Investigations into $GABA_B$ receptor surface stability and molecular interactions

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Abstract

Whereas most G-protein-coupled receptors (GPCRs) are monomeric in structure, γ-Aminobutyric acid type B ($GABA_B$) receptors are heterodimers of two seven transmembrane protein subunits, $GABA_{B(1)}$ and $GABA_{B(2)}$. $GABA_B$ receptor function is dependent upon the co-expression of both these proteins which, when individually expressed, are devoid of receptor activity.

Desensitisation of cell surface receptors allows tissues to rapidly adjust their response to agonist. A conserved mechanism ensures that GPCR signalling is closely followed by desensitisation. This entails the phosphorylation of activated receptors enabling interaction with arrestin proteins and subsequent internalisation. It is not known if heterodimeric $GABA_B$ receptors employ this method of desensitisation. Data presented here result from experiments to determine whether $GABA_B$ receptor cell surface stability is controlled in a similar manner to that of other GPCRs. Also documented is the study of a putative interaction between the protein kinase AMPK and the $GABA_{B(1)}$ subunit.

Initial whole cell labelling studies demonstrated that both $GABA_{B(1)}$ and $GABA_{B(2)}$ are basally phosphorylated. Dissimilar to other GPCRs, agonist did not increase levels of phosphorylation and this remained true upon overexpression of G protein receptor kinases. Because $GABA_B$ receptors lacked the internalisation promoting increase in phosphorylation upon agonist, it was predicted that they might have enhanced surface stability. This was confirmed in heterologous systems where $GABA_B$ receptors did not demonstrably internalise after agonist application even when arrestins were overexpressed.

$GABA_B$ receptors in cultured cortical neurones showed a similar lack of internalisation in response to agonist. Biotinylation of neuronal surface receptors demonstrated that $GABA_B$ receptors reside for an unusually long time at the plasma membrane. Chronic agonist decreased the surface receptor half-life, but this did not correlate with an increase in internalised receptor. Interestingly, chronic agonist did not significantly reduce total receptor protein levels, suggesting $GABA_B$ receptors may not downregulate. Protein kinase A

(PKA) stimulation, both exogenously and through intracellular pathways, counteracted the agonist-induced degradation and demonstrated that this particular kinase can control $GABA_B$ receptor surface numbers. Protection from degradation was correlated with increased phosphorylation at serine 892 within $GABA_{B(2)}$, a residue previously demonstrated to be a PKA substrate.

Subsequent experiments were carried out to identify kinases capable of phosphorylating $GABA_{B(1)}$. Affinity purification assays isolated a kinase from brain able to interact with and phosphorylate a twenty amino acid stretch of the carboxy-terminal domain of $GABA_{B(1)}$. Yeast two-hybrid studies identified the catalytic α1 subunit of AMPK as a putative interacting protein with $GABA_{B(1)}$. AMPK was found to phosphorylate $GABA_{B(1)}$ at serines 917 and 923 within the carboxy-terminal domain. Phosphorylation of serine 917 was further confirmed with a phospho-specific antibody raised to this residue. α1 AMPK affinity purifies with $GABA_{B(1)}$ carboxy-terminal domain GST fusion proteins and also co-immunoprecipitates with $GABA_B$ receptors from brain. Preliminary investigations indicate that AMPK activation increases surface $GABA_B$ receptor levels in cortical neurones and may affect the protein protein interactions of $GABA_{B(1)}$.

The results presented in this thesis suggest $GABA_B$ receptors are highly stable at the neuronal surface. The activation of PKA and AMPK may be mechanisms by which neurones are able to regulate plasma membrane $GABA_B$ receptors.

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List of Abbreviations:

ADP	–	adenosine diphosphate
5'AMP	–	adenosine monphosphate
AMPK	–	AMP activated protein kinase
AMPKK	–	AMP activated protein kinase kinase
AMPA	–	a-amino-3-hydrocy-5-methyl-4-isoxazolepropionic acid
ATP	–	adenosine triphosphate
BSA	–	bovine serum albumin
CamKII	–	Ca^{2+}/calmodulin dependent protein kinase
cAMP	–	cyclic adenosine monophosphate
cDNA	–	complementary deoxyribonucleic acid
CGRP	–	calcitonin gene related peptide
CNS	–	central nervous system
COS-7	–	African green monkey kidney cells
DIV	–	days *in vitro*
EGFP	–	enhanced green fluorescence protein
ER	–	endoplasmic reticulum
et al.	–	and others
FRET	–	Fluorescence Resonance Energy Transfer
GABA	–	γ-aminobutyric acid
GABAA	–	γ -aminobutyric acid receptor type A
GABAB	–	γ -aminobutyric acid receptor type B
GABAC	–	γ -aminobutyric acid receptor type B
GAD	–	glutamic acid decarboxylase
GEF	–	guanine nucleotide exchange factor
GPCR	–	G protein-coupled receptor
G Protein	–	guanine nucleotide binding protein
GRK	–	G protein-coupled receptor kinase
GST	–	glutathione-*s*-transferase
GTP	–	guanosine triphosphate
HEK	–	human embryonic kidney cells

IPSP	–	inhibitory postsynaptic potential
LTP	–	long term potentiation
mGluR	–	metabotropic glutamate receptor
mRNA	–	messenger RNA
NMDA	–	N-methyl-D-aspartate
NP-40	–	nonylphenoxy polyethoxy ethanol
NSF	–	*N*-ethylmaleimide sensitive factor
^{32}PγATP	–	ATP with phosphorous 32 isotope at gamma position
PKA	–	cAMP-dependent protein kinase
PKC	–	Ca^{2+}/phospholipid dependent protein kinase
RGS	–	regulator of G protein signalling
SDS-PAGE	–	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TM	–	transmembrane
TMB	–	tetramethylbenzidine dihydrochloride
TMD	–	transmembrane domain
TRH	–	thyrotropin receptor hormone
TRHR	–	thyrotropin receptor hormaone receptor
WD-40	–	tryptophan aspartate repeat sequence
Y2H	–	yeast two-hybrid

CHAPTER ONE

1.1 The neurone is dedicated to receiving and delivering signals

Of crucial importance to the physiology of multicellular organisms is intercellular signalling. In the central nervous system (CNS) neurones are morphologically and functionally dedicated to delivering and receiving signals, termed neurotransmission. Neurotransmission contains both chemical and electrical components with much of the electrical communication via gap junctions. Chemical synaptic transmission is far more diverse, both in the type and in the nature of delivery of the chemical signal. The amount and identity of neurotransmitter released by pre-synaptic neurones and the class and number of receptors present post-synaptically help determine the manner of the response. In addition, this response is modulated by rates of neurotransmitter uptake and degradation. The effect of transmission on the post-synaptic neurone is to change the membrane potential and alter the probability of action potential firing. Hyperpolarising signals are therefore inhibitory, moving the membrane potential away from the spike threshold for action potentials; whilst excitatory signals will depolarise and cause action potential firing.

1.2 γ-Amino-Butyric Acid (GABA) is the major inhibitory neurotransmitter in brain

The first neurotransmitter reported to have an inhibitory action was GABA, and its discovery opened up the new field of inhibitory neurotransmission. GABA was initially isolated from brain tissue in 1950 independently by both Jorge Awapara and Eugene Roberts, who also demonstrated its synthesis from glutamic acid (Awapara *et al.,* 1950; Roberts & Frankel 1950). For a chemical to be classified as a neurotransmitter it is accepted that it should be demonstrable that it is synthesised, stored, and released from neurones. A site of action should also exist in tandem with a mechanism of removal from that site.

The inhibitory action of GABA was first demonstrated in the crayfish where the exogenous application of a substance from mammalian brain extracts, termed 'Factor I', mimicked the effect of stimulating inhibitory axons at the neuromuscular junction (Elliot *et al.,* 1956). This substance was identified as GABA (Bazemore *et al.,* 1957)

and was observed to be present at high concentrations in crayfish inhibitory axons, but virtually undetectable in excitatory motor fibres (Kravitz *et al.,* 1963). It became appreciated that GABA application had generally inhibitory effects in both vertebrates and invertebrates, and that these effects were matched by changes in ion conductance similar to those observed under inhibitory nerve activation (Kuffler & Edwards 1958; Kuffler 1960). It was subsequently shown that GABA was released from the inhibitory axon in a calcium dependent manner (Otsuka *et al.,* 1966).

GABA is synthesised through de-carboxylation of the amino-acid L-glutamate by the enzyme glutamic acid decarboxylase (GAD). GAD exists as two major isoforms GAD 65 and 67, which differ in their kinetics, sensitivity to the presence of co-factor pyridoxal 5-phosphate and cellular localisation (Martin & Rimvall, 1993). Synthesis occurs in the cytosol of inhibitory neurones from where GABA is loaded into vesicles ready for release by vesicular neurotransmitter transporter (VGATs) (Fon & Edwards 2001).

It has become apparent that most classical neurotransmitters, including GABA, have both rapid and modulatory effects. This is achieved through activity at two different classes of receptors; ionotropic and metabotropic receptors. Ionotropic receptors are responsible for the fast effects of neutrotransmitters, whilst the slower action is through metabotropic receptors.

1.3 The ionotropic GABA response

The ionotropic GABA response was the foremost to be characterised and is elicited through $GABA_A$ receptors (Macdonald & Olsen 1994). $GABA_A$ receptors were the first GABA receptors to be cloned (Schofield *et al.,* 1987). This revealed that they belong to the ligand gated ion channel superfamily of receptors, homologous to nicotinic acetylcholine receptors, glycine receptors and 5-HT_3 receptors amongst others. $GABA_A$ receptors are hetero-oligomers of five subunits, each with four transmembrane domains, a large extracellular N-terminus and an intracellular carboxy-terminal tail (Macdonald & Olsen 1994). The pentameric nature of the $GABA_A$ receptor leaves a central pore lined by the second transmembrane domain of each subunit. The division between each subunit forms extracellular clefts that in conjunction with the N-terminal domain that are thought to form ligand binding sites.

Agonist binding allosterically alters the structure of the receptor, allowing the selective flow of anions through the central pore (Macdonald & Olsen 1994). This selectivity is dictated by the amino-acid composition of the second transmembrane domain of each subunit. The pore of the $GABA_A$ receptor is permeable to Cl^- ions and, to a lesser extent, HCO^{3-} ions (Bormann *et al.,* 1987; Kaila 1994). In the mature neurone the extracellular concentration of Cl- ions is approximately 120mM while intracellularly the concentration is 7mM. Upon activation of $GABA_A$ receptors, chloride ions flow down the concentration gradient, hyperpolarising the neurone. The exception to this is seen in embryonic neurones where the chloride gradient is reversed, resulting in excitation through $GABA_A$ activity.

A large number of different $GABA_A$ receptor subunits are expressed and are grouped according to sequence homology into $\alpha(1\text{-}6),\beta(1\text{-}3),\gamma(1\text{-}3),\delta,\varepsilon,\pi$ and θ subunits (Sieghart & Sperk 2002). Additional variability is also provided by the existence of alternate splice variants of certain subunits. The existence of so many subunits is probably a product of genetic duplication throughout evolutionary history. Although a huge number of receptors are theoretically possible due to different subunit permutations, this does not appear to be the case; and instead certain subunit combinations are favoured (McKernan & Whiting 1996). However, there is still considerable variability in $GABA_A$ receptor composition, leading to heterogeneity in $GABA_A$ expression patterns, targeting and pharmacology.

$GABA_A$ receptor mediated inhibition is fast, i.e. it is rapidly activated and inactivated. $GABA_A$ receptor pharmacology is complex, receptors having a Hill coefficient of 2 for GABA indicating the presence of 2 GABA binding sites, both of which must be occupied for maximal response. The extracellular surface of $GABA_A$ receptors also provides other ligand binding sites including ethanol, benzodiazepines, barbiturates and endogenous neurosteroids. Benzodiazepines act as allosteric modulators enhancing the GABA induced current by increasing the frequency of channel opening, but not acting as agonists in their own right (Study & Barker 1981). At low concentrations barbiturates also enhance GABA induced current, probably by increasing the duration of channel opening, whilst at high concentrations barbiturates act as agonists. Ethanol and neurosteroids such as 5-α-pregnanolone again enhance GABA mediated current through the receptor by allosterically modulating its structure.

$GABA_A$ receptors are inhibited by bicuculline, although sensitivity to other antagonists such as to picrotoxin is modified by receptor subunit composition. The variable composition of each receptor can also dictate differential targeting of the receptor within a neurone, with receptors containing γ subunit being targeted to the synapse whilst others are targeted to extrasynaptic regions. Because different neurones express different subunits, areas of the brain can become pharmacologically distinct.

GABA also activates another class of ionotropic receptors that are closely related to $GABA_A$ receptors and are termed $GABA_c$ receptors. $GABA_c$ receptors are pharmacologically distinct from $GABA_A$ receptors, being insensitive to bicuculline and activated by the selective agonist *cis*-4-aminocrotonic acid (CACA). $GABA_c$ receptors are also stimulated by the $GABA_A$ agonist muscimol and are actually more sensitive to GABA, although they desensitise more slowly and show no modulation by benzodiazepines or neurosteroids (Bormann and Feigenspan, 1995). $GABA_c$ are believed to be structurally similar to $GABA_A$ receptors but are composed of novel subunits designated ρ1 and ρ2 in either a homo- or heteropentameric manner.

$GABA_c$ expression in the brain is limited probably to ρ2 subunits with most $GABA_c$ receptors being found in the retina especially in bipolar cells (Bormann & Feigenspan, 1995).

1.4 The metabotropic GABA response

1.4.1 Discovery of $GABA_B$ receptors

Much of the early work characterising the inhibitory action of GABA utilised the drug bicuculline, which antagonised the effect of GABA application. Resistance to bicuculline antagonism though was described (Straughan *et al.,* 1971), demonstrating that bicuculline was not a universal GABA receptor antagonist. This bicuculline insensitive GABA response appeared to be especially prominent in sympathetic nerve terminals, where it decreased evoked neurotransmitter release (Bowery & Hudson 1979, Brown & Higgins 1979).

Crucial to deducing the nature of the bicuculline insensitive GABA response was the drug (-)β-p-chlorophenyl-GABA (baclofen). Baclofen was initially conceived as a lipophilic gabamimetic, and indeed it proved to have an inhibitory quality (Mott &

Lewis 1994). However, the inhibitory effect of baclofen was also bicuculline insensitive (Curtis *et al.,* 1974). Moreover, baclofen was inactive at sites that had been previously characterised as bicuculline sensitive, yet was as effective as GABA in reducing neurotransmitter release. These inconsistencies meant that it was generally considered that baclofen did not act at GABA receptors. In 1980 Bowery and co-workers demonstrated the presence in the CNS of a receptor to baclofen (Bowery *et al.,* 1980). They determined that baclofen diminished the evoked release of radiolabelled neurotransmitter from cortical and cerebellar slices, an effect that was mimicked by GABA. This led them to propose that both GABA and baclofen may indeed act at the same site to inhibit neurotransmitter release (Bowery *et al.,* 1980). Evidence to support this hypothesis was produced the following year with the demonstration that baclofen could displace tritiated GABA from rat brain membranes (Hill & Bowery 1981). Previous groups had shown that, at best, baclofen could only weakly displace tritiated GABA (Galli *et al.,* 1979; Horng *et al.,* 1979), however Hill and Bowery showed that upon the inclusion of divalent cations in the binding solutions a much larger pool of GABA could be displaced. Indeed, $GABA_B$ receptor binding was the first to be described that required the presence of physiological concentrations of divalent cations (Hill & Bowery 1981). Because the classical GABA agonist isoguvacine could not displace baclofen, the isoguvacine binding site was termed the $GABA_A$ receptor, whilst the novel GABA and baclofen binding site was designated the $GABA_B$ receptor (Hill & Bowery 1981).

1.4.2 $GABA_B$ receptors couple to heterotrimeric G-proteins

Further studies identified a large number of differences, apart from agonist and antagonist sensitivity, between $GABA_A$ and $GABA_B$ receptors. The most salient observation was that GTP potently inhibits $GABA_B$ receptor binding (Hill *et al.,* 1984; Asano *et al.,* 1985). This inhibition is due to a reduction in receptor affinity for agonist and not receptor binding capacity. Sensitivity to GTP was $GABA_B$ specific with no effect of nucleotides at $GABA_A$ receptors; and the effect was confined to GTP, with other nucleotides such as ATP having no effect (Hill *et al.,* 1984; Asano *et al.,* 1985). Sensitivity to GTP had been previously demonstrated with several other receptors including those for dopamine (Freedman *et al.,* 1981), adrenergic receptors

(U'Prichard & Snyder, 1978) and opiate receptors (Pert & Taylor, 1980). These receptors couple to G proteins, and because GTP sensitivity is a hallmark of G protein coupled receptors (GPCRs), it suggested that $GABA_B$ receptors belong to this class of receptor. Further evidence that $GABA_B$ receptors are indeed GPCRs was the effect of solubilisation on $GABA_B$ receptor binding. In contrast to $GABA_A$ receptors, where solubilisation has little effect upon agonist binding, it was noted that solubilisation prevents $GABA_B$ receptor binding. This supported the notion that $GABA_B$ receptors couple to G proteins, with detergents that disrupt the receptor:G protein interaction removing agonist binding. Also demonstrated was the ability of the inhibitory G protein inactivator, pertussis toxin, to decrease $GABA_B$ receptor binding in brain membranes (Asano *et al.,* 1985), indicating that $GABA_B$ receptors couple to inhibitory G proteins. Finally, it was shown that baclofen enhances GTPase activity in brain membrane preparations, with this enhancement greatest in areas with the highest concentration of $GABA_B$ receptors (Hill *et al.,* 1989; Bowery *et al.,* 1989). This indicated that $GABA_B$ receptor activation stimulated GDP:GTP exchange, a property of GPCRs.

1.5 G Protein-Coupled Receptors (GPCR's)

1.5.1 G proteins act as signal transducers

Unlike ionotropic receptors, which are also the effectors of agonist activity, GPCRs utilise a transducer, a G protein, to couple to effector proteins. G proteins are named after their ability to bind guanine nucleotides, specifically GTP and GDP. They have an inherent GTPase activity, catalysing the hydrolysis of GTP to GDP. Binding of GTP to a G protein switches it 'on' and it is able to couple to effectors. The period of coupling is dictatated by the rate of GTP hydrolysis; with hydrolysis terminating effector coupling and leaving the G-protein GDP bound. This rate can be modulated by a host of other proteins including regulators of G-protein signalling proteins (RGS's) and GTP exchange factors (GEF's) (De Vries *et al.,* 2000; Ross & Wilkie 2000). Early purification studies of G-proteins that couple to GPCR's elucidated that they are composed of three independent proteins an α, a β and a γ subunit (Gilman 1987). The α subunit binds guanine nucleotides and possesses GTPase activity. The

activated receptor acts as a GEF to the α subunit, allosterically modulating the G-protein to exchange bound GDP for GTP and so switching it 'on'. Reciprocally, the activated G protein reduces the affinity of the receptor for agonist, promoting ligand removal. This is why a high concentration of GTP can prevent GPCR agonist binding, because by shifting the equilibrium towards GTP bound G-proteins it also shifts the GPCR agonist affinity in a negative direction.

1.5.2 Structural analysis elucidates mechanisms of G-protein action

Crystallisation studies of the heterotrimeric G-proteins have shed further light on their ability to link the GPCR to effector molecules (Lambright *et al.,* 1996). The α subunit is composed of three domains: a Ras-like GTPase domain, a helical domain and an N-terminal helix projecting away from the rest of the protein. The N-terminus is subject to myristoylation aiding the membranous localisation of the α subunit. The GTPase and helical domains form a cleft where guanine nucleotide binding occurs. In a GDP bound state the α subunit binds to the β subunit over two distinct interfaces called the 'switch I' and 'switch II' regions (Lambright *et al.,* 1996). The γ phosphate of GTP is capable of interacting with residues unaffected by GDP and in doing so causes a confirmational change that removes the switch I and II regions from close proximity to the β subunit, thus dissociating the complex (Lambright *et al.,* 1996).

In contrast to the more flexible α subunit, the β and γ subunits form a tightly associated rigid unit. The β subunit is a canonical water filled 'propeller' with seven blades and an approximate seven-fold symmetry (Sondek *et al.,* 1996). The γ subunit partially encircles the β subunit with the N-termini of each subunit forming parallel coiled coils. Post-translational farnesylation of the γ subunit N-terminus aids membranous localisation of the complex. Each blade of the β propeller is a WD repeat motif of approximately 40 amino-acids terminating in a tryptophan-aspartate dipeptide. The γ subunit does not appear to directly interact with the α subunit, the interaction of the βγ complex being mediated by amino-acids at one end of the β propeller. The βγ subunit is also able to interact with the receptor, probably playing a role in the receptor catalysed GTP exchange. Dissociation of the βγ subunit upon GDP-GTP exchange in the α subunit reveals residues at the end of the propeller that

mediate interaction with the switch I and II regions of the α subunit. These residues are implicated in effector coupling and so the $\beta\gamma$ complex is activated.

Both the α and $\beta\gamma$ subunits of the G-protein are capable of coupling to effectors. However, it is the α subunit that determines the activity of the heterotrimer with nucleotide exchange promoting a structural reorganisation. In contrast there is little change in the $\beta\gamma$ subunit on nucleotide exchange, its activity being solely regulated by the confirmation of the α subunit. Binding of the α subunit negatively regulates $\beta\gamma$ activity not only by masking important amino-acids responsible for effector coupling but also by restricting the degrees of freedom of the complex, thus making other interactions impossible. The precise extent to which the heterotrimeric G protein physically dissociates upon activation remains controversial (Klein *et al.,* 2000), but it is clear that to activate effectors some form of detachment occurs.

1.5.3 Heterotrimeric G-proteins couple to a large array of effector proteins

In contrast to the rapid action of an ionotropic receptor, where agonist binding elicits an almost immediate effect through the receptor, the activation of a GPCR is slow. Agonist binding enhances receptor affinity for G proteins, which in turn, bind receptors and exchange GDP for GTP leading to dissociation of G protein subunits. Only then can effectors be activated. Although this may appear to be an inefficient process, it is quite the reverse. Because one given GPCR can couple to more than type of G-protein it allows a receptor to have multiple effects and these can be either temporally and spatially separated or concurrent. Signalling through G-proteins also has other advantages including a multiplicative effect where a small signal can give a large response due to the ability of the receptor to couple to several orders of magnitude higher number of G-proteins. GPCR signalling can act over a longer time frame than ionotropic signalling, due to the property whereby it elicits an accumulation of metabolites which continue to exert an effect after cessation of receptor activity.

1.5.4 GPCRs exhibit a seven-helical transmembrane domain structure

The sequencing of the light sensitive GPCR rhodopsin allowed the first analysis of a GPCR structure (Hargrave *et al.,* 1983; Ovchinnikov 1982; Nathans & Hogness

1983). This showed seven hydrophobic stretches of amino acids that were putative α-helical transmembrane (TM) domains, connected by hydrophilic loops with a proposed extradiscal N-terminal domain and intracellular C-terminus. After the cloning of the β2 adrenergic receptor which showed a remarkably similar composition (Dixon *et al.,* 1986), it became apparent that this structure may be common to all GPCRs. The solving of the crystal structure of rhodopsin has further elucidated this arrangement; demonstrating a 7TM collection of a helices joined by variable length loops arranged, when viewed from outside the cell, in an anti-clockwise manner (Palczewski *et al.,* 2000).

The importance of GPCRs within an organisms signalling repertoire is illustrated by the number of receptors within the genome; with 5% of *c.elegans* genome dedicated to ~1100 receptors (Bargmann 1998). Within vertebrates a similar number of different GPCRs exist, and are involved in processes as diverse as taste and olfacation to regulation of neuronal activity, reflecting the evolutionary effectiveness of such a signalling mechanism.

GPCRs can be subdivided along the lines of sequence similarity into families A,B and C. Family members tend to share over 25% sequence identity across their transmembrane domains as well as possessing other highly conserved familial characteristics (Bockaert & Pin 1999). Between the three families there is very little sequence similarity with possession of predicted 7TM domains being the only common feature (Bockaert & Pin 1999; Pierce *et al.,* 2002).

Family A contains the archetypal GPCRs rhodopsin, and β adrenergic receptors, along with receptors to dopamine and the muscarinic acetylcholine receptors. Other GPCRs belonging to family A include all of the olfactory receptors (~200), making it by far the largest class of GPCR. Family A GPCRs characteristically possess a small N-terminal extracellular domain and a large third intracellular loop, with variable length intracellular carboxy-terminal domains. The majority of family A receptors are activated by small ligands. These are believed to fit into pockets created on the extracellular surface deep between the transmembrane domains. Ligand binding alters receptor confirmation, rearranging transmembrane domains 6 and 3 amongst other movements (Ballesteros *et al.,* 2001; Farahbakhsh *et al.,* 1995). This movement is transmitted to the intracellular side of the receptor and is thought to

unmask G protein binding sites, allowing the catalysis of G protein guanine nucleotide exchange. Mutational analysis of several family A members supports this theory, with mutations of the proposed ligand binding sites able to remove agonist sensitivity. Mutations of putative binding sites can also cause constitutive receptor activity if they place receptors in a state analogous to that adopted when ligand is bound (Bockaert & Pin 1999; Pierce *et al.,* 2002).

Family B is much smaller and consists of receptors sensitive to peptides such as the secretin receptor and the vasoactive peptide receptor. It appears that all members of family B couple to G-proteins to positively regulate adenylyl cyclase. Like family A the transmembrane domains of family B receptors are believed to be involved in ligand binding, as are extracellular the loops. The comparative bulk of peptides to ligands of family A means that the ligand binding site is probably not so deeply buried within the transmembrane domains of the receptor, and there is also evidence to support a role for the N-terminus of certain family B receptors in ligand binding.

When metabotropic glutamate receptors (mGluRs) were initially cloned (Houamed *et al.,* 1991; Masu *et al.,* 1991) it was apparent that they were very dissimilar to previously cloned GPCRs and they were proposed to belong to a separate GPCR family (Nakanishi 1994), which became family C. Family C receptors are the most phylogenetically disparate group of GPCRs. Several other family C receptors have been identified in addition to mGluRs including taste receptors (TiRs), putative pheromone receptors, calcium sensing receptors and also $GABA_B$ receptors (Bockaert & Pin 1999). Whereas most GPCR's are 400-500 amino-acids in length and typically have molecular weights in the region of 50-60kDa, family C receptors are much larger with molecular weights often well over 100kDa. The seven transmembrane domains are found towards the end of family C sequences due to the large (often in excess of 500 amino-acids) N-terminal extracellular domain (NTED) (fig.1). Another feature of the NTED common to family C receptors is the presence of a region showing homology to bacterial periplasmic binding proteins (PBPs), first described in mGluRs (O'Hara *et al.,* 1993). PBPs are involved in the transport of amino-acids and ions across bacterial membranes and their role in family C

receptors is thought to be analogous to this in that they bind the amino-acid or ionic ligands required for receptor activation. This characteristic of family C GPCRs appears to have evolved through the gene fusion of a primordial amino-acid-binding protein and an ancestral 7TM receptor (Nakanishi 1994). Within the mGluRs the amino-acid binding area shows a high degree of similarity to that found in the ionotropic glutamate AMPA and NMDA receptors (O'Hara *et al.,* 1993). Between the amino-acid binding domain and the first transmembrane domain lies a stretch of 70 amino-acids containing 9 highly conserved cysteine residues, reminiscent of those found in transmembrane tyrosine kinase receptors (Aaronson 1991). This cysteine rich region is thought to be important in ligand binding interactions and is lacking in $GABA_B$ receptors. The intracellular loops of family C receptors are small in comparison to those of other GPCRs- often less than 10 amino-acids in length whilst the intracellular carboxy-terminal domains are of moderate length, being normally 100 amino-acids.

1.5.5 GPCRs share a conserved desensitisation mechanism

Evolution preserves successful mechanisms, and as organisms become more complex, these systems are employed over and again. Organisms need to be able to receive and transduce signals and GPCRs are an efficient solution to this, as evidenced by their phylogenetic conservation. In a similar manner a conserved system has developed and is utilised by a wide variety of GPCRs to 'switch off' receptor activity i.e. mediate desensitisation.

The primary step in GPCR desensitisation is phosphorylation. This can be mediated by classical second messenger activated kinases such as PKA and PKC (so called heterologous desensitisation), or by specific G protein receptor kinases (GRKs) (homologous desensitisation). GRKs belong to a gene family encoding 7 kinases (GRKs 1-7), and they specifically phosphorylate G protein coupled receptors (Pitcher *et al.,* 1998). Importantly, GPCRs that are in an active confirmation are far more amenable to GRK phosphorylation, allowing GRKs to preferential 'label' activated receptors. In addition to this the agonist occupied receptors potently stimulate GRK activity (Pitcher *et al.,* 1998). GRKs themselves are subject to palmitoylation, keeping them in close proximity to the membrane, and are able to

directly interact with G protein $\beta\gamma$ subunits (Pitcher *et al.,* 1992) which is important for their activation. GPCRs that are substrates for GRKs usually have multiple serine and threonine residues in regions responsible for coupling to G proteins, usually the second and third intracellular loops in conjunction with the carboxy-terminal intracellular domain. Phosphorylation of these residues may promote uncoupling from G proteins, but more importantly, greatly enhances the affinity of GPCRs for arrestins. These are a class of protein belonging to a gene family containing four different isoforms (visual arrestin, cone arrestin, arrestins 2 & 3 (formerly β-arrestin 1 & 2)) that when bound to phosphorylated GPCRs prevent G protein coupling (Pierce *et al.,* 2002; Luttrell & Lefkowitz 2002). The tertiary step in GPCR desensitisation is the internalisation of the GPCR into clathrin-coated vesicles. Arrestins are crucial to this step, acting as bridging molecules between adaptin proteins (hetero-oligomeric protein complexes involved in the intracellular trafficking of proteins) and phosphorylated GPCRs.

The process of vesicle formation from surface membranes is too complex to be covered in depth here, but briefly involves the formation of clathrin-coated pits around the molecules to be endocytosed. Adaptin 2 (AP2) is thought to be the main adaptin involved in endocytosis, and assists the formation of clathrin coated pits by binding both clathrin and arrestins. Clathrin structure is such that as oligomers of clathrin build-up they cause curvature of the membrane and subsequent invagination. The final step in vesicle formation is the separation of this invaginated pit from the rest of the membrane. The GTPase dynamin, in tandem with other proteins of the endocytotic machinery such as ampiphysin, is central to this step and catalyses the 'pinching' of vesicle necks causing vesicle formation.

GPCR endocytosis can rapidly alter the number of receptors at the surface, thereby causing a desensitisation of the response. Complexed GPCR arrestin molecules can also form intracellular scaffolds that mediate a variety of downstream actions. Once a GPCR has been endocytosed, it can be dephosphorylated by specific phosphatases (Pitcher *et al.,* 1995) and recycled to the surface membrane, or it can be degraded via late endosomes and lysosomes. It is now thought that not all GPCR endocytosis occurs in an arrestin and clathrin dependent manner. GPCR endocytosis can also occur into caveolae, although this appears to be somewhat

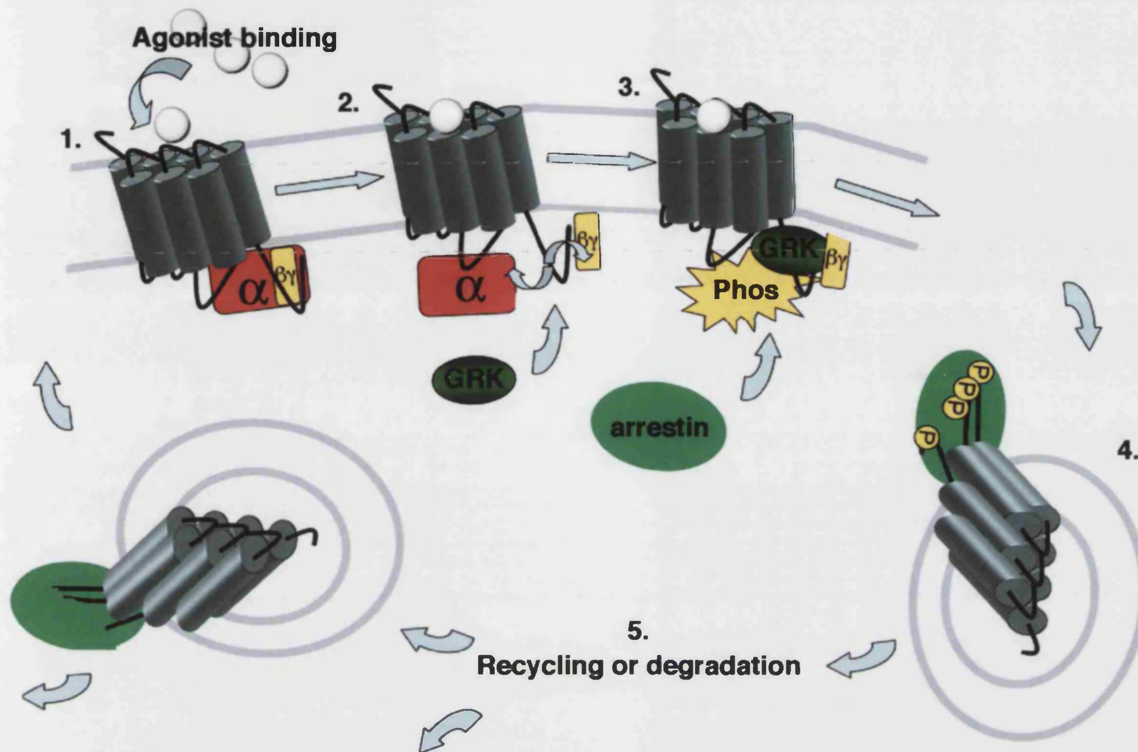


Figure 1: Illustration of the multiple steps in GPCR desensitisation

1. Agonist binding activates receptors which in turn catalyse GTP:GDP exchange within heterotrimeric G proteins.
2. Dissociation of heterotrimeric G protein subunits allows coupling to effector proteins. GRKs are able to interact with activated receptors which reciprocally elicit GRK activity.
3. GRK phosphorylation of multiple residues within the intracellular loops and carboxy-terminal intracellular domain of the GPCR promotes binding to arrestin proteins.
4. Arrestins sterically prohibit further G protein coupling and facilitate internalisation into clathrin coated pits.
5. Internalised GPCRs are dephosphorylated by GPCR specific phosphatases causing arrestin dissociation from followed by recycling to the membrane. Alternatively, receptors are trafficked to late endosomes and lysosomes for degradation.

receptor and cell type specific. It is thought that the caveloae mechanism of GPCR endocytosis is secondary to that mediated through the arrestin pathway, which under physiological conditions predominates (Ferguson 2001).

1.5.6 Post-translational modifications of GPCRs

GPCRs are subject to a number of important post-translational modifications that help to maximise receptor coupling efficiency, and tailor receptor activity to specific cellular demands. The phosphorylation of GPCRs is most closely associated with desensitisation mechanisms, as covered above. Other roles for phosphorylation of GPCRs have also been uncovered outside this context. Notable illustrations of alternative roles of phosphorylation include the ability of phosphorylation of the β2 adrenergic receptor at residue ser216 to switch coupling from G_s G proteins, to which they normally efficiently couple, to G_i G proteins (Daaka *et al.,* 1997). It has also been shown that PKA phosphorylation of $GABA_B$ receptors can paradoxically delay the onset of desensitisation (Couve *et al.,* 2001). Phosphorylation is able to promote changes in the intermolecular interactions of GPCRs. One such example is observed in the interactions of mGluR7 which binds directly to active calmodulin or G protein βγ subunits in a mutually exclusive manner via residues in its intracellular carboxy-terminal domain. The binding of βγ subunits renders mGluR7 unable to couple to Ca^{2+} channels, but this ability is restored in the presence of activated calmodulin. Phosphorylation of ser862 within mGluR7 prevents these interactions and alters mGluR7 activity, permitting coupling to Ca^{2+} channels (El Far & Betz 2002).

Post-translational palmitoylation of specific cysteine residues within many GPCR intracellular carboxy-terminal domains increases the hydrophobicity of this region, and has been proposed to allow the formation of a fourth intracellular loop. Preventing the palmitoylation of β2-adrenergic receptors through mutagenesis of the putative palmitoylation site leads to an uncoupled GPCR (O'Dowd *et al.,* 1989). The observation that the majority of GPCRs have conserved cysteine residues within or at the end of their carboxy-terminal intracellular domain ($GABA_B$ receptors included) means that this may be a highly conserved modification amongst GPCRs to enhance coupling and perhaps modulate targeting.

Post-translational glycosylation is almost universal amongst GPCRs, and indeed the presence of immature glycol groups can be used as a marker of retention within the endoplasmic reticulum (ER) and is also used to trigger ER associated degradation. The precise functions of GPCR glycosylation are poorly understood, but glycosylation may play a role in receptor folding and targeting, and also mediating interactions on the extracellular surface of GPCRs.

1.5.7 Monomeric or polymeric?

The 'classical' view of GPCRs describes them as monomeric entities that contain all the necessary information within their structure to correctly traffick and couple to G proteins. The tenet that GPCRs function solely as monomers has never been conclusively proved though; and data is accruing that challenges the monomeric dogma. It transpires that certain GPCRs require the presence of accessory molecules to confer activity, whilst others form dimers or higher order structures (Bouvier 2001).

The first evidence that a GPCR may require an accessory protein to mediate activity concerned the calcitonin-receptor-like receptor (CRLR). CRLR was initially proposed to be the calcitonin gene related peptide receptor (CGRP) (Njuki *et al.,* 1993) but appeared inactive in heterologous systems. It was subsequently discovered that activity was conferred upon the co-expression of a protein called RAMP (receptor-activity-modifying protein) (McLatchie *et al.,* 1998). RAMP was a member of what emerged to be a family of 3, single transmembrane domain proteins (RAMP1-3) which showed no resemblance to GPCRs. CRLR became the CGRP (calcitonin gene-related peptide) upon the co-expression of RAMP 1, and the adrenomedullin receptor when co-expressed with RAMP2 (McLatchie *et al.,* 1998).

The possibility that GPCRs may form dimeric or even oligomeric structures was entertained in the early days of GPCR research, when ligand binding studies of β2 adrenergic receptors demonstrated co-operativity, i.e. the ability of the presence of one ligand to alter the receptor affinity for the second (Limbird & Lefkowitz 1976). It has since been shown that certain receptors, including the β2 adrenergic and muscarinic receptors, can be immunoprecipitated as homodimers from transfected cell lines (Herbert *et al.,* 1996; Zeng *et al.,* 1999). Endogenously expressed

adenosine A_1 receptor and dopamine receptors have also been observed to form dimers (Ciruela *et al.,* 1995; Ng *et al.,* 1996) and it is now accepted that dimerisation may play a role in GPCR activity. Heterodimerisation between different GPCR subtypes has been proposed as a mechanism whereby novel receptors with altered pharmacology may be formed, an example being the heterodimerisation of δ and κ opioid receptors (Jordan & Devi 1999). Mechanistically it has been argued that the formation of a dimer may allow more efficient coupling to G proteins (Bouvier x); and the idea of 'rafts' of GPCRs complexing with conveniently juxtapositioned effectors is appealing. Whether oligomerisation is a universal feature of GPCR structure is contentious, indeed, the crystal structure of rhodopsin does not necessarily support it forming dimers (Palczewski *et al.,* 2000). It is more probable that dimerisation is a property that has evolved within some GPCR members as receptors have diverged. In family C GPCRs dimerisation is obligate though, and this was first shown with the advent of the heterodimeric $GABA_B$ receptors, which is covered in greater detail below.

1.6 $GABA_B$ receptor molecular biology

1.6.1 Cloning reveals similarities to mGluR receptors

The cloning of the β2 adrenergic receptor signified the beginning of a period in which receptors to most major neurotransmitters and hormones were rapidly identified. This was due in part to the relative sequence similarities of receptors, allowing the use of degenerate primers to clone putative receptors, and also new bioinformatic techniques. Although purification from bovine brain of a putative $GABA_B$ receptor with an M_r 80kDa was reported in 1993 (Nakayasu *et al.,* 1993), no amino-acid sequence was divulged. Indeed $GABA_B$ receptors remained elusive until 1997 (Kaupmann *et al.,* 1997), 11 years after the cloning of the β2 adrenergic receptor. This resistance to cloning was attributable partly to the dearth of ligands that retained high affinity for the receptor under solubilising conditions, and also to the lack of cell lines expressing large amounts of receptor protein.

Antagonists, unlike agonists, can bind to receptors independently of G-protein coupling, allowing antagonist binding to crude membrane preparations. To clone

$GABA_B$ the novel high affinity radiolabelled $GABA_B$ antagonists [^{125}I] CGP64213 and [^{125}I] CGP71872 were designed. These antagonists blocked both the pre and postsynaptic $GABA_B$ response and had dissociation constants of 1.0nM (Kaupmann *et al.,* 1997). [^{125}I] CGP71872 is a photoaffinity ligand, covalently binding proteins upon exposure to UV light. Brain membranes were incubated with [^{125}I] CGP71872, washed thoroughly and then exposed to UV light. The membrane preparation was then analysed using SDS-PAGE. Using this technique two specific bands of approximately 100kDa and 130kDa were radiolabelled, indicating the antagonist bound two separate proteins. Subsequent to this a brain cDNA library was transfected into COS cells which were screened for expression of proteins that would bind the antagonist. Using this technique a clone encoding a 960 amino-acid protein was isolated, designated $GABA_BR1a$ (NC-IUPHAR now designates $GABA_B$ receptors $GABA_{B(X,Y)}$ where X= subunit and Y = splice variant – this nomenclature is adhered to throughout the rest of this thesis). The cDNA for a second 844 amino-acid receptor, $GABA_{B(1b)}$, was isolated by screening a cDNA library with $GABA_{B(1a)}$ cDNA. $GABA_{B(1a)}$ and $GABA_{B(1b)}$ are identical except at the N-terminus with the first 144 amino-acids of $GABA_{B(1a)}$ replaced with 18 different residues in $GABA_{B(1b)}$, indicating that they are alternate splice variants of the same gene. Sequence analysis predicted a 7TM GPCR like structure for $GABA_{B(1a)}$ and $GABA_{B(1b)}$; with their size and large NTED (accounting for over 50% of each protein), placing them in GPCR family C. Similar to other family C GPCRs, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ possess domains with homology to bacterial periplasmic binding proteins (also known as Leucine Isoleucine Valine Binding Proteins (LIVBP's)) in the extracellular N-terminus. It should be noted however that the amino-acid sequence identity of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ with members of the mGluR family is comparatively low with mGluR receptors retaining a similar identity to iontropic glutamate receptor subunits. All mGluRs share 21 highly conserved cysteine residues, including the N-terminal cysteine rich domain, that are considered a hallmark of the receptor, but these are mostly missing in $GABA_B$ receptors. $GABA_{B(1)}$ showed weak similarities to naturetic peptide receptor, selectins and complement receptor type I (Kaupmann *et al.,* 1997).

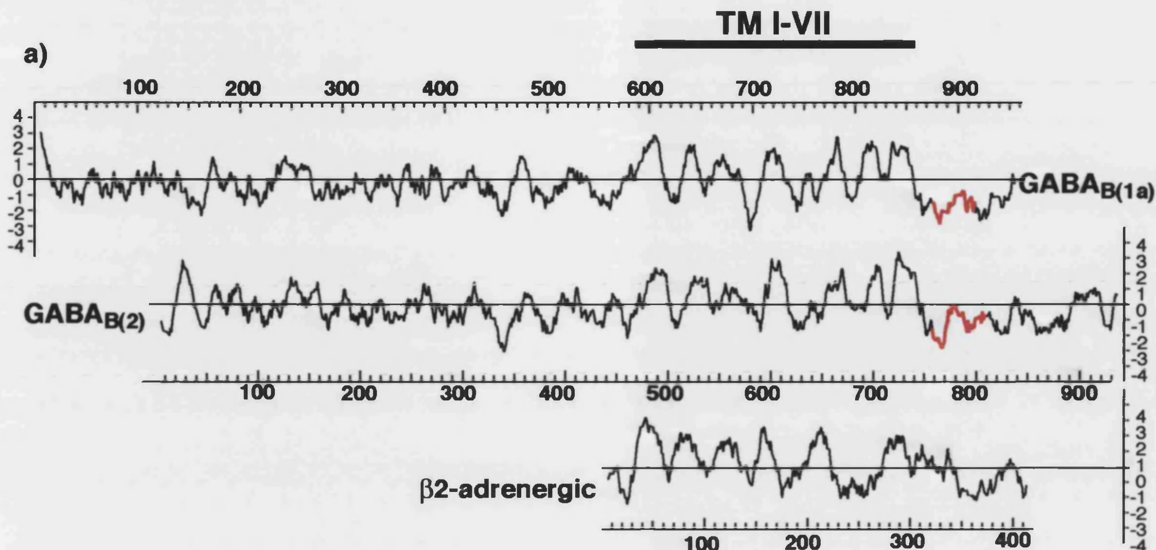
When expressed in recombinant systems $GABA_{B(1a)}$ and $GABA_{B(1b)}$ yielded proteins of M_r 130kDa and 100kDa respectively, identical to the M_r of the proteins

photoaffinity labelled with [^{125}I] CGP71872 in brain membranes. $GABA_{B(1a)}$ and $GABA_{B(1b)}$ also bound antagonists with similar affinities to native proteins, and both were shown to negatively regulate adenylyl cyclase upon exposure to GABA or baclofen in stably transfected HEK cells (Kaupmann *et al.,* 1997). This was strong evidence that the proteins responsible for the metabotropic GABA response had been identified. Anomalous to this supposition was that the agonist affinity of heterologously expressed proteins was 100-150 fold lower than in native systems, and adenylyl cyclase regulation was weak (Kaupmann *et al.,* 1997).

