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Modulation of the $GABA_A$ receptor by CaMK-II dependent phosphorylation.

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ABSTRACT:

Phosphorylation is an important mechanism for the modulation of ligand-gated ion channel function. CaMK-II dependent modulation of $GABA_A$ receptors was investigated through analysis of $GABA_A$ receptor mediated currents recorded by whole-cell patch clamp from non-neuronal and neuronal cell lines and from primary neurones in culture.

Application of pre-activated α-CaMK-II had no effect on recombinant receptors expressed in HEK293 cells. Whole-cell currents recorded from cerebellar granule cells were significantly increased in peak amplitude on application of pre-activated α-CaMK-II. Analysis of spontaneous (s)IPSCs recorded from cerebellar granule cells in culture in the presence of pre-activated α-CaMK-II revealed an increase in sIPSC amplitude and decay time constants. Expression of recombinant $GABA_A$ receptors in the neuronal cell line NG108-15 allowed the functional effect of CaMK-II to be studied in a recombinant system. CaMK-II was able to up-regulate the function of $\alpha1\beta1\gamma2S$ and $\alpha1\beta3\gamma2S$ $GABA_A$ receptors but not $\alpha1\beta2\gamma2$ receptors. This modification of function occurred through phosphorylation of Ser^{383} on the β3 subunit and through a downstream activation of a tyrosine kinase and subsequent phosphorylation of the γ2S subunit at previously identified sites of tyrosine kinase phosphorylation, Tyr^{357} and Tyr^{359}. Tyrosine kinase phosphorylation of γ2S occurred when co-expressed with $\alpha1\beta1$ or $\alpha1\beta3$ but not with $\alpha1\beta2$. Transfection of different $GABA_A$ receptor subunits into cerebellar granule cells was carried out to confirm the importance of these sites in a neuronal environment. Analysis of sIPSCs recorded from cerebellar granule cell cultures prepared from β2 subunit knockout mice revealed α-CaMK-II modification of β2 subunit-containing receptors at the synapse, resulting in an increase in sIPSC amplitude with no change in decay times. This was in contrast to the lack of any functional modulation of $\alpha1\beta2\gamma2S$ receptors observed in NG108-15 cells.

These findings are potentially important for understanding the mechanisms of CaMK-II dependent modulation of the $GABA_A$ receptor and may also have significant implications for the understanding of inhibitory synaptic plasticity.

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AC, adenylate cyclase; **AID**, autoinhibitory domain; **AKAP**, A-kinase anchoring protein; **BAPTA**, 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; **BDNF**, brain-derived neurotrophic factor; **BDZ**, benzodiazepine; **CBD**, calmodulin binding domain; **CaM**, calmodulin; **CaMK-II**, Calcium/calmodulin-dependent protein kinase II; **CaN**, calcineurin; **DARPP-32**, dopamine- and cAMP- regulated phosphoprotein; **EGTA**, Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; **EPSC**, excitatory postsynaptic current; **GABARAP**, $GABA_A$ receptor associated protein; **GFP**, green fluorescent protein; **HEK**, human embryonic kidney; **LTD**, long term depression; **LTP**, long term potentiation; **MAGUK**, membrane associated guanylate kinase; **MAPK**, mitogen-activated protein kinase; **m/nAchR**, muscarinic/nicotinic acetylcholine receptor; **m/sIPSC/P**, miniature/spontaneous inhibitory synaptic current/potential; **NSF**, *N*-ethylmaleimide sensitive factor; **OA**, okadaic acid; **PCR**, polymerase chain reaction; **PKA**, cAMP-dependent protein kinase; **PKB**, protein kinase B; **PKC**, protein kinase C; **PKG**, cGMP-dependent protein kinase; **PLC**, phospholipase C; **PP(1/2)**, protein phosphatase 1/2; **PRIP-1**, PLC-related inactive protein type 1; **PSD**, postsynaptic density; **RACK**, receptor for activated C kinase;

Chapter One: General Introduction:
Modulation of the $GABA_A$ receptor by phosphorylation.

1.1 INTRODUCTION

The γ-aminobutyric acid type A ($GABA_A$) receptor is a pentameric ligand-gated ion channel responsible for the majority of fast synaptic inhibition in the brain. It is related in structure to the cys-loop family of ligand-gated ion channels including the serotonin ($5\text{-}HT_3$) receptors, nicotinic acetylcholine receptors (nAChR) and glycine receptors (Nayeem *et al.*, 1994; Rothlin *et al.*, 1999; Cromer *et al.*, 2002; Korpi *et al.*, 2002). It is formed from five subunits that form a Cl^- permeable ion channel (Jenson *et al.*, 2002) that is opened after binding of the amino acid neurotransmitter GABA (Smith and Olsen, 1995; Moss and Smart, 1996; Mehta and Ticku, 1999; Moss and Smart, 2001; Connolly and Wafford, 2004).

1.1.1 Receptor structure.

Numerous different subunits of the $GABA_A$ receptor have been identified which can be divided into subunit families based on sequence homology. These consist of α1-6, β1-3, γ1-3, δ, π, ε, θ and ρ (Barnard *et al.*, 1998; Whiting *et al.*, 1999; Korpi *et al.*, 2002). There is typically 30-40 % homology between subunit families and 70-90 % between subunit isoforms (Smith and Olsen, 1995; Korpi *et al.*, 2002). In addition, some of these subunits have multiple splice variants the most commonly expressed being the γ2S and γ2L forms of the γ2 subunit in which the γ2L variant has an additional eight amino acids at position 338-345 (Moss and Smart, 1996). This heterogeneity of $GABA_A$ receptors allows for a large amount of spatial and temporal diversity with different cells expressing different subunit combinations at

different times (McKernan and Whiting, 1996; Whiting *et al.*, 1999; Sieghart *et al.*, 1999).

Each $GABA_A$ receptor subunit contains a large extracellular domain, four transmembrane domains (TM) and a large intracellular loop between TM 3 and 4 (Smith and Olsen, 1995; Moss and Smart, 1996). The TM2 domain lines the channel pore (Xu and Akabas, 1996). The TM3-4 intracellular loop can account for up to 20 % of the total mass of a subunit and is known to contain multiple sites of phosphorylation (Smith and Olsen, 1995; Moss and Smart, 1996; See Fig 1.1).

The subunit stoichiometry of $GABA_A$ receptors in the brain is thought to consist of $2\alpha:2\beta:1\gamma$ (Nayeem *et al.*, 1994; Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999). In recombinant systems, it has been determined that the $\beta1$ and $\beta3$ subunits can form functional homomeric channels, although this doesn't appear to be the case for $\beta2$ subunits (Krishek *et al.*, 1996; Connolly *et al.*, 1996a; Connolly *et al.*, 1996b; Wooltorton *et al.*, 1997; Taylor *et al.*, 1999). For the formation of a robust GABA gated Cl^- channel it appears that co-expression of an α and a β subunit is required (Connolly *et al.*, 1996a; Taylor *et al.*, 1999). However $\alpha\beta$ channels have different single channel conductance and gating properties than $\alpha\beta\gamma$ channels and they are not modulated by benzodiazepines (Angelotti and Macdonald, 1993; Angelotti *et al.*, 1993a). The inclusion of the γ subunit increases the amplitude of whole-cell currents recorded in a recombinant system and results in an increase in the EC_{50} for GABA (Angelotti *et al.*, 1993a). The inclusion of $\gamma2$ in the receptor complex has also been reported to alter the kinetic properties of $GABA_A$ receptors (Boileau *et al.*, 2003). For modulation by benzodiazepines, an α and γ subunit are required (Sigel *et al.*, 1990; Angelotti *et al.*, 1993a).

Fig 1.1:

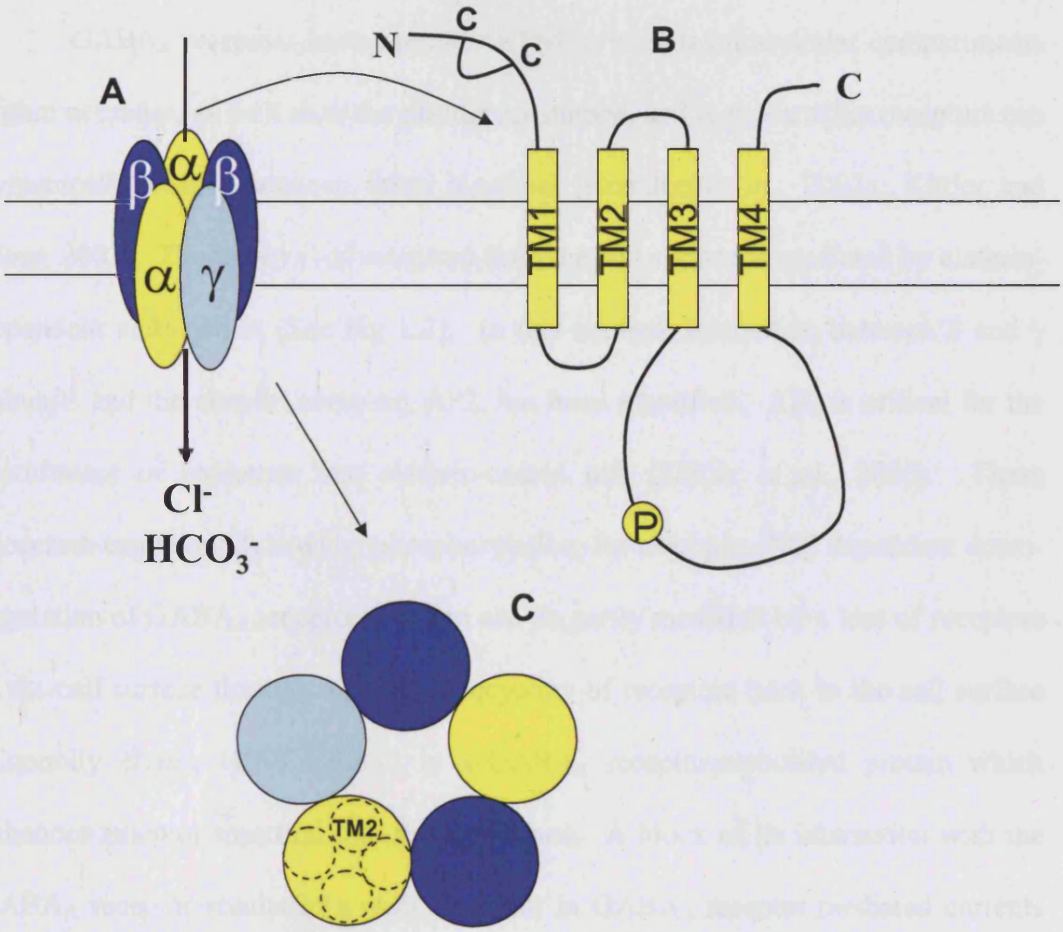


Fig 1.1: Illustration of the proposed structure of the $GABA_A$ receptor.

(A) The $GABA_A$ receptor is thought to be pentameric consisting of 2α, 2β and 1γ subunit. The channel opens in response to GABA binding and is permeable to Cl^- and HCO_3^- ions.

(B) Each subunit is proposed to consist of an N-terminal extracellular domain, four trans-membrane domains (TM), a large intracellular loop between TM3-4 and an extracellular N-terminus in which the cys-loop is situated. The large intracellular loop is thought to contain multiple potential sites of phosphorylation. A small external C-terminus is also evident.

(C) The TM2 region of each subunit is thought to line the channel pore.

Adapted from Moss and Smart (1996).

1.1.2 $GABA_A$ receptor trafficking.

$GABA_A$ receptors have been identified in various intracellular compartments within neurones, as well as at the plasma membrane, and it appears that receptors can dynamically shuttle between these locations (Brandon *et al.*, 2002a; Kittler and Moss, 2003). The removal of receptors from the cell surface is mediated by clathrin-dependent endocytosis (See Fig 1.2). In this context, interaction between β and γ subunits and the adaptin complex, AP2, has been identified. AP2 is critical for the recruitment of receptors into clathrin-coated pits (Kittler *et al.*, 2000). These processes can be modulated by phosphorylation, for example, PKC dependent down-regulation of $GABA_A$ receptor function can be partly mediated by a loss of receptors at the cell surface through a block of recycling of receptors back to the cell surface (Connolly *et al.*, 1999). Plic-1 is a $GABA_A$ receptor-associated protein which enhances receptor insertion into the membrane. A block of its interaction with the $GABA_A$ receptor results in a rapid decrease in $GABA_A$ receptor-mediated currents implying that Plic-1 is required for the replacement of receptors lost by internalisation (Bedford *et al.*, 2001). Alteration in trafficking through perturbation of these pathways results in significant effects on $GABA_A$ receptor-mediated currents and inhibitory synaptic transmission (Kittler and Moss, 2003).

The correct targeting of assembled receptors to synaptic sites is crucial for the proper functioning of inhibitory synaptic transmission. It is thought that an interaction between the $\gamma 2$ subunit and the scaffolding protein gephyrin may be involved in receptor targeting and clustering at the synapse (See Fig 1.2). The loss of the $\gamma 2$ subunit results in a large decrease in the number of receptors at synapses despite the fact that $\alpha\beta$ receptors are capable of trafficking to the cell surface. The loss of $\gamma 2$ also results in a subsequent loss of gephyrin, suggesting an important link

between the two proteins (Essrich *et al.*, 1998). Disruption of gephyrin results in the disruption of α2/3 subunit-containing $GABA_A$ receptor clustering and synaptic targeting of γ2 subunit-containing receptors. However, in gephyrin knockout mice, some $GABA_A$ receptor subtypes are unaffected. Gephyrin is thought to act as a scaffolding molecule important for receptor clustering of certain subtypes of $GABA_A$ receptor, but evidence suggests there must be additional proteins involved in clustering and trafficking (Kneussel *et al.*, 1999; Kneussel *et al.*, 2001).

The $GABA_A$ receptor associated protein GABARAP has been identified to associate with the γ2 subunit and is thought to play a role in intracellular transport and cell surface targeting of the receptor (Chen *et al.*, 2000; Kittler *et al.*, 2001; Kneussel, 2002; Kanematsu *et al.*, 2002; Leil *et al.*, 2004). The correct targeting of receptors to synapses is significant given the large amount of heterogeneity of $GABA_A$ receptors and the apparent precise control of $GABA_A$ receptor subtype expression and localisation (Koulen *et al.*, 1996; Brünig *et al.*, 2002).

Fig 1.2:

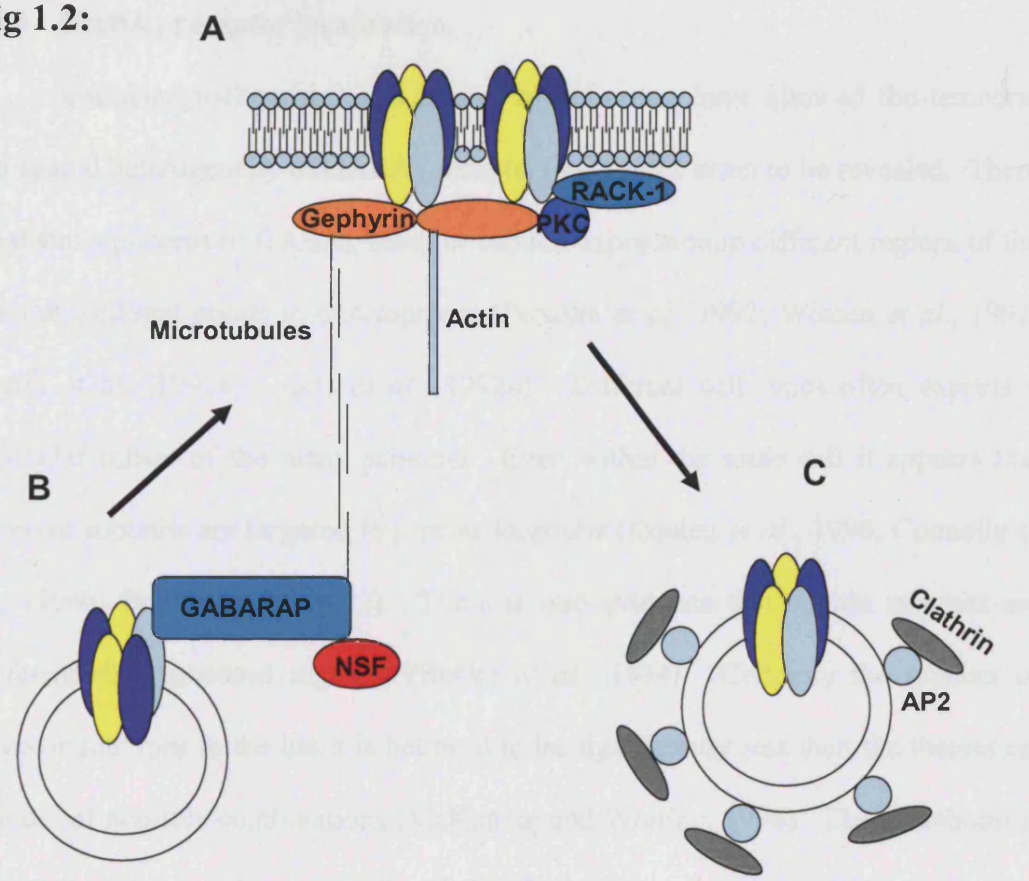


Fig 1.2: Trafficking of the $GABA_A$ receptor and interactions at the plasma membrane.

(A) $GABA_A$ receptor clustering in some cases is thought to be mediated by the protein gephyrin. Associations between gephyrin, the $GABA_A$ receptor and the cytoskeleton have been proposed to anchor and cluster $GABA_A$ receptors at the synapse.

Anchoring proteins can target protein kinases to the $GABA_A$ receptor and can modulate, or be critical for kinase function (e.g. PKC and RACK-1).

(B) GABARAP has been implicated in the trafficking of $GABA_A$ receptors to the plasma membrane through an interaction with the γ subunit of the $GABA_A$ receptor, microtubules and N-ethylmaleimide sensitive factor (NSF). NSF is key protein involved in membrane fusion and is critical for intracellular transport.

(C) $GABA_A$ receptors at synaptic sites undergo constitutive endocytosis via clathrin-mediated endocytosis. This is mediated by an interaction between β and γ subunits and adaptor proteins (e.g. AP2) essential for the recruitment of protein cargo into clathrin-coated pits.

Adapted from Moss and Smart (2001).

1.1.3 $GABA_A$ receptor localisation.

Immunocytochemistry and *in situ* hybridisation have allowed the temporal and spatial heterogeneity of $GABA_A$ receptors within the brain to be revealed. There are distinct patterns of $GABA_A$ receptor subunit expression in different regions of the brain at different points in development (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Laurie *et al.*, 1992a; Laurie *et al.*, 1992b). Different cell types often express a particular subset of the many subunits. Even within the same cell it appears that different subunits are targeted to precise locations (Koulen *et al.*, 1996; Connolly *et al.*, 1996b; Brünig *et al.*, 2002). There is also evidence that certain subunits are preferentially expressed together (Benke *et al.*, 1994). Certainly the number of receptor subtypes in the brain is believed to be significantly less than the theoretical number of possible combinations (McKernan and Whiting, 1996). The $\beta 2$ subunit is widely expressed and accounts for 60 % of all β subunits with a relatively smaller contribution of $\beta 1/3$ (Benke *et al.*, 1994). However, recent evidence suggests that $\alpha 1\beta 2$ subunits prefer to co-assemble and that $\alpha 2/3\beta 3$ subunits also preferentially co-assemble (Benke *et al.*, 1994; Ramadan *et al.*, 2003; Ortinski *et al.*, 2004; Vicini and Ortinski, 2004). The $\alpha 1\beta 2\gamma 2$ receptor and the $\alpha 2\beta 3\gamma 2$ receptor together account for 75-85 % of the diazepam-sensitive receptors in the brain (Benke *et al.*, 1994). Although these associations are not mutually exclusive, they suggest a broad division between these two receptor subtypes with each form being targeted to specific cellular locations (Whiting *et al.*, 1999; Vicini and Ortinski, 2004).

1.1.4 Physiological role and pathology.

Activation of $GABA_A$ receptors in the adult brain causes the rapid influx of Cl^- ions which results in a hyperpolarisation of the cell. As a consequence they are

critical in the control of neuronal excitability (Moss and Smart, 1996). In immature neurones, GABA is excitatory resulting in cell depolarisation and the generation of action potential firing. Independent of the net flow of Cl^- ions, the opening of $GABA_A$ receptors is often enough to shunt excitatory synaptic currents (Ruiz *et al.*, 2003). The shift from excitation to inhibition occurs when the KCC2, K^+ / Cl^- co-transporter is expressed changing the electrochemical gradient for Cl^- (Ben-Ari, 2002).

The $GABA_A$ receptor is also the site of action of a number of important, clinically relevant drug families. The most widely used and characterised of these is the benzodiazepine family, which acts as an allosteric modulator of $GABA_A$ receptors enhancing their function in response to GABA. Disruptions to the normal function of $GABA_A$ receptors have been linked to many pathological conditions (Mehta and Ticku, 1999). The clinical use of benzodiazepines is particularly widespread in anxiety and epilepsy (Lüddens *et al.*, 1995; Basile *et al.*, 2004). One of the most widely studied pathological conditions associated with $GABA_A$ receptors is epilepsy in which disruption or alteration of normal GABAergic synaptic transmission has been observed in a number of different experimental models (Kardos, 1999; Jones-Davis and Macdonald, 2003). Analysis of familial epilepsy has revealed mutations in $GABA_A$ receptor subunit genes which result in altered functional behaviour of these receptors (Bianchi *et al.*, 2002; Buhr *et al.*, 2002; Fisher, 2004; Macdonald *et al.*, 2004). $GABA_A$ receptors are also thought to play an important role in alcohol dependence (Young *et al.*, 2004) and alterations in the normal physiology of inhibitory synaptic transmission has been linked to schizophrenia (Lewis *et al.*, 2005), Alzheimer's disease (Armstrong *et al.*, 2003) and a number of anxiety disorders (Nemeroff, 2003; Basile *et al.*, 2004).

$GABA_A$ receptors have been demonstrated to be critical for the formation of network oscillations crucial to behaviour (Nusser *et al.*, 2001; Gaiarsa *et al.*, 2002). The function of $GABA_A$ receptors is critical to the development and maintenance of normal neuronal network activity. As a result it is of great importance to understand how $GABA_A$ receptor function is regulated within the cell. The cellular signalling mechanism of phosphorylation has been shown to modulate the function of many ligand-gated ion channels including the $GABA_A$ receptor. There is a substantial amount of evidence that phosphorylation can modulate $GABA_A$ receptor function in physiologically relevant ways, however, there is not yet a full understanding of the mechanisms and the significance of this form of cellular signalling and modification.

1.2 Phosphorylation of the $GABA_A$ receptor: recombinant systems.

The process of phosphorylation involves the covalent modification of receptor structure through the transfer of a highly charged phosphate group from adenosine triphosphate (ATP) to a serine, threonine or tyrosine residue within a substrate. This covalent modification is catalyzed by a series of enzymes known as protein kinases. The subsequent cleavage of the phosphate group from a substrate (dephosphorylation) is catalysed by protein phosphatases (Moss and Smart, 1996). The phosphorylation state of a given substrate is often bi-directionally regulated and the functional consequences are determined by a balance between kinase and phosphatase activities. The most common class of protein kinases are the serine/threonine second messenger dependent kinases such as cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). The second major class of

kinases are those that specifically phosphorylate tyrosine residues (Moss and Smart, 1996; Smart, 1997).

1.2.1 Phosphorylation *in vitro.*

Protein kinases catalyse phosphorylation acting at specific sites that can be recognised by the sequence surrounding the site of phosphorylation, which is often specific to a particular kinase. Consensus sites for a number of different kinases have been identified on $GABA_A$ receptor subunits, most commonly on the TM3-4 intracellular loop. However, consensus sites do not act as a guarantee that phosphorylation will actually occur in a living cell. Phosphorylation can occur at sites that do not conform to consensus sequences. As a result consensus sites can only be used as a rough guide for the potential targets and sites of phosphorylation (Moss and Smart, 1996; Sedelnikova and Weiss, 2002).

Consensus sites for a number of Ser/Thr kinases, such as PKA / PKC / cGMP-dependent protein kinase (PKG) and calcium/calmodulin dependent protein kinase II (CaMK-II) have been identified on the large intracellular loop between TM 3 and 4 in a range of $GABA_A$ receptor subunits (Moss and Smart, 1996). These sites, to date, have all been identified on the β and γ subunits. The intracellular domains of $GABA_A$ receptor subunits have been expressed as glutathione-S-transferase (GST) fusion proteins in *E-coli* which have been used to identify the individual sites of phosphorylation *in vitro* (See Table 1.1). The β1 fusion protein can be phosphorylated by PKC/PKG/PKA/CaMK-II at Ser^{409} (Moss *et al.*, 1992a; McDonald and Moss, 1994). The β2 fusion protein can be phosphorylated at the corresponding site, Ser^{410}, by the same kinases (Moss *et al.*, 1992a; McDonald and Moss, 1997) and the β3 fusion protein can be phosphorylated at Ser^{408} and Ser^{409} by

PKA/PKC/PKG/CaMK-II (McDonald and Moss, 1997). CaMK-II can also phosphorylate additional sites in the β1 and β3 fusion proteins at Ser^{384} and Ser^{383}, respectively (McDonald and Moss, 1994; McDonald and Moss, 1997). Both splice variants of γ2 can be phosphorylated by CaMK-II at Ser^{348} and Thr^{350}, and PKC can phosphorylate γ2S/L at Ser^{327} (Moss *et al.*, 1992a; McDonald and Moss, 1994). The γ2L subunit can also be phosphorylated by PKC and CaMK-II at Ser^{343} within the 8 amino acid insertion (Machu *et al.*, 1993; McDonald and Moss, 1994).

Table 1.1:

Subunit	Residue	Kinase	Reference
β1	Ser^{384} Ser^{409}	CaMK-II PKA/PKC/PKG/CaMK-II	McDonald and Moss (1994) Moss *et al.*, (1992)
β2	Ser^{410}	PKA/PKG/PKC/CaMK-II	Moss *et al.*, (1992)
β3	Ser^{383} Ser^{408} Ser^{409}	CaMK-II PKC PKA/PKG/PKC/CaMK-II	McDonald and Moss (1997)
γ2S	Ser^{327} Ser^{348} Thr^{350}	PKC CaMK-II CaMK-II	McDonald and Moss (1994)
γ2L	Ser^{327} Ser^{343} Ser^{348} Thr^{350}	PKC PKC/CaMK-II CaMK-II CaMK-II	McDonald and Moss (1994) Moss *et al.*, (1992) Machu *et al.*, (1993)

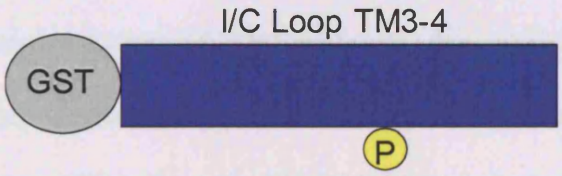


Table 1.1: A summary of the main sites of phosphorylation identified on the TM3-4 loop of the β and γ subunits of the $GABA_A$ receptor.

Each of these potential sites of phosphorylation was identified by expression of GST-fusion proteins of the TM3-4 intracellular loop for different $GABA_A$ receptor subunits and co-incubation, *in vitro,* with various protein kinases. Specific residues that were phosphorylated were identified by site-specific mutagenesis.

1.2.2 Phosphorylation of recombinant $GABA_A$ receptors: PKC

The study of phosphorylation in neurones has been hampered by the heterogeneity of $GABA_A$ receptors between cell types and within the same cell. As a result phosphorylation of $GABA_A$ receptors has been studied in a number of different recombinant systems to attempt to determine the functional consequence of phosphorylation of different receptor subunits. Transfection of relevant subunit cDNA's into a recombinant cell line with no endogenous $GABA_A$ receptor subunits allows the precise subunit combination of the receptor to be controlled within reasonable limits. However, even within recombinant systems there is a considerable degree of variation in the reported effects of phosphorylation (Song and Messing, 2005).

Phosphorylation of the recombinant subunit combination $\alpha1\beta2\gamma2S$ expressed in *Xenopus oocytes* by PKC has been reported to decrease the amplitude of whole-cell currents recorded. This modification was abolished by the site-specific mutation of the residues Ser^{410} site on $\beta2$ and Ser^{327} on the $\gamma2S$ subunit to alanine (Kellenberger *et al.*, 1992). Both sites were thought to be required for a full functional effect. A similar functional result of PKC activation on $\alpha1\beta2\gamma2S$ receptors expressed in *Xenopus oocytes* was also reported that was not mediated by phosphorylation of $\beta2$ Ser^{410}. In this case it was thought that PKC dependent phosphorylation resulted in a decrease in the number of $GABA_A$ receptors at the cell surface (Chapell *et al.*, 1998). PKC activity has also been reported to block receptor recycling of $\alpha1\beta2\gamma2$ receptors expressed in HEK293 cells back to the cell surface thereby reducing receptor number at the cell surface (Connolly *et al.*, 1999).

PKC dependent phosphorylation of $\alpha1\beta1\gamma2S$ expressed in HEK293 cells and in *Xenopus oocytes* results in a decrease of the amplitude of whole-cell currents

recorded that was found to be dependent on phosphorylation of Ser^{409} in the $\beta 1$ subunit, Ser^{327} in the $\gamma 2S$ subunit and Ser^{343} in $\gamma 2L$ (Leidenheimer *et al.*, 1992; Krishek *et al.*, 1994). However, phosphorylation of similar residues in the $\beta 1$ and $\gamma 2L$ subunits has also been shown to mediate a PKC dependent enhancement of the amplitude of whole-cell currents recorded from L929 fibroblasts transfected with $\alpha 1\beta 1\gamma 2L$ receptors (Lin *et al.*, 1994; Lin *et al.*, 1996).

The discrepancies between these different effects may be in part due to the mechanism of activation of PKC. It is difficult to rule out the possibility that activation through different mechanisms may activate different signalling pathways. It is always possible that another downstream kinase or signalling pathway is involved in, or masks an apparent effect of, phosphorylation. In addition, the cell type may alter the response to phosphorylation as different cell lines may contain different endogenous phosphatases and kinases that may alter the basal phosphorylation state of the receptor.

Many kinases have multiple substrates within a cell and play a role in many different cellular processes. There is increasing evidence that the localisation of a kinase, possibly through specific kinase anchoring proteins, has a profound effect on the functional effects of phosphorylation (Pawson and Scott, 1997). Some differences in the response to phosphorylation between cell lines may therefore reflect differences in the anchoring proteins present. The receptor for activated C kinase-1 (RACK-1) has been shown to facilitate phosphorylation and functional modulation of $GABA_A$ receptors in HEK293 cells. In this case PKC dependent phosphorylation of $\beta 1$ Ser^{409} resulted in a depression of whole-cell currents. Blocking the binding of RACK-1 to the $\beta 1$ subunit (with a dominant negative peptide) abolished this PKC dependent effect (Brandon *et al.*, 2002b).

1.2.3 Phosphorylation of recombinant $GABA_A$ receptors: PKA

One further factor that is important concerns the receptor subunit composition. PKA dependent phosphorylation of recombinant receptors has been demonstrated to be dependent on the β subunit present. Within HEK293 cells, PKA increased the amplitude of whole-cell currents recorded from $\alpha1\beta3\gamma2S$ receptors through phosphorylation of $Ser^{408,409}$. PKA dependent phosphorylation of $\beta1$ at Ser^{409} resulted in a depression of whole-cell currents recorded from HEK293 cells expressing $\alpha1\beta1\gamma2S$ (Moss *et al.*, 1992b). The mutation of Ser^{408} on the $\beta3$ subunit converted the observed enhancement into a depression similar to that seen for $\beta1$ as well as reproducing the phosphorylation profile of the $\beta1$ subunit. The $\beta2$ subunit-containing receptors were not functionally modulated at all by PKA in this study (McDonald *et al.*, 1998). Chronic activation of PKA in Cα12 fibroblast cells (L929 cell line over-expressing the active catalytic subunit of PKA) led to an enhancement of $\alpha1\beta1\gamma2S$, receptors, in contrast to the depression observed after acute activation of PKA (McDonald *et al.*, 1998). This enhancement was also dependent on Ser^{409} but in this case was also dependent on the presence of the $\gamma2S$ subunit (Angelotti *et al.*, 1993b). This implied that acute transient activation of a protein kinase may have a different functional effect to that of chronic activation. In addition over-expression of this catalytically active form of PKA (cAMP-independent) in this cell line could be expected to produce levels of PKA activity that far exceeded the levels reached using other protocols.

The effects of PKA dependent phosphorylation on desensitisation have also been studied in a recombinant system through rapid application of GABA to HEK293 cells expressing $\alpha1\beta1\gamma2L$ and $\alpha1\beta2\gamma2L$. The residues Ser^{408} in $\beta1$ and Ser^{409} in $\beta3$ were again identified as critical for PKA dependent modulation. In this

case PKA phosphorylation of both β1 and β3 resulted in alterations in desensitisation and deactivation that prolonged the duration of inhibitory postsynaptic current (IPSC)–like currents (Hinkle and Macdonald, 2003) suggesting that phosphorylation may be capable of modulating IPSC duration in neurones. The relatively less well studied protein kinase B (PKB/Akt), has also been demonstrated to phosphorylate the β2 residue Ser^{410} in HEK293 cells. This appeared to increase the number of $GABA_A$ receptors at the cell surface (Wang *et al.*, 2003b).

1.2.4 Phosphorylation of recombinant $GABA_A$ receptors: tyrosine kinases.

In addition to Ser/Thr kinases, tyrosine kinases are capable of phosphorylating and modulating the function of ligand-gated ion channels (Boxall and Lancaster, 1998). Tyrosine kinase phosphorylation of both β and γ subunits of $GABA_A$ receptors has been reported (Moss *et al.*, 1995; Valenzuela *et al.*, 1995; Wan *et al.*, 1997). Tyrosine phosphorylation of β subunits has been reported to increase the amplitude of whole-cell currents in HEK293 cells (Wan *et al.*, 1997). However, Moss *et al.* (1995) originally reported that the β subunit phosphorylation was functionally silent and that phosphorylation of $Tyr^{365:367}$ on the γ2L subunit alone was responsible for the increase in whole-cell currents. Valenzuela and colleagues (1995) determined that β2 and γ2 $GABA_A$ receptor subunits purified from bovine brain could be phosphorylated by Src as well as the intracellular loop of β1 and γ2L *in vitro*. They also observed in *Xenopus oocytes* expressing α1β1 and α1β1γ2L receptors an inhibition of GABA currents on bath application of genistein suggesting in this case β1 phosphorylation had a functional effect (Valenzuela *et al.*, 1995). However, genistein can have a direct effect on $GABA_A$ receptor function (Dunne *et al.*, 1998). Tyrosine phosphorylation may maintain $GABA_A$ receptor activity and a

loss of basal tyrosine kinase activity may be responsible for the phenomenon of "run-down" (Huang and Dillon, 1998).

The α subunit of the $GABA_A$ receptor is not thought to be a good substrate for phosphorylation. There is a lack of suitable consensus sites within the TM3-4 intracellular loop and the α1 GST-fusion protein cannot be phosphorylated *in vitro* (Moss *et al.*, 1992a; Moss and Smart, 1996). However, CaMK-II dependent phosphorylation of the α1 subunit has been reported using synaptosomal membrane fractions (Churn *et al.*, 2002). In addition, phosphorylation of the α1 TM3-4 intracellular loop, that supports $GABA_A$ receptor activity and prevents receptor run-down during electrophysiological recording, has been observed in acutely dissociated rat neocortical neurones (Laschet *et al.*, 2004).

1.3 Phosphorylation of the $GABA_A$ receptor: neuronal systems.

There is a wide range of evidence that phosphorylation of the $GABA_A$ receptor can modulate function in a neurone in a physiologically significant way. However, there has been a wide range of different functional effects reported. Part of this variation in response to phosphorylation between different cell-types and culture conditions may reflect differences in the $GABA_A$ receptor subunits that are expressed. However, even taking this into account there are still discrepancies between reported effects of phosphorylation in recombinant systems and neurones.

1.3.1 PKC dependent effects in neuronal preparations.

The effects of PKA and PKC dependent phosphorylation have been the most widely studied. Activation of PKC in cultured sympathetic neurones results in a decrease in whole-cell currents recorded, similar to that seen in HEK293 cells

expressing $\alpha 1\beta 1\gamma 2$ receptors (Krishek *et al.*, 1994). PKC activity has been reported to have different effects in different cell types. In CA1 pyramidal neurones, PKC was without effect (Poisbeau *et al.*, 1999); however in dentate gyrus granule cells, PKC increased the peak amplitude of spontaneous (s)IPSCs (Poisbeau *et al.*, 1999). Dentate gyrus cells express pre-dominantly $\beta 3$ subunits (Miralles *et al.*, 1999; Pirker *et al.*, 2000) and the increase in amplitude is consistent with $\beta 3$ subunit mediated increases in $GABA_A$ receptor function observed in other neuronal cell types (Jovanovic *et al.*, 2004). However, in cortical neurones, constitutive phosphorylation of $\beta 3$ $Ser^{408,409}$ by PKC has been reported to decrease the amplitude of whole-cell currents (Brandon *et al.*, 2000).

The anchoring protein RACK-1 has been shown to form complexes with PKC and $GABA_A$ receptors in the adult rat brain. In rat superior cervical ganglion neurones activation of PKC led to a decrease in the amplitude of GABA-mediated currents. Inclusion of a peptide that competes with RACK-1 for binding to the $\beta 1$ subunit reduced the effect of PKC on these $GABA_A$ receptors (Brandon *et al.*, 2002b). The absence or presence of RACK-1 or other similar proteins within a cell-type could significantly alter the apparent effect of phosphorylation and may explain some of the differences reported for PKC modulation within cells that express similar $GABA_A$ receptor subtypes.

Brain-Derived Neurotrophic factor (BDNF) can modulate $GABA_A$ receptor mediated mIPSCs through the activation of PKC, followed by protein phosphatase (PP2A) mediated dephosphorylation of the receptor. There was differential recruitment of PKC, RACK-1 and PP2A according to the phosphorylation state of the $\beta 3$ subunit. Phosphorylation of $\beta 3$ at $Ser^{408:409}$ was associated with an increase in

miniature (m)IPSC amplitude, with no change in the decay time (Jovanovic *et al.*, 2004).

Recombinant studies highlighted potential phosphorylation sites on the γ2 subunit for PKC at Ser^{327} and Ser^{343} within the eight amino acid insert that forms the γ2L splice variant. Within neurones the role of PKC phosphorylation of Ser^{327} is unknown. However, phosphorylation of Ser^{343} has been reported in neurones to facilitate the trafficking of γ2S to the synapse (Meier and Grantyn, 2004). Activation of PKC has also been reported to increase receptor internalisation in cultured cortical neurones and promotes receptor endocytosis in HEK293 cells. This appears to occur via a dynamin endocytic pathway that is dependent on a dileucine motif in the β2 subunit and also requires the presence of the γ2 subunit (Herring *et al.*, 2005).

1.3.2 PKA dependent effects in neuronal preparations.

Modulation of $GABA_A$ receptors by PKA in neurones has also revealed different functional effects in different cell-types (Porter *et al.*, 1990; Kano and Konnerth, 1992; Moss *et al.*, 1992b; Kapur and Macdonald, 1996). Again, some of this may be related to differential effects on different subunits. There is however, some discrepancy between reported effects in a recombinant system and that seen in neurones.

Inclusion of the catalytic domain of PKA in the patch pipette was reported to reduce the amplitude of whole-cell currents recorded from mouse spinal neurones. This reduction was reported to be mediated by a reduction in channel opening frequency (Porter *et al.*, 1990). It has also been reported that in CA1 hippocampal pyramidal cells PKA reduced the amplitudes of IPSCs and altered the fraction of events with a double exponential decay. However, in hippocampal dentate gyrus

granule cells PKA was without effect (Poisbeau *et al.*, 1999). In granule cells of the olfactory bulb which express predominantly the β3 subunit, constitutively-active PKA resulted in an increase in IPSC amplitudes with no change in decay (Nusser *et al.*, 1999). An increase in amplitude would correlate with the up-regulation of function seen in HEK293 cells expressing β3 subunit-containing receptors (McDonald *et al.*, 1998). The lack of a reported effect of PKA on dentate gyrus cells is surprising given this cell-type also expresses predominantly β3 subunit-containing receptors (Miralles *et al.*, 1999; Pirker *et al.*, 2000). However a PKA dependent increase in whole-cell currents recorded from hippocampal dentate granule cells has been reported by another group (Kapur and Macdonald, 1996). Therefore, even within the same cell type, variations in response to phosphorylation occurs possibly as a result of differences in tissue preparation or experimental method of kinase activation. Addition of PKA to cerebellar stellate and basket cells that express predominantly β2 subunit-containing receptors prolonged the time-course of IPSCs without changing the peak amplitude (Nusser *et al.*, 1999). This is in contrast to the lack of any functional modulation observed on β2 subunit-containing receptors in HEK293 cells (McDonald *et al.*, 1998).

Phosphorylation of β3 $Ser^{408,409}$ by PKA can be counteracted by protein phosphatase 1 (PP1). This interaction can also be regulated by additional binding proteins. For instance, phospholipase C (PLC)-related inactive protein type 1 (PRIP-1) binds and inactivates protein phosphatase (PP)1 allowing PKA phosphorylation to occur (Terunuma *et al.*, 2004). The A-kinase anchoring protein (AKAP) 79/150 has been identified which allows PKA to be selectively targeted to β1/3 subunit-containing receptors. This protein binds to β1 and β3 only and is critical for PKA mediated phosphorylation (Brandon *et al.*, 2003). PKA dependent effects on

$GABA_A$ receptors in neurones have been observed in cell-types thought to express predominantly $\beta 2$ subunit-containing receptors (Kano and Konnerth, 1992; Nusser *et al.*, 1999). It may be that other, as yet unidentified, kinase anchoring proteins exist that are able to allow PKA dependent phosphorylation of the $\beta 2$ subunit. Or alternatively, the activation of PKA or PKC in neurones might activate secondary pathways downstream or in parallel which influence $GABA_A$ receptor function. PKA or PKC might be able to phosphorylate proteins associated with the $GABA_A$ receptor which may have different effects to direct phosphorylation of the receptor itself. The considerable variation in response to activation of these kinases in neuronal systems implies that phosphorylation dependent modulation of $GABA_A$ receptors is complex and may well involve multiple kinase anchoring proteins and/or different mechanisms of phosphorylation mediated effects.

1.3.3 PKB and Src.

In addition to PKA and PKC the Ser/Thr kinase PKB has also been demonstrated to phosphorylate the $GABA_A$ receptor in neurones at Ser^{410} in the $\beta 2$ subunit. PKB activity leads to an increase in the number of functional $GABA_A$ receptors at the cell surface, increasing IPSC amplitudes with no change in decay times. The tyrosine kinase Src has also been demonstrated to alter $GABA_A$ receptor function in neurones. Src mediated phosphorylation of $Tyr^{365:367}$ on $\gamma 2L$ leads to an increase in the whole-cell GABA current amplitude recorded from superior cervical ganglion neurones in culture. Single channel recordings revealed an increase in mean open time and probability of opening of the channel after activation of Src (Moss *et al.*, 1995). Src is capable of interacting with β and $\gamma 2$ subunits. The

residues $Tyr^{365,367}$ on $\gamma 2$ have also been shown to be phosphorylated in the adult rat brain (Brandon *et al.*, 2001).

1.3.4 Mechanisms of phosphorylation dependent changes in receptor function.

The process of phosphorylation appears to be more complex than a simple covalent modification of receptor structure. There are a number of potential mechanisms for phosphorylation-dependent effects. The simplest mechanism is direct phosphorylation of the receptor followed by a structural change which alters the ion channel properties. For instance tyrosine kinase dependent changes in mean open time and probability of opening (Moss *et al.*, 1995). These types of changes could also alter the amplitude or decay phase of whole-cell currents or IPSCs. One particular mechanism that has been identified is phosphorylation-dependent modulation of desensitisation (Jones and Westbrook, 1996). This could alter the apparent decay times of IPSCs, a phenomenon that has been observed in response to phosphorylation. Phosphorylation dependent changes in desensitisation of the $GABA_A$ receptor have been demonstrated in response to application of the protein phosphatase calcineurin (Jones and Westbrook, 1997).

Another potential mechanism of phosphorylation-dependent changes is through modulation of receptor trafficking which could occur through direct phosphorylation of the receptor or indirectly through phosphorylation of other proteins involved in the correct targeting and insertion of receptors at the synapse. For instance, PKC can alter the trafficking of $GABA_A$ receptors by blocking receptor recycling to the cell surface thereby reducing the number of functional receptors at the plasma membrane (Connolly *et al.*, 1999). It has also been proposed that PKC activity can induce receptor internalisation through a dynamin-dependent endocytic

pathway (Herring *et al.*, 2005). In addition, PKC phosphorylation of Ser^{343} has been linked to the correct targeting of γ2L subunits to the synapse (Meier and Grantyn, 2004).

Phosphorylation dependent changes to receptor trafficking may also allow the insertion of new receptors in order to potentiate inhibitory synapses. There is evidence that PKB phosphorylation may function in this way (Wang *et al.*, 2003b). The modulation of excitatory AMPA receptors by phosphorylation has been shown to occur by both mechanisms (Lisman *et al.*, 2002). Phosphorylation can directly change the properties of the AMPA channel (Barria *et al.*, 1997; Benke *et al.*, 1998); however, synaptic plasticity driven by phosphorylation can result in the insertion of new AMPA receptors at the synapse (Hayashi *et al.*, 2000; Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Ju *et al.*, 2004).

1.3.5 Perturbation of normal phosphorylation pathways in disease states.

The abnormal function of protein kinases or perturbation of specific signalling pathways can be linked to particular disease states. Mice lacking the γ isoform of PKC have been shown to exhibit decreased anxiety (Bowers *et al.*, 2000). The mutant tottering (tg) mouse, displays seizures similar to human petit-mal epilepsy. It has been reported that there is an abnormal elevation of PKA activity in these mice and a reduction in $GABA_A$ receptor function, indicating that this may contribute to the induction of seizures (Tehrani and Barnes, 1995). Cell membranes isolated from patients with drug-resistant temporal lobe epilepsy were injected into *Xenopus oocytes* and the response of $GABA_A$ receptors to BDNF was determined. BDNF potentiated the epileptic $GABA_A$ receptors probably through activation of tyrosine kinase receptor B and both PLC and PKC. Recombinant $\alpha1\beta2\gamma2$ receptors

and $GABA_A$ receptors transplanted from a non-epileptic brain were unaffected by BDNF (Palma *et al.*, 2005). The onset of epilepsy appears to induce changes in the $GABA_A$ receptor in terms of its response to signalling pathways and phosphorylation.

1.4 Calcium/calmodulin-dependent protein kinase II: structure and function.

Calcium/calmodulin dependent protein kinase II is a serine/threonine 2nd messenger dependent protein kinase that responds to increases in intracellular Ca^{2+} concentration (Fink and Meyer, 2002; Hudmon and Schulman, 2002; Soderling *et al.*, 2002). It is a multifunctional protein that phosphorylates a large number of different substrates and is involved in many different cellular processes (Schulman, 2004; Colbran, 2004b). There are four main families of CaMK-II subunits (α, β, γ, δ) and around 28 different isoforms. The α and β subunits are most prominent in the brain where they are highly expressed (Hudmon and Schulman, 2002). CaMK-II is known to phosphorylate a number of proteins involved in synaptic transmission and is thought to play a key role in synaptic plasticity (Lisman *et al.*, 2002; Colbran and Brown, 2004).

1.4.1 CaMK-II structure and function: autophosphorylation at Thr^{286}.

CaMK-II has a unique complex structure containing self-regulatory properties. Each subunit consists of an N-terminal catalytic domain, a central regulatory domain (containing within it an auto-inhibitory domain), a Ca^{2+}/CaM binding motif and a C-terminal association domain (Shen and Meyer, 1998; Hudmon and Schulman, 2002; Lisman *et al.*, 2002). Subunits can associate together in groups

of 10-12 to form a holoenzyme arranged as two stacked rings (See Fig 1.3, 1.4) with the association domains in the centre and the catalytic domains facing outwards (Kolodziej *et al.*, 2000; Hoelz *et al.*, 2003).

In the absence of Ca^{2+}/CaM, CaMK-II remains relatively inactive. The auto-inhibitory domain (AID) acts as a pseudo-substrate which binds with the catalytic region of the same subunit acting as a gate that inhibits enzyme activity (Mukherji *et al.*, 1994; Mukherji and Soderling, 1994). The AID makes multiple interactions with the catalytic domain. Part of the AID occupies the substrate binding site or the S-site of the kinase whereas a more N-terminal portion of the AID makes an additional interaction on the C-terminal lobe of the catalytic domain (T-site). The interaction at the T-site is thought to induce conformational changes that interfere with ATP binding to the kinase. In this way the kinase remains inactive in the absence of Ca^{2+}/CaM (Hudmon and Schulman, 2002; Lisman *et al.*, 2002; Colbran, 2004b). The Ca^{2+}/CaM binding motif overlaps the AID, so Ca^{2+}/CaM binding is thought to disrupt the interaction between the AID and the catalytic domain allowing the kinase to become active (See Fig 1.4).

After binding of Ca^{2+}/CaM and subsequent activation of the kinase, autophosphorylation can occur at Thr^{286} (α-subunit, Thr^{287} - β-subunit) within the AID. This occurs between adjacent subunits that have bound Ca^{2+}/CaM (Hanson *et al.*, 1994). Autophosphorylation causes the dissociation rate for Ca^{2+}/CaM to fall significantly so that it is effectively bound to the kinase. Even after Ca^{2+}/CaM dissociates the kinase retains partial Ca^{2+}/CaM independent activity (Colbran *et al.*, 1988; Fong *et al.*, 1989), until it is dephosphorylated by a protein phosphatase (Hashimoto *et al.*, 1987). Autophosphorylation at Thr^{286} allows CaMK-II to remain persistently active after the initial stimulus and Ca^{2+} signal has dissipated. Thus, a

transient rise in Ca^{2+} can stimulate long-term kinase activation (See Fig 1.3 and 1.4). It also allows CaMK-II to respond to the frequency of Ca^{2+} oscillations (Hanson *et al.*, 1994; De Koninck and Schulman, 1998). The extent of CaMK-II activity is also dependent on the rate at which subunits are de-phosphorylated. Evidence suggests that in the PSD this dephosphorylation is primarily mediated by PP1 (Hudmon and Schulman, 2002; Lisman *et al.*, 2002).

Fig 1.3:

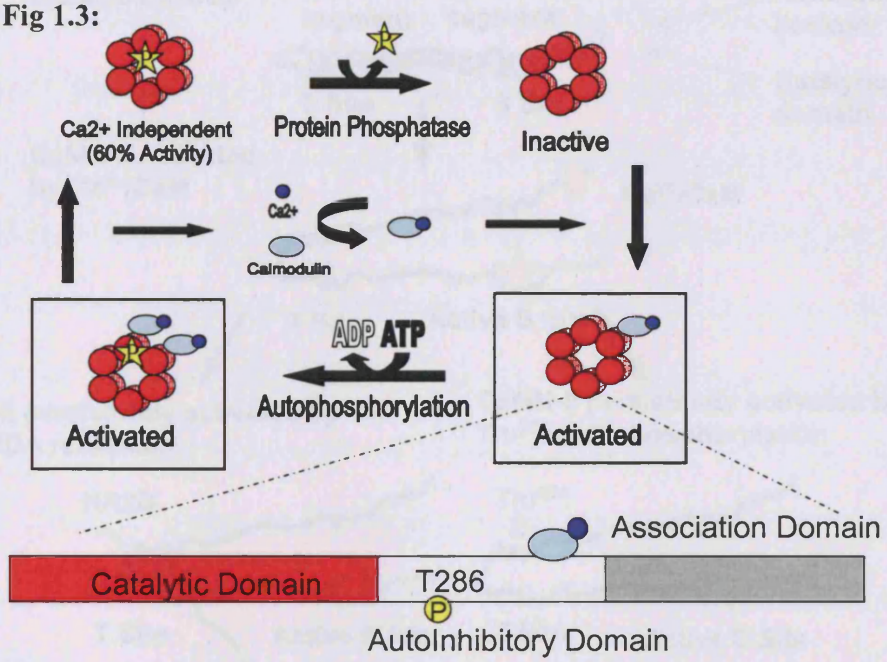


Fig 1.3: A schematic representation of the cycle of activation and deactivation of CaMK-II.

The binding of Ca^{2+}/CaM activates CaMK-II and enables it to phosphorylate substrates. It also allows autophosphorylation to occur at Thr^{286} between adjacent subunits that have bound Ca^{2+}/CaM.

Autophosphorylation reduces the rate of Ca^{2+}/CaM dissociation, trapping Ca^{2+}/CaM to the kinase. When Ca^{2+}/CaM eventually dissociates the kinase retains some Ca^{2+}/CaM independent activity until Thr^{286} is dephosphorylated by a protein phosphatase and the kinase becomes inactive again.

CaMK-II forms holoenzymes of 10-12 subunits. Each subunit consists of three functional domains, a catalytic domain, an autoinhibitory domain and an association domain. Thr^{286} autophosphorylation and Ca^{2+}/CaM binding occurs within the autoinhibitory domain.

Adapted from Molecular Biology of the Cell (1994), Garland Publishing.

Fig 1.4:

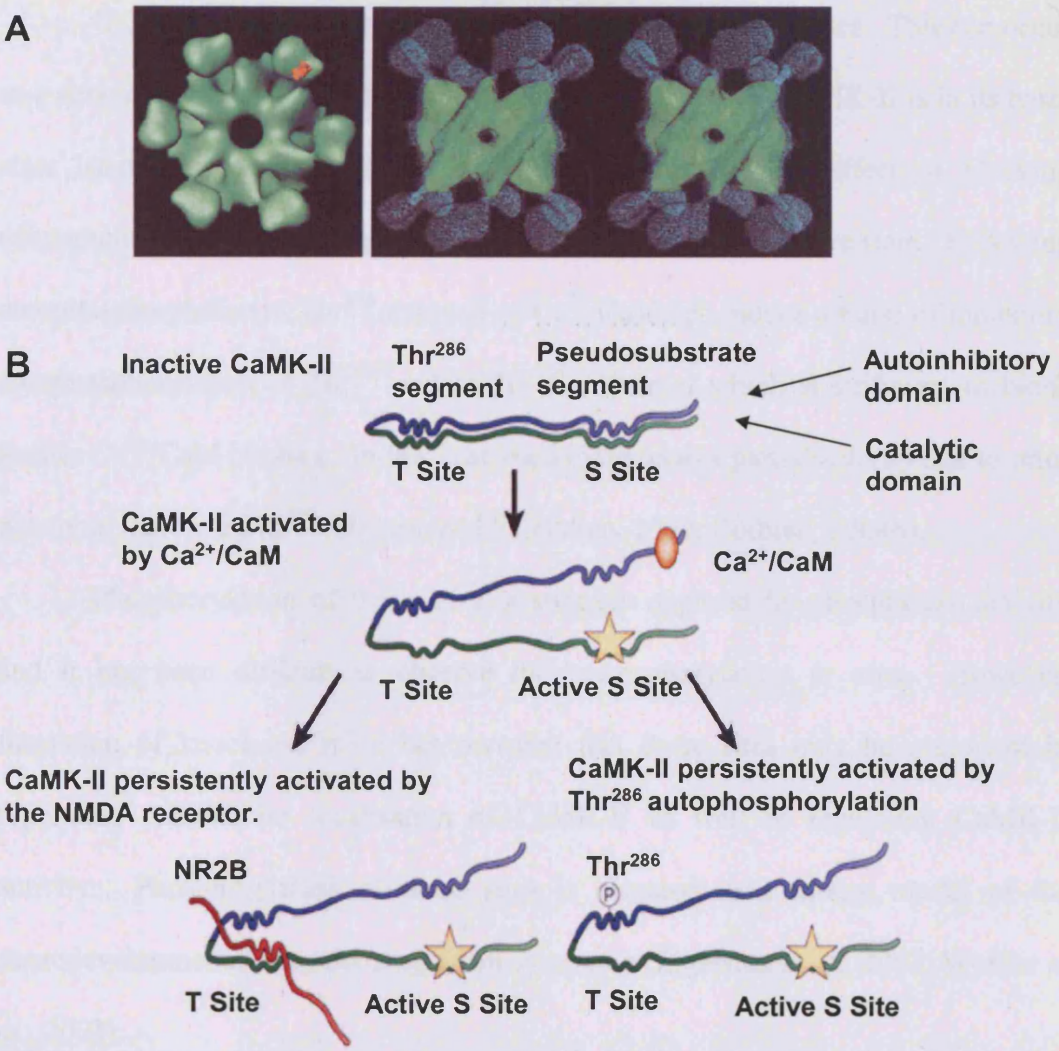


Fig 1.4: Proposed 3D structure of CaMK-II and schematic representation of the interaction between the catalytic and autoinhibitory domains.

(A) Three-dimensional structure of CaMK-II holoenzyme, showing a hexameric ring from a plan-view perspective.

(B) The autoinhibitory domain acts as a gate to regulate catalytic activity. The enzyme is inhibited when the autoinhibitory domain binds at the S and T sites. The binding of Ca^{2+}/CaM disrupts the association and the enzyme becomes active. A site on the NR2B subunit of the NMDA receptor can bind the T site, disrupting the association of the autoinhibitory domain and keeping the enzyme active in the absence of Ca^{2+}/CaM. Autophosphorylation at Thr^{286} is also sufficient to disrupt the association between the catalytic domain and the autoinhibitory domain and to allow CaMK-II to be active after the dissociation of Ca^{2+}/CaM.

Adapted from Lisman *et al.* (2002) and Kolodziej *et al.* (2000).

1.4.2 CaMK-II structure and function: autophosphorylation at $Thr^{305/6}$.

CaMK-II can also undergo autophosphorylation at other sites. This can occur as a slow intra-subunit autophosphorylation that occurs when CaMK-II is in its basal state leading to phosphorylation of $Thr^{305/6}$. This has the effect of blocking subsequent Ca^{2+}/CaM binding and so locks the kinase in an inactive state. Following autophosphorylation at Thr^{286} removal of Ca^{2+}/CaM can induce a burst of inhibitory autophosphorylation at Thr^{305} and/or Thr^{306}, either of which is sufficient to block further Ca^{2+}/CaM binding. In this case the kinase retains partial activity due to prior phsophorylation at Thr^{286} (Hanson and Schulman, 1992; Colbran, 2004b).

Phosphorylation of these sites is strongly opposed by phosphatase activity and it has been difficult to observe their phosphorylation *in vivo*. However, formation of knock-out mice has revealed that these sites may be important in regulating sub-cellular localisation of CaMK-II as well as regulating CaMK-II activity. Phosphorylation of these sites is elevated in a mouse model of the neurodevelopmental disorder Angelman syndrome (Elgersma *et al.*, 2002; Weeber *et al.*, 2003).

1.4.3 Regulation of CaMK-II by protein phosphatases.

It is important to consider how the activity of protein phosphatases regulates CaMK-II activity in the brain (Colbran, 2004a). It has been known for a number of years that PP1, PP2A and PP2C can dephosphorylate Thr^{286} and that in some cell types autophosphorylation of CaMK-II is strongly opposed by phosphatase activity (Fukunaga *et al.*, 1989; Molloy and Kennedy, 1991; Fukunaga *et al.*, 1993). It is thought that PP1 acts to dephosphorylate Thr^{286} at the PSD, whereas PP2A might dephosphorylate CaMK-II in the cytosol (Strack *et al.*, 1997). Therefore the

localisation of the kinase may alter which phosphatase is the more physiologically relevant for dephosphorylation (Strack *et al.*, 1997; Colbran, 2004a). The presence of phosphatases may alter CaMK-II function, as the presence of PP1 results in an ultra-sensitive kinase activation within a tighter range of Ca^{2+} concentrations than that observed for the kinase alone (Bradshaw *et al.*, 2003). The opposing actions of CaMK-II and PP1 have been suggested to be such that the CaMK-II/PP1 system could function as an energy efficient bi-stable switch (Lisman and Zhabotinsky, 2001). Phosphatase activity against CaMK-II has also been shown to be physiologically and pathologically relevant in that PP1 activity modulates LTP induction (Brown *et al.*, 2000) and decreased PP1/PP2A activity has been shown to be involved in the alterations in CaMK-II inhibitory autophosphorylation associated with the mouse model of Angelman syndrome (Weeber *et al.*, 2003; Elgersma *et al.*, 2004).

The binding of Ca^{2+} to CaM and recruitment of the Ca^{2+}/CaM complex to a CaMK-II enzyme is not simply a diffusion of Ca^{2+} and CaM in solution but is a regulated process in itself involving multiple cellular proteins and processes. Ca^{2+} influx to a cell can occur via voltage-gated Ca^{2+} channels or ligand-gated channels (e.g., NMDA receptors). Within the cell release of Ca^{2+} in stores can also provide a stimulus to activate CaMK-II. There are a large number of proteins present in neurones that can bind CaM. Increases in Ca^{2+} cause the release of this stored CaM and phosphorylation of some CaM binding proteins can cause CaM to dissociate, allowing it to participate in other signalling events (Gnegy, 2000; Meldolesi, 2001; Xia and Storm, 2005). As well as activating CaMK-II, CaM is involved in several other cellular signalling cascades and can have direct effects on ligand-gated ion channel function. For example, CaM has been shown to bind to the NR1 subunit of

the NMDA receptor and cause a four-fold reduction in channel open probability (Ehlers *et al.*, 1996; Zhang *et al.*, 1998; Rycroft and Gibb, 2002). It can also activate the phosphatase calcineurin (PP2B). PP2B is not thought to directly dephosphorylate Thr^{286} like PP1/PP2A but could act indirectly through dephosphorylation of DARPP-32 which inhibits PP1 activity. Therefore PP2B activity against DARPP-32 might indirectly increase PP1 activity against CaMK-II (Kawaguchi and Hirano, 2002; Svenningsson *et al.*, 2004; Colbran, 2004a). In addition, activation of PP2B/calcineurin is thought to be involved in some forms of LTD at both excitatory and inhibitory synapses (Winder and Sweatt, 2001; Wang *et al.*, 2003a).

1.5 CaMK-II localisation and role in synaptic plasticity.

CaMK-II is an abundant enzyme in the nervous system with multiple substrates that responds to changes in intracellular Ca^{2+} (Bayer and Schulman, 2001; Hudmon and Schulman, 2002; Griffith *et al.*, 2003; Colbran, 2004b). Calcium itself is a ubiquitous signalling molecule which plays a role in multiple cellular processes. Ca^{2+} signalling is tightly controlled both temporally and spatially (Berridge *et al.*, 1998). Increases in Ca^{2+} usually occur within discrete microdomains, small local regions of Ca^{2+} influx and localisation of Ca^{2+}-sensitive effector proteins to these regions of Ca^{2+} influx is often crucial for the proper function of Ca^{2+}-dependent signalling pathways (Franks and Sejnowski, 2002; Augustine *et al.*, 2003). The localisation of CaMK-II to sites of action and an ability to translocate on stimulation to specific sites of action is thought to be crucial for the proper function of the kinase (Bayer and Schulman, 2001; Griffith *et al.*, 2003; Schulman, 2004; Colbran, 2004b).

1.5.1 The role of α and β subunits of CaMK-II.

Examination of different forms of CaMK-II has revealed the importance of the different subunits of CaMK-II. The α and β isoforms can exist as homomers or can form heteromers with varying amounts of α : β subunits. Within the cerebellum there is a high ratio of β-CaMK-II to α-CaMK-II but within the forebrain α-CaMK-II is dominant (Miller and Kennedy, 1985). The physical properties of these two isoforms of CaMK-II are identical in that homomers of α or β subunits possess the same substrate specificity. However, β-CaMK-II appears to be more sensitive to Ca^{2+} and becomes autophosphorylated at Thr^{287} at much lower Ca^{2+}/CaM levels (Miller and Kennedy, 1985). Half-maximal autophosphorylation of the α subunit occurs at 130 nM CaM whereas the same level of autophosphorylation of β subunits occurs at 15 nM CaM (Brocke *et al.*, 1999).

The β subunit is expressed earlier in development, within the embryo, whereas the α subunit is only expressed postnatally and the ratio of α to β increases throughout synaptogenesis (Burgin *et al.*, 1990; Vallano, 1990; Griffith *et al.*, 2003). The localisation of α and β subunits also appears to differ and it is thought that the proportion of α to β subunits within a heteromer may determine kinase localisation. The α subunit is often found to be cytosolic whereas the β subunit is thought to be more closely localised to the cytoskeleton. The β subunit can bind F-actin whereas the α subunit does not and the presence of a small number of β subunits is enough to localise CaMK-II to the cytoskeleton (Shen *et al.*, 1998; Bayer and Schulman, 2001; Fink *et al.*, 2003; Griffith *et al.*, 2003). The mRNA for α-CaMK-II but not β-CaMK-II can be found at dendrites and an increase in mRNA and *de novo* synthesis of this subunit has been reported in response to synaptic plasticity (Ouyang *et al.*, 1997; Ouyang *et al.*, 1999).

The proportion of α to β subunits is known to change in response to stimulation. The α-subunit increases in expression during increased activity and the β-subunit increases in expression during decreased activity. Over-expression of α-CaMK-II or β-CaMK-II can have opposite effects on synaptic strength. Over-expression of α-CaMK-II leads to an increase in miniature excitatory postsynaptic current (mEPSC) amplitudes and a lengthening of decay times and over-expression of the β-subunit has no effect on amplitude whilst decay time is reduced. Overall, increased expression of α-CaMK-II increased charge transfer of mEPSCs whilst over-expression of β-CaMK-II decreased charge transfer (Thiagarajan *et al.*, 2002). Despite similar substrate specificity (Miller and Kennedy, 1985), α and β isoforms in heteromers may alter cellular localisation and translocation rates (Griffith *et al.*, 2003; Colbran, 2004b) and so differences in the relative access to specific substrates may mediate this differential effect on EPSC synaptic strength. Therefore the ratio of α to β subunits within a cell can play an important role in synaptic transmission and plasticity. In addition, the β subunit appears to be involved in morphological and structural changes associated with synaptogenesis in line with its close association with the cytoskeleton, whereas the α subunit has no morphogenic activity (Fink *et al.*, 2003).

1.5.2 Translocation of CaMK-II to the PSD.

CaMK-II is capable of rapid translocation in response to a stimulus in time scales of seconds / minutes (Gleason *et al.*, 2003). Expression of GFP-tagged α-CaMK-II and β-CaMK-II has revealed that these subunits differ in terms of their translocation in response to a stimulus. NMDA receptor stimulation can cause reversible CaMK-II translocation from an F-actin bound to a postsynaptic density

(PSD) bound state. The time taken for translocation to occur is controlled by the ratio of α : β subunits expressed. F-actin dissociation, PSD translocation and localisation at the PSD can be controlled by Ca^{2+}/CaM binding and autophosphorylation (Shen and Meyer, 1999). Early reports estimated that CaMK-II constituted 20-50 % of the total protein isolated in PSDs. Subsequently it has become clear that in some cases there can be a post-mortem accumulation of CaMK-II at the PSD (Suzuki *et al.*, 1994; Lengyel *et al.*, 2001). In addition, in certain pathological conditions such as during ischemic episodes CaMK-II can translocate and form clusters of CaMK-II within the cytoplasm (Dosemeci *et al.*, 2000; Tao-Cheng *et al.*, 2002).

Association of CaMK-II with the PSD can be dynamically regulated in an activity-dependent way (Griffith *et al.*, 2003; Schulman, 2004; Colbran, 2004b). The α subunit of CaMK-II appears to be cytosolic but can co-localise with the PSD in puncta within 20 s of glutamate addition (Shen and Meyer, 1999). The β subunit can associate with F-actin under basal conditions, but after stimulation slowly dissociates and accumulates at the PSD (Shen and Meyer, 1999). Autophosphorylation at Thr^{286} stabilises the association of CaMK-II with the PSD and PP1 activity encourages dissociation (Strack *et al.*, 1997; Shen and Meyer, 1999; Shen *et al.*, 2000). Autophosphorylation at Thr^{305} and/or Thr^{306} also modulates the interaction of CaMK-II with the PSD. It appears that phosphorylation of these sites leads to CaMK-II dissociation from the PSD (Shen *et al.*, 2000; Elgersma *et al.*, 2002; Weeber *et al.*, 2003).

1.5.3 CaMK-II targeting and/or anchoring proteins.

The importance of kinase anchoring proteins in localising a kinase to its substrate and controlling phosphorylation-dependent signalling has been revealed in recent years (Pawson and Scott, 1997). As a result there has been a great deal of interest in identifying potential CaMK-II anchoring proteins. A number of proteins located at the synapse have been found to bind CaMK-II and may play a role in localistion of the kinase. In some cases these interactions can alter the function of the kinase itself (Bayer and Schulman, 2001; Colbran, 2004b).

The NMDA receptor is capable of binding to CaMK-II (See Fig 1.4). This association is thought to localise CaMK-II to the site of Ca^{2+} entry. In addition, the association of CaMK-II with the NR2B subunit may alter the function of the kinase itself (See Fig 1.4) acting to disrupt the AID and allow Ca^{2+}-independent activity (Strack *et al.*, 2000a; Bayer *et al.*, 2001).

As well as a known association between F-actin and β-CaMK-II (Shen *et al.*, 1998) both α and β CaMK-II can interact with α-actinin at a region within the catalytic domain (Walikonis *et al.*, 2001; Dhavan *et al.*, 2002). CaMK-II also interacts with another synaptic protein, densin-180, which can also interact with α-actinin, suggesting a possible ternary complex at the synapse (Walikonis *et al.*, 2001). α-actinin can bind a number of receptors and ion channels and so this association may act to localise CaMK-II and target its activities to specific sites. Densin-180 is capable of directing CaMK-II localisation within HEK293 cells and α-CaMK-II and densin-180 can be co-immunoprecipitated from brain extracts (Strack *et al.*, 2000b; Walikonis *et al.*, 2001). As densin-180 can interact with other synaptic proteins it is also a strong candidate for a protein that can determine CaMK-II localisation and therefore function (Colbran, 2004b).

Another identified CaMK-II binding protein is a membrane associated guanylate kinase (MAGUK) found in *Drosophila* called Camguk. Camguk is similar to the mammalian CASK protein which is part of a subfamily of MAGUKs which are defined by an N-terminal CaMK-II like domain. In mammalian neurones, CASK has been shown to be localised at synapses both pre- and postsynaptically (Hata *et al.*, 1996). In *Drosophila*, Camguk, associates with CaMK-II in the absence of Ca^{2+}/CaM and Thr^{286} autophosphorylation and promotes phosphorylation of $Thr^{305/6}$ and subsequent inactivation of the kinase. In the presence of Ca^{2+}/CaM, CaMK-II associated with camguk can autophosphorylate at Thr^{286} and became constitutively-active. Camguk in the presence of Ca^{2+}/CaM provides a local source of active CaMK-II but when synaptic activity is low and levels of Ca^{2+}/CaM are low, Camguk promotes autophosphorylation at inhibitory sites that then requires phosphatase activity to dephosphorylate these inhibitory phosphorylation sites and allow subsequent activation of the kinase (Griffith *et al.*, 2003; Lu *et al.*, 2003). This kind of interaction may provide a mechanism for the local control of CaMK-II activity at active and inactive synapses.

1.5.4 CaMK-II and synaptic plasticity.

The most well studied role for CaMK-II at the synapse is in the induction of long-term potentiation (LTP) of AMPA receptor-mediated synaptic currents. This is a process by which a repetitive stimuli at a synapse leads to an increase in the strength of AMPA receptor mediated transmission at that synapse for hours after the initial stimulus has dissipated (Lisman *et al.*, 2002; Rongo, 2002; Collingridge *et al.*, 2004). CaMK-II activity is now known to be crucial for this process to occur. For a number of years the ability of CaMK-II to maintain activity after a Ca^{2+} stimulus

through autophosphorylation at Thr^{286} led to the hypothesis that CaMK-II was a "memory molecule" capable of maintaining long-term changes in synaptic function. CaMK-II was found to phosphorylate the AMPA receptor directly at Ser^{831} on the GluR1 subunit (Barria *et al.*, 1997; Derkach *et al.*, 1999; Lee *et al.*, 2000). This phosphorylation led to an up-regulation of receptor function through a change in the properties of the channel, by increasing the single channel conductance (Benke *et al.*, 1998; Derkach *et al.*, 1999).

Recently it has been demonstrated that CaMK-II dependent changes associated with LTP can also occur through another separate mechanism. CaMK-II dependent activation of a Ras/Rap GTPase pathway (Zhu *et al.*, 2002) and a subsequent insertion of receptors (Lledo *et al.*, 1998) into the synapse is thought to up-regulate AMPA receptor function during LTP. This process does not require direct phosphorylation of the AMPA receptor (Hayashi *et al.*, 2000). However, receptor insertion is thought to be subunit dependent as GluR1/4 subunits are thought to be inserted into the membrane during LTP and this process is dependent on the GluR1 subunit, whereas the maintenance of receptor number through recycling is thought to involve GluR2/3 subunits (Shi *et al.*, 2001; Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Ju *et al.*, 2004). In this way receptor number is increased and then maintained (See Fig 1.5).

1.5.5 CaMK-II, LTP and behaviour.

It is clear that CaMK-II is crucial for certain forms of LTP and that disruption of CaMK-II and subsequently LTP prevents proper memory formation and alters animal behaviour (Lisman *et al.*, 2002; Elgersma *et al.*, 2004; Ehrlich and Malinow, 2004). Mice devoid of α-CaMK-II have been generated which revealed the

importance of this subunit for the generation of NMDA receptor-dependent LTP at the CA1 pyramidal neurone in the hippocampus. In addition these mice exhibited specific spatial learning impairments strengthening the view that LTP forms the cellular basis of spatial learning (Silva *et al.*, 1992a; Silva *et al.*, 1992b). In T286A α-CaMK-II mutant mice, in which the α subunit cannot undergo autophosphorylation and therefore display Ca^{2+}-independent activity, CA1-LTP is completely absent (Giese *et al.*, 1998). These mice also displayed deficits in spatial learning. This illustrated the importance of autophosphorylation for LTP and the subsequent effects on physiology and behaviour.

Within mice which contain the α-CaMK-II T305D mutation (mimicking inhibitory autophosphorylation), CaMK-II activation and autophosphorylation at Thr^{286} is blocked as is subsequent translocation to the PSD (Hanson and Schulman, 1992; Elgersma *et al.*, 2002). In this case a stimulus which has no effect in wild-type mice can induce LTD. Mice which express α-CaMK-II which cannot be phosphorylated at $Thr^{305,306}$; show a marked LTP in response to the same stimulus demonstrating that these sites are important in the regulation of CaMK-II activity and can control the threshold for LTP induction (Elgersma *et al.*, 2002). Alteration in LTP seems to correlate with cognitive impairment. For instance T305D and T286D mice, which lack any LTP at CA1 synapses and show no evidence of β-CaMK-II compensation, have severely impaired spatial learning (Elgersma *et al.*, 2002). A reduction in the level of CaMK-II at the PSD, and in CaMK-II activity as well as impairment of LTP and deficits in context-dependent learning, have been observed within a mouse model of Angelman syndrome (Weeber *et al.*, 2003). The human form of this disease is characterised by severe mental retardation and epilepsy. This forms a clear link between alterations in the normal function of CaMK-II, changes in

hippocampal LTP and subsequent alterations in memory learning and behaviour (Elgersma *et al.*, 2004).

