, activation of $GABA_A$ receptors results in a rapid efflux of chloride ions causing depolarization of the cell (Yamada *et al*., 2004). Between postnatal days 3 and 12, NKCC1 is down-regulated and the expression of the potassium and chloride co-transporter, KCC2 is enhanced. KCC2 pumps chloride ions out of the cell, switching the actions of GABA from excitatory to inhibitory (Rivera *et al*., 1999). Therefore, $GABA_A$ receptors have an important function during development, as, in the absence of glutamatergic synapses at this time, the GABAergic system acts as the major mode of excitatory neurotransmission. Furthermore, during development, GABA also acts as a trophic factor, supporting neuronal migration, cell division and neurite outgrowth (Ben-Ari *et al*., 2002; Cancedda *et al*., 2007).

Dysfunction of GABAergic neurotransmission has been implicated in a number of neurological and psychiatric disorders, including epilepsy, anxiety, schizophrenia, Angleman syndrome and some neurodegenerative conditions such as Huntington's disease and Alzheimer's disease (Armstrong *et al*., 2003; Fritschy and Brünig, 2003; Lüscher and Keller, 2004). Perhaps the most well characterised pathological condition associated with abnormal $GABA_A$ receptor function is epilepsy. Mutations in the $GABA_A$ receptor α1, β3, γ2 and δ subunits have been linked to a wide range of genetic epilepsies, with numerous studies indicating that these mutations can alter both the function and expression of specific receptor subtypes, leading to reduced inhibitory neurotransmission and ultimately an enhancement in neuronal excitability in the affected brain regions (MacDonald *et al*., 2010). Other forms of epilepsy have also been associated with perturbed GABAergic transmission, for example, temporal lobe epilepsy has been linked with the altered expression of a number of receptor subunits, including decreased δ and enhanced α4 and γ2 (Brooks-Kayal *et al*., 1998; Zhang *et al*., 2007), with further studies showing that these changes are sufficient to alter both phasic and tonic inhibition in the dentate gyrus (Zhang *et al*., 2007). The $GABA_A$ receptor also forms the major target for a number of clinically useful drugs (see 1.1.4), including the benzodiazepines, which are commonly used for the treatment of epilepsy, anxiety and insomnia, as well as barbiturates and some general anaesthetics (Korpi *et al*., 2002).

Due to their essential role in controlling neuronal excitability, it is important to elucidate the cellular mechanisms by which $GABA_A$ receptors are regulated *in vivo*, as this will not only provide a further insight into the control of GABAergic transmission under physiological conditions, but may also help in identifying potential therapeutic targets for the treatment of conditions associated with $GABA_A$ receptor dysfunction.

1.1.4 $GABA_A$ receptor modulation

$GABA_A$ receptors are known to be modulated by a number of exogenous and endogenous compounds, the actions of which can result in altered receptor function and/or cell surface expression. Exogenous modulators include a number of clinically important drugs, such as benzodiazepines, barbiturates and some general anaesthetics. The most well characterized example is the benzodiazepines, which are routinely used in the clinic for the treatment of anxiety, insomnia and some forms of epilepsy (Sigel and Buhr, 1997; Bateson, 2004). Therefore, as one of the most widely prescribed drugs in the clinic, the benzodiazepines have been the subject of numerous investigations to determine their mechanism of action. These studies have shown that the benzodiazepines act by enhancing $GABA_A$ receptor function, an effect which is mediated by its binding to a discrete site located within the receptor complex (Sieghart, 1995). Studies using chimeras formed from the benzodiazepine sensitive α1 subunit and the relatively insensitive α6 showed that histidine 101, which is present in α1, but is replaced with an arginine in α6, is essential for benzodiazepine binding (Wieland *et al*., 1992). It was later confirmed that this histidine residue is conserved in the benzodiazepine sensitive α2, α3 and α5 subunits but not in the benzodiazepine insensitive α4, confirming its importance for benzodiazepine modulation (Wieland *et al*., 1992; Benson *et al*., 1998). Furthermore, the presence of a γ subunit within the receptor complex has also been shown to be necessary for benzodiazepine binding (Pritchett *et al*., 1989; Sigel *et al*., 1990), indicating that the benzodiazepine binding site is likely to be located at the interface between the α and γ subunits. Interestingly, studies using knock-in mice which carry the histidine to arginine mutation within specific α subunit isoforms showed that the different actions of benzodiazepines are mediated through different $GABA_A$ receptor subtypes, for example, α1 subunit-containing receptors are responsible for the sedative and anticonvulsant properties, whereas receptors containing α2 are involved in anxiolysis and muscle relaxation (Rudolph and Möhler, 2004). This discovery is particularly important for the development of new benzodiazepine-site drugs, as compounds can now be designed to have more specific therapeutic profiles, eliminating some of the undesired effects which are associated with classical non-specific benzodiazepine drugs, such as diazepam.

A number of studies have reported that ethanol may also act as a positive allosteric modulator at $GABA_A$ receptors, although the evidence for this remains contentious. Low, sobriety-impairing doses of ethanol (10-30mM) have been shown to potentiate $GABA_A$ receptor function in a number of neuronal populations including those from the cortex, cerebellum, dentate gyrus and thalamus (Reynolds *et al*., 1992; Wei *et al*., 2004; Hanchar *et al*., 2005; Glykys *et al*., 2007; Mody *et al*., 2007). This is supported by studies of recombinant receptors which showed that GABA-activated currents recorded from receptors composed of α4β2δ, α4β3δ or α6β3δ subunits were also enhanced by low doses of ethanol (Sundstrom-Poromaa *et al*., 2002; Wallner *et al*., 2003). However, other studies, which have investigated both native and recombinant $GABA_A$ receptors have reported no such effect, with results showing that low concentrations of ethanol had negligible effects upon $GABA_A$ receptor function, although some enhancement was observed with much higher doses (White *et al*., 1990; Borghese *et al*., 2006; Yamashita *et al*., 2006; Casagrande *et al*., 2007). The reasons for these discrepancies are unclear but one potential explanation is that ethanol may only modulate specific subpopulations of $GABA_A$ receptors, for example, those composed of specific subunit-combinations or those located in particular regions of the brain. Offering support for this notion are studies from recombinant systems which showed that, although GABA-activated currents recorded from α4β2δ, α4β3δ and α6β3δ $GABA_A$ receptors were enhanced by ethanol, those recorded from receptors incorporating other subunits, including those containing γ2, were not significantly altered (Sundstrom-Poromaa *et al*., 2002; Wallner *et al*., 2003). Furthermore, Wei *et al*. (2004) demonstrated that, although ethanol acted to enhance $GABA_A$ receptor function in dentate gyrus granule cells, no effect was observed in CA1 neurons of the hippocampus, indicating that ethanol modulation at $GABA_A$ receptors may be neuron specific. However, two independent studies, both utilizing recombinant α4β3δ $GABA_A$ receptors, have also yielded contradictory results (Borghese and Harris, 2007), although it has been suggested that these differences may be due to the problematic expression of these receptors or differences in the expression systems used (Mody *et al*., 2007; Wallner and Olsen, 2008). Ethanol modulation at $GABA_A$ receptors has also been reported to be PKC dependent (Qi *et al*., 2007; Choi *et al*., 2008) and therefore differing levels of PKC activity may also have contributed to these differences in ethanol sensitivity.

Endogenous modulators perhaps play a more important role, acting to regulate and 'fine tune' inhibitory transmission under physiological conditions, ensuring that neuronal excitability is precisely controlled. $GABA_A$ receptors are modulated by numerous endogenous molecules including protons (Wilkins *et al.,* 2002), Zn^{2+} (Smart *et al*., 1994, 2004), neurosteroids (Belelli

and Lambert, 2005) and protein kinases (Moss and Smart, 1996). Proton modulation is complex, with studies reporting that protons can potentiate, inhibit or have no effect upon $GABA_A$ receptor function (Robello *et al*., 1994; Pasternack *et al*., 1996; Krishek and Smart, 2001), depending on the subunit combination of the receptor (Krishek *et al*., 1996). Zn^{2+} acts as an inhibitor at $GABA_A$ receptors, with the extent of this inhibition once again dependent on the receptor's subunit composition (Smart *et al*., 1991; 1994). Neurosteroids and protein kinases are among the most potent modulators of the $GABA_A$ receptor and therefore these compounds play a particularly important regulatory role in the CNS.

1.2 Protein kinase modulation at the $GABA_A$ receptor

The first indication that phosphorylation, by protein kinases, may be an important mechanism for regulating $GABA_A$ receptor function was inferred by the receptor's significant structural homology to that of the nACh receptor, which had previously been shown to be functionally modulated by the activity of protein kinases (Huganir *et al*., 1986; Hopfield *et al*., 1988; Swope *et al*., 1992). This was supported by the presence of consensus sites for phosphorylation within the major intracellular domains of some $GABA_A$ receptor subunits (Ymer *et al*., 1989; Whiting *et al*., 1990) as well as by biochemical studies using purified brain preparations, which showed that $GABA_A$ receptors could be phosphorylated by PKC and PKA, although unequivocally discriminating the subunits which were phosphorylated by these protein kinases proved difficult due to the heterogeneous nature of these preparations (Kirkness *et al*., 1989; Browning *et al*., 1990). Therefore, since these early reports, numerous studies have been conducted in order to elucidate the actions of protein kinases in more detail, with the aim of determining: which protein kinases are able to phosphorylate the $GABA_A$ receptor, which subunits and/or residues act as their substrates in this process and finally, what are the functional consequences for $GABA_A$ receptors *in vivo*.

1.2.1 Identification of the sites for $GABA_A$ receptor phosphorylation

Studies investigating the phosphorylation of $GABA_A$ receptors *in vivo* have proven difficult due to the heterogeneous nature of the receptor and its relatively low abundance in the CNS. Therefore, in order to circumvent these problems, initial studies were carried out using purified GST fusion proteins produced from the large TM3-4 intracellular domains of specific $GABA_A$ receptor subunits, with these experiments indicating that a number of residues located within the β1-3 and γ2 subunits are substrates for specific protein kinases. These studies showed that the β1-3 subunits contain a conserved serine residue, S409 (S410 in β2), which is phosphorylated by protein kinase C (PKC), protein kinase A (PKA), protein kinase G (PKG) and

calcium/calmodulin-dependent protein kinase II (CaMKII; Moss *et al*., 1992a, McDonald and Moss, 1994, 1997). An additional site for CaMKII was also identified in both β1 and β3 subunits, at S384 and S383, respectively (McDonald and Moss, 1994, 1997). Furthermore, the β3 subunit was shown to possess a unique phosphorylation site, at S408, which was phosphorylated by PKC (McDonald and Moss, 1997). Both the γ2S and γ2L subunits were phosphorylated at S327 by PKC and both contain two additional sites for CaMKII, at S348 and T350 (Moss *et al*., 1992a; McDonald and Moss, 1994). The γ2L isoform, which contains an 8 amino acid insertion within its large intracellular domain (Whiting *et al*., 1990; Kofuji *et al*., 1991), was also shown to contain a further site which was a substrate for both PKC and CaMKII (Moss *et al*., 1992a; Machu *et al*., 1993; McDonald and Moss, 1994; summarised in Table 1.1).

Further studies using HEK293 cells expressing α1β1γ2L $GABA_A$ receptors showed that the β1 and γ2 subunits are also substrates for phosphorylation by tyrosine kinases. The γ2 subunit appears to be the major target, with results showing that it is highly phosphorylated at Y365 and Y367 when HEK293 cells are co-transfected with the tyrosine kinase vSRC. By contrast, the β1 subunit, which was phosphorylated at Y384 and Y386, was subject to much lower levels of phosphorylation (Moss *et al*., 1995). Phosphorylation at Y365 and Y367 has also been observed *in vivo* using phospho-specific anti-sera to assess the level of phosphorylation present in whole brain extracts and cultured cortical neurons (Brandon *et al*., 2001).

Subunit	Isoform(s)	Residue(s)	Protein Kinase(s)	References
β	β1, β2, β3	S409 (S410 in β2)	PKC, PKA, CaMKII, PKG	Moss *et al*., 1992a, 1995; McDonald and Moss, 1994, 1997
		Y384, Y386	Tyrosine Kinases	
	β1, β3	S384 (S383 in β3)	CaMKII	
	β3	S408	PKC, PKA	
γ2	γ2S, γ2L	S327	PKC	Moss *et al*., 1992a, 1995; Machu *et al*., 1993; McDonald and Moss, 1994; Brandon *et al*., 2001
		S348, T350	CaMKII	
		Y365, Y367	Tyrosine Kinases	
	γ2L	S343	PKC, CaMKII	

Table 1.1 Summary of the $GABA_A$ receptor phosphorylation sites identified to date
(Sites are conserved in mouse, rat and human DNAs)

Interestingly, although experiments carried out using HEK293 cells expressing recombinant $GABA_A$ receptors have, in the most part, confirmed the results obtained from the study of fusion proteins *in vitro*, some of the results obtained have been contradictory. For example, PKC has been shown to phosphorylate $GABA_A$ receptor β1-3 subunits at S409/S410 (Krishek *et*

al., 1994; McDonald *et al*., 1998; Brandon *et al*., 2000), a result which mimics that obtained from the study of fusion proteins. However, although studies by Moss *et al*. (1992a) showed that β1, β2 and β3 fusion protein constructs could be phosphorylated by PKA, experiments in HEK293 cells indicated that only the β1 and β3 subunits were substrates for PKA, with no phosphorylation observed at β2 (Moss *et al*., 1992b; McDonald *et al*., 1998). This suggests that, in a cell environment, there may be additional factors which can determine whether a protein kinase is able to phosphorylate a specific target.

Studies to investigate phosphorylation at the γ2 subunit in recombinant cell systems have proven to be technically challenging, with biochemical approaches unable to detect the expression of the γ2 subunit protein (Hadingham *et al*., 1992; Krishek *et al*., 1994). This is thought to be due to the γ2 subunit being more susceptible to proteolysis, as demonstrated by its appearance as a diffuse band at approximately 42kD, much smaller than that predicted from the amino acid sequence of the protein (Hadingham *et al*., 1992; Krishek *et al*., 1994). This lack of γ2 detection was not due to a failure in the transfection of this protein as γ2 subunit mRNA was readily detected via Northern blot (Hadingham *et al*., 1992).

Although studies in recombinant cell systems act as a good indicator of phosphorylation *in vivo*, it has been difficult to confirm these observations at native $GABA_A$ receptors. However, with the development of phospho-specific anti-sera it is becoming increasingly easy to determine whether specific subunits and/or residues are substrates for specific protein kinases in intact neurons. Using an antibody which specifically targets phosphorylated β3 at both S408 and S409, it has been possible to confirm that the β3 subunit is phosphorylated by both PKC and PKA in cortical and striatal neurons and furthermore, that this subunit is phosphorylated under basal conditions by PKC in both of these neuronal populations (Brandon *et al*., 2000; Brandon *et al*., 2003; Jovanovic *et al*., 2004).

1.2.2 Functional effects of $GABA_A$ receptor phosphorylation

1.2.2.1 PKC

A number of studies have indicated that phosphorylation by PKC acts to inhibit $GABA_A$ receptor function. Experiments using recombinant receptors showed that the activation of PKC results in a significant decrease in the GABA-activated currents recorded from receptors consisting of both αβ and αβγ subunits, with this effect being observed with multiple receptor subtypes including those incorporating α1, α3, α5, β1, β2 and γ2 subunits (Kellenberger *et al*., 1992;

Sigel *et al*., 1991; Leidenheimer *et al*., 1992; Krishek *et al*., 1994). At α1β2γ2S $GABA_A$ receptors, this inhibition was shown to be mediated through direct phosphorylation at both β2 S410 and γ2S S327, with phosphorylation at both sites thought to be required to produce the full modulatory effect (Kellenberger *et al*., 1992). Studies of α1β1γ2S and α1β1γ2L $GABA_A$ receptors also showed that activation of PKC resulted in a decrease of $GABA_A$ receptor function, with this effect being dependent upon phosphorylation at β1 S409 and γ2 S327, with S343 in the γ2L subunit also playing a role. Although phosphorylation at any of these sites was sufficient to induce a decrease in GABA-activated currents (Leidenheimer *et al*., 1992; Krishek *et al*., 1994), at receptors composed of α1β1γ2L subunits, PKC-mediated phosphorylation at γ2L S343 was shown to produce the largest inhibitory effect (Krishek *et al*., 1994).

PKC activation has also been shown to decrease $GABA_A$ receptor function in a number of neuronal preparations, including cultured superior cervical ganglion (SCG) neurons, cerebellar microsacs and retinal bipolar cells (Leidenheimer *et al*., 1992; Feigenspan and Bormann, 1994; Krishek *et al*., 1994). More recent experiments have shown that, in cultured cortical neurons, a direct correlation exists between the extent of phosphorylation at the $GABA_A$ receptor β3 subunit and receptor function, with results showing that enhancement of PKC-mediated phosphorylation causes a decrease in the peak amplitude of the mIPSCs recorded. Furthermore, it was also shown that the β3 subunit is phosphorylated by PKC under basal cell conditions, which, when inhibited by calphostin C, resulted in a significant increase in $GABA_A$ receptor function indicating that, not only is PKC acting to negatively modulate $GABA_A$ receptors, but also that there is the potential for a bi-directional modulation by PKC in these neurons (Brandon *et al*., 2000). The ability of PKC to phosphorylate the β3 subunit under basal cell conditions is thought to be due to its close association with the receptor, facilitated by the accessory protein, RACK-1 (Receptor for activated-C kinase 1), which binds to $GABA_A$ receptor β subunits and acts to target PKC to the receptor (Brandon *et al*., 1999).

Although the majority of studies have indicated that PKC acts to inhibit $GABA_A$ receptor function, some studies have obtained contradictory results. Lin *et al*. (1996) reported that co-expression of a high dose of PKM, a constitutively active form of PKC, resulted in an increase in the GABA-activated currents recorded from L929 cells expressing α1β1γ2L $GABA_A$ receptors. The reasons for this discrepancy are unclear, but differences in the expression systems and experimental protocols used have been implicated (Moss and Smart, 1996). For example, the availability of RACK-1 may be important. In HEK293 cells expressing recombinant α1β1 $GABA_A$ receptors, activation of PKC reduced the peak amplitude of the GABA-activated current,

however, this effect was abolished by the inclusion of a peptide sequence designed to perturb the interaction between RACK-1 and β1 (Brandon *et al*., 2002b). This indicates that RACK-1 is not only involved in the targeting of PKC to its substrate, but it also acts to facilitate the phosphorylation of $GABA_A$ receptor β subunits by PKC. Therefore, the differential effects of PKC activation in different expression systems may reflect differences in the anchoring proteins present. Activation of PKC has also been shown to enhance or have no effect on $GABA_A$ receptor function in neurons of the dentate gyrus and CA1 region of the hippocampus, respectively (Poisbeau *et al*., 1999). One potential factor which may contribute to the differential effects of PKC in different neuronal populations is the differences in the receptor subunits which are expressed. However, in recombinant studies, subunit combination did not appear to affect the actions of PKC (Sigel *et al*., 1991; Krishek *et al*., 1994) and therefore, it is more likely that these differences are due to other factors. The interaction between PKC and RACK-1 has been shown to be important for the effects of PKC in some neurons, for example, in studies of SCG neurons, perturbation of the interaction between RACK-1 and the β1 subunit resulted in a reduction in the effects of PKC (Brandon *et al*., 2002b). Therefore, the expression of different accessory proteins in different neuronal populations may account for the differences in the effects of PKC observed.

1.2.2.2 PKA

The functional effects of PKA have been shown to be dictated by the identity of the β subunit within the receptor complex. PKA-mediated phosphorylation at receptors containing the β1 subunit has been shown to result in a reduction in $GABA_A$ receptor function, whereas at β3 subunit-containing receptors, phosphorylation by PKA causes an enhancement of GABA-activated responses (Moss *et al*., 1992b; McDonald *et al*., 1998). This PKA-mediated increase in function at receptors incorporating the β3 subunit was shown to be dependent upon phosphorylation at S408, with studies showing that replacement of S408 with a neutral alanine residue resulted in a switch in the functional effects of PKA from enhancing to inhibitory (McDonald *et al*., 1998). Interestingly, at receptors containing the β2 subunit, PKA activation had no functional effects, which is consistent with its inability to phosphorylate this subunit when expressed in HEK293 cells (McDonald *et al*., 1998).

Given that PKA-mediated phosphorylation can cause differential modulation of $GABA_A$ receptors depending on their subunit composition, it is perhaps unsurprising that studies in neurons have yielded conflicting results. For example, activation of PKA has been shown to decrease $GABA_A$ receptor function in SCG neurons, cerebellar granule cells and hippocampal

pyramidal neurons (Moss *et al*., 1992b; Robello *et al*., 1993; Poisbeau *et al*., 1999), whereas an enhancement or no effect was observed in studies of Purkinje neurons and dentate gyrus granule cells, respectively (Kano and Konnerth, 1992; Poisbeau *et al*., 1999). Some of these effects may be due to differences in the β subunits expressed within the different neuronal populations however, studies of olfactory bulb neurons, which have shown to predominately express the β3 subunit (Nusser *et al*., 1999), indicate there may be other factors which act to determine the functional effects of PKA in neurons. The results from recombinant studies would predict that, due to the high level of β3 subunit expression in these neurons, PKA activation would cause an enhancement of $GABA_A$ receptor function. However, PKA has been shown to both positively (Nusser *et al*., 1999) and negatively (Brunig *et al*., 1999) modulate $GABA_A$ receptor function in neurons from the olfactory bulb, although the results reported by Brunig *et al*. (1999) may be confounded by the fact that PKA was activated via D1 receptors, which may modulate $GABA_A$ receptor function through other signalling pathways. Further studies of cerebellar stellate and basket cells, which are thought to predominately express β2 subunits, have shown that PKA acts to prolong the time course of the mIPSCs recorded from these neurons. This is in contrast to earlier recombinant studies which indicated that PKA could not phosphorylate the β2 subunit when expressed in HEK293 cells. The ability of PKA to phosphorylate $GABA_A$ receptor β subunits has been shown to be dependent upon its association with A-kinase anchoring protein 79/150 (AKAP79/150; Brandon *et al*., 2003), a neuronal accessory protein which binds to and targets PKA to its prospective substrate (Colledge and Scott). AKAP79/150 has been shown to interact with the β1 and β3 subunits, but not with β2 in some neuronal populations (Brandon *et al*., 2003). This result is consistent with the recombinant studies, but contradicts the results reported by Nusser *et al*. (1999), which showed that PKA was able to modulate $GABA_A$ receptor function in cerebellar stellate and basket cells. However, it is possible that the effects of PKA in these neurons are facilitated by another member of the AKAP family or alternatively, through the activation of other PKA-dependent signalling pathways.

1.2.2.3 CaMKII

CaMKII can enhance $GABA_A$ receptor function in a number of neuronal populations. Infusion of pre-activated CaMKII (termed α-CaMKII) has been shown to potentiate GABA-activated currents in spinal dorsal horn neurons as well as increase the amplitude of IPSCs recorded from CA1 hippocampal neurons (Wang *et al*., 1995). α-CaMKII also enhances mIPSC amplitudes recorded from cerebellar Purkinje cells (Kano *et al*., 1996), with this process thought to be crucial for the establishment of 'rebound potentiation', a form of inhibitory synaptic plasticity

in which GABAergic inhibition mediated by Purkinje neurons is potentiated by the activation of excitatory climbing fibre inputs (Kano *et al*., 1992). Increasing intracellular Ca^{2+} in mouse cortical neurons has also been shown to cause an enhancement of $GABA_A$ receptor function, an effect which was blocked by the CaMKII inhibitor, KN-93, indicating a role for CaMKII in modulating the $GABA_A$ receptors in these neurons (Aguayo *et al*., 1998). Further studies have focused on the effects of CaMKII at $GABA_A$ receptors containing specific β subunit isoforms. α-CaMKII has been shown to potentiate GABA-activated currents recorded from CGCs in both wild-type and $\beta2^{-/-}$ mice to a similar extent, indicating that these effects are not mediated by phosphorylation at the β2 subunit (Houston and Smart, 1996). CGCs are thought to predominately express β2 and β3 subunits (Wisden *et al*., 1996) and therefore, it was suggested that the CaMKII-dependent enhancement of $GABA_A$ receptor function was mediated by phosphorylation at the β3 subunit-containing receptors. This is consistent with results from recombinant receptors, which showed that GABA-activated currents recorded from cells expressing α1β3 or α1β3γ2S $GABA_A$ receptors, but not those expressing α1β2 or α1β2γ2S, were potentiated by exposure to α-CaMKII (Houston and Smart, 2006). Interestingly, inclusion of the γ2S subunit significantly increased the CaMKII-induced potentiation in both NG108 cells and CGCs, indicating that phosphorylation at the γ2S subunit contributes to this modulation. Further studies showed that, at α1β3 $GABA_A$ receptors, the enhancement of receptor function was due to phosphorylation at S383 alone. At α1β3γ2S receptors, mutation of S383 only partially abolished the α-CaMKII-induced potentiation, supporting notion that phosphorylation of the γ2 subunit is also important for the effects of CaMKII at $GABA_A$ receptors (Houston *et al*., 2007). However, site-specific mutation of γ2 S348 and T350, the residues which were identified as substrates for CaMKII phosphorylation is studies using fusion proteins (McDonald and Moss, 1994), did not affect the potentiation induced. Moreover, α-CaMKII still induced some potentiation at $\alpha\beta^{S408A,S409A,S383A}\gamma2S^{S348A,T350A}$ $GABA_A$ receptors, which contained mutations at all known CaMKII phosphorylation sites, indicating that CaMKII must be acting via other sites on the receptor (Houston *et al*., 2007). Curiously, mutation of Y365 and Y367 to neutral alanine residues abolished this residual potentiation, suggesting that some of the actions of CaMKII were mediated via the activation of tyrosine kinases. This was confirmed by experiments using phospho-specific antibodies, which showed that infusion of α-CaMKII caused an up-regulation of phosphorylation at Y365 and Y367 (Houston *et al*., 2007).

Although studies of recombinant $GABA_A$ receptors indicated that receptors incorporating the β3, but not the β2 subunit were potentiated by CaMKII-dependent phosphorylation (Houston and Smart, 2006, 2007), further studies investigating the effects of CaMKII on synaptic

inhibition showed that the function of both β2 and β3 subunit-containing receptors could be modulated by CaMKII, although these effects occurred via different mechanisms. Studies comparing synaptic inhibition in CGCs from wild-type and $\beta2^{-/-}$ mice identified two separate populations of sIPSCs which possessed different kinetic properties and were differentially modulated by exposure to α-CaMKII (Houston *et al*., 2008). The β2-mediated sIPSCs were large in amplitude and fast decaying, with CaMKII acting to potentiate their amplitude with no effect on decay. By contrast, the sIPSCs mediated by β3 subunit-containing receptors, which had smaller amplitudes and more variable decay, displayed increased decay times in response to CaMKII with no effect on amplitude. Therefore, CaMKII can modulate both β2 and β3 subunit-containing receptors in CGCs, but via different mechanisms. These subunits are likely to be located at distinct synapses within CGCs (Houston *et al*., 2008) allowing differential modulation by CaMKII to occur at these different synapses.

1.2.2.4 Tyrosine kinases

Studies of recombinant $GABA_A$ receptors have indicated that phosphorylation by tyrosine kinases causes a significant enhancement of receptor function. In HEK293 cells expressing α1β1γ2L $GABA_A$ receptors, intracellular dialysis of the tyrosine kinase cSRC, along with the tyrosine phosphatase inhibitor, sodium vanadate, resulted in a significant enhancement of the GABA-activated current. In addition, infusion of tyrosine kinase inhibitors has been shown to decrease GABA-activated currents recorded from HEK293 cells expressing α1β2 or α1β2γ2 $GABA_A$ receptors (Wan *et al*., 1997a) and *Xenopus* oocytes expressing α1β1 or α1β1γ2L receptors (Valenzuela *et al*., 1995). In both cases, the decrease in GABA responses was more pronounced at receptors incorporating γ2 subunits, suggesting that tyrosine kinase-mediated phosphorylation at either the β or γ2 subunit is able to modulate $GABA_A$ receptor function to some extent. This is consistent with studies by Moss *et al*. (1995) which showed that both the β and γ2 subunits are phosphorylated by tyrosine kinases and is also supported by a more recent study which showed that the tyrosine kinase, SRC can directly interact with both β and γ subunits in HEK293 cells (Brandon *et al*., 2001). However, contradictory results were obtained by Moss *et al*. (1995), who reported that, although the β subunit was a substrate for tyrosine kinases, phosphorylation at this subunit did not result in any functional effect. Therefore, this implies that tyrosine phosphorylation at the $GABA_A$ receptor β subunit may act to enhance the functional effect conveyed by the γ2 subunit, rather than producing a direct modulatory effect in itself. This discrepancy could also reflect differences in the subunit composition of the receptors expressed (β1 versus β2 containing receptors) or the expression system used (HEK293 cells versus *Xenopus* oocytes).

Tyrosine kinases have also been shown to enhance $GABA_A$ receptor function in neurons. Inclusion of cSRC in the patch electrode resulted in a significant enhancement of GABA-activated currents recorded from both SCG and spinal dorsal horn neurons (Moss *et al*., 1995; Wan *et al*., 1997a). Furthermore, intracellular dialysis of the tyrosine kinase inhibitor, genistein caused a decrease in the peak response elicited by GABA in both sets of neurons (Moss *et al*., 1995; Wan *et al*., 1997a; Dunne *et al*., 1998). Taken together, this indicates that tyrosine kinases act to positively modulate the function of $GABA_A$ receptors.

1.2.2.5 PKG

The functional effects of PKG-mediated phosphorylation at the $GABA_A$ receptor are not well defined, with very few studies having been conducted to date. In neurons, activation of intracellular PKG via the infusion of cGMP or its analogue 8BrGMP has been shown to both inhibit and enhance $GABA_A$ receptor-mediated currents, depending on the neuronal preparation studied. Activation of PKG resulted in a decrease of the GABA-activated currents recorded from neurons from the nucleus of the tractus solitarus (Glaum and Miller, 1993) and cultured retinal amacrine cells (Wexler *et al*., 1998), whereas an enhancement was reported in studies of bullfrog dorsal root ganglia (Bradshaw and Simmons, 1995). The latter result was mimicked in recombinant studies from *Xenopus* oocytes expressing α1β2γ2L $GABA_A$ receptors (Leidenheimer, 1996), although, curiously, the increase in $GABA_A$ receptor function observed in this study was not due to phosphorylation at β2 S410. This is inconsistent with the results obtained from *in vitro* fusion proteins which showed that β2 S410 was the only residue in the receptor which could be phosphorylated by PKG (McDonald and Moss, 1997). This suggests that PKG may mediate this effect through phosphorylation at an unidentified site or, alternatively, it may alter other intermediary proteins or signaling pathways which subsequently act to modulate the function of $GABA_A$ receptors.

1.2.3 Mechanisms mediating functional modulation by protein kinases

Modulation of GABA-activated currents by protein kinases has been proposed to be mediated via the alteration of intrinsic ion channel function. For example, single channel recordings in SCG neurons showed that tyrosine kinases act to enhance both the mean open time and the probability of the channel opening, resulting in an increase in receptor function (Moss *et al*., 1995). However, studies investigating the actions of PKA have shown that, in mouse spinal neurons, PKA acts to depress receptor function by decreasing the frequency of channel opening, with no effect on mean open time or probability of opening (Porter *et al*., 1990). Therefore, this indicates that different protein kinases may act through different mechanisms

to alter $GABA_A$ receptor function. The mechanism of action may also be dependent upon the subunit-combination of the receptor. In neurons, protein kinases have been shown to modulate the amplitude and/or decay times of synaptic events (e.g. Wang *et al*., 1995; Kano *et al*., 1996; Poisbeau *et al*., 1999), which is consistent with an alteration of $GABA_A$ receptor channel function.

Protein kinases have also been shown to alter desensitization of $GABA_A$ receptors, which may also contribute to their ability to modulate receptor function. PKA activation has been shown to enhance the rate and extent of fast desensitization at α1β1γ2L and α1β3γ2L $GABA_A$ receptors expressed in HEK293 cells (Hinkle and Macdonald, 2003). Furthermore, infusion of pre-activated α-CaMKII into rat spinal dorsal horn neurons was also shown to decrease desensitization (Wang *et al*., 1995). Finally, in rat hippocampal neurons, promotion of phosphorylation via the inhibition of protein phosphatase 2B (calcineurin) enhances the rate and extent of macroscopic desensitization (Jones and Westbrook, 1997).

1.2.4 Regulation of $GABA_A$ receptor trafficking by protein kinases

In addition to altering ion channel function, protein kinases are also thought to modulate the trafficking of $GABA_A$ receptors. $GABA_A$ receptors are dynamically regulated and have been shown to shuttle between the cell surface and endosomal compartments. Therefore, any alterations to this process can result in changes to the number of receptors expressed on the cell surface, a parameter which is a critical determinant of synaptic efficacy (Nusser *et al*., 1997, 1998; Kittler *et al*., 2000; Smith *et al*., 2008; Arancibia-Cárcamo *et al*., 2009; Bannai *et al*., 2009).

Studies have reported that, in both HEK293 cells and *Xenopus* oocytes expressing α1β2γ2L $GABA_A$ receptors, activation of PKC results in a significant depression of the GABA-activated current, which is associated with a decrease in the number of receptors expressed on the cell surface (Chapell *et al*., 1998; Connolly *et al*., 1999; Filipova *et al*., 2000). This decrease in receptor surface expression was thought to be due to PKC preventing the recycling of receptor back to the surface (Connolly *et al*., 1999). Interestingly, these studies indicated that the PKC-mediated internalisation of $GABA_A$ receptors was independent of direct receptor phosphorylation (Chapell *et al*., 1998; Connolly *et al*, 1999).

$GABA_A$ receptors have been shown to directly interact with parts of the endocytic machinery, including the clathrin adaptor protein, AP2, a protein which plays a critical role in recruiting

cargo to clathrin-coated pits ready for internalisation into endocytic vesicles (Schmid, 1997). Initial biochemical experiments showed that $GABA_A$ receptors are co-immunoprecipitated with AP2 (Kittler *et al*., 2000), with further studies showing that the μ2 subunit of AP2 could bind directly to both β and γ $GABA_A$ receptor subunits (Kittler *et al*., 2000; Kittler *et al*., 2005; Smith *et al*., 2008). In the β subunit, the AP2 binding motif overlaps with the major site(s) for phosphorylation, indicating that the actions of protein kinases may be important for controlling the interaction between the $GABA_A$ receptor β subunit and the AP2 adaptor (Kittler *et al*., 2005). This was confirmed by *in vitro* binding assays, which showed that phosphorylation at the β1 or β3 subunits by PKC or PKA resulted in a significant reduction in its ability to bind AP2 (Kittler *et al*., 2005). Moreover, in cortical neurons, interfering with the interaction between β3 and AP2 resulted in an increase in the peak amplitude of the mIPSCs recorded from these neurons, suggesting an enhancement in the number of receptors expressed on the cell surface (Kittler *et al*., 2005).

In the γ2 subunit, the AP2 binding motif encompasses the major tyrosine phosphorylation sites located at Y365 and Y367 (Kittler *et al*., 2008; Smith *et al*., 2008), with phosphorylation at these sites also acting to decrease its ability to bind AP2 (Kittler *et al*., 2008). Furthermore, perturbation of the γ2-AP2 interaction in striatal neurons resulted in an increase in the number of receptors expressed on the cell surface and a concurrent enhancement in the amplitude of the mIPSCs recorded (Kittler *et al*., 2008).

Some extracellular signaling molecules, including insulin and BDNF have also been shown to modulate the number of $GABA_A$ receptors expressed on the cell surface, effects which are thought to be mediated via the activation of specific protein kinases. In hippocampal neurons, insulin signaling has been shown to result in a rapid increase in the number of $GABA_A$ receptors expressed on the cell surface (Wan *et al*., 1997b). This effect is thought to be mediated via the protein kinase, Akt (also called PKB), which has been shown phosphorylate β2 S410 both *in vitro* and *in vivo* (Wang *et al*., 2003). Activation of Akt mimics the effects of insulin, increasing cell surface expression of $GABA_A$ receptors in both HEK293 cells and rat hippocampal neurons (Wang *et al*., 2003). Furthermore, site-specific mutation of β2 S410 abolished the insulin-dependent increase in $GABA_A$ receptor surface expression, indicating that this effect is mediated through direct phosphorylation at the $GABA_A$ receptor (Wang *et al*., 2003).

BDNF signaling via the TrkB receptor has been shown to transiently increase mIPSC amplitude, an effect which is associated with an enhancement of $GABA_A$ receptor cell surface stability

(Jovanovic *et al*., 2004). The increase is evident within 5 min of BDNF stimulation and is thought to be due to the rapid recruitment of PKC and RACK-1 to the receptor which acts to phosphorylate the β3 subunit, enhancing its stability in the cell membrane. The mIPSC amplitude returns to baseline within approximately 30 min, an effect which is associated with dephosphorylation of the receptor by protein phosphatase 2A (PP2A; Jovanovic *et al*., 2004). Therefore, this highlights how phosphorylation dependent modulation of $GABA_A$ receptor trafficking can bi-modally alter synaptic inhibition, providing an ideal mechanism for rapidly controlling neuronal excitability *in vivo*.

1.2.5 Actions of phosphatases at $GABA_A$ receptors

Phosphorylation at the $GABA_A$ receptor is not solely controlled by protein kinases, but also by protein phosphatases, with the balance of activities between the two determining the overall functional effect. In both HEK293 cells and cortical neurons, inhibition of tyrosine phosphatases results in a significant increase in the levels of phospho-tyrosine present, indicating that high intrinsic phosphatase activity keeps basal tyrosine phosphorylation low in these cells (Moss *et al*., 1995; Brandon *et al*., 2001). The activities of phosphatases are also important for controlling phosphorylation by serine/threonine protein kinases, for example, phosphatases PP1 and PP2A have been shown to dephosphorylate the β1 and β3 subunits and are important in determining $GABA_A$ receptor function both *in vitro* and *in vivo* (McDonald *et al*., 1998; Jovanovic *et al*., 2004; Terunuma *et al*., 2004).

Similar to protein kinases, protein phosphatases have also been shown to associate with the $GABA_A$ receptor through their interactions with specific anchoring proteins. For example, in addition to binding and targeting PKA to the $GABA_A$ receptor, AKAP79/150 also binds PP2B (also termed calcineurin), with ternary complexes containing PKA, AKAP79/150 and PP2B being isolated from bovine brain (Coghlan *et al*., 1995). Furthermore, immunocytochemistry experiments have demonstrated a co-localisation between PKA and PP2B in hippocampal neurons, suggesting that they may function co-operatively to control receptor phosphorylation. Interestingly, the binding of AKAP79/150 to PP2B was shown to inhibit its phosphatase activity, implying that AKAP79/150 may be crucial for controlling the balance between protein kinase and phosphatase activities (Coghlan *et al*., 1995).

The phosphatase PP1α can associate directly with the $GABA_A$ receptor, being co-immunoprecipitated along with β3 and PRIP-1 from mouse brain (Terunuma *et al*., 2004). PRIP-1 is important for this interaction, as demonstrated by the reduced levels of PP1α associated

with β3 in PRIP-1$^{-/-}$ mice (Terunuma *et al*., 2004), indicating that PRIP-1 is likely to act as an anchoring protein, facilitating the interaction between PP1α and the $GABA_A$ receptor.

1.2.6 Physiological importance of $GABA_A$ receptor phosphorylation

The importance of protein kinase modulation at $GABA_A$ receptors has been highlighted by the study of knock-out mice, in which specific protein kinases have been ablated. PKCε$^{-/-}$ mice have been shown to exhibit enhanced $GABA_A$ receptor responses (Hodge *et al*., 1999), which is consistent with earlier studies showing that PKC-mediated phosphorylation acts to decrease $GABA_A$ receptor function (Kellenberger *et al*., 1991; Sigel *et al*., 1991; Leidenheimer *et al*., 1992; Krishek *et al*., 1994; Brandon *et al*., 2000). This was coupled with decreased anxiety behaviour, as determined by open field testing and the elevated plus maze, an effect which was restored to normal by treatment with the $GABA_A$ receptor antagonist, bicuculline (Hodge *et al*., 2002). Similar results have been reported for PKCγ$^{-/-}$ mice, which also displayed decreased anxiety behaviours (Bowers *et al*., 2000). Therefore, taken together, these results suggest that the actions of PKC at $GABA_A$ receptor may be important for regulating anxiety *in vivo*.

Studies of knock-in mice in which tyrosine phosphorylation at the $GABA_A$ receptor γ2 subunit is ablated (via Y365/7F mutations) have also provided an insight into the importance of tyrosine phosphorylation at $GABA_A$ receptors *in vivo*. Homozygous knock-in of this mutation was embryonic lethal, perhaps suggesting a crucial role for $GABA_A$ receptor tyrosine phosphorylation during development (Tretter *et al*., 2009). Heterozygous mice exhibited enhanced mIPSCs in hippocampal CA3 pyramidal neurons, which was associated with an increase in cell surface expression of $GABA_A$ receptors (Tretter *et al*., 2009). This was correlated with deficits in spatial object recognition, a behavioural paradigm which has been shown to be dependent upon the CA3 region of the hippocampus (Stupien *et al*., 2003; Hunsaker *et al*., 2007), indicating that tyrosine phosphorylation at the $GABA_A$ receptor is important for spatial cognition.

Dysfunction of protein kinases has also been shown to be a key feature in epilepsy. During status epilepticus, the number of $GABA_A$ receptors expressed on the cell surface is reduced, resulting in a decrease in inhibitory synaptic transmission. This is thought to be mediated by reduced PKC-mediated phosphorylation at the $GABA_A$ receptor β3 subunit due to its decreased association with PKC as well as its enhanced interaction with the phosphatase, PP2A (Terunuma *et al*., 2008). This dephosphorylation of the receptor causes an increase in binding

of the β3 subunit to the AP2 clathrin adaptor, resulting in increased receptor internalisation. Therefore, PKC-mediated phosphorylation at the $GABA_A$ receptor is vital for controlling neuronal excitability *in vivo*.

1.3 Neurosteroid modulation at the $GABA_A$ receptor

Endogenous neurosteroids are among the most potent allosteric modulators of the $GABA_A$ receptor. Their importance as highly effective neuromodulators first came to light when progesterone and some of its metabolites were reported to induce sedation and anaesthesia in rats (Selye, 1941). Later studies provided a feasible molecular mechanism when it was shown that the synthetic anaesthetic steroid, alphaxalone, potently enhanced the function of the $GABA_A$ receptor (Harrison and Simmonds, 1984). It soon became evident that a number of steroids are synthesised *de novo* in the brain, termed neurosteroids, which share this ability to enhance neuronal inhibition via a selective interaction with the $GABA_A$ receptor. These include the progesterone metabolite, 5α-pregnan-3α-ol-20-one (allopregnanolone) and the deoxycorticosterone metabolite, 5α-pregnan-3α-diol-20-one (THDOC), which are considered to be two of the most prevalent neurosteroids in the mammalian CNS (reviewed in Lambert *et al*., 2009). Investigation into the effects of neurosteroids at the $GABA_A$ receptor have shown that, unlike the classical genomic mechanism of action for steroid hormones, neurosteroids mediate their effects over a much faster time course, which is compatible with a direct interaction with the receptor itself, through binding to a novel recognition site. In addition, as with other endogenous regulators of the $GABA_A$ receptor, some neurosteroids act as positive modulators, to enhance $GABA_A$ receptor responses (e.g. allopregnanolone and THDOC), whereas others act in a negative manner to reduce receptor function (e.g. pregnenolone sulphate; Belelli and Lambert, 2005). The actions of the positively modulating neurosteroids are the most well characterised, with these forming the basis of this thesis work.

1.3.1 Neurosteroid synthesis in the CNS

Neurosteroids are synthesised from cholesterol via a series of enzymatic reactions, with the first of these, mediated by the P450 side-chain-cleavage enzyme (P450scc), taking place in mitochondria (Baulieu *et al*., 1998; Compagnone and Mellon, 2000; Chisari *et al*., 2010). Cholesterol is thought to be delivered to the outer mitochondrial membrane by the chaperone, StAR (steroidogenic acute regulatory protein; Sierra *et al*., 2004; Chisari *et al*., 2009), where it is subsequently transferred to the inner membrane by the 18kDa translocator protein (TSPO, also known as the peripheral benzodiazepine receptor; Papadopoulos *et al*., 1997; Rone *et al*., 2009). Once the cholesterol molecule is inside the mitochondrion, P450scc

mediates side-chain cleavage, converting the cholesterol to pregnenolone (Baulieu *et al*., 1998; Compagnone and Mellon, 2000; Fig. 1.3). Due to its hydrophobic nature, cholesterol cannot readily diffuse across the mitochondrial intermembrane space and therefore this requirement for a transporter means that the access of cholesterol to P450scc at the inner membrane is the rate limiting step in neurosteroid biosynthesis (Miller, 1995).

Upon exiting the mitochondrion, pregnenolone can be converted to progesterone by 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD). Subsequent catalysis by the 21β-hydroxylase enzyme, CYP21 converts progesterone to deoxycorticosterone. Reduction by 5α-reductase reduces progesterone and deoxycorticosterone to the pregnane steroids, 5α-dihydroprogesterone and 5α-dihydrodeoxycorticosterone, which undergo further reduction by 3α-hydroxysteroid dehydrogenase (3α-HSD), to form the neurosteroids, 3α5α-tetrahydroprogesterone (allopregnanolone) and 3α5α-tetrahydrodeoxycorticosterone (THDOC), respectively (Baulieu *et al*., 1998; Compagnone and Mellon, 2000; see Fig. 1.3). Peripheral steroids, which are able to cross into the brain via the blood-brain barrier can feed into this synthetic pathway, augmenting the levels of neurosteroids produced, as demonstrated by a partial loss of pregnenolone and some of its metabolites in the brains of rats after the removal of peripheral sources of steroids by gonadectomy and adrenalectomy (Corpéchot *et al*., 1981, 1983, 1993).

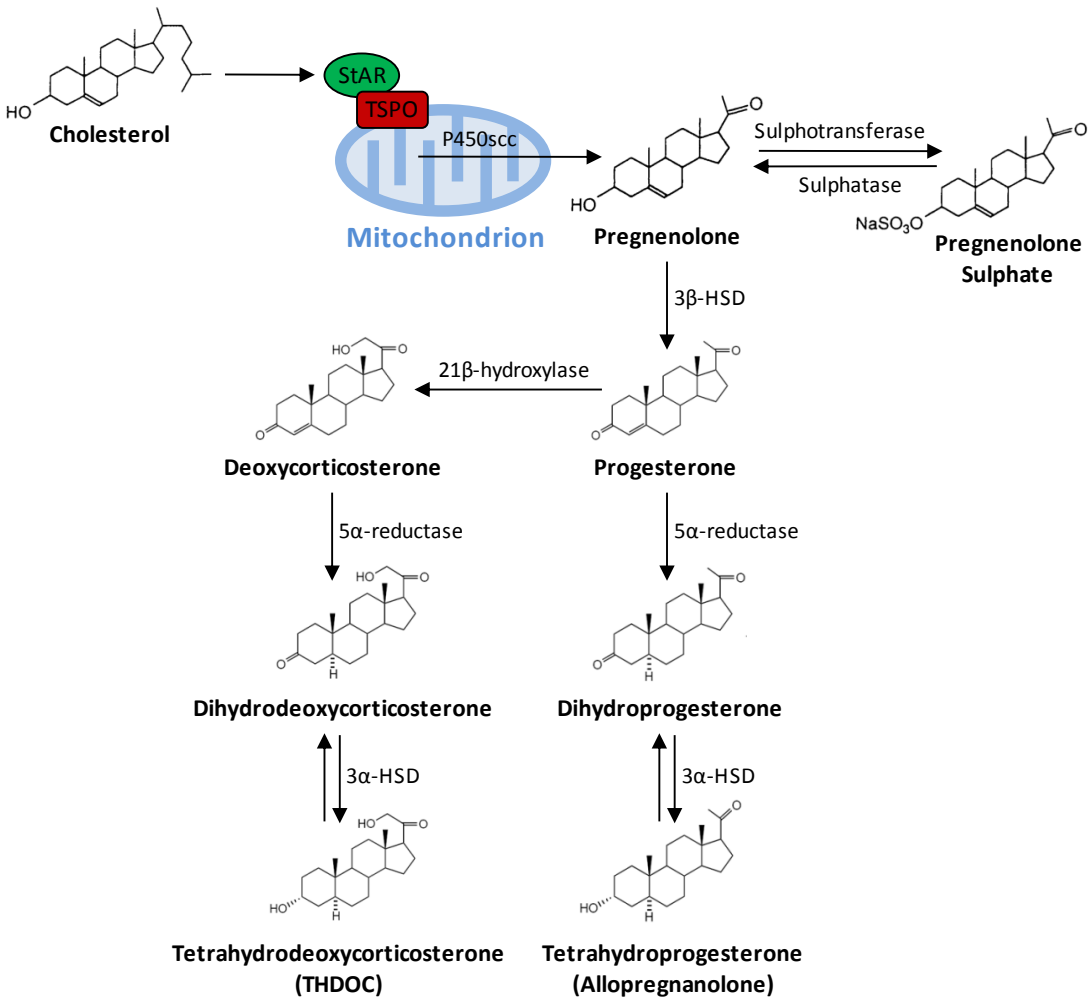

Figure 1.3 Biosynthesis of neurosteroids
Schematic showing the biosynthetic pathway of some of the most prevalent neurosteroids in the CNS including, the positively modulating neurosteroids, allopregnanolone and THDOC and the inhibitory neurosteroid, pregnenolone sulphate. Neurosteroids are synthesised from cholesterol via a series of enzymatic reactions, the first of which occurs in mitochondria, where cholesterol is converted to pregnenolone. Upon exiting the mitochondrion, pregnenolone is subject to further catalysis to form a number of neurosteroids.