1.6.2 $GABA_B$ receptors are heterodimeric in nature

Although initially described as functional (Kaupmann *et al.,* 1997), it became apparent that the pharmacology of the cloned $GABA_B$ receptor did not match that of native $GABA_B$ receptors. Recombinant expression of tagged $GABA_{B(1)}$ in a variety of cell lines failed to demonstrate the surface trafficking of functional receptor with all receptor intracellularly retained within the endoplasmic reticulum (ER) (Couve *et al.,* 1998). ER retention is a common property of proteins that form part of multimeric complexes and prevents the inappropriate surface trafficking of unassembled subunits (Teasdale & Jackson 1996). In an acute prediction, it was suggested that $GABA_{B(1)}$ contained an ER retention element and that additional information, such as the co-expression of another protein, would be needed to allow the surface trafficking of functional receptors in a recombinant system (Couve *et al.,* 1998).

The form of this additional information was indeed another protein; termed $GABA_B$ R2 ($GABA_{B(2)}$) it had a 7TM topology and was concurrently identified by several groups (Jones *et al.,* 1998; Kaupmann *et al.,* 1998; White *et al.,* 1998; Kuner *et al.,* 1999). They demonstrated that only upon co-expression with $GABA_{B(2)}$ could $GABA_{B(1)}$ form a functional receptor with similar pharmacological properties to native receptors. This discovery redefined the GPCR paradigm because in essence it demonstrated that $GABA_B$ receptors were composed of two individual subunits. Moreover, succeeding analysis of $GABA_B$ receptor elicited closure of Ca^{2+} channels in sympathetic neurones verified that coupling was only possible upon coexpression of both subunits (Fillipov *et al.,* 2000). Some groups observed that whilst $GABA_{B(2)}$ alone could couple to adenylyl cyclase (Martin *et al.,* 1999; Kuner *et al.,* 1999), the



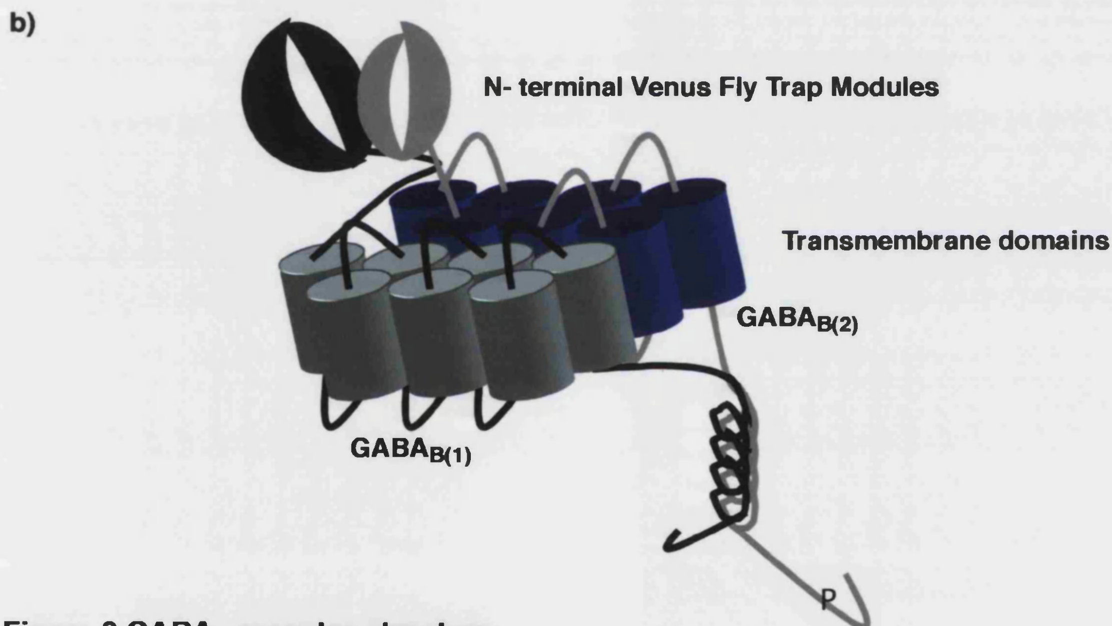


Figure 2 GABA$_B$ receptor structure

a) Kyte-Doolittle hydropathicity indices of both GABA$_B$ receptor subunits aligned along their transmembrane domains. Highlighted in red are the putative coiled coils within each subunit, important for mediating interactions between the intracellular carboxy-terminal domains. Also shown for comparison is the β2 adrenergic receptor, a family A GPCR. Note the far larger N-terminal domains of the family C GABA$_B$ subunits, whilst the β2 adrenergic receptor shows a much larger 3rd intracellular loop.

b) Cartoon of the proposed topology of the heterodimeric GABA$_B$ receptor. Interactions between the two subunits occur within the extracellular N-terminal domains, transmembrane domains and intracellular carboxy-terminal located coiled coils. The N-terminal domains are thought to be composed of venus fly-trap modules, with ligand binding within the groove. GABA$_{B(2)}$ is basally phosphorylated at serine 892.

co-expression of $GABA_{B(1)}$ was required to couple to potassium channels (Kuner *et al.,* 1999). Since these initial findings, further thorough investigations support the concept that only heterodimeric receptors are able to couple to G-proteins and thus activate effectors.

The two subunits interact with each other through putative coiled coil domains found in their intracellular carboxy-terminal domains, and additionally in unmapped regions, probably the transmembrane domains (Calver *et al.,* 2001; Galvez *et al.,* 2001). The interaction between the two subunits is of extremely high affinity, and the receptor is immunoprecipitated as a heterodimer from native tissue (Kaupmann *et al.,* 1998; Benke *et al.,* 1999). Native receptors were further shown to be heterodimers of $GABA_{B(1)}$ and $GABA_{B(2)}$, with the formation of homodimers of either subunit precluded (Benke *et al.,* 1999). Double immunolabelling of thalamocortical neurones has shown that >95% dendrites immunoreactive for $GABA_{B(1)}$ are also immunoreactive for $GABA_{B(2)}$ (Kulik *et al.,* 2002).

1.6.3 Splice variants

Multiple splice variants of $GABA_{B(1)}$ have been discovered, along with those initially reported $GABA_{B(1a)}$ and $GABA_{B(1b)}$. As mentioned earlier, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ differ in their N-terminal domains. Specifically, $GABA_{B(1a)}$ possesses a pair of tandem complement protein modules ('sushi' domains) N-terminal to the region with homology to bacterial amino acid binding proteins (Hawrot *et al.,* 1998). Complement protein modules are usually (although not exclusively) found in proteins associated with the complement, clotting and immune systems, and so appear out of place in a receptor for neurotransmitters. Complement protein modules are involved in mediating protein protein interactions, although N-terminal complement modules are often associated with ligand recognition (Hawrot *et al.,* 1998). Expression of the splice variants $GABA_{B(1a)}$ and $GABA_{B(1b)}$ is regionally and developmentally differential (Fritschy *et al.,* 1999). Western blotting with splice variant specific antibodies for $GABA_{B(1a)}$ and $GABA_{B(1b)}$ demonstrates that between P0 and P10 $GABA_{B(1a)}$ is predominant. At P10 both splice variants are expressed in equal amounts, and total $GABA_{B(1)}$ expression is greatest. In the adult $GABA_{B(1b)}$ is expressed at roughly twice $GABA_{B(1a)}$ concentration, whilst total $GABA_{B(1)}$ expression is halved (Fritschy *et al.,*

1999). Within the CNS the distribution of $GABA_{B(1a)}$ is markedly dissimilar to that of $GABA_{B(1b)}$ with certain brain regions containing exclusively one splice variant (Fritschy *et al.,* 1999) (covered in greater detail in section **1.7.1**).

Another splice variant, $GABA_{B(1c)}$, is also encoded in the human $GABA_{B(1)}$ gene. $GABA_{B(1c)}$ possesses only the first complement domain and, like $GABA_{B(1a)}$, is preferentially expressed in foetal tissue (Calver *et al.,* 2002); hinting at a possible developmental role of the complement modules within $GABA_B$ receptors. Further splice variants of $GABA_{B(1)}$ are thought to exist in rats, including $GABA_{B(1c)}$ (which confusingly is a different splice variant to human $GABA_{B(1c)}$), $GABA_{B(1d)}$, and $GABA_{B(1e)}$. Rat $GABA_{B(1c)}$ contains an insertion prior to the fifth transmembrane domain, $GABA_{B(1d)}$ encodes a truncated receptor missing half of the intracellular carboxy-terminal domain (Calver *et al.,* 2002) and $GABA_{B(1e)}$ only encodes the NTED (Schwarz *et al.,* 2000). Studies show that expression of mRNA transcript $GABA_{B(1e)}$ is much greater in the periphery, where levels of expression of other splice variants are low; however when co-expressed with $GABA_{B(2)}$, $GABA_{B(1e)}$ is unable to couple to G proteins (Schwarz *et al.,* 2000). Other splice variants ($GABA_{B(1f)}$, $GABA_{B(1g)}$) have also been identified (Wei *et al.,* 2001). However, there have been no demonstrations of endogenous protein expression of any of these splice variants and only rat $GABA_{B(1c)}$ has been shown to be able to form a functional $GABA_B$ receptor. Until it can be shown that these splice variants are translated their relevance remains open to query.

Studies of $GABA_{B(2)}$ initially reported three splice variants (Ng *et al.,* 1999), but subsequent analysis of $GABA_{B(2)}$ gene structure has shown that splice variants are not encoded (Martin *et al.,* 2001), and that the initial supposed splice variants were artefactual.

1.6.4 Further insights into $GABA_B$ molecular biology

Supervening the finding that functional $GABA_B$ receptors are heterodimeric, a range of studies has elucidated why activity is dependent upon heterodimerisation. They have also added weight to the notion that exclusively heterodimeric receptors are active. Primarily, $GABA_{B(1)}$ is inactive when expressed alone because it is retained within the ER (Couve *et al.,* 1998). This retention can be attributed to the

presence of an RSR(R) motif within the intracellular carboxy-terminal domain at amino acids 922-925 (Margeta-Mitrovic *et al.,* 2000; Calver *et al.,* 2001; Pagano *et al.,* 2001). This motif is found in other multimeric protein complexes that require assembly within the ER prior to export, such as K_{ATP} channels, and prevents the surface expression of unassembled subunits (Margeta-Mitrovic *et al.,* 2000). Thus, the co-expression of $GABA_{B(2)}$ is required to mask this ER retention motif and allow passage of the combined receptor through the secretory pathway.

Inactivity of $GABA_{B(1)}$ in the absence of $GABA_{B(2)}$ is not solely attributable to ER retention. Substitution of an arginine for an alanine within the RSR motif can relieve ER retention of $GABA_{B(1)}$, with the mutant subunit migrating to the cell surface. This mutant though is incapable of activating $GABA_B$ effector proteins (Margeta-Mitrovic *et al.,* 2000; Pagano *et al.,* 2001), demonstrating that $GABA_{B(1)}$ is not just a functional GPCR that happens to be ER retained. It was subsequently demonstrated that although the NTED of $GABA_{B(1)}$ contains all the structural information necessary and sufficient for agonist binding (Malitshek *et al.,* 1999); $GABA_{B(1)}$ is lacking key residues in the second intracellular loop that are critical for coupling to G proteins and are conserved amongst mGluRs (Robbins *et al.,* 2001). Tellingly, these residues are also present in the second intracellular loop of $GABA_{B(2)}$, and it was consequently proven that $GABA_{B(2)}$ is the subunit that couples to G proteins (Robbins *et al.,* 2001; Galvez *et al.,* 2001).

As mentioned earlier, $GABA_B$ receptors, in common with other family C GPCRs, possess a large NTED with homology to bacterial periplasmic binding proteins. Three dimensional molecular modelling, and crystallisation studies of these regions reveal a structure akin to venus flytraps (fig. 2) and the regions are sometimes referred to as venus flytrap modules (VFTMs) (Galvez *et al.,* 1999). Agonist is purported to bind within the groove created by each lobe, and upon binding brings amino acids of either lobe into close proximity, thus activating the receptor (Kunishima *et al.,* 2000; Galvez *et al.,* 1999; Galvez *et al.,* 2000). Because $GABA_{B(2)}$ can traffick to the surface in the absence of $GABA_{B(1)}$ and it also contains residues responsible for G protein coupling, the possibility that $GABA_{B(2)}$ may form an active receptor when expressed alone is more plausible. $GABA_{B(2)}$ is missing key determinants though that manifest in it lacking activity. Although $GABA_{B(2)}$, like $GABA_{B(1)}$, possesses an N-terminal VFTM,

a number of residues vital to GABA binding within the VFTM of $GABA_{B(1)}$ are absent within the VFTM of $GABA_{B(2)}$ (Kniazeff *et al.,* 2002). This accordingly leaves $GABA_{B(2)}$ unable to bind either agonist or antagonist when expressed alone (Kniazeff *et al.,* 2002). In spite of the inability of $GABA_{B(2)}$ to bind ligand, the VFTM within this subunit is not vestigial. In fact, deletion of the NTED of $GABA_{B(2)}$ results in the removal of $GABA_B$ receptor activity, as do certain point mutations within the VFTM of $GABA_{B(2)}$ (Kniazeff *et al.,* 2002). One possibility is that $GABA_{B(2)}$ binds an altogether different ligand to $GABA_{B(1)}$, conferring activity upon itself and perhaps altering the pharmacology of the complete receptor. Studies of the evolutionary conservation of amino acids reveal that those critical for ligand binding within mGluR receptors and $GABA_{B(1)}$ are conserved between species as distant as *Caenorhabditis elegans* and *Homo Sapiens* (Kniazeff *et al.,* 2002). Evolutionary pressure has maintained certain amino acids at key positions within these receptors so that the ligand binding characteristics are preserved, thus maintaining receptor activity. There appears to be no such conservation of amino acids within the VFTMs in $GABA_{B(2)}$ however. This argues against the existence of a natural ligand for this site and so non-specific mutations over time are permitted, whilst receptor activity remains unaffected (Kniazeff *et al.,* 2002).

Because mRNA and protein expression patterns for individual subunits do not show perfect overlap (Billinton *et al.,* 2000; Calver *et al.,* 2000) the case is still open for roles of monomeric subunits. The possibility of dimerisation with other GPCRs has been proposed (Sullivan *et al.,* 2000); and monomeric surface trafficking could be dependent upon non 7TM proteins similar to RAMPs. This does appear to be increasingly unlikely though, with neurones from mice that have had the intracellular carboxy-terminal domain of $GABA_{B(2)}$ genetically ablated showing no surface expression of $GABA_{B(1a)}$ (unpublished observation), arguing against the existence of other proteins capable of trafficking $GABA_{B(1a)}$. Furthermore, genetic ablation of $GABA_{B(1)}$ massively reduces expression levels of $GABA_{B(2)}$, although mRNA levels are unchanged (Prosser *et al.,* 2001, Schuler *et al.,* 2001). This infers that the presence of $GABA_{B(1)}$, or a protein associated with $GABA_{B(1)}$ is required to stabilise $GABA_{B(2)}$ protein levels.

A comprehensive model of $GABA_B$ receptor activation now exists. Primarily, agonist binds within the VFTM of $GABA_{B(1)}$, causing the closure of the VFTM, and also confirmational changes within the VFTM of $GABA_{B(2)}$, probably through direct physical interactions. This change in structure; which when one considers the sequence of the two subunits, involves the majority of the receptor complex, is propagated through the transmembrane domains. This activates the G protein through specific residues within the intracellular loops of $GABA_{B(2)}$, but probably also involving other determinants within $GABA_{B(1)}$ intracellular domains, and hence allows receptor-effector coupling. Because efficient $GABA_B$ coupling is dependent upon allosteric interactions between all major domains within the two subunits (Galvez *et al.,* 2001), it can be deduced that the two subunits have evolved together so as to co-operatively produce the metabotropic GABA response.

1.6.5 $GABA_B$ interactions

To search for other possible $GABA_B$ subunits several groups have utilised yeast two-hybrid screens of brain cDNA libraries using both the N and Carboxy terminals of $GABA_{B(1)}$ and $GABA_{B(2)}$ as bait proteins. In parallel to yeast two-hybrid screens a bioinformatics approach consisting of mining expressed sequence transcripts (ESTs) for sequences with homology to $GABA_{B(1)}$ or $GABA_{B(2)}$ has been employed. Both these techniques have thus far failed to identify any further mammalian members of the $GABA_B$ subunit family. It is therefore likely that the $GABA_B$ family consists of only two subunits (although interestingly Drosophila possesses a third member with equal homology to $GABA_{B(1)}$ and $GABAB_{B(2)}$ (Calver *et al.,* 2002)).

A by product of the search for other receptors however has been the identification of a large number of potential binding partners to $GABA_{B(1)}$ and $GABA_{B(2)}$. Amongst these are proteins from the 14-3-3 family of signalling molecules that have been shown to interact with the intracellular carboxy-terminal domain of $GABA_{B(1)}$ (Couve *et al.,* 2001). The putative interaction site overlaps amino acids 905-927, a stretch running from the second half of the coiled coil domain to just past the ER retention motif. The precise function of the interaction between 14-3-3 and $GABA_{B(1)}$ remains unknown, but may it be involved with signalling or scaffolding events (Couve *et al.,* 2001; El Far & Betz 2002). Recently it has been demonstrated that the binding of 14-

3-3 overcomes the ER retention of homomeric channels such as the KCNK3 potassium channel (O'Kelly *et al.,* 2002). Misfolded or ER resident proteins that escape the ER are efficiently retrieved by COPI-coated vesicles for retrograde transport due to the presence of dibasic ER retention motifs interacting with COPI proteins, such as β-COP. 14-3-3 binding can mask these sequences and therefore prevent recognition by the COPI complex, allowing forward transport. 14-3-3 binding stabilises and enhances the surface expression of both homomers of α4 subunit and, interestingly, heteromeric α4β2 neuronal nicotinic acetylcholine receptors (Jeanclos *et al.,* 2001). It has been suggested that 14-3-3 binding could act as a general mechanism to allow the escape from the ER of proteins retained, and that this function of 14-3-3 may also apply to heteromeric complexes. So whereas previously it was believed that the masking of the ER retention sequence alone by other proteins in a complex was enough to elicit the ER escape, it maybe that the additional binding of 14-3-3 molecules are also required to facilitate this process by helping prevent β-COP recognition. Whether 14-3-3 plays such a role in the trafficking of the $GABA_B$ complex is not known, however it is tempting to speculate that the overlap of the ER retention sequence and the 14-3-3 binding region are not purely coincidental.

Other proteins that interact with $GABA_B$ have been identified and include the transcription factor ATF4 (CREB2) which has been shown to interact with $GABA_{B(1)}$ (Nehring *et al.,* 2000; White *et al.,* 2000; Vernon *et al.,* 2001). The interaction occurs between the leucine zipper region of ATF4 and the coiled coil of $GABA_{B(1)}$. Complexed $GABA_{B(1)}$ and ATF4 co-immunoprecipitate from transfected cells, but only when $GABA_{B(2)}$ is not expressed, demonstrating that $GABA_{B(1)}$ and ATF4 binding is mutually exclusive with respect to $GABA_{B(2)}$. Baclofen application has been shown to induce trafficking of ATF4, although the direction of this trafficking is unresolved with one group reporting trafficking into the nucleus (White *et al.,* 2000), whilst others detecting nuclear exit of ATF4 (Vernon *et al.,* 2000). It should be noted that this trafficking is unlikely to be due to baclofen causing a direct structural reorganistion of the $GABA_{B(1)}$:ATF4 complex because baclofen is unable to activate the monomeric receptor. A more probable occurrence is that baclofen triggers other signals e.g. altered intracellular metabolite levels which can then result in ATF4 trafficking.

Several other proteins have been reported to interact with $GABA_{B(2)}$ including the multiple PDZ domain protein MUPP-1 and N-ethylmaleimide-sensitive factor (NSF) (Calver *et al.,* 2002). These interactions however only occur in the absence of $GABA_{B(1)}$, and so are probably not involved in the regulation of the complete heterodimeric receptor. Thus far only one protein (apart from heterotrimeric G proteins) has been implicated in altering the function of the dimer and this is PKA. PKA phosphorylates the carboxy-terminal intracellular domain of $GABA_{B(2)}$ and is also able to associate with fusion proteins of this region from brain lysates (Couve *et al.,* 2001). Although this interaction has not been demonstrated to be direct, and indeed could involve kinase-anchoring proteins, it is of relevance to the heterodimer. This is because PKA activation can slow the desensitisation of receptors by phosphorylating serine 892 in the carboxy-intracellular domain of $GABA_{B(2)}$ (Couve *et al.,* 2001). Heterodimerisation is obligate to receptor activity, and therefore this phosphorylation occurs at the complete receptor. Apart from the interaction with PKA, all other reported $GABA_B$ receptor interactions involve the coiled coil domains of either subunit. The region implicated in PKA phosphorylation is considerably downstream of the coiled coil within $GABA_{B(2)}$, and this is probably why the interaction can occur in the dimerised receptor.

1.7 Anatomical studies of $GABA_B$ distribution

1.7.1 Receptor distribution within the brain

Initial descriptions of the distribution of $GABA_B$ receptors within the CNS relied upon the use of radiolabelled agonists, usually tritiated GABA in the presence of isoguvacine to block $GABA_A$ binding sites (Mott & Lewis 1994). In most regions of the brain $GABA_A$ receptors predominate in concentration over $GABA_B$, with $GABA_A$ receptors accounting for 70-80% of overall GABA binding sites (Wilkin *et al.,* 1981; Chu *et al.,* 1990). However the molecular layer of the cerebellum (Wilkin *et al.,* 1981), specific thalamic nuclei and the interpeduncular nucleus of the brainstem show much greater concentrations of $GABA_B$, with $GABA_B$ receptors representing up to 90% of GABA binding sites (Chu *et al.,* 1990). The highest absolute concentrations of $GABA_B$ binding sites are seen in the frontal cortex, olfactory bulb, superficial grey of the

superior colliculus and molecular layer of the cerebellum (Chu *et al.,* 1990; Mott & Lewis 1994). Areas with intermediate levels of binding include the amygdala and certain thalamic nuclei whilst lower levels are observed in the hippocampus, hypothalamus, substantia nigra and neostriatum. Within the hippocampus the dendritic layer shows the most prominent binding, indicative of preferential $GABA_B$ receptor targeting to dendrites as opposed to somatic layers (Mott & Lewis 1994). Unfortunately radioligand binding is of low resolution and has several caveats. These include variable concentrations of endogenous GTP that could alter receptor sensitivity to radiolabelled agonist, and varying concentrations of endogenous GABA which could displace radiolabelled agonist. In fact the number of $GABA_B$ antagonist binding sites in crude brain membranes is approximately 3-fold the number of $GABA_B$ agonist binding sites (Bittiger *et al.,* 1992), reflecting the variable agonist affinity of receptors. This quantative difference is of no concern in whole brain radioligand binding assays if there is an equal distribution of different affinity receptors, but if certain regions of the brain contain more receptors in a low affinity state then the results could be misleading.

$GABA_B$ receptor cloning has enabled mRNA *in situ* hybridisation studies to be performed. This, in conjunction with the design of subunit and splice variant specific antibodies, has allowed for a very accurate map of receptor localisation.

Most data from *in situ* hybridisation studies supports previous radioligand binding assays. Initial studies of $GABA_{B(1)}$ mRNA transcript localisation denoted the presence of high levels in all cerebral cortical layers, the hippocampal pyramidal cell layers, the granular cell layer of the dentate gyrus and in the basal ganglia. In addition high levels of expression were noted in the cerebellum, especially in the Purkinje cell layer and at moderate levels in the granular layer (Kaupmann *et al.,* 1997) supporting the earlier conclusions from radiolabelled agonist. Studies using antibodies to either $GABA_{B(1a)}$ or $GABA_{B(1b)}$ show expression patterns similar to those observed with mRNA transcripts. Intriguingly though, there is a sharp contrast in the distribution patterns of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ within certain areas of the brain (Fritschy *et al.,* 1999). This is most noticeable within the striatum where $GABA_{B(1a)}$ is moderately expressed, whilst $GABA_{B(1b)}$ is virtually absent. Discrepancies are also noted in the hippocampus, with the CA3 region containing exclusively $GABA_{B(1b)}$ whilst the CA1

layer stains for both $GABA_{B(1a)}$ and $GABA_{B(1b)}$, but in a highly delineated manner, with $GABA_{B(1a)}$ in the pyramidal cell layer and $GABA_{B(1b)}$ in the stratum lacunosum pyrimidale (Fritschy *et al.,* 1999). Within the cerebellum immunostaining for $GABA_B$ is particularly strong, with $GABA_{B(1b)}$ selectively present in Purkinje cells and lower levels of $GABA_{B(1a)}$ in granule cells. The level of $GABA_B$ expression within the molecular layer of the cerebellum is not uniform but consists of intensely stained bands of Purkinje cells forming a regular striped appearance. This staining is coincident to that seen with the protein zebrin, and positively correlates with metabolic activity, hinting that $GABA_B$ expression maybe regulated according to metabolic demands (Fritschy *et al.,* 1999). Analysis of the class of neurones immunoreactive for $GABA_B$ demonstrates very high staining in monoaminergic and cholinergic neurones within the CNS, but much lower levels within the GABAergic neurones (Margeta-Mitrovic *et al.,* 1999). This supports the important role of $GABA_B$ receptors in regulating noradrenergic transmission, although questions the importance of $GABA_B$ as an autoreceptor (Margeta-Mitrovic *et al.,* 1999).

Macroscopic staining of brain sections with a $GABA_{B(2)}$ specific antibody demonstrates a very similar pattern of expression to that observed with $GABA_{B(1)}$ specific antibodies. Nonetheless, certain fields poorly overlap with $GABA_{B(1)}$ staining and these included the striatum, nucleus accumbens, olfactory tubercle and the hypothalamus where the staining for $GABA_{B(2)}$ was much lower (Kulik *et al.,* 2002). Studies of the neocortex have also demonstrated the existence of interneurones discretely staining for $GABA_{B(1)}$ and not $GABA_{B(2)}$ (López-Bendito *et al.,* 2002). A common theme emerging from studies of the expression of either subunit in the brain is that most regions immunopositive for $GABA_B$ stain for both subunits, but if there is a discrepancy then it is $GABA_{B(2)}$ which is not expressed. This leaves certain areas expressing either exclusively or much higher levels of $GABA_{B(1)}$. Neurones staining for exclusively $GABA_{B(1)}$ show highest immunoreactivity in the cell body and lack electrophysiologically recordable $GABA_B$ responses (López-Bendito *et al.,* 2002). During development it appears that $GABA_{B(1)}$ is primarily expressed, with $GABA_{B(2)}$ co-expression, which would allow functional receptors, occurring later (López-Bendito *et al.,* 2002). It maybe that $GABA_{B(1)}$ serves an alternative role in certain cells such as acting as an anchoring molecule. Another possibility is that $GABA_{B(1)}$ is expressed

in these cells and is retained in the ER, but in certain circumstances, e.g. after neuronal insult, $GABA_{B(2)}$ expression is upregulated allowing the rapid formation of functional receptors. The reason behind the discrepancies in expression profiles requires further studies to elucidate.

1.7.2 Subcellular receptor distribution

Withinin the cerebellum Purkinje cells show by far the highest immunoreactivity for $GABA_B$, with much lower levels of staining noted in granule cell somata (Fritschy *et al.,* 1999). Electron microscopy analysis of Purkinje cell staining show that $GABA_B$ is predominantly located along the dendritic trees and cell bodies. $GABA_B$ staining was conspicuously absent from dendrites apposed to GAD-positive puncta, indicating a lack of $GABA_B$ localisation at GABAergic synapses, at least in the cerebellum (Fritschy *et al.,* 1999). Ultrastructural localisation of $GABA_{B(1)}$ within Purkinje dendrites shows weak labelling in the dendritic shaft, but intense labelling within dendritic spines that make asymmetric (i.e. excitatory) synapses with parallel fibre and climbing fibre terminals. This staining was extrasynaptic or perisynaptic and maximal ~240nm from the edge of the synapse (Kulik *et al.,* 2002), with pre and postsynaptic densities on the whole immunonegative for $GABA_B$ (Fritschy *et al.,* 1999; Kulik *et al.,* 2002). Staining of $GABA_{B(2)}$ showed a similar ultrastructural localisation within the cerebellum to that of $GABA_{B(1)}$ (Kulik *et al.,* 2002). Therefore it appears that within the cerebellum $GABA_B$ receptors are preferentially expressed in Purkinje cells where they accumulate peri and extrasynaptically in spines making excitatory synapses, and are restricted from the pre and postsynaptic densities.

Studies of the ventrobasal thalamus show $GABA_B$ localisation is also extrasynaptic here. $GABA_B$ receptors in this region were preferentially located extrasynaptic to GABAergic synapses as opposed to glutamergic synapses in a ratio of 2:1 (Kulik *et al.,* 2002). Presynaptic staining of $GABA_B$ was not observed on either glutamergic or GABAergic terminals indicating that it neither functions as an auto or heteroreceptor in this region (Kulik *et al.,* 2002). Common to both regions is the extrasynaptic targeting of $GABA_B$ receptors, but the lack of $GABA_B$ receptors at GABAergic synapses in the cerebellum is not replicated in neurones from the ventrobasal thalamus. Overall, it appears $GABA_B$ receptors are extrasynaptically

targeted, preferentially in dendritic spines. Presynaptic targeting and the class of synapse that $GABA_B$ receptors appose is probably specific to different neuronal types.

1.8 $GABA_B$ receptor function

1.8.1 Physiological roles

As previously mentioned, initial appraisals of the $GABA_B$ response indicated that it could be inhibited by pre-treating tissue with pertussis toxin (Asano *et al.,* 1985; Ohmori *et al.,* 1990). Pertussis toxin specifically inactivates the $G_{i/o}$ class of G proteins. Activation of this class of G proteins triggers inhibitory effectors, primarily the closure of Ca^{2+} channels, inhibition of adenylyl cyclase and the opening of potassium channels (Mott & Lewis 1994) and it is through these effector pathways that $GABA_B$ elicits its actions.

The earliest studies of what would resolve to be $GABA_B$ activity implicated the bicuculline insensitive GABA response in the inhibition of noradrenergic transmitter release (Bowery & Hudson 1979). This effect exemplifies the role of $GABA_B$ receptors at the presynaptic terminal where activation of receptors coupling to G_o G proteins causes the closure of voltage sensitive Ca^{2+} channels (Mott & Lewis 1994). The G protein educed closure of Ca^{2+} channels involves a direct interaction between liberated βγ subunits and the channels (Ikeda 1996). This presynaptic activity of $GABA_B$ receptors is of crucial importance both at GABAergic neurones and also at neurones carrying other classes of neurotransmitter. At the GABAergic neurone it is critical to inhibit further release of GABA, with the $GABA_B$ receptor acting as an autoreceptor. Stimulation of inhibitory interneurones elicits GABA release and an IPSP that quickly starts to diminish in magnitude as the stimulation is repeated (Mott & Lewis 1994). Further studies showed that this reduction in IPSP size could be blocked by $GABA_B$ antagonists (Davies *et al.,* 1990)). It is now recognised that the rapid reduction of IPSP is caused by GABA activation of $GABA_B$ autoreceptors on interneurones which inhibit presynaptic calcium influx and subsequent GABA release. The reduction in GABA release consequences in decreased postsynaptic $GABA_A$ receptor activity, and means that macroscopically this $GABA_B$ action is excitatory.

$GABA_B$ autoreceptors are thought to be important in the induction of long term potentiation (LTP) because the reduction in $GABA_A$ activity permits depolarisation of the postsynaptic membrane great enough to relieve the Mg^{2+} block on NMDA receptors and allow LTP (Davies *et al.,* 1991).

The inhibition of adenylyl cyclase through $GABA_B$ activation confers a modulatory role upon the receptor. It permits the fine tuning of individual neuronal responses by reducing the intracellular concentration of cAMP and therefore PKA activity. PKA phosphorylation can significantly effect the activity of many classes of ion channels and so even minor changes in the level of PKA activity can have profound effects on neuronal firing. $GABA_B$ receptor inhibition of adenylyl cyclase has also recently been revealed to play an unexpected role in the control of neurotransmitter release. Presynaptic vesicles containing neurotransmitter are replenished in a manner dependent upon both local Ca^{2+} and cAMP concentrations. As well as the inhibiton of Ca^{2+} influx covered above, it appears that the activation of $GABA_B$ receptors at presynaptic terminals causes a dip in local concentrations of cAMP, so that vesicles upstream of those released are less amenable to liberation (Sakaba & Neher 2003). This demonstrates dual complementary functions of $GABA_B$ activation at the presynaptic terminal utilising two different effector systems.

As stated, $GABA_B$ activity normally negatively regulates adenylyl cyclase, however a wealth of evidence also exists to support a paradoxical role for $GABA_B$ receptors in the control of cAMP concentration. It transpires that when co-activated with G_s G protein coupling receptors which positively regulate adenylyl cyclase, such as the β adrenergic receptors, the liberated βγ subunit from G_i class of G proteins can synergistically enhance the action of the $G_{\alpha s}$ subunit. This can trigger an accumulation of cAMP far greater than that elicited by the activation of a G_s coupled GPCR alone (Karbon *et al.,* 1984; Karbon & Enna 1989). This property of $GABA_B$ receptor coupling allows a form of the learning event associated coincidence detection in neuronal circuits such that when two different classes of receptor on a neurone are stimulated through different transmitters the effect is far greater than that when either receptor is stimulated singularly.

The other major effect of $GABA_B$ receptors is observed postsynaptically, and is described as the late inhibitory postsynaptic potential (IPSP). Orthodromic stimulation

in the hippocampus primarily elicits a glutamergic excitatory postsynaptic potential, which is followed by a biphasic GABAergic IPSP. The early component of the IPSP is maximal 10-20ms after stimulation and is caused by increased membrane permeability to Cl^- ions through $GABA_A$ receptors ($IPSP_A$) (Mott & Lewis 1994). The second slow component is bicuculline insensitive, peaks 130-200ms after stimulation, and lasts for 0.4-1.5s (Mott & Lewis 1994; Calver *et al.,* 2002). The slow component of the GABA mediated IPSP ($IPSP_B$) is due to $GABA_B$ receptor activity and is inhibited by the $GABA_B$ antagonist phaclofen (Dutar & Nicoll 1988(a)) and can be replicated by baclofen application. $IPSP_B$ is caused by increased membrane permeability to K^+ ions and can be blocked by application of ions known to block K^+ channels activity such as extracellular Ba^{2+} and intracellular Cs^{2+} (Gähwiler & Brown 1985). The increase in membrane permeability allows K^+ ion efflux down an intracellular to extracellular concentration gradient which produces hyperpolarisation of the membrane. This action of $GABA_B$ receptors is elicited through the $\beta\gamma$ subunit activation of G protein coupled inwardly rectifying potassium channels (GIRKs) (Wickman & Clapham 1995). The coupling of $GABA_B$ receptors to GIRKs is thought to be entirely postsynaptic (Lüscher *et al.,* 1997; Couve *et al.,* 2000; Calver *et al.,* 2002). Mice lacking a GIRK channel subunit (GIRK2) are unable to form GIRKs and in these mice the $IPSP_B$ is absent, although there is no change in presynaptic $GABA_B$ properties (Lüscher *et al.,* 1997).

One aspect of $IPSP_B$ that should be mentioned is that it is dependent upon multiple stimuli, and indeed is only evoked by relatively strong repetitive stimulations (Newberry & Nicoll 1984; Dutar & Nicoll 1988(b)). If neurones are treated with a GABA uptake inhibitor such as the anticonvulsant Tiagibine the requirement for strong stimuli is removed and $IPSP_B$ is greatly enhanced (Thompson & Gähwiler 1992). Analysis of the ultrastructural localisation of $GABA_B$ receptors demonstrates the vast majority of both pre and postsynaptic receptors are found extrasynaptically (see above). GABA uptake mechanisms operate to minimise the duration that released GABA resides within the synapse, preventing extrasynaptic GABA spillover and corresponding $GABA_B$ activation. Only when strong stimuli elicit more GABA than can be efficiently taken up are the $GABA_B$ receptors activated, and this can be mimicked by GABA uptake inhibitors. Physiologically, it is has been demonstrated

that certain synchronised firing patterns of interneurones, such as those observed in hippocampal theta rhythm activate $GABA_B$ receptors through GABA spillover (Scanziani 2000; Couve *et al.,* 2000; Calver *et al.,* 2002). $GABA_B$ activity is of crucial importance in keeping the rhythm at a certain frequency, and blockade of $GABA_B$ receptors can dramatically increase the frequency of oscillations (Scanziani 2000).

$GABA_A$ receptor-mediated IPSPs can change the membrane conductance by 90-140nS, whereas $IPSP_B$ only causes an increase of between 13-19nS (Calver *et al.,* 2002). Thus, it would appear that the $IPSP_B$ is minor in comparison to $IPSP_A$; however because the duration of $IPSP_B$ is much greater it can have a profound effect upon the membrane potential causing peak membrane hyperpolarisation of approximately ~10-20mV. $IPSP_B$ is relatively ineffective in preventing strong depolarising signals, but significantly reduces the probability of weak synaptic inputs eliciting action potential firing. This quality of the postsynaptic $GABA_B$ response can help maintain the Mg^{2+} block of NMDA receptors and has the effect of enhancing the signal to noise ratio (Calver *et al.,* 2002).

Although GABA is an inhibitory neurotransmitter and $GABA_B$ couples to effectors that are inhibitory in nature; the precise role of $GABA_B$ receptors has not been easy to elucidate. $GABA_B$ receptor activation could be excitatory if inhibiting GABA release directly by acting as an autoreceptor. Likewise, $GABA_B$ activity at glutamergic neurones may also be excitatory if they are stimulating GABAergic interneurones. Pharmacological studies have been used to try and identify the physiological roles of $GABA_B$ receptors. These have produced conflicting results, with some studies demonstrating that application of baclofen is proconvulsant (Mott *et al.,* 1989) and thus suggesting activation can be excitatory; whilst other studies propose $GABA_B$ activity is anticonvulsant (Morrisett *et al.,* 1993). Mice have recently been generated lacking the $GABA_{B(1)}$ subunit and these lack all baclofen mediated $GABA_B$ responses (Proser *et al.,* 2001; Schuler *et al.,* 2001). This has not only supported previous work showing that the $GABA_B$ response depends upon heterodimer formation, but has also provided a compelling insight into the physiological role of $GABA_B$ receptors within the whole organism. Mice deficient in $GABA_{B(1)}$ subunit lack both pre and postsynaptic $GABA_B$ activity, and so lack the baclofen inhibition in neurotransmitter release, as well as the slow GABAergic IPSP. Phenotypically these mice are prone

to developing intractable tonic-clonic seizures that can be triggered by very slight stimuli (Proser *et al.,* 2001; Schuler *et al.,* 2001). This suggests that the loss of the inhibitory tone provided by $GABA_B$ action leaves neurones closer to the threshold needed for triggering seizures. Other phenotypic traits include increased locomotor activity and also reduced pain thresholds. This implies that $GABA_B$ receptor activity has a basal negative effect on pathways in the brain important for controlling these events. Although these traits are most probably due to the lack of $GABA_B$ responses, it is impossible to discount any effect caused by the absence of $GABA_B$ receptors throughout development. To strictly differentiate between developmental and physiological roles of the receptor requires the generation of inducible $GABA_B$ receptor knockout animals.

1.8.2 Pharmacological Heterogeneity

Early studies of $GABA_B$ receptors proposed a number of pharmacologically distinguishable subtypes mediating different actions of $GABA_B$ receptors in different localisations (Bonanno & Raiteri 1993). The most prominent finding was a purported difference in the sensitivity to the antagonist phaclofen between presynaptic and postsynaptic receptors (Dutar & Nicoll 1988(b)). Studies analysing the $GABA_B$ mediated inhibition of neurotransmitter release in synaptosomal preparations disclosed variations in the antagonism of this effect by phaclofen dependent upon the neurotransmitter release antagonised (Bonnano 1989). It has since been highlighted that phaclofen is a comparatively poor antagonist alongside newer antagonists and is typically used at concentrations of 1mM to exact $GABA_B$ antagonism (Mott & Lewis 1994). At these concentrations phaclofen has non-specific effects at $GABA_A$ receptors and may also act as a partial agonist at $GABA_B$ receptors (Stirling *et al.,* 1989). This leaves much of the work based solely upon this drug open to question.

Pharmacologically it would be beneficial to separate the $GABA_B$ presynaptic response from the postsynaptic response. Differences in sensitivity to both agonists and antagonists are easily attributable to the existence of different subtypes. However, the absence of alternative subunits and the results from knocking out the $GABA_{B(1)}$ subunit suggest that one type of receptor is responsible for mediating both responses. Differential pharmacology can also be explained in varying accessibility to

drugs in different neuronal populations, and disparity in the ratios of receptor to G protein. Post-translational modifications may also affect the sensitivities to agonist and antagonist, and it is also likely that the configuration of the receptor in terms of its molecular interactions with other proteins, both extracellularly and intracellularly influences pharmacology.

1.9 Pathological roles

Dysfunction of the response mediated by $GABA_B$ receptors is associated with several pathological conditions. Because $GABA_B$ receptors provide an important inhibitory tone over neuronal firing, it is probable that they are involved in the pathogenesis of epilepsy, a disease characterised by paroxysmal neuronal depolarisation. Indeed, as mentioned above, the main phenotype of mice lacking $GABA_B$ receptors is a reduced seizure threshold and a tendency to develop generalised intractable tonic clonic seizures (Proser *et al.,* 2001; Schuler *et al.,* 2001). Pharmaceutical preparations used in the clinical treatment of epilepsy do not directly target $GABA_B$ receptors but certain anticonvulsants which increase synaptic and extrasynaptic concentrations of GABA such as GABA uptake inhibitors will increase $GABA_B$ receptor activity. It is likely that an element of the therapeutic effect of GABA uptake inhibitors is mediated through enhanced $GABA_B$ receptor activity. $GABA_B$ receptors are also implicated in a very different form of epilepsy known as absence seizures. Absence seizures consist of periods of non-convulsive inactivity and inattentiveness due to dysfunctional thalamo-cortical circuitry. $GABA_B$ receptor activity is critical in the maintenance of seizures in animal models of absence seizure (Hosford *et al.,* 1992), and baclofen application can provoke these seizures (Vergnes *et al.,* 1997). It is therefore possible that drugs antagonising the $GABA_B$ response may have a therapeutic role in the treatment of absence seizures.

In humans as well as other animals a sudden loud sound normally elicits a startle response. The response is reduced if it follows a weaker prepulse and this is known as prepulse inhibition. Schizophrenics have consistently been shown to have deficiencies in their prepulse inhibition, with subsequent auditory stimuli having a similar startling effect to the initial stimulation (Braff & Geyer 1978). An interesting observation of heterozygote $GABA_B$ receptor knockout mice was that they show an

increase in their prepulse inhibition so that they respond even less to a second startling sound than wild type mice (Proser *et al.,* 2001). An indication as to the effectiveness of an antipsychotic compound is its ability to enhance this prepulse inhibiton (Hunter *et al.,* 2000), and the observation that effectively reducing the $GABA_B$ receptor response may have a similar effect indicates that $GABA_B$ receptors may play a role in either the aetiology of schizophrenia and/or the clinical effectiveness of certain antipsychotics.

$GABA_B$ receptor activation has been demonstrated to decrease the amount of glutamate and the peptides substance P and CGRP (Kangrga *et al.,* 1991; Malcangio & Bowery 1993, 1995) released in the dorsal horn of the spinal cord. These neurotransmitters all have excitatory effects within the dorsal horn and are important in the transmission of acute and chronic pain. In animal models baclofen has been demonstrated to have analgesic actions (Hammond *et al.,* 1993) and baclofen appears an, albeit rarely employed, useful adjunct treatment in such conditions as trigeminal neuralgia (Couve *et al.,* 2000).

Experiments conducted with $GABA_B$ receptor antagonists that are able to cross the blood brain barrier indicate that antagonism of $GABA_B$ receptors is associated with enhanced learning behaviour (Mondadori *et al.,* 1993, 1996 a,b), whilst baclofen treatment limits learning behaviours (DeSousa *et al.,* 1994). The treatment with $GABA_B$ receptor antagonists may lower the threshold for LTP, and this is of clinical relevance because it indicates that $GABA_B$ receptor antagonists may be used as cognitive enhancing drugs in those with dementia.

$GABA_B$ receptor agonists can alter the response of animal and human subjects to drugs of abuse. Baclofen has been demonstrated to reduce the self-administration of cocaine, alcohol and opiates and also reduce cocaine cravings in abusers of this substance (Roberts & Andrews 1997; Addolorato *et al.,* 2000; Xi & Stein 1999; Ling *et al.,* 1998). This anti-abusive potential of $GABA_B$ agonists maybe related to a reduction in dopamine release in the ventral tegmental area of the nucleus accumbens (Kalivas & Duffy 1995). It is also of interest to note it has been recently demonstrated that chronic cocaine administration can cause elevated levels of extracellular GABA in this area of the brain, and this has been linked to a cocaine induced dysfunction of $GABA_B$ receptor activity (Xi *et al.,* 2003). $GABA_B$ receptor

dysfunction may also be involved in the aetiology of depression, and it is likely that many of the antidepressant treatments in current use have considerable downstream effects upon $GABA_B$ receptors (Couve *et al.,* 2000).