1.6 CaMK-II and $GABA_A$ receptor function.

1.6.1 Ca^{2+}-dependent depression of $GABA_A$ receptor function.

There have been a number of studies that have demonstrated Ca^{2+} dependent modulation of $GABA_A$ receptor function. Increases in intracellular calcium levels have been reported to depress GABA currents in sensory neurones of the bullfrog. In this case, Ca^{2+} influx led to a rapid decrease in the affinity of the $GABA_A$ receptor to GABA (Inoue *et al.*, 1986). In CA1 hippocampal pyramidal cells Ca^{2+} entry through NMDA receptors was reported to depress the amplitude of whole-cell currents. This effect was prevented by the addition of a specific protein phosphatase 2B (PP2B) / Calcineurin (CaN) inhibitor (Stelzer and Shi, 1994). Ca^{2+}-dependent phosphatases have been reported to depress $GABA_A$ receptor currents in a number of different cell-types (Akopian *et al.*, 1998; Huang and Dillon, 1998; Mozrzymas and Cherubini, 1998; Wang *et al.*, 2003a; Sanchez *et al.*, 2005). Calcineurin has also been reported to alter desensitisation, acting to alter the decay phase of IPSCs (Martina *et al.*, 1996; Jones and Westbrook, 1997; Mozrzymas and Cherubini, 1998; Sanchez *et al.*, 2005) as well as playing a pivotal role in the process of long-term depression (LTD) of CA1 inhibitory synapses in the hippocampus (Wang *et al.*, 2003a).

1.6.2 Bidirectional plasticity.

At excitatory synapses long-term plasticity is thought to be mediated through phosphorylation (Soderling and Derkach, 2000; Lee *et al.*, 2000; Lisman *et al.*, 2002; Collingridge *et al.*, 2004). The protein phosphatase, CaN, and the protein kinase,

CaMK-II, may act in opposition to each other in response to Ca^{2+} signals that vary in magnitude (Yang *et al.*, 1999; Cormier *et al.*, 2001; Winder and Sweatt, 2001; Collingridge *et al.*, 2004; Xia and Storm, 2005). It is possible that a similar situation exists at inhibitory synapses. Indeed it has been demonstrated that long-term plasticity at inhibitory synapses can be bi-directional (potentiation or depression) and that this is also mediated by Ca^{2+} signals of different magnitudes (McLean *et al.*, 1996; Aizenman *et al.*, 1998).

1.6.3 CaMK-II mediated potentiation of $GABA_A$ receptor function.

As well as depression in response to Ca^{2+} influx there are also a number of studies which have identified up-regulation of $GABA_A$ receptor function which is dependent on Ca^{2+} (See Table 1.2). For instance, Aguayo *et al.* (1998) demonstrated that increases in intracellular Ca^{2+} in mouse cortical neurones could induce a transient increase in the amplitude of whole-cell $GABA_A$ receptor-mediated currents. The addition of CaM and CaMK-II specific inhibitors blocked this potentiating effect suggesting that CaMK-II was a necessary part of the signal cascade.

There are several studies which have reported a CaMK-II dependent modulation of $GABA_A$ receptor function. Wang *et al.* (1995) intracellularly-applied pre-activated (autophosphorylated at Thr^{286}) α-CaMK-II to acutely isolated rat spinal dorsal horn neurones and observed a significant increase in the peak amplitude of whole-cell currents induced by a fixed dose of GABA. They found a similar effect when recording IPSCs from CA1 neurones in a hippocampal slice preparation. As well as an increase in amplitude they also reported a slight change in desensitisation acting to prolong the decay of the currents they recorded. A Ca^{2+} dependent increase in the decay of IPSCs has also been reported in response to amputation of the

dendrites of dentate gyrus granule cells in central neurones (Soltesz and Mody, 1995).

Churn *et al.* (1998) examined how CaMK-II activity towards endogenous substrates in a synaptosomal membrane fraction altered $GABA_A$ receptor agonist binding. Activation of endogenous CaMK-II led to an increase in muscimol binding, without altering binding affinity. The results suggested that the increase in binding was due to an increase in the number of functional $GABA_A$ receptors (Churn and DeLorenzo, 1998). In an additional study they used a similar protocol to demonstrate that CaMK-II activity increased allosteric-modulator binding (Churn *et al.*, 2002). Again this was thought to be due to an increase in the apparent number of receptors available for binding. In this study, CaMK-II was reported to phosphorylate the α1 subunit (Churn *et al.*, 2002).

Alix *et al.* (2002) characterised a Ca^{2+} sensitive GABA mediated chloride (Cl^-) current in cockroach dorsal unpaired median neurones. This current could be potentiated by increases in intracellular Ca^{2+} and the potentiation could be blocked by addition of specific CaMK-II and CaM inhibitors (KN-62 and W7 respectively). Insect GABA-stimulated Cl^- channels are pharmacologically and structurally distinct from vertebrate $GABA_A$ receptors (Alix *et al.*, 2002), but it appears they may be regulated by phosphorylation in a similar way to the vertebrate $GABA_A$ receptors.

During the development of the hippocampus BDNF has been reported to have a potentiating action on GABA currents recorded from CA1 pyramidal neurones isolated from P6 rats. This was prevented by the addition of the CaMK-II inhibitor KN-62 (Mizoguchi *et al.*, 2003). Potentiation of GABA currents has also been reported after mechanical injury of cortical neurones. This potentiation took the

form of an increase in peak current density (pA/pF) that could be blocked by the CaMK-II inhibitor KN-93 (Kao *et al.*, 2004).

It appears possible that $GABA_A$ receptors can be modulated by Ca^{2+}-dependent phosphorylation processes and that in some cases Ca^{2+}-dependent activation of CaMK-II results in a potentiation of $GABA_A$ receptor mediated currents. CaMK-II dependent phosphorylation is critical for excitatory synaptic plasticity, like long-term potentiation (LTP). There is also evidence that CaMK-II may also play a role in inhibitory synaptic plasticity.

Table 1.2:

Cell-Type	Method	Response	Reference
Rat dorsal horn neurones Hippocampal CA1 pyramidal	i/c pre-activated α-CaMK-II	↑ Peak w-c amplitude ↓Desensitisation ↑ IPSC amplitude	Wang *et al.*, (1995)
Mouse cortical neurones.	↑ i/c Ca^{2+}	↑ Peak w-c amplitude Blocked by KN-93	Aguayo *et al.*, (1998)
Rat forebrain synaptosomal membrane fractions.	i/c Ca^{2+}/CaM i/c α-CaMK-II	↑ Agonist binding ↑ Allosteric modulator binding α1 subunit phosphorylation	Churn and DeLorenzo (1998) Churn *et al.*, (2002)
Cerebellar purkinje neurones	↑i/c Ca^{2+} via VGCCs i/c pre-activated α-CaMK-II	↑ Peak w-c Amplitude ↑ sIPSCs amplitude No change in decay. (Rebound Potentiation)	Kano *et al.*, (1996)
Hippocampal CA1 pyramidal neurones	BDNF application	↑ Peak w-c Amplitude (P6 not P14) Blocked by BAPTA and KN-62	Mizoguchi *et al.*, (2003)
Cockroach dorsal unpaired median neurones	↑ i/c Ca^{2+} via depolarisation	↑ w-c amplitude Blocked by calmodulin blocker (W7) and KN-62	Alix *et al.*, (2002)
CA1 hippocampal neurones	↑ Ca^{2+}/CaM	↑ IPSC amplitude. Blocked by CaMK autoinhibitory peptide.	Wei *et al.*, (2004)

Table 1.2: A summary of the published evidence for CaMK-II modulation of the $GABA_A$ receptor.

In a number of neuronal preparations increases in Ca^{2+}/CaM and activation of CaMK-II or inclusion of exogenous pre-activated CaMK-II in the patch pipette has been demonstrated to up-regulate $GABA_A$ receptor function.

i/c = intracellular; w-c = whole-cell GABA current.

1.7 Inhibitory Synaptic Plasticity

A number of Ca^{2+} dependent forms of inhibitory synaptic plasticity have been reported (Komatsu and Iwakiri, 1993; Komatsu, 1994; McLean *et al.*, 1996; Caillard *et al.*, 1999; Gaiarsa *et al.*, 2002; Patenaude *et al.*, 2003; Bauer and LeDoux, 2004). The mechanisms behind plasticity vary with some reports of a presynaptic effect on frequency (Caillard *et al.*, 1999; Ouardouz and Sastry, 2000) and others suggesting a purely postsynaptic effect mediated by an up-regulation of $GABA_A$ receptor function (Patenaude *et al.*, 2003; Bauer and LeDoux, 2004).

1.7.1 Activity and development dependent plasticity.

One of the first forms of inhibitory plasticity to be identified occurs within the visual cortex. High frequency stimulation could induce a long-term increase in the amplitude of IPSPs recorded. This form of LTP of inhibitory synapses shared many of the features of excitatory LTP recorded from CA1 hippocampal neurones (Komatsu and Iwakiri, 1993; Komatsu, 1994). One of the interesting features of this form of plasticity was that LTP was more easily inducible in developing rats rather than mature. A similar form of activity-dependent plasticity of IPSCs mediated by $GABA_A$ receptors has been reported in rat hippocampal slices. Stimulation induced a long-lasting change in both the frequency and amplitude of IPSCs recorded, however, this plasticity could only be induced within a restricted period of development (Gubellini *et al.*, 2001).

Some of these reported forms of inhibitory plasticity are bi-directional (McLean *et al.*, 1996; Aizenman *et al.*, 1998) and rely on differences in magnitude of Ca^{2+} signals in a similar way to the plasticity of excitatory synapses (Yang *et al.*, 1999; Cormier *et al.*, 2001). In the same way that CaMK-II and CaN can bi-

directionally regulate excitatory synaptic transmission it has been proposed that CaMK-II may potentiate inhibitory synapses and CaN may depress them (Morishita and Sastry, 1996; Wang *et al.*, 2003a; Sanchez *et al.*, 2005). Aizenman *et al.* (1998) found that at GABAergic synapses onto deep cerebellar nuclei neurones, a long-term potentiation of IPSC amplitudes could be induced by a strong stimulation and large Ca^{2+} influx into the postsynaptic cell whereas a more moderate stimulation and Ca^{2+} influx resulted in a long-term depression of the IPSC amplitudes.

In neurones of the deep cerebellar nuclei (DCN) long-term potentiation (LTP) of inhibitory $GABA_A$ receptor mediated currents can be evoked by high frequency stimulation of the white matter surrounding the DCN. This potentiation is dependent on NMDA receptor activation and Ca^{2+} influx and is blocked by an NMDA receptor antagonist (APV) and the Ca^{2+} chelator BAPTA, suggesting that Ca^{2+} influx via NMDA receptors stimulated the potentiation. Intracellular application of tetanus toxin, a blocker of exocytosis, also prevented the induction of LTP of inhibitory currents. Suggesting a role for exocytosis and receptor trafficking in this form of Ca^{2+} stimulated inhibitory plasticity (Ouardouz and Sastry, 2000).

Another form of long-term inhibitory plasticity has been identified at inhibitory synapses onto CA1 hippocampal pyramidal neurones (Wei *et al.*, 2004). In this case a Ca^{2+}/CaM dependent increase in the amplitude of IPSCs was prevented by postsynaptic dialysis of an auto-inhibitory peptide of CaM-dependent kinases, suggesting the enhancement observed was CaMK-II dependent. In addition, inhibition of tubulin or actin polymerization attenuated this effect implying an intact cytoskeleton was required for this to occur.

Modelling and functional studies of GABAergic synapses suggests that if GABA is saturating at a given synapse, the most effective mechanism to potentiate

GABA mediated currents would be to increase receptor number at the synapse, as alterations in transmitter concentration and the probability of channel opening would have no impact on the amplitude of the inhibitory currents (Otis *et al.*, 1994; Nusser *et al.*, 1998). In this regard, it is now thought AMPA receptor insertion may be the predominant mechanism operating during excitatory LTP (Hayashi *et al.*, 2000; Zhu *et al.*, 2002).

Experimental models of epilepsy in which chronic epilepsy is induced through a process known as kindling have revealed inhibitory synaptic plasticity in response to the induction of epilepsy. This increase in inhibitory synaptic transmission is thought to be associated with an increase in the number of $GABA_A$ receptors at inhibitory synapses (Otis *et al.*, 1994; Nusser *et al.*, 1998). These kinds of alterations in inhibitory synaptic transmission have been suggested to function as homeostatic plasticity (Turrigiano and Nelson, 2004). Blockade of activity in cortical neuronal cultures by TTX leads to a decrease in the amplitude of IPSCs recorded. This is also accompanied by a reduction in the number of channels at the cell surface (Kilman *et al.*, 2002). A similar treatment caused an increase in the amplitude of mEPSCs, suggesting that the balance between excitatory and inhibitory synaptic currents could be dynamically modulated (Turrigiano *et al.*, 1998).

1.7.2 Rebound potentiation

Kano *et al.* (1992; 1996) characterised a phenomenon known as rebound potentiation in which stimulation of climbing fibre synapses onto cerebellar Purkinje cells could produce a long term potentiation of $GABA_A$ receptor mediated currents (in the same cell). The Ca^{2+} chelator BAPTA can block this rebound potentiation suggesting the process is Ca^{2+} dependent. Calmodulin binding domain (CBD)

synthetic peptide inhibits binding of Ca^{2+}/CaM to CaMK-II and blocks the activation of the kinase. Its introduction into Purkinje cells blocked the potentiation of GABA-mediated currents. The CaM antagonist, calmidazolium, and the selective CaMK-II antagonist, KN-62, had a similar effect. Moreover, the intracellular introduction of purified pre-activated CaMK-II increased the amplitude of whole-cell GABA currents recorded. Addition of CaMK-II inhibitors five minutes after the initial stimulus (Ca^{2+} rise) failed to block the effect, suggesting that CaMK-II activation is involved in the induction not the maintenance of rebound potentiation.

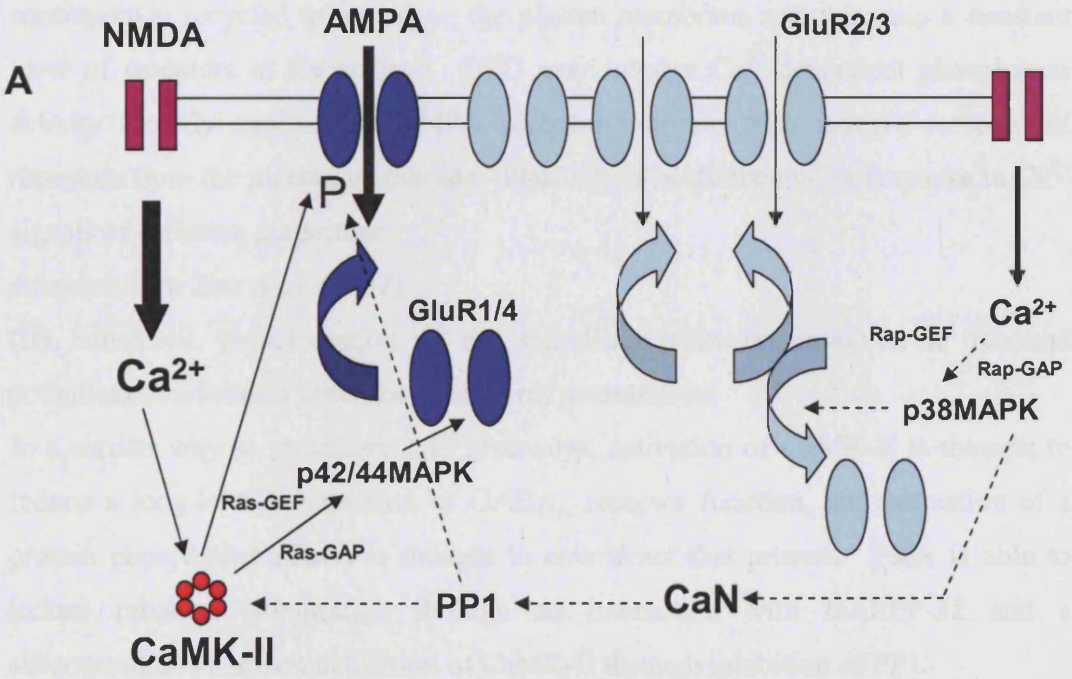
Further investigation has elucidated some of the molecular pathways involved in rebound potentiation. It has been demonstrated that multiple kinase pathways interact to modulate $GABA_A$ receptors in Purkinje cells. Kawaguchi and Hirano (2002) confirmed that inhibition of CaMK-II reduces or blocks rebound potentiation and observed that inhibition of PKA has a similar effect. Inhibition of PP1 or CaN impairs the suppression of potentiation caused by PKA inhibition, but not that caused by CaMK-II inhibition. The substrate of PKA and CaN, DARPP-32, inhibits PP1 when phosphorylated by PKA (Svenningsson *et al.*, 2004). PP1 inhibits CaMK-II, which alters $GABA_A$ receptor function more directly by a mechanism that is yet to be determined (Fig 1.5B).

In ionotropic glutamate receptor subunit δ2 (GluRδ2) knockout mice GABAergic synaptic transmission is enhanced and it is not possible to stimulate rebound potentiation suggesting that rebound potentiation occurs *in vivo,* potentially as a form of homeostatic plasticity (Ohtsuki *et al.*, 2004). Stimulation of metabotropic P2Y purinoceptors and subsequent G-Protein-coupled stimulation of Ca^{2+} release from intracellular stores can induce a long-term enhancement of $GABA_A$ receptor mediated currents recorded from Purkinje cells. This process

appears similar to rebound potentiation in that it is dependent on postsynaptic calcium increases. However, the time course is much slower and does not require Ca^{2+} influx through voltage gated calcium channels indicating that different pathways may be involved (Saitow *et al.*, 2005).

There are a number of similarities between long-term plasticity of excitatory and inhibitory synaptic transmission in that both kinds of synapse can be modified bi-directionally, dependent upon the magnitude of the Ca^{2+} signal. Under certain circumstances Ca^{2+}-dependent potentiation of $GABA_A$ receptor function has been linked to activation of CaMK-II. It appears, therefore, that CaMK-II may be capable of mediating some forms of long-term inhibitory plasticity and as a result CaMK-II dependent modulation of the $GABA_A$ receptor may have important functional consequences for understanding network behaviour.

Fig 1.5:



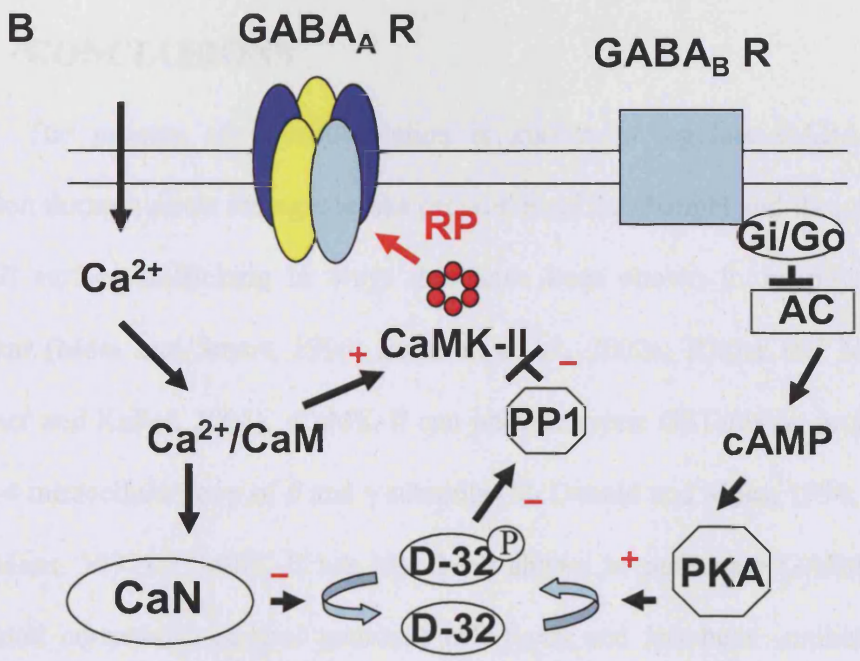


Fig 1.5: Schematic representation of signalling pathways involved in excitatory and inhibitory forms of long-term synaptic plasticity.

(A) Schematic representation of the signalling pathways thought to be involved in excitatory LTP. CaMK-II is able to directly phosphorylate the AMPA receptor and can also drive AMPA receptor insertion into the plasma membrane. GluR1/4 receptors are inserted into the synapse during LTP. GluR2/3 receptors are continuously recycled to and from the plasma membrane and maintain a constant level of receptors at the surface. LTD may involve Ca^{2+}-dependent phosphatase activity directly against the AMPA receptor but may also involve removal of receptors from the plasma membrane. Plasticity is bi-directional in response to Ca^{2+} signals of differing magnitude.

Adapted from Zhu *et al.* (2002).

(B) Schematic representation of the signalling pathways involved in rebound potentiation, a form of inhibitory long-term potentiation.

In a similar way to excitatory LTP pathways, activation of CaMK-II is thought to induce a long-term potentiation of $GABA_A$ receptor function, and activation of a protein phosphatase (CaN) is thought to counteract that process. PKA is able to induce rebound potentiation through an interaction with DARPP-32 and a subsequent downstream activation of CaMK-II through inhibition of PP1.

The process or events downstream of CaMK-II activation that result in modification of $GABA_A$ receptor function, are at present unknown.

Adapted from Kawaguchi and Hirano (2002).

1.8 CONCLUSIONS

The process of phosphorylation is known to regulate $GABA_A$ receptor function through direct changes to the properties of the channel and through changes in cell surface trafficking in ways that have been shown to be physiologically relevant (Moss and Smart, 1996; Brandon *et al.*, 2002a; Kittler and Moss, 2003; Lüscher and Keller, 2004). CaMK-II can phosphorylate GST-fusion proteins of the TM3-4 intracellular loop of β and γ subunits (McDonald and Moss, 1994; McDonald and Moss, 1997). CaMK-II has also been shown to potentiate $GABA_A$ receptor mediated currents in several neuronal cell types and has been implicated in the induction of certain forms of inhibitory synaptic plasticity (Wang *et al.*, 1995; Kano *et al.*, 1996; Wei *et al.*, 2004). It is important to determine the mechanism of CaMK-II dependent modulation of $GABA_A$ receptors as the processes involved may play important physiological roles. For instance, up-regulation of inhibitory synaptic strength may be important in forms of homeostatic plasticity and in the proper formation of inhibitory networks during development.

At present it is unclear whether CaMK-II dependent modulation of $GABA_A$ receptors occurs through direct phosphorylation or through indirect downstream processes. In addition, given the heterogeneity of $GABA_A$ receptors and the differential effects of other kinases on different $GABA_A$ receptor subtypes, it is unknown whether CaMK-II shows any subunit-dependent effects on $GABA_A$ receptors.

1.9 AIMS

- To establish whether CaMK-II dependent modulation of $GABA_A$ receptors occurs through direct phosphorylation of the receptor.
- To determine if CaMK-II dependent modulation of the $GABA_A$ receptor has differential effects on different $GABA_A$ receptor subtypes.
- To establish which specific subunits are required for CaMK-II modulation, and if possible, which specific residues might be phosphorylated.
- To understand more about the possible mechanism(s) of CaMK-II dependent functional modulation of the $GABA_A$ receptor.

Chapter Two:
Materials and Methods

2.1 MOLECULAR BIOLOGY

All molecular biology was carried out in collaboration with Dr A.M Hosie and Ms H.M.A da Silva. In brief the methods were as follows:

2.1.1 Site-directed mutageneis.

Murine α1, β2/3, γ2S $GABA_A$ receptor cDNAs were cloned into the plasmid vector pRK5 (Moss *et al.*, 1991; Connolly *et al.*, 1996). Expression of heterologous subunits in this vector is under the control of the cytomegalovirus promoter. Site-directed mutagenesis was carried out using the Quikchange method (Stratagene). This involved:

- The design of appropriate oligonucleotides containing the desired mutation flanked by an unmodified nucleotide sequence; to be used as mutagenic primers.
- The amplification of the relevant product by polymerase chain reaction (PCR); electrophoresis to check for amplification of the desired product and digestion of unmutated DNA.
- Transformation into *E.Coli.*
- Purification and sequencing of plasmid DNA.

Complementary olignucleotides containing the desired mutation were used as primers for PCR (See Appendix A1.1). The PCR reaction contained 5 µl of 10X reaction buffer, 10-100 ng of dsDNA template (cloned into pRK5), 125 ng primer 1 (Invitrogen) and 125 ng of primer 2 (Invitrogen), 1 µl of deoxynucleotide

triphosphate (dNTP) mix (10 mM of each dNTP), 2.5U of *Pfu turbo* DNA polymerase and double distilled water to a final volume of 50 µl.

The PCR reaction mix underwent thermocycling with the following parameters: 99°C for 30 s, followed by 18 cycles of 95°C for 30 s (denaturation), 55 to 65°C for 30 s to 60 s (annealing), and 72°C for 2 min/kb of plasmid length (extension). The PCR mix was then electrophoresed on a 1 % w/v agarose gel, to check for the formation of a product. The PCR product was then digested with *Dpn I* restriction enzyme (10U-New England Biolabs) to cut the methylated template DNA, leaving only mutated PCR product DNA (1 hr at 37°C).

After digestion, the PCR product was transformed into *E-Coli* using TOP10 one shot Kit (Invitrogen – See Appendix A1.2). Top10 competent cells (Invitrogen) were grown on LB Agar (Sigma) containing 50 µg/ml ampicillin. Bacterial colonies containing the mutated DNA were selected and grown overnight in Lennox L broth base (LB) medium (Sigma) to allow the transformed bacteria to multiply. The plasmid was then purified using the QIAprep Spin Miniprep Kit (Qiagen – See Appendix A1.3).

The sequence of the entire coding region of the cDNA was then determined to ensure the mutation had been correctly incorporated into the template DNA sequence without any ectopic mutations (DNA sequencing service – The Wolfson Institute for Biomedical Research, Cruciform Building, UCL). The analysis of the base sequence was performed by Sequencher 3.1.2 software. If the mutation had been successfully incorporated into the base sequence the bacterial stock was grown to increase the amount of the mutated cDNA using a large volume of LB media (100 ml midi prep; 250 ml, maxi prep Qiagen; grown overnight). As before, cDNA was

purified but this time at higher concentrations (1 μg/μl) that could then be used for transfection of cell lines or neurones.

2.2 CELL CULTURE

2.2.1 HEK293 cells.

HEK293 cells (Graham *et al.*, 1977) were grown in 10 cm diameter culture dishes (Nunc) in media containing Dulbecco's modified Eagle's medium (DMEM - Gibco) supplemented with 10 % v/v foetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and incubated at 37°C, in 95 % air / 5 % CO_2. Cells were passaged when they reached approximately 70 % confluency, usually every 2-3 days. Cells requiring passaging were washed once with 5 ml Hank's balanced salt solution (HBSS) and then incubated in 2 ml trypsin (0.25 % w/v) until the cells floated free off the dish. DMEM (10 ml) was then added to quench the trypsin and the cells were transferred to a 50 ml conical tube for centrifugation at 1000 rpm for 2 min. The supernatant was then removed and cells were re-suspended in 5 ml of media and re-plated at a dilution factor of 1:10 / 1:20 cell-containing media to fresh media (Krishek *et al.*, 1994; Dunne *et al.*, 1998; Thomas and Smart, 2002; Thomas and Smart, 2005). Cells used for transfection by electroporation or calcium phosphate were subsequently triturated to ensure a single-cell suspension and were re-suspended in OPTIMEM (Gibco) at this point.

2.2.2 NG108-15 cells.

NG108-15 cells (Klee and Nirenberg, 1974; Daniels and Hamprecht, 1974; Docherty *et al.*, 1991) were grown in 50 ml culture flasks (Nunc) in DMEM (4.5 g/L glucose - Gibco) supplemented with 10 % v/v FCS, 2 mM glutamine, 100 μM

hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (Sigma) and 20 U/ml penicillin G and 20 mg/ml streptomycin and incubated at 37°C, 90 % air /10 % CO_2. Cells were passaged when they reached approximately 70 % confluency every two-three days at a ratio of 1:3 or 1:6 cell-containing media to fresh media. Cells were detached from the bottom of the flask by a gentle wash and spun at 900 rpm for 5 min; they were then re-suspended in fresh media and re-plated to continue the culture. At this point cells for transfection were further diluted (1:2-3) and drops of cell-containing media were dropped onto pre-prepared (2.2.3) glass coverslips in 35 mm dishes to allow the cells to adhere (Nunc, See Fig 2.1A).

2.2.3 Primary cell culture – rat/mouse cerebellar granule cells.

The cerebella of postnatal day 1 (P1) Sprague-Dawley rats were removed after decapitation and placed in ice-cold dissection media (DM = HBSS – without Ca^{2+}/Mg^{2+} (Gibco) + Gentomycin 10 μg/ml (Sigma). The meninges were then carefully removed and the cerebella transferred to DM that had been heated to 37°C and CO_2 saturated in the incubator. They were then centrifuged at 1000 rpm for 1.5 min; the supernatant was removed and another 10 ml DM added and the tissue centrifuged again. The supernatant was removed and the cerebella were then incubated in trypsin (0.1 % w/v, Sigma) at 37°C for 10 min. The trypsin was removed and the cerebella washed with 10 ml DM. They were centrifuged again for 3 min at 1000 rpm followed by a second wash in DM and centrifuged at 1000 rpm for 1.5 min.

The DM was removed virtually completely using a 200 μl Gilson pipette. Then, 1 ml DNase (0.05 % w/v in 12 mM $MgSO_4$, Sigma) was added and the cerebella were triturated with two glass pipettes that had been fire-polished to small

bore diameters. The final trituration step was carried out with a 200 µl pipette tip until a single-cell suspension had formed. Another 9 ml DM was added and the mixture centrifuged at 1200 rpm for 5 min. Care was taken to remove all the supernatant at this point with a 200 µl pipette and the cells were re-suspended in 1 ml of seeding media (Basal Medium Eagle – BME (Gibco) + 10 % v/v FCS). An aliquot (15 µl) of the suspension were then transferred to a haemocytometer and an estimate of cell number made. Cells were diluted to give approximately 5 x 10^6 cells/ml; 90 µl of this suspension was then placed onto the pre-prepared glass coverslips coated in poly-L-ornithine (Sigma). Coverslips were washed in 100 % v/v ethanol and flamed, twice and coated in 500 µg/ml poly-L-ornithine in borate buffer (50 mM Boric acid, 12.5 mM sodium tetraborate), 0.5 ml per coverslip, overnight at 4°C, then washed three times with sterile water and finally left to air dry before use. Dishes were left in the incubator for 3 hrs to allow cells to adhere to the glass after which the appropriate growth media was added.

[1] Serum free media – 20 mM KCl.

BME (Gibco) supplemented with 0.5 % w/v glucose, 5 mg/L insulin, 5 mg/L transferrin, 5 mg/L selenium (Sigma), 20 U/ml pencillin-G and 20 µg/ml streptomycin, 0.14 mM glutamine, 1.4 mM NaCl , 0.01 mg/ml Bovine Serum Albumin (BSA, Sigma) and 20 mM KCl (See Fig 2.1B).

[2] Serum-containing media – 5 mM KCl.

BME (containing 5mM KCl) supplemented with 0.5 % w/v glucose, 20 U/ml penicillin-G, 20 µg/ml streptomycin, 0.2 mM glutamine, 5 % v/v FCS, 1.2 mM NaCl and 5 mg/ml insulin, 5 mg/ml transferrin and 5 mg/ml selenium (Sigma, See Fig 2.1B).

Cells maintained in serum-containing media were treated with 1-2 μM cytosine arabinoside (Sigma) at 4-5 DIV to control the growth of non-neuronal cells, which otherwise threatened to overwhelm the culture. Serum-free media maintained cells had 1 ml of media replaced every 7-10 days. Cells for transfection were chosen and transfected at 3-4 DIV (Furuya *et al.*, 1998; Leao *et al.*, 2000; Tabata *et al.*, 2000; Ives *et al.*, 2002).

2.2.4 Embryonic cortical neuronal culture.

The cortical neuronal cultures (See Chapter 4, Fig 4.1) were prepared by Dr J.N Jovanovic and Ms K McAinsh as follows:

Cortices were dissected from embryonic day 19 rats and the tissue incubated in 0.25 % w/v trypsin in HBSS for 15 min followed by 3 x 5 min washes in HBSS. The tissue was then dissociated by titration with a fire-polished pipette. Cells were plated on 0.5 mg/ml poly-L-ornithine-coated glass coverslips at a density of 10^5 cells/cm^2 and incubated at 37°C, 95 % air / 5 % CO_2 for 6 days before use (Brandon *et al.*, 1999).

Fig 2.1:

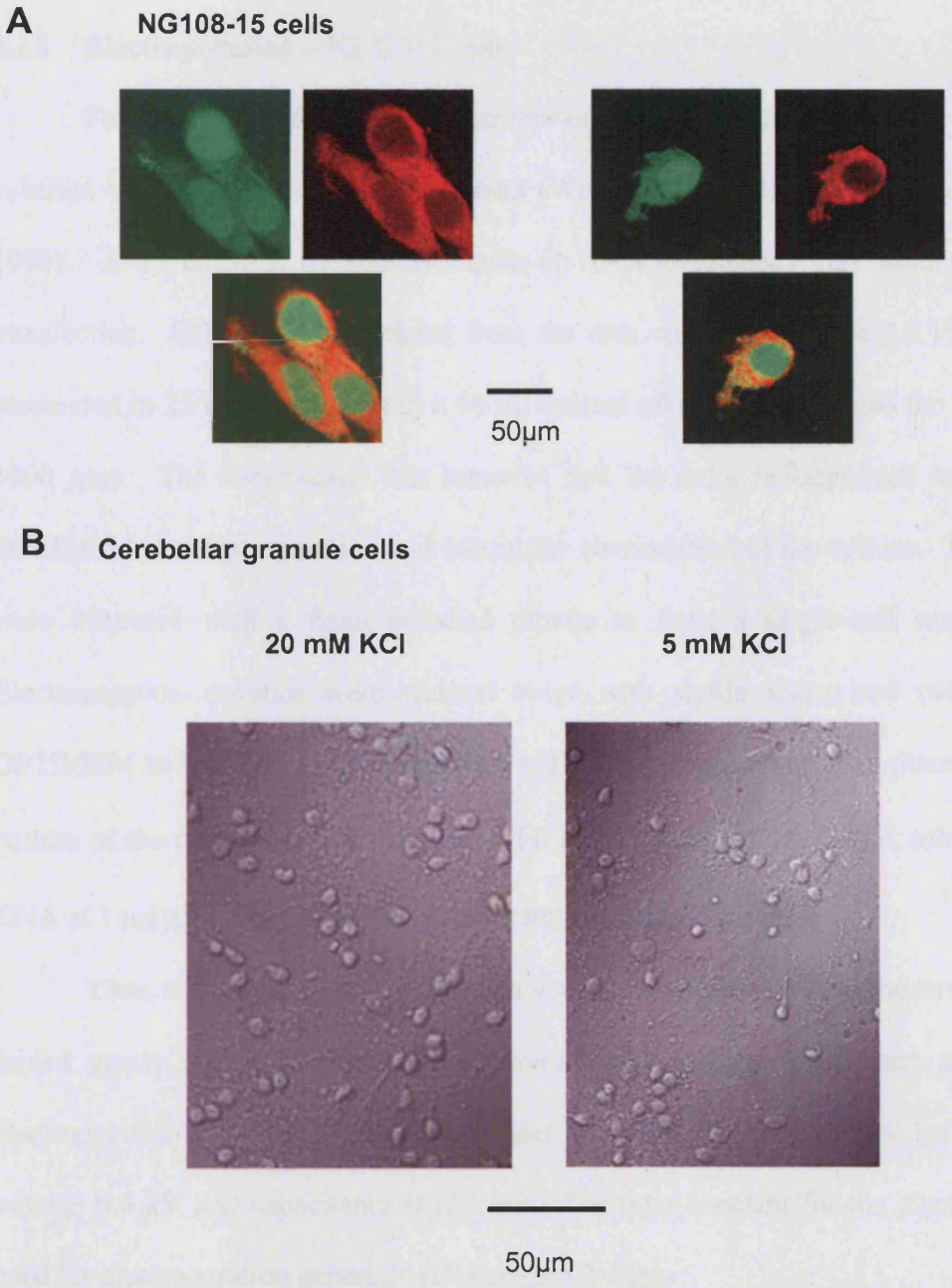


Fig 2.1: Images of NG108-15 cells and cerebellar granule cells in culture.

(A) NG108-15 cells transfected with cDNAs for $\alpha 1\beta 3\gamma 2S$ $GABA_A$ receptor subunits and EGFP (Enhanced Green Florescent Protein). Cells were then stained with a $\beta 3$ specific antibody (red fluorescence - See table 2.1) 48 hrs after transfection.

(B) Differential interference contrast (DIC) images of cerebellar granule cells maintained in culture in 20 mM KCl and 5 mM KCl. Cells in 5 mM KCl tended to form clusters whereas cells in 20 mM KCl had a more uniform distribution.

Images acquired on a Zeiss laser scanning confocal microscope (LSM 510 Meta; See 2.6.2).

2.3 TRANSFECTION

2.3.1 Electroporation – HEK293 cells.

For the production of a large number of dishes expressing the same receptor subunits the following method was used (Wooltorton *et al.*, 1997; Dunne *et al.*, 1998). A 10 cm dish of HEK293 cells at 70 % confluency was used for each transfection. Cells were dissociated from the dish with trypsin (See 2.2.1) and re-suspended in 25 ml OPTIMEM in a 50 ml conical tube and centrifuged for 2 min at 1000 rpm. The supernatant was removed and the cells re-suspended in 0.5 ml OPTIMEM for electroporation and 0.3 ml for continuation of the culture. The cells were triturated with a flame-polished pipette to form a single-cell suspension. Electroporation cuvettes were washed twice with sterile water and twice with OPTIMEM before use. The appropriate cDNA suspension was then placed in the bottom of the cuvette, e.g, α1:β3:γ2S:EGFP at a ratio of 1:1:1:1 (Stock solutions of DNA at 1 μg/μl, 1 μl of each cDNA was used per dish).

Then, 0.5 ml of the cell suspension was added to the cDNA in the cuvette and mixed gently. The cuvette contents were mixed before, in-between and after electroporation with the Biorad Gene pulser II. Settings were "infinite resistance", voltage 0.4 kV and capacitance 0.125 mF. The time constant for the electric field used for electroporation generally fell between 3-4 ms.

Cells were allowed to stand for 10 min after electroporation. Then, 8-10 ml of media (See 2.2.1) was then placed in a 10 ml tube and with a glass pipette the contents of the cuvette were added and the suspension inverted to mix well. After checking the cell density, 0.5 ml of media was placed onto pre-prepared glass coverslips in 35 mm dishes (Nunc) and a suitable number of drops of the cell suspension were added to this. Coverslips were prepared by washing in 100 % v/v

ethanol and flaming, then adding 0.5 ml of poly-L-lysine (100 μg/ml) for 1 hour, followed by three washes with sterile water and then left to dry before use.

Coverslips with cells added were left in the incubator for 10 min at 37°C to allow the cells to adhere to the substratum. Then 1-2 ml of media (DMEM) was added to each dish and they were placed into the incubator (37°C / 95 % air / 5 % CO_2) for at least 24 hrs. These cells could be recorded from for 24-48 hrs after transfection.

2.3.2 Calcium phosphate – HEK293 and NG108-15 cells.

This procedure was used when a number of different cDNAs were to be transfected into different dishes for recording on the same day (Krishek *et al.*, 1994). To 1 μl of cDNA stock solution (1000 ng/μl) per dish per subunit (+ EGFP), 340 mM $CaCl_2$ (20 μl per dish to be transfected) was added. This was left for 20 min to allow a precipitate to form. This suspension was then mixed with double-strength HBSS (24 μl per dish to be transfected; 2 x HBSS = 280 mM NaCl , 2.8 mM Na_2HPO_4, 50 mM HEPES, NaOH to pH 7.2). Once mixed, 45 μl was then applied to each 35 mm dish of cells. Dishes of cells were prepared by dissociating cells from the stock dish they were growing in (when at 70 % confluency) and re-suspending them in their normal media (See 2.2.1 and 2.2.2). Pre-prepared coverslips in 35 mm dishes (Nunc), already coated with poly-L-lysine for HEK293 cells (See 2.3.1) and poly-L-ornithine (See 2.2.3, 1 hr incubation only) for NG108-15 cells, were coated with 0.5 ml of normal media, prior to seeding with drops of cell suspension to obtain the appropriate cell density for recording.

After seeding the cells, the coverslips were left for 10-15 min to allow the cells to adhere. After this, 1.5 ml of fresh media was applied to each dish. At this

point the cells were ready for calcium phosphate transfection. After transfection cells were left overnight and the media changed in the morning. Cells could be recorded from 24 hr after transfection (See Fig 2.1A).

2.3.3 Effectene transfection - NG108-15 cells and cerebellar granule cells.

Both NG108-15 and cerebellar granule cell cultures were transfected using the Effectene transfection reagent (Qiagen, See Fig 2.1A and Fig 7.6-7).

On the day of transfection, NG108-15 cells were seeded onto glass coverslips (See 2.3.2) and left for 10 min in the incubator for cells to adhere. Cerebellar granule cell cultures were transfected at 3-4 DIV. A total of 0.4 μg DNA per dish (0.3 μg for granule cells) was prepared (containing an equal ratio of each subunit and enhanced GFP, 1:1:1:1) and added to EC Buffer (DNA-condensation buffer, 75 μl per 35 mm dish – supplied with reagent). Then, 8μl Enhancer (supplied with reagent) was then added per 1 μg of total DNA. The mixture was then vortexed and allowed to stand for 5 min. Then, 10 μl of Effectene reagent was added per dish for the transfection and the mixture vortexed and allowed to stand for a further 10 min to allow the DNA transfection-complex to form. Finally 0.5 ml of the appropriate media was added for each dish and mixed. The DNA suspension was then added immediately to the previously prepared cells. Cells were left in the transfection reagent containing media for 3-4 hrs and then the media was replaced. NG108-15 cells could be recorded from 24 hrs later (See Fig 2.1A). Cerebellar granule cells were left for a further 3-5 days and recorded from at 7-12 DIV.

2.4 ELECTROPHYSIOLOGY

2.4.1 The equipment.

The cells to be recorded from were identified under a Nikon Eclipse TE300 microscope at 200-400X magnification, with additional X5 magnification, as required sometimes for cerebellar granule cells. Transfected NG108-15 and HEK293 cells could be identified by GFP fluorescence observed using the Nikon epifluorescence unit (See Fig 2.2). Drugs were applied to cells using a fast perfusion system based on a modified Y-tube (Wooltorton *et al.*, 1997) controlled with solenoid valves (Lee products ltd, Buckinghamshire) operated electronically (See Fig 2.4). Cells were voltage-clamped using an Axopatch 1C patch clamp amplifier (Axon Instruments) with a CV-3 headstage (Axon Instruments) at -50mV (HEK293 or NG108-15 cells) or -60mV (cerebellar granule cells). Membrane currents were filtered at 3kHz (8 pole Bessel filter) and digitised via a Digidata 1200 (Axon Instruments) and viewed with a PC (Dell – Optiplex GX260) using the software programme Clampex 8.2 (Axon Instruments - See Fig 2.3).

2.4.2 Whole-cell patch clamp recording.

Cells were cultured on glass coverslips that could be transferred to the recording chamber and anchored in place in the bath. Cells were continuously perfused with Krebs solution at room temperature containing: 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.52 mM $CaCl_2$, 11 mM glucose and 5 mM HEPES, pH adjusted to 7.4 with NaOH and filtered through Whatman paper. Osmolarity was confirmed as 300 ± 10 mOsm with an Osmometer (Automatic – CAMLAB).

Patch pipettes were pulled from thick-walled borosilicate glass (Harvard, GC150-10) and filled with an internal patch pipette solution containing: 120 mM

KCl, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, 1mM $CaCl_2$ and 4mM Na_2ATP, pH adjusted to 7.2 with NaOH (osmolarity confirmed as 280-300 mOsm). This solution was used to record from HEK293 cells in Figs 3.2-4. At all other times the patch pipette solution contained: 150 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM Na_2ATP, 0.1 mM $CaCl_2$ and 1.1 mM EGTA, pH adjusted to 7.2 with CsOH (osmolarity confirmed as 280-300 mOsm). Free $[Ca^{2+}] \leq 100nM$. [1]

The resistance of patch electrodes was monitored using the seal-test option in Clampex 8.2. The resistance of electrodes used to record from HEK293 and NG108-15 cells was 5-6 MΩ, and for cerebellar granule cells, 8-9 MΩ. Electrodes were manoeuvred into position onto the surface of the cell using a Sutter MP-285 micromanipulator.

The seal-test option and the membrane test option in Clampex 8.2 were used to monitor the fidelity of the whole-cell recording (See Fig 2.3B and C). The seal test measured the seal resistance between pipette and ground by repetitive application of -5mV voltage pulses and measuring the current response. This was then used to set the capacitance compensation on the patch clamp amplifier. Series resistance and whole-cell capacitance were optimised before the start of experiments using the shape of the waveform in the seal-test as a guide (See Fig 2.3C).

During patch clamp recording a proportion of the command potential is expected to be lost as it passes across the patch electrode and cell membrane in series (series resistance). Voltage clamp errors are expected to be low if the cell is healthy and the membrane seal around the electrode is high (GΩ). Voltage clamp errors were further reduced by compensating for series resistance by using the patch clamp amplifier to inject current into the cell to compensate what is lost due to series resistance. Voltage clamp errors unavoidably increase for larger membrane currents

1. Calculated using Mathcad 2000i.

(Thomas and Smart, 2002). Thus as a result, membrane currents of over 4 nA, usually recorded from larger NG108-15 cells, were discarded from the analysis.

The stability of the connection between cell and electrode was monitored throughout the course of the experiment using the seal-test and membrane test options to continuously monitor seal resistance, access resistance (Ra) and series resistance (Rs). Series resistance can also vary as access resistance alters which tends to occur as the electrode tip becomes occluded with cellular contents. Cells showing changes in access resistance or series resistance of over 15 % were discarded from the analysis.

The cell's current response after application of GABA was recorded, in the gap-free mode (Clampex 8.2) and stored on the PC for further analysis. Typical values for series resistance were <1MΩ, and for capacitance, 21.7 ± 3.6 pF (n = 6, HEK293), 56.5 ± 7.6 pF (n = 6, NG108-15), 11.1 ± 0.6 pF (n = 15, CGCs – 20mM KCl) and 8.3 ± 0.5 pF (n = 15, CGCs – 5mM KCl). The resting membrane potentials of cells immediately after achieving the whole-cell configuration were recorded as a good approximation of cell health. HEK293 cells and NG108-15 cells generally had typical values -33.5 ± 5.6 mV (n = 6, HEK293 cells), -39.5 ± 4 mV (n = 6, NG108-15). CGCs maintained in depolarising conditions had slightly lower resting potentials on average (-39.2 ± 2.5 mV) as compared to CGCs maintained under physiological conditions (-47.5 ± 5.9 mV; comparable to that reported by Mellor *et al.,* 1998).

Fig 2.2:

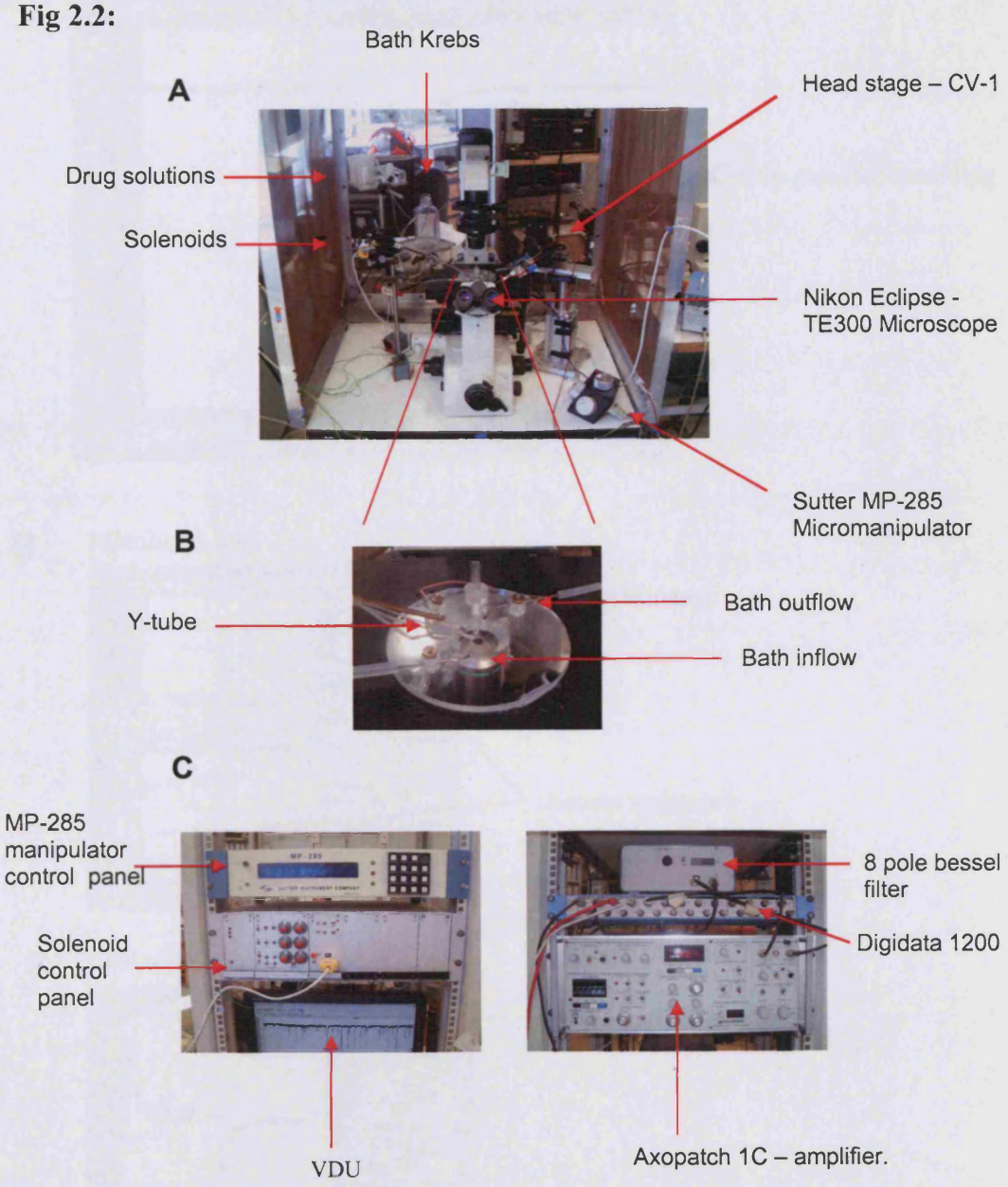
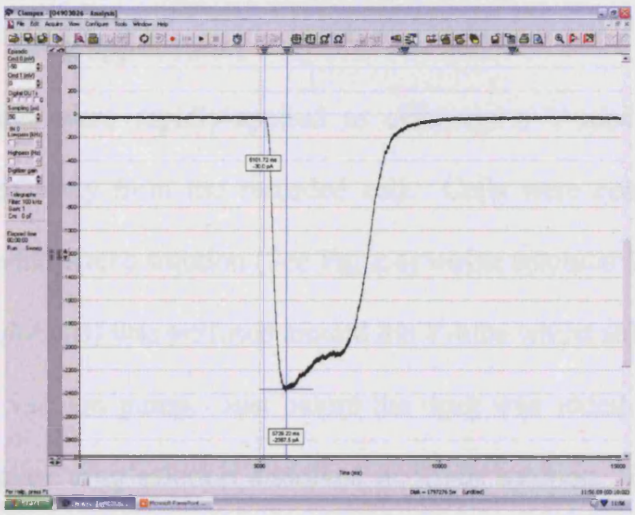


Fig 2.2: Photographs of the electrophysiology rig.

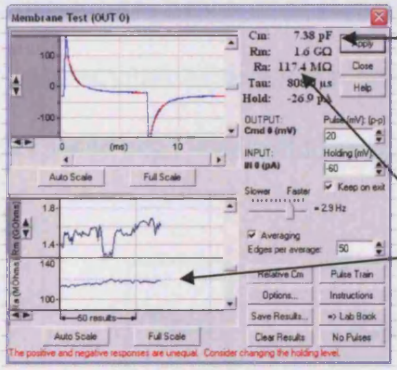
Fig 2.3:

A



pClamp gap-free recording

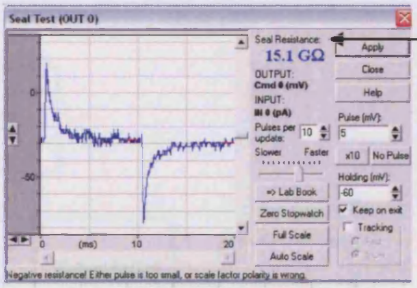
B Membrane test



Capacitance

Access resistance

C Seal test



Seal resistance

Fig 2.3: Software used for data acquisition: pClamp 8.2.

(A) Whole cell currents were recorded in the gap-free mode.

(B/C) Membrane test and seal test in pClamp were used throughout the experiment to monitor Ra and Rs. The seal test waveform was used as a guide for compensation of series resistance on the amplifier.

2.5 DRUG APPLICATION AND SOLUTIONS

2.5.1 Drug application.

Drugs were rapidly-applied to cells via a Y-tube positioned approximately 400 μm away from the recorded cell. Cells were continuously superfused by a gravity-fed Krebs solution (See Fig 2.4) whilst solenoid 1 was open. Water (to save drug solutions) was perfused around the Y-tube whilst solenoid 2 was open, powered by the vacuum pump. Just before the drug was added water was replaced by the appropriate drug solution dissolved in Krebs solution. Both solenoids were closed simultaneously allowing the drug solution to flow rapidly over the cell whilst stopping the normal Krebs from flowing. When both solenoids were opened again (after a fixed amount of time), the normal Krebs could wash-off the drug solution from the exposed cell (Wooltorton *et al.*, 1997).

Fig 2.4:

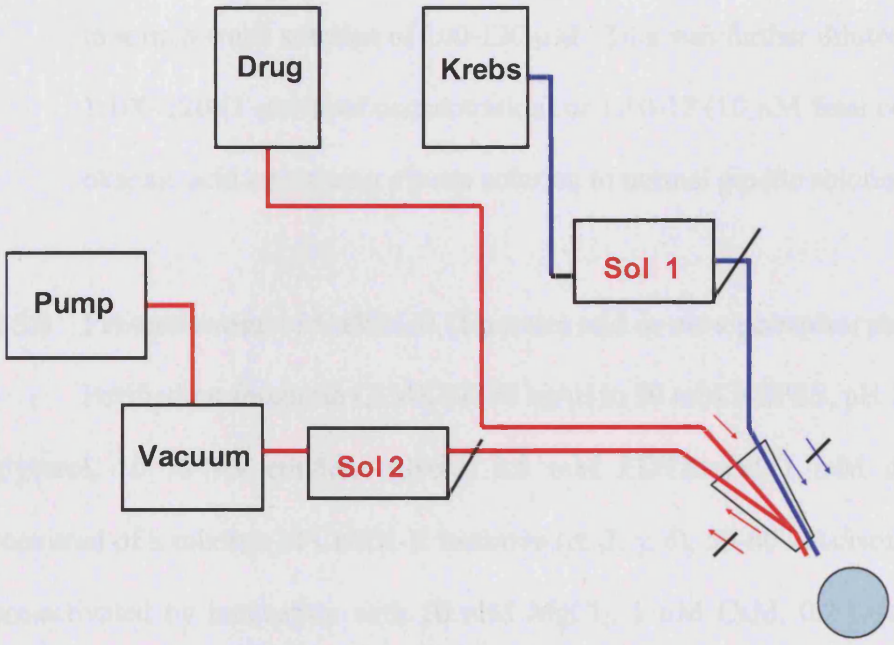


Fig 2.4: Schematic representation of the rapid perfusion Y-tube system.
The flow of a drug was controlled by two solenoids which acted as gates to allow or stop drug application and wash. The solenoids were controlled electronically. The typical rise time (10-90 %) of the 10 μM GABA response in NG108-15 cells was 276.2 ± 28.9 ms (n = 6).

2.5.2 Drug solutions (intracellular ligands).

- **KN-93** (Sigma), an inhibitor of CaMK-II activity (Sumi *et al.*, 1991) was dissolved in DMSO to form a stock concentration of 2.4 mM. KN-93 was added to the pipette solution at a dilution factor of 1 in 4800 to ensure DMSO did not interfere with the stability of recordings.
- **KN-92** (Sigma), an inactive analogue of KN-93 was dissolved in DMSO to form a stock concentration of 1 mM and was dissolved in the pipette solution at a dilution of 1 in 2000.
- **Genistein** a specific inhibitor of tyrosine kinase activity (Akiyama *et al.*, 1987) was dissolved in DMSO to give a final stock solution of 100 mM and diluted 1 in 1000 in the patch pipette solution.
- **Okadaic acid** sodium salt (Sigma), an inhibitor of protein phosphatase activity (Bialojan and Takai, 1988) was dissolved in internal pipette solution to form a stock solution of 100-120 μM. This was further diluted at a ratio of 1:100-120 (1 μM final concentration) or 1:10-12 (10 μM final concentration) okadaic acid-containing pipette solution to normal pipette solution.

2.5.3 Pre-activation of CaMK-II (Upstate) and *in vitro* phosphorylation assay.

Purified rat forebrain CaMK-II (50 ng/μl in 50 mM HEPES, pH 7.5, 50 % v/v glycerol, 10 % v/v ethylene glycol, 2.5 mM EDTA and 1 mM dithiothreitol) consisted of a mixture of CaMK-II isoforms (α, β, γ, δ), 50-60 kDa/isoform and was pre-activated by incubation with 10 mM $MgCl_2$, 1 μM CaM, 0.2 mM EGTA, 0.3 mM $CaCl_2$ for 15 min at 25°C. The pre-activation buffer containing CaMK-II was then diluted in the patch pipette solution to give a final concentration of 60-100 nM CaMK-II.

In vitro phosphorylation of β3 TM3-4 loop GST-fusion proteins by CaMK-II (See Fig 3.4) was carried out in collaboration with Dr J.N Jovanovic as follows:

Purified CaMK-II (Upstate – 10 ng/μl final concentration in pre-activation buffer), was pre-activated (10 min) and incubated with GST-β3 (1.6 μg/μl) and ^{32}P-ATP (0.4mM) at 25°C or 5°C, with or without 150 mM KCl. The reaction was stopped (t = 10 min) by addition of an equal volume of SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) sample buffer. Phosphorylation was analysed by SDS-PAGE and visualized by autoradiography. KCl (150 mM) was added to the pre-activation/autophosphorylation buffer to simulate addition to the patch pipette solution. CaMK-II was pre-activated by incubation in 10 mM $MgCl_2$, 1 μM CaM, 0.2 mM EGTA, 0.3 mM $CaCl_2$ (McDonald and Moss, 1994; McDonald and Moss, 1997).

2.5.4 Pre-activation of recombinant α-CaMK-II (New England Biolabs).

In all other experiments a truncated monomer of rat α-CaMK-II (1-325 amino acid residues) with a molecular weight of 33 kDa (New England Biolabs) obtained from *Spodoptera frugiperda* (Sf9) cells infected with recombinant baculovirus carrying the recombinant α-CaMK-II cDNA (Brickey *et al.*, 1990; Suzuki-Takeuchi *et al.*, 1992) was used. This had a specific activity of ≈5,000,000 units/mg, supplied at 500 units/μl (100 ng/μl). One unit is defined as the amount of activated CaMK-II required to catalyze the transfer of 1 pmol of phosphate to Autocamtide-2 (CaMK-II peptide substrate) KKALRRQETVDAL (NEB – 50 μM) in 1 min at 30°C in a total reaction volume of 30 μl.

α-CaMK-II was supplied in 100 mM NaCl , 50 mM Tris-HCl (pH 7.5 @ 25°C), 0.1 mM Na_2EDTA, 1 mM dithiothreitol, 0.02 % v/v Tween-20 and 50 % v/v

glycerol. Glycerol at this concentration was detrimental to the health of the cells so α-CaMK-II was dialysed with the Slide-A-Lyzer MINI Dialysis unit to reduce the glycerol concentration. Dialysis units were soaked in de-ionised water for 15 min. The dialysis unit was then placed in the microtube supplied, filled with 1.3 ml of dialysate containing 100 mM NaCl , 50 mM Tris-HCl (pH 7.5 @ 25°C), 0.1 mM EDTA and 1 mM dithiothreitol. The α-CaMK-II as supplied was then added just above the membrane and allowed to sink. The dialysis unit was then capped and placed on a shaker at 4°C. CaMK-II and the microtube were kept on ice throughout the procedure. After 1-2 hrs the sample was collected from the dialysis unit and aliquots ready for use in patch clamp recordings were frozen and kept at -70°C. The dilution factor generally fell between 2-2.5 fold giving a final concentration of 1200-1500 nM (40-50 ng/μl). Stocks were defrosted just before use and incubated in a pre-activation/autophosphorylation buffer as suggested by the manufacturers: 8-10 μl CaMK-II stock (40-50 ng/μl = 1200-1500 nM) diluted into CaMK-II buffer as supplied (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na_2EDTA, pH 7.5 @ 25°C) in a total volume of 16-20 μl supplemented with 1.2 μM calmodulin (CaM), 1.5 mM $CaCl_2$, 0.4 mM ATP-γ-S incubated at 25°C for 15 min (McGlade-McCulloh *et al.*, 1993).

The CaMK-II pre-activation buffer was then diluted into the patch pipette solution (McGlade-McCulloh *et al.*, 1993; Lledo *et al.*, 1995; Barria *et al.*, 1997; Nelson *et al.*, 2005) to give a final CaMK-II concentration of 35 nM (1.16 ng/μl), 60 nM (2 ng/μl), 85 nM (2.85 ng/μl) or 100 nM (3.33 ng/μl). At the highest concentration the dilution factor was approximately 1:12 α-CaMK-II stock solution to patch pipette solution. Control recordings were made with the pre-activation mixture without CaMK-II diluted into the patch pipette solution at the same ratio, in

order to ensure there were no non-specific effects on the stability of recordings caused by residual CaM and $CaCl_2$ from the pre-activation buffer or that the buffer diluted into the patch pipette solution did not activate endogenous Ca^{2+}/CaM dependent kinases or phosphatases.

2.6 IMMUNOCYTOCHEMISTRY

2.6.1 Procedure for immunolabelling of cell cultures.

Cells cultured on glass coverslips (See 2.2.3) were washed gently twice with ice-cold phosphate buffered saline (PBS). They were then fixed in 4 % paraformaldehyde (Sigma) in PBS, for 15 min at room temperature. The fixative was then washed with PBS twice and quenched with 50 mM NH_4Cl (in PBS) twice for 5 min. Cells were then washed again with PBS (twice). At this point the fixed cells were kept until required at 4°C.

If the desired antigen was intracellular (anti-β3) the cells were then permeabilised by incubation at room temperature with 0.05 % v/v Triton-X-100, 10 % v/v FCS, 0.5 % w/v BSA (in PBS) for 10 min. Followed by a further two washes in PBS. If the desired antigen was extracellular (anti-myc) this stage was omitted as only cell-surface labelling was required.

Prior to antibody incubation, cells were washed in 10 % v/v FCS, 0.5 % w/v BSA (in PBS). The appropriate antibody was then diluted (See Table 2.1) in the same buffer. Then, 50 µl of the diluted antibody was placed onto a parafilm segment, the coverslip was removed from solution and the edge briefly dried on a tissue before inverting over the drop. Coverslips were covered with the dish lid to prevent drying and left for 45 min at room temperature.

After the primary antibody incubation cells were placed back into 35 mm dishes and washed three times with 10 % v/v FCS, 0.5 % w/v BSA in PBS. The secondary antibody was diluted into the same buffer at the appropriate concentration (See Table 2.1) and again coverslips were inverted over 50 µl of the diluted antibody mixture dropped onto parafilm. Coverslips were covered with the lid of the dish to prevent drying and covered with foil to protect from the light. Cells were incubated in the secondary antibody for 45 min at room temperature.

After incubation, the coverslips were returned to the 35 mm dishes and washed three times in 10 % v/v FCS, 0.5 % w/v BSA (in PBS) and then three times in PBS. Coverslips were fixed to microscope slides using glycerol. The coverslips were lifted from the dish and excess fluid was removed by dipping the edge on a tissue. A drop of (pre-heated) glycerol (30 µl) was placed on the slide and the coverslip was inverted onto it and left to dry (Duguid and Smart, 2004).

Table 2.1:

Antibody	Species	Epitope	Dilution	Source
Anti-myc (1°)	Polyclonal Raised in Rabbit	C-teminus of c-Myc of human origin.	1:250	Santa Cruz Biotechnology
Anti-β3 (1°)	Polyclonal Raised in Rabbit	TM3-4 Loop of the β3 subunit.	1:200	Gift from Prof. W. Sieghart (Vienna)
Anti-rabbit TRITC (2°)	Raised in Goat	IgG pooled rabbit serum	1:250	Sigma

Table 2.1: Primary and secondary antibodies used in immunocytochemistry.

2.6.2 Image acquisition.

Images were acquired using a Zeiss laser-scanning confocal microscope (LSM 510 Meta). An oil-immersion objective lens (x 40 Plan-Neofluar or x 63 Plan-Apochromat) was used to visualise individual cells. Multi-track images were obtained with an Argon laser (488 nM) to visualise EGFP fluorescence and a Krypton (568 nM) laser to visualise tetramethyl rhodamine isothiocyanate (TRITC) fluorescence. Optical sections (z series) of intervals of 0.3-0.4 μm were taken through cells and 8-10 images were reconstructed to yield the final projection image. The precise settings can be seen in Fig 2.5.

Fig 2.5:

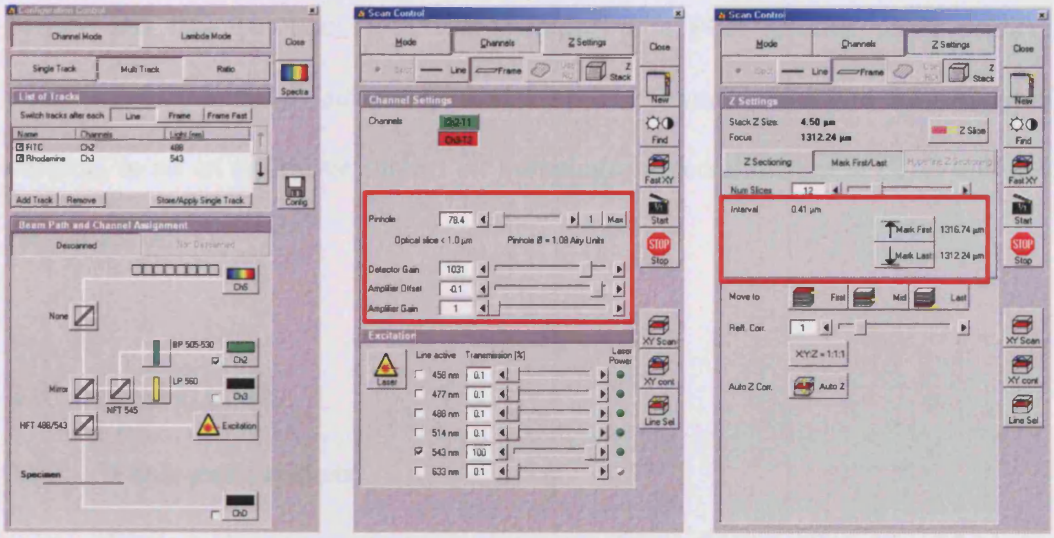


Fig 2.5: Settings of Zeiss LSM 510 Meta laser scanning confocal microscope.

Optimal settings are shown. Detector gain and amplifier offset can be altered to increase the signal to noise ratio for each channel. The pinhole size was set at 1 Airy unit with an optical slice of <1 μm. A series of optical slices (Z series) can be taken through the cell to form a projected image.

2.7 KNOCKOUT MICE

The β2 knockout mouse strain (from Merck Sharp and Dohme, UK) was created by deleting exons 6 and 7 of the β2 subunit gene by homologous recombination (Sur *et al.*, 2001). Heterozygous F3 generation mice were cross-bred to produce β2 -/- and wild-type (wt) strains. The offspring of breeding pairs of the F5-6 generation of wt and β2 -/- mice were taken at P0-2 to prepare cerebellar granule cell cultures (See 2.2.3).

The tails of offspring were removed after dissection for genotyping to confirm that the β2 subunit was missing from the β2 -/- cultures. DNA was extracted from the mouse tails of every pup (See appendix A1.4) in a litter used for each culture and this genomic DNA was included in a PCR reaction using primers complimentary to the β2 subunit gene and a primer complimentary to the neomycin cassette, to act as a positive control for homologous recombination in β2 -/- cultures (Sur *et al.*, 2001).

2.8 ANALYSIS

2.8.1 Whole-cell currents.

Whole-cell currents were analysed in Clampfit 8.2 (See Fig 2.3A) by measuring the peak amplitude and subtracting the baseline. Cells were chosen for analysis if access resistance and series resistance remained stable throughout the period of recording (See 2.5.2). If traces had deflections in their rising phase that current was discarded from the analysis. The peak amplitude recorded at 2 min intervals was normalised to the peak amplitude of the response obtained 3-4 min after achieving the whole-cell configuration.

The peak amplitude of the response to different concentrations of GABA was used to construct a concentration-response curve (See Fig 4.2). The peak amplitudes were normalised to the capacitance of the cell (pA/pF). These values were then plotted against GABA concentration. At least 5 different GABA concentrations were used to define the threshold or heel of the curve, the linear portion, and the clear saturating maximum. These data were then fitted by the Hill equation (shown below) within the program Origin 6.0 (Microcal):

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

where, **I** = the current activated by GABA (normalised to membrane capacitance). $\mathbf{EC_{50}}$ = the concentration of GABA to activate a current that is 50 % of the amplitude of current induced by a maximal dose of GABA and **n** = the Hill coefficient.

2.8.2 MiniAnalysis

The software program MiniAnalysis (Synaptosoft) was used to analyse synaptic currents. Recordings were captured in Clampex 8.2 over 2 min or 5-6 min epochs starting 4-5 min after achieving the whole-cell configuration. Within MiniAnalyis a period of recording could be displayed and synaptic currents were detected automatically with an amplitude threshold of 6-10 pA, depending on the level of baseline noise, which was typically around 5 pA. After automatic detection, each event was manually checked and verified by eye. The program could then analyse and display a mean value for the frequency, the peak amplitude and the half-

width (the time taken to decay to 50 % of the peak amplitude) over that period of recording (See Fig 2.6A).

Events could also be grouped manually and these groups could be used to analyse the decay time constants of each period of recording. Synaptic events were chosen for analysis if there were no deflections in the rising or decaying phases and they decayed back to the baseline. Events of low amplitude (<5 pA) were discarded from this analysis as they were thought to represent events occurring at the most distant synapses and therefore more affected by dendritic filtering or cabling. All the events chosen were grouped together for a given recording and aligned by their mean rise-times (50 %) for analyses of decay time constants. The program calculated an average current from the group. From this the 10-90 % rise-time could be calculated. The average current can then be fitted with a sum of one, two or three exponentials. The best fit could be identified visually but the program also allowed the best fit to be determined by regression analysis.

The resulting best fit curve was described by the following equation:

$$\mathbf{y = A1*exp\ (-x/\tau 1) + A2*\ exp\ (-x\ /\ \tau 2) + baseline,}$$

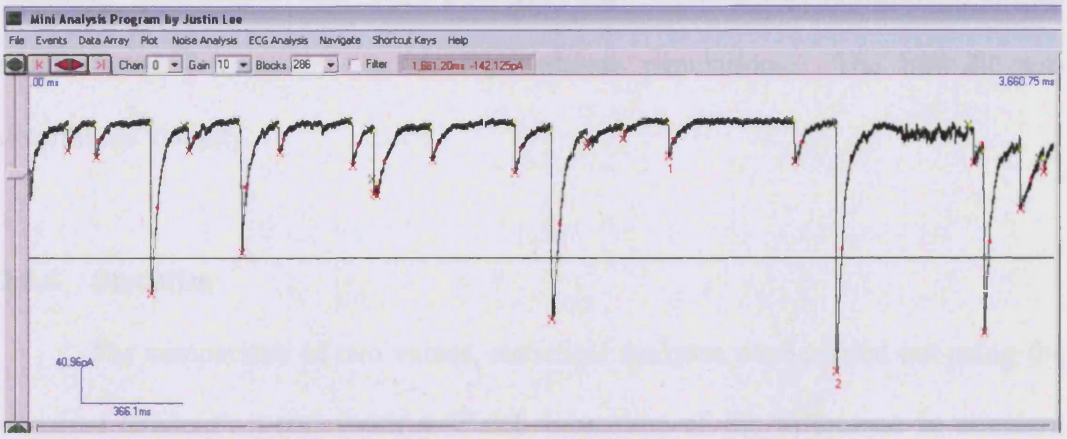
where, A1 and A2 are the fractions of the fast (1) and slow (2) components and $\tau 1$ and $\tau 2$ are their respective decay time constants.

A decay time constant is defined as the time taken for each component to decay to 37 % of its original amplitude. From this, values for the decay time constant of each component could be recorded as well as the relative area of each

component. When several cells had been analysed in this way, mean values could then be determined (See Fig 2.6B).

Fig 2.6:

A



B

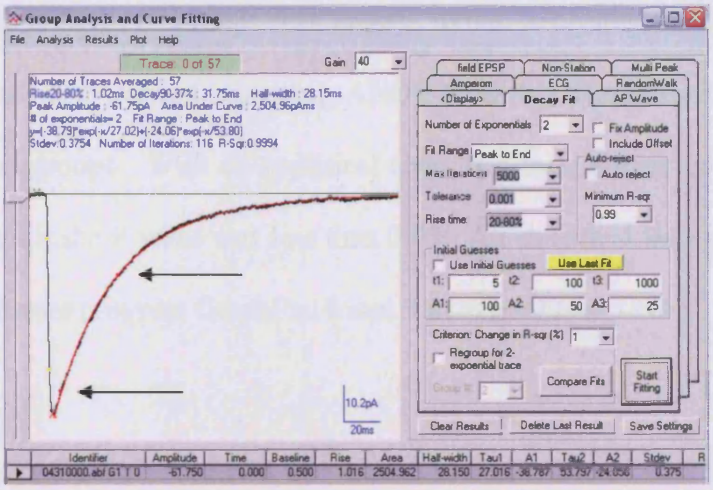


Fig 2.6: Software used for analysis of synaptic currents: MiniAnalysis.

(A) In the main display window the green x indicates the location of the baseline. The yellow dot (●) indicates the starting time of the rising phase of the peak. The red x indicates the maximum point of the peak. The purple dot (●) indicates the decay of the peak (50 %).

(B) 10-90 % rise time is indicated by two yellow dots (●). 90-37 % decay is shown by two purple dots (● , indicated by the arrows). The red x is where the peak is located. The green x and green horizontal line are where the baseline average is located. The peak amplitude is calculated as peak minus baseline average. The area under the curve is calculated from the whole trace with respect to the green line. The equation describing the curve is shown in 2.8.2.