The first evidence that steroids could be synthesised *de novo* in the CNS was obtained from studies by Baulieu and colleagues, who reported that, in rats, levels of some steroids, such as pregnenolone and dehydroepiandrosterone (DHEA), as well as neurosteroids, such as allopregnanolone, were present at higher concentrations in tissue from the nervous system than in the plasma and furthermore, that these steroids remained in the nervous system long after the removal of peripheral steroid sources (Corpéchot *et al*., 1981, 1983, 1993), an observation that was mimicked in other mammalian species including rabbits, dogs and monkeys (Robel and Baulieu, 1985; Robel *et al*., 1987; Jo *et al*., 1989; Mathur *et al*., 1993). Together, these results indicated that steroids were either synthesised within the brain or

were accumulated there. Accumulation of steroids was ruled out by clearance assays, which showed that radiolabelled pregnenolone was cleared from the plasma and brain over similar timecourses (Corpéchot *et al*., 1981). In addition, further studies showed that a number of important steroidogenic enzymes were expressed in the brain (reviewed in Compagnone and Mellon, 2000), strongly suggesting that steroids can be synthesised here.

Immunohistochemical experiments showed that the P450scc enzyme, which is required to convert cholesterol to pregnenolone, is present in the brain and is particularly enriched in the white matter (Le Goascogne *et al*., 1987; Iwahashi *et al*., 1990). Furthermore, cultures of cells dissociated from rat forebrain, the majority of which were oligodendrocytes and glial cells, were also highly immunopositive for P450scc (Jung-Testas *et al*., 1989) and moreover, cholesterol was shown to be converted to pregnenolone in these cells, indicating that the enzyme is active. This suggests that the conversion of cholesterol to pregnenolone may take place in the glial cells of the nervous system. RT-PCR and Western blotting techniques have also identified P450scc mRNA and protein in the brain, particularly in glial cells (Iwahashi *et al*., 1990; Mellon and Deschapper, 1993; Kohchi *et al*., 1998), supporting the notion that pregnenolone may be synthesised in the glial cells of the CNS. However, more recent studies have begun to focus on expression of P450scc in specific neuronal populations, with Ukena *et al*. (1998) demonstrating that both the mRNA and protein are present in the Purkinje neurons of the cerebellum in rats. P450scc has also been found in other neurons including those from dorsal root ganglia, spinal cord dorsal horn and somatosensory cortex (Patte-Mensah *et al*., 2003). Therefore, taken together, these results indicate that steroids can be synthesised *de novo* in both the neurons and glial cells of the mammalian nervous system.

The neurosteroids, allopregnanolone and THDOC, are synthesised from progesterone and deoxycorticosterone, respectively, by the sequential actions of two enzymes, 5α-reductase and 3α-HSD. Studies have shown that these two enzymes are also expressed in the mammalian CNS, indicating that neurosteroids can be synthesised here. RT-PCR and Western blot experiments have determined that both the mRNA and protein corresponding to 5α-reductase and 3α-HSD enzymes are present in the brains of rats, mice and humans (Thigpen *et al*., 1993; Dong *et al*., 2001; Steckelbroeck *et al*., 2001; Stoffel-Wagner *et al*., 1998). The activities of these enzymes have also been investigated in rat brains using cultures of neurons, oligodendrocytes and astrocytes. Cells were treated with radiolabelled progesterone or dihydroprogesterone in order to determine the activities of 5α-reductase and 3α-HSD, respectively. The results showed that 5α-reductase was most active in neurons, although some

activity was observed in both oligodendrocytes and astrocytes. By contrast, 3α-HSD was most active in astrocytes, with less in oligodendrocytes and neurons (Melcangi *et al*., 1994). Therefore, this suggests that both neurons and glia are important for neurosteroid synthesis in the CNS. Recent studies have used immunohistochemistry coupled with *in situ* hybridization in mouse brain slices to determine the expression of 5α-reductase and 3α-HSD mRNA in specific neuronal populations. Interestingly, these enzymes were shown to co-localise in principle glutamatergic neurons of the cortex, hippocampus, and olfactory bulb and in some output of neurons of the thalamus and amygdala. Furthermore, although 5α-reductase and 3α-HSD are significantly expressed in principal GABAergic neurons, such as Purkinje neurons of the cerebellum, no signal was detected in cortical and hippocampal GABAergic interneurons. This indicates that expression of these enzymes is not uniform, which is likely to affect the regional distribution of neurosteroids in the brain (Agís-Balboa *et al*., 2006). Given that neurons express the necessary enzymes for neurosteroidogenesis, it has been proposed that, once synthesised, neurosteroids may diffuse into the extracellular space where they can modulate local synaptic transmission in an autocrine or paracrine fashion.

1.3.2 Dynamic fluctuations of neurosteroids in the brain

A number of techniques have been employed in order to determine the concentrations of neurosteroids present in the brain. In rats and humans, radioimmunolabelling techniques have obtained varied results, with concentrations of allopregnanolone between 0.5 and 20nM being reported, depending upon the brain region being examined (Purdy *et al*., 1991; Bernardi *et al*., 1998; Weill-Engerer *et al*., 2002). However, an additional study in humans observed much higher concentrations of between 30 and 70nM (Bixo *et al*., 1997). This large variance in results has been proposed to be due to a lack of specificity in the antibodies used in the assay, which may result in higher levels of signal being detected (Thienpont *et al*., 1991). Techniques which are considered to be more accurate and have yielded more consistent results are those which identify the neurosteroids present in brain homogenates by three sequential techniques: high performance liquid chromatography (HPLC), followed by gas chromatography and finally, mass fragmentography (termed GC-MF). Studies using this and similar techniques in rats have shown that concentrations of allopregnanolone present in the brain are around 3-10nM (Cheney *et al*., 1995; Uzunov *et al*., 1996; Uzunova *et al*., 2003). Furthermore, these studies showed that the distribution of allopregnanolone across the brain is not uniform, with the highest concentrations observed in olfactory bulb, striatum, hypothalamus and cerebellum and less in the cerebral cortex, hippocampus and amygdala (Cheney *et al*., 1995; Weill-Engerer *et al*., 2002; Uzunova *et al*., 2003). Another interesting observation from these studies is that

in rats which have undergone adrenalectomy and castration, levels of allopregnanolone fall by around 30% compared to sham operated animals, indicating that peripheral steroids contribute around a third of the total neurosteroid concentration in the brain (Cheney *et al*., 1995; Uzunov *et al*., 1996).

More recent experiments have used immunohistochemistry to investigate the presence of neurosteroids within specific subsets of neurons. THDOC and allopregnanolone were found to be present in a number of neuronal populations, including glutamatergic neurons of the cortex, hippocampus and thalamus as well as in projecting GABAergic neurons such as reticular thalamic neurons, striatal cells and cerebellar Purkinje neurons. However, very few local GABAergic interneurons were immunoreactive (Saalmann *et al*., 2007). This distribution of neurosteroids is very similar to the expression pattern of the key neurosteroidogenic enzymes, 5α-reductase and 3α-HSD (Agís-Balboa *et al*., 2006), supporting the notion that neurosteroids are synthesised *de novo* within these neurons. Interestingly, although some studies have suggested that both neurons and glia are able to synthesise neurosteroids, this study reported very few immunopositive glial cells (Saalmann *et al*., 2007), which may suggest that only some of the synthetic pathway takes place in glia or alternatively, could reflect the rapid diffusion of neurosteroids out of glial cells once synthesised, resulting in them being difficult to detect here.

The concentration of neurosteroids present in the brain is not static but instead it undergoes dynamic changes in response to various physiological and pathophysiological conditions. In rats, swim stress has been shown to enhance CNS levels of allopregnanolone and THDOC to as much as 50nM (Purdy *et al*., 1991; Paul and Purdy, 1992). Measurements of allopregnanolone in the brains of pregnant rats showed that levels can increase to 100nM (Paul and Purdy, 1992), an observation which was mimicked in humans, where, during pregnancy, plasma levels of allopregnanolone were reported to rise to a peak of around 60nM just prior to parturition (Parizek *et al*., 2005). Cyclic changes in neurosteroid levels have been observed during ovarian cycle, with concentrations of around 1nM reported during follicular phase, rising to between 4 and 12nM during the luteal phase (Bäckström *et al*., 2003; Maguire and Mody, 2009; Sanna *et al*., 2009). Further studies have shown that CNS concentrations of neurosteroids are significantly decreased with age (Schumacher *et al*., 2003). Concentrations of allopregnanolone were measured in the cerebral cortices of male rats at 6, 12, 16, 18 and 20 months of age, with results showing that levels decreased from 6.5ng/g at 6 months to around 3ng/g at 20 months (Bernardi *et al*., 1998). Neurosteroid concentrations have also been shown to fluctuate in

response to the administration of some psychoactive drugs such as ethanol, γ-hydroxybutyrate (GHB) and anti-depressants such as fluoxetine (Uzunov *et al*., 1996; Sanna *et al*., 2004).

Alterations in neurosteroid levels are also a common feature in some pathological conditions, including depression and Alzheimer's disease. In the olfactory bulbectomised rat model of depression, although an overall increase in allopregnanolone was reported in the whole brain, specific decreases were observed in the amygdala and frontal cortex (Uzunova *et al*., 2003). These two areas are considered to be important in regulating mood and emotion (Akwa et al., 1999; Rupprecht and Holsboer, 1999) and therefore this reduction may contribute to the appearance of depressive behaviours. A study comparing neurosteroid levels in the brains of Alzheimer's and non-demented humans showed that the neurosteroids pregnenolone sulphate and dehydroepiandrosterone sulphate were significantly reduced in the brains of patients with Alzheimer's disease (Weill-Engerer *et al*., 2002).

1.3.3 Neurosteroid actions at $GABA_A$ receptors: potentiation

The first indication that certain steroids could potentiate $GABA_A$ receptor responses came from studies by Harrison and Simmonds (1984) who reported that the synthetic steroid, alphaxalone enhanced stimulus evoked inhibition as well as potentiating the actions of the $GABA_A$ receptor agonist, muscimol in rat cuneate nucleus slices. Subsequent studies showed that endogenously produced neurosteroids could also enhance $GABA_A$ receptor function, with concentrations as low as 10nM able to potentiate GABA-activated currents recorded from a number of preparations including hippocampal and spinal cord neurons from rats, bovine adrenomedullary chromaffin cells and HEK293 cells expressing recombinant $GABA_A$ receptors (Majewska *et al*., 1986; Callachan *et al*., 1987; Morrow *et al*., 1987; Peters *et al*., 1988; Puia *et al*., 1990). Thus, at the low, nanomolar concentrations known to exist *in vivo* (see 1.3.2), neurosteroids can enhance $GABA_A$ receptor responses. Interestingly, more recent experiments have indicated that much lower concentrations of neurosteroid (around 3-5nM) can also potentiate $GABA_A$ receptor function, although these effects take much longer to develop than those elicited by higher concentrations of neurosteroid (Li *et al*., 2007). It was suggested that this was due to the ability of neurosteroids to diffuse between the cell membrane and intracellular compartments, effectively reducing the concentration of neurosteroid present in the membrane and decreasing the modulatory effect observed for a given concentration of neurosteroid. During longer applications, the neurosteroid equilibrates between the membrane and intracellular compartments, thus the neurosteroid is no longer removed from

the membrane and therefore the concentration of neurosteroid present at the $GABA_A$ receptor is more similar to that which has been applied (Li *et al*., 2007). However, more recently, it has been suggested that, due to the lipophilicity of neurosteroids, concentrations present within the membrane may be much higher than those measured in aqueous compartments, implying that the potency of neurosteroids at $GABA_A$ receptors may be much less than that indicated in earlier experiments (Chisari *et al*., 2010).

Following this work, many studies have been carried out in order to characterise the mechanisms by which neurosteroids act to potentiate $GABA_A$ receptor function. Early patch clamp studies showed that neurosteroids had little effect on the single channel conductance of the receptor, but greatly promoted its ion channel open state (Callachan *et al.*, 1987; Lambert *et al.*, 1987; Twyman and Macdonald, 1992). Using outside-out membrane patches from bovine chromaffin cells, Callachan *et al*. (1987) showed that 300nM pregnanolone induced a 272% increase in channel open probability (P_{open}). More recent evidence from studies using HEK293 cells expressing recombinant α1β2γ2L $GABA_A$ receptors has revealed that neurosteroids alter a number of kinetic properties in order to enhance P_{open}. In the absence of neurosteroid, the $GABA_A$ receptor open and close time distributions each contain three components (open times: OT1, OT2, OT3; closed times: CT1, CT2, CT3). Application of the synthetic neurosteroid analogue, (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile (ACN) caused an increase in prevalence and duration of the longest open times (OT3) as well as a decrease in the prevalence of the longest closed time durations (CT3), resulting in an increase in the time the receptor spends in its open state (Akk *et al*., 2004, 2005).

The effects of neurosteroids at synaptic $GABA_A$ receptors have been studied using whole-cell patch clamp electrophysiology to record mIPSCs from neurons in brain slices. Experiments have been conducted in a number of neuronal populations including: CA1 hippocampal neurons (Harney *et al*., 2003), cortical neurons (Puia *et al*., 2003), dentate gyrus granule cells (Cooper *et al*., 1999; Harney *et al*., 2003), the oxytocin-producing neurons of the hypothalamus (Brussaard *et al*., 1997; Fancsik *et al*., 2000; Koksma *et al*., 2003) and the Purkinje, granule and stellate neurons of the cerebellum (Cooper *et al*., 1999; Hamann *et al*., 2002; Vicini *et al*., 2002). In these neurons, neurosteroids (10nM-1μm) had little effect on mIPSC rise times or amplitudes, but instead caused a dramatic prolongation of the decay phase, a modulation which results in the potentiation of GABAergic synaptic transmission.

Interestingly, although neurosteroids had similar effects in all of these neuronal populations,

the effective concentration range was neuron specific. For example, low, physiological concentrations of neurosteroids prolonged mIPSCs in hippocampal CA1 neurons (Harney *et al*, 2003), cerebellar granule cells (Cooper *et al*., 1999; Hamann *et al*., 2002; Vicini *et al*, 2002) and cerebellar Purkinje neurons (Cooper *et al.*, 1999); whereas concentrations above those seen physiologically are required to produce similar effects in hypothalamic neurons (Brussaard *et al.*, 1997; Fancsik *et al*., 2000; Koksma *et al.*, 2003). Thus, *in vivo*, it appears that neurosteroids may only affect $GABA_A$ receptors in particular brain regions and it is not until levels rise that receptors in other brain regions are modulated. Further experiments have shown that even neurons in the same brain structure can be differentially affected, for example, in rat hippocampal slices, mIPSCs recorded from the CA1 region are prolonged by concentrations of allopregnanolone of 10nM and above whereas, in dentate gyrus granule cells, 300nM is required to induce a similar effect (Harney *et al*., 2003).

Neurosteroid sensitivity is also modulated during development, for example, in P10 rats, mIPSCs recorded from dentate gyrus granule cells were prolonged by 100nM THDOC, whereas, at P20, this concentration had no effect (Cooper *et al*., 1999). It has been suggested that these changes may be due to alterations in expression patterns of some $GABA_A$ receptor subunits, which have also been shown to occur during this developmental period (Lambert *et al*., 2003). More rapid alterations in neurosteroid sensitivity have also been described, for example, the magnocellular neurons of the hypothalamus are sensitive to allopregnanolone 1 day prior to parturition, however, after parturition, these neurons become neurosteroid insensitive (Brussaard *et al*., 1997).

In addition to synaptic or phasic inhibition, which is mediated by the rapid, transient release of GABA from pre-synaptic terminals which diffuses rapidly across the synaptic cleft to activate receptors clustered at the postsynaptic site, $GABA_A$ receptors also generate a slower 'tonic' form of inhibition in response to the persistent activation of extrasynaptic receptors by low, ambient concentrations of GABA (Farrant and Nusser, 2005; see Chapter 5). Recent studies have shown that, in some regions of the brain, this tonic inhibition is also modulated by neurosteroids, with results showing that low concentrations of neurosteroids can potentiate the tonic currents recorded from dentate gyrus granule cells and cerebellar granule cells (Hamann *et al*., 2002; Stell *et al*., 2003;). In fact, in the dentate gyrus granule cells of adult rats, physiological concentrations of neurosteroid exclusively modulate the tonic conductance, with no effect at synaptic receptors (Harney *et al*., 2003; Stell *et al*., 2003). By contrast, tonic conductance recorded from the thalamus and hippocampal CA1 region was not sensitive to

neurosteroids (Belelli and Lambert, 2005), indicating that tonic inhibition is subject to neurosteroid modulation in a neuron-specific manner.

1.3.4 Neurosteroid actions at $GABA_A$ receptors: direct activation

At concentrations higher than those required to potentiate GABA-mediated neurotransmission (above 1μm), neurosteroids are capable of directly activating the $GABA_A$ receptor in the absence of GABA (Callachan *et al*., 1987; Peters *et al.*, 1988; Shu *et al.*, 2004). Callachan *et al*. (1987) showed that application of the neurosteroid, pregnanolone to bovine adrenomedullary chromaffin cells resulted in an increase in membrane current, an effect which was abolished by the $GABA_A$ receptor antagonist, bicuculline, indicating that pregnanolone was directly activating the $GABA_A$ receptor. This was supported by single channel recordings which showed that, at concentration in excess of 1μM, pregnanolone induced currents with similar kinetic properties to those induced by GABA (Callachan *et al*., 1987). However, due to this effect being elicited by concentrations of neurosteroid above those that would be expected *in vivo*, this effect is not considered to be physiologically relevant.

1.3.5 Effect of subunit composition

Many of the pharmacological properties of the $GABA_A$ receptor are dependent upon its subunit composition, for example, the isoform of β subunit present in the receptor complex infers its sensitivity to the general anaesthetic, etomidate, whereas the α and γ isoforms present greatly influence the receptors response to benzodiazepines (Korpi *et al*., 2002). However, the affinity with which neurosteroids bind to and modulate the $GABA_A$ receptor appears to be largely independent of subunit composition. Studies investigating the dose-response relationships of allopregnanolone (co-applied with an EC_{10} concentration of GABA) at recombinant receptors consisting of different subunit combinations have shown that the presence of different α subunit isoforms has only modest effects on neurosteroid actions, with the EC_{50}'s for potentiation not differing significantly between receptors expressing α1-6 in combination with β1 and γ2L subunits (Belelli *et al*., 2002; Hosie *et al*., 2007). Some small differences in potentiation were observed with low concentrations of neurosteroid, with results showing that GABA-activated currents recorded from receptors incorporating α1 or α3 subunits, but not those containing α2, α4, α5, or α6, were significantly enhanced by 3nM allopregnanolone, although the extent of this potentiation was small (Belelli *et al*., 2002).

The presence of different γ subunit isoforms also had modest effects on neurosteroid sensitivity, with γ2 subunit-containing receptors being the most sensitive, with an EC_{50} of 89 ±

6nM and γ1-containing receptor the least sensitive (EC_{50} = 559 ± 22nM; Belelli *et al*., 2002). However, the magnitude of potentiation elicited by physiological concentrations of allopregnanolone (3-100nM) did not differ significantly between receptors containing γ1-3 subunits. In addition, the identity of the β subunit expressed did not affect the activity of neurosteroids at the receptor, with the extent of potentiation elicited by concentrations of allopregnanolone between 3nM and 10μM, not differing significantly between receptors containing β1, β2 or β3 subunits (Hadingham *et al*., 1993; Belelli *et al*., 1996, 2002).

Although the majority of synaptic $GABA_A$ receptors are thought to be composed of α, β and γ subunits (Sieghart *et al*., 1999; Whiting, 1999; Olsen and Sieghart, 2008), a number of studies have indicated that some extrasynaptic receptors populations consist of α and β subunits in combination with δ (Farrant and Nusser, 2005; Sieghart, 2008). One early study suggested that inclusion of the δ subunit significantly reduced the magnitude of potentiation induced at $GABA_A$ receptors (Zhu *et al*., 1996). However, a number of more recent studies have shown that, in both HEK293 cells and *Xenopus* oocytes expressing recombinant $GABA_A$ receptors, incorporation of the δ subunit actually enhanced neurosteroid potentiation compared to those containing γ2 (Belelli *et al*., 2002; Brown *et al*., 2002; Wohlfarth *et al*., 2002). This is consistent with results obtained from knock-out mice, which showed that deletion of the δ subunit caused a reduction in behavioural responses to alphaxalone, ganaxolone and pregnenolone (Mihalek *et al*., 1999), implying that, in these mice, the δ subunit-containing receptors convey much of the animal's sensitivity to neuroactive steroids.

Receptors incorporating the δ subunit have been shown to exhibit high affinity, but low efficacy for GABA, that is, GABA acts as a partial agonist at these receptors (Brown *et al*., 2002; Bianchi and Macdonald, 2003; Stórustovu and Ebert, 2006). Using full and partial agonists of both γ and δ subunit-containing receptors, Bianchi and Macdonald (2003) demonstrated that neurosteroids enhance partial agonist activity to a greater extent than full agonist activity, an effect which was independent of receptor subunit composition. Therefore, this indicated that the enhanced neurosteroid sensitivity displayed by δ subunit-containing receptors is a consequence of altered functional properties of the receptor rather than the ability to neurosteroids to bind with greater affinity. As mentioned previously, δ subunit-containing receptors are thought to be located extrasynaptically to inhibitory synapses where they generate a slow 'tonic' inhibition in response to low, ambient concentrations of GABA (Farrant and Nusser, 2005). Therefore, this demonstration that δ subunit-containing receptors are more sensitive to neurosteroids has important implications for the regulation of tonic inhibition *in vivo*.

1.3.6 Neurosteroid binding sites

1.3.6.1 Evidence for the presence of a specific binding site

The first evidence that neurosteroids modulate $GABA_A$ receptors by binding to a discrete site located on the receptor complex was obtained from studies that showed that the actions of neurosteroids were not influenced by other $GABA_A$ receptor modulators such as benzodiazepines (Callachan *et al*., 1987; Cottrell *et al*., 1987) or barbiturates (Gee *et al*., 1988; Peters *et al*., 1988), suggesting that the neurosteroids act through a separate binding site. Further studies investigating the structure-activity relationships of steroids demonstrated that specific features of neurosteroid molecules were required for their actions at $GABA_A$ receptors. In the case of the pregnane-based neurosteroids (e.g. allopregnanolone and THDOC), the presence of a hydroxyl group in the α conformation at C3 of ring A, together with a keto group at C20 in ring D are crucial for their potentiation and direct activation of $GABA_A$ receptors (Simmonds *et al*., 1991; Covey *et al*., 2001; see Fig. 1.4). This structure specificity argued for the existence of a specific binding site on the $GABA_A$ receptor rather than a non-specific action of neurosteroids upon cell membranes, a second theory which had been proposed due to the lipophilic nature of neurosteroids and their ability to accumulate within the membrane.

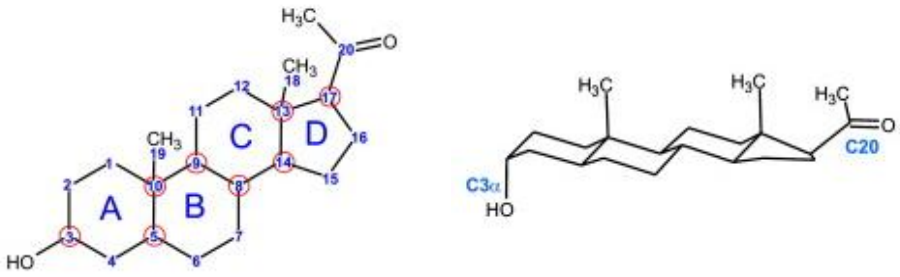

Figure 1.4 Structural requirements for potentiating neurosteroids
Chemical structure of the neurosteroid, allopregnanolone. The 2D image (left) shows the 4 ring structure (labelled A to D), with the 8 chiral centres highlighted with red circles and carbon positions indicated in blue. The 3D image shows the C3 hydroxyl group in the α conformation and the C20 keto group, which are obligatory for potentiation. Figure is adapted from Hosie *et al*., 2007.

Studies using enantiomers of allopregnanolone also provided strong evidence that neurosteroids act by binding to a specific site. Wittmer *et al*., (1996) created an enantiomeric form of (+)-allopregnanolone, by inverting the configuration of all 8 chiral centres (see Fig. 1.4) to produce its mirror image compound, *ent*-allopregnanolone. This compound elicited markedly reduced potentiation of GABA currents compared to (+)-allopregnanolone and was unable to directly activate the receptor (Wittmer *et al*., 1996), indicating the presence of a

specific neurosteroid binding site which has stringent structural requirements. Furthermore, both (+)-allopregnanolone and *ent*-allopregnanolone have been shown to exhibit similar membrane perturbing properties (Alakoskela *et al*., 2007) and, in experiments using fluorescently labeled enantiosteric neurosteroids, these compounds also displayed identical timecourses for membrane accumulation and similar cellular partitioning (Chisari *et al*., 2009). Therefore, their differential abilities to modulate $GABA_A$ receptor responses are not well correlated with their membrane perturbing properties, indicating that neurosteroids are unlikely to act by simply perturbing membrane lipids.

These studies of enantiomeric neurosteroids also revealed the potential for multiple binding sites on the $GABA_A$ receptor. Although different potentiation and direct activation profiles were observed with the (+)- and *ent*- forms of allopregnanolone, this was not the case for all pairs of neurosteroid enantiomers, with both forms of the 5β-reduced neurosteroid, pregnanolone, exhibiting similar abilities to potentiate and directly activate GABA responses (Covey *et al*., 2000). It was therefore suggested that there may be more than one binding site, enabling the receptor to accommodate neurosteroids with different structural conformations. More recent studies investigating the effects of neurosteroids on the single channel properties of $GABA_A$ receptors have also indicated that more than one neurosteroid binding site may exist, with results showing that two different synthetic neurosteroids (ACN and B285 ((3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile)) had different effects on single channel kinetics. For example, ACN increased the duration and prevalence of OT3 (longest open time), whereas B285 increased the prevalence of OT3, but only increased its mean duration at very high concentrations (Akk *et al*., 2004). Therefore, it was proposed that multiple binding sites may exist, each associated with a different functional effect, and thus the ability of each neurosteroid to bind these sites would determine its effects upon single channel kinetics.

1.3.6.2 Identification of neurosteroid binding sites on the $GABA_A$ receptor

The first clues as to the nature of the neurosteroid binding site came from studies of the ligand binding domains present on other steroid binding proteins (reviewed in Hosie *et al*., 2007). Using X-ray crystallography, it was revealed that the ligand binding domains of the nuclear steroid receptor family are wedge-like structures which incorporate a ligand binding cavity at their narrow end. The ligand binding cavities were shown to be lined with hydrophobic residues, which interact with the steroid's ring structure via van der Waals forces as well as containing apical polar residues, which facilitate hydrogen bonding with the C3 and C17/C20 oxygen groups present on the molecule (Weatherman *et al*., 1999). These features are also

conserved within the ligand binding cavities of other steroid binding proteins including the synthetic enzymes, 3α-HSD and 20α-HSD (Jin *et al*., 2001; Nahoum *et al*., 2001), therefore suggesting that the neurosteroid binding site(s) on the $GABA_A$ receptor may contain similar features.

In order to identify the location of neurosteroid binding sites, initial studies utilised chimeras formed from the $GABA_A$ receptor and the relatively neurosteroid insensitive glycine receptor. Rick *et al*. (1998) produced a series of chimeric receptors using the $GABA_A$ receptor α2 or β1 subunits and the glycine receptor α1 subunit and showed that potentiation by the synthetic steroid, alphaxalone was mediated by the N-terminal half of the $GABA_A$ receptor α2 or β1 subunits (N-terminus + TM1 + TM2). Furthermore, the neurosteroid sensitivity of the glycine receptor could be markedly enhanced by replacing TM4 and the C-terminus of the glycine α1 subunit with the corresponding region of the $GABA_A$ receptor α1 (Ueno *et al*., 2004), indicating that the TM4 region of the α1 subunit may also play a part in neurosteroid binding at the $GABA_A$ receptor. Therefore, it was suggested that the transmembrane domains of the $GABA_A$ receptor were likely to be the location for neurosteroid binding.

To locate the specific transmembrane residues that were important for neurosteroid binding, Hosie *et al*. (2006) utilised chimeras formed from the mammalian $GABA_A$ receptor α1 and β2 subunits and the corresponding regions of the *Drosophila melanogaster* RDL GABA receptor, which is homologous to the mammalian $GABA_A$ receptor but is relatively neurosteroid insensitive (Chen *et al*., 1994; Hosie and Sattelle, 1996). Replacement of TM1 and TM2 of the mammalian $GABA_A$ receptor α1 subunit resulted in the abolition of both the potentiation and direct activation effects of neurosteroids. By contrast, similar modification to the β2 subunit had no effect, indicating that the TM1 and TM2 regions of the α1, but not the β2 subunit are important for the actions of neurosteroids. Secondly, given that polar residues were shown to be important for the binding of steroid molecules to nuclear steroid receptors and some steroid synthetic enzymes, Hosie *et al*. (2006) then identified a number of polar residues that were present in the α1 TM1 and TM2 domains but replaced with hydrophobic residues in the RDL receptor. Sequential mutation of these residues to their hydrophobic RDL equivalents showed that Threonine 236 was required for direct activation whereas Glutamine 241 was necessary for both potentiation and direct activation by neurosteroids. In addition, the requirement for polar residues at these positions indicated that hydrogen bonding is important for the actions of neurosteroids. Furthermore, these mutations had no effect on the actions of diazepam, pentobarbitone or mefanamic acid, indicating that these residues are likely to

contribute to a specific neurosteroid binding site rather than modulating allosteric potentiation more generally.

It was suggested that, due to the differential effects of mutating T236 and Q241, these two residues may contribute to separate neurosteroid binding sites. This was confirmed using homology modelling, which suggested that T236 is situated at the α-β subunit interface, whereas Q241 is located at the base of a water-filled cavity between the α1 TM1 and TM4 domains. Interestingly, substituted cysteine accessibility experiments have shown that this pocket can increase in depth and volume following receptor activation by GABA (Williams and Akabas, 1999) and therefore, it has been hypothesized that this increase in volume may facilitate entry of the neurosteroid into the cavity, allowing it to bind Q241, stabilizing the receptor in its open conformation and resulting in potentiation of the GABA current (Hosie *et al*., 2007).

Finally, by estimating the dimensions of the neurosteroid molecule, Hosie *et al*. (2006) identified further residues which may provide potential hydrogen bonding partners at the opposite end of the neurosteroid molecule. In the case of the Q241 site, hydrogen bonding at α1 TM4 Asparagine 407 and Tyrosine 410 were shown to be important for both potentiation and direct activation by neurosteroids. For the T236 site, Tyrosine 284 on TM3 of the β2 subunit was shown to be important for direct activation only (see Fig. 1.5). Therefore, taken together, these data indicate that the $GABA_A$ receptor contains two distinct neurosteroid binding sites, the first is located within the transmembrane region of the α1 subunit and is important for both potentiation and direct activation by neurosteroids and the second is found on the putative interface between the α1 and β2 subunits and is required for direct activation only. Furthermore, for maximal receptor activation by neurosteroids, occupation of both sites is required (Hosie *et al*., 2006). Although this study indicated that hydrogen bonding between polar residues housed within the binding cavity and the neurosteroid molecule was imperative for the actions of neurosteroids, a more recent study has suggested that this may not be the case, with results showing that a synthetic neurosteroid containing a C17 group that cannot form hydrogen bonds can still potentiate α1β2γ2L $GABA_A$ receptors expressed in HEK293 cells (Li *et al*., 2009). However, some hydrogen bonding capabilities still remain on this steroid molecule, at C3, which may therefore be sufficient to induce this potentiation. Furthermore, mutation of N407 and Y410 abolished this potentiation indicating that these residues are still important for the effects of this synthetic neurosteroid (Li *et al*., 2009).

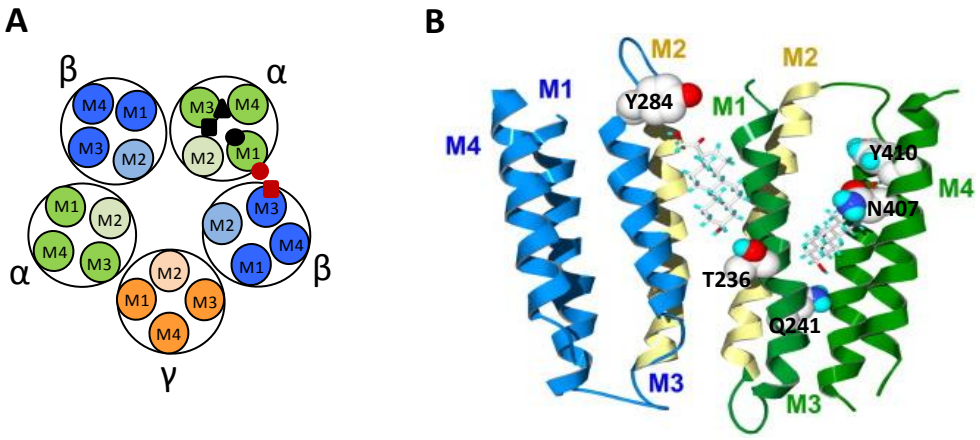

Figure 1.5 Neurosteroid binding sites
(A) Schematic diagram showing the positions of residues which contribute to the neurosteroid potentiation site (black: Q241 (circle), Y410 (square), N407 (triangle)) and the direct activation site (red: T236 (circle), Y284 (square)). (B) Homology model (taken from Hosie *et al*., 2007) showing the $GABA_A$ receptor α1 (green) and β2 (blue) subunit transmembrane domains (TM2 domains are shown in pale yellow). The positions of key residues which form the neurosteroid potentiation and direct activation sites as well as the proposed positions of the neurosteroids within the binding cavities are indicated.

The potentiation site has been shown to be conserved across the α subunit family, with mutation of the glutamine equivalent to Q241 in these subunits, resulting in the abolition of neurosteroid potentiation (Hosie *et al*., 2008). This is consistent with the observation that the identity of the α subunit does not significantly alter the receptor's sensitivity to neurosteroids (Belelli *et al*., 2002). In addition, although some studies have indicated that receptors incorporating the δ subunit are more sensitive to neurosteroids, Hosie *et al*. (2008) showed that the δ subunit is unlikely to contribute to the neurosteroid binding site, with results showing that $\alpha4^{Q246L}\beta3\delta$ $GABA_A$ receptors expressed in HEK293 cells were not potentiated by THDOC. This is consistent with results from previous studies which indicated that the increase in neurosteroid sensitivity observed at δ subunit-containing receptors was due to GABA having a low efficacy interaction with this receptor rather than the presence of the δ subunit acting to modify neurosteroid binding (Bianchi and Macdonald, 2003).

As there are two copies of the α subunit within each pentameric $GABA_A$ receptor, recent studies have used concatemers of subunits to investigate the relative contributions of each potentiation site to the overall neurosteroid effect. Concatemers enable individual subunit cDNAs to be tethered together and transfected as a single construct, allowing for greater control over the subunit arrangement of the receptor, as well as permitting each neurosteroid potentiation site to be mutated individually in order to assess its contribution. Concatemers formed from α1-β2 and α1-β2-γ2 subunits were expressed in *Xenopus* oocytes or HEK293 cells

and were shown to have similar single channel kinetic properties as receptor formed from single subunits, although whole-cell concentration-response relationships indicated that they have a lower affinity for GABA (Akk *et al*., 2009). Mutation of Q241 in a single potentiation site resulted in only a modest decrease in neurosteroid activity, whereas mutation of both sites resulted in a complete abolition of potentiation (Akk *et al*., 2009; Bracamontes and Steinback, 2009). This indicates that neurosteroid binding to a single site is sufficient to induce potentiation and that both sites are functionally equivalent. However, the use of two concatemers encoding α1-β2 and α1-β2-γ2 means that it is not possible to rule out the formation of unnatural $GABA_A$ receptors composed of four or even six subunits which, if functional, may act to confound the results obtained.

1.3.6.3 Neurosteroid access to its binding site

Conventional models of ligand-receptor interactions usually involve a molecule accessing an extracellular binding site on the aqueous accessible domain of the receptor. Early evidence suggested that this may be the case for the neurosteroids with Lambert *et al*. (1990) showing that intracellular application of alphaxalone did not potentiate GABA-activated currents recorded from bovine chromaffin cells, which indicated that the neurosteroid binding site can only be accessed from the extracellular surface of the receptor. However, recent evidence obtained by Akk *et al*. (2005) has indicated that, to the contrary, neurosteroids may access their binding site via the membrane. In cell-attached patch recordings from HEK293 cells expressing α1β2γ2 $GABA_A$ receptors, application of the synthetic neurosteroid, ACN resulted in potentiation of GABA-activated currents, despite the fact that direct aqueous access to the receptor was prevented. Furthermore, in excised patches formed from HEK293 cells after preincubation with ACN, potentiation continued long after excision, indicating that neurosteroid which has accumulated in the membrane is sufficient to induce potentiation (Akk *et al*., 2005). Finally, internal application of neurosteroid was able to potentiate GABA responses to a similar degree to that applied extracellularly and a membrane impermeable neurosteroid analog could only potentiate the $GABA_A$ receptor when applied to the inner membrane leaflet. Therefore, taken together, these results indicate that neurosteroids are likely to access their binding site via a membrane rather than an aqueous route (Akk *et al*., 2005).

1.3.7 Neurosteroid modulation of $GABA_A$ receptor expression

In addition to directly modulating $GABA_A$ receptor ion channel kinetics, other studies have also proposed that neurosteroids may act by altering cell surface expression of some $GABA_A$

receptor subunits, which may, in turn, alter $GABA_A$ receptor sensitivity to neurosteroids and other endogenous modulators. Using RNAase protection assays to quantify mRNA expression, a number of studies have shown that long term exposure to both progesterone and allopregnanolone results in changes to the expression patterns of specific $GABA_A$ receptor subunit mRNAs (reviewed in Biggio *et al*., 2006). Follesa *et al*. (2000) showed that, in rat cerebellar granule cells, exposure to progesterone for 5 days caused a significant decrease in the amount of mRNA encoding α1, α3, α5 and γ2 subunits but had no effect on levels of α2, α4, β1 or β2 subunit mRNAs. This effect was abolished by treatment of the neurons with finasteride, a specific inhibitor of 5α-reductase, indicating that these effects are mediated by the conversion of progesterone to allopregnanolone (Follesa *et al*., 2000). Different patterns of down-regulation have been seen in other neuronal types, with exposure of rodent cortical neurons to neurosteroids having no significant effect on the abundance of mRNA encoding α1, α4, or γ2S (Follesa *et al*., 2001), but instead leading to a decrease in α2, α3, β2 and β3 subunit mRNAs (Yu *et al.*, 1996). It has been postulated that such variations in the levels of $GABA_A$ receptor mRNAs in different types of neurons has the effect of differentially altering the sensitivity of their surface $GABA_A$ receptors to endogenous modulators. In fact, after prolonged exposure to progesterone, cerebellar granule cells exhibited reduced sensitivity to the benzodiazepine, diazepam (Follesa *et al*., 2000). This effect is consistent with the down-regulation of diazepam sensitive α subunits (α1, α3 and α5) and with the decrease in γ2 subunit expression, the presence of which has been shown to be necessary for benzodiazepine modulation (Pritchett *et al*., 1989; Sigel *et al*., 1990).

Further studies using pregnant rats have addressed the effects of prolonged neurosteroid exposure on $GABA_A$ receptor mRNA expression *in vivo*. During the late stages of pregnancy, neurosteroid levels have been shown to rise, peaking at around 100nM just prior to parturition (Paul and Purdy *et al*., 1992). Using RNase protection assays it has been demonstrated that, during pregnancy, γ2L mRNA expression is decreased in the cerebral cortex and hippocampus, an effect which is reversed by finasteride treatment (Concas *et al*., 1998). In addition, mRNAs corresponding to α1-4, β1-3 and γ2S subunits were unaltered, indicating the specificity of this modulation (Concas *et al*., 1998). In the oxytocin-producing neurons of the hypothalamus, there was a specific increase in α1 mRNA expression during pregnancy, which rapidly declined to normal after parturition, a time which is associated with rapid decline in neurosteroid levels (Fenelon and Herbison, 1996).

More recent studies have focussed on investigating the changes in cell surface expression of

$GABA_A$ receptor subunit proteins in response to the chronic increases in CNS neurosteroid concentrations during pregnancy and the briefer, cyclic fluctuations that occur during the ovarian cycle. Maguire *et al*., (2005) showed that, in the dentate gyrus of female mice, fluctuating levels of neurosteroids during the ovarian cycle resulted in cyclic changes in the cell surface expression of δ subunits. When levels of circulating progesterone are high in diestrous, a concurrent increase in membrane localised δ subunits was observed. This was coupled with a decrease in surface expression of γ2 subunits during this time (Maguire and Mody, 2005). Further studies showed that this increase in δ subunit expression resulted in a significant increase in the magnitude of the tonic conductance (Maguire and Mody, 2007), thus, neurosteroid-induced changes in $GABA_A$ receptor subunit expression during the ovarian cycle can have profound effects on neuronal excitability. By contrast, the prolonged enhancement of neurosteroid levels which occurs during pregnancy have been shown to cause a down-regulation of cell surface δ subunit expression in a number of brain regions including the thalamus, striatum and CA1 region of the hippocampus (Maguire *et al*., 2009), an effect which has been suggested to occur as a homeostatic mechanism in order to maintain normal levels of neuronal excitability during this period (Maguire and Mody, 2008; Maguire *et al*., 2009; see 1.3.8).

1.3.8 Physiological importance of neurosteroid modulation

1.3.8.1 Presence of an underlying neurosteroid tone

The importance of the actions of neurosteroids in the CNS has been highlighted by studies in which their synthesis has been perturbed. Injection of mice with the 5α-reductase inhibitor, SKF105111 was shown to cause a reduction in the decay time of mIPSCs recorded *in situ* from cortical neurons (Puia *et al*., 2003), indicating the presence of an underlying neurosteroid tone in these neurons. These mice were also shown to exhibit a reduction in behavioural responses to the $GABA_A$ receptor agonist, muscimol, as demonstrated by a decrease in the induction of the loss of righting reflex (Sanna *et al*., 2004). Therefore, this indicates that this underlying neurosteroid tone is important for regulating GABAergic inhibitory transmission and that synaptic $GABA_A$ receptors are an important physiological target for neurosteroids. An underlying neurosteroid tone has also been observed in the dentate gyrus, but interestingly, treatment of CA1 pyramidal neurons with the 5α-reductase inhibitor, finasteride, did not affect mIPSC decay times however, stimulation of neurosteroid synthesis using a TSPO agonist resulted in a prolongation of mIPSCs, an effect which was blocked by finasteride (Sanna *et al*., 2004). This indicates that different neuronal populations are subject to differential control by

neurosteroids under physiological conditions and that basal synthesis of neurosteroids may play a large role in this process.

1.3.8.2 Role of neurosteroids in neurological and psychiatric disorders

Dysfunction of neurosteroid activity is associated with a number of neurological and psychiatric disorders. Catamenial epilepsy describes the appearance of seizure exacerbations in women during certain phases of the ovarian cycle. As discussed previously, the ovarian cycle is associated with cyclic changes in the levels of circulating neurosteroids in the brain (Paul and Purdy, 1992; Bäckström *et al*., 2003) and these changes in seizure susceptibility have been correlated with these fluctuations in neurosteroid concentrations (Maguire *et al*., 2005). It has been shown that, when neurosteroid levels are high in diestrous, the tonic current present in dentate gyrus granule cells is markedly increased, resulting in decreased neuronal excitability in these neurons, coupled with a significantly increase latency to first seizure appearance and shorter seizure durations after kainic acid injection (Maguire *et al*., 2005). This indicates that neurosteroid modulation at $GABA_A$ receptors is critically important for regulating neuronal excitability and therefore, when neurosteroid levels are low, for example, during estrous, neuronal excitability will be increased, causing decreased seizure thresholds. The increase in tonic current during diestrous has been shown to be due to the enhancement of δ subunit expression, with results showing that treatment of neurons with an antisense δ RNA abolishes this effect (Maguire *et al*., 2005; Maguire and Mody, 2007). Studies using rodent models have also implicated neurosteroids in the aetiology of temporal lobe epilepsy. Induction of status epilepticus via pilocarpine injection or through continuous hippocampal stimulation had the effect of reducing the neurosteroid sensitivity of both phasic and tonic conductance in dentate gyrus granule cells (Peng *et al*., 2004; Sun *et al*., 2007). This decrease in sensitivity is thought to be due to alterations in the cell surface expression of specific subunits, including decreases in extrasynaptic δ subunits (Peng *et al*., 2004) and relocation of α4 subunits from peri-synaptic to synaptic regions (Sun *et al*., 2007) and may contribute to the perturbation of inhibitory transmission, leading to seizure propagation and reoccurrence.

Ovarian cycle-linked changes in neurosteroid levels are also thought to contribute to certain mood disorders, including premenstrual disphoric disorder (PMDD; reviewed by Bäckström *et al*., 2003). Maguire *et al*. (2005) reported that, in female mice, anxiety levels, as tested by the elevated plus maze, were increased during estrous, when neurosteroid levels are low, an effect which correlates with the reduction of tonic conductance in the dentate gyrus granule cells during this time. Therefore, this indicates that physiological changes in neurosteroid

levels during the ovarian cycle can result in increased anxiety and this may contribute to the feeling of dysphoria in PMDD patients. Interestingly, women with PMDD have a higher incidence of catamenial epilepsy than the general population, which implies a shared pathogenesis, thus, decreases in neurosteroid-dependent augmentation of GABAergic inhibition may underlie both of these conditions.

During pregnancy, levels of neurosteroid present in the brain are increased, peaking at around 100nM (Paul and Purdy, 1992). During this time it has been shown that both phasic and tonic inhibition are decreased in the dentate gyrus, an effect which results from the down-regulation of γ2 and δ subunits, respectively (Maguire and Mody, 2008). This decrease in inhibition has been shown to lead to an enhancement of neuronal excitability, as shown by a leftward shift in the input-output curves recorded from the dentate gyrus molecular layer in response to lateral perforant path stimulation (Maguire *et al*., 2009). Addition of allopregnanolone, at the concentration expected during pregnancy (100nM) shifted the input-output relationships back towards those recorded from virgin mice (Maguire *et al*., 2009), indicating that during pregnancy, decreases in δ subunit expression result in enhanced neuronal excitability, an effect which is rectified by the high levels of circulating neurosteroid. Therefore, it has been suggested that the decrease in δ subunit expression during this time may act as a homeostatic mechanism in order to maintain normal levels of neuronal excitability (Maguire and Mody, 2008). Interestingly, studies of $\delta^{-/-}$ mice, which cannot regulate δ subunit expression during pregnancy and the post-partum period, showed that these mice exhibit abnormal maternal behaviours, with the appearance of a depression-like phenotype, which results in decreased pup survival (Maguire and Mody, 2008). Therefore, it has been suggested that a failure to restore normal δ subunit expression following the rapid decline in neurosteroid levels post-partum could lead to hyper-excitability in certain neuronal networks which may contribute to the appearance of post-partum depression in humans (Maguire and Mody, 2008). However, in contrast to these findings, Sanna *et al*. (2009) reported that, in pregnant rats, tonic current recorded from dentate gyrus granule cells was increased and that this effect is mediated by an enhancement of δ subunit expression. The reasons for these discrepancies are unclear but may be due to species differences with Maguire and colleagues recording from mice and Sanna *et al*. from rats.

Puberty is also associated with mood disturbances and recent evidence suggests that neurosteroids may play a substantial role in the appearance of enhanced levels of anxiety during this time. Shen *et al*. (2007) have shown that, in contrast to its anxiolytic effects in adult

mice, allopregnanolone enhances anxiety in pubertal female mice. This effect is thought to be mediated by extrasynaptic α4βδ subunit-containing $GABA_A$ receptors, which are up-regulated in the CA1 region of the hippocampus during puberty. These receptors generate outward currents and, in stark contrast to the reported potentiating actions of neurosteroids, are in fact inhibited by allopregnanolone, resulting in enhanced tonic inhibition and increased neuronal excitability in the hippocampus (Shen *et al*., 2007). This effect was shown to be dependent upon arginine 353, a non-conserved residue located within the large TM3-4 intracellular domain of the α4 subunit (Shen *et al*., 2007).