Although there are a large number of neuropharmacological areas where drugs that act at $GABA_B$ receptors may be of some benefit, currently only one condition is regularly treated by a drug that directly acts at $GABA_B$ receptors and this is the therapeutic use of baclofen in the treatment of spasticity (Meythaler 1999). Spasticity is a highly debilitating condition characterised by an increase in muscle tone in the limbs and is often secondary to cerebrovascular accidents which damage the descending inhibition. Treatment with baclofen, usually intrathecally, is highly effective in relieving spasticity and can be continued for years without apparent loss in efficacy (Meythaler 2001).

1.10 Aims of this thesis:

Our insight into the structure and function of $GABA_B$ receptors has greatly increased over the last few years. It is still unclear however whether the receptor behaves in a similar manner to other GPCRs in terms of desensitisation and internalisation responses. Also unexplored are the effects that interactions with other proteins have upon receptor activity. In this thesis I propose to:

1) Study the effect application of agonist has upon the phosphorylation state and surface stability of $GABA_B$ receptors in heterologous and native systems.

2) Examine the phosphorylation of $GABA_{B(1)}$ and identify kinases able to mediate phosphorylation of this subunit.

CHAPTER 2 Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma (Dorset, UK) unless otherwise stated, and all restriction enzymes were purchased from New England Biolabs. All tissue culture reagents, unless otherwise stated, were from GibcoBRL Lifetechnologies. Baclofen and Br-cAMP were purchased from Sigma. Oligonucleotides were synthesised by Cruachem (Edin, UK), and MWG-Biotech (Germany). Imject maleimide-coupled mcKLH, EZ Link Sulfo-NHS-SS Biotin, EZ Link Sulfo-NHS Biotin and UltraLink NeutrAvidin beads were purchased from Pierce (Rockford, IL). GRK2 was purified from Sf9 insect cells. The PKA catalytic subunit was purchased from Promega (Southampton, UK). Radionucleotides and Hybond nitrocellulose membranes were purchased from Amersham Pharmacia (Buckinghamshire, UK). Peptides and phospho-peptides for purification of phospho-specific antibodies were synthesised by the Rockefeller institute (NY, USA). Primers were synthesised by MWG-Biotech (Ger.). A Biorad phosphorimager was used for data capture and quantification (Hertfordshire, UK).

2.2 Molecular Biology

Most of the techniques described can be found in Sambrook et al. (1989).

2.2.1 DNA constructs

Rat $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunit cDNAs were expressed in the mammalian expression vector pRK5 (Couve *et al* 1998; Couve *et al* 2002). $GABA_{B(1a)}$ was tagged with 9E10 epitope (EQKLISEEDL) between amino acids 4 and 5 as described previously (Couve *et al* 1998), whilst $GABA_{B(2)}$ was N-terminally tagged with the flag epitope (Couve *et al* 2002). The GST fusion protein vectors pGEX-CR1 (CR1, containing the carboxyl terminal domain of $GABA_{B(1)}$), truncations of the carboxyl terminal domain of $GABA_{B(1)}$ (δ1-8) and pGEX-CR2 (CR2, containing the carboxyl terminal domain of $GABA_{B(2)}$) have been described previously (Couve *et al* 1998; Couve *et al* 2002). The cDNAs for THRH, arrestin 2-EGFP and arrestin 3-EGFP have

been described previously (Scott *et al* 2002). All DNA manipulations and fidelity of DNA constructs were verified by DNA sequencing.

2.2.2 Bacterial strains

Subcloning and PCR were performed using the *E.Coli* strains XL1Blue (F'::Tn *10 proA+B+laclq D (lacZ)M15/recA1 end A1 gyrA96*(Nal^r) *thi hsdR17*(rK-mK+) *supE44 rel 1 lac*) .

Production of GST-fusion proteins was performed using the *E.Coli* strain BL21 (F- *ompT [lon} hsdSB*(r_B- m_B- an *E.Coli* B strain) with DE3, a prophage carrying the T7 RNA polymerase gene (Studier *et al* 1990).

2.2.3 Growth media and agar plates

Bacteria were grown in Luria-Bertani medium (LB). For plasmids encoding ampicillin resistance, ampicillin was added to a concentration of 100mg/ml. For growth of BL21 bacteria, chloramphenicol was added to a concentration of 34mg/ml.

For plates, agar was added to 15g/l. All growth was carried out at 37°C.

2.2.4 Preparation of electrocompetent bacterial cells.

Cells were streaked onto an LB agar plate lacking selective antibiotics. A single colony was then used to inoculate 10 mls of LB which was then incubated at 37°C overnight. The overnight culture was added to 1l of LB and grown to an absorbance at OD_{600} of 0.6. Bacteria were centrifuged at 4000 rpm for 10mins and then washed in 500ml sterile, ice-cold water. The bacteria were centrifuged again, washed in 25mls ice-cold glycerol, re-centrifuged and finally resuspended in 2.5ml 10% glycerol. Aliquots of 90µl were stored at –80°C.

2.2.5 Transformation of bacteria with plasmid DNA

20-30µl of electrocompetent bacteria with the DNA of interest were added to a 0.2cm electroporation cuvette on ice. A Biorad genepulser was then used to give a single pulse with the settings of 2.5KV, 200Ω and 25µF. Following this the bacteria were resuspended in 500µl of LB and incubated at 37°C for 1 hour before plating onto LB-agar plates containing the appropriate antibiotics and incubating overnight at 37°C.

2.2.6 Ethanol precipitation of DNA

To precipitate DNA from an aqueous solution, 0.1 volumes of 3M Sodium Acetate pH 5.2 was added to the solution followed by two volumes of 100% ethanol. After incubation of the solution at –20°C for at least 10mins followed by centrifugation at 13,000 rpm for 10 mins, the DNA pellet was washed with 70% ethanol and dried at room temperature. For the precipitation of small amounts of DNA, such as ligations, 1µl of glycogen (1mg/ml) was added before addition of Sodium Acetate and ethanol.

2.2.7 Phenol/chloroform extraction

A 1:1 mixture of Phenol and Chloroform equilibrated with Tris pH 8.0, Phenol/Chloroform (p/c), was obtained from Camlabs. Extraction of DNA samples in a volume of 50-500ml was carried out by vortexing with an equal volume of p/c in a microfuge tube followed by centifugation at full speed for 5 min. The aqueous phase was then transferred to a new microfuge tube and the process repeated with chloroform to remove any traces of phenol. Finally the DNA in the aqueous phase was precipitated with ethanol.

2.2.8 Agarose gel electrophoresis of DNA

See Sambrook et al. (1989) chapter 6.

Briefly, 1% agarose gels were prepared by dissolving agarose in 1xTAE by heating in a microwave. After cooling, ethidium bromide was added to the solution to a concentration of 100ng/ml and the gel pored. 10x loading buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll-400 was added to samples prior to loading on gel and DNA was resolved at 100 Volts. DNA was visualized by placing the gel on a UV transilluminator.

2.2.9 Restriction digestion of DNA, plasmid inserts and PCR products

10µg of plasmid DNA or 20µl of PCR product was digested in a final volume of a 100µl with 5µl each of the appropriate restriction enzyme(s) and 10µl buffer at 37°C for 2 hours or overnight. In the case of single digests of vector DNA self-ligation of the vector was inhibited by adding 1ml of shrimp alkaline phosphatase (Amersham) to the reaction mixture after digestion followed by a further incubation at 37°C for 30

min. Digested DNA was purified by agarose gel electrophoresis followed by extraction of the band from the agarose gel using Qiagen gel purification kits.

2.2.10 Polymerase Chain Reaction (PCR)

PCR was carried out using either Taq polymerase (Promega) or Pfu polymerase (Stratagene) in a final concentration of: 1x the appropriate buffer supplied by the manufacturer, 0.25 mM dNTPs and 1µM forward and reverse primers. Amplification was caried out with 30 cycles of: denaturation at 94°C for 30s, annealing at 52-56°C (depending on the primer melting temperature) for 45s and extension at 72°C for 1 to 2 mins (depending on length of expected product).

2.2.11 Ligations.

A rough estimate of the relative concentrations of vector and insert was made based on the intensity of bands on an agarose gel. Three different insert:vector ratios were used; approximately 2:1, 5:1 and 10:1. A control with no insert was also carried out. The reaction mixture was as follows:

vector
insert
1µl 10mM ATP (pH 7.5)
1µl NEB T4 DNA Ligase Buffer
1µl NEB T4 DNA Ligase
H_2O to a final volume of 10µl

The reaction mixture was then incubated overnight at 16°C. Following incubation, 1µl glycogen was added and the reaction mix ethanol precipitated. The pellet was then resuspended in 10µl water, and this was electroporated into XL-1Blue bacteria as described. Mini-Preps (see next section) were carried out if an enhancement of more than two-fold from the control was seen from the numbers of colonies on the plates.
Mini-Preps were screened for successful ligation by restriction digestion, using the same enzymes which were used to prepare the fragments before ligation.
Plasmids containing inserts were sequenced to verify the correct insertion of the ligation product and in the case of PCR inserts for fidelity of the amplification.

2.2.12 Mini-preparation of plasmid DNA (mini-preps)

Alkaline lysis method: 3ml of bacterial culture was centrifuged at 3500rpm for 5min in a bench-top centrifuge. The medium was removed and the bacteria resuspended in 100µl of solution I. To this was added 200µl solution II (made fresh on the day of use), and mixed by inverting several times. After 5min, 150µl solution III was added. After mixing (not vortexing), and leaving on ice for 10min, the tubes were centrifuged at full speed in a bench-top microfuge for 10min. The supernatant was then removed to a fresh tube, and ethanol precipitated, followed by centrifugation at full speed for 10min. The pellet was washed in 1ml 70% ethanol, dried, and resuspended in 50µl water to which 1µl RNAse was added.

2.2.13 DNA sequencing

Sequencing was carried out using the automated services provided by either MWG-Biotech (Germany) or Cytomyx (UK).

2.2.14 Site directed mutagenesis

This was carried out according to the protocol of the Stratgene 'Quikchange' kit
Briefly, primers complementary to sequence to be mutated were designed containing the mutated residues approximately in the middle of the primer, the primer length being between 25 and 50 base pairs. PCR reactions were carried out using Pfu 'turbo' as follows:

30 sec 95°C (1st cycle - 1min)
45 sec 54°C
2 min 72°C for every kb in plasmid to be mutagensied
12-18 cycles

PCR products were then digested with DpnI to remove all methylated, non-mutagenised backbone DNA.
Products were then transformed into super-competent XL-1B cells using heat shock at 42°C for 45sec followed by ice for 2min. Cells were then incubated at 37°C for 30min in LB and plated as normal.

For ease of selection of mutagenised constructs, primers were designed containing silent mutations that inserted restriction sites- usually PstI. This allowed for mini-prep and digestion and prevented sequencing of false- positives. Prior to mutagenesis it was checked that codon usage would not be significantly altered by introduction of silent mutations.

2.2.15 Maxi-preparation of plasmid DNA by caesium chloride banding

This was carried out as described in Sambrook *et al* (Sambrook *et al* 1989).

1l of bacterial culture in LB ampicillin grown overnight at 37°C was centrifuged at 4,000 rpm for 15 mins. The bacterial pellet was resuspended in 10ml Solution I, to which 20ml Solution II was added and mixed thoroughly. 15ml Solution III was then added and the mixture left on ice for 5min. It was then centrifuged for 10min at 4,000 rpm. The supernatant was removed and added to an equal volume of isopropanol, followed by centrifugation for 10min at 4,000rpm. The pellet was resuspended in 6ml 10X TE to which was added 6g CsCl and 100μl 10mg/ml Ethidium Bromide. This was centrifuged at 100,000 rpm overnight in a Beckman TLN100 ultracentrifuge rotor. Centrifugation promoted formation of DNA bands that were extracted using a 5ml syringe and wide-bore hypodermic needle. Ethidium bromide was removed from the DNA by butanol extraction with water saturated butanol a sufficient number of times to remove all ethidium bromide colouring. DNA was precipitated by adding 2 volumes of ethanol and centrifuged at 4,000rpm for 5min. The DNA pellet was then resuspended in 1ml 10X TE followed by 2X phenol/chloroform extraction and 1X chloroform extraction. The DNA was again precipitated by adding 2 volumes of ethanol and centrifuged at 4,000rpm for 5min. and finally resuspended in TE to a concentration of 1mg/ml. The DNA concentration was determined by reading the absorbance at 260nm.

2.2.15 List of oligonucleotides used

To manufacture site directed mutants the following oligonucleotides were employed:

$GABA_{B(1a)}$ S909A

5' GAG GAG CGC GTC GAT GAA CTG CGC CAT CAG CTG CAG TCT

3' AGA CTG CAG CTG ATG GCG CAG TTC TGC GAC GCG CTC

$GABA_{B(1a)}$ S917A

5' GAA CTG CGC CAT CAG CTG CAG GCT CGG CAG CAA CTC CGC

3' GCG GAG TTG CTG CCG AGC CTG CAG CTG ATG GCG CAG TTC

$GABA_{B(1a)}$ S917D

5' GAA CTG CGC CAT CAG CTG CAG GAC CGG CAG CAA CTC CGC

3' GCG GAG TTG CTG CCG AGC CTG CAG CTG ATG GCG CAG TTC

$GABA_{B(1a)}$ S923A

5' TCT CGG CAG CAA CTC CGC GCA CGG CGC CAC CCC CCA ACA

3' TGT TGG GGG GTG GCG CCG TGA GCG GAG TTG CTG CCG AGA

$GABA_{B(1a)}$ S923D

5' TCT CGG CAG CAA CTC CGC GAC CGG CGC CAC CCC CCA ACA

3' TGT TGG GGG GTG GCG CCG GTC GCG GAG TTG CTG CCG AGA

$GABA_{B(1a)}$ 929'STOP

5' CGG CGC CAC CCC CCA TGA TGA CCA GAT

3' ATC TGG TCA TCA TGG GGG GTG GCG CCG

2.3 Cell Biology

2.3.1 Antibodies

The rabbit anti-$GABA_{B(1a)}$ antibody was raised against a 15 amino acid peptide from the amino terminus $GABA_{B(1a)}$ of 2.5 µg/ml purified according to protocol below. The pan anti α-AMPK antibody was a gift from David Carling (Imperial College, UK). Secondary antibodies were from Molecular Probes and Jackson and used at 1:400. Monoclonal FLAG and VSV antibodies were obtained from Sigma (Dorset, UK). The anti-myc (9E10) antibody was obtained from 9E10 hybridoma cells (Connolly et al., 1996) and used post- protein A purification. The polyclonal anti-myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The guinea-pig anti $GABA_BR1$ and $GABA_BR2$ antibodies and the rabbit anti GluR1 antibody were purchased from Chemicon International (Harrow, UK). The $GABA_A\beta3$ antibody was used as described previously (Kittler *et al.*, 2000). The EEA1 antibody was purchased from BD Biosciences (Palo Alto, CA). The rabbit anti-$GABA_BR2$-P-Ser892 (UCL71) has been described previously (Couve *et al.*, 2002). The secondary anti-mouse, anti-rabbit and anti-guinea pig antibodies conjugated to Cy5, Texas Red (TR) and fluorescein isothiocyanate (FITC) were purchased from Jackson Immuno Research Laboratories (West Grove, PA). The secondary anti-rabbit antibody conjugated to [^{125}I] was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.3.2 Cell line culture

COS-7 cells were most routinely used for transiently expressing proteins of interest. They were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM Glutamine and 10% Foetal Calf Serum.

2.3.3 Transient transfection of COS cells

Two dishes seeded at $2X10^6$ cells/10cm dish of cells in exponential growth at 30-50% confluence were used per transfection. Cells were trypsinised from the dish and washed once in 50ml DMEM. After centrifugation at 1,000rpm for 2min, the cells were washed once in 10ml Optimem (Gibco), and then resuspended in 0.5ml optimem. Cells were transfected by electroporation (400V, infinite resistance, 125-µF Bio-Rad Gene Electropulser II) with up to 20µg of DNA using equimolar ratios of expression

constructs unless otherwise stated. Cells were used 36-48h after transfection. For immunofluorescence studies cells were plated onto poly-L-lysine (10μg/ml) -coated coverslips and analyzed 36-48h after transfection.

2.3.4 Preparation of low density hippocampal cultures

Low density cultures of hippocampal neurones were prepared as described previously (Goslin and Banker, 1991) on poly-L-lysine coated (1 mg/ml) glass coverslips over a glial feeder layer. Briefly, from embryonic day 18 (E18) rats hippocampi were dissected in 1x hepes buffered (10 mM) HBSS under a dissecting microscope. Hippocampi were trypsinised for 15 min in 1xHBSS with 0.25% trypsin followed by 3x5 min washes in 1xHBSS. After the final wash, hippocampi were triturated in 1ml 1xHBSS with 2 fire polished pasteur pipettes (5 passes each), the second pipette having a 60-70% diameter of the first. The number of live cells was then counted in trypan blue and cells were plated on polylysine treated (1mg/ml) coverslips at a density of 2600-10000 cells cm^2 in attachment medium. After allowing the cells to attach for 4 hours or overnight the attachment media was replaced with maintenance media. Half the medium was changed every 7 days.

2.3.5 Preparation cortical neuronal cultures

Cultures of hippocampal neurones were prepared as described previously (Goslin and Banker, 1991) on poly-L-lysine coated (1 mg/ml) 5 or 10 cm plates. Embryonic day 18 (E18) rat cortices were dissected in 1x hepes buffered (10 mM) HBSS under a dissecting microscope. Hippocampi were trypsinised for 15 min in 1xHBSS with 0.25% trypsin followed by 3x5 min washes in 1xHBSS. After the final wash, cortices were triturated by multiple passaging into and out of a 10ml plastic pipette until completely broken up. The number of live cells was then counted in trypan blue and cells were plated on polylysine treated (1mg/ml) coverslips at a density of 2600-10000 cells cm^2 in attachment medium. After allowing the cells to attach for 4 hours or overnight the attachment media was replaced with maintenance media. The neurones were kept for no longer than 7 days.

2.3.6 Imaging

Transfected cells were plated onto 6 cm dishes containing 10 mg/ml poly-L-lysine coated 13 mm coverslips. Cells were washed twice with PBS, fixed for 10 min in 4% paraformaldehyde and blocked for 10 min in immunofluorescence solution (0.25 % BSA, 10% horse serum in PBS). Cells were permeabilised in immunofluorescence solution containing 0.5% NP-40 for 10 min at RT and blocked for another 10 min in immunofluorescence solution containing 0.1% NP-40. Samples were incubated sequentially with primary and secondary antibodies for 1 h at RT in blocking solution. Coverslips were examined using a confocal microscope (MRC1000, Bio-Rad).

2.4 Biochemistry

2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3X SDS PAGE sample buffer (80mM Tris-HCl pH6.8, 100mM DTT, 10% glycerol, 2%SDS and 0.1% bromophenol blue) was added to samples before loading.

Separating gels were made up as follows:

Tris-HCl pH 8.8 1.5M 7.5 ml

Water + PROTOGEL (30% acrylamide) (National Diagnostics) 26.6ml

(relative amounts of water and acrylamide depended upon the size of proteins to be resolved)

10% SDS 0.3 ml

10% Ammonium Persulphate 0.3 ml

N,N,N',N'-tetramethyl-ethylene diamine (TEMED) 12µl

Stacking gels were prepared as follows:

Tris-HCl pH 6.8 1.25M 1.25 ml

Water 6.8 ml

PROTOGEL 1.7 ml

SDS 10% 100µl

10% Ammonium Persulphate 100µl

TEMED 10µl

Gels were run in 1X PAGE Buffer until the dye front reached the bottom of the gel. If proteins were to be visualised directly, they were fixed and stained with 1% Coomassie Blue in 10% acetic acid/20% methanol and destained in 10% acetic acid/20% methanol. If proteins were to be visualised by western blotting, proteins in the PAGE gel were transferred to a nitrocellulose membrane as described below.

2.4.2 Transfer of SDS–PAGE gels

The SDS-PAGE gel was placed against a pre-wetted Hybond nitrocellulose membrane (Amersham) with three pieces of Whatman 3mm filter paper on each side, and the completed "sandwich" placed in a BioRad transfer apparatus cassette. This procedure was carried out with all components submerged in transfer buffer. Transfer was carried out in a BioRad transfer apparatus with 1X Transfer Buffer at 350mA for 2h. After transfer the filter was stained using 0.1% Ponceau S in 5% acetic acid, and the positions of protein lanes and molecular weight markers marked with a ball-point pen. Excess Ponceau S was washed away with water.

2.4.3 Western blotting

Following transfer the filter was blocked with 4% marvel milk in 0.05% Tween-20 in PBS for 1h. Antibodies were diluted to the appropriate concentration in blocking buffer and applied to the filter in a sealed plastic bag overnight with agitation at 4°C. Excess antibody was washed off with 4% marvel milk and 0.05% Tween-20 in PBS (5 X 5 min). Secondary antibodies either conjugated to Horseradish Peroxidase or ^{125}I were added for 1h at room temperature prior to being washed off with 4% marvel milk in 0.05% Tween-20 in PBS (4 X 10 min) followed by 2X 5 min with 0.05% Tween-20 in PBS. HRP conjugated anti-mouse and anti-rabbit secondary antibodies for Western blotting were from Jackson and used at 1:5000 and detected by application of Super Signal Chemiluminescent substrate (Pierce). Immunoblots using ^{125}I conjugated antibody were visualized via Phosphorimager (Bio-Rad) and the radioactive bands were quantified using the Quantity One software (Bio-Rad).

2.4.4 Whole cell labelling studies:

Metabolic [^{35}S]-methionine, [^{32}P]-orthophosphate labelling and immunoprecipitations: For metabolic labelling, COS-7 cells were washed twice with methionine-free DMEM or phosphate-free DMEM and incubated in methionine-free DMEM containing 0.5 mCi [^{35}S]-methionine or in phosphate free DMEM containing 0.5 mCi [^{32}P]-orthophosphate for 4h at 37°C. After the incubation periods, cells were washed twice with Phosphate Buffer Saline (PBS) and lysed in 0.5 ml RIPA buffer (50 mM Tris-Cl, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 10 mM Na-Pyrophosphate, 1 mM Sodium Orthovanadate, 1% NP-40, 0.5% DOC, 0.1% SDS, 0.1% Phenylmethylsulfonyl fluoride, 10 mg/ml Leupeptin, 10 mg/ml Pepstatin, 10 mg/ml Antipain). Nuclei were removed from cell lysates by centrifugation at 22,000g for 5 min at 4°C in a microfuge. Cell lysates were preabsorbed with 25µl protein A- or protein G-Sepharose, previously equilibrated in RIPA buffer, for 1 h at 4°C. Protein A/G-Sepharose beads were then removed by centrifugation at 22,000g for 1 min at 4°C, and lysates were rotated with 5µg of antibodies for 1h at 4°C. Immune complexes were then precipitated with 25µl protein A/G-Sepharose for 1h at 4°C. Sepharose beads were washed twice in RIPA buffer containing 500mM NaCl and once in RIPA buffer containing 150mM NaCl. Immunoprecipitated proteins were eluted in 40µl of SDS-PAGE loading buffer, boiled for 3 min, resolved by SDS-PAGE and visualized by Phosphorimager, Bio-Rad (Hertfordshire, UK). Quantification of radioactive bands was performed using the Quantity One software (Bio-Rad).

2.4.5 GST-Fusion protein production

BL21 bacteria were transformed with pGEX constructs and plated onto LB Agar plates containing Ampicillin and Chloramphenicol. A 10ml LB Chlor/Amp culture was grown overnight at 37°C, and added to 1l LB Amp the following morning. This was allowed to grow to an OD A_{600} of 0.5-0.7 (approx. 2.5 hours), after which isopropylthio-b-D-galactoside (IPTG) was added to a final concentration of 0.5mM. Induction was carried out at room temperature, and allowed to continue for 3-4h. The bacteria were centrifuged at 4,000 rpm, and the resultant pellet washed in 10ml buffer A, re-centrifuged and the pellet was left overnight at -20°C at this stage. The following day the pellet was resuspended in 10ml 1xTE containing 100µM

phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml each of antipain, leupeptin and pepstatin. Triton-X 100 was added to 1% and the mixture was sonicated at full power for 3 X 30s followed by addition of 25ml Buffer C + PMSF and the mixture centrifuged at 35,000 rpm for 30min. To the supernatant was added pre-swollen glutathione-Agarose beads (80mg dry unswollen mass), and the mixture left at 4°C rotating for 2h (Glutathione-Agarose beads were pre-swollen in Buffer C and then washed three times in Buffer C before use). After affinity-purification beads were washed five times with Buffer C + PMSF. GST-fusion proteins were then eluted from the beads in 70mM glutathione containing buffer C (pH adjusted to 7.4), or if fusion proteins were to be used in a kinase reaction, TE. Proteins were eluted in 1ml elutant solution for 15 minutes on ice which was then removed and set aside. A further 1.5 ml of elutant was added for 15 more minutes which was then removed. The eluted proteins were then dialysed for a minimum of 24h @ 4°C in 1xTE or Buffer C depending upon the intended use. The concentration of dialysed protein was determined and then proteins were snap frozen in liquid nitrogen prior to storage at –80°C until required.

2.4.6 Affinity-purification ("pull-down") assays

GST-fusion proteins purified as described above were thawed on ice prior to use. Brain lysate was made by homogenising the tissue in a Down's Homogeniser in 50mM HEPES pH 7.5, 150mM NaCl, 50 mM NaF, 10mM NaPyrophosphate, 1mM EDTA, 2mM EGTA, 0.5% Triton X-100 (or 1% NP-40, 0.5% DOC), plus protease inhibitors PMSF, leupeptin, antipain, pepstatin (10 µg/ml). This was then centrifuged at 50,000 rpm for 30min at 4°C. The supernatant was incubated with the GST-fusion proteins for 30min with rotation at 4°C prior to addition of glutathione agarose beads and incubation for a further 90min. 25µg fusion protein was used per assay and 5mg of brain lysate. After incubation beads were washed five times with 1ml ice-cold lysis buffer, and resuspended in 50µl 2X SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and detected by western blotting.

2.4.7 In vitro kinase assays

10 μg of GST fusion proteins or rod outer segments were mixed with purified GRK2, AMPK or PKA in kinase buffer (20 mM Tris-Cl, pH 7.2, 5 mM $MgCl_2$, 2 mM EDTA, 1mM DTT. For GRK reactions the buffer was supplemented with $G\beta\gamma$ and PIP_2) in a final volume of 25 μl. Agarose beads were pre-warmed in kinase buffer for 1min at 37°C, ATP was then added to the reaction to a final concentration of 0.2 mM containing 1/10 vol of $[^{32}P]$-γATP (10mCi/ml) and the reactions were continued for 15 min at 37°C. Reactions were stopped by the addition of 12.5 μl of 3X SDS-PAGE loading buffer, boiled for 3 min, resolved by SDS-PAGE and visualized by Phosphorimager, Bio-Rad. Quantification of radioactive bands was performed using the Quantity One software (Bio-Rad).

2.4.8 Immunoprecipitation from brain membranes

Brain membranes were prepared as described previously (Couve *et al.*, 2000). They were then solubilised for 2h at 4°C in buffer containing 2% CHAPS. After centrifugation, the lysate was precleared with 50μl of a 1:1 slurry of protein A beads (Pharmacia Biotech) in lysis buffer with 50μg rabbit non-immune IgG (Pierce) for 1h at 4°C, rotating on a wheel. The beads were removed by centrifugation, and to the supernatant was added a further 50μl protein A (for polyclonal antibodies) plus the antibody of interest. Immunoprecipitations were carried out using 5-10 mg of antibody or an equivalent amount of IgG control. This was incubated at 4°C for 4h on a rotating wheel. The beads were then washed four times with 1ml ice-cold lysis buffer, and resuspended in 50μl 3X SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and detected by western blotting.

2.4.9 Biotinylation

5 DIV rat cortical neurones grown on poly-L-lysine coated dishes were incubated for 1h in culture media containing 100μg/ml leupeptin. Dishes were placed on ice and washed twice with ice-cold PBS/Ca^{2+}/Mg^{2+} (PBS containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$). The biotin reagent was freshly dissolved at 1mg/ml in ice-cold PBS/Ca^{2+}/Mg^{2+}, if biotin was to be cleaved for internalization assays then EZ Link Sulfo-NHS-SS Biotin was used, otherwise Sulfo-NHS-biotin was used. Cultures were

incubated with biotin solution for 12 min on ice which was then aspirated and the dishes were blocked by washing 3 x 5 min in PBS/Ca^{2+}/Mg^{2+} containing 0.1% BSA and 2 x 5 min in ice cold PBS/Ca^{2+}/Mg^{2+}. Neurons were returned to the 37°C incubator for the appropriate periods of time (10min to 60h) in the presence of 100μg/ml leupeptin. Dishes were placed on ice, the media removed and 2-5 ml of ice-cold cleaving buffer was added (50 mM Glutathione in 75 mM NaCl, 10 mM EDTA, 1% BSA and 0.075 N NaOH). Dishes were incubated 2 x 15 min at 4°C with constant shaking. Finally neurons were washed twice in ice cold PBS/Ca^{2+}/Mg^{2+}, lysed in 300 ml ice-cold RIPA buffer and solubilised by rotating 1h at 4°C. Nuclear and cellular debris were removed by centrifugation at 14,000 rpm x 5 min at 4°C and the supernatants were precipitated with 100 ml UltraLink NeutrAvidin slurry for 2 h at 4°C. Beads were washed twice in RIPA buffer containing 500 mM NaCl and twice in RIPA buffer containing 150 mM NaCl (one low salt wash followed by two high salt washes and then a final low salt wash). Beads were resuspended in 60μl SDS-sample buffer and resolved on SDS-PAGE and transferred to Hybond-C membranes.

2.4.10 ELISA

Cells were cultured in poly-L-lysine coated six-well plates with each experiment being carried out in triplicate. Cells were removed from the incubator, rinsed twice in cold PBS and then fixed in 4% PFA.. Cells were then blocked using blocking buffer for 15 minutes and then affinity-purified 9E10 antibody was applied for 1h in blocking buffer at 10μg/ml. Cells were then washed extensively and exposed to hrp-conjugated anti-mouse secondary (1 in 2000) in blocking buffer for 45min. Cells were again washed and then TMB was applied for 5 minutes or until significant colour change was noted. TMB was removed placed in cuvettes and read at 455nm using a spectrometer. Nonspecific binding was determined using mock-transfected cells.

2.4.11 Overlay assay

The bait protein of interest was resolved using SDS-PAGE followed by transfer to nitrocellulose membranes. Here a renaturation procedure was enacted incubating the membrane for 15 minutes in 6M guanidine hydrochloride, 50mM Tris pH 7.4, 150 mM NaCl and 0.5% BSA. Renaturation consisted of a halving of the concentration of

guanidine present in the buffer every 15 minutes whilst keeping other solute concentrations fixed until the concentration of guanidine was >50mM, when it was all removed. The probing protein was phosphorylated with PKA for 30 minutes using 32PγATP. Unincorporated ATP was removed from the probe by centrifugation in micro-spin chromatography columns (BioRad). Probe was then applied to nitocellulose membranes in 5% BSA/PBS 0.1% TWEEN overnight at 4°C with agitation. Membranes were then washed thoroughly in blocking solution followed by PBS/TWEEN alone. Membranes were dried and exposed to phosphorimager screen.

2.4.12 Phosphoamino acid analysis

Post-SDS-PAGE gels were fixed in 40% methanol, stained with Coomassie and dried. Post-autoradigroaph exposure bands were excised from gel and rehydrated. They were then digested in either trypsin or thermolysin (0.1mg/ml) for >18h at 37°C. Digests were separated from gel and dryed down in a spin vacuum. They were then hydrolysed in 6N HCl for 2h at 95°C. The solution was redried and then dissolved in 10µl H_20 which was spotted on plastic backed cellulose chromatography paper. Also spotted were standards. Samples were electrophoresed at pH 1.9 (in formic acid, acetic acid, water (1:10:89) solution) and then at pH 3.5 (acetic acid, water). Standards were visualised with ninhydrin. Chromotography paper was then exposed to either phosphorimager screen or autoradiograph.

2.4.13 Peptide maps

Peptides were retrieved from gels as above and then centrally spotted onto cellulose chromatography paper along with phenol red and basic fuschin dyes. Samples were then separated using electrophoresis at pH 3.5. A second dimension was then run using ascending chromatography.

2.4.14 Production and Purification of phospho-specific antibodies

Peptides were conjugated using N-terminal cysteine residues onto maleimide reacted MCKL haemocyanin Imject kit (Pierce). They were then injected into rabbits at set dates following a standard protocol (Cocalbiochem, USA). Bleeds were regularly tested for immunoreactivity to peptides and when this was deemed to be sufficient

purified over peptide conjugated Thiosepharose columns (Pharmacia), initially over a de-phospho peptide column, and then the elute was passed over a phospho-peptide column. After extensive washing in buffer containing 500mM NaCl columns were eluted with 0.1M glycine pH 2.8. Eluant was immediately added to 1M Tris pH8, to a final pH 7.4. Antibody was then dialysed, concentrated and had sodium azide added.

2.4.15 Purification of AMPK and Edman degradation

AMPK was kindly purified by David Carling as described previously (Carling *et al.* 1989). Edman degradation was carried out as previously described.

2.5.0 Commonly Used Buffers

TAE (Tris-Acetate EDTA)	40mM Tris-acetate 1mM EDTA (pH 8.0)
TBE (Tris-Borate EDTA)	90mM Tris-borate 2mM EDTA
TE (Tris EDTA) usually pH 7.6	10mM Tris.HCl (pH 7.6) 1mM EDTA (pH 8.0)
SDS-PAGE buffer (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis Buffer)	25mM Tris 250mM glycine (pH 8.3) 0.1% SDS
Western Blotting Transfer Buffer	50mM Tris 380mM glycine 0.1% SDS 20% methanol
Solution I (For mini-preparation of plasmid DNA)	50mM glucose 25mM Tris pH 8.0 10mM EDTA

		10mM EDTA
Solution II	"	1% SDS 200mM NaOH
Solution III	"	5M potassium acetate (29ml glacial acetic, 50ml H_20 + 10MKOH on ice pH4.8)
Buffer A (For GST fusion protein purification)		50mM Tris pH 8.0 25% sucrose 10mM EDTA
Buffer B	"	10mM Tris pH 7.6 1mM EDTA
Buffer C	"	20mM HEPES pH 7.6 100mM KCl 1mM EDTA 20% glycerol 5mM DTT (Dithiotriol)
Phosphate Buffer pH 7.6		84.5mM Na_2HPO_4 15.5mM Na_2HPO_4

CHAPTER THREE

3.1 Introduction

In the introduction to this thesis I described what is understood with regards to the mechanisms of GPCR receptor desensitisation and subsequent endocytosis. The majority of GPCRs studied desensitise due to receptor phosphorylation; this can be homologous, i.e. triggered by agonist occupation, or heterologous through the activity of other second messenger kinases. Rapid agonist induced phosphorylation is normally carried out by GRKs (Pitcher *et al.,* 1999). Phosphorylation uncouples receptors from G-proteins and promotes interaction with arrestins. Association with arrestins sterically prevents further G-protein coupling and facilitates receptor endocytosis via clathrin-coated pits (Luttrell & Lefkowitz 2002). Endocytosis leads to a decrease in the number of receptors at the cell surface and this is detectable within minutes of GPCR activation. Internalised receptors can be either returned to the surface in a de-phosphorylated and resensitised state, or degraded via late endosomes and lysosomes. Downregulation describes the permanent reduction in the number of receptors that is often observed after prolonged agonist exposure (Tsao *et al.,* 2001). This is the least understood step in the process of GPCR desensitisation, but it is thought to involve degradation of receptor proteins concomitant with a reduction in receptor expression (Ferguson 2001, Lefkowitz 2001, von Zastrow 2001).The separate steps of GPCR desensitisation have been delineated using a small number of 'model' receptor proteins, most commonly the β2 adrenergic receptor, expressed in recombinant systems. It has been subsequently demonstrated that a large number of other class A and class B GPCRs utilise similar systems to the β_2 adrenergic receptor albeit with minor differences such as GRK specificity and arrestin preference (Zhang *et al.,* 1998). Heterologous and homologous phosphorylation of members of the class C mGluR family is also important for desensitisation (Schaffhauser *et al.,* 2000, Cai *et al.,* 2001, Sorenson & Conn 2003). Although structurally quite dissimilar from class A and class B GPCRs, with much smaller intracellular loops and far larger extracellular N-terminal domains, mGluRs can interact with both GRKs and arrestins. In cerebellar Purkinje cells mGluR1 activity is regulated by GRK4 (Sallese *et al.,* 2000) and in HEK 293 cells

GRK2 and GRK5 phosphorylate mGluR1 in an agonist dependent manner (Dale *et al.,* 2000, Kaur Dhami *et al.,* 2002). mGluR1 undergoes both constitutive and agonist induced endocytosis that is enhanced by overexpression of arrestin 2 (Dale *et al.,* 2001) but interestingly arrestin 3 has little effect. It has recently been demonstrated that GRKs can regulate mGluR1 activity in a manner independent of phosphorylation, but instead dependent upon the RGS domains of the GRKs. GRK 2 and GRK 5 have also been shown to regulate the desensitisation of mGluR 5 (Sorenson & Conn 2003).

Heterologous expression of receptors in cell lines such as HEK 293 leads to uniform expression over a cell surface. Neurones show a far higher degree of compartmentalisation and this may lead to variable ratios of receptors to GRKs and arrestins. It is therefore likely that the regulation of cell surface GPCRs is far more complex in neurones, and probably dependent upon the receptor location e.g. synaptic verses extra-synaptic receptors.

$GABA_B$ receptors are, as previously noted, members of the class C GPCRs. The control of $GABA_B$ receptor desensitisation and surface stability is poorly understood in comparison to other GPCRs. It has been established that $GABA_{B(2)}$ is a substrate for PKA, and that phosphorylation by PKA at serine 892 in the intracellular carboxy-terminal domain of $GABA_{B(2)}$ greatly delays the onset of $GABA_B$ desensitisation (Couve *et al.,* 2001). This contrasts with the classical induction of desensitisation through phosphorylation observed with other receptors including those in class C. Chronic agonist activation decreases phosphorylation of $GABA_{B(2)}$ at serine 892 (Couve *et al.,* 2001), but it is not clear whether agonist affects the phosphorylation status of $GABA_{B(1)}$, or other sites within $GABA_{B(2)}$. A role in the desensitisation and control of cell surface stability of $GABA_B$ receptors for GRKs and arrestins has yet to be determined. It is possible that the heterodimeric nature of $GABA_B$ receptors precludes interactions with these proteins.

To further understand the mechanisms behind the control of $GABA_B$ cell surface stability I examined:

1) The effect of agonist on the phosphorylation state of both $GABA_{B(1)}$ and $GABA_{B(2)}$ in heterologous systems.

2) The effects of overexpression of different GRKs upon agonist-induced phosphorylation.

3) The cell surface stability of $GABA_B$ receptors in heterologous systems with and without the overexpression of arrestins.

4) The endocytosis and surface stability of endogenous $GABA_B$ receptors in cultured neurones.

For the heterologous studies I chose to use COS-7 cells. These have no endogenous $GABA_B$ response but readily express both subunits in quantities amenable to biochemical analysis. Moreover, COS-7 cells have formed the basis for much of the current understanding of trafficking events of $GABA_B$ receptors (Margeta Mitrovic *et al.,*. 1999, Calver, *et al.,*. 1999). Another advantage of using COS-7 cells is that they express only low levels of endogenous GRK's and arrestins (Ferguson 2001), allowing for the observation of larger effects upon their overexpression.

For endogenous studies I used embryonically derived cultured cortical and hippocampal neurones, both of which express $GABA_{B(1)}$ and $GABA_{B(2)}$.

3.2 Studies in recombinant systems

3.2.1 $GABA_{B(1)}$ and $GABA_{B(2)}$ are phosphorylated in mammalian cell lines

COS-7 cells were transiently transfected with myc-tagged $GABA_{B(1a)}$ and FLAG-tagged $GABA_{B(2)}$ either singly (10µg per transfection) or together in a ratio of 1:4 $GABA_{B(1a)}$: $GABA_{B(2)}$ (20µg total). This had previously been determined to be the optimal ratio of DNA for maximal surface expression of the receptor complex (Couve *et al.*, 2001) and was kept constant in all experiments unless otherwise stated.

To determine the optimal conditions for immunoprecipitation, cells expressing receptors were incubated in media containing ^{35}S labelled methionine for 4h, 36-48h after transfection. Cells were then rinsed in PBS prior to solubilisation in RIPA buffer for 1h. Cell lysates were then prepared and pre-cleared with IgG and Protein G and receptors were then immunoprecipitated with appropriate antibodies for 2h. After extensive washing in high salt concentration RIPA, immunoprecipitates were resolved using 8% SDS-PAGE, gels dried and exposed to autoradiograph. When expressed in COS-7 cells, myc-tagged $GABA_{B(1a)}$ migrates at approximately 116 kDa, whilst flag-tagged $GABA_{B(2)}$ migrates around 105 kDa (fig.3). Although the use of RIPA as an extraction buffer allows for the maximal solubilisation of $GABA_B$ receptors (Benke *et al.*, 1999) it disrupts the interaction between $GABA_{B(1a)}$ and $GABA_{B(2)}$. If receptor complexes were immunoprecipitated using an antibody to one subunit only, minimal amounts of the other subunit were co-immunoprecipitated; yet to analyse phosphorylation of the receptor it was important to maximise the amount of receptor immunoprecipitated and immunoprecipitate similar amounts of both subunits. Immunoprecipitation was therefore carried out with antibodies to both tagged subunits allowing analysis of similar amounts of both $GABA_{B(1a)}$ and $GABA_{B(2)}$ (fig.3a lane 4).

To determine whether receptor subunits were phosphorylated they were transfected singly or co-transfected into COS-7 cells. 36-48h post-transfection, cells were rinsed in phosphate-free media prior to incubation for 4h in phosphate free media to which was added ^{32}P labelled orthophosphate (0.5mCi ml^{-1}). Receptors were then immunoprecipitated and samples resolved on 8% SDS-PAGE. I observed that the bands derived from cells labelled with ^{32}P are much broader, and it is more

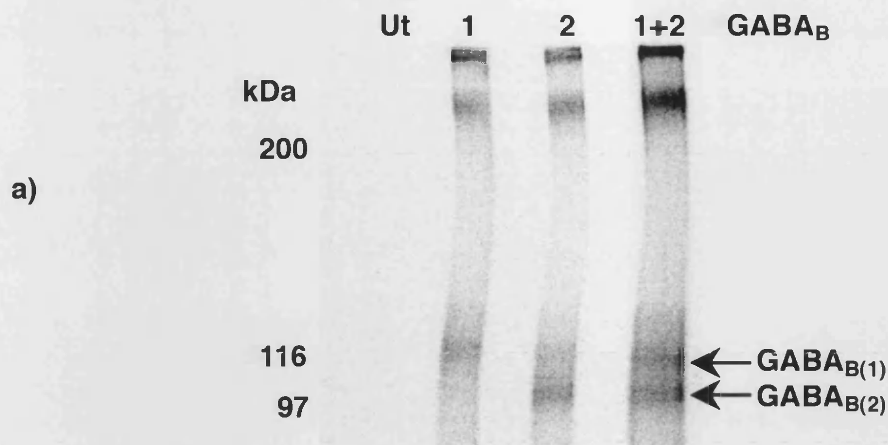
difficult to separate the individual subunits $GABA_{B(1a)}$ and $GABA_{B(2)}$. This is because of the much higher energy beta emission produced by the ^{32}P isotope in comparison to ^{35}S. It is apparent however that both $GABA_{B(1a)}$ and $GABA_{B(2)}$ are highly phosphorylated when expressed either singly or together, and that bands at this molecular weight are not observed to immunoprecipitate from untransfected cells with either flag or myc antibodies (fig. 3b,c).

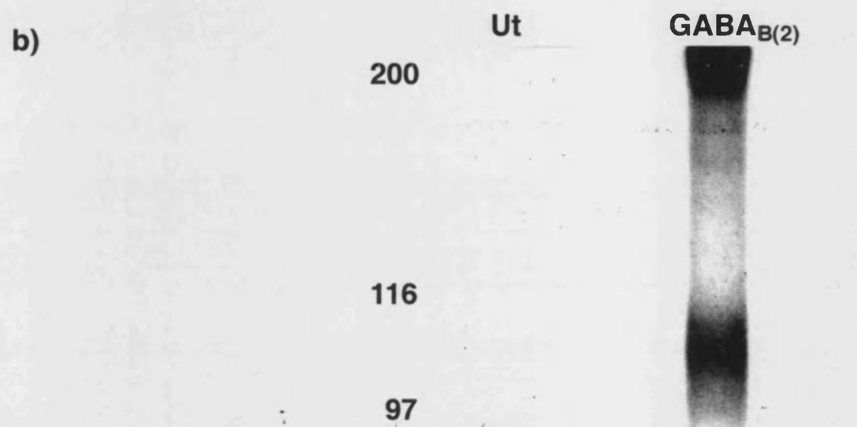
The observation that both $GABA_{B(1)}$ and $GABA_{B(2)}$ are phosphorylated when expressed individually demonstrates that this covalent modification does not require receptor activity (because of receptor inactivity when subunits are expressed individually). It is also worthy of note that $GABA_{B(1)}$ is phosphorylated even when intracellularly retained.

