Identification of interactions between signalling molecules and $\text{GABA}_A$ receptors

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ABSTRACT

GABA<sub>A</sub> receptors are the main sites of inhibitory neurotransmission in the brain. They are also the sites of action of a number of clinically important drugs such as the benzodiazepines and barbiturates. Phosphorylation has been identified as an important mechanism by which neurons control the function of these receptors.

GABA<sub>A</sub> receptors are hetero-pentamers which are assembled from the subunit classes α, β, γ, δ and ε. Subunits have a distinctive topology which includes 4 transmembrane domains, extracellular amino- and carboxy termini, and a large intracellular domain between transmembrane regions 3 and 4. In vivo most benzodiazepine sensitive receptors are believed to be composed of α, β, and γ subunits. Intracellular domains of receptor subunits contain consensus phosphorylation sites for a range of protein kinases, including the cAMP-dependent kinase (PKA), the Ca<sup>2+</sup>-phospholipid dependent kinase (PKC) and the non-receptor tyrosine kinase src. Studies with subunit cDNAs transiently expressed in heterologous cells, has identified principally the β and γ2 subunits as substrates in vivo for these kinases. To date there have been few studies on the phosphorylation of the native receptor. Using polyclonal antibodies, which were raised for this study, the tyrosine phosphorylation of the γ2 subunit has been able to be studied for the first time in neurons. Using an antibody which recognises the first 29 amino-terminal amino-acids of the γ2 subunit, this protein was shown to be tyrosine phosphorylated in adult rat brain lysate. It has been previously shown that the tyrosine kinase src phosphorylates the residues Y365 and Y367 of the γ2 subunit in HEK293 cells, causing up-regulation of receptor function. The second antibody successfully raised in this study recognises the γ2 subunit when it is specifically phosphorylated on these two tyrosine residues. This allowed a more direct analysis of γ2 subunit tyrosine phosphorylation in adult rat brain lysate, and cultured cortical neurons. Results indicated that in cortical neurons phosphorylation of the γ2 subunit, on Y365 and Y367, is under very tight regulation.
At present, there is little known as to how kinases or any other signalling molecules interact with GABA<sub>A</sub> receptors in neurones. Previous studies have identified serine and tyrosine kinase activities co-purifying with the receptor from brain preparations. To identify how protein kinases and related signalling molecules are targeted to the GABA<sub>A</sub> receptor, the intracellular domains of receptor subunits were used as baits to probe rat brain extracts. This approach has identified a very intimate association between PKC, the ‘Receptor for Activated C-kinase’, RACK-1, and the GABA<sub>A</sub> receptor β1 and β3 subunits.

These interactions were initially identified by analysis of proteins bound to receptor fusion proteins in in vitro ‘pull-down’ assays. Both molecules were identified as binding directly and independently to the receptor by ‘gel-overlay’ assays. Confirmation of the in vivo existence of this complex was performed by co-precipitation of RACK-1 and PKC with the GABA<sub>A</sub> receptor from transfected HEK293 cells and from cultured cortical neurons. The functional significance of the RACK-1 interaction was investigated electrophysiologically in HEK293 cells, by disrupting the binding of RACK-1 with a polypeptide corresponding to its binding site on the GABA<sub>A</sub> β1 subunit. Finally, in cortical neurons, the β3 subunit was shown to be highly basally phosphorylated, due to the activity of closely associated PKC.
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKAP</td>
<td>A-Kinase Anchoring Protein</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CACA</td>
<td>cis-4-amino-crotonic acid</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>Concentration of agonist which produces half-maximal response</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABA(_A)R</td>
<td>Gamma-aminobutyric acid type A receptor</td>
</tr>
<tr>
<td>GABA(_B)R</td>
<td>Gamma-aminobutyric acid type B receptor</td>
</tr>
<tr>
<td>GABA(_C)R</td>
<td>Gamma-aminobutyric acid type C receptor</td>
</tr>
<tr>
<td>GlyR</td>
<td>Glycine receptor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>5-HT(_3)R</td>
<td>5-hydroxytryptamine type 3 receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Myristoylated alanine-rich C-kinase substrates</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nAchR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>$^{32}$P-γ-ATP</td>
<td>ATP with phosphorous 32 isotope at the gamma position</td>
</tr>
<tr>
<td>PDBu</td>
<td>Phorbol 12:13 dibutyrate</td>
</tr>
<tr>
<td>PICKs</td>
<td>Proteins that interact with C-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Ca$^{2+}$/phospholipid-dependent protein kinase</td>
</tr>
<tr>
<td>PKCI</td>
<td>PKC inhibitor peptide</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylinserine</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>RACK</td>
<td>Receptor for activated C-kinase</td>
</tr>
<tr>
<td>RICK</td>
<td>Receptor for inactivated C-kinase</td>
</tr>
<tr>
<td>SBP</td>
<td>Substrate binding protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pp60$^{c-src}$</td>
<td>Cellular homolog of pp60$^{v-src}$</td>
</tr>
<tr>
<td>pp60$^{v-src}$</td>
<td>60kD transforming gene product of the Roux Sarcoma virus</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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1.1. INTRODUCTION

1.1.1. γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain

GABA is the main inhibitory neurotransmitter in the brain. The initial observation that it is a neurotransmitter came in 1957, when an extract from mammalian brain was shown to inhibit the activity of the crayfish stretch receptor (Bazemore, 1957). The extract was found to contain GABA. Further studies in crustacea proved that GABA fulfilled all the criteria to be classified as a neurotransmitter, including mechanisms for synthesis, release, postsynaptic action and removal. Studies, principally in cultured chick spinal cord neurons, showed that this was also the case for the vertebrate central nervous system (For review see Farrant, 1990).

The importance of GABA as a neurotransmitter is emphasised by the estimate that 20-50% of all central synapses use GABA as their neurotransmitter (Bloom et al., 1971, Young et al., 1990). Like other neurotransmitters, e.g. L-glutamate and serotonin, GABA activates both ionotropic and metabotropic receptors. The former are ligand-gated ion channels which mediate fast synaptic transmission, while the latter couple to G-proteins and modulate synaptic transmission through intracellular signalling pathways (Bowery et al., 1993; Smith et al., 1995).

1.1.2. Ionotropic and metabotropic GABA receptors

To date three types of GABA receptor have been identified, according to their pharmacological and electrophysiological properties. The initial classification into \( \text{GABA}_A \) and \( \text{GABA}_B \) receptors, came about upon the discovery of a novel GABA receptor which was unaffected by recognised GABA antagonists such as bicuculline, but was activated by baclofen, normally inactive at the classical GABA binding site (Hill et al., 1981). The classical GABA receptor was named the \( \text{GABA}_A \)-subtype receptor, and the novel receptor the \( \text{GABA}_B \) receptor. The \( \text{GABA}_A \)-
receptor has since been shown to be a member of the Ligand Gated ion-channel Super-family. Binding of GABA or structural analogues of GABA e.g. muscimol results in the opening of an integral chloride ion (Cl⁻) channel, causing an influx of Cl⁻ ions, and rapid hyperpolarisation of the post-synaptic cell, leading to stabilisation of the resting potential during the activation of excitatory receptors (Sakmann et al., 1983; Bormann et al., 1987). The GABA\textsubscript{B} receptor upon activation produces a much slower inhibitory current, by activating intracellular G-proteins, which activate other second-messenger systems. In turn these regulate the opening of potassium and calcium channels, resulting in hyperpolarising of the membrane potential. The GABA\textsubscript{B} receptor was only recently cloned and shown to be a seven transmembrane receptor, bearing significant homology to the metabotropic glutamate receptors (Kaupmann et al., 1997). Originally a single subunit, GABA\textsubscript{B}R1 was cloned, but in recombinant systems it did not reproduce the pharmacology of the neuronal receptor. More recently a second subtype was identified by a number of groups, GABA\textsubscript{B}R2, which hetero-dimerises with R1, to produce functional GABA\textsubscript{B} receptors (Jones et al., 1998; White et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999).

A third class of GABA receptor, GABA\textsubscript{C}, was identified by its activation by the GABA analogue, cis-4-aminocrotonic acid (CACA), in a baclofen and bicuculline insensitive manner in binding studies (Drew et al., 1984). Patch-clamp studies later measured a receptor, which showed bicuculline and baclofen insensitive, CACA evoked currents in cultured retinal rod bipolar cells (Feigenspan et al., 1993).

1.1.3. Pharmacologic modulation of the GABA\textsubscript{A} receptor

The GABA\textsubscript{A} receptor is the site of action of many important compounds, of both experimental and clinical value. They are thought to bind to specific sites on the receptor, causing an allosteric change in receptor function. A summary of the better characterised ligands is detailed below.
Benzodiazepines; for example diazepam and librium, enhance the action of GABA at the GABA_\text{A} receptor by increasing the frequency of channel opening (Study et al., 1981). They have important clinical effects which include anti-anxiety and sedative actions. Analogues or related substances to the benzodiazepines have antagonistic (inhibiting benzodiazepine agonists but not GABA) or inverse agonist (inhibiting GABA_\text{A} receptor function) efficacy. For example flumazenil (Ro 15-1788), a benzodiazepine antagonist, inhibits the effects of benzodiazepines, without affecting GABA action. While the inverse agonist, 6,7-dimethyl-4-ethylcarboline-3-carboxylate methyl ester (DMCM), reduces the frequency of channel opening (Rogers, 1994).

Barbiturates; both enhance GABA responses, and mimic GABA by opening the integral ion channel in the absence of GABA (Macdonald and Olsen., 1994), so accounting for their sedative, and anticonvulsant effects. For example phenobarbital and pentobarbital increase the average open duration of GABA_\text{A} receptor single channel currents without altering channel conductance (Barker et al., 1979; Study et al., 1981). It is thought that they alter the intrinsic gating of the channel once GABA is bound, so increasing the proportion of channel openings of longer duration (Macdonald et al., 1989).

Steroids; naturally occurring steroids are seen to enhance GABA_\text{A} receptor function in a manner reminiscent of the barbiturates. The steroids pregnanolone and androsterone are seen to enhance GABA current by increasing both the probability of longer channel openings, as seen with the barbiturates, and also channel opening frequency (Twyman et al., 1992). Other steroids, such as dehydropiandrosterone sulphate, inhibit the GABA current, acting as an allosteric receptor antagonist (Majewska et al., 1990).

Anaesthetics; the major effect of anaesthetics is to shift the GABA-response curve to lower concentrations, with very little effect at high
GABA concentrations (Franks et al., 1994). For example the anaesthetics isoflurane, enflurane and halothane increase GABA currents at low GABA concentrations, but are ineffective at higher GABA levels. It is believed that these effects are due to a specific interaction of the anaesthetic with the receptor, as shown in a study of both GABA\textsubscript{A} and glycine receptors, which identified sites within the receptors necessary for the effects of enflurane and ethanol (Mihic et al., 1997).

Picrotoxin; picrotoxin is a non-competitive inhibitor of the GABA\textsubscript{A} receptor, acting at a site which is distinct from the site of GABA binding, within the ion channel pore. It binds preferentially to an agonist-bound state, stabilising it in a closed conformation (Newland et al., 1992). It decreases the mean average open duration and burst duration, in direct contrast to the effects of barbiturates noted above. Other agents are also thought to act at the picrotoxin site, for example t-butylicyclophosphorothionate (TBPS) (Whiting et al., 1995) and certain benzodiazepines e.g.Ro 5-3663.

Ethanol; ethanol and other alcohols have been shown to have a potentiating effect on GABA\textsubscript{A} receptors (Dietrich et al., 1989). For example ethanol enhances the response of cultured spinal cord neurons to GABA (Celentano et al., 1988). The effects of ethanol at low concentrations were thought to be dependent on phosphorylation of a specific residue on an intracellular domain of the GABA\textsubscript{A} receptor (Wafford et al, 1991; Wafford et al., 1992). But more recently it has been shown that targeted deletion of this region has no effect on the ethanol response (Homanics et al., 1999), while sites within the transmembrane domain of the receptor have been shown to be important for the modulation seen at higher concentrations of ethanol (Mihic et al., 1997).

A number of other compounds have been shown to have modulatory effects on the GABA\textsubscript{A} receptor. These include the anti-convulsant drug
lorectlezo (Wafford et al., 1994), zinc ions (Smart, 1992) and penicillin (Schneiderman et al., 1994).

1.2. Structure of GABA_\textsubscript{A} receptors

1.2.1. Initial discovery of GABA_\textsubscript{A} receptor polypeptides

Isolation of a bovine GABA\textsubscript{A}/Benzodiazepine receptor complex by benzodiazepine affinity chromatography, followed by separation by SDS-PAGE, revealed two polypeptide bands. They were termed the \( \alpha \) subunit, molecular weight of 51kD and \( \beta \) subunit, 56-58kD (Sigel et al., 1983; Mamalaki et al., 1987; Stephenson et al., 1988). Also it was discovered that the 51kD polypeptide could be labelled by \(^{3}\text{H}\)-flunitrazepam, and the larger protein by \(^{3}\text{H}\)muscimol. (Deng et al., 1986; Stauber, et al. 1987). The cDNAs were cloned for the two original subunits, after cyanogen bromide cleavage of the whole receptor and micro-sequencing of the peptides, which allowed the design of oligodeoxyribonucleotide probes to screen bovine cDNA libraries (Schofield et al, 1987). Co-injection of mRNA coding for \( \alpha \) and \( \beta \) into Xenopus oocytes resulted in the formation of functional GABA gated chloride channels, which were insensitive to benzodiazepines (Schofield et al, 1987).

1.2.2. GABA_\textsubscript{A} receptors are members of the ligand-gated ion channel superfamily

Analysis of the cloned \( \alpha \) and \( \beta \) subunits showed that they shared a common structure with other ion channels which allows the grouping of GABA\textsubscript{A} receptors with the nicotinic acetylcholine receptors (nAChR) (Noda et al., 1983), the glycine receptor (Grenningloh et al., 1987) and the serotonin gated 5HT3 ion channel (Maricq et al., 1991), into a superfamily known as the ligand gated ion channel family.
Figure 1 Quaternary structure and subunit topology of the GABA$_A$ receptor

EC - extracellular
IC - intracellular

NH$_2$, COOH

4 TM domains

Major Intracellular domain
All the members of this family share a number of common structural characteristics including: a cleavable signal sequence, a large N-terminal extracellular domain with putative sites for glycosylation and disulphide bonded β-structural loop, four transmembrane domains (TM), and a major intracellular domain between TM3 and TM4 (see figure 1 for a schematic diagram of the GABA$_A$ receptor) (Barnard et al., 1987).

1.2.3. Quaternary structure of the ligand gated ion channel family

The quaternary structure of these ion-channels is largely based by analogy with the nACHR. This receptor was originally shown to have five subunits by gel electrophoresis (Lindstrom et al., 1979). Detailed electron-micrograph work, to a resolution of 9Å, on crystalised nACHRs from the electric organ of the torpedo ray, have shown the receptor is a pentamer of approximately 250kD, with a central cation-specific pore (Unwin et al., 1988; Unwin, 1989). In comparison, other members of the ligand gated ion channel family are not found in such high-density preparations. For example in the brain the GABA$_A$ receptor is of low abundance and high heterogeneity, so such detailed methods cannot be applied to them. Due to the large amount of sequence homology between the family members the same structure is thought to apply. GABA$_A$ receptors, purified by benzodiazepine chromatography from porcine cortex, have been visualized by electron microscopy though, with a similar pentameric arrangement observed (Nayeem et al., 1994).

1.2.4. Diversity of GABA$_A$ receptor subunits

The initial cloning of the α and β subunits from a bovine brain cDNA library, led rapidly to the isolation of a large number of GABA$_A$ receptor subunit isoforms. This included five additional α subunit isoforms, α2, α3 (Levitan et al., 1988), α4 (Ymer et al., 1989) α5 (Khrestchatisky et al., 1989; Malherbe et al., 1990) and α6 (Kato et al., 1990; Luddens et al., 1990) and three additional β subunits, β2, β3 (Ymer et al., 1989) and in chicken a β4 subunit (Lasham et al., 1991). No β1 subunit has been
identified in chicken however (Lasham et al., 1991). Additional subunit classes based upon the deduced amino acid sequence homology now include: \( \gamma(1-4), \delta, \varepsilon \) and \( \pi \). (Macdonald and Olsen., 1994; Rabow et al., 1995; Whiting et al., 1997; Davies et al., 1997; Hedblom et al., 1997). Typically there is 30-40% homology between subunit classes and 70-90% between subunit isoforms. The greatest areas of structural diversity are found within the large intracellular domains between TM3 and TM4 (Macdonald, 1994). Also 3 homologous cDNAs \( \rho 1-3 \), which are expressed principally in the retina have been cloned (Cutting, 1991; Wang, 1994; Ogurusu, 1996). They form receptors, which unlike \( \text{GABA}_A \) receptors are bicuculline-insensitive, have smaller single channel conductances and show little desensitization. They show the greatest divergence amongst the subtypes, and are viewed as a separate class of \( \text{GABA}_A \) receptor, the previously described \( \text{GABA}_C \) receptors (Cutting et al., 1991; Macdonald, 1994; Rabow et al, 1995).

1.2.5. Alternative splicing of \( \text{GABA}_A \) receptor mRNAs

Diversity of structure is further enhanced by alternative splicing of the \( \alpha 6, \beta 2, \beta 4, \gamma 2 \) and \( \rho 1 \) subunits mRNAs in a number of species (Whiting et al., 1990; Bateson et al., 1991; Kofuji et al., 1991; Harvey et al., 1994; Korpi, et al. 1994; McKinley et al., 1995) With the exception of the \( \alpha 6 \) and \( \rho 1 \) subunits, the structural diversity generated by these splicing events occurs within the predicted major intracellular domains of receptor subunits.

1.3. Cell biology of the \( \text{GABA}_A \) receptor

1.3.1. Regional and developmental heterogeneity of \( \text{GABA}_A \) receptor expression in the brain

There is a large developmental and regional heterogeneity of \( \text{GABA}_A \) receptor expression in the brain. In situ hybridisation with subunit specific oligonucleotide probes and immunolocalisation methodologies have been used to examine the diversity of \( \text{GABA}_A \) receptor structure in
the brain (Fritschy et al., 1992; Laurie et al., 1992a; Wisden et al., 1992). In the adult rat the α1, β2, β3, and γ2 subunits are abundantly expressed throughout the brain (Wisden et al., 1992; Laurie et al., 1992a). While for example the α6 subunit is found only in cerebellar granule cells (Kato et al., 1990). The β1 subunit is found at a reduced abundance and more restricted expression compared to β2/3. The γ1 and γ3 subunits though expressed widely, are at a much lower level than γ2 (Shivers and Seeburg, 1989; Herb et al., 1992). The δ subunit is expressed at a high level in granule cells of the cerebellar cortex and the thalamus (Shivers and Seeburg, 1989).

Potential receptor subtype compositions can be predicted by the co-expression of subunits in the same neuronal populations. A major subtype is thought to consist of α1, β2 and γ2 subunits due to their co-expression in many brain regions (Persohn et al., 1992; Fritschy et al., 1995). While α5 and β1 are thought to coexist in the hypothalamus, and α4 and δ in the forebrain (Rabow et al., 1995). As some cell-types express the majority of subunit subtypes e.g. hippocampus, it can be very difficult to predict the final pentamer composition (Rabow et al., 1995).

Expression of GABA<sub>α</sub> receptor subunits changes dramatically during development (Laurie et al., 1992a; Macdonald, 1994; Rabow et al., 1995). In summary, α2, α3, α5, β1-3, and γ1-3 subunits mRNAs form the neonatal complement of receptors expressed, while α1, α4, α6, β2, γ2 and δ mRNAs that of the adult (Laurie et al., 1992a; Laurie et al., 1992b).

### 1.3.2. Recombinant studies of GABA<sub>α</sub> receptors

Expression of receptor cDNAs has been used to determine the minimal subunit requirement for the production of GABA-gated chloride channels which show the full pharmacological repertoire of neuronal GABA<sub>α</sub> receptors. It is generally accepted that the expression of single subunits
alone does not lead to the formation of functional channels (Macdonald and Olsen, 1994; Rabow et al., 1995), though the β1 and β3 subunits are able to form spontaneously open chloride channels that are insensitive to GABA but can be blocked by picrotoxin and enhanced by propofol and pentobarbitol (Connolly et al., 1996; Krishek et al., 1996; Wooltorton et al., 1997; Davies et al., 1997b). Expression of α and β subunits produces GABA-gated currents which are modulated by barbiturates, inhibited by GABA antagonists and zinc ions, but are not enhanced by benzodiazepine (Levitan et al., 1988; Macdonald, 1994). Co-expression of a γ subunit with α and β subunits is necessary for the formation of a benzodiazepine binding site and also the relative insensitivity to Zn²⁺ antagonism (Pritchett et al., 1989; Draguhn et al., 1990; Smart et al., 1991). Replacement of the γ subunit with δ or ε subunits results in benzodiazepine insensitivity of the expressed receptor. Contradictory reports on the function of receptors consisting of α, β and ε subunits have been made with respect to their response to a range of anaesthetics (Davies et al., 1997; Whiting et al., 1997). Therefore the consensus of opinion from these experiments is that in vivo most GABAₐ receptors consist of α, β, and γ subunits.

1.3.3. Subunit stoichiometry and assembly of GABAₐ receptors

The final subunit stoichiometry and mechanism of assembly of the final pentameric receptor complex is still unknown. To maintain the same number of ligand binding sites on each receptor subunit stoichiometry must be regulated. Recombinant studies with α₁, β₂ and γ₂ have shown that only certain co-expressed combinations form functional cell-surface receptors (α/β and α/β/γ), while other are retained in the endoplasmic reticulum (Connolly et al., 1996). This suggests that the assembly of the final complex is an ordered process. This has been confirmed by recent studies looking at how selective subunit interactions are mediated, to produce GABAₐ receptors with defined subunit stoichiometry. Initial studies have identified "assembly boxes" within the N-terminus of receptor β subunits which are important for mediating subunit homo-and
hetero-oligomerisation (Taylor et al., 1999a), and residues in the α subunits important for the formation of a subunit ‘recognition domain’ during assembly (Taylor et al., 1999b).

The actual subunit stoichiometry has been studied in various ways, focusing on αβ and αβγ containing receptors in recombinant systems. For example, the ratio of α/β is believed to be 2:3 and α/β/γ 2:2:1, based on benzodiazepine affinity purification and quantitative western blotting on receptor complexes expressed in HEK293 cells (Tretter et al., 1997). This study also suggested that tetrameric complexes of 2α + 2β subunits may exist, as did a study of virally expressed α and β subunits in fibroblasts (Gorrie et al., 1997). But with the great heterogeneity of receptor expression in the brain it is difficult to detail all combinations seen, but with the identification of assembly domains within distinct subunits the dissection of receptor stoichiometry may be made easier. The combination of expression profiles, and favoured subunit interactions within the expressed pool, may allow better predictions of mature surface receptors.

1.3.4. Intracellular targeting of GABA_α receptors

Another phenomenon becoming apparent is the distinct subcellular distributions of receptor subunits. Madine-Darby canine kidney cells (MDCK) are polarised epithelial cells, with distinct apical and basolateral domains, which are thought to serve as a good model for neuronal polarity. Expression of recombinant GABA_α receptor subunits showed that the final location of the receptor was dependent on the β subunit expressed (Connolly et al., 1996b).

In neurons, electron microscopic immunogold localisation studies, using subunit selective antibodies, has shown that in cerebellar granule cells the α1, α6, β2/3 and γ2 subunits are found at GABAergic Golgi synapses, α6, β2/3 and γ2 at glutamatergic mossy fibre synapses, and α1, α6, β2/3, γ2 and δ subunits at extrasynaptic sites (Nusser et al., 1998). While in hippocampal pyramidal neurons distinct synaptic
localisations for the α1 and α2 subunits has been observed, with α1 seen at all GABAergic synapses over the axo-somato-dendritic domains, and α2 at only at a subset of synapses on the somata and dendrites (Nusser et al., 1996a). This hints at distinct subcellular localisation mechanisms for receptor subtypes. Other studies have shown the clustering of GABA_A receptors at discrete puncta in cultured neurons, suggesting that their subcellular distribution is regulated (Allison et al., 1998; Essrich et al., 1998). Processes to explain such targeting are an area of intense current interest. Using the yeast-two hybrid system to identify proteins which interact with specific GABA receptor subunits has led to the identification of microtubule-associated proteins as binding partners, which may play a role in the subcellular distribution and targeting of distinct receptor subtypes (Wang et al., 1999; Hanley et al., 1999). While disruption of the γ2 gene has been shown to disrupt the localisation of GABA_A receptor clusters at synapses, as does inhibiting the expression of the molecule gephyrin, suggesting that these two molecules play some role in GABA_A receptor clustering (Essrich et al., 1998).

1.3.5. Targeted disruption of GABA_A receptor subunit genes

The contribution of individual receptor subunits to GABA_A receptor function in the brain has been studied by the deletion of defined subunits by homologous recombination. A number of transgenic lines have now been produced lacking single GABA_A receptor subunits by the targeted disruption of GABA_A receptor subunit genes.

Deletion of the γ2 subunit causes a 94% reduction in the number of benzodiazepine binding sites, with a reduction in the single channel conductance and Hill Coefficient to a level consistent with those measured for αβ recombinant receptors (Gunther et al., 1995). The phenotype of the mice is characterized by retarded growth, sensorimotor dysfunction and reduced lifespan. Follow-up studies showed disruption
of the normal clustering of GABA$_A$ receptors in cultured cortical and hippocampal neurons, and brain slices from $\gamma_2$-/- mice, accounting for the observed phenotype (Essrich et al., 1998).

Deletion of the $\beta_3$ subunit causes the density of GABA$_A$ receptors to be approximately halved, and GABA$_A$ receptor mediated transmission is severely impaired (Homanics et al., 1997). The mice that survive to adulthood are hyperactive, have poor co-ordination and suffer epileptic seizures.

Loss of the $\alpha_5$ gene causes the specific loss of zolpidem insensitive benzodiazepine binding sites but with no obvious phenotypic defects (Fritschy et al., 1997).

Disruption of the $\alpha_6$ subunit results in a loss of diazepam insensitive Ro15-4513 binding in the cerebellar granule cell layer, and a selective degradation of the $\delta$ subunit (Jones et al., 1997). The latter suggests that the $\alpha_6$ and $\delta$ subunits specifically associate during receptor assembly.

Overall these transgenic studies how the $\alpha$ and $\beta$ subunits are required for the efficient assembly and cell surface assembly of GABA$_A$ receptors in vivo, and the $\gamma_2$ subunit is critical in the targeting/clustering of the final receptor complex.
1.4. Protein phosphorylation of the GABA\textsubscript{A} receptor

1.4.1. Consensus phosphorylation sites within the GABA\textsubscript{A} receptor subunits

The major intracellular domains of many GABA\textsubscript{A} receptor subunits contain a number of consensus sites for serine/threonine and tyrosine protein kinases (Kennelly and Krebs, 1991; Songyang et al., 1994; Songyang et al., 1995; Moss and Smart, 1996). The \(\alpha6\) subunit is unique amongst GABA\textsubscript{A} receptor \(\alpha\) subunits in encoding a strong consensus site for phosphorylation by a number of kinases, including PKA (Moss, 1996). \(\beta\) subunits contain a conserved site for a number of second messenger dependent protein kinases, including PKA and PKC (Moss et al., 1992a; Moss and Smart, 1996). The \(\rho1\) subunit also contains consensus phosphorylation sites including a number for PKC (Kusama et al., 1998). An interesting facet to this is the prevalence of phosphorylation sites found within alternative spliced regions. For the \(\gamma2\) subunit an insertion of 8 amino acids within the TM3/TM4 cytoplasmic loop distinguishes 2 forms of this subunit termed \(\gamma2S\) and \(\gamma2L\) (Whiting et al., 1990; Kofuji et al., 1991). This insertion contains a serine residue which satisfies the consensus for phosphorylation by a number of protein kinases, including PKC. Alternative splicing of the \(\beta2\) subunit within the TM3/TM4 cytoplasmic loop is found in chicken and human (Harvey et al., 1994). Both insertions contain consensus sites for phosphorylation which in the case of the human insertion encodes a strong consensus for PKA phosphorylation (McKinley et al., 1995).