Alterations in neurosteroid concentrations have also been implicated in stress and depression. Studies in rodents revealed that allopregnanolone levels in the brain were increased during stressful situations to levels sufficient to modulate $GABA_A$ receptors (Purdy *et al.*, 1991). These increases in allopregnanolone have also been correlated with decreased dopamine and noradrenaline levels in the prefrontal cortex, suggesting that allopregnanolone may also influence the mesolimbocortical dopamine pathway, a system that is crucial for the neurochemical stress response (Zimmerberg and Brown, 1998). In an animal study involving social isolation, the development of depressive behaviour was paralleled by a reduction in 5α-reductase activity and consequent reduction in allopregnanolone levels in the frontal cortex and olfactory bulb (Dong *et al*., 2001). These neurochemical perturbations were accompanied by the development of aggressive behaviour, which was rectified by restoration of 'normal' levels of allopregnanolone, indicating a possible role for neurosteroids in depression.

One of the strongest demonstrations of the importance of neurosteroids *in vivo* comes from the study of Niemann-Pick Disease (type c), a fatal childhood condition associated with faulty cholesterol transport (reviewed in Mellon *et al*., 2007). This genetic abnormality produces dramatic reductions in allopregnanolone levels in mouse brain at birth (Griffin *et al*., 2004), a change which is associated with a progressive loss of motor and neuronal function, particularly in Purkinje neurons, resulting in premature death. Administration of allopregnanolone delays the onset of the motor symptoms and extends survival, with the impact of this treatment being more pronounced the earlier the steroid is administered after birth (Griffin *et al*., 2004). The neurosteroid effect on cell survival is bicuculline–sensitive, indicating that modulation of $GABA_A$ receptor function by neurosteroids is crucial for normal brain development, perhaps undertaking a neuroprotective function. Neurosteroid levels have also been shown to be perturbed in some other neurodegenerative conditions, including Alzheimer's and Parkinson's disease (Weill-Engerer *et al*., 2002; Luchetti *et al*., 2010). For example, quantification of

neurosteroid levels in the post-mortem brains of patients with Alzheimer's disease showed that they had significantly lower levels of striatal and cerebellar pregnenolone sulphate and dehydroepiandrosterone sulphate compared to non-demented control subjects (Weill-Engerer *et al*., 2002). The level of pregnenolone sulphate present was negatively correlated with the appearance of cortical β-amyloid plaques, suggesting that this neurosteroid may have neuroprotective effects which are compromised in Alzheimer's disease.

Modulation of $GABA_A$ receptors by neurosteroids has also been suggested to be important for cognitive processing. The level of pregnenolone sulphate present in the brain has been shown to affect the cognitive behaviour of aged rats, with low concentrations correlating with memory impairment. These aged rats gained a temporary improvement in memory tasks after administration of pregnenolone sulphate (Mayo *et al*., 1993). A further study showed that pregnenolone sulphate can enhance hippocampal neurogenesis in young and aged rats and that these effects are dependent upon modulation of the $GABA_A$ receptor (Mayo *et al*., 2005), suggesting that this may be the mechanism by which pregnenolone sulphate enhances cognitive abilities in aged rats. More recently, it has been reported that mental retardation in a number of patients is caused by mutations in the neurosteroid synthetic enzyme, hydroxysteroid (17β) dehydrogenase, which results in decreased or abolished function of this enzyme and a perturbation of neurosteroid metabolism (Yang *et al*., 2009). The severity of the mutation (limited versus no functionality) was closely correlated with the severity of the phenotype observed, with the mutation resulting in a non-functional enzyme proving fatal (Yang *et al*., 2009). This indicates a role for neurosteroids in normal cognitive development, which, when perturbed, can result in severe cognitive deficits as seen in patients with mental retardation.

Taken together, these studies indicate that modulation of $GABA_A$ receptors by neurosteroids plays a considerable role in maintaining neuronal excitability in the brain, an importance that is highlighted by the large number of disorders associated with perturbations in the activity of these compounds. Clarifying the role of endogenous neurosteroids in these processes may therefore aid in the development of effective therapeutic strategies to treat such conditions.

1.3.9 Therapeutic potential of neurosteroids

Consistent with their enhancement of $GABA_A$ receptor function, administration of neurosteroids to rodents exhibits clear behavioural effects including anxiolysis, sedation, hypnosis, anticonvulsion, and at high concentrations, anaesthesia (Gasior *et al*., 1999;

Rupprecht, 2003), all of which are clinically useful properties. The therapeutic potential of steroids has been recognised since the demonstration by Selye in 1941 that some steroids could rapidly induce anaesthesia. This eventually led to the development of a number of steroid based intravenous anaesthetic agents. Potential therapeutic uses for neurosteroids have also been addressed in a number of more recent studies involving the treatment of conditions associated with dysfunction of GABAergic inhibitory neurotransmission, for example epilepsy.

In mice, pre-treatment with allopregnanolone, THDOC or pregnanolone protected against seizures induced by injection of pilocarpine or kainic acid (Kokate *et al*., 1996). Furthermore, these neurosteroids also protected against progression of seizures to generalised status epilepticus. More recent studies have shown that, in rats, the intra-hippocampal administration of allopregnanolone blocked the audiogenic seizures induced by nicotine (Martin-Garcia and Pallares, 2005) and therefore, taken together, this indicates a potential therapeutic role for neurosteroids in the treatment of seizure disorders. Animal models have also been used to investigate potential therapies for catamenial epilepsy (Reddy and Rogawski, 2009). To investigate this, pseudopregnancy is induced for 12-13 days as a means of physiologically elevating progesterone and allopregnanolone levels in order to mimic those found in humans during the luteal phase of the ovarian cycle. This is followed by finasteride injection to rapidly decrease neurosteroid concentrations, similar to that seen during menstruation in humans, a time when seizure exacerbations appear. After neurosteroid withdrawal, seizure susceptibility can then be tested in order to evaluate the effectiveness of various therapeutic interventions (reviewed in Reddy and Rogawski, 2009). Using this model it has been shown that conventional antiepileptic drugs are less effective at protecting against seizures that occur during the period of enhanced seizure susceptibility induced by neurosteroid withdrawal. By contrast, naturally occurring neurosteroids such as allopregnanolone and THDOC as well as the synthetic neurosteroid, ganaxolone exhibited enhanced sensitivity at this time (Reddy and Rogawski, 2000, 2001). Ganaxolone is useful from a clinical point of view as it undergoes only minimal conversion back to its hormonally active derivative, and therefore, administration of this compound will have less impact upon levels of circulating hormones. Interestingly, administration of ganaxolone to rats undergoing neurosteroid withdrawal resulted in increased protection against seizures induced by pentylenetetrazol (Reddy and Rogawski, 2009) compared to control animals, indicating that neurosteroid replacement therapy is a promising candidate for the treatment of catamenial epilepsy.

In clinical studies into potential treatments for schizophrenia, the neurosteroid, dehydroepiandrosterone was given to schizophrenic patients, resulting in significant dose-related improvements in the negative symptoms as well as depressive and anxiety symptoms that are associated with this condition (Strous *et al*., 2003). Furthermore, neurosteroids have also been tested in relation to age-related cognitive dysfunction, with results showing that intra-hippocampal infusion of pregnenolone sulphate in aged, cognitively impaired rats reverses their memory deficits (Vallee *et al*., 1997). Therefore, this may provide a potential starting point for the development of therapies for the prevention or alleviation of age-related cognitive decline.

Neurosteroids have also been suggested to be promising candidates for the treatment of pain. The anti-nociceptive properties of steroids were demonstrated by studies in which intrathecal injection of steroid anaesthetics resulted in spinally mediated anti-nociception, as tested by the tail flick and electrical current nociceptive tests (Goodchild *et al*., 2000). This effect was abolished by treatment with the $GABA_A$ receptor antagonist, bicuculline, indicating that the anti-nociceptive actions of these compounds were mediated by spinal $GABA_A$ receptors (Goodchild *et al*., 2000). In addition, studies investigating the effects of synthetic steroid alphadalone in rats showed that, whereas intravenous injection of this compound resulted in sedation and anaesthesia, intragastric or intraperitoneal injections caused anti-nociceptive effects without any visible signs of sedation (Nadeson and Goodchild, 2001). Further studies of alphadalone in humans showed that, after surgery, oral administration resulted in a significant reduction of patient-controlled morphine use as well as decreased pain scores, indicating that this steroid may be useful for the treatment of post-operative pain (Goodchild *et al*., 2001).

1.4 Co-modulation of the $GABA_A$ receptor

Neurosteroids and protein kinases are among the most potent modulators of the $GABA_A$ receptor, which, as discussed previously, can enhance or depress receptor function depending on the type of neurosteroid or kinase present and the subunit combination of the receptor (Belelli *et al.*, 2005; Moss *et al.*, 1996). However, *in vivo*, these agents cannot be treated as discrete modulators of the $GABA_A$ receptor as it is likely that they will act in concert to achieve a precise fine-tuning of inhibitory neurotransmission.

The activity of protein kinases has been shown to modulate the actions of a number of other $GABA_A$ receptor modulators, including benzodiazepines and ethanol. Studies in *Xenopus* oocytes expressing α1β2γ2L $GABA_A$ receptors showed that pre-treatment with the PKC

activator, PMA resulted in a significant enhancement in the diazepam-mediated potentiation of GABA-activated currents, indicating that PKC acts to positively modulate the actions of benzodiazepines at $GABA_A$ receptors (Leidenheimer *et al*., 1993). However, in contrast to these findings, $PKC\varepsilon^{-/-}$ mice were shown to exhibit increased behavioural responses to benzodiazepines, suggesting that PKCε acts as to negatively modulate the actions of benzodiazepines (Hodge *et al*., 1999; Qi *et al*., 2007). Interestingly, $PKC\gamma^{-/-}$ mice showed no alterations in their behavioural responses to benzodiazepines (Harris *et al*., 1995), indicating that specific isoforms of PKC may modulate benzodiazepine potentiation at $GABA_A$ receptors differently. Although caution must be exercised when interpreting the results from studies of knock-out animals as compensatory mechanisms may alter the actions of benzodiazepines through pathways unrelated to the abolition of PKCε or PKCγ, the lack of specific inhibitors has meant that knock-out animals are the only method to obtain a strictly specific abolition of PKCε activity. However, a recent study has used a chemical genetic approach to produce a mutant ATP-analog sensitive of PKCε which can be expressed in cell lines and selectively inhibited by 1Na (Qi *et al*., 2007). Using this approach it was shown that, in HEK293 cells expressing α1β2γ2L $GABA_A$ receptors, inhibition of PKCε results in an increase in the potency of the benzodiazepine, flunitrazepam, an effect which was abolished in cells expressing $\alpha1\beta2\gamma2L^{S327A}$ $GABA_A$ receptors. This indicates that phosphorylation of γ2L S327 by PKCε causes a decrease in the potency of benzodiazepines at $GABA_A$ receptors, a result which is consistent with the increase in behavioural effects of benzodiazepines observed in $PKC\varepsilon^{-/-}$ mice (Hodge *et al*., 1999; Qi *et al*., 2007).

The effects of ethanol at $GABA_A$ receptors have proved to be extremely variable, an effect that has been suggested to be due to the activities of protein kinases in the chosen preparation, which have been shown to alter the activity of ethanol at $GABA_A$ receptors. Initial studies by Wafford and Whiting (1992) suggested that phosphorylation at S343 in the γ2L subunit was crucial for ethanol modulation with results showing that, in *Xenopus* oocytes, only GABA currents mediated by α1β1γ2L $GABA_A$ receptors could be potentiated by ethanol, with α1β1γ2S and α1β1 receptors remaining unaffected. However, more recent studies have suggested that the activity of protein kinases acts to modulate $GABA_A$ receptor responses to ethanol. Studies of knock-out mice have shown that deletion of PKCγ abolishes the ethanol-mediated potentiation of GABAergic IPSCs recorded from hippocampal neurons whereas, in $PKC\varepsilon^{-/-}$ mice, IPSCs become sensitive to potentiation by ethanol, an effect which was not observed in wild-type littermates (Proctor *et al*., 2003). The negative modulatory effects of PKCε are consistent with studies by Qi *et al*. (2007), who showed that, in HEK293 cells

expressing α1β2γ2L $GABA_A$ receptors, inhibition of a stably transfected ATP-analog sensitive mutant of PKCε caused a significant increase in ethanol potentiation, an effect which was dependent upon phosphorylation at γ2L S327. A further study has also implicated PKCδ in the modulation of ethanol potentiation, with results showing that $PKC\delta^{-/-}$ mice display reduced intoxication when administered ethanol. Furthermore, this study also reported that, in L(tk^-) cells expressing α4β3δ $GABA_A$ receptors, inhibition of a stably expressed ATP-analog sensitive mutant of PKCδ results in a reduction of ethanol potentiation, indicating that, similar to PKCγ, PKCδ acts to positively modulate the actions of ethanol at $GABA_A$ receptors (Choi *et al*., 2008). Therefore, the opposing effects of different PKC isoforms upon the actions of ethanol at $GABA_A$ receptors indicates that the activity of ethanol can be modulated differently, depending on the expression patterns of different PKC isoforms. In addition, different PKC isoforms may also associate with different subsets of $GABA_A$ receptors within the same neuron, enabling ethanol responses to be spatially regulated.

There is also a growing body of evidence that the activity of protein kinases can modulate the potentiating effects of at least some neurosteroids on both recombinant and native $GABA_A$ receptors (see Chapter 3). However, the results presented to date have been complex and often conflicting. For example, Leidenheimer and Chapell (1997) reported that, in *Xenopus* oocytes expressing α1β2γ2L $GABA_A$ receptors, the magnitude of potentiation elicited by THDOC was enhanced after PKC activation. These results were supported by further studies which showed that inhibition of PKC and/or PKA resulted in a reduction of neurosteroid sensitivity in neurons from both the hippocampus (Harney *et al*., 2003) and hypothalamus (Fáncsik *et al*., 2000). Collectively, these results indicated that the actions of neurosteroids at $GABA_A$ receptors can be positively modulated by the activity of protein kinases. However, additional studies have indicated, to the contrary, that protein kinases act to negatively modulate neurosteroid potentiation, with results from studies of both the lamina II neurons of the spinal cord (Vergnano *et al*., 2007) and the hypothalamic neurons of pregnant rats (Koksma *et al*., 2003) showing that enhancement of protein kinase activity causes a reduction in the neurosteroid sensitivity of $GABA_A$ receptors. Inconsistent results have also been reported regarding the involvement of specific protein kinases in modulating the actions of neurosteroids, for example, in the CA1 pyramidal neurons of the hippocampus, inhibition of PKC or PKA resulted in a concurrent decrease in neurosteroid sensitivity (Harney *et al*., 2003), however, in hypothalamic neurons inhibition of PKA had no such effect (Fáncsik *et al*., 2000).

In addition to protein kinases acting to modulate the activity of neurosteroids at $GABA_A$ receptors, neurosteroids have also been shown to alter the actions of protein kinases at the receptor. In the oxytocin neurons of the hypothalamus, PKC activation results in a reduction of the amplitude of sIPSCs, an effect which can be abolished by pre-treating neurons with allopregnanolone (Brussaard *et al*., 2000). This effect is thought to be important for the timing of oxytocin release during parturition, as when allopregnanolone levels are high during late pregnancy, the combination of increasing sIPSC decay times and preventing PKC-mediated decreases in sIPSC amplitude, ensures that a high level of inhibition is present in these neurons, preventing the premature firing of the oxytocin neurons (Brussaard *et al*., 2000). At parturition, oxytocin neurons become less sensitive to allopregnanolone modulation (Brussaard *et al*., 1997), resulting in the disinhibition of oxytocin neurons, the firing of which releases oxytocin to initiate parturition. The oxytocin then acts to enhance PKC activity which continues to reduce inhibition, allowing further release of oxytocin via a positive feedback mechanism (Brussaard *et al*., 2000).

Given the potency and physiological importance of neurosteroids and protein kinases, it is of utmost importance to determine how they act together to regulate GABAergic inhibition *in vivo*. In addition, due to the lack of specificity for any particular $GABA_A$ receptor subtypes, phosphorylation is thought to constitute one of the main ways in which the neuron and brain region specific actions of neurosteroids are achieved. Therefore, due to the conflicting nature of the results obtained to date, the aim of this thesis was to investigate the relationship between neurosteroids and protein kinases at $GABA_A$ receptors in more detail.

1.5 Thesis aims

- To determine whether protein kinases act to modulate neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors.

- To investigate the involvement of specific protein kinases in the modulation of neurosteroid potentiation.

- To identify the mechanism by which protein kinases act to modulate neurosteroid activity at $GABA_A$ receptors, with a particular focus on determining whether direct phosphorylation is required and if so, the subunits and/or residues which are involved.

- To determine whether, in the reverse situation, neurosteroids can modulate the actions of protein kinases at the $GABA_A$ receptor.

- To investigate whether the actions of neurosteroids at extrasynaptic $GABA_A$ receptors are also modulated by protein kinases and to determine whether phosphorylation at specific loci is important for this effect.

Chapter 2

Materials and Methods

2.1 Molecular Biology

2.1.1 cDNA plasmids

cDNA plasmids encoding murine α1, $\alpha1^{Q241W}$, α4, β3, $\beta3^{S408A}$, $\beta3^{S409A}$, $\beta3^{S408A,S409A}$, γ2L, $\gamma2L^{S327A,S343A}$ and δ $GABA_A$ receptor subunits have all been described previously (Moss *et al*, 1991; Connolly *et al*., 1996; McDonald *et al*., 1998; Hosie *et al*., 2006; Hosie *et al*., 2009). These constructs consist of the cDNA of interest cloned into the plasmid vector, pRK5. Additional mutant constructs used in this project were generated in collaboration with Mr. Mike Lumb using the method outlined below.

2.1.2 Site-directed mutagenesis

In order to generate the mutant cDNA construct encoding murine $\alpha4^{S443A}$, site-directed mutagenesis was carried out using plasmids containing the wild-type α4 gene insert. Appropriate oligonucleotides containing the S443A point mutation were designed and obtained from MWG (see Table 2.1). These primers were then used to amplify the relevant DNA product using the polymerase chain reaction (PCR).

Construct	Primer	Sequence
$\alpha4^{S443A}$	Forward	gccactcgccctgcatttggatctag
	Reverse	agctgaccccaaagaagctggc

Table 2.1 Primers used for PCR reactions

The PCR reaction mixture contained:
- 1µl DNA of interest (10µg/ml stock)
- 0.25µl Forward primer (100pM/µl)
- 0.25µl Reverse primer (100pM/µl)
- 10µl HF buffer
- 1µl dNTP mix
- 0.5µl PHusion enzyme (New England Biolabs)
- 37µl H_2O (to make a total volume of 50µl per reaction)

The PCR was carried out in a PTC-200 DNA engine thermal cycler (MJ Research) using the following protocol: 98°C for 30s, followed by 35 cycles of 98°C for 10s (denaturation), 63°C for 30s (annealing) and 72°C for 5 min (extension) and lastly, a final extension step of 72°C for 5 min.

PCR products were run on 1% agarose gels containing 1µl/100ml ethidium bromide and successful PCR was denoted by the presence of a single band approximately 7kb in size. The band was excised from the gel and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen). This was followed by a ligation step in which 10µl of DNA + 6µl H_2O were incubated at 70°C for 5 min then placed on ice to prevent the DNA re-annealing. 2µl of T4 ligase buffer and 1µl of T4 kinase enzyme (New England Biolabs) were then added and the reaction mixture was incubated at 37°C for 30 min. Finally, 1µl of T4 ligase enzyme (New England Biolabs) was added and the mixture was then incubated at room temperature for 15 min before placing at 4°C overnight.

The ligation product was transformed into chemically competent XL1Blue *E. coli* bacteria by heat shocking at 42°C for 45s. Transformed bacteria were plated on LB agar containing 50µg/ml ampicillin and incubated overnight at 37°C to allow bacterial colonies to grow. Bacterial colonies were then selected and grown overnight in LB medium to allow the bacteria to multiply. The mutant plasmid DNA was purified using the QIAprep Spin Miniprep Kit and fully sequenced (DNA sequencing service, The Wolfson Institute for Biomedical Research, UCL) to ensure that the mutation of interest had been incorporated into the DNA and that no additional, ectopic mutations were present. Once the mutation had been verified, the remaining bacterial stock was amplified in 200ml of LB medium overnight at 37°C and the DNA purified using the PureYield Plasmid Maxiprep System (Sigma). The concentration (OD at 260nm) and quality (OD 260nm/OD 280nm and OD at 320nm) of the resulting DNA was determined using a spectrophotometer (Eppendorf). If acceptable results were obtained, the DNA was used for transfection of HEK293 cells.

2.2 Cell culture

2.2.1 HEK293 Cells

HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% v/v foetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin G, 100µg/ml streptomycin and incubated at 37˚C in 95% air/5% CO_2. Cells were passaged two to

three times per week; once they had reached approximately 70-80% confluency. For passage, cells were washed with 5ml Hank's Balanced Salt Solution (HBSS; Gibco) and subsequently incubated with 2ml 0.05% w/v trypsin-EDTA (Gibco) until they detached from the culture dish. Cells were then suspended in 10ml of culture medium to quench the trypsin, transferred to a 50ml falcon tube and centrifuged for 5 min at 1000rpm. Following removal of the supernatant, the cells were re-suspended in 5ml of culture medium and triturated using a fire polished Pasteur pipette to ensure a single cell suspension. Cells were then re-plated at the appropriate dilution onto new 10cm plates (Nunc) containing 10ml of fresh culture medium.

2.2.2 Hippocampal neurons

The hippocampi of postnatal day 5 (P5) Sprague-Dawley rats were removed and placed into ice cold HBSS ($+Ca^{2+}$, $+Mg^{2+}$; Gibco). The tissue was then transferred into a 35mm dish (Sterilin) containing 5ml trypsin (0.1% w/v; Sigma) and incubated at 37˚C in 95% air/5% CO_2 for 10 min. The digested tissue was then placed into a solution of HBSS supplemented with BSA (1mg/ml) and 5% v/v FCS and triturated using a Pasteur pipette. Tissue was then centrifuged for 30s at 1000rpm, before removing 1.5ml of supernatant into a fresh Falcon tube. A further 1.5ml of supplemented HBSS solution was then added to the remaining tissue and triturated once again with a medium bore fire-polished Pasteur pipette. Centrifugation was repeated as before and once again, 1.5ml of supernatant was removed and placed with that transferred previously. This process was repeated once more, with trituration being carried out with a narrow bore (approximately one third) Pasteur pipette. Once the final 1.5ml of supernatant had been transferred to the Falcon tube, this accumulated cell suspension was then centrifuged for 5 min at 1000rpm. The supernatant was discarded and the cell pellet resuspended in Neurobasal medium supplemented with 5% v/v FCS, 0.5mM glutamine and 2% B27 (using 1ml per hippocampus isolated). A final trituration was then completed using a sterile quill tube, before plating the cells onto poly-D-lysine (100µg/ml made up in borate buffer) coated coverslips (0.5ml cell suspension per coverslip), prewashed with FCS. Coverslips contained within individual 35mm culture dishes (Nunc) were then incubated at 37˚C in 95% air/5% CO_2 for 1 hour after which a further 1ml of Neurobasal medium (supplemented as before) was added. After 3 days in vitro (DIV) cells were treated with an anti-mitotic solution containing 5-fluorodeoxyuridine, uridine and cytosine-arabinofuranoside (final concentration = 100nM of each compound) for 5-6 hours, before washing and adding fresh supplemented Neurobasal medium.

2.3 Transfection

2.3.1 Calcium phosphate precipitation

Cells were transfected with 1µg of cDNA expression plasmid encoding each subunit plus 1µg of plasmid encoding enhanced green fluorescent protein (eGFP). The reaction mixture was as follows (per 35mm dish to be transfected):

4µg cDNA plasmid mixture (3 x subunit plasmids + eGFP)
20µl $CaCl_2$ (340mM, sterile filtered)
24µl 2 x HBSS (280mM NaCl, 2.8mM Na_2HPO_4, 50mM HEPES, pH 7.2, sterile filtered)

The transfection mixture was vortexed and immediately pipetted onto HEK293 cells plated at least 2 hours previously, at 20% confluency, onto poly-L-lysine (100µg/ml) coated coverslips. Cells were then used for electrophysiology 24-48 hours later.

Alternatively, cells for biochemistry experiments were plated in 6cm culture dishes (Nunc) at approximately 50% confluency and transfected with 3µg of each subunit cDNA per plate (9µg total cDNA), with the other components of the reaction mixture being scaled up accordingly.

2.4 Electrophysiology

2.4.1 Whole-cell patch clamp recording

Coverslips with cells attached were transferred into the recording chamber and perfused continuously with Krebs solution containing: 140mM NaCl, 4.7mM KCl, 1.2mM $MgCl_2$, 2.52mM Glucose, 11mM HEPES and 5mM $CaCl_2$ (pH 7.4), pre-filtered through Whatman paper (number 1). Patch electrodes were fabricated from thin-walled borosilicate glass (1.5mm internal diameter; Havard apparatus) and filled with an internal solution containing: 120mM KCl, 1mM $MgCl_2$, 11mM EGTA, 10mM HEPES, 1mM $CaCl_2$ and 4mM ATP (pH 7.11; sterile filtered). The osmolarity of the Krebs and internal patch electrode solutions were confirmed as 300 ± 20 mOsm using a vapour pressure osmometer (Wescor).

An Axopatch 200B amplifier (Axon Instruments) was used to record whole-cell membrane currents from HEK293 cells viewed under a Nikon Eclipse TE300 microscope at 200-400X overall magnification. Transfected cells were identified by the presence of eGFP using the Nikon epifluorescence unit. Recordings were routinely made from single or occasionally

doublets of cells to ensure successful voltage clamp and to avoid errors associated with recording from groups of cells which are electrically-coupled via gap junctions. Cells were voltage clamped at -10mV and the membrane currents recorded were filtered at 3kHz (8 pole Bessel filter), digitized via a Digidata 1322A series (Axon Instruments) and viewed on a PC using Clampex 9.2 software (Axon Instruments).

Once the patch electrode had been placed into the bath solution the resistance could be monitored using the seal test facility in Clampex 9.2. Electrodes used for recording exhibited resistances of 4-5MΩ. At this stage, the residual patch electrode capacitance transients were reduced using the fast capacitance compensation. The electrode was then manoeuvred into the correct position on the surface of the cell using a Sutter MP-285 micromanipulator. After whole-cell patch configuration had been achieved, the seal test facility was used to optimize the series resistance compensation and whole-cell capacitance before recording commenced. The resting membrane potential of the cell was determined as an indication of cell health. Cells selected for recording exhibited resting membrane potentials of between -15mV and -40mV.

Once the whole-cell configuration had been established, a period of at least 2 min was allowed in order for the contents of the patch electrode to dialyse into the cell. Membrane currents in response to brief (3s) applications of GABA or GABA + THDOC were recorded using the gap free recording mode in pClamp 9.2 and stored for further analysis. Between each acquisition a voltage step protocol, consisting of three 100ms, -10mV steps, was used to give a measurement of the series resistance. Series resistance is a measure of the resistance resulting from the electrode and cell membrane in 'series' and changes can lead to errors in voltage clamping, resulting in inaccuracy of the membrane currents recorded. Therefore changes >15% in the currents recorded from the voltage step protocol resulted in cessation of the recording and exclusion of the data from subsequent analysis.

2.4.2 Drug application

Rapid application of drugs was achieved by the use of a modified U-tube system (see Fig. 2.1) positioned approximately 500µm from the cell. Under normal conditions (no drug applied), solenoid A was open allowing cells to be continuously perfused with Krebs solution via the auxiliary tube. During this time the U-tube was perfused with water (to preserve drug solutions) and, whilst solenoid B was open, the flow was powered by a vacuum pump, preventing water from exiting through the hole at the apex of the U-tube. Immediately before drug application, the water was replaced by the appropriate drug diluted in Krebs solution. To

apply the drug, firstly solenoid A was closed to stop the continuous flow of Krebs solution. Secondly, solenoid B was closed to isolate the vacuum, resulting in the drug solution exiting the hole in the U-tube and flowing rapidly over the cell undergoing recording. After a pre-determined period of time (usually 3s), both solenoids were re-opened to reverse the flow of drug and to re-instigate the flow of Krebs from the auxiliary tube, resulting in a rapid 'wash-off' of the drug from the cell. Using the U-tube system, the onset of the drug response was approximately 100-150ms.

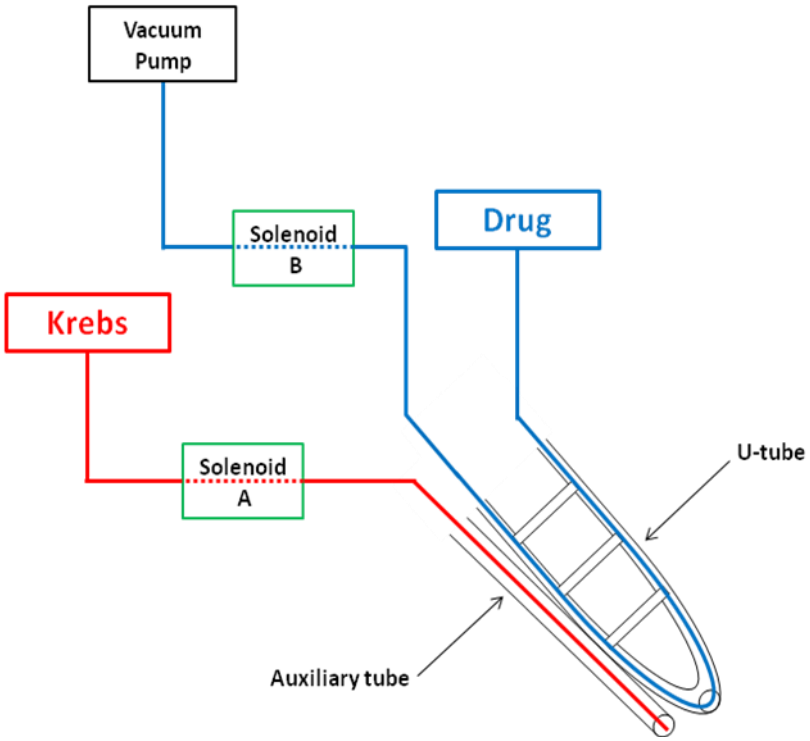

Figure 2.1 Schematic diagram of the modified U-tube system
The U-tube system was used to rapidly apply drugs to the recorded cell. The flow of Krebs solution via the auxiliary tube and the application of drug solution via the U-tube were controlled by solenoids A and B, respectively, which were in turn controlled electronically using a control panel interface.

2.4.3 Protein kinase inhibition/activation experiments

To monitor the effects of protein kinase inhibition/activation on neurosteroid potentiation the cell was first exposed to pulses of EC_{20} GABA until stability was reached with regard to response amplitude, then to two further brief applications of EC_{20} GABA. The EC_{20} GABA concentration was pre-determined by constructing a GABA concentration-response curve in HEK293 cells expressing the same $GABA_A$ receptor subunits as those utilised in the main experiment. New curves were constructed regularly to adjust for any drifts in EC_{20} which may

occur, and were always repeated when a new batch of HEK293 cells were used. This allowed the variation in potentiation due to changes in agonist EC_{20} to be minimized. EC_{20} concentrations were 1-3μM for α1β3γ2L and 30-100nM for α4β3δ $GABA_A$ receptors.

The two applications of EC_{20} GABA were separated by a 2 min interval, allowing the baseline response of the cell to be determined and to ensure the baseline response of the cell was stable. The neurosteroid-mediated potentiation was then measured by co-applying EC_{20} GABA with 50nM THDOC (Sigma Aldrich) in 2 successive applications, again separated by a 2 min interval. EC_{20} GABA alone was then re-applied to ensure the cell's response had returned to baseline. This usually occurred within 4 min (see Fig. 3.1) and recording was ceased and the cell discarded from analysis if the responses did not return to baseline levels within this time. The cell was then perfused with the appropriate protein kinase inhibitor/activator (see Table 2.2), diluted in Krebs solution, applied continuously via the auxiliary tube. The exceptions to this were PKG inhibitor, KT5823 and PKA activator, cAMP, as these drugs were not suitable for external perfusion and so were instead diluted to the appropriate concentration within the patch electrode internal solution and applied intracellularly.

Drug	Kinase Targeted	Action	Source	Stock Concentration/Solvent	Final Concentration
Staurosporine	Broad spectrum	Inhibitor	Calbiochem	1mM in DMSO	200nM
Bisindolylmaleimide I	PKC	Inhibitor	Calbiochem	1mM in DMSO	500nM
Myristoylated PKA inhibitor peptide 14-22 amide	PKA	Inhibitor	Biomol	500μM in H_2O	500nM
KT5823	PKG	Inhibitor	Calbiochem	3mM in DMSO	3μM
Phorbol-12-myristate-13-acetate (PMA)	PKC	Activator	Calbiochem	1mM in DMSO	100nM
cAMP	PKA	Activator	Sigma	10mM in H_2O	300μM

Table 2.2 Protein kinase inhibitors and activators

During the treatment period, the cell's response to EC_{20} GABA alone was monitored every 2 min in order to assess the effects of the inhibitor/activator on the receptor's responses to GABA alone. Once the cell had been treated for the designated amount of time (see Chapters 3-5), the neurosteroid-mediated potentiation was re-measured with 2 further co-applications of EC_{20} GABA + 50nM THDOC, followed by 2 applications of EC_{20} GABA alone to, once again, ensure that the cell's response returned to baseline.

2.4.4 Data analysis

The recorded whole-cell GABA currents were analysed in Clampex 9.2. Peak responses were measured by subtracting the baseline holding current from the peak amplitude of the response. For protein kinase inhibitor/activator experiments, current responses were normalized to the first response elicited by EC_{20} GABA, recorded at t = 2 min.

The potentiation elicited by neurosteroid (see Chapters 3-5) was calculated by dividing the first potentiated response (induced by co-application of EC_{20} GABA + 50nM THDOC) by the cell's preceding response to EC_{20} GABA alone and transformed to a percentage. In doing this, any changes in the receptor's response to agonist alone, caused by the protein kinase inhibitor/activator treatment, could be separated from the effects of the treatment on the neurosteroid-mediated potentiation.

All dose-response curves were constructed by plotting mean peak response amplitude against GABA concentration and fitting this data with the Hill equation:

$$I = 1/(1 + (EC_{50}/[GABA])^{n})$$

Where I = GABA-activated current, EC_{50} = concentration of GABA inducing a current response 50% of the maximal GABA-activated current and n = Hill coefficient.

All graphical representations of the data shown were plotted using Origin version 6.0 (Microcal). Statistical analyses were carried out using GraphPad Instat version 3 employing either a student's t-test (for comparing two values) or an ANOVA (for three or more values) followed by an appropriate post hoc test to compare selected data sets. Data from the same cell was treated as 'paired' whereas data obtained from different cell populations was considered 'unpaired'.

2.5 Biochemistry

2.5.1 Preparation of cell lysates

Post-transfection (24-48 hrs), HEK293 cells were treated with 50nM THDOC or 100nM PMA for the appropriate length of time (see Chapter 4). Before treatment, the culture medium was replaced with Krebs solution (see Electrophysiology 2.4) pre-warmed to 37°C. Once the treatment period was complete, the dishes of cells were placed on ice. Cells were washed

twice with ice-cold phosphate buffer solution (PBS) containing 20nM Calyculin A (Calbiochem). Cells were then lysed using RIPA lysis buffer containing: 150mM NaCl, 50mM Tris pH 8.0, 5mM EDTA, 1% v/v NP-40, 0.5% w/v deoxycholate, 0.1% SDS, supplemented with protease inhibitors (Phenylmethyl sulfonyl fluoride and Benzamidine) and phosphatase inhibitors (20mM NaF, 10mM Sodium pyrophosphate and 20nM Calyculin A). The lysates were transferred to individual Eppendorf tubes and sonicated to ensure complete lysis. Lysates were then centrifuged (14000rpm, 5 min) at 4°C to sediment the cell debris and the supernatants transferred to fresh Eppendorf tubes and stored on ice. The concentration of protein present in the sample was measured using the BCA Protein Assay (Thermo Scientific).

2.5.2 Polyacrylamide gel electrophoresis

Samples (20µg protein) were boiled in SDS-PAGE buffer and loaded onto 10% polyacrylamide gels made up in Tris-HCl with 10% SDS and polymerized using TEMED and 10% APS (Sigma Aldrich). A molecular weight marker (GE Healthcare) was run alongside the samples in order to estimate the size of the proteins present. Gels were run at 300mA for 50 min. The gels were then transferred onto Hybond nitrocellulose transfer membrane (Amersham) using a semi-dry blotter (Camlab) at 0.8mA/cm^2 of transfer membrane for 110 min.

2.5.3 Western blotting

Following protein transfer, membranes were blocked in either 5% milk or 0.2% BSA made up in TBST (Tris buffered saline + 0.1% v/v Tween) for 60 min. Membranes were then incubated with primary antibody (diluted in appropriate blocking solution; see Table 2.3) overnight at 4°C on an orbital shaker. On the following day the membranes were washed for 3 x 10 min in TBST and then incubated with secondary, HRP-conjugated antibody (diluted in TBST; see Table 2.3) for 60 min at room temperature on an orbital shaker. Membranes were then washed for 3 x 10 min and imaged using the ECL detection system (GE Healthcare).

Primary Antibody	**Epitope Location**	**Species**	**Source**	**Dilution Used**
Anti-β3	Intracellular loop (TM3-TM4)	Rabbit	Millipore	1:1000 in 5% milk
Anti-Phosphoβ3	Phosphorylated S408/S409	Rabbit	Gift from Dr. J Jovanavic	1:1000 in 0.2% BSA
Secondary Antibody	**Target**	**Species**	**Source**	**Dilution Used**
Anti-rabbit HRP	Rabbit	Donkey	GE Healthcare	1:2500 in TBST

Table 2.3 Antibodies used for Western blotting

2.5.4 Data analysis

Protein levels were quantified using a GS-800 densitometer. To determine the amount of phosphorylated β3 subunit, each reading was normalized to that from the corresponding band in the total β3 Western blot. For comparison purposes these values were then normalized to the levels of basal phosphorylation (in untreated cells) to determine whether any changes had taken place after treatment.

2.6 Immunocytochemistry

2.6.1 Immunolabelling of cultured hippocampal neurons

After at least 7 DIV, hippocampal neurons were treated with either 10μM allopregnanolone or 100nM diazepam (both obtained from Sigma Aldrich) and incubated at 37°C in 95% air/5% CO_2 for 1 hour along with the untreated control cells. After treatment, cells were washed twice in ice cold PBS and fixed in 4% v/v paraformaldehyde (PFA) for 15 min at room temperature. The fixative was then washed twice in PBS and quenched with 50mM NH_4Cl for 10 min. Following two further washes in PBS, cells were then washed once with PBS supplemented with 10% v/v FCS and 0.4% w/v BSA. Cells were then incubated with primary antibody (see Table 2.4), diluted in the same supplemented PBS solution, for 45 min at room temperature. Following three washes with PBS (+ 10% v/v FCS, + 0.4% w/v BSA), cells were incubated with secondary antibody (see Table 2.4), diluted in the same buffer, for 45 min at room temperature, during which cells were protected from light using a foil lid. Finally, cells were washed three times in PBS and fixed to glass slides using 40μl pre-melted glycerol and stored at 4°C for imaging the following day.

Primary Antibody	**Epitope Location**	**Species**	**Source**	**Dilution Used**
Anti-α1	Extracellular N-terminus	Guinea Pig	Gift from Fritschy	1:200
Secondary Antibody	**Target**	**Species**	**Source**	**Dilution Used**
FITC conjugated anti-guinea pig	Guinea Pig	Donkey	Jackson	1:100

Table 2.4 Antibodies used for immunocytochemistry

2.6.2 Image acquisition

Images were acquired using a Zeiss laser-scanning confocal microscope (LSM 510 Meta), with a 40x oil immersion objective. Light with a wavelength of 543nm was used to visualize

fluorescein isothiocyanate (FITC) fluorescence and images were captured and stored using a computer interface running Zeiss LSM confocal software (version 3.0).

2.6.3 Image analysis

Images were analysed using Igor Pro (version 5.02) with a Batch Image Processor plugin specifically designed for analysing fluorescent puncta (Bergsman *et al*., 2006). The number of puncta present along the entire length of dendrite was quantified for each neuron analysed. The length of the dendrites were then determined using the measuring facility in the LSM Image Browser (version 3.0), allowing the mean puntca density per μm of dendrite to be calculated for each neuron.

Chapter 3

Neurosteroid modulation at $GABA_A$ receptors: regulation by protein kinases

3.1 Introduction

$GABA_A$ receptors are subject to modulation by a number of endogenous compounds, which, by acting to alter receptor function, have a significant impact on neural activity. Neurosteroids and protein kinases are among the most potent modulators of the $GABA_A$ receptor, which, when acting individually, can enhance or depress receptor function depending on the type of neurosteroid or kinase present and the subunit combination of the receptor (Belelli and Lambert, 2005; Moss and Smart, 1996). However, *in vivo*, these agents cannot be treated as discrete modulators of the $GABA_A$ receptor as it is more likely that they will act in concert to achieve a precise fine-tuning of inhibitory neurotransmission.

Some studies have indicated that the activity of protein kinases can modulate the potentiating effects of at least some neurosteroids on both recombinant and native $GABA_A$ receptors. Leidenheimer and Chapell (1997) reported that for *Xenopus* oocytes expressing recombinant α1β2γ2L $GABA_A$ receptors, the potentiation of $GABA_A$ receptor mediated currents by the naturally-occurring neurosteroid, tetrahydrodeoxycorticosterone (THDOC) was enhanced by the activation of PKC, indicating that receptor phosphorylation may alter the modulatory effects of neurosteroids. These results were supported by further studies which showed that inhibition of PKC and/or PKA resulted in a reduction of neurosteroid sensitivity in neurons from both the hippocampus (Harney *et al*., 2003) and hypothalamus (Fáncsik *et al*., 2000). However, whilst many of the studies conducted to date agree that the activity of protein kinases has at least some modulatory effect on the activity of neurosteroids at $GABA_A$ receptors, the results presented are complex and often conflicting. For example, the aforementioned studies collectively showed that the magnitude of neurosteroid potentiation is positively regulated by the activity of protein kinases. In contrast, additional studies in both the lamina II neurons of the spinal cord (Vergnano *et al*., 2007) and the hypothalamic neurons of pregnant rats (Koksma *et al*., 2003) indicated that an inverse relationship exists between neurosteroids and protein kinases, with results showing that enhancement of protein kinase activity causes a reduction in the neurosteroid sensitivity of $GABA_A$ receptors. Inconsistent results have also been reported regarding the involvement of specific protein kinases in modulating the actions

of neurosteroids. Harney *et al.* (2003) reported that, in the CA1 pyramidal neurons of the hippocampus, inhibition of PKC or PKA resulted in a concurrent decrease in neurosteroid sensitivity. However, studies of hypothalamic neurons have shown that, although inhibition of PKC exhibited similar effects to those seen in hippocampal neurons, inhibition of PKA did not affect neurosteroid activity (Fáncsik *et al*., 2000).

The reason for these discrepancies is currently unclear, but one factor which may affect the relationship between neurosteroids and protein kinases is the subunit combination of the $GABA_A$ receptor. Although the neurosteroids appear to display only modest changes in potency across most $GABA_A$ receptor subtypes tested (Belelli *et al*., 2002; Herd *et al*., 2007), phosphorylation, by protein kinases, has been shown to differentially alter $GABA_A$ receptor function, depending on the subunits present within the receptor complex (see Chapter 1; Moss and Smart, 1996). Therefore, when acting together, it could be hypothesized that protein kinases may modulate the activities of neurosteroids at the $GABA_A$ receptor in a manner that is dependent upon the receptor isoform. This may explain the variation in prior results, as many of the studies carried out to date have utilized different neuronal populations, which are likely to express a mixture of different subsets of $GABA_A$ receptor subunits. Therefore, in order to reduce the complexity associated with neurons and ultimately construct a clearer picture of how protein kinases modulate the activity of neurosteroids, the experiments in this investigation were carried out using HEK293 cells expressing specific recombinant $GABA_A$ receptors.

The HEK293 cell line is epithelial in origin but has been extensively used as a tool to study the pharmacology and physiology of heterologously-expressed $GABA_A$ receptors. These cells can be readily transfected with cDNAs encoding for recombinant $GABA_A$ receptors, allowing their pharmacological profiles to be studied using whole-cell patch clamp electrophysiology (Thomas and Smart, 2005). Due to their non-neuronal lineage, HEK293 cells are not thought to express endogenous $GABA_A$ receptors (Thomas and Smart, 2005) making it possible to control the subunit composition of the receptors expressed in these cells, thus allowing functional analyses of the roles of different receptor subunit isoforms. To date, only one study has examined the relationship between neurosteroids and protein kinases at $GABA_A$ receptors containing defined subunits, with Leidenheimer and Chapell (1997) investigating the modulation of neurosteroid activity using α1β2γ2L $GABA_A$ receptors expressed in *Xenopus* oocytes. Interestingly, it has been shown that the effects of phosphorylation at β2 subunit-containing $GABA_A$ receptors differ from those observed at receptors containing other β subunit

isoforms (see Chapter 1; Moss and Smart, 1996). Therefore, the initial aims of these experiments were to establish whether neurosteroid-mediated potentiation of α1β3γ2L subunit-containing $GABA_A$ receptors could also be modulated by protein kinases and if so, which protein kinase(s) were responsible for this modulation. It was anticipated that these results could then be compared to those obtained previously from the α1β2γ2L $GABA_A$ receptors, potentially exposing any differences between the co-modulatory effects of neurosteroids and protein kinases at these different $GABA_A$ receptor subtypes.

3.2 Results

In order to examine the effects of phosphorylation, by protein kinases, on the potentiation of $GABA_A$ receptor function by neurosteroids, HEK293 cells were transfected to express α1β3γ2L subunit-containing $GABA_A$ receptors. Cells were transfected using calcium phosphate precipitation (see Methods and Materials) with expression plasmids encoding each receptor subunit in a 1:1:1 ratio and co-transfected with plasmid DNA for the enhanced green fluorescent protein (eGFP), to enable the identification of transfected cells. Co-expression of α, β and γ subunits has been shown to result in the formation of functional $GABA_A$ receptors at the cell surface (Sigel *et al.*, 1990; Verdoorn *et al*., 1990; Angelotti *et al*., 1993). It is also known that α and β subunits alone can form functional receptors (Levitan *et al*., 1988; Sigel *et al*., 1990; Verdoorn *et al*., 1990; Angelotti *et al*., 1993), however it is thought that the majority of receptors expressed here will contain α and β subunits in combination with γ as this investigation, along with previous studies, have shown that when equal proportions of α, β and γ subunits are transfected, αβγ heteromers are preferentially formed, demonstrated by their robust sensitivity to the benzodiazepine, diazepam (Angelotti *et al*., 1993), a modulation which requires the presence of a γ subunit within the receptor complex (Pritchett *et al*., 1989; Sigel *et al*., 1990; Sigel and Buhr, 1997). In contrast, other studies have reported that a 1:1:10 ratio of α:β:γ cDNAs is optimal for the formation of αβγ heteromers (Boileau *et al*., 2002), however, this was not necessary in the present study.

Whole-cell patch clamp electrophysiology was used to assess the magnitude of neurosteroid-mediated potentiation before and after cell treatment with compounds to inhibit intracellular protein kinase activity. Peak currents were recorded in response to brief (3s) applications of either EC_{20} (the concentration eliciting 20% of the maximal GABA response) GABA alone (baseline control responses) or EC_{20} GABA co-applied with 50nM of the neurosteroid, tetrahydrodeoxycorticosterone (potentiated GABA responses). Tetrahydrodeoxycorticosterone (THDOC) is synthesised within neurons and glial cells in the CNS (Patte-Mensah *et al*., 2003;

Tsutsui, 2003; Agís-Balboa *et al*., 2006; Saalmann *et al*., 2007) and is one of the most potent modulators of the $GABA_A$ receptor *in vivo* (Lambert *et al*., 2001). THDOC can potentiate $GABA_A$ receptor responses at low nanomolar concentrations (Morrow *et al*., 1987; Paul and Purdy, 1992). Levels of neurosteroids present within the brain are thought to fluctuate between 3 and 10nM but can rise in excess of 50nM during acute stress and may be increased to around 100nM during pregnancy (Purdy *et al*., 1991; Paul and Purdy, 1992; Cheney *et al*., 1995; Weill-Engerer *et al*., 2002). Therefore, 50nM THDOC was chosen as it is within the concentration range estimated *in vivo* and so its effects at the $GABA_A$ receptor are physiologically relevant. At this concentration neurosteroids act to potentiate $GABA_A$ receptor currents, with concentrations much larger than this, in the region of 1μM and above, required to induce direct activation of the receptor in the absence of agonist. Therefore, this investigation was solely focussed on the more physiological potentiating actions of THDOC.