2.8.3 Gaussian fits

Raw current data could be transferred from MiniAnalysis into Origin 6.0 for analysis of distributions. If the data was formed from populations described by a Gaussian distribution this program fitted the data (with multiple Gaussian distributions if necessary) to identify separate populations. The best fit was determined visually.

2.8.4 Statistics

For comparison of two values, statistical analyses were carried out using the unpaired Student's t-test (with a Welch correction if the difference in standard deviations between groups was large). When analysing multiple means, statistical analyses were carried out using ANOVA, with a Bonferroni post-test to compare selected groups. With all statistical tests, two means were considered significantly different if the P value was less than 0.05. All statistical tests were carried out using the software program GraphPad Instat 3.01 (GraphPad, USA).

Chapter Three:

CaMK-II modulation of recombinant $GABA_A$ Receptors expressed in HEK293 cells.

3.1 INTRODUCTION

The HEK293 cell line is endothelial in origin so is generally not thought to express any functional neuronal ligand-gated ion channels (Graham *et al.*, 1977). These cells are suitable for whole-cell patch clamp, which allows electrophysiological recordings to be made of membrane currents following activation of recombinant ion channels that are expressed after transfection. In this way the exact isoform/s or combinations of subunits making up a given receptor/ion channel can be largely controlled, allowing functional dissection of the roles of different subunit isoforms, and the signalling processes that alter their function.

Expression of $GABA_A$ receptor subunits in HEK293 cells has been used previously to investigate the role of PKA-induced phosphorylation, revealing functional heterogeneity between different β subunits (McDonald *et al.*, 1998). Similar approaches have been used to investigate PKC-dependent phosphorylation of $GABA_A$ receptors (Brandon *et al.*, 2002b) and tyrosine kinase-Src dependent modulation (Moss *et al.*, 1995).

It has been shown that CaMK-II can phosphorylate GST-fusion proteins formed from the intracellular loop between transmembrane (TM) domains 3 and 4 of β and γ subunits of $GABA_A$ receptors *in vitro* (Machu *et al.*, 1993; McDonald and Moss, 1994; McDonald and Moss, 1997). In neuronal preparations intracellular addition of pre-activated CaMK-II, through inclusion in the patch pipette, led to a significant increase in the peak amplitude of $GABA_A$ mediated currents (Wang *et al.*,

1995; Kano *et al.*, 1996). Synaptosomal membrane fractions have also been used to show CaMK-II dependent modulation of agonist binding to $GABA_A$ receptors and alterations in benzodiazepine binding to these receptors possibly through phosphorylation of the α1 subunit (Churn and DeLorenzo, 1998; Churn *et al.*, 2002).

However, as yet, no attempt has been made to study the functional effects of CaMK-II dependent phosphorylation of $GABA_A$ receptors in a controlled environment such as the HEK293 cell. Such HEK293 cells have been used to successfully demonstrate CaMK-II dependent functional modulation of AMPA receptors (Barria *et al.*, 1997; Derkach *et al.*, 1999). The advantages of using such a system are that any potential differences in effect caused by phosphorylation on different $GABA_A$ receptor subunits may be determined. This is especially important in the case of GABAergic signalling mechanisms as the $GABA_A$ receptor displays considerable heterogeneity in subunit combinations and variable functional effects depending on the different subunits present in a particular recombinant system or neuronal cell type (Moss and Smart, 1996; Nusser *et al.*, 1999; Brandon *et al.*, 2002a). It is also possible, through site-directed mutagenesis, to disrupt phosphorylation of specific residues within subunits and determine the specific sites of phosphorylation and their relative importance. Using these advantages, the initial aim of these experiments was to express different subunit combinations of the $GABA_A$ receptor in HEK293 cells and to induce CaMK-II dependent phosphorylation to study the functional consequences (See Fig 3.1).

Fig 3.1:

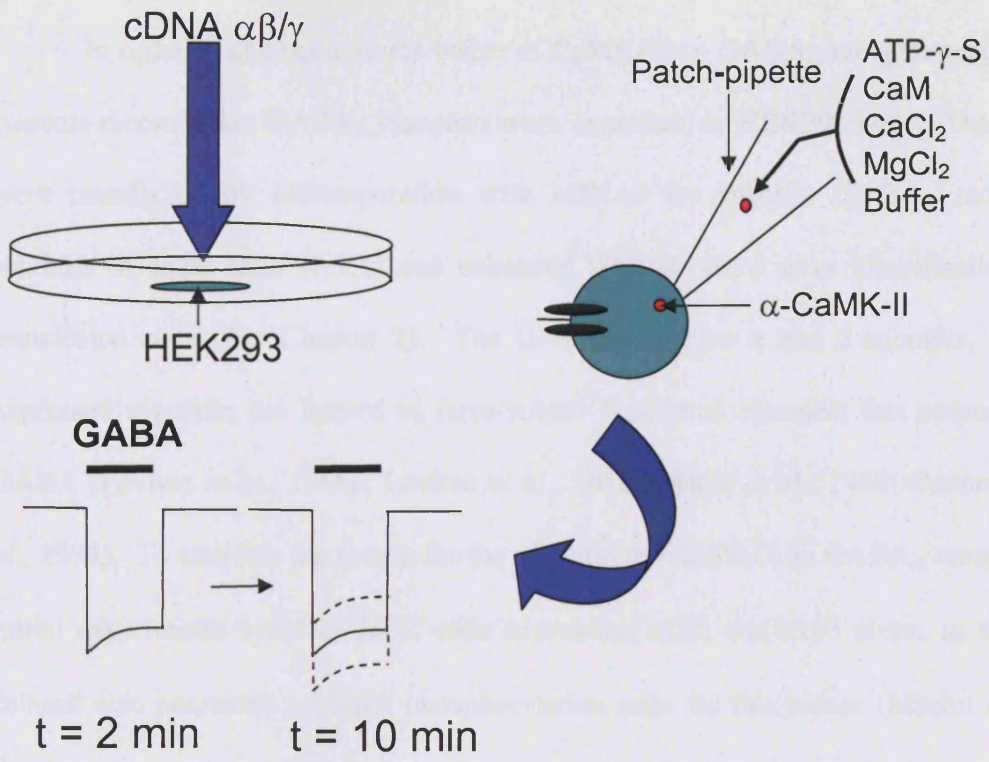


Fig 3.1: Studying CaMK-II dependent effects on $GABA_A$ receptors using HEK293 cells.

The cDNAs of various $GABA_A$ receptor subunits are transfected into HEK293 cells by electroporation (See Chapter 2) along with the plasmid encoding for enhanced GFP to allow identification of transfected cells.

The cells are left for 24 hours to allow expression of the recombinant $GABA_A$ receptors at the cell surface.

Whole-cell currents can then be recorded in response to applied GABA, over time, as pre-activated CaMK-II within the patch pipette dialyses into the cell and modulates the response (dotted lines).

3.2 RESULTS

In order to characterise the effect of CaMK-II on $GABA_A$ receptor mediated currents recombinant $GABA_A$ receptors were expressed in HEK293 cells. The cells were transfected by electroporation with cDNAs for specific $GABA_A$ receptor subunits in equal ratio (1:1:1) and enhanced GFP to allow easy identification of transfected cells (See Chapter 2). The $GABA_A$ receptor α and β subunits, when expressed together, are known to form robust functional channels that respond to GABA (Levitan *et al.*, 1988a; Levitan *et al.*, 1988b; Sigel *et al.*, 1990; Connolly *et al.*, 1996). To simplify the search for the effect(s) of CaMK-II on $GABA_A$ receptors, initial experiments involved HEK cells expressing $\alpha 1\beta 2$ and $\alpha 1\beta 3$ alone, as the $\gamma 2$ subunit also possesses potential phosphorylation sites for this kinase (Machu *et al.*, 1993; McDonald and Moss, 1994).

Whole-cell patch clamp recordings were conducted to measure GABA mediated currents. To assess the stability of these currents over time, a fixed concentration of GABA (10 μM) was applied every 2 min for 20-30 min. The peak amplitude was then normalised to the peak amplitude recorded 3-4 min after achieving the whole cell configuration. Under control conditions, the peak amplitude of the GABA-activated current remained at a relatively constant level for the time of recording with only a slight decrease of about 10-20 % apparent over 30 min (Fig 3.2-3.4). This "run-down" of $GABA_A$ receptor currents has been observed in neurones and in recombinant expression systems and is thought to be associated with a disruption of the phosphorylation state(s) of the receptor. This is possibly through depletion of ATP or an alteration in the concentration of unbuffered Ca^{2+} caused by differences between the internal pipette solution and the normal intracellular milieu

(Stelzer *et al.*, 1988; Chen *et al.*, 1990; Huang and Dillon, 1998; Laschet *et al.*, 2004).

3.2.1 Recombinant $\alpha 1\beta 2$ receptors and α-CaMK-II.

Purified recombinant α-CaMK-II (Brickey *et al.*, 1990; Suzuki-Takeuchi *et al.*, 1992) was pre-incubated in 1.2 μM Calmodulin (CaM), 10 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.4 mM ATP-γ-S to promote autophosphorylation at Thr^{286} (McGlade-McCulloh *et al.*, 1993; Lledo *et al.*, 1995; Wang *et al.*, 1995; Barria *et al.*, 1997; Nelson *et al.*, 2005). This recombinant form of α-CaMK-II represents a structurally simplified form of the kinase that cannot form a holoenzyme but which is capable of phosphorylating the same substrates as the full length form of the kinase (Suzuki-Takeuchi *et al.*, 1992). This recombinant form of α-CaMK-II can be extracted and purified after being expressed at high levels in an appropriate cell line, avoiding the difficulties associated with obtaining purified CaMK-II from whole-brain extracts. In addition this monomeric form of the kinase has a molecular weight of 33 kDa as opposed to the full holoenzyme with a molecular weight of 600 kDa thereby avoiding problems associated with diffusion of such a large molecule out of the patch pipette and into the cell.

ATP-γ-S was used rather than ATP in the pre-activation/autophosphorylation reaction, as thiophosphorylated proteins are less subject to dephosphorylation by the endogenous protein phosphatases that might be present in the HEK cell. The α-CaMK-II and the pre-activation buffer were then diluted into the pipette solution immediately before recording and then maintained on ice (See Chapter 2). To determine that the pre-activation buffer had no effect on GABA mediated currents,

all controls included a mock pre-activation buffer (lacking α-CaMK-II) diluted into the pipette solution at the same ratio.

In previous experiments using a similar form of recombinant α-CaMK-II, 200 nM α-CaMK-II significantly potentiated GABA mediated currents in rat dorsal horn and hippocampal neurones (Wang *et al.*, 1995). Since HEK293 cells are more compact without the large processes of neurones to diffuse through, the concentration of α-CaMK-II was reduced to 100 nM (3.33 ng/μl, See Chapter 2). This concentration should be sufficient to allow phosphorylation of the $GABA_A$ receptor without compromising the quality of recordings. In HEK cells expressing α1β2 subunits, the addition of 100 nM pre-activated α-CaMK-II in the patch pipette induced no significant differences from control GABA currents over a recording period of 30 min. Control currents recorded in response to application of 10 μM GABA at t = 10 min was 88.5 ± 2.3 % (Fig 3.2A, n = 13) and in the presence of α-CaMK-II at t = 10 min was 87.5 ± 3.1 % (Fig 3.2A, n = 5).

In addition, purified rat forebrain CaMK-II (full-length, consisting of a mixture of α, β, γ and δ subunits) was also used and pre-activated in a similar way (co-incubation with 10 mM $MgCl_2$, 1 μM CaM, 0.2 mM EGTA, 0.3 mM $CaCl_2$, 0.4 mM ATP-γ-S; See Chapter 2). This form of CaMK-II (60-100 nM) also had no significant effects on GABA mediated currents which attained 87.8 ± 1.8 % at t = 10 min (n = 4, Fig 3.4A) as compared to control (88.5 ± 2.3 %, n = 13; Fig 3.2A). This form of CaMK-II was tested biochemically and shown to be capable of phosphorylating the β3 subunit intracellular domain *in vitro*, after the pre-activation reaction and in the presence of 150 mM KCl, used to simulate conditions in the patch pipette solution (in collaboration with Dr. J. Jovanovic; Fig 3.4B).

As an alternative method, HEK cells were transfected with $\alpha 1\beta 2$ cDNA and with cDNA for α-CaMK-II in equal ratio. Inclusion of a relatively high CaM (600 nM) concentration in an internal solution with a higher free Ca^{2+} (10 μM) concentration may be expected to activate α-CaMK-II expressed in the HEK cells and thereby modulate GABA mediated currents. However no significant differences were seen between control recordings without CaMK-II co-transfection, CaMK-II co-transfection with normal patch pipette solution, and CaMK-II co-transfection with a high CaM and high free Ca^{2+} patch pipette solution. The free Ca^{2+} concentration in the pipette solution was altered by changing the ratio between EGTA : $CaCl_2$ from 10:1 (10 nM free Ca^{2+}) in the normal pipette solution to 1:1 (10 μM free Ca^{2+}- (Aguayo *et al.*, 1998). After 10 minutes recording, GABA current amplitudes were: Control, 88.5 ± 2.3 % (n = 13); CaMK-II co-transfection, 92.9 ± 5 % (n = 5); and CaMK-II co-transfection with additional Ca^{2+}/CaM, 96.4 ± 6.2 % (n = 9, Fig 3.2B).

Previously, it has been demonstrated that CaM itself can have a functional effect on some ion channels including ligand-gated ion channels like $GABA_A$ receptors. For instance it can have a direct inhibitory effect on NMDA receptor function (Ehlers *et al.*, 1996; Zhang *et al.*, 1998; Rycroft and Gibb, 2002). It can also activate CaM dependent phosphatases such as PP2B/Calcineurin, which may have a direct effect on the $GABA_A$ receptor (Jones and Westbrook, 1997; Wang *et al.*, 2003). In order to determine whether CaM itself was modulating $GABA_A$ receptor function and occluding CaMK-II dependent effects, GABA currents were recorded in the presence of higher concentrations of CaM (1.5 μM) alone. However there were no significant differences observed between control GABA current amplitudes, 88.5 ± 2.3 % (Fig 3.2C, n = 13), and those in the presence of CaM, 90.5 % ± 4.2 % (Fig 3.2C, n = 6) at t = 10 min.

Taken together it appeared that CaMK-II had no functional effect on $\alpha 1\beta 2$ subunit-containing $GABA_A$ receptors expressed in HEK293 cells. Interestingly, cAMP-dependent kinase (PKA) was also unable to functionally modulate $\alpha 1\beta 2$ subunit-containing receptors in HEK cells but had a significant effect on whole-cell currents recorded from $\alpha 1\beta 3$ subunit-containing receptors (McDonald *et al.*, 1998). Therefore the same series of experiments with CaMK-II were repeated in HEK293 cells expressing $\alpha 1\beta 3$ subunits.

Fig 3.2:

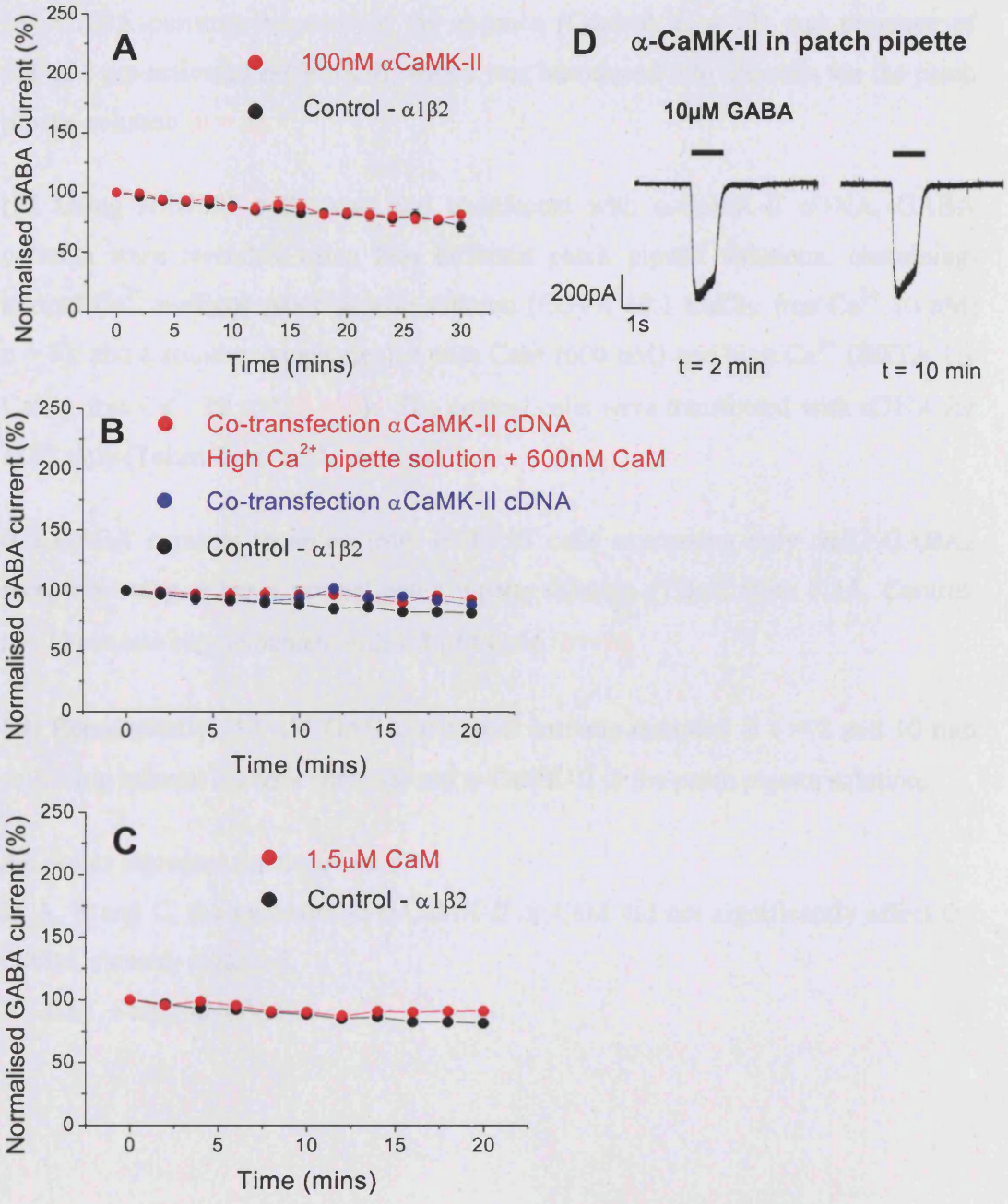


Fig 3.2: α-CaMK-II modulation of whole-cell currents recorded from HEK293 cells expressing α1β2 subunits.

Peak amplitudes of GABA-mediated currents recorded following the application of 10 μM GABA to transfected HEK293 cells expressing α1β2 $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 30 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded in the absence (Control, n = 13) and presence of 100 nM pre-activated α-CaMK-II, which was introduced into the cells via the patch pipette solution (n = 5).

(B) Using HEK293 cells now also transfected with α-CaMK-II cDNA, GABA currents were recorded using two different patch pipette solutions, containing: normal Ca^{2+} buffered patch pipette solution (EGTA 10:1 $CaCl_2$, free Ca^{2+} 10 nM; n = 5); and a solution supplemented with CaM (600 nM) and high Ca^{2+} (EGTA 1:1 $CaCl_2$, free Ca^{2+} 10 μM; n = 9). The control cells were transfected with cDNA for α1β2 only (Taken from 3.2A, n = 13).

(C) GABA currents recorded from HEK293 cells expressing only α1β2 $GABA_A$ receptors using either a normal patch pipette solution (Taken from 3.2A, Control, n = 13) or one supplemented with 1.5 μM CaM (n = 6).

(D) Representative 10 μM GABA-activated currents recorded at t = 2 and 10 min following internal dialysis with 100 nM α-CaMK-II in the patch pipette solution.

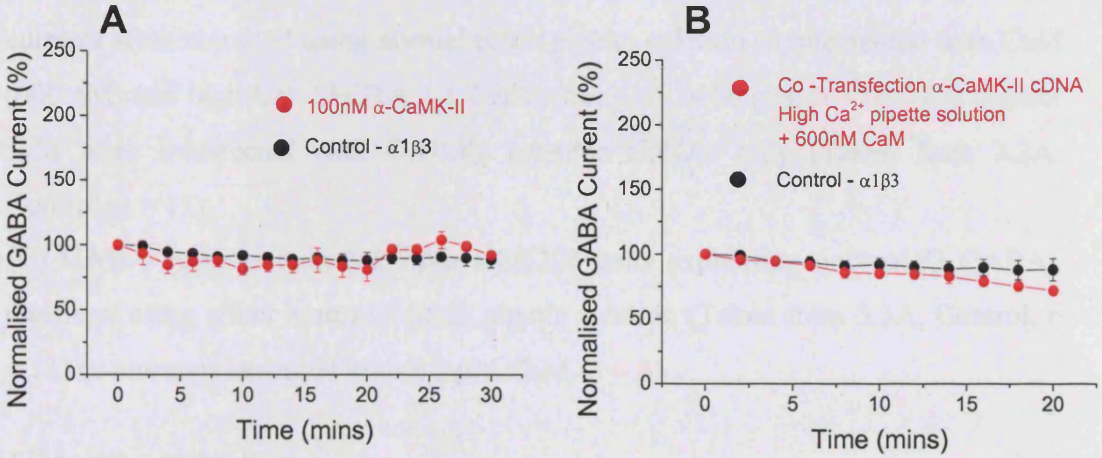
All points represent the mean ± s.e.
In A, B and C, the exposure to α-CaMK-II or CaM did not significantly affect the GABA currents recorded.
$P < 0.05$, t-test, ANOVA.

3.2.2 Recombinant $\alpha 1\beta 3$ $GABA_A$ receptors and CaMK-II

The addition of autophosphorylated α-CaMK-II to the pipette solution also had no significant effects on GABA mediated currents compared to control in cells expressing $\alpha 1\beta 3$ (Control = 92 ± 3.3 %, n = 11; + CaMK-II = 81.7 ± 4.5 %, n = 6; Fig 3.3A, t = 10 min). Furthermore, the co-transfection with α-CaMK-II cDNA and the addition of CaM to a high Ca^{2+} containing patch pipette solution had no significant effects on GABA (10 μM) currents compared to control (Control = 92 ± 3.3 %, n = 11, α-CaMK-II transfection + CaM = 85.2 ± 2.9 %, n = 4, t = 10 min, Fig 3.3A). Finally the addition of 1.5 μM CaM alone to the pipette solution also had no significant effects on the $\alpha 1\beta 3$ $GABA_A$ receptors (Control = 92 ± 3.3 %, n = 11, + CaM = 92.2 ± 3.5 %, n = 6, t = 10 min, Fig 3.3C).

Pre-activated CaMK-II purified from rat forebrain also had no significant effect on GABA mediated currents recorded from HEK293 cells expressing $\alpha 1\beta 3$ (84.4 ± 11.3 %, n = 5, t = 10 min, Fig 3.4A). Taken overall CaMK-II was unable to modulate $\alpha 1\beta 3$ subunit-containing receptors in HEK cells.

Fig 3.3:



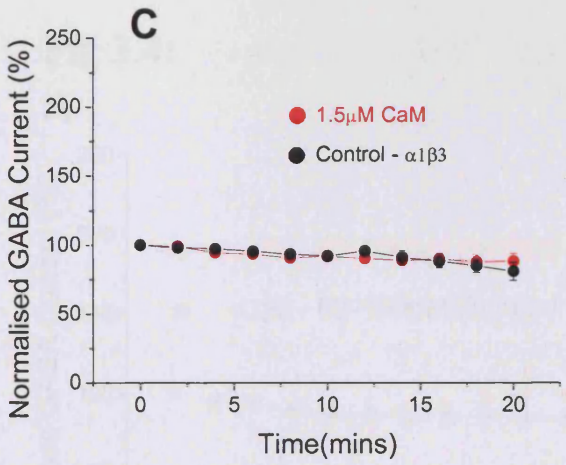


Fig 3.3: α-CaMK-II modulation of whole-cell currents recorded from HEK293 cells expressing α1β3 subunits.

Peak amplitudes of GABA-mediated currents recorded following the application of 10 μM GABA to transfected HEK293 cells expressing α1β3 $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 30 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded in the absence (Control, n = 11) and presence of 100 nM pre-activated α-CaMK-II, which was introduced into the cells via the patch pipette solution (n = 6).

(B) Using HEK293 cells now also transfected with α-CaMK-II cDNA, GABA currents were recorded using normal patch pipette solution supplemented with CaM (600 nM) and high Ca^{2+} (EGTA 1:1 $CaCl_2$: free Ca^{2+} ≈10 μM; n = 4). The control cells were transfected with $GABA_A$ receptor cDNAs only (Taken from 3.3A, Control, n = 11).

(C) GABA currents recorded from HEK293 cells expressing only α1β3 $GABA_A$ receptors using either a normal patch pipette solution (Taken from 3.3A, Control, n = 11) or one supplemented with 1.5 μM CaM (n = 6).

All points = mean ± s.e.

In A, B and C, the exposure to α-CaMK-II or CaM did not significantly affect the GABA currents recorded.

$P < 0.05$, t-test.

Fig 3.4:

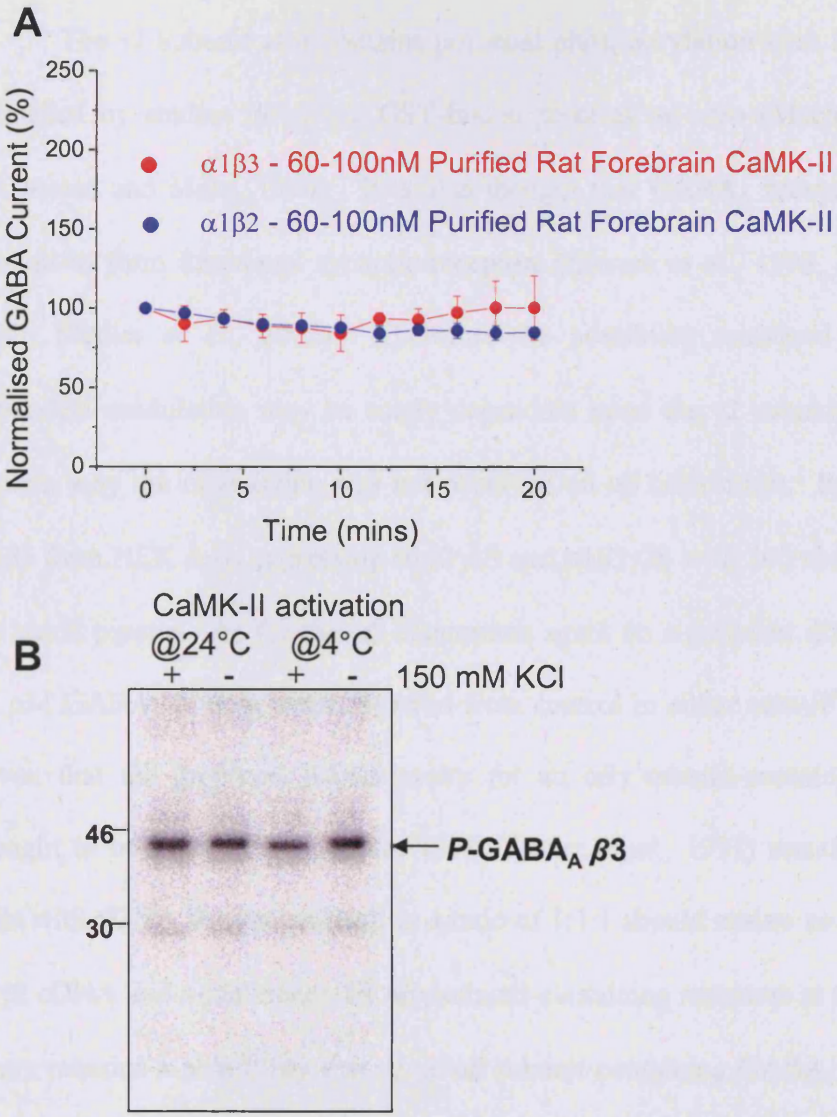


Fig 3.4: Purified rat forebrain CaMK-II phosphorylation of the β3 subunit *in vitro* and modulation of GABA currents in HEK293 cells.

(A) GABA currents recorded with patch pipette solution supplemented with purified pre-activated CaMK-II (60-100 nM) from HEK293 cells expressing α1β2 (n = 4) and α1β3 (n = 5). All points are mean ± s.e.

(B) Purified pre-activated CaMK-II incubated with GST-β3 and ^{32}P-ATP at 25°C or 5°C with or without 150 mM KCl, visualized by autoradiography.
Phosphorylation reaction was carried out in collaboration with Dr J. Jovanovic (See Chapter 2).

3.2.3 Recombinant $\alpha 1\beta 2/3\gamma 2$ $GABA_A$ receptors and CaMK-II.

The $\gamma 2$ subunit also contains potential phosphorylation sites for CaMK-II as identified by studies involving GST-fusion proteins *in vitro* (Machu *et al.*, 1993; McDonald and Moss, 1994). It is also thought that $GABA_A$ receptors require a γ subunit to form functional synaptic receptors (Essrich *et al.*, 1998; Brickley *et al.*, 1999; Studler *et al.*, 2002). Therefore the possibility remained that CaMK-II dependent modulation may be solely dependent upon the $\gamma 2$ subunit, which would explain why the modulation was not observed on $\alpha\beta$ heteromers. Recordings were made from HEK cells expressing $\alpha 1\beta 2\gamma 2S$ and $\alpha 1\beta 3\gamma 2S$ with 100 nM α-CaMK-II in the patch pipette. As for the $\alpha\beta$ heteromers again no significant differences in the 10 μM GABA currents were observed from control in either case (Fig 3.5A, 3.5B). Given that the proposed stoichiometry for an $\alpha\beta\gamma$ subunit-containing receptor is thought to be 2:2:1 (Chang *et al.*, 1996; Tretter *et al.*, 1997) transfection of HEK cells with cDNA for each subunit in a ratio of 1:1:1 should ensure an overabundance of $\gamma 2$ cDNA and a preference for $\alpha\beta\gamma$ subunit-containing receptors at the cell surface. There remains a possibility that some $\alpha\beta$ subunit-containing $GABA_A$ receptors may have been present and there is evidence that this can occur in HEK cells (Boileau *et al.*, 2003). There is also, however, evidence that when $\alpha\beta$ and γ subunits are expressed together within a cell line $\alpha\beta\gamma$ channels are formed preferentially (Farrar *et al.*, 1999) and that the ratio of cDNA has to be increased to $\alpha\beta\gamma$ 1:4:1 before $\alpha\beta$ receptors can be detected alongside $\alpha\beta\gamma$ (Angelotti *et al.*, 1993; Angelotti and Macdonald, 1993). Angelotti *et al.*, (2004) also observed that incorporation of the $\gamma 2$ subunit increased the peak amplitudes of the currents recorded. This was also observed in the current study (Fig 3.2D, Fig 3.5C). For the purposes of this experiment there should have been sufficient expression of $\gamma 2S$ subunit-containing

receptors at the cell surface to determine if CaMK-II can functionally modulate these receptors.

Fig 3.5:

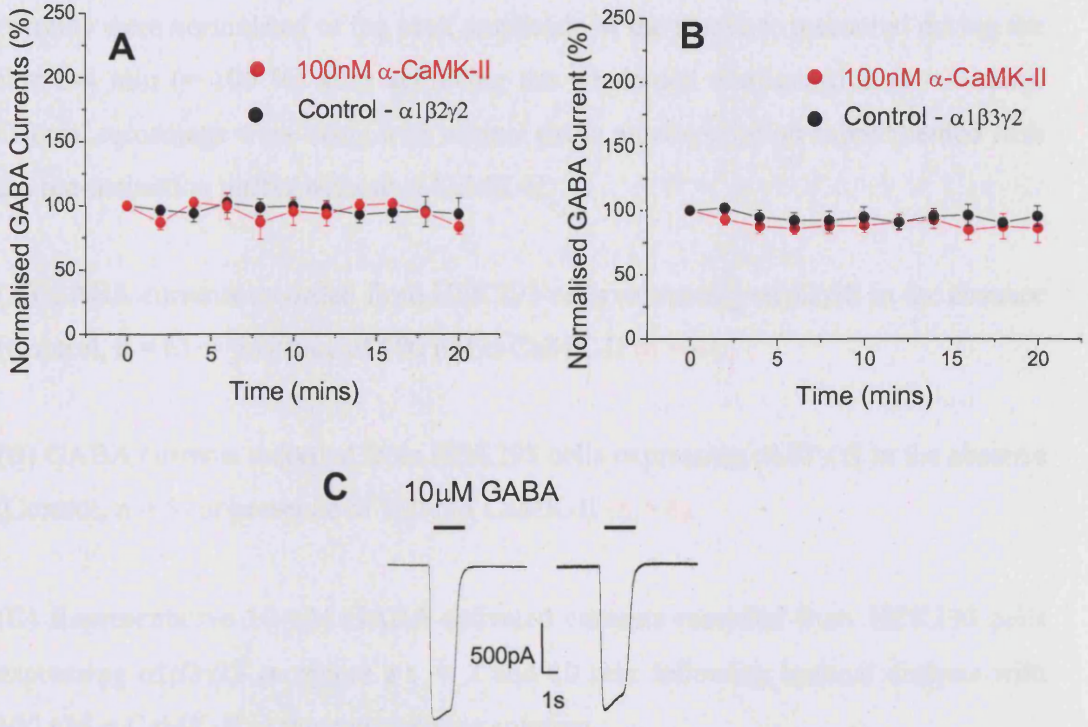


Fig 3.5: α-CaMK-II modulation of whole-cell currents recorded from HEK293 cells expressing α1β2γ2S and α1β3γ2S subunits.

Peak amplitudes of GABA-mediated currents recorded following the application of 10 μM GABA to transfected HEK293 cells expressing α1β2γ2S and α1β3γ2S $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 30 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from HEK293 cells expressing α1β2γ2S in the absence (Control, n = 6) or presence of 100 nM α-CaMK-II (n = 6).

(B) GABA currents recorded from HEK293 cells expressing α1β3γ2S in the absence (Control, n = 5) or presence of 100 nM CaMK-II (n = 8).

(C) Representative 10 μM GABA-activated currents recorded from HEK293 cells expressing α1β2γ2S receptors a t = 2 and 10 min following internal dialysis with 100 nM α-CaMK-II in the patch pipette solution.
Note: Increased current amplitude compared to Fig 3.2C.

All points = mean ± s.e.
In A, B and C, the exposure to α-CaMK-II or CaM did not significantly affect the GABA currents recorded.
$P < 0.05$, t-test.

3.3 DISCUSSION

CaMK-II has previously been shown to modulate $GABA_A$ receptor mediated currents in neurones that are known to express predominantly β2/3 and γ2 subunits (Wang *et al.*, 1995; Kano *et al.*, 1996; Wei *et al.*, 2004). Biochemical analysis of CaMK-II dependent phosphorylation of fusion proteins formed from the intracellular loops of different subunits, indicates that the β and γ subunits are the main candidates for phosphorylation (McDonald and Moss, 1994; Connolly *et al.*, 1996; McDonald and Moss, 1997). However, the present study shows that the expression of receptors containing β2/3 and γ2S in HEK cells are not functionally modulated by CaMK-II.

It is possible that other subunits or subunit combinations that were not tested here are responsible for the CaMK-II mediated effect observed in neurones. For instance there is a potential site for phosphorylation in the 8 amino acid insert in the γ2L splice variant. There are also potential sites for CaMK-II phosphorylation on the β1 subunit (See Chapter 1, Table 1.1). The majority of β subunits expressed throughout the brain are β2 followed by lower levels of β3 and β1 (Benke *et al.*, 1994). The expression of γ2 is widespread with relatively low expression levels of other γ isoforms in a few selected cell types (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Pirker *et al.*, 2000). CaMK-II dependent effects $GABA_A$ receptors have been observed in hippocampal CA1 neurones, which are thought to express β2/3/γ2 subunits. CaMK-II mediated effects have also been observed in cerebellar Purkinje cells that are thought to express predominantly α1β2γ2 receptor subunits (Persohn *et al.*, 1992; Laurie *et al.*, 1992a) and so it would be predicted therefore, that $GABA_A$ receptors containing β2/3/γ2 are good candidates for CaMK-II dependent modulation.

More recently, it has been suggested that the $GABA_A$ receptor α1 subunit may also be phosphorylated by CaMK-II and that this phosphorylation modulates agonist and allosteric-modulator binding (Churn *et al.*, 2002). This subunit was included in all of the combinations tested and there was no evidence of altered $GABA_A$ receptor function. The majority of biochemical studies have identified β and γ and not α as the main sites of phosphorylation suggesting the α subunit is a poor substrate for phosphorylation (Moss *et al.*, 1992; McDonald and Moss, 1994; Moss and Smart, 1996).

Given the contradictory nature of the literature and the present results it is a more likely possibility that CaMK-II dependent modulation of $GABA_A$ receptor mediated currents is not possible in HEK293 cells because of an inherent difference between a neuronal environment and that of a HEK cell which is of epithelial origin. For instance the background of protein kinase and protein phosphatase activity within the intracellular milieu may be quite different. A very high level of phosphatase activity in HEK cells may act to prevent any modulation by CaMK-II by acting to dephosphorylate the $GABA_A$ receptor itself. Recombinant $GABA_A$ receptors expressed within different cell lines have been shown to display different functional properties. Comparison of $\alpha1\beta2\gamma2$ mediated currents recorded in HEK293 cells and QT6 cells reveals that the deactivation kinetics of the receptor can be modulated by the cell type used for expression. Possibly, this may result because of differences in endogenous kinases and phosphatases, or in the proteins linking the receptors to the cytoskeleton (Mercik *et al.*, 2003). In support of this there is evidence that the scaffolding protein GABARAP, which is thought to promote clustering of $GABA_A$ receptors, also affects the channel properties of the receptor

when GABARAP and $GABA_A$ receptors are co-expressed in L929 cells (Everitt *et al.*, 2004).

In addition, there is some evidence that HEK293 cells can express endogenous $GABA_A$ receptor subunits that can co-assemble with subunits transfected into the cell altering the observed properties of the $GABA_A$ receptor mediated currents recorded from HEK293 cells (Ueno *et al.*, 1996). However, this scenario has not been reported by all laboratories using HEK293 cells (Pritchett *et al.*, 1988) and has not been observed using the batches of HEK293 cells used in the current experiments (Connolly *et al.*, 1996; Thomas and Smart, 2005).

Despite these potential reservations, it is also clear that HEK cells have been used quite successfully in the study of PKA, PKC and the tyrosine kinase Src on $GABA_A$ receptor function (Moss *et al.*, 1995; McDonald *et al.*, 1998; Brandon *et al.*, 2002b). The apparent inability of CaMK-II to modulate receptor function in HEK293 cells may simply reflect the inability of CaMK-II to associate with the $GABA_A$ receptor in these cells.

There is a possibility that the diffusion of CaMK-II into the cell was too slow to reach full concentration in the time allowed. However, co-transfection of cDNA for CaMK-II would have produced a large amount of CaMK-II within the HEK cell before electrophysiological recording. The addition of CaM and an increase in free Ca^{2+} still produced no obvious modulation of $GABA_A$ receptors. A recombinant form of α-CaMK-II, identical to the one used in these experiments, has been shown to have a diffusion coefficient of 4-5$\mu m^2/s$ within rat basophilic leukemia cells (RBL 2H3) (Shen and Meyer, 1998). This should be sufficient to allow diffusion throughout the relatively compact HEK cell within the time allowed. Examination of CaMK-II effects on AMPA receptors within HEK cells using a similar form of

recombinant, truncated CaMK-II, activated by a similar autophosphorylation protocol, showed a significant modulation of currents within 10 min (Barria *et al.*, 1997). Another experiment with the same form of CaMK-II and using the *in vitro* autophosphorylation procedure as described in this study also demonstrated a functional effect on excitatory post synaptic currents (EPSCs) and whole-cell responses to AMPA in hippocampal pyramidal cells within 10-20 min (Lledo *et al.*, 1995).

There is also a possibility that CaMK-II activity was low when in the patch pipette solution either due to problems with the pre-activation reaction or due to deactivation within the patch pipette solution. This could be tested functionally by applying the same preparation to a neurone to establish whether neuronal $GABA_A$ receptors could be modulated by CaMK-II. Under such conditions, the same recombinant α-CaMK-II preparation as that used to record from HEK293 cells could functionally modulate GABA mediated currents recorded from cerebellar granule cells (See Chapter 4).

In addition to recombinant α-CaMK-II a second preparation of purified rat forebrain CaMK-II consisting of a mixture of different isoforms of CaMK-II was also pre-activated and added to the patch pipette solution. This preparation of CaMK-II was tested for its ability to phosphorylate a GST-fusion protein of the intracellular loop of the β3 subunit (See Fig 3.4B). This form of CaMK-II was shown to be able to phosphorylate the β3 subunit TM3-4 intracellular loop *in vitro* (McDonald and Moss, 1997) but the same preparation also failed to significantly modulate GABA currents recorded from HEK293 cells expressing $\alpha 1\beta 2/3$ after being included in the patch pipette in the same way as the recombinant α-CaMK-II (See Fig 3.4).

The evidence that CaMK-II can directly phosphorylate $GABA_A$ receptors comes from analysis of GST-fusion proteins (McDonald and Moss, 1994; McDonald and Moss, 1997). The results indicated that CaMK-II could phosphorylate β1/2 and 3 receptor subunits and γ2S/L at previously identified consensus sites on the intracellular loop between TM 3 and 4 (See Chapter 1, Table 1.1). It should be noted however that fusion proteins are not directly comparable to full-length, native receptor subunits. For example the β2 subunit cannot be functionally modulated by PKA within HEK293 cells although it can be phosphorylated *in vitro* (McDonald *et al.*, 1998). It therefore needs to be confirmed that full-length native $GABA_A$ receptors can be phosphorylated by CaMK-II. It is possible that within neurones CaMK-II does not phosphorylate the $GABA_A$ receptor directly, acting instead, through another kinase or signalling pathway.

The signalling cascade involved in forms of excitatory plasticity such as LTP requires CaMK-II for induction. It has been revealed there are two mechanisms of action involved in CaMK-II modulation of AMPA receptors (Poncer *et al.*, 2002). One involves a direct phosphorylation of the GluR1 subunit at Ser^{831} (Barria *et al.*, 1997) and the other involving an insertion of AMPA receptors into the postsynaptic membrane. This insertion of receptors does not occur through direct phosphorylation but through CaMK-II dependent activation of a downstream-signalling cascade involving Ras/Rap GTPases (Hayashi *et al.*, 2000; Zhu *et al.*, 2002). So CaMK-II can act both directly and indirectly to modulate AMPA receptor function. It is interesting, however, that CaMK-II modulation of AMPA receptors can be observed in HEK cells (Barria *et al.*, 1997), suggesting there is a key difference between CaMK-II modulation of AMPA and $GABA_A$ receptors.

CaMK-II is found in high levels throughout the brain and has a large number of potential substrates (Hudmon and Schulman, 2002). It may be that in order to phosphorylate the $GABA_A$ receptor or other targets successfully, it has to be targeted to the correct location. Anchoring proteins can restrict the subcellular location, at which a signalling molecule like CaMK-II exerts its effect, controlling which substrates are phosphorylated, by the location of the kinase (Bayer and Schulman, 2001; Schulman, 2004; Colbran, 2004). Such anchoring proteins can also have a direct effect on the activity of the kinase. The interaction between CaMK-II and the NMDA receptor is one example of this. Binding of CaMK-II to the NMDA receptor acts in the same way as Ca^{2+}/CaM binding, allowing CaMK-II to be active when bound to the NMDA receptor. In this way CaMK-II is localised to a specific location and its activity is altered through an interaction with a binding partner (Bayer *et al.*, 2001).

A number of potential CaMK-II anchoring proteins have been identified which localise the kinase to targets of phosphorylation (See Chapter 1). E.g. CaMK-II binding to the NMDA receptor (Strack *et al.*, 2000; Bayer *et al.*, 2001) localising the kinase at the excitatory post-synaptic density (PSD). In addition a non-catalytic subunit of CaMK-II has been identified which can localise CaMK-II to the sarcoplasmic reticulum in muscle cells (Bayer *et al.*, 1998). It is thought that there are probably a number of other proteins with a similar function that have yet to be identified (Bayer and Schulman, 2001; Griffith *et al.*, 2003; Colbran, 2004).

Other serine/threonine kinases like PKC are known to interact with anchoring proteins at the $GABA_A$ receptor, e.g. RACK-1, which binds to the $GABA_A$ receptor enhancing PKC phosphorylation of Ser^{409} (Brandon *et al.*, 2002b). Furthermore the anchoring protein, AKAP150 can also directly bind the $GABA_A$ receptor and has

been shown to be critical for PKA mediated phosphorylation of the β3 subunit (Brandon *et al.*, 2003). Currently relatively little is known about the scaffolding and signalling complexes that exist for the $GABA_A$ receptor at inhibitory synapses. It may be that in order for CaMK-II to exert its effect, an additional anchoring protein may be required which is not present in HEK cells or alternatively, that CaMK-II phosphorylates another substrate closely linked to the $GABA_A$ receptor which then goes on to modulate $GABA_A$ receptor function.

It may be possible to determine in a neuronal preparation which subunits are important for CaMK-II modulation through transfection of neurones or the use of knock-out animals lacking specific subunits of the $GABA_A$ receptor. It seems a neuronal system or neuronal cell-type environment may be crucial to reliably determine the signalling pathways and mechanisms of action involved in CaMK-II dependent modulation of $GABA_A$ receptors. It is also important to try to determine if CaMK-II directly phosphorylates the $GABA_A$ receptor, or if it acts through another kinase or signalling pathway.

3.4 CONCLUSIONS

- Intracellular dialysis of two different preparations of pre-activated CaMK-II failed to modulate α1β2/3γ2 $GABA_A$ receptors.
- Co-transfection with α-CaMK-II cDNA and intracellular dialysis of CaM and Ca^{2+} fails to modulate $GABA_A$ receptors expressed in HEK293 cells.
- Intracellular dialysis of CaM alone also had no effect on the amplitude of GABA mediated currents.
- Recombinant $GABA_A$ receptors expressed in HEK293 cells cannot be modulated by CaMK-II.

Chapter Four:

CaMK-II dependent modulation of $GABA_A$ receptors in cerebellar granule cells.

4.1 INTRODUCTION

CaMK-II has previously been shown to modulate $GABA_A$ mediated currents in a number of neuronal preparations (Kano *et al.*, 1992; Wang *et al.*, 1995; Kano *et al.*, 1996; Aguayo *et al.*, 1998; Kawaguchi and Hirano, 2002; Mizoguchi *et al.*, 2003; Wei *et al.*, 2004). In order to confirm these results it was necessary to repeat the experiments of the previous chapter in a neuronal cell type, given the lack of significant modulation observed in HEK293 cells. The same protocols that were applied in chapter three were adapted for cerebellar granule cells maintained in culture. The aim was to determine if CaMK-II can modulate $GABA_A$ receptors in this cell type and if so, to characterise this modulation.

Intracellular dialysis of pre-activated α-CaMK-II has been previously shown to increase the peak amplitude of whole-cell GABA currents and IPSCs recorded in rat spinal dorsal horn neurones, hippocampal pyramidal and cerebellar Purkinje cells (Wang *et al.*, 1995; Kano *et al.*, 1996). Calcium dependent increases in the peak amplitude of $GABA_A$ mediated currents that could be blocked by CaMK-II inhibitors have also been observed in mouse cortical neurones and hippocampal pyramidal neurones (Aguayo *et al.*, 1998; Mizoguchi *et al.*, 2003; Wei *et al.*, 2004). As CaMK-II dependent modulation of $GABA_A$ occurs in a range of different neuronal cell types, it seemed reasonable to assume that CaMK-II dependent modulation of $GABA_A$ receptors might be observed in cerebellar granule cells (CGCs). CGCs are thought to express a wide variety of different $GABA_A$ receptor subunits (Wisden *et*

al., 1996) so if only certain subunits of $GABA_A$ receptors could be modulated by CaMK-II there was a high probability that they would exist in CGCs. This array of subunits could be important, since some $GABA_A$ receptors are differentially sensitive to phosphorylation. For example, the expression of different β subunits has been shown using heterologous expression systems and in neuronal preparations to alter the response to PKA phosphorylation (McDonald *et al.*, 1998; Nusser *et al.*, 1999).

Equally important in determining the phosphorylation state of $GABA_A$ receptors, and therefore potential changes in function, are protein phosphatases, which act to dephosphorylate the $GABA_A$ receptor itself and can also regulate CaMK-II activity. Wang and colleagues (1995) demonstrated that in isolated spinal dorsal horn neurones the addition of calyculin A, an inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), caused an increase in the peak amplitude of whole-cell GABA currents recorded similar to that observed after the addition of pre-activated CaMK-II. Although PP1, PP2A and protein phosphatase 2B (PP2B)/calcineurin have been shown to directly modulate $GABA_A$ receptor mediated currents (Wang *et al.*, 2003; Jovanovic *et al.*, 2004; Terunuma *et al.*, 2004; Sanchez *et al.*, 2005), protein phosphatases can also directly modulate CaMK-II activity. For example in neurones, phosphatases can alter the levels of Thr^{286} autophosphorylation and also the inhibitory autophosphorylation sites $Thr^{305/6}$ (Fukunaga *et al.*, 1989; Fukunaga *et al.*, 1993; Kasahara *et al.*, 1999; Colbran, 2004), which will have indirect consequences for $GABA_A$ receptor function.

The dynamic balance between phosphatase and kinase activities will determine the overall phosphorylation state of the receptor and result in altered function accordingly. Therefore the phosphatase inhibitor okadaic acid (OA) was used to determine the role of various phosphatases in cerebellar granule cells in

altering $GABA_A$ receptor function. At different concentrations, OA inhibits various protein phosphatases (Bialojan and Takai, 1988) and so the relative contribution of different protein phosphatases in modulating $GABA_A$ receptor function could be determined. The relative balance between CaMK-II activity and phosphatase activity, under basal conditions, could then be determined in CGCs under these particular culture conditions.

4.2 RESULTS

Rat cerebellar granule cells were cultured in a 20 mM KCl supplemented, serum free medium and recorded from after 10-14 days *in vitro* (DIV, See Fig 2.1B). A recombinant form of α-CaMK-II, (Brickey *et al.*, 1990; Suzuki-Takeuchi *et al.*, 1992) 1200-1500 nM (40-50 ng/μl), was pre-incubated in 2 mM $CaCl_2$, 1.2 μM CaM, 0.4 mM ATP-γ-S, 10 mM $MgCl_2$ in a total volume of 16-20 μl (See Chapter 2 and 3.2.1). This was then transferred to the patch pipette solution at a dilution of at least 1:12 and maintained on ice throughout the recording (Brickey *et al.*, 1990; Suzuki-Takeuchi *et al.*, 1992; McGlade-McCulloh *et al.*, 1993). Controls consisted of using the same patch pipette solution containing pre-activation buffer diluted at the same ratio but without α-CaMK-II present or with heat-inactivated α-CaMK-II (95°C for 15 min). Whole-cell patch clamp recordings were then made in response to 10 μM GABA applied intermittently for a period of 15-20 min. Cerebellar granule cells have very small cell bodies. Their relatively compact morphology results in recordings with very low noise and high fidelity for voltage clamp; however, due to their size, recordings of over 20 min were often difficult to maintain.

4.2.1 Cerebellar granule cells in culture and modulation by α-CaMK-II

The inclusion of 100 nM α-CaMK-II in the patch pipette solution and subsequent dialysis into the cell resulted in a significant increase in the peak amplitude of GABA currents to 204.9 ± 24.7 % (n = 7) as compared to control currents, 96.5 ± 4.2 % (n = 7, Fig 4.1A) 10 min into the recording. A reduction in the concentration of α-CaMK-II to 35 nM resulted in a smaller, but still statistically significant, increase in the peak amplitude of GABA currents recorded to 127.3 ± 4.7 % (n = 7, Fig 4.1A) at t = 10 min. It appeared therefore that the effect of α-CaMK-II was dose-dependent and reached a plateau within 10 min. As a second control, α-CaMK-II was heat-inactivated and applied in the same way (100 nM). In this case no increase was observed compared to control currents (93 ± 7.3 %, n = 7, Fig 4.1A) at t = 10 min.

To confirm that this effect is present in different neuronal cell types, 85 nM pre-activated α-CaMK-II was added to the patch pipette solution and whole-cell currents were recorded from cortical pyramidal neurones in culture (DIV6-8). Reducing the concentration to 85 nM improved the quality and duration of recordings whilst producing an easily observed effect. Intracellular addition of 85 nM α-CaMK-II led to a significant increase in the peak amplitude of GABA currents recorded to 122.3 ± 5.3 % (n = 14, Fig 4.1C) at t = 10 min as compared to the equivalent time in control, 90.2 ± 4.1 % (n = 9, Fig 4.1C). The increase in this case appeared to be proportionately smaller than in CGCs and diminished to control levels within 25 min. In CGCs the effect remained stable and did not decay, although recordings of over 20 min were not routinely possible.

In order to further characterise the nature of this up-regulation of $GABA_A$ receptor function the dose-response relationship to GABA was determined under

control conditions at t = 10 min and in the presence of 85 nM α-CaMK-II. The EC_{50} value for GABA in the presence of α-CaMK-II was 6.3 ± 0.5 μM (n = 13, Fig 4.2), and in control conditions, it was not significantly different, at 5.9 ± 0.7 μM (n = 12, Fig 4.2). Indicating there was no change in the potency of GABA at these receptors. There was also no significant difference in the Hill slope (Control = 0.85 ± 1.7, CaMK-II 0.93 ± 0.6). However the maximal response to GABA appeared to have significantly increased from 127.4 ± 15.1 pA/pF to 212.1 ± 26.1 pA/pF.

Fig 4.1:

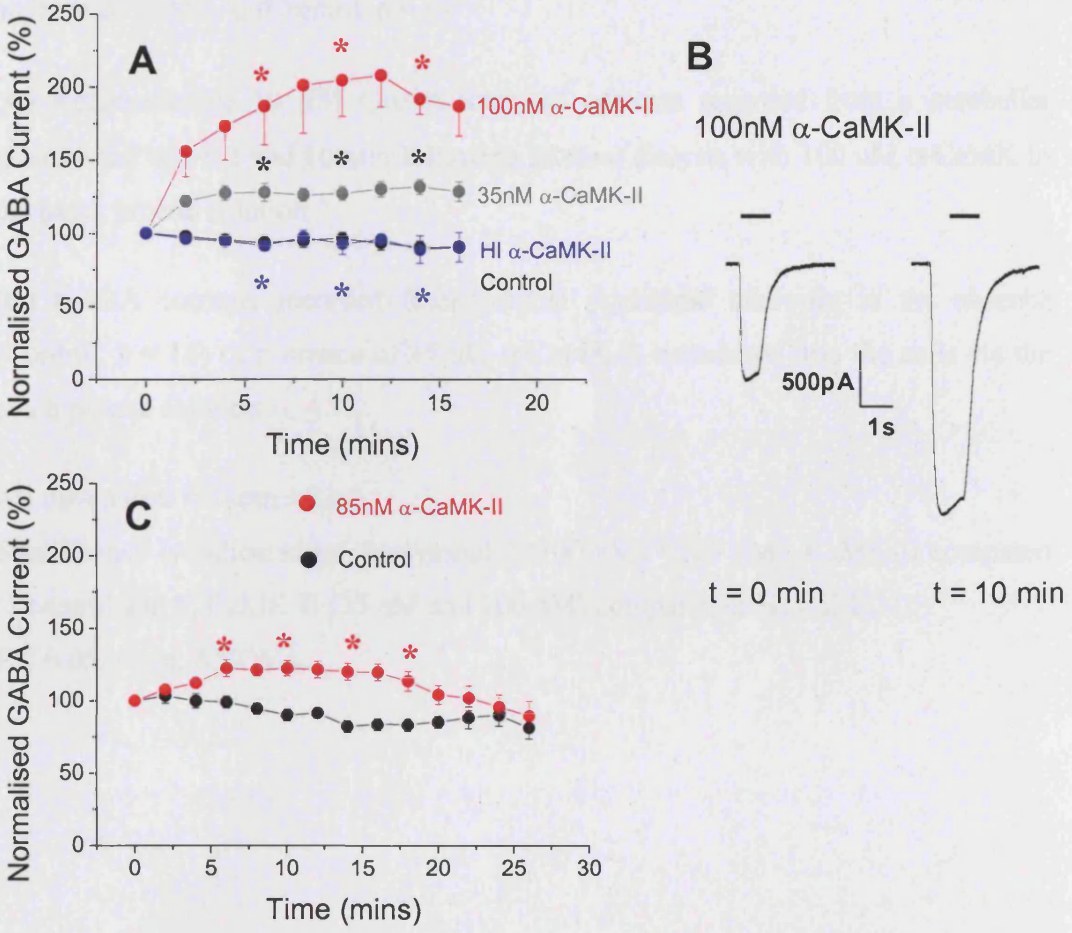


Fig 4.1: α-CaMK-II modulation of whole-cell GABA currents recorded from cerebellar granule and cortical pyramidal cells in culture.

Peak amplitudes of GABA-mediated currents were recorded after application of 10 μM GABA to cerebellar granule or cortical pyramidal neurones in culture. GABA was re-applied at 2 min intervals for up to 30 min. All currents were normalised to the peak response of the GABA current measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from cerebellar granule neurones using patch pipette solution supplemented with 100 nM pre-activated α-CaMK-II (n = 7), 35 nM α-CaMK-II (n = 7), 100 nM heat-inactivated (HI) α-CaMK-II (n = 7) or in the absence of CaMK-II (Control, n = 6).

(B) Representative 10 μM GABA-activated currents recorded from a cerebellar granule cell at t = 2 and 10 min following internal dialysis with 100 nM α-CaMK in the patch pipette solution

(C) GABA currents recorded from cortical pyramidal neurones in the absence (Control, n = 14) or presence of 85 nM α-CaMK-II introduced into the cells via the patch pipette solution (n = 9).

All data points = mean ± s.e.

Significance is indicated by the symbol * (100 nM) * (35 nM), CaMK-II compared to control and *, CaMK-II (35 nM and 100 nM) compared to HI CaMK-II.

$P < 0.05$, t-test, ANOVA.

Fig 4.2:

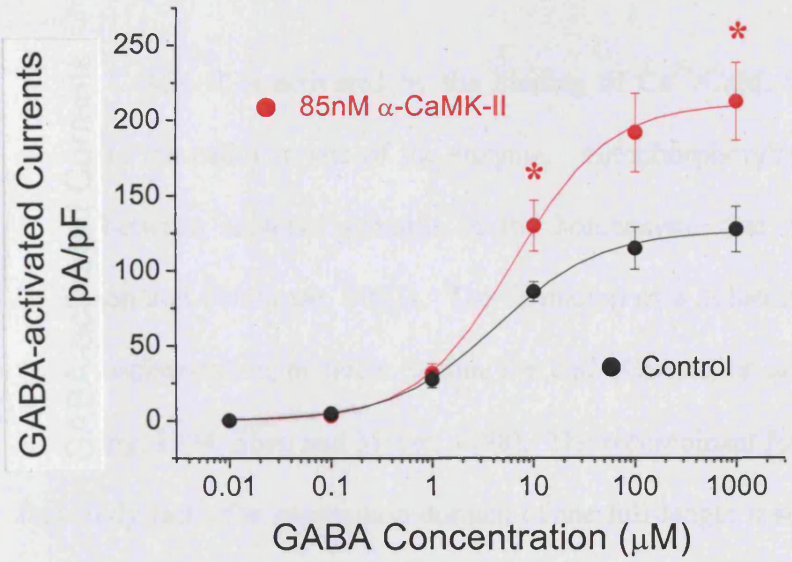


Fig 4.2: The concentration response relationship of GABA in the absence and presence of α-CaMK-II.

Peak amplitudes GABA-mediated currents were recorded in response to various concentrations of GABA normalised to the cerebellar granule cell's membrane capacitance (pA/pF).

GABA currents were recorded with a patch pipette solution supplemented with 85 nM pre-activated α-CaMK-II (n = 13) and normal patch pipette solution supplemented with pre-activation buffer without CaMK-II (Control, n = 12). Currents were recorded at t = 10 min after the effect of α-CaMK-II had reached a plateau.

All data points = mean ± s.e.

Significance is indicated by the symbol *, CaMK-II compared to control.

$P < 0.05$, t-test.

4.2.2 Modulation of $GABA_A$ receptors in cerebellar granule cells by endogenous CaMK-II.

CaMK-II is activated by the binding of Ca^{2+}/CaM, which allows substrate access to the catalytic site of the enzyme. Autophosphorylation at Thr^{286} can then occur between adjacent subunits in the holoenzyme that have bound Ca^{2+}/CaM (Hudmon and Schulman, 2002). The formation of a holoenzyme is critical for this autophosphorylation to occur within the cell (Hanson *et al.*, 1994; Mukherji and Soderling, 1994; Shen and Meyer, 1998). The recombinant form of CaMK-II used in this study lacks the association domain of the full-length α subunit and so it cannot form a holoenzyme. However it has the same catalytic properties as native CaMK-II and can phosphorylate the same substrates (Suzuki-Takeuchi *et al.*, 1992; Lledo *et al.*, 1995). Incubation of Ca^{2+}/CaM and ATP-γ-S *in vitro,* in a small volume, enables autophosphorylation to occur.

The CaMK-II inhibitor, KN-93, prevents Ca^{2+}/CaM binding and so in this way prevents Ca^{2+}/CaM from increasing CaMK-II activity (Sumi *et al.*, 1991). Application of KN-93 to the pre-activation buffer (15-20 μM) should prevent Ca^{2+}/CaM binding and autophosphorylation at Thr^{286} from occurring and therefore prevent CaMK-II activation. When α-CaMK-II and the pre-activation buffer are then diluted into the patch pipette solution the α-CaMK-II that then dialyses into the cell will be in its basal state and therefore inactive. Endogenous Ca^{2+}/CaM within the cell is unlikely to activate the recombinant α-CaMK-II because it cannot form a holoenzyme and in addition KN-93 will still be present in the patch pipette solution at a concentration of 500 nM.

The addition of the pre-activation buffer containing KN-93 (500 nM in patch pipette solution) and α-CaMK-II (35 nM) to the patch pipette solution resulted in a

significant depression of the peak amplitude of whole-cell GABA currents recorded to 71.9 ± 4.6 % at t = 10 min (n = 10, Fig 4.3A), compared to controls of, 96.5 ± 5.1 % (n = 6, Fig 4.3A). This suggested that endogenous CaMK-II is active and maintaining $GABA_A$ receptor function under basal conditions. The addition of KN-93 alone led to a similar significant decrease in the amplitude of currents recorded to 69.3 ± 7.6 % at t = 10 min. The fact that the depression with or without α-CaMK-II present was not significantly different suggests that pre-incubation in KN-93 was able to prevent the activation of exogenously applied, as well as endogenous α-CaMK-II.

As a control the inactive analogue of KN-93, KN-92 was added at the same concentration (500 nM) to the patch pipette. In this case there was no significant difference from controls over 16 min (91.2 ± 8.5 % at t = 10 min). Taken together, this suggests there is a basal level of Ca^{2+}/CaM dependent activity within these cells that can be inhibited by KN-93. Inhibition of CaMK-II activity by KN-93 results in a depression of GABA currents, presumably due to a loss of CaMK-II dependent up-regulation of $GABA_A$ receptor function.

Fig 4.3:

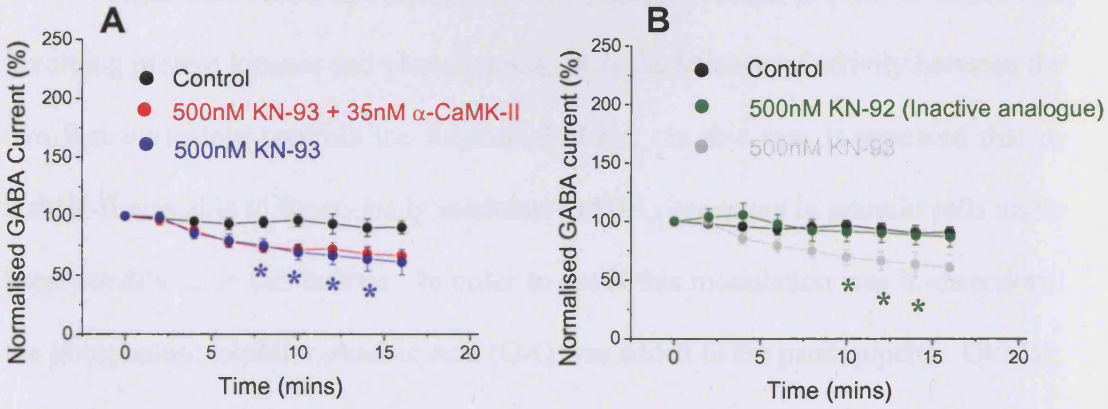


Fig 4.3: Modulation of whole-cell GABA currents recorded from rat cerebellar granule cells in culture by KN-93.

Peak amplitudes of GABA-mediated currents were recorded after application of 10 μM GABA to cerebellar granule cells in culture. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak response of the GABA current measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded with: normal patch pipette solution (Control, n = 6); patch pipette solution supplemented with 500 nM KN-93 (n = 10); and patch pipette solution supplemented with 35 nM CaMK-II and 500 nM KN-93, pre-incubated during the pre-activation reaction (n = 10).

(B) GABA currents recorded with a patch pipette solution supplemented with 500 nM KN-92 (n = 6) compared to normal patch pipette solution (Control, n = 6) and patch pipette solution with 500 nM KN-93 (n = 10, taken from A).

All data points = Mean ± s.e.

Significance is indicated by the symbol *, KN-93 compared to control and the symbol *, KN-92 compared to KN-93.

$P < 0.05$, t-test, ANOVA.

4.2.3 Protein phosphatase activity in cerebellar granule cells in culture.

Regulation of ion channel function by phosphorylation is often bi-directional involving protein kinases and phosphatases. It is the balance of activity between the two that ultimately controls the functional effect. In this case it appeared that α-CaMK-II was able to functionally modulate $GABA_A$ receptors in granule cells under basal conditions in this culture. In order to see if this modulation was bi-directional the phosphatase inhibitor okadaic acid (OA) was added to the patch pipette. Okadaic acid is a general phosphatase inhibitor but at different concentrations affects a different range of phosphatases. At 10 μM, OA blocks the activity of PP1, PP2A and PP2B/calcineurin, but at 1 μM, only the activity of PP1 and PP2A would be affected (Bialojan and Takai, 1988). In this way the relative contribution of different phosphatases to the regulation of $GABA_A$ receptor function could be determined in this cell type (Lieberman and Mody, 1994; Kurz *et al.*, 2001). In addition, inclusion of KN-93 could be used to determine the contribution of CaMK-II activity to the effect of blocking various protein phosphatases.

Including 10 μM OA in the patch pipette resulted in a significant increase in the peak amplitude of whole-cell GABA currents recorded within 4 min to 126 ± 11.5 % as compared to control, 95.6 ± 3.3 % (n = 7, Fig 4.4A). This increase was relatively short-lived and there was no significant difference from control after 15 min. Lowering the concentration of okadaic acid to 1 μM appeared to have no significant effect on the increase in amplitude, 117.1 ± 4.3 % at t = 4 min (n = 6, Fig 4.4A). This suggested that PP2B/calcineuin activity played no significant role in the modulation of GABA currents observed in these cells under basal conditions.

This increase in amplitude could have been caused by a release of protein kinase activity from inhibition by protein phosphatases. A number of different

kinases may be involved. As it had already been established that CaMK-II might be involved in altering $GABA_A$ receptor function in these cells, the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was added to the patch pipette to see what proportion of the effect was related to Ca^{2+} sensitive kinases. Inclusion of 20 mM BAPTA to the patch pipette with 5 μM OA completely blocked the rise in amplitude and resulted in a significant depression to 75.8 ± 5.7 % (n = 5, Fig 4.4B). This indicated that all of the effect produced by blocking phosphatase activity was mediated through Ca^{2+} sensitive processes and confirmed that some of this activity maintained $GABA_A$ receptor function under basal conditions.

In order to determine the role of CaMK-II in increasing $GABA_A$ receptor function after block of phosphatase activity, the CaMK-II inhibitor KN-93 (500 nM) was also added to the patch pipette containing 1 μM of okadaic acid. This led to a decrease in the effect of OA, 99.3 ± 3.5% (n = 6, Fig 4.4C) at t = 4 min so that there was no significant difference from control, 95.6 ± 3.3 % (n = 6, Fig 4.4C). The addition of 500 nM KN-93 previously led to a significant depression of amplitude. This did not occur with OA included in the patch pipette. This suggests that another kinase may also have been involved in increasing $GABA_A$ receptor activity when protein phosphatase activity was blocked. As the addition of 20 mM BAPTA led to a depression of currents recorded this might suggest the additional kinase/phosphatase is Ca^{2+} sensitive. As an additional control, recordings were made with 1 μM OA and the inactive analogue of KN-93, KN-92. In this case the effect of 1 μM OA was not significantly different from 1 μM OA alone, 125 ± 6.7 % at t = 4 min (n = 6, Fig 4.4C).

The increase in amplitude caused by OA addition in all cases was rapid but transient. There were no significant differences from control after 15 min. This is possibly an indication that other OA insensitive phosphatases were present that counteracted the effect of blocking PP1/2A but on a slower time-scale.

Fig 4.4:

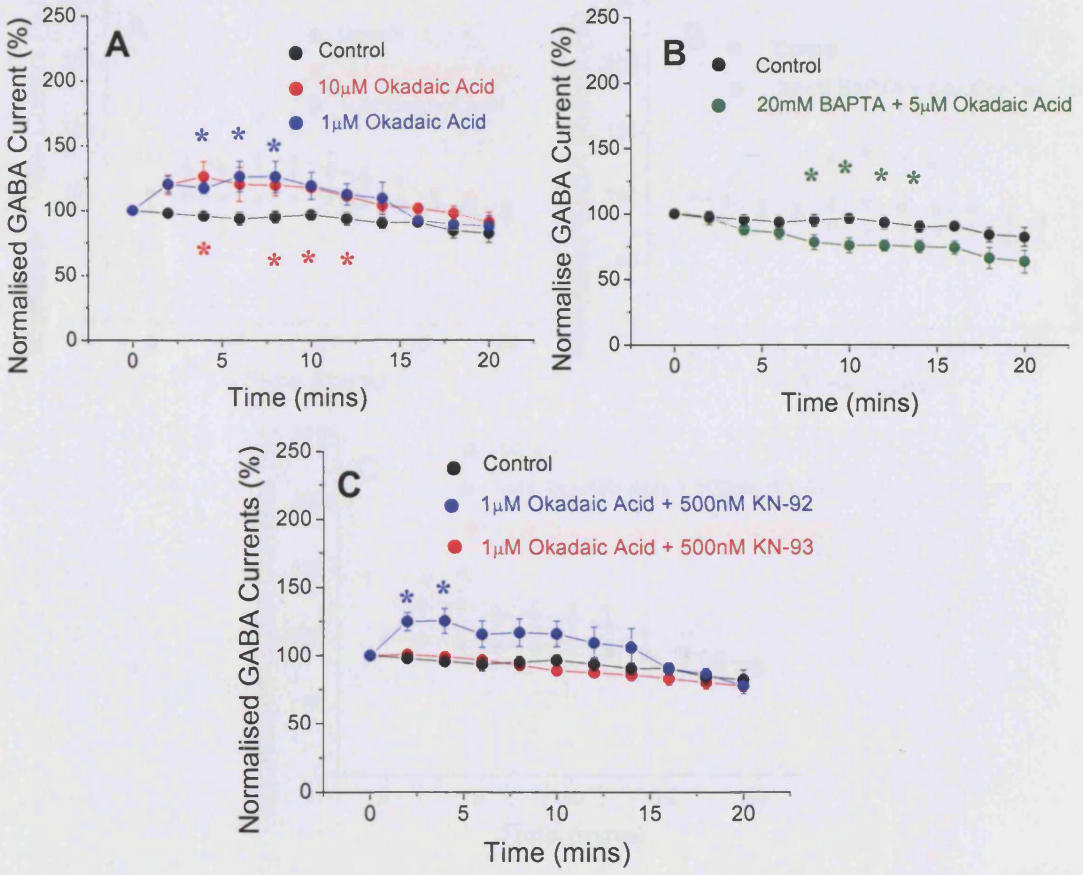


Fig 4.4: Modulation of whole-cell GABA currents recorded from cerebellar granule cells by okadaic acid.

Peak amplitudes of GABA-mediated currents were recorded after application of 10 μM GABA to cerebellar granule in culture. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak response of the GABA current measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded with normal patch pipette solution (Control, n = 6); a patch pipette solution supplemented with 10 μM okadaic acid (n = 7); and with a patch pipette solution supplemented with 1 μM okadaic acid (n = 6).

(B) GABA currents recorded with a patch pipette solution supplemented with 20 mM BAPTA and 5 μM okadaic acid (n = 5) compared to control (n = 6).

(C) GABA currents recorded with a patch pipette solution supplemented with 1 μM okadaic acid and 500 nM KN-93 (n = 6) and with a patch pipette solution with 1 μM okadaic acid and 500 nM KN-92 (n = 6) compared to control (n = 6).

All data points = Mean ± s.e.
Significance is indicated by the symbol *. Each group (indicated by colour) is compared to control, $P < 0.05$, t-test, ANOVA.

4.2.4 Depolarisation of cerebellar granule cells

CaMK-II activity is increased in response to a Ca^{2+} signal. In order to determine if endogenous CaMK-II in CGCs could be modulated by an increase in internal Ca^{2+} concentration, CGCs were depolarised to 0 mV for 2 s to allow Ca^{2+} entry through voltage-gated calcium channels (VGCCs). In this case depolarisation resulted in a slight but significant depression of currents recorded over time to 80 ± 4.5 % (n = 9, Fig 4.5) at t = 10 min. It is possible that this was due to activation of Ca^{2+} sensitive kinases/phosphatases or signalling pathways other than CaMK-II (Wang *et al.*, 2003).

Calcium signalling is complex, controlling many cellular signalling complexes/pathways. Often slight changes in the amplitude and/or localisation of a calcium signal can alter significantly the target-signalling molecule that responds (Franks and Sejnowski, 2002; Augustine *et al.*, 2003). It appeared that depolarisation was not sufficient to increase endogenous CaMK-II activity or that any increase was occluded by activation of other Ca^{2+} sensitive signalling molecules.

Fig 4.5:

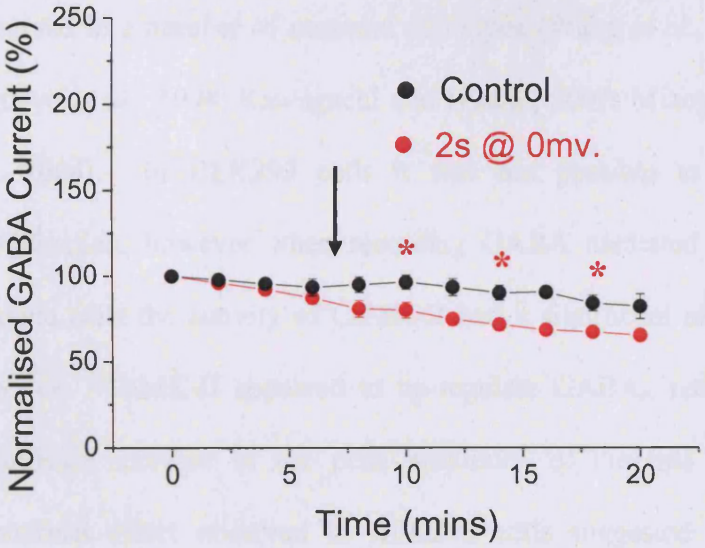


Fig 4.5: Depolarisation of cerebellar granule cells.