3.2.2 Agonist does not enhance phosphorylation of either subunit

Agonist occupation of G-protein coupled receptors can trigger phosphorylation of the receptor. This can be through second messenger stimulated pathways such as PKA and PKC, or it can be through GRK's. To determine whether the basal phosphorylation observed in $GABA_B$ receptors could be modulated by the activity of the receptors, whole cell labelling experiments were carried out to determine the phosphorylation state of both $GABA_B$ subunits pre- and post agonist. COS-7 cells were transiently transfected and then labelled with ^{32}P as before. Thirty minutes before the end of ^{32}P orthophosphate incubation plates were treated with 100μM baclofen, or designated untreated controls. Receptors were then immunoprecipitated and resolved using 8% SDS-PAGE.

Baclofen did not induce a significant enhancement in the phosphorylation levels of either $GABA_{B(1)}$ or $GABA_{B(2)}$ (n=4) (fig 4a,b). The phosphorylation state of both subunits also did not increase after only 5 or 10 minutes of agonist application.





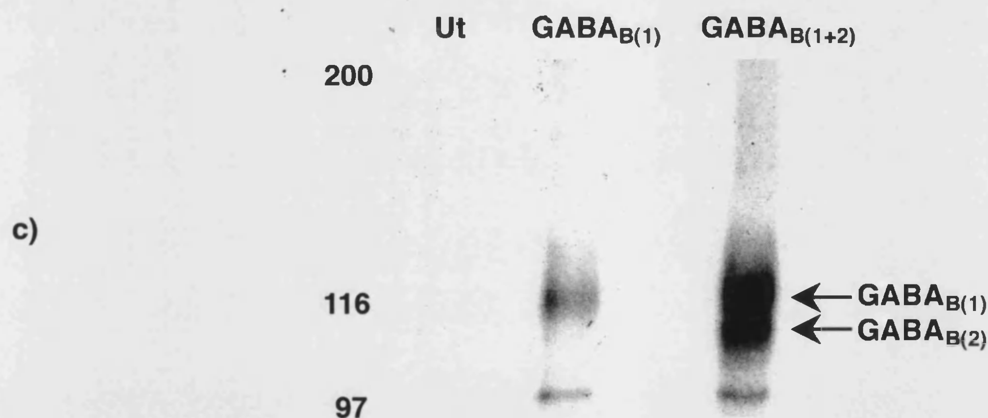


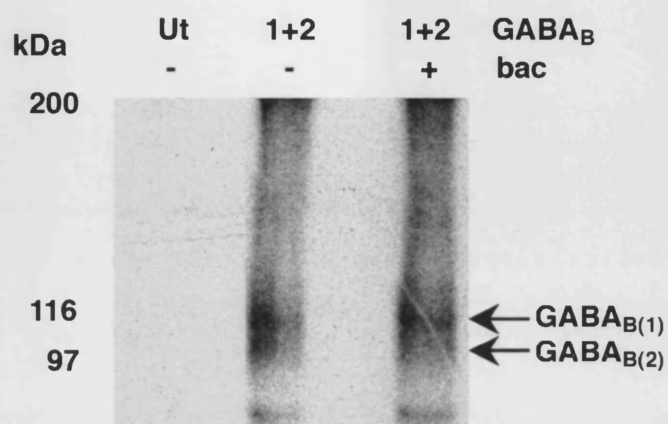
Figure 3 GABA$_B$ receptors are basally phosphorylated

a) COS-7 cells transfected with myc-tagged GABA$_{B(1)}$ and flag-tagged GABA$_{B(2)}$ were incubated with ^{35}S labelled methionine containing media for 4 hours prior to immunoprecipitation with myc, flag or both antibodies. R1 migrates at ~116kDa whilst R2 migrates at 105 kDa.

b) Immunprecipitated GABA$_{B(2)}$ from ^{32}P labelled COS-7 cells demonstrates basal phosphorylation.

c) GABA$_{B(1)}$ is also basally phosphorylated when expressed alone or with R2.

a)



b)

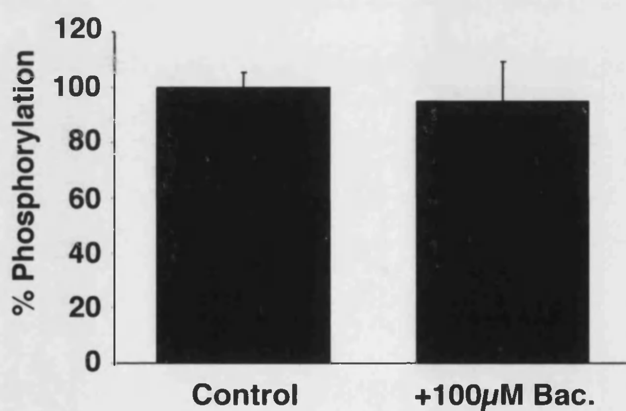


Figure 4 **Agonist does not enhance phosphorylation of either subunit**

Application of baclofen does not enhance the phosphorylation of either subunit when co-expressed in ^{32}P labelled COS-7 cells (n=4)

3.2.3 GRK overexpression does not enhance agonist induced phosphorylation of $GABA_B$ receptors

It is possible that a kinase responsible for agonist induced phosphorylation *in vivo* would be underexpressed in comparison to the expression levels of $GABA_B$ in transfected cells. This would lead to difficulties in measuring agonist induced phosphorylation.

GRKs are responsible for homologous phosphorylation of receptors and overexpression of GRK 2 has been shown to enhance agonist-induced phosphorylation of a multitude of GPCRs including mGluRs. Because phylogenetically the $GABA_B$ receptor is closest to metabotropic glutamate receptors, I decided to determine whether $GABA_B$ receptor phosphorylation could be enhanced by the overexpression of GRK2. Whole cell labelling experiments were carried out as before, with the co-expression of GRK2. Transfection ratios were of 1:4:1 $GABA_{B(1)}$: $GABA_{B(2)}$:GRK2.

Initially I studied solely $GABA_{B(2)}$ phosphorylation in the presence of GRK2 because it is $GABA_{B(2)}$ that is G-protein coupling subunit, and therefore most likely to be the target of uncoupling phosphorylation. $GABA_{B(2)}$ was immunoprecipitated from cells co-expressing $GABA_{B(1)}$ and GRK2 after different exposure times to 100μM baclofen. However, agonist application did not elicit an increase in the phosphorylation of $GABA_{B(2)}$, but the converse (fig. 5a). This response was noted in the presence or absence of GRK2 and may be due to a decrease in PKA activity sequential to baclofen-mediated inhibition of adenylyl cyclase. $GABA_{B(2)}$ phosphorylation is returned to basal levels after 30 minutes, possibly reflecting the importance of $GABA_{B(2)}$ phosphorylation in preventing desensitisation. This rebound in phosphorylation can be explained by $GABA_B$ desensitisation, reducing the negative regulation of adenylyl cyclase and leading to increased PKA activity and a subsequent increase in $GABA_{B(2)}$ phosphorylation.

The effect of overexpression of GRK2 on total receptor phosphorylation by immunoprecipitating both $GABA_{B(1)}$ and $GABA_{B(2)}$ with and without agonist exposure was then analysed. Overexpression of GRK2 did not significantly affect the basal phosphorylation state of the whole receptor, neither the phosphorylation after agonist treatment (fig 5b). It is possible though that GRK2 does interact with the whole

receptor, as is seen in figure 5b, where a band running at approximately 80kDa is only observed when GRK2 is over-expressed. Although this could be an interacting protein that is phosphorylated by GRK2, 80kDa is the molecular weight of GRK2. It may be that GRK2 interacts with $GABA_B$ in a manner not involving phosphorylation, but can modulate signalling through its RGS domains.

Concurrent to investigating the role of GRK2 in $GABA_B$ phosphorylation *in vivo*, an *in vitro* method was also employed. Recombinant GRK2, purified from an SF9 insect cell line (a kind gift from Dr J.Pitcher), was incubated in tandem with G-protein $\beta\gamma$ subunits, PIP2 (both important for optimal GRK2 activity) and $^{32}P\gamma ATP$ with purified GST-fusion proteins encompassing the carboxy-terminal domain of $GABA_{B(1)}$ or $GABA_{B(2)}$. Reactions were carried out in kinase buffer @ 30°C and terminated after 15 minutes. GST alone was not a GRK substrate, and neither were the carboxy-terminal domains of $GABA_{B(1)}$ or $GABA_{B(2)}$ (fig. 5c). *In vivo* the receptor domains are believed to interact through coiled-coil motifs; and this may confer a different confirmation to when alone. To investigate the possibility that together the domains may present a substrate more similar to that *in vivo*, fusion proteins of $GABA_{B(1)}$ and $GABA_{B(2)}$ carboxy-terminal domains were pre-mixed and then incubated with GRK2. This was not the case though and again there was negligible phosphorylation (fig. 5c lane 4). The poor efficiency of GRK2 phosphorylation of $GABA_B$ subunits is illustrated when contrasted with the phosphorylation of $GABA_{B(2)}$ achieved by co-incubation with PKA (approximately 1 mole stoichiometry), or the phosphorylation observed of activated rhodopsin from purified rod outer segments by GRK2.

To explore the possibility that $GABA_B$ receptors are substrates for other GRKs similar whole cell labelling experiments were carried with the co-expression of both $GABA_B$ subunits and either GRK3, GRK5. Again, agonist application in the presence of each of these GRKs did not elicit an increase in the phosphorylation status of $GABA_B$ receptors (fig 5d,e).

3.2.4 Influence of agonist upon stability of cell surface $GABA_B$ receptors in COS-7 cells

Although agonist did not enhance $GABA_B$ phosphorylation, the effect of agonist upon the surface stability of $GABA_B$ remained unknown. To investigate this COS-7 cells were transiently transfected with $GABA_B$ receptors and seeded into 6 X 30mm well plates. Cells were exposed to agonist 36-48 hours post-transfection by addition of 100µM baclofen to the media for either 5,10,15 or 30 minutes whilst incubated at 37°C. Cells were then fixed in paraformaldehyde and blocked in blocking solution. Whole cell ELISAs were carried out with surface myc-tagged $GABA_{B(1)}$ probed for with affinity purified monoclonal anti-myc antibody. After extensive washing cells were exposed to secondary HRP conjugated donkey anti-mouse antibody. Cells were again washed and then incubated with the substrate TMB for 15 minutes. TMB is colourless upon application but exposure to HRP catalyses a colour change to blue. This colour change was quantitated with a photospectrometer set to read absorbance at 655nM. A cuvette containing unexposed TMB was used as a blank, and background antibody adherence to cells was quantitated by carrying out the ELISA on untransfected cells. All experiments were carried out in triplicate. Agonist appeared to cause a decrease in detectable receptors (fig. 6a), but this reduction is too small to be significant and, being similar at 5 minutes and 30 minutes, did not increase with time as would be expected with receptor internalisation. Typical reductions of 40% surface number are observed upon agonist application in similar experiments (Scott *et al.,* 2002). This is illustrated by comparing the $GABA_B$ response to the decrease in surface levels of the TRH receptor in the same system (fig. 6a).

To determine whether the small decrease in surface $GABA_B$ receptor number could be explained by receptor internalisation, or whether receptors were internalising and then rapidly recycling, biotinylation-cleavage assays were performed. These consisted of labelling $GABA_B$ transfected COS-7 cells that were pre-treated for 1 hour with the lysosomal inhibitor leupeptin (100µg/ml), with cell-impermeable, cleavable NHS-SS-biotin in PBS on ice. Post-biotinylation, cell media was returned and plates were replaced in the incubator at 37°C. Cells were then treated with 100µM baclofen, or left untreated. At 5, 10 or 30 minutes after the commencement of treatment, plates were placed back on ice. The remaining surface

biotin was cleaved using glutathione containing cleavage buffer and cells were then solubilised in RIPA. Cell lysates were prepared and biotinylated proteins were precipitated on streptavidin-conjugated beads. After thorough washing, samples were examined using SDS-PAGE followed by western blotting. Streptavidin precipitates from only cells transfected with both $GABA_{B(1)}$ and $GABA_{B(2)}$ that had been kept on ice post-biotinylation and not cleaved showed myc-$GABA_{B(1)}$ at the surface, detectable as a broad band of approximately 115-130 kDa (fig. 6b lane 1). As a control to demonstrate the efficiency of cleavage, cells were kept on ice for 30 minutes, preventing any internalisation, and then cleaved. These cells showed >95% decrease in $GABA_{B(1)}$ present in precipitates (fig. 6b lane 2), illustrating high cleavage efficiency. Precipitates from cells returned to the incubator prior to cleavage showed low myc-immunoreactivity, similar to that in observed in the cleavage control. This demonstrates that $GABA_B$ receptors are not subject to constitutive internalisation in recombinant systems. Application of baclofen did not cause an increase in detectable internalised receptors, demonstrating a lack of agonist induced $GABA_B$ receptor internalisation.

Although the data from cell-surface ELISA assays appears to contradict the biotinylation data, there are other explanations for the small reduction in signal seen after baclofen application. Baclofen binding to $GABA_B$ is thought to allosterically change the confirmation of the extracellular domain of $GABA_{B(1)}$ with the exposed extracellular domain closing in on itself. It is possible that upon fixation of cells with paraformaldehyde a proportion of the receptors are cross-linked in this confirmation leaving the N-terminal myc-tag marginally less accessible to antibody. Receptor number seems to be similar at all time points post baclofen addition, and does not decrease with time as would be expected with internalisation, supporting a change in affinity for anti-myc antibody.

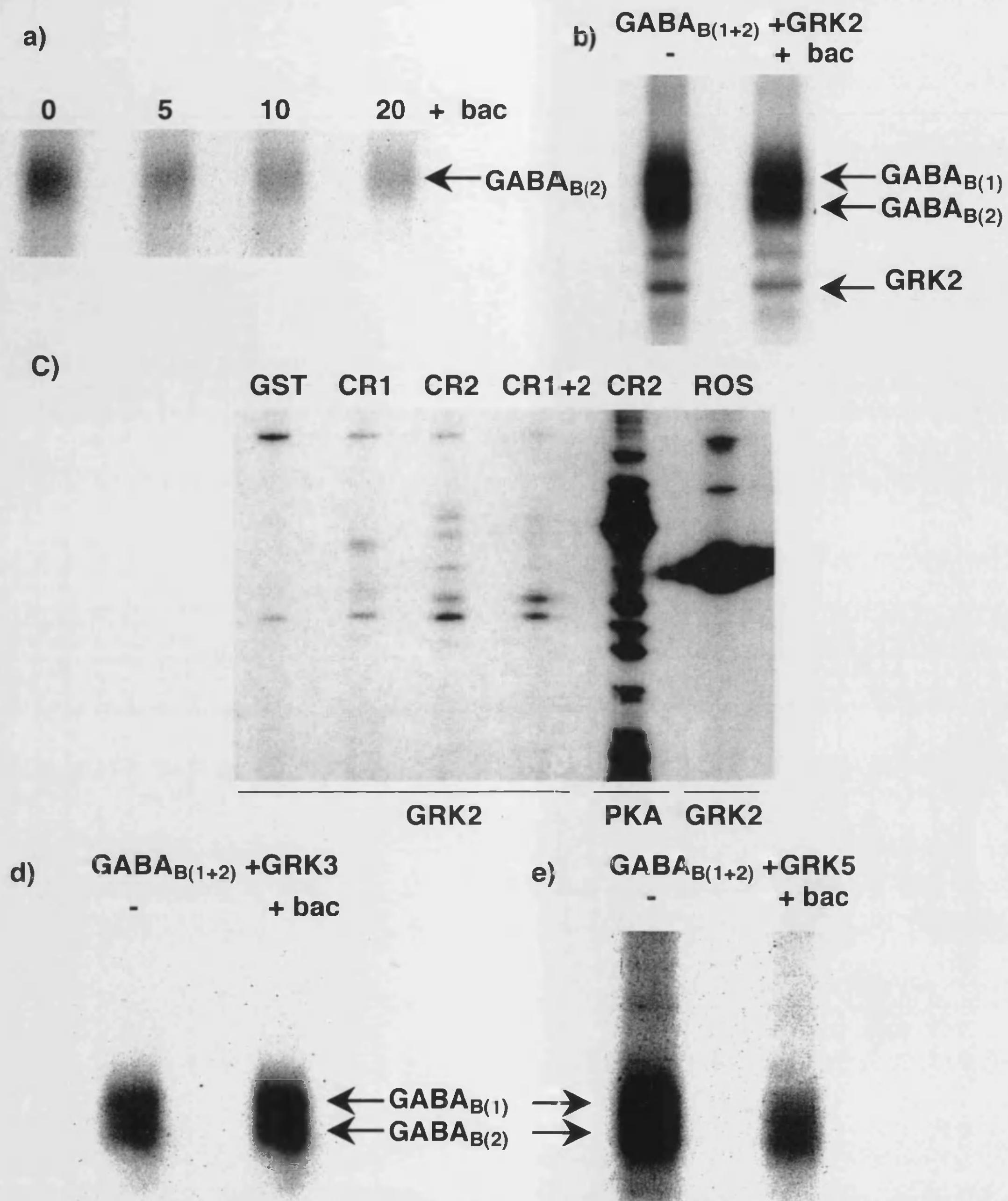


Figure 5 Analysis of effects of GRK over-expression upon $GABA_B$ receptor phosphorylation

a) Timecourse of $GABA_{B(2)}$ phosphorylation in COS-7 cells co-transfected with $GABA_{B(1)}$, $GABA_{B(2)}$, and GRK2 over 20 minutes after baclofen application.

b) $GABA_{B(1)}$ and $GABA_{B(2)}$ co-expressed with GRK 2 were immunoprecipitated from ^{32}P radiolabelled COS-7 cells after exposure to 100μM baclofen for 30 minutes.

c) GST-fusion proteins of the carboxy-terminal tails of $GABA_{B(1)}$ and $GABA_{B(2)}$ were incubated with purified GRK2 and $^{32}P\gamma ATP$ in the presence of PIP2 and G-protein $\beta\gamma$ subunits. Phosphorylation was insignificant in comparison to rhodopsin from rod outer segments, or the phosphorylation of $GABA_{B(2)}$ by PKA.

d) e) Whole cell labelling experiments were carried out over-expressing GRK3 and 5 to analyse whether the overexpression of these kinases lead to agonist induced phosphorylation. No significant effect was observed.

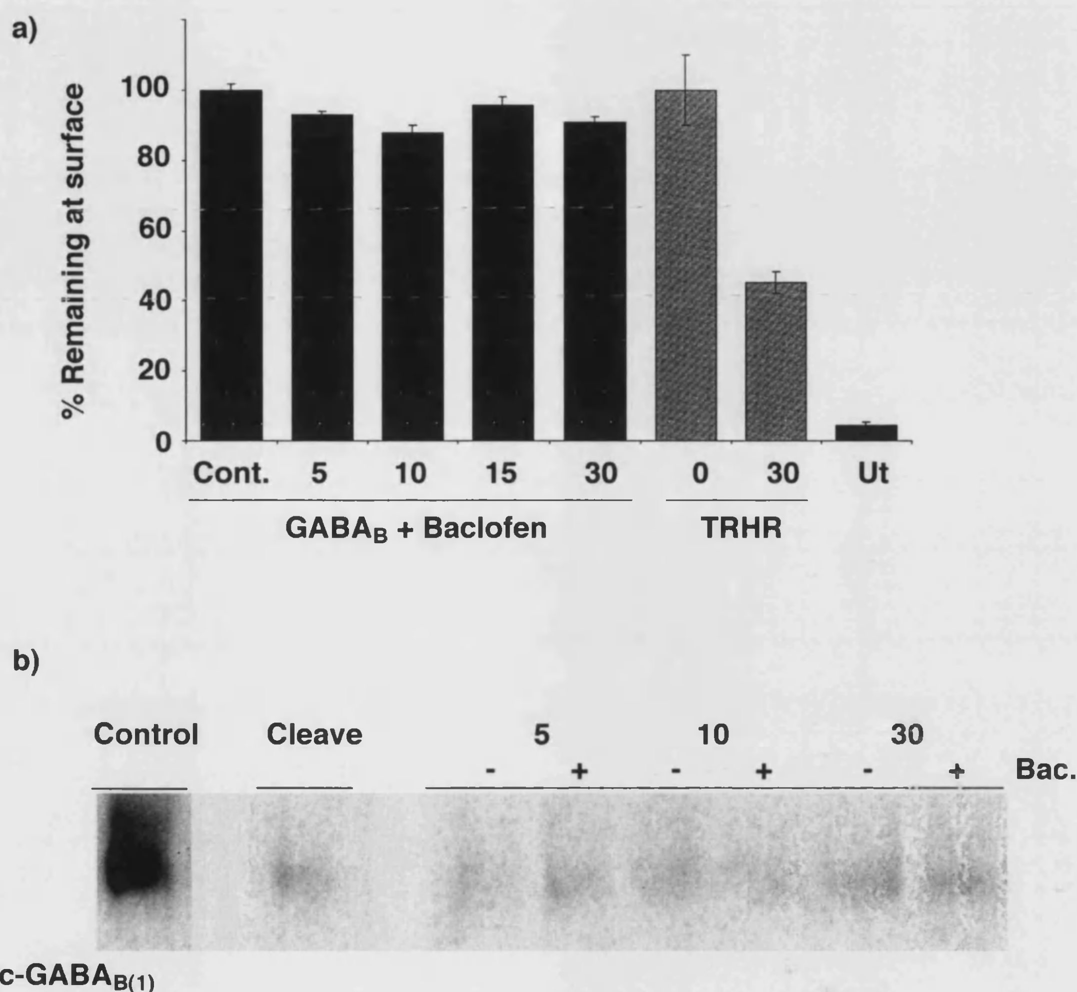


Figure 6 **Surface $GABA_B$ receptors are highly stable in heterologous systems**

a) COS-7 cells co-transfected with myc-$GABA_{B(1)}$ and flag-$GABA_{B(2)}$ were treated for between 5 and 30 minutes with 100μM baclofen before fixation and then assaying the number of surface receptors using a whole cell ELISA. Treatment had no significant effect upon surface $GABA_B$ levels, whilst treatment of cells transfected with TRHR with 30min 10μM TRH significantly reduced surface receptor levels.

b) COS-7 cells co-transfected with myc-$GABA_{B(1)}$ and flag-$GABA_{B(2)}$ were biotinylated with cleavable biotin, and then returned to the incubator for times upto 30 minutes in the presence of 100μM baclofen. Surface biotin was then cleaved and cells solubilised with labelled receptor purified on streptavidin beads. Total surface was assessed from plates left on ice (Control), and efficiency of cleavage was determined by cleaving plates not returned to the incubator but kept on ice (Cleave). Incubation in the presence or absence of baclofen did lead to receptor internalisation.

3.2.5 Analysis of the effect of arrestins upon $GABA_B$ receptors

The apparent stability of $GABA_B$ receptors upon activation could be because arrestin proteins, which are important for GPCR endocytosis, are not present in a stoichiometric ratio to $GABA_B$ receptors. Indeed COS-7 cells express low levels of arrestins in comparison to other cell lines such as HEK-293 (J.Pitcher, personal communication). Although $GABA_B$ phosphorylation does not appear to be mediated by GRKs, certain GPCRs can endocytose in an arrestin dependent manner irrespective of GRK phosphorylation. One such example is mGluR1, which is dependent upon arrestin 2 for both constitutive and agonist induced endocytosis (Dale *et al.,*. 2001). The crystallisation of arrestin 1 demonstrated the presence of a phosphate sensor domain that specifically interacts with phosphorylated rhodopsin (Graznin *et al.,* 1998; Hirsch *et al.,* 1999). That both $GABA_{B(1)}$ and $GABA_{B(2)}$ are basally phosphorylated also means that an interaction with arrestins could be possible, with agonist altering the confirmation of the cytosolic portion of the receptor exposing phosphorylated residues to arrestins.

Activation of GPCRs that interact with arrestins causes a characteristic change in the cellular localisation of arrestins. Prior to receptor activation, arrestins display a mainly cytosolic localisation, but upon receptor activation arrestin distribution is changed and arrestins translocate to the cell surface membrane and interact with with receptors (Luttrell & Lefkowitz 2002). This is rapidly followed by the clustering of receptor/arrestin complexes into clathrin-coated pits, where arrestins can be observed in punctate endosomal compartments. From this stage receptors can be divided into 2 different classes: those that continue to interact with arrestins and those that don't (Oakley *et al.,* 2000). If the arrestin receptor interaction is maintained then arrestins are observed to form large peri-nuclear clusters in tandem with the receptors, otherwise they gradually resume a more cytosolic localisation (Oakley *et al.,* 2000).

An immunofluorescence based approach was utilised to investigate whether $GABA_B$ receptors can interact with arrestins, and also whether any such interaction can direct the internalisation of $GABA_B$ receptors. COS-7 cells were transiently transfected with $GABA_B$ receptors and an EGFP tagged arrestin 3 construct and seeded onto poly-L-lysine coated coverslips. Thyrotropin-releasing hormone

receptors (TRHR) have previously been demonstrated to internalise in response to agonist in an arrestin dependent manner (Scott *et al.,* 2002) and so VSV-tagged TRHRs were also co-expressed with EGFP arrestin 3 as a positive control. Coverslips were incubated at 4°C with antibodies to expressed receptors added to complete media (monoclonal anti-myc for $GABA_{B(1)}$ and monoclonal anti-VSV for TRHR). After thorough washing coverslips returned to the incubator in serum free media to which was added agonist complimentary to the receptor (100μM baclofen or 10μM TRH). After 2, 5 or 30 minutes of incubation coverslips were removed and fixed. Receptors remaining at the surface were stained with Cy5 conjugated anti-mouse antibody, cells were then permeabilised and internalised receptors stained with TRITC conjugated anti-mouse antibody.

Only low levels of TRITC staining were observed in cells expressing $GABA_B$ receptors that had been returned to the incubator, similar to the staining present in cells kept on ice (fig.7 images C,K,S); whilst Cy5 staining of surface receptors remained constant (fig.7 images B,J,R). This supported the data obtained in cell-surface biotinylation assays indicating a lack of agonist-induced internalisation of $GABA_B$ receptors. Furthermore, the localisation of co-expressed arrestin 3 was unchanged throughout agonist exposure (fig.7 images A,I,Q) signifying the lack of a role for arrestins in $GABA_B$ desensitisation. The response of arrestin 3 in cells expressing TRHR contrasts vividly with that seen in $GABA_B$. Here a redistribution of receptor from the surface to an internal pool through an endosomal pathway in conjunction with a tight association of arrestin 3 is observed (fig.7 images D-G,M-P,U-X).

Although there is functional redundancy between arrestin 2 and 3 in regard to the endocytosis of many GPCRs (Luttrell 2002) with similar expression patterns of both, instances of receptor preference for one over the other exist, as is seen for mGluR1 (Dale *et al.,*. 2001). Arrestin 2 was also over-expressed in conjunction with $GABA_B$ receptors to investigate whether it could interact with and direct $GABA_B$ receptor endocytosis. However, these experiments revealed that, similar to arrestin 3, arrestin 2 had no observable effect upon the cell surface stability or internalisation of $GABA_B$ receptors (fig.8).

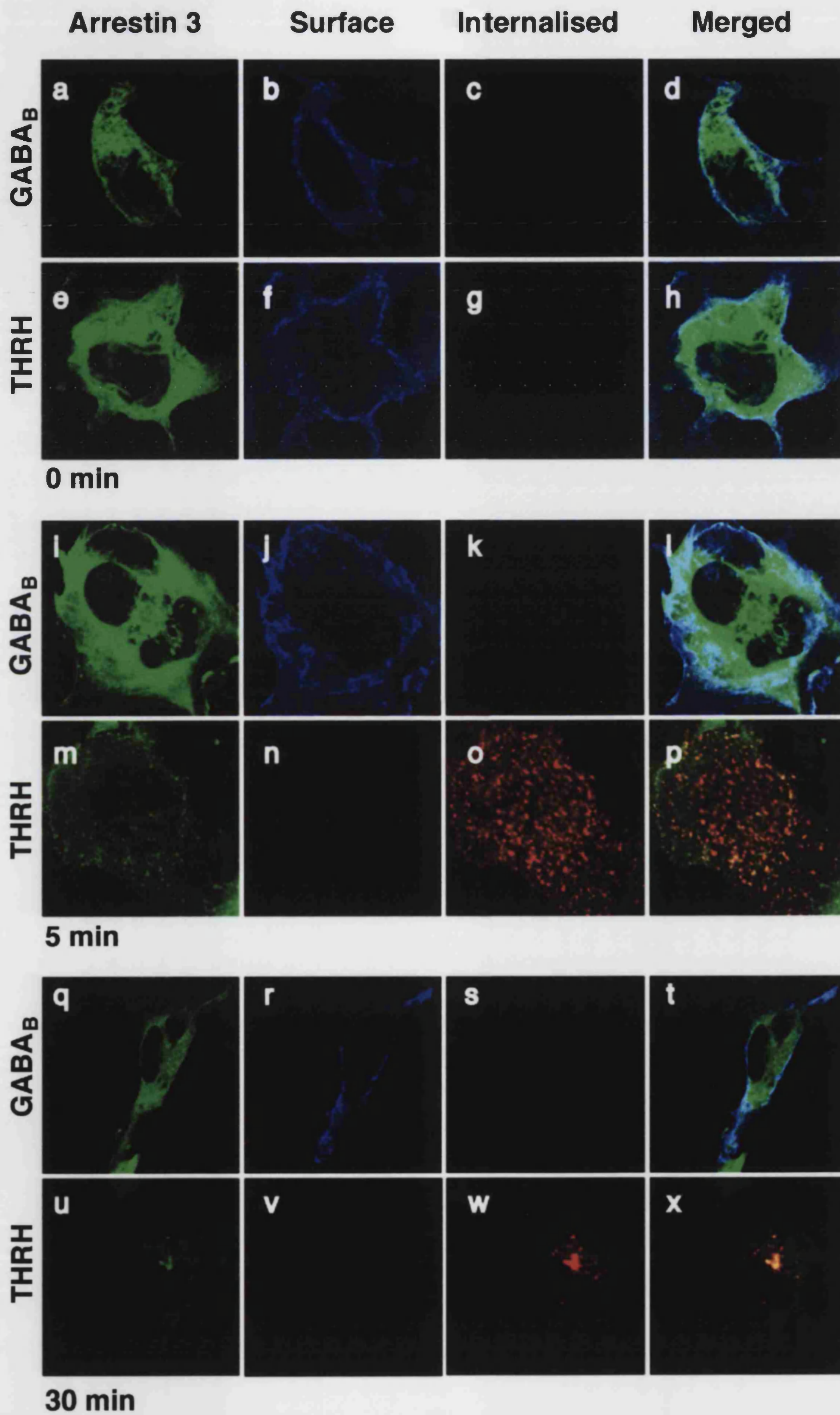
3.2.6 Effects of agonist on the degradation of $GABA_B$ receptors in COS-7 cells

Post-internalisation, GPCRs are either degraded or recycled to the surface. Application of agonist enhances internalisation and therefore increases the probability that a receptor will be degraded. To determine whether chronic agonist treatment enhances degradation of surface $GABA_B$ receptors I used a biotinylation-degradation assay similar to that used by other experimenters in the study of GPCR degradation (e.g. Whistler *et al.,* 2002). Cells co-transfected with $GABA_{B(1)}$ and $GABA_{B(2)}$ were labelled with the non-cleavable biotin derivative succimodobiotin 36-48h post-transfection. This does not have the disulphide group of NHS-SS-biotin, and so is stable even under reducing conditions, allowing for irreversible biotinylation. Post-biotinylation, plates were returned to the incubator to equilibrate for 6h before lysis in RIPA buffer of labelled plates for an initial time point, taken to be time 0h which was snap frozen in liquid N_2. Plates were then treated with 100μM baclofen or left untreated and incubated for a further 20 hours prior to lysis of remaining plates. Lysates were then prepared from all cells and remaining biotinylated proteins purified with streptavidin-conjugated beads. Western blotting of SDS-PAGE subjected samples demonstrates that after 20h of incubation ~40% of $GABA_B$ receptors are remaining (fig. 9a,b), equating to an approximate half life of surface $GABA_B$ in COS-7 cells of 16 hours. This is significantly greater than the 10h half life of $GABA_{B(1)}$ measured by Couve *et al.,*. when expressed in COS-7 cells (Couve *et al.,* 1999). This difference is probably attributable to co-expression of $GABA_{B(2)}$ in my study, allowing me to look purely at surface receptors.

Interestingly, treatment of the cells with baclofen did not significantly alter the receptor degradation rate. This again supports the lack of agonist-induced internalisation observed in this cell line.

Figure 7 Overexpression of arrestin 3 does not alter $GABA_B$ heterodimer cell surface stability

COS cells were transfected with MYC-$GABA_{B(1a)}$ and FLAG-$GABA_{B(2)}$ plus arrestin 3-EGFP (a-d, i-l and q-t) or with TRHR plus arrestin 3-EGFP (e-h, m-p and u-x). Cells were stimulated with 100 μM baclofen for 5 min (i-l) or 30 min (q-t) or with 10 μM TRH for 5 min (m-p) or 30 min (u-x). Cells were then processed for immunofluorescence. Arrestin 3-EGFP is shown on the left panels. The surface receptor pool was detected using Cy5 conjugated secondary antibodies prior to permeabilisation (blue channel). The internalised receptor pool was detected using Texas-Red conjugated secondary antibodies following permeabilisation (red channel). Merged images are shown on the right hand panels.


Arrestin 3
Surface
Internalised
Merged
GABA$_B$
THRH
a
b
c
d
e
f
g
h
0 min
i
j
k
l
m
n
o
p
5 min
q
r
s
t
u
v
w
x
30 min

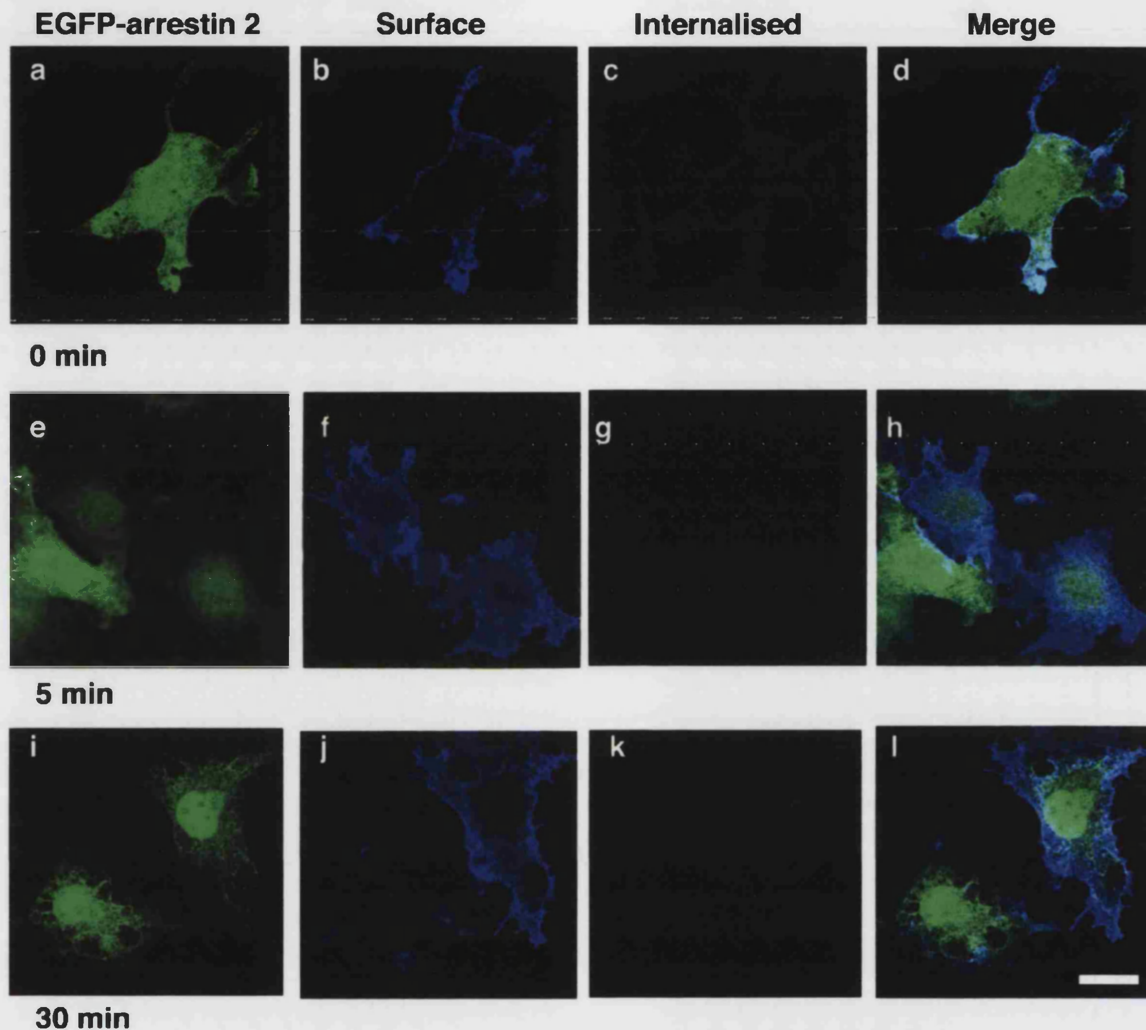
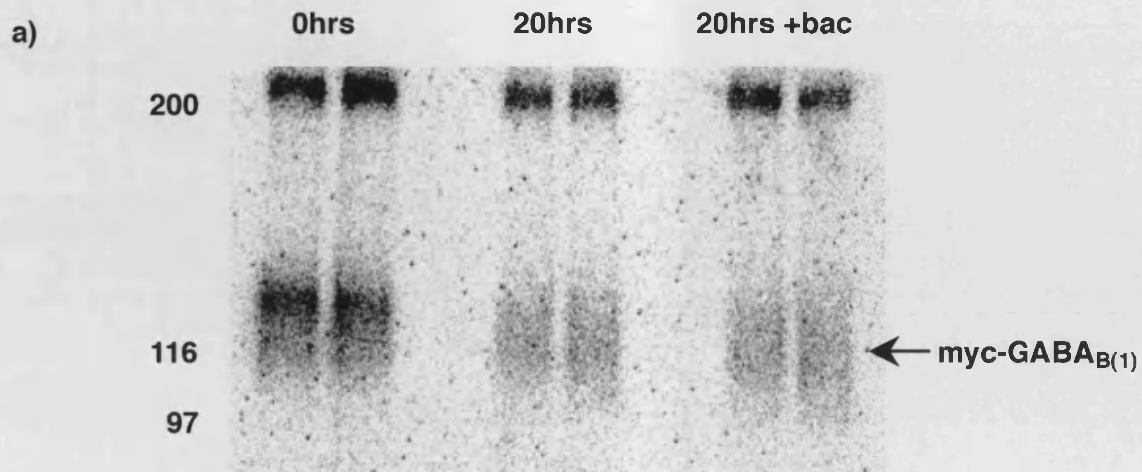


Figure 8: Overexpression of arrestin 2 does not alter GABAB heterodimer cell surface stability

COS-7 cells were transfected with myc-GABA$_{B(1a)}$ and flag-GABA$_{B(2)}$ plus EGFP-arrestin 2 (a-l). Cells were left untreated (a-d) or stimulated with 100 μM baclofen for 5 min (e-h) or 30 min (i-l). Cells were then processed for immunofluorescence. EGFP-arrestin 2 is shown on the left panels. The surface receptor pool was detected using Cy5 conjugated secondary antibodies prior to permeabilisation (blue channel). The internalised receptor pool was detected using Texas-Red conjugated secondary antibodies following permeabilisation (red channel). Merged images are shown on the right hand panels.



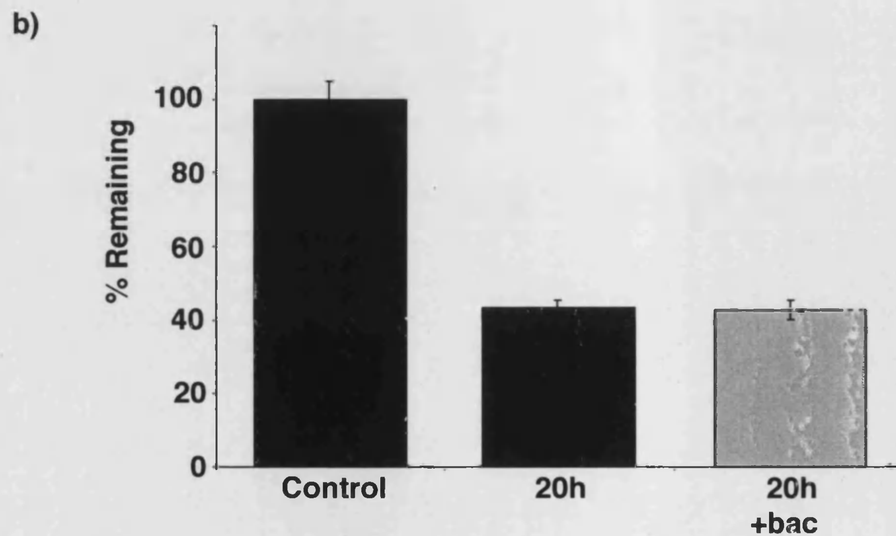


Figure 9 Application of agonist does not enhance $GABA_B$ degradation in heterologous systems

a) COS-7 cells co-transfected with myc-$GABA_{B(1)}$ and flag-$GABA_{B(2)}$ were surface biotinylated and then either lysed (control) or returned to the incubator for 20h with or without 100μM baclofen. Cells were then lysed and biotinylated proteins were streptavidin purified. Samples were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-myc antibody, follwed by [^{125}I] labelled secondary.

b) Blots were quantitated using a phosphorimager. Application of baclofen had no effect upon amount of biotinylated $GABA_B$ remaining after 20h.

3.3 Studies of endogenous receptors

3.3.1 $GABA_B$ receptors are highly stable at the cell surface in cortical neurones

Cortical neurones derived from E18 rat embryos were cultured on 10cm poly-L-lysine plates. At 5 DIV plates were pre-treated for 1 hour with the lysosomal inhibitor leupeptin (100µg/ml), biotinylated with cleavable biotin and then either left on ice or returned to the incubator for defined time periods in the presence or absence of 100µM baclofen. This was to allow observation of $GABA_B$ receptor internalisation in the cultures, and also to determine whether internalisation is enhanced by agonist. After incubation surface biotin was cleaved using glutathione based cleavage buffer and cells were lysed in RIPA. Streptavidin coated beads were used to purify proteins that had been biotinylated and then endocytosed, thus protected from cleavage. The experiments in figure 10 were carried out with Dr A. Couve, and these demonstrate that both $GABA_{B(1)}$ and $GABA_{B(2)}$ are present at the surface of cortical neurones after 5 DIV. Moreover, they are not subject to significant levels of basal endocytosis and neither is there enhancement of endocytosis by treatment of agonist at 10 or 60 minutes (60 min: control = 2.6 ±1.0%, baclofen = 3.5 ± 1.0%, n=3) (fig. 10a,b). Only when cultures are treated for 6h with baclofen is there a small enhancement of the internalisation of $GABA_{B(1)}$. This increase is relatively minor however, and the length of treatment required does not fit with the time course expected from classical agonist induced endocytosis of other GPCRs.

To demonstrate the validity of the system, membranes were immunoblotted for the AMPA receptor subunit GluR1. This has previously been demonstrated to internalise both constitutively and upon treatment with AMPA (Ehlers 2000). Over a 60 minute timecourse in the presence of AMPA ~43% of GluR1 was recovered from an internal pool (fig. 10c).

To visually confirm the biotinylation data, 20 DIV hippocampal neurones grown on coverslips were treated with 100µM baclofen for either 15 minutes or 1 hour. Cultures were then fixed in paraformaldehyde in tandem with untreated controls, and co-stained with antibodies against either $GABA_{B(1)}$ or $GABA_{B(2)}$ and a marker for early endosomes, EEA-1. Because AMPA receptors internalise rapidly into endosomes in

a dynamin and agonist dependent manner (Carroll *et al.,* 1999), I compared the endocytosis of AMPA receptors with that of $GABA_B$. To do this I treated separate coverslips from the same culture with AMPA (100µM) for 15 minutes and then fixed, permeabilised and co-stained for GluR1 and EEA-1 in parallel with untreated coverslips.

Treatment of neurones with AMPA for 15 minutes caused a rapid change in the distribution of GluR1 containing receptors. Prior to treatment they exhibit a dendritic cell membrane distribution. AMPA treatment results in a re-distribution of receptors to the soma of the cell body where the appear very punctate. This occurred in tandem with a large increase in co-localisation of GluR1 and EEA1, indicative of trafficking to early endosomes (fig.11a).

Antibodies to $GABA_{B(2)}$ show membranous distribution over the cell body and also along dendrites (fig. 11b). Receptors are not clustered, with staining appearing quite even throughout the neurones. In the basal state there is no co-localisation of $GABA_{B(2)}$ with EEA1 staining endosomes. Agonist treatment does not change the localisation of $GABA_{B(2)}$, with the same proportion remaining at the membrane. There is also no re-distribution of $GABA_{B(2)}$ to endosomes with there remaining a complete lack of co-localisation of $GABA_{B(2)}$ and EEA1 after both 15 minutes. Even when treated for 60 minutes with baclofen there was no observable change in staining.