3.2.1 THDOC-mediated potentiation at α1β3γ2L $GABA_A$ receptors is decreased after protein kinase inhibition

To investigate whether neurosteroid-mediated potentiation could be modulated by protein kinases in the HEK293 heterologous expression system, cells were treated with staurosporine, a broad-spectrum protein kinase inhibitor. Staurosporine acts to inhibit protein kinase activity by binding to the ATP-binding site (Meggio *et al*., 1995; Lawrie *et al*., 1997); the binding of ATP is crucial for the activity of protein kinases and due to the ATP-binding site being relatively conserved, staurosporine can inhibit a number of protein kinases (Meggio *et al*., 1995; Karaman *et al*., 2008). A major advantage of using staurosporine is that it is cell permeable, which allows the potentiation induced by THDOC to be measured before and after treatment within the same cell, eliminating the variation in results which could arise when comparing separate treated and untreated populations of cells. For each cell, the baseline response to EC_{20} GABA alone and potentiated response after co-application of EC_{20} GABA and 50nM THDOC were established under basal cell conditions (i.e. before protein kinase inhibitor treatment; Fig. 3.1A). The cell was then treated with 200nM staurosporine, applied continuously via the bath perfusion. Staurosporine has been shown to inhibit protein kinase activity at nanomolar concentrations, with IC_{50} values ranging from 3nM for PKC to 8nM for PKA (Rüegg and Burgess, 1989; Meggio *et al*., 1995) and therefore, 200nM was used to ensure full inhibition of protein kinase activity. All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration (designated as t = 0).

During the treatment period the cell's response to EC_{20} GABA was monitored every 2 min in order to examine the effect of the staurosporine treatment on the receptor's response to GABA alone. The effects of phosphorylation at the $GABA_A$ receptor are well documented with a number of studies showing that phosphorylation can result in both enhancement and depression of the GABA-activated current, depending on the protein kinase present and the subunit combination of the receptor (Moss and Smart, 1996). However, in the current study, inhibition of protein kinase activity by staurosporine did not significantly alter the receptor's response to EC_{20} GABA; both untreated and staurosporine treated cells showed no change in the mean peak GABA-activated current recorded at 32 min compared to that recorded at 2 min (change = 5.1 ± 8.8% and 4.1 ± 9.1%, respectively, mean ± s.e.m., $P > 0.05$; n = 5-6; Fig. 3.1B; at t = 0 min, GABA-activated response = 100%). Previous studies of β3 subunit-containing $GABA_A$ receptors have revealed that phosphorylation by PKA and PKC have opposing effects on receptor function; PKA-mediated phosphorylation has been shown to enhance GABA-activated current (McDonald *et al*., 1998) whereas phosphorylation by PKC results in a reduction in the magnitude of receptor responses to agonist (Brandon *et al*., 2000). It is thought that both PKC and PKA are present and active at $GABA_A$ receptors expressed in HEK293 cells, therefore, assuming that the receptors are basally-phosphorylated by both protein kinases under these conditions, it is possible that inhibition of both PKA and PKC simultaneously by staurosporine may mask the effects of each individual protein kinase, resulting in no overall change in the EC_{20} GABA-activated current, as seen in this study.

After 14 min of staurosporine treatment the baseline and potentiated responses were re-measured. To allow a direct comparison of the potentiation before and after staurosporine treatment, each potentiated response was normalised to the cell's preceding response to EC_{20} GABA alone. Control experiments carried out in cells which remained untreated for the duration of the recording showed a negligible change in the potentiation elicited by 50nM THDOC (change in potentiation = 2.5 ± 7.9%; n = 6). In contrast, cells treated with staurosporine exhibited a significant decrease in THDOC-mediated potentiation of 54.8 ± 9.1% ($P < 0.05$, paired t-test; n = 5; Fig. 3.1A). However, the potentiation was not completely abolished with 50nM THDOC still enhancing the EC_{20} GABA-activated response by 45.5 ± 12.7% after staurosporine treatment (Fig. 3.1A). This indicates that THDOC-mediated potentiation of recombinant α1β3γ2L $GABA_A$ receptors is modulated by, but does not require activated protein kinases.

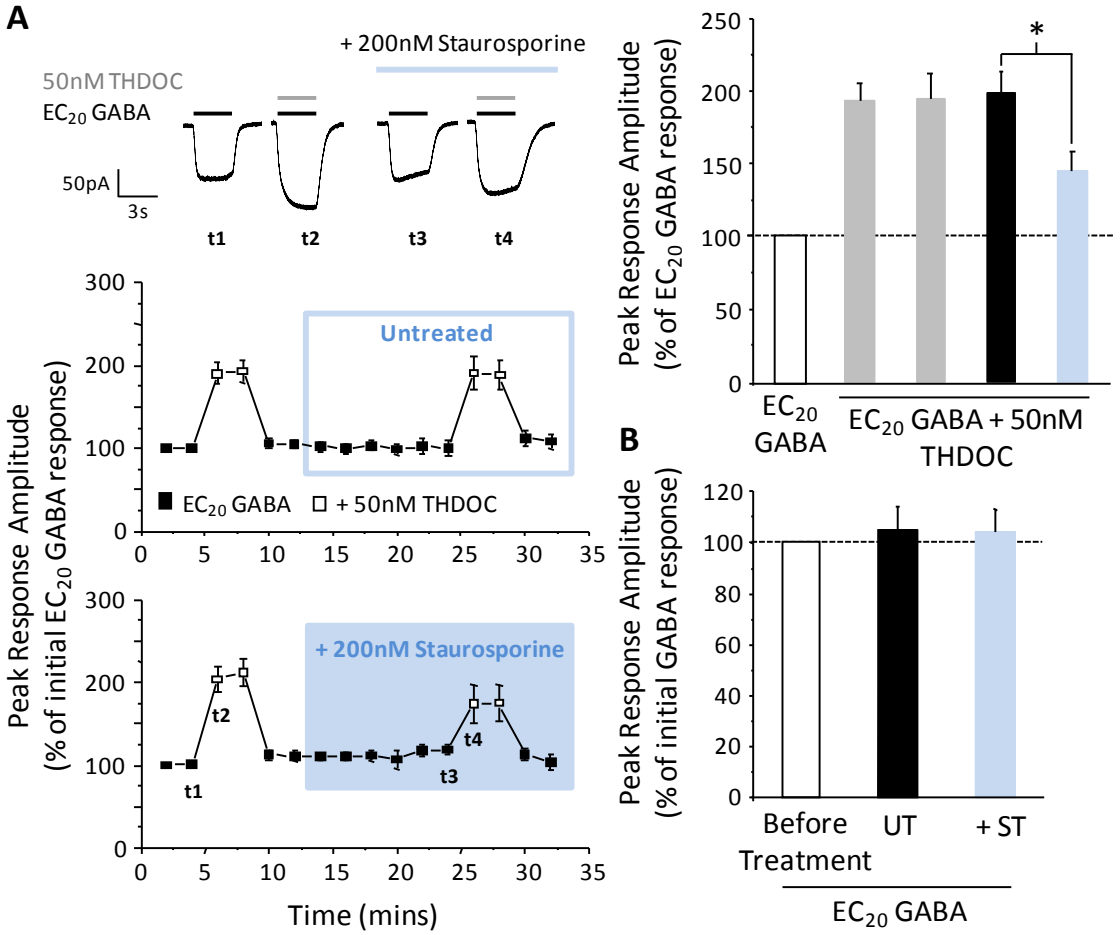

Figure 3.1 Staurosporine decreases THDOC-mediated potentiation at α1β3γ2L $GABA_A$ receptors

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording (upper panel) or were treated with 200nM staurosporine (lower panel; as indicated by light blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0 (= 100%). Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 26 min, respectively; n = 6) or in treated cells before (black bar) and after (light blue bar) 200nM staurosporine treatment (n = 6). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells just treated with 200nM staurosporine (light blue bar). Change was measured between 2 min (before treatment) and 32 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

To ensure that the application of staurosporine did not cause a decrease in THDOC-mediated potentiation due to an alteration of the GABA concentration-response relationship, dose-response curves were constructed before and after staurosporine treatment (Fig. 3.2). Lateral displacement of the GABA dose-response curve would result in a concurrent change in the potentiation of receptor currents by THDOC if the GABA EC_{20} was not adjusted and therefore

could explain the decreased potentiation seen after staurosporine treatment. However, this was not the case, with GABA EC_{50} values not differing significantly before and after staurosporine treatment (2.97 ± 1.15μM and 2.70 ± 0.74μM, respectively; $P > 0.05$; n = 5; Fig. 3.2). This suggests that the decrease in THDOC-mediated potentiation seen after staurosporine treatment was not due to a change in the concentration-response relationship for GABA.

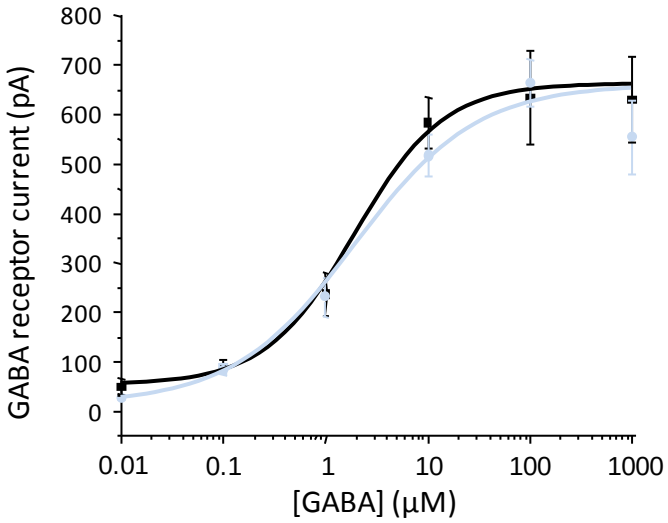

Figure 3.2 Effect of staurosporine on the GABA dose-response relationship for α1β3γ2L $GABA_A$ receptors
GABA dose-response curves were established in HEK293 cells expressing α1β3γ2L $GABA_A$ receptors before (black) and after (light blue) staurosporine treatment (n = 5). Each cell was exposed to brief applications of GABA, at varying concentrations (0.01-1000μM), before being treated with 200nM staurosporine for 14 min. After the treatment period the responses to the same concentrations of GABA, in each cell, were re-measured. All data points represent mean ± s.e.m.

The $GABA_A$ receptor can be phosphorylated by a number of protein kinases, including serine-threonine kinases: PKA, PKC, PKG and CaMKII as well as by protein tyrosine kinases (PTK) such as SRC and PYK (Moss and Smart, 1996; Brandon *et al.*, 2002a). As a broad-spectrum inhibitor, staurosporine cannot distinguish between the effects of individual protein kinases. Therefore, in order to identify the roles of specific protein kinases in modulating potentiation of the $GABA_A$ receptor by neurosteroids, this experiment was repeated using specific protein kinase inhibitors.

3.2.2 THDOC-mediated potentiation is modulated by PKC

Previous studies examining the modulation of neurosteroid-mediated potentiation by protein kinases have yielded complex and often conflicting results. Therefore, this series of

experiments aimed to elucidate whether the activity of a specific protein kinase(s) was responsible for modulating the potentiation of α1β3γ2L $GABA_A$ receptors by neurosteroids. Many of the studies conducted to date have indicated a role for PKC in the modulation of neurosteroid-mediated potentiation (Herd *et al*., 2007) and therefore PKC was the initial focus of this investigation. PKC activity was inhibited by treating cells with 500nM bisindolylmaleimide I (Bis I; Fig. 3.3). Bis I is a potent and selective inhibitor of PKC but with limited isoform specificity. IC_{50} values of 10-20nM for PKCα, βI, βII and γ and 100-200nM for δ and ε have been reported using *in vitro* kinase assays (Toullec *et al*., 1991; Martiny-Baron *et al*., 1993) and therefore, it would be expected that all of these isoforms would be inhibited by the concentration of Bis I used in this experiment. Similar to staurosporine, Bis I is cell-permeable and so was applied via the bath perfusion.

Previous studies have shown that inhibition of PKC causes an enhancement of α1β3γ2L $GABA_A$ receptor currents (Brandon *et al*., 2000). However, this was not the case in these experiments, with receptor responses elicited by EC_{20} GABA not changing significantly after Bis I treatment (percentage change in GABA-activated current measured between 2 and 58 min = 0.2 ± 9.4%; n = 5; Fig. 3.3B). A trend towards a modest increase in the GABA-activated current was evident after around 10 min exposure to 500nM Bis I (19.6 ± 15.3%), however this enhancement was neither significant nor persistent, with the magnitude of the response reverting back to baseline levels by 58 min.

As in the previous experiment, baseline and potentiated responses were measured before and after Bis I treatment and the magnitude of potentiation induced by 50nM THDOC was compared. Untreated cells showed little change in the potentiation elicited by 50nM THDOC (decrease in potentiation = 1.6 ± 8.3%; n = 5; Fig. 3.3A). By contrast, cells treated with 500nM Bis I for 40 min exhibited a significant decrease in THDOC-mediated potentiation (43.5 ± 3.8%, $P < 0.05$, paired t-test; n = 5; Fig. 3.3A), indicating that potentiation of α1β3γ2L $GABA_A$ receptors by THDOC can be modulated by PKC. As before with staurosporine, the potentiation was not completely abolished after Bis I treatment (Fig. 3.3A), providing further support for the idea that protein kinases are not required for the induction of potentiation by neurosteroids.

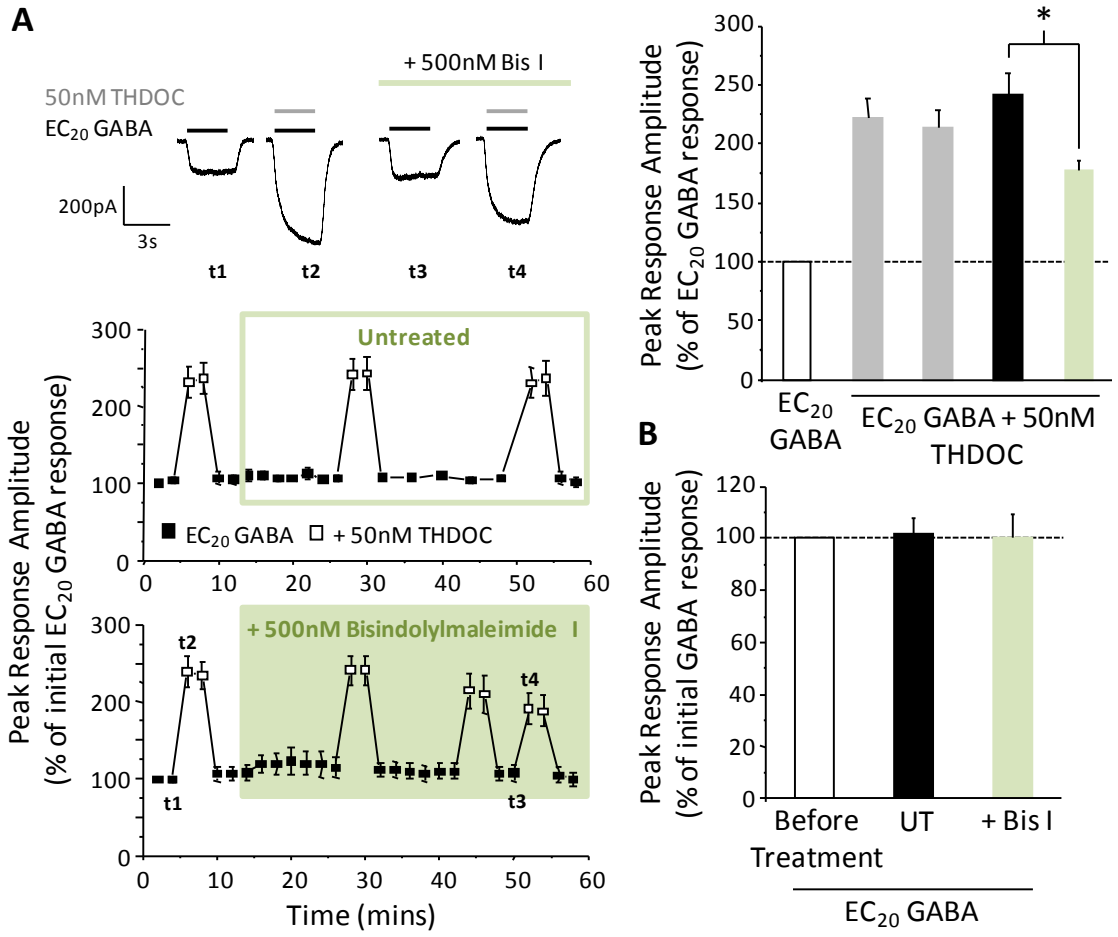

Figure 3.3 Bisindolylmaleimide I decreases THDOC-mediated potentiation at α1β3γ2L $GABA_A$ receptors

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording or were treated with 500nM bisindolylmaleimide I (Bis I, as indicated by green shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after Bis I treatment (green bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 52 min, respectively; n = 6) or in treated cells before (black bar) and after (green bar) 500nM Bis I treatment (n = 5). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells treated with 500nM Bis I (green bar). Change was measured between 2 min (before treatment) and 58 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

3.2.3 Inhibition of PKA or PKG does not affect THDOC-mediated potentiation

The involvement of PKA in modulating the actions of neurosteroids remains confusing, with some studies reporting a role for PKA in modulating the sensitivity of $GABA_A$ receptors to applied neurosteroids (Harney *et al.*, 2003), while others find no such effect (Fáncsik *et al.*, 2000). Therefore, in order to determine whether PKA plays a role in modulating neurosteroid

potentiation at α1β3γ2L $GABA_A$ receptors, PKA was inhibited using a cell-permeable peptide inhibitor (myristoylated PKA inhibitor peptide 14-22 amide; PKAI; Fig. 3.4). There is no evidence to date that indicates that PKG is involved in modulating neurosteroid activity at $GABA_A$ receptors, however, as PKG is able to phosphorylate the β subunit of the $GABA_A$ receptor (McDonald and Moss, 1994; McDonald and Moss, 1997) and is inhibited by staurosporine (Meggio *et al.,* 1995), a specific inhibitor of PKG, KT5823, was also used to investigate the potential involvement of PKG (Fig. 3.5). PKAI is cell-permeable and was applied via the bath perfusion whereas KT5823 is cell-impermeable and so was applied intracellularly via the patch electrode.

Inhibition of PKA caused a modest, but significant decrease in the response elicited by EC_{20} GABA alone, with cells exhibiting a 21.6 ± 8.7% reduction in GABA-activated current after treatment with 500nM PKAI for 14 min ($P < 0.05$, paired t-test; $n = 5$; Fig. 3.4B). This suggests that, in the HEK293 cell, there may be some basal phosphorylation of the α1β3γ2L $GABA_A$ receptor by PKA. In contrast, cells treated with 3μM KT5823 showed no change in the EC_{20} GABA-activated current (Fig. 3.5B), suggesting that PKG does not phosphorylate this receptor isoform under basal conditions in HEK293 cells.

Comparison of the magnitude of potentiation elicited by 50nM THDOC before and after each treatment revealed no significant change in potentiation after 14 min exposure to either 500nM PKAI or 3μM KT5823. In both cases the change in neurosteroid potentiation was 4.1 ± 6.6% ($n = 5$; Fig. 3.4A) and 1.3 ± 4.8% ($n = 6$; Fig. 3.5A) in PKAI and KT5823 treated cells, respectively ($P > 0.05$).

As KT5823 was applied intracellularly via the patch electrode, there was a small possibility that inhibition of PKG could be partially or fully complete before the recording commenced. Therefore, to ensure that the first 'potentiated' response was recorded before the onset of PKG inhibition, the diffusion rate of KT5823 into the cell was estimated. Using a mathematical model (as outlined by Thomas and Smart, 2005) it was estimated that the concentration of KT5823 within the cell would reach equilibrium at around 6 min. The first 'potentiated' response was recorded 6 min after achieving whole-cell recording configuration and therefore, taking into consideration the time lag which is likely to occur between the KT5823 diffusing into the cell and it binding to and inactivating intracellular PKG, this is likely to be before a significant amount of PKG inhibition had occurred. Therefore, the results indicate that neither

PKA nor PKG are involved in modulating THDOC-mediated potentiation of α1β3γ2L $GABA_A$ receptors.

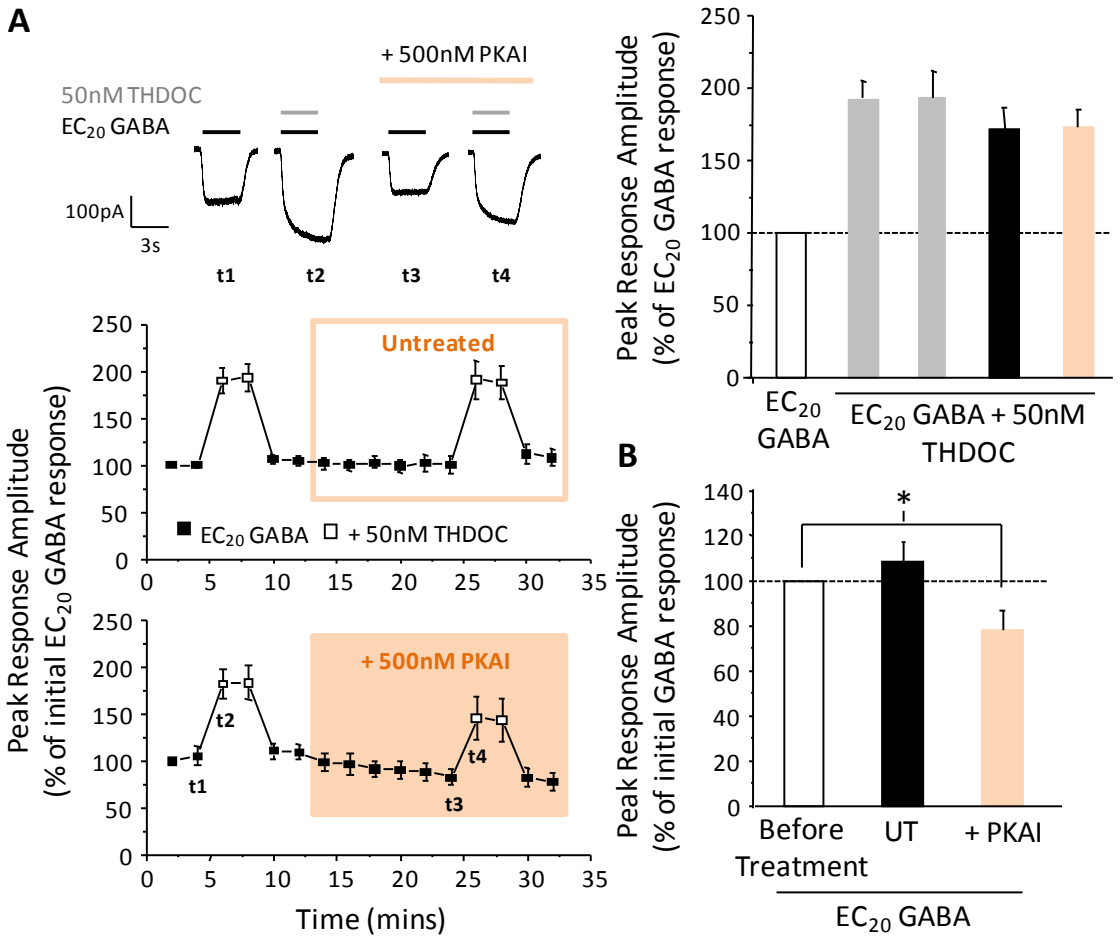

Figure 3.4 Inhibition of PKA does not affect THDOC-mediated potentiation of α1β3γ2L $GABA_A$ receptor currents

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording or were treated with 500nM myristoylated PKA inhibitor peptide 14-22 amide (PKAI; as indicated by orange shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after PKAI treatment (orange bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 26 min, respectively; n = 6) or in treated cells before (black bar) and after (orange bar) 500nM PKAI treatment (n = 5). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells treated with 500nM PKAI (orange bar). Change was measured between 2 min (before treatment) and 32 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

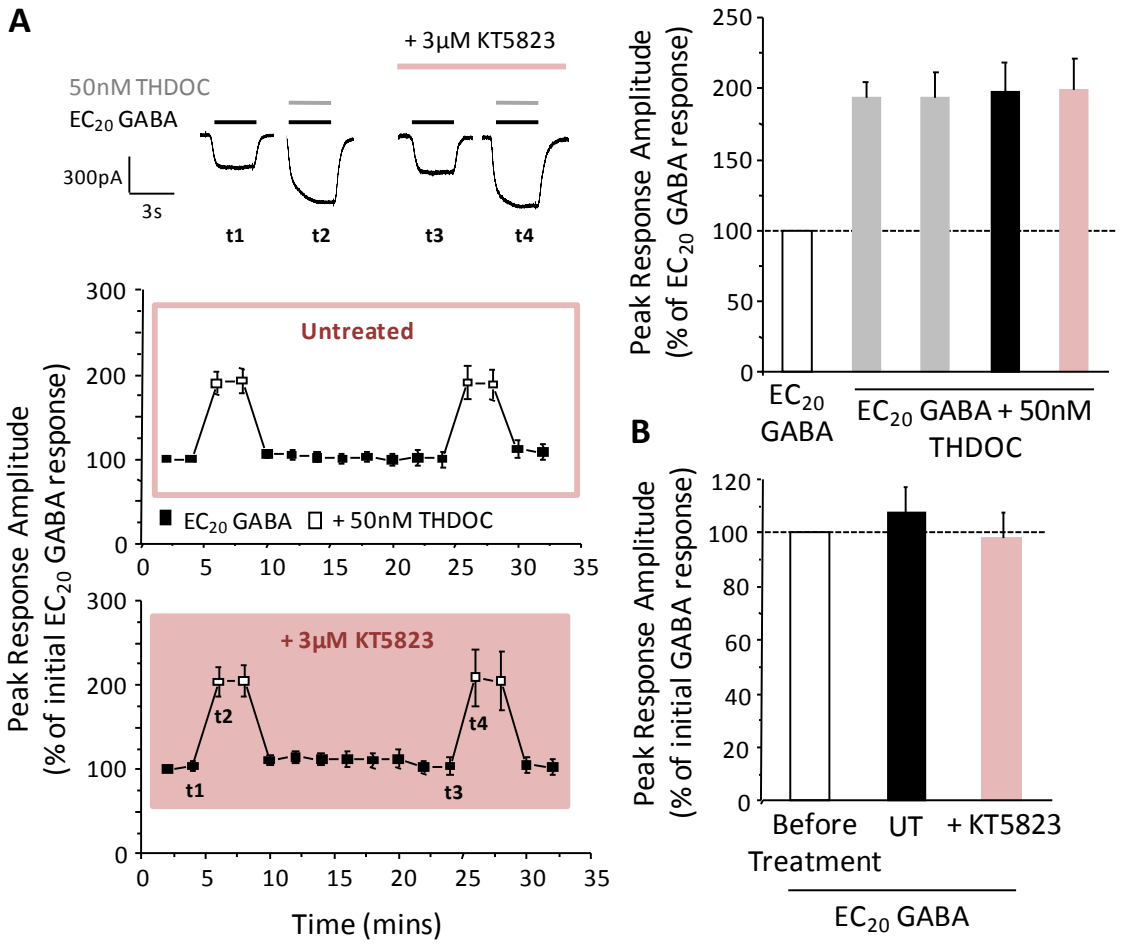

Figure 3.5 PKG inhibition does not affect THDOC-mediated potentiation of α1β3γ2L $GABA_A$ receptor currents

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording or were treated with 3µM KT5823 (as indicated by pink shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after KT5823 treatment (pink bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 26 min, respectively; n = 6) or in treated cells before (black bar) and after (pink bar) 3µM KT5823 treatment (n = 6). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells treated with 3µM KT5823 (pink bar). Change was measured between 2 min (before treatment) and 32 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

In addition to PKA, PKC and PKG, the $GABA_A$ receptor has also been shown to be phosphorylated by CaMKII (Machu *et al.*, 1993; McDonald and Moss, 1994; McDonald and Moss, 1997) and so this could potentially have a role in modulating neurosteroid potentiation. However, in HEK293 cells, $GABA_A$ receptor function has been shown to be unaffected by CaMKII (Houston and Smart, 2006). In these studies, pre-activated CaMKII was infused into the

cells however, due to their non-neuronal lineage, it is thought that these cells may lack the necessary trafficking or scaffolding machinery required to facilitate phosphorylation by CaMKII (Houston and Smart, 2006). For this reason, the role of CaMKII in modulating neurosteroid-mediated potentiation was not investigated.

Taken together, the results indicate that the activity of PKC is important for regulating the potentiation of $GABA_A$ receptor function by neurosteroids. To further examine the relationship between PKC and the actions of neurosteroids, the effects of activating PKC were investigated.

3.2.4 Activation of PKC enhances THDOC-mediated potentiation

As PKC inhibition caused a reduction in neurosteroid potentiation, it was of interest to investigate whether PKC activation might promote enhancement of neurosteroid activity at $GABA_A$ receptors. Accordingly, PKC was activated using phorbol-12-myristate-13-acetate (PMA). PKC activation is the result of a signalling cascade initiated by the activation of phospholipase C, leading to the release of diacylglycerol (DAG) which subsequently binds to and activates PKC. Phorbol esters such as PMA act as a substitute for DAG, thus directly activating PKC (Castagna *et al*., 1982; Sharkey *et al*., 1984; Tanaka and Nishizuka, 1994). Due to its mechanism of action, PMA shows some isoform specificity; the 'atypical' PKC isoforms (ζ and λ) are activated via a DAG-independent mechanism and therefore are insensitive to activation by PMA (Ono *et al*., 1989; Tanaka and Nishizuka, 1994). However, the 'classical' (α, βI, βII and γ) and 'novel' (δ, ε, η and θ) classes of PKC isoforms can be activated by phorbol esters at nanomolar concentrations (Dimitrijević *et al*., 1995) and therefore, in this experiment, during which cells were treated with 100nM PMA, the actions of these individual isoforms cannot be distinguished. PMA is cell-permeable and so was applied via the bath perfusion.

Treatment of cells with 100nM PMA for 36 min resulted in a significant reduction in the EC_{20} GABA-activated current (31.0 ± 8.2%, $P < 0.05$, paired t-test; n = 6; Fig. 3.6B). This supports results from previous studies which, as discussed earlier, have shown that PKC-mediated phosphorylation of β3 subunit-containing $GABA_A$ receptors causes a decrease in receptor function (Brandon *et al*., 2000). The potentiation induced by 50nM THDOC was calculated as in the previous experiments; potentiation was increased by 58.0 ± 18.3% ($P < 0.05$, paired t-test; n = 6; Fig. 3.6A) after treatment with 100nM PMA for 30 min, indicating that activation of PKC can enhance THDOC-mediated potentiation at α1β3γ2L $GABA_A$ receptors.

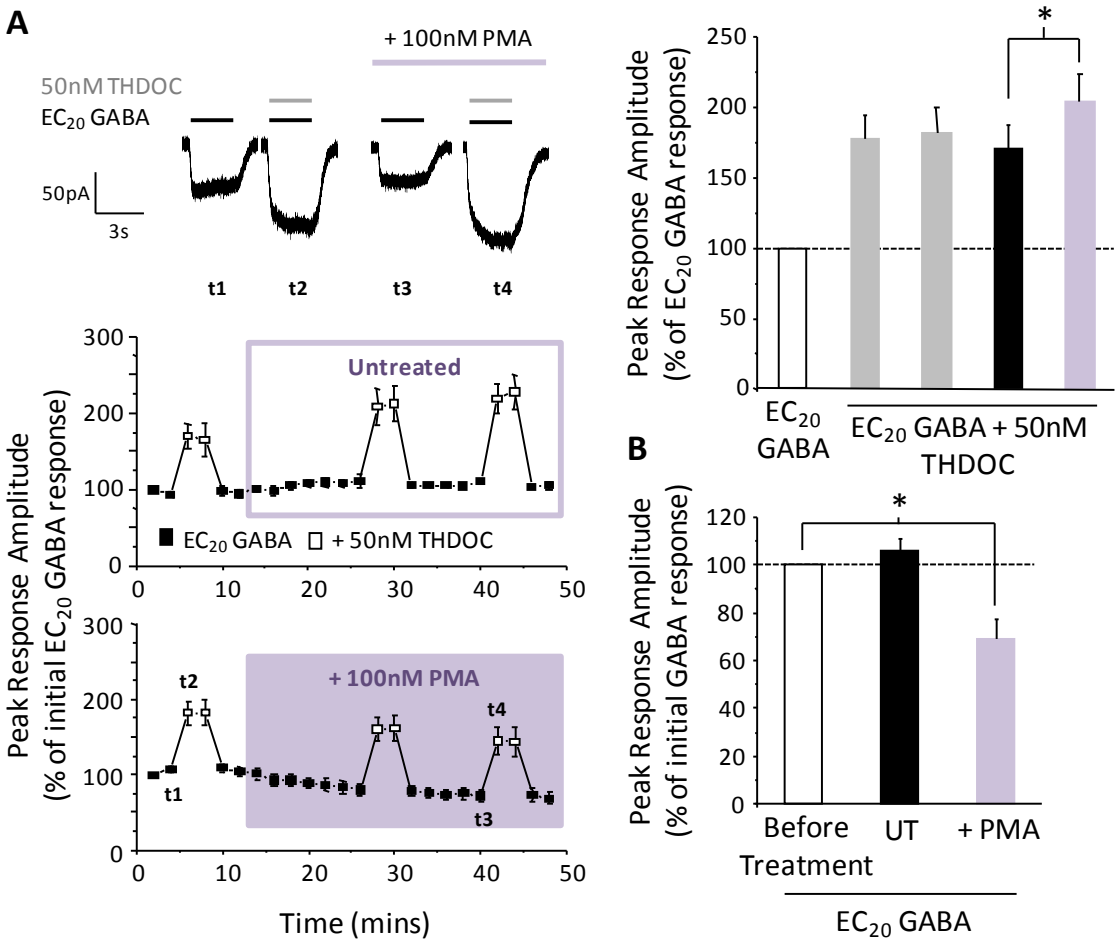

Figure 3.6 PMA increases THDOC-mediated potentiation of α1β3γ2L $GABA_A$ receptor currents
(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording or were treated with 100nM PMA (as indicated by purple shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after PMA treatment (purple bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 42 min, respectively; n = 4) or in treated cells before (black bar) and after (purple bar) 100nM PMA treatment (n = 6). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells treated with 100nM PMA (purple bar). Change was measured between 2 min (before treatment) and 48 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

As PMA treatment resulted in a reduction of responses induced by EC_{20} GABA (Fig. 3.6B), it was possible that PMA caused a shift in the GABA dose-response relationship, which could explain the change in THDOC-mediated potentiation seen after PMA application. To test this, GABA dose-response curves were constructed before and after PMA treatment (Fig. 3.7). The EC_{50} values did not differ significantly before and after PMA treatment (3.22 ± 0.71μM and 3.80 ±

0.58μM, respectively; P > 0.05; n = 4), indicating that the enhancement of THDOC-mediated potentiation seen after PMA treatment was not due to a shift in the GABA concentration-response relationship.

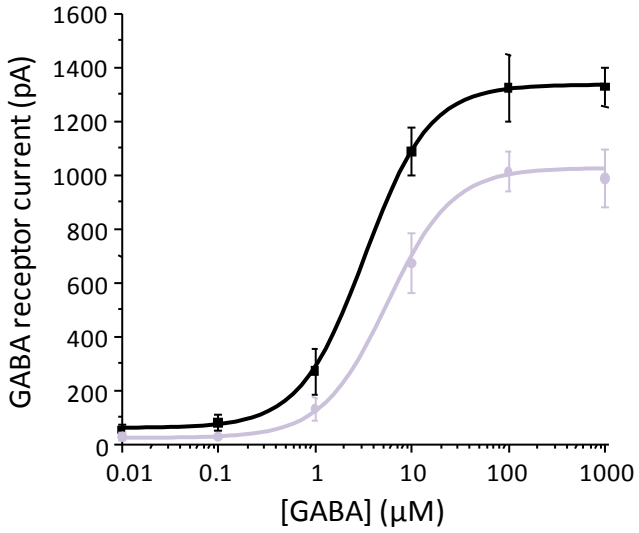

Figure 3.7 Effect of PMA on the GABA dose-response relationship at α1β3γ2L $GABA_A$ receptors.
GABA dose-response curves were established in HEK293 cells expressing α1β3γ2L $GABA_A$ receptors before (black) and after (purple) PMA treatment (n = 5). Each cell was exposed to brief applications of GABA, at varying concentrations (0.01-1000μM), before being treated with 100nM PMA for 30 min. After the treatment period the responses to the same concentrations of GABA, in each cell, were re-measured. All data points represent mean ± s.e.m.

The concentration of neurosteroids present *in vivo* is not static but instead undergoes dynamic changes in response to various physiological and pathophysiological conditions including stress, pregnancy, ovarian cycle and ageing (Purdy *et al*., 1991; Paul and Purdy, 1992; Bäckström *et al*., 2003; Schumacher *et al*., 2003; Maguire and Mody, 2009; Sanna *et al*., 2009), as well as in response to administration of some psychoactive drugs such as ethanol, γ-hydroxybutyrate (GHB) and anti-depressants such as fluoxetine (Uzunov *et al*., 1996; Sanna *et al*., 2004). The experiments thus far have been carried out using a single concentration of neurosteroid, 50nM, which although falls within the concentration range likely to be experienced *in vivo*, does not fully reflect the endogenous environment, as steroid levels will fluctuate both above and below this concentration. To investigate this further, similar experiments were performed using varying concentrations of THDOC (0.1-100nM) in order to elucidate whether PKC can modulate the actions of neurosteroids across a range of concentrations. Consistent with previous data, THDOC concentrations of 10nM and above were able to significantly potentiate $GABA_A$ receptor function (% potentiation = 12.0 ± 3.5

(10nM), 66.2 ± 10.0 (50nM), 111.5 ± 17.2 (100nM); $P < 0.05$, paired t-test; n = 5-11). No potentiation was observed in cells exposed to 1nM THDOC (5.3 ± 4.3%, $P > 0.05$; n = 8) and, as expected, 0.1nM THDOC was also unable to potentiate α1β3γ2L $GABA_A$ receptor responses. After treatment with 100nM PMA for 30 min, potentiation by 1, 10, 50 and 100nM THDOC was significantly enhanced by 102.3 ± 43.2%, 152.7 ± 65.4%, 50.8 ± 14.2% and 34.8 ± 14.2%, respectively ($P < 0.05$, paired t-test; n = 5-11; Fig. 3.8). This indicates that PKC can modulate THDOC-mediated potentiation across the entire range of neurosteroid concentrations likely to be experienced *in vivo*. Interestingly, treatment with PMA revealed that potentiation can be elicited by just 1nM THDOC under conditions of increased PKC activity.

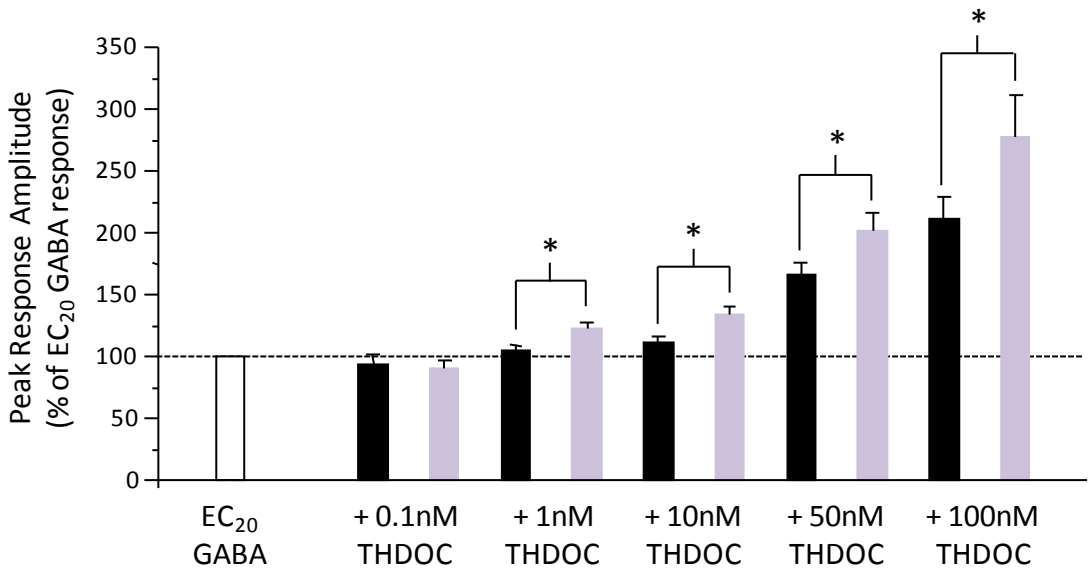

Figure 3.8 Effect of PMA on the potentiation of α1β3γ2L $GABA_A$ receptor currents by varying concentrations of THDOC

Bar chart showing the mean potentiation of EC_{20} GABA-activated responses elicited by various concentrations of THDOC (0.1nM to 100nM) before (black bars) and after (purple bars) 100nM PMA treatment (n = 5-11). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

Although the results from the protein kinase inhibition experiments indicated that PKA was unlikely to be involved in modulating neurosteroid activity at α1β3γ2L $GABA_A$ receptors, other studies have implicated PKA in this modulation (Harney *et al.*, 2003). Therefore, to examine the role of PKA more fully, the effects of activating PKA were also investigated.

3.2.5 Activation of PKA does not alter THDOC-mediated potentiation

PKA is activated by cyclic adenosine monophosphate (cAMP), which is produced endogenously from adenosine triphosphate (ATP) by the enzyme, adenyl cyclase. Therefore, PKA activity was enhanced by intracellularly applying exogenous cAMP via the patch electrode. Previous studies investigating β3 subunit-containing $GABA_A$ receptors have shown that phosphorylation by PKA causes enhancement of GABA-activated responses by approximately 30% (McDonald *et al*., 1998). Consistent with this, intracellular perfusion of the cells with 300μM cAMP caused a significant increase in EC_{20} GABA-activated responses by 35.7 ± 11.8% ($P < 0.05$, paired t-test; n = 4; Fig. 3.9B). The presence of this enhancement was a good indicator that PKA was being activated during the course of the experiment, however there was no concurrent change in the THDOC-mediated potentiation. Treatment of cells with 300μM cAMP appeared to cause a small decrease in the potentiation elicited by 50nM THDOC, but this was not significant. (9.0 ± 4.4%, $P > 0.05$; n = 4; Fig. 3.9A), indicating that activation of PKA does not alter the potentiation by neurosteroids at $GABA_A$ receptors.

Taken together, the results show that specific activation of PKC, but not PKA, results in an enhancement of neurosteroid-mediated potentiation at α1β3γ2L $GABA_A$ receptors. This supports the results from the protein kinase inhibition experiments, indicating that the activity of PKC is important for regulating the magnitude of neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors.

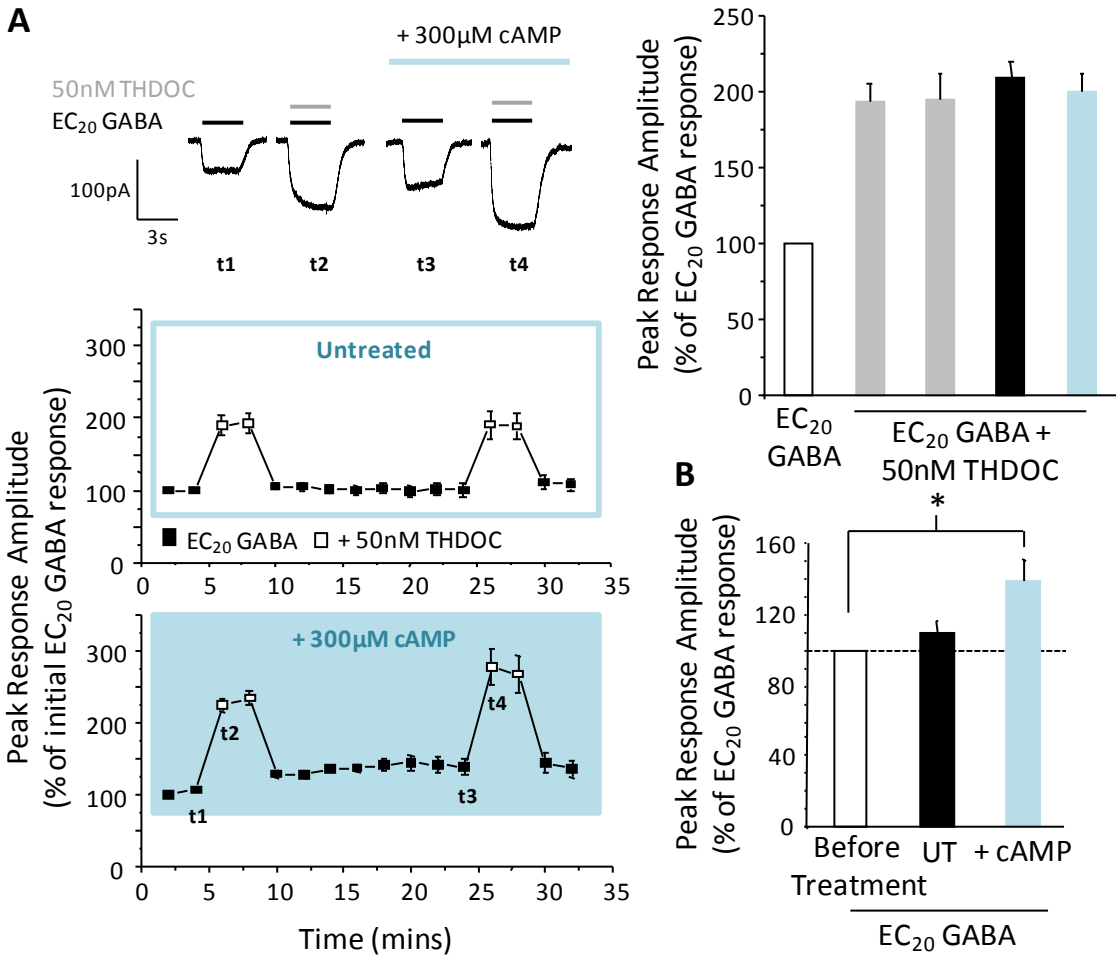

Figure 3.9 Activation of PKA does not affect THDOC-mediated potentiation of α1β3γ2L $GABA_A$ receptor currents

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording or were treated with 300µM cAMP (as indicated by blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after cAMP treatment (blue bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 26 min, respectively; n = 6) or in treated cells before (black bar) and after (blue bar) 300µM cAMP treatment (n = 4). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar) or cells treated with 300µM cAMP (blue bar). Change was measured between 2 min (before treatment) and 32 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

3.3 Discussion

3.3.1 Protein kinases positively modulate neurosteroid potentiation at $GABA_A$ receptors

An increasing number of studies have indicated that the activity of protein kinases can alter the modulatory effects of neurosteroids at $GABA_A$ receptors (see Table 3.1). This is supported by the results from the present study, which showed that modulation of protein kinase activity altered the actions of neurosteroids at α1β3γ2L $GABA_A$ receptors expressed in HEK293 cells. Inhibition of a broad-spectrum of protein kinases by staurosporine treatment resulted in a significant reduction in the potentiation elicited by 50nM THDOC (see Fig. 3.1). This decrease was not caused by a staurosporine-mediated shift in the GABA EC_{50} (see Fig. 3.2), indicating that the magnitude of neurosteroid potentiation, at this receptor subtype, is positively modulated by the activity of protein kinases. This supports observations from previous studies which showed that both recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes (Leidenheimer and Chapell, 1997) and native $GABA_A$ receptors of the hippocampus (Harney *et al*., 2003) and hypothalamus (Fáncsik *et al*., 2000), exhibit a positive correlation between protein kinase activity and the magnitude of neurosteroid-mediated potentiation. Furthermore, a recent behavioural investigation has also demonstrated a similar relationship, with results showing that enhancement of lordosis behaviour by neurosteroids in female rats, can be reversed by inhibition of PKC (Frye and Walf, 2008). This behaviour is, at least in part, thought to be mediated by $GABA_A$ receptors (Frye and Vongher, 1999), once again suggesting that protein kinase activity has a positive modulatory effect on the action of neurosteroids at these receptors. However, other investigations have obtained results which are inconsistent with this notion; studies of both the lamina II neurons of the spinal cord (Vergnano *et al*., 2007) and the hypothalamic neurons of pregnant rats (Koksma *et al*., 2003) showed that enhancement of protein kinase activity resulted in a significant decrease in the sensitivity of $GABA_A$ receptors to applied neurosteroid. This indicates that an inverse relationship exists between the activity of protein kinases and the actions of neurosteroids in these neuronal populations.

The reason for this difference is unclear, but one factor which may affect the modulation of neurosteroid activity by protein kinases is the subunit combination of the receptor. It is widely accepted that the majority of synaptic $GABA_A$ receptors are composed of α, β and γ subunits (MacDonald and Olsen, 1994; Rabow *et al*., 1995). There are multiple isoforms of each of these subunits (α1-α6, β1-β3, γ1-γ3; Sieghart *et al*., 1999) and, although it is thought that nearly all

synaptic receptors contain a γ2 subunit, this still leaves the potential for a large number of different receptor subtypes to be expressed *in vivo*. It is therefore likely that the different neuronal populations utilised in the various studies to date express different complements of $GABA_A$ receptor subunits (Laurie *et al*., 1992a; Wisden *et al*., 1992; Pirker *et al*., 2000; Olsen and Sieghart, 2009). However, both the α1β3γ2L subunit-containing receptor studied in these experiments and the α1β2γ2L receptor investigated previously (Leidenheimer and Chapell, 1997) have shown a similar relationship between neurosteroids and phosphorylation, with neurosteroid-mediated potentiation being positively modulated by the activity of protein kinases. The α1, β2/3 and γ2 subunits are thought to be amongst the most highly expressed in the CNS (McKernan and Whiting, 1996; Olsen and Sieghart, 2009), but additional receptor subtypes, including those containing the α2/3 and/or β1 subunits, are also present, and therefore, it is possible that neurosteroid actions at these receptor subtypes may be inversely regulated by protein kinases, which may provide an explanation for the conflicting results seen in some studies, but this remains to be investigated.