1.4.2. \textit{In vitro} phosphorylation of neuronal GABA\textsubscript{A} receptors

Benzodiazepine affinity-purified preparations of GABA\textsubscript{A} receptors have been shown to be phosphorylated by a number of differing protein kinases. PKA and PKC both appear to phosphorylate "\(\beta\) subunits" based on apparent molecular masses (53-57 kDa) observed by SDS-PAGE.
(Kirkness et al., 1989; Browning et al., 1993; Tehrani, 1994). Consistent with this, antisera directed against the intracellular domain of the β1 subunit blocks the phosphorylation of these bands by both PKA and PKC (Browning et al., 1993). A receptor-associated kinase, which is not stimulated by either phorbol esters or cyclic nucleotides, phosphorylates an "α subunit" (Sweetnam et al., 1988; Bureau and Laschet., 1995). Purified receptors are also substrates for the well-characterized non-receptor tyrosine kinase, vSRC which phosphorylates "β and γ subunits" as determined by migration on SDS-PAGE (Valenzuela et al, 1995). Due to the heterogeneous nature of affinity-purified receptor preparations and low abundance of GABA\(_{A}\) receptors in the brain, the precise identity of the subunits phosphorylated in these studies remains unclear.

1.4.3. Phosphorylation of recombinant GABA\(_{A}\) receptors

To overcome the problems associated with identifying phosphorylation sites within neuronal GABA\(_{A}\) receptor subunits, recombinant subunit expression has been utilized. Receptor intracellular domains have been expressed as soluble glutathione-S-transferase (GST) fusion proteins in \textit{E.coli}, allowing purification under native conditions (Smith and Johnston, 1988). Using this approach combined with site-directed mutagenesis, it has been possible to identify phosphorylation sites for specific kinases within some GABA\(_{A}\) receptor subunits. The murine β1 subunit intracellular domain is phosphorylated by: PKA, PKC, cGMP-dependent protein kinase (PKG), and Ca/Calmodulin protein kinase (CaM KII) on Serine 409 (S409) (Moss et al., 1992a; McDonald et al., 1994). This conserved residue (S410 in the case of the β2 subunit) is also phosphorylated by these kinases in both the β2 and β3 subunits (Mcdonald, 1997). There are additional sites in the murine β1 and β3 subunits for CamKII phosphorylation, identified as S383 and S384 respectively (McDonald and Moss, 1994; Mcdonald and Moss, 1997). The β1 subunit intracellular domain can also be phosphorylated by vSRC \textit{in vitro}; (Valenzuela et al., 1995); however, the precise site(s)
phosphorylated in this subunit intracellular domain have not been identified.

Phosphorylation of both forms of the \( \gamma_2 \) subunit has also been analyzed using similar methodologies (Whiting et al., 1990; Kofuji et al., 1991). Serine 343 within the 8 amino acid insertion that differentiates these 2 forms of the \( \gamma_2 \) subunit is a high affinity substrate of both PKC and CamKII (Whiting et al., 1990; Moss, 1992a; Machu, 1993; McDonald and Moss, 1994). Both isoforms of \( \gamma_2 \) are also phosphorylated on additional residues by both of these kinases. PKC phosphorylates S327, while CamKII phosphorylates both S348 and T350 (Moss et al., 1992a; McDonald and Moss, 1994). The \( \gamma_2L \) intracellular domain can also be phosphorylated by vSRC in vitro, (Valenzuela et al., 1995). Experiments have failed to show significant phosphorylation of any \( \alpha \) subunit intracellular domains by PKA, PKC, PKG, CamKII or vSRC, or the \( \rho_1 \) subunit by PKC, PKA, CamKII and PKG. (Unpublished observations, J.G. Hanley and S.J. Moss). Overall these in vitro studies suggest that the \( \beta \) and \( \gamma_2 \) subunits are the major sites of phosphorylation within GABA\( _A \) receptors in vivo.

In parallel with these in vitro studies, the phosphorylation of GABA\( _A \) receptor subunits has been investigated in heterologous expression systems such as the human embryonic kidney cell line (HEK293). After pre-labeling expressing cells with \(^{32}\)P-orthophosphoric acid, receptor phosphorylation can be assessed by immunoprecipitation with subunit-specific antisera. Receptors can also be co-expressed with constitutively active protein kinases to produce constitutively phosphorylated receptor subunits. Murine GABA\( _A \) receptors composed of either \( \alpha_1 \beta_1 \) or \( \alpha_1 \beta_1 \gamma_2S \) subunits expressed in HEK293 cells are phosphorylated specifically by PKA on S409 within the intracellular domain of the \( \beta_1 \) subunit (Moss et al., 1992b). The \( \beta_3 \) subunit is phosphorylated on two adjacent residues S408 and S409 by PKA. Surprisingly, the \( \beta_2 \) subunit is not phosphorylated at the conserved position S410 by PKA when expressed with \( \alpha_1 \) and \( \gamma_2 \) subunits in HEK293 cells, although it is
phosphorylated on S410 \textit{in vitro} by PKA (Mcdonald and Moss, 1997; McDonald et al., 1998). This data indicates that \textit{in vitro} phosphorylation can only serve as a guide to the likelihood of phosphorylation at particular residues and that the use of whole receptor subunits in phosphorylation studies is vital to ascertain whether or not the sites identified from the \textit{in vitro} studies are indeed phosphorylated \textit{in vivo}. Interestingly S410, in the \(\beta2\) subunit is phosphorylated by PKC when expressed in HEK293 cells with the \(\alpha1\) subunit (McDonald et al., 1998). Receptors composed of \(\alpha1\beta1\) and \(\alpha1\beta1\gamma2\) subunits are also phosphorylated by PKC on S409 within \(\beta1\) (Krishek et al., 1994; McDonald and Moss unpublished data). The \(\beta1\) subunit has also been found to be phosphorylated to a low level by v-SRC on Y385 and Y387 (Moss et al., 1995). In the same study, the \(\gamma2\) subunit when expressed with \(\alpha1\beta1\) and vSRC results in significant tyrosine phosphorylation of the \(\gamma2\) subunit on residues Y365 and Y367. Tyrosine phosphorylation of receptor subunits has also been studied by receptor immunoprecipitation followed by western blotting using phosphotyrosine antibodies. Wan et al., have shown that the \(\beta2/3\) subunits are tyrosine phosphorylated in dorsal horn neurones (Wan et al., 1997).

Thus, the \textit{in vivo} studies of subunit phosphorylation are generally in good agreement with the \textit{in vitro} phosphorylation studies.

1.4.4. Functional significance of GABA\(_{\alpha}\) receptor phosphorylation by cAMP-dependent protein kinase

The effects of PKA phosphorylation on GABA\(_{\alpha}\) receptor function have been examined in a number of different experimental systems. The results have been complex and often contradictory. Thus, PKA appears to regulate GABA\(_{\alpha}\) receptor desensitization in cortical neurones and synaptoneurosomes after activation of endogenous PKA (Harrison and Lambert., 1989; Heuschneider and Schwartz, 1989; Porter et al., 1990; Schwartz et al., 1991; Tehrani et al., 1989; White et al., 1992). Using the
catalytic subunit of PKA it has been possible to demonstrate similar modulation of GABA<sub>A</sub> receptor function in cultured superior cervical ganglia, spinal cord neurones and cerebella granule cells (Moss et al., 1992b; Porter et al., 1990; Robello et al., 1993). GABA induced chloride flux from brain microsacs is also reduced by inclusion of the catalytic subunit of PKA (Leidenheimer et al., 1991) but not in spinal cord neurones (Ticku et al., 1990). However, in the retina and cerebella Purkinje cells, vasoactive intestinal peptide (VIP) and noradrenaline, enhance GABA<sub>A</sub> responses. Both of these neurotransmitters are known to increase the intracellular levels of cAMP (Parfitt et al., 1990; Veruki, 1992; Veruki, 1994). Interestingly, the effects of VIP may be dependent on the species used, since in rabbit retina, VIP inhibits GABA-activated currents (Gillette et al., 1996). Enhancement of GABA<sub>A</sub> responses have also been reported in hippocampal dentate granule neurons, and also in cerebellar Purkinje neurones where the effect can be mimicked by cAMP analogs and blocked by PKA inhibitor peptide (Kano et al., 1992b). Likewise in rat retina, intracellular dialysis with the catalytic subunit of PKA enhances GABA<sub>A</sub> receptor responses (Feigenspan et al., 1994). Interestingly the same study has shown that dopamine but not noradrenaline is capable of enhancing GABA<sub>A</sub> receptor function in the retina (Feigenspan et al., 1994). This observation is of interest as both of these neurotransmitters increase the intracellular levels of cAMP, which suggests that there may be a further restriction on the phosphorylation of receptor subunits within cell compartments.

It seems then that PKA may modulate receptor function in a cell-type specific manner. The variability of the effects may be due to cell type differences, method of kinase activation, differences between species or an underlying heterogeneity of receptor structure. The latter could in turn be dependent on receptor subunit composition. A role for receptor diversity has been suggested (McDonald et al., 1998) by the study of the phosphorylation of the β2 and β3 subunits in HEK293 cells, adding to work on the β1 subunit (Moss et al., 1992b). The latter studied GABA<sub>A</sub> receptors expressed in HEK293 cells, composed of either α1β1 or
α1β1γ2S subunits. Phosphorylation of S409 in the β1 subunit mediated by either intracellular dialysis with cAMP or with the catalytic subunit of PKA, results in a time dependent decrease of GABA-induced currents and a modulation of α1β1 receptor desensitization. These functional effects could be abolished by mutation of S409 to an alanine residue in the β1 subunit, the sole site of PKA phosphorylation in these receptors (Moss et al., 1992b). The β3 subunit differs from the other β subunits in having two adjacent serines at positions 408 and 409 which can be phosphorylated after PKA activation. In contrast, β1 is phosphorylated on a single serine (S409), while the β2 subunit is not phosphorylated by PKA under the same conditions in HEK293 cells (McDonald et al., 1998). An enhanced GABA-activated response is seen in cells expressing α1β3γ2S or α1β3 after intracellular dialysis with cAMP when S408 and S409 are phosphorylated. Mutation of S408 to an alanine in the β3 subunit converts this potentiation to an inhibition, as seen with the β1 subunit which is phosphorylated solely at S409 (Moss et al., 1992b). While the mutation S409A showed no functional effect with cAMP. Furthermore, if the alanine residue preceding S409 in β1 is converted to a serine so giving the β1 subunit the same PKA phosphorylation profile as β3, the inhibitory effect of cAMP was seen to be converted to an enhancement. The double mutant β1 A408S/S409A expressed with α1 and γ2S was insensitive to cAMP (McDonald et al., 1998). Therefore, phosphorylation of both S408 and S409 causes potentiation of receptor function while an inhibition is seen on phosphorylation of S409 alone. Interestingly there does not appear to be any functional consequences of phosphorylating S408 alone. No functional effects of PKA activation were seen for receptors composed of α1β2 or α1β2γ2, consistent with the failure of PKA to phosphorylate any of these receptor subunits in vivo (McDonald et al., 1998). The observations of McDonald et al., (1998) suggest that the differential phosphorylation of distinct β subunits may partially explain the distinct effects of PKA activation on GABAA receptor function. Recent work in rat hippocampal slices supports the recombinant observations (Poisbeau et al., 1999). This study examined the regulation of mIPSCs in hippocampal CA1 pyramidal
neurons and dentate gyrus granule cells. PKA reduced the amplitude of mIPSCs in pyramidal cells. While in dentate gyrus granule cells, PKA was without effect, suggesting the presence of differing β subunits in these distinct neurone types. Interestingly, β subunits show distinct patterns of temporal and spatial expression in the CNS supporting this hypothesis (Benke et al., 1994; Laurie et al., 1992b; Wisden et al., 1992). Furthermore it is also known that β subunits are important for the subcellular localization of GABA<sub>α</sub> receptors in polarized epithelial cells (Connolly et al., 1996b). Therefore if receptors containing different β subunits, are targeted to distinct synapses, PKA-induced phosphorylation may allow for a synapse specific control of GABA<sub>α</sub> receptor function.

1.4.5. Functional significance of GABA<sub>α</sub> receptor phosphorylation by Protein kinase C

The first evidence that GABA<sub>α</sub> receptors were subject to modulation by PKC mediated phosphorylation was from the expression of GABA<sub>α</sub> receptors in *Xenopus* oocytes after injection of either rat or chick brain subunit (Moran et al., 1989; Sigel et al., 1988). Activation of PKC using phorbol esters resulted in a significant inhibition of GABA-induced whole-cell currents in these experiments. This initial observation was followed by extensive studies using the heterologous expression of receptor cDNAs, which have shown that phorbol ester-induced PKC activity can inhibit the function of a range of receptors constructed from: α1-α5, β1-2 and γ2 subunits (Leidenheimer et al., 1993; Leidenheimer et al., 1992; Sigel et al., 1991). In the case of receptors composed of α1β1γ2L subunits, the effects of phorbol esters can be blocked by PKC inhibitory peptide (PKCI, (Leidenheimer et al., 1992). The role of specific phosphorylation sites for PKC within the predicted intracellular domains has been examined by site specific mutagenesis of sites within individual subunits.
PKC exerts its inhibitory effect on receptors composed of α1β1, α1β1γ2S/L, α1β2 and α1β2γ2S/L subunits, occur via phosphorylation of S409 within the β1 subunit, S410 in the β2 subunit, S327 in both the γ2γ2S subunits and S343 within the γ2L subunit (Kellenberger et al., 1992; Krishek et al., 1994). The effects of phosphorylation are also related to the GABA concentration with larger inhibitions apparent with receptors incorporating the γ2L subunit at high concentrations of GABA. Selective mutagenesis revealed that phosphorylation at any of the sites on the β1 or γ2 subunits is sufficient to produce negative modulation (Krishek, 1994). However, phosphorylation at S343 which is contained within the 8 extra amino acids within the γ2L subunit produces the largest inhibitory effect, suggesting that the phosphorylation sites are not functionally equivalent (Krishek et al., 1994). Moreover there was no apparent linear relationship between the degree of inhibition and the number of phosphorylation sites (Krishek et al., 1994). PKC is also seen to modulate the rapid desensitization of α1β1 receptors, as previously described for PKA mediated phosphorylation (Moss et al., 1992a). This is not unexpected as S409 in the β1 subunit represents the sole site of phosphorylation for both PKA and PKC in these receptors (Krishek et al., 1994; Moss et al., 1992b). Phosphorylation of such receptors by PKC gives an increased EC50 for GABA and decreased Imax which are consistent with either a decrease in the affinity for GABA or a decreased probability of channel opening.

A series of other studies though, using the intracellular dialysis of trypsin cleaved rat brain PKC, have given differing functional effects to those using phorbol ester treatment. Cleavage of PKC with trypsin leads to its constitutive activation. Intracellular application of this preparation at a concentration of 40 nM to L929 cells transiently expressing bovine GABA_A receptors composed of α1, β1 and γ2L subunits enhances receptor function (Lin et al., 1994). This enhancement could be blocked by the PKCI peptide, or by mutation of either S409 (β1 subunit), S327 (γ2S or γ2L subunits) or S343 (γ2L subunit) to alanines (Lin, 1996; Lin, 1994). The differences seen in the effects PKC has on receptor function
could be due simply to the differences in the means used to activate PKC (Phorbol esters v PKM) or by the origin of the cDNA i.e. bovine v murine, or possibly recording differences.

Phorbol ester treatment has been used to examine the regulation of neuronal $\text{GABA}_A$ receptors by PKC phosphorylation. Responses in cultured superior cervical ganglion (SCG) neurones are inhibited by phorbol ester treatment but not by $4\alpha$-phorbols, which are incapable of activating PKC (Krishek et al., 1994). Similar results to Krishek et al., (1994) were obtained from rabbit retinal rod neurons, with staurosporine and calphostin C blocking the inhibition by PKC (Gillette et al., 1996). GABA-induced chloride flux from cerebellar microsacs can be selectively inhibited by PKC activators (Leidenheimer et al., 1992), but PKC does not appear to modulate receptor desensitization in spinal cord microsacs (Ticku et al., 1990). Further evidence that PKC inhibits channel function comes from the inhibitory effects elicited on receptor function by substance P and the bradykinins NKA and NKB in bullfrog sensory neurons (Yamada et al., 1996). PKC inhibitors can block this effect while activators can mimic it. Experiments have shown that synaptic currents mediated via $\text{GABA}_A$ receptors can be regulated by a PKC-dependent mechanism in CA1 hippocampal neurones. Inclusion of the specific peptide PKC inhibitor, PKCI, enhances $\text{GABA}_A$ mediated inhibitory postsynaptic potentials (Weiner et al., 1994). This is in good agreement with experiments demonstrating the inhibition of receptor function by direct PKC mediated phosphorylation (Kellenberger et al., 1992; Krishek et al., 1994).

Much discussion has focused on the phosphorylation at S343 in the $\gamma_2L$ subunit. This phosphorylation site is of special significance as ethanol was thought to exert much of its action on $\text{GABA}_A$ receptor function via this residue (Sigel et al., 1993; Wafford et al., 1992; Wafford et al., 1991). It is now apparent that this residue may not be so critical. For example in DRG neurons the ethanol sensitivity of receptor was studied under conditions where S343 should be phosphorylated, but the GABA
response seemed to be insensitive to a wide range of ethanol concentrations (Zhai, et al. 1998). More importantly through the targeted disruption of the extra 8 amino-acids in the γ2L subunit producing a transgenic mouse which only contains the short splice variant of the γ2 subunit, and so lacks S343, has been generated but exhibits normal ethanol responses showing that phosphorylation at S343 is not essential for ethanol modulation of the GABA response (Homanics et al., 1999).

Finally, GABAₐ receptors have been shown to be inhibited by PKC activation. This was initially shown in rat retinal bipolar cells where a PKC dependent process was shown to inhibit channel function (Feigenspan et al., 1994). Further in *Xenopus* oocytes ρ1 homomeric GABAₐ channels were shown to be inhibited by PKC activators (Kusama, 1995). Recent data has shown that the inhibition seen after PKC activation is not due to phosphorylation of PKC consensus sites in the intracellular loop (Kusama et al., 1998), suggesting that the effects of PKC on ρ1 subunit function are not mediated by direct receptor phosphorylation. In agreement with this, results from our laboratory have shown that the intracellular loop of the ρ1 subunit is not an *in vitro* substrate for PKC, PKA, PKG and CaMKII. (S.J.Moss and J.G.Hanley, unpublished observations).

**1.4.6. Functional significance of GABAₐ receptor phosphorylation by cGMP-dependent protein kinase and Ca²⁺/calmodulin-dependent protein kinase II**

The functional effects of these two kinases on GABAₐ receptor function is poorly understood. GABAₐ receptor-mediated currents in neurones from the nucleus of the tractus solitarius of the rat are inhibited by cGMP or on the activation of metabotropic glutamate receptors (Glaum et al., 1993). A similar effect has been reported in retinal amacrine cells where nitric oxide (NO) has been suggested to depress GABAₐ receptor
function by a cGMP dependent pathway (Wexler et al., 1998). A NO mimic or perfusion with cGMP decreased receptor function, the effects of both treatments could be partially blocked by a specific PKG inhibitor peptide. The PKG-independent affects were attributed to a cGMP-stimulated phosphodiesterase. Recently a study in cerebellar granule cells at the single channel level showed PKG activation causes a reduction with time of the mean open time constant of a single channel (Robello et al., 1998). In contrast, in oocytes α1β2γ2L, receptors are enhanced by a cGMP dependent mechanism (Leidenheimer., 1996). Mutation of the major *in vitro* phosphorylation site for PKG within β2 subunit, S410 did not block this effect, suggesting that direct phosphorylation by PKG does not underlie the modulatory effects seen with cGMP.

Ca²⁺ has been shown to inhibit or activate GABA-mediated currents dependent on the neuronal population studied (Stelzer et al., 1992; Stelzer et al., 1995), though the role played by CamKII in these actions of Ca²⁺ is not fully understood. Experiments have shown that constitutively-activated CamKII can activate GABAₐ receptor mediated whole-cell currents in cultured rat spinal cord neurones (Wang et al., 1995), though whether these effects were mediated by direct receptor phosphorylation was not addressed.

Recent experiments have shown that CamKII is essential for the establishment of ‘rebound potentiation’ in cerebellar Purkinje neurons (Kano et al., 1996). In response to an increase in intracellular Ca²⁺ due to glutamatergic stimulation a potentiation of GABAₐ responsiveness is seen in Purkinje neurons (Kano et al., 1992), an effect that is blocked by CamKII inhibitors (Kano et al., 1996). Interestingly, rebound potentiation can be mimicked by intracellular dialysis with PKA or with cAMP analogues (Kano et al., 1992). This effect is reminiscent of the enhancement of β3 containing receptors function on the phosphorylation of S408 and 409 in β3 (McDonald et al., 1998). Given that the β3 subunit is also phosphorylated *in vitro* by CamKII (Mcdonald, 1997),
this may suggest that phosphorylation of the β3 subunit which is predominately expressed in Purkinje neurones (Laurie et al., 1992a; Benke et al., 1994) may underlie rebound potentiation.

1.4.7. Functional significance of GABA<sub>A</sub> receptor phosphorylation by Tyrosine Kinases

The role of tyrosine phosphorylation in modulating receptor function is an area of current intense interest. The effects of tyrosine phosphorylation on GABA<sub>A</sub> receptor function, was first examined by Moss et al., (1995). Receptors composed of α1 β1 and γ2L subunits were co-expressed in HEK293 cells with vSRC. Phosphorylation of the γ2L subunit on residues Y365 and Y367 by vSRC enhances GABA-induced currents (Moss et al., 1995). Tyrosine phosphorylation was also seen at residues Y370 and Y372 of the β1 subunit but had no measurable effect on receptor function. In agreement with this, it has been shown that tyrosine kinase inhibitors reduced the magnitude of GABA-gated currents recorded from Xenopus oocytes expressing GABA<sub>A</sub> receptors composed of α1β1γ2L and α1β1 subunits (Valenzuela et al, 1995). It was suggested that phosphorylation on tyrosine residues in the β1 and γ2L subunits underlie this effect. It has recently been reported that tyrosine phosphorylation of the β subunits is important in the regulation of receptor function (Wan et al., 1997). Intracellular application of pp60-cSRC into cultured spinal dorsal horn neurones caused a progressive increase in current amplitude, which could be prevented by pre-treatment with PTK inhibitors. Immunoprecipitation of β2/3 receptor subunits followed by Western blotting with a phosphotyrosine antibody showed the β subunits to be tyrosine phosphorylated (Wan et al., 1997). In addition neuronal GABA<sub>A</sub> receptors can also be modulated by tyrosine phosphorylation in other preparations. Whole-cell currents recorded from cultured SCG neurones are reduced by exposure to tyrosine kinase inhibitors and are enhanced by tyrosine phosphatase inhibitors (Moss et al., 1995). Furthermore GABA mediated chloride flux from brain microsacs is also reduced by tyrosine kinase inhibitors (Valenzuela et al,
Single channel recording from SCG neurones suggested that tyrosine phosphorylation increased both mean open time and also the probability of channel opening (Moss et al., 1995). These results suggest that tyrosine phosphorylation may be a means of enhancing or maintaining $\text{GABA}_\alpha$ receptor function.

Interestingly, phosphorylation by unidentified kinases (neither PKA nor PKC) or "phosphorylation factors", have been implicated in preventing the wash-out or rundown of $\text{GABA}_\alpha$ receptor responses in a number of neurones (Chen et al., 1990; Gyenes et al., 1994; Gyenes et al., 1988; Stelzer et al., 1988). Interestingly, rundown of $\text{GABA}_\alpha$ receptor current seen with high GABA concentration is dependent on both ATP and Ca\textsuperscript{2+} levels (Huang et al., 1998). The ATP dependence is due in part to the hydrolysis of this compound by PTKs, as addition of tyrosine phosphatase inhibitors decreased rundown normally seen at high GABA concentrations, while PTK inhibitors caused rundown even if the ATP level was artificially high. This suggests that phosphorylation by a PTK prolongs receptor activity. This was also shown in dissociated neurones from the Diagonal Band of Broca, where receptor activation was shown to be dependent on ATP and PTK activity (Jassar et al., 1997).

A note of caution must be advised when interpreting experiments utilizing tyrosine kinase inhibitors though. It has been shown that the PTK inhibitor genestein directly blocks $\text{GABA}_\alpha$ receptor function when applied extracellularly (Dunne et al., 1998b). In recombinant experiments where the major sites for SRC phosphorylation have been mutated, the inhibitor has the same effect as it does on the wild-type receptor. Therefore genestein antagonizes receptor function independently of the action on any PTK, when applied to the outside of cells. No effect was however seen on mutant receptors devoid of SRC phosphorylation sites when genestein was applied via intracellular dialysis (Dunne et al., 1998b). Also a depression of the GABA current was also seen with the genestein inactive analogue "daidzein" when applied to the outside of expressing cells. Together these observations suggest experiments utilizing genetsein application to the extracellular...
domains of GABA\textsubscript{\textalpha} receptors should be treated with caution due to the non-specific effects of this agent on receptor function. Also they emphasize the importance where possible, to use recombinant receptors with mutated phosphorylation sites, to determine if the effects seen with particular kinase activators/inhibitors are due to receptor phosphorylation.

Tyrosine phosphorylation has also be implicated in the effect of insulin on GABA\textsubscript{\textalpha} receptor surface expression. It has been reported that insulin causes the rapid translocation of receptors from intracellular compartments to the surface in transfected HEK293 cells (Wan et al., 1997b). This effect can be blocked by the application of genestein confirming that activation of the insulin receptor tyrosine kinase is essential for this effect. In hippocampal neurones and brain slices insulin causes an increase in GABA\textsubscript{\textalpha} receptor number at the cell surface (Wan et al., 1997b). Whether these effects are due to direct receptor phosphorylation in either of these systems remains unknown.

Overall there is agreement that tyrosine phosphorylation enhances GABA\textsubscript{\textalpha} receptor function, via phosphorylation of tyrosine residues in the \(\gamma2\) and/or \(\beta\) subunits.

1.4.8. Other effects of phosphorylation on the GABA\textsubscript{\textalpha} receptor

In addition to effects on channel function, chronic activation of PKA has been reported to enhance the assembly of GABA\textsubscript{\textalpha} receptors (Angelotti et al., 1993). This observation comes from the expression of GABA\textsubscript{\textalpha} receptors in cell lines that have various levels of constitutive (cAMP-independent) PKA activity. Angelotti et al., used 3 cell lines which have PKA activities of 500, 100, and 5 units/mg protein respectively. Larger GABA responses were seen from receptors composed of \(\alpha1\beta1\) and \(\gamma2S\) subunits in the cells with the higher activity compared to the other 2 cell lines, but not from receptors composed of only \(\alpha1\) and \(\beta1\) subunits. This effect could be blocked by mutation of S409 in the \(\beta1\) subunit, the sole
site of phosphorylation in receptors composed of \( \alpha_1, \beta_1 \) and \( \gamma_{2S} \) subunits (Moss et al., 1992b). Interestingly, forskolin treatment has been shown to increase the level of the \( \alpha_1 \) subunit, concomitantly reducing the level of \( \alpha_6 \) subunit expression in cerebellar granule cells, supporting a role for PKA activity in facilitating \( \Gamma \) receptor assembly (Thompson et al., 1996). If these effects are mediated by changes in protein stability or modifications in the rate of \( \Gamma \) receptor gene transcription remain unknown.