$GABA_{B(1)}$ showed a notable absence of surface staining. Instead, staining was largely somatic with distribution also along the internal compartment of dendrites. This was reminiscent of an ER distribution and is similar to that observed by other experimenters (Vernon *et al.,* 2001). The data obtained using biotin clearly demonstrates the presence of the $GABA_{B(1)}$ at the surface in neurones, and this is supported by an overwhelming wealth of electrophysiological data from other laboratories. The antibody used for immunocytochemistry was raised against a portion of the carboxy-terminal domain of $GABA_{B(1)}$. It is feasible that the epitope is masked when $GABA_{B(1)}$ and $GABA_{B(2)}$ interact, thus explaining the lack of surface staining. Other antibodies available within the laboratory raised to the N-terminal of $GABA_{B(1)}$ were also used, but unfortunately these were not suitable for immunofluorescence.

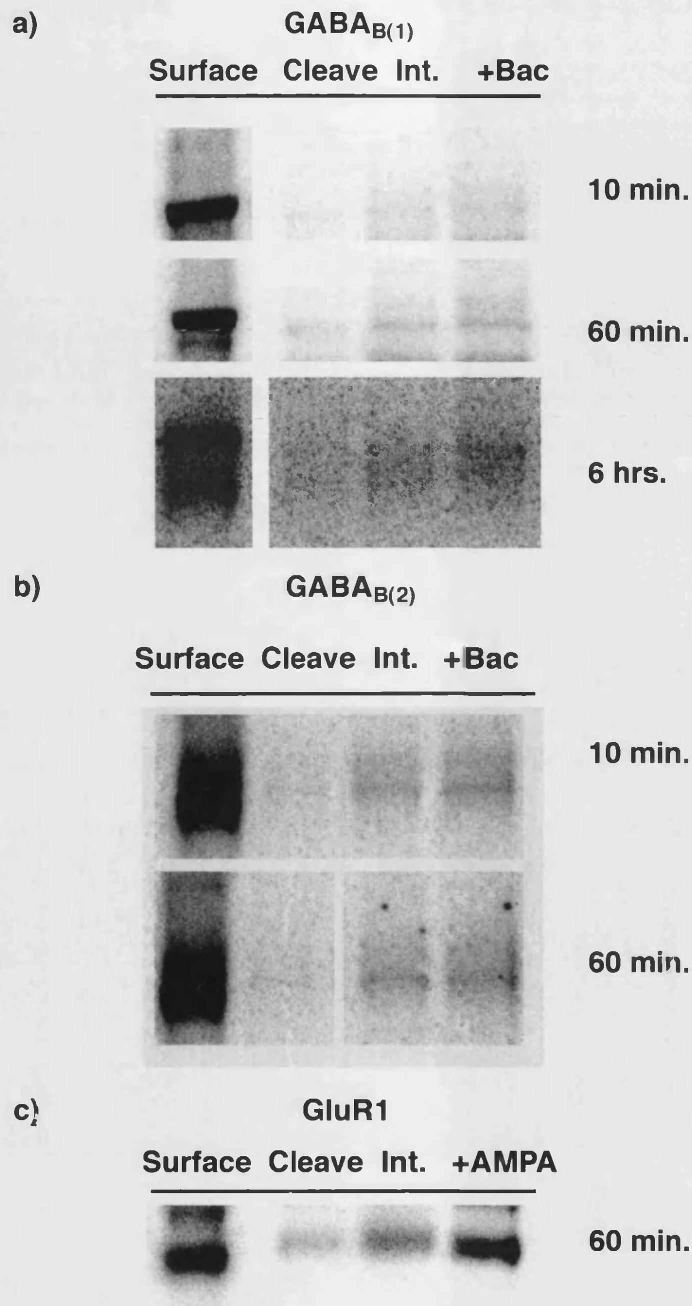


Figure 10: GABA$_B$ receptor cell surface stability in primary neuronal cultures.

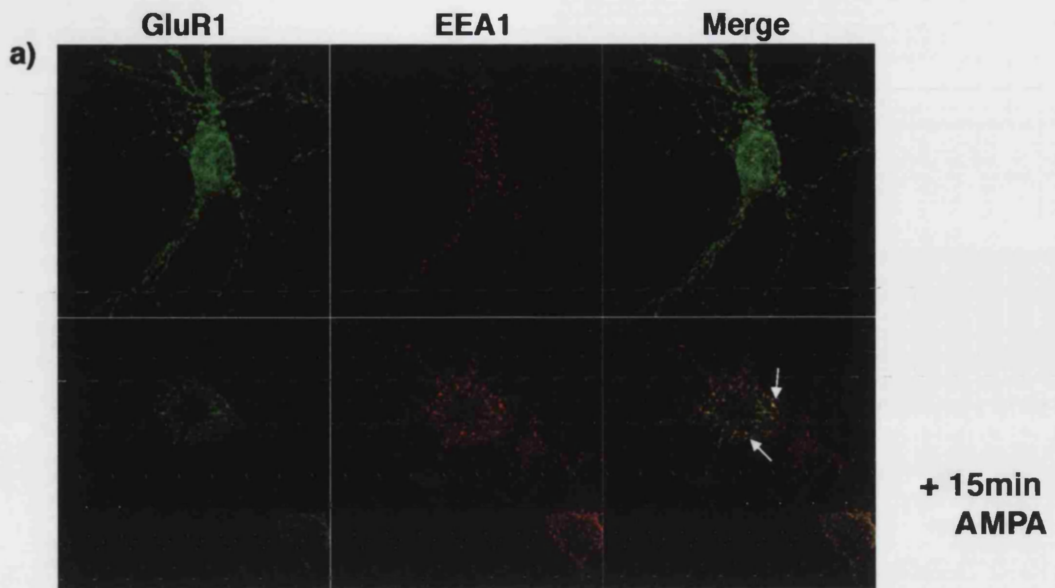
a) Surface proteins in 5 DIV rat cortical neurons were labelled with 1 mg/ml biotin and left untreated (lanes 1-3) or exposed to 100 μM baclofen (lane 4) for the indicated periods of time. Dishes were incubated at 4°C (lanes 1 and 2) or 37°C (lanes 3 and 4). Surface biotin remaining after incubation periods was left uncleaved (lane 1) or removed by cleaving with glutathione (lanes 2-4). Lysates were precipitated with streptavidin beads and samples resolved by SDS-PAGE. Immunoblots were performed with anti-GABA$_{B(1)}$ antibodies and [^{125}I]-conjugated secondary anti-rabbit antibody. The immunoblots were visualized using a phosphorimager.

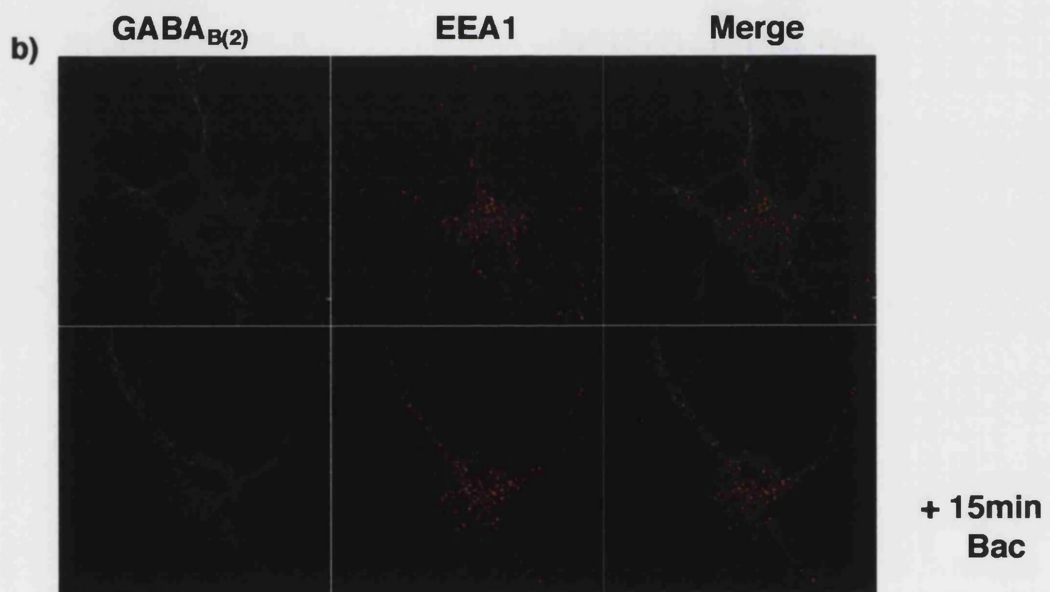
b) Same as above using anti-GABA$_{B(2)}$ antibodies.

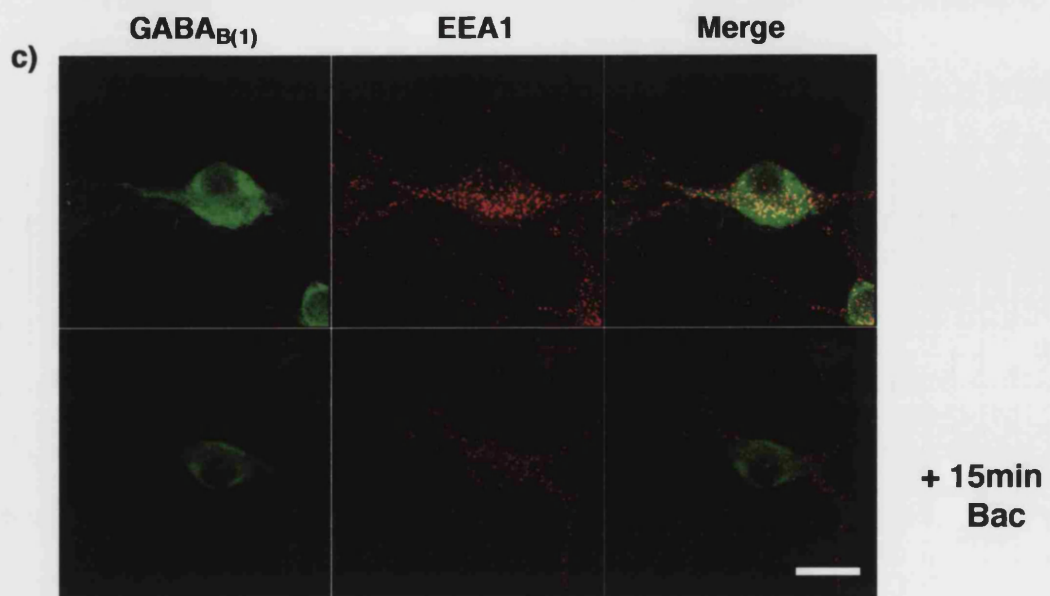
c) Same as above using 100 μM AMPA + 50 μM AP5 for 60 minutes (lane 4) and GluR1 antibodies.

Figure 11 Immunofluorescence analysis of $GABA_B$ receptor surface stability

21 DIV hippocampal neurons were left untreated (a,b,c top panels) or stimulated with 100μM AMPA for 15 minutes (a) or 100μM baclofen for 15 minutes (b,c). Cells were fixed and treated for immunofuorescence using anti GluR1 antibodies (a) or anti $GABA_B$ antibodies (b,c) in combination with anti-EEA1 antibodies. FITC conjugated secondary antibodies were used to visualise the receptors and Texas Red-conjugated secondary antibodies were used to visualize EEA1. All panels correspond to merged images. Examples of colocalization of AMPA receptors and EEA1 are indicated by white arrows.


a)
GluR1
EEA1
Merge
+ 15min
AMPA


b)
$GABA_{B(2)}$
EEA1
Merge
+ 15min
Bac


c)
$GABA_{B(1)}$
EEA1
Merge
+ 15min
Bac

The lack of observable $GABA_{B(1)}$ at the surface makes it difficult to draw any conclusions from $GABA_{B(1)}$ staining. It is possible to say though that although there is a degree of overlap of $GABA_{B(1)}$ and EEA1 staining, there is no accumulation of $GABA_{B(1)}$ within EEA1 staining endosomes. The $GABA_{B(2)}$ data though supports that obtained with biotinylation.

3.3.2 Agonist application enhances $GABA_B$ receptor degradation rate in neurones

Detection of an internalised pool of biotinylated $GABA_B$ receptors was difficult even six hours post-biotinylation. Any endocytosis that occurs is probably matched by the rate of degradation of endocytosed receptors, although the use of leupeptin excludes the possibility that this degradation is lysosomal. If $GABA_B$ receptors are not subject to appreciable levels of endocytosis they may be present at the surface membrane for a long period prior to removal. Alternatively, it is possible that they are subject to very rapid turnover rates, and that they are degraded in a non-lysosomal manner at a similar rate to which they are endocytosed. This could explain the lack of observable endocytosed receptors. Ascertaining the average length of time a $GABA_B$ receptor resides at the surface would allow me to make judgements as to the dynamism of the receptor in neuronal cultures. To do this I labelled surface receptors with non-cleavable biotin and calculated their half-life, i.e. the time taken for the number of receptors retrieved with streptdavidin to decrease by 50%. This technique has been used previously to calculate the cell surface half-life of neuronal AMPA receptors (Mammen *et al.,* 1997; Archibald *et al.,* 1998).

$GABA_B$ receptors have a very slow turnover rate, with 63.1% (±3.8% s.e.m.) remaining 24 hours post-biotinylation (fig. 12a). At all sampled time points up to 24 hours post-biotinylation the addition of baclofen elicited a small increase in the degradation of $GABA_B$ receptors, although this was outside the realms of statistical significance. However, after 60h of baclofen treatment there was a highly significant difference between the number of $GABA_B$ receptors retrieved from treated vs. untreated plates. From untreated cultures 24.3% (±0.4% s.e.m.) of receptors present at time zero were recovered. Treatment with baclofen reduced this to 14.8% (±0.5% s.e.m.) ($n=7$, $p<0.0001$) indicating enhanced degradation rates (fig. 12bi,c). To

determine whether at 60h all receptors retrieved were still at the cell surface, a similar experiment was performed, but using cleavable baclofen. After 60h plates were cleaved prior to strpetdavidin precipitation and western blotting. When compared to an uncleaved control from the same experiment it can be seen that cleavage removes all $GABA_{B(1)}$ immunoreactivity, indicating that all labelled receptor recovered at this time remains at the surface (fig. 12bii) Baclofen did not alter the morphology of the neurones or cause an increase in cell death. To demonstrate that the effect of baclofen was specific to $GABA_B$ receptors, membranes were blotted for the $GABA_A$ receptor subunit β3. There was no difference in the amount of $GABA_A$ receptor β3 remaining 60 hours post-biotinylation between treated and untreated samples (baclofen: 97.1 ± 3.3% control, n=6) (fig. 12c).

Plotting all data showed that $GABA_B$ receptor number decreased according to an exponential decay with $\tau \approx 31.0$h. Application of baclofen decreased this surface half-life to 23.5h (fig. 13a,b).

3.3.3 Chronic agonist application causes a decrease in surface $GABA_B$ receptors but not downregulation

Although chronic agonist enhances the degradation of surface $GABA_B$ receptors, the total number of receptors at the cell surface may not alter. It could be that the enhanced degradation rate is matched by an increase in the rate of receptor insertion, leaving the net number of surface receptors unchanged. To examine whether the total number of receptors at the surface is modulated in response to agonist it was necessary to pre-treat the neurones for set time periods prior to biotinylation and quantification of the number of receptors present. Plates of cultured cortical neurones were prepared and then treated at 4.5 DIV for 60h with 100μM baclofen. At 7 DIV plates were biotinylated in tandem with the biotinylation of untreated control plates. Surface proteins were purified on streptavidin as before and examined by SDS-PAGE and western blotting. Using this technique I found 60h pre-treatment significantly reduced the total number of $GABA_B$ receptors at the surface (baclofen: 75.7 ± 4.7% control $p<0.05$) (fig.14).

Continual or repetitive activation of GPCRs leads to their downregulation. This is defined as a decrease in the total number of receptors present in cells or tissues

(Tsao *et al.,* 2001). Crude lysates were also prepared so that it could be determined whether chronic baclofen treatment affected the internal pool of $GABA_B$ receptors. I also analysed levels of neurofilament to determine whether the treatment *per se* was affecting the vitality of the neurones and having a non-specific effect on $GABA_B$ numbers. Interestingly, I did not observe a significant decrease in the levels of $GABA_{B(1)}$ in lysates from treated neurones, and neurofilament levels remained unchanged (fig.14). This demonstrates that whilst surface $GABA_B$ receptor levels are altered by chronic baclofen, there is no large-scale downregulation of $GABA_B$ receptor protein synthesis. This indicates that the enhanced degradation rates caused by chronic baclofen can alter surface $GABA_B$ receptor numbers independently of changes in total receptor number.

3.3.4 Treatments that result in the enhancement or quiescence of neuronal activity do not effect $GABA_B$ degradation

The chronic application of baclofen is likely to lead to a continued state of enhanced neuronal inhibition through enhanced baclofen induced GIRK currents. I decided to investigate whether this enhanced inhibition *per se* was responsible for the increase in $GABA_B$ receptor degradation rate. The chronic treatment of cultures with the AMPA receptor antagonist CNQX in conjunction with the NMDA antagonist APV prevents spontaneous mEPSCs and elicits a decrease in synaptic activity in cultured neurones and is a well used pharmacological tool for inhibiting neuronal activity in cultures (Okabe *et al.,* 1999, Watt *et al.,* 2000, Ehlers 2003.) I biotinylated cultures as before and then returned them to the incubator in conditioned media and treated with CNQX(10μM) and D-AP5(50μM) for 60h, equivalent to approximately 2 half lives of the receptor under basal conditions. I then retrieved remaining biotinylated protein and quantitated using western blotting. If the inhibition of neuronal activity by baclofen was responsible for enhanced $GABA_B$ degradation then I would expect to see a similar enhancement in degradation with CNQX+APV. This was not the case however, and this pharmacological treatment appeared to have no significant effect on the amount of biotinylated $GABA_B$ receptor protein remaining (fig. 15). I also investigated whether enhanced neuronal activity had an effect on the amount of biotinylated $GABA_B$ remaining. To do this I treated biotinylated cultures

with the $GABA_A$ antagonist bicuculline (40μM). This prevents the firing of $GABA_A$ mediated mIPSCs thus increasing overall excitatory activity within cultures (Ehlers 2003). This treatment again elicited no significant effect on the amount of biotinylated $GABA_B$ receptors retrieved. If cultures were treated with baclofen and bicuculline however then a similar baclofen induced degradation of $GABA_B$ receptors was observed to that seen in cultures treated with baclofen alone (fig. 15). This demonstrates again that it is not the effect of inhibiting neuronal activity that enhances $GABA_B$ receptor degradation, but an effect specific to $GABA_B$ receptor agonist.

3.3.5 Treatment with activators of cAMP dependent protein kinase pathways reduces $GABA_B$ degradation

Chronic activation of $GABA_B$ receptors will lead to chronic inhibition of adenylyl cyclase, causing a decrease in cAMP accumulation and thus PKA activity. A physiological response from the neurones to try and correct this perturbation in PKA activity could involve reducing the number of $GABA_B$ receptors at the surface by enhancing the rate of their degradation. If this is true then the concomitant treatment of activators of PKA and baclofen should reverse such a situation. To investigate this possibility I treated biotinylated neurones with baclofen in conjunction with PKA activator forskolin (500nM) and 8-Br-cAMP (10μM). These drugs both activate PKA but through different pathways. Typically forskolin is used at concentrations of 20-50μM to cause maximal activation of PKA (e.g Couve *et al.,* 2001), whilst 8-Br-cAMP is also used at much higher concentrations (Ehlers 2000). I used sub-optimal concentrations of both because I wanted to subtly increase overall PKA activity over a long time period to counteract the effect of baclofen. Treatment with PKA activators alone caused an increase in $GABA_B$ retrieved over basal levels. This demonstrates that the surface stability of the receptor is controlled as a function of PKA activity. When cultures were treated with both PKA activators and baclofen there was a significant reduction in baclofen-induced degradation (baclofen: 65.1 ±3.5%, baclofen + fsk/Br-cAMP: 95.0 ±8.8%, $n = 4$, $p < 0.05$ (fig. 16)) such that degradation was returned to near basal levels.

Another method of increasing PKA activity is to activate endogenous pathways within the neurones. One such pathway is that through β-adrenoceptors which couple to the G_s class of G-protein, stimulating the activity of adenylyl cyclase and so increasing cAMP accumulation and PKA activity. To activate this pathway I co-treated cultures with isoproterenol (10μM), a non-selective β-adrenergic agonist and baclofen. When cultures were treated with both there was a small but highly significant reduction in the degradation of $GABA_B$, with more biotinylated R1 retrieved from cultures that had been treated with isoproterenol and baclofen than baclofen alone (baclofen: 65.1 ±3.5%, baclofen + isoproterenol: 83.2 ±4.6%, n= 7, $p< 0.01$(fig. 16)). This demonstrates that activation of endogenous systems can influence $GABA_B$ surface stability through receptor cross-talk.

3.3.6 Protection from degradation correlates with increased $GABA_{B(2)}$ ser892 phophorylation

As mentioned earlier, $GABA_{B(2)}$ forms a substrate for PKA where it is basally phosphorylated at residue ser892. This phosphorylation has been demonstrated to delay the onset of desensitisation of $GABA_B$ elicited inwardly rectifying potassium current (Couve *et al.,* 2001). Chronic baclofen leads to a decrease in cAMP accumulation by the negative regulation of adenylyl cyclase by $GABA_B$ receptors. This in turn leads to a reduction in PKA activity and therefore a diminishment of the phosphorylation at ser892; and so increased desensitisation. I noted that stimulators of PKA activity could reduce the baclofen-enhanced degradation. I was interested to see whether this reduction in degradation correlated with protection from the baclofen-induced dephosphorylation at ser892. To investigate this I treated cultures of cortical neurones with 100μM baclofen, or with 100μM baclofen + PKA activators as before. After treatment, crude lysates of these cultures were prepared for western blotting where I probed for phosphorylated $GABA_{B(2)}$ by using an antibody raised to a peptide designed to mimic the phospho-892 epitope within $GABA_{B(2)}$ (Couve *et al.,* 2001). Lysates were also probed for $GABA_{B(2)}$ alone so that the signal from the phospho-specific antibody could be normalised to total $GABA_{B(2)}$ levels. The PKA activators had a significant effect upon the phosphorylation of $GABA_{B(2)}$, and there was a 30.0±2.3% increase compared to cultures treated with baclofen alone (fig 16b).

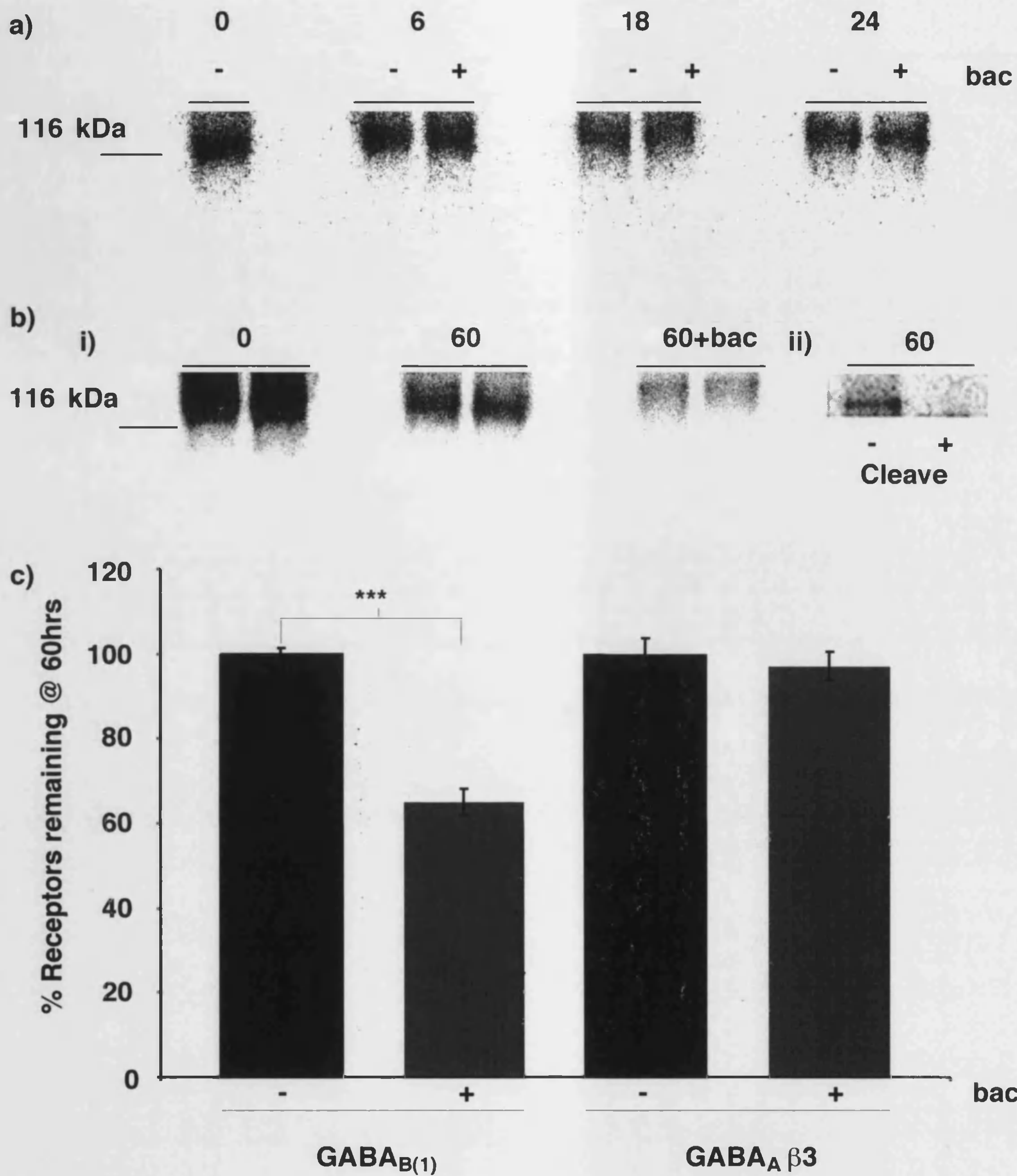
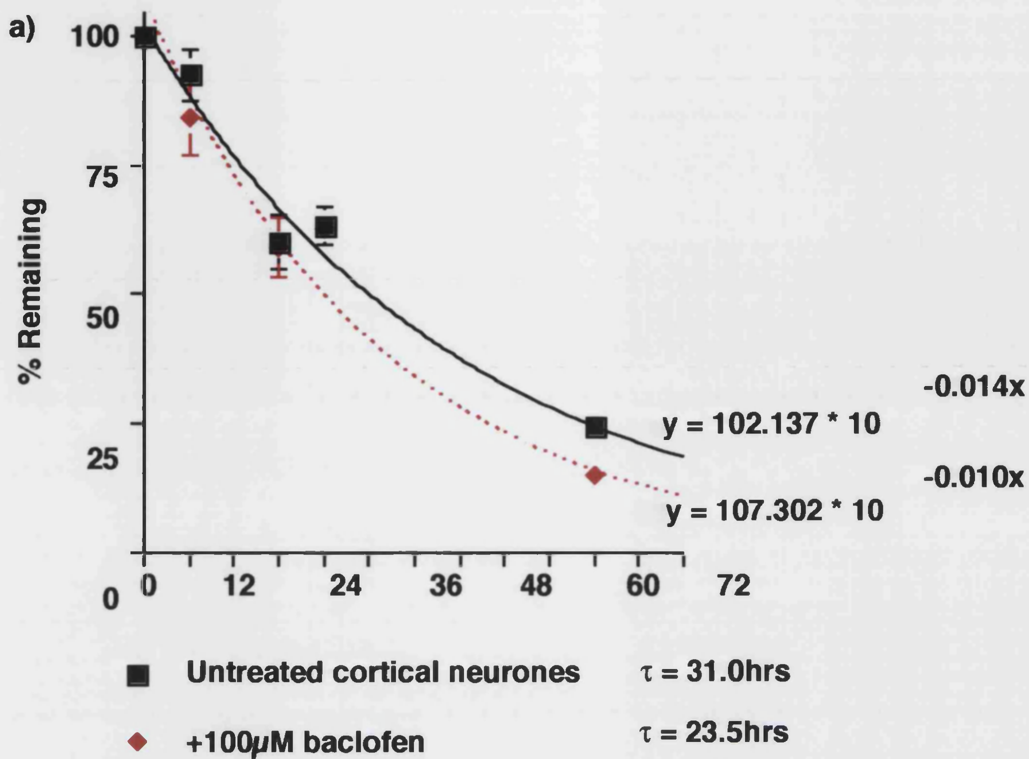


Figure 12 Baclofen specifically enhances surface GABA$_B$ removal

Cortical neurones were surface biotinylated and then incubated for varying times with or without 100μM baclofen. Neurones were then solubilised and biotinylated proteins were purified on streptavidin beads. Samples were resolved using SDS-PAGE and immunoblotted for GABA$_{B(1a)}$ and GABA$_A$β3. Baclofen had little effect upon the amount of biotinylated GABA$_B$ retrieved up to 24h of treatment (a), but 60h treatment caused a significant reduction in retrieval of GABA$_{B(1a)}$ (b i). The use of cleavable biotin suggested that all biotinylated GABA$_{B(1a)}$ retrieved at this time was at the surface (b ii). Data from multiple experiments show that at 60h treatment causes a significant reduction in the amount of biotinylated GABA$_{B(1a)}$ retrieved (basal: 24.33 ± 0.4% n=7, baclofen: 14.75 ± 0.5%, n=7, $p<0.0001$), but no effect on the amount of GABA$_A$β3 (c).



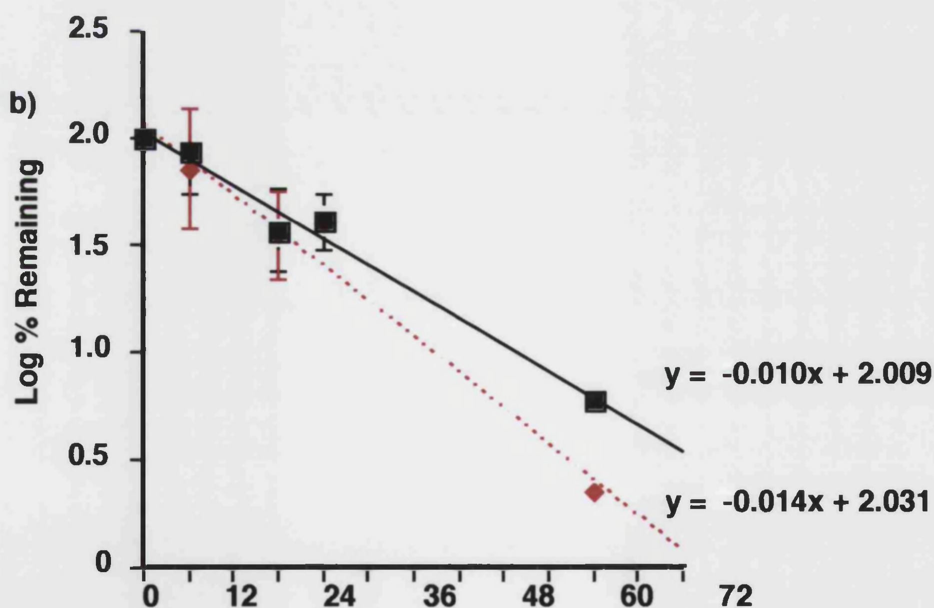


Figure 13 Exponential decay of surface $GABA_B$ receptors

Plotting results from multiple experiments demonstrates that biotinylated surface receptor decreases exponentially with a half life of 31.0h, and this is reduced to 23.5h in presence of 100μM baclofen.

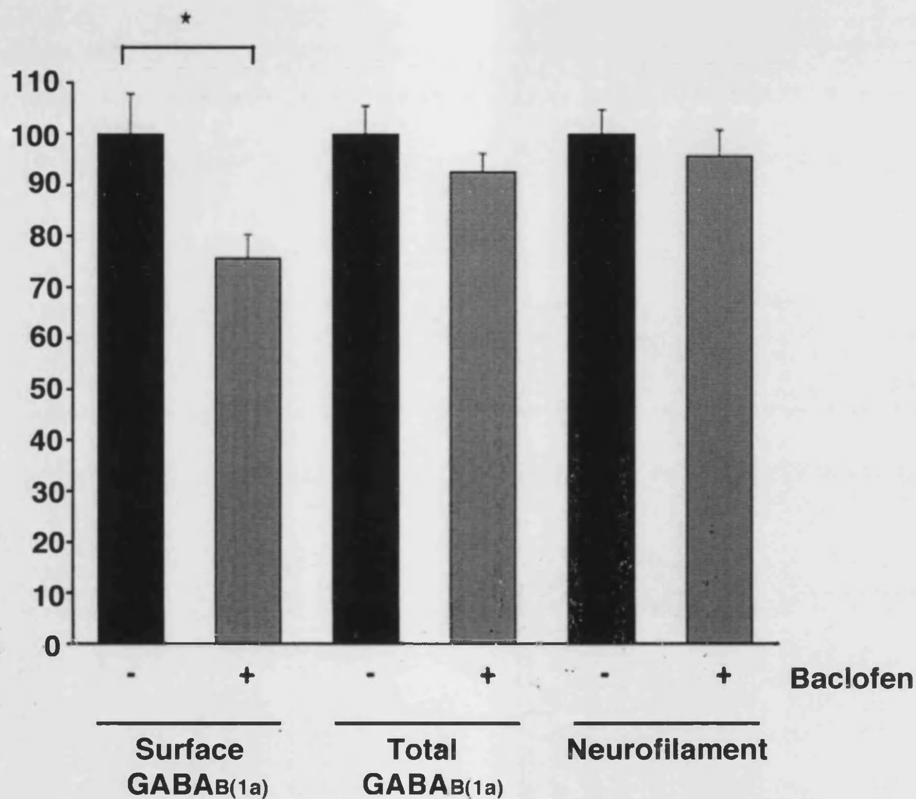


Figure 14 Chronic baclofen reduces surface but not total $GABA_B$ receptor numbers

Total surface numbers of $GABA_B$ receptor in primary cultures of cortical neurons after prolonged agonist stimulation. 5 DIV cortical neurons were left untreated (-, black bars) or exposed to 100 μM baclofen (+, grey bars) for 60 h at 37°C. After agonist treatment neurones were labelled with 1 mg/ml biotin. Lysates were precipitated with streptavidin beads and samples separated by SDS-PAGE. Immunoblots of the biotin and total fractions were performed with anti-$GABA_{B(1a)}$ antibodies, anti-neurofilament and [^{125}I]-conjugated secondary anti-rabbit antibodies. The immunoblots were exposed to phosphorimager and quantified. The data from multiple independent experiments are expressed as % control ± s.e.m. $p= <0.05$

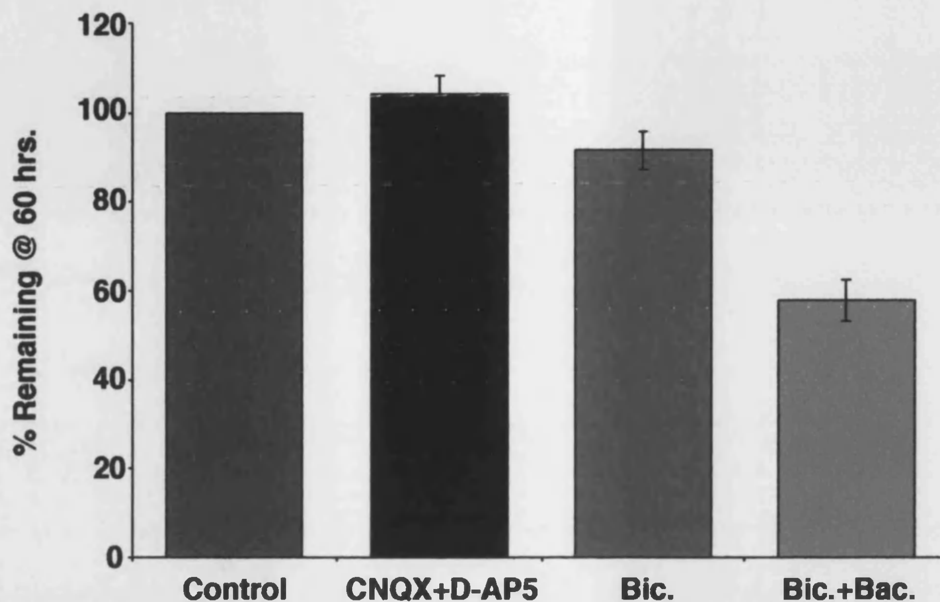


Figure 15 **Effect of activators and inhibitors of synaptic activity upon $GABA_B$ receptor degradation**

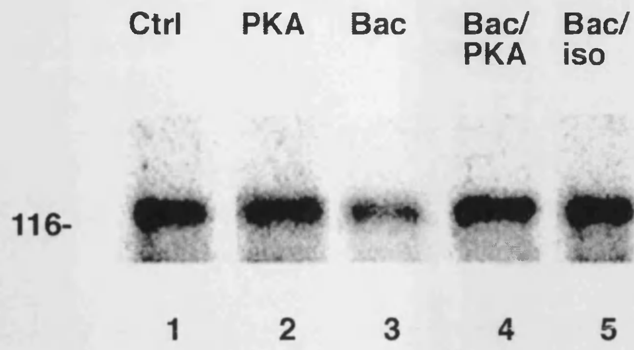
Cortical neurones were surface biotinylated and then incubated for 60h in media alone, or with inhibitors of neuronal activity- either 10μM CNQX + 50μM D-AP5, or activators- 40μM bicuculline. Plates were also incubated with 40μM bicuculline + 100μM baclofen. Neurones were then solubilised and biotinylated proteins were purified on streptavidin beads. Samples were resolved using SDS-PAGE and immunoblotted for $GABA_{B(1a)}$. Both inhibitors (CNQX + D-AP5) and activators (bicuculline) of neuronal activity had no effect upon amount of $GABA_B$ receptor retrieved at 60h. However, the addition of baclofen to bicuculline caused a similar enhancement of degradation as seen previously.

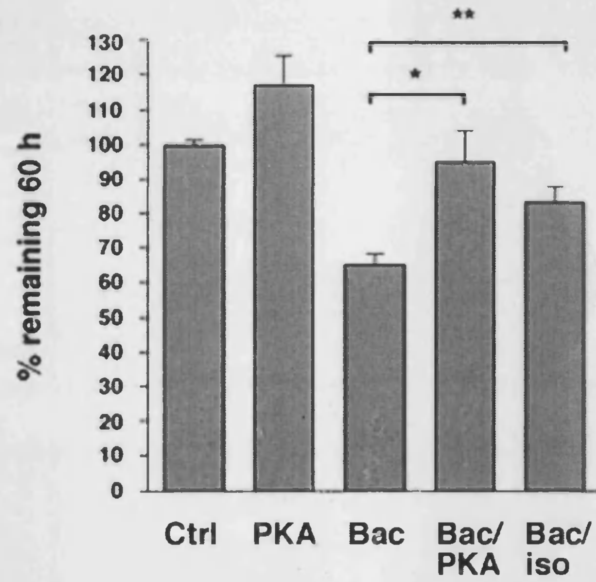
Figure 16 Protection of $GABA_B$ receptor stability in primary cultures of cortical neurons by PKA activation.

a) 5 DIV rat cortical neurons were biotinylated and left untreated (lane 1) or treated with 500 nM forskolin/10μM Br-cAMP (lane 2), 100 μM baclofen (lane 3), 100 μM baclofen + 500 nM forskolin/10μM Br-cAMP (lane 4) or with 100 μM baclofen + 500 nM isoproterenol (lane 5) for 60 h at 37°C. Lysates were precipitated with streptavidin beads and samples separated by SDS-PAGE. Immunoblots were performed with anti-$GABA_{B(1)}$ antibodies and [^{125}I]-conjugated anti-rabbit secondary antibody. Blots were exposed to a phosphorimager.

b) Data collected from multiple experiments was quantified and normalized to surface anti-$GABA_{B(1a)}$ in control neurons. The data is expressed as % control ± s.e.m.

c) 5 DIV rat cortical neurons were treated with 100 μM baclofen (-, black bars) or with 100 μM baclofen + 500 nM forskolin/10 μM Br-cAMP (+, grey bars) for 30 or 60 h at 37°C. Lysates were separated by SDS-PAGE and immunobloted with anti-$GABA_{B(2)}$ antibodies (R2) or with phosphorylation site-specific antibodies (P-Ser892) and [^{125}I]-conjugated secondary. Blots were exposed to a phosphorimager and quantified. Data collected from multiple experiments was averaged and expressed as % control (baclofen treatment) ± s.e.m.





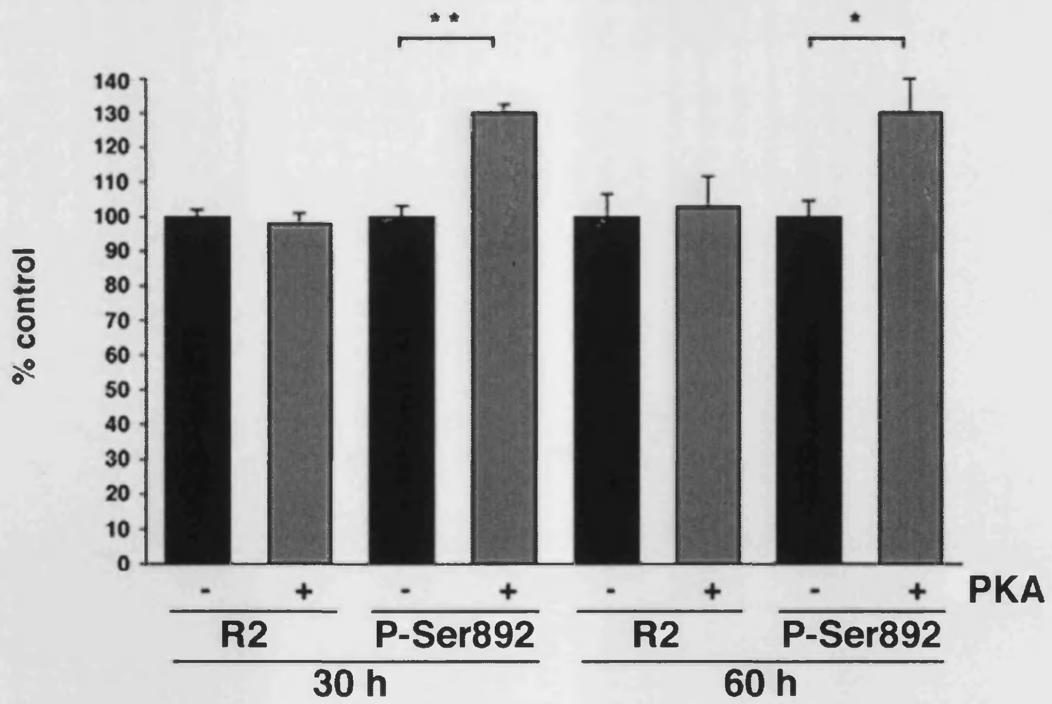


Figure 16

3.4 Discussion

The regulation of cell surface $GABA_B$ receptors is poorly understood at present. The data in this chapter suggests that the cell surface stability of $GABA_B$ receptors is unlikely to be regulated by either GRKs or the arrestin family. Agonist does not promote any GRK mediated phosphorylation of $GABA_B$ receptors, nor interaction between $GABA_B$ receptors and arrestins. Nevertheless, a role for GRKs in the regulation of $GABA_B$ receptor activity cannot be conclusively negated. This is because this study focussed upon the cell surface stability of $GABA_B$ receptors and not the coupling of receptors to G-proteins; and so it is possible that receptors are more rapidly desensitised in conjunction with overexpression of GRKs. During the writing of this thesis a study was published from an independent group containing similar initial observations to those made here (Perroy *et al.,* 2003). By using FRET this group demonstrated that $GABA_B$ receptors do not interact with arrestin 3 and do not internalise in HEK cells. Perroy *et al.,* observed a lack of desensitisation of $GABA_B$ receptors in HEK cells, correlating the surface stability with activity. However, desensitisation was observed in cerebellar granule cells though which, unlike HEK cells, express GRK4. Co-expression of GRK4 and $GABA_B$ receptors in HEK cells led to $GABA_B$ receptor desensitisation. Perroy *et al.,* showed that this GRK induced desensitisation was independent of phosphorylation but relied upon the presence of the RGS domains of GRK4. Although novel with respect to $GABA_B$ receptors, there is precedence for a role of GRKs in desensitisation not involving phosphorylation as observed in the desensitisation of follicle stimulating hormone receptors (Reiter *et al.,* 2001) and mGluR1 (Dhami *et al.,* 2002).