Peak amplitudes GABA-mediated currents were recorded after application of 10 μM GABA to cerebellar granule cells in culture. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak response of the GABA current measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

GABA currents were recorded from cerebellar granule cells with normal patch pipette solution (Control, n = 6). This is compared to cells that were depolarised to 0 mV for 2 s (indicated by the arrow, n = 9).

All data points = Mean ± s.e.

Significance is indicated the symbol *, depolarisation compared to control.

$P < 0.05$, t-test.

4.3 DISCUSSION

CaMK-II dependent modulation of $GABA_A$ receptors has been previously observed in a number of neuronal cell types (Wang *et al.*, 1995; Kano *et al.*, 1996; Aguayo *et al.*, 1998; Kawaguchi and Hirano, 2002; Mizoguchi *et al.*, 2003; Wei *et al.*, 2004). In HEK293 cells it was not possible to observe any functional modification, however when recording GABA mediated currents from cerebellar granule cells the activity of CaMK-II had a significant effect on $GABA_A$ receptor function. CaMK-II appeared to up-regulate $GABA_A$ receptor function causing a significant increase in the peak amplitude of currents recorded. The lack of functional effect observed in HEK293 cells suggested that this modulation by CaMK-II required a "neuronal environment" to be operative.

Intracellular dialysis of pre-activated α-CaMK-II (100 nM) to cerebellar granule cells resulted in a significant potentiation of whole-cell currents recorded. This effect appeared to be concentration-dependent. Heat-inactivated α-CaMK-II had no significant effects compared to control suggesting it was CaMK-II activity that was responsible for the effect and not another non-specific mechanism (See Fig 4.1A). Intracellular dialysis of α-CaMK-II into cortical pyramidal neurones in culture also induced a significant increase in GABA mediated currents (See Fig 4.1C). This effect was comparatively smaller than that observed in granule cells suggesting a difference in sensitivity between the two cell types. This potentially could have been mediated by differences in $GABA_A$ receptor subunits present or differences in the background levels of kinase and phosphatase activities within the two cell types.

Construction of GABA dose-response relationships in control conditions and in the presence of CaMK-II revealed a difference in the maximal response without an

alteration in the GABA EC_{50} (See Fig 4.2). A change in the number of functional receptors at the cell surface, an increase in channel conductance or a change in the probability of opening could mediate this increase in the maximal response. Aguayo and colleagues (1998) studied Ca^{2+} dependent potentiation of $GABA_A$ currents in mouse cortical neurones. This modulation could be blocked by addition of the CaMK inhibitor KN-62 and the CaM inhibitor W-7, indicating the likely involvement of CaMK-II. They examined the GABA concentration-response relationship in the presence of high Ca^{2+} (10 μM) and saw no change in the GABA EC_{50}. Using a saturating concentration of GABA they still observed a Ca^{2+} dependent potentiation, indicating that an increase in the maximal $GABA_A$ receptor function had occurred. In cultured rat cortical neurones it has also been observed that mild stretch injury resulted in a potentiation of the maximal GABA current density (pA/pF - with no change in EC_{50}) that could be blocked by KN-93 (Kao *et al.*, 2004). An increase in the overall charge transfer on activation of $GABA_A$ receptors could have significant effects on neuronal excitability. Modulation of the maximal response to GABA with no change to EC_{50} appears to be a common feature of phosphorylation dependent modulation of $GABA_A$ receptor function. For example PKC dependent down-regulation of receptor function results in a depression of the maximal response to GABA without alteration of the EC_{50} (Krishek *et al.*, 1994; Brandon *et al.*, 2000).

In the present study intracellular dialysis of the CaMK inhibitor KN-93 led to a significant decrease in the amplitude of currents recorded indicating that there is a certain level of Ca^{2+}/CaM dependent CaMK-II activity under basal conditions that acts to enhance $GABA_A$ receptor function.

Cerebellar granule cells in culture are often maintained in depolarising conditions (20 mM KCl) to increase CGC survival (Galli *et al.*, 1995; Borodinsky *et al.*, 2002; Borodinsky *et al.*, 2003; Linseman *et al.*, 2003). There is some controversy over how depolarisation increases cell survival but it seems that maintenance in chronically depolarising conditions significantly alters the development of these neurones. It would appear that basal calcium levels and calcium dynamics are also altered by such culture conditions (Moulder *et al.*, 2003).

Borodinsky *et al.* (2002; 2003) observed after addition of KN-93 that CaMK-II activity was involved in neuritogenesis and neuronal survival of CGCs grown under depolarising conditions. Linesman *et al.* (2003) also identified basal levels of CaMK-II activity in CGCs in culture under depolarising conditions that were responsible for increased neuronal survival through activation of myocyte enhancer factor 2 (MEF2) transcription factors. MEF2 transcription factors are thought to be involved in activity dependent neuronal survival (McKinsey *et al.*, 2002). CGCs cultured under depolarising conditions may therefore have significant levels of basal Ca^{2+}/CaM dependent CaMK-II activity and this activity may mediate part of the observed increase in neuronal survival seen under these conditions (Hack *et al.*, 2004).

CaMK-II within the brain consists of two main isoforms, α/β. These isoforms are able to phosphorylate the same substrates but they differ in their localisation and sensitivity to Ca^{2+}/CaM (Hudmon and Schulman, 2002). CaMK-II isolated from the cerebellum is thought to contain a higher proportion of β subunits compared to that isolated from the forebrain. Cerebellar CaMK-II has a two-fold higher affinity to Ca^{2+} and associates differently with subcellular structures (Miller

and Kennedy, 1985). Under non-saturating levels of CaM, β-CaMK-II has a higher sensitivity to lower levels of Ca^{2+} (Brocke *et al.*, 1999).

Evidence suggests that the ratio of α:β CaMK-II may be important for CaMK-II modulation of AMPA receptors in terms of localisation and translocation (Shen *et al.*, 1998; Shen and Meyer, 1999; Thiagarajan *et al.*, 2002) even though both subunits can phosphorylate the same substrates. The role of the different isoforms of CaMK-II in modulation of inhibitory synaptic transmission has yet to be determined. Previous experiments have involved introduction of pre-activated α-CaMK-II (Wang *et al.*, 1995; Kano *et al.*, 1996). This along with the current study suggests α-CaMK-II when applied to the cell through intracellular dialysis is capable of modulating $GABA_A$ receptor function. However, it is thought that CGCs express a high level of β-CaMK-II and little or no α-CaMK-II. This would suggest that the basal activity observed in cerebellar granule cells on application of KN-93 is mediated through β-CaMK-II. Since β-CaMK-II has a higher affinity for Ca^{2+} it is possible it may be basely active within CGCs which is in line with experiments that suggested basal CaMK-II activity is involved in CGC survival when cultured under depolarising conditions (Borodinsky *et al.*, 2002; Linseman *et al.*, 2003).

It can be predicted that the role of the α and β subunits would have little significance on the effect observed in the current study as this involved pre-activating CaMK-II *in vitro*. Pre-activated recombinant β-CaMK-II applied to the cell through the patch pipette would be predicted to behave in exactly the same way as pre-activated α-CaMK-II. The role of the α:β subunits within a CaMK-II heteromer would be significant in a physiological situation if CaMK-II is activated by an endogenous Ca^{2+} signal.

The evidence suggests that basal CaMK-II activity within CGC cultures under depolarising conditions can modulate $GABA_A$ receptor function. Analysis of CaMK-II activity by Fukanaga *et al.* (1989) in CGCs through examination of the proportion of CaMK activity that is Ca^{2+} independent (presumed to be autophosphorylated at Thr^{286}), revealed that 4-5 % of CaMK-II in CGCs was Ca^{2+} independent. This level could be reduced to 1-2 % (reduction of 50 %) by removal of extracellular Ca^{2+} suggesting basal intracellular Ca^{2+} levels must allow a certain level of CaMK-II autophosphorylation at Thr^{286}.

They also provided evidence for high levels of phosphatase activity within these cells, which could reduce Ca^{2+} independent activity rapidly after an initial stimulus. Application of OA increased the amount of Ca^{2+} independent activity over basal levels but this was not sustained and returned to baseline within 10-15 min. They proposed that PP1/PP2A strongly regulated CaMK-II activity in CGCs and that another OA insensitive protein phosphatase (PP2C) was responsible for the slow return of CaMK-II activity to baseline (Fukunaga *et al.*, 1989; Fukunaga *et al.*, 1993).

Analysis of CaMK-II activity in organotypic hippocampal slices also revealed a significant level of autophosphorylation under basal Ca^{2+} conditions that was reduced by the removal of extracellular Ca^{2+} and increased by treatment with OA. This suggested that the relatively high level of CaMK-II activity in basal conditions could allow for a bi-directional regulation of CaMK-II activity (Molloy and Kennedy, 1991).

Both Fukunaga *et al.* (1989) and Molloy and Kennedy (1991) looked at the relative proportions of Ca^{2+}-independent activity as a measure of CaMK-II activity. This reveals levels of CaMK-II autophosphorylation at Thr^{286} but does not reveal the

absolute levels of CaMK-II activity in the cell which would include Ca^{2+}-independent and Ca^{2+}-dependent activity. The levels of Ca^{2+}-independent activity reported in these studies are low (<10%) however, even maximally autophosphorylated homogenates display only 45 % Ca^{2+}-independent activity using the methods used in these studies (Molloy and Kennedy, 1991). It is interesting though that both of these studies suggest a certain level of basal Ca^{2+}-independent activity (autophosphorylation at Thr^{286}) which must reflect a basal level of CaMK-II activity which can be up or down-regulated by the activity of protein phosphatases.

Applying these details to CaMK-II modulation of CGC GABA currents, it can be speculated that endogenous CaMK-II within cerebellar granule cells, which predominantly express the β isoform of CaMK-II, can be basally active in a Ca^{2+}/CaM-dependent manner and that this basal activity acts to up-regulate $GABA_A$ receptor function.

The amplitude of GABA mediated currents within CGCs in this study was altered by the phosphatase inhibitor OA. Different concentrations of OA were used to determine which protein phosphatases regulated $GABA_A$ receptor function. Inclusion of OA in the patch pipette resulted in an increase in the amplitude of GABA mediated currents that was the same regardless of the concentration of OA used; suggesting that PP1/PP2A modulated $GABA_A$ receptor function but that PP2B did not. The increased amplitude returned to baseline within 15 min indicating that another OA insensitive protein phosphatase may also be involved. This time-course and sensitivity to OA of this effect on $GABA_A$ receptors was very similar to the observed effects of OA on Ca^{2+}-independent CaMK-II activity levels (Fukunaga *et al.*, 1989).

The increased amplitude caused by the block of these phosphatases was removed by the addition of BAPTA. Indeed, there was a decrease similar to that observed with KN-93, suggesting Ca^{2+} sensitive kinase/phosphatase activities mediated the increase in $GABA_A$ receptor function observed on addition of OA. Addition of KN-93 and OA resulted in no significant increase or decrease in amplitude. Suggesting CaMK activity was partly responsible for an increase in amplitude initiated by protein phosphatase blockade but that other Ca^{2+} sensitive kinases/phosphatases were also involved. It appears that $GABA_A$ receptor function can be bi-directionally altered by CaMK-II activity and protein phosphatase activity.

It may be that for CGCs maintained under depolarising conditions, a relatively high basal Ca^{2+} level and/or a proportionately high level of β-CaMK-II, may act to allow a significant level of basal CaMK-II activity, which can modulate $GABA_A$ receptor function. Blocking the activity of protein phosphatases may act to increase the level of CaMK-II activity, e.g, PP1 is known to dephosphorylate CaMK-II (Colbran, 2004), or may act directly on the $GABA_A$ receptor itself. The presence of PP1 has been shown to alter the sensitivity of CaMK-II to Ca^{2+} levels (Bradshaw *et al.*, 2003). Inhibition of PP1 has been linked to increased CaMK-II activity in a number of systems (Brown *et al.*, 2000; Kawaguchi and Hirano, 2002; Colbran, 2004). PP1 is also thought to modulate $GABA_A$ receptor function directly (Terunuma *et al.*, 2004). Moreover, PP2A has also been suggested to modulate CaMK-II activity (Fukunaga *et al.*, 1989; Colbran, 2004) and $GABA_A$ receptor activity directly (Jovanovic *et al.*, 2004).

Basal $GABA_A$ receptor phosphorylation by CaMK-II would require CaMK-II to be localised to inhibitory synapses. Attempts to identify the location of CaMK-II subunits have suggested that the α subunit can be localised to excitatory but not

inhibitory synapses within the CA1 region of the hippocampus, the thalamus and the cerebral cortex (Liu and Jones, 1996; Liu and Jones, 1997). However, in the rat basolateral amygdala α-CaMK-II has been identified postsynaptically at GABAergic synapses (McDonald *et al.*, 2002). Differences between papers may be due to the different antibodies used and slight variations in immunocytochemical techniques. In addition, recent evidence suggests that CaMK-II is capable of very rapid translocation (Shen and Meyer, 1999; Gleason *et al.*, 2003). It has also been suggested CaMK-II can alter localisation and form clusters in response to ischaemic stress and that the process of preparing tissue for staining may alter CaMK-II localisation (Dosemeci *et al.*, 2000; Lengyel *et al.*, 2001). Depolarisation and excitotoxic stress appears to increase the apparent accumulation of α-CaMK-II at the PSD (Tao-Cheng *et al.*, 2002). Early on in development the β-CaMK-II is more prevalent with an increase in α subunit occurring in the rat in the 2nd and 3rd postnatal week (Burgin *et al.*, 1990). There is also variation in the relative proportion of α:β in different tissues (Miller and Kennedy, 1985; Vallano, 1990). Since the ratio of α:β subunits can alter the localisation of the kinase, it may be that at different developmental time points the distribution of CaMK-II observed may also be different.

Maintenance of CGC cultures in depolarising conditions not only alters CaMK-II activity; it can also alter the expression of ligand-gated ion channels (Vallano *et al.*, 1996; Mellor *et al.*, 1998; Ives *et al.*, 2002). Ives *et al.* (2002) studied mRNA levels and protein levels with immunocytochemistry of different $GABA_A$ subunits in mouse cerebellar granule cells cultured in 25 mM KCl, or 5 mM KCl. They reported that under depolarising conditions, α1, α6 and β2 expression was greatly reduced and they failed to detect any β2 subunit protein. In addition there

was an increase in α3 and β3 subunit expression with no difference in α2. Given that β2 shows a preference to assembly with α1 and β3 with α2/3 (Benke *et al.*, 1994) it could be predicted that CGCs grown in 20 mM KCl medium, may express predominantly α2/3β3 subunit-containing receptors. In more physiological conditions (5 mM KCl) there may be a mixture of α1/β2 and α2/3β3 subunit-containing receptors.

The β3 subunit is, in many parts of the brain, expressed earlier in development with β2 expression increasing at later developmental stages (Laurie *et al.*, 1992). In support of a crucial role for β3 in early development β3 knock-out mice display severe developmental impairments (Homanics *et al.*, 1997; DeLorey *et al.*, 1998). Ives *et al.* (2002) suggested that CGCs maintained under depolarising conditions are "arrested" in their development and that they represent an immature stage in CGC development. This is supported by the fact that these cells are generally electrically silent, characteristic of a lack of fully functional synapses. In further support of this idea, depolarisation appears to prevent α6 subunit expression in mouse cerebellar granule cultures which is known to be expressed in mature CGCs (Mellor *et al.*, 1998). It should be noted though that α6 expression has been reported in rat CGC cultures under depolarising conditions (Thompson and Stephenson, 1994). Rat CGC cultures appear to behave slightly differently and so the alteration in $GABA_A$ subunit expression may not be as pronounced as in mouse cultures. However rat CGCs are similar to mouse CGCs in that they are electrically silent and so they may also represent an earlier stage in development and express different levels of $GABA_A$ receptor β subunits accordingly.

The age of the animal used for culture will also have an impact on the $GABA_A$ subunits present. In the current study P0 rats were used. As α6/δ

expression does not occur until the end of the 1st postnatal week (Wisden *et al.*, 1996) the cultures used in these experiments would probably not have substantially expressed these subunits. CaMK-II has been shown to phosphorylate β and γ subunits *in vitro* (Machu *et al.*, 1993; McDonald and Moss, 1994; McDonald and Moss, 1997). It is reasonable to suggest that the cultures used in the current experiments may have expressed predominantly $\alpha 2/3\beta 3\gamma 2$ subunit-containing receptors and so this suggests that $\beta 3$ and $\gamma 2$ may be good candidates for CaMK-II modulation in this cell type.

In the current study, an attempt was also made to depolarise CGCs to encourage Ca^{2+} entry via VGCCs to see if endogenous CaMK-II activity could be increased thereby increasing the amplitude of $GABA_A$ currents. However, depolarisation led to a small decrease in amplitude. There are several possible reasons why depolarisation did not appear to activate endogenous CaMK-II. Calcium signalling is complex, Ca^{2+} signals are often under tight spatial and temporal control to allow a neurone to use multiple Ca^{2+} sensitive signalling pathways (Augustine *et al.*, 2003). The source and localisation of Ca^{2+} entry can have a profound effect on CaMK-II activation (Franks and Sejnowski, 2002). CaMK-II can respond with differing sensitivities to Ca^{2+} signals depending on the proportion of α:β subunits within the heteromer. In addition CaMK-II has been shown to respond not only to the amplitude of a Ca^{2+} signal but also to its frequency (De Koninck and Schulman, 1998). Complex processes within the cell can also regulate the availability of CaM (Gnegy, 2000). Therefore the link between depolarisation and increases in the intracellular Ca^{2+} concentration is not always straightforward and Ca^{2+} entry, in itself, is not a guarantee that CaMK-II will be activated.

Global Ca^{2+} entry may have activated a number of different Ca^{2+} sensitive processes that may have had direct or indirect effects on $GABA_A$ receptor function. Using different concentrations of OA it appeared that PP2B/Calcineurin had little basal effect on $GABA_A$ receptor function. However, Ca^{2+} entry may have activated this phosphatase. Calcineurin has been shown to decrease the amplitude of IPSCs through an interaction with the γ2 subunit (Wang *et al.*, 2003). Previously the alteration of intracellular Ca^{2+} levels has been associated with both increased (Kano *et al.*, 1992; Aguayo *et al.*, 1998) and decreased $GABA_A$ receptor function (Martina *et al.*, 1994; Stelzer and Shi, 1994). This may be due to $GABA_A$ receptor heterogeneity, but also to activation of different downstream signalling pathways. In addition the Ca^{2+} sensitive kinase PKC has also been shown to up or down-regulate $GABA_A$ receptor function depending on the cell type and the $GABA_A$ receptor subtypes present (Krishek *et al.*, 1994; Lin *et al.*, 1994; Lin *et al.*, 1996; Brandon *et al.*, 2000; Jovanovic *et al.*, 2004).

In addition to the problems associated with the complexity of Ca^{2+} signalling it has been reported by Moulder *et al.* (2003) that CGCs maintained in depolarising conditions have a reduced calcium channel density. Ca^{2+} entry on depolarisation may be significantly reduced after maintenance in these conditions. The authors also suggested that reduced Ca^{2+} entry, presynaptically, might be responsible, in part, for the electrical silence of these cells.

In summary it appears that CaMK-II is capable of potentiating GABA currents recorded from cerebellar granule cells. There also appears to be a certain level of basal CaMK-II activity in these cells which acts to enhance $GABA_A$ receptor function under resting conditions. In order for CaMK-II modulation of $GABA_A$ receptors to play a physiological role, CaMK-II activity must be able to significantly

modulate synaptic function. Since CGCs are electrically silent and morphologically immature when maintained under depolarising conditions it was necessary to alter the CGC culture conditions to those that are more physiologically representative (5 mM KCl) in order to determine if CaMK-II activity can indeed modulate synaptic $GABA_A$ receptors.

4.4 CONCLUSIONS

- α-CaMK-II activity was capable of potentiating $GABA_A$ receptor function in cerebellar granule and cortical pyramidal neurones.
- Within CGCs, CaMK-II activity appeared to play a basal role in maintaining $GABA_A$ receptor function.
- Protein phosphatase activity, probably involving PP1/PP2A, modulated CaMK-II activity towards $GABA_A$ receptors either acting on CaMK-II or the $GABA_A$ receptor directly.

Chapter Five:
Modulation of inhibitory synaptic transmission by α-CaMK-II

5.1 INTRODUCTION

$GABA_A$ receptors mediate the majority of fast synaptic inhibition in the brain. Their activity can shape the output of a given neurone and the general excitability of a network and their function is thought to be crucial for the generation of network oscillations relevant to behaviour (Gaiarsa *et al.*, 2002). Inhibitory synaptic transmission has also been shown to be altered in certain pathological conditions such as epilepsy (Macdonald *et al.*, 2004). At the receptor level, covalent modification by phosphorylation has been demonstrated to modulate $GABA_A$ receptor mediated synaptic transmission (Wang *et al.*, 1995; Wang *et al.*, 2003b; Wei *et al.*, 2004; Jovanovic *et al.*, 2004), although there is controversy over the exact effects observed in different cell types (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999). Phosphorylation appears to play an important role in long-term plasticity of both excitatory and inhibitory synapses (Soderling and Derkach, 2000; Kittler and Moss, 2003; Colbran and Brown, 2004; Lüscher and Keller, 2004). For CaMK-II modulation of $GABA_A$ receptors to have any role in shaping the activity of a neurone or network it is important to establish if CaMK-II can modulate inhibitory synaptic transmission. Therefore this chapter addressed this issue by examining CaMK-II dependent effects on $GABA_A$ receptor mediated synaptic transmission in neurones.

There are a number of examples of kinase and phosphatase activities altering fast synaptic inhibition. PKC activity has been shown to increase miniature inhibitory postsynaptic current (mIPSC) amplitudes and protein phosphatase 2A

(PP2A) has been shown to subsequently reverse this effect (Jovanovic *et al.*, 2004), without any changes to mIPSC decay time constants. Using recombinant $GABA_A$ receptors expressed in *Xenopus oocytes*, PKC activity can lead to a depression of GABA currents (Krishek *et al.*, 1994) with no change in the time constants for decay. More recent evidence suggests PKC can have different functional effects in different neuronal cell types (Poisbeau *et al.*, 1999). PKA activity can also have different functional effects in different cell types and has been shown to lead to an increase, no change, or a depression of IPSC amplitudes. The decay time course of IPSCs was in some cases prolonged, but in others remaining unaffected (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999).

Alteration in the decay phase of an IPSC appears to be a common mechanism for phosphorylation-dependent modulation of inhibitory synaptic transmission (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999; Hinkle and Macdonald, 2003). In addition to the lengthening of IPSC decay times by PKC and PKA, the phosphatase calcineurin (CaN)/PP2B has also been shown to regulate IPSC decay times (Martina *et al.*, 1996; Jones and Westbrook, 1997; Sanchez *et al.*, 2005). However under certain conditions CaN/PP2B causes a reduction in mIPSC amplitude without affecting decay times (Wang *et al.*, 2003a). As well as Ser/Thr kinases, tyrosine kinase phosphorylation has been shown to up-regulate $GABA_A$ receptor function (Moss *et al.*, 1995) and can increase mIPSC amplitude without apparent changes in their decay times (Boxall, 2000; Mizoguchi *et al.*, 2003; Ma *et al.*, 2003).

Similarly CaMK-II activity has been shown to alter fast synaptic inhibition. Wang and colleagues (1995) applied pre-activated α-CaMK-II to hippocampal CA1 neurones through inclusion in the patch pipette and observed an increase in evoked IPSP amplitudes. They also observed a reduction in desensitisation, a possible

mechanism for alteration of IPSC decay (Jones and Westbrook, 1996). Kano *et al.* (1996) observed increases in the amplitude of mIPSCs recorded from Purkinje neurones without a change in the decay time constant after application of pre-activated α-CaMK-II. In addition, Wei and colleagues (2004) observed a calcium-dependent increase in spontaneous IPSC (sIPSC) amplitudes that could be blocked by CaMK-II inhibitors.

The evidence suggests that CaMK-II is capable of altering $GABA_A$ receptor mediated synaptic transmission through changes in amplitude and possibly the rate of desensitisation of IPSCs. However phosphorylation-dependent effects on inhibitory synaptic transmission mediated by other kinases/phosphatases have suggested considerable variation in the effect observed amongst different cell types.

Cerebellar granule cells (CGCs) maintained in depolarising conditions do not display synaptic activity and so these cells were cultured in more physiological conditions in order to examine synaptic transmission. Mixed cerebellar cultures maintained in physiological (5 mM KCl) conditions are able to form inhibitory synapses. *In vivo* cerebellar granule cells receive inhibitory inputs from Golgi cells and Purkinje cells receive inhibitory input from stellate and basket cells (Wisden *et al.*, 1996; Nakanishi, 2005). In culture it is possible that GABAergic synapses were formed by all of these interneurones onto the granule cell. Recordings were made from rat cerebellar granule cells with pre-activated α-CaMK-II in the patch pipette using the same protocol as described in the previous chapters (See Chapter 2 and 3.2.2) to determine if there was a CaMK-II dependent effect on inhibitory synaptic transmission, and if so, what form that modulation took.

5.2 RESULTS

Cerebellar granule cells maintained in physiological levels of KCl (5 mM) for 7-14 days (See Chapter 2) had a slightly altered morphology to cells maintained under depolarising conditions (Moulder *et al.*, 2003). Generally they had slightly smaller cell bodies and correspondingly lower cell capacitance (8.3 ± 0.5 pF, n = 15) compared to cells maintained in depolarising conditions (11.1 ± 0.6 pF, n = 15). In 5 mM KCl, CGCs tended to form small clusters of cells (See Fig 2.1B) rather than the uniform distribution of cells maintained in 20 mM KCl (Ives *et al.*, 2002). Most CGCs within clusters displayed spontaneous synaptic activity, although a proportion displayed no activity at all (15-20 %). Both excitatory and inhibitory postsynaptic currents could be observed. Excitatory input from surrounding granule cells could be observed as spontaneous excitatory postsynaptic currents (sEPSCs). Application of the NMDA receptor blocker AP-5 (20 μM) and AMPA receptor blocker CNQX (10 μM), revealed GABAergic currents (IPSCs) that could be blocked by the $GABA_A$ receptor antagonist bicuculline (20 μM; See Fig 5.1). Bath application of tetrodotoxin (TTX – 500 nM) almost completely abolished all IPSCs. This is in line with the observation that most CGC IPSCs in culture and in thin brain slices are action potential dependent (Puia *et al.*, 1994; Leao *et al.*, 2000; Farrant and Brickley, 2003). The Na^{2+} channel blocker QX-314 (5 mM) was included within the patch pipette to prevent action potentials from occurring within the cell being recorded from whilst allowing the activity of the rest of the network to remain relatively unaltered. In this way action potential driven sIPSCs could be recorded.

A tonic $GABA_A$ receptor mediated current has been observed in cerebellar granule cells in culture and in slices, presumed to be mediated by the basal GABA concentration or by GABA spillover acting on $\alpha 6/\delta$ subunit containing extrasynaptic

receptors (Brickley *et al.*, 1996; Leao *et al.*, 2000; Wall, 2002). On application of bicuculline the tonic noise was altered in only a small subset of the cells recorded from these cultures (1 in 20). This is possibly due to the immature nature of the cultures and a possible lack of δ subunit expression, or perhaps due to a reduced number/density of GABA releasing cells in culture. Expression of δ subunits is thought to be negligible until postnatal day 6-7 and does not reach adult levels until the third postnatal week (Wisden *et al.*, 1996). As these cultures were prepared from P0 rats it may be that δ subunit expression never reached high enough levels to support a tonic GABA current.

Fig 5.1:

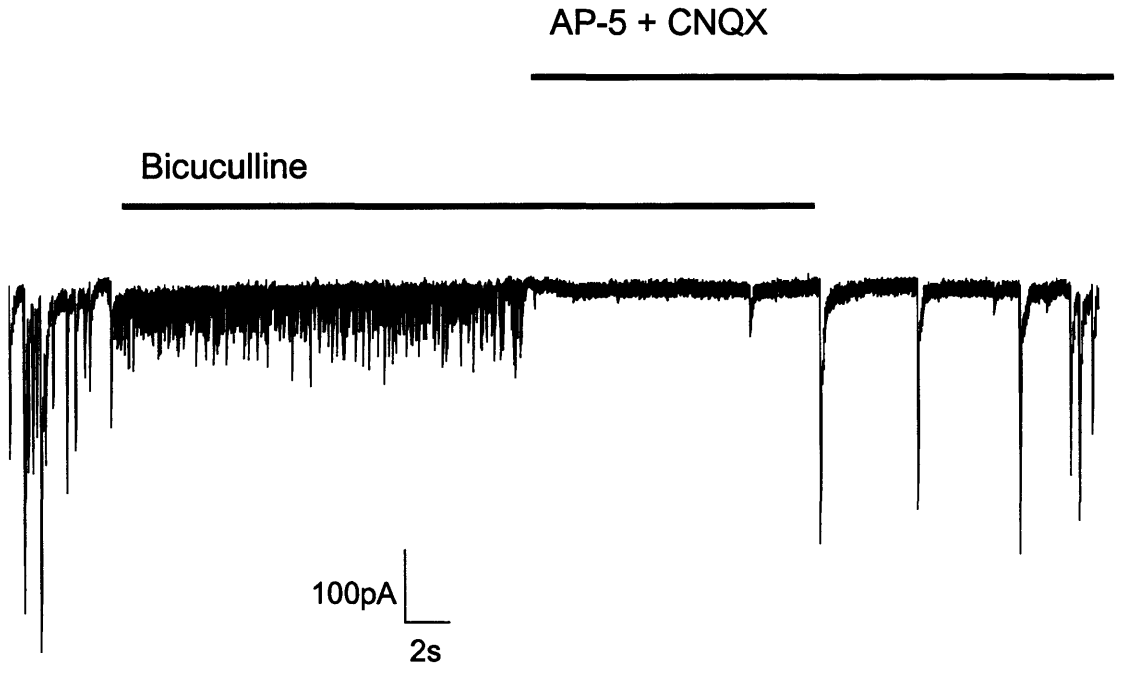


Fig 5.1: sIPSCs recorded from rat cerebellar granule cells in culture.

Representative synaptic currents recorded from rat cerebellar granule cells grown and maintained in a physiological culture medium containing (5 mM) KCl and 5 % FCS.

Application of bicuculline (20 μM) reveals EPSCs. Application of AP-5 (20 μM), CNQX (10 μM) and bicuculline (20 μM) removes all events. Application of AP-5 and CNQX reveals sIPSCs.

5.2.1 CaMK-II modulation of sIPSC amplitude.

Recordings were made with either 85 nM pre-activated α-CaMK-II (See Chapter 2 and 3) within the patch pipette or normal patch pipette solution containing the pre-activation buffer without α-CaMK-II as a control. This was to ensure that the pre-activation buffer (although diluted in the patch pipette solution) did not activate endogenous CaMK-II or other Ca^{2+} sensitive kinases or phosphatases. Continuous recordings were made in 2 min periods starting 4-5 min after achieving the whole-cell configuration (t = 0 min), for a total period of 20 min. Analysis of IPSC kinetics required continuous recordings to be made from t = 6 min to t = 12 min to ensure an adequate number of events for analysis.

The addition of 85 nM α-CaMK-II led to a significant increase in the mean peak amplitude of sIPSCs to 129.7 ± 6.1 % (n = 7) as compared to control, 93.7 ± 7.8 % (n = 9, Fig 5.2A) at t = 6-8 min. This effect reached a steady-state within 6 min. The observed increase in the mean sIPSC amplitude did not necessarily indicate a uniform increase in amplitude of all events within the population. In order to further evaluate the effects of CaMK-II on sIPSC amplitude, an amplitude distribution histogram was constructed. Under control conditions the population was best fitted by three Gaussian populations (n = 7, 7000 events). The presence of more than one population has been previously observed in rat cerebellar cultures (Leao *et al.*, 2000) and is presumed to correspond to action potential dependent (sIPSCs) and action potential independent (mIPSC) forms of GABA release. However, the addition of TTX abolishes almost all IPSCs in this culture so it is difficult to confirm that large amplitude events are action potential driven as there are not enough mIPSCs for reliable analysis. The distribution of amplitudes appeared to be skewed towards

higher amplitude events as has also been described previously (De Koninck and Mody, 1994; Nusser *et al.*, 1997).

The addition of α-CaMK-II led to the appearance of a group of large amplitude IPSCs of over 400 pA (n = 4, 7000 events, Fig 5.2C). There was a large drop in the number of events of around 200 pA and an increase in the number of smaller amplitude events. The population was now composed of four Gaussian populations. The appearance of larger amplitude events appears to have been at the expense of the larger amplitude populations of events seen in controls (around 200 pA). The smallest amplitude population seen in controls shifts slightly to the right (to give rise to a fourth population, around 70 pA) and the number of the smallest events (<70 pA) is increased. This could be partly due to smaller amplitude events that were previously undetectable, increasing in amplitude, and appearing out of the noise. This might be expected to be reflected by an increase in frequency, however, the frequency of events was not significantly altered over the time of recording (Fig 5.2D) or in the presence of α-CaMK-II (n = 7, 2.2 ± 0.6 Hz) as compared to control (n = 8, 2.8 ± 1.2 Hz). It appears though that the effect of CaMK-II is not uniform across the whole population.

Fig 5.2:

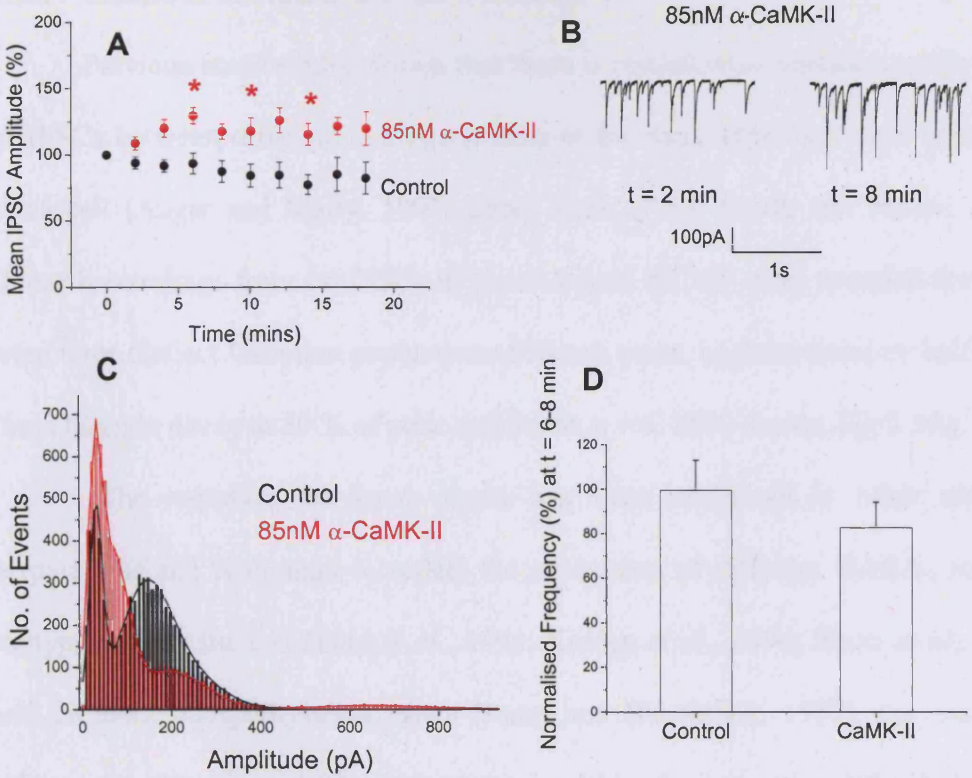


Fig 5.2: α-CaMK-II modulation of sIPSCs recorded from rat cerebellar granule cells.

sIPSCs were recorded from cerebellar granule cells in culture (DIV10-14) maintained in 5 mM KCl and 5 % FCS. Recordings were made in 2 min periods with the first starting 4-5 min after achieving the whole-cell configuration (t = 0-2 min) or in 5-6 min periods starting 6 min after achieving the whole-cell configuration. Control recordings were made using normal patch pipette solution supplemented with pre-activation buffer without α-CaMK-II.

(A) Mean sIPSC peak amplitudes of events within a 2 min recording period were normalised to the mean peak amplitude at t = 0-2 min, in the absence (Control, n = 9) and presence of 85 nM α-CaMK-II (n = 7). All points are mean ± s.e.

(B) Representative recordings of sIPSCs at t = 2 min and t = 8 min with 85 nM α-CaMK-II present within the patch pipette solution.

(C) Amplitude distribution histogram of sIPSCs in the absence (Control, n = 7, 7000 events) and in the presence of 85 nM α-CaMK-II (n = 4, 7000 events) at t = 6-12 min.

(D) Bar chart showing the frequency at t = 6-8 min normalised to the frequency at t = 0-2 min in control conditions (n = 8) and in the presence of 85 nM α-CaMK-II (n = 7).

Significance is indicated by the symbol *, $P < 0.05$, t-test.

5.2.2 CaMK-II modulation of sIPSC decay.

Previous studies have shown that there is considerable variation in the decay of IPSCs between different cell types, cells of the same type, and even within the same cell (Auger and Marty, 1997; Bacci *et al.*, 2003; Mody and Pearce, 2004). Control recordings from rat CGCs in physiological KCl (5 mM) revealed that there were three distinct Gaussian populations of decay times, as determined by half-width (time taken to decay to 50 % of peak amplitude; n = 6, 2600 events, Fig 5.3A).

The variation in decay times has been observed in other neuronal preparations and is thought to reflect the expression of different $GABA_A$ receptor subtypes within the cell (Puia *et al.*, 1994; Koulen *et al.*, 1996; Bacci *et al.*, 2003) and different phosphorylation states (Jones and Westbrook, 1997), but may also reflect variations in GABA concentration within the synaptic cleft of different synapses (Nusser *et al.*, 2001; Mody and Pearce, 2004). A typical example trace taken from a single CGC recording illustrates events with similar rise times but different decay profiles (Fig 5.3A).

The distribution of half-widths of sIPSCs recorded in the presence of α-CaMK-II displays a shift to the right towards a slower decay time (n = 7, 2600 events, Fig 5.3A). There is an increase in the number of events decaying within the 25-45 ms range and a drop in the number of events within the 10-25 ms range.

At t = 6-12 min, sIPSC events (50-100) were averaged from control cells and from those in the presence of α-CaMK-II. Average traces were then used to calculate decay time constants (See Chapter 2, 2.7.2). Events recorded in the absence and in the presence of α-CaMK-II were best fitted by a bi-exponential decay. The decay constant τ_1 significantly increased from 10 ± 0.9 ms (n = 7) to 22.7 ± 3.2 ms in the presence of α-CaMK-II (n = 8, Fig 5.3C). The decay constant τ_2 also

significantly increased from 46.7 ± 6 ms (n = 7) to 78 ± 10 ms in the presence of α-CaMK-II (n = 8, Fig 5.3C). The proportion of area τ_1:τ_2 was not significantly altered (A1 Control = 53.6 ± 4.7 %, n = 7, A1 + CaMK-II = 51.7 ± 3.6 %, n = 7, Fig 5.3D) by the presence of α-CaMK-II.

Variation in IPSC decay times could also result from dendritic filtering, however, CGCs are electrotonically very compact and so the effect of dendritic filtering should be comparatively low (Gabbiani *et al.*, 1994; Rossi and Hamann, 1998). To confirm this, scatter plots of IPSC rise-time against IPSC amplitude were constructed for each cell to check there was no linear relationship between these two variables. Only cells lacking any clear correlation between IPSC rise-time and amplitude were used in the analysis of IPSC decay times (r = 0.15, Fig 5.4). This suggests that variation in decay times in the majority of cases is related to genuine differences in the properties of $GABA_A$ receptors at different synapses and not due to dendritic cabling. Moreover, α-CaMK-II did not affect the mean rise-time of the IPSCs, from 10-90 % of peak amplitude (Control, 1.2 ± 0.2 ms, n = 8, + α-CaMK-II, 1.1 ± 0.1 ms, n = 7, Fig 5.4B).

Fig 5.3:

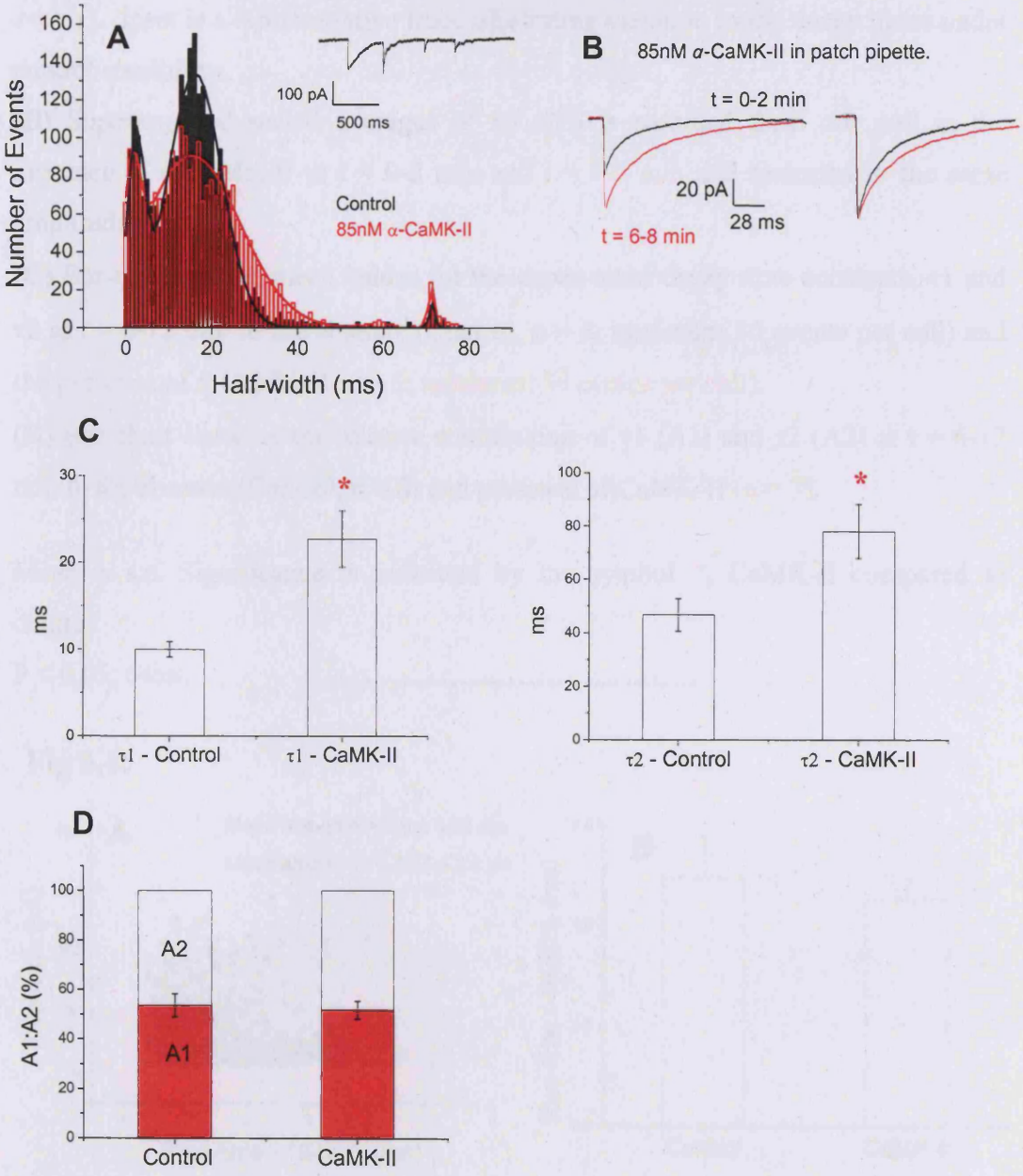

A
Number of Events
Half-width (ms)
100 pA
500 ms
Control
85nM α-CaMK-II
B
85nM α-CaMK-II in patch pipette.
t = 0-2 min
20 pA
28 ms
t = 6-8 min
C
ms
τ1 - Control
τ1 - CaMK-II
τ2 - Control
τ2 - CaMK-II
D
A1:A2 (%)
A2
A1
Control
CaMK-II

Fig 5.3: Modulation of sIPSC decay times by α-CaMK-II.

(A) Frequency distribution histogram of sIPSC half-widths (τ_{50}) at t = 6-8 min in the absence (Control, n = 6, 2600 events) and the presence of α-CaMK-II (n = 7, 2600 events). Inset is a representative trace illustrating variation in the decay times under control conditions.

(B) Superimposed scaled averages of 80 sIPSCs recorded from one cell in the presence of α-CaMK-II at t = 0-2 min and t = 6-8 min and re-scaled to the same amplitude.

(C) Bar charts of the mean values for the exponential decay time constants, τ1 and τ2 at t = 6-12 min in the absence (Control, n = 8, minimum 50 events per cell) and the presence of α-CaMK-II (n = 9, minimum 50 events per cell).

(D) Bar chart showing the relative contribution of τ1 (A1) and τ2 (A2) at t = 6-12 min in the absence (Control, n = 8) and presence of CaMK-II (n = 7).

Mean ± s.e. Significance is indicated by the symbol *, CaMK-II compared to control.

P < 0.05, t-test. .....

Fig 5.4:

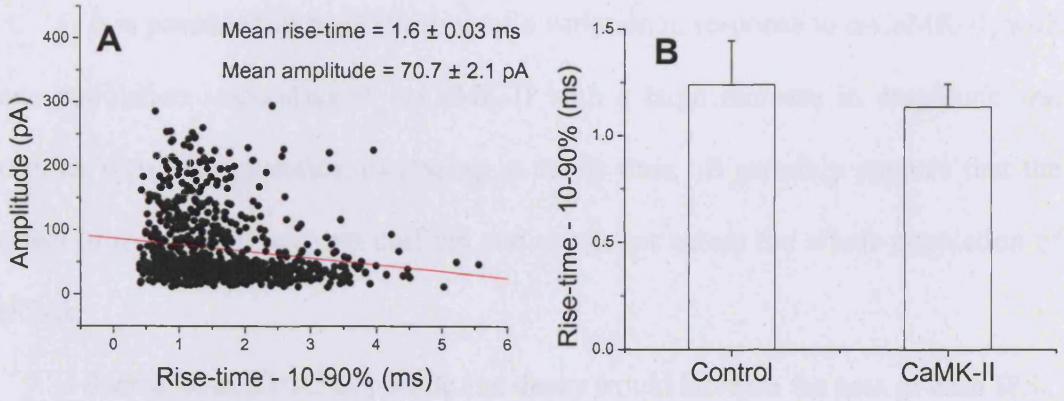


Fig 5.4: sIPSC rise-times in the absence and presence of α-CaMK-II.

(A) Example scatter plot of sIPSC amplitudes against sIPSC rise-times in the absence (Control, n = 3, 780 events) of α-CaMK-II. Note the lack of a linear relationship (r = 0.15).

(B) Bar chart showing the mean sIPSC rise-time in the absence (n = 8) and the presence of 85 nM α-CaMK-II (n = 7). For each cell the mean rise-time was calculated from 50-100 events averaged from recordings, t = 6-12 min.

5.2.3 CaMK-II modulation of the whole population of sIPSCs.

Currently, little is known about CaMK-II dependent modulation of $GABA_A$ receptors, so the possibility remains that CaMK-II preferentially modulates certain $GABA_A$ receptor subtypes or can differentially modulate certain subtypes in different ways. The latter may involve either direct phosphorylation, indirect phosphorylation of $GABA_A$ receptor-associated proteins or possibly may act through downstream signalling pathways. Scatter plots of sIPSC amplitudes against half-widths recorded at t = 6-12 min in the absence (Control, n = 7, 7000 events) and in the presence of 85 nM α-CaMK-II (n = 4, 7000 events, Fig 5.5A) were constructed to examine the effects of α-CaMK-II on the population as a whole. Addition of α-CaMK-II results in a shift of a sub-population of IPSCs to much larger amplitudes with relatively little change in decay. In addition another population of events appears to increase in half-width with relatively little change in amplitude. There is also another sub-population of events that are not significantly altered by α-CaMK-II at all.

It is possible this is a reflection of a variation in response to α-CaMK-II, with one population responding to α-CaMK-II with a large increase in amplitude and another separate population increasing in decay time. It certainly appears that the effect of α-CaMK-II was not uniform and consistent across the whole population of IPSCs.

Increases in sIPSC amplitude and decay would increase the area of each IPSC and therefore the overall charge transfer. The amplitude distribution histogram of sIPSC areas in the absence (n = 6, 2600 events) and the presence of α-CaMK-II (n = 7, 2600 events, Fig 5.4B) at t = 6-12 min displays a significant shift to events with much greater area. This would be consistent with a significant increase in the overall charge transfer. In addition the mean area of sIPSCs significantly increases from

1931 ± 265 fC (n = 14) to 4440 ± 714 fC (n = 9, Fig 5.5C) in the presence of 85nM α-CaMK-II.

Fig 5.5:

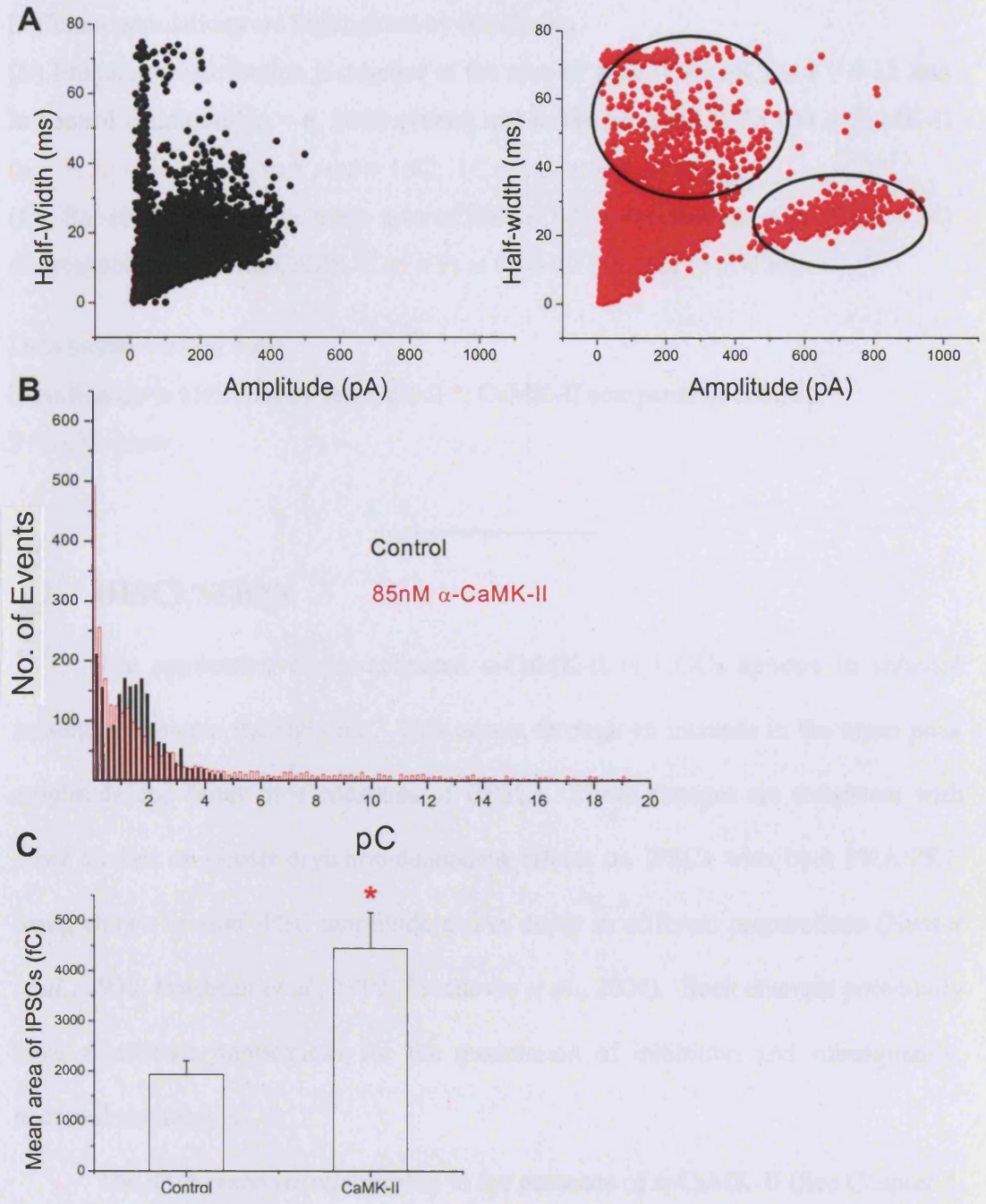


Fig 5.5: CaMK-II modulation of sIPSCs as a population and changes in synaptic charge transfer.

(A) Scatter plots of sIPSC amplitude against half-width at t = 6-12 min in the absence (left, Control, n = 7, 7000 events) and presence (right) of 85 nM α-CaMK-II (n = 4, 7000 events).
Different populations are highlighted by the circles.
(B) Frequency distribution histogram of the area of each IPSC (pC) at t = 6-12 min in control conditions (n = 6, 2600 events) and in the presence of 85 nM α-CaMK-II (n = 7, 2600 events). (1nA . ms = 1pC; 1C = 1 coulomb of charge)
(C) Bar chart showing the mean area of IPSCs (fC) in the absence (Control, n = 14) or presence of 85 nM α-CaMK-II (n = 9) at t = 6-12 min and t = 6-8 min.

Data points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control.
$P < 0.05$, t-test.

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5.3 DISCUSSION

The application of pre-activated α-CaMK-II to CGCs appears to enhance inhibitory synaptic transmission. This occurs through an increase in the mean peak amplitude and decay time constants of sIPSCs. These changes are consistent with other studies on phosphorylation-dependent effects on IPSCs with both PKA/PKC being shown to alter IPSC amplitude and/or decay in different preparations (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999; Jovanovic *et al.*, 2004). Such changes potentially have significant implications for the modulation of inhibition and subsequently, neuronal excitability.

The dose-response relationship in the presence of α-CaMK-II (See Chapter 4, Fig 4.2) revealed an increase in the maximal response to GABA. This implies an increase in the number of functional receptors at the synapse, a change in

conductance or a change in the probability of ion channel opening may be responsible for the up-regulation of $GABA_A$ receptor function observed. A change in the number of receptors at a synapse has been proposed to be the most efficient way of enhancing GABAergic transmission given that GABA is presumably saturating at those synapses (Nusser *et al.*, 1998).

There is some evidence that at certain synapses in certain cell types, GABA is indeed saturating (De Koninck and Mody, 1994; Nusser *et al.*, 1997; Hájos *et al.*, 2000). Non-stationary noise analysis has allowed the numbers of functional receptors at synapses to be estimated (Traynelis and Jaramillo, 1998) and has shown that in some cases up-regulation of $GABA_A$ receptor function is attributable to increases in receptor number (Otis *et al.*, 1994; De Koninck and Mody, 1994; Mizoguchi *et al.*, 2003). These increases are usually not associated with a change in decay. Recently Wang *et al.* (2003) demonstrated that PKB phosphorylation can increase the number of receptors at the cell surface via phosphorylation of the $\beta 2$ subunit at Ser^{410}, which results in an increase in mIPSC amplitude without affecting IPSC decay.

Recent evidence, however, has suggested that GABA is not saturating at all synapses in the CNS (Perrais and Ropert, 1999) and that receptor occupancy can vary between cell types and synapses within the same cell (Nusser *et al.*, 1997; Hájos *et al.*, 2000). Application of benzodiazepines (BDZ's) will increase the amplitude and the decay time constant of IPSCs, but only the decay time constant, if GABA is saturating. A number of studies have shown BDZs can increase IPSC amplitude in a number of cell types at different synapses. This is true for multi-quantal release (sIPSCs) as well as spontaneous miniature release (mIPSCs) of GABA (Rumpel and Behrends, 2000).

In the current study it is not yet apparent whether some or all of the synapses formed in these cultures are saturating. However there is a possibility that, as in other cell types, receptor occupancy is variable (Hájos *et al.*, 2000). Nusser and colleagues (1997) observed that a quantum of GABA results in incomplete receptor occupancy for a sub-population of events recorded from cerebellar stellate cells whilst others were thought to be saturated. Hajos *et al.* (2000) also observed differential receptor occupancy in olfactory bulb granule cells. They observed two populations of events with different decay time constants only one of which appeared to be saturated by a quantum of GABA. In the current study, α-CaMK-II appeared to have different effects on different populations of events; one possible explanation for this could be differences in the level of receptor occupancy after GABA release at different synapses within these cells.

An alteration in the probability of channel opening and the conductance of a channel could account for the apparent changes in amplitude observed (Moss *et al.*, 1995). An increase in the probability of channel opening could increase the apparent amplitude of whole-cell GABA currents and sIPSCs but would only produce large effects if GABA were non-saturating (Nusser *et al.*, 1998). Probability of opening may also affect the decay phase of IPSCs. These parameters could be changed by the covalent addition of a phosphate group (phosphorylation) altering conformation of the receptor, resulting in an alteration of single channel properties (Soltesz and Mody, 1995). Tyrosine kinase phosphorylation of the γ2 subunit has been shown to increase the mean open time and the probability of opening of the $GABA_A$ receptor (Moss *et al.*, 1995) corresponding to an increase in the amplitude of whole-cell currents recorded.

Another potential mechanism for the increase of IPSC amplitude and decay times observed could involve changes in the desensitisation of the receptor. Alterations in desensitisation have been shown to occur through phosphorylation/dephosphorylation (Jones and Westbrook, 1997). This may be a relatively common mechanism to modulate fast synaptic transmission (Jones and Westbrook, 1996). The bi-exponential decay of most IPSCs is thought to reflect the movement of the $GABA_A$ receptor through desensitised states (representing the slower phase of decay). Although no or limited current flows through the desensitised channel, entering into a desensitised state prolongs the amount of time the channel can be active after a transient exposure to GABA.

The number of IPSCs with a bi-exponential decay and the relative contribution of each phase has been shown to be modulated by phosphorylation (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999). The current study reveals an increase in amplitude and a slowing of the decay phase without alteration in the relative contribution of each component, resulting in a significant increase in synaptic charge transfer. Soltesz and Mody (1995) identified a calcium-dependent alteration in decay from a single exponential decay to a prolonged double exponential after amputation of dentate gyrus granule cell dendrites (>66 % of the dendritic tree – dendrotomy), which resulted in a significant increase in synaptic charge transfer. α-CaMK-II has been observed to alter desensitisation in rat spinal dorsal horn neurons acting to prolong decay times (Wang *et al.*, 1995). In addition the Ca^{2+} sensitive phosphatase calcineurin (CaN) has been reported to modulate desensitisation acting to lengthen and in some cases to shorten decay times (Martina *et al.*, 1996; Jones and Westbrook, 1997; Mozrzymas and Cherubini, 1998).

IPSCs in general display considerable variation in decay times between different cell types and even within the same cell (Banks *et al.*, 1998; Bacci *et al.*, 2003). The different populations of decay times seen in the present study were entirely consistent with this. Such variation may have significant functional effects with different $GABA_A$ receptor subtypes and their distinct kinetic properties being incorporated into discrete synapses to perform particular roles in controlling neuronal excitability (Jones and Westbrook, 1996; Somogyi, 2005). Variation can occur as a result of several different reasons. Different $GABA_A$ receptor subunit combinations can produce receptors with different kinetic properties (Puia *et al.*, 1994; Gingrich *et al.*, 1995; Haas and Macdonald, 1999; Bacci *et al.*, 2003). Often these subunit combinations and their corresponding decay time constants can alter during development (Tia *et al.*, 1996; Hollrigel and Soltesz, 1997; Hutcheon *et al.*, 2000; Vicini *et al.*, 2001). For example, $\alpha 2/3\beta 3$ receptors have a slower apparent IPSC decay time and are often expressed earlier in development being replaced by faster decaying $\alpha 1\beta 2$ receptors as synapses reach maturity (Vicini *et al.*, 2001; Jüttner *et al.*, 2001). The $GABA_A$ receptor $\beta 3$ subunit, knock-out mouse displays CGC IPSCs that decay faster than their wild-type counterparts, which may be a result of an associated down-regulation of $\alpha 2/3$ expression (Ramadan *et al.*, 2003). There is evidence that different synapses within the same cell may express different $GABA_A$ receptor subunits and that accordingly, decay time constants at different synapses within the same cell are different (Koulen *et al.*, 1996; Banks *et al.*, 1998; Brünig *et al.*, 2002). This possibly suggests that the slower decaying events in the current study may correspond to $\alpha 2/3\beta 3$ subunit-containing receptors.

Variation in the rate of decay can also result from the activation of perisynaptic receptors, activated by the spillover of GABA from the synaptic cleft

(Wall, 2002; Wei *et al.*, 2003). This phenomenon has been observed at Golgi-granule cell synapses in the cerebellum (Rossi and Hamann, 1998; Hamann *et al.*, 2002). A preferential up-regulation of these perisynaptic receptors by CaMK-II dependent phosphorylation could theoretically increase the apparent decay of synaptic events without involving phosphorylation of the synaptic $GABA_A$ receptors themselves. However, this would affect the rise-times of IPSCs as well as the decay times which was not observed in the current study.

Variations in IPSC decay times can also result at non-saturating synapses as a result of variations in GABA concentration within the synaptic cleft (Nusser *et al.*, 2001). Clustering of receptors and associated scaffolding proteins at the synapse can also alter decay. Co-expression of recombinant $\alpha1\beta2\gamma2L$ receptors with GABARAP in QT6 quail fibroblasts promoted clustering. These clustered receptors deactivated faster and densensitised slower (Chen *et al.*, 2000).

Alteration in the level of variation around the mean (IPSC amplitude and/or decay) can in itself alter neuronal excitability. Increasing event to event variance of peri-somatically injected IPSCs caused either a decrease, an increase or no change in the firing rate of CA1 hippocampal pyramidal cells, depending on the mean around which the scatter was introduced and the degree of scatter (Aradi *et al.*, 2002; Aradi *et al.*, 2004). Alterations in the decays of IPSCs can have significant effects on synaptic inhibition and the excitability of a cell (Jones and Westbrook, 1996). Changes in decay have also been linked to pathological states, for example, receptor mutations that alter the decay phase of IPSCs have been linked to familial epilepsy and insomnia (Bianchi *et al.*, 2002; Buhr *et al.*, 2002; Fisher, 2004).

It is possible that in the current study some of the variation in IPSC decay times observed occurred as a result of dendritic filtering. An inadequate voltage

clamp may alter the profile of IPSCs occurring at distant synaptic sites. It is unlikely this effect is significant in these cells as they are electrotonically compact (Gabbiani *et al.*, 1994; Rossi and Hamann, 1998), so the effect of dendritic filtering should be minimal. To confirm this, scatter plots of sIPSC rise-times were plotted against amplitudes and it was confirmed there was no linear relationship between the two.

Given the large number of potential mechanisms that could result in the changes in sIPSC amplitude and decay times observed in the present study it is not possible to say precisely how α-CaMK-II mediates its effects on IPSCs. It is also not clear whether this effect is through direct phosphorylation or whether modulation occurs indirectly. There is some evidence to suggest that CaMK-II can bind to the β subunit TM3-4 intracellular loop and that phosphorylation of the β3 subunit occurs within embryonic cortical neurones in culture in response to CaMK-II activation (McAinsh K, Jovanovic JN and Moss SJ, unpublished observations). However, the data in the current study suggests that the effect of α-CaMK-II is not uniform across all IPSC events. It may be that more than one process is involved and/or that some $GABA_A$ receptor subunit combinations are functionally modulated in different ways by α-CaMK-II. In addition, variation in synapses such as receptor occupancy, receptor number and GABA concentration within the synaptic cleft following action potential driven release could alter the way an IPSC is modulated by CaMK-II phosphorylation.

It is interesting that α-CaMK-II appears to potentiate $GABA_A$ receptor function when CaMK-II is also involved in potentiation of AMPA receptor function (Lisman *et al.*, 2002; Colbran and Brown, 2004). There are a number of parallels that exist between excitatory and inhibitory long-term synaptic plasticity, in that both respond bi-directionally to Ca^{2+} signals (Aizenman *et al.*, 1998; Yang *et al.*, 1999;

Cormier *et al.*, 2001). These Ca^{2+} signals, at excitatory synapses, are thought to preferentially activate either calcineurin or CaMK-II (Xia and Storm, 2005). GABAergic synapses have also been shown to be depressed by calcineurin and potentiated by CaMK-II (Kano *et al.*, 1996; Wang *et al.*, 2003a). It may be that this form of potentiation of $GABA_A$ receptor synapses occurs as a homeostatic mechanism in response to large increases in excitatory drive (Turrigiano and Nelson, 2004). Long lasting changes in inhibitory synaptic transmission can as a consequence alter the thresholds for inducing excitatory LTP, a process known as metaplasticity (Abraham and Tate, 1997; Chevaleyre and Castillo, 2004).

Kano *et al.* (1992; 1996) identified a phenomenon termed rebound potentiation, involving an α-CaMK-II dependent increase in amplitude (with no change in the decay times) of sIPSCs in cerebellar Purkinje neurones. Purkinje cells are thought to express predominantly α1β2γ2 receptors (Persohn *et al.*, 1992). The CGCs in the current study are thought to express α1/2/3/6, β2/3, γ2 and possibly δ subunit-containing receptors. Maintaining cells in depolarising conditions may shift the expression of $GABA_A$ receptor subunits towards α2/3, β3 subunit-containing receptors (Ives *et al.*, 2002) but in low KCl (5 mM), CGC development may be expected to follow the same pattern of development as *in vivo* and as a result express an increased number of different $GABA_A$ receptor subunits (Wisden *et al.*, 1996). The differential effect of α-CaMK-II on different populations of IPSCs may reflect different $GABA_A$ receptor subtypes at different synapses. As modulation of IPSCs in Purkinje cells by CaMK-II involves an increase in amplitude with no change in decay the sub-population of events in the current study that increases in amplitude with little change in decay times could be proposed to be mediated by the same $GABA_A$ receptor subtype as that in Purkinje cells. The alteration in decay times seen

in the current study may reflect α-CaMK-II action on another $GABA_A$ receptor subtype other than that seen in Purkinje cells.

In order to understand the variations in response to α-CaMK-II observed in the present study and within the literature it is necessary to determine the effect of CaMK-II on defined receptor populations. This has proved effective in investigating the variation in response to PKA dependent phosphorylation of $GABA_A$ receptors (McDonald *et al.*, 1998). The lack of response to α-CaMK-II in HEK293 cells suggests a neuronal environment may be needed to observe any effects. However, it is difficult to control the expression of $GABA_A$ receptor subunits expressed in neuronal cells. A cell line of neuronal origin may allow CaMK-II dependent modulation of the $GABA_A$ receptor to be observed whilst allowing more control over the $GABA_A$ receptor subunits that are expressed.

5.4 CONCLUSIONS

- Application of α-CaMK-II results in a significant potentiation of the amplitudes and an increase in the decay times of sIPSCs recorded from cerebellar granule cells.
- The effect of α-CaMK-II is not uniform across all events in the population suggesting possible differential effects of α-CaMK-II at different synapses possibly expressing different $GABA_A$ receptor subtypes.

Chapter Six:

CaMK-II modulation of recombinant $GABA_A$ receptors expressed in NG108-15 cells.

6.1 INTRODUCTION

CaMK-II is capable of modulating $GABA_A$ receptor mediated currents in a number of neuronal cell types (Wang *et al.*, 1995; Kano *et al.*, 1996; Wei *et al.*, 2004) including cerebellar granule cells (Chapters 4-5). However it appears unable to modulate recombinant $GABA_A$ receptors expressed in HEK293 cells. This is presumed to be because the intracellular environment of a HEK293 cell is unable to support the CaMK-II modulation of $GABA_A$ receptors. However, expression of recombinant receptors in a cell line that does not express endogenous $GABA_A$ receptors is desirable because it allows the exact subunit combination present to be controlled (McDonald *et al.*, 1998). For this reason, and to find an environment that would support CaMK-II modulation, $GABA_A$ receptor subunits were expressed in NG108-15 cells, a cell line of neuronal and glial origin (Klee and Nirenberg, 1974; Daniels and Hamprecht, 1974). In this way, CaMK-II modulation of recombinant $GABA_A$ receptors could be investigated and used to determine the relative effects of the kinase on different subunit types.

The NG108-15 clonal cell line is a hybrid cell line formed from the fusion of mouse neuroblastoma N18TG-2 cells and rat glioma C6BU-1 cells (Klee and Nirenberg, 1974; Daniels and Hamprecht, 1974). Since the formation of this cell line in the 1970s these cells have been used extensively to study neuronal differentiation (Tojima and Ito, 2004). Under basic culture conditions NG108-15 cells proliferate, but after treatment to increase internal cAMP levels, they stop proliferating and

produce extended processes consistent with a neuronal cell-type morphology (Docherty *et al.*, 1991; Wu *et al.*, 1998; Tojima and Ito, 2004). This property has been used as a model for the study of neuritogenesis and synaptogenesis (Nirenberg *et al.*, 1983; Han *et al.*, 1991; Chen *et al.*, 2001; Tojima *et al.*, 2003a; Tojima *et al.*, 2003b).

These cells display a wide range of voltage-dependent currents comparable to those observed in neurones (Docherty *et al.*, 1991; Lukyanetz, 1998; Bowden *et al.*, 1999). They are thought to be comparable to sympathetic neurones of the autonomic nervous system and are known to express choline acetyltransferase (ChAT; Daniels and Hamprecht, 1974). In co-culture with myotubes they form synapses and miniature endplate potentials (mEPPS) can be recorded (Nelson *et al.*, 1976; Christian *et al.*, 1977; Chen *et al.*, 2001). For this reason they have also been used as a basic model of the neuromuscular junction. Furthermore, under certain culture conditions, they can express a number of ligand-gated ion channels. For example, they have been reported to express $5HT_3$ receptors (Chan *et al.*, 1994; Bartrup and Newberry, 1996; Boddeke *et al.*, 1996), δ opioid receptors (Beczkowska *et al.*, 1997; Buzas *et al.*, 1998), NMDA receptors (Ohkuma *et al.*, 1994; Beczkowska *et al.*, 1997) and muscarinic acetylcholine receptors (Fukuda *et al.*, 1988). It should be noted that although this cell line is termed clonal, this refers to the fact that they are derived from a common ancestor and does not indicate that all cells are genetically identical. Over time in culture they can undergo selection depending on the specific culture conditions within a given laboratory. For this reason not all NG108-15s will display the same characteristics and may not express the same neuronal receptors (Docherty *et al.*, 1991).

NG108-15 cells have even been reported to display spontaneous synaptic currents when cultured alone (Tojima *et al.*, 2000a; Tojima *et al.*, 2003a). In general they are able, given the appropriate culture conditions, to form morphological characteristics of neurones as well as certain pre- and post-synaptic structures.

Under non-differentiating conditions when they are allowed to grow freely, they can display some neuronal-type morphological characteristics with some small processes present (Docherty *et al.*, 1991); however the expression of neuronal markers (ChAT, synaptophysin, syntaxin-1), the formation of large processes, and the establishment of synaptic connections, tends to occur after alteration of culture conditions to increase intracellular cAMP levels (Tojima *et al.*, 2000b). Under non-differentiating conditions they can express some cytoskeletal components associated with neurones, such as certain neurofilaments (e.g. NF200), and the actin depolymerising protein, cofilin (Tojima *et al.*, 2000b; Tojima *et al.*, 2003b). Under both proliferating and differentiated conditions they are not thought to express $GABA_A$ receptors (Searles and Singer, 1988; Ishizawa *et al.*, 1997) although they have been reported to express glutamic acid decarboxylase (GAD) and possibly GABA (Searles and Singer, 1988).

NG108-15 cells have been used to study the properties of recombinant ion channels in the same way as HEK293 cells have by transfection with the relevant cDNAs. They have been stably transfected with dopamine D_3 receptors which have been shown to be functionally coupled to G-proteins. Dopamine D_3 receptors only couple weakly with G-proteins in Chinese Hamster Ovary cells, GH_4C_1 pituitary cells and non-differentiated NG108-15 cells (Pilon *et al.*, 1994; Seabrook *et al.*, 1994). NG108-15 cells have also been successfully transfected with $GABA_B$ receptors to study $GABA_B$ receptor function. This approach revealed possible

endogenous but non-functional $GABA_B$ receptor subunits within these cells (Easter and Spruce, 2002).

NG108-15 cells have also been used to investigate Ca^{2+} dependent signalling pathways involving Ca^{2+}/CaM dependent phosphatase and kinase activity. Calcineurin activity has been shown to regulate the function of voltage-operated Ca^{2+} channels and 5-HT_3 receptors in NG108-15 cells (Boddeke *et al.*, 1996; Lukyanetz *et al.*, 1998). It has also been shown that calcineurin and possibly CaMK activity can modulate δ opioid mRNA expression in these cells (Buzas *et al.*, 1998). CaMK-II and CaMK-IV activity has been detected in NG108-15 cells after stimulation by direct depolarisation or following application of bradykinin (Yamakawa *et al.*, 1992; Enslen *et al.*, 1996). Endogenous CaMK-II within NG108-15s exists at a low level but is significantly increased by treatment that induces cellular differentiation (Vallano and Beaman-Hall, 1989). Activation of endogenous CaMK-II has been linked to peripheral actin polymerization and filopodia formation (Chen *et al.*, 2003) and overexpression of α-CaMK-II and autophosphorylation of α-CaMK-II have been shown to alter growth cone motility and neurite outgrowth in this cell line (Goshima *et al.*, 1993).

It seemed, therefore, that because of its neuronal origin and evidence of endogenous Ca^{2+}/CaM signalling pathways, that CaMK-II may be able to modulate the function of recombinant $GABA_A$ receptors expressed within this cell line. In order to eliminate the possibility of endogenous $GABA_{A/B}$ receptors being present or other ligand-gated ion channels, and to limit the contribution of endogenous CaMK-II; proliferating, undifferentiated NG108-15s were transfected with various $GABA_A$ receptor subunit cDNAs. In this way NG108-15s provided a neuronal-like environment where α-CaMK-II modulation of recombinant receptors could be

observed and the relative contribution of different receptor subunits to the action of CaMK-II could be determined.

6.2 RESULTS

Using a similar protocol to that established in the previous chapters, pre-activated recombinant α-CaMK-II (Suzuki-Takeuchi *et al.*, 1992) was included in the patch pipette solution (McGlade-McCulloh *et al.*, 1993) and whole-cell GABA currents were recorded from transfected NG108-15 cells. This was compared to controls which included either pre-activation buffer without α-CaMK-II or heat-inactivated α-CaMK-II (pre-incubated in activation buffer) in the patch pipette. The peak amplitude of GABA currents were recorded over time as α-CaMK-II dialysed into the cell and normalised to the peak GABA current amplitude obtained 3-4 min after achieving the whole-cell configuration.