It has become apparent that the mechanisms by which PKC activation reduces channel function may be more complex than simply effecting the kinetics of receptor gating by direct receptor phosphorylation. PKC activation of oocytes expressing receptors composed of \( \alpha_1, \beta_2, \) and \( \gamma_2 \) or \( \alpha_1 \) and \( \beta_2^{5410A} \) decreases the GABA response with a concomitant internalization of receptor (Chapell et al., 1998). This effect is not due to direct receptor phosphorylation as mutation of previously identified phosphorylation sites for PKC in the \( \beta \) and \( \gamma \) subunits does not block internalization (Chapell et al., 1998). A similar process has been reported in HEK293 cells expressing \( \alpha_1\beta_2\gamma_2 \) receptors and also for native receptors on hippocampal neurons (Connolly et al., 1998). In HEK293 cells down regulation of receptors from the cell surface after PKC activation is independent of receptor phosphorylation but is dependent on the presence of the \( \gamma_2 \) subunit (Connolly et al., 1998). Furthermore the site of PKC action was found to be to in preventing the recycling of receptors back to the cell surface, after constitutive endocytosis (Connolly et al., 1998; Barnes et al., 1996). Therefore PKC activity can, in addition to modifying receptor gating by direct phosphorylation (Kellenberger et al., 1992; Krishek et al., 1994; Lin et al., 1996), also mediate the internalization of receptors, a process that is not dependent upon direct receptor phosphorylation (Chapell et al., 1998; Connolly et al., 1998).

1.4.9. Interactions of ion-channels and protein kinases
The mechanism by which protein kinases are targeted to GABA<sub>A</sub> receptors to ensure subunit specific phosphorylation is an important issue about which has yet to be addressed. Studies of other ion-channels has seen the emergence of two main themes. The first is an increasing number of reports of the direct interaction of protein kinases with ion-channels. This is thought to be very important as it suggests that ion-channels are acting as a 'self-scaffold' for their own set of modulatory enzymes. It allows rapid responses to signals in their environment. To date it is principally protein tyrosine kinases which have been demonstrated binding directly. Fyn and fyk were shown to bind to the nAChR (Swope and Huganir, 1993), fyn and src to the nAChR (Fuhrer and Hall, 1996), src to the voltage dependent potassium channel Kv1.5 (Holmes, 1996), src to co-precipitate with the NR1 subunit of the NMDA-type glutamate receptor (Yu, 1997), and lyn to the AMPA-type glutamate receptor. Recently the protein tyrosine kinase src, and the catalytic subunit of PKA (PKA<sub>cat</sub>) have been shown to bind simultaneously to the Drosophila Slowpoke calcium-dependent potassium channel dSlo (Wang et al., 1999). The fact that an ion-channel is able to participate in a regulatory complex with multiple signalling molecules is of great significance for neuronal function.

Secondly, it is becoming apparent that kinase anchoring and other adaptor molecules play a critical role in mediating substrate phosphorylation by restricting enzyme subcellular distribution (Faux and Scott., 1996a; Faux and Scott., 1996b; Pawson and Scott. 1997). In the case of glutamate receptors, A-kinase binding proteins (AKAPs) are important in mediating PKA regulation of this receptor. The molecule AKAP-79 has been implicated in the control of AMPA receptors (Rosenmund et al, 1994), and the molecule 'yotiao', also an AKAP, in that of the NMDA-type receptors (Westphal et al., 1999a; Westphal et al., 1999b).
For the GABA$_A$ receptor no known protein kinases or other signalling molecules have as yet been identified in close association with the receptor. Studies though have reported an unknown serine kinase co-purifying with receptor from rat cerebellum (Sweetnam et al., 1988), and serine/threonine kinase and tyrosine kinase activity similarly from bovine brain (Bureau and Laschet., 1995; Kannenberg et al., 1999).
1.5. AIMS OF THESIS

Phosphorylation of GABA$_A$ receptors has been identified as a mechanism for controlling receptor function. To date the majority of such studies have focused on recombinant receptors in heterologous cells. This work will therefore look at the phosphorylation of the $\gamma_2$ subunit, on tyrosine residues, in neurones. It has been shown that tyrosine phosphorylation of this subunit, on two distinct residues, causes an enhancement of channel function (Moss et al., 1995). So it is important to look at the relevance of this process in neurones. Raising specific antibodies against the $\gamma_2$ subunit will allow immunoprecipitation and western blotting of the protein to enable detection of phosphotyrosine residues.

Secondly, this study will start to look at the nature of the interactions between GABA$_A$ receptor subunits and signalling molecules. There have been reports of unknown protein kinases co-purifying with GABA$_A$ receptor complexes, while for other ion-channels, there have been identified a number of direct and in-direct interactions between subunits and protein kinases. In this thesis, attempts were made to identify interactions between the GABA$_A$ receptor and specific signalling molecules, with particular focus on tyrosine kinases to complement the studies on the tyrosine phosphorylation of the $\gamma_2$ subunit.
CHAPTER 2: Materials and methods

All chemicals were purchased from Sigma unless otherwise stated, and all restriction enzymes from New England Biolabs. Oligonucleotides were produced by MWG Biotech. Radionucelotides were purchased from Amersham.

2.1. Molecular biology

2.1.1. Bacterial strains
Subcloning and PCR cloning were performed using the E.Coli strain XL1Blue (F'::Tn 10 proA+B+ lacI9 Δ (lacZ)M15/recA1 end A1 gyrA96(Nalr) thi hsdR17(rK-mK+) supE44 rel 1 lac (Bullock et al BioTechniques 1987).

Production of GST-fusion proteins from the pGEX-4T3 or pGEX-2TK vectors was performed using the E.Coli strain BL21 (F-ompT [lon] hsdSB(rB-mB-); an E.coli B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene (Studier, F.W. et al. Meth. Enzymol(1990))

DNA was transformed into both strains of bacteria by electroporation in a Biorad Gene-pulser II.

2.1.2. Growth media and agar plates
Bacteria were grown in Luria-Bertani medium (LB). For plasmids encoding Ampicillin resistance, Ampicillin (amp), was added to a concentration of 70μg/ml. For growth of BL21 bacteria, Chloramphenicol (chlor) was added to a concentration of 30μg/ml.
For plates, agar was added to 15g/l. All growth was carried out at 37 °C.

2.1.3. Preparation of electrocompetent bacterial cells.
Cells were streaked onto an LB agar plate without antibiotics. A single colony was then used to inoculate 10mls of LB grown at 37°C overnight.
This was added to 1 litre of LB and grown to an absorbance at OD$_{600}$ of 0.6 (±0.5). The bacteria were spun down at 4000rpm for 10 mins and washed in 500ml sterile ice-cold water. They were then washed in 25ml sterile ice-cold 10% glycerol, and finally resuspended in 2.5ml 10% glycerol. Aliquots were stored at -80°C.

2.1.4. Transformation of bacteria with plasmid DNA
20μl of electrocompetent bacteria with the DNA of interest were added to a 0.2cm electroporation cuvette kept on ice. A BioRad Genepulser II was used to give a single pulse with the settings of 2.5KV, 200Ω and 25μF. The bacteria were immediately resuspended in 200μl of LB and incubated at 37°C for 30min before plating onto LB-agar plates with the appropriate antibiotics. The plates were then incubated at 37°C overnight.

2.1.5. Ethanol precipitation of DNA
To precipitate DNA from an aqueous solution, 0.1 volume of 3M Sodium Acetate pH 5.2 followed by 2 volumes of 100% ethanol were added, and placed at -20°C for at least 10min. For small amounts of DNA, such as ligations and preparation of fragments prior to ligation, 1μl of glycogen (1mg/ml) was added before the salt and ethanol. After centrifugation at 13,000 rpm for 15 mins, the pellet was washed with 70% ethanol and dried at room temperature for 5 mins.

2.1.6. Phenol/chloroform extraction
Phenol/Chloroform (p/c) is a 1:1 mixture of Phenol and Chloroform, equilibrated with Tris pH 7.5. Extraction was carried out on DNA samples by adding the same volume of p/c in a microfuge tube. After addition of the p/c to the DNA sample, the mixture was vortexed thoroughly and centrifuged at full speed for 1min. The aqueous phase was then transferred to a fresh microfuge tube and the process repeated. A final extraction with chloroform alone was carried out to remove traces of phenol.
2.1.7. DNA electrophoresis


Briefly, agarose was added to 1xTAE, and melted in a microwave oven. The concentration of agarose used depended on the size of fragments to be resolved, on a scale of 0.6% for large fragments (>5kB) up to 2.5% for small fragments (<0.5kB). Ethidium Bromide was added to a concentration of 100ng/ml. A 10x loading buffer of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll-400 was added to samples prior to loading on the gel. DNA was visualised by placing the gel on a UV transilluminator.

2.1.8. Maxi-preparation of plasmid DNA by caesium chloride banding

This was carried out as described in Sambrook et al (1989) pages 1.38-1.39 & 1.42-1.46.

500ml of bacterial culture in LB/amp grown overnight at 37°C was centrifuged at 4,000 rpm for 15 mins. This was resuspended in 10ml Solution I, to which 20ml Solution II was added and mixed well. 15ml Solution III was added and left on ice for 5min. This was centrifuged for 10min at 4,000 rpm. To the supernatant was added an equal volume of isopropanol, followed by centrifugation for 10min at 4,000 rpm. The pellet was resuspended in 3ml 10X TE to which was added 3.5g CsCl and 50µl 10mg/ml Ethidium Bromide. This was centrifuged at 100,000 rpm overnight (or at least 4 hours) in a TLN100 ultracentrifuge rotor. DNA bands were pulled using a 5ml syringe and wide-bore hypodermic needle and the ethidium bromide removed by butanol extraction.

The DNA was precipitated by adding 2 volumes of ethanol and centrifuged at 4,000rpm for 2min. It was often necessary to phenol/chloroform extract the DNA preparation if destined for cell transfection. The pellet was finally resuspended in TE to a concentration of 1mg/ml.

DNA concentration was determined by reading the absorbance at 260nm.
2.1.9. PCR subcloning

To generate fusion proteins containing either truncations of the major intracellular loop of the β1 subunit or the whole of the molecule RACK-1, regions were amplified by the polymerase chain reaction (PCR) using primers to introduce restriction sites into the products which would allow in-frame cloning into the appropriate pGEX vector: pGEX4T3 for the truncations or pGEX-2TK for RACK-1.

-Preparation of vector fragments
5-10μg of plasmid was digested in a volume of 50μl with the appropriate restriction enzymes and buffer. 2μl of each enzyme (approximately 40 units) were used per digest, and digests were carried out at 37°C. 1μl of shrimp alkaline phosphatase (Amersham) was added to the reaction mixture after digestion and incubated at 37°C for a further 30min to reduce the level of self-ligation. 5μl of loading buffer was added to the reaction mixture and this was run on an agarose gel (0.6-1% agarose). The DNA fragments were excised, and purified using the Gene Clean kit (Bio 101).

-Preparation of inserts synthesised by Polymerase Chain Reaction (PCR)
PCR reactions were carried out with the following reaction mixture:
10 mM Tris pH 7, 0.1% Tween-20 (v/v), 50 mM KCl, 1-5 mM MgCl2, 500μM dNTPs, 1ng template plasmid, 0.5μg 5' sense primer, 0.5μg 3' antisense primer, 0.5μl TAQ polymerase. Volume was made up to 20 μls with water.
This reaction mixture was subjected to 30 cycles of the following programme on a BioRad Gene Cycler: 30s @ 94°C, 50s @ 50°C, 70s @ 72°C.
After the reaction, the volume was increased to 500μl with water, phenol/chloroform extracted twice and ethanol precipitated as described in section 2.1.5.
The PCR products for the truncated β1 were digested with the enzymes BamH1 and EcoR1, as was the vector pGEX-4T3. While the product of
the RACK-1 amplification was digested with SmaI and EcoR1, along with the vector pGEX-2TK. This reaction mixture was then incubated for 1h at 37°C. Gel purification was carried out as above using 1.5-2.5% agarose gels.

-List of primers used for synthesising PCR cloning inserts.
All are written 5' - 3'.

β1 (302-365) 5' GTTCCGCGTGGATCCAATTACATTCTTCTTCGGAAAA
3' ACGATGAATTCCACTTACCTGTGAGCACCCTC

β1(366-426) 5' GACGGATCCAAGGCCACCATGTACTCG
3' GGAGAATTCCTTGTCTATGGAGTT

β1(366-394) 5' see β1(366-426) 5'
3' GTGGAATTCGTCAGCCCACGCCGAA

β1(395-426) 5' CTGGGATCCAGGCACCGGGGTACCGGGC
3' see β1(366-426) 3'

β1 (366-404) 5' see β1(366-426) 5'
3' CCTGAATTCTCAGATGCCTGCCTTTGGCCGGTAC

β1 (366-415) 5' see β1(366-426) 5'
3' GGGGAATTCCTCACTTCACTTTGAGCCTGCGA

All the above were cloned into the pGEX-4T3 vector.

RACK1 (full length) 5'TGCCCGCGGCGTGGACCGAGCAGGTAATTGAC
3' AAAGAATTCTTGCGGGGTACCAATAGT
Cloned into pGEX-2TK vector.

2.1.10. Subcloning
pBS-RACK-1 was obtained kindly from the lab of Dr D.M-Rosen (Stanford). To subclone RACK-1 into pGW1, pBS-RACK-1 was digested with EcoRI and partially with XhoI. A XhoI-EcoRl fragment coding for all of RACK-1 was purified on a 1% agarose gel and gene-cleaned.

Preparation of vector: as above (2.1.9.) but the vector pGW1 was digested with EcoRl and XhoI.

2.1.11. Ligations

For all types of subcloning the digested vector and insert were purified by GeneClean.
A rough estimate of the relative concentrations of vector and insert was made based on the intensity of bands on an agarose gel. Two different insert:vector ratios were used; approximately 5:1 and 20:1. A control with no insert was also carried out. The reaction mixture was as follows:

Vector, insert, 1mM ATP (pH 7.5), 1x NEB T4 DNA Ligase Buffer, 200 units NEB T4 DNA Ligase and water to a final volume of 10μl.

The reaction mixture was then left at 14°C for 10-16 hours. After the reaction, the volume was increased to 400μl with water, phenol/chloroform extracted twice and ethanol precipitated as described in section 2.1.5. The pellet was then resuspended in 10μl water, and 1μl of this was electroporated into XL1Blue bacteria as described in section 2.1.4.

Mini-Preps from colonies were screened for successful ligation by restriction digest, using the same enzymes which were used to prepare the fragments before ligation.
All constructs were then sequenced to ensure their fidelity

2.1.12. Mini-preparation of plasmid DNA (mini-preps)

1.5ml of bacterial culture was added to a microfuge tube and centrifuged at 6,000rpm for 5 mins in a bench-top microfuge. The supernatant was
removed and the bacteria resuspended in 100μl of solution I. 200μl of freshly made solution II was added and mixed by inverting several times. This was left on ice for 5 minutes and then 150μl solution III was added. After mixing and leaving on ice for 10min, the tubes were centrifuged at 13,000rpm in a bench-top microfuge for 10min. The supernatant was then removed to a fresh tube, and subjected to phenol/chloroform extraction. 1ml 100% ethanol was added to the samples and incubated at -20°C for 10min, after which they were centrifuged at full speed for 15min. The pellet was washed in 400μl 70% ethanol, dried, and resuspended in 50μl water.

2.1.13. DNA sequencing
This was carried out using the United States Biochemical sequencing kit. 2μl of the mini prep (approx 1μg) was added to 1μl 2M NaOH, 2μl 0.2M EDTA and water to a final volume of 10μl. After a 30 minute incubation at 37°C the DNA was precipitated as above (2.1.5.) and resuspended in 7μl of water. 1μl sequencing primer (21ng/μl) and 2μl sequencing buffer was then added. This was incubated at 70°C for 2min, and then allowed to cool slowly to 30°C. Then proceeded with the labelling reaction of the USB protocol.

2.2. Biochemistry

2.2.1. 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.
-Running gels (7.5-12%(w/v) polyacrylamide)[National Diagnostics] was made up in 500mM Tris-Cl pH8.8, 0.2% SDS). Gel was polymerised with 0.1% Ammonium Persulphate (APS), and 0.05% N,N,N',N'-tetramethyl-ethylene diamine (TEMED).
Stacking gels (4% (w/v) polyacrylamide) were made up in 155mM Tris-Cl pH6.8, 0.2% SDS, and polymerised with 0.1% APS, and 0.05% TEMED as above.

Gels were run in 1X PAGE running buffer. If proteins were to be visualised directly, gel was 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, the following protocol was used.

2.2.2. Western blotting

The SDS-PAGE gel was placed against pre-wetted Hybond nitrocellulose (Amersham) with two pieces of Whatman 3mm filter paper on each side into a BioRad western blot cassette. Transfer was carried out in a BioRad western blotting apparatus with 1X Transfer Buffer at 400mA. Transfer was normally for 90 minutes. After transfer the filter was stained with 0.1% Ponceau S in 5% acetic acid, and the positions marked of protein lanes and molecular weight markers (Biorad). The filter was blocked with 4% Marvel milk in 0.1% Tween-20/PBS for 1h. Antibodies were diluted to the appropriate concentration in blocking buffer and applied to the filter in a sealed plastic bag for 1h with vigorous shaking. Excess antibody was washed off with 0.1% Tween-20 in PBS (3 X 10min). Secondary antibodies were conjugated to Horseradish Peroxidase and detected by application of Super Signal Chemiluminescent Substrate (Pierce).

Primary antibodies used in this study for western blots.

- Anti-phosphotyrosine (monoclonal, UBI)
- Anti-phosphotyrosine (rabbit polyclonal, a kind gift of Rick Huganir)
- Anti-Pan Protein Kinase C (rabbit polyclonal, UBI, recognises α, β, and γ isoforms)
- Anti-Protein Kinase C α isoform (MC5)
- Anti-Protein Kinase C β isoform
- Anti-Protein Kinase C γ isoform (36G9)
- Anti-Protein Kinase C δ isoform (DC01)
- Anti-Protein Kinase C ε isoform (PPA89)
- Anti-Protein Kinase C ξ isoform (FRANK)
- Anti-Protein Kinase C η isoform (PPA022)
- Anti-Protein Kinase C θ isoform (PPA047)
- Anti-Protein Kinase C τ isoform (PPA082)
- Anti-RACK-1 (monoclonal, Transduction Laboratories)
- Anti-GABA<sub>A</sub>R β2/3-subunit (monoclonal, Boehringer)

All PKC isoform antibodies were a kind gift of Dr Peter Parker (ICRF, London).

2.2.3. GST-Fusion protein production
BL21 bacteria were transformed with pGEX constructs and plated onto LB Agar plates containing amp and chlor. A 20ml LB amp/chlor culture was grown overnight at 37°C, and added to 1l LB/amp the following morning. This was allowed to grow to an OD A600 of 0.5-0.7, after which isopropylthio-β-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, washed in 10ml buffer A, centrifuged again and resuspended in 10ml 1X TE containing a batch of protease inhibitors (100μM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml Aprotinin, leupeptin, antipain, pepstatin). Triton-X 100 was added to 1% and the mixture was sonicated at full power for 3 X 30s. 25ml Buffer C + protease inhibitors was added and the mixture centrifuged at 30,000 rpm for 30min. To the supernatant was added 1ml of Glutathione-Agarose beads, and the mixture left at 4°C rotating for 2hrs. Glutathione-Agarose beads were pre-swollen in Buffer C before use. After affinity-purification, the beads were batch-washed four times with Buffer C and protease inhibitors. If required, the protein was eluted from the beads with Buffer C and protease inhibitors supplemented with 10mM reduced glutathione pH 7.5.
2.2.4. Affinity-purification ("pull-down") assays
Brains from adult Sprague-Dawley rats, were homogenised in a Downs' homogeniser in a buffer containing 1% nonidet P-40 (NP40), 0.5% deoxycholate (DOC), 150 mM NaCl, 10 mM triethanolamine pH 7.6, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM Na Orthovanadate 100 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, pepstatin, antipain and aprotinin. Insoluble material was removed by centrifugation at 50K for 30 min. Extracts (0.5-20 mg of protein) were then exposed to receptor fusion proteins (approx 20-50 μg) bound to glutathione agarose beads at 4°C for 2 hours. Beads were washed twice in buffer 1 consisting of 0.4% NP40, 500 mM NaCl, 10 mM triethanolamine pH 7.6, 5 mM EGTA, 5 mM EDTA, 1 mM Na Orthovanadate, 1 mM PMSF and then twice in buffer 1 supplemented with 150 mM NaCl. At this stage the beads were either used in kinase assays or subjected to SDS-PAGE. Proteins binding to the fusions or GST alone were then detected by Western-blotting.

2.2.5. In vitro phosphorylation catalysed by associating kinases
To analyse the capability of associating kinases to phosphorylate bound GABA<sub>A</sub> receptor subunit intracellular domains or whole immunoprecipitated receptor, washed beads from the 'pull-down' assays (2.3.4.) or immunoprecipitations (2.2.11) were washed in kinase buffer (20 mM Tris pH 7.4, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM ouabain, 1 mM sodium orthovanadate, 0.1 mM DTT, 2 mM MnCl<sub>2</sub>) and then incubated at 30°C for up to 30 minutes in kinase buffer containing 3-30 mCi γ<sup>32</sup>P-ATP at a final concentration of 20 mM (Amersham). Beads were then pelleted and bound material was separated by SDS-PAGE followed by autoradiography. To characterise the co-purifying kinase activity, assays were performed in the presence of various kinase inhibitors; 0.1 mM PKA inhibitor/Walsh peptide (Promega), 1 mM CaMKII inhibitor W7 (Calbiochem), 0.1 mM-0.5 mM PKC inhibitor peptide 19-36 (Calbiochem).
2.2.6. *In vitro* kinase assays using purified kinases

Phosphorylation of GST-fusion proteins was carried out in kinase-specific buffers for a number of reasons including the characterisation of phosphospecific antibodies.

**cAMP-dependent protein kinase (PKA):** 20mM HEPES pH 7.0, 20mM MgCl₂.

**PKC:** 20mM HEPES pH 7.5,
10mM MgCl₂, 0.5mM CaCl₂, 40μg/ml phorbol-12,13-dibutyrate.

**v-src:** 20mM Tris pH 7.4, 20mM MgCl₂, 1mM EDTA, 1mM EGTA, 10μM Na Orthovanadate, 10mM MnCl₂

Final reaction volumes varied but generally most reactions were done with a final 'cold' ATP concentration of 100μM. 0.2mM ATP (specific activity 500-1000 cpn/pmol using γ-³²P-ATP; Amersham, 3000Ci/mmol) was added when necessary. 10-50ng purified kinase was used per reaction, which was carried out at 30°C for 30-60min. Assays were terminated by adding 25µl SDS-PAGE sample buffer. Incorporation of phosphate was visualised by separation of the proteins by SDS-PAGE, followed by autoradiography for radiolabelled samples or by western blotting with phosphospecific antibodies.

2.2.7. Quantification of kinase activity interacting with GABA<sub>₆</sub> receptor subunits

To analyse the level of kinase activity co-purifying with each subunit, kinase assays were performed as above (see 2.2.5.) but in the presence of a core substrate peptide derived from neurogranin residues 28-43 (NG 28-43, Promega). NG is a well characterised PKC substrate (Chen et al., 1993). The peptide was added to the reaction at a concentration of 50 mM with or without PKC<sub>(19-36)</sub> inhibitor peptide (10 mM) under the conditions described above. The reaction was stopped by adding an equal volume of ice-cold 150 mM H₃PO₄. The beads were then pelleted and triplicate aliquots of the supernatant were spotted onto Whatmann P-81 phosphocellulose filter papers. Papers were then washed (3x10) minutes with 150 mM H₃PO₄, dried and then subjected to Cherenkov scintillation...
counting. For all experiments, values for control reactions lacking substrate or beads not exposed to lysate were subtracted as blanks.

2.2.8. Phosphopeptide map analysis
Phosphopeptide map analysis was performed on excised gel slices as described previously (Moss et al., 1992a; McDonald and Moss, 1994). Phosphoprotein gel slices were re-hydrated, washed and digested with trypsin (0.1 mg/ml Sigma) for 24-48hrs. The supernatants were dried in a speed-vac. The samples were then resuspended in 10 μl water, heated to 65°C for 1 minute, and centrifuged at 13,000rpm for 15 minutes. Depending on level of radiolabel incorporated into the sample a certain amount was then loaded in the middle of a chromatography plate along with 1μl of the dyes basic fuchsin (10mg/ml) and phenol red (10mg/ml). Samples were then dried. The plate was then pre-wetted with peptide-map buffer (17.4%(v/v) acetic acid, 0.92%(v/v) pyridine, pH3.5), before being submerged in same buffer in chamber and subjected to electrophoresis in the ‘x-dimension’ at 500 volts. Once the dyes reach within 4cm of the plates edge, plate was removed from chamber and dried. This was followed by thin-layer chromatography in the ‘y-dimension’, using chromatography solvent buffer (37.5%(v/v) pyridine, 25% (v/v) n-butanol, 7.5% (v/v) acetic acid), until the buffer reaches the top of the plate. Plates were then dried, wrapped in saran wrap, and the phosphopeptides were visualised by autoradiography.

2.2.9. Phosphoamino acid analysis
Phosphoamino acid analysis was performed on excised gel slices as described previously (Moss et al., 1992a; McDonald and Moss, 1994). As above, phosphoprotein gel slices were re-hydrated, and washed and digested with trypsin. Samples were then hydrolysed with 6N HCl for 1h at 100°C, and then dried. The samples were then resuspended in 10 μl water, heated to 65°C for 1 minute, and centrifuged at 13,000rpm for 15 minutes. The resulting phosphoamino acids were then loaded onto a chromatography plate with the dye basic fuchsin and a set of
phosphostands (1\mu l phosphoserine (10mg/ml), 1\mu l phosphotyrosine (10mg/ml), and 1\mu l phosphothreonine (10mg/ml) and subjected to electrophoresis at 500v in a buffer of 10%(v/v) acetic acid, 1% formic acid, pH1.9. Once dye migrated 4cm the plate was moved to the chamber used for phosphopeptide analysis and the dye was allowed to move a further 7cm. After drying and wrapping in saran wrap the radiolabelled phosphoproteins were visualized by autoradiography.

2.2.10. In Vitro overlay assays
-RACK-1 Overlay
Filter overlay assays were performed as described by Li et al (1992). Equivalent amounts of GST-GABA\alpha receptor fusion proteins encoding the intracellular domains or truncated forms of the intracellular domains were separated by SDS-PAGE and transferred to Hybond nitrocellulose membrane as for a western blot. A denaturing-renaturing process was then carried out by initially incubating the membrane in 7M Guanidine-HCl in 10mM Hepes pH 7.5, 70mM KCl, 5mM EDTA, 1mM beta-mercaptoethanol for ten minutes at 4°C. A series of similar incubations with decreasing Guanidine concentrations (3M, 1.5M, 0.75M, 0.4M, 0.2M, 0.1M, 0M) were then carried out for 10min each. Non-specific protein binding was then blocked by incubation in the Hepes/KCl/EDTA/\beta-mercaptoethanol buffer supplemented with 5% marvel milk and 0.05% Triton X-100 for 1h at 4°C. This was followed by another blocking step with 1% marvel for 1h at 4°C.
The GST- GABA\alpha receptor fusion proteins and GST alone were then probed with a GST-RACK-1 fusion protein produced in pGEX-2TK (Pharmacia), labelled via phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (Promega) to a specific activity in excess of 10^6 cpm/mg. This is possible as the pGEX-2TK vector has a strong PKA consensus phosphorylation site within its polylinker. The kinase reaction mixture was made up as follows;
10X PKA buffer (see section 2.IV.d, above) 6\mu l, GST-RACK-1 (2TK) 10\mu g, [\gamma-^{32}P] ATP redivue (Amersham) 15\mu l, Cold ATP 10\mu M 15\mu l, 10units PKA catalytic subunit, made up to 60\mu l with water.