All GRKs possess RGS domains with very similar homology to one another (Pitcher *et al.,* 1998). Whether the RGS domains of other GRKs could mediate the $GABA_B$ receptor desensitising effects of the RGS domain of GRK4 has not been ascertained. Indeed, it has been shown that for mGluR1 the RGS domains of both GRK2 and GRK5 can mediate desensitisation. Crucially, the expression profiles of GRK4 do not match those of $GABA_B$ receptors. I did not analyse GRK4 mediated phosphorylation of $GABA_B$ receptors because initial reports could demonstrate only significant expression of GRK4 in the testes (Ambrose *et al.,* 1992; Premont *et al.,* 1996). A more recent study using PCR showed expression of mRNA transcript in

cerebellar granule cells (Sallese *et al.,* 2000), but importantly demonstrated the lack of transcript in many other regions of the brain. Comparative sequence analysis of GRK4 shows a high degree of interspecies variation. Only 72% of GRK4 amino acids are identical between mouse and human and 90% between rat and mouse; whereas GRK2 and GRK6 show 96% identity between mouse and human, and GRK5 94% identity between mouse and human (Premont *et al.,* 1999). This large amount of variation indicates a lack of selective pressure that would otherwise prevent non-specific, function interfering changes in the sequence over time. The differences between species in sequence means the importance of GRK4 is questionable, as is whether GRK4 serves the same function in rat as in human. I think it is therefore unlikely that GRK4 is the major determinant of $GABA_B$ receptor desensitisation in the cortex, hippocampus, striatum etc.

The scale of $GABA_B$ desensitisation in neurones is an unresolved issue. Earlier reports suggest that presynaptic $GABA_B$ receptor mediated effects do not desensitise (Mott & Lewis 1994). A more recent study demonstrates that postsynaptic $GABA_B$ responses decrease by ~50% after chronic incubation of neurones with 50µM baclofen for 2h, whereas presynaptic $GABA_B$ receptors responses are unaffected until the duration of treatment is greater than 48h (Wetherington & Lambert 2002). Repetitive baclofen can also greatly diminish $GABA_B$ receptor coupling to potassium channels within 1h (Couve *et al.,* 2001), which is a postsynaptic response (Lüscher *et al.,* 1997). Perroy *et al.,* measured the ability of baclofen to stimulate GTP exchange, an indicator of G-protein coupling, and found that after 1h incubation with baclofen there was a 67.2 ± 4.8% reduction in coupling in cerebellar granule cells (Perroy *et al.,* 2003). It should be noted however that to trigger this reduction neurones were pre-treated with 1mM baclofen, a concentration more than an order of magnitude greater than that needed to elicit maximal $GABA_B$ responses.

The majority of studies of GRK and arrestin activity have assessed their activity against receptors at a monomeric level. It is possible that the formation of a heterodimer confers insensitivity to GRK phosphorylation and arrestin action upon $GABA_B$ receptors. Indeed, the intracellular domains of heterodimerised receptors probably form a markedly different topology to monomeric receptors. In this respect,

the fate of other GPCRs that form functional heterodimers remains to be studied in detail.

The study presented in this chapter clearly differentiates $GABA_B$ receptors from other receptor subtypes in terms of the regulation of surface levels. The lack of agonist-induced internalisation is a remarkable feature of $GABA_B$ receptors and may have implications for their physiological role, being not confined to heterologously expressed receptors but also observed in endogenous receptors. This is of greater significance because in neurones other proteins that control $GABA_B$ surface stability are also likely to be expressed. The calculated surface half-life of receptors in cultured neurones was ≈31.0 h. Because after 60h an internal receptor pool was not detected, it suggests that $GABA_B$ receptors are not recycling during this period. This inherent stability may be a product of the extrasynaptic localisation of $GABA_B$ receptors (Fritschy *et al.,* 1999, Kulik *et al.,* 2002). Wetherington & Lambert demonstrated that the activity of pre-synaptic $GABA_B$ receptors remains unaffected after up to 48h of treatment (Wetherington & Lambert 2002). Only when neurones are treated for 72 hours or longer with baclofen is an apparent change in $GABA_B$ response noted. Although the chronic treatment of neurones with baclofen alters the half-life of $GABA_B$ receptors in my study, it is apparent that this decrease in half -life will not greatly effect total receptor number up to 48h after treatment. It may be that physiologically recordable desensitisation is not appreciable until 72h after initialisation of treatment, because it takes this long to change surface receptor numbers, again illustrating the lack of dynamism within the $GABA_B$ receptor. It is also a possibility that it requires prolonged agonist treatment to 'switch on' a special degradation pathway for $GABA_B$ receptors. The biotinylation of neurones pre-treated with 60h baclofen does demonstrate a reduction in surface $GABA_B$ receptor numbers, supporting the data from Wetherington & Lambert. This effect was only observed at the surface though, with the intracellular pool of $GABA_B$ receptors showing no significant reduction. This demonstrates that as well as being resistant to endocytosis, it appears that $GABA_B$ receptors do not undergo downregulation, at least over a 60h treatment.

Endocytosis plays a role in decreasing the response of a target tissue to agonist and refining a tissues response over time. However, most GPCRs that endocytose

are not basally exposed to agonist, instead agonist is released intermittently. The β3 adrenergic receptor is important in the regulation of thermogenesis and as such needs to be basally and continually activated. Interestingly the β3 adrenergic receptor is not phosphorylated or internalised in response to agonist (Liggett *et al.,* 1993). One can draw parallels between the importance of the basal activity of the β3 receptor and that of the $GABA_B$ receptor. $GABA_B$ receptors are tonically activated by GABA spillover (Scanziani 2000) having an important inhibitory effect on neuronal firing patterns. Maximal $GABA_B$ activation is likely to occur under conditions where GABAergic neurones are firing at high rates altering the GABA release to uptake ratio and enhancing spillover. It is precisely under these sort of conditions that $GABA_B$ receptor activity needs to be maximal. At GABAergic synapses it will limit further GABA release, whilst at excitatory synapses it will increase the threshold for neuronal firing. One can envisage that if the receptor were to endocytose under these conditions then it may have dramatic and deleterious effects on neuronal firing.

As mentioned in the introduction to this thesis, intrathecal administration of baclofen is used to treat intractable spasticity (Meyerthaler 1999). Using this treatment regime a regular bolus of baclofen supplied directly into the spinal cord can alleviate spasticity. Interestingly, a fixed dose of baclofen can be used for long periods (years) without seeing a diminshment in therapeutic properties. This contrasts with other classes of drugs acting at GPCRs e.g. opiates, where an escalation of dose to achieve the same results is often inescapable. The intrinsic stability and lack of downregulation of $GABA_B$ receptors observed in this study may also help to explain how this is possible

This study also suggests that $GABA_B$ receptors are degraded in a membrane-delimited manner, or at a rate that is equal to their internalisation. This is because an internalised pool of receptors was not observed. The endocytic pathway leads directly from early endosomes to late endosomes and then lysosomes. In a set of further experiments designed to examine the effect of inhibiting the lysosome on $GABA_B$ degradation rates I observed no effect of the lysosomal inhibitor leupeptin upon the degradation of surface biotinylated receptor in the presence or absence of agonist over a 25h period (basal plus leupeptin: 96.3 ± 7.8% control, n=3; baclofen plus leupeptin: 95.5 ± 8.5% baclofen, n=3). This suggests that degradation takes

place in a non-lysosomal manner. The presence of either $GABA_B$ receptor subunit in compartments staining for EEA1 was never observed in confocal studies regardless of agonist exposure time. Both of these observations imply that direct surface regulation of $GABA_B$ receptors does not involve the endocytic pathway. It is possible that $GABA_B$ receptors are targeted directly to the proteasome from the surface, preventing the build-up of an internalised pool. However, β2 adrenoceptors are also subject to non-endocytic proteolysis that is insensitive to proteasomal inhibitors (Jockers *et al.,* 1999) and the vasopressin receptor can be degraded in a membrane-delimited manner via metalloproteases (Kojro & Fahrenholz 1995). Therefore other uncharacterised pathways for GPCR degradation exist through which $GABA_B$ receptors may be processed.

Lastly, it was observed that activating PKA directly or through cAMP producing pathways protected $GABA_B$ receptors from degradation. It is tempting to speculate that the degradation of $GABA_B$ receptors and their stabilisation depends solely on the cAMP levels in neurons. These results support this model with agonist occupancy lowering cellular cAMP, resulting in enhanced degradation of the surface population of receptors. Interestingly, $GABA_{B(2)}$ displays a high basal phosphorylation in its single phosphorylated serine residue that is a PKA substrate (Couve *et al.,* 2002). Thus, the high stoichiometry of receptor phosphorylation provides an explanation for the stability of the resting population of receptors in cortical neurons. My results show that phosphorylation at this residue is correlated with protection from degradation, highlighting the importance of this residue in $GABA_B$ function. Whether or not specific proteins associate with the receptor in a phosphorylation dependent manner and alter the receptor stability remains to be determined. A number of associated partners of the $GABA_B$ receptors have been described recently but their role in $GABA_B$ receptor function remain, for the most part, unexplored (Couve *et al.,* 2001; El Far & Betz, 2002; Nehring *et al.,* 2000; Vernon *et al.,* 2001; White *et al.,* 2000).

CHAPTER FOUR GABA$_{B(1)}$ Phosphorylation

4.1.1 Introduction

Whole cell labelling assays performed to analyse GABA$_B$ phosphorylation demonstrated that both GABA$_{B(1)}$ and GABA$_{B(2)}$ are phosphoproteins. As covered in the previous chapter, phosphorylation of GABA$_{B(2)}$ is largely attributable to protein kinase A activity at serine 892 (Couve *et al.,* 2002). However, the sites of phosphorylation within GABA$_{B(1)}$ and the kinases responsible for this phosphorylation remain unknown.

Utilising GST fusion proteins of the carboxy-terminal domain of GABA$_{B(1)}$ it was possible to demonstrate that an unknown kinase associated with and phosphorylated this domain from brain lysates. The use of different kinase inhibitors revealed that this unknown kinase did not fit the pharmacological profile of well-characterised second-messenger kinases. Particularly intriguing was the observation that heparin, normally a kinase inhibitor, robustly stimulated kinase activity.

I proposed to identify this unknown kinase by mapping the minimal region required for binding and phosphorylation. I then intended to use a truncated fusion protein encoding this region to affinity purify enough kinase for identification through mass spectrometry or Edman degradation techniques. However, prior to me carrying this approach out the α1 catalytic subunit of AMP activated protein kinase (AMPK) was isolated as an interacting protein with the GABA$_{B(1)}$ tail in yeast two-hybrid studies at GSK, Harlow. Subsequent to this I focussed on determining whether AMPK can phosphorylate GABA$_B$ receptors and the sites involved in phosphorylation.

4.1.2 5'AMP activated protein kinase: a regulator of metabolic activity

5'AMP activated protein kinase was initially identified as a kinase able to co-purify with and phosphorylate the cholesterol synthesising enzyme HMG-CoA reductase, which lead to it being called HMG-CoA reductase kinase (HRK). Phosphorylation reduced the activity of HMG-CoA reductase leading to a cessation in cholesterol synthesis. In terms of metabolism this can be envisaged as the switching off of an anabolic pathway so as to conserve energy. It was subsequently discovered that HRK phosphorylated a number of other substrates and that its activity was potently

stimulated by the presence of 5'AMP. This lead to the kinase being fittingly renamed 5'AMP activated protein kinase (AMPK) (Hardie *et al.,* 1998).

ATP hydrolysis leads to the formation of inorganic phosphate ions and ADP. ADP rarely accumulates though because it is rapidly converted back to ATP under the action of adenylate kinase. This reaction also produces AMP which is formed in a 1:1 ratio with every molecule of ATP. In the cell the ratio of ATP:AMP is normally ~100:1 (Hardie *et al.,* 1998), but in metabolic stress ATP levels fall and ADP levels rise which leads to dramatic changes in the ratio of ATP:AMP (Hardie *et al.,* 1998). AMPK is exquisitely sensitive to such changes because it is independently influenced by both falling ATP levels and rising AMP levels. Activation of AMPK depends upon the direct allosteric modulation of the kinase by AMP binding (Carling *et al.,* 1989) and phosphorylation of the kinase by an upstream AMPK kinase (AMPKK) which is also activated by AMP (Weekes *et al.,* 1994). ATP is thought to compete for the AMP binding site within AMPK and when bound leaves the kinase in an inactive state (Hardie *et al.,* 1998). This means that when cellular ATP levels are normal AMPK is kept in an inactive state, but perturbations in the ratio between ATP and AMP can trigger rapid activation. Protein phosphatase dephosphorylation of AMPK leads to inactivation, however when AMP levels are continually raised AMPK remains AMP bound and AMPK dephosphorylation is prevented (Davies *et al.,* 1995).

The main effect of AMPK activation within the cellular environment is to conserve energy (Hardie *et al.,* 1998). AMPK does this by 'switching off' anabolic biosynthetic processes that consume ATP and 'switching on' ATP producing catabolic pathways. A well studied example is the activity of AMPK against Acetyl-CoA carboxylase, where phosphorylation by AMPK on multiple residues within Acetyl-CoA carboxylase inhibits fatty acid synthesis and stimulates fatty acid oxidation (Hardie *et al.,* 1998). In the heart AMPK has been shown to be important in initialising the Pasteur effect whereby anaerobic conditions stimulate glycolysis (Marsin *et al.,* 2000), thus protecting cardiac myocytes in times of ischaemia by producing ATP.

Biochemical purification of AMPK revealed a heterotrimeric complex with a larger α subunit of approximately 63 kDa and two smaller β and γ subunits of 38 and 35 kDa respectively. The subunits were subsequently cloned and it was demonstrated that there are 2 closely related α isoforms, two β and three γ isoforms. The α subunits

possess classical protein kinase active sites flanked by the amino acids Asp-Phe-Gly (DFG) and Ala -Pro-Glu (APE) and are responsible for the catalytic activity of the complex. The relevance of the different subunit isoforms is as yet poorly understood, although there have been reported differences in substrate preferences between the $\alpha1$ and $\alpha2$ catalytic subunits. Sequence comparisons with known yeast proteins showed a striking homology between the SNF (sucrose non-fermenting) proteins and AMPK subunits, with $\alpha1$ AMPK showing 47% sequence identity to SNF1. This demonstrates the highly conserved nature of AMPK across phyla. The α subunit is responsible for AMPK catalytic activity whilst the β subunit is believed to act as a bridging molecule between the α and γ subunits (Woods *et al.,* 1996). The precise roles of the β and γ subunits remain unclear, however they are thought to be involved in both regulating kinase activity and modifying kinase localisation.

As mentioned previously, phosphorylation of AMPK takes place upon sites in the α and β subunits by an upstream AMPKK and is important for kinase activity. One well characterised critical site which must be phosphorylated prior to activation is found within the active site of the catalytic α subunits at threonine residue 172 (Hawley *et al.,* 1996; Stein *et al.,* 2000). Antibodies that specifically recognise an epitope incorporating a phosphorylated threonine 172 are commercially available and provide a good indication of intracellular AMPK activation.

Although AMPK is expressed in neuronal tissue, relatively little is known about its activity in the brain. Reports have demonstrated that AMPK is basally active in cortical neurones and especially so in astrocytes, where it plays an important role in regulating ketogenesis (Blázquez *et al.*, 1999). Ligation of the coronary arteries mimics a heart attack by causing myocardial ischaemia, with an increase in heart AMP levels coupled with ATP depletion activating AMPK (Kudo *et al.*, 1995). In the brain the closest analogy to such an event is a 'stroke' whereby occlusion of an artery supplying the brain leads to ischaemia. Similar to in the heart, it is likely that such an event will cause rapid AMPK activation. AMPK is also activated by non-pathological events in tissues that have variable demands in energy consumption. The most obvious example of such a tissue is skeletal muscle which can remain unused for long durations and then subjected to intense activity. Indeed, studies have demonstrated that AMPK is significantly activated during muscle contraction (Hutber

et al., 1997). Transgenic mice expressing a dominant negative AMPK construct specifically in muscle tissue have a 'lazy' phenotype and are reluctant to exercise, demonstrating a physiological role for AMPK in muscle contraction outside of pathological events (Mu *et al.,* 2001). Neuronal tissue is also subject to extreme and rapid changes in activity patterns. LTP formation is thought to involve repetitive neuronal firing so as to depolarise postsynaptic neurones enough to release the Mg^{2+} block on NMDA receptors. These activity patterns may cause disturbances in the intracellular concentrations of Na^+ and K^+ ions, which will lead to increased Na^+/K^+ ATPase activity in attempt to rectify the perturbance. It is probable that increased Na^+/K^+ ATPase activity will also lead to transiently raised AMP levels that may activate AMPK. Pathologically it is likely that AMPK is even more readily activated in neural tissue than other tissues such as liver and muscle because of the inherently high metabolic demands of neurones coupled to their inability to store glucose in the form of glycogen.

4.2.1 $GABA_{B(1)}$ carboxy-terminal domain forms a major kinase substrate

GPCR's are phosphorylated at residues along the intracellular loops and the intracellular carboxy-terminal domain. Because family C GPCRs characteristically have relatively small intracellular loops, the carboxy-terminal domains are the most probable sites for phosphorylation. Sequence analysis of $GABA_{B(1)}$ shows a total of 26 serines and threonines within the putative intracellular loops and carboxy-terminal. Of these, 17 are found within the carboxy-terminal domain, making this the most likely site for phosphorylation. To investigate this further I labelled plates of COS-7 cells transfected with a truncated 'tail-less' myc-tagged $GABA_{B(1)}$ (C^-) or full length myc-tagged $GABA_{B(1)}$ with either ^{35}S labelled methionine or ^{32}P orthophosphate. Subunits were immunoprecipitated and resolved using 8% SDS-PAGE with gels being dried and exposed to autoradiograph. Expression levels of ^{35}S labelled $GABA_{B(1)}$ C^- were several fold higher than wild type $GABA_{B(1)}$ (fig. 17a). This is probably because deletion of the carboxy-terminal domain results in the removal of the main 'RSRR' ER-retention motif at amino acids 922-925 (Margeta-Mitrovic *et al.,* 2000; Pagano *et al.,* 2001). Without this motif the $GABA_{B(1)}$ subunit is no longer retained within the ER when expressed alone, thus preventing ER accumulation which is probably a limiting factor on synthesis. When proteins are labelled with ^{32}P however, the full length $GABA_{B(1)}$ subunit gives a much stronger signal, even though there is far less expressed (fig. 17b). This supports the supposition that the carboxy-terminal domain is the most highly phosphorylated region of the receptor.

4.2.2 A kinase associates with and phosphorylates $GABA_{B(1)}$ carboxy-terminal domain from brain lysates

In an attempt to isolate kinases that interact with the carboxy-terminal domain of $GABA_{B(1)}$ a GST-fusion protein of this domain was prepared (CR1). This was added as an eluted protein to lysates produced from rat brain homogenised in 'pull-down' buffer. 25 μg of fusion protein was incubated with 1ml of lysate (5 mg/ml) for 90 minutes with rotation at 4°C. A 50:50 slurry of glutathione agarose beads was then added for 30 minutes to bind the fusion proteins. This was then washed extensively for 30 minutes in low (150mM) and high salt (350mM) containing pull-down buffer. The beads were washed a further 5 times in kinase buffer to remove all traces of

detergent and salt. Beads were then added to pre-warmed kinase buffer containing 200μM cold ATP, spiked with 5μCi ^{32}PγATP. Using this protocol a kinase was observed to specifically associate with and phosphorylate CR1 (fig. 18a). The kinase did not interact with GST alone, and importantly no kinase activity was registered if CR1 had not been pre-exposed to brain lysates. This ruled out the possibility that the associating kinase was bacterially derived and interacted during purification of the fusion protein.

In an attempt to identify the kinase the same experiment was repeated but to the kinase buffer inhibitors of either PKA (PKA peptide), PKC (PKC inhibitor peptide) or CamKII (KN-93) were added at concentrations in excess of the IC_{50}. As an additional controls GTP and heparin (7μM) were also included. Heparin is a poly-anionic amino-glycan, synthesised in the liver and certain white blood cells. In addition to being a powerful anti-coagulant, heparin is a non-specific kinase inhibitor. GTP (2mM) was used because certain kinases such as casein kinase, are able to use GTP as a phosphate donor molecule in preference for ATP. The added GTP was not radioactively labelled and so if used as a phospho-donor it would appear to inhibit kinase activity. Inhibitors of PKA, CamKII and casein kinase had no effect on kinase activity and neither did GTP (fig. 18b). A small reduction in phosphorylation was noted when PKC inhibitors were used, but because this was used at a concentration in excess of predicted IC_{100}, it was likely to be non-specific inhibition. Intriguingly, heparin caused a strong and reproducible stimulation of kinase activity. Searches of scientific literature revealed that only two identified kinases have been documented to be stimulated by heparin. These are GRK 6 (Hall *et al.,* 1999) and heparan binding FGF receptor. The associated kinase was unlikely to be GRK 6 because previous studies (see Chapter 3) had indicated that $GABA_B$ receptors do not form GRK substrates. Heparan binding FGF receptors are tyrosine kinase receptors (Pellegrini 2001). Interestingly, $GABA_{B(1)}$ possesses a single tyrosine residue in its intracellular domain (Y959) which follows a dileucine motif and is the penultimate amino acid within $GABA_{B(1)}$. Phosphorylation of this residue might be expected to have major effects upon protein-protein interactions. A phospho-amino acid analysis was performed to identify the amino acids that are phosphorylated by hydrolysing the phosphorylated fusion protein and then separating the hydrolysed amino acids using

thin layer chromatography. These were run alongside phospho-threonine, phospho-serine and phospho-tyrosine control samples. Exposure of the chromatography plate to autoradiograph demonstrated which amino acid had incorporated the labelled phosphate group. This indicated that the associating kinase was phosphorylating exclusively serine residues (fig. 18c).

4.2.3 Sites of binding and phosphorylation map to amino acids 905-925

To further map the site of kinase binding and also narrow down possible sites of phosphorylation a similar pull-down with subsequent *in vitro* kinase assay was performed using fusion proteins comprising truncated regions of the $GABA_{B(1)}$ intracellular domain. The carboxy-terminal intracellular domain of $GABA_{B(1)}$ extends from the predicted end of the last transmembrane domain at amino acids 857 to the terminal amino acid 960. The putative coiled coil region lies from amino acids 876 to 916 (Couve *et al.,* 2001). The truncated fusion proteins used included fusion proteins of each half of the tail (δ2 aa:857-908, δ3 aa:909-960) and the coiled coil region alone (δ5 aa:876-917) (Couve *et al.,* 2001). Out of seven truncations only 2 bound kinase and formed a substrate (fig 19). These were δ3 and δ7, which allowed me to infer that the minimum region required for kinase binding and phosphorylation was from amino acid 909 to 925. This region contains 3 possible sites of phosphorylation, S909, S917 and S923. Previously this region has been reported to be responsible for mediating the binding between $GABA_{B(1)}$ and 14-3-3 proteins (Couve *et al.,* 2001) and also partially overlaps the binding site of ATF4/CREB2 (Nehring *et al.,* 2000; Vernon *et al.,* 2001; White *et al.,* 2000). Interestingly when truncation δ8 was used phosphorylation was not observed, although this construct is lacking only 4 amino acids present in δ7 922-925, the ER retention motif. This implies that the phosphorylating kinase must bind to these four amino acids, and/or phosphorylate S923.

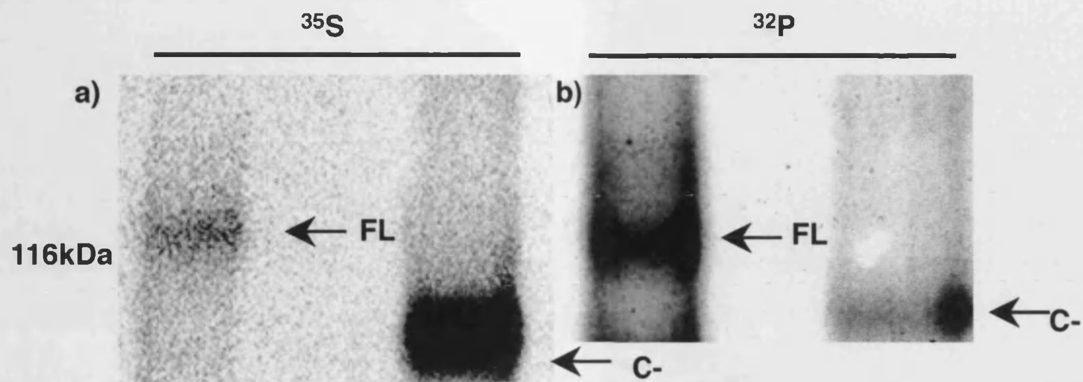


Figure 17 Removal of the carboxy-terminal domain greatly reduces $GABA_{B(1)}$ phosphorylation

a) COS-7 cells transfected with either myc-$GABA_{B(1a)}$ or a mutated version of the same construct lacking the carboxy-terminal intracellular domain were incubated in ^{35}S labelled methione containing medium for 4h and then immunoprecipitated. Upon SDS-PAGE full length myc-$GABA_{B(1a)}$ migrated just above 116kDa, whilst the trucated subunit migrated at ~105kDa. Removal of the carboxy-terminal intracelluar domain greatly enhances the amount of subunit immunoprecipitated.

b) As a), but medium labelled with ^{32}P orthophosphate instead of ^{35}S methionine. Although far higher amounts of 'tailess' receptor are precipitated, it appears that removal of the carboxy-terminal intracellular domain dramatically reduces the extent of subunit phosphorylation.

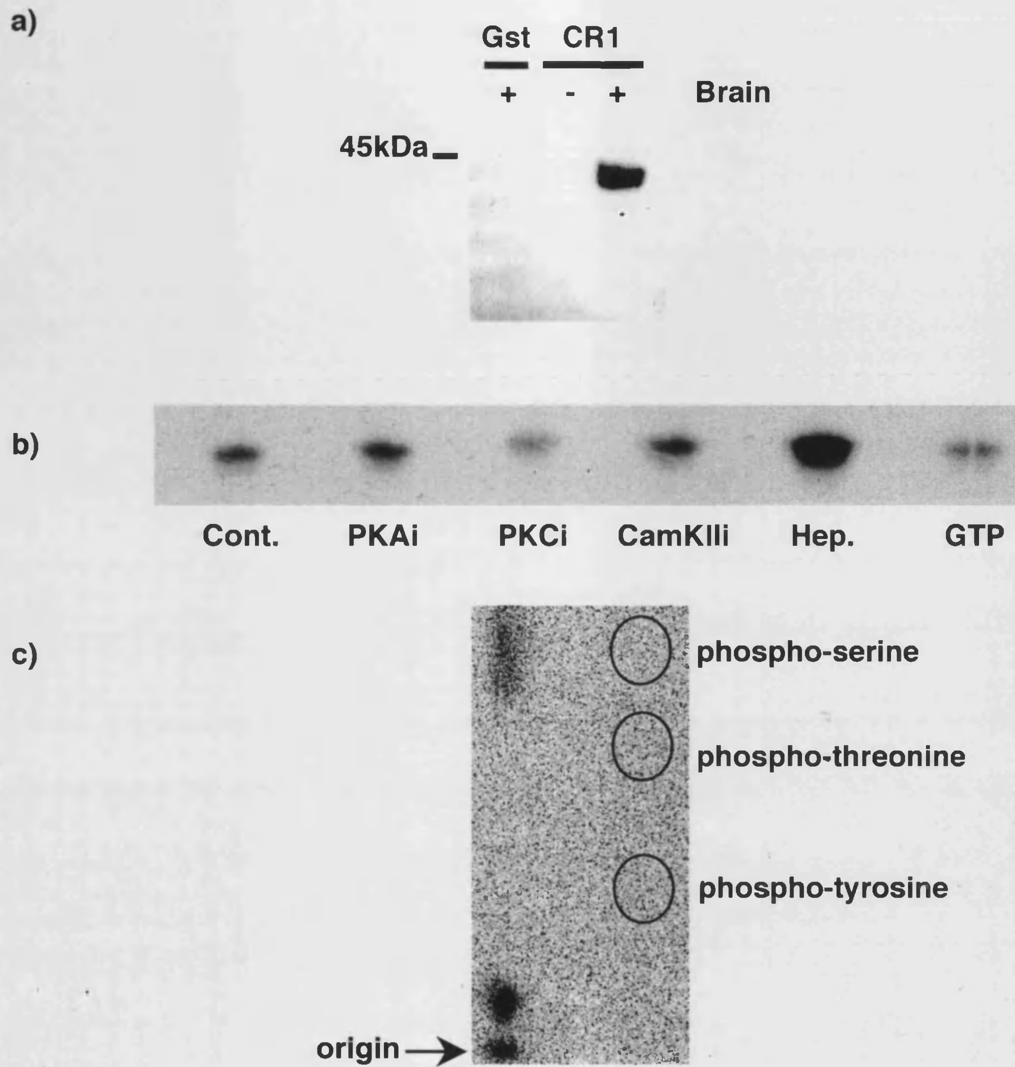


Figure 18 Association of a kinase with the $GABA_{B(1)}$ intracellular carboxy-terminal domain from brain

a) Gst-fusion proteins of the $GABA_{B(1)}$ intracellular carboxy-terminal domain (CR1) were exposed to rat brain lysate, washed extensively and then incubated in kinase buffer @ 30˚C with $^{32}P\gamma ATP$ spiked ATP. A kinase associates with and is able to phosphorylate CR1. No phosphorylation is noted of either Gst nor CR1 not previously exposed to brain.

b) CR1 exposed to brain was incubated in kinase buffer as before, but also added were inhibitors of different classical second messenger activated kinases. No significant effect was noted except for the addition of heparin.

c) A phospho-amino acid analysis was performed upon phosphorylated CR1. This demonstrated that the kinase that associates from brain phosphorylated CR1 solely at serine residues. (Signal above origin from incompletely hydrolysed amino acids)

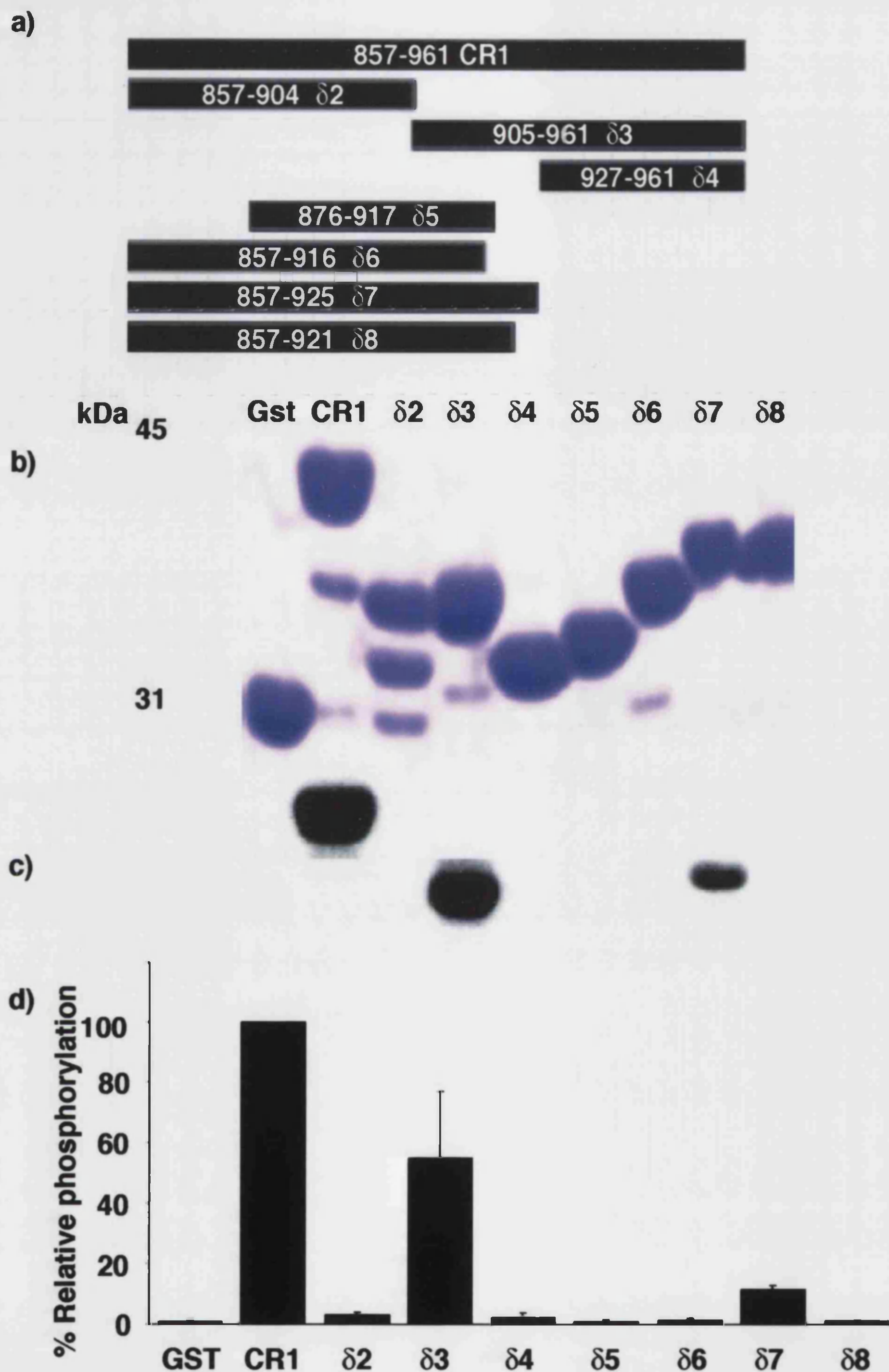


Figure 19 **Mapping the region of phosphorylation within CR1**

a) Gst fusion proteins of truncations of CR1 used to map phosphorylation sites. δ5 corresponds to the coiled coil region within the subunit.

b) Coomassie stained SDS-PAGE gel of fusion proteins.

c) Autoradiograph of SDS-PAGE resolved fusion proteins in same order as b) post-exposure to brain lysate and kinase assay. Full length CR1 is highly phosphorylated as is δ3 and to a lesser extent δ7.

d) Results from multiple experiments demonstrating that CR1,δ3 and δ7 can all associate with and be phosphorylated by kinase(s) from brain.

4.2.4 Isolation of AMPK as a potential interacting protein with $GABA_{B(1)}$

At the start of this study I set up a yeast two-hybrid (Y2H) screen using the carboxy-terminal domain of $GABA_{B(1)}$ as a bait protein. However, this was abandoned when collaborators with our group at the Neurology Centre for Excellence in Drug Discovery, GlaxoSmithkline, Harlow kindly let us have access to data obtained during screens that they had carried out. At GlaxoSmithkline Dr Katie Freeman and her group had carried out Y2H screening with the intracellular carboxy-terminal domain of $GABA_{B(1)}$ on an industrial scale, isolating ~160 potential interacting proteins. Because the aim of their screen was to discover other unidentified $GABA_B$ subunits, other hits were by-products of little interest to industry but of major interest to us. One hit of particular interest was the $\alpha 1$ catalytic subunit of AMPK. The region of interaction involved the second half of the catalytic subunit from amino acids 289-550 (fig 20). The catalytic domain within the $\alpha 1$ subunit consists of the majority of the N-terminal half, and ends around amino acid 250, just upstream of the interacting portion. It has been established that the portion of the $\alpha 1$ subunit isolated from the Y2H with $GABA_{B(1)}$ interacts with the β and γ subunits of AMPK (Woods *et al.,* 1996, Crute *et al.,* 1998); but although the precise amino acids necessary have not been mapped, the interaction is thought to be mediated by amino acids c-terminal to aa392 (Crute *et al.,* 1998).

4.2.5 AMPK robustly phosphorylates $GABA_{B(1)}$ carboxy-terminal domain

If AMPK interacted with $GABA_{B(1)}$ in yeast, it was plausible that $GABA_{B(1)}$ may form a substrate for AMPK. Because proving or disproving the veracity of yeast two-hybrid interactions can be a long and often fruitless process, I decided to primarily resolve whether AMPK could phosphorylate $GABA_B$ receptors.

In vitro kinase assays were carried out using immunopurified AMPK from rat liver (a kind gift from Dr David Carling, Imperial College London) and CR1 fusion protein. GST alone was also used in the assay to control for non-specific phosphorylation. Prior to *in vitro* kinase assays, fusion proteins were eluted and then extensively dialysed against 20mM Tris-EDTA pH 7.4 to remove all salts and impurities that could affect AMPK activity. To further control that any observed kinase activity was due to

AMPK and not non-specifically immunopurified kinases I also used pre-immune IgG that had been exposed to the same lysates from which AMPK was purified.

Using this assay I observed that CR1 formed a specific AMPK substrate (fig 21 a,b). Carrying out a similar experiment using truncated fusion proteins encoding either the first or second half of $GABA_{B(1)}$ carboxy-terminal intracellular domain (δ2 and δ3) demonstrated that, in the same manner as the kinase associating from brain, the second half of $GABA_{B(1)}$ carboxy-terminal domain formed the AMPK substrate. AMPK showed no activity towards δ2 (which includes 9 serine and threonine residues) indicating that AMPK does not just randomly phosphorylate any serine or threonine present but shows specificity to certain sites within CR1. Because the second half of CR1 was phosphorylated the sites of phosphorylation were narrowed down to between amino acids 905 and 960 (fig 21 c). The addition of heparin to the kinase assay at a concentration identical to that used in the kinase assays after exposure to brain lysates elicited a profound increase in AMPK phosphorylation of CR1 and δ3. Multiple experiments revealed that the heparin stimulation was highly reproducible and increased phosphorylation of CR1 by ~250% over 30 minutes. This increase was identical to that observed with the kinase from brain (fig. 21d). Stimulation of AMPK by heparin has not previously been documented in the literature, although one study (Ahmad *et al.,* 1985) noted the stimulation of a ~70kDa kinase purified from rabbit muscle which phosphorylated glycogen synthase and was inhibited by of glycogen, both properties of AMPK, with heparin. Experiments performed using SAMS, an AMPK substrate peptide revealed that heparin increased the activity of AMPK against this by ~30%. It therefore appears that the heparin stimulation is somewhat substrate specific. The presence of poly-anionic heparin may involve the stabilisation of CR1 into a confirmation more accessible for kinase activity, perhaps mimicking endogenous protein interactions.

To determine whether the kinase isolated from brain and AMPK phosphorylate the same residues I carried out two-dimensional peptide maps of tryptically digested, SDS-PAGE resolved CR1, phosphorylated (in the presence of radiolabelled ATP) either by the kinase associating from brain extracts or AMPK. Digested peptides were dried and then spotted onto cellulose chromatography plates where they were separated along a horizontal dimension according to their charge to mass ratio. The

second ascending dimension separated peptides according to their hydrophobicity. Chromatography plates were then dried and exposed to film, with only the radiolabelled peptides producing an image on the film. Peptide maps produced in this manner demonstrated very similar patterns produced from tryptically digested CR1 phosphorylated by AMPK or the kinase associating from brain, indicating that both kinases phosphorylated the same residues (fig. 21e).

4.2.6 Serine 917 is the primary site of AMPK activity

To gain an understanding into the possible role of $GABA_{B(1)}$ phosphorylation by AMPK it was necessary to identify the major site(s) of kinase activity within the fusion protein. To do this phosphorylated CR1 was digested with the proteases trypsin, chymotrypsin or thermolysin. Digested peptides were then separated on thin layer chromatography plates in two different dimensions according to their charge/mass ratio and also their hydrophobicity index. It was found that thermolysin gave the most efficient digestion of the phospho-protein, and when peptides were separated a single major phospho-peptide was present. This indicated that there was probably one major site of phosphorylation. I decided to mutate putative phosphorylation sites present in the second half of CR1 after amino acid 905 from serine to alanine. I then repeated the peptide mapping to see if I lost the major peptide. Using this methodology I observed that mutagenesis of the residue S917 lead to the removal of the major peptide within the map. This indicated that S917 was the major site for AMPK activity within the CR1 fusion protein. Somewhat more perplexing though was that the mutation S917A lead to only a very small decrease in CR1 phosphorylation (see **4.2.7** below). Analysis of the peptide map produced from peptides of CR1 S917A demonstrates a large number of new peptides created that don't exist, or are insignificant in maps from wild type CR1. Subsequent mutagenesis of the next serine S923 to an alanine within the CR1 S917A fusion protein to make a double mutant CR1 S917A,S923A removes the majority of these extra spots (fig. 22e). This loss is not observed if S909 is mutated in addition to S917 though (fig. 22d). The peptide maps suggest that S917 is the major site of phosphorylation within CR1, but that if this site is no longer present, 'silent' sites are revealed. The implications of this are somewhat uncertain, for CR1 S917A is an artificial protein and as such is an artificial substrate. Nonetheless, this data could also imply that if for some reason S917 is

masked by, for example, other proteins; S923 and other downstream residues to a lesser extent may serve as phosphate acceptors.

To further confirm the data obtained during from maps and mutagenesis, wild type CR1 was digested with chymotrypsin and subject to chromatography followed by Edman degradation. This experiment, conducted in conjunction with Dr D.Carling demonstrated that within wild type CR1 there is only one major site of phosphorylation and Edman degradation confirmed that this was S917 (fig 23).

4.2.7 *In vitro* phosphorylation of mutated CR1

Subsequent to experiments mapping the sites of phosphorylation within CR1, the efficiency with which AMPK could phosphorylate mutated CR1 fusion proteins was analysed. As well as the mutant fusion proteins previously used, a CR1 S917D mutant was also studied. Here the major site of phosphorylation is mutated to an aspartic acid residue, thus mimicking the negative charge conferred by the addition of a phosphate group. I hypothesised that if S917 was mutated in such a manner as to give the appearance of a phosphorylated residue, the kinase activity observed against a S917A mutant would no longer occur. All kinase reactions took place in the presence of heparin (7μM). In the control assay using IgG no phosphorylation of CR1 was registered (fig 21c). As observed previously, there was robust phosphorylation of CR1, and this was decreased to ~30% of wild type when S917 was substituted for either an alanine or an aspartic acid residue (fig 24). If S917 was mutated to an alanine in conjunction with mutation of S909 then there was no further decrease in total phosphorylation indicative that S909 was not a site of AMPK activity. Mutation of S923 in conjunction with S917 though significantly decreased phosphorylation. This observation in combination with the results from the peptide maps confirms that whilst S917 is indeed the major site of AMPK phosphorylation within CR1, S923 may form a secondary site or be of importance for interaction with AMPK. When both S917 and S923 are mutated to alanines there remains residual phosphorylation within CR1. The significance of this remaining phosphorylation is questionable because the rate of phosphorylation is very low and the peptide maps from this double mutant show no well defined peptides (fig. 22e). A stop codon at amino acid 929 prevents further phosphorylation of S917,923A mutant showing any residual phosphorylation takes place at downstream sites.

a)

	Control	α1 AMPK
Control	---	---
CR1	---	+++
CR2	---	---

b)

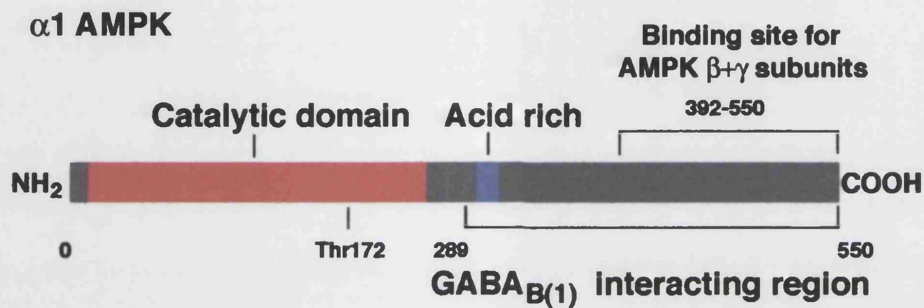


Figure 20 $GABA_{B(1)}$ interacts with α1 AMPK in the yeast-two-hybrid system

a) Table showing interaction of α1 AMPK subunit with the carboxy-terminal domain of $GABA_{B(1)}$ (CR1) in yeast. Plasmid backbone is represented by control. Interaction was specific to CR1

b) Cartoon illustrating the specific region of α1 AMPK isolated using the yeast-2-hybrid screen.

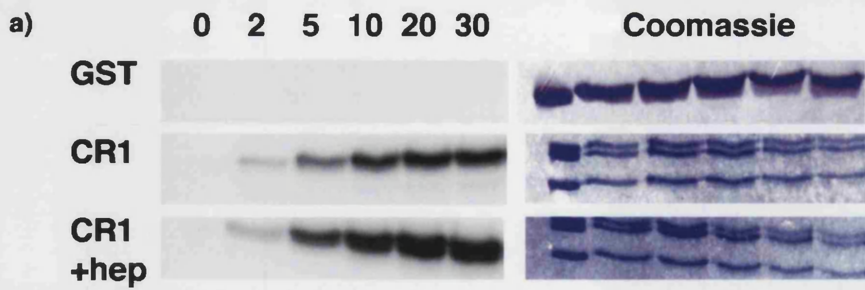
Figure 21 **AMPK phosphorylates CR1**

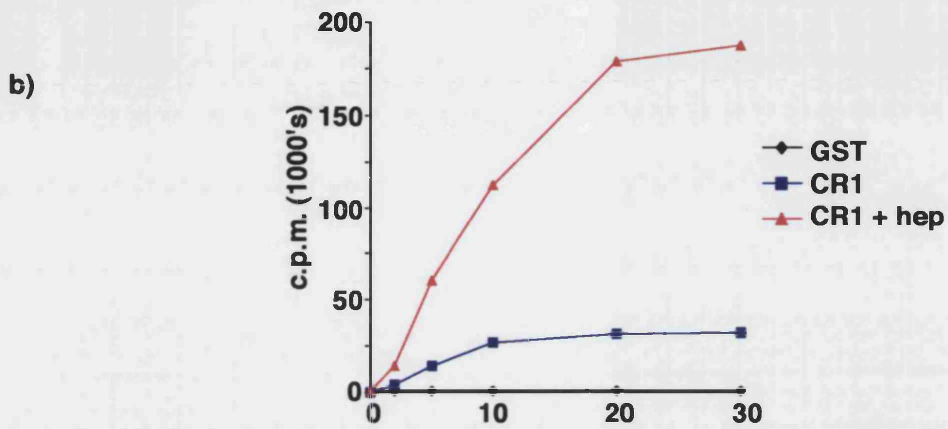
a+b) Timecourse of CR1 phosphorylation by AMPK. Note there is no phosphorylation of Gst, whilst addition of heparin greatly enhances the rate of phosphorylation.