As NG108-15 cells in their undifferentiated state have a more compact round morphology (Docherty *et al.*, 1991) compared to cerebellar granule cells, and as in previous experiments 85-100 nM α-CaMK-II produced large increases in amplitude, the concentration was reduced to 60 nM α-CaMK-II to produce an easily observable effect whilst maintaining stable recordings. After stimulation to undergo differentiation NG108-15 cells can form large processes that make it difficult to establish an ideal voltage clamp of membrane currents (Docherty *et al.*, 1991). Using undifferentiated cells therefore, had the added advantage of avoiding problems of inadequate space clamp, as in this state, cellular processes are generally small.

NG108-15 cells were transfected with an equal ratio of α:β:γ subunit and enhanced GFP cDNAs using the transfection reagent Effectene (See Chapter 2). Transfected cells were subsequently identified by their fluorescence. As NG108-15

cells tended to form aggregates, they were plated at a low density following transfection and recorded from 24-72 hrs later. Cells were selected for electrophysiology if they were isolated from other cells, had only a few small processes and displayed low levels of fluorescence. As the cells were large and generally transfection efficiency was high, some GABA currents recorded were over 4000pA in amplitude even with a 10 μM GABA application (EC_{50}). These currents were discarded from further analysis due to the potential for insufficient voltage clamp and series resistance problems.

6.2.1 NG108-15 cells did not express functional endogenous $GABA_A$ receptors.

It is thought that NG108-15 cells do not express $GABA_A$ receptors, however as these cells differ in their characteristics depending on the culture conditions and there was no systematic or thorough demonstration of this lack of expression in the literature, cells were transfected with cDNAs for GFP alone or for GFP and α1 subunits alone, and GABA was applied to untransfected and transfected cells. As can be seen in Fig 6.1 no response to GABA was seen in any control situation (Un-transfected, n = 9, GFP only, n = 5, α1 subunit only, n = 4). This confirmed that there were no functional $GABA_A$ receptors expressed at the cell surface of un-transfected cells. As α1 subunit transfected cells also failed to respond to GABA this confirmed that there were no endogenous β subunits present as if there had been, it would be predicted that the co-expression of α1 would allow α1β receptors to be formed as has been observed in some cases with HEK293 cells (Ueno *et al.*, 1996; Davies *et al.*, 2000). As transfection with cDNAs for αβ/γ subunits produced large currents in response to GABA it appeared that recombinant $GABA_A$ receptors were

expressed with high efficiency. It was thought, therefore, that recombinant subunits would dominate any endogenous protein that might be present (Davies *et al.*, 2000).

Fig 6.1:

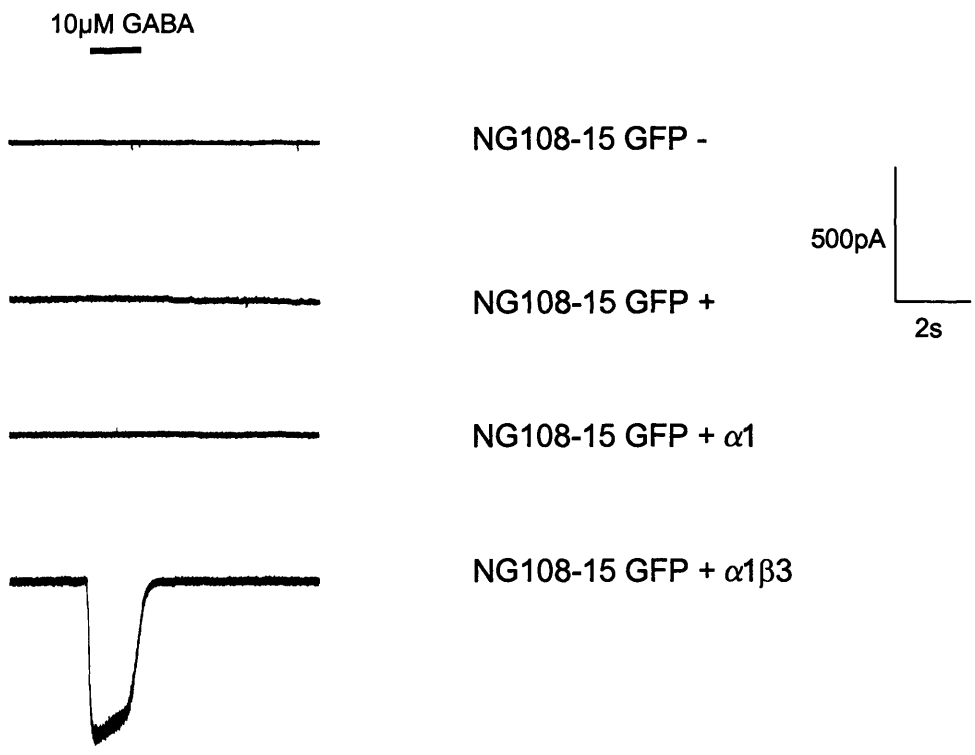

Fig 6.1: Expression of $GABA_A$ receptors in NG108-15 cells.

Representative membrane currents showing response to applied GABA (10 μM) recorded from:

(A) Untransfected NG108-15 cell (n = 9).

(B) Enhanced GFP (EGFP) positive NG108-15 cell transfected with EGFP alone (n = 5).

(C) EGFP positive NG108-15 cell transfected with the EGFP and the α1 subunit only (n =5).

(D) EGFP positive NG108-15 cell transfected with enhanced EGFP and α1β3.

6.2.2 Recombinant $\alpha\beta$ receptors and CaMK-II.

Application of 60 nM α-CaMK-II to cells transfected with $\alpha1\beta1$ produced no significant effects (107.4 ± 7.3 %, n = 7) as compared to control (93.8 ± 3.7 %, n = 3, Fig 6.2A) at t = 6 min. Although a slight increase seemed apparent in the first few minutes this was not statistically significant and was indistinguishable from control at t = 8 min. Application of 60 nM CaMK-II to $\alpha1\beta2$ receptors also produced no significant differences (85.5 ± 4.2 %, n = 5) from control GABA currents (91 ± 6.3 %, n = 4, Fig 6.2B) at t = 6 min.

However, application of 60 nM α-CaMK-II to $\alpha1\beta3$ receptors produced a statistically significant increase in the peak amplitude of currents recorded to 118 ± 4.8 % (n = 7) as compared to control, 96.4 ± 4.6 % (n = 7, Fig 6.2C) at t = 6 min which reached a plateau within 6-8 min.

6.2.3 Recombinant $\alpha\beta\gamma$ receptors and CaMK-II.

Application of 60 nM α-CaMK-II to $\alpha1\beta1\gamma2S$ receptors expressed in NG108-15 cells resulted in a significant potentiation of the whole cell currents recorded to 125.6 ± 6.4 % (n = 11) as compared to control, 97.1 ± 3.9 % (n = 5) at t = 6 min. The inclusion of heat inactivated α-CaMK-II though, had no significant effects (89.2 ± 3.1 % at t = 6 min, n = 7, Fig 6.3A).

As for the $\alpha1\beta2$ receptor, application of 60 nM α-CaMK-II to $\alpha1\beta2\gamma2S$ expressing NG108-15 cells had no significant effect (102.5 ± 5.3 %, n = 11) as compared to control (105.1 ± 6.7 %, n = 6, Fig 6.3B). However, application of 60 nM α-CaMK-II to $\alpha1\beta3\gamma2S$ receptors significantly increased the peak amplitude of whole-cell currents to 145.8 ± 10.8 % (n = 7) at t = 6 min as compared to control, 102.8 ± 5 % (n = 4). Heat inactivated α-CaMK-II again had no significant effect

(90.5 ± 11.6, n = 5, Fig 6.3C) as compared to control, but was significantly different from addition of pre-activated CaMK-II, at t = 6 min. The response to α-CaMK-II for α1β3 receptors was significantly increased by the inclusion of the γ2S subunit.

Transfection with cDNA for αβγ subunits in equal ratio should ensure that the majority of receptors at the surface of the cell are αβγ containing receptors as opposed to αβ (Angelotti *et al.*, 1993; Angelotti and Macdonald, 1993). In agreement with this the amplitudes of currents recorded from α1β3γ2S receptors were larger than those recorded from αβ receptors (Angelotti *et al.*, 1993). In addition, application of 10 μM diazepam (Herring *et al.*, 2003) routinely increased the amplitude of the currents recorded indicating the presence of the γ2 subunit (Fig 6.3D).

Fig 6.2:

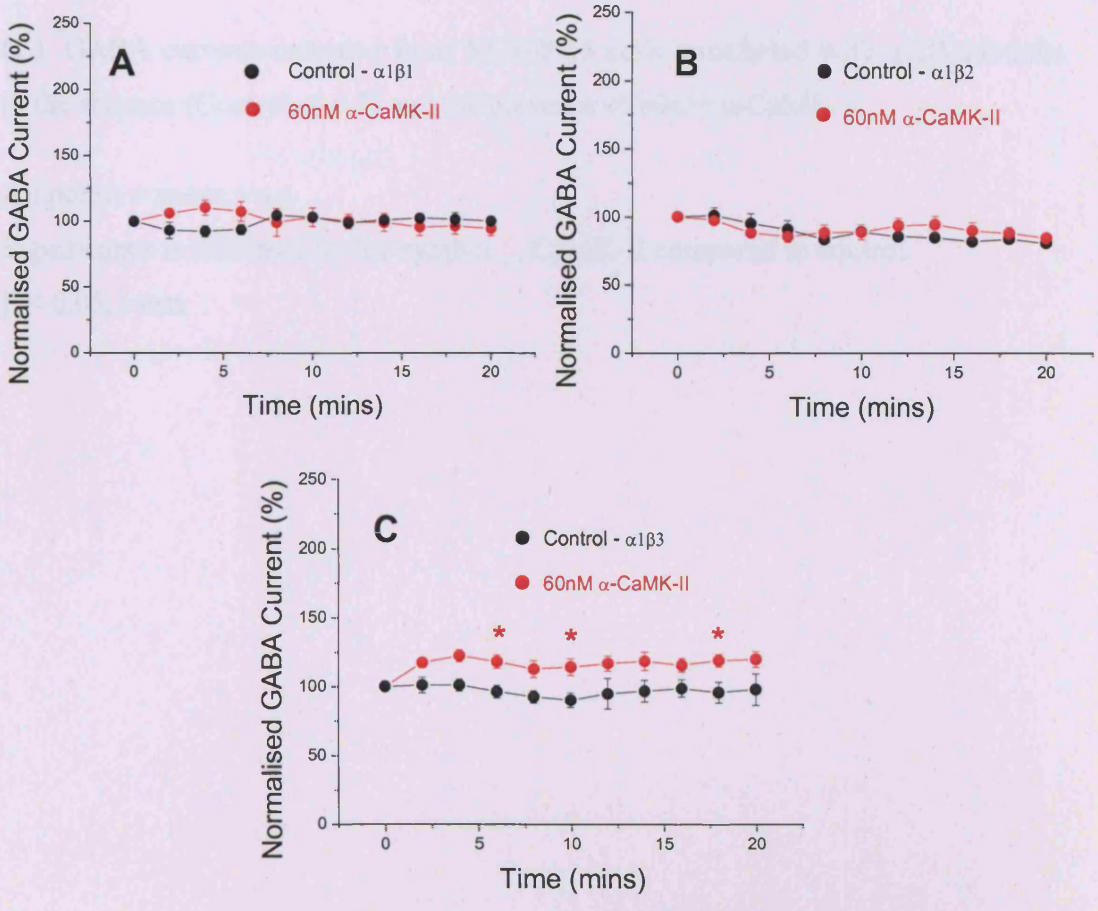

Fig 6.2: Whole-cell currents recorded from NG108-15 cells expressing α1β1-3 subunits were differentially modulated by α-CaMK-II.

Peak amplitude of GABA mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing α1β1, α1β2 and α1β3 $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with α1β1 subunits in the absence (Control, n = 3) and presence of 60 nM pre-activated α-CaMK-II, which was introduced into the cells via the patch pipette solution (n = 7).

(B) GABA currents recorded from NG108-15 cells transfected with α1β2 subunits in the absence (Control, n = 4) and the presence of 60 nM α-CaMK-II (n = 5).

(C) GABA currents recorded from NG108-15 cells transfected with α1β3 subunits in the absence (Control, n = 7) and the presence of 60nM α-CaMK-II (n = 7).

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control.
$P < 0.05$, t-test.

Fig 6.3:

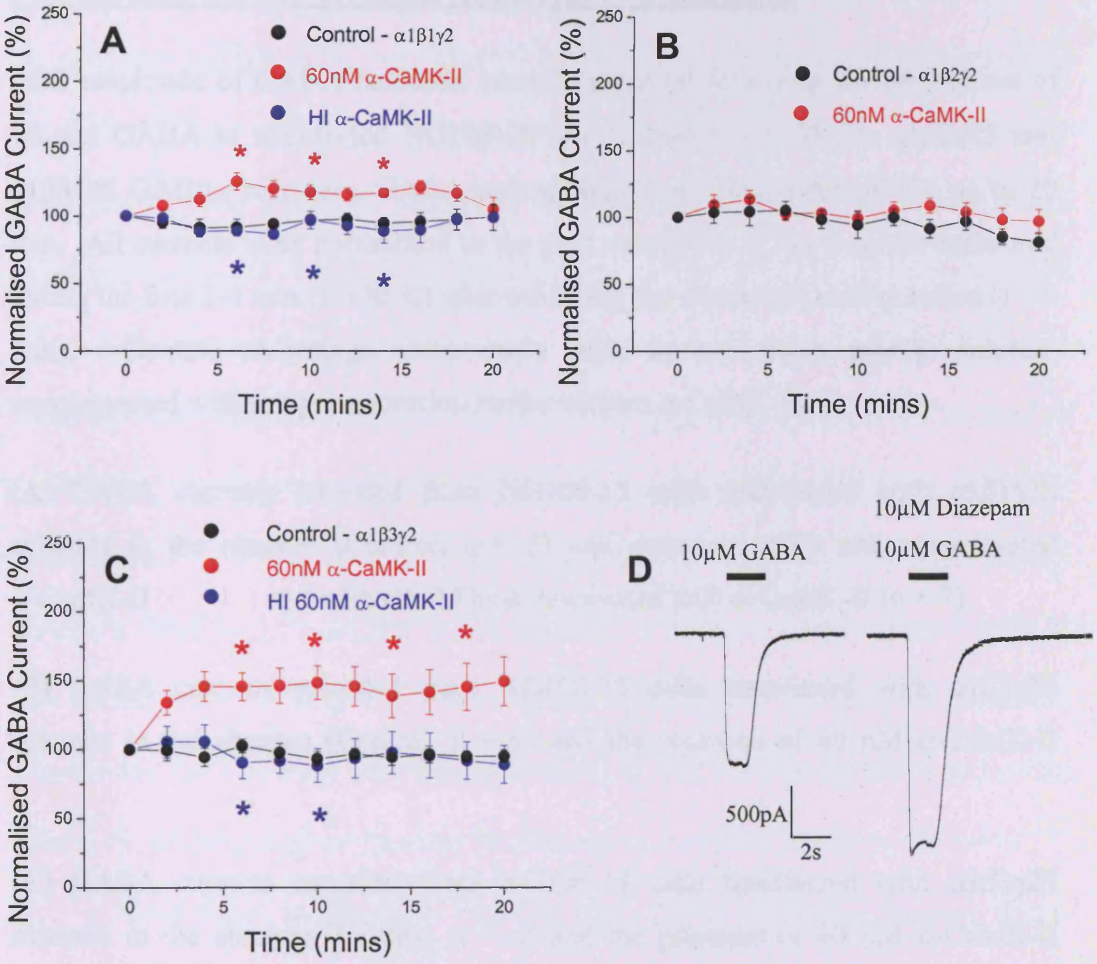
A
Control - α1β1γ2
60nM α-CaMK-II
HI α-CaMK-II
Normalised GABA Current (%)
Time (mins)
B
Control - α1β2γ2
60nM α-CaMK-II
C
Control - α1β3γ2
60nM α-CaMK-II
HI 60nM α-CaMK-II
D
10μM GABA
10μM Diazepam
10μM GABA
500pA
2s

Fig 6.3: Whole-cell currents recorded from NG108-15 cells expressing α1β1-3γ2S subunits were differentially modulated by α-CaMK-II.

Peak amplitude of GABA mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing α1β1γ2S, α1β2γ2S and α1β3γ2S $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with α1β1γ2S subunits in the absence (Control, n = 5) and presence of 60 nM pre-activated α-CaMK-II (n = 11) and with 60 nM heat inactivated (HI) α-CaMK-II (n = 7).

(B) GABA currents recorded from NG108-15 cells transfected with α1β2γ2S subunits in the absence (Control, n = 6) and the presence of 60 nM α-CaMK-II (n = 11).

(C) GABA currents recorded from NG108-15 cells transfected with α1β3γ2S subunits in the absence (Control, n = 4) and the presence of 60 nM α-CaMK-II (n = 7) and in the presence of 60 nM heat inactivated (HI) α-CaMK-II (n = 5).

(D) Representative membrane currents recorded from NG108-15 cells expressing α1β3γ2S receptors before and after the bath application of 10 μM diazepam.

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control and the symbol *, CaMK-II compared to HI CaMK-II.
$P < 0.05$, t-test, ANOVA.

6.3 DISCUSSION

Recombinant $GABA_A$ receptors expressed in NG108-15 cells can be functionally modulated by α-CaMK-II in a manner similar to that observed in cerebellar granule cells. However, control over the subunits that are expressed revealed different CaMK-II dependent effects depending on the precise subunit combination present. The only αβ heteromer that could be modulated by α-CaMK-II was α1β3 suggesting a significant role for the β3 subunit in the α-CaMK-II modulation of $GABA_A$ receptors. Addition of the γ2S subunit increased this level of modulation significantly and revealed a lower, but still significant, level of modulation of α1β1 subunit-containing receptors. However, both α1β2 and α1β2γ2S receptors failed to be modulated by α-CaMK-II. It appears, therefore, that CaMK-II has a differential effect on $GABA_A$ receptors depending on the subunits present, a phenomena that has been observed in relation to other phosphorylation-dependent effects on $GABA_A$ receptor function (McDonald *et al.*, 1998; Nusser *et al.*, 1999).

The different responses to CaMK-II mediated by different β subunits shows some similarity to the effects seen on $GABA_A$ receptors mediated by PKA (McDonald *et al.*, 1998). In this case PKA phosphorylation of β3 is thought to increase the peak amplitude of GABA currents and have no effect on β2 subunit-containing receptors. However, phosphorylation of the β1 subunit by PKA caused a depression of receptor function. In addition PKC is thought to have different effects on different $GABA_A$ receptor subtypes. Again phosphorylation of β3 subunit-containing receptors is thought to increase $GABA_A$ receptor function (Jovanovic *et al.*, 2004) whilst PKC dependent phosphorylation of β1 subunit-containing receptors depresses $GABA_A$ receptor function (Leidenheimer *et al.*, 1992; Krishek *et al.*, 1994;

Brandon *et al.*, 2002b). Although there is some discrepancy in the functional effect observed (Lin *et al.*, 1994; Lin *et al.*, 1996).

The variation in response of native $GABA_A$ receptors to activation of these kinases in neurones is thought, in part, to be mediated by these differential effects of kinases on different receptor subunit combinations. PKA and PKC have both been shown in neurones to have slightly different effects on $GABA_A$ receptor mediated mIPSCs (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999). However the response in neurones does not always correlate to that seen in recombinant systems. For instance, PKA appears able to modulate IPSCs in cells that predominantly express the $\beta 2$ subunit (Nusser *et al.*, 1999) despite a lack of response of the $\beta 2$ subunit-containing $GABA_A$ receptors in HEK293 cells to PKA (McDonald *et al.*, 1998).

In different recombinant systems there is also reported variation in the effects of phosphorylation. For example PKC activity has been reported to increase the amplitude of GABA currents mediated by $\alpha 1\beta 1\gamma 2L$ receptors expressed in L929 fibroblasts (Lin *et al.*, 1996) although in *Xenopus oocytes* and HEK293 cells this has been reported to result in a depression of receptor function (Krishek *et al.*, 1994). In addition, examination of desensitisation rates of $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ receptors expressed in HEK293 cells in response to fast application of GABA revealed identical effects of PKA phosphorylation on macroscopic desensitisation rates (Hinkle and Macdonald, 2003) regardless of the β subunit present.

An important observation in the current study is that the addition of $\gamma 2S$ to the $\alpha 1\beta 3$ heteromer increased the response to α-CaMK-II. It appeared that both $\beta 3$ and $\gamma 2S$ subunits might be important for CaMK-II modulation and that both subunits are required for the full effect to be observed. It is thought that $\alpha\beta\gamma$ receptors form the majority of $GABA_A$ receptors in neurones (Benke *et al.*, 1994) and the $\gamma 2S$

subunit is thought to be required for $GABA_A$ receptors to be correctly targeted to the synapse (Essrich *et al.*, 1998). It is significant, therefore, that α-CaMK-II modulates γ2S subunit-containing receptors to a greater degree than αβ heteromers, as these are the synaptic and therefore more physiologically relevant receptor subtype. This finding also supports data from the previous chapter that α-CaMK-II is capable of significant modulation of synaptic GABA currents in cerebellar granule cells.

Modulation of $GABA_A$ receptors by PKA and PKC has also been suggested to be dependent on both β and γ subunits, with both being required for the full effect to be observed (Krishek *et al.*, 1994). Ser^{409} on the β1 subunit with $Ser^{327/343}$ on the γ2L were found to be important for PKC modulation in L929 fibroblasts (Krishek *et al.*, 1994; Lin *et al.*, 1996). Ser^{410} and Ser^{327} were also found to be important when looking at PKC modulation of α1β2γ2S receptors in *Xenopus oocytes* (Kellenberger *et al.*, 1992). In both cases it was suggested that phosphorylation of both β and γ subunits were required for the full PKC mediated effect to be apparent.

The β and γ subunits are thought to be the main targets of phosphorylation of $GABA_A$ receptors. It may be that phosphorylation of both subunits is required in some cases to mediate a functional effect. Little is currently known about how the intracellular loops of different subunits might interact. There is evidence that γ2 intracellular loops can interact with β subunit intracellular loops (Nymann-Andersen *et al.*, 2002). It is also thought intracellular loops between neighbouring receptors may be able to interact as the γ2 TM3-4 intracellular loop can also associate with other γ2 TM3-4 intracellular loops expressed as GST fusion proteins and this may play a role in receptor clustering (Nymann-Andersen *et al.*, 2002). It has become apparent that phosphorylation can be regulated by the targeting of kinases to specific sites and that anchoring proteins can significantly alter how a kinase behaves

(Brandon *et al.*, 2002a). It may be that the phosphorylation of β and γ subunits is intricately linked within the receptor complex.

In the current study, β2 subunit-containing receptors are not modulated by α-CaMK-II (similar to PKA). The presence of γ2, therefore, is not enough on its own to ensure CaMK-II dependent modulation. In the case of β1 subunit-containing receptors CaMK-II modulation is only observed with γ2S present. This implies that individual subunits cannot be regarded in isolation, but that the receptor as a whole and all its subunits together, determine the effects of phosphorylation. It might be that the β subunit acts in a permissive manner to allow γ2S related phosphorylation.

Cerebellar granule cells maintained in depolarising or more physiological conditions may both express β2/β3 subunits. In depolarising conditions it has been suggested there may be a higher level of β3 subunit expression (Ives *et al.*, 2002a) possibly reflecting the immature nature of these cultures. This would be consistent with the large response to α-CaMK-II in these cultures and the large response to α-CaMK-II seen in NG108-15 cells expressing α1β3γ2S. In more physiological conditions, it is likely that the expression of $GABA_A$ receptor subunits more closely follows that seen *in vivo* (Laurie *et al.*, 1992a; Wisden *et al.*, 1996). As a result β2 and β3 expression may both be significant, although β1 levels would be expected to be low (Persohn *et al.*, 1992; Laurie *et al.*, 1992b). IPSCs in CGCs were modulated by α-CaMK-II but there was evidence for some variation in the response to α-CaMK-II of different populations of sIPSCs. It is possible this may reflect differences in response to α-CaMK-II of β3 versus β2 subunit-containing receptors.

Between subunit isoforms of the $GABA_A$ receptor, there is typically around 70-90 % sequence homology (Moss and Smart, 1996). The β subunits are similar in structure with the main differences between isoforms related to the TM 3-4

intracellular domain (Moss and Smart, 1996). It is known that the β2 subunit is the most prevalent throughout the CNS (Benke *et al.*, 1994), but the β3 subunit is thought to be expressed more predominantly at early stages in development in certain brain regions (Laurie *et al.*, 1992b). This is possibly significant given that β3 appears to be strongly regulated by phosphorylation by a number of kinases. In the adult brain there are clear differences in the pattern of β2 and β3 subunit expression (Miralles *et al.*, 1999). As another example of the differences between these two subunits, β2 subunit knock-out mice are apparently normal in development and behaviour (Sur *et al.*, 2001) whereas β3 knock-out mice are severely developmentally impaired (Homanics *et al.*, 1997; DeLorey *et al.*, 1998).

It is surprising that recombinant receptors containing β2 do not appear to be modulated by PKA (McDonald *et al.*, 1998) or CaMK-II given the large number of these receptors within the brain. There are also surprisingly few examples of β2 subunit dependent phosphorylation in the literature, however, phosphorylation of Ser^{410} by PKB causing an up-regulation of receptor number has been reported (Wang *et al.*, 2003). Ser^{410} appears, so far, to be the main identified Ser/Thr kinase site of phosphorylation on this subunit (McDonald and Moss, 1997). Interestingly when β2 Ser^{410} is phosphorylated by PKC it appears to result in a down-regulation in receptor function (Kellenberger *et al.*, 1992) when receptors are expressed in *Xenopus oocytes*. Chapell *et al.* (1998) also observed this down-regulation and related it to a loss of receptors from the cell surface, however, in this case, mutation of Ser^{410} had no effect on PKC modulation. Both of these studies involved activation of PKC via addition of phorbol 12-myristate 13-acetate (PMA). Differences may reflect variations in experimental protocol (application of PMA) or may of course reflect

another example of the cellular environment, in this case *Xenopus oocytes*, affecting the functional consequences of phosphorylation.

The trafficking of receptors to the synapse can also be altered by phosphorylation and alterations in the number of receptors at the synapse have been suggested to mediate changes in synaptic strength (Kittler and Moss, 2003; Lüscher and Keller, 2004). PKC dependent phosphorylation of β2 subunit-containing $GABA_A$ receptors has been linked to alterations in the cell surface stability of the receptors (Chapell *et al.*, 1998; Connolly *et al.*, 1999). PKB dependent phosphorylation at Ser^{410} has also been suggested to alter receptor number at the cell surface. PKA activity has been shown to differentially regulate trafficking and cell surface expression of β2 and β3 subunit-containing receptors in CGCs (Ives *et al.*, 2002b). Moreover, there is evidence that β2 and β3 subunits can be targeted differently within cells to different locations (Connolly *et al.*, 1996). Interestingly, β2 and β1/3 subunits are reported to require different assembly signals within α1 and γ2 to be correctly assembled and trafficked to the cell surface (Bollan *et al.*, 2003). It is possible that phosphorylation may differentially affect the trafficking of these subunits and some of the variation in response to phosphorylation, observed in neurones, may be due to different levels of activation of mechanisms that alter the number of receptors at the synapse through changes in cell surface stability, or insertion, and those that alter the channel properties of the receptors directly. This type of model has also been proposed for modulation of AMPA receptors by CaMK-II (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Malinow and Malenka, 2002).

The phenomena of rebound potentiation of $GABA_A$ receptor function that occurs in Purkinje cells of the cerebellum, is also CaMK-II dependent (Kano *et al.*, 1992; Kano *et al.*, 1996; Kawaguchi and Hirano, 2002). This cell type is thought to

express predominantly $\alpha1\beta2\gamma2$ receptors (Persohn *et al.*, 1992; Laurie *et al.*, 1992b), however, this subunit combination, in a recombinant system, is not modulated by α-CaMK-II. As yet there is no apparent explanation for this; however, the lack of response to phosphorylation seen in recombinant systems expressing $\beta2$ subunit-containing receptors may reflect unique functional properties of such receptors. A possible explanation is the need for additional co-factors scaffolding/anchoring proteins for $\beta2$ subunit phosphorylation to occur, or that phosphorylation dependent effects on $\beta2$ subunit-containing receptors are indirect, occurring through downstream pathways or phosphorylation of associated proteins. Interestingly the insertion of AMPA receptors stimulated by CaMK-II activity is dependent on GluR1 receptors but not by their direct phosphorylation (Hayashi *et al.*, 2000). It could be speculated that modulation of $\beta2$ subunit-containing receptors by CaMK-II is similar in that modulation of function occurs via downstream pathways / indirect effects and that this is difficult to observe in recombinant systems.

Expression of recombinant receptors in NG108-15 cells suggests that CaMK-II modulation of $GABA_A$ receptors is mediated mainly through $\alpha1\beta3\gamma2S$ receptors and partially through $\alpha1\beta1\gamma2S$. Within the $\beta1/\beta3$ and $\gamma2S$ subunits there are a number of potential sites for phosphorylation by α-CaMK-II (McDonald and Moss, 1994; McDonald and Moss, 1997). Mutation of these residues so they cannot be phosphorylated will allow determination of which sites are the most important on each subunit and also if CaMK-II mediates its effects through direct phosphorylation or whether it can act indirectly through other downstream signalling pathways.

6.4 CONCLUSIONS

- Recombinant $\alpha 1\beta 3$ receptors expressed in NG108-15 cells can be functionally up-regulated by α-CaMK-II.
- Recombinant $\alpha 1\beta 1$ and $\alpha 1\beta 2$ receptors are not functionally modulated by α-CaMK-II.
- Inclusion of the $\gamma 2S$ subunit to $\alpha 1\beta 3$ subunit-containing receptors enhances the modulation by α-CaMK-II.
- Inclusion of the $\gamma 2S$ subunit to $\alpha 1\beta 1$ subunit-containing receptors reveals a functional modulation by α-CaMK-II that is smaller than the effect observed for $\alpha 1\beta 3\gamma 2S$ receptors.
- Inclusion of the $\gamma 2S$ subunit to $\alpha 1\beta 2$ subunit-containing receptors has no effect. $GABA_A$ receptors containing the $\beta 2$ subunit do not appear to be regulated by α-CaMK-II in a recombinant system.

Chapter Seven:

Sites of CaMK-II phosphorylation on recombinant $GABA_A$ receptors expressed in NG108-15 cells and cerebellar granule cells.

7.1 INTRODUCTION

Modulation of the $GABA_A$ receptor by α-CaMK-II was observed in cerebellar granule cells and modulation of recombinant $GABA_A$ receptor subunits was also observed in the neuronal cell line NG108-15 (See Chapter 6). α-CaMK-II was capable of potentiating GABA currents recorded from NG108-15 cells expressing α1β3 / α1β1γ2S and α1β3γ2S receptors implying that the β1/3 and γ2S subunits were required for the effect. It was, however, unclear if α-CaMK-II mediated its effect through: direct phosphorylation of these subunits; phosphorylation of accessory proteins associated with them; or through activation of further downstream signalling pathways. In order to address these questions, potential phosphorylation sites on the β and γ2 subunits were altered through site-directed mutagenesis to prevent phosphorylation. These mutated recombinant receptors were then expressed in NG108-15 cells, which do not express endogenous $GABA_A$ receptors, and the effect on α-CaMK-II modulation was determined. To confirm the importance of potential sites of phosphorylation recombinant receptors were also expressed in cerebellar granule cells in culture.

The use of site-directed mutagenesis and expression of recombinant receptors in a cell line with no endogenous receptors has been used successfully to determine the sites of phosphorylation important for modulation of $GABA_A$ receptors by

PKC/PKA and the tyrosine kinase, Src (Kellenberger *et al.*, 1992; Moss *et al.*, 1995; Lin *et al.*, 1996; McDonald *et al.*, 1998; Chapell *et al.*, 1998). In addition, GST fusion proteins, formed from the TM3-4 intracellular loop of different subunits, have been used to identify potential sites of phosphorylation by different kinases on $GABA_A$ receptors (Machu *et al.*, 1993; McDonald and Moss, 1994; McDonald and Moss, 1997). The fusion proteins formed from the β1 subunit could be phosphorylated by PKG, PKA, PKC and CaMK-II on Ser^{409}, and on Ser^{384} by CaMK-II only (Moss *et al.*, 1992; McDonald and Moss, 1994). The Ser^{409} site was found to be important for PKC dependent functional modulation of $GABA_A$ receptors (Krishek *et al.*, 1994; Lin *et al.*, 1996; McDonald *et al.*, 1998; Brandon *et al.*, 2002b; Hinkle and Macdonald, 2003).

The β2 subunit has one main site of phosphorylation at Ser^{410}, (the equivalent of Ser^{409} in β1/3). PKG, PKC, PKA and CaMK-II were all capable of phosphorylating this site on a GST fusion protein of the TM3-4 intacellular loop of the β2 subunit (McDonald and Moss, 1997). This site has been shown to be important in PKC dependent modulation of whole-cell currents recorded from recombinant α1β2γ2S receptors expressed in *Xenopus oocytes* (Kellenberger *et al.*, 1992). It has also been shown to be required for PKB mediated increases in the number of β2 subunit-containing receptors at the cell surface of HEK293 cells and hippocampal neurones (Wang *et al.*, 2003a).

The use of GST-fusion proteins has also identified Ser^{409} as the major site of phosphorylation in the β3 subunit. Using such constructs PKC, PKA, PKG and CaMK-II all phosphorylated this site. Moreover, Ser^{408} was additionally phosphorylated by PKC, and Ser^{383} was also subject to phosphorylation, but only by CaMK-II (See Table 1.1). In a recombinant system, Ser^{408} and Ser^{409}

phosphorylation of β3 by PKA was required for an up-regulation of function and mutation of Ser^{408} converted the up-regulation into a down-regulation similar to that observed for PKA modulation of β1 subunit-containing receptors (McDonald *et al.*, 1998). It appears, therefore, that there are three potential sites of CaMK-II phosphorylation on the β3 subunit, $Ser^{383,408,409}$.

The γ2S subunit intracellular loop, expressed as a fusion protein, can be phosphorylated by PKC at Ser^{327}, and at Ser^{348} and Thr^{350} by CaMK-II (Moss *et al.*, 1992; McDonald and Moss, 1994). Both PKC and CaMK-II were capable of phosphorylating Ser^{343} within the 8 amino acid insert that forms the γ2L splice variant (Moss *et al.*, 1992; Machu *et al.*, 1993; McDonald and Moss, 1994). PKA did not phosphorylate the γ2S subunit intracellular loop fusion protein (Moss *et al.*, 1992). Phosphorylation of Ser^{327} and Ser^{343} in γ2L by PKC have both been shown to be functionally important for $GABA_A$ receptors expressed in heterologous systems (Kellenberger *et al.*, 1992; Krishek *et al.*, 1994; Lin *et al.*, 1996).

As well as Ser/Thr kinases, tyrosine kinases have also been shown to phosphorylate the $GABA_A$ receptor and modulate function in both heterologous expression (Moss *et al.*, 1995; Wan *et al.*, 1997) and neuronal systems (Moss *et al.*, 1995; Valenzuela *et al.*, 1995). Tyrosine kinase activity has been shown to potentiate GABA currents (Moss *et al.*, 1995; Huang and Dillon, 1998) including IPSC amplitudes (Boxall, 2000; Ma *et al.*, 2003). It has been reported that application of $pp60^{c\text{-}src}$ to HEK293 cells resulted in phosphorylation of β2/3 subunits (Wan *et al.*, 1997). Modulation of α1β1 receptors expressed in *Xenopus oocytes* by tyrosine kinase activity has also been reported (Valenzuela *et al.*, 1995). In addition phosphorylation of tyrosines 384 and 386 within the β1 subunit has also been reported, but in this case, phosphorylation appeared to be functionally silent (Moss *et*

al., 1995). Mutation of the tyrosine residues at 365 and 367 on the γ2L subunit (corresponding to Y357 and Y359 on γ2S) was found to block functional modulation by Src of $GABA_A$ receptors in HEK293 cells (Moss *et al.*, 1995). It appears therefore that these two residues may be the most functionally relevant sites of phosphorylation on the γ2 subunit. These sites have been shown to be phosphorylated within the brain and can be dynamically regulated by tyrosine phosphatase activity in cultured cortical neurones (Brandon *et al.*, 2001).

Thus, there are a number of sites identified on $GABA_A$ receptors that may be directly phosphorylated by CaMK-II. Expression of αβ heteromers in NG108-15 cells suggests that β3 is the only β subunit that could be directly phosphorylated by α-CaMK-II. In addition, γ2S subunits appeared to play a role in α-CaMK-II modulation of α1β1γ2S and α1β3γ2S receptors. A series of mutant receptor subunits were prepared, with potential sites of phosphorylation ablated so they could not be phosphorylated, using site-specific mutagenesis (See Chapter 2.1). These mutant receptors were expressed in NG108-15 cells and cerebellar granule cells to determine the functional effect on α-CaMK-II modulation.

7.2 RESULTS

In each case, NG108-15 cells and cerebellar granule cells were transfected with αβ or αβγ subunit cDNA combinations, in equal ratios, along with enhanced GFP for identification of transfected cells. 60 nM α-CaMK-II (pre-activated) was introduced into the cells via the patch pipette solution (See Chapter 2 and 3.2.1). Control recordings consisted of a normal patch pipette solution containing the pre-activation buffer without α-CaMK-II. Whole-cell currents were recorded in response to GABA and the percentage change in the peak current amplitude was recorded over

time. Current amplitudes were then normalised to the peak amplitude recorded 3-4 min after first achieving the whole-cell configuration.

7.2.1 α-CaMK-II modulation of $GABA_A$ receptor mutant αβ heteromers.

The α1β3 receptor was selected as this exhibited the most significant modulation by α-CaMK-II (See Fig 6.2). All three potential phosphorylation sites, $Ser^{383,408,409}$ were converted to alanines within the β3 subunit. It was presumed that this would remove all the potential sites of CaMK-II phosphorylation within this subunit. On the sequential application of 10 μM GABA over 20 min in control conditions, the amplitude of GABA currents recorded was quite stable with little run-down.

On application of 60 nM α-CaMK-II, no significant effect was seen on the amplitude of the currents recorded (93.2 ± 7.6 %, n = 8) as compared to the controls (101.3 ± 4.6 %, n = 5 at t = 6 min, Fig 7.1A) indicating that phosphorylation of at least one of these residues was capable of mediating the α-CaMK-II modulation of the α1β3 receptor. PKA phosphorylation of β3 has been reported to increase the amplitude of $GABA_A$ receptor mediated currents through phosphorylation of $Ser^{408,409}$. However, the application of 60 nM α-CaMK-II to $\alpha1\beta3^{S408A,S409A}$ receptors resulted in a significant potentiation of the amplitude of currents recorded to 120.6 ± 6.2 % (n = 7) as compared to control, 93.9 ± 3.8 % (n = 4) at t = 6 min, which was not significantly different from the effect of 60 nM α-CaMK-II on α1β3 wild-type receptors (118 ± 4.8 %, n = 7, Fig 7.1B). This effect of α-CaMK-II had a similar time-course to the effect on wild-type receptors reaching a steady-state within 6 min.

Although $Ser^{408,409}$ appeared unimportant for the α-CaMK-II effect, application of 60 nM α-CaMK-II to $\alpha1\beta3^{S383A}$ receptors resulted in no significant

increase in amplitude (94 ± 6.8 %, n = 5) as compared to control (101.7 ± 1.5 %, n = 6 at t = 6 min, Fig 7.1C). This indicates that α-CaMK-II modulation of the α1β3 receptor is mediated solely through phosphorylation of Ser^{383}.

Fig 7.1:

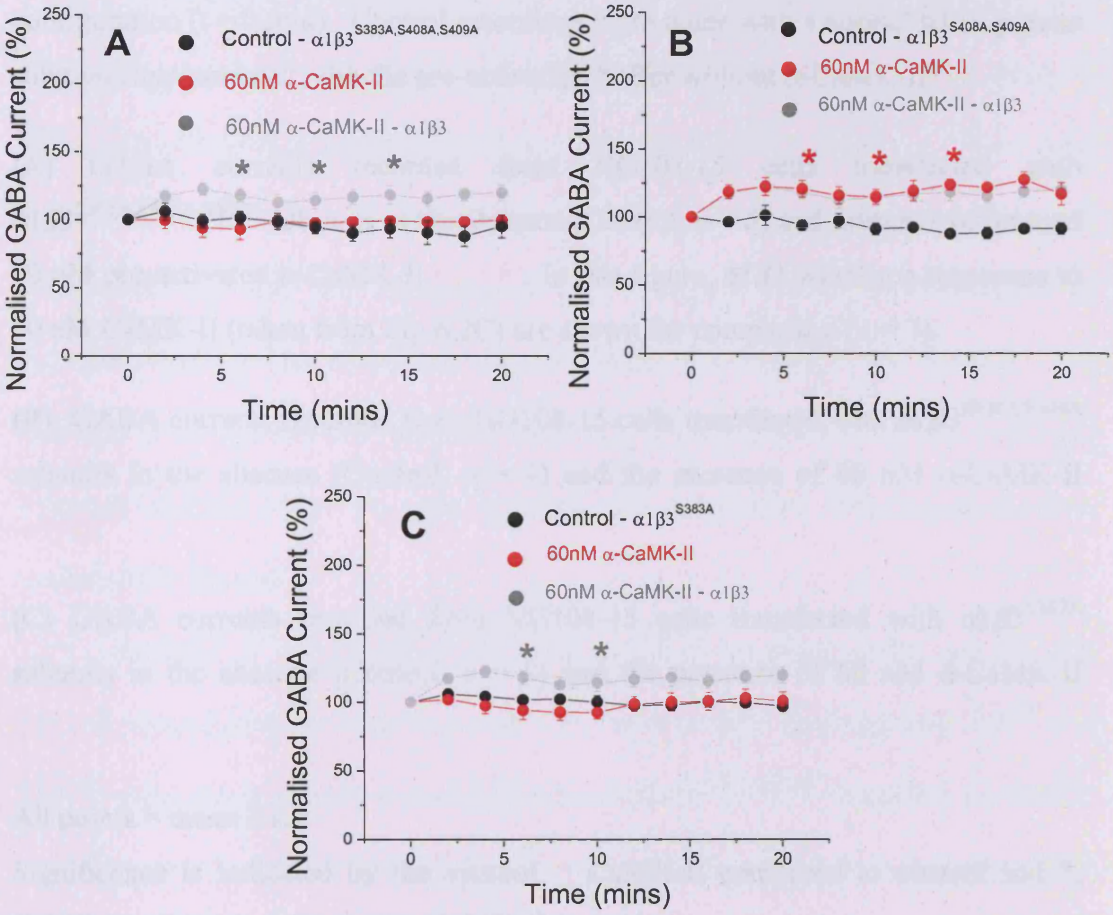

Fig 7.1: α-CaMK-II modulation of α1β3 heteromers is mediated through phosphorylation of Ser^{383}.

Peak amplitudes of GABA-mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing: $\alpha1\beta3^{S383A,S408A,S409A}$; $\alpha1\beta3^{S408A,S409A}$ or $\alpha1\beta3^{S383A}$ $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with a normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3^{S383A,S408A,S409A}$ subunits in the absence (Control, n = 5) and presence of internal 60 nM pre-activated α-CaMK-II (n = 8). In this figure, α1β3 wild-type responses to 60 nM CaMK-II (taken from Fig 6.2C) are shown for comparison (n = 7).

(B) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3^{S408A,S409A}$ subunits in the absence (Control, n = 4) and the presence of 60 nM α-CaMK-II (n = 7).

(C) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3^{S383A}$ subunits in the absence (Control, n = 6) and the presence of 60 nM α-CaMK-II (n = 5).

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control and *, CaMK-II wild-type compared to CaMK-II mutant.
$P < 0.05$, t-test, ANOVA.

7.2.2 α-CaMK-II modulation of mutant α1β3γ2S subunit-containing receptors.

CaMK-II modulation of α1β3γ2S receptors appeared significantly larger when compared to the modulation of α1β3 receptors (Figs 6.2C, 6.3C) indicating that α-CaMK-II modulation may be partly mediated through the γ2S subunit. Application of 60 nM α-CaMK-II to $\alpha1\beta3^{S383A,S408A,S409A}\gamma2S$ receptors still resulted in a significant potentiation of the amplitude of GABA (10 μM) currents recorded to 117.8 ± 5 % (n = 8) as compared to control, 94.9 ± 4 % (n = 4, Fig 7.2A) at t = 6 min. However, this effect was significantly smaller (107.5 ± 3.5 %, n = 8) than the effect of α-CaMK-II on α1β3γ2S wild-type receptors (145.8 ± 10.8 %, n = 7) at t = 4 min. This confirmed the likelihood that α-CaMK-II mediated part of its functional modulation via the γ2S subunit and that mutation of all known sites of phosphorylation in the β3 subunit did not remove the CaMK-II modulation. The time-course of the residual response appeared slightly slower than for wild-type receptors and the effect was also transient with amplitudes returning to baseline levels within 20 min.

Analysis of the potential sites for CaMK-II phosphorylation on the γ2S subunit, using fusion proteins, suggested that the main sites involved were Ser^{348} and Thr^{350}. Application of 60 nM α-CaMK-II to $\alpha1\beta3\gamma2S^{S348A,T350A}$ receptors resulted in a significant potentiation of GABA currents recorded to 142.7 ± 11.2 % (n = 8) as compared to control, 101.3 ± 6.7 % (n = 5, Fig 7.2B) at t = 6 min. Interestingly, this was not significantly different from the potentiation seen when CaMK-II was applied to wild-type α1β3γ2S receptors indicating that these residues are not functionally important in modulating the amplitude of α1β3γ2S receptor mediated currents via CaMK-II phosphorylation.

The application of 60 nM α-CaMK-II to the combined mutant receptor $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S^{S348A,T350A}$ also resulted in a significant potentiation to 127.4 ± 9 % (n = 8) as compared to control, 104.2 ± 2.8 % (n = 4, Fig 7.2C) at t = 6 min. This was not significantly different from that seen on $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S$ receptors, further indicating that phosphorylation of Ser^{348} and Thr^{350}, in this case, does not mediate the effect of α-CaMK-II. Again, the effect was transient and by t = 10 min the effect of α-CaMK on $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S^{S348A,T350A}$ receptors was significantly different (114.2 ± 6.4 %, n = 8) from the effect on wild-type (148.5 ± 12.2 %, n = 7) but was not significantly different from control (102.7 ± 5.8 %, n = 4, Fig7.2C).

Application of 10 μM diazepam to $\alpha 1\beta 3\gamma 2S^{S348A,T350A}$ receptors resulted in potentiation of GABA currents, indicating that the $\gamma 2S^{S348A,T350A}$ subunit was expressed at the cell surface (Fig 7.2D). In addition, if this subunit was not expressed, it would be predicted that α-CaMK-II would be without effect as previous results indicated α-CaMK-II could not functionally modulate $\alpha 1\beta 3^{S383A,S408A,S409A}$ receptors (Fig 7.1A), however, a significant increase in amplitude was observed on α-CaMK-II application to $\alpha 1\beta 3^{S383A:S408A:S409A}\gamma 2S^{S348A,T350A}$ receptors suggesting an αβγ heteromer was correctly assembled and expressed (Fig 7.2C).

Fig 7.2:

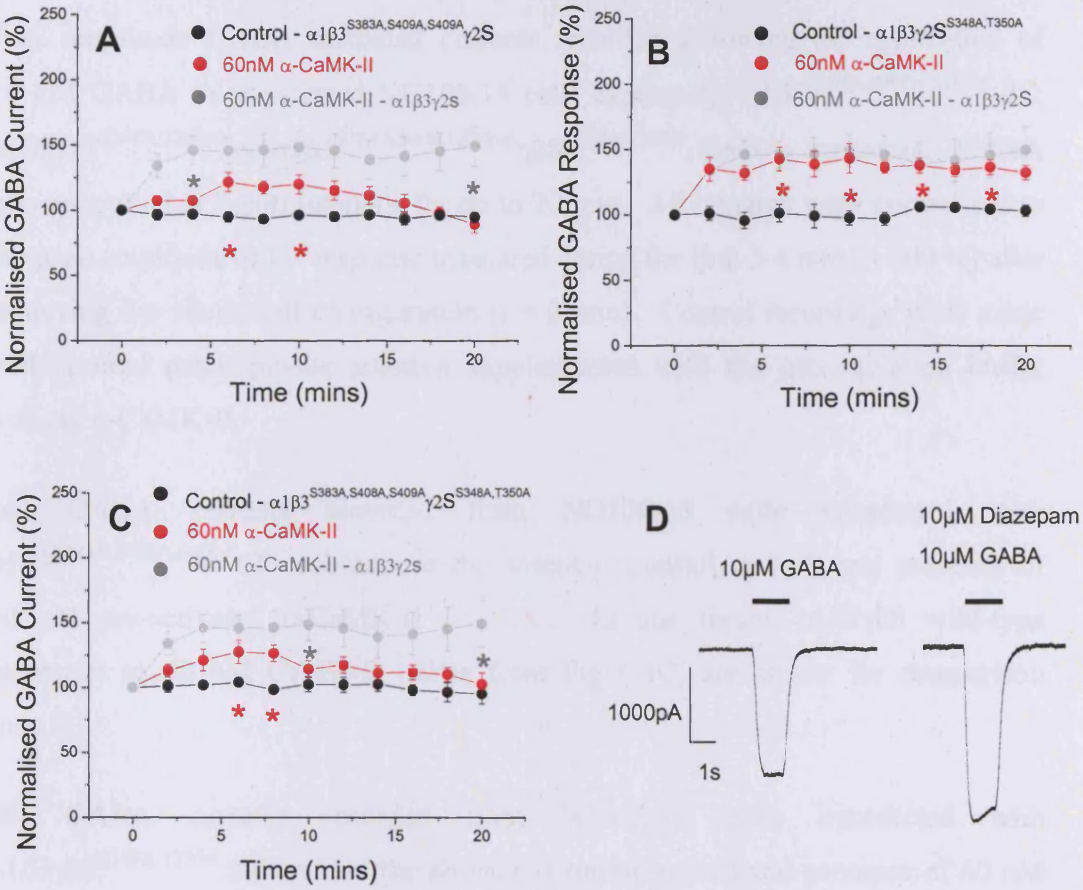
A
Control - α1β3S383A,S409A,S409Aγ2S
60nM α-CaMK-II
60nM α-CaMK-II - α1β3γ2s
Normalised GABA Current (%)
Time (mins)
B
Control - α1β3γ2SS348A,T350A
60nM α-CaMK-II
60nM α-CaMK-II - α1β3γ2S
Normalised GABA Response (%)
Time (mins)
C
Control - α1β3S383A,S408A,S409Aγ2SS348A,T350A
60nM α-CaMK-II
60nM α-CaMK-II - α1β3γ2s
Normalised GABA Current (%)
Time (mins)
D
10μM GABA
10μM Diazepam
10μM GABA
1000pA
1s

Fig 7.2: CaMK-II modulation of γ2S subunit-containing receptors is not mediated through phosphorylation of $GABA_A$ Ser^{348} and Thr^{350}.

Peak amplitude GABA mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing: $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S$; $\alpha 1\beta 3\gamma 2S^{S348A:T350A}$ or $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S^{S348A,T350A}$ $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S$ subunits in the absence (Control, n = 4) and presence of 60 nM pre-activated α-CaMK-II (n = 8). In this figure, $\alpha 1\beta 3\gamma 2S$ wild-type responses to 60 nM CaMK-II (taken from Fig 6.3C) are shown for comparison (n = 7).

(B) GABA currents recorded from NG108-15 cells transfected with $\alpha 1\beta 3\gamma 2S^{S348A,T350A}$ subunits in the absence (Control, n = 5) and presence of 60 nM α-CaMK-II (n = 8).

(C) GABA currents recorded from NG108-15 cells transfected with $\alpha 1\beta 3^{S383A,S408A,S409A}\gamma 2S^{S348,T350A}$ subunits in the absence (n = 4) and the presence of 60 nM α-CaMK-II (n = 8).

(D) Representative currents recorded from NG108-15 cells expressing $\alpha 1\beta 3\gamma 2S^{S348A,T350A}$ receptors, before and after bath application of 10 μM diazepam.

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control and the symbol *, CaMK-II wild-type compared to CaMK-II mutant.
$P < 0.05$, t-test, ANOVA.

7.2.3 Cross-talk between CaMK-II and tyrosine kinase phosphorylation of the α1β3γ2S receptor.

Analysis of fusion proteins *in vitro* suggested that Ser^{348} and Thr^{350} were the main sites of CaMK-II phosphorylation on the γ2S subunit however mutation of these sites to alanines clearly had no effect on α-CaMK-II modulation of α1β3γ2S receptors. This left three possibilities, either CaMK-II was capable of phosphorylating γ2S at another site in an NG108-15 cell that it could not phosphorylate *in vitro*; or CaMK-II was acting on the γ2S subunit through phosphorylation of an accessory protein; or CaMK-II was acting through the subsequent activation of another downstream kinase. It has previously been shown that phosphorylation of two tyrosine residues in the γ2S subunit can result in an increase in whole-cell current amplitudes similar in magnitude to that observed following the action of α-CaMK-II on γ2S subunit-containing receptors (Moss *et al.*, 1995). Therefore, 100 μM genistein an inhibitor of tyrosine kinase activity (Akiyama *et al.*, 1987), was co-applied in the patch pipette solution with 60 nM α-CaMK-II. It was not possible to pre-apply genistein in the bath, because if applied extracellularly, genistein is capable of directly inhibiting the $GABA_A$ receptor (Dunne *et al.*, 1998). Application of 60 nM CaMK-II and 100 μM genistein to α1β3γ2S receptors resulted in a significant increase in the amplitude of currents recorded to 120.3 ± 7.6 % (n = 7) as compared to control, 100.2 ± 2.3 % (n = 4, Fig 7.3A) at t = 4 min. This potentiation however was significantly smaller than that seen on application of α-CaMK-II alone (145.8 ± 10.8 %, n = 7). It should also be noted in this case that application of genistein alone had no effect on the amplitude of currents recorded over 20 min. This indicated that, in this cell type, the γ2S subunit is not subject to significant basal phosphorylation by tyrosine kinases. Application

of 60 nM α-CaMK-II and 100 μM genistein to $\alpha 1\beta 3^{S383A}\gamma 2S$ receptors resulted in no significant changes in amplitude (103.6 ± 2.8 %, n= 7) over 20 min as compared to control (97 ± 7.1 %, n = 4 at t = 6 min, Fig 7.3B). This suggested that phosphorylation at Ser^{383} in the β3 subunit and α-CaMK-II dependent activation of tyrosine kinase activity was fully responsible for the modulation of $GABA_A$ receptors by α-CaMK-II.

The application of 60 nM α-CaMK-II to $\alpha 1\beta 3\gamma 2S^{Y357F,Y359F}$ receptors resulted in a significant potentiation of the amplitude of currents recorded to 126.2 ± 4 % (n = 7) as compared to control, 107.6 ± 6.5 % (n = 4, Fig 7.3C) at t = 4 min. However, as before, this potentiation was significantly less than the effect of α-CaMK-II on $\alpha 1\beta 3\gamma 2S$ receptors (145.7 ± 10.2 %, n = 7) at t = 6 min. However after 8-10 min of recording, there was no significant difference in response to α-CaMK-II as compared to wild-type. This coincides with the observation that the response to α-CaMK-II mediated by the γ2S subunit is transient and is no longer evident after 10-15 min.

Finally, application of 60 nM α-CaMK-II to $\alpha 1\beta 3^{S383A}\gamma 2S^{Y357F,Y359F}$ receptors abolished the response to α-CaMK-II with no significant effect on the amplitude of currents recorded over 20 min (103.3 ± 3.4 %, n = 6, at t = 6 min) as compared to control (108.4 ± 4.3 %, n = 6, at t = 6 min, Fig 7.3D). This indicated that CaMK-II dependent phosphorylation of β3 Ser^{383} and γ2S $Tyr^{357,359}$ mediates α-CaMK-II dependent modulation of $\alpha 1\beta 3\gamma 2S$ receptors. This suggested a possible direct site of phosphorylation by α-CaMK-II and the activation of a second signalling pathway, acting through tyrosine kinase phosphorylation of the γ2S subunit.

Application of 10 μM diazepam to $\alpha 1\beta 3\gamma 2S^{Y357F,Y359F}$ receptors resulted in a potentiation of the current recorded indicating that the $\gamma 2S^{Y357F,Y359F}$ subunit was expressed and correctly assembled at the cell surface (Fig 7.4A). It has been

observed that the addition of the γ subunit to αβ heteromers increases the amplitudes of the GABA currents recorded (Angelotti *et al.*, 1993). The current density recorded from NG108-15 cells expressing α1β3 (20.2 ± 11.5 pA/pF, n = 6) was significantly increased for α1β3γ2S receptors (94.1 ± 19 pA/pF, n = 6, Fig 7.4B). There were no significant differences in current density between α1β3γ2S, $\alpha1\beta3\gamma2S^{S348A,T350A}$ (87.1 ± 21.3 pA/pF, n=7) and $\alpha1\beta3\gamma2S^{Y357F,Y359F}$ (82.1 ± 15.6 pA/pF, n = 6, Fig 7.4B) receptors, indicating that site-specific mutation of phosphorylation sites in the γ2S subunit was unlikely to interfere with subunit expression or trafficking to the cell surface.

Fig 7.3:

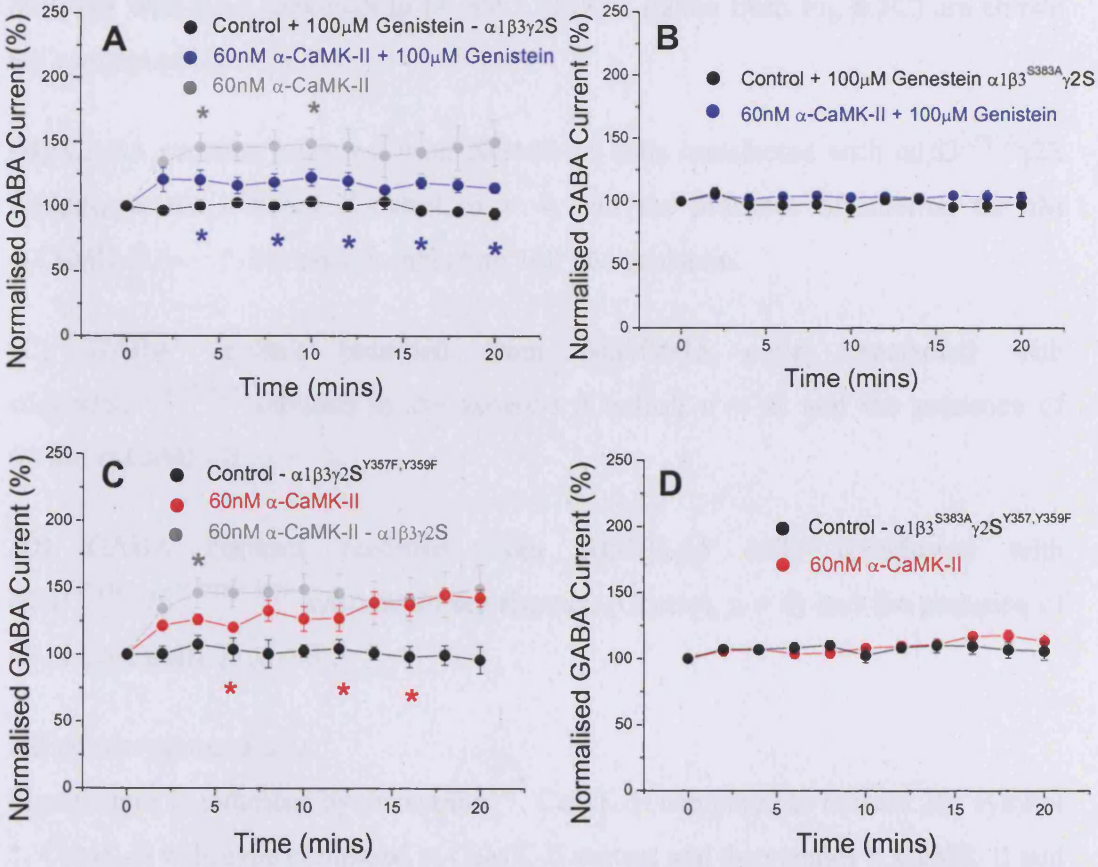

Fig 7.3: α-CaMK-II modulation of α1β3γ2S subunit-containing $GABA_A$ receptors is mediated partly through tyrosine kinase phosphorylation of $Tyr^{357,359}$.

Peak amplitudes of GABA mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing: α1β3γ2S; $\alpha1\beta3^{S383A}\gamma2S$; $\alpha1\beta3\gamma2s^{Y357F,Y359F}$ or $\alpha1\beta3^{S383A}\gamma2S^{Y357F,Y359F}$ $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with α1β3γ2S subunits in the absence (Control, n = 4) and presence of 60 nM pre-activated α-CaMK-II (n = 7) which also contained 100 μM genistein. In this panel and C α1β3γ2S wild-type responses to 60 nM CaMK-II (taken from Fig 6.3C) are shown for comparison (n = 7).

(B) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3^{S383A}\gamma2S$ subunits in the absence (Control, n = 4) and the presence of internal 60 nM α-CaMK-II (n = 7) but always including 100 μM genistein.

(C) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3\gamma2S^{Y357F,Y359F}$ subunits in the absence (Control, n = 4) and the presence of 60 nM α-CaMK-II (n = 7).

(D) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta3^{S383A}\gamma2S^{Y357F,Y359F}$ subunits in the absence (Control, n = 6) and the presence of 60 nM α-CaMK-II (n = 6).

All points = mean ± s.e.

Significance is indicated by the symbol *, CaMK-II compared to control, the symbol *, CaMK-II wild-type compared to CaMK-II mutant and the symbol *, CaMK-II and inhibitor compared to control.

$P < 0.05$, t-test, ANOVA.

Fig 7.4:

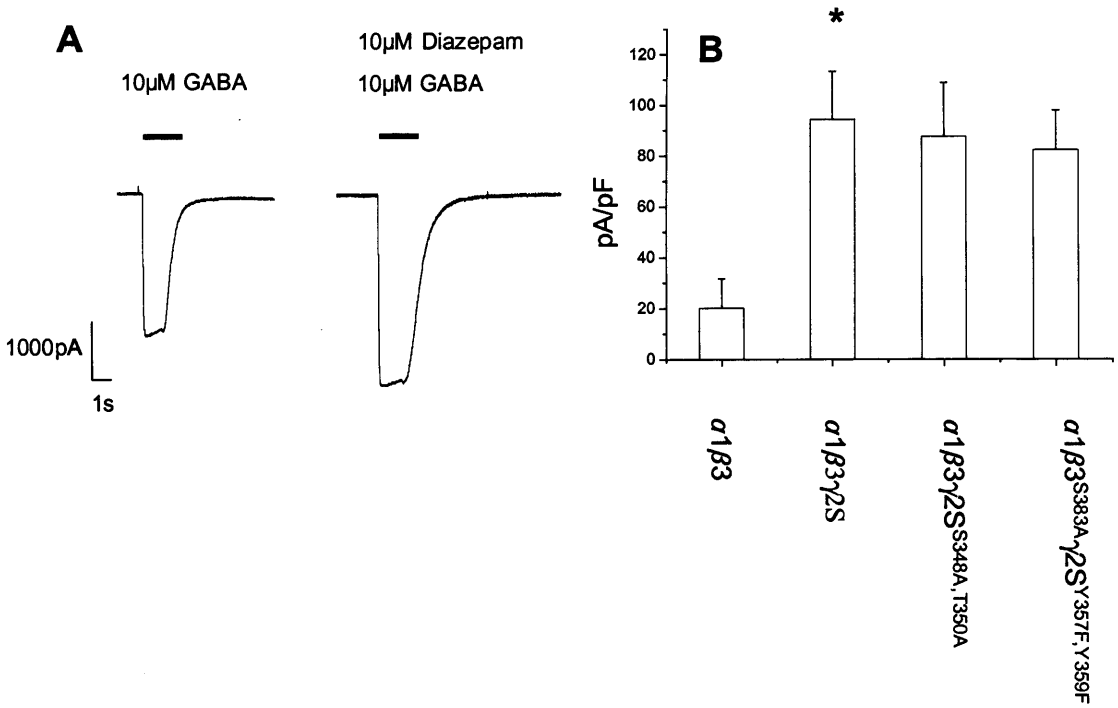

Fig 7.4: $\gamma 2S^{S348A,T350A}$ and $\gamma 2S^{Y357F,Y359F}$ receptor subunits are expressed at the cell surface.

(A) Representative GABA currents recorded from NG108-15 cells expressing $\alpha 1\beta 3\gamma 2S^{Y357F,Y359F}$ receptors before and after bath application of 10 μM diazepam.

(B) Bar chart showing the mean current density (pA/pF) of NG108-15 cells expressing $\alpha 1\beta 3$ (n = 6), $\alpha 1\beta 3\gamma 2S$ (n = 6), $\alpha 1\beta 3\gamma 2S^{S348A,T350A}$ (n = 7) and $\alpha 1\beta 3^{S383A}\gamma 2S^{Y357F,Y359F}$ (n = 6) GABA receptors after application of 10 μM GABA at t = 0 min in control conditions.

Significance is indicated by the symbol *, $\alpha 1\beta 3$ compared to $\alpha 1\beta 3\gamma 2S$.
$P < 0.05$, t-test.

7.2.4 Cross-talk between CaMK-II and tyrosine kinases acting on $\alpha1\beta1\gamma2S$ receptors.

α-CaMK-II was capable of modulating $\alpha1\beta1\gamma2S$ receptors, but not $\alpha1\beta1$ receptors, suggesting that modulation of $\alpha1\beta1\gamma2S$ receptors occurred primarily through the $\gamma2S$ subunit. In order to confirm if the same tyrosine kinase dependent mechanism revealed for $\alpha1\beta3\gamma2S$ receptors, was also involved, 60 nM α-CaMK-II and 100 μM genistein were internally applied to $\alpha1\beta1\gamma2S$ receptors. In this case there was no significant effect on the amplitude of currents recorded (98.5 ± 5 %, n = 6) as compared to control (105.1 ± 2.5 %, n = 4, Fig 7.5A) at t = 6 min. The application of genistein in control conditions again had no significant effect on the amplitude of the GABA currents recorded, suggesting a lack of any basal tyrosine kinase phosphorylation of these receptors in NG108-15 cells.

Application of 60 nM α-CaMK-II to $\alpha1\beta1\gamma S^{Y3576F,Y359F}$ receptors abolished the effect of α-CaMK-II, with no significant difference in the amplitude of GABA currents (99.7 ± 5.5 %, n = 7) compared to control (102.1 ± 3.5 %, n = 4, Fig 7.5B) at t = 6 min. This indicated that the effect of α-CaMK-II on $\alpha1\beta1\gamma2S$ receptors was entirely mediated by CaMK-II dependent activation of tyrosine kinase activity.

The current density of $\alpha1\beta1\gamma2S$ receptors (87.9 ± 16.3 pA/pF, n = 6) was significantly higher than the current density of $\alpha1\beta1$ receptors (19.5 ± 4.8 pA/pF, n = 6) and the current density of $\alpha1\beta1\gamma2S^{Y357F,Y359F}$ receptors was not significantly different (75.2 ± 32 pA/pF, n = 6, Fig 7.5C) from $\alpha1\beta1\gamma2S$. This indicated that the $\gamma2s^{Y357F,Y359F}$ subunit was most likely expressed at the cell surface and assembled correctly with $\alpha1\beta1$.

Fig 7.5:

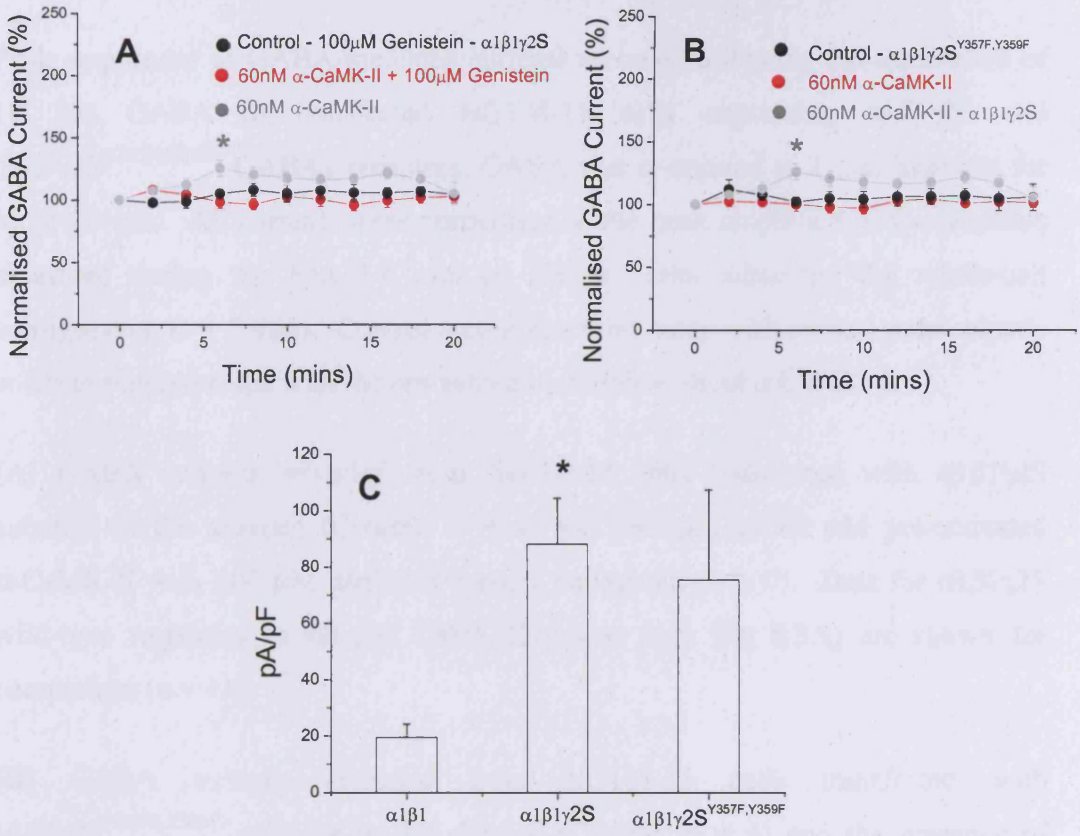
A
Control - 100μM Genistein - α1β1γ2S
60nM α-CaMK-II + 100μM Genistein
60nM α-CaMK-II
Normalised GABA Current (%)
Time (mins)
B
Control - α1β1γ2S Y357F,Y359F
60nM α-CaMK-II
60nM α-CaMK-II - α1β1γ2S
Normalised GABA Current (%)
Time (mins)
C
pA/pF
α1β1
α1β1γ2S
α1β1γ2S Y357F,Y359F

Fig 7.5: CaMK-II modulation of $\alpha1\beta1\gamma2S$ subunit-containing $GABA_A$ receptors is mediated through tyrosine kinase phosphorylation of $Tyr^{357,359}$.

Peak amplitudes of GABA-mediated currents recorded following the application of 10 μM GABA to transfected NG108-15 cells expressing $\alpha1\beta1\gamma2S$ and $\alpha1\beta1\gamma2S^{Y357F,Y359F}$ $GABA_A$ receptors. GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta1\gamma2S$ subunits in the absence (Control, n = 4) and presence of 60 nM pre-activated α-CaMK-II with 100 μM genistein present throughout (n = 6). Data for $\alpha1\beta1\gamma2S$ wild-type responses to 60 nM CaMK-II (taken from Fig 6.3A) are shown for comparison (n = 11).

(B) GABA currents recorded from NG108-15 cells transfected with $\alpha1\beta1\gamma2S^{Y357F,Y359F}$ subunits in the absence (Control, n = 4) and the presence of 60 nM α-CaMK-II (n = 7).

(C) Bar chart showing the mean current densities (pA/pF) of NG108-15 cells expressing: $\alpha1\beta1$ (n = 6), $\alpha1\beta1\gamma2S$ (n = 6), $\alpha1\beta1\gamma2S^{Y357,Y367F}$ (n = 6) $GABA_A$ receptors after application of 10 μM GABA at t = 0 min in control conditions.

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II wild-type compared to CaMK-II mutant or CaMK-II and inhibitor.
$P < 0.05$, t-test, ANOVA.

7.2.5 Recombinant $GABA_A$ receptors expressed in cerebellar granule cells are expressed at the cell surface.

There is a certain amount of discrepancy between the reported effects of phosphorylation in recombinant systems and that observed in neuronal systems (See Chapter 1). In addition, it has been suggested that the expression of recombinant receptors in different cell lines can alter the properties of the receptors expressed (Ueno *et al.*, 1996; Mercik *et al.*, 2003; Everitt *et al.*, 2004) as has also been demonstrated in the current study (See Chapter 3). In order to confirm the importance of the sites of phosphorylation identified in NG108-15 cells in a neuronal system, recombinant $GABA_A$ receptors were expressed in cerebellar granule cells in culture.

A number of studies have reported that expression of single $GABA_A$ receptor subunits in neurones allows expression of these recombinant receptors at the surface through assembly with endogenous subunits (Okada *et al.*, 2000; Cheng *et al.*, 2001). However a number of other studies have reported that recombinant single subunits cannot be expressed at the cell surface without co-transfection of the other recombinant $GABA_A$ receptor subunits required for cell surface expression (Gorrie *et al.*, 1997; Kittler *et al.*, 2000).

Cerebellar granule cells maintained in depolarising conditions were transfected using Effectene (See Chapter 2) with α1 $GABA_A$ receptor subunit cDNA co-expressed with β2 subunit cDNA containing a functionally silent extracellular myc epitope (See Chapter 2) inserted at the N terminus (Connolly *et al.*, 1996a; Connolly *et al.*, 1996b; Farrar *et al.*, 1999). The presence of recombinant receptors at the cell surface was determined by immunocytochemistry. Cultures were immunolabeled with a primary antibody directed against the myc epitope. As these

cells were not permeabilised and the myc epitope was inserted in a part of the subunit expected to be extracellular, the presence of fluorescence would indicate cell surface expression of the recombinant subunit.

In order to observe strong cell surface expression in the current study it was observed that β subunits had to be co-expressed with a recombinant α subunit. Co-expression of α1 with β2 and α1 with β3 resulted in strong cell surface expression suggesting that these granule cells now expressed a large number of $\alpha\beta$ heteromers at the cell surface most likely in excess of endogenous receptors given the strong viral promoter within the plasmid cDNA (See Fig 7.6A and B).

Co-expression of α1 with $\beta3^{S408A,S409A}$ and $\beta3^{S383A}$ revealed that despite the disruption to the phosphorylation sites in these recombinant subunits they were still strongly expressed at the cell surface of cerebellar granule cells after transfection (See Fig 7.6 C and D). Co-expression of $\gamma2S^{myc}$ with α1 and β2 or β3 resulted in strong surface expression suggesting that these cells now predominantly express recombinant $\alpha\beta\gamma$ $GABA_A$ receptors (See Fig 7.7 A and B). Co-expression of α1 and β3 with $\gamma2S^{Y357F,Y359F,myc}$ also resulted in expression of the recombinant $\alpha1\beta3\gamma2^{Y357F,Y359F,myc}$ receptors at the cell surface (See Fig 7.7C).