Secondly, there is a small possibility that the changes in neurosteroid potentiation seen after the modulation of endogenous protein kinase activity are not due to direct changes in receptor phosphorylation, but may be the result of altering the levels of phosphorylation upon an accessory protein(s), which could then interact with the $GABA_A$ receptor to modulate neurosteroid potentiation. The actions of accessory proteins cannot be ruled out for any of the previous studies to date as none have demonstrated that the changes in neurosteroid activity observed were due to a direct change in the levels of phosphorylation present at the $GABA_A$ receptor. Therefore, it is possible that the results reported from experiments in some neuronal populations may be confounded by the actions of such proteins. However, the present investigation goes on to show a direct link between the levels of phosphorylation at the receptor and the magnitude of neurosteroid potentiation observed (see Chapter 4), suggesting that accessory proteins are unlikely to play a major role in altering neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors expressed in HEK293 cells. It may be interesting to determine whether accessory proteins are having any effect at other $GABA_A$ receptor subtypes, as the presence or absence of these proteins *in vivo* may provide an explanation for the conflicting results from studies to date concerning the nature of the relationship between neurosteroids and protein kinases at $GABA_A$ receptors in different neuronal populations.

Brain Region/Expression System	Neuron/Cell Type/Receptor	Protein Kinase/Phosphatase		Neurosteroid potentiation of GABA response	Species/Stage of development	Citation
		Type	Inhibition or Activation?			
Hippocampus	CA1 Pyramidal neurons	PKC	Activation	None	Rat P16-22	Harney *et al.*, (2003)
			Inhibition	Decreased		
		PKA	Inhibition	Decreased		
	Dentate gyrus granule neurons	PKC	Activation	Increased		
Hypothalamus	Magnocellular neurons	PKC	Inhibition	Decreased	Rat Adult	Fáncsik *et al.*, (2000)
		PKA	Inhibition	None		
		PKG	Inhibition	None		
	SON neurons	PKC	Activation	Decreased	Rat Pregnancy day 20	Koksma *et al.*, (2003)
		PP1/2A	Inhibition	Decreased		
	Ventral tegmental area neurons	PKC	Inhibition	Decreased (Behavioural assessment only)	Rat Adult	Frye and Walf (2008)
Spinal Cord	Lamina II neurons	PKC	Activation	Decreased	Rat P15-20	Vergnano *et al.*, (2007)
Xenopus Laevis heterologous expression system	Oocytes expressing recombinant $\alpha1\beta2\gamma2L$ $GABA_A$ receptors	PKC	Activation	Increased	N/A	Leidenheimer and Chapell (1997)
PKCε KO mouse	Whole animal behaviour	PKCε	Knock-out	Increased	Mouse Adult	Hodge *et al.*, (2002)

Table 3.1 Summary of the publications to date examining the actions of neurosteroids and protein kinases at the $GABA_A$ receptor

P – Post-natal day

PP1/2A – Protein Phosphatase 1 or 2A

3.3.2 Neurosteroid activity at α1β3γ2L $GABA_A$ receptors is modulated by PKC, but not PKA

Contradictory results have also been reported regarding the involvement of specific protein kinases in modulating the actions of neurosteroids (see Table 3.1). For example, studies in CA1 pyramidal neurons of the hippocampus showed that inhibition of PKC or PKA resulted in a concurrent decrease in neurosteroid sensitivity (Harney *et al*., 2003). However, Fáncsik *et al.* (2000) showed that, in hypothalamic neurons, the activity of neurosteroids can be modulated by PKC, but inhibiting PKA had no effect. The results from the present investigation demonstrated that the magnitude of potentiation elicited by neurosteroids at α1β3γ2L $GABA_A$ receptors is regulated by PKC, but not PKA or PKG. The results showed that inhibition or activation of PKC caused a significant reduction or enhancement of THDOC-mediated potentiation, respectively (see Fig. 3.3 and 3.6). In contrast, inhibition or activation of PKA or inhibition of PKG had no effect (see Fig. 3.4, 3.5 and 3.9). This mimics very closely the results obtained by Fáncsik *et al.* (2000), as they showed that the activities of both PKA and PKG did not affect the actions of neurosteroids. To the contrary, Harney *et al*. (2003) reported that the activity of PKA was also important and that, although inhibition of PKC caused a reduction in neurosteroid sensitivity at $GABA_A$ receptors, activation of PKC had no effect.

The latter discrepancy may be explained by considering that there may be differences in the levels of endogenous protein kinase activity in the CA1 pyramidal neurons of the hippocampus and the HEK293 cells used in this investigation. It is possible that, if the level of PKC in the pyramidal neurons is high, near saturating, any further activation of PKC will have no effect. This would explain why inhibiting PKC is still effective in modulating the activity of neurosteroids in these neurons, even though they appear to be insensitive to PKC activation. In support of this idea, a previous study examining the effects of PKC-mediated phosphorylation on $GABA_A$ receptor function in hippocampal CA1 pyramidal neurons showed that activation of PKC had no effect on receptor function, whereas activation of PKA caused a significant reduction in the amplitudes of mIPSCs recorded from these neurons (Poisbeau *et al*., 1999). However, this is not the case for all neuronal populations as results from another investigation showed that activation of PKC caused a significant reduction of $GABA_A$ receptor function in cortical neurons (Brandon *et al*., 2000). This suggests that PKC activity may be higher in the CA1 pyramidal neurons compared to neurons from other regions of the brain, resulting in activation of PKC being ineffective in modulating the $GABA_A$ receptor both directly, by phosphorylation and indirectly, by regulating the activity of neurosteroids. It may be interesting to determine, under conditions whereby these neurons have been pre-incubated

with a PKC inhibitor to reduce levels of active PKC, whether activation of PKC would subsequently become effective in modulating the actions of neurosteroids at the $GABA_A$ receptors, mimicking the results seen in this investigation.

The inconsistencies in the results concerning the role played by PKA in the regulation of neurosteroid activity at $GABA_A$ receptors are, perhaps, more difficult to explain. A factor which may affect the ability of PKA to modulate the activity of neurosteroids is, once again, the subunit combination of the receptor. Studies have revealed that neurosteroids do not appear to exhibit any specific subunit selectivity at $GABA_A$ receptors containing α, β and γ subunits (Belelli *et al*., 2002; Herd *et al*., 2007), however, protein kinases have been shown to differentially modulate $GABA_A$ receptor function, depending on the subunit-combination of the receptor (Moss and Smart, 1996). A well-documented example of this is the disparity between the effects of phosphorylation, by PKA, upon β1, β2 and β3 subunit-containing $GABA_A$ receptors; PKA-mediated phosphorylation at receptors containing the β1 subunit results in a reduction in $GABA_A$ receptor function, whereas at β3 subunit-containing receptors, phosphorylation by PKA causes enhancement of GABA-activated responses. Interestingly, at $GABA_A$ receptors containing the β2 subunit, PKA phosphorylation has no effect (McDonald *et al*., 1998). Therefore, when considering the actions of protein kinases in conjunction with the activity of neurosteroids, it could be hypothesized that different protein kinases may also modulate the actions of neurosteroids differently, depending on the subunit-combination of the receptor. Many of the studies carried out to date have utilised neurons from distinct regions of the brain, which, as discussed earlier, are likely to express different combinations of $GABA_A$ receptor subunits. Therefore, if subunit combination does indeed play a role in determining the co-regulatory relationship between neurosteroids and protein kinases at the $GABA_A$ receptor, this may explain the variation in the results seen. The results from this investigation failed to show any role for PKA in the regulation of neurosteroid-mediated potentiation at α1β3γ2L subunit-containing $GABA_A$ receptors, which, as discussed earlier, is in contrast to a study in hippocampal CA1 pyramidal neurons which showed that PKA positively modulates the activity of neurosteroids at the $GABA_A$ receptors in these neurons (Harney *et al*., 2003). This suggests that the modulatory effects of PKA seen in these neurons are unlikely to be mediated by receptors containing the β3 subunit, which is supported by immunocytochemical studies showing β3 subunit expression to be relatively low in hippocampal CA1 pyramidal neurons (Pirker *et al*., 2000). Leidenheimer and Chapell (1997) examined the co-regulation of α1β2γ2L subunit-containing $GABA_A$ receptors by protein kinases and neurosteroids and showed that PKC could regulate the magnitude of neurosteroid-

mediated potentiation at these receptors however, this study did not examine the effects of PKA and therefore it cannot be ruled out that the effects of PKA seen in the hippocampal neurons are mediated by α1β2γ2L $GABA_A$ receptors, which are known to be highly expressed in these neurons (Wisden *et* al., 1992; Pirker *et al*., 2000). In addition, it is possible that receptors containing other subunits, such as α2/3 and β1, which have not been investigated here, may also mediate the modulatory effects of PKA seen in some neurons. Therefore, in order to create a clearer picture of how different neuronal populations can be differentially regulated by specific protein kinases, further study, similar to that carried out here, is required to examine the co-modulation, by neurosteroids and protein kinases, of the multiple $GABA_A$ receptor subtypes which are likely to be expressed *in vivo*.

Another factor which may affect the ability of specific protein kinases to modulate neurosteroid potentiation at $GABA_A$ receptors in some neuronal populations and/or cell lines is the relative availability of anchoring proteins for these specific protein kinases. Scaffold proteins such as the A-kinase anchoring proteins (AKAPs) or the receptors for activated C-kinase (RACKs) are required to mediate proximity of the protein kinase with its substrate, as well as ensuring specificity of action through compartmentalization of specific protein kinases with the receptor complex at the cell surface. (Pawson and Scott, 1997; Colledge and Scott, 1999). In the case of PKA, the presence of AKAP79 or its murine ortholog AKAP150 has been shown to be important for mediating phosphorylation of the $GABA_A$ receptor. Studies have shown that AKAP79/150 can directly interact with both β1 and β3 receptor subunits, facilitating phosphorylation of these two subunits by PKA. Furthermore, in the case of the β3 subunit, it was reported that this interaction is crucially required for phosphorylation by PKA to occur (Brandon *et al*., 2003). Therefore, the levels of endogenous AKAPs may be of importance when considering whether PKA will affect $GABA_A$ receptor currents within different neuronal populations and ultimately whether PKA will play a role in modulating the actions of neurosteroids at the $GABA_A$ receptors within these neurons. Interestingly, it has been shown that AKAP70/150 is differentially expressed throughout the brain; relatively high levels have been detected in the hippocampus and cerebral cortex, whereas neurons of the thalamus and hypothalamus exhibited much lower immunoreactivities (Glantz *et al*., 1992). Therefore, this may offer a potential explanation for the discrepancies in results concerning the role played by PKA in modulating neurosteroid-mediated potentiation at $GABA_A$ receptors in hippocampal versus hypothalamic neurons. Given that the levels of AKAP79/150 have been shown to be much lower in hypothalamic neurons, the inability to detect a modulatory role for PKA may be due to its inability to interact with the receptors in these neurons, resulting in no PKA-

mediated modulation of neurosteroid activity at hypothalamic $GABA_A$ receptors. Ectopic expression of AKAP79/150 in hypothalamic neurons could help to solve this controversy. Also, it cannot be ruled out that another member of the AKAP family may mediate the actions of PKA in these neurons.

In the present study, the results showed that PKA did not play a role in modulating neurosteroid-mediated potentiation at α1β3γ2L subunit-containing $GABA_A$ receptors. However, modulation of the receptor currents by PKA-mediated phosphorylation was evident (see Fig. 3.4 and 3.9), indicating that PKA is able to phosphorylate the $GABA_A$ receptors expressed in these cells. This suggests that appropriate scaffold protein(s) are present within HEK293 cells allowing this phosphorylation process to occur successfully and thus, it seems extremely unlikely that PKA plays a role in modulating neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors. Therefore, it is highly likely that the modulatory effects of PKA seen in hippocampal neurons are mediated by a different $GABA_A$ receptor subtype. As discussed earlier, the β2 subunit is highly expressed in the neurons of the hippocampus (Wisden *et* al., 1992; Pirker *et al*., 2000) but, as this subunit has been shown to be unaffected by PKA *in vivo* (McDonald *et al*., 1998), it is difficult to envisage that the effects of PKA are mediated via receptors containing this subunit. However, this cannot be completely ruled out as it is possible, as mentioned previously, that PKA may act indirectly by phosphorylating an accessory protein which could then modulate neurosteroid potentiation at β2 subunit-containing $GABA_A$ receptors.

3.3.3 The roles of specific PKC isoforms

This investigation did not specifically address the potential for differential involvement of specific PKC isoforms in the modulation of neurosteroid activity. A previous study examining the effects of neurosteroids in PKCε knock-out mice showed that the $GABA_A$ receptors in these mice exhibit enhanced neurosteroid sensitivity, resulting in a reduction of anxiety-like behaviour (Hodge *et al*., 2002). At the concentrations used in this experiment, the PKC inhibitor bisindolylmaleimide I (500nM), and activator PMA (100nM), are likely to show only limited isoform specificity. *In vitro* assays have shown that the IC_{50} of bisindolylmaleimide I is 10-20 nM for inhibiting PKCα/βI/βII/γ and 100-200 nM for PKCδ/ε isoforms (Martiny-Baron *et al*., 1993). Therefore, it would be expected that all of these isoforms would be inhibited in the present study. PMA is also likely to activate all PKC isoforms, with the exception of the DAG-independent atypical class (Dimitrijević *et al*., 1995). Therefore, this investigation cannot distinguish between the effects of the different PKC isoforms; in order to examine this, each

isoform must be assessed in isolation, which may prove difficult with the limited complement of isoform specific PKC inhibitors and activators that are currently available.

3.3.4 Neurosteroid-mediated potentiation does not require protein kinases.

An important conclusion that can be drawn from the results is that neurosteroid-mediated potentiation is modulated by, but does not require protein kinase activity. Cells exposed to staurosporine or bisindolylmaleimide I exhibited significant decreases in the potentiation elicited by 50nM THDOC, but potentiation was still evident after both of these treatments (see Fig. 3.1 and 3.3). It is possible that this may be because the staurosporine or bisindolylmaleimide I treatments were not successful in completely abolishing the activity of their target protein kinases. However, the concentrations used (5-20 times the IC_{50}'s) and durations of treatments were in excess of those reported to cause inhibition of the target protein kinases and therefore it is unlikely that this result is due to incomplete kinase inhibition. In addition, later experiments in this study, utilising mutant $GABA_A$ receptor constructs, have shown that abolition of all candidate phosphorylation sites from the receptor complex does not eradicate potentiation by THDOC (see Chapter 4). This strongly suggests that the actions of protein kinases are not required for neurosteroid-mediated potentiation at α1β3γ2L subunit-containing $GABA_A$ receptors, but instead act to modulate the magnitude of potentiation that is elicited.

3.3.5 The physiological significance of receptor co-modulation

Neurosteroids can be synthesised *de novo* in the CNS with a number of neuronal populations known to possess the necessary synthetic enzymes. Studies have shown that neurons of the cerebellum, hippocampus, cortex, olfactory bulb and thalamus are immunoreactive for 5α-reductase (type 1) and 3α-hydroxysteroid dehydrogenase, the enzymes required for the synthesis of allopregnanolone and THDOC (Agís-Balboa *et al*., 2006; Tsutsui, 2006). In addition, P450scc, another key steroidogenic enzyme responsible for converting cholesterol into pregnenolone, has been detected in neurons of both sensory and pain pathways, including dorsal root ganglia, spinal cord dorsal horn and somatosensory cortex (Patte-Mensah *et al*., 2003). $GABA_A$ receptor expression is relatively ubiquitous throughout the CNS and, with neurosteroids exhibiting no clear receptor isoform specificity, it might be expected that the actions of neurosteroids would be globally experienced, causing a generalised potentiation, rather than achieving a more localized 'fine-tuning' of inhibitory neurotransmission. Such a non-specific action would be incompatible with neurosteroids having a physiological role, however, a number of studies have shown that neurosteroid action is both brain region and

neuron specific. It has been hypothesized that the differing activities of protein kinases within these different brain areas and/or neuronal populations may contribute to the differential sensitivity to neurosteroids. Indeed, results from a previous study indicated that the neurons of the dentate gyrus are relatively insensitive to physiological concentrations of neurosteroids under basal conditions, but when PKC activity is increased these neurons become much more sensitive to the effects of applied neurosteroids. In contrast, the same study showed that neurons of the CA1 area of the hippocampus were sensitive to neurosteroids under basal conditions with activation of PKC having no effect (Harney *et al*., 2003). This may indicate that PKC activity is lower in neurons of the dentate gyrus compared to those in the hippocampal CA1 area, which could result in their differing sensitivity to neurosteroids under basal conditions. This may offer a potential mechanism by which different populations of neurons can be differentially sensitive to the actions of neurosteroids. Interestingly, a similar result was obtained from the present investigation with results showing that under basal cell conditions, the α1β3γ2L $GABA_A$ receptor was insensitive to concentrations of THDOC below 10nM. However, after PKC activation, 1nM THDOC was able to induce a modest, although significant, potentiation at the $GABA_A$ receptor (see Fig. 3.8). This highlights a situation whereby enhancing protein kinase activity can cause $GABA_A$ receptors to become sensitive to previously sub-threshold concentrations of neurosteroid, which would be expected to have a significant impact on inhibitory neurotransmission *in vivo*.

As discussed earlier, the actions of protein kinases are reliant upon the availability of appropriate scaffold proteins, such as AKAPs and RACKs, which enable the protein kinase to be anchored close to its substrate, within a defined compartment, allowing rapid and successful transfer of the phosphate moiety to the target protein. Therefore, the relative expression levels of these scaffold proteins across different brain regions as well as their distribution within the neurons themselves will act to determine the location(s) that will be subjected to the modulatory effects of protein kinases, thus limiting the brain regions and/or neuronal populations in which neurosteroid activity will be regulated by protein kinases. This offers an additional mechanism by which neurosteroid actions can become both brain region and neuron specific, despite showing no clear $GABA_A$ receptor isoform specificity.

The ability of protein kinases to positively modulate the actions of neurosteroids at $GABA_A$ receptors will have important implications under conditions in which activity of protein kinases is reduced or elevated. For example, decreased PKC levels have been observed in the brains of patients with a number of neurodegenerative diseases, including Alzheimer's disease (Masliah

et al., 1990), Parkinson's disease (Nishino *et al*., 1989) and Huntingdon's disease (Hashimoto *et al*., 1992) as well as in some forms of epilepsy (Terunuma *et al*., 2008) and enhanced PKC activity has been seen after administration of some psychoactive drugs such as ethanol (Stubbs and Slater, 1999) and cocaine (Steketee, 1996; Steketee *et al*., 1998). Therefore, in situations where protein kinase activity is perturbed, particularly PKC, effects will be seen both directly, in the alteration of $GABA_A$ receptor phosphorylation and indirectly, by modulating the activity of neurosteroids at these receptors.

3.3.6 Effects of phosphorylation on $GABA_A$ receptor function

It is well documented that phosphorylation alone can affect the function of $GABA_A$ receptors. The receptor has been shown to be phosphorylated by a number of protein kinases including serine-threonine kinases: PKA, PKC, PKG and CaMKII as well as tyrosine kinases SRC and PYK (Moss and Smart, 1996; Brandon *et al*., 2002a; Houston *et al*., 2009). Studies have shown that phosphorylation of the $GABA_A$ receptor can result in both enhancement and reduction of GABA-activated currents, depending on the protein kinase present and the subunit combination of the receptor (Moss and Smart, 1996). However, in this investigation, inhibition of protein kinase activity by staurosporine did not result in an alteration of EC_{20} GABA-activated responses (see Fig. 3.1). This may be explained by evidence from previous studies which have shown that PKA and PKC have opposing effects at β3 subunit-containing $GABA_A$ receptors; phosphorylation by PKA has been shown to enhance GABA-activated currents (McDonald *et al*., 1998) whereas phosphorylation by PKC results in a reduction of $GABA_A$ receptor function (Brandon *et al*., 2000). Staurosporine would be expected to inhibit both of these protein kinases simultaneously and therefore, this may mask the effects of each individual protein kinase, resulting in no overall effect on the EC_{20} GABA-activated response. However, treatment of cells with the specific PKC inhibitor, bisindolylmaleimide I, also did not result in a significant change in receptor function (see Fig. 3.3), whereas, in contrast, cells treated with the PKA peptide inhibitor (14-22 amide) exhibited a significant reduction in EC_{20} GABA-activated currents (see Fig. 3.4). This may indicate that PKA, but not PKC-mediated phosphorylation exists at the α1β3γ2L $GABA_A$ receptor under basal conditions. However, this is not consistent with the reduction in THDOC-mediated potentiation observed after bisindolylmaleimide I treatment, which suggests that the receptor is basally phosphorylated by PKC. Offering support to this notion, Western blot analysis examining the levels of phosphorylated β3 in HEK293 cells expressing α1β3γ2L $GABA_A$ receptors showed that the levels of phosphorylation could be reduced by exposing cells to bisindolylmaleimide I (see Chapter 4), suggesting that the β3 subunit is indeed phosphorylated by PKC under basal

conditions in the HEK293 cell. Examining the results from the electrophysiology, it is interesting to note that during the initial period of bisindolylmaleimide I treatment, a modest increase in EC_{20} GABA-activated current was observed (see Fig. 3.3), however this enhancement was not persistent, with the magnitude of the response reverting back to baseline levels after 58 min. This suggests that PKC-mediated basal phosphorylation may have been present, but that later in the recording some further changes to signalling pathways may have occurred, for example, up-regulation of other protein kinases to compensate for the depleted activity of PKC, which may have caused the response to EC_{20} GABA to return to baseline levels, however this remains to be investigated.

3.4 Conclusions

- Protein kinase activity positively modulates the actions of neurosteroids at α1β3γ2L subunit-containing $GABA_A$ receptors.

- The magnitude of neurosteroid-mediated potentiation is modulated by the activity of PKC, but not by PKA or PKG.

- Potentiation of α1β3γ2L $GABA_A$ receptor currents by neurosteroids does not require protein kinase activity per se.

Chapter 4

Modulation of neurosteroid activity by PKC: a role for direct receptor phosphorylation

4.1 Introduction

An increasing number of studies have reported that the activity of PKC can alter the modulatory effects of neurosteroids at $GABA_A$ receptors (Leidenheimer and Chapell, 1997; Fáncsik *et* al., 2000; Harney *et al*., 2003; Frye and Walf, 2008). This was supported by the results from the previous chapter, which showed that, at α1β3γ2L subunit-containing receptors, the magnitude of potentiation elicited by the naturally-occurring neurosteroid, THDOC, is positively modulated by the activity of PKC. However, it remains to be determined whether PKC mediates these effects by directly phosphorylating the $GABA_A$ receptor or whether it acts indirectly, though the phosphorylation of receptor-associated accessory proteins or via the modulation of other downstream signalling pathways. Therefore, the aim of experiments in this chapter was to investigate the mechanism by which PKC modulates the actions of neurosteroids at α1β3γ2L $GABA_A$ receptors, with the objective of establishing whether direct receptor phosphorylation is required.

The intracellular domains of $GABA_A$ receptor β1-3 and γ2 subunits are known to contain a number of consensus phosphorylation sites, which can be targeted by numerous Ser/Thr and Tyr protein kinases (Moss *et al.*, 1992a, 1995; McDonald and Moss, 1994, 1997). At the α1β3γ2L $GABA_A$ receptor, previous studies report that PKC is able to phosphorylate consensus sites on both the β3 and γ2L subunits. Studies utilising GST fusion proteins produced from the TM3-4 intracellular loops of these receptor subunits showed that PKC can phosphorylate the β3 subunit at serines 408 and 409 (McDonald and Moss, 1997) and the γ2L subunit at serines 327 and 343 (Moss *et al*., 1992a). Therefore, this indicates that, at the α1β3γ2L $GABA_A$ receptor, there are four potential sites through which PKC could act to modulate neurosteroid activity.

To assess whether phosphorylation at one or more of these residues is required for PKC to modulate the actions of neurosteroids, a series of experiments was carried out using mutant β3 and γ2L cDNA constructs in which candidate phosphorylation sites had been mutated to neutral alanine residues, a mutation that has been shown to be effective in preventing

phosphorylation and its functional effects (Moss *et al*., 1992a; McDonald and Moss, 1997). Therefore, these constructs are a reliable method for eliminating phosphorylation at specific loci in order to determine whether phosphorylation at a specific receptor site(s) is responsible for modulating the actions of neurosteroids at α1β3γ2L $GABA_A$ receptors. It was hypothesised that, if PKC-mediated phosphorylation at a particular site(s) is important for neurosteroid-mediated potentiation, under conditions where this site was no longer phosphorylated (i.e. when the phosphorylation site had been mutated), any subsequent enhancement or reduction in PKC activity would no longer affect the magnitude of the potentiation.

4.2 Results

In order to explore the mechanism by which PKC acts to modulate the actions of neurosteroids at $GABA_A$ receptors and, in particular, to examine the involvement of direct receptor phosphorylation in this process, HEK293 cells were transfected to express α1β3γ2L $GABA_A$ receptors containing single or multiple point mutations of consensus phosphorylation sites known to be targeted by PKC. As before, cells were co-transfected with eGFP to enable the identification of transfected cells. Experiments were carried out using the protocol described in the previous chapter, enabling a direct comparison between the results obtained from wild-type receptors (see Chapter 3) and the mutant receptors used in these experiments. Briefly, whole-cell patch clamp electrophysiology was used to assess the magnitude of potentiation elicited by 50nM THDOC, before and after cell treatment with compounds to inhibit or activate protein kinase activity. For each cell, baseline responses to EC_{20} GABA and potentiated responses after co-applications of EC_{20} GABA and 50nM THDOC were established under basal cell conditions and after the treatment period. To allow a direct comparison of the potentiation elicited before and after treatment, each potentiated response was normalised to the cell's preceding response to EC_{20} GABA alone (see Methods and Materials). In order to ensure that the results from wild-type receptors presented in the previous chapter were valid as controls for the mutant receptor experiments in this chapter, each set of experiments (wild-type and mutant receptors) for a given protein kinase inhibitor or activator were carried out in parallel, with wild-type control data being collected throughout the experimental period. These results were then collated to form the 'wild-type' experimental group which is presented in this and the previous chapter.

4.2.1 Phosphorylation at the β3 subunit is important for modulating THDOC-mediated potentiation

The effects of phosphorylation at the β3 subunit are well documented, with a number of studies reporting that phosphorylation can result in both enhancement and reduction of the GABA-activated current, depending on the protein kinase present (Moss and Smart, 1996). For example, PKC-mediated phosphorylation at β3 subunit-containing $GABA_A$ receptors has been shown to induce a reduction in receptor function (Brandon *et al.*, 2000), whereas phosphorylation by PKA results in an enhancement of GABA-activated responses (McDonald *et al.*, 1998). Therefore, it was hypothesised that phosphorylation at the β3 subunit may also act to modulate the potentiation of $GABA_A$ receptor function by neurosteroids. To investigate this, cells were transfected to express $GABA_A$ receptors containing either $\beta3^{S408A}$, $\beta3^{S409A}$ or $\beta3^{S408A,S409A}$ in combination with wild-type α1 and γ2L subunits. After establishing the baseline and potentiated responses to GABA under basal cell conditions, cells were treated with the broad-spectrum protein kinase inhibitor, staurosporine (200nM; Fig. 4.1). As in the previous experiments, during the treatment period, the cell's response to EC_{20} GABA was monitored every 2 min in order to examine the effects of staurosporine on the receptor's response to agonist alone. In cells expressing $\alpha1\beta3^{S408A}\gamma2L$, $\alpha1\beta3^{S409A}\gamma2L$ or $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, treatment with 200nM staurosporine for 20 min caused no significant change in the magnitude of the response elicited by EC_{20} GABA (percentage change measured between 2 and 32 min = 6.1 ± 7.3%, 2.9 ± 8.4% and 9.4 ± 7.2%, respectively for each receptor isoform, mean ± s.e.m., $P > 0.05$; n = 4-5; Fig. 4.4C).

After treatment with staurosporine, the baseline and potentiated responses were re-measured and the potentiation elicited by 50nM THDOC, before and after protein kinase inhibition, was compared. Similar to the results obtained for wild-type receptors, THDOC-mediated potentiation at both $\alpha1\beta3^{S408A}\gamma2L$ and $\alpha1\beta3^{S409A}\gamma2L$ $GABA_A$ receptors was significantly reduced after treatment with 200nM staurosporine for 14 min (decrease in potentiation = 36 ± 7.5% and 54 ± 6.9%, respectively, $P < 0.05$, paired t-test; n = 5; Fig. 4.1A,B,C). In contrast, cells expressing $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors showed no change in the magnitude of potentiation elicited (change = 1.74 ± 8.7%, $P > 0.05$; n = 5; Fig. 4.1A,B,C). This indicated that phosphorylation at the β3 subunit is important for the PKC-mediated modulation of neurosteroid potentiation at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors. Furthermore, the requirement for both S408 and S409 to be mutated in order to abolish the staurosporine-induced decrease in THDOC-mediated potentiation, suggests that phosphorylation at either β3 S408 or S409 is sufficient to modulate the actions of neurosteroids at these receptors (Fig. 4.1C).

Interestingly, the decrease in potentiation observed with staurosporine at $\alpha1\beta3^{S408A}\gamma2L$ receptors was less than that seen at both wild-type and $\alpha1\beta3^{S409A}\gamma2L$ $GABA_A$ receptors. This suggests that phosphorylation at β3 S408 is more effective at modulating THDOC-mediated potentiation. Another notable observation from this experiment was that, under basal cell conditions (i.e. before staurosporine treatment), the magnitude of potentiation elicited by 50nM THDOC at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors was significantly reduced compared to that induced at wild-type receptors (48.6 ± 10.8% versus 99.1 ± 13.8%, $P < 0.05$, ANOVA with Tukey's post hoc test; n = 5-6; Fig. 4.1C, see also Fig. 4.5). This decrease may be due to the β3 subunit being phosphorylated under basal cell conditions, a post-translational modification which cannot occur at the mutated subunit. Therefore, as the magnitude of potentiation was reduced under conditions where this subunit cannot be phosphorylated, this supports the notion that phosphorylation at the β3 subunit is important for regulating neurosteroid activity at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors.

The use of staurosporine in this experiment was a rapid way of determining that phosphorylation of the β3 subunit is important for modulating the actions of neurosteroids at $GABA_A$ receptors. However, the results from the previous chapter demonstrated that THDOC-mediated potentiation at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors was specifically modulated by PKC (see Chapter 3) and therefore, in order to investigate the actions of PKC alone, this experiment was repeated using compounds that specifically activate or inhibit PKC.

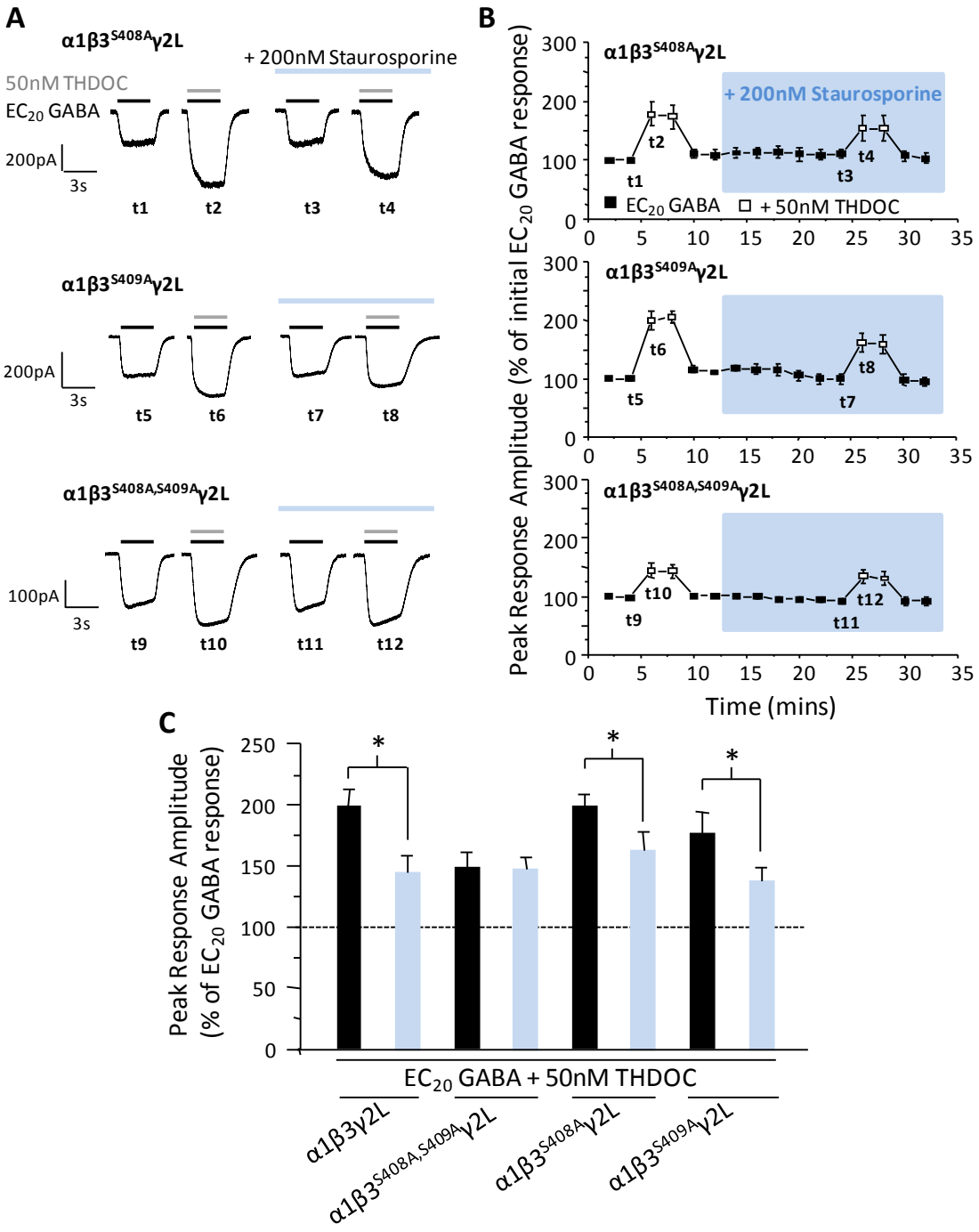

Figure 4.1 Staurosporine decreases THDOC-mediated potentiation at α1β3^{S408A}γ2L and α1β3^{S409A}γ2L, but not at α1β3^{S408A,S409A}γ2L GABA$_A$ receptors

(A,B) Mean peak GABA currents recorded from HEK293 cells expressing α1β3^{S408A}γ2L (upper panel; n = 5), α1β3^{S409A}γ2L (middle panel; n = 5) or α1β3^{S408A,S409A}γ2L (lower panel; n = 5) GABA$_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells were treated with 200nM staurosporine (light blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0 (= 100%). Example whole-cell currents (panel A) show the potentiation elicited by 50nM THDOC (grey bars) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated in B. (C) Bar chart showing the potentiation of EC_{20} GABA-activated currents by 50nM THDOC before (black bars) and after (light blue bars) staurosporine treatment in cells expressing α1β3γ2L (n = 6) α1β3^{S408A}γ2L (n = 5), α1β3^{S409A}γ2L (n = 5) or α1β3^{S408A,S409A}γ2L (n = 5) GABA$_A$ receptors. Data displayed for α1β3γ2L receptors is as shown previously in Fig. 3.1. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

4.2.2 Phosphorylation by PKC at β3 S408 or S408 is sufficient to modulate neurosteroid activity

To confirm whether PKC does indeed modulate the actions of neurosteroids through direct phosphorylation of the β3 subunit, cells were treated with either 100nM PMA or 500nM bisindolylmaleimide I (Bis I), in order to specifically activate or inhibit PKC, respectively. After treatment with 100nM PMA, cells expressing $\alpha1\beta3^{S408A}\gamma2L$ or $\alpha1\beta3^{S409A}\gamma2L$ subunit-containing $GABA_A$ receptors exhibited significant enhancements in THDOC-mediated potentiation of 54.6 ± 13.8% and 61.1 ± 37%, respectively ($P < 0.05$, paired t-test; n = 4; Fig. 4.2A,B), which were similar in magnitude to the increase observed at wild-type receptors (see Fig. 3.6). In contrast, potentiation at $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors was unaffected by treatment with either PMA or Bis I (change = 7.3 ± 9.0% and 1.6 ± 8.3%, respectively, $P > 0.05$; n = 4-5; Fig. 4.2A,B and Fig. 4.3A,B), indicating that these residues are important for the modulatory effects of PKC observed previously. These results support those obtained from the staurosporine experiment, demonstrating that PKC-mediated phosphorylation at β3 S408 or S409 is sufficient to modulate the magnitude of potentiation elicited by 50nM THDOC at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors.

The results from the PKC inhibition experiment showed once again that, under basal cell conditions, the magnitude of THDOC-mediated potentiation induced at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors was significantly reduced compared to that observed at wild-type receptors (140.6 ± 19.2% versus 45.7 ± 12.9%, $P < 0.05$, paired t-test; n = 4-5; Fig. 4.3A,B). This supports the idea that basal phosphorylation at the wild-type receptor, which is eliminated from the mutant receptor, may result in an apparent enhancement in the level of potentiation elicited by 50nM THDOC. Curiously, in the PKC activation experiment, no significant difference was observed between the potentiation elicited at wild-type and $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors. However, the results do reveal a trend towards a decrease in the magnitude of potentiation elicited at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, with 50nM THDOC inducing only a 48.8 ± 6.5% enhancement of the EC_{20} GABA-activated current at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors compared to the 70.2 ± 17.3% potentiation elicited at wild-type receptors (Fig. 4.2B).

Previous studies have reported that PKC-mediated phosphorylation of β3 subunit-containing $GABA_A$ receptors causes a decrease in receptor function (Brandon *et al*., 2000). This was supported by results from the present study which showed that, at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors, activation of PKC induces a significant reduction in the EC_{20} GABA-activated current (see Fig. 3.6). Similar results were obtained for $\alpha1\beta3^{S408A}\gamma2L$ and $\alpha1\beta3^{S409A}\gamma2L$ $GABA_A$ receptors, with

both exhibiting significant decreases in the receptor's response to EC_{20} GABA after treatment with 100nM PMA for 36 min (decrease = 22.6 ± 4.5% and 26.5 ± 7.6%, respectively, $P < 0.05$, paired t-test; n = 4; Fig. 4.2C). Interestingly, the reductions in function observed at these receptors were slightly diminished compared to that seen at wild-type receptors (35 ± 8%; Fig. 4.3C, see also Fig. 3.6), which may reflect the reduction in potential phosphorylation sites from four to three, reducing the receptor's capacity for modulation by PKC. In contrast, at $\alpha1\beta3^{S408A,S409A}\gamma2L$ subunit-containing $GABA_A$ receptors, the change in EC_{20} GABA-activated current was not statistically significant, indicating that phosphorylation at the β3 subunit plays an important role in modulating the receptor's response to agonist. However, a closer look at the results reveals that, although not significant, a trend towards a decrease in the responses elicited by EC_{20} GABA was evident at $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors after treatment with 100nM PMA (decrease = 25.3 ± 11.6%, $P > 0.05$; n = 5; Fig. 4.2C). This indicates that, at receptors containing the mutated $\beta3^{S408A,S409A}$ subunit, some reduction in receptor function can still be induced after activation of PKC, implying that phosphorylation at sites independent of those contained on the β3 subunit may also contribute to this effect. In addition to β3 serines 408 and 409, PKC can also phosphorylate two residues on the γ2L subunit; serines 327 and 343. Therefore, phosphorylation at these sites may also be important for regulating the function of $\alpha1\beta3\gamma2L$ $GABA_A$ receptors and thus, could also play a role in modulating the actions of neurosteroids at these receptors.

Curiously, the results from the present study showed that, at both wild-type and $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, inhibition of PKC caused no significant alteration of EC_{20} GABA-activated responses (Fig. 4.4C). Therefore, it was important to confirm whether Bis I was really inhibiting PKC in these experiments. To investigate this, Western blot analysis was used to examine the levels of phosphorylated β3 before and after treatment with 500nM Bis I for 40 min. Lysates were prepared from HEK293 cells expressing $\alpha1\beta3\gamma2L$ $GABA_A$ receptors and were probed in duplicate with β3 and phospho-β3 anti-sera (see Methods and Materials). To enable a direct comparison to be made between the untreated and treated cell populations the level of phosphorylated β3 was normalized to the corresponding amount of total β3 present. As expected, exposure of cells to Bis I resulted in a clear reduction in the extent of β3 subunit phosphorylation (Fig. 4.4D), indicating that this compound is indeed effective at inhibiting PKC.

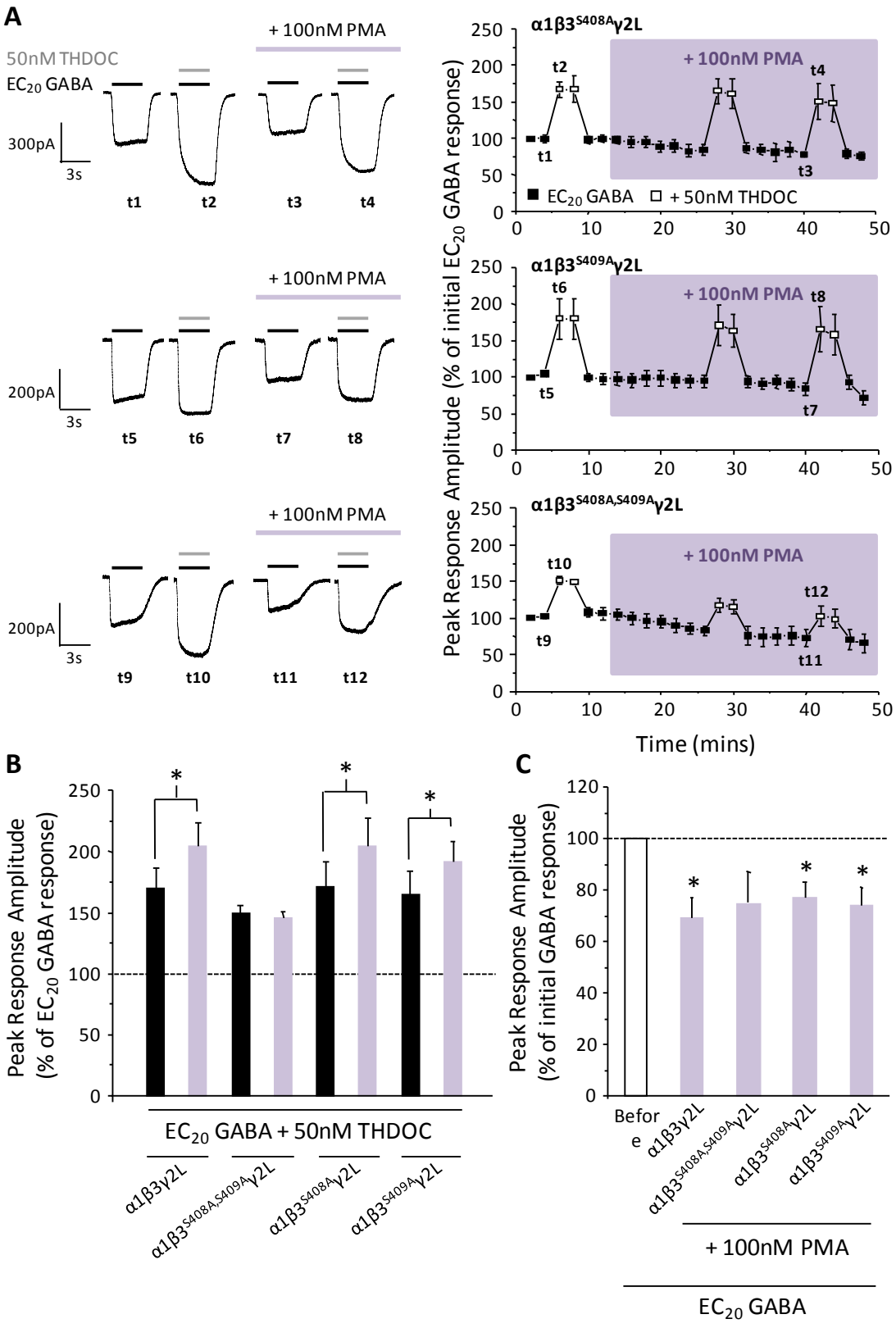
A
+ 100nM PMA
50nM THDOC
EC20 GABA
300pA
3s
t1
t2
t3
t4
+ 100nM PMA
200pA
3s
t5
t6
t7
t8
+ 100nM PMA
200pA
3s
t9
t10
t11
t12
α1β3S408Aγ2L
α1β3S409Aγ2L
α1β3S408A,S409Aγ2L
+ 100nM PMA
EC20 GABA
+ 50nM THDOC
Peak Response Amplitude (% of initial EC20 GABA response)
Time (mins)
B
Peak Response Amplitude (% of EC20 GABA response)
EC20 GABA + 50nM THDOC
α1β3γ2L
α1β3S408A,S409Aγ2L
α1β3S408Aγ2L
α1β3S409Aγ2L
C
Peak Response Amplitude (% of initial GABA response)
Before
α1β3γ2L
α1β3S408A,S409Aγ2L
α1β3S408Aγ2L
α1β3S409Aγ2L
+ 100nM PMA
EC20 GABA

Figure 4.2 PMA enhances THDOC-mediated potentiation at $\alpha1\beta3^{S408A}\gamma2L$ and $\alpha1\beta3^{S409A}\gamma2L$, but not at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors

(A) Mean peak GABA currents recorded from HEK293 cells expressing $\alpha1\beta3^{S408A}\gamma2L$ (upper panel; n = 4), $\alpha1\beta3^{S409A}\gamma2L$ (middle panel; n = 4) or $\alpha1\beta3^{S408A,S409A}\gamma2L$ (lower panel; n = 5) $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells were treated with 100nM PMA (purple shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bars) before and after PMA treatment (purple bar). Current traces are representative of recordings at the time points indicated. (B) Bar chart showing the potentiation of EC_{20} GABA-activated currents by 50nM THDOC before (black bars) and after (purple bars) PMA treatment in cells expressing $\alpha1\beta3\gamma2L$ (n = 6), $\alpha1\beta3^{S408A}\gamma2L$ (n = 4), $\alpha1\beta3^{S409A}\gamma2L$ (n = 4) or $\alpha1\beta3^{S408A,S409A}\gamma2L$ (n = 5) $GABA_A$ receptors. Data displayed for $\alpha1\beta3\gamma2L$ receptors is as shown previously in Fig. 3.6. (C) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA before (open bar) or after treatment with 100nM PMA (purple bars) in cells expressing $\alpha1\beta3\gamma2L$ (n = 6), $\alpha1\beta3^{S408A}\gamma2L$ (n = 4), $\alpha1\beta3^{S409A}\gamma2L$ (n = 4) or $\alpha1\beta3^{S408A,S409A}\gamma2L$ (n = 5) $GABA_A$ receptors. Change was measured between 2 min (before treatment) and 48 min (after treatment). Responses were normalised to the peak current recorded at 2 min. Data displayed for $\alpha1\beta3\gamma2L$ receptors is as shown previously in Fig. 3.6. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

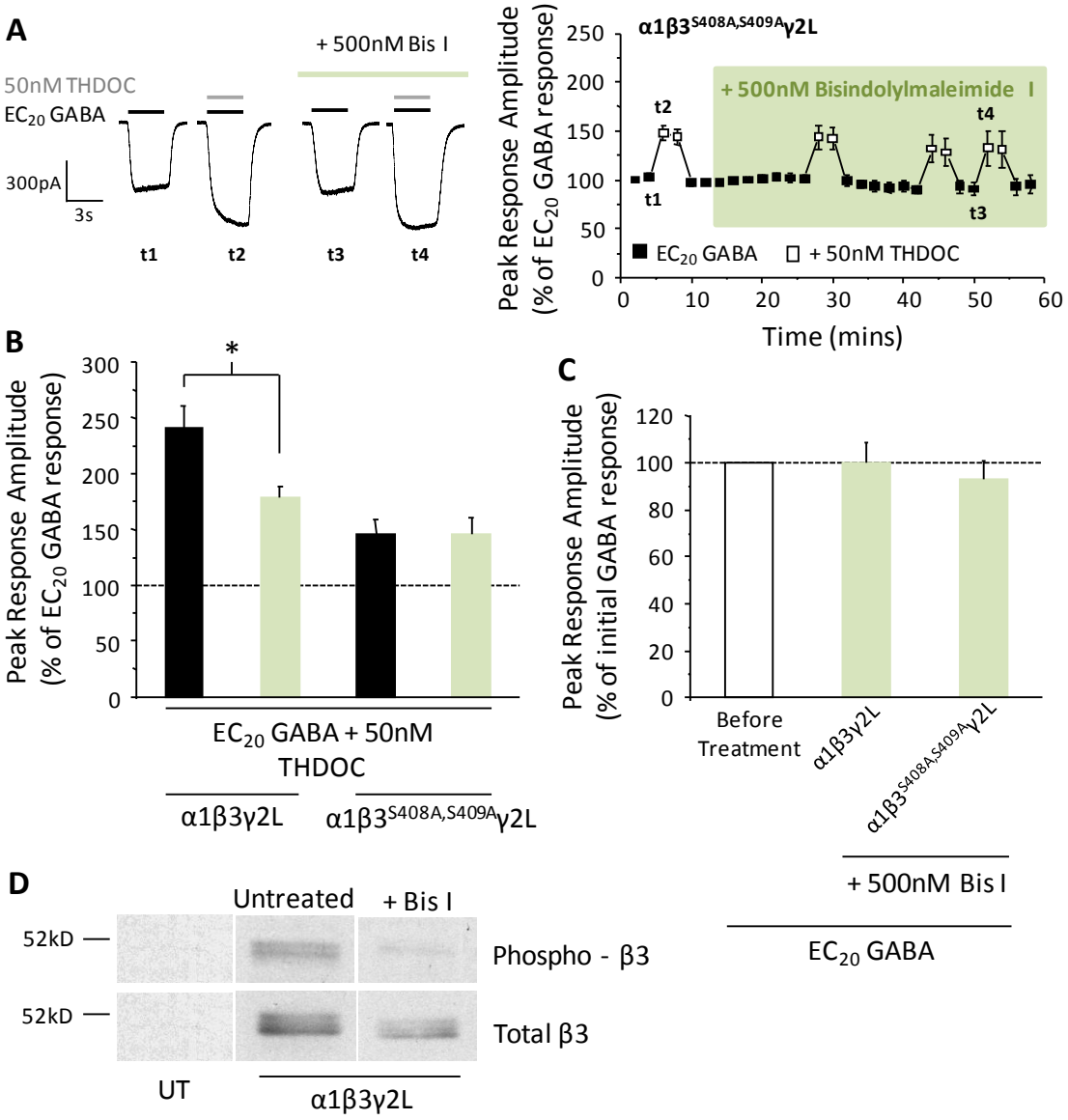

Figure 4.3 Bisindolylmaleimide I does not decrease THDOC-mediated potentiation at α1β3^{S408A,S409A}γ2L GABA$_A$ receptors

(A) Mean peak GABA currents recorded from HEK293 cells expressing α1β3^{S408A,S409A}γ2L (n = 4) GABA$_A$ receptors in response to EC$_{20}$ GABA (filled squares) or EC$_{20}$ GABA + 50nM THDOC (open squares). Cells were treated with 500nM Bisindolylmaleimide I (Bis I; green shading). All responses were normalised to the peak current of the first EC$_{20}$ GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bars) before and after Bis I treatment (green bar). Current traces are representative of recordings at the time points indicated. (B) Bar chart showing the potentiation of EC$_{20}$ GABA-activated currents by 50nM THDOC before (black bars) and after (green bars) Bis I treatment in cells expressing α1β3γ2L (n = 5) or α1β3^{S408A,S409A}γ2L (n = 4) GABA$_A$ receptors. Data displayed for α1β3γ2L receptors is as shown previously in Fig. 3.3. (C) Bar chart showing the change in peak receptor response elicited by EC$_{20}$ GABA before (open bar) or after treatment with 500nM Bis I (green bars) in cells expressing α1β3γ2L (n = 5) or α1β3^{S408A,S409A}γ2L (n = 4) GABA$_A$ receptors. Change was measured between 2 min (before treatment) and 58 min (after treatment). Responses were normalised to the peak current recorded at 2 min. Data displayed for α1β3γ2L receptors is as shown previously in Fig. 3.3. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test). (D) Western blot showing the level of GABA$_A$ receptor phosphorylation before and after treatment with Bis I. HEK293 cells expressing α1β3γ2L GABA$_A$ receptors were treated for 40 min with 500nM Bis I and probed with β3 and phospho-β3 anti-sera (see Methods and Materials). Untransfected (UT) cells were used as a negative control.