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This was incubated at 30°C for 20min, after which EDTA was added to a final concentration of 20mM, and glycerol to 50%. To separate the unincorporated counts from the labelled protein, a column of Sephadex G-25 beads (Pharmacia Biotech) in Hepes/KCl/EDTA/β-mercaptoethanol buffer was set up in a long glass pasteur pipette. The kinase reaction mixture was added to the top of the column and allowed to run in, with additional buffer added afterwards as required. Buffer emerging from the column was monitored for radioactivity and collected when radioactivity was detected. Unincorporated $^{32}$P remained in the column.

Radiolabelled RACK-1 was added to the second blocking buffer (1% Marvel) in a sealed plastic bag, and incubated overnight at 4°C with vigorous shaking. The membrane was then washed 3 X 10min with Hepes/KCl/EDTA/β-mercaptoethanol/0.05% Triton X-100, and wrapped in saran wrap. Autoradiography was then used to visualise binding of RACK-1 to the fusion proteins on the membrane.

-PKC Overlay
PKC overlays were performed essentially as described by Ron et al (1994) using PKC, purified from rat brain, a generous gift from Rick Huganir (Johns Hopkins School of Medicine). PKC-β1 was detected using an antisera specific for this PKC isoform (Marais et al, 1989; Kiley et al, 1995). Equivalent amounts of GST fusion proteins encoding the intracellular domains of receptor or the full length RACK-1 molecule were separated by SDS-PAGE and transferred to Hybond nitrocellulose membrane. The filter was then incubated for minimum one hour in ‘Overlay block’ (0.2M NaCl, 50mM Tris-HCl pH 7.5, 3% w/v BSA, 0.1% polyethylene glycol 15-20, 000). PKC was diluted in ‘Overlay’ buffer (0.2M NaCl, 50mM Tris-HCl pH 7.5, 0.1% w/v BSA, 0.1% PEG, 12mM 2-mercaptoethanol, 20ug/ml leupeptin/PMSF) and incubated at room temperature for one hour. After 4 x 5minutes washes in ‘Overlay wash’ buffer (0.2M NaCl, 50mM Tris-HCl pH 7.5, 0.1% PEG, 12mM 2-mercaptoethanol), the filter was processed as for a Western blot with PKC antibodies used to identify bound enzyme on the filter.
2.2.11. Immunoprecipitation

-[\textsuperscript{35}S]-labelled HEK 293 cells; cells were transfected with the appropriate constructs and left overnight. The cells were then, approx 16 hrs later, starved of methionine by washing three times in DMEM lacking methionine (met-) (Gibco) and incubating in this medium for 30min. After this time, fresh met- medium supplemented with 0.2mCi \textsuperscript{35}S-methionine was added to the cells, and incubated for 4 hours. The medium was then removed from the cells, which were then washed in PBS, and lysed in IP buffer (50mM NaF, 10mM Na Pyrophosphate, 1mM Na Orthovanadate, 5mM EGTA, 5mM EDTA, 16.9mM Na\textsubscript{2}HPO\textsubscript{4}, 3.1mM NaH\textsubscript{2}PO\textsubscript{4}, 0.5% Na Deoxycholate, 1% NP-40, 1mM PMSF, 10µg/ml Leupeptin, Antipain, and Pepstatin). Cell lysate was then spun at 13,000K for 10 minutes to remove nuclei. The lysate was pre-cleared by incubation with 50µl of a 1:1 slurry of protein A-sepharose beads for 1h rotating at 4°C. These beads were removed by centrifugation, and to the resulting supernatant was added a further 50µl protein A sepharose slurry along with 5-10µg of antibody. This was incubated for 2-10hrs rotating on a wheel at 4°C. The beads were washed with IP buffer supplemented with 250mM NaCl, by three incubations at 4°C for 10min rotating on a wheel. A final wash was carried out with 150mM NaCl. The beads were resuspended in 30µl SDS-PAGE sample buffer and analysed by SDS-PAGE.

The gel was stained with coomassie blue to visualise molecular weight markers and then incubated in 1M Sodium Salicylate at room temp for 20min with gentle shaking. This acts to enhance the signal by emitting light as well as beta-radiation. The salicylate was washed off with water, the gel dried, and exposed to film.

-unlabelled HEK293 cells. A modification of above was used to look for co-precipitating proteins. Cells were transfected as above and left for 12-18 hours. The cells were then directly lysed and processed as above.
Samples were only washed under conditions of low salt so as to preserve interactions. The gels were processed by western blotting to detect any co-precipitating proteins.

$^{35}$S-labelled cortical neurons. After cells had been in culture for 7-10 days they were starved of methionine by washing three times in DMEM lacking methionine (met-) (Gibco), and incubating in this medium for 2 hours. After this time, fresh met- medium supplemented with 1mCi $^{35}$S methionine was added to the cells, and incubated for approx 14 hours. Cells were then lysed in a buffer of 40mM Tris (pH 7.4), 5mM EGTA, 5mM EDTA, 10mM Na Pyrophosphate, 1mM Na Orthovanadate, 50mM NaF, 1% triton X-100, 0.5% DOC, 100µM PMSF, 10µg/ml Leupeptin, Antipain, and Pepstatin, and the protocol as for HEK293 cells was followed. Samples were analysed similarly by radiography.

$^{32}$P-labelled cortical neurons. Cells after 7 days in culture were washed in pre-warmed phosphate-free medium (Gibco) and then incubated for 4 hours in phosphate free medium containing 0.5mCi/ml $^{32}$P-orthophosphate ($^{32}$P-orthophosphate in dilute HCl, 10 mCi/ml; Amersham). Protein kinases were activated/inhibited as described. Cells were then lysed and proteins immunoprecipitated as above. Samples were again analysed by autoradiography, with phosphorylated proteins analysed by phosphopeptide mapping and phosphoamino analysis (See 2.2.8/9.)

-unlabelled cultured neurones; cells were processed as for transfected HEK293 cells.

-brain lysate

Brain lysate was prepared as in section 2.2.4. Approximately 1-10mg of lysate was used per immunoprecipitation.
2.3. Cell culture

2.3.1. Maintenance of HEK293 cells

Human embryonic kidney cell fibroblasts were grown at 37°C with 5% CO2 in Dulbecco’s Modified Eagles Medium (DMEM; Gibco) containing 10% (v/v) foetal calf serum (FCS; Gibco), 0.3%mg/ml glutamine (Gibco), 60μg/ml penicillin (Sigma) and 150μg/ml streptomycin (Gibco). At approx 90% confluence, cells were passaged by treatment in prewarmed trypsin (0.05% w/v), EDTA (0.02%) in Modified Pucks saline A (Sigma). Once in suspension the cells were diluted with pre-warmed DMEM (as above), and seeded out onto 90mm dishes.

2.3.2. Transient transfection of HEK293 cells

One 10cm dish of cells at 30-50% confluence was used per transfection. Cells were trypsinised from the dish and washed once in 10ml DMEM. After centrifugation at 1,000rpm for 2min, the cells were washed once in 10ml OptiMem (Gibco), and then resuspended in 0.5ml OptiMem. The cells were transferred to a 0.4cm electroporation cuvette (BioRad), and 10μg total plasmid DNA was added. Electroporation was carried out with the settings 400V, 250μF, Ω. The cells were then added to 5ml DMEM in a 60 mm dish. If the cells were to be viewed by immunofluorescence microscopy, 1cm coverslips coated with poly-L-lysine (10ug/ml) were used.

2.3.3. Culturing of cortical and hippocampal neurons

Corticies or hippocampai were dissected from embryonic day 19 rats and the tissue incubated in 0.25% trypsin in HEPES buffer saline (HBSS; Gibco) for 15 min followed by 3, 5 min washes in HBSS. The tissue was then dissociated by tituration with a fire polished glass pipette. Cortical neurons were then plated on 0.1 mg/ml poly (L) lysine treated 10 cm tissue culture dishes at a density of 10⁵ cells/cm² for biochemical
analysis and on coverslips for the purposes of immunofluorescence, and grown for 7 days before use.

2.4. Immunofluorescence microscopy
Cells on coverslips were washed once with PBS and then fixed with 4% paraformaldehyde in PBS for 10min. After washing again in PBS, the cells were blocked in 10% horse serum (Gibco) and 0.5% Bovine Serum Albumin in PBS for 2 X 10min. If intracellular antigens were to be visualised, the cells were permeabilised with 0.1% NP-40. After another wash in PBS, cells were quenched with 50mM NH$_4$Cl for 10min, followed by a further PBS wash. Antibodies were diluted in blocking buffer at the appropriate concentration and applied to the cells for one hour. Between primary and secondary antibody applications, the cells were washed 3 X 5min in blocking buffer. Fluorophore-conjugated secondary antibodies (Jackson immunochemicals) were used at a concentration of 1:250 in blocking buffer. The cells were finally washed three times in PBS and mounted in N-propyl gallate on glass slides. These were then viewed with an MRC 1000 confocal microscope.

2.5. Antibody Production
2.5.1. Immunisation protocols
Immunisations were carried out by Cocalico Biologicals, Inc. Anti-$\gamma_2$ N-terminal, and Anti-$\gamma_2$ phosphospecific antibodies were produced in both guinea pigs and rabbits. The $\gamma_2$ N-terminal antigen was a peptide consisting of the first 29 amino acids of the protein (QKSDDDYEDYTSNKTWVLTPKPEGDVTC; synthesised by Alta Biosciences, Birmingham, UK). The phosphospecific antisera were against the peptide ERDEEYGY(p)ECLDG or ERDEEY(p)GY(p)ECLDG (p denotes phosphorylated residue). Peptides produced in the Rockefeller University Protein/DNA Technology Centre, New York, USA. Peptides were conjugated to keyhole limpet haemocyanin with glutaraldehyde, and administered intradermally at multiple sites in each animal. Primary immunisation was administered in Complete Freunds adjuvant with boosts in Incomplete Freunds Adjuvant.
Animals were immunised according to the following protocol:
Day 0: Prebleed and initial inoculation
Day 14: Boost
Day 21: Boost.
Day 35: Test Bleed.
Day 49: Boost (as above)
Day 56: Final Bleed.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)
To assay the specificity of the IgG being produced test bleeds were assayed by ELISA. ELISA plates were coated with approx 10µg per well of either antigen coupled to BSA, or BSA alone. Plates were sealed with Parafilm and left at 4°C overnight. Wells were washed three times with Phosphate-buffered saline /0.5% Tween w/v (PBS/T), and similarly with PBS, before a 60 minute incubation in blocking buffer (PBS/T, 4% Marvel). After washing as before, each well was incubated in primary antibody for 60 minutes, washed out, and incubated in secondary antibody for 45 minutes. Both antibodies were in blocking buffer. To develop the reactions, a tablet of o-phenyldiazmine dihydroxychloride was dissolved in phospho-citrate. 100µl was added to each well. The plate was wrapped in foil to wait for a colour change. To stop reaction 50µl HCl (5M) was added, and colour change read on a plate-reader at λ490.

2.5.3. Protein-A purification of polyclonal antibodies
Protein A-sepharose beads were swollen in TBS/tween pH7.5 (50mM Tris, 150mM NaCl, 0.1% Tween 20 w/v) to give a final packed column volume of 1ml. The column was washed with approx 30mls TBS/tween pH7.5. Filtered crude serum (filtered through 0.45µm filter) was passed through the column twice. Column was then washed with 30mls TBS/tween pH7.5, 30mls BBS/tween pH7.5 (25mM Na Borate, boric acid, 1M NaCl, 0.1% Tween 20 w/v), 30 mls ACS/tween pH5.5 (50mM
Na acetate, 1M NaCl, 0.1% Tween 20) and finally 40 mls TBS. Bound antibodies were eluted with 0.1M glycine pH2.5. Elutions were neutralised with 50µl 1M Tris pH7.4 per ml eluate, and then dialysed overnight against 50mM Tris pH7.4 with 150mM NaCl, at 4°C. Antibody concentration was then determined by reading OD$_{280}$ (OD$_{280}$1.4=1mg/ml IgG). If necessary antibody was concentrated using Centriprep-30 concentrators.

2.5.4. Affinity purification of polyclonal antibodies
Peptides were coupled covalently to an activated gel matrix (CH Sepharose®4B; Pharmacia) according to the manufacturers instructions. Column was washed in 25mls TBS/tween pH7.5 (50mM Tris, 150mM NaCl, 0.1% Tween 20 w/v). Serum was then passed over the column and the same procedure as for protein-A purification was followed (see section 2.5.3.). Similar elution conditions were used, but with some antibodies different conditions were required, e.g. lower pH. After dialysis overnight against 50mM Tris pH7.4/150mM NaCl at 4°C, antibody concentration was then determined by reading OD$_{280}$.

2.5.5. Covalent coupling of antibodies to Protein-A sepharose
Affinity-purified antibody was bound to protein A beads. Beads were washed and left resuspended in 0.2M sodium borate (pH 9.0). Dimethylpimelidate was added to a final concentration of 20mM, and gently mixed for 30 minutes. Beads were then washed and incubated for 2 hours in 0.2M ethanolamine. The bound antibody was finally stored in PBS.

2.6. Whole-cell patch clamp electrophysiology
For electrophysiology, HEK cells were electroporated (400V, infinite resistance, 125µF; Biorad Gene Electropulser II) with cDNAs for GABA$_A$ receptor subunits (1µg/µl) and the reporter plasmid encoding for the S65T mutant of jellyfish green fluorescent protein (GFP; 1µg/µl) (Heim et al., 1995) and maintained in culture for 24 hrs prior to use. Membrane currents were measured from single cells using the whole-cell
patch clamp configuration in conjunction with an Axopatch 200B amplifier (Axon instruments). Patch pipettes (resistance 1-5 MΩ) were fabricated from thin-walled borosilicate glass (Clark electromedical) and filled with a solution containing (in mM): 140 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 11 EGTA and 2 adenosine triphosphate, pH 7.1. The cells were subjected to continuous background perfusion with a Krebs solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 HEPES and 11 glucose, pH 7.4. Membrane currents were filtered at 5-10 kHz and displayed on Gould 2200S ink-pen chart recorder and stored on a Racal Store 4 FM tape recorder (DC to 5Hz) for analysis. HEK cells were used 24 to 72 hrs after transfection and possessed membrane potentials of -30 to -55mV. Single fluorescing HEK cells were visualised using a Nikon Diaphot 300 and epifluorescence with filter block B-2A. Drugs and Krebs solution were rapidly-applied to single cells using a modified Y-tube positioned to within 100-200 μm of the recorded cell as previously reported (Wooltorton et al., 1997). Activation of PKC was achieved by applying 0.5μM PMA from a rapid perfusion pipette placed 200μm from the recorded cell.
### 2.7. Commonly Used Buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
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<tr>
<td>TAE (Tris-Acetate EDTA)</td>
<td>40mM Tris-acetate, 1mM EDTA (pH 8.0)</td>
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<tr>
<td>TBE (Tris-Borate EDTA)</td>
<td>90mM Tris-borate, 2mM EDTA</td>
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<tr>
<td>TE (Tris EDTA) usually pH 7.6</td>
<td>10mM Tris.Cl (pH 7.6), 1mM EDTA (pH 8.0)</td>
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<tr>
<td>SDS-PAGE buffer (Sodium Dodecyl Sulphate</td>
<td>25mM Tris, 250mM glycine (pH 8.3), 0.1% SDS</td>
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<td>PolyAcrylamide Gel Electrophoresis Buffer</td>
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<tr>
<td>Western Blotting Transfer Buffer</td>
<td>50mM Tris, 380mM glycine, 0.1% SDS, 20% methanol</td>
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<tr>
<td>Solution I (For mini-preparation of plasmid DNA)</td>
<td>50mM glucose, 25mM Tris pH 8.0, 10mM EDTA</td>
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<tr>
<td>Solution II</td>
<td>&quot;</td>
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<tr>
<td></td>
<td>1% SDS, 200mM NaOH</td>
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<tr>
<td>Buffer A</td>
<td>(For GST fusion protein purification)</td>
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Solution III: 5M potassium acetate (made by adding 29 ml glacial acetic acid to 50 ml H₂O and, on ice, adding 10 M KOH to pH 4.8)
Chapter 3
Production and characterisation of polyclonal antibodies to investigate the tyrosine phosphorylation of the \( \gamma_2 \) subunit in the brain

3.1 Introduction

Protein tyrosine phosphorylation has been shown to be critical in a wide range of intracellular signalling pathways moreover, it has been implicated as a key mechanism in the modification of neuronal function (Tananka et al., 1994, Nicholls, 1997). The latter includes roles in long-term potentiation (Terlau et al., 1989; O'Dell et al., 1991), synaptogenesis (Catarsi et al., 1993), and in certain neuronal pathologies such as epilepsy (Stratton et al., 1991). Phosphorylation studies of the GABA\(_A\) receptor has principally focused on the phosphorylation of serine residues. More recently, tyrosine phosphorylation has been implicated as an important mechanism for channel regulation (Moss et al., 1995; Valenzuela et al, 1995; Wan et al., 1997; Wan et al., 1997b), with \( \beta \) and \( \gamma_2 \) subunits suggested as being the specific substrates for tyrosine kinases. Previous studies have shown that when \( \gamma_2L \) is co-expressed with \( \alpha_1, \beta_2 \) and \( v\text{-src} \) in HEK293 cells, it is phosphorylated at a consensus site for tyrosine phosphorylation, centred on the residues Y365 and Y367 (Moss et al., 1995). This phosphorylation causes an enhancement of the GABA current. Single-channel recordings from cultured rat sympathetic neurons in the presence of cSRC and sodium orthovanadate, suggested that the increase in channel function is due to increases in the mean open time and probability of opening of the channel (Moss et al., 1995).

Biochemical analysis of the phosphorylation of the \( \gamma_2 \) subunit in a cellular environment has been limited to recombinant, epitope-tagged receptors in HEK293 cells, due to the unavailability of high titre \( \gamma_2 \) specific antibodies, which would allow the study of endogenous \( \gamma_2 \) subunit phosphorylation in neurons. Since the cloning of the GABA\(_A\)
receptor subunits and publication of the primary amino acid sequences, a number of specific GABA_A receptor subunit antibodies have been raised. The γ2 subunit was cloned from a human fetal brain library (Pritchett et al., 1989b), and has two splice variants, γ2S and γ2L, which differ by the insertion of eight amino-acids in the major intracellular loop (Whiting et al., 1990; Kofuji et al., 1991). A number of antibodies have been raised against the subunit, recognising epitopes in the intracellular loop or amino-terminus (Stephenson et al., 1990; Benke et al., 1991; Khan et al., 1993; Somogyi et al., 1996). For the purposes of this study, two approaches were taken to produce specific antibodies to study tyrosine phosphorylation of the γ2 subunit in neurons.

The first involved raising antisera in two species (rabbit and guinea-pig), to a peptide which corresponded to the first 29 amino-terminal amino-acids of the γ2 subunit. This epitope has been used successfully in an earlier study to raise antibodies against the γ2 subunit (Somogyi et al., 1996). After characterisation of the antisera by immunofluorescence, western blotting and immunoprecipitation, they were used to immunoprecipitate the γ2 subunit from neurons and whole brain lysate, to look for tyrosine phosphorylation of the subunit. After the initial immunoprecipitation, the samples are separated by SDS-PAGE and blotted with an anti-phosphotyrosine antibody. But this two-step process is still quite unrefined, and can give results which are hard to interpret. This is mainly due to cross-reactivity between the two antibodies used.

To get around these problems, polyclonal antibodies were also raised which recognise the γ2 subunit when it is specifically phosphorylated on residues Y365 and Y367, and solely on Y367, as these sites have been shown to be the major sites for tyrosine phosphorylation (Moss et al., 1995). Phosphospecific antibodies have the great advantage that samples can be directly probed by western blotting, and there is no need for prior immunoprecipitation, or pre-radiolabelling of proteins to look for phosphorylation.
3.2. Characterisation of polyclonal antibodies raised against the amino-terminus of the γ₂ subunit

3.2.1. Enzyme-linked immunosorbent assay (ELISA) to measure antisera reactivity of γ₂ N-terminal antibody

Enzyme-linked immunosorbent assay (ELISA) was used to look at the reactivity of the sera for the antigen they were raised against, i.e. the first 29 amino acids of the γ₂ amino-terminus. ELISA has the advantage over other methods such as ‘dot-blots’, in that it produces numerical data correlating to the strength of interaction between antigen and antibody. Figure 2 (A & B) shows that both rabbits UCL17, and-18 showed high immune responses, with the production bleed for UCL17 being slightly superior. This is emphasised by Figure 2B, where a dilution series of both sera, shows UCL17 to give a higher immune response. Both animals showed a positive response though down to a dilution of 1:10 000.

Figure 2C shows the results for a similar dilution series for four guinea pigs, GP1-4, which had been immunised with the same antigen. Again good immune responses were seen with the production bleeds of all animals, with GP1 being slightly better, as shown by the maintained response as the crude sera was diluted. Both GP-1, and -2, showed a positive immune response at a 1:100 000 dilution of the crude sera.

3.2.2. Subunit specificity of γ₂ N-terminal antibodies determined by immunofluorescence

The next test for the rabbit and guinea-pig sera was to see if either could specifically detect the γ₂ subunit, especially given the relatively high homology between subunits, when either transiently transfected into HEK293 cells, or when expressed endogenously in neurons, by immunofluorescence. The sera had to be initially affinity purified, on a resin of the original antigen, as the background seen with crude sera.
Figure 2 Elisa Data to measure the titre of $\gamma^2$
N-terminal specific antibodies
A) Rabbits UCL17/18 were immunised with a peptide corresponding to the amino acids 1-29 of γ2. Titer measured by ELISA against immunising peptide. 1-prebleed. 2-Second bleed, 3-Production bleed, 4-Affinity purified UCL17. All sera at 1/100 dilution. B) Dilution series of production bleeds of UCL17/18. C) Guinea-pigs 1-4 were immunised similarly. Dilution series of production bleeds measured as for UCL17/18.
was very high. Aliquots of UCL17 and GP1 were purified as stated, and tested initially in ELISA. Though the titre was slightly reduced compared to crude sera it was still high. Figure 2A shows ELISA data for affinity-purified UCL17.

cDNAs encoding α1, β2, γ2S, γ2L, γ2SMYC and γ2LMYC in the mammalian expression vector pGW1, were transfected in combinations into HEK293 cells. The γ2 subunits were transfected in both a wild-type, and a 9E10-modified form, to test if the 9E10 epitope (Evan et al., 1985), inserted between residues four and five at the amino-terminus (Connolly et al., 1996), interferes with the epitope recognised by the γ2 antibodies (amino-acids 1-29).

When expressed alone, GABA_A subunits are in general unable to access the cell-surface (Connolly, 1996). α1 and β2 subunits expressed together are able to hetero-oligomerise and reach the cell-surface, while the γ2 subunit requires the presence of both the α and β subunit to reach the cell-surface. Single subunits of α1, β2 or γ2 or combinations of αγ or βγ are seen to be retained in the ER (Connolly et al., 1996). The γ2 antisera raised in this study recognise the N-terminus of the protein, and as seen in figure 1, this is predicted to be extracellular. So if the antibody recognises γ2 in non-permeabilised cells, we can presume that the subunit is at the cell-surface. If it is trapped in the ER, the antibody will only be able to recognise the epitope under cell-permeabilising conditions.

Affinity purified forms of UCL17 and GP1, used at a concentration of 5μg/ml, were able to detect γ2S and γ2L on the cell surface of HEK293 cells when co-expressed with α1 and β2 [see Figures 3A, 3B (UCL17), 4A, 4D (GP1)]. The specificity of this was shown by blocking the staining by pre-adsorbing the antibody, prior to application, with the immunising peptide (see 3D, 3E, 4B, 4E). Figures 3G and 3H also show that UCL17 is able to recognise both the modified proteins, γ2SMYC and γ2LMYC, indicating that the insertion of the 9E10 epitope does not disrupt
the epitope recognised by the antibodies. As expected, expression of γ2S or γ2L alone gave staining typical of retention in the endoplasmic reticulum, upon permeabilisation (see Figures 3C, 3F, 4C, 4F). Also no staining was seen when α1 and β2 were transfected without the γ2 subunit, Figure 3I, showing clearly the specificity of the antibody.

The affinity-purified UCL17 antibody was also able to stain endogenous γ2 in neurones. Hippocampal neurons were prepared by Mr Josef Kittler (LMCB, UCL). They were processed for immunofluorescence as for HEK293 cells, with the γ2 antibody used at a concentration of 5μg/ml, in permeabilising conditions. The antibody stained distinct clusters of GABA_A receptors (see figure 5), as seen previously in other studies (Allison et al., 1998; Essrich et al., 1998). This staining was not seen when the antibody was pre-adsorbed with the immunising peptide, showing that it is specific for the γ2 subunit.

### 3.2.3. Subunit specificity of γ2 N-terminal antibodies determined by western blotting

To see if the antibodies could detect the γ2 subunit in western blotting, HEK293 cells were transfected with combinations of α1, β2 and γ2 subunit cDNAs. The cells were harvested after 24 hours in culture in SDS-PAGE reducing sample buffer. Samples were separated by SDS-PAGE, transferred to nitrocellulose and western-blotted by a standard procedure. Initial attempts with both crude sera and affinity-purified forms of UCL17 and GP1 were unsatisfactory. Both sera were then purified by a Protein A-Sepharose method. This allows purification of the whole IgG pool, without reducing the yield of higher titre antibodies, which is seen with affinity purification. Surprisingly only
HEK293 cells were transfected with following combinations of expression constructs: α1/β2/γ2s (A & D), α1/β2/γ2L (B & E), α1/β2/γ2sMYC (G), α1/β2/γ2LMYC (H), γ2s alone (C), γ2L alone (F) and (l) α1/β2. Cells were stained with affinity purified UCL17 (5μg/ml). D & E were preabsorbed with immunising peptide. All staining was in non-permeabilising conditions except C & F, where 0.5% NP-40 was used. Scale Bar =10μm. Transfection efficiency was approximately 25%.
Figure 4 Immunofluorescence detection of the $\gamma_2$ subunit with affinity purified GP1

HEK293 cells were transfected with the following combinations of expression constructs $\alpha_1/\beta_2/\gamma_2s$ (A & B), $\alpha_1/\beta_2/\gamma_2L$ (D & E), $\gamma_2s$ alone (C), $\gamma_2L$ alone (F) and mock. Cells were stained with affinity purified GP1 (10µg/ml). B & E were preabsorbed with immunising peptide. All staining was in non-permeabilising conditions except C & F, where 0.5% NP-40 was used. Scale Bar = 10µm. Transfection efficiency was approximately 25%
Figure 5 Immunofluorescence detection of the $\gamma_2$ subunit within culture hippocampal neurons with affinity purified UCL17

Cultured hippocampal neurons were stained with affinity purified UCL17 (10\mu g/ml). B was preabsorbed with immunising peptide. All staining was in permeabilising conditions of 0.5% NP-40. Cells had been in culture for 10 days. Scale Bar = 15\mu m.
GP1 gave Protein A purified antibody suitable for blotting. Figure 6A shows whole cell lysates from HEK293 cells transfected with α1, β2 and a γ2 subunit, blotted with protein-A purified GP1. A specific band of approximately 48kD was seen (Lanes 1-4), as expected. As for immunofluorescence the insertion of the 9E10 tag did not disrupt the epitope recognised by the antibody (Lanes 3 & 4). This band was not seen though when the γ2 subunit was omitted from the transfection (Lane 5), or in the case of mock transfection (Lane 6). To confirm that this band was the γ2 subunit, extract as resolved in lane 1 was blotted with antibody which had been pre-adsorbed with the γ2 1-29 peptide. In lane 7 the band is clearly not seen. Further, this same extract was blotted for the α1 subunit (Lane 8) and the β2 subunit (BD17:Lane 9), showing that the band in lanes 1-4 is unlikely to be α1 or β2.