Fig 7.6:

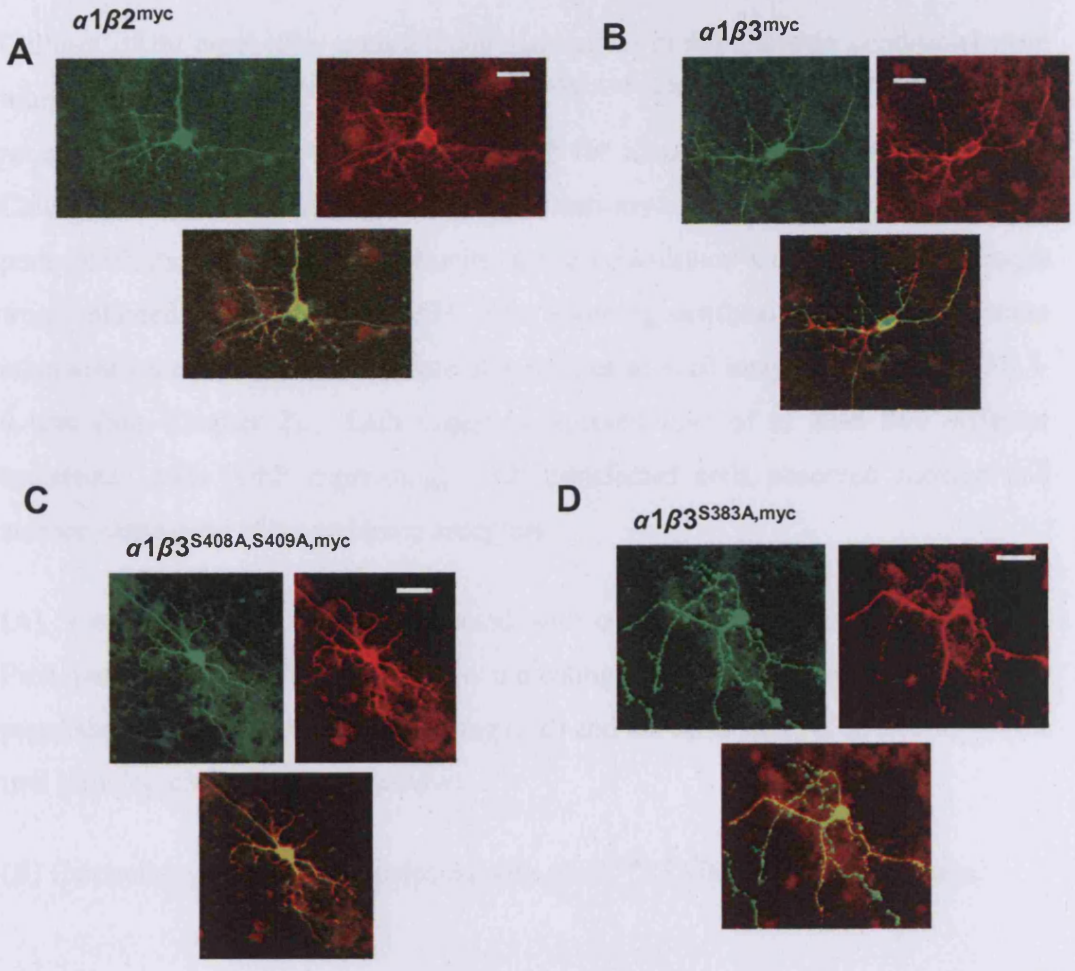

Fig 7.6: Expression of recombinant $\alpha\beta$ subunit-containing $GABA_A$ receptors at the cell surface of transfected cerebellar granule cells:

Cultures of rat cerebellar granule cells maintained in depolarising conditions were transfected with $\alpha1\beta2^{myc}$, $\alpha1\beta3^{myc}$, $\alpha1\beta3^{S408A,S409A,myc}$ and $\alpha1\beta3^{S383A,myc}$ $GABA_A$ receptor subunits along with enhanced GFP for identification of transfected cells. Cells were immunolabelled with an anti-myc epitope antibody without permeabilisation so that only subunits at the cell-surface were detected. Images were obtained on a Zeiss Meta 510 laser scanning confocal microscope. Unless otherwise stated each image consists of a Z series of 8-10 images at intervals of 0.3-0.4μm (See Chapter 2). Each image is representative of at least five different transfected cells (GFP expressing). All transfected cells observed showed cell surface expression of recombinant receptors.

(A) Cerebellar granule cell transfected with $\alpha1\beta2^{myc}$ $GABA_A$ receptor subunits. First panel shows GFP fluorescence indicating transfection (Green), the second panel shows anti-myc immunolabelling (red) and the third panel is an overlay of the two showing co-localisation (yellow).

(B) Cerebellar granule cell transfected with $\alpha1\beta3^{myc}$ $GABA_A$ receptor subunits.

(C) Cerebellar granule cell transfected with $\alpha1\beta3^{S408A,S409A,myc}$ $GABA_A$ receptor subunits.

(D) Cerebellar granule cell transfected with $\alpha1\beta3^{S383A,myc}$ $GABA_A$ receptor subunits. (Calibration bar = 10 μm)

Fig 7.7:

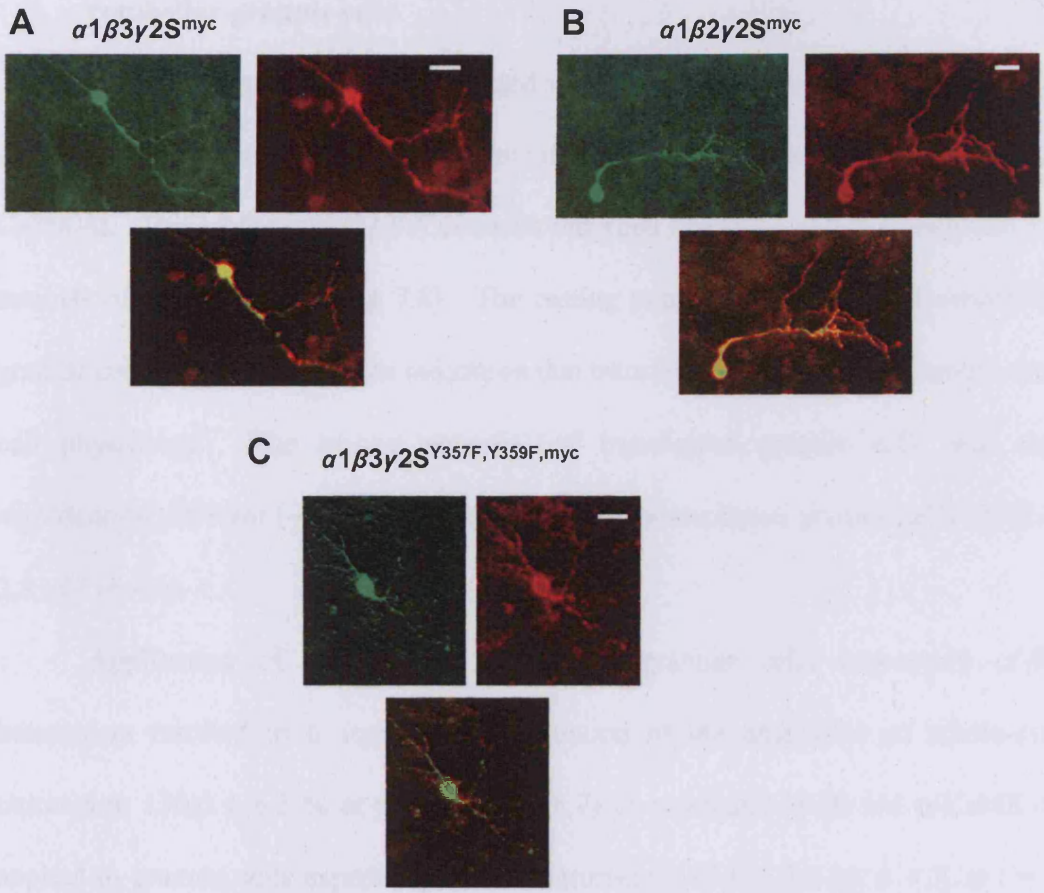

Fig 7.7: Expression of recombinant $\alpha\beta\gamma$ subunit-containing $GABA_A$ receptors at the cell surface of transfected cerebellar granule cells:

Cultures of rat cerebellar granule cells maintained in depolarising conditions were transfected with $\alpha1\beta3\gamma2S^{myc}$, $\alpha1\beta2\gamma2S^{myc}$ and $\alpha1\beta3\gamma2S^{Y357F,Y359F,myc}$ $GABA_A$ receptor subunits along with enhanced GFP for identification of transfected cells. Cells were immunolabelled with an anti-myc epitope antibody without permeabilisation so that only subunits at the cell-surface were detected (See Chapter 2). Each image is representative of at least five different transfected cells (GFP expressing). All transfected cells observed showed cell surface expression of recombinant receptors.

(A) Cerebellar granule cell transfected with $\alpha1\beta3\gamma2S^{myc}$ $GABA_A$ receptor subunits.
(B) Cerebellar granule cell transfected with $\alpha1\beta2\gamma2S^{myc}$ $GABA_A$ receptor subunits.
(C) Cerebellar granule cell transfected with $\alpha1\beta3\gamma2S^{Y357F,Y359F,myc}$ $GABA_A$ receptor subunits. (Calibration bar = 10 μm)

7.2.6 α-CaMK-II modulation of recombinant αβ heteromers expressed in cerebellar granule cells.

Cerebellar granule cells transfected with GFP only were recorded from with normal patch pipette solution supplemented with pre-activation buffer without CaMK-II. The whole-cell GABA currents recorded remained stable throughout 16 minutes of recording (See Fig 7.8). The resting potential of transfected cerebellar granule cells was recorded as an indication that transfection did not significantly alter cell physiology. The resting potential of transfected granule cells was not significantly different (-43.9 ± 2.8 mV, n = 8) to untransfected granule cells -39.2 ± 2.5 mV (n = 6).

Application of 60 nM α-CaMK-II to granule cells expressing α1β3 heteromers resulted in a significant potentiation of the amplitude of whole-cell currents to 130.6 ± 6.3 % at t = 4 min (n = 7) as compared to 60 nM α-CaMK-II applied to granule cells expressing α1β2 heteromers (105.6 ± 2.6 %, n = 7, at t = 4 min, Fig 7.8A). Application of 60 nM α-CaMK-II to $\alpha1\beta3^{S408A,S409A}$ expressing granule cells resulted in a significant increase in amplitude to 127.1 ± 8 % (n = 4) at t = 6 min as compared to application of α-CaMK-II to $\alpha1\beta3^{S383A}$ expressing granule cells (104.2 ± 2.6 %, n = 4, at t = 6 min, Fig 7.8B), which for $\alpha1\beta3^{S408A,S409A}$, was not significantly different from the application of α-CaMK-II to wild-type α1β3 heteromers.

The response of cerebellar granule cells expressing recombinant $GABA_A$ receptors to α-CaMK-II was similar to that observed in NG108-15 cells (See Fig 7.1). Again, the results indicated that α1β2 heteromers were not modulated by α-CaMK-II in this system and that Ser^{383} on the β3 subunit appeared to mediate the α-CaMK-II dependent effect on α1β3 heteromers. The extent of α-CaMK-II

modulation was also similar to that observed in NG108-15 cells, suggesting that in granule cells the γ2 subunit may also mediate part of the response to CaMK-II. It also suggested that the expressed recombinant αβ heteromers dominated over endogenous receptors in granule cells which would be expected to consist of αβγ receptors.

7.2.7 α-CaMK-II modulation of αβγ receptors expressed in cerebellar granule cells.

Application of 60 nM α-CaMK-II to granule cells expressing α1β3γ2S recombinant receptors resulted in a significant potentiation of the amplitude of whole-cell currents to 167.8 ± 13.7 % (n = 4) at t = 6 min, as compared to granule cells expressing α1β2γ2S recombinant receptors (112.8 ± 8 %, n = 7, at t = 6 min, Fig 7.9A). In addition this increase was significantly greater than the effect of α-CaMK-II on α1β3 expressing granule cells at t = 6 min (127.1 ± 8 %, n = 7, Fig 7.8A). This indicated that similar to NG108-15 cells, the γ2S subunit mediated part of the $GABA_A$ receptor response to α-CaMK-II. In contrast to NG108-15 cells (Fig 6.3), application of α-CaMK-II to α1β2γ2S expressing granule cells resulted in a significant but transient potentiation of whole cell currents to 115.6 ± 4.2 % (n = 7) at t = 4 min as compared to control (96.4 ± 6.1 %, n =7, at t = 4 min, Fig 7.8A). This was possibly as a result of CaMK-II modulation of a population of endogenous $GABA_A$ receptors. Again, this suggests that the recombinant receptor expression dominates over the endogenous receptors when measuring whole-cell currents from transfected cells.

Application of 60 nM α-CaMK-II to granule cells expressing $\alpha1\beta3\gamma2S^{Y357F,Y359F}$ resulted in a significant increase of the amplitude of whole-cell

currents measured to 120.4 ± 8 % (n = 7) at t = 6 min as compared to control (92.3 ± 5.8 %, n = 7, at t = 6 min, Fig 7.9B). This increase, however, was significantly smaller than the increase observed after application of α-CaMK-II to α1β3γ2S expressing granule cells (167.8 ± 13.7 %, n = 4, at t = 6 min, Fig 7.9B). This indicated that, as in NG108-15 cells (See Fig 7.3), a proportion of the effect of α-CaMK-II on α1β3γ2S receptors was mediated through tyrosine kinase phosphorylation of the γ2S subunit. In addition, similar to that observed in NG108-15 cells (See Fig 7.3C), this proportion of the effect was transient as there was no significant difference between α1β3γ2S and α1β3γ2S^{Y357F,Y359F} in the presence of α-CaMK-II after 10 min. The overall similarity between results obtained in NG108-15 cells and cerebellar granule cells confirms that NG108-15 cells are an appropriate model for the study of CaMK-II phosphorylation of $GABA_A$ receptors. The mechanisms and signalling pathways involved in CaMK-II modulation of $GABA_A$ receptors in NG108-15 cells must, therefore, be comparable to that observed in cerebellar granule cells (maintained in depolarising conditions).

Fig 7.8:

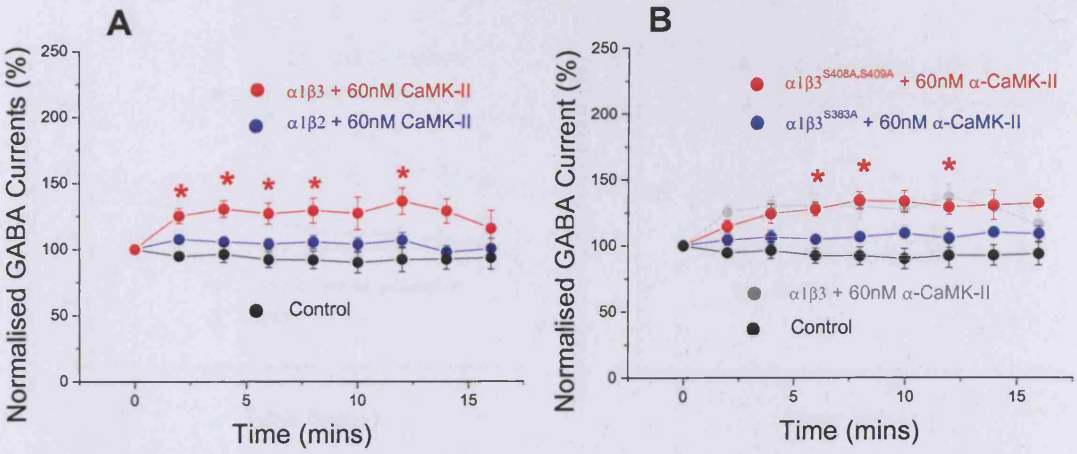

Fig 7.8: CaMK-II dependent modulation of whole-cell currents recorded from transfected cerebellar granule cells.

Peak amplitude of GABA mediated currents recorded following the application of 10 μM GABA to cerebellar granule cells transfected with $\alpha1\beta2$, $\alpha1\beta3$, $\alpha1\beta3^{S408A,S409A}$ and $\alpha1\beta3^{S383A}$ $GABA_A$ receptor subunits. GABA was re-applied at 2 min intervals for up to 16 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from cerebellar granule cells transfected with GFP only in the absence of α-CaMK-II (Control, n = 7) and transfected with $\alpha1\beta3$ (n = 7) and $\alpha1\beta2$ (n = 7) $GABA_A$ receptor subunits in the presence of 60 nM α-CaMK-II.

(B) GABA currents recorded from cerebellar granule cells transfected with GFP only in the absence of α-CaMK-II (Control, n = 7, taken from A) and transfected with $\alpha1\beta3^{S408A,S409A}$ (n = 4), $\alpha1\beta3^{S383A}$ (n = 4) and $\alpha1\beta3$ (n = 7, taken from A) $GABA_A$ receptor subunits in the presence of 60 nM α-CaMK-II.

All data points mean ± s.e.

Significance is indicated by the symbol *, comparing different subunit combinations and mutants in the presence of CaMK-II.

P<0.05%, t-test, ANOVA.

Fig 7.9

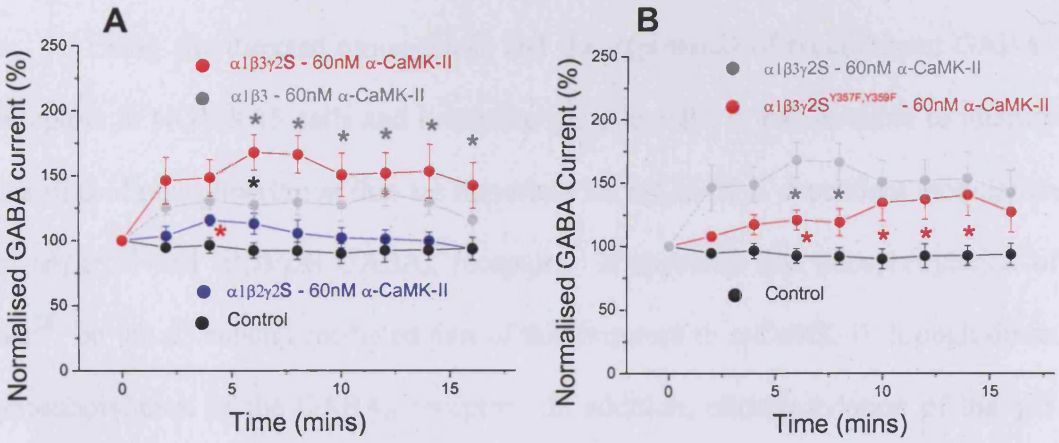

Fig 7.9: CaMK-II dependent modulation of whole-cell currents recorded from cerebellar granule cells transfected with recombinant αβγ receptors.

Peak amplitude of GABA mediated currents recorded following the application of 10 μM GABA to cerebellar granule cells transfected with α1β2γ2S, α1β3γ2S and α1β3γ2s^{Y365F,Y367F} $GABA_A$ receptor subunits. GABA was re-applied at 2 min intervals for up to 16 min. All currents were normalised to the peak amplitude of the response measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from cerebellar granule cells transfected with GFP only in the absence of α-CaMK-II (Control, n = 7) and transfected with α1β2γ2S (n = 7), α1β3γ2S (n = 6) and α1β3 (n = 7, taken from A) $GABA_A$ receptor subunits in the presence of 60 nM α-CaMK-II.

(B) GABA currents recorded from cerebellar granule cells transfected with GFP only in the absence of α-CaMK-II (Control, n = 7) and transfected with α1β3γ2S (n = 8, taken from A) and α1β3γ2S^{Y357F,Y359F} (n =7) $GABA_A$ receptor subunits in the presence of 60 nM α-CaMK-II.

All data points mean ± s.e.

Significance is indicated by the symbol *, comparing α1β3γ2S to α1β2γ2S and α1β3γ2S^{Y357F,Y359F} in the presence of α-CaMK-II. The symbol * indicates α1β3γ2S compared to α1β3 and the symbol *, indicates α1β2γ2S and α1β3γ2S^{Y357F,Y359F} α-CaMK-II compared to control.

$P < 0.05$, t-test, ANOVA.

7.3 DISCUSSION

Using site-directed mutagenesis and the expression of recombinant $GABA_A$ receptors in NG108-15 cells and cerebellar granule cells, it was possible to identify the sites of phosphorylation that are important for α-CaMK-II dependent modulation of α1β1γ2S and α1β3γ2S $GABA_A$ receptors. It appeared that phosphorylation of Ser^{383} on the β3 subunit mediated part of this response to α-CaMK-II through direct phosphorylation of the $GABA_A$ receptor. In addition, phosphorylation of the γ2S subunit contributed to the effect, but in this case via CaMK-II dependent activation of tyrosine kinase activity and phosphorylation of $Tyr^{357;359}$ in the γ2S subunit.

To date, the Ser^{383} site on the β3 subunit is the only site reported to be phosphorylated by α-CaMK-II and no other Ser/Thr kinases when the TM3-4 intracellular loop is expressed as a fusion protein (McDonald and Moss, 1997). The same comparable site, Ser^{384}, can be phosphorylated by CaMK-II on the β1 subunit, when expressed as a fusion protein (McDonald and Moss, 1994), but phosphorylation of this site does not seem to be involved in mediating the functional effect of α-CaMK-II on α1β1γ2S receptors, as α1β1 heteromers do not respond to α-CaMK-II. It appears that the tyrosine kinase-dependent phosphorylation of the γ2S subunit must also be dependent on the β subunit present in the receptor complex, as α1β2γ2S receptors are not modulated by α-CaMK-II at all in NG108-15 cells, or in transfected cerebellar granule cells.

This may be an emerging theme in phosphorylation dependent modulation of $GABA_A$ receptors as differences between β1/3 and β2 subunits have previously been demonstrated. PKA appears able to phosphorylate β1/3 in a recombinant system whereas β2 is not (McDonald *et al.*, 1998), even though in a neuronal environment PKA can modulate mIPSCs thought to be mediated by α1β2γ2S receptors (Kano and

Konnerth, 1992; Nusser *et al.*, 1999). This is comparable to the fact that recombinant $\alpha1\beta2\gamma2S$ receptors cannot be modulated by CaMK-II in NG108-15 cells or cerebellar granule cells, but CaMK-II dependent modulation of $GABA_A$ receptors involved in rebound potentiation (Kano *et al.*, 1996; Kawaguchi and Hirano, 2002) may be mediated by $\alpha1\beta2\gamma2$ receptors (Persohn *et al.*, 1992). Despite the high level of homology in the β subunit family, it has been demonstrated that $\beta1/3$ and $\beta2$ subunits require different assembly signals on the α / γ subunits for correct assembly and trafficking to the cell surface (Bollan *et al.*, 2003). In addition, different β subunits appear to be targeted to different specific regions of a cell (Connolly *et al.*, 1996b). This implies that $GABA_A$ receptors containing different β subunits are assembled, trafficked and targeted differentially to different synaptic sites. It is not surprising therefore that they might also be modulated by phosphorylation in different ways.

It is also becoming increasingly clear that for many multi-functional kinases with multiple substrates to function efficiently, correct targeting within the cell or localization to points of action is necessary (Pawson and Scott, 1997; Brandon *et al.*, 2002a). The action of kinase anchoring proteins that can bind both kinase and receptor substrate may provide explanations for some of the conflicting reports regarding the effects of phosphorylation on $GABA_A$ receptors. For example, the kinase anchoring protein RACK-1, which potentiates PKC dependent phosphorylation of the β subunit of the $GABA_A$ receptor (Brandon *et al.*, 2002b), has been shown to associate with $\beta1/3$ subunits (Brandon *et al.*, 1999; Brandon *et al.*, 2002b). Interestingly, RACK-1 is able to bind tyrosine kinases as well, in particular the cytosolic Src family (Yaka *et al.*, 2002). The action of PKA has also been reported to be dependent on AKAP 79/150 which only binds $\beta1$ and $\beta3$ and not $\beta2$

subunits (Brandon *et al.*, 2003) giving a possible mechanism for the observed lack of functional modulation of β2 subunit-containing $GABA_A$ receptors in HEK293 cells (McDonald *et al.*, 1998). It is possible that another, as yet unidentified, anchoring protein mediates PKA dependent effects on α1β2γ2 receptors observed in some neuronal cell-types (Kano and Konnerth, 1992; Nusser *et al.*, 1999). The differential effect of α-CaMK-II on α1β1/3γ2S and α1β2γ2S receptors may be mediated by an anchoring protein with differential binding affinities for the different subunits. This model of phosphorylation may also explain why α-CaMK-II modulation was not observed at all in HEK293 cells, if this cell line lacks the relevant anchoring protein(s).

The fact that $\gamma2S^{Y357F,Y359F}$ mutant subunit-containing $GABA_A$ receptors had their response to α-CaMK-II reduced or abolished, suggests that α-CaMK-II is capable of directly or indirectly activating a tyrosine kinase which can then phosphorylate the γ2S subunit. As there did not appear to be any basal tyrosine phosphorylation of $GABA_A$ receptors in this cell line, as has been reported elsewhere (Moss *et al.*, 1995; Brandon *et al.*, 2001), phosphorylation of these residues would be expected to result in an increase in the whole-cell GABA current amplitude. Moss *et al.* (1995) determined that phosphorylation of these residues led to an increase in whole-cell GABA currents primarily due to an increase in the single GABA channel mean open time and probability of opening. They also suggested that in neurones, these residues are basally phosphorylated. Using phospho-specific antibodies directed against Y365 and Y367 on the γ2L subunit, Brandon *et al.* (2001) demonstrated that phosphorylation of these residues occurred in rat brain extracts and further demonstrated a dynamic regulation of phosphorylation of these sites through tyrosine kinase / phosphatase activities in cortical neurones in culture.

In cells where tyrosine phosphorylation has already occurred under basal conditions, this may be expected to reduce the effect of CaMK-II modulation. Interestingly, in cortical neuronal cultures, addition of α-CaMK-II had a less profound effect on GABA currents than that observed in cerebellar granule cell cultures (See Chapter 4, Fig 4.1). Cortical neuronal cultures of this type have been reported to have a very high level of tyrosine phosphatase activity and subsequently low levels of basal phosphorylation of Y365 and Y367 even after stimulation to activate tyrosine kinases (Brandon *et al.*, 2001). This may also explain why the response of $GABA_A$ receptors to α-CaMK-II in cortical neurones (Chapter 4, Fig 4.1) appeared comparably lower. The importance of these sites of tyrosine kinase phosphorylation on the γ2 subunit has been recently demonstrated by the formation of a transgenic mouse where these tyrosine residues have been mutated to prevent tyrosine kinase phosphorylation of the γ2 subunit. This mutation is fatal with -/- mice not surviving beyond birth (V. Tretter, C. Houston, T. Smart and S. Moss, unpublished observations).

It is also possible that mutation of these tyrosine residues to phenylalanines altered the normal behaviour of the receptor such that it appeared to reduce the response to α-CaMK-II, but in fact, was simply an indirect effect, altering the receptors basal phosphorylation state. In addition it is always possible that in different neuronal cell types phosphorylation of other residues that have not yet been identified might also be important. However, these hypotheses are not the simplest ones to adopt. Within the γ2S subunit the only residue that was not tested was Ser^{327} the residue thought to be partially responsible for a PKC dependent depression (Kellenberger *et al.*, 1992; Krishek *et al.*, 1994) and a potentiation of α1β1/2γ2S containing receptors (Lin *et al.*, 1996). In addition a reduction in the level of

phosphorylation of this residue was reported after induction of a calcineurin-dependent form of LTD of inhibitory synapses (Wang *et al.,* 2003). Given that CaMK-II appeared unable to phosphorylate this residue *in vitro* (fusion proteins) it seems α-CaMK-II action on the γ2S subunit is unlikely to be through direct phosphorylation of this residue. Downstream activation by CaMK-II of PKA and/or PKC is also possible but $Ser^{408,409}$ mutants in β3 subunits seemed to have no effect, arguing against this. The residues Ser^{348} and Thr^{350}, identified in fusion proteins, were thought to be main the main sites of phosphorylation by CaMK-II in γ2S subunits (McDonald and Moss, 1994). Mutation of these residues to alanine abolished CaMK-II dependent phosphorylation *in vitro*. However, mutation of these residues had no functional effect on α-CaMK-II modulation of α1β3γ2S $GABA_A$ receptors in NG108-15 cells. The evidence therefore suggests that CaMK-II modulation of $GABA_A$ receptors is dependent on both the β and γ2 subunits, but not through direct phosphorylation by CaMK-II of the γ2S subunit. The depression of CaMK-II mediated effects by tyrosine kinase inhibitors and mutation of sites of tyrosine kinase phosphorylation on the γ2S subunit both suggest that CaMK-II may activate a tyrosine kinase which in turn phosphorylates the γ2S subunit and up-regulates receptor function.

An important question relating to the above hypothesis is can CaMK-II activate a tyrosine kinase? There is some evidence that Ca^{2+} sensitive tyrosine kinases exist (Rusanescu *et al.*, 1995; Boxall and Lancaster, 1998). In addition, it has been determined that PKC can influence the activity of the non-receptor tyrosine kinase Src, suggesting that an interaction between Ser/Thr kinases and the Src family of tyrosine kinases is possible (Lu *et al.*, 1999; Ali and Salter, 2001). It is generally thought that activation of Src through PKC is mediated by activation of PYK2 (Dikic

et al., 1996), a member of the focal adhesion kinase family highly expressed in the nervous system (Sasaki *et al.*, 1995; Lev *et al.*, 1995), which responds to calcium (Lev *et al.*, 1995). Activation of PYK2 leads to autophosphorylation which creates an SH2 ligand that can bind the SH2 domain of Src, thereby activating it (Dikic *et al.*, 1996; Ali and Salter, 2001).

Activation of PYK2, and subsequently, Src has been demonstrated in some cases to be Ca^{2+}/Calmodulin dependent, but not PKC dependent (Della Rocca *et al.*, 1997; Heidinger *et al.*, 2002). Indeed, there is evidence for CaMK-II dependent activation of Src in glomerular mesangial cells (Wang *et al.*, 2003b) and HEK293 cells (Lo and Wong, 2004). There is also evidence for CaMK-II dependent activation of PYK2 and Src in vascular smooth muscle (Ginnan and Singer, 2002; Ginnan *et al.*, 2004), PC12 cells (Zwick *et al.*, 1999) and in hippocampal neurones following ischemia (Guo *et al.*, 2004). It is unknown if NG108-15 cells express PYK2, but given that this protein is abundant in the CNS (Sasaki *et al.*, 1995; Lev *et al.*, 1995) and the neuronal origin of the cell line, it seems a plausible possibility especially considering how NG108-15 cells are also used as a model of neuronal differentiation and neuritogenesis.

Src activation has been implicated in the regulation of excitatory synaptic plasticity through the action of Src on the NMDA receptor (Lu *et al.*, 1999; Huang and Hsu, 1999; Salter and Kalia, 2004). PYK2 induced Src activation is thought to be necessary for certain forms of excitatory LTP (Huang *et al.*, 2001). At the NMDA receptor, Src kinase acts as a point of convergence for a number of different signalling pathways (Salter and Kalia, 2004).

For Src and also other tyrosine kinases, binding to the NMDA receptor is thought to occur via association with PSD-95, or possibly another adaptor protein

rather than directly interacting with the receptor itself (Tezuka *et al.*, 1999; Gingrich *et al.*, 2004). For the present study, it may be that binding of the PYK2:Src complex, either directly or through an intermediate protein to the $GABA_A$ receptor, may be dependent on the subunits present. Binding may be $\beta 1/3$ subunit dependent and unable to occur if $\beta 2$ is present within the receptor complex. This would explain the lack of a tyrosine kinase dependent effect seen on $\alpha 1\beta 2\gamma 2S$ receptors.

The actions of Src on $GABA_A$ receptors in neurones suggest that Src can increase whole-cell current amplitudes (Moss *et al.*, 1995; Wan *et al.*, 1997). There is also evidence for a Src mediated increase in the amplitude of IPSCs without a change in their decays (Boxall, 2000). This was considered to be in accord with the findings of Moss *et al.* (1995) and the observed increase in mean open time and probability of channel opening. Activation of PYK2 and Src in prefrontal cortical neurones was also shown to increase the amplitude of IPSCs without changing their decays (Ma *et al.*, 2003). The known association between Ca^{2+} and PYK2 (Lev *et al.*, 1995) and the activation of Src (Rusanescu *et al.*, 1995; Dikic *et al.*, 1996), together with the evidence for CaMK-II dependent activation of Src (Wang *et al.*, 2003b; Lo and Wong, 2004; Ginnan *et al.*, 2004) possibly through PYK2 (Zwick *et al.*, 1999; Ginnan and Singer, 2002), links several signal transduction pathways. It seems that a CaMK-II dependent activation of PYK2 and subsequent activation of Src tyrosine kinase is the most plausible, potential signalling pathway for CaMK-II dependent, tyrosine kinase dependent, phosphorylation of the $GABA_A$ receptor.

The application of α-CaMK-II to cerebellar granule cells (maintained in physiological conditions) led to an increase in IPSC amplitude and a change in decay. It appeared that CaMK-II did not have a uniform effect across all populations of synaptic events. It is possible that the analysis of the effect of CaMK-II on sIPSCs

is further complicated by the fact that there may be two different effects being mediated by phosphorylation of the β3 subunit and the γ2S subunit.

In conclusion, the evidence suggests that α-CaMK-II mediates its effects on α1β1γ2S and α1β3γ2S receptors in NG108-15 cells and cerebellar granule cells through a direct phosphorylation of the β3 subunit at Ser^{383} and through CaMK-II activation of a tyrosine kinase that phosphorylates γ2S at $Tyr^{357,359}$. This tyrosine kinase dependent phosphorylation is partially dependent on the identity of the β subunit, as it is only observed if the β1/3 subunit is present and not when β2 is present. It is assumed that the modulation of $GABA_A$ receptors expressed in NG108-15 cells is comparable to that of native receptors in neurones; however, there remain unanswered questions about the role of the β2 subunit-containing $GABA_A$ receptors.

7.4 CONCLUSIONS

- The modulation of α1β3 heteromers expressed in NG108-15 cells and cerebellar granule cells by α-CaMK-II is mediated through phosphorylation of the Ser^{383} residue.
- Modulation of α1β3γ2S receptors expressed in NG108-15 cells and cerebellar granule cells is mediated through phosphorylation of β3 Ser^{383} and through tyrosine kinase mediated phosphorylation of $Tyr^{357,359}$ on the γ2S subunit.
- Modulation of α1β1γ2S receptors expressed in NG108-15 cells is mediated through tyrosine kinase phosphorylation of $Tyr^{357,359}$.
- CaMK-II modulation of the $GABA_A$ receptor can occur through direct phosphorylation of the receptor and through activation of a second downstream kinase signalling pathway.

Chapter Eight:

CaMK-II modulation of sIPSCs in cerebellar granule cells from β2 knockout mice.

8.1 INTRODUCTION

Analysis of the effects of α-CaMK-II on recombinant $GABA_A$ receptors expressed in NG108-15 cells and cerebellar granule cells suggested that there was a differential effect of CaMK-II on $GABA_A$ receptors depending on the combination of β and γ subunits assembled within the receptor. The evidence suggested that β2 subunit-containing receptors could not be functionally modulated by α-CaMK-II in a recombinant system or in neurones expressing recombinant receptors but that β1/3 subunit-containing recombinant receptors can be up-regulated by a direct phosphorylation of the β3 subunit and a downstream activation of a tyrosine kinase and subsequent phosphorylation of the γ2S subunit. However, a lack of PKA dependent modulation of recombinant receptors containing the β2 subunit has been observed in HEK293 cells (McDonald *et al.*, 1998), but PKA activity in neurones can alter IPSCs within cells that express predominantly α1β2γ2 receptors (Kano and Konnerth, 1992; Nusser *et al.*, 1999). A lack of functional modulation of β2 subunit-containing receptors in a recombinant system is therefore not necessarily an indication that modulation cannot occur for native $GABA_A$ receptors at the synapse.

In order to determine more clearly how CaMK-II might modulate β2 versus β3 subunit-containing receptors in a neuronal environment it was necessary to make use of a $GABA_A$ receptor β2 subunit knockout mouse (Sur *et al.*, 2001). In any neuronal preparation it is difficult to study the effects of phosphorylation on a discrete receptor population because of heterogeneity. However, cultures prepared from β2 knockout mice will always lack this subunit and so after comparison with

wild-type mice it may be possible to determine if there are any differences between modulation of receptors that contain different β subunits.

Knockout mice have been used to study the relative importance and contribution of different $GABA_A$ receptor subunits to synaptic transmission. At present knockout mice lacking the α1, α5, α6, β2, β3, δ and γ2S/L subunits have been generated (Vicini and Ortinski, 2004; Rudolph and Möhler, 2004). The γ2 subunit knockout mouse revealed the importance of this subunit in receptor clustering at the synapse (Essrich *et al.*, 1998) and for survival as homozygotes have a high level of morbidity. By contrast, the α1 knockout mouse has provided insight into the observed reduction in decay time of IPSCs during development observed in some cell types (Vicini *et al.*, 2001; Jüttner *et al.*, 2001; Goldstein *et al.*, 2002; Ortinski *et al.*, 2004). This is thought to be a result of increased α1 expression later in development (Ortinski *et al.*, 2004). The α1 knockout mouse phenotype is relatively normal; except for an occasional handling induced tremor, these mice are indistinguishable from wild-type (Sur *et al.*, 2001) in many behavioural tests.

The β3 subunit knockout mouse suffers from severe neurological impairments including epilepsy (Homanics *et al.*, 1997) and has many of the phenotypic characteristics of the human neurodevelopmental disorder, Angelman syndrome (DeLorey *et al.*, 1998). Interestingly Angelman syndrome has also been linked to deficits in normal CaMK-II signalling pathways (Weeber *et al.*, 2003).

The removal of the β3 subunit reduces IPSC duration in β3 -/- mouse cortical neurones in culture and also decreases expression of the α2/3 subunits (Ramadan *et al.*, 2003). This supports the idea that there is a preferential association of α2/3 with β3 and α1 with β2. Disruption of β3 has been shown in β3 -/- mice to alter network oscillations in the olfactory bulb (Nusser *et al.*, 2001)

demonstrating that specific β subunit-containing receptors can play specific physiological roles.

The β2 knockout mouse unlike the β3 knockout mouse is behaviourally normal with no evidence of spontaneous seizures. The only distinguishable difference from wild-type is a slight increase in locomotor activity (Sur *et al.*, 2001). The difference between the β2 and β3 knockout mice is striking given the comparable loss in receptor density across the brain seen in the two lines of mice (Homanics *et al.*, 1997; Sur *et al.*, 2001; Vicini and Ortinski, 2004). There was no observed compensatory increase in β1/3 subunits observed in the β2 knockout mouse but there was a decrease in expression of all six α subunits (Sur *et al.*, 2001). The generation of knockout mice lacking subunits of the $GABA_A$ receptor has started to reveal differences in the function of different $GABA_A$ receptor subtypes. Although compensatory increased expression of subunits can sometimes mask the role(s) of particular receptor subtypes, it appears that β2 and β3 subunit-containing receptors must play very different roles in development (Homanics *et al.*, 1997; DeLorey *et al.*, 1998; Sur *et al.*, 2001; Sinkkonen *et al.*, 2003) and potentially in pathological states such as epilepsy (Brooks-Kayal *et al.*, 1998; DeLorey *et al.*, 1998; Sur *et al.*, 2001; Jones-Davis and Macdonald, 2003). Knock-in mice which lack the developmental changes that can occur in whole-subunit knockouts have revealed that different effects of anaesthetics are mediated through specific β2 or β3 subunit-containing $GABA_A$ receptors (Jurd *et al.*, 2003; Reynolds *et al.*, 2003; Rudolph and Möhler, 2004). Using the β2 knockout mouse, changes in the response of $GABA_A$ receptors to phosphorylation may reveal how distinct receptor populations are modulated by protein kinases.

Application of α-CaMK-II to rat cerebellar granule cells in culture under depolarising conditions (20 mM KCl) led to a significant increase in the whole-cell currents recorded (see Chapter 4). Application of α-CaMK-II to cerebellar granule cells cultured under more physiological conditions (5 mM KCl) led to a significant modulation of the amplitude and decay times of sIPSCs. This modulation of sIPSCs was not consistent across the whole population suggesting that there were possibly different effects of phosphorylation at different synapses (See Chapter 5). This could be related to differential effects of α-CaMK-II on different $GABA_A$ receptor subunit combinations.

Cerebellar granule cells are thought to express a number of different $GABA_A$ receptor subunits. Including α1/2/3/6, β2/3, γ2 and δ subunits (Persohn *et al.*, 1992; Laurie *et al.*, 1992b; Wisden *et al.*, 1996; Pirker *et al.*, 2000). They are thought to express very low levels of the β1 subunit (Persohn *et al.*, 1992; Laurie *et al.*, 1992b). The β2 subunit knockout mouse causes a concurrent decrease in the expression of all six α subunits but there has been no reported compensatory increase in the expression of different β subunits (Sur *et al.*, 2001). Therefore cultures obtained from β2 knockout mice may be expected to consist predominantly of β3 subunit-containing receptors as compared to wild-type mice which may be expected to express both β2 and β3.

Mouse cerebellar cultures maintained under depolarising conditions have been reported to express high levels of β3 subunits and a decreased expression of β2 subunits as compared to cultures maintained under more physiological conditions (Ives *et al.*, 2002a). The α-CaMK-II dependent effect observed on whole-cell currents under depolarising conditions therefore may reflect CaMK-II action on β3 subunit-containing receptors. Cells maintained under physiological conditions are

thought to express both β2 and β3 subunits (Laurie *et al.*, 1992b; Ives *et al.*, 2002a; Ortinski *et al.*, 2004) so the effect of CaMK-II on sIPSCs could potentially be mediated by receptors containing either or both of these subunits. Analysis of the effects of α-CaMK-II cultures prepared from β2 knockout mice maintained under both conditions should allow the determination of the relative contribution of the β2 subunit to GABA currents and reveal whether α-CaMK-II can modulate β2 subunit-containing receptors at the inhibitory synapse.

8.2 RESULTS

Cerebellar cultures were prepared from β2 knockout and wild-type mice at P0-2 using the same protocol as that used in the culture of rat cerebellar granule cells (See methods). The β2 knockout mouse was generated by deletion of exons 6 and 7 of the β2 subunit gene by homologous recombination (Sur *et al.*, 2001). Heterozygous F3 generation mice were bred to produce β2 -/- and wild-type strains of mice of the same genetic background (50 % C57BL6, 50 % 129SvEv; Sur *et al.*, 2001). Litters of mice were taken at P0-2 from F5-7 generation wild-type (wt) or β2 knockout (-/-) mice to produce cerebellar cultures. Tissue (in the form of tail snips) was taken from each mouse pup used for culturing and PCR was used for genotyping (See Chapter 2).

Recombinant α-CaMK-II (Suzuki-Takeuchi *et al.*, 1992) pre-activated by incubation with CaM, $CaCl_2$, and ATP-γ-S (McGlade-McCulloh *et al.*, 1993) was applied to cerebellar granule cells through inclusion in the patch pipette solution (See Chapter 2 and 3.2.1). The pre-activated α-CaMK-II was diluted into the internal solution (85 nM) and maintained on ice prior to recording. Control recordings were

made by supplementing normal patch pipette solution with the pre-activation buffer but without α-CaMK-II.

Whole-cell currents were recorded from cerebellar granule cells maintained in depolarising conditions in response to application of 10 μM GABA every 2 min for 20 min (See Chapter 4). Recordings of sIPSCs were made in the presence of 10 μM CNQX, 20 μM AP-5 to block excitatory postsynaptic currents (EPSCs). All the remaining synaptic currents could be blocked by 20 μM bicuculline indicating they were $GABA_A$ receptor mediated. In a small proportion of cells (15 %) there was evidence of a small tonic current (Fig 8.1) revealed by a shift in baseline current after the application of bicuculline (Brickley *et al.*, 1996; Leao *et al.*, 2000). Application of 500 nM TTX abolished nearly all IPSCs recorded indicating that the majority of IPSCs in this cell-type are action potential driven as has been previously described (Puia *et al.*, 1994; Leao *et al.*, 2000; Farrant and Brickley, 2003). Recordings were made with 5 mM QX-314 in the patch pipette solution to allow recordings of action potential driven sIPSCs whilst suppressing action potentials within the cell being recorded from (See Chapter 5). Continuous 5 min recordings were made starting 5 min after achieving the whole-cell configuration. Cerebellar granule cells were identified by their rounded cell body morphology measuring <8 μm in diameter (See Fig 2.1 and Fig 7.6).

Fig 8.1:

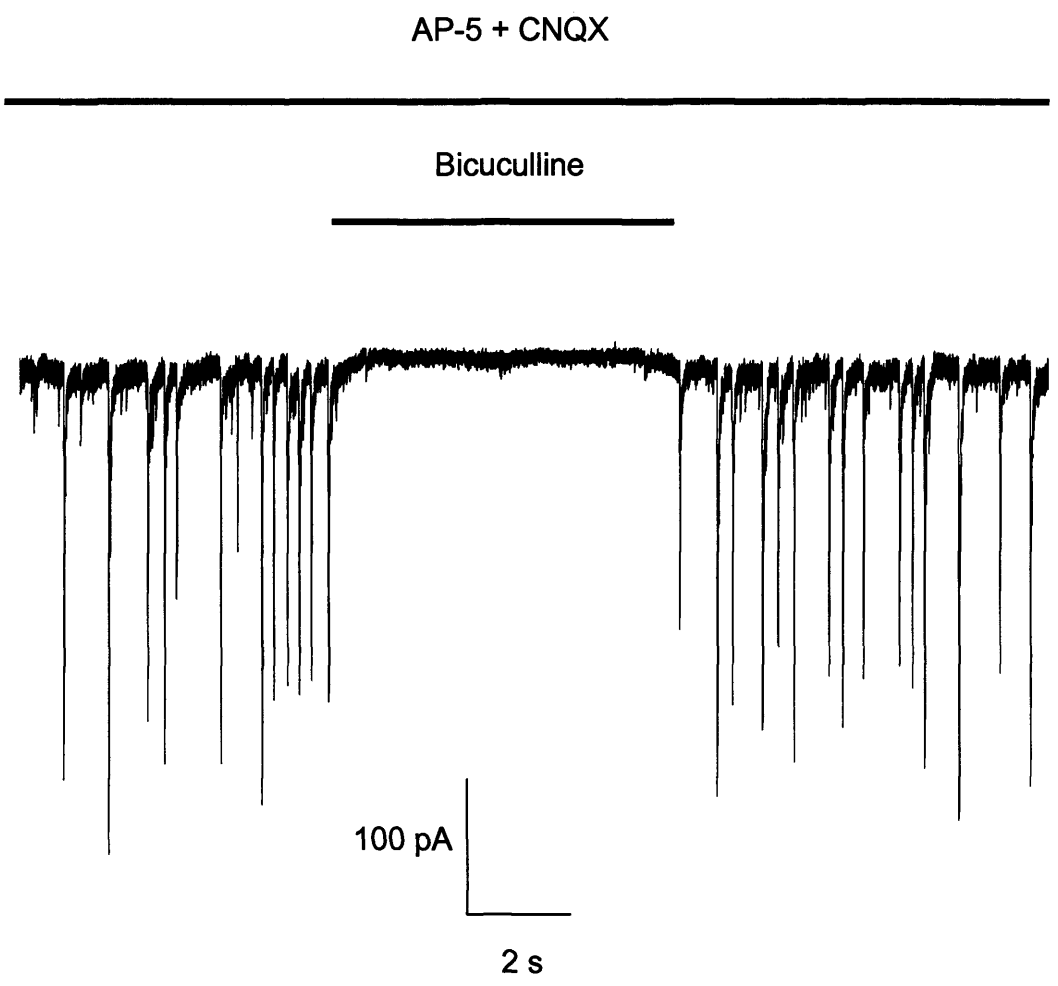

Fig 8.1: sIPSCs recorded from mouse cerebellar granule cells in wt cultures.

Representative synaptic currents recorded from mouse cerebellar granule cells grown and maintained in a physiological culture medium (5 mM KCl). Neurones were cultured from P0-2 wt mice.

Recordings were made with 5 mM QX-314 within the patch pipette solution. Bath applied AP-5 (20 μM) and CNQX (10 μM) reveals sIPSCs. Additional application of bicuculline (20 μM) reveals a tonic $GABA_A$ receptor-mediated current.

8.2.1 CaMK-II modulation of whole-cell currents recorded from wt and β2 -/- mice.

Application of 85 nM α-CaMK-II to cerebellar granule cells grown in depolarising conditions (20 mM KCl) resulted in a significant potentiation of the amplitude of whole-cell currents recorded from wt cultures to 146 ± 4.6 % (n = 8) as compared to control, 95.4 ± 4 % (n = 6, Fig 8.2A) at t = 8 min. Application of 85 nM α-CaMK-II was also significantly potentiated in β2 -/- cultures to the same extent, where the amplitude was potentiated to 142.8 ± 9 % (n = 10) as compared to control, 100.6 ± 5.7 % (n = 9, Fig 8.2B) at t = 8 min. This indicated that either the β2 subunit-containing receptors are not functionally modulated by α-CaMK-II in cerebellar granule cells under these culture conditions or that there was a low level of β2 subunits present even in wild-type cultures. There was no significant difference in the current density of GABA currents recorded in response to 10 μM GABA in either culture (wt, n = 8, β2 -/-, n = 10, Fig 8.2C) suggesting that there was a comparable number of functional $GABA_A$ receptors in wt and β2 -/- cultures.

8.2.2 CaMK-II modulation of sIPSCs.

Application of 85 nM α-CaMK-II to wt cerebellar granule cells grown in physiological (5 mM KCl) conditions resulted in a significant increase in the mean peak amplitude of sIPSCs from 44.9 ± 7.6 pA (n = 9) to 95.4 ± 22.6 pA (n = 12, Fig 8.3A). Application of 85 nM α-CaMK-II to β2 -/- cerebellar granule cells resulted in no significant change in mean peak amplitude (Control, 47.1 ± 8 pA, n = 8, + CaMK-II, 46.7 ± 5.6 pA, n= 9, Fig 8.3B and Table 8.1). There was also no significant difference between the mean peak amplitude of wt and β2 -/- mice in control conditions indicating that there was relatively little β2 subunit expression even under

physiological culture conditions (5 mM KCl) or that there was a compensatory increase in the expression of β1/3 subunit-containing receptors.

Application of α-CaMK-II significantly increased the half-width (time taken to decay to 50 % amplitude) of sIPSCs recorded from wt cultures from 9.8 ± 1.4 ms (n = 9) to 17.7 ± 2.9 ms (n = 12, Fig 8.3B). Application of α-CaMK-II to β2 -/- cultures also resulted in a significant increase in the half-width of sIPSCs from 10.4 ± 1.3 ms (n = 8) to 17.8 ± 3 ms (n = 9, Fig 8.3B and Table 8.1). The half-width was not significantly different from wt and β2 -/- cultures in control conditions. There was also no significant difference between half-widths after application of α-CaMK-II in either culture, indicating that removal of the β2 subunit had little effect on the lengthening of decay by α-CaMK-II. Unlike α1 knockout mice (Vicini *et al.*, 2001; Goldstein *et al.*, 2002; Ortinski *et al.*, 2004) removal of β2 does not significantly alter the mean decay time of IPSCs in control conditions.

Application of α-CaMK-II to wt cultures had no significant effect on sIPSC frequency (Control, 3.5 ± 0.8 Hz, n = 9, + CaMK-II, 2.4 ± 0.8 Hz, n = 12). There was no significant difference between wt and β2 -/- cultures in control conditions and there was no significant effect on β2 -/- cultures after application of α-CaMK-II (Control, 2.6 ± 1 Hz, n = 8, + CaMK-II -/-, 2.5 ± 0.7 Hz, n = 9, Fig 8.3C).

Fig 8.2:

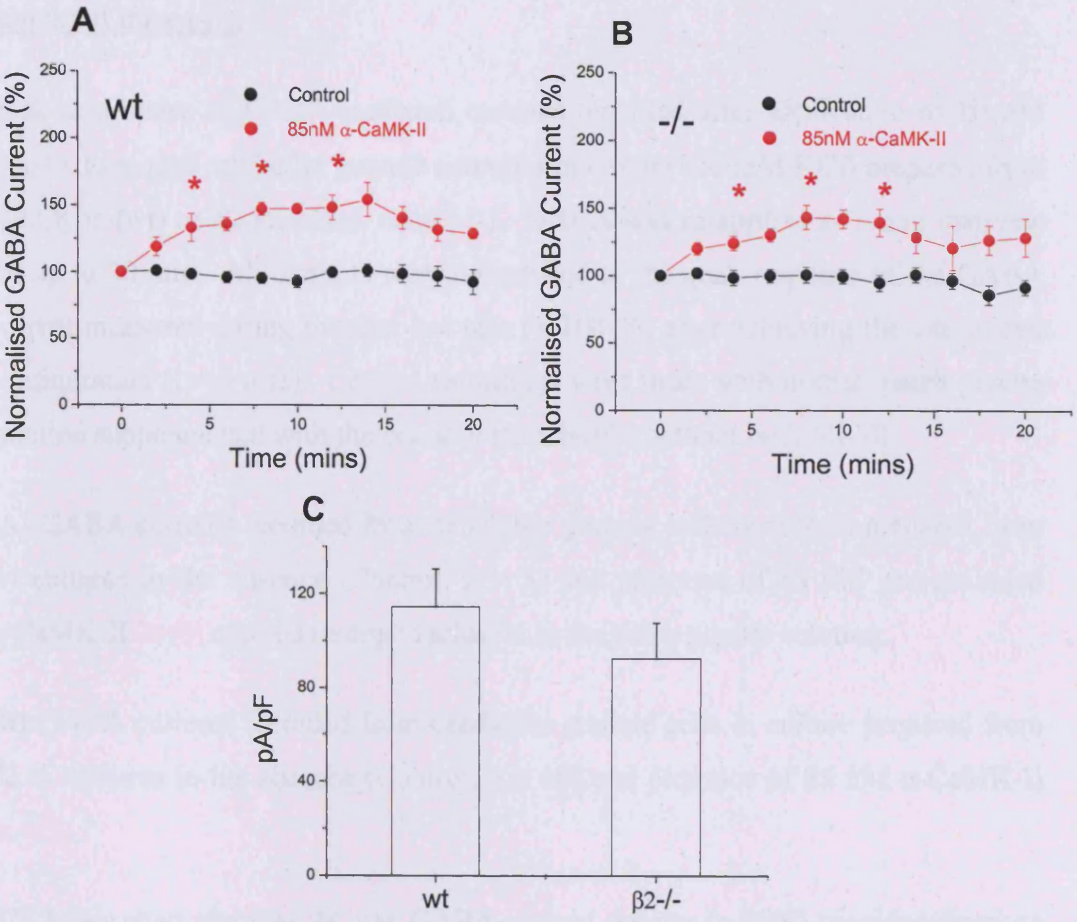
A
wt
Control
85nM α-CaMK-II
Normalised GABA Current (%)
Time (mins)
B
-/-
Control
85nM α-CaMK-II
Normalised GABA Current (%)
Time (mins)
C
pA/pF
wt
β2-/-

Fig 8.2: Modulation of whole-cell currents by α-CaMK-II recorded from cerebellar granule cells in culture (20 mM KCl) from wild-type (wt) and β2 knockout mice (-/-).

Peak amplitudes of GABA-mediated currents recorded after application of 10 μM GABA to mouse cerebellar granule neurones in culture (20 mM KCl) prepared from wild-type (wt) or β2 knockout mice (-/-). GABA was re-applied at 2 min intervals for up to 20 min. All currents were normalised to the peak response of the GABA current measured during the first 3-4 min (= 100 %) after achieving the whole-cell configuration (t = 0 min). Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) GABA currents recorded from cerebellar granule cells in culture prepared from wt cultures in the absence (Control, n = 8) and presence of 85 nM pre-activated α-CaMK-II (n=6) applied through inclusion in the patch pipette solution.

(B) GABA currents recorded from cerebellar granule cells in culture prepared from β2 -/- cultures in the absence (Control, n = 10) and presence of 85 nM α-CaMK-II (n = 9).

(C) A bar chart showing 10 μM GABA current density (pA/pF) recorded from wt (n = 8) and β2 -/- (n = 10) mouse cultures at t = 0 min in control conditions.

All points = mean ± s.e.
Significance is indicated by the symbol *, CaMK-II compared to control.
$P < 0.05$, t-test.

Fig 8.3:

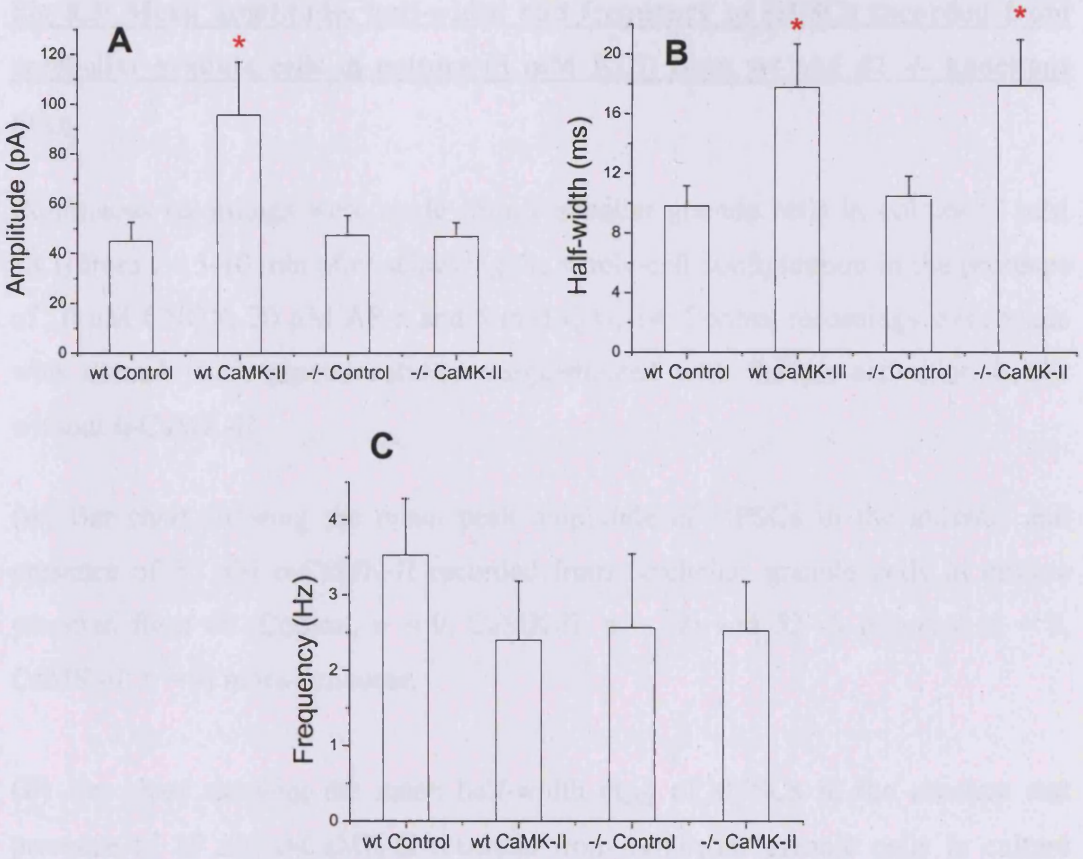
A
Amplitude (pA)
0
20
40
60
80
100
120
*
wt Control
wt CaMK-II
-/- Control
-/- CaMK-II
B
Half-width (ms)
0
4
8
12
16
20
*
*
wt Control
wt CaMK-III
-/- Control
-/- CaMK-II
C
Frequency (Hz)
0
1
2
3
4
wt Control
wt CaMK-II
-/- Control
-/- CaMK-II

Fig 8.3: Mean amplitude, half-width and frequency of sIPSCs recorded from cerebellar granule cells in culture (5 mM KCl) from wt and β2 -/- knockout mice.

Continuous recordings were made from cerebellar granule cells in culture (5 mM KCl) from t = 5-10 min after achieving the whole-cell configuration in the presence of 10 μM CNQX, 20 μM AP-5 and 5 mM QX-314. Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) Bar chart showing the mean peak amplitude of sIPSCs in the absence and presence of 85 nM α-CaMK-II recorded from cerebellar granule cells in culture prepared from wt (Control, n = 9, CaMK-II, n = 12) and β2 -/- (Control, n = 8, CaMK-II, n = 9) mouse cultures.

(B) Bar chart showing the mean half-width (τ_{50}) of sIPSCs in the absence and presence of 85 nM α-CaMK-II recorded from cerebellar granule cells in culture prepared from wt (Control, n = 9, CaMK-II, n = 12) and β2 -/- (Control, n = 8, CaMK-II, n = 9) mouse cultures.

(C) Bar chart showing the mean frequency of sIPSCs in the absence and presence of 85 nM α-CaMK-II recorded from cerebellar granule cells in culture prepared from wt (Control, n = 9, CaMK-II, n = 12) and β2 -/- (Control, n = 8, CaMK-II, n = 9) mouse cultures.

Mean ± s.e. Significance is indicated by the symbol *, CaMK-II compared to control.
$P < 0.05$, t-test.

8.2.3 CaMK-II modulation of decay times.

To look at decay times more carefully groups of 50-100 events were selected from each cell that had no deflections in either the rising or decay phases, and which decayed back to the baseline. These average traces were best fitted by a bi-exponential decay (See Chapter 2, 2.7.2). The mean $\tau 1$ (fast component of decay) of sIPSCs was significantly increased to 29.5 ± 5.3 ms (n = 8) as compared to control, 16.1 ± 2 ms (n = 8) on application of α-CaMK-II to wt cultures. Application of α-CaMK-II to $\beta 2$ -/- cultures also resulted in a significant increase in $\tau 1$ to 22.6 ± 2.2 ms (n = 7) as compared to control, 11.7 ± 1.3 ms (n = 7, Fig 8.4A,E and Table 8.1). There was no significant difference between $\tau 1$ in control conditions in wt or $\beta 2$ -/- cultures, or after the application of α-CaMK-II (Fig 8.4B).

Analysis of $\tau 2$ values for wt cultures also showed a significant increase on application of α-CaMK-II to 109 ± 15.9 ms (n = 8) as compared to control, 55.2 ± 7 ms (n = 8). Application of α-CaMK-II to $\beta 2$ -/- cultures also resulted in a significant increase in $\tau 2$ to 78.8 ± 5.1 ms (n = 7) as compared to control 55.2 ± 4.3 ms (n = 7, Fig 8.4B and Table 8.1). There was no significant difference between $\tau 2$ in control conditions in wt or $\beta 2$ -/- cultures or on application of α-CaMK-II between wt and $\beta 2$ -/- cultures (Fig 8.4B).

Phosphorylation has been reported to alter the relative area or contribution of fast and slow decay time constants. However, the proportional area of $\tau 1$ in wt cultures was unaffected by the application of α-CaMK-II (69.5 ± 1.8 %, n =8, as compared to control; 58.7 ± 5.1 %, n = 8). Application of α-CaMK-II to $\beta 2$ -/- cultures also did not affect the relative areas ($\tau 1$ area control, 45.5 ± 7.3 %, n = 7; CaMK-II, 59.5 ± 3.1 %, n = 7, Fig 8.4C,E). The data obtained from rat cerebellar cultures also showed no observable change in the proportional area of $\tau 1$ on

application of α-CaMK-II (Fig 5.3D). In addition there was no significant change between the proportional area of τ1 from wt cultures in control conditions (58.7 ± 5.1 %, n = 8) and that for β2 -/- cultures (45.5 ± 7.3 %, n = 7, Fig 8.4C and Table 8.1) in control conditions. However, the relative contribution of τ2 on application of α-CaMK-II to β2 -/- cultures was significantly greater than when α-CaMK-II was applied to wt cultures (Fig 8.4C).

Changes in IPSC decay in control conditions can also result from dendritic filtering. In order to avoid problems associated with dendritic filtering when analysing decay constants only events with fast rise-times (<3 ms) and clear peak amplitudes were used in the analyses. As cerebellar granule cells are electrotonically compact the effect of filtering should have been relatively small (Gabbiani *et al.*, 1994; Rossi and Hamann, 1998). Synaptic events for analysis were only chosen if there were no deflections in the rising or decaying phase from cells and there was no correlation between rise-times and amplitudes (See Chapter 5, Fig 5.4). The mean rise-times calculated from the grouped average traces used to analyse kinetics showed no significant differences between application of α-CaMK-II (0.76 ± 0.06 ms, n = 8) and control (0.73 ± 0.08 ms, n = 8) in wt cultures. In β2 -/- cultures there was no change in the rise-times on application of α-CaMK-II (0.75 ± 0.06 ms, n = 7) as compared to control (0.59 ± 0.06 ms, n = 7, Fig 8.4D,E) and there was no significant difference between rise-times after application of α-CaMK-II when comparing wt and β2 -/- cultures.

Fig 8.4:

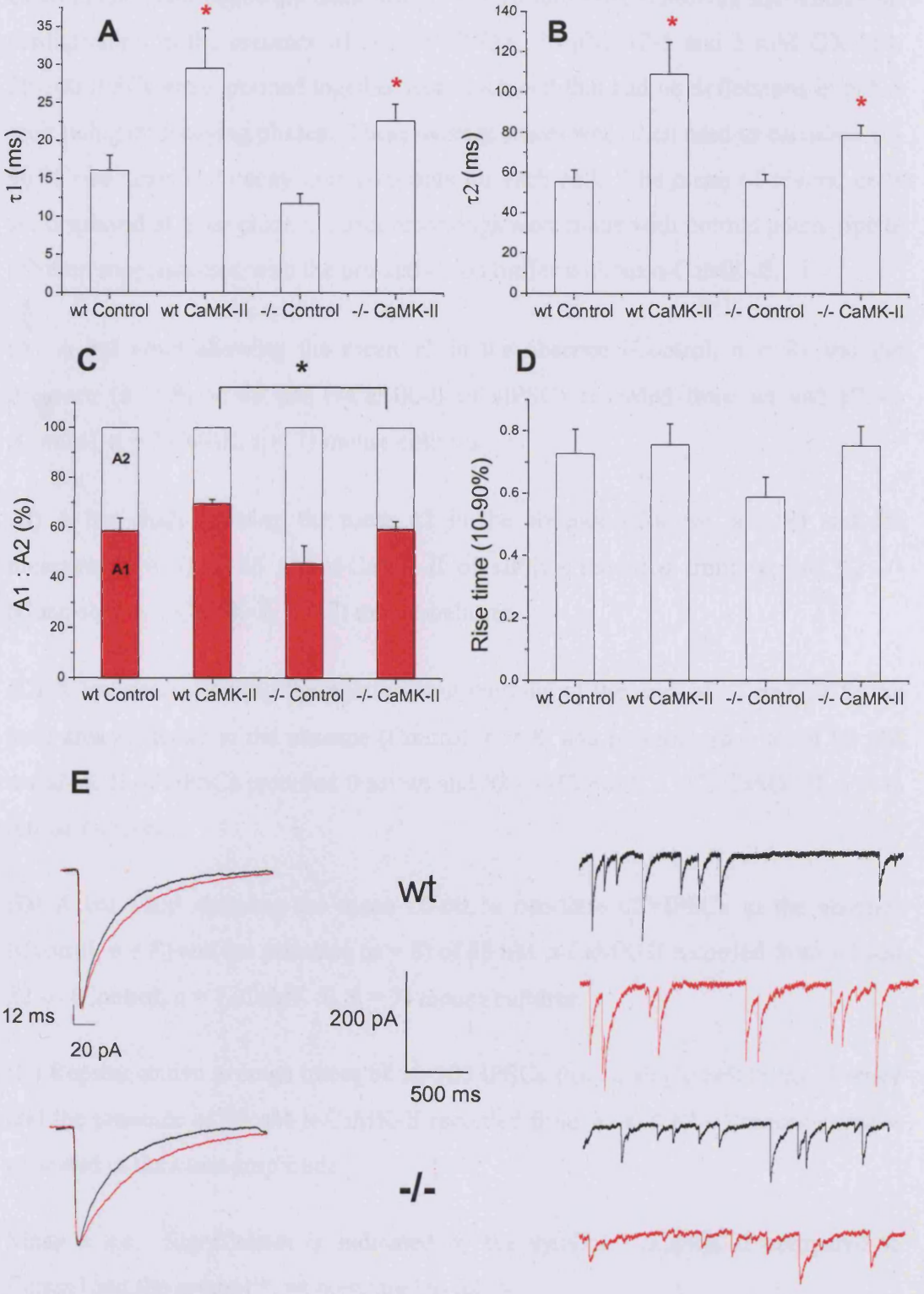
A
τ1 (ms)
wt Control
wt CaMK-II
-/- Control
-/- CaMK-II
B
τ2 (ms)
C
A1 : A2 (%)
A2
A1
D
Rise time (10-90%)
E
12 ms
20 pA
wt
200 pA
500 ms
-/-

Fig 8.4: Modulation of sIPSC decay times by α-CaMK-II in wt and β2 -/- mouse cultures.

Continuous recordings were made from t = 5-10 min after achieving the whole-cell configuration in the presence of 10 μM CNQX, 20 μM AP-5 and 5 mM QX-314. 50-100 IPSCs were grouped together from each cell that had no deflections in either their rising or decaying phases. These average traces were then used to calculate 10-90 % rise-times and decay time constants for each cell. The mean of several cells are displayed as a bar chart. Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) A bar chart showing the mean τ1 in the absence (Control, n = 8) and the presence (n = 8) of 85 nM α-CaMK-II of sIPSCs recorded from wt and β2 -/- (Control, n = 7,CaMK, n = 7) mouse cultures.

(B) A bar chart showing the mean τ2 in the absence (Control, n = 8) and the presence (n = 8) of 85 nM α-CaMK-II of sIPSCs recorded from wt and β2 -/- (Control, n = 7, CaMK-II, n = 7) mouse cultures.

(C) A bar chart showing the relative contributions of the area of τ1 and τ2 to the total area of decay in the absence (Control, n = 8) and presence (n = 8) of 85 nM α-CaMK-II of sIPSCs recorded from wt and β2 -/- (Control, n = 7, CaMK-II, n = 7) mouse cultures.

(D) A bar chart showing the mean 10-90 % rise-time of sIPSCs in the absence (Control, n = 8) and the presence (n = 8) of 85 nM α-CaMK-II recorded from wt and β2 -/- (Control, n = 7, CaMK-II, n = 7) mouse cultures.

(E) Representative average traces of 50-100 IPSCs from a single cell in the absence and the presence of 85 nM α-CaMK-II recorded from wt and β2 -/- mouse cultures re-scaled to the same amplitude.

Mean ± s.e. Significance is indicated by the symbol *, CaMK-II compared to Control and the symbol *, wt compared to β2 -/-.
$P < 0.05$, t-test.

Table 8.1:

	Amplitude (pA)	H-W (ms)	τ1 (ms)	τ2 (ms)	A1 (%)
Wt Control	44.9 ± 7.6	9.8 ± 1.4	16.1 ± 2	55.2 ± 7	58.7 ± 5.1
Wt CaMK-II	* 95.4 ± 22.6	* 17.7 ± 2.9	* 29.5 ± 5.3	* 109 ± 15.9	69.5 ± 1.8
β2 -/- Control	47.1 ± 8	10.4 ± 1.3	11.7 ± 1.3	55.2 ± 4.3	45.5 ± 7.3
β2 -/- CaMK-II	46.7 ± 5.6	* 17.8 ± 3	* 22.6 ± 2.2	* 78.8 ± 5.1	* 59.5 ± 3.1

Table 8.1: A table summarising sIPSC characteristics.

The mean values of peak amplitude, half-width (H-W), time constants τ1 and τ2 and the proportional area of τ1, in control conditions and in the presence of 85 nM α-CaMK-II from wt and β2 -/- cultures. Significance is indicated by the symbol *, CaMK-II compared to control and the symbol *, Wt compared to β2 -/- in the presence of CaMK-II. $P < 0.05$, t-test.

8.2.4 CaMK-II amplitude and decay time changes across the whole population of IPSCs.

As previously demonstrated, in rat cerebellar cultures the population of sIPSCs did not respond uniformly to α-CaMK-II (See Chapter 5) it was therefore possible that different changes in different sub-populations of events between wt and β2 -/- in control conditions or on application of α-CaMK-II, might mask or alter the apparent effect of α-CaMK-II. Therefore, a more beneficial approach would be to look at the whole population of IPSC events.

An IPSC half-width histogram was constructed for wt and β2 -/- cultures for control conditions and following the response to application of 85 nM α-CaMK-II.

In wt cultures there did appear to be a slight shift in half-width values from under 15 ms to over 15 ms (Control, n = 8, 6600 events, CaMK-II, n = 11, 6300 events, Fig 8.5A). The histogram constructed from β2 -/- cultures also showed a shift to higher values of half-width (over 15 ms) but in this case it appeared much more pronounced (Control, n = 9, 5500 events, CaMK-II, n = 10, 5500 events, Fig 8.5B).

The more pronounced effect on addition of α-CaMK-II could indicate that the increase in half-width observed in wt cultures is partly masked by the presence of the β2 subunits which are not affected by CaMK-II. There is also the indication that the loss of the β2 subunit also creates a small group of events with large half-widths of over 30 ms which are not present in the wild-type, under control conditions. It should be noted from the previous analysis though, that this slight shift in the population was not enough to alter the mean half-width. This population of IPSCs with increased half-widths in β2 -/- cultures could possibly be a result of a loss of α1 (faster decaying) subunit-containing receptors alongside β2 in favour of α2/3/6 containing receptors which are slower decaying (Gingrich *et al.*, 1995; Tia *et al.*, 1996; Ortinski *et al.*, 2004). It is important to note that analysis of decay time constants and proportional area's required selection of events with no deflections during the rise or decay phase. Slower decaying events are more likely to be superimposed by other events so these slower events may have been under-represented in the group averages used to calculate τ values and proportional areas.

Amplitude histograms were constructed of sIPSCs recorded from wt cultures in control conditions and after application of α-CaMK-II. In control conditions (n = 8, 6500 events) there appeared to be two populations of events: one under 100 pA and the other of 100-300 pA. On application of α-CaMK-II (n = 11, 6300 events, Fig 8.6A) both populations shifted slightly to the right with an increase in the number of

events at around 100 pA and the appearance of events over 300 pA in amplitude (Fig 8.6A, arrow). The amplitude histogram of sIPSCs constructed from events recorded from β2 -/- cultures showed a slightly different pattern. In control conditions (n = 9, 5500 events) the distribution of amplitudes was similar to that seen in wild-type with two populations of under 100 pA and between 100-300 pA. On application of α-CaMK-II (n = 10, 5500 events, Fig 8.6B) there was an increase in the number of events around 100 pA; however, in this case, there was no appearance of larger IPSCs around 400pA. This suggested that a sub-population of events did increase in amplitude but that the removal of the β2 subunit prevented the appearance of another sub-population of much larger amplitude IPSCs. This is in contrast to the more pronounced effect of α-CaMK-II on half-widths observed in β2 -/- cultures. Despite the increase in the number of IPSCs around 100pA in β2 -/- cultures this was not enough to significantly alter the mean peak amplitude. The loss of the larger amplitude events is probably responsible for the lack of apparent effect on the mean peak amplitude after α-CaMK-II application to β2 -/- cultures.