4.2.3 Phosphorylation at the γ2L subunit does not modulate THDOC-mediated potentiation

In addition to β3 serines 408 and 409, PKC has also been shown to phosphorylate two residues on the γ2L subunit. Therefore, further experiments were carried out to investigate whether phosphorylation at the γ2L subunit contributes towards the modulatory actions of neurosteroids at α1β3γ2L $GABA_A$ receptors. Accordingly, HEK293 cells were transfected to express $GABA_A$ receptors containing $\gamma2L^{S327A,S343A}$ subunits in combination with wild-type α1 and β3, in order to specifically determine whether phosphorylation of γ2L is important for the regulation of neurosteroid activity by protein kinases. After treatment with 200nM staurosporine for 20 min, cells expressing $\alpha1\beta3\gamma2L^{S327A,S343A}$ $GABA_A$ receptors exhibited no significant change in the current response elicited by EC_{20} GABA (3.7 ± 9.1%, $P > 0.05$; $n = 4$; Fig. 4.4C). In contrast, THDOC-mediated potentiation was significantly reduced by 50.3 ± 7.5% after staurosporine treatment ($P < 0.05$, paired t-test; $n = 4$; Fig. 4.4A,B), a decrease very similar to that observed for wild-type receptors (Fig 4.4. A,B see also Fig. 3.1). This suggests that phosphorylation at γ2L S327 and/or S343 does not play a significant role in the modulation of neurosteroid activity at α1β3γ2L $GABA_A$ receptors.

Taken together, the results indicate that, at α1β3γ2L $GABA_A$ receptors, PKC acts to modulate THDOC-mediated potentiation through direct phosphorylation at the β3, but not the γ2L subunit. Furthermore, it appears that PKC-mediated phosphorylation at either S408 or S409 is sufficient for this modulation to occur.

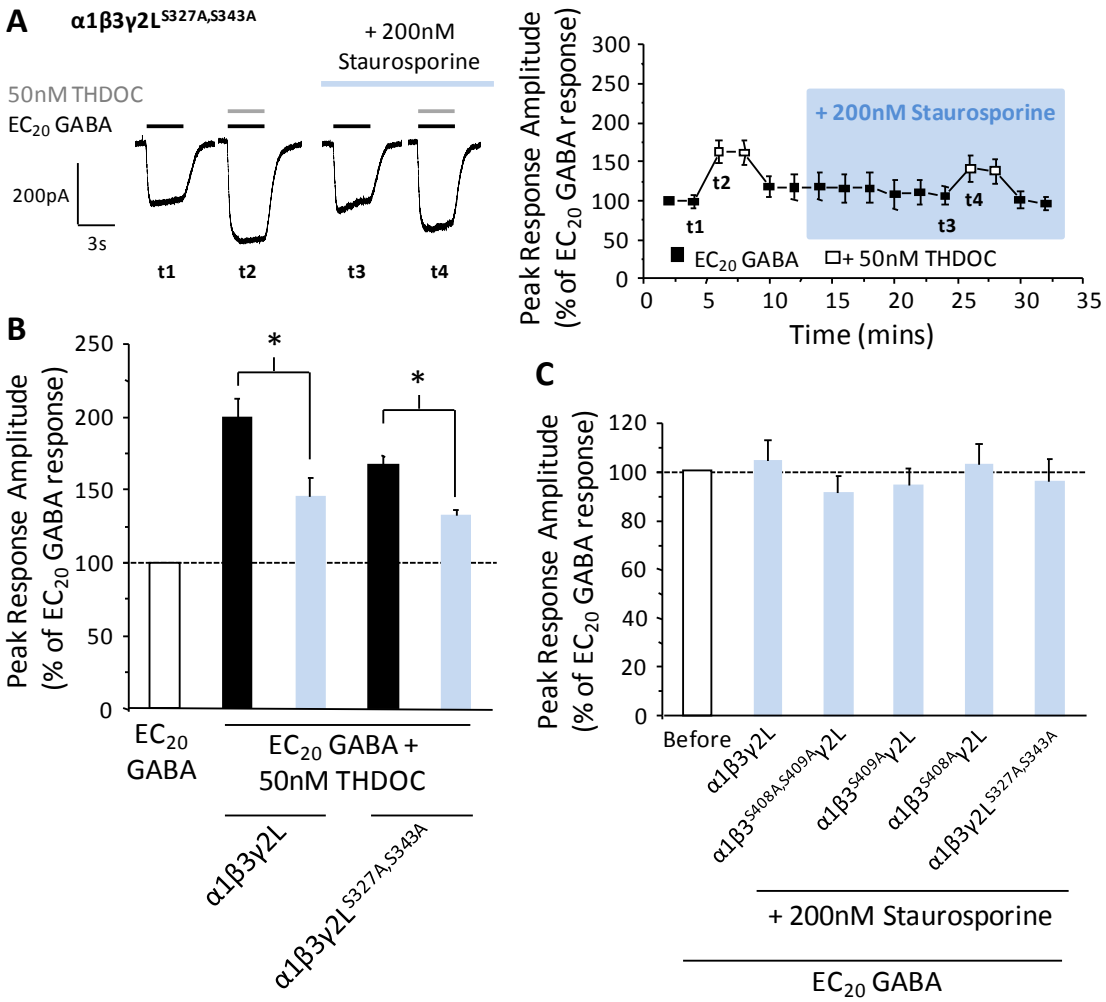

Figure 4.4 Staurosporine decreases THDOC-mediated potentiation at α1β3γ2L^{S327A,S343A} GABA$_A$ receptors

(A) Mean peak currents recorded from HEK293 cells expressing α1β3γ2L^{S327A,S343A} (n = 4) GABA$_A$ receptors in response to EC$_{20}$ GABA (filled squares) or EC$_{20}$ GABA + 50nM THDOC (open squares). Cells were treated with 200nM staurosporine (light blue shading). All responses were normalised to the peak current of the first EC$_{20}$ GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bars) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated. (B) Bar chart showing the potentiation of EC$_{20}$ GABA-activated currents by 50nM THDOC before (black bars) and after (light blue bars) staurosporine treatment in cells expressing α1β3γ2L (n = 6) or α1β3γ2L^{S327A,S343A} (n = 4) GABA$_A$ receptors. Data displayed for α1β3γ2L GABA$_A$ receptors is as shown previously in Fig. 3.1 (C) Bar chart showing the change in peak receptor response elicited by EC$_{20}$ GABA before (open bar) or after treatment with 200nM staurosporine (light blue bars) in cells expressing α1β3γ2L (n = 6), α1β3^{S408A}γ2L (n = 5), α1β3^{S409A}γ2L (n = 5), α1β3^{S408A,S409A}γ2L (n = 5) or α1β3γ2L^{S327A,S343A} GABA$_A$ receptors. Change was measured between 2 min (before treatment) and 32 min (after treatment). Responses were normalised to the peak current recorded at 2 min. Data displayed for α1β3γ2L receptors is as shown previously in Fig. 3.1. All data points represent mean ± s.e.m. Significant results are indicated by * (P < 0.05, paired t-test).

4.2.4 Phosphorylation is not required for THDOC-mediated potentiation

An interesting conclusion from the previous chapter was that neurosteroid-mediated potentiation is modulated by, but does not absolutely require protein kinase activity. This was demonstrated by the fact that potentiation was still evident after cells had been exposed to protein kinase inhibitors (see Fig. 3.1 and 3.3). However, it remains possible that this may be because the staurosporine or bisindolylmaleimide I treatments did not completely abolish the activity of their target protein kinases, resulting in potentiation being induced due to residual protein kinase activity. Therefore, in order to investigate this further, the magnitude of potentiation elicited by 50nM THDOC was assessed in cells expressing $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ $GABA_A$ receptors, effectively eliminating PKC-induced phosphorylation at all known sites within the receptor. Consistent with the results presented in the previous chapter, potentiation was still observed at these receptors (potentiation induced by 50nM THDOC = 45.7 ± 7.1%; Fig. 4.5), once again indicating that phosphorylation, by protein kinases, is not required for the potentiation of $GABA_A$ receptor function by neurosteroids.

The magnitude of the neurosteroid potentiation induced at $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ $GABA_A$ receptors was significantly reduced compared to that observed at wild-type receptors (45.7 ± 7.1% versus 99.1 ± 13.8%, $P < 0.05$, ANOVA with Tukey's post hoc test; n = 4-5; Fig. 4.5, see also Fig. 4.1). This is, perhaps, unsurprising given that the results from the present study have indicated that the level of basal phosphorylation of the receptor may be an important determinant of the extent of potentiation. However, it is interesting to note that the level of potentiation elicited at these receptors is very similar to that seen at $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors (45.7 ± 7.1% versus 48.6 ± 10.8%, $P > 0.05$; Fig. 4.5, see also Fig. 4.1), indicating that additional mutation of γ2L serines 327 and 343 does not reduce the magnitude of potentiation beyond that previously observed for $\alpha1\beta3^{S408A,S409A}\gamma2L$ receptors. This supports the results obtained in earlier experiments, suggesting that phosphorylation at the β3, but not the γ2L subunit, is important for regulating neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors.

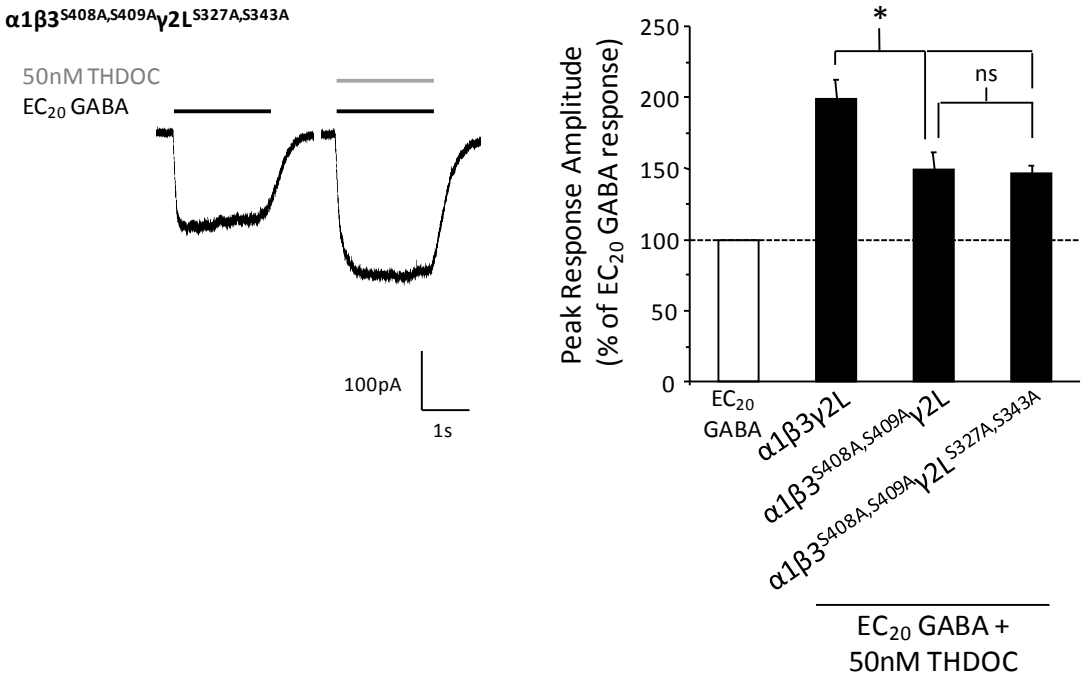

Figure 4.5 THDOC-mediated potentiation is still evident at $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ $GABA_A$ receptors

Mean peak currents recorded from HEK293 cells expressing $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ (n = 4) $GABA_A$ receptors in response to EC_{20} GABA or EC_{20} GABA + 50nM THDOC (as indicated). Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in cells expressing α1β3γ2L (n = 6), $\alpha1\beta3^{S408A,S409A}\gamma2L$ (n = 5) or $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ (n = 4) $GABA_A$ receptors. Data displayed for α1β3γ2L and $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors is as shown previously in Fig. 3.1 and 4.1, respectively. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, ANOVA with Tukey's post hoc test).

4.2.5 THDOC enhances β3 subunit phosphorylation via an interaction with the $GABA_A$ receptor

The experiments conducted throughout this study have focused on the ability of phosphorylation, by protein kinases, to modulate the actions of neurosteroids at $GABA_A$ receptors. However, this reflects just one aspect of the co-regulatory relationship and does not address the potential for this modulation to be bi-directional. Therefore, in order to investigate whether, in the reverse situation, neurosteroids can act to modulate the activities of protein kinases, Western blot analysis was used to assess the extent of β3 subunit phosphorylation in HEK293 cells expressing α1β3γ2L $GABA_A$ receptors, before and after exposure to a neurosteroid. Cells either remained untreated or were exposed to 50nM THDOC for 5, 10 or 20 min. To enable direct comparisons to be made between the separate cell populations undergoing different treatment protocols, lysates were probed in duplicate with both β3 and phospho-β3 anti-sera and for each treatment group, the level of phosphorylated β3 was normalized to the corresponding amount of total β3 present.

To verify the specificity of the antibodies, control experiments were carried out in HEK293 cells expressing either $\alpha1\beta3^{S408A,S409A}\gamma2L$ or α1 and γ2L subunits in the absence of β3. As expected, no phosphorylated β3 was detected in cells expressing $\alpha1\beta3^{S408A,S409A}\gamma2L$ (Fig. 4.6A lane 1) and signals for both β3 and phosphorylated β3 were absent in cells expressing just α1 and γ2L subunits (Fig. 4.6A lane 2), confirming that the antibodies are indeed specific for their respective targets.

Experiments carried out in untreated cells confirmed that there is some basal phosphorylation of the β3 subunit when it is co-expressed with α1 and γ2L in HEK293 cells (Fig. 4.6A lane 3). After treatment of cells with 50nM THDOC for 5 min, there was little change in the level of β3 subunit phosphorylation (10.7 ± 11.6% increase, $P > 0.05$; n = 3; Fig. 4.6A lane 4, Fig. 4.6C). A tendency towards increased phosphorylation was detected in cells treated with THDOC for 10 min (increase = 78.7 ± 34.9%, $P > 0.05$; n = 3; Fig. 4.6A lane 5, Fig. 4.6C), and cells exposed to THDOC for 20 min exhibited a significant enhancement in the extent of β3 subunit phosphorylation of 96.3 ± 27.9% ($P < 0.05$, ANOVA with Dunnett's post hoc test; n = 3; Fig. 4.6A lane 6, Fig. 4.6C). This indicates that THDOC can enhance β3 subunit phosphorylation, implying the presence of a 'reverse' transduction pathway in which neurosteroids can modulate the phosphorylation of the $\alpha1\beta3\gamma2L$ $GABA_A$ receptor.

To gauge the extent to which THDOC can increase β3 subunit phosphorylation, the effects of the neurosteroid were compared to those of PMA, a potent activator of PKC. Cells treated with 100nM PMA for 30 min exhibited a 193.3 ± 34.9% increase in the level of phosphorylated β3 (Fig. 4.6A lane 7), an enhancement twice as large as that observed for THDOC, indicating that neurosteroids are less effective at enhancing β3 subunit phosphorylation when compared to compounds which can directly activate PKC.

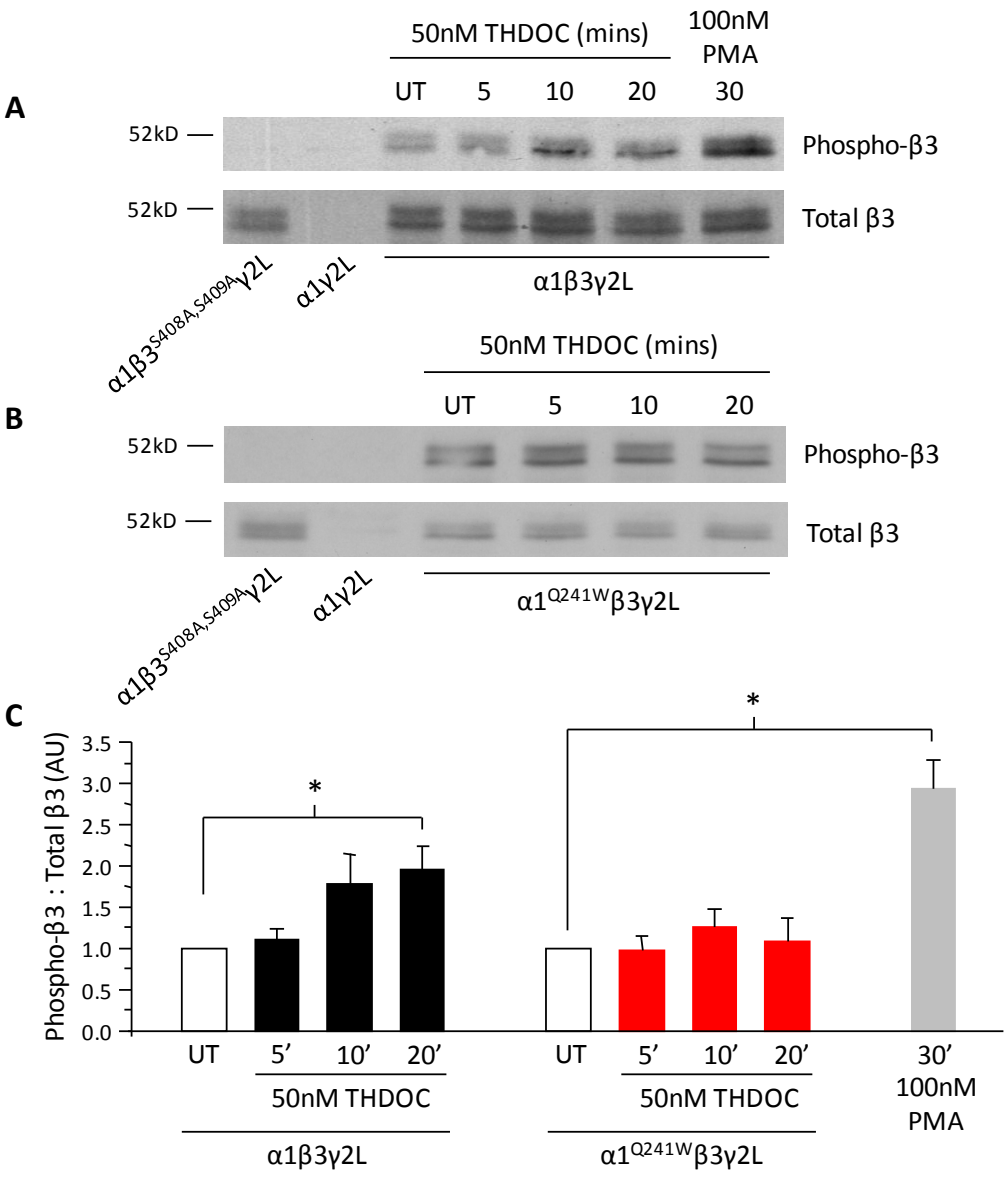

Figure 4.6 THDOC enhances phosphorylation of β3 S408/S409 by binding to the $GABA_A$ receptor.

(A,B) Western blots showing the level of β3 subunit phosphorylation in HEK293 cells expressing (A) α1β3γ2L or (B) $\alpha1^{Q241W}$β3γ2L $GABA_A$ receptors. Cells either remained untreated (UT) or were exposed to 50nM THDOC for 5, 10 or 20 min or 100nM PMA for 30 min (as indicated) and probed with β3 or phospho-β3 anti-sera. Cells transfected with $\alpha1\beta3^{S408A,S409A}\gamma2L$ or α1γ2L $GABA_A$ receptor subunits were used as negative controls. (C) Bar chart shows the levels of phosphorylated β3 subunits present in cells expressing α1β3γ2L (black/grey bars; n = 3) or $\alpha1^{Q241W}$β3γ2L (red bars; n = 3), in untreated cells (UT) or cells treated with 50nM THDOC for 5, 10 or 20 min or 100nM PMA for 30 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, ANOVA with Dunnett's post hoc test).

Although this experiment demonstrated that THDOC is able to modulate the levels of phosphorylation at the $GABA_A$ receptor, it remained unclear whether neurosteroids are mediating this effect through a direct interaction with the $GABA_A$ receptor. Neurosteroids are thought to potentiate $GABA_A$ receptor function by binding at a discrete site located within the transmembrane region of the α subunit (Hosie *et al.*, 2006; Hosie *et al.*, 2008). Three key

residues have been identified to date, Q241, N407 and Y410, which when replaced with hydrophobic residues, resulted in the attenuation, or in the case of Q241 mutants, the complete ablation of potentiation by neurosteroids (Hosie *et al*., 2006). Therefore, in order to examine whether THDOC acts to enhance receptor phosphorylation through an interaction with the $GABA_A$ receptor, the previous experiment was repeated in cells expressing $\alpha1^{Q241W}\beta3\gamma2L$ $GABA_A$ receptors. In untreated cells, basal phosphorylation was still evident at the β3 subunit (Fig. 4.6B lane 3), indicating that these receptors can be phosphorylated as normal, however, after treatment of cells with 50nM THDOC for 5, 10 or 20 min, no significant alteration in β3 subunit phosphorylation was observed (Changes = -2.7 ± 18.4%, 25.0 ± 22.7% and 9.3 ± 27.4%, respectively, $P > 0.05$; $n = 3$; Fig. 4.6B lanes 4-6). This suggests that THDOC acts to modulate $GABA_A$ receptor phosphorylation through a direct interaction with the receptor complex. Furthermore, as the mutation of Q241 is sufficient to eliminate any enhancement in receptor phosphorylation, this indicates that occupancy of the neurosteroid binding site is likely to be facilitating this modulation.

Taken together, the results show that a complex, bi-directional relationship exists between neurosteroids and protein kinases at the $\alpha1\beta3\gamma2L$ $GABA_A$ receptor, with phosphorylation at the receptor acting to modulate the actions of neurosteroids and, in the reverse situation, exposure to neurosteroids causing an alteration in the extent of receptor phosphorylation.

4.3 Discussion

4.3.1 PKC-mediated phosphorylation at the β3 subunit regulates neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors

Using recombinant $GABA_A$ receptors containing mutations designed to prevent phosphorylation at one or more candidate sites on the receptor, it has been possible to identify the residues at which PKC acts to modulate the potentiating actions of neurosteroids. The results from this study showed that, at $GABA_A$ receptors incorporating mutant $\beta3^{S408A,S409A}$ subunits, the magnitude of potentiation elicited by 50nM THDOC was significantly reduced compared to that observed at wild-type receptors. Furthermore, the potentiation induced at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors was unaffected by treatment with either staurosporine, bisindolylmaleimide I or PMA (see Fig. 4.1, 4.2, 4.3 and 4.5). This indicates that phosphorylation at the β3 subunit is important for regulating the potentiation of $GABA_A$ receptor function by neurosteroids. In addition, the apparent decrease in the magnitude of potentiation induced at $\alpha1\beta3^{S408A,S409A}\gamma2L$ versus wild-type $GABA_A$ receptors suggests that, in HEK293 cells, the β3 subunit is likely to be phosphorylated under basal cell conditions, which, when eliminated from the mutant receptor, results in the reduced level of neurosteroid-mediated potentiation observed in these experiments. This was confirmed by Western blot analysis which demonstrated that the β3 subunit is indeed phosphorylated under basal cell conditions (see Fig. 4.4 and 4.6) and, moreover, that this phosphorylation is mediated, at least in part, by PKC (see Fig. 4.4), which is consistent with the previous results demonstrating that the actions of neurosteroids at α1β3γ2L $GABA_A$ receptors are modulated exclusively by PKC (see Chapter 3).

Basal phosphorylation at the $GABA_A$ receptor β3 subunit has also been detected in a number of neuronal populations, including those from the cortex, hippocampus and striatum (Brandon *et al*., 2000; Brandon *et al*., 2003; Terunuma *et al*., 2008). Therefore, the results from the present study provide a good indication that, in these neurons, the actions of neurosteroids could be both enhanced and reduced, depending on the activity of PKC, which may provide an ideal mechanism for the rapid fine-tuning of $GABA_A$ receptor function *in vivo*, particularly at inhibitory synapses.

Curiously, the results from the PMA experiment differed slightly from those obtained from cells treated with either staurosporine or bisindolylmaleimide I in that, although there was a trend towards a decrease in the magnitude of potentiation elicited at $\alpha1\beta3^{S408A,S409A}\gamma2L$ versus

wild-type $GABA_A$ receptors, this did not reach statistical significance (see Fig. 4.2). The reason for this is unclear but one potential explanation is that, in the PMA experiment, the results obtained from the cells expressing wild-type receptors (see Fig. 3.6) may have been unintentionally skewed. A number of studies have shown that the activation of PKC results in a reduction in $GABA_A$ receptor function (Krishek *et al*., 1994; Brandon *et al*., 2000). Therefore, in order to ensure that PKC was definitely being activated in this experiment, those cells which exhibited no clear decrease in EC_{20} GABA-activated current after treatment with PMA were excluded from analysis. It can be hypothesized that the cells in which PMA appeared to have no effect on $GABA_A$ receptor function are likely to be those which contained the highest levels of basal receptor phosphorylation, meaning that further increases in PKC activity would have no further effect on GABA-activated responses. Therefore, considering the results from the present study, these cells are also likely to be those which display the highest levels of neurosteroid potentiation, thus eliminating these cells from analysis could have resulted in a slight decrease in the mean potentiation calculated for the wild-type cell group, hence the magnitude of neurosteroid-mediated potentiation induced in these cells does not differ significantly from that observed at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors.

4.3.2 Phosphorylation at β3 S408 or S409 is sufficient to modulate neurosteroid activity

A notable observation from the present study was that, in contrast to $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, cells expressing either $\alpha1\beta3^{S408A}\gamma2L$ or $\alpha1\beta3^{S409A}\gamma2L$ receptors exhibited similar changes in neurosteroid-mediated potentiation, after treatment with either staurosporine or PMA, to those observed at wild-type receptors (see Fig. 4.1 and 4.2). This indicates that PKC-mediated phosphorylation at either S408 or S409 is sufficient to modulate the actions of neurosteroids at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors. Interestingly, after treatment with staurosporine, the decrease in potentiation observed at $\alpha1\beta3^{S408A}\gamma2L$ $GABA_A$ receptors appeared to be less than that seen at $\alpha1\beta3^{S409A}\gamma2L$ receptors (see Fig. 4.1). This may suggest that phosphorylation at S408 is slightly more effective at modulating the actions of neurosteroids. Alternatively, this discrepancy may be due to the presence of higher levels of basal phosphorylation at S408 which could result in this site having a greater potential for modulation by protein kinases.

Although the β3 subunit contains two potential phosphorylation sites, the results from the present study showed that phosphorylation at either site was sufficient to modulate neurosteroid-mediated potentiation (see Fig. 4.1 and 4.2). This suggests that the mechanism by which phosphorylation acts to alter the actions of neurosteroids at $\alpha1\beta3\gamma2L$ $GABA_A$

receptors most likely requires the addition of just a single phosphate moiety at the β3 subunit. Therefore, it may be interesting to explore whether $GABA_A$ receptors containing β1 or β2 subunits are modulated to a similar extent as β3 subunit-containing receptors, as previous studies have shown that, although all members of the β subunit family contain a conserved phosphorylation site at S409 (S410 in the β2 subunit; Moss *et al*., 1992a; McDonald and Moss, 1997), the β3 subunit also contains a unique site at S408, which is not present in either β1 or β2 (see Fig. 4.7; McDonald and Moss, 1997; McDonald *et al*., 1998). Given that the extent of receptor phosphorylation appears to be important for regulating the actions of neurosteroids at $GABA_A$ receptors, this may indicate that, at β3 subunit-containing $GABA_A$ receptors, the actions of protein kinases may act to modulate neurosteroid-mediated potentiation to a greater extent than at receptors containing either β1 or β2. However, if phosphorylation at a single site is sufficient to induce an alteration of neurosteroid activity, then phosphorylation of β1 or β2 may induce similar levels of modulation to that seen at β3 subunit-containing $GABA_A$ receptors.

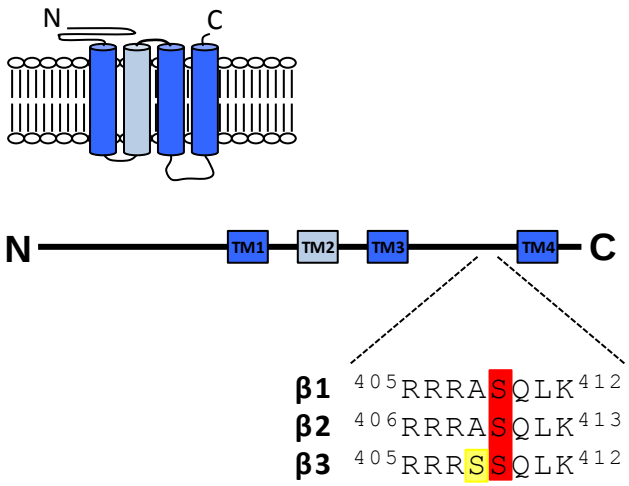

Figure 4.7 Conserved phosphorylation sites on the $GABA_A$ receptor β subunit family
Schematic diagram showing the proposed topology of the major transmembrane domains of the $GABA_A$ receptor β subunit along with the locations of the phosphorylation sites identified to date. Highlighted are the conserved phosphorylation site, at S409/S410 (red) and the additional phosphorylation site, at S408, found only on the β3 subunit (yellow).

In support of this notion, preliminary results from HEK293 cells expressing α1β2γ2L $GABA_A$ receptors showed that, after treatment with staurosporine, the magnitude of potentiation elicited by 50nM THDOC was reduced by 65.6 ± 4.6% ($P < 0.05$, paired t-test; n = 4; Fig. 4.8), a decrease very similar to that observed at α1β3γ2L receptors (see Fig. 4.8). This indicates that, at β2 subunit-containing $GABA_A$ receptors, the activity of protein kinases can modulate the actions of neurosteroids to a similar extent to that seen at receptors incorporating the β3

subunit. Furthermore, as the β2 subunit contains just one phosphorylation site (S410), this suggests that phosphorylation at a single site may be sufficient to induce this modulation.

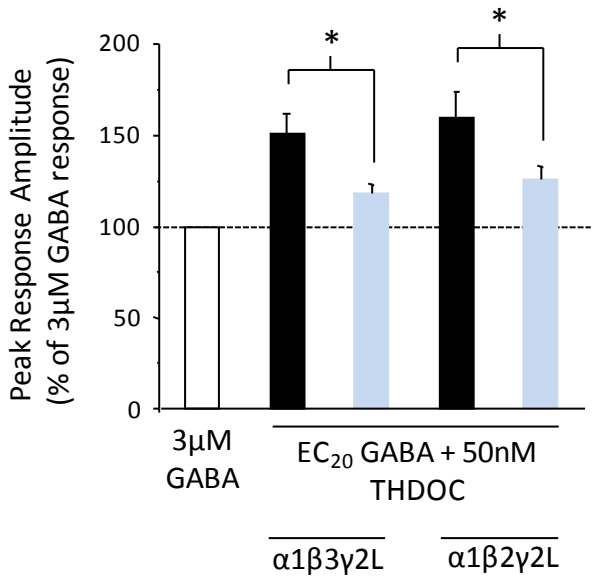

Figure 4.8 Staurosporine decreases THDOC-mediated potentiation at α1β2γ2L and α1β3γ2L subunit-containing $GABA_A$ receptors to a similar extent
Bar chart showing the potentiation of 3µM GABA-activated currents by 50nM THDOC before (black bars) and after (light blue bars) staurosporine treatment in cells expressing α1β3γ2L (n = 5) or α1β2γ2L (n = 4) $GABA_A$ receptors. Cells were treated with 200nM staurosporine for 14 min and the potentiation elicited by 50nM THDOC is expressed as a percentage of the cell's preceding response to 3µM GABA alone (=100%). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

4.3.3 Phosphorylation at the γ2L subunit is not important for the modulation of neurosteroid-mediated potentiation

In addition to investigating the involvement of β subunit phosphorylation in the regulation of neurosteroid activity at $GABA_A$ receptors, it was also important to examine whether the magnitude of potentiation elicited by neurosteroids could be altered by phosphorylation of other subunits within the receptor complex. Studies utilising purified fusion proteins have demonstrated that the intracellular domains of both the γ2 short (γ2S) and γ2 long (γ2L) subunits are phosphorylated by PKC at serine 327 (Moss *et al*., 1992a). In addition, the γ2L subunit, which differs from the γ2S by the presence of an 8 amino acid insertion within the major TM3-4 intracellular domain (Whiting *et al*., 1990; Kofuji *et al*., 1991), contains an additional consensus site for PKC, at serine 343 (Moss *et al*., 1992a; Machu *et al*., 1993). Further studies exploring the functional effects of phosphorylation at the γ2 subunit have shown that phosphorylation at S327 and/or S343 causes a reduction in $GABA_A$ receptor function (Kellenberger *et al*., 1992; Krishek *et al*., 1994). Furthermore, the results have also

indicated that phosphorylation at S343 appears to produce the largest modulatory effect (Krishek *et al*., 1994). However, an additional study reported that, to the contrary, PKC-mediated phosphorylation at the γ2L subunit results in an enhancement of GABA-activated responses (Lin *et al*., 1996). The reason for this discrepancy is unknown but it has been suggested that it may be due to disparities in experimental conditions, with different heterologous expression systems and experimental protocols being employed (Moss and Smart, 1996). Although these studies yielded different results, taken together, they indicate that PKC-mediated phosphorylation at the γ2L subunit plays a role in the functional modulation of $GABA_A$ receptors.

Further to its effects on GABA-activated currents, phosphorylation at the γ2 subunit has also been shown to alter the actions of some $GABA_A$ receptor modulators, including ethanol and benzodiazepines (Wafford and Whiting, 1992; Qi *et al*., 2007). However, the results from the present study demonstrated that phosphorylation at the γ2L subunit is not required for the modulation of neurosteroid-mediated potentiation by protein kinases. At $\alpha1\beta3\gamma2L^{S327A,S343A}$ receptors, exposure to staurosporine resulted in a significant reduction in the magnitude of potentiation elicited by 50nM THDOC. Thus, in contrast to $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, the elimination of phosphorylation at the γ2L subunit does not affect neurosteroid-mediated potentiation, suggesting that phosphorylation at this subunit is not important for the modulation of neurosteroid activity at α1β3γ2L $GABA_A$ receptors. This is supported by further results which showed that, although the magnitude of potentiation elicited by neurosteroids at $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ $GABA_A$ receptors was significantly reduced compared to that observed at wild-type receptors, it was no different to that seen at $\alpha1\beta3^{S408A,S409A}\gamma2L$ subunit-containing receptors, indicating that additional mutation of γ2L serines 327 and 343 does not further modulate the actions of neurosteroids at these receptors.

4.3.4 Phosphorylation is not required for the induction of potentiation by neurosteroids

The results from the previous chapter indicated that neurosteroid-mediated potentiation at $GABA_A$ receptors is modulated by, but does not require, protein kinase activity. This was demonstrated by the fact that potentiation was still evident after cells were treated with either staurosporine or bisindolylmaleimide I. Furthermore, neurosteroid-mediated potentiation was still apparent at receptors containing mutations at all known phosphorylation sites ($\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$), indicating that phosphorylation is not required for the potentiation of $GABA_A$ receptor function by neurosteroids. However, there is a slight possibility

that phosphorylation of, as yet, unidentified sites may be contributing to the induction of neurosteroid-mediated potentiation, causing the residual level of potentiation observed at $\alpha1\beta3^{S408A,S409A}\gamma2L^{S327A,S343A}$ $GABA_A$ receptors in the present study. It has been suggested that additional phosphorylation sites may exist at the $GABA_A$ receptor although their location is yet to be determined. A study by McDonald and Moss (1997) reported that a further site for PKC may exist on the β3 subunit and, although the levels of phosphorylation present at this site were thought to be minimal, it is still possible that phosphorylation at this or another unidentified site may be affecting the actions of neurosteroids at α1β3γ2L $GABA_A$ receptors. Identifying these novel phosphorylation sites may prove difficult if, as suggested by McDonald and Moss (1997), these sites are only subject to low levels of phosphorylation. A more effective method may be to examine the actions of neurosteroids at receptors containing truncated subunits which lack the large TM3-4 intracellular domain, which is thought to be the major target for protein kinase activity. Such subunits have been successfully constructed for $GABA_A$ ρ1 receptors, with Jansen *et al* (2008) demonstrating that replacement of the large intracellular loop of the ρ1 subunit with the comparatively short loop present in the prokaryotic homolog from *Gloeobacter violaceus* (Glvi) yielded functional receptors with similar pharmacological profiles to their wild-type counterparts. Therefore, it is conceivable that similar constructs could be created for the β3 and γ2L subunits, which could then be used to investigate whether neurosteroid-mediated potentiation can be induced at $GABA_A$ receptors when phosphorylation is completely and undisputedly eliminated.

4.3.5 Modulation of $GABA_A$ receptors by neurosteroids and protein kinases is bi-directional

The results from the present investigation, together with those obtained from a number of previous studies, have demonstrated that levels of receptor phosphorylation can alter the modulatory effects of neurosteroids at $GABA_A$ receptors. Interestingly, the results from this study also showed that neurosteroids can modulate the phosphorylation of the receptor. Western blot analysis showed that, after treatment with neurosteroid for 20 min, cells expressing α1β3γ2L $GABA_A$ receptors exhibited a significant increase in the levels of β3 subunit phosphorylation, suggesting the presence of a 'reverse' signalling pathway in which neurosteroids can alter the extent of $GABA_A$ receptor phosphorylation. This effect was mediated via a direct interaction with the neurosteroid-binding site, since cells expressing mutant $\alpha1^{Q241W}$ subunit-containing receptors exhibited no change in the level of β3 subunit phosphorylation after treatment with neurosteroid. Glutamine 241 is the most important of three key residues identified to date which are thought to form part of a neurosteroid binding

site at the $GABA_A$ receptor. Replacement of the polar glutamine residue with a hydrophobic tryptophan (Q241W) was sufficient to eliminate neurosteroid-mediated potentiation at $GABA_A$ receptors, suggesting that this residue is a crucial determinant for the neurosteroid-receptor interaction (Hosie *et al*., 2006). As mutation of α1 Q241 was sufficient to prevent the neurosteroid-mediated enhancement of receptor phosphorylation, this indicates that a direct interaction between the neurosteroid and $GABA_A$ receptor complex was necessary and sufficient for its effect. However, the signalling pathway downstream of this interaction, which results in the enhancement of β3 subunit phosphorylation, is yet to be determined (see General Discussion).

4.3.6 Neurosteroids enhance $GABA_A$ receptor phosphorylation to a lesser extent than direct activation of PKC

The extent to which neurosteroids could enhance β3 subunit phosphorylation was less than that induced by PMA, indicating that neurosteroids are less effective at promoting $GABA_A$ receptor phosphorylation than via the direct activation of PKC. This may suggest that neurosteroids are unlikely to be increasing receptor phosphorylation by directly activating PKC. This is, perhaps, unsurprising as this mechanism would be difficult to reconcile with the results from the mutant $\alpha1^{Q241W}\beta3\gamma2L$ $GABA_A$ receptors. The difference in the effects of neurosteroids versus PMA may also be explained by the fact that cells were exposed to PMA for 30 min whereas the duration of neurosteroid treatment was shorter, at just 20 min, which may have resulted in the larger increase in receptor phosphorylation observed. Furthermore, it is unclear whether the neurosteroid-receptor interaction acts to directly activate protein kinases or whether it simply recruits active protein kinase molecules to the receptor microdomain or makes the kinase binding site on the receptor more accessible, thus facilitating phosphorylation. It may, therefore, be interesting to determine whether neurosteroids can enhance β3 subunit phosphorylation under conditions where the activation of protein kinases is perturbed, for example, by inhibiting the activities of phospholipase C or diacylglycerol. The experiments conducted in the present study have focused on the ability of neurosteroids to modulate phosphorylation at the β3 subunit. Therefore, it would also be of interest to discover whether this effect is specific to β3 or if neurosteroids can also enhance phosphorylation at the γ2L subunit. This may also provide further insight into whether the co-modulatory relationship between neurosteroids and protein kinases at $GABA_A$ receptors is mediated via interplay between neurosteroid-binding and the extent of phosphorylation at the β3 subunit, or whether the γ2L subunit may also play a role. In addition, as the results from the present study indicated that neurosteroid activity at $\alpha1\beta3\gamma2L$ $GABA_A$ receptors is modulated by the

activity of PKC alone, it is also important to determine whether the enhancement in receptor phosphorylation observed here is also mediated by PKC or whether the activities of other protein kinases can also be similarly modulated.

4.3.7 Physiological significance of bi-directional modulation

Perhaps the most intriguing question concerns the physiological significance of this modulation and the possible implications for $GABA_A$ receptors *in vivo*. A number of studies have indicated that, in addition to modulating receptor function, the activity of protein kinases, particularly PKC, may also be involved in the regulation of $GABA_A$ receptor trafficking. Activation of PKC promotes receptor internalization, causing a reduction in the number of receptors expressed at the cell surface (Chapell *et al*., 1998; Connolly *et al*., 1999; Filippova *et al*., 2000; Kittler *et al*., 2000). Although it is thought that this PKC-mediated endocytosis is independent of receptor phosphorylation (Chapell *et al*., 1998; Connolly *et al*., 1999), it may still be interesting to determine whether the enhancement of receptor phosphorylation by neurosteroids may be acting to modulate receptor trafficking. To investigate this, the levels of β3 subunit phosphorylation, before and after neurosteroid treatment, must be probed in the membrane and cytosolic cell fractions independently. A discrepancy in the extent of phosphorylation present at cell surface versus intracellular receptors may suggest that the neurosteroid-mediated enhancement of receptor phosphorylation could be acting to modulate the trafficking of α1β3γ2L $GABA_A$ receptors, which will have important consequences for synaptic inhibition.

In support of this idea, preliminary experiments examining the effects of neurosteroids on the trafficking of $GABA_A$ receptors showed that, in hippocampal neurons, treatment with 10μM allopregnanolone for 1hr significantly reduced the number of $GABA_A$ receptor α1 subunits expressed on the cell surface (decrease = 30.6 ± 2.6%, $P < 0.05$, ANOVA with Tukey's post hoc test; n = 16; Fig. 4.9). This indicates that, in these neurons, exposure to neurosteroids can modulate the trafficking of $GABA_A$ receptors. This decrease was comparable to that induced by exposure to 100nM diazepam (decrease = 32.3 ± 1.3%, $P < 0.05$; n = 15; Fig. 4.9), which is consistent with results from a number of previous studies which have reported that exposure to benzodiazepines can induce a down-regulation of $GABA_A$ receptors from the cell surface (Miller *et al*., 1988; Brown and Bristow, 1996). This benzodiazepine-induced trafficking of receptors has been proposed to be mediated by protein kinases, with results showing that the decrease in cell surface expression of $GABA_A$ receptors can be abolished by treating cells with the protein kinase inhibitor, staurosporine (Brown and Bristow, 1996). Therefore, this, taken

together with the fact that neurosteroids have been shown to promote phosphorylation at the $GABA_A$ receptor (see Fig. 4.6), may suggest the neurosteroid-induced enhancement of phosphorylation may indeed act to modulate the trafficking of $GABA_A$ receptors. However, in order to confirm this, further study is required to investigate whether the effects of neurosteroids observed in hippocampal neurons can be abolished when cells are treated with protein kinase inhibitors. Furthermore, in these preliminary experiments, 10µM neurosteroid was used, a much higher concentration than that experienced *in vivo*. Therefore, it is also important to repeat these experiments with a lower, more physiological concentration of neurosteroid to determine whether at lower concentrations, neurosteroids can still induce a reduction in cell surface expression of $GABA_A$ receptor α1 subunits.