To test if the GP1 antibody could recognise the γ2 subunit in neurones by western blotting, extracts of cultured rat cerebellar granule neurones and whole adult rat brain were processed as for the HEK293 cell extracts. Figure 6B, Lane 1 shows a band at approximately 50 kD, which is not seen when the antibody is pre-adsorbed with the γ2 1-29 peptide (Figure 6B, Lane 2). A second band of approximately 45kD is shown to be non-specific as it remains in the peptide blocked lane. In whole brain lysate a much more diffuse 46-50 kD band is seen (Figure 6C, Lane 3). Again it is not seen after peptide pre-adsorption. These results show that the protein A-Sepharose purified GP1 antibody is suitable for the specific identification of the γ2 subunit by western blotting. The molecular weight for the γ2 subunit reported here is very similar to earlier studies, with antibodies raised against similar peptides or fusion proteins of the major intracellular loop (Stephenson et al., 1990; Benke et al., 1991; Khan et al., 1993; Somogyi et al., 1996). The reason for the diffuse band seen in whole brain lysate may be due to the fact that both long and short forms of the subunit, generated by alternative mRNA splicing (Whiting et al., 1990; Kofuji et al., 1991), will be present in this preparation, with both being recognised due to their common amino-terminus. Differential glycosylation states of the
Figure 6 Western blot detection of the $\gamma^2$ subunit in transfected HEK293 cells, cultured neurons and brain lysate

A) HEK293 cells were transfected with following combinations of expression constructs $\alpha_1/\beta_2/\gamma^2$ (1, 7, 8, 9), $\alpha_1/\beta_2/\gamma^2$s (2), $\alpha_1/\beta_2/\gamma^2s^{MYC}$ (3), $\alpha_1/\beta_2/\gamma^2L^{MYC}$ (4), $\alpha_1/\beta_2(5)$, and mock (6). Total cell lysate was separated by SDS-PAGE, Lanes 1-6 were blotted with protein A purified GP1 (5μg/ml), Lane 7, as lanes 1-6 but antibody preadsorbed with immunising peptide. Lane 8 with RH15 (anti $\alpha_1$ antibody) and lane 9 with BD17 (anti $\beta 2/3$ antibody).

Total cell lysate from cultured granule cells (B) and whole adult rat brain lysate (C) was separated by SDS-PAGE. Lanes 1 & 3 were blotted with protein A purified GP1 (5μg/ml). Lanes 2 & 4 were processed similarly but after pre-adsorption of the antibody with the original immunising peptide.
subunit may also account for the smear. It has previously been noted that the \( \gamma_2 \) subunit runs anomalously on SDS-PAGE. It has an expected non-glycosylated MR of (49,848) but is always seen to run at an apparent lower MR. In the cerebellar granule cell lysate a tighter band is seen. Antibodies which distinguish the \( \gamma_2S \) and \( \gamma_2L \) forms (Khan et al., 1994), and mRNA distribution studies (Whiting et al., 1990; Wang et al., 1991; Bovolin et al., 1992) have suggested that the \( \gamma_2L \) variant is found at a much greater abundance than \( \gamma_2S \) in the cerebellum. A \( \gamma_2L \) specific antibody recognises a 47kD protein in cerebellar lysates (Khan et al., 1994), which may correspond to the single band seen here.

### 3.2.4 Immunoprecipitation of \( \gamma_2 \) subunits from HEK293 cells

To be able to study the tyrosine phosphorylation of the whole \( \gamma_2 \) subunit, it is essential that an antibody is able to immunoprecipitate native \( \gamma_2 \) subunit. Samples can then be western blotted with an anti-phosphotyrosine specific antibody, to look for phosphorylated tyrosine residues on the subunit (Derkinderen, 1997). Initially the \( \gamma_2 \) amino-terminal antibodies were tested to see if they could immunoprecipitate overexpressed \( \gamma_2 \) subunit from HEK293 cells. Cells were transfected with \( \alpha_1 \), \( \beta_2 \) and a \( \gamma_2 \) subunit. Sixteen hours later they were metabolically labelled with \([^{35}S]\)-methionine for 4 hours and immunoprecipitated with antibody immobilised on protein A-sepharose, Immune complexes were resolved by SDS-PAGE and visualised by autoradiography. Figure 7 shows that the affinity purified version of UCL17 was able to successfully immunoprecipitate \( \gamma_2S \) (Lane 1) or \( \gamma_2L \) (Lane 2) containing receptors. Other subunits, much more distinguishable than the \( \gamma_2 \) subunits, can be seen to co-precipitate (See arrows). \( \beta_2 \) can be see as 2 bands, running at approximately 55 and 57kD, and \( \alpha_1 \) at 50kD. \( \alpha_1 \) is probably masking \( \gamma_2S \) in lanes 1 and 3. These observations correspond to values reported previously (Krishek et al., 1994; Benke et al., 1994; Connolly et al., 1996).
Figure 7B shows that the GP1 antibody performs similarly to UCL17, in being able to immunoprecipitate γ2 subunit containing receptors (Lanes 7 & 8). To identify the γ2 subunits specifically, immunoprecipitations were also performed under non-native conditions, with cells lysed in the same buffer but with 1% SDS, to break interactions with co-precipitating subunits. Figure 7B, Lanes 9 & 10, show the result obtained in such an experiment with GP1. γ2S is seen as a tight band at about 48kD (Lane 9), above the smeary γ2L band, which is seen to run at 40-44kD (Lane 10). The reasons for the differences in molecular weight are unclear, especially as by western blotting a band of similar MR was observed for both splice-variants (see Figure 6A). In previous studies, though, differences between the two spliced forms have been seen. For example γ2L was seen to run at 47kD, compared to 45kD for the γ2s form in one study (Khan, 1994). Also the smeary band seen for γ2L may be due to the previously reported susceptibility of γ2 to degradation (Moss et al., 1995; Moss and Smart, 1996). Lanes 3 and 4 are very similar to lanes 1 and 2 except that the cells were transfected with 9E10-tagged versions of the γ2 subunit, and immunoprecipitated with the 9E10 anti-myc antibody (Evan et al., 1985). As expected, a similar band pattern is seen. Lanes 5 and 6 shows that the bands are specific for receptor proteins, as no corresponding bands are seen in untransfected cells with either the γ2 antibody (Lane 5), or 9E10 anti-myc antibody (Lane 6).

3.2.5. Immunoprecipitation from neurones and detection of phosphotyrosine residues on γ2

To determine if the γ2 subunit was phosphorylated on tyrosine residues in the brain, the initial approach was to look in cultured cerebellar granule cells. This involved treating one plate of cells with activated sodium orthovanadate (NaVan), and sham treating a second set. The cells were then lysed as described in section 2.2.11. Lysates were then subjected to immunoprecipitation with 10μg of UCL17, which had been covalently coupled to Protein-A. The latter procedure was due to the IgG heavy chain running at a relative MR similar to γ2, and to reduce cross-
reactivity with the phosphotyrosine antibody in the western blot procedure to follow. The results for both treated and untreated cells were negative (Figure 8, Lanes 3-6). One reason for this result which can be discounted is the failure to immunoprecipitate the γ2 subunit, as blotting the samples with Protein-A purified GP1, at 10μg/ml, showed a band at approx 47kD, as seen previously by direct western blotting of cerebellar granule cell lysate (Figure 8, Lane 9).

A similar procedure was then used to look in whole rat brain lysate. 10mgs of lysate, prepared as in section 2.3.11., was immunoprecipitated with 10μg of UCL17, again covalently coupled to Protein-A. The samples were separated by SDS-PAGE, and then subjected to western blotting with a monoclonal phosphotyrosine antibody, or the guinea pig purified γ2 N-terminal antibody. Fig 8, Lane 1 shows a tyrosine phosphorylated band at approximately 46kD, which migrates similarly to the band recognised by the amino terminal antibody (Lane 7). Both of these bands are not seen when the precipitating antibodies are preincubated with the immunising peptide (Figure 8, Lanes 2 and 8). This does not conclusively show that the subunit is phosphorylated in whole brain, but the peptide block result, combined with the molecular weight of the tyrosine phosphorylated band is quite suggestive. It also brings up the interesting question of why γ2 is phosphorylated in whole brain lysate, but not in cultured neurons. It could be that in culture a factor is missing. The lack of synaptic contacts in these cultures could account for this. This could be analogous to what is seen with the AChR at the neuromuscular junction, where a molecule, Agrin, stimulates phosphorylation of the β-subunit (Ferns et al., 1996; Colledge et al., 1998).
Figure 7 Immunoprecipitation of the γ2 subunit from transfected HEK293 cells with affinity purified UCL17 and GP1

HEK293 cells were transfected with following combinations of expression constructs; α1/β2/γ2s (1 & 7), α1,β2/γ2L (2 & 8), α1/β2/γ2sMYC (3), α1/β2/γ2LMYC (4) and mock (5 & 6). After metabolic labelling with [35S]-methionine, NP-40/DOC lysates were immunoprecipitated with 10μg UCL17 (1, 2, 5), 5μg 9E10 anti-MYC (3, 4, 6) or 10μg GP1 (7-10), all bound to protein-A. Lanes 9 and 10 are duplicate of 7 and 8, but performed under non-native conditions (1% SDS). Samples were separated by SDS-PAGE and analysed by autoradiography.
Figure 8 Tyrosine phosphorylation of the γ2 subunit in whole adult rat brain lysate

Blot

Phosphotyrosine

γ2

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Peptide block

NaVan

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Whole brain (Lanes 1-2, 7-8) or CGC (Lanes 3-6 and 9) lysates, treated with NaVan as shown, were immunoprecipitated with 10 μg of affinity purified UCL17 covalently coupled to protein A, which had been preadsorbed with immunising peptide where indicated. Precipitates were separated by SDS-PAGE, and western blotted with either a murine phosphotyrosine antibody or protein A purified GP1.
3.3. Characterisation of γ2 subunit phosphotyrosine specific polyclonal antibodies

3.3.1. ELISA to test the reactivity of γ2 phosphorylation-state specific antibodies

To identify the specific residues in γ2 which are tyrosine phosphorylated, polyclonal antibodies were raised which specifically recognise the subunit when it is phosphorylated on both residues Y365 and Y367, or just on Y367. The rationale for this stems from Moss et al (1995). In HEK293 cells these residues are tyrosine phosphorylated by overexpressed v-src, causing an enhancement of channel function, while unpublished data suggest the second tyrosine is the most important functionally (Dr Chris Connolly, Personal Communication). For reference the peptide with both Y365 and Y367 in a phospho-state will be called Peptide 1, with just Y367 in a phospho-state will be called Peptide 2 and a dephospho-peptide of the same region will be known as Peptide 3. Guinea pigs, GY1 & GY2, were immunised with either peptide 1 or 2 respectively. ELISAs were performed to test the titre of the serum. Figure 9A/B show that high titre antibodies were found in both animals immunised. This immunogenicity was not blocked by O-phospho-L-tyrosine, peptide 3, or BSA. Both sera were blocked very specifically though by either peptide 1 or 2. This suggests that both GY1 and GY2 have some preference for an epitope containing phospho-Y367. When further characterisation was attempted of both sera, after protein A-Sepharose or affinity purification, problems were encountered which were suggestive of too low a titre. So a further immunisation protocol was performed in a single rabbit (RY1) using peptide 1. Figure 9C shows the ELISA results for the immune response for this animal. The production bleed gave good titre, which as for the guinea-pig sera was not blocked by O-phospho-L-tyrosine, peptide 3 or BSA. It was comprehensively blocked by the peptide it was raised against though, peptide 1.
Figure 9 ELISA data to measure the titer of γ2 phosphotyrosine specific antibodies

A)  

OD 490

0  0.5  1  1.5

1  2  3  4  5  6  7  8

GY1

B)  

OD 490

0  0.5  1  1.5  2

1  2  3  4  5  6  7  8

GY2
A) Guinea-pig GY1 was immunised with phosphopeptide 1-(Y365 & phospho Y365/7). B) Guinea-pig GY2 was immunised with phosphopeptide 2(Y367). Titer measured by ELISA against immunising peptide. 1-prebleed, 2-First bleed, 3-Production bleed, 4-as 3 but pre-adsorbed with phosphoY, 5-as 3 but preadsorbed with peptide 3, 6-as 5 but preadsorbed with peptide 1, 7- as 5 but preadsorbed with peptide 2, 8-preadsorbed with BSA. C) Rabbit RY1 was immunised with phosphopeptide 1. 1-prebleed, 2-bleed 1, 3-bleed-2, 4-production bleed, 5-PY block. 6-peptide 3 block, 7-peptide 1 block, 8-BSA block.

All sera at 1/100 dilution.
3.3.2. Further characterisation of RY1

RY1 crude sera was purified by both affinity, and protein A-Sepharose methods, and attempts were made to further characterise it. The first test was to ensure that the antibody was recognising phospho-γ2. Purified v-Src is able to phosphorylate a fusion protein, containing the intracellular-loop of γ2L, at residues Y365 and Y367 (S.J.Moss, personal communication). Such phospho-protein was used to screen RY1 antibody by western blotting. Approximately 2 μg of phosphorylated and unphosphorylated GST-γ2L, and GST were separated by SDS-PAGE and subjected to western blotting with the RY1 Protein A-Sepharose purified antibody. Figure 10A shows that the protein A purified form of RY1 only recognises the phosphorylated γ2 fusion protein (Lane 1), and not the dephosphorylated form (Lane 2) or either phospho/dephospho-GST (Lanes 3 and 4). Lanes 5-8 are a duplicate gel of Lanes 1-4 which was stained with coomassie blue to identify the position of the fusion proteins. Thus, the RY1 antibody seems to be γ2 (Y365/Y367)-phospho-specific.

3.3.3. The tyrosine phosphorylation of the γ2 subunit is under tight regulation in cultured cortical neurons

The antibody was then tested on lysate from cortical neurons which had been in culture for 10 days. One plate of cells was treated with activated sodium orthovanadate (NaVan) for 30 minutes, to inhibit protein tyrosine phosphatases, while a second was left untreated. Cells were harvested into SDS reducing sample buffer. Lysate from treated cells (Lane 1) and untreated cells (Lane 2) were blotted with an anti-phosphotyrosine monoclonal antibody to look at the effects of NaVan. Lane 1 shows a vast increase in tyrosine phosphorylated proteins over Lane 2, showing there is high tyrosine-phosphatase activity in these cells at resting state. The paired lanes 3-4 and 5-6 are duplicates of 1-2, with the former blotted with RY1 blocked with O-phospho-L-tyrosine and peptide 3, while the latter is blocked similarly but also by the
A) GST-γ2 and GST proteins was phosphorylated *in vitro* by purified v-src, and separated by SDS-PAGE. Sham kinase assays were done in parallel minus kinase. These phosphorylated and de-phosphorylated proteins were separated by SDS-PAGE and blotted with protein-A purified RY1 at a concentration of 10μg/ml. Lane 1-phospho GST-γ2L, Lane 2-dephospho GST-γ2L, Lane 3-phospho-GST, Lane 4-dephospho GST. Lanes 5-8 are a coomassie stained duplicate gel of lanes 1-4.

B) Cultured cortical neurons were treated with NaVan (Lanes 1, 3, 5) or sham treated (Lanes 2, 4, 6). Whole cell lysates were separated by SDS-PAGE and blotted as follows. Lanes 1-2: anti-phosphotyrosine, Lanes 3-4: RY1 (preadsorbed with PY & peptide 3), Lanes 5-6, as 3-4 but also blocked with peptide 1. Lane 7 is a blot of NaVan treated same sample with the γ2 N-terminal specific antibody. *-highlights 2 bands blocked by peptide 1 in NaVan treated cells.
Whole adult rat brain lysate was separated by SDS-PAGE and blotted with protein A purified RY1 antibody (Lanes 3 & 4). Lane 3-pre-adsorbed with peptide 3 and phosphotyrosine. Lane 4- as 3 but also preadsorbed with peptide 1. Lanes 1-2 are as Figure 6C, blotting for γ2 with the N-terminal specific antibody.
immunising peptide 1. In lane 3, two bands (*) are apparent which are absent when blocked with peptide 1 (Lane 5), and are only apparent after NaVan treatment. If cortical lysate is blotted with the protein A purified γ2 N-terminal antibody these same two bands are apparent, lending support to the argument that the additional bands in lane 3 are the γ2 subunit, phosphorylated at residues Y365 and/or Y367. If this is true it is interesting that the receptor is not tyrosine phosphorylated on these residues under basal conditions.

Figure 11 shows that when whole adult rat brain lysate is blotted with RY1, a specific band is recognised (Lane 3-arrow), which can be blocked with peptide 1. This band runs similarly to the smeary band recognised by the γ2 amino-terminal antibody (lane 1-arrow).

3.4. Conclusions

Tyrosine phosphorylation has been implicated in many important neuronal processes, from plasticity (O'Dell et al., 1991), to disease (Stratton et al., 1991; Yokota et al., 1994). Recent studies have shown it may also play a critical role in the modulation of GABA_A receptors (Moss et al., 1995; Valenzuela et al., 1995; Wan et al., 1997; Wan et al., 1997b). This study provides the first biochemical evidence that the γ2 subunit is tyrosine phosphorylated in neurons. This subunit has been shown to be the critical substrate for modulation of receptor function by the tyrosine kinase v-src in HEK293 cells (Moss et al., 1995). This means that the receptor may be subject to regulation in vivo by tyrosine phosphorylation.

Previous attempts to study the phosphorylation of GABA_A receptors in cell lines or neurones has been hampered by the lack of good subunit-specific antibodies. The biochemical studies of GABA_A receptor phosphorylation to date have been limited to recombinant receptors in HEK293 cells (Moss et al., 1992; Moss et al., 1995; McDonald et al., 1998). This has involved prelabeling the cells with ^{32}P-orthophosphate,
prior to kinase activation and immunoprecipitation of subunits. The γ2 subunit has been particularly troublesome to study, due not only to the paucity of suitable antibodies, but also the susceptibility of the protein itself to degradation. So the main goal of this section was simply to tackle these problems, with the direct aim of being able to look at the tyrosine phosphorylation of γ2 in the brain. Prior to these studies it was not even known if the native subunit was tyrosine phosphorylated.

This study reports the characterisation of two sets of antibodies, which allow for the question of γ2 tyrosine phosphorylation, to be addressed by two approaches. The first method was by raising antibodies against amino-acids 1-29 of the amino-terminus of γ2, in both guinea pigs and rabbits. Affinity-purified forms of these antibodies were able to recognise the γ2 subunit specifically in immunofluorescence, and able to immunoprecipitate it from both transfected HEK293 cells and neuronal preparations. While a Protein A-Sepharose purified form of the guinea-pig antisera was suitable for western blot detection of the protein. The immunoprecipitation of γ2 from whole adult rat brain lysate, followed by western blotting with a phosphotyrosine antibody, showed that the γ2 subunit was tyrosine phosphorylated (Figure 8). But by a similar method, no such phosphorylation was seen of the subunit in cultured cerebellar granule cells.

The second approach was to raise phosphospecific antibodies which recognise the γ2 subunit when residues Y365 and/or Y367 are phosphorylated. A specific, high titre antibody was raised in rabbit, which in blotting extract from adult rat brain recognised a specific band, again suggestive of the subunit being tyrosine phosphorylated. Intriguingly the antibody also recognised specific bands in the lysates of cortical neurons which had been treated with NaVan, which could be blocked by the immunising peptide. It was apparent that the γ2 subunit showed no basal tyrosine phosphorylation in this cell type, perhaps indicating that this modification is under very tight control by an unknown protein tyrosine phosphatase. This result is different from that
obtained by the cruder two step assay with cultured cerebellar granule cells. This could be explained by the difference in cell type. For example there are a greater number of synaptic connections in the cortical cultures. It is possible that a synaptic signal may be required for the modification, as is seen with AChR at the developing neuromuscular junction. The motor-neuron secreted molecule ‘agrin’ causes a rapid increase in the tyrosine phosphorylation of the AChR β subunit, which is important for the clustering of AChR at the end-plate (Wallace et al., 1991; Ferns et al., 1996; Colledge et al., 1998). Also the difference could be due simply to the greater sensitivity of the phosphospecific antibodies.

Both sets of antibodies made here will be very useful in future studies of GABA_α receptor regulation and function, especially for in vivo studies. The N-terminal antibody has already been put to use in recent studies (Hanley et al., 1999; Wang et al., 1999). The phospho-state specific antibody though, should prove to be of immense value. It will now allow for a range of questions to be asked, which current methodologies can’t attempt to answer. For example which first messengers (e.g. growth-factors) affect the tyrosine phosphorylation state of the GABA_α γ2 subunit, in what regions of the brain is the γ2 subunit tyrosine phosphorylated and if the latter changes during development or disease.
Chapter 4
Identification of signalling molecules which interact with GABAₐ receptors

4.1. Introduction

There is considerable interest in understanding the molecular mechanisms used by neurones to regulate GABAₐ receptor function, with much emphasis focusing on the role of receptor phosphorylation. Studies on recombinant receptors have revealed that receptor β and γ subunits are the substrates for a range of protein kinases (Moss and Smart, 1996). Specifically, the β1-3 subunits are phosphorylated on a conserved serine residue (S409 or S410) by PKC, whilst PKA will differentially phosphorylate β subunits on S409 in vivo (Moss et al., 1992a,; Moss et al., 1992b; Krishek, 1994; Mcdonald and Moss., 1997; McDonald, 1998). There are additional phosphorylation sites for PKC, Ca²⁺/Calmodulin type 2 dependent protein kinase (Cam KII) and cGMP dependent protein kinase (PKG) within the β1, β3 and γ2 subunits (Moss et al., 1992a; McDonald and Moss, 1994; Mcdonald and Moss, 1997). The prototypic tyrosine kinase SRC will also phosphorylate specific sites within the γ2 and β1 subunits (Moss et al., 1995). In agreement with these observations purified preparations of neuronal GABAₐ receptors are phosphorylated in vitro by PKA, PKC and SRC (Kirkness et al., 1989; Browning et al., 1990; Valenzuela et al, 1995). GABAₐ receptor phosphorylation can cause diverse functional effects, ranging from enhancements to inhibitions depending upon the identity and location of the sites phosphorylated (Moss and Smart, 1996; Lin et al., 1996; Kapur, et al. 1996; McDonald et al., 1998; Brunig et al., 1999; Poisbeau et al., 1999).

While much progress has been made on identifying which receptor subunits are protein kinase substrates, such as described in chapter 3, little is presently understood about how specific kinases are targeted to GABAₐ receptors to ensure rapid, subunit specific phosphorylation. In
cultured cortical neurons, tyrosine phosphorylation of the γ2 subunit was only observed after treatment with NaVan, suggestive that this process was under tight regulation. This might involve a close relationship with a protein tyrosine kinase. Indeed tyrosine kinase activity has been reported to co-purify with GABA_A receptors purified from bovine brain (Bureau and Laschet, 1995). For other ion-channel families there is growing evidence that kinases can directly bind to the channel (Swope and Huganir., 1993; Fuhrer and Hall, 1996; Holmes et al., 1996; Yu et al., 1997; Wang et al., 1999), or are maintained very close to their substrate by means of an intermediate anchoring or adaptor molecule (Rosenmund et al., 1994; Gray et al., 1998; Westphal et al., 1999b; Fraser et al., 1999).

In order to further investigate, and identify molecules that are involved in regulating the phosphorylation state of the γ2 subunit, subunit intracellular domains were utilised to look for interacting molecules that mediate GABA_A receptor phosphorylation. Such an approach was used by Fuhrer and Hall (1996), in a study using GST fusion proteins of AChR subunits to probe mammalian C2 myotube lysates for interacting kinases. To identify interacting kinases, bound material was probed with specific antibodies. They showed that the tyrosine kinase src binds specifically to the β-subunit and phosphorylates it (Fuhrer and Hall, 1996). A similar approach was taken here, with adult rat brain extract used as the source of protein kinases/signalling molecules. As the phosphorylation of GABA_A receptor intracellular loops have been very well characterised in a number of studies (Moss et al., 1992a; McDonald and Moss, 1994; Mcdonald and Moss, 1997), it should be possible to correlate phosphorylation by interacting kinases, with specific residues in the fusion proteins.
4.2.1. Phosphorylation of the GABA\textsubscript{A} \(\beta1\) subunit intracellular domain by brain extracts

To identify molecules that interact with GABA\textsubscript{A} receptors and mediate phosphorylation, the intracellular domains of a range of different subunits were expressed as GST fusion proteins (Moss et al., 1992a; McDonald and Moss, 1994; Mcdonald and Moss, 1997). Purified fusion proteins immobilised on glutathione beads were then exposed to detergent solubilized adult rat brain extracts. After extensive washing bound material was then subjected to an \textit{in vitro} kinase assay. Reactions were stopped over a time course of thirty minutes by the addition of SDS-PAGE sample buffer. Phosphorylation was assessed by SDS-PAGE and autoradiography (Figure 12). Using this regime \(\beta1\)-GST (Panel 2) was found to be phosphorylated rapidly to high stoichiometry (\(-0.2\) mol/mol). \(\alpha1\)-GST (panel 1), \(\gamma2S\)-GST (Panel 3) and the GST backbone alone (Panel 4) were not significantly phosphorylated.

4.2.2. GABA\textsubscript{A} \(\beta1\) subunit is phosphorylated by a kinase from brain extracts, whose activity can be inhibited by the PKC inhibitor peptide PKCI\(_{(19-36)}\)

Previous studies have revealed that the \(\beta1\) subunit can be phosphorylated by PKC, PKA, Cam KII, and PKG (Moss et al., 1992a; Moss et al., 1992b; Krishek et al., 1994; McDonald and Moss, 1994; Mcdonald and Moss, 1997; McDonald et al., 1998). To determine if any of these kinases were binding to, and phosphorylating \(\beta1\)-GST, kinase inhibitors were utilised. Fusion proteins of the \(\beta1\) subunit were again exposed to adult rat brain extract, and subjected to a fifteen minute kinase assay, but in the presence of various specific kinase inhibitors: Walsh peptide, a specific inhibitor of PKA, W7, an inhibitor of Cam KII and PKCI\(_{(19-36)}\), a specific inhibitor of PKC). As Figure 13 shows PKA and CaMKII inhibitors (Lanes 2 & 4 respectively) were without effect on \(\beta1\)-GST phosphorylation. However, the specific peptide inhibitor of PKC, PKCI\(_{(19-36)}\), used at a concentration of 100 nm (Lane 3), abolishes
Figure 12 Time course of phosphorylation of GABA_{A}R subunits by associating kinases

\[ \begin{align*}
\alpha 1 & \quad \beta 1 & \quad \gamma 2s & \quad \text{GST} \\
\text{TIME (mins)} & 0 & 5 & 10 & 15 & 30 \\
45kD & - & - & - & -
\end{align*} \]

\( \alpha 1 \)-GST, \( \beta 1 \)-GST, \( \gamma 2 \)-GST or GST alone were exposed to solubilised neuronal extracts. After extensive washing, bound material was subjected to an in vitro kinase assay for various time periods and reaction products were subjected to SDS-PAGE. The upper panels show autoradiograms whilst the lower panels represent coomassie staining of the same gels to demonstrate equal loading.
Figure 13 Protein kinase C inhibitor peptide abolishes the phosphorylation of the β1 subunit by the brain extract derived kinase

The phosphorylation of β1-GST by neuronal extracts was analysed with specific kinase inhibitors. Material associating with β1-GST from neuronal extracts was subjected to in vitro kinase assays alone (lane 1) or in the presence of a specific PKA inhibitor peptide (Walsh peptide 0.1 mM; lane 2) a specific inhibitor of PKC (PKC(19-36) 0.1 mM; lane 3) or an inhibitor of CamKII (W7, 1 mM lane 4).
the phosphorylation. This suggests that PKC is binding to, and phosphorylating the β1 intracellular domain.