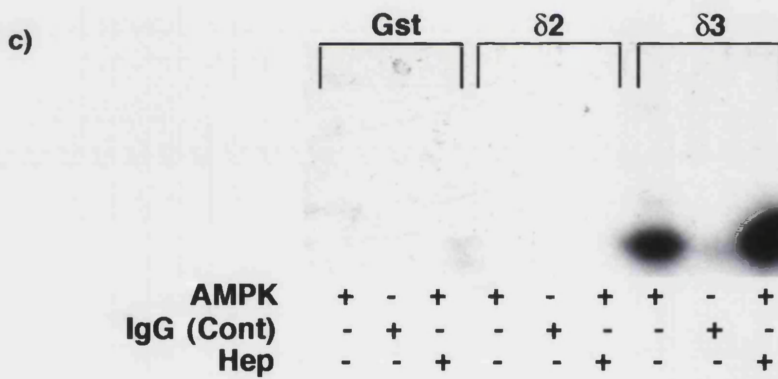
c) AMPK specifically and robustly phosphorylates the second half of the carboxy-terminal of R1 (δ3).

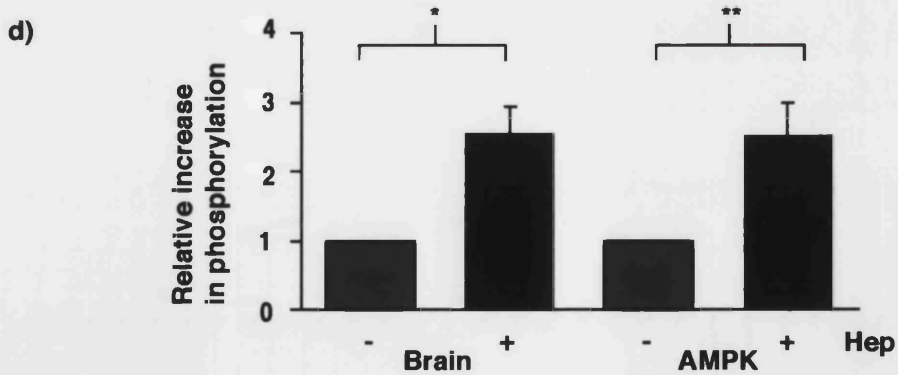
d) Comparison of the enhancement in phosphorylation achieved through the addition of heparin to the kinase buffer by AMPK and the kinase associating from brain.

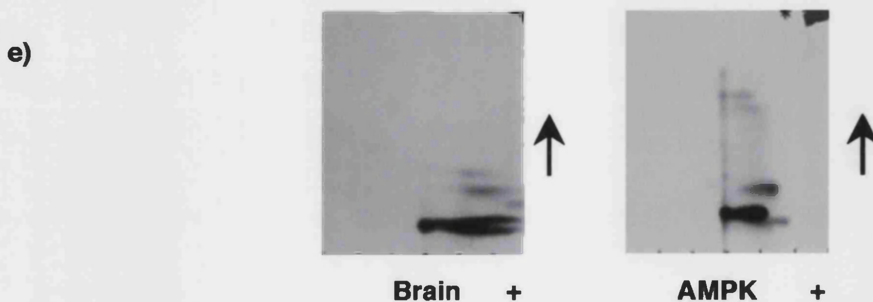
e) Similar tryptic peptide maps of phosphorylated CR1 by i) Kinase from brain and ii) AMPK suggest AMPK and kinase from brain phosphorylate the same residues.


a)
0 2 5 10 20 30
Coomassie
GST
CR1
CR1 +hep


b)
c.p.m. (1000's)
200
150
100
50
0
0
10
20
30
GST
CR1
CR1 + hep


c)
Gst
δ2
δ3
AMPK + - + + - + + - +
IgG (Cont) - + - - + - - + -
Hep - - + - - + - - +


d)
Relative increase in phosphorylation
4
3
2
1
0
*
**
- Brain +
- AMPK +
Hep


e)
Brain +
AMPK +

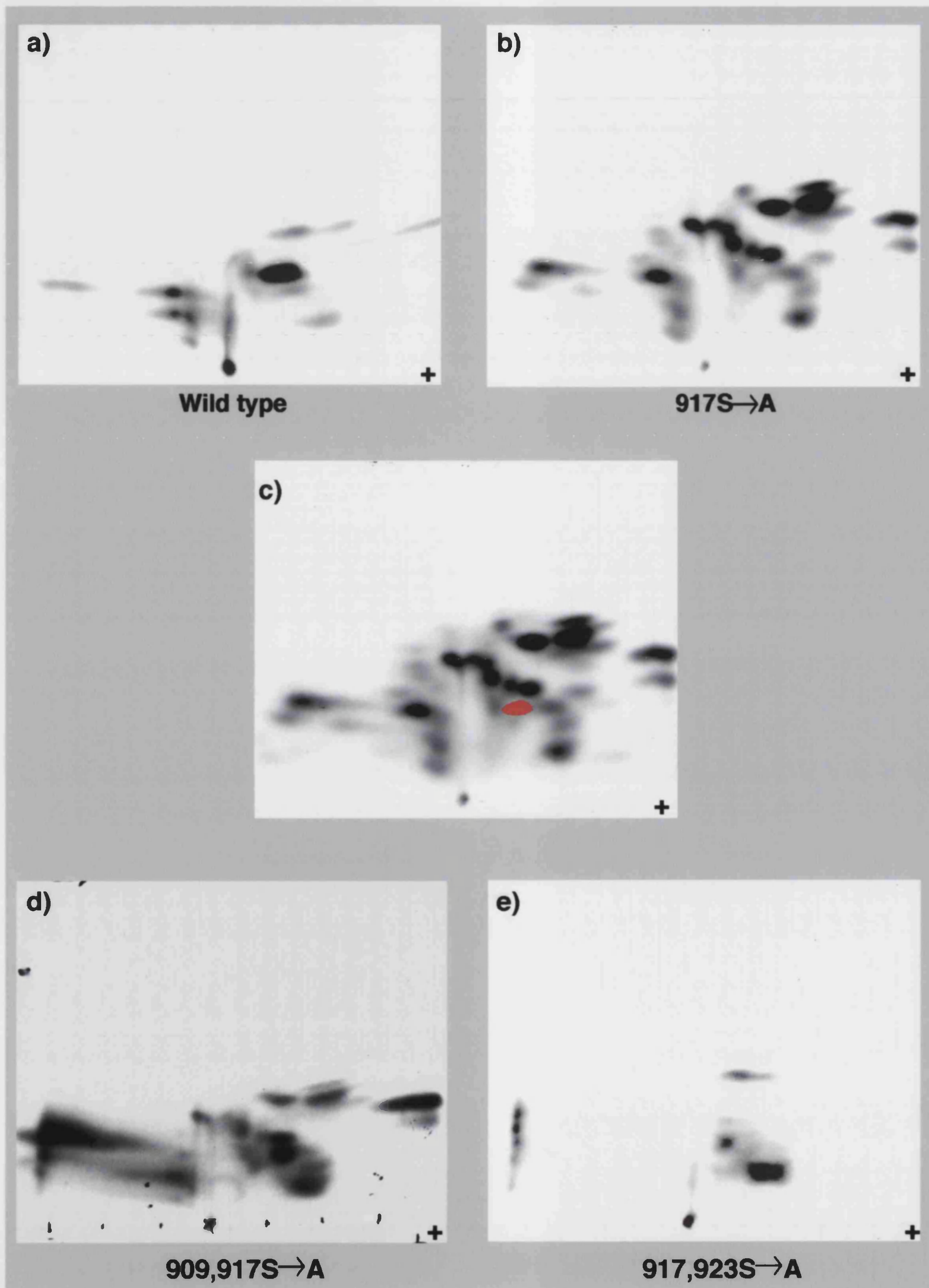


Figure 22 Peptide maps
Fusion proteins of wild type or serine to alanine mutants of the carboxy-terminal intracellular domain of $GABA_{B(1)}$ were phosphorylated by AMPK using ^{32}P labelled ATP and then digested with thermolysin. Peptides were separated using 2D chromatography and then plates exposed to film. a) Wild type shows one major peptide. b) S917A mutation causes loss of major peptide revealing novel peptides.c) Superimposition of wild type peptide (red) upon S917A mutant map.d) Additional mutation of S909A has no major effect, whilst additional mutation of S923A (e) causes loss of all major peptides.

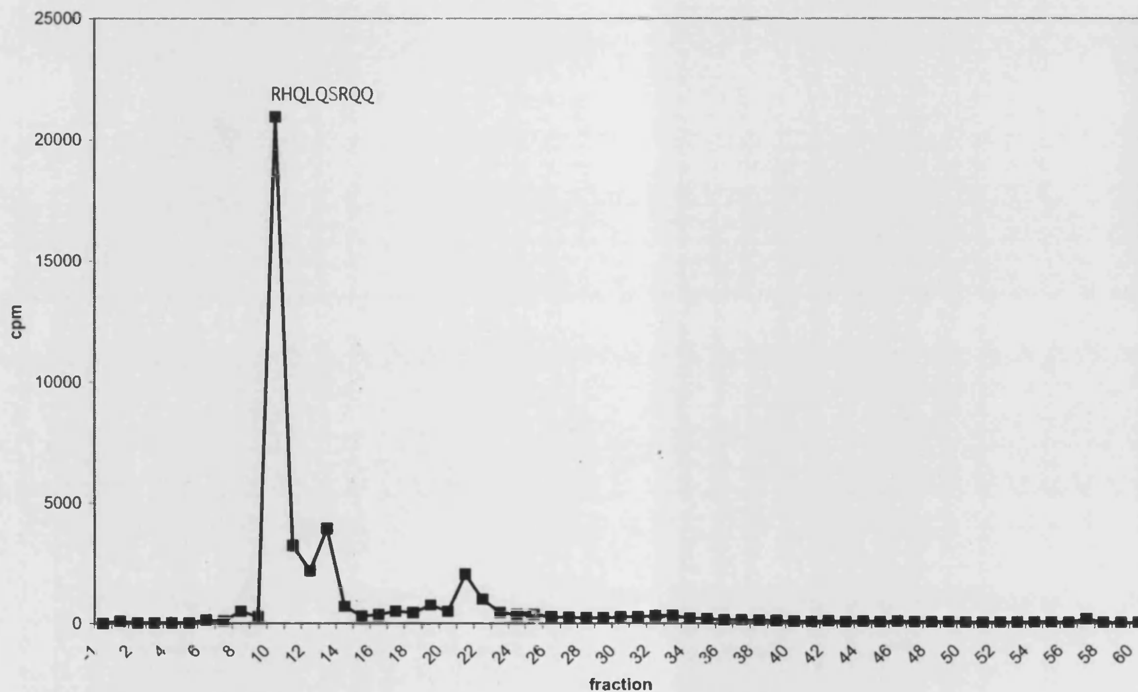
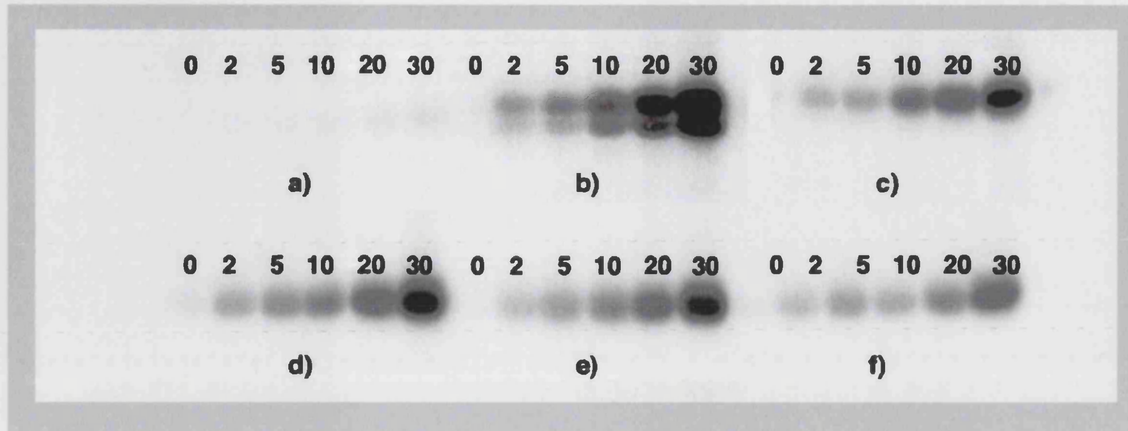


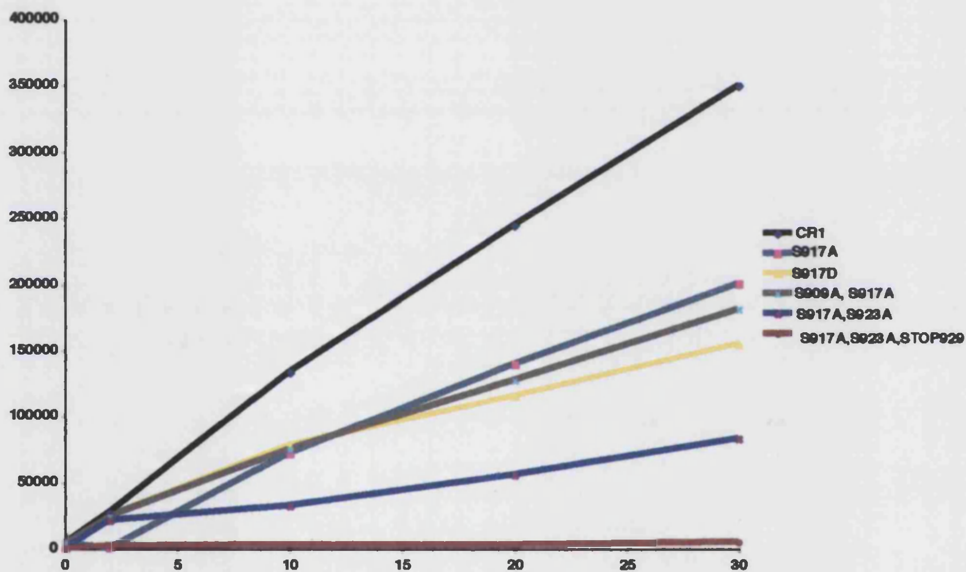
Figure 23 **Edman degradation of phosphorylated CR1**

A GST fusion protein of the GABA$_{B(1)}$ carboxy-terminal intracellular domain (CR1) was phosphorylated with ^{32}PγATP spiked ATP to saturation point by AMPK and then resolved using SDS-PAGE. Radiolabelled CR1 was excised from the gel and then digested with Chymotrypsin. Peptides were separated using a chromatography column. Edman degradation of the maximally radiolabelled peptide revealed the sequence RHQLQSRQQ, with the serine showing high levels of radiolabelling. This region corresponds to Ser917 within the carboxy-terminal intracellular domain of GABA$_{B(1)}$

Figure 24



i)



ii)

Figure 24 **Analysis of mutant phosphorylation**

i) Example time course phosphorylations of a)CR1, control IgG, b) CR1, c) CR1 S917D, d) CR1 S917A, e) CR1 S909,917A and f) CR1 S917,923A

ii) Quantification of experiment, also shown is CR1 S917,923A with stop codon at 929.

These experiments show that although peptide maps and Edman degradation show S917 is AMPK preferred substrate residues within CR1, in the absence of this site other downstream residues form substrates.

4.3 Discussion

Proceeding with an *in vitro* kinase assay after a 'pull-down' is a convenient test as to whether your candidate protein: a) binds kinases, and b) forms a substrate. The use of radioactively labelled ATP allows for greater sensitivity than western blotting. However, this experiment is only a preliminary screen. The lack of phosphorylation of a candidate protein may mean kinases are unable to bind even if the protein forms an excellent substrate. Conversely a kinase may interact but if the protein does not form a substrate it will be impossible to ascertain. Other possible drawbacks include reduction of kinase activity by extraction conditions and incorrect folding of fusion proteins.

Notwithstanding these limitations, this technique has demonstrated that a kinase both associates with and phosphorylates CR1 from brain lysates. In conjunction with the data obtained from the whole cell labelling analysis this data indicates that the carboxy-terminal domain may form a substrate for kinase activity in the brain. Mapping of the kinase associating region demonstrates that the first half of the carboxy-terminal domain (δ2 aa 876-904), containing multiple serine and threonine residues and the majority of the coiled coil motif, is not phosphorylated by associated kinases from brain. Instead it is the distal portion of the carboxy-terminal domain that associates with kinases. The minimum region required to confer kinase activity from brain upon the CR1 fusion protein covers the amino acids 905-925. This stretch of 20 amino acids contains the last 12 amino acids of the coiled coil and also the ER-retention motif RSRR. Removal of the RSRR region prevents association with kinases, indicating that this may form the major kinase binding site. The association of kinases with the end of the carboxy-terminal domain as opposed to the proximal portion probably reflects greater accessibility of this region to other proteins within the heterodimerised receptor because it projects away from the coiled coil. In the complete receptor the proximal portion of the carboxy-terminal domain is likely to be in closer association with the plasma membrane and $GABA_{B(2)}$.

The chief determinant in the interaction between a phosphorylation motif and kinase is the ability of the motif to fit into the substrate-binding pocket of the kinase, found in the kinase catalytic domain. A Y2H interaction between a bait protein and a kinase could conceivably take place between a consensus phosphorylation motif

within the bait protein binding to the active site of the kinase. The interaction between AMPK and $GABA_{B(1)}$ though must involve regions outside the active site of AMPK because the non-catalytic carboxy-terminal half of α1AMPK was isolated as the interacting region in the Y2H screen. The fact that AMPK also phosphorylates the carboxy-terminal tail of $GABA_{B(1)}$ can therefore be viewed as additional support of an interaction, because it involves a separate domain of AMPK. Further characterisation of the interaction between $GABA_{B(1)}$ and α1 AMPK is provided in the next chapter.

The introduction of an S917A mutation did not elicit a dramatic reduction in the phosphorylation of CR1. However, the peptide maps in conjunction with the Edman degradation, clearly demonstrate that S917 is the main site (>90% of total phosphorylation is at this site in wild type). It could be that, as previously mentioned, mutation reveals silent sites that would be phosphorylated if S917 were to be obscured. As discussed in the introduction to this chapter, the consensus sequence for AMPK phosphorylation is poorly defined (Hardie *et al.,* 1998). However, studies using synthetic peptides and of known AMPK phosphorylation sites have demonstrated that three or four sites are conserved between substrates (Weekes *et al.,* 1993, Hardie *et al.,* 1998). The conserved sites being hydrophobic residues at P-5 and P+4, with a basic residue found at either P-3 or P-4. Also, often noted is the presence of a basic residue at P-6. Although none of the possible serine and threonine residues within the second half of the carboxy-terminal domain of $GABA_{B(1)}$ forms a perfect AMPK consensus phosphorylation site, S917 satisfies a number of the determinants. Crucially at P+4 the residue is a leucine fulfilling the requirement for a hydrophobic residue at this position. A histidine is found 4 amino acids upstream of serine 917, meeting the requirement for a basic amino acid at position P-3. Most AMPK substrates have a hydrophobic residue at P-5, but in the case of S917 a hydrophobic residue (leucine) is found at P-6. Although this differs from classical AMPK phosphorylation sites, it should be kept in mind that this particular stretch of residues putatively forms a coiled coil ending at S917. It is probable that the tertiary structure imposed by the coiled coil allows for a hydrophobic residue at P-6 instead of P-5. The additional mutation S923A reduces the phosphorylation by a much greater amount than S917A alone, and this is not observed when S909 is mutated in addition to S917. This implies that S923 is a secondary site, but another explanation is that

the area around S923 is important for interaction with AMPK, and this mutation affects the binding of the kinase and subsequent phosphorylation of other serines. Indeed, the addition of a stop codon at amino acid 929 truncates CR1 and removes all further phosphorylation in a S917A, S923A mutant, indicating that any other phosphorylation occurs downstream of these sites. Although the relevance of consensus sites for kinase activity is questionable, it is clear that S923 does not appear to be an optimal AMPK phosphorylation site, and indeed the most likely other site within CR1 is at S953, although this lacks a basic residue at P-3/4. It is possible that an S917A mutant containing S923 but no further serines would demonstrate whether S923 is a true AMPK site. This though would also be a substrate much removed from the wild-type CR1, where clearly only one site serves as an AMPK substrate.

CHAPTER FIVE

5.1 Introduction

Having established that $GABA_{B(1)}$ is an *in vitro* substrate for AMPK it was important to further investigate the interaction between $GABA_{B(1)}$ and AMPK and determine whether AMPK could associate with as well as phosphorylate $GABA_{B(1)}$. I also wanted to further investigate the role of phosphorylation of $GABA_{B(1)}$ by AMPK. Although serine 917 is a substrate for AMPK *in vitro* it remained unproven as to whether serine 917 forms a substrate *in vivo.* One approach to ascertaining whether serine 917 is an *in vivo* kinase substrate was to raise a phospho-specific antibody to this site and then use it to blot brain lysates. This would allow me to determine whether phosphorylation occurs *in vivo.*

The addition of a negatively charged phosphate group to a protein affects the net charge of a protein as well as the distribution of charge within a protein. An additional phosphate group can also result in the creation of motifs responsible for protein-protein interactions. Phosphorylation of GPCRs is normally associated with desensitisation and internalisation events. However, as shown in chapter 3 of this thesis, the phosphorylation of $GABA_{B(1)}$ does not increase upon agonist exposure, making an involvement in desensitisation events unlikely. I therefore focussed upon other possible consequences of $GABA_{B(1)}$ phosphorylation. Phosphorylation of residues within the intracellular carboxy-terminal domain of $GABA_{B(1)}$ might be expected to have an effect on the trafficking of the receptor due to the presence of the ER retention motif in this region, in addition to the coiled coil domain. Primarily I decided to study the effect of mimicking or inhibiting the phosphorylation of $GABA_{B(1)}$ upon cell surface trafficking of the receptor through the site-directed mutagenesis of putative phosphorylation sites to either alanines or aspartates. I then assayed the ability of these mutant receptors to traffic to the cell surface in COS-7 cells using ELISA. Subsequently the effect of treating cortical neurones with activators of AMPK upon the cell surface levels of $GABA_{B(1)}$ was analysed.

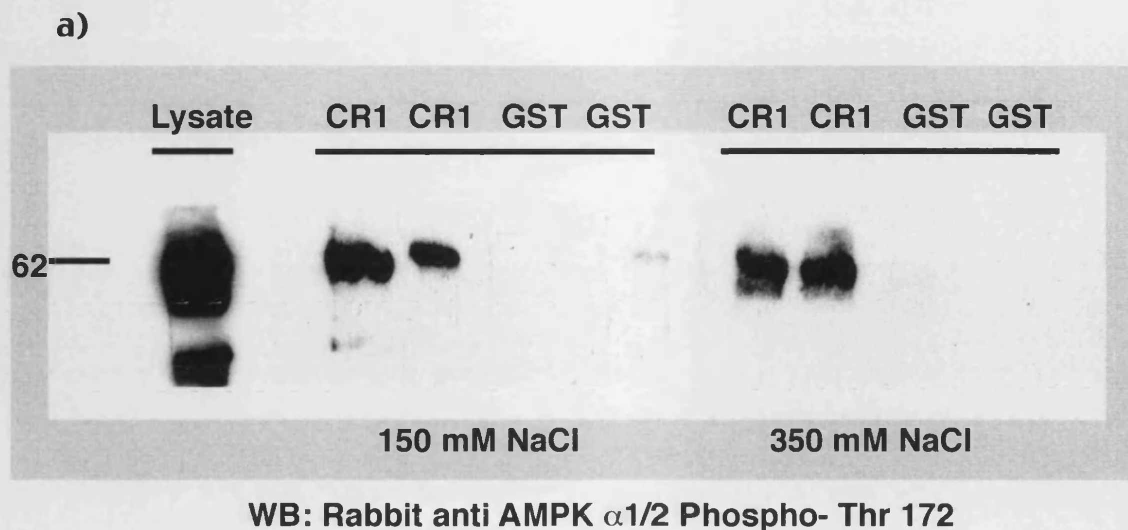
The other area investigated was the ability of phosphorylated $GABA_{B(1)}$ to interact with other proteins. Rather than trying to find novel binding partners to phosphorylated $GABA_{B(1)}$, which would be a long process outside the realms of this

investigation, I concentrated upon two proteins that had been previously shown to interact, namely $GABA_{B(2)}$ and the transcription factor CREB2.

5.2.1 Confirmation that AMPK associates with CR1 from brain

To confirm that the kinase co-purifying from brain lysates with CR1 was indeed AMPK it was important to immunoblot pull-downs from brain lysates that had been transferred to nitrocellulose membranes. It was empirically determined that AMPK from brain was far more soluble in buffer containing the detergents NP-40 (1%) and de-oxycholic acid (DOC) (0.5%) than in Triton X-100 containing buffers. Highly efficient solubilisation of AMPK from muscle and liver is obtained in buffers containing 0.5% Triton X-100 (D.Carling, personal communication), and so this differing solubility could be indicative of differences in the nature of AMPK found in brain compared to that observed in muscle. Pull-downs using 25µg of eluted CR1 fusion protein or GST were carried out in lysate (5mg protein) from rat brain homogenised in a buffer that allowed maximal solubilisation of AMPK. After incubation with rotation for 90 minutes at 4°C, a 50:50 slurry of glutathione agarose beads was added and lysates were incubated for a further 30 minutes. Beads were then washed 4 times in pull down buffer with 5 minutes of rotation per wash. To determine the stringency of the interaction the experiment was also carried out using pull-down buffer containing 350mM salt for bead washing. Loading buffer was added to the samples which were then boiled followed by resolution using 12% SDS-PAGE. Following electrotransfer of gels to nitrocellulose, membranes were probed with an antibody that specifically recognised the catalytic subunit of AMPK when phosphorylated at threonine 172. As previously mentioned, phosphorylation at this site is imperative for the catalytic activity of AMPK. Activated AMPK was observed to associate with CR1 (figure 25a), and this association was not attenuated by 350mM salt washes signifying the interaction was of high affinity. To confirm that the kinase associating in these conditions could phosphorylate CR1 pulldowns were washed extensively in kinase buffer and then incubated with ATP spiked with $^{32}\gamma$ATP to which was also added 200µM 5'AMP. CR1 was robustly phosphorylated in these conditions and this phosphorylation was significantly stimulated by the addition of 5'AMP (fig 25b).

a)



b)

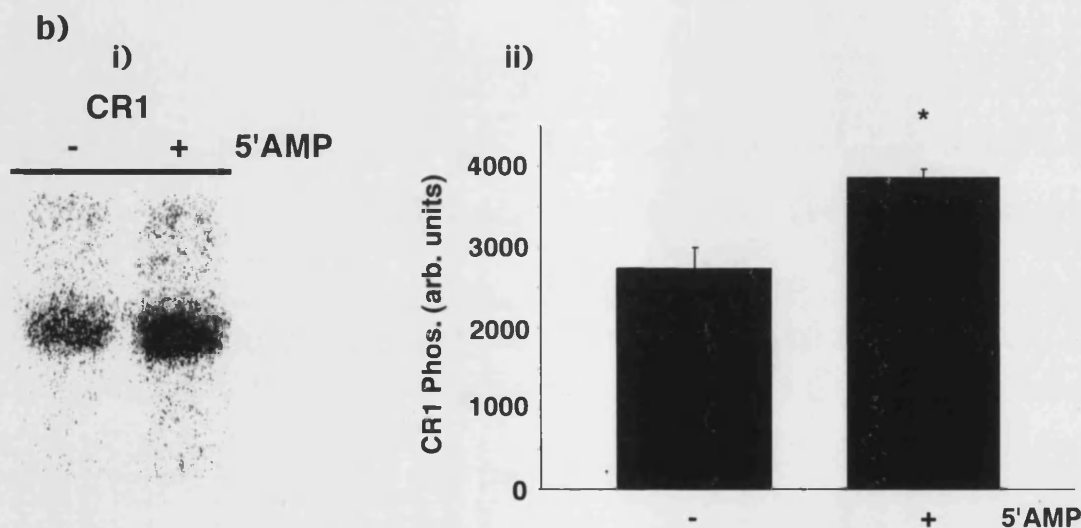


Figure 25: The intracellular carboxy-terminal domain of $GABA_{B(1)}$ associates with AMPK catalytic subunit

a) GST-CR1 or GST alone were exposed to brain lysate. After extensive washing in low (150mM) or high (350mM) salt buffer, bound material was resolved by SDS-PAGE and analysed by immunoblotting with an anti α1/2 Phospho-Thr172 antibody. Binding was detected under both wash conditions to GST-CR1 but not GST alone.

b)i) GST-CR1 exposed to brain as above was then incubated with ATP (200μM) spiked with $^{32}P\gamma$ATP, with or without 5'AMP (200μM) for 20 minutes. Samples were then resolved using SDS-PAGE and exposed to phospho-imager for quantification.
ii) Inclusion of 5'AMP significantly enhanced phosphorylation (* = $p \leq 0.05$)

5.2.2 Immunofluoresence

Few studies detailing the subcellular localisation of AMPK exist in the literature, and those have been performed are in cell lines. Although it is known that both catalytic subunits of AMPK are expressed in neurones (Culmsee *et al.,* 2001), the precise neuronal localisation of the kinase has never been ascertained. For the interaction between $GABA_{B(1)}$ and AMPK subunits to be of physiological relevance both $GABA_{B(1)}$ and AMPK are must be co-expressed in the same subcellular compartments. To analyse the distribution of AMPK relative to $GABA_B$ receptors, neurones were doubly immunolabelled with antibodies specific to both $GABA_B$ subunits and also to antibodies raised to the catalytic subunit of AMPK. 21 DIV cultured hippocampal neurones were fixed and blocked in blocking solution containing 0.2% Triton X-100 to allow cell permeabilisation. They were then stained for the catalytic subunit of AMPK using a rabbit polyclonal anti $\alpha1/2$ subunit AMPK antibody (gift from Dr D. Carling) or the activated AMPK recognising Thr172 phospho-specific $\alpha1/2$ antibody.

$\alpha1/2$ AMPK exhibits a somatodenditic staining pattern, with the majority of dendrites and the axon strongly staining for $\alpha1/2$ AMPK whilst the cell soma shows diffuse AMPK staining throughout, with the occasional aggregate. There is a small amount of nuclear staining. As noted in chapter 3, the commercially available $GABA_{B(1)}$ used in this study shows very little $GABA_{B(1)}$ staining at the surface, possibly because the antigenic epitope is obscured in the complete receptor. Use of this antibody shows intracellular $GABA_{B(1)}$ staining that is most probably ER resident. Comparisons of this intracellular $GABA_{B(1)}$ pattern and the staining obtained with the anti $\alpha1/2$ subunit AMPK antibody show a high degree of colocalisation throughout the internal bodies of neuronal dendrites, with certain dendrites showing almost complete overlap of staining (example staining fig 26 a-c). Within the soma of the neurone pockets of colocalisation do exist but overall it is much lower. Because intracellularly retained $GABA_{B(1)}$ is unlikely to represent the complete receptor, this staining pattern indicates that AMPK is found in similar intracellular regions to $GABA_{B(1)}$ and may associate with the non-dimerised subunit *in vivo.*

As mentioned in chapter 3, the staining pattern obtained with an antibody raised to $GABA_{B(2)}$ is quite different to that seen with $GABA_{B(1)}$. Use of this

antibody gives very strong surface staining although no clustering of the subunit is evident. $GABA_{B(2)}$ immunoreactivity is probably representative of the complete receptor because of the extremely high degree of interaction between the two subunits *in vivo* (Benke *et al.,* 1999). When comparing co-distribution of AMPK and $GABA_{B(2)}$ it is apparent that, similar to $GABA_{B(1)}$, there is a large degree of colocalisation between the two proteins especially in the dendrites of the neurone. Within the dendrites there is strong staining just beneath the membrane suggesting that AMPK may be able to phosphorylate surface receptors (fig. 26 b1-b6).

Immunolabelling of active AMPK with the phospho-specific thr172 antibody gives a similar staining pattern to the pan α subunit antibody in the neuronal soma although nuclear staining is increased. However the dendritic labelling is quite different with most dendrites being only weakly immunoreactive for activated AMPK, where it is observed in punctate clusters, possibly at synapses. However, many neurones analysed do appear to posess one strongly non-punctate staining dendrite (fig. 26 c,d). This is most probably the axon and suggests that in this region AMPK is active under basal conditions. When comparing the co-staining of activated AMPK with $GABA_{B(2)}$ there appears to be a far lower degree of colocalisation. The staining patterns observed in this study may be interpreted to indicate that under basal conditions AMPK is situated in close proximity to $GABA_B$ receptors but is not activated and so will not phosphorylate the receptor. The fact that the two strongly colocalise though suggests that upon activation of AMPK, phosphorylation of the receptor may occur rapidly.

5.2.3 AMPK co-immunoprecipitates with $GABA_{B(2)}$

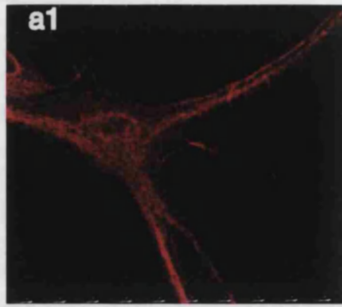
To further qualify the nature of the interaction between $GABA_{B(1)}$ and AMPK, the possibility that the two proteins could co-immunoprecipitate was investigated. This has the advantage of demonstrating an interaction between complete proteins, and strongly suggests that the proteins interact *in vivo.* Unknown motifs within the carboxy-terminal half of AMPK α subunits limit their heterologous expression of this subunit (Crute *et al.,* 1998). High expression levels are only possible when transfecting an α subunit truncated at amino acid 312 (Crute *et al.,* 1998). Because the carboxy-terminal half of AMPK was isolated as an interacting protein from the

yeast two-hybrid system it was not feasible to use a truncated α subunit. Therefore efforts were focussed upon trying to demonstrate a direct interaction between native AMPK and native $GABA_{B(1)}$ from brain. Initially, experiments were conducted utilising available antibodies to either immunoprecipitate $GABA_{B(1)}$ and blot for a AMPK or vice versa. Unfortunately this technique elicited extremely high levels of background antibody cross reactivity such that autoradiographs of western blots 'blacked out' making it impossible to discern whether the two proteins co-immunoprecipitated.

Interestingly, immunoprecipitations using pan α AMPK antibodies from solubilised rat brain membrane fractions were immunopositive for $GABA_{B(2)}$ when blots were probed with a monoclonal mouse anti-$GABA_{B(2)}$ antibody (fig. 27). No signal was noted in the control rabbit IgG. Because in yeast $GABA_{B(2)}$ is unable to interact directly with the α1 AMPK subunit, coupled to the fact that almost all $GABA_{B(2)}$ is bound to $GABA_{B(1)}$ in brain membranes, the co-immunoprecipitation of $GABA_{B(2)}$ with AMPK suggests that it is probably the complete receptor that is co-immunoprecipitating with AMPK. It may be that $GABA_{B(1)}$ is acting as a bridging protein interacting with both AMPK and $GABA_{B(2)}$ concurrently.

5.2.4 Site-directed mutagenesis of $GABA_{B(1)}$ carboxy-terminal

Because S917 is close to the RSR ER retention motif of $GABA_{B(1)}$ and S923 is in the middle of this motif, investigations were undertaken to determine whether the mutation of these sites could effect the cell surface trafficking of either the single subunit or the heterodimerised receptor. Serines 909, 917 and 923 were mutated to either alanine or aspartate residues and used either individually or in permutations. Mutant constructs were sequenced prior to experimentation. Determination of the efficiency of cell surface trafficking was carried out using whole cell ELISAs in COS-7 cells. Mutant $GABA_{B(1)}$ was transfected alone initially to determine whether the mutation allowed cell surface trafficking. Mutated $GABA_{B(1)}$ was then co-transfected with $GABA_{B(2)}$. For maximal efficiency of receptor surface trafficking, co-transfections of the two subunits are carried out in a ratio of $GABA_{B(1)}$ to $GABA_{B(2)}$ that is 1:4. In these experiments though a submaximal ratio of $GABA_{B(1)}$ to $GABA_{B(2)}$ at 1:2 was used so that any enhancement in the trafficking efficiency was more apparent.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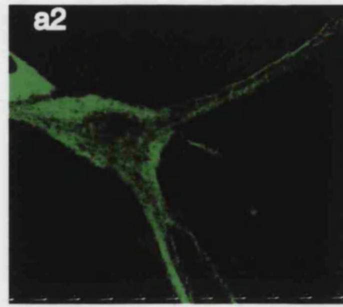
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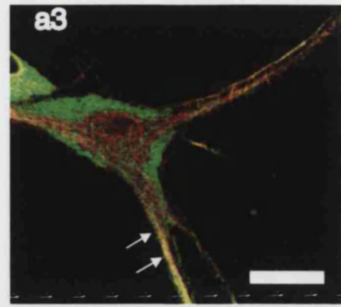
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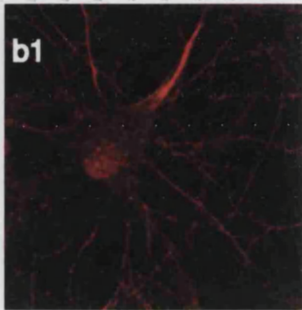
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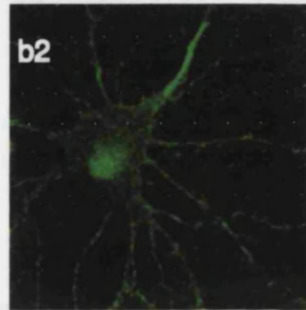
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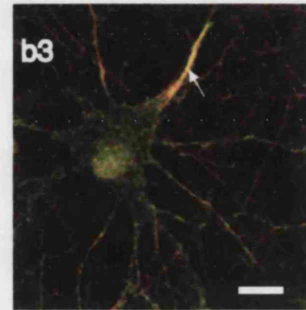
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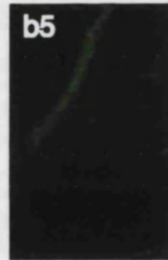
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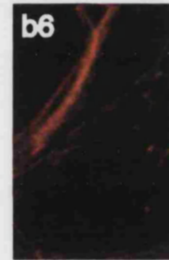
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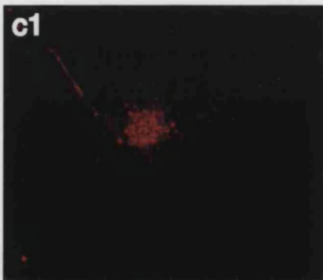
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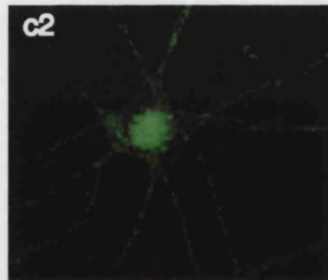
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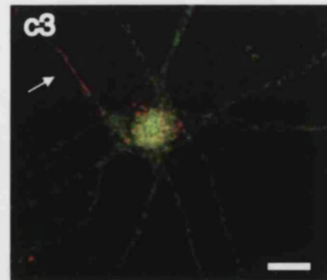
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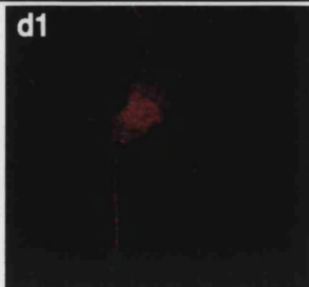
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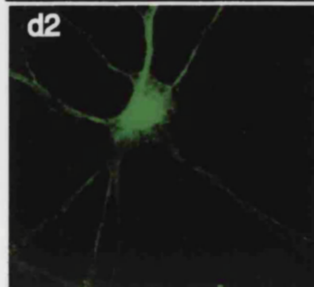
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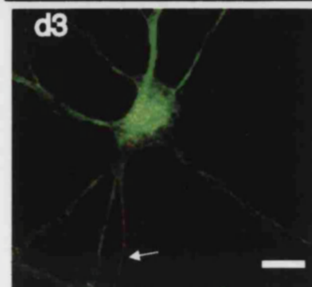
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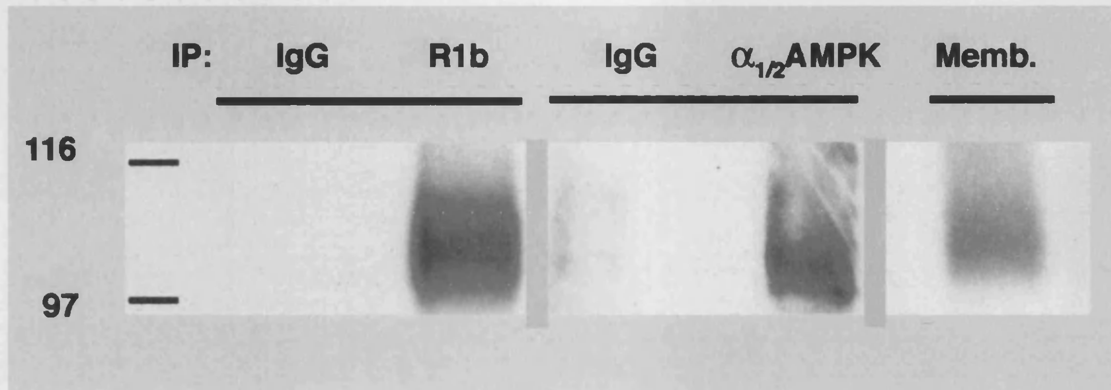
Figure 26 Co-localisation of $GABA_B$ and AMPK in neurones

21 DIV hippocampal neurones were stained for $GABA_B$ receptor subunits and AMPK in neurones. Neurones were then visualised using confocal microscopy. Scale = 10μM

a) 1) pan α -AMPK staining, 2) $GABA_{B(1)}$ staining, 3) merge. Areas of strong colocalisation are highlighted with arrows.

b) 1) pan α -AMPK staining, 2) $GABA_{B(2)}$ staining, 3) merge, 4-6 close up of of arrowed area in 3.

c) +d) 1) phospho-AMPK staining, 2) $GABA_{B(2)}$ staining, 3) merge. Note single dendrite per neurone strongly stains for phospho(active) AMPK (arrowed). There is less co-localisation of active AMPK and $GABA_{B(2)}$ than total AMPK



WB: Mouse anti $GABA_{B(2)}$

Figure 27 $GABA_{B(2)}$ co-immunoprecipitates with the catalytic subunit of AMPK

Crude brain membranes were prepared and then detergent solubilised in buffer containing 2% CHAPS. From this solution extracts were immunoprecipitated using a rabbit anti AMPK α1/2 subunit specific antibody, or control rabbit IgG. As a positive control a goat $GABA_{B(1b)}$ subunit specific antibody or goat IgG were also used in immunoprecipitations. Antibody protein complexes were purifed on Protein A agarose and, after extensive washing, were resolved on SDS-PAGE and analysed by immunoblotting with a mouse anti $GABA_{B(2)}$ antibody. $GABA_{B(2)}$ subunit was observed to immunoprecipitate with antibodies to either $GABA_{B(1b)}$ or α1/2 AMPK, but not control IgG.

Mutations S909A, S917A and S923A did not significantly effect surface trafficking of the receptor. Mutations of serines to aspartates mimicking the phosphorylation at these sites were also tested. The mutation S917D alone had no detectable effect on the trafficking of either $GABA_{B(1)}$ or the complete receptor, but the mutation S917D S923D caused an increase in the surface trafficking of the $GABA_{B(1)}$ in the presence of $GABA_{B(2)}$ and also allowed ER exit of $GABA_{B(1)}$ alone (fig. 28).

5.2.5 Effect of activators of AMPK on neuronal surface numbers of $GABA_{B(1)}$

The data obtained from mutating residues within the intracellular carboxy-terminal of $GABA_{B(1)}$ suggested that the sequential phosphorylation of S917 and S923 may alter the surface trafficking of the receptor and possibly allow the ER exit of monomeric $GABA_{B(1)}$ subunit. To investigate whether compounds known to activate AMPK could alter the surface receptor number in neurones, experiments were carried out treating neurones with oligomycin and metformin. Oligomycin has been demonstrated to potently activate AMPK in many different cell types (Marsin *et al.*, 2000) and is thought to work by uncoupling oxidative phosphorylation and thus depleting intracellular ATP. Because of the mechanism of action it rapidly depletes ATP, but is toxic over longer periods. 5 DIV cultured cortical neurones were treated for up to 80 minutes with 200nM oligomycin at 37°C in tandem with an untreated control. Cells were then placed on ice where they were biotinylated prior to solubilisation in RIPA. Biotinylated proteins were streptavidin precipitated and then resolved using 8% SDS-PAGE. Proteins were electrotransferred to nitrocellulose and then immunoblotted with anti-$GABA_{B(1a)}$ antibodies. A secondary ^{125}I labelled anti-rabbit antibody was then used and proteins were quantitated using a phosphorimager. Twenty minute treatment with oligomycin caused a ~65% increase in the amount of surface $GABA_B$ receptor, however this fell outside the boundaries of statistical significance. Other time points also showed a general trend towards an increase in surface levels of the receptor but at no time point was this increase significant even when n=9. This variability was probably attributable to the inherent toxicity of oligomycin.