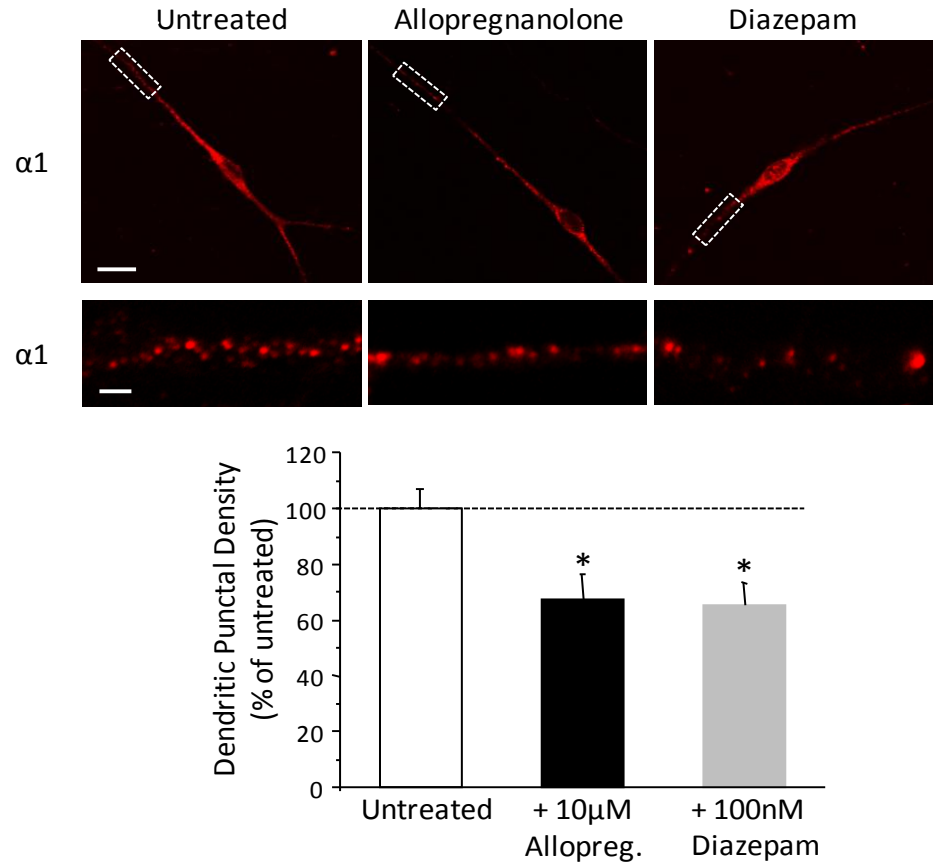

Figure 4.9 Neurosteroids reduce cell surface expression of α1 $GABA_A$ receptor subunits in cultured hippocampal neurons

Immunofluorescence labeling for the $GABA_A$ receptor α1 subunit in cultured hippocampal neurons (7-11DIV). Cells either remained untreated (left panel) or were exposed to either 10µM allopregnanolone (centre panel) or 100nM diazepam (right panel) for 1hr at 37°C. After treatment cells were fixed with paraformaldehyde (PFA) and labelled using an α1 antibody specific for the extracellular N-terminus of the protein (Alomone). Images in the bottom row are enlargements of the areas indicated. Scale bars = 15µm (upper) and 3µm (lower). Bar chart shows the number of fluorescent, α1-positive puncta present per µm of dendrite in hippocampal neurons which were either untreated (white bar; n = 30) or exposed to 10µM allopregnanolone (allopreg; black bar; n = 16) or 100nM diazepam (grey bar; n = 15). Data is expressed as a % of the puncta present in untreated cells (=100%). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, ANOVA with Tukey's post hoc test).

4.3.8 Neurosteroid-induced enhancement of receptor phosphorylation may act as a positive feedback mechanism

Given that previous results have indicated that the magnitude of neurosteroid-mediated potentiation can be enhanced by phosphorylation at the β3 subunit, neurosteroids increasing receptor phosphorylation may act as a kind of 'positive feedback' mechanism, promoting an enhancement in the magnitude of the potentiation induced. Indeed, a form of positive feedback, termed 'auto-modulation' has been reported in a study by Wegner *et al* (2007), showing that, at a number of $GABA_A$ receptor subtypes (including α1β3γ2L), repetitive co-applications of GABA and neurosteroid, resulted in a 'run-up' of neurosteroid-mediated potentiation. This progressive enhancement in potentiation could be prevented by treatment of cells with Protein Kinase Inhibitor (PKI), indicating that this increase is likely to be mediated by PKA. Although this study did not examine whether direct receptor phosphorylation was required for this 'auto-modulation' to occur, the results from the present study suggest that this could be a feasible explanation. However, the study by Wegner *et al* also showed that this 'auto-modulation' was only observed when GABA was co-applied with neurosteroid concentrations of 300nM and above and therefore, this is inconsistent with the results from the present study, where 50nM THDOC was able to induce a significant enhancement of receptor phosphorylation. This discrepancy may be explained by considering the time-course of the effects observed in this and the previous study. The 'auto-modulatory' effects described by Wegner *et al* were induced by repeated co-applications of GABA and neurosteroid, lasting around 2-5s, whereas, cells exposed to 50nM THDOC did not exhibit a significant enhancement of β3 subunit phosphorylation until cells had been exposed to the neurosteroid for 20 min. Therefore, it could be hypothesized that, if the 'run-up' of potentiation is indeed mediated by direct receptor phosphorylation, then two or three applications of 30nM alphaxalone, lasting a maximum of around 10-15s, is unlikely to be sufficient to cause receptor phosphorylation, which could explain the lack of 'auto-modulation' seen in cells exposed to this concentration of neurosteroid.

4.3.9 Effects of phosphorylation on $GABA_A$ receptor function

Phosphorylation has been shown to play an important role in the regulation of $GABA_A$ receptor function, with numerous studies demonstrating that this modulation can result in both enhancement and reduction of GABA-activated responses, depending on the protein kinase present and the subunit combination of the receptor (Moss and Smart, 1996). However, the results from the previous chapter showed that inhibition of protein kinase activity by staurosporine did not alter the magnitude of the EC_{20} GABA-activated currents recorded from

cells expressing α1β3γ2L $GABA_A$ receptors (see Chapter 3). It is, therefore, unsurprising that, in the present study, $GABA_A$ receptors containing mutations to prevent phosphorylation at one or more candidate sites also did not exhibit significant changes in the receptor's response to EC_{20} GABA after treatment with staurosporine. As discussed previously, this may be due to the disparate functional effects of PKC and PKA at β3 subunit-containing $GABA_A$ receptors, which, as staurosporine would be expected to inhibit both of these protein kinases, may result in a masking of the effects mediated by each individual protein kinase.

However, this does not explain the results obtained at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, as, although the sites which remain intact on the γ2L subunit are only subject to phosphorylation by PKC (Moss *et al*., 1992a), there is still no obvious modulation of the EC_{20} GABA-activated currents after staurosporine treatment. A potential explanation for this is that there may be no basal phosphorylation present at the γ2L subunit, thus, any subsequent inhibition of protein kinases will have no functional effect. Another reason for this anomaly could be that the γ2L subunit may be targeted for phosphorylation by other protein kinases which may act to mask the modulatory effects mediated by PKC. In order to identify the protein kinases which are able to phosphorylate the γ2L subunit, many of the experiments carried out to date have utilised purified fusion proteins (Moss *et al*., 1992a), which, although offering a good indication of the protein kinases acting at this subunit, may not fully reflect the complex environment present within a native neuronal system. Therefore, this method may have failed to detect the effects of other protein kinases, however this remains to be determined.

In contrast to the results from the staurosporine experiments, specific activation of PKC induced a significant reduction of EC_{20} GABA-activated currents recorded from cells expressing both wild-type and mutant $GABA_A$ receptors. Previous studies have demonstrated that phosphorylation at β3 subunit-containing $GABA_A$ receptors by PKC results in a decrease of receptor function (Brandon *et al*., 2000). Interestingly, at $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors, the PMA-induced reduction of GABA-activated current was not statistically significant, most likely reflecting the reduction in potential phosphorylation sites from four to two, resulting in a reduced capacity for modulation by PKC. This is supported by observations from both $\alpha1\beta3^{S408A}\gamma2L$ and $\alpha1\beta3^{S409A}\gamma2L$ $GABA_A$ receptors, which also exhibited slight reductions in the functional effects of PKC.

Curiously, treatment of cells with the specific PKC inhibitor, bisindolylmaleimide I, caused no significant alteration of EC_{20} GABA-activated responses recorded from cells expressing either

wild-type or mutant $GABA_A$ receptors. This is inconsistent with the results from Western blot analysis which showed that the level of basal phosphorylation present at the β3 subunit could be reduced by treating cells with bisindolylmaleimide I, indicating that PKC-mediated phosphorylation is present at the $GABA_A$ receptor under basal cell conditions and that bisindolylmaleimide I is effective at ablating this phosphorylation within the duration of the experiment. Therefore, this suggests that there may be additional modifications occurring which could compensate for the effects of the PKC inhibitor. For example, it is possible that there may be an up-regulation of phosphorylation at other sites, such as those on the γ2L subunit, which may act to oppose the changes in $GABA_A$ receptor function caused by the decrease in β3 subunit phosphorylation.

4.3.10 Are all $GABA_A$ receptor subtypes modulated similarly?

The experiments carried out in this study so far have focussed on the co-modulatory relationship between neurosteroids and protein kinases at typical 'synaptic-type' $GABA_A$ receptors. However, other receptor subtypes have been shown to exist outside of inhibitory synapses (Somogyi *et al*., 1989; Nusser *et al*., 1998; Wei *et al*., 2003; Jia *et al*., 2005). These 'extrasynaptic' receptors mediate a slow 'tonic' form of neuronal inhibition thought to play an important role in the regulation of CNS excitability (Semyanov *et al*., 2004; Farrant and Nusser, 2005; Belelli *et al*., 2009). Therefore, it was of interest to determine whether the actions of neurosteroids at typical 'extrasynaptic-type' $GABA_A$ receptors could also be modulated by protein kinase activity.

4.4 Conclusions

- PKC acts to modulate the actions of neurosteroids at α1β3γ2L subunit-containing $GABA_A$ receptors via direct phosphorylation of the β3 subunit.

- Phosphorylation at β3 S408 or S409, but not γ2L S327 or S343 modulates neurosteroid potentiating activity.

- Induction of neurosteroid-mediated potentiation does not require direct receptor phosphorylation.

- THDOC enhances phosphorylation at the β3 subunit via a direct interaction with the $GABA_A$ receptor.

Chapter 5

Neurosteroid modulation of extrasynaptic-type $GABA_A$ receptors: regulation by protein kinases via direct receptor phosphorylation

5.1 Introduction

Activation of $GABA_A$ receptors has been shown to generate two distinct forms of inhibition. Synaptic or 'phasic' inhibition is mediated by the rapid, transient release of GABA from pre-synaptic terminals which diffuses rapidly across the synaptic cleft to activate receptors clustered at the postsynaptic site. In contrast, $GABA_A$ receptors located extrasynaptically to inhibitory synapses are responsible for mediating a slower 'tonic' form of inhibition in response to their persistent activation by low, ambient concentrations of GABA (Farrant and Nusser, 2005). The differing spatial and temporal characteristics of these two modes of inhibition results in them having profoundly different effects on neuronal network activity. Synaptic inhibition mediates its effects in a precise, neuron-specific manner, whereas, prolonged activation of spatially diverse extrasynaptic receptors generates a more widespread inhibitory effect (Semyanov *et al*., 2004; Farrant and Nusser, 2005).

The presence of tonic inhibition was initially discovered in cerebellar granule cells, with studies showing that the application of $GABA_A$ receptor antagonists resulted in a decrease in the 'holding current' recorded from these neurons, implying the presence of a 'background' conductance which is mediated by the persistent activation of $GABA_A$ receptors (Kaneda *et al*., 1995; Brickley *et al*., 1996). Subsequent studies have indicated that tonic inhibition is also present in a number of other neuronal populations including: thalamic relay neurons (Porcello *et al*., 2003; Cope *et al*., 2005; Jia *et al*., 2005), dentate gyrus granule cells (Nusser and Mody, 2002) and neurons from the CA1 region of the hippocampus (Bai *et al*., 2001; Semyanov *et al*., 2003). Moreover, the tonic inhibition present in these neurons has been shown to be mediated by $GABA_A$ receptors located outside of synapses, with studies from knock-out mice showing that deletion of the extrasynaptically located δ subunit (Nusser *et al*., 1998; Wei *et al*., 2003; Jia *et al*., 2005), significantly reduces the tonic conductance recorded from cerebellar granule cells (Brickley *et al*., 2001; Stell *et al*., 2003), dentate gyrus granule cells (Stell *et al*., 2003) and thalamic relay neurons (Porcello *et al*., 2003). In accordance with this, extrasynaptic receptors have been shown to contain specific subsets of receptor subunits which convey biophysical properties consistent with their involvement in tonic inhibition. In contrast to

synaptic receptors, which are thought to consist of γ2 subunits, in combination with α1, α2 or α3, it has been suggested that the majority of extrasynaptic receptors contain the δ subunit in association with α4 or α6, possibly supplemented with α5βγ and also αβ receptors (Farrant and Nusser, 2005; Mortensen and Smart, 2006; Glykys *et al*., 2008; Olsen and Sieghart, 2008). The inclusion of these subunits within extrasynaptic receptors results in their enhanced sensitivity to agonist as well as a reduction in the speed and extent to which they undergo desensitisation (Saxena and Macdonald, 1994; Fisher and Macdonald, 1997; Haas and Macdonald, 1999; Brown *et al*., 2002) making them ideally specialised for prolonged exposure to low concentrations of GABA which receptors positioned outside of the synapse are likely to encounter. However, recently, αβδ receptors have been shown to desensitise to a far greater extent than originally envisaged (Mortensen *et al*., 2010).

Although the effects mediated by tonic inhibition are thought to be more widespread, it is still considered to be an important mechanism for the regulation of neuronal excitability *in vivo*. In fact, the dysfunction of tonic inhibition has been shown to be a common feature of a number of neurological disorders including epilepsy (Dibbens *et al*., 2004; Maguire *et al*., 2005; Naylor *et al*., 2005; Scimemi *et al*., 2005; Feng *et al*., 2006; Zhang *et al*., 2007; Cope *et al*., 2009), stress (Maguire and Mody, 2007), Fragile X syndrome (Curia *et al.*, 2009) and mood disorders associated with the ovarian cycle (Maguire *et al*., 2005; Shen *et al*., 2007) and pregnancy (Maguire and Mody, 2008; Maguire *et al*., 2009). Therefore, it is important to understand the mechanisms by which extrasynaptic receptors are modulated *in vivo* as this may provide further insight into the pathogenesis of these conditions as well as identifying potential therapeutic targets.

Studies of recombinant $GABA_A$ receptors have shown that neurosteroids can potentiate GABA-activated currents at typical 'extrasynaptic-type' receptors, although early studies indicated that inclusion of the exclusively extrasynaptic δ subunit significantly reduced the magnitude of potentiation induced (Zhu *et al*., 1996). However, more recent studies showed that, to the contrary, receptors incorporating the δ subunit exhibited enhanced neurosteroid potentiation compared to those containing γ2 (Belelli *et al*., 2002; Brown *et al*., 2002; Wohlfarth *et al*., 2002). Moreover, studies of native $GABA_A$ receptors have shown that neurosteroids can potentiate GABA-mediated tonic currents in a number of neuronal populations including cerebellar granule cells (Hamann *et al*., 2002; Stell *et al*., 2003) and dentate gyrus granule cells (Stell *et al*., 2003), suggesting that neurosteroids may play an important role in the regulation of tonic inhibition. In fact, in both of these sets of neurons, physiological concentrations of

neurosteroid (10nM) have been shown to selectively enhance tonic currents, with little or no effect observed at synaptic $GABA_A$ receptors (Stell *et al*., 2002; Harney *et al*., 2003). Furthermore, the enhancement of tonic currents by neurosteroids was abolished in δ knock-out mice (Stell *et al*., 2002). Collectively, these results suggest that the actions of neurosteroids at extrasynaptic $GABA_A$ receptors may play a significant role in the regulation of tonic inhibition and therefore, it is important to understand how the presence of other endogenous modulators, such as protein kinases, may affect this regulatory relationship *in vivo*.

5.2 Results

In order to investigate whether the activity of protein kinases is important for regulating the magnitude of neurosteroid potentiation at 'extrasynaptic-type' receptors, HEK293 cells were transfected to express α4β3δ subunit-containing $GABA_A$ receptors. Cells were transfected with cDNAs encoding each receptor subunit in a 1:1:1 ratio and, as in previous experiments, were co-transfected with eGFP to enable the identification of expressing cells. The δ subunit has proved difficult to express in recombinant cell systems, however, a number of previous studies have shown that co-expression of δ, in combination with α4 and β3 subunits, produces functional receptors at the cell surface that exhibit pharmacological profiles consistent with the incorporation of the δ subunit (Brown *et al*., 2002; Stórustovu and Ebert, 2006; Hosie *et al*., 2008; Meera *et al*., 2009). To ensure that the receptors expressed in the present study did indeed contain the δ subunit, sensitivity to the hypnotic drug, 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol (THIP) was examined. THIP has been shown to exhibit 'super-agonist' activity at δ subunit-containing receptors compared to its partial agonism at αβγ receptors (Brown *et al*., 2002; Stórustovu and Ebert, 2006; Mortensen *et al*., 2010). Therefore, this drug can be used as a reliable indicator of the presence of δ subunits within the receptor complex. To investigate the agonist activity of THIP, peak currents were recorded in response to brief (usually 3s) applications of either, near saturating 100μM GABA or 100μM THIP (Fig. 5.1A). The results showed that 100μM THIP induced currents that were significantly larger than those elicited by 100μM GABA (increase = 35.6 ± 4.1%, mean ± s.e.m., $P < 0.05$, paired t-test; n = 15; Fig. 5.1B), with the magnitude of the THIP responses being indicative of receptors incorporating α4β3δ, rather than α4β3 subunits. Therefore, this indicates that the receptors expressed here contain the δ subunit. This process was completed in all cells prior to the experimental period to ensure that each cell expressed functional α4β3δ subunit-containing $GABA_A$ receptors at the cell surface. After THIP sensitivity was assessed, cells were exposed to brief applications of EC_{20} GABA at 2 min intervals until a stable current response was achieved.

After this period, further experiments investigating the effects of phosphorylation, by protein kinases, on the potentiation of $GABA_A$ receptor function by neurosteroids were carried out.

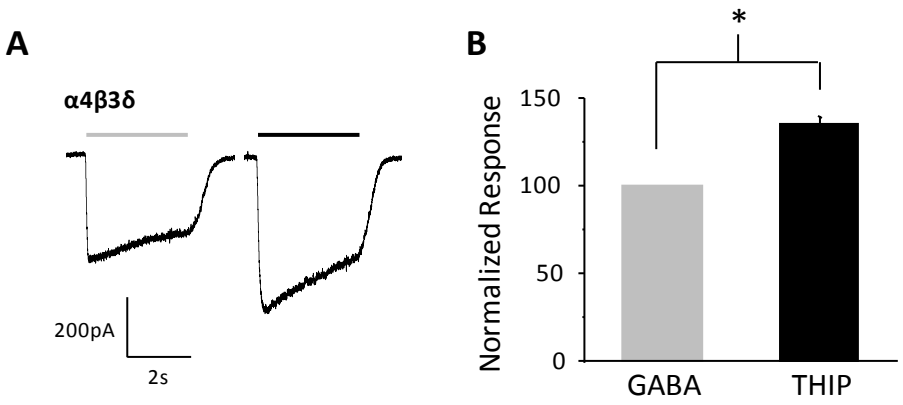

Figure 5.1 HEK293 cells expressing α4β3δ $GABA_A$ receptors exhibit THIP 'super-agonist' activity
(A) Peak currents recorded from HEK293 cells expressing α4β3δ subunit-containing $GABA_A$ receptors in response to either 100μM GABA (grey bar) or 100μM THIP (black bar). (B) Bar chart showing the mean agonist-activated currents recorded (n = 15). Each response elicited by 100μM THIP was normalised to the peak current induced by 100μM GABA in the same cell (= 100%). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

Experiments were conducted as in the previous two chapters, with whole-cell patch clamp electrophysiology being used to assess the magnitude of potentiation elicited by 50nM THDOC, before and after cell treatment with compounds to inhibit protein kinase activity. For each cell, baseline responses to EC_{20} GABA and potentiated responses after co-applications of EC_{20} GABA and 50nM THDOC were established under basal cell conditions and after the treatment period. Each potentiated response was normalised to the cell's preceding response to EC_{20} GABA alone in order to allow direct comparisons to be made between the potentiation elicited before and after the treatment period.

5.2.1 THDOC-mediated potentiation at α4β3δ $GABA_A$ receptors is decreased after protein kinase inhibition

To determine whether the actions of neurosteroids at α4β3δ $GABA_A$ receptors could be modulated by the activity of protein kinases, cells were treated with the broad-spectrum protein kinase inhibitor, staurosporine. After establishing the baseline and potentiated responses elicited by GABA under basal cell conditions, cells were treated with 200nM staurosporine, applied extracellularly via the bath perfusion. During the treatment period the cell's response to EC_{20} GABA was monitored every 2 min in order to examine the effects of staurosporine on the receptor's response to agonist alone. In cells expressing α4β3δ $GABA_A$

receptors, inhibition of protein kinase activity by staurosporine did not significantly alter the EC_{20} GABA-activated response, with a negligible change of only 10.2 ± 6.8% being observed, which did not differ significantly from that seen in untreated cells (3.4 ± 9.2%, mean ± s.e.m., $P > 0.05$; n = 5-9; Fig. 5.2B).

After staurosporine treatment, the baseline and potentiated responses were re-measured and the potentiation elicited by 50nM THDOC before and after protein kinase inhibition was compared. Control experiments carried out in cells which remained untreated for the duration of the experiment showed no change in THDOC-mediated potentiation (change = 4.7 ± 7.9%, $P > 0.05$; n = 5; Fig. 5.2A). By contrast, cells treated with 200nM staurosporine for 14 min exhibited a significant reduction in the magnitude of potentiation induced by 50nM THDOC (decrease = 38.1 ± 5.8%, $P < 0.05$, paired t-test; n = 9; Fig. 5.2B). This indicates that the actions of neurosteroids at α4β3δ $GABA_A$ receptors can be modulated by the activity of protein kinases. Furthermore, the results showed that the protein kinases are not required for the induction of THDOC-mediated potentiation at these receptors, with 50nM THDOC still enhancing the EC_{20} GABA-activated current by 83.7 ± 18.5% after staurosporine treatment (Fig. 5.2B).

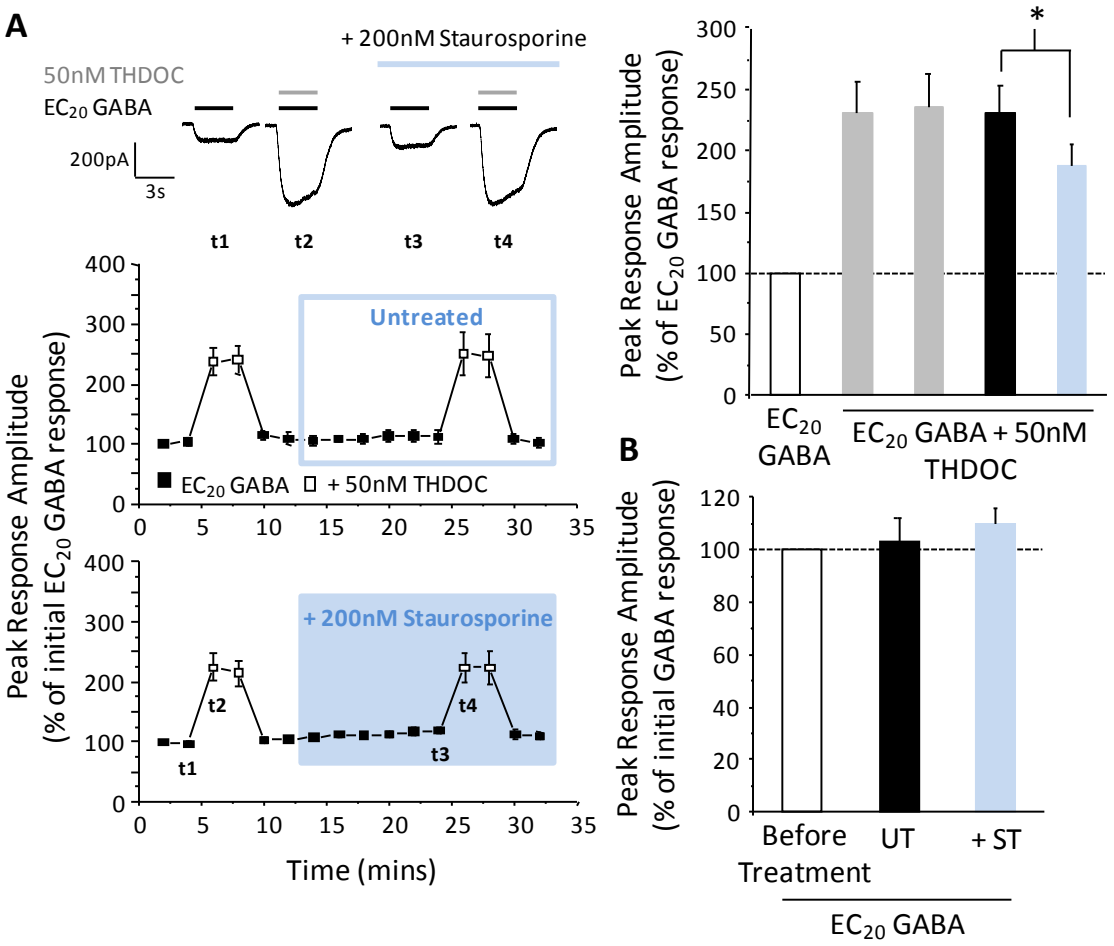

Figure 5.2 Staurosporine decreases THDOC-mediated potentiation at α4β3δ $GABA_A$ receptors
(A) Mean peak currents recorded from HEK293 cells expressing α4β3δ $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells either remained untreated for the duration of the recording (upper panel) or were treated with 200nM staurosporine (lower panel; light blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving the whole-cell recording configuration, designated as t = 0 (= 100%). Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in untreated cells (grey bars, measured at 6 min and 26 min, respectively; n = 5) or in treated cells before (black bar) and after (light blue bar) 200nM staurosporine treatment (n = 9). (B) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA in untreated cells (UT, black bar; n = 5) or cells treated with 200nM staurosporine (light blue bar; n = 9). Change was measured between 2 min (before treatment) and 32 min (after treatment or at corresponding time points in untreated cells). Responses were normalised to the peak current recorded at 2 min. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

Interestingly, staurosporine induces a smaller decrease in THDOC potentiation at α4β3δ $GABA_A$ receptors compared to that observed at α1β3γ2L receptors (38.1 ± 5.8% versus 54.8 ± 9.1% reduction, respectively; Fig. 5.3). This indicates that, although the activity of protein kinases acts to modulate neurosteroid potentiation at extrasynaptic $GABA_A$ receptors, this occurs to a lesser extent than at synaptic-type receptors. Another potential explanation for this

discrepancy is that there may be a reduced level of basal phosphorylation present at α4β3δ receptors, reducing its capacity for modulation by staurosporine.

A number of studies have reported that $GABA_A$ receptors incorporating the δ subunit are more sensitive to the potentiating actions of neurosteroids (Belelli *et al*., 2002; Brown *et al*., 2002; Wohlfarth *et al*., 2002). However, in the present study, the magnitude of potentiation elicited by 50nM THDOC at α4β3δ $GABA_A$ receptors did not differ significantly from that observed previously at α1β3γ2L receptors (potentiation = 128 ± 23.6% and 99.1 ± 13.8%, respectively, P > 0.05; n = 5-9; Fig. 5.3).

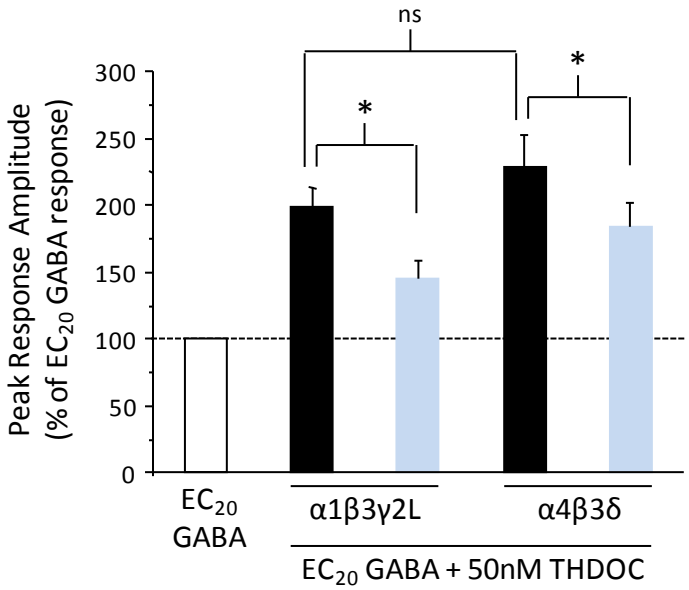

Figure 5.3 Comparison between the effects of staurosporine at α4β3δ versus α1β3γ2L $GABA_A$ receptors
Bar chart showing the potentiation of EC_{20} GABA-activated currents by 50nM THDOC in HEK293 cells expressing either α1β3γ2L or α4β3δ $GABA_A$ receptors, before and after treatment with 200nM staurosporine for 14 min. Responses were normalised to the peak response elicited by EC_{20} GABA alone (= 100%). All data points represent mean ± s.e.m. Significant results are indicated by * (P < 0.05, paired t-test) and ns = not statistically significant.

Although the results from this experiment indicated that the actions of neurosteroids at extrasynaptic-type $GABA_A$ receptors are positively modulated by the activity of protein kinases, it remained to be determined whether, similar to synaptic-type receptors, this effect was mediated by direct phosphorylation of the receptor complex. Therefore, mutant receptors were examined to explore whether phosphorylation at a specific site(s) was important for the regulation of neurosteroid potentiation at α4β3δ $GABA_A$ receptors.

5.2.2 Phosphorylation of β3 subunits is insufficient to account for protein kinase modulation of THDOC potentiation at α4β3δ $GABA_A$ receptors

The results presented in the previous chapter showed that, at α1β3γ2L $GABA_A$ receptors, the extent of β3 subunit phosphorylation regulates the magnitude of neurosteroid potentiation. Therefore, it was hypothesized that phosphorylation at the β3 subunit may also be important for the modulation of neurosteroid activity at α4β3δ receptors. To investigate this, cells were transfected to express $GABA_A$ receptors containing mutant $\beta3^{S408A,S409A}$ subunits, in combination with wild-type α4 and δ. As discussed previously, serine to alanine amino acid substitutions have been shown to be effective at preventing phosphorylation at the target site (Moss *et al*., 1992; McDonald and Moss, 1997). After staurosporine treatment, cells expressing $\alpha4\beta3^{S408A,S409A}\delta$ $GABA_A$ receptors exhibited no significant change in EC_{20} GABA-activated currents (percentage change measured between 2 and 32 min = 6.5 ± 4.7%; n = 10; Fig. 5.6C). By contrast, THDOC-mediated potentiation was significantly reduced after staurosporine treatment (decrease = 27.2 ± 4.6%, $P < 0.05$, paired t-test; n = 9; Fig. 5.4), implying that phosphorylation at the β3 subunit is not required for the modulation of neurosteroid potentiation by protein kinases. However, staurosporine was notably less effective at $\alpha4\beta3^{S408A,S409A}\delta$ $GABA_A$ receptors, with a smaller decrease in potentiation being observed at these compared to wild-type receptors (27.2 ± 4.6% versus 38.1 ± 5.8%; Fig. 5.4). This suggests that phosphorylation at the β3 subunit may play a role in the modulation of neurosteroid activity at α4β3δ receptors, but that phosphorylation at this subunit is not sufficient to completely account for the modulatory effects of protein kinases observed at wild-type receptors. Therefore, this indicates that phosphorylation at other residues and/or subunits may also be important for the regulation of neurosteroid potentiation at this receptor subtype.

Interestingly, there was a modest decrease in the potentiation elicited by 50nM THDOC at $\alpha4\beta3^{S408A,S409A}\delta$ versus wild-type $GABA_A$ receptors under basal cell conditions (potentiation = 107 ± 30.3% and 128 ± 23.6%, respectively, $P > 0.05$; n = 9-10; Fig. 5.4), which, once again, implies that phosphorylation at the β3 subunit may be involved in the regulation of neurosteroid potentiation at α4β3δ $GABA_A$ receptors. However, as this decrease was much less than that observed at wild-type receptors after protein kinase inhibition, this indicates that phosphorylation at other residues and/or subunits must also contribute to this effect.

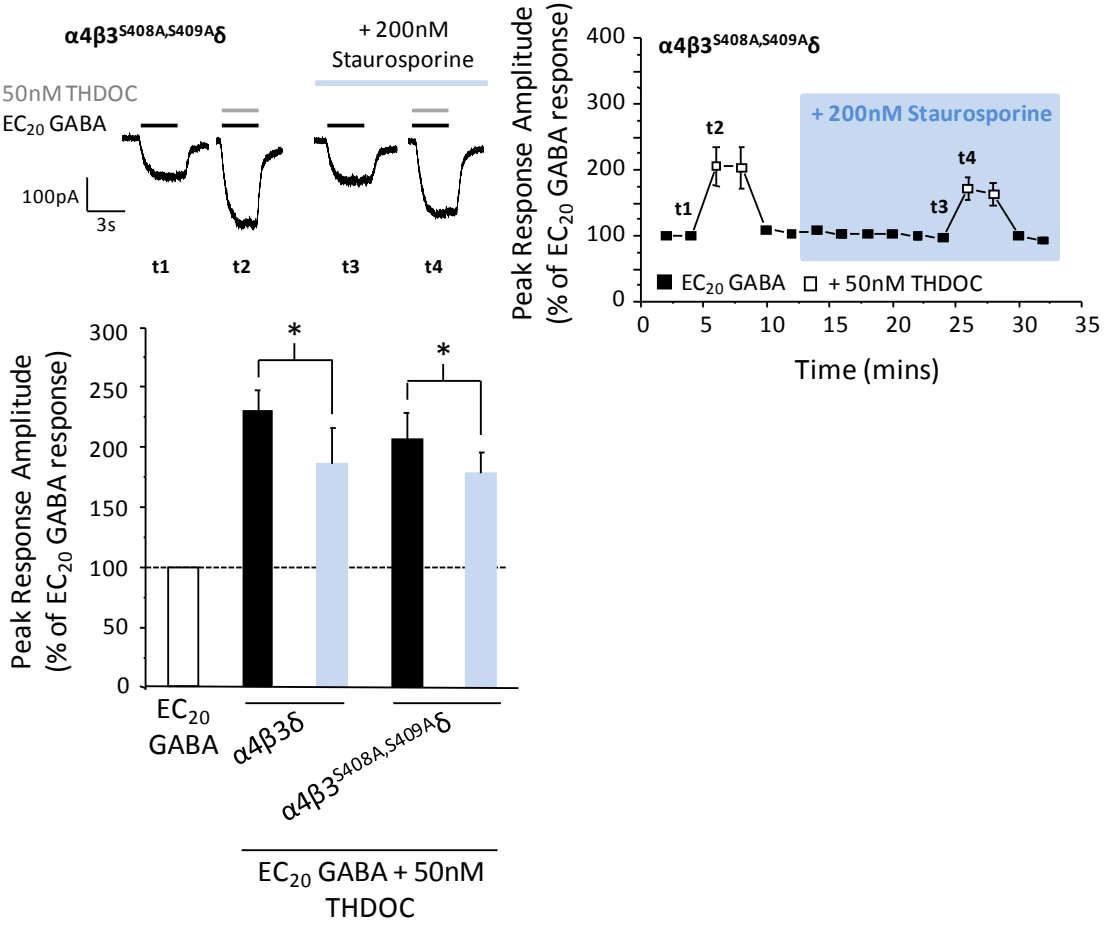

Figure 5.4 Staurosporine decreases THDOC-mediated potentiation at α4β3^{S408A,S409A}δ $GABA_A$ receptors

Mean peak currents recorded from HEK293 cells expressing α4β3^{S408A,S409A}δ $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells were treated with 200nM staurosporine (as indicated by light blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated. Bar chart shows the potentiation of EC_{20} GABA-activated currents by 50nM THDOC before (black bars) and after (light blue bars) staurosporine treatment in cells expressing α4β3δ (n = 9) or α4β3^{S408A,S409A}δ (n = 7) $GABA_A$ receptors. Data displayed for α4β3δ receptors is as shown previously in Fig. 5.2. All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

5.2.3 Presence of a novel phosphorylation site on the $GABA_A$ receptor α4 subunit

As discussed previously, the $GABA_A$ receptor has been shown to possess a number of consensus phosphorylation sites which can be targeted by numerous Ser/Thr and Tyr protein kinases (Moss *et al.*, 1992a, 1995; McDonald and Moss, 1994, 1997). To date, sites have been identified on the β and γ2 subunit families, indicating that, at α4β3δ $GABA_A$ receptors, there are only two potential phosphorylation sites (β3 serines 408 and 409) through which protein kinases could act to modulate the actions of neurosteroids. However, this is inconsistent with

the results from the present study which showed that abolition of phosphorylation at the β3 subunit was not sufficient to prevent the staurosporine-induced decrease in neurosteroid potentiation at this receptor subtype. Interestingly, however, recent experiments have identified a novel PKC phosphorylation site on the α4 subunit, at serine 443 (Abramian and Moss, unpublished; Fig. 5.5), which is not thought to be present on other members of the α subunit family (Moss *et al*., 1992a; Moss and Smart, 1996). Therefore, further experiments were carried out in order to examine the role of phosphorylation at α4 S443 in the regulation of neurosteroid activity at α4β3δ $GABA_A$ receptors.

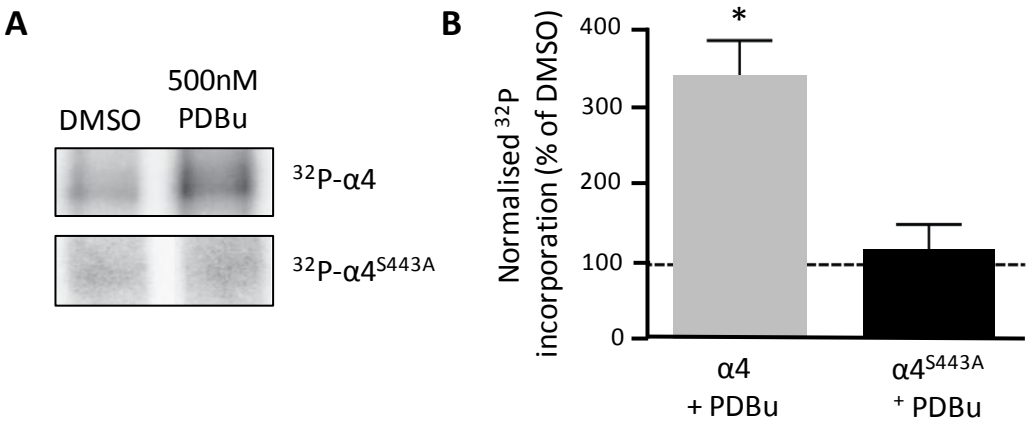

Figure 5.5 Existence of a novel PKC phosphorylation site, at S443 on the $GABA_A$ receptor α4 subunit
(A) COS7 cells expressing either α4β3 (top panel) or $α4^{S443A}$β3 (bottom panel) $GABA_A$ receptors were pre-labelled with 0.5 mCi/ml [^{32}P]-orthophosphoric acid for 4 hours. After the labeling period, cells were incubated with 500nM PDBu (or DMSO for control cells) for 10 min. Cells were then lysed and subject to immunoprecipitation using α4 anti-sera. Bead samples were separated using SDS-PAGE followed by autoradiography to assess ^{32}P incorporation. (B) Bar chart showing the amount of ^{32}P incorporated in COS7 cells expressing either α4β3 or $α4^{S443A}$β3 in response to treatment with 500nM PDBu for 10 min. The levels of phosphorylation were normalised to the amount detected in the corresponding DMSO treated control cell group (= 100%). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test). Data presented in this figure was obtained from experiments carried out by Matthew Abramian in the laboratory of Professor Stephen J Moss at Tufts University School of Medicine, Boston, USA.

5.2.4 Phosphorylation of both the α4 and β3 subunits regulates neurosteroid activity at α4β3δ $GABA_A$ receptors

To explore the involvement of α4 subunit phosphorylation in the modulation of neurosteroid potentiation, site-directed mutagenesis was used to substitute serine 443 with a neutral alanine residue in order to prevent phosphorylation at this site (see Materials and Methods). The resulting $α4^{S443A}$ cDNA construct was expressed in HEK293 cells in combination with either wild-type β3 and δ or with mutant $β3^{S408A,S409A}$ and wild-type δ subunits. The magnitude of

potentiation elicited by 50nM THDOC was then assessed before and after staurosporine treatment. Similar to the results obtained for α4β3^{S408A,S409A}δ $GABA_A$ receptors, cells expressing α4^{S443A}β3δ receptors exhibited a significant decrease in THDOC-mediated potentiation after treatment with 200nM staurosporine for 14 min (decrease = 24.5 ± 4.3%, $P < 0.05$, paired t-test; n = 7; Fig. 5.6A,B). The effects of staurosporine were notably less effective at receptors containing α4^{S443A} subunits compared to wild-type receptors (decrease = 24.5 ± 4.3% versus 38.1 ± 5.8%, respectively; Fig. 5.6A,B). This suggests that phosphorylation at α4 S443 is likely to be involved in regulating neurosteroid activity at α4β3δ $GABA_A$ receptors, although, again, it is not sufficient to induce the full modulation observed at wild-type receptors. By contrast, in cells expressing α4^{S443A}β3^{S408A,S409A}δ receptors the magnitude of potentiation elicited by 50nM THDOC was unaffected by staurosporine treatment (change = 0.9 ± 6.5%, $P > 0.05$; n = 7; Fig. 5.6A,B), thus, phosphorylation at both the α4 and β3 subunits must be abolished in order to fully prevent the staurosporine-induced decrease in THDOC potentiation. This indicates that phosphorylation at both α4 and β3 is important for regulating neurosteroid activity at α4β3δ $GABA_A$ receptors. However, the fact that a reduced, but significant reduction of THDOC-mediated potentiation can be induced by the inhibition of protein kinase activity at α4^{S443A}β3δ and α4β3^{S408A,S409A}δ $GABA_A$ receptors, suggests that phosphorylation at either α4 S443 or β3 S408/S409 is able to modulate the actions of neurosteroids to some extent, although it appears that phosphorylation at both subunits is required to induce the full modulatory effect.

Interestingly, the decrease in potentiation observed at α4^{S443A}β3δ and α4β3^{S408A,S409A}δ $GABA_A$ receptors was not additive compared to that induced at wild-type receptors, with results showing that, at α4^{S443A}β3δ and α4β3^{S408A,S409A}δ receptors, respectively, staurosporine treatment induced a decrease in neurosteroid potentiation that was 64.3% and 71.4% of that observed at α4β3δ receptors (Fig. 5.4 and 5.6B). This indicates that phosphorylation at each subunit can induce approximately two thirds of the total modulation observed at wild-type receptors and that additional phosphorylation at the second subunit acts to enhance this effect by around 50% of its own modulatory capacity. Curiously, it appears that phosphorylation at either subunit is capable of inducing the initial two thirds of this modulation, with the actions of protein kinases at one subunit not appearing to be more effective at regulating the activity of neurosteroids than the other.

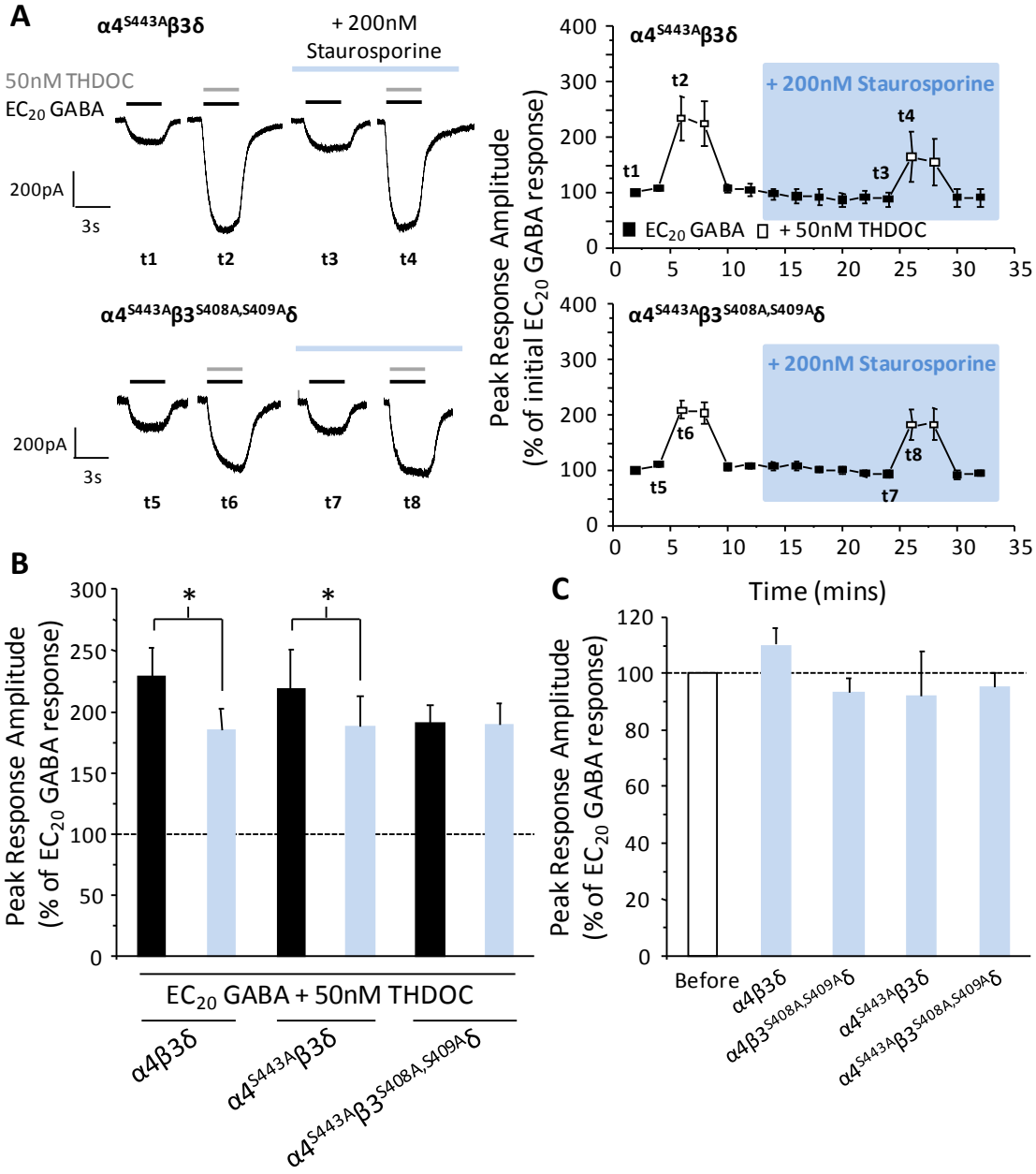

Figure 5.6 Staurosporine decreases THDOC-mediated potentiation at $\alpha4^{S443A}\beta3\delta$, but not at $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ $GABA_A$ receptors

(A) Mean peak currents recorded from HEK293 cells expressing $\alpha4^{S443A}\beta3\delta$ (n = 7) or $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ (n = 7) $GABA_A$ receptors in response to EC_{20} GABA (filled squares) or EC_{20} GABA + 50nM THDOC (open squares). Cells were treated with 200nM staurosporine (light blue shading). All responses were normalised to the peak current of the first EC_{20} GABA-activated response, recorded 2 min after achieving whole-cell recording configuration, designated as t = 0. Example whole-cell currents show the potentiation elicited by 50nM THDOC (grey bar) before and after staurosporine treatment (light blue bar). Current traces are representative of recordings at the time points indicated. (B) Bar chart showing the mean potentiation of EC_{20} GABA-activated currents by 50nM THDOC before (black bars) and after (light blue bars) staurosporine treatment in cells expressing α4β3δ (n = 9), $\alpha4^{S443A}\beta3\delta$ (n = 7) or $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ (n = 7) $GABA_A$ receptors. Data displayed for α4β3δ receptors here, and in (C), is as shown previously in Fig. 5.2. (C) Bar chart showing the change in peak receptor response elicited by EC_{20} GABA before (open bar) or after treatment with 200nM staurosporine (light blue bars) in cells expressing α4β3δ, $\alpha4^{S443A}\beta3\delta$ or $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ $GABA_A$ receptors. Change was measured between 2 min (before treatment) and 32 min (after treatment). All data points represent mean ± s.e.m. Significant results are indicated by * ($P < 0.05$, paired t-test).

Another notable observation from this experiment was that, similar to α4β3^{S408A,S409A}δ $GABA_A$ receptors, the magnitude of potentiation elicited at α4^{S443A}β3δ receptors, under basal cell conditions, did not differ significantly from that observed at wild-type receptors, implying, once again, that phosphorylation at either subunit can sustain the majority of the THDOC potentiation at α4β3δ $GABA_A$ receptors. Intriguingly, although the results showed a trend towards a decrease in the magnitude of basal THDOC potentiation induced at α4^{S443A}β3^{S408A,S409A}δ versus wild-type receptors, this was not statistically significant (potentiation = 89.7 ± 15.3% at α4^{S443A}β3^{S408A,S409A}δ compared to 128 ± 23.6% at α4β3δ receptors; Fig. 5.2A and 5.6A,B). However, this modest decrease of approximately 30% is consistent with the relatively low levels of staurosporine-induced modulation observed at α4β3δ receptors (38.1 ± 5.8%; Fig. 5.2). Therefore, this may suggest that the level of basal phosphorylation at this receptor subtype is low compared to that present at α1β3γ2L receptors, resulting in a reduced potential for modulation after either protein kinase inhibition or the abolition of phosphorylation through the mutation of consensus sites.

Interestingly, the results from the mutant receptor experiments showed that neurosteroid potentiation is still evident at α4^{S443A}β3^{S408A,S409A}δ $GABA_A$ receptors, with 50nM THDOC still inducing a 89.7 ± 15.3% enhancement of the EC_{20} GABA-activated current (Fig. 5.6C). This supports the results from the protein kinase inhibition experiment and demonstrates that phosphorylation, by protein kinases is not required to induce THDOC-mediated potentiation at α4β3δ $GABA_A$ receptors.