4.2.3. The GABA\textsubscript{A} β1 mutant protein β1\textsuperscript{(S409A)} is not phosphorylated by brain-extract derived kinase

Previous studies have identified the residue Serine 409 (S409) as the major PKC substrate in the β1 subunit both \textit{in vitro} and \textit{in vivo} (Moss et al., 1992a; Moss et al., 1992b; Krishek et al., 1994). To see if the phosphorylation seen in the assays above on β1 is at this well characterised serine residue, a mutated version of the β1 subunit, with the serine converted to an alanine was similarly exposed to brain extract and put into a kinase assay. As seen in Figure 14 Lane 2, the mutant fusion protein was not significantly phosphorylated. It is possible though that the mutation may have disrupted binding of the kinase, but as seen later (Figure 17, Lane 6), PKC is still able to bind to the mutant, so confirming that the phosphorylation is at S409.

4.2.4. Phosphorylation by the interacting kinase is reminiscent of phosphorylation in vitro by PKC

The inhibitor studies above suggest that PKC is responsible for the phosphorylation of the β1-GST protein. It is possible to analyse this further by performing phosphoamino and phosphopeptide mapping on the phosphorylated proteins. If PKC from brain lysate is binding and phosphorylating β1-GST, the phosphorylated protein should be comparable, in the mentioned analyses, to β1-GST phosphorylated \textit{in vitro} by a purified PKC preparation (Figure 15A). The latter is known to be phosphorylated specifically on serine 409 (Moss et al., 1992a). Figure 15B shows that the phosphorylation in both cases is on serine residues only, as you would predict from the studies above with the S409A mutant. Tryptic phosphopeptide maps, Figure 16, show the generation of two major positively charged phosphopeptides (a & b) from β1-GST phosphorylated \textit{in vitro} by PKC (Panel 2). β1-GST
Figure 14 The $\beta_1$ mutant $\beta_1^{S409A}$ is not phosphorylated by the brain extract derived kinase

The ability of the associating kinase to phosphorylate a wild type (lane 1) or a mutant form of the $\beta_1$ subunit, $\beta_1^{S409A}$ (lane 2) was also assessed. Lanes 3 and 4 represent control coomassie blue stains of lanes 1 and 2.
Figure 15 Phosphoamino analysis of β1 fusion protein.

A) β1-GST phosphorylated in vitro by purified PKC preparation
B) Gel slices containing β1-GST phosphoprotein were subject to tryptic digestion followed by acid hydrolysis. The resulting phosphoamino acids were then separated by thin layer chromatography. 1) β1-GST exposed to brain lysate and subjected to an in-vitro kinase assay 2) β1-GST phosphorylated in vitro by purified PKC (from A)

The migration of phosphoserine (pSER), phosphothreonine (pTHR) and phosphotyrosine (pTYR) are indicated.
Figure 16 Phosphopeptide analysis of β1 fusion proteins

Gel slices containing the β1-GST phosphoprotein were subject to tryptic. The resulting phosphopeptides were then separated by thin-layer chromatography in the first dimension and by electrophoresis in the second, as labelled 1) β1-GST exposed to brain lysate and subject to an in vitro kinase assay 2) β1-GST phosphorylated in vitro by purified PKC.
phosphorylated by the interacting kinase gives a similar peptide map (Panel 1). The phosphorylation of \( \beta_1 \)-GST within the same tryptic phosphopeptides, on serine residues confirms that it is likely that PKC is binding to, and phosphorylating the \( \beta_1 \)-GST fusion protein.

4.2.5. \( \beta_1 \)-GST binds significantly more kinase activity, independent of substrate preference

To control for possible differences in PKC substrate preferences between the various intracellular domains, material binding to the \( \alpha_1 \)-GST, \( \beta_1 \)-GST, \( \gamma_2 \)-GST and GST from brain extract were all exposed to a PKC substrate peptide derived from neurogranin (NG) (Chen et al., 1993). A pilot experiment was initially conducted to look at the phosphorylation kinetics of NG by \( \beta_1 \)-GST bound kinases over 15 minutes. Figure 17 shows that the reaction was linear over this period, so it was decided to conduct all future experiments for a five minute period under the same conditions. The neurogranin peptide was phosphorylated by kinase activity binding to all three intracellular domains (Figure 17B). However, the highest level of neurogranin phosphorylation was seen with \( \beta_1 \)-GST. In addition, only kinase activity associated with \( \beta_1 \)-GST could be inhibited by PKC inhibitor peptide (Figure 17B). This approximate fifty-percent inhibition causes the level of kinase activity associated with \( \beta_1 \)-GST to fall to a level comparable to the other subunits. There is kinase activity common to/associating with all the subunits, which does not seem to be PKC, and does not phosphorylate the fusion proteins. There have been previous reports of kinase(s) associating with purified \text{GABA}_\alpha receptors, which seem to preferably phosphorylate the \( \alpha \) subunits (Sweetnam et al., 1988; Bureau and Laschet., 1995). The identity of the kinase responsible for the phosphorylation of neurogranin, which is not inhibited by PKC inhibitor, is currently unknown. It may be related to the earlier identified activities, though the \( \alpha_1 \) subunit is not significantly phosphorylated in the assay used here.
Figure 17 Phosphorylation of neurogranin peptide by PKC activity specifically associating with β1-GST

A) 1 µg β1-GST was exposed to brain extract and bound material was subjected to an in vitro kinase assay with a substrate peptide derived from neurogranin (50 µM). Incorporation of $^{32}$P into this peptide was measured and normalised to the protein input over the time course shown. 5' time point (phosphorylation linear) was then used in B) where material binding to α1-GST(1), β1-GST(2), γ2-GST(3) or GST alone(4) was subjected to similar in vitro kinase assay in the presence (black bars) or absence (white bars) of PKC inhibitor peptide (PKCl19-36, 1 mM). * Significantly different from control (p>0.05) as measured using the Students-t-test.
4.2.6. The PKC β1 isoform specifically interacts with the GABA_α receptor β1-GST subunit intracellular domain.

To further characterise the interaction of PKC with GABA_α receptors, fusion proteins were exposed to brain extracts and bound material was then subjected to western blotting using a pan PKC antisera. This antisera recognises the α, β1, β2 and γ isoforms of PKC. Whole brain lysate was blotted as a positive control, with bands seen around 82-83kD running at the expected molecular weight for PKC (Figure 18A, Lane 5). The smear of bands is to be expected as the antisera recognises a number of isoforms. In the experimental lanes a band of 82 KDa was seen binding to GABA_α β1-GST (Lane 2), but not to α1-GST, γ2S-GST or GST alone. This is in agreement with the results from the previous experiments, where the β1-GST fusion protein only, was significantly phosphorylated by a kinase which could be inhibited by a PKC specific inhibitor. Material binding to the β1-GST(S409A) fusion protein was also subjected to blotting using the pan PKC antisera (Figure 18A, Lane 6). As shown PKC was still capable of binding to β1-GST(S409A).

To further identify the isoform of PKC interacting with β1-GST, bound material was probed with isoform specific antibodies. Using an antibody directed against PKC-β1, a band of identical molecular mass was seen as with the pan-PKC antisera (Figure 18B). In addition small amounts of the α isoform of PKC were also detected binding to β1-GST (Panel 2). In contrast, the γ, δ, ε, ζ, η and θ PKC isoforms did not appear to interact with β1-GST as determined by western blotting, using isoform specific antibodies (Figure 18B).

4.2.7. RACK-1 associates directly with the β1-GST intracellular domain

Previous studies have shown that the PKC-β1 isoform is targeted to substrates by anchoring proteins, such as RACK-1, a homologue of G-protein β subunits (Ron et al., 1994; Pawson and Scott, 1997; Mochly-Rosen and Gordon, 1998). This molecule binds a number of PKC
Proteins from brain extract binding to fusion proteins (as indicated) were separated by SDS-PAGE and blotted with A) pan-PKC antibody or B) PKC isoform specific antibodies (as indicated to left and right of panel).
isoforms, but has a significant preference for $\beta_1$, and is thought to bind PKC when in an active form, and to potentiate this activity (Ron et al., 1994). To examine whether RACK-1 has a role in targeting PKC activity to GABA$_A$ receptors, material binding to receptor intracellular domains from brain extracts was blotted using antisera specific for RACK-1. Using this antibody a major band of 36 kD and a degradation product of 34 kD were seen in brain extract (Figure 19A, Lane 5). The 36 kD band representing RACK-1 could be observed binding to $\beta_1$-GST (Lane 2) but not to either $\alpha_1$-GST, $\gamma_2$S-GST or GST alone (Lanes 1, 3, 4).

To determine if the interaction between RACK-1 and receptor intracellular domains was direct, gel overlay assays were utilised (Modified from Li, et al. 1992). This method has been used in a number of other studies to show that an interaction between two proteins is direct (Hanley et al., 1999). Receptor GST-fusion proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with [$^{32}$P]-labelled RACK-1 expressed as a GST fusion protein. This latter construct is made in the vector pGEX-2TK, which has an engineered consensus site for PKA just upstream of the subcloned sequence, RACK-1 in this case. This allows for tagging of the protein by phosphorylation at this site in vitro by the PKA catalytic subunit. Figure 20B-Lane 1, shows purified RACK-1 fusion protein which has been labelled with [$^{32}$P], separated by SDS-PAGE, and visualised by autoradiography, while Lane 2 is a coomassie blue stain of the same protein. After exposure of this radiolabelled protein to the GABA$_A$ receptor fusion proteins, RACK-1 could be detected binding to both $\beta_1$-, and $\alpha_1$-GST, but not to $\gamma_2$-GST or GST alone (Figure 20A). This suggests that RACK-1 is capable of binding directly to the intracellular domains of the $\alpha_1$ and $\beta_1$ subunits in vitro. Reason for the interaction with $\alpha_1$ in this assay but not in the 'pull-down' assays from brain lysate is that it may be weaker than the interaction with $\beta_1$-GST. This may have important consequences, as differential binding to two GABA$_A$ receptor subunits may allow for a mechanism of regulation for PKC targeting to the channel complex.
Figure 19 RACK-1 binds specifically to the \( \text{GABA}_A \) \( \beta_1 \) subunit

A) \( \alpha_1 \)-GST (Lane 1), \( \beta_1 \)-GST (Lane 2), \( \gamma_2 \)-GST (Lane 3) or GST alone (Lane 4) were exposed to solubilised neuronal extracts. After extensive washing bound material was separated by SDS-PAGE, transferred to nitrocellulose and western blotted with a RACK-1 antibody. Lane 5 represents 5% of the starting material.

B) To test the RACK-1 specific antibody, RACK-1 cDNA in the pGW1-vector was overexpressed in HEK293 cells. 10\( \mu \)g of transfected (Lane 1), and 10 \( \mu \)g of untransfected whole cell lysate (Lane 2) was separated by SDS-PAGE and western blotted with the RACK-1 antibody. 50 \( \mu \)g of whole brain lysate was loaded in Lane 3.
Figure 20 RACK-1 can bind directly to $\text{GABA}_A$ receptor subunit intracellular domains.

A) $\alpha_1$-GST (lane 1), $\beta_1$-GST (lane 2), $\gamma_2$-GST (lane 3), or GST (lane 4) were transferred to a membrane and overlaid with RACK-1-GST that have been labelled with $^{32}$P to high specific activity with the catalytic subunit of PKA. Bound RACK-1 was detected by autoradiography. Lanes 5-8 represents a coomassie stain of an identical gel to show the equivalence in loading of the various proteins.

B) Lane 1 shows 5µg of the phosphorylated RACK-1 fusion protein, while Lane 2 is a coomassie stained gel of an equivalent amount of RACK-1 fusion protein.
4.2.8. PKC-β1 is capable of binding directly to GABA_α receptor intracellular domains

PKC and RACK-1 both interact with the GABA_α receptor in \textit{in vitro} 'Pull-down' assays from brain extract. By gel-overlay assay the RACK-1 interaction seems to be direct. It is known that RACK-1 binds activated PKC, and targets it to substrate (Ron et al., 1994; Mochly-Rosen and Gordon, 1998). To test whether the PKC-β1/ GABA_α β1 interaction was direct, or whether it was indirect and mediated possibly via RACK, gel overlay assays were again utilised. A range of receptor intracellular domains were transferred to a membrane and then exposed to PKC purified from rat brain, that had been activated \textit{in vitro}. Material binding to the GABA_α receptor intracellular domains was then visualised with antisera directed against PKC-β1. As a positive control, a RACK-1 GST fusion protein was included in the assay. PKC-β1 could be seen binding directly to β1-GST (Figure 21A, Lane 2) and also to RACK-1 (Lane 3), but not to α1-GST (Lane 1) or GST alone (Lane 4). Importantly, western blotting of the PKC preparation used for these experiments failed to detect RACK-1 (Figure 21C, Lower Panel), so the interaction observed is not mediated by contaminating RACK-1. But it cannot be ruled out that another adaptor molecule could be mediating the interaction. Together, these observations strongly suggest that PKC-β1 is capable of binding directly to the intracellular domain of the GABA_α receptor β1 subunit, independent of the molecule RACK-1, which is also seen to interact directly.

4.2.9. The RACK-1/PKC complex also associates \textit{in vitro} with the GABA_α receptor β3-subunit

To determine if the association of PKC and RACK-1 is common to other β subunit isoforms, the intracellular domain of the β3 subunit was exposed to solubilised neuronal extracts. In a kinase assay, β3-GST was phosphorylated (Figure 22A, Lane 1), and this phosphorylation could be specifically inhibited by PKC\textsubscript{I(19,36)} (Figure 22A, Lane 2). Previous
**Figure 21** The $\beta_1$ isoform of PKC can interact and bind directly to the intracellular domain of the GABA$_A$ $\beta_1$ subunit.

A) $\alpha_1$-GST (lane 1), $\beta_1$-GST (lane 2), RACK-1-GST (lane 3) or GST alone (lane 4) were transferred to a membrane and probed with PKC purified from rat brain. PKC binding to fusion proteins was then visualised with an antisera against PKC $\beta_1$ by western blotting. B) Lanes 5-8 represents a coomassie stain of an identical gel to show the equivalence in loading of the various proteins. C) Purified protein kinase C preparation was immunoblotted for PKC $\beta_1$ (top section of gel) and RACK-1 (bottom section of gel) to ensure preparation was lacking RACK-1. Lane (1)-1$\mu$g of protein kinase C, Lane (2)-0.1 $\mu$g of protein kinase C, Lane (3)-10 $\mu$g of adult rat brain.
studies have determined that the major in vitro substrate for PKC within the intracellular domain of the β3 subunit are the residues S408 and S409 (McDonald and Moss., 1997). A mutant form of the β3 subunit intracellular domain in which S408 and S409 had been mutated to alanine residues β3(S408/409A)-GST was not phosphorylated in assays with neuronal extracts (Figure 22A, Lane 3). Together these observations suggest that PKC can interact with, and phosphorylate the β3 subunit intracellular domain. To confirm the binding of PKC to the β3 subunit intracellular domain, material binding to β3-GST and β3(S408/409A)-GST was blotted with a pan-PKC antisera. In common with β1-GST, PKC isoforms could be detected binding to both of these proteins (Fig 22B, Lanes 1 & 2). Similarly, western blots were conducted with an antibody to RACK-1. As for β1-GST, the 36kD protein recognised by this antibody in brain lysate, could be seen to bind to the β3-GST subunit (Figure 22C, Lane 1).

Together these results suggests that PKC isoforms are capable of interacting and phosphorylating the intracellular domains of GABA_A receptor β1 and β3 subunits.
Figure 22 Protein Kinase C and RACK-1 bind to the intracellular domain of the \( \beta_3 \) subunit with phosphorylation of S408/409.
A) β3-GST was exposed to neuronal extracts and material binding was subject to an in vitro kinase assay alone (lane 1) or in the presence a specific inhibitor of PKC (PKCI19-36) 0.1 mM; lane 2). Phosphorylation was then assessed by SDS-PAGE followed by autoradiography. Lane 3, β3-GST(S408/409A) was substituted for β3-GST.

B) Material binding to β3-GST(Lane 1), β3-GST S408/409A(lane 2), or GST alone (lane 3) was probed by western blotting with a pan PKC antibody. Lane 4 represents 5% of the starting material.

(C) Material binding to β3-GST(Lane 1), or GST alone (lane 2) was probed by western blotting with RACK-1 antibody. Lane 3 represents 5% of the starting material.
4.3. Conclusions

Phosphorylation is now well characterised as an important mechanism for regulating GABA_A receptors. Studies have identified sites which are phosphorylated by PKA, PKC, CamKII, PKG, and src, _in vitro_, in a number of different subunits (Moss and Smart, 1996). Furthermore, some of these sites have been shown to be phosphorylated _in vivo_, with a direct effect on receptor function. The β1 and β3 subunits are seen to be phosphorylated by PKA with differential functional effects (Moss et al., 1992b; McDonald et al., 1998), β1 is also phosphorylated by PKC causing a reduction in channel function (Krishek et al., 1994), and γ2 is seen to be phosphorylated by src, causing an enhancement in the GABA current (Moss et al., 1995). This study has also presented evidence for tyrosine phosphorylation of the γ2 subunit _in vivo_, which in cultured cortical neurons seems to be under tight regulation. It is not understood though, how protein tyrosine kinases, or any other kinase, are targeted to the GABA_A receptor complex, to allow rapid responses to stimuli. Studies with other receptors have started to address this question, and two main lines of evidence have started to emerge.

The direct binding of kinases to ion-channel subunits has been shown in a number of cases, for example the binding of the protein tyrosine kinases src and fyn to the acetylcholine receptor (Fuhrer and Hall, 1996), the tyrosine kinase lyn to the AMPA-type glutamate channel, and the simultaneous binding of the tyrosine kinase src and the catalytic subunit of PKA to a Ca^{2+}-dependent potassium channel (Wang et al., 1999). Secondly, kinases (and phosphatases) have been shown to be located near to, or at their substrate, an ion-channel in this case, by an intermediate anchoring molecule (Faux and Scott, 1996a; Faux and Scott, 1996b; Pawson et al., 1997). The A-Kinase Anchoring Proteins (AKAPs) are the best characterised of these molecules (Colledge and Scott, 1999). AKAP-79, enriched in the post-synaptic density targets the molecules PKA, PKC and the phosphatase calcineurin to the AMPA/Kainate type glutamate receptors (Rosenmund et al., 1994; Klauck, 1996), while the AKAP 'yotiao' targets PKA and the
phosphatase PP1 to the NMDA receptor (Westphal et al., 1999b). A number of different molecules have been identified to restrict the subcellular location of PKC. ‘Proteins that interact with C-kinase’, PICKs (Staudinger et al., 1995), and ‘Receptors for Activated C-Kinase’, RACKs, are just two examples. The latter are thought to target specific isoforms of PKC to their substrates (Ron et al., 1994; Mochly-Rosen and Gordon, 1998).

In this study the intracellular domains of the GABA\(_A\) receptor were used to identify signalling molecules from the brain, specifically kinases, with which they interact. This was performed with an expectation of looking for tyrosine kinases, due to the apparent tight regulation seen of the phosphorylation of the \(\gamma_2\) subunit, and the preeminence of tyrosine kinase and ion channel interactions already reported. Using a method adapted from Fuhrer and Hall (1996), the intracellular domains expressed as GST fusion proteins were exposed to adult rat brain lysate, and used as ‘baits’ to capture interacting molecules. A serine-kinase was seen to specifically bind to the intracellular domains of GABA\(_A\) \(\beta_1\) and \(\beta_3\) subunits and to phosphorylate the proteins (Figures 12 & 22). There was no evidence of significant tyrosine kinase activity precipitating with any of the subunits tested. It may be that it is at too low a level to be picked up by the assays used here. The phosphorylation of \(\beta_1\) and \(\beta_3\) subunits could be inhibited by a specific PKC inhibitor peptide (Figure 13 and 22A). Furthermore the mutant \(\beta\) fusion proteins \(\beta_1^{S409A}\) and \(\beta_3^{S409/410A}\) were unable to be phosphorylated in an identical assay, indicating that the interacting kinase is phosphorylating these residues (Figure 14 & 22A). Serine 409 is conserved between all GABA\(_A\) \(\beta\) subunits, and is known to be a critical residue in the modulation of \(\beta\) containing receptors by phosphorylation (Moss, et al. 1992b; Krishek et al., 1994; McDonald et al., 1998). Blotting the bound material with specific antisera, the \(\beta_1\) isoform of PKC was principally identified as binding to the receptor (Figure 18). Blotting also showed that the mutant fusion proteins still bound PKC immunoreactivity (Figure 14 & 22B). It may have been possible that the lack of \([\text{\textsuperscript{32}P}]\) incorporation into the
mutants was due to the point mutation disrupting kinase association. This emphasizes the fact that PKC is binding to the β subunits, phosphorylating the residue serine 409.

It is thought that the PKC anchoring molecule RACK-1 targets specifically the β1 isoform to its target substrates (Ron et al., 1994; Mochly-Rosen and Gordon, 1998). RACK-1 only binds PKC in an active form, and is thought to potentiate its activity (Ron et al., 1994). This makes RACK-1 a good candidate for mediating the interaction seen in this study between PKC and the GABAₐ receptor complex. Other PKC binding molecules e.g. AKAP-79 (Klauck et al., 1996) bind the PKC β1 isoform but inhibit its activity. This makes it an unlikely candidate for mediating the interaction observed in these studies. In affinity-purification 'pull-down' and gel overlay assays, RACK-1 is indeed seen to bind directly and specifically to the β1 subunit (Figure 19 & 20). Interestingly in the overlay assay it also bound to the GABAₐ α1 subunit. Reasons for this discrepancy may be a differential affinity of RACK-1 for the different subunits. In the overlay assay there is a vast excess of purified RACK-1 available to bind to the fusion proteins, so the α1 interaction is observed. While in the pull down assay, where the concentration of RACK-1 is much less, only the higher affinity β1 interaction would be seen. This could be analysed by the ‘Biacore’ system, where the affinity of interactions between molecules can be accurately measured. Such a variable affinity model may for example allow the α1 subunit to bind excess RACK-1, and provide a readily available pool of enzyme-bound scaffold protein. Also it is known that the GABAₐ γ2 subunit is phosphorylated by PKC (Moss et al., 1992a), so the α1 subunit may provide a source of PKC to phosphorylate this subunit.

Similar overlay experiments with purified PKC showed that the PKC-receptor interaction can occur independently of RACK-1. This was confirmed by analysing the PKC preparation for contaminating RACK-1. Though no RACK-1 was seen by western blotting, it does not rule out
that another adaptor protein could be mediating the interaction. Similar results were observed for the GABA$_A$ $\beta$3 subunit (Figure 22), suggesting that a signalling complex of PKC and RACK-1 binds to the intracellular domains of the GABA$_A$ receptor $\beta$ subunits. Experiments to further characterise these interactions will be described in the next chapter.

4.4. The Ca$^{2+}$-phospholipid dependent kinase, PKC, in the nervous system

PKC is very abundant in the nervous system, where it plays a role in a wide range of neuronal functions on both sides of the synapse. For example at the presynaptic terminal it is involved in neurotransmitter release and vesicle recycling, while post-synaptically it plays a critical role in the regulation of channel and receptor function (Huganir et al., 1990; Nicholls, 1997; Tananka et al., 1994). There are multiple isozymes of this kinase family found in the cell, each performing specific functions. The location of the different isoforms in the brain varies with brain region and cell type (Tananka et al., 1994).

Broadly this family can be divided into 3 subfamilies based on sequence homology and sensitivity to various activators (Dekker, 1997; Tananka, 1994).

Classical PKC (cPKC) isozymes: $\alpha$, $\beta$1, $\beta$2, and $\gamma$ contain 4 homologous domains (C1-C4), separated by isozyme-unique/variable (V) domains. For activation they require calcium (Ca$^{2+}$), phosphatidylserine (PS), and diacylglycerol (DAG) or phorbol esters (Huang et al., 1986; Kikkawa et al., 1987).

Novel PKC (nPKC) isozymes: $\delta$, $\epsilon$, $\eta$, $\theta$ don't have typical C2 homologous domain, and don't require Ca$^{2+}$ for activation (Ono et al., 1989; Ono et al., 1988; Konno et al., 1989; Bacher et al., 1991). Differ from cPKCs in overall organisation, but not in basic modular composition (Dekker, 1997).
Atypical PKC (aPKC) isoymes: λ/τ and ζ lack the classical C2 and half of the C1 homologous domains. Are dependent on PS as they are insensitive to DAG, phorbol esters and Ca\(^{2+}\) (Ono et al., 1989; Nakanishi et al., 1992).

μ PKC doesn’t fall into any of the above subfamilies. It contains 2 unique hydrophobic domains in its amino-terminus, a putative pleckstrin-homology (PH) domain and a distinct catalytic domain.

The β1 isoform observed as binding to the GABA\(_A\) β subunits is a member of the classical PKCs. This isoform is expressed widely in tissues, but is seen to be concentrated in amino-acid neurons for example in cortical and hippocampal pyramidal cells, and striatal neurons (Tanaka et al., 1992; Tananka et al., 1994). In neurons it is found localised to the trans face of the golgi apparatus and the proximal dendrites (Tanaka et al., 1991). The latter is intriguing, as this is where the GABA\(_A\) receptor is located.

4.5. Regulation of PKC by restricted distribution

Among PKC isoymes there is very limited differences in substrate specificity and sensitivity to activators. So to explain how different isoymes mediate distinct functions, restrictions on their subcellular distribution has been implicated (Mochly-Rosen et al., 1995; Jaken et al., 1997). Upon agonist stimulation PKC undergoes both activation, and translocation from the cell cytosol to the particulate fraction, as shown by simple centrifugation studies. If the subcellular distribution of PKC isoymes are monitored, before and after agonist application, an increase in PKC activity/immunoreactivity is seen in pelleted membrane fractions, with a comparable loss in the supernatant (Mochly-Rosen and Gordon, 1998). After activation the enzyme is seen to be associated with the plasma membrane, cytoskeleton and other subcellular components (Kiley, 1995). Microscopy studies show the various isoymes to have specific
locations at rest, and then to translocate to new subcellular sites upon activation (Goodnight et al., 1995). This implies that PKC isoforms are restricted to certain sites before stimulation and then translocate to specific regions where they are again maintained (Mochly-Rosen and Gordon, 1998; Mochly-Rosen, 1998). A very early study showed that pretreatment of membranes with proteases abolishes the phorbol ester induced binding of PKC to these membranes, suggesting that they may be held there by membrane associated PKC-binding proteins. A number of different types have now been categorised, including: Substrate binding proteins (SBPs), receptors for activated C-kinase (RACKS), receptors for inactive C-kinase (RICKS) and proteins that interact with C-kinase (PICKS) (Mochly-Rosen, 1998).

a) RACKS-proteins known as RACKS have been shown to be important for PKC mediated function. Criteria to be met to be classified as a RACK include: saturable and isozyme selective binding of PKC, binding should be independent of an interaction between the catalytic domain of PKC and a phosphorylation site on the proposed anchoring molecule, and the interaction should be a direct protein-protein interaction. RACK-1, a homologue of the heterotrimeric G-protein β-subunit and RACK-2, a β’-COP homologue have been well characterised. RACK-1 was cloned by screening a rat brain expression library for molecules that bind activated PKC in a PKC overlay assay (Ron et al., 1994). RACK-2 was identified by expression cloning, using a fragment of ε-PKC containing the RACK binding site (V1 region) (Csukai et al., 1997).