The majority of other AMPK activators also have a high degree of toxicity except for the anti-diabetic agent metformin. Metformin has recently been demonstrated to

activate AMPK (Zhou *et al.*, 2001) in a manner independent of changes to AMP:ATP ratios (Fryer *et al.*, 2002; Hawley *et al.*, 2002) and has far lower toxicity than other AMPK activators. Metformin is slower to activate AMPK than the metabolic poisons, probably because it is very hydrophilic and thus takes a long time to cross the cell membrane and accumulate intracellularly to an optimal concentration. Neurones were treated with 2mM metformin, this concentration previously being demonstrated to be the optimal for rapid AMPK activation (Zhou *et al.*, 2001). Biotinylation experiments were carried out as with oligomycin, but at 3 and 5h after commencing treatment. Multiple experiments demonstrated that 5h treatment with metformin significantly increased surface $GABA_{B(1)}$ by 50.7±14.7% ($p<0.05$) (fig. 29). Western blotting of lysates from treated neurones for activated AMPK and quantitation with ^{125}I labelled secondary demonstrated an increase in signal that was maximal 4h after commencement of treatment. This showed that AMPK is activated in neurones by metformin (example experiment is shown in fig. 29).

5.2.6 Purification of an antibody specific to phosphorylated $GABA_{B(1)}$

To confirm whether S917 is phosphorylated *in vivo* an antibody (UCL-89) was raised to a phospho-peptide designed around the S917 (fig. 30). Peptides were designed with an N-terminal Cysteine so that they could be coupled via a sulphydryl group to maleimide activated keyhole limpet haemocyanin. Rabbits were immunised with peptide conjugated carrier protein and then re-immunised until significant immunoreactivity with peptide was noted using dot-blots of peptide. After the final bleed had been taken antibodies were purified by initially running serum over thiopropyl-sepharose columns to which a non-phosphorylated form of the antigenic peptide had been coupled. This was to minimise the purification of antibodies that recognised epitopes within the antigenic peptide that did not include the phospho-serine. The serum was then purified over columns to which had been conjugated the immunising phospho-peptide and antibody was eluted with glycine pH 2.5.

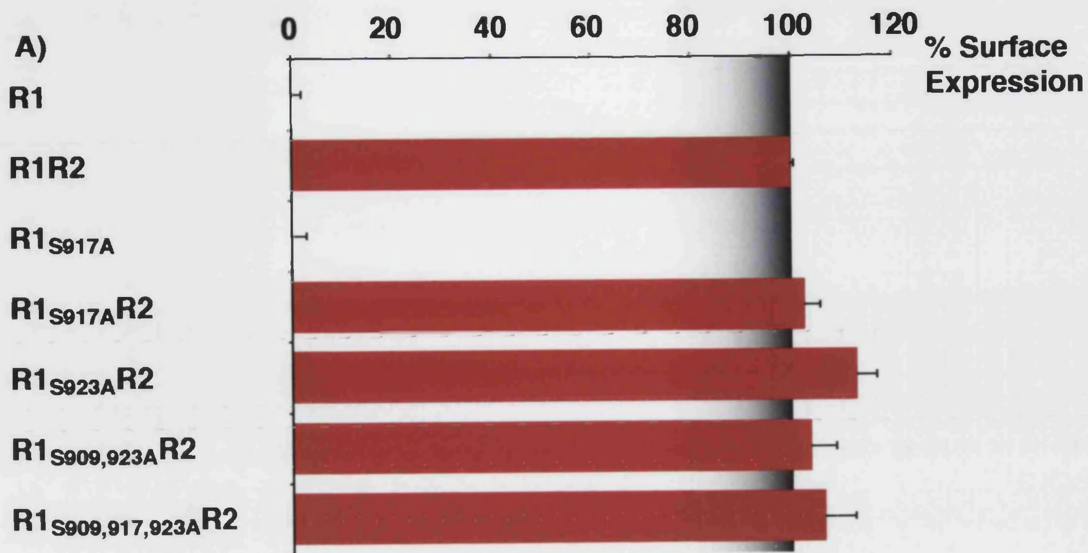
5.2.7 UCL-89 specifically recognises phosphorylated CR1

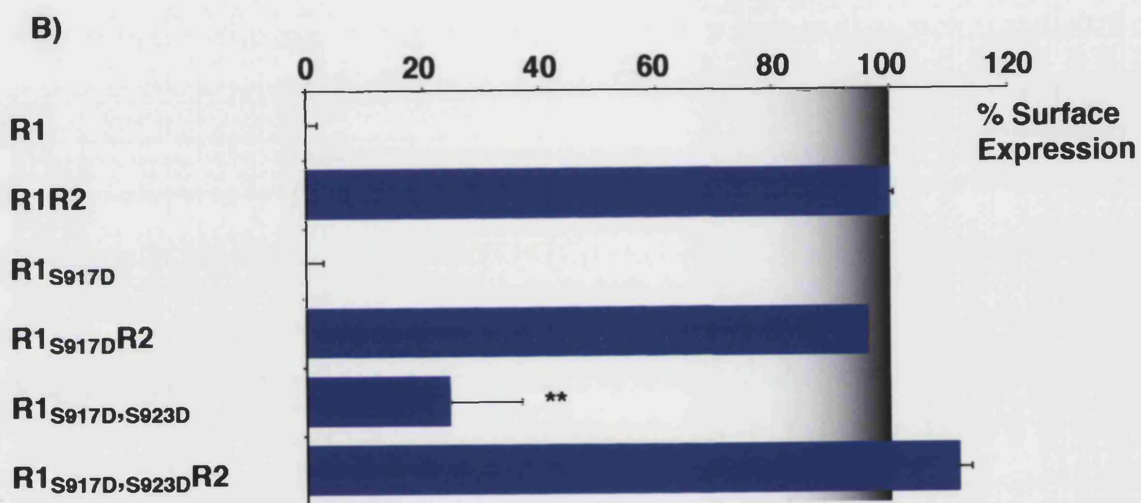
Initial experiments were conducted to determine whether UCL-89 could cross-react with GST fusion proteins of the carboxy-terminal domain of $GABA_{B(1)}$ (CR1)

Figure 28 **Mutations of phosphorylation sites affect surface trafficking**

A+B GABA$_{B(1)}$ was mutated at S909, S917 and S923 to either alanines or aspartate residues. Constructs were then transfected into COS cells and assayed for surface trafficking using whole cell ELISA.

C Confocal microscopy confirms that whilst GABA$_{B(1)}$ wild tye (a) and S917D mutant (b) do not traffick to surface, constructs with both S917D and S923D mutations are efficiently trafficked and can be visualised using anti-myc (c i,ii)


A)
0
20
40
60
80
100
120
% Surface Expression
R1
R1R2
R1S917A
R1S917AR2
R1S923AR2
R1S909,923AR2
R1S909,917,923AR2


B)
0
20
40
60
80
100
120
% Surface Expression
R1
R1R2
R1S917D
R1S917DR2
R1S917D,S923D
**
R1S917D,S923DR2

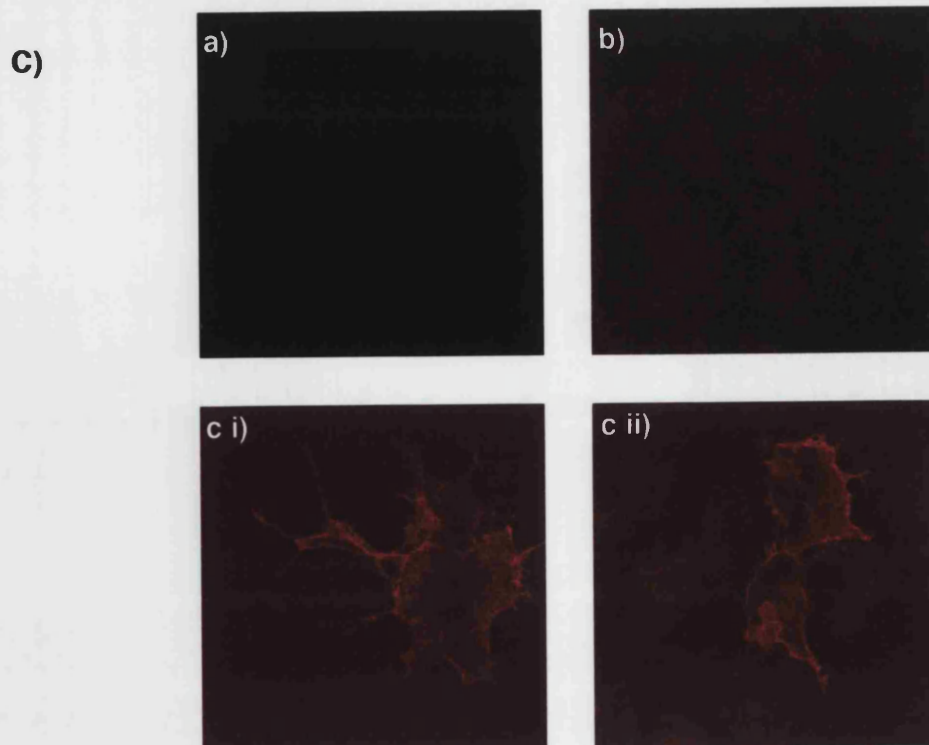
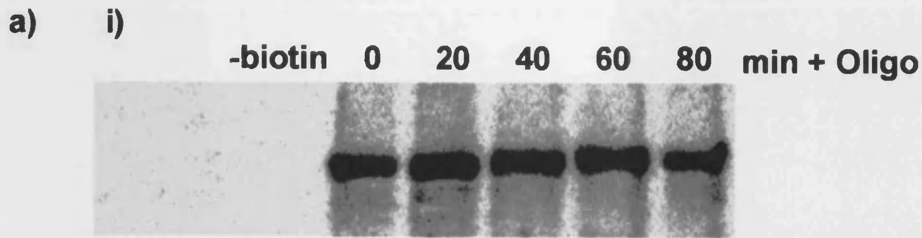

C)
a)
b)
c i)
c ii)

Figure 29 **Effect of activators of AMPK on neuronal surface numbers of $GABA_{B(1)}$**

a) Cortical neurones were treated with the AMPK activating toxin oligomycin (200nM) for times between 0 and 80 minutes. They were then biotinylated and surface receptors streptavidin purified prior to resolution using SDS-PAGE and western blotting. I125 secondary was used to allow quantification of bands. Although oligomycin appears to enhance surface receptor numbers this is not significant (n=9).

b) Neurones were treated with the less potent but non-toxic AMPK activator, metformin (2mM) prior to biotinylation and purification using streptavidin beads. Metformin caused a consistent increase in phospho-AMPK levels highest approximately 4h after treatment began (i). There was a significant increase in surface $GABA_B$ receptors 5h post metformin (5h vs. cont = 150.7±14.7% ($p<0.05$))

a) i)



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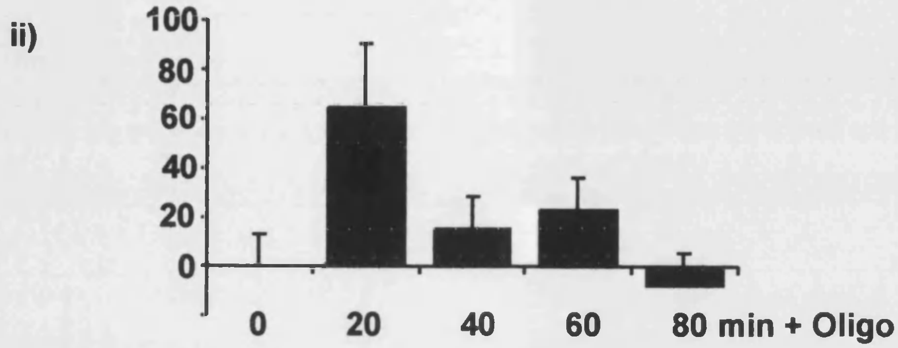

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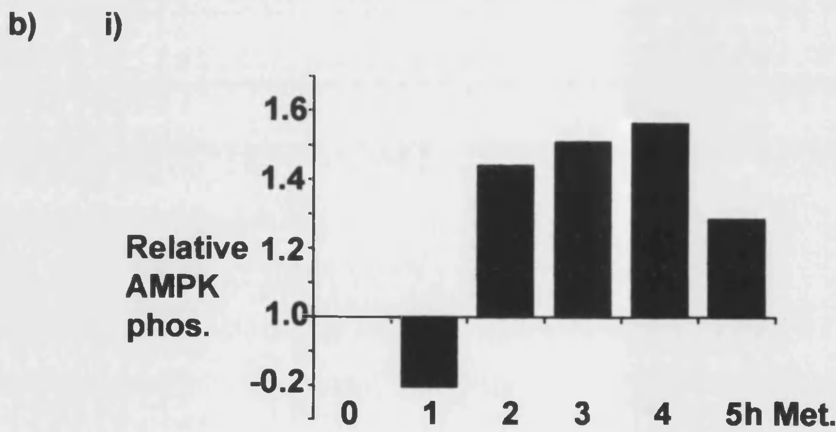
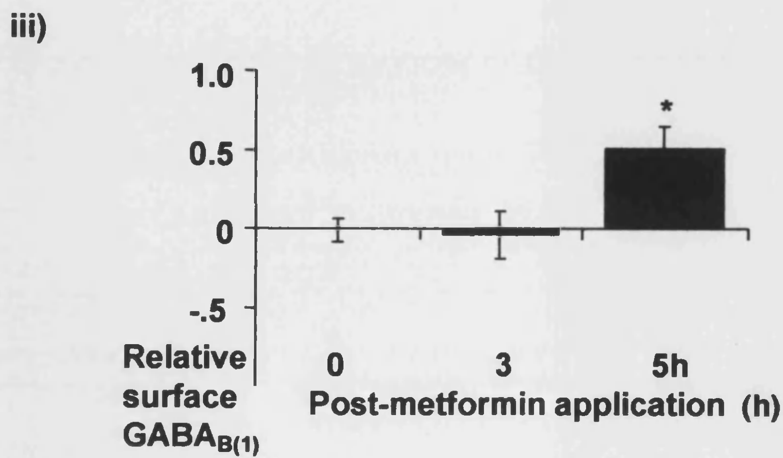


ii)

iii)



when phosphorylated. Preliminary investigations determined that optimal UCL-89 reactivity occurred when membranes had been blocked in 5% BSA, 1xTBS and immunoblotting was carried out overnight at 4°C. CR1 was co-incubated with AMPK at 30°C in the presence or absence of ATP. Kinase reactions were terminated by the addition of SDS loading buffer. 50ng of fusion protein was then loaded per lane and resolved using 12% SDS-PAGE. Proteins were electrotransferred and membranes immunoblotted with UCL-89 (5µg/ml). UCL-89 was observed to cross reacted with CR1 post AMPK phosphorylation and did not recognise GST (fig. 30). There was a small amount of cross-reactivity with unphosphorylated CR1 but this was trivial in comparison to the enhancement in signal achieved through phosphorylation. To conclusively demonstrate that UCL-89 was recognising S917 phosphorylation peptide blocking assays were performed. These showed that addition of even 50ng of phospho-peptide to the antibody containing solution dramatically reduced UCL-89 recognition of phosphorylated CR1, whereas addition of up to 1µg of dephosphorylated peptide had little effect upon immunoreactivity.

5.2.8 UCL-89 cross reacts with a ~110-120kDa doublet from rat brain membranes

To investigate whether S917 is phosphorylated *in vivo* rat brain membranes were prepared including the phosphatase inhibitors sodium fluoride (50mM) and sodium pyrophosphate (10mM) in all buffers. 200µg of membranes were then boiled per sample in SDS loading buffer for 5 minutes prior to resolution using 8% SDS-PAGE. Proteins were electrotransferred and membranes were immunoblotted with UCL-89 in the presence or absence of 100ng phospho-peptide. A doublet band was detected at between 110kDa and 120kDa that was completely blocked by co-incubation of the antibody with 100ng phospho-peptide. These bands correspond with the predicted molecular weights of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ respectively.

5.2.9 Other effects of phosphorylation

S917 forms the final amino acid of the coiled coil motif within the receptor. It was therefore tempting to speculate that phosphorylation of this residue might alter the binding properties of the coil and thus the subunit. To determine whether this might occur I analysed the effect that phosphorylation had upon the interaction of $GABA_{B(1)}$ with $GABA_{B(2)}$. To do this overlay assays were performed where 10µg of either GST, AMPK phosphorylated or mock-phosphorylated CR1 or CR1 with a phosphorylation mimicking S917D mutation were resolved on a 12% SDS-PAGE gel. Proteins were then electrotransferred to nitrocellulose and re-natured in the presence of decreasing concentrations of guanidine hydrochloride. A GST fusion protein of the carboxy-terminal of $GABA_{B(2)}$ (CR2) was then phosphorylated with PKA using $^{32}P\gamma$ATP spiked ATP and used to probe the membranes in an overlay assay. After extensive washing the membrane was exposed to phosphorimager and binding between the different preparations of CR1 and CR2 was quantitated. The GST control showed no detectable binding but the intracellular domains strongly bound to one another. Interestingly there was no significant change in the affinity of the domains for one another irrespective of either phosphorylation or mutation of S917 (fig. 31). This suggests that phosphorylation at this residue has little effect upon the interaction between $GABA_{B(1)}$ and $GABA_{B(2)}$.

CREB 2 /ATF 4 is a transcription factor that has been demonstrated to strongly interact with the carboxy-terminal domain of $GABA_{B(1)}$ by several independent groups (Nehring *et al.,* 2000; Vernon *et al.,* 2001; White *et al.,* 2000). The precise function of this interaction is as yet undetermined as is a mechanism by which the interaction is regulated. The site of interaction has been thoroughly mapped in yeast and involves the coiled coil of $GABA_{B(1)}$. To determine whether phosphorylation of S917 could alter the binding of $GABA_{B(1)}$ to CREB2 pulldowns were carried out using either GST or CR1 that had been either phosphorylated or not by AMPK. To ensure that the effect of phosphorylation on the fusion protein and not the actual kinase assay *per se* (30min incubation in kinase buffer at 30°C) was being tested the negative control involved carrying out the same kinase assay in the presence of AMPK but the absence of ATP. Fusion proteins were then removed from the kinase assay and added to brain lysates prepared according to Vernon *et al.*, (Vernon *et al.*, 2001).

Pulldown assays were performed and fusion purifying proteins resolved using 12% SDS-PAGE. Gels were electrotransferred and probed with an antibody specific to CREB2 (Santa Cruz). CREB2 was observed to strongly associate with CR1 alone and not with GST. Phosphorylation of CR1 by AMPK prevented the interaction between CREB2 and $GABA_{B(1)}$ (fig. 31b).

Figure 30 **Purification of phospho-specific antibody to S917**

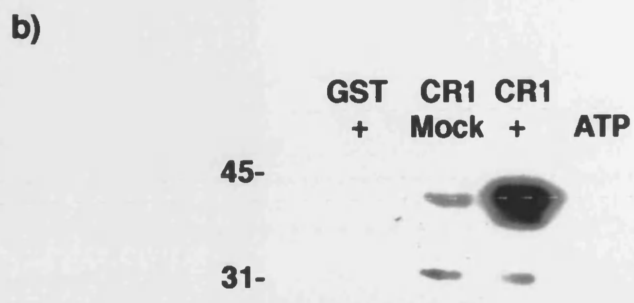
a) Immunising peptide

b) UCL-89 specificallly recognises CR1 after AMPK phosphorylation

c+d) Immunoreactivity is blocked by phospho-peptide but not de-phospho peptide demonstrating phospho-specificity of UCL-89

e) Preliminary blots from brain membranes show doublet of immunoreactivity at 115kDa blocked only by phospho-peptide

a)



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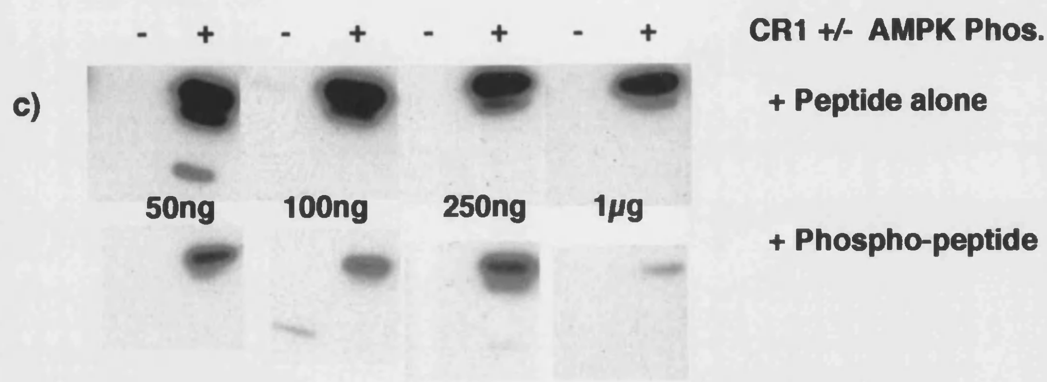
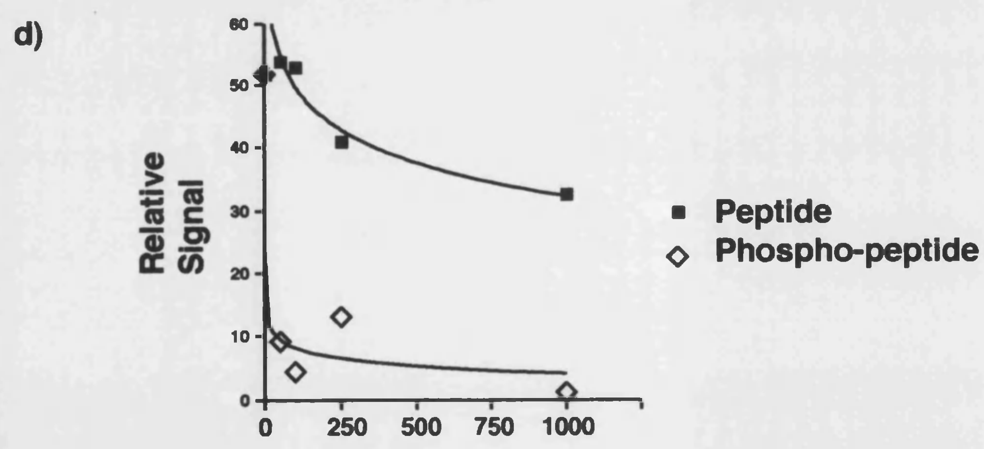


c)

d)



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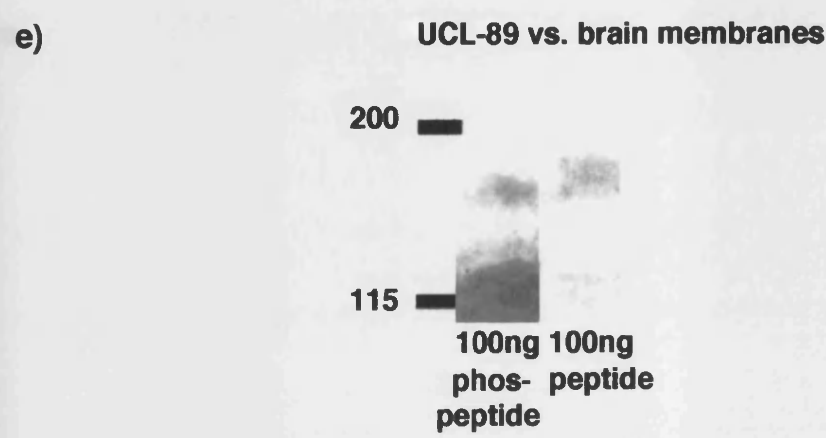

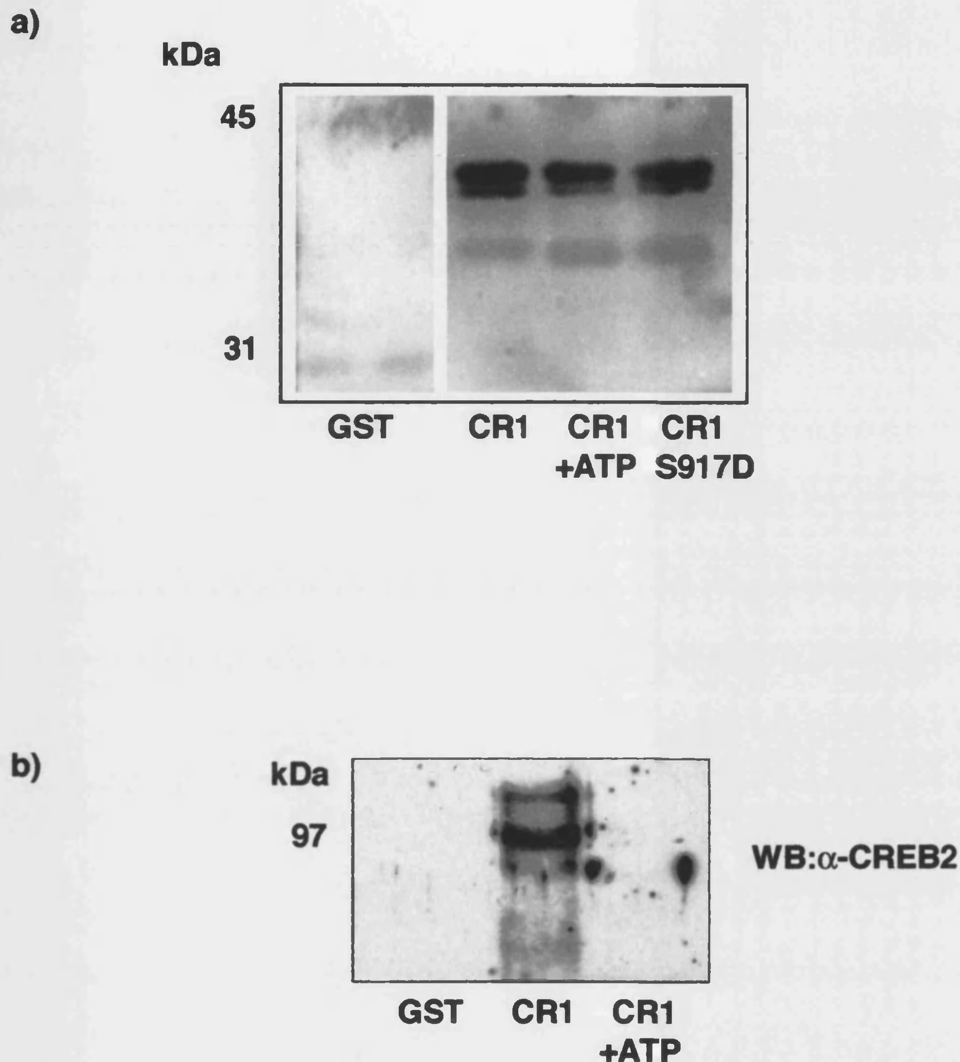

Figure 31 Other effects of phosphorylation

a) Overlay assay was performed against Gst, CR1, CR1 post-ampk phosphorylation and CR1 S917D, probing with CR2. Phosphorylation by AMPK or mutation of S917 to aspartate did not affect binding of CR2 (replicative experiment)

b)Pulldown assays were performed from brain lysates with CR1 that had previously been either mock-phosphorylated or phosphorylated by AMPK. Proteins were resolved using SDS-PAGE and transferred to nitrocellose membranes. These were then blotted with α-CREB2 antibody. CREB2 was observed to run as a dimer and associated exclusively with non-phosphorylated CR1 (replicative experiment).

5.3 Discussion

The yeast two-hybrid interaction between $\alpha1$ AMPK and the carboxy-terminal tail of $GABA_{B(1)}$ involved the carboxy-terminal half of the $\alpha1$ subunit. Further delineation of putative interaction site using *in vitro* translated $\alpha1$ AMPK as a bait was carried out, but significant binding between the fusion protein and the translation product was unachievable. This was observed over a range of different conditions using a variety of buffers. However, as demonstrated above, active AMPK strongly associates with the carboxy-terminal tail of $GABA_{B(1)}$ from brain. This indicates that other factors not present in the translation reaction help to confer binding. It could be that post-translational modifications of $\alpha1$ AMPK are important to determine the correct folding and therefore binding to $GABA_{B(1)}$. Another possibility is that AMPK only binds to $GABA_{B(1)}$ when activated, i.e. phosphorylated at threonine 172, and other possible sites. Activation is unlikely to occur in reticulocyte lysates, for not only is the presence of the upstream AMPKK unknown in this lysate, but also reticulocyte contains an ATP-regenerating system that would maintain AMPK in its inactive state (Woods *et al.,* 1996). Whether the entire heterotrimeric AMPK complex binds to $GABA_{B(1)}$ is not clear, but varying expression patterns of different subunits suggest that AMPK may exist in confirmations other than the heterotrimeric form (Woods *et al.*, 1996). Because the molecular weight of the β subunit of AMPK is 38kDa I was able to probe the same transferred membranes from pull-downs that had shown an association with the α subunit. However, I was unable to obtain a signal from pull-down lanes, and moreover the signal present from lysates itself was very weak. Empirically I observed strong β AMPK subunit immunoreactivity in western blots from crude brain membranes when loading a similar amount of protein to that in the lysates. This indicates differences in the solubility of the β subunit, which can be myristoylated at its N-terminus (Mitchelhill *et al.,* 1997) and the α subunit, and allows for the possibility of an α subunit fraction not bound to β subunit. This would not be entirely without precedent, for within skeletal muscle the amount of α subunit greatly exceeds that present for either β and γ, denoting a large proportion of the α subunit is not complexed with β and γ. It does not appear that the presence of the β and γ subunits are essential for catalytic activity, but instead they act as modulators of both activity and cellular localisation. Because the binding sites on α AMPK of $GABA_{B(1)}$ and β

AMPK subunit overlap it may be that the binding of either β AMPK or $GABA_{B(1)}$ is mutally exclusive to α AMPK. Unfortunately time did not allow for further investigation of this possibility.

A further controlling experiment to analyse the specificity of UCL-89 would be to blot brain homogenates obtained from $GABA_{B(1)}$ knockout mice. There should be no cross-reactivity with proteins in these homogenates, verifying that the bands observed in wild type brain are from $GABA_B$ receptors.

Throughout this study on the activity I have been hampered by the lack of a non-toxic method by which to manipulate the activity of AMPK. Unlike PKA specific activators of AMPK do not as yet exist. I experimented with several toxic activators including oligomycin, but it is difficult to tell whether effects observed are due to AMPK activation. Metformin however is non-toxic even at relatively high concentrations is non-toxic. However it is not as potent as the metabolic poisons (Zhou *et al.*, 2001; Fryer *et al.*, 2002), and in neurones I did not record an increase in phosphorylation of more than ~80% at T172. This increase in T172 phosphorylation was prolonged over several hours (fig. 29) and was great enough to have an apparent effect. Although time considerations meant that investigations into the physiological effects of activating AMPK were of a preliminary nature metformin significantly increases the surface levels of $GABA_B$ receptors probably through the insertion of new receptors. Similar to in COS cells where the mimicry of phosphorylation of both S917 and S923 enhanced trafficking of the receptor, it may be that phosphorylation in neurones helps overcome ER retention and may more efficient trafficking of the complete receptor. Further work of an electrophysiological nature is needed to confirm that these additional receptors are functional. If inserted receptors are functional however, this may represent a novel mechanism for modulating neuronal function in the onset of metabolic stress. An enhancement in $GABA_B$ activity might be expected to lead to a decrease in neuronal firing, and thus decrease the metabolic demand. Analogous to this possible mechanism, it has been previously demonstrated that phosphorylation of NMDA receptors channels can enhance surface delivery, most probably by helping to overcome ER retention (Scott *et al.*, 2001).

Ideally the most appropriate experiments to carry out to determine whether AMPK has an effect upon $GABA_B$ receptor surface level and signalling would be to carry out cell surface studies and electrophysiology in neurones from mice deficient in the catalytic subunit of AMPK. Recently data has been reported from mice deficient in the $\alpha2$ catalytic subunit of AMPK (Viollet *et al.,* 2003). Although the study concentrated on measuring the metabolic function of these mice, it was noted that they had altered autonomic responses consistent with enhanced catecholamine release. This was proposed to be due to a central defect in the control of sympathetic activty, (Viollet *et al.,* 2003) indicating AMPK activity may modulate the sympathetic nervous system.

The observation that CREB-2 no longer associates with phosphorylated CR1 was interesting and may provide a possible mechanism controlling this interaction. The fact that phosphorylation did not affect the affinity of CR1 for CR2 shows that this site may be phosphorylated within the complete receptor. This, in combination with the immunoprecipitation data suggests that as well as possibly regulating receptor trafficking, it is feasible that AMPK may interact with and regulate the function of the heterodimerised receptor in response to cell stress.

CHAPTER SIX Final Discussion

6.1 Summary

Regulation of GPCR signalling is crucially important in controlling neuronal activity. An intricate system involving kinase activation and subsequent arrestin mediated receptor sequestration has evolved to allow the desensitisation of activated GPCRs (Pierce *et al.*, 2002). Much of this mechanism has been delineated in recombinant expression systems, but the use of knockout animals has confirmed its importance in the control of GPCR activity in neurones (Luttrell & Lefkowitz 2001). This thesis describes work undertaken to identify pathways that can control the surface number and hence the signalling of $GABA_B$ receptors in heterologous and native systems. In addition I have also undertaken the preliminary characterisation of a novel interaction between the stress activated kinase AMPK and $GABA_{B(1)}$. This interaction may have importance in governing surface $GABA_B$ receptor numbers and activity.

Initial studies in COS-7 cells demonstrated that both $GABA_B$ receptor subunits are basally phosphorylated and that, surprisingly, agonist has no effect upon the level of this phosphorylation. Overexpression of several different GRK constructs did not confer an agonist-induced phosphorylation upon $GABA_B$ receptors and this suggested that the classical agonist-induced increase in phosphorylation observed in a large number of GPCRs was not relevant to $GABA_B$ receptor signalling. In support of this I noted no interaction between the arrestin proteins and $GABA_B$ receptors, regardless of agonist application, and no significant internalisation of the receptor in COS-7 cells.

Further studies in cultured neurones showed that the lack of internalisation observed in a recombinant system was replicated with native receptors. Furthermore, it was demonstrated that surface $GABA_B$ receptors have an unusually long half-life, in excess of 30h. Application of the agonist baclofen significantly and specifically reduced the half-life of surface $GABA_B$ receptors but this was only observed over long time periods. Interestingly the presence of lysosomal inhibitors did not alter degradation rates, and coupled to the lack of a detectable internalised

pool, it is probably that $GABA_B$ receptors are either degraded at the surface membrane or are rapidly degraded upon internalisation so as to prevent the accumulation of an internalised pool.

Modulation of baclofen induced degradation was achieved by activating PKA or pathways leading to PKA activation. Activation of $GABA_B$ receptors negatively regulates adenylyl cyclase activity and so leads to a decrease in intracellular cAMP and hence a reduction in PKA activity. Thus stimulation of PKA by the application of exogenous activators would be expected to compensate for the effect of baclofen. Analysis of the phosphorylation state of S892 in $GABA_{B(2)}$ has previously shown that chronic baclofen can reduce phosphorylation at this site (Couve *et al.*, 2002). In the study presented here it was possible to correlate the protective effect of PKA activation with increased phosphorylation at S892. This strongly infers that phosphorylation at S892 is not only important in the relatively short-term control of desensitisation events, but also in the long-term modulation of surface receptor number. In concordance with the increase in degradation rate elicited by baclofen, it was found that chronic baclofen treatment decreased the number of $GABA_B$ receptors at the neuronal surface. Interestingly though, chronic baclofen treatment did not trigger downregulation as measured by total protein levels of $GABA_B$ receptor. It thus appears that insensitivity to agonist-induced downregulation is another distinguishing feature of $GABA_B$ receptors. This is in agreement with the fact that GPCR endocytosis is an important preliminary step to downregulation (Tsao *et al.*, 2001). This lack of downregulation helps to explain why, in the clinical context of treating spasticity, $GABA_B$ receptors remain sensitive to baclofen after years of administration (Meyerthaler *et al.*, 2001).

The remainder of this thesis focussed upon studying the phosphorylation of $GABA_{B(1)}$ subunit. $GABA_{B(1)}$ is phosphorylated to a high degree in COS-7 cells and this phosphorylation appears confined to the intracellular carboxy-terminal domain. I then observed that fusion proteins encoding the carboxy-terminal domain of $GABA_{B(1)}$ provide a substrate to a kinase associating from brain homogenates. This kinase appeared insensitive to inhibitors of classical second-messenger activated kinases, but was unexpectedly robustly activated by heparin. Yeast two-hybrid screens carried out by collaborators at GlaxoSmithline identified the catalytic subunit of AMPK as a

putative interacting protein with the carboxy-terminal domain of $GABA_{B(1)}$. In this thesis I have confirmed that $GABA_{B(1)}$ can form an *in vitro* substrate for AMPK. Intriguingly the AMPK phosphorylation of $GABA_{B(1)}$ was also stimulated by heparin to a similar degree to the kinase associating from brain suggesting they are one and the same. Use of site-directed mutagenesis allowed me to map the major site of AMPK phosphorylation to S917 within the carboxy-terminal domain, although there appeared to be residual phosphorylation at other sites, most probably S923.

Pull-down assays performed from brain lysates demonstrated that as well as interacting in yeast, AMPK was able to interact with $GABA_{B(1)}$ in brain. Western blotting demonstrated that specifically the activated α subunit of AMPK was able to associate with $GABA_{B(1)}$. Kinase assays performed after pulldowns demonstrated an enhancement in $GABA_{B(1)}$ phosphorylation upon the addition of 5'AMP, confirming the kinase was in an active state. Immunoprecipitations confirmed that AMPK can associate with $GABA_{B(2)}$ in brain. Because these two proteins are unable to interact in yeast, coupled to the fact that almost all $GABA_{B(2)}$ is heterodimerised with $GABA_{B(1)}$ in brain (Benke *et al.*, 1999), the co-immunoprecipitation of the two proteins is coherent with AMPK interacting with the complete $GABA_B$ receptor. It is notable that this is the first reported yeast 2-hybrid hit with $GABA_{B(1)}$ that probably associates with the dimerised receptor.

Aspartate mutations of $GABA_{B(1)}$ S917 and S923 suggest that sequential phosphorylation of both S917 and S923 can enhance surface delivery of the dimerised receptor, implicating phosphorylation in $GABA_B$ trafficking events. It was also noted that mutation of both these residues permits the ER exit of $GABA_{B(1)}$ in the absence of $GABA_{B(2)}$ co-expression. I then went on to affinity purify an antibody specific to phospho-S917 – UCL-89. UCL-89 strongly cross-reacted with the carboxy-terminal domain of $GABA_{B(1)}$ but only after phosphorylation by AMPK. Preliminary investigations using UCL-89 suggested that $GABA_{B(1)}$ is phosphorylated at S917 site *in vivo*. Early data also indicates that activators of AMPK can influence the surface receptor complement of neurones.

Phosphorylation of $GABA_{B(1)}$ by AMPK has no effect upon the interaction of $GABA_{B(1)}$ with $GABA_{B(2)}$, but it does prevent the interaction of $GABA_{B(1)}$ with CREB2. The lack of effect upon the interaction between $GABA_{B(1)}$ and $GABA_{B(2)}$ by

phosphorylation suggests that this residue can be phosphorylated in the complete receptor implying that phosphorylation of S917 may affect receptor activity. The demonstration that CREB2 is no longer able to interact from brain with phosphorylated $GABA_{B(1)}$ points towards a mechanism controlling the interaction between these two proteins. It is highly probable that the position of S917 at the end of the coiled coil allows phosphorylation at this site to regulate other protein interactions through the coiled coil.

6.2 Implications of $GABA_B$ receptor surface stability

Recent work demonstrates that $GABA_A$ receptors are highly dynamic and that their surface number is tightly regulated by endocytotic pathways (Kittler *et al.,* 2000; Kittler & Moss 2003). This means that changes in receptor number can rapidly and critically influence synaptic efficacy, leading to continual variations in $IPSP_A$. The inhibitory response provided by $GABA_B$ receptor activation is markedly different both in onset and duration from that of $GABA_A$ inhibitory activity. The role of $GABA_B$ receptors coupling to inwardly rectifying potassium channels is to provide an inhibitory current that is longer lasting than that provided by $GABA_A$ receptors. The inherent stability of $GABA_B$ receptors observed gives a molecular basis for this physiological action. It is conceivable that if $GABA_B$ receptors were rapidly trafficked in a manner akin to other GPCRs upon activation then the receptors would be unable to provide the prolonged inhibitory $IPSP_B$. It is plausible that the heterodimeric configuration of $GABA_B$ receptors precludes interactions with GRKs and arrestins that may otherwise occur. An interesting hypothesis is that $GABA_B$ receptors have evolved a heterodimeric nature precisely for this reason.

Although this study demonstrated an inherent surface stability of $GABA_B$ receptors, I did note increases in their degradation in response to chronic agonist application. This identifies a novel mechanism by which neurones can tailor the complement of $GABA_B$ receptors over longer time periods. The observation that activation of PKA or PKA stimulating pathways in the form of β adrenoceptors reduces degradation suggests baclofen triggered degradation is allied to prolonged dampening of PKA activity elicited through baclofen application. It could be argued that the increase in $GABA_B$ degradation and the concomitant decrease in surface

$GABA_B$ receptors should be viewed as a cellular attempt to rectify this perturbation. Interestingly, the protective effect of PKA activation was correlated to an increase in the phosphorylation of S892 in $GABA_{B(2)}$, which otherwise is reduced in response to chronic agonist (Couve *al* 2002). Previously it had been proposed that phosphorylation of S892 prevented the internalisation of the receptor (Couve *et al.,* 2002). However, the results presented in this thesis and by other groups show that $GABA_B$ receptors do not readily internalise. It is more likely that phosphorylation of S892 prevents other post-receptor activation sequelae occurring, thus leading to delayed desensitisation in tandem with protection from surface degradation over chronic periods. S892 phosphorylation may be controlled as function of total PKA activity. A large number of drugs used to treat psychiatric conditions such as depression and anxiety act either directly or indirectly at β- adrenoceptors which activate adenylyl cyclase. It is likely that they have downstream effects upon $GABA_B$ receptor degradation, possibly by influencing the phosphorylation at S892. In agreement with this possibility it has been recently been shown that chronic cocaine greatly decreases $GABA_{B(2)}$ receptor phosphorylation and is correlated to reduced $GABA_B$ receptor activity (Xi *et al.*, 2003).

6.3 Regulation of $GABA_B$ receptor function in response to metabolic demands

Immunolabelling studies of $GABA_B$ localisation demonstrate that in the cerebellum $GABA_{B(1)}$ stains in a zebrin (aldolase-C) like pattern (Fritschy *et al.*, 1999). Aldolase-C is a marker of metabolic acitivity and this implies that $GABA_B$ receptor expression can be regulated as a function of metabolic activity. The interaction between $GABA_{B(1)}$ and AMPK is particularly exciting because it provides additional evidence that events affecting the neuronal metabolic status may have downstream effects upon inhibitory neurotransmission. The data presented suggests that AMPK is able to associate with dimerised $GABA_B$ receptors, and therefore it is likely that AMPK phosphorylates sites is able to act to modify receptor G-protein coupling. Mutagenesis studies indicate that phosphorylation at S917 and S923 may affect trafficking of the receptor. This is most probably due to change of charge at S923 interupting the RSRR ER retention motif which it lies in. Metformin causes an increase in surface $GABA_B$ receptor number in

neurones. This is unlikely to be due to an effect on protein synthesis because activators of AMPK have catabolic as opposed to anabolic activities. Whether this increase correlates with phosphorylation requires further investigation. UCL-89 was raised to monitor the phosphorylation state of $GABA_{B(1)}$ in the face of metabolic stress but unfortunately time-constraints meant that this study could not be contained within this thesis.

6.4 Future Directions

It is apparent that surface $GABA_B$ receptors are exceptionally stable, but eventually they are degraded in a non-lysosomal manner. The mechanism behind $GABA_B$ degradation requires further clarification; receptors may be degraded by membrane associated proteases or they may internalise (albeit extremely slowly) and be rapidly cleaved so that an intracellular pool is not observed. The protective effect of phosphorylation also needs further analysis. It could be due to the promotion of interactions of $GABA_{B(2)}$ with anchoring molecules or by targeting $GABA_B$ receptors to less dynamic regions of the cell. Further work will be useful to clarify the precise role of phosphorylation at serine 892 and to screen for proteins that interact with this region.

Mechanisms regulating AMPK function in neurones have not been investigated and little is known about how neuronal AMP levels are regulated. Future work should determine whether other neuronal phenomena such as LTP formation can activate AMPK *in vivo*. It is also conceivable that a downstream effect of cAMP accumulation is an increase in AMP levels due to the action of phosphodiesterases. Because it is very difficult to pharmacologically manipulate AMPK function without causing non-specific effects, future studies of AMPK regulation of $GABA_B$ activity could ideally take place in neurones from AMPK knockout mice using electrophysiological and cell biology techniques. It is possible that the charge change through phosphorylation may also alter receptor to G protein coupling and this could be studied using mutants and FLIPR assays. The intracellular domains of $GABA_B$ receptor subunits may interact directly with G proteins in a phospho-dependent manner as observed in mGluR receptors (El Fae & Betz 2002) and this could be investigated with binding studies.

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