Fig 8.5:

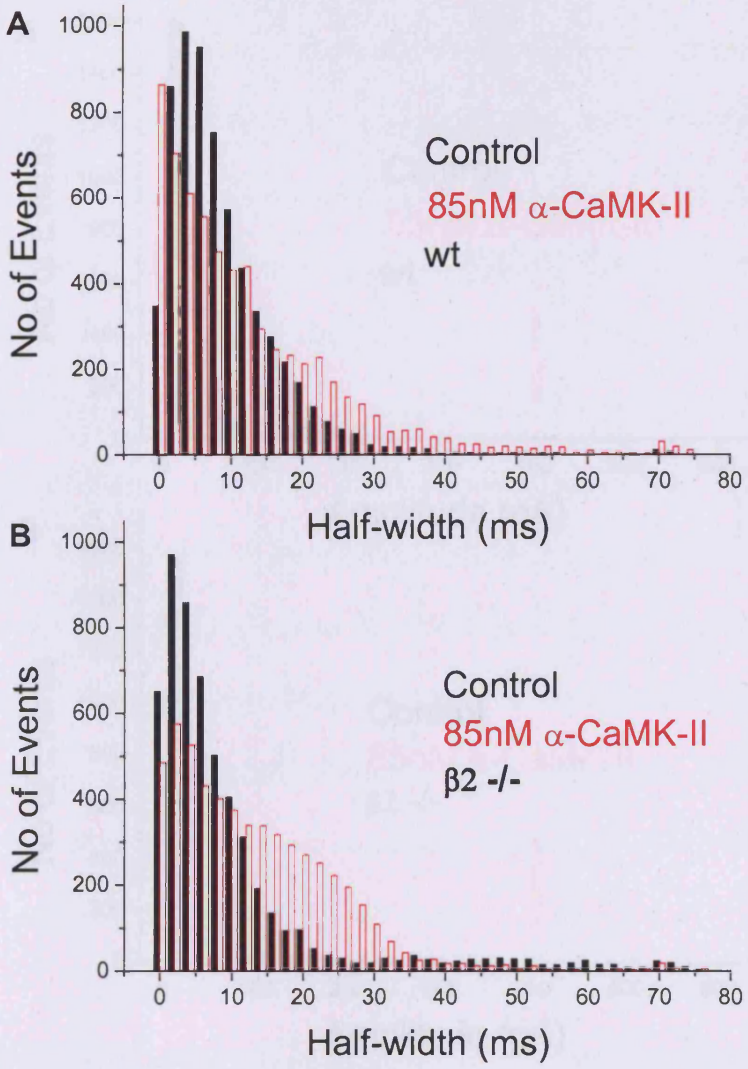

Fig 8.5: Frequency histograms of sIPSC half-widths recorded from wt and β2 -/- mouse cultures.

Continuous recordings were made from t = 5-10 min after achieving the whole-cell configuration in the presence of 10 μM CNQX, 20 μM AP-5 and 5 mM QX-314. All the half-widths of sIPSCs from 10-11 cells were grouped together and frequency histograms were constructed. Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) Half-width distribution histogram of sIPSCs in the absence (Control, n = 8, 6600 events) and the presence (n = 11, 6300 events) of 85 nM α-CaMK-II recorded from wt mouse cultures.

(B) Half-width distribution histogram of sIPSCs in the absence (Control, n = 9, 5500 events) and the presence (n = 10, 5500 events) of 85nM α-CaMK-II recorded from β2 -/- mouse cultures.

Fig 8.6:

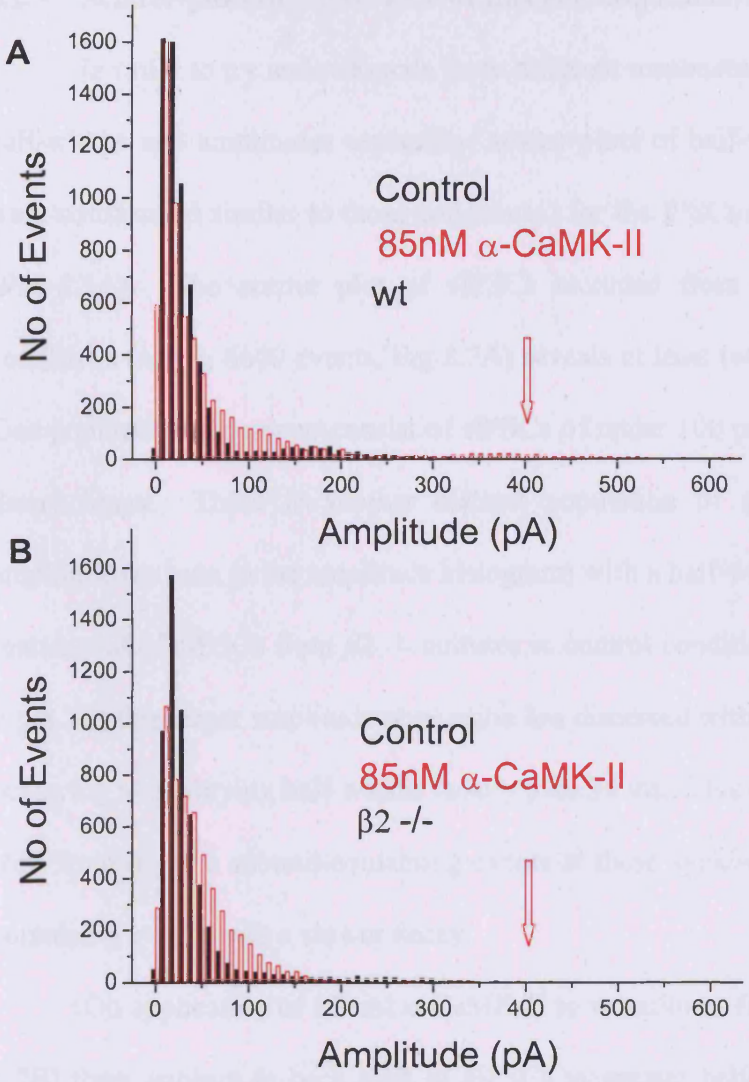

Fig 8.6: Frequency histograms of sIPSC amplitudes recorded from wt and β2 -/- mouse cultures.

Continuous recordings were made from t = 5-10 min after achieving the whole-cell configuration in the presence of 10 μM CNQX, 20 μM AP-5 and 5 mM QX-314. All the peak amplitudes of sIPSCs from a series of cells were grouped together and frequency histograms were constructed. Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) Amplitude distribution histogram of sIPSCs in the absence (Control, n = 8, 6600 events) and the presence (n = 11, 6300events) of 85 nM α-CaMK-II recorded from wt mouse cultures.

(B) Amplitude distribution histograms of sIPSCs in the absence (Control, n = 9, 5500 events) and the presence (n = 10, 5500 events) of 85 nM α-CaMK-II recorded from β2 -/- mouse cultures. The red arrow depicts large amplitude events of over 300 pA.

8.2.5 Scatter-plots of sIPSC half-widths and amplitudes.

In order to try and reconcile these different responses when examining sIPSC half-widths and amplitudes separately, scatter-plots of half-width against amplitude were constructed similar to those constructed for the IPSCs measured in rat cultures (Fig 5.5A). The scatter plot of sIPSCs recorded from wt cultures in control conditions (n = 8, 6600 events, Fig 8.7A) reveals at least two populations of events. One population appears to consist of sIPSCs of under 100 pA with a broad range of decay times. There is another distinct population of sIPSCs of 100-300 pA amplitude (as seen in the amplitude histogram) with a half-width of 10-30 ms. In the scatter-plot of sIPSCs from $\beta 2$ -/- cultures in control conditions (n = 9, 5500 events, Fig 8.7C) this larger amplitude population has dispersed with larger amplitude events occurring with varying half-widths mostly over 30 ms. This could be interpreted as a shift from $\alpha 1\beta 2\gamma 2$ subunit-containing events at these synapses to $\alpha 2/3\beta 3\gamma 2$ subunit-containing events with a slower decay.

On application of 85 nM α-CaMK-II to wt cultures (n = 11, 6300 events, Fig 8.7B) there appears to be a shift of sIPSCs to greater half-widths and amplitudes. The population of sIPSCs of 100-300 pA shifts to even higher amplitudes with little effect on half-width. If these events can indeed be interpreted as $\beta 2$ subunit-containing events this implies an α-CaMK-II dependent effect on $\beta 2$ subunit-containing receptors that is only revealed in a native environment. The addition of α-CaMK-II to rat cerebellar granule cultures revealed a similar effect on the population of sIPSCs to that observed in wt mouse cultures (See Fig 5.5A).

On addition of α-CaMK-II to $\beta 2$ -/- cultures (n = 10, 5500 events, Fig 8.7D) there is a shift of events, or the appearance of another population of events, at 75-200 pA amplitude and 15-35 ms that was not apparent in control conditions. There is,

however, no shift in amplitude of the larger amplitude events and there are no sIPSCs of over 300 pA amplitude. Again this could be interpreted as a result of the loss of $\beta 2$ subunit-containing events. It cannot be concluded with certainty that this is a specific $\beta 2$ subunit dependent effect due to the possible changes in the expression levels of other subunits on removal of the $\beta 2$ subunit however, it does appear that in this neuronal culture, synaptic $\beta 2$ subunit-containing receptors may be modulated by α-CaMK-II which contrasts with the lack of response to α-CaMK-II of $\alpha 1\beta 2\gamma 2S$ receptors expressed in NG108-15 cells.

Fig 8.7:

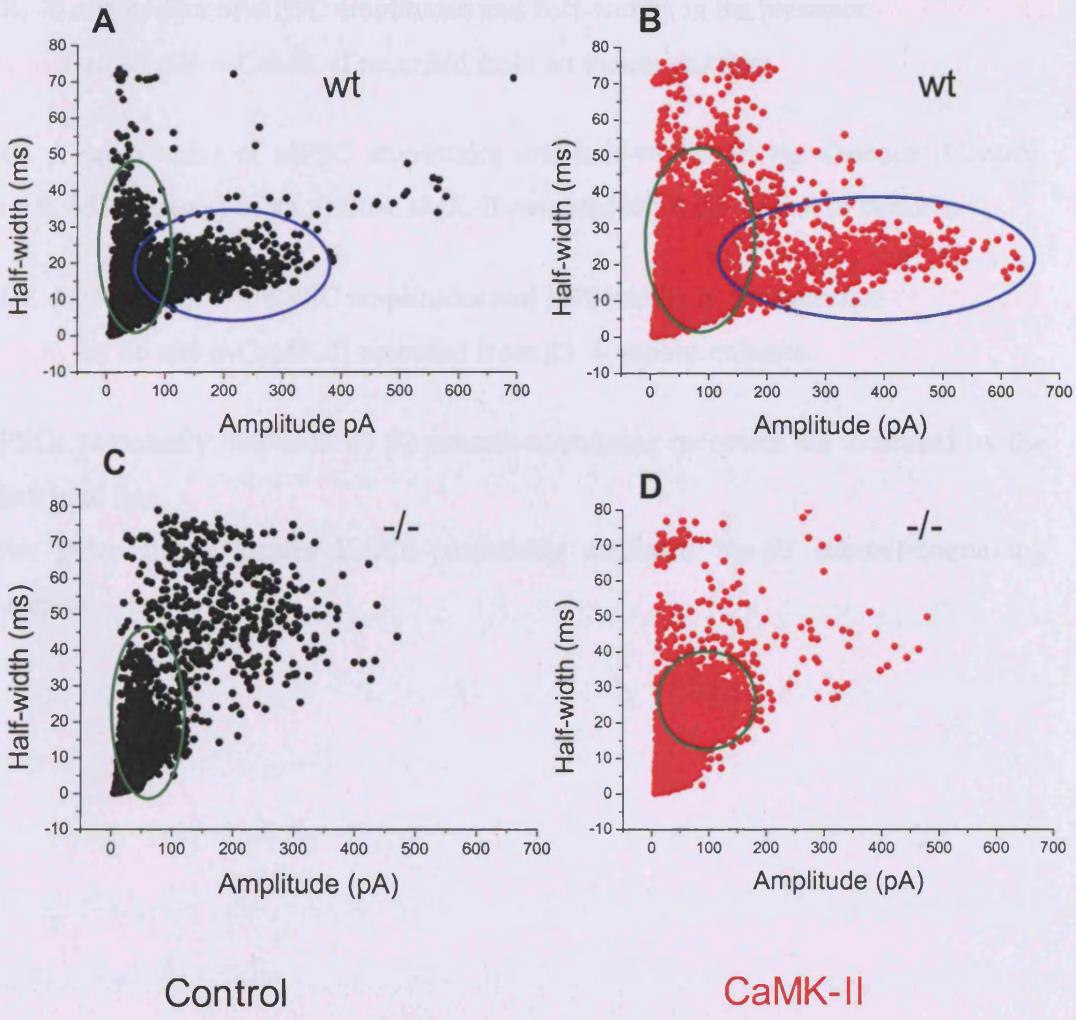

Fig 8.7: Scatter plots of sIPSC amplitude against half-width recorded from wt and β2 -/- mouse cultures.

Continuous recordings were made from t = 5-10 min after achieving the whole-cell configuration in the presence of 10 μM CNQX, 20 μM AP-5 and 5 mM QX-314. All the peak amplitudes and half-widths of sIPSCs from a series of cells were grouped together and scatter-plots of the amplitudes and half-widths for the IPSCs were constructed. Control recordings were made with normal patch pipette solution supplemented with the pre-activation buffer without α-CaMK-II.

(A) A scatter-plot of sIPSC amplitudes and half-widths in the absence (Control, n = 8, 6600 events) of 85 nM α-CaMK-II recorded from wt mouse cultures.

(B) A scatter-plot of sIPSC amplitudes and half-widths in the presence (n = 11, 6300 events) of 85 nM α-CaMK-II recorded from wt mouse cultures.

(C) A scatter-plot of sIPSC amplitudes and half-widths in the absence (Control, n = 9, 5500 events) of 85 nM α-CaMK-II recorded from β2 -/- mouse cultures.

(D) A scatter-plot of sIPSC amplitudes and half-widths in the presence (n = 9, 5500 events) of 85 nM α-CaMK-II recorded from β2 -/- mouse cultures.

IPSCs potentially mediated by β2 subunit-containing receptors are indicated by the dark blue line.
The green line indicates IPSCs potentially mediated by β3 subunit-containing receptors.

8.3 DISCUSSION

Spontaneous IPSCs recorded from cerebellar granule cells lacking the β2 subunit display slightly different properties compared to sIPSCs in wild-type cells. The population of sIPSCs from β2 -/- cultures has more events with slower decay times although this is not enough to significantly alter the mean half-width. There is however a significant difference in the relative contribution of τ2 on application of α-CaMK-II between wt and β2 -/- cultures indicating a more pronounced effect on decay times which can be observed in the half-width distribution histogram.

On application of α-CaMK-II to wild-type cultures there appears to be two broad trends. An increase in decay time for one population of IPSCs and an increase in amplitude with little change in decay times for another set of IPSCs. By comparing populations of sIPSCs in control conditions for wild-type and β2 -/- cells it can be predicted that the larger amplitude sIPSCs with faster decay times that change in amplitude, but not decay, on α-CaMK-II application, may be β2 subunit-containing $GABA_A$ receptor mediated.

This raises an interesting possibility, that β2 subunit-containing $GABA_A$ receptors at the synapse can be modulated by α-CaMK-II in a native neuronal environment. It appeared that expression of recombinant receptors in a neuronal cell line or in native neurones was not sufficient to allow α-CaMK-II modulation of α1β2γ2 receptors to occur. This may be because recombinant receptors expressed in neurones are not targeted to the correct subcellular location. For instance αβ heteromers would not be expected to be targeted to the synapse without the γ2 subunit (Essrich *et al.*, 1998). It is also possible that recombinant receptors do not bind to the relevant kinase anchoring proteins and this disrupts their modulation by phosphorylation. The current study seems to suggest that α1β2γ2 receptors need to

be expressed in their native neuronal environment at the synapse in order for α-CaMK-II modulation of $GABA_A$ receptors to be observed. It should be noted that recombinant α1β2γ2S receptors were transfected into cerebellar granule cells maintained in depolarising conditions, which display no synaptic activity and are thought to be representative of immature neurones (Ives *et al.*, 2002a). It could therefore be that expression of a mature functional synapse and the associated scaffolding and kinase anchoring proteins is critical for modulation of α1β2γ2 $GABA_A$ receptors by CaMK-II.

The functional effect of α-CaMK-II modulation appeared to be different for β2 and β3 subunit-containing receptors with the β3 subunit-containing receptors changing in decay time and β2 subunit-containing receptors increasing in amplitude with no change in decay time. It should be noted that a sub-population of IPSCs in β2 -/- cultures also increased in amplitude as can be seen from the amplitude distribution (Fig 8.6) but this shift was not enough to alter the mean IPSC amplitude (Fig 8.3). There was also a slight reduction in the number of large amplitude events on application of α-CaMK-II to β2 -/- cultures which may have acted to mask this change in amplitude.

It appeared from the study of recombinant receptors expressed in NG108-15 cells and cerebellar granule cells that in looking at α-CaMK-II dependent modulation it is necessary to consider the whole receptor and all subunits together. For instance it appeared that β and γ subunits interacted in different ways to modulate the response to CaMK-II. In a neuronal situation it appears that the loss of the β2 subunit may alter α subunit expression. A number of studies have suggested that α1 may preferentially co-assemble with β2, over α2/3 (Benke *et al.*, 1994; Ramadan *et al.*, 2003; Ortinski *et al.*, 2004). The loss of faster decaying large amplitude events

in β2 -/- cultures may reflect not only the loss of β2 but also a concurrent loss of α1, so it is difficult to say with absolute certainty that the β2 subunit is modulated directly by CaMK-II as it is possible α1 may be involved as well. Indeed, CaMK-II has been reported to phosphorylate α1 subunits (Churn *et al.*, 2002) although generally, they are thought to be poor substrates for phosphorylation (Moss *et al.*, 1992; Moss and Smart, 1996). The increase in the number of slower decaying events in control conditions in β2 -/- cultures may reflect a shift from α1 to α2/3 after a loss of α1 together with β2. In support of this, in β3 knockout mice, there is a decrease in α2/3 subunit expression and a reduction in IPSC decay times (Ramadan *et al.*, 2003).

It appears that β2 subunit-containing receptors predicted to consist of α1β2γ2 subunits may be preferentially targeted to large synapses. The size of the amplitudes of these IPSCs suggests that they were produced by multi-quantal release of GABA. One other possibility is that at these large amplitude synapses, β2/3 subunits are co-assembled in the same receptor complex and that β3 still mediates the CaMK-II effect. However if direct phosphorylation of β3 causes an increase in decay time this should have been observable in $GABA_A$ receptors containing both β2 and β3 subunits. The lack of effect on decay time of the large amplitude IPSCs suggests this effect was not mediated by β3.

It is always difficult when interpreting data from knockout mice to say for certain if a loss or change in synaptic transmission is due to the loss of the subunit removed or unforeseen indirect compensatory mechanisms. Despite these potential problems the evidence does suggest that it is possible that β2 subunit-containing receptors may be modulated at the synapse by α-CaMK-II. Modulation of β2 subunit-containing receptors by α-CaMK-II in a recombinant system is clearly not

possible (See Chapter 6) suggesting the mechanisms for modulation of both subtypes could be dependent on cell type.

Evidence in the literature suggests that phosphorylation in neurones can have distinctly different functional effects depending on the receptor subtypes present in a cell (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999). There is evidence for PKC dependent down-regulation of receptor function via phosphorylation of $\beta 2$ Ser^{410} in a recombinant system (Kellenberger *et al.*, 1992). A similar modulation by PKC has also been reported to be independent of phosphorylation of this subunit (Chapell *et al.*, 1998). In this case the down-regulation observed was thought to be associated with a loss of receptors from the cell surface and phosphorylation of receptor associated proteins rather than the $\beta 2$ subunit directly. PKC has also been reported to modulate receptor endocytosis in HEK293 cells and cortical neurones in culture. This process involved a dynamin endocytic pathway and was dependent on a dileucine motif present on the $\beta 2$ subunit (Herring *et al.*, 2005).

In a neuronal environment, PKA can modulate IPSCs recorded from cerebellar stellate, basket and Purkinje neurones that are thought to predominantly express the $\beta 2$ subunit (Persohn *et al.*, 1992; Kano and Konnerth, 1992; Nusser *et al.*, 1999). In addition, CaMK-II is thought to play a role in rebound potentiation (Kano *et al.*, 1992; Kano *et al.*, 1996; Kawaguchi and Hirano, 2002), a phenomenon that results in an increase in the amplitude of IPSCs without a change in decay times. This occurs in Purkinje cells of the cerebellum that are thought to express predominantly $\alpha 1\beta 2\gamma 2$ subunits (Persohn *et al.*, 1992; Laurie *et al.*, 1992a). Wang and colleagues (2003) have demonstrated an increase in the number of receptors at the cell surface related to PKB phosphorylation of $\beta 2$ Ser^{410} in HEK293 cells and in neurones using immunocytochemistry. Thus, functional changes due to

phosphorylation of β2 by PKB can be detected in HEK293 cells, in contrast to the present study with CaMK-II, and in contrast to the lack of PKA dependent functional modulation of $GABA_A$ receptors in HEK293 cells (McDonald *et al.*, 1998). Therefore, phosphorylation of receptors may not only be dependent on cell type and receptor subunit composition, but also on the kinase under investigation.

In neurones the effect of PKB induced an increase in the amplitude of IPSCs without a change in their decay, which was related to an increase in β2 subunit-containing receptors at the synapse (Wang *et al.*, 2003). This is comparable to the β2 subunit-containing population of sIPSCs in the current study increasing in amplitude without a change in decay time after exposure to α-CaMK-II. This raises the possibility that β2 subunit mediated effects may involve insertion of new receptors rather than a change in their single channel properties. It may be that a lack of functional effect on β2 subunit-containing receptors in recombinant systems is related to a lack of the appropriate cellular machinery needed to insert new receptors. Direct modification of channel properties by phosphorylation may be easier to observe and measure in a recombinant environment. However, insertion of receptors into the cell membrane has been observed in recombinant systems in response to phosphorylation (Wan *et al.*, 1997; Wang *et al.*, 2003).

It might simply be that β2 subunit phosphorylation is under much tighter control than that of β1/3, requiring multiple kinase anchoring proteins or $GABA_A$ receptor scaffolding proteins to occur. There is evidence for differences in β2 and β3 binding to kinase anchoring proteins as well as differences in the trafficking and targeting of these receptors to the synapse. For example AKAP 79/150 has been shown to directly bind the β1/3 subunits but not β2 and is critical for PKA-mediated phosphorylation of the β3 subunit (Brandon *et al.*, 2003) and RACK-1 has been

associated with binding to and facilitation of phosphorylation of β1/3 subunits (Brandon *et al.*, 2002; Jovanovic *et al.*, 2004). The $GABA_A$ receptor-associated protein (GRIF-1) has been identified which binds to the β2 subunit but not β1/3, although the function of this protein is unknown, it has been proposed as another subunit trafficking factor (Beck *et al.*, 2002).

There is some evidence that the trafficking and targeting of β2 and β3 subunit-containing receptors to the cell surface are different (Connolly *et al.*, 1996) and that these processes can be differentially affected by phosphorylation (Ives *et al.*, 2002b). The β2 and β3 subunits require different processes for correct assembly of a receptor and trafficking to the cell surface (Bollan *et al.*, 2003). In addition, β3 subunits alone can form functional homomeric channels, whereas β2 subunits cannot (Connolly *et al.*, 1996; Wooltorton *et al.*, 1997). Moreover, PKC phosphorylation can modulate receptor endocytosis of α1β2γ2 receptors in HEK293 cells and in neurones (Connolly *et al.*, 1999; Herring *et al.*, 2005) as well as directly affecting the properties of the ion channel (Krishek *et al.*, 1994; Lin *et al.*, 1996).

The activity of CaMK-II itself is thought to be controlled through its localisation to targets where it exerts an effect on proteins (Bayer and Schulman, 2001; Schulman, 2004; Colbran, 2004). For instance, it can bind to the NMDA receptor to maintain the activity of the kinase in a calcium-dependent manner (Bayer *et al.*, 2001). A CaMK-II anchoring protein, formed by alternative splicing of the α-CaMK-II gene that is stably integrated into the CaMK-II holoenzyme and influences CaMK-II localisation and function, has been identified in skeletal muscle (Bayer *et al.*, 1998; Bayer and Schulman, 2001) although not yet in neurones. Although a few potential CaMK-II anchoring proteins have been characterised (See Chapter 1) there are probably others that are yet to be found (Colbran, 2004). It is probable, therefore,

that CaMK-II dependent modulation of $GABA_A$ receptors may also be dependent on, or altered by, appropriate anchoring proteins and that some of the differences seen in the present study between recombinant and neuronal cultures may be due to the presence or absence of critical CaMK-II anchoring proteins.

It is possible to interpret the effects of α-CaMK-II in the current study on sIPSCs as a direct β3 phosphorylation and possibly an indirect tyrosine kinase phosphorylation of γ2S (See Chapter 7) leading to a possible increase in IPSC amplitude and a change in decay time seen in both wt and β2 -/- cultures. This would also explain the strong effect of α-CaMK-II on whole-cell currents recorded from cerebellar granule cells cultured in depolarising conditions when cells are thought to express predominantly β3 subunit-containing receptors (Ives *et al.*, 2002a) and would also be in accord with the modulation of recombinant α1β3γ2S receptors seen in NG108-15 cells. The lack of a significant change in the extent of the effect of CaMK-II on β2 -/- cerebellar cultures, maintained in depolarising conditions, could indicate that there is little β2 in these cells (Ives *et al.*, 2002a), or that β2 modulation by α-CaMK-II is only possible at a functional synapse since these cultures generally display no synaptic activity (Mellor *et al.*, 1998).

On application of exogenous GABA, the time to reach the peak amplitude of whole-cell current is relatively slow (See Chapter 2, 200-300ms). Direct changes on $GABA_A$ channel function altering probability of opening or the extent of desensitisation could increase the apparent amplitude of whole-cell currents. It has been demonstrated that phosphorylation of the γ2 subunit by tyrosine kinases alters the mean open time and probability of opening of the $GABA_A$ receptor (Moss *et al.*, 1995) and that this tyrosine kinase phosphorylation of γ2S can increase whole-cell currents recorded from HEK293 cells and neurones (Moss *et al.*, 1995).

Tyrosine kinase phosphorylation of γ2 has been reported to increase the amplitude of IPSCs, although not the decay time (Boxall, 2000; Ma *et al.*, 2003). So it could be speculated that Ser^{383} phosphorylation of the β3 subunit and phosphorylation of γ2S by down-stream activation of a tyrosine kinase together, alters directly the properties of the channel in such a way as to lengthen the decay times and increase the amplitudes of a sub-population of IPSCs. The β2 subunit mediated effect may be related to increased numbers of functional receptors and therefore results in an increase in amplitude with no change in decay times. The β2 subunit-containing receptors appear to be located at synapses responsible for much larger amplitude IPSCs in the present study (See Fig 8.7), possibly reflecting the fact that they are situated near to the soma or that they are located at specific, large, multi-quantal synapses.

There is evidence that the extent of receptor occupancy on release of GABA varies at different synapses within the same cell (Hájos *et al.*, 2000). Changes in channel properties would be expected to increase decay times and amplitudes of sIPSCs if GABA is not saturating, but would have no effect on amplitude at synapses where GABA is saturating. This may mean that phosphorylation dependent changes in channel function have slightly different effects depending on the degree of receptor occupancy at a given synapse. This may explain why α-CaMK-II appears to increase the amplitude of some IPSCs in β2 -/- cultures (See Fig 8.6), but has little effect on the mean IPSC amplitude (See Fig 8.3), as some IPSCs increase in amplitude and decay time and others only increase in decay time dependent on the level of receptor occupancy by GABA. At synapses where GABA is saturating an increase in receptor number is predicted to be the most effective way of up-regulating inhibitory transmission (Otis *et al.*, 1994; De Koninck and Mody, 1994;

Nusser *et al.*, 1998). It is possible therefore that different receptor subtypes are modulated by phosphorylation in different ways. This may allow the most appropriate form of modulation to occur at different synapses.

Changes in the decay times of IPSCs can be the result of changes in desensitisation of the receptor (Jones and Westbrook, 1996). The bi-exponential decay observed at many GABAergic synapses has been proposed to be related to desensitisation. The slower component of decay may relate to $GABA_A$ receptors that have bound GABA that are briefly desensitised before re-opening, prolonging the decay phase of the IPSC (Jones and Westbrook, 1996). The alteration in the apparent contribution of $\tau 1$ and $\tau 2$ between wt and $\beta 2$ -/- cultures on application of α-CaMK-II may suggest that CaMK-II dependent phosphorylation can alter the desensitisation of the receptor. In support of this, the calcium-dependent phosphatase calcineurin has been proposed to alter the desensitisation of the $GABA_A$ receptor (Jones and Westbrook, 1997).

The presence of perisynaptic receptors can alter the apparent rise-time and decay time of IPSCs (Mody and Pearce, 2004). Spillover-mediated transmission has been identified at golgi cell to granule cell synapses in brain slice preparations (Rossi and Hamann, 1998; Wall, 2002; Hamann *et al.*, 2002). This is thought to be related to the presence of high affinity $\alpha 6$ subunits in these cells which have been identified at cerebellar granule cell synapses (Nusser *et al.*, 1996; Ortinski *et al.*, 2004). Within rat cultures, under physiological conditions, there was little evidence of a tonic current, however, in these mouse wild-type and $\beta 2$ -/- cultures, a proportion of cells did express a tonic current indicating the likely presence of $\alpha 6$ subunit-containing receptors. Up-regulation of the function of perisynaptic receptors could alter the apparent decay times and rise-times of IPSCs. In the current study, a slight tendancy

to change the rise-time was observed in cells from β2 -/- cultures on application of α-CaMK-II which could be related to an effect on perisynaptic receptors, however, this effect did not reach statistical significance ($P < 0.1$, Fig 8.4D).

In conclusion the analysis of knockout mouse data can be complicated by compensatory changes and any differences observed could potentially be non-specific. But the evidence does indicate that an α-CaMK-II dependent effect occurs on a population of events that disappear in the β2 knockout mouse. The most parsimonious explanation is that α-CaMK-II can modulate β2 subunit-containing receptors, but only in the native neuronal synaptic environment. It is not clear why this does not occur in a recombinant system, but it could be because specific kinase anchoring proteins, synaptic scaffolding proteins and/or receptor trafficking machinery is needed for α-CaMK-II to modulate these receptors. This has important implications for the functional role of phosphorylation of different $GABA_A$ receptors at inhibitory synapses.

8.4 CONCLUSIONS:

- There was no significant difference in the modulation of whole-cell GABA currents by α-CaMK-II recorded from cerebellar granule cells maintained in depolarising conditions lacking the β2 subunit. Suggesting a lack of β2 in wild-type cultures or a lack of an α-CaMK-II dependent effect on β2 subunit-containing receptors under these culture conditions.
- Application of α-CaMK-II had a different effect on two populations of sIPSCs recorded from cerebellar granule cells from wt mice. One population of sIPSCs increased in decay time and another population of larger amplitude sIPSCs increased significantly in amplitude with no change in decay.

- The larger amplitude population of sIPSCs was no longer present in β2 -/- cultures, suggesting that they were mediated by β2 subunit-containing receptors and that these sIPSCs could be modulated by α-CaMK-II in the wild-type cultures.
- The loss of the β2 subunit resulted in subtle changes to the population of sIPSCs observed in control conditions. There was an apparent loss of large amplitude fast decaying events which were replaced by large amplitude events with slower decay times. This may coincide with a loss of α1 subunit-containing receptors alongside the loss of β2.

Chapter Nine:
Physiological significance of CaMK-II modulation of $GABA_A$ receptors

9.1 INTRODUCTION

Modulation of $GABA_A$ receptor mediated currents through activation of CaMK-II has been reported in a number of neuronal cell types (Kano *et al.*, 1992; Wang *et al.*, 1995; Kano *et al.*, 1996; Kawaguchi and Hirano, 2002; Wei *et al.*, 2004). The current study provides evidence for the potential mechanisms behind this modulation. Use of NG108-15 cells allowed sites of CaMK-II dependent phosphorylation to be determined revealing that Ser^{383} on the β3 subunit is likely to be a direct site of CaMK-II phosphorylation on the $GABA_A$ receptor. In addition, a CaMK-II dependent phosphorylation of tyrosine residues 357 and 359 on the γ2S subunit was also observed (See Fig 9.1). The evidence suggested that CaMK-II can activate a down-stream tyrosine kinase which goes on to phosphorylate β1 and β3 subunit-containing $GABA_A$ receptors. However, β2 subunit-containing receptors are not modulated at all by CaMK-II suggesting a specific kinase anchoring protein may be required for any effect on β2 subunit-containing receptors to be observed (See Chapters 6 and 7). In addition, in the HEK293 cell line, it was not possible to identify any functional modulation of $GABA_A$ receptors by CaMK-II (See Chapter 3), again suggesting kinase anchoring proteins may be important in regulating CaMK-II dependent phosphorylation of $GABA_A$ receptors. The importance of these sites of phosphorylation was confirmed by expression of recombinant receptors in cerebellar granule cells maintained in depolarising conditions. However it was still not possible to observe any α-CaMK-II dependent modulation of $\alpha1\beta2\gamma2S$ receptors.

CaMK-II is capable of increasing whole-cell current amplitudes in a neuronal culture preparation (cerebellar granule cells in depolarising conditions) that is predicted to express predominantly β3 subunit-containing receptors (See Chapter 4). Analysis of synaptic currents recorded from cerebellar granule cell cultures predicted to express both β2 and β3 subunits (physiological conditions) confirms that CaMK-II can significantly increase the amplitude and decay times of IPSCs. However, these effects are not uniform across the whole population of IPSCs recorded. One population of IPSCs significantly increased in decay time and another population of IPSCs significantly increased in amplitude with no affect on decay time (See Chapter 5).

Analysis of synaptic currents in a culture preparation lacking β2 subunit-containing receptors inferred that CaMK-II can indeed modulate β2 subunit-containing $GABA_A$ receptor mediated IPSCs. The effect of CaMK-II on β2 subunit-containing receptors involves an increase in IPSC peak amplitudes with no change in decay times (See Chapter 8). It is proposed that the mechanism of CaMK-II action on β2 subunit-containing receptors may involve insertion of receptors into the plasma membrane rather than a direct effect on channel properties, consistent with other evidence in the literature regarding β2 subunit phosphorylation. Whereas, CaMK-II dependent effects on β3 subunit-containing receptors may involve direct phosphorylation and alteration of channel properties which is consistent with the lengthened decay times of IPSCs.

Fig 9.1:

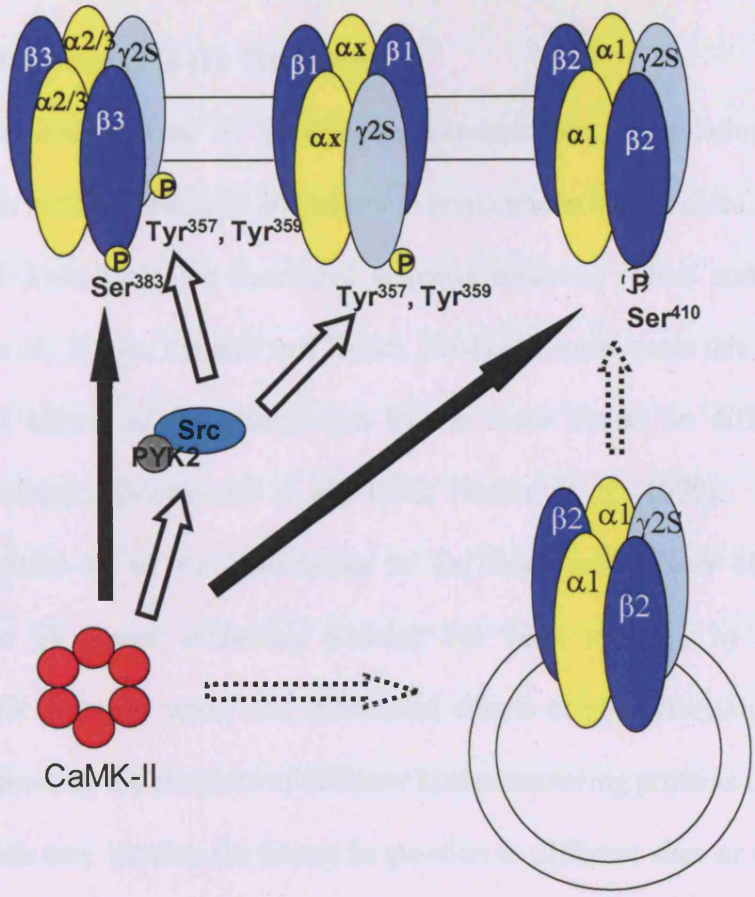

Fig 9.1: Summary of the proposed action of CaMK-II on different $GABA_A$ receptor subtypes.

CaMK-II can modulate $GABA_A$ receptor function through direct phosphorylation of the β3 subunit at Ser^{383} and through activation of a tyrosine kinase that can phosphorylate the γ2S subunit at $Tyr^{357, 359}$. PYK2 and Src tyrosine kinase (the most likely candidates) have been proposed to be activated by Ca^{2+} dependent signalling pathways and in some cases CaMK-II in other cell types.

CaMK-II modulation of the β2 subunit-containing receptors could occur through direct phosphorylation of the subunit or through an indirect mechanism. It is proposed that CaMK-II modulation of β2 subunit-containing receptors may occur through insertion of receptors into the plasma membrane.

Arrows in black indicate potential sites of direct phosphorylation. Block arrows indicate sites of phosphorylation mediated by a downstream kinase. Dashed line arrows indicate pathways involving changes to receptor trafficking.

9.2 Limitations in studying phosphorylation.

9.2.1 Discrepancies in the literature.

The investigation of phosphorylation-dependent modulation of $GABA_A$ receptors in both recombinant and neuronal preparations has revealed a considerable amount of diversity in the functional response observed (Moss and Smart, 1996; Brandon *et al.*, 2002a; Lüscher and Keller, 2004). In some cases this may be due to differential effects of phosphorylation by the same kinase on different $GABA_A$ receptor subtypes (McDonald *et al.*, 1998; Nusser *et al.*, 1999). However, this cannot explain all of the differences in the functional effects observed. The importance of kinase anchoring proteins has been revealed by a number of experiments in recent years, and differential effects of phosphorylation may be, in part, explained by the presence of different kinase anchoring proteins in different cell types, which may localise the kinase in question to different sites or enable binding to different $GABA_A$ receptor subtypes (Brandon *et al.*, 2002b; Brandon *et al.*, 2003; Terunuma *et al.*, 2004).

The mechanisms of functional modification by phosphorylation may also vary depending on the kinase involved or again the target $GABA_A$ receptor subtype. Covalent modification of receptor structure and alteration of channel properties, such as desensitisation (Jones and Westbrook, 1997) or probability of channel opening (Moss *et al.*, 1995), have been shown to modulate $GABA_A$ receptor function. In addition, phosphorylation can alter the trafficking of $GABA_A$ receptors to the synapse and dynamic modulation of receptor number has been shown to be an important mechanism in synaptic plasticity (Nusser *et al.*, 1998; Wang *et al.*, 2003b; Kittler and Moss, 2003).

When interpreting data relating to phosphorylation dependent changes in $GABA_A$ receptor function it is important to note that different experimental approaches can reveal different functional effects (See Chapter 1). For instance, the mechanism of activation of a particular kinase or the time-scale of activation can have a significant influence on the effect observed (Angelotti *et al.*, 1993; Krishek *et al.*, 1994; Lin *et al.*, 1996; McDonald *et al.*, 1998). When activating any kinase signalling pathway it is very difficult to discount downstream activation of other kinases, as has been demonstrated in the current study (See Chapter 7). In addition, phosphorylation must be considered in the context of a dynamically changing balance of activity between different kinases and phosphatases. The background basal phosphorylation state of the $GABA_A$ receptor in a given cell type may also alter the response observed in any given experiment (Brandon *et al.*, 2000; Brandon *et al.*, 2001).

The method of analysis also has significant implications in that measurement of whole-cell current amplitudes *per se*, may not identify subtle changes or differences in channel behaviour which could have a significant effect on synaptic currents (Hinkle and Macdonald, 2003). When analysing the effects of phosphorylation on a heterogeneous population of $GABA_A$ receptor mediated IPSCs within a neuronal population, it is possible a significant effect on different subpopulations of events may be missed if only mean values are measured (See Chapters 5 and 8).

9.2.2 CaMK-II

When looking at CaMK-II dependent modification of receptor function these problems are pronounced by the complexity of the processes that modulate the

kinase activity itself (Griffith *et al.*, 2003; Colbran, 2004). The current study indicates that pre-activated recombinant α-CaMK-II is capable of modulating $GABA_A$ receptor function. However, in cerebellar granule cells maintained in depolarising conditions, endogenous β-CaMK-II may also be capable of basal phosphorylation of $GABA_A$ receptors (See Chapter 4) as this is the predominant isoform in this cell type (Miller and Kennedy, 1985; Burgin *et al.*, 1990). It is currently not known what the role of different ratios of α to β CaMK-II subunits and/or translocation of CaMK-II to inhibitory as opposed to excitatory synapses might be in relation to $GABA_A$ receptor modification.

The recombinant α-CaMK-II used in the current study can phosphorylate the same substrates as the full-length form of this subunit (Suzuki-Takeuchi *et al.*, 1992) but cannot form a holoenzyme (Shen and Meyer, 1998). Pre-activation of this recombinant form of α-CaMK-II and inclusion within the patch pipette has been used successfully to reveal CaMK-II dependent effects on the function of AMPA receptors (Lledo *et al.*, 1995; Barria *et al.*, 1997). Application of a pre-activated recombinant form of β-CaMK-II would be expected to have a similar effect using this protocol because of the similarity in substrate specificity between the two isoforms (Miller and Kennedy, 1985). The α and β subunits differ in their sensitivity to activation by Ca^{2+}/CaM (Miller and Kennedy, 1985; Brocke *et al.*, 1999) which in this case is not relevant as CaMK-II was pre-activated *in vitro*.

It should be considered though, that localisation and targeting of recombinant constructs of CaMK-II may not be the same as that under physiological conditions after activation of an endogenous CaMK-II holoenzyme by Ca^{2+} signalling which, in itself, is a complex and highly regulated process (Meldolesi, 2001; Franks and Sejnowski, 2002; Augustine *et al.*, 2003). Pre-activated recombinant α-CaMK-II can

be expected to phosphorylate CaMK-II substrates within the cell but the role of α:β subunits within an endogenous heteromer cannot be determined using this protocol.

9.3 The roles of specific $GABA_A$ receptor subtypes.

9.3.1 Specific synapses express discrete $GABA_A$ receptor subtypes.

The current study has revealed a difference in the response to CaMK-II dependent phosphorylation of different $GABA_A$ receptor subtypes (See Chapter 6 and Chapter 8). This finding is similar to the observed differences in response of different $GABA_A$ receptor subtypes to PKA dependent phosphorylation (McDonald *et al.*, 1998). Such differences have been proposed to partly explain some of the cell type dependent differences in functional response to phosphorylation by PKA and PKC (Nusser *et al.*, 1999; Poisbeau *et al.*, 1999).

Recent evidence also suggests that different $GABA_A$ receptor subtypes could be targeted to different locations within the same cell (Connolly *et al.*, 1996; Koulen *et al.*, 1996; Brünig *et al.*, 2002). Specific synapses within the same neurone may express specific $GABA_A$ receptor subtypes (See Fig 9.2). Within rat or rabbit retinae, α1, α2 and α3 subunits were shown to not be co-localised within the same clusters. Individual neurones could express several isoforms of $GABA_A$ receptor and these were targeted to specific postsynaptic sites (Koulen *et al.*, 1996; Brünig *et al.*, 2002). In dissociated cultures of hippocampal neurones, specific α subunits were targeted to discrete cellular regions in a different way depending on the cell type (Brünig *et al.*, 2002). Although these studies focused on α subunit expression it is known that certain α and β subunits preferentially assemble suggesting that the subcellular location of β subunits may also be tightly controlled (Benke *et al.*, 1994; Whiting, 2003). In support of this, in a recombinant system β subunits have been

shown to target to different subcellular locations (Connolly *et al.*, 1996). Furthermore, specific targeting of $GABA_A$ receptor subtypes to particular synapses appears to occur in the current study, in that $\beta 2$ subunit-containing receptors are targeted to form large IPSC amplitude synapses whereas the other β subunits, presumed to be predominantly $\beta 3$ in cerebellar granule cells, are targeted to smaller amplitude IPSC synapses (See Chapter 8).

Interneurones are able to form synapses at precise locations on the target cell (McBain and Fisahn, 2001; Di Cristo *et al.*, 2004; Farrant and Nusser, 2005; Somogyi, 2005). Different interneurones can form discrete synapses onto hippocampal pyramidal cells with different $GABA_A$ receptor subtypes located postsynaptically (See Fig 9.2), even within a similar domain of the pyramidal cell. Pyramidal cells clearly have mechanisms to target $GABA_A$ receptor subtypes to distinct synapses depending on the presynaptic cell type (Nyíri *et al.*, 2001).

The targeting of specific $GABA_A$ receptor subtypes to specific sites is important because the channel properties of $GABA_A$ receptors can differ according to the subunits within the receptor (See Fig 9.2). For example, the presence of different α subunits has been shown to alter the kinetics of a given receptor (Gingrich *et al.*, 1995). Different receptor subtypes may be targeted to specific locations so that the functional properties of a $GABA_A$ receptor may be tailored to suit the specific synapse and functional role(s) that they play. In addition, these functional properties can be differentially modulated by phosphorylation depending on the $GABA_A$ receptor subtype present (See Fig 9.2).

In the current study, $\beta 2$ subunit-containing receptors could not be modulated by CaMK-II in a recombinant system, whereas it was possible to observe modulation by CaMK-II of $\beta 3$ subunit-containing $GABA_A$ receptors (Chapter 6). This suggests a

possible difference in the way $GABA_A$ receptor subtypes are modulated by CaMK-II. CaMK-II dependent effects on $\beta 3$ subunit-containing receptors were thought to involve a change in decay time of IPSCs consistent with a change in the single channel properties of the receptor, whereas effects on $\beta 2$ subunit-containing receptors appeared to involve an increase in amplitude with no change in decay consistent with alterations in receptor number (See Chapter 8). The heterogeneity of response of different β subunits to phosphorylation may provide a mechanism to alter the function of specific synapses in precise ways according to their location and physiological role.

9.3.2 The physiological roles of $\beta 2$ and $\beta 3$ subunit-containing receptors.

Evidence suggests that certain α and β subunits preferentially assemble together (Benke *et al.*, 1994). It is thought that $\alpha 1\beta 2\gamma 2$ and $\alpha 2/3\beta 3\gamma 2$ receptors comprise the majority of receptors in the brain (Benke *et al.*, 1994; Whiting, 2003; Vicini and Ortinski, 2004). Although the $\alpha 1\beta 2\gamma 2$ receptor is the most widely expressed recent evidence suggests both $\beta 2$ and $\beta 3$ subunit-containing receptors have important physiological roles. The use of knock-in mice with mutations that render specific subtypes of receptor insensitive to selected drugs has enabled their contribution to the effects of these drugs to be ascertained (Vicini and Ortinski, 2004; Rudolph and Möhler, 2004; Wafford *et al.*, 2004; Wafford, 2005). Knock-in mutation of the $\beta 2$ and $\beta 3$ subunits, with mutations known to be critical for the action of general anaesthetics *in vitro,* has revealed that $\beta 2$ and $\beta 3$ subunit-containing receptors mediate different physiological effects of anaesthetics (Rudolph and Möhler, 2004). The $\beta 2$ subunit appears to be involved in mediating the sedative

properties of anaesthetics whereas the β3 subunit appears to mediate a loss of consciousness (Jurd *et al.*, 2003; Reynolds *et al.*, 2003).

The β2 subunit also appears to mediate a significant proportion of the hypothermic effect of the general anaesthetic etomidate (Cirone *et al.*, 2004). The drug oleamide has sleep promoting effects in rodents but administration of this drug to β3 knockout mice fails to have any affect suggesting that the action of this drug is solely mediated through actions on β3 subunit-containing receptors (Laposky *et al.*, 2001). The use of α1 subunit and β2 subunit knockout mice has also revealed that the behavioural effects of ethanol may be mediated by distinct $GABA_A$ receptor subtypes (Blednov *et al.*, 2003).

9.3.3 Network oscillations.

Inhibitory synaptic transmission plays a critical role in the generation of network oscillations which are associated with behaviour (Gaiarsa *et al.*, 2002). During active exploration rat hippocampal neurones exhibit rhythmic theta and gamma frequency activity (McBain and Fisahn, 2001). Simulations involving interneuronal networks with fast and slow $GABA_A$ receptor kinetics (fast and slow decaying IPSCs) have been shown to produce theta-gamma rhythmic activity (White *et al.*, 2000; McBain and Fisahn, 2001). The kinetics of $GABA_A$ receptors have been shown to be important in shaping network oscillations in the cortex (Whittington *et al.*, 1995). Alteration of the decay times of IPSCs through phosphorylation may therefore be important in generating or modulating this rhythmic activity.

Subunit specific effects of phosphorylation on different subtypes may modulate receptor properties changing the decay times of IPSCs and therefore altering oscillations in the network. In addition mutations that affect the decay times

of IPSCs have been linked to pathological conditions such as epilepsy (Bianchi *et al.*, 2002; Fisher, 2004; Macdonald *et al.*, 2004) and have been shown to alter behaviour (Homanics *et al.*, 2005).

Specific $GABA_A$ receptor subunits have been shown to play precise roles in the generation of oscillatory activity. The role of the β3 subunit in network oscillations of the olfactory bulb has been investigated using the β3 subunit knockout mouse. Alteration of normal synaptic inhibition resulted in a significant increase in the amplitude of theta and gamma oscillations recorded from this region in freely moving mice resulting in an alteration of normal olfactory discrimination (Nusser *et al.*, 2001). This implied an important role for β3 subunit-containing receptors in modulating theta and gamma oscillations. In addition use of a knock-in mouse with a β3 mutation rendering this subunit insensitive to the anaesthetic etomidate revealed that β2 and β3 subunit-containing receptors could exert opposing actions on theta activity in neocortical slice cultures (Drexler *et al.*, 2005).

It appears specific synapses formed at particular locations by specific interneurones may play precise roles in shaping network activity and oscillations (McBain and Fisahn, 2001; Klausberger *et al.*, 2004; Farrant and Nusser, 2005; Somogyi, 2005). It is becoming clear that the function of $GABA_A$ receptors is not simply to provide "blanket" inhibition. If different inhibitory synapses play precise roles then modification of these synapses by phosphorylation may also differ in mechanism and functional effect. Phosphorylation dependent effects may occur through several different mechanisms with the functional effect on $GABA_A$ receptor subtypes varying according to the subtype present in order to modulate and control the function of specific synapses and therefore patterns of activity related to behaviour.

Fig 9.2:

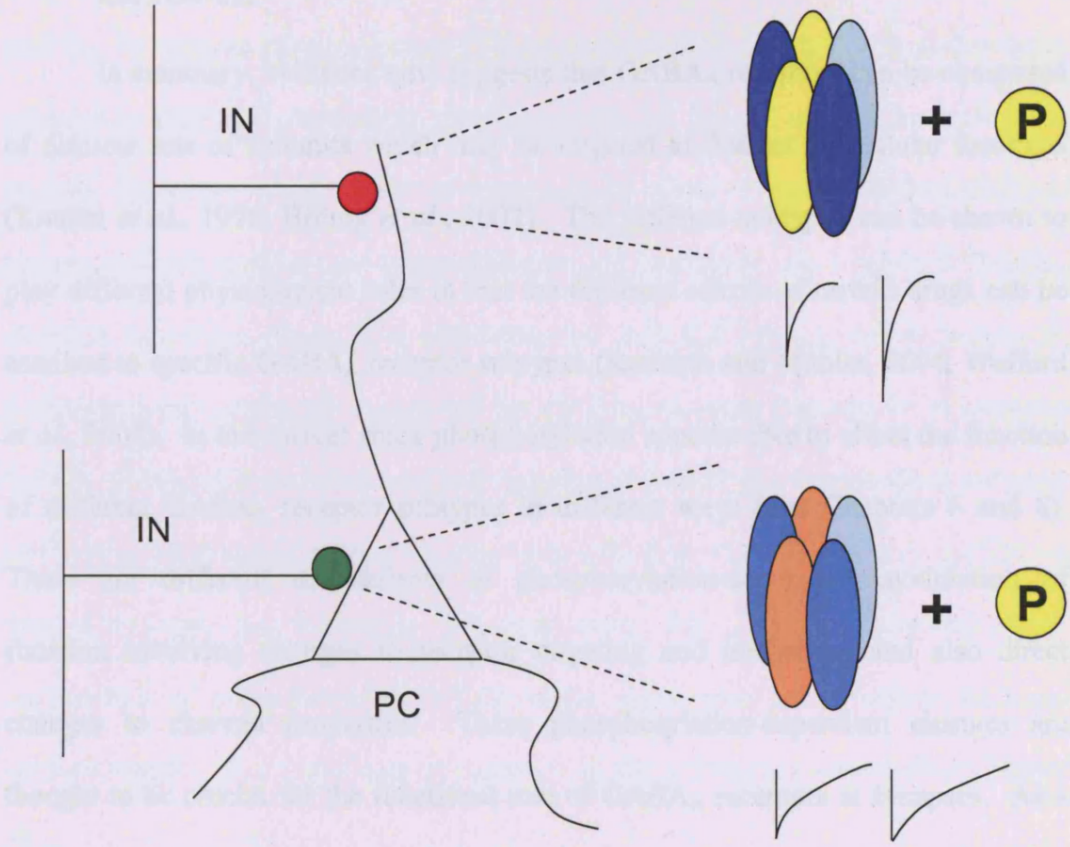

Fig 9.2: Specific $GABA_A$ receptor subtypes are targeted to discrete subcellular locations.

Different classes of interneurone have been shown to form synapses at particular locations on the target cell. In addition there is evidence that specific $GABA_A$ receptor subtypes are expressed at different synapses.

Different $GABA_A$ receptor subtypes have different kinetic properties which shape the profile of an IPSC. Phosphorylation can have a differential effect on different $GABA_A$ receptor subtypes.

Network oscillations relevant to behaviour are differentially affected by changes in activity at these different synapses.

IN = Interneurones. PC = Principal cell

9.4 Physiological role of $GABA_A$ receptor modulation by CaMK-II.

In summary, evidence now suggests that $GABA_A$ receptors can be composed of discrete sets of subunits which may be targeted to distinct subcellular locations (Koulen *et al.*, 1996; Brünig *et al.*, 2002). The different subtypes can be shown to play different physiological roles in that the different effects of certain drugs can be ascribed to specific $GABA_A$ receptor subtypes (Rudolph and Möhler, 2004; Wafford *et al.*, 2004). In the current study phosphorylation appears able to affect the function of different $GABA_A$ receptor subtypes in different ways (See Chapters 6 and 8). There are different mechanisms of phosphorylation-dependent modulation of function involving changes in receptor targeting and trafficking and also direct changes to channel properties. These phosphorylation-dependent changes are thought to be crucial for the functional role of $GABA_A$ receptors at synapses. As a result CaMK-II dependent modulation of $GABA_A$ receptors may play important roles in neurodevelopment, inhibitory plasticity and certain disease states.

9.4.1 Development.

The role of the β3 subunit in neurodevelopment was highlighted by the severe behavioural deficits of the β3 subunit knockout mouse (Homanics *et al.*, 1997; DeLorey *et al.*, 1998). In contrast, the β2 subunit knockout mouse phenotype was relatively normal given the comparable loss of receptor number found in these mice (Sur *et al.*, 2001) suggesting each β subunit plays very different physiological roles in neurodevelopment. In support of this the β3 subunit is the predominant subunit expressed early on in development (Laurie *et al.*, 1992).

The current study indicated that β3 subunit-containing receptors can be phosphorylated by CaMK-II resulting in a potentiation of function (See Chapters 6 and 7). In support of this electrophysiological evidence, biochemical studies have revealed a CaMK-II dependent phosphorylation of the β3 subunit in embryonic cortical neurones. Application of muscimol in these cultures activates $GABA_A$ receptors which, at this point in development, are excitatory. As a result of $GABA_A$ receptor activation, there is a depolarisation and Ca^{2+} influx which results in phosphorylation of the β3 subunit of the $GABA_A$ receptor that can be blocked by KN-93. Further biochemical evidence suggests CaMK-II can bind to the β subunits of $GABA_A$ receptors *in vitro* at a region within the first 30 amino acids of the TM3-4 loop (McAinsh K, Jovanovic JN and Moss SJ, personal communication). Application of pre-activated CaMK-II to the same embryonic cortical neuronal cultures resulted in a significant potentiation of the whole-cell current (See Chapter 4).

It appears from the biochemical evidence that CaMK-II dependent modulation of β3 subunit-containing receptors may play a role in strengthening $GABA_A$ receptor synaptic transmission early on in development possibly playing a role in activity dependent strengthening of inhibitory synaptic transmission. Certain Ca^{2+}-dependent forms of inhibitory plasticity have been suggested to be dependent on the stage of development of a particular cell-type and may play a role in the functional maturation of GABAergic synaptic transmission (Komatsu and Iwakiri, 1993; Komatsu, 1994; Gubellini *et al.*, 2001). It could be speculated that CaMK-II dependent potentiation of $GABA_A$ receptor function could provide a mechanism for these Ca^{2+}-dependent forms of inhibitory plasticity.

9.4.2 Homeostatic plasticity and metaplasticity.

The role of excitatory synaptic plasticity, in particular LTP, in learning and memory has been extensively studied in recent years. However, it is now becoming apparent that inhibitory synapses are also capable of long-lasting synaptic plasticity and that changes in inhibition can modulate excitatory plasticity. Activity-dependent changes in synaptic plasticity altering the threshold of excitatory LTP induction has been termed metaplasticity (Abraham and Tate, 1997; Elgersma *et al.*, 2004). For example in CA1 pyramidal neurones induction of excitatory LTP activated a calcineurin-dependent LTD of $GABA_A$ receptor-mediated synaptic transmission which was found to enhance the LTP of EPSPs (Lu *et al.*, 2000).

Inhibitory synapses have been demonstrated to change in strength in such a way as to enhance changes in excitatory transmission, but they have also been shown to adjust in opposition to changes in excitatory transmission in homeostatic forms of plasticity (Turrigiano and Nelson, 2004). Certain forms of inhibitory plasticity have been shown to be Ca^{2+}-dependent and bidirectional (Aizenman *et al.*, 1998) in a manner similar to excitatory LTP. CaMK-II has been shown to be critical in the potentiation of AMPA receptor-mediated synaptic transmission. It has also been shown that CaMK-II may play a role in inhibitory synaptic plasticity. CaMK-II modulation of $GABA_A$ receptors may represent a possible mechanism of postsynaptic inhibitory plasticity that could be involved in homeostatic or metaplasticity.

Rebound potentiation is a form of CaMK-II dependent long-term synaptic plasticity that occurs in Purkinje cells in response to a strong stimulation of excitatory inputs onto the same cell. In this way it could be thought of as a homeostatic form of plasticity acting to increase GABAergic synaptic transmission

after strong excitation. Purkinje cells are thought to express pre-dominantly β2 subunits over β3 and β1 (Persohn *et al.*, 1992). Depolarisation and Ca^{2+} entry after strong excitation activates CaMK-II which goes on to modify IPSCs by increasing their amplitude with no change in decay times (Kano *et al.*, 1992; Kano *et al.*, 1996; Kawaguchi and Hirano, 2002). This is consistent with the current studies observation of β2 subunit-containing receptor mediated IPSCs increasing in amplitude with no change in decay times (See Chapter 8). It has been shown that rebound potentiation does not occur in the β2 knockout mouse supporting the role of this subunit in this kind of plasticity (Duguid and Smart, personal communication).

9.5 The role of CaMK-II dependent modulation of $GABA_A$ receptors in disease states.

9.5.1 Angelman syndrome and Autism.

The β3 knockout mouse displays epilepsy and behavioural deficits that are similar to the phenotype of Angelman syndrome (DeLorey *et al.*, 1998) a neurodevelopmental disorder affecting humans that results from a maternal deletion/mutation of chromosome 15q11-13. In this region, there are two candidate genes thought to be potentially responsible for the neurodevelopmental deficits seen. The UBE3A gene which encodes a ubiquitin ligase, a protein involved in protein degradation (Weeber *et al.*, 2003) and the GABRB3 gene encoding the β3 subunit of the $GABA_A$ receptor. Both of these genes have been demonstrated to have reduced expression in Angelman syndrome patients (Samaco *et al.*, 2005) and disruption of both genes in mice has generated models of Angelman syndrome with many of the same behavioural deficits as that seen in humans (DeLorey *et al.*, 1998; Weeber *et al.*, 2003; Sinkkonen *et al.*, 2003).

The role of the β3 subunit early on in development suggested that Angelman syndrome was caused by dysfunction in the normal development of inhibitory synaptic transmission. However, in another mouse model of Angelman syndrome involving maternal deletion of UBE3A, a reduction in CaMK-II activity and the total amount of CaMK-II associated with the postsynaptic density has been proposed to underlie the deficits in normal function associated with Angelman syndrome (Weeber *et al.*, 2003). It is interesting to speculate that disruption of an interaction between CaMK-II and the β3 subunit of the $GABA_A$ receptor may be involved in the neurodevelopmental deficits seen in this syndrome. This implies an interaction between CaMK-II and the β3 subunit may be important in the development of inhibitory synaptic transmission.

Other conditions such as Rett syndrome, an X-linked dominant disorder caused by MECP2 (a transcriptional repressor protein) gene mutation and autism have also been demonstrated to involve a reduction in UPE3A and GABRB3 gene expression (Samaco *et al.*, 2005). If CaMK-II modulation of β3 subunit-containing $GABA_A$ receptors in the early stages of development plays a role in neurodevelopement this signalling pathway may be a potentially important target for future drug therapies.

9.5.2 Epilepsy

A number of mutations in $GABA_A$ receptor subunit genes have been identified in humans that have been associated with epilepsy (Jones-Davis and Macdonald, 2003; Macdonald *et al.*, 2004). Some of these mutations alter the properties of the channel itself and are proposed to alter the decay times of IPSCs (Bianchi *et al.*, 2002; Fisher, 2004). In addition, epilepsy has been associated with

up and/or down-regulation of specific $GABA_A$ receptor subunit mRNAs (Brooks-Kayal *et al.*, 1998; Meguro *et al.*, 2004) and increases in the number of $GABA_A$ receptors at specific synapses (Nusser *et al.*, 1998).

During periods of enhanced neural activity the intracellular Ca^{2+} load would be expected to increase and subsequently CaMK-II activity may also be increased. Observed compensatory increases in $GABA_A$ receptor function (Brooks-Kayal *et al.*, 1998; Nusser *et al.*, 1998) could be related to CaMK-II dependent modification of receptor function. The decay times of IPSCs and the number of $GABA_A$ receptors at the synapse could both potentially be modulated by CaMK-II dependent phosphorylation.

In support of this epilepsy has also been linked to a decrease in the expression of α-CaMK-II. In human tissue of patients undergoing surgery who suffer from severe seizures immunocytochemical staining revealed a decrease in the expression of α-CaMK-II and α-CaMK-II autophosphorylated at Thr^{286} (Battaglia *et al.*, 2002). In hippocampal neuronal cultures, complimentary oligonucleotides for α-CaMK-II were used to decrease the expression of this subunit. Reduction in α-CaMK-II expression resulted in increased neuronal excitability and epileptiform discharges. The magnitude of the reduced α-CaMK-II expression could be correlated with increased neuronal excitability (Churn *et al.*, 2000). In addition, in a pilocarpine-induced rat model of epilepsy it has been demonstrated that there is a decrease in the activity of cortical and hippocampal CaMK-II (Singleton *et al.*, 2005). These results are in contrast to the fact that increased α-CaMK-II activity and expression is associated with increases in excitatory synaptic transmission (Ouyang *et al.*, 1997; Colbran and Brown, 2004; Collingridge *et al.*, 2004). It could be speculated that

CaMK-II dependent effects on $GABA_A$ receptor mediated inhibitory synaptic transmission might be involved in epilepsy in a causative or compensatory manner.

9.6 CONCLUSIONS

The evidence presented in this study suggests that CaMK-II can have a different effect on the function of specific $GABA_A$ receptor subtypes and that these effects may be mediated by different mechanisms. Although CaMK-II dependent effects on $\beta 1/3$ subunit-containing receptors appear to be related to direct phosphorylation of the receptor it is not yet known if CaMK-II modulation of $\beta 2$ subunit-containing receptors occurs through direct phosphorylation or another indirect process (See Fig 9.1). The different effects on IPSCs may be tailored to form specific roles. As $\beta 2$ and $\beta 3$ subunit-containing receptors appear to cluster at specific sets of synapses with specific physiological roles, phosphorylation may alter $GABA_A$ receptor function of different subtypes according to the function they perform.

Modulation of $GABA_A$ receptors by CaMK-II may represent a mechanism of synaptic plasticity that is involved in activity-dependent formation of inhibitory synapses during development. It may also represent a way of altering the decay of IPSCs in order to alter network oscillations crucial to behaviour. Alteration in IPSC decay times appears to form an important part of development and alteration of IPSC decay times has important pathological and physiological consequences (Macdonald *et al.*, 2004; Homanics *et al.*, 2005).

Long-term changes in the amplitude of IPSCs, as seen in rebound potentiation, may have important implications in the processes of learning and memory. Understanding the role of different $GABA_A$ receptor subtypes and their modulation by phosphorylation is crucial to understanding many physiological and pathological

forms of inhibitory plasticity. In addition, understanding these processes may highlight important potential therapeutic targets for a number of diseases linked to alterations in the normal behaviour of $GABA_A$ receptors and inhibitory synapses.

Reference List:

Abraham WC, Tate WP (1997) Metaplasticity: A new vista across the field of synaptic plasticity. *Progress in Neurobiology* **52**: 303-323.

Aguayo LG, Espinoza F, Kunos G, Satin LS (1998) Effects of intracellular calcium on $GABA_A$ receptors in mouse cortical neurons. *Pflügers Archiv - European Journal of Physiology* **435:** 382-387.

Aizenman CD, Manis PB, Linden DJ (1998) Polarity of long-term synaptic gain change is related to postsynaptic spike firing at a cerebellar inhibitory synapse. *Neuron* **21:** 827-835.

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S-I, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *The Journal of Biological Chemistry* **262:** 5592-5595.

Akopian A, Gabriel R, Witkovsky P (1998) Calcium released from intracellular stores inhibits $GABA_A$-mediated currents in ganglion cells of the turtle retina. *The Journal of Neurophysiology* **80:** 1105-1115.

Alberts S, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Cell Signalling. In: *Molecular Biology of the Cell.* Garland Publishing. (Eds. Alberts S, Bray D, Lewis J, Raff M, Roberts K, Watson JD) 751

Ali DW, Salter MW (2001) NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Current Opinion in Neurobiology* **11:** 336-342.

Alix P, Grolleau F, Hue B (2002) Ca^{2+}/calmodulin-dependent protein kinase regulates GABA-activated Cl^- current in cockroach dorsal unpaired neurons. *The Journal of Neurophysiology* **87:** 2972-2982.

Angelotti TP, Macdonald RL (1993) Assembly of $GABA_A$ receptor subunits: $\alpha 1\beta 1$ and $\alpha 1\beta 1\gamma 2S$ subunits produce unique ion channels with dissimilar single-channel properties. *The Journal of Neuroscience* **13:** 1429-1440.

Angelotti TP, Uhler MD, Macdonald RL (1993a) Assembly of $GABA_A$ receptor subunits: Analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *The Journal of Neuroscience* **13:** 1418-1428.

Angelotti TP, Uhler MD, Macdonald RL (1993b) Enhancement of recombinant γ-aminobutyric acid type A receptor currents by chronic activation of cAMP-dependent protein kinase. *Molecular Pharmacology* **44:** 1202-1210.

Aradi I, Santhakumar V, Chen K, Soltesz I (2002) Postsynaptic effects of GABAergic synaptic diversity: regulation of neuronal excitability by changes in IPSC variance. *Neuropharmacology* **43:** 511-522.

Aradi I, Santhakumar V, Soltesz I (2004) Impact of heterogeneous perisomatic IPSC populations on pyramidal cell firing rates. *The Journal of Neurophysiology* **91:** 2849-2858.

Armstrong DM, Sheffield R, Mishizen-Eberz AJ, Carter TL, Rissman RA, Mizukami K, Ikonomovic MD (2003) Plasticity of glutamate and $GABA_A$ receptors in the hippocampus of patients with Alzheimer's disease. *Cellular and Molecular Neurobiology* **23:** 491-505.

Auger C, Marty A (1997) Heterogeneity of functional synaptic parameters among single release sites. *Neuron* **19:** 139-150.

Augustine GJ, Santamaria F, Tanaka K (2003) Local calcium signaling in neurons. *Neuron* **40:** 331-346.

Bacci A, Rudolph U, Huguenard JR, Prince DA (2003) Major differences in inhibitory synaptic transmission onto two neocortical interneuron subclasses. *The Journal of Neuroscience* **23:** 9664-9674.

Banks MI, Li T-B, Pearce RA (1998) The Synaptic Basis of $GABA_A$ slow. *The Journal of Neuroscience* **18:** 1305-1317.

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

Barria A, Derkach V, Soderling T (1997) Identification of the Ca^{2+}/calmodulin-dependent protein kinase II regulatory phosphorylation site in the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. *The Journal of Biological Chemistry* **272:** 32727-32730.

Bartrup JT, Newberry NR (1996) Electrophysiological consequences of ligand binding to the desensitized 5-HT_3 receptor in mammalian NG108-15 cells. *The Journal of Physiology* **490:** 679-690.

Basile AS, Lippa AS, Skolnick P (2004) Anxioselective anxiolytics: can less be more? *The European Journal of Pharmacology* **500:** 441-451.

Battaglia G, Pagliardini S, Ferrario A, Gardoni F, Tassi L, Setola V, Garbelli R, LoRusso G, Spreafico R, Di Luca M, Avanzini G (2002) α-CaMKII and NMDA-receptor subunit expression in epileptogenic cortex from human periventricular nodular heterotopia. *Epilepsia* **43:** 209-216.

Bauer EP, LeDoux JE (2004) Heterosynaptic long-term potentiation of inhibitory interneurons in the lateral amygdala. *The Journal of Neuroscience* **24:** 9507-9512.

Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H (2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. *Nature* **411:** 801-805.

Bayer KU, Harbers K, Schulman H (1998) αKAP is an anchoring protein for a novel CaM Kinase II isoform in skeletal muscle. *The EMBO Journal* **17:** 5598-5605.

Bayer KU, Schulman H (2001) Regulation of signal transduction by protein targeting: the case for CaMK-II. *Biochemical and Biophysical Research Communications* **289:** 917-923.

Beck M, Brickley K, Wilkinson HL, Sharma S, Smith M, Chazot PL, Pollard S, Stephenson FA (2002) Identification, molecular cloning, and characterization of a novel $GABA_A$ receptor-associated protein, GRIF-1. *The Journal of Biological Chemistry* **277:** 30079-30090.

Beczkowska IW, Gracy KN, Pickel VM, Inturrisi CE (1997) Detection of delta opioid receptor and N-methyl-D-aspartate receptor-like immunoreactivity in retinoic acid-differentiated neuroblastoma x glioma (NG108-15) cells. *Journal of Neuroscience Research* **47:** 83-89.

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ (2001) $GABA_A$ receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nature Neuroscience* **4:** 908-916.

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

Benke D, Fritschy JM, Trzeciak A, Bannwarth W, Mohler H (1994) Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *The Journal of Biological Chemistry* **269:** 27100-27107.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. *Nature* **393:** 793-797.

Berridge MJ, Bootman MD, Lipp P (1998) Calcium-a life and death signal. *Nature* **395:** 645-648.

Bialojan C, Takai A (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *The Biochemical Journal* **256:** 283-290.

Bianchi MT, Song L, Zhang H, Macdonald RL (2002) Two different mechanisms of disinhibition produced by $GABA_A$ receptor mutations linked to epilepsy in humans. *The Journal of Neuroscience* **22:** 5321-5327.

Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris RA (2003) $GABA_A$ receptor $\alpha 1$ and $\beta 2$ subunit null mutant mice: behavioral responses to ethanol. *The Journal of Pharmacology and Experimental Therapeutics* **305:** 854-863.

Boddeke HWGM, Meigel I, Boeijinga P, Arbuckle J, Docherty RJ (1996) Modulation by calcineurin of $5\text{-}HT_3$ receptor function in NG108-15 neuroblastoma x glioma cells. *The British Journal of Pharmacology* **118:** 1836-1840.

Boileau AJ, Li T, Benkwitz C, Czajkowski C, Pearce RA (2003) Effects of $\gamma 2S$ subunit incorporation on $GABA_A$ receptor macroscopic kinetics. *Neuropharmacology* **44:** 1003-1012.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ, Connolly CN (2003) $GABA_A$ receptor composition is determined by distinct assembly signals within α and β subunits. *The Journal of Biological Chemistry* **278:** 4747-4755.

Borodinsky LN, Coso OA, Fiszman ML (2002) Contribution of Ca^{2+}/calmodulin-dependent protein kinase II and mitogen-activated protein kinase kinase to neural activity-induced neurite outgrowth and survival of cerebellar granule cells. *The Journal of Neurochemistry* **80:** 1062-1070.

Bowden SEH, Selyanko A, Robbins J (1999) The role of ryanodine receptors in the cyclic ADP ribose modulation of the M-like current in rodent m1 muscarinic receptor-transformed NG108-15 cells. *The Journal of Physiology* **519:** 23-34.

Bowers BJ, Collins AC, Tritto T, Wehner JM (2000) Mice lacking PKCγ exhibit decreased anxiety. *Behavior Genetics* **30:** 111-121.

Boxall AR (2000) GABAergic mIPSCs in rat cerebellar Purkinje cells are modulated by TrkB and mGluR1-mediated stimulation of Src. *The Journal of Physiology* **524:** 677-684.

Boxall AR, Lancaster B (1998) Tyrosine kinases and synaptic transmission. *The European Journal of Neuroscience* **10:** 2-7.

Bradshaw JM, Kubota Y, Meyer T, Schulman H (2003) An ultrasensitive Ca^{2+}/calmodulin-dependent protein kinase II-protein phosphatase 1 switch facilitates specificity in postsynaptic calcium signaling. *Proceedings of the National Academy of Sciences, USA* **100:** 10512-10517.

Brandon NJ, Delmas P, Hill J, Smart TG, Moss SJ (2001) Constitutive tyrosine phosphorylation of the $GABA_A$ receptor γ2 subunit in rat brain. *Neuropharmacology* **41:** 745-752.

Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG, Moss SJ (2000) $GABA_A$ receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. *The Journal of Biological Chemistry* **275:** 38856-38862.