RACK-1: Structure and function
The RACK-1 clone identified was shown to have high homology to chicken and human genes which had been previously identified as homologs of the G-protein β subunits (Ron et al., 1994). The superfamily of proteins to which RACK-1 belongs is characterised by the presence of multiple repeats of what has been termed the WD40
motif, with RACK-1 seen to contain 7 such motifs. The presence of multiple putative protein-protein interaction WD motifs may allow RACK-1 to function as a scaffold protein as will be discussed below. This 36kD molecule is seen to fulfill all the criteria to be classified as a RACK: it binds PKC in a specific and saturable manner in the presence of PKC activators, it is not a PKC substrate, the binding occurs on a site on PKC distinct from the catalytic site of the enzyme, it increases phosphorylation of PKC substrates e.g. histone H1 when present in a complex with the enzyme, and from studies in cardiac myocytes it seems to have a preference for binding of the β1 isozyme i.e. shows isozyme specificity (Ron et al., 1994a; Ron et al., 1994b). The developmental expression of RACK-1 mirrors that of the majority of PKC isoforms in rat cerebral cortex (Battaini et al., 1997). Principally it is expressed at a low level at birth, and increases up to three weeks post-natally. Also very intriguingly in aged rats there is a very striking deficiency in the ability of PKC to translocate to substrate. This is thought to be partly due to the concomitant fifty-percent decrease in the level of RACK-1 protein seen in aged rats (Battaini et al., 1997).

In this study, the β1 isoform is seen to bind the GABAα receptor β subunits. When bound the kinase is in a state of high activity, as demonstrated by the robustness of the phosphorylation in Figure 12. These observations were the main reasons for considering the RACK-1 molecule as a possible adaptor molecule in this interaction. ‘Pull-down’ assays showed that PKC β1 and RACK-1, from neuronal extracts, are both able to bind to the receptor. Moreover gel overlay assays indicated that both these interactions are direct, and can occur apparently independently of each other. This may indicate a novel aspect to the PKC β1:RACK-1 interaction, which will be discussed in the next chapter, as this potential tripartite complex is studied in more detail.

**RACK-1 does not only bind PKC** : various techniques to identify protein-protein interactions have identified a range of molecules which
bind RACK-1. This could be of great importance as it may bring in a wide range of different molecules to the GABA$_A$ receptor.

**PLC-γ1:** phospholipase γ1 hydrolyses phosphatidylinositol 4,5-bisphosphate to generate DAG and inositol 1,4,5-trisphosphate. Epidermal growth factor (EGF) stimulation causes phosphorylation of PLC-γ1 and a 'PKC-like' translocation of the enzyme to the particulate fraction in A431 cells. The PKC-RACK-1 interaction is partially mediated through the C2 domain of PKC. C2-domains are found in a range of other molecules including PLC-γ1. PLC-γ1 has been shown in vitro by overlay technique to interact with RACKs (Disatnik et al., 1994), and unlike for PKC binding the interaction is independent of calcium and phospholipids, but shown to be enhanced by tyrosine phosphorylation after stimulation of the EGF-receptor. PLC-γ1 is also seen to inhibit the PKC-RACK-1 interaction, but PKC does not block the opposite PLC-γ1-RACK interaction i.e. the affinity of PLC-γ1 for RACKs is greater than for the corresponding RACK-1-PKC interaction or the PLC-γ1 binding may hinder the binding of PKC (Disatnik et al., 1994). What is of interest though is the possibility of PLC-γ1 and PKC binding sequentially to the same molecule, with the former providing DAG for activation of the latter, and consequent binding to RACK-1.

**-integrin β subunits:** integrins are αβ heterodimeric adhesion receptors which are important in attachment of the cytoskeleton to the ECM. Integrin binding to the ECM causes clustering of integrins which triggers a number of cell signalling cascades including the activation of PKC. Integrin subunits consist of an extracellular domain, a single transmembrane domain and a short cytoplasmic domain. Using the two-hybrid system with the cytoplasmic domain as a bait the integrin β1, β2, and β5 subunits have been shown to bind RACK-1 (Liliental et al., 1998). WD repeats 5-7 on RACK-1 are seen to be essential for this direct interaction, while in vivo the interaction is dependent on phorbol ester treatment. These results imply that RACK-1 may link activated
PKC to integrins, and their potential downstream effectors (Liliental et al., 1998).

-src-family tyrosine kinases: it has been shown that the src-family of non-receptor tyrosine kinases including src, lyn and fyn interact with RACK-1 (Chang et al., 1998). This interaction was again identified by using the two-hybrid system to screen a human lung fibroblast cDNA library with a bait consisting of a domain of src. The SH2 domain of src is seen to be essential for the interaction, while the binding site on RACK-1 is as yet unknown. When complexed together RACK-1 inhibits the kinase activity of the src family members, contrasting with its role as a receptor for activated PKC. So in this respect it acts like AKAP-79, which binds PKA, PKC and PP2B and suppresses their activity (Klauck et al., 1996).

This interaction could be of great interest as a number of studies, including this one, have shown the tyrosine phosphorylation of GABA<sub>A</sub> receptor subunits (Valenzuela et al, 1995; Moss et al., 1995; Wan et al., 1997), but there have been no reports of tyrosine kinases binding to the GABA<sub>A</sub> receptor. While the latter has been seen with a range of other ion-channels e.g. potassium channels (Holmes et al., 1996), AMPA and NMDA receptors (Yu et al., 1997) and the acetylcholine receptor (Swope and Huganir, 1993). RACK-1 may bring the kinase into the receptor complex. Similar procedures as used here could address this.

-cAMP-specific phosphodiesterase isoform PDE4D5: this recently cloned isoform was shown by the two-hybrid system to interact with RACK-1 (Yarwood, 1999). The enzyme hydrolyses cAMP, so could potentially regulate PKA activity in the vicinity of a scaffold complex based around RACK-1. This would be very interesting in the context of the GABA<sub>A</sub> receptor, which is known to be phosphorylated by PKA, with differential functional effects (Moss et al., 1992b; McDonald et al., 1998).
The possibility that RACK-1 could be the focus of a large signalling complex around the GABA_A receptor is very reminiscent of some AKAPS. AKAP-79 is known to bind PKC, PKA and calcineurin (Klauck et al., 1996), and to play a role in the regulation of glutamate and Ca^{2+} channels (Gao et al., 1997; Rosenmund, 1994). It is possible that RACK-1 may be crucial for GABA_A receptor modulation.

b)SBPs-Substrate binding proteins were identified by Jaken and colleagues using the PKC overlay protocol, as also used in this study (Chapline et al., 1993). These proteins are all PKC substrates and bind PS directly, with PS mediating the interaction possibly. Examples include talin, MARCKS (Hyatt et al., 1994), AKAP-79 (Klauck et al., 1996) and AKAP-250/gravin (Nauert et al., 1996). As PS alone is sufficient for these interactions it is thought that full activation of PKC is not required. It is possible that the interaction observed between PKC β1 and the β1 subunit of the GABA_A receptor may be a simple kinase-substrate interaction.

c)PICKs-proteins that interact with C-kinase (PICKs) were identified by using the 2-hybrid system with the catalytic core of the α isoform as the bait. The molecule PICK-1 was originally cloned by this strategy (Staudinger et al., 1995), and has more recently been shown to play a potential role in the clustering of excitatory glutamate receptors (Xia et al., 1999).

RICKS- are proposed to bind PKC in a saturable and isozyme-specific manner, as isozymes are seen to be differentially localized to distinct subcellular locations when inactive (Jaken et al., 1997; Mochly-Rosen and Gordon, 1998). They don’t necessarily have to be PKC substrates but should show a preference for binding inactive PKC. Upon activation these proteins should release the bound PKC. This is somewhat analogous to the A-kinase anchoring proteins, the AKAPS. The
Drosophila eye protein Inad has been seen to bind inactive PKC (Tsunoda et al., 1997). It binds the enzyme close to its site of action in the rhabdomere but it is not yet known if it fulfills all criteria to be known as a RICK.

A potential trimolecular complex of an ion-channel, the protein kinase PKC and a molecule which was initially identified by its ability to bind activated PKC has been identified in vitro. Such a complex could be very important in the regulation of GABA\textsubscript{A} receptor regulation. It may provide a means of specifically targeting the kinase to not only the receptor complex but to specific subunits, and potentially to specific residues in the intracellular domain. Furthermore this would also allow for rapid and efficient phosphorylation in response to physiological stimuli. To show the absolute importance of these interactions though, they need to be investigated further, especially with direct reference to their physiological relevance.
CHAPTER 5
Further investigations of the interactions between the GABA_A receptor, PKC and RACK-1

5.1. Introduction

PKC is abundantly expressed in the nervous system, and has been implicated in a broad range of neuronal functions (Tananka, 1994). Activation of this enzyme has for a long time known to be associated with the modulation of ion-channel function (Huganir et al., 1990; Moss and Smart, 1996). The effects of PKC on the function of GABA_A receptors remains controversial, with both up-, and down-regulation of channel function reported (Kellenberger et al., 1992; Krishek et al., 1994; Lin et al., 1996; Moss and Smart, 1996; Smart et al., 1997). In heterologous systems, phorbol ester induced PKC activity is seen to inhibit receptors composed of α1β1, α1β1γ2S/L, α1β2 and α1β2γ2S/L by phosphorylation of S409 on β1, S410 on β2, S327 in both γ2S and γ2L and S343 in γ2L reported (Kellenberger et al., 1992; Krishek et al., 1994). In other studies, with receptors composed of bovine α1, β1 and γ2L subunits, phosphorylation catalysed by constitutively active trypsin cleaved PKC (PKM), resulted in an enhancement of channel function, which could be inhibited by mutation of either S409 on β1, S327 on γ2S/L or S343 on γ2L (Lin et al., 1996). Potential explanations for such differences are the nature of PKC activation and the origins of the cDNAs used.

The PKC family of kinases have very similar substrate specificities, but in vivo are seen to phosphorylate specific molecules. Such specificity is possible through restrictions on the distribution of isozymes in vivo. The main mechanism by which this is achieved is by interacting with specific PKC binding/anchoring proteins (Mochly-Rosen, 1995; Faux and Scott, 1996a; Faux and Scott, 1996b; Pawson and Scott., 1997; Jaken, 1997). The enzyme at rest is compartmentalised close to its substrate, so upon activation it can rapidly and preferentially

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phosphorylate this substrate. Recently a number of different PKC interacting molecules have been discovered. The PKC overlay method and the two-hybrid system for detecting interacting molecules have played the prominent role in such studies (Mochly-Rosen et al., 1991; Staudinger et al., 1995; Jaken, 1997; Mochly-Rosen, 1998). One of the best characterised molecules is RACK-1 (Ron et al., 1994). It belongs to a group of proteins known as 'Receptors for Activated C-Kinase' (Mochly-Rosen et al., 1991). It binds preferentially to the active form of the PKC β1 isoform, and is seen to enhance and maintain its activity, while targeting it to substrates (Ron et al., 1994; Ron et al., 1994; Faux and Scott, 1996a; Faux and Scott, 1996b; Pawson and Scott, 1997).

This study has provided evidence for a possible tripartite complex, focussed around the GABA\textsubscript{A} β subunits, of the PKC β1 isoform and the molecule RACK-1. In vitro assays show that both of these molecules bind to the β-subunits directly and, from the data presented here, do so independently of each other. To find out more about these interactions an important first step is to try and study the nature of PKC and RACK-1 binding to the receptor i.e. do the molecules bind concomitantly, or are the two interactions mutually exclusive. An approach which would give much useful information is the mapping of the binding sites of the two molecules on the GABA\textsubscript{A} β1 subunit. Truncations of the intracellular loop of this subunit can be utilised to sequentially narrow down smaller and smaller regions of binding on the loop, using in vitro 'Pull-down' and overlay assays. Such studies then open up the possibility of trying to disrupt such interactions with peptides corresponding to the binding site on the β1 subunit. If the interaction can be shown biochemically in vivo, for example in HEK293 cells, the effects of disrupting the interactions can be studied. The functional consequences of PKC activation on α1β1 recombinant receptors in HEK293 cells is well characterised (Krishek, 1994). If the disrupting peptides have an effect, it should be straightforward to identify the nature of it.
The interactions identified, have so far only be analysed *in vitro*, so it is of paramount importance that they are also shown *in vivo*. If they are physiologically relevant, it should be possible to immunoprecipitate the receptor, co-precipitating the kinase and RACK-1. Such an approach can use either overexpressed proteins in HEK293 cells, but more crucially endogenous proteins from a neuronal preparation. Cultured cortical neurons are ideal for this purpose. They can be grown at such a density as to provide sufficient material for biochemical analysis, and are easily treated with drugs to modulate kinase activity.

5.2.1. Identification of the binding site of PKC on the GABA₁ β₁ subunit

To map the binding site of PKC on the intracellular domain of the GABA₁ β₁ subunit, the full-length intracellular loop was systematically cut into smaller portions and made as GST fusion proteins, so as to narrow down the region of binding. At each stage, the fusion proteins were exposed to adult rat brain extracts (see 4.2.6.). The bound material was analysed by western blotting with a PKC β₁ specific antibody, to identify regions of the intracellular domain PKC binds to. Initially the loop was cut in half to give the constructs: β₁(302-365) and β₁(366-426). PKC was seen clearly to bind to the latter. The next step was to then cut this region into two creating: β₁(366-394) and β₁(395-426). As Figure 23 shows, PKC binds to the quarter 395-426. This is not too surprising as this region contains the conserved PKC phosphorylation site, serine 409. Two more constructs were then made which included the region 366-394, which does not bind PKC, plus a ten and twenty residue increment, giving the two constructs: β₁ (366-404) and β₁(366-415) respectively. PKC only binds to the latter, allowing the conclusion that it binds to a region in between residues 405-415. Serine-409 is in the middle of this proposed binding region, so it seems the kinase is
Figure 23 Identification of the binding site of PKC on the GABA\textsubscript{A} \textbeta{1} subunit

Various deletion constructs of the GABA\textsubscript{A} \textbeta{1} intracellular domain (numbers indicate residues of each construct) were exposed to solubilised neuronal extracts. After extensive washing bound material was separated by SDS-PAGE, transferred to nitrocellulose and western blotted with a specific PKC \textbeta{1} antibody. GST alone was included as a negative control. IN represents 5\% of the starting material.
at, or very close to its phospho-acceptor-site. The serine itself is not critical for the interaction though as PKC is still seen to bind to the mutant fusion protein β1S409A (see section 4.2.6.).

5.2.2. Identification of the binding site of RACK-1 on the GABA\textsubscript{A} β1 subunit

The same set of truncations used to identify the site of PKC binding on the GABA\textsubscript{A} β1 subunit were used in gel-overlay assays to map the site at which the molecule RACK-1 binds. The overlay assay has the advantage over using 'pull-down' assays for such experiments in that it involves the direct binding of proteins, and removes the possibility that the interaction is mediated by an intermediate protein. Figure 24 shows that the RACK-1 fusion protein binds to a region immediately upstream of the PKC β1 enzyme. Lanes 1-7 are a coomassie stained version of the gel in lanes 8-14, which was transferred to nitrocellulose and overlaid with \textsuperscript{32}P-labelled RACK-1. The latter can be clearly seen by autoradiography to bind to the truncations β1(395-426), (366-404) and (366-415) [Lanes 11-13]. The binding of RACK-1 is independent of S409 as shown by the positive interaction with the 366-404 truncation, which does not contain this site. PKC was able to bind to β1(395-426) and (366-415), but not the construct which terminates at residue 404 (Figure 24). This is summarised in Figure 25A.

This allows the model as seen in Figure 25B to be proposed. RACK-1 binds just upstream of PKC, with the latter binding to a region containing the PKC phosphorylation consensus site. These findings agree with the observation that the two molecules can bind independently of each other. If for example RACK-1 mediated the binding of PKC a single shared binding site would be expected. The arrangement seen here allows for the two molecules to bind independently, but also simultaneously. This opens up the possibility that RACK-1 may guide PKC to its target substrate, and maintain it there, in a state of high activity. The RACK-1 site is not as highly
Figure 24 Identification of the binding site of RACK-1 on the GABA\(_A\) \(\beta1\) subunit

A) Various deletions of the GABA\(_A\) \(\beta1\) subunit (numbers below gel indicate residues of each construct) were separated by SDS-PAGE, transferred to nitrocellulose and overlaid with \(^{32}\)P-labelled RACK-1-GST. Bound RACK-1 was detected by autoradiography (Lanes 8-14). Lanes 1-7 are a coomassie stain to show the equivalence in loading of the various proteins.
Figure 25 Schematic representation of PKC and RACK-1 binding sites on the GABA$_A$ $\beta_1$ subunit

A)

<table>
<thead>
<tr>
<th>PKC</th>
<th>RACK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>302</td>
<td>426</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>365</td>
<td></td>
</tr>
<tr>
<td>366</td>
<td>426</td>
</tr>
<tr>
<td>394</td>
<td></td>
</tr>
<tr>
<td>395</td>
<td>426</td>
</tr>
<tr>
<td>404</td>
<td></td>
</tr>
<tr>
<td>415</td>
<td></td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th></th>
<th>S409</th>
</tr>
</thead>
<tbody>
<tr>
<td>395</td>
<td>405</td>
</tr>
<tr>
<td>415</td>
<td></td>
</tr>
</tbody>
</table>

RGLD RHGVPGKGRIRRRA SQLKV KI PDLT

RACK-1 (395-404) PKC (405-415)
Figure 25C Comparison of the RACK-1 and PKC binding sites between the β–subunits

**RACK-1 site**

<table>
<thead>
<tr>
<th>β1</th>
<th>R H G V P G K G R I</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>• • V A Q K • S • L</td>
</tr>
<tr>
<td>β3</td>
<td>• S I P H K • T H L</td>
</tr>
<tr>
<td>α1</td>
<td>R G D P G P G L A T I</td>
</tr>
</tbody>
</table>

RACK-1 binds α1 *in vitro* in gel overlay assays. A sequence similar to the β1 binding site is found at the residue 353.

**PKC site**

<table>
<thead>
<tr>
<th>β1</th>
<th>R R R A S Q L K V K I</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>• • • • • • • • • I T•</td>
</tr>
<tr>
<td>β3</td>
<td>• • • S • • • • • I • •</td>
</tr>
</tbody>
</table>

137
conserved between the $\beta$-subunits as that of the PKC binding site (figure 25C). Further experiments will be required to identify the precise motif in the other $\beta$ subunits that bind RACK-1.

5.2.3. Identification of a tripartite complex of the $\text{GABA}_A$ receptor, PKC and RACK-1 in HEK293 cells

It is crucial for protein-protein interactions, as described in this study, that they are seen to occur in a cellular environment. The initial approach taken here was to overexpress the proteins of interest in HEK293 cells, and then to immunoprecipitate the receptor. The material co-precipitating with the receptor can then be western blotted for PKC and RACK-1.

HEK293 cells were transfected with cDNAs for the PKC $\beta_1$, RACK-1 and the $\text{GABA}_A$ receptor subunits $\alpha_1$ and $\beta_1$. It is known that these receptor subunits are able to oligomerise and form functional channels at the cell-surface (Connolly, 1996). Receptors were then solubilised and immunoprecipitated with a polyclonal antibody specific for the $\beta_1$ and $\beta_3$ subunits (McDonald et al., 1998). The coprecipitating material was then probed with antibodies specific for RACK-1 and PKC $\beta_1$. Figure 26 (B & C) clearly shows that PKC $\beta_1$ (B, Lane 1) and RACK-1 (C, Lane 1) coprecipitate with the $\text{GABA}_A$ receptor complex. Controls where the receptor subunits were not included in the transfection (Lane 2), or pre-immune antisera was used for immunoprecipitation (Lane 3), show the bands to be specific for the receptor. To control for the efficacy of the immunoprecipitation HEK293 cells were transfected as above and metabolically labelled with $^{35}$S-methionine. Receptors were immunoprecipitated as before, and visualised by autoradiography. Figure 26A shows that bands of 56 and 50kd are precipitated (Lane 1), and by comparison to earlier studies, the former is likely to be the $\beta_1$ subunit and the latter $\alpha_1$ (Krishek et al., 1994; McDonald et al., 1998).
Figure 26 Identification of an interaction between PKC, RACK-1, and the GABA\textsubscript{A} receptor in HEK293 cells

Two duplicate sets of HEK293 cells were transfected with cDNAs for GABA\textsubscript{A} receptor subunits $\alpha_1/\beta_1/\gamma_2s$, PKC $\beta_1$, and RACK-1 (Lane 1 and 3) or RACK-1 only (Lane 2). A) One set of cells was metabolically labelled with $[^{35}\text{S}]$-methionine. Cell lysates were immunoprecipitated with 10$\mu$g UCL17 (Lane 1 and 2) or 10$\mu$g pre-immune sera (Lane 3) bound to protein-A. Samples were separated by SDS-PAGE and visualised by autoradiography. Arrow indicates precipitated $\beta_1$-subunit. B) & C) The second set of cells was lysed and immunoprecipitated as A). Samples were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was cut into two sections and western blotted with a PKC $\beta_1$ antibody (B), and a RACK-1 antibody (C). Lane 4 represents 10% of the input. Arrows indicate co-precipitated proteins in Lane 1.
5.2.4. *In vitro* phosphorylation of the GABA$_A$ $\beta$1 subunit by co-precipitating PKC from HEK293 cells

HEK293 cells were transfected as in Section 5.2.3. Cells were treated as indicated in Figure 27, lysed and receptors immunoprecipitated. The precipitated material was then put in an *in vitro* kinase assay with $\gamma^{32}$P-ATP, and where indicated with the specific PKC inhibitor peptide PKCl$_{(19-36)}$. Lane 2 shows that with untreated cells the $\beta$1 subunit (as marked) and potentially other proteins are phosphorylated by a co-precipitating kinase. If the cells are pre-treated with phorbol ester the level of phosphorylation is increased (Lane 1), while staurosporine (broad-range kinase inhibitor) decreases the level (Lane 3). If the PKCl$_{(19-36)}$ is included in the kinase assay the phosphorylation is totally abolished (Lane 4). PKC, which in section 5.2.3. was seen to co-precipitate with receptor, is active and can phosphorylate the $\beta$1 subunit, presumably at the conserved PKC phosphorylation site, serine 409.

5.2.5. Disruption of functional regulation of the GABA$_A$ receptor by PKC phosphorylation by a polypeptide corresponding to the RACK-1 binding site

PKC activation leads to inhibition of GABA$_A$ receptor function in HEK293 cells expressing $\alpha$1 and $\beta$1 subunits (Krishek et al., 1994). To gain further insight into the importance of the interaction between the GABA$_A$ receptor and RACK-1, this functional assay was utilised to look at the effects of disrupting the binding of RACK-1 to the receptor. All electrophysiological recordings were performed by Professor Trevor Smart of the London School of Pharmacy. HEK293 cells transiently expressing $\alpha$1 and $\beta$1 subunits were subjected to whole-cell recording. The GABA activated response was observed as seen in the first two traces of each panel in Figures 28-30. Upon activation of PKC with phorbol esters, the GABA activated current is reduced as seen in the last two traces of Figure 28A. To disrupt the interaction between RACK-1
Figure 27 In vitro phosphorylation of immunoprecipitated GABA$_{A}$ $\beta$1 subunit by coprecipitating PKC in HEK293 cells

HEK293 cells were transfected with cDNAs for GABA$_{A}$ subunits $\alpha$1 and $\beta$1. Cells were lysed, and solubilised receptors were immunoprecipitated with the polyclonal $\beta$1/3 antisera. Precipitated material was put into a kinase assay with $\gamma^{32}$P-ATP for 30 minutes, and treated as indicated. Lane 1: Phorbol ester, Lane 2: untreated, Lane 3: Staurosporine, and Lane 4: PKC inhibitor peptide. Samples were separated by SDS-PAGE and visualised by autoradiography.
Figure 28 Disruption of the functional PKC regulation of the GABA$_{A}$R by the polypeptide corresponding to the residues 366-426 of the $\beta$1 subunit

A) 10mM GABA
   Control
   +0.5mM PMA

B) +$\beta$1 loop 366-426

Whole cell 10um GABA activated currents were recorded from HEK293 cells expressing $\alpha$1$\beta$1 subunits. The patch pipette solution was either control (A) or supplemented with 200 ug/ml peptide 366-426. Control GABA-activated currents (solid lines) were recorded to ascertain stability. Following the addition of 0.5um PMA, GABA-activated currents were monitored at 5’ and 15’ intervals (broken lines).
Figure 29 Polypeptide corresponding to the residues 302-365 of the β1 subunit has no effect on the functional regulation of the GABA$_A$R by PKC.

A) 10μM GABA +0.5μM PMA

Control

B) +β1 loop 302-365

+β1 loop 302-365

100pA

300pA

5s

Whole cell 10μm GABA activated currents were recorded from HEK293 cells expressing α1β1 subunits. The patch pipette solution was either control (A) or supplemented with 200 μg/ml peptide 302-366. Control GABA-activated currents (solid lines) were recorded to ascertain stability. Following the addition of 0.5μm PMA, GABA-activated currents were monitored at 5' and 15' intervals (broken lines).
Figure 30 Disruption of the functional PKC regulation of the GABA<sub>AR</sub> by the region corresponding to the RACK-1 binding site on the β<sub>1</sub> subunit (residues 395-404)

Whole cell 10μm GABA activated currents were recorded from HEK293 cells expressing α1β1 subunits. The patch pipette solution was either supplemented with (A) 200 μg/ml peptide 366-394 or (B) peptide 366-404. Control GABA-activated currents (solid lines) were recorded to ascertain stability. Following the addition of 0.5μm PMA, GABA-activated currents were monitored at 5’ and 15’ intervals (broken lines).
and the receptor, fusion proteins, as utilised to identify the PKC and RACK-1 binding sites, were included in the patch pipette. When the polypeptide $\beta_1$ (366-426) was applied, which includes both PKC and RACK-1 binding sites, PKC regulation is abolished, as shown in the last two traces of Figure 28B. It is quite clear that upon PMA treatment there is no inhibition of channel function (*). The fusion protein $\beta_1$ (302-365), which contains neither binding site, has no effect, as seen in the final two traces of Figure 29B. The effects of PKC activation in this case on the GABA current are as with control cells. The fusion protein $\beta_1$ (366-426) could be having multiple effects though, especially as it contains the PKC phosphorylation site (S409), and the PKC binding site. The fusion protein $\beta_1$ (366-404) which contains neither of these sites but does include the RACK-1 site, is seen to prevent inhibition of channel function by PMA (Figure 30 B, *). This result is critical, as it shows that the binding of RACK-1 has a crucial role in some aspect of the interaction between PKC and receptor. A similar fusion protein $\beta_1$(366-394), lacking the proposed 10 amino-acid RACK-1 binding site, has no effect (Figure 30A).