As in previous experiments, the EC_{20} GABA-activated current was monitored throughout the staurosporine treatment period in order to assess the effects of protein kinase inhibition on the receptor's response to agonist alone. Similar to the results obtained for wild-type receptors, cells expressing α4^{S443A}β3δ or α4^{S443A}β3^{S408A,S409A}δ $GABA_A$ receptors exhibited no change in the magnitude of responses elicited by EC_{20} GABA after treatment with 200nM staurosporine for 20 min (change = 7.7 ± 15.6% for α4^{S443A}β3δ and 5.5 ± 5.5% for α4^{S443A}β3^{S408A,S409A}δ receptors, $P > 0.05$; $n = 7$; Fig. 5.6C).

5.3 Discussion

5.3.1 Protein kinases positively modulate the actions of neurosteroids at 'extrasynaptic-type' $GABA_A$ receptors

$GABA_A$ receptors containing the δ subunit are located extrasynaptically to inhibitory synapses and are responsible for mediating a widespread 'tonic' inhibition in response to low, ambient concentrations of GABA. Modulation of tonic inhibition has been shown to alter the overall excitability of neurons (Semyanov *et al*., 2004; Farrant and Nusser, 2005) and therefore, endogenous agents that modulate extrasynaptic receptors will have important consequences for neural network activity. To date, studies investigating the relationship between neurosteroids and phosphorylation have predominantly focussed on their effects at typical 'synaptic' $GABA_A$ receptors, however, studies have shown that receptors located extrasynaptically are also subject to modulation by both neurosteroids and protein kinases (Herd *et al*., 2007; Smith *et al*., 2007; Bright and Smart, unpublished). Therefore, it was hypothesised that, a similar co-modulatory relationship may exist between neurosteroids and protein kinases at extrasynaptic $GABA_A$ receptors. This was confirmed by results from the present study which showed that, at α4β3δ receptors, inhibition of protein kinase activity caused a significant decrease in the magnitude of potentiation elicited by 50nM THDOC. (see Fig. 5.2). $GABA_A$ receptors incorporating α4 and δ subunits have been shown to be located outside of inhibitory synapses in a number of brain regions including the dentate gyrus and thalamus (Sperk *et al*., 1997; Sur *et al*., 1999; Pirker *et al*., 2000; Peng *et al*., 2004; Jia *et al*., 2005) and therefore, these results indicate that the actions of neurosteroids at extrasynaptic receptors are positively modulated by the activity of protein kinases.

5.3.2 Protein kinases are less effective at modulating neurosteroid activity at extrasynaptic- compared to synaptic-type $GABA_A$ receptors

An interesting observation from the present study was that, although staurosporine treatment induced a significant decrease in neurosteroid potentiation at α4β3δ $GABA_A$ receptors, this decrease was less than that seen at α1β3γ2L subunit-containing receptors (see Chapter 3), indicating that the modulatory effects mediated by protein kinases at synaptic and extrasynaptic $GABA_A$ receptors may not be equivalent. This may have important implications for the regulation of phasic and tonic inhibition *in vivo*, suggesting that the phasic inhibition mediated by synaptically located $GABA_A$ receptors has a greater potential to undergo 'fine-tuning' through the co-operative actions of neurosteroids and protein kinases. However, another reason for this discrepancy is that α4β3δ $GABA_A$ receptors may be subject to reduced

levels of basal phosphorylation, resulting in a reduced potential for modulation by protein kinase inhibitors such as staurosporine. The actions of protein kinases are reliant upon the availability of appropriate accessory proteins, such as RACK-1 and AKAP79/150, which enable the protein kinase to be anchored to the receptor, within a defined compartment, facilitating the phosphorylation process (Pawson and Scott, 1997; Colledge and Scott, 1999). Therefore, one factor which will determine whether $GABA_A$ receptors are phosphorylated under basal cell conditions is the presence of these proteins and their ability to bind to the receptor, which could differ between the synaptic and extrasynaptic receptor populations. It is possible that, given the mobility of extrasynaptic receptors in the membrane (Thomas *et al*., 2005), as well as, for the majority of extrasynaptic receptors, the inability to detect interactions with any defined scaffolding proteins, they may be less amenable to rigid interactions with protein kinases and their associated proteins, resulting in them being phosphorylated to a lesser extent.

5.3.3 THDOC potentiates α4β3δ and α1β3γ2L subunit-containing $GABA_A$ receptors to a similar extent

A number of studies have reported that $GABA_A$ receptors incorporating the δ subunit exhibit enhanced sensitivity to neurosteroids (Belelli *et al*., 2002; Brown *et al*., 2002; Wohlfarth *et al*., 2002). However, the results from the present study showed that the magnitude of potentiation elicited by 50nM THDOC at α4β3δ $GABA_A$ receptors was not significantly different from that induced at receptors containing α1β3γ2L subunits (see Fig. 5.3). The δ subunit is considered difficult to express in recombinant cell systems and therefore it is possible that this lack of difference between the potentiation observed at δ versus γ2L subunit-containing receptors may be due to the receptors not incorporating the δ subunit. However, the results from the present study showed that co-expression of δ, in combination with α4 and β3 subunits, produced functional receptors which incorporated the δ subunit. This was determined by the magnitude of THIP 'super-agonist' activity, which was indicative of receptors incorporating α4β3δ, rather than α4β3 subunits (Stórustovu and Ebert, 2006; Krogsgaard-Larsen *et al*., 2002). This is consistent with a number of previous studies which have also shown that expression of α4, β3 and δ subunits produces functional receptors at the cell surface which exhibit pharmacological profiles indicative of them incorporating the δ subunit (Belelli *et al*., 2002; Brown *et al*., 2002; Stórustovu and Ebert, 2006; Hosie *et al*., 2008; Meera *et al*., 2009; see Fig. 5.1). Therefore, in the present study, the absence of enhanced neurosteroid sensitivity at α4β3δ $GABA_A$ receptors is not likely to be due to the receptors failing to incorporate the δ subunit.

Another factor which may affect the relative sensitivities of α1β3γ2L and α4β3δ $GABA_A$ receptors to neurosteroids is the presence of different α subunit isoforms. It is possible that, any enhancement in neurosteroid sensitivity conveyed by the presence of the δ subunit may be masked by opposing effects mediated by the incorporation of the α4 subunit. Consistent with this notion, some studies have reported that the extent of neurosteroid potentiation induced at $GABA_A$ receptors can be modestly altered depending on the α subunit isoform present (Belelli *et al*., 2002). However, this effect was not evident at concentrations of neurosteroid between 30 and 100nM, with no significant difference between the magnitudes of potentiation induced at receptors containing α1-α6 being observed (Belelli *et al*., 2002). Furthermore, Hosie *et al*. (2009) demonstrated that the binding site which is responsible for the potentiating effects of neurosteroids at $GABA_A$ receptors is conserved across all α subunits. Therefore, in the present study, it is unlikely that the expression of different α subunits acts to modulate the extent of neurosteroid potentiation.

It is interesting to note that, although a number of studies have reported that neurosteroids display enhanced sensitivity at receptors containing the δ subunit, a closer look at the results reveals that the overall efficacy of the neurosteroid was increased, but the EC_{50} for potentiation remained unchanged (Belelli *et al*., 2002; Brown *et al*., 2002; Wohlfarth *et al*., 2002). In addition, for THDOC, a significant enhancement in potentiation at δ versus γ2 containing receptors was only observed at concentrations of 100nM and above, indicating that, at low, physiological concentrations, the difference between the sensitivities of δ and γ2 subunit-containing receptors is unlikely to be significant. This is consistent with studies of dentate gyrus granule cells, which have shown that, during development, THDOC sensitivity is reduced in these neurons (Cooper *et al*., 1999), despite the concurrent increase in δ subunit expression (Laurie *et al*., 1992b). Therefore, this reinforces the conclusion that, under physiological conditions, δ subunit-containing receptors may not exhibit enhanced sensitivity to neurosteroids, thus, indicating that it is unlikely that this effect will be observed at the concentration of neurosteroid used in the present study.

However, experiments carried out using knock-out mice have shown that deletion of the δ subunit results in diminished behavioural responses to alphaxalone, ganaxolone and pregnenolone (Mihalek *et al*., 1999), suggesting that, in these mice, the δ subunit-containing receptors convey much of the animal's sensitivity to neuroactive steroids. However, this study focused on the effects of therapeutic doses of steroids, which are likely to be much higher than those present under physiological conditions, in fact, this study reports no difference in the

basal levels of anxiety between $\delta^{-/-}$ and wild-type mice, indicating that the actions of the endogenous neurosteroids in the knock-out mice may be normal. It might be expected that if neurosteroid sensitivity was blunted, these mice would exhibit behaviours consistent with enhanced anxiety, although it cannot be discounted that compensatory mechanisms attempting to counteract the loss of δ subunits may be masking this effect.

Therefore, taken together, these results suggest that, under physiological conditions, when low concentrations of neurosteroid are likely to be present, there may be no significant difference between neurosteroid sensitivities at δ versus γ2 subunit-containing $GABA_A$ receptors, however, when concentrations of neurosteroids rise, for example during the late stages of pregnancy (Paul and Purdy, 1992), δ subunit-containing receptors may become more sensitive to neurosteroids than those containing γ2 subunits, which will have important implications for the regulation of tonic inhibition *in vivo*.

5.3.4 Phosphorylation of β3 subunits is not sufficient to induce complete modulation of neurosteroid potentiation

The results from the previous chapter indicated that phosphorylation at the β3 subunit is important for regulating neurosteroid activity at α1β3γ2L $GABA_A$ receptors, with results showing that the effects of inhibiting protein kinase activity were absent in cells expressing $\alpha1\beta3^{S408A,S409A}\gamma2L$ $GABA_A$ receptors (see Chapter 4). By contrast, a significant decrease in neurosteroid potentiation was still observed after staurosporine treatment in cells expressing $\alpha4\beta3^{S408A,S409A}\delta$ receptors (see Fig. 5.4), implying that phosphorylation at the β3 subunit is not absolutely required for the modulation of neurosteroid activity at α4β3δ $GABA_A$ receptors. However, staurosporine was notably less effective at $\alpha4\beta3^{S408A,S409A}\delta$ receptors, which suggests that phosphorylation at the β3 subunit may be involved in regulating neurosteroid potentiation at α4β3δ receptors, although it is not sufficient to induce the full modulatory effect observed. This was supported by further observations which showed that there was only a modest decrease in the magnitude of neurosteroid potentiation induced under basal cell conditions at $\alpha4\beta3^{S408A,S409A}\delta$ compared to wild-type $GABA_A$ receptors. Therefore, under conditions where the β3 subunit cannot be phosphorylated, the magnitude of potentiation elicited by 50nM THDOC is only moderately affected. This suggested that, in addition to phosphorylation at the β3 subunit, phosphorylation at other residues/subunits must also contribute to neurosteroid potentiation.

To date, consensus sites for phosphorylation have been identified on the $GABA_A$ receptor β and γ2 subunit families (Moss *et al.*, 1992, 1995; McDonald and Moss, 1994, 1997), which suggests that, at α4β3δ receptors, phosphorylation can only occur at the β3 subunit. This indicates that there are just two potential sites (serines 408 and 409) through which protein kinases could act to modulate neurosteroid potentiation. This is inconsistent with the results from the present study which showed that phosphorylation at the β3 subunit was not sufficient to induce the full modulation of neurosteroid potentiation that was observed at wild-type receptors. However, a recent study has identified a novel phosphorylation site, at serine 443 on the α4 subunit, which can be phosphorylated by PKC (Abramian and Moss, unpublished; see Fig. 5.5). This site is not thought to be present on the other members of the α subunit family (Moss *et al*., 1992; Moss and Smart, 1996; see Fig. 5.7), which is, perhaps, unsurprising given that, not only are the large TM3-4 intracellular domains of the $GABA_A$ receptor the least conserved portion of the protein, but the α4 subunit has also been shown to contain an exceptionally long intracellular loop when compared to the other α subunits (Ymer *et al*., 1989). In fact, the novel phosphorylation site appears to be located within a seemingly expanded region of the TM3-4 loop that is almost completely absent from the other members of the α subunit family (see Fig. 5.7). The presence of an additional phosphorylation site on the α4 subunit has important implications for the functional regulation of $GABA_A$ receptors incorporating this subunit, suggesting that they may have an enhanced potential for modulation by protein kinases. As the α4 subunit is thought to preferentially co-assemble with β and δ subunits at extrasynaptic sites (Sur *et al*., 1999; Peng *et al*., 2002), this enhanced modulation by protein kinases provides a promising mechanism for additional 'fine-tuning' of tonic inhibition *in vivo*. Furthermore, phosphorylation at the α4 subunit may also act to modulate the actions of neurosteroids at α4β3δ $GABA_A$ receptors.

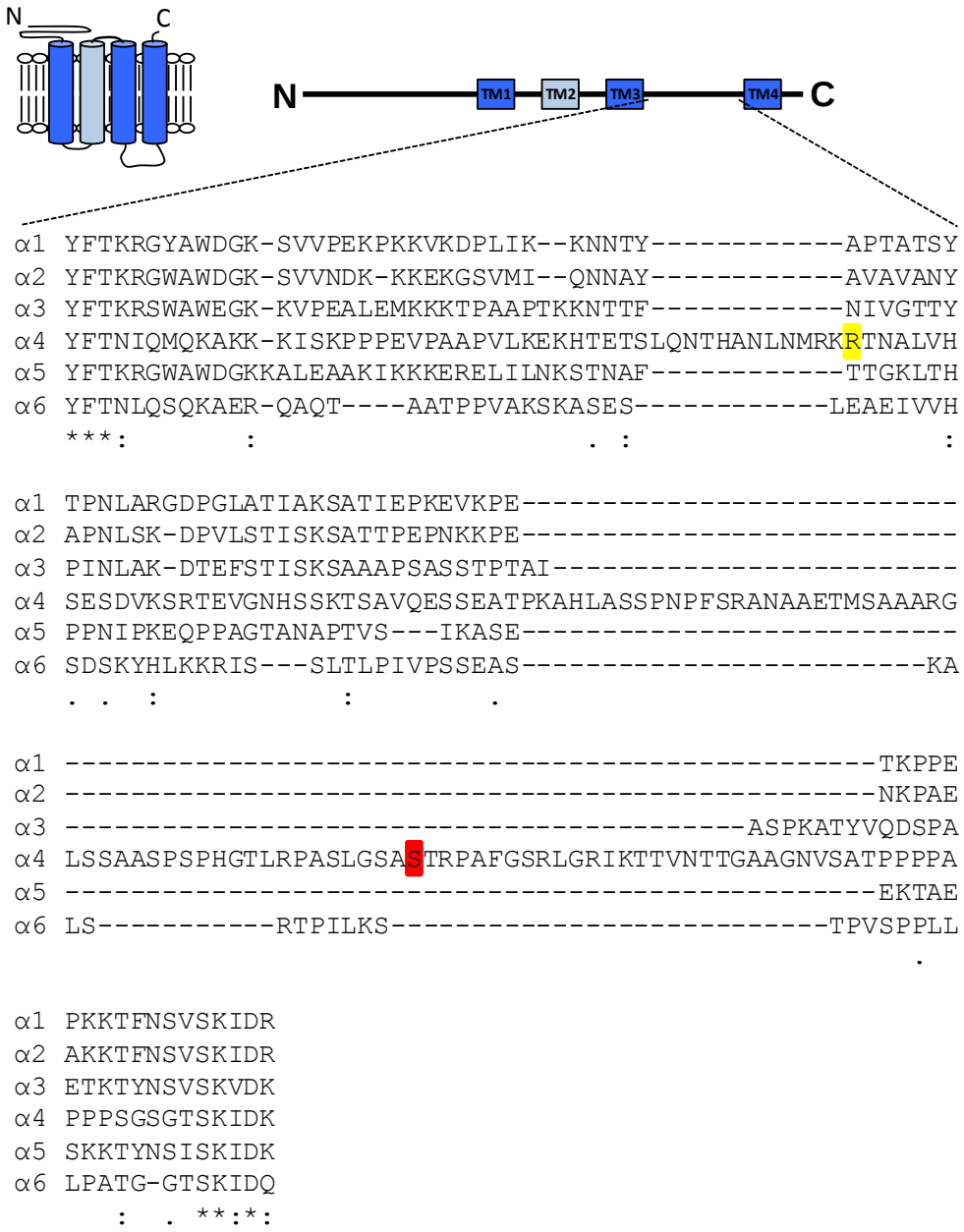

Figure 5.7 The major TM3-4 intracellular domains of the GABA$_A$ receptor α subunit family are poorly conserved

Sequence alignment of the TM3-4 intracellular domain of murine GABA$_A$ receptor α1-6. Fully conserved residues are indicated by *, with : and . symbols depicting strongly and weakly conserved residues, respectively. Highlighted in red is the novel phosphorylation site present on the α4 subunit (serine 443), which is present in the expanded region of the α4 intracellular domain, a region that appears to be mostly absent from the other members of the α subunit family. Yellow box highlights arginine 353, the residue crucial for the inhibitory effects of allopregnanolone at α4βδ GABA$_A$ receptors (Shen *et al*., 2007). Sequence alignment performed using clustalw.

5.3.5 Phosphorylation at β3 S408/S409 and novel α4 site, S443 is important for regulating neurosteroid activity at α4β3δ GABA$_A$ receptors

In order to determine whether phosphorylation at the α4 subunit was important for regulating neurosteroid potentiation at α4β3δ GABA$_A$ receptors, S443 was mutated to a neutral alanine

residue to prevent phosphorylation at this site. Interestingly, the results obtained from $\alpha4^{S443A}\beta3\delta$ $GABA_A$ receptors were very similar to those observed at $\alpha4\beta3^{S408A,S409A}\delta$ receptors, with staurosporine still inducing a significant decrease in neurosteroid potentiation, although to a notably lesser extent than observed at wild-type receptors (See Fig. 5.6). This implies that phosphorylation at α4 S443 may play a role in regulating the actions of neurosteroids at α4β3δ $GABA_A$ receptors, but once again, it is not sufficient to induce the complete modulatory effect. By contrast, at $\alpha4^{S443A}\beta3^{S408A,S409A}\delta$ $GABA_A$ receptors, the staurosporine-induced reduction in neurosteroid potentiation was completely abolished (see Fig. 5.6), indicating that phosphorylation at both the α4 and β3 subunits is important for regulating neurosteroid activity at 'extrasynaptic-type' α4β3δ $GABA_A$ receptors.

Intriguingly, these results suggest that phosphorylation at at least two sites is required to achieve a complete modulation of neurosteroid potentiation at α4β3δ receptors, which is in contrast to α1β3γ2L subunit-containing receptors, where phosphorylation at just one residue was sufficient (see Chapter 4). This is a curious result which implies that, when the $GABA_A$ receptor contains α1, phosphorylation at the β3 subunit is able to initiate a modulatory mechanism, probably via either a conformational change or through the alteration of other signalling pathways to alter the potentiating actions of neurosteroids. However, when the α1 subunit is replaced with the α4, this mechanism is re-routed, with phosphorylation at the β3 subunit no longer sufficient to induce a complete modulation of neurosteroid potentiation, but instead, phosphorylation at the α4 subunit is also required. The reason for this is unclear but, as discussed previously, it is known that the α4 subunit has an exceptionally long TM3-4 intracellular domain (Ymer *et al*., 1989; see also Fig. 5.8) and therefore, it is plausible that it may hold a rather different conformation compared to the shorter loop present in the α1 subunit. Therefore, if phosphorylation at the β3 subunit acts to alter the actions of neurosteroids via a conformational change, this additional structure present in the α4 subunit might act to perturb this alteration, resulting in an incomplete modulation of neurosteroid potentiation, as seen for α4β3δ $GABA_A$ receptors.

5.3.6 Phosphorylation at either α4 or β3 can modulate neurosteroid activity to some extent, although their effects are not additive

A notable observation from the present study was that, although the effects are reduced, phosphorylation at the α4 or β3 subunit alone can significantly alter the actions of neurosteroids at α4β3δ $GABA_A$ receptors (see Fig. 5.4 and 5.6). Interestingly, the modulation induced by phosphorylation at each subunit was not linearly additive, with phosphorylation at

either the α4 or β3 subunit resulting in a decrease in neurosteroid potentiation of approximately 27% and 25%, respectively, the combined total of which is much greater than the 38% reduction observed at wild-type receptors. This suggests that there may be some redundancy in the modulatory mechanism, with phosphorylation at a single subunit able to compensate to some degree for the lack of phosphorylation at the other. Alternatively, there may be a maximum threshold for this modulation which is less than the sum of the individual regulatory abilities of each subunit.

The non-additive nature of this modulation is not unusual, for example, Krishek *et al*. (1994) showed that the effects of phosphorylation at the β1 and γ2L subunits individually were not linearly additive compared to the modulation of GABA responses seen when both could be phosphorylated by PKC at wild-type α1β1γ2L $GABA_A$ receptors. Similarly, the results presented in the previous chapter showed that the sum of the decreases in GABA-activated current induced by PMA at $\alpha1\beta3^{S408A}\gamma2L$ and $\alpha1\beta3^{S409A}\gamma2L$ receptors was not equal to the reduction observed at wild-type receptors.

5.3.7 Direct receptor phosphorylation is not required for the induction of neurosteroid potentiation at α4β3δ $GABA_A$ receptors

The results from the present study have indicated that neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors is modulated by, but does not require, the activity of protein kinases. This was also the case for α4β3δ receptors, with results showing that neurosteroid potentiation was still evident after treatment with the broad-spectrum protein kinase inhibitor, staurosporine. Furthermore, potentiation could still be elicited at receptors containing mutations at all known phosphorylation sites ($\alpha4^{S443A}\beta3^{S408A,S409A}\delta$). However, as discussed previously, there is a small possibility that phosphorylation at, as yet, unidentified sites may also contribute to the induction of neurosteroid potentiation, with some previous studies reporting that there may be an additional site(s) located on the β3 subunit (McDonald and Moss, 1997). Moreover, as initial studies examining the α1 subunit reported that phosphorylation was not observed (Moss *et al*., 1992), it was inferred that other members of the α subunit family were also unlikely to be phosphorylated, leading to a lack of studies investigating potential phosphorylation sites on the α2-6 subunits. However, the recent discovery of a novel phosphorylation site on the α4 subunit suggests that there may be further sites present on other α subunit isoforms. In addition, the δ subunit has received relatively little attention in studies investigating potential phosphorylation sites and therefore

experiments to determine whether this subunit can also be phosphorylated could prove interesting.

5.3.8 Roles of specific protein kinases

This investigation did not specifically address the roles of individual protein kinases in the modulation of neurosteroid activity at α4β3δ $GABA_A$ receptors. For α1β3γ2L receptors, results showed that the actions of neurosteroids are regulated by PKC alone (see Chapter 3), although other studies have reported that, in some neuronal populations, other protein kinases may also be involved (Harney *et al*., 2003). At α4β3δ $GABA_A$ receptors, it is likely that PKC is involved in the modulation of neurosteroid activity as all three phosphorylation sites present on these receptors can be phosphorylated by PKC (McDonald and Moss, 1997; Abramian and Moss, unpublished). However, as both the α4 and β3 subunits are substrates for phosphorylation by other protein kinases (Moss and Smart, 1996; Abramian and Moss, unpublished), it is possible that any of these could be involved in the modulation of neurosteroid potentiation. Therefore, in order to identify the roles of specific protein kinases in the regulation of neurosteroid activity at α4β3δ $GABA_A$ receptors, further study is required using specific protein kinase inhibitors/activators.

5.3.9 Physiological significance of co-modulation at extrasynaptic $GABA_A$ receptors

The actions of GABA at extrasynaptic receptors have been shown to generate a widespread, tonic inhibition, which is an important regulator of neuronal excitability *in vivo*. Therefore, endogenous agents that can modulate the function of extrasynaptic receptors will have important consequences for neural network activity. The results from the present investigation showed that, at extrasynaptic-type, α4β3δ $GABA_A$ receptors, a co-modulatory relationship exists between neurosteroids and protein kinases and therefore, this provides important insight into how tonic inhibition may be regulated *in vivo*.

Interestingly, a number of neurological and psychiatric disorders that are associated with the dysfunction of neurosteroid activity may be attributable to an alteration in their actions at extrasynaptic receptors. For example, it has been shown that, in the dentate gyrus, fluctuating levels of neurosteroids during the ovarian cycle (Paul and Purdy, 1992; Bäckström *et al*., 2003), results in cyclic changes in δ subunit expression and a concurrent modulation of tonic inhibition (Maguire and Mody, 2007). These changes have been associated with altered seizure susceptibility in catamenial epilepsy as well as the appearance of enhanced anxiety during certain phases of the menstrual cycle (Maguire *et al*., 2005). Furthermore, the alterations in δ

subunit expression have been shown to be induced rapidly, appearing after exposure to neurosteroid for just 30 min. Therefore, these changes may also be initiated by episodes of stress, which have been also shown to rapidly enhance levels of the neurosteroid, THDOC (Purdy *et al*., 1991). During pregnancy, levels of neurosteroids are thought to increase to around 100nM (Paul and Purdy, 1992) and therefore, a concurrent decrease in δ subunit expression is thought to occur as a homeostatic mechanism in order to maintain normal levels of neuronal excitability (Maguire and Mody, 2008; Maguire *et al*., 2009). Interestingly, studies of $\delta^{-/-}$ mice, which cannot regulate δ subunit expression during pregnancy and the post-partum period, showed that these mice exhibit abnormal maternal behaviours, with the appearance of a depression-like phenotype (Maguire and Mody, 2008). Therefore, it has been suggested that a failure to restore normal δ subunit expression following the rapid decline in neurosteroid levels post-partum could lead to hyper-excitability in certain neuronal networks which may contribute to the appearance of post-partum depression in humans (Maguire and Mody, 2008).

Collectively, these studies demonstrate that the actions of neurosteroids at extrasynaptic $GABA_A$ receptors are crucially important for regulating tonic inhibition *in vivo*, with perturbations in this process contributing to a number of debilitating disorders. Therefore, as the results from the present study indicated that the actions of neurosteroids at extrasynaptic receptors can be modulated by the extent of receptor phosphorylation, the basal activities of protein kinases as well as conditions where these activities are enhanced or reduced may also affect the manifestation of these disorders. Furthermore, the existence of a co-regulatory relationship between neurosteroids and protein kinases at extrasynaptic receptors provides an increased number of potential targets for therapeutic interventions.

Another disorder associated with abnormal neurosteroid modulation at extrasynaptic $GABA_A$ receptors is the appearance of enhanced anxiety during puberty. Curiously, although administration of the neurosteroid, allopregnanolone, produces anxiolytic effects in adult mice, paradoxically, it increases anxiety in pubertal female mice (Shen *et al*., 2007). This effect has been attributed to the increased expression of extrasynaptic, α4βδ subunit-containing $GABA_A$ receptors in the CA1 region of the hippocampus during puberty, which generate outward currents and, in stark contrast to the reported potentiating actions of neurosteroids, are in fact inhibited by allopregnanolone, resulting in enhanced tonic inhibition and increased neuronal excitability in the hippocampus (Shen *et al*., 2007). This effect is thought to be unique to receptors containing the α4 subunit as it has been shown to be dependent upon arginine

353, a non-conserved residue located within the large TM3-4 intracellular domain of the α4 subunit (see Fig. 5.8). Given the position of this residue and its proximity to the recently identified consensus phosphorylation site, S443, it may be interesting to investigate whether the actions of protein kinases can modulate the generation of anxiety during puberty, as this may present additional targets for treatment of mood disorders associated with puberty.

$GABA_A$ receptors consisting of α4βδ subunits have a well-defined expression within the CNS, with studies showing that they are predominately expressed at extrasynaptic sites in the thalamus and dentate gyrus (Sperk *et al*., 1997; Sur *et al*., 1999; Pirker *et al*., 2000; Peng *et al*., 2004; Jia *et al*., 2005). Therefore, the results from the present study provide an insight into the regulatory mechanisms controlling tonic inhibition in these regions of the brain. Interestingly, previous studies have shown that, in the dentate gyrus, physiological concentrations of neurosteroid exclusively modulate the tonic conductance, with no effect at synaptic receptors (Harney *et al*., 2003; Stell *et al*., 2003). This highlights the importance of understanding the regulatory mechanisms controlling the actions of neurosteroids at extrasynaptic receptors in these neurons. At α4β3δ $GABA_A$ receptors, neurosteroid potentiation can be modulated by the activities of protein kinases. This implies that the tonic conductance present in the dentate gyrus is also likely to be subject to a similar co-modulation, which could provide an ideal mechanism by which the actions of two distinct modulators act in concert to achieve a rapid and precise fine-tuning of neuronal excitability. Furthermore, the presence of an additional consensus phosphorylation site on the α4 subunit also provides the potential for an added level of regulation, which may indicate that extrasynaptic receptors can undergo 'fine-tuning to a greater extent than synaptic receptors, although the relevance of this remains to be determined *in vivo*.

5.4 Conclusions

- Protein kinase activity positively modulates the actions of neurosteroids at 'extrasynaptic-type', α4β3δ $GABA_A$ receptors.

- Protein kinases act to modulate the actions of neurosteroids via direct phosphorylation of the α4 and β3 subunits.

- Phosphorylation at either α4 or β3 can alter neurosteroid potentiation, but phosphorylation at both subunits is required to induce the full modulatory effect.

- Induction of neurosteroid potentiation at α4β3δ $GABA_A$ receptors does not require direct receptor phosphorylation.

Chapter 6

General Discussion

$GABA_A$ receptors are subject to modulation by a number of endogenous compounds, which, by acting to alter receptor function, have a significant impact on neural activity. Neurosteroids and protein kinases are among the most potent modulators of the $GABA_A$ receptor, which, when acting individually, can enhance or depress receptor function depending on the type of neurosteroid or kinase present and the subunit combination of the receptor (Belelli and Lambert, 2005; Moss and Smart, 1996). However, *in vivo*, these agents are most likely to act in concert to achieve a precise fine-tuning of inhibitory neurotransmission by regulating $GABA_A$ receptor function.

Studies of both recombinant and native $GABA_A$ receptors have indicated that the activity of protein kinases can modulate the potentiating actions of neurosteroids (Leidenheimer and Chapell, 1997; Harney *et al*., 2003; Fáncsik *et al*., 2000). This is supported by the results from the present study, which showed that the magnitude of potentiation induced by neurosteroids at α1β3γ2L $GABA_A$ receptors is positively modulated by the activity of PKC. PKC was shown to mediate this effect through direct phosphorylation of the β3 subunit, with phosphorylation at either S408 or S409 being sufficient. Furthermore, at α1β3γ2L $GABA_A$ receptors, the co-modulatory relationship between neurosteroids and protein kinases was shown to be bi-directional, with phosphorylation at the receptor acting to modulate the actions of neurosteroids and, in the reverse situation, exposure to neurosteroids causing an increase in the extent of receptor phosphorylation. These findings provide an important insight into the regulation of $GABA_A$ receptors *in vivo*, and into the mechanisms by which fine-tuning of GABAergic inhibitory transmission may be achieved via the actions of two endogenous neuromodulators.

Interestingly, results showed that the co-modulatory relationship between neurosteroids and protein kinases was not identical for all receptor subtypes, with neurosteroid potentiation at extrasynaptic-type α4β3δ $GABA_A$ receptors being modulated to a lesser extent than synaptic-type α1β3γ2L receptors. This may have important implications for the regulation of phasic and tonic inhibition *in vivo*, suggesting that the phasic inhibition mediated by synaptically located $GABA_A$ receptors has a greater potential to undergo 'fine-tuning' through the cooperative actions of neurosteroids and protein kinases. Furthermore, at α4β3δ receptors,

phosphorylation on both the β3 and α4 subunits was required to achieve a complete modulation of neurosteroid potentiation, which is in contrast to α1β3γ2L subunit-containing receptors, where phosphorylation at just one residue was sufficient. Therefore, this suggests that, although neurosteroid potentiation at α4β3δ receptors may be modulated to a lesser extent by protein kinases, the actions of neurosteroids at this receptor may be under greater incremental control due to the effect being regulated by phosphorylation at multiple residues, as opposed to the 'all or none' effect mediated by phosphorylation at a single residue on the α1β3γ2L receptor.

6.1 Mechanism of co-modulation

The mechanism by which phosphorylation influences the interaction between neurosteroids and the $GABA_A$ receptor is unknown. However, a number of possible mechanisms have been proposed. One such theory is that the $GABA_A$ receptor is phosphorylated under basal cell conditions and this is required for neurosteroid binding and subsequent potentiation (Tasker, 2000). However, the results from the present study are inconsistent with this notion, showing that neurosteroid potentiation is still evident at receptors containing mutations at all known phosphorylation sites, indicating that phosphorylation is not absolutely required for the potentiation of $GABA_A$ receptor function by neurosteroids.

A second potential mechanism is that the binding of the neurosteroid to the receptor causes a conformational change which unmasks a PKC consensus site, allowing it to be phosphorylated (Tasker, 2000). Although it is accepted that phosphorylation can occur in the absence of neurosteroid, this could provide a possible explanation for the enhancement of phosphorylation after neurosteroid treatment, perhaps suggesting that the binding of neurosteroid causes the sites for phosphorylation to become more accessible. However, exposure of receptors to the PKC activator, PMA resulted in a greater enhancement of receptor phosphorylation than observed after neurosteroid treatment, perhaps implying that the sites for phosphorylation are fully accessible in the absence of neurosteroids. Furthermore, as neurosteroids were less effective at promoting $GABA_A$ receptor phosphorylation than PMA, this suggests that neurosteroids are unlikely to increase receptor phosphorylation by directly activating protein kinases. This is supported by the results obtained from $\alpha1^{Q241W}\beta3\gamma2L$ $GABA_A$ receptors, which showed that neurosteroids up-regulate phosphorylation through a direct interaction with the receptor, indicating that the increase in phosphorylation is more likely to be mediated via an alteration of receptor-associated signalling pathways. An alternative mechanism by which neurosteroids may facilitate $GABA_A$ receptor phosphorylation is by

recruiting active protein kinase molecules to the receptor microdomain, however further experiments would be required to elucidate this in more detail, perhaps using biochemical techniques to investigate the association between protein kinases and the receptor in the presence and absence of neurosteroids.

The results from the present investigation showed that, at α1β3γ2L $GABA_A$ receptors, phosphorylation by PKC at either β3 S408 or S409 was sufficient to modulate neurosteroid potentiation, indicating that phosphorylation at just one of these residues is required to regulate the actions of neurosteroids at this receptor subtype. Similar results were also obtained in preliminary studies of α1β2γ2L subunit-containing receptors, where phosphorylation at S410 was sufficient to modulate neurosteroid potentiation to a similar extent to that observed at receptors incorporating the β3 subunit. This indicates that only a small negative charge is required to enhance neurosteroid activity, which may be in the form of a conformational change or modification of the signal transduction mechanism through which neurosteroids act to potentiate $GABA_A$ receptors. In addition, the fact that, at α1β3γ2L receptors, phosphorylation at the second PKC site does not further modulate the magnitude of neurosteroid potentiation indicates that the addition of a single phosphate moiety induces the full modulatory effect. This has interesting implications for the modulation of $GABA_A$ receptors *in vivo*, suggesting that the actions of neurosteroids at receptors containing different β subunits are not subject to differential regulation by PKC.

One possible mechanism by which phosphorylation may act to enhance neurosteroid potentiation is by inducing a conformational change which increases the accessibility of the neurosteroid binding site. Glutamine 241, one of the three key residues identified as being important for the interaction between neurosteroid molecules and the $GABA_A$ receptor (Hosie *et al*., 2006), is proposed to be located at the base of a water-filled cavity formed between TM1 and TM4 of the α subunit (Hosie *et al*., 2007). Interestingly, studies using substituted cysteine accessibility methods have shown that this cavity increases in volume in the presence of GABA (Williams and Akabas, 1999), leading to the hypothesis that this GABA-induced increase in volume may facilitate entry of the neurosteroid into the cavity, allowing it to bind Q241, stabilizing the receptor in its open conformation and resulting in potentiation of the GABA current (Hosie *et al*., 2007). Therefore, it is a possibility that phosphorylation may act to increase the volume of this cavity further, promoting even greater access to the neurosteroid binding site, which may lead to an increase in neurosteroid binding, resulting in an enhancement of its potentiating effects. To investigate this in more detail, further experiments

could be conducted using the substituted cysteine accessibility method in order to identify any further conformational changes which may occur within this cavity in the presence of phosphorylation. Williams and Akabas (1999) reported that, at α1β1γ2 receptors, the presence of GABA causes an increase in the depth and volume of this cavity as demonstrated by the exposure of four previously inaccessible residues on the TM3 domain, residues which are deeper within this cavity. Therefore, in order to determine whether this cavity enlarges further in the presence of both GABA and phosphorylation, accessibility of residues not exposed by GABA alone could be investigated. It can be hypothesised that, if additional residues present on the TM3 domain become accessible in the presence of GABA and phosphorylation, this may indicate that further conformational changes are occurring in this cavity which may facilitate the interaction between neurosteroids and their binding site. In addition, if the presence of phosphorylation does indeed increase the accessibility of the neurosteroid binding site, it would be expected that the potency of neurosteroids would be increased. Therefore, this could be investigated by constructing neurosteroid dose-response curves in the presence and absence of phosphorylation. A curve shift would be indicative of a change in neurosteroid potency with a leftward shift reflecting an increase in the neurosteroid interaction.

6.2 Relative contribution of each neurosteroid potentiation site

Given that phosphorylation on the β3, but not the γ2L subunit is important for regulating neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors, it may be interesting to consider the relative positioning of the neurosteroid binding sites within the receptor complex. Neurosteroids are thought to mediate their potentiating actions through a discrete site located within the transmembrane region of the α subunit, of which there are two copies within each pentameric $GABA_A$ receptor assembly. Interestingly, studies investigating the stoichiometry and relative positioning of subunits within the $GABA_A$ receptor complex have suggested that one α subunit is flanked by two β subunits whereas the second is located between a β and a γ (Sieghart *et al*., 1999). Therefore, although the use of concatemeric receptors has suggested that these sites are functionally equivalent with respect to their ability to induce neurosteroid potentiation (Akk *et al*., 2009; Bracamontes and Steinback, 2009), it is possible that one site may be subject to greater modulation by phosphorylation at its surrounding β subunits. To investigate this, experiments similar to those conducted in the present study could be conducted using concatemeric receptors which contain mutations within one of the neurosteroid binding sites, thus allowing the influence of phosphorylation at each site to be determined.

6.3 Additional mechanistic complexity for α4β3δ $GABA_A$ receptors

Interestingly, although phosphorylation at the β3 subunit was shown to be sufficient to modulate neurosteroid potentiation at α1β3γ2L $GABA_A$ receptors, this was not the case for α4β3δ receptors, with results showing that phosphorylation at both the α4 and β3 subunits was important for regulating neurosteroid potentiation at this receptor subtype. Therefore, this suggests that phosphorylation at at least two sites is required to achieve the complete modulatory effect at α4β3δ receptors, which is in contrast to α1β3γ2L subunit-containing receptors, where phosphorylation at just one residue was sufficient. This indicates that phosphorylation sites present on the α subunit also play a role in modulating neurosteroid potentiation at $GABA_A$ receptors and therefore this introduces the potential for differential regulation depending upon the identity of the α subunit. For example, when the receptor contains the α1 subunit, which is not thought to possess any consensus sites for PKC (Moss *et al*., 1992), phosphorylation at the β3 subunit is able to initiate the modulatory mechanism which results in the enhancement of neurosteroid activity. However, when the α1 subunit is replaced with the α4, which contains a phosphorylation site at S443 (Abramian and Moss, unpublished), this mechanism is either perturbed or re-routed, with phosphorylation at the β3 subunit no longer sufficient to induce a complete modulation of neurosteroid potentiation. The reason for this is unclear but as the α4 subunit has been shown to possess an exceptionally long TM3-4 intracellular domain (Ymer *et al*., 1989), it is possible that it may hold a different conformation compared to the shorter loop present in the α1 subunit, with this additional structure acting to perturb the mechanism by which β3 subunit phosphorylation alters neurosteroid activity at this receptor. In order to investigate this further, it may be interesting to determine how neurosteroid potentiation at receptors containing different α subunits is modulated by protein kinases, particularly those which also contain long intracellular domains such as α5 and α6. Furthermore, experiments investigating the co-modulatory relationship between neurosteroids and protein kinases at αβ $GABA_A$ receptors may help to determine whether the δ or γ subunits are involved in modulating neurosteroid potentiation, either through direct phosphorylation or by contributing to or modifying the mechanism by which phosphorylation acts to alter neurosteroid potentiation.

6.4 Specificity of co-modulation

The results from the present study indicate the existence of a co-modulatory relationship between neurosteroids and protein kinases at $GABA_A$ receptors. However, previous studies have shown that the actions of protein kinases can also modify the activities of other $GABA_A$ receptor modulators including, ethanol (Proctor *et al*., 2003; Qi *et al*., 2007; Choi *et al*., 2008)

and benzodiazepines (Leidenheimer *et al*., 1993; Hodge *et al*., 1999; Qi *et al*., 2007). Therefore, to extend the present study, it may be interesting to determine whether phosphorylation at the β3 and/or α4 subunits specifically acts to modulate the actions of neurosteroids or whether the activities of other modulators are also affected. If phosphorylation at these subunits also modulates the actions of other compounds at the $GABA_A$ receptor, this may suggest that phosphorylation at the β3 or α4 subunits acts to modify a common modulatory pathway, thus providing a further insight into the mechanism by which phosphorylation acts to regulate the actions of neurosteroids at the receptor.

The present study investigated the effects of protein kinases on the actions of THDOC, a potentiating neurosteroid which is endogenously produced within the CNS. However, a second class of neurosteroid is also synthesised within the mammalian CNS, which have inhibitory effects on $GABA_A$ receptors, resulting in an increase in neuronal excitability. Therefore, it may be interesting to determine whether the actions of these inhibitory neurosteroids are also modulated by protein kinases, and if so, whether the same residues which modify the actions of potentiating neurosteroids are involved.

6.5 Role of phosphatases

Phosphorylation of the $GABA_A$ receptor is controlled by both protein kinases and phosphatases, with the balance of activities between the two determining the overall functional effect. This study examined the roles of protein kinases in modulating the activity of neurosteroids at $GABA_A$ receptors, however, the role of phosphatases was not investigated. Some previous studies have suggested that the activity of phosphatases may also be important, with blockade of phosphatases 1 and 2A resulting in a decrease in neurosteroid sensitivity at $GABA_A$ receptors in the hypothalamus (Koksma *et al*., 2003). It is likely that both protein kinases and phosphatases will be present and active in neurons and therefore, it may be instructive to extend this study to examine the effects of phosphatase inhibitors on neurosteroid-mediated potentiation, enabling a clearer picture of the co-modulatory relationship between neurosteroids and protein kinases at $GABA_A$ receptors *in vivo* to be determined.

6.6 Involvement of other protein kinases

In addition to PKC, PKA and PKG, the $GABA_A$ receptor can also be phosphorylated by CaMKII (Machu *et al*., 1993; McDonald and Moss, 1994; McDonald and Moss, 1997; Houston *et al*., 2008), the effects of which were not investigated in this study. The use of HEK293 cells meant

that it was impossible to assess the potential effects of CaMKII on neurosteroid-mediated potentiation at $GABA_A$ receptors as CaMKII is not thought to be active in this cell line (Houston and Smart, 2006). The reason for this is unclear, but it is thought that due to their non-neuronal lineage, HEK293 cells may lack the necessary trafficking or scaffolding machinery required for CaMKII to phosphorylate the receptor. Therefore, any further studies to examine the potential role of CaMKII, would need to be conducted using a neuronal cell line, such as NG108-15 cells, in which phosphorylation by CaMKII has been shown to mediate functional effects at $GABA_A$ receptors (Houston and Smart, 2006).

6.7 Physiological importance of co-modulation

The ability of protein kinases to positively modulate the actions of neurosteroids at $GABA_A$ receptors has important implications under conditions in which the activity of protein kinases is reduced or elevated. For example, decreased PKC levels have been observed in the brains of patients with a number of neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntingdon's disease (Nishino *et al*., 1989; Masliah *et al*., 1990; Hashimoto *et al*., 1992), as well as in some forms of epilepsy (Terunuma *et al*., 2008). By contrast, enhanced PKC activity has been seen after the administration of some psychoactive drugs including ethanol (Stubbs and Slater, 1999) and cocaine (Steketee, 1996; Steketee *et al*., 1998). Therefore, in situations where protein kinase activity is perturbed, particularly PKC, effects will be seen both directly, in the alteration of $GABA_A$ receptor phosphorylation and indirectly, by modulating the activity of neurosteroids at these receptors. These changes will inevitably lead to alterations of GABAergic inhibition, which may contribute to disease aetiology or exacerbate symptomatic profiles.

Interestingly, the results from the present study showed that, at α1β3γ2L $GABA_A$ receptors, the co-modulatory relationship between neurosteroids and protein kinases is bi-directional with phosphorylation at the receptor acting to modulate the actions of neurosteroids and, in the reverse situation, exposure to neurosteroids causing an alteration in the extent of receptor phosphorylation. Therefore, any variations in neurosteroid concentrations, such as those observed during pregnancy, stress or at certain phases of the ovarian cycle will not only modulate $GABA_A$ receptor function directly, but also indirectly through the alteration of receptor phosphorylation. Furthermore, the bi-directional nature of this modulation provides an ideal mechanism for the fine-tuning of GABAergic inhibition. The function of this 'reverse' modulation in which neurosteroids alter the extent of receptor phosphorylation remains to be determined, however, there is some evidence to suggest that it may have a role in regulating

the trafficking of $GABA_A$ receptors or could be involved in the generation of 'automodulation', a kind of positive feedback whereby repetitive co-applications of GABA and neurosteroid causes a 'run-up' of neurosteroid potentiation (Wegner *et al*., 2007; see Chapter 4).

$GABA_A$ receptor expression is relatively ubiquitous throughout the CNS and, with neurosteroids exhibiting no clear receptor isoform specificity (Hadingham *et al*., 1993; Belelli *et al*., 1996, 2002), it could be hypothesised that the actions of neurosteroids would be globally experienced, causing a generalised potentiation, rather than achieving a more localized 'fine-tuning' of inhibitory neurotransmission. Such a non-specific action is incompatible with neurosteroids having a physiological role, however, studies have shown that the actions of neurosteroids are both brain region and neuron specific. For example, low, physiological concentrations of neurosteroids have been shown to prolong mIPSCs recorded from hippocampal CA1 neurons (Harney *et al*, 2003), cerebellar granule cells (Cooper *et al*., 1999; Hamann *et al*., 2002; Vicini *et al*, 2002) and cerebellar Purkinje neurons (Cooper *et al.*, 1999); whereas concentrations above those seen physiologically are required to produce similar effects in hypothalamic neurons (Brussaard *et al.*, 1997; Fancsik *et al*., 2000; Koksma *et al.*, 2003). Further experiments have shown that even neurons within the same brain structure can be differentially modulated, for example, in rat hippocampal brain slices approximately 30 times higher concentrations of allopregnanolone are required to prolong mIPSCs in dentate gyrus granule cells than in CA1 pyramidal neurons (Harney *et al*., 2003). It has been suggested that the differing activities of protein kinases within these different brain areas and neuronal populations may contribute to their differential sensitivity to neurosteroids. The results from a previous study showed that, under basal conditions, dentate gyrus granule cells are relatively insensitive to physiological concentrations of neurosteroids. However, when PKC activity is increased these neurons become much more sensitive to the effects of applied neurosteroids (Harney *et al*., 2003). A similar result was obtained in the present study, with results showing that activation of PKC caused α1β3γ2L $GABA_A$ receptors to become sensitive to lower, previously ineffective concentrations of THDOC (1nM). Therefore, taken together, these results indicate that differing activities of protein kinases offers a plausible mechanism for regulating the effects of neurosteroids within different neuronal populations.

6.8 Implications for the treatment of pathological conditions

Dysfunction of GABAergic neurotransmission has been implicated in a number of neurological and psychiatric disorders including epilepsy, anxiety and some neurodegenerative conditions such as Huntington's and Alzheimer's disease (Armstrong *et al*., 2003; Fritschy and Brünig,

2003; Lüscher and Keller, 2004). Therefore, given their essential role in controlling neuronal excitability, it is important to elucidate the cellular mechanisms by which $GABA_A$ receptors are regulated *in vivo*. The results from the present study provide an important insight into the mechanisms by which GABAergic inhibition may be regulated by the co-operative actions of neurosteroids and protein kinases and therefore these results may also help to identify potential therapeutic targets for conditions associated with $GABA_A$ receptor dysfunction.

Dysfunction of neurosteroid activity is also associated with a number of pathological conditions such as catamenial epilepsy, stress, depression, as well as mood disturbances associated with the ovarian cycle and pregnancy (Bäckström *et al*., 2003; Maguire *et al*., 2005; Maguire and Mody, 2007; Maguire *et al*., 2009). In the majority of these conditions, alterations in the concentrations of CNS neurosteroids have been shown to cause a perturbation of inhibitory transmission. This can lead to hypo- or hyper-excitability in certain neuronal networks, which may contribute to the appearance of these conditions or exacerbate the associated symptoms. Therefore, as the results from the present study indicate that the actions of neurosteroids at $GABA_A$ receptors can be modulated by the extent of receptor phosphorylation, this provides an increased number of potential therapeutic targets for the treatment and prevention of these debilitating conditions.

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