Brandon NJ, Jovanovic JN, Colledge M, Kittler JT, Brandon JM, Scott JD, Moss SJ (2003) A-kinase anchoring protein 79/150 facilitates the phosphorylation of $GABA_A$ receptors by cAMP-dependent protein kinase via selective interaction with receptor β subunits. *Molecular and Cellular Neuroscience* **22:** 87-97.

Brandon NJ, Jovanovic JN, Moss SJ (2002a) Multiple roles of protein kinases in the modulation of $GABA_A$ receptor function and cell surface expression. *Pharmacology and Therapeutics* **94:** 113-122.

Brandon NJ, Jovanovic JN, Smart TG, Moss SJ (2002b) Receptor for activated C kinase-1 facilitates protein kinase C-dependent phosphorylation and functional modulation of $GABA_A$ receptors with the activation of G-protein-coupled receptors. *The Journal of Neuroscience* **22:** 6353-6361.

Brandon NJ, Uren JM, Kittler JT, Wang H, Olsen R, Parker PJ, Moss SJ (1999) Subunit-specific association of protein kinase C and the receptor for activated C kinase with GABA type A receptors. *The Journal of Neuroscience* **19:** 9228-9234.

Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* **40:** 361-379.

Brickey DA, Colbran RJ, Fong Y-L, Soderling TR (1990) Expression and characterization of the α-subunit of CaMK-II using the baculovirus expression system. *Biochemical and Biophysical Research Communications* **173:** 578-584.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. *The Journal of Physiology* **497:** 753-759.

Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic $GABA_A$ receptors suggests differential targeting of receptor subtypes. *The Journal of Neuroscience* **19:** 2960-2973.

Brocke L, Chiang LW, Wagner PD, Sculman H (1999) Functional implications of the subunit composition of neuronal CaM Kinase II. *The Journal of Biological Chemistry* **274:** 22713-22722.

Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Coulter DA (1998) Selective changes in single cell $GABA_A$ receptor subunit expression and function in temporal lobe epilepsy. *Nature Medicine* **4:** 1166-1172.

Brown GP, Blitzer RD, Connor JH, Wong T, Shenolikar S, Iyengar R, Landau EM (2000) Long-term potentiation induced by θ frequency stimulation is regulated by a protein phosphatase-1-operated gate. *The Journal of Neuroscience* **20:** 7880-7887.

Brünig I, Scotti E, Sidler C, Fritschy J-M (2002) Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons *in vitro*. *The Journal of Comparative Neurology* **443:** 43-55.

Buhr A, Bianchi MT, Baur R, Courtet P, Pignay V, Boulenger JP, Gallati S, Hinkle DJ, Macdonald RL, Sigel E (2002) Functional characterization of the new human $GABA_A$ receptor mutation β3(R192H). *Human Genetics* **111:** 154-160.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990) *In Situ* hybridization histochemistry of Ca^{2+}/calmodulin-dependent protein kinase in developing rat brain. *The Journal of Neuroscience* **10:** 1788-1798.

Buzas B, Rosenberger J, Cox BM (1998) Ca^{2+}/calmodulin-dependent transcriptional activation of δ-opioid receptor gene expression induced by membrane depolarization in NG108-15 cells. *The Journal of Neurochemistry* **70:** 105-112.

Caillard O, Ben-Ari Y, Gaiarsa J-L (1999) Long-term potentiation of GABAergic synaptic transmission in neonatal rat hippocampus. *The Journal of Physiology* **518:** 109-119.

Chan RJ, McBride AW, Crabb DW (1994) Seven transmembrane domain receptor subtypes identified in NG108-15 cells by reverse transcription-polymerase chain reaction. *Biochemical and Biophysical Research Communications* **205:** 1311-1317.

Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant $GABA_A$ receptor. *The Journal of Neuroscience* **16:** 5415-5424.

Chapell R, Bueno OF, Alvarez-Hernandez X, Robinson LC, Leidenheimer NJ (1998) Activation of protein kinase C induces γ-aminobutyric acid type A receptor internalization in *Xenopus oocytes*. *The Journal of Biological Chemistry* **273:** 32595-32601.

Chen L, Wang H, Vicini S, Olsen RW (2000) The γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein (GABARAP) promotes $GABA_A$ receptor clustering and modulates the channel kinetics. *Proceedings of the National Academy of Sciences, USA* **97:** 11557-11562.

Chen N, Furuya S, Doi H, Hashimoto Y, Kudo Y, Higashi H (2003) Ganglioside/calmodulin kinase II signal inducing cdc42-mediated neuronal actin reorganization. *Neurosicence* **120:** 163-76.

Chen QX, Stelzer A, Kay AR, Wong RK (1990) $GABA_A$ receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurones. *The Journal of Physiology* **420:** 207-221.

Chen X-L, Zhong Z-G, Yokoyama S, Bark C, Meister B, Berggren P-O, Roder J, Higashida H, Jeromin A (2001) Over-expression of rat neuronal calcium sensor-1 in rodent NG108-15 cells enhances synapse formation and transmission. *The Journal of Physiology* **532:** 649-659.

Chevaleyre V, Castillo PE (2004) Endocannabinoid-mediated metaplasticity in the hippocampus. *Neuron* **43:** 871-881.

Christian CN, Nelson PG, Peacock J, Nirenberg M (1977) Synapse formation between two clonal cell lines. *Science* **196:** 995-998.

Churn SB, DeLorenzo RJ (1998) Modulation of GABAergic receptor binding by activation of calcium and calmodulin-dependent kinase II membrane phosphorylation. *Brain Research* **809:** 68-76.

Churn SB, Rana A, Lee K, Parsons JT, De Blas A, DeLorenzo RJ (2002) Calcium/calmodulin-dependent kinase II phosphorylation of the $GABA_A$ receptor α1 subunit modulates benzodiazepine binding. *Journal of Neurochemistry* **82:** 1065-1076.

Churn SB, Sombati S, Jakoi ER, Severt L, DeLorenzo RJ (2000) Inhibition of calcium/calmodulin kinase II alpha subunit expression results in epileptiform activity in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences, USA* **97:** 5604-5609.

Cirone J, Rosahl TW, Reynolds DS, Newman RJ, O'Meara GF, Hutson PH, Wafford KA (2004) γ-Aminobutyric acid type A receptor β2 subunit mediates the hypothermic effect of etomidate in mice. *Anesthesiology* **100:** 1438-1445.

Colbran RJ, Brown AM (2004) Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. *Current Opinion in Neurobiology* **14:** 318-327.

Colbran RJ, Fong Y-L, Schworer CM, Soderling TR (1988) Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca^{2+}/calmodulin-dependent protein kinase II. *The Journal of Biological Chemistry* **263:** 18145-18151.

Colbran RJ (2004a) Protein phosphatases and calcium/calmodulin-dependent protein kinase II-dependent synaptic plasticity. *The Journal of Neuroscience* **24:** 8404-8409.

Colbran RJ (2004b) Targeting of calcium/calmodulin-dependent protein kinase II. *The Biochemical Journal* **378:** 1-16.

Collingridge GL, Isaac JTR, Wang YT (2004) Receptor trafficking and synaptic plasticity. *Nature Reviews Neuroscience* **5:** 952-962.

Connolly CN, Kittler JT, Thomas P, Uren JM, Brandon NJ, Smart TG, Moss SJ (1999) Cell surface stability of γ-aminobutyric acid type A receptors. Dependence on protein kinase C activity and subunit composition. *The Journal of Biological Chemistry* **274:** 36565-36572.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ (1996a) Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. *The Journal of Biological Chemistry* **271:** 89-96.

Connolly CN, Wafford KA (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochemical Society Transactions* **32:** 529-534.

Connolly CN, Wooltorton JRA, Smart TG, Moss SJ (1996b) Subcellular localization of γ-aminobutyric acid type A receptors is determined by receptor β subunits. *Proceedings of the National Academy of Sciences, USA* **93:** 9899-9904.

Cormier RJ, Greenwood AC, Connor JA (2001) Bidirectional synaptic plasticity correlated with the magnitude of dendritic calcium transients above a threshold. *The Journal of Neurophysiology* **85:** 399-406.

Cromer BA, Morton CJ, Parker MW (2002) Anxiety over $GABA_A$ receptor structure relieved by AChBP. *Trends in Biochemical Sciences* **27:** 280-287.

Daniels MP, Hamprecht B (1974) The ultrastructure of neuroblastoma glioma somatic cell hybrids. Expression of neuronal characteristics stimulated by dibutyryl adenosine 3',5' cyclic monophosphate. *The Journal of Cell Biology* **63:** 691-699.

Davies PA, Hoffmann EB, Carlisle HJ, Tyndale RF, Hales TG (2000) The influence of an endogenous β3 subunit on recombinant $GABA_A$ receptor assembly and pharmacology in WSS-1 cells and transiently transfected HEK293 cells. *Neuropharmacology* **39:** 611-620.

De Koninck P, Schulman H (1998) Sensitivity of CaM Kinase II to the frequency of Ca^{2+} oscillations. *Science* **279:** 227-230.

De Koninck Y, Mody I (1994) Noise analysis of miniature IPSCs in adult rat brain slices: Properties and modulation of synaptic $GABA_A$ receptor channels. *The Journal of Neurophysiology* **71:** 1318-1335.

Della Rocca GJ, Biesen TV, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. *The Journal of Biological Chemistry* **272:** 19125-19132.

DeLorey TM, Handforth A, Anagnostaras SG, Homanics GE, Minassian BA, Asatourian A, Fanselow MS, Delgado-Escueta A, Ellison GD, Olsen RW (1998) Mice lacking the $\beta 3$ subunit of the $GABA_A$ receptor have the epilepsy phenotype and many of the behavioural characteristics of Angelman syndrome. *The Journal of Neuroscience* **18:** 8505-8514.

Derkach V, Barria A, Soderling TR (1999) Ca^{2+}/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proceedings of the National Academy of Sciences, USA* **96:** 3269-3274.

Dhavan R, Greer PL, Morabito MA, Orlando LR, Tsai L-H (2002) The cyclin-dependent kinase 5 activators p35 and p39 interact with the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and α-actinin-1 in a calcium-dependent manner. *The Journal of Neuroscience* **22:** 7879-7891.

Di Cristo GD, Wu C, Chattopadhyaya B, Ango F, Knott G, Welker E, Svoboda K, Huang ZJ (2004) Subcellular domain-restricted GABAergic innervation in primary visual cortex in the absence of sensory and thalamic inputs. *Nature Neuroscience* **7:** 1184-1186

Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J (1996) A role for PYK2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383:** 547-550.

Docherty RJ, Robbins J, Brown DA (1991) NG108-15 neuroblastoma x glioma hybrid cell line as a model system. In: *Cellular Neurobiology: A Practical Approach* Oxford University Press. (Eds. Chad J and Wheal HV) 75-95.

Dosemeci A, Reese TS, Peterson J, Tao-Cheng J-H (2000) A novel particulate form of Ca^{2+}/calmodulin-dependent protein kinase II in neurons. *The Journal of Neuroscience* **20:** 3076-3084.

Drexler B, Roether CL, Jurd R, Rudolph U, Antkowiak B (2005) Opposing actions of etomidate on cortical theta oscillations are mediated by different γ-aminobutyric acid type A receptor subtypes. *Anesthesiology* **102:** 346-352.

Duguid IC, Smart TG (2004) Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nature Neuroscience* **7:** 525-533.

Dunne EL, Moss SJ, Smart TG (1998) Inhibition of $GABA_A$ receptor function by tyrosine kinase inhibitors and their inactive analogues. *Molecular and Cellular Neurosciences* **12:** 300-310.

Easter A, Spruce AE (2002) Recombinant $GABA_B$ receptors formed from $GABA_{B1}$ and $GABA_{B2}$ subunits selectively inhibit N-type Ca^{2+} channels in NG108-15 cells. *European Journal of Pharmacology* **440:** 17-25.

Ehlers MD, Zhang S, Bernhardt JP, Huganir RL (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84:** 745-755.

Ehrlich I, Malinow R (2004) Postsynaptic density 95 controls AMPA receptor incorporation during long-term potentiation and experience-driven synaptic plasticity. *The Journal of Neuroscience* **24:** 916-927.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMK-II controls PSD association, plasticity, and learning. *Neuron* **36:** 493-505.

Elgersma Y, Sweatt JD, Giese PK (2004) Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase function in plasticity and cognition. *The Journal of Neuroscience* **24:** 8410-8415.

Enslen H, Tokumitsu H, Stork PJS, Davis RJ, Soderling TR (1996) Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. *Proceedings of the National Academy of Sciences, USA* **93:** 10803-10808.

Essrich C, Lorez M, Benson JA, Fritschy J-M, Lüscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the $\gamma 2$ subunit and gephyrin. *Nature Neuroscience* **1:** 563-571.

Fagiolini M, Fritschy J-M, LöW K, Möhler H, Rudolph U, Hensch TK (2004) Specific $GABA_A$ circuits for visual cortical plasticity. *Science* **303:** 1681-1683.

Farrant M, Brickley SG (2003) Properties of $GABA_A$ receptor-mediated transmission at newly formed Golgi-granule cell synapses in the cerebellum. *Neuropharmacology* **44:** 181-189.

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

Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *The Journal of Biological Chemistry* **274:** 10100-10104.

Fink CC, Meyer T (2002) Molecular mechanisms of CaMK-II activation in neuronal plasticity. *Current Opinion in Neurobiology* **12:** 293-299.

Fink CC, Bayer KU, Myers JW, Ferrell JE, Jr., Schulman H, Meyer T (2003) Selective regulation of neurite extension and synapse formation by the β but not the α isoform of CaMKII. *Neuron* **39:** 283-297.

Fisher JL (2004) A mutation in the $GABA_A$ receptor α1 subunit linked to human epilepsy affects channel gating properties. *Neuropharmacology* **46:** 629-637.

Fong Y-L, Taylor WL, Means AR, Soderling TR (1989) Studies of the regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase II: mutation of threonine 286 to alanine and aspartate. *The Journal of Biological Chemistry* **264:** 16759-16763.

Franks KM, Sejnowski TJ (2002) Complexity of calcium signaling in synaptic spines. *BioEssays* **24:** 1130-1144.

Fukuda K, Higashida H, Kubo T, Maeda A, Akiba I, Bujo H, Mishina M, Numa S (1988) Selective coupling with K^+ currents of muscarinic acetylcholine receptor subtypes in NG108-15 cells. *Nature* **335:** 355-358.

Fukunaga K, Kobayashi T, Tamura S, Miyamoto E (1993) Dephosphorylation of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II by protein phosphatase 2C. *The Journal of Biological Chemistry* **268:** 133-137.

Fukunaga K, Rich DP, Soderling TR (1989) Generation of the Ca^{2+}-independent form of $Ca^{2+/}$calmodulin-dependent protein kinase II in cerebellar granule cells. The *Journal of Biological Chemistry* **264:** 21830-21836.

Furuya S, Makino A, Hirabayashi Y (1998) An improved method for culturing cerebellar Purkinje cells with differentiated dendrites under a mixed monolayer setting. *Brain Research Protocols* **3:** 192-198.

Gabbiani F, Midtgaard J, Knöpfel T (1994) Synaptic integration in a model of cerebellar granule cells. *The Journal of Neurophysiology* **72:** 999-1009.

Gaiarsa J-L, Caillard O, Ben-Ari Y (2002) Long-term plasticity at GABAergic and glycinergic synapses: mechanisms and functional significance. *Trends in Neurosciences* **25:** 564-570.

Galli C, Meucci O, Scorziello A, Werge TM, Calissano P, Schettini G (1995) Apoptosis in cerebellar granule cells is blocked by high KCl, forskolin, and IGF-1 through distinct mechanisms of action; the involvement of intracellular calcium and RNA synthesis. *The Journal of Neuroscience* **15:** 1172-1179.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr^{286} of the α-calcium-calmodulin kinase II in LTP and learning. *Science* **279:** 870-873.

Gingrich JR, Pelkey KA, Fam SR, Huang Y, Petralia RS, Wenthold RJ, Salter MW (2004) Unique domain anchoring of Src to synaptic NMDA receptors via the mitochondrial NADH dehydrogenase subunit 2. *Proceedings of the National Academy of Sciences, USA* **101:** 6237-6242.

Gingrich KJ, Roberts WA, Kass RS (1995) Dependence of the $GABA_A$ receptor gating kinetics on the alpha-subunit isoform: implications for structure-function relations and synaptic transmission. *The Journal of Physiology* **489:** 529-543.

Ginnan R, Pfleiderer PJ, Pumiglia K, Singer HA (2004) PKC-δ and CaMKII-δ2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. *American Journal of Physiology - Cell Physiology* **286:** C1281-C1289.

Ginnan R, Singer HA (2002) CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *American Journal of Physiology - Cell Physiology* **282:** C754-C761.

Gleason MR, Higashijima S, Dallman J, Liu K, Mandel G, Fetcho JR (2003) Translocation of CaM kinase II to synaptic sites *in vivo*. *Nature Neuroscience* **6:** 217-218.

Gnegy ME (2000) Ca^{2+}/calmodulin signaling in NMDA-induced synaptic plasticity. *Critical Reviews in Neurobiology* **14:** 91-129.

Goldstein PA, Elsen FP, Ying S-W, Ferguson C, Homanics GE, Harrison NL (2002) Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the $GABA_A$ receptor α1 subunit. *The Journal of Neurophysiology* **88:** 3208-3217.

Goshima Y, Ohsako S, Yamauchi T (1993) Overexpression of Ca^{2+}/calmodulin-dependent protein kinase II in Neuro2a and NG108-15 neuroblastoma cell lines promotes neurite outgrowth and growth cone motility. *The Journal of Neuroscience* **13:** 559-567.

Graham FL, Smiley J, Russell WC, Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *The Journal of General Virology* **36:** 59-74.

Griffith LC, Lu CS, Sun XX, (2003) CaMK-II, an enzyme on the move: regulation of temporospatial localization. *Molecular Interventions* **3:** 386-403.

Gubellini P, Ben-Ari Y, Gaiarsa JL (2001) Activity- and age-dependent GABAergic synaptic plasticity in the developing rat hippocampus. *The European Journal of Neuroscience* **14:** 1937-1946.

Guo J, Meng F, Fu X, Song B, Yan X, Zhang G (2004) N-methyl-D-aspartate receptor and L-type voltage-gated Ca^{2+} channel activation mediate proline-rich tyrosine kinase 2 phosphorylation during cerebral ischemia in rats. *Neuroscience Letters* **355:** 177-180.

Haas KF, Macdonald RL (1999) $GABA_A$ receptor subunit $\gamma 2$ and δ subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. *The Journal of Physiology* **514:** 27-45.

Hack N, Hidaka H, Wakefield MJ, Balazs R (2004) Promotion of granule cell survival by high K^+ or excitatory amino acid treatment and Ca^{2+}/calmodulin-dependent protein kinase activity. *Neuroscience* **57:** 9-20.

Hájos N, Nusser Z, Rancz EA, Freund TF, Mody I (2000) Cell type- and synapse-specific variability in synaptic $GABA_A$ receptor occupancy. *The European Journal of Neuroscience* **12:** 810-818.

Hamann M, Rossi D, Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* **33:** 625-633.

Han H-Q, Nichols RA, Rubin MR, Bähler M, Greengard P (1991) Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. *Nature* **349:** 697-700.

Hanson PI, Meyer T, Stryer L, Schulman H (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron* **12:** 943-956.

Hanson PI, Schulman H (1992) Inhibitory autophosphorylation of multifunctional Ca^{2+}/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. *The Journal of Biological Chemistry* **267:** 17216-17224.

Hashimoto Y, Schworer CM, Colbran RJ, Soderling TR (1987) Autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II. Effects on total and Ca^{2+}-independent activities and kinetic parameters. *The Journal of Biological Chemistry* **262:** 8051-8055.

Hata Y, Butz S, Sudhof TC (1996) CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *The Journal of Neuroscience* **16:** 2488-2494.

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287:** 2262-2267.

Heidinger V, Manzerra P, Wang XQ, Strasser U, Yu S-P, Choi DW, Behrens MM (2002) Metabotropic glutamate receptor 1 - induced up-regulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. *The Journal of Neuroscience* **22:** 5452-5461.

Herring D, Huang R-Q, Singh M, Dillon GH, Leidenheimer NJ (2005) PKC modulation of $GABA_A$ receptor endocytosis and function is inhibited by mutation of a dileucine motif within the receptor $\beta 2$ subunit. *Neuropharmacology* **48:** 181-194.

Herring D, Huang R, Singh M, Robinson LC, Dillon GH, Leidenheimer NJ (2003) Constitutive $GABA_A$ receptor endocytosis is dynamin-mediated and dependent on a dileucine AP2 adaptin-binding motif within the $\beta 2$ subunit of the receptor. *The Journal of Biological Chemistry* **278:** 24046-24052.

Hinkle DJ, Macdonald RL (2003) β subunit phosphorylation selectively increases fast desensitization and prolongs deactivation of $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ $GABA_A$ receptor currents. *The Journal of Neuroscience* **23:** 11698-11710.

Hoelz A, Nairn AC, Kuriyan J (2003) Crystal structure of a tetradecameric assembly of the association domain of Ca^{2+}/calmodulin-dependent kinase II. *Molecular Cell* **11:** 1241-1251.

Hollrigel GS, Soltesz I (1997) Slow kinetics of miniature IPSCs during early postnatal development in granule cells of the dentate gyrus. The *Journal of Neuroscience* **17:** 5119-5128.

Homanics GE, DeLorey TM, Firestone LL, Quinlan JJ, Handforth A, Harrison NL, Krasowski MD, Rick CE, Korpi ER, Makela R, Brilliant MH, Hagiwara N, Ferguson C, Snyder K, Olsen RW (1997) Mice devoid of γ-aminobutyrate type A receptor $\beta 3$ subunit have epilepsy, cleft palate, and hypersensitive behavior. *Proceedings of the National Academy of Sciences, USA* **94:** 4143-4148.

Homanics GE, Elsen FP, Ying S-W, Jenkins A, Ferguson C, Sloat B, Yuditskaya S, Goldstein PA, Kralic JE, Morrow AL, Harrison NL (2005) A gain-of-function mutation in the $GABA_A$ receptor produces synaptic and behavioral abnormalities in the mouse. *Genes, Brain and Behavior* **4:** 10-19.

Huang C-C, Hsu K-S (1999) Protein tyrosine kinase is required for the induction of long-term potentiation in the rat hippocampus. *The Journal of Physiology* **520:** 783-796.

Huang R-Q, Dillon GH (1998) Maintenance of recombinant type A γ-aminobutyric acid receptor function: role of protein tyosine phosphorylation and calcineurin. *The Journal of Pharmacology and Experimental Therapeutics* **286:** 243-255.

Huang Y-Q, Lu W-Y, Ali DW, Pelkey KA, Pitcher GM, Lu YM, Aoto H, Roder JC, Sasaki T, Salter MW, MacDonald JF (2001) CAKβ/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* **29:** 485-496.

Hudmon A, Schulman H (2002) Neuronal CaMK-II: the role of structure and autoregulation in cellular function. *Annual Review of Biochemistry* **71:** 473-510.

Hutcheon B, Morley P, Poulter MO (2000) Developmental change in $GABA_A$ receptor desensitization kinetics and its role in synapse function in rat cortical neurons. *The Journal of Physiology* **522:** 3-17.

Inoue M, Oomura Y, Yakushiji T, Akaike N (1986) Intracellular calcium ions decrease the affinity of the GABA receptor. *Nature* **324:** 156-158.

Ishizawa Y, Furuya K, Yamagishi S, Dohi S (1997) Non-GABAergic effects of midazolam, diazepam and flumazenil on voltage-dependent ion currents in NG108-15 cells. *Neuropharmacology and Neurotoxicology* **8:** 2635-2638.

Ivankovic-Dikic I, Grönroos E, Blaukat A, Barth B-U, Dikic I (2000) Pyk2 and FAK regulate neurite out-growth induced by growth factors and integrins. *Nature Cell Biology* **2:** 574-581.

Ives JH, Drewery DL, Thompson CL (2002a) Neuronal activity and its influence on developmentally regulated $GABA_A$ receptor expression in cultured mouse cerebellar granule cells. *Neuropharmacology* **43:** 715-725.

Ives JH, Drewery DL, Thompson CL (2002b) Differential cell surface expression of $GABA_A$ receptor $\alpha 1$, $\alpha 6$, $\beta 2$ and $\beta 3$ subunits in cultured mouse cerebellar granule cells influence of cAMP-activated signalling. *The Journal of Neurochemistry* **80:** 317-327.

Jenson ML, Timmermann DB, Johansen TH, Schousboe A, Varming T, Ahring PK (2002) The β subunit determines the ion selectivity of the $GABA_A$ receptor. *The Journal of Biological Chemistry* **277:** 41438-41447.

Jones MV, Westbrook GL (1996) The impact of receptor desensitization on fast synaptic transmission. *Trends in Neurosciences* **19:** 96-101.

Jones MV, Westbrook GL (1997) Shaping of IPSCs by endogenous calcineurin activity. *The Journal of Neuroscience* **17:** 7626-7633.

Jones-Davis DM, Macdonald RL (2003) $GABA_A$ receptor function and pharmacology in epilepsy and status epilepticus. *Current Opinion in Pharmacology* **3:** 12-18.

Jovanovic JN, Thomas P, Kittler JT, Smart TG, Moss SJ (2004) Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating $GABA_A$ receptor phosphorylation, activity, and cell-surface stability. *The Journal of Neuroscience* **24:** 522-530.

Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC (2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nature Neuroscience* **7:** 244-253.

Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, Rudolph U (2003) General anesthetic actions *in vivo* strongly attenuated by a point mutation in the $GABA_A$ receptor $\beta 3$ subunit. *The FASEB Journal* **17:** 250-252.

Jüttner R, Meler J, Grantyn R (2001) Slow IPSC kinetics, low levels of α1 subunit expression and paired-pulse depression are distinct properties of neonatal inhibitory GABAergic synaptic connections in the mouse superior colliculus. *The European Journal of Neuroscience* **13:** 2088-2098.

Kanematsu T, Jang I-S, Yamaguchi T, Nagahama H, Yoshimura K, Hidaka K, Matsuda M, Takeuchi H, Misumi Y, Nakayama K, Yamamoto T, Akaike N, Hirata M, Nakayama K-I (2002) Role of the PLC-related, catalytically inactive protein p130 in $GABA_A$ receptor function. *The EMBO Journal* **21:** 1004-1011.

Kano M, Kano M, Fukunaga K, Konnerth A (1996) Ca^{2+}-induced rebound potentiation of γ-aminobutyric acid-mediated currents requires activation of Ca^{2+}/calmodulin-dependent kinase II. *Proceedings of the National Academy of Sciences, USA* **93:** 13351-13356.

Kano M, Konnerth A (1992) Potentiation of GABA-mediated currents by cAMP-dependent protein kinase. *NeuroReport* **3:** 563-566.

Kano M, Rexhausen U, Dreessen J, Konnerth A (1992) Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature* **356:** 601-604.

Kao C-Q, Goforth PB, Ellis EE, Satin LS (2004) Potentiation of $GABA_A$ currents after mechanical injury of cortical neurons. *Journal of Neurotrauma* **21:** 259-270.

Kapur J, Macdonald RL (1996) Cyclic AMP-dependent protein kinase enhances hippocampal dentate granule cell $GABA_A$ receptor currents. *The Journal of Neurophysiology* **76:** 2626-2633.

Kardos J (1999) Recent advances in GABA research. *Neurochemistry International* **34:** 353-358.

Kasahara J, Fukunaga K, Miyamoto E (1999) Differential effects of a calcineurin inhibitor on glutamate-induced phosphorylation of Ca^{2+}/calmodulin-dependent protein kinases in cultured rat hippocampal neurons. *The Journal of Biological Chemistry* **274:** 9061-9067.

Kawaguchi S-Y, Hirano T (2002) Signaling cascade regulating long-term potentiation of $GABA_A$ receptor responsiveness in cerebellar Purkinje neurons. *The Journal of Neuroscience* **22:** 3969-3976.

Kellenberger S, Malherbe P, Sigel E (1992) Function of the $\alpha 1\beta 2\gamma 2S$ γ-aminobutyric acid type A receptor is modulated by protein kinase C via multiple phosphorylation sites. *The Journal of Biological Chemistry* **267:** 25660-25663.

Kilman V, van Rossum MCW, Turrigiano GG (2002) Activity deprivation reduces miniature IPSC amplitude by decreasing the number of postsynaptic $GABA_A$ receptors clustered at neocortical synapses. *The Journal of Neuroscience* **22:** 1328-1337.

Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, Moss SJ (2000) Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *The Journal of Neuroscience* **20:**7972-7977.

Kittler JT, Moss SJ (2003) Modulation of $GABA_A$ receptor activity by phosphorylation and receptor trafficking: Implications for the efficacy of synaptic inhibition. *Current Opinion in Neurobiology* **13:** 341-347.

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

Klausberger T, Márton LF, Baude A, Roberts JDB, Magill PJ, Somogyi P (2004) Spike timing of dendrite-targeting bistratified cells during hippocampal network oscillations *in vivo*. *Nature Neuroscience* **7:** 41-47.

Klee WA, Nirenberg M (1974) A neuroblastoma x glioma hybrid cell line with morphine receptors. *Proceedings of the National Academy of Sciences, USA* **71:** 3474-3477.

Kneussel M (2002) Dynamic regulation of $GABA_A$ receptors at synaptic sites. *Brain Research Reviews* **39:** 74-83.

Kneussel M, Brandstätter JH, Gasnier B, Feng G, Sanes JR, Betz H (2001) Gephyrin-independent clustering of postsynaptic $GABA_A$ receptor subtypes. *Molecular and Cellular Neuroscience* **17:** 973-982.

Kneussel M, Brandstätter JH, Laube B, Stahl S, Müller U, Betz H (1999) Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. *The Journal of Neuroscience* **19:** 9289-9297.

Kolodziej SJ, Hudmon A, Waxham MN, Stoops JK (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIα and truncated CaM kinase IIα reveal a unique organization for its structural core and functional domains. *The Journal of Biological Chemistry* **275:** 14354-14359.

Komatsu Y (1994) Age-dependent long-term potentiation of inhibitory synaptic transmission in rat visual cortex. *The Journal of Neuroscience* **14:** 6488-6499.

Komatsu Y, Iwakiri M (1993) Long-term modification of inhibitory synaptic transmission in developing visual cortex. *NeuroReport* **4:** 907-910.

Korpi ER, Gründer G, Lüddens H (2002) Drug interactions at $GABA_A$ receptors. *Progress in Neurobiology* **67:** 113-159.

Koulen P, Sassoè-Pognetto M, Grünert U, Wässle H (1996) Selective clustering of $GABA_A$ and glycine receptors in the mammalian retina. *The Journal of Neuroscience* **16:** 2127-2140.

Krishek BJ, Moss SJ, Smart TG (1996) Homomeric $\beta 1$ γ-aminobutyric acid A receptor-ion channels: evaluation of pharmacological and physiological properties. *Molecular Pharmacology* **49:** 494-504.

Krishek BJ, Xie X, Blackstone C, Huganir RL, Moss SJ, Smart TG (1994) Regulation of $GABA_A$ receptor function by Protein Kinase C phosphorylation. *Neuron* **12:** 1081-1095.

Kurz J, Sheets D, Parsons JT, Rana A, DeLorenzo RJ, Churn SB (2001) A significant increase in both basal and maximal calcineurin activity in the rat pilocarpine model of status epilepticus. *The Journal of Neurochemistry* **78:** 304-315.

Laposky AD, Homanics GE, Basile A, Mendelson WB (2001) Deletion of the $GABA_A$ receptor $\beta 3$ subunit eliminates the hypnotic actions of oleamide in mice. *NeuroReport* **12:** 4143-4147.

Laschet JJ, Minier F, Kurcewicz I, Bureau MH, Trottier S, Jeanneteau F, Griffon N, Samyn B, Beeuman JV, Louvel J, Sokoloff P, Pumain R (2004) Glyceraldehyde-3-phosphate dehydrogenase is a $GABA_A$ receptor kinase linking glycolysis to neuronal inhibition. *The Journal of Neuroscience* **24:** 7614-7622.

Laurie DJ, Seeburg PH, Wisden W (1992a) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *The Journal of Neuroscience* **12:** 1063-1076.

Laurie DJ, Wisden W, Seeburg PH (1992b) The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *The Journal of Neuroscience* **12:** 4151-4172.

Leao RM, Mellor JR, Randall AD (2000) Tonic benzodiazepine-sensitive GABAergic inhibition in cultured rodent cerebellar granule cells. *Neuropharmacology* **39:** 990-1003.

Lee H-K, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional plasticity. *Nature* **405:** 955-959.

Leidenheimer NJ, McQuilkin SJ, Hahner LD, Whiting P, Harris RA (1992) Activation of protein kinase C selectively inhibits the γ-aminobutyric acid (A) receptor: role of desensitization. *Molecular Pharmacology* **41:** 1116-1123.

Leil TA, Chen Z-W, Chang C-SS, Olsen RW (2004) $GABA_A$ receptor-associated protein traffics $GABA_A$ receptors to the plasma membrane. *The Journal of Neuroscience* **24:** 11429-11438.

Lengyel I, Cammarota M, Brent VA, Rostas JAP (2001) Autonomous activity and autophosphorylation of CaMPK-II in rat hippocampal slices: effects of tissue preparation. *The Journal of Neurochemistry* **76:** 149-154.

Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B, Schlessinger J (1995) Protein tryosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature* **376:** 737-745.

Levitan ES, Blair LA, Dionne VE, Barnard EA (1988a) Biophysical and pharmacological properties of cloned $GABA_A$ receptor subunits expressed in *Xenopus oocytes*. *Neuron* **1:** 773-781.

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

Lewis DA, Hashimoto T, Volk DW (2005) Cortical inhibitory neurons and schizophrenia. *Nature Reviews Neuroscience* **6:** 312-324.

Lieberman DN, Mody I (1994) Regulation of NMDA channel function by endogenous Ca^{2+}-dependent phosphatase. *Nature* **369:** 235-239.

Lin Y-F, Angelotti TP, Dudek EM, Browning MD, Macdonald RL (1996) Enhancement of recombinant $\alpha 1\beta 1\gamma 2L$ γ-aminobutyric acid A receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both $\beta 1$ and $\gamma 2L$ subunits. *Molecular Pharmacology* **50:** 185-195.

Lin Y-F, Browning MD, Dudek EM, Macdonald RL (1994) Protein kinase C enhances recombinant bovine $\alpha 1\beta 1\gamma 2L$ $GABA_A$ receptor whole-cell currents expressed in L929 fibroblasts. *Neuron* **13:** 1421-1431.

Linseman DA, Bartley CM, Le SS, Laessig TA, Bouchard RJ, Meintzer MK, Li M, Heidenreich KA (2003) Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca^{2+}/calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. *The Journal of Biological Chemistry* **278:** 41472-41481.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMK-II function in synaptic and behavioural memory. *Nature Reviews Neuroscience* **3:** 175-190.

Lisman JE, Zhabotinsky AM (2001) A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. *Neuron* **31:** 191-201.

Liu X, Jones EG (1996) Localization of alpha type II calcium/calmodulin-dependent protein kinase at glutamatergic but not γ-aminobutyric acid (GABAergic) synapses in thalamus and cerebral cortex. *Proceedings of the National Academy of Sciences, USA* **93:** 7332-7336.

Liu X, Jones EG (1997) Alpha isoform of calcium-calmodulin dependent protein kinase II (CAM II kinase-α) restricted to excitatory synapses in the CA1 region of rat hippocampus. *NeuroReport* **8:** 1475-1479.

Lledo P-M, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC, Nicoll RA (1995) Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proceedings of the National Academy of Sciences, USA* **92:** 11175-11179.

Lledo P-M, Zhang X, Südhof TC, Malenka RC, Nicoll RA (1998) Postsynaptic membrane fusion and long-term potentiation. *Science* **279:** 399-403.

Lo RKH, Wong YH (2004) Signal transducer and activator of transcription 3 activation by the δ-opioid receptor via Gα14 involves multiple intermediates. *Molecular Pharmacology* **65:** 1427-1439.

Lu CS, Hodge JJL, Mehren J, Sun X, X, Griffith LC (2003) Regulation of the Ca^{2+}/CaM-responsive pool of CaMK-II by scaffold-dependent autophosphorylation. *Neuron* **40:** 1185-1197.

Lu W-Y, Xiong Z-G, Lei S, Orser BA, Dudek E, Browning MD, MacDonald JF (1999) G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nature Neuroscience* **2:** 331-338.

Lu YM, Mansuy IM, Kandel ER, Roder J (2000) Calcineurin-mediated LTD of GABAergic inhibition underlies the increased excitability of CA1 neurons associated with LTP. *Neuron* **26:** 197-205.

Lüddens H, Korpi ER, Seeburg PH (1995) $GABA_A$/benzodiazepine receptor heterogeneity: neurophysiological implications. *Neuropharmacology* **34:** 245-254.

Lukyanetz EA (1998) Diversity and properties of calcium channel types in NG108-15 hybrid cells. *Neuroscience* **87:** 265-274.

Lukyanetz EA, Piper TP, Sihra TS (1998) Calcineurin involvement in the regulation of high-threshold Ca^{2+} channels in NG108-15 (rodent neuroblastoma x glioma hybrid) cells. *The Journal of Physiology* **510:** 371-385.

Lüscher B, Keller CA (2004) Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacology and Therapeutics* **102:** 195-221.

Ma X-H, Zhong P, Gu Z, Feng J, Yan Z (2003) Muscarinic potentiation of $GABA_A$ receptor currents is gated by insulin signaling in the prefrontal cortex. *The Journal of Neuroscience* **23:** 1159-1168.

Macdonald RL, Gallagher MJ, Feng H-J, Kang J (2004) $GABA_A$ receptor epilepsy mutations. *Biochemical Pharmacology* **68:** 1497-1506.

Machu TK, Firestone JA, Browning MD (1993) Calcium/calmodulin-dependent protein kinase II and protein kinase C phosphorylate a synthetic peptide corresponding to a sequence that is specific for the γ2L subunit of the $GABA_A$ receptor. *The Journal of Neurochemistry* **61:** 375-377.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annual Review of Neuroscience* **25:** 103-126.

Martina M, Kilic G, Cherubini E (1994) The effect of intracellular Ca^{2+} on GABA-activated currents in cerebellar granule cells in culture. *The Journal of Membrane Biology* **142:** 209-216.

Martina M, Mozrzymas JW, Boddeke HWGM, Cherubini E (1996) The calcineurin inhibitor cyclosporin A-cyclophilin A complex reduces desensitization of $GABA_A$-mediated responses in acutely dissociated rat hippocampal neurons. *Neuroscience Letters* **215:** 95-98.

McBain CJ, Fisahn A (2001) Interneurons unbound. *Nature Reviews Neuroscience* **2:** 11-23.

McDonald AJ, Muller JF, Mascagni F (2002) GABAergic innervation of alpha type II calcium/calmodulin-dependent protein kinase immunoreactive pyramidal neurons in the rat basolateral amygdala. *The Journal of Comparative Neurology* **446:** 199-218.

McDonald BJ, Amato A, Connolly CN, Benke D, Moss SJ, Smart TG (1998) Adjacent phosphorylation sites on $GABA_A$ receptor β subunits determine regulation by PKA. *Nature Neuroscience* **1:** 23-28.

McDonald BJ, Moss SJ (1994) Differential phosphorylation of intracellular domains of γ-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. *The Journal of Biological Chemistry* **269:** 18111-18117.

McDonald BJ, Moss SJ (1997) Conserved phosphorylation of the intracellular domains of $GABA_A$ receptor β2 and β3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin type II-dependent protein kinase. *Neuropharmacology* **36:** 1377-1385.

McGlade-McCulloh E, Yamamoto H, Tan S-E, Brickey DA, Soderling TR (1993) Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* **362:** 640-642.

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

McKinsey TA, Zhang CL, Olsen EN (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends in Biochemical Sciences* **27:** 40-47.

McLean HA, Caillard O, Ben-Ari Y, Gaiarsa JL (1996) Bi-directional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. *The Journal of Physiology* **496:** 471-477.

Meguro R, Lu J, Gavrilovici C, Poulter MO (2004) Static, transient and permanent organization of $GABA_A$ receptor expression in calbindin-positive interneurons in response to amygdala kindled seizures. *The Journal of Neurochemistry* **91:** 144-154.

Mehta AK, Ticku MK (1999) An update on $GABA_A$ receptors. *Brain Research - Brain Research Reviews* **29:** 196-217.

Meier J, Grantyn R (2004) Preferential accumulation of $GABA_A$ receptor γ2L, not γ2S, cytoplasmic loops at rat spinal cord inhibitory synapses. *The Journal of Physiology* **559:** 355-365.

Meldolesi J (2001) Rapidly exchanging Ca^{2+} stores in neurons: molecular, structural and functional properties. *Progress in Neurobiology* **65:** 309-338.

Mellor JR, Merlo D, Jones A, Wisden W, Randall AD (1998) Mouse cerebellar granule cell differentiation: electrical activity regulates the $GABA_A$ receptor α6 subunit gene. *The Journal of Neuroscience* **18:** 2822-2833.

Mercik K, Pytel M, Mozrzymas JW (2003) Recombinant $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors expressed in HEK293 and in QT6 cells show different kinetics. *Neuroscience Letters* **352:** 195-198.

Miller SG, Kennedy MB (1985) Distinct forebrain and cerebellar isozymes of type II Ca^{2+}/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *The Journal of Biological Chemistry* **260:** 9039-9046.

Miralles CP, Li M, Mehta AK, Khan ZU, De Blas AL (1999) Immunocytochemical localization of the β3 subunit of the γ-aminobutyric acid A receptor in the rat brain. *The Journal of Comparative Neurology* **413:** 535-548.

Mizoguchi Y, Ishibashi H, Nabekura J (2003a) The action of BDNF on $GABA_A$ currents changes from potentiating to suppressing during maturation of rat hippocampal CA1 pyramidal neurons. *The Journal of Physiology* **548:** 703-709.

Mizoguchi Y, Kanematsu T, Hirata M, Nabekura J (2003b) A rapid increase in the total number of cell surface functional $GABA_A$ receptors induced by brain-derived neurotrophic factor in rat visual cortex. *The Journal of Biological Chemistry* **278:** 44097-44102.

Mody I, Pearce RA (2004) Diversity of inhibitory neurotransmission through $GABA_A$ receptors. *Trends in Neurosciences* **27:** 569-575.

Molloy SS, Kennedy MB (1991) Autophosphorylation of type II Ca^{2+}/calmodulin-dependent protein kinase in cultures of postnatal rat hippocampal slices. *Proceedings of the National Academy of Sciences, USA* **88:** 4756-4760.

Morishita W, Sastry BR (1996) Postsynaptic mechanisms underlying long-term depression of GABAergic transmission in neurons of the deep cerebellar nuclei. *The Journal of Neurophysiology* **76:** 59-68.

Moss SJ, Doherty CA, Huganir RL (1992a) Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the β1, γ2S and γ2L subunits of the γ-aminobutyric acid type A receptor. *The Journal of Biological Chemistry* **267:** 14470-14476.

Moss SJ, Gorrie GH, Amato A, Smart TG (1995) Modulation of $GABA_A$ receptors by tyrosine phosphorylation. *Nature* **377:** 344-348.

Moss SJ, Ravindran A, Mei L, Wang JB, Kofuji P, Huganir RL, Burt DR (1991) Characterization of recombinant $GABA_A$ receptors produced in transfected cells from murine α1, β1, and γ2 subunit cDNAs. *Neuroscience Letters* **123:** 265-268.

Moss SJ, Smart TG (1996) Modulation of amino acid-gated ion channels by protein phosphorylation. *International Review of Neurobiology* **39:** 1-52.

Moss SJ, Smart TG (2001) Constructing inhibitory synapses. *Nature Reviews Neuroscience* **2:** 240-250.

Moss SJ, Smart TG, Blackstone CD, Huganir RL (1992b) Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation. *Science* **257:** 661-665.

Moulder KL, Cormier RJ, Shute AA, Zorumski CF, Mennerick S (2003) Homeostatic effects of depolarization on Ca^{2+} influx, synaptic signaling, and survival. *The Journal of Neuroscience* **23:** 1825-1831.

Mozrzymas JW, Cherubini E (1998) Changes in intracellular calcium concentration affect desensitization of $GABA_A$ receptors in acutely dissociated P2-P6 rat hippocampal neurons. *The Journal of Neurophysiology* **79:** 1321-1328.

Mukherji S, Brickey DA, Soderling TR (1994) Mutational analysis of secondary structure in the autoinhibitory and autophosphorylation domains of calmodulin kinase II. *The Journal of Biological Chemistry* **269:** 20733-20738.

Mukherji S, Soderling TR (1994) Regulation of Ca^{2+}/calmodulin-dependent protein kinase II by inter- and intrasubunit-catalyzed autophosphorylations. *The Journal of Biological Chemistry* **269:** 13744-13747.

Nakanishi S (2005) Synaptic mechanisms of the cerebellar cortical network. *Trends in Neurosciences* **28:** 93-100.

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

Nelson AB, Gittis AH, du Lac S (2005) Decreases in CaMKII activity trigger persistent potentiation of intrinsic excitability in spontaneously firing vestibular nucleus neurons. *Neuron* **46:** 623-631.

Nelson P, Christian C, Nirenberg M (1976) Synapse formation between clonal neuroblastoma x glioma hybrid cells and striated muscle cells. *Proceedings of the National Academy of Sciences, USA* **73:** 123-127.

Nemeroff CB (2003) The role of GABA in the pathophysiology and treatment of anxiety disorders. *Psychopharmacology Bulletin* **37:** 133-146.

Nirenberg M, Wilson S, Higashida H, Rotter A, Krueger K, Busis N, Ray R, Kenimer JG, Adler M (1983) Modulation of synapse formation by cyclic adenosine monophosphate. *Science* **222:** 794-799.

Nusser Z, Cull-Candy SG, Farrant M (1997) Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. *Neuron* **19:** 697-709.

Nusser Z, Hajos N, Somogyi P, Mody I (1998) Increased number of synaptic $GABA_A$ receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* **395:** 172-177.

Nusser Z, Kay LM, Laurent G, Homanics GE, Mody I (2001a) Disruption of $GABA_A$ receptors on GABAergic interneurons leads to increased oscillatory power in the olfactory bulb network. *The Journal of Neurophysiology* **86:** 2823-2833.

Nusser Z, Naylor D, Mody I (2001b) Synapse-specific contribution of the variation of transmitter concentration to the decay of inhibitory postsynaptic currents. *Biophysical Journal* **80:** 1251-1261.

Nusser Z, Sieghart W, Mody I (1999) Differential regulation of synaptic $GABA_A$ receptors by cAMP-dependent protein kinase in mouse cerebellar and olfactory bulb neurones. *The Journal of Physiology* **521:** 421-435.

Nusser Z, Sieghart W, Stephenson FA, Somogyi P (1996) The α6 subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. *The Journal of Neuroscience* **16:** 103-114.

Nyíri G, Freund TF, Somogyi P (2001) Input-dependent synaptic targeting of α2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. *The European Journal of Neuroscience* **13:** 428-442.

Nymann-Andersen J, Wang H, Sawyer GW, Olsen RW (2002) Interaction between $GABA_A$ receptor subunit intracellular loops: implications for higher order complex formation. *The Journal of Neurochemistry* **83:** 1164-1171.

Ohkuma S, Katsura M, Chen DZ, Chen SH, Kuriyama K (1994) Presence of N-methyl-D-aspartate (NMDA) receptors in neuroblastoma x glioma hybrid NG108-15 cells-analysis using [$^{45}Ca^{2+}$]influx and [^{3}H]MK-801 binding as functional measures. *Brain Research - Molecular Brain Research* **22:** 166-172.

Ohtsuki G, Kawaguchi S-Y, Mishina M, Hirano T (2004) Enhanced inhibitory synaptic transmission in the cerebellar molecular layer of the GluRδ2 knock-out mouse. *The Journal of Neuroscience* **24:** 10900-10907.

Ortinski PI, Lu C, Takagaki K, Fu Z, Vicini S (2004) Expression of distinct α subunits of $GABA_A$ receptor regulates inhibitory synaptic strength. *The Journal of Neurophysiology* **92:** 1718-1727.

Otis TS, De Kononck Y, Mody I (1994) Lasting potentiation of inhibition is associated with an increased number of γ-aminobutyric acid type A receptors activated during miniature inhibitory postsynaptic currents. *Proceedings of the National Academy of Sciences, USA* **91:** 7698-7702.

Ouardouz M, Sastry BR (2000) Mechanisms underlying LTP of inhibitory synaptic transmission in the deep cerebellar nuclei. *The Journal of Neurophysiology* **84:** 1414-1421.

Ouyang Y, Kantor D, Harris KM, Schuman EM, Kennedy MB (1997) Visualization of the distribution of autophosphorylated calcium/calmodulin-dependent protein kinase II after tetanic stimulation in the CA1 area of the hippocampus. *The Journal of Neuroscience* **17:** 5416-5427.

Ouyang Y, Rosenstein A, Kreiman G, Schuman EM, Kennedy MB (1999) Tetanic stimulation leads to increased accumulation of Ca^{2+}/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. *The Journal of Neuroscience* **19:** 7823-7833.

Palma E, Torchia G, Limatola C, Trettel F, Arcella A, Cantore G, Di Gennaro G, Manfredi M, Esposito V, Quarato PP, Miledi R, Eusebi F (2005) BDNF modulates $GABA_A$ receptors microtransplanted from the human epileptic brain to *Xenopus oocytes*. *Proceedings of the National Academy of Sciences, USA* **102:** 1667-1672.

Patenaude C, Chapman A, Bertrand S, Congar P, Lacaille J-C (2003) $GABA_B$ receptor- and metabotropic glutamate receptor- dependent cooperative long-term potentiation of rat hippocampal $GABA_A$ synaptic transmission. *The Journal of Physiology* **553:** 155-167.

Pawson T, Scott JD (1997) Signaling through scaffold, anchoring, and adapter proteins. *Science* **278:** 2075-2080.

Perrais D, Ropert N (1999) Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. *The Journal of Neuroscience* **19:** 578-588.

Persohn E, Malherbe P, Richards JG (1992) Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *The Journal of Comparative Neurology* **326:** 193-216.

Pilon C, Lévesque D, Dimitriadou V, Griffon N, Martres M-P, Schwartz J-CSP (1994) Functional coupling of the human dopamine D3 receptor in a transfected NG108-15 neuroblastoma-glioma hybrid cell line. *The European Journal of Pharmacology* **268:** 129-139.

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

Poisbeau P, Cheney MC, Browning MD, Mody I (1999) Modulation of synaptic $GABA_A$ receptor function by PKA and PKC in adult hippocampal neurons. *The Journal of Neuroscience* **19:** 674-683.

Poncer JC, Esteban JA, Malinow R (2002) Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by α-Ca^{2+}/calmodulin-dependent protein kinase II. *The Journal of Neuroscience* **22:** 4406-4411.

Porter NM, Twyman RE, Uhler MD, Macdonald RL (1990) Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. *Neuron* **5:** 789-796.

Puia G, Costa E, Vicini S (1994) Functional diversity of GABA-activated Cl^- currents in Purkinje versus granule neurons in rat cerebellar slices. *Neuron* **12:** 117-126.

Ramadan E, Fu Z, Losi G, Homanics GE, Neale JH, Vicini S (2003) $GABA_A$ receptor β3 subunit deletion decreases α2/3 subunits and IPSC duration. *The Journal of Neurophysiology* **89:** 128-134.

Reynolds DS, Rosahl TW, Cirone J, O'Meara GF, Haythornthwaite A, Newman RJ, Myers J, Sur C, Howell O, Rutter AR, Atack J, Macaulay AJ, Hadingham KL, Hutson PH, Belelli D, Lambert J, Dawson GR, McKernan R, Whiting PJ, Wafford KA (2003) Sedation and anesthesia mediated by distinct $GABA_A$ receptor isoforms. *The Journal of Neuroscience* **23:** 8608-8617.

Rongo C (2002) A fresh look at the role of CaMKII in hippocampal synaptic plasticity and memory. *BioEssays* **24:** 223-233.

Rossi D, Hamann M (1998) Spillover-mediated transmission at inhibitory synapses promoted by high affinity $\alpha 6$ subunit $GABA_A$ receptors and glomerular geometry. *Neuron* **20:** 795.

Rothlin CV, Katz E, Verbitsky M, Elgoyhen AB (1999) The $\alpha 9$ nicotinic acetylcholine receptor shares pharmacological properties with type A γ-aminobutyric acid, glycine, and type 3 serotonin receptors. *Molecular Pharmacology* **55:** 248-254.

Rudolph U, Möhler H (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annual Review of Pharmacology and Toxicology* **44:** 475-498.

Ruiz A, Fabian-Fine R, Scott R, Walker MC, Rusakov DA, Kullmann DM (2003) $GABA_A$ receptors at hippocampal mossy fibers. *Neuron* **39:** 961-973.

Rumpel E, Behrends J-C (2000) Postsynaptic receptor occupancy during evoked transmission at striatal GABAergic synapses *in vitro*. *The Journal of Neurophysiology* **84:** 771-779.

Rusanescu G, Qi H, Thomas SM, Brugge JS, Halegoua S (1995) Calcium influx induces neurite growth through a Src-Ras signaling cassette. *Neuron* **15:** 1415-1425.

Rycroft BK, Gibb AJ (2002) Direct effects of calmodulin on NMDA receptor single-channel gating in rat hippocampal granule cells. *The Journal of Neuroscience* **22:** 8860-8868.

Saitow F, Murakoshi T, Suzuki H, Konishi S (2005) Metabotropic P2Y purinoceptor-mediated presynaptic and postsynaptic enhancement of cerebellar GABAergic transmission. *The Journal of Neuroscience* **25:** 2108-2116.

Salter MW, Kalia LV (2004) Src kinases: a hub for NMDA receptor regulation. *Nature Reviews Neuroscience* **5:** 317-328.

Samaco RC, Hogart A, LaSalle JM (2005) Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human Molecular Genetics* **14:** 483-492.

Sanchez RM, Dai W, Levada RE, Lippman JJ, Jensen FE (2005) AMPA/kainate receptor-mediated downregulation of GABAergic synaptic transmission by calcineurin after seizures in the developing rat brain. *The Journal of Neuroscience* **25:** 3442-3451

Sasaki H, Nagura K, Ishino M, Tobioka H, Kotani K, Sasaki T (1995) Cloning and characterization of cell adhesion kinase β, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *The Journal of Biological Chemistry* **270:** 21206-21219.

Schulman H (2004) Activity-dependent regulation of calcium/calmodulin dependent protein kinase II localization. *The Journal of Neuroscience* **24:** 8399-8403.

Seabrook GR, Kemp JA, Freedman SB, Patel S, Sinclair HA, MaAllister G (1994) Functional expression of human D_3 dopamine receptors in differentiated neuroblastoma x glioma NG108-15 cells. *The British Journal of Pharmacology* **111:** 391-393.

Searles CD, Singer HS (1988) The identification and characterization of a GABAergic system in the cholinergic neuroblastoma x glioma hybrid clone NG108-15. *Brain Research* **448:** 373-376.

Sedelnikova A, Weiss DS (2002) Phosphorylation of the recombinant ρ1 GABA receptor. *International Journal of Developmental Neuroscience* **20:** 237-246.

Shen K, Meyer T (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284:** 162-166.

Shen K, Teruel MN, Connor JH, Shenolikar S, Meyer T (2000) Molecular memory by reversible translocation of calcium/calmodulin-dependent protein kinase II. *Nature Neuroscience* **3:** 881-886.

Shen K, Teruel MN, Subramanian K, Meyer T (1998) CaMKII β functions as an F-actin targeting module that localizes CaMKII α/β heterooligomers to dendritic spines. *Neuron* **21:** 593-606.

Shen K, Meyer T (1998) *In vivo* and *in vitro* characterization of the sequence requirement for oligomer formation of Ca^{2+}/calmodulin-dependent protein kinase IIα. *The Journal of Neurochemistry* **70:** 96-104.

Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105:** 331-343.

Sieghart W, Fuchs K, Tretter V, Ebert V, Jechlinger M, Höger H, Adamiker D (1999) Structure and subunit composition of $GABA_A$ receptors. *Neurochemistry International* **34:** 379-385.

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

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in α-calcium-calmodulin kinase II mutant mice. *Science* **257:** 206-211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257:** 201-206.

Singleton MW, Holbert II WH, Ryan ML, Lee AT, Kurz JE, Churn SB (2005) Age dependence of pilocarpine-induced status epilepticus and inhibition of CaM Kinase II activity in the rat. *Developmental Brain Research* **156:** 67-77.

Sinkkonen ST, Homanics GE, Korpi ER (2003) Mouse models of Angelman syndrome, a neurodevelopmental disorder, display different brain regional $GABA_A$ receptor alterations. *Neuroscience Letters* **340:** 205-208.

Smart TG (1997) Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. *Current Opinion in Neurobiology* **7:** 358-367.

Smith GB, Olsen RW (1995) Functional domains of $GABA_A$ receptors. *Trends in Pharmacological Sciences* **16:** 162-168.

Soderling TR, Chang B, Brickey D (2002) Cellular signaling through multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. *The Journal of Biological Chemistry* **276:** 3719-3722.

Soderling TR, Derkach VA (2000) Postsynaptic protein phosphorylation and LTP. *Trends in Neurosciences* **23:** 75-80.

Soltesz I, Mody I (1995) Ca^{2+}-dependent plasticity of miniature inhibitory postsynaptic currents after amputation of dendrites in central neurons. *The Journal of Neurophysiology* **73:** 1763-1773.

Somogyi P (2005) Defined types of cortical interneurone structure space and spike timing in the hippocampus. *The Journal of Physiology* **562:** 9-26.

Song M, Messing RO (2005) Protein kinase C regulation of $GABA_A$ receptors. *Cellular and Molecular Life Sciences* **62:** 119-127.

Stelzer A, Kay AR, Wong RK (1988) $GABA_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science* **241:** 339-341.

Stelzer A, Shi H (1994) Impairment of $GABA_A$ receptor function by N-methyl-D-aspartate-mediated calcium influx in isolated CA1 pyramidal cells. *Neuroscience* **62:** 813-828.

Strack S, Choi S, Lovinger DM, Colbran RJ (1997) Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. *The Journal of Biological Chemistry* **272:** 13467-13470.

Strack S, McNeill RB, Colbran RJ (2000a) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *The Journal of Biological Chemistry* **275:** 23798-23806.

Strack S, Robison AJ, Bass MA, Colbran RJ (2000b) Association of calcium/calmodulin-dependent kinase II with developmentally regulated splice variants of the postsynaptic density protein densin-180. *The Journal of Biological Chemistry* **275:** 25061-25064.

Studler B, Fritschy J-M, Brünig I (2002) GABAergic and glutamatergic terminals differentially influence the organization of GABAergic synapses in rat cerebellar granule cells *in vitro*. *Neuroscience* **114:** 123-133.

Sumi M, Kiuchi K, Ishikawa T, Ishii A, Hagiwara M, Nagatsu T, Hidaka H (1991) The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochemical and Biophysical Research Communications* **181:** 968-975.

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack JR, Dawson GR, McKernan RM, Whiting PJ, Rosahl TW (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. *The Journal of Neuroscience* **21:** 3409-3418.

Suzuki T, Okumura-Noji K, Tanaka R, Tada T (1994) Rapid translocation of cytosolic Ca^{2+}/calmodulin-dependent protein kinase II into postsynaptic density after decapitation. *The Journal of Neurochemistry* **63:** 1529-1537.

Suzuki-Takeuchi E, Tanaka T, Hink WF, King MM (1992) High-level expression using baculovirus, purification, and characterization of a monomeric form of type II calmodulin-dependent protein kinase. *Protein Expression and Purification* **3:** 160-164.

Svenningsson P, Nishi A, Fisone G, Girault J-A, Nairn AC, Greengard P (2004) DARPP-32: an integrator of neurotransmission. *Annual Review of Pharmacology and Toxicology* **44:** 269-296.

Tabata T, Sawada S, Araki K, Bono Y, Furuya S, Kano M (2000) A reliable method for culture of dissociated mouse cerebellar cells enriched for Purkinje neurons. *The Journal of Neuroscience Methods* **104:** 45-53.

Tao-Cheng J-H, Vinade L, Pozzo-Miller LD, Reese TS, Dosemeci A (2002) Calcium/calmodulin-dependent protein kinase II clusters in adult rat hippocampal slices. *Neuroscience* **115:** 435-440.

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

Tehrani MHJ, Barnes EM (1995) Reduced function of γ-aminobutyric acid (A) receptors in tottering mouse brain: role of cAMP-dependent protein kinase. *Epilepsy Research* **22:** 13-21.

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

Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T (1999) PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proceedings of the National Academy of Sciences, USA* **96:** 435-440.

Thiagarajan TC, Piedras-Renteria ES, Tsien RW (2002) α- and β-CaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. *Neuron* **36:** 1103-1114.

Thomas P, Smart TG (2002) Use of electrophysiological methods in the study of recombinant and native neuronal ligand-gated ion channels. In: *Current Protocols in Pharmacology.* (Eds. Enna SJ, Williams M, Ferkany JW, Kenakin T, Porsolt RD, Sullivan JP. John Wiley and sons, inc.) **2:** 11.4.1-11.4.34

Thomas P, Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods* **51**: 187-200.

Thompson CL, Stephenson FA (1994) $GABA_A$ receptor subtypes expressed in cerebellar granule cells: a developmental study. *The Journal of Neurochemistry* **62:** 2037-2044.

Tia S, Wang J-F, Kotchabhakdi N, Vicini S (1996) Developmental changes of inhibitory synaptic currents in cerebellar granule neurons: role of $GABA_A$ Receptor α6 Subunit. *The Journal of Neuroscience* **16:** 3630-3640.

Tojima T, Ito E (2004) Signal transduction cascades underlying *de novo* protein synthesis required for neuronal morphogenesis in differentiating neurons. *Progress in Neurobiology* **72:** 183-193.

Tojima T, Kobayashi C, Ito E (2003a) Dual role of cyclic AMP-dependent protein kinase in neuritogenesis and synaptogenesis during neuronal differentiation. *The Journal of Neuroscience Research* **74:** 829-837.

Tojima T, Takahashi M, Ito E (2003b) Dual regulation of LIM kinase 1 expression by cyclic AMP and calcium determines cofilin phosphorylation states during neuritogenesis in NG108-15 cells. *Brain Research* **985:** 43-55.

Tojima T, Yamane Y, Takagi H, Takeshita T, Sugiyama T, Haga H, Kawabata K, Ushiki T, Abe K, Yoshioka T, Ito E (2000a) Three-dimensional characterization of interior structures of exocytotic apertures of nerve cells using atomic force microscopy. *Neuroscience* **101:** 471-481.

Tojima T, Yamane Y, Takahashi M, Ito E (2000b) Acquisition of neuronal proteins during differentiation of NG108-15 cells. *Neuroscience Research* **37:** 153-161.

Traynelis SF, Jaramillo F (1998) Getting the most out of noise in the central nervous system. *Trends in Neurosciences* **21:** 137-145.

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

Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* **391:** 892-896.

Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. *Nature Reviews Neuroscience* **5:** 97-107.

Ueno S, Zorumski C, Bracamontes J, Steinbach JH (1996) Endogenous subunits can cause ambiguities in the pharmacology of exogenous γ-aminobutyric acid A receptors expressed in human embryonic kidney 293 cells. *Molecular Pharmacology* **50:** 931-938.

Valenzuela CF, Machu TK, McKernan RM, Whiting PJ, VanRenterghem BB, McManaman JL, Brozowski SJ, Smith GB, Olsen RW, Harris RA (1995) Tyrosine kinase phosphorylation of $GABA_A$ receptors. *Brain Research - Molecular Brain Research* **31:** 165-172.

Vallano M (1990) Developmental regulation of type II calcium/calmodulin-dependent kinase isoforms in rat cerebellum. *The Journal of Neurobiology* **21:** 1262-1273.

Vallano M, Beaman-Hall CM (1989) Differentiation increases type II calmodulin-dependent protein kinase in the neuroblastoma/glioma cell line 108CC15 (NG108-15). *The Journal of Neuroscience* **9:** 539-547.

Vallano M, Lambolez B, Audinat E, Rossier J (1996) Neuronal activity differentially regulates NMDA subunit expression in cerebellar granule cells. *The Journal of Neuroscience* **16:** 631-639.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE (2001) $GABA_A$ receptor $\alpha 1$ subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *The Journal of Neuroscience* **21:** 3009-3016.

Vicini S, Ortinski P (2004) Genetic manipulations of $GABA_A$ receptor in mice make inhibition exciting. *Pharmacology and Therapeutics* **103:** 109-120.

Wafford KA (2005) $GABA_A$ receptor subtypes: any clues to the mechanism of benzodiazepine dependence? *Current Opinion in Pharmacology* **5:** 47-52.

Wafford KA, Macaulay AJ, Fradley R, O'Meara GF, Reynolds DS, Rosahl TW (2004) Differentiating the role of γ-aminobutyric acid type A ($GABA_A$) receptor subtypes. *Biochemical Society Transactions* **32:** 553-556.

Walikonis RS, Oguni A, Khorosheva EM, Jeng C-J, Asuncion FJ, Kennedy MB (2001) Densin-180 forms a ternary complex with the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and α-actinin. *The Journal of Neuroscience* **21:** 423-433.

Wall MJ (2002) Furosemide reveals heterogeneous $GABA_A$ receptor expression at adult rat Golgi cell to granule cell synapses. *Neuropharmacology* **43:** 737-749.

Wan Q, Man HY, Braunton J, Wang W, Salter MW, Becker L, Wang YT (1997a) Modulation of $GABA_A$ receptor function by tyrosine phosphorylation of β subunits. *The Journal of Neuroscience* **17:** 5062-5069.

Wan Q, Xiong ZG, Man HY, Ackerley CA, Braunton J, Lu WY, Becker LE, MacDonald JF, Wang YT (1997b) Recruitment of functional $GABA_A$ receptors to postsynaptic domains by insulin. *Nature* **388:** 686-690.

Wang J, Liu S, Haditsch U, Tu W, Cochrane K, Ahmadian G, Tran L, Paw J, Wang Y, Mansuy I, Salter MM, Lu Y (2003a) Interaction of calcineurin and type-A GABA receptor γ2 subunits produces long-term depression at CA1 inhibitory synapses. *The Journal of Neuroscience* **23:** 826-836.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang Y-T (2003b) Control of synaptic strength, a novel function of Akt. *Neuron* **38:** 915-928.

Wang RA, Kolaj CM, Randic M (1995) α-subunit of CaMK-II enhances GABA and inhibitory synaptic responses of rat neurons *in vitro*. *The Journal of Neurophysiology* **73:** 2099-2106.

Wang Y, Mishra R, Simonson MS (2003c) Ca^{2+}/calmodulin-dependent protein kinase II stimulates c-fos transcription and DNA synthesis by a Src-based mechanism in glomerular mesangial cells. *The Journal of the American Society of Nephrology* **14:** 28-36.

Weeber EJ, Jiang Y-H, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL, Sweatt JD (2003) Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of Neuroscience* **23:** 2634-2644.

Wei J, Zhang M, Zhu Y, Wang J-H (2004) Ca^{2+}-calmodulin signalling pathway up-regulates GABA synaptic transmission through cytoskeleton-mediated mechanisms. *Neuroscience* **127:** 637-647.

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

White JA, Banks MI, Pearce RA, Kopell NJ (2000) Networks of interneurons with fast and slow γ-aminobutyric acid type A ($GABA_A$) kinetics provide substrate for mixed gamma-theta rhythm. *Proceedings of the National Academy of Sciences, USA* **97:** 8128-8133.

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

Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Thompson SA, Wafford KA (1999) Molecular and functional diversity of the expanding $GABA_A$ receptor gene family. *Annals of the New York Academy of Sciences* **30:** 645-653.

Whittington MA, Traub RD, Jefferys JG (1995) Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation. *Nature* **16:** 612-615.

Winder DG, Sweatt JD (2001) Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. *Nature Reviews Neuroscience* **2:** 461-474.

Wisden W, Korpi ER, Bahn S (1996) The Cerebellum: a model system for studying $GABA_A$ receptor diversity. *Neuropharmacology* **35:** 1139-1160.

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

Wooltorton JRA, Moss SJ, Smart TG (1997) Pharmacological and physiological characterization of murine homomeric β3 $GABA_A$ receptors. *The European Journal of Neuroscience* **9:** 2225-2235.

Wu G, Fang Y, Lu Z-H, Ledeen RW (1998) Induction of axon-like and dendrite-like processes in neuroblastoma cells. *The Journal of Neurocytology* **27:** 1-14.

Xia Z, Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nature Reviews Neuroscience* **6:** 267-276.

Xu M, Akabas MH (1996) Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor α1 subunit. *The Journal of General Physiology* **107:** 195-205.

Yaka R, Thornton C, Vagts AJ, Phamluong K, Bonci A, Ron D (2002) NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. *Proceedings of the National Academy of Sciences, USA* **99:** 5710-5715.

Yamakawa T, Fukunaga K, Higashida H, Miyamoto E (1992) Activation of Ca^{2+}/calmodulin-dependent protein kinase II by stimulation with bradykinin in neuroblastoma x glioma hybrid NG108-15 cells. *Brain Research* **597:** 220-226.

Yang SN, Tang YG, Zucker RS (1999) Selective induction of LTP and LTD by postsynaptic $[Ca^{2+}]i$ elevation. *The Journal of Neurophysiology* **81:** 781-787.

Young RM, Lawford BR, Feeney GFX, Ritchie T, Noble EP (2004) Alcohol-related expectancies are associated with the D_2 dopamine receptor and $GABA_A$ receptor $\beta 3$ subunit genes. *Psychiatry Research* **127:** 171-183.

Zhang S, Ehlers MD, Bernhardt JP, Su C-T, Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. *Neuron* **21:** 443-453.

Zhu J, Qin Y, Zhao M, Van Aelst L, Malinow R (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110:** 443-455.

Zwick E, Wallasch C, Daub H, Ullrich A (1999) Distinct calcium-dependent pathways of epidermal growth factor receptor transactivation and PYK2 tyrosine phosphorylation in PC12 cells. *The Journal of Biological Chemistry* **274:** 20989-20996.

Appendix 1:

A1.1 Oligonucleotide design for site-directed mutagenesis and knockout mouse genotyping.

Carried out in collaboration with Dr. A.M Hosie and Ms. H.M.A da Silva as follows:

Two complimentary oligonucleotides were designed containing the desired mutation for use as mutagenic primers in a PCR reaction. The design of the 5'-3' primer is shown in Table A1. Primers were synthesised by Invitrogen Life Technologies. The $\gamma 2S^{T357F,\ T359F}$ cDNA was a gift from Prof. S.J. Moss. As required some subunits were tagged with the 9E10 epitope (EQKLISEEDL) from c-myc, inserted between the fourth and fifth amino acid of the mature protein by site-directed mutagenesis (Connolly *et al.*, 1996).

Subunit mutation	**Sequence of primer #1: 5' – 3'**	**Primer #2:**
$\beta 3^{S383A}$	gtataggaaacaggccatgcccaaggaag	cttccttgggcatggcctgtttcctatac
$\beta 3^{S408A,S409A}$	ctacggaggagggctgcacagctcaaaatc	gattttgagctgtgcagccctcctccgtag
$\gamma 2S^{T348A,T350A}$	gatattcgtcccagagcagcagccattcaaatgaac	gttcatttgaatggctgctgctctgggacgaatatc
$\gamma 2S^{Y357F,Y359F}$	gagggatgaagaatttggctttgagtgtttggatg	catccaaacactcaaagccaaattcttcatccctc

Table A1: Primers designed for the construction of $GABA_A$ receptor subunit cDNAs containing the desired single point mutations.

Primers were designed by by Dr A Hosie. Letters indicate: a = adenine, g = guanine, c = cytosine, t = thymine.

A1.2 Transformation of competent *E-Coli* cells using TOP10 one shot Kit (Invitrogen).

Carried out by Ms. H.M.A da Silva as follows:

- An aliquot of cDNA PCR product was centrifuged and placed on ice.
- An aliquot of frozen competent cells was thawed and kept on ice.
- 0.5-1 µl of cDNA PCR product was mixed with the competent cells (25 µl) in a sterile microcentrifuge tube and this was left on ice for 30 min.
- The tube was then transferred to a 42°C water bath for 30 s and then placed back on ice.
- 80 µl of pre-warmed SOC media (Sigma, 0.5 % w/v yeast extract, 2.0 % w/v tryptone, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose) was added to the cells and they were incubated for 60-90 min at 37°C shaking at ~900 rpm.
- 100 µl/transformation was then plated out on LB-agar plates containing 50 µg/ml ampicillin (Sigma) and left overnight for colonies to develop.
- At least 2 colonies per mutant were selected from the agar plates and grown overnight in 2ml LB Broth base media (Sigma) with 10 % w/v ampicillin, the plasmid from these *E. Coli* cultures was purified before sequencing.

A1.3 Purification of plasmid DNA using the QIAPrep Spin Miniprep kit (Qiagen).

Carried out by Ms H.M.A da Silva as follows:

- 2 ml of bacterial suspension were transferred to a microcentrifuge tube and centrifuged for 1-2 min at 13,200 rpm and the supernatant discarded.

- The pellet of bacterial cells was then re-suspended in 250 µl of Buffer P1 (as supplied).
- 250 µl of buffer P2 (as supplied) was added and again inverted 4-6 times to mix.
- 350 µl of buffer N3 (as supplied) was added and inverted 4-6 times.
- The mixture was then centrifuged at 16,000 rpm for 10 min, and the supernatant decanted into a spin column.
- The column was centrifuged at 13,200 rpm for 30-60 s.
- The flow through the column was discarded and 750 µl buffer PE was added and centrifuged for 30-60 s for an additional 2 min, to remove any residual washing buffer.
- The QIAprep column was placed into a clean microcentrifuge tube. To elute the DNA, 50 µl buffer EB (10 mM Tris-Cl @ pH 8.5) or water was added to the centre of the QIAprep column, allowed to stand for 1 min, centrifuged for 1 min.
- The concentration of purified cNDA was measured using a BioPhotometer (Eppendorf).

A1.4 Preparation of mouse tail DNA.

Carried out by Ms H.M.A da Silva as follows:

- Tails were placed into a 1.5 ml microcentrifuge tube and 700 µl of NTES (50 mM Tris HCl @ pH 8, 50 mM EDTA, 100 mM $NaCl_2$ and 1 % w/v SDS) was added along with 7.5 µl of 20 mg/ml Proteinase K (Sigma) per tube.
- This was incubated overnight at 55°C on a thermomixer at 300 rpm.

- The following day 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the tubes were left to shake for 10-15 min. They were then spun at 11,400 rpm in a microcentrifuge. The top phase (DNA) was transferred into a fresh tube avoiding the interface.
- Then 2.5 µl of 20 mg/ml RNase was added and incubated for 1 hr at 37°C.
- Phenol/chloroform/iosamyl alcohol (25:24:1) was added for 5-10 min and then the tubes were microfuged again for 5 min.
- The top phase was transferred to a fresh tube and this was filled with 1 ml isopropanol. This was inverted a few times until a precipitate had formed and centrifuged for a further 10 min.
- The supernatant was aspirated and 100 µl 70 % v/v ethanol was added, followed by centrifugation for 5 min (12,000 rpm). The supernatant was aspirated again and the pellet left to air dry.
- 300 µl of TE buffer was added and left at 37°C at 300 rpm on a thermomixer overnight. The next day the solution could be transferred to the fridge until required.