5.2.6. Characterisation of a neuronal system to study the interaction of RACK-1 and PKC with the GABA$_A$ receptor

Evidence of a tripartite complex of recombinant GABA$_A$ receptor, RACK-1 and PKC in HEK293 cells, which can be manipulated by peptides corresponding to the binding sites on the subunit, was shown in sections 5.2.4. and 5.2.5. The overexpression of proteins in these cells though creates a very artificial situation, as they come into contact in vast excess, in potentially foreign intracellular compartments, compared to the situation in neurons. For the interaction to be credible, it must be studied in a neuronal preparation. Cultured cortical neurons provide a very good system for such a study. To ensure that this cell-type is suitable for the purposes of this study, the GABA$_A$ receptors of
Figure 31 Characterisation of GABA<sub>A</sub> β1/3 containing receptors from cultured cortical neurons by immunofluorescence and immunoprecipitation

A) Cortical pyramidal neuron stained under permeabilising conditions with anti-β1/3 antibody (10μg/ml).

B) Lanes 1) & 2) Two plates of cortical neurons were metabolically labelled with 35S-methionine for 12-16 hours. Tx-100/DOC cell-lysates were immunoprecipitated with 10μg anti-β1/3 antibody (Lane 1) or 10μg pre-immune sera (Lane 2) bound to protein-A. Samples were separated by SDS-PAGE and visualised by autoradiography. Lanes 3) and 4) Two duplicate plates of cortical neurons were lysed, and immunoprecipitated with 10μg anti-β1/3 antibody (Lane 3) or 10μg pre-immune sera (Lane 4) bound to protein-A. Samples were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was western blotted with BD17 (recognises β2/3). Migration of β3 is indicated (Arrow).
interest must be expressed. Their receptor expression profile has been characterised and are known to express the subunits: $\alpha(1-5)$, $\beta1$, $\beta3$ and $\gamma2$ (Benke et al., 1994; Macdonald and Olsen, 1994). So the system was initially characterised by immunofluorescence and immunoprecipitation, using the polyclonal $\beta1/3$ antisera. Figure 31A shows a cortical pyramidal neuron stained with the $\beta1/3$ antibody. Clusters of GABA$_A$ receptors are clearly seen, as observed by staining for the $\gamma2$ subunit in section 3.2.2. and Figure 5, and in other studies (Allison et al. 1998; Essrich et al., 1998). Though the $\beta1/3$ antibody has already been shown to immunoprecipitate the recombinant $\beta1$-subunit from transfected HEK293 cells, it does not mean that it will be able to do the same from neurones. To test this, cortical neurones were metabolically labelled with $[^{35}\text{S}]$-methionine, and the receptors were immunoprecipitated with either the $\beta1/3$ or pre-immune sera. As Figure 31B shows, bands of 47, 55 and 57kD are immunoprecipitated with the $\beta1/3$ antibody from the metabolically labelled cortical neurons (Lane 1). Furthermore, similar unlabelled precipitates were western blotted with the BD17 monoclonal antibody, to try and identify the bands in lane 1. BD17 recognises both $\beta2$ and $\beta3$ subunits (Benke et al, 1994), and in Lane 3 identifies the 57kD band. Given that most GABA$_A$ receptors only contain a single $\beta$ subunit isoform (Benke et al., 1994; Li and De Blas, 1997), it is very likely that this band is $\beta3$.

5.2.7. Co-precipitation of PKC and RACK-1 with the GABA$_A$ $\beta1/3$ containing receptors from cortical neurons

The possibility of a tripartite complex of PKC, RACK-1 and GABA$_A$ $\beta$ subunits existing in neurones was tested by precipitating receptors from cortical neurons as in section 5.2.6., and western blotting for the other two molecules. In a previous study, of the interaction of RACK-1 with the $\beta$-integrins, it was necessary to treat cells with phorbol esters to show an interaction by co-precipitating RACK-1 (Liliental et al., 1998). Therefore cortical neurons were processed in parallel, with one set treated with phorbol esters for thirty minutes, and the other untreated.
Figure 32 Co-immunoprecipitation of PKC and RACK-1 with the GABA<sub>A</sub> β1/3 subunits from cultured cortical neurons

A)

97kD
66kD

1 2 3 4 5

PKC

B)

45kD
31kD

1 2 3

RACK-1

A) Cortical neurones were treated with PdBu for 30 minutes (Lanes 2 & 4) or sham treated (Lanes 1 & 3), and solubilised in CHAPS buffer. Receptors were immunoprecipitated using an antisera specific for the β1 and β3 subunits (Lanes 1 & 2) or pre-immune IgG (Lanes 3 & 4) coupled to protein A-sepharose. Precipitated material was then separated by SDS-PAGE, and western blotted with a pan-PKC antibody. Lane 5 represents 10% of input used for the immunoprecipitation.

B) Cortical neurones were treated with PdBu for 30 minutes, solubilised in a CHAPS buffer. Solubilised receptors were immunoprecipitated using β1/3 antisera (Lanes 1) or pre-immune IgG (Lanes 2). Precipitated material processed as A) but western blotted with a RACK-1 antibody. Lane 3 represents 10% of input used for the immunoprecipitation.
Figure 32A shows that PKC is co-precipitated with GABA_A β1/3 subunits from untreated cells (Lane 1), but prior treatment with phorbol esters leads to a dramatic increase in this amount (Lane 2). The interaction with RACK-1 was not seen in unstimulated cells, but was with phorbol ester treatment (Figure 32B, Lane 1). In HEK293 cells such treatment was not required to see this interaction. In this instance we do not have a vast excess of over-expressed protein though, but are in a system which is under many more restrictions, and such prior stimulation may be required for RACK-1 to bind to the GABA_A receptor. Under resting conditions PKC interacts with the receptor at a low stoichiometry. Upon activation of PKC by phorbol esters, a lot more of the kinase binds, along with the RACK-1 protein. We do not know if these two changes are independent of each other or whether for example, stimulation leads to a direct enhancement of the RACK-1 interaction, which in turn pulls in more molecules of PKC, or vice versa. Also, though RACK-1 can bind the β1 subunit directly, it is not known if the interaction seen here is direct, or whether PKC, upon activation and translocation to the receptor, is bringing RACK-1 with it.

5.2.8. In vitro phosphorylation of β1/3 subunits by co-precipitating PKC

Immunoprecipitations, as described in sections 5.2.6. and 5.2.7. from cortical neurons, phorbol ester treated as indicated in Figure 33, were put into an in vitro kinase assay with γ^32P-ATP and the PKC inhibitor peptide as indicated. This was to see if PKC, which is shown to co-precipitate with the receptor is capable of phosphorylating it. Figure 33, Lane 3 shows a major phosphorylated band, which from the reasoning in Section 5.2.7. is very likely to be the β3 subunit. If cells are phorbol ester treated, a small increase in phosphorylation is seen (Lane 1). Such a small increase is surprising, considering the large increase observed in PKC binding (Figure 32). This may indicate that the amount of kinase bound when PKC is not activated is sufficient for near maximal receptor phosphorylation. Addition of the specific PKC inhibitor, PKC19-36,
Cortical neurones were treated with PdBu, as indicated, for 30 minutes and solubilised in a buffer containing 1% CHAPS. Receptors were immunoprecipitated using antisera specific for the β1/3 subunits (Lanes 1-3) or pre-immune serum (Lanes 4-6), coupled to protein A-sepharose. Precipitated material was subject to in vitro kinase assays, with the specific PKC-inhibitor, PKI_{19-36}, as indicated. Beads were washed extensively in kinase buffer before the addition on γ^{32}P-ATP, and incubated at 30°C for 30 min. Reaction products were then separated by SDS-PAGE and visualised by autoradiography.
completely abolishes the phosphorylation, confirming the fact that PKC is the kinase responsible.

5.2.9. GABA\textsubscript{A} \(\beta1/3\) subunits are highly basally phosphorylated in cultured cortical neurons

To study the \textit{in vivo} phosphorylation of the \(\beta1\) and \(\beta3\) subunits in cultured cortical neurons, cells were labelled with \([^{32}\text{P}]\)-orthophosphate and receptor complexes were immunoprecipitated with the \(\beta1/3\) antisera as in sections 5.2.6-5.2.8. As Figure 34A shows, there is high basal phosphorylation of the receptor, especially of the 57kD band assumed to be \(\beta3\). To further characterise the nature of this phosphorylation the phosphoprotein, marked (*), was excised from the gel and subject to phosphopeptide mapping and phosphoamino acid analysis. The phosphorylation was seen to be mainly on serine residues (Figure 34B), and the peptide map showed the generation of three phosphopeptides [Figure 35B (a-c)]. The phosphopeptide map looks very similar to that of the recombinant \(\beta3\) subunit (Figure 35B, right hand panel), which is seen to be basally phosphorylated when expressed in HEK293 cells with the \(\alpha1\) subunit (Figure 35A). It is known that the latter is due to phosphorylation at serines 408 and 409 (B.J.McDonald and S.J.Moss, unpublished data).

5.2.10. PKC is responsible for the high basal phosphorylation of GABA\textsubscript{A} \(\beta1/3\) subunits in cultured cortical neurons

To identify the kinase(s) responsible for the basal phosphorylation of the \(\beta3\) subunit in 5.2.9., cells were again prelabelled with \([^{32}\text{P}]\)-orthophosphate and then treated with specific kinase inhibitors for thirty minutes prior to lysis and immunoprecipitation. Inhibiting CamKII and PKA (Figure 36, Lanes 2 & 3 respectively) does not have a significant effect on the level of phosphorylation. Inhibiting PKC with Calphostin
Figure 34 Immunoprecipitation of β1/3 subunits from prelabelled cortical neurons

A) Cortical neurons were prelabelled for 4 hours with $[^{32}P]$-orthophosphate, lysed and receptors immunoprecipitated with β1/3 antisera (Lanes 1) or pre-immune serum (Lane 2). Precipitated material was then separated by SDS-PAGE, and visualised by autoradiography. Gel slices containing the phosphoprotein (*) were either: (B) subject to tryptic digestion followed by acid hydrolysis. The resulting phosphoamino acids were then separated by thin layer chromatography. The migration of phosphoserine (pSER), phosphothreonine (pTHR) and phosphotyrosine (pTYR) are indicated.
Figure 35 GABA\textsubscript{A} \(\beta 3\) subunit is basally phosphorylated in cortical neurones and HEK293 cells

A) HEK293 cells were transfected with \(\alpha 1\beta 3\) (Lane 2) or sham transfected (Lane 2). Cells prelabelled for 4 hours with \(^{32}\text{P}\)-orthophosphate, lysed and receptors immunoprecipitated with \(\beta 1/3\) antisera. Precipitated material was then separated by SDS-PAGE, and visualised by autoradiography.

B) Phosphoprotein from these cells and from cortical neurones was subjected to tryptic digestion. The resulting phosphopeptides were then separated by electrophoresis in the first dimension and by thin layer chromatography in the second.
Cortical neurons were prelabelled for 4 hours with $[^{32}\text{P}]$-orthophosphate and treated with specific kinase inhibitors for thirty minutes. Lane 1: untreated, Lane 2: CamKII inhibitor KN-93 (2μM), Lane 3: PKA inhibitor Rp-8-Br-cAMPS (1mM) and Lane 4: PKC inhibitor Calphostin C (2μM) [All Calbiochem]. Cells were then lysed, and receptors immunoprecipitated using antisera specific for the β1/3 subunits. Precipitated material was then separated by SDS-PAGE, and visualised by autoradiography.
C results in a dramatic decrease in the level of phosphorylation observed of the β3 subunit (Lane 4). It is clear that the basal phosphorylation of the β3 subunit is due to an intimate association with PKC. It is interesting that such a high level of phosphorylation is observed, even though there is only a low level of PKC binding to the receptor complex at rest (Section 5.2.7.). As suggested previously, the level of PKC bound at rest may be sufficient to maintain phosphorylation of the β subunits, and the excess which binds upon PKC stimulation is able to phosphorylate other subunits e.g. γ2 or other subunit associated proteins. It will now be of great interest to try to dissect out the factors which are responsible for this basal level of PKC phosphorylation. Candidates include activity, which can be blocked with tetrodotoxin, GABA_A receptor agonists, which can be outcompeted with antagonists and certain growth factors. This should be feasible by both biochemical and electrophysiological analysis.

5.3. Discussion

PKC has been shown to phosphorylate a number of GABA_A subunit intracellular loops in vitro. These include the β1 subunit at S409, β3 at S408 and S409 and the γ2 subunit at S327 (Moss et al., 1992a; Mcdonald and Moss, 1997). In HEK293 cells PKC is able to phosphorylate any of the following sites to cause an inhibition of channel function; β1 at S409, β2 at S410, S327 in γ2S/L and S343 in γ2L (Kellenberger et al., 1992; Krishek et al., 1994). It is not known how PKC is targeted to the receptor, though as described earlier the PKC β1 isoform and the RACK-1 protein specifically interact with the GABA_A β subunits in vitro. It was apparent from these studies that the two molecules were able to bind independently of each other, but this does not rule out the possibility that they may interact in some manner at the receptor. To investigate this possibility, the binding sites of the two proteins on the β1 subunit were mapped. The model arising from the results is very interesting, with PKC binding around the conserved phosphorylation site at serine 409, and RACK-1 to a region immediately
upstream of this (Figure 25). Peptides corresponding to the binding sites were able to inhibit these interactions, by outcompeting the normal interactions. This was studied from a functional perspective. Initially, by overexpressing all the relevant proteins in HEK293 cells, a tripartite complex of β1 subunit, PKC β1 and RACK-1 was shown to be present by co-immunoprecipitation studies (Figure 27). HEK293 cells transfected with GABA\(_A\) α1 and β1 subunits were then subject to whole-cell patch-clamp analysis. Cells were treated with phorbol esters with and without the presence of polypeptides of the PKC and RACK-1 binding sites. Dramatically, blocking the RACK-1 binding site causes a very significant decrease in the normal inhibition seen upon PKC activation (Figure 30B). This observation is of significance as it may suggest a new role for RACK-1. Previous studies have indicated that it is essential for the targeting of PKC to substrate (Ron et al., 1994a; Ron et al., 1994b). But in this instance it is apparent that RACK-1 and PKC are interacting with a common molecule independently of each other, but RACK-1 still has a critical role in mediating the PKC interaction, as suggested by the functional studies. It could perhaps have some form of allosteric function. It will be important now to analyse the effects of these peptides in cultured neurons.

To identify if a complex of RACK-1 and PKC is associated with neural GABA\(_A\) receptors, this complex was studied in cultured cortical neurons by immunoprecipitation. Co-immunoprecipitation studies show that a small amount of PKC is associated with the receptor, which is greatly increased upon PKC activation. The latter also gives rise to RACK-1 association with the receptor. The increase in the amount of PKC binding may reflect the well known translocation of PKC from the soluble fraction to the membranes of cells (Goodnight et al., 1995; Mochly-Rosen, 1998), or it may be due to an increase in affinity of the kinase for its substrate upon activation. The need for PKC activation for an interaction with RACK-1 to be observed has been seen in other studies (Liliental et al., 1998), and may reflect either an independent translocation of RACK-1 due to direct action of phorbol esters, or co-
translocation with PKC. This should be able to be resolved utilising the peptides corresponding to the specific binding sites of the two molecules.

Pre-labelling cortical neurons with $^{32}$P-orthophosphate, and immunoprecipitating the receptor with the β1/3 specific antibody showed that β3 has high levels of basal phosphorylation. The phosphorylation was solely on serines, with the phosphopeptide map characteristic of the sites S408 and S409 being phosphorylated. The latter comes from comparisons to the phosphopeptide maps generated from recombinant phospho-β3, after PKC activation (B.J.McDonald and S.J.Moss, unpublished data). This phosphorylation was blocked by treating the cells with the cell-permeable PKC inhibitor Calphostin C. This finding is of major significance as it demonstrates that the GABA$_{	ext{A}}$ β3 subunit is highly basally phosphorylated, by a relatively small amount of bound PKC. These studies also demonstrate for the first time that neural GABA$_{	ext{A}}$ receptors are phosphorylated in vivo, confirming the importance of this process in regulating receptor function from recombinant studies. Patch-clamp analysis of GABA$_{	ext{A}}$ mediated currents in these cortical cultures using kinase inhibitors and phosphatase inhibitors will establish the role of the basal phosphorylation in controlling receptor function.

PKC is important for many neuronal processes, and is shown in this study to have an intimate relationship with the GABA$_{	ext{A}}$ receptor. This potentially has many important consequences for the regulation of neuronal excitability.
6. General Discussion

GABA_A receptors are essential in the control of neuronal excitability and as the sites of action for many important clinical drugs (Macdonald and Olsen, 1994; Rabow et al, 1995). The mechanisms used by neurones to regulate these receptors is not well characterised. However in recent years the role of phosphorylation has been extensively analysed (Moss and Smart, 1996; Smart, 1997). Recombinant studies have shown that phosphorylation on principally the β and γ2 subunits results in functional modulation of the receptor (see introduction for further discussion). Study of γ2 subunit phosphorylation in brain preparations has been restricted though, due to the lack of good, high-titre antibodies against the subunit and due to the subunits' apparent susceptibility to degradation. To address the phosphorylation of the γ2 subunit in neurons two sets of antibodies were raised which allowed the tyrosine phosphorylation of the γ2 subunit in neurons to be analysed. The first approach was to produce an antisera which recognises the amino-terminal 29 amino-acids of the subunit. Antibodies against this epitope were raised in both rabbits and guinea-pigs. These antibodies were initially tested by ELISA, and production bleeds from a single rabbit (UCL17) and a guinea-pig (GP1) were characterised further. Affinity-purified forms of both antibodies were shown to recognise the γ2 subunit specifically by immunofluorescence in transfected HEK293 cells and were able to immunoprecipitate receptor complexes containing γ2 from similarly transfected cells. A protein A-sepharose purified form of GP1 was able to recognise γ2 by western blotting from either transfected cells or neuronal lysates. UCL17 was also shown to recognise γ2 in cortical and hippocampal neurons by immunofluorescence. The affinity purified form of UCL17 was used to immunoprecipitate γ2 from neuronal lysates, with the immune-complexes blotted with a phosphotyrosine antibody. This showed for the first time that the γ2 subunit is phosphorylated on tyrosine residues, as predicted from recombinant studies (Moss et al., 1995).
of the sites at which this phosphorylation occurs. It has been demonstrated in recombinant systems that phosphorylation by v-src at residues Y365 and Y367 on this subunit causes an up-regulation in channel function (Moss, 1995), so on this basis an antibody was raised which recognises the γ2 subunit when it is specifically phosphorylated on residues Y365 and Y367. Guinea pigs were initially immunised with a γ2 tyrosine phosphopeptide, but the titre of the sera was insufficient. A single rabbit was similarly immunised and gave rise to an antisera which by ELISA seemed to be very specific for the phosphotyrosine γ2 epitope. This was further characterised by western blotting against the γ2 intracellular loop expressed as a GST fusion protein. The antibody only recognised tyrosine-phosphorylated γ2. Finally this antibody was used to probe neuronal preparations. Western blotting of whole brain lysate showed a band which was of a molecular weight expected for γ2, and was blocked by preincubating the antibody with the immunising peptide. More intriguingly though were the results obtained from blotting against lysates from cortical neurons. In untreated cells the γ2 subunit was apparently not phosphorylated on Y365 and Y367. If the cells were pretreated with NaVan (a potent protein tyrosine phosphatase inhibitor), a band identical in molecular weight to γ2 was recognised, again this could be blocked by preincubating the antibody with the immunising peptide. This observation suggests that there is tight regulation of tyrosine phosphorylation of the γ2 subunit on Y365 and Y367. Interestingly the NMDA receptor has recently been shown to be tightly associated with, and regulated by, a tonically active form of the phosphatase PP1 (Westphal et al., 1999b). It should be possible to identify any such tightly associated signalling molecules with the γ2 subunit. Furthermore, it will be important to identify first messengers that are able to elicit the phosphorylation of γ2 in culture, mimicking the action of NaVan.

It is unknown though what tyrosine kinase(s) or phosphatases are responsible for the tyrosine phosphorylation of the γ2 subunit, indeed, little is known about the relationship between the GABA$_{A}$ receptor and
such signalling molecules in neurons. Recently there have been many reports of the tight association of protein kinases with a number of ion channels. These interactions have either been shown to be direct or mediated by an adaptor/anchoring protein. The majority of the former have shown the interaction of a non-receptor protein tyrosine kinase and a specific ion-channel subunit. Examples include the interaction of src with the acetylcholine receptor β subunit (Fuhrer, 1996) and the human Kv1.5 potassium channel (Holmes et al., 1996). The best characterised of the anchoring proteins are the family of A-kinase anchoring proteins (AKAPs) (Colledge and Scott, 1999). These include the interaction of the NMDA R1 subunit with PKA and PP1, which is mediated by the AKAP 'yotiao' (Westphal et al., 1999a; Westphal et al., 1999b), and the interaction of both Ca\(^{2+}\) and Na\(^+\) channels with AKAP15/18 (Gray et al., 1997; Tibbs et al., 1998). There have been a number of studies which have identified kinase activity co-purifying with GABA\(_\alpha\) receptors. The first report identified an unknown serine kinase which preferentially phosphorylates the α1 subunit (Sweetnam et al., 1988), which was associated with receptor immunoprecipitated from rat cerebellum. A similar activity, along with a little tyrosine kinase activity was found associated with receptor immunoprecipitated from bovine brain. Again the serine kinase activity preferred α subunits, while it was suggested that the tyrosine kinase activity phosphorylated the γ2 subunit (Bureau and Laschet., 1995). Much more recently though a novel serine kinase of predicted molecular mass 34kD, has been identified by co-precipitation with receptor from bovine brain (Kannenberg et al., 1999). Furthermore it preferentially phosphorylates the β3 subunit. This is more in keeping with recombinant work where the α1 subunit has never been shown to be phosphorylated in vivo, while the β-subunits are seen to be very good substrates for a range of protein kinases (Moss and Smart, 1996). To investigate the interaction of kinases with the GABA\(_\alpha\) receptor the intracellular domains of a range of receptor subunits, expressed as GST fusion proteins, were used as baits to probe rat brain lysate for interacting kinases. Fusion proteins and bound material were then exposed to an in vitro kinase assay and the phosphorylation of the
fusion proteins was analysed. Though the principal aim was to identify interacting protein tyrosine kinases, none of the fusion proteins seemed to be phosphorylated on tyrosine residues. Instead the β subunits were stoichiometrically phosphorylated on serine residues, while α1 and γ2 were not significantly phosphorylated. This phosphorylation of the β-subunits was abolished by the addition of the PKC inhibitor peptide to the kinase assays, suggesting that PKC was binding to and phosphorylating the receptor. Western blotting confirmed that PKC, and in particular the β1 and α isoforms were interacting with the β1 and β3 subunit intracellular domains. The GABAₐ β-subunits have a conserved serine at position 409/410. S409 in β1 has been shown to be phosphorylated by both PKA and PKC, causing a decrease in channel function (Moss et al., 1992b; Krishek et al., 1994), while in β3 the phosphorylation of this residue and the adjacent residue S408 enhances receptor function (McDonald et al., 1998). Mutant constructs, with the serine converted to an alanine were still able to bind PKC, but were not phosphorylated. This allowed the site of phosphorylation to be identified in the β1 and β3 subunits as S409 in β1 and S408 and S409 in β3. As well as the PKC activity there was a kinase activity associated with α1, β1 and γ2 subunits which was not inhibited by the PKC inhibitor peptide. This activity did not seem to be able to phosphorylate any of the fusion proteins though. It is possible that this activity could be related to the serine kinase activities previously reported to be associated with the receptor (Sweetnam et al., 1988; Bureau and Laschet., 1995; Kannenberg et al., 1999).

There have been no reports to date of PKC interacting with an ion-channel, though there are now a wide range of PKC binding molecules which are able to target PKC to its distinct subcellular sites. One of the better characterised of these molecules is the molecule 'Receptor for activated C-kinase', RACK-1. RACK-1 was originally identified in a screen to identify molecules which bind activated PKC (Mochly-Rosen et al., 1991; Ron et al., 1994). More recently a whole range of other signalling molecules have been reported to bind to RACK-1. These
include the protein tyrosine kinase src, integrin β-subunits and the cAMP phosphodiesterase PDE4D5 (Chang et al., 1998; Liliental et al., 1998; Yarwood et al., 1999). RACK-1 is shown to have a binding preference for the β1 and α isoforms of PKC, which are bound in a state of high activity. Due to the PKC isoforms identified binding to the GABA_4, β1 subunits and the high stoichiometry of the phosphorylation, the presence of RACK-1 was probed for, and identified in ‘pull-down’ assays with GABA_4, β1 and β3 subunit intracellular domains from adult rat brain lysate. With two molecules, PKC and RACK-1, whose own interaction, is well characterised, binding to a single ion-channel subunit it was possible that one molecule could be interacting as an adaptor molecule for the other. In this study this was not found to be the case, as gel-overlay assays showed that these two molecules both bound directly to the β1 subunit. To further characterise this potential signalling complex the binding sites of PKC and RACK-1 on the β1 subunit were identified by deletion analysis. The two sites were narrowed down to adjacent 10 amino-acid regions around the functionally important consensus phosphorylation site at S409. PKC was found to bind to a region including this site, with RACK-1 immediately upstream. This is of interest as it suggests that the two molecules are able to interact when bound, even though their binding is independent of each other. To confirm the trimolecular interaction in a mammalian cell context, PKC and RACK-1 were shown to co-immunoprecipitate with the GABA_4 receptor in heterologous cells. The in vivo relevance of this complex was confirmed in cultured cortical neurons, by similar co-immunoprecipitation studies. In the latter the complex was dynamically regulated. In unstimulated cortical neurons low levels of PKC were associated with the receptor. No RACK-1 was found to bind under these conditions. Upon PKC stimulation the amount of PKC binding to the receptor increases dramatically, with a concomitant binding of RACK-1. As previously discussed the latter may be due to RACK-1 being targetted to the receptor by PKC, or by a direct effect of phorbol esters on RACK-1. From the in vitro phosphorylation of immunoprecipitated receptor by co-precipitated PKC the activation of PKC, and consequent increased
binding, does not cause a dramatic increase in phosphorylation of the β subunit. In agreement with this, in vivo labelling demonstrated high basal receptor phosphorylation that was mediated by PKC. Therefore the low level of PKC associated with GABA_A receptors is sufficient to produce high basal phosphorylation. This tonic receptor phosphorylation by PKC may therefore play a major role in controlling receptor function. The additional PKC bound upon PKC activation with phorbol esters may be available to phosphorylate other subunits, in particular the γ2 subunit, which is known to be phosphorylated by PKC in vivo (Kellenberger et al., 1992; Krishek et al., 1994).

The functional consequence of RACK-1 binding was investigated in HEK293 cells. A peptide corresponding to the RACK-1 binding site on the β1 subunit was able to block the normal inhibition of channel function seen upon PKC activation (Krishek et al., 1994), when applied to a cell in the patch pipette. So it is apparent that RACK-1 plays an important role in channel regulation by PKC. It will be critical though to test these peptides in a neuronal context, before more credence can be given to the physiological relevance of the RACK-1 interaction. RACK-1 could also bring other signalling molecules into the GABA_A receptor complex. In relation to this study the recently identified interaction of RACK-1 and src-family tyrosine kinases could be of great importance (Chang et al., 1998). This study has shown that the γ2 subunit is tyrosine phosphorylated and it would be great interest if RACK-1, or another scaffold-like molecule, was involved in bringing the relevant enzymes into close contact with the receptor.

In summary, this study shows for the first time that the γ2 subunit of the GABA_A receptor, which is critical for receptor modulation by benzodiazepines, is phosphorylated on tyrosine residues. The antibodies from this study should now allow for the upstream events in this regulation to be dissected. To gain an insight into the interaction of kinases with the receptor, this work has also identified a very intimate relationship between the GABA_A β subunits, PKC and the molecule
RACK-1. It is likely that this complex plays a critical role in the maintenance and inhibition of channel function.
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